Loss of GATA-6 and GATA-4 in Granulosa Cells Blocks Folliculogenesis, Ovulation, and Follicle Stimulating Hormone Receptor Expression Leading to Female Infertility

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Single GATA-6 ($G6^{gcko}$), GATA-4 ($G4^{gcko}$), and double GATA-4/6 ($G4/6^{gcko}$) granulosa cell-specific knockout mice were generated to further investigate the role of GATA transcription factors in ovarian function *in vivo*. No reproductive defects were found in $G6^{gcko}$ animals. $G4^{gcko}$ animals were subfertile as indicated by the reduced number of pups per litter and the release of significantly fewer oocytes at ovulation. In marked contrast, $G4/6^{gcko}$ females fail to ovulate and are infertile. Furthermore, $G4/6^{gcko}$ females had irregular estrous cycles, which correlate with the abnormal ovarian histology found in unstimulated adult $G4/6^{gcko}$ females showing lack of follicular development and increased follicular atresia. Moreover, treatment with exogenous gonadotropins did not rescue folliculogenesis or ovulation in double-knockout $G4/6^{gcko}$ mice. In addition, ovary weight and estradiol levels were significantly reduced in $G4^{gcko}$ and $G4/6^{gcko}$ animals when compared with control and $G6^{gcko}$ mice. Aromatase, P450scc, and LH receptor expression was significantly lower in $G4^{gcko}$ and $G4/6^{gcko}$ mice when compared with control animals. Most prominently, FSH receptor (FSHR) protein was undetectable in granulosa cells of $G4^{gcko}$ and $G4/6^{gcko}$. Accordingly, gel shift and reporter assays revealed that GATA-4 binds and stimulates the activity of the FSHR promoter. These results demonstrate that GATA-4 and GATA-6 are needed for normal ovarian function. Our data are consistent with a role for GATA-4 in the regulation of the *FSHR* gene and provide a possible molecular mechanism to explain the fertility defects observed in animals with deficient GATA expression in the ovary. **(*Endocrinology* 153: 2474–2485, 2012)**

Follicle selection and atresia are highly regulated pathways controlled by genes involved in differentiation, cell survival, steroidogenesis, hormone production, and apoptosis. Several genes included in these diverse processes contain within their regulatory elements the motif WGATAR (1–3). This motif is recognized by, and gives name to, a small family of transcription factors identified as GATA (1, 4, 5). Of the six members of this family only GATA-4 and GATA-6 are highly expressed in ovarian granulosa cells (2, 6, 7). GATA-4 and/or GATA-6 have been shown to directly regulate the expression of genes involved in follicle growth and steroid synthesis (1). Furthermore, GATA factors mediate the stimulatory effects of FSH on several ovarian genes (2). For instance, silencing of GATA-4 in primary rat granulosa cells blunts the stimulation of aromatase by FSH (9). Aromatase is essential for estradiol production and a hallmark of granulosa cell differentiation in preovulatory follicles (10). Likewise, mutation of a GATA-binding site on the steroidogenic acute regulatory protein (StAR) promoter prevents both basal and FSH-driven activity (11), whereas forced expression of GATA-4 in porcine granulosa cells increases StAR promoter activity (2, 12). GATA-4 and GATA-6 can also activate the type II 3β-hydroxysteroid dehydrogenase promoter (13). Overexpression studies have demonstrated that GATA-4 can also activate the promoter of ovarian

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Abbreviations: Cre, Cyclic recombinase; Cyp, cytochrome P450; FSHR, FSH receptor; GATA-RE, GATA response element; hCG, human chorionic gonadotropin; PMSG, pregnant mare serum gonadotropin; StAR, steroidogenic acute regulatory protein.

hormones including inhibin α (14) and anti-Müllerian hormone (15), both of which are produced by the granulosa cells (16, 17). This evidence suggests that GATA-4 and GATA-6 may have a crucial role in the regulation of granulosa cell function.

In fact, a recent report demonstrated that conditional deletion of exons 3, 4, and 5 of the *GATA-4* gene in reproductive tissues results in impaired fertility (18). In addition, transgenic mice expressing a small interfering RNA against GATA-4 develop ovarian tumors, suggesting that this factor is also involved in the function and integrity of the ovary (19). The role of GATA-6 in the regulation of ovarian function *in vivo*, however, has yet to be examined.

GATA-4 and GATA-6 are coexpressed in granulosa cells, recognize identical binding motifs, and regulate the expression of similar target genes (20–23). Therefore, we hypothesized that these factors have redundant functions in the ovary and predicted that loss of both GATA-4 and GATA-6 should result in a significant decrease in ovarian function. Here we test this hypothesis and investigate the roles of GATA factors in folliculogenesis by silencing the expression of GATA-4, GATA-6, or GATA-4 and GATA-6 in granulosa cells using a cyclic recombinase (Cre)-Lox system. Our results show, for the first time, that GATA-4 and GATA-6 expression in granulosa cells is essential for normal folliculogenesis and female fertility. In addition, we have demonstrated that the FSH receptor (*FSHR*) is a GATA-4 target gene in the ovary, providing a novel molecular mechanism to explain the fertility defects observed in animals with deficient GATA expression in the ovary.

Materials and Methods

Reagents

DMEM/F12 medium was purchased from Invitrogen (Carlsbad, CA). Pregnant mare serum gonadotropin (PMSG), estradiol, human chorionic gonadotropin (hCG), and all buffer components were purchased from Sigma (St. Louis, MO). The antibodies for GATA-4 (sc9053 and sc1237) and GATA-6 (sc9055 and AF1700) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and R&D systems (Minneapolis, MN); β-actin (antibody 8227) and Lamin B1 (antibody 16048) were purchased from Abcam (Cambridge, MA). FSHR (S0522) was purchased from Epitomics (Burlingame, CA). Cleaved caspase 3 (catalog no. 9661) was purchased from Cell Signaling Technology (Danvers, MA). Secondary antibodies (6721 and 305-035-045) were purchased from Abcam and Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Animals

Animals were treated in accordance with the NIH Guide for Care and Use of Laboratory Animals, and all protocols were approved by the University of Illinois at Chicago Animal Care Committee. GATA-6$^{F/F}$ and ZP3-Cre animals were obtained from The Jackson Laboratory (Bar Harbor, ME). GATA-4$^{F/F}$ animals were kindly provided by Dr. William Pu. The generation of Cyp19-Cre has been previously described (24, 25).

Estrous cycling

Vaginal smears were collected daily from adult females (~d 55) over the course of 12 d. The vaginal smears were evaluated microscopically.

Superovulation

Adult females (~d90) were injected sc with 7.5 IU of PMSG followed by 7.5 IU of hCG 48 h after to promote ovulation. Animals were killed 17 h after hCG and oocytes from each oviduct were collected and counted.

Ovarian weight determination

After dissection, the surrounding fat was removed from each ovary, and the wet weight was determined on an analytical balance.

Hormone assessments

Truncal blood was collected using lithium heparin tubes (Sarstedt, Newton, NC), and plasma was isolated following manufacturer's instructions. Estradiol and progesterone plasma levels were determined by using estradiol and progesterone EIA kits (Cayman Chemical Co., Ann Arbor, MI), respectively. Estradiol concentration was determined in undiluted plasma samples whereas progesterone plasma levels were used at a 1:10 dilution. FSH and LH plasma levels were determined by The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core.

Granulosa cell isolation

Immature female mice (d 21–d 25) were injected sc with 7.5 IU PMSG, and ovaries were harvested after 48 h. Animals were anesthetized with isoflurane and then cervically dislocated. Ovaries were dissected and placed in DMEM/F12. Large antral follicles were punctured to extrude granulosa cells, which were then strained and spun down. Cells were resuspended in either Trizol-reagent for RNA isolation or radioimmune precipitation assay buffer for protein isolation (see below).

RNA isolation and quantitative real-time PCR analysis

Total RNA from primary mouse granulosa cells was isolated using Trizol-Reagent (Invitrogen) following the manufacturer's instructions. For mRNA analysis by RT-PCR, 1μg of the total RNA was reverse transcribed at 42 C using Moloney murine leukemia virus reverse transcriptase and later diluted to a final volume of 100 μl. To generate standard curves, the cDNA of our genes of interest was cloned into the pCR 2.1 vector (Invitrogen), sequenced, and excised by restriction enzyme. Purified cDNA was diluted to concentrations ranging from 10×10^2 to 6×10^6 copies/μl. Aliquots (5 μl) of standard cDNA or sample cDNA were combined with SYBR Green I (Bio-Rad Laboratories, Inc., Hercules, CA) and primers specific for the gene of interest. Only

intron-spanning primers were used for PCR amplification. The sequence of the primers used is available upon request.

Real-time quantification of the PCR product in each cycle was carried out in an iQcycler Real Time PCR machine (Bio-Rad) with the following cycling conditions: preincubation at 95 C for 2 min, followed by 40 cycles of denaturation at 95 C for 5 sec, annealing at 60 C for 10 sec, and extension at 72 C for 40 sec. The melting peak of each sample was routinely determined by melting curve analysis to ascertain that only the expected products had been generated. The minimal number of cycles sufficient to produce detectable levels of fluorescence (Ct) was calculated using MyiQ software. For each gene of interest the number of mRNA molecules was calculated using a standard curve and expressed as copies per nanogram of total RNA. The results are expressed as the ratio between the copies per nanograms of total RNA of the gene of interest and ribosomal L19 protein.

Western blot analysis

Primary mouse granulosa cells were homogenized in an ice-cold radioimmune precipitation assay lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Nonidet P-40; 0.25% sodium deoxycholate; 1 mM phenylmethylsulfonyl fluoride; 150 mM EDTA; 1 × protease inhibitor cocktail (Sigma); 1 mM NaF; and 1 mM Na_3VO_4). Protein concentration was determined using BSA as a standard. The samples were denatured by adding sample buffer (0.555 M bis-Tris, 4.44% sodium dodecyl sulfate, 0.333 M HCl 30% glycerol, 2.22 mM EDTA, 10% β-mercaptoethanol, 0.04% bromophenol blue), followed by boiling at 90 C for 10 min. Protein (12 μg) was separated on 12% bis-Tris-PAGE gels in 250 mM 3[*N*-morpholino]propanesulfonic acid, 250 mM Tris, 5 mM EDTA, 1 M sodium bisulfite, and 0.5% sodium dodecyl sulfate buffer. Samples were transferred to nitrocellulose membranes followed by incubation in 5% nonfat dry milk for 2 h at room temperature to block unspecific binding. After several washes in Tris-buffered saline-Tween 20, the membranes were incubated overnight at 4 C with anti-GATA-4 (1:500), anti-GATA-6 (1:100), anti-B-actin (1:500), anti-Lamin B1 (1:500), or anti-FSHR (1:1000) antibody. Membranes were washed and incubated with a secondary antibody conjugated to horseradish peroxidase (1:10,000 dilution) for 2 h at room temperature. Protein-antibody complexes were visualized using Immoblon western chemiluminescent horseradish peroxidase substrate (Millipore Corp., Billerica, MA) or Supersignal Westfento Maximum Senstitivity Substrate (Thermo Scientific, Rockford, IL) depending on the abundance of the protein and sensitivity of the antibody.

Immunohistochemistry

Ovaries were fixed in Bouin's Solution before paraffin embedding. Sections (5 μm) were dewaxed and rehydrated. This was followed by antigen retrieval using citrate buffer solution (10 mM citric acid and sodium citrate, pH 6) and microwaved on high for 30 sec until boiling and then at low for 8 more minutes. After cooling, slides were placed in 1% H_2O_2. Sections were then blocked using the Avidin/Biotin Blocking kit (Vector Laboratories, Burlingame, CA) followed by 30 min of blocking in superblock blocking buffer (Pierce Chemical Co., Rockford, IL) before the addition of the primary antibody diluted in PBS (GATA-4, 1:500; GATA-6, 1:200; active caspase 3, 1:200). After washes with Tris-PBS, slides were incubated in secondary antibody for 30 min at room temperature followed by washing. Tissues were stained through use of the Vectastain elite ABC kit and 3,3′diaminobenzidine (Vector laboratories) following manufacture's recommendations. Slides were counterstained with Gill's hematoxylin before mounting.

Hematoxylin and eosin stain

Dewaxed and rehydrated tissue sections were dipped in Harris Hemotoxylin (Fisher Scientific, Rockford, IL) followed by rinsing in running water. Slides were next dipped in Define Reagent (Fisher Scientific) followed by rinsing in running water. Next, Bluing Reagent (Fisher Scientific) was used to sharpen the staining of the nucleus followed by rinsing in running water. Slides were then placed in 95% ethanol followed by staining in Eosin-Y and Phloxine (Thermo Scientific) to stain the plasma.

Plasmid and reporter constructs and cell transfection

The region between −311 to −1 of the *FSHR* gene, where +1 is the transcription initiation site gene, was cloned from rat genomic DNA using the following primers: forward, GCG GTA CCA AAT ATG CAC CAA GTT TCT CTT TTC TG; reverse, GCC TCG AGC CTT ATT TAT CCA TTC ACC GAC TTT C. PCR products were cloned into the pGL4 Basic luciferase report vector (Promega Corp., Madison WI) and confirmed by sequencing. This reporter construct was named rFSHRp-Luc. The GATA-binding site found at position −82 of the FSHR promoter was mutated using QuikChange II XL Site-Directed Mutagenesis (Stratagene, La Jolla, CA). Both wild-type and mutant rFSHRp-Luc were transfected into primary granulosa cells along with a mouse GATA-4 expression plasmid or an empty plasmid (pcDNA) as previously described (9). Luciferase activity was assessed 48 h after transfection using the Dual Luciferase assay kit (Promega).

Gel shift analysis

Nuclear protein extracts were isolated from primary granulosa cells (9). Radioactive labeled double-stranded oligonucleotide probes containing the GATA-binding sites were used as probes. The shift band obtained with this probe was competed against 50× excess of unlabeled wild-type oligonucleotides or mutant oligonucleotides containing a TATC to T*gg*C mutation on the GATA-binding site. Supershift experiments were carried out by adding 2 μl of the GATA-4 antibody (sc9053/H-112X), 2 μl of GATA-6 antibody (sc-7245/N-18 X), or 2 μl of normal serum to the binding reaction 30 min before the addition of the labeled probe. Both GATA-4 and GATA-6 antibodies have been shown to be effective in supershift reactions (12). Reactions were performed as previously described (9).

Statistics

Data are expressed as the mean ± SEM. Multiple group statistical analyses were performed by one-way ANOVA followed by the Tukey *t* test for multiple comparisons. Two-group comparisons were performed using a *t* test for independent samples. Statistics were calculated with GraphPad Prism 5 (GraphPad, La Jolla, CA).

Results

Granulosa cell-specific disruption of the *GATA-4* and *GATA-6* genes

A Cre-Lox system was used to knockdown GATA-4 and GATA-6 expression in ovarian granulosa cells. Exon II of the *GATA-4* and *GATA-6* genes was targeted for deletion because it codes for the majority of both GATA proteins (26, 27). To maximize the recombination of the floxed genes, we generated animals carrying one floxed allele and one null allele using zona pellucida glycoprotein-3 Cre animals (28). GATA-$4^{F/-}$ and GATA-$6^{F/-}$ animals are fertile and have normal ovarian structure and function (data not shown).

To selectively disrupt GATA expression in granulosa cells, GATA-$4^{F/-}$ and/or GATA-$6^{F/-}$ mice were crossed with transgenic mice expressing Cre driven by the cytochrome P450 (Cyp)19 proximal promoter (Cyp19-Cre) (24, 25). In CYP19-Cre animals, Cre is expressed in granulosa cells of antral follicles and has not been detected in the theca, epithelial cells, or the oocyte throughout postnatal development (25). In this study, we used GATA-$4^{F/-}$-CYP19-Cre ($G4^{gcko}$), GATA-$6^{F/-}$-CYP19-Cre ($G6^{gcko}$), or GATA-$4^{F/-}$/GATA-$6^{F/F}$-CYP19-Cre ($G4/6^{gcko}$) animals in all experiments unless a specific genotype is indicated. Controls were CYP19-Cre-GATA-$4^{F/+}$, CYP19-Cre-GATA-$6^{F/+}$, or GATA-$4^{F/+}$/GATA-$6^{F/+}$-CYP19-Cre.

Immunostaining for GATA-4 and GATA-6 in $G4^{gcko}$ and $G6^{gcko}$ ovaries confirmed GATA knockdown in granulosa cells (Fig. 1A). Strong staining for GATA-4 was found in both control (data not shown) and $G6^{gcko}$ ovaries, whereas little or no staining was observed in $G4^{gcko}$. The opposite findings were observed when staining for

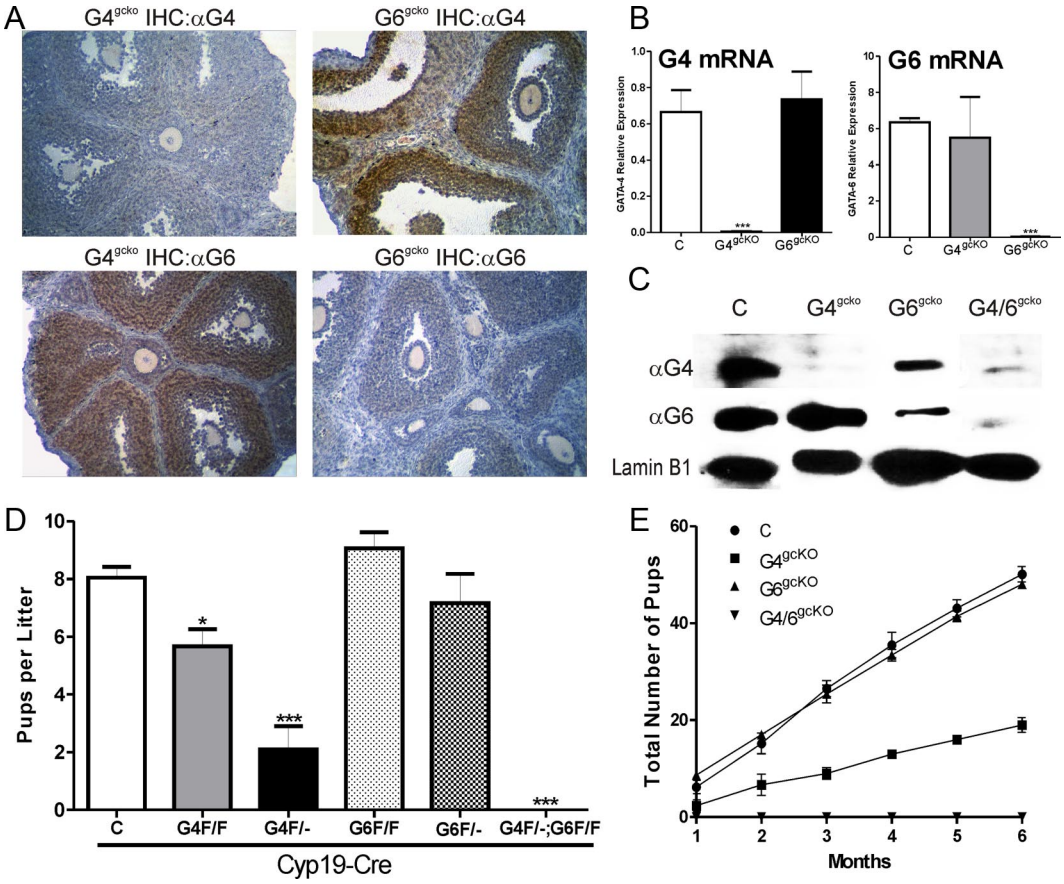

FIG. 1. GATA-4 and GATA-6 expression in granulosa cells is necessary for normal fertility. A, ×10 magnification of IHC for GATA-4 protein in $G4^{gcko}$ (*top left*) ovary compared with GATA-4 protein in the $G6^{gcko}$ (*top right*) ovary from d21–25 PMSG-stimulated mice. GATA-6 protein expression in $G4^{gcko}$ (*bottom left*) ovary compared with in $G6^{gcko}$ (*bottom right*) ovary (n = 3 for each genotype; only a representative is shown). B, mRNA expression of GATA-4 (*left*) and GATA-6 (*right*) from PMSG-treated animals. Expression is expressed relative to mouse ribosomal L19 (the average of six or more samples per genotype is shown). ***, $P < 0.01$. C, Protein expression of GATA-4 (*top*) and GATA-6 (*middle*) from PMSG-treated females. Lamin B1 was used as a loading control (*bottom*). A representative experiment of three or more samples for each genotype is shown. D, The number of pups per litter was determined in control mice (C) and experimental animals (G4F/F; G4F/−; G6F/F; G6F/− and G4F/−; G6F/F). All animals carried the CYP19-Cre expression cassette. Four or more females were used for each genotype. *Columns* represent the average ± SEM of the number of pups per litter. *Asterisk* denotes significance compared with control (*, $P < 0.05$; ***, $P < 0.01$). E, Continuous breeding assessment showing the cumulative number of progeny per female. Values represent the mean ± SEM of litters derived from three or more females for each genotype. IHC, Immunohistochemistry.

GATA-6 was performed. No staining for GATA-4 or GATA-6 was found in G4/6^{gcko} animals (data not shown). The knockdown of GATA-4 and GATA-6 at the level of mRNA was confirmed using granulosa cells isolated from preovulatory follicles. As shown in Fig. 1B, GATA-4 mRNA levels were undetectable in granulosa cells obtained from G4^{gcko} animals, whereas no differences in GATA-4 expression were found between control and GATA-6 knockout cells. Similarly, GATA-6 mRNA was undetectable in granulosa cells from G6^{gcko} animals, but it remained highly expressed in control and GATA-4 knockout cells. Neither GATA-4 nor GATA-6 mRNA was detectable in G4/6^{gcko} animals (data not shown). Western blot (Fig. 1C) confirmed these results and showed very low levels of GATA-4 protein in GATA-4 knockout cells as well as in the double GATA-4 and GATA-6 knockout cells. GATA-6 protein levels in single and the double-knockout animals were significantly lower than in control and G4^{gcko} mice.

GATA-4 and GATA-6 are essential for female fertility

The fertility of mice lacking GATA-4 and/or GATA-6 in granulosa cells was tested by mating control or experimental (G4F/F, G4F/−, G6F/F, G6F/−, and G4F/−;G6F/F)-Cyp19-Cre-females with males of proven fertility for 6 months. No differences were found in the number of pups per litter between control, G6F/F, and G6F/− (Fig. 1D); however, both G4F/− and G4F/F animals had a significant decrease in the number of pups per litter when compared with controls. Fertility defects were more significant in the G4F/− females, which produced not only less pups per litter than G4F/F females but also had a high prevalence of sterility. Thus, six of 11 (54%) G4F/− females failed to produce offspring, suggesting that the reproductive capacity of G4F/− females ranges from subfertility to infertility. Even more striking, all double-knockout females were completely infertile. The total number of pups produced by females of each genotype was monitored over the course of a 6-month breeding period; as shown in Fig. 1E, no differences in the progressive accumulation of pups was observed between control and G6^{gcko} animals. In contrast, the total number of pups produced by G4^{gcko} females was significantly lower whereas G4/6^{gcko} females produced no pups. These results demonstrate that the expression of both GATA-4 and GATA-6 in ovarian granulosa cells is essential for female fertility.

Ovarian granulosa cells play a central role in the regulation of the murine estrous cycle; therefore, we next compared the length of the various phases of the estrous cycle in control, G4^{gcko}, G6^{gcko}, and G4/6^{gcko} animals. The results showed that G4^{gcko} and G4/6^{gcko} have significantly fewer complete cycles when compared with control (C) and G6^{gcko} mice (Fig. 2). Accordingly, the average

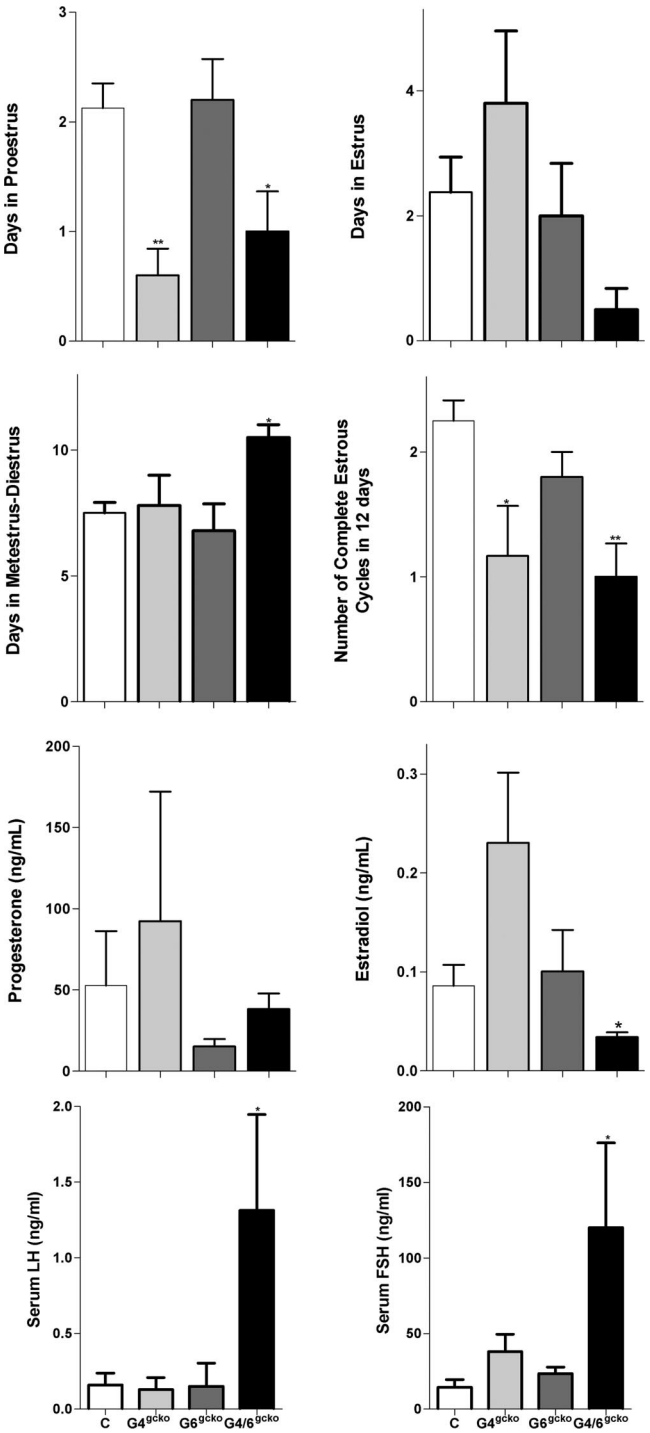

FIG. 2. Abnormal estrous cycling in G4^{gcko} and G4/6^{gcko} mice. The estrous cycle was tracked over the course of 12 d in control, G4^{gcko}, G6^{gcko}, and G4/6^{gcko} adult females. Each estrous cycle was broken into three stages of proestrus (P), estrus (E), and metestrus/diestrus (M/D). The *columns* represent the average number of days spent at each stage ± SEM. Estradiol, progesterone, LH, and FSH levels were determined at the proestrous stage. *Bars* represent the mean ± SEM of measurements from at least four animals per genotype. *, $P < 0.05$; **, $P < 0.01$ *vs.* control.

number of days in proestrus was significantly decreased in G4^{gcko} and G4/6^{gcko} animals, suggesting a defective follicular phase. There was also a decrease in the amount of days that double GATA-4/6 mutant animals spent in estrus when compared with all the other phenotypes, although this was not statistically significant. Accordingly, G4/6^{gcko} animals stayed in metestrus/diestrus for an extended period of time. Based on these results, it is not surprising that estradiol and progesterone levels were highly variable in G4^{gcko} animals. Because of this variability, no significant differences in the serum levels of these steroids were found between control, G4^{gcko}, and G6^{gcko}. However, estradiol levels were significantly lower in G4/6^{gcko} mice when compared with control animals (Fig. 2). On the other hand, gonadotropin levels (FSH and LH) were significantly higher in G4/6^{gcko} mice when compared with all other genotypes including control. Taken together, these results show that G4^{gcko} and G4/6^{gcko} animals have abnormal estrous cycles, suggesting impaired folliculogenesis and/or irregular ovulation.

To investigate whether folliculogenesis was defective, ovaries of adult 50- to 70-d-old animals at the proestrous stage were microscopically examined (Fig. 3A). This study indicated that the ovarian morphology of G4^{gcko} and G6^{gcko} animals appears normal, showing the presence of follicles at all stages of development and corpora lutea (n = 3 for each genotype). In marked contrast, ovaries of double G4/6^{gcko} animals contained only a few small antral follicles and no large/preovulatory follicles or corpora lutea, suggesting that both GATA-6 and GATA-4 factors are needed for normal folliculogenesis and luteal formation.

The presence of large antral follicles in control, G6^{gcko}, and G4^{gcko} animals was confirmed at higher magnification (×20) (Fig. 3B and data not shown). At this magnification, it was also evident that the granulosa layer of G4/6^{gcko} follicles is disorganized and shows morphological characteristics of atresia (29, 30) including granulosa cells with pyknotic nuclei (Fig. 3B, *right panel*). Furthermore, G4/6^{gcko} follicles showed increased staining for cleaved caspase 3 (Fig. 3C, *right panel*), which is known to be a key executioner of apoptosis (31). Negligible cleaved caspase 3 staining was observed in controls (Fig. 3C, *left panel*), G6^{gcko}, and G4^{gcko} animals (data not shown). These results confirm the abnormal follicular development suggested by the acyclicity of mice lacking GATA-6 and GATA-4 in granulosa cells and demonstrate an increase in follicular atresia in these animals.

Based on these results, we examined whether follicle growth in G4/6^{gcko} animals could be rescued by treatment with exogenous gonadotropin. Imma-

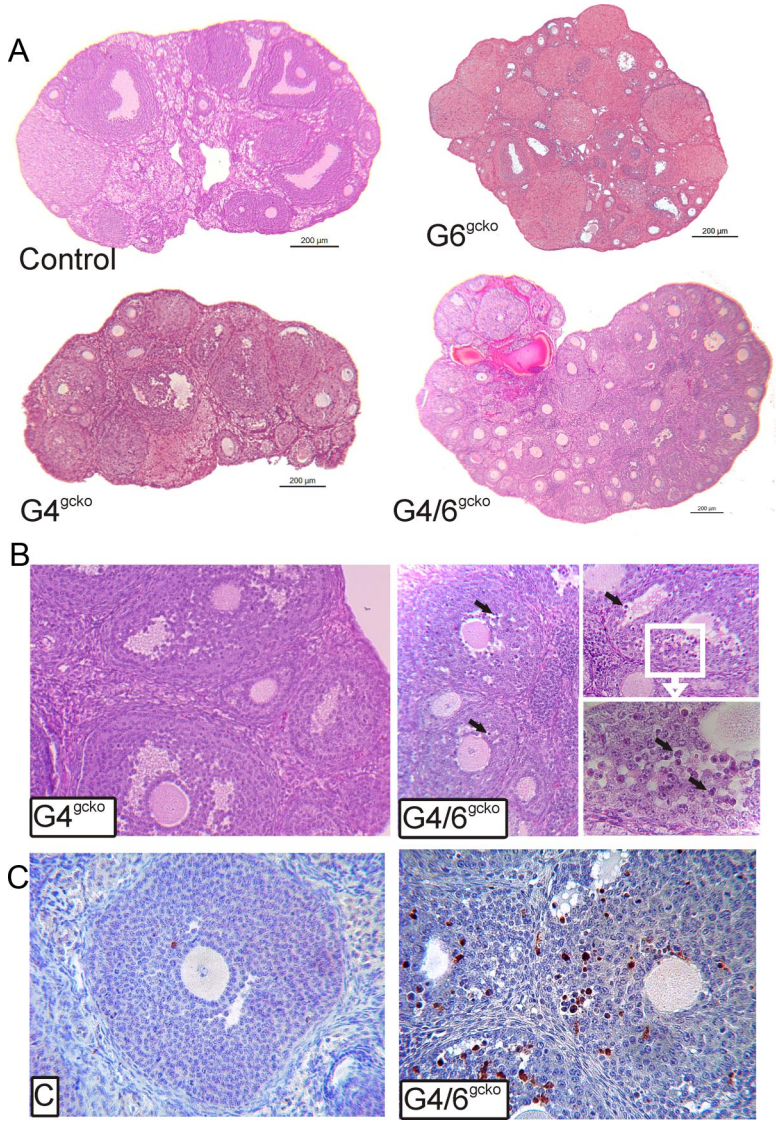

FIG. 3. A, Abnormal folliculogenesis in G4/6^{gcko}. Representative hematoxylin and eosin staining of ovaries of control, G4^{gcko}, G6^{gcko}, and G4/6^{gcko} (average age 60 d) females at proestrus. B, Follicles in G4/6^{gcko} animals show morphological characteristics of atresia. Hematoxylin and eosin staining of representative ovaries from G4^{gcko} and G4/6^{gcko} animals at a ×20 magnification. High magnification (×40) is included for G4/6^{gcko} animals. *Arrows* point to granulosa cells with pyknotic nuclei, which were found only in G4/6^{gcko} ovaries. Control and G6^{gcko} animals showed no sign of atresia (data not shown). C, Cleaved caspase 3 staining in control and G4/6^{gcko} animals. Four animals for each genotype were included in all experiments but only a representative picture is shown.

ture (d 21–d 25) mice received a single dose of PMSG to stimulate follicle growth and were killed 48 h later to collect ovaries for histological analysis. Control and single-knockout mice responded to PMSG stimulation by producing a large number of antral follicles (Fig. 4A). Hemorrhagic follicles were frequently found in G4^{gcko} but rarely in G6^{gcko} ovaries. No hemorrhagic follicles were found in control animals. Strikingly, the ovaries of G4/6^{gcko} animals did not contain antral or preovulatory follicles after PMSG treatment and weighed less than control, G4^{gcko}, and G6^{gcko} ovaries (Fig. 4B). These results demonstrate that follicular development cannot be rescued by exogenous gonadotropins in conditional GATA-knockout animals.

After PMSG treatment, progesterone levels were not significantly different between knockouts and control animals (ANOVA I, Fig. 4C), although there was a trend suggesting lower progesterone levels in double-knockout animals. In fact, when progesterone levels in control and double-knockout animals were analyzed separately using a *t* test they were found to be significantly different ($P < 0.05$). On the other hand, both G4^{gcko} and G4/6^{gcko} females had a significant reduction in serum estradiol levels when compared with control and G6^{gcko} (Fig. 4D), suggesting that estrogen production in the ovary is mainly regulated by GATA-4.

Because follicle growth is defective in G4/6^{gcko} mice, the response of control and mutant animals to a superovulation protocol was examined. Adult (average age 90 d) control, G4^{gcko}, G6^{gcko}, and G4/6^{gcko} females were stimulated with PMSG (an FSH equivalent) for 48 h followed by hCG administration. The presence of oocytes in the oviducts was determined 17 h after hCG treatment. Control and G6^{gcko} females released a similar amount of oocytes: 27.7 ± 2.8 and 25.16 ± 3.5, respectively (Fig. 4E). By contrast, the average number of oocytes produced by G4^{gcko} females was significantly lower (6.5 ± 3; $P < 0.01$). Remarkably, in six of nine (66%) G4^{gcko} females ovulation did not occur. In contrast, all double-knockout G4/6^{gcko} females (n = 8) failed to ovulate. Thus, the reduced fertility of G4^{gcko} and the infertility of G4/6^{gcko} animals correlates with impaired or lack of ovulation, respectively.

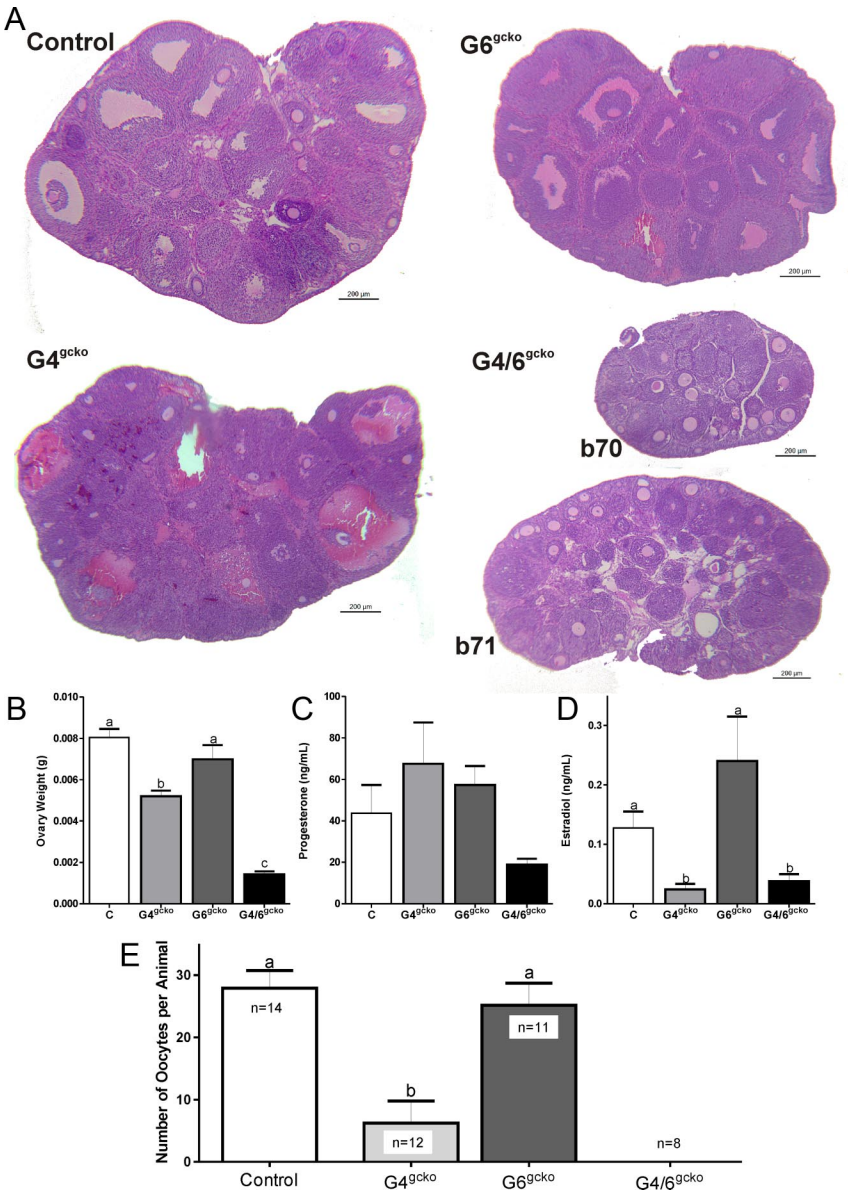

FIG. 4. Effect of PMSG treatment on follicle development. A, Representative hematoxylin and eosin staining of ovaries from animals treated with PMSG for 48 h. Four or more animals were used per group; a representative ovary is shown for control, G4^{gcko}, and G6^{gcko} animals. The varies of two different G4/6^{gcko} animals (b70 and b71) are shown. B, Ovarian weight; C, progesterone, and D, estradiol levels, all after 48 h treatment with PMSG. Seven or more animals were included in each group. *Bars* represent mean ± SEM, and *different letters* denote differences between groups (a and b and b and c, $P < 0.05$; a–c, $P < 0.01$). E, Defective or lack of ovulation in G4^{gcko} and G4/6^{gcko} mice, respectively. Ovulation was induced in immature (d 22–d 23) animals by a sc injection of 7.5 IU of PMSG, followed 48 h later by administration of 7.5 IU of hCG. Oviducts and ovaries were harvested 17 h after hCG; oocytes found in the oviduct at this time were counted (n = number of animals included in each group). *Bars* represent mean ± SEM, and *different letters* denote differences between genotypes (a and b, $P < 0.05$). No oocytes were found in the oviducts of G4/6^{gcko} animals.

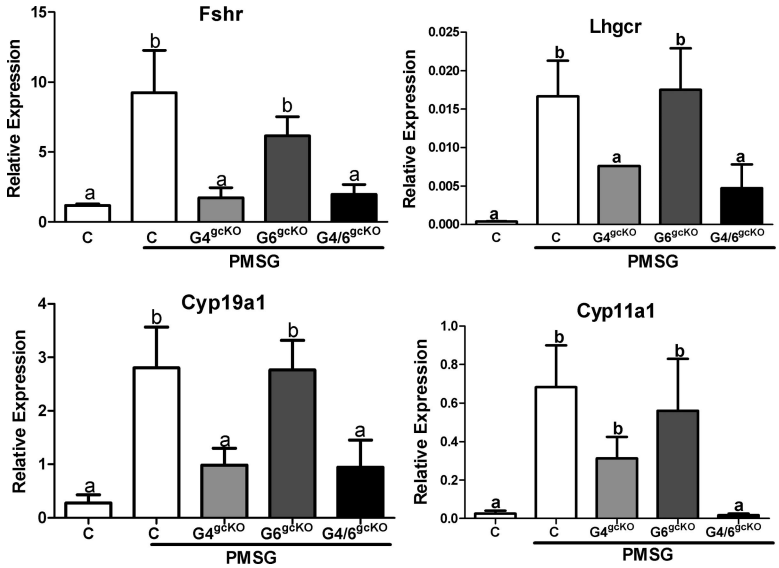

FIG. 5. Relative expression of key ovarian genes in the different genetic backgrounds. Total RNA was extracted from granulosa cells isolated after PMSG treatment by puncturing only large antral follicles in control, G4^{gcko}, and G6^{gcko} females or only large secondary follicles in G4/6^{gcko} mice. Three or more animals were included for each genotype. *Columns* represent the mean ± SEM. *Different letters* denote significance (a and b, $P < 0.05$; a–c, $P < 0.01$).

Lack of GATA-4 is associated with a decrease in FSHR expression in granulosa cells

In view of the elevated levels of gonadotropins found in the G4/6^{gcko} and the impaired response of both G4^{gcko} and G4/6^{gcko} to PMSG/hCG, we examined the responsiveness of granulosa cells to gonadotropins by determining the expression of the receptors for FSH and LH in granulosa cells. The expression of aromatase, StAR, and P450scc, which are known to increase during the differentiation of granulosa cells, was also examined. Granulosa cells were isolated from large antral follicles of immature (d 21–d 25) single-knockout and control female mice treated with PMSG (7.5 IU) for 48 h. Double-knockout animals do not produce large antral follicles in response to PMSG; however, primary granulosa cells were obtained from large secondary follicles for comparison with control or single-mutant animals.

As shown in Fig. 5, treatment with PMSG increased Fsh receptor (Fshr) mRNA expression levels in control and G6^{gcko} animals. *Fshr* stimulation was completely prevented by the lack of GATA-4 expression and by the lack of both GATA-4 and GATA-6. As expected, a strong induction of the LH receptor (Lhcgr), a key target of FSH in the granulosa cells, was observed in control and G6^{gcko} granulosa cells but not in cells lacking GATA-4 or both GATA-4 and GATA-6. Moreover, the expression of classical markers of granulosa cell differentiation, such as aromatase (Cyp19a1) was dramatically down-regulated in G4^{gcko} and GATA-4 and GATA-6 mutant ovaries, suggesting that the differentiation of granulosa cells to the preovulatory stage is impaired. The stimulation of Cyp11a1 by PMSG observed in control, G4^{gcko}, and G6^{gcko} animals did not occur in the double knockout G4/6^{gcko} mice. These changes in aromatase and Cyp11a1 expression are in good agreement with the serum level of estradiol and progesterone described in Fig. 4, B and C. The stimulation of StAR by PMSG was not affected by GATA-4 or GATA-6 knockdown (data not shown).

Confirming RT-PCR results, Western blot analyses showed that the FSHR protein was undetectable in GATA-4-knockout granulosa cells but remained highly expressed in granulosa cells of control and G6^{gcko} animals (Fig. 6A). As expected, FSHR protein was nondetectable in granulosa cells of double-knockout G4/6^{gcko} mice (data not shown). These *in vivo* results demonstrate that GATA-4 is necessary to maintain FSHR expression in the ovary. This conclusion is supported by the presence of an inverted GATA response element (GATA-RE) in *Fshr* promoter (Fig. 6B).

Next, we examined whether either GATA-4 or GATA-6 interact with this GATA-RE using gel shift assays. A prominent shift band was observed when labeled oligonucleotides containing the GATA-RE found in the *Fshr* promoter and nuclear extracts of primary granulosa cells were used (Fig. 6C). The addition of an antibody against GATA-4 to the binding reaction caused the formation of a supershift band demonstrating that GATA-4 binds to the GATA-RE oligonucleotide. The presence of an anti-GATA-6 antibody in the gel shift reaction, however, did not affect the mobility of the shift band, suggesting that GATA-6 does not form part of the complex formed between granulosa cell nuclear proteins and the GATA-RE oligonucleotide. Moreover, the addition of a 50-fold excess of unlabeled wild-type oligonucleotide prevented the formation of the GATA-4/DNA complex whereas the addition a 50-fold excess of oligonucletides carrying a mutation on the GATA-RE (TATC to T*gg*C) had no effect (Fig. 6D). These results demonstrate that GATA-4 recognizes the GATA-RE found in the *Fshr* promoter, further suggesting a role of this transcription factor in the regulation of *Fshr* expression in granulosa cells.

To investigate whether GATA-4 regulates the activity of the *Fshr* promoter, this regulatory region was cloned into a luciferase reporter vector (pGL4). The *Fshr* promoter reporter (FSHr-Luc) was transfected into primary

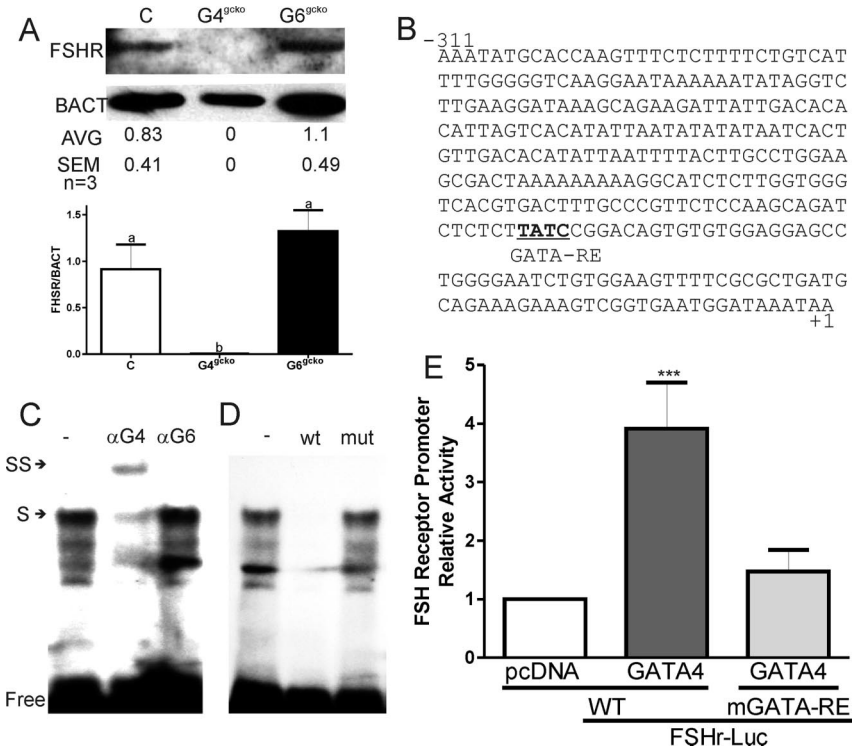

FIG. 6. GATA-4 regulates FSHR expression. A, FSHR protein (FSHR) levels in granulosa cells of control, $G6^{gcko}$, and $G4^{gcko}$ animals. BACT, β-actin protein. FSHR/BACT intensity ratio, SEM: SE, n = 3, *Columns with different letters* differ significantly (a and b, $P < 0.001$). B, Sequence of the proximal promoter of the *Fshr* gene indicating the location of a putative inverted GATA-binding motif (GATA-RE). C, Gel shift assay using labeled oligonucleotides containing the GATA-RE and nuclear extracts of primary granulosa cells in the presence of normal serum (NS), an anti-GATA-4 antibody (αG4), or an anti-GATA-6 antibody (α-G6). D, Gel shift assay using the nuclear extracts from primary granulosa cells in the presence of a 50-fold excess of unlabeled wild-type (wt) probe or 50-fold excess of unlabeled mutant (mut) probe. E, Primary granulosa cells were transfected with a wild-type (WT) *Fshr* promoter reporter construct (FSHr-Luc) or an *Fshr* promoter construct with a mutated GATA-RE (mGATA-RE) in addition to a mouse GATA-4 expression vector (GATA4) or an empty vector (pcDNA). Luciferase activity was determined 48 h after transfection. *Bars* represent the mean ± SEM (n = 4). ***, $P < 0.01$ *vs.* GATA-4 or pcDNA. AVG, Average.

granulosa cells simultaneously with either an empty vector (pcDNA) or a GATA-4 expression vector. GATA-4 overexpression stimulated the activity of the FSHr-Luc reporter vector in a dose-dependent manner (data not shown). The stimulation of the *Fshr* promoter activity by GATA-4 was prevented by the mutation of the GATA-RE (Fig. 6E). These results demonstrate that the expression of GATA-4 alone is enough to stimulate the transcriptional activity of the *Fshr* promoter and that this stimulatory effect is mediated by the GATA-RE found in this promoter.

Discussion

Folliculogenesis involves several sequential stages including initiation, growth, selection, and ovulation. The transition between stages and the change in direction toward follicular atresia depends on the timely activation or repression of specific genes. The transcription factors GATA-4 and GATA-6 are expressed in granulosa and theca cells of growing follicles (2, 6, 7), suggesting that they could play a crucial role in the normal progress of folliculogenesis. Using single and double GATA-4 and GATA-6 granulosa cell conditional knockout animals, we document that both factors are necessary for normal folliculogenesis and female fertility. Our findings provide further evidence supporting the conclusion that deletion of GATA-4 leads to subfertility (18) and demonstrate that animals lacking GATA-6 in granulosa cells have normal ovarian function. These results prove our initial hypothesis, which suggests that silencing both factors leads to a stronger ovarian phenotype when compared with the single knockouts.

The lack of an abnormal phenotype in GATA-6 mutants may be due to a functional compensation by GATA-4. In fact, the DNA-binding domains of mouse GATA-4 and GATA-6 proteins are 85% identical, allowing these factors to recognize similar DNA sequences (1–3). This characteristic may also explain the functional redundancy between GATA-4 and GATA-6 found in liver bud formation (32) and cardiac myocyte differentiation (33). Overlapping functions for GATA-4 and GATA-6 have also been described in the jejunum and duodenum where conditional deletion of GATA-6 has no effect; however, a strong phenotype was observed when both GATA-6 and GATA-4 were deleted (34). Interestingly, similar to our findings, conditional deletion of GATA-4 in the jejunum has profound effects even in the presence of GATA-6 (35). Taken together, this evidence suggests that GATA-4 can regulate specific GATA-6 gene targets. Considering that double-knockout animals are infertile whereas single GATA-4 knockout are subfertile, it is also possible to conclude that GATA-6 is able to compensate, at least partially, for the lack of GATA-4. This finding also suggests that GATA-6 plays a crucial role in ovarian function. The precise roles of GATA-6 in ovarian function warrant future studies. The mechanisms involved in the full or partial compensation observed between GATA-4 and GATA-6

also remain to be determined. These mechanisms are most likely complex and probably specific for each GATA target gene.

Lack of ovulation due to an ovarian defect seems to be the major cause of infertility in G4/6^{gcko} mice because these animals have elevated levels of FSH, and PMSG treatment does not stimulate the formation of preovulatory follicles. In the ovaries of nontreated double-knockout animals, however, small antral follicles were regularly found. These puzzling differences can be attributed to a much stronger and earlier activation of the Cyp19 promoter by PMSG, as previously demonstrated (25). It is possible that in PMSG-treated mice the silencing of GATA factors occurs before the formation of antral follicles, leading to a complete halt in follicle growth.

Granulosa differentiation to the preovulatory stage is impaired in G4^{gcko} and G4/6^{gcko} females. The low expression of aromatase, a classical marker of granulosa cell differentiation, supports this conclusion and suggests an attenuated response to FSH. In fact, our findings demonstrate that in the absence of GATA-4 or GATA-4 and GATA-6, the FSHR protein was undetectable by Western blot. We also demonstrated that GATA-4, by interacting with a GATA-RE, increases the activity of the *Fshr* promoter. FSH is crucial for follicle growth and granulosa cell differentiation, as pointed out by the phenotype observed in FSHR (36) and FSHβ subunit (37) knockout mice. FSHR- and FSH-deficient females are infertile due to a block in folliculogenesis before antral follicle formation. This block in folliculogenesis also occurs in the double GATA-4/6^{gcko} animals treated with PMSG, in which follicles do not grow beyond the multilayered preantral stage. At this point in follicular development, FSH responsiveness is essential for the formation of the antrum and growth to the preovulatory stage (38). Therefore, the down-regulation of the FSHR in the granulosa cells provides a molecular mechanism that explains the abnormal follicle growth and infertility of GATA conditional knockout animals.

On the other hand, FSH seems to be necessary to maintain GATA expression in the gonads of humans and rodents. Women carrying an inactivating mutation of the *Fshr* gene express little GATA-4 protein in their ovaries (39). As expected, the ovaries of these women lack significant follicular development. In mice, PMSG and FSH enhance the expression of GATA-4 and GATA-6 transcripts (7, 9). Moreover, we have previously demonstrated that FSH signaling leads to the phosphorylation of GATA-4 on serine 105 (9), a modification that is known to increase the transcriptional activity of GATA-4 (40). This evidence, along with the reduction in *Fshr* expression after loss of GATA function, suggests the presence of a positive feedback system between FSH and GATA in ovarian granulosa cells. We propose that this positive feedback is important for the differentiation of granulosa cells and the rapid growth of preantral and early antral follicle to the preovulatory stage.

Low FSHR expression accounts for the poor ovarian response to gonadotropin stimulation found in approximately 25% of women undergoing *in vitro* fertilization (8, 41, 42). There is no evidence suggesting that GATA factors could be involved in the regulation of FSHR expression in humans. It would be of great interest, then, to examine whether low levels of FSHR in poor-responding women correlate with defects in GATA-4 expression and/or activity.

Collectively, these data provide strong evidence that GATA-4 and GATA-6 have overlapping but essential roles in the ovary and both are needed to ensure proper follicle growth, granulosa cell differentiation, and female fertility. Additionally, our *in vivo* and *in vitro* findings are consistent with a role for GATA-4 in the regulation of FSHR expression in the ovary. Considering that FSH signaling is crucial for normal folliculogenesis, GATA-4 stimulation of the FSHR may be crucial for normal follicle development, granulosa cell differentiation, and, ultimately, female fertility. This evidence provides a likely molecular mechanism to explain the subfertility of single G4^{gcko}-knockout mice (this report and Ref. 18) and the infertility phenotype of the double GATA-4 and GATA-6 conditional knockout mice.

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References

1. **Viger RS, Guittot SM, Anttonen M, Wilson DB, Heikinheimo M** 2008 Role of the GATA family of transcription factors in endocrine development, function, and disease. Mol Endocrinol 22: 781–798
2. **LaVoie HA** 2003 The role of GATA in mammalian reproduction. Exp Biol Med (Maywood) 228:1282–1290
3. **Molkentin JD** 2000 The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. J Biol Chem 275:38949–38952
4. **Molkentin JD, Lin Q, Duncan SA, Olson EN** 1997 Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. Genes Dev 11:1061–1072
5. **Arceci RJ, King AA, Simon MC, Orkin SH, Wilson DB** 1993 Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. Mol Cell Biol 13:2235–2246
6. **Laitinen MP, Anttonen M, Ketola I, Wilson DB, Ritvos O, Butzow R, Heikinheimo M** 2000 Transcription factors GATA-4 and GATA-6 and a GATA family cofactor, FOG-2, are expressed in human ovary and sex cord-derived ovarian tumors. J Clin Endocrinol Metab 85:3476–3483
7. **Heikinheimo M, Ermolaeva M, Bielinska M, Rahman NA, Narita N, Hutaniemi IT, Tapanainen JS, Wilson DB** 1997 Expression and hormonal regulation of transcription factors GATA-4 and GATA-6 in the mouse ovary. Endocrinology 138:3505–3514
8. **González-Fernández R, Peña O, Hernández J, Martín-Vasallo P, Palumbo A, Avila J** 2010 FSH receptor, KL1/2, P450, and PAPP genes in granulosa-lutein cells from in vitro fertilization patients show a different expression pattern depending on the infertility diagnosis. Fertil Steril 94:99–104
9. **Kwintkiewicz J, Cai Z, Stocco C** 2007 Follicle-stimulating hormone-induced activation of Gata4 contributes in the up-regulation of Cyp19 expression in rat granulosa cells. Mol Endocrinol 21:933–947
10. **Stocco C** 2008 Aromatase expression in the ovary: hormonal and molecular regulation. Steroids 73:473–487
11. **Silverman E, Eimerl S, Orly J** 1999 CCAAT enhancer-binding protein β and GATA-4 binding regions within the promoter of the steroidogenic acute regulatory protein (StAR) gene are required for transcription in rat ovarian cells. J Biol Chem 274:17987–17996
12. **Gillio-Meina C, Hui YY, LaVoie HA** 2003 GATA-4 and GATA-6 transcription factors: expression, immunohistochemical localization, and possible function in the porcine ovary. Biol Reprod 68: 412–422
13. **Martin LJ, Taniguchi H, Robert NM, Simard J, Tremblay JJ, Viger RS** 2005 GATA factors and the nuclear receptors, steroidogenic factor 1/liver receptor homolog 1, are key mutual partners in the regulation of the human 3β-hydroxysteroid dehydrogenase type 2 promoter. Mol Endocrinol 19:2358–2370
14. **Tremblay JJ, Viger RS** 2001 GATA factors differentially activate multiple gonadal promoters through conserved GATA regulatory elements. Endocrinology 142:977–986
15. **Tremblay JJ, Viger RS** 1999 Transcription factor GATA-4 enhances Mullerian inhibiting substance gene transcription through a direct interaction with the nuclear receptor SF-1. Mol Endocrinol 13: 1388–1401
16. **Findlay JK, Drummond AE, Dyson M, Baillie AJ, Robertson DM, Ethier JF** 2001 Production and actions of inhibin and activin during folliculogenesis in the rat. Mol Cell Endocrinol 180:139–144
17. **Knight PG, Glister C** 2006 TGF-β superfamily members and ovarian follicle development. Reproduction 132:191–206
18. **Kyrönlahti A, Vetter M, Euler R, Bielinska M, Jay PY, Anttonen M, Heikinheimo M, Wilson DB** 2011 GATA4 deficiency impairs ovarian function in adult mice. Biol Reprod 84:1033–1044
19. **Thurisch B, Liang SY, Sarioglu N, Schomburg L, Bungert J, Dame C** 2009 Transgenic mice expressing small interfering RNA against Gata4 point to a crucial role of Gata4 in the heart and gonads. J Mol Endocrinol 43:157–169
20. **Morrisey EE, Ip HS, Tang Z, Parmacek MS** 1997 GATA-4 activates transcription via two novel domains that are conserved within the GATA-4/5/6 subfamily. J Biol Chem 272:8515–8524
21. **Nemer G, Nemer M** 2003 Transcriptional activation of BMP-4 and regulation of mammalian organogenesis by GATA-4 and -6. Dev Biol 254:131–148
22. **Divine JK, Staloch LJ, Haveri H, Jacobsen CM, Wilson DB, Heikinheimo M, Simon TC** 2004 GATA-4, GATA-5, and GATA-6 activate the rat liver fatty acid binding protein gene in concert with HNF-1α. Am J Physiol Gastrointest Liver Physiol 287:G1086–G1099
23. **Flück CE, Miller WL** 2004 GATA-4 and GATA-6 modulate tissue-specific transcription of the human gene for P450c17 by direct interaction with Sp1. Mol Endocrinol 18:1144–1157
24. **Fan HY, Liu Z, Cahill N, Richards JS** 2008 Targeted disruption of Pten in ovarian granulosa cells enhances ovulation and extends the life span of luteal cells. Mol Endocrinol 22:2128–2140
25. **Fan HY, Shimada M, Liu Z, Cahill N, Noma N, Wu Y, Gossen J, Richards JS** 2008 Selective expression of KrasG12D in granulosa cells of the mouse ovary causes defects in follicle development and ovulation. Development 135:2127–2137
26. **Sodhi CP, Li J, Duncan SA** 2006 Generation of mice harbouring a conditional loss-of-function allele of Gata6. BMC Dev Biol 6:19
27. **Pu WT, Ishiwata T, Juraszek AL, Ma Q, Izumo S** 2004 GATA4 is a dosage-sensitive regulator of cardiac morphogenesis. Dev Biol 275:235–244
28. **de Vries WN, Binns LT, Fancher KS, Dean J, Moore R, Kemler R, Knowles BB** 2000 Expression of Cre recombinase in mouse oocytes: a means to study maternal effect genes. Genesis 26:110–112
29. **Devine PJ, Payne CM, McCuskey MK, Hoyer PB** 2000 Ultrastructural evaluation of oocytes during atresia in rat ovarian follicles. Biol Reprod 63:1245–1252
30. **Jolly PD, Smith PR, Heath DA, Hudson NL, Lun S, Still LA, Watts CH, McNatty KP** 1997 Morphological evidence of apoptosis and the prevalence of apoptotic versus mitotic cells in the membrana granulosa of ovarian follicles during spontaneous and induced atresia in ewes. Biol Reprod 56:837–846
31. **Elmore S** 2007 Apoptosis: a review of programmed cell death. Toxicol Pathol 35:495–516
32. **Holtzinger A, Evans T** 2005 Gata4 regulates the formation of multiple organs. Development 132:4005–4014
33. **Zhao R, Watt AJ, Battle MA, Li J, Bondow BJ, Duncan SA** 2008 Loss of both GATA4 and GATA6 blocks cardiac myocyte differentiation and results in acardia in mice. Dev Biol 317:614–619
34. **Beuling E, Baffour-Awuah NY, Stapleton KA, Aronson BE, Noah TK, Shroyer NF, Duncan SA, Fleet JC, Krasinski SD** 2011 GATA factors regulate proliferation, differentiation, and gene expression in small intestine of mature mice. Gastroenterology 140: 1219–1229
35. **Bosse T, Piaseckyj CM, Burghard E, Fialkovich JJ, Rajagopal S, Pu WT, Krasinski SD** 2006 Gata4 is essential for the maintenance of jejunal-ileal identities in the adult mouse small intestine. Mol Cell Biol 26:9060–9070
36. **Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P** 1998 Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proc Natl Acad Sci USA 95:13612–13617
37. **Kumar TR, Wang Y, Lu N, Matzuk MM** 1997 Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 15:201–204

38. **Richards JS, Russell DL, Ochsner S, Hsieh M, Doyle KH, Falender AE, Lo YK, Sharma SC** 2002 Novel signaling pathways that control ovarian follicular development, ovulation, and luteinization. Recent Prog Horm Res 57:195–220
39. **Vaskivuo TE, Aittomäki K, Anttonen M, Ruokonen A, Herva R, Osawa Y, Heikinheimo M, Huhtaniemi I, Tapanainen JS** 2002 Effects of follicle-stimulating hormone (FSH) and human chorionic gonadotropin in individuals with an inactivating mutation of the FSH receptor. Fertil Steril 78:108–113
40. **Liang Q, Wiese RJ, Bueno OF, Dai YS, Markham BE, Molkentin JD** 2001 The transcription factor GATA4 is activated by extracellular signal-regulated kinase 1- and 2-mediated phosphorylation of serine 105 in cardiomyocytes. Mol Cell Biol 21:7460–7469
41. **Cai J, Lou HY, Dong MY, Lu XE, Zhu YM, Gao HJ, Huang HF** 2007 Poor ovarian response to gonadotropin stimulation is associated with low expression of follicle-stimulating hormone receptor in granulosa cells. Fertil Steril 87:1350–1356
42. **González-Fernández R, Peña Ó, Hernández J, Martin-Vasallo P, Palumbo A, Ávila J** 2011 Patients with endometriosis and patients with poor ovarian reserve have abnormal follicle-stimulating hormone receptor signaling pathways. Fertil Steril 95:2373–2378