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In Vitro Growth and Ovulation of Follicles from Ovaries of Estrogen Receptor (ER)α and ERβ Null Mice Indicate a Role for ERβ in Follicular Maturation

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Both estrogen receptor (ER) α and β are expressed within the ovary and lack of either of these receptors affects ovarian function. In this study, the role of ERα and ERβ in folliculogenesis and ovulation was further analyzed. Evaluation of ovarian follicle populations in wild-type and ERβ knockout (βERKO) ovaries revealed reduced late antral growth and ovulatory capacity of βERKO follicles, indicated by reduced numbers of large antral follicles and corpora lutea and increased atresia of large antral follicles. An *in vitro* culture system was used to study growth, rupture, and luteinization of wild-type, ERα knockout (αERKO) and βERKO ovarian follicles. αERKO follicles exhibited wild-type-like growth and ovulation rates but an increased capacity to synthesize estradiol. In contrast, βERKO follicles showed a significant lack of progression from early antral to large antral stage, decreased estradiol production, and reduced ovulation. Expression patterns of several genes involved in follicle maturation and ovulation were analyzed in follicles grown *in vitro*. *Ar*, *Pgr*, and *Has2* mRNA expression levels were the same among the three genotypes. However, βERKO follicles showed reduced expression of *Cyp19* mRNA during follicle maturation and reduced *Lhcgr* and *Ptgs2* mRNA expression after human chorionic gonadotropin stimulus. Luteinization occurs normally in αERKO and βERKO follicles, shown by increased progesterone secretion and increased *cdkn1b* mRNA expression after human chorionic gonadotropin. Collectively, these data indicate that ERβ, but not ERα, plays a direct role in folliculogenesis. ERβ appears to facilitate follicle maturation from the early antral to the preovulatory stage. (*Endocrinology* 146: 2817–2826, 2005)

ESTROGENS PLAY A KEY role in female reproduction, with the ovary serving as both the primary site of estrogen synthesis and a major estrogen target. The importance of estrogen actions in the ovary have recently been illustrated by the phenotypes of mice lacking functional estrogen receptors (ER) α and/or β [ER knockout (ERKO)] and those lacking the capacity to produce estradiol, the *Cyp19* [aromatase knockout (ArKO)] null mice (1, 2). Adult αERKO and ArKO females are infertile and exhibit ovaries that possess large, hemorrhagic and cystic follicles and no evidence of spontaneous ovulation, whereas adult βERKO females are subfertile and possess ovaries that are best characterized as exhibiting a paucity of growing follicles and corpora lutea (2–5). These findings, along with the expression pattern of each nuclear ER form within the ovary suggest a critical role for intraovarian estrogen signaling in ovarian function (6–8).

In the rodent ovary, estrogen is primarily produced by preovulatory follicles under the influence of FSH (9). The endocrine actions of estrogen in the female reproductive tract and hypothalamic-pituitary axis are well documented and critical to reproduction (1). However, estrogen is also thought to fulfill a number of intraovarian roles during folliculogenesis. For example, in granulosa cells of maturing follicles, estradiol has little effect alone but is required for maximum FSH stimulation of 1) *Cyp19* expression and estradiol synthesis (10–13); 2) LH receptor (*Lhcgr*) expression and LH responsiveness (14–16); 3) antrum formation (17–19); 4) gap-junction formation (20); and 5) prevention of atresia (21). In thecal cells of growing follicles, estradiol modulates androgen synthesis by negatively regulating *Cyp17* expression in thecal cells (22, 23).

Estrogens signal via two nuclear estrogen receptors, ERα and ERβ, and both are present in the ovary but in different cell types. Granulosa cells of follicles from the primary to large antral stage abundantly express ERβ-encoding transcripts and protein, whereas expression is low or absent in interstitial and luteal cells (7, 24, 25). In contrast, ERα-encoding transcripts and protein are predominantly localized to thecal and interstitial cells with little or no expression in granulosa cells (24, 26–28). Whether oocytes express ER remains controversial; however, because oocytes from both ER null mice and ArKO mice are able to undergo fertilization and yield viable embryos, neither ER nor estrogen appears critical to oocyte integrity (4, 29, 30). The divergent pattern of expression of ERα and ERβ in the ovary and the distinct ovarian phenotypes exhibited by αERKO and βERKO females indicate that the two ER forms have distinct roles in ovarian function. Although αERKO ovaries contain normal

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Abbreviations: ArKO, Aromatase knockout; COC, cumulus oocyte complex; ER, estrogen receptor; ERKO, ER knockout; hCG, human chorionic gonadotropin; PGE_2, prostaglandin E_2; PMSG, pregnant mare serum gonadotropin.

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follicles up to the small antral stage, the predominant features are multiple, large hemorrhagic follicles and the absence of corpora lutea, indicating an anovulatory phenotype (5, 31). We have previously shown that this dramatic phenotype in the αERKO ovary is secondary to chronically elevated levels of plasma LH that result from the loss of ERα-mediated negative feedback in the hypothalamus (23, 29). In contrast, βERKO female mice exhibit normal levels of circulating gonadotropins and ovaries that possess all stages of folliculogenesis, including corpora lutea (4, 23). However, when bred, βERKO female mice yield significantly fewer litters and offspring compared with wild-type counterparts (4). Furthermore, the oocyte yield from βERKO female mice after gonadotropin-induced ovulation is approximately 15% that of wild-type females (4, 32). These data suggest that ERβ plays a more direct role in ovarian function, although the exact mechanism has yet to be determined.

Detailed studies of the intraovarian events during follicular development and ovulation are difficult to carry out *in vivo* because of the various endocrine factors known to influence ovarian function, including gonadotropins, prolactin, steroids, and various growth factors. This is especially true for the αERKO mouse because ovaries of these mice exist in a severely abnormal hormonal environment (23). To gain further insight into the role(s) of ERα and ERβ in ovarian function we have established an *in vitro* culture system using follicles from αERKO and βERKO ovaries. This system allows us to compare growth, steroidogenesis, and ovulation rates between wild-type, αERKO, and βERKO follicles in a controlled hormonal environment. In addition, expression patterns of several genes known to be involved in follicular maturation, ovulation, or luteinization were evaluated in follicles grown *in vitro*. Using *in vitro* culture of follicles, we have shown that ERβ, and not ERα, is most critical to follicle growth and maturation leading up ovulation.

Materials and Methods

Animals

All animal procedures were approved by the National Institute of Environmental Health Sciences Animal Care and Use Committee. Generation of αERKO and βERKO null mice has been previously described by Lubahn *et al.* (3) and Krege *et al.* (4), respectively. Mice were generated from αERKO and βERKO heterozygous breeding pairs on a background of C57BL6 obtained from Taconic Farms (Germantown, NY). Offspring were genotyped by PCR analysis of tail-snip genomic DNA as previously described (23).

Ovarian follicle counts in vivo

Ovaries were collected from immature (28–30 d) wild-type and βERKO female mice 48 h after a single injection of 2.2 IU of pregnant mare serum gonadotropin (PMSG) (Sigma Chemical Co., St. Louis, MO) or vehicle (0.85% saline). In addition, ovaries were collected from adult (~109 d) wild-type and βERKO female mice that were untreated. The ovaries were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned (5 μm), and stained with hematoxylin and eosin. One ovary per animal was used for follicle counts. Follicles were counted in every 10th section, beginning with the fifth section, through the entire ovary. Only follicles with a visible nucleus were counted to avoid double counting. Follicles were categorized as primordial if they contained an intact oocyte surrounded by a single layer of predominantly squamoid, flattened epithelial cells, lacking a connective tissue or thecal investment. Follicles were categorized as primary if they had an enlarged oocyte surrounded by a single layer of predominantly cuboidal granulosa cells. Small preantral follicles were identified as having an oocyte surrounded by two layers of granulosa cells, whereas large preantral follicles were identified as having an oocyte surrounded by a multilayered (more than two), solid mantle of granulosa cells. Follicles were categorized as small antral if they had a central oocyte surrounded by layered granulosa cells and containing fluid-filled areas within the granulosa cell layer. Large antral follicles were identified as having a single cavity containing follicle fluid surrounded by a granulosa cell layer of even thickness and a cumulus-cell enclosed oocyte. In addition, the total number of corpora lutea was counted. Atretic follicles, defined as follicles containing one or more apoptotic cells with pyknotic nuclei, were not included in the above categories but counted and categorized separately. The presence of apoptotic cells was verified using the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling assay (Upstate Biotechnology, Charlottesville, VA) according to the manufacturer's protocol. For each ovary, total number of follicles (per follicle class) present in the scored sections was normalized to the total number of scored sections from that ovary to obtain a mean follicle count per section. The average number of scored sections per ovary was the same between βERKO animals and the respective wild-type controls.

No data on follicle counts were collected from αERKO ovaries, because these ovaries exhibit extensive formation of hemorrhagic cystic follicles that distort the overall morphology and make it difficult to obtain representative follicle counts.

In vitro culture of follicles

Follicles were isolated from whole ovaries of 21- to 25-d-old female mice (wild-type, αERKO, and βERKO) according to the method of Nayudu and Osborne (33). In brief, mice were killed by CO_2 asphyxiation, and the ovaries were removed and placed in Leibovitz's L-15 medium (Life Technologies, Inc., Invitrogen, Carlsbad, CA) supplemented with insulin (5 μg/ml; Life Technologies), transferrin (10 μg/ml; Sigma), selenium (2 ng/ml; Sigma), ascorbic acid (50 μg/ml; Sigma), and 0.3% BSA (Sigma) at 37 C. Large preantral follicles (200 ± 20 μm in diameter) were then mechanically dissected from the ovaries using 25-gauge syringe needles. Isolated follicles were transferred to four-well multidishes (Nalge, Nunc International, Rochester, NY) containing α-MEM (Life Technologies) supplemented with insulin, transferrin, selenium, and ascorbic acid as above as well as penicillin (100 IU/ml; Life Technologies), streptomycin (100 μg/ml; Life Technologies), 5% fetal bovine serum (Sigma), and 100 mIU recombinant human FSH (kindly donated by Ares Serono, Geneva, Switzerland). The culture dishes were placed in a humidified incubator at 37 C in 5% CO_2 in air until a sufficient number of follicles had been collected.

The harvested preantral follicles were evaluated, and those exhibiting an intact basement membrane, a high density of granulosa cells, a centrally located oocyte, and some thecal cells attached were selected for further culture. The culture method used for *in vitro* evaluation of follicle growth and androstenedione, testosterone, and estradiol secretion was based on that of Nayudu and Osborne (33) with modifications according to Rose *et al.* (34). Follicles were individually cultured in Millipore CM culture plate inserts (Millipore, Bedford, MA) filled with 250 μl α-MEM supplemented as described above, at 37 C in 5% CO_2 in air. Medium was changed on culture d 1, 3, and 5. Spent medium collected on culture d 5 was stored at −80 C until assayed. Follicular growth was monitored daily by measuring follicle diameter using a precalibrated ocular micrometer. Follicles that did not survive the 5-d culture period, those with obvious darkening of the granulosa cell mass and/or signs of disintegration or that had spontaneously extruded their oocyte, were not included in the analysis.

For *in vitro* ovulation and progesterone production assays, follicles were cultured in Costar Ultra Low flat-bottom 96-well plates (35). After isolation and selection, follicles were transferred individually into wells containing 40 μl α-MEM, supplemented as described above. The culture dishes were placed in a humidified incubator at 37 C in 5% CO_2 in air. Medium was changed every 48 h. When follicles reached a diameter of at least 340 μm, after approximately 4 or 5 d of culture, the medium was replaced by medium containing human chorionic gonadotropin (hCG) (2–32 IU/ml) (kindly donated by Ares Serono, Geneva, Switzerland) to induce ovulation. Follicles were checked for ovulation at 16 h after hCG stimulus and were considered to have ovulated when the follicle was visibly ruptured and the cumulus oocyte complex (COC) extruded.

The size at which large antral follicles were exposed to hCG was determined as follows. Large antral wild-type follicles were divided into three groups: follicles that had reached a diameter of 320–340 μm, 340–360 μm, or more than 360 μm. After treatment with 2 IU/ml hCG, 35% of follicles with a diameter of 320–340 μm, 60% of follicles with a diameter between 340 and 360 μm, and 70% of follicles with a size greater than 360 μm ovulated. Initial follicle growth studies indicated a reduction in the number of βERKO follicles reaching a size of 340 μm or more. To collect sufficient late antral βERKO follicles for the ovulation assays, these assays were performed with follicles that had reached a diameter of at least 340 μm.

In some of the cultures, the effect of prostaglandin on ovulation was evaluated by *in vitro* ovulation assays and by histology. Prostaglandin E_2 (PGE_2) (500 ng/ml) (36) dissolved in ethanol was added to the medium 4 h after the hCG pulse, and follicles were checked for ovulation 16 h later. For histological analysis, follicles were collected before hCG or 16 h after incubation with hCG alone or a combination of hCG and PGE_2, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin. At least five follicles per experimental group per genotype were studied, and representative photomicrographs are presented for histology.

RIA

Androstenedione, testosterone, and estradiol were measured in spent medium collected on culture d 5, after incubation for 48 h. Androstenedione and testosterone were assayed in a single assay using direct RIA kits from Diagnostics Systems Laboratories (Webster, TX), according to the manufacturer's protocol. The intraassay coefficients of variation were 4.3% for androstenedione and 8.5% for testosterone, and the assay sensitivities were 0.02 ng/ml for androstenedione and 0.08 ng/ml for testosterone. Estradiol was measured using a double-antibody RIA kit (Diagnostics Systems Laboratories) with inter- and intraassay variations of 8.5 and 4.6%, respectively, and a sensitivity of 4.7 pg/ml. Progesterone production was measured in spent medium collected just before or 24 h after hCG using a direct RIA kit (Diagnostics Systems Laboratories). The inter- and intraassay coefficients of variation were 11.7 and 6.6%, respectively, and the assay sensitivity was 0.12 ng/ml. For each hormone, the level in medium that had not been in contact with follicles was below the level of detection.

Analysis of mRNA expression

Follicles that reached a diameter of at least 340 μm were collected either immediately (no hCG) or after incubation with 2 IU/ml hCG for 3 or 24 h. Total RNA was extracted from individual follicles using PicoPure RNA isolation kit (Arcturus, Mountain View, CA) according to the manufacturer's protocol. To remove any potential genomic DNA contamination, samples were incubated with 2 μl RNase-free DNase I (2 U/μl) (Ambion Inc., Austin, TX) for 15 min at room temperature. Semiquantitative RT-PCR was performed as described previously (23) with some modifications. Total RNA (0.1 μg) was reverse transcribed in a total volume of 25 μl using the Superscript first-strand synthesis system (Invitrogen Life Technologies Inc.). PCR amplification was carried out with 1 μl of the RT reaction in a 15-μl reaction at 95 C for 30 sec, 58 C for 45 sec, and 72 C for 30 sec for 28 cycles. All samples were electrophoresed on agarose gels containing ethidium bromide and electroblotted to BrightStar nylon membrane (Ambion). The membranes were hybridized with ^{33}P-labeled probes and washed, and results were quantified by using a PhosphorImager and ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). Per experiment, the intensity values for each transcript were normalized to the respective expression level of *Actb*, a housekeeping gene that is nondifferentially expressed, and then expressed as fold induction over the wild-type no-hCG value by dividing each value by the wild-type no-hCG mean value. The primers and probes used for the semiquantitative RT-PCR are listed in Table 1.

Statistical analysis

Within the follicle count studies, differences between the mean numbers of follicles per section were evaluated for each follicle class per genotype using the nonparametric Mann-Whitney *U* test. Data of αERKO and βERKO follicles cultured *in vitro* obtained in separate experiments were analyzed as separate groups. Each experiment always included follicles derived from wild-type ovaries as controls. Follicle growth *in vitro* was analyzed by comparison of daily follicular sizes between genotypes using repeated-measures ANOVA followed by two-sample *t* tests comparing mean size at each day. Differences between genotypes in reaching a large antral size or ovulation rates were assessed by χ^2 tests. Differences in estradiol secretion among genotypes were identified by nonparametric Kruskal-Wallis ANOVA followed by Mann-Whitney *U* tests. Comparisons of the secreted amount of progesterone and of the gene expression data between the genotypes were done using the Mann-Whitney *U* test. All results are presented as mean ± SE of the mean (SEM). Differences were considered significant at $P < 0.05$.

Results

βERKO ovaries exhibit a reduced number of large antral follicles and corpora lutea

The follicle distribution in wild-type and βERKO ovaries is shown in Fig. 1. Ovaries from untreated 28-d-old wild-type and βERKO mice revealed similar numbers of primordial and primary follicles per section (Fig. 1A). Among the pool of growing follicles in immature mice, only the number of large preantral follicles per section in βERKO ovaries *vs.*

TABLE 1. Primer pairs and probes used for semiquantitative RT-PCR

Gene	Primer sequence	Size (bp)	Probe (bp)	GenBank accession no.
Actb	5′-GTGGGCCGCTCTAGGCACCAA-3′ 5′-CTCTTTGATGTCACGCACGATTTC-3′	540	422–466	M12481
Ar	5′-TGCCTCCGAAGTGTGGTACC-3′ 5′-GCTTACGAGCTCCCAGAGTCA-3′	401	1475–1811	M37890
Cdkn1b	5′-TTTTCCGGAGAGAGGCGAG-3′ 5′-GCAGTGCTTCTCCAAGTCCC-3′	196	278–298	NM009875
Cyp19	5′-AGGGTTACTTTAACGCCTGGC-3′ 5′-CTTTCAGGATTGCTGCTTCG-3′	351	882–911	D00659
Has2	5′-GGTTGGAGGTGTTGGAGGAGA-3′ 5′-TGGACCGAGCCGTGTATTTAG-3′	302	1407–1436	NM_008216
Lhcgr	5′-AACCCGGTGCTTTTTACAAACC-3′ 5′-TCCCATTGAATGCATGGCTT-3′	246	410–433	NM_013582
Pgr	5′-TTACCATGTGGCAAATCCCA-3′ 5′-GTCCGGGATTGGATGAATGTA-3′	304	2481–2513	M68915
Ptgs2	5′-TGATCGAAGACTACGTGCAACA-3′ 5′-AATGGAGGCCTTTGCCACT-3′	326	1222–1251	M64291
Timp1	5′-TCTTGGTTCCCTGGCGTACT-3′ 5′-AGCAAAGTGACGGCTCTGGT-3′	191	545–574	BC008107

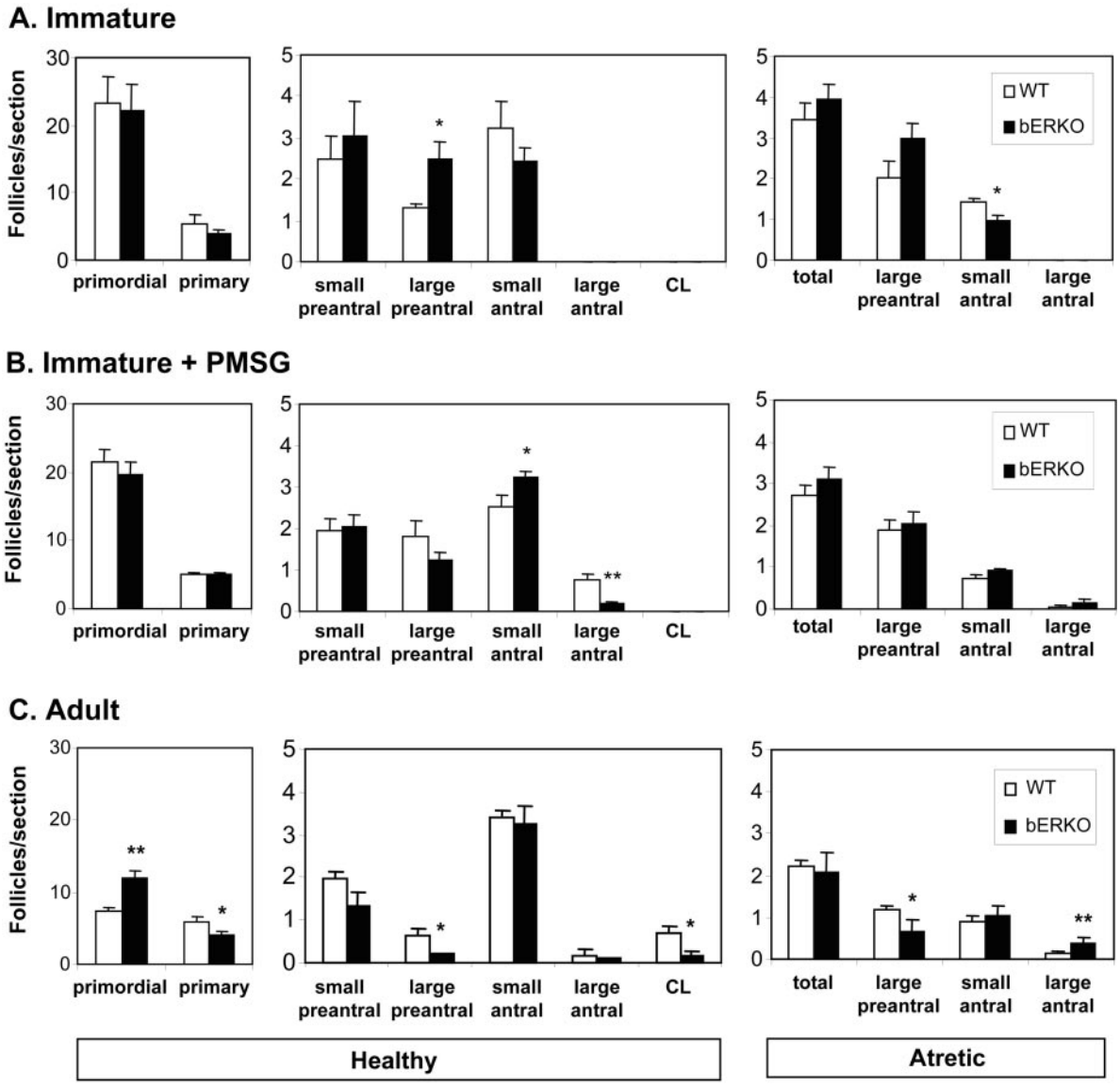

FIG. 1. Distribution of the follicle populations in wild-type and βERKO ovaries. A, Follicle population (healthy and atretic) in ovaries of immature wild-type (WT) and βERKO mice 28–30 d of age after treatment with vehicle for 48 h (n = 4 per group). B, Follicle population (healthy and atretic) in ovaries of immatureWT and βERKO mice 28–30 d of age after treatment with PMSG for 48 h (n = 7 wild type; n = 9 βERKO). C, Follicle population (healthy and atretic) in ovaries of adult WT and βERKO approximately 109 d of age (n = 6 per group). Shown is the mean number of follicles per section for each follicle class per genotype (± SEM). *, $P < 0.05$ *vs.* respective WT value; **, $P < 0.01$ *vs.* respective WT value.

wild-type ovaries was significantly increased ($P < 0.05$). Although the total number of atretic follicles per section did not significantly differ between immature wild-type and βERKO ovaries (Fig. 1A), there were fewer atretic small antral follicles per section in βERKO ovaries compared with wild-type ovaries ($P < 0.05$).

PMSG treatment for 48 h had no marked effect on the pool of primordial or primary follicles in immature ovaries of either genotype (Fig. 1B). There were also no differences in the number of preantral follicles per section between βERKO and wild-type PMSG-treated ovaries. Treated βERKO ovaries did exhibit an increased number of small antral follicles per section compared with treated wild-type ovaries ($P < 0.05$). As expected, PMSG treatment led to the presence of large antral follicles that were not observed in ovaries of untreated animals (compare Fig. 1, A with B; $P < 0.01$, untreated *vs.* treated wild-type ovaries; $P < 0.05$, untreated *vs.* treated βERKO ovaries). Interestingly, PMSG-treated βERKO females exhibited significantly fewer large antral follicles per section ($P < 0.01$) compared with treated wild type. No differences were observed in the number of atretic follicles per section, total or per category, between PMSG-treated wild-type and PMSG-treated βERKO ovaries (Fig. 1B).

A comparison of follicle counts per section between adult wild-type and βERKO ovaries revealed that βERKO ovaries contain significantly more primordial ($P < 0.01$) but significantly fewer primary follicles per section ($P < 0.05$; Fig. 1C). βERKO ovaries also exhibited a significantly reduced number of large preantral follicles and corpora lutea per section ($P < 0.05$). Although no difference was observed in the total

number of atretic follicles between adult wild-type and βERKO ovaries, βERKO ovaries contained fewer atretic large preantral follicles and more atretic large antral follicles per section than wild-type ovaries.

βERKO follicles fail to reach maximum size in vitro

Figure 2 shows the growth profiles of *in vitro* cultured follicles from αERKO (Fig. 2A) and βERKO (Fig. 2B) ovaries compared with follicles from wild-type ovaries. At the start of culture (d 0), preantral follicles from wild-type, βERKO, and αERKO ovaries exhibited a similar mean diameter, and each continued to grow and exhibit visible antrum formation by d 2. The overall growth profile of αERKO follicles did not differ from their respective wild-type controls ($P = 0.08$; Fig. 2A), but a significant difference in size was observed between wild-type and αERKO follicles on culture d 1 ($P < 0.001$) and d 5 ($P < 0.01$). Nonetheless, the percentage of αERKO follicles (77%) that reached 340 μm did not differ from wild type (91%; Table 2). In contrast, there was a significant difference between the overall growth profiles of wild-type and βERKO follicles ($P < 0.0001$; Fig. 2B) such that βERKO follicles exhibited retarded growth that first became apparent on d 2 ($P < 0.01$). On subsequent days, the difference in follicle diameter between wild-type and βERKO follicles became more pronounced ($P < 0.001$ on d 3, 4, and 5). As a result, the percentage of βERKO follicles reaching a large antral size (designated as a diameter ≥ 340 μm) after 5 d in culture was significantly lower relative to wild-type, 60 *vs.* 92%, respectively ($P < 0.0001$; Table 2).

The steroidogenic capacity of follicles in culture was assessed by measuring the level of androstenedione, testosterone and estradiol that accumulated in the culture medium over a 48 h interval, between days 3 and 5 of culture. Androstenedione and testosterone in the medium were undetectable. Although we observed a considerable degree of variation in estradiol release among follicles within each genotype, a significant difference among the genotypes was evident when comparing the mean levels (Table 3). Estradiol secretion by αERKO follicles significantly exceeded that of wild-type follicles by at least 4-fold ($P < 0.05$), whereas βERKO follicles exhibited a significantly lower rate of secretion compared with wild-type follicles ($P < 0.05$).

TABLE 2. Percentage of wild-type, αERKO, and βERKO follicles that reached a large antral size

Genotype	n[a]	%≥340 μm (n)[b]
Wild-type	46	91 (42)
αERKO	26	77 (20)
Wild-type	118	92 (109)
βERKO	134	60 (80)[c]

[a] Number of follicles at d 5 of culture.
[b] Follicles that reached a diameter ≥340 μm within 5 d of culture in percentages and in absolute numbers (in *parentheses*).
[c] $P < 0.0001$ *vs.* respective wild type.

βERKO follicles do not fully respond to hCG

Wild-type, αERKO, and βERKO follicles that had reached a large antral size of at least 340 μm were induced to ovulate by treatment with hCG and checked for ovulation at 16 h after the hCG stimulus (Table 4). Initially, a dose of 2 IU/ml hCG was used, because this was shown to be the optimal dose to induce ovulation *in vitro* by Rose *et al.* (34). After exposure to 2 IU/ml hCG, a similar percentage of wild-type (47%) and αERKO (66%) follicles exhibited successful rupture. However, only 10% of βERKO follicles that had reached a diameter of at least 340 μm ovulated in response to hCG, a significant reduction ($P < 0.0001$) relative to their wild-type (53%) counterparts. Increased hCG doses ranging from 4–32 IU/ml did not improve the percentage of ovulating follicles among wild-type, αERKO, or βERKO follicles. Regardless of genotype, released oocytes were consistently surrounded by expanded, mucified cumulus cells and appeared to resume

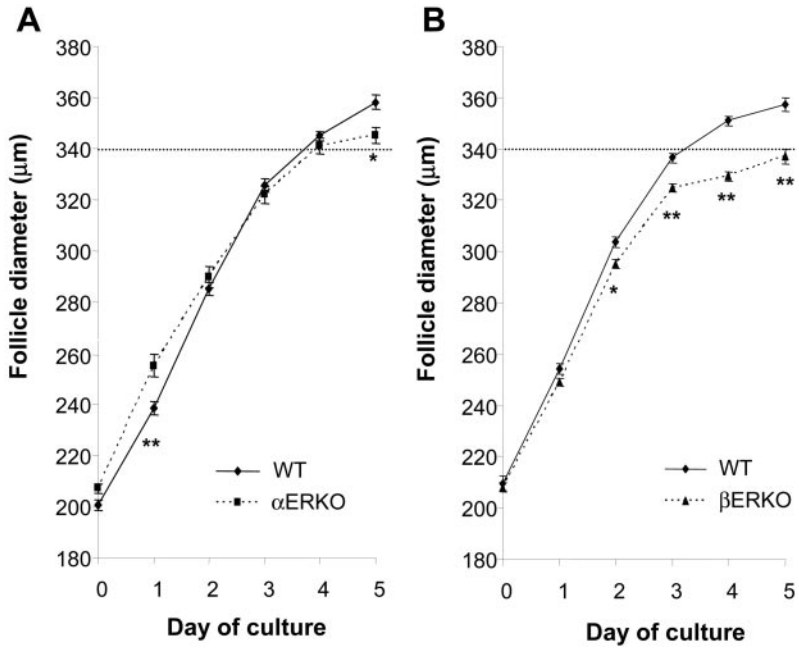

FIG. 2. *In vitro* growth of follicles from wild-type (WT), βERKO, and αERKO ovaries. Preantral follicles were grown *in vitro* in the presence of 100 mIU/ml recombinant human FSH. The follicle diameter was measured each culture day. Follicles that exceeded a diameter of 340 μm (*dotted line*) were classified large antral follicles. A, Growth profiles of WT and αERKO follicles (n = 7–46 follicles per data point). The growth of αERKO follicles was similar to that of WT follicles ($F_{1,218} = 3.1$; $P = 0.08$). There was a significant difference between the size of WT and αERKO follicles on d 1 and 5 of culture. B, Growth profiles of WT and βERKO follicles (n = 11–133 follicles per data point). The growth profile of βERKO follicles was different from WT follicles ($F_{1,630} = 181.1$; $P < 0.001$). From culture d 2 onward, WT follicles were significantly bigger in diameter than βERKO follicles at each culture day. Shown is the mean follicle diameter (μm) ± SEM for each culture day per genotype. *, $P < 0.01$ *vs.* WT; **, $P < 0.001$ *vs.* WT.

TABLE 3. Estradiol secretion by follicles *in vitro*

Genotype	n	Estradiol (pg/ml·48 h)[a]
Wild-type	3	137.5 ± 47.3[b]
	0	
αERKO	2	563.6 ± 177.9[b]
	7	
βERKO	2	89.1 ± 61.3[b]
	4	

[a] Secretion per follicle over a 48-h interval, measured on culture d 5, expressed as mean ± SEM.
[b] $P < 0.05$ among the three genotypes.

meiosis as evident by germinal vesicle breakdown and occasionally polar body extrusion. No additional evaluation of oocyte competence was performed. Luteinization of the follicle after hCG exposure was monitored by assessing the release of progesterone into the culture medium 24 h after hCG treatment. Before the hCG stimulus, progesterone secretion by large antral follicles was low in all genotypes but rose rapidly by 24 h after the induction of ovulation (Table 5).

To extend the above findings, we selected genes known to show coordinated expression during antral growth and ovulation, including *Lhcgr*, *Cyp19*, *Ar*, *Timp1*, *Cdkn1b*, *Pgr*, *Ptgs2*, and *Has2* for evaluation of their expression patterns in wild-type, αERKO, and βERKO follicles grown *in vitro*. Semiquantitative RT-PCR analysis for these genes was performed on total mRNA from large antral follicles that reached a diameter of at least 340 μm and were collected just before or 3 or 24 h after incubation with hCG (2 IU/ml). Because some of selected genes show a rapid but transient in response to the LH surge (37), collecting follicles at the indicated time points enabled us to determine whether this rapid changes also occur in follicles of all three genotypes.

Representative blots for each transcript and bar graphs of those transcripts that showed differences between genotypes are shown in Fig. 3. Gene expression assays from αERKO follicles indicate no significant differences in the temporal pattern or level of expression for *Lhcgr*, *Cyp19*, *Ar*, *Cdkn1b*, *Ptgs2*, and *Has2* when compared with similarly treated wild-type follicles (Fig. 3A). αERKO follicles did have reduced levels of *Pgr* expression just before and 24 h after hCG ($P < 0.01$); however, the expected hCG induction of *Pgr* mRNA expression occurred normally in αERKO follicles. Moreover, αERKO follicles showed a reduction in *Timp1* expression before the hCG pulse ($P < 0.05$).

Gene expression assays in βERKO follicles indicated no significant differences in the temporal expression pattern or level of expression for *Ar*, *Cdkn1b*, *Pgr*,and *Has2* when compared with similarly treated wild-type follicles (Fig. 3A). In contrast, *Cyp19* mRNA levels were reduced relative to wild-type follicles of similar size just before the hCG pulse ($P < 0.01$). *Lhcgr* mRNA levels in βERKO follicles differed from wild-type levels 3 and 24 h after hCG ($P < 0.05$). The dramatic and transient induction of *Ptgs2* by hCG in wild-type follicles (>200-fold) was significantly reduced in βERKO follicles ($P < 0.05$). Moreover, βERKO follicles showed a reduction in *Timp1* expression before and 3 h after the hCG pulse ($P < 0.05$).

TABLE 4. Percentage of follicles ovulating *in vitro*

Genotype	% Ovulation per hCG dose (IU/ml)[a]				
	2	4	8	16	32
Wild-type	47	49	73	50	50
αERKO	66	60	40	78	62
Wild-type	53	55	63	67	75
βERKO	10[b]	9[b]	15[b]	14[b]	12[b]

[a] Follicles (≥340 μm diameter) were incubated with hCG and checked for ovulation 16 h later; n = 11–44, wild-type and αERKO; n = 24–99, wild-type and βERKO.
[b] $P < 0.0001$ *vs.* respective wild type.

Exogenous PGE_2 does not restore ovulation in βERKO follicles

Davis *et al.* (38) showed that *Ptgs2*-null mice have a reduced ovulation rate that could be restored to normal by treatment with exogenous PGE_2. Therefore, the effect of PGE_2 on ovulation rates of wild-type and βERKO follicles *in vitro* was examined. For this, large antral follicles (diameter ≥ 340 μm) were incubated with PGE_2 (500 ng/ml) 4 h after hCG administration. As shown in Fig. 4, PGE_2 had no effect on the ovulation rates of either wild-type follicles or βERKO follicles, but histological analysis of unruptured follicles treated with a combination of hCG and PGE_2 indicated the presence of an expanded COC and thinning of the follicle wall. The majority of wild-type follicles showed this thinning and expansion after treatment with hCG only. βERKO follicles, however, often failed to undergo this thinning and expansion in response to hCG only but did show it in response to hCG combined with PGE_2.

Discussion

In the present study, we have used the method of *in vitro* follicle culture to gain further insight into the intraovarian role of ERα and ERβ in follicle growth, ovulation, and luteinization. βERKO females exhibited an abnormal growth pattern, decreased estradiol production, and an attenuated response to an ovulatory dose of hCG, whereas follicles from αERKO ovaries exhibited wild-type-like growth and ovulatory efficiency but an elevated capacity to synthesize estradiol. These data confirm as well as advance our previous descriptions of ovarian phenotypes in ERKO mice that were derived from *in vivo* studies. The capacity of αERKO follicles to behave normally when placed in culture, removed from the chronically high levels of LH, estradiol, and androgens that are characteristic of this animal, supports our previous hypothesis that the more dramatic phenotypes in the αERKO ovary are secondary to the loss of ERα-mediated negative feedback in the hypothalamic-pituitary axis (23, 29). The reduced efficiency of βERKO follicles to ovulate *in vitro* after hCG exposure corroborates our earlier *in vivo* superovulation data (4) and confirms that reduced fertility in these animals is primarily a result of the loss of ERβ actions in the ovary.

A sizeable percentage of βERKO follicles, both *in vivo* and *in vitro*, fail to reach maximal size after gonadotropin exposure. The reduced size of mature βERKO follicles is not a result of a dramatic decrease in the granulosa cell population, although no quantitative assays for cell number were carried out. Instead, the *in vitro* growth curve demonstrates that βERKO follicles exhibit wild-type-like increases in diameter

TABLE 5. Progesterone secretion of follicles *in vitro* upon hCG stimulus

Genotype	Progesterone (ng/ml) per hCG dose (IU/ml)[a]					
	0	2	4	8	16	32
Wild-type	0.4 ± 0.1	50.3 ± 7.1	50.7 ± 2.4	51.8 ± 2.5	44.7 ± 3.4	51.0 ± 2.2
αERKO	0.5 ± 0.3	48.1 ± 5.7	41.1 ± 3.7[b]	53.8 ± 1.9	41.7 ± 1.1	49.6 ± 3.2
Wild-type	0.7 ± 0.3	50.8 ± 2.0	46.8 ± 2.6	47.1 ± 4.2	45.4 ± 5.2	50.0 ± 1.3
βERKO	0.6 ± 0.1	45.5 ± 2.2	34.0 ± 4.0	48.6 ± 2.9	51.0 ± 1.2	46.8 ± 4.4

[a] Follicles (≥340 μm diameter) were incubated with hCG, and medium was collected for progesterone measurement 24 h later. Data are expressed as mean ± SEM; n = 3–9, wild-type and αERKO; n = 4–42, wild-type and βERKO.
[b] $P < 0.05$ *vs.* respective wild type.

up to the small antral stage, when granulosa cell proliferation is most rapid and the primary contributor to increased follicle size (19). However, during the later stages of follicular growth when granulosa cell proliferation is slowed but follicle size continues to increase because of antrum formation (19), βERKO follicles begin to exhibit retarded growth. The reduced number of large antral follicles in immature βERKO ovaries after gonadotropin stimulation observed *in vivo* corroborates the *in vitro* findings. Dupont *et al.* (32) also described follicles with an underdeveloped antrum after go-

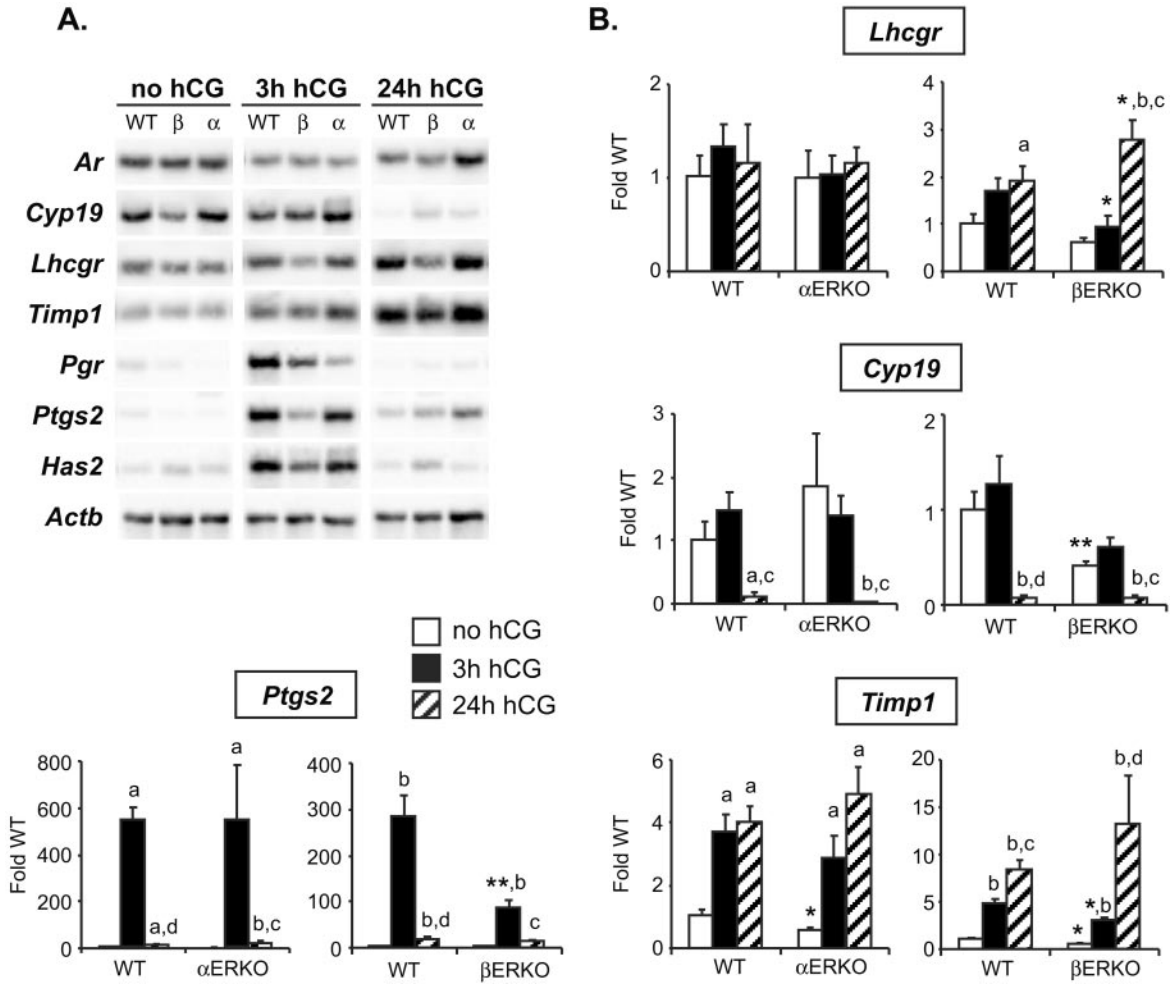

FIG. 3. Analysis of *Lhcgr*, *Cyp19*, *Ar*, *Timp1*, *Cdkn1b*, *Pgr*, *Ptgs2*, and *Has2* mRNA expression patterns in follicles from wild-type (WT), αERKO, and βERKO ovaries. Expression was measured in follicles grown *in vitro* that exceeded a diameter of 340 μm and were collected immediately (no hCG) or 3 or 24 h after incubation with hCG (2 IU/ml). A, PhosphorImage analysis of representative RT-PCR assays for *Lhcgr*, *Cyp19*, *Ar*, *Timp1*, *Cdkn1b*, *Pgr*, *Ptgs2*, and *Has2* mRNA in WT, βERKO, and αERKO follicles at indicated time points (no hCG, 3 h hCG, and 24 h hCG). *Actb* mRNA analysis was included as control. B, *Bar graphs* for *Lhcgr*, *Cyp19*, *Timp1*, and *Ptgs2* mRNA levels in WT, αERKO, and βERKO follicles. Expression levels have been normalized to *Actb* expression levels and expressed as fold induction over the WT no-hCG value. Data are expressed as the mean ± SEM (three to six follicles per data point in WT *vs.* αERKO graphs; three to13 follicles per data point in WT *vs.* βERKO graphs). *Asterisk* denotes statistical significance between genotypes at a time point: *, $P < 0.05$ *vs.* respective WT value; **, $P < 0.01$ *vs.* respective WT value. *Letters* denote statistical significance between time points within a genotype; a, $P < 0.05$ *vs.* no-hCG value; b, $P < 0.01$ *vs.* no-hCG value; c, $P < 0.05$ *vs.* 3-h hCG value; d, $P < 0.01$ *vs.* 3-h hCG value.

A

Percentage of follicles ovulating *in vitro* in the presence or absence of PGE_2

	% ovulation (n)	
Genotype	hCG	hCG + PGE_2
Wild-type	46 (48)	58 (43)
βERKO	13 (46)*	20 (41)*

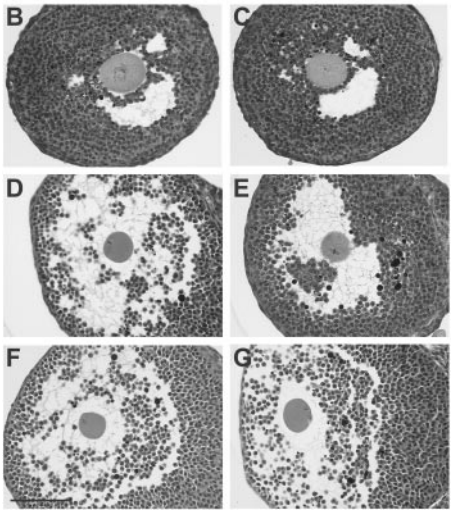

FIG. 4. Effect of PGE_2 on *in vitro* ovulation of wild-type and βERKO follicles. A, Percentage of follicles with a diameter of at least 340 μm that ovulated within 16 h in response to treatment with hCG (2 IU/ml) alone or hCG plus PGE_2 (500 ng/ml PGE_2 added 4 h after hCG). *, $P < 0.001$ *vs.* respective wild-type value. The number of follicles used for each group is shown in *parentheses*. B–G, Histological analysis of follicles cultured for 5 d. B, Wild-type follicle; C, βERKO follicle; D, nonovulated wild-type follicle 16 h after stimulation with hCG (2 IU/ml) (note the expanded COC and thinning of the follicle wall); E, nonovulated βERKO follicle 16 h after hCG (2 IU/ml) (less than robust COC expansion and thinning of the wall compared with wild-type follicle); F, nonovulated wild-type follicle treated with hCG plus PGE_2, 16 h after hCG pulse; G, nonovulated βERKO follicle treated with hCG plus PGE_2, 16 h after hCG pulse showing similar COC expansion and thinning of the follicle wall. *Scale bar*, 100 μm. These data are representative of results obtained from analyzing at least five follicles per experimental group per genotype.

nadotropin treatment in the ovaries of a separate strain of ERβ-null mice. Granulosa cell proliferation and antrum development are predominantly under the positive regulation of FSH and estradiol (19, 39). ERβ is localized to the granulosa cells of growing follicles and expression peaks during the progression from small to large antral size (24, 25, 28, 40). Therefore, our combined *in vitro* and *in vivo* data indicate that ERβ may contribute little to granulosa cell proliferation but is important to antrum formation during follicle maturation. This is consistent with the findings of Burns *et al.* (41) showing that ERβ is not a determining factor in the growth of granulosa cell tumors in mice. Because some but not all βERKO follicles do progress through the large antral stage, ERβ is not absolutely required for antrum formation.

In addition to gonadotropin-stimulated growth of follicles *in vitro*, we also compared the ability of mature follicles from all three genotypes to respond to a surge of hCG and successfully expel the oocyte *in vitro* as well as demonstrate the expected changes in gene expression and steroid production. Ovulation is a tightly coordinated process involving multiple factors and signaling pathways (37). *In vivo*, ovulation is triggered by a surge of LH upon the ovary, which simultaneously stimulates follicular somatic cells to cease proliferation and terminally differentiate toward a luteinized cell type as well as the processes necessary to rupture the follicle and release the oocyte into the extraovarian environment. βERKO follicles that reach a large antral size are less likely to ovulate in response to hCG than wild-type follicles, whereas αERKO follicles showed no deficit. Increasing the dose of hCG could not improve the poor ovulation rate of βERKO follicles. Although most βERKO follicles failed to rupture in response to hCG, the somatic cells of the follicles did luteinize as shown by the rapid increase in progesterone secretion regardless of ovulatory success and increased expression of *Cdkn1*b, a gene that is known to be increased in luteinized granulosa cells (42). And although initially decreased in βERKO and αERKO follicles, *TIMP-1*, another gene that is likely to play a role in corpus luteum formation (43), is properly up-regulated 24 h after hCG in ERKO follicles. These findings are consistent with earlier *in vivo* studies demonstrating that hCG-stimulated βERKO ovaries possessed corpora lutea and luteinized follicles that still contained trapped oocytes (4, 32). Therefore, we conclude that ERβ facilitates follicle rupture but not luteinization.

Ovulation is preceded by rapid and transient increases in gene expression in the follicle, including several genes whose products are obligatory to follicle rupture via the study of gene-targeted mice, including *Pgr* (44) and *Ptgs2* (38). HAS2 (*Has2*), the enzyme necessary for synthesis of glycosaminoglycan hyaluronan, the main component of the expanded COC, is also rapidly induced by LH just before ovulation (45). To determine whether the abnormal failure rate among βERKO follicles to ovulate was because of a lack of necessary gene expression, we evaluated the transient expression of *Pgr*, *Ptgs2*, and *Has2* mRNA in wild-type and ERKO follicles *in vitro*. Whereas *Pgr* and *Has2* induction by hCG was similar in wild-type, αERKO, and βERKO follicles, hCG-induced *Ptgs2* levels among βERKO follicles were significantly reduced. *Ptgs2* encodes the enzyme prostaglandin-endoperoxide synthase 2 necessary for synthesis of prostaglandins, including PGE_2, which are critical to COC expansion and follicle rupture (38). The less than robust COC expansion observed herein among βERKO follicles *in vitro* correlates with a lack of sufficient *Ptgs2* induction. In addition, we and others have previously reported impaired mucification of the COC among βERKO oocytes *in vivo* after superovulation of ERβ-null females (4, 32). Therefore, the reduced induction of *Ptgs2* after hCG exposure in βERKO follicles may be a causal factor in reduced ovulation rates observed both *in vitro* and *in vivo*. The ovulatory defect in *Ptgs2*-null mice can be restored by treatment with PGE_2 during superovulation (38). Herein, similar treatment of βERKO follicles *in vitro* improved expansion of the COC and thinning of the follicle wall, but no improvement of follicle rupture was observed. This suggests that βERKO follicles may have reduced levels of PGE_2 but intact prostaglandin-receptor signaling path-

ways. However, no quantitative assays for PGE_2 content or PGE_2 target genes were carried out to elucidate this.

Although *Ptgs2* was the only hCG-regulated gene evaluated herein to show a reduced response, we did observe that preovulatory βERKO follicles *in vitro* exhibit deficits in granulosa cell differentiation, as indicated by a significant reduction in preovulatory level of *Cyp19*, as was estradiol output. *Cyp19* expression in granulosa cells of large antral follicles is primarily dependent on FSH, but this requires estradiol (11). A similar role for estradiol has been shown to occur in the FSH regulation of *Lhcgr* mRNA expression (14–16), which was slightly reduced in preovulatory βERKO follicles *in vitro* but within normal range. These data suggest that the long-known synergistic effect of estradiol on FSH actions may be mediated by ERβ. Additional support for this hypothesis comes from Boa *et al.* (40) and their report of increased ERβ expression just before initiation of *Cyp19* and *Lhcgr* mRNA expression in granulosa cells of preovulatory follicles. Another indication of a failure of ERβ-null granulosa cells to fully differentiate is reports of elevated androgen receptor expression in antral βERKO follicles (46), which is normally reduced in differentiated granulosa cells (10). However, we were unable to verify this abnormality in individual βERKO follicles *in vitro*.

Interestingly, *in vitro* cultured ERα-null follicles behaved similarly to wild-type follicles among all of the parameters evaluated with the exception of estradiol synthesis. Thus, ERα has a minimal intrafollicular role in terms of regulating growth and ovulatory capacity of the individual follicle. However, the increased estradiol output by individual αERKO follicles when placed in a controlled hormonal environment was surprising and in contrast to our earlier hypotheses (23). We have previously shown that by reducing the chronically high plasma LH levels in the αERKO female via a GnRH antagonist, several aspects of the αERKO ovarian phenotype are prevented, including the increased *Cyp19* expression and estradiol synthesis (23, 29). Although the present *in vitro* data further support the *in vivo* data in terms of rescuing αERKO follicle growth when placed in a more physiological hormonal milieu, the continued elevated level of estradiol secretion was puzzling. No increase in level of *Cyp19* mRNA was observed between wild-type and αERKO follicles. However, the availability of androgen precursors from the theca is considered limiting to estradiol synthesis by granulosa cells in preovulatory follicles (9). We have previously shown that αERKO ovaries have increased *Cyp17* mRNA expression, and this may persist *in vitro* (23). Alternatively, the explanation for this observation may be that the follicles were prepared from 21- to 25-d-old ovaries, at which time αERKO females already show elevated levels of serum LH (29). Exposure of αERKO follicles to higher endogenous LH before removal and culture may enhance the steroidogenic capacity of these follicles when compared with wild type.

In summary, our combined use of *in vitro* follicle culture methods with ERKO mice has proved to be a powerful tool to expand our knowledge of the role of ERα and ERβ in follicular development and ovulation. Our *in vitro* data suggest that folliculogenesis and ovulation can normally occur in the absence of ERα but not in the absence of ERβ. Several indicators of follicle maturity, *e.g.* antrum formation as well as acquisition of *Cyp19* expression, fail to fully develop in ERβ-null follicles. These findings suggest that ERβ may not be directly mediating the ovulatory response to the LH surge but instead is most important to propagating the necessary differentiation of the follicle that leads up to the LH surge and successful ovulation.

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