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The transcription factor GATA4 is required for follicular development and normal ovarian function

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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.

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 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 upregulated 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

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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 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

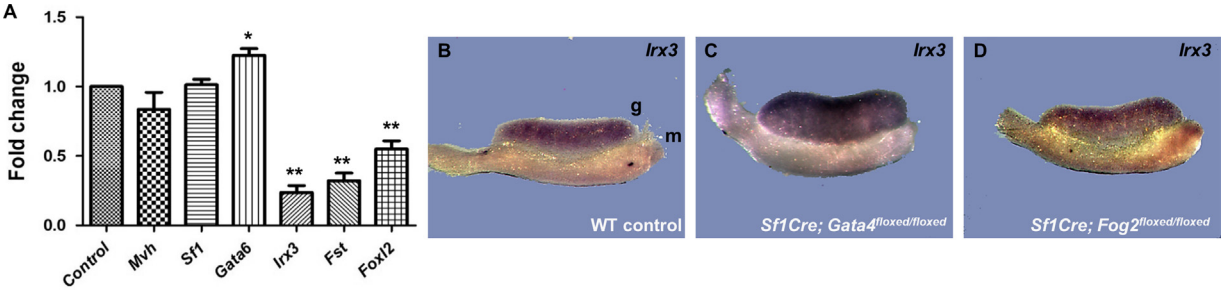

Fig. 1. Embryonic expression of ovarian-specific genes upon *Sf1Cre*-mediated deletion of *Gata4* and *Fog2*. (A) Quantitative changes in gene expression of *Sf1Cre*; $Gata4^{floxed/floxed}$ ovaries at E13.5. Genes examined were *Mvh*, *Sf1*, *Gata6*, *Irx3*, *Fst* and *Foxl2* and results are shown as 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.