Activin-A and Myostatin Response and Steroid Regulation in Human Myometrium: Disruption of Their Signalling in Uterine Fibroid

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Context: Investigation of activin-A (A) and myostatin (M) in human myometrium (HM) and leiomyoma (HL) will explain their involvement in human myometrial pathophysiology.

Objective: We aimed to investigate A and M response and steroid regulation in HM. We also evaluated A and M expression and response in HL.

Design: Tissues were analyzed and cultured.

Patients: Patients included fertile (in proliferative phase) and menopausal women undergoing hysterectomy.

Interventions: HM explant cultures were treated with A and M (for Smad-7 mRNA quantification) or estrogen and progesterone (for A and M mRNA quantification). A and M expression levels were also evaluated in menopausal (physiological absence of steroids) HM specimens. A and M and their receptors were evaluated in HL (n = 8, diameter 5–8 cm) compared with their matched HM. HL explants cultures were treated with A and M (for Smad7 mRNA quantification), and, to explain the absence of response, the levels of follistatin, follistatin-related gene (FLRG), and Cripto were evaluated.

Results: A and M increased Smad7 expression in HM explants. A and M mRNAs were both reduced after estradiol treatment, unchanged after progesterone treatment, but were higher in menopausal than fertile (in proliferative phase) specimens. A, M, and FLRG were expressed at higher levels in HL compared with adjacent HM, whereas the receptors, follistatin, and Smad7 mRNAs resulted unchanged. Cripto mRNA was expressed only in HL.

Conclusions: A and M act on human HM and are regulated by steroids. In HL there is an increase of A, M, FLRG, and Cripto expression. **(*J Clin Endocrinol Metab* 96: 755–765, 2011)**

Activin and myostatin are important growth factors belonging to the TGF-β family and represent key regulators of cell growth and differentiation. Activins were first identified by virtue of their ability to regulate FSH secretion from the anterior pituitary. Activins are also powerful regulators of gonadal functions and are involved in fibrosis, inflammation, and neurogenesis (1). Myostatin, also called growth differentiation factor (GDF)-8, is specifically expressed in the skeletal muscle lineage and is a candidate muscle chalone negatively regulating the growth of myoblasts (2).

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2010-0501 Received March 1, 2010. Accepted November 8, 2010.
First Published Online December 22, 2010
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Abbreviations: ActR, Activin receptor; ALK, activin receptor-like kinase; EGF, epidermal growth factor; FLRG, follistatin-related gene; FST, follistatin; GDF, growth differentiation factor; RT, reverse transcription.

Activin and myostatin share similar receptors and intracellular signaling. Activin initiates signaling by binding two types of transmembrane serine/threonine receptor kinases classified as type II [activin receptor (ActR) type II or ActRIIB] and type I [ActRIB; activin receptor-like kinase (ALK)-4]. ActRII binds activin with high affinity and facilitates activin binding to ALK4 (3). In the receptor complex, the constitutively active type II receptor kinase phosphorylates ALK4, and this phosphorylation leads to activation of the ALK4 kinase (4). Once activated, ALK4 phosphorylates Smad2 and Smad3, which form part of the postreceptor signal transduction system (5). Myostatin, like activin, stimulates target cells by assembling a cell surface receptor complex containing type I and II receptors. Myostatin binds the type II Ser/Thr kinase receptor, ActRIIB, and then partners with a type I receptor, either ALK4 (or ActRIB) or ALK5 (TGF-β receptor I). These complexes induce phosphorylation of Smad2 and Smad3 and activate an activin/TGF-β-like signaling pathway (6). Smad7 functions as an inhibitory downstream modulator of activin/TGF-β-like signaling, and its expression is induced by both activin (7) and myostatin (8).

Extracellular activin-binding proteins control activin signaling. Follistatin (FST) is a prototype of activin-binding proteins that binds and inhibits both activins and myostatin (9). The follistatin-related gene (*FLRG*) is a follistatin domain-containing protein structurally similar to FST (10, 11). Whereas FST has three follistatin domains, FLRG has only two. Like FST, FLRG binds and neutralizes activins and also myostatin (9, 11).

Cripto is an epidermal growth factor (EGF)-Cripto, FGF receptor-like-1, Cryptic protein that was first isolated as a putative oncogene from human teratocarcinoma cell line (12). Cripto modulates the signaling of multiple TGF-β family members, and we have previously shown that Cripto can antagonize TGF-β and activin signaling (13–15).

We recently demonstrated that activin-A and myostatin act on uterus and on myometrial cells. Specifically we found that myometrial cells are activin-A and myostatin sensitive, that activin-A and myostatin can regulate myometrial cell proliferation, and that their expression levels are modulated *in vivo* in rats during the estrous cycle and in response to steroid deprivation and replacement (16, 17).

Because no data are available on human tissues, in the present paper, we investigated whether human myometrial specimens are responsive to activin and myostatin and tested the hypothesis brought up recently in animal models that their expression is regulated by steroid hormones (16).

Myometrial mass is greatly modified in tumoral conditions including malignant leiomyosarcoma and benign leiomyoma. Uterine leiomyoma, or fibroid, is the most common benign neoplasia in women, one of the most frequent causes of infertility in reproductive years, and the leading cause for hysterectomy. The pathophysiology of uterine leiomyomas is uncertain. Therefore, therapeutic approaches have been primarily empirical. However, recently the presence of growth substances in uterine tissues suggested that the role of sex steroid hormones in the pathophysiology of leiomyomas may be mediated by substances influencing the proliferation of smooth muscle cells and fibroblasts (18, 19).

Using the Eker rat model, an animal model extensively characterized for uterine leiomyoma studies that carries a mutation in the *Tsc2* gene (20), Laping *et al.* (21) demonstrated that the type I TGF-β receptor kinase inhibitor, SB-525334, is able to block TGF-β signaling in uterine leiomyoma cells. Specifically, SB-525334 significantly decreased tumor incidence and multiplicity and reduced the size of these mesenchymal tumors (leiomyoma).

Many microarray studies have analyzed differential gene expression in uterine fibroids, and a very large number of genes including TGF-β family components have been reported to either down- or up-regulated. Whereas the microarray analyses remain to be confirmed/validated using real-time PCR and whereas there is considerable variation and lack of overlap between studies, the apparent disparities in gene profiles cannot be fully explained by methodological differences between studies and may be attributable in part to genetic variability between patients, differences in the hormonal milieu, or tumors size (22–24).

Our previous data indicate that activin and myostatin may represent important factors regulating human myometrial pathophysiology (16, 17). Therefore, in the present paper, we aimed also to evaluate their expression in paired myometrium/leiomyoma and their activity in uterine fibroids.

Patients and Methods

Sample collection

All patients gave their informed consent and the permission of the Human Investigation Committee was granted. Samples of fibroid and adjacent normal myometrium were excised from women undergoing hysterectomy for fibroids. Fibroid tissue was defined based on the well-established histopathological criteria. Considering the high variability that could occur with different age, race, hormonal milieu, tumor size, and location of tumors, we included in the study the most homogeneous sample possible. All patients (n = 8) were Caucasian (age range 41–49 yr) and in proliferative phase of the menstrual cycle. The location of the leiomyomas was intramural, and their size range was 7–10 cm in diameter.

Histological evaluation of the endometrium and the patient's last menstrual period were used to determine the phase of the menstrual cycle.

In Fig. 1, as well as in Fig. 2, A–D, the patients were in proliferative phase of the menstrual cycle. For the results shown in Fig. 2,

E and F the patients were in menopause (age range 60–70 yr), while for all the following results (Figs. 3–6), the patients were not in menopause. Menopausal status was confirmed by serum FSH levels greater than 40 mIU/ml and serum estradiol less than 30 pg/ml.

Tissue samples were taken only from women who had not received exogenous hormones for the previous 3 months.

Myometrial short-term tissue culture

All myometrial samples were placed into Hanks' balanced salt solution (Euroclone, Milan, Italy) immediately after surgery and then washed several times in Dulbecco's PBS (Invitrogen, Paisley, UK) to remove the excess blood. The myometrium was then cut into small explants (10–15 mg). Myometrial explants were placed in every well of a 24-well plate, each of which contained 1 ml of the culture medium (Dulbecco's serum-free medium supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin; Invitrogen). After stabilization at 37 C for 45 min in a humidified atmosphere containing 95% O_2-5% CO_2, the culture medium was changed and tissue explants were incubated with activin (4 nM), myostatin (4 nM), estrogen (10^{-7} to 10^{-5} M), or progesterone (10^{-7} to 10^{-5} M).

Materials

Recombinant human activin-A was generated using a stable activin-expressing cell line generously provided by Dr. J. Mather (Genentech, Inc., South San Francisco, CA) and was purified by Wolfgang Fischer (Peptide Biology Laboratory, The Salk Institute, La Jolla, CA). Myostatin was produced as previously shown (16). Recombinant Cripto, follistatin, and FLRG antibodies were purchased from R&D Systems (Minneapolis, MN), whereas estrogen and progesterone was purchased by Sigma-Aldrich (Steinheim, Germany). NuPAGE gels were purchased from Invitrogen.

RNA extraction

For reverse transcription (RT) experiments performed to investigate basal activin and myostatin related proteins, myometrial tissue was snap frozen in liquid nitrogen at the operating theater immediately after washing in Dulbecco's PBS and was stored at −80 C. Additionally, myometrial explants from tissue cultures were snap frozen in liquid nitrogen at the end of incubation and stored at −80 C.

Total RNA was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. Samples were digested with a ribonuclease-free deoxyribonuclease (Promega Corp., Madison, WI), and the RNA was cleaned up and concentrated using an RNeasy microkit (QIAGEN, Milan, Italy).

Quantitative RT-PCR

We performed the RT using the high-capacity cDNA RT kit (Applied Biosystems, Foster City, CA) with 1 μg RNA, and we performed the TaqMan real-time PCR for all the genes analyzed. We used the TaqMan gene expression assays (Applied Biosystems) reported in Table 1, performing the following thermal cycle protocol (initial denaturation at 95 C for 20 sec, followed by 40 cycles of 95 C for 1 sec and 60 C for 20 sec) using 100 ng cDNA in a final reaction volume of 20 μl. The blank for each reaction, consisting of amplifications performed in the absences of RT enzyme, was performed. All results were normalized against either β-actin and hypoxanthine-guanine phosphoribosyl transferase expression to correct for differences in concentration of the starting template.

Western blotting

Tissues were flash frozen on dry ice, stored at −20 C, until crushed while frozen, and suspended in ice-cold radioimmunoprecipitation assay buffer [50 mm Tris-HCl (pH 7.4), 150 mm NaCl, 1 mm EDTA, 1% Igepal CA-630, 0.25% Na-deoxycholate] supplemented with protease inhibitors (set III; Calbiochem, San Diego, CA). Soluble protein was quantified using a Bradford protein assay (Bio-Rad, Richmond, CA), and equal amounts of proteins were loaded onto 4–12% NuPAGE gels (Invitrogen) and resolved by SDS-PAGE. Proteins were transferred to 0.2-μm nitrocellulose membranes in an X-cell II apparatus (Invitrogen) according to the manufacturer's instructions. After blocking of the membrane with 5% (wt/vol) nonfat milk powder in Tris-buffered saline with Tween 20 [50 mm Tris-HCl (pH 7.4), 150 mm NaCl, 0.05% Tween 20], membranes were incubated overnight with 1:1000 dilutions of primary antibodies. Membranes were washed in Tris-buffered saline with Tween 20 and incubated with 1:10,000 dilutions of horseradish peroxidase-conjugated antigoat IgG (Pierce, Rockford, IL) for 2 h. Immunoreactive proteins were visualized using Super Signal West Pico chemiluminescent substrate (Pierce). Protein levels were assessed by densitometric analysis using Chemidoc and the Quantity-One program (Bio-Rad Laboratories, Milan, Italy).

Luciferase assays

Luciferase assays were carried out using an A3-luciferase construct containing three copies of the activin response element from the *Xenopus laevis Mix.2* promoter linked to a basic TATA box and a luciferase reporter gene. Human embryonic kidney 293T cells were plated on poly-L-lysine-coated 24-well plates at 1×10^5 cells/well and transfected (Perfectin, Genlantis, San Diego, CA) in triplicate less than 24 h later with 500 ng of DNA/well: 400 ng of either mouse Cripto in pcDNA3.0 or empty vector together with 50 ng FAST2 (FoxH1), 25 ng of A3-luciferase, and 25 ng of cytomegalovirus-β-galactosidase. Cells were treated less than 24 h after the transfection and then harvested less than 16 h after treatment. Cells were incubated in solubilization buffer [1% Triton X-100, 25 mm glycylglycine (pH 7.8), 15 mm $MgSO_4$, 4 mm EGTA, and 1 mm dithiothreitol] for 30 min on ice, and luciferase reporter activity was measured and normalized relative to cytomegalovirus-β-galactosidase activities.

Data analysis

Data are presented as the mean ± SEM, and a two-tailed *t* test was used for data analysis. Differences were considered significant when $P < 0.05$. Linear correlations were calculated by nonparametric correlation (Spearman) performed using GraphPad Prism version 4.01 for Windows (GraphPad, San Diego, CA). All experiments were done in triplicate and repeated either two or three times.

Results

Activin-A and myostatin responsiveness of myometrial tissues

To determine whether human myometrium is activin and myostatin responsive, we first tested whether myometrium explants exhibit detectable Smad signaling on

TABLE 1. TaqMan gene expression assays (Applied Biosystems) used to perform the real-time PCR

Gene name	Alias	Gene symbol	Reference sequence	Assay ID	Amplicon length
Inhibin, βA	EDF FRP	INHBA	NM_002192.2	Hs00170103_m1	61
Follistatin-like 3 (secreted glycoprotein)	FLRG FSRP	FSTL3	NM_005860.2	Hs00610505_m1	84
Actin, β	PS1TP5BP1	ACTB	NM_001101.3	Hs99999903_m1	171
Myostatin	GDF8	MSTN	NM_005259.2	Hs00193363_m1	118
Activin-A receptor, type IIA	ACTRII ACVR2	ACVR2A	NM_001616.3	Hs01012007_m1	110
Activin-A receptor, type IB	ACTRIB ACVRLK4 ALK4 SKR2	AcvR1B	NM_004302.3	Hs00923299_m1	74
TGF, β receptor 1	AAT5 ACVRLK4 ALK-5 ALK5 LDS1A LDS2A SKR4 TGFR-1	TGFBR1	NM_004612.2	Hs00610318_m1	92
Follistatin	FS	FST	NM_006350.2	Hs00246260_m1	105
Hypoxanthine phosphoribosyltransferase 1	HGPRT HPRT	HPRT1	NM_000194.2	Hs99999909_m1	100
Activin-A receptor, type IIB	ACTRIIB ActR-IIB MGC116908	ACVR2B	NM_001106.3	Hs00609603_m1	101
Cripto, FGF receptor-like-1, cryptic family 1	CFC1	CRYPTIC FLJ77897 HTX2 MGC133213	NM_032545.2	Hs00414425_m1	90
SMAD family member 7	SMAD7	CRCS3 FLJ16482 MADH7 MADH8	NM_005904.2	Hs00178696_m1	61

activin-A and myostatin treatment. Myometrial explants were treated with vehicle or 4 nM of either activin-A or myostatin for 2 h, and the expression levels of Smad2/3 targets such as Smad7 were evaluated. As shown in Fig. 1, both activin-A (Fig. 1A) and myostatin (Fig. 1B) were able to increase Smad7 expression in human myometrial explants, demonstrating that these explants did respond to activin-A and myostatin.

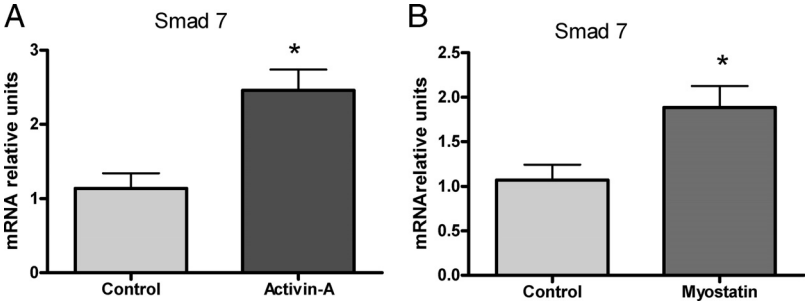

FIG. 1. Representative results of real-time PCR for the relative amounts of Smad7 mRNA in myometrial explants in response to 4 nM activin-A (A) and in response to 4 nM myostatin (B) treatment for 2 h. Control, Left untreated. *, $P < 0.05$.

Regulation of activin-A and myostatin mRNA expression by sexual steroids in human myometrium

To determine whether activin-A and myostatin expression are regulated by steroids in human myometrium, we treated human myometrial explants with estradiol (10^{-7}, 10^{-6}, and 10^{-5} M) and progesterone (10^{-7}, 10^{-6}, and 10^{-5} M). As shown in Fig. 2, both activin-A (Fig. 2A) and myostatin (Fig. 2B) mRNA levels were reduced after 3 h estradiol treatment, and treatment for 24 h gave similar results (data not shown). By contrast, treatment of myometrial explants for 3 h (Fig. 2, C and D) and 24 h with progesterone did not result in significant variation of activin-A (Fig. 2C) or myostatin (Fig. 2D) mRNA expression.

To further explore the effects of sexual steroids on activin and myostatin expression in physiological conditions, we evaluated activin and myostatin mRNA expression levels in menopausal myometrial

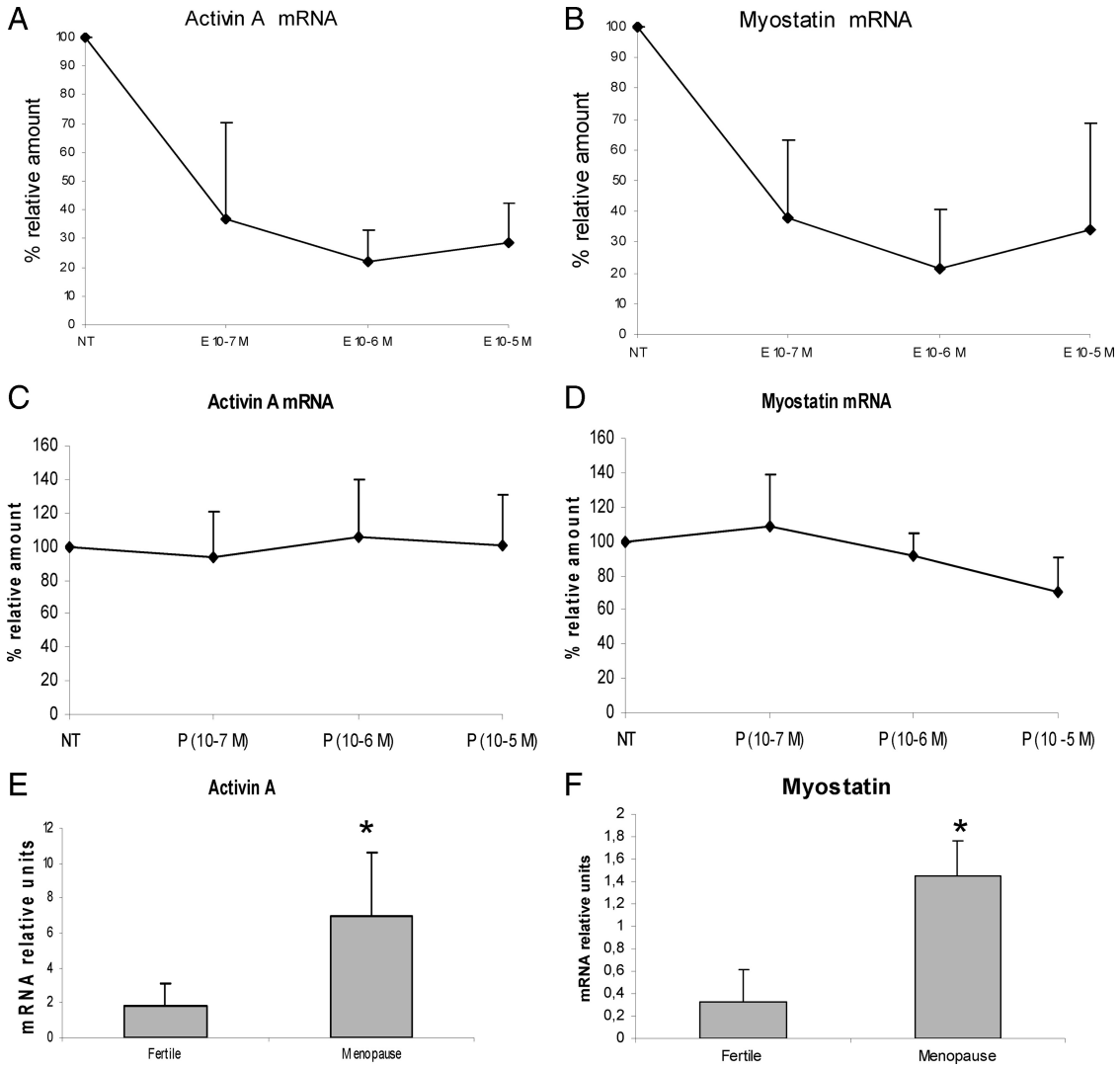

FIG. 2. Regulation of activin-A and myostatin mRNA expression by steroids in human myometrium. Activin-A (A) and myostatin (B) mRNA percentage relative amount in explants treated with estradiol (E) were compared with untreated samples. Activin-A (C) and myostatin (D) mRNA percentage relative amount in explants treated with progesterone (P) compared with untreated samples. Activin-A (E) and myostatin (F) mRNA expression levels in myometrium from fertile were compared with menopausal women. n = 5. *, $P < 0.05$.

specimens compared with myometrial specimens from fertile (proliferative) women. As shown in Fig. 2, E and F, under physiological conditions in which sex steroids are absent, the expression levels of activin-A (Fig. 2 E) and myostatin (Fig. 2 F) are both higher.

Activin-A and myostatin expression in leiomyoma

Because our data show that activin-A and myostatin act on myometrium and are regulated by sexual steroids, we hypothesized that they may represent gene candidates responsible for pathologies that are very frequent in women such as leiomyoma. To test this, we evaluated the expression levels of activin-A and myostatin in fibroid compared with matched myometrium specimens. We found that activin-A (Fig. 3A) and myostatin (Fig. 3B) mRNA levels are higher expressed in fibroid specimens compared with adjacent myometrium.

Expression levels of activin-A and myostatin receptors and Smad 7 in leiomyoma

Next we tested whether the higher levels of activin-A and myostatin expression found in leiomyoma corresponded to a higher expression of the activin/myostatin signaling components. We evaluated the expression levels of all the receptors (Fig. 3, C–F) and the basal expression levels of Smad 7 (Fig. 4A) and, as shown in Figs. 3, C–F (columns I and II) and 4A, we found that all the receptors and Smad 7 mRNA levels were not different in leiomyoma compared with matched healthy myometrium. Column III (Fig. 3) shows the correlation of activin-A levels with receptors levels, whereas column IV shows the correlation of myostatin levels with those of its receptors. The statistical analysis (nonparametric correlation, Spearman) revealed no correlation of both activin and myostatin levels with receptor levels.

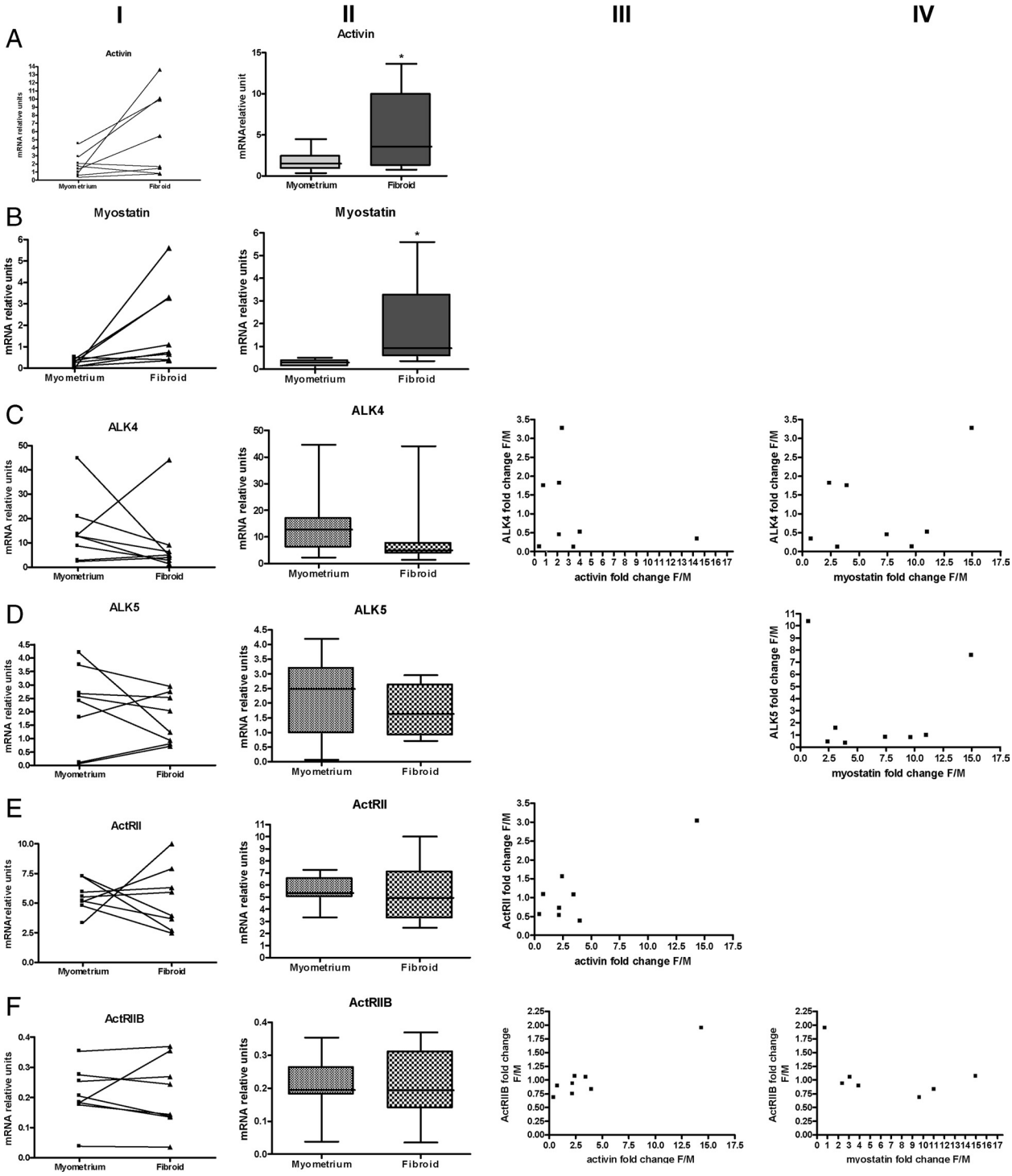

FIG. 3. A and B, Activin and myostatin levels of mRNA expression in eight paired fibroid and myometrial specimens. C–F, Levels of mRNA expression in eight paired fibroid and myometrial specimens for the four receptor genes ALK4, ALK5, ActRII, and ActRIIB. Graphs on column I are line graphs linking paired specimens, and those on column II are box-and-whisker plots. In column III is reported the correlation of activin-A with its receptors, whereas in column IV is reported the correlation of myostatin with its receptors. The nonparametric correlation (Spearman) revealed no correlation of both activin and myostatin levels with receptor levels. *, $P < 0.05$.

Activin-A and myostatin responsiveness of fibrotic tissues

Our result showing that Smad7 mRNA levels were not higher in leiomyoma than in normal myometrium (Fig. 4A) suggested to us that higher activin-A and myostatin expression levels did not correspond a higher activin-A and myostatin actions in leiomyoma. To test this, we treated leiomyoma explants for 2 h with activin-A or myostatin and then measured resulting effects on Smad7 expression. As shown in Fig. 4B, neither activin-A (Fig. 4A)

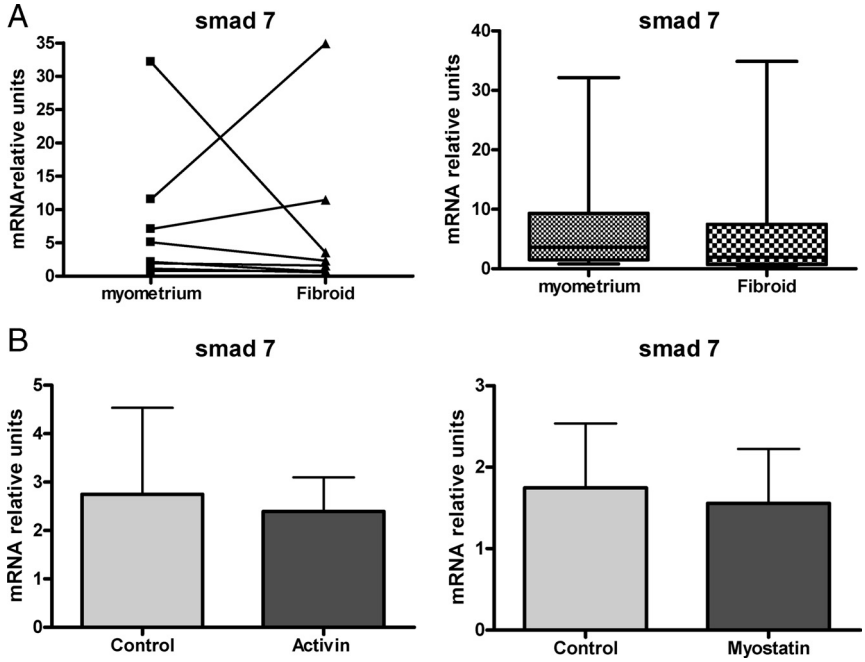

FIG. 4. A, Smad7 levels of mRNA expression in eight paired fibroid and myometrial specimens. Graphs on the *left* are line graphs linking paired specimens, and those on the *right* are box-and-whisker plots. B, Representative results of real-time PCR for the relative amounts of Smad7 mRNA in fibroid explants in response to 4 nM activin-A and in response to 4 nM myostatin treatment for 2 h. Control, Left untreated.

nor myostatin (Fig. 4B) were able to increase Smad7 expression, demonstrating that, unlike normal myometrium (Fig. 1), leiomyoma explants do not respond to these ligands. We had the same result with 4 and 10 nM of protein concentration.

Activin-A and myostatin binding proteins follistatin and FLRG expression levels in leiomyoma

To explain the previous finding, we hypothesized that one or more inhibitors of activin and myostatin may display an altered expression that could interfere with them by disrupting their signaling. We tested follistatin and FLRG mRNA expression, and we found that although follistatin did not change, FLRG expression was higher in leiomyoma compared with matched healthy myometrium (Fig. 5A). As shown in Fig. 5B, activin and myostatin evaluated as ratio with follistatin were still higher in leiomyoma compared with matched healthy myometrium. On the other hand, activin and myostatin evaluated as ratio with FLRG were not significantly higher in leiomyoma compared with matched healthy myometrium. To better understand the role of follistatin and FLRG in blocking activin and myostatin, we also evaluated their respective protein levels. As shown in Fig. 5C, FLRG protein was increased in the leiomyoma compared with matched healthy myometrium, with a pattern similar to the RNA, whereas follistatin protein resulted unchanged.

Identification of Cripto expression in leiomyoma

Because we previously identified Cripto as factor that is able to antagonize activin (13, 14), we also tested its expression in the leiomyoma. As shown in Fig. 6A, Cripto was expressed in almost all the fibrotic specimens examined but was not detected in healthy myometrium.

Cripto inhibits myostatin signaling

We have previously shown that Cripto inhibits activin and TGF-β signaling (15). Because myostatin uses the activin/TGF-β signaling pathway, we tested whether Cripto also functions as an antagonist of myostatin signaling. We transfected 293T cells with a Smad2-responsive luciferase reporter in the presence of empty vector or Cripto and then treated cells with a range of doses of myostatin. As shown in Fig. 6A, myostatin induced Smad2-dependent luciferase expression in a dose-dependent manner, and this myostatin response was inhibited by Cripto. The inhibitory effect of Cripto on myostatin signaling appeared to be similar to Cripto antagonism of activin signaling (15). This suggests that Cripto blocks both ligands via a similar mechanism and is consistent with the ability of Cripto to bind the activin/myostatin type I receptor ALK4.

Discussion

In the present paper, we provide the first evidence that human myometrial tissues are able to respond to activin and myostatin as measured by their induction of Smad7. This finding is consistent with what we have seen before in a myometrial cell line, PHM1, and in rat uterine explants (16, 17). In these previous studies, we also reported that the expression of activin and myostatin in rat uterine explants (endometrium + myometrium) is regulated by steroid hormones. Similar to what was observed in rat, progesterone did not change activin or myostatin expression in human uterine myometrium. By contrast, although estrogen treatment drastically decreased expression of activin and myostatin in human myometrium, it abrogated myostatin expression but increased expression of activin in rat uterus (endometrium + myometrium) (16). The reason for this discrepancy is not clear but may be due to

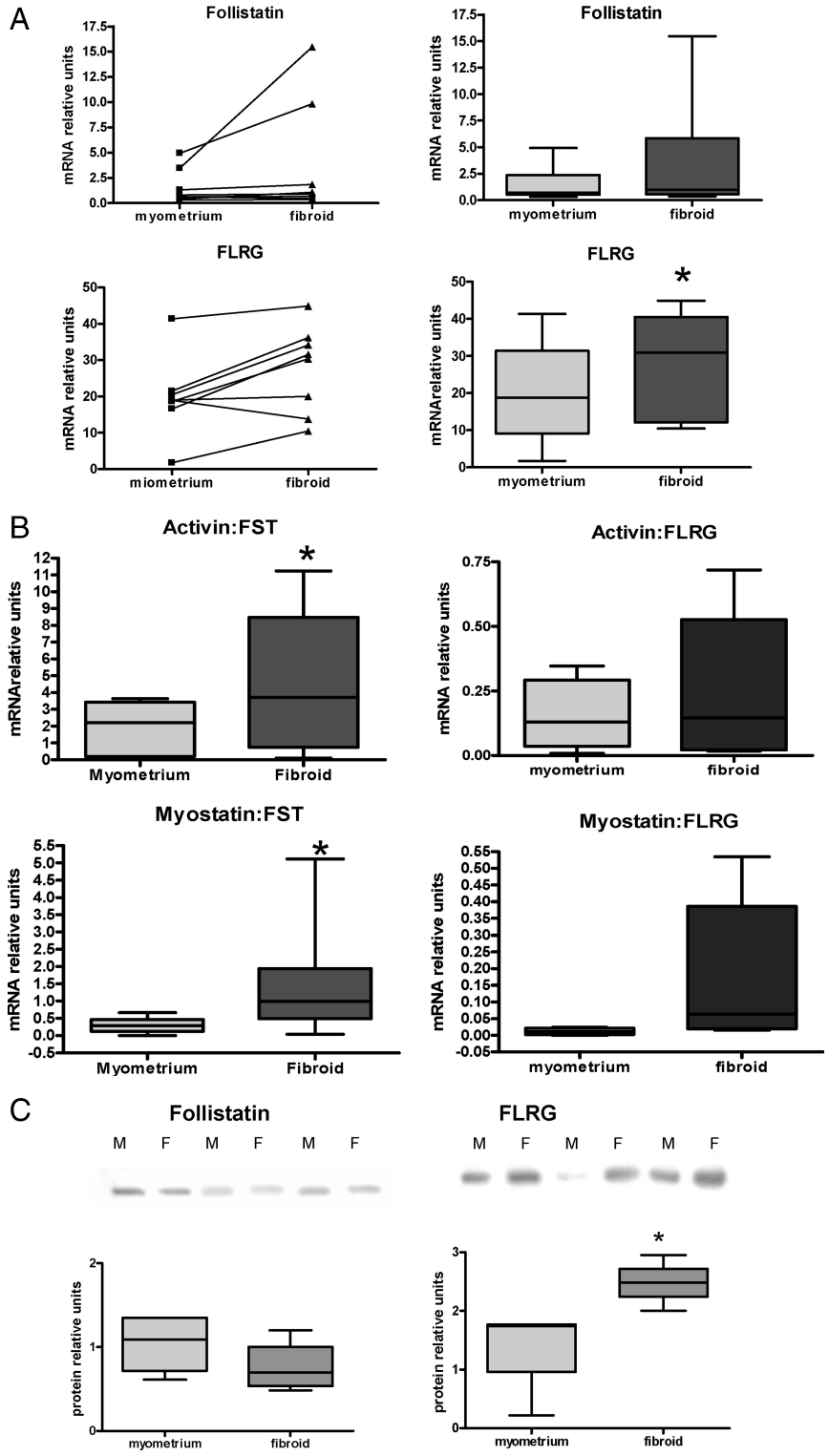

FIG. 5. A, Levels of mRNA expression in paired fibroid and myometrial specimens for the two binding proteins FST (n = 8) and FLRG (n = 6). Graphs on the *left* are line graphs linking paired specimens, and those on the *right* are box-and-whisker plots. B, Activin and myostatin mRNA levels normalized with FST (on *left*) and FLRG (on *right*) in paired fibroid and myometrial specimens. C, Western blotting and densitometric analisys of FST, FLRG, and proteins. M, Myometrium; F, fibroid. *, $P < 0.05$.

species-specific and/or tissue-specific regulatory mechanisms and/or instead methods specific (*in vivo vs.* explants culture). Of note, we found increased activin and myostatin expression in menopausal tissues in the absence of steroids providing further evidence that estrogen acts to suppress the levels of both these ligands in humans.

After having established that activin and myostatin target human myometrium explants and are regulated by steroid hormones, we evaluated their relative expression levels in fibroid specimens and adjacent healthy myometrium. Considering the high variability that has been reported for many factors, including levels of estrogen and progesterone receptors (25, 26) and gene profiles (23) between patients, we chose to use restricted inclusion criteria to keep group mean variation to a minimum (same race, menstrual phase, size and location of the tumor). Our finding that activin-A and myostatin expression levels were higher in leiomyoma than in normal myometrium was unexpected because these growth factors have cytostatic effects on the human myometrial PHM1 cell line (16, 17). On the other hand, basal Smad7 expression levels and the expression levels of all the receptors did not change between normal and fibroid tissue. We tested whether activin-A and myostatin target the fibrotic tissue, and, not surprisingly, neither protein was able to increase Smad7 expression in leiomyoma specimens. We hypothesized that the inability of fibroid to respond to activin and myostatin was due to the presence of signaling inhibitors, *i.e.* higher expression of FLRG in fibroid specimens compared with adjacent healthy myometrium and the selective expression of Cripto in leiomyoma.

When activin and myostatin mRNA levels were corrected relative to follistatin mRNA levels, we still observed significantly higher expression of their mRNAs in leiomyoma compared with healthy adjacent myometrium. By contrast, FLRG expression differences between normal tissue and leiomyoma were sufficient to explain loss of signaling in leiomyoma. Furthermore, evaluating activin and myostatin mRNA expression levels corrected to FLRG mRNA levels showed

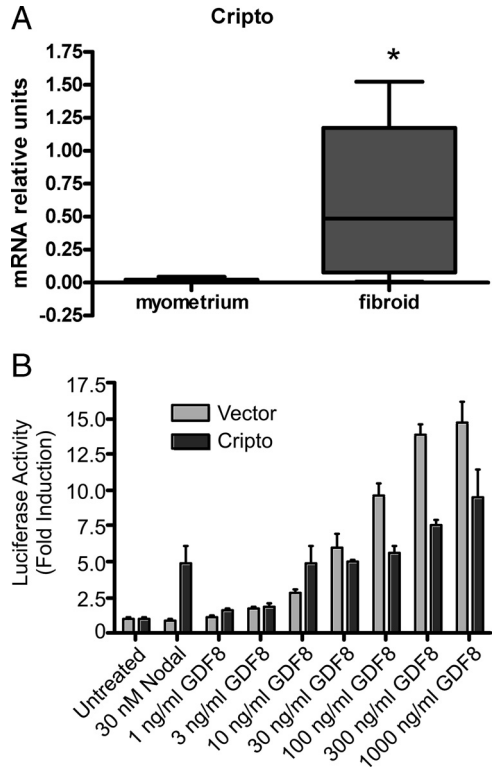

FIG. 6. A, Identification of mRNA Cripto expression in leiomyoma by real-time PCR. B, Cripto disruption of myostatin (GDF8) signaling. In the x-axis is reported the treatment and the doses used for the luciferase assay. *, $P < 0.05$.

that their relative amounts were not different between the healthy and pathological specimens.

Similar to previous studies, our experimental data support the concept that follistatin and FLRG are functionally redundant despite differences in their expression and transcriptional regulation (27). Indeed, we have observed this phenomenon in other reproductive systems, such as placenta (28), endometrium (29, 30), and breast (31).

Activin and myostatin are members of the TGF-β superfamily and the presence of TGF-βs and their receptors in human myometrium and leiomyoma was first shown in 1994 by Chegini *et al.* (32). The same authors later also showed that GnRH-α decreased expression of TFG-β receptors and TGF-β receptor intracellular signaling molecules (33).

Cripto is a GPI-anchored signaling protein that controls the activity of multiple TGF-β superfamily members (34). Cripto binds directly to the activin/myostatin type I receptor ALK4 (35) and inhibits activin signaling (13, 14). This led us to hypothesize that Cripto may also function as an inhibitor of myostatin signaling. Indeed, we have now shown that Cripto causes a reduction in myostatin signaling similar to that previously shown for activin. Cripto is predominantly expressed during normal developmental processes and in cancer. Cripto is expressed at high levels in human breast, colon, stomach, pancreas, lung, ovary endometrial, testis, bladder, prostate, and myometrial tumors but is absent or expressed at lower levels in their normal counterparts (34, 36).

Recently leiomyosarcoma of the uterus was observed in Cripto-overexpressing transgenic mice (36). Regarding human myometrium, Cripto has been reported to be expressed, by immunohistochemistry, in leiomyosarcoma and only in very few samples of leiomyoma (36). Here, using real-time PCR, we were also able to show the presence of Cripto mRNA in leiomyoma, suggesting that it may be a contributing cause of benign tumor transformation.

The data presented in this paper support the hypothesis that alterations in the activin- and myostatin-related protein systems may produce loss of sensitivity to the antiproliferative effects of activin and myostatin and that increased expression of FLRG and Cripto may contribute to the growth of these tumors. Therefore, activin and myostatin need to be considered as factors involved in myometrial functionality in addition to the other growth factors already known to have this function such as EGF, heparin-binding EGF-like growth factor, platelet-derived growth factor (37), IGF (38), vascular endothelial growth factor, and TGF-β (39).

Regarding activin-A and myostatin, we evaluated their expression and tested their ability to act on fibrotic tissues. Although activin-A and myostatin are overexpressed in leiomyoma, they are unable to induce Smad7, likely due to overexpression of inhibitors including FLRG and/or Cripto.

Thus, it is possible that in fibrotic myometrium there is an initial activation of Cripto expression and an increased FLRG expression that render activin and myostatin less active. The reduction of activin and myostatin signaling may then cause a compensatory increase in their expression.

In conclusion, our findings suggest that activin-A and myostatin act on human myometrium, that they are regulated by steroid hormones, and that the disruption of their signaling may contribute to the fibroid growth.

Acknowledgments

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This work was supported by the Grants4Targets initiative from Bayer Schering Pharma (to P.Ci.) and the "Fondazione Cassa di Risparmio di Fabriano e Cupramontana" (to M.C.). This work was also supported by Award 2R56 CA107420-06 from the National Cancer Institute (to P.C.G.). This work was

also supported in part by the Clayton Medical Research Foundation, Inc. (to W.V. and P.C.G.). W.V. is a CMRF Senior Investigator.

The content of this work is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health.

Disclosure Summary: W.V. is a cofounder, consultant, equity holder, and member of the Board of Directors of Neurocrine Biosciences and Acceleron Pharma. All the other authors have nothing to declare.

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