p155抗体は悪性腫瘍合併例で高率とされる。その他の膠原病を合併するオーバーラップ（重複）症候群では，抗PM-1抗体（強皮症），抗Ku抗体（強皮症・全身性エリテマトーデス），抗nRNP抗体（混合性結合組織病）が陽性となることがある。

4．診断

BohanとPeterの診断基準や皮膚筋炎・多発筋炎の改訂診断基準（厚生省特定疾患自己免疫疾患調査研究班平成4年度研究報告，pp25-28，1993）が汎用される。

5．治療方針

多発筋炎あるいは皮膚筋炎の確定診断後は，プレドニン®1～1.5 mg/kg/日を1～2ヵ月間連日投与する。筋力の改善，血清CK値の減少がみられれば2週間に10％の割合でプレドニン®を減量し，2～3年程度は維持療法を行う。筋症状は早期治療例ほど回復がよい。改善がないときは同量を1～2ヵ月間投与するか，ステロイドパルス療法を2～3クール行う（メチルプレドニゾロン1 g/回の3日間連続投与）。効果がない場合はメトトレキサート（メソトレキセート®，5～25 mg/週，経口あるいは筋肉内投与），アザチオプリン（イムラン®，50～100 mg/日，経口投与）などを併用する。ステロイド，免疫抑制薬の無効例では，追加療法として免疫グロブリン療法を併用する。

6．治療のポイント

筋症状増悪時には筋炎の再燃か，ステロイドミオパチーの合併かの鑑別が重要である。血清CK値上昇，筋電図で線維性収縮電位や陽性鋭波の出現頻度が上昇した場合には再燃を疑う。ステロイドミオパチーは，プレドニン内服を4週間以上続けた場合に発症し，下肢近位筋優位の筋力低下・筋萎縮を呈するが，顔面筋および頸部伸展筋は保たれ，血清CK値は低下する。

7．患者指導とリハビリテーション

急性期は等尺性収縮以外の運動は避ける。安定後は誤嚥性肺炎，廃用性筋萎縮，関節拘縮予防のための理学療法を早期に開始する。

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5. 骨格筋

骨格筋は傷害を受けると再生する（図 1）．筋傷害シグナルにより骨格筋特異的幹細胞である筋衛星細胞（muscle satellite cells）が活性化され，分裂・増殖し，やがてお互いに融合，あるいは既存の筋線維と融合して筋線維を再生する．デュシェンヌ型筋ジストロフィー（Duchenne muscular dystrophy；DMD）等の重篤な遺伝性筋疾患に対して筋・幹細胞を移植する再生医療が期待されているが，その確立のためには，筋組織の再生がどのように制御されているかを理解することが重要である．

a． 骨格筋衛星細胞

筋衛星細胞は筋基底膜と筋線維の間にある単核の細胞で 1961 年に Alexander Mauro によって初めてその存在を記載された．通常，細胞周期の G0 の状態にあるが，筋傷害時に活性化され，増殖して筋線維を再生する．生直後は骨格筋組織の中の核の 30 %程度が筋衛星細胞の核であるが，成体になると 5 %程度とほぼ一定になる．体幹と四肢の骨格筋の発生学的な起源は沿軸中胚葉由来の体節であり，その中に形成される dermomyotome に出現する Pax3，Pax7 陽性の筋前駆細胞（muscle progenitor cells）が増殖し，やがて Myf5，MyoD 等の筋分化制御遺伝子を発現して筋芽細胞（myoblast）となり，次に増殖を止め，融合して，筋線維を形成する．筋衛星細胞はその過程で派生してく

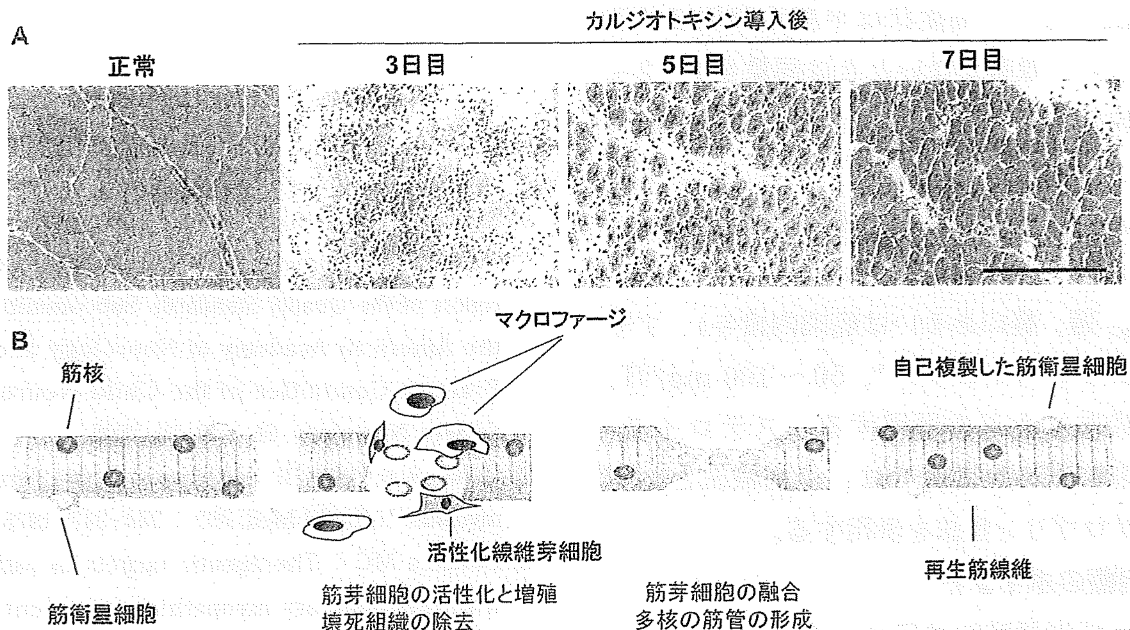

図 1　骨格筋の再生

A） C57Bl/6 マウス骨格筋に蛇毒であるカルジオトキシンを導入して筋傷害を引き起こした後の組織修復過程を示す．ヘマトキシリン・エオジン染色．スケールバー：200 ミクロン．

B） 骨格筋特異的幹細胞である筋衛星細胞は，静止期の状態では筋基底膜と筋線維の間に存在するが，筋傷害時には活性化し，増殖する（筋芽細胞）．やがてお互いに融合し，あるいは既存の筋線維と融合して筋再生が完了する．この過程には好中球やマクロファージ等による壊死組織の貪食機能が重要である．活性化した筋衛星細胞の一部は，元の筋衛星細胞の状態に戻る（自己複製）．

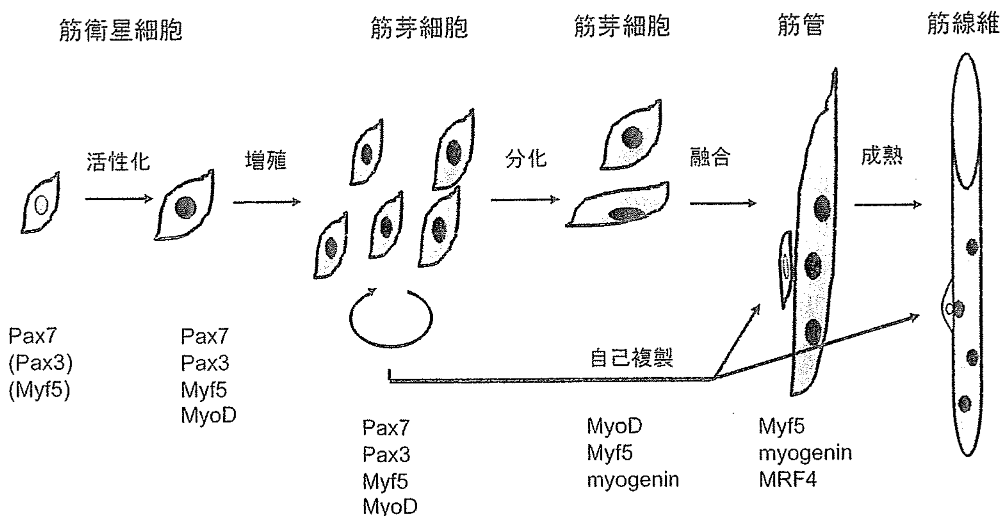

図 2　筋衛星細胞の分化過程とその制御因子
筋衛星細胞の筋分化は，発生過程の筋分化と類似しているが，その維持にはPax7が重要な働きを持つ．筋衛星細胞が不均等分裂により自己複製すると考えられているが，そのタイミングや制御分子に関しては不明な点が多い．

る．c-Met，Pax7，Myf5，M-cadherin，CD34等が筋衛星細胞特異的マーカーとして知られているが，昨今の網羅的遺伝子発現研究等の結果，新しいマーカー（カルシトニン・レセプター，odz4 等）がリストに加わってきた．筋衛星細胞研究には実験動物の骨格筋から筋衛星細胞を高い純度で分離する方法が有用だが，従来は，線維芽細胞との培養皿への接着性の違いを利用した preplating 法，現在は各種細胞表面マーカーで染色しセル・ソーターで分離する方法が用いられている．筋衛星細胞は自己複製することで，一生涯にわたって筋再生能を維持する（図 1，図 2）．その機構として不均等分裂が提唱されているが，その分裂様式，制御因子等，不明な点が残されている．

b．筋衛星細胞の活性化，増殖，分化

骨格筋が傷害されると nitric oxide synthase（NOS）が活性化され，nitric oxide（NO）が産生され，hepatocyte growth factor（HGF；肝細胞増殖因子）を活性化し，c-Met レセプターへ結合する．c-Met を介したシグナルが筋衛星細胞を活性化し，筋衛星細胞は活発に増殖する．筋衛星細胞の増殖能は分裂を繰り返すと徐々に低下する．とくに筋ジストロフィー等の，筋変性・壊死，再生を繰り返す筋疾患では，筋衛星細胞の増殖能は徐々に低下し，筋再生が筋壊死に追いつかなくなり，筋線維が脱落し，筋力が低下していく．筋衛星細胞は筋細胞の他に脂肪細胞，骨細胞にも分化することが報告されているので，筋疾患の進行した段階で認められる脂肪変性や，徐々に筋組織の骨化が進行する進行性骨化性線維異形成症等の遺伝性の疾患の発症に関与する可能性がある．

c．筋・幹細胞と再生医療

1990 年代前半，近親者から得た筋衛星細胞を培養後，DMD 患者の骨格筋へ移植する筋芽細胞移植が行われたが，その効率は低かった．移植直後に多くの筋芽細胞が死んでしまうこと，移植後筋芽細胞があまり移動しないこと，免疫抑制が不十分であったこと等が原因であったと推察されている．1998 年，骨髄細胞が筋線維へ分化し，さらに筋衛星細胞へ分化することが示され[1]，造血幹細胞の可塑性との関連で，DMD への治療応用が期待されたが，その筋線維再生への寄与率はわずかであり，またその分化機序は依然不明で，大部分は細胞融合に

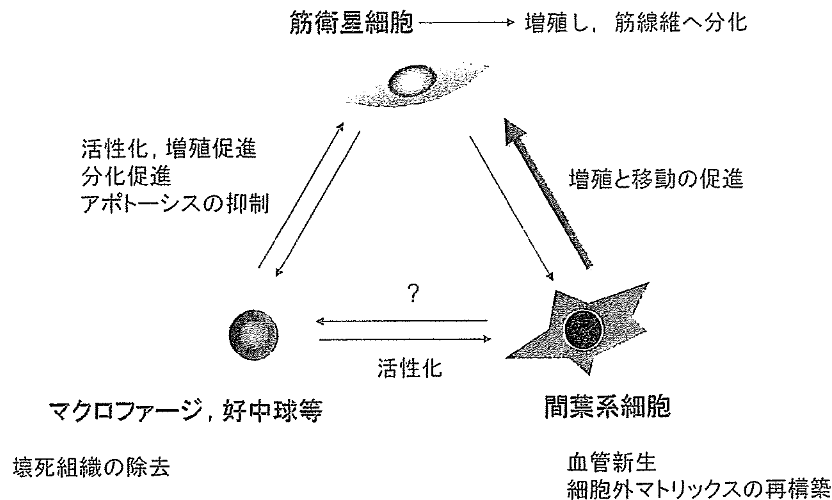

図3 筋再生を制御する細胞とそのネットワーク
筋再生過程では，筋前駆細胞である筋衛星細胞が中心的な役割を果たすが，その他に，壊死組織の除去を担うマクロファージ，好中球等が重要な細胞である．さらに間葉系細胞が間質に存在し，筋傷害時に活性化し，細胞外マトリックスの分解と再構築を促進し，血管新生を制御することで筋再生を制御している．これらの細胞は，直接相互作用する他に，サイトカイン等を介してお互いの活性化，増殖や移動，生存，分化を制御している．

よると思われた．しかし血中のAC133陽性細胞は移植すると効率よく筋線維に分化するという報告もあり，循環している細胞の中に筋分化能を持つ特別な細胞が存在する可能性は否定できない．一方，骨格筋組織の間質や血管周囲にも，多能性を持ち，筋細胞へも分化する細胞が数多く報告されているが，これらの細胞の相互関係ははっきりしない．それらは，ヘキスト色素を排出する能力に富むside population細胞(SP cells)，血管周囲に存在するペリサイト(pericyte)，同じく血管組織に由来するメソアンギオブラスト（mesoangioblast)，muscle-derived stem cells，myo-endothelial cells等である．数量的には筋衛星細胞が筋線維再生に最も寄与していることは広く認められているが，筋変性疾患に対する移植治療という観点では，移植後の生存率が低く，局所にしか生着しない筋衛星細胞に対して，経動脈的，あるいは経静脈的に移植可能なこれらの多能性幹細胞の利用が期待されている[2]．

d. 筋再生におけるマクロファージや線維芽細胞の役割

筋再生はさまざまな細胞間の相互作用によって完了する．なかでもとくに重要な細胞はマクロファージと間質の線維芽細胞様の間葉系細胞であろう（図3)．マクロファージは壊死組織の除去の他に，筋衛星細胞の活性化やアポトーシスの抑制，筋分化の促進等の機能があると考えられており[3]，その機能不全で筋再生は障害される．間葉系細胞も筋再生時に活性化され，増殖し，MMPs等のプロテアーゼを分泌し，細胞移動の促進，細胞外マトリックスの分解・再構築，血管新生，各種成長因子の活性化に関わっている．また，各種ケモカインを分泌しており，炎症細胞，免疫担当細胞の制御にも関与していると思われる．間葉系細胞はin vitroでも脂肪細胞へ分化しやすい傾向を持ち，この細胞の機能低下や異常な活性化が，筋再生の遅延，筋組織の線維化，脂肪変性に関わっていると考えられるので，再生医療の良き標的である．
［鈴木友子，武田伸一］

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Review

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Activin signaling as an emerging target for therapeutic interventions

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Abstract

After the initial discovery of activins as important regulators of reproduction, novel and diverse roles have been unraveled for them. Activins are expressed in various tissues and have a broad range of activities including the regulation of gonadal function, hormonal homeostasis, growth and differentiation of musculoskeletal tissues, regulation of growth and metastasis of cancer cells, proliferation and differentiation of embryonic stem cells, and even higher brain functions. Activins signal through a combination of type I and II transmembrane serine/threonine kinase receptors. Activin receptors are shared by multiple transforming growth factor-β (TGF-β) ligands such as myostatin, growth and differentiation factor-11 and nodal. Thus, although the activity of each ligand is distinct, they are also redundant, both physiologically and pathologically *in vivo*. Activin receptors activated by ligands phosphorylate the receptor-regulated Smads for TGF-β, Smad2 and 3. The Smad proteins then undergo multimerization with the co-mediator Smad4, and translocate into the nucleus to regulate the transcription of target genes in cooperation with nuclear cofactors. Signaling through receptors and Smads is controlled by multiple mechanisms including phosphorylation and other posttranslational modifications such as sumoylation, which affect potein localization, stability and transcriptional activity. Non-Smad signaling also plays an important role in activin signaling. Extracellularly, follistatin and related proteins bind to activins and related TGF-β ligands, and control the signaling and availability of ligands.

The functions of activins through activin receptors are pleiotrophic, cell type-specific and contextual, and they are involved in the etiology and pathogenesis of a variety of diseases. Accordingly, activin signaling may be a target for therapeutic interventions. In this review, we summarize the current knowledge on activin signaling and discuss the potential roles of this pathway as a molecular target of therapy for metabolic diseases, musculoskeletal disorders, cancers and neural damages.

Signaling of activins and related growth factors through activin receptors

Biosynthesis of activin and related growth factors

Activins belong to the transforming growth factor-β (TGF-β) family of growth and differentiation factors [1,2]. They form dimers composed of two inhibin β subunits. Four β subunits have been identified in mammals (βA, βB, βC and βE), whereas only a single inhibin α-subunit has been discovered so far. The βA and βB transcripts are found in nearly all tissues, whereas βC and βE subunits are expressedpredominantly in the liver. Both β and α subunits are synthesized as precursor polypeptides. After dimerization of the precursors, prodomains are cleaved by furin and/or related proprotein convertases in the endoplasmic reticulum and a mature dimeric polypeptide is released. Homodimers of inhibin βA or βB subunits, activin A and activin B, respectively, or heterodimeric activin AB exist in various tissues. Inhibins, heterodimeric proteins composed of an α-subunit linked to β-subunits by disulfide bonds, act as activin antagonists. In the case of myostatin, another TGF-β family protein related to activins, cleavage and maturation of the ligand may occur extracellularly in a tissue-specific manner [3].

Activin receptors

Activin signals are transmitted through two types of transmembrane serine/threonine kinase receptors, type I and type II activin receptors in target cells [1,4]. Activin receptors are prototypes of single-pass transmembrane serine/threonine kinases. Intriguingly, activin receptors are shared by other TGF-β family proteins, such as myostatin, growth and differentiation factor 11 (GDF11) and nodal. Therefore, several activities of these ligands are redundant with those of activins. Myostatin has been characterized as a skeletal muscle-specific cytokine regulating skeletal muscle mass [5]. GDF11 is structurally similar to myostatin, and is involved in neurogenesis in the spinal cord and olfactory bulb [6]. GDF11 also regulates kidney development and endocrine pancreas development [7,8]. Nodal is a central player in patterning the early embryo during the induction of mesoderm and endoderm [9], and acts as an authentic mesoderm inducer in mammalian species. Some of these activities are shared with activins.

Activin type II receptor, ACVR2 or ActRIIA, has been identified and characterized as a transmembrane serine/threonine kinase for activin A [10]. A second activin type II receptor, ACVR2B or ActRIIB, has also been identified [4]. In addition, TGF-β type II receptor, BMP type II receptor and Müllerian duct inhibiting substance type II receptor specific to each ligand have been characterized [2]. To date, seven type I receptors, activin receptor-like kinases 1 to 7 (ALK1-7), have been characterized for the TGF-β family [11]. Like type II receptors, type I receptors possess a serine/threonine kinase domain. However, different from type II receptors, type I receptors have a unique GS domain near the intracellular juxtamembrane regions preceding the kinase domain. The amino acid sequences of L45 loops of type I receptors located between the kinase subdomains IV and V are responsible for the preference of Smad proteins and determine the specificity between the activin/TGF-β subgroup (ALK4, 5, 7) and BMP subgroup (ALK1, 2, 3, 6) [2,11]. ALK4 is known as activin type IB receptor, ACVR1B or ActRIB, whereas ALK7 is known as activin type IC receptor, ACVR1C. ALK4 and ALK7 are type I receptors for activins and nodal, and ALK4 and ALK5 are receptors for myostatin and GDF11 (Table S1; additional file 1) [1,2]. Once activins bind to ActRIIA or ActRIIB, type I receptors are recruited to the ligand/ActRII complex, and the GS domains of type I receptors become phosphorylated by ActRII kinases. Activin/TGF-β-specific Smad, Smad2 and Smad 3, are phosphorylated by activated type I receptors (Figure 1). In the case of nodal, the co-receptor Cripto and related factors are required for the complete activation [9]. Cripto facilitates nodal signaling by binding to both nodal and activin receptors. Interestingly, Cripto may also act as an inhibitory factor for activin signaling when overexpressed [12](Table S1; additional file 1).

A pseudo-receptor BMP and activin membrane-bound inhibitor, BAMBI, has been identified [13]. BAMBI interacts with multiple type I receptors for TGF-β family ligands and inhibits the formation of the active receptor signaling complex. Thus, BAMBI serves as an endogenous dominant negative receptor [13]. BAMBI is characterized as a β-catenin target in colorectal tumors [14].

Regulation of activin receptors

Regulatory proteins for activin receptors control the signaling activity of activins and related growth factors. A FYVE domain-containing protein, the Smad anchor for receptor activation (SARA), interacts with both the type I receptor and Smads.

Complex formation of activin receptors with SARA and Smad in EEA-1 positive early endosomes may be an essential step for efficient activin/TGF-β signaling [15,16]. Activin type II receptors (ActRIIA and ActRIIB) have consensus amino acids for PSD-95/Discs-large/ZO-1 (PDZ) protein interaction at their COOH-terminus [1]. This characteristic is unique among receptors of the TGF-β family [17]. Activin-receptor interacting proteins (ARIPs), which have PDZ domains, associate with the COOH-terminus of ActRIIs and regulate activin signaling. ARIP1 has multiple WW and PDZ domains for protein-protein interactions, and regulates the localization of activin receptors and negatively controls signaling [17]. Intriguingly, ARIP-1 acts as a scaffold for N-methyl-D-aspartate (NMDA) receptor activation in hippocampal neurons, and is also

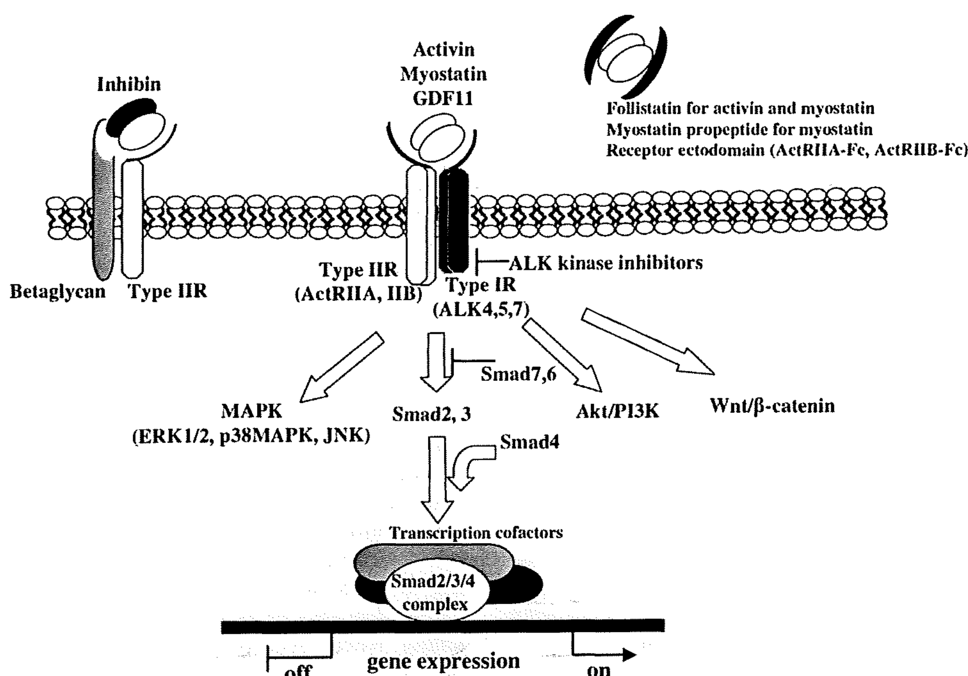

Figure 1
Signal transduction through activin receptors. Activin, myostatin and GDF11 signal through type II and type I serine/threonine kinase receptors. Type IIR is the principal ligand binding receptors, and ligand/typeIIR complexes recruit and associate with type IR. Type IR is phosphorylated and activated by type IIR kinase. Smad2 and 3, activin/TGF-β specific Smads, are phosphorylated by activated type IR. In the nucleus, Smad2/3/4 complexes regulate gene expression with additional transcriptional cofactors. Smad-independent pathway such as MAPK is also activated downstream of activin receptors. Inhibin acts antagonistic to activin by forming high affinity complexes with ActRII and betaglycan. Follistatin, myostatin propeptide and receptor ectodomain inhibit the activities of activin and related factors in the extracellular space to prevent ligand/receptor interaction. Chemical type IR kinase inhibitors act in the cell to disrupt receptor/intracellular signaling.

known as synaptic scaffolding protein, S-SCAM [18]. A recent study showed that activin induces long-lasting NMDA receptor activation by ARIP1 in hippocampal neurons [19]. ARIP2 is a small protein that has one PDZ domain [20]. Several ARIP2 splicing isoforms exist, and, depending on the isoform, ARIP2 either augments or inhibits activin signaling [21]. Gene trapping analysis identified the RasGAP-binding protein Dok-1, which acts downstream of receptor tyrosine kinases as an essential adapter molecule for activin-induced apoptotic signaling in B cells. Dok-1 interacts simultaneously with activin receptors and Smads. Stimulation by activin induces association of Dok-1 and Smad3 [22].

Posttranslational modification of the activin/TGF-β receptor is an additional important mechanism for the regulation of receptor activation. The ubiquitin-proteasome pathway tightly regulates TGF-β family signaling. HECT-type E3 ubiquitin ligases, Smad ubiquitin regulatory factor 1 (Smurf1) and Smurf2 have been implicated in Smad degradation. Smurfl and Smurf2 bind to TGF-β family receptors via the inhibitory Smads, Smad6 and Smad7, to induce their ubiquitin-dependent degradation [23]. In addition, TGF-β type I receptor is sumoylated in response to ligand stimulation. Posttranslational receptor sumoylation, the covalent attachment of a small ubiquitin-like modifier (SUMO) is required for the kinase activities of both the TGF-β type I and type II receptors, and enhances receptor function by facilitating the recruitment and phosphorylation of Smad3 [24].

Regulation of activin signaling through Smads

Smad signaling in the cytoplasm and the nucleus is under tight control. Smads consist of an NH_2-terminal MH1 and a COOH-terminal MH2 domain. The L45 loop of type I receptors directly interacts with the MH2 domain of receptor-regulated Smad (R-Smad), and determines Smad specificity [2]. Type I receptors phosphorylate Smads at their COOH-terminal two serine residues. Smad2 and 3, R-Smads for activin and TGF-β undergo constant shuttling

between the cytoplasm and nucleus, and the activation of R-Smads triggers nuclear accumulation [2]. PPM1A may act as a Smad COOH-terminal phosphatase [25]. Linker regions between MH1 and MH2 domains of Smads are phosphorylated by mitogen-activated protein kinase (MAPK). This phosphorylation enhances the binding of ubiquitin ligase to Smad, resulting in polyubiquitination and degradation [26].

Smads have intrinsic DNA-binding activity [2]. However, to fully activate target genes, Smad physically associates with a diverse set of DNA-binding cofactors such as CBP/p300, TGIF, c-Ski and Evi-1 [11]. This characteristic determines the cell type-specific transcription and complexity of activin/TGF-β signaling. A number of transcription factors including forkhead proteins, bHLH family, AP1 family, homeodomain protein family and nuclear receptors act as Smad-interacting transcription factors [2]. Once activated, Smad complexes recruit additional transcriptional activators or repressors to regulate target genes (Figure 1).

Negative feedback regulation by the inhibitory Smads, Smad6 and Smad7 is an important shutoff system for signaling by the TGF-β family including activins [2,11].

Smad-independent activin signaling and receptor crosstalk

In addition to the canonical Smad pathway, activin signaling through activin receptors regulates other intracellular pathways. p38 MAPK, ERK1/2 and JNK are activated by activin in a cell type-specific manner [27,28]. For example, activin synergizes with basic fibroblast growth factor to activate tyrosine hydroxylase expression via the ERK1/2 pathway [27]. Activin negatively regulates the pituitary transcription factor Pit-1 through p38 MAPK-dependent and Smad-independent pathways [28]. Independently of Smad4, ActRIB/Smad2 acts as a co-activator of the canonical Wnt signaling pathway. Upon activation, Smad2 physically interacts with Tcf4, β-catenin and the co-activator p300 to enhance transcriptional activity of β-catenin/Tcf4 through the histone acetyltransferase activity of p300 [29]. Transactivation by Smad2 is independent of the Smad binding element. Furthermore, recent characterization revealed that TGF-β stimulates phosphorylation of BMP-specific Smad1 independently of BMP receptors [30-32]. Smad-independent activin signaling and receptor crosstalk increase the complexity of activin/TGF-β signaling.

Ligand binding proteins

Extracellular activin-binding proteins control activin signaling [1]. Follistatin (FST) is a prototype of activin-binding proteins. FST is a cysteine-rich single chain glycoprotein that does not possess sequence similarity to the TGF-β family [33]. Structural analysis of FST with activin showed that two FST molecules encircle activin, and neutralize the ligand by burying one-third of its residues and both type II and type I receptor binding sites [34-36] (Figure 1). FST not only binds and inhibits activins, but also binds and neutralizes the actions of myostatin and GDF11 [1,37]. Mice with a disrupted follistatin gene have musculoskeletal and cutaneous abnormalities, reflecting the abnormal signaling of activins, myostatin and GDF11 [38]. The follistatin-related gene, FLRG, is a follistatin domain-containing protein structurally similar to FST [39,40]. Whereas FST has three follistatin domains, FLRG has only two. Like FST, FLRG binds and neutralizes activins, myostatin and GDF11 [37,39]. Proteomics analyses indicate that FLRG associates with myostatin in sera [37]. Although functionally redundant, expression and transcriptional regulation of FST and FLRG are different [39-41]. FLRG gene deleted mice show dysregulated glucose metabolism and fat homeostasis [42](see below).

Biological activities and roles of activin signaling as a target of therapeutic interventions

After the purification and identification of activins as regulators of follicle-stimulating hormone secretion from the anterior pituitary, important roles of activins in the hypothalamus-pituitary-gonadal axis have been described [1]. However, activin activity is not limited to reproductive tissues. Activins and related factors have pleiotropic actions in extragonadal tissues. In this section, we focus on selective actions of activins and related growth factors from a therapeutic point of view.

Activins and their regulators in metabolic disorders

Activin signaling is required for proper development of the endocrine and exocrine pancreas, and dysregulation of the activin signaling pathway contributes to the genesis of metabolic diseases. In human embryonic stem cells, activin B mediates the induction of homeoprotein Pdx1, a key regulator of endocrine pancreas development [43]. ActRIIA mutant mice show hypoplasia of the pancreas and develop diabetes [44]. ActRIIB and Smad2 activity use the same signaling pathway to regulate pancreas islet formation [45]. ALK7, a type I receptor for activin B, activin AB and nodal, is expressed abundantly in pancreatic β cells and adipose tissues, and regulates insulin biosynthesis and secretion [46-48]. Recent characterization revealed that ALK7 transmits signals of GDF3, another TGF-β family member [49,50]. GDF3, ALK7 and co-receptor Cripto are all expressed in adipose tissues, and Gdf3(-/-) null mice and ALK7(-/-) null mice showed reduced fat accumulation and resistance to diet-induced obesity [49,50].

The expression of activin receptors, myostatin and their binding protein FLRG can be modulated in adipose tissue and skeletal muscle by chronic obesity. In subcutaneous and visceral fats, myostatin and ActRIIB mRNA levels in

ob/ob mice are 50- to 100-fold higher than that in wild-type mice [51]. By contrast, FLRG mRNA levels are increased in subcutaneous fat, but decreased in visceral fat of ob/ob mice compared to wild-type mice [51]. In humans, myostatin was shown to increase in skeletal muscle and plasma of obese and insulin resistant women [52].

FLRG gene disrupted mice showed an increased pancreatic islet number and size, β cell hyperplasia, decreased visceral fat mass, improved glucose tolerance, and enhanced insulin sensitivity. This phenotype is caused through increased signaling by activin or myostatin in a tissue-specific manner [42].

Myostatin and activin in muscular diseases

Myostatin, the skeletal muscle specific member of the TGF-β family, restricts muscle growth and determines skeletal muscle mass [5]. Myostatin signals through activin type I receptors (Alk4 and 5) and type II receptors [5]. Mice with a targeted deletion of the myostatin gene have a 25–30% increased muscle mass resulting from hypertrophy and hyperplasia [53]. Double muscling phenotypes upon inactivation of the myostatin gene have been observed in cattle, sheep, race dogs, fish and even in humans [54-59]. Myostatin is regarded as a good drug target since therapeutics that stimulate skeletal muscle growth may be useful for muscle-wasting conditions such as muscular dystrophy, sarcopenia and cachexia. Whereas activins and TGF-β function in almost every cell type, myostatin specifically affects skeletal muscle growth. Thus, targeting myostatin is a rational therapeutic strategy to increase skeletal muscle mass. Several myostatin inhibitors such as monoclonal antibodies and myostatin propeptide, as well as FST and its derivatives are promising candidates for the treatment of muscle wasting disorders [60-67] (Table S2; Additional file 2). Skeletal muscle fibrosis is also ameliorated by myostatin inhibition [68]. The effectiveness of myostatin inhibition has been studied using various muscular dystrophy animal models. Monoclonal antibody-mediated myostatin blockade results in an increase of muscle mass and absolute muscle strength in *mdx* mice, an animal model of Duchenne-type muscular dystrophy [60]. Muscles in *mdx* mice with myostatin inhibition showed less fibrosis, reduced fatty remodeling and an improved regeneration process [61]. Myostatin circulates in the serum in a latent form complexed with multiple binding proteins. NH_2-terminal myostatin propeptide is a major myostatin-binding protein and non-covalently associates with myostatin [5,37]. Myostatin propeptide, stabilized by fusion to IgG-Fc, has been shown to be effective in ameliorating dystrophic pathophysiology [62]. Muscle atrophy caused in mutant caveolin-3 transgenic mice, a model of limb-girdle muscular dystrophy (LGMD) 1C, was reduced dramatically by crossing these mice with myostatin propeptide transgenic mice [63]. In calpain 3-deficient LGMD2A model mice, both muscle mass and muscle force were recovered upon gene therapy using myostatin propeptide [64]. Myostatin blockage at an early stage in a model of δ-sarcoglycan-deficient muscular dystrophy was effective in reducing muscle loss and fibrosis, and in improving regeneration [65]. It is of note that the elimination of myostatin did not suppress the phenotype of a laminin-α2-deficient mice, but increased postnatal lethality due to fat loss [69]. Soluble forms of an extracellular domain of ActRIIB fused with IgG-Fc may block myostatin effectively *in vivo*, and have strong muscle mass increasing activities [70]. In addition to myostatin, activin and GDF11 are recognized by soluble forms of ActRIIB [71]. FST and FST-derived myostatin inhibitors are also effective for increasing muscle mass and ameliorating muscular dystrophy [66,67]. It is worth noting that neurogenic muscle atrophy caused by amyotrophic lateral sclerosis and spinal muscular atrophy may be ameliorated by myostatin inhibition either by myostatin antibody or follistatin [72,73].

The expression of activin, myostatin, TGF-β, activin receptors, and FST in cardiac muscle is also deregulated in pathological conditions such as cardiac failure and cardiomyopathy [74,75]. However, in contrast to the observations in skeletal muscle, myostatin does not counteract cardiac hypertrophy or fibrosis [75].

Roles of activin and BMP signaling in osteoporosis and bone formation

Although both BMP and activin regulate bone formation, their modes of action are distinct. BMPs are potent inducers of osteoblast differentiation. Activins are expressed abundantly in bone tissues, and regulate bone formation by controlling both osteoblast and osteoclast functions. Different from the activity of BMP, activins enhance the receptor activator of NF-κB ligand (RANKL)-mediated osteoclast differentiation, and act as commitment factors for osteoclastogenesis [76]. Both antiresorptive and anabolic drugs are useful for the treatment of osteoporosis [77]. Bisphosphonates, selective estrogen-receptor modulators and estrogen are currently available antiresorptive drugs, whereas recombinant human parathyroid hormone is an anabolic drug. Intriguingly, the extracellular domain of ActRIIA stabilized by fusion to IgG-Fc increases bone mass and strength by activin inhibition, and is a novel promising agent for osteoporosis in early human trials [77,78] (Table S2; Additional file 2).

As mentioned above, the extracellular domain of ActRIIB fused to IgG-Fc increases muscle mass. Thus, two activin type II receptor decoys have different clinical uses. Consistent with the activity of activin in bone formation, inhibin A, an activin antagonist, works as an endocrine stimulator of bone mass *in vivo* by increasing osteoblast-

ogenesis [79]. Inhibin antagonizes activin by forming a complex of ActRIIs and betaglycan [2,4](Figure 1).

Fibrodysplasia ossificans progressive (FOP), a genetic disorder of progressive heterotypic ossification, is caused by missense mutations in ACVR1A (ALK2), a BMP type I receptor, which increase BMP signaling [80]. A recurrent activating mutation in the juxtamembrane GS domain of ACVR1A was reported in sporadic and familial cases of classic FOP [80]. Thus, the activin and BMP pathway are therapeutic targets for the treatment of low bone mass.

Roles of activins and related growth factors in cancer

Inhibition of cancer cell growth is one of the activities of activins in the early phase of cancer development. Facilitating activin signaling either by Cripto silencing or FLRG silencing inhibits human breast cancer cell growth [81,82](Table S2; Additional file 2). Mutations in several genes involved in the activin signaling pathway have been characterized in cancers. Two 8-bp polyadenine tracts of the ACVR2 gene were targets for frameshift mutations in gastrointestinal cancers with microsatellite instability [83]. Somatic ACVR1B gene mutations have been found in pancreatic carcinoma [84] and Smad2 and Smad4 are mutated in colorectal and pancreatic carcinomas [85]. Thus, dysregulation of activin receptors and activin/TGF-β Smads is directly involved in carcinogenesis.

Interestingly, inhibin-deficient mice develop gonadal sex cord-stromal tumors [86]. They develop adrenal cortical tumors when gonadectomized. Therefore, inhibins act as secreted tumor suppressors in gonads and adrenal glands. Supraphysiological levels of activins in inhibin-deficient mice are responsible for the development of tumors. Overproduction of activins was observed in a cachexia-like wasting syndrome that includes hepatocellular necrosis and metastasis [86-88]. Thus, the actions of activin in tumor development are highly context-dependent.

Myofibroblasts present in tumor stroma facilitate tumor development and invasion [2]. TGF-β and activin stimulate the differentiation of myofibroblasts from mesenchymal progenitors, suggesting the facilitation of invasive properties of cancers.

Regarding metastasis, inhibition of activin and/or TGF-β suppresses experimental metastasis to multiple organs including lung, liver and bone [89,90](Table S2; Additional file 2). Chemical inhibitors for type I receptor kinases for activin/TGF-β (ALK4, 5 and 7) are promising cancer therapies [89,91]. They may offer an option for preventing tumor angiogenesis, the motility of cancer cells, fibrosis and metastasis [92].

TGF-β and TGF-β type I receptor are upregulated at the tumor-bone interface and modulate RANKL-dependent osteolysis, and TGF-β inhibition reduces mammary tumor-induced osteolysis [93]. Since activin works as a cofactor for RANKL, similar to TGF-β, activin may modulate osteoclastogenesis in the tumor-bone interaction.

TGF-β produced by cancer cells has immunosuppressive effects, resulting in the evasion of cancers from destruction by the immune system. A novel TGF-β kinase inhibitor reverses this effect, inhibits cell growth and enhances the immunogenicity of cancer cells [94]. Whether activins also act as regulators in immunosuppression in cancers has not yet been determined.

Activities of activins in the brain

Activins and activin receptors are expressed highly in the central nervous system and have crucial roles in neuronal development [95,96]. However, compared with classical neurotrophic factors, our knowledge about the functions of activins in the brain is limited. Importantly, the expression of inhibin βA mRNA, which encodes activin A, is induced by excitatory synaptic input [97,98]. It is induced in granule cell neurons of the hippocampus by high-frequency synaptic stimuli that produce long term potentiation (LTP). This induction is NMDA receptor-dependent [97,98]. Activin increases the number of synaptic contacts by modulating actin dynamics in the spine of the neurons, which may be responsible for the establishment of LTP [99]. This modulation is mediated by the classical MAP kinase cascades via Erk1/2 [99]. Similarly, inhibin βA mRNA is transiently induced in dentate gyrus neurons through NMDA receptor activation after unilateral mechanical brain injury by saline injection [100]. Inhibin βA mRNA is also induced during amygdala kindling, and accurately marks excitatory neurons with synaptic alterations from seizures [101].

Accumulating evidence indicates that activin also has neurotrophic and neuroprotective effects on selective neurons [102]. Treatment with recombinant activin following ischemic injury rescues neurons from damage [103]. Overexcited neurons are protected by the neurotrophic effect of basic fibroblast growth factor, which depends on the induction of activin A [104] (Table S2; Additional file 2). It is also of note that activin and fibroblast growth factor act in synergy in dopaminergic neurons [27].

Neuronal-specific transgenic approaches using the αCaMKII promoter revealed further functions of activins [105,106]. Hippocampal neurons in αCaMKII promoter-driven dominant negative ActRIB transgenic mice were more vulnerable to kainate injection [105]. These mice also showed a reduced NMDA current with an impaired LTP. Reciprocally, activin potentiates NMDA receptor-mediated signaling by forming complexes with activin receptors, NMDA receptors and Fyn on postsynaptic scaffolding proteins [19]. Interestingly, activins tune pre- and

postsynaptic GABAergic transmission affecting anxiety [107]. αCaMKII promoter-driven activin and FST transgenic mice are affected in their anxiety-related behavior by modulation of their postnatal neurogenesis in the subgranular zone of the dentate gyrus in the hippocampus [106]. Infusion of activin into the dentate gyrus of the hippocampus produces an antidepressant-like effect in the forced swim test. Conversely, antidepressants such as fluoxetine and desipramine increase Smad2 phosphorylation [108]. These data suggest that the activin signaling pathway may be a novel target for neuroprotection and psychopharmacological therapy.

Role of activins in embryonic stem cells

Activin A is a potent mesoderm inducer in *Xenopus* embryos, and numerous tissues can be differentiated from *Xenopus* animal cap cells and embryonic stem cells [109]. A sophisticated strategy to differentiate mouse embryonic stem cells into insulin-producing cells or other cell types by activin has been developed [110,111]. Intriguingly, activin signaling is indispensable to maintain self-renewal and the stemness of human embryonic stem cells [111]. Activin signaling sustains the expression of pluripotency-associated genes such as nanog and inhibits BMP signaling, which promotes self-renewal in human embryonic stem cells [112].

Conclusion

Activin signaling as a target for therapeutic intervention

Although activins were first discovered as powerful factors to stimulate follicle-stimulating hormone production from the anterior pituitary, activins act on almost all cell types and have diverse roles. Furthermore, activin receptors are shared by other TGF-β family members such as myostatin, GDF11, nodal and a subset of BMPs. The TGF-β family members are key regulators of myogenesis, neurogenesis and organogenesis, left-right asymmetry and bone formation. Actions of activins through activin receptors and Smads are pleiotropic and context-dependent, and alterations in signaling through activin receptors are the cause of a variety of disorders. In this review, we focused on recently characterized aspects of activin signaling in relationship to metabolic diseases, musculoskeletal diseases, cancers and neuroprotection.

Various strategies have been designed for the inhibition of activin signaling through receptors. Soluble forms of the extracellular domains of activin receptors, FST and related ligand binding proteins, chemical kinase inhibitors for activin receptors, and siRNAs either for ligand or signaling molecules interfere with activin signaling. Intriguingly, histone deacetylase inhibitors or nitric oxide have been demonstrated to inhibit the progression of muscular dystrophy in a mouse model by transcriptional activation of FST [113,114].

In muscle wasting disorders, the inhibition of myostatin is a possible therapeutic strategy. Soluble ActRIIB-Fc, FST and its derivatives, myostatin propeptide, monoclonal myostatin antibodies and myostatin siRNA are myostatin inhibitors that have been shown to be beneficial for preventing muscle loss. Cachexia from cancers and neurogenic muscle atrophy are also targets for myostatin inhibition [72,73,115](Table S2; Additional file 2).

In cancers, activins have multiple roles such as regulation of cancer cell growth, promotion of organ-specific cancer progression and metastasis. Soluble ActRIIA-Fc is a novel promising drug for osteoporosis, cancer-related bone loss and cachexia [77,78,88]. Activin also has neuroprotective functions, and the augmentation of activins may have favorable protective effects on neurons (Table S2; Additional file 2).

Although targeting activin and related factors may become part of future therapies, given the complexity of their action, some side-effects of such therapies are certainly possible. The dysregulation of activin may affect functions of gonads and adipose tissues [4,42]. It is also possible that activation or targeting activin/TGF-β may in some contexts cause uncontrollable tumor growth or detrimental cellular apoptosis [22,86].

Once promising proteins or chemicals targeting activin signaling are discovered, methods of the drug delivery system are important issues for effective treatment. The stabilization of peptides by fusion with IgG-Fc or other stable proteins is a strategy for targeting activin signaling. Delivery of genes by adeno-associated viral vectors is also potentially promising [64,116]. Finally, nanoparticles such as liposomes and atellocollagen are efficient delivery vehicles for siRNA and proteins [117], and may be useful in delivering agents that target activin signaling.

In summary, therapeutic interventions targeted to signaling through activin receptors may provide novel strategies for the development of effective treatments against a variety of diseases.

Abbreviations

TGF-β: transforming growth factor-β; GDF11: growth and differentiation factor 11; ACVR2 or ActRIIA: activin type II receptor; ACVR2B or ActRIIB: activin type IIB receptor; BMP: bone morphogenetic protein; ALK: activin receptor-like kinase; ACVR1B or ActRIB: activin type IB receptor; ACVR1C: activin type IC receptor; BAMBI: BMP and activin membrane-bound inhibitor; PDZ: PSD-95/Discs-large/ZO-1; ARIP: activin receptor interacting protein; NMDA: N-methyl-D-aspartate; MAPK: mitogen-activated protein kinase; FST: follistatin; FLRG: follistatin-related gene; LGMD: limb-girdle muscular dystrophy; RANKL:

receptor activator of NF-κB ligand; FOP: fibrodysplasia ossificans progressive; ACVR1A: activin type IA receptor; LTP: long term potentiation; αCAMKII: α calmodulin kinase II.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MN participated in the analysis of MSTN/activin signaling and muscle diseases. KH participated in the analysis of growth factor signaling and the interaction of growth factors. AU participated in the analysis of skeletal muscle differentiation. YS participated in therapy for muscular dystrophy. HA and KI participated in the functions of activins in the central nervous system. KT conceived of the study, and participated in its coordination. All authors approved the manuscript.

Additional material

Additional file 1

Table S1. Ligand/receptor combination for activin and related factors. The table provided represents the ligand/receptor combination for activins, inhibins, myostatin, GDF11 and nodal.
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Additional file 2

Table S2. Activin signaling as a target for therapeutic interventions. The table provided represents activin signaling as a target for therapeutic interventions and lists the disease, therapeutic strategy, methods and references.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1478-811X-7-15-S2.pdf]

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

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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.

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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

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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/

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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.

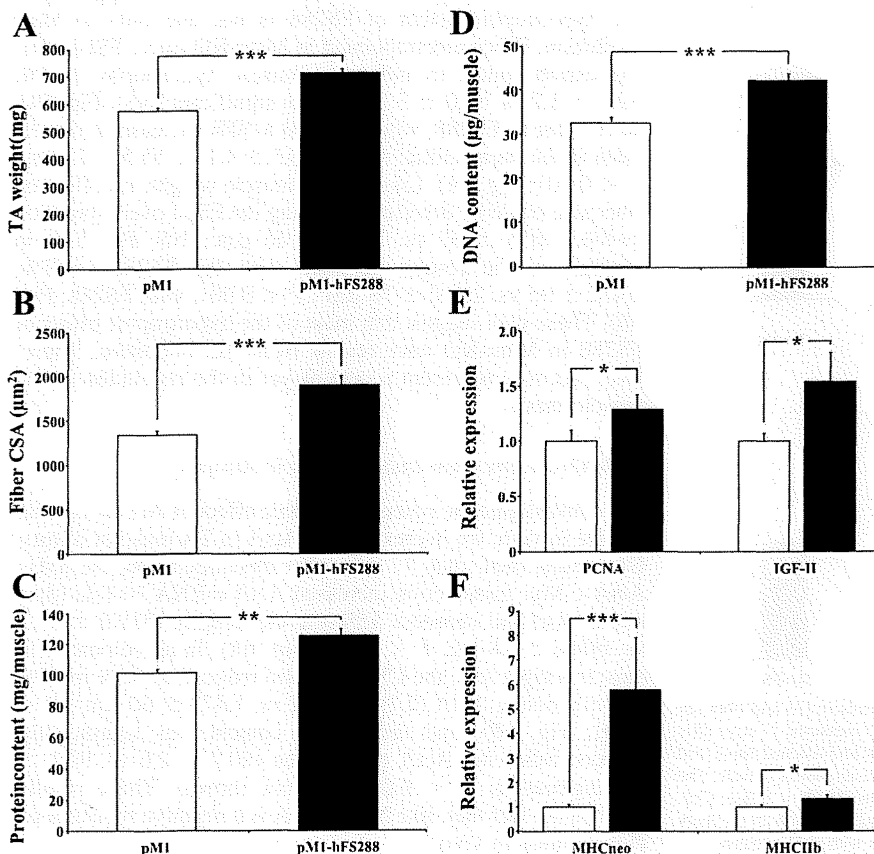

Fig. 1. Overexpression of follistatin (FS) in tibialis anterior (TA) muscle induces muscle (*A*) and fiber (*B*) hypertrophy in rat. It also increases the protein (*C*) and DNA (*D*) contents and stimulates the expression of PCNA and IGF-II (*E*) as well as myosin heavy chain (MHC) [neonatal (MHCneo) 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.

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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).

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.

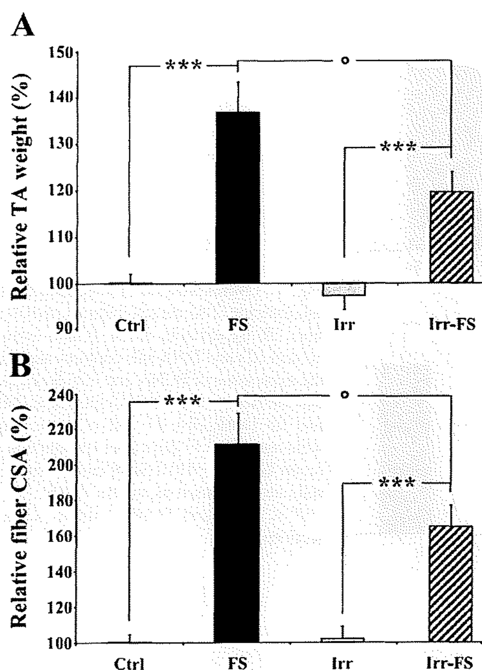

Fig. 2. γ-Irradiation partially blunts the FS-induced muscle (*A*) and fiber (*B*) hypertrophy in mice. TA mass and the fiber CSA were measured 17 days after transfection of pM1 (open bars) or pM1-FS288 (black and hatched bars), and the data are normalized as percentages of the contralateral control 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.

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.

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