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Foxl2 is required for commitment to ovary differentiation

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Genetic control of female sex differentiation from a bipotential gonad in mammals is poorly understood. We find that mouse XX gonads lacking the forkhead transcription factor *Foxl2* form meiotic prophase oocytes, but then activate the genetic program for somatic testis determination. Pivotal *Foxl2* action thus represses the male gene pathway at several stages of female gonadal differentiation. This suggests the possible continued involvement of sex-determining genes in maintaining ovarian function throughout female reproductive life.

INTRODUCTION

Reproductive lifespan in mammals shows a marked sexual dimorphism, with female fertility that is restricted in time and ends with menopause. In contrast to the tight clustering of menopause ~50 years of age for most women, ~1–3% show much earlier menopause, premature ovarian failure (POF), with ovarian dysgenesis as its most extreme form (1,2). In females, the stock of germ cells is usually thought to be fixed at birth and progressively depleted until menopause occurs and is lower or depleted more rapidly in POF (1–4). It is currently under discussion whether the adult mammalian ovary harbors germ stem cells capable of limited self-renewal (5–7), but several lines of evidence indicate that perinatal formation of the ovarian follicle pool and the ensuing follicle dynamics are the most critical determinants of female fertility (4,8,9).

An entry point to the study of follicle dynamics and its relation to menopause was provided by the finding that *FOXL2* is mutated in patients with blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) and POF (10). We have recently shown that *Foxl2* is required for ovarian follicle formation. When it was ablated in XX mice, the sexually undifferentiated cords failed to fragment to yield ovarian follicles (11). An abnormal pattern of oocyte growth ensued and females were sterile (11,12). Here, we report further analysis of *Foxl2* function, characterizing its effect on ovarian soma development and finding that it is also required to stabilize female sex determination.

Ovarian soma development, including the identification of putative gene(s) responsible for female sex determination, has remained poorly understood. During embryonic and fetal development, sexually dimorphic features of the mammalian ovary include mitotic amplification of germ cells followed by their massive entry into meiosis (3,9). Development of the somatic component of the ovary is relatively inconspicuous for a time period that varies across mammalian species but culminates with follicle formation, which occurs concomitant with the progression of oocytes through the last stages of meiotic prophase (9). A number of genes required for meiosis and other aspects of early oocyte differentiation have been described, but only a few genes expressed by the embryonic ovary soma have been characterized functionally (13,14). Furthermore, they have proved to be either primarily involved in oocyte maintenance (*Wnt4* and *Fst*) or required for testis but not for ovary differentiation (*Dax1*) (14–17).

In addition to its role in follicle dynamics, a role for *Foxl2* in repressing male sex determination was previously hinted by findings in goats in which the orthologous locus is associated with both a lack of horns (thought to be homologous to the eyelid anomalies in BPES) and XX maleness (polled-intersex, *PIS*) (18). Here, we demonstrate that a male differentiation program is indeed initiated in $Foxl2^{-/-}$ female mice, indicating that *Foxl2* may function as a conserved repressor of the

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genetic program for somatic testis determination in mammals. The results raise the possibility that the sex determination pathway continues to be involved throughout ovarian development and at subsequent stages of ovarian differentiation and function.

RESULTS

Foxl2$^{-/-}$ ovaries ectopically activate nuclear Sox9 and Fgfr2 perinatally

In *Foxl2*$^{-/-}$ ovaries, the earliest morphological anomalies occurred during the first week postnatum and involved a lack of fragmentation of fetal sex cords into individual follicles in the presence of well-differentiated oocytes (11). Immunohistochemical and *in situ* hybridization results performed by us and others had previously shown that somatic cell markers associated with follicle growth and steroidogenesis were downregulated in *Foxl2*$^{-/-}$ ovaries (11,12). This fetal-like somatic expression profile associated with persistent cords in *Foxl2*$^{-/-}$ mouse ovaries could represent a pure developmental arrest; but we have now investigated whether they also reflect partial gonadal sex-reversal.

A critical test examined the expression of the HMG-box transcription factor *Sox9*, which is necessary for testis determination in humans and mice (19,20) and is itself sufficient to induce ovary-to-testis sex reversal in transgenic mice (21). By quantitative RT–PCR, *Sox9* mRNA expression was sharply upregulated in *Foxl2*$^{-/-}$ ovaries between birth and 1 week postnatum (Fig. 1A). Immunohistochemistry confirmed the expression of Sox9 in testis (Fig. 2C, F I, L and O) and in postnatal *Foxl2*$^{-/-}$ ovaries (Fig. 2K and N), but not in *Foxl2*$^{-/-}$ ovaries before birth (Fig. 2B, E and H) or in wild-type ovaries (except for some signal in the transient rete ovarii; Fig. 2A, D, G, J and M) (discussed subsequently). Strikingly, Sox9 protein localization was nuclear in *Foxl2*$^{-/-}$ supporting cells, a feature characteristic of male supporting (Sertoli) cells in sex determination (22).

Although it was often stronger in the medullary portions of the ovary, nuclear Sox9 localization extended throughout the gonads of all *Foxl2*$^{-/-}$ XX mice that survived (11,12) beyond birth (Fig. 3A–F). As indicated by co-immunostaining with basal lamina-specific antibodies, nuclear Sox9 was confined to the epithelial compartment on *Foxl2*$^{-/-}$ ovaries (Fig. 4B) similar to Foxl2 protein in the wild-type (Fig. 4A), thus confirming that the switch from Foxl2 to Sox9 expression occurred in the somatic lineage that includes Sertoli and granulosa cells. An identical expression pattern was found for Fgfr2 protein (Fig. 4C and D), which is localized to the nucleus of supporting cells specifically in the male gonad (23). In the wild-type ovary, staining was weak and restricted to the cell membrane of granulosa cells of small growing follicles (Fig. 4C); but throughout *Foxl2*$^{-/-}$ ovaries, supporting cells showed nuclear localization of Fgfr2 (Fig. 4D).

Genes involved in male sex determination are activated in *Foxl2*$^{-/-}$ ovaries

Concomitant with *Sox9, Foxl2*$^{-/-}$ supporting cells also upregulated other genes that are required for male sex determination or are considered strong candidates for such a role (reviewed in 24)—including *Fgf9*, *Dhh, Dmrt1, Wt1, Sf1/Nr5a1* and *Gata4* (Fig. 1C–H) (data not shown). Two of these genes *(Sf1* and *Gata4*) were also upregulated in wild-type growing follicles, with mRNA levels that were intriguingly and consistently higher in knockout and wild-type ovaries than in heterozygotes at 1 week (Fig. 1D and F) (data not shown). This pattern of expression suggests that these genes participate in distinct pathways in wild-type and *Foxl2*$^{-/-}$ ovaries (presumably ovarian in wild-type and testicular in the mutant mice), whereas heterozygotes activated the wild-type ovarian pathway but with some delay. Consistent with this notion, heterozygotes also showed lower expression than wild-type at 1 week for several other genes involved in follicle growth (Fig.1I and J) (data not shown).

Among genes that are still insufficiently characterized or are placed downstream of the male sex determination cascade (24,25), some genes, such as *Ppt1*, were selectively upregulated in *Foxl2*$^{-/-}$ ovaries (Fig. 1E), whereas others, such as *Amh*, were activated to a much lesser extent (Fig. 1H). It is of interest that *Dax1* (17) showed a high expression level in mRNA in *Foxl2*$^{-/-}$ ovaries that was comparable to its expression in postnatal testis (Fig. 1G) (17). As for Amh, the protein is known to be expressed from embryonic stages onward in testis, but only postnatally in wild-type ovaries. Consequently, its expression is not informative *per se* to assess any postnatal sex reversal in the ovary. However, Amh is differently regulated in the *Foxl2*-null ovary. It was always absent in wild-type primordial follicles, i.e. around resting oocytes (Fig. 5A, D and G), but was expressed around oocytes of all sizes in *Foxl2*-null ovaries [including both growing (Fig. 5E) and resting (Fig. 5H, white arrows) oocytes]. The lack of correlation of expression with germ cell growth in *Foxl2*$^{-/-}$ mice is reminiscent of Amh expression in the testis, where it is uniformly expressed in supporting cells and thus independent of the differentiation status of male germ cells (Fig. 5C and F).

The sum of the data indicate that the granulosa cell lineage, which selectively expresses *Foxl2* (11,12,26), activates the testis sex determination pathway when *Foxl2* is ablated. The activation extends to the range of markers known for the process, though some downstream effectors like Amh were only partially activated. As expected, we observed marked alterations in the perinatal timing profiles of two female-specific genes (15,16), *Wnt4* and *Fst* (Fig. 1I and J).

Oocyte differentiation is not grossly altered in *Foxl2*$^{-/-}$ ovaries at the time of sex reversal

We have extended previous observations (11,12) indicating that germ cell differentiation was not altered in *Foxl2*$^{-/-}$ newborn ovaries, except for an initially reduced growth rate. Indeed, we found similar estimates of total oocyte counts in *Foxl2*$^{-/-}$ mutants relative to wild-type at birth and at 1 week postnatum ($N = 14$) (data not shown). In addition, we had observed that oocyte growth rates were reduced from the earliest phase of growth, which starts perinatally. As assessed by the fraction of oocytes with a diameter >20 μm, we confirmed that the growth rate was strongly reduced in all ovaries aged 1 week ($N = 9$). We also found that at the same age, mRNA levels for

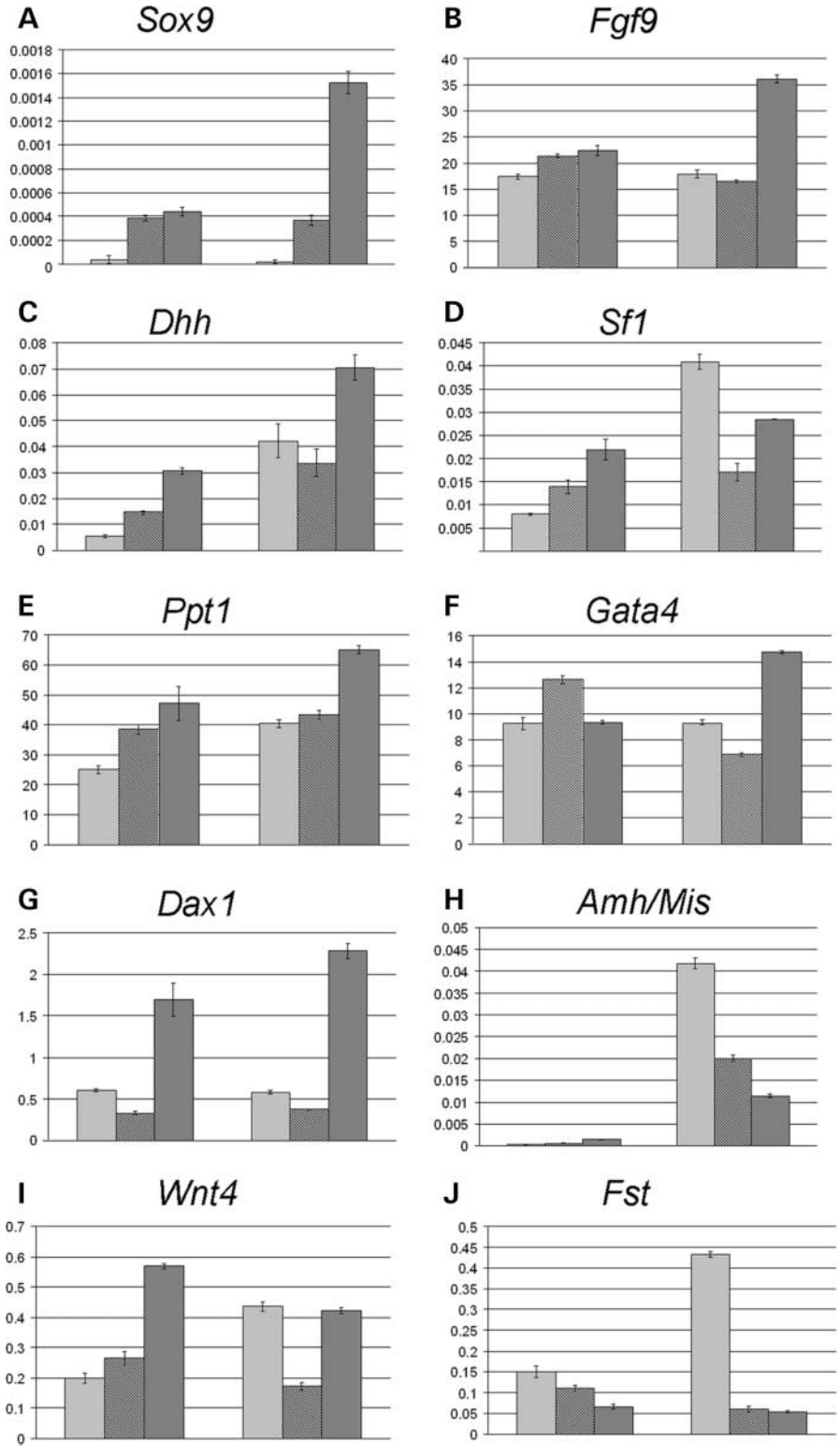

Figure 1. Quantitative RT-PCR of genes with sexually dimorphic expression and function, including SRY-HMG box-like 9 (**A**, *Sox9*), fibroblast growth factor 9 (**B**, *Fgf9*), desert hedgehog (**C**, *Dhh*), steroidogenic factor 1 (**D**, *Sf1/Nr5a1*), palmitoyl-protein thioesterase 1 (**E**, *Ppt-1*), GATA-binding protein 4 (**F**, *Gata4*), dosage sensitive sex reversal/adrenal hypoplasia congenita gene 1 (**G**, *Dax1/Nr0b1/Ahc*), anti-mullerian hormone (**H**, *Amh/Mis*), wingless-type MMTV integration site family, member 4 (**I**, *Wnt4*) and follistatin (**J**, *Fst*). mRNA expression levels are shown for normal ovaries aged 1 day (left bars) and 1 week (right bars). Light color bars left, labeled 'W' for *Sox9* represent wild-type; shaded bars middle, labelled 'He' for *Sox9*, $Foxl2^{+/-}$ and dark color bars right, labeled 'KO' for *Sox9*, $Foxl2^{-/-}$. Error bars represent 1 SD calculated from three replicates and the *y*-axis indicates relative levels compared with *Hprt.*

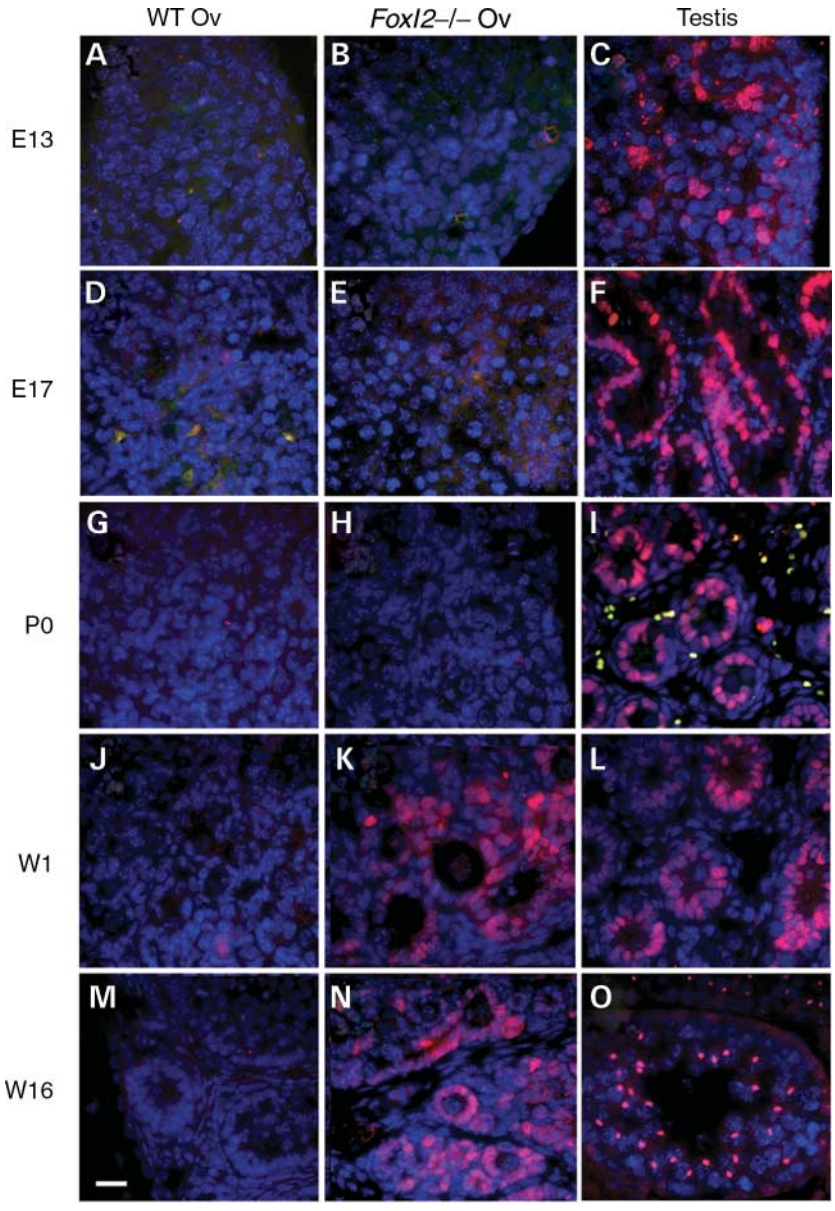

Figure 2. Immunohistochemical detection of Sox9 at high magnification showing partial sex reversal in *Foxl2*$^{-/-}$ ovaries. Columns from left to right represent wild-type ovary (**A, D, G, J** and **M**); Foxl2$^{-/-}$ ovary (**B, E, H, K** and **N**) and testis (**C, F, I, L** and **O**). Rows from top to bottom represent 13 dpc (E13, A–C), 17 dpc (E17, D–F), birth (P0, G–I), 1 week (W1, J–L) and 16 weeks (W16, M–O). Protein expression pattern is for Sox9 (pink) with nuclear counterstaining (DAPI, blue). Bar, 20 μm.

two genes required for early oocyte growth (13), *Gdf9* and *Bmp15*, were increased to a significantly lesser degree in *Foxl2*$^{-/-}$ ovaries than in wild-type littermates (data not shown). In addition, accelerated or fully derepressed growth affected only a small fraction of older *Foxl2*$^{-/-}$ ovaries (3/27 aged 2–16 weeks). Although another study had reported derepressed growth for all ovaries tested in a distinct *Foxl2*$^{-/-}$ model (12), the data that we have collected so far reinforce our initial suggestion that, on at least two genetic backgrounds, derepressed oocyte growth is not a primary consequence of *Foxl2* loss (11). In order to confirm that oocyte defects were confined to their growth phase, we extended our study to the immunohistochemical characterization of additional genes that are functionally relevant for early female gametogenesis.

First, expression of several genes involved in oocyte differentiation (reviewed in 13) was normal in *Foxl2*$^{-/-}$ female mice, including *Fig-alpha* mRNA by *in situ* hybridization, as well as Msy2 and c-kit proteins by immunohistochemistry (11) (Supplementary Material, Fig. S1A–D). Secondly, the mRNA levels of several early oocyte differentiation markers were also unchanged between *Foxl2*$^{-/-}$ and wild-type ovaries at birth and at 1 week (data not shown). Thirdly, the expression of an oocyte marker reported to correlate with follicle formation, i.e. Cyclin D3, was also conserved (27). Cyclin D3 protein nuclear localization, from nearly absent at

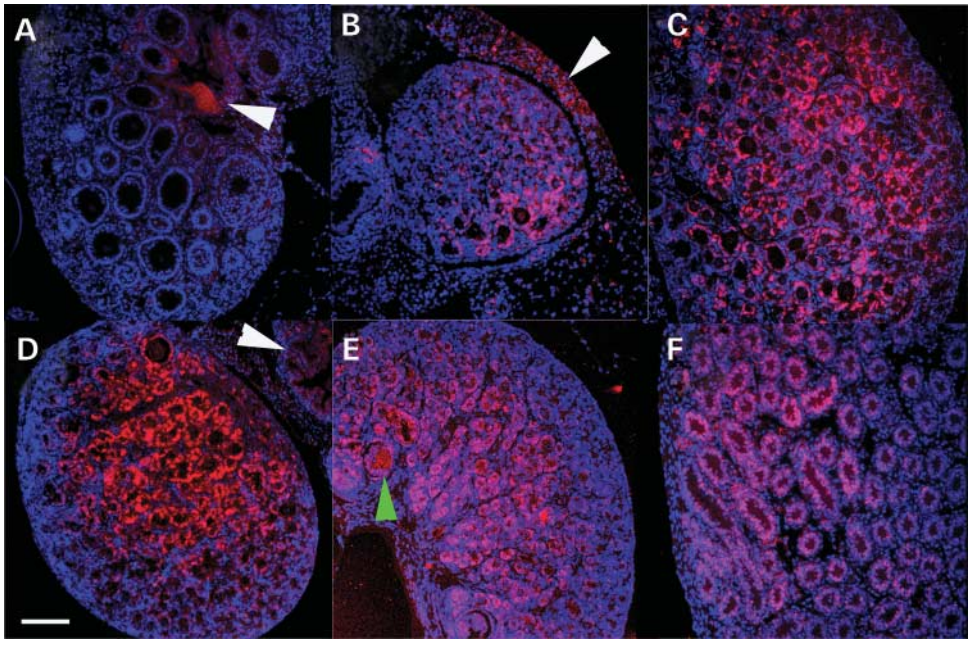

Figure 3. Immunohistochemical detection of Sox9 at low magnification showing partial sex reversal in *Foxl2*$^{-/-}$ ovaries at several developmental stages. Examples are provided for *Foxl2*$^{-/-}$ ovaries aged 1 (**B**), 2 (**C**), 7 (**D**) and 16 weeks (**E**) and compared with wild-type ovary (aged 1 week, **A**) and testis (aged 1 week, **F**). White arrows represent non-specific fluorescence due to red blood cells or border effects. Green arrow (E) represents non-specific fluorescence in large atretic oocytes. Bar, 100 μm.

birth, became widespread at 1 week postnatum both in wild-type and in *Foxl2*$^{-/-}$ oocytes (Supplementary Material, Fig. S1E–H). Somatic support cells expressed cyclin D3 throughout that time interval thus providing an internal control. Finally, we found conserved, biphasic expression of a well-known meiotic marker (28), gamma-H2AX protein, which started to be expressed in the fetus (Supplementary Material, Fig. S2A and B) and was transiently downregulated perinatally both in *Foxl2*$^{-/-}$ and in wild-type ovaries (Supplementary Material, Fig. S2C–H). Meiotic chromosome spreads, specifically stained with Mlh1 and Scp3, showed indistinguishable chromosome pairing and chiasma formation in wild-type and in *Foxl2*-null fetal ovaries (Fig. 6A and B). This indicates that meiotic prophase was not grossly altered in *Foxl2*$^{-/-}$ mice. We thus confirm and extend previous studies indicating that *Foxl2*$^{-/-}$ oocytes do not show overt morphological or molecular defects perinatally (11,12), although oocyte growth is delayed (11). However, we cannot exclude that specific differentiation pathways of the oocytes, still to be identified, may be selectively altered in fetal and neonatal *Foxl2*$^{-/-}$ oocytes before the growth phase.

Ultrastructural appearance of *Foxl2*-null ovaries

Additional immunohistochemical and electron microscopy data have confirmed and extended initial observations (11) of an apparently complete absence of ovarian follicles in *Foxl*$^{-/-}$ ovaries (Fig. 7A–F) (Supplementary Material, Fig. S3A–H). Electron microscopy helped to discern further the status of oocytes and supporting cells in *Foxl2*$^{-/-}$ ovaries. No ovarian follicles were seen, but a well-demarcated basal lamina delineated anastomotic cords that contained multiple oocytes (Fig. 7B and E) (Supplementary Material, Fig. S3A, C, E and G). Oocytes were growing and differentiating with the formation of a thick zona pellucida (Fig. 6C and D). Interestingly, in wild-type ovaries, the zona pellucida was crossed by cellular processes originating from both oocytes and granulosa cells (Fig. 6C), whereas in *Foxl2*$^{-/-}$ ovaries, only oocyte processes were present, suggesting selective defects in the supporting cell lineage (Fig. 6D). In wild-type follicles, myoid cells are well separated from the follicle basal lamina by intervening stroma or steroidogenic theca cell layer. However, similar to testis (Fig. 7C and F), *Foxl2*-null cords were directly apposed to myoid cells (Fig. 7B and E). Supporting cells delimited by the cord basal lamina were very pleiomorphic, ranging in morphological appearance from typical female granulosa to male Sertoli-like (Fig. 7B and E). Sertoli-like features included a large clear cytoplasm, low nuclear electron density and the presence of specific nucleolar satellite organelles. The variable extent of testis differentiation was usually more complete in the cords that lacked oocytes (compare Fig. 7B and E). A morphological pattern similar to *Foxl*$^{-/-}$ ovaries, where oocyte loss correlated with enhanced testis tubule-like morphology, was reported for transplanted fetal mouse ovaries (29) and seen occasionally in *AMH*-transgenic mice (30).

Follicle trans-differentiation into testis-like structures is associated with loss of Foxl2 expression in wild-type ovaries

Occasionally, we found epithelial cells with a Sertoli-like morphology expressing nuclear Sox9 and Fgfr2 in wild-type ovaries; they occupied portions of preantral growing follicles.

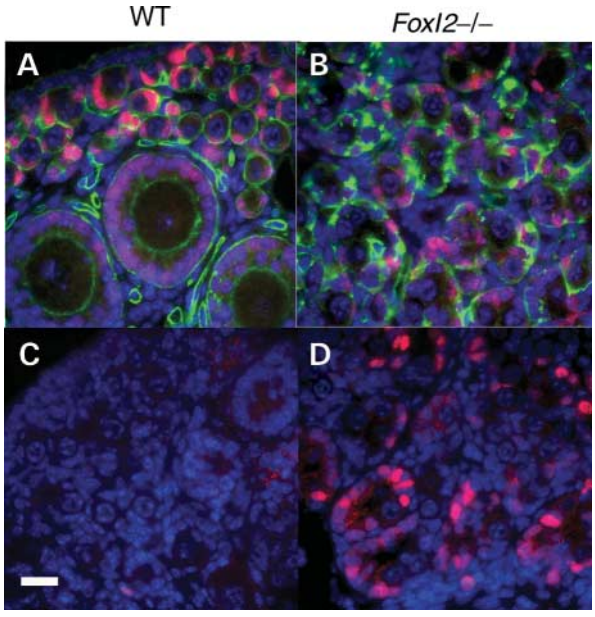

Figure 4. Immunohistochemical detection of Foxl2, Sox9 and laminin A1 and nuclear Fgfr2 in the *Foxl2*$^{-/-}$ and wild-type ovary. Comparison of wild-type (left, **A** and **C**) and *Foxl2*$^{-/-}$ ovaries (right **B** and **D**), aged 1 week (A and B) and 2 weeks (C and D). Protein expression pattern is for Foxl2 (pink, A), Sox9 (pink, B), laminin A1 (green, A and B) and Fgfr2 (pink, C and D) with nuclear counterstaining (DAPI, blue). Bar, 20 μm.

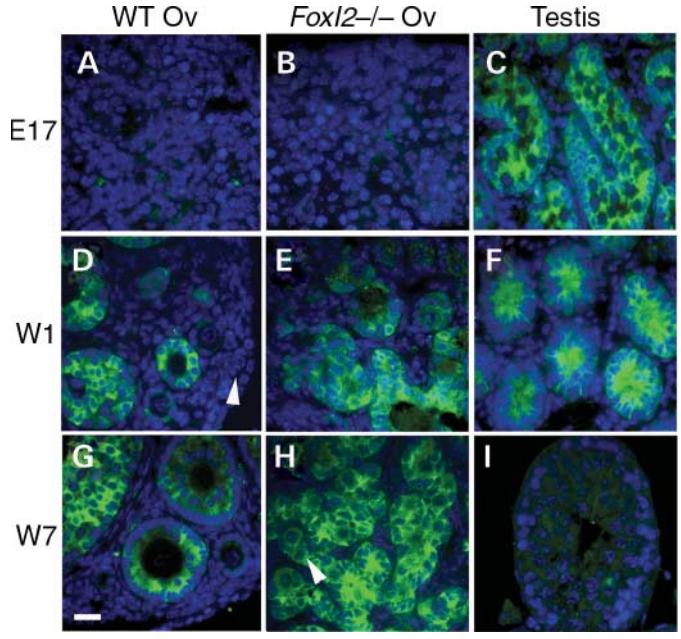

Figure 5. Immunohistochemical detection of Amh/Mis in wild-type and *Foxl2*$^{-/-}$ ovaries when compared with wild-type testis at several developmental stages. Columns from left to right represent wild-type ovary (**A, D** and **G**); Foxl2$^{-/-}$ ovary (**B, E** and **H**) and testis (**C, F** and **I**). Rows from top to bottom represent 17 dpc (E17, A–C), 1 week (W1, D–F) and 7 weeks (W7, G–I). Staining is for Amh (green) with nuclear counterstaining (DAPI, blue). Bar, 20 μm.

Postnatal trans-differentiation of growing follicles into testis-like tubules was previously reported in aromatase and estrogen receptor double knock-outs (31,32). Interestingly, we observed that Sox9 activation correlated with selective Foxl2 downregulation in a complementary pattern (Fig. 8), indicating that Foxl2 and the male differentiation pathway maintain antagonistic roles throughout folliculogenesis. Similarly, nuclear Fgfr2 and Foxl2 were also expressed in a complementary pattern (data not shown).

Sertoli-like cells are a defining feature of a fraction of human ovarian tumors and are reported in some mouse models of ovarian dysfunction (33,34). However, we found no evidence for tumoral formations in *Foxl2*$^{-/-}$ mutants at all ages tested. i.e. ovary size was consistently small (over 90% reduced at 3–4 months) and staining for the proliferation marker Ki67 was inconspicuous in all ovaries (35). Thus, neoplastic transformation, if any, cannot be considered a direct result of *Foxl2*-deficiency in mice, consistent with absent reports in BPES patients.

DISCUSSION

We have previously shown that *Foxl2*$^{-/-}$ mice cannot form ovarian follicles (11), accounting for female sterility (11,12). We now provide evidence that concomitant with the earliest defects detected in follicle formation, *Foxl2* expression in granulosa cells is required to repress the testis determination gene pathway in the postnatal ovary. This inference is supported by the observed activation of all known relevant genes (reviewed in 24). These include demonstrated and likely testis-determining genes that show sexually dimorphic expression patterns (*Sox9, Fgf9, Wt1, Gata4, Dhh, Sf1, Dmrt1* and *Fgfr2*): all were upregulated in *Foxl2*$^{-/-}$ ovaries, along with a number of testis-specific differentiation genes (Figs 1–4) (data not shown). In addition, *Foxl2*-null ovaries contain cords (but not follicles) that show some male-specific features detectable by electron microscopy (as shown in Fig. 7). Further studies should clarify whether this closely parallels fetal testis cord formation as has been suggested for the occasional cords observed in Amh-transgenic mice and fetal ovary grafting experiments (29,30). Our working notion is that in newborn ovaries, as in the embryo, sex determination can be dissociated from sex differentiation, consistent with a bona fide 'turn-on' of the male pathway in *Foxl2*-null mutants. In the postnatal ovary, the sex determination pathway would be directly under *Foxl2* control, with some downstream sex differentiation genes presumably depending on additional factors. Taken together with the observation of Foxl2 downregulation in wild-type follicles that acquire testis tubule-like features (Fig. 8), the data indicate that mammalian female sex determination is labile and may require *Foxl2* activity in the gonadal soma throughout ovary development and maturation.

Other cases of postnatal sex reversal have been reported. However, in those instances, oocyte loss was observed (discussed subsequently); oocytes are thus considered necessary, though not sufficient, for maintaining the female sex postnatally (36). In contrast, in the phenotype observed in *Foxl2*-null mice, several lines of evidence argue against a role of oocyte loss. First, somatic male differentiation was initiated in the presence of a full complement of meiotic

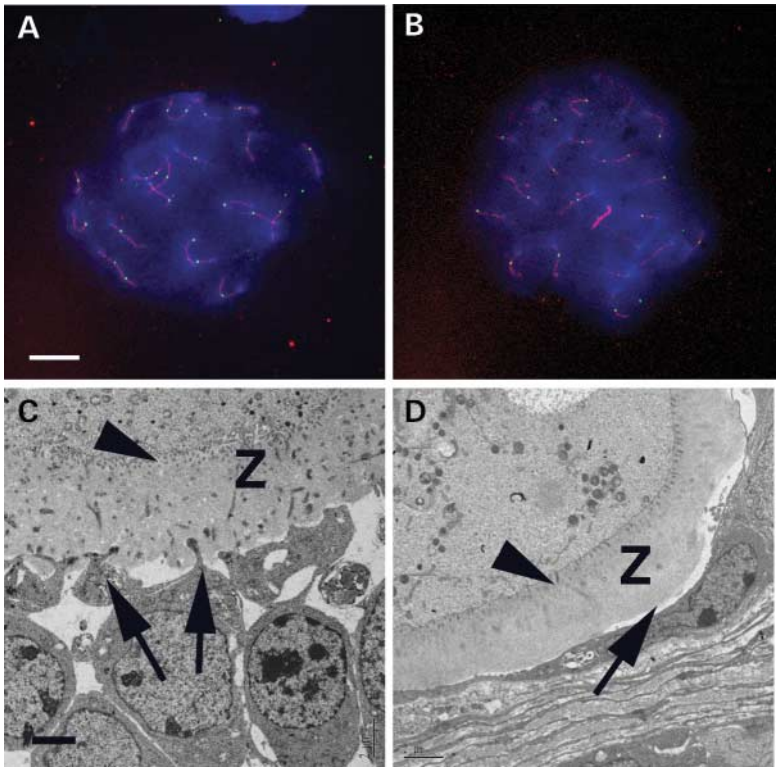

Figure 6. Normal progression through female meiotic prophase (**A** and **B**) and production of a zona pellucida (**C** and **D**) in oocytes of wild-type (left, A–C) and Foxl2-null mice (B–D). Meiotic chromosome spreads from 17 dpc ovaries of wild-type (A) and Foxl2-null ovaries (B) stained for Scp3 (red) and Mlh1 (green) show that the formation of synaptonemal complex and chiasmata, respectively, are not grossly altered in mutant ovaries (DAPI counterstain for chromatin, blue). Electron microscopy evidence that zona pellucida (Z) is formed in both wild-type (C) and *Foxl2*-null (D) oocytes and that oocyte cellular processes are observed through the zona pellucida in both cases (arrowheads). However, contrary to wild-type granulosa cells (C, arrows), *Foxl2*-null somatic cells do not produce processes through the zona pellucida (D, arrow). Bars: 10 μm (A and B) and 2 μm (C and D).

prophase oocytes (11,12). Secondly, oocytes maintained a normal pattern of protein and mRNA expression for all differentiation markers tested and progressed through meiotic prophase with no timing or morphological anomalies. Thirdly, many postnatal oocytes, although with delay compared with wild-type, expressed genes required for oocyte growth, attained large size and formed a thick zona pellucida (11) (Fig. 6D). Finally, the rate of oocyte loss was not increased when compared with wild-type during the first week after birth (11,12). These findings indicate that perinatally, *Foxl2*$^{-/-}$ oocytes are not grossly abnormal and are not lost. Subsequent oocyte degeneration is thus apparently late compared with sex reversal. Further work should determine conclusively whether oocytes in *Foxl2*-null mice are fully competent—for example, by ovary reaggregation or chimera analyses or cell lineage-specific *Foxl2* knock-outs. However, multiple lines of evidence indicate that *Foxl2* acts in granulosa cells both as a morphogenetic factor for ovary and as a repressor of an intrinsic male differentiation pathway. This repression is required for ovary maintenance and may function either independently or downstream of oocyte signals (discussed subsequently).

However, activity of *Foxl2* or upstream regulators, such as oocyte factors, does not account for the observed timing of sex reversal. *Foxl2* starts to be expressed in the mouse bipotential gonad (26), much earlier than birth. Yet its absence does not lead to an early form of sex reversal, affecting the sex fate of germ cells and genitalia. In contrast, ovaries are not sex reversed until the first week postnatum in the *Foxl2*-null mice. Interestingly, a similarly delayed (perinatal) sex reversal in the support cell lineage was reported for *Wnt4*-null mouse ovaries subsequent to massive oocyte loss during mid-fetal life (15). To account for these results, a simple formulation hypothesizes that Foxl2 is able to repress maleness in the fetus; but in perinatal life, XX gonads produce a putative male-promoting factor, '*M*', that induces sex reversal in the absence of *Foxl2* (or upstream activators). *M* would likely be produced at the time when massive reorganization of ovarian architecture leads to follicle formation in wild-type newborn mice (9).

In striking contrast to rodents, where late-onset, partial sex reversal occurs in the presence of well-differentiated female germ cells and genitalia (11,12 and our data) in *PIS* goats, *Foxl2* downregulation is associated with early-onset, nearly complete female-to-male sex reversal (18). A role for Foxl2 loss in sex reversal was previously questioned, precisely because the observations in goat had no obvious parallel in mice or human (18), but the findings here show that *Foxl2* loss reasonably accounts for sex reversal in mice and in goats (the situation in humans remains to be studied; discussed subsequently). The divergent outcomes of *Foxl2* deficiency might then be accounted for by differentially timed expression

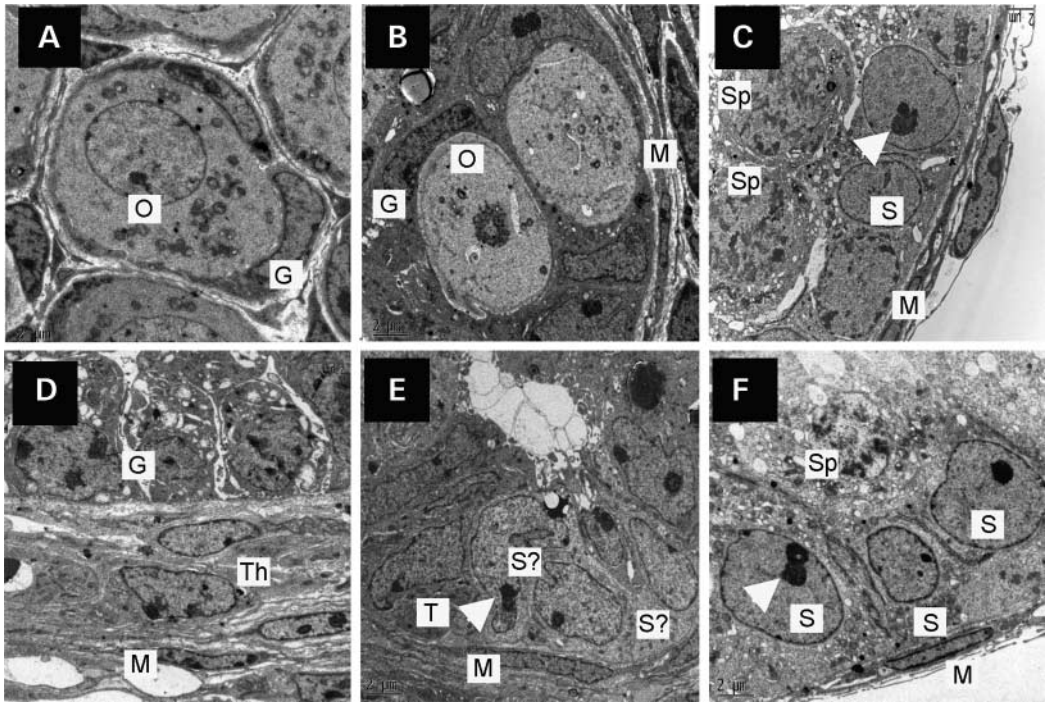

Figure 7. Electron micrographs of wild-type (**A** and **D**) and *Foxl2*$^{-/-}$ ovary (**B** and **E**) and wild-type testis (**C** and **F**). Wild-type: (A), primordial follicle at 1 week and (D), detail of stroma cells surrounding a growing follicle at 3 weeks. *Foxl2*$^{-/-}$ (3 weeks): (B), cross-section of an epithelial cord containing multiple oocytes and (E), testis tubule-like structure. Wild-type testis tubules (2 weeks, C and F). Labels indicate granulosa or pregranulosa cells (G), Sertoli cells (S, followed by a question mark '?' for the putative Sertoli cells seen in mutants), transitional cells between granulosa and Sertoli-like (T), myoid (M), theca (Th), spermatocyte (Sp), oocyte (O) and perinucleolar chromatin (arrowhead) typical of Sertoli cells. Bars, 2 μm.

of *M* across species, related to the different timing of ovarian development in murine and human when compared with ovine species (37). Female-to-male sex reversal of the gonadal soma and early initiation of *Foxl2* expression in both species (26) is thus consistent with early timing of *Foxl2* antagonism of the male gene pathway. *Foxl2* could then satisfy the requirements for '*Od/Z*', the long-sought embryonic ovary determining gene (38,39)—although with the qualification that *Od/Z* would not be required for the initiation of female gametogenesis. In agreement with that qualification, available evidence is consistent with female gametogenesis from primordial germ cells occurring independent of the somatic environment (except in the presence of Sertoli cells) (40 and references therein).

Although oocyte degeneration is unlikely to be a critical feature of *Foxl2*$^{-/-}$ sex reversal, it is possible that support cells lacking *Foxl2* are unresponsive to specific female-promoting signal(s) originated from oocytes. In fact, considerable evidence indicates that oocytes are required to maintain female somatic differentiation. For example, ovaries of aged rats and some mouse models of POF show a progressively larger fraction of follicles that trans-differentiate into testis-like tubules and usually contain either atretic or no oocytes (15,16,31,32,41). Some studies have revealed a possibly related high frequency of testis-like structures in human normal ovaries as well (42,43). We detected a similar process in wild-type mouse ovaries, and in the affected follicles, Foxl2 levels were selectively downregulated in a pattern complementary to Sox9 activation (Fig. 8).

It is possible that oocytes repress male differentiation through multiple pathways, one of which may be independent of *Foxl2*. This would rationalize the incomplete ovary-to-testis sex reversal observed in *Foxl2*$^{-/-}$ mice and the enhanced testis tubule-like morphology in germ-less epithelial cords, also reported in other systems (29). Notably, in organotypic cultures, oocytes were shown to antagonize tubule formation even though the 'testis-determining' transcription factor Sox9 was turned on in embryonic pre-Sertoli cells (44). We are currently using transgenic models to test for effects of oocyte loss on the degree of sex reversal as part of a study of the possible involvement of germline-soma interactions in differential regulation of male and female gametogenesis (see Introduction).

In summary, our data show that *Foxl2* is required for a commitment to female sex gonadal differentiation that appears to be labile during reproductive life (41–43). Other candidates for determinants of mammalian female sex determination have either demonstrated complex effects in both sexes (*Wnt4*) (14,15) or proven to be necessary only for testis differentiation (*Dax1*) (17). *Foxl2* is the first female-specific gene that affects postnatal sex determination without directly involving oocyte survival or differentiation.

Furthermore, *Foxl2* loss in mice leads to sex-reversed cords perinatally and is associated with postnatal follicle sex reversal, but in goats, it produces complete embryonic female-to-male sex reversal. Further studies of Foxl2 action may thus help to clarify the range of reported sex reversals, from primary embryonic sex determination to late cases of 'follicle trans-differentiation'. In addition to identifying other genes

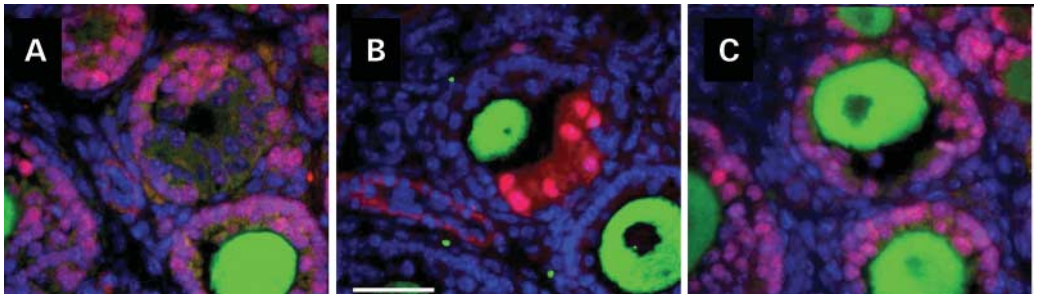

Figure 8. Immunohistochemical detection of Sox9 and Foxl2 in wild-type ovarian follicles showing complementary expression pattern of Foxl2 (**A** and **C**, pink) and Sox9 (**B**, red) in successive 5 μm sections at 15 μm intervals of a 2 week wild-type ovary; oocytes stained with Hsp90 antibody (green). DAPI nuclear counterstaining (blue). Bar, 40 μm.

required for female sex determination, analyses should detect any abnormal activity of the male sex determination pathway across the full spectrum of anomalies associated with *Foxl2* deficiency. These include subfertility in $Foxl2^{+/-}$ heterozygotic mice [we have found ~40% reduced litter size in female heterozygotes from both strains tested, NIHS-BC ($N = 315$, $P < 0.0001$), and C57/B6 ($N = 153$, $P < 0.001$)]. Speculatively, there may also be comparable effects on POF in heterozygotic $FOXL2^{+/-}$ BPES female patients (10), some of whom harbor dysgenetic ovaries with few or no oocytes (45). Unbalanced activity of *Foxl2* and other sex determination genes might be involved in a wider range of conditions affecting female fertility throughout folliculogenesis and reproductive life.

MATERIALS AND METHODS

Mice were euthanized ethically according to ACUC-approved NIA Animal Protocols and genotyped as described (11). The *Foxl2*-null allele was created by deleting the entire *Foxl2* coding region (11).

RNA expression analysis

Pools of ovaries from pups sacrificed at birth and 1 week postnatum were used for each genotype (14–24 ovaries per sample). Total RNA samples were obtained from dissected ovaries (annexa were removed) after homogenization and purification (RNEasy™, Qiagen) followed by linear RNA amplification using Ovation™ (NuGEN). One-step quantitative RT–PCR with Taqman probes and primers (ABI Prism™ 7700 Sequence Detection System, Applied Biosystems) was compared with *Gapdh, Hprt* and *beta2-microglobulin. Sox9* and *Dax1* were also tested directly on unamplified RNA samples. Reagents and PCR conditions are given in Supplementary Material, Table S1.

Immunohistochemistry

Samples were fixed in either 4% paraformaldehyde-phosphate-buffered saline (PBS) or Histochoice (Amresco), which gave comparable immunohistochemical results, as described (11). Primary antibodies were as follows: FOXL2 as reported (11); FGFR2 (23), AMH/MIS and Hsp90 (Santa Cruz Biotechnology); gamma-H2AX (Upstate); Cyclin D3 (Pharmingen) and SOX9 (Abcam). The anti-Msy2 antibody was a generous gift of Dr Richard Schultz (University of Pennsylvania).

Chromosome spreads

Meiotic spreads and immunostaining were performed according to a standard protocol (46) with the modification that a step of heating at 90°C for 5 min to unmask antigenic sites was added before the incubation with primary antibodies (Scp3, Santa Cruz Biotechnology and Mlh1, Pharmingen). Briefly, dissected ovaries were placed in a hypotonic solution and then minced, spread on a slide covered with 1% paraformaldehyde, dried and finally washed with Photo-Flo 0.4% (Kodak). The slides were processed for immunostaining immediately or after 24 h storage at 4°C.

Electron microscopy

Samples were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide and embedded in Spurr resin (Serva). Electron micrographs were taken with a Hitachi H-7000 equipped with a Gatan digital camera.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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