Awakening the oocyte: controlling primordial follicle development

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Abstract

Oocytes are sequestered in primordial follicles before birth and remain quiescent in the ovary, often for decades, until recruited into the growing pool throughout the reproductive years. Therefore, activation of follicle growth is a major biological checkpoint that controls female reproductive potential. However, we are only just beginning to elucidate the cellular mechanisms required for either maintenance of the quiescent primordial follicle pool or initiation of follicle growth. Understanding the intracellular signalling systems that control oocyte maintenance and activation has significant implications for improving female reproductive productivity and longevity in mammals, and has application in domestic animal husbandry, feral animal population control and infertility in women.

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Introduction

In mammals, the functional oocytes that give rise to offspring are sequestered in primordial follicles before birth and remain quiescent in the ovary, often for decades, until recruited into the growing pool throughout the reproductive years (Reynaud & Driancourt 2000, Choi & Rajkovic 2006a). Many years of research have demonstrated that control of primordial follicle activation requires complex bidirectional signalling between the oocyte and the surrounding somatic cells, involving specific cytokines and growth factors (Skinner 2005, Hutt *et al.* 2006b). In contrast, the intracellular signalling pathways activated during follicle development remain largely uncharacterized and are fundamental to understanding the molecular systems responsible for ensuring timely delivery of functional oocytes for fertilization. An improved understanding of these basic cellular mechanisms of ovarian physiology will inform biotechnology strategies for the manipulation of reproductive productivity in domesticated livestock (Hunter *et al.* 2004), the enhancement of protocols for the retention of fertility in endangered native species (Pukazhenthi *et al.* 2006) and the development of novel humane methods of pest animal population control (McLaughlin *et al.* 2003). Similarly, new insights into these fundamental processes have the potential to be therapeutically employed to improve reproductive health and productivity in women choosing to start families in later life (Baird *et al.* 2005), those who

are likely to experience premature ovarian failure (POF; Mahmoud *et al.* 2007) and those women seeking to reserve healthy oocytes for future use, for instance, prior to chemotherapy (Demeestere *et al.* 2007).

Ovarian folliculogenesis and reproductive aging

Oocytes in mammals develop from primordial germ cells (PGCs) that migrate early in development to the naive gonad to become oogonia. Folliculogenesis begins with recruitment of pregranulosa cells to the oocyte to form the primordial follicle in the foetal or neonatal ovary (see Fig. 1; Picton *et al.* 1998). At this stage, the oocyte is arrested in meiotic prophase I and only re-enters meiosis or germinal vesicle breakdown upon ovulation (Picton *et al.* 1998). From the pool of primordial follicles, some are stimulated to grow and enter development into primary, secondary or antral follicles (Skinner 2005; see Fig. 1). By the time the follicle has reached the pre-antral stage, the oocyte has enlarged considerably, synthesized the extracellular zona pellucida and is surrounded by proliferating granulosa cells. During the final stages of antral follicle development, the oocytes become competent enough to resume the meiotic process (Zheng & Dean 2007) and prior to ovulation, a LH surge causes nuclear maturation, culminating in the completion of the first meiotic division and extrusion of the first polar body, followed by re-arrest at metaphase II of meiosis II (Hutt & Albertini 2007). In mammals, the advent of reproductive

Figure 1 Confocal images of murine ovarian tissue triple labelled with rhodamine-conjugated *Pisum sativum* lectin (red), FITC-conjugated wheat germ agglutinin lectin (green) and the DNA-specific dye TOPRO3 (blue). (A) Neonatal ovary containing both primordial and primary follicles. (B) Enlarged image of primordial follicle.

senescence reveals that the loss of ovarian function (or menopause) occurs at a relatively young age compared with other physiological systems. As the primary determinant of reproductive performance in female mammals is the size and longevity of the primordial follicle pool, methods of estimating the reproductive age, opposed to chronological age, have determined that the rate of decline in quiescent functional follicles in the adult ovary increases with age (Hansen *et al.* 2008).

Timing and control of primordial follicle formation

Migration of PGCs from the epithelium of the yolk sack, through the embryonic hindgut, to eventually colonize the gonadal ridge is controlled and directed by the secretion of pleiotrophic cytokines and growth factors, such as SCF and CXCL12 (for reviews see (Molyneux & Wylie 2004, Kunwar *et al.* 2006)). On arrival at the genital ridge the PGCs migrate inwards to form the primitive medullary and sex cords, where they lose their motility and in females become oogonia (Baker & Franchi 1967, Sathananthan *et al.* 2000). Dividing oogonia are found clustered together in oogonial nests, which are connected by intercellular bridges (Pepling & Spradling 1998, 2001, Pepling 2006). On cessation of mitosis, the oogonia enter the early stages of meiosis I as primary oocytes. Somatic cells invade the cortex from the medullary and now surround the oogonial nests (Gondos 1987a, 1987b). Interestingly, those cells in the inner gonad appear to be the first oocytes to enter meiosis while those located at the periphery are the last to do so (Edwards *et al.* 1977, van Wezel & Rodgers 1996) and this may have consequences for the timing of primordial follicle activation. Oocytes then lose their intercellular bridges and become enclosed in a layer of flattened pregranulosa cells, thought to be derived from mesonephric, or surface epithelial, cells (McNatty *et al.* 2000) though this is a matter of some debate (see (Maheshwari & Fowler 2008)). Finally, the somatic cell-surrounded oocytes arrest in meiotic prophase I at diplotene, becoming the primordial follicles (Baker &

Franchi 1967). In the human ovary, the first primordial follicles are detected in mid-gestation and in rodents 2–4 days post-birth (Hirshfield 1991, Sforza & Forabosco 1998) and recent studies have suggested that changes in the levels of maternal and foetal oestradiol and progesterone regulate primordial follicle assembly (Kezele & Skinner 2003, Britt *et al.* 2004, Nilsson *et al.* 2006b, Chen *et al.* 2007).

Transcriptional control of primordial follicle formation

Phenotypic analysis of mice null for a number of transcription factors has been central to our ability to identify concomitant crosstalk between signalling pathways in oogenesis and early folliculogenesis and these transcription factors have been postulated to be candidate genes for POF in humans (Suzumori *et al.* 2007).

One of the first transcription factors found to have a role in primordial follicle formation was factor in the germ-line α (*Fig α* or *Figla*), which is a basic helix-loop-helix transcription factor that binds as a heterodimer to the Ebox elements found in the promoters of the ZP genes to control their expression (Liang *et al.* 1997). Primordial follicle formation appears to be directed by the transcription factor *Figla*, which is expressed by the oocyte (Soyal *et al.* 2000). *Figla* is expressed in the female mouse gonad at embryonic day 13 but its expression is reduced between post-natal day 7 and 14; however, residual transcripts are found in the adult ovary. Phenotypic analysis of the homozygous null female mouse confirmed sterility while the male was fertile. Histological assessment revealed that the female ovary was found to contain no germ cells and that this was a result of the inability of the germ cell nests to break down and form follicles within the post-natal day 0 ovary. In late embryogenesis, the oocytes did not proceed to the diplotene stage of meiosis, and instead these germ cells died. Gene expression analysis indicated that the oocytes of the null mice did not express any zona pellucida (ZP) gene (Soyal *et al.* 2000).

More recent studies have revealed that *Figla* has also been found to up-regulate several genes including *Pou5f1*, *Zp2*, *Ivns1abp*, *Vbp1*, *Padi6* and *Rbpms2* and to down-regulate *Sp3*, *Hdac2* and *Ogt*. Several testis-specific genes are also down-regulated by the presence of *Figla* in oocytes that if expressed might disrupt normal oogenesis (Joshi *et al.* 2007). *Foxc1* is a forkhead/winged helix transcription factor, and while disruption of this gene does not interfere with the assignment of PGCs, the migration of these cells is disrupted with only a quarter of the population reaching the genital ridges (Mattiske *et al.* 2006). However, once in the ovary the PGCs proliferate normally but the supporting tissue is not arranged correctly, and the primordial follicles are not organized correctly (Mattiske *et al.* 2006). These mutants die at birth but analysis of transplanted ovaries in ovariectomized females determined that the germ cells undergo folliculogenesis; however, the theca and the granulosa cells are not well organized and the antrum does not form. Since *Foxc1* is not expressed in the oocyte, the authors surmised that it may be required by the granulosa cells to respond to signals from the oocyte (Mattiske *et al.* 2006).

Post-natal oogenesis in mammals

In 2004, the prevailing dogma that the primordial follicle population is finite (Zuckerman 1951), was challenged (Johnson *et al.* 2004). Initial reaction included the re-appraisal of data pertaining to older studies (Byskov *et al.* 2005) and new studies in traditional (Liu *et al.* 2007c, Begum *et al.* 2008) transplantation (Johnson *et al.* 2005, Lee *et al.* 2007a), parabiotic (Eggan *et al.* 2006) and null mouse models systems (Lee *et al.* 2007b). This was accompanied by the publication of a number of review and commentary articles (Telfer *et al.* 2005, Hutt & Albertini 2007) with the existence and function of these 'Tilly' follicles fiercely contested (Johnson *et al.* 2005, Skaznik-Wikiel *et al.* 2007, Tilly & Johnson 2007, Tilly & Rueda 2008, Tilly *et al.* 2008). Several researchers have sought to produce independent evidence of the existence of post-natal oogenesis in adults with conflicting results (Kerr *et al.* 2006) and while most studies conclude that these newly formed follicles do not contribute to the pool of ovulated oocytes there is some conjecture that these 'follicles' may play a role in providing support for the depleted ovarian reserve (Lee *et al.* 2007a, 2007b).

Regulation of activation and apoptosis in the primordial follicle population

Many questions remain definitively unanswered regarding the regulation of primordial follicle population dynamics. Of key interest are the mechanisms by which a particular follicle is selected to grow while

those immediately adjacent remain quiescent. One popular proposition is known as the 'production-line' hypothesis (Henderson & Edwards 1968), in which the first oocytes to enter meiotic arrest during embryonic gonad development are, in fact, also the first oocytes to activate in adult life. The rationale behind this hypothesis is that mature oocytes produced in late mammalian female life are more likely to be chromosomally imbalanced or aneuploid as they are the last oocytes to enter meiotic arrest and are dysfunctional due to the presence of fewer chiasmata and univalent pairs (Henderson & Edwards 1968). Observation of neonatal ovarian development supports this hypothesis as the oocytes deposited closest to the corticomedullary junction undergo rapid meiotic progression, become primordial follicles and are among the first to activate, whereas oocytes located further within the cortex undergo slower meiotic progression and activate later in life (Hirshfield 1991). Initial evidence using radio-labelling to support this hypothesis that follicle do not activate and mature at random (Polani & Crolla 1991, Hirshfield 1992) conflicted with the *in vitro* data indicating that in culture the majority of the primordial follicles would activate simultaneously (Wandji *et al.* 1996) and thus initiation of growth of primordial follicle was probably subject to extrinsic factors (Eppig & O'Brien 1996). Attempts to solve this conundrum using 5-bromo-2'-deoxyuridine (BrdU) labelling of follicles *in utero* met with limited success (Meredith & Doolin 1997), though did partially support the 'production-line' hypothesis. The situation is further complicated by the evidence that the primordial follicle population is heterogeneous based on the mitotic activity of the lineages of the follicle somatic cells. Growth initiation may depend upon the proportion of each of these cell types in the follicle, as each type may have a different threshold for responding to activation or inhibitory cues (Hirshfield 1992).

In the species studied so far, both the age and cyclicity of the animals is crucial, as multiple reports concur that both follicle activation and development are accelerated during the initial waves of folliculogenesis when compared with those in adult cycling animals (Pedersen 1969, Hage *et al.* 1978). Interestingly, using unilateral ovariectomy, Brook and colleagues (Brook *et al.* 1984) found in mice that the biological age, rather than the chronological age, had an important influence on oocyte quantity and quality. This is reflected in the studies of human ovarian reserve, where the proportion of follicles that activate appears to depend on the size of the ovarian follicle reserve as there is an apparent inverse correlation between the fraction of growing follicles and the size of the primordial follicle store. Importantly, these histomorphometric analyses have consistently shown an increase in follicle loss in the perimenopausal years in humans (Gougeon *et al.* 1994, Faddy & Gosden 1996, Faddy 2000, Hansen *et al.* 2008), with few of the other

mathematical models of primordial follicle growth dissenting (Mandl & Zuckerman 1951).

Elegant work using oocytes and granulosa cells to reconstitute follicles has demonstrated that once activated to grow that oocytes orchestrate and coordinate the development of mammalian ovarian follicles and that the rate of follicle development is controlled by the oocyte (Eppig 2001, Eppig *et al.* 2002). Thus began the search for intrinsic factor(s) that determined how many follicles begin growing each day (Gougeon & Chainy 1987) and led to the hypothesis that the accelerated follicle loss observed in neonatal and juvenile animals is due to the absence of an inhibitor produced by mature follicles (Edwards *et al.* 1977). Moreover, slightly increased serum gonadotrophin levels observed in neonates, juveniles and perimenopausal women may be responsible for the accelerated follicle activation particularly, as recent reports suggest that FSH activity can significantly influence the size of the primordial and early pre-antral follicle populations (Roy & Albee 2000, Allan *et al.* 2006), which is in contrast with the widely accepted view of gonadotrophin-independent early folliculogenesis (McGee & Hsueh 2000).

The vast majority of oogonia and oocytes are lost during embryonic, neonatal and adult life through apoptosis (Kim & Tilly 2004) and are not destined to produce mature oocytes for fertilization. Arguments that this is a selection mechanism designed to remove abnormal oocytes from the follicle pool are cogent but observations of single cell atresia in oocyte nests during primordial follicle formation (Pepling & Spradling 2001) indicates that other factors such as somatic cell support may regulate this process.

Knockout mice null for specific transcription factors has illuminated some of the pathways involved in primordial follicle formation and survival. Preferentially expressed in oocytes in the mouse ovary is LHX8, a LIM-homeobox transcription factor family member. Mice null for LHX8 fail to maintain the primordial follicles that disappear in the first week of life (Choi *et al.* 2008b) and appears to be caused by a marked reduction in the expression of KIT and KITL in LHX8 null ovaries. POU5F1 (or OCT4) is a transcription factor whose precise targets are not known and its role in oogenesis has only recently been ascribed (Pangas & Rajkovic 2006). POU5F1 is expressed in PGCs until after they migrate to the naïve gonad and expression is then repressed after the onset of meiotic prophase I in oocytes. POU5F1 is re-expressed after birth and coincides with the growth period of the oocytes (Parfenov *et al.* 2003). As the phenotype of the POU5F1 null mouse is embryonic lethal, little was known about its post-natal role in the ovary, but analysis of the conditional knockout targeted to the germ cells demonstrates a role in PGC survival. When the POU5F1 conditional oocyte-specific female mice are born, their ovaries contain some oocytes; however, a progressive increase in infertility is observed corresponding to the loss of primordial follicles. At 6 weeks of age, there are almost

no follicles within the ovary and this has been attributed to the absence of POU5F1 resulting in significant premature apoptosis in the PGCs prior to colonization of the gonads (Kehler *et al.* 2004).

Transcript profiling in folliculogenesis

While ovarian follicle development has been well characterized histomorphologically and the occurrence of naturally occurring KIT mutant mice, such as *steel panda* (Slpan/Slpan; Hutt *et al.* 2006b) have given insights into the process of folliculogenesis, it is the advent of state-of-the-art genomic and proteomic approaches that have identified key paracrine and autocrine signalling pathways, associated with the initial signs of primordial follicle activation.

To identify the essential mediators of follicle activation, researchers have obtained and compared ovarian transcriptomes during the crucial phase of primordial to primary follicle transition in the mammalian ovary (Arraztoa *et al.* 2005, Kezele *et al.* 2005b, Serafica *et al.* 2005, Holt *et al.* 2006). These innovative approaches provided new insights into the candidate regulatory factors and the development of novel mechanistic models for this critical biological process. Key findings included the identification of ligands and their receptors in primordial follicles, which play important roles in the control of the primordial to primary follicle transition and maintenance of quiescence.

Functional analysis using ovarian neonatal *in vitro* culture systems has confirmed that an ever increasing number of cytokines and pleiotrophic growth factors are known to enhance the rate of activation of primordial follicles (see Fig. 2) including KIT ligand (KITL; Hutt *et al.* 2006b), leukaemia inhibitory factor (LIF; Nilsson *et al.* 2002), bone morphogenic proteins (BMP4 and BMP7; Lee *et al.* 2001, Nilsson & Skinner 2003, Craig *et al.* 2007), platelet-derived growth factor (Nilsson *et al.* 2006a), keratinocyte growth factor (Kezele *et al.* 2005a), basic fibroblast growth factor (FGF2; Nilsson *et al.* 2001), glial-derived neurotrophic factor (Dole *et al.* 2008) and the neurotrophins (NGF, NTF5 and BDNF; Disen *et al.* 2002, Romero *et al.* 2002, Spears *et al.* 2003, Paredes *et al.* 2004, Dole *et al.* 2008).

In addition, *in vitro* culture studies have indicated that AMH acts to inhibit the primordial to primary follicle transition (Durlinger *et al.* 2002), with the exception of a recent report in the human where AMH initiates follicle development (Schmidt *et al.* 2005). Recently, our genome-wide array analyses further identified the chemoattractive chemokine CXCL12, as a second inhibitor of primordial follicle transition (Holt *et al.* 2006).

Similar to primordial follicle assembly, an endocrine model of the primordial to primary follicle transition has been suggested for the rodent involving the actions of progesterone and oestrogen. *In vivo*, steroid levels are high in prenatal ovaries due to the maternal influence,

Figure 2 Ovarian cytokine and growth factor signalling factors – roles in primordial to primary follicle transition. Green arrows are promoters of development and red arrows are inhibitors of follicle activation. Abbreviations: AMH, anti Müllerian hormone; BMP, bone morphogenic protein; FGF, fibroblast growth factor; GDF, growth differentiation factor; GDNF, glial-derived neurotrophic factor; KGF, keratinocyte growth factor; KL, KIT ligand; LIF, leukaemia inhibitory factor; SDF, stromal cell-derived factor.

and decline during the first post-natal wave of folliculogenesis in mice. *In vitro* both hormones act to decrease primordial follicle recruitment during the first wave following birth (Kezele & Skinner 2003). As progesterone levels have been noted to drop in monkey foetuses at around the time of follicle growth initiation, creating a plausible mechanism for the control of premature recruitment in other species (Kezele & Skinner 2003). This hypothesis is further supported by studies in the oestrogen-deficient aromatase knockout mouse that display higher numbers of pre-antral and antral follicles by 6 weeks of age than wild-type mice. This observation suggests that the onset of primordial follicle recruitment is temporally retarded by oestrogen (Britt *et al.* 2004).

Multiple activator and repressor pathways converge to regulate activation of the primordial follicle

Altogether four lines of evidence suggest that multiple activator and repressor pathways converge to regulate activation of the primordial follicle. First, primordial follicles spontaneously activate *in vitro*. Incubation with exogenous cytokines and growth factors increases the proportion of activated follicles and treatment with function blocking antibodies will suppress but not abolish primordial follicle activation suggesting other endogenous mechanisms (Kezele *et al.* 2002a, Hutt *et al.* 2006a, b). This is corroborated by the inability of AMH and CXCL12 treatment to completely suppress follicle activation (Holt *et al.* 2006, Nilsson *et al.* 2007).

Secondly, studies of mice null for the ligands and their receptors implicated in follicle activation have indicated that their presence is not essential. Examples include the FGF2 knockout mouse, which is viable, fertile and has normal ovarian folliculogenesis (Dono *et al.* 1998). Similarly LIF knockout mouse females while infertile because of a

defect in implantation, have normal folliculogenesis, ovarian morphology and oocytes that fertilize and implant in wild-type recipients (Stewart *et al.* 1992).

Thirdly, crosstalk between signalling pathways is crucial as knockout studies have also revealed that the neurotrophin, nerve growth factor (NGF) and its tyrosine receptor kinase NTRK1 are important in regulating follicular activation (Dissen *et al.* 2002, Romero *et al.* 2002). Two other neurotrophins (NTF5 and BDNF) and their receptor NTRK2 also appear to be highly influential in the survival of oocytes in neonatal mice with a role in primordial follicle maintenance (Romero *et al.* 2002, Paredes *et al.* 2004). Interestingly, treatment with FGF2 can overcome the effects of blocking NTRK2 signalling through rescuing oocytes from apoptosis, suggesting that at least two alternative coordinated pathways exist to ensure oocyte survival (Spears *et al.* 2003). Similarly, treatment of neonatal ovaries with the inhibitor AMH regulated the TGF β signalling pathway, resulting in inhibition of primordial follicle development (Nilsson *et al.* 2007).

Fourthly, the identification of ligands with multiplicity of roles in folliculogenesis, such as the TGF β ligands, GDF9 and BMP15, which are well recognized oocyte-derived regulatory proteins largely thought to have a role during pre-antral follicle development in addition to their facilitation of primordial follicle activation in some species. GDF9 ligand binds to the BMPRII and ALK type I receptor (Mazerbourg *et al.* 2004); however, the intracellular signalling cascades mediated by GDF9 are yet to be fully identified. The *Gdf9* mRNA and protein have been located to the oocytes of primary follicles onwards in a number of species including the mouse (McGrath *et al.* 1995, Dong *et al.* 1996), rat (Hayashi *et al.* 1999, Jaatinen *et al.* 1999) and human (Aaltonen *et al.* 1999), whilst in the sheep and cow the primordial follicles also express GDF9 (Bodensteiner *et al.* 2000). The function of GDF9 in

primordial follicle growth remains somewhat obscure. Treatment of immature rats with recombinant GDF9 *in vivo* leads to an increased rate of follicular activation (Vitt *et al.* 2000). Similarly, addition of recombinant rat GDF9 to human ovarian cortical slices *in vitro* increases the proportion of activated follicles as well as improving survival of follicles (Hreinsson *et al.* 2002). However, GDF9 null mice are infertile as ovarian follicles do not progress beyond the primary stage of folliculogenesis as granulosa cell proliferation is limited and the theca cell layer fails to develop (Dong *et al.* 1996). However, the oocyte growth is accelerated. This suggests that GDF9 may have a role in recruiting granulosa cells while limiting the growth of the oocyte. While BMP15 null mice are subfertile (Yan *et al.* 2001), sheep with BMP15 mutations have primary follicle stage arrest (McNatty *et al.* 2007). BMP15 has also been implicated in the regulation of KITL (Hutt & Albertini 2007).

SMADS are downstream intracellular signalling molecules of the TGF β ligands (BMP4 and BMP7) and several of the SMAD knockouts generated have been found embryonically lethal; however, the SMAD3 null mouse is viable. The SMAD3 null mouse has impaired folliculogenesis as the ovaries of young mice are normal while the adults have increased numbers of primordial follicles and decreased numbers of higher order follicles. SMAD3 deficiency slows follicle growth, causes atretic follicles, and lowers expression of the anti-apoptotic protein BCL2 (Kaivo-oja *et al.* 2006).

In light of the above, it is clear that these cytokine and growth factor-activated pathways interact to generate an intracellular balance of signals, ensuring maintenance of the quiescent primordial oocyte pool and controlled activation of primordial follicles from this finite population. However, the intracellular pathways by which these pleiotrophic cytokines influence follicle growth are only just being characterized.

Intracellular signalling in oocytes and pregranulosa cells in primordial follicles

Recently, one of the upstream transcription factors in the oocyte responsible for controlling expression of key genes in primordial to primary follicle transition was identified. *Sohlh1* is a basic helix-loop-helix transcription factor, which is expressed in the germ cell clusters and in newly assembled primordial follicles. However, SOHLH1 protein expression disappears when the oocyte is activated to become a primordial follicle. The *Sohlh1* null female mouse is infertile with the oocyte numbers decreasing from about normal at birth to none by 7 weeks of age. Further investigation revealed that there was an apparent defect at the primordial to primary follicle transition (Pangas *et al.* 2006).

As the time frame for oocyte loss is similar to that seen in *Figla* null mouse, and *Sohlh1* null mice show a significant reduction in *Figla* transcripts and their targets

(including the zona pellucida genes (Pangas *et al.* 2006), the authors speculated that SOHLH1 positively cooperates with this second transcription factor (FIGLA) in the transcriptional regulation of two of the zona pellucida genes (ZP1 and ZP3), hence the failure to transit into primary follicles (Pangas *et al.* 2006).

More recently the *Sohlh2*-deficient mouse has been described in which this germ cell-specific gene deletion also results in infertile adult mice in which the primordial follicles are abnormal. Again there is no differentiation of the surrounding pregranulosa cells into cuboidal and multilayered structures. Although there is limited growth, the primordial oocytes are abnormal in that several genes including *Sohlh1*, *Nobox*, *Figla*, *Gdf9*, *Pou5f1*, *Zp1*, *Zp3*, *Kit*, *Oosp1*, *Nlrp14*, *H1foo*, and *Stra8* are all misexpressed. The resultant phenotype is the rapid loss of oocytes in early post-natal life (Choi *et al.* 2008a).

In addition, there were several molecular similarities between the *Sohlh1* and *Sohlh2* null mice and the *Nobox* null mouse (newborn ovary homeobox gene) (Rajkovic *et al.* 2004), in that the *Gdf9*, *Pou5f1*, *Zar1*, *Moss* and *H1foo* gene expression was down-regulated. Gene expression studies confirmed that the *Sohlh1* and two null mice also had a significant reduction in the expression of the transcription factor gene, *Nobox* and that the *Lhx8* (LIM homeodomain protein) was also down-regulated; however, the function of *Lhx8* is unknown. *Lhx8* knockouts have an identical ovarian phenotype to that of the *Sohlh1* (Pangas *et al.* 2006). Chromatin immunoprecipitation assay (ChIP) analysis demonstrated that SOHLH1 bound directly to the promoters of *Lhx8*, *Zp1* and 3 but not to *Nobox* or *Zp2*. It is likely that SOHLH1 binds directly to the Ebox found in the promoters of *Lhx8*, *Zp1* and *Zp3* and regulates expression while it is unlikely that SOHLH1 directly controls the expression of *Figla* as this gene does not contain an Ebox in its promoter (Pangas *et al.* 2006). NOBOX binds to cis-acting NOBOX DNA-binding elements (NBEs) TAATTG, TAGTTG, TATTA and the promoters of *Pou5f1* and *Gdf9* both contain NBEs. This suggested that NOBOX regulates their transcription along with the finding that *Nobox* expression closely precedes expression of *Pou5f1* and *Gdf9* (Choi & Rajkovic 2006b). *Nobox* null female mice are infertile with ovaries lacking oocytes by 6 weeks of age. However, the ovaries of *Nobox* null mice are normal before birth, in that ovarian development, germ cell proliferation and initial primordial follicle population was histomorphologically similar to the wild-type siblings. However, development of primary and secondary follicles did not occur in the null mice. Even though some of the oocytes were surrounded by cuboidal granulosa cells, the oocytes were very small and are continually lost from post-natal day 3 until only a few oocytes remained in the post-natal day 14 ovary.

Transcripts for *Pou5f1*, *Gdf9* and *Bmp 15* were down-regulated in the null ovaries indicating that NOBOX may

have a role in their regulation (Rajkovic *et al.* 2004). Recent analysis of the promoter activities of *H1foo*, *Npm2* and *Zar1* maternal genes in oocytes and pre-implantation embryos showed transcriptional activity in oocytes, but not in fertilized embryos. A putative Ebox region in the *H1oo* and the *Npm2* promoters were required for basal transcriptional activity in oocytes and a putative NBE motif was shown to enhance basal transcriptional activity of the *Npm2* promoter (Tsunemoto *et al.* 2008). In addition, a comparison of the expression level of genes regulated in LHX8 null and NOBOX null newborn ovaries indicated a common subset of genes was affected in both LHX8- and NOBOX-deficient mice (Choi *et al.* 2008b).

Does a single pathway control primordial follicle activation?

Of great current interest is the intracellular oocyte signalling pathway that controls FOXO3, a member of the forkhead transcription family, which has been implicated in a range of cellular functions including embryogenesis, tumorigenesis and maintenance of differentiated cell states through regulation of such cellular processes as metabolic cell cycle arrest, cellular stress response and apoptosis (Kaufmann & Knochel 1996, Hosaka *et al.* 2004). Homozygous mutant mice display increased numbers of growing follicles, a lack of primordial follicles by 2 weeks of age and a subsequent increase in oocyte degeneration of newly growing follicles (Castrillon *et al.* 2003, Hosaka *et al.* 2004). Originally discovered to be expressed by the granulosa cells (Richards *et al.* 2002), the mechanisms by which FOXO3 mediated its suppressive effects on primordial follicle recruitment remained unclear. Brenkman and Burgering proposed a model whereby FOXO transcription factors arrest the cell cycle by increasing the expression of a cell cycle inhibitor, CDKN1B, whilst concurrently decreasing cyclin D1 and D2 expression (Brenkman & Burgering 2003).

However, recent studies of a CDKN1B-deficient mouse model indicated that post-natal follicle assembly was accelerated, the initial population of primordial follicles was substantially increased and primordial follicle recruitment was prematurely activated (Rajareddy *et al.* 2007). This group determined that CDKN1B (or *Cdkn1b*) was also expressed in the oocyte and controlled oocyte development not by the usual control of the cell cycle progression but by suppressing the functions of *Cdk2/CDC2A*–cyclin A/E1 in diplotene-arrested oocytes (Rajareddy *et al.* 2007). Simultaneously, through activation of a caspase-mediated apoptotic cascade the authors propose that CDKN1B also induces follicle atresia (Rajareddy *et al.* 2007).

Upstream control of FOXO3 activation of protein kinase B (Akt) via extracellular signalling pathways, such as that induced by insulin signalling, results in FOXO phosphorylation, and inactivation as a result of nuclear exclusion (Junger *et al.* 2003). The reported effect of insulin on follicle

activation (Kezele *et al.* 2002b) may therefore involve FOXO3 inhibition and allow for the transcription of genes required for follicle activation. The presence of FOXO3 in follicles may also provide an explanation for how the quiescent primordial follicle is protected from oxidative stress as FOXO3 up-regulates expression of the superoxide-scavenger manganese superoxide dismutase (MnSOD) in mammalian cells (Kops *et al.* 2002).

Signal transduction – the phosphatidylinositol 3-kinase (PI3K) pathway

By contrast, studies of oocyte intracellular signalling pathways suggest that the KL produced by the granulosa cell binds to oocyte c-KIT receptor and the signal transduced through the PI3K pathway (Reddy *et al.* 2005) and this is important for the regulation of early follicular development (Liu *et al.* 2006). Components of the PI3K pathway, including Akt, FOXO3, GSK3A and GSK3B (Liu *et al.* 2007b), FOXO3 and CDKN1B have been shown to be present in growing mouse oocytes and the PI3K pathway is stimulated in KL-treated *in vitro* cultured mouse and rat oocytes. These authors theorize that this signalling cascade, initiated by KL-activated oocyte surface c-KIT and followed by the subsequent activation of PI3K by c-KIT, may enhance oocyte growth and also the production of oocyte factors, which in turn stimulate the proliferation and differentiation of the surrounding granulosa cells. Simultaneously, KL-stimulated phosphorylation and functional suppression of FOXO3 may release the oocytes from their quiescent state allowing oocyte growth and development secretion (Liu *et al.* 2007a). This hypothesis was plausible given that FOXO3 has been shown to be capable of suppressing the production of BMP15, connexin 37 and connexin 43 in mouse oocytes, which are important for both oocyte granulosa and between granulosa cell communications, and to facilitate nuclear expression of p27 in the oocyte, which all lead to the arrest of oocyte growth and follicular development (Liu *et al.* 2006).

In 2008, an important piece of this signalling pathway was confirmed when Reddy, Liu, and colleagues published their findings on the ovary-specific conditional knockout of PTEN (phosphatase and tensin homolog deleted on chromosome 10) in oocytes, a major negative regulator of PI3K (Reddy *et al.* 2008). Like the FOXO3 null mouse, the entire primordial follicle pool becomes activated and all primordial follicles become depleted in early adulthood, causing POF. The authors concluded that this collective set of results suggested that mammalian oocyte is the initiator of follicle activation and that the oocyte PTEN-PI3K pathway governs follicle activation through control of initiation of oocyte growth (see Fig. 3).

Development of an oocyte-specific inducible conditional null (Vasa-Cre(ERT2) mouse has confirmed that

Figure 3 The oocyte KL – PTEN-PI3K – FOXO3 pathway governs follicle activation through control of initiation of oocyte growth. Abbreviations: KL, KIT ligand; KIT, CD117 (c-KIT); PIP2, phosphatidyl inositol bisphosphatel; PI3K, phosphoinositide 3-kinase; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PDK1-3, phosphoinositide-dependent protein kinase; Akt, protein kinase B; PTEN, phosphatase and tensin homolog; FOXO3, forkhead box O3A; GSK3, glycogen synthase kinase 3; CDKN1B, cyclin-dependent kinase inhibitor 1B (CDKN1); BMP15, bone morphogenic protein 15; Cx37, connexin 37; GDF9, growth differentiation factor 9.

the phosphoinositide 3-kinase (PI3K) signalling pathway does control primordial follicle activation (John *et al.* 2008), and that this is mediated through the forkhead transcription factor FOXO3. Evidence suggests that in oocytes during primordial follicle assembly the transcription factor FOXO3 is imported into the nucleus, and is re exported upon primordial follicle activation. Using oocyte-specific loss of PTEN to induce PI3K activation of Akt activation resulted in FOXO3 hyperphosphorylation, and FOXO3 nuclear export and this was concomitant with primordial follicle activation thus indicating that PI3K pathway and FOXO3 do in fact control primordial follicle activation (John *et al.* 2008).

Conclusions

Elegant work by Eppig and colleagues has demonstrated that once activated to grow, that oocytes orchestrate and coordinate the development of mammalian ovarian follicles and that the rate of follicle development is controlled by the oocyte (Eppig *et al.* 2002). However,

primordial follicle activation appears to require close communication with somatic cells and therefore we conclude that since factors such as KL clearly demonstrate a role for granulosa cells in releasing oocytes into the growing pool, then if the trigger is oocyte generated, an early response must include suppression of FOXO3 activity. This further underscores the necessity of understanding not only the oocyte and granulosa pathways but also the signals between the cells. This has led to the speculation that a 'folliculogenesis clock' exists that is set by the oocyte (Matzuk *et al.* 2002). The discovery of more factors involved in the initiation of folliculogenesis will further aid in defining what triggers the mammalian follicle to leave the resting pool.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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