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Gene Expression Profiling Reveals Cyp26b1 to Be an Activin Regulated Gene Involved in Ovarian Granulosa Cell Proliferation

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Activin, a member of the TGF-β superfamily, is an important modulator of FSH synthesis and secretion and is involved in reproductive dysfunctions and cancers. It also regulates ovarian follicle development. To understand the mechanisms and pathways by which activin regulates follicle function, we performed a microarray study and identified 240 activin regulated genes in mouse granulosa cells. The gene most strongly inhibited by activin was *Cyp26b1*, which encodes a P450 cytochrome enzyme that degrades retinoic acid (RA). *Cyp26b1* has been shown to play an important role in male germ cell meiosis, but its expression is largely lost in the ovary around embryonic d 12.5. This study demonstrated that *Cyp26b1* mRNA was expressed in granulosa cells of follicles at all postnatal developmental stages. A striking inverse spatial and temporal correlation between *Cyp26b1* and activin-βA mRNA expression was observed. *Cyp26b1* expression was also elevated in a transgenic mouse model that has decreased activin expression. The *Cyp26* inhibitor R115866 stimulated the proliferation of primary cultured mouse granulosa cells, and a similar effect was observed with RA and activin. A pan-RA receptor inhibitor, AGN194310, abolished the stimulatory effect of either RA or activin on granulosa cell proliferation, indicating an involvement of RA receptor-mediated signaling. Overall, this study provides new insights into the mechanisms of activin action in the ovary. We conclude that *Cyp26b1* is expressed in the postnatal mouse ovary, regulated by activin, and involved in the control of granulosa cell proliferation. **(*Endocrinology* 152: 303–312, 2011)**

Mammalian ovarian follicle formation and development involves establishment of the initial follicle pool, follicle growth, proper maturation of eggs, and timely production and release of hormones (1, 2). This process is critical for propagation of the species as well as for development and homeostasis. Regulation of follicle formation and development requires intrinsic and endocrine factors as well as interactions between multiple cell types within the ovary (1–5). Among the many intraovarian factors, substantial evidence indicates that activin and inhibin, members of the TGF-β superfamily, play important autocrine/paracrine roles in this process (6–9). Activin and inhibin were first isolated from gonadal sources as endocrine factors that either stimulate (activin) or suppress (inhibin) the synthesis and secretion of FSH by the pituitary gland (10–16). Later studies indicated that activin acts predominantly as a local paracrine and autocrine factor (17, 18). Effects of activin on many biological processes, including wound repair (19, 20), inflammation (20–22), renal tubule morphogenesis (23), hair follicle development (24), neuroprotection (19), glucose metabolism (25), and stem cell growth and differentiation (25,

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Abbreviations: ER, Estrogen receptor; MT, metallothionein-I promoter; NLM, normal litter mate; RA, retinoic acid; RAR, RA receptor.

26), have been reported. In the ovary, activin can regulate granulosa cell proliferation and differentiation (6, 27–30), increase FSH receptor expression in undifferentiated granulosa cells (31, 32), and stimulate oocyte maturation (33). However, the genes regulated by activin in the ovary, and how the products of these genes may affect ovarian follicle formation and development, remain largely unknown.

Like other members of the TGF-β superfamily, activin signals through a receptor serine-threonine kinase/Smad protein pathway (34, 35). The βA- and βB-subunits of activin as well as the α-subunit of the activin antagonist inhibin are all expressed in the ovary and localized predominantly to granulosa cells (36–40). Components of the activin signaling system, including activin receptors, signaling Smad proteins, and activin binding protein and antagonist follistatin, are also expressed within the granulosa cells (6, 41–43). Expression of activin subunits and signaling proteins within the follicle further supports an autocrine/paracrine role of activin in this cell type and suggests granulosa cells are targets of activin action in the ovary.

Through a microarray study of activin-treated primary cultured mouse granulosa cells, we obtained a global view of activin effects on ovarian gene expression/pathways and identified *Cyp26b1* (cytochrome P450, family 26, subfamily b, polypeptide 1) as a novel activin down-regulated gene that was expressed in the postnatal ovary. Because *Cyp26b1* encodes an enzyme that degrades the potent morphogen retinoic acid (RA) (44, 45), we further discovered that RA and a Cyp26 inhibitor stimulated granulosa cell proliferation. We show that RA receptor (RAR)-mediated signaling is involved in both RA- and activin-induced granulosa cell proliferation. Our findings provide new insights into the mechanisms of activin action in the ovary and suggest *Cyp26b1* and RA to be novel candidates for regulating postnatal follicle formation and development.

Materials and Methods

Animals

Wild-type or MT-α inhibin transgenic mice on a CD-1 background (Harlan, Indianapolis, IN) (46) were maintained on a 12-h light, 12-h dark cycle (lights off at 1700 h) with food and water available *ad libitum*. Animals were cared for in accordance with all federal and institutional guidelines.

Primary granulosa cell collection, culture, and treatment

Granulosa cells were collected from ovaries from 22- to 23-d-old immature mice through follicle puncture and cultured as described previously (47). 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% CO_2 in a DMEM/F-12 medium (Invitrogen, Grand Island, NY) 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% fetal bovine serum (Invitrogen) (47). Cultured cells were given treatments at the following concentrations: activin A, 2 nM (R&D Systems, Minneapolis, MN); follistatin, 3 nM (provided by Dr. Teresa Woodruff, Northwestern University, Chicago, IL); TGF-β1, 100 pM (R&D Systems); *all-trans* RA, 0.007, 0.07, or 0.7 μM (Sigma, St. Louis, MO); R115866, 0.007, 0.07,or 0.7 μM (provided by Johnson and Johnson, Beerse, Belgium); or AGN193109, 1 μM (Santa Cruz Biotechnology, Santa Cruz, CA). Treatments were given for 24 h for microarray or real-time PCR studies and 72 h for cell proliferation assays. The doses of treatments were based on reported studies (45, 47).

RNA isolation

Total RNA was isolated either from primary granulosa cells or from whole ovaries or testes using a QIAGEN RNeasy minikit (QIAGEN, Valencia, CA). RNA was subjected to on column deoxyribonuclease digestion and analyzed for quality using a bioanalyzer. Before microarray assays, a small portion of the samples was used for real-time RT-PCR analysis to confirm a consistent effect of treatments across replicate experiments on marker genes, including estrogen receptors (ERs) (47) and inhibin-α (43).

Microarray and data analysis

An Illumina BeadArray (Illumina, Inc., San Diego, CA) was used for gene expression profiling (48, 49). RNA from PBS- (P, control), activin A- (A), and activin A plus its antagonist follistatin (A+F)-treated groups was isolated from four independent experiments. About 100 ng of each RNA sample was amplified using a modified Eberwine T7-based amplification protocol and hybridized to the Illumina Sentrix Mouse-6 Expression BeadChip (Illumina) that contains 48,000 sets of oligonucleotides (48, 49). Among these probes, 14,641 gave positive signals in the granulosa cells, and the genes with expression values less than the background levels were removed from further analysis. A Bioconductor *lumi* package with the variance-stabilizing transform + robust spline normalization scheme was used to preprocess the data (50, 51). Because each treatment condition was repeated four times, samples from experiment 1 were assigned as P1, A1, and A+F1, samples from experiment 2 as P2, A2, and A+F2, and so on. For each comparison, *i.e.* A-P, the package computed A1-P1, A2-P2, A3-P3, and A4-P4 first. Next, to evaluate the statistical significance of these differences, the empirical Bayes shrinkage moderated t statistics in *lumi* Bioconductor package was applied (52). To estimate the false discovery rate, the Bonferroni method was used. Only the genes with a false discovery rate less than 0.05 and with a 1.5- or more-fold change in gene expression were considered as significant.

Real-time PCR

Total RNA from granulosa cells or whole tissues was reverse transcribed with avian myeloblastosis virus-reverse transcriptase (Fisher Scientific, Pittsburgh, PA). Real-time PCR was then performed on a 7300 real-time PCR system using SYBR Green PCR master mix (Applied Biosystems, Warrington, UK). 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. Ribo-

TABLE 1. List of primers used in this study

Genes	PCR primers (5′–3′)	Product size (bp)
Rpl19	CTG AAG GTC AAA GGG AAT GTG	195
	GGA CAG AGT CTT GAT GAT CTC	
Nap1l5	AAG ATC GAG GCC AAG TTT GA	143
	TCA TCA TCC TCT CCC TCC AG	
Mycl1	ACG GCA CTC CTA GTC TGG AA	136
	ACG GTC ACC ACG TCA ATC TC	
Fst	GAG TGA CTT ACT CCA GCG CC	266
	AGC TTC CTT CAT GGC ACA CT	
Mid1ip1	GGC CTG AGT CAC TTG GAG AG	149
	AAG CCG ATC TCC TGC TTG TA	
Ihh	GAG CTC ACC CCC AAC TAC AA	118
	TGA CAG AGA TGG CCA GTG AG	
Rgs2	GAG GAG AAG CGG GAG AAA AT	150
	TTC CTC AGG AGA AGG CTT GA	
Nr1h4	AAG GGG ATG AGC TGT GTG TT	120
	TGT ACA CGG CGT TCT TGG TA	
Figf	GTA TGG ACT CAC GCT CAG CA	137
	GGC GAC TTC TAC GCA TGT CT	
Slit2	CGT TTG GAA AAT GTT CAG CA	103
	TGA AAC TGT CGT TCC CAA CA	
Itgb1	GTC AGC AAC GCA TAT CTG GA	100
	ACA TTC CTC CAG CCA ATC AG	
Cyp26b1	AGC TAG TGA GCA CCG AGT GG	146
	GGG CAG GTA GCT CTC AAG TG–	
Inha	CTC CCA GGC TAT CCT TTT CC	112
	TGG CCG GAA TAC ATA AGT GA	
Esr1	ACC ATT GAC AAG AAC CGG AG	170
	CCT GAA GCA CCC ATT TCA TT	
Esr2	TGT GTG TGA AGG CCA TGA TT	138
	TCT TCG AAA TCA CCC AGA CC	
Cyp19a1	GAC ACA TCA TGC TGG ACA CC	100
	TGC CAG GCG TTA AAG TAA CC	
Inhba	GAT CAT CAC CTT TGC CGA GT	143
	TGG TCC TGG TTC TGT TAG CC	

somal protein L19 was used as an internal control for all measurements. Specificity of all the real-time PCR reactions was also confirmed by a single peak in the melt curves and a single band of the predicted size after agarose gel electrophoresis of the PCR products (data not shown).

Cell proliferation assay

Granulosa cells were plated at $4–5 \times 10^4$ cells/well, and HeLa cells were plated at 3×10^3 cells/well into 96-well plates. After treatments, cell proliferation assays were performed using a CellTiter96 AQueous One Solution cell proliferation assay reagent (Promega, Madison, WI) following the manufacturer's instructions. Absorbance at 490 nm was obtained using a 96-well plate reader. Cell numbers were calculated according to readings from standards plated in the same plates.

In situ hybridization

Ovaries collected from CD-1 mice at different ages were fixed in 4% paraformaldehyde and embedded in paraffin. Five-micrometer serial sections were obtained and mounted on Superfrost-Plus slides (Fisher Scientific). Digoxigenin-labeled RNA antisense and sense probes for *Cyp26b1* were kindly provided by Dr. David Page (Massachusetts Institute of Technology, Boston, MA). Digoxigenin-labeled RNA antisense and sense probes for activin-βA (Inhba) were generated by cloning of a 143-bp PCR product (see primers in Table 1) into the SP6/T7 dual-promoter pCR II vector using a TA cloning kit (Invitrogen) and labeling of the transcript with a digoxigenin RNA labeling kit (Roche, Indianapolis, IN). The probes were hybridized to the sections at 50 C overnight and visualized with an alkaline phosphatase-conjugated antidigoxigenin antibody (Santa Cruz) and 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoyl-phosphate, 4-toluidine salt chromogen (Sigma). Sections were counterstained with nuclear Fast Red (Aldrich Chemical Corp., Milwaukee, WI).

Western blot

Protein homogenates were collected from primary cultured granulosa cells or ovaries or testes of 20-d-old mice. Protein homogenates were prepared in a buffer containing 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10% glycerol, 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (Roche Molecular Biochemicals, Indianapolis, IN) and 0.1 mM bacitracin (Sigma) (pH 7.4) at 4 C. Proteins were electrophoresed and transferred to nitrocellulose membranes. Blots were incubated overnight at 4 C with goat anti-Cyp26b1 antibody (Everest Biotech, Oxfordshire, UK; 1:1000 dilution), followed by a 1-h incubation at room temperature with horseradish peroxidase-labeled antigoat secondary antibody (Zymed Laboratories, South San Francisco, CA; 1:8000 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 (National Institutes of Health; 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.

Statistics

Data are presented as the means ± SE. For comparisons between two groups, a Student's two-tailed *t* test was used. For comparisons among multiple groups, one-way ANOVA followed by a Tukey's test was used. $P < 0.05$ was considered significant.

Results

Gene expression profiles

To obtain gene expression profiles of activin targets, primary granulosa cells were collected from d 22–23 immature mice and treated with activin A (2 nM), activin A (2 nM) plus its antagonist follistatin (3 nM), or PBS (control) for 24 h. RNA from each treatment group was then isolated for microarray analysis. The results revealed that a total of 240 genes were significantly regulated by activin A compared with control (A-P). Among those 240 activin-regulated genes, 236 were specific because their regulation was abolished or reversed in the activin A plus follistatin treatment group (Supplemental Fig. 1, pub-

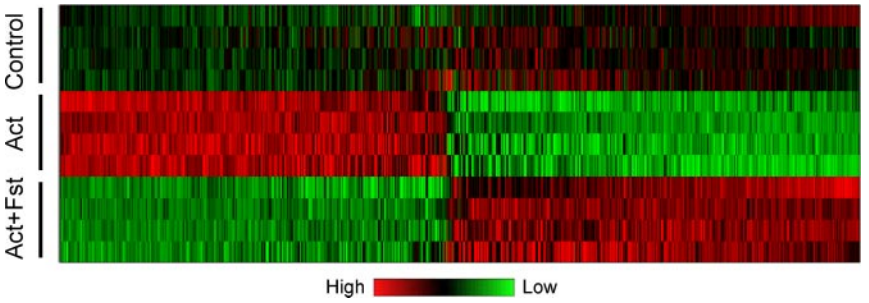

FIG. 1. A heat map overview of all the genes that are significantly regulated by activin A as identified by Illumina BeadArray (n = 4). *Red*, Up-regulation; *green*, down-regulation. Act, Activin A; Fst, follistatin.

lished on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Among these 236 genes, 127 genes were stimulated (range 1.5–18 fold) and 109 genes were inhibited (range 1.5–11 fold) by activin (Fig. 1). Cotreatment with follistatin almost totally reversed activin A's effects on the expression of these genes (Fig. 1). In most cases, the combination of activin A and follistatin resulted in a change opposite to that of activin A alone, suggesting that follistatin (which was present in access) was acting to suppress endogenous activin action. This is consistent with the fact that these cells are known to produce activin A in culture (53). Among the activin-stimulated genes, follistatin was induced 5.72-fold and inhibin-α was induced 3.17-fold, consistent with previous reports and therefore serving as validation for the microarray results (54, 55).

Using Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com), we found that activin regulated genes in granulosa cells are strongly associated with functions including cell signaling, cell death, cellular growth and proliferation, cancer, cell movement, development, cell cycle, and tissue morphology (Supplemental Fig. 2). This is consistent with the known direct actions of other TGF-β superfamily ligands (56–60). To further understand mechanisms of activin action and validate our data, we also performed signaling pathway analysis according to the Kyoto Encyclopedia of Genes and Genomes pathway definitions. The MAPK signaling pathway was the pathway that was most significantly regulated by activin A (Supplemental Fig. 3A). The MAPK signaling pathway is critical for cell proliferation, differentiation, apoptosis, and development (for a review see Ref. 61). Consistent with this finding, we observed that activin A stimulated mouse granulosa cell proliferation in primary culture, and this stimulatory effect was reversed by follistatin (Supplemental Fig. 3B). Follistatin alone suppressed granulosa cell proliferation (Supplemental Fig. 3B).

Quantitative PCR verification

To confirm the results from the microarray assay, seven genes induced by 2- to 15-fold and four repressed by 2- to 6-fold on the array were selected for validation by quantitative RT-PCR. These genes were selected based on a combination of criteria to include genes expected to be regulated by activin (*Fst* and *Inha*) and to include genes that were regulated over the dynamic range observed in the array study. Real-time PCR was performed, and these results were consistent with the microarray measurements, although there was some variance in the magnitude of the induction or repression (Fig. 2A). All of these genes also showed rapid activin responses in experiments in which granulosa cells were treated with activin A for only 4 h. The activin responses after 4 h were generally similar to the corresponding longer-term activin responses after 24 h, although, as expected, some differences were observed between the two time points with several genes further increasing or decreasing by 24 h (Fig. 2B).

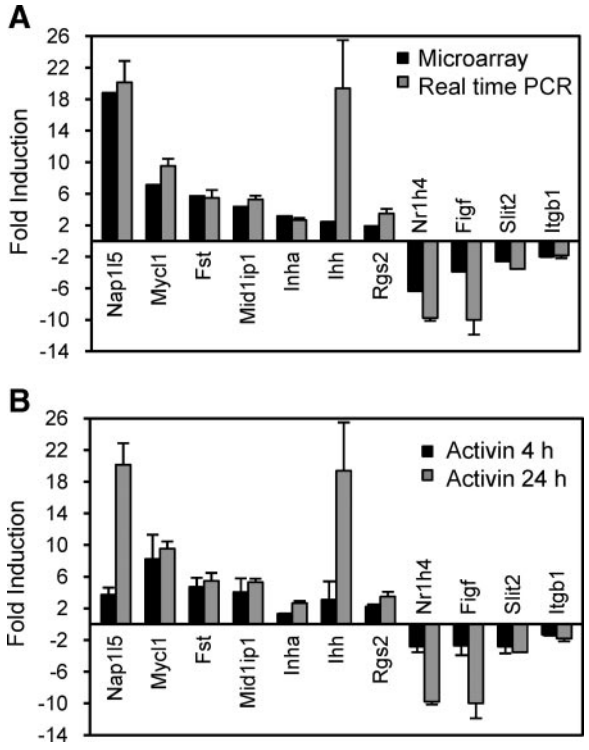

FIG. 2. Validation of microarray results and time-course study of activin effect on selected genes. A, Validation of select activin up- and down-regulated genes obtained from microarray results with real-time RT-PCR ($P < 0.05$ for all; microarray, n = 4; real-time RT-PCR, n = 3–5). B, Time-course study of activin effect on selected genes. The same data for 24 h from A are plotted here as part of the time-course comparison. Primary cultured granulosa cells were treated with activin A or PBS (control) for 4 or 24 h, and mRNA levels were measured with real-time RT-PCR ($P < 0.05$ for all, n = 3–5).

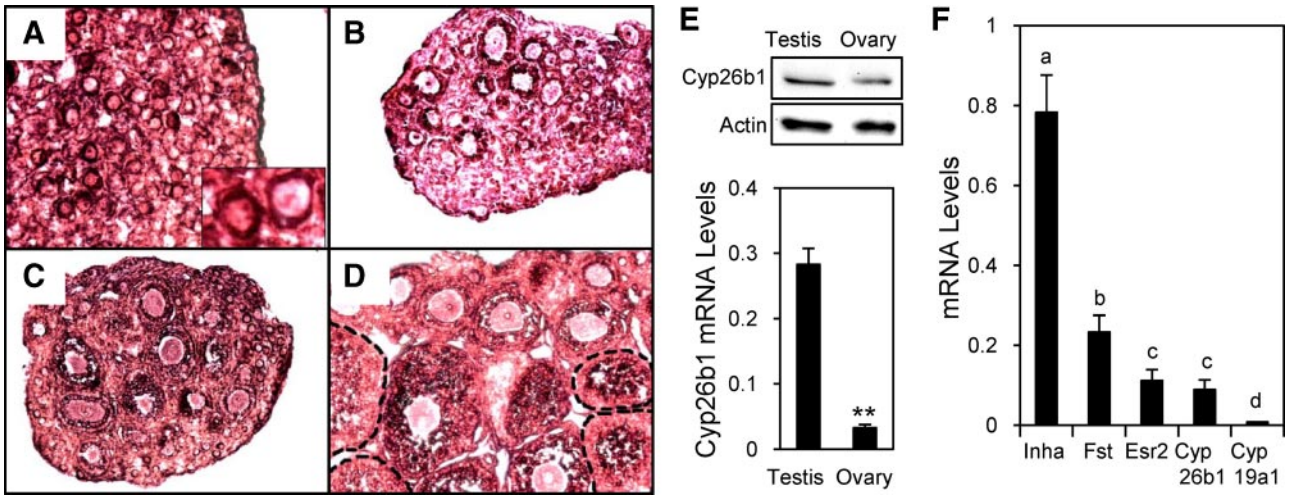

FIG. 3. Localization and relative expression levels of *Cyp26b1* in the mouse ovary. A–D, *In situ* hybridization of *Cyp26b1* in postnatal d 1 (A), 6 (B), 10 (C), and 20 (D) ovaries. *Insert* (A), Enlarged picture of two primordial follicles. E, *top panel*, Western blot showing the *Cyp26b1* protein levels in d 1 testes and ovaries. Actin was detected as a loading control. *Bottom panel,* Comparison of *Cyp26b1* mRNA levels in d 1 ovaries and testes as measured by real-time PCR. **, $P < 0.005$ (n = 4). F, Comparison of *Cyp26b1* mRNA levels with inhibin-α (*Inha*), follistatin (*Fst*), ERβ (*Esr2*), and *Cyp19a1* in d 22 mouse granulosa cells as measured by real-time PCR. Consistent amplification efficiency was observed for the primers for *Inha*, *Fst*, and *Esr2* in the d 22 granulosa cell samples and for *Cyp19a1* in adult ovaries (efficiency = 100 ± 3%, $R^2 = 0.990–0.999$). *Different letters* indicate statistically significant differences, according to ANOVA followed by Tukey's test ($P < 0.05$, n = 3–8).

A novel activin-regulated gene: *Cyp26b1*

Cyp26b1 is the gene that was most significantly down-regulated by activin (−11-fold), suggesting that it may be an important factor mediating the intraovarian functions of activin. It has been shown to play an important role in male germ cell meiosis and survival, but its expression is reduced in the ovary around embryonic d 12.5–13.5 (44, 45). To confirm and further localize the expression of *Cyp26b1* in the postnatal ovary, we performed *in situ* hybridization and showed that *Cyp26b1* mRNA was localized to pregranulosa cells of primordial follicles in the d 1 ovary and to granulosa cells of follicles at all developmental stages in d 6, 10, and 20 ovaries (Fig. 3, A–D). No staining was observed in ovaries probed with a control sense probe for *Cyp26b1* (Supplemental Fig. 4). At postnatal d 1, the mRNA levels of *Cyp26b1* were substantially higher in the testis than in the ovary (Fig. 3E, *bottom panel*); similar results were observed in d 6, 10, and 20 ovaries. The protein levels of *Cyp26b1* were also higher in the testis than in the ovaries (relative intensity 1.51:1), although the difference was not as large as observed for mRNA levels (Fig. 3E, *top panel*). The relative mRNA levels of *Cyp26b1* were compared with select well-characterized ovarian genes in mouse granulosa cells. *Cyp26b1* mRNA was expressed at a comparable level with the ERβ mRNA, the predominant form of ER in the mouse ovary (Fig. 3F). As expected, inhibin-α and follistatin mRNAs showed high levels of expression, while Cyp19A1 (aromatase) mRNA was almost undetectable in the granulosa cells from immature animals due to lack of FSH stimulation (Fig. 3F), consistent with reports by others (62, 63).

Activin regulation of *Cyp26b1* mRNA levels

Activin suppression of *Cyp26b1* mRNA levels was confirmed with real-time PCR analysis of RNA collected from primary cultured granulosa cells at both 4 and 24 h of treatments. This suppression was reversed when an excess amount of follistatin was given together with activin A, and follistatin alone significantly increased *Cyp26b1* mRNA levels because these cells produce endogenous activin (36–38, 64). TGF-β1 also decreased *Cyp26b1* mRNA levels but to a lesser extent (Fig. 4A), suggesting a common effect among related TGF-β superfamily members. The suppressive effect of activin on *Cyp26b1* expression was also observed at the protein level (the relative intensity of *Cyp26b1* protein band in activin *vs.* PBS-treated samples is 1:0.44) (Fig. 4B). To confirm the importance of activin in regulating ovarian *Cyp26b1* levels *in vivo*, we examined *Cyp26b1* expression in a transgenic mouse model that overexpresses the inhibin-α subunit gene from a metallothionein-I promoter (MT-α inhibin) and as a consequence has decreased activin levels (65). These mice show decreased fertility and exhibit a variety of ovarian pathologies (46, 66). The mRNA and protein levels of *Cyp26b1* were significantly increased in the MT-α inhibin transgenic mouse ovary (the relative intensity of *Cyp26b1* protein band in the normal litter mate (NLM) *vs.* MT-α inhibin transgenic mouse ovary is 1:1.64) (Fig. 4, C and D), consistent with a suppressive effect of activin on *Cyp26b1* expression.

In situ hybridization studies revealed that *Cyp26b1* was lost in more mature follicles that express high levels of activin-βA subunit mRNA (Fig. 4, E and F) and that even within a single follicle, the expression of *Cyp26b1* was

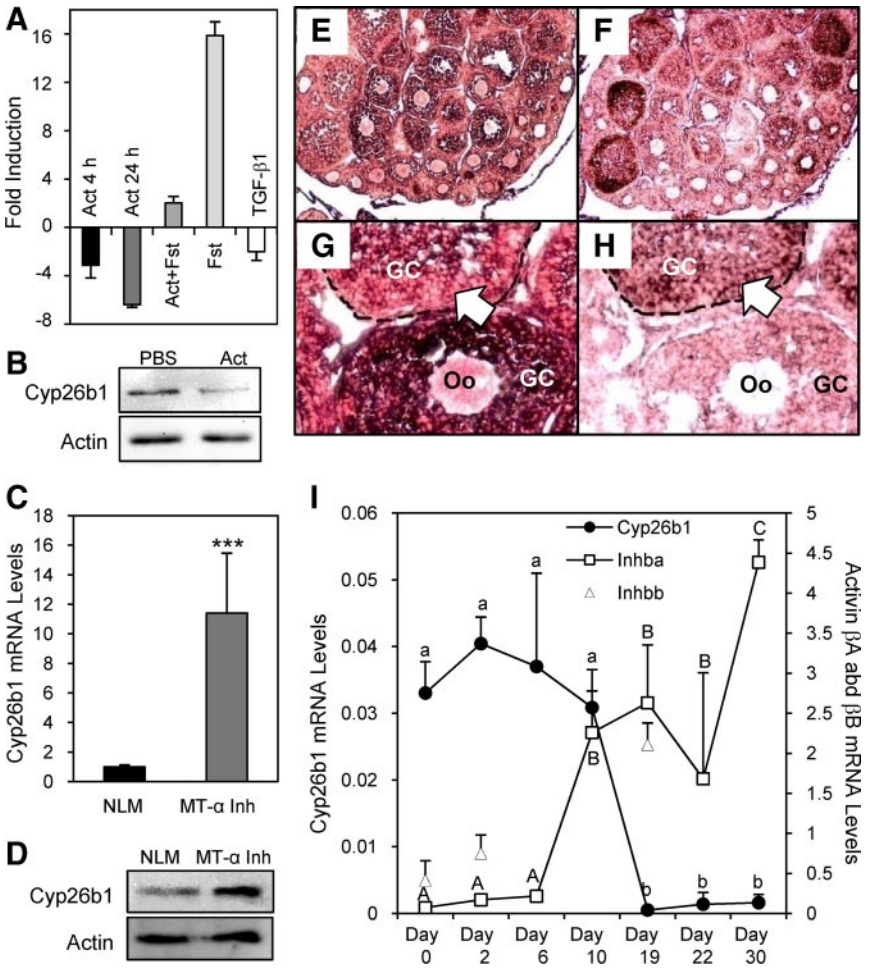

FIG. 4. Activin regulation of Cyp26b1 gene expression and the inverse correlation of *Cyp26b1* and activin βA mRNA expression. A, Effects of activin A (Act), activin A+follistatin (Act+Fst), follistatin (Fst) alone, and TGF-β1 on *Cyp26b1* mRNA levels in primary cultured granulosa cells ($P < 0.05$ for all, n = 3). B, Effect of activin A (Act) on Cyp26b1 protein levels. Actin was detected as a loading control. C, Comparison of *Cyp26b1* mRNA levels in MT-α inhibin transgenic (MT-α Inh) mouse ovaries with those in the NLM ovaries. ***, $P < 0.0001$ (n = 7). D, Western blot pictures showing Cyp26b1 protein levels in NLM and MT-α Inh mouse ovaries. Actin was detected as a loading control. E–H, *In situ* hybridization of Cyp26b1 (E and G) and activin-βA (F and H) in postnatal d 20 ovaries. E and F show lower magnification; G and H show higher magnification. Complementary expression patterns of Cyp26b1 and activin-βA mRNA within one follicle is indicated by *open arrows*. GC, Granulosa cells. Oo, oocytes. I, Comparison of mRNA levels of Cyp26b1, activin-βA (Inhba), and activin-βB (Inhbb) in developing ovaries as measured by real-time PCR. *Different letters* indicate statistically significant differences, according to ANOVA followed by Tukey's test (*lowercase letters* for Cyp26b1; *uppercase letters* for activin βA). $P < 0.05$ (n = 3–5).

decreased in regions in which activin-βA mRNA was highly expressed (Fig. 4, G and H, indicated by *open arrows*). This pattern was more pronounced in d 20 ovaries than in early ovaries, possibly due to low activin production at earlier times (see data below). No staining was observed in ovarian sections probed with a control sense probe for *Cyp26b1* (Supplemental Fig. 5). A striking inverse correlation between *Cyp26b1* expression and activin-βA expression was also observed during postnatal ovary development. *Cyp26b1* mRNA levels remained high from d 0 to d 10 and decreased in d 19–30 ovaries, whereas activin-βA mRNA levels were low from d 0 to d 6 and increased starting at d 10 (Fig. 4I). We have also examined activin-βB mRNA levels in d 0, 2, and 19 mouse ovaries. The results revealed that activin-βB mRNA levels were lower at postnatal d 0 and 2 and increased at d 19, suggesting an inverse correlation between *Cyp26b1* mRNA and activin-βB mRNA levels as well (Fig. 4I). Future studies will also examine the effect of activin B on *Cyp26b1* expression.

RA and the Cyp26 inhibitor R115866 increase mouse granulosa cell proliferation

Cyp26b1 encodes an enzyme that degrades RA, and changes in *Cyp26b1* expression can alter RA levels and result in significant biological consequences (44, 45, 67). To investigate functions of *Cyp26b1* and RA in the ovary, we examined effect of RA on the proliferation of primary cultured mouse granulosa cells. RA significantly stimulated granulosa cell proliferation in a dose-response manner (Fig. 5A). As a control, we also examined the effects of RA on HeLa cell proliferation. Consistent with reports by others (68, 69), RA moderately suppressed HeLa cell proliferation at high concentrations, although the effect was not significant (Supplemental Fig. 6). These results demonstrate that RA is a proliferation-stimulating factor for mouse granulosa cells and suggest that *Cy26b1* may act to suppress granulosa cell proliferation. To examine this hypothesis, we treated primary cultured granulosa cells with the specific Cyp26 inhibitor R115866 (45) and found that R115866 increased granulosa cell numbers in a dose-response manner (Fig. 5B).

A pan-RAR inhibitor attenuates RA and activin-induced granulosa cell proliferation

To examine signaling mechanisms involved in the stimulatory effect of RA, we treated mouse granulosa cells with RA in the presence of the pan-RAR inhibitor AGN193109 (70, 71). AGN193109 completely blocked the stimulatory effect of RA on granulosa cell proliferation (Fig. 6A), whereas no cell toxicity was observed (76 *vs.* 75% cell viability in control and AGN193109 treated groups,

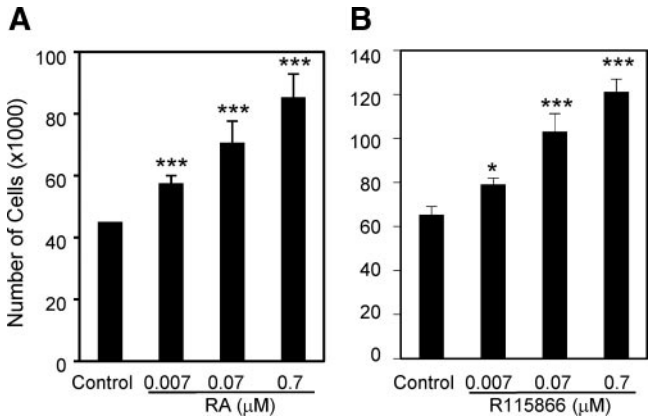

FIG. 5. Effects of RA (A) and the Cyp26 inhibitor, R115866 (B) on proliferation of primary cultured mouse granulosa cells. *, $P < 0.05$; ***, $P < 0.005$ (n = 3–7).

respectively), suggesting that the effect was mediated through RARs. Because activin stimulates mouse granulosa cell proliferation and also suppresses *Cyp26b1* expression, some of the proliferative effects of activin may be mediated by decreased *Cyp26b1*, leading to increased RA levels. We tested this and found that AGN194310 also abolished the stimulatory effect of activin on granulosa cell proliferation (Fig. 6B), indicating an involvement of RAR-mediated signaling in activin-induced granulosa cell proliferation. To examine the specificity of the effect of AGN194310 on activin-induced cell proliferation, we measured the mRNA levels of select activin regulated genes including inhibin-α (55), follistatin (54), ERα (*Esr1*) (47), and *Cyp26b1* after AGN194310 treatment. The mRNA levels of these genes were not altered by AGN194310, suggesting that the drug is not acting as a general inhibitor of activin signaling (Fig. 6C).

Discussion

To understand the intraovarian functions of activin, we used global gene expression profiling to identify networks of genes that are either stimulated or inhibited by activin.

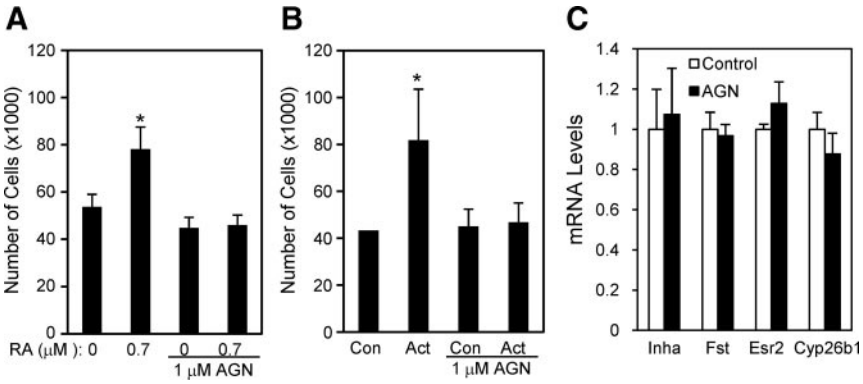

FIG. 6. Effects of the pan-RAR inhibitor, AGN193109, on RA- (A) or activin A (B)-induced proliferation of primary cultured mouse granulosa cells and lack of effect of AGN193109 on select activin target genes (C). Con, Control; Act, activin A. *, $P < 0.05$ (n = 3–5).

By treating primary granulosa cells with activin and activin plus follistatin, we were able to identify a group of genes whose expression is significantly regulated by activin compared with PBS and whose regulation is abolished or reversed when activin is given together with excess follistatin (by comparing activin plus follistatin *vs.* activin). Because excess follistatin was used in the study, it is likely that endogenous activin signaling was suppressed. Treatment with follistatin alone would help to further distinguish the extent to which endogenous activin signaling contributes to follistatin regulation of the activin regulated genes that we have identified.

Stimulatory effects of activin on granulosa cell proliferation have been reported previously in rat (30) and cow (72), although it has also been reported that proliferation and differentiation effects of activin in rat are not apparent unless FSH is present (73). Our study revealed a stimulatory effect of activin on proliferation of primary cultured mouse granulosa cells. We did not detect any changes in most of the key proteins involved in the execution of apoptosis, the caspases, with the exception of a decrease in caspase-6 expression (−1.75 fold) after activin treatment, suggesting suppression of apoptosis in the mouse granulosa cells. It has been reported that activin suppresses apoptosis in granulosa cells of cultured preantral follicles (74), although it induces apoptosis in liver cells (75), B cells (76, 77), and decidual cells (78).

The microarray study revealed that *Cyp26b1* is expressed in postnatal granulosa cells and is the gene most significantly suppressed by activin. It has been reported that *Cyp26b1* expression is largely lost in female embryonic gonads around embryonic d 12.5, and this is believed to be critical for maintaining proper RA levels and ultimately allowing meiosis to progress (44, 45). Expression of *Cyp26b1* in the postnatal ovary has not been previously investigated. Although *Cyp26b1* mRNA is expressed in much less abundance in the ovary than in the testis, it can be detected in pregranulosa cells at d 1 and is maintained at relatively high levels in the ovary during the first 10 d after birth before decreasing by d 19. The higher expression levels of *Cyp26b1* during the first 10 d after birth coincide with active germ cell nest breakdown, massive oocyte apoptosis, somatic cell proliferation, and establishment of the initial follicle pool (3). Activin has been shown to be involved in early follicle formation and development (39, 65), and here we demonstrate that it is a regulator of *Cyp26b1*, suggesting that *Cyp26b1* may mediate some of activin's actions in the ovary.

The suppressive effect of activin on Cyp26b1 expression was detected not only at 24 h treatment but also at 4 h

treatment, suggesting a rapid regulation. Activin signals predominantly through Smad proteins, and Smads 3 and 4 can directly bind to Smad binding elements to regulate target gene expression (79). We have identified multiple potential Smad binding elements in the promoter region of the Cyp26b1 gene. However, activin can also function through other signaling pathways. Related to our finding that activin regulates the MAPK pathway, the MAPK pathway can in turn regulate Smad proteins. The nuclear factor-κB or phosphatidylinositol 3-kinase/AKT pathways can modulate or be activated by Smad pathways as well (80). Whether activin regulation of Cyp26b1 represents a direct transcriptional effect or an indirect effect through other signaling pathways requires further study.

In *Cyp26b1* knockout mice, male germ cells undergo massive apoptosis when meiosis takes place prematurely at embryonic d 13.5 in the testis, whereas female germ cell numbers appear to be normal at birth in the ovary (67). This observation is not surprising because *Cyp26b1* expression is already absent in the normal embryonic ovary at embryonic d 12.5 and meiosis can be initiated normally. No further analysis of postnatal ovary development was carried out in the *Cyp26b1* knockout mice because they die shortly after birth. We observed that the Cyp26 inhibitor R115866 stimulated granulosa cell proliferation, suggesting that *Cyp26b1* inhibits granulosa cell proliferation in the ovary. The stimulatory effect of R115866 on granulosa cell proliferation is consistent with the moderate stimulatory effect of activin on granulosa cell proliferation, suggesting that activin may induce cell proliferation in part through suppressing *Cyp26b1* expression. These observations suggest that in addition to its recently reported roles in determining male germ cell fate and survival in embryos, *Cyp26b1* may be a novel factor that mediates the autocrine/paracrine functions of activin in regulating ovarian follicle formation and development in the postnatal ovaries.

The importance of RA in ovary development has been suggested by several studies. Knockout of stimulated by retinoic acid gene 8 (*Stra8*), a RA-inducible gene whose expression is negatively regulated by *Cyp26b1*, results in depletion of oocytes at 8 wk after birth (81). Vitamin A and 9-*cis*-RA stimulate oocyte development and maturation (82, 83). In this study, we discovered that RA is a potent stimulator of granulosa cell proliferation. The pan-RAR inhibitor, AGN194310, abolished the stimulatory effect of either RA or activin on granulosa cell proliferation, indicating an involvement of RAR-mediated signaling in both RA- and activin-induced granulosa cell proliferation.

Overall, this study provides new insights into activin functions in the female reproductive system. It demonstrates that *Cyp26b1* is expressed in the postnatal mouse ovary and is regulated by activin and suggests that the activin may regulate RA pathways to modulate mouse granulosa cell proliferation and ovarian functions.

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