Ovarian development in mice requires the GATA4-FOG2 transcription complex

Nikolay L. Manuylov, Fatima O. Smagulova*, Lyndsay Leach and Sergei G. Tevosian†

We have demonstrated previously that mammalian sexual differentiation requires both the GATA4 and FOG2 transcriptional regulators to assemble the functioning testis. Here we have determined that the sexual development of female mice is profoundly affected by the loss of GATA4-FOG2 interaction. We have also identified the Dkk1 gene, which encodes a secreted inhibitor of canonical β-catenin signaling, as a target of GATA4-FOG2 repression in the developing ovary. The tissue-specific ablation of the β-catenin gene in the gonads disrupts female development. In Gata4ki/ki; Dkk1+/− or Fog2−/−; Dkk1−/− embryos, the normal ovarian gene expression pattern is partially restored. Control of ovarian development by the GATA4-FOG2 complex presents a novel insight into the cross-talk between transcriptional regulation and extracellular signaling that occurs in ovarian development.

KEY WORDS: Fog2 (Zfpm2), Gata4, Dkk1, Ovary, β-catenin, Mouse

INTRODUCTION

Male sex determination in the majority of mammals is initiated by Sry, the Y chromosome-linked testis-determining gene (reviewed by Capel, 2000; Swain and Lovell-Badge, 1999; Wilhelm et al., 2007). By contrast, genetic mechanisms of development remain enigmatic for mammalian females. The idea that ovarian differentiation requires its own set of genes and occurs shortly after (Eicher and Washburn, 1986), or even precedes (McElreavey et al., 1993), that of the testes was put forward a number of years ago. However, sexually dimorphic expression in the developing ovary was not identified until fairly recently. This new evidence clearly demonstrates that the female pathway of development engages a number of dedicated genes. The two alternative sex fates are thought to emerge through the antagonistic activities of sex-specific transcription factors in a restricted number of gonadal cells. This initial cell-fate decision is further expanded by extracellular non-cell-autonomous signals that promote one developmental program, while at the same time suppressing the other (Brennan and Capel, 2004; Capel, 2006; Kim and Capel, 2006; Kim et al., 2006).

We have previously demonstrated an in vivo requirement for the transcription factor GATA4 and its co-factor FOG2 (ZFP2 – Mouse Genome Informatics) in testis differentiation (Tevosian et al., 2002). Gata4ki/ki mutants [ki is a V217G mutation in GATA4 that specifically cripples the interaction between GATA4 and FOG proteins (Crispino et al., 2001)], as well as Fog2-null embryos (Tevosian et al., 2000), exhibit a profound early block in testis differentiation. Here, we demonstrate that a deficiency in GATA4-FOG2 interaction leads to a block in ovarian development coincident with a drastic alteration in the female gene expression program.

To avoid referring each time to both FOG2 null and GATA4ki mutants we will sometimes refer to them collectively as ‘GATA4/FOG2’ mutants (and to the phenotype as the ‘GATA4-FOG2 interaction/complex loss’). This is justified, as the abrogation of GATA4-FOG2 interaction by a Gata4ki mutation (Gata4ki/ki) or Fog2 loss (Fog2−/−) results in equivalent defects in mouse gonadal differentiation in every experiment we have performed so far. It is formally possible, however, that these mutations have non-overlapping roles in gonadogenesis (for example, FOG2 could have a GATA4-independent function; and the ‘ki’ mutation in GATA4 also renders it incapable of interacting with FOG1). Hence, we performed experiments with both mutants to eliminate this possibility. Importantly, the Gata4ki mutation is not a Gata4 loss of function; deletion of Gata4 gene in gonads may have a different outcome that does not necessarily phenocopy Fog2 gene loss or the Gata4ki/ki phenotype.

MATERIALS AND METHODS

Animals

The generation and genotyping of Fog2-null and Gata4ki-targeted animals have been described previously (Crispino et al., 2001; Tevosian et al., 2000). These strains, as well as Wnt4−/− mice (Vainio et al., 1999), were maintained on the C57BL/6 background. β-catenin (Ctnnb1) mutant mice were obtained from the Jackson Laboratory. The Flk-1-lacZ strain has been genotyped, as previously described (Shalaby et al., 1997). The Dkk1−/− genotype was determined using primers dkk1 (5′-CTTCCGACACAAACACTCCC-3′) and dkk1rev (5′-GTAAACCAAACTCTCGTTCAGC-3′). Sf1-Cre mice were genotyped with Cre-specific primers as previously described (Bingham et al., 2006). Axin2−/− mice (Yu et al., 2005) were obtained from the EMMA repository.

Affymetrix microarray analysis of gene expression

Gonad-mesonephros complexes were dissected from E12.5 XX wild-type and Gata4ki/ki mutant embryos and Affymetrix oligonucleotide arrays were used for RNA expression analysis (Chee et al., 1996; Lipshutz et al., 1999). The array experiment was performed by Dartmouth Genomic and Microarray Laboratory according to a standard protocol. The microarray data have been deposited at the GEO database (GSE11134) and were analyzed using Gene Traffic (lobion Informatics).

In situ hybridization

In situ hybridization (ISH) analysis was carried out essentially as previously described (Manuylov et al., 2007a). Sos9, Mis (Amh), Wnt4, Cyp17a1, Hsd3b1 and Cyp11a1 RNA probes have been described (Tevosian et al., 2002); the fxs3-fragment-containing vector was a gift of Dr Nef (Nef et al., 2005) and the Gng12-containing vector was a gift of Dr Arango (Fujino et al., 2007). Other probes were generated with cDNA obtained from the embryonic total or gonadal RNA by RT-PCR (Table 1).
Table 1. PCR primers and hybridization probes

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*Ken Allbritton, personal communication.

Real-time PCR

Individual gonad-mesonephros complexes were dissected from E11.5-14.5 embryos and RNA was isolated using the RNeasy Mini Kit (Qiagen). All real-time PCR assays were carried out using the SYBR Green I Kit (Applied Biosystems) as previously described (Manuylov et al., 2007b; Smagulova et al., 2008). The relative expression level for each sample was determined in the same run and was expressed as the ratio of the quantity of RNA of interest to that of a control RNA (Gapdh). Gene-specific primers and probes were designed using Primer Express (Perkin Elmer); primer sequences are shown in Table 2 and are also available upon request.

Immunofluorescence

For protein detection, the following antibodies and dilutions were used: mouse anti-DKK1 (R&D Systems; 1:300); rabbit anti-FOXL2 (a gift of Drs Fellous and Veitia, Université Denis, Diderot, Paris, France; 1:300); rat anti-PECAM1 (BD Biosciences; 1:500); rabbit anti-β-PECAM1 (Upstate; 1:300); rabbit anti-SY1 (a gift of Drs Moens and Spyropoulos, York University, Toronto, Canada; 1:300); and rabbit anti-Fog2 (a gift of Drs Fellous and Veitia, Université Denis, Diderot, Paris, France; 1:300) and rabbit anti-mouse anti-DKK1 (R&D Systems; 1:300); rabbit anti-FOXGL2 (a gift of Drs Mann and Page, 2002). The slides were mounted in Vectashield with DAPI (4’,6-diamidino-2-phenylindole, Vector Labs) and photographed. The confocal analysis of anti-PECAM1-stained gonads was performed as described (Manuylov et al., 2007a).

Germ cell depletion

Germ cell depletion was performed as described (Yao et al., 2003). The depletion was confirmed by anti-PECAM1 ISH and alkaline phosphatase staining. To generate W/Wv embryos depleted of germ cells, male mice carrying the c-Kit mutation, dominant white spotting (W), were mated to females carrying viable dominant spotting (W') (Mintz and Russell, 1957). Double heterozygotes depleted of germ cells were identified by immunostaining one gonad from each pair with the germ cell marker PECAM1.

Table 2. PCR primers for real-time PCR

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*Jorgensen and Gao, 2005.

RESULTS

Normal ovarian differentiation requires GATA4-FOG2 interaction

In order to identify the targets of GATA4-FOG2 during ovarian development, we performed an Affymetrix microarray analysis of gene expression, comparing RNA samples from Gata4–/– null and wild-type (control) E12.5 ovaries. Two independent probe sets corresponding to the follistatin (Fst) gene on the microarray showed the most dramatic downregulation among all the differentially expressed genes (~12-fold). The Fst gene encodes a secreted protein that blocks the function of multiple members of the TGFβ superfamily (Patel, 1998). Although Fst expression in embryonic gonads had been reported a number of years ago (Feijen et al., 1994), its sexually dimorphic pattern was not appreciated until recently (Menke and Page, 2002). Both in situ hybridization (ISH) with an antisense Fst probe (Fig. 1A, B) and real-time RT-PCR (Fig. 1L) confirmed the loss of Fst expression in Gata4–/–Fog2 mutants.

Fst is a downstream component of Wnt4 signaling (Yao et al., 2004). Wnt4 controls Fst and Bmp2 (bone morphogenetic protein 2) expression in embryonic ovaries; in Wnt4–null XX E12.5 gonads, expression of both Fst and Bmp2 is lost. The Wnt4-Fst pathway
opposes the formation of the male-specific vasculature in the female and ensures the survival of meiotic germ cells; this constitutes the first established signaling pathway important for early development of the mammalian ovary (Yao et al., 2004).

Wnt4 expression was lost in the gonad of E12.5 XX Fog2-null mutants (Fig. 1D). Importantly, although E12.5 Gata4/Fog2 mutant XX gonads did not express Wnt4, weak but detectable Wnt4 expression reappeared in the E13.5 mutant gonads (Fig. 1F). Wnt4 expression is also observed in XY E13.5-14.5 Fog2 mutant gonads (Tevosian et al., 2002).

Based on the previously established epistatic relationship between Wnt4 and Bmp2, we expected Bmp2 expression to be similarly absent from the Gata4/Fog2 mutant gonads. This, however, was not the case: ISH with a Bmp2 probe and real-time PCR analysis revealed no change in Bmp2 expression in either the Fog2 (Fig. 1H,K,L) or Gata4/Fog2 (Fig. 1L) mutant sample and, in agreement, our microarray analysis showed no difference for the Bmp2 probe set (data not shown). Interestingly, a recently described XX Rspo1–/– mutation demonstrates a similar relationship between Wnt4 and Bmp2: Wnt4 expression is temporarily lost in the XX E12.5 gonad (but not mesonephros), whereas Bmp2 levels remain unchanged (Chassot et al., 2008). In summary, loss of GATA4-FOG2 interaction in the XX gonad results in the loss of Fst expression and a failure to activate Wnt4 in the gonad at E12.5; however, residual Wnt4 expression appears to be sufficient for maintaining the normal level of Bmp2.

GATA4/FOG2 loss affects multiple aspects of early ovarian differentiation

In addition to affecting Wnt4 and Fst expression, as described above, loss of the GATA4-FOG2 complex disrupts the expression of numerous other genes that have been implicated in ovarian development. Expression of the dimorphically expressed genes, Spry2 (small proline-rich 2d) (Beverdam and Koopman, 2006) and Foxl2 (forkhead box L2), was lost in Gata4/Fog2 mutants (Fig. 2A-I) and expression of Gng13 (guanine nucleotide binding protein, gamma 13) (Beverdam and Koopman, 2006; Fujino et al., 2007) was strongly downregulated (Fig. 2K). By contrast, Sf1 (Nr5a1 – Mouse Genome Informatics) expression does not require the GATA4-FOG2 complex (Fig. 2L,M) (see Tevosian et al., 2002).

The current view of mammalian sex determination emphasizes the notion that the two alternative fates, female and male, arise as closely intertwined parities that are determined by antagonistic activities (Kim and Capel, 2006); hence, suppression of one developmental program could result in the emergence of the other.
Examination of XX gonads with GATA4/FOG2 loss showed no signs of testis cord formation (see Fig. S1 in the supplementary material) and markers of Sertoli cell differentiation (Sox9, Mis and Dhh) were absent (Fig. 3A,B). The GATA4-FOG2 transcription complex is required for Sox9 activation and testis differentiation (Manuylov et al., 2007a; Tevosian et al., 2002), so it is not surprising that the loss of GATA4-FOG2 interaction does not result in the activation of Sertoli cell markers (Sox9, Mis and Dhh) in XX gonads upon GATA4/FOG2 loss, whereas expression of genes encoding steroidogenic enzymes (e.g. Hsd3b1) is relaxed. Scale bar: 1 mm. (C)ISH was performed with an Inha probe on XX and XY wild-type and Fog2–/– gonads. Expression of Inha is derepressed in the XX Fog2–/– gonad. (D) The coelomic blood vessel does not form in Fog2–/– gonads. XX and XY gonads from E12.5 mouse embryos carrying Flk1-lacZ with wild-type or homozygous mutant Fog2 were stained with X-Gal, which marks the Flk1-lacZ-positive endothelial cells. The coelomic blood vessel in the testis is indicated by arrows.

One of the early signs of the active testis-specific program is the development of the male-specific vascular pattern. In mouse mutants with a loss of female-specific genes (Wnt4, Fst and Rspo1), a male-specific coelomic vessel appears in XX gonads (Brennan et al., 2002; Brennan et al., 2003; Chassot et al., 2008; Jeays-Ward et
mutants developed similarly to the control XX gonad (ovary) and lacked the coelomic male-specific vessel that is clearly observed in the normal testis (Fig. 3D, arrows).

**Dkk1 is a downstream target of the GATA4-FOG2 transcription complex**

One of the microarray probe sets identified Dkk1 as a target for GATA4-FOG2 regulation in the developing ovary. Gonadal Dkk1 expression has not been previously characterized. Quantitative (q) RT-PCR analysis demonstrated that Dkk1 expression is detectable in both sexes in the developing gonad as early as E11.5, and that the Dkk1 level increases in the developing testis at E12.5. (Fig. 4A). qRT-PCR also confirmed a strong upregulation of Dkk1 expression in E12.5 XX Fog2 mutant gonads (Fig. 4B). ISH with a Dkk1 antisense RNA probe demonstrated that Dkk1 is overexpressed in the developing gonad but not mesonephros (Fig. 4C-L). In the control samples, the expression became visible only in the males at E12.5 and was localized to the central region of the gonad (Fig. 3H). By contrast, in the Gata4/Fog2 mutants strong Dkk1 expression was already apparent at E11.5 in the posterior region of the gonad (Fig. 4E,F). In summary, this analysis established that Dkk1 is upregulated in the embryonic gonads as early as E12.5 and that this expression is dramatically increased upon GATA4-FOG2 complex loss.

**Wnt pathway genes in the Gata4/Fog2 mutants**

At present, a preponderance of data defines DKK1 function mainly within the context of the antagonism of canonical Wnt/β-catenin signaling (Mukhopadhyay et al., 2001) (reviewed by Kikuchi et al., 2007; Niehrs, 2006). The Axin2 lacZ reporter has proven to be effective in monitoring the activity of the β-catenin signaling pathway in the ovary (Chassot et al., 2008); Axin2–/– animals carry the β-galactosidase (lacZ) gene in (knocked-in) the Axin2 locus (Yu et al., 2005). Axin2 is considered to be one of the two (the other being Sp5, see below) candidates for a ‘universal’ Wnt target gene (Clevers, 2006). Although the Axin2 expression level in embryonic gonads is low (data not shown), a sensitive X-Gal assay in the E13.5 gonads of Axin2–/– embryos clearly shows activation of this gene in ovaries but not testes (Fig. 5A,B), as reported previously (Chassot et al., 2008). To examine the Axin2 lacZ expression upon Fog2 loss, we generated XX Fog2 null embryos with an Axin2 lacZ reporter. In the XX Fog2 null gonads, Axin2 lacZ expression was lost (Fig. 5A-D).

A list of Wnt/β-catenin target genes is available from the Wnt homepage (http://www.stanford.edu/~rnusse/wntwindow.html). We compared this list to the list of genes differentially expressed in Gata4 null mutants. Although transcriptional outputs of the Wnt pathway are thought to be cell-specific (Clevers, 2006), one of the best ‘universal’ Wnt/β-catenin target genes is considered to be Sp5 (Clevers, 2006; Weidinger et al., 2005). Microarray analysis detected the downregulation of Sp5 expression in the Gata4 null mutant (~3-fold). Likewise, expression of the other Wnt target gene that encodes a transcription factor, Irx3 (Braun et al., 2003), is reduced. ISH confirmed that Sp5 and Irx3 are downregulated in XX E13.5 mutants with GATA4-FOG2 complex loss (Fig. 5E-H). This decrease in the Sp5 and Irx3 levels in the mutants was confirmed by qRT-PCR (Fig. 5M). Both Sp5 and Irx3 are expressed in an XX-enriched, sexually dimorphic manner (Bouma et al., 2007a; Jorgensen and Gao, 2005; Nef et al., 2005).

Recent work has established that Rspo genes, another gene family that activates the β-catenin pathway, also play a role in female sexual development (Chassot et al., 2008; Parma et al., 2006; Tomizuka et al., 2008). Rspon1 expression is normal in Gata4 null and Fog2 mutants (Fig. 5I-M).

Fig. 4. Dkk1 is a downstream target of the GATA4-FOG2 transcription complex. (A,B) qRT-PCR analysis of Dkk1 expression was performed with wild-type (A) or wild-type and Fog2–/– (B) gonad-mesonephros samples. (C-L) ISH was performed with a Dkk1 RNA probe on XX and XY gonads from wild-type, Gata4ki/ki and Fog2–/– E11.5-13.5 mouse embryos as indicated.
GATA4-FOG2 and WNT4 regulate a partially overlapping set of genes

Our data suggest that in Gata4/Fog2 mutants, gonadal Wnt4 expression is strongly downregulated during the critical time (E12.5) for ovarian development, as is the WNT4 target gene Fst. At the same time, the expression of another WNT4 gonadal target, Bmp2, remains unchanged. To examine whether the GATA4-FOG2 and WNT4 pathways overlap with respect to any other gene targets, we performed qRT-PCR in the Wnt4 XX mutants (Fig. 5N). Both Fst and Bmp2 were severely downregulated in the absence of Wnt4, as previously reported (Yao et al., 2004). The targets of canonical β-catenin signaling, Sp5 and Irx3, were also downregulated in the Wnt4 mutants. However, Irx3 expression was reduced to a greater extent in the Wnt4-null than in the Gata4/Fog2 XX mutant gonads (Fig. 5, compare Irx3 in M and N). By contrast, Dkk1 was not upregulated upon Wnt4 loss. Similarly, another target of GATA4-FOG2 regulation, Foxl2, was expressed normally in the Wnt4-null gonads (Fig. 5N).

Dkk1 protein accumulates in Gata4/Fog2 mutant gonads

So far, our data suggest that canonical β-catenin signaling regulates the gene expression program in somatic cells and that ectopic Dkk1 activation in Gata4/Fog2 mutants interferes with this signaling (Figs 4 and 5). Although ISH indicated that Dkk1 is expressed by the somatic cells (data not shown), DKK1 is a secreted protein that acts outside of the cell (Glinka et al., 1998). To investigate the localization of DKK1 protein in the developing gonads, we performed immunofluorescence analysis. In accordance with the RNA expression data (Fig. 4), DKK1 staining in the E12.5 gonads was faint and appeared marginally enhanced in the testes (Fig. 6A, arrowheads) as compared with the ovaries (Fig. 6B). By contrast, we observed intense DKK1 staining in both XY and XX mutant gonads, as predicted from our ISH experiments (Fig. 6C,D). No staining was observed in the gonads of XY Dkk1–/– embryos (Fig. 6E).

Dkk1 acts cell-autonomously in the somatic cells of the developing ovary

Immunofluorescence analysis demonstrated that DKK1 accumulates in the vicinity of germ cells (Fig. 6C,D); the localization of DKK1 in other settings, at or near plasma membranes, has been reported previously (Caneparo et al., 2007; Maekawa et al., 2005; Mao et al., 2002). This finding was unexpected, as GATA4 and FOG2 are not expressed in germ cells during embryogenesis. Although this expression pattern could be indicative of germ cell-derived DKK1, DKK1 is a secreted protein and so its accumulation pattern does not necessarily reflect its expression origin. To investigate the origin of gonadal Dkk1 expression we used busulfan, an alkylating agent that can be used in rodents to deplete embryonic gonads of germ cells (Menke and Page, 2002; Merchant, 1975). Staining for expression of the germ cell-specific POU transcription factor Oct4 (Pou5f1 – Mouse Genome Informatics) confirmed that most germ cells were eliminated in the busulfan-treated E12.5 XX gonads (Fig. 6F,G). By contrast, the expression of Dkk1 was not affected by busulfan in either the control XY sample (Fig. 6H,I) or in XX Gata4/Fog2 mutants (Fig. 6J-M). Therefore, Dkk1 expression is not dependent on the presence of germ cells.
DKK1 acts through binding to the LRP receptors (LRP5 or LRP6) with high affinity (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001). Lrp6 (but not Lrp5, data not shown) is expressed in the developing gonad at E13.5 (Fig. 6N) and its expression was unaffected in E13.5 XX W/Wv (Kit-mutant) germ cell-deficient gonads (Fig. 6O). In summary, these data suggest that somatic cells in the ovaries are the primary source of Dkk1 expression as well as being the recipients of canonical β-catenin signaling.

GATA4/FOG2 loss does not affect the initiation of germ cell sexual differentiation in XX gonads

Dkk1 accumulation in the vicinity of germ cells in Gata4/Fog2 mutants presented the possibility that germ cell differentiation could be affected by loss of GATA4-FOG2 interaction. Germ cell status in Gata4/Fog2 mutants has not been analyzed previously. Alkaline phosphatase staining of E13.5 gonads detected no change in germ cell number in these mutants. Expression of the germ cell-specific transcription factor Oct4 also appeared normal (Fig. 7A and data not shown). In XX Fog2 mutants, germ cells embarked normally on the female differentiation (meiosis) pathway as defined by the upregulation of histone γH2AX (Fig. 7B-D). Similarly, qRT-PCR analysis of Stra8 (Baltus et al., 2006) and Scp1 (Sycp1 – Mouse Genome Informatics) (Dobson et al., 1994) expression detected no difference between the control and XX Gata4/Fog2 mutant samples (Fig. 7E). Not surprisingly, XY germ cells in the Gata4/Fog2 mutants upregulated Stra8 and Scp1 expression similarly to their XX counterparts, as male differentiation is blocked in these embryos (Tevosian et al., 2002) (Fig. 7E). As Gata4/Fog2 mutants are embryonic lethal at E13.5-14.5, further development of the XX germ cells was not analyzed.

Fig. 6. Dkk1 acts cell-autonomously in the somatic cells of the developing ovary. (A-E) Immunofluorescent staining of frozen sections with an anti-DKK1 antibody (red). Embryonic germ cells are detected by the anti-PECAM1 antibody (green). Note the DKK1 staining in the wild-type testis (A, arrowheads) and mutant gonads (C,D), but not in wild-type ovaries (B) or Dkk1-null testis (E). Magnification: 200×. (F-O) ISH was performed with Oct4 (F,G), Dkk1 (H-M) and Lrp6 (N,O) RNA probes on gonads from wild-type, Gata4ki/ki, Fog2–/– and W/Wv E12.5-13.5 mouse embryos as indicated. (G,I,K,M) Samples were derived from in vivo busulfan-treated embryos.

Fig. 7. GATA4/FOG2 loss does not affect the initiation of germ cell sexual differentiation. (A) ISH was performed with an Oct4 RNA probe on XX gonads from wild-type or Fog2–/– mouse embryos as indicated. (B-D) Immunofluorescent staining of frozen sections with an anti-γH2AX antibody (red). Embryonic germ cells are detected by the anti-PECAM1 antibody. Note the anti-γH2AX staining in the normal ovaries (C) and mutant gonads (D), but not in the control testis (B). (E) qRT-PCR analysis of Stra8 and Scp1 gene expression in wild-type and Fog2–/– E13.5-14.5 XX and XY gonads. The γ-axis shows values for both genes normalized to Gapdh RNA copy number.
Analysis of the sexual differentiation phenotype in doubly homozygous mutant mice

We reasoned that if an abnormally high level of DKK1 in Gata4/Fog2 mutants results in the downregulation of canonical β-catenin pathway targets (Fig. 5), then these same genes could be activated in XX Dkk1−/− embryonic gonads. Indeed, we observed that several canonical β-catenin pathway targets (Irx3, Sp5 and Wnt9a) are upregulated in E12.5 Dkk1−/− ovaries (Fig. 8A,C). Interestingly, in addition to a ‘β-catenin set’, the expression of Foxl2 was also increased; this gene has not previously been described as a target for canonical β-catenin signaling (Fig. 8A,C). These data support the previous assertion, by us and others, that the canonical β-catenin pathway functions during ovarian development and, additionally, identify the Foxl2 gene as a novel target of the canonical β-catenin pathway in the ovary.

Given the importance of canonical β-catenin signaling for the ovarian developmental fate (Chassot et al., 2008; Maatouk et al., 2008; Tomizuka et al., 2008), a profound block in female development observed upon GATA4/FOG2 loss could be explained solely by a dramatic increase in its secreted inhibitor, DKK1, in Gata4/Fog2 mutants. Alternatively, an intact GATA4-FOG2 complex could be independently (i.e. regardless of its role in Dkk1 repression) required for ovarian differentiation and development. To examine XX gonads that are incapable of activating Dkk1 expression in response to the ablation of the GATA4-FOG2 complex, doubly homozygous Dkk1−/−; Fog2−/− and Dkk1−/−; Fog2−/− embryos were generated. We isolated RNA/cDNA from the E12.5 XX control and mutant gonad samples and examined the expression of genes that we have previously confirmed as targets of GATA4-FOG2 regulation in the ovary. As double mutants are no longer capable of upregulating Dkk1, we predicted that the expression of a subset of GATA4-FOG2-dependent genes, previously inhibited through DKK1, would be (at least partially) restored. The expression of Sp5, Irx3 and Wnt9a increased in the double mutants as compared with Fog2 mutants, whereas Inha expression decreased (Fig. 8C). We conclude that the GATA4-FOG2 complex is required to establish the requisite normal level of canonical β-catenin signaling in the ovary by repressing Dkk1. By contrast, Fst and Foxl2 expression in the XX Gata4/Fog2 mutants was not restored by deleting Dkk1, and neither was Wnt4 expression at E12.5 (Fig. 8B,C). These data argue that the GATA4-FOG2 complex regulates these genes independently of its role in maintaining the normal level of canonical β-catenin signaling through repressing Dkk1.

Analysis of the sexual differentiation phenotype in mutants with somatic cell loss of β-catenin

The GATA4-FOG2 transcription complex is required for maintaining a normal level of β-catenin signaling; however, it is also essential for maintaining ovary-specific Wnt4 expression. Either of these GATA4-FOG2-dependent genes (β-catenin or Wnt4) could potentially regulate Fst transcription. Fst levels do not increase in Dkk1−/− XX gonads and Fst expression was not restored in the Dkk1−/−; Fog2−/− double mutants (Fig. 8C). To independently assess the contribution of canonical β-catenin signaling to ovarian Fst expression, we performed a conditional excision of the β-catenin gene in the ovary. A Cre line of mice based on the BAC harboring the Sf1 locus has recently been described (Bingham et al., 2006). This Sf1-Cre is robustly expressed during early gonadogenesis (see Fig. S3 in the supplementary material) and hence is ideal to excise...
β-catenin. Sf1-Cre excision led to the loss of somatic β-catenin expression (see Fig. S4 in the supplementary material) and to a ~7-fold reduction in the *Fst* expression level in the XX gonads, whereas *Gata4*, *Fog2* and *Dkk1* were not affected (Fig. 9A). This experiment demonstrates that β-catenin regulates *Fst* transcription without affecting the GATA4-FOG2 level. In addition to *Fst*, loss of gonadal β-catenin affected the expression of many other genes. The canonical β-catenin target, *Irx3*, and the essential regulators of ovarian development, *Foxl2* and *Wnt4*, were severely downregulated (Fig. 9A and see Fig. S5 in the supplementary material). Loss of *Wnt4* and *Fst* expression was likely to be responsible for a dramatic reduction in the survival of female germ cells in the XX E18.5 β-catenin mutant gonads (Fig. 9D,E) (see Vainio et al., 1999; Yao et al., 2004).

However, the dimorphic gene expression program was not completely extinguished in β-catenin mutants. For example, *Sprr2d* expression was normal, whereas in *Gata4*/*Fog2* mutants *Sprr2d* expression was completely abolished (compare Fig. 9A, bottom panels, with Fig. 2A-D). Although *Dkk1* activation through the canonical β-catenin feedback loop has been reported in some settings (Chamorro et al., 2005; Gonzalez-Sancho et al., 2005; Niida et al., 2004), *Dkk1* expression was not affected (Fig. 9C). β-catenin deletion in the ovary did not result in the activation of the alternative male pathway (sex reversal), as shown by the lack of expression of the Sertoli cell-specific genes *Sox9*, *Mis* and *Dhh* at either E13.5 or E18.5 (Fig. 9B; see Fig. S6 in the supplementary material; data not shown). Similar to the *Gata4*/*Fog2* mutants, the expression of some male-specific genes was derepressed in β-catenin mutants (e.g. *Cyp11a1* and *Inha*, Fig. 9B,C). However, in contrast to the *Gata4*/*Fog2* mutants (but similar to the XX *Rspo1*-null mutation), XX gonads with β-catenin loss developed a coelomic vessel (Fig. 10H,K). In summary, β-catenin is required for normal ovarian development, whereas testis development in the absence of β-catenin proceeds apparently as normal (Fig. 10G and see Fig. S6 in the supplementary material) (see Chang et al., 2008).

**DISCUSSION**

Previously, we determined that the GATA4-FOG2 transcription complex is required for testis differentiation, and visual inspection of the XX *Fog2* mutant gonads at that time suggested that *Fog2* was likely to be needed for female development as well (Tevosian et al., 2002). In the interim period, work by many laboratories has uncovered a number of sexually dimorphic genes in the developing mouse ovary and a functional relationship between them is beginning to emerge (e.g. Bouma et al., 2007a; Chassot et al., 2008;
Jorgensen and Gao, 2005; Menke et al., 2003; Menke and Page, 2002; Nef et al., 2005; Yao et al., 2004). This new body of data has provided a sufficient foundation to integrate the GATA4-FOG2 complex into the transcriptional cascade orchestrating ovarian development in mammals.

GATA4, FOG2 and the canonical β-catenin pathway

We demonstrate here that in the absence of GATA4-FOG2 interaction, the expression of both Wnt4 and Fst is lost. Although several Wnt family members are expressed in the developing gonads (Cederroth et al., 2007), it is primarily Wnt4 that has been associated with sexual development in mammals. Wnt4-null females are masculinized, as demonstrated by the absence of Müllerian ducts and the retention of Wolffian ducts (Vainio et al., 1999). Wnt4 is also required to repress steroidogenic and vascular endothelial cell migration into the developing XX gonad; absence of Wnt4 leads to both ectopic steroid (e.g. testosterone) production and formation of a male-specific coelomic blood vessel (Heikkila et al., 2005; Jeays-Ward et al., 2003). Four known XY human subjects with duplications of the chromosome 1p35 that includes the WNT4 locus exhibit symptoms that range from isolated cryptorchidism to severe genital ambiguity (Jordan et al., 2001; Jordan et al., 2003). A recent report demonstrates that in the ovary, Wnt4 partially acts through canonical β-catenin signaling (Chassot et al., 2008).

Our analysis of Gata4/Fog2 mutant ovaries revealed a dramatic activation of Dkk1 expression in the absence of GATA4-FOG2 interaction, concomitant with the downregulation of genes linked to canonical Wnt/β-catenin signaling. DKK1, the founding member of the DKK family (Krupnik et al., 1999; Monaghan et al., 1999), is a secreted protein and a potent Wnt signaling inhibitor (Glinski et al., 1998). It binds to the LRP receptors (LRP5 or LRP6) and prevents interaction between the Wnt ligand and the Fz-LRP receptor complex (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001). The mechanism of Dkk1 activation as a result of GATA4/FOG2 loss is not clear. The c-JUN transcription factor and JNK signaling have been reported to positively regulate Dkk1 transcription (Colla et al., 2007; Grotewold and Ruther, 2002); however, this is an unlikely explanation because the c-Jun RNA level is unaffected in Gata4/Fog2 XX mutant gonads, and an active form of JNK is undetectable (data not shown).

GATA4-FOG2 complex loss affects multiple aspects of ovarian development

One of the genes requiring the GATA4-FOG2 complex is Foxl2. FOXL2, a forkhead transcription factor, is essential for reproductive development in females (reviewed by Uhlenhaut and Treier, 2006). In mice [as in humans (Coquet et al., 2002)] Foxl2 is one of the earliest genes expressed in a female-specific fashion (Loffler et al., 2003) and Foxl2 homozygous mutants recapitulate female infertility in humans (Schmidt et al., 2004). Although Foxl2 is required in granulosa cell function in postnatal ovaries, embryonic ovarian development initiates and proceeds apparently normally in its absence (Ottolenghi et al., 2005; Schmidt et al., 2004). Importantly, in contrast to the loss of Foxl2 expression in Gata4/Fog2 XX mutants (Fig. 2) and in β-catenin deficiency (Fig. 9 and see Fig. 5S in the supplementary material), Foxl2 expression is normal in the Wnt4-null (Fig. 5N) and Rspo1-null (Chassot et al., 2008) mutants, underscoring the specific requirement for the GATA4-FOG2 complex and β-catenin protein in the control of Foxl2 transcription.

Although GATA4-FOG2 complex loss affects several key elements of the ovarian gene expression program, some dimorphically expressed ovarian genes, such as Bmp2, retain their wild-type levels. Unexpectedly, Bmp2 levels remain unchanged in the XX E12.5 Gata4/Fog2 mutants despite the loss of gonadal Wnt4 expression; Wnt4 is epistatic to Bmp2 and in the Wnt4-null XX gonads Bmp2 expression is dramatically reduced (Fig. 5N) (Yao et al., 2004). The mesonephric expression of Wnt4 that persists in Gata4/Fog2 mutants (e.g. Fig. 1C-F) could be responsible for maintaining the wild-type level of gonadal Bmp2. Alternatively, it is possible that the early (~E11.5) expression of Wnt4 that is independent of GATA4-FOG2 regulation is sufficient to trigger the activation of Bmp2 transcription.

Expression of Rspo1 is also normal in Gata4/Fog2 mutants (Fig. 5). It was proposed that Rspo1 functions to relieve the DKK1-imposed inhibition of the β-catenin pathway by antagonizing DKK1-dependent LRP6 receptor internalization (Binnerts et al., 2001; Semenov et al., 2001). The mechanism of Dkk1 activation in the absence of GATA4-FOG2 complex loss affects multiple aspects of ovarian development.
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Our data suggest that in the XX gonads of the Gata4/Fog2 mutants, excessive DKK1 is no longer adequately antagonized by normal concentrations of the RSPO1 protein. This results in an outcome similar to that of Rspo1 deficiency in that a downregulation of female-specific Wnt4 expression is observed, whereas Bmp2 is unaffected (Fig. 1) (Chassot et al., 2008).

**Fst expression requires multiple regulatory inputs**

Gata4/Fog2, Wnt4 and Rspo1 mutations all converge on the β-catenin signaling pathway and Fst expression is severely reduced or lost in these mutants. Hence, it was tempting to speculate that Fst expression critically depends on the nuclear β-catenin pathway in the ovary. The regulation of an Fst promoter by canonical β-catenin signaling has been reported in cell culture (Miyanaga and Shimasaki, 1993). Mutation of the putative TCF binding site (CTTTGAT) at –223 to –217 relative to the start of Fst transcription led to the abrogation of the WNT3A response (Willert et al., 2002).

A conditional knockout of the β-catenin gene in the gonad results in a drastic reduction in Fst expression, validating the essential requirement for canonical β-catenin signaling in Fst regulation in vivo. A recent report on the Rspo1 knockout (Chassot et al., 2008) also suggests that RSPO1 regulates Fst expression through β-catenin; constitutively active β-catenin is sufficient to rescue the ovarian development of the Rspo1-null mice, although Fst expression was not directly examined in the rescued ovaries. By contrast, Fst expression is not upregulated in Dkk1-/- gonads and is not restored in the Fog2 mutant by Dkk1 ablation. This demonstrates that, in addition to its reliance on intact β-catenin signaling, Fst also requires a functional GATA4-FOG2 complex for its expression. Whether Wnt4 regulates Fst expression via (or independently of) β-catenin is currently unclear and will require restoring (or ectopically stabilizing) β-catenin signaling in the Wnt4-null gonad.

**A pivotal role for the GATA4-FOG2 complex in sexual differentiation**

Previous work demonstrated the importance of the GATA4 and FOG2 proteins in testis development. Loss of GATA4-FOG2 interaction leads to a block in the male pathway because the upregulation of Sox9 gene expression, which is necessary for testicular development, does not occur in mutant XY gonads (Tevosian et al., 2002). Moreover, a threshold concentration of the functional GATA4-FOG2 complex is required to mount an adequate Sox9 expression level that will tip the scale towards testis differentiation (Bouma et al., 2007b; Manuylou et al., 2007a). We have now shown that interaction between these protein partners is also required for the ovarian pathway.

As Gata4 and Fog2 are expressed in gonads of both sexes, their involvement in both testicular and ovarian development is not entirely surprising. The most parsimonious explanation is that GATA4 and FOG2 control the developmental program or programs common to both fates. The block in the proliferation or survival of the pre-Sertoli/pre-granulosa cells could, in principle, account for the observed loss of gene expression. FOG2-GATA4 function is required to maintain sufficient numbers of SOX9-positive cells in the developing testis (Bouma et al., 2007b; Manuylou et al., 2007a). However, a reduction in cell number is unlikely to play a major role in the ovarian pathway block; in this respect, the dramatic increase in coelomic epithelial proliferation in XY E11.5 gonads is not detected in XX gonads (Schmahl et al., 2000). Correspondingly, we observed no significant reduction in proliferation, as assessed by staining for phosphorylated histone H3 and the proliferation-associated protein Ki67 (MKI67), in the E11.5-12.5 XX gonads of the Gata4/Fog2 mutants; the TUNEL assay did not register an increase in apoptosis either. Moreover, whereas Fog2 haploinsufficiency leads to a measurable decrease in the number of SOX9-positive-positive cells in the tests (Manuylou et al., 2007a), we observe no decrease in the number of FOXL2-positive cells in Fog2-/- ovaries (data not shown). Finally, the normal expression of the early ovarian markers Bmp2 (Fig. 1) and Rspo1 (Fig. 5), which are expressed by the somatic support cells (Bouma et al., 2007a; Chassot et al., 2008), argues strongly against a generalized block in the proliferation or survival of a pre-granulosa cell population.

Loss-of-function mutations in male-specific genes such as Sox9 (Chaboissier et al., 2004) or Fog9 (Colvin et al., 2001; Kim et al., 2006) result in activation of the ovarian-specific expression pattern. Similarly, loss-of-function mutations in ovarian-specific genes (Wnt4, Fst, Rspo1 and now β-catenin; Figs 9 and 10) launch the expression of the testis-specific program in the XX gonad (for example, ectopic formation of a male-specific coelomic blood vessel is observed in these knockouts). By contrast, a ‘battle of the sexes’ that lacks its GATA4 and FOG2 pieces results in an earlier tie, as neither side can win. Loss of Gata4/Fog2 does not, however, preclude the initiation of female-specific development of germ cells: in Gata4/Fog2 mutants (XX or XY), germ cells enter meiotic prophase normally, beginning at around E13.5 (Fig. 7).

Many of the crucial events in gonadal (especially ovarian) development can only be realized postnatally, and conditional deletion of Gata4 and Fog2 will be required to analyze the mutants after the time of birth. This will be informative for gaining further insight into the function of GATA4-FOG2 in ovarian development, as only once the mutant XX cells have had to march through the major competence test of folliculogenesisa can the function of the early ovarian genes be truly exposed.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/22/3731/DC1

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