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REVIEW

# Mechanisms controlling germline cyst breakdown and primordial follicle formation

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**Abstract** In fetal females, oogonia proliferate immediately after sex determination. The progress of mitosis in oogonia proceeds so rapidly that the incompletely divided cytoplasm of the sister cells forms cysts. The oogonia will then initiate meiosis and arrest at the diplotene stage of meiosis I, becoming oocytes. Within each germline cyst, oocytes with Balbiani bodies will survive after cyst breakdown (CBD). After CBD, each oocyte is enclosed by pregranulosa cells to form a primordial follicle (PF). Notably, the PF pool formed perinatally will be the sole lifelong oocyte source of a female. Thus, elucidating the mechanisms of CBD and PF formation is not only meaningful for solving mysteries related to ovarian development but also contributes to the preservation of reproduction. However, the mechanisms that regulate these phenomena are largely unknown. This review summarizes the progress of cellular and molecular research on these processes in mice and humans.

**Keywords** Preservation of fertility · Premature ovarian failure · Apoptosis · Autophagy · Reconstituted follicle

**Abbreviations**

| | |
|---|---|
| PF | Primordial follicle |
| CBD | Cyst breakdown |
| POI | Primary ovarian insufficiency |
| POF | Premature ovarian failure |
| MOF | Multi-oocyte follicle |
| JNK | c-Jun amino-terminal kinase |
| mTORC1 | Mechanistic target of rapamycin complex 1 |
| PI3K | Phosphatidyl inositol 3-kinase |
| PGCs | Primordial germ cells |
| Dpc | Day post coitus |
| Wpc | Week post conception |
| FOXL2 | Forkhead box L2 |
| WNT4 | Canonical wingless-type MMTV integration site family member 4 |
| RA | Retinoic acid |
| RARs | RA receptors |
| RXRs | Retinoid X receptors |
| RALDHs | Retinaldehyde dehydrogenases |
| Act | Activin |
| TGF | Transforming growth factor |
| DSBs | DNA double-strand breaks |
| PAR6 | Partitioning-defective Protein 6 |
| AJs | Adherens junctions |
| Cads | Cadherins |
| GJs | Gap junctions |
| NICD | Notch intercellular domain |
| ADAM10 | A disintegrin and metalloproteinase domain10 |
| Bax | Bcl2-associated X protein |
| Casp2 | Caspase 2 |
| FSH | Follicle-stimulating hormone |
| PCD | Programmed cell death |
| SOHLH2 | Spermatogenesis- and oogenesis-specific helix-loop-helix transcription factor 2 |
| AQP8 | Water channel aquaporin-8 |
| Fst | Follistatin |
| Inh | Inhibin |
| LGR5 | Leucine-rich repeat-containing G-protein-coupled receptor 5 |
| Bcl-2 | B-cell lymphoma 2 |

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## Preface

In mammals, a fixed population of primordial follicles (PFs) is generally established in the ovaries during early life that serves as the sole source of developing follicles and oocytes throughout reproductive life [1, 2]. Despite substantial prolongation of female life expectancy during the past century, the female reproductive lifespan remains approximately 51 years. The physiological reason for this limit is the gradual and irreversible exhaustion of the PF pool and decreased egg quality with increasing age. In general, because of the adverse effects of environmental, genetic or other unknown factors, the PF pool shrinks steadily, thus shortening the reproductive lifespan [3]. Primary ovarian insufficiency (POI), the clinical term for premature ovarian failure (POF), which is characterized by the disappearance of menstrual cycles associated with premature follicular depletion, is a condition affecting at least 1% of women under the age of 40 years worldwide [4, 5]. Most recent reports show that upon mutation, many genes responsible for the formation of PFs are also associated with POI [6, 7]. Therefore, clarifying the mechanisms underlying the PF reserve is one of the most important topics in female reproductive research. Alternatively, because cancer therapy can adversely affect germ cell survival and cause POF and infertility [8], the requirement for fertility preservation in young cancer patients has increased significantly since the beginning of this century, although a better prognosis has been achieved after cancer therapy [9]. Clinically, cryopreservation of ovarian tissue before cancer therapy is becoming one of the most effective ways to preserve fertility [10, 11]. Nearly, 80% of human PFs were shown to survive after ovarian tissue cryopreservation using the slow-freezing method in the 1990s [12, 13], although this method is not currently the most effective.

Excitingly, with the progress of research on the molecular mechanisms of PF activation, in vitro activation of PFs has become feasible. Mammalian target of rapamycin complex 1 (mTORC1) signaling in the granulosa cells of PFs is responsible for secreting the Kit Ligand (KITL) to activate phosphatidyl inositol 3-kinase (PI3K) within oocytes, which in turn activates PF development in neonatal mouse ovaries and human ovarian cortical tissue in vitro [14–17]. This novel in vitro activation approach has been successfully used in human POF patients, and one healthy baby has been delivered in 2013 [18].

Applying pluripotent stem cells to reproduce functional gametogenesis in vitro is currently one of the key goals in developmental and reproductive biology. Physiologically, functional oocytes derive from intact follicles, implying that the communication between germ cells and ovarian somatic cells within follicles is pivotal for full oocyte development. However, the mechanisms underlying this process are not fully elucidated. Thus, the identification of effective ways to preserve PFs to the greatest extent may not only ensure the preservation of fertility for cancer patients in the future, but may also help to postpone ovarian aging in healthy women. Understanding the formation mechanism of PFs will become the basis for better controlling the in vitro reconstitution of oogenesis, cryopreservation, and the activation of PFs within ovarian tissues. This review aims to summarize the recent progress regarding PF formation in mice and humans to provide a better understanding of the mechanisms controlling female fertility reserves.

## Asynchronous PF formation

In mice, immediately after the primordial germ cells (PGCs) migrate to the gonad at 10.5 days post coitus (10.5 dpc) during embryonic development, they form into cysts (multi-nucleated syncytial clones) by rapidly dividing until 13.5 dpc. In humans, germ cell mitosis begins at 5 week post conception (5 wpc) and ends at 10–16 wpc [19–22]. The number of germ cell divisions in *Drosophila* is markedly different from that in mice. In *Drosophila*, germ cells undergo four rounds of mitosis, whereas the number of divisions is variable in mice [23]. Unlike spermatogonia, which initiate meiosis postnatally, the oogonia (now referred to as oocytes) within cysts initiate meiosis at approximately 13.5 dpc in mice and 13 wpc in humans [20, 24].

In mammals, the initiation of meiosis is strikingly asynchronous both temporally and spatially [6]. First, along with the increasing number of germ cells initiating meiosis, some oogonia still express stem cell markers and continue to proliferate until at least 16 wpc in humans [21, 25]. Second, this asynchronous development reflects the initiation of meiosis via anterior-to-posterior patterns along the axis of the ovary beginning at 12.5 dpc in the mouse [26, 27]. Third, this asynchronous development is also reflected in the depth of maturation of both germ cells and pre-granulosa cells in the cortical and medullar regions of the ovary. The onset of programmed germ cell cyst breakdown (CBD) starts in the medullary (dorsal) region and moves to the cortical (ventral) side; that is, CBD starts in the medullary region prior to the time of birth and expands toward the cortical surface region of the ovaries in humans and rodents [28–30]. Fewer mature germ cells are located at the periphery of the ovary, with progressively more mature germ cells being found in the medulla area of fetal ovarian tissue [31].

Accordingly, asynchrony also occurs in ovarian somatic cell development. Forkhead box L2 (FOXL2)-positive pre-granulosa cells undergo two waves of differentiation that contribute to two discrete populations

of follicles [32]. The source of both populations of pre-granulosa cells is the ovarian surface epithelium. The first wave of differentiation supports bipotential somatic cell differentiation, which is involved in forming the earliest PFs that develop within the medulla region. Consequently, such PFs undergo rapid maturation and die. The second wave of differentiation occurs at the ovarian cortex, where PFs are formed and arrested until puberty and beyond [32]. Additional studies support the conclusion that there are two waves of perinatal PF formation in the rodent and human ovary [17, 33–36]. Based on a visual in vivo follicle tracing system, Zheng et al. demonstrated that the fates of the two classes of PFs follow distinct, age-dependent developmental paths, and play different roles throughout the reproductive lifespan [37]. Pre-granulosa cells recruited in fetal mouse ovaries form the initial PFs in the medullary region. Once formed, these PFs are synchronously activated and become the first wave of follicles activated after birth [38, 39], which contributes to the onset of puberty and to fertility until 3 months of age. Pre-granulosa cells recruited in postnatal mouse ovaries form PFs in the cortical region, which are gradually activated as a means of providing mature oocytes over the entire course of the reproductive life of the animal [32, 38, 40, 41]. As a result, PFs from the second wave gradually replace those from the first until they become the sole source of follicles from 3 months onward [37]. The spatiotemporal patterns of major cellular events that occur during CBD and PF formation in the mouse ovary are shown in Fig. 1.

## Prerequisite cellular events for the establishment of PFs

In mammals, PF formation consists of a series of cellular events, including the formation of germline cysts through oogonia mitosis, meiosis initiation, CBD, and PF assembly when germ cells are arrested at the dictyate stage of meiosis I. The entire process involves at least two cell types: the germ cells within the cyst and the surrounding pre-granulosa cells [42]. Studies have demonstrated that timely, synchronized development of ovarian somatic cells and oocytes as well as their mutual communication determine PF formation and oocyte survival in mice [43, 44]. Communication occurs not only between ovarian somatic cells and oocytes but also between the somatic cells themselves [45, 46]. Accordingly, the basic criteria for the formation of PFs in mouse include two prerequisites: sufficient numbers of squamous pre-granulosa cells that express the FOXL2 protein and a readily available oocyte arrested at the diplotene stage [32, 47–53].

## Pre-granulosa cell recruitment and differentiation

Ovarian differentiation is a coordinate event, possibly driven by secreted factors, including canonical wingless-type MMTV integration site family member 4 (WNT4) and Rspondin 1 [RSPO1, an LGR5 (Leucine Rich Repeat Containing G Protein-Coupled Receptor 5) receptor ligand], transforming growth factor (TGF) beta superfamily-binding proteins, such as Follistatin (Fst), and transcriptional regulators, such as β-Catenin and FOXL2 [54]. FOXL2 is a winged helix/forkhead domain transcription factor that is extensively expressed within somatic cells from as early as 12.5 dpc until PF formation in the mouse. It participates in various molecular events in fetal ovary development [55, 56]. FOXL2 is considered as one of the most important agents during granulosa cell differentiation and ovary maintenance [52, 55–57]. The first hint that FOXL2 was involved in ovarian development came from an analysis in 2001 of human patients suffering from eyelid malformation and POF, which was found to be due to a mutation of the *Foxl2* gene [58]. In the mouse, *Foxl2* deficiency impairs CBD by affecting granulosa cell differentiation and proper formation of the basal lamina around forming follicles [49].

Physiologically, at 17.5 dpc, only FOXL2-positive pre-granulosa cells start to invade cysts and separate germ cells to form the PF structure [30]. However, the development of pre-granulosa cells is complex. A series of studies have demonstrated that the ovarian surface epithelium, which is LGR5 positive, may be a major source of pre-granulosa cells [32, 59, 60]. Activated WNT4 and RSPO1 are pivotal for ovarian pre-granulosa cell differentiation [61, 62]. LGR5 not only enhances WNT/RSPO1/β-Catenin signaling pathways in various morphogenetic processes, and it is also a marker of stem cells in the ovarian surface epithelium [60, 63]. In general, LGR5-positive cells are restricted to the cortical region of ovaries from 12.5 dpc to perinatal stages. Based on the specific expression patterns of *Lgr5* and *Foxl2*, it has been confirmed that LGR5-positive cells are recruited to differentiate into FOXL2-positive pre-granulosa cells, starting at the initiation of folliculogenesis (approximately 1 dpp) [60, 64–66]. Accordingly, FOXL2-positive cells first emerge in the medulla and then gradually emerge in the cortical region of the ovary (Fig. 1) from 16.5 dpc to 4 dpp in mice [30].

## Oocyte meiosis

In mice, meiosis in the germ cells starts at approximately 13.5 dpc and progresses from leptotene to diplotene stages until arrest at 18.5 dpc. Retinoic acid (RA) derived from the somatic environment of either mesonephroi or the fetal ovary is the key signal for the induction

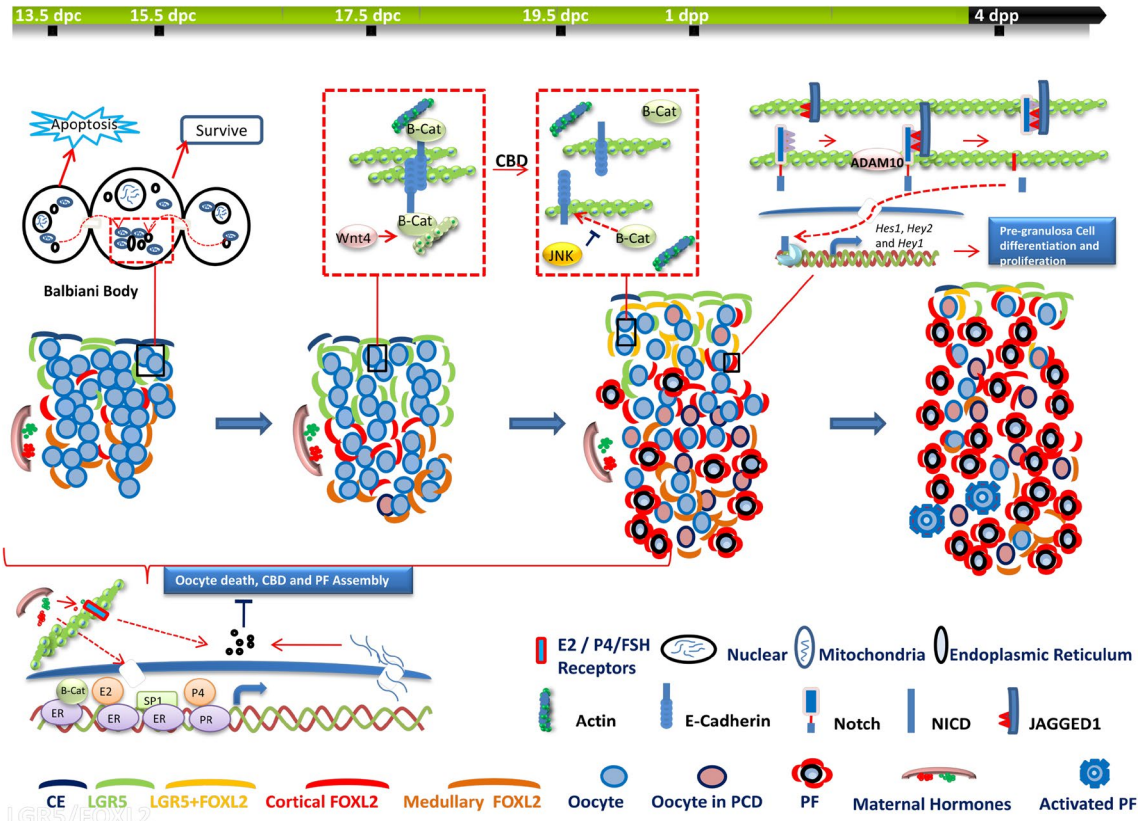


**Fig. 1** Schematic representation showing the spatiotemporal patterns of major cellular events during cyst breakdown (CBD) and primordial follicle (PF) formation in the mouse ovary. **a** After cyst formation, the oocyte with an established Balbiani body in its cytoplasm will survive, whereas the nurse cells surrounding the oocyte may subsequently undergo apoptosis. **b** Adherens junctions (AJs) between oocytes are sustained by the Wnt4/β-Catenin (β-Cat) signaling pathway before CBD and are disassembled via c-Jun amino-terminal kinase (JNK) signaling after CBD. **c** Before PF assembly, oocyte-derived factors, such as Jagged 1, induce Notch signaling activation in pre-granulosa cells to promote their differentiation and proliferation. After Jagged1 binds to the extracellular domain of Notch, the proteolytic cleavage of Notch2 is mediated by a disintegrin and metalloproteinase domain 10 (ADAM10). After cleavage, the Notch intercellular domain (NICD) is released and translocates to the nucleus to interact with the transcriptional complex to activate target genes. **d** Maternal hormones, including estrogen (E2), progesterone (P4), and follicle-stimulating hormone (FSH), exert their inhibitory actions on oocyte apoptosis, CBD and PF formation through classical (nuclear receptor) or non-classical (membrane receptor) pathways. Forkhead box L2 (FOXL2) is one of the most important agents driving granulosa cell differentiation and ovary maintenance. Only FOXL2-positive pre-granulosa cells start to invade cysts and separate germ cells to form PF structures. The differentiation of FOXL2-positive pre-granulosa cells derives from G protein-coupled receptor (LGR5)-positive cells. LGR5-positive cells are restricted to the cortical region of the ovaries from 12.5 dpc to perinatal stages. FOXL2-positive cells first emerge in the medulla and then gradually emerge in the cortical region of the ovary from 16.5 dpc to 4 dpp in the mice

of meiosis in mice as well as humans [67–71]. RA is the active derivative of vitamin A, and participates in the regulation of embryonic development, cellular proliferation, and differentiation, as well as reproductive systems [71, 72]. By binding to intracellular RA receptors (RARs) and retinoid X receptors (RXRs), RA stimulates the expression of *Stra8* (stimulated by retinoic acid 8), which promotes both the replication of germ cell chromatin and the transition of oogonia to meiotic division [67, 68, 73, 74] (Fig. 2a). As a result, various signals that regulate the homeostasis between RA synthesis by retinaldehyde dehydrogenases (RALDHs) and RA degradation by CYP26 (CYP26A1, CYP26B1, and CYP26C1) control *Stra8* expression and the timing of meiosis initiation [75, 76] (Fig. 3a).

In this context, TGF beta superfamily-binding proteins have been demonstrated to participate in the initiation of oogonium meiosis. Activin A (Act A) and RA may cooperate in promoting meiosis in female germ cells [77] (Figs. 2, 3). In vitro, Act A accelerates the progression of oocytes throughout meiotic prophase I stages, which is related to increased expression of premeiotic and meiotic genes (including *Dazl, Spo11, Stra8, Scp3*, and *Rec8*) in ovarian tissues [77]. An earlier study also suggested that CYP26B1 is a novel target of Act [78], because Act A-dependent SMAD3 signaling downregulates the expression of

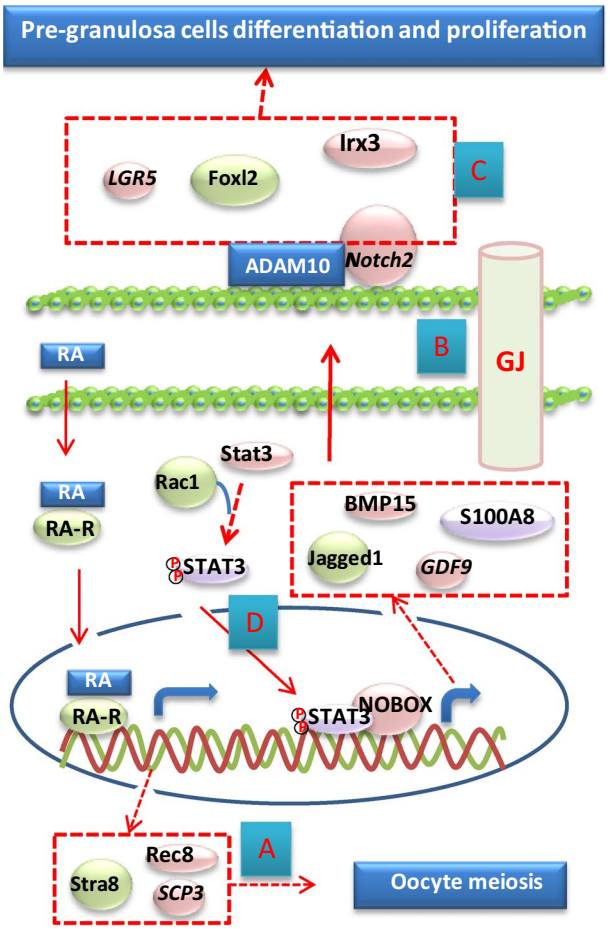


**Fig. 2** Molecular events in developing oocytes and pre-granulosa cells before PF formation. **a** Retinoic acid (RA) secreted from either ovarian somatic cells or mesonephric tissue is responsible for the initiation of meiosis in oocytes. **b** Gap junctions (GJs) established between oocytes and pre-granulosa cells may be more important for controlling the development of ovarian somatic cells, instead of oocytes, during CBD and PF assembly. **c** In the pre-granulosa cells, ADAM10 is responsible for the cleavage of the Notch receptor in both LGR5-positive and FOXL2-positive cells and thus controls the development of pre-granulosa cells in perinatal ovaries. **d** Small GTPase Rac1 may facilitate the import of STAT3 to the nucleus to activate the expression of oocyte-specific proteins that are secreted from the oocyte

*Cyp26b1* and upregulates the expression of *Rars* [67, 68, 78, 79].

In addition to RA and Act, WNT4 and Rspondin signals from ovarian somatic cells have been demonstrated to promote not only the meiotic initiation of female germ cells but also oogonial proliferation and survival [54, 80, 81] (Fig. 3b). Nuclear β-Catenin is involved in ovarian development through either RSPO1-mediated pathways or WNT4 signaling [82, 83], thereby inhibiting *Sox9* expression or promoting *Wnt4, Bmp2*, and *Fst* expression, respectively [54]. In somatic cells, RSPO1 promotes ovarian differentiation via the activation of WNT/β-Catenin signaling. In Rspo1-deficient mouse ovaries, germ cell proliferation, expression of Stra8, and entry into meiosis are all impaired [81].

Is oocyte entry into meiosis and arrest at the dictyate stage necessary for PF formation? The answer seems to be yes. Meiotic progression is not merely concomitant with CBD but also causative [84]. During meiosis, germ cells experience DNA double-strand breaks (DSBs) to undergo recombination, which potentiates genetic diversity in progeny. The extraordinary DSB tolerance of each oocyte could be one of the characteristics of oocytes in a meiotic state [85]. For instance, mutations in genes involved in the creation and repair of DSBs, such as MutS homolog genes (*MSHs*) and disrupted meiotic cDNA 1 (*DMC1*), negatively affect fertility and lead to POI [86, 87]. Premature loss of mouse germ cells is observed by 4 dpp in *Msh4*-deficient ovaries and by 2 months in *Msh5*-deficient ovaries [88, 89]. *Dmc1* deficiency causes a reduction in mouse follicle numbers and follicles, resulting from the failure of chromosome synapsis [90]. *Spo11* (*S. cerevisiae* homolog)-deficient female mice exhibit a smaller PF pool, showing evident germ cell defects by 15.5 dpc [91]. In the ovaries of *Stra8*-deficient mice, although some germ cells evidently survived embryonic development despite the meiotic initiation block at E13.5–E14.5, the attrition of the germ cells accelerates from the fetal to postnatal stages, and the ovary is devoid of germ cells by 6–8 weeks of age in this mouse model [92].

In addition, it has been demonstrated that PF formation is closely related to the progress of meiosis. SCP1 (synaptonemal complex protein-1) is expressed specifically in oocytes in females, and its expression declines precipitously within 24 h after birth when CBD begins. Furthermore, Paredes et al. have shown that loss of SCP1 in rat oocytes facilitates follicle assembly [93]. TAF4B, a gonad-enriched subunit of the TFIID complex is critical for oocyte and granulosa cell survival and PF formation in the mouse [94, 95]. TAF4B correlates with ovarian health and oocyte survival in women as well [96, 97]. In addition, *TAF4b*-deficient female mice are infertile and suffer from POF, which includes persistent estrous, elevated serum FSH levels, and reduced PF numbers [94, 95, 98]. Accordingly, the TAF4B protein has been demonstrated to be responsive to both estrogen and FSH receptors and acts to orchestrate the correct timing of germ cell CBD and PF pool establishment during a critical window of development [95, 99, 100]. Interestingly, TAF4B stimulates the expression of *c-Jun* in the granulosa cells of rats [101], whereas c-Jun Amino-terminal Kinase (JNK) is important for oocyte survival and CBD, which will be discussed later. Notably, Grive et al. demonstrated that PF assembly may not only follow the arrest of meiosis I progression but also be dependent upon the proper completion of these steps [95]. In accordance

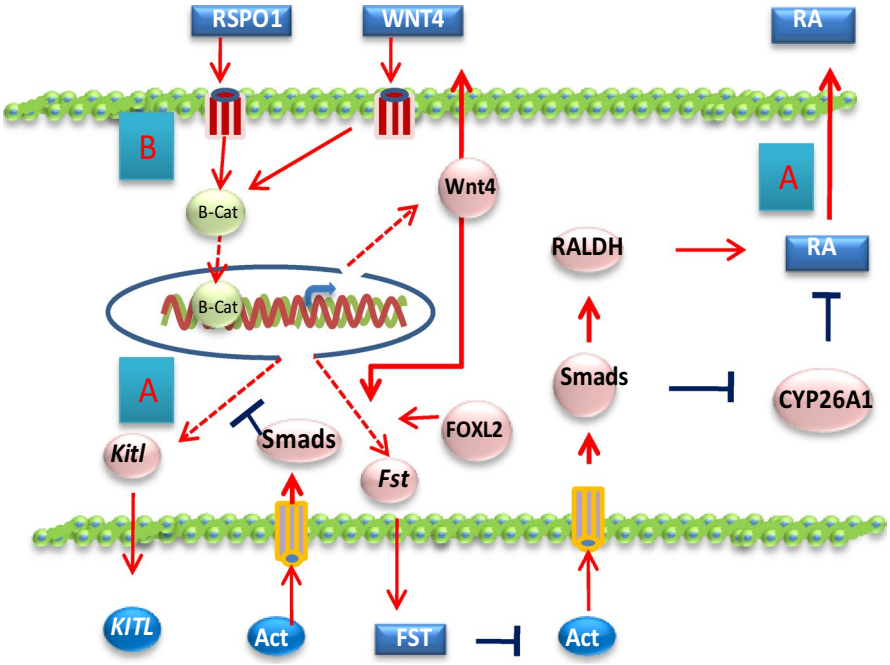


**Fig. 3** Actions of Act, WNT4, and RSPO1 in pre-granulosa cell development. **a** Oocyte-secreted Act acts on ovarian somatic cells to prevent *kitl* expression as well as regulates retinoic acid (RA) synthesis and degradation. **b** In pre-granulosa cells, WNT4 and RSPO1 signals activate the β-Catenin protein, which is responsible for the expression of many genes during CBD and PF formation, including *Kitl, Fst*, and *Wnt4*

with this hypothesis, the most recent study on this topic proposed that proper oocyte meiosis is essential for PF formation under physiological conditions. The meiotic arrest of mouse oocytes at the dictyate stage is indispensable for the formation of PFs [102].

## Germ cell cyst formation

The germ line cyst is a structure that is highly conserved among species. The peak number of cysts occurs at 14.5 dpc in mice and 20 wpc in humans, the point at which meiosis begins. At the end of mitosis, each cyst is composed of up to 30 germ cells (oogonia), which are connected via intercellular bridges due to incomplete cytokinesis in mice [103–106]. Contrary to the notion that intercellular bridges are likely restricted to cells of the same genotype [32], some of the growing cysts will break down into smaller cysts by 17.5 dpc and then associate with other unrelated cysts to form aggregated cysts prior to meiosis [33, 107–109]. Although the role of the cysts is still not well understood, it is clear that the structure of the cysts facilitates organelle exchange between germ cells. According to Lei's and Pepling's results, mouse oogonia develop into oocytes through both organelle enrichment from sister cyst (nurse-like) germ cells and Balbiani body establishment, whereas the other nurse-like germ cells die [106, 110] (Fig. 1a).

Cyst structure may contribute to the development of perfect oocytes with high quality, which is vital for PF formation [95, 106]. The Balbiani body (named after the nineteenth century Dutch microscopist), or mitochondrial cloud, in the cytoplasmic region near the nucleus is a large, distinctive organelle aggregate that has been found in the developing oocytes of many species, including humans [111, 112]. The advantage of the acquisition of additional organelles may be to ensure that oocytes grow into the largest cells in the mammalian body despite possessing four copies of the DNA complement. Although evidence is lacking, the Balbiani body may also serve a physiological secretory function in oocytes [106]. In addition, cytoplasmic RNA transfer between germ cells may enrich reprogramming factors and protective factors, such as piRNAs that repress transposition in the oocyte [113–115]. Alternatively, through intercellular cytoplasmic bridges, the oocyte nucleus may be relieved of metabolic and biosynthetic duties, which could either contribute to inactivation of the nucleus or reduce its susceptibility to mutagenesis [116].

Although it is difficult to anticipate which germ cell within the cyst will undergo apoptosis, or what kind of germ cells are selected to form into PFs, studies have provided some clues about the possible fate of the living germs cells that are doomed to perinatally form PFs. For example, the size of an oocyte that escapes apoptosis could be relatively larger than the surrounding nurse cells, although this size difference is difficult to identify based on appearance [106]. In relation to this possibility, the Balbiani body within the living oocyte could be a candidate biomarker, according to Pepling's research; the oocyte within a PF generally contains a Balbiani body [112]. Regarding molecular biomarkers, partitioning-defective protein 6 (PAR6), one of the proteins that is important for cell

polarity, could be a potential marker for identifying germ cells that will be selected to form PFs in the mouse ovary [117]. PAR6 plays a role in the asymmetric distribution of cytoplasmic determinants and the regulation of cytoskeleton positioning and asymmetric division [118]. In *Drosophila*, only the germ cell expressing *PARs* within a cyst becomes the oocyte, whereas the others undergo degeneration. Mutated *PAR* disrupts the early polarization of the oocyte and leads to failure to maintain its identity in both *C. elegans* and *Drosophila* [119–121]. In the mouse, from 17.5 dpc onward, PAR6 expression shifts from the ovarian somatic cells to the oocytes, and its expression level continues to rise in the nuclei of some oocytes at 19.5 dpc and in of all oocytes at 3 dpp. Importantly, the expression of *PAR6* decreases gradually after the PF pool is established. During PF pool establishment, the number of PAR6-positive germ cells remains steady and is consistent with the number of follicles formed at 3 dpp. Wen et al. suggested that the unknown signals inducing oocyte-expressing PAR6 may derive from somatic cells through gap junctions [117]. However, because detailed information is absent, substantial research will be needed to explain the mechanism of PAR6 involvement in PF formation via shifting expression from pre-granulosa cells to oocytes.

## Cell–cell adhesion during CBD and PF formation

Cell–cell adhesion is critical for various aspects of multicellular existence, such as morphogenesis, tissue integrity, and differentiation, including in the fetal ovary [105, 122]. Adherens junctions (AJs) are intercellular structures responsible for cell–cell adhesion mediated by Cadherins (Cads) through the calcium-dependent homophilic interaction of their extracellular domains. AJ formation is associated with cytoplasmic Catenin (α, β and p120) proteins, as the cytoplasmic tail of Cad itself exhibits no catalytic activity [123]. Therefore, any possible signaling for the activation of cell–cell adhesion must occur upon the recruitment of signaling molecules to the site of the Cad-Catenin complex [124].

During embryonic development, the dynamic cellular behaviors, such as rearrangement, movement, and shape changes, are regulated by AJs, which constitute a physical bridge between the Cad complex and the cortical actin filaments [125]. In the fetal ovary, elongated pre-granulosa cells extend between germ cells within the same cyst, often making direct contact with intercellular bridges, indicating that they are important for CBD and PF formation [23, 126].

A proper spatiotemporal expression of Cads is required for CBD [127]. Among the Cads, E-Cad was first determined to mediate AJ formation by recruiting PI3K-p85 to the cell membrane, which contributes to activation of the PI3K/AKT pathway and up-regulation of epithelial ovarian cancer growth [128, 129]. Physiologically, E-Cad is intensely expressed during the cyst period, but its expression decreases when CBD occurs in the fetal ovaries of mice [127, 130] and human [131] (Fig. 1b). Along with CBD and PF formation, a V-shaped E-Cad expression pattern is observed in mice. E-Cad is intensely expressed at oocyte–oocyte contact sites inside cysts in 17.5 dpc ovaries. Its expression soon decreases and diffuses at 19.5 dpc but rises again from 2 dpp until 4 dpp. Subsequently, E-Cad is widely expressed in oocytes as well as somatic cells [130]. In hamsters, blocking E-Cad accelerates CBD and PF formation, which is in consistent with the finding that overexpression of E-Cad suppresses CBD in the mouse fetal ovary [127, 130].

Gap junction (GJ) establishment is also essential for murine PF assembly (Fig. 2b). The GJ structure was first observed in fetal ovaries at 17.5 dpc [132]. Earlier studies indicated that GJs formed between pre-granulosa and germ cells and that they at least contained GJ1 (also known as Connexin 43) [132, 133]. According to the most recent report on this topic, GJ structures between the oocyte and surrounding pre-granulosa cells are formed at approximately 19.0 dpc, and communication between them is established from 19.5 dpc to 1 dpp [134]. Among the 20 *GJ*s (*Gja* through *Gjd*), 12 elevated genes belong to three distinct patterns that rise and fall from 15.5 dpc to 5 dpp, which imply that GJs are closely related to PF formation and that complex compensatory actions may occur between them [134, 135]. Consistent with this view, global inhibition of GJs not only prevented PF assembly in vitro in perinatal ovaries but also resulted in the failure of antral follicle development in kidney transplant cultures [134]. However, deficiency of either *Gja1* or *Gja4*, the two most essential *GJ*s for follicle growth [136, 137], did not affect PF formation [130, 138, 139]. After systematic inhibition of GJs, ovarian somatic cell-specific genes (such as *Notch2, Foxl2*, and *Irx3*) were found to be down-regulated, whereas oocyte-specific genes (such as *Ybx2, Nobox*, and *Sohlh1*) and the progress of oocyte meiosis were not, demonstrating that GJ communication may be more important for somatic cell differentiation than for oocyte development [134]. Because of the complexity of GJ genes, future studies should concentrate on the cell-type-dependent functions of each GJ during the transformation of oogonia into oocytes and PF formation [44, 60].

## Cross-talk between germ cells and pre-granulosa cells

Mutual communication between oocytes and surrounding pre-granulosa cells is pivotal for CBD and PF formation

[140–142]. The contact between germ cells and somatic cells is established around ring canals connecting two oocytes within the cyst as early as 13.5 dpc in the mouse ovary [110]. Disruption of either germ cell- or somatic cell-specific factors (*FIG1α* and *Wnt4*, respectively) leads to defects in follicle formation [143, 144]. Studies have shown that many molecules are involved in this process [105]. Unfortunately, it is still unclear how such factors participate in the reorganization of the two distinct cell types into one functional unit through cross-talk.

Notch signaling could be one of the most important pathways participating in folliculogenesis in the perinatal mouse ovary. Notch signaling involves four receptors, Notch1, 2, 3, and 4, and their ligands, Jagged1 and 2 and delta-like 1, 3, and 4 [145]. Jagged1 and Notch2 are expressed in the oocyte and pre-granulosa cells, respectively, from 0 to 6 dpp, suggesting that germ cell CBD is partially coordinated through cellular interactions via Notch signaling [146–150]. Immediately after Jagged1 binds to the extracellular domain of Notch, proteolytic cleavage of the Notch2 receptor begins [145, 150]. Following cleavage, the *Notch* intercellular domain (NICD) is released and translocates to the nucleus to interact with the transcriptional complex to activate target genes, such as *Hes1, Hey2*, and *Hey1* [151] (Figs. 1c, 2c). Thus, deficiency of Notch, Notch receptor modulator Lunatic fringe (*Lfng*) or *Hes1* results in subfertility and consequent multi-oocyte follicles (MOFs) [147–150, 152].

The regulatory roles of a disintegrin and metalloproteinase domain 10 (ADAM10), the main physiological α-secretase [153], are responsible for the cleavage of the Notch receptor in both LGR5-positive and FOXL2-positive cells, and hence control the development of pre-granulosa cells in perinatal ovaries [66, 154–157] (Fig. 1c). However, it is possible that not all the components of Notch signaling are involved in the regulation of CBD and PF formation, as Notch3, Notch4, and Delta-like 3 mutants are fertile, and Delta-like 1 and Delta-like 4 are not expressed in neonatal ovaries [105]. Although oocyte-specific factors (GDF9 and BMP15) have been demonstrated to regulate the translation of Notch2 via mTORC1 activation in pre-granulosa cells [157], there is a lack of detailed information about the exact action of Notch signaling. Specifically, conditional genetically modified animals will be necessary to evade the problems of embryonic lethality and complex complementary actions between Notch signaling that have occurred in the past studies [105].

Several studies have noted cross-talk between somatic cells and oocytes via the receptor tyrosine kinase c-kit (KIT), which together with the KITL, is important for the control of oocyte reawakening. Activation of KITL in granulosa cells passes signals to oocytes to stimulate PF activation [6, 158]. However, as KIT signaling occurs in the fetal ovary as early as 7.5 dpc and is continuously expressed during ovary development [159, 160], it must exhibit additional functions in fetal ovary development. Indeed, KIT signaling has been demonstrated to play an important role in perinatal oocyte CBD, the determination of oocyte numbers, and PF formation in mice and humans [161, 162]. The regulation of KITL may be related to Act, one of several identified TGFβ family members with major roles in folliculogenesis [163–166]. In humans, oocytes express Act A before CBD, which repress the expression of membrane-bound KITL (KITL2) in neighboring somatic cells, thus preventing the expression of *c-Kit* in oocytes, because KITL itself can induce the expression of *c-kit* in germ cells [84]. After CBD, along with the decrease in Act expression in the oocyte, KITL2 expression is increased, which in turn induces the expression of KIT and may subsequently promote PF assembly [161, 167]. Lower KIT levels are then proposed to block either meiotic arrest or PF activation [6, 84, 166].

JNK is involved in the regulation cell proliferation, migration, and apoptosis [167–169]. Studies have demonstrated that the JNK signaling pathway is one of the candidate pathways participating in CBD [170–172], possibly by regulating E-Cad junctions between oocytes in the mouse [130] (Fig. 1b). JNK specifically localizes to oocytes, and its activity is increased as CBD progresses. Interestingly, the action of JNK is inversely correlated with WNT4 expression in the perinatal ovary [130]. In *Drosophila* epithelial cells, activated JNK relates to E-Cad inactivation and loss of cell polarity [173, 174]. In *Xenopus*, JNK signaling antagonizes the canonical Wnt3a pathway by regulating β-Catenin transport [175]. JNK was recently found to regulate AJ formation, as the inhibition of JNK kinase activity promoted localization of the E-Cad/β-Catenin complex to cell–cell contact sites [176, 177]. In the mouse, when JNK signaling activity increases, the WNT4 level simultaneously decreases. In vitro, attenuation of JNK signaling leads to WNT4 upregulation, but knockdown of WNT4 does not significantly rescue JNK inhibition-induced CBD failure, as WNT4 regulates *E-Cad* expression, whereas JNK signaling does not. Therefore, the reciprocal change leads to E-cad junction disassembly and gradual germline CBD. JNK signaling is maintained at high levels until 4 dpp when the oocytes are released from the cysts completely and surrounded by pre-granulosa cells to form PFs [130]. Hence, the homeostasis between JNK and WNT4 activity is fine tuned for cyst structure maintenance and breakdown during the early stage of ovarian development.

The small GTPase family member Rac1 may also play an indispensable role in controlling PF formation in the mouse ovary (Fig. 2d). Rac1 acts as a molecular switch by cycling between an active GTP-bound state

and an inactive GDP-bound state. In its active state, Rac1 actively regulates cell shape, adhesion, movement, endocytosis, secretion, and growth [178, 179]. Rac1 is involved in meiotic spindle stability and anchoring in oocytes as well as embryo implantation and embryonic epithelial morphogenesis [180–183]. In the fetal mouse ovary, Rac1 is exclusively expressed in germ cells prior to follicle assembly and in the oocytes of PFs [157]. In vitro, disruption of Rac1 retards CBD, whereas Rac1 overexpression accelerates the formation of PFs. In vivo inhibition of Rac1 results in the formation of MOFs which are similar to those associated with *Gdf9* and *Bmp* mutations in mice [184]. Rac1 may induce the nuclear import of STAT3 through physical binding to activate the expression of oocyte-specific genes, including *Jagged1, GDF9, BMP15*, and *Nobox* [157, 185, 186], indicating that signals from the oocyte to the pre-granulosa cells are vital for CBD and PF formation [187]. The action of Rac1 in CBD and PF formation may also be related to E-Cad, as E-Cad can regulate the localization and function of Rho GTPases, including Rac and Cdc42, through a mechanism described as 'outside-in signaling' [181]. However, more animal models are needed to clearly elucidate the mechanism of Rac1 in this process.

S100A8, an oocyte-specific chemokine and calcium-binding S100 protein family member, has been demonstrated to direct the migration of ovarian somatic cells during mouse PF assembly [188]. Regarding the initial motivation for PF assembly, apoptosis within cysts is important for yielding isolated oocytes for PF formation, as demonstrated by the BCL2-associated X protein (Bax) mutant mouse model [106, 189]. However, studies of Bax and caspase 2 (Casp2) mutant mice indicate that the formation of PFs may be organized by some stimulus other than apoptosis, such as the migration or invasion of pre-granulosa cells from outside-to-inside the cysts to envelop the oocyte [190, 191]. In contrast to observations made in the 1990s showing that the oocyte itself is motile [192, 193], a recent in vitro study demonstrated that oocytes are functionally immotile during PF formation [188]. S100A8 is significantly expressed in the oocytes of cysts at 19.0 dpc, the point at which PF assembly is about to begin. In addition, S100A8 significantly promotes the number of migrating ovarian somatic cells in vitro, the majority of which are FOXL2-positive cells, implying that pre-granulosa cells are motile. In addition, knockdown of S100A8 not only inhibits follicle reconstruction in a dose-dependent manner but also prevents PF assembly in mice [188]. S100A8 exhibits multiple receptors for performing various functions [194, 195]. Hence, future work should concentrate on defining the ovarian cell-specific receptors of S100A8 to explore its roles in ovarian development.

## Hormones regulating CBD and PF formation

In the mouse, circulating hormones and steroid factors, such as diethylstilbestrol [196], bisphenol-A [197] or phytoestrogen genistein [198], as well as estrogen, estradiol, and progesterone [199–202], have negative roles in regulating CBD and PF formation (Fig. 1d). In addition, neonatal mice treated with steroids exhibit a significantly higher incidence of MOFs in adult ovaries, implying that the occurrence of these aberrant follicles results from incomplete CBD [198]. Consequently, under physiological conditions, CBD may be initiated by a dramatic neonatal decrease of estrogen and progesterone, which are derived from the maternal milieu [84, 105, 199] of pregnancy, or from the fetal ovary itself [203]. Estrogen and progesterone may act through classical and non-classical mechanisms to modulate CBD and PF formation [105]. Through binding to estrogen receptors, elevated estrogen, trans-acting transcription factor 1 (SP1), and β-Catenin are involved in regulation of the expression of various genes and cause POF [204, 205]. Estrogen receptor inhibition assures the formation of PF-like structures in vitro [205, 206]. In the hamster and baboon, however, estrogen appears to have positive effects on PF formation [207–211]. Similarly, although estrogen receptors are found in human germ cells at the time of PF formation [212], the situation is quite different in humans compared to mice, as steroid hormones are maintained at high levels during PF formation in the second trimester of embryonic development [213–215]. These differences may be related to the different concentrations before and after CBD or to species specificity [105, 216]. Unfortunately, the exact cause is still unclear.

Follicle-stimulating hormone (FSH) may coordinate the action of estrogen in the fetal mouse ovary, while PFs are being established [5, 105, 217, 218]. FSH has been detected in the serum of neonatal mice and hamsters [219, 220]. The FSH receptor, possibly stimulated by FSH or estrogen, is expressed in the fetal ovaries of both mice [221] and hamsters [218]. In the hamster, FSH is involved in inducing PF formation and cAMP production in the ovary [218], the latter of which is important for early oogenesis and folliculogenesis in mice [102]. Neutralization of FSH results in the inhibition of PF formation [222]. These observations are in line with the findings in the mouse, in which PF formation is improved by FSH in vitro [223], and the oocyte-specific genes *Fig1α* and *Nobox* are promoted in a low-estrogen environment [217]. Therefore, the coordination of FSH and estrogen may be crucial for the proper timing of CBD and PF formation [5].

The downstream targets of estrogen and progesterone may be the TGFβ family member hormones, such as Inhibin (Inh), Act, and Fst [84]. First, these factors are involved in PF formation. In the human fetal ovary, Act is produced by

germ cells in cysts. The level of Act is reduced when CBD begins and is undetectable in oocytes in PFs [215, 224]. Treatment of neonatal mice with Act A results in a greater number of PFs entering the initial follicle pool in vitro and in vivo [77, 225]. Although Act receptors are expressed by both germ and somatic cells in the human fetal ovary [215], the active forms of their downstream transcriptional regulator SMADs are detectable only in the somatic cells, indicating that these cells are the targets of Act signaling [224]. In addition, as the natural antagonist of Act, Fst is specifically expressed in fetal ovary. Conditional knockout of *Fst* in the granulosa cells of the mouse ovary results in an obvious loss of fertility and reduction of litter numbers [226]. FST288, the strongest Act-neutralizing isoform of Fst, may be involved in germ cell CBD and PF assembly by inhibiting somatic cell proliferation via Notch signaling [227]. Second, an appropriate ratio between Inh-Act-Fst plays a powerful role in regulating germline CBD and PF formation. Acts and Inhs are homo- or hetero-dimeric cysteine knot proteins that share a common β subunit [228]. Exposure of neonatal mice to estrogens suppresses the levels of both Act in the ovary and Inh A in serum [200]. Similarly, in the baboon, estrogen regulates fetal ovarian folliculogenesis by controlling the ratio of Act:Inh within the ovary throughout the second half of gestation [211]. Disruption of the Inh-Act-Fst pathway is observed in *TAF4b*-deficient mice, indicating that these hormones function downstream of TAF4b in regulating PF formation [229]. Therefore, the effects of Act on germ cell survival and/or proliferation occur indirectly through juxtacrine/paracrine interactions with surrounding somatic cells rather than through autocrine interactions with germ cells [166] (Fig. 3).

## Germ cell survival

In many mammalian species, large numbers of oocytes die along with the CBD before individual PFs assemble [25, 110, 230, 231]. During this process, the cytoplasmic bridges between the remaining nuclei are either retracted or cleaved, likely through the protease action of the surrounding somatic cells [84]. In mice, the maximum number of germ cells is observed in the mouse ovary at the time at which oocytes enter meiotic prophase [232], whereas oocyte death is most pronounced at the time of birth, the point at which when PF formation is at its peak. As a result, by 4 dpp in the mouse, approximately 6.4 germ cells on average in each cyst survive from the initial 30 (20%) as primary oocytes [109, 110, 232–234]. In humans, attrition of the ovarian reserve begins at approximately 24 wpc when the number of oocytes starts to decline significantly [235]. Consequently, the ovarian lifespan is perinatally determined through a delicate balance of oocyte survival and apoptosis [87, 161].

An earlier depletion of the ovarian reserve results in earlier loss of estrogen production by the ovary. Estrogen is of great importance for bone, cardiovascular, and cognitive health as well as overall mortality in women [236]. Hence, understanding the mechanism of germ cell loss during this time is pivotal for identifying suitable strategies to preserve the size of the ovarian pool. However, detailed information regarding oocyte death remains limited.

Programmed cell death (PCD) is an important physiological process during embryonic development. Germ cell death can be apoptotic or nonapoptotic, depending on the stimulus or stage of development [237]. There are three types of PCD: type I (apoptosis), type II (autophagy), and type III (non-lysosomal vesiculate degradation) [232, 238]. According to in vivo and in vitro findings, fetal germ cell death occurs via a number of mechanisms during CBD, including Caspase 2-dependent apoptosis [34, 110, 239–243], highly conserved cytoprotective autophagy [241, 244–247], and direct extrusion from the ovaries (ovarian shedding) [245, 246].

Apoptotic and autophagic proteins appear to coexist in fetal oocytes [85] (Fig. 4). Recent reviews showed that autophagy exhibits an unresolved close relationship with apoptosis, as the initiation of either autophagy or apoptosis requires disruption of the same B-cell lymphoma/leukemia-2 (Bcl-2)/Bcl-xL complexes, which bind to either Beclin1 (autophagy pathway) or Bax/Bak (apoptosis pathway) [248, 249]. However, the autophagy protein p62 has been shown to interact with caspase-8, TRAF, and ERK [248]. Autophagy degrades damaged mitochondria and caspases and provides membranes for caspase processing in the regulation of apoptosis [249]. In addition, JNK1 is involved in the regulation of oocyte apoptosis and autophagy via phosphorylation of Bcl-2 [250]. The mutual relationship between autophagy and apoptosis before and after PF formation requires substantial further study.

The regulators of apoptosis consist of two groups of proteins: proapoptotic (Bax, Bak, Bad, Bid, PUMA, Noxa, Nix, Xiap, and Bim) and antiapoptotic (Bcl2, Bcl-xL, and Mcl1) [251]. In most cases, survival or apoptosis results from a balance between the expression of survival (antiapoptotic) and proapoptotic factors [84, 235]. Apoptosis has been recognized as the main cause of the attrition of the ovarian PF pool in both humans and mice [84, 191, 252, 253]. Freshly formed cysts sustain ongoing apoptosis and later fragment into smaller cysts [106]. Apoptosis is triggered by either physiological (developmental cues) or cellular (environmental in origin) stressors [232]. For instance, plummeting levels of estrogen immediately after birth in mice may be responsible for oocyte apoptosis [65, 198, 217, 254]. Most recently, deficiency of *Mcl-1* in the

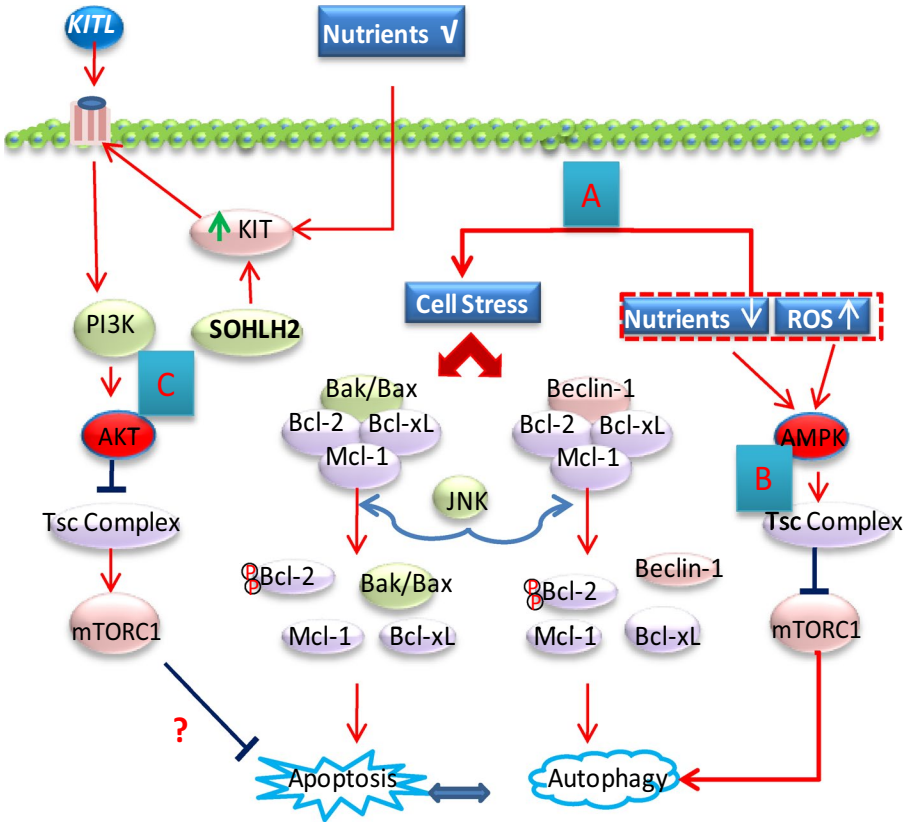


**Fig. 4** Schematic representation of autophagy and apoptosis regulation as well as the meiosis initiation mechanism during CBD and PF formation. **a** Cellular stress induced by starvation, ROS (reactive oxygen species), and other factors causes the displacement of Bcl-2 from either Beclin-1 or Bax, thereby triggering autophagy or apoptosis, respectively. Activated JNK is responsible for the phosphorylation of Bcl-2. The autophagy–apoptosis interactions are not fully understood. **b** Amino acid deprivation, hypoxia, and an elevated AMP/ATP ratio activate AMPK (5′-AMP-activated protein kinase), which in turn activate TSC2 (tuberous sclerosis complex 2), thus preventing the action of mTORC1 from inhibiting autophagy. **c** Increase of KIT levels in oocytes induced by KITL or SOHLH2 may activate mTORC1 to inhibit apoptosis through the PI3K/AKT signaling pathway

mouse oocyte was shown to result in premature exhaustion of the ovarian reserve, characterized by early PF loss due to activation of apoptosis [161, 255]. In humans, *Mcl-1* is expressed in germ cells of different sizes at the periphery of the ovary at 14–16 wpc and in the oocytes within newly formed PFs at 21 wpc, indicating a possible role of *Mcl-1* in PF formation [256]. Mcl-1 may be essential for the conservation of the postnatal PF pool as well as the survival of growing follicles and effective oocyte mitochondrial function [255]. The roles of other apoptosis-related proteins in PF formation also require further study.

As the mitochondrial load has been positively correlated with the prevention of apoptosis [257], intercellularly transferred proteins, nutrients, or mitochondria may themselves serve as the trigger for apoptosis of the nurse cell, and conversely, for protection of oocytes with a Balbiani body from apoptosis. In addition to the proteins that could be involved in the regulation of oocyte death, recent studies have indicated that miRNAs are involved in PF assembly by regulating either pre-granulosa cell proliferation [217, 258] or oocyte apoptosis [259]. These findings provide insight into new possibilities for systematically evaluating the roles of novel miRNAs in the regulation of target genes during CBD and PF assembly.

The term “autophagy” comes from the Greek words “auto” (self) and “phagein” (to eat). Autophagy is a highly conserved intracellular process that maintains cellular homeostasis through the removal of useless or damaged cytoplasmic organelles and large molecules [260]. Autophagy is both a unique cell-death pathway and an adaptation to stress that promotes cell survival [249]. Recently, autophagy has been demonstrated to be active in the life and death decisions of oocytes, particularly around the time of PF assembly [246, 247, 261]. Compromised autophagy within the perinatal ovary due to deficiency of autophagy-related genes (i.e., *Atg7* or *Beclin1*) results in the premature loss of female germ cells by 1 dpp in mice, which is similar to POI in humans [247, 262]. Therefore, autophagy appears to be a cell protective method for maintaining the endowment of female germ cells prior to establishing PF pools in the ovary [247].

Importantly, if starvation is prolonged before oocytes are enclosed in a PF, the lack of nutrients or growth factors may activate protective autophagy or even result in cell death [85, 262] (Fig. 4a). In addition, the mTOR protein, which senses the availability of nutrients and energy substrates within the intracellular milieu, is the major regulator of autophagy in response to nutritional factors (Fig. 4b). A decrease in the above-mentioned factors results in the inactivation of mTOR and, thus, initiates Beclin1-mediated autophagy [241, 260]. The inducer of

autophagy and the prerequisite for activating the process during CBD and PF formation remains a mystery.

KIT is directly involved in and important to the early survival of oocytes (Fig. 4c). As noted above, *c-Kit* is only expressed in oocytes during CBD, but KITL is expressed in both oocytes and ovarian somatic cells when PFs are formed. The inhibition of KIT results in a reduction in CBD, an increase in oocyte numbers, and a reduction of cell death. Activation of KIT promotes CBD and decreases oocyte numbers [161]. In vitro, KIT inhibitors reduce CBD and the growth of oocytes in both the fetal and neonatal ovary [224, 263]. Previous reports have indicated that naturally mutated KIT ligands in the mouse result in the formation of ovaries with fewer germ cells [264, 265]. When activated by KITL, KIT is autophosphorylated at tyrosine 719, which is also the primary binding site for the p85 of PI3K. Autophagy is regulated through the PI3K/AKT-mTOR pathway as well [260, 266]. Alternatively, spermatogenesis- and oogenesis-specific helix-loop-helix transcription factor 2 (SOHLH2) is effective in upregulating *c-kit* expression and inhibiting oocyte apoptosis, possibly through activation of the KIT/PI3K/AKT/Foxo3a signaling pathway [267].

In addition, the presence of a particular number of associated granulosa cells is important for sustaining oocyte survival [66, 268]. For example, neurotrophins, a family of growth factors, are involved in cell survival and ovary development. Surviving oocytes are contained in follicles that exhibit sufficient granulosa cells to provide the oocyte with sufficient neurotrophins [66, 269]. Feng et al. (2016) reported that the proper differentiation and proliferation of ovarian supporting cells are essential for the establishment of the PF pool. In particular, the recruited number of somatic cells and speed of recruitment may determine the final size of the female reproductive reserve [66]. Furthermore, water channel aquaporin-8 (AQP8), which is a major plasma membrane water channel protein that is exclusively expressed in granulosa cells and in the neonatal mouse ovary, has been reported to be responsible for the formation of MOFs [270]. Several studies have demonstrated that deficiency of *AQP8* may regulate pre-granulosa cell function during folliculogenesis by decreasing apoptosis and impairing cell migration [270–272].

Death receptor signaling factors, such as Tumor Necrosis Factor (TNF)/TNF receptor 1 and Fas ligand/Fas, are expressed in neonatal rodent ovaries and regulate oocyte death [232]. A lack of TNF in ovarian somatic cells results in malfunction of CBD and PF formation in rodents, whereas the deletion of TNFα or Fas in mice increases the number of PFs at birth [273–275].

Finally, Wnt4 and Fst are both specifically expressed in somatic cells of the fetal ovary. Based on Wnt4 acting as an initiator of *Fst* expression, FOXL2 and Bmp2 cooperate to ensure the correct expression of *Fst* [276]. Fst may be responsible for preventing the action of Act B, which causes masculinization and female germ cell loss [54]. Germ cells in the ovarian cortex are almost completely lost in both *Wnt4*- and *Fst*-deficient gonads before birth, indicating that Wnt4 acts through Fst in the early ovary to regulate vascular boundaries and endothelial cell migration from the mesonephros and to maintain germ cell survival at the pachytene and diplotene stages (approximately 16.5 dpc) in the ovary [276, 277].

## Summary

PF formation in mammals is systematically engineered by signals inside and outside the ovary. Previous studies have elucidated many important hormones and factors that play roles in CBD and PF assembly through endocrine, paracrine and autocrine actions. However, additional in vivo assays are needed to accurately elucidate what occur under physiological conditions. Moreover, it is still unclear which organizers are the most important in disassembling germline cysts and regulating PF formation. According to the most recent reports, reconstituted follicles, fertile oocytes, and full-term developed pups have been obtained from in vitro-generated PGCs [205, 206]. However, the low rate of generation of pups produced from the MII oocytes (ranging from 3.5% [205] to 14–40% [206] in vitro vs 61.7% in vivo) implies that the physiological mechanism of oogenesis is far from being well understood. Future work focusing on the mechanism that precisely controls this process is pivotal for potential translation of these findings to produce high-quality PFs in vitro for clinical applications.

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