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REVIEW

The developmental origins of the mammalian ovarian reserve

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ABSTRACT

The adult mammalian ovary is devoid of definitive germline stem cells. As such, female reproductive senescence largely results from the depletion of a finite ovarian follicle pool that is produced during embryonic development. Remarkably, the crucial nature and regulation of follicle assembly and survival during embryogenesis is just coming into focus. This developmental pathway involves the coordination of meiotic progression and the breakdown of germ cell cysts into individual oocytes housed within primordial follicles. Recent evidence also indicates that genetic and environmental factors can specifically perturb primordial follicle assembly. Here, we review the cellular and molecular mechanisms by which the mammalian ovarian reserve is established, highlighting the presence of a crucial checkpoint that allows survival of only the highest-quality oocytes.

KEY WORDS: Cyst breakdown, Meiosis, Oocyte, Ovary, Primordial follicle

Introduction

Unlike the adult mammalian testes, in which spermatogonial stem cells support long-term spermatogenesis, the adult mammalian ovary is devoid of germline stem cells. Primordial follicles, each of which contains an oocyte surrounded by a single layer of somatic pre-granulosa cells, thus represent the entire ovarian reserve that a female mouse, or a woman, will ever possess. In humans, these follicles are produced from a pool of primordial germ cells (PGCs), which are localized to the developing gonad early in gestation. These germ cells progress through mitotic divisions with incomplete cytokinesis, producing an excess of interconnected oogonia. Mitotic divisions then cease and the germ cells enter meiosis I, progressing through the first few stages of prophase I before arrest. The clusters of germ cells, or 'cysts', then begin to undergo 'breakdown', in which most of the oocytes are lost through apoptotic cell death, and the remaining oocytes become surrounded by a layer of somatic pre-granulosa cells (Fig. 1), forming 'primordial follicles' during mid-gestation (Cohen and Holloway, 2010; Gondos et al., 1986; Motta et al., 1997). Bidirectional communication and exchange of signaling molecules between oocytes and the surrounding granulosa cells are necessary for both the growth of the oocyte and the development of the follicle after birth (Fig. 1). After onset of puberty, matured follicles can be 'activated' by a surge of luteinizing hormone (LH), which results in breakdown of the germinal vesicle, nuclear maturation and completion of the first meiotic division. These oocytes then re-arrest in metaphase II of meiosis II (MII) and are ovulated (Coticchio et al., 2015). The faithful regulation of primordial follicle assembly during the fetal and neonatal periods therefore distinctly determines the long-term reproductive capacity of female mammals.

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Recent studies suggest that the initial assembly of these follicles encompasses a crucial developmental checkpoint, allowing only the highest-quality oocytes to further develop and be fertilized in the adult. Furthermore, seminal work has revealed the key cellular mechanisms by which primordial follicles are formed from a pool of germ cell cysts that undergo a stereotyped 'breakdown' in mammals. Although less well understood, these important developmental benchmarks appear to be largely conserved during human fetal ovarian development. Remarkably, this breakdown event resembles that occurring during oocyte development in *Drosophila*, in which supporting nurse cells, in the context of a syncytium, support proper oocyte development. In this Review, we provide a comprehensive picture of the predominant mechanisms by which the mammalian ovarian reserve is established and maintained.

A timeline of mammalian primordial follicle development

The embryonic timeline of mammalian primordial follicle assembly is well-documented and involves germ cell fate commitment, migration and arrival at the genital ridge, as well as male or female sex specification (Motta et al., 1997; Pepling, 2012; Tingen et al., 2009). Upon arrival at the gonad, all PGCs enter synchronous mitotic divisions with incomplete cytokinesis, forming clonal cell clusters (Fig. 2). Recent work (Mork et al., 2012) has demonstrated that, in addition to bridges between clonal cells, aggregation that is probably mediated by cell adhesion molecules is responsible for a proportion of cyst formation. After the cessation of mitotic divisions in the developing ovary, germ cells enter meiosis at embryonic day 13.5 in mice (Pepling, 2012; Tingen et al., 2009) and at 11-12 weeks gestation in humans (Cohen and Holloway, 2010; Gondos et al., 1986; Motta et al., 1997), eventually becoming 'oocytes'. These cells ultimately arrest in the diplotene stage of prophase I before birth (Pepling, 2012), immediately after the resolution of meiotic DNA double-strand breaks (DSBs) (McLaughlin and McIver, 2009).

The importance of fetal germ cell cysts in female mammals has been widely speculated upon. Such clusters of interconnected germ cells formed by incomplete cytokinesis are highly conserved structures that are found in organisms ranging from *Drosophila* (de Cuevas et al., 1997) and *Xenopus* to mice (Pepling et al., 1999) and humans (Gondos, 1973; Motta et al., 1997). These cysts have been well-studied in invertebrate models and are defined by key characteristics, including synchronous division, shared cytoplasm between germ cells and morphological similarities. In the *Drosophila* ovary, synchronous divisions produce 16 germ cells within a cyst, from which one cell forms the oocyte, while the others differentiate into nurse cells that support the development of the gamete (de Cuevas et al., 1997). Although the role of these mammalian clusters is still not well understood, it is known that organelles can be exchanged between interconnected germ cells, and that mitochondria and endoplasmic reticulum reorganize just prior to murine cyst breakdown (Pepling and Spradling, 2001). Furthermore, recent work (Lei and Spradling, 2013b) has used

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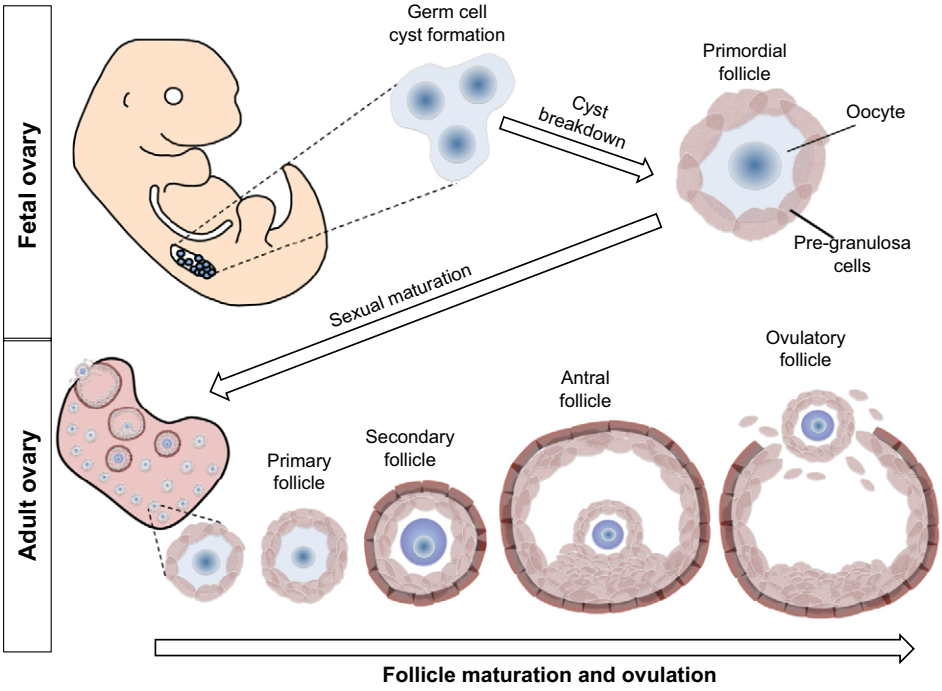

Fig. 1. Fetal origins of the adult ovarian reserve. Primordial follicles are produced from a pool of primordial germ cells, which are localized to the somatic gonad during gestation and undergo mitotic divisions to form germ cell cysts. These cysts then undergo 'breakdown' to form the primordial follicle pool, which comprises oocytes surrounded by a layer of somatic pre-granulosa cells. During sexual maturation, primordial follicles can mature into the primary and secondary follicle stages, eventually acquiring a fluid-filled antral space. After the onset of puberty, matured follicles can be activated by a surge of luteinizing hormone, which promotes further maturation of the oocyte and subsequent ovulation.

lineage tracing to observe primordial germ cell dynamics during cyst formation prior to meiosis. Notably, it was demonstrated that cyst fragmentation occurs prior to meiotic onset and that clonal cells can become components of an average of five germ cell cysts. These data suggest a relationship between clonal divisions, the number of cysts produced and the resulting number of primordial follicles. Furthermore, this work posits that each cyst present at the time of meiotic onset produces a single oocyte, which is quite similar to dynamics of oocyte and nurse cell production in *Drosophila*. Importantly, however, mammalian cysts are fragmented completely early in development, with the ovary possessing only intact follicles during adulthood (Lei and Spradling, 2013b); this is in contrast to *Drosophila* cysts, which are formed and utilized throughout the life of the animal (Roth and Lynch, 2009). Interestingly, it was also demonstrated that mouse oocytes possess a Balbiani body, or mitochondrial cloud of organelles localized next to the developing oocyte nucleus (Pepling et al., 2007). Whereas these structures had been observed in *Drosophila* and *Xenopus*, and are known to form via the intercellular bridges between cyst cells, the presence of this structure was thought to be absent in mammals (Kloc and Etkin, 2005). This recent work emphasizes an additional function for mouse germline cysts, including the transport of organelles from dying oocytes to the developing gamete, resulting in Balbiani body formation (Lei and Spradling, 2013b; Pepling et al., 2007).

Around the time of birth in mice (Pepling and Spradling, 2001), and around 16 weeks gestation in humans (Motta et al., 1997), germ cell cysts undergo 'breakdown' (Fig. 2), during which time most of the oocytes are lost through caspase 2-dependent apoptotic cell death (Bergeron et al., 1998; Morita et al., 2001; Pepling, 2012). The excess production and then culling of germ cells might thus represent a means of 'germ cell selection', analogous to that seen in *Drosophila*, in which only oocytes of the highest quality can further

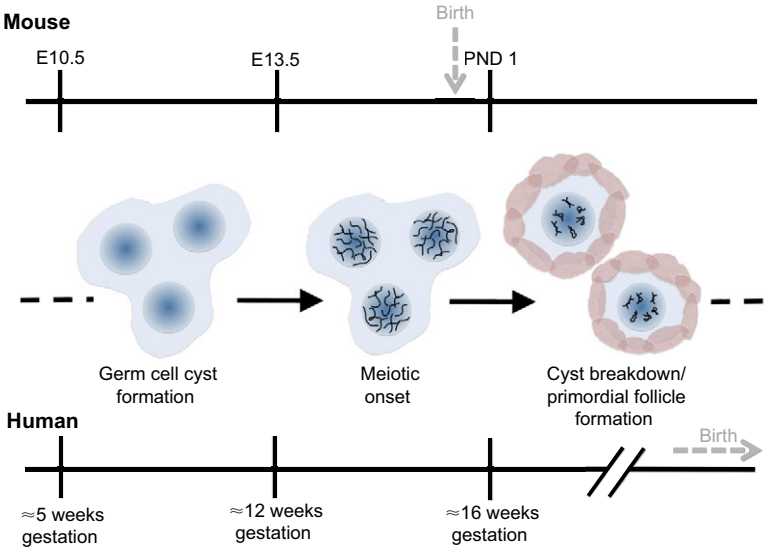

Fig. 2. Comparative timelines of primordial follicle formation in mouse and humans. Representative timelines of cyst formation, meiotic onset and primordial follicle formation in mice and humans. Primordial germ cells colonize the somatic gonad at about E10.5 in mice and ~5 weeks gestation in humans. These cells undergo mitotic divisions, form cysts, and then cease mitosis and enter meiosis I around E13.5 in mice and between 10 and 12 weeks gestation in humans. Finally, these cysts break down via apoptosis of germ cells to form the primordial follicle pool. Whereas this event takes place around the time of birth in mice, it begins during mid-gestation (around 16 weeks) in humans. Abbreviations: E, embryonic day; PND, post-natal day.

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develop into viable gametes (Mork et al., 2012). These remaining oocytes, which are surrounded by pre-granulosa cells, constitute the 'primordial follicle pool' and the complete ovarian reserve for the adult life of the animal or woman. This finite limit on the primordial follicle pool is in direct contrast to the more extensive production of male gametes, despite the common formation of interconnected germ cell cysts (Chiarini-Garcia and Russell, 2001) and the presence of testis-expressed protein 14 (TEX14) at intercellular bridges (Greenbaum et al., 2006, 2009; Mork et al., 2012). Whereas TEX14 does not appear to be essential for female fertility, as it is for male fertility, it is known to stabilize intercellular bridges between oocytes. Furthermore, despite the ability to produce litters, TEX14-null female mice possess fewer oocytes than wild-type mice, suggesting that stable intracellular bridges contribute to germ cell survival (Greenbaum et al., 2009). Thus, despite ample embryonic similarities between male and female germline cyst establishment and function, the definitive male mammalian stem cell population remains a fundamental difference in the sex-specific regulation of reproductive potential and senescence.

It should be noted that, although recent work has suggested the presence of ovarian germline stem cells which can support follicle replenishment in adult female mice and women (Johnson et al., 2005, 2004; White et al., 2012), these cells apparently do not contribute to the fertility of the mouse under normal physiological conditions or after germ cell ablation (Byskov et al., 2011; Eggan et al., 2006; Kerr et al., 2012; Lei and Spradling, 2013a). In addition, recent work utilizing careful lineage-labeling of germ cells in adult ovaries demonstrated an absence of germline stem cell production and quantitatively identified the half-life of murine follicles *in vivo*. This analysis was also performed after oocyte ablation (with the drug busulfan) with similar outcomes, consistent with the notion that the follicles produced around the time of birth are sufficient to satisfy the lifetime reproductive requirements of the animal, and that germline stem cells do not normally contribute to this production (Lei and Spradling, 2013a). As the ovarian reserve has a finite pool of viable gametes produced long before they are needed, the fidelity and stability of each step of primordial follicle formation is therefore essential for the proper completion of oogenesis and the developmental potential of the future embryo that arises from this oocyte.

Genetic determinants of primordial follicle development: from signaling pathways to transcriptional networks

Primordial follicle development is intricately regulated through the coordination of signaling pathways (including the Notch and KIT pathways), transcription factors (including FIGLA, NOBOX and TAF4b) and transposon repression (most notably by Maelstrom). Meiotic fidelity, particularly that controlled by synaptonemal complex protein 1 (SCP1), might also play a crucial role in this process. This complex coordination allows the fine-tuned regulation and quality control of meiotic progression and oocyte survival, allowing only the best of the gametes to constitute the ovarian reserve (Fig. 3, Table 1).

The transcriptional control of primordial follicle development

The regulation of gene expression is a crucial aspect of any developmental program including primordial follicle development. A number of transcription factors play crucial roles in germ cell cyst breakdown in the mouse. For example, factor in the germline alpha (FIGLA), a basic helix-loop-helix transcription factor, was originally found to coordinate the expression of oocyte-specific zona pellucida genes (Liang et al., 1997). Subsequently, FIGLA was

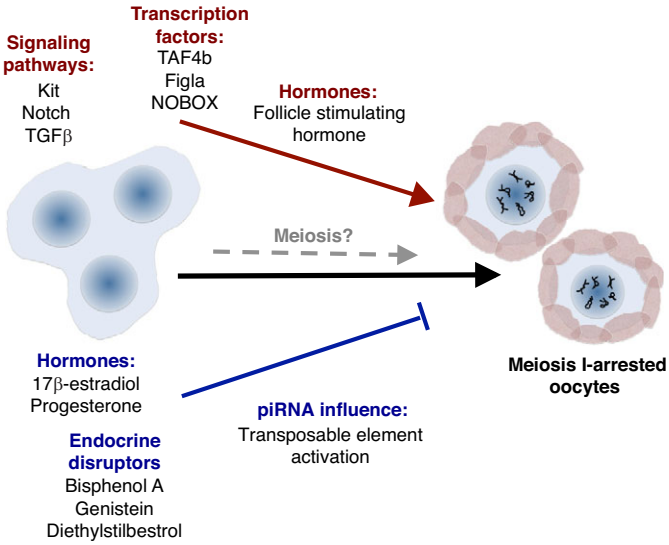

Fig. 3. Regulators of primordial follicle development. Red text and arrow indicate factors that promote the transition between germline cyst and primordial follicles, whereas blue text and arrows indicates inhibitors of primordial follicle formation. Abbreviations: FSH, follicle stimulating hormone; TE, transposable element.

also found to be essential for the formation of primordial follicles in the mouse. Whereas germ cell numbers in *Figla*-null female mice are normal during mid-embryogenesis, primordial follicles are never properly formed and germ cells are lost shortly after birth, resulting in sterility (Soyal et al., 2000). Notably, a *Figla* ortholog was identified in the human genome (Huntriss et al., 2002) and later found to be highly expressed in the primordial follicles of human ovaries. Furthermore, higher *Figla* expression was correlated with primordial follicle development (Bayne et al., 2004), and loss of *Figla* was associated with premature ovarian failure in women (Tosh et al., 2015; Zhao et al., 2008).

The homeobox-containing transcription factor newborn ovary homeobox (*Nobox*) gene has also been implicated in primordial follicle development. *Nobox* was identified using expressed sequence tags from neonatal mouse cDNA databases (Suzumori et al., 2002). Subsequent work (Rajkovic et al., 2004) discovered that Nobox is essential not only for oocyte survival, but also for the proper timing of cyst breakdown and primordial follicle assembly in the mouse. In *Nobox*-null mouse ovaries, defects in this process result from aberrant signaling between oocytes and somatic cells, causing impaired somatic cell invasion into cysts (Lechowska et al., 2011). Oocyte-specific gene expression is also significantly perturbed in these ovaries, with dramatic downregulation of *Pouf51* (*Oct4*) and *Sall4*, among other, more widely expressed genes such as *Jagged1*, a NOTCH ligand (Choi et al., 2007). Work over the last ten years has identified a human ortholog of *Nobox* (Huntriss et al., 2006), and also found significant correlations between reduced or mutated *Nobox* and premature ovarian failure in women (Bouilly et al., 2015, 2011; Qin et al., 2007), thus emphasizing the conserved and crucial role for this transcription factor in oocyte and ovarian development.

In addition to sequence-specific DNA binding proteins, germ cell-specific transcriptional programs are regulated by selective components of the general transcription machinery. Originally identified in *Drosophila* (Crowley et al., 1993; Hiller et al., 2004), gonadal-enriched general transcription factor variants have now been documented in a diverse array of vertebrates, including

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Table 1. Factors that modulate primordial follicle development and disruption

Gene category	Murine gene	Mouse mutant phenotype	References
Transcription factors	Factor in the germline alpha (*Figla*)	Germ cell death at time of birth; absence of primordial follicles	(Liang et al., 1997; Soyal et al., 2000)
	Newborn ovary homeobox gene (*Nobox*)	Impaired cyst breakdown; loss of primordial follicles	(Lechowska et al., 2011; Rajkovic et al., 2004; Suzumori et al., 2002)
	TBP-associated Factor 4b (*Taf4b*)	Impaired cyst breakdown; loss of primordial follicles	(Falender et al., 2005; Freiman et al., 2001; Grive et al., 2014; Lovasco et al., 2010; Voronina et al., 2007)
Signaling factors	*Notch2*	Increased oocytes; multi-oocyte follicles	(Chen et al., 2014; Xu and Gridley, 2013)
	Kit-ligand (*Kitl*)	Inability of primordial follicles to develop	(Jones and Pepling, 2013; Parrot and Skinner, 1999)
	Smad4	Reduced fecundity when ablated at primordial follicle stage	(Li et al., 2012)
	Follistatin (*Fst*)	Global null is embryonic lethal; FST288-only results in impaired cyst breakdown and increased oocytes before birth; accelerated oocyte depletion after birth	(Kimura et al., 2011)
	Growth and differentiation factor 9 (*Gdf9*)	Reduced primordial follicles	(Vitt et al., 2000)
Transposon and meiotic control	Maelstrom (*Mael*)	Extensive loss of oocytes and primordial follicles around the time of birth	(Malki et al., 2014; Soper et al., 2008)
	Synaptonemal complex protein 1 (*Scp1*)	Increased oocytes and accelerated primordial follicle assembly	(Paredes et al., 2005)
Hormonal regulators	Aromatase (*Cyp19a1*)	Decreased oocyte density	(Britt et al., 2004; Dutta et al., 2014)
	3-beta-hydroxysteroid-dehydrogenase (*3βhsd*)	Decreased oocyte density	(Dutta et al., 2014)

Xenopus (Han et al., 2003; Xiao et al., 2006), mice (Freiman et al., 2001; Martianov et al., 2001; Zhang et al., 2001) and humans (Ozer et al., 2000; Upadhyaya et al., 1999). One of the best-characterized selective subunits of the general transcriptional complex TFIID is TBP-associated factor 4b (TAF4b), a paralog of TAF4, originally identified in a human B-cell line and found to be primarily enriched in the mouse ovary and testis (Freiman et al., 2001). *Taf4b*-deficient female mice are viable but infertile and suffer from many hallmarks of premature ovarian failure, including follicle depletion, persistent estrous and high serum levels of the gonadotropin follicle stimulating hormone (FSH) (Falender et al., 2005; Freiman et al., 2001; Lovasco et al., 2010; Voronina et al., 2007). Recent work has demonstrated that *Taf4b*-deficient ovaries experience dramatic germ cell loss by apoptosis immediately after birth, the time at which the ovarian reserve is established (Grive et al., 2014). Furthermore, *Taf4b*-deficient females experience delayed cyst breakdown and defective primordial follicle assembly. These data indicate that TAF4b regulates the establishment of the ovarian reserve in the mouse. Interestingly, *TAF4B* has also been correlated with ovarian health and oocyte survival in women, suggesting that this transcription factor functions similarly in humans (Di Pietro et al., 2008; Knauff et al., 2009). The extensive array of transcriptional components and specialized components of the basal transcription machinery that are required for cyst breakdown underscore the intricate regulation involved in primordial follicle pool establishment.

Signaling during primordial follicle formation

Similarly, the integration of several well-known signaling cascades is required for proper ovarian follicle development, from the time of primordial germ cell specification through to ovulation and fertilization. Disruption of any of these signaling cascades through either genetic or environmental perturbations affects not only that cascade, but all cross-talking pathways as well. These disruptions can have dramatic consequences on germ cell migration, development and reproductive potential within the developing oocyte. The Notch pathway, a universal developmental signaling pathway first identified in *Drosophila*, has been particularly well-studied in the context of neuronal development during embryogenesis (Xiao et al., 2009). However, recent work highlights the role of Notch signaling in oogenesis as well. For example, it was demonstrated that culturing fetal and neonatal ovaries in the presence of the gamma secretase inhibitors DAPT or L-685,458 dramatically reduced primordial follicle formation and delayed prophase I progression (Chen et al., 2014; Feng et al., 2014). Furthermore, the requirement for NOTCH2, specifically in the granulosa cell compartment, is essential for proper cyst breakdown and follicle formation; mice deficient for granulosa cell-NOTCH2 exhibit increased oocyte numbers and multi-oocyte follicles (Xu and Gridley, 2013). Interestingly, ovaries in which NOTCH signaling was inhibited also exhibited downregulation of key transcription factors, including *Figla*, *Nobox* and *Sohlh2*, providing additional evidence for the interdependence of signaling and transcriptional regulation during primordial follicle formation (Chen et al., 2014).

KIT signaling is well-known for its roles in cell proliferation and survival, and recent work (Jones and Pepling, 2013) has demonstrated a regulatory role for KIT signaling during mouse primordial follicle formation. This research found that KIT-ligand (also called stem cell factor, SCF; or Steel factor) is highly expressed at the oocyte membrane, specifically during the window of cyst breakdown and primordial follicle formation, after which KIT-ligand is observed more uniformly in both oocytes and granulosa cells. Furthermore, peptide-inhibition of KIT signaling during ovary culture reduced primordial follicle formation, whereas the supplementation of KIT-ligand enhanced their formation. Downstream activation of the MAPK pathway was observed after KIT-ligand supplementation, and further study is warranted to test the role of this signaling cascade in establishing the ovarian reserve in humans.

One of the best-studied signaling networks in gamete development is that of the transforming growth factor beta (TGFβ) superfamily of receptors and ligands, which includes the bone morphogenetic proteins (BMPs), growth and differentiation

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factors (GDFs), and activin and inhibin subfamilies (Knight and Glister, 2006). All of these subfamily members play essential roles in oogenesis from primordial germ cell recruitment to ovulation and luteinization. Primordial follicle formation also relies on the fidelity of TGFβ signaling; however, little is known about the role that TGFβ members play during this crucial stage of ovarian development. It has been demonstrated that the oocyte-specific ablation of SMAD4, a transcriptional regulator downstream of TGFβ signaling, reduces fertility when the conditional ablation occurs at the primordial follicle stage using GDF9-Cre-recombinase (Li et al., 2012). By contrast, the ablation of SMAD4 after primordial follicle development (e.g. using a ZP3-Cre-recombinase) did not alter fertility or litter size. Interestingly, the GDF9-Cre-induced reduction in fecundity did not correlate with reduced primordial follicle numbers in the ovary. SMAD4 might thus be important not for primordial follicle survival, but for the developmental potential of follicles. Follistatin, an antagonist of activin, has also been shown to play crucial roles in cyst breakdown and primordial follicle assembly. Follistatin exists in three isoforms, and whereas global follistatin deletion is embryonic-lethal, conditional ablation of all but the shortest isoform, FST288, highlights the essential roles of follistatin in oogenesis. In female FST288-only mice, germ cell apoptosis around the time of birth is significantly reduced, leading to impaired cyst breakdown and excess germ cells. Despite this retention of germ cells at birth, the mice later experience accelerated depletion by postnatal day 5, resulting in premature ovarian failure and subfertility (Kimura et al., 2011). Additional research will be necessary to disentangle the complicated roles of the TGFβ signaling pathways and its downstream mediators during ovarian reserve establishment and maintenance.

Coordinating transposon repression and meiotic progression

DNA methylation is highly dynamic in gametes during embryonic development; this is necessary for proper gene expression during oogenesis and spermatogenesis but also allows for the potential de-repression of transposable elements (TEs) (Aravin and Bourc'his, 2008). These TEs, when aberrantly expressed, can cause meiotic errors and germ cell death, leading to sterility (Bourc'his and Bestor, 2004; Carmell et al., 2007). To counteract this de-repression, *de novo* DNA methylation, as well as the production of PIWI-interacting RNAs (piRNAs), provides fidelity during germ cell development in a changing chromatin environment (Aravin and Bourc'his, 2008). The protein Maelstrom (MAEL) is a key player in piRNA-mediated transposon silencing and is conserved from *Drosophila* (Clegg et al., 1997) to mice (Aravin et al., 2009; Soper et al., 2008). *Mael*-null male mice are infertile due to defects in chromosome synapsis during meiosis I, de-repression of TEs and accumulation of non-meiotic DSBs (Soper et al., 2008). MAEL has also been studied in the context of oocyte survival and primordial follicle development in mice. Recent work (Malki et al., 2014) has demonstrated that *Mael*-null female mice express significantly elevated levels of LINE-1 (L1) retrotransposon mRNA as well as of the L1ORF1p protein, which is encoded by L1. Quantitative analysis of oocyte numbers from *Mael*-null fetal and neonatal mice showed a dramatic loss of oocytes by E18.5, asynapsis during prophase I, accumulation of non-meiotic DSBs and the appearance of L1ORF1p foci in oocyte nuclei. This research suggests that L1 expression is a key determinant of the primordial follicle pool, with low L1-expressing oocytes successfully progressing through meiotic prophase I and surviving to contribute to the ovarian reserve.

Meiotic prophase I progression in the embryonic ovary, which results in diplotene arrest, occurs during the same developmental window as the establishment of the primordial follicle reserve, although the relationship between these events is not well understood. It remains unclear whether these processes simply occur concurrently or whether perhaps primordial follicle formation is tied to proper meiotic progression. Evidence for this second possibility has recently been presented, via the analysis of follicle formation in rats deficient for SCP1. SCP1 is a crucial component of the synaptonemal complex and assembles between lateral chromosomal elements, which are coated in synaptonemal complex protein 3 (SCP3), during zygotene. Breakdown of the synaptonemal complex and removal of SCP1 normally occurs after diplotene arrest, when homologs repel each other during diakinesis (Cohen and Holloway, 2010). By using small-RNA knockdown of *Scp1* to achieve a premature and false 'diplotene', Paredes et al. tested the relationship between diplotene arrest and follicle formation (Paredes et al., 2005). Their findings demonstrated that ovaries with reduced SCP1 levels formed primordial follicles earlier and in greater numbers than untreated ovaries, suggesting an intricate relationship between diplotene arrest and primordial follicle assembly.

Contrasting evidence also supports independence between the fidelity of prophase I and follicle formation. For example, recent work (Dokshin et al., 2013) analyzing ovarian development in stimulated by retinoic acid (*Stra8*)-deficient mice demonstrated that meiotic onset is not necessary for follicle differentiation; whereas STRA8 expression is known to be necessary for meiotic initiation and all chromosomal events of prophase I, it was shown to be unnecessary for the development of 'oocyte-like cells' and intact follicle structures, suggesting that these two processes are uncoupled. Despite the formation of 'follicles' and the successful ovulation of these cells, however, these oocytes are developmentally incompetent and the mice are infertile. Although the mechanisms underlying this phenotype are not well understood, this evidence suggests that, whereas meiotic progression is not essential for follicle formation, it is crucial for additional developmental cues that confer reproductive potential of the oocyte (Baltus et al., 2006). Other evidence for the independence of meiosis and follicle development comes from analysis of the fetal human ovarian timeline. Primordial follicle formation in humans apparently occurs over a wider developmental window than in the mouse, with many of the germ cells forming follicles prior to diplotene arrest (Cohen and Holloway, 2010; Motta et al., 1997). This further supports the notion that follicle formation is independent of meiosis, although it could indicate that slightly different mechanisms govern these processes in mice and humans. Together, this research presents the possibility that, whereas meiotic progression might not be essential for follicle assembly, certain signaling or transcriptional cues might enhance the viability of resulting primordial follicles by affecting multiple processes. More research is needed to understand better the relationship between these processes, and any contributions that successful meiosis might have on follicle formation or vice versa.

Hormonal and environmental factors influencing primordial follicle development

Multiple hormones are known to influence and regulate various aspects of normal primordial follicle development. Accordingly, a number of endocrine-disrupting compounds – many of which are found in everyday products – perturb follicle formation. Recent studies analyzing such compounds have provided key insights into the hormonal and environmental control of primordial follicle formation in rodents and primates.

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Hormonal control of follicle formation

Estrogens are steroid hormones that play important roles in normal ovarian development, including promoting granulosa cell division and differentiation. 17β-estradiol (henceforth referred to as 'E_2') is the most bioactive form of endogenous estrogen and is produced by granulosa cells of the ovary. By regulating follicle growth and maturation, E_2 modulates the action of FSH and facilitates further estradiol production (Sarraj and Drummond, 2012). Estrogen signaling takes many forms, both through the classical pathway in which estrogens bind to nuclear estrogen receptors (ERs) α and β to activate transcription of estrogen response element (ERE)-containing promoters, as well as through non-classical pathways in which estrogen signaling is mediated via membrane-bound or cytoplasmic receptors (Björnström and Sjöberg, 2005; Cheskis et al., 2007). Estrogens, in particular E_2, have been found to signal through a variety of pathways to modulate the process of germ cell cyst breakdown and primordial follicle assembly. For example, E_2 supplementation in neonatal ovary cultures has been shown to inhibit the process of cyst breakdown and promote oocyte survival (Chen et al., 2007), although the signaling mechanisms mediating these effects are not well understood. It is now known that both ERα and ERβ are expressed at the mRNA and protein levels in neonatal mouse ovaries and are localized to oocytes and granulosa cells during the window of cyst breakdown. Treatment with the ER agonists PPT and DPN, and the pan-ER antagonist fulvestrant, demonstrates that signaling through both estrogen receptors regulates establishment of the ovarian reserve. Furthermore, BSA-conjugated E_2 was also able to inhibit cyst breakdown, but is restricted to membrane signaling due to its size. Therefore, cyst breakdown might be regulated through non-classical membrane-bound estrogen receptors as well as the classical nuclear receptors (Chen et al., 2009).

Interplay between estrogens and other hormonal signaling cascades has also been observed during primordial follicle formation. In mice, high levels of maternal estrogen prior to birth might maintain germ cell cysts until the post-partum separation from this maternal influence (Chen et al., 2007; Lei et al., 2010). Concurrently, neonatal serum FSH has been shown to increase during the first few postnatal days while primordial follicles are being established. Interestingly, when treated in culture, ovaries exposed to FSH undergo follicle formation despite high or low E_2 levels; however, FSH is better able to facilitate follicle assembly in a low E_2 environment. Gene expression was also examined in these cultured ovaries; it was found that low E_2, especially in the presence of high FSH, permits the upregulation of essential oogenesis factors, including *Figla* and *Nobox* (Lei et al., 2010). The coordination of E_2 and FSH therefore seems to be crucial for the proper timing of cyst breakdown and follicle assembly as well as for the expression of crucial oogenesis regulators.

The relationship between estrogens and progesterone (P_4) has also been examined in the context of the neonatal ovary. P_4 is known to inhibit murine cyst breakdown similarly to E_2 (Chen et al., 2007). Recent work (Dutta et al., 2014) tested the hypothesis that, in addition to circulating maternal steroid hormones, fetal mice produce their own steroid hormones to coordinate primordial follicle development. The steroidogenic enzymes aromatase, which is responsible for E_2 production, and 3-beta-hydroxysteroid-dehydrogenase (3βHSD), which is responsible for the production of P_4, were detected at the mRNA and protein levels in fetal mouse ovaries. Furthermore, intraovarian P_4 and E_2 were demonstrated to peak at E15.5 and E17.5, respectively, just prior to the onset of cyst breakdown. Interestingly, the inhibition of either aromatase or 3βHSD resulted in lower oocyte density in the ovary, but did not appear to affect cyst breakdown and follicle formation, suggesting a role for these hormones in fetal oocyte survival.

The relationship and crosstalk between steroidogenic enzymes, steroid hormones and gonadotropins is complex but is also highly species-specific. Notably, E_2 promotes primordial follicle formation in hamsters (Mukherjee and Roy, 2013; Wang and Roy, 2007), in contrast to its inhibitory role in mice (Chen et al., 2009, 2007; Dutta et al., 2014; Lei et al., 2010). Similarly, E_2 appears to facilitate follicle formation in at least a subset of non-human primates (Bocca et al., 2008; Pepe et al., 2006). Whereas the normal role of estrogen signaling during human folliculogenesis is currently unknown, it has been shown that second-trimester fetal ovaries upregulate the expression of ERα and ERβ (also known as ESR1/2, respectively) and steroidogenic enzymes, including aromatase, during the timeframe of primordial follicle formation (Fowler et al., 2011). Despite these species differences, the importance of proper steroid and gonadotropin signaling in the establishment of the ovarian reserve is indisputable. Further research will be required to elucidate the ways in which endocrine signaling integrates with paracrine signaling and gene expression to facilitate follicle assembly.

Environmental factors that influence primordial follicle development

Given the ways in which proper hormonal signaling crucially regulates follicle assembly, it is not surprising that the chemical disruption of these pathways can lead to detrimental effects on the ovarian reserve. Some of the best-studied of these chemical influences are the synthetic estrogens bisphenol A (BPA) and diethylstilbestrol (DES), and the phytoestrogen genistein. Research from the past ten years, particularly, has focused on the effects of these compounds on a number of developmental events, including prophase I progression, cyst breakdown and formation of a proper number of primordial follicles.

BPA, which can be found ubiquitously in plastics, the lining of canned goods and boxed wine, and on printed receipts (Rubin, 2011), is one of the most common environmental factors linked to reproductive disruption (Hunt et al., 2003, 2012; Peretz et al., 2014; Susiarjo et al., 2007; Zhang et al., 2012). Although BPA has been removed from a number of plastic products, particularly infant health products, its use is still widespread (Rochester, 2013). Recent work has studied the effects of BPA on a number of developmental processes, including the establishment of the primordial follicle pool in organisms ranging from mice to non-human primates. For example, it was demonstrated that fetal mice whose pregnant mothers had been injected with physiologically relevant doses of BPA experienced inhibited cyst breakdown and, at higher doses, greater oocyte survival (Zhang et al., 2012). Furthermore, oocytes suffered from delayed prophase I progression, as well as differentially methylated CpG islands within the *Stra8* locus, suggesting that BPA-induced repression of *Stra8* might be one mechanism for delayed meiosis. These processes have been similarly investigated in rhesus macaques (Hunt et al., 2012), demonstrating an increase in meiotic crossing-over events in BPA-treated ovaries as well as the appearance of multi-oocyte follicles, indicating defective cyst breakdown. Furthermore, even a single daily dose of BPA, in contrast to continuous exposure, produced these effects on follicle disruption.

DES has been shown to produce similar effects on gene expression and follicle formation (Iguchi et al., 1990; Kim et al., 2009a,b; Pepling and Karavan, 2012). DES was widely prescribed to women for almost 40 years, during which time it was thought to contribute to healthy pregnancies and to reduce the incidence of

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miscarriage. However, DES was later pulled from use in 1971, after development of the rare vaginal clear-cell adenocarcinoma had been found to be correlated with its use (Lauver et al., 2005). In addition to contributing to cancers, DES is now understood to affect detrimentally early reproductive development and oogenesis. Analogous to BPA exposure, DES treatment results in multi-oocyte follicles with reduced developmental competence (Iguchi et al., 1990). Interestingly, DES treatment also reduces the expression of the essential oogenesis genes *Figla* and *Nobox* (Kim et al., 2009a,b) as well as the TGFβ family members *Gdf9* and *Bmp15* (Kim et al., 2009a,b).

Genistein, a phytoestrogen derived from soybeans, is often highly enriched in infant soy formulas far beyond what is consumed as part of an adult soy-containing diet (Setchell et al., 1997). It can bind to both nuclear estrogen receptors, but its action is predominantly associated with ERβ (Kuiper et al., 1998). As is the case with the other estrogenic compounds described here, genistein significantly delays cyst breakdown in treated neonatal mouse ovaries and contributes to oocyte retention and multi-oocyte follicles (Chen et al., 2007; Cimafranca et al., 2010; Jefferson et al., 2002, 2006).

Although the combined effects of these endocrine disruptors on the establishment of the human ovarian reserve is not well understood, this evidence from mice and non-human primates provides cause for concern. Whereas many of these exposures are commonplace, the efforts to remove BPA from food-grade plastics, particularly those used for infants, and the now uncommon use of DES, are both encouraging. An increased understanding of the short- and long-term effects of everyday exposure to BPA and genistein from foods and other goods will inform future policy changes to help ensure the development of a healthy ovarian reserve.

Understanding reproductive senescence in women

Recent work, studying the fetal and neonatal developmental periods during which the primordial follicle reserve is established, has highlighted the importance of these key early time points in providing quality control for the development of healthy oocytes. In women, these oocytes are not utilized until much later in life; however, it is clear that the fidelity of early developmental events is essential for ensuring long-term reproductive health and preventing follicle depletion and associated pathologies. A better understanding of the genetic and environmental influences on this intricately regulated process might aid the development of improved assisted reproductive technologies (ART) and the preservation of this finite pool of oocytes for future use in reproductive age women.

Primary ovarian insufficiency (POI), the clinical term for premature ovarian failure, is a condition affecting at least 1% of women worldwide. Whereas the genetic causes responsible for a small proportion of POI cases have been identified (including deficiency in *Bmp15* and aromatase), the etiology of this condition is poorly understood. One overarching hallmark, however, is infertility resulting from accelerated depletion or reduced follicle reserve (Cordts et al., 2011; Nelson, 2009). As described in this Review, excessive follicle depletion often takes place very early in life and can result from genetic perturbations, altered hormonal signaling or environmental toxicants. Interestingly, many of the candidate factors identified in mice, including *Nobox* (Bouilly et al., 2015, 2011; Qin et al., 2007), *Figla* (Huntriss et al., 2002; Tosh et al., 2015; Zhao et al., 2008) and *Taf4b* (Di Pietro et al., 2008; Knauff et al., 2009), have since been implicated in POI in humans. Whereas a better understanding of ovarian reserve establishment and maintenance might not lead to therapies in all cases, knowledge of the causes of this condition can help prospective parents to make informed choices about their fertility options and the reproductive health of their daughters (Cordts et al., 2011; Nelson, 2009).

Polycystic ovary syndrome (PCOS) is another disease of endocrine dysfunction in women, affecting ∼5-7% of the female population worldwide. In addition to characteristic androgen excess and disrupted menstruation, women with PCOS develop – as the name suggests – ‘cysts’ of immature follicles on the ovaries, often resulting in infertility. Although the etiology of this condition is not well understood, recent studies have identified genetic predispositions to development of PCOS, including single nucleotide polymorphisms in the luteinizing hormone receptor (*Lhr*) gene, as well as environmental influences (Jayasena and Franks, 2014). Interestingly, BPA exposure in neonatal rats has been linked to a PCOS-like phenotype during adult life (Fernández et al., 2010). Although this is probably due to effects on a variety of developmental processes, the persistence of germ cell cysts in BPA-treated ovaries (Hunt et al., 2003, 2012; Susiarjo et al., 2007; Zhang et al., 2012) presents an excellent opportunity to study the proper development of the ovarian reserve as well as the reproductive pathology of PCOS.

With more women than ever before delaying pregnancy (Te Velde and Pearson, 2002), *in vitro* fertilization (IVF), intra-cytoplasmic sperm injection (ICSI) and other therapies are becoming increasingly utilized. Despite this, our ability to select ‘quality’ oocytes for fertilization remains fairly limited, and relies primarily on morphological inspection so as not to disrupt the integrity of this important cell. Unfortunately, this type of assessment cannot determine the expression levels of essential genes, nor can it consistently predict the developmental potential of the egg (Rienzi et al., 2011; Wang and Sun, 2007). Recent work from Reich et al. (2011) suggests that this hurdle can be overcome by biopsy of the first polar body followed by single-cell transcriptomics to determine the expression of genes known to be crucial for oogenesis. As gene expression in the polar body closely reflects expression in the oocyte from which it derived, the polar body can act as a proxy when choosing oocytes for ART. The more the field understands about essential regulators of the initial ovarian reserve, the more informed women will be about the potential causes of infertility and the better poised clinicians will be to treat diverse reproductive pathologies in these women.

Conclusion

The establishment of the mammalian primordial follicle pool is a striking developmental process, given its intricate regulation as well as its timing in the life of the animal or woman. Incredibly, the germ cells which form this ovarian reserve remain arrested in meiosis I until ovulation, which in humans first occurs during puberty, and usually are not used for fertilization until decades later. Although the timescale is shorter in mice, the general developmental mechanisms remain the same – extensive coordination of gene expression, signaling pathways and hormonal cues must occur properly early in development to achieve a stable and populated ovarian reserve (Fig. 3). Research from the last 15 years has drawn intriguing parallels between the formation of mammalian germline cysts, from which the primordial follicle pool is created, to germline cysts seen in other vertebrates as well as invertebrates, suggesting a potentially conserved function for this developmental transition. Future research in this field will continue to refine our understanding of this remarkable developmental process, and also help to inform therapeutic interventions for women with ovarian and fertility disorders.

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