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GATA4 Deficiency Impairs Ovarian Function in Adult Mice[1]

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ABSTRACT

Transcription factor GATA4 is expressed in granulosa cells and, to a lesser extent, in other ovarian cell types. Studies of mutant mice have shown that interactions between GATA4 and its cofactor, ZFPM2 (also termed FOG2), are required for proper development of the fetal ovary. The role of GATA4 in postnatal ovarian function, however, has remained unclear, in part because of prenatal lethality of homozygous mutations in the *Gata4* gene in mice. To circumvent this limitation, we studied ovarian function in two genetically engineered mouse lines: C57BL/6 (B6) female mice heterozygous for a *Gata4*-null allele, and 129;B6 female mice in which *Gata4* is deleted specifically in proliferating granulosa cells using the Cre-loxP recombination system and *Amhr2-cre*. Female B6 *Gata4*$^{+/-}$ mice had delayed puberty but normal estrous cycle lengths and litter size. Compared to wild-type mice, the ovaries of gonadotropin-stimulated B6 *Gata4*$^{+/-}$ mice were significantly smaller, released fewer oocytes, produced less estrogen, and expressed less mRNA for the putative GATA4 target genes *Star*, *Cyp11a1*, and *Cyp19*. *Gata4* conditional knockout (cKO) mice had a more severe phenotype, including impaired fertility and cystic ovarian changes. Like *Gata4*$^{+/-}$ mice, the ovaries of gonadotropin-stimulated cKO mice released fewer oocytes and expressed less *Cyp19* than those of control mice. Our findings, coupled with those of other investigators, support the premise that GATA4 is a key transcriptional regulator of ovarian somatic cell function in both fetal and adult mice.

developmental biology, fertility, gene regulation, granulosa cells, ovary, ovulation, puberty, transcription factor

INTRODUCTION

Transcription factor GATA4 is implicated in the development and function of the mammalian ovary [1–3]. GATA4 is expressed in pregranulosa and stroma cells of the fetal ovary and in granulosa and theca cells of the postnatal ovary [4–6]. In adult mice, GATA4 is thought to play a role in the maintenance or maturation of granulosa cells; *Gata4* is expressed in potentially mitotic and proliferating granulosa cells but is downregulated when proliferation ceases during ovulation, apoptosis, or luteinization [4, 7]. Follicle-stimulating hormone (FSH), the gonadotropin that initiates follicular growth, increases both the expression level and the intrinsic activity of GATA4 in granulosa cells [4, 8, 9]. Conversely, loss-of-function mutations in the FSH receptor are associated with reduced ovarian expression of GATA4 in both mice [10] and humans [11]. GATA4 binds and activates genes essential for ovarian steroidogenesis, including *Star* [12, 13], *Cyp11a1* [14], and *Cyp19* (aromatase) [9, 15–18].

Analysis of genetically engineered mice has shown that interactions between GATA4 and its cofactor, ZFPM2 (also termed FOG2), are necessary for early ovarian development [3, 19]. *Zfpm2*$^{-/-}$ mice and *Gata4*$^{ki/ki}$ mice, which bear a knockin mutation that abrogates the interaction of GATA4 with ZFPM2, exhibit identical fetal ovarian phenotypes [3, 19] that include decreased expression of genes essential for early ovarian development, such as *Wnt4* [20] and *Foxl2* [21]. The ovaries of *Zfpm2*$^{-/-}$ and *Gata4*$^{ki/ki}$ fetal mice also express excess DKK1, a secreted inhibitor of the canonical WNT/β-catenin pathway, a signaling cascade critical for female gonadogenesis [3, 19].

Although GATA4 appears to be essential for proper fetal ovarian development, the role of this transcription factor in postnatal ovarian function remains unclear, in part because of prenatal lethality of homozygous mutations in the *Gata4* gene in mice [22–27]. Adult transgenic mice expressing a tetracycline-inducible small interfering RNA (siRNA) directed against GATA4 appear to have intact ovarian steroidogenesis, insofar as circulating levels of FSH and luteinizing hormone (LH) are normal [28]. Approximately 10% of GATA4 siRNA transgenic mice develop ovarian teratomas, suggesting that GATA4 functions as a tumor suppressor in this tissue [28].

In the present study, we examine the impact of GATA4 deficiency on ovarian physiology using two complementary mouse models: C57BL/6 (B6) female mice heterozygous for a germline deletion mutation in *Gata4*, and 129;B6 female mice in which *Gata4* is conditionally ablated in proliferating granulosa cells using the Cre-loxP recombination system.

MATERIALS AND METHODS

Experimental Mice

Procedures involving mice were approved by the Washington University Institutional Committee for Laboratory Animal Care and were conducted in

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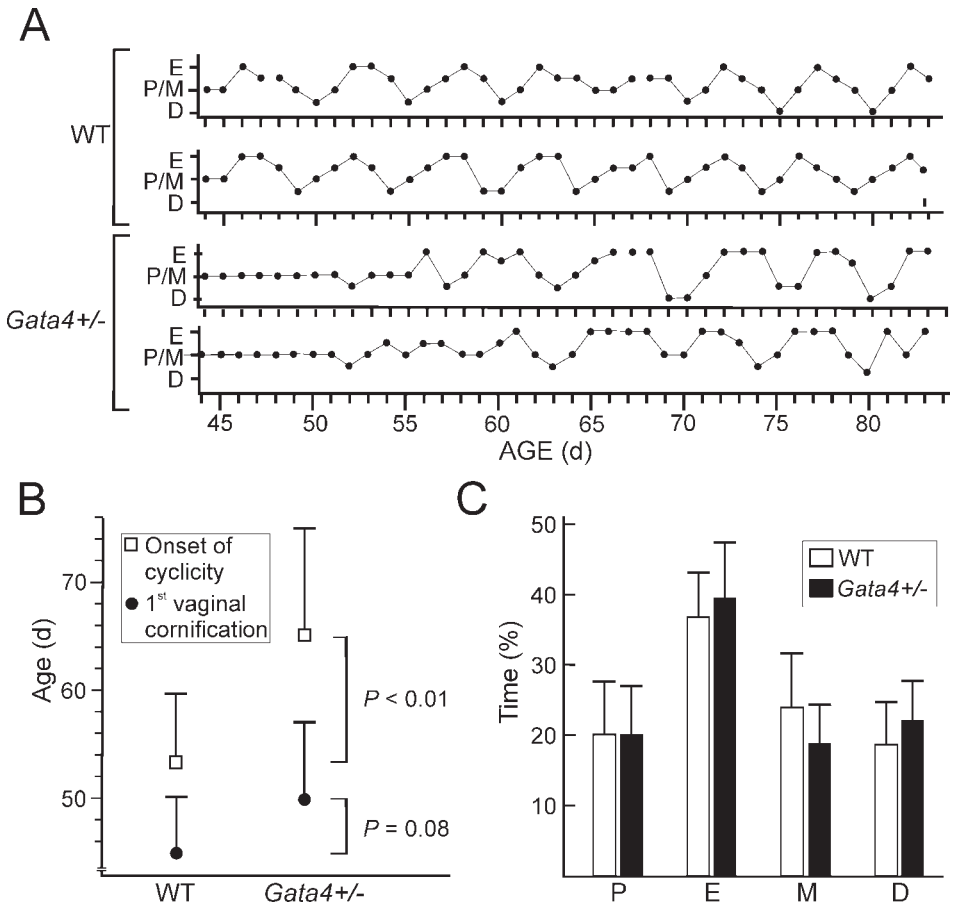

FIG. 1. Delayed puberty in B6 *Gata*$^{+/-}$ female mice. **A**) Representative estrous cycles from WT and *Gata*$^{+/-}$ mice. **B**) Average ages for first vaginal cornification and the onset of cyclicity. Note the significant delay in the onset of cyclicity in *Gata*$^{+/-}$ mice. **C**) Proportion of time spent in each estrous stage (n = 10 for each genotype). Error bars represent SD. D, diestrus; E, estrus; M, metestrus; P, proestrus.

accordance with the National Research Council publication *Guide for Care and Use of Laboratory Animals*. All mice had free access to water and standard rodent chow and were exposed to 12L:12D photoperiods. *Gata4*$^{+/-}$ mice, originally produced by William Pu, were genotyped as described previously [29, 30]. These mice were backcrossed with B6 mice (Charles River Laboratories) for a minimum of 10 generations. *Gata4*$^{Flox/Flox}$ mice (also termed *Gata4*$^{tm1.1Sad}$/J), originally generated by Stephen Duncan [27, 31], were purchased from the Jackson Laboratory and genotyped as described previously [27, 31]. The 129;B6 *Amhr2*$^{cre/+}$ mice (also termed B6;129S7-*Amhr2*$^{tm3(cre)Bhr}$/Mmnc), originally produced by Richard Behringer, were obtained from the Mutant Mouse Regional Resource Centers (MMRRC) and genotyped as described previously [32, 33]. *ROSA26* Flox-stop-Flox *LacZ* reporter mice (also termed B6.129S4-*Gt(ROSA)26Sor*^{tm1Sor}/J), developed by P. Soriano [34], were obtained from the Jackson Laboratory. To generate conditional knockout (cKO) mice, *Gata4*$^{Flox/Flox}$ mice were mated with *Amhr2*$^{cre/+}$ mice, and the resultant *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ mice were mated with *Gata4*$^{Flox/Flox}$ mice to produce *Gata4*$^{Flox/Flox}$;*Amhr2*$^{cre/+}$ mice.

Measurement of Pubertal Events

Daily vaginal lavage was begun on the day of vaginal opening and continued for the duration of the present study [35]. Cytologic analysis was performed on air-dried, Giemsa-stained smears. Estrous cycle stage was determined by the presence of nucleated epithelial cells (NEC), cornified epithelial cells (CEC), and polymorphonuclear cells (PMN), with estrus characterized by CEC and some NEC without PMN, metestrus by CEC and PMN, diestrus by PMN with few NEC, and proestrus mainly by NEC and few CEC and PMN. Ambiguous cycle status was ascertained based on determinations for the preceding and subsequent day and were labeled as an intermediate value. The age of first vaginal cornification and the onset of estrous cyclicity were determined as described previously [35]. Cycle length was defined as the time between onsets of estrus.

Fertility Studies

Reproductive performance was assessed by housing female mice with proven male stud mice and measuring litter size over successive pregnancies. Because some *Gata4*$^{+/-}$ mice die within days of birth because of developmental defects in the heart, lungs, or diaphragm [36–38], we assessed litter size on the morning of birth.

Tissue Isolation and Histological Analyses

At specified times, mice were killed by carbon dioxide inhalation. Blood was collect by cardiac puncture. Ovaries or uteri were harvested, fixed overnight in 4% paraformaldehyde in PBS, and embedded in paraffin for routine histology. Paraffin sections (thickness, 5 μm) were stained with hematoxylin-and-eosin or subjected to immunoperoxidase staining with goat anti-mouse GATA4 immunoglobulin (Ig) G (sc-1237; Santa Cruz Biotechnology, Inc.) at a 1:200 dilution. The secondary antibody employed for immunoperoxidase staining was donkey anti-goat biotinylated IgG (Jackson ImmunoResearch) at a 1:1000 dilution. The avidin-biotin immunoperoxidase system (Vectastain Elite ABC Kit; Vector Laboratories, Inc.) and diaminobenzidine (Sigma-Aldrich Corp.) were used to visualize the bound antibody; slides were then counterstained with toluidine blue. For X-gal staining, frozen tissue sections (thickness, 10 μm) were prepared after embedding mouse ovaries in O.C.T. (Tissue-Tek). Sections were fixed with 0.2% glutaraldehyde for 10 min; permeabilized with 100 mM potassium phosphate (pH 7.4), 0.02% NP-40, and 0.01% sodium deoxycholate for 5 min; and then incubated in 0.5 mg/ml of X-gal (Sigma-Aldrich Corp.) with 10 mM $K_3[Fe(CN)_6]$, 10 mM $K_4[Fe(CN)_6]$, 100 mM potassium phosphate (pH 7.4), 0.02% NP-40, and 0.01% sodium deoxycholate at 37°C overnight [25]. X-gal-stained sections were counterstained with eosin.

TABLE 1. Normal litter size in *Gata4*$^{+/-}$ female mice.[a]

Genotype	Litter no. 1	Litter no. 2	Litter no. 3
WT	6.0 ± 1.9 (n = 8)	6.6 ± 2.1 (n = 8)	7.4 ± 1.2 (n = 7)
Gata4$^{+/-}$	5.7 ± 1.4 (n = 6)	7.0 ± 2.2 (n = 6)	6.8 ± 0.8 (n = 5)

[a] None of the differences was statistically significant.

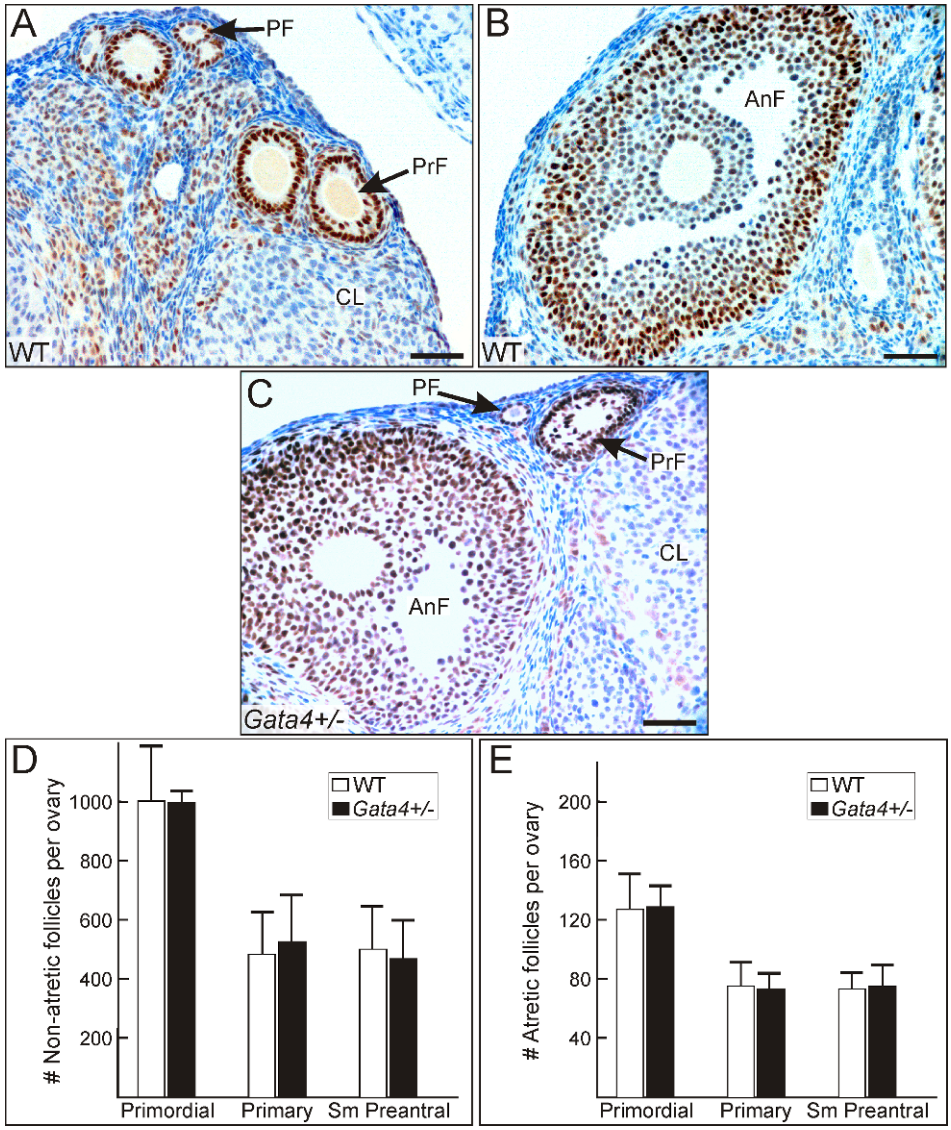

FIG. 2. Ostensibly normal follicular development in the ovaries of randomly cycling adult *Gata*$^{+/-}$ mice. **A**–**C**) Ovaries from 3-mo-old WT (**A** and **B**) or *Gata4*$^{+/-}$ (**C**) mice were subjected to immunoperoxidase staining to assess ovarian morphology and to visualize the distribution of GATA4 within this tissue. Note that GATA4 is expressed in granulosa and theca cells associated with primordial, preantral, and antral follicles of both WT and *Gata4*$^{+/-}$ mice. Bar = 75 μm. **D** and **E**) Histomorphometric analysis of follicle development in WT (n = 10) and *Gata4*$^{+/-}$ (n = 6) mice. Serial ovarian sections from 7-wk-old WT and *Gata4*$^{+/-}$ mice were processed, and the numbers of healthy (nonatretic; **D**) or atretic (**E**) follicles were estimated. Error bars represent SD. AnF, antral follicles; CL, corpora lutea; PF, primordial and primary follicles; PrF, preantral follicles.

Ovarian Follicle Counts

Oocyte-containing follicles were scored as described previously [39, 40]. Briefly, ovaries were collected from mice shortly after the onset of puberty, fixed (0.34 N glacial acetic acid, 28% ethanol, and 10% formalin), embedded in paraffin, serially sectioned (thickness, 6 μm), and stained with hematoxylin-picric acid methyl blue. The number of ostensibly healthy and atretic primordial, primary, and small preantral follicles was determined in every fifth section through the entire ovary. Primordial follicles contained a compact oocyte enveloped by a single layer of flattened granulosa cells. Primary follicles had an enlarged oocyte surrounded by a single layer of cuboidal granulosa cells. Small preantral follicles had an enlarged oocyte surrounded by two to four layers of cuboidal granulosa cells but no visible antrum. Only those follicles in which the oocyte nucleus was visible were scored, and follicles were designated atretic if the oocyte was degenerated, using criteria delineated elsewhere [39, 40]. The total number of healthy and atretic follicles per ovary was then calculated as described previously [39, 40].

Gonadotropin and Estradiol Treatment

Immature (age, 19–25 days) mice were induced to ovulate using equine chorionic gonadotropin (eCG; 5 IU i.p.), followed by human chorionic gonadotropin (hCG; 5 IU i.p.) 48 h later. Four treatment groups were analyzed: 1) no stimulation; 2) eCG for 48 h (follicular growth); 3) eCG for 48 h, followed by hCG for 18 h (postovulation); and 4) eCG for 48 h, followed by hCG for 5 days (luteal glands). Harvested ovaries were processed for histological analysis or RNA isolation. To quantify ovulation, mice were killed 18 h after hCG administration, and oocytes were isolated from oviducts [41].

RNA Isolation and First-Strand cDNA Synthesis

Total RNA was isolated from ovaries using TRIzol (Invitrogen). First-strand cDNA was produced with the SuperScript VILO cDNA Synthesis Kit (Invitrogen) using 700 ng of RNA as a template. An aliquot (1.5 μl) of cDNA was subjected to real-time RT-PCR using SYBR GreenER qPCR SuperMix (Invitrogen). Conditions for all quantitative RT-PCR (qRT-PCR) reactions were optimized in a light cycler (Stratagene Mx3005), which was programmed as follows: denaturation at 95°C for 10 min, followed by 40 cycles of amplification and quantification at 95°C for 30 sec and 60°C for 1 min with single fluorescent measurement, followed by melting at 60–95°C with a heating rate of 0.1°C/sec and continuous fluorescence measurement, followed by a final cooling step to 55°C. Melting curves did not reveal any significant contamination. The relative expression of target genes was calculated using the relative standard curve method as described in *Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR* (Applied Biosystems) and in our prior publication [42].

To compensate for variation among runs, the target gene expression was normalized to the expression of ribosomal protein L19. Primer pairs used for qRT-PCR assays were as follows: *L19*, NM_005084, (forward) 5′-GAAATCGCCAATGCCAACTC-3′, (reverse) 5′-TCTTAGACCTGCGAGCCTCA-3′, 405 bp; *Cyp19*, NM_007810, (forward) 5′-ATGTTCTTGGAAATGCTGAACCC-3′, (reverse) 5′-AGGACCTGGTATTGAAGACGAG-3′, 150 bp; *Star*, NM_011485, (forward) 5′-GCAGCAGGCAACCTGGTG-3′, (reverse) 5′-TGATTGTCTTCGGCAGCC-3′, 246 bp; *Cyp11a1*, NM_19779.3, (forward) 5′-CGATACTCTTCTCATGCGAG-3′, (reverse) 5′-CTTTCTTCCAGGCATCTGAAC-3′, 125 bp; *Gata4*, NM_008092.3, (for-

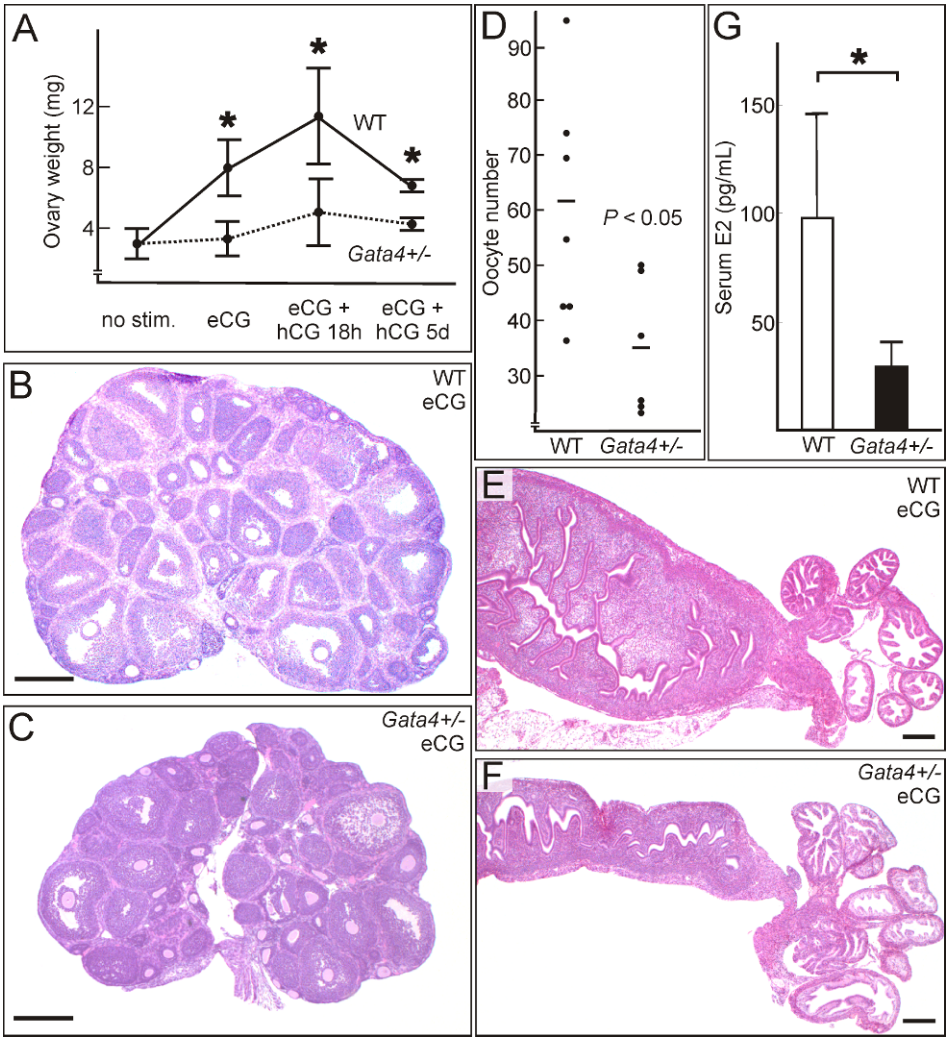

FIG. 3. Impaired ovarian response to gonadotropins in *Gata*$^{+/-}$ mice. **A**) Weanling WT (solid line) or *Gata*$^{+/-}$ (dashed line) mice were treated with one of the following regimens: 1) no stimulation; 2) eCG for 48 h; 3) eCG for 48 h, followed by hCG for 18 h; or 4) eCG for 48 h, followed by hCG for 5 days. Ovaries were harvested and weighed (n = 8 for each genotype). *$P < 0.05$. Error bars represent SD. **B** and **C**) Tissue sections of WT (**B**) and *Gata*$^{+/-}$ (**C**) ovaries 48 h after eCG stimulation. The *Gata*$^{+/-}$ ovary is smaller and contains fewer antral follicles. Bar = 0.3 mm. **D)** Oocyte yields after gonadotropin-induced ovulation. WT and *Gata*$^{+/-}$ mice were treated with eCG, followed 48 h later by hCG. Oocytes were harvested from oviducts and counted 16 h after hCG treatment. Each data point represents the oocyte yield of an individual mouse. Horizontal lines represent mean values. **E** and **F**) Sections of WT (**E**) and *Gata*$^{+/-}$ (**F**) uteri 48 h after eCG stimulation. Note the hypoestrogenic appearance of the heterozygous uterus. Glandular elements of the endometrial layer are less complex, and only scattered glands are detected in the stroma. No difference was found in mean body weight between the WT and *Gata*$^{+/-}$ mice (13.8 ± 1.6 and 14.4 ± 1.6 g, respectively). Bar = 0.3 mm. **G**) Serum E_2 levels in WT (white bar, n = 7) and *Gata*$^{+/-}$ (black bar, n = 7) uteri 48 h after eCG stimulation. *$P < 0.05$. Error bars represent SD.

ward) 5′-CCCTACCCAGCCTACATGG-3′, (reverse) 5′-ACATATCGAGATTGGGGTGTCT-3′, 138 bp.

Semiquantitative RT-PCR for Detection of Cre-Mediated Recombination

First-strand cDNA was subjected to RT-PCR using a forward primer from exon 2 of the *Gata4* gene, 5′-CCCTACCCAGCCTACATGG-3′, and a reverse primer from exon 7 of the same gene, 5′-GAGCTGGCCTGCGATGTCTGAGTG-3′, and the following reaction conditions: denaturation at 95°C for 10 min; followed by 35 cycles of amplification and quantification at 95°C for 30 sec, 55°C for 1 min, and 72°C for 1 min; followed by a final cooling step to 55°C. The intact *Gata4*^{Flox} allele gave rise to a 782-bp band, whereas the recombined allele yielded a 401-bp band, reflecting deletion of exons 3–5.

Hormone Measurements

Serum estradiol (E_2) levels were determined using a commercial radioimmunoassay (BioCheck). Serum antimüllerian hormone (AMH) levels were measured by ELISA as described elsewhere [43].

In Situ Hybridization

Tissue cryosections (thickness, 10 μm) were processed and subjected to mRNA in situ hybridization as described previously [25]. The sections were incubated with 1.2×10^6 cpm [^{33}P]αUTP-labeled (1000–3000 Ci/mmol; Amersham) antisense riboprobe in a total volume of 80 μl. The riboprobe for mouse GATA4 was prepared as described previously [25].

Statistical Analyses

The effects of genotype on the age of first vaginal cornification, onset of estrous cyclicity, estrous cycle length, litter size, ovarian weight, uterine weight, steroid hormone levels, and prevalence of infertility were determined by unpaired, two-tailed *t*-tests. Statistical significance was set at $P < 0.05$. Two-way ANOVA was used to analyze the proportion of time in each estrous stage, follicle counts, and gene expression levels. Two-population-proportion testing was used to analyze the incidence of cystic ovarian changes. All numerical data are presented as the mean ± SD.

RESULTS

Delayed Puberty but Normal Estrous Cycle Length and Litter Size in Gata4$^{+/-}$ *Female Mice*

We first analyzed B6 mice heterozygous for a deletion in exon 2 of the *Gata4* gene [29]. This germline mutation removes the translation start site and N-terminal activation domain of GATA4. Studies have shown that mice heterozygous for this allele, hereafter referred to as *Gata4*$^{+/-}$ mice, express wild-type (WT) *Gata4* mRNA at half-normal levels in heart and other tissues in which this transcription factor is expressed [29, 36].

Vaginal cytology specimens were analyzed to determine the impact of *Gata4* haploinsufficiency on the timing of two pubertal events: the first vaginal cornification and the onset of estrous cyclicity [44]. Previous studies have shown that vaginal

cornification is dependent on an increase in circulating E_2, whereas the onset of estrous cyclicity is dependent on both a preovulatory increase in plasma E_2 and the ability of the pituitary to respond with a preovulatory surge of LH, which stimulates ovulation and luteinization [44]. Representative estrous cycles from WT and *Gata4*$^{+/-}$ females are shown in Figure 1A. The onset of vaginal cornification appeared delayed in *Gata4* heterozygotes compared to WT mice (50 ± 6.8 vs. 45 ± 5.5 days, n = 10 per group), although this difference did not reach statistical significance (Fig. 1B). The onset of estrous cyclicity was delayed an average of 12 days in *Gata4*$^{+/-}$ females compared with WT females ($P < 0.01$) (Fig. 1B). Average cycle length, however, did not differ between *Gata4*$^{+/-}$ and WT females (5.3 ± 0.5 and 5.4 ± 0.4 days, respectively), nor did *Gata4*$^{+/-}$ and WT mice differ in the proportion of time spent in any stage of the estrous cycle (Fig. 1C). The timing of puberty is known to correlate with mouse growth [44]. WT and *Gata4*$^{+/-}$ female mice had similar growth curves, implying that the delay in puberty in *Gata4*$^{+/-}$ females was not the result of impaired growth (data not shown). We conclude that *Gata4* is a genetic determinant of the onset of puberty in female B6 mice.

The reproductive performance of *Gata4*$^{+/-}$ female mice was assessed by mating 2-mo-old WT or *Gata4*$^{+/-}$ female mice with normal males and measuring litter size over successive pregnancies. No differences were found between WT and *Gata4*$^{+/-}$ mice in either litter size (Table 1) or the frequency of parturition (data not shown).

Ovaries from young adult mice were examined to determine whether *Gata4* haploinsufficiency disrupts follicular development. In both WT (Fig. 2, A and B) and *Gata4*$^{+/-}$ (Fig. 2C) females, nuclear GATA4 immunoreactivity was evident in theca cells and in granulosa cells of primary, preantral, and antral follicles (Fig. 2, A–C), but not in atretic follicles or corpora lutea (Fig. 2, A and C). Consistent with a published report [45], no significant differences were found between WT and *Gata4*$^{+/-}$ ovaries in the abundance of primordial, primary, or small preantral follicles (healthy or atretic) (Fig. 2, D and E).

Ovaries of Gata4$^{+/-}$ *Mice Exhibit an Impaired Response to Exogenous Gonadotropins*

Immature WT and *Gata4*$^{+/-}$ female mice were treated with eCG, which mimics the effect of FSH, followed by hCG, which mimics the effect of LH, to induce synchronized follicular growth and ovulation. Unstimulated WT and *Gata4*$^{+/-}$ ovaries had similar weights, whereas gonadotropin-stimulated WT ovaries weighed significantly more than their *Gata4*$^{+/-}$ counterparts (Fig. 3A). Histological analysis confirmed that ovaries of eCG-stimulated WT mice (Fig. 3B) were consistently larger than those of *Gata4*$^{+/-}$ mice (Fig. 3C).

In response to superovulation, *Gata4*$^{+/-}$ mice released significantly fewer oocytes into the oviducts than WT mice (Fig. 3D). Thus, *Gata4* haploinsufficiency decreases oocyte release in response to exogenous gonadotropins without impairing fertility.

Compared to WT mice, the uteri of eCG-stimulated *Gata4*$^{+/-}$ mice weighed significantly less (0.32 ± 0.05 [n = 8] vs. 0.20 ± 0.4 [n = 7] mg/g body weight, $P < 0.05$) and appeared to be hypoestrogenic (Fig. 3, E and F). Following eCG stimulation, serum E_2 levels were significantly greater in WT mice than the *Gata4*$^{+/-}$ mice (Fig. 3G). In contrast, basal serum E_2 levels did not differ significantly between WT (8.8 ± 1.1 pg/ml) and *Gata4*$^{+/-}$ mice (9.5 ± 0.3 pg/ml).

Quantitative RT-PCR was used to measure the relative expression of transcripts for *Gata4* (Fig. 4A) and three of its putative target genes, *Star* (Fig. 4B), *Cyp11a1* (Fig. 4C), and *Cyp19* (Fig. 4D), in ovaries from gonadotropin-stimulated immature WT and *Gata4*$^{+/-}$ mice. To confirm a reduction in *Gata4* expression in the heterozygous ovaries, we performed qRT-PCR using primers specific for the WT *Gata4* mRNA. The ovaries of unstimulated and gonadotropin-stimulated *Gata4*$^{+/-}$ mice contained approximately half the amount of *Gata4* mRNA (normalized to the housekeeping gene *L19*) as their WT counterparts (Fig. 4A). Basal levels of mRNA for *Star* (Fig. 4B), *Cyp11a1* (Fig. 4C), and *Cyp19* (Fig. 4D) were similar in WT and *Gata4*$^{+/-}$ mice. Following gonadotropin stimulation, however, the levels of *Star*, *Cyp11a1*, and *Cyp19* mRNA were significantly higher in WT than in *Gata4*$^{+/-}$ ovaries. The target gene most profoundly affected by *Gata4* haploinsufficiency was *Cyp19*, the key gene of estrogen

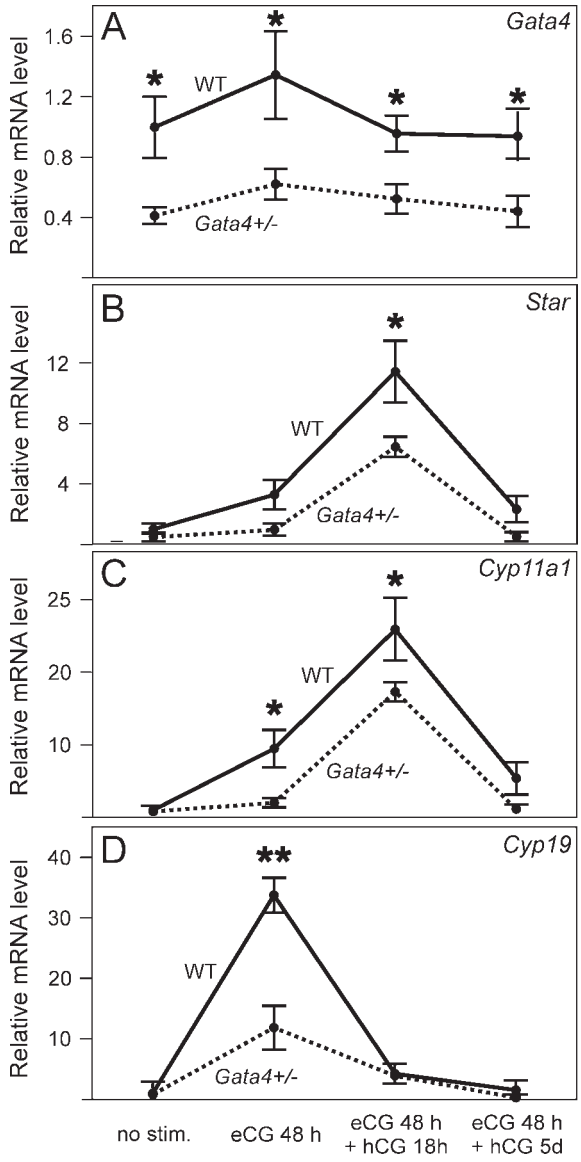

FIG. 4. Reduced expression of mRNA for *Gata4* and steroidogenic factors in the ovaries of gonadotropin-stimulated *Gata*$^{+/-}$ mice. Weanling WT (solid line, n = 5) or *Gata*$^{+/-}$ (dashed line, n = 5) mice were treated with one of the following regimens: 1) no stimulation; 2) eCG for 48 h; 3) eCG for 48 h, followed by hCG for 18 h; or 4) eCG for 48 h, followed by hCG for 5 days. Ovarian RNA was subjected to qRT-PCR for *Gata4* (**A**), *Star* (**B**), *Cyp11a1* (**C**), and *Cyp19* (**D**). Error bars represent SD. *$P < 0.05$, **$P < 0.01$.

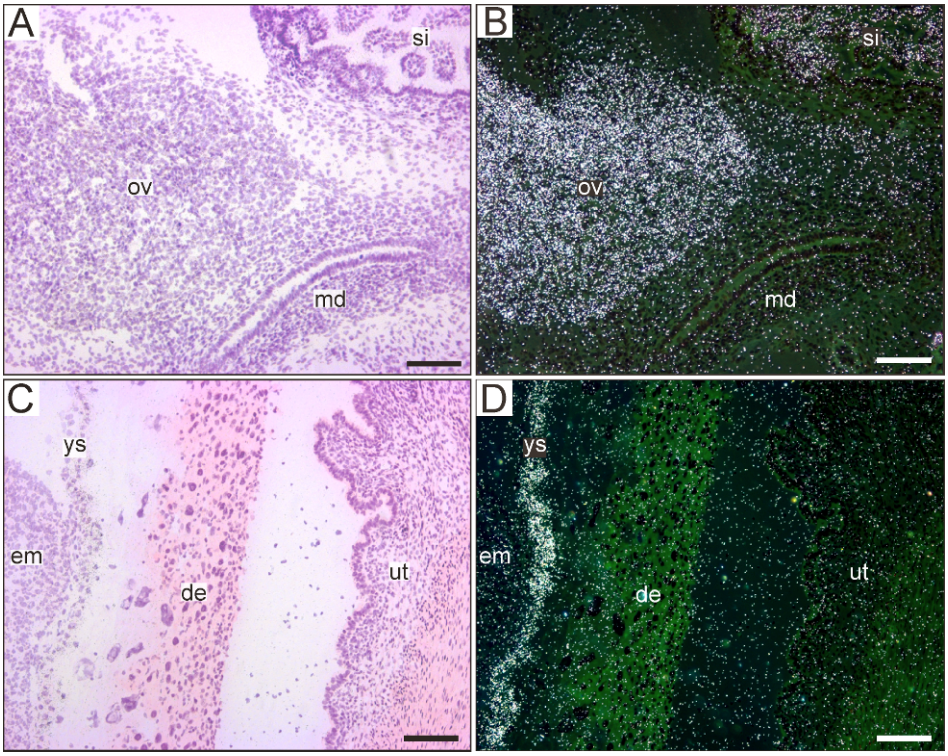

FIG. 5. *Gata4* mRNA is not expressed in the müllerian duct or adult mouse uterus. Cryosections of Embryonic Day 18.5 mouse embryo (**A** and **B**) and Embryonic Day 9.5 gravid uterus (**C** and **D**) were subjected to in situ hybridization for GATA4 using radiolabeled riboprobe. Shown are corresponding bright-field (**A** and **C**) and dark-field (**B** and **D**) photomicrographs. Note that *Gata4* mRNA is expressed in fetal ovary (ov) and in endodermal cells of the small intestine (si) and yolk sac (ys), but not in the müllerian duct (md), embryonic ectoderm (em), decidua (de), or uterus (ut). Bar = 100 μm.

biosynthesis. Previous studies have shown that CYP19 is essential for follicular growth and coordination of the ovulatory process [46–48].

Conditional Deletion of Gata4 *in Granulosa Cells*

To further study the physiological function of GATA4 during folliculogenesis, we used the Cre-loxP recombination system to facilitate granulosa cell-specific deletion of the *Gata4* gene. We hypothesized that conditional deletion of both *Gata4* alleles in granulosa cells would produce a more severe reproductive phenotype than germline deletion of a single allele. The 129;B6 mice homozygous for a floxed allele of *Gata4* were intercrossed with 129;B6 $Amhr2^{cre/+}$ mice. Previous studies have shown that Cre-mediated excision of the region between exons 3 and 5 of the *Gata4* gene converts the floxed allele into a recombined allele no longer capable of encoding a functional GATA4 protein [27, 31]. The *Amhr2-cre* knockin transgene has been widely used to target gene deletion in proliferating granulosa cells and in the mesenchyme-derived cells of the müllerian duct, oviduct, and uterus [33, 49–58]. Because GATA4 is not expressed in the müllerian duct (Fig. 5, A and B), oviduct [59], or adult uterus (Fig. 5, C and D), Cre-mediated deletion of the *Gata4* gene should not have a direct impact on these tissues.

ROSA26 flox-stop-flox *lacZ* reporter (*R26R*) mice were used to assess the expression of *Amhr2-cre* within ovaries. Consistent with published reports [33, 50, 52], the *Amhr2-cre* transgene directed expression of Cre to granulosa cells in secondary follicles (Fig. 6A). Interestingly, Cre expression within secondary follicles was not uniform, as evidenced by the presence of both lacZ-positive (Fig. 6A, arrow) and lacZ-negative (Fig. 6A, arrowhead) granulosa cells. Such variegated Cre expression may contribute to the variable penetrance described below.

Intercrosses of $Gata4^{Flox/Flox}$ mice and $Gata4^{Flox/+}$; $Amhr2^{cre/+}$ mice yielded ($Gata4^{Flox/Flox}$;$Amhr2^{cre/+}$) mice, henceforth referred to as cKO mice, and $Gata4^{Flox/+}$; $Amhr2^{cre/+}$ mice in the expected mendelian ratios. The growth of cKO mice did not differ from that of $Gata4^{Flox/+}$;$Amhr2^{cre/+}$ mice or $Amhr2^{+/+}$ controls (data not shown).

To verify Cre-mediated deletion of the *Gata4* gene in the mouse ovary, we employed an RT-PCR strategy that distinguishes transcripts derived from the intact ($Gata4^{Flox}$) and recombined alleles ($Gata4^{\Delta ex3-5}$). RT-PCR analysis of ovaries from $Gata4^{Flox/Flox}$;$Amhr2^{+/+}$ mice yielded a single band corresponding to the intact allele (Fig. 6B). In contrast, analysis of RNA from the ovaries of cKO mice demonstrated both the intact $Gata4^{Flox}$ allele and the recombined $Gata4^{\Delta ex3-5}$ allele (Fig. 6B). The relative intensities of the $Gata4^{Flox}$ and $Gata4^{\Delta ex3-5}$ bands differed among cKO mice (Fig. 6B). Variability in the extent of Cre-mediated recombination has been observed in other granulosa cell-specific knockout mice made with *Amhr2-cre* and may reflect inefficient recombination in granulosa cells (see [52] and references therein).

To confirm reduced expression of GATA4 in the cKO ovaries, we performed qRT-PCR using primers specific for the WT GATA4 mRNA. The ratio of GATA4 mRNA expression in immature cKO versus $Gata4^{Flox/Flox}$;$Amhr2^{+/+}$ mice was 0.44 ± 0.02 ($P < 0.001$). Moreover, the ratio of GATA4 mRNA expression in immature cKO versus $Gata4^{Flox/+}$; $Amhr2^{cre/+}$ mice was 0.72 ± 0.02 ($P < 0.05$).

Next, we compared the patterns of GATA4 immunostaining in ovaries of postpubertal $Gata4^{Flox/+}$;$Amhr2^{cre/+}$ (Fig. 6C) and cKO (Fig. 6D) mice. In both genotypes, GATA4 protein was detected in theca cells and in granulosa cells of primary, preantral, and antral follicles. The residual GATA4 expression in the immunohistochemical analysis presumably reflects cells in which the *Amhr2-cre* did not fully inactivate both $Gata4^{Flox}$ alleles. Nevertheless, we infer from the reproductive defects described below that GATA4 expression is silenced in enough granulosa cells to elicit a phenotype.

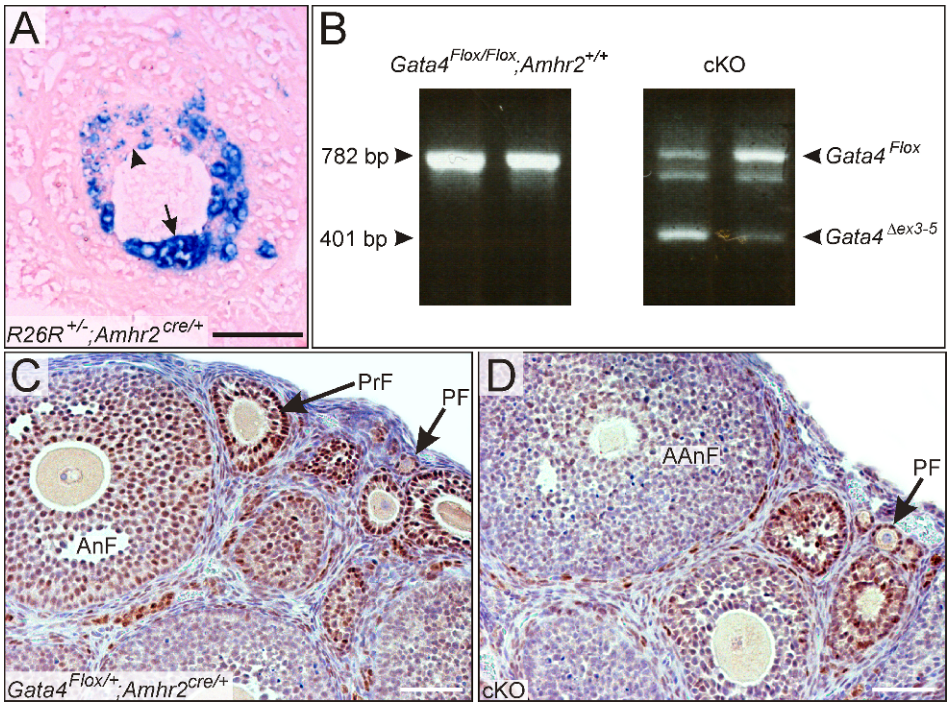

FIG. 6. *Amhr2-cre*-mediated deletion of *Gata4*^{Flox} in the mouse ovary. **A**) *Amhr2*$^{cre/+}$ mice were crossed with homozygous *R26R* mice to generate *R26R*$^{+/-}$;*Amhr2*$^{cre/+}$ mice. Ovaries were cryosectioned and stained with X-gal. Note the variegated pattern of Cre activity in a secondary follicle; both lacZ-positive (arrow) and lacZ-negative (arrowhead) granulosa cells are evident. **B**) Ovarian RNA was isolated from pairs of 2-mo-old control (*Gata4*$^{Flox/Flox}$;*Amhr2*$^{+/+}$) or cKO mice and subjected to RT-PCR analysis with primers that distinguish the intact floxed allele from the recombined allele lacking exons 3–5 (*Gata4*$^{\Delta ex3-5}$). Note that a transcript derived from recombined allele is seen in the cKO mice but not the control mice. The identity the third band in the cKO mice is unclear. **C** and **D**) Ovaries from 7-wk-old *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ (**C**) or cKO (**D**) mice were sectioned and subjected to immunoperoxidase staining to visualize the distribution of GATA4 within this tissue. GATA4 immunoreactivity was seen in granulosa and theca cells of both the *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ and cKO mice. Bar = 75 μm. AAnF, atretic antral follicles; AnF, antral follicles; PF, primordial and primary follicles; PrF, preantral follicles.

Reduced Fertility in Gata4 *cKO Mice*

To assess the impact of conditional deletion of *Gata4* on fertility, female 2-mo-old cKO and *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ mice were housed with proven male studs. Both strains gave birth and raised pups to weanlings, but fertility defects were evident in the cKO females. Six of 18 (33%) cKO females failed to produce offspring, compared to only 1 of 20 (5%) *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ females ($P < 0.05$). Even more striking, the average litter size of cKO mice was reduced ($P < 0.005$) to approximately half that of *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ mice (Table 2).

Although cKO mice were clearly subfertile, no differences were detected in the onset of puberty or in the length of the estrous cycle among cKO mice, *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ mice, and *Amhr2*$^{+/+}$ littermate mice (data not shown). That puberty was delayed in B6 *Gata4*$^{+/-}$ mice (Fig. 1) but not in 129;B6 cKO or *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ mice might reflect background strain influences [35] or the timing/efficiency of Cre-mediated ablation. Alternatively, the B6 *Gata4*$^{+/-}$ mice may have extragonadal defects that delay puberty.

Ovaries of young adult *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ (Fig. 7A) and cKO (Fig. 7B) mice were analyzed to determine the impact of conditional ablation of *Gata4* on follicular development. On gross inspection, the ovaries of young adult *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ and cKO mice were indistinguishable. On histological examination, the number of primordial, primary, and small antral follicles (healthy or atretic) was similar in cKO and *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ ovaries (Fig. 7, C and D).

By 6 mo of age, however, differences were found in the gross and microscopic appearance of ovaries from *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ (Fig. 7, E and G) and cKO (Fig. 7, F and H) mice. Very large, nonhemorrhagic cysts were seen in six of seven (86%) ovaries from cKO mice and in 0 of 10 (0%) ovaries from *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ mice ($P < 0.001$). The cysts were lined by a flattened epithelium containing scattered ciliated cells (data not shown).

Next, we measured serum AMH levels in *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ and cKO mice at different ages (Fig. 7I). Studies of WT mice have shown that serum levels of AMH, a protein secreted by granulosa cells within growing follicles [60], remain relatively constant until 8 mo of age and then decline

TABLE 2. Reduced litter size in B6;129 *Gata4* cKO mice.

Genotype	Litter no. 1	Litter no. 2	Litter no. 3
Gata4$^{Flox/+}$; *Amhr2*$^{cre/+}$	8.0 ± 2.8 (n = 13)[a]	9.4 ± 2.0 (n = 12)	10.3 ± 2.2 (n = 9)
cKO	4.3 ± 2.7* (n = 14)	4.2 ± 2.0** (n = 12)	3.7 ± 2.4* (n = 7)

[a] The litter size of *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ mice did not differ from that of *Amhr2*$^{+/+}$ controls (8.6 ± 3.4 for litter no. 1, n = 5).
* $P < 0.005$; ** $P < 0.001$.

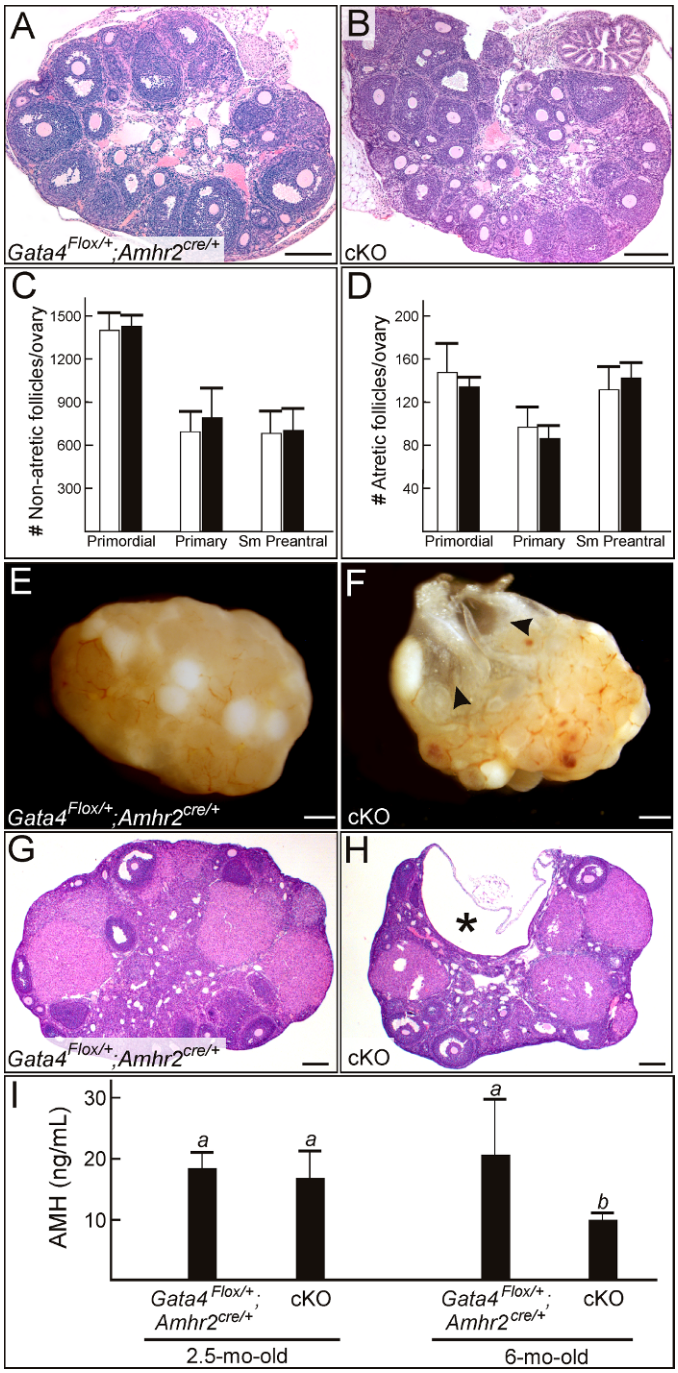

FIG. 7. Morphological and functional abnormalities in the ovaries of cKO mice. **A** and **B**) Hematoxylin-and-eosin-stained sections of ovaries from 7-wk-old *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ and cKO mice, respectively. Bar = 200 μm. **C** and **D**) Histomorphometric analysis of follicle development in 7-wk-old *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ (n = 10) and cKO (n = 6) mice ovaries. Serial ovarian sections were processed, and the numbers of healthy (nonatretic; **D**) or atretic (**E**) follicles were estimated. **E**–**H**) Ovaries from 6-mo-old *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ (**E** and **G**) and cKO (**F** and **H**) mice. The arrowheads and asterisk highlight large cysts in the cKO ovary. Bar = 200 μm. **I**) Serum AMH levels in *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ (n = 3–7) and cKO (n = 5–7) mice of varying ages. Error bars represent SD. The bars denoted by *a* differ significantly ($P < 0.01$) from that denoted by *b* but not from one another.

steadily over the ensuing 10 mo [43]. This age-related decline in serum AMH correlates with decreases in both the number of growing follicles and the size of the primordial follicle pool [43], making AMH a useful marker to quantify ovarian reserve

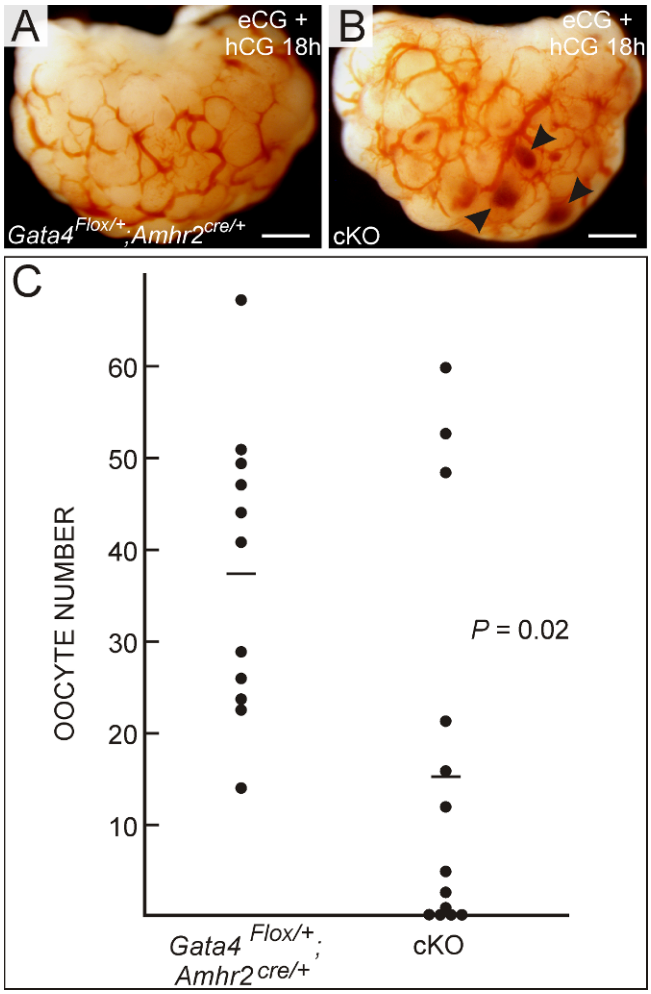

FIG. 8. Aberrant response to superovulation in cKO mice. Immature mice were treated with eCG, followed 48 h later by hCG. Tissue samples were harvested for analysis 18 h after hCG treatment. **A** and **B**) Ovaries from eCG/hCG-treated (**A**) *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ and (**B**) cKO mice. Note the presence of hemorrhagic follicles (arrowheads) in the cKO ovary. **C**) Oocyte yields after superovulation. Each data point represents the oocyte yield of an individual mouse. The horizontal lines represent the mean values. The sample sizes were *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ (n = 11) and cKO (n = 13).

[61]. AMH levels were similar in 2.5-mo-old *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ and cKO mice. In contrast, AMH levels were significantly lower ($P < 0.01$) in 6-mo-old cKO mice. Thus, AMH levels decline prematurely in *Gata4* cKO mice, indicating that ovarian granulosa cell function is compromised. In light of these results, we also measured AMH levels in the serum of older B6 WT and *Gata4*$^{+/-}$ mice but found no significant differences (data not shown).

Gata4 *cKO Mice Exhibit an Impaired Response to Exogenous Gonadotropins*

Next, we assessed the response of immature *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ and cKO mice to superovulation. No significant differences were found in the weight or gross appearance of unstimulated *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ and cKO mice (data not shown). However, the ovaries of cKO mice treated with eCG plus hCG were significantly smaller than those of *Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$ mice (4.8 ± 2.0 mg [n = 10] and 7.9 ± 2.0 mg [n = 8], respectively; $P < 0.05$) and had hemorrhagic follicular

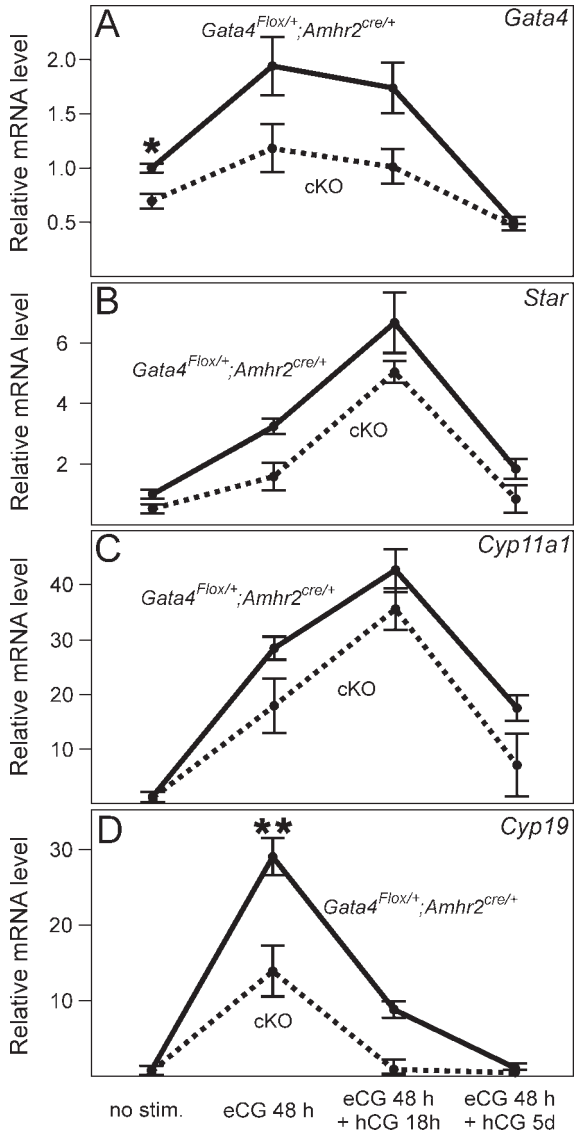

FIG. 9. Reduced expression of mRNA for *Gata4* and steroidogenic factors in the ovaries of gonadotropin-stimulated cKO mice. Weanling $Gata4^{Flox/+};Amhr2^{cre/+}$ (solid line, n = 5) or cKO (dashed line, n = 5) mice were treated with one of the following regimens: 1) no stimulation; 2) eCG for 48 h; 3) eCG for 48 h, followed by hCG for 18 h; or 4) eCG for 48 h, followed by hCG for 5 days. Ovarian RNA was subjected to qRT-PCR for *Gata4* (**A**), *Star* (**B**), *Cyp11a1* (**C**), and *Cyp19* (**D**). Error bars represent SD. $*P < 0.05$, $**P < 0.01$.

cysts on their surface (Fig. 8, A and B). A significant decrease ($P < 0.05$) was observed in the yield of oocytes from cKO mice compared with $Gata4^{Flox/+};Amhr2^{cre/+}$ mice (Fig. 8C), although considerable variability occurred in the response of the cKO mice. One-third of the cKO mice tested released no oocytes following superovulation. Collectively, these data show that the *Gata4* granulosa cell-specific knockout mice are subfertile and have an impaired response to gonadotropin-induced superovulation.

Quantitative RT-PCR was used to measure the relative expression of transcripts for *Gata4* (Fig. 9A) and its target genes *Star* (Fig. 9B), *Cyp11a1* (Fig. 9C), and *Cyp19* (Fig. 9D) in ovaries from gonadotropin-stimulated, immature $Gata4^{Flox/+};Amhr2^{cre/+}$ and cKO mice. Basal levels of mRNA for *Star*, *Cyp11a1*, and *Cyp19* were similar in $Gata4^{Flox/+};Amhr2^{cre/+}$ and cKO mice. Following eCG stimulation, however, the level of *Cyp19* mRNA was significantly higher in $Gata4^{Flox/+};Amhr2^{cre/+}$ than in cKO ovaries. Additionally, a trend, albeit statistically insignificant, was observed toward decreased expression of *Star* and *Cyp11a1* in the ovaries of gonadotropin-stimulated cKO mice. We did not, however, detect a significant difference in the serum E_2 levels of eCG-treated juvenile mice.

DISCUSSION

Expression of GATA4 occurs in potentially mitotic and proliferating granulosa cells, but expression is lost once these cells become either terminally differentiated (from granulosa to luteal cells) or apoptotic during follicular atresia [4, 7]. On the basis of these expression patterns, it was suggested [4] that GATA4 might control genes involved in the maintenance or maturation of granulosa cells within early follicles (i.e., before ovulation). Consistent with its proposed role in granulosa cell maturation, the expression level of GATA4 in granulosa cells was subsequently shown to be enhanced by FSH, and the transcriptional activity of GATA4 was found to be induced by phosphorylation via signaling through the FSH receptor and the downstream mediators cAMP and protein kinase A [8, 9]. The abrupt decrease in GATA4 associated with ovulation or apoptosis led investigators to propose that this factor is not required for the later stages of follicular development, apoptosis, or luteinization, although GATA4 might serve to prime early follicular cells for the transition to late maturation or apoptosis [4]. In keeping with these previous observations and predictions, the present data demonstrate that germline and conditional deletions in the *Gata4* gene cause defects in granulosa cell function that impair ovarian function in adult mice.

The B6 $Gata4^{+/-}$ female mice were fertile but had delayed puberty and a blunted ovarian response to exogenous gonadotropins; the ovaries of gonadotropin-stimulated $Gata4^{+/-}$ mice were smaller, released fewer oocytes, and expressed less mRNA for the steroidogenic genes *Star*, *Cyp11a1*, and *Cyp19* (Table 3). In the absence of a fertility defect, the physiological significance of a reduced response to ovarian superstimulation is arguable. On the other hand, our data show that provocative stimulation can be used to highlight ''subclinical'' defects in hormonal responsiveness that shed light on the function of GATA4 in vivo. The steroidogenic gene most profoundly affected by *Gata4* haploinsufficiency was *Cyp19*, the key gene of estrogen biosynthesis and a known target for activation by GATA4 [8, 9]. Consistent with reduced *Cyp19* expression, serum E_2 levels were significantly reduced in $Gata4^{+/-}$ mice (Fig. 3G), and the uteri of eCG-stimulated $Gata4^{+/-}$ mice appeared to be hypoestrogenic. Like $Gata4^{+/-}$ mice, *Cyp19* knockout mice have impaired ovulation and hypoestrogenic uteri [46–48], suggesting that the abnormal phenotype of gonadotropin-stimulated *Gata4* haploinsufficient mice may reflect, at least in part, attenuated *Cyp19* expression and a concomitant decrease in E_2 production. We speculate that the delay in the onset of puberty in $Gata4^{+/-}$ female mice could reflect a slight delay in the production of E_2 in these mice, although we have no direct evidence of this and cannot exclude an extragonadal effect of *Gata4* haploinsufficiency. That $Gata4^{+/-}$ female mice have a normal estrous cycle length suggests that these animals eventually achieve adequate plasma levels of E_2.

The *Gata4* cKO females have a more profound phenotype, including impaired fertility, cystic ovarian changes, and an attenuated ovulation response to exogenous gonadotropins (Table 3). As with the *Gata4* haploinsufficient mice, the

TABLE 3. Comparison of the phenotypes of the two models of GATA4 deficiency used in this study.

Parameter	*Gata4*$^{+/-}$	*Gata4*$^{Flox/Flox}$;*Amhr2*$^{cre/+}$
Nature of gene deletion	Germline	Conditional
Genetic background	B6 (pure)	129;B6 (mixed)
Experimental control	WT	*Gata4*$^{Flox/+}$;*Amhr2*$^{cre/+}$
Onset of puberty	Delayed	Normal
Fertility	Normal	Decreased
Expression of *Cyp19* mRNA response to exogenous gonadotropins	Decreased	Decreased
Ovulation in response to exogenous gonadotropins	Decreased	Decreased
Cystic ovarian changes	Absent	Present
Serum AMH	Normal for age	Decreased for age

steroidogenic gene most profoundly affected by conditional deletion of the *Gata4* gene was *Cyp19*. That most cKO mice were subfertile rather than completely infertile might reflect an inherent limitation of using *Amhr2-cre* to drive tissue-specific recombination. As shown by studies of β-catenin cKO mice generated using *Amhr2-cre* [52], the importance of a given gene for ovarian function may be masked by compensatory responses that promote selective proliferation of granulosa cells in follicles that escape Cre-mediated recombination. Indeed, a hallmark of the *Gata4* cKO mice and other knockout mice generated using *Amhr2-cre* is animal-to-animal variability, which may reflect inefficient recombination in granulosa cells (see [52] and references therein).

In *Gata4* cKO, but not *Gata4*$^{+/-}$, mice, serum AMH levels declined earlier than in age-matched controls. Studies have shown that AMH, which is produced by granulosa cells in growing follicles, suppresses the development of primordial follicles [62–64]. In the absence of AMH, the primordial follicle pool depletes prematurely [62]. Serum AMH levels correlate with the size of the primordial follicle pool, making it a useful serum marker of ovarian reserve in both mice and women [61]. Of note, the *Amh* gene has been shown to be a direct target for activation of GATA4 in the testis and ovary (see [1] and references therein). Thus, it remains unclear whether the decrease in serum AMH in the cKO mice reflects a decrease in ovarian reserve, reduced GATA4-dependent activation of the *Amh* promoter, or both. Regardless of the mechanism, the premature decline in AMH levels underscores that ovarian granulosa cell function is compromised in the cKO mice.

Studies by Tevosian and colleagues [3, 19, 22] have established that GATA4 and its cofactor, ZFPM2, mediate early events in ovarian development. In particular, these transcriptional regulators appear to modulate the response of embryonic ovarian tissue to extracellular signals, such as the WNT/β-catenin pathway. We presume, based on the appearance of unstimulated ovaries from young mice, that fetal ovarian development proceeds normally in both *Gata4* haploinsufficient mice and granulosa cell-specific cKO mice. This is in accordance with the results of studies on combined haploinsufficiency of *Nr5a1* and *Gata4*, which did not reveal a genetic interaction in mouse gonadal development [45]. In the case of *Gata4* cKO mice, we speculate that fetal ovarian development is normal, because efficient Cre-mediated recombination does not take place until robust expression of *Amhr2-cre* occurs in secondary and small antral follicles [52, 65].

In summary, the present study establishes a role for GATA4 in the regulation of adult ovarian function. These results, coupled with those of other investigators [9], show that GATA4 regulates the response of postnatal ovarian tissue to gonadotropins. Similarities in the postnatal ovarian phenotypes of *Gata4* cKO mice and mice harboring granulosa cell-specific knockouts of genes in the WNT/β-catenin pathway [52, 57] raise the possibility that GATA4 regulates this signaling pathway not only in fetal mice [3, 19, 22] but also in adults; further experiments will explore this possibility. In a complementary study [66], we show that GATA4 also regulates testicular function in adults; male mice in which *Gata4* was conditionally deleted in Sertoli cells using *Amhr2-cre* exhibit impaired fertility and age-dependent testicular atrophy.

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