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## The Intraovarian Actions of Estrogen Receptor- $\alpha$ (ER $\alpha$ ) are Necessary to Repress the Formation of Morphological and Functional Leydig-like Cells in the Female Gonad

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### Abstract

The predisposition of the testis and ovary to primarily synthesize testosterone and estradiol, respectively, is due to gonadal-specific cell types that differentially express the various hydroxysteroid (17 $\beta$ ) dehydrogenase (HSD17B) isoforms. In testes, Leydig cells rely on LH stimulation to maintain expression of the type 3 (*HSD17B3*) isoform, which specifically converts androstenedione to testosterone. In ovaries, thecal-interstitial cells also rely on LH to induce androgen synthesis but lack HSD17B3 and therefore secrete androgens of low biological activity. Therefore, thecal cells may possess a mechanism to repress the Leydig cell phenotype and *HSD17B3* expression. Estradiol is known to inhibit experimentally Leydig cell function and proliferation. In the current study, we provide evidence that estradiol prevents the development of functional Leydig-like cells in the murine ovary; and that this action is mediated by estrogen receptor- $\alpha$  (ER $\alpha$ ). ER $\alpha$ -null ( $\alpha$ ERKO) female mice exhibit testis-like levels of *Hsd17b3* expression in the ovaries and male-like levels of plasma testosterone. Herein, we demonstrate that *a*) *Hsd17b3* expression in  $\alpha$ ERKO ovaries is a primary effect of the loss of intraovarian ER $\alpha$  actions, *b*)  $\alpha$ ERKO ovarian cells produce substantial levels of testosterone *in vitro* and this is blocked by a HSD17B3 specific inhibitor, *c*) *Hsd17b3* expression in  $\alpha$ ERKO ovaries is LH regulated and localized to the secondary/thecal interstitial cells, and *d*)  $\alpha$ ERKO secondary/thecal interstitial cells possess Leydig-like ultrastructural features. These data indicate that intraovarian ER $\alpha$  actions are required to repress *Hsd17b3* expression in the ovary and may be important to maintaining a female phenotype in secondary/thecal interstitial cells.

### Keywords

17 $\beta$ -hydroxysteroid dehydrogenase; steroidogenesis; thecal cells

### INTRODUCTION

The well-conserved sexually dimorphic pattern of gonadal steroid hormone secretion in mammals is vital to the proper development and function of reproductive tissues and behaviors.

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The predisposition of the testis and ovary to primarily produce testosterone and estradiol, respectively, is due primarily to the development of gonadal-specific cell types that differentially express enzymes involved in the final stages of steroid hormone synthesis, namely isoforms of the hydroxysteroid (17 $\beta$ ) dehydrogenase (HSD17B) family (1–3). The gonads of both sexes possess the enzymatic capacity to convert C<sub>27</sub> sterols (*e.g.*, cholesterol) to C<sub>19</sub> steroids, primarily dehydroepiandrosterone and androstenedione, which have low biological activity but are precursors for conversion to the more biologically active steroids, testosterone and estradiol. In mammalian males, the Leydig cells of the testes are especially proficient at reducing androstenedione to testosterone because they specifically express the androgenic or type 3 (HSD17B3) isoform (2,4–7). Furthermore, because testes possess a relatively modest level of aromatase (CYP19A1), which converts androstenedione and testosterone to estrone and estradiol, respectively, the latter androgen is allowed to accumulate and be secreted. In contrast, granulosa cells in adult female ovaries possess substantially high levels of CYP19A1 but lack HSD17B3 (1,2,8), thereby favoring aromatization of androstenedione to estrone rather than reduction to testosterone. The accumulating estrone is then reduced to estradiol by the estrogenic or type 1 (HSD17B1) isoform, which is highly expressed in the granulosa cells of growing follicles, as well as in the uterus, adrenals and mammary gland (1).

The importance of sexual dimorphic expression of *HSD17B1* and *HSD17B3* in female and male gonads, respectively, is well appreciated yet poorly understood. This is especially true of the mechanisms that restrict *HSD17B3* expression to testicular Leydig cells. Leydig cells constitutively express the LH-receptor and rely on LH stimulation to maintain *HSD17B3* expression and testosterone synthesis (6,9,10). Thecal-interstitial cells of the ovary, however, also constitutively possess LH-receptor and rely on LH stimulation to maintain steroidogenesis (11,12) but are limited to the secretion of C<sub>19</sub> steroids of low biological activity because they lack *HSD17B3* expression (13). This divergence between Leydig and thecal-interstitial cells, which are thought to arise from a common primordial cell during gonadal differentiation, suggests that the latter cell type either lack an LH-stimulated transcription factor(s) specific to the induction of *HSD17B3* expression or possess a mechanism(s) to actively repress *HSD17B3* expression. Estradiol has long been considered a leading candidate to fulfill this postulated inhibitory mechanism since it exists at significantly higher levels in ovaries than testes and inhibits Leydig cell function and proliferation in experimental studies (14,15), but has received little investigative attention in this regard. However, our recent studies provide the first definitive evidence of estradiol-mediated repression of *Hsd17b3* expression in the ovary and suggest that receptor-mediated actions of estradiol may play an important physiological role during ovarian development and function. We have previously shown that the ovaries of mice lacking functional estrogen receptor- $\alpha$  ( $\alpha$ ERKO) exhibit testis-like levels of *Hsd17b3* expression and male-like levels of plasma testosterone (16). These findings are corroborated by a report that female mice null for CYP19A1 and therefore lacking endogenous estradiol synthesis exhibit comparable ovarian *Hsd17b3* expression that is abolished by exogenous estradiol treatments (17). These data indicate that ligand-dependent ER $\alpha$  actions are fundamental to the repression of *Hsd17b3* expression in the ovary. In the current study, we expand our description of this unique  $\alpha$ ERKO ovarian phenotype by demonstrating that *a*) ectopic *Hsd17b3* expression is fundamentally due to the loss of intraovarian ER $\alpha$  actions and not a secondary effect of the hypergonadotropism that follows the neuroendocrine loss of ER $\alpha$  functions, *b*) ovarian HSD17B3 activity in  $\alpha$ ERKO females is responsible for their characteristic male-like testosterone burden, *c*) *Hsd17b3* expression in  $\alpha$ ERKO ovaries exhibits a pattern of LH regulation comparable to that of Leydig cells in the testes, *d*) ectopic *Hsd17b3* expression in  $\alpha$ ERKO ovaries is localized to the secondary/thecal interstitial cells, and *e*) these same cells exhibit ultrastructural features that are generally considered unique to Leydig cells.

## MATERIALS AND METHODS

### Animals

The Animal Care and Use Committee of the NIEHS preapproved all procedures involving animals. Animals were maintained in plastic cages in a temperature-controlled room (21–22°C) under a 12-h light:12-h dark schedule and given NIH 31 mouse chow and fresh water *ad libitum*. Wild type (*Esr1*<sup>+/+</sup>) and  $\alpha$ ERKO (*Esr1*<sup>-/-</sup>) mice were of C57BL/6 strain and obtained from our colony at Taconic Farms, Germantown, NY. LH-CTP transgenic mice were a mixed background strain (C57BL/6 x CF-1) and obtained from our in-house colony established with breeders from the original colony at Case Western Reserve University, Cleveland, OH (as a gift from Dr. John H. Nilson). Compound hypogonadal *Gnrh*<sup>hpg</sup>/*Esr1*<sup>+/+</sup> (wild type<sup>hpg</sup>) or *Gnrh*<sup>hpg</sup>/*Esr1*<sup>-/-</sup> ( $\alpha$ ERKO<sup>hpg</sup>) mice were obtained from our colony that was established by crossing heterozygous *Gnrh*<sup>hpg</sup> males (C3H/HeH x 101/H; Jackson Laboratories, Bar Harbor, ME) with *Esr1*<sup>+/-</sup> females (C57BL/6), and maintained at Charles River Laboratories (Wilmington, MA). All animals were genotyped by PCR on DNA extracted from tail biopsies using the Wizard SV 96 Genomic DNA extraction kit (Promega, Madison, WI). The procedures for genotyping *Esr1*<sup>-/-</sup> (16) and LH-CTP (18) mice have been previously described. All potential *Gnrh*<sup>hpg</sup> mice were genotyped using a 3-primer PCR scheme based on the original description of the mutant *Gnrh*<sup>hpg</sup> allele (19). This PCR method used the following common forward primer and two reverse primers to differentiate the wild type and mutant *Gnrh* alleles: forward 5'-ATGATGCTGCCCAATCG (bp 2446–2464) to reverse 5'-TCCAGACAGGAGTGAAGTGC (bp 2847–2827), these primers flank the mutant breakpoint at bp 2700 and produce a 402 bp amplicon from a wild type *Gnrh* allele and no amplicon from a mutant *Gnrh* allele since sequences homologous to the reverse primer are deleted; and the above forward primer paired with the following reverse primer 5'-TTTCTACTCTCTGAAACAGGCAAATT, which is homologous to sequences 47 bp beyond the mutant breakpoint and therefore produces a 301 bp amplicon that is unique to the mutant *Gnrh*<sup>hpg</sup> allele. All PCR results were evaluated by agarose gel electrophoresis.

### Animal treatments and tissue collection

All animals were 2–5 mo of age at the time of use. Animals were killed via CO<sub>2</sub> asphyxiation, whole blood was immediately drawn from the inferior vena cava, mixed with heparin and the plasma later separated by centrifugation; gonads were promptly removed, trimmed of surrounding tissue and processed according to the intended downstream analysis (as described below). Two experiments required the treatment of animals with gonadotropins prior to tissue collection. In the first (described in Fig. 3), animals were implanted (s.c.) with an Alzet® osmotic pump (Durect Corporation, Cupertino, CA) rated for drug delivery of 0.5  $\mu$ l/h for 7 days. Pumps were filled with either *a*) vehicle (0.85% saline), *b*) purified human LH (hLH) at 0.83 I.U./ $\mu$ l or *c*) recombinant human FSH (hFSH) at 0.83 I.U./ $\mu$ l to provide a calculated delivery of 10 I.U. gonadotropin per day. Gonadal tissues were collected as described above on the morning of the 7<sup>th</sup> day. Human LH and hFSH (hFSH) preparations were purchased from A.F. Parlow via the National Hormone and Peptide Program (Torrance, CA). In the second experiments involving gonadotropin treatment (described in Figs. 5 and 6), animals were injected (s.c.) twice daily for 3 consecutive days with vehicle (0.85% saline), 5 I.U. pregnant mares' serum gonadotropin (PMSG) (Sigma, St. Louis, MO) or 5 I.U. human chorionic gonadotropin (hCG) (Sigma). Gonadal tissues were then collected as described above on the morning of the 6<sup>th</sup> day, approximately 1 h after the final treatment.

### Granulosa – thecal cell isolation

Ovaries were immediately removed from adult wild type and  $\alpha$ ERKO females upon death by CO<sub>2</sub> asphyxiation and pooled according to genotype in 100 mm cell culture dishes containing ice-cold M199 medium (Invitrogen, Carlsbad, CA). Granulosa cells were then expressed into

the medium by manual puncture of the ovaries with a 25-gauge needle and by applying slight pressure with a sterile spatula. The suspended granulosa cells in the medium were then concentrated via centrifugation at 250 x g for 5 min at 4°C and washed two times in M199 medium; the pellets were stored at -70°C for later RNA isolation. The remaining ovarian fragments were considered to represent an enriched stromal/thecal-interstitial cell fraction and as such were washed several times in M199 medium and concentrated by centrifugation; the pellets were stored at -70°C for later RNA isolation.

### RNA isolation and gene expression assays

Total RNA was isolated from snap-frozen tissues or cell pellets using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. The concentration and integrity of all final preparations was calculated via an A<sub>260</sub> reading using a Molecular Devices Spectramax (Sunnyvale, CA) spectrophotometer and agarose gel electrophoresis of a 1 µg aliquot.

Northern blots were generated from 20 µg total RNA per sample using NorthernMax formaldehyde based reagents and BrightStar (Ambion, Austin, TX) positively charged nylon membrane according to the manufacturer's protocol. Blots were sequentially probed, stripped and reprobed for serial gene expression analyses using StripEZ (Ambion) generated riboprobes and stripping reagents. Radiolabeled anti-sense riboprobes for *Hsd17b3* (bp 363–729 of U66827) and *Cyp17a1* (bp 522–932 of M4863) mRNAs were generated from previously described cDNA clones (16) using the StripEZ transcription reagents (Ambion) and [<sup>32</sup>P]α-UTP (Amersham Biosciences, Piscataway, NJ). Hybridizations were carried out in ULTRAhyb hybridization solution (Ambion) with approximately 1 x 10<sup>7</sup> cpm riboprobe per ml in a 68°C rotisserie oven (Thermo-Hyaid, Franklin, MA) and then washed according to the manufacturer's protocol. Blots were exposed to a phosphorimager screen and the data analyzed with a Storm 860 and accompanying ImageQuant Software (Molecular Dynamics, Sunnyvale, CA).

RT-PCR was carried out on total RNA that was first rid of contaminating DNA by use of the DNA-free® reagents (Ambion) according to the manufacturer's protocol. For each sample, cDNA was generated from 1 µg RNA in a 25 µl reaction using random hexamers and the Superscript cDNA synthesis system (Invitrogen) according to the manufacturer's protocol. Traditional PCR reactions were then prepared from the equivalent of 1 µl cDNA per 15 µl reaction for each respective primer set using PCR reagents and Platinum *Taq* Polymerase (Invitrogen) as previously described. PCR was carried out in a Thermo Hyaid Multiblock System (Thermo-Hyaid) with the following cycling conditions: 95°C/30 sec (1X); 95°C/30 sec, 58°C/45 sec, 72°C/30 sec (28–35X); 72°C/7 min. The primer sets for assessing *Hsd17b3*, *Hsd17b1* and *Actb* mRNAs have been previously described (16). All samples were electrophoresed on an agarose gel (2% NuSieve/0.7% SeaKem, BMA Bioproducts, Rockland, ME) in 1X Trisborate-EDTA buffer, stained with ethidium bromide and photographed using an EC3 Imaging System (UVP, Upland, CA).

For real-time RT-PCR assessment of gene expression, Applied Biosystems Primer Express (Applied Biosystems, Foster City, CA) software was used to select primers specific for the amplification of murine *Hsd17b1*, *Hsd17b3*, *Cyp17a1* and *Cyp19a1* cDNAs (Table I). All primer sets were a) designed to lie in separate exons to avoid erroneous amplification of contaminating genomic DNA, b) confirmed to amplify a single product via dissociation analysis and gel electrophoresis, and c) confirmed to amplify the expected sequences by restriction enzyme mapping of the amplified product. Each sample was assayed in duplicate using the equivalent of 0.01 µl cDNA (prepared as described above), 10 pmoles of each primer, and 1X SYBR Green Master Mix (Applied Biosystems) in a total reaction volume of 25 µl. For normalization purposes, an identical set of reactions were prepared using primers specific for ribosomal 18S RNA (*Rn18s*) (Table I). Amplification was carried out in an ABI PRISM

7700 Sequence Detection System (Applied Biosystems) as follows: 50°C/2 min, 95°C/10 min (1X); 95°C/15 sec, 60°C/30 sec (40X). Quantitative differences in the cDNA target between samples were determined using the mathematical model of Pfaffl (20) in which an expression ratio was determined for each sample by calculating  $(E_{\text{target}})^{\Delta C_t(\text{target})}/(E_{Rn18s})^{\Delta C_t(Rn18s)}$ , where E is the efficiency of the primer set and  $\Delta C_t = C_{t(\text{normalization cDNA})} - C_{t(\text{experimental cDNA})}$ . The amplification efficiency of each primer set was calculated from the slope of a standard amplification curve of log  $\mu\text{l cDNA/reaction}$  vs. Ct value over at least 4 orders of magnitude ( $E = 10^{-(1/\text{slope})}$ ); *Hsd17b3* primers, E = 1.97 (vs. testis cDNA); *Hsd17b1* primers, E = 2.02 (vs. ovary cDNA); *Cyp17a1* primers, E = 2.16 (vs. ovary cDNA); *Cyp19a1* primers, E = 2.13 (vs. ovary cDNA).

### In situ hybridization

Phylogeny Inc. (Columbus, OH) conducted all *in situ* hybridization experiments under paid contract. Gonadal tissues were collected from adult wild type and  $\alpha$ ERKO animals immediately upon death, fixed overnight in Phylogeny's proprietary fixative, then dehydrated and infiltrated with paraffin. Serial sections of 5–8  $\mu\text{m}$  were mounted on gelatin-coated slides, deparaffinized in xylene, and rehydrated through a series of graded ethanol baths in 1X phosphate-buffered saline, pH 7.5 (PBS). The sections were treated with proteinase K, then triethanolamine/acetic anhydride, washed and dehydrated. Radiolabeled sense and anti-sense riboprobes for *Hsd17b3* mRNA (bp 363–729 of U66827) were generated from a previously described cDNA clone (16) using Maxiscript reagents (Ambion) and  $^{35}\text{S}$ - $\alpha$ UTP (>1000 Ci/mmol; Amersham) according to the manufacturer's protocol. Sections were hybridized overnight at 55°C in a solution of 50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 10 mM  $\text{NaPO}_4$ , 10% dextran sulfate, 1X Denhardt's, 50  $\mu\text{g/ml}$  total yeast RNA, and radiolabeled riboprobe at 5–8  $\times 10^5$  cpm/ $\mu\text{l}$ . The sections were then subjected to stringent washing at 65°C in a solution of 50% formamide, 2X sodium chloride/sodium citrate (SSC) buffer with 10 mM dithiothreitol; washed in 1X PBS; then treated with 20  $\mu\text{g/ml}$  ribonuclease A at 37°C for 30 min. The slides were washed in 2X SSC, then 0.1X SSC for 10 min per wash at 37°C; dehydrated in a series of graded ethanol baths; dipped in Kodak NTB-2 nuclear track emulsion; exposed for 21 days in light-tight boxes with desiccant at 4°C. All slides were then developed in Kodak D-19, lightly counterstained with hematoxylin and eosin (H & E), and viewed and photographed under both light- and dark-field microscopy.

### In vitro acute steroidogenic assays

The acute steroidogenic capacity of dispersed ovarian cells from wild type,  $\alpha$ ERKO and LH-CTP animals was assessed *in vitro* using a method first described by Magoffin and Erickson (11,21), with some modifications. In brief, ovaries were collected from 5–6 untreated or hCG-treated adult females of each genotype and pooled according to genotype and *in vivo* treatment in Medium 199 with 100 mg/l L-glutamine and 25 mM HEPES (M199; Invitrogen) on ice. The pooled ovaries were minced into 8–10 pieces each and the fragments gently washed in M199. The ovarian fragments were incubated in M199 supplemented with 4 mg/ml collagenase (Sigma), 10 mg/ml deoxyribonuclease I (Sigma) and 10 mg/ml bovine serum albumin (Sigma) at 37°C for 90 min in an ambient atmosphere. Several times during this incubation, the fragments were flushed through sterile pipettes with successively smaller orifices to obtain a preparation of dispersed cells. Each cell preparation was washed 3 times in M199 and the final cell pellet suspended in 0.16 ml M199 per pair of ovaries in the original pool.

Acute steroidogenic assays were conducted in sterile 12 x 75 polystyrene culture tubes (BD Falcon, Bedford, MA). Each assay consisted of 25  $\mu\text{l}$  dispersed ovarian cells in a 500  $\mu\text{l}$  final volume of M199 supplemented with 10 I.U./ml hCG or an equivalent volume of vehicle (0.85% saline), and/or a HSD17B3 inhibitor (DP3-3) or equivalent volume of vehicle (100% ethanol). The generation and characterization of DP3-3 ( $3\beta$ -((N-cyclohexylmethyl)-N-

cyclopropylcarbonyl)aminomethyl)-3 $\alpha$ -hydroxy-5 $\alpha$ -androstan-17-one)) has been previously described (22) as compound 213 and was provided for these studies by D.P. All assays were prepared at a minimum of duplicate per genotype, per *in vivo* treatment, per *in vitro* treatment. Immediately following preparation, all assays were incubated for 4 h in a 37°C water bath in an ambient atmosphere. The cells were then pelleted by centrifugation at 8,000 x g for 5 min and the medium transferred to a fresh tube and stored at -70°C for later analyses of androstenedione and testosterone content by radioimmunoassay (RIA). These experiments were repeated in 3 independent trials.

### Hormone radioimmunoassays (RIA)

Plasma and media androgen levels were assessed using the Active Androstenedione RIA and Active Testosterone RIA kits (Diagnostic Systems Laboratories, Webster, TX) according to the manufacturer's protocol. All plasma hormone assays were performed in duplicate (when sample volume allowed) on samples collected from individual animals. All media hormone assays were performed on individual experimental samples and always in duplicate. Final assay samples were quantified using an Apex Automation Gamma Counter (Micromedic Systems, Seattle, WA) and accompanying software. For the androstenedione RIA, the least detectable concentration was 0.03 ng/l, the average intra-assay coefficient of variation was 2.5%, and the inter-assay coefficient of variation was 9.2%. For the testosterone RIA, the least detectable concentration was 0.08 ng/l, the average intra-assay coefficient of variation was 3.0%, and the inter-assay coefficient of variation was 11.6%. RIAs on an equivalent volume of M199 supplemented with 10 I.U./ml hCG or an equivalent volume of vehicle (0.85% saline), and/or 10  $\mu$ M HSD17B3 inhibitor (DP3-3) or equivalent volume of vehicle (100% ethanol) indicated that the androstenedione and testosterone content was below the level of detection for each steroid.

### Transmission electron microscopy

Adult animals were administered a sufficient dose of pentobarbital to induce deep anesthetization and then promptly perfused (whole-body) with a modified Karnovsky's fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde, buffered to pH 7.4 in 0.1 M sodium cacodylate (Electron Microscopy Sciences, Hatfield, PA). Gonads were excised from the animals, placed whole in the above fixative for approximately 2 h, cut into 1–2 mm cubes and then stored in the above fixative at 4°C until further processing. The tissues were then washed in 0.1 M sodium cacodylate, postfixed in 0.1 M sodium cacodylate-buffered 1% OsO<sub>4</sub> (Electron Microscopy Sciences), rinsed in distilled water, dehydrated through a series of graded ethanol baths followed by propylene oxide, and embedded in Polybed epoxy resin (Polysciences, Warrington, PA). Ultrathin (90 nm) sections were prepared and stained on-grid with 5% uranyl acetate and Reynold's lead citrate. All sections were examined in a FEI Tecnai 12 (Hillsboro, OR) transmission electron microscope operated at 80 kV and equipped with Microsoft Windows 2000 (Redmond, WA) and the MegaView III Soft Imaging System (Lakewood, CO). Several electron micrographs were taken for each sample to give an adequate sampling and representative overview.

### Statistical analysis

All data were analyzed for statistical significance ( $P < 0.05$ ) using JMP software (SAS Institute, Cary, NC). Data sets were first tested for homoscedasticity of variance using the Levene's test and if failed were log-transformed prior to further statistical analysis. All data sets were then evaluated by a one-way ANOVA followed by the Tukey-Kramer HSD post-hoc test when applicable.

The testosterone/androstenedione (T/A<sub>4</sub>) ratios were calculated for each individual animal or *in vitro* assay sample using the formula first described by Weibe and Morris (23) to evaluate

the endocrine profiles of women diagnosed with ovarian androgen excess. This value more effectively compares the degree of elevated testosterone above normal (vs. a simple ratio) and employs the following formula: (plasma T  $\div$  95% upper limit normal T)/(plasma A<sub>4</sub>  $\div$  95% upper limit normal A<sub>4</sub>). For determining the *in vivo* T/A<sub>4</sub> ratios (Fig. 1), the individual plasma levels from a group of adult wild type female mice (*n* = 24) were used to determine the 95% upper limit for T and A<sub>4</sub> as 0.10 ng/ml and 0.25 ng/ml, respectively. For determining the *in vitro* T/A<sub>4</sub> ratios (Figs. 5 and 6), the acute *in vitro* steroid synthesis by dispersed cells from untreated wild type ovaries (*n* = 3 replicates) were used to determine the 95% upper limit for T and A<sub>4</sub> as 0.20 ng/ml and 0.60 ng/ml, respectively.

## RESULTS

### $\alpha$ ERKO females exhibit abnormally elevated plasma androgens

Adult  $\alpha$ ERKO females possess levels of circulating androstenedione (A<sub>4</sub>) and testosterone (T) that are more than 3- and 50-fold that of wild type females, respectively (Fig. 1). Wild type females treated with hCG twice daily for 3 consecutive days exhibited significantly increased plasma androgen levels, but only androstenedione and not testosterone approached the levels characteristic of  $\alpha$ ERKO females (Fig. 1). Similar treatment of  $\alpha$ ERKO females had no additive effect on plasma androgen levels. Female LH-CTP mice, a transgenic line that possess chronically elevated LH levels that are more comparable to those of  $\alpha$ ERKO females (16,24), also exhibited significantly increased plasma androstenedione levels relative to wild type and even  $\alpha$ ERKO females, but much lower testosterone levels compared to the latter genotype (Fig. 1). Therefore,  $\alpha$ ERKO females largely differ from other models of ovarian LH-hyperstimulation in their unique capacity to produce much greater levels of testosterone. This male-like capacity of  $\alpha$ ERKO ovaries to synthesize testosterone is especially revealed when T/A<sub>4</sub> ratios among the different genotypes are compared (Fig. 1). Wild type females exhibited a T/A<sub>4</sub> ratio of 0.82, which was significantly increased to 1.17 (*P* < 0.05) following 3 days of hCG treatment. Remarkably, LH-CTP females exhibited a similar T/A<sub>4</sub> ratio (1.11) despite a life-long elevation in circulating LH. In contrast,  $\alpha$ ERKO females exhibited a striking T/A<sub>4</sub> ratio of 17.2 and showed no further increase following hCG treatment (17.0). These data indicate that chronic, excessive LH stimulation alone does not lead to male-like efficiency of testosterone synthesis in murine ovaries; but instead indicate that this phenotype is a primary effect of the loss of intraovarian ER $\alpha$ -mediated actions.

### $\alpha$ ERKO ovaries exhibit testis-like expression and regulation of the *Hsd17b3* gene

Comparative Northern blot analyses of gonadal RNAs indicated that  $\alpha$ ERKO ovaries repeatedly possess levels of *Hsd17b3* expression that are equal to or even greater than those observed in the testes of normal adult males, while levels in wild type ovaries were below the level of detection (Fig. 2a). These data were confirmed by quantitative real-time RT-PCR, which indicated the level of *Hsd17b3* expression in  $\alpha$ ERKO ovaries was 2-fold that of adult testes (Fig. 2c). Similar analyses of LH-CTP ovaries also indicated a lack of *Hsd17b3* expression but increased *Cyp17a1* expression (Figs. 2b and c), the latter being a known ovarian marker of LH stimulation.  $\alpha$ ERKO ovaries also exhibited increased levels of *Cyp17a1* expression that were 6- and 3-fold higher than those of wild type and LH-CTP ovaries, respectively (Fig. 2c).

Because LH is the principle hormone for the induction and maintenance of *Hsd17b3* expression in Leydig cells of adult testes (25–28), we sought to determine if *Hsd17b3* expression in  $\alpha$ ERKO ovaries exhibits a similar, testis-like pattern of gonadotropin regulation. These experiments were conducted by treating hypogonadal (*hpg*) wild type (wild type<sup>*hpg*</sup>) and  $\alpha$ ERKO ( $\alpha$ ERKO<sup>*hpg*</sup>) females, which lack circulating endogenous gonadotropins, with a continuous infusion of exogenous hFSH or hLH for a period of one week. Wild type<sup>*hpg*</sup> males

were similarly treated to provide data on the expected response in testes. As shown in Fig. 3, hLH increased *Hsd17b3* expression almost 40-fold in the testes of wild type<sup>hpg</sup> males, while the response to hFSH was measurable but conspicuously lower (< 5-fold). Surprisingly,  $\alpha$ ERKO<sup>hpg</sup> ovaries exhibited a similar response to hLH by increasing *Hsd17b3* expression over 25-fold, while wild type<sup>hpg</sup> ovaries exhibited a measurable but notably less robust increase in *Hsd17b3* expression (Fig. 3). This difference in the induction of *Hsd17b3* expression in the gonads of wild type and  $\alpha$ ERKO females is not due to a disparity in the effectiveness of hLH treatment since *Cyp17a1*, a known LH-induced gene, was increased 23- and 80-fold in each genotype, respectively (data not shown). hFSH treatment had no effect on *Hsd17b3* expression in the ovaries of either genotype and only a minimal effect in the testes. However, the effectiveness of hFSH treatment in the ovaries of wild type and  $\alpha$ ERKO females was illustrated by induction of *Hsd17b1* (Fig. 3), a known FSH regulated gene in the ovary (29,30). FSH induced *Hsd17b1* expression 4-fold in both wild type<sup>hpg</sup> and  $\alpha$ ERKO<sup>hpg</sup> ovaries, while LH had minimal effect (Fig. 3). Another known FSH-induced gene in the ovary, *Cyp19a1*, was also increased over 200-fold in both wild type and  $\alpha$ ERKO ovaries (data not shown). Neither gonadotropin induced *Hsd17b1* expression in the testes of wild type<sup>hpg</sup> males, demonstrating that this gene is distinctly expressed in ovaries.

### Ectopic *Hsd17b3* expression occurs in the interstitial cells of $\alpha$ ERKO ovaries

To gain insight into the functional compartment responsible for ectopic *Hsd17b3* expression in  $\alpha$ ERKO ovaries, total RNA was prepared from ovaries that were first partitioned into fractions of enriched granulosa and stromal-interstitial cells. Northern blot analysis clearly indicated that *Hsd17b3* mRNAs were concentrated in the stromal-interstitial compartment of  $\alpha$ ERKO ovaries with only nominal detection in the granulosa cell fraction, most likely due to cross-contamination by small amounts of stromal material (Fig. 4). The relative purity of the fractions was illustrated by reprobating the blot for *Cyp19a1* mRNA, which are generally localized to granulosa cells of large follicles (Fig. 4).

The above findings were further supported by *in situ* hybridization analyses of adult  $\alpha$ ERKO ovaries. As shown in Fig. 4, *Hsd17b3* mRNAs were distinctly localized to the stromal-interstitial regions of  $\alpha$ ERKO ovaries, while hybridization levels were below background in the granulosa cells of follicles of all sizes. More precisely, *Hsd17b3* expression in  $\alpha$ ERKO ovaries was concentrated in the secondary interstitial (SI) cells, which populate the areas between the follicles and medullary portions of the ovary. SI cells are thought to have once been thecal interstitial (TI) cells, but following follicle atresia they become no longer associated with growing follicles and populate the ovarian stroma (11). Because growing follicles in  $\alpha$ ERKO ovaries characteristically exhibit poorly organized layers of TI cells, whether *Hsd17b3* expression was distinct to the SI or shared between the two interstitial cell types was difficult to discern. No substantial hybridization of the *Hsd17b3* probe was observed among any cell types in wild type ovaries (Fig. 4). As expected, parallel experiments in testes from adult wild type males indicated significant *Hsd17b3* expression that was distinctly localized to Leydig cells (Fig. 4). Furthermore, hCG-mediated induction of *Hsd17b3* expression in the testis of an adult wild type<sup>hpg</sup> male was again specifically localized to the interstitial (extratubular) compartments of the testis where Leydig cells are located.

### Ectopic HSD17B3 activity mediates testosterone synthesis in $\alpha$ ERKO ovaries

Adult wild type and  $\alpha$ ERKO females were first treated with vehicle or hCG for 3 days to stimulate ovarian androgen production *in vivo*. Dispersed ovarian cell preparations were then generated and their acute steroidogenic capacity was assessed during a 4 h *in vitro* incubation in the presence or absence of hCG and/or a HSD17B3 inhibitor (DP3-3). The results of these experiments are summarized in Fig. 5. As expected,  $\alpha$ ERKO ovarian cells produced significantly more androstenedione and testosterone compared to wild type cells, regardless



of *in vivo* or *in vitro* hCG exposure (Fig. 5a). *In vivo* hCG treatments enhanced *in vitro* androstenedione and testosterone synthesis in both genotypes; however, only  $\alpha$ ERKO ovarian cells exhibited an additive increase in overall androgen synthesis during *in vitro* hCG exposure. More importantly, these experiments provided two critical findings. First, consistent with the *in vivo* phenotype, testosterone was clearly the predominant androgen produced by  $\alpha$ ERKO ovarian cells *in vitro*, yielding an average T/A<sub>4</sub> ratio of  $3.32 \pm 0.5$  vs.  $0.47 \pm 0.1$  in wild type cells (untreated both *in vivo* and *in vitro*). Secondly, inclusion of the HSD17B3 specific inhibitor (DP3-3) consistently reduced testosterone synthesis by 50-60% in  $\alpha$ ERKO ovarian cells and decreased the average T/A<sub>4</sub> ratio to  $0.73 \pm 0.2$ , providing strong evidence that testosterone synthesis in  $\alpha$ ERKO ovaries is largely due to ectopic HSD17B3 activity. In contrast, the minimal but detectable testosterone synthesis by wild type cells was not affected by the HSD17B3 inhibitor (T/A<sub>4</sub> ratio =  $0.45 \pm 0.02$ ), indicating that it is likely mediated by the androgenic activity of rodent HSD17B1, which is innate to ovarian granulosa cells. A representative dose-response curve for HSD17B3 inhibition of testosterone synthesis by  $\alpha$ ERKO ovarian cells *in vitro* is shown in Fig. 5b.

Similar experiments were conducted to compare the steroidogenic capacity of dispersed ovarian cells from LH-CTP and  $\alpha$ ERKO females since these two models are more comparable in terms of chronic LH hyperstimulation of the ovary. Acute *in vitro* steroidogenic assays on dispersed cells from LH-CTP ovaries indicated substantial androstenedione production that was 2-fold that observed in  $\alpha$ ERKO ovarian cells (Fig. 6), consistent with their *in vivo* androgen profile (Fig. 1). Only when  $\alpha$ ERKO females were treated with exogenous hCG prior to tissue collection did the *in vitro* level of androstenedione synthesis become comparable to that of LH-CTP cells. This overabundance of androstenedione in LH-CTP cells is indicative of the increased *Cyp17a1* expression shown earlier (Fig. 2). Still, even the highest level of *in vitro* androstenedione synthesis achieved in LH-CTP cells did not provide for  $\alpha$ ERKO-like levels of testosterone production, which were 3-fold higher in the latter genotype. Furthermore, the HSD17B3 inhibitor had no measurable effect on testosterone synthesis in LH-CTP cells, indicating it is likely mediated by the androgenic activity of HSD17B1.

### $\alpha$ ERKO ovaries harbor stromal Leydig-like cells

To determine if the interstitial portions of  $\alpha$ ERKO ovaries possessed Leydig-like ultrastructural features, we employed transmission electron microscopy (TEM) to compare normal Leydig cells of mouse testes to stromal-interstitial cells of adult  $\alpha$ ERKO ovaries. The typical whorl-arranged SER that is innate to Leydig cells of mouse testes (31) is illustrated in Fig. 7(a and b). The Leydig cell shown possesses multiple whorls of SER, one of which surrounds a lipid droplet. Also prominent are random tubular SER and numerous mitochondria, the latter possessing the tubular cristae that are characteristic of steroidogenic cells (Fig. 7b) (31). Remarkably, a parallel survey of stromal-interstitial regions of multiple  $\alpha$ ERKO ovaries identified cells that possessed similarly arranged SER, clearly organized as whorls and interspersed with large lipid droplets and tubular SER (Fig. 7c-e). The whorl-like SERs found in  $\alpha$ ERKO ovarian stroma-interstitial cells usually occupied a smaller intracellular area and were less structured relative to that in testicular Leydig cells (Fig. 7c and d) but were clearly Leydig-like in appearance. In addition, the whorled SERs observed among  $\alpha$ ERKO ovarian stromal-interstitial cells were often vesiculated (Fig. 7f), remarkably similar to that which occurs during the natural involution of Leydig cells in fetal or aged testes. These vesiculated structures in  $\alpha$ ERKO ovarian stromal-interstitial cells are likely sites of lipid degradation and may account for the substantial intracellular prevalence of lipofuscin observed in H & E stained tissue sections (Fig. 7h), which is also a common cytoplasmic component of Leydig cells in multiple species (31). Furthermore, the cells possessing Leydig-like SER and lipofuscin were limited to the interstitial regions in  $\alpha$ ERKO ovaries, the normal site of secondary interstitial

cells and correlating with the areas of *Hsd17b3* expression indicated by *in situ* hybridization. No similar structures were found in wild type ovaries (data not shown).

Along with the above findings, TEM revealed an additional noteworthy characteristic of  $\alpha$ ERKO ovarian secondary interstitial cells. As illustrated in Fig. 7(c and g), numerous crystalline-like cytoplasmic inclusions were found to repeatedly coexist in cells that harbored whorled, Leydig-like SER. These inclusions were long, cylindrical bodies of 0.1-2  $\mu$ m in length that were often present in great numbers and almost always associated with whorled SER. Crystalline cytoplasmic inclusions are a common feature of normal Leydig cells in the testes of several species and are considered a definitive attribute of human Leydig cells and Leydig cell tumors, within which they are referred to as crystals of Reinke (31). However, there are no reports of similar cytoplasmic crystalline inclusions in Leydig cells or ovarian secondary interstitial cells of mice.

## DISCUSSION

The collective findings from studies as early as the mid 1970's impart a convincing argument for an inhibitory role of estrogens on Leydig cell development, differentiation and function (as reviewed in (14). For example, estrogens are documented to inhibit testosterone synthesis in adult rodent Leydig cells (14) as well as disrupt Leydig cell proliferation and differentiation in fetal and neonatal testes (32). Furthermore, estradiol inhibits the natural regeneration of Leydig cells that occurs in rodent testes following their destruction by acute exposure to ethane dimethylsulfonate (EDS), a Leydig cell-specific toxicant (33). In addition, the precisely timed periods of substantial Leydig cell proliferation and differentiation during testis development correlate inversely with periods of low endogenous estradiol levels (15). Remarkably, the concept that these findings might reveal a parallel, physiological role for estradiol-mediated suppression of Leydig cell differentiation and testosterone synthesis in ovaries has received little attention. Herein, we provide definitive evidence that estradiol is principally involved in preventing the development of functional Leydig-like cells in the stromal-interstitial portions of the mouse ovary and that these actions are exclusively mediated by ER $\alpha$ .

Our assertion that  $\alpha$ ERKO ovaries inappropriately possess functional Leydig-like cells is based on their exhibiting traits that are regarded as exclusive to testicular Leydig cells, including *a*) substantial *Hsd17b3* expression and enzymatic activity, *b*) whorl-like SER and *c*) crystalline-like structures comparable to crystals of Reinke. Furthermore, the ectopic *Hsd17b3* expression in  $\alpha$ ERKO ovaries is LH-dependent and thus exhibits a pattern of gonadotropin regulation that is comparable to that of mature testicular Leydig cells. Similar *Hsd17b3* expression and cells possessing Leydig-like features are reportedly present in the ovaries of mice lacking endogenous estradiol due to targeted disruption of the *Cyp19a1* gene; and the former phenotype is abolished by exogenous estradiol treatments (17). However, because estradiol treatment of CYP19A1-null females also results in normalization of the heightened LH levels that are present in these animals (17), these experiments do not discern whether the loss of ovarian *Hsd17b3* expression is due to activation of intraovarian ER $\alpha$  or decreased gonadotropin stimulation. In the current study, we provide definitive evidence that ectopic *Hsd17b3* expression in  $\alpha$ ERKO females is due solely to the loss of ER $\alpha$ -mediated actions within the ovary and simply requires LH stimulation to manifest. For example, the ovaries of transgenic LH-CTP female mice categorically lack *Hsd17b3* expression and activity (18) and Fig. 2 and 6 herein) despite being chronically exposed to substantially high levels of circulating LH (34,35). The ovaries of estrogen receptor (ER $\beta$ )-null female mice also lack ectopic *Hsd17b3* expression (16) even when harboring the LH-CTP transgene to increase circulating LH levels (18). Likewise, reports of transgenic mice that constitutively express hCG, an LH analog, describe ovaries exhibiting interstitial-thecal cell hypertrophy but make no reference to *Hsd17b3* expression or Leydig-like cells (36,37). In contrast, Heikkilä *et al.* (38) recently

reported that the ovaries of newborn *Wnt4*-null female mice exhibit ectopic induction of *Hsd17b3* expression that is concurrent with a 8-fold reduction in ER $\alpha$  expression but no change in ER $\beta$  levels. All of these findings support our conclusion that ER $\alpha$  is critical to repressing the expression of this Leydig cell-specific steroidogenic gene in the ovary.

Evidence supporting the presences of substantial HSD17B3 enzymatic activities in  $\alpha$ ERKO ovaries was revealed by several findings. Foremost was the remarkably high T/A<sub>4</sub> ratio in the plasma of adult  $\alpha$ ERKO females that is more comparable to that of normal male mice (39, 40) and adult men (41) as well as women clinically diagnosed with ovarian androgen excess (23). We have previously shown that increased androgen levels in  $\alpha$ ERKO females are at least partially due to hyperstimulation of the ovary by LH (16). Still, when wild type female mice were forced to possess a comparable hypergonadotropic condition via either daily injections with hCG, an LH analog, or transgenic methods, as achieved in LH-CTP mice, the resulting androgenic phenotype was markedly less severe and qualitatively different than that of  $\alpha$ ERKO females as androstenedione, and not testosterone, was the predominant steroid produced in the former models. Secondly, we conducted specific experiments to discern the individual contributions of type 1 and type 3 activities to the overall testosterone load in  $\alpha$ ERKO females. This was necessary because the rodent ortholog of HSD17B1, which is normally expressed in the ovary and mediates the reduction of estrone to estradiol (2,42,43), is equally efficient at reducing androstenedione to testosterone (44,45). Furthermore, *Hsd17b1* expression is increased almost 2-fold above normal in  $\alpha$ ERKO ovaries (16). By employing a method first described by Magoffin and Erickson that allows for the evaluation of the acute steroidogenic capacity of dispersed ovarian cells *in vitro* (21,46) with the use of a specific inhibitor of HSD17B3 enzymatic activities (22), we were able to successfully discriminate type 1 and type 3 mediated HSD17B testosterone synthesis in dispersed wild type and  $\alpha$ ERKO ovarian cells. The resulting data strongly indicated that the male-like capacity for testosterone synthesis in  $\alpha$ ERKO ovaries is due predominantly to the presence of substantial HSD17B3 activities, whereas the minimal level of testosterone synthesis observed in hCG-treated wild type or LH-CTP ovaries is due to the androgenic activities of HSD17B1 that occur in the presence of increased androstenedione. Testosterone synthesis in  $\alpha$ ERKO females is undoubtedly further promoted by a parallel increase in CYP17A1 activities that provide for ample synthesis of androstenedione, the substrate for HSD17B3.

The mechanisms by which ER $\alpha$  actions suppress the testicular pathway of testosterone synthesis in the ovary are unclear. Ligand-activated ER $\alpha$  may act in a dynamic manner within thecal/secondary interstitial cells to persistently inhibit *Hsd17b3* expression. ER $\alpha$  is indeed amply expressed in both testicular Leydig cells (47,48) and ovarian thecal/secondary interstitial cells (49–51). Furthermore, estrogens are known to inhibit testosterone synthesis in both thecal and Leydig cells *in vitro*, although most evidence indicates that the upstream steroidogenic enzyme, CYP17A1, is the primary target (11,14,52). Indeed, CYP17A1 expression and activity are also increased in the ovaries (Fig. 2, (16), *in vitro* cultured follicles (F. Taniguchi, J.F. Couse and K.S. Korach, manuscript in preparation) and testes (53) of  $\alpha$ ERKO but not  $\beta$ ERKO mice. However, ours is the first study to demonstrate that ER $\alpha$  represses ovarian testosterone synthesis via robust negative modulation of *Hsd17b3* expression as well. Interestingly, a recent report that  $\alpha$ ERKO males exhibit a 2-fold increase in *Hsd17b3* expression and type 3 activity (53) indicates that ER $\alpha$  may play an analogous role in testes.

In contrast to the above postulated dynamic mechanism for ER $\alpha$ -mediated inhibition of *Hsd17b3* expression and testosterone synthesis in the ovary is a potential organizational role by which ER $\alpha$  might repress the development of a Leydig cell phenotype among the secondary/thecal interstitial cells. Support for such an organizational role for ER action during gonadal differentiation comes from multiple descriptions of permanently disrupted Leydig cell function in adult rodents following developmental exposure to estrogens, as well as estrogen-mediated

inhibition of Leydig cell regeneration and proliferation in EDS-exposed testis (14). Herein, we employed TEM to produce evidence of Leydig-like cells in  $\alpha$ ERKO ovaries. The “single most identifying characteristic of the Leydig cell” is their unique, and often species-specific arrangements of smooth endoplasmic reticulum (SER) (31). The Leydig cells of adult mouse testes, as well as other species, characteristically exhibit a portion of SER that is arranged as “whorls” often surrounding a lysosome or lipid droplet (31 and references therein). This swirled form of SER is continuous with the tubular form and may comprise up to 7% of the total SER within a single Leydig cell (31). In the current study, we found that  $\alpha$ ER ovaries possess secondary-interstitial cells that exhibit similar “whorl”-arranged SER as well as other Leydig cell features, including crystalline-like cytoplasmic inclusions and an accumulation of lipofuscin. Furthermore, the  $\alpha$ ERKO ovarian cells exhibiting these Leydig-like features were located in the same ovarian regions that possessed significant levels of *Hsd17b3* expression as indicated by *in situ* hybridization. These findings raise the prospect that a loss of ER $\alpha$  actions during secondary/thecal-interstitial cell differentiation in the ovary may allow for the inappropriate development of cells possessing a Leydig-like phenotype. The capacity of adult hypogonadal  $\alpha$ ERKO ( $\alpha$ ERKO<sup>hpg</sup>) ovaries to rapidly acquire *Hsd17b3* expression following acute exogenous hLH treatment suggests that the Leydig-like cells may develop in an environment void of gonadotropins and are present as early as birth or prior to puberty.

Our finding that the Leydig-like cells in adult  $\alpha$ ERKO ovaries were located in the stroma and often bordered by secondary/thecal-interstitial cells distinguishes them from hilar interstitial (HI) cells, which are natural to the ovary and considered structurally and functionally similar to testicular Leydig cells (11). HI cells are normally limited to the connective tissues in the hilus of the ovary (11); whereas, the Leydig-like cells observed in  $\alpha$ ERKO ovaries are located in the stroma, surrounded by thecal/secondary interstitial cells and usually in close proximity to a follicle. Admittedly, poor organization of the medullary regions is characteristic of adult  $\alpha$ ERKO ovaries (54,55) and may have altered the natural position of HI cells. Still, there is no evidence of *Hsd17b3* expression in the HI cells of normal ovaries (8,11), providing further evidence that the Leydig-like cells in  $\alpha$ ERKO ovaries are not of hilar origin.

The stromal location of the Leydig-like cells in  $\alpha$ ERKO ovaries in conjunction with their close proximity to hypertrophied secondary/thecal-interstitial cells suggests that they may have once been part of the latter cell population but have since acquired a male-like phenotype via *transdifferentiation*. This phenomenon is thought to underlie the formation of stromal-Leydig cell tumors and non-hilar, pure Leydig cell tumors in women (56,57). Although extremely rare, these ovarian tumors are invariably androgenic and often bordered by hypertrophied ovarian stroma (56–59). Furthermore, there are clinical reports of HSD17B3-like activity in the ovaries of women suffering from ovarian androgen excess (60,61), androgenic HSD17B immunoreactivity in a pure Leydig cell tumor (62), and *HSD17B3* transcripts in a Sertoli-Leydig cell tumor and surrounding luteinized thecal tissue (63). In contrast, ovarian tissue and cell lines from women diagnosed with polycystic ovarian syndrome have been found to lack detectable *HSD17B3* expression (64–66), indicating that this malady is not likely related to the ER $\alpha$ -null phenotype described here.

Thecal cell androgen synthesis is critical to ovarian function as androgens serve as both important hormonal signals during granulosa cell differentiation in the early stages of folliculogenesis and later, as substrates for estradiol synthesis in mature preovulatory follicles (67). However, when androgen levels and activity are allowed to surpass that required to support estradiol synthesis during the latter stages of folliculogenesis, the follicle invariably becomes atretic (68,69). This condition is likely exacerbated if testosterone, rather than androstenedione, is the predominant androgen present, as the former steroid is a more potent androgen receptor agonist. Therefore, stringent regulation of the quantity and nature of thecal/secondary interstitial cell androgen synthesis is critical to the generation of healthy follicles

capable of ovulation and fertilization. Our data provide strong evidence that ligand-dependent ER $\alpha$  actions are fundamental to this physiology of thecal/secondary interstitial cells by repressing their acquisition of HSD17B3 activities and perhaps their differentiation to a Leydig-like phenotype. Our description of ectopic *Hsd17b3* expression and Leydig-like cells in the ovaries of ER $\alpha$ -null female mice represents the first data to support an integral intraovarian role for ER $\alpha$ . Further studies are required to discern whether ER $\alpha$  operates via activational or organizational mechanisms to repress the acquisition of a testis-like phenotype in the secondary/thecal interstitial cells of the ovary. A similar phenotype of granulosa to Sertoli cell *transdifferentiation* occurs in the ovaries of compound ER-null (ER $\alpha$ -null; ER $\beta$ -null) female mice (70,71). From these collective data, we may infer that ER-mediated estradiol signaling is critical to the development and/or maintenance of the female phenotype in the endocrine somatic cell types of the ovary.

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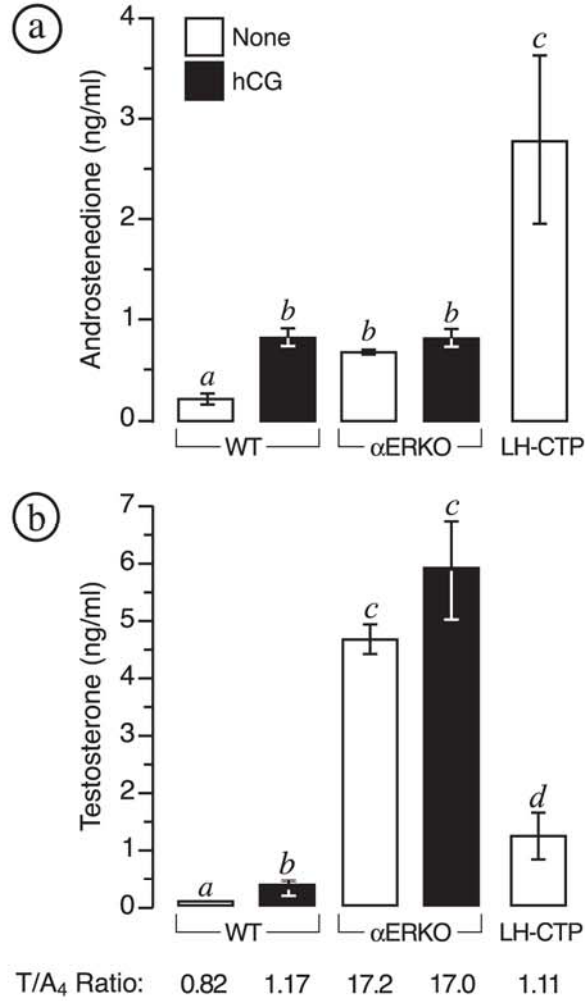
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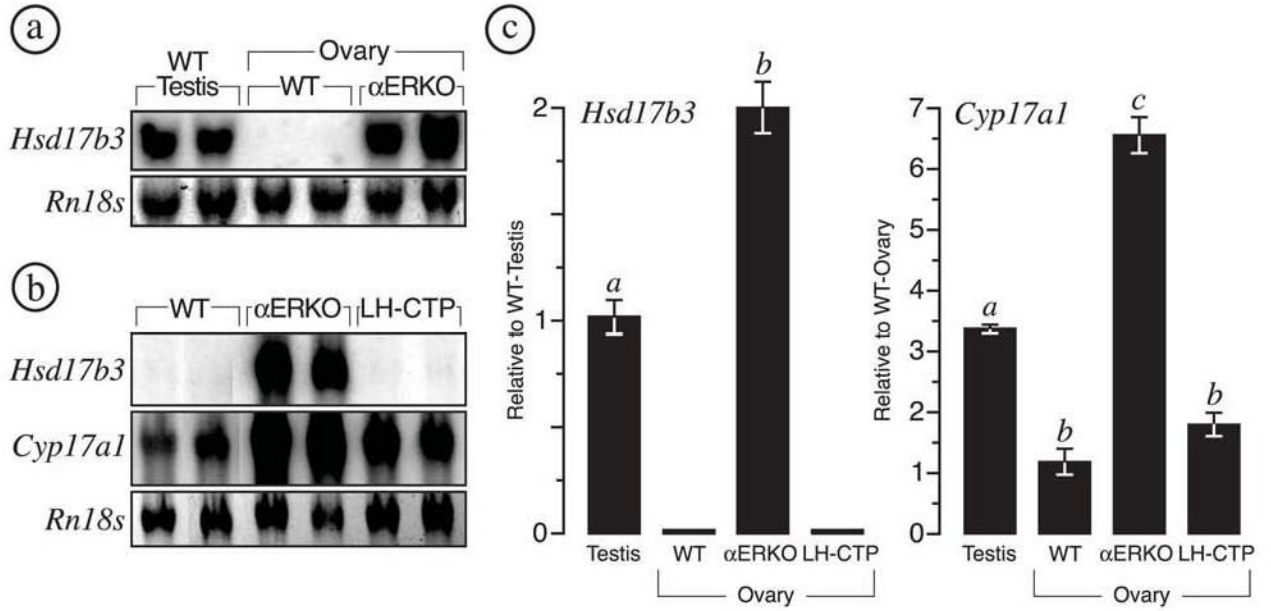
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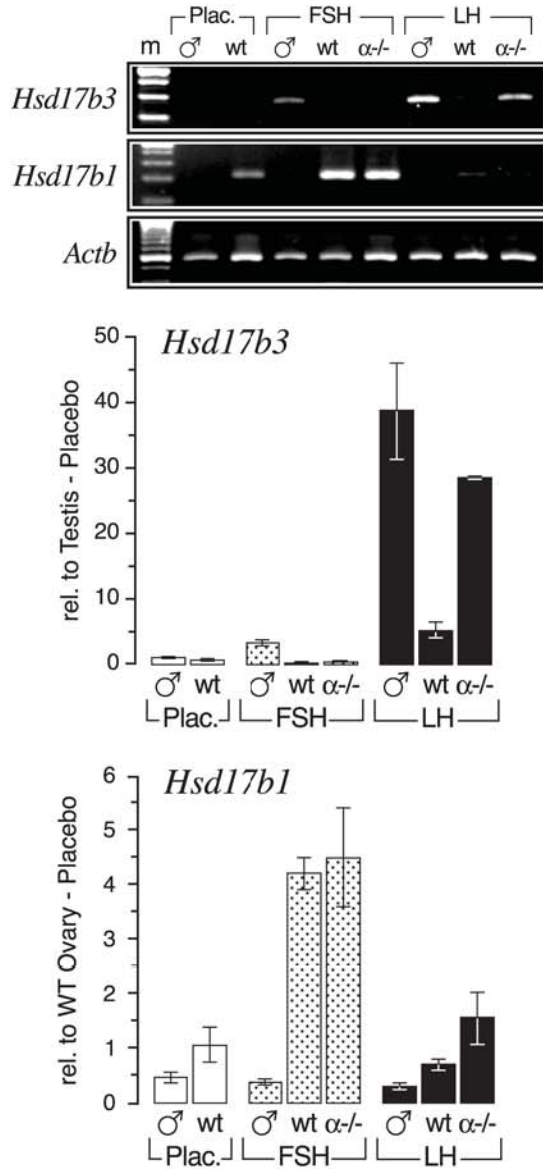


**FIG 1.**  $\alpha$ ERKO females exhibit elevated plasma androgens. Shown are the average ( $\pm$  SEM) plasma levels for (a) androstenedione and (b) testosterone in untreated adult wild type,  $\alpha$ ERKO and LH-CTP females; and wild type (WT) and  $\alpha$ ERKO females treated twice daily with 5 I.U. human chorionadotropin (hCG) for 5 consecutive days (see Materials and Methods). Sample numbers (*n*) for each group were: wild type, 24; wild type + hCG, 22;  $\alpha$ ERKO, 28;  $\alpha$ ERKO + hCG, 8; LH-CTP, 8. Bars that do not share the same letter are significantly different ( $P < 0.05$ ). (bottom) Shown is the average testosterone to androstenedione (T/A<sub>4</sub>) ratio for each genotype and treatment group as calculated using the formula of Weibe and Morris (23).



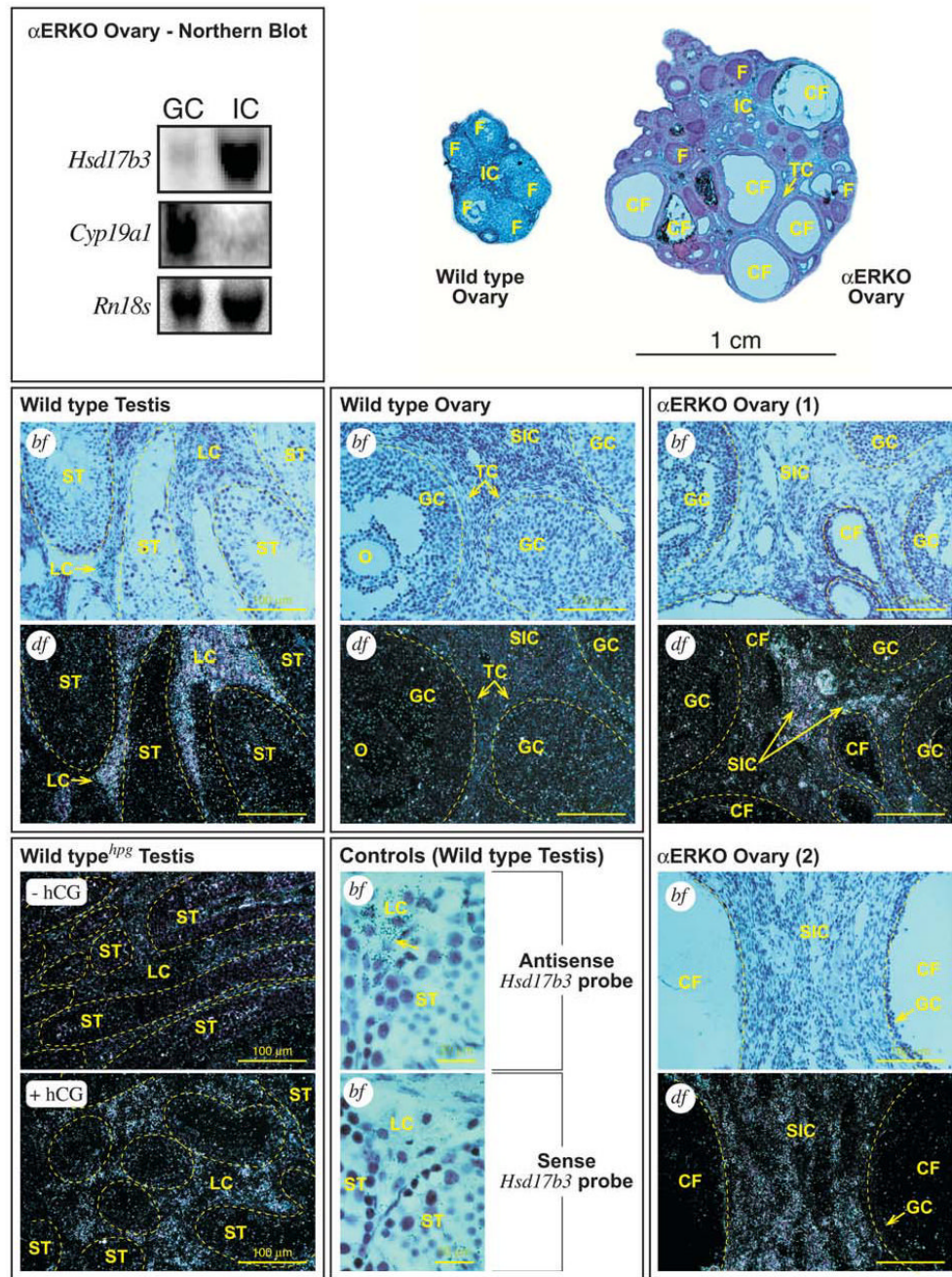
**FIG 2.**

Ectopic *Hsd17b3* expression is unique to  $\alpha$ ERKO ovaries. (a) Northern blot analysis for *Hsd17b3* transcripts in adult wild type (WT) testes, WT ovaries, and  $\alpha$ ERKO ovaries. The level of *Hsd17b3* expression in adult  $\alpha$ ERKO ovaries is comparable to that of WT testes; but undetectable in WT ovaries. (b) Northern blot analysis for *Hsd17b3* transcripts in adult WT,  $\alpha$ ERKO and LH-CTP ovaries indicates that ectopic expression of *Hsd17b3* does not occur in the ovaries of hypergonadotropic LH-CTP females. The same Northern blot was reprobed for *Cyp17a1* transcripts, a known LH-regulated gene in the ovary. All Northern blots were probed for ribosomal *Rn18s* (18S rRNA) to demonstrate equal loading of total RNA among samples. (c) Data from real-time RT-PCR (average  $\pm$  SEM) for *Hsd17b3* and *Cyp17a1* expression in adult WT testes, WT ovaries,  $\alpha$ ERKO ovaries, and LH-CTP ovaries. *Hsd17b3* expression in  $\alpha$ ERKO ovaries is 2-fold that of WT testes.  $\alpha$ ERKO ovaries also exhibit significantly increased *Cyp17a1* expression relative to WT testes and ovaries. Sample numbers (*n*) for each group were as follows: WT testes, 3 males; wild type ovaries, 5 females;  $\alpha$ ERKO, 3 females; LH-CTP, 3 females. Bars that do not share the same letter are significantly different ( $P < 0.05$ ).



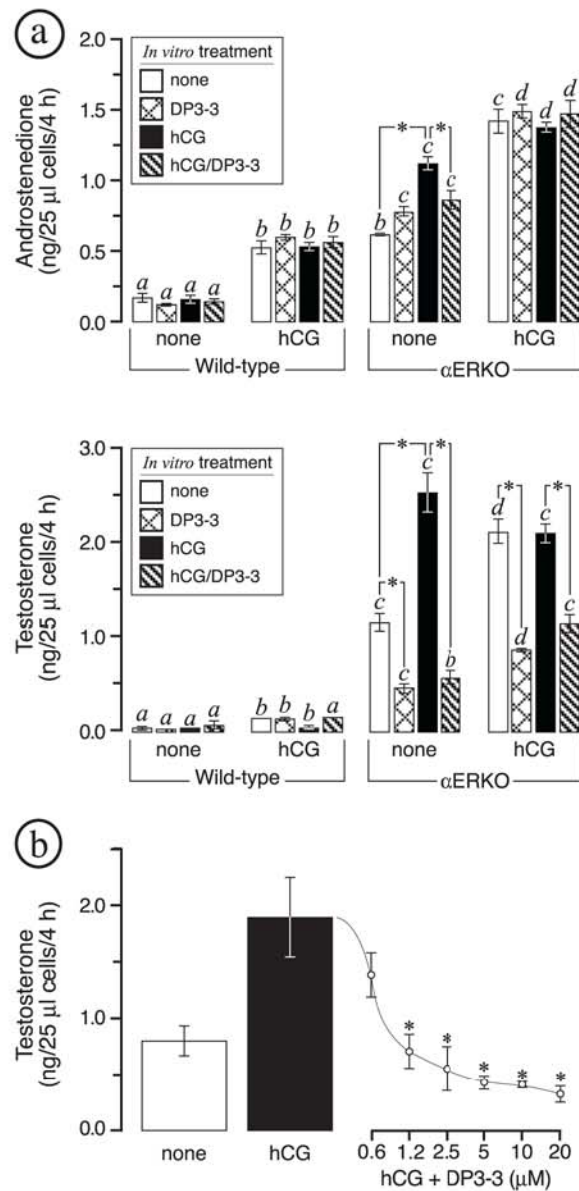
**FIG 3.** Gonadotropin induction of *Hsd17b3* in hypogonadal  $\alpha$ ERKO ovaries. Adult wild-type<sup>hpg</sup> (WT<sup>hpg</sup>) males and females, and  $\alpha$ ERKO<sup>hpg</sup> females were treated for 1 week with purified human LH or recombinant human FSH (see Materials and Methods). (top) Shown is a representative agarose gel of semi-quantitative RT-PCR for *Hsd17b3* and *Hsd17b1* transcripts in the gonads of each genotype and treatment. *Hsd17b3* expression is relatively unique to testes and maximally induced by hLH, while *Hsd17b1* is relatively unique to ovaries and maximally induced by hFSH. Also apparent is the testis-like induction of *Hsd17b3* expression in the ovaries of  $\alpha$ ERKO<sup>hpg</sup> females. A small amount of *Hsd17b3* expression is detectable by RT-PCR in the ovaries of LH-treated WT<sup>hpg</sup> females. (bottom) Shown are the results of real-time RT-PCR quantitative assays (average  $\pm$  SEM) for *Hsd17b3* and *Hsd17b1* transcripts in the gonads of each genotype and treatment. Sample numbers (*n*) for each group were: WT<sup>hpg</sup> males

(all groups), 4 per group; WT<sup>hpg</sup>-none, 2 pools (3–4 ovary pairs per pool); WT<sup>hpg</sup>-hFSH, 6; WT<sup>hpg</sup>-hLH, 3;  $\alpha$ ERKO<sup>hpg</sup> (all groups), 2 per group.



**FIG 4.** Northern blot and *in situ* hybridization (ISH) for *Hsd17b3* mRNA in ovary and testes. (*top, left*) Northern blot analysis on total RNA from pooled αERKO ovaries that were fractionated into granulosa (GC) and stromal-interstitial (IC) cell compartments, demonstrating that *Hsd17b3* expression in αERKO ovaries is localized to the stromal-interstitial compartment. The blot was stripped and reprobbed for transcripts of a granulosa cell-specific gene (*Cyp19a1*) to indicate the cellular purity of each ovarian fraction. A photograph of ethidium-stained *Rn18s* (18S rRNA) bands demonstrates equal loading levels between lanes. (*top, right*) Cross-sections from representative adult wild type (WT) and αERKO ovaries photographed at low magnification. The WT ovary exhibits several healthy, large follicles (F)

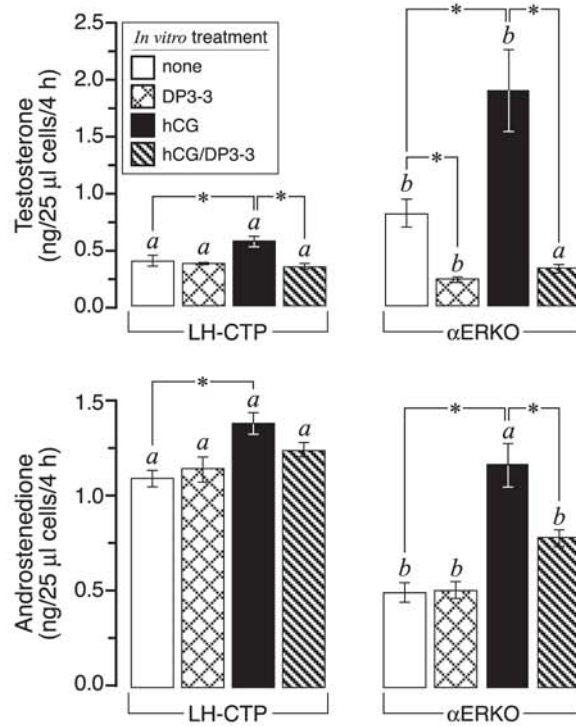
and a normal stromal-interstitial region (IC). In contrast, the  $\alpha$ ERKO ovary exhibits some smaller, relatively healthy follicles (F) but several enlarged, cystic follicles (CF). Also indicated in the  $\alpha$ ERKO ovary is a region of hypertrophied thecal cells (TC). (*middle, bottom*) Shown are representative photomicrographs (*bf*, bright field photomicrograph; *df*, dark-field photomicrograph) of tissue sections from wild type testes, wild type ovaries and  $\alpha$ ERKO ovaries (as labeled) that were subjected to ISH for *Hsd17b3* mRNAs. [Wild type Testis] Photomicrographs of serial sections from a representative adult wild type testis show specific hybridization of the *Hsd17b3* probe to the Leydig cells (LC) only, while hybridization levels among cells within the seminiferous tubules (ST), as indicated by dashed border, are at or below background. [Wild type Ovary] Photomicrographs of serial sections from a representative wild type ovary indicate no specific hybridization of the *Hsd17b3* probe in the oocyte (O), granulosa (GC), secondary interstitial (SIC), thecal (TC) cells or any other cell type. Follicles are indicated by dashed border. [ $\alpha$ ERKO Ovary] Photomicrographs of a pair of serial sections from two representative  $\alpha$ ERKO ovaries indicates specific hybridization of the *Hsd17b3* probe to the secondary interstitial cells (SIC) of the ovary, while hybridization levels among the granulosa cells of normal and cystic follicles (CF) are at or below background. Follicles are indicated by dashed border. [Wild type<sup>hpg</sup> Testis] Dark-field photomicrographs of testes sections from WT<sup>hpg</sup> males following treatment with either vehicle (-hCG) or human choriongonadotropin (+hCG) to induce *Hsd17b3* expression in the Leydig cells (see Materials and Methods). The untreated WT<sup>hpg</sup> testis exhibits little detectable *Hsd17b3* mRNA whereas the testis of the hCG-treated WT<sup>hpg</sup> male exhibits significant induction of *Hsd17b3* expression that is localized to the interstitial areas where Leydig cells (LC) are found. Seminiferous tubules are indicated by dashed border. [Controls - Wild type Testis] Photomicrographs of serial sections from a WT testis probed with either the *Hsd17b3* sense (negative control) or anti-sense (positive control) probes to illustrate specific hybridization of the anti-sense probe only to the Leydig cells (LC), as indicated by the numerous silver grains (arrow).

**FIG 5.**

*In vitro* evaluation of HSD17B3 activity in dispersed  $\alpha$ ERKO ovarian cells. All data are from *in vitro* acute steroidogenic assays on dispersed ovarian cells (see Materials and Methods). These experiments were repeated three times and found to yield comparable results, shown are the results of one independent trial. Wild type (WT) and  $\alpha$ ERKO females were first treated twice daily for 3 days with vehicle or human chorionadotropin (hCG) as indicated along the x-axis; dispersed ovarian cells were then prepared from each genotype/treatment group and incubated for 4 h in the indicated *in vitro* treatments. (a) *In vitro* androstenedione (top) and testosterone (bottom) synthesis indicates that *in vivo* hCG treatments led to increased androstenedione production in both WT and  $\alpha$ ERKO ovarian cells but increased testosterone synthesis in  $\alpha$ ERKO cells only. *In vitro* testosterone synthesis in dispersed  $\alpha$ ERKO ovarian cells was inhibited by the HSD17B3 inhibitor (DP3-3) at 10  $\mu$ M. (b) Shown is a dose-response curve illustrating the effect of the HSD17B3 inhibitor (DP3-3) on testosterone synthesis in

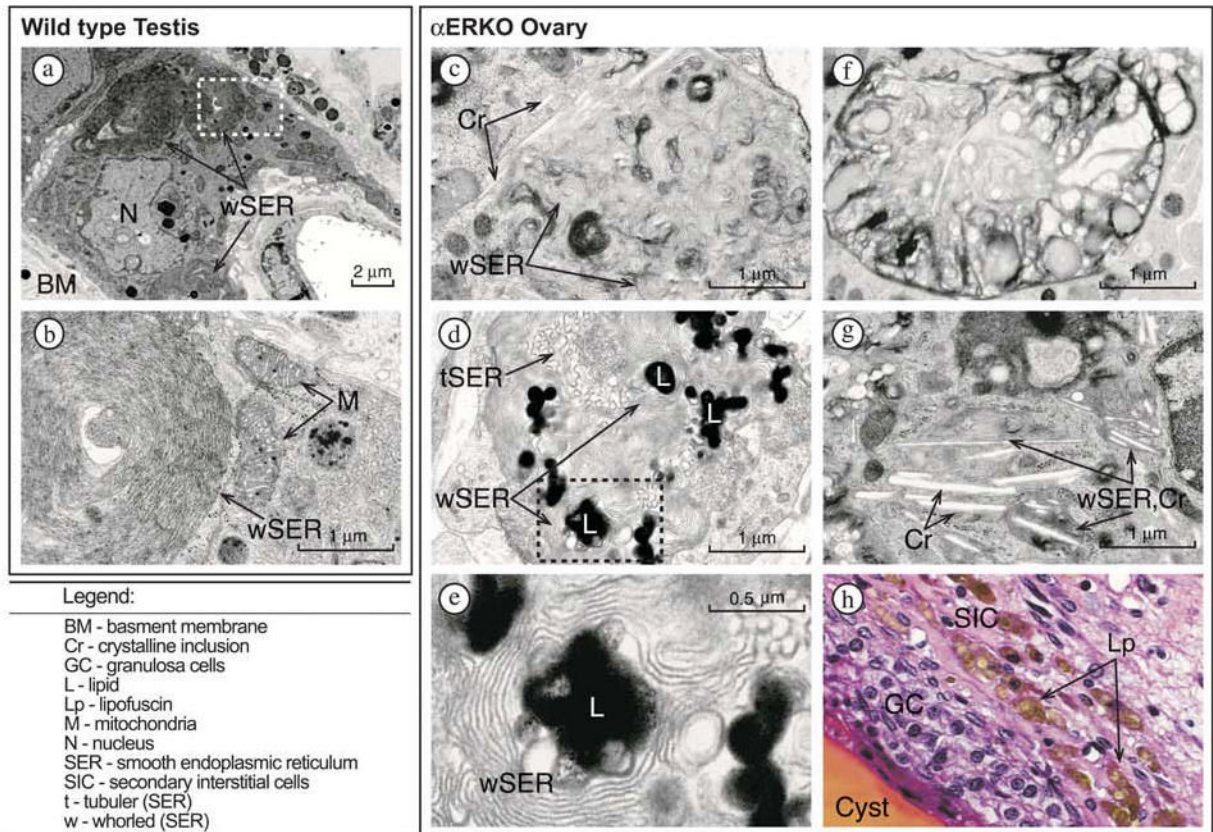


dispersed  $\alpha$ ERKO ovarian cells. All data are from dispersed ovarian cells from untreated  $\alpha$ ERKO females that were incubated in medium alone, medium with hCG (10 I.U./ml), or medium with hCG (10 I.U./ml) plus increasing concentrations of DP3-3. Each bar or point represents the average of 3 replicates, each of which was assayed in duplicate for each steroid. Bars that do not share the same letter are significantly different ( $P < 0.05$ ). Significant differences ( $P < 0.05$ ) within each genotype/treatment group are indicated by asterisks.



**FIG 6.**

*In vitro* evaluation of HSD17B3 activity in dispersed LH-CTP and  $\alpha$ ERKO ovarian cells. All data are from *in vitro* acute steroidogenic assays on dispersed ovarian cells (see Materials and Methods). Dispersed ovarian cells were prepared from untreated adult LH-CTP and  $\alpha$ ERKO females and incubated for 4 h in the indicated *in vitro* treatments. These experiments were repeated two times and found to yield comparable results, shown are the results of one independent trial. Although LH-CTP ovarian cells produce increased amounts of androstenedione relative to  $\alpha$ ERKO ovarian cells, they produce much less testosterone due to the absence of HSD17B3 activity. Bars that do not share the same letter are significantly different ( $P < 0.05$ ). Significant differences ( $P < 0.05$ ) within each genotype/treatment group are indicated by asterisks. Each bar represents the average of 4 ( $\alpha$ ERKO) or 3 (LH-CTP) replicates, each of which was assayed in duplicate for each steroid.

**FIG 7.**

Transmission electron microscopy indicates secondary interstitial cells with Leydig-like ultrastructural features in  $\alpha$ ERKO ovaries. [Wild type Testes] (a) A representative Leydig cell in a wild type testis possesses 3 clusters of whorled smooth endoplasmic reticulum (wSER). (b) A higher magnification of panel a (indicated by outlined area) illustrates a wSER and two juxtaposed mitochondria (M) with tubular cristae that are characteristic of testicular Leydig cells. [ $\alpha$ ERKO Ovary] (c and d) Representative examples of Leydig-like wSER found in the secondary-thecal interstitial cells of  $\alpha$ ERKO ovaries. Numerous electron-dense lipid droplets (L) are surrounded by the wSER; tubuler SER (tSER) that is typical of ovarian steroidogenic cells is also present. (e) Shown is a higher magnification of panel d (indicated by outlined area). (f) Shown is an example of vesiculated wSER in a  $\alpha$ ERKO ovary. (g) A secondary-interstitial cell in a  $\alpha$ ERKO ovary containing wSER with multiple large crystalline (Cr) inclusions throughout the cytoplasm. (h) Photomicrograph of the follicle wall of a cystic follicle in a  $\alpha$ ERKO ovary section stained with H&E, illustrating the considerable accumulation of lipofuscin (brown-pigmented material) in the cytoplasmic vacuoles among the secondary-interstitial cells.

**TABLE I**

Primers used for RT-PCR

Gene	Accession #	Amplified Sequences (bp)	Forward (5'-3')	Reverse (5'-3')
<i>Cyp17a1</i>	NM 007809	1356-1436	GATCGGTTTATGCCTGAGCG	TCCGAAGGGCAAATAACTGG
<i>Cyp19a1</i>	D00659	135-210	TGATCATGGGCCTCCTTCTC	CCCAGACAGTAGCCAGGACCT
<i>Hsd17b1</i>	NM 010475	689-765	CTGCGTGGTTATGAGCAAGC	CGCATTGCAGTCAAGAAGAGC
<i>Hsd17b3</i>	NM 008291	560-635	ATGGAGTCAAGGAGGAAAGGC	GGCTGTAAAGAGGCCAGGG
<i>Rn18s</i>	X56974	1271-1351	GAAACTGCGAATGGCTCATTA	GAATTACCACAGTTATCCAAGTAGGA