Characterizing the neuroendocrine and ovarian defects of androgen receptor-knockout female mice

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A HEALTHY PREOVULATORY FOLLICLE produces a maturing oocyte and secretes estradiol (E2), leading to a preovulatory surge, which in turn triggers the ovulatory luteinizing hormone (LH) surge and the subsequent follicular rupture and release of the oocyte (18). Throughout most of the murine estrous cycle, E2 exerts negative feedback on gonadotropin-releasing hormone (GnRH) and LH secretion until the afternoon of proestrus, when there is a switch to positive feedback, with rising follicular E2 secretion evoking an abrupt release of the preovulatory GnRH surge and then the LH surge that triggers ovulation (22, 30). Although follicle development and ovulation are physiologically well-characterized events, the underlying molecular pathways are still being unraveled.

The androgen receptor (AR), a member of the nuclear receptor superfamily encoded by the X chromosome (31), is expressed in the ovary of many mammalian species (pig: 9; cow: 19; human: 24; rat: 58; primate: 69), and in vivo and in vitro pharmacological studies have demonstrated direct androgen effects on follicle growth (16, 35, 61–63). Findings from several androgen-resistant female mouse models [AR-knockout (ARKO)] have confirmed a role for AR-mediated androgen actions in female reproduction (25, 48, 56, 67). ARKO females are subfertile with fewer pups per litter (25, 48, 64), and dysfunctional ovulation was identified as a key defect, with females exhibiting reduced corpora lutea numbers (25, 48, 64). Reciprocal ovary transplant experiments between ARKO and normal females demonstrated that both intra- and extraovarian defects contribute to the subfertility observed in ARKO female mice (65). Within ARKO ovaries, follicular health was reduced with an increase in follicular atresia (25, 48, 64) and a dissociation of cumulus cells from the oocyte in preovulatory follicles (25). Furthermore, a delayed first litter (64), longer estrous cycles (25, 64), and the finding that reduced naturally ovulated oocyte numbers observed in ARKO females were overcome by gonadotropin (pregnant mare serum gonadotropin/human chorionic gonadotropin) hyperstimulation suggests an extraovarian defect in gonadotropin regulation (64). Additionally, disrupted steroidal feedback signaling was observed in ARKO females with an increase in estrus serum FSH levels and hypersensitivity to negative E2 feedback on LH secretion (65).

AR is also widely expressed in the brain, notably in the preoptic area, hypothalamus, and other limbic structures (44), where it is regulated by testosterone (T) and E2 (29). Hence, AR signaling is likely to influence both negative and positive steroidal feedback mechanisms in the HPG axis governing pulsatile hypothalamic GnRH secretion and consequential pituitary LH and FSH release, notably the ovulatory LH surge.

Although hypothalamic GnRH secretion is tightly responsive to negative feedback from circulating gonadal steroids, it is controversial whether GnRH neurons express steroid receptors (7, 26, 52), so their steroidal control is likely to involve intermediary neurons (22). Kisspeptin (Kp; a product of the Kiss1 gene) is vital for reproduction, with a disruption of its expression or its receptor [G protein-coupled receptor 54 (GPR54)] causing reproductive failure due to gonadotrophin deficiency (38). Kp neurons express AR and estrogen receptor (ER) and directly innervate GnRH neurons (38). Kp also displays a potent stimulatory effect on gonadotrophin release, with the administration of Kp resulting in an abrupt and sustained LH secretion (15, 38). Substantial evidence supports Kp-GPR54 signaling in generating the estrogen-induced pro-ovulatory LH surge (15), and AR expression in Kp neurons implicates them as an AR target. Recent findings implicate AR in the regulation of ovulation, with expression of ovulation-related genes (Cox-2 and Areg) increased in mouse ovaries after treatment with dihydrotestosterone (DHT) but suppressed by the AR antagonist flutamide (72). A role for AR-mediated androgen actions in triggering ovulation is supported by pharmacological evidence in hens, where flutamide administration blocked proovulatory surges of E₂ and LH and ovulation (43). However, in women, administration of high levels of T represses LH secretion, implying that an optimal level of AR activation is required to maintain normal patterns of LH secretion (47). Finally, a positive effect of androgens on the number of follicles available for ovulation is supported by the findings that administration of T or DHT during the follicular phase of follicles available for ovulation is supported by the findings that administration of T or DHT during the follicular phase increases preovulatory follicle and corpora lutea numbers within porcine ovaries (10, 11).

In summary, there is strong evidence supporting a role for intraovarian and neuroendocrine androgen actions via the AR in the control of female fertility; however, the precise pathways underlying these fundamental mechanisms are yet to be elucidated. Because ovulation is a key defect in ARKO females, using our ARKO mice (64), this study set out to determine the specific AR-mediated mechanisms involved in the control of late follicle development, ovulatory pathways, and the subsequent viability of the released oocytes.

**MATERIALS AND METHODS**

**Mice**

Mice were maintained under standard housing conditions (ad libitum access to food and water in a temperature- and humidity-controlled 12-h light cycle environment) at the ANZAC Research Institute. All procedures were performed under ketamine-xylazine anesthesia. All procedures were approved by the Sydney South West Area Health Service Animal Welfare Committee within National Health and Medical Research Council (NHMRC) guidelines for animal experimentation.

**Generation and Genotyping of ARKO Mice**

Female homozygous ARKO mice were generated by crossing ARflox mice (37) with either CMV-Cre (64) or Sox2-Cre mice (21, 49) as the transgene carrier. Specimen Collection

Dissected ovaries were weighed (diestrous: WT (n = 10) and ARKO (n = 9); proestrus: WT (n = 16) and ARKO (n = 16)), fixed in 4% paraformaldehyde at 4°C overnight, and stored in 70% ethanol before histological processing. Ovaries for histological assessment (collected at 2 mo of age) were randomly selected and processed through graded alcohols into glycol methacrylate resin (Technovit 7100; Heraeus Kulzer, Chatswood, Australia). Ovaries were serially sectioned at 20 μm, stained with periodic acid Schiff, and counterstained with hematoxylin. Blood was collected from sexually mature females by cardiac exsanguination under ketamine-xylazine anesthesia, rested at room temperature for 20 min, and centrifuged at 5,000 rpm for 5 min, and then collected serum was stored at −20°C.

**Assessment of Estrous Cycle**

Estrous cycle stage was determined in sexually mature females by light microscope analysis of vaginal epithelial cell smears collected daily (10 AM each morning) in 20 μl of sterile PBS and then transferred to glass slides, air-dried, and stained with 0.05% trypan blue for microscopy (66).

**Hormone Measurements in Ovary-Intact Mice**

Mice were maintained on a 12:12-h light cycle, with lights turned on at 0600 and off at 1800. The ovulatory LH surge in mice typically occurs around the time when lights are turned off (8). To determine the optimal time to collect blood samples to assess the LH surge in our mouse colony, blood was collected by cardiac exsanguination under ketamine-xylazine anesthesia from wild-type (WT) females (2–4 mo of age) at 0900, 1730, 1800, 1830, and 1900 and at proestrus (6–10 females/time point). Serum was stored at −20°C until assay. Timing of the LH surge was also assessed in ARKO females (2–4 mo of age) at the same time points (6–12 females/time point). Because the ovulatory LH surge occurs at 1830 in our colony of WT mice (Fig. 1A), this time was chosen as the optimum time to assess the natural LH surge in our WT and ARKO females at proestrus. WT and ARKO blood samples were collected at diestrus [WT (n = 10), ARKO (n = 9)] and proestrus [WT (n = 16), ARKO (n = 15)].

**Ovariectomy and Estrogen Replacement**

Serum LH and FSH were determined from blood samples collected from intact WT and ARKO females at 0800–0900 [basal group; WT (n = 5), ARKO (n = 6)], as described previously (49, 64, 66). 

**Negative feedback.** To evaluate estrogen sensitivity of negative feedback, serum LH levels were evaluated after ovariectomy (OVX) with and without E2 (Sigma) administration, as described previously (12, 65) with the following minor modification. On the day of surgery (day 0), 8-wk-old females were anesthetized between 0800 and 1100 to undergo OVX and administered (subdermal) at the same time with either an empty silastic tube implant [OVX, AM group; WT (n = 5), ARKO (n = 6)] or a 1-cm implant filled with 2.5 μg of E2 mixed into Silicone Type A Medical Adhesive [OVX + E2, AM group; WT (n = 6), ARKO (n = 6)] (Dow Corning). On day 6 post-OVX, mice received 1 μg of estradiol benzoate (EB; Sigma) in 0.1 ml of sesame oil injected (sc) between 0900 and 0930. Blood samples were collected at 0800–0900 on day 7.
Positive feedback. The LH surge response to administration of E2 was assessed in 8-wk-old females that were ovariectomized and treated with E2 and EB, as described previously (12). On the day of surgery (day 0), 8-wk-old females were anesthetized between 0800 and 1100 to undergo OVX and administered (subdermal) at the same time with a 1-cm implant filled with 2.5 μg of E2 mixed into Silicone Type A Medical Adhesive (Dow Corning) [OVX was assessed in 8-wk-old females that were ovariectomized and implanted with 2.5 μg of E2 (mixed into silicone Type A Medical Adhesive Dow Corning)]. On day 6 post-OVX, mice received 1 μg of EB (Sigma) in 0.1 ml of sesame oil injected (sc) between 0900 and 0930. Blood samples were collected at 1830 on day 7.

Pituitary Response to GnRH Stimulation

Pituitary response to GnRH stimulation was studied in 2- to 3-mo-old females, as described previously (12, 17). Females were ovariectomized and implanted with 2.5 μg of E2 implants, as described above. On day 6 post-OVX, mice received 1 μg of EB (mixed into silicone Type A Medical Adhesive Dow Corning) [OVX was assessed in 8-wk-old females that were ovariectomized and implanted with 2.5 μg of E2 mixed into silicone Type A Medical Adhesive Dow Corning]. On day 6 post-OVX, mice received 1 μg of EB (Sigma) in 0.1 ml of sesame oil injected (sc) between 0900 and 0930. Blood samples were collected 10 min after GnRH or saline injection [saline: WT (n = 6), ARKO (n = 8)]. On day 6 post-OVX, mice received 1 μg of EB (Sigma) in 0.1 ml of sesame oil injected (sc) between 0900 and 0930. Blood samples were collected at 1830 on day 7.

Hormone Assays

Mouse serum LH and FSH were determined using a species-specific immunofluorometric assay, as described previously (27, 60, 66). All immunoassays were performed in a single batch.

Serum levels [diestrus: WT (n = 10), ARKO (n = 8); proestrus: WT (n = 16), ARKO (n = 15)] of estrone (E1), E2, T, and DHT and its two principal metabolites, 5α-androstane-3α,17β-diol (3αdiol) and 5α-androstane-3β,17β-diol (3βdiol), were measured in extracts of 100 μl of mouse serum by liquid chromatography-tandem mass spectrometry (20), as adapted for mouse serum (32). The levels of detection for E1, E2, T, DHT, 3αdiol, and 3βdiol were 0.5 pg/ml, 0.01 ng/ml, 0.03 ng/ml, 0.04 ng/ml, and 0.04 ng/ml, respectively. To characterize T metabolism, the sum of DHT and its two major primary metabolites, 3αdiol and 3βdiol, was calculated.

Kiss1 In Situ Hybridization

In situ hybridization with radiolabeled (35S) Kiss1 riboprobes was performed as described previously (52, 54). The Kiss1-specific cDNA template spanned bases 76-486 of the mouse cDNA sequence (GenBank accession no. AF472576) and was generated by PCR with primers containing promoters for T7 RNA polymerase in the antisense direction and T3 RNA polymerase in the sense direction. Kiss1 riboprobes were applied (55°C for 16 h) and slides dipped in Ilford K5 photographic emulsion (Ilford Imaging), stored at 4°C, and developed 2 wk later.

Kiss1 mRNA-containing cells were identified under dark-field illumination, and image analysis was carried out using randomly coded slides with software designed to count the total number of cells and the number of silver grains per cell (gpc), an index of Kiss1 mRNA content per cell (Image-Pro Plus). Cells were counted when the silver grain density was greater than three times the background. For each animal, the number of Kiss1 mRNA-positive cells grouped by their location was counted [diestrus: WT (n = 5), ARKO (n = 5); proestrus: WT (n = 5), ARKO (n = 6)]. Data are expressed within areas of the brain as the mean number of identifiable cells and the mean number of silver gpc.

Follicle Classification, Enumeration, Growth, and Health

Total numbers of growing follicles per ovary at different developmental stages for each of the WT (control) and ARKO ovaries [WT (n = 5), ARKO (n = 4)] were determined as described previously (36, 64). The follicle classification system was based on the system used by Myers et al. (36). Briefly, follicles classified as small preantral follicles contained an oocyte with 1.5 to two layers of cuboidal granulosa cells, large preantral follicles were classified by having an oocyte surrounded by more than two and up to five layers of cuboidal granulosa cells, small antral follicles contained an oocyte surrounded with more than five layers of cuboidal granulosa cells and/or one or two small areas of follicular fluid, whereas large antral follicles contained a single large antral cavity, and preovulatory follicles possessed a single large antrum and an oocyte surrounded by cumulus...
cells at the end of a stalk of mural granulosa cells. Corpus lutea were identified by morphological properties consistent with luteinized follicles and by being visible throughout several serial sections. Follicles were counted on all serial sections throughout each ovary using an Olympus microscope with Stereo Investigator software (MicroBrightField, Williston, VT). For all histological analysis, repetitive counting of follicles was avoided by counting/measuring only follicles containing an oocyte with a visible nucleolus. To avoid bias, all ovaries were analyzed without knowledge of genotype. Oocyte/follicle diameter ratio was calculated at each developmental stage by measuring diameters of follicles and their contained oocyte in two perpendicular axes using a light microscope at ×20, calibrated using Stereo Investigator computer software (MicroBrightfield). Follicles were classified as unhealthy if they contained a degenerate oocyte and/or >10% of the granulosa cells were pyknotic in appearance, as described previously (59, 64). The proportion of unhealthy follicles/ovary was estimated as the percentage of all follicles at that developmental stage.

Quantitative Real-Time RT-PCR

Total RNA was extracted from whole ovaries at the proestrus stage of the estrous cycle [8–10 wk of age; WT (n = 5), ARKO (n = 6)] using Tri Reagent (Sigma) according to the manufacturer’s protocol. Reverse transcription was performed with total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR analysis of ovarian cDNAs was performed on a Corbett RotorGene 6000 (Corbett Research, Sydney, Australia) using the SensiMix SYBR Kit (Bioline) as recommended and as described previously (64–66). A standard curve was generated for each gene from five serial dilutions of purified PCR product from the same primers designed for quantitative PCR using Wizard DNA Clean-Up System (Promega). Standards (dilutions used for each gene were 10⁻² to 10⁻⁶) were assigned an arbitrary value, and mean relative mRNA expression of samples determined in duplicate was standardized to mouse values, and mean relative mRNA expression of samples determined in triplicate was standardized to mouse Rpl19 values, and mean relative mRNA expression of samples determined in triplicate was standardized to mouse Rpl19 values, and mean relative mRNA expression of samples determined in triplicate was standardized to mouse Rpl19 values, and mean relative mRNA expression of samples determined in triplicate was standardized to mouse Rpl19 values, and mean relative mRNA expression of samples determined in triplicate was standardized to mouse Rpl19 values, and mean relative mRNA expression of samples determined in triplicate was standardized to mouse Rpl19 values, and mean relative mRNA expression of samples determined in triplicate was standardized to mouse Rpl19 values, and mean relative mRNA expression of samples determined in triplicate was standardized to mouse Rpl19 values, and mean relative mRNA expression of samples determined in triplicate was standardized to mouse Rpl19 levels (housekeeping gene), as described previously (64–66). There was no significant differences in Rpl19 mRNA levels between treatment groups (P > 0.05). No template controls, substituting water for cDNA, and a negative reverse transcription were included in each run. Gene expression was detected with specific commercially purchased SYBR Green primers for Lhcgr or the following primers pairs and annealing temperatures: Star (CTTGGCT-GCTCAGTAGTGGC and TGGTGGACAGTCTCACTAAAC, 55°C) and Cyp11a1 (CGAGACTCCTCTCATGGCGAG and CCTTCTCCAG- GCATCTGAAAC, 55°C). Reaction steps were 15 min at 95°C, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s.

Uterine Flushing and Blastocyst Nuclear Staining

Six- to 10-wk-old female mice [WT (n = 60), ARKO (n = 7)] were housed with a proven fertile WT male (AR+/?). Cages were monitored daily for the identification of a copulatory plug. The identification of a plug was recorded as day 1, and 3 days after this female mice were collected and embryos flushed from the uterus using M2 medium (Sigma). The number of corpora lutea present on ovaries, embryo retrieval [(no. of embryos collected/no. of corpora lutea present) × 100], and stages of embryonic development were recorded. Embryo potential was further assessed by collecting blastocysts and determining their total cell numbers, as described previously (42) with modification [WT (n = 49), ARKO (n = 28)]. Blastocysts were washed in Dulbecco’s phosphate-buffered saline solution (PBS; Sigma), fixed in 2% paraformaldehyde-PBS for 30 min, washed in PBS, stained in 8 μg/ml Hoechst 33342 (Sigma) for 10 min, washed in PBS, mounted onto slides, and visualized under UV light.

Statistical Analysis

Statistical analysis was performed using NCSS 2007 software (NCSS Statistical Software, Kaysville, UT). Data that was not normally distributed was transformed prior to analysis using a log transformation. Statistical differences were tested by ANOVA with post hoc test using Fisher’s least significant difference multiple comparison test. All parametric tests were confirmed by the analogous nonparametric tests. The effects of genotype (ARKO, WT) and estrous stage (diestrus, proestrus) on serum sex steroids were analyzed by ANOVA, where all or nearly all samples were detectable (T, 3αdiol), or by Fisher’s test, where a significant proportion of samples were below the limits of detection (DHT, E1, E2, 3αdiol). Fisher’s test compared the proportions of detectable vs. nondetectable samples according to genotype and estrus stage using a Mantel-Haenszel analysis for stratified 2 × 2 tables. Analysis of the effect of genotype at diestrus and proestrus was determined using Kruskal-Wallis one-way ANOVA on Ranks with adjustment for ties, where nondetectable steroid samples were treated as the value set for the limit of detection. P values <0.05 were considered statistically significant.

RESULTS

Serum LH and FSH Levels in Ovary-Intact Mice

Compared with baseline levels (collected at 0900 on the morning of proestrus), the onset of the ovulatory surge in WT females occurred 30 min after lights off at 1830 on the evening of proestrus (P < 0.05) (Fig. 1A). As expected for a normal LH surge, LH levels in WT females were still elevated at 1900. ARKO females exhibited an irregular elevation in LH levels, with a significant premature elevation in LH levels observed at 1730 as well as 1830 compared with baseline LH levels (P < 0.01; Fig. 1B).

Because our mouse colony (as assessed in WT females) exhibited the onset of the ovulatory surge 0.5 h into the dark cycle, at 1830 on the evening of proestrus (P < 0.05; Fig. 1A), this was chosen as the time to assess the natural LH surge in WT and ARKO females at proestrus. Ovulatory surge LH levels were decreased significantly by 49% at proestrus in ARKO females compared with WT females (ARKO: 4.0 ± 1.1 ng/ml, WT: 7.9 ± 1.8 ng/ml, P < 0.05; Fig. 1C). Analysis of LH levels also revealed a significant effect of estrous stage (P < 0.01; Fig. 1C). LH levels at diestrus or FSH levels at diestrus and proestrus were not significantly different between WT and ARKO females (Fig. 1, C and D).

Serum steroid levels in ovary-intact mice

Serum E2 was more frequently detectable in WT (17/26) than ARKO females (8/23) (P < 0.05) and in proestrus than diestrus for both WT (13/16 vs. 4/10; P < 0.05) and ARKO females (8/15 vs. 0/8; P < 0.01). At proestrus, ARKO females had significantly lower levels of E2 than WT females (P < 0.01; Fig. 2A). Serum E1 was undetectable in all samples at diestrus and in ARKO females at proestrus but was more frequently detectable in WT females at proestrus than at diestrus or in ARKO females at proestrus (10/14, both P < 0.01). At proestrus, ARKO females had significantly lower levels of E1 than WT females (P < 0.01; Fig. 2B). Serum T was significantly higher in ARKO than WT females (P < 0.05) and in proestrus vs. diestrus (P = 0.05). There was no significant difference between genotypes at diestrus or proestrus (Fig. 2C). Serum DHT levels were significantly more frequently detectable in ARKO females compared with WT females at diestrus (8/8 vs. 4/10, P < 0.01) but not at proestrus (6/15 vs. 12/16, P = 0.07). Frequency of detectable serum DHT samples did not change between diestrus and proestrus in
WT mice, but the samples were significantly less detectable at proestrus in ARKO mice (8/8 vs. 6/15, P < 0.01). At diestrus, ARKO females had significantly higher levels of DHT than WT females (P < 0.01; Fig. 2D). Serum 3α-diol was significantly higher in ARKO than WT females (P < 0.01) but did not differ according to estrous cycle stage. At diestrus (P < 0.01) and proestrus (P < 0.01), ARKO females had significantly higher levels of 3α-diol than WT females (Fig. 2E). Serum 3β-diol was undetectable in the majority of diestrus samples (2/18) but was more frequently detectable at proestrus (14/31, P < 0.05; Fig. 2F). ARKO females had significantly higher levels of 3β-diol than WT females (P < 0.05; Fig. 2E). ARKO females exhibited an increase in T metabolism, as the combined levels of DHT and its two major primary metabolites 3α-diol (3α-diol; P < 0.01) and 5α-androstane-3β,17β-diol (3β-diol; P < 0.05) (F) at diestrus and proestrus. Diestrus: WT (n = 10), ARKO (n = 8); proestrus: WT (n = 16), ARKO (n = 15). *Significant difference.

**Kiss1 mRNA Expression**

*Kiss1* mRNA was present in the anteroventral periventricular nucleus (AVPV) of all animals (Fig. 3, B–E). In both WT and ARKO mice, the number of *Kiss1*-expressing cells was greater in the afternoon of proestrus compared with diestrus (P < 0.001). During proestrus, there was no difference between ARKO and WT females in the number of *Kiss1* cells (P = 0.06; Fig. 3F). However, *Kiss1* mRNA content per cell was significantly decreased by 39% at proestrus in ARKO females compared with WT females (P < 0.01; Fig. 3G). *Kiss1* mRNA was also present in the arcuate nucleus of all animals, but this did not differ with either the genotype or stage of estrous cycle (no. of *Kiss1* cells; diestrus: WT 215 ± 63 cells, ARKO 186 ± 83 cells; proestrus: WT 166 ± 37 cells, ARKO 150 ± 24 cells; *Kiss1* mRNA content per cell; diestrus: WT 59 ± 10 gpc, ARKO 67 ± 17 gpc; proestrus: WT 58 ± 5 gpc, ARKO 63 ± 10 gpc) (data not shown).

**Serum LH Response to OVX**

To determine the effect of AR loss upon negative feedback, LH response was assessed after OVX for 7 days. Ovariectomized WT females exhibited a significant increase in serum LH (1.7 ± 0.24 ng/ml) compared with basal LH levels in ovari-intact WT mice (0.1 ± 0.03 ng/ml, P < 0.01; Fig. 4A). In contrast, there was no significant difference in LH levels between OVX (0.6 ± 0.15 ng/ml) and ovari-intact ARKO females (0.2 ± 0.04 ng/ml) (Fig. 4A).

**E2-Negative Feedback**

E2 replacement in OVX WT females repressed levels of LH significantly post-OVX (WT OVX, AM: 1.7 ± 0.23 ng/ml; WT OVX + E2, AM: 0.2 ± 0.09 ng/ml, P < 0.01). However, there was no significant reduction in levels of LH after E2 replacement in OVX ARKO females, (ARCO OVX, AM: 0.6 ± 0.14 ng/ml; ARKO OVX + E2, AM: 0.1 ± 0.03 ng/ml) (Fig. 4A).

**E2-Positive Feedback**

Assessment of the LH surge by collection late in the afternoon revealed that both WT and ARKO females (WT OVX +
Fig. 3. Kiss1 mRNA expression in the anteroventral periventricular nucleus (AVPV) during the evening of proestrus. A: the distribution of Kiss1 mRNA expression in a brain map (bregma +0.25 mm), modified with permission from Paxinos and Franklin (40). Kiss1 mRNA-expressing neurons are represented by black circles in a squared region indicating the location of the photomicrographs. mPOA, medial preoptic area; MnPO, median preoptic nucleus; SHy, septohypothalamic nucleus; MS, medial septal nucleus. B–E: representative dark-field photomicrographs showing Kiss1 mRNA-expressing neurons (as reflected by the presence of white clusters of silver grains) in the AVPV from WT and ARKO mice during diestrus (B and C) or late in the afternoon of proestrus (D and E). F and G: open bars, WT mice; black bars, ARKO mice. F: no. of Kiss1 mRNA-expressing cells (P < 0.05). G: Kiss1 mRNA content/cell (P < 0.01). Diestrus: WT (n = 5), ARKO (n = 5); proestrus: WT (n = 5), ARKO (n = 6). Data expressed as means ± SE. Different letters denote statistically significant differences.

E2, AM: 0.2 ± 0.09 ng/ml; WT OVX + E2, PM: 1.3 ± 0.48 ng/ml; ARKO OVX + E2, AM: 0.1 ± 0.03 ng/ml; ARKO OVX + E2, PM: 2.1 ± 0.44 ng/ml, P < 0.01) had significant increases in LH levels compared with females collected on the morning of day 7 (Fig. 4A).

Pituitary Response to Exogenous GnRH

Both WT and ARKO OVX + E2-treated mice given exogenous GnRH responded with a significant elevation of serum LH (WT saline: 0.4 ± 0.09 ng/ml; WT GnRH: 1.1 ± 0.22 ng/ml; ARKO saline: 0.8 ± 0.19 ng/ml; ARKO GnRH: 1.5 ± 0.23 ng/ml, P < 0.05; Fig. 4B).

Ovary Weights, Follicle Populations, Development, and mRNA Expression of Key Markers Involved in Follicle Development

Ovary weight was significantly decreased by 51% at diestrus in ARKO females compared with WT controls (ARKO: 4.2 ± 0.4 mg, WT: 8.6 ± 0.5 mg, P < 0.01) and by 38% at proestrus (ARKO: 3.8 ± 0.2 mg, WT: 6.1 ± 0.4 mg, P < 0.01) (Fig. 5A). At the proestrus stage, ARKO ovaries collected from 6- to 8-wk-old females exhibited a greater than twofold (P < 0.05) decrease in preovulatory follicle numbers (ARKO: 2.3 ± 0.8; WT: 5.2 ± 0.9, P < 0.05) and an approximately fourfold reduction (P < 0.01) in corpora lutea numbers (ARKO: 2.0 ± 0.9; WT: 7.8 ± 1.0, P < 0.01) when compared with WT controls (Fig. 5B). The average number of small and large antral follicles did not differ between ARKO and WT ovaries (Fig. 5B). Real-time RT-PCR analysis on WT and ARKO ovaries collected at proestrus revealed no significant difference between WT and ARKO ovaries in Lhcgr mRNA expression levels or expression of key markers of the shift in steroidogenesis (Star, Cyp11a1; Fig. 5C). Furthermore, ARKO ovaries collected at the proestrus stage exhibited a 143% increase in the percentage of unhealthy large antral follicles present compared with WT ovaries (ARKO: 67.9 ± 11.8, WT: 27.9 ± 11.2, P < 0.05; Fig. 5D). Histological analysis of ovarian sections identified an increase in the number of pyknotic granulosa cells present in ARKO large antral follicles (Fig. 5D). Small antral follicles within ARKO ovaries also exhibited a 15% reduction in oocyte/follicle diameter ratio compared with WT controls (ARKO: 0.3 ± 0.004, WT: 0.4 ± 0.007, P < 0.01; Fig. 5E). Histological analysis of ovarian sections showed that development of the antral cavity appeared delayed in ARKO follicles, with ARKO antral follicles of a similar diameter exhibiting smaller antral cavities compared with WT antral follicles (Fig. 5E).

Embryo Development and Quality

Compared with WT females, ARKO females collected 4 days after the identification of a copulatory plug exhibited a 41% decrease (P < 0.01) in the total number of corpora lutea present (ARKO: 5.7 ± 1.0; WT: 9.7 ± 0.5). There was no significant difference in the percentage of embryos retrieved (ARKO: 81.4% ± 6.0, WT: 69.7% ± 8.0; confirming that of the oocytes ovulated, the majority were collected for assessment), the percentage to have developed to the blastocyst stage (ARKO: 81.2 ± 10.1%, WT: 94.9 ± 4.0%), or the total cell counts per blastocyst (ARKO: 46.0 ± 1.9, WT: 44.8 ± 2.0) between ARKO and WT controls (data not shown).

DISCUSSION

In this study, we reveal that AR-mediated actions are involved in regulating gonadotropin secretion, in particular positive feedback, by ovarian steroids. Furthermore, we demonstrate that AR-mediated actions are involved in optimal antral
of the surge appeared reduced. Previously, in another ARKO mouse model, no differences in hormone levels of E$_2$, progesterone, T, LH, or FSH were identified at the proestrus stage (48). However, unlike in our study, it is not clear when samples were collected during proestrus (48), and hence, the later study may have missed differences in the ovulatory LH surge or the associated surge in serum T (3, 4, 28, 32) at proestrus. Our current findings imply that along with defective late follicle development contributing to the reduced ovulations observed in ARKO females, altered timing, reduced magnitude, and duration of the ovulatory LH surge in ARKO females also play a role.

Expression of Kiss1 mRNA was reduced in the AVPV of ARKO females during the afternoon of proestrus, consistent with the diminished preovulatory surge. Kisspeptin, derived from the Kiss1 gene, is vital for the stimulation of GnRH secretion (51), and its AVPV expression is critical for the GnRH/LH surge in mice (14, 53). Moreover, the surge in E$_2$, observed at proestrus and essential for the release of the LH surge (33), is also significantly decreased in ARKO females compared with WT females. Therefore, the reduced Kiss1 expression and preovulatory LH levels observed in ARKO females at proestrus may be a consequence of a decreased and/or absent E$_2$ surge rather than their response to E$_2$-positive feedback. This is supported by our previous findings that Cyp19a1 (aromatase) expression is significantly decreased in ARKO females (64). Furthermore, as expected with a reduction in the aromatization of androgens to E$_2$, we have also identified an increase in serum levels of androgens in ARKO mice and an increase in testosterone metabolism, as the combined levels of DHT and its two major primary metabolites, 3αdiol and 3βdiol, were significantly increased at proestrus in ARKO females compared with WT females. There is no difference between genotypes in the ability to induce a LH surge after OVX and E$_2$ priming, showing that ARKO females are capable of generating an LH surge in response to an adequate dose of E$_2$. Likewise, the pituitary response to exogenous GnRH that is required for the LH surge (13, 34, 39, 45, 70), does not differ between WT and ARKO females. These findings reveal that the AR has a role in the priming of ovulation. Our results imply that AR regulates negative and positive steroidal feedback mechanisms, in particular the preovulatory E$_2$ surge, which impacts the control of the kisspeptin/GnRH/LH cascade and thus the timing and magnitude of the ovulatory LH surge.

ARKO females in the current study exhibited significantly fewer corpora lutea numbers per ovary, indicative of fewer ovulations. In agreement, we and other groups have shown that ARKO female mice exhibit major disruptions in ovulation rates (25, 48, 64). Previous reports on follicle populations in ARKO ovaries have grouped preovulatory follicles with small and large antral stages (25, 48) or have been carried out at the diestrus stage (64), so specific changes to preovulatory follicles remain undefined. Hence, to determine whether the reduction in ovulation rates in ARKO females is due to reduced numbers of preovulatory follicles present in ARKO ovaries, we assessed follicle populations at the proestrus stage. In the present study, we have revealed that preovulatory follicle numbers within ARKO ovaries are reduced significantly, implying that fewer follicles develop to the final stages of follicle development. Gene expression of LH receptor and key markers of the shift in

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**Fig. 4.** Hormone levels in intact and ovariectomized (OVX) mice with and without E$_2$ treatment. Open bars, WT mice; black bars, ARKO mice. A: LH serum levels in ovary-intact (baseline samples collected between 0800 and 0900) and OVX females ± E$_2$ collected on day 7 post-OVX in the morning (0800–0900) for negative feedback and late afternoon (1830) for positive feedback ($P < 0.01$); $n = 5–8$ females/group/genotype. B: serum LH levels in OVX + E$_2$-treated females given either saline (control) or gonadotropin-releasing hormone (GnRH) between 0800 and 0900 on day 7 post-OVX and collected 10 min later ($P < 0.05$); $n = 6–7$ females/group/genotype. Data expressed as means ± SE. Different letters denote statistically significant differences.

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folic development and health and thus the ripening of the maturing follicle to the preovulatory stage.

Previously, a central role for AR actions in the control of female fertility has been proposed (1, 2, 41, 43, 47, 55, 56, 65), but the precise mechanisms involved remain to be confirmed. An extraovarian defect in ARKO females is indicated by the presence of longer estrous cycles (25, 65) and delayed time to first litter (64). Reciprocal ovary transplantation between ARKO and WT females identified extraovarian defects as playing a role in the observed subfertility of ARKO females (65). In the current study, basal levels of serum LH at diestrus are not different between WT and ARKO females. However, unlike in WT females, following the loss of ovarian feedback by OVX, ARKO females do not exhibit an increase in LH levels. This decrease in the ability of ARKO females to regulate hormonal control implies an AR-dependent damping or desensitizing of the ARKO response to negative feedback signaling by E$_2$.

In the present study, we revealed that intact ARKO females collected at late proestrus exhibit significantly reduced levels of serum LH relative to the normal ovulatory LH surge in WT females. Furthermore, the timing of the emergence of the LH surge in ARKO females was often premature, and the duration
steroidogenesis (Star and Cyp11a1) from E2 to progesterone dominance at proestrus do not differ between genotypes. However, oocyte/follicle diameter ratios are reduced in ARKO small antral follicles, indicating a possible disruption in the connection between oocyte and somatic cells and an altered pattern of follicle growth. Furthermore, ARKO follicles appear to have impaired/delayed antrum development, and there is a significant increase in the percentage of morphologically unhealthy large antral ARKO follicles. Hence, dysfunctional late follicle health may lead to the development of fewer preovulatory follicles and thus reduced E2 levels, which will contribute to the reduced ovulations observed in ARKO females. However, reduced E2 levels may also be due to impaired follicles secreting less E2. In vivo and vitro pharmacological studies have demonstrated that androgens have direct effects on early follicle growth (35, 62, 68, 71) and follicle health (6, 23), with exogenous androgens exerting both inhibitory and stimulatory effects at different follicle developmental stages (reviewed in Ref. 63). Findings from the current study provide direct evidence for AR-mediated actions playing a vital role in maintaining follicle health and normal antral follicle development.

The maintenance of AR expression in cumulus cells of the oocyte cumulus complex (57) and the reduced ability of immature murine oocytes to mature and undergo embryonic development after T exposure (5) suggests a role for AR-mediated action in oocyte viability. Results from the current study show that development to the blastocyst stage is not altered in naturally mated ARKO females, implying that AR-mediated actions do not play a vital role in early embryo development. This is in agreement with our previous findings of unaltered fertilization and progression to the two-cell stage rates in ARKO females (64); hence, the subfertility observed in ARKO females is a consequence of reduced ovulations and not altered embryo quality.

In conclusion, our findings demonstrate that AR-mediated actions play a role in regulating the timing and magnitude of the ovulatory LH surge and thus ovulation rates. Our results imply that AR regulates ovulation priming by mediating negative and positive steroidal feedback mechanisms, which in turn trigger the kisspeptin/GnRH/LH cascade. Furthermore, we have identified that AR actions play a positive role in the late stages of follicle development by maintaining antral follicle health and promoting preovulatory follicle development.

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DISCLOSURES

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

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