Specificity of the requirement for Foxo3 in primordial follicle activation

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Abstract

Primordial follicles are long-lived structures assembled early in life. The mechanisms that control the balance between the conservation and the activation of primordial follicles are critically important for fertility and dictate the onset of menopause. The forkhead transcription factor Foxo3 serves an essential role in these processes by suppressing the growth of primordial follicles, thereby preserving them until later in life. While other factors regulating primordial follicle growth have been described, most serve multiple functions at several stages of female germ cell or follicle development, and corresponding mouse mutants exhibit pleiotropic phenotypes with disruption of multiple stages of follicle assembly, development, or survival. To investigate the possibility that Foxo3 also functions in other aspects of ovarian development beyond its known role in primordial follicle activation (PFA), we performed detailed analyses of mouse ovaries including electron microscopy to study primordial follicle structure, assembly, and early growth. These analyses revealed that the timing of primordial follicle assembly, early oocyte survival, and the expression of early germ line markers were unaffected in early *Foxo3* ovaries. Taken together, these studies demonstrate that the phenotype associated with Foxo3 deficiency is remarkably specific for PFA and further support the placement of *Foxo3* in a unique phenotypic class among mammalian female sterile mutants. Lastly, we discuss the implications of the specificity of this mutant phenotype with regard to the hypothesis that oocyte regeneration may occur in adults and serves as a means to replenish oocytes lost via natural physiological processes.

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Introduction

Primordial follicle activation (PFA), also known as follicle initiation, is the metered process by which primordial follicles are removed from the long-lived reserve pool to enter into the growing follicle pool (Skinner 2005). Since follicle growth is irreversible (Peters *et al.* 1975, Lintern-Moore & Moore 1979, McGee & Hsueh 2000), the mechanisms which regulate PFA ensure that some number of growing follicles is available during each estrus/menstrual cycle, but at the same time serve to forestall premature depletion of primordial follicles and reproductive senescence. Spontaneous oocyte apoptosis is another major physiological mechanism that also contributes to follicle depletion, particularly during development and in the early postnatal period, but also throughout adult life (Tilly 2001). Elucidation of the molecular mechanisms that regulate PFA and early follicle growth and preserve primordial follicles are important remaining challenges in reproductive biology (Matzuk *et al.* 2002).

PFA, unlike later stages of follicle maturation including ovulation, appears to be regulated largely within the ovary itself, and is independent of pituitary gonadotropins, as evidenced by several observations: 1) it occurs in a subset of primordial follicles in newborn mouse ovaries explanted and maintained *in vitro* (Eppig & O'Brien 1996), 2) PFA begins well before the onset of sexual maturity – in fact soon after birth in both mice and humans, and 3) PFA is not impeded by hypophysectomy, treatment with exogenous gonadotropins, or in mice bearing knockout mutations in the genes encoding follicle-stimulating hormone, luteinizing hormone, or their receptors (Peters *et al.* 1973, Mason *et al.* 1986, Elvin & Matzuk 1998). Although apparently regulated predominantly via ovarian-intrinsic mechanisms, there is also evidence of modulation of PFA by systemic factors (Nelson *et al.* 1985, Fortune *et al.* 2000).

The molecular pathways controlling PFA remain poorly understood (Braw-Tal 2002). Several studies have implicated an interaction between kit ligand (KL), produced by granulosa cells, and its receptor kit, expressed on the

oocyte membrane (Driancourt *et al.* 2000). Genetic evidence for this comes from studies of mutants of the *Steel* locus encoding KL. Female mice homozygous for the hypomorphic allele *Steel panda* form ovaries with an apparent primordial follicle growth arrest (i.e., failure to undergo PFA; Huang *et al.* 1993, Bedell *et al.* 1995). Other combinations of *Steel* alleles have similar phenotypic consequences (Kuroda *et al.* 1988). However, these mutations also result in pleiotropic effects due to the diverse roles of kit signaling in germ cells and result in ovaries that are severely atrophic with very few primordial follicles, clouding interpretation of these phenotypes. *In vitro* studies have also implicated kit and KL in PFA (Packer *et al.* 1994, Yoshida *et al.* 1997, Parrott & Skinner 1999, Reynaud *et al.* 2000). Nonetheless, a clear picture of how KL triggers PFA has not emerged, or if indeed KL is necessary and sufficient for this process. Other growth signals may serve such a role (Skinner 2005).

The forkhead transcription factor Foxo3 has a specific and essential role in PFA. Foxo3 is dispensable for embryonic development, but primordial follicles in *Foxo3*^{-/-} ovaries undergo global activation within a few days of birth, resulting in a characteristic syndrome of ovarian hyperplasia and early follicle depletion with consequent premature ovarian failure/secondary infertility (Castrillon *et al.* 2003, Hosaka *et al.* 2004). Remarkably, a distantly related forkhead transcription factor, Foxl2, is also involved in follicle growth. Mutation of a single allele of the human *FOXL2* gene (haploinsufficiency) results in the autosomal dominant blepharophimosis, ptosis, epicanthus inversus syndrome (BPES) syndrome associated with eyelid anomalies and premature ovarian failure (Crisponi *et al.* 2001). *Foxl2*^{-/-} female mice are sterile due to widespread follicular atresia and an apparent block in early follicle growth (Schmidt *et al.* 2002, Uda *et al.* 2004). This abnormal follicle maturation may be secondary to earlier defects, since primordial follicle individualization (also known as cyst breakdown) is disrupted in *Foxl2*^{-/-} ovaries (Uda *et al.* 2004). Thus, Foxl2 acts earlier in follicle development than Foxo3 and may serve distinct functions at multiple stages of folliculogenesis (Ottolenghi *et al.* 2005). Here, we present a more detailed phenotypic characterization of the ovarian defects associated with Foxo3 lack of function, with the aim of more precisely delineating the stage(s) at which Foxo3 functions in the ovary, and an eye to the question of whether Foxo3 indeed functions specifically in PFA or serves additional functions in follicle assembly or maturation.

Materials and Methods

Mouse husbandry

The Foxo3 null allele was generated and backcrossed to FVB for six generations as previously described (Castrillon *et al.* 2003). For breeding assays, mating pairs consisting of 21-day-old experimental *Foxo3*^{-/-} or

Foxo3^{+/+} sibling control females derived from heterozygous intercrosses (Castrillon *et al.* 2003) and FVB stud males were placed in cages (one pair per cage) and inspected every morning. Tail genomic DNA was prepared using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA) and mice were genotyped for *Foxo3* alleles using a multiplex PCR (primers available upon request). Animals were fed *ad libitum* on standard chow with standard light cycling conditions. All mice were housed in a pathogen-free animal facility in microisolator cages and experiments were conducted with the approval of the UT Southwestern Institutional Animal Care and Use Committee.

Electron microscopy

Ovaries were fixed in 2% glutaraldehyde + 0.1 M sodium cacodylate and post fixed in 1% OsO₄ and 2% uranyl acetate. The samples were then dehydrated and rinsed with propylene oxide and embedded in Epon (EMbed-812, DDSA, NMA, and DMP30) and polymerized overnight in a 60°C oven. Sections of 50–70 nm were obtained using a Reichert ultracut E microtome and mounted on formvar-carbon coated copper grids. Images were obtained on a JEOL 1200 EX transmission electron microscope.

Immunolocalization and TUNEL studies

Ovaries were immediately placed in 10% buffered formalin and fixed overnight, paraffin-embedded, and cut into 5 µm sections. At least ten serial sections from four different ovaries were immunostained and evaluated for each time point and each antibody. Antibodies used were laminin Ab-1 (rabbit polyclonal, catalog # RB-082 – A0) purchased from Neo Markers/Lab Vision Corporation (Fremont, CA, USA) and an anti-mouse vasa rabbit polyclonal, courtesy of T Noce (Tanaka *et al.* 2000). The laminin antibody was used at 1:1000 dilution; antigen retrieval was performed at 37 °C in pepsin for 5–10 min. The vasa antibody was used at 1:10 000 dilution; antigen retrieval was performed at 95–100 °C in 100 mM sodium citrate buffer (pH 6.0) for 15 min with 20 min of cooling. The detection system employed was Impress anti-rabbit IG (cat. no. MP-7401) from Vector Laboratories (Burlingame, CA, USA) with diaminobenzidine as the substrate–chromogen. Slides were counterstained with hematoxylin. TUNEL was performed with the Apoptag kit from Chemicon International (Temecula, CA, USA) as per the manufacturer's instructions.

Results

Normal timing of primordial follicle assembly in Foxo3 mice

We had previously shown that nullizygosity for *Foxo3* results in secondary infertility due to global PFA soon

after birth (Castrillon *et al.* 2003). To study the progress of primordial follicle assembly and individualization in greater detail, we performed immunolocalization of the germ cell marker vasa. Prior to individualization, oogonia form syncytial clusters, sharing a common cytoplasm linked by intercellular bridges. During the process of individualization, the specialized stromal cells that will become pregranulosa cells (PGs) 'invade' these clusters, extending intercellular processes to physically separate the oogonia, a process known as programmed cyst breakdown. Vasa localization in the cytoplasm is continuous in syncytial clusters but becomes interrupted following individualization because of the interpolation of these somatic cell processes. Immunostaining for vasa is thus a useful tool to track the individualization of primordial follicles (Pepling & Spradling 2001, Jefferson *et al.* 2006).

At postnatal day (PND) 1, most vasa-positive cells in wild-type control ovaries were associated together in

syncytial clusters of two or more cells, sharing a contiguous vasa-positive cytoplasm (Fig. 1A). By PND3, individualization was largely complete, although occasional clusters remained. In contrast, by PND7, individualization was essentially complete, and even closely apposed oocytes at PND7 and PND14 were clearly separated by a distinct rim of vasa-negative cytoplasm. In PND1-14 *Foxo3*^{-/-} ovaries, the timing and completion of primordial follicle individualization as assessed by vasa immunostaining were unaffected, proceeding in an identical fashion as observed in the wild-type controls (Fig. 1A).

To confirm these findings, we also studied the expression of laminin, an abundant component of the basement membrane surrounding ovarian follicles. This basement membrane undergoes extensive reorganization during primordial follicle individualization and subsequent follicle growth (Lee *et al.* 1996). In both primordial and growing follicles, laminin is localized to

Figure 1 Programmed cyst breakdown in mouse ovaries is unaffected by *Foxo3* deficiency. (A) Immunohistochemistry against vasa shows shared cytoplasm in syncytial germ cell clusters prior to individualization/cyst breakdown. (B) Immunohistochemistry against laminin shows basement membrane surrounding either germ cysts or fully individualized follicles. These studies revealed that the timing of cyst breakdown (arrows) is identical in *Foxo3*^{+/+} and *Foxo3*^{-/-} ovaries and is completed by PND7 in both genotypes. For A and B, arrows point to cysts in the process of breakdown (PND1 and 7) and to fully individualized follicles (PND7 and 14). Asterisks identify germ cell clusters. Bars=20 μm for all panels.

the external aspect of the outermost layer of granulosa cells, making it a useful marker to delineate both germinal cysts and individualized follicles. Consistent with the results obtained with vasa, laminin immunohistochemistry at PND1–PND14 showed essentially complete individualization by PND7, with no delay or abnormality in *Foxo3*^{-/-} ovaries relative to controls.

Polyovular follicles, abnormal follicles with more than one oocyte, are abundant in the setting of exposure to specific teratogens (genistein and diethylstilbestrol; Iguchi *et al.* 1990, Jefferson *et al.* 2002) and in some mutants affecting follicle development (e.g. *Bmp15*; Yan *et al.* 2001), and presumably arise through defects in programmed cyst breakdown or other aspects of folliculogenesis. We note that such polyovular follicles were undetectable in serially sectioned *Foxo3*^{-/-} ovaries (data not shown).

To determine if deletion of *Foxo3* affects the number of syncytial clusters when compared with wild-type controls, we performed quantitation in serially sectioned, vasa immunostained PND1 ovaries (*N*=3 per genotype). There was no significant difference in the mean number of syncytial clusters between the *Foxo3*^{+/+} (41 ± 6.1 (S.E.M.)) and *Foxo3*^{-/-} (45 ± 6.2 (S.E.M.)) ovaries at PND1.

To test whether the oocyte enlargement in *Foxo3*^{-/-} ovaries resulted in a concomitant increase in the number of somatic cells surrounding each oocyte, we performed a quantitative assessment of the mean number of somatic cells surrounding oocytes in *Foxo3*^{+/+} and *Foxo3*^{-/-} ovaries at PND7 and PND14 (*N*=3 per genotype). A total of 50 primordial follicles were counted from each ovary. While there was no significant increase in the mean number of somatic cells surrounding the oocyte at PND7 (*Foxo3*^{+/+} = 3.76 ± 0.08 (S.E.M.), *Foxo3*^{-/-} = 3.86 ± 0.12 (S.E.M.)), we observed a relatively minor increase at PND14 (*Foxo3*^{+/+} = 3.98 ± 0.10 (S.E.M.), *Foxo3*^{-/-} = 4.4 ± 0.11 (S.E.M.)). This finding is consistent with the absence of mitotic activity in follicles that have not undergone a transition to cuboidal granulosa cells. We conclude on the basis of these studies that *Foxo3* is not required for the timing or other aspects of follicle individualization/cyst breakdown.

Ultrastructural analyses: *Foxo3* deficiency does not affect primordial follicle assembly or subcellular ultrastructure

Ovarian follicles are highly complex and undergo intricate structural changes during their formation and subsequent development (Wassarman & Josefowicz 1978). Since global PFA in *Foxo3*^{-/-} ovaries closely coincides with the completion of follicle individualization (Castrillon *et al.* 2003), we considered the possibility that *Foxo3*^{-/-} primordial follicles may be structurally abnormal and that such abnormalities might correlate with or be a direct cause of the global activation phenotype. To determine whether there were any such

structural abnormalities in primordial follicles, we studied *Foxo3*^{+/+} and *Foxo3*^{-/-} ovaries at PND1, 3, 7, and 14 (*N*=3 females per genotype, a total of 24 samples). These time points correspond to follicle development prior to individualization (PND1); complete assembly/individualization and the earliest manifestation of the *Foxo3*^{-/-} phenotype (PND3–7); and early follicle growth (PND14).

Consistent with the vasa and laminin immunolocalization studies, all aspects of follicle assembly appeared unaffected in the mutant ovaries, resulting in fully individualized primordial follicles consisting of ultrastructurally normal oocytes surrounded with squamoid PGs by PND7 (Fig. 2A and B). We also compared a number of ultrastructural features and organelles at each time point as discussed below; representative images are shown for PND7 (Fig. 2).

In resting primordial follicles, the interface between oocytes and PGs consists of an elaborate complex of interdigitating microvilli with occasional gap-junctional contacts. This interface, believed to facilitate direct communication and nutrient exchange between oocytes and granulosa cells (Anderson & Albertini 1976), was structurally unaltered in *Foxo3*^{-/-} ovaries (Fig. 2C and D). We also did not observe differences in the morphology, distribution, or number of gap junctions between oocytes and PGs (not shown). Mitochondria, which are numerous in oocytes and likely serve multiple functions in metabolism and apoptosis (Perez *et al.* 1999, Perez *et al.* 2000, Tilly 2001), also were unaltered in *Foxo3*^{-/-} oocytes and exhibited an identical pattern of tubular cristae and often a single vacuole at PND7 (Fig. 2E and F). Large multilamellar golgi networks with bud-like extensions and multiple endocytic vesicles, which are abundant in the ooplasm, were also unaltered (Fig. 2G and H). We failed to observe differences in the morphology of other organelles or substructures including the nucleus and nucleolus, ribosomes, or smooth endoplasmic reticulum, either in oocytes or granulosa cells.

At PND7 and PND14, *Foxo3*^{-/-} oocytes appeared enlarged relative to controls, consistent with growth secondary to global follicle activation by this time point as previously documented by histomorphometry (Castrillon *et al.* 2003). In summary, we conclude that *Foxo3*^{-/-} primordial follicles are morphologically normal and that the *Foxo3*^{-/-} phenotype is not associated with distinct morphologic abnormalities preceding its onset.

***Foxo3* lack of function does not affect oocyte apoptosis**

Programmed cell death is an important physiological mechanism that reduces oocyte numbers both during embryonic development and in postnatal life (Tilly 2001). Primordial follicle individualization coincides with a high rate of oocyte apoptosis, and it has been

Figure 2 *Foxo3* deficiency does not result in primordial follicle ultrastructural defects, representative electron microscopic images from PND7 *Foxo3*^{+/+} and *Foxo3*^{-/-} sibling females. (A and B) Low magnification showing portion of individual primordial follicles including a single pregranulosa cell (PG) and oocyte (Oo). (C and D) High magnification of interface between oocyte and pregranulosa cell; asterisk indicates interdigitating microvilli at interface. (E and F) Mitochondria with identical patterns of cristae and characteristic single large vacuoles (asterisks). (G and H) Large golgi complexes (asterisks) that occur in large numbers in the ooplasm. Bar=2 μ m (A and B) or 500 nm (C–H).

proposed that apoptosis contributes to the process of cyst breakdown (Pepling & Spradling 2001). Numerous studies link the Foxos, including *Foxo3*, to the regulation of cell death via transcriptional control of apoptotic factors such as Bim & FasL (Brunet *et al.* 1999, Moller

et al. 2005) among others, prompting us to consider the possibility that *Foxo3*-deficient ovaries might exhibit defects in oocyte apoptosis in addition to the observed defects in PFA. TUNEL analysis performed on serial sections at PND3 ($N=4$ ovaries per genotype), revealed no differences either in the overall pattern of TUNEL positive oocytes (Fig. 3A and B) or in the apoptotic index of oocytes in *Foxo3*^{+/+} and *Foxo3*^{-/-} ovaries (Fig. 3C); we also did not observe significant differences at PND1 or 7 (not shown).

***Foxo3* lack of function does not impair the progress of follicle maturation**

Foxo3^{-/-} females have viable litters prior to total follicle depletion at ~ 15 weeks of age, demonstrating that subsequent steps of follicle maturation following PFA are not completely disrupted by *Foxo3* lack of function. In an attempt to uncover subtle defects or delays in follicle maturation or other processes, we determined the earliest age at which females could give birth in matings of virgin females starting at 3 weeks of age. This serves as an indirect measurement of several aspects of reproductive function, including sexual maturation, follicle growth, and gestation. The minimum time interval required to complete the process of follicle maturation (from PFA to ovulation) is difficult to measure directly, but has been estimated at 3–5 weeks (Oakberg 1979, Hirshfield 1991), consistent with the fact that the first waves of follicle activation normally begin soon after birth and that female mice normally begin to ovulate at 4–5 weeks of age. Thus, in the mouse, in contrast to longer-lived mammals, the onset of ovulation/sexual maturity coincides closely with the minimum time required for follicle maturation. The age at first litter in *Foxo3*^{-/-} closely matched that of *Foxo3*^{-/+} or *Foxo3*^{+/+} sibling controls (Fig. 4A), arguing against a significant delay or lag in follicle maturation. We do note, however, that granulosa cells in only a subset of follicles assume a cuboidal morphology and grow in the *Foxo3*^{-/-} ovaries, demonstrating that oocyte and granulosa cell growth are not completely coupled in *Foxo3*^{-/-} ovaries.

To study the progression of follicle maturation in more detail, we analyzed a number of markers known to be turned on or differentially expressed at different stages of follicle maturation. Inhibin is undetectable in primordial follicles and is first expressed in the granulosa cells of primary follicles. p70 S6 kinase, which phosphorylates the S6 protein of the 40S ribosomal subunit and is involved in translational control of 5'-oligopyrimidine tract mRNAs (Manning 2004), is phosphorylated during the transition from primary to secondary follicles, presumably serving to promote the protein synthesis required for subsequent oocyte growth (unpublished data). Connexin-43, which is required for gap-junction formation and communication between granulosa cells

Figure 3 Oocyte apoptosis is unaffected by Foxo3 deficiency. TUNEL staining was performed on ovarian tissue sections from sibling female mice at PND3. (A) Control *Foxo3*^{+/+} ovary. (B) *Foxo3*^{-/-} ovary. Apoptotic oocytes are observed in characteristic cortical pattern similar in both genotypes. (C) Apoptotic index (percentage of TUNEL positive oocytes) in *Foxo3*^{+/+} versus *Foxo3*^{-/-} ovaries at PND3; data are the mean from *N*=3 females per genotype. Error bars represent the S.E.M.; at least 500 oocytes were counted per ovary.

(Ackert *et al.* 2001, Teilmann 2005), is first detectable in primary follicles, and its levels continue to increase during the progression to the secondary and antral stages. For all of these markers, the timing of these changes in expression was identical, stage-for-stage, in *Foxo3*^{+/+} and *Foxo3*^{-/-} ovaries (Fig. 4B). We also did not observe stage-for-stage alterations in the expression of other markers of follicular differentiation such as p27^{KIP1} or WT1 (data not shown).

Discussion

These studies demonstrate that the requirement for Foxo3 in ovarian function is remarkably specific for PFA. We were unable to detect abnormalities in cyst breakdown/follicle individualization, spontaneous oocyte apoptosis, primordial follicle ultrastructure, or in the timing of expression of representative markers of follicle maturation. These findings further support the placement of *Foxo3* in a unique phenotypic class among female sterile mutants, and highlight the importance of Foxo3 in the regulation of PFA.

It is not known whether Foxo3 serves a similar, highly specific function in PFA suppression in other mammals. If such a function were conserved in humans, it is interesting to speculate that mutation or naturally occurring sequence variation at the *FOXO3* locus might account for some cases of premature ovarian failure (menopause prior to the age of 40) or idiopathic primary amenorrhea, two female infertility syndromes associated with premature depletion of primordial follicles (Nakano *et al.* 1982, Anasti 1998). Despite evidence that these conditions have a hereditary component, the relevant hereditary factors remain to be identified. Extrapolating from the mouse phenotype, homozygous *FOXO3* complete loss of function mutations should result in follicle depletion prior to puberty (primary amenorrhea), given the prolonged interval between birth and sexual maturity in girls. Another (not mutually exclusive) hypothesis is that heterozygosity for *FOXO3* could result in a more subtle increase in PFA, culminating over many years or decades in premature ovarian failure. This possibility is suggested by the finding that many forkhead transcription factors are haploinsufficient, resulting in clinical disease following mutation of a single allele, as is the case for *FOXL2* in the BPES premature ovarian failure syndrome (Crisponi *et al.* 2001, Erickson 2001). Nonetheless, such *FOXO3* mutations have not yet been identified (Watkins *et al.* 2006).

This and our previous studies of the ovarian defects associated with Foxo3 deficiency support the following model, shown in Fig. 5. Early steps of germ cell development, including migration, and gonadal colonization are unaffected by Foxo3 lack of function. This has not been assessed directly, but can be inferred from the observation that testis size and spermatogenesis are normal in *Foxo3*^{-/-} males, and the finding that primordial follicle counts are also normal in *Foxo3*^{-/-} females at birth (Castrillon *et al.* 2003). Oogenesis, including cyst formation, cyst breakdown, and follicle individualization proceed normally in *Foxo3*^{-/-} ovaries (this study). Immediately after follicle individualization is completed by PND7, global PFA is triggered in *Foxo3*^{-/-} females, leading to increased numbers of follicles progressing through subsequent stages of follicle maturation. Despite global oocyte growth, granulosa

Figure 4 Evidence against constitutional defects in the progression of follicle maturation subsequent to global follicle activation in *Foxo3*^{-/-} ovaries. (A) Maternal age at the time of first litter. Females at 3 weeks of age (prior to sexual maturity) were placed in cages with stud males. Numbers of females analyzed were *Foxo3*^{+/+} (N=5); *Foxo3*^{-/+} (N=4); *Foxo3*^{-/-} (N=4). There was no lag in *Foxo3*^{-/-} females relative to *Foxo3*^{+/+} and *Foxo3*^{-/+} controls, arguing against a significant disruption in all subsequent stages of follicle maturation. Error bars represent the s.e.m. (B) Immunolocalization of ovarian markers at 4–5 weeks of age that characterize transitions to more advanced follicle stages, including inhibin (undetectable in primordial follicles (asterisks) and first expressed in primary follicles), S6 kinase (which becomes phosphorylated during the transition from primary to secondary follicles), and connexin-43, which is expressed at low levels in secondary follicles but becomes strongly induced by the antral follicle stage. The timing of expression of these markers in *Foxo3*^{-/-} ovaries closely match that observed in *Foxo3*^{+/+} controls. Bars=50 µm for all panels.

cells in only a subset of follicles transition to cuboidal morphology and begin to proliferate. *Foxo3*^{-/-} follicles appear to either undergo essentially normal growth or exhibit the abnormal pattern of continued oocyte growth in the absence of granulosa cell growth, in a rather 'all or none' manner, implying that oocyte and granulosa cell growth are only partially coupled in *Foxo3*^{-/-} ovaries. We hypothesize that in the normal ovary, there are repressive feedback mechanisms which restrict early follicle growth and which operate normally in *Foxo3*^{-/-} ovaries despite global PFA.

Increased PFA also results in striking ovarian hypertrophy by PND14 (due to a vast increase in the number of growing follicles). *Foxo3*^{-/-} females initially have normal litters despite global PFA, but undergo a rapid decrease in fecundity that correlates with massive follicle atresia secondary to global PFA. By 15 weeks,

Foxo3^{-/-} ovaries are completely devoid of follicles and sterility ensues. We stress that *Foxo3*^{-/-} females have normal progeny until the time that follicle depletion is total, indicating that other aspects of oogenesis including meiosis and all stages of follicle maturation subsequent to PFA including ovulation are unaffected. Furthermore, progeny are viable, demonstrating that 1) *Foxo3*^{-/-} oocytes support normal embryogenesis and 2) *Foxo3* serves no essential function in other aspects of female reproductive function including placentation, parturition, lactation, etc.

Lastly, the exceptional phenotype associated with *Foxo3* mutation presents a unique opportunity to reflect upon recent reports that *de novo* oocyte formation occurs in the adult mouse ovary, where evidence has been presented that a continuous influx of germline stem cells into the adult gonad replenishes oocytes lost by

Figure 5 Schematic illustrating the *Foxo3* null phenotype. Colonization of the gonad by germ line stem cells occurs normally during embryonic development (Castrillon *et al.* 2003), as do subsequent stages of female germline development up to cyst breakdown leading to the formation of primordial follicles (this study). Immediately following primordial follicle individualization (within a few days of birth), all primordial follicles in *Foxo3*^{-/-} ovaries undergo activation in a synchronized manner, resulting in dramatically increased ovarian size by PND14. Despite this global activation, maturation of some follicles proceeds normally and females have viable progeny until total follicle atresia occurs by 15 weeks. Since follicle growth is irreversible and activated, growing follicles have a finite lifespan (Elvin & Matzuk 1998, McGee & Hsueh 2000) and *Foxo3* deficiency results in premature ovarian failure due to depletion of the follicle pool.

normal physiological mechanisms such as apoptosis and PFA (Johnson *et al.* 2004, 2005), although recent studies have provided evidence against this hypothesis (Byskov *et al.* 2005, Eggan *et al.* 2006). Furthermore, the *Foxo3*^{-/-} phenotype would appear to be inconsistent with such a model. If *de novo* primordial follicle synthesis did occur in the adult ovary, such newly synthesized follicles in *Foxo3*^{-/-} ovaries should undergo activation at the time of their assembly, leading to ovaries with follicles at different stages of maturation (albeit possibly in reduced numbers) and female infertility would thus not be anticipated from a pure defect in the repression of PFA. However, infertility in *Foxo3*^{-/-} females occurs by 15 weeks of age and we do not observe any evidence of *de novo* follicle regeneration. *Foxo3*^{-/-} ovaries after 15 weeks are always completely devoid of follicles (Fig. 5; Castrillon *et al.* 2003). On the other hand, we cannot exclude the possibility that *Foxo3* is required in the bone marrow for the survival or function of putative germline stem cells or in the somatic ovary to sustain them. Nonetheless, it is not clear why such defects would be manifest only in adult life, since as discussed above, it is apparent from the *Foxo3*^{-/-} phenotype that *Foxo3* is not required for early germline or gonadal development, including the initial colonization of the gonad by germline stem cells.

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