Activins regulate 17β-hydroxysteroid dehydrogenase type I transcription in murine gonadotrope cells

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Abstract

Activins are pleiotropic members of the TGFβ superfamily and were initially characterized based on their abilities to stimulate FSH synthesis and secretion by gonadotrope cells of the anterior pituitary gland. Here, we identified the gene encoding the steroidogenic enzyme, 17β-hydroxysteroid dehydrogenase type I (17β-HSD1; *Hsd17b1*), as an activin-responsive gene in immortalized gonadotrope cells, LβT2. 17β-HSD1 catalyzes the conversion of estrone to the more active 17β-estradiol, and activin A stimulated an increase in this enzymatic activity in these cells. We demonstrated that activins signaled via the type I receptor, activin receptor-like kinase (ALK4), and the intracellular signaling protein, SMAD2, to regulate *Hsd17b1* transcription in immediate-early fashion. Critical *cis*-elements, including a minimal SMAD-binding element, were mapped to within 100 bp of the start of transcription. Activin/ALK4 signaling also regulated *Hsd17b1* transcription in both immortalized and primary cultured murine granulosa cells. The promoter regions mediating basal and activin/ALK4-regulated promoter activity were generally conserved across the different cell types. The data show that activin A rapidly regulates *Hsd17b1* transcription in gonadotrope and granulosa cells and may thereby regulate local 17β-estradiol synthesis.

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Introduction

Activins, members of the transforming growth factor-β (TGFβ) superfamily, were initially purified and characterized based on their abilities to selectively stimulate FSH secretion by primary pituitary cell cultures (Ling *et al.* 1986*a*,*b*, Vale *et al.* 1986). The ligands were subsequently shown to act largely in an autocrine/paracrine fashion in gonadotrope cells to regulate transcription of the FSHβ (*Fshb*) subunit gene, the rate-limiting step in the synthesis of dimeric FSH (Attardi & Miklos 1990, Weiss *et al.* 1995, Pernasetti *et al.* 2001, Suszko *et al.* 2003). Activins' actions are not limited to the pituitary as they play important pleiotropic roles in a variety of tissues during development and in different physiological and pathophysiological processes in adulthood (Matzuk *et al.* 1996, Reis *et al.* 2004, Werner & Alzheimer 2006). Most recently, activins were implicated in maintaining pluripotency of the human embryonic stem cells in culture (Beattie *et al.* 2005, Xu *et al.* 2008).

In addition to their effects on FSH synthesis, activins also regulate transcription of other genes in the gonadotrope cells, including the type I GnRH receptor (*Gnrhr*) and the activin bioneutralizing protein, follistatin (*FST*) (Fernandez-Vazquez *et al.* 1996, Blount *et al.* 2008). These effects provide a means for activins to regulate gonadotropin synthesis and secretion indirectly, in addition to their direct effects on the *Fshb* subunit. To gain a better appreciation for the range of activin effects on gonadotrope cell function, we examined the patterns of gene expression in control and activin A-treated immortalized murine gonadotrope cells, LβT2, using cDNA microarrays. Similar analyses have recently been reported by others (Mazhawidza *et al.* 2006, Zhang *et al.* 2006).

Consistent with one of the previous reports (Zhang *et al.* 2006), we observed a marked upregulation of the mRNA encoding the steroidogenic enzyme 17β-hydroxysteroid dehydrogenase type I (17β-HSD1; *Hsd17b1*) in these cells. Activin A was previously shown to stimulate *Hsd17b1* mRNA expression in isolated rat granulosa cells (Ghersevich *et al.* 2000). Because 17β-HSD1 catalyzes the conversion of estrone (E_1) to the more biologically active estrogen, 17β-estradiol (E_2), the data suggested a mechanism whereby activins might alter gonadotrope sensitivity to estrogen feedback. Indeed, activin A was reported to enhance E_1 regulation of an estrogen-responsive promoter–reporter in

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LβT2 cells (Zhang *et al.* 2006). Here, we investigated the intracellular signaling mechanisms through which activin A regulates *Hsd17b1* transcription in LβT2 cells and then examined the conservation of these mechanisms in immortalized and primary murine granulosa cells.

Materials and Methods

Reagents

Activins A, B, and AB were purchased from R&D Systems (Minneapolis, MN, USA). Inhibin A was from DSL (Webster, TX, USA). Follistatin-288 was either purchased from R&D or generously provided by Dr Tom Thompson (University of Cincinnati). SB431542 and cycloheximide were from Sigma. SMAD2, SMAD4, and C/EBPα antibodies were purchased from Santa Cruz Biotechnology Inc. The phospho-SMAD2 antibody was from Cell Signaling Technology (Danvers, MA, USA). *SMAD4* and control siRNAs were from Dharmacon. DMEM, Dulbecco's PBS, and gentamycin were from Wisent Bioproducts (St. Bruno, QC, Canada). DMEM/F12 was from Fisher-Hyclone (Logan, UT, USA). FBS, Trizol, and Lipofectamine 2000 were from Invitrogen (Carlsbad, CA, USA).

Constructs

To generate murine *Hsd17b1* promoter–reporters, NIH3T3 cell genomic DNA was subjected to PCR using *Pfu* Taq (Stratagene, Cedar, Greek, TX, USA) and the five sense primers listed in Table 1, each with the −13/+9 antisense primer. The start of the transcription (+1) was determined by comparing available murine *Hsd17b1* cDNA (GenBank accession # NM_010475) and genomic sequences (Chr. 11; GenBank accession # AC069014). *Mlu1* and *Xho1* restriction sites (underlined) were included in the primers for cloning purposes. PCR products and the destination vector, pGL3-Basic (Promega), were digested with *Mlu1* and *Xho1* and ligated with T4 DNA ligase (Promega). The −64/+9 *Hsd17b1* reporter was generated using −106/+9 *Hsd17b1*-luc as PCR template. Mutant *Hsd17b1* reporters were produced by PCR-based site-directed mutagenesis of the −106/+9 *Hsd17b1*-luc construct using the mutagenesis primers in Table 1 and the QuikChange protocol (Stratagene). SMAD2 and SMAD3 shRNAs, and SMAD2, SMAD3, and activin receptor-like kinase (ALK4)-T206D expression vectors were described previously (Bernard 2004, Lamba *et al.* 2006). pcDNA3·0 was from invitrogen. The D268A mutation in ALK4-T206D was introduced by site-directed mutagenesis.

Cell line cultures

LβT2 cells were provided by Dr Pamela Mellon (University of California, San Diego, CA, USA) and were cultured in (DMEM, Wisent Bioproducts) supplemented with 10% (v/v) FBS (Invitrogen) and 4 μg/ml gentamycin (Wisent) at 37 °C/5% CO_2. For transfection experiments, cells were seeded in 24-well plates at a density of $2{\cdot}5\times10^5$ cells/well ~72 h prior to transfection. For RNA extractions, cells were plated in 6-well plates at a density of $0{\cdot}5–1\times10^6$ cells/well and allowed to grow to 80–90% confluence before treatment. For nuclear protein extractions, cells were seeded in 10 cm dishes and treated when 70–100% confluent. KK-1 cells were provided by Dr Stephen Franks (Imperial College of London) and were cultured in DMEM/F12 supplemented with 10%

Table 1 PCR primers, and gel shift and DNA-affinity pull-down (DNAP) probe sequences

PCR primers (restriction sites are underlined)	
−1019/−998 sense	5′ GCGACGCGTGACCTCTCCAAGAAGATCTTCC3′
−517/−496 sense	5′ GCGACGCGTAACCTCAGCTCCATAAGGAGTC 3′
−253/−232 sense	5′ GCGACGCGTAACCTACCGATCTAGCTGCTAC 3′
−106/−85 sense	5′ GCGACGCGTCCTTGAGATTGCCAGCAGACAC 3 ′
−64/−44 sense	5′ GCGACGCGTTGGGCAGGAGCAGAGCCAAGC 3′
−13/+9 antisense	5′ GCGCTCGAGAGCAAGCAAGCGAGCATGAAGG 3′
Mutagenesis primers (sense strand only)	
−89/−90 (Chen *et al.*) mut *Hsd17b1*	5′ CCTTGAGATTGCCAGCCTACACAACAAGGGGTGG 3′
−87/−85mut *Hsd17b1*	5′ GAGATTGCCAGCAGAGGGAACAAGGGGTGGGG 3′
−100/−98mut *Hsd17b1*	5′ CTTACGCGTCCTTGATCGTGCCAGCAGACACAAC 3′
ALK4-D268A	5′TTGGGTTTATTGCTGCTGCCAATAAAGACAATGGCACC 3′
qRT-PCR primers	
Hsd17b1 (sense)	5′ GTTATGAGCAAGCCCTGAGC 3′
Hsd17b1 (antisense)	5′ AAGCGGTTCGTGGAGAAGTA 3′
Rpl19 (sense)	5′ CGGGAATCCAAGAAGATTGA 3′
Rpl19 (antisense)	5′ TTCAGCTTGTGGATGTGCTC 3′
EMSA and DNAP probes (sense strand only)	
−106/−77 *Hsd17b1*	5′ CCTTGAGATTGCCAGCAGACACAACAAGGG 3′
−86/−57 *Hsd17b1*	5′ ACAACAAGGGGTGGGCCGCTGTGGGCAGG 3′
Southwestern probes (sense strand only)	
−106/−77 *Hsd17b1*	5′ CCTTGAGATTGCCAGCAGACACAACAAGGG 3′
−569/−543 *Id3*	5′ CATTGTAACCTCAGCTTCACCGCAAT 3′

FBS, and 4 μg/ml gentamycin at 37 °C/5% CO_2. For transfection experiments, cells were seeded in 24-well plates at a density of 10^5 cells/well, ~24 h prior to transfection.

Transfections and luciferase assays in cell lines

Reporter and expression plasmids were transfected at 450 and 100 ng/well respectively, using Lipofectamine 2000 as per the manufacturer's instructions. Total amounts of DNA were balanced across treatments. Approximately 24h post transfection, cells were washed with PBS and then treated with 25 ng/ml activin A, B, or AB in DMEM or with DMEM alone. For inhibitor experiments, 10 μM SB431542 was introduced 30 min prior to treatment with activins. For time-course experiments, activin A was introduced at the indicated number of hours, prior to protein extraction. After the treatments, cells were washed with cold PBS and proteins were extracted using 100 μl/well of 1× passive lysis buffer (Promega). Twenty microliters of each sample were assayed for luciferase activity using an Orion II Microplate Luminometer (Berthold Detection Systems, Bad Wildbad, Germany). All experiments were performed in duplicate or triplicate a minimum of three times.

Thin layer chromatography

17β-HSD1 activity was measured using a method based on that previously described in Singh & Reed (1991). In brief, LβT2 cells were plated in 24 well plates and cultured in growth media until 70–80% confluent. Cells were then washed and incubated overnight in media plus 2% (v/v) FBS in the presence or absence of 25 ng/ml activin A. The intact monolayers were then incubated at 37 °C with 5 nM tritiated [6,7-^{3}H] E1 (50 Ci/mmol, NEN-Perkin-Elmer, Waltham, MA, USA) for the indicated times. Blank incubations were carried out in parallel by incubating labeled steroid in cell-free wells. After incubation, 0·5 ml of the medium was removed and diethyl ether (2 ml) was used to extract steroids from the medium. The diethyl ether was transferred to a new glass tube and the organic layer was dried under nitrogen. Steroids were separated chromatographically on thin layer plates (Silica Gel IB-F, JT Baker, Phillipsburg, NJ, USA) in dichloromethane/ethyl acetate (4:1, v/v), and the radioactivity was measured using a scanning radiometer (System AR2000, Bioscan Inc., Washington, DC, USA). The percentage conversion of E_1 to E_2 was calculated by dividing the radioactive counts identified as E_2 by the total counts associated with E_1 plus E_2. All treatments were performed in triplicate and the experiment was repeated at least twice.

Quantitative RT-PCR

LβT2 cells were washed with PBS and then treated with serum-free DMEM ±25 ng/ml activin A for the indicated periods of time. When included, cycloheximide (5 μg/ml) or SB431542 (10 μM) was applied for 15 or 30 min respectively, prior to the addition of activin A. The cells were then washed with PBS and total RNA was extracted using Trizol following the manufacturer's instructions. Concentrations were determined by spectrophotometry. One microgram of each RNA sample was DNased using RQ1 DNase, and reverse transcribed into cDNA using 50 ng random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Promega). Each reverse transcription reaction was diluted 1:10 for PCR analysis. A relative five-point standard curve was prepared using 1:10 serial dilutions of cDNA from a sample with high *Hsd17b1* expression. The PCR was performed using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) and 0·4 pmol of each primer (see Table 1) on a Corbett Rotor-Gene 6200 HRM, as per Invitrogen's real-time cycling protocol. PCR primers were designed to span intron sequences to distinguish amplified mRNA (cDNA) from contaminating genomic DNA. Melting curve analysis confirmed amplification of a single amplicon. The *Hsd17b1* primers had an efficiency between 0·7 and 1·0 for all experiments. All samples were analyzed in triplicate, and each experiment was performed at least twice. *Hsd17b1* transcripts levels were normalized by a similar qPCR analysis of the housekeeping gene ribosomal protein L19 (*Rpl19*) on the same cDNA samples, but run in separate tubes. The *Rpl19* primers are shown in Table 1 and amplified with an efficiency of 0·7–1·0.

Electrophoretic mobility shift assays

LβT2 cells were washed with PBS and treated with serum-free DMEM ±25 ng/ml activin A for the indicated periods of time. The cells were then washed with cold PBS, and nuclear protein lysates were prepared as described previously (Lamba *et al.* 2006). Protein concentrations were measured by Bradford assay (Biorad). Following the collection of nuclear extracts, gel shift experiments were performed as previously described (Lamba *et al.* 2006), with minor modifications to the protocol. Two micrograms of nuclear proteins were incubated with 50 fmol of ^{32}P γ-ATP (Perkin-Elmer) end-labeled double-stranded probes corresponding to −106/−77 and −86/−57 (Table 1) of the murine *Hsd17b1* promoter for 20 min at room temperature. Binding reactions were incubated with the following components: 25 mM HEPES (pH 7·2), 150 mM KCl, 5 mM dithiothreitol (DTT), 10% (v/v) glycerol, and either 500 ng salmon sperm DNA or 1 μg polydIdC. In competition experiments, reactions were incubated for 10 min at room temperature with 100-fold molar excess unlabeled competitor probes prior to the addition of the radio-labeled probe.

Ion exchange chromatography

LβT2 cells (3×10^9) were harvested for preparation of nuclear protein extracts using the protocol in Yaneva & Tempst (2006) with some modifications. In brief, LβT2 cells were collected and centrifuged at 800***g*** for 10 min at 4 °C. The pellet was

washed twice with 5 ml cold PBS, then resuspended in buffer A (10mM Tris–HCl, pH 8, 10 mM KCl, 0·1 mM EDTA, 0·1 mM EGTA, 1 mM DTT, 0·5 mM PMSF, 1 μg/ml aprotonin, 1 μg/ml pepstatin A, and 0·2 mM sodium vanadate), and transferred to a Dounce homogenizer. Cells were homogenized with 15 strokes of the pestle and incubated for 15 min on ice. Lysates were centrifuged at 3000 ***g*** for 15 min at 4 °C, supernatant aspirated, and the pellet resuspended in minimal volume of low-salt extraction buffer (20 mM Tris–HCl, pH 7·5, 20 mM KCl, 1·5 mM $MgCl_2$, 0·2 mM EDTA, and 25% (v/v) glycerol). Lysates were homogenized with 15 more strokes of the pestle, then transferred to a small beaker with a magnetic stir bar. The solution was gently stirred at 4 °C, while 0·5 volumes of high-salt extraction buffer (20 mM Tris–HCl, pH 7·5, 1·2 M KCl, 1·5 mM $MgCl_2$, 0·2 mM EDTA, and 25% glycerol (v/v)) were added drop wise. The solution was stirred at 4 °C for an additional 30 min, then transferred to 1·5 ml tubes and centrifuged at 15 000***g*** for 30 min at 4 °C. Supernatants were collected and dialyzed overnight in 50 volumes of low-salt buffer D (20 mM HEPES-KOH pH 7·9, 0·2 mM EDTA, 10% glycerol (v/v), 0·01% Nonidet-P40 (v/v), and 0·075 M NaCl) in preparation for ion exchange chromatography. A HiTrap SP Sepharose FF (GE Healthcare) column was used to fractionate protein lysates. Proteins were eluted with a linear gradient of 0·075–0·85 M NaCl in buffer D, over 20 column volumes, at a flow rate of 0·4 ml/min. Fourteen microliters of alternating fractions were analyzed for binding activity by gel shift using the −106/−77 probe. Active fractions were pooled and concentrated using Amicon Ultra centrifuge tubes (Millipore).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was carried out using the ChIP-IT Express kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. LβT2 cells were grown to 70–80% confluence in 10 cm plates, and either untreated or treated with 25 ng/ml activin A for 1 h. The cells were fixed using 10 ml of fixation solution per plate (0·27 ml of 37% formaldehyde in 10 ml of cell culture medium), and incubated for 10 min on a shaker at room temperature. The cells were washed with 10 ml cold 1× PBS followed by 10 ml of glycine stop-fix solution. The cells were then scraped in 1·2 ml of cell scraping solution and collected in 15 ml conical tubes on ice. Cells were centrifuged for 10 min at 2500 r.p.m. at 4 °C. Supernatants were discarded and pellets were resuspended in 1 ml cold lysis buffer, followed by incubation on ice for 30 min. The cells were transferred to a Dounce homogenizer on ice, and homogenized with 10 strokes. Lysates were transferred to 1·5 ml microcentrifuge tubes and centrifuged at 3500 r.p.m. for 10 min at 4 °C. Supernatants were discarded, pellets resuspended in 200 μl of digestion buffer and incubated at 37 °C for 5 min. Ten microliters of enzymatic shearing cocktail were added to each pellet, and samples were vortexed followed by incubation for 15 min at 37 °C. The reaction was stopped by adding 4 μl of ice-cold 0·5 M EDTA, and chilling on ice for 10 min. Samples were centrifuged at 15 000 r.p.m. at 4 °C for 10 min. Supernatants (containing the sheared chromatin) were collected for immunoprecipitation.

Ten microliters of the chromatin from each plate were put aside as 'input DNA'. ChIP reactions were set up as follows: 25 μl protein G magnetic beads, 10 μl ChIP buffer 1, 25 μl sheared chromatin, 1 μl protease inhibitor cocktail, and 3 μg SMAD2 (Zymed, Cat. # 51–1300) or rabbit IgG (Millipore, 12–370) in a final volume of 100 μl. Reactions were incubated overnight at 4 °C on the rotator. Magnetic beads were collected on magnets, and supernatants discarded. The beads were washed once with 800 μl ChIP buffer 1, and twice with 800 μl ChIP buffer 2. The final wash was removed, and the beads were resuspended in 50 μl elution buffer AM2. Beads were incubated at room temperature for 15 min while flicking the tubes to mix. Fifty microliters of reverse cross-linking buffer were added and tubes were placed on a magnetic stand. Supernatants (containing chromatin) were transferred to 0·2 ml PCR tubes. 'Input DNA' samples were mixed with 88 μl ChIP buffer 2, and 2 μl of 5 M NaCl (final volume 100 μl). ChIP and 'Input DNA' samples were incubated at 95 °C for 15 min. Samples were cooled to room temperature, and 2 μl proteinase K (0·5 μg/μl) was added. Samples were incubated at 37 °C for 1 h. Tubes were cooled to room temperature and 2 μl proteinase K stop solution was added. Five microliters of each template were analyzed using the GoTaq PCR Core system (Promega), in a final volume of 25 μl. Fifteen microliters of each reaction were subject to electrophoresis on a 2% (w/v) agarose gel, using the −253/−232 sense and −13/+9 primers in Table 1.

DNA-affinity pull-down

Fifty microliters of M-280 streptavidin-coated Dynabeads (Invitrogen) were washed twice in 500 μl of 2× B&W buffer (10 mM Tris–HCl pH7·5, 1 mM EDTA, and 2 M NaCl). Five hundred microliters of 1× B&W buffer were added, along with 20 μl of 10 μM double-stranded biotinylated −106/−77 probe. Beads were incubated with probe for 30 min at room temperature with gentle mixing. Beads were then washed thrice in 500 μl of 1× B&W buffer, and once in 500 μl of 1× binding buffer (20 mM Tris–HCl, pH 7·5, 1 mM EDTA, 1 mM DTT, 5% glycerol (v/v), 100 mM NaCl, 0·15% Triton X-100 (v/v), and 4 mM $MgCl_2$). Beads were incubated in 500 μl of 1× binding buffer containing 1% BSA (w/v) for 30 min at room temperature with gentle mixing. Beads were collected on a magnet and resuspended in 50 μl of 1× binding buffer. Ninety microliters of 5× binding buffer, 10 μl of 1 μg/μl poly dIdC, and 100 μg LβT2 whole-cell lysates (in 100 μl lysis buffer: 300 mM NaCl, 20 mM Tris–HCl, pH7·5, 1% Triton X-100 (v/v), 1 mM PMSF, 20 μg/ml leupeptin, and 20 μg/ml aprotonin) in a total volume of 500 μl were added. The beads were incubated at

4 °C with gentle mixing overnight and then washed five times in 1× binding buffer, collected in 25 µl of loading buffer, and boiled at 100 °C for SDS-PAGE (10% Bis–Tris). The proteins were transferred to nitrocellulose membranes. Western blots were performed as described previously (Bernard 2004).

Southwestern blots

Protein fractions from ion exchange chromatography that showed binding activity in gel shifts were denatured and run on SDS-PAGE (12% Bis–Tris) under reducing conditions, followed by transfer onto nitrocellulose membranes. Membranes were incubated in renaturing buffer (20 mM HEPES pH7·9, 50 mM KCl, 1 mM DTT, 10% glycerol (v/v), and 0·1% Nonidet P-40 (v/v)) for 45 min at room temperature on a rocking platform. The membrane was then incubated for 3 h at room temperature in blocking buffer (10 ml of renaturing buffer containing 5% (w/v) nonfat milk). The blots were then cut into sections and incubated in the appropriate hybridization mix. Hybridization mixes were prepared by adding 10^6 c.p.m./ml of −106/−77 radiolabeled probe alone or with 100-fold molar excess of unlabeled homologous or heterologous competitor probes (Table 1) to renaturing buffer containing 0·5% nonfat milk (w/v) and 5 µg/ml of polydIdC or 5 µg/ml ssDNA. Blots were hybridized for 1 h at room temperature, followed by three washes in binding buffer, 20 min per wash. Membranes were blotted dry with Whatman paper, wrapped in plastic wrap, and exposed on X-ray film at −80 °C with intensifying screens.

Primary granulosa cell culture

Cluster of differentiation-1 mice (*Mus musculus*) were killed on postnatal days 22–23, and ovaries from 10 to 20 animals were pooled for granulosa cell collection. Granulosa cells were collected through follicle puncture as previously described with slight modification (Kipp *et al.* 2007). Oocytes were filtered out with a 40 µm cell strainer (BD Falcon, Bedford, MA, USA). Granulosa cells were cultured in a humidified incubator at 37 °C/5% CO_2 in a DMEM/F12 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% FBS (v/v) (Invitrogen) for 3 days before transfection. All animals were handled in accordance with institutional and federal guidelines.

Primary granulosa cell transfection and luciferase assays

Transient transfections were performed with 450 ng of pGL3-basic or the indicated murine *Hsd17b1* constructs plus 100 ng of pcDNA3·0 or ALK4-T206D per well of a 24-well culture plate using cationic liposomes in 500 µl Opti-MEM (Invitrogen; Burkart *et al.* 2005). After 6 h of transfection, cells were allowed to recover in the medium without serum for 16–20 h. At the end of recovery, cells were washed with PBS and lysed on ice for 20 min. The lysis buffer contained 25 mM HEPES, pH 7·8, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT, and 0·1% Triton X-100 (v/v). Cell lysates (100 µl) were then added to 400 µl of reaction buffer (25 mM HEPES (pH 7·8), 15 mM $MgSO_4$, 4 mM EGTA, 2·5 mM ATP, 1 mM DTT, 1 µg/ml BSA, and 100 µl of 1 mM luciferin (sodium salt) (Analytical Bioluminescence, San Diego, CA, USA)) and emitted luminescence measured using a 2010 luminometer (Analytical Bioluminescence) for 10 s. Relative light units were normalized for total protein content measured with the Bio-Rad protein assay reagent, and data presented as fold change from the promoter-less vector in the presence of pcDNA3·0.

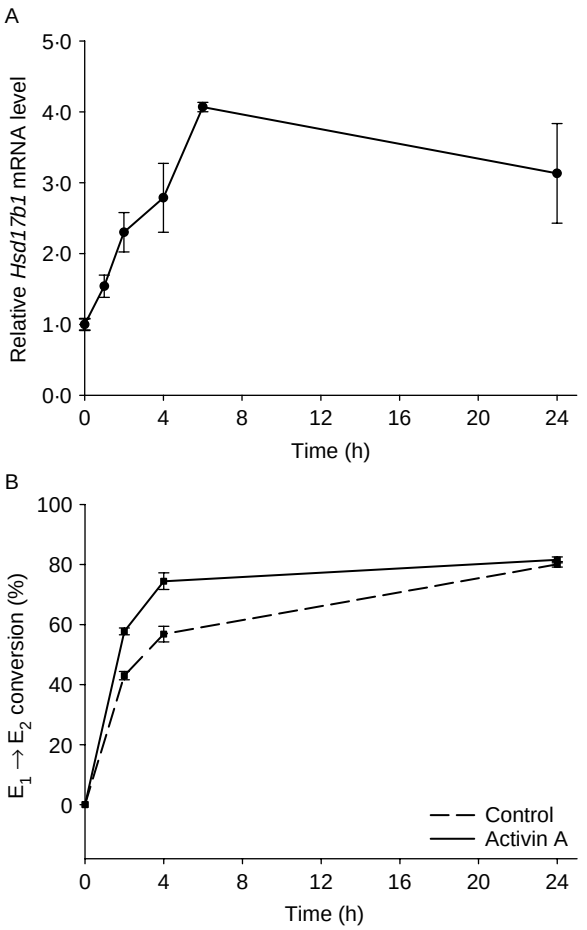


Figure 1 (A) LβT2 cells were treated with 25 ng/ml of activin A for 0, 1, 2, 4, 6, and 24 h. *Hsd17b1* mRNA levels were measured by qRT-PCR. Data are the mean relative mRNA levels (±S.D.) with the control condition (no treatment) set to 1. The analysis was repeated twice with comparable results. (B) LβT2 cells were incubated in control or activin A-containing media overnight (25 ng/ml). The percent conversion of estrone (E_1) to estradiol (E_2) was measured by TLC as described in the Materials and Methods. All treatments were performed in triplicate.

Results

Activin A stimulates an increase in Hsd17b1 mRNA and 17β-HSD1 enzymatic activity

Total RNA from LβT2 cells cultured in serum-free DMEM for 24 h in the presence or absence of 25 ng/ml activin A was subjected to analysis with Affymetrix 430 gene arrays. Based on the results of duplicate samples, the gene for the steroidogenic enzyme 17β-HSD1 (*Hsd17b1*) appeared to be upregulated to the greatest extent (data not shown). Quantitative RT-PCR analysis of LβT2 cells treated with activin A confirmed a rapid and sustained induction of *Hsd17b1* mRNA (Fig. 1A). *Hsd17b1* mRNA expression in adult male and female mouse pituitaries was verified by RT-PCR (data not shown).

To determine whether the increased mRNA was associated with increased protein levels, we measured 17β-HSD1 enzymatic activity in LβT2 cells. After overnight incubation with 1 nM activin A, cells were incubated with 5 nM tritiated E_1 and conversion to E_2 measured by thin-layer chromatography after 2, 4, or 24 h. By 24 h, LβT2 cells in both conditions had converted 80% of E_1 to E_2. At the earlier time points, however, activin A shifted the curve to the left relative to control, indicative of enhanced 17β-HSD1 enzymatic activity (Fig. 1B).

Activin A directly stimulates Hsd17b1 transcription through a proximal promoter region

Activin A induction of *Hsd17b1* mRNA levels was rapid, with increases observed within 1 h (Fig. 1A). This suggested that *Hsd17b1* might be an immediate-early response gene. To test this idea, we pretreated the cells with the translation inhibitor, cycloheximide, for 15 min prior to activin A treatment for 1 h. Cycloheximide affected neither basal nor activin A-stimulated *Hsd17b1* mRNA levels (Fig. 2A), indicating that protein synthesis was not required for the activin A effect. To further confirm that activin A regulates *Hsd71b1* at the transcriptional level, we ligated −1019 to +9 of the murine *Hsd71b1* 5′ flanking region upstream of a luciferase reporter gene. When transfected into LβT2 cells, the reporter conferred significant basal and activin A-stimulated activity relative to the promoter-less parental vector (data not shown). 5′ deletions to −517, −253, or −106 did not significantly alter reporter activity; however, truncating the reporter to −64 abolished both the basal and activin A-dependent activity (Fig. 2B and data not shown).

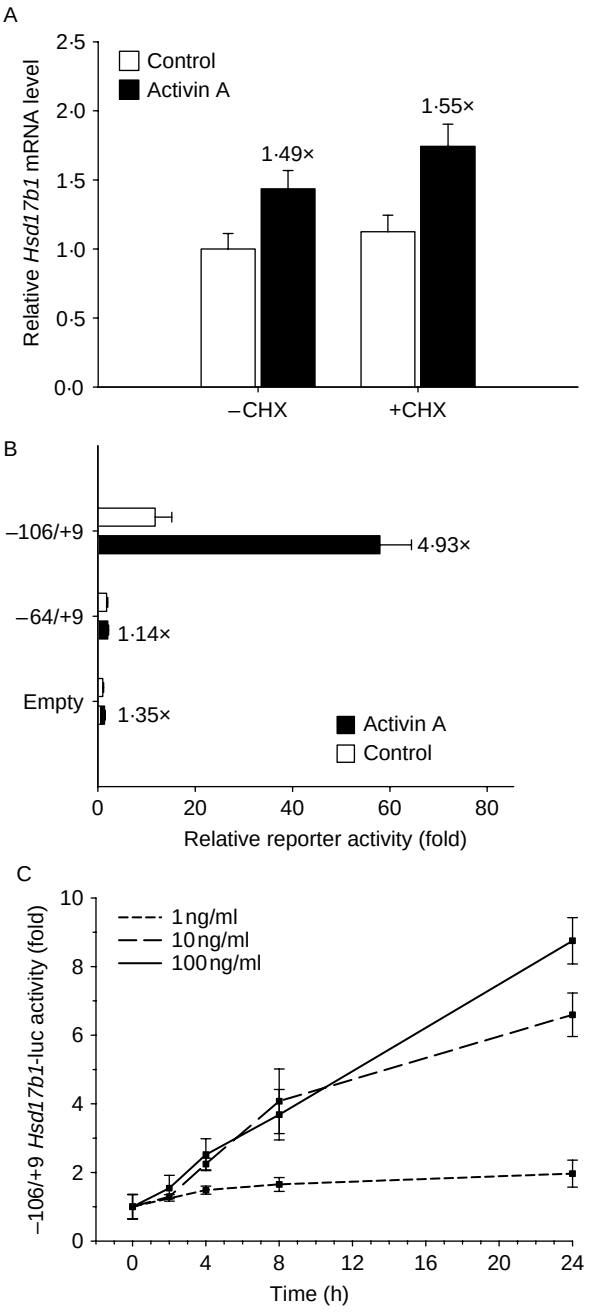
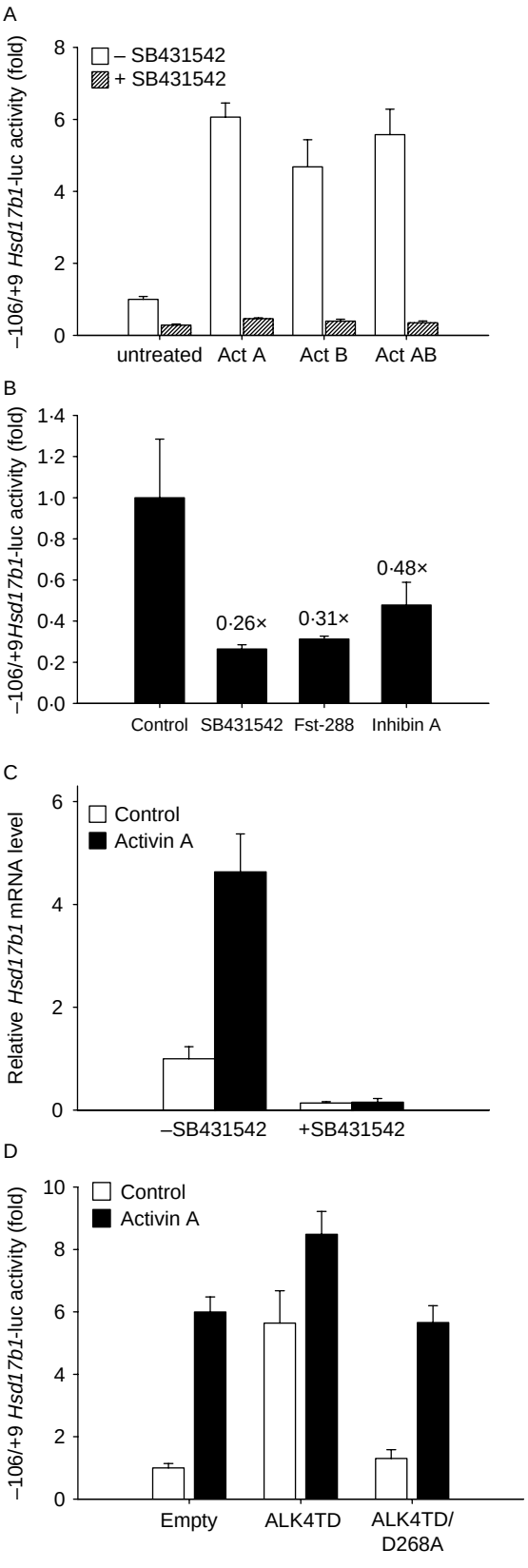


Figure 2 (A) LβT2 cells were treated with 5 μg/ml cycloheximide (CHX) 15 min prior to 1 h treatment with 25 ng/ml activin A. *Hsd17b1* mRNA levels were measured by qRT-PCR. Results were normalized to the untreated samples (−CHX, control). Data are means (±S.E.M.) of triplicate samples. The analysis was repeated twice with comparable results. (B) LβT2 cells were transfected with 450 ng/well of the indicated murine *Hsd17b1*-luc reporters, followed by 24 h treatment with 25 ng/ml activin A. Data show luciferase activity, normalized relative to the empty pGL3-Basic vector. The fold increase induced by activin A is shown for each reporter. The data are means (+S.D.) of triplicate samples. The experiment was repeated thrice with comparable results. (C) LβT2 cells transfected with −106/+9 *Hsd17b1*-luc were treated for the indicated time periods with 1, 10, or 100 ng/ml activin A. Relative luciferase activity is shown relative to untreated (0 h) cells. Data are means (±S.D.) of duplicate samples, and the experiment was repeated thrice with comparable results.



Therefore, in subsequent analyses, we used the minimally responsive −106/+9 *Hsd17b1*-luc. This reporter was both time- and dose-dependently regulated by activin A, with increased activity observed within 2 to 4 h at 10 and 100 ng/ml concentrations (Fig. 2C). Because reporter activity requires both transcription and translation of the luciferase gene, this timing was consistent with an immediate-early response as seen with the endogenous gene.

Activins signal through the type I receptor, ALK4, to stimulate Hsd17b1 transcription

In the canonical activin signaling pathway, activins bind to a type II receptor serine/threonine kinase (ACVR2A or ACVR2B), which then phosphorylates a type I receptor, (ALK4, also known as ACVR1B). Activated ALK4 then propagates intracellular signals, which are classically mediated by SMAD proteins. SB431542 is a selective and potent small molecule inhibitor of ALKs 4, 5, and 7 (Laping *et al.* 2002). Thirty-minute pretreatment with this compound abolished the 24 h effects of three forms of activin (activin A, B, or AB), and significantly reduced basal reporter activity (Fig. 3A). These data indicated that ALKs 4, 5, and/or 7 were required for exogenous ligand action, regardless of activin subtype. The effect on basal activity suggested that endogenous signaling via one or more of these receptors might contribute to the high 'basal'*Hsd17b1* expression and 17β-HSD1 enzymatic activity in these cells.

We previously showed that the *Inhbb*, but not *Inhba* subunit is expressed in LβT2 cells (Lee *et al.* 2007). Homodimers of the INHBB protein form activin B. Activin B was implicated as the physiologically relevant activin in gonadotropes *in vivo* (Roberts *et al.* 1989, Corrigan *et al.* 1991, DePaolo *et al.* 1992). Therefore, the 70% decrease in basal reporter activity

Figure 3 (A) LβT2 cells were transfected with 450 ng/well of −106/+9 *Hsd17b1*-luc reporter. The following day, cells were treated with 10 μM SB431542 for 30 min followed by 24-h treatment with 25 ng/ml activin A, B, or AB. Data show the mean of triplicates (+S.D.), normalized relative to the untreated group. The experiment was repeated thrice with similar results each time. (B) Cells were transfected with 450 ng/well of −106/+9 *Hsd17b1*-luc reporter. The next day, cells were treated with 10 μM SB431542, 100 ng/ml follistatin, or 100 ng/ml inhibin A for 24 h. Treatments were performed in triplicate and data show the mean (+S.D.) reporter activity relative to control set to 1. The experiment was repeated thrice. (C) Cells were treated with 10 μM SB431542 for 30 min followed by 24-h treatment with 25 ng/ml activin A. *Hsd17b1* mRNA levels were measured by qRT-PCR. Data show means of triplicate samples (+S.D.), normalized relative to the untreated group. The experiment was repeated twice with similar results. (D) LβT2 cells were transfected with 450 ng/well of −106/+9 *Hsd17b1*-luc reporter and 100 ng/well ALK4-T206D or ALK4-T206D/D268A expression vectors. The following day, cells were treated for 24 h with 25 ng/ml of activin A. Data show the mean of triplicate samples (+S.D.), normalized to the control pcDNA3·0 vector in the absence of ligand. The experiment was repeated thrice.

with SB431542 treatment might reflect the inhibition of endogenous activin B signaling. We observed that exogenous activin B was as effective as activin A in stimulating reporter activity and was similarly inhibited by SB431542 (Fig. 3A). Therefore, to assess a potential role for endogenous activin B in basal *Hsd17b1* expression, the cells were transfected with the minimal reporter and treated overnight with SB431542, follistatin-288 (Fst-288), or inhibin A. Fst-288 binds and bioneutralizes activins, whereas inhibins are competitive antagonists of activin type II receptors (Harrison *et al.* 2005). All three inhibitors suppressed basal activity to 30–50% of the control (Fig. 3B). We then measured endogenous *Hsd17b1* mRNA levels in the presence of SB431542. As with the reporter, the inhibitor both strongly suppressed basal gene expression and completely blocked the 24 h effect of activin A (Fig. 3C).

Though recent data indicated that activin AB and B signal via ALK4 and/or 7, activin A signals through ALK4 alone (Tsuchida *et al.* 2004, Bernard *et al.* 2006). Activin signaling via ALK5 has not been reported. Therefore, the effects of SB431542, at least in the case of exogenous activin A, were most likely attributable to antagonism of ALK4 signaling. To confirm that ALK4 was sufficient for the induction of *Hsd17b1* reporter activity, we transfected the cells with a constitutively active form of ALK4 (T206D (Attisano *et al.* 1996)), which can signal in the absence of ligands. The receptor stimulated *Hsd17b1* reporter activity to the same extent as exogenous activin A (Fig. 3D). Cells transfected with ALK4-T206D and then treated with activin A showed the highest reporter activity, though the effect was less than additive. Together, the data suggested that ALK4 was sufficient and, perhaps, necessary for endogenous and exogenous activin-mediated *Hsd17b1* transcription.

Activins signal through SMAD2, but not SMAD3, to stimulate Hsd17b1 transcription

ALK4 propagates intracellular signals via phosphorylation of effector substrates. Although the receptor-regulated SMADs, SMAD2 and SMAD3, are the most thoroughly described effectors, ALKs can also generate SMAD-independent signals in cell-specific fashion (Derynck & Zhang 2003). We therefore examined whether activin A and ALK4 use SMAD proteins to signal to the *Hsd17b1* promoter. First, we introduced a point mutation, D268A, into the kinase domain of the constitutively active ALK4-T206D. The comparable mutation in ALK5 (D226A) inhibited the ability of the receptor to stimulate SMAD2 phosphorylation (Itoh *et al.* 2003). Whereas ALK4-T206D strongly stimulated the SMAD-responsive reporter, $CAGA_{12}$-luc, ALK4-T206D/D268A had no effect (data not shown). Therefore, the D268A mutation successfully impaired SMAD-dependent signaling by ALK4-T206D in LβT2 cells. ALK4-T206D/D268A also failed to stimulate *Hsd17b1* reporter activity, suggesting that ALK4 might signal, at least in part, through SMADs to regulate transcription (Fig. 3D).

To more definitively demonstrate the roles for SMADs, we co-transfected SMAD2 or SMAD3 expression vectors with the *Hsd17b1* reporter followed by overnight activin A treatment. SMAD over-expression can both mimic and potentiate the effects of ligand, when the response is SMAD dependent. SMAD2 increased basal reporter activity twofold and potentiated the activin A response (Fig. 4A). SMAD3 over-expression had no effect. Next, we knocked down the expression of SMAD2 or SMAD3 using previously validated short-hairpin (sh) RNA-expressing plasmids (Bernard 2004). The *SMAD2* shRNA decreased activin A-stimulated reporter activity (Fig. 4B) by about 40%. The magnitude of the effect was the same regardless of the amount of shRNA vector transfected. There was no notable effect on basal reporter activity. The *SMAD3* shRNA did not inhibit basal or activin A-regulated reporter activity (Fig. 4C).

Because SMAD2 does not bind DNA directly (Dennler *et al.* 1999, Yagi *et al.* 1999), but can bind indirectly through partnering with SMAD4, we asked whether the latter might play a role in *Hsd17b1* transcription. LβT2 cells were transfected with the −106/+9 reporter alone or with one of three siRNAs: one control and two directed against SMAD4. Relative to cells without siRNA, transfection of *SMAD4* siRNA #2 significantly inhibited basal reporter activity (Fig. 4D). The control siRNA and *SMAD4* siRNA #1 had no effect. Importantly, we observed that *SMAD4* siRNA #2, but not #1, inhibited SMAD4 expression in these cells (Wang, unpublished; data not shown). When controlling for differences in basal activity, none of the siRNAs inhibited the fold activin A response. These data suggested that SMAD4 plays a role in basal reporter activity (which may depend on endogenous activin B signaling), but not in exogenous activin A-regulated activity.

SMADs 3 and 4 can regulate transcription by binding directly to DNA via low-affinity SMAD-binding elements (SBE), AGAC or GTCT. When we screened the activin-responsive −106 to −64 promoter region for candidate SBEs, we identified AGAC at −90/−87. To assess the functional importance of this sequence, we introduced mutations into the first 2 bp at −90/−89 (AG→CT), which would be predicted to inhibit SMAD binding (Shi *et al.* 1998). When transfected into LβT2 cells, the mutant promoter showed a complete loss of both basal and activin A-induced reporter activity (Fig. 4E).

To determine whether SMADs bind this element basally and/or in response to activin A treatment, we performed DNA-affinity pull-down coupled with western blots. A biotinylated double-stranded DNA probe, corresponding to −106/−77 of the *Hsd17b1* promoter, was incubated with lysates from control or activin A-treated (1 h) cells. Western blots of whole-cell extracts demonstrated both basal and activin A-stimulated SMAD2 phosphorylation (pSMAD2), with the signal enhanced in the latter (Fig. 4F, lanes 1 and 2). The DNA probe precipitated both pSMAD2 and a smaller protein, likely to be the SMAD2 splice variant, SMAD2-Δexon3 (Bernard 2004). Importantly, the latter can bind

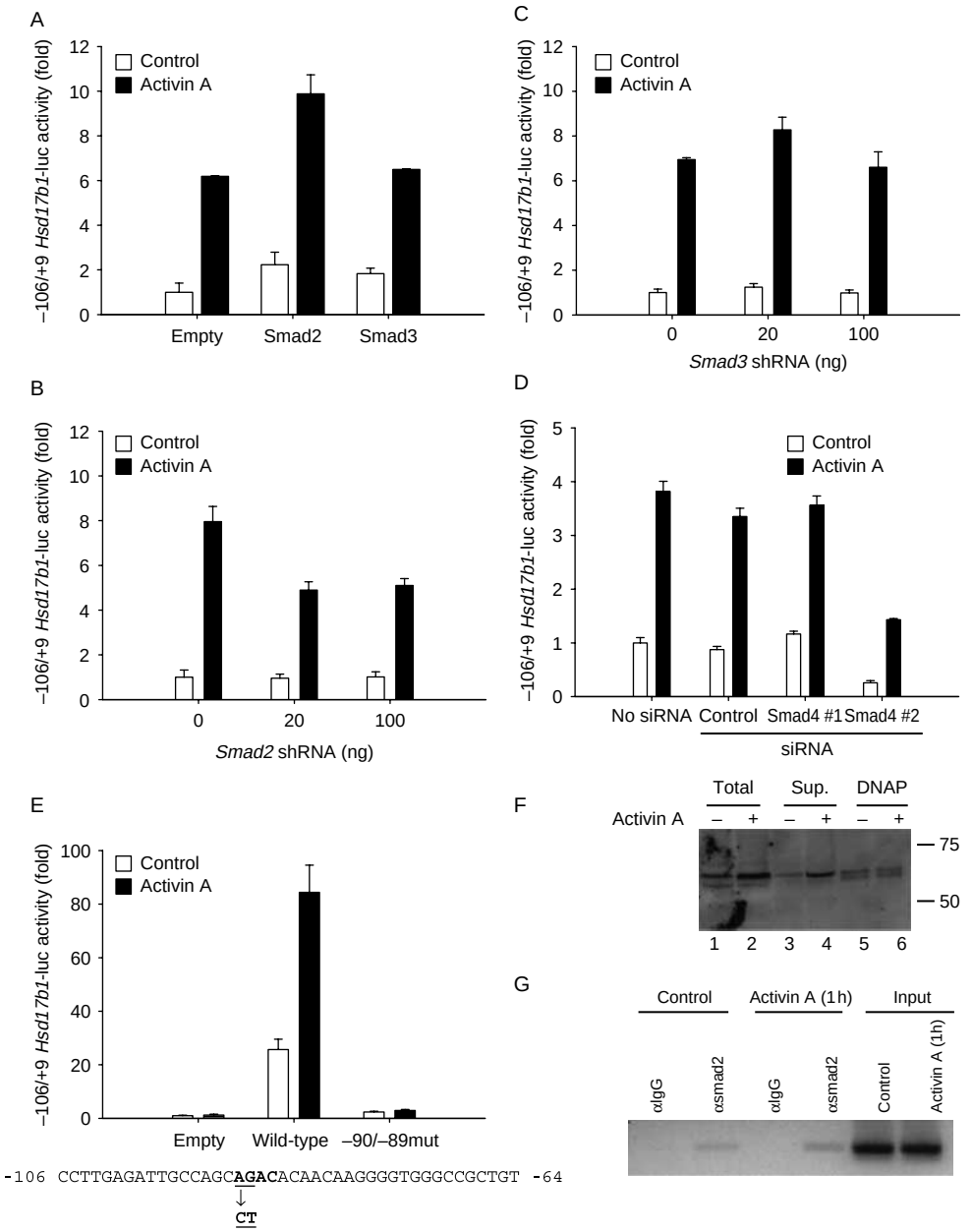


Figure 4 (A) LβT2 cells were transfected with 450 ng/well of −106/+9 *Hsd17b1*-luc and 100 ng/well of either pcDNA3·0, SMAD2, or SMAD3 expression vectors, followed by 24 h treatment with 25 ng/ml activin A. Data show means of duplicate samples (+S.D.), normalized relative to the empty vector without ligand. The experiment was repeated four times. (B and C) Cells were transfected with 450 ng/well of −106/+9 *Hsd17b1*-luc and 0, 20, or 100 ng/well of *SMAD2* or *3* shRNA in pBS/U6 constructs. Empty pBS/U6 vector was used as control. The data are means of triplicates (+S.D.), normalized relative to the empty pBS/U6 vector without ligand. The experiments were repeated four times. (D) LβT2 cells were transfected with 450 ng/well of −106/+9 *Hsd17b1*-luc and 5 nM of either control, or two different *SMAD4* siRNAs, followed by 24-h treatment with 25 ng/ml activin A. Data show means of triplicate samples (+S.E.M.), normalized to −106/+9 *Hsd17b1*-luc basal activity in the absence of any siRNA. The experiment was repeated twice with comparable results. (E) Wild-type and −90/−89 mutant −106/+9 *Hsd17b1*-luc reporters were transfected into LβT2 cells followed by 24-h treatment with 25 ng/ml activin A. Data show means of duplicates (+S.D.), normalized relative to pGL3-Basic (empty) vector. The experiment was repeated thrice. (F) LβT2 cells were cultured for 1 h in the presence or absence of activin A. Protein lysates were subjected to DNA-affinity pull-down (DNAP) with a biotinylated −106/−77 probe. Total (lanes 1 and 2), supernatant (Sup., lanes 3 and 4), and precipitated proteins (lanes 5 and 6) were subjected to western blot with a phospho-SMAD2 antibody. (G) Chromatin immunoprecipitation (ChIP) analysis of control or activin A-treated (1 h) LβT2 cells. Chromatin was precipitated with control IgG or SMAD2 antibody. DNA was amplified with primers flanking the SBE in the *Hsd17b1* gene.

DNA directly (Dennler *et al.* 1999, Yagi *et al.* 1999). The two proteins were pulled down in equal proportions (lanes 5 and 6), despite clear differences in their abundance as seen in whole-cell extracts (lanes 1 and 2) and as reported previously (Bernard 2004). The amount of pSMAD2 pulled down was not affected by activin A treatment. A probe containing the 2 bp mutation at −90/−89 failed to precipitate pSMAD2 (data not shown). SMAD2 interaction with this part of the *Hsd17b1* promoter in LβT2 cells was confirmed by ChIP (Fig. 4G). Here, we detected a modest increase in the interaction following activin A treatment.

Several nuclear proteins bind within the minimal Hsd17b1 promoter region

The reporter data point to the promoter region between −106 and −64 as critical for both basal and activin-induced *Hsd17b1* transcriptional activity in LβT2 cells. SMADs are low-affinity DNA-binding proteins and typically bind with co-factors to mediate their effects on transcription. Therefore, to identify other critical *cis*-elements and their associated binding proteins, we performed gel shift analyses with two overlapping probes, −106/−77 and −86/−57, which encompassed the −106/−64 interval. Both probes were radio-labeled and incubated with nuclear extracts from control and activin A-treated (4 h) LβT2 cells. We performed the binding reactions under a variety of conditions and noted significant differences in binding activity when altering the nonspecific DNA competitor, polydIdC versus salmon sperm (ss) DNA (Fig. 5A, compare lanes 1–4 and 5–8; and data not shown). With the −106/−77 probe and ssDNA, we noted one prominent complex (labeled c in Fig. 5A), which was competed by 100-fold molar excess unlabeled homologous and −86/−57 probes (lanes 3 and 4). These data suggested that complex c bound within the overlapping region between the two probes, −86/−77. Indeed, we observed a complex with the same mobility when using the radio-labeled −86/−57 probe, and this was competed by both cold −106/−77 and −86/−57 probes (data not shown). No other specific complexes binding to the −86/−57 probe were observed, and activin A treatment did not significantly alter complex binding to either probe. With the −106/−77 probe and polydIdC as nonspecific competitor, we failed to detect complex c, but now observed three new complexes (lane 5, labeled a, b, and ⋆). The fastest mobility complex (⋆) appeared to be non-specific as it was not displaced by cold homologous probe (lane 7). By contrast, both complexes a and b were displaced by cold −106/−77 (lane 7), but not −86/−57 (lane 8), suggesting that the binding of both occurred specifically and within the unique −106/−87 interval.

To determine the specific bases mediating binding of complexes a, b, and c, we prepared a series of competitor −106/−77 probes, in which 3 bp mutations were systematically introduced from the 5′ to 3′ end (Fig. 5B). These mutant (Mut) probes were tested for their abilities to displace binding to the wild-type radio-labeled −106/−77 probe in the presence of polydIdC (Fig. 5C, lanes 1–9; for complexes a/b) or ssDNA (Fig. 5C, lanes 10–15; for complex c). Mut1–6 spanned the interval unique to −106/−87, which was predicted to bind complexes a and b. Each of these mutant probes (lanes 3–8), with the exception of Mut3 (lane 5; bp −100/−98, GAT→TCG), could displace complex binding as well as wild type (lane 2). These data suggested that bp −100/−98 was important for complex a/b binding, though the mutant probe could still displace binding to some extent (compare lanes 1 and 5) and a radio-labeled Mut3 probe could still bind complexes a/b, though less well than wild type (data not shown). Mutants 7 to 10 spanned the interval common to both probes, where complex c was predicted to bind. Indeed, Mut8 (lane 13; bp −85/−83, CAA→ACC) and, to a lesser extent, Mut7 (lane 12; −88/−86, ACA→CAC) were impaired in their abilities to compete for binding to complex c relative to wild type (lane 11) or Mut9 and 10 (lanes 14 and 15). The putative SBE (boxed in Fig. 5B) mapped to −90/−87, which was mutated in Mut6 and 7. Mut6 did not affect complex a/b binding. Mut7 only partially disrupted binding to complex c and did so less than Mut8, which did not affect any bases in the putative SBE. Therefore, it seemed unlikely that complexes a, b, or c contained SMAD proteins. Indeed, inclusion of antibodies against SMADs 2 or 4 did not alter complex binding (data not shown). The same antibodies were shown to supershift or displace their targets in previous analyses (Lamba *et al.* 2006).

We next examined whether the bp mediating complex a/b or c binding played roles in basal and/or activin A-regulated promoter activity. We introduced the 3 bp mutation at −100/−98 (Mut3) in the context of −106/+9 *Hsd17b1*-luc and observed a complete loss of reporter activity, akin to what was observed with the SBE mutation (Fig. 5D, compare with Fig. 4E). This suggested that the proteins in complexes a/b might be necessary for *Hsd17b1* promoter activity in gonadotropes. Neither Mut7 nor 8 completely inhibited complex c binding, suggesting that some combination of bp in these mutants might maximally mediate binding. We therefore introduced a novel mutation (bp −87/−85, CAC→GGG), which encompassed bp represented in both mutants. Interestingly, this mutation significantly reduced basal reporter activity without affecting the fold activin A response (Fig. 5D). Therefore, the protein(s) in complex c might be critical specifically for basal promoter activity.

Characterization of proteins binding the proximal Hsd17b1 promoter

We used the *in silico* transcription factor binding program PROMO (Messeguer *et al.* 2002, Farre *et al.* 2003) to predict the identity of proteins binding to the elements identified in Fig. 5. We examined specifically those proteins predicted to bind to the wild type, but not mutant sequences. In the case of Mut3 (bp −100/−98), we observed a loss of predicted C/EBPα binding. This protein was intriguing for a few

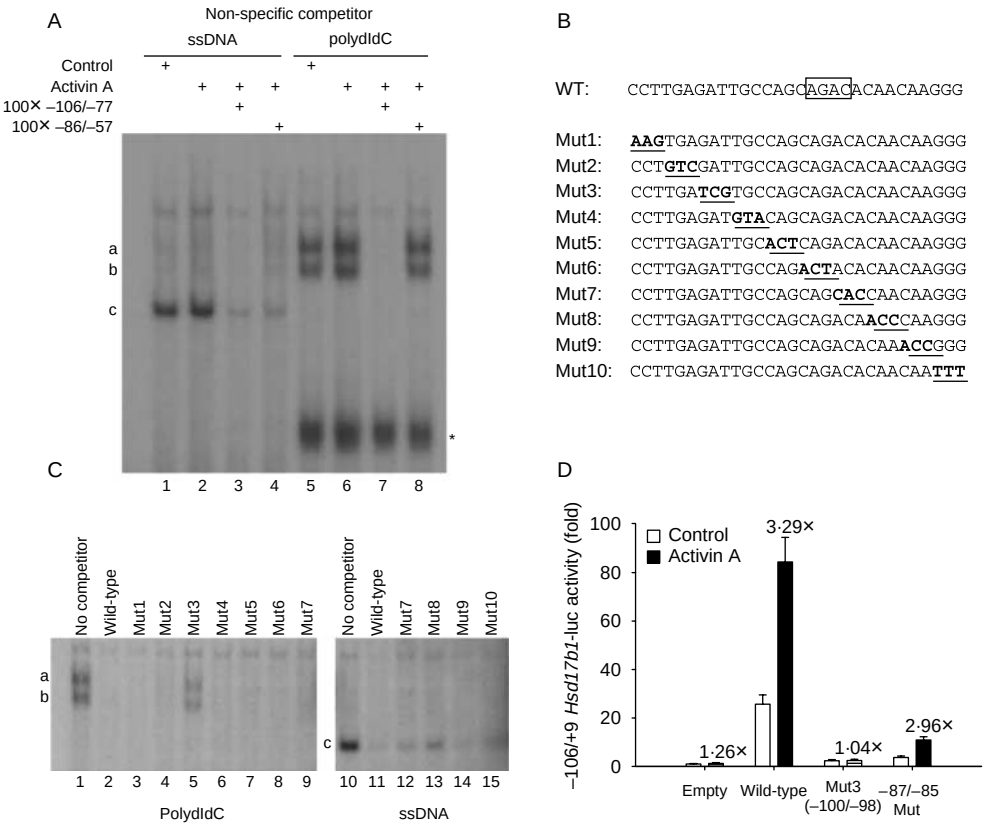


Figure 5 (A) A radio-labeled probe corresponding to −106/−77 of the murine *Hsd17b1* promoter was incubated with LβT2 nuclear protein extracts in the presence of two nonspecific competitor DNAs, ssDNA (lanes 1–4) or polydIdC (lanes 5–8). Binding specificity was determined by incubation with 100× molar excess of unlabeled homologous (lanes 3 and 7) or heterologous (−86/−57 *Hsd17b1*, lanes 4 and 8) probes. Complexes formed under the two conditions are labeled on the left. (B) Sequences of mutagenized −106/−77 probes (sense strand only) used as competitors in gel shifts with the radio-labeled wild-type (WT) −106/−77 probe. (C) Competition assays in the presence of polydIdC (left, lanes 1–9) or ssDNA (right, lanes 10–15). In all the cases, 100× molar excess of mutant probes was used to compete for binding to radio-labeled WT −106/−77 *Hsd17b1* probe. (D) LβT2 cells were transfected with wild-type or the indicated mutant −106/+9 *Hsd17b1*-luc reporters and then treated with 25 ng/ml activin A for 24 h. Data reflect the means of duplicate samples (+S.D.), normalized relative to pGL3-Basic. The experiment was repeated thrice.

reasons. For example, we had already confirmed its expression in these cells (data not shown). Moreover, it is expressed in two major forms (42 and 30 kDa) through alternative translation initiation (Calkhoven *et al.* 1994), which could explain the complex a/b-binding pattern we observed. However, complex binding was not altered by the inclusion of a C/EBPα antibody in gel shift analyses (data not shown). In addition, we observed the same two complexes with nuclear extracts from αT3-1 cells, which do not express C/EBPα (data not shown). In the case of complex c, the *in silico* analysis showed that a putative binding site for homeobox A3 (HOXA3) was disrupted in both probes Mut7 and 8; but this was not pursued further given the results of later experiments indicating the likely size of the protein in complex c (see below).

In the absence of viable candidates, we attempted to further characterize the binding proteins through experimental means. We fractionated LβT2 cell nuclear extracts by cation exchange chromatography and examined the fractions for their binding activities in the established gel shift paradigm with the −106/−77 probe using either polydIdC or ssDNA as nonspecific competitor, in order to isolate complexes a/b and complex c (data not shown). We next collected proteins from fractions exhibiting high binding activity by SDS-PAGE and transferred them to nitrocellulose membranes for southwestern blot analyses with the radio-labeled −106/−77 probe. Protein fractions containing complex a/b produced at least 12 hybridizing bands (Fig. 6A, labeled at right). Most were non-specific, as they were not competed when excess unlabeled homologous or heterologous probes were included in the binding reactions (bands 1, 3, 5–12), or they were also observed in chromatographic fractions that had no binding activity in EMSAs (data not shown). We therefore focused on the two

specific bands (labeled 2 and 4 in Fig. 6A, arrows) whose binding to the probe was diminished in the presence of cold homologous (lane 2) but not heterologous competitor probe (lane 3). Upon performing southwestern blot analysis with protein fractions containing complex c, we observed a specific complex of ~35 kDa (HOXA3 would be expected to migrate at ~46 kDa, making it significantly larger than the protein we observed here) (Fig. 6B). We are currently attempting to purify all three proteins for identification by mass spectrometry.

Regulation of Hsd17b1 transcription in immortalized and primary cultured murine granulosa cells

Having discerned part of the mechanism through which activins regulate *Hsd17b1* transcription in gonadotropes, we next asked whether this mechanism might be conserved across cell types. Previously, activin A was shown to stimulate *Hsd17b1* mRNA levels in primary cultured rat granulosa cells (Ghersevich *et al.* 2000). Activin A also stimulated *Hsd17b1* mRNA levels in primary cultured mouse granulosa cells from a microarray study (Kipp and Mayo, unpublished results). We therefore transfected the murine granulosa cell line, KK-1 (Kananen *et al.* 1995), with the *Hsd17b1* 5′ deletion constructs described above. Though the fold activin A response was smaller than in LβT2 cells, the pattern of results was similar between the two cell types. That is, both basal and activin A promoter activities were lost with the truncation from −106 to −64 (Fig. 7A). As in LβT2 cells, basal activity was significantly inhibited by SB431542 or Fst-288 (Fig. 7B).

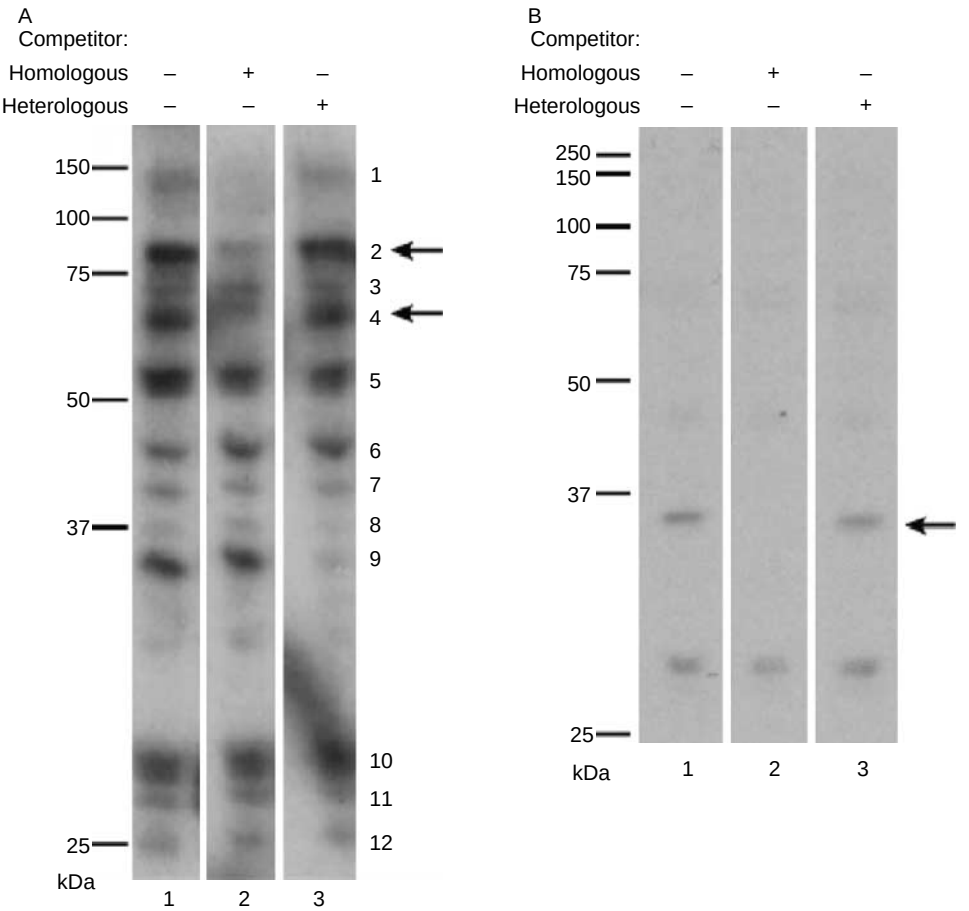


Figure 6 (A) LβT2 nuclear protein fractions were collected after ion exchange chromatography and subjected to EMSA to identify fractions containing complex a/b-binding activity. These fractions were pooled, concentrated, and then used for southwestern blotting. Fractions were run in triplicate on SDS-PAGE, transferred to nitrocellulose membranes, and renatured to determine approximate molecular weights of the binding proteins. Membranes were incubated with radio-labeled −106/−77 *Hsd17b1* probe alone (lane 1) or with 100-fold molecular excess of unlabeled homologous (lane 2, −106/−77 *Hsd17b1*) or heterologous (lane 3, −569/−543 *Id3*) probe. PolydIdC was used as a nonspecific competitor. (B) The same analysis as described in (A) was repeated here, using LβT2 nuclear protein fractions containing complex c-binding activity and ssDNA used as a nonspecific competitor.

Finally, to determine whether the results observed in immortalized cells reflect regulation in non-transformed cells, we transfected primary murine granulosa cells with the *Hsd17b1* 5′ deletion constructs and ALK4-T206D. Similar to what we observed in both KK-1 and LβT2 cells, the promoter conferred significant basal reporter activity in primary cells, and this effect was lost with the truncation from −106 to −64 (Fig. 7C). Again, ALK4-T206D stimulated an increase in reporter activity in most of the reporters. However, where the stimulated response was lost between −106 and −64 in the cell lines, the effect was lost between −253 and −106 in primary cells (Fig. 7C).

Discussion

Activins regulate diverse aspects of reproductive function, and *Fshb, Gnrhr,* and *Fst* are established activin target genes in gonadotrope cells of the anterior pituitary. To identify other targets in these cells, we and others (Mazhawidza *et al.* 2006, Zhang *et al.* 2006) examined activin A-stimulated changes in gene expression in immortalized murine gonadotropes, LβT2, using cDNA microarrays. We observed marked upregulation of *Hsd17b1* expression, consistent with one of the earlier reports (Zhang *et al.* 2006). Here, we demonstrate that activin A regulates *Hsd17b1* transcription in immediate-early fashion, downstream of the type I receptor ALK4, via SMAD2. SMAD4 appears critical for basal reporter activity, which is dependent upon endogenous signaling through ALK4/5/7, but is not required for the response to exogenous activin A (Fig. 4D). Basal and activin A-regulated activity appears to be independent of SMAD3, though we have previously demonstrated that activin A rapidly stimulates SMAD3 phosphorylation in these cells (Bernard 2004). We identified a minimal SBE within the activin A-responsive region of the promoter, which can interact with SMAD2 and SMAD2Δexon3. We have not yet determined whether these proteins are in complex together, but the DNA-affinity pull-down experiments suggest this may be the case. That is, the SBE can only bind one SMAD at a time, and yet both proteins were precipitated. In the case of full-length SMAD2, which does not bind DNA directly, partnering with SMAD2-Δexon3 most likely explains its association with the activin-responsive promoter fragment.

Four bp SBEs are common in the genome, and SMADs 3 and 4 (and SMAD2Δexon3) bind these sequences with low affinity (Shi *et al.* 1998). Therefore, SMAD-dependent signaling is thought to depend on the interactions of activated SMAD complexes with high-affinity DNA-binding partners that bind in the proximity of SBEs (Chen *et al.* 1996, 1997). Along these lines, we mapped two other *cis*-elements in the vicinity of the SBE, which are required for basal and/or activin A-regulated activity. Biochemical analyses suggest that proteins of ∼ 85, 68, and 35 kDa bind to one or more of these sites, though we have not yet determined their identities. Future analyses will characterize these proteins,

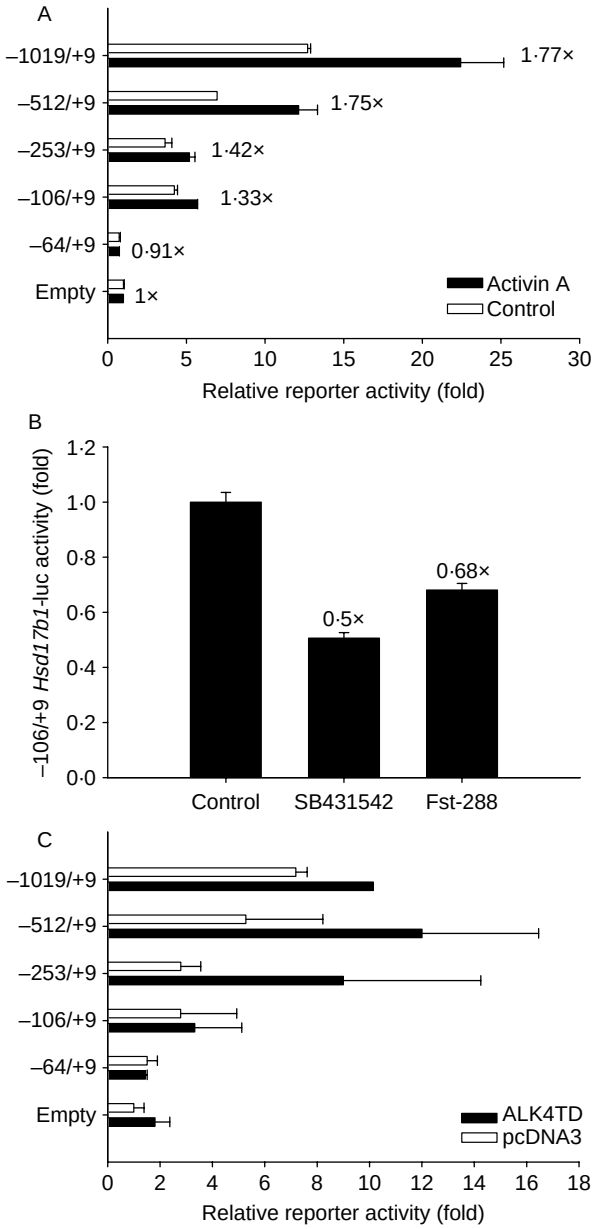


Figure 7 (A) KK1 cells were transfected with 450 ng/well of the indicated murine *Hsd17b1*-luc promoter reporters, followed by 24 h treatment with activin A. Data show means of duplicates, normalized relative to pGL3-Basic (empty) vector. The experiment was repeated thrice, with comparable results each time. (B) KK-1 cells were transfected with 450 ng/well of −106/+9 *Hsd17b1*-luc reporter. Cells were then treated with 10 μM SB431542 or 100 ng/ml follistatin for 24 h. Treatments were performed in triplicate and data show the mean reporter activity relative to control set to 1. (C) Primary murine granulosa cells were transfected with 450 ng/well of pGL3-Basic or the indicated *Hsd17b1*-luc reporters with 100 ng/well of pcDNA3 or ALK4-T206D expression constructs. Cells were harvested the next day. Data show the mean (+S.D.) fold stimulation of duplicate samples relative to the empty vector/pcDNA3 condition. The data were compiled from four to five experiments.

assess their roles in basal and activin A-regulated *Hsd17b1* transcription, and their functional/physical interactions with SMAD proteins. Interestingly, the sequences mediating binding of complexes a, b, and c, as well as the putative SBE, are conserved in the human and rat *HSD17B1/Hsd7b1* genes. In fact, in preliminary analyses, we observed that the human *HSD17B1* proximal promoter is activin A responsive in LβT2 cells (data not shown). Therefore, the mechanisms of activin A-regulated *Hsd17b1* transcription observed here may be evolutionarily conserved.

Both the mRNA and protein analyses indicate a high basal level of *Hsd17b1* gene expression in LβT2 cells. Treatment with an inhibitor of ALK4 kinase activity or knockdown of SMAD4 both strongly inhibited basal promoter activity. SMAD2 knockdown did not inhibit basal activity, but the shRNA used targets of only full-length SMAD2 (Bernard 2004). Because these experiments were done in the absence of serum, the results suggest that a TGFβ superfamily ligand (or ligands) produced by the cells regulates *Hsd17b1* expression in autocrine/paracrine fashion. The most obvious candidate is activin B. We, and others, previously showed that these cells synthesize the *Inhbb*, but not *Inhba,* subunit, allowing them to produce activin B, but not activin A or AB (Pernasetti *et al.* 2001, Lee *et al.* 2007, Antenos *et al.* 2008). Fst-288 or inhibin A, two known antagonists of activins, inhibited basal reporter activity similarly to the ALK4 inhibitor, further implicating activin B as the endogenous regulator in this system. However, recent data indicate that follistatins and inhibins can antagonize other TGFβ superfamily ligands (Wiater & Vale 2003, Schneyer *et al.* 2008). Therefore, we cannot conclude definitively that endogenous activin B is the sole modulator of basal *Hsd17b1* promoter activity. Future analyses are required to assess the full complement of TGFβ ligands expressed in these cells to determine the endogenous regulator(s).

Because LβT2 cells are immortalized gonadotropes, it is important to assess whether the results gathered here reflect *in vivo* regulation of *Hsd17b1*. We confirmed the expression of the gene in both adult male and female murine pituitaries (data not shown), and *Hsd17b1* mRNA is also detectable in purified murine gonadotropes (Gore and Miller, personal communication). Therefore, expression of the gene in a gonadotrope-like cell does not appear to be an artifact of the LβT2 cell model. Still, we do not yet know whether it is similarly regulated by activins in gonadotropes *in vivo* and the difficulty of purifying gonadotropes complicates such analyses. However, activin A was previously shown to stimulate *Hsd17b1* mRNA levels in primary rat granulosa cells (Ghersevich *et al.* 2000), and here we show that activin A and/or a constitutively active activin type I receptor, ALK4-T206D, stimulates *Hsd17b1* promoter activity in immortalized and primary murine granulosa cells. Promoter deletions show that the promoter region (−106/−64) mediating basal reporter activity in LβT2 cells similarly mediates promoter activity in the granulosa cells. The same promoter region also mediates the exogenous activin A/ALK4-T206D regulation of the gene in LβT2 and KK-1 cells. In primary granulosa cells, however, the ALK4-T206D-responsive region maps more distally to −253/−106. Whether this reflects differences between transformed and non-transformed cells is not yet clear. However, there was significantly more variability in the primary than immortalized cells, leaving open the possibility that we may have missed ALK4-T206D regulation of the −106/+9 reporter in the former.

A final obvious question is what role activin-regulated *Hsd17b1* expression might be playing in gonadotrope cells *in vivo.* In granulosa cells, activins potentiate FSH-regulated (Suszko *et al.*) aromatase and 17β-HSD1 activities, leading to increases in 17β-estradiol synthesis (Xiao *et al.* 1990, Hillier & Miro 1993, Ghersevich *et al.* 2000). Therefore, it is possible that activins might regulate 17β-estradiol synthesis in gonadotropes by potentiating the reduction of systemic estrone. Because estrogens inhibit gonadotropin production/secretion at the pituitary level (Glidewell-Kenney *et al.* 2008), this might form part of a negative feedback loop. That is, activins stimulate FSH production, which then stimulates (among other things) ovarian estrogen synthesis. Increased estrone may then travel to the pituitary where it is converted to the more active 17β-estradiol and thereby inhibits or attenuates further FSH synthesis and secretion. Indeed, activin A was shown to increase LβT2 sensitivity to estrone as assessed by the activity of an estrogen-responsive reporter, ERE-tk-luc (Zhang *et al.* 2006). We similarly saw an activin A-induced increase in 17β-HSD1 enzymatic activity in these cells. We therefore assessed the effects of estrogens on murine *Fshb* promoter reporter activity in the presence or absence of activin A, but neither estrone nor 17β-estradiol had any effects in these assays. This was the case in the presence or absence of co-transfected ERα expression vector. Also, we were unable to replicate the results in Zhang *et al.* (2006) with ERE-tk-luc (data not shown). Recently, it was reported that some batches of LβT2 cells are unresponsive to estrogens (Eertmans *et al.* 2007) and this seems to be the case with ours. Therefore, an assessment of the effects of activin-regulated *Hsd17b1* expression on gonadotropin synthesis must await an estrogen-sensitive batch of LβT2 cells or a different cell system.

Declaration of interest

The authors have no conflict of interest to declare.

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