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MINI-REVIEW ***Developments in reproductive biology and medicine***

Understanding follicle growth *in vivo*

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ABSTRACT: Ovarian reserve is determined by the number of primordial follicles in the ovary. Quiescent primordial follicles are activated for growth and pass through stages of development before they reach the antral stage. Then a cohort of antral follicles is recruited for further growth, dominance and ovulation under the cyclic stimulation of gonadotrophins. What triggers the initiation of growth in primordial follicles has remained a mystery for decades. However, recent studies on mutant mouse models have shown that primordial follicles are maintained in a dormant state by the actions of various inhibitory molecules to preserve the follicle pool, such as the transcription factor Foxo3a, PTEN (phosphotase and tensin homolog deleted on chromosome 10) and Tsc-1 (tumour suppressor tuberous schlerosis complex). Mice with deletions of these oocyte-specific genes exhibit premature activation of dormant primordial follicles, and all primordial follicles become depleted in early adulthood, causing premature ovarian failure. Other oocyte and somatic cell-derived growth factors are also involved in the early, gonadotrophin-independent phase of follicle growth via autocrine and paracrine interactions. Interestingly, some of these factors also play critical roles at later stages of follicle growth, such as the process of selecting the dominant follicle, by modifying the response of the follicles to gonadotrophins and inhibiting premature luteinization. Therefore, a thorough understanding of the molecular aspects of folliculogenesis is of paramount importance in the context of translational medicine and future clinical applications in human reproduction.

Key words: germ cells / oogenesis / folliculogenesis / follicle growth / oocyte

Primordial germ cells: the origin of oocytes

The earliest primordial form of germ cells (primordial germ cells, PGCs) are differentiated from the proximal epiblast adjacent to the extra-embryonic ectoderm under the influence of signals from extra-embryonic ectoderm-derived bone morphogenetic proteins (BMP) 4 and 8b and extra-embryonic endoderm-derived BMP2 (Ying and Zhao, 2001; Ying *et al*., 2001). Recently, it has been shown that germ cell fate in the epiblast is a direct consequence of BMP4 signaling from the extra-embryonic ectoderm, which is antagonized by the anterior visceral endoderm. Interestingly, BMP8b from the extra-embryonic ectoderm restricts development of the anterior visceral endoderm, thereby contributing to BMP4 signaling. In response to BMP4, the epiblast activates key transcriptional regulators and acquires germ cell features (Ohinata *et al*., 2009). The onset of germ cell competence is marked by the expression of an interferon inducible trans-membrane protein (fragilis) on the germ cells. Fragilis subsequently induces the expression of Stella, a gene expressed exclusively in lineage-restricted germ cells, allowing escape from somatic cell fate and retention of pluripotency as they migrate through the hindgut mesentery and arrive in the gonad (Saitou *et al*., 2002; Lange *et al*., 2003). PGCs first appear as a cluster of ∼100 cells in the endoderm of the dorsal wall of the yolk sac near the allantois between the third and fourth weeks of gestation in the human (Mc *et al*., 1953). PGCs then migrate to the hindgut and dorsal mesentery during the fourth and fifth weeks of gestation, respectively (Mc *et al*., 1953). By the seventh week of gestation, colonization of gonadal tissue by germ cells is complete. Germ cells are essential for the formation and maintenance of the ovary: in their absence the gonad degenerates into cord-like structures (Merchant-Larios and Centeno, 1981). Once the PGC have arrived in the gonad, they undergo more extensive proliferation such that their number rapidly increases from merely 10 000 at the sixth week of gestation to 600 000 at the eighth week. With rapid mitotic activity, their number further rises to 6 million at the 20th week of gestation; thereafter the rate of oogonial mitosis progressively declines and ends at ∼28 weeks with an almost equally increasing rate of oogonial atresia, which peaks at 20 weeks of gestation (Fig. 1).

Of the 1 million germ cells present in a newborn ovary, only 3000–4000 will remain at puberty. The vast majority of germ cells will undergo atresia with <1% (300) reaching the ovulatory state before menopause, after which only 1000 will remain in the ovary (Oktem and Oktay, 2008). The genes, transcription factors and signaling pathways involved in germ cell formation and survival are summarized in Table I.

Transition of oogonia to oocyte

PGCs are called oogonia once they reach the gonads. The oogonia exhibit higher mitotic activity compared with PGCs and undergo several rounds of mitotic divisions prior to meiosis. *Mitotic activity of*

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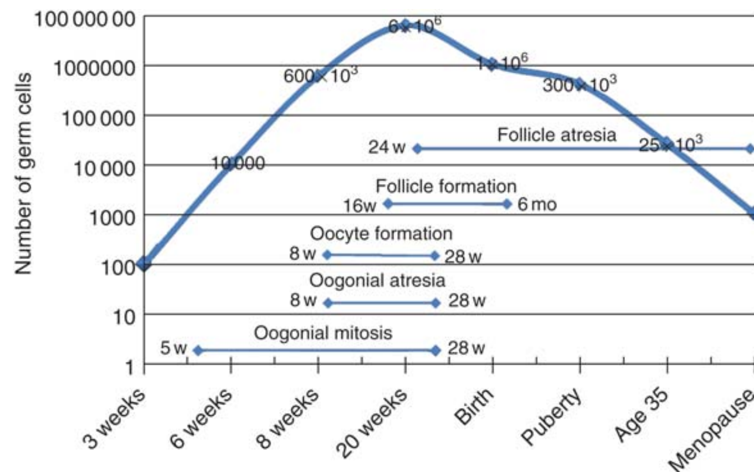

Figure 1 The life history of germ cells. Once specified as a cluster of 100 cells, germ cells begin to migrate, proliferate and colonize the prospective gonads. With exponential growth their number dramatically increases from 600 000 at 8 weeks, and to 6–7 million at 20 weeks of gestation. Thereafter their number progressively declines after 20th weeks owing to atresia. It remains an unsolved puzzle in reproductive biology why millions of germ cells are wastefully lost in order to select only 300–400 (<1%) for ovulation.

the oogonia is the major determinant of the oocyte pool. Oogonial mitosis ceases at ~28th weeks of gestation with an increasing rate of atresia as mentioned above. Therefore, not only mitosis but also atresia of oogonia is important in the formation of the final ovarian reserve.

The last several rounds of mitosis before the initiation of meiosis gives rise to the formation of a syncytium (clusters of oogonia) in which oogonia are connected to each other by their cell membranes, the cytoplasmic bridges caused by incomplete cytokinesis during cell division. In oogonia connected to each other with cell bridges, meiosis begins simultaneously, suggesting propagation of the signal triggering meiotic entry through these bridges. Initiation of premeiotic DNA synthesis marks the end of the oogonial stage whereafter germ cells are called primary oocytes. Entry into meiosis begins between 8 and 13 weeks of gestation, well before follicle formation. The gene Stra8 appears to play a crucial role during this transition as mitotic development of germ cells in the mice null for this gene is normal, but these cells are then unable to undergo premeiotic DNA replication, meiotic chromosome condensation, cohesion, synapsis and recombination (Baltus *et al*., 2006).

The idea of post-natal oogenesis

It is a central dogma in reproductive biology that during the life of the individual there neither is nor can be any increase in the number of primary oocytes beyond those originally laid down when the ovary was formed. This widely accepted doctrine was first described in 1870 (Waldeyer, 1870) and crystallized in 1951 (Zuckerman, 1951). However, a series of recent studies have challenged this dogma by showing regeneration of oocytes from putative germ cells in bone marrow and peripheral blood (Johnson *et al*., 2004, 2005; Lee *et al*., 2007). These results not only triggered an enormous amount of interest among reproductive biologists but also a great deal of debate. While the number of commentaries on this topic continues to grow, recently oocytes and offspring were produced from female germ stem cells (FGSCs) in mice (Zou *et al*., 2009). In the study by Zou *et al*. (2009), FGSCs were isolated from mice and cultured for more than 6 months. Then these FGSCs were infected with green fluorescent protein (GFP) virus and transplanted into the ovaries of chemotherapy-sterilized mice. Transplanted cells underwent oogenesis and the mice produced offspring that had the GFP transgene. These results provided compelling evidence for the existence of FGSCs in adult mammalian females which, at least under the experimental conditions, are fully capable of generating oocytes that can be fertilized and yield viable offspring. Currently, we do not know the biological significance of these germ cells in relation to ovarian function and failure, whether the reported isolation of FGSCs from adult mouse ovaries is a reproducible observation or whether there are similar germ stem cells in the human ovary. The potential implications of these findings for human reproduction could be significant not only for the preservation of the fertile status but also for the prevention of the diverse spectrum of health problems that emerge in women after depletion of ovarian reserve, which is progressive and irreversible with aging, and accelerated and premature in patients who are exposed to gonadotoxic chemotherapy and radiation regimens (Oktem and Oktay, 2007a). Future studies may address whether the dogma in human reproduction that mammalian females lack the ability to replenish their oocyte reserve will be refutable or not.

Formation of primordial follicles

The first primordial follicles appear in the human fetus as early as 15th week of gestation and are complete by 6 months after birth (Baker, 1963; McGee and Hsueh, 2000; Maheshwari and Fowler, 2008). Primordial follicles are composed of diplotene oocytes (30–60 μm) surrounded by flattened pregranulosa cells (Fig. 2). Transcriptomic studies in human and rodents have identified a variety of genes involved in primordial follicle assembly, such as transcription factors (Fig. 1 alpha), zona proteins, meiosis-specific enzymes and nerve growth factors (Table I). Initiation of meiosis in the oogonia (becoming oocytes) with investment of granulosa cells to form the primordial

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Table I The genes and signaling pathways which are involved in the formation of germ cells, follicle assembly and follicle activation.

Gene	Role	Function
PGC formation		
BMP-2 (bone morphogenetic protein-2)	TGF-β member. Extracellular growth factor	PGC formation (Ying *et al.*, 2001)
BMP-4 (bone morphogenetic protein-4)	TGF-β member. Extracellular growth factor	PGC formation (Lawson *et al.*, 1999; Ying *et al.*, 2001)
BMP-8B (bone morphogenetic protein-8b)	TGF-β member. Extracellular growth factor	PGC formation (Ying *et al.*, 2001; Ohinata *et al.*, 2009)
Fragilis	An interferon inducible gene	Germ cell competence (Lange *et al.*, 2003)
Stella	A protein with a SAP-like domain and a splicing factor motif-like structure	Retention of germ cell fate and pluripotency (Saitou *et al.*, 2002)
Smad-1	Signaling molecule of TGF-β ligands	PGC formation (Tremblay *et al.*, 2001)
Smad-5	Signaling molecule of TGF-β ligands	PGC formation (Chang and Matzuk, 2001)
Nanos3	RNA-binding zinc-finger protein	Maintenance of germ cell lineage during migration (Tsuda *et al.*, 2003)
Blimp1 (Prdm1)	A transcriptional repressor	PGC formation (Ohinata *et al.*, 2005)
Prdm14	A transcriptional regulator	PGC formation (Yamaji *et al.*, 2008)
TIAR	A RNA recognition motif/ ribonucleoprotein-type RNA-binding protein	PGC formation (Beck *et al.*, 1998)
Pog	Unknown	PGC proliferation (Agoulnik *et al.*, 2002)
Stra8	Cytoplasmic factor	Premeiotic DNA synthesis and meiotic progression (Baltus *et al.*, 2006)
W (c-kit receptor) and steel (KL)	Tyrosine kinase receptor growth factor	PGC migration and proliferation (Huang *et al.*, 1993; Manova *et al.*, 1993)
LIF	A cytokine with pleiotropic actions	PGC proliferation (Cheng *et al.*, 1994)
Primordial follicle formation and activation		
Fig alpha	Transcription factor	Primordial follicle formation (Soyal *et al.*, 2000). Expression of zone pellucida genes (Liang *et al.*, 1997)
Notch	Signaling pathway	Primordial follicle formation (Trombly *et al.*, 2009)
Dazl1a	Cytoplasmic protein	Primordial follicle formation (Ruggiu *et al.*, 1997)
Nerve growth factor	Growth factor	Primordial follicle formation (Dissen *et al.*, 2001)
SPO11 (sporulation protein homology)	Meiotic proteins	Primordial follicle formation (Di Giacomo *et al.*, 2005)
DMC1 [disrupted meiotic cDNA 1 homolog (human)]	Meiotic proteins	Primordial follicle formation (Dissen *et al.*, 2001)
MSH5 [mutS homolog 5 (*Escherichia coli*)]	Meiotic proteins	Primordial follicle formation (Dissen *et al.*, 2001)
Zfx	The zinc-finger protein	Primordial follicle formation. Oocyte survival and proliferation (Luoh *et al.*, 1997)
ATM	A member of the phosphatidylinositol 3-kinase -like kinases	Meiotic recombination. Mitotic cell cycle regulator kinase DNA damage-induced mitotic cell-cycle checkpoints (Plug *et al.*, 1997)
Nobox	An oocyte-specific homeobox gene	Follicles are replaced by fibrous tissue in female mice lacking nobox. Survival factor for primordial follicles. Also transition of primordial follicles to primary stage is abolished (Rajkovic *et al.*, 2004)
Foxo3	Forkhead transcription factor	Primordial follicle activation (John *et al.*, 2008) Foxo3a−/− female mice exhibit global follicular activation leading to oocyte death, early depletion of functional ovarian follicles, and secondary infertility (Castrillon *et al.*, 2003)
AMH	TGF-β member	There is an increased recruitment of primordial follicles into growing pool in AMH null mice suggesting a negative effect of AMH on primordial-to-primary follicle transition (Durlinger *et al.*, 1999, 2002) A hormonal marker of ovarian reserve used at clinical settings

Continued

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Table I *Continued*

Gene	Role	Function
PTEN–PI3K	PTEN, tumor suppressor gene, a major negative regulator of phosphatidylinositol 3-kinase	In mice lacking PTEN the entire primordial follicle pool becomes activated and all primordial follicles become depleted in early adulthood, causing POF (Reddy *et al.*, 2008) Germline mutations in the PTEN gene cause Cowden disease, a rare autosomal dominant syndrome characterized by multiple hamartomas of the skin, intestine, breast and thyroid and with increased risk of breast, uterus, thyroid, or brain tumors (Liaw *et al.*, 1997)
Tsc/mTORC1 signaling	Tumor suppressor Tsc1,	Negatively regulates mammalian target of rapamycin complex 1 (mTORC1), and keeps primordial follicles quiescencent In mutant mice lacking the Tsc1 gene in oocytes, the entire pool of primordial follicles is activated prematurely due to elevated mTORC1 activity in the oocyte, ending up with follicular depletion in early adulthood and causing POF (Adhikari *et al.*, 2010)
p27	Cyclin-dependent kinase inhibitor 1B [commonly known as p27(kip1)]	Premature activation of the primordial follicle pool initiated by oocyte growth and proliferation and differentiation of pregranulosa cells. Early follicular depletion and POF (Rajareddy *et al.*, 2007)
Foxl2	Winged-helix transcription factor	Premature growth of oocytes; arrested proliferation and differentiation of pregranulosa cells; lack of primary follicles (Schmidt *et al.*, 2004)

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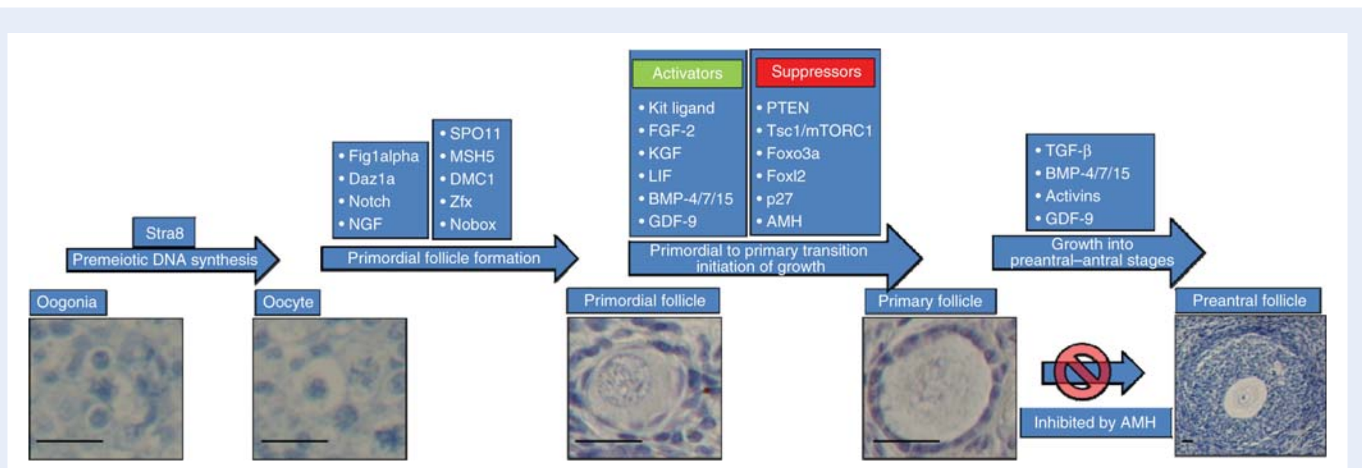

Figure 2 The factors involved in folliculogenesis. Entry into meiosis marks the transition of oogonia to oocytes, which occurs between 8 and 13 weeks of gestation, well before the formation of primordial follicles. The oocyte is arrested at the diplotene stage of prophase after proceeding through the leptotene, zygotene and pachytene stages. At the time of ovulation the first meiotic division is completed in which the oocyte finally assumes the haploid stage, albeit still possessing 2c DNA. Then the oocyte proceeds to the second meiotic division and is arrested at metaphase. The second meiotic division is completed at the time of fertilization resulting in 1n chromosomes and 1n with reestablishment of the diploid state. The first primordial follicles begin to appear at around 16 weeks of gestation, and their formation is complete by 6 months after birth in the human ovary. Our current understanding suggests that the earlier stages of follicle growth, from primordial follicle formation to pre-antral–antral stage, are mainly controlled by ligands of several different signaling pathways acting at the paracrine–autocrine level, such as the TGF-β superfamily (scale bars 25 microns). BMP, bone morphogenetic protein; PTEN, tumor suppressor gene (phosphatase and tensin homolog deleted on chromosome 10); LIF, leukemia inhibitory factor; GDF-9, growth differentiation factor-9; NGF, nerve growth factor; KGF, keratinocyte-growth factor; AMH, anti-Mullerian hormone; FGF-2, basic fibroblast growth factor.

follicle appears to provide protection from atresia, as they cannot persist beyond the seventh month of gestation without entering meiosis. Therefore, ovaries in the newborn are usually devoid of oogonia (Abir *et al.*, 2002). The reproductive life span of women is determined by the number of primordial follicles in the ovary. At present there is no hormonal or any other marker of primordial follicles to be used clinically for the prediction of ovarian reserve.

Transition of primordial follicles into primary follicles (initiation of follicle growth)

Primordial follicles remain in a dormant phase until being recruited into the primary stage for growth. Traditional thinking proposes that rather

than a single signaling pathway, an orchestrated multi-directional communication among the oocytes and somatic cells (granulosa cells and thecal cells), and certain extra-cellular matrix components and growth factors, acting in an autocrine and paracrine manner, play roles in this transition and subsequent growth of follicles (Oktay *et al*., 1997, 2000; Eppig, 2001; Skinner, 2005). However, recent studies on genetically modified mice have taught us that there are indeed some inhibitory signals that maintain primordial follicles in the dormant state. Loss of function of any of the inhibitory molecules for follicular activation, including tumor suppressor tuberous sclerosis complex 1 (Tsc-1), phosphatase and tensin homolog deleted on chromosome 10 (PTEN), Foxo3a, p27 and Foxl2, leads to premature activation of the primordial follicle pool (Castrillon *et al*., 2003; Rajareddy *et al*., 2007; Reddy *et al*., 2008; Adhikari *et al*., 2010). Such a global activation of all primordial follicles inevitably causes early exhaustion of the follicle pool and premature ovarian failure (POF). However, of the genes investigated only the Foxl2 mutation has been linked to POF in humans (De Baere *et al*., 2003). Mutation of the Foxl2 gene also causes blepharophimosis ptosis epicanthus inversus syndrome in human (Crisponi *et al*., 2001). Whereas the Foxo3a knockout mouse is infertile owing to total depletion of follicles (Castrillon *et al*., 2003), mutations or common single nucleotide polymorphisms detected in the Foxo3a gene are not associated with POF in humans (Gallardo *et al*., 2008).

PTEN is a tumor suppressor gene located in a region of human chromosome 10 that is often deleted in many human tumors. Germline mutations in the PTEN gene cause Cowden's disease, a rare autosomal dominant syndrome characterized by multiple hamartomas of the skin, intestine, breast and thyroid and an increased risk of breast, uterus, thyroid or brain tumors (Liaw *et al*., 1997). However, POF has not been reported in any of these patients. No data are available regarding a role for p27 in human POF.

Anti-Mullerian hormone (AMH, also known as Mullerian inhibiting substance) is a member of the transforming growth factor-beta (TGF-β) family and is released from granulosa cells of growing follicles. There is an increased recruitment of primordial follicles into the growing pool in AMH null mice, suggesting a negative effect of AMH on the primordial-to-primary follicle transition (Durlinger *et al*., 1999, 2002). AMH is produced by the granulosa cells of growing pre-antral and small antral follicles as a dimeric glycoprotein (Visser and Themmen, 2005). In fetal ovaries, AMH is first detected at 36 weeks of gestation in granulosa cells of developing pre-antral follicles (Rajpert-De Meyts *et al*., 1999) and reaches its highest levels in puberty and becomes undetectable after menopause (Hudson *et al*., 1990; de Vet *et al*., 2002). AMH has recently emerged as an important marker of ovarian reserve. A growing body of evidence now suggests that AMH can be used both for assessment of ovarian reserve and prediction of IVF outcome. AMH has the least inter- and intra-cycle variability, thus making it a good marker for evaluation in random blood samples, and correlates well with the number of antral follicles in the ovary and the number of oocytes retrieved (van Rooij *et al*., 2002; Ebner *et al*., 2006).

Flattened granulosa cells of primordial follicles become cuboidal during transition into the primary stage along with an increase in oocyte diameter (>60 μm) and acquisition of zona pellucida (Rankin *et al*., 1996). The transcription factor Foxl2 appears to play a critical role in this transition. In mice in which the *Foxl2* gene was interrupted by replacing sequences encoding amino acids 62–375 and part of the 3′-untranslated region with *lacZ* (so-called *Foxl2lacZ* mice), although primordial follicles were formed normally, the pregranulosa cells did not complete their squamous-to-cuboidal transition, which led to the absence of secondary follicles. Also, no signs of granulosa cell differentiation could be seen in *Foxl2^{lacZ}* ovaries. These findings indicate that Foxl2 in pregranulosa cells is important for the differentiation of pregranulosa cells into granulosa cells during follicular activation. In addition, oocytes in almost all primordial follicles had started to grow by 2 weeks after birth and express growth differentiation factor-9 (GDF-9), which is expressed by the primary stage and onward (Schmidt *et al*., 2004).

The initial recruitment of quiescent primordial follicles into the growing pool as primary follicles starts in fetal life and continues postnatally until the ovarian reserve is depleted, leaving behind only ~1000 primordial follicles in the ovary at the time of menopause (Oktem and Oktay, 2008). The process is continuous and different from the cyclic recruitment of a cohort of antral follicles under the actions of FSH.

In addition to inhibitory signals that inhibit premature activation of primordial follicles, there are some other signals in the ovary that promote the transition of primordial follicles to primary follicles. With coordinated and synergistic actions of these signals arising from different compartments, such as oocytes, somatic cells and stroma, growth is initiated in primordials. This could also explain why isolated primordial follicles do not survive in culture, but grow *in situ* in ovarian tissue culture (O'Brien *et al*., 2003). Furthermore, FSH is not required for this transition as primordial follicles do not express FSH receptors (Oktay *et al*., 1997).

According to studies on transgenic animal models and on the human ovary, several members of the TGF-β super family, such as BMP-4 and BMP-7 (expressed by ovarian stromal cells and/or theca cells) (Lee *et al*., 2001; Nilsson and Skinner, 2003) and GDF-9 (expressed on oocytes) (Dong *et al*., 1996; Carabatsos *et al*., 1998; Vitt *et al*., 2000), play critical roles in this process. Even though mice null for the GDF-9 gene are infertile owing to arrested follicle development at the primary stage with no further growth beyond that stage (Dong *et al*., 1996; Carabatsos *et al*., 1998), the *in vitro* effects of GDF-9 in the primordial-to-primary transition are controversial. One study showed increased transition of primordial follicles to the primary stage in the rat model (Vitt *et al*., 2000), whereas another study using the same model found no evidence of increased transition (Nilsson and Skinner, 2002).

It should be noted that there could be wide species differences in the actions of TGF-β members, as in the case of oocyte-derived BMP-6 and BMP-15 (also known as GDF-9B). For instance, mice with null mutations in BMP-6 and BMP-15 genes have normal follicle development and fertility (Solloway *et al*., 1998; Yan *et al*., 2001). By contrast, mutations in the BMP-15 gene are associated with infertility in sheep and POF in the human (Galloway *et al*., 2000; Di Pasquale *et al*., 2004).

There are other growth factors and cytokines that act at a paracrine level in the formation of primary follicles, such as kit-ligand (KL, also known as stem cell factor expressed on granulosa cells, its receptor c-kit expressed on oocyte and theca cells) (Nilsson *et al*., 2002; Nilsson and Skinner, 2004) and the leukemia inhibitory factor (LIF expressed on granulosa cells) (Nilsson *et al*., 2002). LIF can also stimulate proliferation of PGCs, oocyte growth and recruitment of theca cells from the surrounding stromal tissue (Cheng *et al*., 1994; Nilsson *et al*., 2002; Nilsson and Skinner, 2004).

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Theca cell and stroma derived keratinocyte-growth factor (KGF) (also known as fibroblast growth factor-7, FGF-7) and FGF-2 (also known as basic fibroblast growth factor) have been implicated recently as positive regulators of the primordial-to-primary follicle transition and they exert their effects by up-regulating KL expression in granulosa cells (Nilsson and Skinner, 2004; Kezele *et al*., 2005).

Insulin has been shown to promote the primordial-to-primary follicle transition in a rat ovarian organ culture model with some additive effects with KL and LIF (Kezele *et al*., 2002; Nilsson *et al*., 2002). Other recently discovered genes that play a role in follicle growth initiation are nobox [expressed in germ cells (newborn ovary homeobox)] and forkhead transcription factor Foxo3 (Rajkovic *et al*., 2004; John *et al*., 2008).

Follicle growth to pre-antral and antral stages

With mitotic expansion of granulosa cells, single-layered primary follicles are transformed into multi-layered ones (also known as secondary follicles). An increase in oocyte diameter, and formation of basal lamina, zona pellucida and theca cell layer are among the other changes that characterize this developmental stage (Knight and Glister, 2006). During this phase follicle diameter increases from 40–60 μm at the primary stage to 120–150 μm at the pre-antral stage. With further growth, follicles reaching a diameter of 200 μm enter the antral stage. It is also during this stage that the follicle begins to exhibit some fluid-filled spaces within the granulosa cell layers, which will coalesce to form the antral cavity, along with increased vascularization of the theca layer, continued growth of oocytes and proliferation of granulosa and theca cells.

Development of a multi-layered secondary follicle from a primary follicle with a single layer of granulosa cells is a lengthy process that takes months in humans. This slow process appears to be not mediated by the actions of gonadotrophins. Even though pre-antral follicles may express FSH receptors (Oktay *et al*., 1997), FSH may have a permissive role rather than being essential in pre-antral follicle growth since multi-layer follicle development has been observed rarely on histological examination of the ovaries from women with anosmia and hypogonadotropic hypogonadism (Goldenberg *et al*., 1976). On the other hand, FSH has been shown to enhance the survival of ovarian grafts transplanted into immune-deficient hypogonadal mice and induce follicle growth up to the antral stage (Oktay *et al*., 1998). Interestingly, FSH is devoid of such an effect on growth or survival *in vitro* when isolated pre-antral follicles are cultured with FSH in the absence of serum supplementation (McGee *et al*., 1997). Interactions of FSH with locally produced factors in the ovary *in vivo* could be a plausible explanation for this discrepancy.

In contrast to the debated role of FSH in pre-antral follicle growth, there is firm evidence that certain members of the TGF-β super family locally produced from granulosa cells (activins), theca cells (BMP-4 and BMP-7) or both (TGF-β); or oocytes (GDF-9) and BMP-15, play crucial roles in the growth of primary follicles into pre-antral and antral stages (Fig. 2).

In vitro exposure of ovarian cortical samples to oocyte-derived recombinant GDF-9 has been shown to increase the number of primary and secondary follicles in human and rodents suggesting an important role, at least under *in vitro* conditions, for this growth factor in the initiation and progression of follicle growth (Hayashi *et al*., 1999; Hreinsson *et al*., 2002; Nilsson *et al*., 2002; Wang and Roy, 2004). Arrested follicle growth at the primary stage in mice null for the GDF-9 gene and in sheep with an inactivating mutation in the GDF-9 gene is further evidence that confirms the growth-promoting effects of GDF-9 on follicles beyond the primary stage (Dong *et al*., 1996; Juengel *et al*., 2002; Hanrahan *et al*., 2004).

Another positive regulator of follicle growth into pre-antral and antral stages is BMP-15. This oocyte-derived growth factor stimulates the proliferation of granulosa cells in an FSH-independent manner (Otsuka *et al*., 2000). This finding suggests that BMP-15 can stimulate granulosa cell mitosis in pre-antral follicles during the FSH-independent stages of early follicular growth. It is noteworthy that the expression of BMP-15 mRNA first appears in the oocytes of primary follicles transiting into secondary follicles, whereas GDF-9 expression begins earlier in primordial stage oocytes in the human ovary (Shimasaki *et al*., 2004). Currently we do not know whether BMP-15 also regulates granulosa cell proliferation during antral and pre-ovulatory follicle development, but BMP-15 can inhibit FSH receptor expression (Otsuka *et al*., 2000). Interestingly follistatin can neutralize the action of BMP-15 and there is a strong expression of follistatin in the dominant follicle, suggesting that follistatin ensures that sufficient FSH receptors are expressed on the granulosa cells during selection of the dominant follicle (Otsuka *et al*., 2001).

Theca cells play an important role in follicle growth in the ovary from many different perspectives. Firstly, they are the main source of androgen synthesis in the ovary providing steroidogenic precursors for estrogen synthesis in the granulosa cells. Secondly, BMP-4 and -7 of thecal origin are able to promote follicle growth beyond the primary stage in both *in vivo* and *in vitro* rodent models (Nilsson and Skinner, 2003; Lee *et al*., 2004). Thirdly, theca cells communicate bi-directionally with granulosa cells through the production of the hepatocyte growth factor (HGF) and the KGF, which promote granulosa cells to induce KL. KL in turn promotes the expression of HGF and KGF on theca cells. KGF is also involved in primordial-to-primary follicle transition as mentioned previously (Kezele *et al*., 2005). Interestingly, recruitment of theca cells to surround the basement membrane of the follicle is impaired in mice null for GDF-9 gene (Carabatsos *et al*., 1998). Fourthly, during rapid growth of pre-antral–antral follicles, BMP-4 and BMP-7 modulate FSH signaling in a way that promotes estradiol (E_2) production (aromatization) while inhibiting progesterone synthesis, acting as a *luteinization inhibitor*. However, BMP-4 and BMP-7 do not affect granulosa cell steroidogenesis in the absence of FSH at least in the rat model (Shimasaki *et al*., 1999).

Among the other identified positive regulators of growth of pre-antral and antral follicles are activins from the granulosa cells and TGF-β from both granulosa and theca cells. Even though there are three different activin isoforms (activin A, AB and B), most of the data on bioactivity and functions of activins are for activin A. Activin A has been shown to promote pre-antral follicle growth and granulosa cell proliferation in rat and mouse models through local paracrine/autocrine effects (Smitz *et al*., 1998; Liu *et al*., 1999; Oktay *et al*., 2000; Zhao *et al*., 2001; Oktem and Oktay, 2007b).

TGF-β has three different isoforms (TGF-β1, β2 and β3). The pattern of expression of TGF-ß3 has not been determined. TGF-β bioactivity is likely to be derived from both the thecal and granulosa

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cells with expression first observed during pre-antral follicular growth and intensifying as the follicle matures (Chegini and Flanders, 1992; Roy and Kole, 1998; Juengel and McNatty, 2005). In humans TGF-β1 is expressed in oocytes of primary follicles and in theca and granulosa cells of antral follicles, whereas TGF-β2 is expressed by theca cells of large pre-antral/antral follicles and by granulosa cells of larger antral follicles. Type-1 and -2 receptors are expressed by both granulosa and theca cells and oocytes, making it difficult to interpret their roles in follicle development. Furthermore, there are considerable variations in TGF-β actions among species. The actions of TGF-β documented at least in rodent models include proliferation of granulosa cells, and progesterone production and FSH-induced E_2 production (Dodson and Schomberg, 1987; Adashi *et al*., 1989; Saragueta *et al*., 2002). In humans granulosa–luteal cells, TGF-β1 and 2 have also been shown to stimulate the expression of the inhibin/activin βB subunit mRNA without affecting the inhibin or inhibin/activin βA subunit mRNA (Eramaa and Ritvos, 1996). It also appears that TGF-β suppresses steroidogenesis in thecal cells from most species (Juengel and McNatty, 2005). TGF-β may promote proliferation of granulosa cells and pre-antral follicle growth in rodents (Liu *et al*., 1999), but there are no data regarding a role in pre-antral follicle growth in humans.

AMH inhibits FSH-driven growth of late pre-antral follicles *in vitro* in mice (Durlinger *et al*., 2001). AMH appears to have a negative effect on pre-antral follicle development beyond primordial-primary transition. Primordial follicles do not express AMH. Its expression is first detected on granulosa cells of primary follicles and continues until mid-antral stages of follicle development humans. The highest level of expression is observed on granulosa cells of secondary, pre-antral and small antral follicles ≤4 mm in diameter (Weenen *et al*., 2004; Visser and Themmen, 2005). These findings suggest a negative effect of AMH on pre-antral follicle development beyond the primordial-primary transition.

Granulosa cells have no direct blood supply. The basal lamina works as a blood-follicle barrier and separates granulosa cells from the vascularized thecal cell layer necessitating an intimate contact between neighboring granulosa cells and the oocytes. An extensive network of gap junctions between granulosa cells couples them into an integrated functional syncytium. These junctions are composed of connexin proteins arranged in a hexameric configuration and allow not only an effective communication, but also efficient metabolic exchange and transportation of molecules between granulosa cells. Granulosa cells also communicate with the oocytes via the gap junctions projecting through the zona pellucida to the plasma membrane of the oocytes. Connexin proteins appear to play important roles in follicle development since follicle development is arrested in primary and pre-antral stages in mice deficient for connexin 43 and 37, respectively (Simon *et al*., 1997; Juneja *et al*., 1999).

Follicle growth after the antral stage and the process for selection of the dominant follicle

Follicle growth after the antral stage is characterized by further proliferation of granulosa and theca cells, increased vascularization, oocyte growth and formation of the antral space, a fluid-filled space which will coalesce to form the antral cavity. FSH becomes a critical determinant of further follicle growth and survival at this stage. Cyclic recruitment of a cohort of antral follicles for further growth and the selection of a dominant follicle from among this cohort are the most characteristic features of this phase. Although traditional thinking proposes a single wave of cyclic follicular recruitment and growth, recently it has been suggested that multiple waves of follicle development may occur in the human ovary (Baerwald *et al*., 2003).

When a selected cohort of antral follicles grow, modulation of their steroidogenetic activity and their response to gonadotrophins and the prevention of premature luteinization during this rapid growth phase are required to sensitize certain follicles for further growth and selection of a dominant follicle. Current evidence suggests that these goals are being accomplished at the autocrine/paracrine level with the influences of certain locally produced growth factors, such as granulose-derived activin and BMP-6, oocyte-derived GDF-9, BMP-15 and BMP-6 and theca-derived BMP-2, -3b, -4 and -7 exhibiting spatiotemporal expression patterns.

For example, activin A has been shown to promote FSH receptor expression in cultured rat granulosa cells (Xiao *et al*., 1992) and suppress the growth of primary follicles while inducing follicular growth at later stages (Liu *et al*., 1998; Mizunuma *et al*., 1999; Oktem and Oktay, 2007b). Activin A is also involved in the regulation of aromatase activity, estrogen synthesis, LH receptor expression and oocyte maturation (Findlay, 1993). When activin signaling is disrupted, as in the mice with null mutations in activin receptor ActRIIB (Matzuk *et al*., 1995) or in transgenic mice overexpressing follistatin (binds activin irreversibly and neutralizes its bioactivity) (Guo *et al*., 1998), follicle development is arrested, implying a role for activin in the cyclic recruitment of follicles. By contrast, AMH negatively affects cyclic recruitment and dominant follicle selection by reducing the responsiveness of pre-antral and small antral follicles to FSH (Durlinger *et al*., 1999, 2002; Visser and Themmen, 2005).

Another characteristic of follicular development is a switch from an activin A dominant to inhibin A dominant environment. Small follicles tend to produce more activin A relative to inhibin A, whereas larger selected antral follicles secrete more inhibin A (Yamoto *et al*., 1992). Activin-A can attenuate LH-dependent androgen production by theca cells of small pre-antral follicles (Hsueh *et al*., 1987). On the other hand, inhibin A released in large quantities by large selected antral follicles can counteract the inhibitory effects of activin A and increase LH-induced androgen secretion from theca cells. This provides a sufficient supply of androgen for conversion into estrogens by the aromatase enzyme in granulosa cells as there will be a great demand for estrogen synthesis during the peri-ovulatory period. The expression pattern of activin A subunits (expressed on cumulus granulosa cells surrounding oocytes) and its receptors (expressed on oocytes) suggests a role for activin in acquisition of maturational competence in oocytes of growing pre-antral follicles (Hsueh *et al*., 1987). In accordance with this finding, activin A has been shown to accelerate *in vitro* maturation of human, primate and rodent oocytes (Sadatsuki *et al*., 1993; Alak *et al*., 1996, 1998). By contrast inhibin A can function as a meiotic inhibitor and therefore impair oocyte maturation and developmental competence as shown in rat and bovine oocytes (O *et al*., 1989; Silva *et al*., 1999).

The effects of TGF-β during pre-antral follicle growth appear to be similar to activin A, namely stimulation of FSH receptor expression,

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aromatase activity and inhibin production in granulosa cells, progesterone production and LH receptor expression and suppression of androgen production in theca cells (Knight and Glister, 2006).

BMP-6 of granulosa cell origin and theca-derived BMP-4 and -7 can attenuate LH-dependent androgen output by theca cells of small pre-antral follicles. By restricting androgen output from theca cells, the growth of small pre-antral follicles will be impaired as androgen supply for estrogen synthesis will diminish during the growth phase of follicles in a selected cohort.

Inhibition of premature luteinization

Another critical issue during the growth of antral follicles is the prevention of premature luteinization while maintaining cell proliferation and follicle growth. In this context, the oocyte-derived BMP-6, BMP-15 and GDF-9 act within the follicle to inhibit premature luteinization and limit progesterone biosynthesis by suppressing gonadotrophin-driven progesterone synthesis (Otsuka *et al*., 2001). The effects of these oocyte-derived luteinization inhibitors will be lost after the release of the oocyte at ovulation, and luteinization will commence. While BMP-15 acts by suppressing FSH receptors (Otsuka *et al*., 2000; Shimasaki *et al*., 2004), BMP-6 inhibits its adenylate cyclase activity (Erickson and Shimasaki, 2003). Noteworthy, at least in the rat ovary, is that BMP-6 mRNA is lost during selection of the dominant follicle since the selection process is mediated by the actions of FSH. Despite their suppressive effect on progesterone production, neither BMP-6 nor BMP-15 inhibits the stimulatory action of FSH on P450 aromatase mRNA expression and E_2 production (Shimasaki *et al*., 2004). BMP-15 and GDF-9 are also able to promote follicle survival by stimulating cell proliferation and inhibiting luteinization, perhaps in concert with the actions of granulosa cell-derived BMP-2, -5 and -6 (Shimasaki *et al*., 2004).

Other paracrine regulators of follicle growth at this stage are theca cell-derived BMP-4 and -7. Enhanced basal and insulin-like growth factor-stimulated E_2 synthesis (bovine), FSH-stimulated E_2 synthesis (rat), cell proliferation and induction of inhibin A, activin A and follistatin secretions are among the actions of BMP-4 and -7. Again both BMP-4 and -7 can suppress progesterone and androgen production (Knight and Glister, 2006).

Taken together, the growth of pre-antral and early antral follicles is marked by up-regulated expression of FSH and LH receptors and FSH-induced aromatase activity, mainly a result of the actions of activin-A and TGF-β. Activin-A, TGF-β, BMP-6 of granulosa origin, and theca-derived BMP-4 and -7 released from larger pre-antral and antral follicles limit the androgen output from theca cells of smaller follicles, providing a limited supply of androgens for conversion into estrogens. With further growth of the follicle, more inhibin A and follistatin are released from follicles along with a switch from an activin-dominant to inhibin-dominant milieu as the follicle matures. There is also a concomitant decline in the FSH level with rising inhibin A levels. LH-induced androgen secretion is up-regulated by inhibin A since high levels of E_2 secretion are required during the preovulatory phase while suppressing FSH secretion. Follistatin will counteract the actions of activin A. Therefore, on the way to dominance, a challenging situation is being created in such a manner that only those follicles that express higher levels of FSH and LH receptors and FSH-induced aromatase activity during the early growth phase under the actions of activin A, TGF-β and other growth factors, such as GDF-9, will survive this stage where less available substrates have to be utilized in a more efficient way in order to escape atresia and be selected for dominance. Modulation of the follicular response to gonadotrophins and inhibition of luteinization by the actions of GDF-9, BMP-6 and BMP-15 are other features of this stage of follicle growth.

Conclusion

Over the past decades significant progress has been made toward understanding the factors that control folliculogenesis. While initial thinking proposed that there is a long list of factors playing a role in activation of follicle growth, some of them are without validation from genetic studies. Furthermore, recent studies have shown us that there are indeed some inhibitory signals, such as PTEN and Tsc1/mTOR, that maintain primordial follicles in the dormant phase and prevent their premature activation, early exhaustion and POF. Coordinated actions of these suppressor and activator signals are required for the initiation and progression of follicle growth. While gonadotrophins take precedence over paracrine factors in follicle growth at the antral stage and beyond, the early stages of follicle development are mainly controlled by some locally produced factors. Moreover, some of these factors may have roles at the gonadotrophin-dependent phase of follicle growth, such as selection of the dominant follicle and inhibition of luteinization. Some of these locally produced factors may have diagnostic or therapeutic potential in the future, such as those which control activation of primordial follicles. Drugs that antagonize the inhibitory signals may help to activate primordial follicles *in vitro*: these follicles can then be matured *in vitro* and fertilized. Fertilized eggs can then either be transferred to the patient or cryopreserved for fertility preservation purposes.

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