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# Estrogen receptor-α mediates an intraovarian negative feedback loop on thecal cell steroidogenesis via modulation of CYP17A1 (cytochrome P450, steroid 17α-hydroxylase/17,20 lyase) expression

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## Abstract

Excess androgen synthesis by thecal cells is invariably detrimental to preovulatory follicles in the ovary and considered a fundamental characteristic of polycystic ovary syndrome in women. Investigators have long postulated that granulosa cell-derived estrogens modulate thecal cell steroidogenesis via a short negative-feedback loop within the follicle. To test this hypothesis, we assessed the steroidogenic capacity of individual wild type and estrogen receptor-α (ERα)-null follicles when cultured *in vitro* under comparable conditions. Late-stage ERα-null follicles exhibited markedly increased expression of the thecal cell enzyme CYP17A1 and secreted much greater amounts of its end product, androstenedione. This phenotype was reproduced in wild type follicles when exposed to an aromatase inhibitor or ER-antagonist, and prevented when the former treatment was supplemented with an ERα-specific agonist. ERα-null follicles also exhibited increased testosterone synthesis due to ectopic expression of hydroxysteroid (17β) dehydrogenase type 3 (HSD17B3), a testis-specific androgenic enzyme. These data indicate that ERα functions within thecal cells to negatively modulate the capacity for androgen synthesis by repressing *Cyp17a1* expression, and the biological activity of androgens produced by inhibiting *Hsd17b3* expression; and hence provide novel evidence of an intraovarian ERα function that may be critical to the latter stages of folliculogenesis and overall ovarian function.

### Keywords

hydroxysteroid (17β) dehydrogenase; hyperandrogenemia; folliculogenesis; aromatase

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## INTRODUCTION

Ehrmann *et al.* (1) once characterized androgens as a "necessary evil" in the ovary, referring to their obligatory role as intermediates in estradiol synthesis *vs.* their conspicuous atretogenic properties in late stage follicles. During folliculogenesis, androgens are synthesized by the primary thecal cells of growing follicles in response to the pituitary gonadotropin, luteinizing hormone (LH). The androgens then diffuse across the basement membrane of the follicle and into the granulosa cells, where they act in dual roles, first as a hormone via the androgen receptor (AR) to augment Follicle stimulating hormone (FSH) induction of the estrogenic enzymes; and second as the immediate substrates for conversion to estrogens by these same enzymes (2). The subsequent rise in intrafollicular estradiol levels leads to activation of estrogen receptor (ER) signaling, which assumes the role of augmenting FSH actions and results in rapid follicle growth and differentiation (2). Hence, follicle maturation from the preantral to preovulatory stage is marked by a shift in the role of androgens from hormone to substrate (2). If this transition fails to occur, the intrafollicular androgen levels rise above the steroidogenic capacity of the granulosa cells and invariably cause atresia (3). Therefore, progression of viable preovulatory follicles and hence female fertility depends on stringent regulation of thecal cell androgen synthesis during the later stages of folliculogenesis. In fact, there is strong evidence that ovarian hyperandrogenism may be a leading cause of polycystic ovary syndrome (PCOS), which is estimated to account for 75% of anovulatory infertility in women (1).

This need to limit androgen synthesis in preovulatory follicles implies the existence of specific mechanisms that modulate thecal cell function (4). During the late follicular phase of the ovarian cycle, the endocrine actions of estradiol are well described to elicit negative-feedback on the hypothalamic-pituitary (H-P) axis and thereby decrease LH secretion and further stimulation of thecal cell steroidogenesis. However, studies over 25 years ago demonstrated that estrogens can also directly inhibit androgen synthesis in rodent ovaries and isolated thecal cells, leading to speculation that granulosa cell-derived estrogens (*e.g.* estradiol) may also mediate a short, intrafollicular feedback loop to negatively modulate thecal cell steroidogenesis (4). Supporting evidence indicates that estradiol specifically targets CYP17A1 ($P450_{17\alpha}$-hydroxylase:$C_{17,20}$-lyase), the thecal cell-specific enzyme that converts $C_{21}$- to $C_{19}$-steroids (*e.g.* progesterone to androstenedione; pregnenelone to dehydroepiandrosterone) (4,5), yet estradiol does not appear to directly inhibit substrate binding or CYP17A1 enzymatic activity (6), nor their capacity of thecal cells to respond to LH (7,8). Instead, descriptions that estradiol repression of CYP17A1 activity is blocked by an estrogen receptor (ER)-antagonist (8,9) and that estrogens reduce the level of gonadal *CYP17A1* expression (10,11) indicate that regulation may occur via receptor-mediated mechanisms at the transcriptional level.

Our understanding of the direct actions of estradiol in the ovary has historically been impeded by the inherent difficulties of studying the effect of a hormone within the tissue it is synthesized. This is further complicated by the discovery of ERβ and its extraordinarily high expression in the granulosa cells of mammalian ovaries; while ERα, the originally discovered isoform, is largely limited to thecal cells (2). However, the development of ER-null animal models and isoform-specific ER-agonists present new opportunities to better study the contribution of each ER isoform to mediating the intraovarian functions of estradiol. We have previously shown that the ovaries and thecal cells of ERα-null (αERKO) but not ERβ-null (βERKO) mice exhibit abnormally high *Cyp17a1* expression and activity despite a milieu of elevated estradiol (12, 13). These data are consistent with a modulating action of estradiol on thecal cell steroidogenesis and suggest that ERα is primarily involved. However, further insight from these data is confounded by the endocrine effects that follow the systemic loss of ERα functions, more specifically the chronically high LH levels and subsequent hyperstimulation of the ovarian theca that invariably results from the loss of estradiol-mediated negative-feedback in

the H-P axis of αERKO females (12). Therefore, to better investigate the putative intraovarian feedback loop of estradiol on thecal cell androgen synthesis in growing follicles, we compared the steroidogenic capacity of individual wild type and αERKO follicles when grown *in vitro* under normalized gonadotropin levels. Follicles of each genotype were exposed to an aromatase inhibitor (AI) to allow androgen accumulation and more accurate assessment of synthesis rates. The resulting data definitively show that αERKO follicles possess an increased capacity for androgen synthesis that correlates with abnormally high *Cyp17a1* expression, and that this phenotype is innate to the loss of ERα within the follicle. Furthermore, this phenotype was reproduced in wild type follicles when acutely treated with an aromatase inhibitor or ER-antagonist; and abated by co-treatment with estradiol or an ERα-specific agonist. These data provide convincing support for the long-standing hypothesis that estradiol mediates a short feedback loop within the follicle to prevent overproduction of androgens, and definitively demonstrates this mechanism is dependent on functional ERα.

## MATERIALS AND METHODS

### Animals

The Animal Care and Use Committee of the NIEHS pre-approved all protocols and procedures involving animals. Animals were maintained in plastic cages under a 12-h light:12-h dark schedule in a temperature-controlled room (21–22ºC), fed NIH 31 mouse chow and fresh water *ad libutum*. The generation of *Esr1*$^{-/-}$ (αERKO) mice has been described previously (14,15). Wild type (*Esr1*$^{+/+}$) and αERKO female mice were generated via heterozygous (*Esr1*$^{+/-}$) breeding pairs of C57BL/6 strain. A tail biopsy was collected from female offspring at 19 d of age for genotyping as previously described (12).

### Chemicals

The aromatase inhibitor (AI), 4-(imidazolylmethyl)-1-nitro-9H-9-xanthenone, was purchased from Calbiochem, Inc. (San Diego, CA). The non-specific ER antagonist ICI 182,780 was purchased from Zeneca Pharmaceuticals (Cheshire, UK). 17β-Estradiol ($E_2$) was purchased from Steraloids (Newport, RI). The ERα-specific agonist, 4, 4′, 4″-(propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT) and ERβ-specific agonist, 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) were purchased from Tocris Cookson, Inc. (Ellisville, MO). The 17β-HSD3 inhibitor, 3β-propyl-androsterone (DP3-1), was generated and previously characterized by D.P. (16).

### *In vitro* follicle culture

Individual mouse follicles were isolated and cultured *in vitro* as previously described (17,18). In brief, female mice of 21–25 d of age were killed by $CO_2$ asphyxiation and the ovaries immediately dissected and removed to Leibovitz's L-15 Medium (Invitrogen, Carlsbad, CA) supplemented with insulin (5 μg/ml; Invitrogen), transferrin (10 μg/ml; Sigma, St. Louis, MO), selenium (2 ng/ml; Sigma), ascorbic acid (50 μg/ml; Sigma) and 0.3% bovine serum albumin (Sigma) that was pre-warmed and maintained at 37ºC. Individual preantral follicles of 190–210 μm in diameter were isolated by manual dissection using 25 gauge needles and then transferred to α-minimal essential medium (α-MEM; Invitrogen) supplemented with Pen/Strep (Invitrogen), insulin (5 μg/ml; Invitrogen), transferrin (10 μg/ml; Sigma, St. Louis, MO), selenium (2 ng/ml; Sigma), ascorbic acid (50 μg/ml; Sigma), 5% fetal bovine serum (containing 1.2 ng LH/ml according to supplier, Hyclone, Logan, UT) and 100 mIU recombinant human FSH (Serono Inc., Rockland, MA). After harvesting, follicles were transferred to Millipore CM (Millipore Corp., Bedford, MA) culture plate inserts pre-filled with 0.25 ml α-MEM medium containing the above supplements, and maintained in a humidified incubator with a 95% $O_2$/5% $CO_2$ atmosphere at 37ºC. As shown in Fig. 1, follicles were cultured for a total of 5 days, reevaluated daily and allowed to remain in culture only if they continued to exhibit an

intact basement membrane, a dense complement of granulosa cells, a centrally located oocyte and attached thecal cells. The medium was replaced after 1 and 3 days of culture and follicle diameter was measured and recorded daily. On the 4$^{th}$ day of culture, all or 60% of the medium was replaced with fresh media containing one or more of the chemical treatments. After an additional 24 h incubation period, the media and follicle were collected separately and stored at −70ºC for later analysis of steroid content and gene expression, respectively.

### Steroid enzyme immunoassays (EIAs)

Estradiol, androstenedione and testosterone content in collected media were assessed using the respective Active EIA kits (Diagnostics Systems Laboratories, Webster, TX) according to the manufacture's protocol. Due to limited sample volume, samples were measured in singlicate for each steroid. The least detectable concentration, intra-assay coefficient of variation and inter-assay coefficient of variation for each EIA were as follows: estradiol, 7 pg/ml, 7%, 15%; androstenedione, 0.03 ng/ml; 4%, 8%; and testosterone, 0.04 ng/ml; 2.5%, 12%. The level of each steroid in fresh α-MEM medium was below the level of detection.

### RNA isolation and gene expression assays

Total RNA was isolated from individual follicles using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA) according to the manufacturer's protocol. All RNA preparations were rid of contaminating DNA using the DNA-free® reagents (Ambion, Austin, TX) according to the manufacturer's protocol and the concentration of each preparation was determined from an $A_{260/280}$ reading using a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). A cDNA preparation was generated from each sample using the whole preparation of RNA (a volume of 10 μl) in a 25 μl reaction using random hexamers and the Superscript cDNA synthesis system (Invitrogen) according to the manufacturer's protocol. Traditional (semi-quantitative) PCR reactions were 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 Hybaid Multiblock System (Thermo-Hybaid) as follows: 95°C/30 sec (1X); 95°C/30 sec, 58°C/45 sec, 72°C/30 sec (32X); 72°C/7 min. All samples were electrophoresed on an agarose gel (2% NuSieve/0.7% SeaKem, BMA Bioproducts, Rockland, ME) in 1X Tris-borate-EDTA buffer, stained with ethidium bromide and photographed using an EC3 Imaging System (UVP, Upland, CA). Primers used for the detection of murine *Cyp17a1*, *Cyp11a1* and *Hsd17b3* transcripts have been described previously (19); primers for the detection of murine *Actb* transcripts were purchased from Clonetech (Mountain View, CA).

Real-time RT-PCR assessment of *Cyp17a1* and *Hsd17b3* expression employed primers described previously (13). Each sample was assayed in duplicate using the equivalent of 1 μl cDNA, 10 pmoles 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*) as described previously (13). 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 $(E_{target})^{\Delta Ct(target)}/(E_{Rn18s})^{\Delta Ct(Rn18s)}$, where E is the efficiency of the primer set and $\Delta Ct = Ct_{(Rn18s)} - Ct_{(experimental\ cDNA)}$. The amplification efficiency of each primer set was calculated from the slope of a standard amplification curve of log μl cDNA/reaction *vs.* Ct value over at least 4 orders of magnitude ($E = 10^{-(1/slope)}$); *Hsd17b3* primers, E = 1.97 (*vs.* wild type testis cDNA); *Cyp17a1* primers, E = 2.16 (*vs.* wild type ovary cDNA); *Cyp11a1* primers, E = 2.13 (*vs.* wild type ovary cDNA).

### Statistics

All data sets 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.

## RESULTS

### αERKO follicles exhibit elevated androgen synthesis in culture

Steroid secretion by individual wild type and αERKO follicles was assessed over the course of two consecutive 24 h periods between days 3–5 of culture (Fig. 1). Pilot studies indicated that the period between culture days 3–4 and 4–5 was marked by a 5-fold increase in steroid production by follicles of both genotypes (data not shown). Therefore, all subsequent experiments and treatments were conducted during the latter 24 h period. As shown in Fig. 2, αERKO follicles exhibited an over 5-fold increase in estradiol synthesis relative to wild type follicles ($P < 0.05$). These data are consistent with earlier descriptions of increased circulating estradiol levels exhibited by adult αERKO female mice *in vivo* (12) and ER α-null follicles *in vitro* (17). Given that estradiol synthesis is limited by the availability of androgen precursors, αERKO follicles accordingly exhibited a 3- and 4.5-fold increase in androstenedione and testosterone secretion, respectively, relative to wild type follicles grown under comparable conditions (Fig. 2). To better compare the rates of androstenedione and testosterone synthesis in wild type and αERKO follicles, *in vitro* cultures of each were exposed to an aromatase inhibitor (AI) to block CYP19A1-mediated aromatization of $C_{19}$-steroids, thereby allowing the precursors to accumulate in the medium. The AI employed is reported to specifically inhibit CYP19A1 with minimal effect on CYP17A1 enzymatic activity (21). The lowest concentration of AI (0.5 μm) used eliminated all detectable estradiol synthesis in wild type follicles and reduced estradiol synthesis in αERKO follicles by approximately 95% (Fig. 2). A 10-fold increase in AI (5 μm) totally inhibited all detectable estradiol synthesis in αERKO follicles (Fig. 2). As expected, inhibition of CYP19A1 activity led to a dose-dependent increase in androgen accumulation in follicles of both genotypes but αERKO follicles continued to exhibit a 3–5-fold higher rate of androstenedione and testosterone synthesis relative to wild type at all three AI concentrations (Fig. 2). These data indicate that αERKO follicles possess a marked increase in their capacity for androgen synthesis and that this phenotype is innate to the follicle rather than a consequence of increased gonadotropin stimulation.

### αERKO follicles exhibit aberrantly increased *Cyp17a1* expression

The above data strongly indicate that individual αERKO follicles continue to possess increased CYP17A1 activities even when maintained in conditions of controlled gonadotropin stimulation. Therefore, we sought to compare the level of *Cyp17a1* expression in individual wild type and αERKO follicles following 5 days in culture. As shown in Fig. 3, *Cyp17a1* expression in αERKO follicles was 3-fold higher than that of wild type follicles, suggesting this phenotype is inherent to the loss of ER α functions within the follicle. To test this hypothesis, *Cyp17a1* expression was evaluated in wild type follicles following acute, *in vitro* exposure to an AI or ER-antagonist (ICI 182,780), both of which were expected to pharmacologically mimic the loss of ER α function. Interestingly, both treatments increased *Cyp17a1* expression in wild type follicles ($P < 0.05$ *vs.* untreated wild type) to levels that approximated those observed in untreated αERKO follicles (Fig. 3). Therefore, acute inhibition of ER-mediated actions via either removal of activating ligand or direct repression of receptor function leads to increased *Cyp17a1* expression in wild type follicles, hence reproducing the αERKO phenotype. Similar *in vitro* exposure of αERKO follicles to the AI or ER-antagonist had no additive effect on *Cyp17a1* expression (Fig. 3).

Androgens are reported to down-regulate thecal cell androgen synthesis via an AR-mediated auto-regulatory loop (22,23). To determine if increased androgen accumulation in the presence of the AI or loss of ER α may provide for some repression of *Cyp17a1* expression, wild type and αERKO follicles were exposed to the AI plus an AR-antagonist (Flutamide, 10 μM). *Cyp17a1* expression in AI-exposed wild type follicles treated with Flutamide was actually reduced by 30%, a measurable but not statistically significant decline compared to wild type follicles exposed to the AI alone (data not shown). A similar decrease in *Cyp17a1* expression was observed in αERKO follicles exposed to the AI plus Flutamide (data not shown).

Similar assays for *Cyp11a1*, another steroidogenic enzyme that is LH regulated in thecal cells, indicated little difference in expression between wild type and αERKO follicles and minimal changes following all of the above treatments. Therefore, the inhibitory effect of ER α is specific to *Cyp17a1* expression (Fig. 3).

## ERα mediates the inhibitory effect of estradiol on *Cyp17a1*

A phenotype of elevated ovarian *Cyp17a1* expression and activity in αERKO but not βERKO females (12), along with the predominance of ERα in thecal cells, strongly suggests that estradiol modulation of *Cyp17a1* expression is ERα-mediated. To test this hypothesis, *in vitro* cultured wild type follicles were exposed to an AI to eradicate endogenous synthesis of ER ligand (*i.e.* estradiol) while simultaneously exposed to either exogenous estradiol, an ERα-specific agonist (PPT) or an ERβ-specific agonist (DPN) for 24 h. As shown in Fig. 4, the increased *Cyp17a1* expression elicited by removal of endogenous estradiol synthesis was completely abated by exogenous estradiol replacement at 0.2 nM, indicating the inhibitory effect is specific to estrogen action. Furthermore, this effect of estradiol was fully mimicked by the ERα-agonist but not the ERβ-agonist, indicating that ERα solely mediates estradiol repression of *Cyp17a1* expression (Fig. 4). None of the treatments affected *Cyp11a1* expression (Fig. 4), demonstrating the specificity of ERα-mediated actions to *Cyp17a1* regulation.

## Elevated testosterone synthesis in αERKO follicles is mediated by ectopic HSD17B3 activity

In addition to increased androstenedione synthesis, αERKO follicles also exhibited remarkably high rates of testosterone secretion *in vitro*, exhibiting a $T/A_4$ ratio of 3.4 (± 0.7) *vs.* 1.6 (± 0.1) in wild type follicles ($P < 0.05$). An over abundance of precursor, *i.e.* androstenedione, could provide the basis for increased testosterone synthesis in αERKO follicles. However, we have previously described that adult αERKO females exhibit male-like plasma testosterone levels *in vivo* due to ectopic ovarian expression of HSD17B3 (12,13), a testis-specific enzyme that specifically reduces androstenedione to testosterone (24,25). In the current study, *Hsd17b3* transcripts continued to be detected in individual αERKO follicles but not wild type follicles following five days in culture (Fig. 5), indicating the *in vivo* ovarian phenotype is preserved in αERKO follicles under *in vitro* conditions. In contrast to the effect on *Cyp17a1* expression, however, acute exposure to the AI or ER-antagonist did not lead to a αERKO-like induction of *Hsd17b3* expression in wild type follicles (Fig. 5). Some wild type follicles exposed to the AI exhibited a detectable rise in *Hsd17b3* expression but this was neither reproducible nor comparable to the levels detected in αERKO follicles. Therefore, ectopic *Hsd17b3* expression is innate to αERKO follicles and exists prior to *in vitro* culture.

The above findings indicate that increased testosterone synthesis in αERKO follicles is due to ectopic HSD17B3 activity. However, a definitive conclusion is precluded by reports that HSD17B1, a related family member that functions to reduce estrone to estradiol and is highly expressed in granulosa cells, is also capable of reducing androstenedione to testosterone in rodents (26,27). Therefore, to discern the contributions of HSD17B type 1 and type 3 activities to the overall capacity for testosterone synthesis in αERKO follicles, follicles of each genotype were exposed to an HSD17B3-specific inhibitor (DP3-1) in the presence or absence of the AI.

In the absence of the AI, DP3-1 inhibited testosterone synthesis by more than 85% in αERKO follicles (Fig. 6). When the AI was included to allow for the accumulation of androstenedione, the common substrate for HSD17B types 1 and 3, DP3-1 still inhibited testosterone synthesis in αERKO follicles by > 60% ($P < 0.05$) (Fig. 6). Furthermore, DP3-1 treatment led to a measurable accumulation in androstenedione (Fig. 6), indicating that decreased testosterone synthesis was not due to parallel reductions in available precursor. The failure of DP3-1 to inhibit testosterone synthesis in wild type follicles indicates this synthesis is likely mediated by the androgenic actions of HSD17B1 (Fig. 6).

## DISCUSSION

The *two-cell, two-gonadotropin* model of steroidogenesis in ovarian follicles states that androgens are synthesized solely by primary thecal cells in response to LH and then diffuse across the basement membrane to serve as immediate substrates for estradiol synthesis by granulosa cells in response to FSH (5). Hormonal actions of androgens also promote estradiol synthesis in preantral follicles by enhancing FSH-induction of *CYP19A1* (2). This notwithstanding, elevated androgen synthesis and/or accumulation during the later stages of folliculogenesis is undoubtedly detrimental to the follicle (3,5) and is an invariable characteristic in women diagnosed with PCOS (28). Therefore, follicle integrity relies on a delicate balance between the steroidogenic capacities of the theca and granulosa cells. It has long been speculated that this balance is achieved by granulosa cell-derived estradiol acting in a paracrine loop to negatively modulate thecal cell function (3–5). Indeed, estradiol is known to inhibit androgen synthesis in thecal cells under experimental conditions (4,29) and ERα is highly expressed in the primary thecal cells of growing follicles in multiple species (2). However, the generation of definitive experimental evidence to support this hypothesis has been precluded by the lack of appropriate investigative tools. Herein, we employed *in vitro* follicle culture and ERα-null mice to demonstrate that the loss of functional ERα within growing follicles leads to markedly elevated rates of androstenedione synthesis that can be attributed to increased expression of CYP17A1, the thecal cell-specific enzyme that is directly involved in androstenedione synthesis. We also provide evidence that ERα μ functions to repress testosterone synthesis in the ovary by inhibiting expression of HSD17B3, an enzyme that efficiently reduces androstenedione to testosterone but is normally testis-specific. These data indicate that ERα functions within thecal cells to maintain the proper steroidogenic environment of growing follicles by *a*) controlling the overall capacity for androgen and estrogen synthesis by negatively modulating *Cyp17a1* expression, and *b*) inhibiting the synthesis of the more biological active androgen, testosterone, by repressing *Hsd17b3* expression.

The current data are consistent with earlier our reports that adult αERKO but not βERKO female mice exhibit increased ovarian *Cyp17a1* and ectopic *Hsd17b3* expression and correlating levels of circulating androstenedione and testosterone (12,13). However, gonadal *Cyp17a1* and *Hsd17b3* expression are highly dependent on LH stimulation (4,30) and therefore any inference from *in vivo* observations must consider the effects of chronically increased LH secretion that results of the loss of ERα-mediated actions in the H-P axis (12). Herein, we have overcome this caveat by comparing the phenotypes of individual wild type and αERKO follicles when propagated *in vitro* under a normalized gonadotropin milieu. The preservation of increased *Cyp17a1* and ectopic *Hsd17b3* expression, and increased rates of androstenedione and testosterone synthesis, in individually cultured αERKO follicles indicates these traits are inherent to the loss of intrafollicular ERα functions and not the secondary effects of LH-hyperstimulation. Indeed, even when wild type or βERKO female mice are forced to possess comparably elevated LH levels via possession of the LH-CTP transgene, they do not exhibit comparable increases in ovarian *Cyp17a1* expression, presumably because the inhibitory actions of ERα within the ovary remain intact (19). Interestingly, Heikkilä *et al.* recently

reported that ovaries of newborn *Wnt4*-null mice exhibit an over 60-fold increase in *Cyp17a1* expression that is concurrent with a 8-fold reduction in ERα expression but no change in ERβ levels (31). Therefore, increased *Cyp17a1* expression in αERKO ovaries is likely the compound effect of the loss of ERα functions in both the ovary and H-P axis. Recent reports that estradiol down-regulates *Cyp17a1* expression in the testes of rats (10) and fish (11); and that the testes of αERKO males exhibit aberrantly high *Cyp17a1* expression and activity (32) indicates ERα likely plays a comparable role in the male gonad.

The putative auto-regulatory actions of androgens on thecal cell steroidogenesis (22,23) were not observed in the current studies using *in vitro* follicle culture. In fact, αERKO follicles continued to exhibit elevated *Cyp17a1* expression despite their self-generation of an environment rich in testosterone. Furthermore, treatment of wild type and αERKO follicles with an AR-antagonist actually led to a slight decrease in *Cyp17a1* expression, an effect that is opposite that which could be expected if AR-mediated androgen actions repress *Cyp17a1* expression. These data suggest that either ERα is involved in the postulated AR-mediated auto-regulatory loop on thecal cell function or that ERα is the more predominant negative modulator of thecal cell steroidogenesis.

The αERKO phenotype of increased *Cyp17a1* expression could be reproduced in wild type follicles during acute withdrawal of endogenous estrogenic ligand or inhibition of ERα action. Furthermore, only exogenous estradiol or the ERα-specific agonist (PPT) prevented the increase in *Cyp17a1* expression in wild type follicles following withdrawal of endogenous estrogen synthesis. These data indicate that estradiol repression of *Cyp17a1* expression is clearly ERα-mediated as well as acute and reversible in nature. Interestingly, AI or ICI treatment did not elicit ectopic *Hsd17b3* expression in wild type follicles, suggesting this phenotype is fixed in αERKO follicles prior to culture. This divergence in CYP17A1 and HSD17B3 regulation in the ovary is consistent with the role of the former enzyme in the synthesis of androstenedione, which is obligatory for estradiol synthesis; whereas continuous repression of *Hsd17b3* expression in the ovary is conducive to (1) shunting the available thecal cell-derived androstenedione toward the path of estrogen rather than testosterone synthesis, and (2) preventing the generation of testosterone to potentially harmful levels.

There is considerable divergence in *CYP17A1* expression patterns among different species and steroidogenic tissues (24), making it difficult to speculate on the mechanism by which ERα represses expression in thecal cells. Tissue-specific *CYP17A1* expression is at least partly achieved by differential receptor expression among the steroidogenic tissues. For example, LH is necessary to stimulate *CYP17A1* expression in the gonads whereas adrenocorticotropic hormone stimulates expression in the adrenal glands (24). In contrast, mechanisms that actively repress *CYP17A1* expression are gaining attention as another important regulatory mode of *CYP17A1* expression, and several nuclear factors and signaling pathways have been implicated, including UBC9 (33), RIP-140 (34), protein kinase-c (PKC) (23,35), *Src*-tyrosine kinases (36) and transforming growth factor-β (TGF-β) (37–40); the latter of which is estrogen regulated in thecal cells (41). Indeed, isolated thecal cells from women with PCOS exhibit an aberrant increase in basal CYP17A1 activity *in vitro* (42,43), which is currently attributed to a loss of mechanisms that normally repress *CYP17A1* expression (44–46). Although a comparison of ERα and ERβ expression levels in normal *vs.* PCOS human ovaries found that ERα levels are in fact increased in thecal cells from the diseased ovaries (47), an intronic *PvuII* single nucleotide polymorphism in the *ESR1* (ERα) gene is associated with increased androstenedione levels in postmenopausal women (48). Furthermore, we found marked levels of *Cyp17a1* transcripts in the adrenal glands of αERKO females (J.F. Couse and K.S. Korach, unpublished observations) despite reports that rodent adrenal glands are normally void of CYP17A1 (24), thereby providing further evidence that ERα functions are critical to the repression of *CYP17A1* expression in steroidogenic tissues.

In summary, direct effects of estradiol on the ovary were first demonstrated over 60 years ago (49,50) yet in depth studies toward understanding the mechanisms of intraovarian estrogen actions are impeded by difficulties inherent to investigating hormone action within the source tissue. The discovery of ERβ and its marked expression in the ovary, the generation of ER-null and CYP19A1-null mice, and the development of ER-specific agonists has led to a resurgence in the field of intraovarian estrogen actions. We employed an *in vitro* follicle culture method using follicles from ERα-null mice along with recently developed ER-specific compounds to demonstrate an ERα-dependent paracrine loop within late-stage follicles that allows granulosa cell-derived estradiol to negatively modulate thecal cell androgen synthesis by specifically reducing *Cyp17a1* expression, confirming a role for estradiol that was first postulated over 25 years ago (4,29,51).

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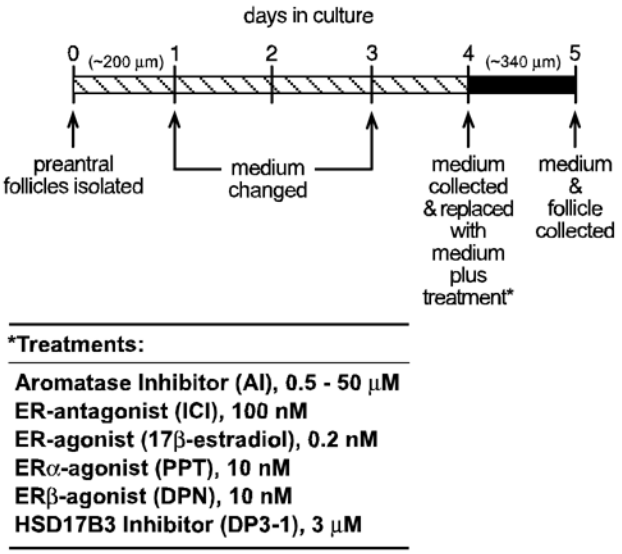


**Figure 1.**

Scheme used for *in vitro* culture of wild type and αERKO follicles for the assessment of steroidogenesis and gene expression. Large preantral follicles of approximately 200 μm in diameter were isolated from immature (21–24 d) wild type and αERKO females and individually propagated in a 250 μl volume of medium for 5 days, during which they grow to approximately 340 μm in diameter. Media was changed and or collected on the indicated days. Follicles were inspected for integrity and health every 24 h and removed from the study if they did not satisfy the conditions described in the Materials and Methods. The 24 h period between days 4–5 was found to be the period of peak steroid synthesis by follicles and was therefore selected for all experimental treatments. Media was changed on day 4 of culture and replaced with media containing vehicle or a combination of the indicated treatments. The media was then collected after 24 h and stored for evaluation of androstenedione, testosterone and estradiol content by EIA. The follicle was also collected for later evaluation of gene expression.

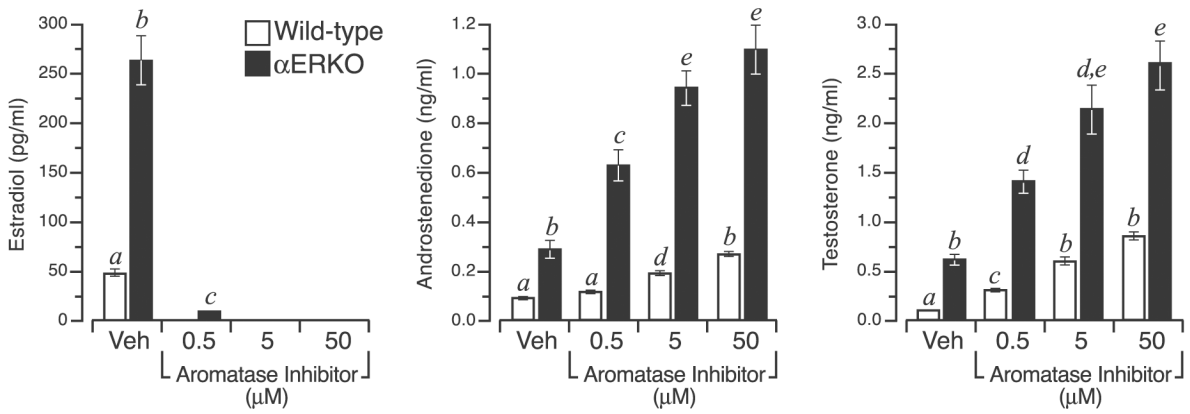


**Figure 2.**

αERKO follicles exhibit elevated steroidogenesis *in vitro*. Shown is the average (± SEM) levels of estradiol, androstenedione and testosterone synthesized by wild type (open bar) and αERKO (filled bar) follicles during the 24 h culture period between days 4–5. Follicles were exposed to either vehicle (Veh) or increasing amounts of an aromatase inhibitor to allow thecal cell-derived androgens to accumulate and be measured. Individual αERKO follicles clearly synthesize greater amounts of all three steroids when cultured under a controlled hormonal milieu. Furthermore, by inhibiting aromatization of androstenedione to estrone, or testosterone to estradiol, the increased level of androgen synthesis in αERKO follicles becomes even more apparent. Bars that do not share a letter are significantly different ($P < 0.05$). The data shown represents the pooled values of four independent experiments, and a total of 14–47 follicles per genotype, per treatment.

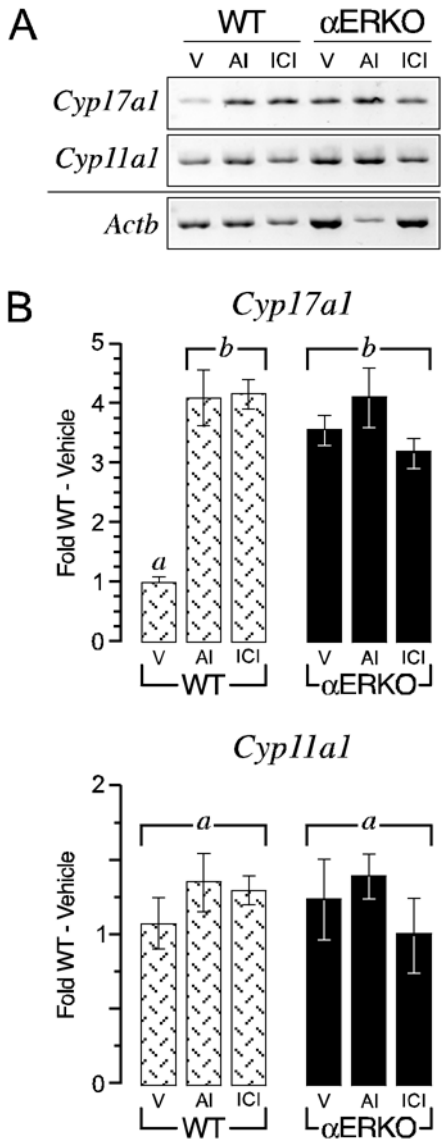


**Figure 3.**

Loss or inhibition of intrafollicular ERα functions leads to increased *Cyp17a1* expression in individually cultured follicles. A. Shown is a representative ethidium bromide stained agarose gel (inverted) of semi-quantitative RT-PCR for *Cyp17a1*, *Cyp11a1* and *Actb* transcripts in wild type (WT) and αERKO day 5 follicles following 24 h of treatment with either vehicle (V), an aromatase inhibitor (AI) or an ER-antagonist (ICI). B. Shown is quantitative data (average ± SEM) from real-time RT-PCR for *Cyp17a1* and *Cyp11a1* expression from these same experiments. αERKO follicles clearly exhibited increased *Cyp17a1* expression relative to vehicle treated wild type follicles and this phenotype was reproduced in the latter genotype following acute withdrawal of endogenous estradiol synthesis (via an AI) or direct repression of ER action (via ICI). In contrast, *Cyp11a1* expression does not differ between genotypes and was not affected by the various treatments. Bars that do not share a letter are significantly different ($P < 0.05$). The data shown is one of two independent experiments that yielded comparable results. Sample sizes were 8–9 follicles per genotype, per treatment, per experiment.

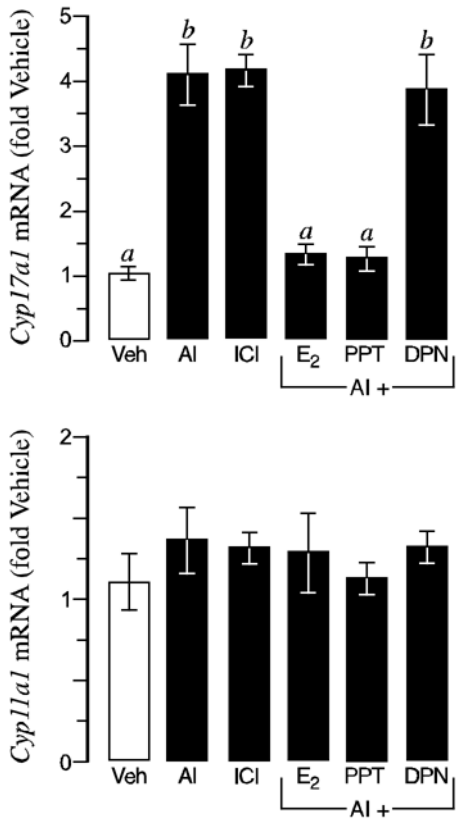


**Figure 4.**

Estradiol suppression of *Cyp17a1* expression is mediated by ERα. Shown is quantitative data (average ± SEM) from real-time RT-PCR for *Cyp17a1* and *Cyp11a1* transcripts in wild type day 5 follicles following 24 h of treatment with either vehicle (Veh), an aromatase inhibitor (AI), an ER-antagonist (ICI); or an AI plus estradiol ($E_2$), an ERα-agonist (PPT) or an ERβ-agonist (DPN). As expected, acute withdrawal of endogenous estradiol synthesis (via an AI) or direct repression of ER action (via ICI) leads to increased *Cyp17a1* expression (*top*). However, this can be prevented by co-treatment with exogenous $E_2$ or an ERα-specific agonist (PPT), indicating that ERα mediates the estradiol suppression of *Cyp17a1* expression in growing follicles. In contrast, *Cyp11a1* expression (*bottom*) was not affected by the various treatments, indicating the effects of ERα-mediated estradiol are specific to *Cyp17a1*. Bars that do not share a letter are significantly different ($P < 0.05$). The data shown is one of two independent experiments that yielded comparable results. Sample sizes were 8–9 follicles per genotype, per treatment, per experiment.

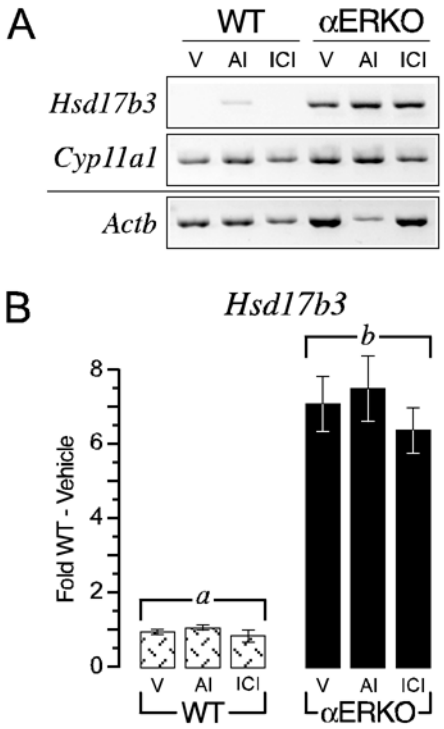


**Figure 5.**

αERKO follicles exhibit ectopic expression of the Leydig cell specific gene *Hsd17b3*. A. Shown is a photograph of a representative ethidium bromide stained agarose gel (inverted) of semi-quantitative RT-PCR for *Hsd17b3*, *Cyp11a1* and *Actb* transcripts in wild type (WT) and αERKO day 5 follicles following 24 h of treatment with either vehicle (V), an aromatase inhibitor (AI) or an ER-antagonist (ICI). B. Shown is quantitative data (average ± SEM) from real-time RT-PCR for *Hsd17b3* expression from these same experiments. αERKO follicles clearly exhibit ectopic *Hsd17b3* expression relative to wild type follicles and this phenotype cannot be reproduced in wild follicles following acute withdrawal of endogenous estradiol synthesis (via an AI) or direct repression of ER action (via ICI), indicating it is a fixed phenotype in αERKO follicles prior to culture. Bars that do not share a letter are significantly different ($P < 0.05$). The data shown is one of two independent experiments that yielded comparable results. Sample sizes were 8–9 follicles per genotype, per treatment, per experiment.

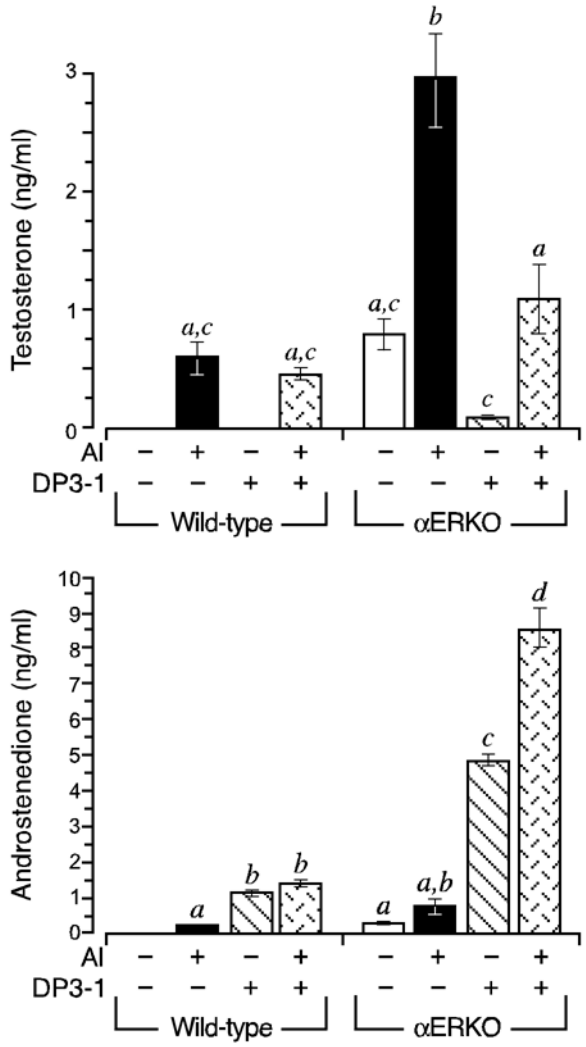


**Figure 6.**

Ectopic HSD17B3 activity accounts for the aberrantly elevated capacity for testosterone synthesis in αERKO follicles. Wild type and αERKO follicles were grown as described in Fig. 1. During the 24 h culture period between days 4–5, follicles were exposed to an aromatase inhibitor (AI) and/or an HSD17B3-specific inhibitor (DP3-1). Shown is the average (± SEM) amount of testosterone (*top*) and androstenedione (*bottom*) synthesized during the 24 h period. As was first shown in Fig. 2, both wild type and αERKO follicles synthesized increased amounts of androstenedione and testosterone when exposed to an AI, however, the levels of both androgens are much higher in the αERKO follicles. Furthermore, testosterone synthesis in αERKO follicles is inhibited by DP3-1, indicating it is mediated by HSD17B3, a Leydig cell-specific enzyme. In contrast, DP3-1 had no effect on testosterone synthesis in wild type follicles, indicated this is likely mediated by the androgenic properties of rodent HSD17B1. The data shown is one of two independent experiments that yielded comparable results. Sample sizes were 8–9 follicles per genotype, per treatment, per experiment.