Activin Regulates Estrogen Receptor Gene Expression in the Mouse Ovary*

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Activin, a member of the transforming growth factor-β superfamily, is an important modulator of follicle-stimulating hormone synthesis and secretion in the pituitary and plays autocrine/paracrine roles in the regulation of ovarian follicle development. From a microarray study on mouse ovarian granulosa cells, we discovered that the estrogen receptor β (ERβ) is inducible by activin. We previously demonstrated that estrogen suppresses activin gene expression, suggesting a feedback relationship between these two follicle-regulating hormones. The purpose of this study was to investigate fully activin A regulation of ER expression. Real time reverse transcription-PCR assays on cultured granulosa cells showed that both ERα and ERβ mRNAs were induced by activin A at 4, 12, and 24 h in a dose-responsive manner. Western blots confirmed an increase in their protein levels. Consistent with increased ERα and ERβ expression, activin A stimulated estradiol-induced estrogen response element promoter activity. Activin A stimulation of ER expression was a direct effect at the level of gene transcription, as it was not abolished by cycloheximide but was abolished by actinomycin D, and in transfected granulosa cells activin A stimulated ERα promoter activity. To investigate the effect of activin in vivo and, thus, its biological significance, we examined ER expression in inhibin transgenic mice that have decreased activin expression and discovered that these mice had decreased ERα and ERβ expression in the ovary. We also found that ER mRNA levels were decreased in Müllerian inhibiting substance promoter (MIS–Smad2) dominant negative mice that have impaired activin signaling through Smad2, and small interfering RNAs targeting Smad2 or Smad3 suppressed ERα promoter activation, suggesting that Smad2 and Smad3 are involved in regulating ER levels. Therefore, this study reveals an important role for activin in inducing the expression of ERs in the mouse ovary and suggests important interplay between activin and estrogen signaling.

Activin and its functional antagonist inhibin were originally isolated from gonadal sources as endocrine factors regulating the synthesis and secretion of follicle-stimulating hormone by the pituitary gland (1–8). More recent studies have indicated that activin acts predominantly as a local paracrine and autocrine factor (9, 10). Consistent with the fact that activin is a member of the transforming growth factor-β (TGF-β) superfamily, activin has a variety of functions and is involved in many physiological processes, including embryonic development, wound repair, inflammation, renal tubule morphogenesis, and neuroprotection. In the reproductive system, in addition to regulating gonadotropin release, activin and inhibin have been shown to play an important role in regulating the formation and development of ovarian follicles in the female (10–15) and development and function of the testis in the male (16). Ovarian follicle formation and development is a dynamic process finely regulated by various intrinsic and endocrine factors, and it involves interactions between multiple cell types within the ovary. It is not well understood how activin may interplay with other ovarian factors to maintain ovarian homeostasis and regulate proper development.

Activin and inhibin share structural features and impact a common signaling pathway. Activins are dimers of two shared β subunits, βA or βB, to form activin A (βAβA), activin B (βBβB), or activin AB (βAβB) (4–7). Inhibin is a heterodimer of a unique inhibin α subunit with either of two shared β subunits, βA or βB, to form inhibin A or inhibin B, respectively (4–7). Activin signals through a receptor serine-threonine kinase/Smad protein pathway, which involves activin binding receptors (ACTR IIA and ACTR IIB), a signaling receptor (ACTR IIB), signaling co-activators (Smads 2, 3, and 4), and an inhibitor (Smad7). All three inhibin/activin subunit mRNAs are expressed in the ovary and are localized predominantly to granulosa cells (17–20). In addition to the inhibin and activin ligands, most components of their signaling systems are expressed in the rodent ovarian follicle both in the oocyte and somatic cells (12, 21–23).

The steroid hormone estrogen is also produced by the ovary. In addition to its functions in many extragonadal tissues, similar to activin, estrogen also plays an intraovarian role in regulating follicle development and function (for reviews, see Refs. 24–26). In an estrogen-deficient mouse model, the aromatase knock-out mouse, there is a blockage of follicle development at

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the antral stage, absence of corpora lutea, as well as a decrease in primordial and primary follicle numbers (27, 28).

Estrogen signals through estrogen receptors (ERs), which are members of the nuclear receptor family. Two major forms of ERs have been identified, ERα (29–33) and ERβ (34–36). Both ERα and ERβ are expressed in the mouse or rat ovary, where ERβ is the most abundant form of ER and is expressed predominantly in the granulosa cells, whereas ERα is expressed mostly in the theca cells (37, 38). Estrogen receptor α, β, or compound knock-out mice show various defects in follicle development and/or ovulation (39–41).

Estrogen and activin both play a role in the early rodent ovary. During follicle formation and development (the first few days after birth in mice), ERs and activin subunits are both expressed, whereas other members of the TGF-β superfamily, inhibin, or follicle-stimulating hormone receptors are not readily detectable until after primary follicles have formed (38, 42–44), suggesting potential functional interactions and regulation between ER and activin in the early ovary.

In an effort to examine the effects of activin on ovarian gene expression, we performed a microarray study, and ERβ was identified as a gene that is significantly up-regulated. This is particularly interesting as we have shown earlier that estrogen can negatively regulate activin subunit expression (45). Cross-talk between ER signaling and TGF-β can negatively regulate activin subunit expression (45). Cross-talk between ER signaling and TGF-β superfamily members has been reported in other systems (46–49). Therefore, this study is aimed at investigating further a novel role for activin in regulating ER expression. The results suggest an important interplay between activin and estrogen signaling in the mouse ovary.

EXPERIMENTAL PROCEDURES

Animals—CD-1 mice (Harlan, Indianapolis, In), MT-α inhibit transgenic mice, and MIS-Smad2 dominant negative mice, both on a CD-1 background, were maintained on a 12:12-h light/dark cycle (lights off at 17:00) with food and water available ad libitum. Breeders (90–180 days old) were fed with a soy-free mouse chow (Harlan 7926, Harlan, Indianapolis, IN) to limit exogenous phytoestrogen intake through food. At the time of delivery (day 1), 8 pups were kept with each female to minimize the possible difference in pup development caused by nutrient availability. Animals were cared for in accordance with all federal and institutional guidelines.

Primary Granulosa Cell Collection, Culture, and Treatment—Wild type or MIS-Smad2 dominant negative mice on a CD-1 background were sacrificed on postnatal days 22–23, and ovaries from 6–10 animals were pooled for granulosa cell collection. Granulosa cells were collected through follicle puncture as described previously by our laboratory in rats (50–54). Oocytes were filtered out with a 40-μm cell strainer (BD Falcon, Bedford, MA). Granulosa cells were either used directly for RNA isolation or cultured in a humidified incubator at 37 °C and 5% CO2 in a phenol red-free Dulbecco’s modified Eagle’s medium/F-12 medium (Invitrogen) supplemented with 2 μg/ml insulin, 5 nM sodium selenite, 5 μg/ml transferrin, 0.04 μg/ml hydrocortisone, 50 μg/ml sodium pyruvate, and 10% charcoal/dextran-treated fetal bovine serum (Hyclone, Logan, UT) for 3 days before treatments. Estrogen-free culture conditions were used to minimize any interference from this steroid as this study measures ER levels. All treatments were done in phenol red-free and serum-free Dulbecco’s modified Eagle’s medium-Ham’s F-12 medium to eliminate endogenous growth factors including activin. The treatments included 25, 50, 100, or 200 ng/ml activin A (equivalent to 0.96, 1.92, 3.84, and 7.96 nM, respectively; R&D Systems, Inc., Minneapolis, MN), 100 ng/ml activin A plus 400 ng/ml follistatin (produced in the Woodruff laboratory), 400 ng/ml follistatin, 100 ng/ml inhibin A (Diagnostic Systems Laboratories, Inc., Webster, TX), 100 μM TGF-β1 (R&D Systems, kindly provided by Dr. Boris Pasche from Northwestern University), 100 ng/ml BMP-2 (R&D Systems, Inc., Minneapolis, MN), or vehicle (PBS) for 1, 4, 12, or 24 h. The doses of treatments were based on reported studies (55–57). Cycloheximide or actinomycin D was used at a concentration of 4 μg/ml for 30 min before treatment with activin A.

Primary Granulosa Cell Transfection—Primary granulosa cells cultured in the above estrogen-deprived conditions were transiently transfected with an ERE promoter-luciferase reporter construct that contains two EREs (58) (kindly provided by Dr. Larry Jameson from Northwestern University). Transfection was performed with 250 ng of DNA per well of a 24-well culture plate using cationic liposomes in a phenol red-free Opti-MEM (Invitrogen) (59). After 12–16 h of transfection, fresh medium containing vehicle, 100 ng/ml activin A, 100 ng/ml activin A plus 400 ng/ml follistatin, or 400 ng/ml follistatin alone was given to the cells for 4–8 h followed by the addition of E2 (100 nM) or ethanol for another 24 h. Cell lysates were then collected for luciferase assays as well as for RNA isolation to measure ER expression levels.

RNA Isolation and Real Time PCR—Total RNA was isolated from primary granulosa cells or from ovaries of 19-day-old MT-α inhibit transgenic mice and their normal littersmates (NLM) using a Qiagen RNA isolation kit (Qiagen, Valencia, CA). Ovaries were collected on postnatal day 19 because at this age all follicles through the antral stage can be observed and yet the animals are pre-pubertal. On-column DNase digestion was performed using an RNase-Free DNase Set (Qiagen) to eliminate DNA contamination. The resulting RNA was reverse-transcribed with avian myeloblastosis virus reverse transcriptase (Fisher). Real time PCR was then performed on a Bio-Rad iCycler using SyberGreen SuperMix to quantitatively measure the mRNA levels of ERα, ERβ, inhibin α, cAMP response element-binding protein (CREB), glyceraldehyde-3-phosphate dehydrogenase, JunB, pS2/TFF1, inhibin βA, and inhibin βB. Primers were designed according to the complete mouse cDNA sequences of the above genes. A list of the primers used is shown in Table 1. Ribosomal protein L19 (L19) was used as an internal control for all reactions. The threshold cycle (Ct) numbers of L19 were not altered by any of the treatments (data not shown). The amplicons from reactions for ERα and ERβ were sequenced to confirm correct products. Specificity of all the real time PCR reactions was also confirmed by a single peak in the melt curves and by a single band of the predicted size after agarose gel electrophoresis of the PCR products (data not shown).

Western Blot—Protein homogenates were collected from the treated primary cultured granulosa cells, ovaries of 19-day-old
MT-α inhibit transgenic mice and their NLM, or transfected GRMO2 cells. Protein homogenates were prepared in GBA buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂, 10% glycerol, 0.5 mM 4-(2-aminoethyl)-benzene-sulfonyl fluoride (Roche Applied Science), and 0.1 mM bacitracin (Sigma), pH 7.4, at 4 °C). Proteins were electrophoresed under reducing conditions in 13% SDS-PAGE gels and transferred to nitrocellulose membranes. Blots were incubated overnight at 4 °C with primary antibody followed by a 1-h incubation at room temperature with horseradish peroxidase-labeled donkey anti-rabbit or goat anti-mouse secondary antibody (all from Zymed Laboratories Inc., South San Francisco, CA; donkey anti-rabbit or goat anti-mouse secondary antibody (all from Bio-Rad), 1:2000 dilution). Proteins were visualized with a fluorescent microscope. For ERα detection, tissue sections were incubated with anti-ERα antibody (39) (kindly provided by Dr. Pierre Chambon, Collège de France, France; 1:500 dilution) overnight at 4 °C. Sections were then counterstained with 4',6-diamidino-2-phenylindole (not shown) and visualized with a fluorescent microscope.

**Immunohistochemistry**—Ovaries collected from 19-day-old MT-α inhibit transgenic mice and their NLM were immediately fixed in 4% paraformaldehyde overnight and embedded in paraffin. Five-μm serial sections were obtained and mounted on Superfrost-Plus slides (Fisher). Slides were deparaffinized and rehydrated. Antigen retrieval was performed using 0.01 M sodium citrate. For ERα detection, tissue sections were incubated overnight with anti-ERα antibody (MC-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-ERβ (PA1–311, Affinity BioReagents, Golden, CO), anti-c-Myc (Sc-40, Santa Cruz Biotechnology, Inc.), anti-phospho-Smad2 (#3101, Cell Signaling Technology, Danvers, MA), anti-phospho-Smad3 (#9514, Cell Signaling Technology, Danvers, MA), and anti-actin (Sigma), all at 1:1000 dilution except for anti-actin, which was at 1:2000 dilution. Proteins were then visualized by chemiluminescence. The blots were scanned by densitometry. The intensities of the protein bands were analyzed using the public domain NIH Image program (rsb.info.nih.gov/nih-image). The pixel intensity of each protein band was normalized against that of the corresponding loading control, which was actin. The relative intensity of the protein band was then obtained from the ratio of the experimental group over the control.

**GRMO2 Cell Culture and DNA/siRNA Transfection**—GRMO2 cells are a mouse granulosa cell line provided by N. V. Innogenetics, Ghent, Belgium (59). Culture of GRMO2 cells was performed as described previously in a humidified incubator at 37 °C and 5% CO₂ (59). Three days before transfection, an estrogen-free culture condition was applied that contained a phenol red-free Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 5 μg/ml insulin, 5 ng/ml sodium selenite, 10 μg/ml transferrin, 50 μg/ml sodium pyruvate, and 2% charcoal/dextran-treated fetal bovine serum. Cells were transiently transfected with a mouse ERα promoter-β-galactosidase construct (60) (MER II βGAL, kindly provided by Dr. Alessandro Weisz from Seconda Università degli Studi di Napoli, Italy) at a dose of 500 ng DNA per well of a 12-well culture plate using cationic liposomes (59). After 20 h of transfection, fresh medium containing vehicle or various concentrations of activin A was given to the cells for 4 or 24 h followed by cell lysate collection and β-galactosidase assays. For the siRNA study, 250 ng of the mouse ERα promoter-β-galactosidase construct was co-transfected with 100 nm control siRNA or siRNAs targeting Smad2 or 3 (all from Dharmacon, Lafayette, CO) in a 24-well culture plate. The co-transfection was performed for 24 h using Lipofectamine (Invitrogen) following the manufacturer’s instructions. Cells were then cultured in a phenol red- and serum-free medium for an additional 16–18 h, and cell lysates were collected for β-galactosidase assays.

**Luciferase Assays and β-Galactosidase Assays**—Transfected cells were washed with PBS and lysed on ice for 20 min. For luciferase assay, the lysis buffer contained 25 mM HEPES, pH 7.8, 15 mM MgSO₄, 4 mM EGTA, 1 mM dithiothreitol, and 0.1% Triton X-100. Cell lysates (100 μl) were added to 400 μl of reaction buffer (25 mM HEPES, pH 7.8, 15 mM MgSO₄, 4 mM EGTA, 2.5 mM ATP, 1 mM dithiothreitol, 1 μg/ml bovine serum albumin), 100 μl of 1 mM luciferin (sodium salt) (Analytical Bioluminescence, San Diego, CA) were added using an automatic injector, and emitted luminescence was measured using a 2010 luminometer (Analytical Bioluminescence) for 10 s. For β-galactosidase assay, a Galacto-Light Plus Systems kit (Applied Biosystems, Bedford, MA) was used following the manufacturer’s instructions. For both assays relative light units were normalized for total protein content measured with the Bio-Rad protein assay reagent.

**List of primers used in this study**

| Genes                  | PCR primers Product size | bp     |
|------------------------|--------------------------|--------|
| GAPDH                  | 5'–CTG CAC TGG CAT ATT CTT CTG CAG AG–3' | 195    |
| ERα                    | 5'–CTG CAG AGT TTG GAT CAG GAC AG–3'  | 170    |
| ERβ                    | 5'–TCA TAG TTC CAA TAG TGC GGA AG–3'  | 138    |
| Inhibin α              | 5'–CCT CCA GGC TAC CCT TCT CTT CTT C–3' | 112    |
| CREB                   | 5'–GCC GAT ACC ACC GAA ACC ACC AG–3'  | 119    |
| GAPDH                  | 5'–ACA TCC GTC AAG GCA CAT ATT GGC AG–3' | 145    |
| JunB                   | 5'–GAC GAC CTT CAC AAG ATC AGA AA–3'  | 130    |
| pS2/TF1                | 5'–CAG GGC CAC GAA GAA CAT C–3'       | 122    |
| Inhibin βA             | 5'–GAT CAT CCT CTT CTC GGA CTA GT–3'  | 143    |
| Inhibin βB             | 5'–GAG GAT TGC GGT GGC GGC CAC–3'     | 308    |
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**RESULTS**

**Activin Increases ERα and ERβ mRNA and Protein Levels in Cultured Granulosa Cells**—In an effort to examine the effects of activin on ovarian gene expression, we performed a microarray study using RNAs collected from mouse granulosa cells maintained in primary culture and treated with PBS, 100 ng/ml activin A, or 100 ng/ml activin A plus 400 ng/ml follistatin for 24 h. Among the genes that are regulated by activin, ERβ was identified as an inducible gene. To verify the microarray results, we treated cultured mouse primary granulosa cells with activin A, follistatin, a combination of the two, or inhibin A for 24 h and collected RNA for quantitative real time PCR analysis. Activin A treatment significantly increased the mRNA levels of both ERα and ERβ (Fig. 1, A and B). Follistatin alone decreased the mRNA levels of ERβ (Fig. 1B), whereas inhibin A had no effect on the expression of either ERα or ERβ (Fig. 1, A and B, respectively). The expression level of ERα was much lower than ERβ in the granulosa cells (Fig. 1A, inset). This may explain why ERα was not identified as an activin-regulated gene in the initial microarray study and also why no further suppression by follistatin treatment was observed. The lack of effect of inhibin A was an interesting observation and indicated that at this concentration inhibin A was not able to reverse endogenous activin action. This may also relate to expression levels of β-glycan in the cultured granulosa cells, as β-glycan is a co-receptor for inhibin and is critical for inhibin action in many systems (61, 62). As a positive control, inhibin α mRNA levels were also examined under the same treatment conditions (Fig. 1C), as inhibin α is known to be induced by activin after a 48-h treatment in cultured rat granulosa cells (63) and our microarray data also revealed an up-regulation of inhibin α by activin A. As a negative control, we examined CREB mRNA levels, which were not altered by any of the treatments (Fig. 1D). We also examined glyceraldehyde-3-phosphate dehydrogenase mRNA levels as an additional negative control and found no regulation by any of the treatments (data not shown).

To compare the effect of activin to the other members of the TGF-β superfamily, primary granulosa cells were also treated with TGF-β1, which is a major functional form of TGF-β, and BMP-2, which is a member of the bone morphogenic protein family and is produced by both granulosa cells and theca cells in the ovary (64). TGF-β1 had no effect on the ERα, ERβ, inhibin α, or CREB mRNA levels, consistent with a previous report on the lack of a significant effect of TGF-β1 on ERα gene transcription in breast cancer cells (65). Although BMP-2 did not alter the mRNA levels of the other genes, it increased ERα mRNA levels by about 50%, which is less robust than the effect of activin A (Fig. 1, A–D).

We next compared the effect of activin A on ER expression at different treatment time points (1, 4, 12, and 24 h). Stimulation of the mRNA levels of ERα and ERβ by activin A was observed starting at 4 h of treatment, indicating a relatively rapid induction of ER expression by activin (Fig. 2, A and B). This stimulation increased with time and persisted until at least 24 h. No significant change in the mRNA levels of inhibin α was observed until at 12 h of treatment of activin A (Fig. 2C), indicating that this gene responds more slowly. In contrast, JunB, another known activin target gene, responded to activin A treatment in a fast and transient manner, consistent with the observation by others (Fig. 2D) (66).

The effect of activin A was also examined at different concentrations as shown in Fig. 3. The range of concentrations was selected based on a previous study in Sertoli cells (67). Activin A stimulated ERα, ERβ, and inhibin α mRNA levels starting at 25, 50, and 100 ng/ml, respectively, and the stimulatory effect increased with dose. Because ER levels increased, we examined if this would affect expression of a well characterized estrogen-inducible gene pS2/TFF1. Expression of pS2/TFF1 is regulated through ERα (68). Our results showed that pS2/TFF1 mRNA levels did increase corresponding to the increased ERα levels (Fig. 3D).

3 J. L. Kipp, S. M. Kilen, J. Avraham, and K. E. Mayo, in manuscript in preparation.
To examine protein levels of ERs, protein homogenates from primary granulosa cells treated 24 h with PBS, activin A, or activin A plus follistatin were collected for Western blot analy-

FIGURE 2. Time course study of activin A stimulation of the mRNA levels of ERα (A), ERβ (B), Inhibin (C), and JunB (D). Primary granulosa cells were collected from 22–23-day-old mice and deprived of estrogen for 3 days before the treatments. The mRNA levels were measured with real time PCR after reverse transcription. Results are an average of three independent experiments each performed in triplicate. PBS, control; ActA, activin A (100 ng/ml). *, p < 0.05.

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FIGURE 3. Activin A (ActA) shows dose-dependent effect on the mRNA levels of ERα (A), ERβ (B), Inhibin α (C), and the estrogen target gene pS2/TFF1 (D). Primary granulosa cells were collected from 22–23-day-old mice and deprived of estrogen for 3 days before the activin A treatments at indicated concentrations for 24 h. The mRNA levels were measured with real time PCR following reverse transcription. *, p < 0.05; **, p < 0.01. n = 3.
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Activin Increases Functional Estrogen Receptor Activity and Stimulates ERE Gene Transcription—To examine the effect of activin on functional ER activity, we compared ER-dependent promoter activity in activin plus E2-treated cells versus cells treated with E2 alone. Primary cultured mouse granulosa cells were transfected with either empty vector (EV) or ERE-luciferase constructs, and the transfected cells were treated with various compounds as indicated in Fig. 5. Before performing luciferase assays, we first examined expression levels of ERα and ERβ under these transfection/treatment conditions. As expected, activin A increased ERα and ERβ mRNA levels in both empty vector- and ERE-luciferase-transfected cells. In ERE-luciferase-transfected cells, when activin A was given together with E2, a more robust induction of the ERα and ERβ mRNA levels was observed, suggesting an additive effect of these two hormones, which is consistent with the slight although not significant stimulatory effect of E2 on ERβ expression (Fig. 5, A and B). When follistatin was given together with activin A, the stimulatory effect was abolished, and follistatin alone also decreased ERβ mRNA levels (Fig. 5, A and B), thus confirming the results shown in Fig. 1. Luciferase activity assays revealed that activin A-treated cells showed higher ERE promoter activity than the nontreated cells. E2 was able to induce ERE promoter activity, and the ERE promoter activity was higher in cells treated with activin A plus E2 than in cells treated with E2 alone. All of these are consistent with an increase in ER expression after activin treatment that in turn mediates a higher response of the ERE promoter to E2 (Fig. 5C). The effect of activin on enhancing E2 stimulation of ERE promoter activity was abolished when activin A was given together with excess amount of follistatin (Fig. 5C). Follistatin alone also suppressed E2 stimulation of ERE promoter activity (Fig. 5C).

When added to cultured primary granulosa cells 30 min before a 4-h treatment with activin A, the protein synthesis inhibitor cycloheximide did not abolish the stimulatory effect of activin A on ERα and ERβ mRNA levels (Fig. 6, A and B), indicating that this regulation does not require ongoing protein synthesis and is likely at the transcriptional level. In agreement with this, treatment with the RNA synthesis inhibitor actinomycin D did abolish the stimulatory effect of activin A on ERα and ERβ mRNA levels (Fig. 6, C and D). With actinomycin D treatment, we also observed that the basal expression levels of ERs decreased by about 1.5-fold as compared with the non-actinomycin D-treated samples (not shown).

To further investigate if activin regulation of ER expression represents a direct transcriptional effect, we examined activin regulation of ERα promoter activity. GRMO2 granulosa cells were transfected with a mouse ERα promoter-β-galactosidase construct (~5 kilobases) and then treated with activin A for 4 or 24 h. β-Galactosidase activity measurements revealed that a 4-h activin A (100 ng/ml) treatment increased ERα promoter activity by 2.0-fold (p < 0.001) and a 24-h activin A treatment at 50, 100, and 200 ng/ml increased ERα promoter activity by 1.4-, 1.7 (p < 0.05), and 2.2- (p < 0.01)-fold, respectively (Fig. 7). These results are consistent with the stimulatory effect of activin on ERα mRNA levels at 4 or 24 h.

The results showed that activin A increased protein levels of ERα and ERβ by about 2-fold, and this effect was abolished when activin was given together with excess follistatin (Fig. 4, A–C). These results are consistent with the mRNA measurements. c-Myc is an estrogen-inducible gene (69). Protein levels of c-Myc were elevated in activin A-treated samples and follistatin partially reduced this elevation (Fig. 4A), indicating that increased ER expression after activin A treatment indeed resulted in an increase in an estrogen-responsive target gene.
ERα and ERβ Expression Levels are Decreased in an Animal Model of Activin Deficiency—To investigate the importance of activin in regulating ovarian ER levels in vivo, we examined ER expression in a transgenic mouse model that overexpresses the inhibin α-subunit gene from a metallothionein-1 promoter (MT-α inhibin subunit), and as a consequence of inhibin overexpression has decreased activin levels (70). These mice show decreased fertility and exhibit a variety of ovarian pathologies, including development of ovarian cysts (70, 71).

We first confirmed that expression of the βA and βB subunits of activin were decreased in the ovaries from the MT-α inhibin transgenic mice using quantitative real time PCR measurements (Fig. 8A). Further studies revealed that in the MT-α inhibin transgenic mice, the mRNA levels of both ERα and ERβ were significantly decreased in the ovary (Fig. 8B). In the uterus, ERα mRNA levels were also decreased in the MT-α inhibin transgenic mice, whereas ERβ mRNA was not detectable, which confirmed an earlier report by others (data not shown) (38). This observation is consistent with the fact that the inhibin α transgene is broadly expressed in these transgenic mice. Consistent with decreased mRNA levels, ERα and ERβ protein levels were lowered by about 42 and 52%, respectively, in the MT-α inhibin transgenic mouse ovary compared with the NLM ovary (Fig. 8, C and D). The minor differences between mRNA level measurements and protein level measurements may result from a difference in sensitivity between real time PCR and Western blots. Immunohistochemical studies confirmed decreased ERα expression (mostly in the thecal cells) and decreased ERβ expression (mostly in the granulosa cells) in the MT-α inhibin transgenic mouse ovary as compared with the NLM mouse ovary (Fig. 8E). These results were observed in ovaries from 19-day-old mice. The effect of decreased activin levels on ER expression in the ovary compared with the NLM ovary (Fig. 8, A and B).

The effect of decreased activin levels on ER expression in the ovarian tissue was also examined by performing Northern blotting using RNA isolated from the ovaries of 22-33-day-old mice. Consistent with decreased mRNA levels, ERα mRNA levels were decreased in the ovaries from the MT-α inhibin transgenic mice, whereas ERβ mRNA was not detectable, which confirmed an earlier report by others (data not shown) (38). This observation is consistent with the fact that the inhibin α transgene is broadly expressed in these transgenic mice. Consistent with decreased mRNA levels, ERα and ERβ protein levels were lowered by about 42 and 52%, respectively, in the MT-α inhibin transgenic mouse ovary compared with the NLM ovary (Fig. 8, C and D). The minor differences between mRNA level measurements and protein level measurements may result from a difference in sensitivity between real time PCR and Western blots. Immunohistochemical studies confirmed decreased ERα expression (mostly in the thecal cells) and decreased ERβ expression (mostly in the granulosa cells) in the MT-α inhibin transgenic mouse ovary as compared with the NLM mouse ovary (Fig. 8E). These results were observed in ovaries from 19-day-old mice. The effect of decreased activin levels on ER expression in the ovary compared with the NLM ovary (Fig. 8, A and B).

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Activin Regulates Estrogen Receptors

In Vivo and In Vitro Studies Confirm Smad2 and Smad3 Regulation of ER Expression—To further examine involvement of Smad proteins in ER expression, we performed in vivo studies in a Smad2 dominant negative (Smad2 DN) transgenic mouse model and in vitro studies in granulosa cells co-transfected with the ERα promoter and siRNAs targeting Smad2 or Smad3. We first examined ER mRNA levels in the Smad2 DN transgenic mice. This transgenic mouse model expresses a non-phosphorylatable Smad2 protein from the Müllerian inhibiting subunit promoter (MIS-Smad2 DN) and has impaired activin signaling through Smad2, as phospho-Smad2 levels are significantly decreased in the transgenic mouse ovaries (71).

In addition to the above observations, we also found that in 19-day-old mice, both ovary and uterus weights were decreased in the MT-α inhibit transgenic mice as compared with the NLM although their body weights were the same, consistent with decreased estrogen responsiveness in the reproductive system (particularly shown by the uterus weight) (Table 2).

In Vivo and In Vitro Studies Confirm Smad2 and Smad3 Regulation of ER Expression—To further examine involvement of Smad proteins in ER expression, we performed in vivo studies in a Smad2 dominant negative (Smad2 DN) transgenic mouse model and in vitro studies in granulosa cells co-transfected with the ERα promoter and siRNAs targeting Smad2 or Smad3. We first examined ER mRNA levels in the Smad2 DN transgenic mice. This transgenic mouse model expresses a non-phosphorylatable Smad2 protein from the Müllerian inhibiting subunit promoter (MIS-Smad2 DN) and has impaired activin signaling through Smad2, as phospho-Smad2 levels are significantly decreased in the transgenic mouse ovaries (71). The transgene is predominantly expressed in the ovary, and these transgenic mice have decreased fertility and develop ovarian epithelial cysts (71). Because the MIS-Smad2 DN transgene is expressed predominantly in granulosa cells and the surface epithelium, we used primary cultured granulosa cells for this study. Granulosa cells were collected from MIS-Smad2 DN transgenic and NLM mouse ovaries, and ER mRNA levels were measured with real-time RT-PCR. The results showed that basal mRNA levels of ERα and ERβ were significantly decreased in the MIS-Smad2 DN transgenic mice compared with the NLM mice (Fig. 9A), consistent with the above observations in the MT-α inhibit transgenic mice and indicating that activin expression and signaling through Smad2 is important in maintaining ER levels.

To further investigate involvement of Smad2 and Smad3 in maintaining ER levels, we examined ERα promoter activity in granulosa cells that were co-transfected with siRNAs against Smad2 or Smad3. β-Galactosidase assays showed that the promoter activity was decreased by either of these two siRNAs as compared with the control siRNA, suggesting that both Smad proteins are important for its activation (Fig. 9B). Specificity of knockdown of Smad expression by these siRNAs were confirmed by Western blot analysis of phosphorylated Smad2 and phosphorylated Smad3, which are the activated forms of Smad2 and Smad3 that mediate activin signaling (Fig. 9C). Quantification of the protein band intensity after normalization against the loading controls revealed a 67% decrease in phosphorylated Smad2 protein levels in siMad2-transfected samples and a 55% decrease in phosphorylated Smad3 protein levels in siMad3-transfected samples.

DISCUSSION

Activin is an important modulator of follicle-stimulating hormone synthesis and secretion and is also involved in reproductive dysfunctions and reproductive cancers (72). Recently, autocrine and paracrine roles for activin have been described in the regulation of ovarian follicle development (10–15, 70, 71). The mechanisms that mediate these functions are not fully understood. This study demonstrates that ERα and ERβ expression can be positively regulated by activin and that activin is important in maintaining ER levels in the mouse ovary.

Our previous study revealed that neonatal estrogen exposure decreases activin βA and βB subunit mRNA levels in the mouse ovary. This may be mediated by a direct action of estrogen through ER on activin gene expression, as E2 suppresses activin βA and βB subunit promoter activities and this suppression can be neutralized by the anti-estrogen ICI182,780 (45). Results from the current study, thus, describe an important feedback mechanism in that activin increases ER levels and enhances estrogen action. Increased estrogen action in turn suppresses activin expression and may eventually return ER levels to basal. This novel interplay between activin and estrogen signaling in the mouse ovary may be critical in maintaining a balance between these two hormones in their expression levels and, hence, their actions.

Interactions between ERs and other members of the TGF-β family, including TGF-β and BMPs, have been documented previously (46–49). Activin and TGF-β share a common set of
signaling proteins: Smad2, Smad3, and Smad4. Among them, Smad3 is a transcriptional protein that directly binds to DNA to modify target gene expression (73). It has been reported that through a physical association between ERs and Smad3, ERs act as transcriptional co-repressors to suppress TGF-β signaling, whereas TGF-β stimulates the transcriptional activity of ERs (46). This feedback loop is very similar to our findings on the reciprocal regulation between ERs and activin. Estrogen also suppresses BMP expression and inhibits BMP actions in cultured oviduct cells (48) and osteoblasts (49). In addition, ERs can directly interact with Smad1 to suppress BMP-induced gene transcription (47). In this study we also showed that BMP-2 increased ERα mRNA levels by about 50%, further suggesting an interaction between ER and BMP signaling.

In both MT-α inhibin transgenic mice and MIS-Smad2 DN transgenic mice, ERα and ERβ expression were decreased in the ovary or granulosa cells as compared with the wild type mice, although to a greater extent in MT-α inhibin mice. These observations indicate that intra-ovarian activin levels and signaling are critical in maintaining ER expression. Activin signaling through phosphorylated Smad2 protein is compromised in the MIS-Smad2 DN transgenic mice (71); therefore, our results indicate that Smad2 is involved in mediating activin regulation of ER expression levels. Involvement of Smad2 is further supported by the siRNA study. The siRNA study also demonstrated an important role for Smad3 in ER expression. Consistent with our results, in a study investigating involvement of Smad3 in ovarian surface epithelium (OSE) proliferation, it was shown that ERα expression is dramatically decreased in OSE of Smad3 homozygous knock-out mice compared with wild type mice (74). Smad3 can bind to DNA directly, and an 8-bp palindromic sequence 5'-GTCTAGAC-3' has been identified as a Smad binding element for the highly conserved MH1 domain of human Smad3 and -4 (73). Smad2 does not normally directly bind to DNA because of an additional sequence encoded by exon 3, although the binding can be restored after this additional sequence is removed (73, 75). Using bioinformatics tools, we have identified a perfect 8-bp palindromic Smad binding element (SBE) site in the promoter region of the mouse ERα gene and several 5'-GTCT-3' SBE sites in the 5'-untranslated region and/or promoter region of both ERα and ERβ. Future study will investigate Smad protein binding to these sites in the ER promoters.

Our data are consistent with a direct transcriptional regulation of ER expression by activin, and we have demonstrated that Smad proteins are involved in maintaining ER levels. In addition to a direct regulation by activin through Smad proteins, ER expression can also be regulated by many other factors. For instance, FoxO3a (Forkhead box class O, 3a) and FoxM1 (Forkhead box M1), two members of the Forkhead transcription factor family, positively regulate ERα expression (76, 77). It has also been reported that Sp1 binds to the ERα promoter region and regulates ERα gene expression (78, 79). FoxO3 is a target of AKT (80), and activin can activate the AKT signaling pathway (81, 82). Interaction between Sp1 and Smad proteins (Smad2, -3, and -4) has also been documented in hepatic cells (83) and in epithelial cells (84). Therefore, it is possible that the Forkhead family members and Sp1 may mediate activin regulation of ER

**FIGURE 9. Smad2 and Smad3 are important for ER gene expression.** A, ERα and ERβ expression is decreased in the granulosa cells from the MIS-Smad2 DN transgenic mouse ovary. Primary granulosa cells were collected from 22–23-day-old control or Smad2 DN mice. The mRNA levels of ERα and ERβ were measured using real-time PCR after reverse transcription and are shown as a ratio over L19. n = 5–8; siRNAs targeting Smad2 or Smad3 suppress ERα promoter activity. GRMO2 cells were deprived of estrogen for 3 days before being co-transfected with a mouse ERα promoter-β-galactosidase construct and siRNAs for 24 h and then cultured in a phenol red- and serum-free medium for an additional 16–18 h. Cell lysates were collected for β-galactosidase activity assays; n = 4. C, representative Western blot pictures showing specificity and degree of knockdown of Smad2 or Smad3 by siRNAs. *, p < 0.05; **, p < 0.01; ***, p < 0.001. RLU, relative light units; p-Smad, phosphorylated Smad.
expression. In addition, the mitogen-activated protein kinase pathway and the NF-κB or phosphatidylinositol kinase/AKT pathways can modulate or be activated by Smad pathways (85), and activin is able to interact with the Wnt signaling pathway to regulate dorsal mesoderm induction in Xenopus (86). Further studies are required to investigate if these signaling pathways may be involved in activin stimulation of ER expression.

Overall, we have shown that activin stimulates ER expression and is important in maintaining ER levels. Our study suggests that ERs are activin target genes and may mediate some of the effects of activin in the ovary. This study provides new insights into activin functions in the reproductive system. Understanding how activin and ER signaling interact also promises to increase our understanding of mechanisms of diseases that are important to human health such as cancer.

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