Follicular assembly: mechanisms of action

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Abstract

The differentiation of primordial germ cells (PGCs) into functional oocytes is important for the continuation of species. In mammals, PGCs begin to differentiate into oocytes during embryonic development. Oocytes develop in clusters called germ line cysts. During fetal or neonatal development, germ cell cysts break apart into single oocytes that become surrounded by pregranulosa cells to form primordial follicles. During the process of cyst breakdown, a subset of cells in each cyst undergoes cell death with only one-third of the initial number of oocytes surviving to form primordial follicles. The mechanisms that control cyst breakdown, oocyte survival, and follicle assembly are currently under investigation. This review describes the mechanisms that have been implicated in the control of primordial follicle formation, which include programmed cell death regulation, growth factor and other signaling pathways, regulation by transcription factors and hormones, meiotic progression, and changes in cell adhesion. Elucidation of mechanisms leading to formation of the primordial follicle pool will help research efforts in ovarian biology and improve treatments of female infertility, premature ovarian failure, and reproductive cancers.

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Introduction

Mammalian females have a reproductive lifespan that is determined by the time of birth. Establishment of the pool of primordial follicles, each consisting of an oocyte surrounded by a layer of somatic cells, is essential for fertility. This begins during embryonic development when germ cells arrive at the gonad and divide to form clusters of cells known as germ cell cysts. As the oocytes develop, these cysts begin to break apart, allowing each oocyte to become surrounded by a layer of somatic cells, forming primordial follicles. This process is referred to as primordial follicle formation or assembly. At the same time, there is a loss of approximately two-thirds of oocytes. Mechanisms regulating cyst breakdown, germ cell numbers, and primordial follicle assembly are currently under investigation. This review focuses on recent findings in the molecular regulation of cyst breakdown and primordial follicle formation.

The process of follicular assembly

In the mouse, primordial germ cells (PGCs) migrate from outside the embryo to the genital ridge at 10.5 days post coitus (dpc) and divide by mitosis until 13.5 dpc (Monk & McLaren 1981). The germ cells are called oogonia as they undergo these divisions and were described many years ago as developing in clusters or nests (Gondos 1973). However, more recently, these germ cell clusters were shown to exhibit the key characteristics of germ

line cysts that have been described in *Drosophila* and other invertebrates (Pepling & Spradling 1998). Germline cysts divide synchronously and are connected by intercellular bridges due to incomplete cytokinesis of oogonial division (de Cuevas *et al.* 1997). Thus, in this review, we will refer to the nests as germ cell cysts. The germ cell cysts are loosely surrounded by somatic cells and this grouping of germ cells and somatic cells is called the ovigerous cords (Byskov 1986). Examples of germ cell cysts within ovigerous cords are shown in Fig. 1. Germ cell cysts have been most well studied in *Drosophila* where the cells undergo four mitotic divisions to form 16 cell cysts (de Cuevas *et al.* 1997). One cell of the cyst becomes the oocyte while the remaining 15 become nurse cells and supply nutrients to the oocyte. In the mouse, the number of divisions is variable and the function of cysts is unclear (Pepling & Spradling 1998). Oogonia become oocytes when they enter meiosis beginning at 13.5 dpc. Oocytes progress through the stages of meiotic prophase I (leptotene, zygotene, and pachytene) and arrest in the diplotene stage beginning at 17.5 dpc with most cells reaching diplotene by postnatal day 5 (PND5) (Borum 1961).

Oocytes separate by a process called cyst breakdown and become enclosed in primordial follicles consisting of one oocyte and several granulosa cells (Fig. 2; Pepling & Spradling 2001). During cyst breakdown, some cells in each cyst die by programmed cell death with only one-third of the total surviving. In one model, one cell of

Figure 1 Germ cell cysts within ovigerous cords in a PND1 Oct4-GFP mouse ovary confocal section. (A) Oocytes expressing GFP (green), (B) stained for DNA (propidium iodide, red) and (C) overlay. Ovigerous cords consist of germ cell cysts and surrounding somatic cells. Two cysts within ovigerous cords are indicated by brackets. Scale bar = 10 μ m. PND1, postnatal day 1; GFP, green fluorescent protein; Oct4 (POU5F1), POU class 5 homeobox 1.

a cyst dies and the large cyst breaks into two smaller cysts. This is repeated until a few individual oocytes remain. The cyst cells may also support oocytes and eventually die analogous to nurse cells in *Drosophila*. Oocytes in primordial follicles represent the entire pool available during a female's reproductive life. After sexual maturity, cohorts of follicles are recruited to grow by a process called follicle activation (McGee & Hsueh 2000). Some follicles that are activated eventually die by a process called follicular atresia while oocytes in surviving follicles undergo ovulation.

Regional differences in germ cell cyst breakdown, oocyte loss, and follicle formation have been observed. Oocyte loss and cyst breakdown begin after birth in the cortical region of the ovary, but in the medullary region, these processes begin as early as 17.5 dpc (Pepling *et al.* 2010). This is similar to other studies on the mouse and also on humans where oocyte loss has been observed during fetal development (De Pol *et al.* 1997, De Felici *et al.* 1999, McClellan *et al.* 2003, Ghafari *et al.* 2007). In the rat, follicles are first observed in the core of the ovary and formation gradually shifts toward the surface (Rajah *et al.* 1992). The follicles closer to the medullary region are also the follicles that begin to develop first (Hirshfield & DeSanti 1995). In addition, oocytes located in the inner cortex and medullary region enter meiosis and start to grow first (Peters 1969, Nandedkar *et al.* 2007). This regional pattern is set up between 13.5 and 16.5 dpc in the mouse as the germ cells are entering meiosis (Byskov *et al.* 1997).

Interactions between germ cells and somatic cells

As the oogonia divide and form germ cell cysts, the cell clusters become enclosed in ovigerous cords consisting of germ cell cysts and somatic pregranulosa cells arranged in irregular branches (Byskov 1986). The cords are surrounded by a basal lamina (Mazaud *et al.* 2005) and are well defined in some species but not as prominent in the mouse (Byskov 1986). The ovigerous cords become fragmented as the oocytes separate and individual oocytes become enclosed in a layer of granulosa cells. The basal lamina of the ovigerous cords is remodeled as the cords fragment and follicles are formed (Mazaud *et al.* 2005). The process of follicle formation likely involves communication between

oocytes and granulosa cells (potential signals are discussed below). In addition, processes extending from somatic cells have been observed between oocytes suggesting that somatic cells may physically separate oocytes (Pepling & Spradling 2001).

Programmed cell death

In the mouse, cyst breakdown and oocyte loss occur concurrently, suggesting they are linked. Mechanisms governing oocyte death during cyst breakdown are not well understood but are thought to involve apoptosis-mediated programmed cell death. Table 1 shows programmed cell death regulators with putative roles in primordial follicle assembly. B-cell lymphoma/leukemia-2 (BCL2) family members regulate apoptosis and can be divided into two groups, pro-apoptotic (such as BAX, BAK, and BAD) and anti-apoptotic (such as BCL2, BCLX, and MCL1) (Kim & Tilly 2004). In the ovary, several BCL2 family members have been implicated in the regulation of oocyte survival. Adult female *Bcl2* knockout mice have fewer oocytes than wild-type mice, suggesting that BCL2 is important for oocyte survival though it is not known when the oocytes are lost in the knockout mice (Ratts *et al.* 1995). Overexpression of BCL2 in germ cells results in ovaries with more oocytes at PND8; however, by PND60, transgenic ovaries have the same number of oocytes as wildtype ovaries, suggesting that there is a mechanism to detect excess oocytes (Flaws *et al.* 2001). There is also evidence that the pro-apoptotic family member BAX is involved in regulating oocyte numbers (Perez *et al.* 1999). Neonatal *Bax* mutant ovaries have more oocytes than wild-type mice and more oocytes still in germ cell cysts linking

Figure 2 Diagram of cyst breakdown and primordial follicle assembly. Blue ovals are oocyte nuclei. Yellow indicates oocyte cytoplasm. Red moons are granulosa cells. As cysts break apart, some oocytes are lost by programmed cell death. Granulosa cells surround each surviving oocyte forming primordial follicles.

Table 1 Programmed cell death regulators that affect oocyte survival or cyst breakdown during follicle assembly.

Genes	Protein/function	Evidence for role in follicle assembly	References
<i>Atg7</i>	Involved in autophagy	Loss of germ cells by PND1	Gawriluk <i>et al.</i> (2011)
<i>Bax</i>	Bcl2 family of apoptosis regulators	Increased oocyte survival, reduced cyst breakdown and follicle formation	Perez <i>et al.</i> (1999) and Greenfeld <i>et al.</i> (2007)
<i>Bcl2</i>	Bcl2 family of apoptosis regulators	Mutation results in reduced oocyte numbers, over-expression results in increased oocyte numbers	Ratts <i>et al.</i> (1995) and Flaws <i>et al.</i> (2001)
<i>Becn1</i>	Involved in autophagy	Reduced oocyte numbers at PND1	Gawriluk <i>et al.</i> (2011)
<i>Casp2</i>	Cysteine protease	Increased number of primordial follicles	Bergeron <i>et al.</i> (1998)
<i>Mcl1</i>	Bcl2 family of apoptosis regulators	Expressed in oocytes during follicle formation	Hartley <i>et al.</i> (2002)

apoptosis and cyst breakdown (Greenfeld *et al.* 2007). These results support the model that apoptosis is required for germ cell cyst breakdown. MCL1 has been detected in human oocytes at the time of follicle formation though its role in the control of oocyte numbers has not been characterized yet (Hartley *et al.* 2002). Caspases act downstream of the BCL2 apoptosis regulators to execute programmed cell death. There are several caspases and one, caspase 2, when disrupted, results in an increased number of primordial follicles (Bergeron *et al.* 1998).

Programmed cell death can occur by mechanisms other than apoptosis, such as autophagy and necrosis. While there is evidence that apoptosis occurs in perinatal oocytes, recent work has also implicated autophagy as a cell death mechanism during cyst breakdown. Autophagy involves the degradation of intercellular components using the lysosomal machinery (Edinger & Thompson 2004). Lysosomal amplification has been observed in oocytes at the time of primordial follicle formation, suggesting that autophagy may occur in newborn ovaries (Rodrigues *et al.* 2009). Newborn female mice with targeted disruption of two different genes (*Atg7* and *Becn1*) encoding proteins involved in autophagy have a greater decrease in oocyte number than wild-type mice supporting a role for autophagy in the regulation of oocyte survival (Gawriluk *et al.* 2011).

Growth factors and signaling molecules

Several growth factors appear to be important for follicle formation (Table 2). Ovary organ culture studies have revealed a potential role for stem cell factor (SCF) in primordial follicle formation. When SCF is added to neonatal hamster ovaries in organ culture, primordial follicle formation is accelerated while an antibody against SCF inhibits follicle formation (Wang & Roy 2004). SCF and its receptor, the c-KIT receptor tyrosine kinase, have been well studied during several phases of ovarian development. Mice mutant for either *Scf* or *c-Kit* have defects in PGC migration and proliferation (Reith *et al.* 1990, Huang *et al.* 1993, Bedell *et al.* 1995). KIT signaling is also important for follicle activation and later follicular survival (Yoshida *et al.* 1997, Parrott & Skinner 1999, Reynaud *et al.* 2001). Injection of neonatal mice with ACK2, an antibody against c-KIT that blocks its action, beginning at PND1 had no effect on cyst

breakdown or primordial follicle formation (Yoshida *et al.* 1997). However, recent work has shown that cyst breakdown begins as early as 17.5 dpc (Pepling *et al.* 2010), so blocking KIT at PND1 may be too late to affect cyst breakdown and follicle formation.

Another group of growth factors, neurotrophins, have also been implicated in primordial follicle formation. Nerve growth factor (NGF) signals through its receptor, neurotrophic tyrosine kinase receptor, type 1 (NTRK1) also known as TRKA. *Ngf* or *Ntrk1* homozygous mutants have fewer primordial follicles and more oocytes still in germ cell cysts (Disen *et al.* 2001, Kerr *et al.* 2009). Two related growth factors are neurotrophin 4 (NT4) and brain-derived neurotrophic factor (BDNF) and both signal through NTRK2 also known as TRKB. Blocking NT4 or BDNF in organ culture results in a reduction of neonatal oocyte survival (Spears *et al.* 2003). *Ntrk2* mutants also have fewer oocytes and, in addition, primordial follicle formation is reduced (Kerr *et al.* 2009).

There is evidence that several signaling pathways are important for follicle assembly (Table 2) including members of the transforming growth factor beta (TGFB) superfamily (Trombly *et al.* 2009). Mutants for either bone morphogenetic protein 15 (*Bmp15*) or growth differentiation factor 9 (*Gdf9*) have ovaries with a significant proportion of multiple oocyte follicles (MOFs; Yan *et al.* 2001). Normal follicles consist of one oocyte enclosed in granulosa cells while MOFs are abnormal follicles with more than one oocyte per follicle (Kent 1960). MOFs are thought to be oocyte cysts that did not completely break apart during cyst breakdown (Jefferson *et al.* 2006). Both BMP15 and GDF9 are secreted by the oocyte early in ovarian differentiation, suggesting that signals from the oocyte to the granulosa cells are important for cyst breakdown and follicle formation (Elvin *et al.* 2000). Supporting the involvement of GDF9 in follicle formation, addition of GDF9 to hamster ovaries in organ culture promotes the formation of follicles (Wang & Roy 2004). Mouse ovaries exposed to another TGFB family member, activin A, have an increased number of primordial follicles (Bristol-Gould *et al.* 2006). Supporting the role of activin in primordial follicle formation, more MOFs are observed in mice that overexpress the activin antagonist, inhibin B (McMullen *et al.* 2001). In addition, activin subunit expression is

Table 2 Growth factors and signaling molecules implicated in follicle assembly.

Genes	Protein/function	Evidence for role in follicle assembly	Expressed in	References
<i>Activin A</i>	TGFB family member	Promotes follicle formation	Oocytes and granulosa cells	McMullen <i>et al.</i> (2001) and Bristol-Gould <i>et al.</i> (2006)
<i>Akt1</i>	Serine/threonine kinase, also known as protein kinase B (PKB)	Multiple oocyte follicles	Oocytes and granulosa cells	Brown <i>et al.</i> (2010)
<i>Amh</i>	Anti-Mullerian hormone, TGFB family member	Follicle formation is reduced and oocyte number increased in mutants	Stromal cells	Nilsson <i>et al.</i> (2011)
<i>Bdnf</i>	Brain-derived neurotrophic factor, neurotrophin signaling	Blocking in organ culture results in a reduction of neonatal oocyte survival	ND	Spears <i>et al.</i> (2003)
<i>Bmp15</i>	Bone morphogenetic protein 15, TGFB family member	Mutants have multiple oocyte follicles	Oocytes	Yan <i>et al.</i> (2001)
<i>Ctgf</i>	Connective tissue growth factor, CCN protein family member	Promotes follicle formation	Oocytes	Schindler <i>et al.</i> (2010)
<i>Follistatin</i>	Activin antagonist, TGFB family member	Reduced fertility, reduced follicle formation	Oocytes, granulosa, and stromal cells	Kimura <i>et al.</i> (2011)
<i>Gdf9</i>	Growth differentiation factor 9, TGFB family member	Mutants have multiple oocyte follicles	Oocytes	Yan <i>et al.</i> (2001)
<i>Inhibin α</i>	Activin antagonist, TGFB family member	Overexpression causes multiple oocyte follicles	ND	McMullen <i>et al.</i> (2001)
<i>Jagged1</i>	Notch ligand	Inhibition of Notch signaling reduces follicle formation	Oocytes	Trombly <i>et al.</i> (2008)
<i>Jagged2</i>	Notch ligand	Inhibition of Notch signaling reduces follicle formation	ND	Trombly <i>et al.</i> (2008)
<i>Lunatic fringe</i>	Regulator of Notch signaling	Mutants have multiple oocyte follicles	Granulosa cells	Hahn <i>et al.</i> (2005)
<i>Ngf</i>	Nerve growth factor, neurotrophin signaling	Follicle formation is reduced in mutants	Oocytes and granulosa cells	Dissen <i>et al.</i> (2001) and Abir <i>et al.</i> (2005)
<i>Notch1</i>	Notch receptor	Inhibition of Notch signaling reduces follicle formation	ND	Trombly <i>et al.</i> (2008)
<i>Notch2</i>	Notch receptor	Inhibition of Notch signaling reduces follicle formation	Granulosa cells	Trombly <i>et al.</i> (2008)
<i>NT4</i>	Neurotrophin 4, neurotrophin signaling	Blocking in organ culture results in a reduction of neonatal oocyte survival	ND	Spears <i>et al.</i> (2003)
<i>Ntrk1</i>	NGF receptor, neurotrophin signaling	Follicle formation is reduced in mutants	Oocytes and somatic cells	Kerr <i>et al.</i> (2009)
<i>Ntrk2</i>	Receptor for NT4 and BDNF, neurotrophin signaling	Follicle formation and germ cell number is reduced in mutants	Oocytes	Spears <i>et al.</i> (2003) and Kerr <i>et al.</i> (2009)
<i>p27</i>	Cyclin-dependent kinase inhibitor 1, downstream of PI3K signaling	Primordial follicle formation is accelerated in mutants	Oocytes and granulosa cells	Rajareddy <i>et al.</i> (2007)
<i>Scf</i>	Stem cell factor, kit signaling	Promotes follicle formation in hamster	Granulosa cells	Wang & Roy (2004) and Nandedkar <i>et al.</i> (2007)

ND, detected in ovary by RT-PCR but cell type not determined.

reduced in neonatal mice treated with estrogen, which blocks cyst breakdown (see Hormones section; Kipp *et al.* 2007). Mutants of another activin antagonist, follistatin, also have defects in oocyte development including a delay in cyst breakdown and follicle formation (Kimura *et al.* 2011). The TGFB family member, TGFB1, itself does not appear to play a role in follicle assembly but does interact with connective tissue growth factor (CTGF), which promotes follicle assembly in rat ovary organ culture (Schindler *et al.* 2010). The final TGFB family member that has been implicated in follicle assembly is anti-Mullerian hormone (AMH). Primordial follicle assembly is reduced in newborn rat ovaries treated with AMH while oocyte number is greater than controls (Nilsson *et al.* 2011). This suggests that AMH would normally maintain oocytes in cysts before follicle formation. Interestingly, AMH is

expressed in stromal cells that have not previously been linked to follicle formation.

There is growing evidence that Notch signaling is important for the regulation of primordial follicle formation. The first suggestion that Notch signaling might be involved in follicle formation came from analysis of lunatic fringe (*Lfng*) mutants (Hahn *et al.* 2005). *LFNG* is part of the Fringe family of proteins that regulate Notch signaling (Bruckner *et al.* 2000, Hicks *et al.* 2000). *Lfng* mutants are sterile and have ovaries with MOFs. The role of Fringe appears to be conserved as *fringe* mutants in *Drosophila* also have egg chambers with more than one oocyte (Grammont & Irvine 2001). There are four Notch receptors (Notch 1–4) and five Notch ligands (Jagged1 and 2 and Delta-like (DLL) 1, 3, and 4) in mammals (Bray 2006). Mouse knockouts have been generated for all of these genes (Conlon *et al.* 1995,

Hrabe de Angelis *et al.* 1997, Jiang *et al.* 1998, Xue *et al.* 1999, Krebs *et al.* 2000, 2003, 2004, McCright *et al.* 2001, Dunwoodie *et al.* 2002) but have not shed light on their role in follicle formation because they are either embryonic lethal or do not affect fertility at all. Primordial follicle formation is reduced in organ culture when Notch signaling is inhibited (Trombly *et al.* 2008). *Notch3*, *Notch4*, and *Dll3* mutants are fertile and *Dll1* and *Dll4* are not expressed in neonatal ovaries, suggesting that these molecules do not play a role in follicle assembly. Generations of *Notch1*, *Notch2*, *Jagged1*, and *Jagged2* tissue-specific knockouts will be necessary to determine which Notch ligands and receptors are important for follicle formation.

Signaling through the phosphoinositide-3-kinase (PI3K) pathway may be a mechanism used in follicle formation. The PI3K pathway activates AKT and AKT in turn can phosphorylate several proteins including TSC1/2, MTOR, FOXO3, and CDK1/p27. *Akt1* mouse knockout ovaries contain MOFs, indicating a role for the PI3K pathway in cyst breakdown (Brown *et al.* 2010). Further, inhibition of AKT in neonatal rat ovary organ culture resulted in decreased CDK1/p27 expression and decreased phosphorylated FOXO3a and was associated with reduced oocyte apoptosis (Liu *et al.* 2009). It is not, however, likely that FOXO3a is involved in cyst breakdown as *FOXO3a* knockouts demonstrate normal primordial follicle formation (John *et al.* 2007). However, *CDK1/p27* knockout mice show accelerated primordial follicle formation and CDK1/p27 is expressed in somatic cells surrounding cysts (Rajareddy *et al.* 2007). TSC1/2 and MTOR may be activated during cyst breakdown within oocytes. Few studies have examined the role of these molecules in follicle development, but TSC1 was recently shown to be important in primordial follicle activation using *Tsc1* knockout mice (Adhikari *et al.* 2010). Oocyte-specific knockouts of *Tsc1* or *Pten* (a negative regulator of PI3K signaling) undergo normal primordial follicle formation (Reddy *et al.* 2008, Adhikari *et al.* 2010). However, in these studies, the *Gdf9* promoter was used to drive Cre recombinase in oocytes, and this promoter is not active until PND3 after

most cysts have already broken down (Lan *et al.* 2004). Therefore, more work is needed to determine the effects of the PI3K pathway in cyst breakdown.

Transcription factors

Several nuclear factors appear to be important for primordial follicle formation (Table 3). Mutants of the gene encoding the aryl hydrocarbon receptor (AHR), a basic helix loop helix (BHLH) transcription factor, form follicles at a faster rate than normal (Benedict *et al.* 2000, Robles *et al.* 2000). Mutants of factor in germ line alpha (*Figla/Mhm*), also encoding a BHLH protein, begin to lose oocytes at birth and oocytes still present are not enclosed in primordial follicles (Soyal *et al.* 2000). Disruption of *Nobox*, an oocyte-specific homeobox gene, results in increased oocyte loss and a delay in cyst breakdown (Suzumori *et al.* 2002, Rajkovic *et al.* 2004). Mutants in *Foxl2*, a winged-helix forkhead transcription factor, are sterile with germ cell cysts that do not break down (Uda *et al.* 2004). Finally, siRNA knockdown of heterogeneous nuclear ribonucleoprotein K (HNRNPK) in rat ovary organ culture caused a block in cyst breakdown and follicle formation (Wang *et al.* 2011). Identifying the targets of these transcription factors will greatly add to our understanding of the mechanisms of primordial follicle assembly.

Hormones

Neonatal female mice treated with estradiol (E_2 ; Iguchi *et al.* 1986), with synthetic estrogens such as diethylstilbestrol (DES; Iguchi *et al.* 1990) or bisphenol-A (BPA; Suzuki *et al.* 2002) or with the phytoestrogen genistein (Jefferson *et al.* 2002), have more MOFs as adults, which are likely oocyte cysts that did not separate and became enclosed in follicles (Gougeon 1981, Iguchi & Takasugi 1986, Iguchi *et al.* 1986). One model is that normally, high levels of E_2 in the fetal ovary keeps oocytes in cysts and that late in fetal development, E_2 levels drop resulting in cyst breakdown (Chen *et al.* 2007). When oocytes are exposed to estrogens, cyst breakdown is

Table 3 Transcription factors active during follicle assembly.

Genes	Protein/function	Evidence for role in follicle assembly	Expressed in	References
<i>Ahr</i>	Aryl hydrocarbon receptor, basic helix-loop-helix transcription factor	Primordial follicle formation is accelerated in mutants	Oocytes and granulosa cells	Benedict <i>et al.</i> (2000) and Robles <i>et al.</i> (2000)
<i>Figla</i>	Factor in the germ line alpha, folliculogenesis-specific basic helix-loop-helix	Mutants are defective in follicle formation and exhibit perinatal oocyte loss	Oocytes	Soyal <i>et al.</i> (2000)
<i>Foxl2</i>	Forkhead box L2, winged helix transcription factor	Mutants are defective in follicle formation and exhibit perinatal oocyte loss	Granulosa cells	Schmidt <i>et al.</i> (2004) and Uda <i>et al.</i> (2004)
<i>Hnrnpk</i>	Heterogeneous nuclear ribonucleoprotein K	siRNA knockdown in rat ovary organ culture caused a block in cyst breakdown and follicle formation	Oocytes and granulosa cells	Wang <i>et al.</i> (2011)
<i>Nobox</i>	Newborn ovary homeobox-encoding gene	Mutants have delayed follicle formation and oocyte loss	Oocytes	Rajkovic <i>et al.</i> (2004)

inhibited. Supporting this, genistein-treated mice had more oocytes in cysts compared with controls (Jefferson *et al.* 2006). These findings are consistent with the idea that exposure to estrogens inhibits cyst breakdown.

Estrogens function via nuclear receptors, members of the steroid hormone receptor superfamily. Mammals have at least two estrogen receptors (ERs), ER α and ER β , containing a conserved DNA-binding domain (Pettersson & Gustafsson 2001). After ligand binding, a conformational change leads to ER dimerization and interaction with the target DNA sequence called the estrogen response element (ERE). The receptor dimer then recruits coregulators that interact with basal transcription factors and either stimulate or inhibit transcription of target genes. Besides 'classical' steroid hormone signaling, several other signaling mechanisms can occur including tethered, ligand-independent, and membrane signaling (Heldring *et al.* 2007). In tethered signaling, estrogen activates ERs, which then interact with other transcription factors that bind to target sequences but the ER does not directly bind to the DNA (Kushner *et al.* 2000, Saville *et al.* 2000). For ligand-independent signaling, ERs are phosphorylated by activation of other signaling pathways and then bind to EREs to modulate target gene transcription without being activated by ligand binding (Kato *et al.* 1995). There are at least two ways estrogen can signal through the membrane. First, nuclear ERs can be translocated to and act at the plasma membrane (Pedram *et al.* 2006). Secondly, estrogen may signal through membrane-bound receptors (mERs) including a G protein-coupled membrane receptor called GPR30 (Revankar *et al.* 2005). GPR30 is thought to be coupled to G α_s (Thomas *et al.* 2005) and estrogen acting through GPR30 activates MAPK and PI3K signaling (Maggiolini & Picard 2010). Another mER agonist, STX (diphenyl acrylamide), selectively targets a G α_q -associated protein and also activates MAPK and PI3K signaling (Qiu *et al.* 2006, Lin *et al.* 2009). It is unclear whether STX is a GPR30 agonist or whether it signals via another unidentified mER (Lin *et al.* 2009). Evidence for other estrogen-responsive G protein-coupled receptors exists but they have not yet been well characterized (Hasbi *et al.* 2005).

Neonatal genistein treatment causes the appearance of MOFs in adult ovaries. To determine which ER genistein was signaling through, Jefferson *et al.* (2002) exposed ER mutants to genistein as neonates and examined effects on the adult ovary. MOFs were still observed in ESR1 but not ESR2 mutant adult females, suggesting that these effects are mediated through ESR2 (Jefferson *et al.* 2002). ESR2 mutant adult females have reduced fertility with fewer litters and pups per litter (Krege *et al.* 1998). Mutant adult ovaries have more atretic follicles and fewer corpora lutea, suggesting that more oocytes are dying. ESR1 mutants are sterile with ovaries lacking corpora lutea and containing hemorrhagic follicles (Lubahn *et al.* 1993). It is not known

whether defects in the adult ovaries of either ER mutant result from problems with follicle development or earlier, during cyst breakdown. ESR2 is expressed in granulosa cells and ESR1 in theca cells of adult ovaries, and mRNAs corresponding to both are detected in developing ovaries (Jefferson *et al.* 2000).

When neonatal oocytes are exposed to estrogens, cyst breakdown is inhibited but it is unclear which estrogen receptor is used (Chen *et al.* 2007). During cyst breakdown, ESR1 is expressed in pregranulosa cells and ESR2 in some oocyte nuclei (Chen *et al.* 2009). In addition, ESR1 or ESR2 agonists inhibit cyst breakdown, suggesting that estrogen can signal through either receptor to regulate cyst breakdown. However, ER knockouts have no effect on neonatal oocyte development (F Tang & M Pepling, unpublished observations). These results suggest that estrogen signals via another receptor to maintain oocytes in cysts. E₂ can signal at the membrane to block cyst breakdown supporting the idea that a membrane receptor is involved (Chen *et al.* 2009). Gpr30 knockout mice are fertile with no observable reproductive defects making it an unlikely candidate as the mER in neonatal ovaries (Otto *et al.* 2009).

The source of E₂ maintaining oocytes in cysts is unknown. Cyst breakdown begins a few days before birth in the inner cortex of the ovaries (Pepling *et al.* 2010), and thus, E₂ levels should drop at this time. The source of E₂ may be the maternal circulation. However, in humans, E₂ levels are rising during primordial follicle formation, and in cows, levels are low prior to follicle formation suggesting that maternal circulating E₂ is not the source in these species (Senger 2003). Alternatively, fetal ovaries could produce E₂ and cyst breakdown could begin when levels of fetal E₂ secretion fall. Fetal cow ovaries produce E₂ and levels drop when primordial follicles begin to form (Yang & Fortune 2008, Nilsson & Skinner 2009).

Unlike rodents and cows, estrogen seems to have a positive effect on follicle formation in some species. In the hamster, estrogen has been shown to promote follicle assembly (Wang & Roy 2007, Wang *et al.* 2008). In the baboon, when estrogen production is blocked, cyst breakdown and follicle assembly are disrupted (Zachos *et al.* 2002). It is not known why estrogen promotes follicle formation in some species and inhibits follicle formation in others. It may be that high estrogen concentrations inhibit follicle assembly while low concentrations promote assembly (Nilsson & Skinner 2009). Alternatively, there may be species differences in estrogen signaling.

Neonatal treatment with progesterone also results in more MOFs in adult females (Iguchi *et al.* 1986). Subsequently, neonatal progesterone treatment was shown to reduce primordial follicle assembly in rats supporting the idea that progesterone, like estrogen, acts during neonatal oocyte development (Kezele & Skinner 2003). Progesterone treatment of neonatal mouse ovaries reduced cyst breakdown (Chen *et al.* 2007). Since progesterone can be converted to estrogen, its

effect could be exerted either directly or via conversion to estrogen. However, a non-metabolizable version of progesterone, promegestone, also inhibited cyst breakdown and follicle assembly. A non-metabolizable progesterone analog blocked follicle formation in rat ovaries as well (Nilsson *et al.* 2006). Like estrogen, there are several possible sources of progesterone. Two studies using the bovine model have demonstrated that progesterone is produced by the fetal ovary (Yang & Fortune 2008, Nilsson & Skinner 2009). One study reported a drop in fetal ovarian progesterone while progesterone in the fetal or maternal circulation did not change (Nilsson & Skinner 2009).

Progesterone can signal through the nuclear progesterone receptor (PR) that has two isoforms, PR-A and PR-B that are transcribed from a single gene using two different promoters (Conneely *et al.* 1989, Kastner *et al.* 1990). Both PR isoforms are found in the adult ovary, in theca cells, and in granulosa cells (Gava *et al.* 2004). The PR knockout results in female infertility with defects in reproductive tissues including the ovary, uterus, and mammary glands (Lydon *et al.* 1995). PR-A-specific knockouts exhibit defects in ovarian and uterine function while PR-B-specific knockouts have defects in mammary gland function (Mulac-Jericevic *et al.* 2000, 2003). While progesterone inhibits follicle development (Kezele & Skinner 2003), follicles develop normally in PR knockouts suggesting an alternative signaling mechanism (Lydon *et al.* 1996). Progesterone can also signal through membrane receptors by at least three mechanisms (Peluso 2006). First, one of the nuclear isoforms could be translocated to the nucleus. Secondly, a seven-transmembrane spanning family of PRs called progestin and adipoQ receptors (PAQR) has been identified with members in three groups, α , β , and γ . Thirdly, two single membrane-spanning proteins called progesterone receptor membrane component (PGRMC) 1 and PGRMC2 can act as PRs in complex with other proteins. While there is evidence that these proteins are present in the adult ovary (Cai & Stocco 2005, Peluso 2006), their expression in the developing mouse ovary has not been examined. In a recent study, PGRMC1 and PGRMC2 were detected in neonatal rat ovaries by microarray analysis and RT-PCR (Nilsson *et al.* 2006).

Exposure of neonates to testosterone also causes more MOFs in adult ovaries (Iguchi *et al.* 1986). However, this may be due to conversion of testosterone to estrogen because inhibiting estrogen synthesis from testosterone eliminated the higher incidence of MOFs (Iguchi *et al.* 1988). Effects of testosterone directly on cyst breakdown have not been tested. Testosterone signals through the androgen receptor (AR) belonging to the nuclear steroid hormone receptor family (Roy *et al.* 1999). Like other steroid hormone receptors, testosterone can also signal through receptors at the membrane (Heinlein & Chang 2002).

Follicle-stimulating hormone (FSH) may also be involved in follicle assembly. FSH is present in the serum of neonatal mice and hamsters (Vomachka & Greenwald 1979, Halpin *et al.* 1986) and the FSH receptor is detected using RT-PCR in neonatal mouse ovaries (O'Shaughnessy *et al.* 1994). Inactivation of FSH using an anti-FSH antibody results in the inhibition of primordial follicle formation in the hamster (Roy & Albee 2000), while addition of FSH to cultured ovaries results in acceleration of primordial follicle formation (Wang & Roy 2004). A recent study using mouse ovary organ culture also implicates FSH in promoting cyst breakdown and primordial follicle formation (Lei *et al.* 2010). However, *Fsh* or *Fsh* receptor knockout female mice while infertile undergo normal primordial follicle formation and follicle development is not arrested until the preantral stage (Kumar *et al.* 1997, Dierich *et al.* 1998, Abel *et al.* 2000).

Links between meiotic progression and follicle formation

Though diplotene arrest and follicle formation occur during the same time frame, their relationship is unclear. Two structural components of the synaptonemal complex found at the pachytene stage, synaptonemal complex protein (SYCP) 1 and SYCP3, are important for normal oocyte development. Inhibition of SYCP1 in rats resulted in premature arrival of oocytes at the diplotene stage and premature primordial follicle assembly suggesting a link between cell cycle stage and primordial follicle formation (Paredes *et al.* 2005). In mice, *Sycp1* mutants are sterile and females lack oocytes but follicle formation was not examined (de Vries *et al.* 2005). *Sycp3* mutants are subfertile and while oocytes appear to develop normally, chromosome segregation is defective (Yuan *et al.* 2002). In cows, while follicles form before the oocytes reach diplotene arrest, they do not develop to the primary follicle stage until they arrest in diplotene again linking follicle development and meiotic stage. Primordial follicle activation can be inhibited by estrogen, which fetal bovine ovaries produce around the time of follicle formation (Yang & Fortune 2008). In the cow, estrogen may block follicle activation by inhibiting meiotic progression. Supporting the idea that estrogen can affect meiotic progression, bisphenol A (BPA) treatment of mouse embryos caused defects in meiosis (Susiarjo *et al.* 2007). *ESR2* mutants had meiotic defects similar to BPA-treated animals, suggesting that BPA acts as an *ESR2* antagonist.

Cell adhesion molecules

Cell adhesion molecules (CAMs) likely play important roles in oocyte development. Before birth, CAMs may be important in keeping oocytes in germ cell cysts.

These CAMs would be downregulated to allow cysts to break apart. A different set of CAMs might be important for adhesion between oocytes and granulosa cells during primordial follicle formation. Recent work using the hamster model has implicated N- and E-cadherin in follicle formation (Wang & Roy 2010). E-cadherin was detected in oocytes within germ cell cysts and in primordial follicles. Blocking E-cadherin using an inhibitory antibody resulted in accelerated follicle formation suggesting that, normally, E-cadherin is involved in maintaining oocytes in cysts. N-cadherin, on the other hand, was detected in granulosa cells as primordial follicles formed and treatment of ovaries in organ culture with an N-cadherin-blocking antibody resulted in reduced follicle formation.

Conclusions and perspectives

The pool of primordial follicles, which is formed early in the life of mammalian species, is important for reproductive success. Progress has been made in elucidating the mechanisms by which primordial follicles are assembled. Proteins classified as programmed cell death regulators, growth factors, signaling molecules, transcription factors, hormones, meiotic regulators, and CAMs have been implicated in primordial follicle formation. Understanding how all these factors work together to establish the primordial follicle pool will shed light on female reproductive disorders such as premature ovarian failure, reproductive lifespan, menopause, and ovarian cancer.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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