

# Signaling pathways in the development of female germ cells

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***To my family***



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# 1. Abstract

Primordial follicles are the first small follicles to appear in the mammalian ovary. Women are born with a fixed number of primordial follicles in the ovaries. Once formed, the pool of primordial follicles serves as a source of developing follicles and oocytes. The first aim of this thesis was to investigate the functional role of the intra-oocyte signaling pathways, especially the phosphatidylinositol-3 kinase (PI3K) and mammalian target of rapamycin complex 1 (mTORC1) pathways in the regulation of primordial follicle activation and survival. We found that a primordial follicle remains dormant when the PI3K and mTORC1 signaling in its oocyte is activated to an appropriate level, which is just sufficient to maintain its survival, but not sufficient for its growth initiation. Hyperactivation of either of these signaling pathways causes global activation of the entire pool of primordial follicles leading to the exhaustion of all the follicles in young adulthood in mice.

Mammalian oocytes, while growing within the follicles, remain arrested at prophase I of meiosis. Oocytes within the fully-grown antral follicles resume meiosis upon a preovulatory surge of leutinizing hormone (LH), which indicates that LH mediates the resumption of meiosis. The prophase I arrest in the follicle-enclosed oocyte is the result of low maturation promoting factor (MPF) activity, and resumption of meiosis upon the arrival of hormonal signals is mediated by activation of MPF. MPF is a complex of cyclin dependent kinase 1 (Cdk1) and cyclin B1, which is essential and sufficient for entry into mitosis. Although much of the mitotic cell cycle machinery is shared during meiosis, lack of *Cdk2* in mice leads to a postnatal loss of all oocytes, indicating that *Cdk2* is important for oocyte survival, and probably oocyte meiosis also. There have been conflicting results earlier about the role of *Cdk2* in metaphase II arrest of *Xenopus* oocytes. Thus the second aim of the thesis was to identify the specific Cdk that is essential for mouse oocyte meiotic maturation. We generated mouse models with oocyte-specific deletion of *Cdk1* or *Cdk2* and studied the specific requirements of Cdk1 and Cdk2 during resumption of oocyte meiosis. We found that only Cdk1 is essential and sufficient for the oocyte meiotic maturation. Cdk1 does not only phosphorylate the meiotic phosphoproteins during meiosis resumption but also phosphorylates and suppresses the downstream protein phosphatase 1, which is essential for protecting the Cdk1 substrates from dephosphorylation.

## 2. Abbreviations

AC	adenylyl cyclase
AMH	anti-Müllerian hormone
APC/C	anaphase promoting complex/cyclosome
ATP	adenosine triphosphate
BMP	bone morphogenic protein
BPES	blepharophimosis ptosis epicanthus inversus syndrome
CAM	chorioallantoic membrane
cAMP	cyclic adenosine 3',5'-monophosphate
Cdk	cyclin dependent kinase
cGMP	cyclic guanosine monophosphate
CL	corpora lutea
CSF	cytostatic factor
Cx	connexin
Emi2	early mitotic inhibitor 2
FSH	follicle stimulating hormone
GC	granulosa cell
GDF	growth and differentiation factor
GPCR	G-protein-coupled receptors
GV	germinal vesicle
GVBD	germinal vesicle breakdown
IBMX	isobutyl methyl xanthine
IVF	in vitro fertilization
KL	kit ligand
LH	luteinizing hormone
MI	metaphase I
MPF	maturation promoting factor
mTORC	mammalian target of rapamycin complex
Nppc	natriuretic peptide precursor type C
Npr2	Nppc receptor 2
OA	okadaic acid
PDE	phosphodiesterase
PI3K	phosphatidylinositol 3 kinase
PKA	protein kinase A
POF	premature ovarian failure
PP	protein phosphatase
PTEN	phosphatase and tensin homolog deleted on chromosome ten
rpS6	ribosomal protein S6
rpS6K	ribosomal protein S6 kinase
TGF	transforming growth factor
TSC	tuberous sclerosis complex
Zp3	zona pellucida 3

### 3. List of publications

The thesis is based on the following papers:

- I. Adhikari D, Zheng W, Shen Y, Gorre N, Ning Y, Halet G, Kaldis P, Liu K. Cdk1, but not Cdk2, is the sole Cdk that is essential and sufficient to drive resumption of meiosis in mouse oocytes. *Hum Mol Genet.* 2012 Jun 1;21(11):2476-84.
- II. Adhikari D, Zheng W, Shen Y, Gorre N, Hämäläinen 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 Feb 1;19(3):397-410.
- III. Reddy P, Liu L, Adhikari D, Jagarlamudi K, Rajareddy S, Shen Y, Du C, Tang W, Hämäläinen T, Peng SL, Lan ZJ, Cooney AJ, Huhtaniemi I, Liu K. Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool. *Science.* 2008 Feb 1;319(5863):611-3.

Paper I, II and III were reprinted with kind permission from the publishers

Papers not included in this thesis:

- a. Adhikari, D., Risal, S., Liu, K., and Shen, Y. (2013). Pharmacological inhibition of mTORC1 prevents over-activation of the primordial follicle pool in response to elevated PI3K signaling. *PLoS one* 8, e53810.
- b. Zhang, H., Zheng, W., Shen, Y., Adhikari, D., Ueno, H., and Liu, K. (2012). Experimental evidence showing that no mitotically active female germline progenitors exist in postnatal mouse ovaries. *Proceedings of the National Academy of Sciences of the United States of America* 109, 12580-12585.
- c. Adhikari, D., Gorre, N., Risal, S., Zhao, Z., Zhang, H., Shen, Y., and Liu, K. (2012). The safe use of a PTEN inhibitor for the activation of dormant mouse primordial follicles and generation of fertilizable eggs. *PLoS one* 7, e39034.
- d. Adhikari, D., Flohr, G., Gorre, N., Shen, Y., Yang, H., Lundin, E., Lan, Z., Gambello, M.J., and Liu, K. (2009). Disruption of Tsc2 in oocytes leads to overactivation of the entire pool of primordial follicles. *Molecular human reproduction* 15, 765-770.
- e. Jagarlamudi, K., Liu, L., Adhikari, D., Reddy, P., Idahl, A., Ottander, U., Lundin, E., and Liu, K. (2009). Oocyte-specific deletion of Pten in mice reveals a stage-specific function of PTEN/PI3K signaling in oocytes in controlling follicular activation. *PLoS one* 4, e6186.
- f. Reddy, P., Adhikari, D., Zheng, W., Liang, S., Hamalainen, T., Tohonen, V., Ogawa, W., Noda, T., Volarevic, S., Huhtaniemi, I., et al. (2009). PDK1 signaling in oocytes controls reproductive aging and lifespan by manipulating the survival of primordial follicles. *Human molecular genetics* 18, 2813-2824.



## **4. Aim of the thesis**

The aim of my PhD, and therefore this thesis, was to characterize the signaling pathways controlling the development of female germ cells that would increase our understanding of normal physiology as well as find new ways of treating female infertility. Novel mice models were generated and studied for this purpose.

Specific aims:

1. To reveal the key signals and molecules those control the dormancy, survival and activation of primordial follicles.
2. To study the novel signaling pathways those regulate the oocyte meiotic maturation to generate healthy and fertilizable eggs.

## **5. Introduction**

### **5.1. Introduction to the mammalian ovary**

The mammalian ovary is a heterogeneous organ containing follicles and corpora lutea (CL) at various stages of development (Fig. 1) (McGee and Hsueh, 2000). The ovary is responsible for the development, maturation, and release of mature egg for fertilization. It is also responsible for synthesizing and secreting hormones that are essential for follicular development, menstrual/estrous cyclicity, maintenance of the reproductive tract and its function, development of female secondary sex characteristics, and metabolic functions. The functions of the mammalian ovary are integrated into the continuous repetitive process of follicular development, ovulation, CL formation, and regression (McGee and Hsueh, 2000; Vanderhyden, 2002).

To produce mature oocytes, primordial follicles develop through primary and secondary stages before reaching to the antral stage (Fig. 1). At the antral stage, most follicles undergo atretic degeneration, whereas a few of them grow further and reach the preovulatory stage under the cyclic gonadotropin stimulation that occurs after puberty. In response to preovulatory surge of gonadotropins during each cycle, the dominant Graffian follicle ovulates to release the mature oocyte for fertilization, whereas the residual follicle undergoes luteinization to become the CL. These cyclic and sequential changes are regulated by the hypothalamus-pituitary-ovary axis (Matzuk et al., 2002; McGee and Hsueh, 2000; Richards et al., 2002). The final stage of follicular development is mainly regulated by follicle stimulating hormone (FSH) and it has been used as a therapeutic agent to develop early antral to preovulatory follicles for treatment of infertility (Macklon et al., 2006). Compared to the relatively well-

characterized later events of gonadotropin-regulated follicular development, the intrinsic ovarian factors that regulate oocyte and follicular development before the follicles can respond to FSH are less well understood (Adhikari and Liu, 2009). It has been shown that the bidirectional communication between oocytes and surrounding somatic cells plays an important role in ovarian follicular development (Eppig et al., 2002; Wigglesworth et al., 2013).

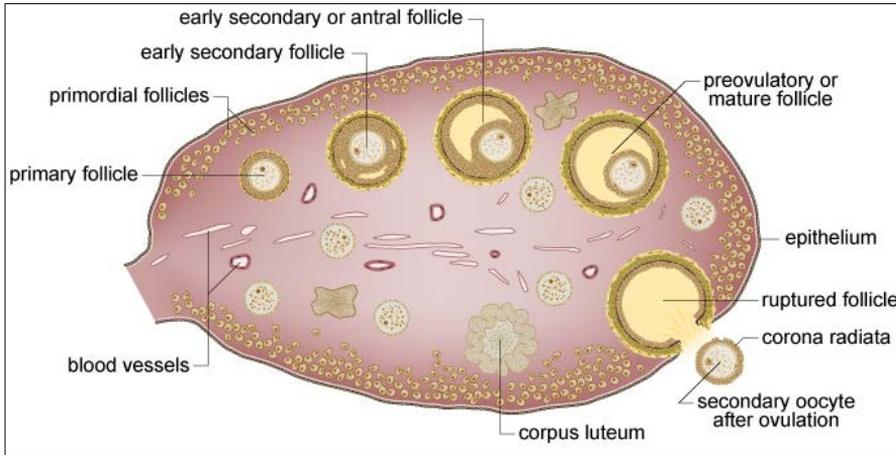


Figure 1. An overview of the mammalian ovary  
(Adapted from <https://www.ehd.org>)

## 5.2. Primordial follicles and their developmental fates

Primordial follicles are the first small follicles to appear in mammalian ovaries, which consist of an oocyte arrested at the diplotene stage of meiosis I and surrounded by several flattened somatic cells, termed pregranulosa cells (Fig. 2) (Borum, 1961; Peters, 1969). It has been shown that the first primordial follicle in human fetuses is formed at around 15–22 weeks of gestation, when oocytes are enclosed by a single layer of pregranulosa cells (Maheshwari and Fowler, 2008) and this process continues until just after birth (Baker, 1963; McGee and Hsueh, 2000). At 20 weeks of gestation, there are about 6–7 million of oocytes in total, but due to a massive oocyte loss in the ovary through multiple mechanisms of cell death (Pepling and Spradling, 2001; Rodrigues et al., 2009; Tingen et al., 2009), this number falls radically to less than 1 million at birth (Hansen et al., 2008). In contrast to humans, the formation of primordial follicles occurs within a few days of birth in rats and mice (Hirshfield, 1991).

It has been generally accepted that mammalian oocytes do not renew themselves once the follicles have formed in fetal or neonatal ovaries and the pool of primordial follicles serves as a non-renewable source of developing

follicles and oocytes, which declines with age (Broekmans et al., 2007; Hansen et al., 2008). An alternative view, promoted in recent years proposes that new oocytes are formed during adult life (Johnson et al., 2005; Johnson et al., 2004). It was also claimed that stem cells with potential to give rise oocytes have been identified in mouse and human ovaries (White et al., 2012; Zou et al., 2009). However, these results have been challenged by studies showing that no postnatal follicular renewal occurs in mammals (Lei and Spradling, 2013; Zhang et al., 2012), favoring the generally accepted view that females are born with a finite number of oocytes that declines with age (Broekmans et al., 2007; Hansen et al., 2008; Zhang et al., 2013).

Theoretically, although every primordial follicle has the potential to grow, mature, and ovulate; this is not the case in reality. Each primordial follicle may have three possible fates: to remain quiescent, to begin the development but later undergo atresia, or to develop, mature, and ovulate (Greenwald, 1972). Interestingly, not all of these endowed primordial follicles give rise to a mature fertilizable egg; the total number of eggs ovulated throughout the reproductive period is far less than the total number of primordial follicles initially endowed. For example, a woman ovulates less than 500 eggs in total throughout her reproductive life, but a young girl has about 300,000 to 400,000 oocytes per ovary at birth. So, roughly speaking only 1 in 1,600 primordial follicles undergoes ovulation. Most of the others die out directly without starting to grow. As a result of continuous activation and death, the pool of primordial follicles gradually shrinks with age. During the reproductive years in humans, the decline in the number of primordial follicles remains steady at about 1,000 follicles per month and accelerates after the age of 37, causing ovarian aging. The general belief is that when the available pool of primordial follicles has become depleted, reproduction ceases and women enter menopause (Broekmans et al., 2007; Hirshfield, 1991). At the time of menopause, the number of follicles remaining drops below 1,000 (Broekmans et al., 2007; Faddy and Gosden, 1996; Hansen et al., 2008).

### **5.2.1. Activation of primordial follicles**

The activation of primordial follicles is defined by a dramatic growth of the oocyte itself, accompanied by proliferation and differentiation of the surrounding pregranulosa cells (Adhikari and Liu, 2009; McGee and Hsueh, 2000) (Fig. 2). Although the activation of primordial follicles has been regarded as gonadotropin-independent event, very little is known about the underlying mechanism—especially why some primordial follicles enter the growth phase while others remain quiescent.

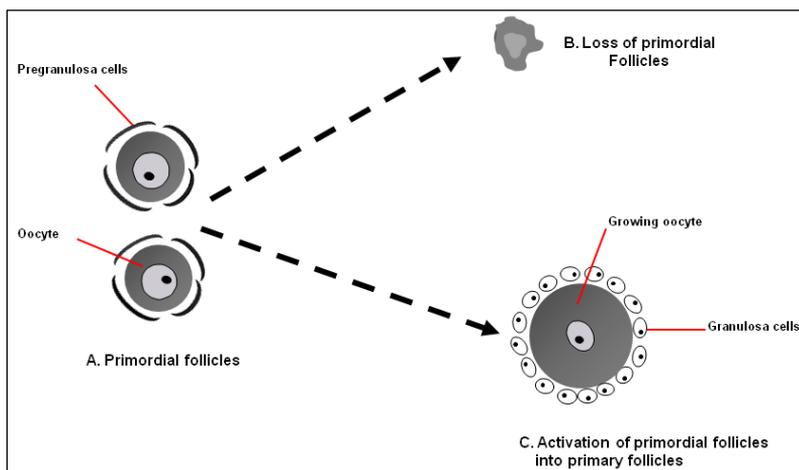


Figure 2. Schematic illustration of the possible fates of primordial follicles. Reproduced with permission from (Adhikari and Liu, 2013) with the permission of Elsevier.

### 5.2.2. Initial studies on primordial follicle activation

In order to obtain follicular growth *in vitro*, the whole ovaries from neonatal mice that contain only the primordial follicles were organ cultured (Eppig and O'Brien, 1996). After 8 days of culture, many oocytes had grown, and these growing oocytes were surrounded by 1-3 layers of granulosa cells. This *in vitro* development was similar to the follicular development observed *in vivo* in 8-day old mice (Eppig and O'Brien, 1996). However, similar approach to culture intact ovaries is not suitable in larger mammals due to two major reasons. Firstly, the ovaries are too large to be cultured as intact organs. Secondly, the formation of primordial follicles begins during fetal development and occurs over a long period of time, so that some primordial follicles are already activated while other follicles are still being formed. Due to this wide window for the primordial follicle formation in larger animals, it is not possible to have an ovary that contains only primordial follicles at any age (Fortune et al., 2000). Thus, for the larger mammals like cow and baboon, small fragments of the ovarian cortex that are rich in primordial follicles were used for *in vitro* culture (Braw-Tal and Yossefi, 1997; Wandji et al., 1997; Wandji et al., 1996). Quite different from the intact neonatal mice ovaries, when these cortical pieces were placed in organ culture, most follicles activated by day 2 of culture (Wandji et al., 1997; Wandji et al., 1996). Under these conditions, most primordial follicles became activated between 12 and 24 hours from the start of culture; their granulosa cells changed shape from flattened to cuboidal and began to express proliferating

cell nuclear antigen, a marker for cell growth and proliferation (Fortune et al., 2000; Wandji et al., 1997; Wandji et al., 1996). What makes an overall activation of primordial follicles in cortical pieces of baboon and cow ovary, but more regulated in intact mouse ovary though both are cultured in similar medium and condition?

These differences in the pattern of primordial follicle activation between intact mouse ovary and cortical pieces of bovine and baboon ovaries led to the hypothesis that the primordial follicles may be subject to an inhibition of growth initiation, possibly secreted from the medullary region of the ovary. To check this possibility, bovine ovary cortical pieces were co-cultured with medulla, however, it still did not hold the global activation of primordial follicles (Derrar et al., 2000). Although this result does not support the hypothesis that an intra-ovarian inhibitor(s) secreted from medulla inhibits the primordial follicle activation, it is possible that the concentration of such paracrine factor is too low to generate its effects as it might get diluted by the greater volume of culture medium.

### **5.2.3. Inhibition of primordial follicle activation by AMH**

It was found that anti-Müllerian hormone (AMH) (also called Müllerian inhibitory substance, MIS) might be such an inhibitory factor in mouse ovary (Durlinger et al., 1999). AMH belongs to the transforming growth factor (TGF)- $\beta$  superfamily of growth factors, which also includes inhibins, activins, bone morphogenic proteins (BMPs), and growth and differentiation factors (GDFs) (Massague and Chen, 2000). The function of AMH as a regulator of primordial follicles was not realized until it was reported that ovaries of *Amh*<sup>-/-</sup> mice show a significant depletion of the primordial follicle pool (Durlinger et al., 1999). This observation was further strengthened by *in vitro* culture of neonatal mouse ovaries (Durlinger et al., 2002a; Durlinger et al., 2002b), human ovarian cortical biopsy (Carlsson et al., 2006) and rat ovaries (Nilsson et al., 2007) in the presence of AMH, which significantly inhibited the activation of primordial follicles.

In order to provide a better environment for the follicular development, the pieces of ovarian cortex from bovine or baboon fetuses were grafted beneath the developing chorioallantoic membrane (CAM) of chick embryos (*in ovo*) (Cushman et al., 2002; Fortune et al., 2000). As this site was already known to support xenografted tissues, these ovarian pieces were also rapidly vascularized. However, histological analysis of pieces recovered after 2, 4, 7, or 10 days "*in ovo*" gave surprising results. There was no increase in the number of primary follicles, and the original primordial follicles were

maintained. Thus, the spontaneous activation of primordial follicles that occurred *in vitro* was completely inhibited *in ovo* (Cushman et al., 2002; Fortune et al., 2000). These findings suggest that chick embryos contain a specific inhibitor that inhibits primordial follicle activation.

When newborn mouse ovaries were grafted beneath the CAM of chick embryos, the primordial to primary transition was found to be suppressed (Gigli et al., 2005) in a similar way to the previously observed suppression of follicular activation in CAM-grafted bovine ovarian cortex (Cushman et al., 2002; Fortune et al., 2000). However, the identity of the inhibitor present in the chick embryo was unknown until it was revealed that primordial follicle activation occurred when ovaries of AMH receptor II KO mice were grafted beneath the CAM (Gigli et al., 2005). These data showed that AMH was the factor in the chick embryo's circulation that inhibited the activation of primordial follicles in grafted mouse ovaries.

#### **5.2.4. Regulation of follicular activation by *Foxl2* in pregranulosa cells**

FOXL2 belongs to the family of winged-helix/forkhead transcription factors (Crisponi et al., 2001). Mutations of the *FOXL2* gene cause blepharophimosis ptosis epicanthus inversus syndrome (BPES), an autosomal dominant disorder (Crisponi et al., 2001; De Baere et al., 2003; Udar et al., 2003). Affected female and male individuals show typical characteristics with dysplasia of the eyelids. It has been reported that mutations leading to a truncation of the FOXL2 protein in the forkhead domain (BPES type I) are at high risk of developing premature ovarian failure (POF) (De Baere et al., 2003). POF was found in type I BPES females, whereas males who were affected were fertile (Zlotogora et al., 1983).

In mice, *Foxl2* is expressed throughout ovarian development. It is detected mainly in somatic cells of the fetal mouse ovary, but is absent from germ cells (Cocquet et al., 2002; Pisarska et al., 2004; Schmidt et al., 2004; Uda et al., 2004). Studies in *Foxl2*<sup>-/-</sup> mice have shown that *Foxl2* maintains the quiescence of primordial follicles (Schmidt et al., 2004; Uda et al., 2004). When *Foxl2* gene was inactivated, the pregranulosa cells did not complete their squamous to cuboidal transition, which led to the absence of secondary follicles. However, oocytes in almost all primordial follicles had started to grow by two weeks after birth, as evidenced by the expression of GDF-9 (Schmidt et al., 2004), which is expressed in oocytes from primary follicles (Dong et al., 1996; Elvin et al., 1999). By 8 weeks of age, most of the oocytes had grown to nearly full size, indicating total depletion of the primordial follicles (Schmidt et al., 2004).

Thus, these findings provided evidence that *Foxl2* in pregranulosa cells is essential for inhibition of oocyte growth to maintain the quiescence of primordial follicles. In the absence of *Foxl2*, the whole pool of primordial oocytes gets prematurely activated without synchronizing with differentiation and division of the surrounding granulosa cells. These findings show that the surrounding pregranulosa cells are not only required for oocyte growth, but also for maintenance of follicular quiescence. It is likely that as a transcription factor, *Foxl2* in pregranulosa cells provides an inhibitory mechanism via gap junction or paracrine secretion, and maintains the primordial follicles in a dormant state. Sequence comparison of *Foxl2* genes from several vertebrate species has shown that they are highly conserved, suggesting that this molecule may have a similar role in the development of human ovaries (Uhlenhaut and Treier, 2006). Thus, the fact that loss of functional *FOXL2* in BPES type I patients causes POF (Crisponi et al., 2001) may be explained in a similar way. However, the downstream effectors of this transcription factor remain largely unknown.

#### **5.2.5. Suppression of primordial follicle activation by oocyte PTEN**

*PTEN* (phosphatase and tensin homolog deleted on chromosome ten) is a tumor suppressor gene located in a region of human chromosome ten that is often deleted in many human tumors. It is a negative regulator of the phosphatidylinositol 3 kinases (PI3Ks) (Fig. 3) (Cantley, 2002; Cully et al., 2006; Stokoe, 2001). PI3K pathway had been found to be operational in the mouse oocytes (reviewed in: Adhikari and Liu, 2009). In order to study the functional roles of the PI3K pathway in oocytes, *Pten* gene was deleted with an efficient oocyte-specific Cre transgenic mouse line driven by the *growth differentiation factor 9 (Gdf-9)* promoter, which mediates deletion of floxed genes in oocytes of primordial and further developed follicles (Lan et al., 2004). Although it had been speculated that teratoma may occur upon deletion of *Pten* in oocytes, this was not the case. Deletion of *Pten* from oocytes of primordial follicles led to the global activation of immature oocytes, resulting in premature activation of the entire primordial follicle pool (Reddy et al., 2008). The accelerated growth of oocytes was found to be mediated by enhanced activation of Akt and elevated phosphorylation (indicating activation) of ribosomal protein (rp) S6.

Independent work from another group has confirmed the role of PTEN in controlling the growth initiation of oocytes in primordial follicles, as an identical phenotype was observed when *Pten* was deleted from oocytes using the germ cell-specific *Vasa* promoter-driven Cre (John et al., 2008).

It is, however, worth noting that *Pten* in oocytes has stage-specific roles in controlling follicular development. When *Pten* was deleted from the oocytes of primary and further developed follicles by using an oocyte-specific Cre transgenic mouse line driven by the *Zona pellucida 3 (Zp3)* promoter, which mediates deletion of floxed genes in oocytes of primary and more developed follicles (de Vries et al., 2000), the female mice showed normal fertility, indicating that PTEN may not be essential for the development of oocytes in already activated follicles (Jagarlamudi et al., 2009).

As a proof of principle, a PTEN inhibitor has been successfully used *in vitro* to activate primordial follicles in both mouse and human ovaries (Li et al., 2011; Li et al., 2010). Because the original protocols for the *in vitro* culture of neonatal mouse ovaries (Eppig and O'Brien, 1996; O'Brien et al., 2003) do not efficiently generate germinal vesicle breakdown (GVBD)-competent oocytes, these protocols have recently been modified to also include a PTEN inhibitor and a PI3K activator in the first step of ovarian culture (Li et al., 2011). After 13 days of culturing neonatal ovaries in this modified medium, the isolated pre-antral follicles were cultured on an ovarian stromal cell feeder layer. This led to the generation of a significantly higher number of GVBD-competent oocytes (Li et al., 2011) than the original culture protocol (Eppig and O'Brien, 1996; O'Brien et al., 2003).

These results suggest that PTEN inhibitors could potentially be used in human ovarian culture medium to trigger the activation of primordial follicles. However, one must be extremely cautious while using PTEN inhibitors because PTEN has been shown to be a tumor suppressor (Cully et al., 2006; Salmena et al., 2008) and metabolic regulator (Ortega-Molina et al., 2012) in various tissues. To study the safety and efficacy of the use of a PTEN inhibitor, primordial follicles from neonatal mouse ovaries were activated by transient treatment with an inhibitor. The treated ovaries were then transplanted under the kidney capsules of recipient mice to generate mature oocytes. The mature oocytes were fertilized *in vitro* and progeny mice were obtained after transfer of the embryos back into female mice. Long-term monitoring up to the second generation of progeny mice showed that the mice were reproductively active and were free from any overt signs or symptoms of chronic illnesses (Adhikari et al., 2012a). These results suggest that PTEN inhibitors could also be safely and effectively used for generating mature human oocytes for use in novel *in vitro* fertilization (IVF) techniques. Recently, PI3K activator has been used to reawaken the primordial follicles that failed to activate and grow normally in the patients with primary ovarian insufficiency. The surgically removed ovarian fragments from these patients were briefly treated with Akt activator *in vitro* and were grafted back into women. In some women, rapid follicle growth occurred and mature eggs could be retrieved for IVF and embryo transfer. One of the women has given birth to a healthy baby (Kawamura et al., 2013). These findings altogether show that manipulation of signaling pathways can be a potential treatment strategy for obtaining mature eggs for IVF.

### **5.2.6. Suppression of follicular activation by Foxo3a in oocytes**

FOXO3a is a member of FOXO family of forkhead transcription factors (Brunet et al., 1999). It is a substrate of Akt, which acts downstream of PI3K/PTEN signaling (Fig. 3). In *Foxo3a*<sup>-/-</sup> mice, primordial follicles undergo spontaneous global activation so that the primordial follicle reserve is totally depleted by 2 weeks after birth (Castrillon et al., 2003). Thus, Foxo3a suppresses the activation of primordial follicles. It has been noticed in mice that Foxo3a is expressed mainly in the nuclei of oocytes of primordial follicles and early primary follicles and that its expression in oocytes of larger primary follicles and further developed follicles is dramatically downregulated (Liu et al., 2007). This finding is in accordance with the role of Foxo3a in suppressing primordial follicle activation, and suggests that downregulation of Foxo3a expression in oocytes may be a prerequisite for follicular activation (Liu et al., 2007).

Foxo3a is a substrate of Akt, the function of which is regulated by PI3K and PTEN. It has been shown that kit ligand (KL) leads to phosphorylation of Foxo3a through PI3K in cultured mouse and rat oocytes (Reddy et al., 2005), indicating that Foxo3a is located downstream of PI3K/PTEN in oocytes. In oocytes lacking *Pten*, Foxo3a was found to be phosphorylated constantly by the elevated Akt activity, keeping it in a suppressed state (Reddy et al., 2008). In addition, double-mutant mice lacking both *Pten* in oocytes and *Foxo3a* overall did not show synergistically enhanced follicular activation, showing that activation of the entire primordial follicle pool in oocyte-specific *Pten* knockout ovaries is at least partially mediated by the constant suppression of Foxo3a function in oocytes (Reddy et al., 2008). It was shown by an independent group that the loss of *Pten* from oocytes resulted in increased levels of PIP<sub>3</sub>, promoted membrane recruitment and activation of Akt, and subsequently resulted in hyperphosphorylation and nuclear transport of Foxo3a (John et al., 2008). It was confirmed that when *Pten* was deleted from oocytes Foxo3a was phosphorylated at the Akt phosphorylation sites, leading to its export from the nucleus to the cytoplasm (John et al., 2008).

### **5.2.7. Suppression of follicular activation by Tsc in oocytes**

Heterodimeric complex of tuberous sclerosis complex 1 (TSC1 or hamartin) and TSC2 (or tuberin) is one of the most important sensors involved in the regulation of mammalian target of rapamycin complex 1 (mTORC1) activity

(Huang and Manning, 2009; Inoki et al., 2002). mTORC1 is a serine/threonine kinase that positively regulates cell growth and proliferation by promoting many anabolic processes, including biosynthesis of proteins, lipids and organelles (Laplante and Sabatini, 2012; Wullschleger et al., 2006) and by limiting catabolic processes such as autophagy (Codogno and Meijer, 2005). TSC1 stabilizes TSC2 and protects it from ubiquitination and degradation (Chong-Kopera et al., 2006). When *Tsc1* (Adhikari et al., 2010) or *Tsc2* (Adhikari et al., 2009) is specifically deleted from primordial follicles, it causes a global activation of all primordial follicles around the time of puberty, ending up with follicular depletion in early adulthood and causing POF in mice. These studies also showed that increased mTORC1 activity was responsible for the loss of quiescence when *Tsc1* or *Tsc2* was absent from the primordial follicles. Increased mTORC1 activity in *Tsc1* or *Tsc2*-null oocytes was obvious as phosphorylation (that indicates activation) of the p70 ribosomal protein S6 kinase 1–(S6K1) was highly increased in these oocytes (Adhikari et al., 2009; Adhikari et al., 2010). Thus *Tsc* in oocytes plays an indispensable role in preserving the primordial follicles in their dormant state.

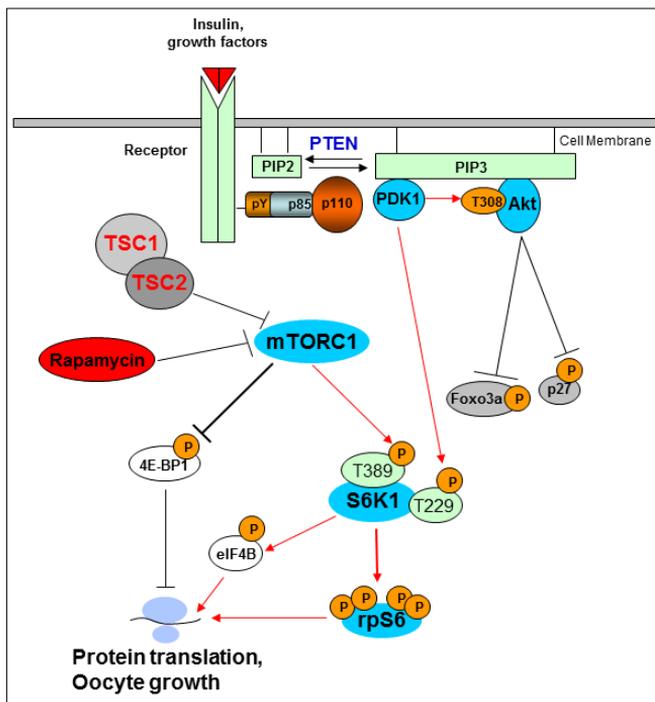


Figure 3. Simplified illustration of PI3K and mTORC1 signaling pathways. Reproduced with permission from (Adhikari and Liu, 2013) with the permission of Elsevier

Interestingly, although Tsc/mTORC1 signaling has been proposed to act downstream of PTEN/PI3K signaling in some cell types (Yang and Guan, 2007), the functions of both PTEN and Tsc in maintaining the quiescence of primordial follicles do not seem to be in an upstream and downstream arrangement in mouse oocytes. Instead, PTEN and Tsc appear to suppress follicular activation in parallel, in a synergistic and collaborative way. This notion is supported by the results that double deletion of *Tsc1* and *Pten* leads to further synergistically enhanced oocyte growth as compared to singly mutated mice (Adhikari et al., 2010). These results indicate that in order to maintain the quiescence of primordial follicles, phosphorylation of both T389 and T229 of S6K1 in oocytes must be suppressed by Tsc and PTEN, respectively (Adhikari et al., 2010), through which the activation of S6K1–rpS6 signaling is suppressed (Fig. 3). As a result, primordial follicles are prevented from being activated.

#### **5.2.8. Suppression of follicular activation by p27**

p27 is a member of the Cip/Kip family of Cdk inhibitors and is a negative regulator of the mammalian cell cycle and cell growth (Fero et al., 1996; Kaldis, 2007; Kiyokawa et al., 1996). In postnatal mouse ovaries, p27 is expressed in the pregranulosa cells and oocyte nuclei of primordial, primary, and secondary follicles (Rajareddy et al., 2007). In further developed follicles, although p27 is constantly expressed in granulosa cells, its expression in partially grown oocytes is reduced, indicating that the initiation of oocyte growth may require downregulation of p27 (Rajareddy et al., 2007). This hypothesis has been confirmed by the finding that in *p27<sup>-/-</sup>* mice, the primordial follicle pool is prematurely activated once it is endowed. In early adulthood in these mice, the overactivated follicular pool is largely depleted, leading to POF (Rajareddy et al., 2007). These results indicate that in oocytes and pregranulosa cells, p27 is important for the negative regulation of follicular activation.

As p27 is an important downstream molecule of the PI3K pathway (Kaldis, 2007), the effect of the simultaneous loss of both *p27* and *Foxo3a* was studied by generating double-knockout mice that lack both *p27* and *Foxo3a*. When *p27<sup>-/-</sup>* mice were crossed with *Foxo3a<sup>-/-</sup>* mice to generate double-knockout mice that lack both *p27* and *Foxo3a*, the activation of primordial follicles was synergistically accelerated by the simultaneous loss of *p27* and *Foxo3a* (Rajareddy et al., 2007). Thus, p27 and Foxo3a appear to suppress follicular activation independently of each other.

#### **5.2.9. Other molecules/pathways regulating the activation of primordial follicles**

Insulin has been found to promote the activation of primordial follicles in cultured fetal hamster ovaries (Yu and Roy, 1999), neonatal rat ovaries (Kezele et al., 2002) and the bovine ovarian cortex (Fortune et al., 2011; Wandji et al., 1996). Various growth factors like basic fibroblast growth factor (Nilsson et al., 2001; Nilsson and Skinner, 2004), keratinocyte growth factor (Kezele et al., 2005), platelet-derived growth factor (Nilsson et al., 2006), glial-derived neurotrophic factor (Dole et al., 2008), leukemia inhibitory factor (Nilsson et al., 2002), BMP-7 (Lee et al., 2001; Lee et al., 2004), BMP-4 (Nilsson and Skinner, 2003), and GDF-9 (Hreinsson et al., 2002; Martins et al., 2008; Vitt et al., 2000) have been found to enhance the activation of primordial follicles *in vitro*. Similarly, Smad3 (Tomic et al., 2004) and growth hormone (Slot et al., 2006) also enhance follicular activation in mice.

### **5.3. Oocyte maturation**

Meiosis consists of two rounds of successive cell divisions after a single round of DNA replication that halves the chromosome complement in male (sperm) or female (oocyte) gametes. In mice, oocytes begin to arrest at prophase of meiosis I (prophase I) at embryonic day 17.5 and most oocytes reach this stage by postnatal day 5 (Borum, 1961). In mammals, oocyte maturation occurs over a prolonged period of time, consisting of stops and starts at various steps (Mehlmann, 2005b). The preovulatory oocytes are arrested at the prophase of meiosis I until a surge of luteinizing hormone (LH) triggers the meiosis resumption. In mice, oocytes also resume meiosis spontaneously when released from the follicles and cultured *in vitro* (Eppig, 1989; Jones, 2004). Germinal vesicle (GV) breakdown (GVBD) is a hallmark of meiosis resumption of the oocytes, which is followed by chromosome condensation and spindle formation. Meiosis I is completed by extruding a polar body containing one set of chromosomes. The other set of chromosomes is retained in the oocyte, and the oocytes subsequently enter into the second round of meiosis but arrest at metaphase of meiosis II until fertilization (Adhikari and Liu, 2014; Jones, 2004) (Fig. 4).

Errors in oocyte maturation can cause aneuploidy in the resulting eggs and embryos, and this is one of the leading causes of spontaneous abortions and developmental abnormalities in humans (Hassold and Hunt, 2001; Jones, 2008). A proper understanding of the mechanisms underlying oocyte maturation would also improve the quality of fertility treatments.

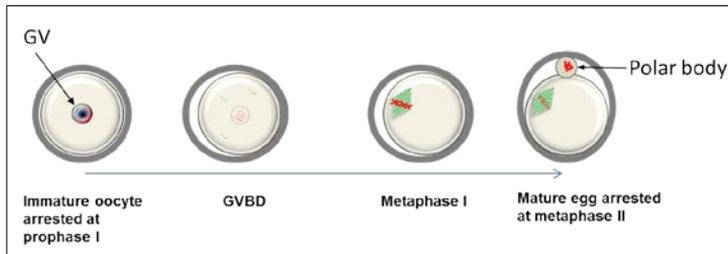


Figure 4. Schematic representation of the mammalian oocyte meiotic maturation process. Reproduced from (Adhikari and Liu, 2014) with the permission of Elsevier.

### 5.3.1. Mechanism of oocyte meiosis arrest at prophase I

Mammalian oocytes within fully-grown antral follicles remain arrested at prophase I and they do not resume meiosis until there is a preovulatory surge of LH, which indicates that LH mediates the resumption of oocyte meiosis. However, it was noticed early on that fully-grown oocytes also resumed meiosis spontaneously and without the requirement for any hormonal stimulation when freed from the follicle and cultured *in vitro* (Pincus and Enzmann, 1935). This observation led to the hypothesis that the somatic cells of the follicle function to prevent the resumption of meiosis in the oocyte and that the LH surge removes this inhibitory factor rather than providing any positive stimulus for the resumption of meiosis (Channing and Tsafiri, 1977). Although the preovulatory LH surge overcomes this inhibitory action, LH cannot have a direct effect on the oocyte because LH receptors are not expressed on the oocyte surface (McGee and Hsueh, 2000; Peng et al., 1991; van Tol et al., 1996). It was believed for many years that factors present in the follicular fluid were essential for maintaining the prophase I arrest in oocytes. Accumulating evidence suggests that a high level of cAMP (cyclic adenosine 3',5'-monophosphate) in the oocyte is responsible for maintaining oocyte meiotic arrest at prophase I.

The meiotic resumption of mouse oocytes is linked to the activation of maturation promoting factor (MPF) (Dekel, 2005; Jones, 2004). MPF is considered to be a universal regulator of G2-M transition in eukaryotic cell cycle (Nurse, 1990). *In vitro* experimental approaches, mostly by using *Xenopus* and starfish (Dunphy and Newport, 1989; Gautier et al., 1988; Labbe et al., 1989) eggs as models have shown that MPF is a complex of cyclin dependent kinase 1 (Cdk1, also called Cdc2) and cyclin B1. In such experiments, to identify Cdk1 as the kinase part of MPF, mainly two kinds of reagents have been extensively used: anti-PSTAIRE antibody raised against the 16 amino acid sequence EGVSTAIREISLLKE of yeast Cdk1 (Gautier et al., 1988) and a fission yeast protein, p13<sup>Suc1</sup>, which can bind to Cdk1 (Dunphy and Newport, 1989). Both of these reagents are able to immuno

deplete the activities of MPF-associated kinase across wide species (Dunphy and Newport, 1989; Gautier et al., 1988; Rosenblatt et al., 1992).

Despite only about 65% homology in amino acid sequences between Cdk1 and its close relative Cdk2 (cyclin dependent kinase 2), antigenically these two kinases are closely related. As a result, Cdk2 can also be recognized equally well by the anti-PSTAIRE antibody (Yamashita et al., 1991) in human cell lines (Merrick et al., 2008; Rosenblatt et al., 1992). Similarly, p13<sup>Suc1</sup>, another reagent which is often used to detect CDK1, can also recognize CDK2 in human cells (Rosenblatt et al., 1992). Moreover, specific sera developed against the C-terminal peptides from CDK1 and CDK2 can both recognize proteins of similar molecular weight (~34-kDa) (Rosenblatt et al., 1992). Not only Cdk1 but also Cdk2 can form active complex with cyclin B1 and drive cell cycle progression (Aleem et al., 2005). Clearly, due to these similarities between CDK1 and CDK2, identifying their relative contribution for the specific cell cycle events had always been a challenge (Bashir and Pagano, 2005; Krasinska et al., 2008; Rosenblatt et al., 1992).

The link between MPF activation and mouse oocyte meiotic maturation has been purely correlational and direct evidence showing whether Cdk1 only or Cdk2 also is responsible for MPF activation remains unknown. Although Cdk1 is considered as a universal regulator of G2-M transition in eukaryotic cell cycle (Nurse, 1990), Cdk2 is essential for MPF activation during *Xenopus* embryonic development (Guadagno and Newport, 1996) and mitosis of certain types of human cells (Mitra and Enders, 2004). In mouse oocytes, the histone H1 kinase activity rises shortly before GVBD, reaches a plateau at metaphase I (MI). After MI, the activity is down-regulated, which increases again before MII (Gavin et al., 1994), and Cdk1 is presumed to be responsible for this histone H1 phosphorylation despite the fact that histone H1 can also be phosphorylated by Cdk2 (Krasinska et al., 2008). Interestingly, meiotic resumption still occurs without a noticeable increase in histone H1 phosphorylation when oocytes are treated with okadaic acid (OA), a protein phosphatase inhibitor (Gavin et al., 1994). These findings show the cell-type and species-specific requirement of Cdks during cell cycle and thus raise the questions about the sole sufficiency of the Cdk1 for MPF activation in mouse oocyte meiosis. Thus, neither there is sufficient evidence to unequivocally demonstrate a sole role for Cdk1 nor there is an evidence to rule out the strong possibility for the requirement of Cdk2 in the regulation of mouse oocyte meiotic maturation.

Subsequently, mature mouse oocyte is once again arrested at MII by an activity known as cytostatic factor (CSF), until fertilization (Masui, 2001; Tunquist and Maller, 2003). The identity of such CSF molecules is only

poorly known in mouse oocytes; currently, c-Mos (Araki et al., 1996; Verlhac et al., 1996) and Emi2 (Madgwick et al., 2006) are known to be involved. In non-murine oocytes, Cdk2 kinase has also been studied as a possible CSF candidate (Furuno et al., 1997; Gabrielli et al., 1993) and as a regulator for MII entry (Sugiura et al., 2005a). However, its involvement has been disputed due to inconsistencies in results obtained by *in vitro* experiments in *Xenopus* eggs (Furuno et al., 1997; Gabrielli et al., 1993).

Deletion of *Cdk1* leads to embryonic lethality in mice (Santamaria et al., 2007), and oocytes from *Cdk2*-deficient mice die at the diplotene stage of prophase I, leading to an extinction of oocytes in 2-week old mice (Berthet et al., 2003; Ortega et al., 2003). Thus, there has been no possibility to study functional roles of these kinases during oocyte meiosis *in vivo*. In addition, downstream effectors of MPF that govern mammalian oocyte meiosis resumption are not well characterized. In order to circumvent these technical difficulties, we generated mouse models with *Cdk1* or *Cdk2* deleted specifically from oocytes for the study of the specific requirements of Cdk1 or Cdk2 during oocyte meiosis. We found that Cdk1 is the sole Cdk that is essential and sufficient to drive resumption of meiosis in mouse oocytes.

### **5.3.2. Regulation of CDK1 by cAMP**

The prophase I arrest in the follicle-enclosed oocyte is the result of low MPF activity, and resumption of meiosis upon the arrival of hormonal signals is mediated by activation of MPF (Adhikari and Liu, 2014; Eppig et al., 2004; Jones, 2004). Cho et al (Cho et al., 1974) reported that the spontaneous meiotic maturation of mouse oocytes is prevented when they are cultured in the presence of the cAMP analog dibutyryl cAMP. Similarly, the cAMP phosphodiesterase (PDE) inhibitor isobutyl methyl xanthine (IBMX) reversibly inhibits spontaneous resumption of meiotic maturation *in vitro* in mammalian oocytes. PDE inhibitors maintain high levels of oocyte cAMP by preventing its degradation (Schultz et al., 1983). Accordingly, the oocytes from mutant mice lacking PDE3A are permanently arrested at the GV stage leading to female infertility (Masciarelli et al., 2004a) and indicating that the maintenance of high cAMP in the oocyte is necessary for keeping the oocytes arrested at the GV stage. However, whether the cAMP is generated by the oocyte itself or it is transported to the oocyte from its surrounding somatic cells has not been clearly established and the issue remains controversial (Edry et al., 2006).

### **5.3.3. Production of cAMP by somatic cells**

Adenylyl cyclase (AC) catalyzes the formation of cAMP from ATP, but when denuded rat oocytes are cultured with forskolin, an agonist of AC, spontaneous resumption of meiosis still occurs (Dekel et al., 1984). There are

two types of granulosa cells: those which surround the oocyte are called cumulus cells and those which line the basement membrane are called mural granulosa cells. However, oocytes remain arrested at GV stage when cumulus-enclosed oocytes are cultured in the presence of forskolin (Dekel et al., 1984). This observation led to the hypothesis that cAMP is generated by the somatic follicular cells and is transported to the oocyte through gap junctions to maintain the meiotic arrest (Dekel, 1988). According to this model, LH initiates the resumption of meiosis by interrupting the communication between somatic cells and the oocyte and by blocking cAMP transport from the surrounding somatic cells into oocyte (Dekel et al., 1981).

Gap junctions between granulosa cells and gap junctions between cumulus cells and oocytes play an important role in supplying cAMP produced by somatic cells to the oocyte (Dekel, 1996; Granot and Dekel, 1994; Kidder and Mhawi, 2002). Gap junction channels are composed of proteins belonging to the connexin (Cx) family, and Cx43 is the most predominant Cx in the gap junctions between cumulus cells (Juneja et al., 1999; Kidder and Mhawi, 2002). In rat ovarian follicles, LH stimulation can mediate the phosphorylation and decrease in the expression of Cx43 leading to the disruption of the gap junction communication between the cells (Granot and Dekel, 1994). Incubation of rat follicle-enclosed oocytes with the gap junction blocker carbenoxolone also reduces cAMP concentrations in the oocytes and leads to the resumption of meiosis in follicle-enclosed oocytes (Sela-Abramovich et al., 2006). These results support the notion that the cAMP required for the maintenance of prophase I arrest of oocyte is synthesized by follicular somatic cells and is transported into the oocyte through gap junctions.

#### **5.3.4. Production of cAMP by the oocyte**

In contrast to the above hypothesis, when denuded mouse oocytes were cultured with forskolin, the cAMP level in the oocytes increased and GVBD was delayed (Schultz et al., 1983). It has also been found that mouse oocytes that cannot express the AC3 isoform of AC can no longer be arrested at the GV stage *in vivo* (Horner et al., 2003). Thus an alternative hypothesis proposes that oocytes synthesize their own cAMP through G-protein-coupled receptors (GPCR) present in the oocyte plasma membrane (Mehlmann, 2005b). Moreover, mouse oocytes are equipped with all of the necessary molecular machinery for the synthesis of cAMP. Gs protein, which is stimulated by GPR3 present on the oocyte, has been shown to stimulate AC3. Therefore, blocking  $\alpha$  subunit of Gs in follicle-enclosed mouse oocytes prevents cAMP synthesis and causes meiosis resumption spontaneously (Kalinowski et al., 2004; Mehlmann et al., 2002). Similarly, GPR3-null mouse oocytes also resume meiosis within the follicles *in vivo* (Ledent et al., 2005; Mehlmann, 2005a; Mehlmann et al., 2004). GPR12 has been reported to play a similar role in rat oocytes as GPR3 in mouse oocytes during meiotic arrest (Hinckley et al., 2005). Thus, the issue of whether the cAMP responsible for maintaining prophase I arrest is autonomously generated by

the oocyte or is transported from the surrounding granulosa cells still remains controversial.

### **5.3.5. Role of cGMP in maintaining a high cAMP level in the oocyte**

It has been shown that cAMP and cyclic guanosine monophosphate (cGMP) signaling cooperate in the maintenance of the high level of cAMP that is essential for oocyte meiotic arrest (Vaccari et al., 2009). cGMP is produced by the surrounding follicular cells and it passes into the oocyte through gap junctions where it inhibits cAMP hydrolysis by PDE3A (Norris et al., 2009). LH causes closure of gap junctions throughout the follicle halting the supply of cGMP to the oocyte. This in turn increases the cAMP-hydrolytic activity of PDE3A and the resumption of meiosis (Norris et al., 2009). Thus, the control of cGMP synthesis by GCs might be essential for maintaining meiotic arrest in fully-grown oocytes. Mural GCs of mouse follicles express a natriuretic peptide precursor type C (Nppc) and the cumulus GCs surrounding the oocyte express Nppc receptor 2 (Npr2), which is a guanylyl cyclase. Nppc peptide can inhibit the spontaneous GVBD in cumulus cell-enclosed oocytes but not in denuded mouse oocytes. In support of these *in vitro* findings, oocytes from Npr2 or Nppc-null mice also resume meiosis spontaneously *in vivo* (Zhang et al., 2010). These results show that cGMP signaling plays an important role in maintaining the high cAMP concentration in the oocyte during prophase I arrest (Fig. 5).

### **5.3.6. Regulation of CDK1 activity**

Mouse oocytes resume meiosis spontaneously when they are released from the antral follicles and cultured *in vitro*. However, microinjection of the catalytic subunit of protein kinase A (PKA) can maintain their prophase I arrest (Bornslaeger et al., 1986). Similarly, although mouse oocytes lacking PDE3A fail to undergo GVBD, inhibition of PKA signaling can induce GVBD in these oocytes (Masciarelli et al., 2004a). These results show that prophase I arrest by cAMP is mediated through PKA activation. PKA, however, does not phosphorylate CDK1 directly but it regulates and balances the activities of the Wee1B kinase and Cdc25 phosphatase in such a way that CDK1 remains inactive. Following the preovulatory LH surge, or after release from the follicle, the reduction in cAMP ultimately leads to activation of CDK1. The Cdc25 phosphatase and the Wee1B kinase have been proposed to be the direct substrates of PKA in mouse oocytes (Han and Conti, 2006).

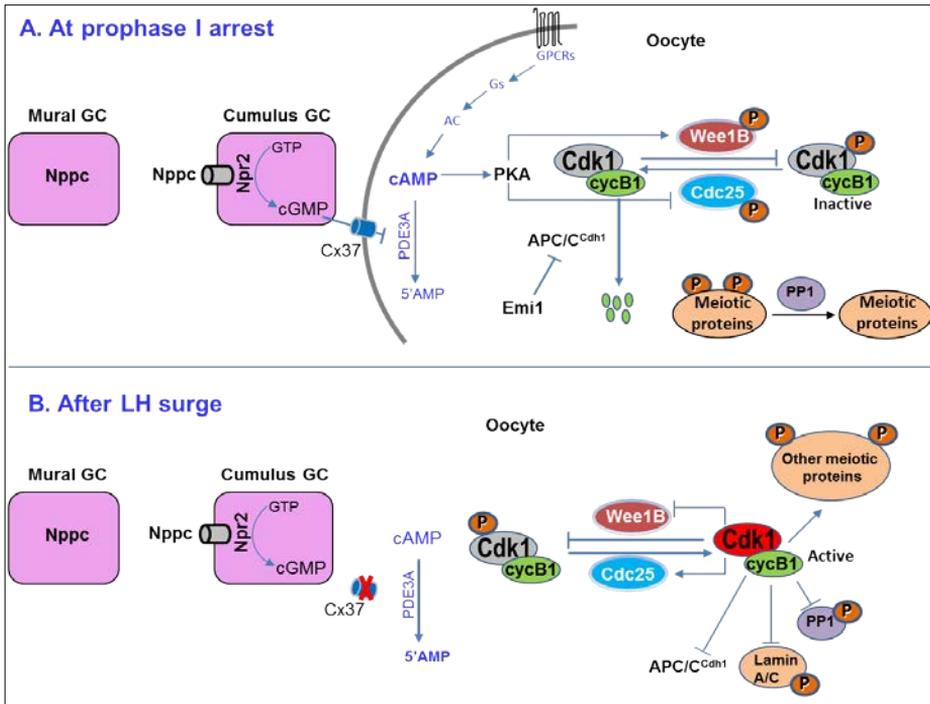


Figure 5. Model showing the regulation of MPF during prophase I arrest and after a preovulatory surge of LH. Reproduced from (Adhikari and Liu, 2014) with the permission of Elsevier.

### 5.3.7. Inhibition of CDK1 by Wee1B

CDK1 is inactivated when it is phosphorylated on Thr14 and Tyr15 by the Wee1/MYT1 kinases (Gautier et al., 1989; Mueller et al., 1995). In mouse oocytes, Wee1B has been shown to be a key CDK1 inhibitory kinase that phosphorylates CDK1 at Tyr15 (Han et al., 2005). Wee1B mRNA is translated in GV-arrested oocytes and down regulation of its expression enhances early resumption of meiosis both *in vitro* and *in vivo* (Han et al., 2005). Inhibition of Wee1B also causes GVBD in PDE3A-null mouse oocytes that normally fail to undergo GVBD upon release from the follicle. This also indicates that Wee1B acts downstream of PKA in maintaining prophase I arrest (Han et al., 2005; Masciarelli et al., 2004b). PKA has been found to directly phosphorylate Wee1B at Ser15 and enhance its kinase activity *in vitro* (Han et al., 2005). Reduction of the MYT1 kinase also enhances the early resumption of meiosis in mouse oocytes. Wee1B localizes to the oocyte nucleus whereas MYT1 inhibits CDK1 activity in the cytoplasm (Oh et al., 2010). When both Wee1B and MYT1 are down regulated, the effect on the resumption of meiosis is additive compared to the down regulation of Wee1B or MYT1 alone (Han et al., 2005).

### 5.3.8. Inactivation of Cdc25B by PKA inhibits CDK1

Three isoforms of the dual specificity phosphatase Cdc25 (Cdc25A, Cdc25B, and Cdc25C) function in the regulation of the mammalian cell cycle to reverse the inhibitory phosphorylation on CDK1 by the Wee1/MYT1 kinases (Rudolph, 2007). PKA inactivates Cdc25 in *Xenopus* oocytes by phosphorylating it on Ser287. When a mutant version of Cdc25 that cannot be phosphorylated by PKA is overexpressed in the oocyte, PKA is no longer able to maintain the prophase I arrest (Duckworth et al., 2002). In mouse oocytes, Cdc25B has been shown to be essential for CDK1 activation and the resumption of meiosis (Lincoln et al., 2002). During the meiotic arrest of mouse oocytes, PKA has been suggested to inactivate Cdc25B by phosphorylating it on Ser321 (Pirino et al., 2009; Zhang et al., 2008). When Cdc25B is phosphorylated by PKA, it binds to 14-3-3 proteins and is transported to the cytoplasm away from its substrate i.e. away from the phosphorylated CDK1 in the nucleus (Duckworth et al., 2002; Pirino et al., 2009; Zhang et al., 2008). Accordingly, when a mutant version of Cdc25B that cannot be phosphorylated by PKA is expressed in mouse oocytes it localizes to the nucleus and accelerates GVBD (Oh et al., 2010; Pirino et al., 2009). Upon LH surge, Cdc25B cannot be phosphorylated and inactivated by PKA and this active Cdc25B can enhance CDK1 activity by removing the inhibitory phosphorylation caused by Wee1B/MYT1 kinase (Fig. 5).

### 5.3.9. Regulation of cyclin B1 levels

In the prophase I-arrested mouse oocyte, cyclin B1 is already present in an adequate amount for resumption of meiosis (Kanatsu-Shinohara et al., 2000) and no new protein synthesis is required (Fulka et al., 1986). Activation of CDK1 kinase by removing its inhibitory phosphorylation is sufficient to cause the resumption of meiosis. However, cyclin B1 is constantly degraded by the anaphase promoting complex/cyclosome (APC/C) to maintain the prophase I arrest in the mouse oocytes (Reis et al., 2006). Failure to continuously degrade cyclin B1 during the prophase I arrest increases its concentration leading to MPF activation and spontaneous resumption of meiosis (Holt et al., 2013; Holt et al., 2011; Reis et al., 2006). APC/C is a large multisubunit E3 ubiquitin ligase that tags its substrates by polyubiquitination, and these are then identified and degraded by the 26S proteasome (Castro et al., 2005; Peters, 2006). One of the two positive regulators – Cdc20 or Cdh1 – is required for the substrate specificity and activation of APC/C.

In GV stage mouse oocytes, APC/C<sup>Cdh1</sup> activity itself is inhibited by early mitotic inhibitor 1 (Emi1). Reduction of Emi1 in GV-stage mouse oocytes by injection of morpholinos delays GVBD by preventing the accumulation of cyclin B1 whereas Emi1 overexpression leads to cyclin B1 accumulation and GVBD (Marangos et al., 2007) (Fig.5). Thus, besides the inactivation of CDK1 by phosphorylation, destruction of cyclin B1 also keeps MPF inactive during the meiotic arrest in oocytes (Jones, 2011).

A spindle assembly checkpoint protein, BubR1, has also been shown to regulate prophase I arrest in mouse oocytes (Homer et al., 2009). Downregulation of BubR1 expression by morpholinos in oocytes causes spontaneous GVBD in the presence of IBMX. In the BubR1-depleted oocytes, the Cdh1 level is significantly reduced (Homer et al., 2009). However, the GVBD rate in the BubR1-depleted oocytes decreases when the Cdh1 level is restored by injecting Cdh1 cRNA into the oocyte.

The dual-specificity phosphatase Cdc14B has been shown to be required for preventing the resumption of meiosis. Oocytes depleted of Cdc14B spontaneously resume meiosis whereas oocytes that overexpress Cdc14B are significantly delayed in resuming meiosis (Schindler and Schultz, 2009). Such a delay in GVBD, however, is corrected when Cdh1 is concomitantly depleted in Cdc14B-overexpressing mouse oocytes (Schindler and Schultz, 2009). Thus, Cdc14B functions to prevent resumption of meiosis by activating Cdh1 and thereby enhancing cyclin B1 proteolysis. Another protein, securin, is also an APC/C substrate and has a well-established role in binding and inhibiting separase to ensure the appropriate timing of sister chromatid separation (Kudo et al., 2006; Wirth et al., 2006). In mouse oocytes, excess securin causes accumulation of cyclin B1 and premature entry into meiosis, whereas securin depletion leads to cyclin B1 degradation and delayed GVBD (Marangos and Carroll, 2008). These results also show that constant degradation of cyclin B1 is required to maintain meiotic arrest in oocytes by inhibiting the activation of MPF.

### **5.3.10. Roles of protein phosphatases**

Protein phosphatases (PPs) that dephosphorylate the CDK1 substrates have been found to be important in regulating both mitosis and meiosis (Domingo-Sananes et al., 2011; Mochida and Hunt, 2012; Mochida et al., 2010; Wang et al., 2004). Concomitant inhibition of CDK1-opposing PPs is required together with the activation of CDK1 kinase during oocyte meiotic maturation (Adhikari et al., 2012b; Smith et al., 1998; Swain et al., 2003; Wang et al., 2004). GV-stage mouse oocytes contain both PP1 and PP2A (Smith et al., 1998), and PP1 is localized to the nucleus and PP2A is localized to the cytoplasm (Smith et al., 1998; Swain et al., 2003). CDK1 inactivates PP1 in somatic cells by phosphorylating it on Thr320 (Dohadwala et al., 1994; Kwon et al., 1997). PP1 phosphorylation is also increased following GVBD in mouse oocytes, and this phosphorylation is sensitive to roscovitine (Wang et al., 2004). These results indicate that CDK1 might be responsible for phosphorylation of PP1 during GVBD in mouse oocytes as well.

Okadaic acid, an inhibitor of both PP1 and PP2A (Cohen, 1989) can induce GVBD in mouse oocytes even when they are treated with substances that are known to prevent MPF activation and spontaneous resumption of meiosis (Alexandre et al., 1991; Gavin et al., 1994; Gavin et al., 1991; Rime and Ozon, 1990; Schwartz and Schultz, 1991). In mouse oocytes, the inhibition of GVBD by roscovitine can be overcome by microinjection of an anti-PP1 antibody,

but not by an anti-PP2A antibody (Wang et al., 2004), indicating that PP2A might not play a role during GVBD. A recent study in mice with CDK1-null oocytes has also shown that CDK1 plays a major role in GVBD by phosphorylating PP1 (Adhikari et al., 2012b). Thus, at the GV stage low CDK1 kinase activity favors hypophosphorylation and activation of PP1 (Fig. 5), which in turn maintains meiotic phosphoproteins in their hypophosphorylated states (Adhikari et al., 2012b) (Fig. 5).

Lamins maintain the structural integrity of the nuclear envelope (Holaska et al., 2002) and CDK1 promotes the disassembly of nuclear lamina by phosphorylating specific sites on lamins (Heald and McKeon, 1990; Peter et al., 1990; Ward and Kirschner, 1990). In the prophase I arrested mouse oocytes, lamin A/C remains unphosphorylated and CDK1-mediated phosphorylation causes disassembly of nuclear lamina during GVBD (Adhikari et al., 2012b) (Fig. 5). The suppressed PP1 activity in oocytes facilitates to maintain the phosphorylated status of lamin A/C during oocyte GVBD, which is required for disassembly of the nuclear envelope (Adhikari et al., 2012b). Recently, hyperactive PP2A has also been shown to be the cause for the permanent GV-stage arrest of mouse oocytes that lack meiosis arrest female 1 expression (Su et al., 2012). Thus, further studies are needed to identify which PP type is involved during prophase I arrest of mouse oocytes.

## 6. Results

(The figures cited correspond to those in original papers)

### Paper I

In order to identify which Cdk is essential for oocyte meiotic resumption, we generated conditional knockout mice with oocyte-specific deletions of *Cdk1* and *Cdk2*. It was found that the *OoCdk1*<sup>-/-</sup> females were completely infertile (Fig. 1A, c) despite their normal folliculogenesis (Fig. 1A, d) and ovulation (Fig. 1A, d and e). On the other hand, the *OoCdk2*<sup>-/-</sup> mice were completely fertile (Fig. 1B, a) and the meiotic resumption (Fig. 1B, d), progression to MII stage of *OoCdk2*<sup>-/-</sup> oocytes was normal (Fig. 1B, e), indicating that Cdk2 is dispensable for oocyte meiotic maturation.

However, we found that all the ovulated *OoCdk1*<sup>-/-</sup> oocytes were permanently arrested at the GV stage (Fig. 2A, b, arrows). As a comparison, the control ovulated *OoCdk1*<sup>+/+</sup> oocytes had emitted the first polar body (Fig. 2A, a, arrows), indicating entry into the MII stage. Moreover, instead of two-cell embryos, GV-stage oocytes were recovered from oviducts of *OoCdk1*<sup>-/-</sup> females at embryonic day (E) 1.5 after mating with stud males. These oocytes failed to develop further during a 24-h culture period (Fig. 2B, a and b). Control two-cell embryos were recovered from oviducts of *OoCdk1*<sup>+/+</sup> females at E1.5 (Fig. 2B, c), which subsequently developed into 4- to 8-cell embryos over a 24-h culture period (Fig. 2B, d). These results indicate that *OoCdk1*<sup>-/-</sup> oocytes are permanently arrested at the GV stage and are not able to resume meiosis.

To ascertain that the GV-stage arrest of *OoCdk1*<sup>-/-</sup> oocytes was indeed caused by the lack of *Cdk1*, we injected *in vitro* transcribed *Cdk1* mRNA into *OoCdk1*<sup>-/-</sup> oocytes. Water was injected into *OoCdk1*<sup>+/+</sup> and *OoCdk1*<sup>-/-</sup> oocytes as controls. As shown in Fig. 2D, after the introduction of *Cdk1* mRNA, 78.2±16.1% of the injected *OoCdk1*<sup>-/-</sup> oocytes underwent GVBD, a rate comparable to the GVBD rate for *OoCdk1*<sup>+/+</sup> oocytes injected with water (88.5±3.4%). None of the *OoCdk1*<sup>-/-</sup> oocytes injected with water underwent GVBD (Fig. 2D). These results showed that the GV-stage arrest as observed in *OoCdk1*<sup>-/-</sup> oocyte was solely caused by the lack of Cdk1.

In *OoCdk1*<sup>-/-</sup> oocytes no apparent phosphorylation of lamin A/C was observed during the culture period of 0 to 14 h (Fig. 3A). This result suggests that the phosphorylation and disassembly of nuclear lamina are downstream of Cdk1 during GVBD. After re-introduction of *Cdk1* mRNA into *OoCdk1*<sup>-/-</sup> oocytes, phosphorylation of lamin A/C was recovered in *OoCdk1*<sup>-/-</sup> oocytes that had been supplied with *Cdk1* mRNA (Figure 3B). We found that in *OoCdk1*<sup>+/+</sup> oocytes, phosphorylation of PP1 at Thr320 was elevated along with the progress of oocyte meiotic maturation (Fig. 4A, 1-6 h), and was reduced during the MI-MII transition (Fig. 4A, 10 h). In *OoCdk1*<sup>-/-</sup> oocytes,

however, phosphorylation of PP1 (Thr320) was completely absent during a culture period of 14 h (Fig. 4A), indicating that Cdk1 is the upstream kinase that regulates PP1 activity during resumption of oocyte meiosis. To confirm that Cdk1 mediates resumption of oocyte meiosis through phosphorylation of PP1, we re-introduced *Cdk1* mRNA into *OoCdk1*<sup>-/-</sup> oocytes. As shown in Figure 4B, phosphorylation of PP1 was recovered in *OoCdk1*<sup>-/-</sup> oocytes that were supplied with *Cdk1* mRNA.

After treatment with OA GVBD was resumed in *OoCdk1*<sup>-/-</sup> oocytes. Notably, lamin A/C phosphorylation in *OoCdk1*<sup>-/-</sup> oocytes was also elevated 2 h after OA treatment (Fig. 5), a time point when most of the oocytes were still at the GV stage. These results indicate that inhibition of PP1 is required for GVBD, and that a major role of Cdk1 in oocyte GVBD is to inactivate PP1. The suppressed PP1 activity in oocytes then facilitates to maintain the phosphorylated status of lamin A/C during oocyte GVBD, which is required for disassembly of the nuclear envelope.

In summary, we have shown that Cdk1 is indispensable for triggering of resumption of meiosis in mammalian oocytes. This is achieved through phosphorylation and suppression of PP1, and subsequent phosphorylation of lamin A/C and possibly other key proteins required for GVBD. The information obtained will also provide useful knowledge for treatment of female infertility that is caused by meiosis-arrested oocytes, which are often seen in *in vitro* fertilization clinics.

## Paper II

To determine the role of Tsc/mTORC1 signaling in oocytes for the regulation of the development of primordial follicles, in this study we deleted the *Tsc1* gene from mouse oocytes in primordial and further developed follicles.

Although no apparent morphological difference was found in postnatal day (PD) 5 ovaries of *OoTsc1*<sup>-/-</sup> and *OoTsc1*<sup>+/+</sup> mice (Fig. 3, A–C), the *OoTsc1*<sup>-/-</sup> ovaries (Fig. 3E) appeared larger than the *OoTsc1*<sup>+/+</sup> ovaries (Fig. 3D) by PD23. All primordial follicles were activated in *OoTsc1*<sup>-/-</sup> ovaries with enlarged oocytes (Fig. 3F, arrows; Fig. 3H), whereas 74% of the follicles in control ovaries were still at the primordial stage (Fig. 3D, arrows; Fig. 3H). This leads to higher numbers of activated follicles in *OoTsc1*<sup>-/-</sup> ovaries (Fig. 3H, Acti). Thus, with *Tsc1* deleted in oocytes, the entire pool of primordial follicles in *OoTsc1*<sup>-/-</sup> ovaries had been activated prematurely. By 3 months of age, *OoTsc1*<sup>-/-</sup> ovaries were smaller and did not have any healthy follicular structure (Fig. 4, H and I). As a control, the *OoTsc1*<sup>+/+</sup> ovaries were larger, showing healthy follicles (Fig. 4G). Thus, premature activation of the primordial follicle pool led to follicle depletion and POF in *OoTsc1*<sup>-/-</sup> mice in early adulthood.

In *OoTsc1*<sup>-/-</sup> oocytes the activity of mTORC1 was enhanced, as indicated by the elevated levels of phosphorylated S6K1 (Fig. 5A, p-S6K1, T389) and

phosphorylated 4E-BP1 (Fig. 5A, p-4E-BP1, S65) in Oo*Tsc1*<sup>-/-</sup> oocytes. The elevated phosphorylation of S6K1 at T389 had apparently led to an increase in its activity, as phosphorylation of S6K1 substrate, rpS6, was dramatically elevated in Oo*Tsc1*<sup>-/-</sup> oocytes (Fig. 5A, p-rpS6, S240/4 and S235/6). We found that in PD23 Oo*Tsc1*<sup>-/-</sup> mice that had been treated with mTORC1 inhibitor, rapamycin, the ovaries were smaller (Fig. 6D) and typical primordial follicles were found (Fig. 6E, arrows). These results clearly showed that elevated mTORC1 activity in oocytes is the major driving force that activates the entire pool of primordial follicles in Oo*Tsc1*<sup>-/-</sup> mice. At the same time, this result also indicates that suppression of mTORC1 activity in oocytes is necessary for preservation of primordial follicles in a dormant state.

To determine the functional relationship between the PTEN/PI3K signaling and the Tsc/mTORC1 signaling within the oocyte in regulating follicular activation, we crossed Oo*Tsc1*<sup>-/-</sup> mice with Oo*Pten*<sup>-/-</sup> mice and studied follicular development in progeny mice with concurrent loss of *Tsc1* and *Pten* in oocytes (referred to as Oo*Tsc1*<sup>-/-</sup>;*Pten*<sup>-/-</sup> mice). We found that in Oo*Tsc1*<sup>-/-</sup>;*Pten*<sup>-/-</sup> mice, the rate of oocyte growth was synergistically enhanced further (as shown in the ovaries of PD 23 mice; Fig. 7C, arrows) as compared to that in single mutant Oo*Pten*<sup>-/-</sup> ovaries (Fig. 7A, arrows) or Oo*Tsc1*<sup>-/-</sup> ovaries (Fig. 7B, arrows).

Our results show that loss of *Tsc1* or *Pten* in oocytes both lead to overactivation of rpS6, but through distinct signaling pathways. PTEN in oocytes suppresses follicular activation through negative regulation of the PI3K signaling, which subsequently inhibits phosphorylation of S6K1 at its T229 (Fig. 9, T229 of S6K1); while *Tsc1* in oocytes suppresses follicular activation by negative regulation of mTORC1 signaling, which subsequently suppresses the phosphorylation of S6K1 at its T389 (Fig. 9, T389 of S6K1). Our results thus indicate that the function of either *Tsc1* or PTEN is necessary, but not sufficient, to maintain the quiescence of primordial follicles.

### **Paper III**

The functional roles of the PI3K pathway in oocytes in controlling follicular activation were first revealed by deletion of *Pten* from oocytes in mice. By using an oocyte-specific Cre transgenic mouse line driven by the *growth differentiation factor 9* (*Gdf-9*) promoter, which mediates deletion of floxed genes in oocytes of primordial and further developed follicles (Lan et al., 2004), the *Pten* gene was deleted from oocytes of primordial follicles.

Deletion of *Pten* from oocytes of primordial follicles led to accelerated growth of immature oocytes, resulting in premature activation of the entire primordial follicle pool. Mechanistically, the accelerated growth of oocytes was found to be mediated by enhanced activation of Akt and elevated phosphorylation (indicating activation) of rpS6. Moreover, the activation of

rpS6 in oocytes was also largely dependent on mTORC1. This work showed that the PI3K/PTEN pathway in the oocyte governs follicular activation through control of initiation of oocyte growth.

No apparent morphological difference in PD5 ovaries of *Pten<sup>loxP/loxP</sup>;GCre<sup>+</sup>* and *Pten<sup>loxP/loxP</sup>* mice were observed and the ovaries of both genotypes had mostly primordial follicles containing small oocytes surrounded by flattened pregranulosa cells (Fig. 2, A to C, arrows) and some activated follicles containing enlarged oocytes (Fig. 2, A to C, arrowheads). By PD23, virtually no primordial follicles could be identified in mutant ovaries (figs. S2E and S3C), whereas 69.2% of the follicles in control ovaries were still at the primordial stage (fig. S2D, inset, and fig. S3C). Therefore, the entire pool of primordial follicles had been activated in *Pten<sup>loxP/loxP</sup>;GCre<sup>+</sup>* ovaries. Such a global activation of the whole pool of primordial follicles leads to follicle depletion; and POF in *Pten<sup>loxP/loxP</sup>;GCre<sup>+</sup>* mice.

We found that the level of phospho-Akt (p-Akt, Ser<sup>473</sup>) was elevated in *Pten<sup>loxP/loxP</sup>;GCre<sup>+</sup>* oocytes that were cultured in vitro and starved of serum (Fig. 4A). In addition, Kit ligand, which can activate the PI3K pathway in growing oocytes through its oocyte surface receptor Kit, activated Akt to a greater extent in *Pten<sup>loxP/loxP</sup>;GCre<sup>+</sup>* oocytes than in *Pten<sup>loxP/loxP</sup>* oocytes (Fig. 4B). Thus, the loss of *Pten* in oocytes leads to enhanced oocyte PI3K-Akt signaling, which led to increased activation of ribosomal protein S6 (rpS6) (Fig. 4C, PD12 to 14). Thus, PTEN in the oocyte is required for the maintenance of primordial follicle quiescence and the female fertility lifespan.

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