The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance

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Summary

Human Blepharophimosis/ptosis/epicanthus inversus syndrome (BPES) type I is an autosomal dominant disorder associated with premature ovarian failure (POF) caused by mutations in FOXL2, a winged-helix/forkhead domain transcription factor. Although it has been shown that FOXL2 is expressed in adult ovaries, its function during folliculogenesis is not known. Here, we show that the murine Foxl2 gene is essential for granulosa cell differentiation and ovary maintenance. In Foxl2lacZ homozygous mutant ovaries granulosa cells do not complete the squamous to cuboidal transition leading to the absence of secondary follicles and oocyte atresia. We further demonstrate that activin-βA and anti-Müllerian inhibiting hormone expression is absent or strongly diminished in Foxl2lacZ homozygous mutant ovaries. Unexpectedly, two weeks after birth most if not all oocytes expressed Gdf9 in Foxl2lacZ homozygous mutant ovaries, indicating that nearly all primordial follicles have already initiated folliculogenesis at this stage. This activation, in the absence of functional granulosa cells, leads to oocyte atresia and progressive follicular depletion. In addition to providing a molecular mechanism for premature ovarian failure in BPES, these results suggest that granulosa cell function is not only crucial for oocyte growth but also to maintain follicular quiescence in vivo.

Key words: Transcription factor, Forkhead, Winged-helix, Folliculogenesis, Premature ovarian failure

Introduction

Fertility in women depends on complex molecular events that begin in utero with ovarian organogenesis and primordial follicle formation and, after birth, involves the highly coordinated interactions of the hypothalamus, pituitary and ovaries (Apter, 1997; Matzuk and Lamb, 2002). Ovarian failure can vary from a complete failure in germ cell development, resulting in lack of secondary sexual pubertal development (primary amenorrhea), to a reduction in number of germ cells (secondary amenorrhea or premature ovarian failure). Premature ovarian failure (POF) is defined as menopause before age 40 and affects an estimated 1% of women (Pal and Santoro, 2002; Simpson and Rajkovic, 1999).

Several different genetic mechanisms can lead to ovarian failure, including X-chromosomal abnormalities, autosomal recessive genes causing various types of XX gonadal dysgenesis, and autosomal dominant genes (Schlessinger et al., 2002; Sherman, 2000). In contrast to the multiplicity of possible X-linked genes, a single autosomal dominant locus, associated with Blepharophimosis/ptosis/epicanthus inversus syndrome [BPES (MIM 110100)], has been implicated in ovarian failure. BPES type I is characterized by POF and a complex eyelid malformation, whereas BPES type II patients manifest only the eyelid defect. Mutations in FOXL2, a forkhead domain/winged-helix (WH) transcription factor, have recently been shown to cause both types of BPES (Crisponi et al., 2001; De Baere et al., 2001). The infertility is sexually dimorphic. Males remain fertile and transmit the trait to the next generation.

The duration of fertility in a female is determined by the size of the primordial follicle pool formed during fetal life and by the rate of depletion of the pool after birth (Erickson, 2001). Primordial follicles are formed perinatally, as pregranulosa cells encase individual oocytes within the ovary, and the entire germ cell pool available to a female for reproduction is established. The initial stages of folliculogenesis are independent of gonadotropins and involve both cell-autonomous and non-autonomous factors (Hillier, 2001; Kendall et al., 1995). Periodically, several primordial follicles simultaneously enter a growth phase that ultimately leads to ovulation of a mature egg. Little is known about the molecular interaction between germ and somatic cells during primordial follicle formation, or the mechanisms that trigger the selective growth of particular follicles in vivo (Epifano and Dean, 2002; Fortune et al., 2000; Kezele et al., 2002; Smits and Cortvrindt, 2002); although many novel insights into ovarian follicle development have come from the study of relevant knockout mouse models (Burns and Matzuk, 2002; Matzuk et al., 2002).

We have previously reported isolation of the murine P-Frk/Foxl2 gene (Treier et al., 1998). Here we demonstrate that the sexually dimorphic ovarian-specific murine Foxl2 gene is...
essential for the granulosa cell differentiation and ovary maintenance. In the absence of Foxl2, granulosa cell differentiation is blocked at the squamous to cuboidal transition and no secondary follicles are formed. Furthermore, we show that most if not all primordial follicles are activated in Foxl2lacZ homozygous mutant ovaries as demonstrated by activation of Gdf9 expression. Concurrently, expression of two inhibitors of primordial follicle activation, activin-βA and anti-Müllerian inhibiting substance (Amh), is absent or strongly diminished in Foxl2lacZ homozygous mutant ovaries. We further provide evidence that follicles in Foxl2lacZ homozygous mutant ovaries, once activated, undergo apoptosis in the absence of functional granulosa cells which leads ultimately to progressive follicular depletion and ovary atresia. Thus, our results suggest that follicular activation is regulated by granulosa cell function in vivo and provide a mechanism to explain the etiology of BPES.

Materials and methods

Foxl2 targeting vector

Four overlapping Foxl2-positive BAC clones were isolated from a 129 mouse BAC library (Invitrogen/ResGen). A 12.2 kb genomic Asp718 fragment containing Foxl2 was subcloned. The targeting vector consists of a 3.6 kb 5'-homologous region (Nhel-NcoI fragment) and a 5.6 kb 3'-homologous region (XhoI-BsaBI fragment). The coding region (amino acids 62-375) was replaced with a nstlacZ cassette (obtained from S. Tajbakhsh) followed by the self-excisable neo'-cassette (ACN) (obtained from K. R. Thomas). For negative selection, a MC1-DTA (diphtheria toxin A) cassette was placed at the end of the 3'-homologous region.

Mouse genetics

Mice were housed in specific pathogen-free and light, temperature (21°C) and humidity (50-60% relative humidity) controlled conditions. Food and water were available ad libitum. The procedures for performing animal experiments were in accordance with the principles and guidelines of the LAR/EMBL.

The targeting vector was linearized with PmeI and 30 μg vector was electroporated into E14 embryonic stem cells according to standard protocols. Cells were selected with G418 after 24 hours. Positive clones were confirmed by Southern blot using 5'- and 3'- probes outside probes. Two positive clones were microinjected into C57Bl/6J embryos. One positive clone was used for further analysis was performed on a mixed (129/BlackSwiss/CD1) background. The Foxl2lacZ mutation has been kept by now for nine generations without any change in the phenotypic appearance on this background. Offspring were genotyped either by Southern blot or with PCR using the following primers: wild-type (WT) allele 5'-CAC-GATGGCCAGCTACCCCGAGC-3' and 5'-GTTGTTGCGGAT-GCTATTCGCGGCC-3'; mutant allele 5'-TGAGATGCGGCCGAC-TGTGAACCGTGCC-3'. We intercrossed heterozygous offspring to obtain homozygous mutant mice.

Production of peptide antibody against Foxl2

A peptide antibody was produced against the first 15 aa of Foxl2 (MMASYPEPEDITAGTL) coupled to KLH and injected into rabbits.

In situ hybridization

Tissues were fixed in 10% formalin. Hybridization with 35S-labeled antisense RNA probes was done as previously described (Treier et al., 1998) on 20 μm cryosections. Hybridization signals were detected by autoradiography using Kodak NTB-2 liquid emulsion. Autoradiographic exposure was for 21 days.

β-galactosidase staining and histology

β-galactosidase staining was performed according to standard protocols. For histological analysis tissues were fixed overnight with 4% paraformaldehyde at 4°C, dehydrated and embedded in paraffin wax (Vogel). Sections that were 6 μm thick were stained with Hematoxylin/Eosin.

Immunofluorescence and immunohistochemistry

We fixed tissues in 4% paraformaldehyde at 4°C overnight. Sections that were 6 μm thick were hydrated and nonspecific binding was blocked in 5% serum corresponding to the secondary antibody in 1xTBS + 0.4% Triton X-100. Reactions were performed with the following primary antibodies: rabbit polyclonal antibody against MSY2 (1:4000 dilution; kindly provided by R. Schultz, University of Pennsylvania), rabbit polyclonal antibody against lacZ (dilution 1:400; ICN), mouse monoclonal antibody against PCNA (dilution of 1:200; DAKO) and rat monoclonal antibody against GCNA (undiluted; kindly provided by G. Enders, University of Kansas Medical Center). Primary antibodies were incubated overnight at 4°C. Successful staining for PCNA required antigen retrieval methods. Sections were treated for 20 minutes with 2 N HCl. Slides were washed twice with TBS+0.4% Triton X-100 followed by an incubation for one hour at room temperature with one of the following antibodies: Alexa 488, Alexa 594 and Rhodamine Red (dilution 1:400 for all; Molecular Probes). PCNA detection was accomplished using the M.O.M. Peroxidase Kit (Vector Laboratories).

TUNEL assay

Ovaries were stained for apoptotic cells by a modified TUNEL method using the ApoTag Peroxidase In Situ Apoptosis Detection Kit (Intergen) following the manufacturer’s instructions. Sections were counterstained with Methyl Green (Vector Laboratories).

Protein analysis

P1 ovaries were suspended in lysis buffer [100 mM Pipes pH 6.5, 150 mM NaCl, 1% IGEPAL (Sigma), 0.05% β-mercaptoethanol, Complete Mini (Roche) and 10 μg/ml pepstatin (Chemicon)]. Extracts were then sonicated, spun down on centricron columns (Millipore) and washed twice (100 mM Pipes, 100 mM NaCl, 0.05% β-mercaptoethanol and Complete Mini). Washed extracts were diluted in washing buffer and directly subjected to western blot analysis. For western blot analysis, protein samples were separated by SDS-PAGE, blotted onto nitrocellulose, and immunodetection was performed with an enhanced chemiluminescence system (Amersham Biosciences).

Results

Targeted disruption and ovarian expression of Foxl2

A Foxl2lacZ allele was generated by homologous recombination in ES cells, replacing sequences encoding amino acids 62-375 and part of the 3'-untranslated region with lacZ in frame with the first ATG (Fig. 1A). This results in almost complete deletion of the WH domain and also allows convenient localization of Foxl2 expression. Positive clones were verified by Southern blot analysis (Fig. 1B). Two positive ES cell clones were injected and germline transmission was obtained for both of them. To verify the functional inactivation of Foxl2, we analysed whole cell protein extracts from neonatal ovaries of Foxl2lacZ homozygous mutant mice and littermate controls. Western blot analysis of WT extract with an antibody directed against the N-terminus of Foxl2 showed a doublet band that was absent in ovarian extracts from neonatal Foxl2lacZ homozygous mutant mice; instead the Foxl2-lacZ fusion protein is detected (Fig. 1C).
Previous studies have detected Foxl2 transcripts in the granulosa cells of the adult ovary (Crisponi et al., 2001). Using the Foxl2lacZ allele, we determined that Foxl2 expression is activated in the gonads of female mice already around 12.5 dpc, exactly at the time when sex-specific cord-like structures become apparent in the indifferent gonad in males (Fig. 2A,B). This implies that signals must exist in the gonad around 12.5 dpc, which either induce the expression of Foxl2 specifically in females or suppress its expression in males. Foxl2 continues to be expressed in mesenchymal pregranulosa cells, which start to break down the oocyte syncytium to form primordial follicles around birth (Fig. 2C,D). Foxl2 expression is maintained in granulosa cells of early follicles but declines at later stages of folliculogenesis (Fig. 2E,F). The expression pattern of Foxl2 suggests that determination of somatic cells to a granulosa cell fate occurs very early in ovary organogenesis. 35S-in situ hybridization experiments confirmed the expression patterns observed with β-galactosidase staining (data not shown).

**Ovarian failure in Foxl2lacZ homozygous mutant female mice**

Intercrosses of Foxl2lacZ heterozygotes generated homozygous Foxl2lacZ mutants which were born at the expected Mendelian frequency. On a 129/BlackSwiss/CD1 mixed background 95% of mutant animals were lost before weaning, most probably because of craniofacial defects (to be described elsewhere). However, the remaining 5% of mutant animals lived up to one year. Heterozygous Foxl2lacZ mutant female mice were subfertile, whereas homozygous mutant Foxl2lacZ female mice were infertile because of ovarian failure. In contrast, homozygous mutant male animals were fully fertile.

To elucidate the ovarian defect in Foxl2lacZ homozygous mutant female mice, we first performed a histological comparison between neonatal WT and Foxl2lacZ homozygous mutant ovaries. Around birth, the somatic cells are recruited by germ cells and shortly after birth, the oocytes have formed well-defined primordial follicles in which the 12-15 μm diameter germ cells are surrounded by a single layer of squamous or flattened granulosa cells, enclosed together within an outer basal lamina. Comparable numbers of germ cells were present in WT and Foxl2lacZ homozygous mutant neonatal ovaries and primordial follicles were developed. Furthermore, we counted the number of primordial follicles/oocytes during the first three postnatal days in WT and Foxl2lacZ homozygous mutant ovaries and did not observe any significant statistical difference (Fig. 3A,B and data not shown). Oocytes remain in the prophase of the first meiotic division until shortly after birth when they arrest at the late diplotene or dictyate stage of the first meiotic division. To see if the formed primordial follicles have progressed through prophase of the first meiotic division, immunofluorescence was performed with antibodies that distinguish specific meiotic stages of oocyte development. Gcn1 (germ cell nuclear antigen 1) is a marker of the germ cell lineage until they reach the diplotene/dictyate stage of the first meiotic division, an arrested stage that persists from shortly after birth until just prior to ovulation (Enders and May, 1994). In contrast, expression of MSY2, encoding a cytoplasmic RNA-binding protein, starts after oocytes have entered the diplotene stage and persists into the dictyate stage (Yu et al., 2001). Antibodies for each marker were added simultaneously to ovarian sections. Confocal analysis revealed no difference between Foxl2lacZ homozygous mutant and WT ovaries (Fig. 3C). In addition, no difference in FIGα expression, a germ cell-specific transcription factor required for ovarian follicle formation, was detected in Foxl2lacZ heterozygous or homozygous mutant ovaries (Soyal et al., 2000) (data not shown).

In mice and rats, the oocyte starts to grow, when it is surrounded by approximately 10, mostly cuboidal granulosa cells in the largest cross-section. The presence of predominantly cuboidal granulosa cells seems to be a precondition for oocyte growth (Arendsen de Wolff-Exalto and Groen-Klevant, 1980; Braw-Tal and Yossefi, 1997; Lintern-Moore and Moore, 1979). The first wave of follicular growth
is initiated in the inner part of the cortex, at the corticomedullary junction (Hirshfield, 1992). In ovaries of 2-week-old WT mice many secondary follicles containing two or more layers of cuboidal granulosa cells were seen (Fig. 4A). In contrast, only few follicles where the oocyte had substantially grown were seen close to the rete ovarii in Foxl2lacZ homozygous mutant ovaries and these never contained two layers of granulosa cells (Fig. 4B,C). These ‘mutant follicles’ contained oocytes that had grown but undergone atresia and contained many less granulosa cells compared with WT follicles with a similar oocyte size (Pedersen and Peters, 1968). Most importantly, the granulosa cells in these ‘mutant follicles’ did not complete the squamous to cuboidal transition which is highly characteristic for granulosa cells at this developmental stage. At 8 weeks of age, almost all follicles had entered this ‘mutant follicle stage’ (Fig. 4E,F). Although oocytes were surrounded by only a few flattened or squamous-like granulosa cells, most of them had grown to nearly full size at this stage (Fig. 5C,D). Quiescent primordial follicles were not found in serially sectioned Foxl2lacZ homozygous mutant ovaries at this stage, indicating a total depletion of the primordial follicle pool. In contrast to the 2-week-old mutant ovaries, widespread follicular atresia was observed in 8-week-old Foxl2lacZ ovaries (Fig. 5E-H). In addition, an increase in zona pellucida remnants was found.

**Fig. 2.** Foxl2lacZ expression during organogenesis of the ovary. β-galactosidase staining of 12.5 dpc gonads (A,B), newborn (C,D) and adult ovaries (E,F) from Foxl2lacZ heterozygous animals. (A,B) Foxl2lacZ expression is sexually dimorphic. No expression is detected in male gonads, whereas Foxl2lacZ expression starts at around 12.5 dpc in female gonads. (C,D) In newborns Foxl2lacZ expression is detected in the somatic cells of the ovary. (E,F) In adult ovaries Foxl2lacZ expression is seen in granulosa cells with the highest levels in early follicles (black arrowheads), whereas expression declines during later stages of folliculogenesis (white arrowheads).

**Fig. 3.** Maturation state of primordial follicles in WT and Foxl2lacZ homozygous mutant ovaries. (A) Foxl2lacZ expression determined by β-galactosidase staining in newborn heterozygous and homozygous Foxl2lacZ ovaries. β-galactosidase staining is more intense in Foxl2lacZ homozygous mutant ovaries than in Foxl2lacZ heterozygous ovaries because of the two lacZ alleles present showing that there is no somatic cell loss in mutant ovaries. The inset section shows a representative primordial follicle from a double immunofluorescence staining of a mutant newborn ovary with anti-lacZ (green) and anti-GCNA (red) antibodies. LacZ expression was only detected in somatic cells but not in oocytes. (B) Hematoxylin and Eosin staining of P1 WT and mutant ovaries. Formation of primordial follicles was observed in WT and mutant ovaries (black arrowhead marking squamous granulosa cells; white arrowhead marking oocyte). (C) Immunofluorescence for Gcn1 (red) and MSY2 (green) on WT and mutant newborn ovaries. Gcn1 is expressed in germ cells until the pachytene stage of the prophase of Meiosis I, whereas MSY2 expression starts at the diplotene stage of Meiosis I. Comparison of WT and mutant neonatal ovaries shows no apparent difference in the expression profile of the two proteins, providing evidence that primordial follicles have progressed through the prophase of Meiosis I. Corresponding pictures are photographed at the same magnification.
Molecular characterization of the follicle defect

To further investigate the molecular defects underlying the Foxl2lacZ homozygous mutant phenotype, we used 35S-in situ hybridization analysis to visualize the expression of molecules crucial for folliculogenesis (Fig. 6). Foxl2 itself is expressed in granulosa cells during folliculogenesis. Using an in situ probe localized in the 5′-non-deleted region of Foxl2, we followed its expression in WT and Foxl2lacZ homozygous mutant ovaries (Fig. 6A). Foxl2 expression was indistinguishable between WT and mutant newborn ovaries at P1. In 2-week-old ovaries, Foxl2 expression was strong in preantral follicles. In contrast, mutant ovaries displayed a more uniform expression consistent with the altered morphological appearance. In 16-week-old mutant ovaries Foxl2 expression declined to low levels.

The factors regulating initiation of follicle growth are still poorly understood, however in vitro and in vivo data have implicated activins and Amh in this process (Durlinger et al., 2002; Mizunuma et al., 1999). Therefore, we investigated the expression pattern of activin-βA, activin-βB and Amh in WT and Foxl2lacZ homozygous mutant ovaries at different time points of ovary organogenesis. It has been demonstrated that follicles with multi-layers of granulosa cells co-incubated with primordial/primary follicles arrest the development of the smaller follicles in vitro (Mizunuma et al., 1999). Activin-βA message is not detectable in the one-layered follicles and only becomes expressed in the secondary follicle stage. Consistent with the observed absence of secondary follicles in Foxl2lacZ homozygous mutant ovaries, activin-βA expression was not detectable at any stage in Foxl2lacZ homozygous mutant ovaries (Fig. 6B). In contrast, activin-βB was still expressed in
Foxl2\textsuperscript{lacZ} homozygous mutant ovaries (Fig. 6C). The activin-binding protein follistatin is expressed in overlapping patterns with activin-\(\beta A\) and activin-\(\beta B\) in the WT ovary. As early as P1 we found a significant decrease in follistatin expression in Foxl2\textsuperscript{lacZ} homozygous mutant ovaries that stayed just above the detection limit in older mutant ovaries (Fig. 6D). In contrast, we saw no change in the expression pattern for inhibin-\(\alpha\) (Fig. 6E). It has been postulated that Amh may play an important inhibitory role in primordial follicle recruitment, such that more primordial follicles are recruited in Amh-deficient mice than in WT animals (Durlinger et al., 2002). Amh expression in Foxl2\textsuperscript{lacZ} homozygous mutant ovaries was significantly diminished compared with WT ovaries because of the lack of secondary follicles (Fig. 6F). Thus, factors implicated in the suppression of primordial follicle activation by in vitro studies, most notably activin-\(\beta A\) and Amh, are absent or strongly diminished in Foxl2\textsuperscript{lacZ} homozygous mutant ovaries.

It has been shown that murine folliculogenesis is regulated by the interplay of Kit ligand (Kitl) and growth differentiation factor 9 (Gdf9), a TGF-\(\beta\) family member, once a primordial follicle is activated. Gdf9 is an oocyte-derived growth factor synthesized from the primary one-layer or type 3a follicle stage until after ovulation and required for somatic cell function in vivo (Dong et al., 1996; Elvin et al., 1999). Unexpectedly, at P14 most if not all oocytes in Foxl2\textsuperscript{lacZ} homozygous mutant ovaries had activated Gdf9 expression (Fig. 6G). This indicates that almost all follicles have already initiated folliculogenesis at this stage (Elvin et al., 1999).

Kitl is expressed in pregranulosa and granulosa cells and is essential for survival and proliferation of primordial germ cells and has also been implicated in initial primordial follicle activation (Bedell et al., 1995; Huang et al., 1993; Klinger and De Felici, 2002). Interestingly, Kitl was strongly expressed throughout all analysed stages and even persisted at high levels in 16-week-old Foxl2\textsuperscript{lacZ} homozygous mutant ovaries (Fig. 6H).

Several structural cell surface molecules (e.g. E-cadherin, connexin37, connexin43, \(\alpha 6\) and \(\beta 1\) integrins) have been implicated in early ovarian folliculogenesis (Ackert et al., 2001; Burns et al., 2002; Di Carlo and De Felici, 2000; Simon et al., 1997). RT-PCR did not reveal any qualitative differences in the expression of these molecules between Foxl2\textsuperscript{lacZ}.

Fig. 5. Proliferation status of WT and Foxl2\textsuperscript{lacZ} homozygous mutant ovaries. (A-D) High magnifications of Hematoxylin and Eosin stainings showing WT and Foxl2\textsuperscript{lacZ} homozygous mutant follicles 2 weeks and 8 weeks after birth. (A) Secondary follicle in WT ovaries 2 weeks after birth (black arrow, cuboidal granulosa cells; o, oocyte). (B) Primordial follicles in mutant ovaries 2 weeks after birth (white arrow, squamous granulosa cells; o, oocyte). (C) Preantral follicle in WT ovaries 8 weeks after birth (black arrow, cuboidal granulosa cells; o, oocyte). (D) Atretic follicles in mutant ovaries 8 weeks after birth (black arrow, squamous-like granulosa cells; o, oocyte). (E-H) Detection of apoptosis by TUNEL assay in WT and Foxl2-deficient follicles. (E,F) There is almost no apoptosis detectable in WT or in Foxl2\textsuperscript{lacZ} homozygous mutant ovaries 2 weeks after birth. (G) An antral follicle in an 8-week-old WT ovary showing apoptotic nuclei in the granulosa cells. (H) Atretic follicles in 8-week-old mutant ovaries stain positive in the squamous-like granulosa cells. (I-L) Staining of control and Foxl2\textsuperscript{lacZ} homozygous mutant ovaries with an anti-PCNA antibody. (I) Two weeks after birth granulosa cells in primary and secondary follicles stain positive for PCNA, whereas no staining is detectable in Foxl2\textsuperscript{lacZ} homozygous mutant ovaries (J). (K) Positive PCNA staining in granulosa cells of an antral follicle in the WT ovaries. (L) In contrast, no staining was observed in the mutant ovary.
**Discussion**

*Foxl2* is expressed throughout ovarian development. However, Foxl2 function in the ovary first becomes essential during initial primordial follicle recruitment after birth. The failure of granulosa cells in *Foxl2* mutant ovaries to complete the squamous to cuboidal transition indicates that Foxl2 is essential for granulosa cell differentiation. It has been shown that granulosa cells only start to proliferate once they reach the stage of primordial follicle recruitment.

homozygous mutant ovaries and WT ovaries at P1 (data not shown).

**Fig. 6.** $^{35}$S-in situ hybridization analysis of *Foxl2*, activin-βA, activin-βB, follistatin, inhibin-α, *Amh* (anti-Mullerian inhibiting hormone), *Gdf9* and *Kitl*, at different stages of ovary organogenesis (P, postnatal day; w, weeks). (A) *Foxl2* is expressed in granulosa cells at early stages of folliculogenesis, whereas its expression declines to low levels in adult mutant ovaries. (B) In 2-week-old ovaries activin-βA is localized to granulosa cells of antral follicles, whereas it is absent in *Foxl2*-deficient ovaries. (C) In contrast, activin-βB expression is unaltered in mutant ovaries compared with WT ovaries. (D) Expression of follistatin was significantly downregulated in *Foxl2* homozygous mutant ovaries after P1. (E) Expression of inhibin-α was not affected in mutant ovaries. (F) The expression pattern of *Amh*, which is implicated in the activation of primordial follicles, exhibits significantly reduced expression levels in *Foxl2* homozygous mutant ovaries. (G) *Gdf9* expression starts at the type 3a follicle stage in WT oocytes. In the 2-week-old *Foxl2* homozygous mutant ovary all oocytes express *Gdf9*, including regions that correspond histologically to Fig. 5B. (H) *Kitl* is expressed in pregranulosa and granulosa cells during folliculogenesis. In *Foxl2* homozygous mutant ovaries *Kit* ligand expression is maintained at all stages. At least three different ovaries were analysed per time point.
have changed their shape from squamous to cuboidal (Meredith et al., 2000). Consistent with this observation we hardly detect any granulosa cell proliferation in Foxl2lacZ homozygous mutant follicles. Surprisingly, most of the oocytes grow and subsequently undergo atresia because of the absence of follicular cell support, suggesting that granulosa cell function is not only required for oocyte growth but also to maintain follicular quiescence. The observed general oocyte activation correlates with the absence of secondary follicles in Foxl2lacZ homozygous mutant ovaries, leading to a complete depletion of the primordial follicle reserve pool in 8-week-old Foxl2lacZ homozygous mutant females.

Factors that trigger the primordial to primary follicle transition in vivo are largely unknown, although somatic cell-derived Kitl and Amh are implicated in the regulation of this process (Durlinger et al., 2002; Klinger and De Felici, 2002; Parrott and Skinner, 1999). Furthermore, it has been suggested that factors secreted from late stage follicles modulate the activation of primordial follicles (Durlinger et al., 2002; Mizunuma et al., 1999). Most notably Amh and activin-βA have been proposed as inhibitory factors, whereas follistatin secreted by cuboidal granulosa cells would protect early follicles from inhibition (Braw-Tal, 2002). The absence or significant downregulation of the inhibitory factors, activin-βA and Amh, coincides with the precocious activation of almost all primordial follicles in Foxl2lacZ homozygous mutant ovaries two weeks after birth, as demonstrated by activation of Gdf9 expression in oocytes. Neonatal rat ovaries cultured in the presence of Kitl display a higher rate of primordial to primary follicle transition (Klinger and De Felici, 2002; Parrott and Skinner, 1999). Thus, the high expression level of Kitl in the absence of inhibitory factors in Foxl2lacZ homozygous mutant neonatal ovaries is consistent with the proposed role for Kitl in primordial follicle activation in vivo. Together these data indicate that suppressors of initial primordial follicle activation such as activin-βA and Amh expressed by advanced follicles may regulate the primordial to primary follicle transition in vivo, supporting the proposed model that initially oocyte growth follows, rather than precedes, the changes in granulosa cells (Braw-Tal, 2002; Fortune et al., 2000; Kezele et al., 2002). However, further studies will have to show if additional, as yet unknown Foxl2-regulated suppressors of primordial follicle activation exist that are absent in Foxl2lacZ homozygous mutant ovaries. In this respect it is noteworthy that another distantly related WH/forkhead transcription factor Foxo3a has also recently been implicated as a suppressor of ovarian follicle activation (Castrillon et al., 2003). It will be interesting to determine in the future if Foxo3a and Foxl2 share common target genes that are required to maintain primordial follicles in a dormant state.

Subsequent to the initial primordial follicle recruitment, bidirectional signaling between oocytes and surrounding somatic cells is critical for the progression beyond the primary follicle stage (Matzuk et al., 2002). Molecules important in this cross-talk include Gdf9, which is secreted by oocytes, and again Kitl, which is produced by the granulosa cells (Driancourt et al., 2000). It has been shown that the mammalian oocyte orchestrates the rate of ovarian follicle development once the paracrine oocyte factors Gdf9 and BMP-15 have been activated (Eppig and O’Brien, 1996; Eppig et al., 2002; Yan et al., 2001). Despite the high expression of Gdf9 and Kitl in Foxl2lacZ homozygous mutant ovaries, proliferation of granulosa cells is almost absent, suggesting that only cuboidal granulosa cells may be responsive to mitogenic factors such as Gdf9 secreted from the oocyte. However, the high level of Kitl expression maintained in the mutant ovaries may explain the slow degeneration of the oocytes and the presence of very few degenerated oocyte remnants in 16-week-old Foxl2lacZ homozygous mutant ovaries.

Although folliculogenesis in Gdf9 mutant ovaries is arrested around the same stage as in Foxl2lacZ homozygous mutant ovaries, there are noticeable phenotypic differences. In the Gdf9 mutant ovaries the oocyte overgrows and is surrounded by up to two layers of cuboidal granulosa cells before atresia occurs (Dong et al., 1996; Elvin et al., 1999). In contrast, we never observe follicles with a healthy-looking oocyte surrounded by two layers of cuboidal granulosa cells in Foxl2lacZ homozygous mutant ovaries. However, both factors, Gdf9 and Foxl2, are required for granulosa cell proliferation. Nevertheless, the more severe ovarian phenotype of Foxl2lacZ homozygous mutant mice compared with Gdf9 mutant mice suggests that Foxl2 is involved in additional transcriptional networks. Indeed, molecules such as bFGF and EGF/TGF-α may play complementary roles in these processes and Foxl2 may be the signaling integrator in the nucleus (Nilsson et al., 2001; Qu et al., 2000).

It has recently been reported that Foxl2 is not only expressed in somatic cells but also in female germ cells and oocytes during ovarian development (Loffler et al., 2003). However, we cannot detect any expression of Foxl2 in germ cells/oocytes similar to what has been observed in other studies (Cocquet et al., 2002). In addition, we also do not observe lacZ expression of our Foxl2lacZ allele in germ cells/oocytes, which otherwise faithfully recapitulates the Foxl2 in situ expression pattern in the whole embryo during development.

Foxl2, in combination with Gata2 and follistatin, is one of the earliest sexually dimorphic genes expressed specifically in the ovary (Menke and Page, 2002; Siggers et al., 2002) and one of two genes affected in the goat polled intersex syndrome (PIS) (Pailhoux et al., 2001). BPES female patients clearly show no sex reversal. However, they are only haploinsufficient for the FOXL2 mutation. Our results clearly demonstrate that complete loss of Foxl2 protein function in the mouse is not sufficient to lead to XX sex reversal in female mice. However, it will be interesting to see whether a role for Foxl2 in primary sex determination will be revealed on a sensitized background for sex determination. Nevertheless, the expression pattern of Foxl2 together with the results obtained from the Wnt4 knockout model, supports the current trend toward a change in the dogma that ovary development is the default pathway of gonadal differentiation (Vainio et al., 1999).

In conclusion, the observed infertility in Foxl2lacZ homozygous mutant females can be explained by the early depletion of the primordial follicle pool. This depletion is because of a premature activation of almost all primordial follicles as a result of granulosa cell differentiation failure and leads to widespread follicular atresia. The absence of activin-βA and Amh, two suppressors of initial follicle recruitment, may contribute to the observed general loss of follicular quiescence in Foxl2lacZ homozygous mutant ovaries.

So far, mutations in eight different forkhead genes have been associated with human developmental disorders (Carlsson and
Malahapuu, 2002). BPES is, to date, the only human autosomal dominant disorder found associated with POF, a disease that affects 1% of women (Pal and Santoro, 2002; Schlessinger et al., 2002).

Our analysis of Foxl2lacZ homozygous mutant ovaries has provided novel insights into the activation of primordial follicles and ovarian maintenance in vivo and may explain the underlying pathophysiological mechanisms leading to POF in BPES patients. Mouse models of POF such as the Foxl2lacZ knockout mouse described here will facilitate the identification of additional factors regulating ovarian follicle activation, a process crucial in reproductive biology, and the development of improved contraceptives and potential therapeutic treatments for POF.

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References

Ackert, C. L., Gittens, J. E., O'Brien, M. J., Epigee, J. J. and Kidder, G. M. (2001). Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse. Dev. Biol. 233, 258-270.

Apter, D. (1997). Development of the hypothalamic-pituitary-ovarian axis. Ann. New York Acad. Sci. 816, 9-21.

Arendsen de Wolff-Exalto, E. and Groen-Klevant, A. C. (2002). Oocyte growth in the immature rat. J. Reprod. Fertil. 99, 187-192.

Bedell, M. A., Brannan, C. I., Evans, E. P., Copeland, N. G., Jenkins, N. A. and Donovan, P. J. (1996). Growth differentiation factor-9 is required during early follicle development. Mol. Endocrinol. 10, 1018-1034.

Bedell, M. A., Brannan, C. I., Evans, E. P., Copeland, N. G., Jenkins, N. A. and Donovan, P. J. (1996). Characterization of integrin expression in the mouse ovary. Biol. Reprod. 67, 743-751.

Carlsson, P. and Mahlapuu, M. (2002). Forkhead transcription factors: key players in development and metabolism. Dev. Biol. 250, 1-23.

Castillo, D. H., Miao, L., Kolllipara, R., Horner, J. W. and DePinho, R. A. (2003). Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science 301, 215-218.

Coquet, J., Pailloux, E., Jaubert, F., Servel, N., Xia, X., Pannetier, M., De Baere, E., Messiaen, L., Cotinot, C., Fellous, M. et al. (2002). Evolution and expression of FOXL2. J. Med. Genet. 39, 916-922.

Crisponi, I., Delama, M., Loi, A., Chiappe, F., Uda, M., Amati, P., Bisciglia, L., Zelante, L., Nagaraja, R., Porcu, S. et al. (2001). The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/potisis/epicanthus inversus syndrome. Nat. Genet. 27, 159-166.

De Baere, E., Dixon, M. J., Small, K. W., Jabs, E. W., Leroy, B. P., Devrient, K., Gillerot, Y., Mortier, G., Meire, F., Van Maldegen, L. et al. (2001). Spectrum of FOXL2 gene mutations in blepharophimosis-potisos/epicanthus inversus (BPE) families demonstrates a genotype-phenotype correlation. Hum. Mol. Genet. 10, 1591-1600.

Di Carlo, A. and De Felici, M. (2000). A role for E-cadherin in mouse primordial germ cell development. Dev. Biol. 226, 209-219.

Dong, J., Albertini, D. F., Nishimori, K., Kumar, T. R., Lu, N. and Matzuk, M. M. (1996). Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 383, 531-535.

Driancourt, M. A., Reynaud, K., Cortvriendt, R. and Smits, J. (2000). Roles of KIT and KIT-LIGAND in ovarian function. Rev. Reprod. 5, 143-152.

Duan, A. L., Grujicic, M., Kuroki, P., Puelles, L., Ingramah, H. A., Nachtigal, M. W., Uilenbroek, J. T., Grootegoed, J. A. and Themmen, A. P. (2002). Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology 143, 1076-1084.

Elvin, J. A., Yan, C., Wang, P., Nishimori, K. and Matzuk, M. M. (1999). Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient mouse. Mol. Endocrinol. 13, 1018-1034.

Enders, G. C. and May, J. J. (1994). Developmentally regulated expression of a mouse germ cell nuclear antigen examined from embryonic day 11 to adult in male and female mice. Dev. Biol. 163, 331-340.

Epifano, O. and Dean, J. (2002). Genetic control of early folliculogenesis in mice. Trends Endocrinol. Metab. 13, 169-173.

Epigee, J. J. and O'Brien, M. J. (1996). Development in vitro of mouse oocytes from primordial follicles. Biol. Reprod. 54, 197-207.

Epigee, J. J., Wigglesworth, K. and Pendola, F. L. (2002). The mammalian oocyte orchestrates the rate of ovarian follicular development. Proc. Natl. Acad. Sci. USA 99, 2890-2894.

Erickson, G. F. (2001). Role of growth factors in ovary organogenesis. J. Soc. Gynecol. Invest. 8, S13-S16.

Fortune, J. E., Cushman, R. A., Wahl, C. M. and Kito, S. (2000). The primordial to primary follicle transition. Mol. Cell Endocrinol. 163, 53-60.

Hillier, S. G. (2001). Gonadotropin control of ovarian follicular growth and development. Mol. Cell. Endocrinol. 179, 39-46.

Hirshfield, A. N. (1992) Heterogeneity of cell populations that contribute to the formation of primordial follicles in rats. Biol. Reprod. 47, 466-472.

Huang, E. J., Manova, K., Packer, A. L., Sanchez, S., Bachvarova, R. F. and Besmer, P. (1993). The murine steel pan mutation affects kis ligand expression and growth of early ovarian follicles. Dev. Biol. 157, 100-109.

Kendall, S. K., Samuelson, L. C., Saunders, T. L., Wood, R. I. and Camper, S. A. (1995). Targeted disruption of the pituitary glycoprotein hormone alpha-subunit produces hypogonadotrophic and hypothalamic mice. Genes Dev. 9, 2007-2019.

Kreitz, P., Nilsson, E. and Skinner, M. K. (2002). Cell-cell interactions in primordial follicle assembly and development. Front. Biosci. 7, 1990-1996.

Klinger, F. G. and De Felici, M. (2002). In vitro development of growing oocytes from fetal mouse oocytes: stage-specific regulation by stem cell factor and granulosa cells. Dev. Biol. 244, 85-95.

Lintermo-Moore, S. and Moore, G. P. (1979). The initiation of follicle and oocyte growth in the mouse ovary. Biol. Reprod. 20, 773-778.

Loffler, K. A., Zarkover, D. and Koopman, P. (2003). Endocrinology and oocyte development. Endocrinology 119, 43-48.

Mizunuma, H., Liu, X., Andoh, K., Abe, Y., Kobayashi, J., Yamada, K., Yokota, H., Ikubu, Y. and Hasegawa, Y. (1999). Activin from secondary follicles causes small preantral follicles to remain dormant at the resting stage. Endocrinology 140, 37-42.

Nilsson, E., Parrott, J. A. and Skinner, M. K. (2001). Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. Mol. Cell. Endocrinol. 175, 123-130.

Pailhoux, E., Vigier, B., Chaffaux, S., Servel, N., Taourit, S., Furet, J. P., Fellous, M., Grosclaude, S., Crihbiu, E. P., Cotinot, C. et al. (2001). A 1.7-kb deletion triggers intersexuality and polledness in goats. Nat. Genet. 29, 453-458.

Pal, L. and Santoro, N. (2002). Premature ovarian failure (POF): discordance between somatic and reproductive aging. Aging Res. Rev. 1, 413-423.

Parrott, J. A. and Skinner, M. K. (1999). Kit-ligand/ stem cell factor induces primordial follicle development and initiates folliculogenesis. Endocrinology 140, 4262-4271.

Pedersen, T. and Peters, H. (1968). Proposal for a classification of oocytes and follicles in the mouse ovary. J. Reprod. Fertil. 17, 555-557.
Peters, H., Byskov, A. G., Himelstein-Braw, R. and Faber, M. (1975). Follicular growth: the basic event in the mouse and human ovary. *J. Reprod. Fertil.* 45, 559-566.

Qu, J., Nisolle, M. and Donnez, J. (2000). Expression of transforming growth factor-alpha, epidermal growth factor, and epidermal growth factor receptor in follicles of human ovarian tissue before and after cryopreservation. *Fertil. Steril.* 74, 113-121.

Schlessinger, D., Herrera, L., Crisponi, L., Mumm, S., Percesepe, A., Pellegrini, M., Pillai, G. and Forabosco, A. (2002). Genes and translocations involved in POF. *Am. J. Med. Genet.* 111, 328-333.

Sherman, S. L. (2000). Premature ovarian failure in the fragile X syndrome. *Am. J. Med. Genet.* 97, 189-194.

Siggers, P., Smith, L. and Greenfield, A. (2002). Sexually dimorphic expression of Gata-2 during mouse gonad development. *Mech. Dev.* 111, 159-162.

Simon, A. M., Goodenough, D. A., Li, E. and Paul, D. L. (1997). Female infertility in mice lacking connexin 37. *Nature* 385, 525-529.

Simpson, J. L. and Rajkovic, A. (1999). Ovarian differentiation and gonadal failure. *Am. J. Med. Genet.* 89, 186-200.

Smitz, J. E. and Cortvrindt, R. G. (2002). The earliest stages of folliculogenesis in vitro. *Reproduction* 123, 185-202.

Soyal, S. M., Amleh, A. and Dean, J. (2000). FlGalpha, a germ cell-specific transcription factor required for ovarian follicle formation. *Development* 127, 4645-4654.

Treier, M., Gleiberman, A. S., O’Connell, S. M., Szeto, D. P., McMahon, J. A., McMahon, A. P. and Rosenfeld, M. G. (1998). Multistep signaling requirements for pituitary organogenesis in vivo. *Genes Dev.* 12, 1691-1704.

Vainio, S., Heikkila, M., Kispert, A., Chin, N. and McMahon, A. P. (1999). Female development in mammals is regulated by Wnt-4 signalling. *Nature* 397, 405-409.

Yan, C., Wang, P., DeMayo, J., DeMayo, F. J., Elvin, J. A., Carino, C., Prasad, S. V., Skinner, S. S., Dunbar, B. S., Dube, J. L. et al. (2001). Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Mol. Endocrinol.* 15, 854-866.

Yu, J., Hecht, N. B. and Schultz, R. M. (2001). Expression of MSY2 in mouse oocytes and preimplantation embryos. *Biol. Reprod.* 65, 1260-1270.