Doctoral thesis for the Degree of Doctor of Philosophy

Molecular mechanisms of ovarian follicular development and early embryogenesis

Nagaraju Gorre

Department of Chemistry and Molecular Biology
University of Gothenburg
Gothenburg, Sweden

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Department of Chemistry and Molecular Biology,

University of Gothenburg, SE-40530,

Gothenburg, Sweden.

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To my family

ABSTRACT

In the mammalian ovary, the dormant primordial follicles are the source of developing follicles and fertilizable ova for the entire reproductive life. In addition, the duration of fertility of a female is determined by the initial size of her pool of primordial follicles and by the rate of its activation and depletion. Menopause (the end of female reproductive life), also known as ovarian senescence occurs when the pool of primordial follicles is exhausted. However, the molecular mechanisms underlying the reproductive aging and menopausal age in females are poorly understood. In this thesis, by generating the oocyte-specific deletion of *Rptor*, *Tsc2* and *Pdk1* in mice, I have thus studied PI3K-mTORC1 signaling in oocytes in physiological development of follicles and early embryogenesis of mice.

We provided *in vivo* evidence that deletion of *Rptor* in oocytes of primordial and further developed follicles leads to the ablation of mTORC1 signaling. However, upon the loss of mTORC1 signaling in oocytes, follicular development and fertility of mice lacking *Rptor* in oocytes were not affected. Interestingly, PI3K signaling was found to be elevated upon the loss of mTORC1 signaling in oocytes, and become essential to maintain normal physiological development of ovarian follicles and fertility of females. Therefore, it indicates that the loss of mTORC1 signaling in oocytes triggers a compensatory activation of the PI3K-Akt signaling that maintains normal ovarian follicular development and fertility.

However, the female mice lacking *Tsc2*, a negative regulator of mTORC1, in oocytes produced at most two litters of normal size and then became infertile in young adulthood. We found that the mTORC1–S6K1–rpS6 signaling is elevated upon the deletion of *Tsc2* in oocytes, leading to the overactivation of pool of primordial follicle in ovaries of mice lacking *Tsc2* in oocytes. Consequently, the ovaries lacking *Tsc2* in oocytes were observed to be completely devoid of follicles, causing POF in early adulthood. Therefore, we identified the *Tsc2* gene as an essential factor in oocytes to preserve the female reproductive lifespan by suppressing the activation of primordial follicles.

Furthermore, we had shown that blockage of maternal PI3K signaling by deletion of *Pdk1* from primary oocytes leads to the arrest of resultant embryos at the two-cell stage, which is most probably a consequence of suppressed EGA and a defective G2/M phase at the two-cell stage. Surprisingly, concurrent loss of maternal *Pten* recovered the impaired Akt activation, rescued the suppressed EGA and two-cell arrest of embryos, and restored the fertility of double-mutant females. We therefore identified the maternal PI3K/Pten–Pdk1–Akt signalling cascade as an indispensable maternal effect factor in triggering EGA and sustaining preimplantation embryogenesis in mice.

In summary, Tsc2/mTORC1 signaling in oocytes is essential for the maintenance of quiescence and the survival of primordial follicles, and thereby controls the reproductive aging and menopausal age in females. Furthermore, the molecular network involved in PI3K/Pten–Pdk1–Akt signalling is crucial for EGA and preimplantation embryogenesis in mice.

Key words: ovary, primordial follicles, Embryogenesis, PI3K-mTORC1 signaling.
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3. Zheng, W., **Gorre, N**., Shen, Y., Noda, T., Ogawa, W., Lundin, E., and Liu, K. (2010). Maternal phosphatidylinositol 3-kinase signalling is crucial for embryonic genome activation and preimplantation embryogenesis. EMBO reports 11, 890-895.

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3. Adhikari, D., Zheng, W., Shen, Y., **Gorre, N**., Ning, Y., Halet, G., Kaldis, P., and Liu, K. (2012). Cdk1, but not Cdk2, is the sole Cdk that is essential and sufficient to drive resumption of meiosis in mouse oocytes. Human molecular genetics *21*, 2476-2484.

4. Adhikari, D., Zheng, W., Shen, Y., **Gorre, N**., Hamalainen, T., Cooney, A.J., Huhtaniemi, I., Lan, Z.J., and Liu, K. (2010). Tsc/mTORC1 signaling in oocytes governs the quiescence and activation of primordial follicles. Human molecular genetics *19*, 397-410.

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ABBREVIATIONS

CL	Corpora lutea
PGCs	Primordial germ cells
NOBOX	Newborn ovary homeobox protein
SYCP-1	Synaptonemal complex protein 1
FOXL2	Forkhead box protein L2
COC	Cumulus oocyte complex
POF	Premature ovarian failure
MTOC	Microtubule-organizing center
EGA	Embryonic genome activation
H1FOO	H1 histone family member oocyte specific
ICM	Inner cell mass
EPI	Pluripotent epiblast
PE	Primitive endoderm
UTR	Untranslated region
CPE	Cytoplasmic polyadenylation element
BrUTP	5-bromouridine 5'-triphosphate
PI3K	Phosphatidylinositol 3-kinase
RPTK	Receptor protein tyrosine kinase
GPCR	G-protein coupled receptors
IGF-1	Insulin-like growth factor 1
PDGF	Platelet-derived growth factor
EGF	Epidermal growth factor
PH	Pleckstrin homology
mTORC1	Mammalian target of rapamycin complex1
mTORC2	Mammalian target of rapamycin complex2
SH2	Src homology 2
MMAC	Mutated in multiple advanced cancers
Pten	Phosphatase and tensin homolog
PKB	Protein kinase B
Akt	V-akt Murine Thymoma Viral Oncogene Homolog
TGF	Transforming growth factor
S6K	Ribosomal S6 kinase
SGK	Serum-and glucocorticoid-induced protein kinase
Pdk1	3-phosphoinositide dependent protein kinase 1
Tsc	Tuberous sclerosis complex
ERK	Extracellular signal regulated kinase
PIKK	Phosphoinositide 3-kinase related kinase
Raptor	Regulatory associated protein of mTOR
PRAS40	Proline-rich AKT substrate 40
Deptor	DEP domain-containing mTOR-interacting protein
FRB	FKBP12-rapamycin-binding
FKBP12	FK506-binding protein 12 kDa
AMPK	AMP-activated protein kinase
4e-bp1	4e-binding protein 1
ATG13	Autophagy-related 13
hESC	Human embryonic stem cell
RBD	Ras binding domain

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1 INTRODUCTION

1.1 The mammalian ovary

The mammalian ovary is a heterogeneous organ containing follicles at various stages of development and corpora lutea (CL). The mammalian ovary is not only the female gonad supplying germ cells to produce the next generation but also the female reproductive gland controlling many aspects of female development and physiology (McGee and Hsueh, 2000; Edson et al., 2009). 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; Richards et al., 2002; Vanderhyden, 2002). The individual follicles in mammalian ovaries consist of an innermost oocyte that is surrounded by granulosa cells, and outer layers of thecal cells (Edson et al., 2009) (Fig. 1).

1.1.1 Formation of follicles

In mice, oocytes are developed from primordial germ cells (PGCs), which are first discernible in the extra-embryonic mesoderm at embryonic day 7.5 (E7.5) under the influence of signals from extra embryonic ectoderm-derived bone morphogenetic protein 4 (BMP4), BMP8b, and extra embryonic endoderm-derived BMP2 (Lawson and Hage, 1994; Ying et al., 2000; Ying and Zhao, 2001). The PGCs migrate through hindgut and dorsal mesentery to colonize the genital ridge at E10.5. The PGCs undergo several mitotic divisions and multiply in number throughout their migration (Ginsburg et al., 1990). The dividing PGCs are connected together by intercellular bridges due to the incomplete cytokinesis, resulting in the formation of many germ cell clusters or oogonia in female gonad (Pepling and Spradling, 1998). By E13.5, oogonia begin to enter meiosis and are then referred to as oocytes (McLaren and Southee, 1997; McLaren, 2000). Oocytes within clusters progress through the stages of prophase I of meiosis and arrest in the diplotene stage at approximately E17.5 (Borum, 1967). The germ cell cysts are surrounded by a few numbers of somatic cells that are also called as pregranulosa cells. The primordial follicles are formed when the pregranulosa cells invade the germ cell cysts and break apart the oocytes (McNatty et al., 2000; Pepling and Spradling, 2001). During the primordial follicle formation, proper communication between the pregranulosa cells that surround the cysts and the oocyte is essential (Guigon and Magre, 2006) and the pregranulosa cells take an active part during the follicle formation (Epifano and Dean, 2002). However, there is a massive loss of oocytes when the oocyte clusters begin to break down to form primordial follicles (Pepling and Spradling, 2001). The exact molecular mechanisms behind this loss remain elusive.

The primordial follicles in mice are visible by postnatal day 3 (PD3) and by PD7 the formation of primordial follicles is virtually completed (Choi and Rajkovic, 2006). It has been demonstrated that during the process of cysts breakdown and primordial follicle formation, hormones play an important role. For instance, treatment of PD1 mouse ovaries with progesterone, estradiol or genistein prevents the cysts breakdown and primordial follicle formation (Chen et al., 2007). The maternal estrogen and progesterone prevent the cysts from breaking down before birth and prevent the process of initial follicle formation (Kezele and Skinner, 2003; Chen et al., 2007).

During the past decade, many genetic studies have shed light on the different molecules that are expressed in granulosa cells and oocytes, and play a major role during the formation of primordial follicles. For example, Figla (Factor in the germline alpha), a transcription factor expressed in both germ cells and postnatal oocytes, is required for the expression of zona pellucida proteins. The female mice that lack Figla do not have any primordial follicles, and all the oocytes are depleted after birth (Soyal et al., 2000). The germ cell-specific marker NOBOX (Newborn ovary homeobox protein) is expressed in oocytes, and in its absence there is a delay in germ cell cysts breakdown and increased oocyte loss (Rajkovic et al., 2004). Furthermore, the oogonia that lack synaptonemal complex protein-1 (SYCP-1) complete meiotic prophase-1 early and primordial follicles are formed early (Paredes et al., 2005). In addition, the Foxl2 gene is specifically expressed in the eyelids and ovaries of mammals (Crisponi et al., 2001; Cocquet et al., 2002). In mice, Foxl2 is expressed in pregranulosa cells of primordial follicles; the expression level is reduced in granulosa cells of preantral follicles (Uda et al., 2004). Interruption of Foxl2 gene in mice is found to prevent the pregranulosa cells from undergoing squamous to cuboidal transition (Schmidt et al., 2004).

1.1.2 Classification of follicles

There are various classification methods that have been used to describe the stages of ovarian follicle development. The ovarian follicles were classified based on the shape and the number of layers of the granulosa cells surrounding the oocyte (Adams and Hertig, 1964); diameter or the volume of the follicles (Paesi, 1949); a combination of the number of cell layers and follicle diameter (Ingram, 1959). However, variations in the size of the oocyte and the follicle had rarely been taken into account to describe the follicle development. Terms like primordial follicles, small follicles and primary follicles had been used to describe follicles with a single layer of cells attached to the oocytes. Secondary and growing follicles were the follicles with several layers of cells surrounding the oocyte. Tertiary, large, vesicular, Graafian or preovulatory follicles were some of the terms used for various later stages of follicle development.

The well-known method of classification of ovarian follicles was based on the size of the oocyte in follicles of different stages of development, the size of the follicle defined by the number of cells constituting the follicular envelope and the morphology (Pedersen and Peters, 1968). Based on this method, the follicles were divided into three main groups: small, medium and large follicles. These are further subdivided according to the number of follicle cells counted on the largest cross-section of the follicle and taking its morphological appearance into account. They are small follicles (type 1), type 2 and type 3a (transient follicles), medium-sized follicles (type 3b), type 4, type 5a, type 5b, type 6, type 7 and type 8. The type 1 follicles are composed of a small oocyte with no follicle cells attached to its surface while Type 2 follicle consists of a small oocyte that has a few cells attached to its cell surface. The Type 3a has a complete ring of follicle cells that surrounds the oocyte, which is usually small or might have started to grow and there are not more than 20 follicle cells on the largest cross-section, whereas the medium-sized follicles (type 3b) have one complete ring of follicle cells that surrounds a growing oocyte and there are 21 to 60 cells on the largest cross-section. The follicles where two layers of follicle cells surround a growing oocyte are called as type 4 follicles. The type 4 follicles have 61 to 100 cells on the largest cross-section. Type 5a follicles are the follicles that are in transitory stage between medium-sized and large follicles. This type has three layers of follicle cells and 101 to 200 cells on the largest cross-section. Large follicles (type 5b) have a fully-grown oocyte that is surrounded by many layers

of follicle cells. There are 201 to 400 cells on the largest cross-section. Type 6 follicles have a large oocyte with many layers of cells. Scattered areas of fluid separate the follicle cells. On the largest cross-section, there are 401 to 600 cells. Type 7 follicles are follicle with a single cavity containing follicle fluid. There are more than 600 cells on the largest cross-section. The formation of cumulus oocyte complex (COC) takes place. Type 8 (preovulatory follicle) is a large follicle with a single cavity with follicle fluid and a well-formed cumulus oocyte complex.

1.1.3 Primordial follicles-the dormant ovarian follicles

The first small follicles to be appeared in the mammalian ovary are termed primordial follicles. The primordial follicles remain dormant and surviving, and are mainly localized at the cortex of the ovary. The primordial follicle consists of an oocyte arrested at the diplotene stage of meiosis I, which is surrounded by several flattened pregranulosa cells (Borum, 1961; Peters, 1969). It is not well known that when the first primordial follicle appears in the mammalian ovary. However, it has been shown that the first primordial follicle is formed at around 15–22 wk gestation in human fetuses when a single layer of pregranulosa cells encloses oocyte (Maheshwari and Fowler, 2008). This process continues until just after birth (Baker, 1963; McGee and Hsueh, 2000). In contrast, the formation of primordial follicles takes place within a few days of birth in rats and mice (Hirshfield, 1991). The pool of dormant primordial follicles serves as the source of all developing follicles and fertilizable ova for the entire duration of reproductive life. Each primordial follicle, however, has three possible developmental fates: (i) to remain quiescent (i.e. to survive in dormancy for various lengths of time throughout the reproductive period); (ii) to be activated into the growing follicle pool, that is either followed by atresia at a later stage of follicular development or by ovulation; (iii) to undergo death directly from the dormant state, contributing to female reproductive aging (McGee and Hsueh, 2000; Broekmans et al., 2007; Hansen et al., 2008).

During the reproductive years in humans, the decline in the number of primordial follicles remains steady at about 1000 follicles per month and accelerates after the age of 37 yr, causing ovarian aging. The general belief is that when the available pool of primordial follicles has become depleted, reproduction ceases and women enter menopause (Hirshfield, 1991; Broekmans et al., 2007). At the time of menopause, the number of follicles remaining drops below 1000 (Faddy and Gosden, 1996; Broekmans et al., 2007; Hansen et al., 2008).

The activation of primordial follicles is generally subdivided into two broad categories: initial activation of primordial follicles, which occurs throughout life until menopause; and, after puberty, cyclic recruitment of a limited number of small follicles from the growing cohort, from which a subset is selected for dominance and ovulation. The initial recruitment of primordial follicles starts soon after the completion of formation of the follicles (McGee and Hsueh, 2000). The activation of primordial follicles is defined by a dramatic growth of the oocyte itself, which is accompanied by differentiation and proliferation of the surrounding pregranulosa cells (McGee and Hsueh, 2000). In contrast, it had earlier been suggested that during the activation of primordial follicles to primary follicles, there is first a change in the pregranulosa cells, and then oocyte growth follows (Lintern-Moore and Moore, 1979). However, both the oocyte and the granulosa cells are interdependent for their growth and survival. The bidirectional communication between oocyte and granulosa cells is essential for the follicular development in the mammalian ovary, from the primordial follicle formation, activation and ovulation (Eppig, 2001). During the activation of a primordial follicle to a

primary follicle, the growth of the oocyte *per se* is remarkable. For instance, in the mouse, during follicular activation and early development the oocyte grows aggressively with an almost 300-fold increase in volume during this 2 to 3-wk growth phase (Peters et al., 1975; Elvin and Matzuk, 1998; Matzuk et al., 2002). This growth phase is also accompanied by a 300-fold increase in RNA content (Sternlicht and Schultz, 1981) and a 38-fold increase in the absolute rate of protein synthesis, as calculated per hour and per oocyte (Schultz et al., 1979). These events are indicative of a period of robust growth of the oocyte cell, with intense metabolic activity.

The activated primordial follicles pass through different stages of follicular development and the fate of growing follicle is defined by many endocrine and paracrine factors (McGee and Hsueh, 2000). The recruitment of follicles is a continuous process that is controlled by the hypothalamus-pituitary-ovary axis (Elvin and Matzuk, 1998; McGee and Hsueh, 2000; Matzuk et al., 2002; Richards et al., 2002; Vanderhyden, 2002). Based on the number of growing follicles in the ovary throughout life, it has been estimated that more than 90% of the dormant primordial follicles die during infancy and early adulthood (Gougeon et al., 1994; Gougeon, 1996). The dormant primordial follicles leave the resting pool continuously, either by entering into the growing phase or by undergoing death. Once the mammalian ovary becomes devoid of the primordial follicles, the reproductive life comes to an end and menopause follows. Moreover, the reproductive lifespan and timing of menopause in a woman are decided by the duration of survival and the rate of loss of primordial follicles.

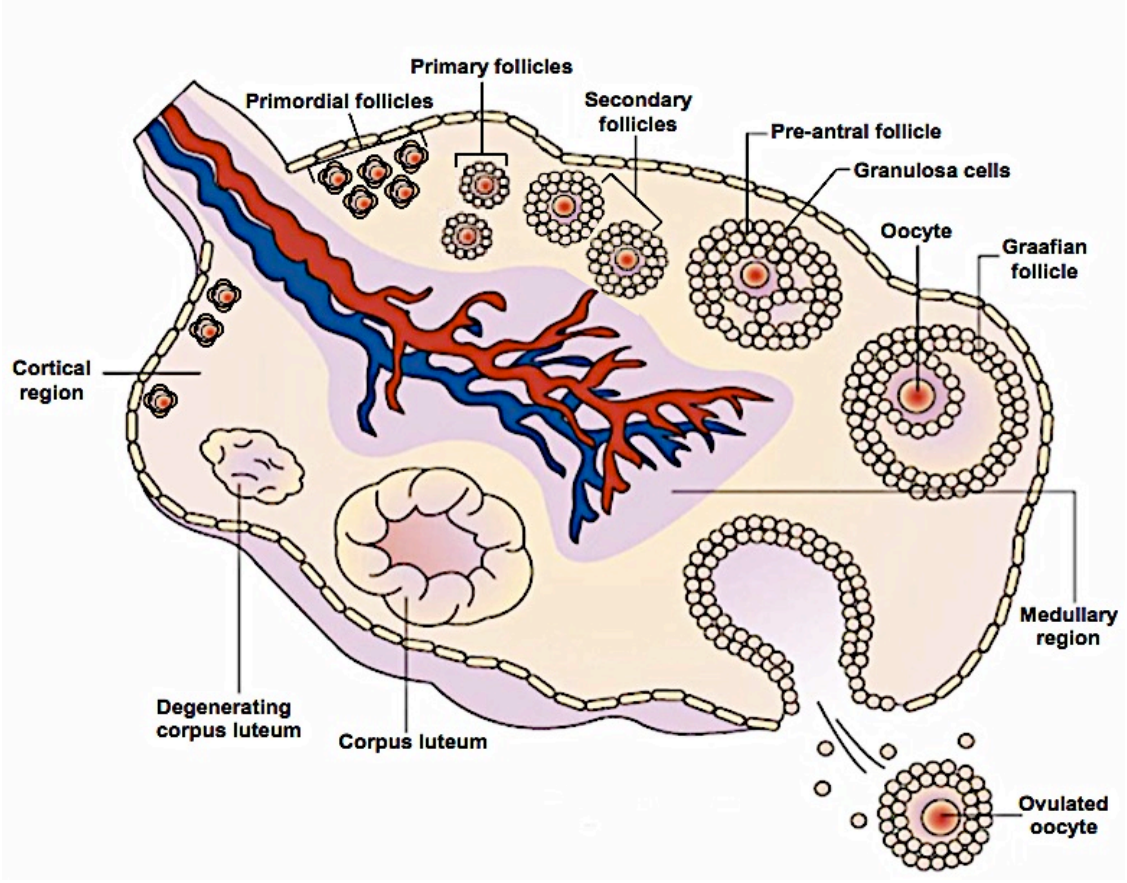

Figure 1. An overview of the mammalian ovary.
The mammalian ovary is a heterogeneous organ containing follicles at various stages of development and corpora lutea (CL).

1.2 Early embryogenesis

Mammalian gametes share an unequal burden in ensuring the successful initiation of development. During fertilization, the sperm fuses with the plasma membrane of the egg and is incorporated into the cytoplasm. The haploid sperm provides DNA for the male pronucleus and is essential for egg activation (Latham, 1999). However, the sperm mitochondria, the microtubule-organizing center (MTOC) precursors and the stored cellular components of the sperm play no major role in embryogenesis (Schatten et al., 1985; Shitara et al., 1998; Sutovsky and Schatten, 2000). Thus, the early embryo is almost entirely dependent on the egg for its initial complement of the subcellular organelles and macromolecules that are required for survival until the robust activation of the embryonic genome at cleavage-stage development takes place. After fertilization, the one cell zygote starts moving towards the uterus through oviduct. During this period, the embryo undergoes three principle phases: (1) from fertilization to the two-cell stage, which is mainly controlled by the maternal factors stored in the oocyte; (2) from the late two-cell stage after the embryonic genome activation (EGA) to the formation of compacted morula, which is controlled by declining maternal factors and increasing newly synthesized factors from the embryonic genome; (3) formation of the blastocyst with fluid-filled blastocyst cavity and two distinct cell lineages: the inner cell mass (ICM), which is pluripotent and gives rise to the proper embryo, and the trophectoderm (TE), which forms the extraembryonic tissues (Fig. 2).

1.2.1 From egg to embryo

During intraovarian growth, the diameter of mouse oocytes increases drastically due to the accumulation of maternal RNAs and proteins that are derived from the maternal genome, with a concomitant ~300-fold increase in volume (Liu et al., 2006). Some of these oocyte-derived macromolecules are believed to be dispensable for oocyte development and fertilization, but are essential for sustaining the early embryogenesis, at least prior to the robust transcription from the embryonic genome. This phenomenon is therefore called the maternal effect (Schultz, 2002; Li et al., 2010). Upon fertilization, the sperm-specific histone-like proteins (protamines) are rapidly replaced by the oocyte-specific linker histone H1Foo (H1 histone family, member O, oocyte- specific) in the mouse (Becker et al., 2005). After this reorganization of paternal chromatin, the maternal and paternal pronuclei migrate to the center of the zygote for DNA replication. Within 24 hours of fertilization, male and female pronuclei replicate their DNA in the 1-cell zygote and then their chromosomes congress on a metaphase plate prior to first mitosis. It has been demonstrated that a series of epigenetic modifications occurs during the one-cell stage in mice prior to the first round of DNA replication.

During gametogenesis, the haploid genomes of the male and female germ cells are highly methylated (Reik, 2007). However, after fertilization, the male PN is actively demethylated by a group of demethylases in the cytoplasm of the oocyte whereas the female PN is only passively demethylated during the subsequent cleavage events (Mayer et al., 2000; Oswald et al., 2000; Morgan et al., 2005). Therefore, it has been proposed that the male PN supports a

significantly higher level of transcription than the female PN in one-cell zygotes (Aoki et al., 1997). Nevertheless, the asymmetry of transcriptional activity between male and female genomes is found to be lost upon exit from the first mitotic cell cycle. The second and third embryonic divisions occur at ~12-hour intervals, and the resultant blastomeres appear to be morphologically symmetric. However, prior to the next cell division, the 8-cell embryo undergoes a Ca2+-mediated compaction to form the morula, where individual blastomeres greatly increase their area of cell-cell contact (Ziomek and Johnson, 1980). Subsequent asymmetric cell divisions result in two distinct cell populations: cells positioned inside the embryo develop into the inner cell mass (ICM), whereas outside cells develop into the first extraembryonic tissue, the trophectoderm, that will give rise to the placenta (Tarkowski and Wroblewska, 1967; Johnson and Ziomek, 1981). Subsequently, ICM differentiate into two distinct populations: the pluripotent epiblast (EPI) that generates cells of the future body and the second extraembryonic tissue, primitive endoderm (PE) (Gardner, 1982). However, it has not yet been well established how this second cell fate decision is made. In addition, the formation of adhesion complexes (adherens, gap and tight junctions) between outer cells enables the directional translocation of ions via basolateral Na+/K+-ATPase into the embryonic interior. The concomitant passage of water forms a fluid-filled blastocoel at the 32-cell stage that defines the early blastocyst, and at embryonic day 4.5 (E4.5) the fully formed blastocysts implants into the wall of the uterus (Madan et al., 2007; Wang et al., 2008).

1.2.2 Maternal factors

Oocytes, the female germ cells, carry all the messenger RNAs and also proteins that are indispensable to start a new life after fertilization. The oocyte-zygote transition occurs in the absence of transcription and therefore depends on the maternal mRNAs and proteins that are accumulated in the oocyte during follicular development (Seydoux, 1996). The transition from oocyte to zygote involves many changes, including protein synthesis, protein and RNA degradation, and organelle remodeling. Translational activation of these dormant mRNAs is initiated by cytoplasmic polyadenylation, which requires the presence of two cis-acting elements in the 3' untranslated region (UTR) of the mRNAs: a nuclear polyadenylation signal and a UA-rich cytoplasmic polyadenylation element (CPE) (Fox et al., 1989; McGrew and Richter, 1990; Salles et al., 1992). Activation of the stored maternal messages appears to occur at oocyte meiotic maturation and after fertilization (Fox et al., 1989; McGrew et al., 1989; Simon and Richter, 1994). However, maternal factors (RNAs and proteins) are rapidly degraded by means of various mechanisms once their functions have been accomplished, providing a pool of basic materials to generate embryonic macromolecules (Schultz, 1993; Li et al., 2010). Even though a progressive destruction of maternal RNAs is started off after the resumption of meiosis, the majority (60%) of the maternal RNAs are degraded by the late one cell stage. This leads to the minor embryonic genome activation at late one cell zygote. Moreover, over 90% of maternal factors including proteins is degraded by the mid two-cell stage, leading to the major embryonic genome activation (Bachvarova, 1985; Paynton et al., 1988; Schultz, 1993; Latham and Schultz, 2001; Alizadeh et al., 2005).

1.2.3 Embryonic genome activation

Embryonic genome activation (EGA) is the critical event that governs the transition from maternal to embryonic control of development. The onset of EGA must depend on maternally inherited proteins. The epigenetic modifications such as DNA demethylation and chromatin remodelling by maternal factors play a major role at the beginning of EGA prior to the activation of RNA polymerase II (pol II) and a range of transcription factors (Latham, 1999). In the absence of appropriate activation of the embryonic genome, the mammalian embryo fails to develop further. Thus, EGA is likely to be one of the first critical events in early development. EGA is involved in replacing maternal transcripts that are common to both the oocyte and early embryo, as well as generating novel ones that are likely to be involved in early embryogenesis prior to implantation. It is well known that EGA comprise a period of minor wave of gene activation that occurs in late one-cell embryo and the major wave of gene activation that takes place in the G2 phase of two-cell embryo (Schultz, 1993; Latham and Schultz, 2001; Schultz, 2002). Most recent studies have demonstrated that the one cell embryo is transcriptionally active, and the RNA polymerase I, II and III are functional in one cell embryo (Nothias et al., 1996). For example, luciferase activity is detected in G2 of the one cell embryo when luciferase reporter gene is injected into male pronucleus during early S phase of the one cell zygote (Ram and Schultz, 1993). In addition, it has been shown in another study that BrUTP (5-bromouridine 50-triphosphate) is able to get incorporated into the newly synthesized RNA and then visualized by immunofluorescence in the one-cell mouse zygote. Moreover, RNA synthesis is thought to be 30–40 % of that in two-cell embryos, based on the quantification of fluorescence intensity (Bouniol et al., 1995; Aoki et al., 1997). Inhibition of first round of DNA replication in one cell embryo results in 40% decrease in transcription in the one cell embryo as assessed by BrUTP incorporation. This indicates that DNA replication is linked to the initiation of transcription in one cell embryo (Aoki et al., 1997).

The robust transcription from the embryonic genome starts at the G2 phase of the second mitotic cell cycle and is crucial for embryonic development beyond the two- cell stage. It has been shown using RNA arrays that genes involved in ribosome biogenesis and assembly, protein synthesis, RNA metabolism and transcription are transcribed during this major wave of EGA. They are mostly housekeeping genes and are critical for maintaining the cleavage-stage development that follows further (Hamatani et al., 2004; Zeng et al., 2004; Zeng and Schultz, 2005). Later, the embryo, derived from two terminally differentiated gametes, is endowed with totipotency through the reprogramming of its DNA by several pluripotent transcription factors such as OCT4, SOX2. It has recently been reported that depletion of Oct4 mRNA at the 1-cell stage causes embryonic arrest during cleavage-stage development. The data suggest that maternal OCT4 both facilitates zygotic genome activation and enhances maternal RNA degradation, including that of Zar1 and Nobox, two known oocyte-specific transcription factors (Foygel et al., 2008).

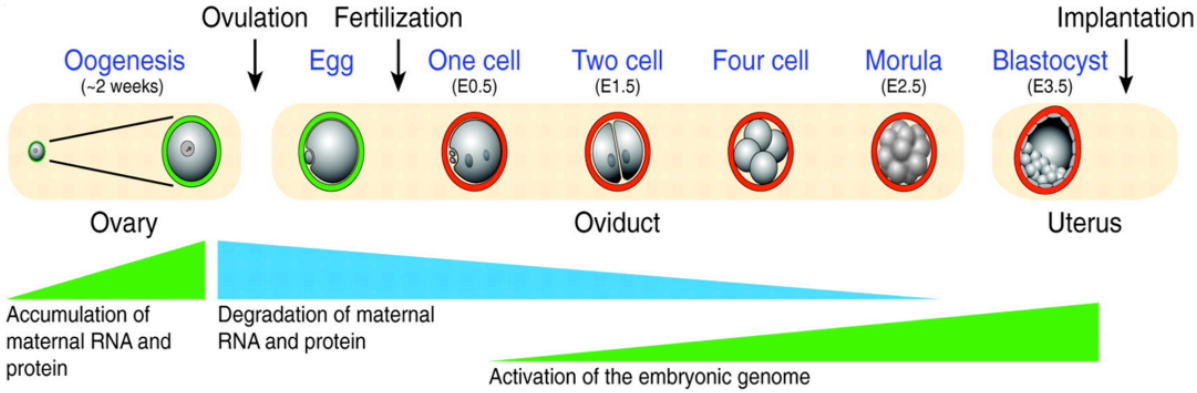

Figure 2. Illustration of the early embryonic development of mice. The Zona pellucida surrounds growing oocytes and ovulated oocytes (green in color) and is modified following fertilization (red in color) to prevent polyspermy and to protect embryo as it passes through the oviduct. The embryo reaches to blastocyst stage at embryonic day 3.5 (E3.5) followed by implantation at E4.5. (Note: Adapted and modified from Lei Li et al., Development, 2010).

1.3 The PI3K-mTORC1 signaling pathway

Phosphatidylinositol 3-kinases (PI3Ks) are lipid kinases that phosphorylate the 3'-OH group on the inositol ring of inositol phospholipids. In general, PI3K isoforms have been divided into three classes (class I, class II, and class III) based on their substrate preferences and sequence homology. In terms of their physiological functions, class I PI3K among them is well characterized to regulate glucose homeostasis, cell migration, growth, and proliferation in mammals (Cantley, 2002; Engelman et al., 2006; Vanhaesebroeck et al., 2010). Class I PI3Ks are further sub-grouped into class IA and class IB, depending on the receptors through which they are activated. Class IA PI3K is a heterodimer composed of regulatory (p85) and catalytic (p110) subunits, which is activated by receptor protein tyrosine kinases (RPTKs) whereas class IB PI3K consists of regulatory (101) and catalytic (110γ) subunits, which is activated by G-protein-coupled receptors (GPCRs) (Engelman et al., 2006). In general, class IA PI3Ks phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP2) at the 3´-OH position of the inositol ring and then produce phosphatidylinositol-3,4,5-triphosphate (PIP3) at the plasma membrane in response to numerous growth factors such as insulin, insulin-like growth factor I (IGF-I), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF). Subsequently, PIP3s that are produced by class IA PI3Ks interact with molecules containing pleckstrin homology (PH) domain such as PDK1, AKT and recruit them to the cell membrane area from the cytoplasm (Vanhaesebroeck et al., 2001). Furthermore, PIP3 also indirectly activates the mammalian target of rapamycin complex 1 (mTORC1), a protein kinase that is involved in the control of a diverse range of cellular processes such as protein synthesis, ribosome biogenesis, the cell cycle, cell growth, gene transcription, autophagy and metabolism (Gschwind et al., 2004) (Fig. 3).

1.3.1 Class IA PI3K

In mammals, there are three isoforms of the class IA PI3K catalytic subunit (p110 α, p110β and p110δ) whereas the regulatory subunits, collectively known as p85s, are generated by

three genes, PIK3R1, PIK3R2 and PIK3R3, which encode the p85α, p85β and p55γ isoforms, respectively. The PIK3R1 gene also encodes two shorter isoforms, p55α and p50α (Engelman et al., 2006). Class IA PI3Ks are regulated through the inhibitory action of the p85 subunit on the p110 catalytic subunit. P85 subunits contain two Src homology 2 (SH2) domains. The region between these two domains binds to the catalytic subunit. In resting cells, p85 stabilizes the overall confirmation of p110 and protects it from thermal inactivation in vitro. P85-p110 heterodimers are activated when the p85 SH2 domains bind to phosphorylated tyrosine residues on activated tyrosine kinase receptors, resulting in a conformational change in p85 that releases inhibition of p110. In the case of class IA PI3Ks, the active p110 catalytic subunit catalyzes the conversion of PIP2 to PIP3 (Yu et al., 1998). This reaction is negatively regulated by PTEN, which converts PIP3 back to PIP2 (Maehama and Dixon, 1998).

1.3.2 Pten

Pten, also referred to as MMAC (mutated in multiple advanced cancers) phosphatase, is a tumor suppressor that is encoded by *Pten* gene, which is located in the frequently altered chromosomal region 10q23 (Wu et al., 1998). The Pten protein is composed of an amino-terminal phosphatase domain, a lipid binding C2 domain, and a 50-amino-acid C-terminal domain (the tail). The Pten tail possesses three phosphorylation sites (Ser380, Thr382, and Thr383) that regulate Pten stability and function (Vazquez et al., 2000). The main function of Pten is to dephosphorylate PIP3 at position 3 on the inositol ring and convert them back to PIP2, and thereby negatively regulates the PIP3 mediated downstream signaling of PI3K (Maehama and Dixon, 1998). It has been shown that overexpression of Pten suppresses growth in a glioma cell line and inhibits cell migration and spreading in fibroblasts due to the lack of adequate number of PIP3s (Furnari et al., 1997; Tamura et al., 1998). In the absence of functional Pten, however, the PIP3 levels are increased at the inner cell membrane, resulting in constant active Akt signaling (Stocker et al., 2002; Cicenas et al., 2005; Robertson, 2005; Salmena et al., 2008). The hyperactive signaling through Akt favors the cells to proliferate more, increase in size, reduced cell death and altered migration; all of these conditions are conducive to the formation of tumors (Vivanco and Sawyers, 2002; Luo et al., 2003; Parsons, 2004; Renner et al., 2008; Tokunaga et al., 2008). Mutation or loss of functional Pten leads to a wide range of cancers in humans including cancers of the prostate, breast, endometrium, glioblastoma, and melanoma. Furthermore, germline mutation of Pten results in Cownden syndrome, Lhermitte-Duclos disease and Bannayan-Riley-Ruvalcaba syndrome, which are characterized by the presence of multiple hamartomatous lesions in different organs and systems such as skin, central nervous system, intestines, eyes and bones (Knobbe et al., 2008).

The functional role of Pten *in vivo* is well studied by means of Pten knock out mice. The homozygous Pten knockout mice are embryonic lethal whereas the heterozygous mice develop tumors in various parts of the body (Suzuki et al., 1998; Di Cristofano et al., 1999; Podsypanina et al., 1999). The deletion of Pten led to the formation of cancer in breast (Li et al., 2002), prostate (Wang et al., 2003), thyroid (Yeager et al., 2007), lung (Yanagi et al., 2007), pancreas (Stanger et al., 2005) and testis (Kimura et al., 2003). In addition, deletion of Pten from primordial oocytes of the mice ovary results in the overactivation of primordial follicles from the pool, causing premature ovarian failure (POF) (Reddy et al., 2008). However, ovarian folliculogenesis in mice is not affected by the deletion of Pten from primary oocytes (Jagarlamudi et al., 2009). Thus, it indicates that Pten has a stage specific role during folliculogenesis of mice ovary. It has been shown that the transforming growth factor β (TGFβ) down regulates the transcription of Pten (Li and Sun, 1997). In humans, *Pten*

promoter contains a unique p53-binding site, which is essential for inducible transactivation of Pten by p53 (Stambolic et al., 2001). Furthermore, the RAS-MAPK pathway and MEKK4 and JNK pathway are shown to be indispensable for the transcription of Pten (Chow et al., 2007; Xia et al., 2007).

1.3.3 Pdk1

Pdk1 is a 556 amino-acid protein encoded by PDPK1 gene in mammals. Pdk1 is composed of two domains, the kinase or catalytic domain and the PH domain. The kinase domain has three ligand binding sites: the substrate binding site, the ATP binding site, and the docking site (also known as PIF pocket) (Alessi et al., 1997; Stephens et al., 1998; Currie et al., 1999). Pdk1 phosphorylates and activates the AGC kinase members, which include isoforms of protein kinase B (PKB)/Akt, p70 ribosomal S6 kinase (S6K), serum- and glucocorticoid-induced protein kinase (SGK) and protein kinase C (PKC) (Mora et al., 2004). The recruitment of Pdk1 to the cell membrane area by PIP3 enables Pdk1 to phosphorylate Akt at T-loop residue, T308 (Vanhaesebroeck and Alessi, 2000), which is prerequisite for the phosphorylation of Akt at S473 by mammalian target of rapamycin complex 2 (mTORC2) (Sarbassov et al., 2005b). Pdk1 also activates p70 S6 kinase-1 (S6K1) and serum- and glucocorticoid- induced protein kinase-1 (SGK1) by phosphorylation at T-loop residue; the phosphorylation on hydrophobic motifs of S6K1 and SGK1 is required for providing the docking site for Pdk1 (Alessi et al., 1998; Pullen et al., 1998; Kobayashi and Cohen, 1999; Park et al., 1999). Furthermore, MAP kinases ERK1 and ERK2 phosphorylate the hydrophobic motif on ribosomal S6 kinase (RSK) and RSK thereby binds to the Pdk1-interacting fragment (PIF) pocket of Pdk1 and gets phosphorylated at T-loop by Pdk1 (McManus et al., 2004). Pdk1 with a mutated PH domain is unable to bind to PIP3 and thereby cannot phosphorylate Akt. However the activation of other AGC protein kinases such as RSK is apparently not affected. Therefore, it indicates that PIP3 binding to Pdk1 is required for Akt but not RSK activation (McManus et al., 2004; Bayascas et al., 2008). In contrast, Pdk1 mutants in which PIF pocket has been disrupted cannot phosphorylate and activate S6K1 and SGK1 (Biondi et al., 2001).

It has been shown that conventional Pdk1 knockout mice are embryonic lethal at embryonic day 9.5 (E9.5) and the hypomorphic mice that only have 10% of Pdk1 are viable with 40-50% smaller in size as compared to controls (Lawlor et al., 2002). The functional role of Pdk1 in different tissues was studied using tissue specific deletions, where conditional deletion of Pdk1 from the heart, T cells, liver and pancreas resulted in heart failure (Mora et al., 2003), impaired T cell differentiation (Hinton et al., 2004), liver failure (Mora et al., 2005) and diabetes (Hashimoto et al., 2006) respectively. In addition, in mice lacking the Pdk1-encoding gene Pdk1 in primordial oocytes, the majority of primordial follicles are depleted around the onset of sexual maturity, causing POF during early adult- hood (Reddy et al., 2009).

1.3.4 Akt

Akt, also known as Protein Kinase B (PKB), is a serine/threonine protein kinase that plays a key role in multiple cellular processes such as cell growth and proliferation, cell migration, transcription, glucose metabolism and apoptosis (Manning and Cantley, 2007). Akt is normally maintained in an inactive state through an intramolecular interaction between the PH

and kinase domains. However, the interaction between the PH domain of Akt and PIP3 not only recruits Akt to inner cell membrane but also induces a conformational change in Akt, which provides the accessibility for PDK1 to phosphorylate at threonine site (T308) (Calleja et al., 2007). The phosphorylation of Akt at serine 473 (S473) and threonine 308 (T308) sites is required for the complete activation of Akt. Upon Akt phosphorylation and activation, Akt dissociates from the membrane and translocates to the cytosol and nucleus where it activates downstream signaling pathways through phosphorylation of a plethora of Akt substrates (Hers et al., 2011).

There are three isoforms of Akt—Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ, which are encoded by different genes localized on different chromosomes. The isoforms are 80% similar in their amino acids and domain structure. They have a highly conserved domain structure; an N-terminal pleckstrin homology (PH) domain, a kinase domain and a C-terminal regulatory tail containing a hydrophobic motif (Vanhaesebroeck and Alessi, 2000). Akt1 has a wide tissue distribution and is implicated in cell growth and survival (Chen et al., 2001; Cho et al., 2001b), whereas Akt2 is highly expressed in muscle and adipocytes and contributes to insulin-mediated regulation of glucose homeostasis (Cho et al., 2001a; Garofalo et al., 2003). The expression of Akt3 is more restricted to the testes and brain (Brodbeck et al., 1999; Nakatani et al., 1999). $Akt1^{-/-}$ mice are viable and smaller in size but not diabetic (Chen et al., 2001; Cho et al., 2001b), whereas $Akt2^{-/-}$ mice have lost the ability to lower their blood glucose level and they show some important features of type 2 diabetes mellitus in humans (Cho et al., 2001a). $Akt3^{-/-}$ mice have smaller brains due to reduced cell numbers and cell size (Tschopp et al., 2005). In contrast to Akt1-/- and Akt2-/- mice, Akt3-/- mice have no defects in growth and glucose metabolism. Due to the compensatory roles of different Akt isoforms, knockout of two genes at the same time has been done to determine the role of these different isoforms. Akt1/2 double mutants die shortly after birth due to defects in the development of skin and bone, and skeletal muscle atrophy (Peng et al., 2003). Akt1/3 double mutant mice are embryonic lethal by E11-12 due to severe defects in the cardiovascular and nervous systems (Yang et al., 2005). $Akt1^{-/-}$; $Akt3^{+/-}$ mice display multiple defects in the thymus, heart and skin, and die a few days after birth, whereas $Akt1^{+/-}$; $Akt3^{-/-}$ mice are viable and normal. Thus, it indicates that Akt1 is indispensable in the absence of Akt3 during embryonic development (Yang et al., 2005).

1.3.5 Tuberous sclerosis complex (Tsc)

Tuberous sclerosis complex (Tsc) is an autosomal dominant disorder that causes symptoms including hamartomas in brain, kidney, heart, lung and skin (Sparagana and Roach, 2000). The tumor suppressor genes *Tsc1* and *Tsc2* encode Tsc1/hamartin and Tsc2/tuberin respectively (van Slegtenhorst et al., 1997). Tsc1 and Tsc2 form a functional complex, where Tsc1 prevents the degradation of Tsc2 by ubiquitination and thereby stabilizes the Tsc1/Tsc2 complex (Chong-Kopera et al., 2006). The Tsc1/Tsc2 complex is involved in numerous cellular activities such as vesicular trafficking, regulation of the G1 phase of the cell cycle, steroid hormone regulation, Rho activation and anchoring neuronal intermediate filaments to the actin cytoskeleton (Xiao et al., 1997; Henry et al., 1998; Plank et al., 1998; Lamb et al., 2000; Tapon et al., 2001; Haddad et al., 2002). The combination of genetic, biochemical and cell-biological studies demonstrate that the Tsc1/Tsc2 complex functions as a GTPase-activating protein for the Ras-related small G protein, Rheb. The GTPase activity of Tsc2 converts active Rheb–GTP, which is the positive regulator of mTORC1, to inactive Rheb–GDP and thereby inhibits mTORC1 (Yang and Guan, 2007; Huang and Manning, 2008). The

Tsc1/2 complex controls cell growth and protein synthesis by negatively regulating mTORC1 activity. Activation of Akt and extracellular signal regulated kinase (ERK) upon stimulation with external growth factors leads to phosphorylation and functional inactivation of TSC2 (Inoki et al., 2002; Manning et al., 2002; Ma et al., 2005). In the presence of growth factors, the active Akt/PKB directly phosphorylates Tsc2 at Thr1462 (Manning et al., 2002). Upon phosphorylation, Tsc2 loses its GTPase activity and releases the inhibitory effect on Rheb (Garami et al., 2003). Active Rheb prevents the binding of FKBP38 (FK506–binding protein) to mTORC1, thereby releasing the inhibitory effect on mTORC1 (Bai et al., 2007). The functional role of Tsc1/Tsc2 complex in vivo has been investigated by means of knockout mice. The mice that are homozygous for either Tsc1 or Tsc2 are embryonic lethal and heterozygous mice develop renal and extra-renal tumors such as hepatic hemangiomas (Kobayashi et al., 1999; Onda et al., 1999; Kobayashi et al., 2001).

1.3.6 Mammalian target of rapamycin complex 1

The mTOR, serine/threonine kinase, is a member of the phosphoinositide 3-kinase (PI3K) related kinase (PIKK) family. This conserved protein integrates diverse upstream signals to regulate growth related processes, including mRNA translation, ribosome biogenesis, autophagy, and metabolism (Sarbassov et al., 2005a; Zoncu et al., 2011). The mTOR nucleates two large physically and functionally distinct signaling complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Guertin and Sabatini, 2007). The mTORC1 consists of mTOR, raptor (regulatory associated protein of mTOR), PRAS40 (proline-rich AKT substrate 40 kDa), mLST8 (mammalian lethal with sec-13 protein 8; also known as GβL) and Deptor (DEP domain-containing mTOR-interacting protein) (Zoncu et al., 2011). The mTOR protein, which consists of multiple HEAT repeats at its N-terminal half followed by the FKBP12-rapamycin-binding (FRB) and serine-threonine protein kinase domains near its C-terminal end, has no known enzymatic functions besides its kinase activity. It has been shown that raptor is involved in mediating mTORC1 assembly, recruiting substrates, and regulating mTORC1 activity and subcellular localization. The strength of the interaction between mTOR and raptor can be modified by nutrients and other signals that regulate the mTORC1 pathway (Hara et al., 2002; Kim et al., 2002; Sancak et al., 2008). The role of mLST8 in mTORC1 function is also unclear, as the chronic loss of this protein does not affect mTORC1 activity in vivo. However, the loss of mLST8 can perturb the assembly of mTORC2 and its function (Guertin et al., 2006). PRAS40 and Deptor have been characterized as negative regulators of mTORC1 (Wang et al., 2007; Peterson et al., 2009). The mTOR pathway is activated by a variety of divergent stimuli. mTOR senses cellular energy levels by monitoring cellular ATP:AMP levels via the AMP-activated protein kinase (AMPK), growth factors such as insulin and insulin-like growth factor 1 (IGF-1) via the insulin receptor and the IGF-1 receptor respectively, amino acids via Rag GTPases, and signals from the Wnt family via glycogen synthase kinase 3 (GSK3) (Yang and Guan, 2007; Avruch et al., 2009). Rapamycin, in complex with its intracellular receptor FKBP12 (FK506-binding protein of 12 kDa), acutely inhibits mTORC1 by binding to the FRB domain of mTOR (Sarbassov et al., 2005a). Previous biochemical studies indicated that binding of FKBP12-rapamycin to mTORC1 induces a conformational change that weakens the mTOR-raptor interaction (Kim et al., 2002). However, it has recently been shown that the initial binding of one FKBP12-rapamycin to mTORC1 does not suffice to disrupt the dimeric architecture even though it weakens the mTOR-raptor interaction. Over time, either amplified structural strain caused by the first FKBP12-rapamycin or, perhaps, the binding of a second rapamycin complex leads to a fast disintegration of the already weakened mTORC1, resulting in the complete abolishment

of mTORC1 activity (Yip et al., 2010).

The mTORC1 controls protein synthesis through the direct phosphorylation and inactivation of a repressor of mRNA translation, eukaryotic initiation factor 4E-binding protein 1 (4e-bp1), and through phosphorylation and activation of S6 kinase (S6K1 or p70S6K), which in turn phosphorylates the ribosomal protein S6 (Hay and Sonenberg, 2004). In addition, the mTORC1 actively suppresses autophagy by the phosphorylation of autophagy related 13 (Atg13) and ULK1 and, conversely, inhibition of mTORC1 strongly induces autophagy (Noda and Ohsumi, 1998; Jung et al., 2009). In mice, embryonic homozygous deletion of mTOR or raptor leads to a developmental arrest at E5.5, and knockout of mLST8 results in developmentally delayed embryos that die by embryonic day 10.5–11.5. Thus, it indicates the mTORC1 is critical for the embryonic development (Gangloff et al., 2004; Guertin et al., 2006). The mTORC1 integrates signals from extrinsic pluripotency supporting factors and represses the transcriptional activities of a subset of developmental and growth inhibitory genes in human embryonic stem cells (hESCs). Repression of the developmental genes by mTOR is necessary for the maintenance of hESC pluripotency (Lee et al., 2010). On the other hand, it has been proposed that mTOR-mediated activation of S6K1 induces differentiation of pluripotent hESCs (Easley et al., 2010). The mTORC1 pathway is emerging as a key regulator of aging as inhibition of mTOR by rapamycin or genetic deletion has been shown to expand life span of invertebrates, including yeast, nematodes, fruit flies and mice (Hands et al., 2009; Harrison et al., 2009).

1.3.7 Mammalian target of rapamycin complex 2

The mTORC2 is composed of mTOR, rapamycin insensitive companion of mTOR (RICTOR), mLST8/GβL, mammalian stress activated protein kinase interacting protein 1 (mSIN1), Deptor and protor (Pearce et al., 2007; Zoncu et al., 2011). Rictor is a 192 kDa protein that forms a rapamycin insensitive complex with mTOR and acts as regulatory subunit of mTORC2 (Jacinto et al., 2004; Sarbassov et al., 2004). It has been demonstrated by a knockout approach that mLST8 stabilizes the interaction between rictor and mTOR (Guertin et al., 2006). mSIN1 contains an RBD (Ras binding domain) and a PH domain (pleckstrin homology domain) important for the localization of this protein at the plasma membrane (Schroder et al., 2007). Thus, mSIN1 is critical for the function of mTORC2 related to the phosphorylation of Akt at serine 473 (Jacinto et al., 2006). In addition, mSin1 has been proposed as a determinant in the dynamic localization of mTORC2, explained by putative lipid interactions established by the PH domain and protein interactions dependent on the RBD (Frias et al., 2006). Protor is also known as PRR5 (proline-rich protein5), which interacts with Rictor (Pearce et al., 2007). Protor1 and 2 knockout mice show no defects on the phosphorylation of Akt or PKCα at their hydrophobic or turn motifs, but are unable to properly phosphorylate SGK1, at least in the kidney (Pearce et al., 2011). Deptor is a 48 kDa protein with two tandem DEP domains at the amino terminus and a PDZ domain at the carboxyl-terminus. Deptor inhibits mTOR by interacting with the mTOR-FAT domain (Peterson et al., 2009). The mTORC2 is activated by growth factors and chemokines. Reported activators include Rac1, PIP3, direct interaction with Tsc1-Tsc2 complex, the heat shock protein of 70 kDa (Hsp70) and growth factor dependent association to ribosomes (Huang et al., 2008; Martin et al., 2008; Gan et al., 2011; Saci et al., 2011; Zinzalla et al., 2011).

The mTORC2 plays a critical role in the regulation of AGC family kinases such as Akt,

PKCα/β and SGK (Sarbassov et al., 2005b; Facchinetti et al., 2008; Garcia-Martinez and Alessi, 2008). The stabilization and full activation of Akt requires its phosphorylation at threonine 450 and serine 473, and at threonine 308 by Pdk1. Active Akt phosphorylates numerous substrates promoting cell migration, survival, metabolism, growth, proliferation and angiogenesis. The mTORC2 regulates actin polymerization by phosphorylation or direct interaction of several regulators of cytoskeleton. For instance, Akt phosphorylated by mTORC2 phosphorylates other substrates such as PAK, Girdin and integrin β3, which stimulate rearrangement of the actin cytoskeleton and cell migration (Kirk et al., 2000; Zhou et al., 2003; Enomoto et al., 2005).

The mTORC1 and mTORC2 signaling networks are connected at different levels. Even though mTORC1 keeps controlled different upstream regulators of mTORC2, mTORC1 is itself a downstream effector of mTORC2. The interplay between these complexes involves positive regulation of signaling intermediates and negative regulation of others. For example, keeping down mTORC1, with either rapamycin or Deptor overexpression, enhances mTORC2 signaling (Peterson et al., 2009). This effect is due to inhibition of the well-known feedback control mechanism by which mTORC1-dependent effectors prevent PI3K activation. The phosphorylation and activation of S6K1 by mTORC1 inhibits the activity of PI3K and also mTORC2 (Hsu et al., 2011). In addition, Grb10, identified as an mTORC1 substrate, inhibits the response to insulin and other growth factors upon phosphorylation by mTORC1 (Yu et al., 2011). On the other hand, mTORC1 positively regulates mTORC2 by the activation of S6K1, which in turn activates rpS6 involved in ribosome biogenesis (Zinzalla et al., 2011).

1.3.8 p70S6K1

Protein70 S6 kinase is a mitogen activated Ser/Thr protein kinase that is required for cell growth and G1 cell cycle progression. A second isoform, p85 S6 kinase, is derived from the same gene and is identical to p70 S6 kinase except for 23 extra residues at the amino terminus, which encode a nuclear localizing signal. Both isoforms lie on a mitogen activated signaling pathway downstream of phosphoinositide-3 kinase (PI3K) and the mTORC1, a pathway distinct from the Ras/MAP kinase cascade (Pullen and Thomas, 1997). The activity of p70 S6 kinase is controlled by multiple phosphorylation events located within the catalytic, linker and pseudosubstrate domains. Phosphorylation of Thr229 in the catalytic domain by Pdk1 and Thr389 in the linker domain by mTORC1 are most critical for kinase function (Pullen and Thomas, 1997). The phosphorylation of Thr389 by mTORC1 is a prerequisite for the phosphorylation of p70S6K1 at Thr229 in the catalytic domain (Alessi et al., 1998; Pullen et al., 1998). Thus, Phosphorylation of Thr389 most closely correlates with p70 kinase activity *in vivo* (Weng et al., 1998). The phosphorylation of p70S6K1 by Pdk1 and mTOC1 is stimulated by growth factors such as insulin, EGF and FGF, as well as by serum and some G-protein-coupled receptor ligands, and is blocked by wortmannin, LY294002 (PI3K inhibitor) and rapamycin (FRAP/mTOR inhibitor) (Pullen and Thomas, 1997; Polakiewicz et al., 1998; Fingar et al., 2002). Moreover, the phosphorylation of Ser411, Thr421 and Ser424 within a Ser-Pro-rich region located in the pseudosubstrate region is thought to activate p70 S6 kinase via relief of pseudosubstrate suppression (Pullen and Thomas, 1997; Dufner and Thomas, 1999).

S6K1 regulates protein translation and cell growth by phosphorylating downstream substrates such as rpS6, which are needed for ribosome and protein synthesis (Engelman et al., 2006;

Yang and Guan, 2007). Several other substrates for the S6 kinases have been identified, including other proteins that are involved in mRNA translation: eIF4B (eukaryotic initiation factor 4B), PDCD4 (programmed cell death protein 4), an inhibitor of eIF4A and eEF2 (eukaryotic elongation factor 2) (Wang et al., 2001; Raught et al., 2004; Dorrello et al., 2006; Shahbazian et al., 2006). S6K1 is actually recruited to translation initiation complexes (Holz et al., 2005) and also to newly synthesized mRNA, by its interaction partner and substrate, SKAR (S6K1 Aly/REF-like substrate) (Ma et al., 2008). S6 kinases can impair PI3K/Akt signaling through the phosphorylation of insulin receptor substrate 1 (IRS1) (Um et al., 2004). Treating cells with rapamycin can therefore actually promote Akt signaling by abrogating this feedback. Given the oncogenic properties of Akt, mTORC1 inhibition might actually promote tumorigenesis in some contexts. However, it has recently been shown that mTORC1 signaling is to be the major route by which Akt promotes cell proliferation and tumorigenesis (Skeen et al., 2006). S6K1 knockout mice are significantly smaller, whereas S6K2 knockout mice tend to be slightly larger. However, mice lacking both genes showed a sharp reduction in viability due to perinatal lethality. Thus, the absence of S6K1 and S6K2 profoundly impairs animal viability but does not seem to affect the proliferative responses (Pende et al., 2004).

1.3.9 4e-bp1

The human 4e-bp family comprises three members: 4e-bp1, 4e-bp2, and 4e- bp3. 4e-bp1 undergoes phosphorylation at several sites. Seven phosphorylation sites have been identified in 4e-bp1, four of which (Thr37/46, Ser65 and Thr70) directly or indirectly control its binding to eukaryotic translation initiation factor 4E (eIF4E), which is a limiting component of the multisubunit complex that recruits 40S ribosomal subunits to the 5' end of mRNAs (Ferguson et al., 2003). 4e-bp1 (also known as PHAS-1) inhibits cap-dependent translation by binding to the translation initiation factor eIF4E (Pause et al., 1994). The phosphorylation of the two sites (Ser65 and Thr70) nearest in the primary sequence to the eIF4E-binding site decreases the association of 4e-bp1 with eIF4E (Ferguson et al., 2003). However, two more N-terminal sites, Thr37/46, are required for the phosphorylation of Thr70 and Ser65 (Gingras et al., 1999). In addition, phosphorylation of Ser65 and Thr70 requires the TOR signalling (TOS) motifs, which interact with raptor; but mutation of residues in the TOS motif only has a modest effect on the phosphorylation of Thr37/46 (Beugnet et al., 2003). In accordance with this, transient depletion of raptor by siRNA markedly decreases the phosphorylation of 4e-bp1 at Ser65 and Thr70 with only a modest effect on Thr37/46 phosphorylation (Fonseca et al., 2007). Phosphorylation of Thr37/46 requires a region in the N-terminal part of 4e-bp1 that contains the residues Arg-Ala-Ile-Pro (hence ‘RAIP’ motif) (Tee and Proud, 2002). Recent data show that this region plays a role in the interaction of 4e-bp1 with Raptor: although variants of 4e-bp1 where the RAIP motif is replaced by four alanines (AAAA) can still bind to raptor, such binding is markedly decreased as compared to the wild-type protein (Lee et al., 2008).

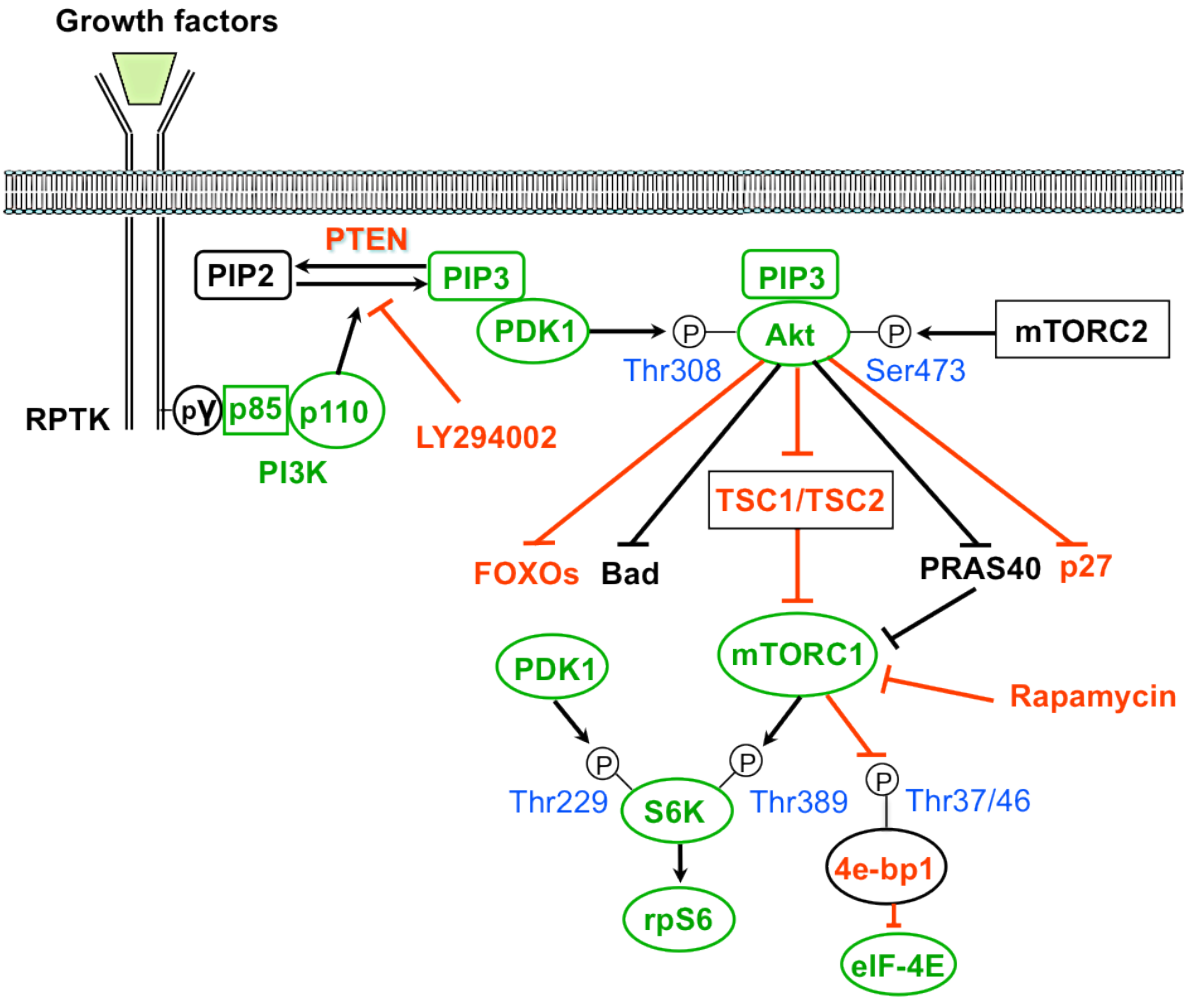

Figure 3. Illustration of the class IA PI3K-mTOR signaling pathway.
Note that green color indicates the molecules for positive signaling and red color highlights the molecules for negative signaling.

2 AIM OF THE THESIS

The main aim of this thesis is to study PI3K-mTORC1 signaling in oocytes in physiological development of follicles and early embryogenesis of mice by generating the oocyte-specific deletion of *Rptor*, *Tsc2* and *Pdk1* in mice.

This thesis is organized into three scientific reports, and intended to address the following questions:

Paper I:

How does mTORC1 signaling in oocytes regulate the physiological development of ovarian follicles and fertility of females?

Paper II:

How does *Tsc2* gene, a negative regulator of mTORC1, function in oocytes in the regulation of activation and development of follicles and fertility of females?

Paper III:

What are the functions of maternally derived PI3K signaling in mice preimplantation embryogenesis?

3 RESULTS AND DISCUSSION

(The figures cited corresponds to those in the original papers)

4.1 Paper I

mTORC1 signaling in oocytes is dispensable for the survival of primordial follicles and female fertility

The molecular mechanisms underlying reproductive aging in females are poorly understood. Mammalian target of rapamycin complex 1 (mTORC1) is a central controller of cell growth and proliferation. In this study, to investigate whether mTORC1 signaling in oocytes directly plays a role in physiological follicular development and fertility of female mice, we conditionally deleted the specific and essential mTORC1 component, *Rptor (regulatory associated protein of mTORC1)* gene from oocytes of primordial and further developed follicles by using transgenic mice expressing *growth differentiation factor 9 (Gdf-9)* promoter-mediated Cre recombinase.

Upon the deletion of *Rptor* from primordial oocytes, the mTORC1 signaling was found to be abolished in oocytes, as indicated by the loss of phosphorylations of its well-known substrates, S6K1 and 4e-bp1 at T389 and S65 respectively (Fig. 1B). However, the female mice lacking *Rptor* in primordial oocytes produced normal litter sizes, indicating that loss of mTORC1 signaling in oocytes does not affect the fertility of female mice (Fig. 2). In recent years, the PI3K-Akt signaling in oocytes has been shown to have important roles in controlling the activation and development of ovarian follicles and fertility. To explore the molecular mechanisms underlying the normal fertility of females lacking *Rptor* in oocytes, we thus analyzed the phosphorylation of Akt in oocytes lacking *Rptor*. We found that the activity of Akt is enhanced, as indicated by the hyperphosphorylation of Akt at S473 and T308 in oocytes lacking *Rptor* (Fig. 3). Thus, it indicates that the loss of mTORC1 signaling in oocytes leads to hyperactivation of PI3K-Akt signaling. We then studied the morphology of ovaries of mice lacking *Rptor* in primordial oocytes to investigate whether elevated PI3K-Akt signaling leads to normal follicular development in females lacking *Rptor* in primordial oocytes. At postnatal day (PD) 35, follicles at various developmental stages from primordial to preovulatory were found to be normal in mutant mice ovaries (Fig. 4 B and D, arrows) as compared to control mice (Fig. 4 A and C, arrows). In addition, we found healthy corpora lutea (Fig.4 F and H, CL) along with all types of follicles (Fig. 4 F and H, arrows) in mutant mice ovaries at 16 weeks of age, which is comparable to control mice ovaries (Fig. 4 E and G, CL and arrows). Therefore, it demonstrates that elevated PI3K-Akt signaling in oocytes leads to normal follicular development of females lacking *Rptor* in primordial oocytes.

In this study, the PI3K-Akt signaling was found to be elevated upon the loss of mTORC1 signaling in oocytes. Our results demonstrated that the enhanced PI3K-Akt signaling in the absence of mTORC1 signaling in oocytes is essential to compensate and support physiological development of ovarian follicles, and female fertility. We therefore concluded that loss of mTORC1 signaling in oocytes triggers a compensatory activation of the PI3K-Akt signaling that maintains normal ovarian follicular development and fertility.

4.2 Paper II

Disruption of Tsc2 in oocytes leads to overactivation of the entire pool of primordial follicles

In this study, to determine whether the Tsc2 plays a major role in the regulation of follicular activation and development, we deleted the *Tsc2* gene from oocytes of primordial and further developed follicles by using transgenic mice expressing *Gdf-9* promoter-mediated Cre recombinase.

The female mice lacking *Tsc2* in the oocytes of primordial follicles produced at most two litters of normal size and then became infertile in young adulthood (Fig 2). To explore the molecular mechanisms underlying the infertility of females lacking *Tsc2* in oocytes, we analyzed mTORC1 signaling, which is negatively regulated by Tsc1-Tsc2 complex, in oocytes lacking Tsc2. We found that loss of *Tsc2* from primordial oocytes leads to elevated mTORC1 activity, as indicated by the enhanced phosphorylation of its substrate, S6K1 (Fig. 3, p-S6K1, T389), resulting in elevated phosphorylation of rpS6 (Fig. 3, p-rpS6, S240/4). However, the pool of primordial follicles was formed normally in ovaries of mice lacking *Tsc2* in oocytes when compared to the ovaries of control mice. At PD13, no apparent morphological difference was observed in ovaries of mutant (Fig. 4B) as compared to control (Fig. 4A) mice, where ovaries of both mutant and control mice had clusters of primordial follicles (Fig. 4A and B, inset, arrows). However, at PD23, the mutant ovaries (Fig. 4D) appeared somewhat larger than the control ovaries (Fig. 4C). By this age, all primordial follicles were activated with enlarged oocytes in ovaries lacking *Tsc2* in oocytes (Fig. 4D, inset, red arrows) as compared to the control (Fig. 4C, inset, arrows). At PD35, in the mutant ovaries, there were many activated follicles with enlarged oocytes (Fig. 4F, inset, red arrows) as compared to the control (Fig. 4E), which contained clusters of primordial follicles (Fig. 4E, inset, arrows). By 4 months of age, no healthy follicular structure was observed in mutant ovaries (Fig. 4H), and only unhealthy dying oocytes were identified (Fig. 4H, inset, arrows) whereas the control mice ovaries contained healthy follicles and corpus luteum (CL) (Fig. 4G).

In this study, we provided experimental evidence showing that the deletion of *Tsc2* from primordial oocytes leads to the elevated mTORC1– S6K1–rpS6 signaling, resulting in the overactivation of primordial follicle pool in the ovaries of mice lacking *Tsc2* in oocytes. Consequently, the mutant ovary was observed to be completely devoid of follicles, causing POF in early adulthood. Therefore, we identified the *Tsc2* gene as an essential factor in oocytes to preserve the female reproductive lifespan by suppressing the activation of primordial follicles.

4.3 Paper III

Maternal phosphatidylinositol 3-kinase signalling is crucial for embryonic genome activation and preimplantation embryogenesis

In this study, we deleted the *Pdk1* gene (also known as *Pdpk1* or *Pkb kinase*) and, both the *Pdk1* and *Pten* genes from primary oocytes with the help of transgenic mice carrying *Zp3* promoter-mediated Cre recombinase and studied the development of these embryos to determine the functions of maternally derived PI3K signaling in preimplantation embryogenesis.

Deletion of *Pdk1* from the primary oocytes resulted in the arrest of embryos at two-cell stage (Fig 1A,C) even though key events such as oocyte maturation, ovulation and fertilization are intact (Fig S1C, D). Consequently, mutant females were sterile (Fig S2B). To investigate the various phases of the second mitotic cell cycle (G1, S, G2 and M), mutant and control embryos were cultured for different lengths of time after hCG injection, corresponding to different stages of the second mitotic cell cycle. There was no effect of deletion of maternal *Pdk1* in the kinase profiles for S phase (38 h after hCG injection), including the expression level of cyclin E and kinase activities of CDK2– cyclin A complex, and DNA synthesis (Fig 2A, B). However, upon the deletion of maternal *Pdk1*, the de novo synthesis of TRC (transcription-requiring complex) and de novo RNA synthesis were attenuated to 15.2% and 26.4% respectively as compared to control (Fig 2D, E). In addition, the kinase activity of CDC2 and cyclin B1 (maturation-promoting factor (MPF) activity) was significantly reduced as compared to control embryos (Fig 2C). Therefore, the mutant embryos were arrested due to the suppressed EGA and defective G2-to-M transition of the second mitotic cell cycle, which were caused by the deletion of maternal *Pdk1*.

Nevertheless, the concurrent loss of maternal *Pten* rescued the two-cell arrest of maternal *Pdk1* mutant embryos (Fig 3A) and completely restored the fertility of double-mutant females (Fig S2A, B). The concurrent loss of *Pten* has largely rescued the suppressed EGA, as shown by the elevated TRC synthesis (Fig 3B) and global RNA transcription (Fig 3C) in double mutant embryos. Furthermore, *in vitro* kinase assays showed recovered the kinase activity of CDC2 and cyclin B1 in late double mutant embryos (Fig 3D), indicating a normal G2-to-M transition. Upon the concurrent loss of maternal *Pten*, the phosphorylation of Akt at Ser 473 was fully recovered in double mutant embryos, in contrast with the absence of phosphorylation of Akt at Ser 473 in *Pdk1* mutant embryos (Fig 4A). The recovery of Akt phosphorylation at Ser 473 in double mutant embryos led to at least partly restored Akt kinase activity, as the phosphorylation of several known Akt substrates, including Foxo3a, GSK3β and Tsc2 was restored in double mutant embryos (Fig 4A). With elevated Akt activity, the loss of maternal Pten probably allows the double mutant embryos to initiate EGA and to develop beyond the two-cell stage.

In this study, we have shown that blockage of maternal PI3K signaling by deletion of *Pdk1* from oocytes leads to the arrest of resultant embryos at the two-cell stage, which is most probably a consequence of suppressed EGA and a defective G2/M phase at the two-cell stage. Furthermore, concurrent loss of maternal *Pten* recovered the impaired Akt activation, rescued the suppressed EGA and two-cell arrest of embryos, and restored the fertility of double-mutant females. We therefore identified the maternal PI3K/Pten–Pdk1–Akt signalling cascade as an indispensable maternal effect factor in triggering EGA and sustaining preimplantation embryogenesis in mice.

4 CONCLUSIONS

The existence of primordial follicles in the mammalian ovary is crucial to maintain the normal reproductive lifespan in females. The Tsc/mTORC1 signaling in oocytes was found be essential for the physiological development of follicles in the ovary.

Deletion of the specific and essential mTORC1 component, *Rptor* from the oocytes of primordial follicles led to the ablation of mTORC1 signaling, resulting in the compensatory activation of PI3K signaling in oocytes. Consequently, the follicular development and fertility of mice lacking *Rptor* in oocytes were not affected. However, deletion of negative regulator of mTORC1, *Tsc2* from the oocytes of primordial follicles led to the elevation of mTORC1 signaling, leading to the complete activation of the primordial follicle pool in early adulthood, which is followed by a pathological condition resembling POF in humans. The constant active mTORC1 signaling in oocytes of primordial follicles is thus deleterious. Therefore, Tsc2 in oocytes is indispensable for the maintenance of dormancy, and for the prevention of premature activation of primordial follicles.

Furthermore, deletion of *Pdk1*, where the considerable proportion of PI3K signaling converges, from primary oocytes led to the arrest of resultant embryos at the two-cell stage. This was found to be due to the suppressed EGA and a defective G2/M phase at the two-cell stage. Surprisingly, concurrent loss of *Pten* restored the fertility of double-mutant females by recovering the impaired Akt activation and suppressed EGA. Thus, the maternal PI3K/Pten–Pdk1–Akt signalling cascade is considered as an indispensable maternal effect factor in triggering EGA and sustaining preimplantation embryogenesis in mice.

In conclusion, it is clear that Tsc/mTORC1 signaling in oocytes is essential for the maintenance of quiescence and the survival of primordial follicles, and thereby controls the reproductive aging and menopausal age in females. Furthermore, the molecular network involved in PI3K/Pten–Pdk1–Akt signalling is crucial for EGA and preimplantation embryogenesis in mice

5 CLINICAL PERSPECTIVES

Our growing knowledge of the molecular mechanisms involved in the physiological development of follicles in the ovary is crucial to open up new avenues for a better understanding of ovarian physiology and pathology.

Currently, the emerging technique to treat the pathological conditions of the ovary, including POF and infertility is to develop the healthy mature oocytes from ovarian cortical tissues with dormant primordial follicles. Perhaps, it might be feasible to trigger the activation of dormant oocytes in primordial follicles by blocking the molecules that suppress the activation of primordial follicles such as Tsc. In addition, pharmacological inhibitors of mTORC1 such as rapamycin may be useful to rescue the follicular development in the ovaries of women who are otherwise at risk of POF.

Cancer patients, who cannot undergo a full course of hormonal treatment for collection and preservation of mature oocytes, can have their ovarian cortical tissues cryopreserved before undergoing for chemo-or radiotherapy. The cryopreserved ovarian cortical tissues with primordial follicles can be used later for *in vitro* growth (IVG), followed by *in vitro* maturation (IVM) of oocytes and *in vitro* fertilization (IVF) (Jeruss and Woodruff, 2009).

Therefore, our growing knowledge of the molecules that maintain the dormancy, the survival and the activation of primordial follicles in the mammalian ovary would help us to better understand the physiology and pathophysiology of mammalian ovary, and in the development of more promising methods to obtain mature fertilizable oocytes from primordial follicles.

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