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Determine the developmental dynamics of primordial follicles in the mouse ovary

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To my family

Don't do fashionable science.

-- Max Delbrück

ABSTRACT

Ovarian follicles are the basic functional units of the mammalian ovary. Progressive activation of primordial follicles serves as the source of fertilizable ova. In this thesis, by generating the *Foxl2-CreER*^{T2} and *Sohlh1-CreER*^{T2} mouse models, I have specifically labeled and traced the *in vivo* development of two classes of primordial follicles, the first wave of primordial follicles that are activated immediately after they are formed and the adult primordial follicles that are activated gradually in later life. The time-lapse tracing study has shown that the first wave of primordial follicles exist in the ovaries for about 3 months and contribute to the onset of puberty and to early fertility, whereas the adult primordial follicles gradually replace the first wave and dominate the ovary after 3 months of age, providing fertility until the end of reproductive life. Moreover, the two follicle populations also exhibit diverged minimal and maximal *in vivo* ripening times. Thus the two classes of primordial follicles follow distinct, age-dependent developmental paths and play different roles in the mammalian reproductive lifespan. Next I have verified whether primordial follicles can be regenerated from the purported female germline stem cells in the postnatal mouse ovary. We have created a multiple fluorescent *Rosa26*$^{rbw/+}$*;Ddx4-Cre* germline reporter mouse model for *in vivo* and *in vitro* tracing the development of female germline cell lineage. Through live cell imaging and neo-folliculogenesis experiments, we have shown that the Ddx4-expressing cells from postnatal mouse ovaries do not divide during the *in vitro* culture, nor do they differentiate into oocytes following transplantation into the recipient mouse. Such experimental evidence supports the classic view that there is neither follicular replenishment nor female germline stem cell in the postnatal mammalian ovary. In summary, I have determined the developmental dynamics of two distinct populations of primordial follicles in the mouse ovary and confirmed that there is no spontaneous follicle regeneration. Such knowledge will hopefully lead to a more in-depth understanding of how different types of primordial follicles contribute to physiological and pathological alterations of the mammalian ovary.

Key words: ovary, primordial follicles, germline stem cell, cell lineage tracing.

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ABBREVIATIONS

AMH	anti-Müllerian hormone
bFGF	basic fibroblast growth factor
bHLH	basic helix–loop–helix
BIRC	baculoviral inhibitors of apoptosis repeat-containing
Blimp1	B-lymphocyte-induced maturation protein 1
BMP	bone morphogenetic protein
BPES	blepharophimosis/ptosis/epicanthus inversus syndrome
CCN	connective tissue growth factor, cystein rich protein, and nephroblastoma
CL	corpora lutea
Ddx4	DEAD box polypeptide 4
DEAD	Asp-Glu-Ala-Asp
Dmrt1	doublesex and mab-3 related transcription factor 1
dpc	days post coitum
Dppa3	developmental pluripotency-associated 3
EBs	embryoid bodies
EGF	epidermal growth factor
eIF5B	eukaryotic translation initiation factor 5B
EpiLCs	epiblast-like cells
ESCs	embryonic stem cells
FACS	fluorescence-activated cell sorting
Figla	factor in the germ-line alpha
Foxl2	forkhead box L2
FSH	follicle-stimulating hormone
Gdf9	growth and differentiating factor 9
GSK3	glycogen synthase kinase 3
hCG	human chorionic gonadotropin
H-P-O	hypothalamus-pituitary-ovary
Ifitm3	interferon-induced transmembrane protein 3
iPSCs	induced pluripotent stem cells
LH	luteinizing hormone
Lhx8	LIM homeobox 8
LIF	lymphocyte inhibitory factor
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinase

NHL	NCL-1, HT2A and Lin-41
Nobox	newborn ovary homeobox
OCT	octamer-binding transcription factor
OSCs	oogonial stem cells
PD	postnatal day
Pdk1	phosphoinositide-dependent kinase 1
PGC	primordial germ cells
PGCLCs	primordial germ cell–like cells
PI3K	phosphoinositide 3-kinase
piRNAs	Piwi-interacting RNAs
PMSG	pregnant mare's serum gonadotropin
Prdm14	PR domain-containing protein 14
Pten	phosphatase and tensin homolog deleted on chromosome 10
RA	retinoic acid
SCF	stem cell factor
Sohlh1	spermatogenesis and oogenesis specific basic helix-loop-helix 1
TGF	transforming growth factor
TRIM	tripartite motif-containing
WT	wild-type
YAP	Yes-associated protein

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1 INTRODUCTION

1.1 Mammalian ovary

The mammalian ovary is a reproductive and endocrine organ. It generates fertilizable ova and produces steroid hormones to facilitate the development of the female secondary sexual characteristics and support the pregnancy (Hirshfield, 1991; McGee and Hsueh, 2000). The basic functional units of the ovary are ovarian follicles. Each follicle is composed by a meiotic arrested oocyte and surrounding pregranulosa cells or granulosa cells. Growing follicles with multiple layers of granulosa cells contain an additional theca cell layer outside the basement membrane (Eppig et al., 2003; Hirshfield, 1991; McGee and Hsueh, 2000).

The majority of the follicles in the mammalian ovary are primordial follicles. The female reproductive lifespan is determined by the size of primordial follicle pool, which is fixed early in life (Zuckerman, 1951). It has been shown that the human ovary contains around 2 million follicles at birth, and this number drops below 1000 at approximately 51 years of age. Most of the follicles undergo degeneration and only about 400 oocytes reach ovulation (Kaipia and Hsueh, 1997). Thus the gradual diminution of ovarian follicle reserve is associated with reproductive aging and menopause (Adhikari and Liu, 2009; McGee and Hsueh, 2000).

Unlike tissues in many other organs, the follicles in the mammalian ovary do not follow a uniform growth pattern. They display unique developmental fates and represent independent functional units within the ovary (McGee and Hsueh, 2000). Recent studies using knockout mouse models have demonstrated that the intra-oocyte phosphoinositide 3-kinase (PI3K) pathway determines the fates of primordial follicles. As summarized in Figure 1, an optimal PI3K activity is needed for maintaining the dormancy of the majority of primordial follicles in the mouse ovary (Zheng et al., 2012) (Fig. 1A), whereas suppressed PI3K activity leads to the death of follicles (Reddy et al., 2009) (Fig. 1C). In a progressive manner, a small proportion of primordial follicles with elevated PI3K pathway are activated and enter into the growing follicle pool (Adhikari and Liu, 2009; Reddy et al., 2008) (Fig. 1B). The activation of primordial follicles involves the differentiation of squamous pregranulosa cells into cuboidal granulosa cells, proliferation of granulosa cells, and the enlargement of oocytes (Adhikari and Liu, 2009; Hirshfield, 1991).

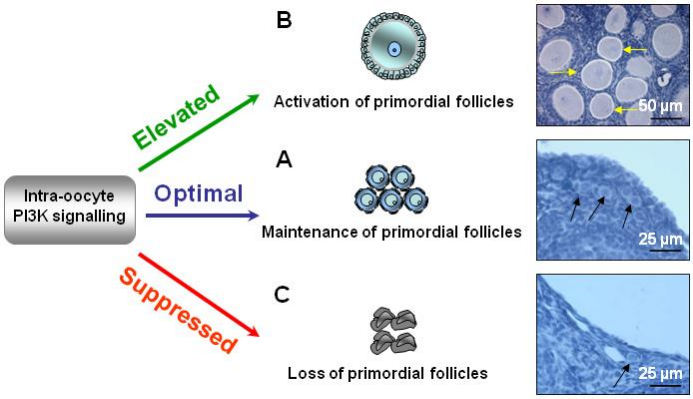

Fig. 1 Roles of intra-oocyte PI3K signaling

(A) Optimal PI3K signaling in oocytes maintains the survival of primordial follicles during their long dormancy. (B) Elevated PI3K signaling leads to oocyte growth and follicular activation. Shown is the premature follicle activation in mice where the PI3K negative regulator Pten (phosphatase and tensin homolog deleted on chromosome 10) is depleted in oocytes of primordial follicles. (C) Suppressed PI3K signaling leads to loss of primordial follicles initiated by the death of inner oocytes. Shown is the premature follicle depletion in mice where the PI3K master kinase Pdk1 (phosphoinositide-dependent kinase 1) is depleted from oocytes of primordial follicles. Yellow arrows: overactivated primordial follicles. Black arrows: dormant primordial follicles. The schema is obtained from Zheng et al., 2012.

Once activated, the primordial follicles develop through primary and secondary stage before acquiring an antral cavity. During this period, the follicular development is believed to be independent on the cyclic follicle-stimulating hormone (FSH) that is secreted by the pituitary gland (McGee and Hsueh, 2000). Instead, inter-follicle communication has been postulated to play a key role in regulating the follicular development at this phase. One example is the inhibition of neighboring follicles by the dominant growing follicles (Baker and Spears, 1999). Accumulating evidence has indicated that the anti-Müllerian hormone (AMH), which is secreted by the granulosa cells of large growing follicles, suppresses the activation of primordial follicles and the development of small growing follicles within the adjacent region (Durlinger et al., 2001; Durlinger et al., 1999; Nilsson et al., 2007). Moreover, a quite recent study on both mouse and human ovary has uncovered the role of inter-follicle mechanical stress in regulating the development of small pre-antral follicles in the mouse ovary (Kawamura et al., 2013). It has been shown that fragmentation of ovarian tissues increases actin polymerization and suppressed the conserved Hippo signaling pathway, leading to the

nuclear translocation of Yes-associated protein (YAP) in the somatic cells of ovarian follicles. Consequently, the expression of CCN (connective tissue growth factor, cystein rich protein, and nephroblastoma overexpressed gene) growth factors and BIRC (baculoviral inhibitors of apoptosis repeat-containing) apoptosis inhibitors are upregulated to allow the rapid expansion of granulosa cells (Kawamura et al., 2013; Reddy et al., 2013). Interestingly, the expression of AMH is unaltered in fragmented ovarian tissues, indicating that AMH and Hippo pathway are probably two independent systems regulating the growth of small pre-antral follicles (Kawamura et al., 2013).

The cyclic recruitment of antral follicles to the preovulatory stage is tightly controlled by the hypothalamus-pituitary-ovary (H-P-O) axis (McGee and Hsueh, 2000; Ojeda et al., 1986; Richards et al., 2002). In the mouse ovary, most antral follicles undergo atresia in each estrus cycle, whereas a few of them develop to preovulatory stage in response to the cyclic FSH. Granulosa cells of these dominant antral follicles undergo a fast proliferation and maturation process. They start to express the LH (luteinizing hormone, another pituitary gonadotropin) receptor and to produce inhibin and estrogens (Burns and Matzuk, 2002; McGee and Hsueh, 2000). The inhibin and estrogens in turn provide a negative feedback and suppress the secretion of FSH, balancing the H-P-O axis (Burns and Matzuk, 2002; McGee and Hsueh, 2000).

Finally the ovulation is triggered by the surge of LH and the meiosis II arrested oocytes are released into the oviduct for fertilization (Burns and Matzuk, 2002; Hirshfield, 1991). The remaining granulosa and theca cells then differentiate into corpora lutea (CL). The CL secret progesterone to prepare the uterus for embryo implantation. If no successful implantation occurs, the CL undergo a controlled degenerative process named luteal regression (also known as luteolysis) (Niswender et al., 1994).

1.2 Classification of ovarian follicles

As demonstrated in Figure 2, the rodent ovarian follicles can be divided into the following 8 categories based on the size of oocytes, the number of granulosa cells and the morphology of the follicles (Pedersen and Peters, 1968).

The type 1 are small dormant naked oocytes (< 20 μm in diameter) without surrounding supporting cells. The type 2 are quiescent primordial follicles containing small oocytes (< 20 μm in diameter) and squamous pregranulosa cells. The type 3 are primary follicles that are composed by growing oocytes (≥ 20 μm in diameter) and a complete layer of cuboidal granulosa cells. The type 4 and 5 are secondary follicles containing two or more layers of granulosa cells, with no visible antrum. The type 6 are early antral follicles containing scattered areas of fluid. The type 7 are antral follicles consisting of cumulus oophorus and a single cavity. The type 8 are preovulatory follicles in which the cumulus stalk and a large single antrum are well formed.

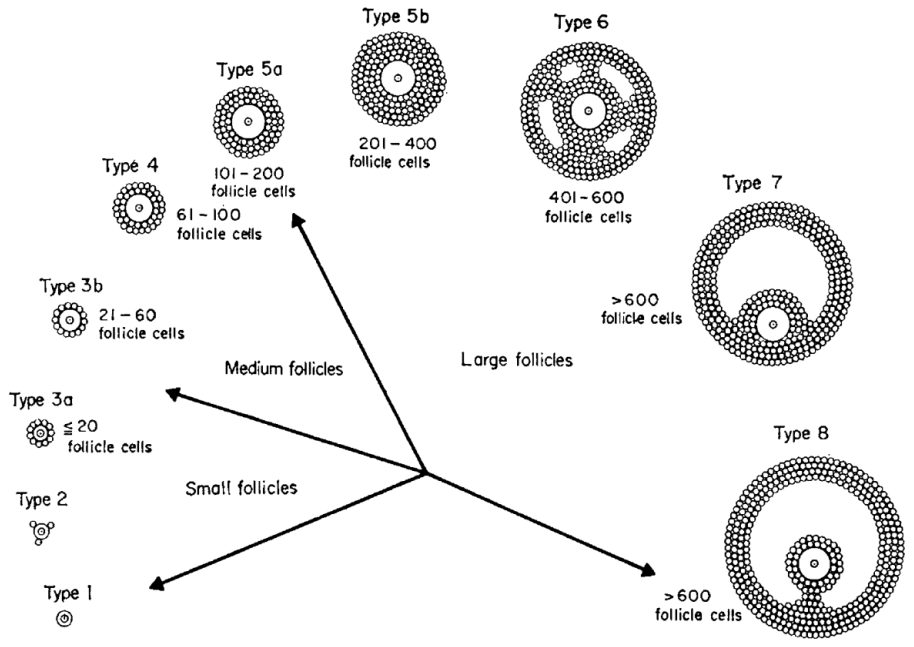

Fig. 2 Classification of ovarian follicles

Eight categories of follicles are classified according to the size of oocytes, the number of granulosa cells and the morphology of the follicles. This schema is obtained from Pedersen and Peters, 1968.

1.3 Formation of primordial follicles

In the mouse, the precursors of primordial germ cells (PGCs) arise from the most proximal epiblasts at 6.25 to 6.5 days post coitum (dpc) (Saitou and Yamaji, 2012). They are expressing two key transcriptional regulators B-lymphocyte-induced maturation protein 1 (Blimp1) and

PR domain-containing protein 14 (Prdm14) in response to the bone morphogenetic protein (BMP) 4 secreted from the extra-embryonic ectoderm (Fujiwara et al., 2001; Lawson et al., 1999; Ohinata et al., 2005; Vincent et al., 2005; Yamaji et al., 2008). At 7 to 7.25 dpc, around 45 founder PGCs are established as a cluster of alkaline phosphatase-positive cells in the extraembryonic mesoderm (Bendel-Stenzel et al., 1998; Saitou et al., 2002). The expression of two PGC-specific genes, *Fragilis* (also known as *interferon-induced transmembrane protein 3*, *Ifitm3*), and *Stella* (also known as *developmental pluripotency-associated 3*, *Dppa3*) highlights the successful specification of PGCs, but they are dispensable for accomplishing this germline cell lineage establishment (Lange et al., 2008; Payer et al., 2003; Saitou et al., 2002).

The PGCs actively proliferate and migrate through primitive streak and hindgut to finally settle in the genital ridge at 10.5 to 11.5 dpc (Ginsburg et al., 1990). In the female XX embryo, the PGCs continue to proliferate until 13.5 dpc, when they reach the number of ~ 25,000 and are clustered to form germline cysts (Hilscher et al., 1974; McLaren and Southee, 1997; Tam and Snow, 1981). From 17.5 dpc to postnatal day (PD) 5, the germ cells enter into diplotene stage of meiosis I and become oocytes (Borum, 1967; Pepling, 2006; Pepling and Spradling, 1998). These oocytes are arrested at this stage for the rest of the developmental process, until ovulation (Hirshfield, 1991; McGee and Hsueh, 2000). Next, the germline cysts start to breakdown to form primordial follicles. This process involves a death of one third of the germ cells within the cysts, and the invasion of the somatic pregranulosa cells to encapsulate individual oocytes (Pepling and Spradling, 2001). It has been postulated that the massive loss of germ cells is associated with the quality control system for elimination of compromised germ cells (Pepling and Spradling, 2001; Tingen et al., 2009).

The communication between the pregranulosa cells that surround the cysts and the oocytes is believed to be essential for the formation of primordial follicles (Eppig, 1991; Guigon and Magre, 2006; Kezele et al., 2002). For example, the KIT and KIT ligand (also known as Stem Cell Factor, SCF) are expressed in the perinatal ovary from 16.5 dpc. The KIT signaling pathway has been shown to play a role in promoting cyst breakdown and maintaining the survival of oocytes during the assembly of primordial follicles in the mouse ovary (Jones and Pepling, 2013). In addition, several *in vitro* and *in vivo* studies have indicated that perinatal exposure of steroid hormones, including estrogen and progesterone, may inhibit the cyst

breakdown and interfere with the coordinated loss of germ cells during cyst breakdown (Chen et al., 2009; Chen et al., 2007; Kezele and Skinner, 2003; Tingen et al., 2009). Such studies provide insight into how maternal hormones may regulate the primordial follicle formation in the offspring ovary during the pregnancy.

In mice, the primordial follicles are firstly formed in the ovarian medulla from 17.5 dpc (Hirshfield and DeSanti, 1995; Pepling, 2012; Pepling et al., 2010). They are activated immediately after their formation and are thus named the first wave of primordial follicles (Hirshfield, 1992; Mork et al., 2012). It is generally believed that most of the follicles in the first wave are anovulatory due to the insufficient gonadotropins before the puberty onset (Eppig and Handel, 2012; Hirshfield, 1992; Matzuk et al., 2002). The primordial follicles in the ovarian cortex are formed shortly after birth and this process lasts until PD5 to 8, depending on the genetic background of the mouse strain (Mork et al., 2012; Pepling, 2012). Such cortical primordial follicles are activated gradually in later life and provide fertility till the end of reproductive life (Hirshfield, 1992; Hirshfield and DeSanti, 1995; Zheng et al., 2014). In the humans, the formation of primordial follicles starts from the fourth month of fetal life and is completed by birth (Gougeon, 1996; Konishi et al., 1986).

1.4 Two distinct populations of primordial follicles

A pioneering study by Hirshfield for the first time proposed the hypothesis that there are two populations of primordial follicles in the postnatal rat ovary (Hirshfield, 1992). By autoradiographic labeling of the somatic cells in the embryonic gonad, Hirshfield found that the medullary granulosa cells had all developed from the mitotically quiescent progenitor cells during mid- to late gestation. The cortical pregranulosa cells, however, were specified after birth, and their progenitor cells were still actively proliferating during mid- to late gestation. Such distinct origins of supporting cells strongly suggested that there were two separate populations of primordial follicles (Fig. 3). The medullary follicles (referred to as the first wave of primordial follicles) start to grow as soon as they are formed, whereas the cortical follicles (referred to as the adult primordial follicles) mature gradually over the reproductive lifespan of the animal (Hirshfield, 1992; Hirshfield and DeSanti, 1995).

In the human ovary, following the completion of primordial follicle formation before birth, most of the primordial follicles enter into a quiescent state (with some of them having reached

the primary stage), but a sub-population of primordial follicles start to grow immediately after they are formed (Gougeon, 1996). These actively growing follicles are present in the human ovarian medulla throughout infancy and childhood (Lintern-Moore et al., 1974; Peters et al., 1976). The number of pre-antral follicles in the ovarian medulla is even higher in ovaries of 3- to 9-year-old girls than in older post-puberty girls and women (Kristensen et al., 2011). Therefore, there might also be two distinct populations of primordial follicles in the human ovary. They might exhibit different developmental dynamics as seen in the mouse ovary as well.

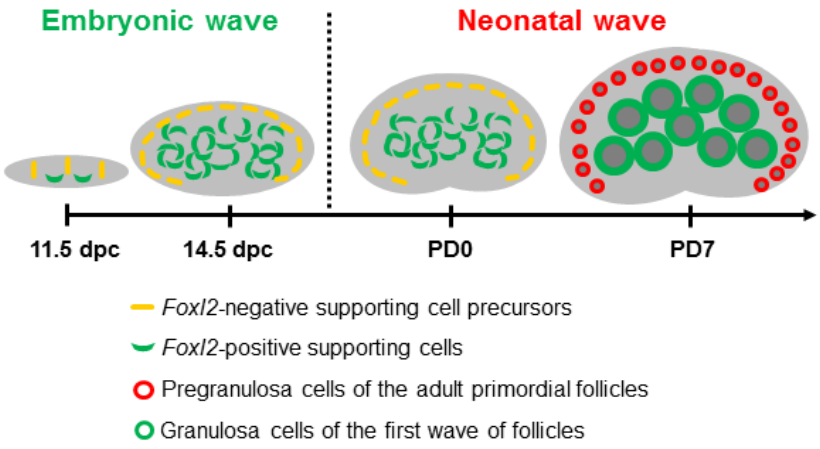

Fig. 3 Two classes of primordial follicles are formed by two waves of *Foxl2*-expressing supporting cells.
In the embryonic wave, the *Foxl2*-negative precursors migrate from the epithelium to the medulla of the gonad and differentiate into *Foxl2*-expressing supporting cells from 11.5 dpc. They develop to granulosa cells of the first wave of follicles after birth. The ingression lasts until about 14.5 dpc. The other group of *Foxl2*-negative precursors continue proliferating at the cortex of the gonad until birth. The expression of *Foxl2* is then elevated in these cells after the formation of adult primordial follicles, and this constitutes the neonatal wave of *Foxl2*-expressing supporting cells. This figure is adapted from Mork et al. (2012).

The roles of these two populations of primordial follicles to female fertility are yet to be differentiated. It was generally believed for many years that most of the first wave of follicles undergo atresia and are anovulatory due to the lack of cyclic gonadotropins before sexual maturity (Eppig and Handel, 2012; Hirshfield and DeSanti, 1995; McGee and Hsueh, 2000), although oocytes from the first wave of follicles can mature *in vitro* and generate live mice and rats (Eppig et al., 2009; O'Brien et al., 2003; Popova et al., 2002). On the other hand, the

adult primordial follicles in the ovarian cortex are thought to be the solitary source for producing fertilizable ova during the reproductive life (Eppig and Handel, 2012; Hirshfield and DeSanti, 1995; McGee and Hsueh, 2000). However, this notion has been challenged by the study presented in this thesis (Zheng et al., 2014).

1.5 Past studies on the development of ovarian follicles

Although the molecular mechanisms regulating the activation of primordial follicles have been extensively studied in genetically modified mouse models in recent years (Adhikari and Liu, 2009; Reddy et al., 2010; Zheng et al., 2012), much less is known about the timelines for the maturation of individual ovarian follicles *in vivo* under the physiological conditions.

In 1960s, Pedersen for the first time labeled the proliferating granulosa cells with the radioactive tritiated thymidine and traced the development of labeled follicles in immature and adult mice respectively. He determined that the follicular development from primary (type 3) to antral (type 7) stage took 10 to 16 days in immature mice (Pedersen, 1969) and 19 days in adult cyclic mice (Pedersen, 1970). Based on the same labeling strategy, it was calculated that the ripening time of primary follicle was 15 to 17 days in immature rats (Hage et al., 1978) and 22 days in adult cyclic rats (Groen-Klevant, 1981). However, by autoradiographic labeling of the zona pellucida with L-[^{3}H]fucose, Oakberg suggested that the development of ovarian follicles from the primary stage to ovulation took around 5 to 6 weeks in adult mice, almost twice the time proposed by Pedersen (Oakberg, 1979; Oakberg and Tyrrell, 1975). These tracing studies represent the earliest trials attempting to describe the developmental dynamics of ovarian follicles in mammals.

Despite the promise of these early studies, it was not possible to distinguish the first wave of primordial follicles from the adult primordial follicles by autoradiographic labeling. In addition, the radioactive labels can only be integrated into the proliferating granulosa cells or newly synthesized zona pellucida in the methods mentioned above, thus only primary or further developed follicles can be labeled. Moreover, it has been reported that the radioactivity of isotopes caused massive follicular atresia in the labeled ovaries and consequently influenced the accuracy of the tracing study (Hoage and Cameron, 1976; Oakberg, 1979). Therefore it is necessary to develop novel labeling strategies that can

differentially label the two populations of primordial follicles and trace their development without disturbing their growth.

1.6. Markers for tracing the development of ovarian follicles

To trace the developmental dynamics of ovarian follicles under the physiological conditions, it is essential to utilize endogenous molecular markers that are specifically expressed in germline cells or supporting cells.

1.6.1 Foxl2, a pregranulosa and granulosa cell-specific marker

Forkhead box L2 (Foxl2) is one of the 44 members of forkhead box transcription factors identified in both mice and humans. *Foxl2* heterozygous mutations in humans lead to the autosomal dominant blepharophimosis/ptosis/epicanthus inversus syndrome (BPES) (Crisponi et al., 2001). There are two types of BPES, both of which cause the eyelid malformations. Type I BPES is also associated with the premature ovarian insufficiency, and the patients encounter an early onset of menopause before the age of 40 years (De Baere et al., 2001). A single point mutation (402C to G) on the *Foxl2* allele has been found to cause granulosa cell tumor in women, which represents 5 to 10% of all ovarian cancers (Schumer and Cannistra, 2003; Shah et al., 2009).

In mice, the expression of *Foxl2* is detected in the supporting cells of female gonad as early as 12.5 dpc, but the formation of primordial follicles is not impaired in *Foxl2*$^{-/-}$ mouse ovaries (Schmidt et al., 2004; Uda et al., 2004). However, in the newborn mouse ovary, the *Foxl2* null mutation arrests the squamous to cuboidal transition of pregranulosa cells during the primordial follicle activation. Meanwhile, there is an extensive expression of *growth and differentiating factor* 9 (*Gdf9*) in *Foxl2*$^{-/-}$ mouse ovaries, indicating an early derepression of oocyte growth. The follicles with such abnormal growing oocytes (> 20 μm in diameter) and squamous granulosa cells can´t reach the secondary stage. Finally the massive oocyte apoptosis leads to the premature ovarian insufficiency (Schmidt et al., 2004; Uda et al., 2004). Based on these findings, Foxl2 is proposed to be essential for maintaining the dormancy of primordial follicles in the mouse ovary (Jagarlamudi and Rajkovic, 2012; Reddy et al., 2010).

Moreover, genes that are required for male sex determination, including *Sox9*, are expressed in the supporting cells of *Foxl2*$^{-/-}$ mouse ovaries, turning the female supporting cells to Sertoli-

like cells (Ottolenghi et al., 2005). Co-deletion of *Wnt4* and *Foxl2* even generates tubules with well differentiated spermatogonia in the medulla of new born XX mouse ovaries (Ottolenghi et al., 2007). When *Foxl2* is deleted in adult mice, granulosa cells are transdifferentiated to Sertoli cells, and structures reminiscent of seminiferous tubules are formed in the ovary (Uhlenhaut et al., 2009). On the other hand, loss of the transcription factor doublesex and mab-3 related transcription factor 1 (Dmrt1) in the adult mouse testis turns on the expression of *Foxl2* in Sertoli cells and transdifferentiates the Sertoli cells to granulosa-like cells (Matson et al., 2011). Combining these findings with the fact that *Foxl2* is expressed in supporting cells at all developmental stages in the mouse ovary (Schmidt et al., 2004; Uda et al., 2004), it is highly possible that a central role of Foxl2 is the lifetime maintenance of granulosa cell identity.

The specific expression pattern of *Foxl2* makes it a potential candidate for labeling ovarian follicles. As demonstrated in Figure 3, the two populations of primordial follicles in the mouse ovary can be distinguished by the sequential expression of *Foxl2* in their pregranulosa cells (Mork et al., 2012). It has been shown that *Foxl2*-negative supporting cell precursors constantly ingress from the epithelium of the fetal gonad from 11.5 to 14.5 dpc. These precursors continue proliferating before the expression of *Foxl2* is elevated. Then these *Foxl2*-expressing supporting cells remain quiescent in the ovarian medulla during fetal development and resume their mitotic cycle along with the development of the first wave of primordial follicles. The second population of precursors arise from the ovarian surface epithelium from 15.5 dpc to PD4, and the ingression is accomplished by PD7. This batch of *Foxl2*-negative supporting cell precursors continue proliferating in the peripheral region of the neonatal ovary, and then differentiate into *Foxl2*-expressing pregranulosa cells at the end of the assembly of adult primordial follicles, which occurs a few days after birth (Mork et al., 2012). This finding provides strong evidence supporting the proposal by Hirshfield (Hirshfield, 1992; Hirshfield and DeSanti, 1995) that there are two populations of primordial follicles formed by two waves of supporting cells (pregranulosa cells) in the postnatal mouse ovary.

It is worthy of noticing that for the first time, the pregranulosa cells have been specifically labeled in the tamoxifen-inducible *Foxl2-CreER*^{T2} mouse model developed by Mork et al. (2012). However, the labeling efficiency of *Foxl2*-expressing cells was fairly low, and

consequently it was not possible to label all *Foxl2*-expressing supporting cells in the fetal gonad and then trace their development in the ovaries of born pups (Mork et al., 2012).

1.6.2 Sohlh1 and Sohlh2, primordial follicle oocyte-specific markers

Spermatogenesis and oogenesis specific basic helix-loop-helix 1 (*Sohlh1*) was initially identified as a novel germ-cell-specific transcription factor expressing in the prespermatogonia and Type A spermatogonia of mouse testes (Ballow et al., 2006a). The mRNA of *Sohlh1* is readily detected in the male gonad from 12.5 dpc till adulthood. Spermatogonia are embryonic as well as adult germline stem cells that on one hand self-renew themselves and on the other hand differentiate into spermatocytes. Loss of *Sohlh1* impairs the differentiation of spermatogonia into spermatocytes and finally causes the male infertility (Ballow et al., 2006a).

Sohlh1 is also indispensable for the development of mouse ovaries. The *Sohlh1* mRNA is first detected at 13.5 dpc and then enriched at 15.5 dpc in the female gonad, when the female germ cells start to enter into the prophase of the first meiotic division and become oocytes (Pangas et al., 2006). In the postnatal mouse ovary, the *Sohlh1* mRNA is expressed in oocytes of primordial follicles, and the Sohlh1 protein is detected in oocytes of both primordial and primary follicles (Pangas et al., 2006).

There is no obvious defect in the newborn *Sohlh1*$^{-/-}$ mouse ovary, indicating that Sohlh1 is dispensable for germline cyst breakdown and the formation of primordial follicles. However, the activation of primordial follicles is suppressed in the absence of Sohlh1. It has been shown that no primary follicle exists in *Sohlh1*$^{-/-}$ ovaries at PD3, and only empty primary follicles without live oocytes are found in *Sohlh1*$^{-/-}$ ovaries at PD7. Finally no live follicle is left in *Sohlh1*$^{-/-}$ ovaries at 3 weeks of age. Further gene expression profiling analysis has revealed that the transcription of a female germ-cell-specific transcription factor, LIM homeobox 8 (Lhx8), is suppressed. The expression of two key oocyte-specific transcription factors, newborn ovary homeobox gene (Nobox), factor in the germ-line alpha (Figlα), are also strikingly downregulated in the absence of Sohlh1 (Pangas et al., 2006). The phenotype of *Sohlh1*$^{-/-}$ ovaries is reminiscent of *Figlα*$^{-/-}$, *Nobox*$^{-/-}$ and *Lhx8*$^{-/-}$ ovaries, indicating that these 4 transcription factors are probably interactive with each other and are all essential for early

folliculogenesis in the mouse ovary (Pangas et al., 2006; Rajkovic et al., 2004; Soyal et al., 2000).

Sohlh2 shares a highly conserved basic helix–loop–helix (bHLH) domain with Sohlh1 (Ballow et al., 2006b). In mice, the expression patter of *Sohlh2* is quite similar to that of *Sohlh1* in both male and female gonads (Ballow et al., 2006b; Hao et al., 2008; Toyoda et al., 2009). Sohlh1 and Sohlh2 form homodimers and heterodimers to control the expression of KIT in early differentiating germ cells (Barrios et al., 2012; Toyoda et al., 2009). It has been shown that blocking KIT signaling pathway impairs the differentiation of spermatogonia in the mouse testis (Yoshinaga et al., 1991) and the activation of primordial follicles in the mouse ovary (Yoshida et al., 1997). Therefore Sohlh1 and Sohlh2 are probably both upstream regulators that coordinate the maturation of germ cells in mouse testes and ovaries (Barrios et al., 2012; Suzuki et al., 2012; Toyoda et al., 2009).

Given that the expression of *Sohlh1* and *Sohlh2* are both restricted to the oocytes of primordial follicles in the mouse ovary (Ballow et al., 2006b; Pangas et al., 2006), they are ideal endogenous markers to target primordial follicles. A mouse model harboring a *Sohlh1-mCherryFlag* transgene has been developed recently (Suzuki et al., 2013). The red fluorescence (mCherry) resembles the expression patter of endogenous *Sohlh1* in the mouse ovary, confirming the fidelity of labeling primordial follicles with *Sohlh1* as a molecular marker (Suzuki et al., 2013).

1.6.3 Ddx4, a germline cell marker

DEAD (Asp-Glu-Ala-Asp) is a highly conserved protein family that has been reported in various prokaryotic and eukaryotic species ranging from *Escherichia coli* to human (Linder, 2006; Wassarman and Steitz, 1991). Many of them have been shown to have RNA-dependent ATPase and ATP-dependent RNA helicase activities, including DEAD box polypeptide 4 (Ddx4, also known as VASA) (Gustafson and Wessel, 2010). *Ddx4* was originally identified as one of the three maternal-effect genes on the second chromosome of Drosophila that control the germ cell specification (Hay et al., 1988; Schüpbach and Wieschaus, 1986). It is a highly conserved germline cell-specific gene in invertebrate and vertebrate species, including *Caenorhabditis elegans*, *Crassostrea gigas*, *Parhyale hawaiensis*, Xenopus, zebrafish, chicken, monotremes, marsupials, mouse, rat, human and etc. (Castrillon et al., 2000; Fabioux

et al., 2009; Fujiwara et al., 1994; Gruidl et al., 1996; Hickford et al., 2011; Ikenishi and Tanaka, 1997; Komiya and Tanigawa, 1995; Ozhan-Kizil et al., 2009; Tsunekawa et al., 2000; Yoon et al., 1997).

In Drosophila, *Ddx4*-null females fail to produce differentiated oocytes and nurse cells. This phenotype has been linked to the malfunction of Gurken, a TGF (transforming growth factor)-α-like protein. Gurken is secreted by the oocyte for the communication with adjacent follicle cells in order to establish the germ cell dorsal-ventral polarity (Ghabrial and Schupbach, 1999). Ddx4 protein binds to eukaryotic translation initiation factor 5B (eIF5B) to regulate the expression of *Gurken* whereas the accumulation of Gurken is impaired in the absence of Ddx4 (Carrera et al., 2000; Johnstone and Lasko, 2004; Styhler et al., 1998; Tomancak et al., 1998). In addition, Ddx4 also promotes the translation of *Mei-P26* by interaction with eIF5B. *Mei-P26* is one of the 221 different mRNAs that directly bind to Ddx4 (Liu et al., 2009). It encodes a TRIM (tripartite motif-containing)-NHL (NCL-1, HT2A and Lin-41) domain protein that interacts with Argonaute-1 to repress miRNA-mediated gene silencing, promoting differentiation of early-stage committed germline cells (Neumuller et al., 2008). Therefore Ddx4 regulates at the translational level the expression of genes that are required for germ cell specification in Drosophila.

In mice, the expression of Ddx4 starts from 12.5 dpc in PGCs and lasts until the post-meiotic stage in both males and females (Toyooka et al., 2000; Tsunekawa et al., 2000). Confocal imaging by immunofluorescence has shown that in the adult mouse testis, Ddx4 protein is exclusively localized in the cytoplasm of spermatogenic cells, and some granular staining is observed in late pachytene spermatocytes and round spermatids. In the adult mouse ovary, Ddx4 has been found to be expressed in the oocytes of follicles from primordial stage to pre-antral stage, but not in the oocytes of antral follicles. The subcellular localization of Ddx4 in female germ cells is cytoplasmic but is not restricted to particular cellular organelles (Toyooka et al., 2000).

Unlike the ortholog in Drosophila, the murine Ddx4 is dispensable for germline cell specification in the female embryonic gonad. *Ddx4*-null females are normal in fertility in terms of sexual behavior and the litter size. No defect in oogenesis or folliculogenesis has been found in *Ddx4*-null females. However, the proliferation of PGCs in the *Ddx4*-null male

embryonic gonad is impaired with reduced expression of OCT (octamer-binding transcription factor)-3/4 expression. Then all premeiotic spermatogenic cells beyond the postmeiotic stage undergo apoptotic death, resulting in no sperm production in the adult testis (Tanaka et al., 2000). Moreover, Ddx4 is involved in the *de novo* DNA methylation and subsequent silencing of the retrotransposons in fetal male germ cells through regulating the production of Piwi-interacting RNAs (piRNAs) (Kuramochi-Miyagawa et al., 2010). It thus can be concluded that Ddx4 is essential for the proliferation and differentiation of germ cells in the male but not female mouse gonad.

In humans, the expression of Ddx4 is also restricted to ovary and testis (Castrillon et al., 2000). The subcellular localization of human Ddx4 in PGCs and oocytes is also cytoplasmic, the same as its murine ortholog (Castrillon et al., 2000). Although the expression of murine Ddx4 in oocytes is decreasing along with the growth of the ovarian follicles, and finally is gone in oocytes of mouse antral follicles (Toyooka et al., 2000), the human Ddx4 is still detectable in human antral follicles (Castrillon et al., 2000).

To sum up, Ddx4 is a highly specific germline cell marker and is a potential marker for labeling female germline cell lineage. The promoter region of murine *Ddx4* has been cloned to drive the expression of Cre and $CreER^{T2}$ recombinases in transgenic mouse models (Gallardo et al., 2007; John et al., 2008). The expression of Cre and $CreER^{T2}$ recombinases is restricted in germline cells from PGCs to oocytes and spermatocytes, which is reminiscent of the expression pattern of endogenous Ddx4, proving that *Ddx4-Cre* and *Ddx4-*$CreER^{T2}$ are reliable mouse models for targeting germline cells (Gallardo et al., 2007; John et al., 2008; Zhang et al., 2012).

1.7 Regeneration of primordial follicles

It has been a golden rule for more than half a century that in mammals the number of primordial follicles is fixed at birth and there is no replenishment of ovarian follicles in the postnatal life (Zuckerman, 1951). Therefore much effort has been made by researchers and clinicians to explore novel methods that may hopefully lead to the regeneration of primordial follicles either *in vivo* or *in vitro* for treating human infertility (Zhang et al., 2013).

1.7.1 Follicle regeneration from putative oogonial stem cells

The existence of oogonial stem cells (OSCs) in the postnatal mouse ovary were first proposed in 2004 (Johnson et al., 2004). The authors claimed that OSCs were originated from the ovarian epithelium, serving as the source of follicle replenishment at a rate of 77 follicles per day in each ovary. The germline cell marker, Ddx4, was found to be expressed in such OSCs. This work attracted much criticism concerting the research methodologies and the quality of the data immediately after the publication (Albertini, 2004; Gosden, 2004; Telfer, 2004; Telfer et al., 2005). None of the major findings in this work can be reproduced by other researchers (Begum et al., 2008; Bristol-Gould et al., 2006; Kerr et al., 2012). Later the same authors, Johnson et al, amended their previous conclusions and postulated that the Ddx4-positive OSCs were actually originated from the bone marrow and peripheral blood (Johnson et al., 2005). This follow-up study was soon overwhelmed by another straightforward and convincing work (Eggan et al., 2006). Eggan et al. (2006) stitched together the circulation systems of a wild-type (WT) mouse and a mouse expressing GFP ubiquitously, and found that no GFP-positive eggs had ever been ovulated from the WT mouse, proving that no female germ cell precursors exist in bone marrow or peripheral blood.

However, the disputation on the existence of OSCs in the postnatal mouse ovary had not been settled down. Based on a polyclonal anti-human DDX4 antibody, Zou et al. (2009) claimed that they isolated a population of OSCs (referred to as female germline stem cells in their study) from neonatal and adult mouse ovaries by magnetic-activated cell sorting (MACS). Such OSCs were spherical and were 12-20 μm in diameter. They were positive for several germline cell markers and were mitotically active during the *in vitro* culture (Zhang et al., 2011; Zou et al., 2009), resembling the features of spermatogonial stem cells in postnatal mouse testes. The authors further reported that when transplanted to the chemotherapy drug-sterilized mouse ovaries, these OSCs were capable of recruiting supporting cells to regenerate follicles in the recipient ovary and restored the fertility of host females, as evidenced by the birth of live pups (Zou et al., 2009). Two other groups repeated this work independently but failed to obtain the same results as described by Zou et al. They did obtain a population of cells morphologically resembling the OSCs, but no meiotic competent oocyte had ever been generated from such cells (Hu et al., 2012; Pacchiarotti et al., 2010).

Then White et al. (2012) modified the original method by Zou et al. (2009) and utilized the fluorescence-activated cell sorting (FACS) instead of MACS to purify both mouse and human OSCs with the same anti-human DDX4 antibody. Strangely, the OSCs purified by White et al. were in square shape and were only 5-8 μm in diameter. They were much smaller than embryonic primordial germ cells (10-20 μm in diameter) (Donovan et al., 1986), male germline stem cells (10-20 μm in diameter) (Spiegelman and Bennett, 1973), and the OSCs obtained by Zou et al. (12-20 μm in diameter) (Zou et al., 2009). The mouse OSCs obtained by White et al. (2012) differentiated into fertilizable oocytes after transplantation and generated viable blastocysts. The human OSCs obtained in this work also initiated the neo-folliculogenesis when they were injected into the human ovarian cortical tissues that were xeno-transplanted to the immunodeficient female mice. However, no functional human eggs or live mouse pups were generated in this study (White et al., 2012).

The major concern of this DDX4 antibody-based OSC sorting system is that both mouse Ddx4 and human DDX4 are germ cell-specific RNA helicase and they are cytoplasmic proteins (Tanaka et al., 2000). No convincing data has been provided by either Zou et al. or White et al. to elucidate how DDX4 antibodies bind to intracellular Ddx4/DDX4 proteins and then isolate the OSCs. In addition, White et al. (2012) reported the presentence of haploid oocyte-like cells during the in *vitro* culture of OSCs. As the extrusion of the Meiosis II chromosomes only occurs after fertilization, the nature of these purported OSCs, therefore, is highly questionable (Oatley and Hunt, 2012).

Quite recently, several independent studies in mice and monkeys have arisen to deny the existence of OSCs. By inducible cell lineage tracing, Lei and Spradling have shown that the primordial follicle pool established in a 4-week-old mouse ovary provides the eggs for ovulation throughout the reproductive life and there is no replenishment of ovarian follicles. Nor is OSC observed in the adult mouse ovary (Lei and Spradling, 2013). This finding is consistent with the study presented in this thesis showing that there is no mitotically active female germline stem cell in the postnatal mouse ovary (Zhang et al., 2012). Moreover, Yuan et al. analyzed the expression of mitotic and germline cell markers in the ovaries of rhesus monkeys at different ages, and confirmed the absence of proliferating germline stem cells in the non-human postnatal primate ovary (Yuan et al., 2013).

Collectively, there is still a lack of comprehensive validation for the existence of OSCs in the postnatal mammalian ovary. More studies are needed before the OSCs can be safely used to regenerate ovarian follicles for ovarian rejuvenation and treating infertility.

1.7.2 Follicle regeneration from embryonic stem cells

Reconstitution of the mammalian gametogenesis *in vitro* has been a subject of interest for several decades. Efficient and reliable protocols for *in vitro* generation of both male and female mature functional gametes from PGCs, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have been developed recently.

PGCs are the progenitors of germ cell lineages. Hashimoto et al. for the first time obtained live mouse offspring following the transplantation of PGC-containing XX fetal ovary reaggregates into the ovarian capsules of adult females (Hashimoto et al., 1992). This method was later improved, so that the PGCs and somatic cells from both XX and XY embryonic gonads can be reaggregated to form reconstituted ovaries and testes that are subjected to ectopic transplantation in the kidney capsules of adult mice. Fertilizable oocytes and spermatids were eventually obtained and their viabilities were testified by the birth of live mouse pups, (Matoba and Ogura, 2011). These studies have established protocols for regeneration of primordial follicles from PGCs, as well as methods for the validation of *in vitro*-derived germline cells.

In vitro differentiation of germline cells from ESCs is more challenging (Childs et al., 2008). The ESCs derived from the inner cell mass of mouse blastocysts are capable of differentiating into germline cells *in vivo* (Bradley et al., 1984), yet co-culture of mouse ESCs with gonadal somatic cells failed to generate Ddx4-positive germline cells, indicating that germ cell specification cannot be induced by signals from the embryonic gonad (Toyooka et al., 2000). Several studies have shown that oocytes can be spontaneously generated during the *in vitro* culture of ESCs or somatic stem cells. However, none of the oocyte-like-cells generated in these studies have been proved to be meiotic competent and to be capable of generating live offspring (Danner et al., 2007; Dyce et al., 2006; Hubner et al., 2003).

On the other hand, it has been reported that embryoid bodies (EBs) can be obtained by culturing ESCs with lymphocyte inhibitory factor (LIF)-free medium. Then the PGCs derived

from the EBs are capable of generating fertilizable male gametes following transplantation into adult testes (Geijsen et al., 2004; Nayernia et al., 2006; Toyooka et al., 2003). Retinoic acid (RA) and BMP 4 or 8b have been shwon to be beneficial for promoting the differentiation of PGCs from ESCs, and for maintaining the survival and proliferation of EB-derived PGCs *in vitro*. Although live mouse pups have been obtained, the efficiency of obtaining viable germ cells is as low as one in one million starting cells with this method (Geijsen et al., 2004).

Recently, a more efficient two-step guided induction method has been developed to generate mouse male and female gametes from ESCs and iPSCs *in vitro* (Hayashi et al., 2012; Hayashi et al., 2011). The ESCs are first maintained at the ground state of pluripotency in a serum- and feeder-free medium supplied with a MAPK (mitogen-activated protein kinase) inhibitor (PD0325901), a GSK3 (glycogen synthase kinase 3) inhibitor (CHIR99021), and LIF (Saitou and Yamaji, 2012; Ying et al., 2008). Then the ESCs are stimulated by Activin A, bFGF (basic fibroblast growth factor), and knockout serum replacement to generate epiblast-like cells (EpiLCs). Finally primordial germ cell–like cells (PGCLCs) are induced by a medium supplied with BMP4, BMP8b, SCF, LIF and EGF (epidermal growth factor). In this method, approximately 40% of the EpiLCs can be differentiated into PGCLCs in a relatively large number (10^5–10^6), showing a robust PGCLC induction efficiency (Hayashi et al., 2012; Hayashi et al., 2011; Hayashi and Saitou, 2013). Following the transplantation into adult mouse testes, the male PGCLCs develop into fertilizable spermatozoa (Hayashi et al., 2011). On the other hand, the female PGCLCs have to be co-transplanted with somatic cells of female embryonic gonads in order to regenerate follicles in the recipient ovary (Hayashi et al., 2012; Hayashi and Saitou, 2013). Both male and female gametes obtained in this method are capable of generating live pups, indicating that this *in vitro* guided differentiation system faithfully replicates genetic and epigenetic programs of the gametogenesis *in vivo* (Hayashi et al., 2012; Hayashi et al., 2011). This method thus makes it possible for regeneration of primordial follicles from ESCs or iPSCs in mammals, including humans.

2 AIMS

The main aims of this dissertation are to provide a comprehensive timeline for the full-course development of two distinct populations of primordial follicles in the mouse ovary, and to verify whether primordial follicles can be regenerated spontaneously in the postnatal mouse ovary. More specifically, I would like to:

1. Determine the minimal and maximal lifespans of the first wave and the adult wave of primordial follicles, respectively.
2. Quantify how the proportions of the two populations of primordial follicles change in the mouse ovary from birth to adulthood.
3. Find out whether the first wave of primordial follicles contributes to fertility, and how long does it last for.
4. Determine whether the postnatal mouse ovary is permissive for neo-oogenesis and neo-folliculogenesis.
5. Verify whether the germline cells and the somatic cells within the postnatal mouse ovary are involved in the neo-folliculogenesis.
6. Label the Ddx4-positive ovarian cells and monitor their development *in vivo* and *in vitro*, so as to determine whether Ddx4-positive ovarian cells are mitotically active.
7. Verify whether other mitotically active ovarian cells can develop into germline cells in the postnatal mouse ovary.

3 RESULTS

3.1 Paper I

Two classes of ovarian primordial follicles exhibit distinct developmental dynamics and physiological functions

In this study, we first showed that the *Foxl2-CreER^{T2};mT/mG* mouse model can be used to label the first wave of primordial follicles in the mouse ovary by injecting tamoxifen to the pregnant females at 16.5 dpc. The labeled first wave of primordial follicles dominated the growing follicle pool at early age, and diminished gradually. They contributed to the ovulation in young adulthood and were exhausted from the mouse ovary by 3 months of age. On the other hand, the unlabeled adult wave of primordial follicles progressively replaced the first wave of primordial follicles in the growing follicle pool and became the only source of ovulated follicles by 3 months of age.

We next labeled the adult wave of primordial follicles in the *Sohlh1-CreER^{T2};R26R* mouse ovary by tamoxifen administration at 3 months of age. We traced the development of labeled follicles and calculated 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 stage. We also found that the primordial follicle pool labeled at 3 months of age persisted in the ovary until the end of productive life.

Collectively, we have depicted the lifelong developmental dynamics of ovarian primordial follicles under the physiological conditions, and have clearly shown that two classes of primordial follicles follow distinct, age-dependent developmental paths and play different roles in the mammalian reproductive lifespan.

3.2 Paper II

Experimental evidence showing that no mitotically active female germline progenitors exist in postnatal mouse ovaries

In this study, we first transplanted EGFP-expressing ovarian cells from 12.5 dpc *Rosa26*$^{rbw/+}$ fetuses into the ovaries of 2-month-old WT C57BL/6 female mice. Four weeks later, EGFP-positive follicles at various developmental stages were observed in the recipient ovary, and these regenerated follicles persisted there for at least four more weeks. However, all EGFP-positive follicles were found to be composed by EGFP-positive oocytes and granulosa cells, indicating that these neo follicles were all formed by cells that were derived from the transplanted embryonic gonadal cells, whereas no oocytes or granulosa cells from the recipient ovary contributed to the neo-folliculogenesis. We then pre-conditioned the adult mouse ovary with chemotherapy drugs busulfan and cyclophosphamide and repeated the above mentioned transplantation experiment. Still all neo follicles were found to contain EGFP-positive oocytes and granulosa cells. Therefore, the adult mouse ovary was permissive for neo-folliculogenesis, but no cell from the adult mouse ovary can be enclosed into the neo follicles, even if the ovary was pre-sterilized by chemotherapy drugs.

Next, we cultured the ovarian cells from the *Rosa26*$^{rbw/+}$*;Ddx4-Cre* postnatal mouse ovary and studied the mitotic division of Ddx4-positive cells *in vitro* by live-cell imaging. The testicular cells from *Rosa26*$^{rbw/+}$*;Ddx4-Cre* males were used as the positive control. It was found that none of the 1517 Ddx4-positive ovarian cells monitored underwent mitosis during the 72-h culture, whereas 263 Ddx4-positive testicular cells (spermatogonial stem cells) examined divided 2 to 3 times under the same culture condition. This result showed that the Ddx4-positive cells in the postnatal mouse ovary were mitotically inactive.

We then cultured the ovarian cells for long term. We observed that only Ddx4-negative cells were mitotically active and formed cell colonies during the *in vitro* culture. Semi-quantitative RT-PCR showed that no pluripotent stem cell marker *Sox2* or germline markers *Oct4*, *Stella*, or *Ddx4*, were expressed in these clonal cells. To verify whether such Ddx4-negative clonal cells can differentiate into germline cells *in vivo*, we first transplanted these cells into adult mouse ovaries and found that no transplanted cell differentiated into oocyte or other Ddx4-positive germline cell in the recipient ovary. We next mixed these clonal cells with 14.5 dpc

embryonic ovarian cells to generate reconstituted ovarian tissues and transplanted them under the kidney capsules of recipient mice. Still no oocyte or Ddx4-positive germline cell was found to be derived from the clonal cells. These results showed that the mitotically active Ddx4-negative ovarian cells that can form colonies during the *in vitro* culture were neither germline cells nor germline cell progenitors.

Collectively, our results showed that the postnatal mouse ovary contained no mitotically active female germline progenitors.

4 CONCLUSIONS AND PERSPECTIVES

The concept of a first wave of primordial follicles that is distinct from the adult primordial follicles raises an issue that has been overlooked by researchers for decades. In most studies on meiosis or early embryonic development, it is quite common to collect oocytes by stimulating follicle growth with pregnant mare's serum gonadotropin (PMSG) and priming the ovulation with human chorionic gonadotropin (hCG) at around 3.5 to 4 weeks. However, the majority of the oocytes obtained with such a protocol are from the first wave of follicles. It still remains unclear whether the first wave of follicles fully represents the genetic and epigenetic features of adult primordial follicles. More studies are needed to evaluate the differences between the two populations of follicles at both the system and molecular levels.

Although it was postulated in early studies that all growing follicles in the human ovary undergo atresia before the onset of puberty (Lintern-Moore et al., 1974; Peters et al., 1976; Valdes-Dapena, 1967), there is a lack of direct evidence to ascertain whether or not the first wave of follicles contribute to ovulation. Based on the recent follicular tracing study presented in this thesis (Zheng et al., 2014), it is possible that the fertility of women from puberty onset through young adulthood might rely on the first wave of follicles that are already activated at the fetal stage and that fertility in adulthood might rely on adult primordial follicles. The quality of human oocytes deteriorates with age (Gougeon, 1996). However, it has been reported that the dormant follicles in the ovarian cortex (i.e., the adult primordial follicles) of pre-pubertal girls exhibit compromised *in vitro* developmental potential compared to those from pubertal and adult women (Anderson et al., 2014). It also remains to be determined whether the oocytes of the first wave of follicles are of higher quality than those that develop from the adult primordial follicles. A recent study has proposed the use of medullary growing follicles for preserving fertility in young female cancer patients (Kristensen et al., 2011). The outcome of such studies might address questions about the oocyte viability of the first wave of primordial follicles.

Based on the work to date, it is highly possible that no spontaneous regeneration of primordial follicles occurs to replenish the ovarian follicle pool in the mammalian ovary after birth. There is also a lack of comprehensive validation for the existence of OSCs in mammals. So far the strategies to generate patient-specific iPSCs have been well developed and

standardized (Maherali and Hochedlinger, 2008; Wu and Hochedlinger, 2011). In contrast, even if OSCs do exist in postnatal human ovaries, it is technically more demanding to isolate sufficient OSCs from limited ovarian biopsies of patients. From this point of view, it is more promising to regenerate of primordial follicles from ESCs or iPSCs, rather than OSCs, for combating ovarian aging and treating female infertility.

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