MOLECULAR PATHWAYS MEDIATING THE DEVELOPMENT OF FEMALE GERM CELLS

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TO MY FAMILY

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1. Abstract

In mice, primordial germ cells (PGCs) dramatically increase in their number from 200 cells at 9.5 dpc to 10 000 cells at 12.5 dpc with doubling time of 12.6 hours. Thus, cell proliferation of PGCs seems a unique compared to proliferation of other cell types. How Mastl (microtubule-associated serine/threonine kinase-like) is involved in the rapidly dividing PGCs is not known. PGC-specific deletion of *Mastl* resulted in a significant loss of PGCs by interfering with cell cycle progression beyond metaphase. This mitotic defect further led to the activation of DNA damage and the apoptotic cell death of *Mastl*-null PGCs by 12.5 dpc. Therefore, indicating that Mastl-mediated molecular events are indispensable for cell cycle progression in PGCs. However, the metaphase-anaphase transition defects observed in the *Mastl*-null PGCs is rescued by simultaneous deletion of *Ppp2r1a* (α subunit of PP2A). Collectively, our results indicated that the Mastl-PP2A axis plays a fundamental role in controlling PGCs proliferation. At 13.5 dpc, PGCs started to differentiate into female germ cells by entering prophase I of meiosis. Around the time of birth, the primordial follicles assembly occurs in mammalian ovary. In mice, there exist two classes of primordial follicles: the first wave of primordial follicles develops immediately after birth, whereas the second wave of primordial follicles activated later in adult life. To label and trace the *in vivo* development and activation of two waves (or classes) of primordial follicles, we used two different mouse models: *Foxl2-CreERT*^{2} and *Sohlh1-CreERT*^{2}. Our study showed that the first wave of primordial follicles get exhausted around three months of age in mice. Additionally, this class of primordial follicles contributes to the onset of puberty and to early fertility in mice. On the other hand, the primordial follicles in the cortical region of the ovary are activated after three months of age of mice contributing to fertility until the end of reproductive life. One of the hallmarks of the activation of primordial follicles is a squamous-cuboidal transition of primordial follicle granulosa cells (pfGCs). However, how pfGCs–oocytes dialog regulate the activation of primordial follicles and determines their developmental fates are still unclear. To study this, we used *Foxl2-CreERT*^{2} mouse model to show that for maintenance of quiescence of primordial follicles, inhibition of mammalian target of rapamycin complex 1 (mTORC1) signaling in pfGCs is imperative. For this purpose, we deleted *Tsc1* and *Rptor* from pfGCs, which caused over-activation and suppression of activation of primordial follicles respectively. Furthermore, our study also demonstrated that mTORC1-KITL cascade trigger the awakening of dormant oocytes in primordial follicles as initiated by cellular and molecular changes in pfGCs. Mammalian females are endowed with fixed number of primordial follicles in the ovaries, which are depleted gradually and menopause ensued with the exhaustion of pool of primordial follicles. However, many ovarian pathological conditions lead to premature ovarian failure due to early depletion

of follicles pool. Therefore, the existence of OSCs (oogonial stem cells) in ovaries generated excitement in many females suffering from infertility resulting from various reproductions related ailments. This also changed the notion that OSCs might contribute to renewable of follicles as shown by OSCs purified from human and mouse ovaries by DDX4 antibody-based FACS. However, technically the isolation of OSCs by DDX4 antibody-based FACS is in controversy. To verify this claim, we followed the published reported method to isolate OSCs from human and mouse ovaries. Our results showed that isolated DDX4 positive cells from human ovary did not express any *DDX4* mRNA as shown by single-cell mRNA sequencing analysis. Additionally, when these DDX4-positve cells were injected into SCID mice, no follicle formation was noted, thus refuting the reported evidence. Similarly, Ddx4-antibody based FACS sorted mouse ovarian cells also displayed an absence of *Ddx4* mRNA and unable to generate follicles. Our results claimed that previously reported DDX4 positive ovarian cells purified from human and mouse are neither DDX4 expressing cells nor are they functional OSCs.

Keywords: Ovary, primordial follicles, primordial germ cells, Oogonial stem cells, metaphase-anaphase transition

http://hdl.handle.net/2077/46598

2. Abbreviations

AMH	anti-Müllerian hormone
APC/C	anaphase promoting complex/cyclosome
ATP	adenosine triphosphate
BMP	bone morphogenic protein
BPES	blepharophimosis ptosis epicanthus inversus syndrome
Cdk	cyclin-dependent kinase
CL	corpora lutea
Dppa3	developmental pluripotency associated 3
FACS	Fluorescence activated cell sorting
FSH	follicle stimulating hormone
GC	granulosa cell
GDF	growth and differentiation factor
Gwl	Greatwall kinase
KL	kit ligand
LH	luteinizing hormone
mTORC	mammalian target of rapamycin complex
Mastl	microtubule-associated serine/threonine kinase-like
pfGC	primordial follicle granulosa cells
PGC	primordial germ cells
PI3K	phosphatidylinositol 3 kinase
PKA	protein kinase A
POF	premature ovarian failure
PP	protein phosphatase
PTEN	phosphatase and tensin homolog deleted on chromosome ten
OSC	oogonial stem cell
rpS6	ribosomal protein S6
rpS6K	ribosomal protein S6 kinase
TGF	transforming growth factor
TSC	tuberous sclerosis complex
Zp3	zona pellucida
DDX4	DEAD box polypeptide 4

3. List of publications

The thesis is based on the following papers:

Risal, S., Zhang, J., Shao, J., Kaldis, P., and Liu, K. (2016). *Mastl is essential for the metaphase-anaphase transition during proliferation of primordial germ cells in mice* (manuscript)

Zhang, H., Panula, S., Petropoulos, S., Edsgard, D., Busayavalasa, K., Liu, L., Li, X., **Risal, S**., Shen, Y., Shao, J.*, et al.* (2015). *Adult human and mouse ovaries lack DDX4-expressing functional oogonial stem cells*. ***Nature medicine*** *21*, 1116-1118.

Zhang, H., **Risal, S**., Gorre, N., Busayavalasa, K., Li, X., Shen, Y., Bosbach, B., Brannstrom, M., and Liu, K. (2014). *Somatic cells initiate primordial follicle activation and govern the development of dormant oocytes in mice*. ***Curr Biol*** *24*, 2501-2508.

Zheng, W., Zhang, H., Gorre, N., **Risal, S.**, Shen, Y., and Liu, K. (2014). *Two classes of ovarian primordial follicles exhibit distinct developmental dynamics and physiological functions*. ***Hum Mol Genet*** *23*, 920-928.

Papers not included in this thesis:

Adhikari, D., Busayavalasa, K., Zhang J., Hu M., **Risal, S**., Singh M., Diril M.K., Kaldis P., and Liu K. (2016). *Inhibitory phosphorylation of Cdk1 mediates prolonged prophase I arrest in female germ cells and is essential for female reproductive lifespan.* ***Cell Research*** (accepted).

Adhikari, D., Diril, M.K., Busayavalasa, K., **Risal, S**., Nakagawa, S., Lindkvist, R., Shen, Y., Coppola, V., Tessarollo, L., Kudo, N.R.*, et al.* (2014). *Mastl is required for timely activation of APC/C in meiosis I and Cdk1 reactivation in meiosis II.* ***The Journal of Cell Biology*** 206, 843-853.

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.

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

4. Aim of the thesis:

The aim of my Ph.D., and therefore this thesis, was to study the different signaling pathways involved in the development of female germ cells. The study would facilitate the understating of normal physiology and ways to implement a new strategy to treat infertility in the female. For these purposes, novel mice models were generated and studied.

Specific aims:

1. To study the key signaling pathways involve in primordial follicles activation
2. To characterize two waves of primordial follicle activation and development in mice.
3. To study the cell cycle regulation of the primordial germ cells in female embryos before the onset of meiosis.
4. To validate the existence of oogonial stem cells (OSCs) in human and mouse.

5. Introduction

5.1. Mammalian Ovary

The mammalian ovary is complex organ containing follicles at various stages of development and corpora lutea (CL) (Fig.1). The ovary serves two-fold functions: supply the germ cells to produce the next generation and secretes hormones those are essential for follicular development and female secondary sexual characters (McGee and Hsueh, 2000). Therefore, the fate of individual follicle is modulated by paracrine as well as endocrine factors.

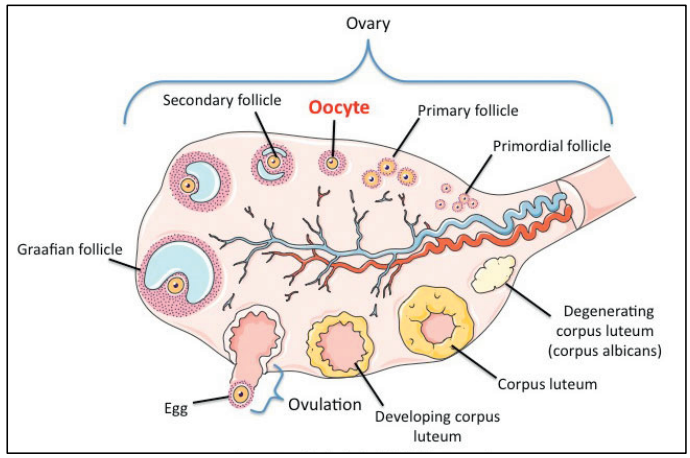

Figure 1. An overview of mammalian ovary (Adapted from https://www.repropedia.org)

Developing follicles passage through primordial, primary, secondary stages and eventually reaches the antral stage (Fig. 1). Thus formed antral follicles have two fates a) the majority of them undergo atretic degeneration and b) a few of them reach preovulatory stage under the influence of cyclic gonadotrophins surge that occurs after puberty (McGee and Hsueh, 2000). In response to a preovulatory surge of cyclic gonadotrophins during each reproductive cycle, the dominant Graafian follicle ovulates to release the mature oocyte for fertilization, whereas the remnant of follicles is luteinized to become CL. These cyclic and sequential changes in follicular development are regulated by hypothalamic-pituitary-ovarian axis (Matzuk et al., 2002; McGee and Hsueh, 2000). The final stage of follicular development is mediated by follicle stimulating hormone (FSH). The essential functions of FSH during antral folliculogenesis are to prevent granulosa cells (GC) apoptosis and follicular atresia, to enhance GC proliferation, to produce estradiol, and promote expression of LH receptor (Edson et al., 2009). After fertilization (the union of oocyte and sperm), a zygote emerges, which after implantation and differentiation, give rise to embryos. The

precursors of primordial germ cells (PGCs) originate in embryo, which serves as the progenitor of germ cells in both male and female.

5.2. Development of primordial germ cells

In mice, PGCs are a precursor of the germ cells in both males and females. The establishment of germ cell fate appears to exist in two pathways in metazoans: 'preformation', which predominates in *Drosophila, Xenopus laevis,* and *C. elegans*. In these models, the embryonic cells inherit the maternal determinants for the development of germ cell lineage (Extavour and Akam, 2003; Saitou, 2009). The second model is 'inductive or epigenesis,' which is common in mammalian PGCs development (Extavour and Akam, 2003; Saitou, 2009). In mice and presumably in all mammals, PGCs are specified through epigenesis mechanism. In this mechanism, the bone morphogenetic protein (BMP) signals from the extraembryonic tissues induced the cells in proximal epiblasts to form precursors of PGCs (Extavour and Akam, 2003).

The genetic studies in mice uncovered that the synergistic actions of bone morphogenetic protein 4 (Bmp4) and Bmp8b are essential for the formation of PGCs. The expression of *Bmp4* and *Bmp8b* occur around 5.5 dpc in extraembryonic ectoderm. Bmp4 and Bmp8b secreted from the extraembryonic ectoderm are essential for the pluripotent proximal epiblast cells to differentiate into PGCs and the allantois (Lawson et al., 1999; Ying et al., 2002).

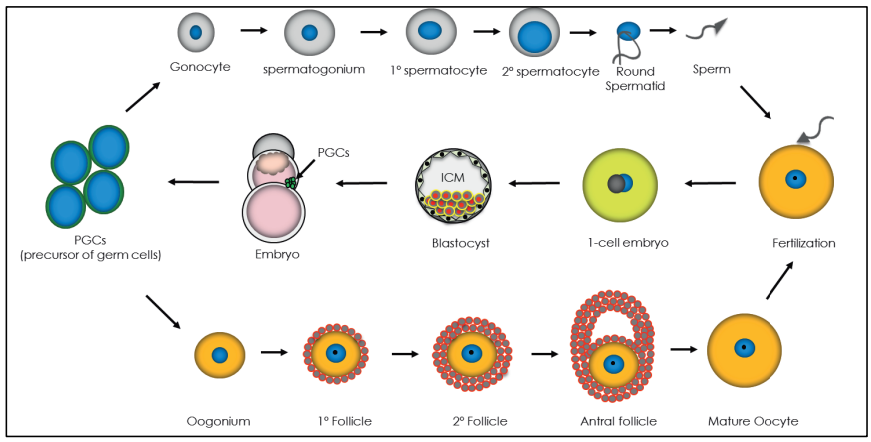

Figure 2. An overview of development of mammalian PGC and germ cells. (Adapted from Schuh-Huerta et al., 2011)

At 5.5–5.75 dpc Bmp4–Smad1/5 signaling pathway directly induced the proximal epiblasts cells leading to the activation of B-lymphocyte-induced maturation protein 1(Blimp1) at 6.0 dpc and PR domain-containing protein 14

(Prdm14) at 6.5 dpc, marking the PGC specification (Ohinata et al., 2009). The expression of *Wnt3* around 5.75 dpc also facilitates PGC specification may be by priming the epiblast cells to express *Blimp1* and *Prdm14* in response to Bmp4 (Ohinata et al., 2009; Ohinata et al., 2005). Moreover, the activation of Wnt3/β-catenin signaling upregulates the expression of pluripotent genes and thus prevents the differentiation of PGCs (Saitou and Yamaji, 2012). Once specified at 7.25 dpc, the expression of *Dppa3* gene (*developmental pluripotency associated 3*; also known as *Pgc7* or *Stella*) marks the establishment of PGCs, which continue to express in PGCs until 13.5 dpc in female and 15.5 dpc in males (Hirota et al., 2011). Then PGCs initiate migration to the developing hindgut (7.75 dpc), into the mesentery (9.5 dpc), and colonize the genital ridges (10.5 dpc) (Molyneaux et al., 2001; Saitou and Yamaji, 2012). During migration into hindgut (7.75–8.75 dpc) the majority of PGCs are arrested at G2 phase of cell cycle (Seki et al., 2007), and PGCs start to proliferate rapidly when they come out from mesentery after 9.5 dpc (Kagiwada et al., 2013). After 9.5 dpc, PGCs maintain the constant rapid proliferative profile at least until 12.5 dpc with doubling time of ~12.6 hours, suggesting that PGCs divide twice per day (Kagiwada et al., 2013). Around 13.5 dpc, female PGCs undergo meiosis, whereas male PGCs are arrested at the G0/G1 phase of cell cycle and resume meiosis only after birth around postnatal day (PD)10 (Miles et al., 2010; Sung et al., 1986). Once PGCs differentiate into oocytes in female or arrested at G0/G1 in male, PGCs lose their PGC properties (Western et al., 2008).

5.3. Development of female germ cells

In female mice, when PGCs differentiate into oocytes, they will go through different sub-stages of meiotic prophase I and eventually are arrested at diplotene around at 17.5 dpc to PD 5 (Borum, 1961; Pepling and Spradling, 2001). These meiotically arrested oocytes are enclosed by somatic cells, known as primordial follicle granulosa cells (pfGCs). The communication between the pfGCs and oocytes in germline cyst is believed to be essential for the formation of primordial follicles (Borum, 1961; Leung and Adashi, 2004; Pepling and Spradling, 2001). The healthy nulliparous woman ovulates 13 times per year from the age of 15 to 50 years, which is less than 500 of the originally endowed follicles (Hansen et al., 2008; Moghissi, 1992). Therefore, the mammalian ovaries contain the finite number of non-growing follicles known as primordial follicles.

Primordial follicles are the earliest follicles formed in the mammalian ovary. These follicles are comprised of an immature oocyte arrested at diplotene stage of meiosis I, enclosed by a flattened pfGC that is presumably the progenitors of GCs (Knobil and Neill, 1988). There are a fixed number of primordial follicles endowed in the mammalian ovaries during the early life, for example, the

neonatal mouse ovaries are populated with 8000 primordial follicles at birth, whereas human ovaries contain 300 000–400 000 primordial follicles at birth per ovary (Hansen et al., 2008; McGee and Hsueh, 2000). This pool of primordial follicles serves as the source of developing follicles and oocytes. The hallmark of primordial follicle activation, the transition from primordial to the primary follicle, is a dramatic growth of oocyte and proliferation and squamous-to-cuboidal differentiation of pfGCs (McGee and Hsueh, 2000; Schmidt et al., 2004).

Primordial follicle has three possible fates: (1) remain dormant; (2) to become activated, mature, and undergo ovulation; (3) or to undergo atresia-either directly from quiescence or after activation (Greenwald, 1972). A primordial follicle activation is an irreversible event, and it can be divided into two parts (Fig. 3): (1) the first wave of activation, where primordial follicles grow right from their birth. The medullary follicles formed during the perinatal period is known as the first wave of primordial follicles (Hirshfield, 1991) (2) the second wave of activation, where primordial follicles remain quiescent for months or years and wait for the enigmatic activation signal. The primordial follicles in the cortical region of the ovary are known as the second wave of or adult primordial follicles (Hirshfield, 1991; Hirshfield and DeSanti, 1995).

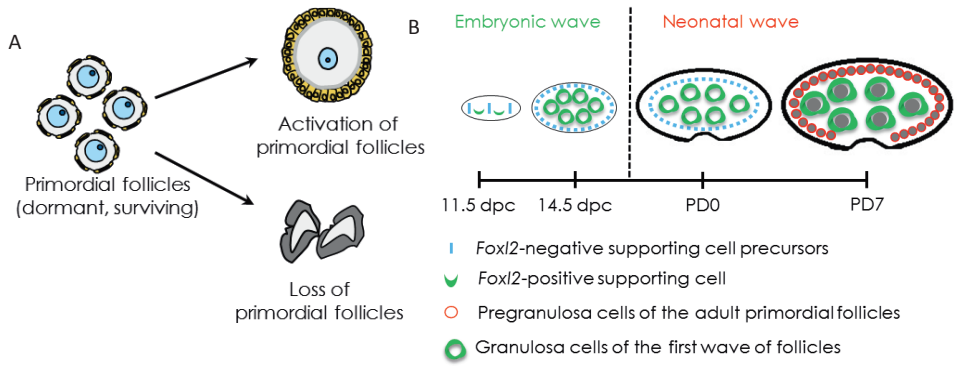

Figure 3. Illustrations are showing (A) the possible fates of primordial follicles. (B) An overview of two waves of primordial follicles activation. (Adapted from Adhikari and Liu, 2009, and Mork et al., 2012)

The pioneer study by Lintern-Moore and Moore (Lintern-Moore and Moore, 1979) suggested that during the activation of primordial follicles, first, a change occurs in pfGCs, and then follows the oocyte growth. The oocyte nucleolar RNA polymerase activity measures the oocyte growth, whereas increased in GCs number determines the transition from flattened to cuboidal morphology

(Lintern-Moore and Moore, 1979). In mouse, when nine GCs surround the oocytes of non-growing follicles, oocytes growth is initiated. Thus, the number of somatic cells enclosing oocyte is a crucial determining factor for the growth of oocyte and is species specific (Cahill and Mauleon, 1981; Gougeon and Chainy, 1987; Lintern-Moore and Moore, 1979). Conversely, the follicular area also increases during growing phases of the oocyte, which marked proliferation and differentiation of pfGCs to GCs (Lintern-Moore and Moore, 1979; McGee and Hsueh, 2000). Thus formed GCs proliferate and become multilayered to form secondary, pre-antral and antral follicles (Hirshfield and DeSanti, 1995). This phenomenon underscored that the oocyte is a passive recipient of developmental signals from the surrounding somatic cells. However, this perspective has been changed and now it is well known that the bidirectional dialog between oocyte and GCs exist, which is necessary for the folliculogenesis (Eppig, 2001; Liu et al., 2006). Moreover, the variable fate of follicles is modulated by the complex interplay of paracrine as well as endocrine factors. The *in vivo* and *in vitro* studies had shown that intra-ovarian factors are regulating the somatic cells and germ cells compartments during the activation of primordial follicles (Moghissi, 1992). Kit/Kit ligand (KL) (Kissel et al., 2000), platelet-derived growth factor (PDGF) (Pinkas et al., 2008), and other growth factors establish the modulatory system in the development of primordial follicles.

5.4. Growth factors involved in activation of primordial follicles

5.4.1. KIT and KIT Ligand

In mice, the receptor tyrosine kinase KIT and its ligand, KITL are the products of *W* (white spotting) and *Sl* (steel) loci respectively (Manova et al., 1990). Both KIT and KIT L are actively expressed in distinct cell lineages during embryogenesis and in adult life. In the ovaries of mouse, rat, and human, KIT is expressed in oocytes of all stages of follicles. However, the KIT L is exclusively voiced by the GCs of growing follicles, although the expression is low in primordial follicles and cumulus cells (Ismail et al., 1996; Laitinen et al., 1995; Manova et al., 1993). The significant number of studies has indicated that the KIT / KIT L signaling is essential for follicular growth. For example, the genetic study of deletion mutants of KIT or KIT L had shown defects in PGCs development and absence of germ cells in their gonads at birth (Yoshida et al., 1997). Additionally, other alleles of *Sl* mutants had more pronounced defects in the postnatal follicular development (Yoshida et al., 1997). *In vitro* study by using KIT -blocking antibodies in ovarian culture concluded the importance of KIT / KIT L in activation of primordial follicles and early folliculogenesis (Parrott and Skinner, 1999). On the other hand, the point mutation study in KIT revealed that the autophosphorylation at Y719 is necessary for docking of the

p85 subunit of phosphatidylinositol 3′ kinase (PI3K) and activation of PI3K pathway. The impairment in the phosphorylation of this site of KIT resulted to compromised and delayed follicular development, leading to infertility in female (Kissel et al., 2000). Therefore, the balance between these intra-ovarian molecules might be involved in regulating the functions of GCs to achieve bi-directional dialog between oocytes and GCs.

5.4.2. Platelet-derived growth factor

PDGF is a putative paracrine molecule regulating the activation of primordial follicles (Kezele et al., 2005b). It exists in different isoforms, namely, PDGF-AA, PDGF-BB homodimers, and PGDF-AB heterodimers. All these isoforms effectively bind to the PDGF receptor alpha (PDGFRα), while PDGF-BB binds specifically to PDGFRβ (Fredriksson et al., 2004; Tallquist and Kazlauskas, 2004). Immunohistochemical localization study and *in vitro* culture of rat ovaries had shown that PDGF and its receptors are expressed in granulosa cells and oocytes of both primordial and early developing follicles (Nilsson et al., 2006; Sleer and Taylor, 2007). Furthermore, in cultured rat ovaries, it was found that PDGF has an active role in the activation of primordial follicles and the upregulation of KL expression (Nilsson et al., 2006). Hence, PDGF synthesized by oocytes may act upon its cognate receptors present in pfGCs and GCs to enhance their KL production, which may promote the follicular activation (Hutt et al., 2006; Nilsson et al., 2006).

5.4.3 Other growth factors

Accumulating *in vitro* studies showed that many other growth factors might have stimulatory functions in the activation of the primordial follicle. The ovarian tissues rich in primordial follicles have been cultured with or without a wide range of putative growth factors in the culture medium. For example, oocyte-derived basic fibroblast growth factor (bFGF) acts on the GCs and theca cells to promote activation of primordial follicles (Nilsson et al., 2001). Similarly, keratinocyte growth factor (KGF) (Kezele et al., 2005a), glial-derived neurotrophic factor (GDNF) (Dole et al., 2008), leukemia inhibitory factor (LIF) (Nilsson et al., 2002), bone morphogenic protein-7 (BMP-7) (Lee et al., 2001), and BMP-4 (Nilsson and Skinner, 2003) have been shown to enhance the activation of primordial follicles *in vitro*.

5.5. Genetic studies in primordial follicles activation in mice

5.5.1. Role of AMH in primordial follicle activation

In mouse and human ovaries, anti-Müllerian hormone (AMH) is produced by granulosa cells of growing follicles and belongs to transforming growth factor β family (Durlinger et al., 2002a). The main physiological function of AMH appeared to regulate primordial follicles activation temporally, as shown in *Amh* $^{-/-}$ mice (Durlinger et al., 1999). In this study, the *Amh* $^{-/-}$ ovaries show a significant depletion of primordial follicles pool by four months of age, indicating that AMH suppresses activation of primordial follicles (Durlinger et al., 1999). By using a neonatal mouse, rat ovaries, and human ovarian cortical tissue, it has been shown that AMH is a negative regulator of primordial follicles activation (Carlsson et al., 2006; Durlinger et al., 2002b; Nilsson et al., 2007). Thus, these *in vitro* findings complemented the *in vivo* results showing that AMH retards the primordial to primary follicle transition.

5.5.2. Oocyte-specific roles of PTEN and PDK1in primordial follicle activation

PTEN (*phosphatase and tensin homolog deleted on chromosome 10*) is a tumor suppressor gene discovered in human, which is often deleted in many human cancers (Cantley and Neel, 1999). The main role of PTEN is to dephosphorylate the 3′ position of the phosphatidylinositol-3, 4, 5-triphosphate (PIP_3) and inactive the PI3K, a main negative regulator of the PI3K pathway (Cantley and Neel, 1999). It has been found that PI3K pathway is operational in mouse oocytes and acts as a suppressor of the activation of primordial follicles (Reddy et al., 2008). *Pten* was deleted from mouse oocytes by using the growth differentiation factor 9 (*Gdf-9)* promoter-driven Cre. *Gdf-9* Cre mediates specific deletion of *Pten* gene in primordial follicles and in further developed follicles. Because of the loss of *Pten* from oocytes of primordial follicles, the resting pool of primordial follicle undergoes premature activation leading premature ovarian failure (POF) and infertility in early adulthood in mice (Reddy et al., 2008). The enhanced growth of *Pten*-null oocytes was due increased activity of Akt as well as elevated phosphorylation of ribosomal protein (rp) S6 (Reddy et al., 2008). This genetic study warrant two issues: (1) the intra-oocyte PTEN-PI3K signaling is an imperative factor to initiate growth of oocytes directly from the quiescence (2) the different fates of primordial follicles are regulated by the activation of PI3K pathway in individual oocytes (Reddy et al., 2008). The hallmark of activation of PI3K pathway is phosphorylation of Akt at two different phospho-sites, namely threonine 308 (T308) and serine 473 (S473) by 3′-phosphoinositide-dependent kinase-1 (PDK1) and mammalian target of rapamycin (mTOR) complex 2 (mTORC2) respectively, which render partial and full activation of Akt. The active Akt then phosphorylates its substrates either activating or inhibiting them. *Pten* was deleted from embryonic ovaries by using *Vasa* promoter-driven Cre, confirming the role of PTEN in regulating the initiation of growth of the oocytes in

primordial follicles (John et al., 2008). However, when *Pten* was deleted in primary and more developed oocytes by using the Zona pellucidia 3 (*Zp3*) promoter-driven Cre, the female mice showed the normal fertility. This study demonstrated that PTEN is indispensable only in primordial follicle oocytes activation, not in primary or further developed oocytes (Jagarlamudi et al., 2009).These *in vivo* observations in mice that the loss of *Pten* gene resulted in premature activation of primordial follicles were further supported by *in vitro* study. The culture of both mouse and human ovaries in the presence of a PTEN inhibitor activate the primordial follicles (Li et al., 2010).

The signaling mediated by PI3K converges primarily at PDK1, and it activates Akt by phosphorylating at T308. Besides Akt, PDK1 also phosphorylates and activates S6K1 and serum- and glucocorticoid-induced protein kinase 1 (SGK1). S6K1 is a key kinase that activates the rpS6 that is essential for protein translation. The ablation of *Pdk1* from mouse oocytes revealed its indispensability in maintaining the survival of primordial follicles, as the majority of primordial follicles were found to be depleted directly from the dormant state around the onset of sexual maturity, resulting in POF during early adulthood (Reddy et al., 2009). Furthermore, this genetic study also underscored that the basal level of PI3K in oocytes is essential to maintain the survival of the primordial follicles (Reddy et al., 2009).

Notably, the combined deletion of *Pten* and *Pdk1* in oocytes prevents the premature activation of all primordial follicles as seen in *Pten*-null oocytes, indicating that PDK1 mostly mediates the excessive follicular activation observed in *Pten*-null oocytes. Therefore, both the activation and survival of primordial follicles are dependent on PDK1 signaling in oocytes. Moreover, these studies emphasized the molecular link involving PI3K/PTEN-PDK1 signaling in oocytes that controls the survival, loss, and activation of primordial follicles.

5.5.3. Oocyte-specific roles of Foxo3a in follicular activation

The forkhead transcription factor 3a (Foxo3a) is a substrate of Akt, which is a downstream effector of the PI3K/PTEN pathway (Castrillon et al., 2003). Foxo3a is mainly expressed in the nucleus of mouse oocytes of primordial follicles and its nuclear expression decrease in oocytes of primary or fully-grown follicles (Castrillon et al., 2003). In *Foxo3a* $^{-/-}$ mice, it has been shown that loss of Foxo3a resulted in the global activation of primordial follicles, thus depleting primordial follicle completely by two weeks after birth (Castrillon et al., 2003). This study showed that the functional role of Foxo3a in maintaining the quiescence of primordial follicle, and further suggest that the down-

regulation of nuclear Foxo3a is pre-requisite for follicular activation (Castrillon et al., 2003).

In vitro culture data of mouse and rat oocytes demonstrated that KL phosphorylates Foxo3a through PI3K, indicating that Foxo3a is downstream of PI3K/PTEN in oocytes (Reddy et al., 2005). To confirm this notion, when *Pten* was deleted from mouse oocytes, Foxo3a was found to be constitutively phosphorylated (T32) by high Akt activity, keeping it in the cytoplasmic state (Reddy et al., 2008). However, the double-mutant mice for oocyte-specific *Pten* and overall *Foxo3a* deletion did not lead to synergistic enhanced follicular activation. This study indicated that Foxo3a suppression contributes partially to the premature activation of primordial follicles in *Pten*-null oocytes (Reddy et al., 2008).

5.5.4. Oocyte-specific roles of Tsc1 and Tsc2 in suppression of follicular activation

In human, the heterodimeric complex of tuberous sclerosis complex 1 (TSC1, hamartin) and TSC2 (tuberin) negatively regulates the mTOR complex 1 (mTORC1) (Huang and Manning, 2009). TSC2 is one of the key substrates of Akt, which upon phosphorylation by it undergoes proteasomal degradation and loses the functional capacity to form the heterodimeric complex with TSC1. Therefore, the function of TSC1 is to stabilize TSC2 and protect it from ubiquitination and degradation (Huang and Manning, 2009). Oocyte-specific deletion of *Tsc1* (Adhikari et al., 2010) or *Tsc2* (Adhikari et al., 2009) from primordial follicles showed that the increased activation of mTORC leads to the premature activation of the primordial follicles and follicular depletion in early adulthood (Adhikari et al., 2010). These studies also showed that low mTORC1 activity is crucial for the maintenance of dormancy of primordial follicles. The increased mTORC1 activity in these studies has been demonstrated by high phosphorylation of the p70 ribosomal protein S6 kinase 1 (S6K1). Thus, these genetic studies in mice revealed that Tsc in oocytes plays a major role in maintaining quiescence of primordial follicles. Although the over-activation of primordial follicles observed in mice by deleting *Tsc1* is phenotypically similar to the oocyte-specific loss of *Pten*. However, these signaling operate distinctly in oocytes to maintain dormancy of primordial follicles, as shown by double knockout mice model of *Tsc1* and *Pten* (Adhikari et al., 2010).

5.5.5. Role of p27 in follicular activation

In mammals, p27, also known as Kip1, regulates cell cycle by binding with cyclin-dependent kinases (Cdks). The expression of p27 is detected in pfGCs and oocyte nuclei of primordial, primary, and secondary follicles (Rajareddy et

al., 2007). However, in the fully-grown follicle, p27 is only expressed in GC but not in oocyte nuclei. In *p27* $^{-/-}$ mice, the loss of p27 resulted in the premature activation of endowed primordial follicles. In early adulthood in these mice developed POF due to depletion of the overactivated follicular pool. Thus, the results showed p27 in oocytes and pfGCs negatively regulates the follicular activation (Rajareddy et al., 2007).

5.5.6. Role of Foxl2 in ovary

Foxl2 is a member of the winged helix or forkhead family of transcription factors and essential for female reproduction. In human, the loss of *FOXL2* caused blepharophimosis/ptosis/epicanthus inversus syndrome (BPES), an autosomal dominant disorder (Crisponi et al., 2001). BPES affected male and female individual show drooping eyelids, a small skinfold running inward from the lower lid, and a broad nasal bridge. BPES type I is characterized by dysplasia of eyelids associated with POF in the female, whereas affected males remain fertile (De Baere et al., 2003). In this type of BPES, the truncation of FOXL2 protein occurs due to a mutation in the forkhead domain of the gene (De Baere et al., 2003). Consistent with human BPES in which ovary and development of eyelid are affected (Cocquet et al., 2002); the mouse *Foxl2* gene was found to be expressed in eyelids and ovarian follicles too (Schmidt et al., 2004). The *Foxl2* expression is detected from 12.5 dpc female embryonic gonads, whereas it is absent in male. Moreover, in the postnatal ovary, *Foxl2* expression is detected in granulosa cells of the ovary (Schmidt et al., 2004; Uda et al., 2004). Genetic studies in *Foxl2* $^{-/-}$ mice have elucidated that Foxl2 is involved in maintaining the dormancy of primordial follicles (Uda et al., 2004). Furthermore, these studies also showed that Foxl2 is imperative for differentiation of pfGCs from squamous to cuboidal state, as a loss of *Foxl2* blocked the differentiation of pfGCs and no secondary follicles are formed. Thus, these findings provided evidence that Foxl2 in pfGCs is essential for inhibition of oocyte growth to maintain quiescence of primordial follicles (Uda et al., 2004).

5.6. Germline Stem Cells in female

The adult stem cells in the gonads destined to be gametes are also known as germline stem cells (GSCs). The GSCs are extensively characterized in *C. elegans* gonads, *Drosophila* testes and ovaries, and mice testes (Lehmann). Male GSCs are often called as SSCs (spermatogonial stem cells) and undergo continuous self-renewal and differentiation into mature spermatozoa, whereas female GSCs, also known as oogonial stem cells (OSCs), are rare across the species (Spradling et al., 2011).

In mammals, a fixed population of primordial follicles is established in the ovary before birth that declines with age, with no new oocytes formed in the adult life (Zuckerman, 1951). This view of a fixed ovarian reserve was challenged in 2004 by proposing that existence of OSCs in postnatal ovaries. This assumption is based on quantitative data indicating that primordial follicles in mice could be renewed at a rate of 77 follicles per day per ovary. Moreover, authors claimed that a group of OSCs residing in ovarian surface epithelium replenished follicle pool continuously in adulthood (Johnson et al., 2004). The detail characterization of OSCs showed that these cells were mitotically active and were positive for the germline marker dead box polypeptide-4 (Ddx4). However, in a subsequent study, the authors amended their previous conclusion and reported that the OSCs were originated from transplanted bone marrow or peripheral blood, not from ovarian surface epithelium (Johnson et al., 2005). Nevertheless, the surgical joining of the circulatory systems of a wild-type mouse and a mouse ubiquitously expressing GFP refuted this claim. Thus, findings revealed that no GFP-positive female germ cells were generated in a wild-type mouse (Eggan et al., 2006), proving that no OSC exist in bone marrow.

As the disputation on the existence of OSC was going on, however, Zou et al. (2009) took a step forward and claimed that they successfully isolated OSCs (which authors named as female germline stem cells in their study) measuring 12-20μm in diameter from neonatal and adult mouse ovaries (Zou et al., 2009). The authors isolated putative OSCs by using a polyclonal anti-human DDX4 antibody and magnetic-activated cell sorting (MACS) technique. Thus, isolated OSCs expressed both pluripotent and germ cell markers. Moreover, these cells also showed normal karyotype with maternal imprinting. Transplanting OSCs in chemotherapy conditioned mouse ovaries has generated the competent oocytes. In the recipient ovaries, OSCs were capable of recruiting supporting cells to form follicles. The oocytes isolated from the recipient female were capable of giving live GFP-positive offspring (Zou et al., 2009). However, two other groups were unable to repeat the work laid by Zou et al. (2009). The authors are isolated putative OSCs by using different techniques, though with a limited demonstration of oocyte competency (Hu et al., 2012; Pacchiarotti et al., 2010).

Recently, the new evidence for the existence of OSCs has been reported based on Zou et al., work by using an adult mouse and human ovaries (White et al., 2012). However, this study used fluorescence-activated cell sorting (FACS) with a DDX4 antibody staining for cell surface expression of DDX4 protein to isolate a small population of cells from adult mouse and human ovaries. It is widely accepted notion that DDX4 localized mainly in the cytoplasm of the cells (Noce et al., 2001; Tanaka et al., 2000), based on which DDX4 antibody-based FACS purification of human and mouse OSC was a controversial method. Notably, the

cell diameter of purified OSC was only 5-8 μm. In context to cell sizes, the OSCs were smaller than 12.5dpc PGCs (10-15 μm) (Mayanagi et al., 2003) and OSC isolated by Zou et al. (12–20 μm) (Zou et al., 2009). Moreover, the purified human and mouse OSCs were able differentiated into oocyte-like cells in culture and able to generate functional oocytes after transplantation/xenograft (White et al., 2012). However, this study failed to demonstrate the polar body extrusion as indicative of meiosis II completion and haploid status of oocytes (Handel et al., 2014). The live cell imaging pinpoints that the Ddx4 expressing cells from postnatal mouse ovaries did not contribute in de novo folliculogenesis (Zhang et al., 2012).

Recently, independent studies led by Lei and Spradling rule out the claimed of the existence of OSCs contributing to follicle renewable in mice (Lei and Spradling, 2013). By using the inducible sensitive lineage-tracing technique, they showed that the pool of primordial follicles established in four weeks is a reservoir for oocytes for ovulation throughout the reproductive life, and there is no replenishment of ovarian follicles. Moreover, this study also indicated that there is no existence of OSCs in adult mouse ovary (Lei and Spradling, 2013). This study is consistent with work of Zhang et al. work that there are no mitotically active germline stem cells in the postnatal mouse ovary (Zhang et al., 2012). Thus, still, there is a lack of comprehensive validation of OSCs in adult mouse and human ovaries, which can change the reproductive dogma.

5.7. Cell cycle of PGCs

The mammalian PGCs are developmentally distinct from other cell types. PGCs are the first lineages to be established in embryos after receiving BMP signals from extra-embryonic (Extavour and Akam, 2003). Rapidly dividing PGCs are unique embryonic cells with doubling time of 12.6 hours, and expand their population from ~200 PGCs at 9.5dpc to ~10000 PGCs at 12.5dpc (Kagiwada et al., 2013). However, there are limited studies revealing mechanisms underlying proliferation of PGCs. The germ-line loss of *Pin1* (*peptidyl-prolyl isomerase*) resulted in depletion of germ cells in both adult male and female mice. The loss of *Pin1*-null PGCs was because of proliferation defects due to prolonged cell cycle, which eventually affect the final pool of germ cells (Atchison et al., 2003). Additionally, the specific deletion of *Pten* (*phosphatase and tensin homolog deleted from chromosome 10*) in PGCs caused the formation of testicular teratoma in male mice. Also, *Pten*-null PGCs showed enhanced the formation of embryonic germ cells as compared to control PGCs (Kimura et al., 2003). This genetic study indicated that *Pten* is involved in the cell cycle of PGCs, and deficiency of *Pten* caused differentiation of PGCs into malignant cells (Kimura et al., 2003). Recently, it has been shown that *Prmt5* (*protein arginine methyltransferase 5*) is essential for maintaining cell cycle in PGCs in

mice. The PGC-specific deletion of *Prmt5* caused cell cycle exit in PGCs and thus preventing the developmental switch occurring between 9.5dpc and 10.5dpc as marked by expression of *Mvh* (Li et al., 2015). Therefore, molecular mechanisms regulating the cell cycle of proliferating PGCs from 9.5dpc to 12.5dpc is not well known and how *Mastl* is involved in proliferation is not studied.

A sequential activation of different types of cyclin-dependent kinases (Cdks) drives the mammalian cell cycle (Santamaria et al., 2007). However, in mice it had been shown that Cdk1 is the only essential Cdk for mitotic entry and progression (Diril et al., 2012; Santamaria et al., 2007). An increase in Cdk1 activity alone is not sufficient to progress through mitosis (Virshup and Kaldis, 2010), and a simultaneous suppression of phosphatases activity is equally important, as shown in *Xenopus* egg extracts and *Drosophila melanogaster* (Bollen et al., 2009; Castilho et al., 2009). Studies in mouse embryonic fibroblasts (MEFs), human cell lines, and *D. melanogaster* showed that Greatwall kinase (Gwl) or its mammalian orthologue microtubule-associated serine/threonine kinase-like (Mastl) activation is essential for mitotic entry and progression (Alvarez-Fernandez et al., 2013; Burgess et al., 2010; Yu et al., 2004). It has been shown in *Xenopus* egg extracts that active Gwl phosphorylates endosulfine α (Ensa) and cAMP regulated phosphoprotein 19 (Arpp19) and convert them into an inhibitor of PP2A (protein phosphatase 2A). Thus, the PP2A activity is suppressed by binding of phosphorylated Ensa or Arpp19 to PP2A-B55 (PP2A with its regulatory subunit B55), which occurs at the same time when Cdk1 activity peaks (Gharbi-Ayachi et al., 2010; Mochida et al., 2009). These regulatory events ensure the maximal phosphorylation of Cdk1 substrates to complete mitosis as shown in *Xenopus* egg extracts (Mochida et al., 2009). The current understanding is that Gwl or Mastl maintains the mitotic state by negative regulation of PP2A activity in *D. melanogaster*, *Xenopus* egg extracts, and MEFs (Alvarez-Fernandez et al., 2013; Gharbi-Ayachi et al., 2010; Yu et al., 2004).

6. Results

Paper I

To investigate the function of *Mastl* in the proliferation of mouse PGCs, we generated PGC-specific knockout mice with specific deletion of floxed allele of *Mastl*, *Ppp2r1a,* and *Cdk1* by using tamoxifen-inducible *Dppa3-CreMER*. To delete *Mastl, Ppp2r1a* and *Cdk1* in PGCs, the tamoxifen was injected at once to 9.5 dpc pregnant female. The deletion of *Mastl* in PGCs resulted in a nearly complete loss of germ cells in ovaries and testes. Moreover, we found that the average numbers of *Mastl* $^{-/-}$ PGCs were significantly lower compared to *Mastl* $^{+/+}$ PGCs at 12.5 dpc. The FACS analysis of 11.5 dpc PGCs in female gonads showed that 58% of the *Mastl* $^{-/-}$ PGCs were arrested at the G2 phase of cell cycle compared to 39% of the *Mastl* $^{+/+}$ PGCs. However, 11.5 dpc *Mastl* $^{-/-}$ PGCs entered mitosis normally as shown by condensed chromosomes in tissues sections as well as in metaphase spread. Thus, our results suggest that deletion of *Mastl* from PGCs at 9.5 dpc resulted in a G2 arrest, but *Mastl* $^{-/-}$ PGCs were able to enter and reach metaphase normally.

To determine whether the *Mastl* $^{-/-}$ PGCs can proceed to anaphase normally, we arrested 12.0 dpc *Mastl* $^{+/+}$ and *Mast* $^{-/-}$ PGCs in prometaphase by culturing female embryonic gonads in nocodazole for 4 h. By 60 minutes after release from nocodazole, 26.5% of the *Mastl* $^{+/+}$ PGCs were in metaphase, and 18.5% of the *Mastl* $^{+/+}$ PGCs were in anaphase. In contrast, 23.2% of the *Mastl* $^{-/-}$ PGCs reached metaphase by 60 minutes after release from nocodazole, but these mutant PGCs failed to proceed to anaphase. Instead, *Mastl* $^{-/-}$ PGCs exhibited abnormal cellular morphology with fragmented DNA, indicating a failed metaphase-anaphase transition. Even at 90 minutes after nocodazole release, the *Mastl* $^{-/-}$ PGCs did not proceed into anaphase demonstrating that *Mastl* $^{-/-}$ PGCs could enter metaphase normally but could not undergo the metaphase-anaphase transition and suffered from fragmented DNA as a result of the mitotic catastrophe. We also observed that *Mastl* $^{-/-}$ PGCs formed micronuclei in addition to binucleate and giant cells and suffered from DNA damage and the apoptotic pathway by 12.5dpc.

To investigate the co-contribution of PP2A in PGC development, we generated double mutant mice lacking both *Ppp2r1a* (the PP2A α-subunit) and *Mastl* in PGCs. The simultaneous deletion of *Mastl* and *Ppp2r1a* appeared to rescue the phenotype observed in *Mastl* $^{-/-}$ PGCs and resulted in normal-looking populations of follicles in PGC-*Mastl* $^{-/-}$; *Ppp2r1a* $^{-/-}$ ovaries. We further confirmed that PGCs in PGC-*Mastl* $^{-/-}$; *Ppp2r1a* $^{-/-}$ female embryonic gonads develop and normally survive at 12.5 dpc, and there were no abnormal nuclear structures, especially micronuclei such as those seen in *Mastl* $^{-/-}$ PGCs at 12.5

dpc. These results strongly indicate that PP2A is a downstream target of Mastl in mouse PGCs.

As expected, 12.0 dpc *Mastl* $^{-/-}$; *Ppp2r1a* $^{-/-}$ female embryonic gonads 60 min after release from nocodazole arrest 10% of the *Mastl* $^{-/-}$; *Ppp2r1a* $^{-/-}$ PGCs entered anaphase, indicating that the loss of PP2A in *Mastl* $^{-/-}$ PGCs at least partially rescued the metaphase-anaphase transition defect. Therefore, the results indicated that the *Mastl* $^{-/-}$ phenotype is likely to be mediated by increased PP2A activity.

Paper II

To study the development of two classes of primordial follicles, we had used two different mouse models: *Forkhead box L2* (*Foxl2)-CreERT2; mT/mG* and *Spermatogenesis and oogenesis-specific basic helix-loop-helix 1(Sohlh1)-CreERT2, R26R*. These mouse models specifically label the first wave of activated follicles in the medulla of the ovary and the primordial follicles in the cortex, respectively. To label the first wave of follicles, we gave a single injection of tamoxifen to pregnant females at16.5 dpc. In this way, we specifically labeled only follicles in the medullary region, whereas the follicles in the cortical remained unlabeled. Thus, labeled the first wave of follicles remained dominant at an early age and dwindled gradually. This batch of follicles was exhausted in ovary by three months of age. After three months of age, the pool of unlabeled first wave of primordial follicles replaced the labeled first batch of follicles.

Tamoxifen was injected at three months of age to label the adult wave of primordial follicles in mice. In this way, the primordial follicles in the cortical regions were specifically labeled as shown by β-galactosidase staining in the oocytes. The developmental tracing of labeled follicles revealed that it took at least 7 days, 23 days, 37 days, and 47 days for adult primordial follicles to reach primary, secondary, early antral and antral stages. Our study also indicated that the pool of primordial follicles labeled at 3 months of age remained in the ovary until the end of reproductive life in mice. The study clarifies the existence of distinct developmental dynamics of two classes of primordial follicles.

Paper III

In this study, we deleted *Rptor* (regulatory-associated protein of MTOR, complex 1) in pfGCs by using tamoxifen-inducible *Foxl2* promoter-mediated *CreERT*[2] fusion protein (*Foxl2-CreERT2* mice), to determine how follicular activation and the quiescence and awakening of dormant oocytes might be regulated by pfGCs in primordial follicles. Although no apparent morphological difference was found in PD5 pfGC-*Rptor* $^{-/-}$ and pfGC-*Rptor* $^{+/+}$ ovaries, which contained mostly with primordial follicles. This result indicated that the loss of *Rptor* from pfGC does not affect the primordial follicles formation postnatally. The pfGC-*Rptor* $^{-/-}$ ovaries appeared much smaller than pfGC-*Rptor* $^{+/+}$ ovaries at PD13, indicating that the oocytes in the mutant ovaries remained in a dormant state in primordial follicles. At PD35, ovaries of pfGC-*Rptor* $^{-/-}$ remained smaller compared to pfGC-*Rptor* $^{+/+}$ ovaries, as the mutant ovaries contained larger number of primordial follicles. Thus, these results showed that the ablation of *Rptor* in pfGCs resulted in the suppression of primordial follicles activation and preventing the awakening of the dormant oocytes in mice. The deletion of *Tsc1* (*tuberous sclerosis complex1*), a negative regulator of mTORC1 signaling, in pfGCs resulted in the activation of the primordial follicles and awakening of the dormant oocytes in mice. When *Tsc1* was deleted from pfGCs of neonatal ovaries, no apparent morphological difference in the development of oocytes was seen at PD10. However, by PD23 pfGC-*Tsc1* $^{-/-}$ ovaries were found to be larger than pfGC-*Tsc1* $^{+/+}$ ovaries. These results showed that premature activation of primordial follicles ensued, with the awakening of the dormant oocytes. All primordial follicles were activated in pfGC-*Tsc1* $^{-/-}$ ovaries compared to pfGC-*Tsc1* $^{+/+}$ ovaries, which still contained the primordial follicles. By 4 months of age, pfGC-*Tsc1* $^{-/-}$ ovaries were smaller and did not have any healthy follicular structure. As a control, pfGC-*Tsc1* $^{+/+}$ ovaries were larger, showing healthy follicles. Therefore, these results demonstrate that the enhanced mTORC1 signaling caused premature differentiation and proliferation of pfGCs into granulosa cells and the awakening of quiescent oocytes in primordial follicles.

The loss of *Tsc1* in pfGCs also caused enhanced intra-oocyte PI3K signaling, as shown by immunofluorescence staining for FOXO3 (forkhead box O3) in pfGC-*Tsc1* $^{-/-}$ ovaries. The results demonstrated a clear localization of FOXO3 in pfGC-*Tsc1* $^{-/-}$ oocytes, indicating constitutively active PI3K signaling. However, a nuclear localization of FOXO was seen in pfGC-*Tsc1* $^{+/+}$ oocytes of primordial follicles. Therefore, our results showed the awakening of pfGC-*Tsc1* $^{-/-}$ oocytes in primordial follicles were initiated via activation of PI3K pathway.

In vitro culture of pfGC-*Tsc1* $^{-/-}$ ovaries with KIT inhibitor prevented the awakening of oocytes in primordial follicles. The results indicated that the

network between KITL in pfGCs and KIT in oocytes might involve in the premature awakening of dormant oocytes in pfGC-*Tsc1* $^{-/-}$ ovaries. Moreover, these results were further supported by *in vivo* studies by using double mutant pfGC- *Tsc1* $^{-/-}$; Kit^{Y719F}/Kit^{Y719F} mice that carry a point mutation in which KIT Y719 is substituted by a phenylalanine (F) with simultaneous deletion of *Tsc1*. This study revealed that *Kitl* expression in pfGCs is regulated by mTORC1 signaling in mice pfGCs and the suppressed of the awakening of oocytes in primordial follicles in pfGC- *Tsc1* $^{-/-}$; Kit^{Y719F}/Kit^{Y719F} ovaries was due to the failure of activation of PI3K signaling in oocytes.

Based on these results, the activation of mTORC1 in pfGCs mediates the proliferation and differentiation of pfGCs to granulosa cells and expression of KITL. The binding of KIT and KITL results in the phosphorylation of KIT Y719 leading to the activation of PI3K in oocytes, which initiates the awakening of oocytes from a dormant state.

Paper IV

In this study, we first isolated human ovarian cells by DDX4-based FACS approach. However, our results showed that the isolated cells did not show any *DDX4* mRNA expression by validated qPCR as well as by single-cell mRNA sequencing. Additionally, the single-cell mRNA sequencing results revealed that these cells also expressed the genes found in differentiated ovarian somatic cells. Both cultured DDX4-positive, as well as DDX4-negative cells, showed no DDX4 expression as revealed by immunofluorescence staining for DDX4. Taken together, these data demonstrated that DDX4 is not expressed in the sorted DDX4-positive human ovarian cells. Xenografting of human cortical ovarian slice injected with cultured GFP positive DDX4-positive cells in SCID mice did not develop into GFP- positive oocytes as reported. Thus, the DDX4-positive human ovarian cells are not functional stem cells, as these cells cannot regenerate oocytes. Similar results were obtained from the Ddx4-positive mouse ovarian cells. As Ddx4 is a well-established marker for germ cells, however, Ddx4-positive cells were isolated from non-ovarian tissues like liver, spleen, and kidney; indicate a non-specific reactivity of Ddx4 antibody. Based on these results, the use of the DDX4-specific antibody for FACS sorting of OSCs in human and mice is not viable, as this method does not select for the DDX4-expressing cells.

7. Conclusion

The mammalian PGCs are developmentally distinct from other cells and are precursor cells for oocyte and sperm. PGCs are specified and establish after receiving bone morphogenetic protein signals from the extraembryonic tissues. Moreover, these cells are among the first lineages to be established in mammalian embryos (Extavour and Akam, 2003). In addition, PGCs are unique embryonic cells dividing rapidly twice per day, thus expanding their population from ~45 cells at 7.5 dpc to ~10 000 cells at 12.5 dpc in mice (Kagiwada et al., 2013). However, only a limited number of studies have focused on the mechanisms underlying the proliferation of PGCs. The loss of *Pin1* (*peptidyl-prolyl isomerase*) results in proliferation defects in *Pin1*-null PGCs due to a prolonged cell cycle, leading to germ cells deficient gonads in the adult mice (Atchison et al., 2003). Additionally, the loss of *Pten* causes the differentiation of PGCs into malignant cells and leads to testicular teratoma in male mice and formation of the embryonic germ cells formation in *Pten*-null PGCs in culture (Kimura et al., 2003). Recently, it has been shown that *Prmt5* (*protein arginine methyltransferase 5*) is essential for maintaining the cell cycle in PGCs in mice. The PGC-specific deletion of *Prmt5* causes cell cycle exit in PGCs and thus prevents the developmental switch that occurs between 9.5 dpc and 10.5 dpc as marked by the expression of *Mvh* (Li et al., 2015). These findings are valuable for studying cell cycle regulation in PGCs in the context of the germ cells development.

Our study showed that the first wave of activated primordial follicles in mouse ovaries is exhausted by 3 months after birth. These follicles are mainly involved in the onset of puberty and to fertility in early adulthood. The second wave of primordial follicles is formed after birth and located in the ovarian cortex. This population of follicles gradually replaces the first wave of follicles and serves as the sole source of the follicles until the end of the reproductive life. Therefore, recent studies in mice had revealed the unique fates and physiological functions of the first wave of primordial follicles and adult primordial follicles in ovaries. These findings in rodent may help to understand the development of individual follicle in human, especially in pre-pubertal and pubertal girls. The primordial follicles in human start from the fourth month of fetal life and completed by birth (Gougeon, 1996). Therefore, there might be two distinct populations of primordial follicles in the human ovary, as seen in the mouse ovary.

The follicles in mammalian ovaries are basic functional units of reproduction. The development of follicles starts from fetal life, and primordial follicles are first follicles to be found in ovaries. The elucidation of how the pfGCs regulate the primordial follicle activation is the most important topic in reproductive biology. In this thesis, the study demonstrated that the essential networking

between mTOR and KIT–KL signaling regulates the size of the ovarian follicles in mice. mTOR signaling is governed by nutrition, stress, oxygen, energy, and growth factors and KIT ligand signaling are downstream of the mTOR pathway. Therefore, to investigate the roles of local environmental factors on the primordial follicle activation will be interesting. Moreover, it is possible that by using an mTOR activator or KIT ligand together will be helpful to treat infertility.

The existence of OSCs and their involvement in oogenesis is debated topic in the field of reproductive biology. If normal OSCs exist, might prove useful for advancing reproductive health and for stem-cell-based therapies. However, the existence of OSCs in human and mouse remained to be surreal. After following the reported protocols published by White et al. (White et al., 2012), we could not recapitulate their claim of the existence of OSCs in human and mouse ovaries. The major limitation in reported study (White et al., 2012) was using the DDX4-antibody based FACS sorting of OSCs, which isolate DDX4-positive unspecific cells, and thus failed to give fertilizable oocytes. Therefore, a method that is more robust needs to be developed for finding of putative OSCs in ovaries.

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