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Abstract

The factors governing maintenance of the non-renewable reservoir of primordial follicles in female mammals remain largely elusive. During the early stages of fetal development, oocytes grow in nests or clusters known as germ cell cysts. Cysts breakdown into individual oocytes in the perinatal period and become enclosed by somatic pregranulosa cells to form primordial follicles. Steroid hormones have been shown by numerous studies to be one of the important factors which critically govern the process of cyst breakdown and follicle formation. There has been earlier evidence from this laboratory to indicate that exogenous exposure of neonatal mice ovaries to estradiol (E₂), progesterone (P₄) or E₂ mimicking chemicals known as xenoestrogens such as Diethylstilbestrol (DES) and Bisphenol-A (BPA) delay cyst breakdown and follicle formation. The overall goal of this dissertation project is centered on the pivotal question: “What is the source of steroid hormone signaling and its role in meiotic progression during fetal oocyte development in mice?” One of the two objectives of this dissertation was to identify the sources of the steroid hormones (maternal circulation or fetal ovaries) which regulate fetal oogenesis. Our studies showed prominent expression of both mRNA and protein in the fetal ovaries for aromatase and 3-beta-hydroxysteroid dehydrogenase (3βHSD), cardinal steroidogenic enzymes required for E₂ and P₄ synthesis respectively. The mRNA levels for both aromatase and 3βHSD in the fetal ovaries detected by qPCR were found to decrease prior to cyst breakdown. These results align to our previous model that high levels of steroid hormones keep oocytes in cysts during fetal development and the drop in hormone levels is required to trigger cyst breakdown. To
analyze the functional significance of this local steroid action, we used aromatase and 3βHSD inhibitors (letrozole and trilostane respectively) in organ culture to block hormone production by fetal ovaries. We find that the total number of oocytes was reduced in treated ovaries compared to controls. The second objective of the dissertation was to examine the relation between two temporal events in mice oogenesis: progression to the diplotene stage and primordial follicle formation. We performed a thorough quantitative analysis by nuclear morphological observations of diplotene versus pre-diplotene nuclei of hematoxylin and eosin stained serial sections of ovaries at different ages. Interestingly, we observed that primordial follicle formation occurs irrespective of the meiotic stage of the oocyte nuclei. Thus oocytes in follicles were found both at diplotene and pre-diplotene stages. We also wanted to understand the role of steroid hormone signaling in meiotic progression of oocytes. Our data indicate that exogenous treatment of P₄ and not E₂ decrease the number of follicles containing oocytes at diplotene. Such insights from the murine research models significantly contribute to our knowledge of the meiotic defects caused due to E₂ or P₄ exposure during fetal oogenesis in the case of human pregnancies (which often results from exposure to environmental estrogens or xenoestrogens). Aneuploidy is one of the prevalent causes for genetic disorders in humans and it arises from anomalies in the chromosome content of the gametes (sperms and ova). Any disruption in the normal meiotic events during fetal gametogenesis may be amplified along the way to give rise to aneuploid gametes. Meiotic studies in model organisms are therefore indispensable to our understanding of human aneuploidy. In summary, this dissertation project has focused on the critical role
of steroid hormone regulation of fetal mouse oocyte development and its role on meiotic progression, thus contributing to our understanding of early ovarian differentiation.
Steroid hormone regulation of fetal mouse oocyte development

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biology in the Graduate school of Syracuse University

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This thesis is dedicated to Ma and Baba. Thank you for your unconditional love, support and sacrifices throughout my education.
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Table of Contents
Abstract ............................................................................................................................... i
List of Tables .................................................................................................................... xii
List of figures ................................................................................................................... xii
List of abbreviations ...................................................................................................... xvi

CHAPTER 1: Introduction and Objectives .......................................................................1

1.1. Significance ................................................................................................................. 2
1.2. Ovarian development ................................................................................................. 3
1.3. Germline cysts ............................................................................................................ 5
1.4. Germ cell attrition .................................................................................................... 9
1.5. Meiotic progression and folliculogenesis ................................................................. 12
1.6. Diplotene arrest and follicle formation .................................................................... 14
1.7. Endocrine Disruptors ............................................................................................... 16
1.8. Estrogen (E\(_2\)) and Progesterone (P\(_4\)) receptors .............................................. 18
1.9. Influence of hormones on cyst breakdown and primordial follicle formation .... 20
1.10. Aromatase and 3-beta-hydroxysteroid dehydrogenase ....................................... 22
1.11. Figures ...................................................................................................................... 27
1.12. Objectives/ Specific aims ....................................................................................... 35

CHAPTER 2: Source of steroid hormone signaling regulating fetal mouse oocyte
development .................................................................................................................... 36

2.1. Abstract ...................................................................................................................... 37
2.2. Introduction ............................................................................................................... 38
2.3. Materials and Methods ...........................................................................................................41

2.4. Results .....................................................................................................................................48

2.4.1. Circulating maternal and perinatal steroid hormone levels during gestation ...... 48

2.4.2. Aromatase and 3βHSD present in perinatal ovaries .....................................................49

2.4.3. E₂ and P₄ found locally in perinatal ovaries .................................................................50

2.4.4. Effects of inhibiting E₂ and P₄ production in the fetal ovary ..................................51

2.5. Discussion ..........................................................................................................................52

2.6. Figures ...................................................................................................................................56

Chapter 3: The role of steroid hormones in murine oocyte meiotic progression ........64

3.1. Abstract .................................................................................................................................65

3.2. Introduction ..........................................................................................................................66

3.3. Materials and Methods .......................................................................................................68

3.4. Results ...................................................................................................................................73

3.4.1. Meiotic progression and diplotene arrest in developing mouse ovaries .................73

3.4.2. Primordial follicle formation and diplotene arrest are independent processes ....74

3.4.3. All oocytes in a cyst do not reach diplotene together .......................................................75

3.4.4. Progesterone decreases the number of follicles at diplotene ........................................75

3.5. Discussion ............................................................................................................................77

3.6. Table .....................................................................................................................................80

3.7. Figures ...................................................................................................................................81

Chapter 4: Conclusion and Future Directions 85

4.1. Conclusion ............................................................................................................................86

4.2. Future Directions ................................................................................................................90
Bibliography .......................................................................................................................... 94
Biographical Sketch .................................................................................................................. 116

List of Tables
Chapter 3
Table 3.1: Analysis of meiotic stage within 5 individual germ cell cysts ..................... 81

List of Figures
Chapter 1
Figure 1.1: Migration of Primordial Germ Cells (PGC)s in mice ................................. 27
Figure 1.2: Cyst breakdown and germ cell attrition ......................................................... 28
Figure 1.3: Stages of follicle development ...................................................................... 29
Figure 1.4: Structure of an adult mammalian ovary ......................................................... 30
Figure 1.5: Early timeline of fetal mouse oogenesis ......................................................... 31
Figure 1.6: Estrogen (E$_2$) can bind to three different receptors ................................. 32
Figure 1.7: Formation of Multiple Oocyte Follicles (MOF)s ........................................ 33
Figure 1.8: Steroid Synthesis Pathway ............................................................................. 34

Chapter 2
Figure 2.1: Circulating maternal and circulating perinatal levels of E$_2$ and P$_4$ .......... 56
  Figure 2.1 A: Maternal serum levels of E$_2$ measured in pg/ml and P$_4$ measured in
  in ng/ml during gestation and during birth ................................................................. 56
  Figure 2.1 B: Perinatal serum levels of E$_2$ measured in pg/ml and P$_4$ measured in
  ng/ml ............................................................................................................................... 56
Figure 2.2: mRNA and protein levels of steroidogenic enzymes in perinatal mouse
ovaries ............................................................................................................................... 57
Figure 2.2 A: Aromatase mRNA levels during the perinatal period ......................57
Figure 2.2 B: 3βHSD mRNA levels during the perinatal period ......................57
Figure 2.2 C: Western blot analysis of aromatase in fetal ovaries ......................57
Figure 2.2 D: Quantification of aromatase protein ...........................................57
Figure 2.2 E: Western blot analysis of 3βHSD in fetal ovaries ......................57
Figure 2.2 F: Quantification of 3βHSD protein .............................................57
Figure 2.3: Expression of aromatase protein in the fetal ovary ......................58
Figure 2.4: Expression of 3βHSD protein in the fetal ovary ......................59
Figure 2.5: Colabeling of 3βHSD and mitochondria or ER .............................60
Figure 2.6: Concentration of E₂ and P₄ in fetal and neonatal ovaries ..............61
  Figure 2.6 A: E₂ levels in ovaries from 15.5 dpc to PND7 ..............................61
  Figure 2.6 B: E₂ levels in ovaries from 15.5 dpc to PND3 ..............................61
  Figure 2.6 C: P₄ levels in ovaries from 15.5 dpc to PND7 ..............................61
  Figure 2.6 D: P₄ levels in ovaries from 15.5 dpc to PND3 ..............................61
Figure 2.7: Effects of inhibiting aromatase in fetal ovaries .............................62
  Figure 2.7 A: percent single oocytes ..........................................................62
  Figure 2.7 B: number of oocytes ..............................................................62
  Figure 2.7 C: percent primordial and primary follicles relative to the total number of follicles per confocal section ..........................................................62
Figure 2.8: Effects of inhibiting 3βHSD in fetal ovaries ...............................63
  Figure 2.8 A: percent single oocytes ..........................................................63
  Figure 2.8 B: number of oocytes ..............................................................63
  Figure 2.8 C: percent primordial and primary follicles relative to the total number
of follicles per confocal section .....................................................63

Figure 2.9: Effects of inhibiting aromatase and 3βHSD in fetal ovaries ..................64

Figure 2.9 A: percent single oocytes .................................................64

Figure 2.9 B: number of oocytes .......................................................64

Figure 2.9 C: percent primordial and primary follicles relative to the total number 
of follicles per confocal section .....................................................64

Chapter 3

Figure 3.1: Diplotene oocytes first detected at 17.5 dpc ..........................82

Figure 3.1 A: Percentage of oocytes at the diplotene stage meiotic prophase I 
in perinatal mouse ovaries from 15.5 dpc to PND5 ................................82

Figure 3.1 B: Relative measurements of mRNA corresponding to MSY2, a specific 
marker for diplotene stage oocytes as determined by qPCR in perinatal mice 
oviducts at different gestational ages ..............................................82

Figure 3.1 C-Figure 3.1F: Representative histological images of perinatal mouse 
oviducts stained with hematoxylin and eosin showing different meiotic stages .... 82

Figure 3.2: Follicle formation and meiotic progression in perinatal mouse ovaries ......83

Figure 3.2 A: Percentage of oocytes in follicles over time .........................83

Figure 3.2 B: Percentage of follicles at the pre-diplotene stage out of total follicles 
in perinatal mice ovaries ..............................................................83
Figure 3.2 C-Figure 3.2 F: Representative histological images of perinatal mouse ovaries stained with hematoxylin and eosin .............................................83

Figure 3.3: Representative histological images showing paraffin sections of fetal mouse ovaries at 18.5 dpc stained with hematoxylin and eosin .............................................84

Figure 3.3 A: ovarian section showing one cyst labeled Cyst 1 with five oocytes, one oocyte in diplotene and four in pre-diplotene .............................................84

Figure 3.3 B: ovarian section showing one cyst labeled Cyst 2 with three oocytes, two in diplotene and one in pre-diplotene .............................................84

Figure 3.3 C: ovarian section showing Cyst 3 with two oocytes, one in diplotene and one in pre-diplotene ..................................................................................84

Figure 3.3 D: ovarian section showing one cyst labeled Cyst 5 with five oocytes three in diplotene and two in pre-diplotene ........................................................................84

Figure 3.4: Effects of E₂ and P₄ treatment in organ culture on meiotic stage ............. 85

Figure 3.4 A: Total number of oocytes .............................................................................85

Figure 3.4 B: Percent single oocytes .............................................................................85

Figure 3.4 C: Percent of diplotene oocytes .....................................................................85

Figure 3.4 D: percent of follicles containing diplotene stage oocytes in control
ovaries or ovaries treated with $10^{-6}$ M $E_2$, $10^{-6}$ M $P_4$ or $10^{-6}$ M $E_2 + 10^{-6}$ M $P_4$ for 4 days in organ culture .................................................................85

List of Abbreviations

Aromatase enzyme knockout .......................................................... ArKO

3-beta-hydroxysteroid dehydrogenase ........................................ 3βHSD

Bisphenol-A .............................................................. BPA

Days post coitum .............................................................. dpc

Diethylstilbestrol .............................................................. DES

Estradiol ................................................................. $E_2$

Estrogen receptor .......................................................... ER

Estrogen receptor $\alpha$ ................................................... ER$\alpha$

Estrogen receptor ...................................................... ER$\beta$

Estrogen Response Element ................................................ ERE

Germinal vesicle breakdown ................................................ GVBD

Hematoxylin and Eosin .......................................................... H&E

Luteinizing Hormone .......................................................... LH

Meiosis ........................................................................ MI
Meiosis II .......................................................... MII

Multiple Oocyte Follicle .................................................. MOF

Polychlorinated biphenyls.................................................. PCB

Post Natal Day .................................................................. PND

Primordial Germ Cell ......................................................... PGC

Progesterone .................................................................. P₄

Progesterone receptor ..................................................... PR

Quantitative Polymerase Chain Reaction .............................. qPCR

Stimulated by retinoic acid 8 ................................................. Stra8

Synaptonemal complex ...................................................... SC

Synaptonemal complex protein .......................................... SYCP
Chapter I
Introduction and Objectives
1.1. Significance

A critical but poorly understood aspect of ovarian biology is the assembly of primordial follicles early in development. This process gives rise to the finite reservoir of oocytes available to a female mammal during her entire reproductive life (Kezele et al., 2002). Defects in the process of follicular assembly or inappropriate activation during primordial follicle development can give rise to a series of reproductive diseases. This can lead to disorders like premature ovarian failure or physiological changes like menopause in humans (Kezele et al., 2002). The tight regulation of the size of the primordial follicle pool is critical for the maintenance of female fertility (Pepling, 2006). Thus, studies elucidating the factors responsible for primordial follicle assembly and activation are critical to develop potential therapeutic treatments for these conditions. The primordial follicle is composed of an oocyte primarily arrested at the diplotene stage of prophase I of meiosis and is surrounded by squamous epithelial or pre-granulosa cells. The primordial follicles typically assemble around the time of birth in rodents and by the third trimester in humans and cattle (Gondos et al., 1971; Russe, 1983). This dissertation primarily focuses on two main aspects of early mouse oogenesis, synthesis of steroid hormones by perinatal ovaries and the link between diplotene arrest and follicle formation.
1.2. Ovarian Development

In mammals, oogenesis initiates during early fetal development and takes months to years to complete (Eppig, 2004) (Figure 1.1). Precursors for germ cell populations are established during the embryonic period (Hirshfield, 1991). In the mouse, the germ cell lineage derives from a founder population of approximately 45 primordial germ cells (PGCs) (Lawson and Hage, 1994). The PGCs are motile and highly responsive to chemotaxic stimuli that enable them to migrate from their site of origin (i.e., the embryonic epiblast) to the genital ridge. In mouse, PGCs are first observed in the yolk sac around 7.5 days postcoitum (dpc) (Snow, 1983). The migratory events occur between 9.5 dpc and 11.5 dpc (Figure 1.1).

During early embryonic life, the ovary develops from the indifferent gonad. The indifferent gonad is composed of mesenchymal cells derived from the genital ridge (Yoshinaga et al., 1988). PGCs enter the indifferent gonad around 10.5 dpc and are henceforth termed oogonia. After colonization, they rapidly proliferate to increase in number (generation time is around 15-16 hours in mouse) (Snow, 1983; Tam, 1981). As a result, the number of germ cells increases from 25,000 to 83,000 (Kezele et al., 2002; Snow, 1983). The somatic cells of the gonad which later give rise to the granulosa, theca, endothelial cells of the mature follicles also proliferate extensively like the germ cells.

The mitotic division of PGCs coupled with incomplete cytokinesis, produce clusters of oogonia known as ovarian germ cell cysts or nests (Pepling, 2006; Wylie, 1999). Germ cell cysts are composed of a pocket of germ cells surrounded by a layer of stromal cells. In certain species like cattle and pig, the stromal cells organize into histologically distinct
“ovigerous cords” surrounding the oogonia (Hirshfield, 1991). The origin of the stromal cells or cords enclosing the germ cell nests is not known (Hirshfield, 1991; Jost, 1988; Sawyer et al., 2002). Some evidence indicate that the cords may be derived from coelomic epithelium (Sawyer HR, 2002) though most evidences support that ovigerous cords arise from ovarian rete (Byskov and Lintern-Moore, 1973; Hirshfield, 1991; Kezele et al., 2002; Wenzel, 1985). The stromal cells or the ovigerous cords later form the pre-granulosa cells surrounding the primordial follicle (Kezele et al., 2002). The ovigerous cords, present in the ovaries of certain species like cattle, pig and cat divide the embryonic ovary into two distinct compartments separated by the basement membrane. The inner compartment containing the germ cells is known as epithelioid compartment and the outer one is called interstitial or mesenchymal compartment (Hirshfield, 1991).

The population of germ cells expands through mitotic divisions and undergoes one last round of DNA replication, before entering meiosis around 13.5 dpc. The germ cells are henceforth known as oocytes (Franchi, 1973; McLaren, 2000a). The two events, expansion of the germ cell population by mitosis and entry into meiosis are fairly synchronized with 19.5 % of germ cells entering meiosis and 80% proliferating at 13.5 dpc which rapidly decreases to only 23.5 % proliferating cells at 14.5 dpc (Evans et al., 1982). In humans, meiotic oocytes are first observed during the second month of gestation even though at 5 months, 28.6% of the germ cells are still mitotically active (Baker, 1963). By the seventh month of gestation, the last germ cells enter meiosis (Peters, 1976).
1.3. Germline cysts

Germline cysts were first studied in a wide variety of insects (e.g., Drosophila, beetles, bees, aphids) where female germ cells develop in clusters of interconnected cells that share several distinctive characteristics (Brunet and Verlhac, 2011; Bu¨ning, 1994). During cyst formation, there is an individual founder cell also referred to as a germline stem cell. The germline stem cell divides to form a daughter stem cell and a cystoblast. The cystoblast gives rise to the cyst. Cysts grow from the progenitor cell or cystoblast progressively by mitosis without undergoing any cytokinesis. The daughter cells known as cystocytes are normally formed in clusters of cells corresponding to $2^n$ (n can vary from 1 to 8 depending on the species) (Bu¨ning, 1994). Due to incomplete cytokinesis, the individual cystocytes are interconnected with one another by intercellular bridges known as ring canals. Germ cell clusters exhibiting characteristics of cysts have been observed in several vertebrate ovaries which indicate that it is a conserved trait across the vertebrate kingdom (Gondos, 1973). Small clusters of premeiotic germ cells have been found in mice, rabbits, rats, hamsters and humans. A detailed examination by electron microscopy revealed that intercellular bridges strongly resemble ring canals that connect those premeiotic germ cells. The germ cells in those organisms are also found to divide synchronously in small groups similar to germline cysts (Franchi and Mandl, 1964; Gondos, 1987; Gondos et al., 1971; Gondos and Zamboni, 1969; Pepling, 2013; Ruby et al., 1969; Weakly, 1967; Zamboni and Gondos, 1968).

Drosophila oogenesis has long been a favorite system to study cyst formation. Drosophila has two ovaries and each ovary consists of 16 ovarioles. Each ovariole represents an independent egg assembly line. The anterior region of each ovariole is
known as the germarium which consists of 2 germline and somatic stem cells which gives rise to mature egg chambers (follicles) (Spradling, 1993). A typical germarium has the following structures: one cystoblast (cyst founder cell), three successive stages of forming cysts, six cysts of sixteen cells and one egg chamber at the posterior end. Egg chambers leave the germarium from the posterior end. The egg chamber consists of a sixteen cell cyst and is surrounded by follicle cells. Egg chambers move posteriorly within each ovariole as they are developing. Each egg chamber develops from a 16-cell germline cyst. Cyst formation is initiated by the asymmetric division of one of the two germline stem cells in the germarium to produce a cystoblast. The cystoblast compared to its sister stem cell exhibits a unique pattern of cell division. It undergoes four additional cell divisions without any cytokinesis causing the daughter cells or cystocytes to be interconnected with one another by intercellular bridges or ring canals. One cell from the 16-cell germline cyst develops into an oocyte and the rest serve as nurse cells. Electron microscopic studies reveal a continuous flow of cytoplasmic organelles like mitochondria, ribosomes, maternal mRNAs as well as other nutrients through the ring canals from the nurse cells to the oocyte (Spradling, 1993). The characteristic “dumping” of cytoplasmic contents by the nurse cells occurs by increased cytoplasmic oscillations and streaming in the nurse cells and oocytes (Gutzeit, 1982). The nurse cells when treated with a drug called colchicine which inhibits microtubule polymerization exhibits complete loss of cytoplasmic transport into the oocyte (Gutzeit, 1986). This suggests that microtubules are present inside the ring canals or intercellular bridges and they facilitate the intercellular transport between nurse cells and oocyte.
One of the striking features of ring canals is the presence of an unusual vesicle rich cytoplasmic organelle known as the fusome. The mitotic spindle fibers, in dividing cells orient their poles adjacent to the fusome. Mutants defective in fusome formation do not undergo cyst formation and are unable to form functional gametes (Brunet and Verlhac, 2011). Fusomes are composed of membrane skeletal proteins like α-spectrin, β-spectrin, and the adducin like product of the *hu-li tai shao* (*hts*) gene, motor protein dynein, and the protein expressed by the *bag-of-marbles* (*bam*) gene (de Cuevas et al., 1996; Deng and Lin, 1997; Lin et al., 1994). The fusome arises from a precursor known as spectrosome present in the Drosophila germline stem cells (Lin and Spradling, 1997; Lin et al., 1994). Mutations in *α-spec* and *hts* result in abnormally small cysts, which do not have 2ⁿ cells (de Cuevas et al., 1996; Deng and Lin, 1997; Lin et al., 1994; Yue and Spradling, 1992). *α-spec* and *hts* are essential components of fusome and their mutations result in a nonfunctional fusome. This highlights the fact that the fusome plays a pivotal role in cell divisions (Brunet and Verlhac, 2011).

There has been evidence that germ cell clusters connected by intercellular bridges are present in 17 or 18 day fetal mice ovaries (Ruby et al., 1969). It was unclear at that time whether the observed intercellular bridges are true “germline cysts” like those in Drosophila. Electron microscopic studies first revealed the presence of microtubule bundles in the intercellular bridges connecting germ cell clusters in mice ovaries (Vegetti and Alagna, 2006). Moreover, mitochondria were found in those intercellular bridges reinforcing the fact that intercellular transport occurs via the intercellular bridges (Vegetti and Alagna, 2006). Another important feature of a germline cyst is that they are mitotically synchronized, the daughter cells known as cystocytes are normally formed in
cluster of cells corresponding to $2^n$. If the germ cell clusters were formed by cell aggregation, their numbers would have been unsynchronized. A high degree of mitotic synchrony was found in the germ cell clusters in 10.5 dpc-15.5 dpc ovaries when most of the germ cells are at premeiotic stage. Most of the clusters contained 2, 4, 8 or 16 mitotic cells. This indicates that the clusters are formed by rounds of synchronous divisions with incomplete cytokinesis (Vegetti and Alagna, 2006). To examine another important hallmark, fusomes of germline cysts, ovaries were labeled with anti-EMA (a marker for germ cells). EMA labeling images indicated the presence of a fusome-like structure which was associated with the poles of mitotic spindles (Vegetti and Alagna, 2006). However, it was not asymmetrically distributed during cell divisions like Drosophila fusomes.

Alternatively, there have been reports supporting the “aggregation theory” to document the formation of germ cell clusters (Gomperts et al., 1994). PGCs are often connected by long processes known as filopodia while they are migrating to the gonad. The filopodial connections are generally lost once the PGCs reach the gonads around 11.5 dpc. Germ cells at 10.5 dpc when cultured in vitro, were found to aggregate. These findings led the authors to postulate that germ cell clusters arise by aggregation of germ cells instead of incomplete cytokinesis (Bendel-Stenzel et al., 2000; Gomperts et al., 1994). Recent observations confirmed that all germ cell clusters are not formed by incomplete cytokinesis, some may be formed by aggregation too (Mork et al., 2012).

A defined role for murine germline cysts has not been found yet. Only one-third of the oocytes within each cyst survive to form primordial follicles and rest are lost by germ cell attrition (Pepling and Spradling, 2001). Germ cell attrition has been observed
germ cells serving as nurse cells in the Drosophila ovary (Brunet and Verlhac, 2011). One or more oocytes within each murine germline cyst may accumulate materials transported from the others that would determine them as oocytes (Vegetti and Alagna, 2006). It has been postulated that in mammalian oogenesis, all premeiotic germ cells might not be oogonia, some may be nurse cells too (Gondos, 1973). The oocytes which undergo attrition within each murine cyst might be analogous in function to nurse cells in Drosophila. Those oocytes serving as nurse cells in mice might transport their cellular organelles like mitochondria, mRNAs, centrioles, ribosomes to the future oocytes and undergo attrition (Vegetti and Alagna, 2006).

1.4. Germ Cell Attrition

Extensive loss of germ cells occur as they enter meiosis (Baker, 1972; Coucouvanis, 1993; Ratts, 1995). Germ cell degeneration appears to occur in four waves during rodent oogenesis reducing the oocyte number from 75,000 on 18.5 dpc to only 27,000 at Postnatal Day (PND)2 (Beaumont, 1962). During the postnatal period (days 1-6), some oocytes migrate through the germinal epithelium and are lost by elimination into the peritoneal cavity (Hiura and Fujita, 1977). Germ cell attrition occurs mostly during the first 30 days postnatal and is seldom observed in older animals (Edwards et al., 1977).

The majority of the germ cell cysts in mouse break down to form primordial follicles within two days after birth (PND2) (Pepling and Spradling, 2001). Recent findings from our laboratory revealed that cyst breakdown and follicle formation can start two days prior to birth at 17.5 dpc particularly deeper in the ovarian tissue (medullary region) (Pepling et al., 2010). During the process of cyst breakdown, about two-thirds of the cells
within each cyst die by programmed cell death and the rest (one-third) of the oocytes
ultimately develop into primordial follicles (Figure 1.2). Thus, programmed cell death is
a requisite for bigger cysts to break down to smaller cysts and ultimately form primordial
follicles. The two important events during oogenesis, cyst breakdown and oocyte death
occur within the same temporal time frame. However, there is no evidence to address
whether they are developmentally linked processes. There are no definite reasons yet
known to explain the huge oocyte loss associated with germ cell cyst breakdown
(Pepling, 2006). Another wave of apoptosis occurs during sexual maturity when cohorts
of follicles are recruited to grow by a process called follicle activation (McGee and
Hsueh, 2000). Some of the activated follicles eventually die by a process called follicular
atresia while oocytes in surviving follicles undergo ovulation (Pepling, 2012). We still do
not know the exact function and mechanism of germ cell attrition. Apoptosis or
programmed cell death is believed to play the major role. Two major hallmarks of
apoptosis, germ cell shrinkage and nuclear chromatin condensation were both found in
the mouse ovaries between 13.5 dpc and PND1 (Coucouvanis, 1993). Furthermore, dying
oocytes from mouse ovaries aged between 15.5 dpc and PND1 had the characteristic
DNA laddering pattern of apoptosis (Ratts, 1995).

Programmed cell death of oocytes is found to be conserved throughout the animal
kingdom. In C. elegans, 50% of the germ cells are lost by apoptosis of oocytes arrested
during the pachytene stage of meiosis (Gumienny et al., 1999). In many invertebrate
females, only a fraction of the cells within each cyst ultimately give rise to mature
oocytes (Bu´ning, 1994). In Drosophila, only one of a 16-cell cyst stage becomes the
oocyte and the rest of the 15 cells known as cystocytes serve as nurse cells. They help in
the growth of the oocyte by supplying mRNAs, proteins, and organelles to the oocyte. At the end of oogenesis, there is rapid, bulk transport of nurse cell cytoplasm leading to the growth of the oocyte and the nurse cells then die by apoptosis (Spradling, 1993).

Apoptosis occurs in other systems like the nervous and immune systems during the course of development. The principle for cell death in the ovary may be due to similar functions like those systems. During both early neuro- and lymphogenesis, cells are overproduced. They are subjected to at least two different waves of apoptosis both in utero and during the postnatal period (Elmore, 2007). Various factors like neurotrophic factors may restrict the number of developing neurons during the proliferative period and post-mitotically. This process helps to determine the number of neurons and also appropriate, functional synaptic connections to form between neurons (Yuan and Yankner, 2000). In the lymphatic system, young lymphocytes are overproduced. The initial cell numbers are reduced due to limited cytokine availability. Further apoptosis may occur on the reduced cell population to generate lymphocytes with functional antigen receptors. This process ensures that the lymphocytes are equipped with the appropriate level of responsiveness required for the efficient functioning of the immune system (Opferman, 2008). The underlying theme is that cells in the above two systems undergo apoptosis to regulate their population as their modules of functionality are assembled. This process ensures that the two systems function with highest efficiency. In the ovary, analogous to nervous and immune systems, oocyte numbers decrease as the functional units of the ovary, the follicles get assembled.
1.5. Meiotic progression and folliculogenesis

In mouse, the majority of primordial follicles are formed after birth. Primordial follicles consist of small oocytes (~ 20 µm in diameter) enclosed in a single layer of squamous or flat somatic cells known as the pre-granulosa cells. The pre-granulosa cells do not express follicle stimulating hormone (FSH) receptor and do not respond to hormonal stimulation (Oktay et al., 1997). The primordial follicles remain in the resting stage, until they either degenerate or enter the growth phase. By PND7, most of the oocytes are enclosed in primordial follicles with very few cysts remaining. During the first week of development, an initial synchronous wave of follicle recruitment takes place. Once recruited into the pool of primary follicles, oocytes grow in size and the surrounding pre-granulosa cells become cuboidal and proliferative. In female mammals, the recruitment of primordial follicles continues throughout the reproductive lifespan. The process of transition of resting stage-oocytes to subsequent growth and differentiation is known as folliculogenesis (Figure 1.3). Primary follicles increase in size both by the proliferative action of granulosa cells and an increase in size of the oocyte. When the follicles reach the secondary (or preantral) stage, they are characterized by two or more layers of granulosa cells surrounding the oocytes. Primary and preantral follicles are responsive to gonadotropins and therefore optimal development of preantral follicles require gonadotropin stimulation (Cortvrindt et al., 1997; Fortune and Eppig, 1979). A fluid-filled antrum forms between the layers of somatic cells as granulosa cells continue to divide and the oocyte enlarges to form the antral/Graafian follicle. In the
mouse, the first cohort of growing follicles reaches this stage between PND 14 and 24. The granulosa cells are divided into two separate compartments by the growing antrum. Those closest to the oocyte are called cumulus cells and those lining the follicle wall are the mural granulosa cells. The first wave of follicles formed before puberty eventually degenerate in absence of appropriate gonadotropin surge.

An oocyte within the primary or preantral follicle is arrested at diplotene stage of prophase I of meiosis and hence meiotically incompetent. It still does not have the regulatory machinery to drive meiotic progression. It cannot mature spontaneously if removed from the follicle and placed in supportive medium. During the transition from preantral to antral stage, a critical developmental change takes place in the oocyte. The oocyte reaches its full size (~ 75 µm diameter in the mouse). This is accompanied by an increase in both the rate of protein synthesis and total cellular protein content. Once the oocyte reaches the optimal size, it acquires the capacity to undergo germinal vesicle breakdown (GVBD) and complete the first meiotic division (Sorensen and Wassarman, 1976). Henceforth, it attains the capacity to support fetal development (Kono et al., 1996). An oocyte removed from the antral follicle and placed in appropriate medium will spontaneously resume meiosis. Luteinizing hormone (LH) receptors develop on mural granulosa cells as the follicle enlarges. The granulosa cells, in response to the midcycle surge of LH undergo changes in gene expression that indirectly stimulate oocyte meiotic maturation (Hernandez-Gonzalez et al., 2006). In most mammals, before ovulation oocytes resume meiosis and progress to metaphase II. In response to a LH surge, the large antral/preovulatory follicle ruptures resulting in ovulation of a metaphase II-arrested oocyte which is competent to undergo fertilization. Lastly, the mural granulosa cells
remaining in the ovary undergo a differentiation event known as luteinization. These cells together with the adjacent theca cells form the corpus luteum, essential for the maintenance of pregnancy (Figure 1.4).

1.6. Diplotene arrest and follicle formation

The overall goal of folliculogenesis is to produce eggs competent to resume meiosis and undergo complete nuclear maturation, to produce haploid gametes capable of supporting embryonic development (Brunet and Verlhac, 2011). Meiosis is a unique cellular process consisting of one round of DNA replication and two rounds of cell division, producing the haploid sperm or egg. It consists of two successive cell divisions. Meiosis I separates homologous chromosomes, producing two haploid cells with n chromosomes. Meiosis I is therefore termed as reductional division. In Meiosis II, equational division by mitosis occurs whereby the sister chromatids are finally split, generating a total of 4 haploid cells (two from each daughter cell from the first division). Prophase of the first meiotic division (MI) is the longest phase and has five different stages (leptotene, zygotene, pachytene, diplotene, diakinesis) (Freeman, 2005) (Figure 1.5). During meiotic prophase I, homologous chromosomes by a process known as meiotic recombination undergo reciprocal DNA exchange. The paternal and maternal allelic combinations are thus shuffled along the chromosome, generating genetic diversity. After recombination, the homologous chromosomes move to opposite poles of the spindle and segregate. Leptotene is the first stage of meiotic prophase I during which progressive condensation and coiling of chromosome fibers takes place. Zygotene occurs next during which pairing known as synopsis of homologous chromosomes takes place.
The pairing is highly specific and exact. The homologous chromosomes are of identical length and pairing ensures that they are correctly positioned around their centromeres. The paired chromosomes are known as bivalents. Pachytene is the next stage to follow. Nonsister chromatids of homologous chromosomes exchange segments over regions of homology by a process known as crossing over. Sex chromosomes (X and Y) are of unequal length and undergo crossing over around a small region of homology. A protein structure known as the synaptonemal complex (SC) forms between the homologous chromosomes which serve as a scaffold to facilitate interacting chromatids to undergo meiotic crossover activities. The points along the chromosomes where crossing over has occurred are known as chiasmata. It represents the actual break of the phosphodiester bond during crossing over. Diplotene occurs next where the homologous chromosomes separate from one another due to the degradation of the synaptonemal complex. They are held tightly mainly at the points of crossing over or chiasmata till they are severed in anaphase I. During mammalian oogenesis, all developing oocytes develop to the diplotene stage and remain suspended until ovulation (Rosenbusch, 2006). This resting phase of the oocytes is also known as the dictyate stage. The final stage of meiotic prophase I is diakinesis. More condensation of the chromosomes occurs, the tetrads become more prominent with the chiasmata clearly visible. The nuclear membrane and nucleolus disintegrate along with the formation of meiotic spindle in preparation of metaphase I. In the second meiotic division (MII), sister chromatids segregate, similar to a mitotic division, giving rise to haploid daughter cell(s).

Diplotene arrest and follicle formation occur during the same temporal period. Stra8 is essential for meiotic entry of oocytes and germ cells in Stra8−/− mice do not enter
meiosis (Baltus et al., 2006). However, follicles are observed in *Strad* mutant mice ovaries at puberty with the oocyte nuclei at mitosis (Dokshin et al., 2013). In rats, inhibition of synaptonemal complex protein (SYCP)1 resulted in premature arrival of oocytes at the diplotene stage and precocious assembly of primordial follicles suggesting a link between cell cycle stage and primordial follicle formation (Paredes et al., 2005). *Sycp1* mice mutants are sterile and females lack oocytes though follicle formation was not examined (de Vries et al., 2005). SYCP3 mutants have defective chromosome segregation. Their oocytes appear to develop normally though they are subfertile (Yuan et al., 2002). In cattle, the primordial follicles are at the pre-diplotene stage. It has been shown that activation of the primordial follicles to form primary follicles is related to their ability to attain diplotene stage. Furthermore, estrogen can block follicle activation by inhibiting meiotic progression (Yang and Fortune, 2008). In mouse, bisphenol A (BPA) treatment of mouse embryos caused defects in meiosis. Estrogen receptor (ER) β mutants had meiotic defects like the BPA-treated animals, suggesting that BPA acts as an ERβ antagonist (Susiarjo et al., 2007).

### 1.7. Endocrine Disruptors

The post-World War II era saw a massive explosion in industrial growth. Thousands of man-made compounds were introduced into the environment as industrial waste. In the US, more than 80,000 chemicals are found in products ranging from toys and food packaging to detergents and pesticides. Recently, there has been an increasing concern among the scientific community, general public and the policy makers regarding the
potential reproductive hazards of a range of environmental chemicals known as ‘endocrine disruptors.’

An endocrine disruptor is defined as an exogenous agent that can interfere with the synthesis, secretion, transport, metabolism, binding, action, or elimination of natural blood-borne hormones in the body that are responsible for reproduction, and developmental processes (Kavlock and Ankley, 1996). A developing embryo or a fetus is more sensitive than adults because of underdeveloped DNA repair mechanisms and incomplete functions of detoxifying enzymes (Trubo, 2005). Thus, endocrine disruptors at this stage can produce disruptions in reproductive development at a concentration much lower than which can produce effects in adults. Some of the widely used chemicals used on a daily basis and act as endocrine disruptors are phthalates (widely used plasticizers), polychlorinated biphenyls (PCB) (used in transformers and electric equipment), and the insecticide DDT. The best-documented case of estrogen exposure in humans has come from the use of diethylstilbestrol (DES) widely prescribed by the physicians in the United States between 1940s and 1970s to prevent miscarriages (Smith, 1949). DES was not only ineffective in preventing miscarriages but had long-term and multigenerational effects in causing reproductive disorders. Ultimately, it was banned in 1971 by the U.S. Food and Drug Administration (FDA).

Many studies suggest the potential relationship between human exposure to environmental factors and increased nondisjunction of chromosomes in germ cells. But the risks still remain unclear and limited. More studies are thus necessary to establish a definitive impact of endocrine disruptors on human reproduction. One approach is to utilize the mouse as a mammalian model to study such relationship.
1.8. Estrogen (E₂) and Progesterone (P₄) receptors

Both E₂ and P₄ are endogenous hormones with important physiological roles targeting the reproductive tissues, brain, bone, and cardiovascular system. In the classical steroid signaling pathway, E₂ action is mediated through binding to nuclear receptors (ERs) that regulate transcription of estrogen responsive genes (Figure 1.6). In mammals, there are two main estrogen receptors (ER) s - ERα and ERβ, containing a conserved DNA-binding domain (Pettersson and Gustafsson, 2001). The ligand, E₂ on binding with the receptor induces its conformational changes. This leads to ER dimerization and the receptor dimer then recruits a multi-component coactivator or corepressor complex. The coactivator or corepressor complexes then interact with basal transcription factors and induce either stimulation or inhibition of target genes containing the estrogen response element (ERE). Apart from the ‘classical’ steroid hormone signaling, estrogen can signal through other mechanisms like tethered, ligand-independent, and membrane signaling (Heldring et al., 2007). In tethered signaling, ER does not directly bind to the DNA unlike classical signaling. E₂ activates ERs, which in turn, interact with other transcription factors and bind to target DNA sequences (Kushner et al., 2000; Saville et al., 2000). For ligand-independent signaling, ERs are not activated by ligand binding. They are phosphorylated by activation of other signaling pathways and then bind to EREs to modulate target gene transcription (Kato et al., 1995). E₂ can signal through the membrane via two ways. First, nuclear ERs can directly translocate to act at the plasma membrane (Pedram et al., 2006). Secondly, E₂ can also signal through membrane bound
receptors (mERs) including a G protein-coupled membrane receptor called GPR30 (Revankar et al., 2005). Evidence for other G protein-coupled receptors for estrogen exists but they have not been well characterized (Hasbi et al., 2005).

ERα mutants are sterile with ovaries lacking corpora lutea and containing hemorrhagic follicles (Lubahn et al., 1993). ERβ mutant adult females have reduced fertility with fewer litters and fewer pups per litter. Their ovaries have many dying oocytes in atretic follicles and fewer corpora lutea (Krege et al., 1998). This observation suggests that in absence of ERβ, ERα may compensate for the lack of ERβ and thus ERβ mutant females are not completely infertile. Mice lacking both ERα and ERβ receptors (αβERKO) are infertile. They have normal early follicular growth and development but the growth is arrested prior to the antral stage (Couse and Korach, 1999; Dupont et al., 2000). The complete sterility of ERα females may be mainly due to lack of estrogen-mediated negative feedback on pituitary LH secretion (Couse et al., 2003; Schomberg et al., 1999). ERβ mutant adult females show aberrant expression of markers of granulosa cell differentiation such as the LH receptor and aromatase. Their granulosa cells exhibit a reduced response to FSH, and are not able to respond appropriately to LH-induced cumulus expansion, follicle rupture, and ovulation (Couse et al., 2005). Thus, the role of ERβ as the primary regulator in follicle development is more defined (Woodruff and Mayo, 2005).

P₄ signals through the nuclear progesterone receptor (PR) that has two isoforms, PR-A and PR-B. They are transcribed from a single gene using two different promoters (Conneely et al., 1989; Kastner et al., 1990). Both PR isoforms are found in the thecal and granulosa cells of the adult ovary (Gava et al., 2004). PR-A-specific knockouts have
defects in ovarian and uterine function and PR-B-specific knockouts have defects in mammary gland function (Mulac-Jericevic et al., 2003; Mulac-Jericevic et al., 2000). The PR knockout females lacking both PR-A and PR-B receptors are sterile (Lydon et al., 1995). Progesterone also signals through membrane receptors by at least three mechanisms (Peluso, 2006). First, the nuclear isoforms, PR-A and PR-B can be translocated to the nuclear membrane. The second type is a seven-transmembrane spanning family of PRs called progestin and adipoQ receptors (PAQR) present with members in three groups- $\alpha$, $\beta$, and $\gamma$. The third types identified are two single membrane-spanning proteins called progesterone receptor membrane component (PGRMC) 1 and PGRMC2 can act as PRs in complex with other proteins. Their presence has been confirmed in the adult mouse ovary and their expression in the neonatal mouse ovary is yet to be examined (Cai and Stocco, 2005; Peluso, 2006). In neonatal rat ovaries, both PGRMC1 and PGRMC2 are detected by microarray and qPCR analysis (Nilsson et al., 2006).

1.9. Influence of hormones on cyst breakdown and primordial follicle formation

High levels of steroid hormones (both $E_2$ and $P_4$) maintain oocytes in cysts and the drop in their levels trigger cyst breakdown and formation of follicles (Chen et al., 2007). When female neonatal mice are treated with $E_2$ (Iguchi et al., 1986), with xeno estrogens DES (Iguchi et al., 1990) or BPA (Suzuki et al., 2002) or with the phytoestrogen genistein (Jefferson et al., 2002), they have multiple oocyte follicles (MOF)s in their ovaries at the adult stage. MOFs are likely oocyte cysts that did not separate and became enclosed in follicles (Gougeon, 1981; Iguchi and Takasugi, 1986; Iguchi et al., 1986)
To examine whether receptors for steroid hormones are present in the neonatal ovaries, immunocytochemistry was performed on the neonatal ovaries. ERα was found to be expressed in pregranulosa cells and ERβ in some oocyte nuclei during cyst breakdown (Chen et al., 2009). For further investigation, neonatal ovaries were treated with ERα or ERβ agonists and were seen to have reduced cyst breakdown compared to controls (Chen et al., 2009). This suggests that E2 can signal through either receptor to regulate cyst breakdown. ER knockouts though do not exhibit any defects in neonatal oocyte development (Tang & Pepling, unpublished observations). These results indicate that E2 signals via another receptor to maintain oocytes in cysts. E2 conjugated with BSA cannot enter the cell because of its size but shows the same effect as free E2 to block cyst breakdown. This supports the idea that a membrane receptor might be potentially involved in cyst breakdown (Chen et al., 2009). Gpr30 knockout mice are fertile and have no reproductive defects making it an unlikely candidate function as the membrane receptor in neonatal ovaries (Otto et al., 2009).

Unlike mice, E2 seems to have a positive effect on follicle formation in some species. There are species differences in E2 signaling (Pepling, 2012). There is no evidence to indicate why E2 promotes follicle formation in some species and inhibits follicle formation in others. E2 has been shown to promote follicle assembly in hamster (Wang et al., 2008; Wang and Roy, 2007). In baboons, cyst breakdown and follicle assembly are disrupted if E2 production is inhibited (Zachos et al., 2002).

The source of E2 and P4 maintaining oocytes in cysts during the fetal period in mice is unknown. In humans, maternal E2 levels are high during primordial follicle formation. Whereas in cattle, maternal E2 levels are low before follicles form during gestation.
suggesting that maternal circulating E\textsubscript{2} is not the source in these species (Senger, 2003). It has already been shown that fetal bovine ovaries can synthesize E\textsubscript{2} and P\textsubscript{4} and the levels subside when primordial follicles begin to form (Nilsson and Skinner, 2009; Yang and Fortune, 2008).

Neonatal treatment with P\textsubscript{4} can also give rise to MOFs in adult females (Iguchi et al., 1986). Neonatal treatment of mice and rat ovaries with P\textsubscript{4} reduces cyst breakdown and primordial follicle formation (Chen et al., 2007; Kezele and Skinner, 2003). P\textsubscript{4} can be converted to E\textsubscript{2} and its effect could be exerted either directly or via conversion to E\textsubscript{2}. To investigate that possibility, a non-metabolizable analog of P\textsubscript{4} (promegestone) was also found to block follicle formation in rat and mice ovaries (Chen et al., 2007; Nilsson et al., 2006). Two studies confirmed that fetal bovine ovaries are capable of synthesizing P\textsubscript{4} to regulate their own folliculogenesis (Nilsson and Skinner, 2009; Yang and Fortune, 2008).

Testosterone (T) when given as a neonatal treatment can give rise to MOFs in the adult stage (Iguchi et al., 1986). The direct effect of T on cyst breakdown has not been yet investigated; it could due to its conversion to E\textsubscript{2}. One study indicated that inhibiting E\textsubscript{2} synthesis from T can reduce the incidence of MOFs (Iguchi et al., 1988). T signals through the androgen receptor (AR) belonging to the nuclear steroid hormone receptor family (Roy et al., 1999) and can also signal through receptors at the membrane (Heinlein and Chang, 2002).

1.10. Aromatase and 3-beta-hydroxysteroid dehydrogenase

The aromatase enzyme is localized in the endoplasmic reticulum of the cells that synthesize E\textsubscript{2} (Sebastian and Bulun, 2001). Aromatase P450 (CYP19) is an enzyme
which catalyzes the conversion of androgens into estrogens (Figure 1.8). In humans, aromatase is expressed in a number of cells, such as the ovarian granulosa cells, the placental syncytiotrophoblast, the testicular Leydig cells, brain and skin fibroblasts (Simpson et al., 1994). The tissue-specific expression of this gene is controlled using alternative splicing by means of tissue-specific promoters. The protein translated from the message is the same in all tissues (Simpson et al., 1997).

Aromatase is mostly restricted to the gonads and brain in rodents (Stocco, 2008). In female adults, aromatase is mostly present in the follicle and the corpus luteum of the ovaries. The expression of this gene is controlled in a cell-specific and temporal manner. This expression pattern ensures that E<sub>2</sub> synthesis occurs only in mural granulosa cells of healthy large antral follicles and luteal cells (Stocco, 2008). In several species like humans, rabbit and cattle, ovaries acquire the enzymatic capacity to synthesize E<sub>2</sub> during embryonic life (George and Ojeda, 1987; Vigier et al., 1989). Fetal ovaries in humans and rabbits have been demonstrated to exhibit aromatase activity by their ability to convert radiolabeled androgen to estrone and estradiol (George and Wilson, 1978; Milewich et al., 1977; Vigier et al., 1989). ERα and ERβ knockout mice show no defects on cyst breakdown and early follicle development and thus the role of E<sub>2</sub> during fetal ovarian development is still unclear (Couse et al., 1999). LH levels are found to be elevated in ERα and ERβ knockout mice which cause premature maturation of the ovary and development of advanced stage follicles in prepubertal females. This indicates that the main function of E<sub>2</sub> in pre-pubertal females is to maintain normal levels of gonadotropins via its regulatory negative feed-back inhibition on the pituitary gland (Couse et al., 1999).
The earliest indication of aromatase activity and presence of aromatase mRNA are found at PND5 and PND8 in the granulosa cell layer of growing follicles (George and Ojeda, 1987; Guigon et al., 2003; Mendelson and Kamat, 2007). Aromatase expression progressively increases in preantral and antral follicles of prepubertal ovaries. Around PND21, it becomes mostly restricted to healthy large antral follicles (Guigon et al., 2003; Stocco, 2008). In a mature follicle such as a pre-antral follicle which possess both granulosa and thecal cells, LH acts on the thecal cells to produce androgen precursors and FSH acts on the granulosa cells to synthesize estradiol from the androgen precursors on gonadotrophin surge. However, follicles in the neonatal ovaries do not possess all the components of the ‘two cell (thecal/granulosa cells), two gonadotropins (LH/FSH)’ model and thus E$_2$ production during the neonatal stage remains mostly elusive (Stocco, 2008).

Mice lacking a functional aromatase enzyme (ArKO) can be generated by targeted disruption of the cyp19 gene (Fisher et al., 1998). Female ArKO mice at 9 weeks of age have underdeveloped external genitalia and uteri. The ovaries of ArKO females have follicles of all types (primordial, primary, secondary, and antral) but do not undergo ovulation. ArKO ovaries have more primary follicles compared to wild-type at 21–23 weeks and to heterozygous ovaries both at 10–12 weeks and 21-23 weeks. There were significantly fewer secondary follicles in the ArKO ovaries around 10-12 weeks and by 1 year of age, no secondary or antral follicles were observed in the ArKO ovaries. The ovaries do not have any corpora lutea (Britt et al., 2000; Britt et al., 2004). This observation suggests that estrogen is not an absolute requirement for the growth of
primordial follicles but is required for the development of antral follicles and subsequent steps in folliculogenesis which gives rise to ovulation (Britt et al., 2000).

When the ovarian follicles are not ovulated, they undergo atretic degeneration by apoptosis. This leads to the depletion of ovarian follicles at a relatively early stage of life in ArKO females compared to wild-type females (Toda et al., 2001). The presence of haemorrhagic cysts on the surface of ovaries is a very characteristic feature of aged ArKO females. It is speculated that the absence of estrogens (or excess of androgens) in ArKO females might lead to the invasion of macrophages into the follicular antrum. The macrophages then phagocytose and produce an empty space in the follicle, into which blood flows and accumulates, which gives rise to the haemorrhagic cystic follicles (Toda et al., 2001). ArKO males of the same age have relatively normal internal anatomy but have enlarged male accessory glands. Their testes look normal and they are capable of breeding and producing litters of average size. They have highly elevated serum levels of testosterone, follicle stimulating hormone and luteinizing hormone (Toda et al., 2001).

The enzyme 3beta-hydroxysteroid dehydrogenase (3βHSD) is present both in microsomes and mitochondria of mouse ovaries (Figure 1.8). Six different isoforms of 3βHSD are present in mice (Abbaszade et al., 1997). During the follicular phase of the estrous cycle, the 3βHSD activity is predominant in the microsomes and on LH stimulation during the luteal phase it increases in the mitochondria. It could be due to the fact that 3βHSD is preferentially inserted into the mitochondrial membrane after LH stimulation (Chapman et al., 2005). 3βHSD uses NAD+ as cofactor for converting pregnenolone and dehydroepiandrosterone (DHEA) to progesterone and androstenedione respectively (Chapman et al., 2005). 3βHSD I is expressed in the gonads and adrenal
glands of adult mouse and synthesizes adrenal and gonadal steroid hormones. 3βHSD II and III are expressed in the liver and kidney. 3βHSD IV is expressed exclusively in the kidney and is the major isoform expressed in the kidney of both sexes (Payne et al., 1997). During diestrus (luteal phase), the specific activity of mitochondrial 3βHSD is 80% higher than that of microsomal 3βHSD (Chapman et al., 1992). During the other three stages of estrus, microsomal 3βHSD has the highest specific activity (Chapman et al., 1992). The rate-limiting step in steroidogenesis is considered to be cholesterol side-chain cleavage enzyme P450_scc (Miller, 1995). It is found that 3βHSD and P450_scc have a strong binding affinity for each other and are simultaneously synthesized (Chapman et al., 2005). 3β-HSD and P450_scc, bound together as a complex has an added advantage in functioning together as a unit. They can together control the rate-limiting step, or steps, in conversion of cholesterol to progesterone (Cherradi et al., 1995). The coupling of 3βHSD and P450_scc as an enzyme complex has further three advantages- first, cholesterol can be converted to progesterone in a single step without any shuttling of steroid intermediates from one organelle to another. Second, the rate of synthesis of progesterone is considerably increased if the reactions for both enzymes are coupled together. Finally, the end-product inhibition of P450_scc by pregnenolone is eliminated because pregnenolone can be almost immediately converted to progesterone (Chapman et al., 2005). Thus, the synthesis of progesterone during the luteal phase involves one cell type and two enzymes in contrast to the follicular phase where steroid precursors are shuttled from one cell type to another and one organelle to another organelle (Chapman et al., 2005).
Figure 1.1. Migration and development of Primordial Germ Cells (PGCs) in mice.

The PGC precursors are derived from epiblast. They are first detected during gastrulation at the base of the allantois around 7.5 dpc (~75 cells). They expand in number by mitosis and migrate through the hindgut and dorsal mesentery ultimately reaching the genital ridges around 10.5 dpc (~25,000 cells/each gonad primordium). Sexual differentiation occurs at 12.5 dpc. The oocytes undergo growth and maturation to form follicles. At puberty, adult females begin to release mature eggs (ova) for fertilization.
Figure 1.2. Cyst breakdown and germ cell attrition. Within each cyst, surviving oocytes are represented by the yellow color; dying oocytes are represented in green. Along with cyst breakdown, two-thirds of the oocytes within each cyst dies and one-third survives to form primordial follicles. The primordial follicles are the finite reservoir of germ cells available to the female mammal during her entire reproductive life span.

Modified from: Jefferson et al., 2006
Figure 1.3. Stages of follicle development. Representative confocal sections of germ cell cysts and ovarian follicles. Nuclei of oocytes and granulosa (somatic) cells are labeled with a nuclear marker, propidium iodide. A) A germ cell cyst showing several oocyte nuclei sharing the same cytoplasm. B) A primordial follicle containing an oocyte (arrowhead) surrounded by flattened granulosa cells (arrow). C) A primary follicle containing an oocyte (arrowhead) surrounded by cuboidal granulosa cells (arrow). D) A secondary follicle containing an oocyte surrounded by several layers of cuboidal granulosa cells (arrow) and a layer of theca cells (double arrows) Scale bars= 10μm.(Pepling, 2013)
Figure 1.4. Structure of an adult mammalian ovary. Stages of follicle development:

- Primordial follicle (oocyte surrounded by a flattened layer of granulosa cells)
- Primary follicle (oocyte surrounded by a cuboidal layer of granulosa cells)
- Secondary follicle (oocyte surrounded by several layers of granulosa cells)
- Pre-antral follicle (presence of a fluid filled cavity called antrum adjacent to the oocyte)
- Antral follicle (contains the pre-ovulatory ova which is at Meiosis I arrest)
- Ovulation (mature ova released)
- Corpus luteum (temporary endocrine structure formed in the ovary after ovulation from the follicular thecal and granulosa cells and secretes progesterone)

(Niswender, 2002; Vegetti and Alagna, 2006)
Figure 1.5. Early timeline of fetal mouse oogenesis. PGCs migrate to the gonad at 10.5 dpc. They divide by mitosis without undergoing cytokinesis to form cysts. They are termed as oogonia. They start entering meiotic prophase I from 13.5 dpc onwards. Leptotene, zygote, and pachytene begin approximately from 13.5 dpc, 14.5 dpc and 16.5 dpc onwards. Oocytes start entering diplotene from 17.5 dpc and it may take five days after birth for all oocytes to enter diplotene. Cyst breakdown may start as early as 16.5 dpc and is associated with huge germ cell loss. (Adapted from Pepling, 2006.)
Figure 1.6. Estrogen ($E_2$) can signal through several different receptors. Membrane-bound G-protein coupled receptor (GPR30), a putative membrane receptor (not yet characterized) and cytosolic ERα or ERβ (which are transcription factors). In the classical steroid signaling pathway, ligand ($E_2$) binds to cytosolic ERα or ERβ which dimerizes on ligand binding and enters the nucleus. On entering the nucleus, it can bind to the DNA and with the help of co-activators can initiate transcription of genes having estrogen responsive elements (EREs).
**Fig 1.7. Formation of Multiple Oocyte Follicles (MOF).** A) A normal secondary follicle with an oocyte surrounded by several layers of granulosa cells B) Three oocyte nuclei surrounded by the same layers of granulosa cells forming a MOF. Oocytes are labeled with an oocyte marker VASA (green) and propidium iodide (red) is used to label nuclei. Scale bar=20µm (Pepling, 2006).
Figure 1.8. Steroid Synthesis Pathway. Cholesterol is shuttled across the mitochondrial membrane by steroidogenic acute regulatory protein (StAR), the rate-limiting step for steroid synthesis. On entering the mitochondria, cholesterol is acted by side chain cleavage P450 enzyme (P-450scc) to form pregnenolone. 3βHSD acts on pregnenolone to synthesize progesterone. 17-hydroxylase/17-20 lyase acts on the intermediate steps to synthesize androstenedione (precursor for estradiol). Cytochrome P-450 aromatase then acts on androstenedione to synthesize estradiol.
1.12. Objectives

The overall goal of the dissertation project was to elucidate the source of steroid hormone signaling and its role in meiotic progression during murine fetal oocyte development. The first aim of this dissertation study was to identify the sources of the steroid hormones (maternal circulation or perinatal ovaries) which regulate fetal oogenesis. To investigate that, the goal was to identify the presence of the cardinal steroidogenic enzymes required for \( E_2 \) and \( P_4 \) synthesis, aromatase and \( 3\beta\text{HSD} \) respectively. Finally, we sought to determine the role of \( E_2 \) and \( P_4 \) synthesis during the perinatal period by inhibiting the enzymes- aromatase and \( 3\beta\text{HSD} \) by letrozole and trilostane respectively in organ culture treatment of perinatal ovaries.

We next sought to examine as the second objective of the dissertation, the relation between two temporal events during mice oogenesis, attainment of the diplotene stage by the oocytes and primordial follicle formation. A thorough quantitative analysis was performed to characterize and classify ovaries at different ages by nuclear morphological observations of diplotene versus pre-diplotene nuclei by examining H&E stained serial sections. We next sought to understand the role of steroid hormone signaling in meiotic progression of oocytes. This was carried out by organ culture treatments of perinatal ovaries with \( 10^{-6} \, \text{M} \, E_2 \), \( 10^{-6} \, \text{M} \, P_4 \) and \( 10^{-6} \, \text{M} \, E_2 + 10^{-6} \, \text{M} \, P_4 \).
Chapter 2

Source of steroid hormone signaling regulating fetal mouse oocyte development

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2.1. ABSTRACT

Primordial follicle assembly is essential for reproductive success in mammalian females. Oocytes develop in clusters called germ cell cysts. Beginning in late fetal development, cysts break down into individual oocytes that are surrounded by pregranulosa cells to form primordial follicles. Exposure to estradiol, progesterone or estrogen mimicking chemicals delays cyst breakdown and follicle formation. One model for cyst breakdown regulation is that steroid hormones in the maternal circulation keep cells in cysts during fetal development but late in pregnancy hormone levels drop triggering cyst breakdown. However, here we found that while maternal circulating levels of progesterone drop during late fetal development, maternal estradiol levels remain high. We hypothesized that fetal ovaries were the source of hormones and late in fetal development their production stops. To test this, mRNA and protein levels of steroidogenic enzymes required for the final step of estradiol and progesterone synthesis were measured. We found aromatase and 3-beta-hydroxysteroid dehydrogenase mRNA levels drop prior to cyst breakdown and corresponding proteins were also present. To determine the levels of estradiol and progesterone present, the steroid content of fetal and neonatal ovaries was analyzed using radioimmunoassay. Both hormones were detected in fetal ovaries prior to cyst breakdown. To determine the role of steroid hormones in oocyte development we examined effects of blocking steroid hormone production in organ culture and found that oocyte number was reduced. These results support our model that steroid hormone signaling is important for fetal oocyte development and contribute to the understanding of ovarian differentiation.
2.2. INTRODUCTION

Mammalian females are born with a pool of primordial follicles that represents the total population of germ cells available during their entire reproductive life and thus establishment of this primordial follicle pool is essential for fertility (Pepling, 2006). Oocytes start out as primordial germ cells and migrate from extra embryonic tissue to the genital ridge. Subsequently, they increase by mitotic division as they colonize the ovary between 10 days post coitum (dpc) and 12.5 dpc in the mouse (McLaren and Monk, 1981). After arriving at the ovary, the germ cells classified as oogonia, undergo incomplete cytokinesis and develop in clusters of interconnected cells called germ cell cysts until the initiation of primordial follicle formation (Pepling and Spradling, 2001). At approximately 13.5 dpc, oogonia begin to enter meiosis and are henceforth classified as oocytes (McLaren, 2000a). They progress through the stages of prophase I of meiosis and then begin to arrest at the diplotene stage starting at 17.5 dpc though some oocytes may take up to five days after birth to reach this stage (Borum, 1961). Cysts break apart and most of the oocytes undergo apoptosis with only one third surviving to become surrounded by pregranulosa cells to form primordial follicles (Pepling and Spradling, 2001). While a few follicles are observed as early as 17.5 dpc, most of the follicles are formed within the first 3 days after birth (Pepling et al., 2010). The basic mechanisms regulating cyst breakdown, germ cell numbers and formation of primordial follicles as well as how these normal processes are interrupted in reproductive disorders are still poorly understood.
The role of sex steroids during the formation of primordial follicles has recently been explored in various mammals. A study in rats showed that neonatal exposure to progesterone ($P_4$) but not estradiol ($E_2$) inhibited cyst breakdown and follicle formation (Kezele and Skinner, 2003). However, in mice both hormones blocked cyst breakdown (Chen et al., 2007). To test the idea that maternal hormones might be involved in maintaining oocytes in cysts before birth, 16.5 dpc mouse ovaries were grown in organ culture media for 3 days which is equivalent to the day of birth at 19.5 dpc. In the absence of maternal hormones increased cyst breakdown and follicle formation was observed compared to PND1 ovaries in vivo (Chen et al., 2007). These observations helped to postulate the model that birth removes the developing mice from high levels of maternal circulating steroid hormones, and this in turn facilitates cyst breakdown and follicle formation during the early neonatal period (Chen et al., 2007).

Studies from other species suggest that maternal circulating hormone levels may not be the source of hormones regulating cyst breakdown. In humans and cattle, follicle formation starts during gestation, earlier than in mice (Gondos et al., 1971; Russe, 1983). In humans maternal $E_2$ levels are rising at the time of primordial follicle formation and in cows levels are low prior to follicle formation suggesting maternal circulating $E_2$ is not the source of $E_2$ in these species. In addition, fetal bovine ovaries produce $E_2$ and $P_4$, which inhibit precocious follicle formation (Nilsson and Skinner, 2009; Yang and Fortune, 2008). Furthermore, while the majority of follicles form after birth in the mouse, some oocytes are found in follicles as early as two days before birth (Pepling et al., 2010). These findings necessitate a revision in the endocrine model of follicle formation in mice.
Steroid hormones are synthesized from precursors starting with cholesterol in the mitochondria (Miller, 2013). In the final step of E2 synthesis, androgens are converted to estrogens by the enzyme aromatase encoded by the Cyp19a gene (Stocco, 2008). In adult ovaries, E2 is synthesized in granulosa cells of preovulatory follicles (Stocco, 2012). Aromatase has also been detected in fetal mouse and human ovaries and E2 has been proposed to play a role in ovarian fetal development (Fowler et al., 2011; Greco and Payne, 1994). Synthesis of P4 occurs earlier than synthesis of E2 in the biosynthetic pathway and is synthesized from its precursor by 3-beta-hydroxysteroid dehydrogenase (3βHSD) (Miller and Auchus, 2011). There are several 3βHSD isoforms in the mouse encoded by the HSD3B1-6 genes. Similar to aromatase, 3βHSD has been detected in the fetal ovary (Payne et al., 1997).

The goal of the current study was to investigate the source of steroid hormones during the fetal period in mice before cyst breakdown and follicle formation. Our hypothesis was that fetal ovaries synthesize their own steroid hormones to regulate oocyte development. We first measured levels of maternal circulating hormones as well as hormone levels in fetal ovaries. Second, we investigated the presence of the necessary components of steroid biosynthetic machinery to facilitate hormone production in fetal ovaries. Finally, we examined perinatal oocyte development in ovaries where hormone production is blocked by inhibitors. These studies will enrich our understanding of the factors regulating fetal oocyte development in mice providing useful insights in ameliorating human infertility.
2.3. MATERIALS AND METHODS

2.3.1. Animals

Adult male and female CD1 outbred mice were obtained from Charles River Laboratories (Wilmington, MA, USA). They were maintained in accordance with the policies of Syracuse University’s Institutional Animal Care and Use Committee. Mice were housed and bred in a controlled photoperiod (14 h light, 10 h dark), temperature (21-22°C), and humidity with food and water available ad libitum. Timed pregnant females were either generated by setting up matings in house or purchased timed pregnant from Charles River Laboratories. For in house matings, CD1 females were mated with males of the same strain and checked daily for vaginal plugs. Noon on the day of vaginal plug detection was designated as 0.5 dpc. Birth usually occurred at 19.5 dpc and was designated as postnatal day (PND) 1. Pregnant mice were euthanized by CO₂ asphyxiation for fetal ovary collection. For neonatal ovary collection, female pups were sacrificed by decapitation on the appropriate day.

2.3.2. Steroid assays

Maternal and perinatal blood were collected for evaluation of estradiol and progesterone content in the circulation. Whole blood was allowed to clot briefly at room temperature before centrifugation at 10,000g for 15 minutes. The serum was then collected for assays. Perinatal ovaries were harvested and homogenized in phosphate buffered saline (PBS) for measurement of tissue steroid hormone levels. Steroid hormone radioimmunoassays (RIAs) were performed on serum samples and ovarian homogenates by the Assay Core Laboratory, Center for Reproductive Biology, Washington State
University. An organic phase extraction procedure was used to concentrate samples for the assays. The extracted samples were then reconstituted into the same buffer as used with the assay standards. The steroid recovery was greater than 95% and the linearity was within the acceptable criteria for the Assay Core Laboratory. Intra assay coefficient of variation (CV) for the estradiol assay (Kit KE2D1, Siemens Healthcare Diagnostics Inc., Los Angeles, CA) was 5.6% and for the progesterone assay (Kit TKPG1, Siemens Healthcare Diagnostics Inc., Los Angeles, CA) was 3.8%.

2.3.2. RNA isolation

Fetal (13.5 dpc-18.5 dpc) and neonatal (PND1-PND3) ovaries were dissected and stored in RNAlater at -80°C until further use. Total RNA was isolated using an RNeasy Mini kit (n=3; approximately 50 neonatal or 100 fetal ovaries were used per pool). Ovaries were briefly lysed, homogenized using a motor pestle and then applied to a QIAshredder column. The ovarian tissue sample in the QIAshredder column was then centrifuged at 14,000 g for 2 minutes. To isolate the RNA, the resulting flow-through was applied to an RNeasy mini column allowing the RNA to bind to the filter cartridge. RNA was eluted by washing from the filter and was concentrated using an RNeasy MinElute kit. Isolated RNA was briefly applied to an RNeasy MinElute spin column and washed, then the RNA was eluted using 14 μl of RNase-free water. The RNA concentration in the elutant was determined using an ND-1000 Spectrophotometer (λ= 260/280 nm; Nanodrop Technologies, Inc., Wilmington, DE).
2.3.3. First strand cDNA synthesis and real-time polymerase chain reaction (PCR)

Total RNA (0.5 µg) was reverse transcribed using the Superscript III One-Step RT-PCR System into cDNA. cDNA was diluted in RNase-free water (1:25). 2 µl of diluted cDNA was amplified on a Rotor-Gene 3000 using Quantitect™ SYBR Green PCR kit and custom designed primers for Aromatase (Cyp 19) (forward primer: 5’ GGCCAAATAGCGCAAGATGTTCTT 3’; reverse primer: 5’ GACTCTCATGAATTCTCCATACATCT 3’; NCBI Genbank accession number NM_007810), 3βHSD (forward primer: 5’ CTC TCT TTA ACC GCC ACT CG 3’; reverse primer: 5’ TAC CTG CCC TTT TTC CAT CA 3’; NCBI Genbank accession number NM_013821) and β-actin (forward primer: 5’ AGT GTG ACG TTG ACA TCC GTA 3’; reverse primer: 5’ GCC AGA GCA GTA ATC TAA TTA T 3’; NCBI Genbank accession number NM_007393). The cycling program consisted of a 15 min hold at 95°C and 45 cycles of: denaturing at 95°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 20 s at which point data were acquired. Determination of product melt conditions were done using a temperature gradient from 72°C to 99°C with a 1°C increase at each step. β-actin expression remained constant across all ages and each sample was normalized to β-actin before quantification.

2.3.4. Protein Isolation

Ovaries were lysed in lysis buffer (1% Triton X-100, 50mM HEPES, 150mM NaCl, 10% glycerol, 50mM NaF, 2mM EDTA and 0.1% SDS). Supernatants were collected after centrifugation and stored at -80°C until further use. The protein content was quantified using Bradford’s Reagent (Biorad). Protein concentrations were calculated
from a BSA protein standard curve. Emission absorbance values were detected with a $\lambda = 595$ nm excitation with a microplate reader (Beckman Coulter, Fullerton, CA).

2.3.5. Western blot analysis

Proteins were separated on 10% SDS-PAGE gels (30 µg protein/lane) for each time point and subsequently electroblotted onto Immobilon-PVDF membranes (Millipore). For $3\beta$HSD, 5% milk in PBS with 0.05% Tween 20 (PBST) was used as the blocking agent. Blots were blocked overnight while shaking at 4°C and exposed to an antibody against $3\beta$HSD (K0607, Cosmo Bio, Carlsbad, CA) at a dilution of 1:100. Blots were stripped using a stripping buffer (0.25M Tris pH=6.8, 40% glycerin, 20% β-mercaptoethanol and 10% SDS) and then reprobed using an antibody against aromatase (ab35604, Abcam Inc., Cambridge, MA) diluted 1:100. For aromatase, the blots were blocked overnight with shaking at 4°C in 5% BSA in Tris buffered saline with 0.05% Tween 20 (TBST). The membranes were incubated with primary antibodies overnight at 4°C in their respective blocking agents. Western blots were detected by chemiluminescence using Pierce (Rockford, IL) HRP-conjugated secondary IgG antibodies (1:10,000) in 5% milk in PBST at room temperature for 1 hr. For aromatase, the HRP-conjugated secondary antibody used was goat α-rabbit IgG (Pierce, catalog # 31460) and for $3\beta$HSD, the secondary antibody used was rabbit α-goat IgG (Pierce, catalog # 31402) The membrane was then washed three times by PBST and the signal visualized using Bio-Rad ChemiDoc™ XRS+ system. Blots were stripped and reprobed for GAPDH (sc166545, Santa Cruz Biotechnology, Inc., La Jolla, CA) diluted 1:5000 as a loading control. For quantitative analysis, blot images were saved as PICT files using
Science Lab 99 Image Gauge software version 3.3 (Fujifilm Inc.) and pixel intensities were measured. Protein levels were normalized to the corresponding GAPDH protein levels and expressed as relative percentage of the earliest age, 15.5 dpc which was set to 100%.

2.3.6. Whole-mount immunohistochemistry and fluorescence microscopy

Ovaries were dissected and fixed in 5% EM-grade paraformaldehyde (Ted Pella, Inc., Redding, CA) in PBS for 1 h at room temperature followed by several washes in 5% BSA and 0.1% Triton X-100 in PBS. Whole ovaries were immunostained as previously described (Murphy et al., 2005). Signal transducer and activator of transcription (STAT)-3 (C20) antibody, (sc-482, Santa Cruz Biotechnology, La Jolla, CA) was used as an oocyte marker at a dilution of 1:500 (Murphy et al., 2005). Propidium iodide (P3566, Invitrogen, Carlsbad, CA) was used to label nuclei. Anti-aromatase antibody (ab18995, Abcam Inc., Cambridge, MA) and anti-3βHSD (P-18) antibody (sc-30820, Santa Cruz Biotechnology, La Jolla, CA) were both used at a 1:100 dilution. For double labeling of mitochondria and endoplasmic reticulum (ER) with anti-3βHSD antibodies, cytochrome C (ab 18738) antibody and calnexin (ab22595) antibody (Abcam Inc., Cambridge, MA) were used. Secondary antibodies, donkey anti-rabbit Alexa 488 (A-21206) and donkey anti-goat Alexa 568 (A-11057, Invitrogen, Carlsbad, CA) were used at a dilution of 1:200. TOTO-3 (T3604, Life Technologies, Carlsbad, CA) was used as a nuclear marker in case of double labeling. Samples were imaged on a Zeiss LSM 710 confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY).
2.3.7. *In vitro ovary organ culture*

Letrozole (Sigma Chemical Co., St. Louis, MO) and trilostane (a gift from Dr. Gavin Vinson, Queen Mary, University of London) were both dissolved in dimethylsulfoxide (DMSO) at a concentration of 0.1 M. They were added to culture media to achieve the desired final concentrations (see below). DMSO was added to media at the same percent as the chemical to serve as vehicle control. Ovaries were cultured in 4-well culture plates in drops of media on 0.4 µM floating filters (Millicell CM; Millipore Corp., Bedford, MA) in 0.4 ml DMEM-Ham’s F-12 media supplemented with penicillin-streptomycin, 5X ITS-X (Life Technologies, Inc., Grand Island, NY), 0.1% BSA, 0.1% albumax, and 0.05 mg/ml L-ascorbic acid. Ovaries were placed in culture at 16.5 dpc and exposed daily to letrozole, trilostane or both inhibitors for 5 days (equivalent to PND3 in vivo) at $10^{-10}$, $10^{-8}$, $10^{-6}$ or $10^{-4}$ M or in DMSO alone as a vehicle control (n=5-8 ovaries per treatment group). Ovaries from both control and treatment groups were processed for whole mount immunocytochemistry.

2.3.8. *Analysis of cyst breakdown, primordial follicle assembly and follicle development*

Whole ovaries labeled with an antibody against STAT3, a specific marker for germ cells (Murphy et al., 2005) and propidium iodide as nuclei marker were imaged using confocal microscopy. For each ovary, two cores were randomly selected and visualized (Jones and Pepling, 2013). A core was defined as a region of 134.7 µm x 134.7 µm consisting of four optical sections at four different depths of the ovary with each optical section 15 µm apart. To ensure ovarian development was assessed across all regions of the ovary, the first section was typically chosen at the outer cortex and the last section
was at the medullar region. Thus, for each ovary, a total of eight optical sections were analysed. To determine whether or not oocytes were in cysts, a z-stack of images each 1μm apart was obtained with five images above the section and five images below the section being analyzed. The same procedure was repeated for each of the four optical sections in a core. This allowed us to determine whether an oocyte was part of a germ cell cyst above or below the plane of focus. Oocytes were considered unassembled or in cysts if STAT3 antibody labeling showed oocytes having continuous cytoplasm. The oocytes surrounded by a layer or layers of granulosa cells were considered to be in follicles.

The number of oocytes in follicles relative to the total number of oocytes was determined for each ovary and reported as percent single oocytes. Follicle development was determined by counting the number of primordial (oocyte surrounded by several flattened granulosa cells) and primary follicles (oocyte surrounded by one layer of cuboidal granulosa cells) present in relation to the total number of follicles counted and reported as percent primordial or primary follicles. More advanced staged follicles were not observed.

2.3.9. Statistical analysis

Statistical analysis of qPCR data was performed using Statview 5.0.1 (SAS Institute Inc., Cary, NC). Gene expression from 13.5 dpc to PND3 was evaluated by one-way ANOVA followed by Bonferroni-Dunn’s post hoc test (P< 0.0014). Analysis of fetal ovary hormone levels and effects of hormone inhibitors on oocyte development were performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA). One-way ANOVA followed by Newman-Keuls multiple comparison test was used to determine if
there were significant differences in hormone levels of fetal and neonatal ovaries. One-way ANOVA followed by Dunnett’s post hoc test (P< 0.05) was used to assess the effects of letrozole and trilostane treatments on oocyte number, percent single oocytes and follicle development. Percentage data by nature is not normal and arcsine transformation on the percentage data was performed before doing statistical analysis. All results are presented as mean ± SEM of nontransformed data.

2.4. RESULTS

2.4.1. Circulating maternal and perinatal steroid hormone levels during gestation

Our initial model was that steroid hormones in the maternal circulation maintained oocytes in cysts in female fetuses. Germ cell cysts begin to break down at 17.5 dpc and thus we expected maternal steroid hormone levels to drop at that time. RIAs were performed with maternal serum to test whether the drop in maternal circulating hormones correlated with cyst breakdown. E\textsubscript{2} levels in the maternal circulation rose from 5 pg/ml to 25 pg/ml between 13.5 and 17.5 dpc (Figure 2.1A). However, maternal E\textsubscript{2} levels did not drop until the day of birth which is two days after cysts begin to break down. P\textsubscript{4} levels were steady from 13.5 to 16.5 dpc and began to drop at 17.5 dpc. Thus unlike E\textsubscript{2}, the drop in P\textsubscript{4} levels appeared to correlate with the beginning of cyst breakdown.

**Figure 2.1B** shows the steroid hormone levels in the perinatal circulation from 17.5 dpc to PND7. E\textsubscript{2} levels were high at 17.5 dpc and continually dropped until birth. P\textsubscript{4} levels rose slightly from 17.5 dpc to 18.5 dpc and then began to drop from PND1 to PND3. After PND3 the levels were constant. The source of the steroid hormones in the perinatal circulation could be maternal, fetal or both.
2.4.2. Aromatase and 3\(\beta\)HSD present in perinatal ovaries

To investigate the possibility of a fetal contribution to steroid hormone levels, the presence of steroidogenic enzymes were examined in developing ovaries using qPCR and Western blotting. Figure 2.2A and 2.2B shows the relative changes in aromatase and 3\(\beta\)HSD mRNA levels from 13.5 dpc to PND3 as determined by qPCR analysis. Aromatase mRNA levels are relatively low from 13.5 to 15.5 dpc and then peak at 16.5 dpc (Figure 2.2A). The aromatase levels then drop at 17.5 dpc and do not significantly increase thereafter. Figure 2.2B shows the relative changes in 3\(\beta\)HSD mRNA levels from 13.5 dpc to PND3. The mRNA levels are relatively high from 13.5 dpc to 15.5 dpc followed by a dramatic drop. Thus, both ovarian aromatase and 3\(\beta\)HSD mRNA levels drop just before the beginning of cyst breakdown and follicle formation. Aromatase protein was detected by Western blotting in ovaries from 15.5 dpc to 18.5 dpc (Figure 2.2C). A 50 kd protein was detected at all timepoints. The presence of 3\(\beta\)HSD protein was also detected by Western blotting in 15.5 dpc to 18.5 dpc ovaries (Figure 2.2D). A 42 kd band corresponding to 3\(\beta\)HSD was observed in all four samples.

To determine what cell types express aromatase and 3\(\beta\)HSD protein in fetal ovaries, whole mount immunocytochemistry was performed on ovaries from 15.5 dpc to 18.5 dpc (Figures 2.3 and 2.4). At 15.5 dpc aromatase was detected in all ovarian cells (Figure 2.3A-C). Aromatase protein appears to be expressed more strongly in oocytes than in somatic cells at 16.5 dpc (Figure 2.3D-F), 17.5 dpc (Figure 2.3G-I) and 18.5 dpc (Figure 2.3J-L). Like aromatase, 3\(\beta\)HSD protein was found in all ovarian cells at 15.5 dpc (Figure 2.4A-C). At 16.5 dpc, 3\(\beta\)HSD appears to be expressed more strongly in
oocytes than somatic cells and is much stronger in some of the oocytes (Figure 2.4D-F). This trend continues at 17.5 dpc (Figure 2.4G-I) and 18.5 dpc (Figure 2.4J-L). In addition, at 18.5 dpc, in oocytes that are strongly labeled for 3βHSD, the protein is detected in oocyte cytoplasm with a very speckled appearance. The speckles are likely mitochondria as 3βHSD is known to be expressed in the mitochondria and also in the endoplasmic reticulum (Pelletier et al., 2001). To determine if the speckled bodies correspond to either mitochondria or ER, we co-labeled neonatal ovaries with 3βHSD and either mitochondrial marker (Figure 2.5A-D) or an ER marker (Figure 2.5E-H). While there is some overlap of 3βHSD with mitochondria and with ER they do not completely correspond with one another.

2.4.3. E₂ and P₄ found locally in perinatal ovaries

Steroid assays were performed on ovarian tissue to determine the levels of E₂ and P₄ present in perinatal ovaries. Ovarian E₂ was detected from 15.5 dpc to 18.5 dpc and again from PND5 to PND7 (Figure 2.6A). There was significant increase in the levels of E₂ at PND6 and PND7. Figure 2.6B focuses only on the perinatal time-period from 15.5 dpc to PND3 for ovarian E₂ levels. Ovarian E₂ was detected at 15.5 dpc and 16.5 dpc with an increase at 17.5 dpc. The E₂ level decreased at 18.5 dpc though the difference from 17.5 dpc was not statistically different. E₂ was undetectable between PND1 and PND3. Ovarian P₄ was detected at all time-points from 15.5 dpc to PND7 (Figure 2.6C). Figure 2.6D focuses on the perinatal time period of 15.5 dpc to PND3 for intraovarian P₄ levels. P₄ levels fluctuate from 15.5 dpc to PND3 but the differences were not significant. For both E₂ and P₄, the statistically significant increase at PND7 correlates to previous studies.
showing that on gonadotrophin surge starting around PND7, cultured mouse ovaries have the ability to synthesize significant levels of steroid hormones (Fortune and Eppig, 1979).

2.4.4. Effects of inhibiting $E_2$ and $P_4$ production in the fetal ovary

Our hypothesis is that $E_2$ and $P_4$ produced in fetal ovaries are important for regulating cyst breakdown. To test this we examined the effects of blocking $E_2$ and $P_4$ production in organ culture. We expected that cyst breakdown and associated oocyte loss would occur prematurely when $E_2$ or $P_4$ synthesis was inhibited. We used a series of doses of the aromatase inhibitor, letrozole to decrease the production of $E_2$. The $IC_{50}$ of letrozole is $2 \times 10^{-8}$ M for hamster ovaries cultured in vitro (Bhatnagar et al., 1990). We treated fetal mouse ovaries with $10^{-10}$ M (100 fold below the $IC_{50}$), $10^{-8}$ M (half of the $IC_{50}$), $10^{-6}$ M (50 times the $IC_{50}$) and $10^{-4}$ M (5,000 times the $IC_{50}$) letrozole. Ovaries were collected at 16.5 dpc, when $E_2$ levels were expected to be high, and cultured for 5 days. We found that the percent of single oocytes (a measure of cyst breakdown) was not significantly different from control ovaries at any of the concentrations tested (Figure 2.7A). However, at the lowest concentration of letrozole ($10^{-10}$ M) the number of oocytes per section was significantly lower than in controls (Figure 2.7B). Follicle development was not affected by letrozole treatment with no significant difference in the percent of growing follicles between the control and all four concentrations of letrozole used (Figure 2.7C). To block $P_4$ production we used trilostane, a $3\beta$HSD inhibitor (Figure 2.8). The $IC_{50}$ of trilosane in rat sciatic nerves has been reported as $4 \times 10^{-6}$ M (Coirini et al., 2003). Here, we used the same range of concentrations as for aromatase and similar results were obtained except that oocyte number per section was reduced at $10^{-8}$ M and
$10^{-4}$ M but not at $10^{-10}$ M or $10^{-6}$ M (Figure 2.8B). We used letrozole and trilostane together to block production of both E$_2$ and P$_4$ and found that oocyte number was reduced at all concentrations tested but cyst breakdown and follicle development were not significantly different from controls (Figure 2.9).

2.5. DISCUSSION

We found that the drop in maternal P$_4$ but not E$_2$ corresponded to the initiation of cyst breakdown. This suggested that there was another source of hormones possibly the fetal ovary as in the bovine model (Nilsson and Skinner, 2009; Yang and Fortune, 2008). Here we showed that the corresponding mRNA and protein of enzymes necessary for the production of E$_2$ and P$_4$, aromatase and 3βHSD, respectively were present in fetal ovaries. In addition, we detected E$_2$ and P$_4$ locally in fetal ovaries. Finally, we carried out inhibitor studies blocking aromatase and 3βHSD in organ culture and demonstrated that oocyte number was reduced at certain inhibitor concentrations for each hormone while oocyte number was reduced at all concentrations when both inhibitors were used.

Our data bring into question the idea that maternal hormones are the only source of hormones that regulate fetal ovary development. We previously showed that some follicles assemble before birth during the fetal period in mice and the timing of follicle assembly is more similar to the bovine model (Pepling et al., 2010). Fetal bovine ovaries synthesize their own E$_2$ and P$_4$ (Nilsson and Skinner, 2009; Yang and Fortune, 2008) and here, we detected E$_2$ and P$_4$ in fetal mouse ovaries as well. Supporting the idea that fetal mouse ovaries can produce steroid hormones, we also detected the enzymes responsible for final steps of E$_2$ and P$_4$ biosynthesis. Aromatase mRNA levels peaked at 16.5 dpc and
then declined correlating with start of cyst breakdown. Likewise, 3βHSD mRNA levels were high from 13.5 to 15.5 dpc and then declined. In the steroid biosynthetic pathway, 3βHSD acts upstream of aromatase and synthesizes the precursor for E<sub>2</sub> production (Miller and Auchus, 2011). High levels of 3βHSD from 13.5 to 15.5 dpc may allow the synthesis of E<sub>2</sub> precursors before aromatase peaks at 16.5 dpc. Protein levels of these two enzymes were also detected in ovaries but did not fluctuate across timepoints.

E<sub>2</sub> peaked one day after aromatase at 17.5 dpc and then dropped at 18.5 dpc, though this was not a statistically significant decrease and P<sub>4</sub> while present did not significantly change across timepoints. Likewise, P<sub>4</sub> levels produced by fetal bovine ovaries in culture did not fluctuate over time (Yang and Fortune, 2008). However, in studies where P<sub>4</sub> levels were determined in fetal bovine tissue, levels dropped right at the time when follicles were forming (Nilsson and Skinner, 2009). One reason that fetal E<sub>2</sub> and P<sub>4</sub> levels don’t correlate with follicle formation in some studies is that proteins that bind to steroid hormones such as α-fetoprotein might also be present and limit hormone activity or availability. Another possibility is that the hormones present in fetal ovaries come from multiple sources including local hormone production, maternal circulation or from the placenta.

Steroid hormones can promote or inhibit follicle formation depending on species. This could be due to species differences in response to steroid hormone signaling. Alternatively, a basal low concentration of steroid hormones might be necessary for follicle formation and only at high concentrations have inhibitory effects (Kezele and Skinner, 2003; Wang and Roy, 2007). In mice, exposure to exogenous E<sub>2</sub> and P<sub>4</sub> blocks primordial follicle formation (Chen et al., 2007; Kezele and Skinner, 2003). Similarly, in
cultured fetal bovine ovaries follicle formation is blocked by E2 and P4 (Nilsson and Skinner, 2009; Yang and Fortune, 2008). However, in hamsters, E2 promotes follicle formation (Wang and Roy, 2007). In baboons, follicle formation is disrupted if E2 production is inhibited suggesting that normally as in the hamster, E2 promotes follicle formation (Zachos et al., 2002). Further studies are necessary to understand species differences in response to steroid hormones.

We found that inhibiting synthesis of E2 and P4 in organ culture reduced the number of oocytes per section but did not alter cyst breakdown and follicle formation. We expected that cyst breakdown would be accelerated based on the model that a drop in E2 and P4 would cause cyst breakdown and associated oocyte loss to begin. Our data raise the possibility that in mouse, estrogen is important for oocyte survival during fetal ovarian development but is not directly involved in cyst breakdown. Our results are similar to data obtained from studies of mice lacking aromatase (Britt et al., 2004). Although fetal and neonatal oocyte development was not examined, aromatase knockout mice had fewer primordial and primary follicles at 10 weeks of age. Since earlier timepoints were not examined it is not known when this loss occurred or if follicle formation was affected. Fetal ovary development has been examined in baboons treated with letrozole during late gestation (Zachos et al., 2002). Female fetuses collected from mothers treated with letrozole had a reduced number of primordial follicles compared to controls. However, unlike our studies, an increased number of interfollicular nests containing oocytes that are presumably in cysts were found suggesting that inhibition of E2 synthesis led to a block in cyst breakdown. An increase in primary-like follicles was
also observed. It is not known if the total oocyte number was affected because the overall number of oocytes was not determined.

Many questions remain regarding the role of steroid hormones in the fetal ovary as well as the signaling mechanisms involved. It is unclear what receptors are important during fetal development and future studies identifying the relevant receptors are necessary. It will also be informative to investigate cyst breakdown and follicle formation in aromatase knockout animals. Understanding the role of steroid hormone signaling in fetal oocyte differentiation will elucidate mechanisms leading to formation of the primordial follicle pool and thus fertility.
Figure 2.1. Maternal and perinatal circulating levels of E\textsubscript{2} and P\textsubscript{4}:

(A) Maternal serum levels of E\textsubscript{2} measured in pg/ml and P\textsubscript{4} measured in ng/ml during gestation and after birth (n ≥ 5). (B) Perinatal serum levels of E\textsubscript{2} measured in pg/ml and P\textsubscript{4} measured in ng/ml (n=3). Data are presented as the mean ± SEM.
Figure 2.2. mRNA and protein levels of steroidogenic enzymes in perinatal mouse ovaries: Aromatase (A) and 3βHSD (B) mRNA levels during the perinatal period. Levels of aromatase and 3βHSD mRNAs are normalized to the levels of mRNA for a housekeeping gene, β-actin in the same sample. The normalized values are expressed relative to the mRNA levels at 13.5 dpc with 13.5 dpc set at 1. * indicates a significant difference as determined by one-way ANOVA followed by a Bonferroni-Dunn’s post hoc test (p< 0.0014; n= 3). Western blot analysis of aromatase and 3βHSD in fetal ovaries (C-F). Ovary extracts from 15.5 dpc, 16.5 dpc, 17.5 dpc and 18.5 dpc were probed for (C) aromatase (50 kd) or (E) 3βHSD (42 kd). All extracts were also probed for the loading control GAPDH (38 kd). Quantification of aromatase (D) or 3βHSD (F) protein expression normalized to GAPDH and reported as % relative pixel intensity compared to 15.5 dpc set at 100%.
Figure 2.3. Expression of aromatase protein in the fetal ovary.

Confocal sections from 15.5 dpc (A–C), 16.5 dpc (D–F), 17.5 dpc (G–I) and 18.5 dpc (J–L) ovaries labeled for aromatase (green) (A, D, G and J) and the nuclear marker propidium iodide (red) (B, E, H and K) with overlay shown in C, F, I and L. White arrowheads indicate oocytes, white arrows indicate somatic cells. Scale bar, 20 µm.
**Figure 2.4. Expression of 3βHSD protein in the fetal ovary.** Expression of 3βHSD protein in the fetal ovary. Confocal sections from 15.5 dpc (A–C), 16.5 dpc (D–F), 17.5 dpc (G–I) and 18.5 dpc (J–L) ovaries labeled for 3βHSD (green) (A, D, G and J) and thenuclear marker propidium iodide (red) (B, E, H and K) with overlay shown in C, F, I and L. White arrowheads indicate oocytes (upper arrowhead in D-F and J-L indicates a strongly labeled oocyte while the lower arrowhead indicates a weakly labeled oocyte), white arrows indicate somatic cells. Scale bar, 20μm. Inset shown in panel L is an enlarged view of the area within the white dashed box.
Figure 2.5. Colabeling of 3βHSD and mitochondria or ER. Confocal sections from a PND6 ovary (A-D) labeled for the mitochondrial marker CYTC (green) (A), 3βHSD (red) (B), overlay (C) and DNA marker TOTO3 (white) (D). Confocal sections a PND5 ovary (E-H) labeled for the ER marker Calnexin (green) (E), 3βHSD (red) (F), overlay (G) and the DNA marker TOTO3 (white) (H). Scale bar, 10 µm.
Figure 2.6. Concentration of E\(_2\) and P\(_4\) in fetal and neonatal ovaries.

(A) E\(_2\) levels (pg/mg protein) in ovaries from 15.5 dpc to PND7 (B) E\(_2\) levels (pg/mg protein) in ovaries from 15.5 dpc to PND3 (C) P\(_4\) levels (ng/mg protein) in ovaries from 15.5 dpc to PND7 and (D) P\(_4\) levels (ng/mg protein) in ovaries from 15.5 dpc to PND3 (n=3, each n~ 300 ovaries).

Data are presented as the mean ± S.E.M. ND- Not Detected. Different letter subscripts indicate a significant difference. * indicates PND7 significantly different from other ages. (One-way ANOVA followed by Newman-Keuls multiple comparison test, P<0.05).
Figure 2.7. Effects of inhibiting aromatase in fetal ovaries.

(A) percent single oocytes (B) number of oocytes and (C) percent primordial and primary follicles relative to the total number of follicles per confocal section in 16.5 dpc ovaries cultured with the aromatase inhibitor letrozole or in control media for 5 days. Data are presented as the mean ± S.E.M. *Indicates a significant difference between control and treated ovaries (One-way ANOVA followed by Dunnett’s post hoc test, P<0.05). n ≥ 5 ovaries per group.
Figure 2.8. Effects of inhibiting 3βHSD in fetal ovaries.

Effects of inhibiting 3βHSD in fetal ovaries. (A) percent single oocytes, (B) number of oocytes and (C) percent primordial and primary follicles relative to the total number of follicles per confocal section in 16.5 dpc ovaries cultured with the 3βHSD inhibitor trilostane or in control media for 5 days. Data are presented as the mean ± S.E.M.

*Indicates a significant difference between control and treated ovaries (One-way ANOVA)
Figure 2.9. Effects of inhibiting aromatase and 3βHSD in fetal ovaries.

(A) percent single oocytes (B) number of oocytes and (C) percent primordial and primary follicles relative to the total number of follicles per confocal section in 16.5 dpc ovaries cultured with the aromatase inhibitor letrozole and the 3βHSD inhibitor trilostane or in control media for 5 days. Data are presented as the mean ± S.E.M.

*Indicates a significant difference between control and treated ovaries (One-way ANOVA followed by Dunnett’s post hoc test, P<0.05). n ≥ 5 ovaries per group.
Chapter 3
The Role of Steroid Hormones in Murine Oocyte Meiotic Progression
3.1. ABSTRACT

In mammalian females, reproductive capacity is determined by the size of the primordial follicle pool. During embryogenesis, oogonia divide by mitosis but cytokinesis is incomplete so that the oogonia remain connected in germ cell cysts. Oogonia begin to enter meiosis at approximately 13.5 days postcoitum (dpc) and over the next several days, oocytes progress through the stages of meiotic prophase I and arrest in the diplotene stage. At the same time, germ cell cysts begin to break down and individual oocytes become surrounded by granulosa cells forming primordial follicles. In rats, inhibition of a synaptonemal complex protein caused premature arrival at the diplotene stage and premature assembly of primordial follicles suggesting that diplotene arrest might trigger primordial follicle formation. Cyst breakdown and primordial follicle formation are blocked by exposure to exogenous hormones but effects on the timing of diplotene arrest are unclear. Here, we asked: (1) if oocytes were required to be in diplotene before follicles formed, (2) if all oocytes within a germ cell cyst arrested at diplotene synchronously, and (3) if steroid hormones could affect progression through prophase I of meiosis. Meiotic stage and follicle formation were assessed in histological sections. We found that oocytes within primordial follicles were at diplotene or pre-diplotene stages suggesting that meiosis and primordial follicle formation are independent. In addition, we found that germ cell cysts contain oocytes at both diplotene and pre-diplotene stages. To determine if steroid hormones affect the rate of progression to the diplotene stage, 17.5 dpc ovaries were placed in organ culture with control media or media containing estradiol, progesterone or both hormones. We found that progesterone delayed meiotic progression.
3.2. INTRODUCTION

In female mammals, reproductive capacity is determined at birth by the non-renewable pool of primordial follicles present representing the total population of germ cells available for reproductive purposes (Pepling, 2006). In mice, the primordial germ cells migrate to the genital ridge and divide by mitosis until 13.5 days postcoitum (dpc) (McLaren and Monk, 1981). During these divisions the germ cells are known as oogonia and develop in germ cell cysts due to incomplete cytokinesis following each cell cycle (Pepling, 2006). Oogonia start to enter meiosis at approximately 13.5 dpc and are then referred to as oocytes (McLaren, 2000b). Oogonia do not appear to enter meiosis synchronously (Borum, 1961; Peters, 1970). However, meiosis proceeds from anterior to posterior suggesting that local factors diffuse from the mesonephros at the anterior side of the ovary to promote meiosis (Bullejos and Koopman, 2004). Most germ cells are in meiosis by 15.5 dpc (Borum, 1961). After entering meiosis oocytes progress through the initial stages of meiotic prophase I and remain arrested at diplotene stage until just prior to ovulation. Some oocyte arrive at the diplotene stage by 17.5 dpc but it takes several days until all oocytes are in diplotene (Borum, 1961).

Mutations in genes such as disrupted meiotic cDNA 1 (Dmc1) and meiosis-specific sporulation protein (Spo11) responsible for the initial stages of meiosis result in loss of oocytes and an inability to form follicles which ultimately gives rise to sterility (Villeneuve and Hillers, 2001). This time point is marked by the expression of synaptonemal complex proteins (SYCPs) that make up the synaptonemal complex which is required for DNA synapsis and meiotic recombination between homologous chromosomes. In rats, it was found that inhibition of SYCP1 accelerated the attainment
of the diplotene stage along with premature assembly of those oocytes into primordial follicles (Paredes et al., 2005). A large number of oocytes that fail to attain diplotene arrest but continue on through diakinesis are lost by attrition during early folliculogenesis (Ohno and Smith, 1964). A crucial factor for the survival of germ cells may be their ability to be enclosed within follicles (Byskov, 1986). This evidence reflects the possibility of a relation between two temporal events during mouse oogenesis, diplotene stage and primordial follicle formation.

Both estradiol (E$_2$) and progesterone (P$_4$) in mouse can delay cyst breakdown and primordial follicle formation (Chen et al., 2007). Estrogens have also been shown to affect meiotic progression of oocytes. Studies were performed using a synthetic estrogen, bisphenol A (BPA) on pregnant mice during mid-gestation. The oocytes from the female fetuses of exposed mothers had synaptic defects and recombination aberrations. In adults, those aberrations gave rise to aneuploid eggs and embryos (Susiarjo et al., 2007). In cattle, it is thought that primordial follicles cannot be activated until after the oocyte reaches diplotene arrest. In vitro treatment of bovine ovaries with estradiol (E$_2$) or progesterone (P$_4$) decreased the number of follicles that were activated and may affect progression to the diplotene stage (Yang and Fortune, 2008).

MSY2 is a germ cell specific maternal mRNA binding protein and is developmentally regulated in mouse oocytes and spermatocytes. In oocytes, it is upregulated as the oocytes enter the diplotene stage and persists until after fertilization (Gu et al., 1998). Its role as a diplotene arrest marker was shown in cattle when the mRNA levels for $Ybx2$ increased 2.3 fold after Day 141 of gestation when primordial
folicles are activated and form primary follicles containing oocytes at the diplotene stage (Yang and Fortune, 2008).

The main objectives of the present study were to determine if diplotene arrest is linked with primordial follicle formation. Secondly, we wanted to elucidate the role of steroid hormone signaling in meiotic progression of oocytes. The molecular mechanisms involved in fetal ovarian development are still poorly understood. Future studies elucidating events during fetal ovarian development will increase our understanding of the factors that control oocyte quality and quantity and thus help in improvement of currently available infertility treatments.

3.3. MATERIALS AND METHODS

3.3.1. Animals

Adult CD1 male and female outbred mouse strain was obtained from Charles River Laboratories (Wilmington, MA, USA). They were maintained in accordance with the policies of Syracuse University’s Institutional Animal Care and Use Committee. Mice were housed and bred at a controlled photoperiod (14 h light, 10 h dark), temperature (21-22° C), and humidity with food and water available ad libitum. CD1 females were mated with males of same strain and checked daily for vaginal plugs. The noon on the day of vaginal plug detection was designated as 0.5 dpc. Birth usually occurred at 19.5 dpc and was designated as postnatal day (PND) 1. Pregnant mice were euthanized by CO₂ asphyxiation for fetal ovary collection. For neonatal ovary collection, only the female pups were dissected after their birth at 19.5 dpc.
3.3.2. Histological Methods

Fetal and neonatal ovaries were dissected and trimmed of any extra tissue. They were fixed in Bouin’s solution for 2 hours at room temperature, followed by dehydration through an ethanol series. Histological processing of the ovaries was performed at Electron Microscopy & Histology Core Facility, Weill Cornell Medical College, New York. Based on the standard protocols for paraffin-embedded sections, the ovaries were serially sectioned at 5 µm and stained with hematoxylin and eosin. Images were taken on an Olympus BX50 microscope with an Olympus DP71 digital camera. Every fifth section was marked for direct counts of oocytes at pre-diplotene and diplotene stages and at least five ovaries were analyzed at each age. A total of 10-12 sections per ovary were used for counting. To avoid bias, all ovaries were analyzed without knowledge of age. To avoid double counting of oocytes, only oocytes having a visible nucleus were counted. Oocytes at the pre-diplotene stage were characterized at the leptotene stage by the speckled appearance of their nuclei, zygotene nuclei were be distinguished by their thicker chromosome strands and pachytene nuclei have chromosomes with a beaded appearance. Oocytes at the diplotene stage are recognized by areas of condensed chromatin interspersed with clear areas (Challoner, 1974). The total number of pre-diplotene and diplotene oocytes for each ovary was determined by multiplying the number of pre-diplotene and diplotene oocytes by 5 to account for every 5<sup>th</sup> section being used in the analysis. Oocytes were counted as cysts if they were present in clusters of at least two oocytes without any intervening somatic cells. Oocytes were counted to be in primordial follicles if they contained an oocyte surrounded by flattened granulosa cells.
3.3.3. RNA isolation

Fetal (13.5 dpc-18.5 dpc) and neonatal (PND1-PND7) ovaries were dissected and stored in RNAlater at -80°C until further use. Total RNA was isolated using Qiagen’s RNeasy Mini kit (n=3; 50/100 ovaries per pool for neonatal and fetal ovaries respectively). Ovaries were briefly lysed and homogenized using a motor pestle and the mixture was then applied onto a QIAshredder column. The ovarian tissue sample in the QIAshredder column was then centrifuged at 11,000g for 2 minutes. To isolate the RNA, the resulting flow-through was applied to an RNeasy mini column which allowed the RNA to bind to the filter cartridge. RNA was eluted by washing from the filter and was concentrated using an RNeasy MinElute kit. The RNA which was isolated was briefly applied to an RNeasy MinElute spin column and after washing, RNA was eluted using 14µl of RNase-free water. The RNA concentration in the elutant was determined using an ND-1000 Spectrophotometer (λ= 260/280 nm; Nanodrop Technologies, Inc., Wilmington, DE).

3.3.4. First strand cDNA synthesis and real-time polymerase chain reaction (PCR)

Total RNA (0.5µg) was reverse transcribed using the Superscript III One-Step RT-PCR System into cDNA. cDNA was diluted in RNase-free water (1:25). 2 µl of diluted cDNA was amplified on a Rotor-Gene 3000 using Quantitect™ SYBR Green PCR kit and custom designed primers for Msy2 (forward primer: 5' CCC TGG CAA CCA GGC GAC GG 3'; reverse primer: 5' TGA CTG TGC CCA GGA CTT GGA TTG 3'; NCBI Genbank accession number NM_016875), and β-actin (forward primer: 5’ AGT GTG ACG TTG ACA TCC GTA 3’; reverse primer: 5’ GCC AGA GCA GTA ATC TAA
TTA T 3’; NCBI Genbank accession number NM_007393). The cycling program consisted of a 15 min hold at 95°C and 45 cycles of: denaturing at 95°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 20 s at which point data were acquired. Determination of product melt conditions were done using a temperature gradient from 72°C to 99°C with a 1°C increase at each step. β-actin expression remained constant across all ages and so each sample was normalized to β-actin before quantification.

3.3.5. In vitro ovary organ culture

Ovaries dissected at 17.5 dpc were placed in culture. Ovaries were cultured in 4-well culture plates in drops of media on 0.4 µM floating filters (Millicell- CM; Millipore Corp., Bedford, MA) in 0.4 ml DMEM-Ham’s F-12 media supplemented with penicillin-streptomycin, 5X ITS-X (Life Technologies, Inc., Grand Island, NY), 0.1 % BSA, 0.1% albumax, and 0.05 mg/ml L-ascorbic acid. E\textsubscript{2} and P\textsubscript{4} were purchased from Sigma Chemical Co., St. Louis, MO and dissolved in dimethylsulfoxide (DMSO) at a concentration of 0.1 M and then added to culture media to achieve the desired final concentration. DMSO was added to media at the same percent as the chemical to serve as vehicle control. Ovaries were placed in culture and exposed daily to E\textsubscript{2}, P\textsubscript{4} or both hormones at 10\textsuperscript{-6} M or DMSO alone as a vehicle control (n= 5 ovaries per treatment group). The ovaries from control and treatment were fixed in Bouin’s fixative and histologically processed.
3.3.6. Statistical analysis

The percent of oocytes in diplotene, percent of oocytes in follicles and percent of follicles containing pre-diplotene oocytes over time were calculated using four to five ovaries at each time point. Data are represented as mean ± SEM of nontransformed data. Counted data by nature is not normal and logarithmic transformation was performed on the data before doing statistical analysis. Statistical analyses using transformed data were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Statistical differences (P<0.05) among the means were evaluated using one-way ANOVA followed by Dunnett’s multiple comparison test. Effects of E\textsubscript{2} and P\textsubscript{4} on oocyte number, percent single oocytes, percent oocytes in diplotene and percent of follicles containing diplotene oocytes were analyzed in the same manner as the time course above using 5 ovaries per treatment group. Statistical analyses of Real time PCR data were performed using Statview 5.0.1 (SAS Institute Inc., Cary, NC). Differences between fold increases in gene expression over various time points were evaluated by one-way ANOVA followed by Bonferroni-Dunn’s post hoc test (P<0.0005). All results are presented as mean ± SEM of nontransformed data.
3.4. RESULTS

3.4.1. Meiotic progression and diplotene arrest in developing mouse ovaries

Borum in 1961 reported that mouse oocytes begin to reach the diplotene stage at 17.5 dpc (Borum, 1961). More recently, it has been reported that the first diplotene oocytes were observed at 18.5 dpc (Di Carlo et al., 2000). For that study, the sample size was limited to only 200 nuclei in ovaries of each age. In contrast, we did a thorough quantitative study counting histological sections for each timepoint from 15.5 dpc to PND5. This resulted in a consideration of approximately 1000 nuclei at each age. The earliest diplotene oocytes were observed at 17.5 dpc with about 8% of the oocytes at the diplotene stage (Figure 3.1A). A statistically significant increase to 32% diplotene oocytes was observed at PND1 and to 49% at PND2. No further increase was observed from PND3 to PND5.

Representative histological sections with examples of the different meiotic stages are shown in Figure 3.1C-3.1F. Figure 3.1C shows a section from a 13.5 dpc ovary with oogonia in mitosis characterized by dense nuclei and leptotene oocytes recognized by speckled appearance of their chromatin (Challoner, 1974; Yang and Fortune, 2008). Figure 3.1D shows a section from a 15.5 dpc ovary with oocytes at zygotene characterized thick strands of entangled chromatin (Challoner, 1974). Figure 3.1E shows a PND1 ovary with oocytes at pachytene distinguished by the beaded appearance of condensed chromatin (Challoner, 1974). Figure 3.1F shows a PND4 ovary with oocytes at diplotene characterized by areas of condensed chromatin separated by clear areas (Challoner, 1974).
Mammalian female germ cells express a germ cell-specific Y-Box protein, MSY2, which is a cytoplasmic marker for diplotene oocytes. MSY2 is upregulated at the diplotene stage (Gu et al., 1998). In addition to the morphological assessment of meiotic stage discussed above, we also measured the levels of Msy2 mRNA by qPCR during the same time period (Figure 3.1B). A statistically significant increase of Msy2 mRNA is observed at 18.5 dpc correlating with the increase in diplotene oocytes observed one day earlier at 17.5 dpc.

3.4.2. Primordial follicle formation and diplotene arrest are independent processes

Previous studies from our lab have indicated that in the medullary region, primordial follicles can be found as early as 17.5 dpc (Pepling et al., 2010). Here we performed a thorough investigation analyzing serial sections 5 μm apart covering the entire ovary thereby enabling us to obtain a more accurate estimate regarding the presence of primordial follicles. The earliest primordial follicles were detected at 16.5 dpc where we found 2% of the oocytes in enclosed in follicles (Figure 3.2A). An example of a primordial follicle in a 16.5 dpc ovary is shown in Figure 3.2C. We hypothesized that oocytes needed to reach diplotene before becoming enclosed in follicles. However, we observed follicles with pre-diplotene oocytes at all ages examined. Figure 3.2B shows the percentage of follicles containing pre-diplotene oocytes from 15.5 dpc to PND5. All of the follicles observed at 16.5 dpc were at the pre-diplotene stage. For the later stages, only a small percentage of follicles contained pre-diplotene oocytes with the majority already at diplotene. Figure 3.2D shows a 17.5 dpc ovary with a primordial follicle containing a pre-diplotene oocyte. We also observed oocytes still in germ cell cysts that
were at the diplotene stage (Figure 3.2E). Finally, at PND5 we observed a small proportion of pre-diplotene oocytes (Figure 3.2F). Thus, meiotic progression and primordial follicle formation do not appear to be linked.

3.4.3. All oocytes in a cyst do not reach diplotene together

Oocytes enter meiosis in a wave from anterior to posterior in the mouse ovary. However, it is not known whether in individual cysts oocytes enter or progress through meiosis and arrest at diplotene synchronously. To determine whether all oocytes in a germ cell cyst were at the same meiotic stage, a quantitative analysis of the meiotic stages of the oocytes present within individual cysts was performed at 18.5 dpc. We chose this age because many pachytene and diplotene stage oocytes are present and most oocytes are still in germ cell cysts. We examined five representative cysts from five different ovaries labeled Cysts 1 - 5 shown in Figure 3.3. Each cyst contained two to five oocytes visible in the section. It is important to note that only oocytes in individual sections were analyzed and that the cysts likely contained more oocytes not visible in the section. For each cyst we determined whether an oocyte was at the pre-diplotene or diplotene stage of meiosis. Each cyst examined had both pre-diplotene and diplotene oocytes (Figure 3.3 and Table 3.1). These findings suggest that oocytes within individual cysts do not progress through meiosis synchronously.

3.4.4. Progesterone decreases the number of follicles at diplotene

There is some evidence that steroid hormones can cause delays in meiotic progression. We wanted to know if E₂ or P₄ could delay arrival at the diplotene stage in
mouse ovaries. To test this, 17.5 dpc fetal ovaries were placed in organ culture for four days with control media or media containing $10^{-6}$ M E$_2$, $10^{-6}$ M P$_4$ or both $10^{-6}$ M E$_2$ and $10^{-6}$ M P$_4$. After culture, serial sections were prepared, stained with hematoxylin and eosin and analyzed. There was no difference in the number of oocytes between control and treated ovaries (Figure 3.4A). There was also no difference in the percent of single oocytes, a measure of primordial follicle formation (Figure 3.4B). In addition, there was also no statistically significant difference in the percentage of oocytes at the diplotene stage of prophase I (Figure 3.4C). However, when only oocytes already assembled in primordial follicles were examined, there were significantly fewer follicles containing diplotene oocytes in the P$_4$ treated ovaries (Figure 3.4D).
3.5. DISCUSSION

Here we investigated the relationship between two critical events in mammalian oogenesis, primordial follicle formation and diplotene arrest. We confirmed that the earliest diplotene oocytes were observed at 17.5 dpc and in accordance with this Msy2 mRNA levels increase one day later. In previous studies from our lab the earliest age that primordial follicles were reported was 17.5 dpc (Pepling et al., 2010). However, here we observed a small percentage of follicles even earlier at 16.5 dpc. We also found primordial follicles containing pre-diplotene oocytes supporting the idea that oocytes do not need to reach diplotene arrest before they can become enclosed in primordial follicles. Furthermore, oocytes within individual germ cell cysts do not appear to proceed through meiosis synchronously. Finally, progesterone treatment delayed meiotic progression of oocytes in follicles.

There have been some reports that mouse oocytes arrest in the diplotene stage of meiosis I starting at 17.5 dpc while others do not observe diplotene oocytes until 18.5 dpc (Borum, 1961; Di Carlo et al., 2000). We reexamined this question and found oocytes at diplotene arrest as early as 17.5 dpc in agreement with the original studies of Borum. Further, we see an increase in the number of diplotene oocytes at PND1 and PND2 but surprisingly no further increase to PND5. Previous reports suggest that all oocytes have arrested in the diplotene stage by PND5. It may be that a small number of oocytes do not reach diplotene until later.

Inhibition of the synaptonemal complex protein SYCP1 in rats led to premature arrival of oocytes at the diplotene stage and also resulted in accelerated primordial follicle assembly (Paredes et al., 2005). These observations suggested that diplotene
arrest is developmentally linked with follicle formation in mice. However, our results here suggest that meiotic stage and primordial follicle formation are independent. *Stra8* deficient female mice have been previously shown to have meiotic initiation block at 13.5 dpc to 14.5 dpc. (Baltus et al., 2006). The authors postulated that if oocyte growth and differentiation were required for meiotic entry then *Stra8* deficient ovarian germ cells would not undergo folliculogenesis. Contrary to postulations, the germ cells in *Stra8* deficient ovaries did become enclosed in granulosa cells and develop to advanced stages (Dokshin et al., 2013). Thus, entry into meiosis or progression to the diplotene stage is not a prerequisite for oocytes to form follicles in mice. In cattle, however, the activation of primordial follicles to form primary follicles is correlated with the achievement of meiotic arrest by the oocyte (Yang and Fortune, 2008).

Here, we examined the effects of E₂ and P₄ on progression of oocytes to the diplotene stage. Surprisingly, P₄ but not E₂ delayed meiotic progression, decreasing the percent of follicles containing diplotene oocytes. In previous studies, exposure of pregnant C57BL/6 females to the estrogenic compound BPA, disrupted the processes of meiotic prophase (Susiarjo et al., 2007). Oocytes from female fetuses had defects in synapsis and increased recombination resulting in higher levels of aneuploidy. This may be due to differences in the specific time period examined or route of hormone exposure. BPA exposure was from 11.5 dpc until 18.5 dpc when the fetal ovaries were harvested while in our study 17.5 dpc ovaries were exposed to hormones in organ culture for 4 days. There may also be differences depending on which estrogenic compound is used. Finally, Susiarjo and colleagues examined chromosomal defects while we examined progression to the diplotene stage.
Previous work from our lab demonstrated that exposure to P₄ or E₂, as well as synthetic estrogens, BPA, diethylstilbestrol or ethinyl estradiol delays germ cell cyst breakdown and primordial follicle formation (Chen et al., 2007; Karavan and Pepling, 2012). In addition, synthetic estrogens at some concentrations also alter the oocyte loss that accompanies cyst breakdown. Here we found that P₄ or E₂ had no effect on cyst breakdown, primordial follicle formation or oocyte survival. The previous studies began hormone exposure at PND1 while in the current work hormone treatment began earlier at 17.5 dpc and this maybe outside the window of sensitivity. Interestingly, in a previous study using rats P₄ but not E₂ significantly inhibited primordial follicle assembly (Kezele and Skinner, 2003). While in our study neither hormone affected follicle assembly we also observed an effect with P₄ not on follicle formation but on meiotic progression.

In summary, the processes of primordial follicle formation and meiotic progression to the diplotene stage do not appear to be linked. P₄ reduced the percent of oocytes within primordial follicles that had reached diplotene arrest. Diplotene arrested oocytes often remain dormant for many years before being activated. Mechanisms controlling progression of oocytes through prophase I and arrest at diplotene are not well understood but this knowledge will be essential to prevent defects in meiosis such as aneuploidy.
### Table 3.1. Analysis of meiotic stage within 5 individual germ cell cysts

<table>
<thead>
<tr>
<th>Cyst</th>
<th># of oocytes at pre-diplotene</th>
<th># of oocytes at diplotene</th>
<th>Total # of oocytes</th>
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<tr>
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<td>1</td>
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<td>3</td>
</tr>
<tr>
<td>Cyst 3</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
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<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Cyst 5</td>
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<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>8</td>
<td>17</td>
</tr>
</tbody>
</table>
3.7. Figures

**Figure 3.1. Diplotene oocytes first detected at 17.5 dpc.** (A) Percentage of oocytes at the diplotene stage meiotic prophase I (± SEM) in perinatal mouse ovaries from 15.5 dpc to PND5. Asterisks indicate a significant difference (P < 0.05; n=4-5 ovaries per time point) as determined by one-way ANOVA followed by Dunnett’s Multiple Comparison Test. (B) Relative measurements of mRNA corresponding to MSY2, a specific marker for diplotene stage oocytes as determined by qPCR in perinatal mice ovaries at different gestational ages. The levels of MSY2 mRNA are normalized to the levels of mRNA for a housekeeping gene, β-actin in the same sample. Asterisks indicate a significant difference (P<0.0005) as determined by one- way ANOVA with Bonferroni-Dunn’s post hoc test (n=3). (C-F) Representative histological images of perinatal mouse ovaries stained with hematoxylin and eosin showing different meiotic stages. (C) 13.5 dpc ovary showing oogonia in mitosis and oocytes at leptotene. (D) 15.5 dpc ovary showing oocytes at zygotene. (E) PND1 ovary showing oocytes at pachytene. (F) PND4 ovary showing oocytes at diplotene. Scale bar = 50 µM.
Figure 3.2. Follicle formation and meiotic progression in perinatal mouse ovaries.

(A) Percentage of oocytes in follicles (± SEM) over time. Different letters indicate a significant difference between groups (one-way ANOVA, P<0.05). (B) Percentage of follicles at the pre-diplotene stage out of total follicles (± SEM) in perinatal mice ovaries. Asterisks indicate a significant difference (P<0.05; n=4-5 ovaries per time point) as determined by one-way ANOVA followed by Dunnett’s Multiple Comparison Test. (C-F) Representative histological images of perinatal mouse ovaries stained with hematoxylin and eosin. (C) 16.5 dpc ovary showing a primordial follicle with a pre-diplotene oocyte indicated by an arrow. (D) 17.5 dpc ovary showing a primordial follicle containing a pre-diplotene oocyte indicated by an arrow. (E) PND3 ovary showing diplotene oocytes not yet enclosed in primordial follicles. (F) PND5 ovary showing a pre-diplotene oocyte. Scale bar = 50 μM.
Figure 3.3. Representative histological images showing paraffin sections of fetal mouse ovaries at 18.5 dpc stained with hematoxylin and eosin.

(A) ovarian section showing one cyst labeled Cyst 1 with five oocytes, one oocyte in diplotene and four in pre-diplotene. (B) ovarian section showing one cyst labeled Cyst 2 with three oocytes, two in diplotene and one in pre-diplotene. (C) ovarian section showing Cyst 3 with two oocytes, one in diplotene and one in pre-diplotene. (D) ovarian section showing one cyst labeled Cyst 5 with five oocytes three in diplotene and two in prediplotene. Diplotene oocytes are indicated by an arrow and pre-diplotene oocytes are indicated by an arrowhead. Scale bar = 20 µM.
Figure 3.4. Effects of E\textsubscript{2} and P\textsubscript{4} treatment in organ culture on meiotic stage.

(A) Total number of oocytes, (B) percent single oocytes (C) percent of diplotene oocytes and (D) percent of follicles containing diplotene stage oocytes in control ovaries or ovaries treated with 10\textsuperscript{-6} M E\textsubscript{2}, 10\textsuperscript{-6} M P\textsubscript{4} or 10\textsuperscript{-6} M E\textsubscript{2} + 10\textsuperscript{-6} M P\textsubscript{4} for 4 days in organ culture. Data are presented as the mean ± SEM. * indicates a significant difference (P < 0.05; n=5 ovaries per treatment) as determined by one-way ANOVA followed by Dunnett’s Multiple Comparison Test.
Chapter 4
Conclusions and Future Directions
4.1. Conclusions

The overarching theme of this dissertation is to elucidate the sources of steroid hormones in perinatal ovaries and investigate their role on meiotic progression. Fertility and reproductive lifespan of a female mammal is limited to the germ cell reserve present at the time of birth (Kezele et al., 2002). A lot remains elusive about the signaling pathways responsible for maintaining the early germ cell reserve as well as factors causing germ cell apoptosis. It has been observed that in higher mammals like cattle, fetal oogenesis is regulated by the autocrine and paracrine actions of the hormones produced by the fetal ovaries themselves (Nilsson and Skinner, 2009; Yang and Fortune, 2008). We sought to determine whether the same phenomenon occurs in perinatal mice ovaries. We first measured the circulating maternal E₂ and P₄ levels during gestation. E₂ levels were considerably low during 13.5dpc to 15.5 dpc when cysts are being actively formed. This suggests that maternal E₂ did not play any role in maintaining oocytes in cysts in the fetal ovaries. Additionally, the earliest primordial follicles were detected at 16.5 dpc by the present studies. The circulating maternal P₄ level is highest at this time-point and it drops after that. The maternal circulating level of E₂ did not seem to play a role in maintaining oocytes in cysts in the fetal ovaries. We then sought to explore the possibility of perinatal ovaries synthesizing steroid hormones. The presence of steroidogenic enzymes, aromatase and 3βHSD (both mRNA and protein), were confirmed in the perinatal ovaries. The intraovarian steroid content of the perinatal ovaries were examined by RIA. E₂ levels were highest in the fetal ovaries at 17.5 dpc and then decreased and became undetectable at birth. The levels of P₄ on the other hand did not vary much from 15.5 dpc
to PND3. The intraovarian steroid hormone levels detected here seem to correlate well with studies done with neonatal ovaries in other rodents like hamsters. In hamsters, a basal level of E$_2$ is found to be essential for normal primordial follicle development and somatic cell differentiation (Wang and Roy, 2007). The authors also conclude from their observations that it is the dosage of the steroid hormones rather than their presence which determines whether it has a inhibitory or supportive role in follicle formation (Wang and Roy, 2007). During fetal oogenesis in humans, apoptosis of oocytes decreases considerably when the maternal and fetal E$_2$ levels begin to rise from 22 weeks of gestation (Casey, 1998; Shutt et al., 1974; Vaskivuo et al., 2001). We conjecture from our findings that the low levels of steroid hormones found in the perinatal mice ovaries help to support the healthy development of primordial follicles during that period.

We then treated fetal ovaries at 16.5 dpc in organ culture for five days with aromatase and 3βHSD inhibitors (letrozole and trilostane respectively) and collected them at PND3. We found that the treated ovaries have significantly fewer oocytes per section compared to control (untreated). This is in accordance to what is found in ArKO mice which have significantly fewer number of primordial and primary follicles compared to the wildtype mice aged 10 weeks (Britt et al., 2004). This underscores the importance of E$_2$ and P$_4$ in early follicle formation. Our findings with the in vitro inhibitor experiments during the perinatal period extend our understanding regarding the low follicle count found at puberty in ArKO mice. The ArKO mice are deficient in E$_2$ and this deficit might account for the fewer number of oocytes to begin with during the neonatal development which results in the reduced follicle count in the pubertal ovary.
A similar phenomenon occurs in higher mammals like baboons. In one particular study, pregnant baboons were treated with aromatase inhibitor and ovaries from the female fetuses of those pregnant dams were analyzed (Zachos et al., 2002). On analysis, it was found that fetal ovaries from the pregnant dams who were deprived of E\textsubscript{2} by inhibitor treatment have their primordial follicle count reduced by 50% compared to fetuses from untreated dams (Zachos et al., 2002). The fetuses from pregnant dams who were treated with inhibitor plus estradiol benzoate and have their E\textsubscript{2} levels restored to about 30% of normal levels have a follicle count similar to controls (untreated fetuses)(Zachos et al., 2002). Collectively, these findings emphasize a critical role of E\textsubscript{2} in maintaining the oocyte pool during the perinatal period in higher mammals.

In Chapter 3, we sought to examine the role of steroid hormones in the meiotic progression of oogenesis. There are two arrests during meiosis in mammalian oogenesis, diplotene arrest and metaphase II arrest during meiosis II. Diplotene arrest primarily occurs around the time of primordial follicle formation. There was no definitive evidence till date to link the two temporally occurring events. We did a thorough quantitative analysis to examine the possibility of a link between the two processes and categorized the oocytes into four classes while counting in cysts at pre-diplotene, in cysts at diplotene, in follicles at pre-diplotene and in follicles at diplotene. A significant finding was that primordial follicles are observed as early as 16.5 dpc. The oocytes begin to enter diplotene from 17.5 dpc onwards. This underscores the fact that primordial follicle formation is not dependent on the diplotene stage of oocyte nuclei. Our counting data correlate well with observations in Stra8\textsuperscript{−/−} mice. Stra8 is essential for meiotic entry of oocytes and thus germ cells in ovaries of Stra8\textsuperscript{−/−} mice are observed to have a block in
meiotic entry at 13.5 dpc to 14.5 dpc (Baltus et al., 2006). It was observed that the germ cells continue to grow and differentiate to form follicles in a manner consistent with normal follicle development (Dokshin et al., 2013). Collectively, these observations reinforce the fact that primordial follicle formation in mice does not depend on the meiotic stage of the oocyte nuclei.

Germ cell meiosis is a critical event during mammalian oogenesis and any aberrations in this process can lead to aneuploid eggs. Xenoestrogens, like BPA in particular have been shown by various studies to cause various meiotic prophase defects (Susiarjo et al., 2007). Our second objective was to examine the effects of E$_2$ and P$_4$ on meiotic progression of oocytes. To test this, ovaries at 17.5 dpc were cultured for four days and collected at PND3 in three separate experimental sets treated with (10$^{-6}$M E$_2$, 10$^{-6}$M P$_4$ and 10$^{-6}$M E$_2$ & P$_4$). Only P$_4$ had an effect in decreasing the percent of diplotene follicles from 66% in control ovaries to 45.6% in P$_4$ treated ovaries (P< 0.05). In cattle, both E$_2$ and P$_4$ had an effect in reducing the number of primary follicles at diplotene stage by 90% and 85%, respectively (Yang and Fortune, 2008).

A critical factor which governs the fertility of a female mammal at puberty is the finite pool of primordial follicles formed during the perinatal period (Kezele et al., 2002). Infertility has been recognized as a public health concern by the World Health Organization (WHO). According to the report of a survey done from 2006-2010 by National Survey of Family Growth, CDC about 7.4 million women or 11.9% of women in the population have sought infertility treatments at some point during their reproductive life span. The delay in the maternal age of childbearing and exposure to environmental toxins are particularly held responsible in decreasing the follicle count in
the ovaries leading to infertility. The two predominant disorders which interfere with the formation of healthy follicles and ovulation in women are premature ovarian failure or primary ovarian insufficiency when the ovaries fail to respond to circulating FSH by synthesizing E2 and develop mature follicles and primary amenorrhea which is the absence of menstruation. (Coulam et al., 1986; Yen, 1991). Studies in this dissertation have primarily addressed the role of steroid hormone signaling which plays a critical role in the formation of the reservoir of primordial follicles. For advanced reproductive technologies like in vitro fertilization (IVF), live birth from donor embryo transfer, it is important to maintain the reservoir of healthy follicles for ovulation. While this dissertation study addresses basic mechanisms in the formation of primordial follicles during the perinatal period, translational implications are enormous.

4.2. Future Directions

An interesting area to explore for future research is to examine whether placenta, which remains intimately associated with fetal growth and metabolism, might have an effect on the formation of follicles in the fetal ovary. The placenta is the site of synthesis for various hormones like chorionic gonadotropin, estrogen and progesterone. Estriol is the predominant estrogen produced during pregnancy by the placenta. It would be helpful to collect the placenta particularly during the time period of 13.5 dpc to 15.5 dpc when cysts are being formed. A timecourse of placental hormone levels (mainly estriol and progesterone) might be useful to address whether placental hormones play a role in oocyte development in fetal ovaries.
In Chapter 2, we analyzed the intraovarian steroid content of the perinatal ovaries. A more direct approach would be to cultivate the perinatal ovaries in organ culture and collect the media for hormone assays. Our initial attempts to grow the ovaries \textit{in vitro} for a few days and collect the media for hormone assays proved unsuccessful. The hormone content of the media was below the detection limit of RIA. There are several ways to circumvent the problem. One ideal way would be to concentrate the media by changing only half of the volume (e.g. 200 µl) of the total 400µl volume daily with fresh media. In that way, half of the old media would already have the hormones secreted by the ovaries and the other half of the fresh media would provide sufficient fresh nutrients to support growth of the ovaries. Another way is to increase the number of ovaries grown on each filterpaper in order to increase the amount of steroid hormones secreted in the media. In our present organ culture protocol, we usually cultivate 3-4 ovaries per filterpaper. An ideal way would be to do a trial set with 4, 6, 8 and 12 ovaries per filterpaper. Plotting the amount of hormone secreted in the media (Y axis) vs. the number of ovaries placed in each filterpaper (X axis) would ideally give rise to an exponential curve. The point where the curve becomes a plateau is where the number of ovaries per filterpaper has exceeded the optimal number of ovaries such that feedback inhibition on the hormone secretion occurs. Ideally, the optimal number of ovaries to be placed in the filterpaper would be a number in the exponential region of the curve.

In this dissertation study, \textit{in vitro} inhibitor studies in organ culture were carried out with letrozole and trilostane to block aromatase and 3βHSD action respectively. The earliest age at which the ovaries of ArKO mice have been analyzed is 10 weeks (Britt et
al., 2004). They have fewer number of follicles developing in their ovaries compared to age-matched wildtype females (Britt et al., 2004). An interesting area for future research would be to collect neonatal ovaries of three possible genotypes by mating ArKO hetrozygotes (+/-) parents. The three expected genotypes of the offsprings would be wildtype (+/+), heterozygote (+/-) and homozygous knockout (-/-). In heterozygous (+/-) ovaries, with one copy of the aromatase gene it is predicted to have half the amount of E$_2$ synthesis as opposed to wildtype (+/+) ovaries. Whereas, in homozygous knockout (-/-) ovaries, lacking both the copies of aromatase gene, there would be no E$_2$ synthesis . It would be useful to grow the ovaries of three expected genotypes in organ culture media for a few days and analyze the percent of cyst breakdown of each genotype. This would enable us to derive a comparative estimate of the role of 50% E$_2$ synthesis in perinatal ovaries of heterozygotes (+/-) as opposed to total absence of E$_2$ synthesis in homozygous knockouts (-/-) and also 100% E$_2$ synthesis in wildtype (+/+). In *in vitro* conditions, it would also be possible to grow perinatal ovaries of homozygous knockouts (-/-) in presence of exogenous E$_2$ treatment. By comparing the results of percent of cyst breakdown from such exogenous E$_2$ treatment in (-/-) ovaries to the percent of cyst breakdown in wildtype or heterozygote ovaries- would enable us to get better insights about the role of E$_2$ synthesis during perinatal mice oogenesis. Also, it would help to extend our understandings of our previous *in vitro* inhibitor results.

Another interesting direction to consider for future research might be to gain better insights whether estradiol and progesterone have any effects in inhibiting cyst breakdown when the ovaries are treated during the fetal period. In Chapter 3, we treated the ovaries as early as 16.5 dpc with E$_2$ and P$_4$ and grew them for five days to be collected at PND3.
Our main objective was to examine whether the hormone treatment had any effect on diplotene arrest of the oocytes. It was indeed surprising to observe though hormonal treatment (only P₄) reduced the number of primordial follicles at diplotene, both E₂ and P₄ had no effect in inhibiting cyst breakdown during that period. Our previous findings (Chen et al., 2007) clearly demonstrate an inhibitory effect of E₂ and P₄ on cyst breakdown thus reducing the total number of primordial follicles when treated from PND1 to PND7. This apparently contradictory report might require more detailed examination whether hormonal treatments have effects in inhibiting when treated only during a specific time-frame. Overall, this dissertation study has contributed in opening several interesting avenues to pursue for future research.
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BIOGRAPHICAL SKETCH

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