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The Transcription Factor GATA4 is required for Follicular Development and Normal Ovarian Function

Evgeni Efimenko[a,*], **Maria B. Padua**[b,*], **Nikolay L. Manuylov**[a], **Shawna C. Fox**[a,b], **Deborah A. Morse**[b], and **Sergei G. Tevosian**[a,b,c,d]

[a]Department of Genetics, Dartmouth Medical School, Hanover, NH 03755, USA

[b]Department of Physiological Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL, 32610, USA

[c]Norris Cotton Cancer Center, Lebanon, NH, 03756, USA

Abstract

Sex determination in mammals requires interaction between the transcription factor GATA4 and its cofactor FOG2. We have recently described the function of both proteins in testis development beyond the sex determination stage; their roles in the postnatal ovary, however, remain to be defined. Here, we use gene targeting in mice to determine the requirement of GATA4 and FOG2 in ovarian development and folliculogenesis. The results from this study identify an essential role of the GATA4 protein in the ovarian morphogenetic program. We show that in contrast to the sex determination phase, which relies on the GATA4-FOG2 complex, the subsequent regulation of ovarian differentiation is dependent upon GATA4 but not FOG2. The loss of *Gata4* expression within the ovary results in impaired granulosa cell proliferation and theca cell recruitment as well as fewer primordial follicles in the ovarian cortex, causing a failure in follicular development. Preantral follicular atresia is observed within the few follicles that develop despite *Gata4* deficiency. The depletion of the follicular pool in GATA4 deficient ovary results in the formation of ovarian cysts and sterility.

Keywords

GATA4; FOG2; ovarian development

Introduction

GATA-binding proteins have been implicated as key regulators of ovarian gene expression. In mammals, six GATA-binding proteins (designated GATA1 to GATA6) have been

[d]Corresponding author: Dr. Sergei G. Tevosian, Department of Physiological Sciences, Basic Science Bldg. 206; Rm. B3-3, College of Veterinary Medicine, University of Florida, Gainesville, FL, 32610. Ph: (352) 294-4364; stevosian@ufl.edu.
[*]E.E. and M.B.P. contributed equally to this work

described. This family of structurally related transcription regulators use their double zinc finger domain to recognize the consensus sequence (A/T)GATA(A/G), known as the "GATA" motif, in target promoters. Ovarian expression of three GATA factors, GATA2, GATA4, and GATA6, has been reported. Among these three proteins, GATA4 has been studied most extensively because of its contribution to ovarian development and function. *Gata4* is detectable in somatic cells of the bipotential genital ridge (Anttonen et al., 2003; Defalco et al., 2011; Heikinheimo et al., 1997) and its expression continues in somatic cells upon sex determination of both XX and XY gonads (Ketola et al., 2000). At embryonic day (E)13.5, *Gata4* expression becomes sexually dimorphic: in the testis, GATA4 is notably up-regulated in the Sertoli cells compared to the interstitial cells, whereas in the ovary moderate levels of the protein are present in most somatic cells. In postnatal ovaries, GATA4 is conspicuously present in somatic cells and is prominently expressed within granulosa cells; however, GATA4 expression is downregulated in luteal cells of the corpus luteum (Anttonen et al., 2003).

Another GATA family member, *Gata6*, is also expressed in both the somatic and germ cells of the developing ovaries (Heikinheimo et al., 1997; Lavoie et al., 2004). It has been hypothesized that GATA4 and GATA6 carry (at least partially) overlapping functions in gonadal somatic gene regulation [(Robert et al., 2006) and, most recently, (Bennett et al., 2012)]. Alternatively, these GATA proteins may play non-overlapping roles in ovarian function. In contrast, *Gata2* expression has been documented only in germ cells during a narrow window of embryonic development (Bhardwaj et al., 2008; Siggers et al., 2002). Embryos carrying germline homozygous mutations of *Gata* genes are lethal before ovarian development can be examined (Kuo et al., 1997; Molkentin et al., 1997; Morrisey et al., 1998; Tsai and Orkin, 1997).

The multitype zinc-finger proteins of the FOG (Friend of GATA) family can modulate GATA regulatory activities (Tevosian et al., 2000). FOG1 and FOG2 are the two members of the FOG family in vertebrates, and FOG2 is the only member present in ovarian somatic cells. The expression of *Fog2* in the developing mouse gonads starts as early as E11.5 (Svensson et al., 1999; Tevosian et al., 1999). Subsequent characterization demonstrated that *Fog2* expression generally follows that of *Gata4* in both sexes until E13.5, when the expression of *Fog2* notably decreases in the fetal testis (Anttonen et al., 2003; Manuylov et al., 2007a). Although the expression of GATA4 and FOG2 is evident in fetal ovarian somatic cells, the number of cells expressing FOG2 is less than the number expressing GATA4 (Anttonen et al., 2003). In the postnatal ovary, FOG2 is expressed in the granulosa and theca cells of growing follicles in addition to the luteal cells of the corpus luteum (Anttonen et al., 2003).

Mouse fetuses carrying null alleles for *Fog2* ($Fog2^{-/-}$) die of cardiac defects at mid-gestation (Svensson et al., 2000; Tevosian et al., 2000). Because $Fog2^{-/-}$ embryos survive until approximately E14.0, analysis of early gonadal development in the absence of FOG2 has been previously described (Manuylov et al., 2008; Tevosian et al., 2002). Such analysis is not possible in $Gata4^{-/-}$ mutants because embryos die at E7.0–E9.5, before the appearance of the gonadal anlagen. A modified *Gata4* knock-in allele ($Gata4^{ki}$, a V217G amino acid substitution) that abrogates the interaction between GATA4 and FOG2 (Crispino

et al., 2001) was instrumental in establishing the role of GATA4 in mouse fetal development, including gonadogenesis. Homozygous *Gata4*^{ki} embryos survive until E13.0, when they die of cardiac abnormalities similar to those reported for the *Fog2*$^{-/-}$ embryos. Analysis of early gonadogenesis in the *Fog2*$^{-/-}$ and *Gata4*$^{ki/ki}$ mutants confirmed that the GATA4 and FOG2 proteins and their interaction are absolutely required for the sexual determination of both testes (Tevosian et al., 2002) and ovaries (Manuylov et al., 2008).

Although the *Gata4*^{ki} allele provided important insight into the function of the GATA4 protein during organogenesis, this knock-in mutation does not result in a loss of *Gata4* function. The GATA4^{ki} protein is specifically modified to hinder the binding of FOG partners, while all other GATA4 functions are expected to remain intact. Furthermore, the embryonic lethality of the *Gata4; Fog2* mutants precludes the examination of gonad development after birth. This lethality is particularly restrictive for the analysis of the ovarian developmental pathway because the full outcome of a gene loss in ovarian development may not be functionally appreciated until sometime after birth. In addition, the study of GATA4^{ki} mutants established that GATA4 and FOG2 worked as partners during the sex determination phase (Manuylov et al., 2008) but provided little insight into whether these proteins have separate functions later in ovarian development. Thus, the consequences of GATA4 loss in ovarian development remain to be determined. In the present study, we evaluated the roles of the GATA4 and FOG2 proteins and their interaction in ovarian development using conditional mouse models. In these mice, the steroidogenic factor 1/Nr5a1 (*Sf1*)-driven Cre recombinase (Bingham et al., 2006) limits the deletion of *Gata4* or *Fog2* to a subset of progenitor somatic cells within the gonads. We also generated double *Gata4; Fog2* mutants carrying simultaneous deletions of both genes. We then inspected the importance of the interaction between GATA4 and FOG2 using the *Sf1Cre; Gata4*$^{floxed/ki}$ mice. In these animals, the *Gata4*^{ki} allele serves as the sole source for the GATA4 protein (GATA4^{ki}) in ovarian somatic cells once the recombination of the *Gata4* floxed allele encoding the wild-type GATA4 protein takes place. Finally, we took advantage of another mouse strain that carries an inducible *CreERT2* under the control of the Wilms' tumor 1 (*Wt1*) promoter (Zhou et al., 2008) to determine the need for GATA4 at different stages of ovarian development.

Here, we provide evidence that firmly establishes the importance of GATA4 in ovarian and follicular development beyond the sex determination stage. In the absence of GATA4, folliculogenesis is markedly diminished, which leads to a drastic reduction in the number of developing follicles shortly after birth, the formation of ovarian cysts and sterility.

Results

Deletion of *Gata4* by *Sf1Cre* produced subtle changes in early ovarian development

Previous studies of *Gata4*^{ki} and *Fog2* null mutants demonstrated that a deficiency in the GATA4-FOG2 interaction leads to a block in ovarian sex determination and a down-regulation of the canonical Wnt signaling pathway (Manuylov et al., 2008). However, how GATA4 and FOG2 act during subsequent ovarian development and whether their functions diverge at a later stage both remain unknown.

To address these questions, we performed Cre-assisted deletions of the *Gata4* and *Fog2* genes using a Cre recombinase under control of the *Sf1* promoter. In these experiments, *Sf1Cre*-induced deletion of the *Gata4* and *Fog2* floxed alleles was highly effective during ovarian development. In the *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries, the reduction in the amount of the GATA4-positive cells was noticeable as early as E11.5 (data not shown); at E12.5, the GATA4 protein was practically absent from the somatic cells of the gonad. In contrast, the amount of cells positive for both PECAM-1/CD31, a protein expressed in endothelial and germ cells, and FOXL2, the earliest marker of granulosa cells (Schmidt et al., 2004), appeared similar in wild-type controls and mutant ovaries (Supplemental Fig. S1A–D). In the *Sf1Cre; Fog2*$^{floxed/floxed}$, the excision of *Fog2* was not apparent at E11.5, but the expression of the FOG2 protein was already diminished at E12.5 (data not shown) and markedly reduced by E13.5 (Supplemental Fig. S1E–F). These data are in agreement with our previous observations of *Gata4* and *Fog2* deletions upon *Sf1Cre* excision in the testis (Manuylov et al., 2011).

To determine the effect of *Gata4* or *Fog2* deletion during embryonic ovarian development, ovaries at E13.5 from wild-type controls and both conditional mutants were analyzed to determine the expression of ovarian-specific genes. Quantitative analysis by RT-PCR confirmed no significant difference in the relative expression of *Sf1* and *Mvh* genes compared to wild-type controls. Real-time PCR experiments also demonstrated a significant increase ($P < 0.05$) in the relative expression of *Gata6* and significant decrease ($P < 0.05$) in the relative expression of *Fst*, *Irx3* and *Foxl2* in the *Sf1Cre; Gata4* $^{floxed/floxed}$ ovaries (Fig. 1A). Similarly, *in situ* hybridization with RNA probes for *Irx3*, *Stra8*, *Wnt4*, *Fst*, *Foxl2* and *Sf1* did not reveal drastic changes in gene expression in the embryonic ovaries of *Gata4* and *Fog2* conditional mutants (Fig. 1B–D and data not shown).

The entrance of oogonia into meiosis is a defining event in ovarian gonadogenesis. Staining of postnatal day (PND) 1-ovarian sections for the meiotic marker Synapsin 1 (SYN1) (Dobson et al., 1994) showed a similar pattern of expression of SYN1 between oocytes from wild-type control and mutant ovaries (Supplemental Fig. S2A–C). Likewise, no apparent difference in the pattern of expression of WT1, another protein expressed in granulosa cells (Pelletier et al., 1991), was observed between controls and conditional mutants (Supplemental Fig. S2D–F). In addition, inspection of the developing gonads up to PND 1 did not uncover any notable changes in either size or morphology between conditional mutant ovaries and wild-type controls.

In summary, subsequent to the sex determination phase (as early as E13.5), *Gata4* deletion resulted in a detectable alteration in the expression of genes involved in the ovarian developmental pathway, but embryonic ovarian development in *Gata4* mutants appeared to proceed essentially normal.

GATA4 is absent in the early postnatal hypothalamus and pituitary gland

Sf1 regulatory elements target Cre expression to the somatic cells of the gonads and to other tissues, including the anterior pituitary and the ventromedial hypothalamic nucleus (Bingham et al., 2006). While embryonic ovarian development is independent of hormonal regulation (Couse et al., 1999; Simpson et al., 2002), in our analysis, it was essential to rule

out the possibility that extra-gonadal *Gata4* deletion could have an effect on the ovarian postnatal phenotype (i.e., folliculogenesis). Therefore, we examined GATA4 expression in organs where Sf1Cre is known to be active. To visualize Sf1Cre activity, we used the *ROSA26mT/mG* "switch" mice, in which a membrane-targeted red fluorescent protein (mT) is ubiquitously expressed before Cre-mediated excision and a membrane-targeted green fluorescent protein (mG) is expressed upon Cre excision (Muzumdar et al., 2007). Cells in which Sf1Cre was active were distinguishable by the expression of the membrane-bound eGFP. Anti-GATA4 antibody produced no specific staining in either the hypothalamus or pituitary at PND 1–8 (Supplemental Fig. S3A–F and data not shown). The integrity of the pituitary sample was further corroborated by staining for protein hormones such as α-GSU, TSH and LH, which are already produced by the gland at this time (Supplemental Fig. S3G–L and data not shown). In contrast, specific GATA4 nuclear staining was easily observed in other tissues (i.e., testis, specifically in Sertoli cells of the seminiferous tubule from an XY littermate, Supplemental Fig. S3M–O). These data corroborate that the early ovarian phenotype observed in *Sf1Cre; Gata4*$^{floxed/floxed}$ mutants (described below) is most parsimoniously explained by the loss of *Gata4* expression within the ovary and not elsewhere.

Only GATA4 is required for ovarian morphogenesis after birth

The *Sf1Cre*-induced deletion of *Gata4* does not affect viability, and mice lacking expression of GATA4 within the gonads can be analyzed throughout their lifetimes. Ovaries of *Sf1Cre; Gata4*$^{floxed/floxed}$ mice remained in their typical position inside the peritoneal cavity, although they were substantially smaller and the number of follicles was greatly reduced compared to wild-type control littermates (Fig. 2A–D). Moreover, when *Sf1Cre; Gata4*$^{floxed/floxed}$ adult females (n = 10) were housed for 2–8 months with fertile C57BL/6 males, none of the females developed visible pregnancies or delivered litters. Mice carrying simultaneous deletions of both the *Gata4* and *Fog2* genes were analyzed, and the ovarian phenotype observed for the *Sf1Cre; Gata4*$^{floxed/floxed}$*; Fog2*$^{floxed/floxed}$ (conditional double mutants) was found to be similar to that of *Sf1Cre; Gata4*$^{floxed/floxed}$ mice (Fig. 2I–J).

In contrast to the ovarian phenotype observed in *Sf1Cre; Gata4*$^{floxed/floxed}$ and *Sf1Cre; Gata4*$^{floxed/floxed}$*; Fog2*$^{floxed/floxed}$ mice, females with *Fog2* deletion alone (*Sf1Cre; Fog2*$^{floxed/floxed}$) were fertile and indistinguishable from the control littermates (Fig. 2E–F).

The importance of the GATA4-FOG2 interaction during sex determination has been established by analyzing fetuses with the *Gata4*$^{ki/ki}$ mutation (Manuylov et al., 2008; Tevosian et al., 2002). Analysis *Sf1Cre; Gata4*$^{ki/floxed}$ ovaries (in which the remaining GATA4^{ki} isoform expressed by the *Gata4*^{ki} allele is incapable of interacting with FOG2) showed no morphological differences with the wild-type littermates and females were fertile (Fig. 2G–H).

Considering that embryonic ovaries from the *Sf1Cre; Gata4*$^{floxed/floxed}$ mice had some changes in gene expression but no apparent changes in meiotic initiation, we hypothesized that the presence of functional *Gata4* becomes essential following birth. To test this hypothesis, we used the tamoxifen-inducible Wt1CreERT2 mouse (Zhou et al., 2008). Timed-pregnant *Wt1CreERT2; Gata4*$^{flox/flox}$ females were injected at E18.5 (just before

birth and initiation of ovarian folliculogenesis) with tamoxifen to induce Cre excision and ovaries from the control and mutant offspring were collected four weeks later. Staining of ovarian sections from *Wt1CreERT2; Gata4$^{floxed/floxed}$* at PND 28 for GATA4 demonstrated that tamoxifen-induced Cre recombination was highly effective and no GATA4 expression was detected (Fig. 2O, P). Hematoxylin and eosin staining of *Wt1CreERT2; Gata4$^{floxed/floxed}$* sections revealed an ovarian phenotype similar to the one observed for the *Sf1Cre; Gata4$^{floxed/floxed}$*, with smaller ovaries and reduced follicular population compared with controls (compare Fig. 2C, D to 2M, N).

In summary, these data indicate that GATA4 has an essential function in postnatal ovarian development and differentiation. In contrast, neither the GATA4-FOG2 partnership nor FOG2 function itself are necessary for the subsequent ovarian morphogenetic program.

Loss of *Gata4* function affects normal follicular development

It has been documented that major structural and developmental changes within the postnatal mouse ovaries happen during the first two weeks of life. The breaking of the oogonial nests initiates immediately after the time of birth, and most oocytes become surrounded by squamous granulosa cells by PND 3. This process is described as the formation of primordial follicles, an important step in the ovarian developmental program that depends on the intimate interaction between oocytes and pregranulosa cells. Granulosa cells of primordial follicles undergo a squamous-to-cuboidal transition and completely encircle the oocyte to advance to the next (primary) follicular stage. An intermediate (transitional) stage also exists and is defined as a follicle containing a mixture of cuboidal and squamous granulosa cells. Primary follicles further progress to a secondary stage in which two to three layers of granulosa cells accumulate and surround the developing oocyte and the outermost theca cell layer is recruited. The timing of follicular recruitment into the pool of actively developing follicles is not well understood. For example, while it is clear that the transition from primordial to primary follicle represents a regulated, irreversible maturation step, it has also been suggested that all unilaminar follicles (primordial, transitional and primary) represent the resting population (Da Silva-Buttkus et al., 2008; Meredith et al., 2000; Parrott and Skinner, 1999).

Because ovaries from *Sf1Cre; Gata4$^{floxed/floxed}$* females already harbor a greatly reduced number of mature follicles at PND 30 (Fig. 2D, J), we examined the dynamics of folliculogenesis in these mice at earlier stages of development. At PND 3, sections from wild-type controls and *Sf1Cre; Gata4$^{floxed/floxed}$* ovaries revealed follicles at the primordial stage, in which oocytes are surrounded by flattened granulosa cells, and at the primary stage, in which the granulosa cells are cuboidal (Fig. 3A–D). No significant difference, however, was observed in the number of primordial follicles between wild-type controls (43 ± 6) and *Sf1Cre; Gata4$^{floxed/floxed}$* ovaries (35 ± 2) (Fig. 3O). In contrast, the number of advanced follicles (containing cuboidal cells) was significantly reduced ($P < 0.001$) in the *Sf1Cre; Gata4$^{floxed/floxed}$* ovaries (5 ± 0.3) compared to those of wild-type littermates (15 ± 1) (Fig. 3O). Overall, oocyte development in PND 3 mutants appeared normal, and the expression of FOXL2 and the oocyte-specific protein mouse vasa homolog (MVH) were comparable between follicles from wild-type control and *Sf1Cre; Gata4$^{floxed/floxed}$* follicles (Fig. 3E–H).

At PND 7, numerous primary follicles were present in the wild-type control ovaries (Fig. 3I, K). In contrast, while follicles containing cuboidal granulosa cells could be easily observed in the mutant *Sf1CreGata4*$^{floxed/floxed}$ ovaries, most of them appeared abnormal and exhibited a transitional phenotype characterized by the persistence of squamous cells (Fig. 3J, L). Furthermore, while numerous secondary and higher-order multilayered follicles were present in the ovarian sections of wild-type controls, few such follicles were evident in the conditional mutants (Fig. 3I–L). Quantitatively, ovaries at PND 7 revealed a significant reduction ($P < 0.001$) in the number of developing follicles (13 ± 0.8 vs. 27 ± 1.4) between the *Sf1Cre; Gata4*$^{floxed/floxed}$ and wild-type control ovaries, whereas the number of primordial follicles in conditional mutants was significantly higher ($P < 0.001$) compared to wild-type controls (48 ± 2.2 and 20 ± 1.8, respectively) (Fig. 3P).

Previously, GATA4 has been shown to act as a regulator of cell growth and proliferation in tissues other than the gonads (Agnihotri et al., 2009; Rojas et al., 2008; Singh et al., 2010; Trivedi et al., 2010; Zeisberg et al., 2005). To determine whether ovaries from conditional mutants have altered cell proliferation or apoptosis, we performed BrdU-labeling (Fig. 3M, N) and TUNEL analysis. In the *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries at PND 7, the number of proliferating somatic cells was significantly reduced ($P < 0.001$) (86 ± 5.7) compared to controls (225 ± 9) (Fig. 3Q). There was, however, no difference in the number of apoptotic cells between wild-type controls and *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries (data not shown).

To explore whether *Gata4* deletion by *Sf1Cre* directly affected granulosa cells or had an indirect effect on oocyte development, we examined the expression of different oocyte and granulosa cell markers. Anti-Müllerian hormone (AMH) is expressed at specific follicular stages and is first detected in primary follicles; its expression peaks in granulosa cells of preantral and small antral follicles (Durlinger et al., 2002a; Durlinger et al., 1999; Durlinger et al., 2002b; Visser et al., 2006). Staining of sections with an antibody against AMH showed that wild-type ovaries possessed numerous AMH-positive follicles, while very few cells in the *Sf1Cre; Gata4*$^{floxed/floxed}$ mutants were positive for AMH (Fig. 4A, B). 3-beta hydroxysteroid dehydrogenase (3βHSD) was also found to be robustly expressed by granulosa cells in wild-type control ovaries (Fig. 4C), in which the expression was low in primary follicles but increased in follicles that had become multilayered. In contrast, in the *Sf1Cre; Gata4*$^{floxed/floxed}$ ovary, the expression of 3βHSD was hardly detectable (Fig. 4D).

Oocyte development appeared normal in the conditional mutant, and the expression of oocyte-specific proteins (MVH and GDF-9) was comparable between wild-type controls and *Sf1Cre; Gata4*$^{floxed/floxed}$ oocytes (Fig. 4A, B, E, F). However, the ovarian cortex of the *Sf1Cre; Gata4*$^{floxed/floxed}$ was depleted of primordial follicles compared to wild-type controls (Fig. 4A, B).

At PND 9, follicles in the wild-type ovaries continued to express AMH abundantly whereas in *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries, AMH staining was restricted to a few transitional primary follicles that were able to form in the absence of *Gata4* (Fig. 5A–F). We also noted that in wild-type ovaries, WT1 and 3βHSD were abundantly expressed by granulosa cells of multilayered follicles (Fig. 5G, I, K). Moreover, expression of 3βHSD in theca cells surrounding preantral follicles was also clearly visible in control ovarian sections (Fig. 5I,

K). This observation suggests that the majority of the follicles had advanced out of the quiescent (primordial) pool. However, in the mutant *Sf1Cre; Gata4*$^{floxed/floxed}$ sections, most follicles were still in the primordial and transitional primary stage, and multilayered follicles surrounded by positive 3βHSD-theca cells were very rare (Fig. 5H, J, L) because transitional primary follicles do not express 3βHSD. While *Gata4* loss in *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries was comprehensive, rare somatic cells retain GATA4 expression (Supplemental Fig. S4A–F).

In summary, morphological and molecular analysis indicates that formation of primordial follicles initiates normally, but subsequent follicular development is severely constrained by the loss of GATA4. Moreover, the failure of ovarian development in the conditional GATA4 mutant is associated with a decrease in cell proliferation, a decreased number of primordial follicles in the cortex and a marked reduction in the somatic ovarian expression of AMH and 3βHSD.

Characterization of adult ovaries at PND 90, 120 and 180

Inspection of adult ovaries from wild-type controls, *Sf1Cre; Fog2*$^{floxed/floxed}$ and *Sf1Cre; Gata4*$^{floxed/ki}$ mice showed a normal and healthy gross appearance (Fig. 7A, C). By contrast, ovaries from *Sf1Cre; Gata4*$^{floxed/floxed}$ mice were smaller and had visible cavities apparently filled with follicular fluid (Fig. 7E). Histological analysis of the *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries at PND 90, 120 and 180 revealed a few remaining follicles at the preantral and antral stages as well as hemorrhagic follicles and large cystic structures (Figs. 6C, E, F and 7F). These remaining follicles were not the result of the incomplete excision of *Gata4*, as they were negative for GATA4 staining (Supplemental Fig. S4A–D). Thus, the deletion of *Gata4* by *Sf1Cre* results in an extensive block in follicular development and the subsequent loss of follicles.

We also noted an abundance of autofluorescent particles in the *Gata4* conditional mutant ovaries. These inclusion bodies could be detected as early as PND 30 (data not shown) but were especially prominent during adulthood and resembled the accumulation of lipofuscin (Supplemental Fig. S5B). Lipofuscin is known as an aging pigment composed of highly oxidized and cross-linked proteins that accumulate in aged, postmitotic cells (Zimon et al., 2006). The presence of lipofuscin inclusions was further confirmed by staining sections of *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries with Sudan Black (Supplemental Fig. S5D).

Early follicular atresia in the *Gata4* conditional mutant ovaries

Once follicles reach the antral stage, they are either selected for ovulation or become atretic and undergo cell death (Matsuda et al., 2012). To determine the stage when follicles undergo atresia, adult ovarian sections (PND 90 and 120) from wild-type controls and *Sf1Cre; Gata4*$^{floxed/floxed}$ mice were stained for active caspase-3 (CASP3) and AMH. At both time points examined, preantral follicles of wild-type control ovaries had very few CASP3 positive cells, in which the majority of apoptotic cells were localized among granulosa cells of atretic antral follicles and luteal cells of regressing corpora lutea (Fig. 8A, B, E, F and data not shown). In contrast, in the *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries, active CASP3 positive cells were easily observed among granulosa cells, theca cells of preantral follicles, in the

few follicles that reached the antral stage and in the luteal cells of the corpus luteum (Fig. 8C, D, G, H and data not shown). Likewise, in sections of the *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries, we noticed the presence of cystic structures as well as autofluorescent particles that presumably contained lipofuscin.

Characterization of the cysts in the *Gata4* conditional mutants

Inclusion cysts, which are frequently observed in ovarian epithelial cancer, are generally thought to arise from either the coelomic-derived surface epithelium or the interconnecting epithelial cords and tubules of the intra-ovarian rete located within the hilum of the ovary (Tan et al., 2005). Previous studies demonstrated that GATA4 is strongly expressed in epithelial cells of ovarian surface epithelium (OSE) (Capo-chichi et al., 2003). To determine the origin of cysts observed in conditional mutants (Figs. 6C, E, F and 7F), we used antibodies against laminin and pan-cytokeratin, the latter of which recognizes a common epitope on the Type II cytokeratins. Epithelial cells typically express cytokeratin and are lined by a layer of laminin-positive basement membrane. We observed positive staining for both proteins (Fig. 9E, F), consistent with the epithelial origin of these cells. Unlike OSE, which is devoid of cilia, cells of the ovarian rete epithelium expose multiple motile cilia on their apical surfaces (Long, 2002; Tan et al., 2005). Staining for acetylated α-tubulin, a well-known marker of the ciliary axoneme, demonstrated that epithelial cells underlying the ovarian cysts in mutant ovaries expressed cilia on their apical surface (Fig. 9C, D). We also observed the internalization of ciliated crypts in the region of the ovarian hilum as early as PND 30 (Fig. 9A, B). Together, these data strongly suggest that the ovarian cysts observed in the *Gata4* conditional mutants originate not from the OSE but instead from epithelial cells of the intra-ovarian rete. Finally, to show that the cysts were not immature antral follicles, ovarian sections were stained for FOXL2, which was detected in the granulosa cells of the remaining follicles as well as in the luteal cells of the corpus luteum, but not in the cells underlying ovarian cysts in the *Gata4* mutants (Supplemental Fig. S6). During the reproductive cycle in mammals, ovarian inclusion cysts form periodically and are subsequently eliminated by apoptosis (Ghahremani et al., 1999). Apoptosis in the ovarian epithelial cells is under follicular control (Slot et al., 2006a; Slot et al., 2006b). Given the drastic decrease in the number of follicles in the *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries, we hypothesize that the formation of at least some cysts likely originates from the failure of this epithelial turnover process when it is no longer guided by the normal follicular cycle.

Follicular development in the *Gata4* conditional mutant ovaries cannot be rescued by exogenous gonadotropins

To assess the response of wild-type controls and *Sf1Cre*, *Gata4*$^{floxed/floxed}$ ovaries to exogenous gonadotropins, we performed a super-stimulation protocol in sexually immature mice (PND 30) by treating them with PMSG, followed by hCG 48 hours later. Ovaries from *Sf1Cre*, *Gata4*$^{floxed/floxed}$ were notably smaller compared to those of wild-type controls (Fig. 10A–D). Moreover, control ovaries responded to exogenous gonadotropins as was expected by producing a large number of preantral and antral follicles and several corpora lutea (Fig. 10A, B). In contrast, in the *Sf1Cre*, *Gata4*$^{floxed/floxed}$ ovaries, a great number of follicles were still at the primordial and primary stage of folliculogenesis and few follicles reached the antral stage (Fig. 10C, D). Few corpora lutea developed and some hemorrhagic follicles

were observed in the conditional mutant ovaries, whereas no hemorrhagic follicles were found in control ovaries. These results demonstrate that normal follicular development cannot be induced (rescued) by exogenous gonadotropins in the *Gata4* conditional mutant ovaries.

Discussion

We have previously shown that the GATA4-FOG2 complex and the FOG2 cofactor are required for embryonic ovarian development. Specifically, ovarian differentiation was arrested at the sex determination stage, and genes involved in ovarian somatic cell differentiation, including *Foxl2*, *Wnt4* and *Fst*, were not expressed in gonads from either $Fog2^{-/-}$ mice or $Gata4^{ki/ki}$ fetuses (Manuylov et al., 2008). In the present work, the roles of GATA4 and FOG2 in ovarian development were evaluated by the use of conditional deletions of both *Gata4* and *Fog2* genes and the outcomes were compared. We now demonstrate that in contrast to the sex determination phase, which relies on the functional GATA4-FOG2 complex, postnatal regulation of ovarian development is contingent upon GATA4 but not FOG2. The loss of *Gata4* expression results in impaired granulosa cell proliferation and the failure of follicular development. We also demonstrate that, unlike germline $Gata4^{ki}$ or *Fog2* null mutations, conditional deletion of *Gata4* by *Sf1Cre* produces negligible effects during ovarian embryonic differentiation. We observed no apparent changes in either size or gross morphology of embryonic and neonatal *Gata4* conditional mutant ovaries, although the expression of genes involved in ovarian differentiation was subtly altered. Meiosis appeared to initiate and proceed normally in the *Gata4* conditional mutants (Supplemental Fig. S2).

Ovarian differentiation is believed to initiate concurrently with that of the testis at approximately E11.5, coincidental with the expression of FOXL2 in a subset of XX somatic cell precursors. During this time, GATA4 is still present in the *Sf1Cre;* $Gata4^{floxed/floxed}$ gonads (Manuylov et al., 2011), and it is likely that this residual GATA4 activity is adequate to ensure the commitment of a sufficient number of somatic progenitor cells to become granulosa cells. To characterize the phenotype of GATA4 loss of function during the sex determination period, an approach different from the *Sf1Cre*-mediated deletion will be required. Nonetheless, the data presented here strongly argue that GATA4 is no longer required in embryonic ovaries once the sex determination checkpoint is cleared. This finding is notably different from the situation in the male gonads, where GATA4 is required to maintain the expression of specific genes in Sertoli cells and testis development throughout embryogenesis (Manuylov et al., 2011).

In contrast to the embryonic stage, postnatal gonads from female *Sf1Cre;* $Gata4^{floxed/floxed}$ mice revealed prominent consequences of GATA4 loss within the ovary, where delayed follicular development becomes noticeable as early as day 3. To reveal the molecular mechanism for the disruption in follicular development in *Sf1Cre;* $Gata4^{floxed/floxed}$ ovaries, we examined protein expression, cell proliferation and cell death between PND 7 and 9. At these stages, notable decreases in both ovarian size and follicular development were observed. Markers of differentiated granulosa cells, such as AMH and 3βHSD, were restricted to the few follicles that could develop in the conditional mutant ovaries. The

reasons for the partial rescue of follicular development in *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries are not clear; however, another GATA family member, GATA6, is expressed in ovarian somatic cells and may be able to partially rescue follicle development in the absence of GATA4 (Bennett et al., 2012). In this respect, *Gata6* expression in the *Sf1Cre; Gata4*$^{floxed/floxed}$ embryonic ovaries was significantly upregulated. Additionally, the few follicles in *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries could be derived from a recently proposed alternative source of granulosa cells in the adult female (Mork et al., 2011). Furthermore, results from the BrdU labeling experiments revealed a significant decrease in somatic cell proliferation in the *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries. Together, these data suggest that *Gata4* is required in the ovary to promote granulosa cell proliferation and to generate granulosa and theca cells in sufficient numbers to advance follicle development beyond the primary stage. Similarly, *Gata4* has been shown to regulate cardiomyocyte proliferation by directly binding and activating the *Cyclin D2* and *Cdk4* promoters (Rojas et al., 2008).

As described above, folliculogenesis in the GATA4 conditional mutant ovaries is severely reduced but not completely abolished. The few follicles that do persist achieve preantral and small antral stages and express AMH and 3βHSD, but not GATA4 (Figs. 3, 4 and Supplemental Fig. S4). This observation supports the conclusion that in *Sf1Cre; Gata4*$^{floxed/floxed}$ early postnatal ovaries, the absence of AMH/3βHSD-positive follicles represents a comprehensive failure for the majority of the granulosa cells to advance to the next developmental stage, not a loss of GATA4-dependent AMH- or 3βHSD transcriptional regulation in otherwise normal granulosa cells. This suggests a broad function of GATA4 in regulating the maturation of postnatal granulosa cells. Genes expressed at this developmental stage of follicular development have not been well-characterized (e.g., *3βHsd*; (Lavoie and King, 2009)). Given the reduction in AMH expression in the conditional mutant ovaries, the data presented here are consistent with previous observations that AMH could act to protect the primordial follicle niche in the ovarian cortex (Durlinger et al., 2002b).

During subsequent stages of development (e.g., PND 30), the *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries were notably smaller and contained few follicles; after PND 90, the ovary size decreased approximately threefold. Moreover, inclusion bodies, hemorrhagic follicles and large ovarian cysts were easily detected in ovaries of animals older than three months. Not surprisingly, *Sf1Cre; Gata4*$^{floxed/floxed}$ females were sterile. Similar phenotypes have been described in several mouse models with ovarian deficiency, including mice with *Cyp19* (aromatase) gene deletion (Britt et al., 2000) or mice carrying a constitutively active FSH receptor (Peltoketo et al., 2010), which likely reflects the presence of macrophages during massive ovarian tissue degeneration in these animals. Similarly, ovaries with hemorrhagic follicles have been previously described in mice with follicular degeneration (Peltoketo et al., 2010).

Ovarian cells are thought to undergo cell death by several mechanisms, including apoptosis, autophagy, necrosis or cornification (Young and McNeilly, 2010). Apoptosis occurs in antral follicles not selected for ovulation that become atretic (Matsuda et al., 2012). In this study, we showed that follicles from *Sf1Cre; Gata4*$^{floxed/floxed}$ adult ovaries underwent early atresia. As detected by an active CASP3 antibody, apoptotic cells were identified among

granulosa and theca cells of preantral follicles. Moreover, numerous apoptotic cells were detected among the granulosa cells of rare follicles that reached the antral stage. While we cannot define the ultimate fate of the primordial and primary follicles that form in the absence of *Gata4*, it is likely that once their development is arrested, they are eliminated through apoptosis.

In contrast to mice with the *Gata4* deletion, *Sf1Cre*-mediated loss of *Fog2* within the ovary does not result in any discernible phenotype. The *Sf1Cre; Fog2*$^{floxed/floxed}$ females are fertile and deliver normal, fertile offspring. In comparison, their male littermates carrying the *Sf1Cre*-mediated deletion of *Fog2* are sterile and undergo sex reversal (Manuylov et al., 2011). Similarly, in contrast to *Sf1Cre; Gata4*$^{floxed/floxed}$ mice, *Sf1Cre; Gata4*$^{/floxed/ki}$ females are fertile. Absence of the phenotype in these animals carrying a *Gata4*^{ki} mutation in addition to the *Sf1Cre*-mediated *Gata4* loss further strengthens the conclusion that FOG2 and GATA4-FOG2 interaction are dispensable for postnatal ovarian development. Indeed, it could be argued that the absence of an ovarian phenotype in female *Sf1Cre; Fog2*$^{floxed/floxed}$ mice compared to *Gata4* deletion is due to a delay or incomplete deletion of *Fog2* at E12.5 (Supplemental Fig. S1E–F). However, in the *Sf1Cre; Gata4*$^{floxed/ki}$ mice, it is the “flox” *Gata4* allele that is deleted, while the remaining *Gata4*^{ki} allele produces a protein incapable of binding FOG2.

We have previously described the constitutional *Fog2* null and *Gata4*^{ki} (where GATA4-FOG2 complex never forms) genotypes. In these animals, a loss of the early ovarian gene expression program is followed by a concomitant block in the pathway of granulosa cell differentiation (Manuylov et al., 2008). Those data define the GATA4-FOG2 complex as a key constituent in the initiation of ovarian-specific gene expression program, ovarian sex determination and the establishment of the granulosa cell fate. However, once the sex determination stage is completed, neither protein is required to maintain gene expression or embryonic development of the ovary. A similar situation occurs during the SOX9-dependent regulation of testis development where, despite its central role in the initiation of the male program, *Sox9* becomes dispensable after E14.5 (Barrionuevo et al., 2009; Chang et al., 2008). After birth, however, the process of folliculogenesis imposes an extensive, novel set of demands on ovarian cells requiring activation and possibly reemergence of transcriptional regulatory proteins to control gene expression, extensive cell proliferation and organ remodeling. At this time, GATA4 is called upon to regulate genes required for granulosa cell proliferation and theca cell recruitment. The loss of GATA4 caused a reduction in the number of developing follicles shortly after birth with subsequent cellular loss, formation of hemorrhagic follicles, ovarian cysts and therefore sterility.

Recently, Bennett et al. (2012) and Kyrönlahti et al. (2011) deleted *Gata4* in proliferating granulosa cells using *Cyp19*- or *Amhr2-Cre*, respectively (the recombination mediated by these Cre drivers is inefficient within the ovary until after birth) (Bennett et al., 2012; Kyrönlahti et al., 2011). Intriguingly, the *Amhr2Cre; Gata4*$^{floxed/floxed}$ adult ovaries had a similar phenotype to the *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries with cyst formation and other reproductive defects such as impaired fertility (Kyrönlahti et al., 2011). Immature *Amhr2Cre; Gata4*$^{floxed/floxed}$ mice exposed to exogenous gonadotropins had small ovaries with hemorrhagic follicles and cystic structures on the ovarian surface (Kyrönlahti et al.,

2011). A similar phenotype was observed in ovaries from immature *Sf1Cre; Gata4$^{floxed/floxed}$* mice treated with gonadotropins. Furthermore, follicular mice, as development was not rescued by exogenous gonadotropins in *Sf1Cre; Gata4$^{floxed/floxed}$* their ovaries had a great number of follicles that were still at the primordial and primary stage of folliculogenesis. In agreement, low levels of estradiol were detected in *Cyp19Cre; Gata4$^{floxed/floxed}$* females after stimulation with gonadotropins (Bennett et al., 2012). These results, taken together with the data obtained in this study, clearly demonstrate the important role of GATA4 in ovarian development and function.

Studies using genetically modified animals have identified genes associated with ovarian dysfunction in humans, including *Fmr1*, *Diaph2*, *Pof1b*, *Foxl2*, *Bmp15*, *Nobox*, *Figla* and *Sf1* (reviewed in (Persani et al., 2010)). These studies also suggest a direct link between fetal development and adult disease, as mutations in these genes disrupted the assembly and maturation of follicles in the developing ovary.

Conclusions

In this study, we show that the loss of GATA4, a key transcription factor that controls embryonic ovarian differentiation, results in sterility in female mice. We have demonstrated that GATA4 is essential for the regulation of specific steps during ovarian morphogenesis independent of its interaction partner, FOG2. Conditional deletion of *Gata4* within the ovary induced a failure of normal follicular development and differentiation, with reduced numbers of primordial follicles in the ovarian cortex and a defect in granulosa cell and theca cell recruitment. Mice with a conditional deletion of *Gata4* represent a novel genetic model for studying ovarian cystogenesis. Exploring downstream targets of GATA4 regulation should reveal novel genes critical to ovarian differentiation and development.

Materials and methods

Generation of mouse strains

Gata4$^{flox/flox}$(Zeisberg et al., 2005), *Fog2$^{flox/flox}$* (Manuylov et al., 2007b) and *Gata4^{ki}* (Crispino et al., 2001) mice were maintained on a mixed 129/C57BL/6 genetic background. Both Cre lines were maintained on a mixed 129/C57BL/6 background. The *Sf1Cre* transgenic mice (a kind gift from Dr. Keith Parker), in which a bacterial artificial chromosome (BAC) harboring *Sf1* regulatory elements directs Cre expression to the somatic cells of the gonads (as well as to a limited number of extra-gonadal tissues), have been previously described (Bingham et al., 2006). To produce strains with *Sf1Cre*-mediated deletions, “flox” mice were crossed with *Sf1Cre*-containing animals. The resulting *Sf1Cre; Gata4$^{floxed/+}$* and *Sf1Cre; Fog2$^{floxed/+}$* males were backcrossed with *Gata4$^{flox/flox}$* and *Fog2$^{flox/flox}$* females to generate conditional mutants with gonad-specific deletions (*Sf1Cre; Gata4$^{floxed/floxed}$* and *Sf1Cre; Fog2$^{floxed/floxed}$*). To obtain mutants with gonad-specific loss of the GATA4-FOG2 interaction, *Sf1Cre; Gata4$^{ki/+}$* females were crossed with *Gata4$^{flox/flox}$* males. Upon the excision of *Gata4* by Sf1Cre, the *Gata4^{ki}* allele remained as the only functional *Gata4* allele in the somatic cells of the progeny. The *Wt1CreERT2* line was obtained from the Jackson Laboratories and was previously described (Zhou et al., 2008). *Wt1CreERT2* mice were used to obtain the *Wt1CreERT2; Gata4$^{floxed/floxed}$* inducible line of

conditional mutants via an approach similar to the one described above for *Sf1Cre* animals. Mice were genotyped with primers listed in the Supplemental Table S1.

For timed pregnancies, noon of the day of plug observation was designated as E0.5. Timed-pregnant *Wt1CreERT2; Gata4*$^{flox/flox}$ females were injected at E18.5 (just before birth and initiation of ovarian folliculogenesis) intraperitoneally with tamoxifen (Sigma-Aldrich, St. Louis, MO) dissolved in corn oil at a dose of 0.2 mg/g of body weight. Four weeks later, ovaries from the offspring were collected in 4% (w/v) paraformaldehyde. All experiments were performed under approved animal protocols in accordance to the guidelines established by the University of Florida, Institutional Animal Care and Use Committees (IACUC).

In situ hybridization

In situ hybridization analysis was performed as previously described (Manuylov et al., 2007a). The *Wnt4* and *Fst* RNA probes have been described (Manuylov et al., 2008; Tevosian et al., 2002) and the *Irx3*-fragment-containing vector was a gift from Dr. Nef (Nef et al., 2005).

RNA Extraction

Total RNA was extracted from *Sf1Cre; Gata4*$^{floxed/floxed}$ E13.5 ovaries using TRIzol® reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, and concentration was determined using a NanoDrop Lite (Thermo Fisher Scientific Inc., Waltham, MA). Genomic DNA contamination was removed by treating RNA samples with DNase I (New England Biolabs, Ipswich, MA) according to the vendor's specifications.

Quantitative RT-PCR (qPCR)

The M-MLV (Moloney Murine Leukemia Virus) Reverse Transcriptase kit (Invitrogen) was utilized to synthesize complementary DNA from total RNA with oligo-dT primers, following the manufacturer's recommendations. The primers used for qPCR were manufactured by Integrated DNA Technologies (San Diego, CA) and are listed in Supplemental Table S2. qPCR reactions were performed with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) in a total volume of 20 μl containing 1 μl of cDNA. Transcript levels were analyzed on an ABI7500 real-time PCR system (Applied Biosystems) over 40 cycles of 95°C for 15 sec and 60°C for 1 min in a 2-step thermal cycle, preceded by two initial steps: 2 min at 50°C and 10 min at 95°C. Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) served as the normalizing gene to standardize qPCR data. Samples were analyzed in duplicates from at least 3 biological replicates, and the fold change was calculated by the ΔΔCt method. The Student's t test (two-tailed) was utilized for statistical analysis with significance considered at $P > 0.05$. The data analyzed were ΔCt values and results were plotted as fold-change differences using the GraphPad Prism (5.02 version) software.

Immunofluorescence

Immunofluorescence experiments were carried out on 7 μm cryosections of ovaries. After permeabilization and blocking [5% (w/v) nonfat dry milk; 0.15% (v/v) Triton X-100 dissolved in Dulbecco's phosphate buffered saline (Sigma-Aldrich)], sections were

incubated for 1 hour with the following primary antibodies: AMH, 3βHSD, GDF-9 (1:500) and acetylated g-tubulin (1:1000) from Santa Cruz Biotechnologies; GATA6 and MVH (1:300) from Abcam; Cytokeratin, pan (1:500; Sigma-Aldrich); FOXL2 (1:300; Everest Biotech); GATA4 N-terminal antibody (1:500; R&D); Laminin (1:500; Molecular Probes); WT1 (1:500; Epitomics) PECAM-1/CD31 (1:500; BD Biosciences); SYN1 (1:300; a gift from P. B. Moens). Sections were then incubated for 1 hour with the corresponding Alexa Fluor 488- and Alexa Fluor 555-conjugated secondary antibodies (1:500; Invitrogen). All antibodies were diluted in Dako antibody diluent (Dako). Slides were mounted in Vectashield containing DAPI (Vector Laboratories) and images of nuclear staining were taken on an Olympus BX51 microscope. Pictures shown are representative images of n = 3 from each genotype.

Hematoxylin and eosin (H&E) staining

Ovarian section of 6 μm of thickness were dewaxed and rehydrated followed by staining with modified Harris Hemotoxylin (Thermo Fisher Scientific Inc, Waltham, MA). After staining differentiation with acid-alcohol [1% (v/v) HCl in 70% (v/v) ethanol], Eosin-Y (Thermo Scientific) was used for counterstaining and Permount (Fisher Scientific) as mounting media. Pictures shown are representative images of either n = 2 or 3 from each genotype.

Quantification of ovarian follicles

Follicle quantification was achieved by serial-sectioning at 10 μm of thickness snap-frozen ovaries from each genotype at PND 3 and 7. Ovarian sections were stained with hematoxylin and eosin (H&E), photographed and saved as Adobe Photoshop files. Only follicles containing oocytes were classified as either primordial (an oocyte partially or completely encapsulated by flattened squamous pregranulosa cells) or developing (containing cuboidal granulosa cells in layers around the oocyte). Every fifth ovarian section was selected for follicle quantitation. The final data represent total follicle numbers per section. Comparison between wild-type controls and *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries was performed by the two-tailed *t* test using Microsoft Excel software. The data are presented as the means ± S.E.M. with n = 3 for both controls and *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries, and $P < 0.05$ was considered statistically significant.

Cell proliferation and cell death detection assays

Cell proliferation was analyzed by using the synthetic thymidine analog bromodeoxyuridine (BrdU). Mice were injected with BrdU (Sigma-Aldrich) at a dose of 0.1 mg/g of body weight two hours before euthanasia. BrdU incorporation was detected by using anti-BrdU Alexa Fluor 488-conjugated antibody (Invitrogen). Cell death was determined either by the terminal deoxynucleotide UTP nick-end labeling (TUNEL) in situ cell death detection kit (Roche) or a rabbit-anti mouse active CASP3 antibody (1:300; Abcam). Nuclei were counterstained with DAPI (Vector Labs).

Super-stimulation protocol

Wild-type controls (n = 4) and *Sf1Cre; Gata4*$^{floxed/floxed}$ (n = 4) immature mice (PND 30) were subjected to a super-stimulation protocol with pregnant mare serum gonadotropin (PMSG; 5IU; Sigma-Aldrich) followed by human chorionic gonadotropin (hCG; 5IU; Sigma-Aldrich) 48 hours later. After 24 hours of hCG administration, animals were euthanized and one ovary was fixed in Bouin's (Sigma-Aldrich) for histological analysis and the other ovary was fixed for 24 hours in 4% (w/v) paraformaldehyde for immunofluorescence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We report a key role for GATA4 protein in the ovarian morphogenetic program in mice.
- Subsequent to sex determination, ovarian development requires GATA4, but not FOG2.
- The loss of *Gata4* leads to a block in a key transition during follicular development.
- Depletion of the follicular pool results in the formation of ovarian cysts and sterility.
- *Gata4* might be considered as a candidate gene for ovarian dysfunction in humans

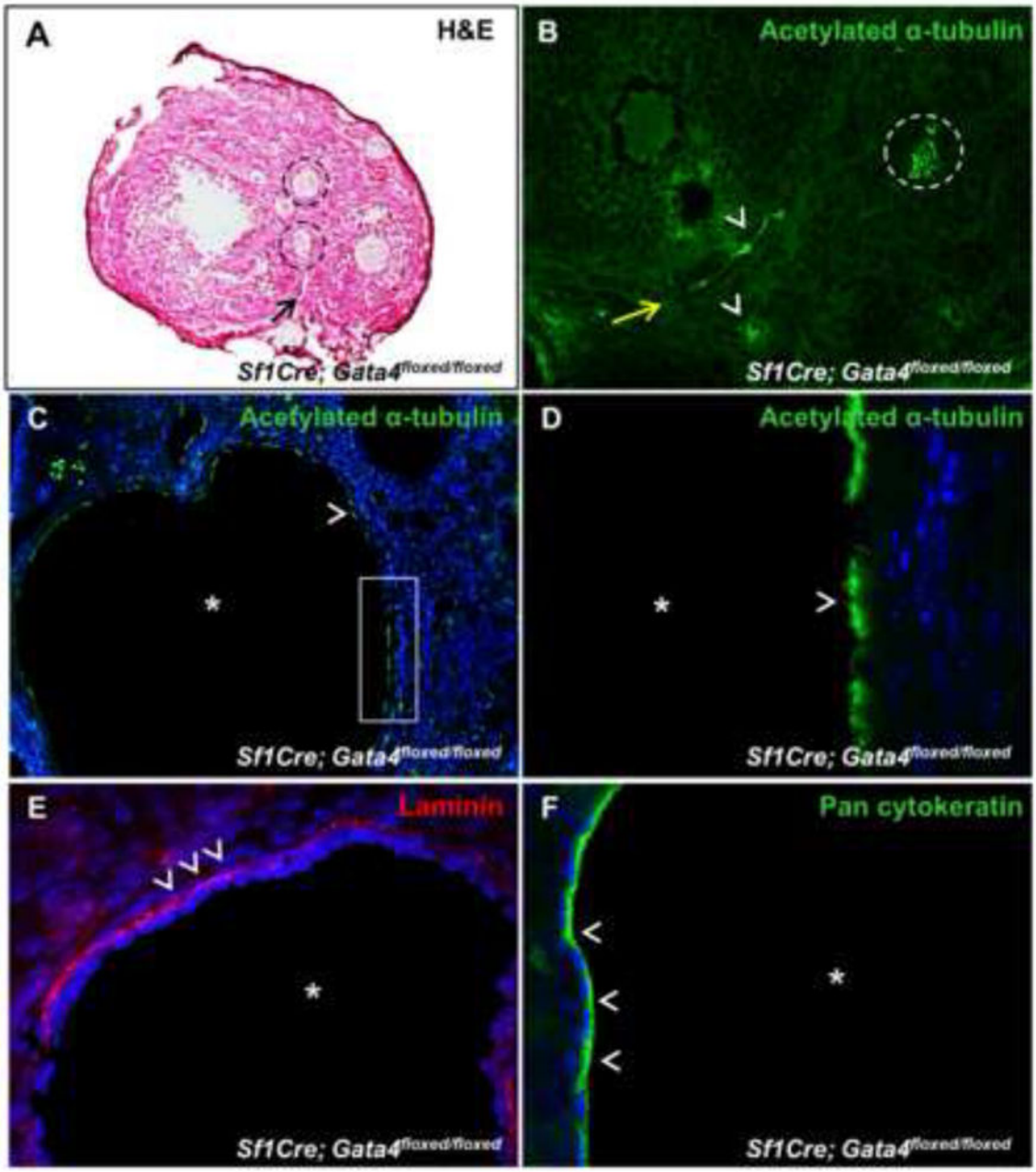

Figure 1. Embryonic expression of ovarian-specific genes upon *Sf1Cre*-mediated deletion of *Gata4* and *Fog2*

(A) Quantitative changes in gene expression of *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries at E13.5. Genes examined were *Mvh*, *Sf1*, *Gata6*, *Irx3*, *Fst* and *Foxl2* and results are shown as means ± S.E.M of fold change relative to wild-type controls. Wild-type control (B), *Sf1Cre; Gata4*$^{floxed/floxed}$ (C) and *Sf1Cre; Fog2*$^{floxed/floxed}$ (D) E13.5 ovaries were hybridized with RNA probes to *Irx3*. Gonad (g)-mesonephros (m) are oriented with the anterior facing the right.

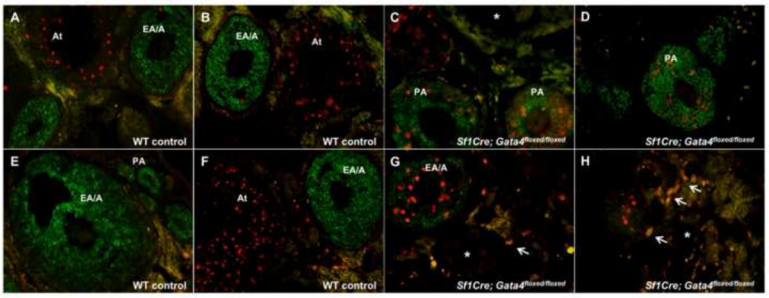

Figure 2. Histomorphological appearance of wild-type control and conditional mutant ovaries at PND 30

Gross morphological appearance and histological sections stained with hematoxylin-eosin (H&E) of wild-type control (A, B, I), *Sf1Cre; Gata4*$^{floxed/floxed}$ (C, D), *Sf1Cre; Fog2*$^{floxed/floxed}$ (E, F), *Sf1Cre; Gata4*$^{floxed/ki}$ (G, H) and the double mutant, *Sf1Cre; Gata4*$^{floxed/floxed}$*; Fog2*$^{floxed/floxed}$ (J) ovaries. Note that the double mutant ovary exhibits a similar phenotype to the *Sf1Cre; Gata4*$^{floxed/floxed}$ ovary. Pregnant females were treated with tamoxifen at E18.5 and ovaries from female offspring were analyzed at PND 28. Gross morphological appearance and histological sections stained with H&E of control (K, L) and *Wt1CreERT2; Gata4*$^{floxed/floxed}$ (M, N) ovaries at PND 28. Efficiency of the tamoxifen-induced CreERT2 recombinase-*Gata4* deletion (O, P). Numerous GATA4-positive somatic cells (*red*) were observed in the section of the Cre-negative control ovary (O), whereas no staining was observed in the somatic cells of the *Wt1CreERT2; Gata4*$^{floxed/floxed}$ ovary (P). Scale bars are 200 μm. Nuclear staining (DAPI) is blue.

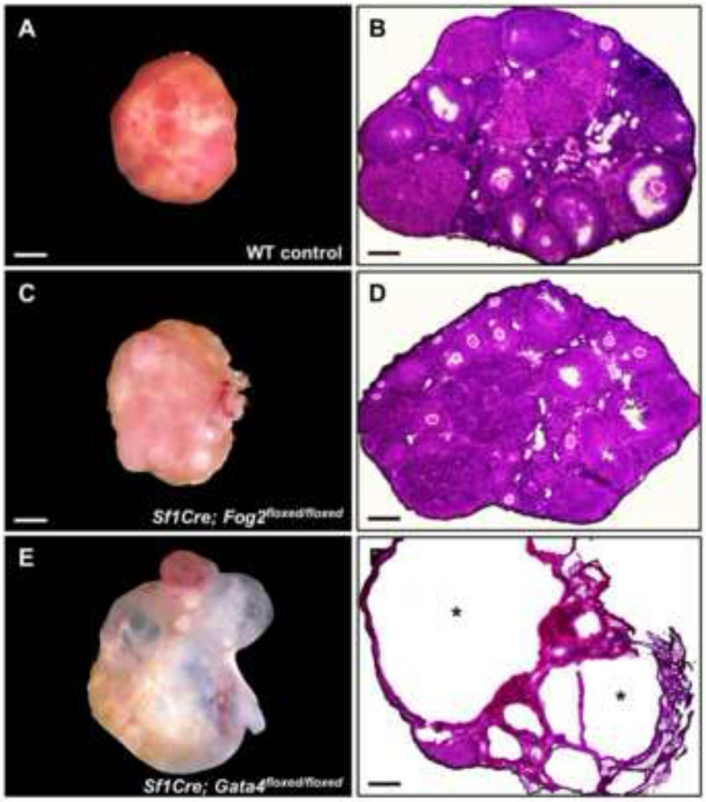

Figure 3. *Gata4* loss disrupts normal follicular development

Sections (A–H) of wild-type control (*left*; A, C, E, G) and *Sf1Cre; Gata4*$^{floxed/floxed}$ (*right*; B, D, F, H) ovaries at PND 3 were stained either with H&E (A–D) or for the germ cell marker, MVH (*red*; E, F) or the granulosa cell marker, FOXL2 (*green*; G, H). Panels C and D are higher magnification of sections A and B, respectively. Sections (I–N) of wild-type controls (I, K, M) and *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries (J, L, N) at PND 7. Sections in panels I and J were stained with H&E and panels K and L are higher magnifications of sections I and J, respectively. Primordial and primary follicles are shown by arrowheads. PA, preantral follicle. Analysis of cell proliferation in the wild-type control (M) and *Sf1Cre; Gata4*$^{floxed/floxed}$ (N) ovaries from mice treated with BrdU. Sections were stained with an antibody against BrdU (*green*) and DAPI (*blue*) for nuclear staining. Note the decrease in BrdU-positive cells in the conditional mutant ovary (N). Scale bars in panels C, D, K and L are 20 μm and in all other panels 100 μm. Quantitation of follicles and proliferating cells in wild-type control and *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries. (O) Total number of primordial and developing follicles at PND 3 in wild-type (WT) control (black) and *Sf1Cre; Gata4*$^{floxed/floxed}$ (grey) ovaries. (P) Total number of primordial and developing follicles at PND 7 in wild-type control and *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries. (Q) Total number of BrdU-positive cells in wild-type control and *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries. The data are presented as means ± S.E.M. from three independent samples (n = 3) of each genotype (wild-type control and *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries). $^{**}P < 0.001$.

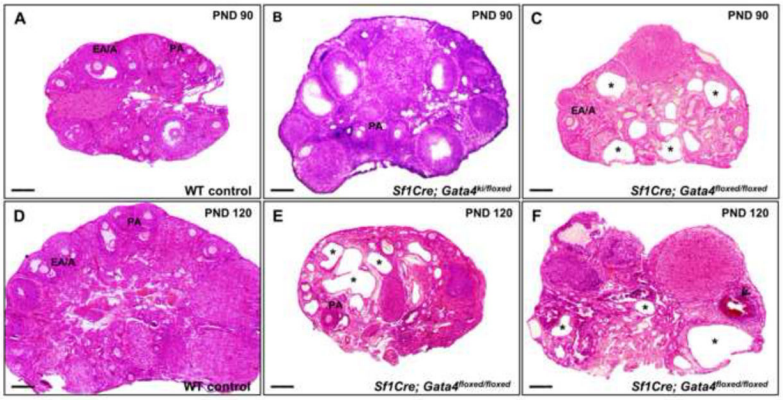

Figure 4. Ovarian expression of granulosa and germ cell proteins at PND 7

Sections of wild-type control (*left*; A, C, E) and *Sf1Cre; Gata4*$^{floxed/floxed}$ (*right*; B, D, F) ovaries were stained for AMH (*red*) and MVH (*green*) (A and B); 3βHSD (*red*) and GATA6 (*green*) (C and D) or GDF-9 (*green*; G and H). Note the reduction in expression of AMH and 3βHSD in the ovaries from the conditional mutant animals. Additionally, numerous follicles are present in the ovarian cortex of the wild-type control (white arrows in panel A) but absent in the conditional mutant, which also exhibits a decrease in advanced-stage follicles (B). The expression of MVH, GATA6 and GDF-9 in germ cells appeared similar between wild-type controls and conditional mutant ovaries.

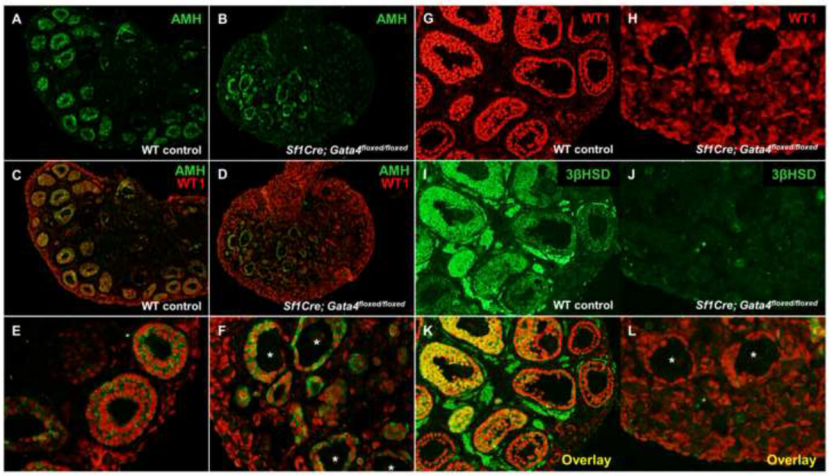

Figure 5. Arrested follicles in ovaries of the conditional mutants express AMH, but not 3βHSD at PND 9

Sections of wild-type control (A, C, E) and *Sf1Cre; Gata4*$^{floxed/floxed}$ (B, D, F) ovaries stained for AMH (*green*; A–F) and WT1 (*red*; C–F). Panels E and F are higher magnifications of C and D, respectively. Staining of ovarian sections from wild-type controls (G, I, K) and *Sf1Cre; Gata4*$^{floxed/floxed}$ (H, J, L) with antibodies against WT1 (*red*; G, H, K, L) and 3βHSD (*green*; I, J, K, L). Note that few transitional follicles in the *Sf1Cre; Gata4*$^{floxed/floxed}$ (asterisks in panels F and L) express AMH (B, D, F), but not 3βHSD (J, L). Additionally, note in Panels J and L the lack of theca cells surrounding preantral follicles.

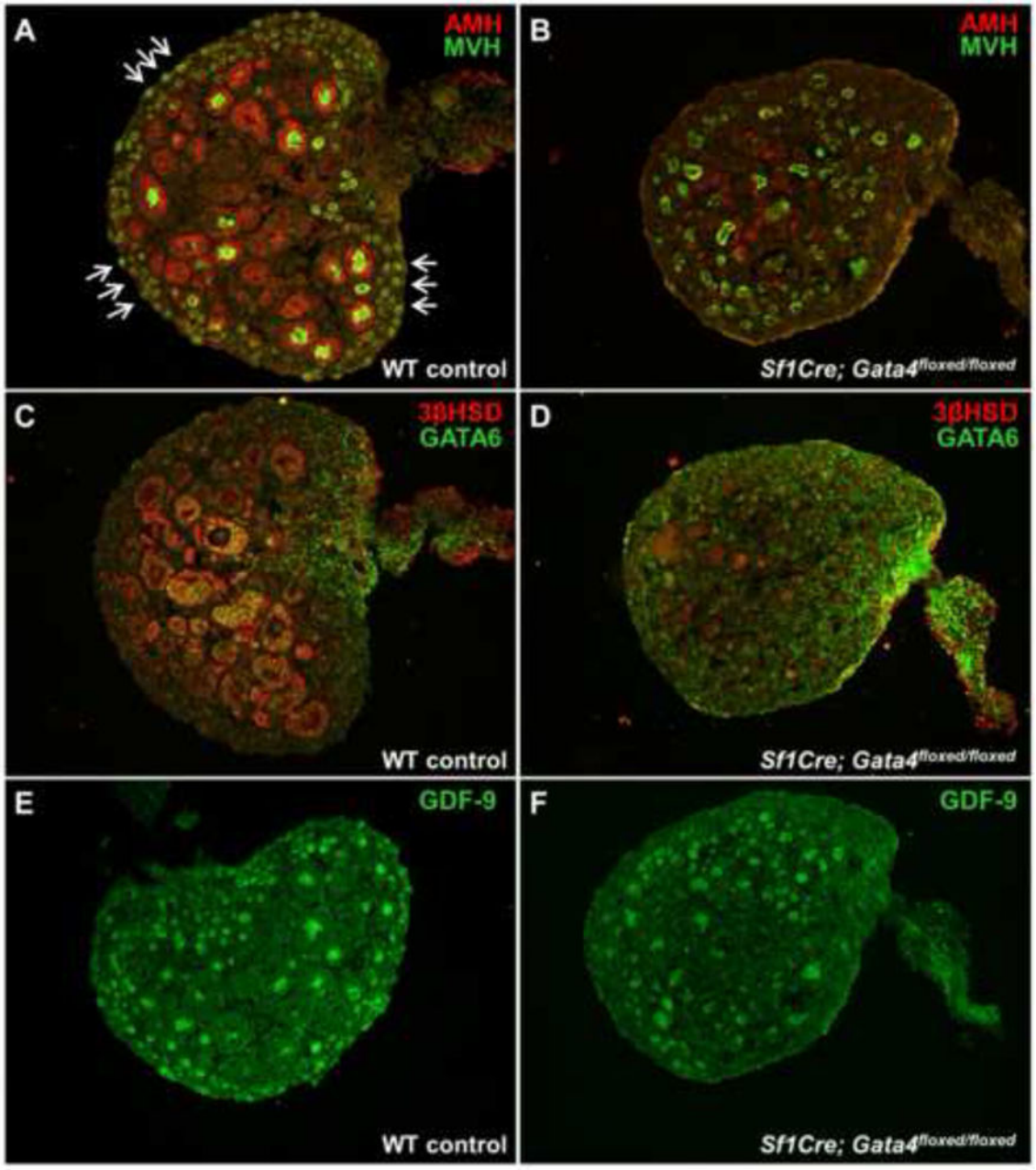

Figure 6. Histological sections of wild-type control and conditional mutant ovaries at PND 90 and 120

Representative histological sections stained with hematoxylin-eosin (H&E) of wild-type controls (A, D), *Sf1Cre; Gata4*$^{floxed/ki}$ (B) and *Sf1Cre; Gata4*$^{floxed/floxed}$ (C, E, F). Asterisks denote ovarian cysts in panels C, E and F. Arrowhead shows a hemorrhagic follicle. PA, preantral follicle; EA/A, early antral/antral follicle. Scale bars are 200 μm.

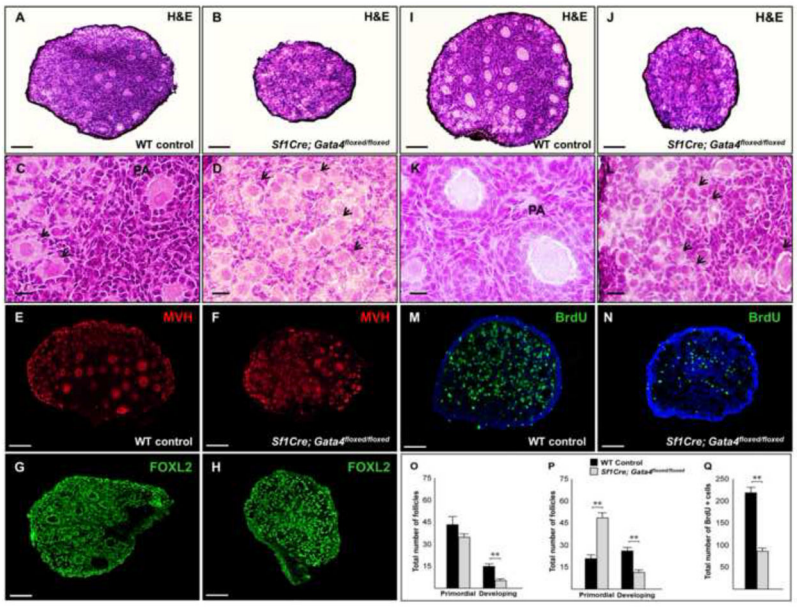

Figure 7. Histomorphological appearance of wild-type control and conditional mutant ovaries at PND 180

Gross morphological appearance and histological sections stained with hematoxylin-eosin (H&E) of wild-type control (A, B), *Sf1Cre; Fog2*$^{floxed/floxed}$ (C, D) and *Sf1Cre; Gata4*$^{floxed/floxed}$ (E, F) ovaries. Asterisks denote ovarian cysts in panel F.

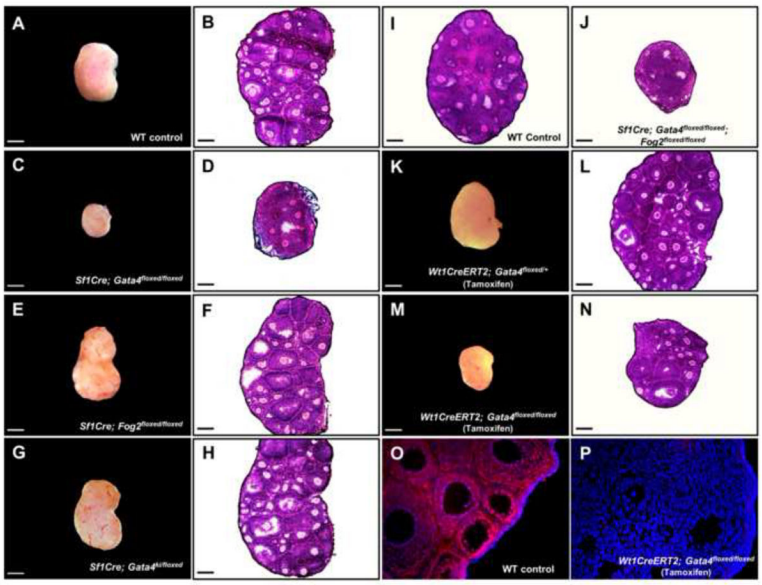

Figure 8. Preantral follicular atresia in the *Gata4* conditional mutant ovaries

Representative sections of wild-type controls (A, B, E, F) and *Sf1Cre; Gata4*$^{floxed/floxed}$ (C, D, G, H) ovaries at PND 90 (A, B, C, D) and 120 (E, F, G, H) stained for active CASP3 (*red*) and AMH (*green*). In control ovaries, active CASP3-positive cells were localized in granulosa cells of atretic antral follicles and very few CASP3 positive cells in early antral follicles. In the *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries active CASP3-positive cells were detected in granulosa and theca cells of preantral follicles. In Panels G and H, arrows indicate autofluorescent particles (presumably lipofuscin) and asterisks denote cystic structures in sections of the *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries. PA, preantral follicle; EA/A, early antral/antral follicle; At, atretic antral follicle. Nuclear staining (DAPI) is blue.

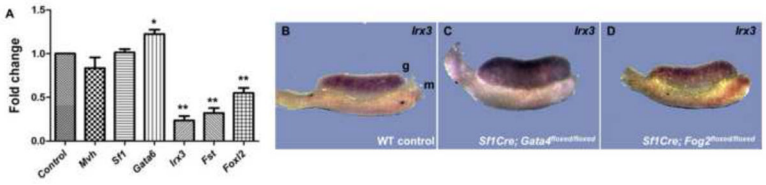

Figure 9. Epithelial cysts in the *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries are derived from the ovarian rete

Sections of *Sf1Cre; Gata4*$^{floxed/floxed}$ ovaries at PND 30 stained with either H&E (A) or an antibody against acetylated α-tubulin (B). Arrowheads indicate ciliated epithelial cells, arrows point the direction of epithelial invagination and dashed circles denote cysts. Ovarian sections of *Sf1Cre; Gata4*$^{floxed/floxed}$ at PND 180 stained for either acetylated α-tubulin (*green;* C), laminin (red; F) or pan-cytokeratin (*green*; E). Arrowheads in panels C–F show ovarian epithelial cells and asterisks denote ovarian cysts. Panel D shows a higher magnification of a boxed area inside panel C.

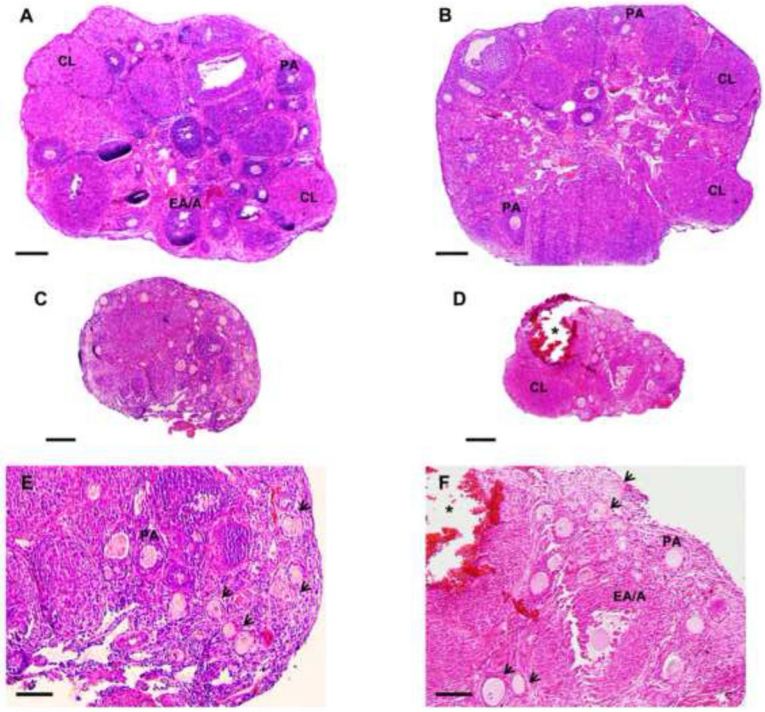

Figure 10. Follicular development cannot be rescued by exogenous gonadotropins in the *Gata4* conditional mutant ovaries

Representative histological sections stained with hematoxylin-eosin (H&E) of ovaries from wild- type controls (A, B) and *Sf1Cre; Gata4*$^{floxed/floxed}$ (C, D) immature mice (PND 30) that were subjected to a super-stimulation protocol with PMSG followed by hCG 48 hours later. Panels E and F are higher magnifications of C and D. Primordial and primary follicles were present in the conditional mutant ovaries (shown by arrowheads). Asterisk denotes a hemorrhagic follicle. PA, preantral follicle; EA/A, early antral/antral follicle; CL, corpus luteum. Scale bars are 200 μm (A, B, C, D) and 100 μm (E, F).