

Understanding follicle growth *in vivo*

Ozgur Oktem* and Bulent Urman

Women's Health Center, Assisted Reproduction Unit, Vehbi Koc Foundation American Hospital, Guzelbahce Sok.
No. 20 Nisantasi, 34365 Istanbul, Turkey

*Correspondence address. Tel: +90-212-444-3-777; E-mail: ozgurok@amerikanhastanesi.org

Submitted on May 15, 2010; resubmitted on September 4, 2010; accepted on September 8, 2010

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 (phosphatase and tensin homolog deleted on chromosome 10) and Tsc-1 (tumour suppressor tuberous sclerosis 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 transmembrane 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 1.

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*

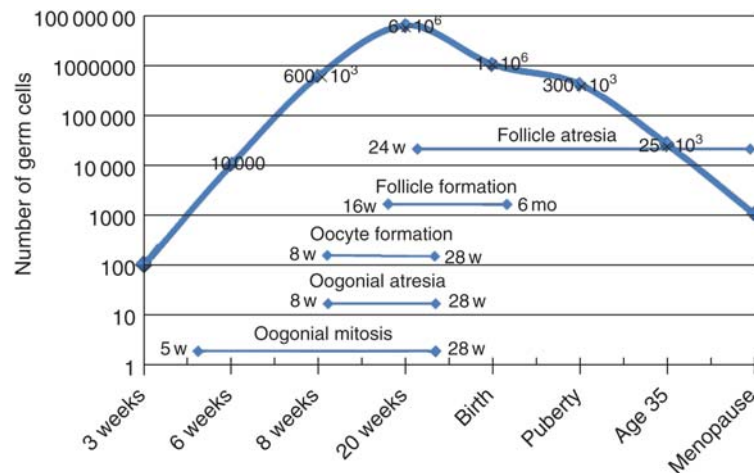


Figure 1 The life history of germ cells. Once specified as a cluster of 100 cells, germ cells begin to migrate, proliferate and colonize the prospective gonads. With exponential growth their number dramatically increases from 600 000 at 8 weeks, and to 6–7 million at 20 weeks of gestation. Thereafter 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 oögonia is the major determinant of the oocyte pool. Oögonial mitosis ceases at ~28th weeks of gestation with an increasing rate of atresia as mentioned above. Therefore, not only mitosis but also atresia of oögonia 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 oögonia) in which oögonia are connected to each other by their cell membranes, the cytoplasmic bridges caused by incomplete cytokinesis during cell division. In oögonia 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 oögonial 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 oögenesis

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 (Waldayer, 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 oögenesis 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 oögonia (becoming oocytes) with investment of granulosa cells to form the primordial

Table 1 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 <i>et al.</i> , 2001) |
| BMP-4 (bone morphogenetic protein-4) | TGF- β member. Extracellular growth factor | PGC formation (Lawson <i>et al.</i> , 1999; Ying <i>et al.</i> , 2001) |
| BMP-8B (bone morphogenetic protein-8b) | TGF- β member. Extracellular growth factor | PGC formation (Ying <i>et al.</i> , 2001; Ohinata <i>et al.</i> , 2009) |
| Fragilis | An interferon inducible gene | Germ cell competence (Lange <i>et al.</i> , 2003) |
| Stella | A protein with a SAP-like domain and a splicing factor motif-like structure | Retention of germ cell fate and pluripotency (Saitou <i>et al.</i> , 2002) |
| Smad-1 | Signaling molecule of TGF- β ligands | PGC formation (Tremblay <i>et al.</i> , 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 <i>et al.</i> , 2003) |
| Blimp1 (Prdm1) | A transcriptional repressor | PGC formation (Ohinata <i>et al.</i> , 2005) |
| Prdm14 | A transcriptional regulator | PGC formation (Yamaji <i>et al.</i> , 2008) |
| TIAR | A RNA recognition motif/ribonucleoprotein-type RNA-binding protein | PGC formation (Beck <i>et al.</i> , 1998) |
| Pog | Unknown | PGC proliferation (AgoulNIK <i>et al.</i> , 2002) |
| Stra8 | Cytoplasmic factor | Premeiotic DNA synthesis and meiotic progression (Baltus <i>et al.</i> , 2006) |
| W (c-kit receptor) and steel (KL) | Tyrosine kinase receptor growth factor | PGC migration and proliferation (Huang <i>et al.</i> , 1993; Manova <i>et al.</i> , 1993) |
| LIF | A cytokine with pleiotropic actions | PGC proliferation (Cheng <i>et al.</i> , 1994) |
| Primordial follicle formation and activation | | |
| Fig alpha | Transcription factor | Primordial follicle formation (Soyal <i>et al.</i> , 2000). Expression of zone pellucida genes (Liang <i>et al.</i> , 1997) |
| Notch | Signaling pathway | Primordial follicle formation (Trombly <i>et al.</i> , 2009) |
| Dazl a | Cytoplasmic protein | Primordial follicle formation (Ruggiu <i>et al.</i> , 1997) |
| Nerve growth factor | Growth factor | Primordial follicle formation (Disson <i>et al.</i> , 2001) |
| SPO11 (sporulation protein homology) | Meiotic proteins | Primordial follicle formation (Di Giacomo <i>et al.</i> , 2005) |
| DMC1 [disrupted meiotic cDNA 1 homolog (human)] | Meiotic proteins | Primordial follicle formation (Disson <i>et al.</i> , 2001) |
| MSH5 [mutS homolog 5 (<i>Escherichia coli</i>)] | Meiotic proteins | Primordial follicle formation (Disson <i>et al.</i> , 2001) |
| Zfx | The zinc-finger protein | Primordial follicle formation. Oocyte survival and proliferation (Luoh <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 2004) |
| Foxo3 | Forkhead transcription factor | Primordial follicle activation (John <i>et al.</i> , 2008) Foxo3a ^{-/-} female mice exhibit global follicular activation leading to oocyte death, early depletion of functional ovarian follicles, and secondary infertility (Castrillon <i>et al.</i> , 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 <i>et al.</i> , 1999, 2002) A hormonal marker of ovarian reserve used at clinical settings |

Continued

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 <i>et al.</i> , 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 <i>et al.</i> , 1997) |
| Tsc/mTORC1 signaling | Tumor suppressor Tsc1, | Negatively regulates mammalian target of rapamycin complex I (mTORC1), and keeps primordial follicles quiescent 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 <i>et al.</i> , 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 <i>et al.</i> , 2007) |
| Foxl2 | Winged-helix transcription factor | Premature growth of oocytes; arrested proliferation and differentiation of pregranulosa cells; lack of primary follicles (Schmidt <i>et al.</i> , 2004) |

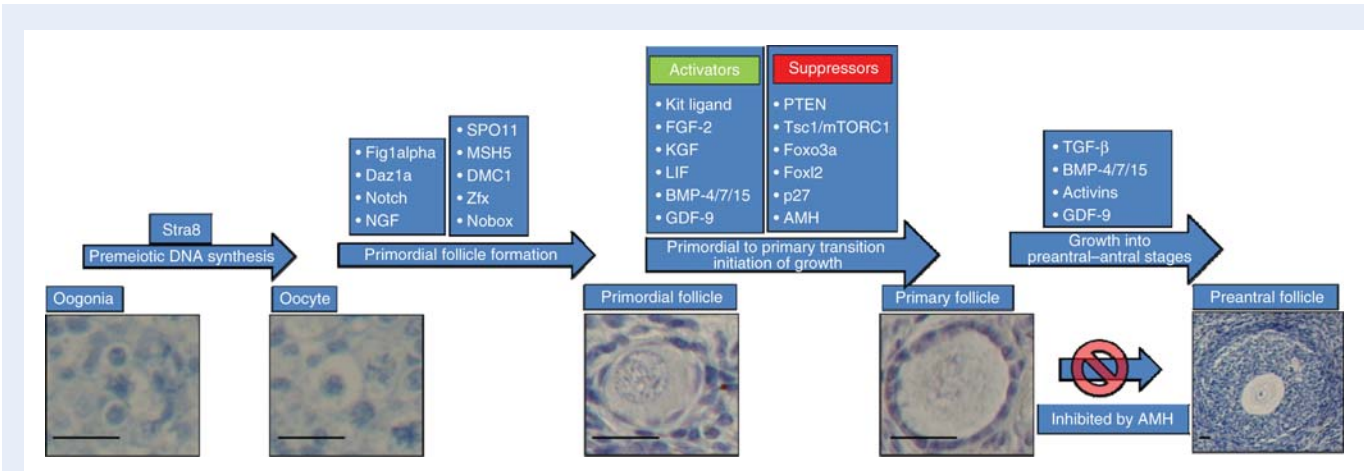


Figure 2 The factors involved in folliculogenesis. Entry into meiosis marks the transition of oogonia to oocytes, which occurs between 8 and 13 weeks of gestation, well before the formation of primordial follicles. The oocyte is arrested at the diplotene stage of prophase after proceeding through the 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-Müllerian 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 (Oktaý *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. Germ-line 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-Müllerian hormone (AMH, also known as Müllerian 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 ($>60\ \mu\text{m}$) 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 Oktaý, 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 (Oktaý *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).

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 Glistler, 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- β 1, β 2 and β 3). The pattern of expression of TGF- β 3 has not been determined. TGF- β bioactivity is likely to be derived from both the thecal and granulosa

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- β 1 is expressed in oocytes of primary follicles and in theca and granulosa cells of antral follicles, whereas TGF- β 2 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 E₂ production (Dodson and Schomberg, 1987; Adashi et al., 1989; Saraguet et al., 2002). In humans granulosa-luteal cells, TGF- β 1 and 2 have also been shown to stimulate the expression of the inhibin/activin β B subunit mRNA without affecting the inhibin or inhibin/activin β A 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 (Veenen 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 granulosa-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 (Yamamoto 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,

aromatase activity and inhibin production in granulosa cells, progesterone production and LH receptor expression and suppression of androgen production in theca cells (Knight and Glistler, 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 E₂ 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 E₂ synthesis (bovine), FSH-stimulated E₂ 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 Glistler, 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 E₂ 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.

References

- Abir R, Orvieto R, Dicker D, Zukerman Z, Barnett M, Fisch B. Preliminary studies on apoptosis in human fetal ovaries. *Fertil Steril* 2002;**2**:259–264.
- Adashi EY, Resnick CE, Hernandez ER, May JV, Purchio AF, Twardzik DR. Ovarian transforming growth factor-beta (TGF beta): cellular site(s), and mechanism(s) of action. *Mol Cell Endocrinol* 1989;**2**:247–256.
- Adhikari D, Zheng W, Shen Y, Gorre N, Hamalainen T, Cooney AJ, Huhtaniemi I, Lan ZJ, Liu K. Tsc/mTORC1 signaling in oocytes governs the quiescence and activation of primordial follicles. *Hum Mol Genet* 2010;**3**:397–410.
- AgoulNIK AI, Lu B, Zhu Q, Truong C, Ty MT, Arango N, Chada KK, Bishop CE. A novel gene, Pog, is necessary for primordial germ cell proliferation in the mouse and underlies the germ cell deficient mutation, gcd. *Hum Mol Genet* 2002;**24**:3047–3053.
- Alak BM, Smith GD, Woodruff TK, Stouffer RL, Wolf DP. Enhancement of primate oocyte maturation and fertilization *in vitro* by inhibin A and activin A. *Fertil Steril* 1996;**4**:646–653.
- Alak BM, Coskun S, Friedman CI, Kennard EA, Kim MH, Seifer DB. Activin A stimulates meiotic maturation of human oocytes and modulates granulosa cell steroidogenesis *in vitro*. *Fertil Steril* 1998;**6**:1126–1130.
- Baerwald AR, Adams GP, Pierson RA. Characterization of ovarian follicular wave dynamics in women. *Biol Reprod* 2003;**3**:1023–1031.

- Baker TG. A quantitative and cytological study of germ cells in human ovaries. *Proc R Soc Lond B Biol Sci* 1963;**158**:417–433.
- Baltus AE, Menke DB, Hu YC, Goodheart ML, Carpenter AE, de Rooij DG, Page DC. In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nat Genet* 2006;**12**:1430–1434.
- Beck AR, Miller IJ, Anderson P, Streuli M. RNA-binding protein TIAR is essential for primordial germ cell development. *Proc Natl Acad Sci USA* 1998;**5**:2331–2336.
- Carabatsos MJ, Elvin J, Matzuk MM, Albertini DF. Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. *Dev Biol* 1998;**2**:373–384.
- Castrillon DH, Miao L, Kolipara R, Horner JW, DePinho RA. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science* 2003;**5630**:215–218.
- Chang H, Matzuk MM. Smad5 is required for mouse primordial germ cell development. *Mech Dev* 2001;**1**:2:61–67.
- Chegini N, Flanders KC. Presence of transforming growth factor-beta and their selective cellular localization in human ovarian tissue of various reproductive stages. *Endocrinology* 1992;**3**:1707–1715.
- Cheng L, Gearing DP, White LS, Compton DL, Schooley K, Donovan PJ. Role of leukemia inhibitory factor and its receptor in mouse primordial germ cell growth. *Development* 1994;**11**:3145–3153.
- Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, Amati P, Bisceglia L, Zelante L, Nagaraja R, Porcu S. et al. The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. *Nat Genet* 2001;**2**:159–166.
- De Baere E, Beysen D, Oley C, Lorenz B, Cocquet J, De Sutter P, Devriendt K, Dixon M, Fellous M, Fryns JP. et al. FOXL2 and BPES: mutational hotspots, phenotypic variability, and revision of the genotype-phenotype correlation. *Am J Hum Genet* 2003;**2**:478–487.
- de Vet A, Laven JS, de Jong FH, Themmen AP, Fauser BC. Antimüllerian hormone serum levels: a putative marker for ovarian aging. *Fertil Steril* 2002;**2**:357–362.
- Di Giacomo M, Barchi M, Baudat F, Edelmann W, Keeney S, Jasin M. Distinct DNA-damage-dependent and -independent responses drive the loss of oocytes in recombination-defective mouse mutants. *Proc Natl Acad Sci USA* 2005;**3**:737–742.
- Di Pasquale E, Beck-Peccoz P, Persani L. Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. *Am J Hum Genet* 2004;**1**:106–111.
- Dissen GA, Romero C, Hirshfield AN, Ojeda SR. Nerve growth factor is required for early follicular development in the mammalian ovary. *Endocrinology* 2001;**5**:2078–2086.
- Dodson WC, Schomberg DW. The effect of transforming growth factor-beta on follicle-stimulating hormone-induced differentiation of cultured rat granulosa cells. *Endocrinology* 1987;**2**:512–516.
- Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM. Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* 1996;**6600**:531–535.
- Durlinger AL, Kramer P, Karels B, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen AP. Control of primordial follicle recruitment by anti-Müllerian hormone in the mouse ovary. *Endocrinology* 1999;**12**:5789–5796.
- Durlinger AL, Gruijters MJ, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JT, Grootegoed JA. et al. Anti-Müllerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. *Endocrinology* 2001;**11**:4891–4899.
- Durlinger AL, Gruijters MJ, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JT, Grootegoed JA, Themmen AP. Anti-Müllerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. *Endocrinology* 2002;**3**:1076–1084.
- Ebner T, Sommergruber M, Moser M, Shebl O, Schreier-Lechner E, Tews G. Basal level of anti-Müllerian hormone is associated with oocyte quality in stimulated cycles. *Hum Reprod* 2006;**8**:2022–2026.
- Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. *Reproduction* 2001;**6**:829–838.
- Eramaa M, Ritvos O. Transforming growth factor-beta 1 and -beta 2 induce inhibin and activin beta B-subunit messenger ribonucleic acid levels in cultured human granulosa-luteal cells. *Fertil Steril* 1996;**5**:954–960.
- Erickson GF, Shimasaki S. The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during the estrous cycle. *Reprod Biol Endocrinol* 2003;**1**:9.
- Findlay JK. An update on the roles of inhibin, activin, and follistatin as local regulators of folliculogenesis. *Biol Reprod* 1993;**1**:15–23.
- Gallardo TD, John GB, Bradshaw K, Welt C, Reijo-Pera R, Vogt PH, Touraine P, Bione S, Toniolo D, Nelson LM. et al. Sequence variation at the human FOXO3 locus: a study of premature ovarian failure and primary amenorrhea. *Hum Reprod* 2008;**1**:216–221.
- Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW. et al. Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nat Genet* 2000;**3**:279–283.
- Goldenberg RL, Powell RD, Rosen SW, Marshall JR, Ross GT. Ovarian morphology in women with anosmia and hypogonadotropic hypogonadism. *Am J Obstet Gynecol* 1976;**1**:91–94.
- Guo Q, Kumar TR, Woodruff T, Hadsell LA, DeMayo FJ, Matzuk MM. Overexpression of mouse follistatin causes reproductive defects in transgenic mice. *Mol Endocrinol* 1998;**1**:96–106.
- Hanrahan JP, Gregan SM, Mulsant P, Mullen M, Davis GH, Powell R, Galloway SM. Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (Ovis aries). *Biol Reprod* 2004;**4**:900–909.
- Hayashi M, McGee EA, Min G, Klein C, Rose UM, van Duin M, Hsueh AJ. Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles. *Endocrinology* 1999;**3**:1236–1244.
- Hreinsson JG, Scott JE, Rasmussen C, Swahn ML, Hsueh AJ, Hovatta O. Growth differentiation factor-9 promotes the growth, development, and survival of human ovarian follicles in organ culture. *J Clin Endocrinol Metab* 2002;**1**:316–321.
- Hsueh AJ, Dahl KD, Vaughan J, Tucker E, Rivier J, Bardin CW, Vale W. Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. *Proc Natl Acad Sci USA* 1987;**14**:5082–5086.
- Huang EJ, Manova K, Packer AI, Sanchez S, Bachvarova RF, Besmer P. The murine steel panda mutation affects kit ligand expression and growth of early ovarian follicles. *Dev Biol* 1993;**1**:100–109.
- Hudson PL, Douglas I, Donahoe PK, Cate RL, Epstein J, Pepinsky RB, MacLaughlin DT. An immunoassay to detect human müllerian inhibiting substance in males and females during normal development. *J Clin Endocrinol Metab* 1990;**1**:16–22.
- John GB, Gallardo TD, Shirley LJ, Castrillon DH. Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth. *Dev Biol* 2008;**1**:197–204.
- Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL. Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature* 2004;**6979**:145–150.
- Johnson J, Bagley J, Skaznik-Wikiel M, Lee HJ, Adams GB, Niikura Y, Tschudy KS, Tilly JC, Cortes ML, Forkert R. et al. Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. *Cell* 2005;**2**:303–315.

- Juengel JL, McNatty KP. The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development. *Hum Reprod Update* 2005;**2**:143–160.
- Juengel JL, Hudson NL, Heath DA, Smith P, Reader KL, Lawrence SB, O'Connell AR, Laitinen MP, Cranfield M, Groome NP. et al. Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular development in sheep. *Biol Reprod* 2002;**6**:1777–1789.
- Juneja SC, Barr KJ, Enders GC, Kidder GM. Defects in the germ line and gonads of mice lacking connexin43. *Biol Reprod* 1999;**5**:1263–1270.
- Kezele PR, Nilsson EE, Skinner MK. Insulin but not insulin-like growth factor-I promotes the primordial to primary follicle transition. *Mol Cell Endocrinol* 2002;**1**–2:37–43.
- Kezele P, Nilsson EE, Skinner MK. Keratinocyte growth factor acts as a mesenchymal factor that promotes ovarian primordial to primary follicle transition. *Biol Reprod* 2005;**5**:967–973.
- Knight PG, Glistler C. TGF-beta superfamily members and ovarian follicle development. *Reproduction* 2006;**2**:191–206.
- Lange UC, Saitou M, Western PS, Barton SC, Surani MA. The fragilis interferon-inducible gene family of transmembrane proteins is associated with germ cell specification in mice. *BMC Dev Biol* 2003;**3**:1.
- Lawson KA, Dunn NR, Roelen BA, Zeinstra LM, Davis AM, Wright CV, Korving JP, Hogan BL. Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev* 1999;**4**:424–436.
- Lee WS, Otsuka F, Moore RK, Shimasaki S. Effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat. *Biol Reprod* 2001;**4**:994–999.
- Lee WS, Yoon SJ, Yoon TK, Cha KY, Lee SH, Shimasaki S, Lee S, Lee KA. Effects of bone morphogenetic protein-7 (BMP-7) on primordial follicular growth in the mouse ovary. *Mol Reprod Dev* 2004;**2**:159–163.
- Lee HJ, Selesniemi K, Niikura Y, Niikura T, Klein R, Dombkowski DM, Tilly JL. Bone marrow transplantation generates immature oocytes and rescues long-term fertility in a preclinical mouse model of chemotherapy-induced premature ovarian failure. *J Clin Oncol* 2007;**22**:3198–3204.
- Liang L, Soyal SM, Dean J. FGFalpha, a germ cell specific transcription factor involved in the coordinate expression of the zona pellucida genes. *Development* 1997;**24**:4939–4947.
- Liaw D, Marsh DJ, Li J, Dahia PL, Wang SI, Zheng Z, Bose S, Call KM, Tsou HC, Peacocke M. et al. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat Genet* 1997;**1**:64–67.
- Liu X, Andoh K, Yokota H, Kobayashi J, Abe Y, Yamada K, Mizunuma H, Ibuki Y. Effects of growth hormone, activin, and follistatin on the development of preantral follicle from immature female mice. *Endocrinology* 1998;**5**:2342–2347.
- Liu X, Andoh K, Abe Y, Kobayashi J, Yamada K, Mizunuma H, Ibuki Y. A comparative study on transforming growth factor-beta and activin A for preantral follicles from adult, immature, and diethylstilbestrol-primed immature mice. *Endocrinology* 1999;**6**:2480–2485.
- Luoh SW, Bain PA, Polakiewicz RD, Goodheart ML, Gardner H, Jaenisch R, Page DC. Zfx mutation results in small animal size and reduced germ cell number in male and female mice. *Development* 1997;**11**:2275–2284.
- Maheshwari A, Fowler PA. Primordial follicular assembly in humans—revisited. *Zygote* 2008;**4**:285–296.
- Manova K, Huang EJ, Angeles M, De Leon V, Sanchez S, Pronovost SM, Besmer P, Bachvarova RF. The expression pattern of the c-kit ligand in gonads of mice supports a role for the c-kit receptor in oocyte growth and in proliferation of spermatogonia. *Dev Biol* 1993;**1**:85–99.
- Matzuk MM, Kumar TR, Bradley A. Different phenotypes for mice deficient in either activins or activin receptor type II. *Nature* 1995;**6520**:356–360.
- Mc KD, Hertig AT, Adams EC, Danziger S. Histochemical observations on the germ cells of human embryos. *Anat Rec* 1953;**2**:201–219.
- McGee EA, Hsueh AJ. Initial and cyclic recruitment of ovarian follicles. *Endocr Rev* 2000;**2**:200–214.
- McGee E, Spears N, Minami S, Hsu SY, Chun SY, Billig H, Hsueh AJ. Preantral ovarian follicles in serum-free culture: suppression of apoptosis after activation of the cyclic guanosine 3',5'-monophosphate pathway and stimulation of growth and differentiation by follicle-stimulating hormone. *Endocrinology* 1997;**6**:2417–2424.
- Merchant-Larios H, Centeno B. Morphogenesis of the ovary from the sterile W/Wv mouse. *Prog Clin Biol Res* 1981;**59B**:383–392.
- Mizunuma H, Liu X, Andoh K, Abe Y, Kobayashi J, Yamada K, Yokota H, Ibuki Y, Hasegawa Y. Activin from secondary follicles causes small preantral follicles to remain dormant at the resting stage. *Endocrinology* 1999;**1**:37–42.
- Nilsson EE, Skinner MK. Growth and differentiation factor-9 stimulates progression of early primary but not primordial rat ovarian follicle development. *Biol Reprod* 2002;**3**:1018–1024.
- Nilsson EE, Skinner MK. Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. *Biol Reprod* 2003;**4**:1265–1272.
- Nilsson EE, Skinner MK. Kit ligand and basic fibroblast growth factor interactions in the induction of ovarian primordial to primary follicle transition. *Mol Cell Endocrinol* 2004;**1**–2:19–25.
- Nilsson EE, Kezele P, Skinner MK. Leukemia inhibitory factor (LIF) promotes the primordial to primary follicle transition in rat ovaries. *Mol Cell Endocrinol* 2002;**1**–2:65–73.
- O WS, Robertson DM, de Kretser DM. Inhibin as an oocyte meiotic inhibitor. *Mol Cell Endocrinol* 1989;**2**:307–311.
- O'Brien MJ, Pendola JK, Eppig JJ. A revised protocol for *in vitro* development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biol Reprod* 2003;**5**:1682–1686.
- Ohinata Y, Payer B, O'Carroll D, Ancelin K, Ono Y, Sano M, Barton SC, Obukhanych T, Nussenzweig M, Tarakhovsky A. et al. Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature* 2005;**7048**:207–213.
- Ohinata Y, Ohta H, Shigeta M, Yamanaka K, Wakayama T, Saitou M. A signaling principle for the specification of the germ cell lineage in mice. *Cell* 2009;**3**:571–584.
- Oktay K, Briggs D, Gosden RG. Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles. *J Clin Endocrinol Metab* 1997;**11**:3748–3751.
- Oktay K, Newton H, Mullan J, Gosden RG. Development of human primordial follicles to antral stages in SCID/hpg mice stimulated with follicle stimulating hormone. *Hum Reprod* 1998;**5**:1133–1138.
- Oktay K, Karlikaya G, Akman O, Ojakian GK, Oktay M. Interaction of extracellular matrix and activin-A in the initiation of follicle growth in the mouse ovary. *Biol Reprod* 2000;**2**:457–461.
- Oktem O, Oktay K. Quantitative assessment of the impact of chemotherapy on ovarian follicle reserve and stromal function. *Cancer* 2007a;**10**:2222–2229.
- Oktem O, Oktay K. The role of extracellular matrix and activin-A in *in vitro* growth and survival of murine preantral follicles. *Reprod Sci* 2007b;**4**:358–366.
- Oktem O, Oktay K. The ovary: anatomy and function throughout human life. *Ann NY Acad Sci* 2008;**1127**:1–9.
- Otsuka F, Yao Z, Lee T, Yamamoto S, Erickson GF, Shimasaki S. Bone morphogenetic protein-15. Identification of target cells and biological functions. *J Biol Chem* 2000;**50**:39523–39528.

- Otsuka F, Moore RK, Iemura S, Ueno N, Shimasaki S. Follistatin inhibits the function of the oocyte-derived factor BMP-15. *Biochem Biophys Res Commun* 2001;**5**:961–966.
- Plug AW, Peters AH, Xu Y, Keegan KS, Hoekstra MF, Baltimore D, de Boer P, Ashley T. ATM and RPA in meiotic chromosome synapsis and recombination. *Nat Genet* 1997;**4**:457–461.
- Rajareddy S, Reddy P, Du C, Liu L, Jagarlamudi K, Tang W, Shen Y, Berthet C, Peng SL, Kaldis P. et al. p27kip1 (cyclin-dependent kinase inhibitor 1B) controls ovarian development by suppressing follicle endowment and activation and promoting follicle atresia in mice. *Mol Endocrinol* 2007;**9**:2189–2202.
- Rajkovic A, Pangas SA, Ballow D, Suzumori N, Matzuk MM. NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression. *Science* 2004;**5687**:1157–1159.
- Rajpert-De Meyts E, Jorgensen N, Graem N, Muller J, Cate RL, Skakkebaek NE. Expression of anti-Mullerian hormone during normal and pathological gonadal development: association with differentiation of Sertoli and granulosa cells. *J Clin Endocrinol Metab* 1999;**10**:3836–3844.
- Rankin T, Familiar M, Lee E, Ginsberg A, Dwyer N, Blanchette-Mackie J, Drago J, Westphal H, Dean J. Mice homozygous for an insertional mutation in the Zp3 gene lack a zona pellucida and are infertile. *Development* 1996;**9**:2903–2910.
- Reddy P, Liu L, Adhikari D, Jagarlamudi K, Rajareddy S, Shen Y, Du C, Tang W, Hamalainen T, Peng SL. et al. Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool. *Science* 2008;**5863**:611–613.
- Roy SK, Kole AR. Ovarian transforming growth factor-beta (TGF-beta) receptors: *in vitro* effects of follicle stimulating hormone, epidermal growth factor and TGF-beta on receptor expression in human preantral follicles. *Mol Hum Reprod* 1998;**3**:207–214.
- Ruggiu M, Speed R, Taggart M, McKay SJ, Kilanowski F, Saunders P, Dorin J, Cooke HJ. The mouse Dazl gene encodes a cytoplasmic protein essential for gametogenesis. *Nature* 1997;**6646**:73–77.
- Sadatsuki M, Tsutsumi O, Yamada R, Muramatsu M, Taketani Y. Local regulatory effects of activin A and follistatin on meiotic maturation of rat oocytes. *Biochem Biophys Res Commun* 1993;**1**:388–395.
- Saitou M, Barton SC, Surani MA. A molecular programme for the specification of germ cell fate in mice. *Nature* 2002;**6895**:293–300.
- Saraguetta PE, Lanuza GM, Baranao JL. Autocrine role of transforming growth factor beta1 on rat granulosa cell proliferation. *Biol Reprod* 2002;**6**:1862–1868.
- Schmidt D, Ovitt CE, Anlag K, Fehsenfeld S, Gredsted L, Treier AC, Treier M. The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. *Development* 2004;**4**:933–942.
- Shimasaki S, Zachow RJ, Li D, Kim H, Iemura S, Ueno N, Sampath K, Chang RJ, Erickson GF. A functional bone morphogenetic protein system in the ovary. *Proc Natl Acad Sci USA* 1999;**13**:7282–7287.
- Shimasaki S, Moore RK, Otsuka F, Erickson GF. The bone morphogenetic protein system in mammalian reproduction. *Endocr Rev* 2004;**1**:72–101.
- Silva CC, Groome NP, Knight PG. Demonstration of a suppressive effect of inhibin alpha-subunit on the developmental competence of *in vitro* matured bovine oocytes. *J Reprod Fertil* 1999;**2**:381–388.
- Simon AM, Goodenough DA, Li E, Paul DL. Female infertility in mice lacking connexin 37. *Nature* 1997;**6616**:525–529.
- Skinner MK. Regulation of primordial follicle assembly and development. *Hum Reprod Update* 2005;**5**:461–471.
- Smitz J, Cortvrindt R, Hu Y, Vanderstichele H. Effects of recombinant activin A on *in vitro* culture of mouse preantral follicles. *Mol Reprod Dev* 1998;**3**:294–304.
- Solloway MJ, Dudley AT, Bikoff EK, Lyons KM, Hogan BL, Robertson EJ. Mice lacking Bmp6 function. *Dev Genet* 1998;**4**:321–339.
- Soyal SM, Amleh A, Dean J. FGFalpha, a germ cell-specific transcription factor required for ovarian follicle formation. *Development* 2000;**21**:4645–4654.
- Tremblay KD, Dunn NR, Robertson EJ. Mouse embryos lacking Smad1 signals display defects in extra-embryonic tissues and germ cell formation. *Development* 2001;**18**:3609–3621.
- Trombly DJ, Woodruff TK, Mayo KE. Suppression of Notch signaling in the neonatal mouse ovary decreases primordial follicle formation. *Endocrinology* 2009;**2**:1014–1024.
- Tsuda M, Sasaoka Y, Kiso M, Abe K, Haraguchi S, Kobayashi S, Saga Y. Conserved role of nanos proteins in germ cell development. *Science* 2003;**5637**:1239–1241.
- van Rooij IA, Broekmans FJ, te Velde ER, Fauser BC, Bancsi LF, de Jong FH, Themmen AP. Serum anti-Mullerian hormone levels: a novel measure of ovarian reserve. *Hum Reprod* 2002;**12**:3065–3071.
- Visser JA, Themmen AP. Anti-Mullerian hormone and folliculogenesis. *Mol Cell Endocrinol* 2005;**1**:2:81–86.
- Vitt UA, McGee EA, Hayashi M, Hsueh AJ. *In vivo* treatment with GDF-9 stimulates primordial and primary follicle progression and theca cell marker CYP17 in ovaries of immature rats. *Endocrinology* 2000;**10**:3814–3820.
- Waldeyer W. *Eierstock und Ei* Engleman. Leipzig, 1870.
- Wang J, Roy SK. Growth differentiation factor-9 and stem cell factor promote primordial follicle formation in the hamster: modulation by follicle-stimulating hormone. *Biol Reprod* 2004;**3**:577–585.
- Weenen C, Laven JS, Von Bergh AR, Cranfield M, Groome NP, Visser JA, Kramer P, Fauser BC, Themmen AP. Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. *Mol Hum Reprod* 2004;**2**:77–83.
- Xiao S, Robertson DM, Findlay JK. Effects of activin and follicle-stimulating hormone (FSH)-suppressing protein/follistatin on FSH receptors and differentiation of cultured rat granulosa cells. *Endocrinology* 1992;**3**:1009–1016.
- Yamaji M, Seki Y, Kurimoto K, Yabuta Y, Yuasa M, Shigeta M, Yamanaka K, Ohinata Y, Saitou M. Critical function of Prdm14 for the establishment of the germ cell lineage in mice. *Nat Genet* 2008;**8**:1016–1022.
- Yamoto M, Minami S, Nakano R, Kobayashi M. Immunohistochemical localization of inhibin/activin subunits in human ovarian follicles during the menstrual cycle. *J Clin Endocrinol Metab* 1992;**5**:989–993.
- Yan C, Wang P, DeMayo J, DeMayo FJ, Elvin JA, Carino C, Prasad SV, Skinner SS, Dunbar BS, Dube JL. et al. Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Mol Endocrinol* 2001;**6**:854–866.
- Ying Y, Zhao GQ. Cooperation of endoderm-derived BMP2 and extraembryonic ectoderm-derived BMP4 in primordial germ cell generation in the mouse. *Dev Biol* 2001;**2**:484–492.
- Ying Y, Qi X, Zhao GQ. Induction of primordial germ cells from murine epiblasts by synergistic action of BMP4 and BMP8B signaling pathways. *Proc Natl Acad Sci USA* 2001;**14**:7858–7862.
- Zhao J, Taverne MA, van der Weijden GC, Bevers MM, van den Hurk R. Effect of activin A on *in vitro* development of rat preantral follicles and localization of activin A and activin receptor II. *Biol Reprod* 2001;**3**:967–977.
- Zou K, Yuan Z, Yang Z, Luo H, Sun K, Zhou L, Xiang J, Shi L, Yu Q, Zhang Y. et al. Production of offspring from a germline stem cell line derived from neonatal ovaries. *Nat Cell Biol* 2009;**5**:631–636.
- Zuckerman S. The number of oocytes in the mature ovary. *Recent Prog Horm Res* 1951;**6**:63–109.