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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 break down 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 (E_2), progesterone (P_4) or E_2 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 (3 β HSD), cardinal steroidogenic enzymes required for E_2 and P_4 synthesis respectively. The mRNA levels for both aromatase and 3 β HSD 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 3 β HSD 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

Department of Biology

SYRACUSE UNIVERSITY

June 2014

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Dedication

This thesis is dedicated to Ma and Baba. Thank you for your unconditional love, support and sacrifices throughout my education.

Acknowledgement

As I look back at the last seven years of my life, I owe my gratitude to a lot of wonderful people without whose support this dissertation would never have been possible. While it is impossible to mention all, several deserve special attention for their contribution.

I would begin by thanking my parents for unconditionally supporting me throughout my Ph.D. career. Living half-way across the globe was not easy. My parents tolerated me being away on occasions when they needed me most. My Mom, Gita Dutta constantly kept me reminding of the deadlines through every step of graduate school and always adding things to my to-do list. My Dad, Mantu K. Dutta inspired me to keep my focus whenever I tended to wander. This dissertation would not have been possible without them.

I would especially like to thank my advisor, Dr. Melissa Pepling for giving me the independence to work in my own style and pace. Because of her, I could interact with several post docs and professors belonging to different universities. She also gave me the opportunity to travel to several conferences. I am grateful to her for providing me comments and suggestions during my presentations. I believe that a true advisor ensures an atmosphere in which a student can learn, thrive, and prosper on her own. I am grateful that I could get such a working environment under her tutelage.

Next I would like to thank Dr. Donna Korol for helping me prepare for the postdoctoral interviews, giving me leads about my job applications and sparing her invaluable time reviewing my manuscripts. She guided me enormously in developing my

scientific writing. I am also grateful to Dr. Sandra Hewett for agreeing to serve on my committee. Her breadth and depth of knowledge is truly awe-inspiring. She was always there for me whenever I had any question regarding my experiments when I had difficulties with. I would just walk to her door and bug her with my queries. And invariably, she would provide an answer or at least a direction towards it. I have always been inspired by her working style and her endless creativity and enthusiasm. I can only hope that I will be able to imbibe some percentage of those qualities in me in the forthcoming years.

I would also like to thank Dr. Patricia Hoyer at University of Arizona. It was an honor for me to collaborate with her. Her critical insights helped me to develop my project along the way. She is the epitome of an ideal mentor for me. Several other people worth mentioning who immensely helped me provide directions and guidance to overcome several roadblocks along the way are Drs. Joanne Fortune and Ming Yang at Cornell University for teaching me to characterize nuclei at different meiotic stages, Dr. Joshua Johnson at Yale University for his helpful suggestions, Dr. Debanjan Goswami at Stanford University, who has been a father like figure, for providing me advice about science and life beyond lab.

My committee member, Dr. Allen Silverstone deserves a special mention for his endless patience to listen to my research updates, constructively criticize my data and provide me helpful insights. I would also like to extend my thanks to my other committee members- Dr. Eleanor Maine and Dr. John Belote. I would specially like to thank Dr. Torsten Wöllert and research technicians, Valarie Vought and Xia Xu of my department for countless discussions on research and helping me with troubleshooting tips for my

experiments. Graduate school would have been difficult for me without them. I also owe my gratitude to the biology departmental staff- Dave Aldhinger, Paul Logue, Kelly Pierson, Katherine Geraghty, Sally Hallahan, the late Evelyn Lott and LAR staff for their technical assistance all these years. I would also like to extend my gratitude to National Science Foundation (NSF) for funding the studies.

Life thousands of miles away from home would have been impossible if I did not have the company of some amazing people at Syracuse who were family to me. I am especially grateful to Uncle Suresh and Aunty Ramani for being like foster parents for me. I would swing by at their doorstep whenever I needed the comfort, love and support of a family and also a shoulder to cry in times of distress. I would cherish the time spent with them. I would also like to thank my friend, Kaushik for the much needed laughs and for bringing charm and energy to my life during the stressful days of graduate school. His companionship had been truly a personally rewarding experience of my life. I am also grateful to my friend, Mahmuda to be supportive of my needs and get past the finishing line in style.

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List of Abbreviations

| | |
|---|---------------|
| Aromatase enzyme knockout | ArKO |
| 3-beta-hydroxysteroid dehydrogenase | 3 β HSD |
| Bisphenol-A | BPA |
| Days post coitum | dpc |
| Diethylstilbestrol | DES |
| Estradiol | E_2 |
| Estrogen receptor | ER |
| Estrogen receptor α | ER α |
| Estrogen receptor | ER β |
| 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 | P ₄ |
| 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; Büning, 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) (Büning, 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 2^n 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 in

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 (Burning, 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 synapsis 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 *Stra8*⁻ 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 ER β 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 (E_2) and Progesterone (P_4) receptors

Both E_2 and P_4 are endogenous hormones with important physiological roles targeting the reproductive tissues, brain, bone, and cardiovascular system. In the classical steroid signaling pathway, E_2 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 - ER α and ER β , containing a conserved DNA-binding domain (Pettersson and Gustafsson, 2001). The ligand, E_2 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. E_2 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). E_2 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, E_2 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).

ER α mutants are sterile with ovaries lacking corpora lutea and containing hemorrhagic follicles (Lubahn et al., 1993). ER β 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 ER β , ER α may compensate for the lack of ER β and thus ER β mutant females are not completely infertile. Mice lacking both ER α and ER β receptors ($\alpha\beta$ ERKO) 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 ER α females may be mainly due to lack of estrogen-mediated negative feedback on pituitary LH secretion (Couse et al., 2003; Schomberg et al., 1999). ER β 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 ER β 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- α , β , and γ . 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)

(Figure 1.7). To examine whether receptors for steroid hormones are present in the neonatal ovaries, immunocytochemistry was performed on the neonatal ovaries. ER α was found to be expressed in pregranulosa cells and ER β in some oocyte nuclei during cyst breakdown (Chen et al., 2009). For further investigation, neonatal ovaries were treated with ER α or ER β 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_2 is not the source in these species (Senger, 2003). It has already been shown that fetal bovine ovaries can synthesize E_2 and P_4 and the levels subside when primordial follicles begin to form (Nilsson and Skinner, 2009; Yang and Fortune, 2008).

Neonatal treatment with P_4 can also give rise to MOFs in adult females (Iguchi et al., 1986). Neonatal treatment of mice and rat ovaries with P_4 reduces cyst breakdown and primordial follicle formation (Chen et al., 2007; Kezele and Skinner, 2003). P_4 can be converted to E_2 and its effect could be exerted either directly or via conversion to E_2 . To investigate that possibility, a non-metabolizable analog of P_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_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 be due to its conversion to E_2 . One study indicated that inhibiting E_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_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 E₂ 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 E₂ 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). ER α and ER β knockout mice show no defects on cyst breakdown and early follicle development and thus the role of E₂ during fetal ovarian development is still unclear (Couse et al., 1999). LH levels are found to be elevated in ER α and ER β 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 E₂ 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 E₂ 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 (3 β HSD) is present both in microsomes and mitochondria of mouse ovaries (**Figure 1.8**). Six different isoforms of 3 β HSD are present in mice (Abbaszade et al., 1997). During the follicular phase of the estrous cycle, the 3 β HSD 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 3 β HSD is preferentially inserted into the mitochondrial membrane after LH stimulation (Chapman et al., 2005). 3 β HSD uses NAD⁺ as cofactor for converting pregnenolone and dehydroepiandrosterone (DHEA) to progesterone and androstenedione respectively (Chapman et al., 2005). 3 β HSD I is expressed in the gonads and adrenal

glands of adult mouse and synthesizes adrenal and gonadal steroid hormones. 3β HSD II and III are expressed in the liver and kidney. 3β HSD 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 3β HSD is 80 % higher than that of microsomal 3β HSD (Chapman et al., 1992). During the other three stages of estrus, microsomal 3β HSD has the highest specific activity (Chapman et al., 1992). The rate-limiting step in steroidogenesis is considered to be cholesterol side-chain cleavage enzyme $P450_{\text{sc}}$ (Miller, 1995). It is found that 3β HSD and $P450_{\text{sc}}$ have a strong binding affinity for each other and are simultaneously synthesized (Chapman et al., 2005). 3β -HSD and $P450_{\text{sc}}$, 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 3β HSD and $P450_{\text{sc}}$ 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 $P450_{\text{sc}}$ 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).

1.11. Figures

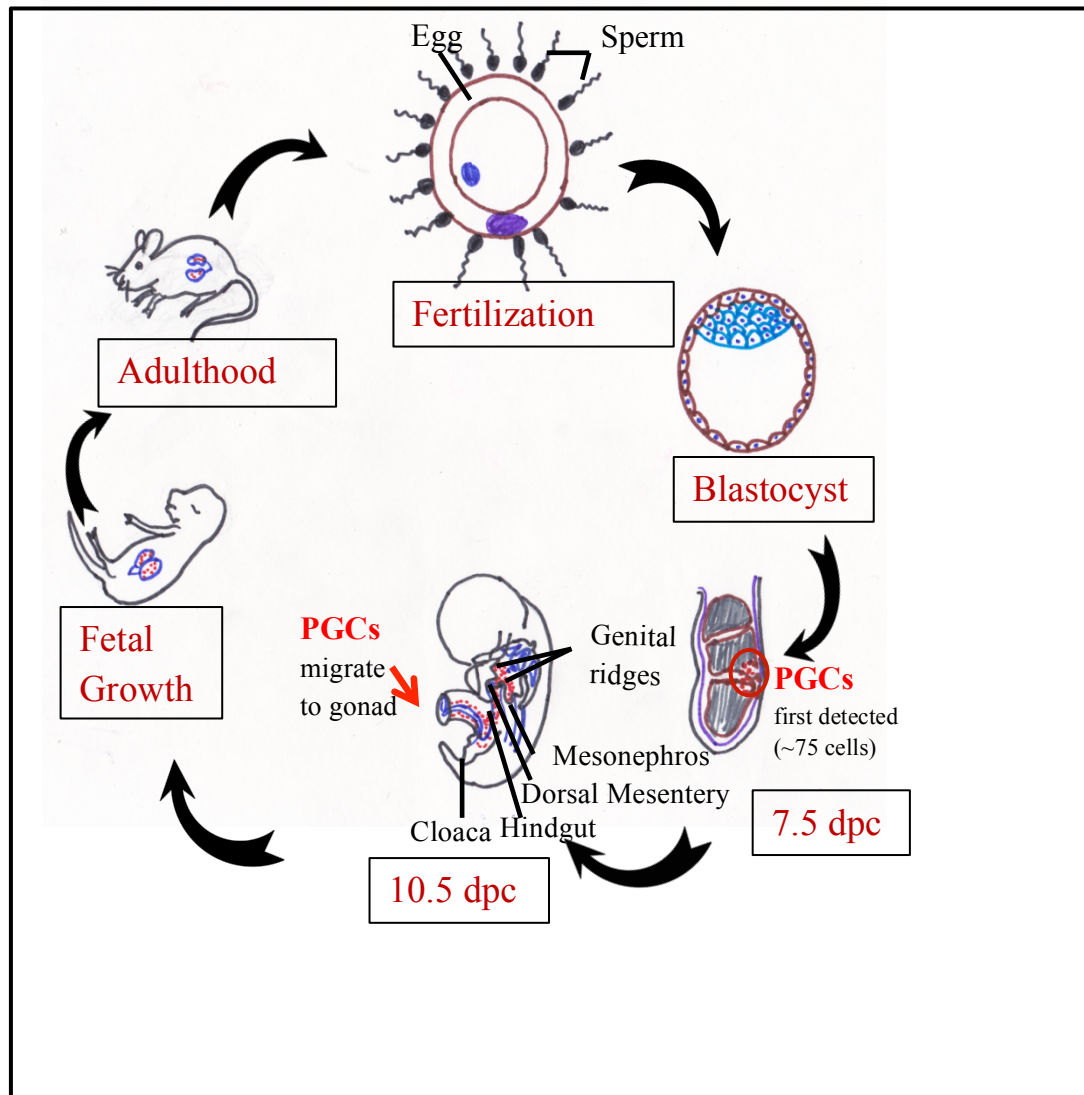


Figure 1.1. Migration and development of Primordial Germ Cells (PGCs) in mice.

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.

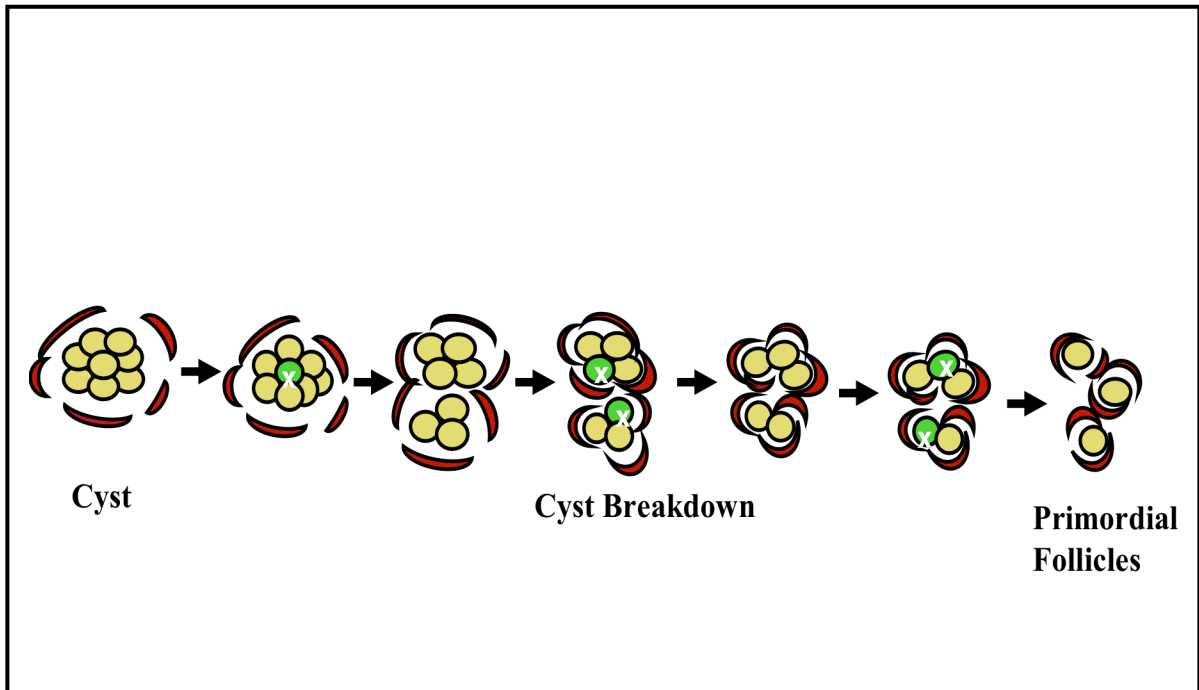


Figure 1.2. Cyst breakdown and germ cell attrition. Within each cyst, surviving oocytes are represented by the yellow color; dying oocytes are represented in green. Along with cyst breakdown, two-thirds of the oocytes within each cyst dies and one-third survives to form primordial follicles. The primordial 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

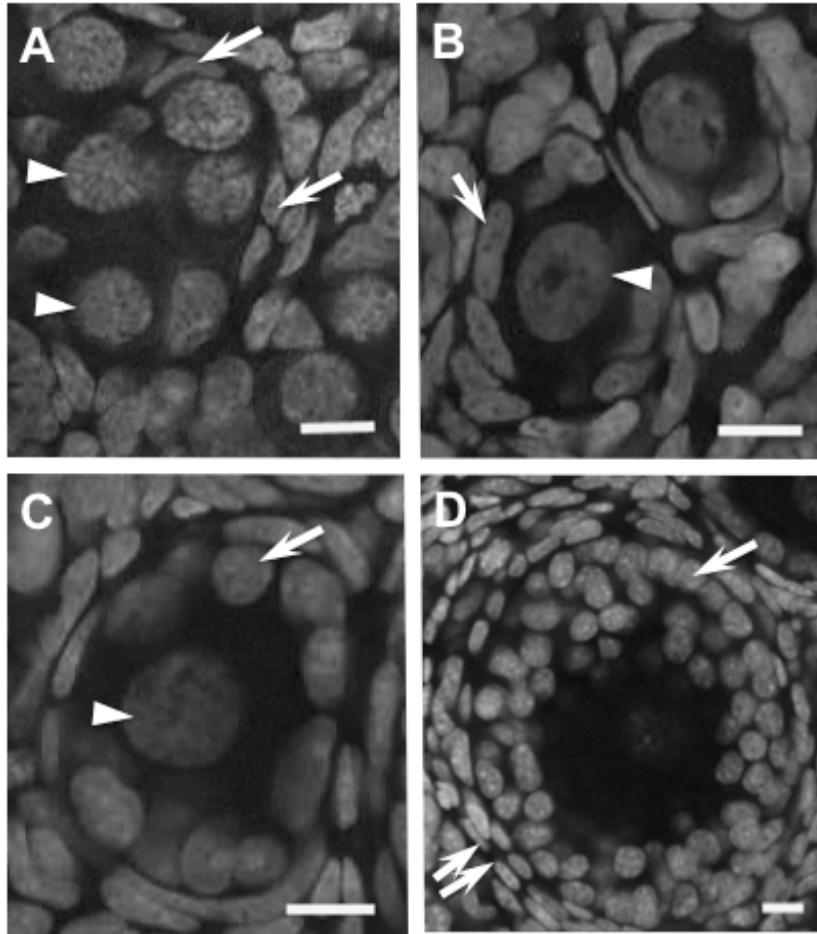


Figure 1.3. Stages of follicle development. Representative confocal sections of germ cell cysts and ovarian follicles. Nuclei of oocytes and granulosa (somatic) cells are labeled with a nuclear marker, propidium iodide. **A)** A germ cell cyst showing several oocyte nuclei sharing the same cytoplasm. **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= 10 μ m.(Pepling, 2013)

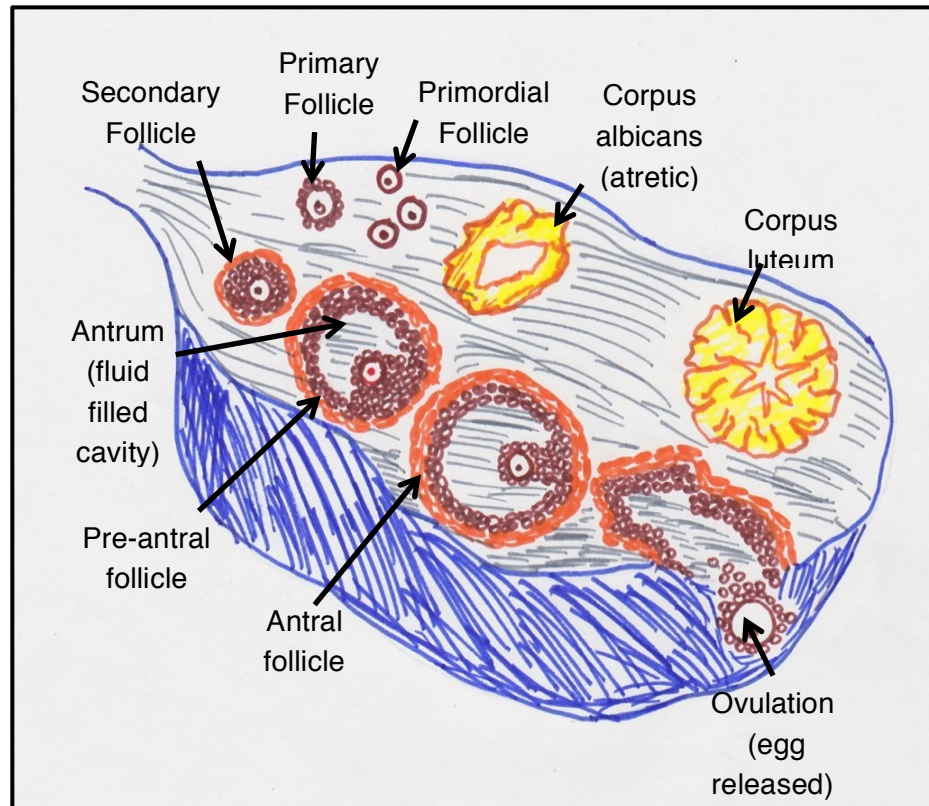


Figure 1.4. Structure of an adult mammalian ovary. Stages of follicle development: primordial follicle (oocyte surrounded by a flattened layer of granulosa cells) → primary follicle (oocyte surrounded by a cuboidal layer of granulosa cells) → secondary follicle (oocyte surrounded by several layers of 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) → the follicular thecal and granulosa cells and secretes progesterone) (Niswender, 2002; Vegetti and Alagna, 2006)

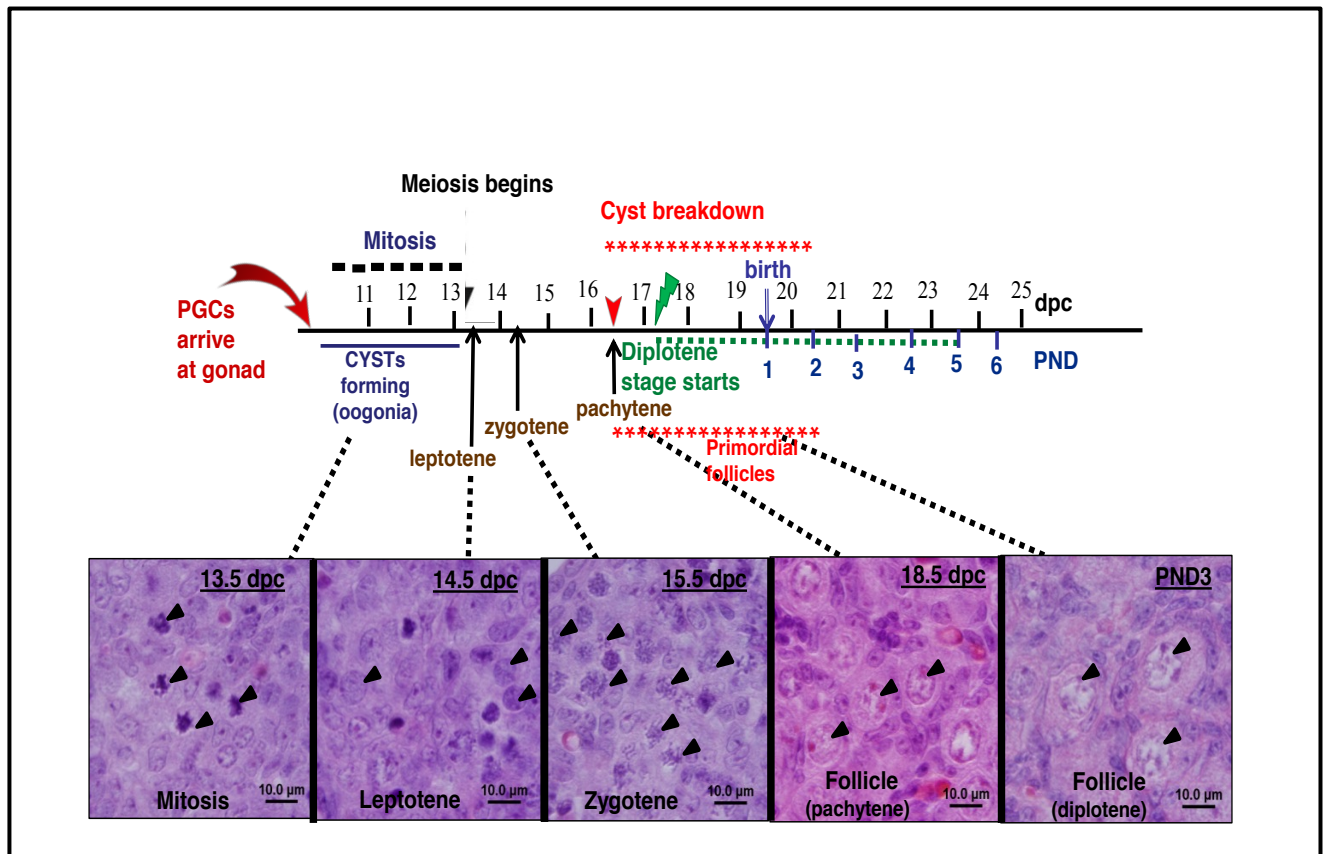


Figure 1.5. Early timeline of fetal mouse oogenesis. PGCs migrate to the gonad at 10.5 dpc. They divide by mitosis without undergoing cytokinesis to form cysts. They are termed as oogonia. They start entering meiotic prophase I from 13.5 dpc onwards. Leptotene, zygotene and pachytene begin approximately 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.)

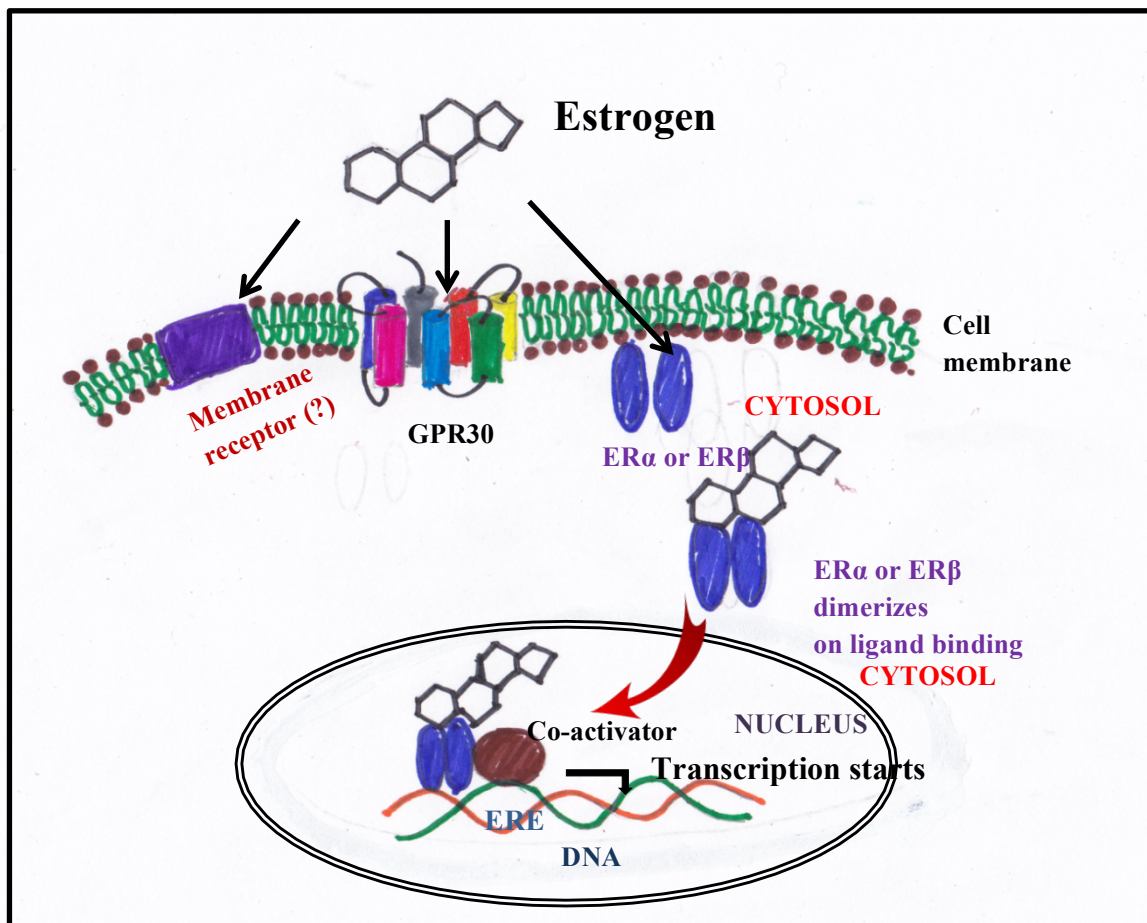


Figure 1.6. Estrogen (E_2) can signal through several different receptors. Membrane-bound G-protein coupled receptor (GPR30), a putative membrane receptor (not yet characterized) and cytosolic ER α or ER β (which are transcription factors). In the classical steroid signaling pathway, ligand (E_2) binds to cytosolic ER α or ER β 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).

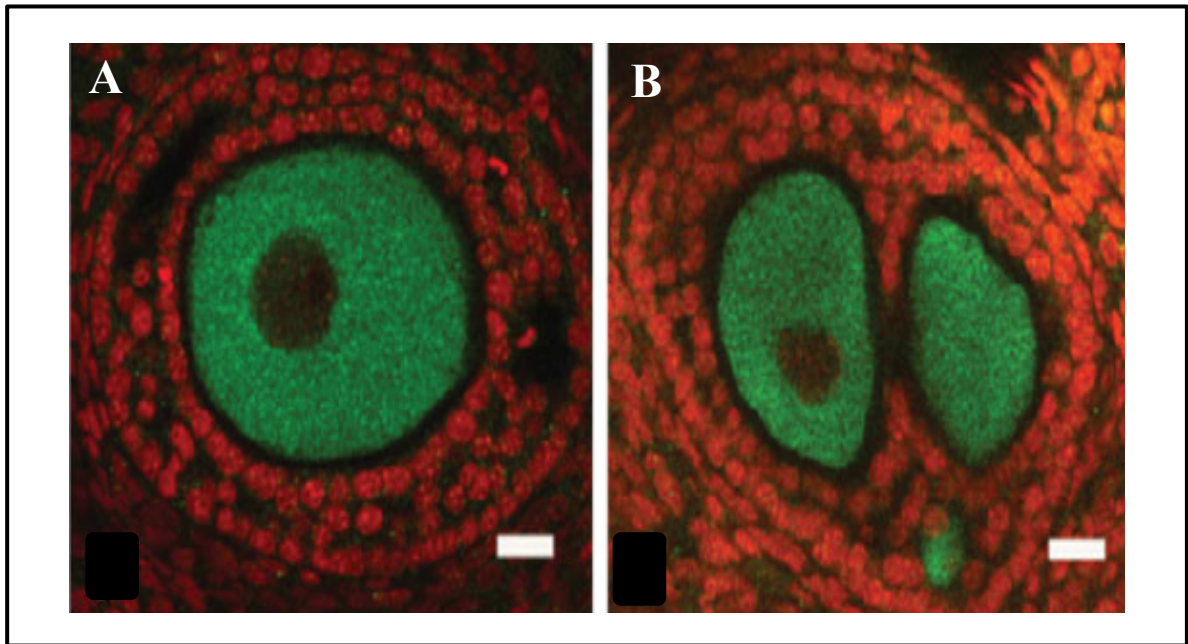


Fig 1.7. Formation of Multiple Oocyte Follicles (MOF). A) A normal secondary follicle with an oocyte surrounded by several layers of granulosa cells B) Three oocyte nuclei surrounded by the same layers of granulosa cells forming a MOF. Oocytes are labeled with an oocyte marker VASA (green) and propidium iodide (red) is used to label nuclei. Scale bar=20 μ m (Pepling, 2006).

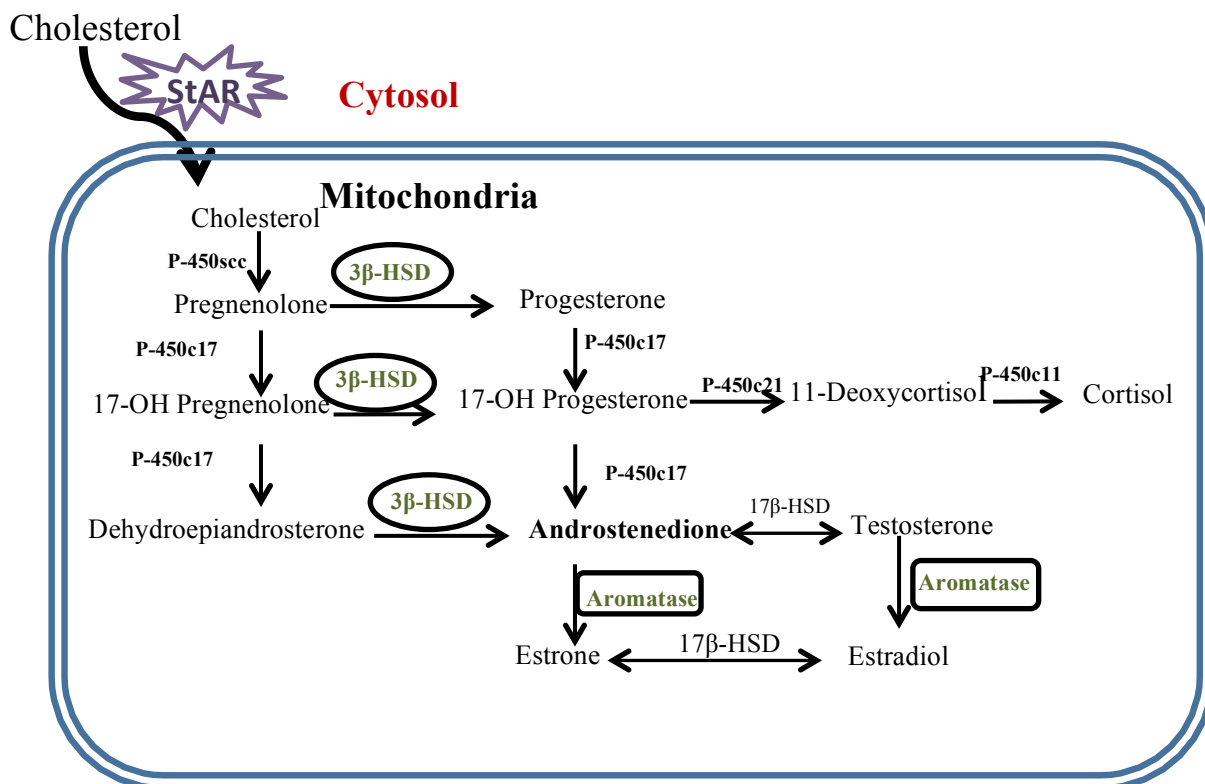


Figure 1.8. Steroid Synthesis Pathway. Cholesterol is shuttled across the mitochondrial membrane by steroidogenic acute regulatory protein (StAR), the rate-limiting step for steroid synthesis. On entering the mitochondria, cholesterol is acted by side chain cleavage P450 enzyme (P-450_{scc}) to form pregnenolone. 3 β HSD 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 E₂ and P₄ synthesis, aromatase and 3βHSD respectively. Finally, we sought to determine the role of E₂ and P₄ synthesis during the perinatal period by inhibiting the enzymes- aromatase and 3βHSD 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 M E₂, 10⁻⁶ M P₄ and 10⁻⁶ M E₂ + 10⁻⁶ M P₄.

Chapter 2

Source of steroid hormone signaling regulating fetal mouse oocyte development

A part of this chapter has been accepted with revision as “*Source of steroid hormone signaling regulating fetal mouse oocyte development*” authored by Sudipta Dutta, Connie J Mark-Kappeler, Patricia B Hoyer and Melissa E. Pepling in *Biology of Reproduction*, April, 2014.

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 (P₄) but not estradiol (E₂) 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 E₂ levels are rising at the time of primordial follicle formation and in cows levels are low prior to follicle formation suggesting maternal circulating E₂ is not the source of E₂ in these species. In addition, fetal bovine ovaries produce E₂ and P₄, 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 ($\lambda= 260/280$ 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). 2µl 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 μ g protein/lane) for each time point and subsequently electroblotted onto Immobilon-PVDF membranes (Millipore). For 3 β 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 β 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 β 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 ChemiDocTM 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-3 β HSD (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-3 β HSD 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 μ M 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 μ m x 134.7 μ m consisting of four optical sections at four different depths of the ovary with each optical section 15 μ m 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 \pm 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_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_2 levels did not drop until the day of birth which is two days after cysts begin to break down. P_4 levels were steady from 13.5 to 16.5 dpc and began to drop at 17.5 dpc. Thus unlike E_2 , the drop in P_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_2 levels were high at 17.5 dpc and continually dropped until birth. P_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 β 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 β 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 β 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 β 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 β 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 β HSD was observed in all four samples.

To determine what cell types express aromatase and 3 β 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 β HSD protein was found in all ovarian cells at 15.5 dpc (**Figure 2.4A-C**). At 16.5 dpc, 3 β 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 3 β HSD, the protein is detected in oocyte cytoplasm with a very speckled appearance. The speckles are likely mitochondria as 3 β HSD 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 3 β HSD and either mitochondrial marker (**Figure 2.5A-D**) or an ER marker (**Figure 2.5E-H**). While there is some overlap of 3 β HSD 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 E₂ and P₄ production in the fetal ovary

Our hypothesis is that E₂ and P₄ produced in fetal ovaries are important for regulating cyst breakdown. To test this we examined the effects of blocking E₂ and P₄ production in organ culture. We expected that cyst breakdown and associated oocyte loss would occur prematurely when E₂ or P₄ synthesis was inhibited. We used a series of doses of the aromatase inhibitor, letrozole to decrease the production of E₂. The IC₅₀ of letrozole is 2×10^{-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 IC₅₀), 10^{-8} M (half of the IC₅₀), 10^{-6} M (50 times the IC₅₀) and 10^{-4} M (5,000 times the IC₅₀) letrozole. Ovaries were collected at 16.5 dpc, when E₂ 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 P₄ production we used trilostane, a 3 β HSD inhibitor (**Figure 2.8**). The IC₅₀ of trilostane in rat sciatic nerves has been reported as 4×10^{-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 3β HSD, 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 3β HSD 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, 3 β HSD mRNA levels were high from 13.5 to 15.5 dpc and then declined. In the steroid biosynthetic pathway, 3 β HSD acts upstream of aromatase and synthesizes the precursor for E₂ production (Miller and Auchus, 2011). High levels of 3 β HSD from 13.5 to 15.5 dpc may allow the synthesis of E₂ 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.

E₂ 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 P₄ while present did not significantly change across timepoints. Likewise, P₄ levels produced by fetal bovine ovaries in culture did not fluctuate over time (Yang and Fortune, 2008). However, in studies where P₄ 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 E₂ and P₄ 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 E₂ and P₄ 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.

2.6. Figures

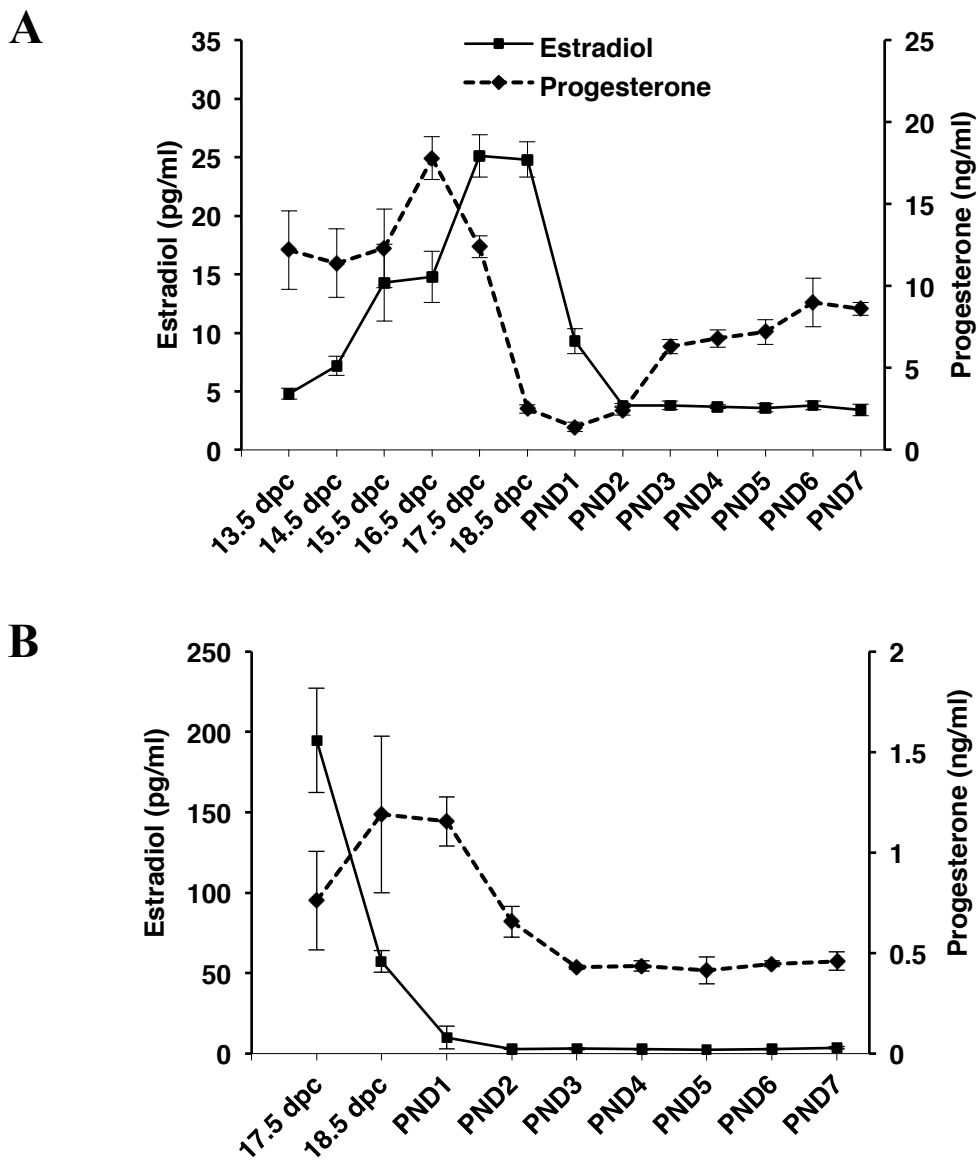


Figure 2.1. Maternal and perinatal circulating levels of E_2 and P_4 : (A) Maternal serum levels of E_2 measured in pg/ml and P_4 measured in ng/ml during gestation and after birth ($n \geq 5$). (B) Perinatal serum levels of E_2 measured in pg/ml and P_4 measured in ng/ml ($n=3$). Data are presented as the mean \pm SEM.

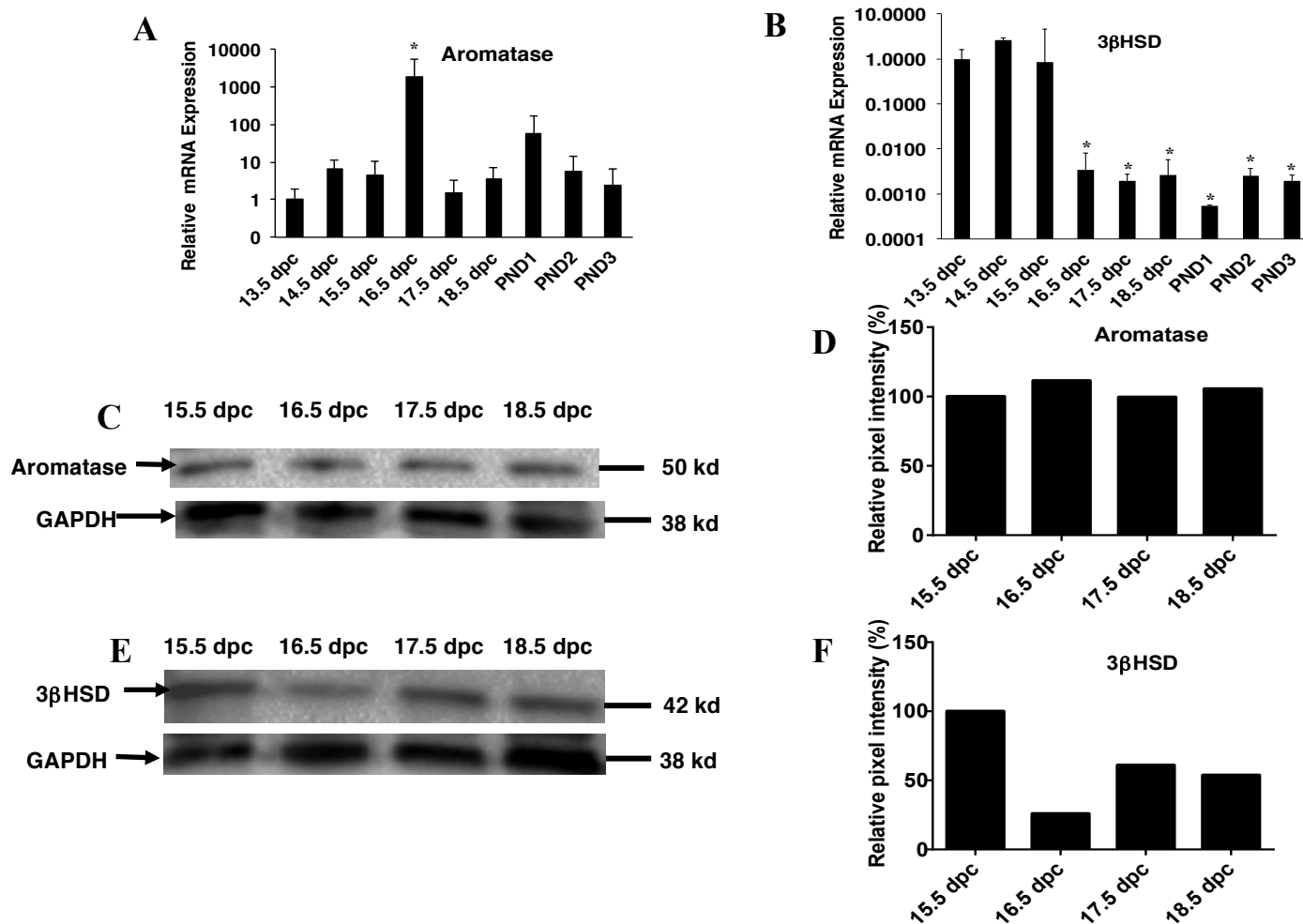


Figure 2.2. mRNA and protein levels of steroidogenic enzymes in perinatal mouse ovaries: Aromatase (A) and 3βHSD (B) mRNA levels during the perinatal period. Levels of aromatase and 3βHSD mRNAs are normalized to the levels of mRNA for a housekeeping gene, β-actin in the same sample. The normalized values 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%.

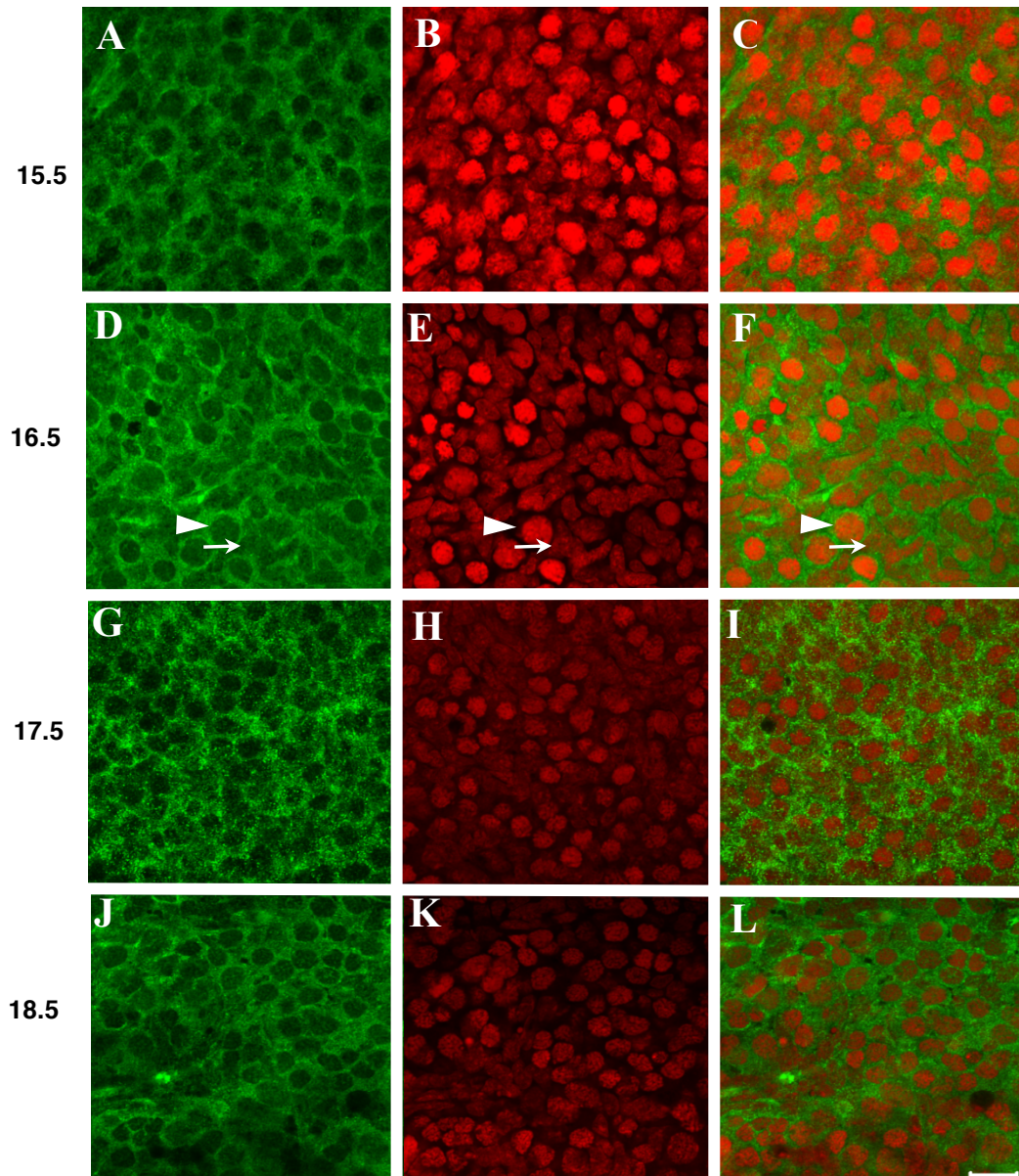


Figure 2.3. Expression of aromatase protein in the fetal ovary.

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 .

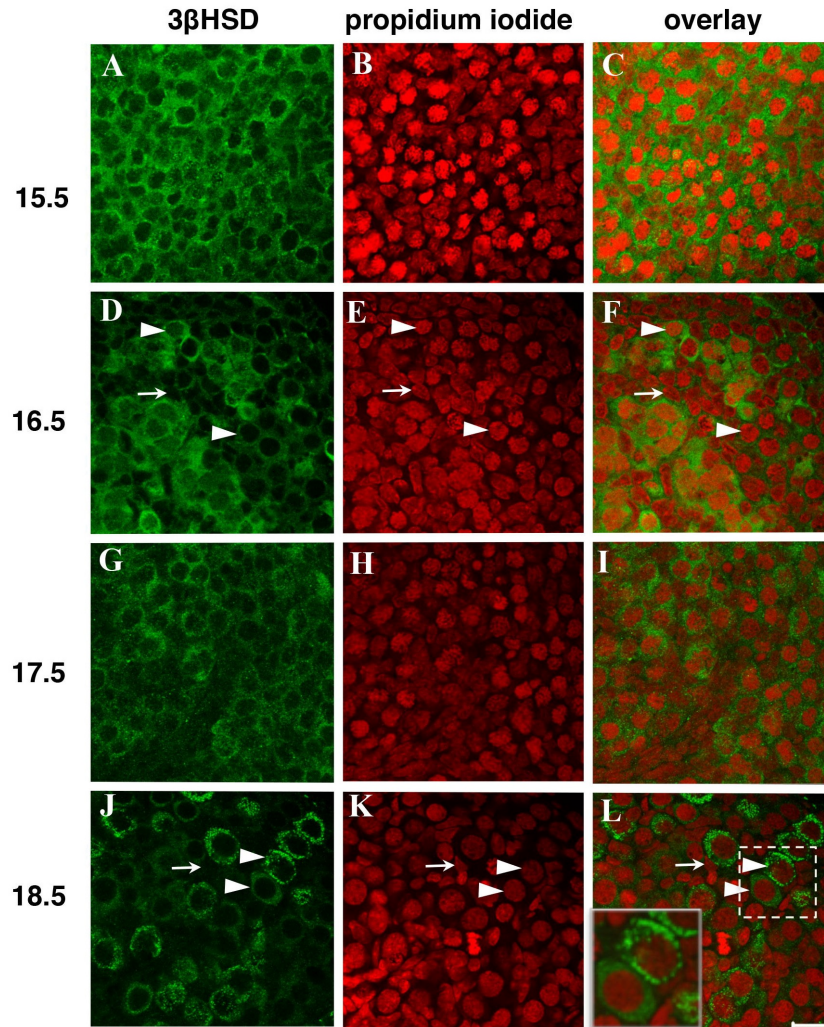


Figure 2.4. Expression of 3 β HSD protein in the fetal ovary. Expression of 3 β HSD protein in the fetal ovary. Confocal sections from 15.5 dpc (A–C), 16.5dpc (D–F), 17.5 dpc (G–I) and 18.5 dpc (J–L) ovaries labeled for 3 β HSD (green) (A, D, G and J) and thenuclear marker propidium iodide (red) (B, 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, 20 μ m. Inset shown in panel L is an enlarged view of the area within the white dashed box.

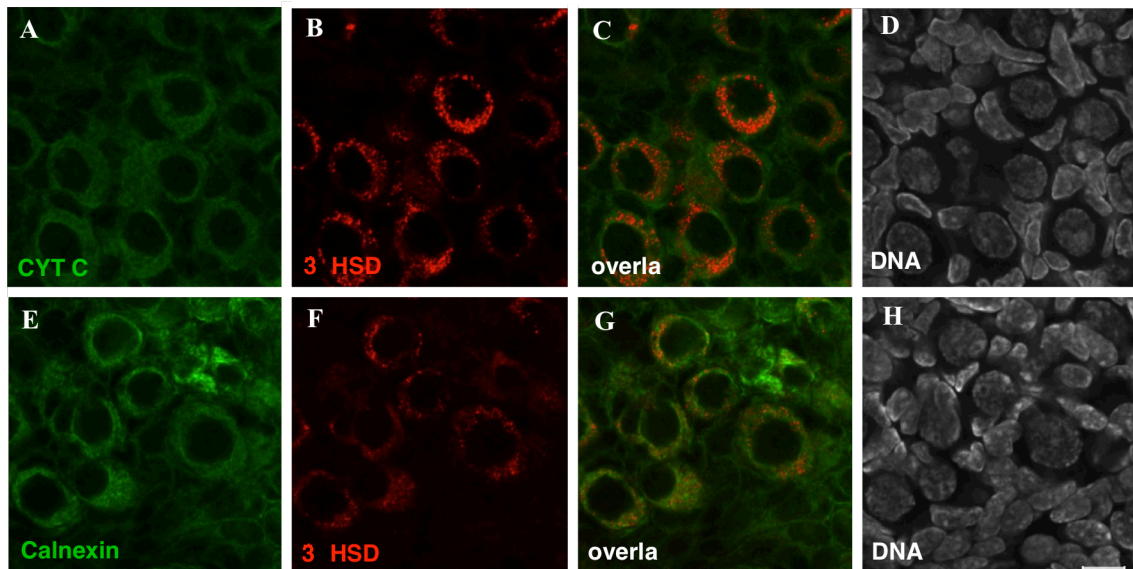


Figure 2.5. Colabeling of 3 β HSD and mitochondria or ER. Confocal sections from a PND6 ovary (A-D) labeled for the mitochondrial marker CYTC (green) (A), 3 β HSD (red) (B), overlay (C) and DNA marker TOTO3 (white) (D). Confocal sections a PND5 ovary (E-H) labeled for the ER marker Calnexin (green) (E), 3 β HSD (red) (F), overlay (G) and the DNA marker TOTO3 (white) (H). Scale bar, 10 μ m.

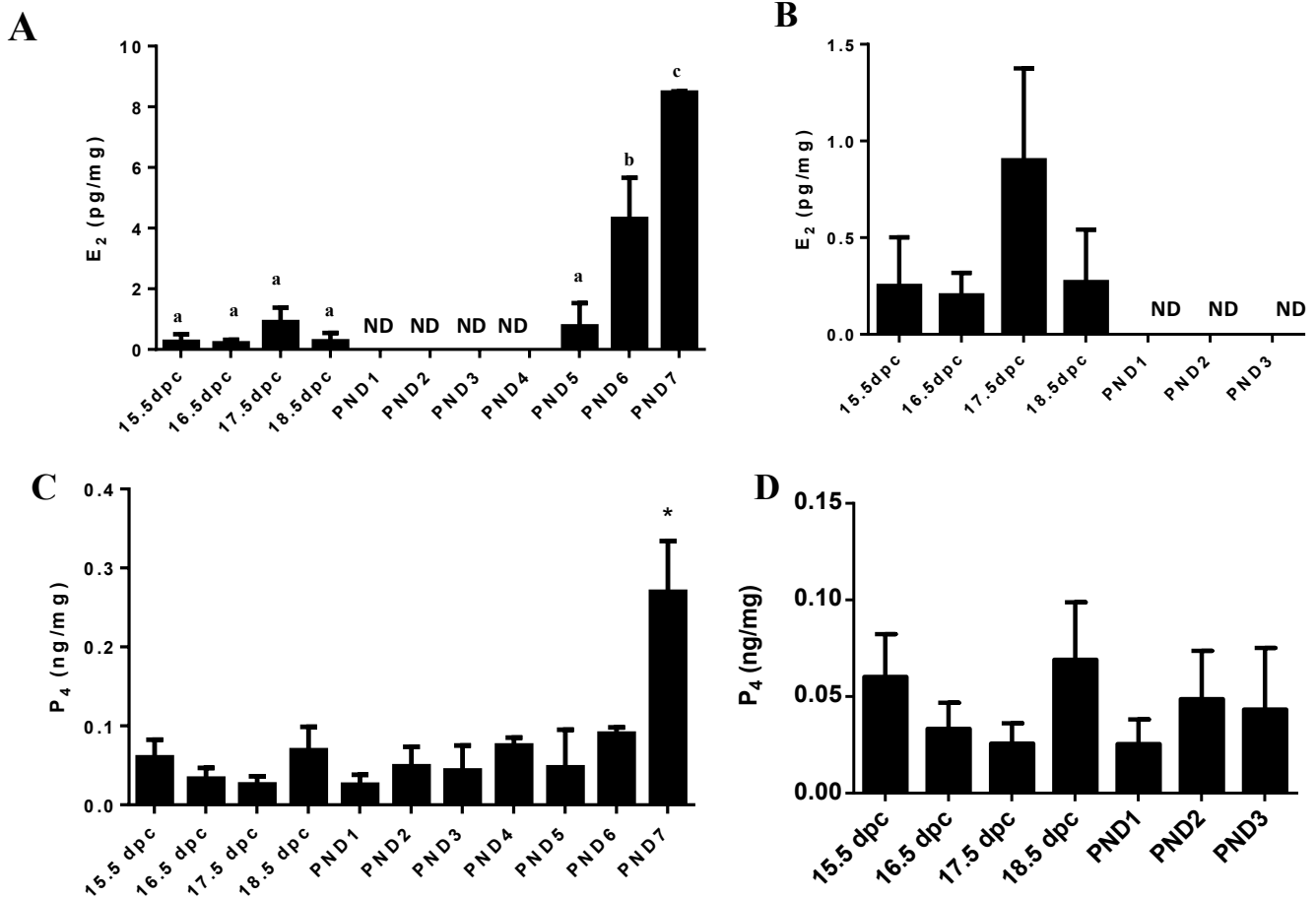


Figure 2.6. Concentration of E₂ and P₄ in fetal and neonatal ovaries.

(A) E₂ levels (pg/mg protein) in ovaries from 15.5 dpc to PND7 (B) E₂ levels (pg/mg protein) in ovaries from 15.5 dpc to PND3 (C) P₄ levels (ng/mg protein) in ovaries from 15.5 dpc to PND7 and (D) P₄ levels (ng/mg protein) in ovaries from 15.5 dpc to PND3 (n=3, each n~ 300 ovaries).

Data are presented as the mean \pm 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, $P < 0.05$).

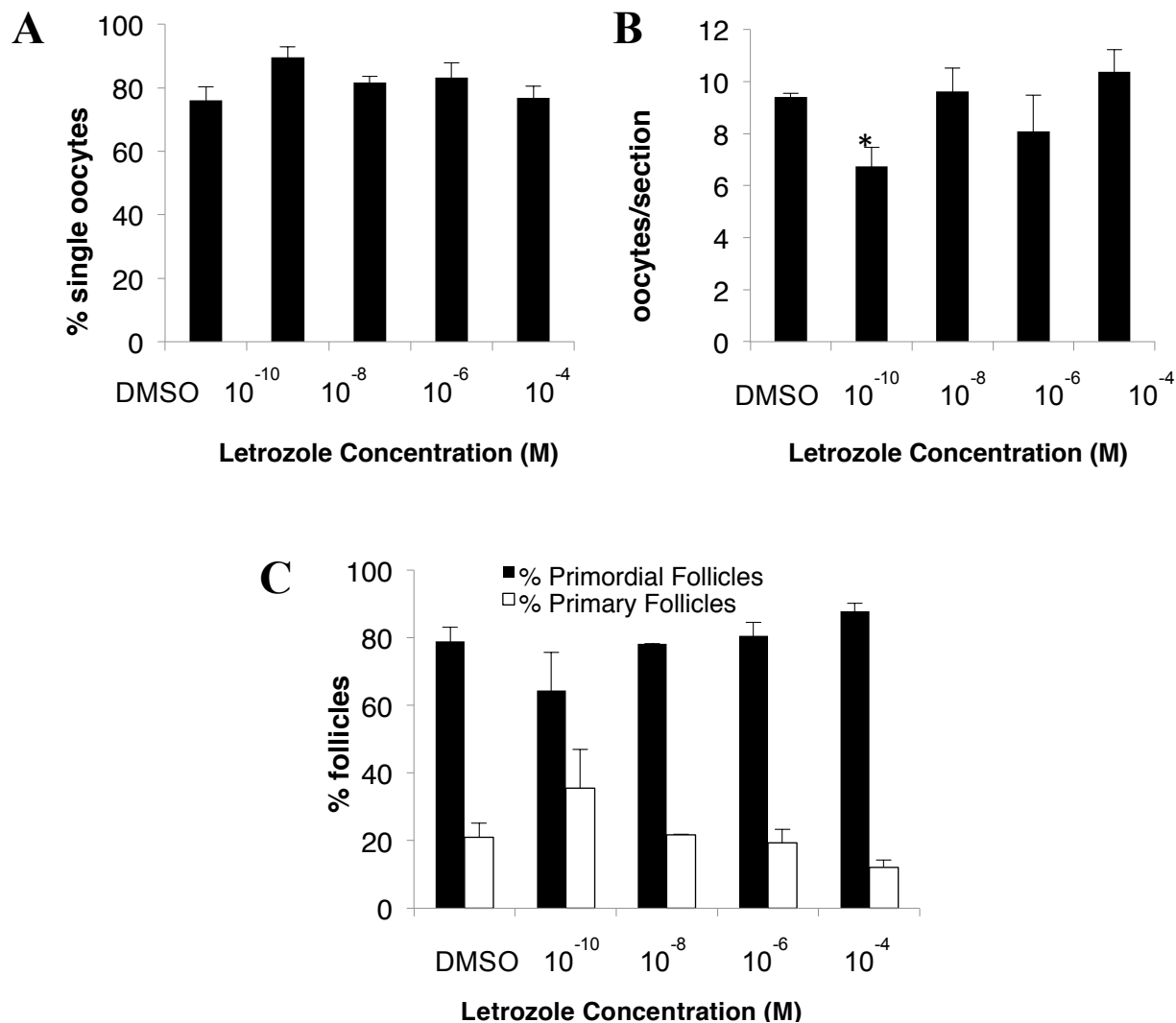


Figure 2.7. Effects of inhibiting aromatase in fetal ovaries.

(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 \pm S.E.M. *Indicates a significant difference between control and treated ovaries (One-way ANOVA followed by Dunnett's post hoc test, $P < 0.05$). $n \geq 5$ ovaries per group.

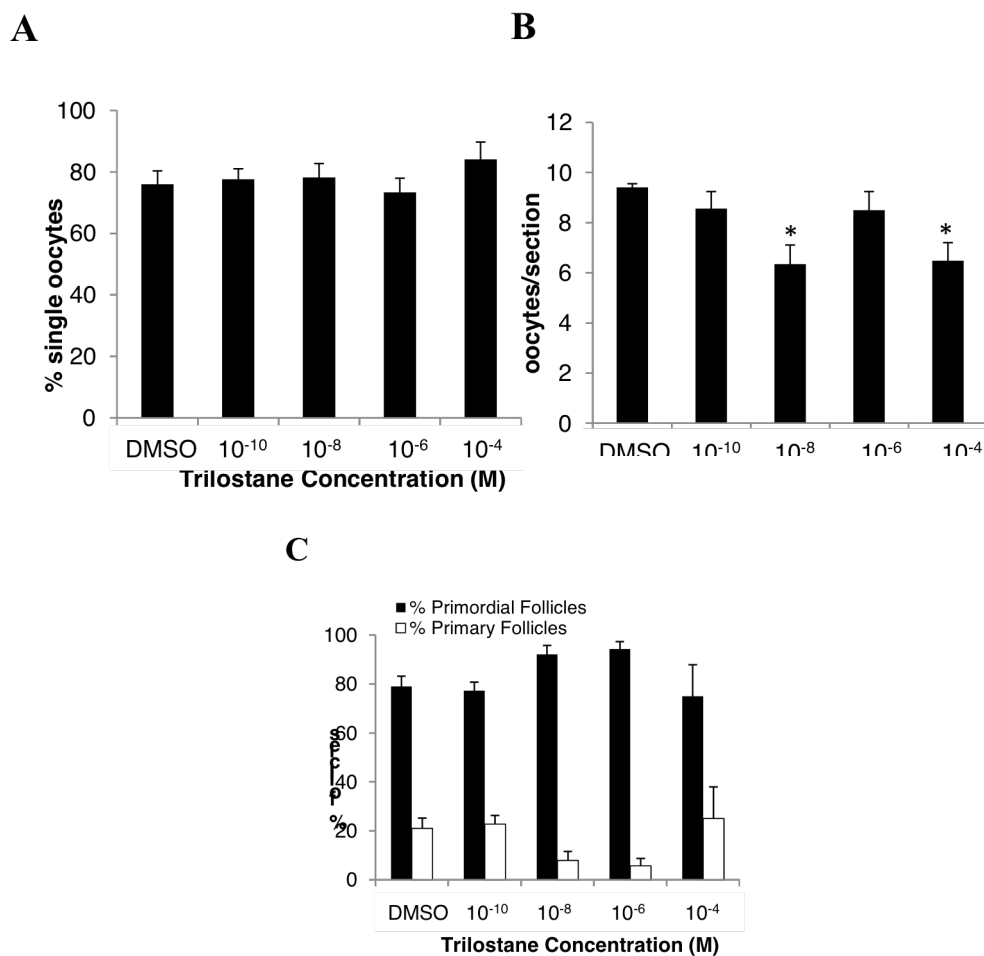


Figure 2.8. Effects of inhibiting 3βHSD in fetal ovaries.

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

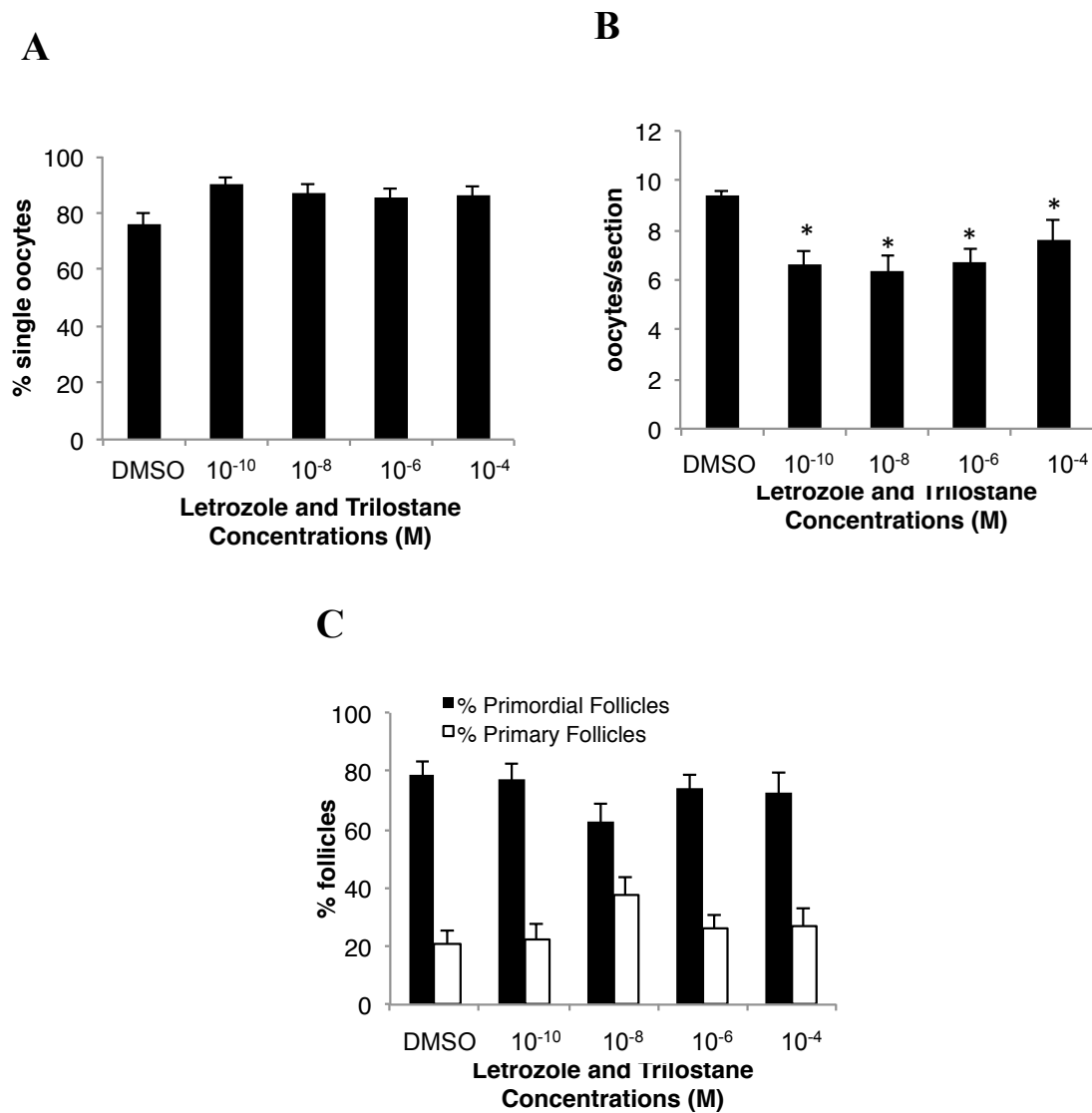


Figure 2.9. Effects of inhibiting aromatase and 3 β HSD in fetal ovaries.

(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 3 β HSD inhibitor trilostane or in control media for 5 days. Data are presented as the mean \pm S.E.M.

*Indicates a significant difference between control and treated ovaries (One-way ANOVA followed by Dunnett's post hoc test, $P < 0.05$). $n \geq 5$ 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

follicles 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 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 5th 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 ($\lambda= 260/280$ 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 μ M 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. E₂ and P₄ 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 E₂, P₄ or both hormones at 10⁻⁶ 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 \pm 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_2 and P_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 \pm 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 E₂, 10^{-6} M P₄ or both 10^{-6} M E₂ and 10^{-6} M P₄. 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 P₄ 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 E_2 and P_4 on progression of oocytes to the diplotene stage. Surprisingly, P_4 but not E_2 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.

3.6. Table*Table 3.1. Analysis of meiotic stage within 5 individual germ cell cysts*

| Cyst | # of oocytes at pre-diplotene | # of oocytes at diplotene | Total # of oocytes |
|--------|-------------------------------|---------------------------|--------------------|
| Cyst 1 | 4 | 1 | 5 |
| Cyst 2 | 1 | 2 | 3 |
| Cyst 3 | 1 | 1 | 2 |
| Cyst 4 | 1 | 1 | 2 |
| Cyst 5 | 2 | 3 | 5 |
| Total | 9 | 8 | 17 |

3.7. Figures

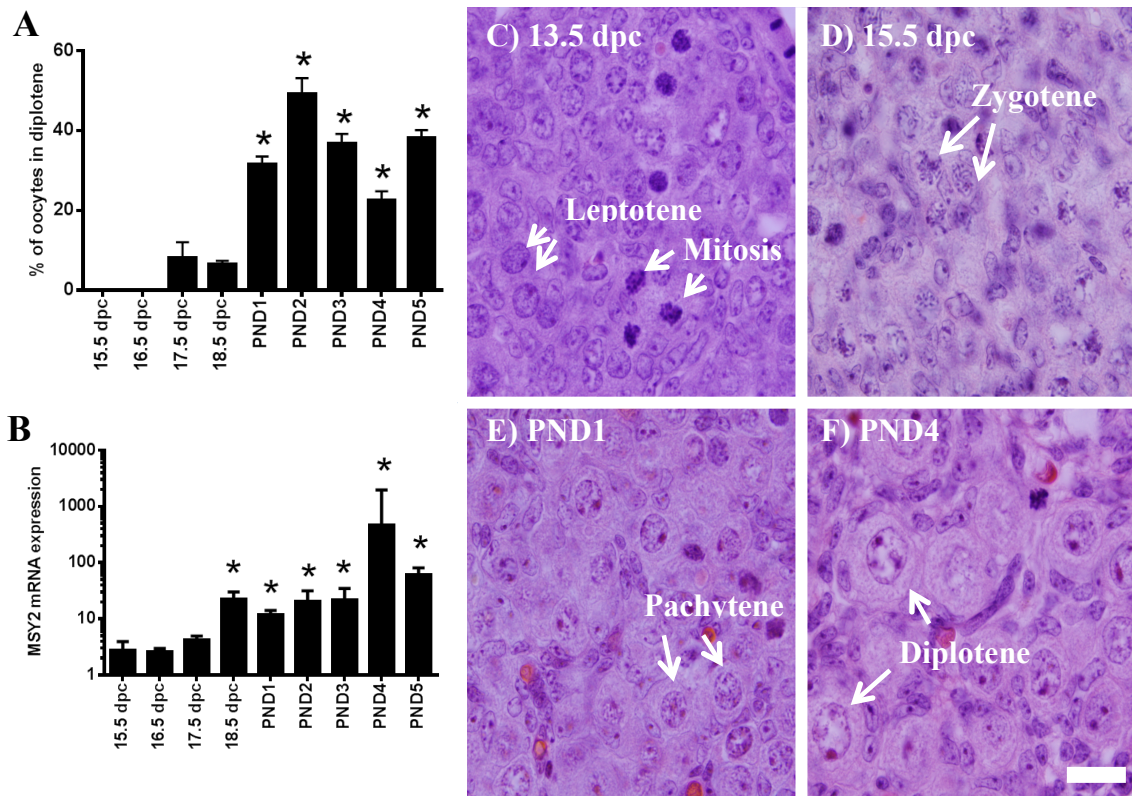


Figure 3.1. Diplotene oocytes first detected at 17.5 dpc. (A) Percentage of oocytes at the diplotene stage meiotic prophase I (\pm SEM) in perinatal mouse ovaries from 15.5 dpc to PND5. Asterisks indicate a significant difference ($P < 0.05$; $n=4-5$ ovaries per time point) as determined by one-way ANOVA 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 μ M.

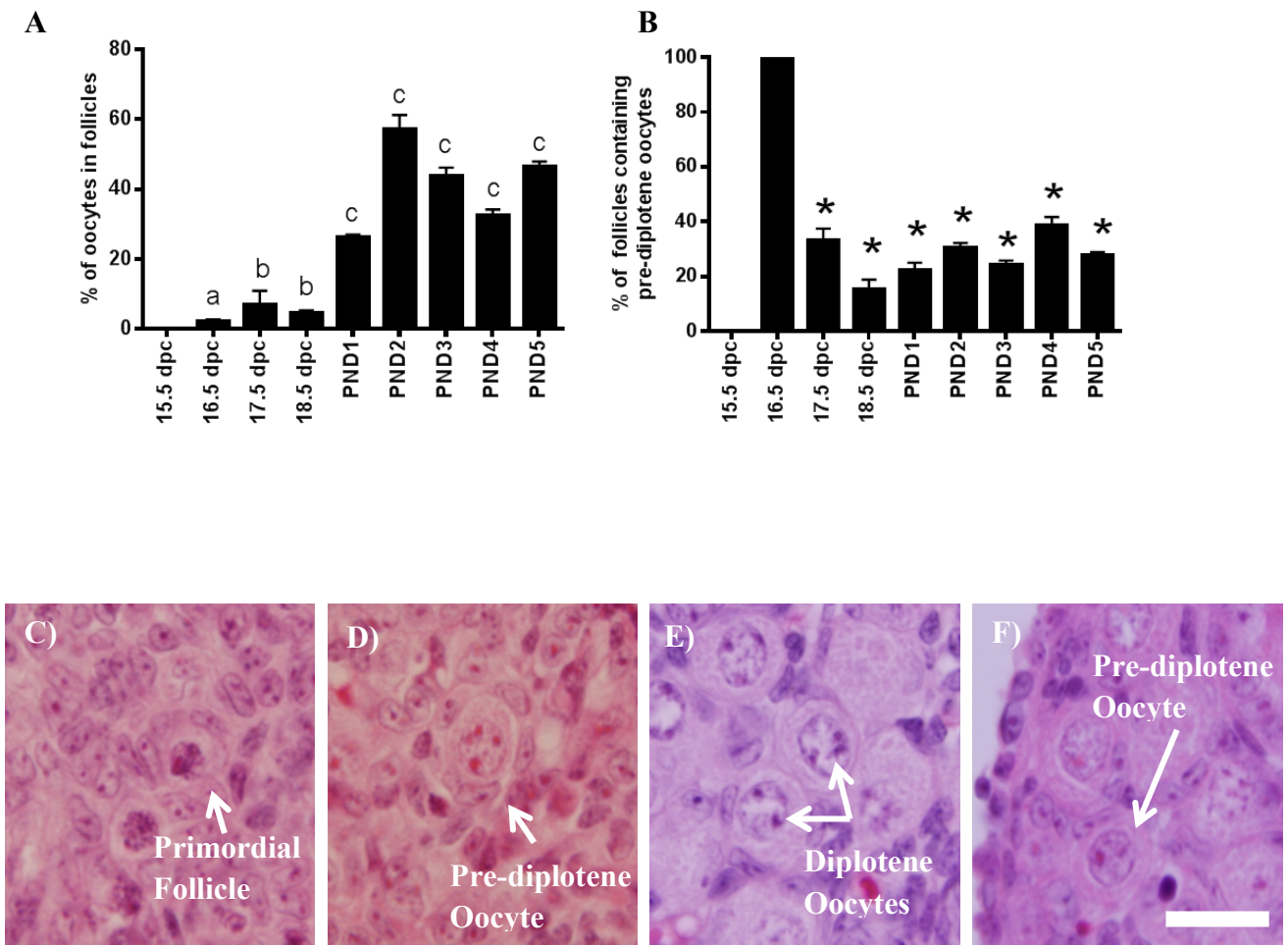


Figure 3.2. Follicle formation and meiotic progression in perinatal mouse ovaries.

(A) Percentage of oocytes in follicles (\pm 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 (\pm 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 μ M.

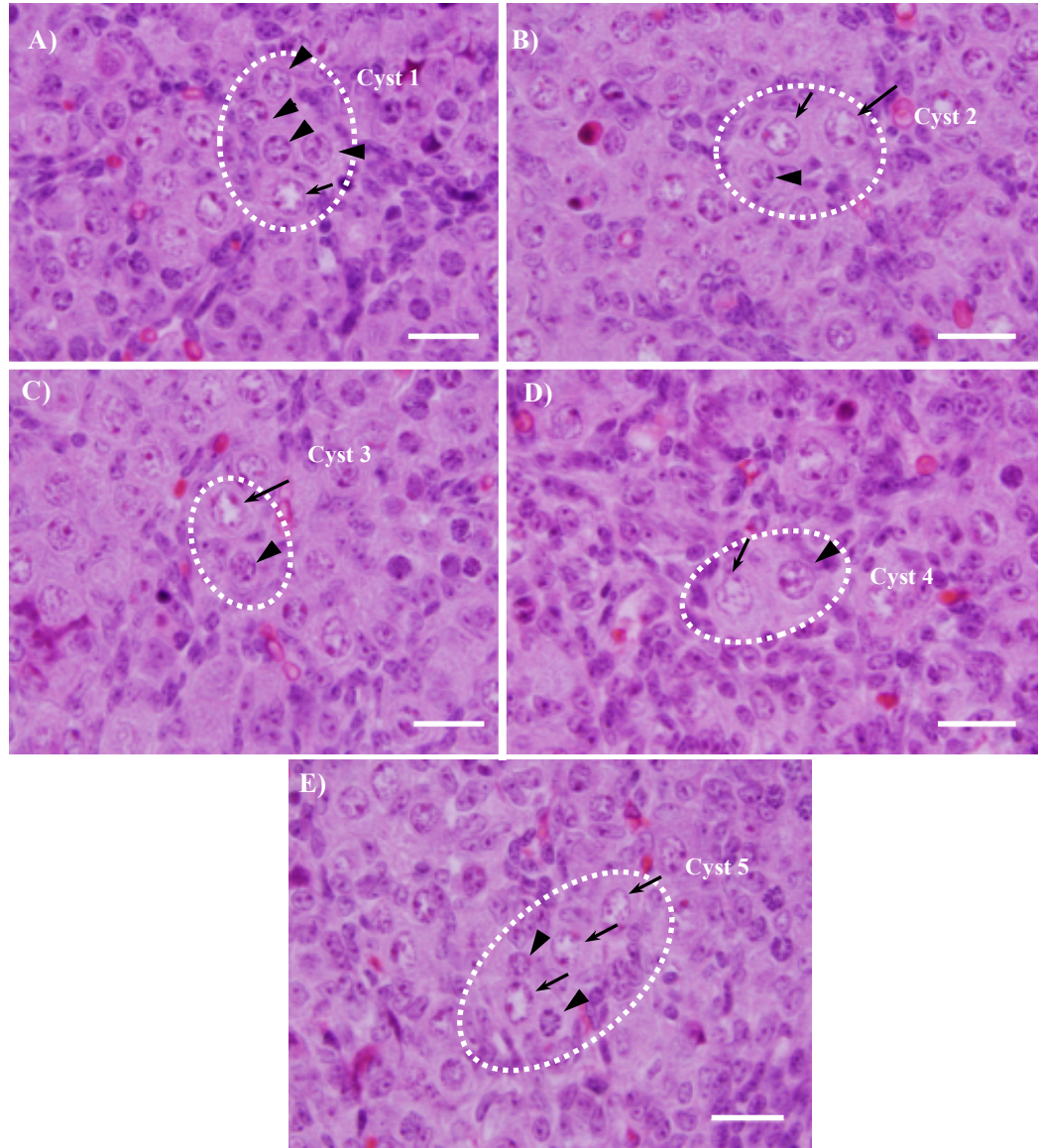


Figure 3.3. Representative histological images showing paraffin sections of fetal mouse ovaries at 18.5 dpc stained with hematoxylin and eosin.

(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 μ M.

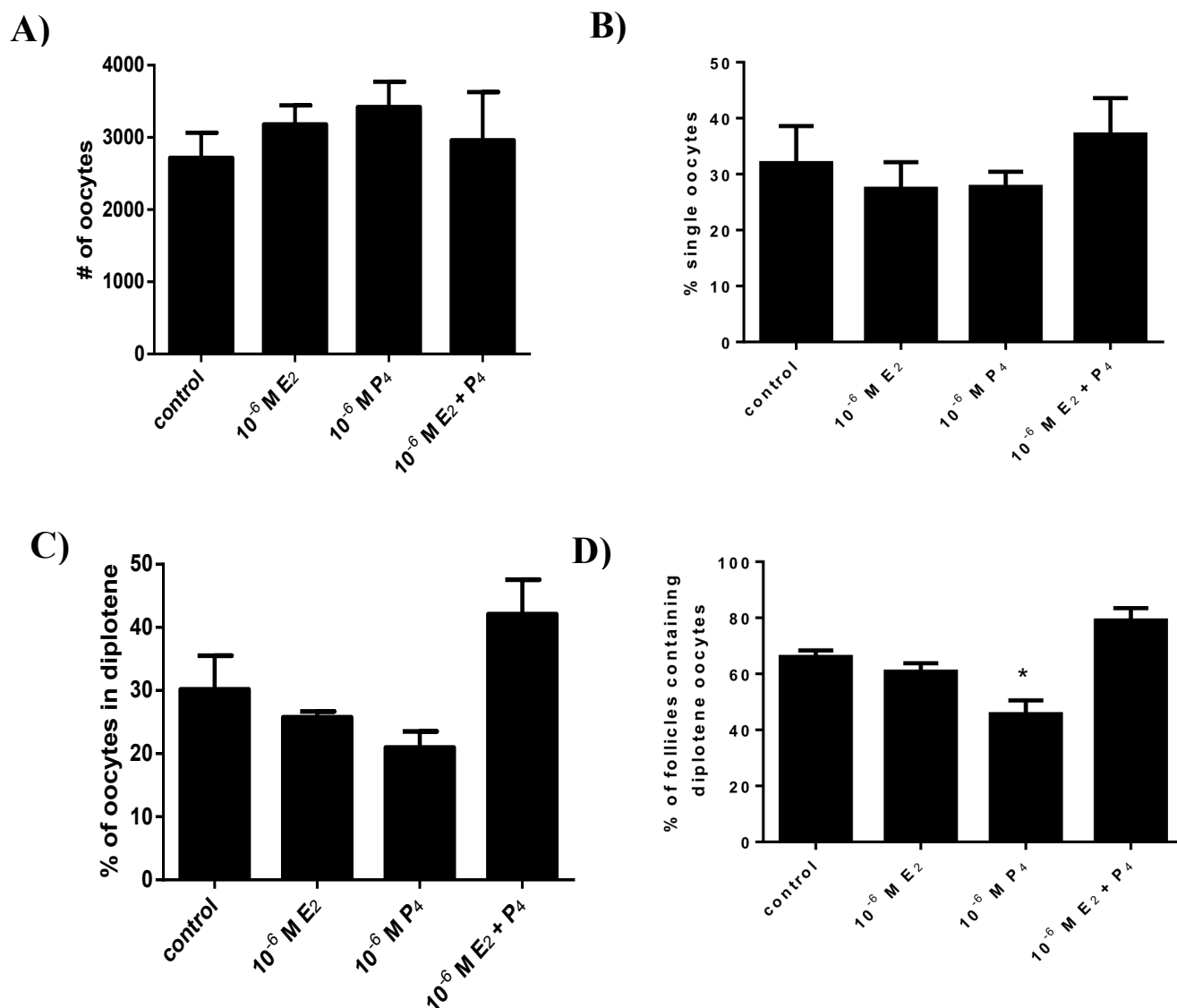


Figure 3.4. Effects of E₂ and P₄ treatment in organ culture on meiotic stage.

(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^{-6} M E₂, 10^{-6} M P₄ or 10^{-6} M E₂ + 10^{-6} M P₄ for 4 days in organ culture. Data are presented as the mean \pm 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 E_2 and P_4 levels during gestation. E_2 levels were considerably low during 13.5dpc to 15.5 dpc when cysts are being actively formed. This suggests that maternal E_2 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 P_4 level is highest at this time-point and it drops after that. The maternal circulating level of E_2 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 3β HSD (both mRNA and protein), were confirmed in the perinatal ovaries. The intraovarian steroid content of the perinatal ovaries were examined by RIA. E_2 levels were highest in the fetal ovaries at 17.5 dpc and then decreased and became undetectable at birth. The levels of P_4 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 an 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 3β HSD 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 E₂ 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 E₂ 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 E₂ 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*⁻ mice. *Stra8* is essential for meiotic entry of oocytes and thus germ cells in ovaries of *Stra8*⁻ 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 E₂ and P₄ 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 (10⁻⁶M E₂, 10⁻⁶M P₄ and 10⁻⁶M E₂ & P₄). Only P₄ had an effect in decreasing the percent of diplotene follicles from 66% in control ovaries to 45.6% in P₄ treated ovaries (P < 0.05). In cattle, both E₂ and P₄ 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 *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 μ l) of the total 400 μ l 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, *in vitro* inhibitor studies in organ culture were carried out with letrozole and trilostane to block aromatase and 3 β HSD 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 heterozygotes (+/-) 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 E₂ synthesis as opposed to wildtype (+/+) ovaries. Whereas, in homozygous knockout (-/-) ovaries, lacking both the copies of aromatase gene, there would be no E₂ 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% E₂ synthesis in perinatal ovaries of heterozygotes (+/-) as opposed to total absence of E₂ synthesis in homozygous knockouts (-/-) and also 100% E₂ synthesis in wildtype (+/+). In *in vitro* conditions, it would also be possible to grow perinatal ovaries of homozygous knockouts (-/-) in presence of exogenous E₂ treatment. By comparing the results of percent of cyst breakdown from such exogenous E₂ 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 E₂ 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 E₂ and P₄ 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.

Bibliography

REFERENCES:

- Abbaszade, I.G., Arensburg, J., Park, C.H., Kasa-Vubu, J.Z., Orly, J., Payne, A.H., 1997. Isolation of a new mouse 3beta-hydroxysteroid dehydrogenase isoform, 3beta-HSD VI, expressed during early pregnancy. *Endocrinology* 138, 1392-1399.
- Baker, T.G., 1963. A quantitative and cytological study of germ cells in human ovaries. *Proc R Soc Lond B Biol Sci* 158, 417-433.
- Baker, T.G., 1972. *Reproductive biology*. Amsterdam: Excerpta Medica.
- Baltus, A.E., Menke, D.B., Hu, Y.C., Goodheart, M.L., Carpenter, A.E., de Rooij, D.G., Page, D.C., 2006. In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nature genetics* 38, 1430-1434.
- Beaumont, H.M., 1962. The radiosensitivity of germ-cells at various stages of ovarian development. *International journal of radiation biology* 4, 581-590.
- Bendel-Stenzel, M.R., Gomperts, M., Anderson, R., Heasman, J., Wylie, C., 2000. The role of cadherins during primordial germ cell migration and early gonad formation in the mouse. *Mechanisms of development* 91, 143-152.
- Bhatnagar, A.S., Hausler, A., Schieweck, K., Lang, M., Bowman, R., 1990. Highly selective inhibition of estrogen biosynthesis by CGS 20267, a new non-steroidal aromatase inhibitor. *The Journal of steroid biochemistry and molecular biology* 37, 1021-1027.
- Borum, K., 1961. Oogenesis in the mouse. A study of the meiotic prophase. *Exp Cell Res* 24, 495-507.

- Britt, K.L., Drummond, A.E., Cox, V.A., Dyson, M., Wreford, N.G., Jones, M.E., Simpson, E.R., Findlay, J.K., 2000. An age-related ovarian phenotype in mice with targeted disruption of the Cyp 19 (aromatase) gene. *Endocrinology* 141, 2614-2623.
- Britt, K.L., Saunders, P.K., McPherson, S.J., Misso, M.L., Simpson, E.R., Findlay, J.K., 2004. Estrogen actions on follicle formation and early follicle development. *Biology of reproduction* 71, 1712-1723.
- Brunet, S., Verlhac, M.H., 2011. Positioning to get out of meiosis: the asymmetry of division. *Human reproduction update* 17, 68-75.
- Buñing, J., 1994. *The Insect ovary: Ultrastructure, previtellogenic growth and evolution*. New York: Chapman and Hall.
- Bullejos, M., Koopman, P., 2004. Germ cells enter meiosis in a rostro-caudal wave during development of the mouse ovary. *Mol Reprod Dev* 68, 422-428.
- Byskov, A.G., 1986. Differentiation of mammalian embryonic gonad. *Physiol Rev* 66, 71-117.
- Byskov, A.G., Lintern-Moore, S., 1973. Follicle formation in the immature mouse ovary: the role of the rete ovarii. *J Anat* 116, 207-217.
- Cai, Z., Stocco, C., 2005. Expression and regulation of progesterin membrane receptors in the rat corpus luteum. *Endocrinology* 146, 5522-5532.
- Casey, M.L., MacDonald, P.C., 1998. Endocrine changes of pregnancy. In: Wilson JD, Foster DW, Kronenberg HM, Larsen PR, eds *Williams textbook of endocrinology*. 9th ed. Philadelphia: WB Saunders; 1259-1271.
- Challoner, S., 1974. Studies of oogenesis and follicular development in the golden hamster 1. A quantitative study of meiotic prophase in vivo. *J Anat* 117, 373-383.

- Chapman, J.C., Polanco, J.R., Min, S., Michael, S.D., 2005. Mitochondrial 3 beta-hydroxysteroid dehydrogenase (HSD) is essential for the synthesis of progesterone by corpora lutea: an hypothesis. *Reprod Biol Endocrinol* 3, 11.
- Chapman, J.C., Waterhouse, T.B., Michael, S.D., 1992. Changes in mitochondrial and microsomal 3 beta-hydroxysteroid dehydrogenase activity in mouse ovary over the course of the estrous cycle. *Biology of reproduction* 47, 992-997.
- Chen, Y., Breen, K., Pepling, M.E., 2009. Estrogen can signal through multiple pathways to regulate oocyte cyst breakdown and primordial follicle assembly in the neonatal mouse ovary. *The Journal of endocrinology* 202, 407-417.
- Chen, Y., Jefferson, W.N., Newbold, R.R., Padilla-Banks, E., Pepling, M.E., 2007. Estradiol, progesterone, and genistein inhibit oocyte nest breakdown and primordial follicle assembly in the neonatal mouse ovary in vitro and in vivo. *Endocrinology* 148, 3580-3590.
- Cherradi, N., Chambaz, E.M., Defaye, G., 1995. Organization of 3 beta-hydroxysteroid dehydrogenase/isomerase and cytochrome P450_{scc} into a catalytically active molecular complex in bovine adrenocortical mitochondria. *The Journal of steroid biochemistry and molecular biology* 55, 507-514.
- Coirini, H., Guezou, M., Delespierre, B., Liere, P., Pianos, A., Eychenne, B., Schumacher, M., Guennoun, R., 2003. Characterization and regulation of the 3beta-hydroxysteroid dehydrogenase isomerase enzyme in the rat sciatic nerve. *Journal of neurochemistry* 84, 119-126.

Conneely, O.M., Kettelberger, D.M., Tsai, M.J., Schrader, W.T., O'Malley, B.W., 1989.

The chicken progesterone receptor A and B isoforms are products of an alternate translation initiation event. *The Journal of biological chemistry* 264, 14062-14064.

Cortvrindt, R., Smitz, J., Van Steirteghem, A.C., 1997. Assessment of the need for follicle stimulating hormone in early preantral mouse follicle culture in vitro. *Human reproduction* 12, 759-768.

Coucovanis, E.C., Sherwood, S.W., Carswell-Crumpton, C., Spack, E.G., Jones, P.P., 1993. Evidence that the mechanism of prenatal germ cell death in the mouse is apoptosis. *Exp Cell Res* 209, 238-247.

Coulam, C.B., Adamson, S.C., Annegers, J.F., 1986. Incidence of premature ovarian failure. *Obstetrics and gynecology* 67, 604-606.

Couse, J.F., Hewitt, S.C., Bunch, D.O., Sar, M., Walker, V.R., Davis, B.J., Korach, K.S., 1999. Postnatal sex reversal of the ovaries in mice lacking estrogen receptors alpha and beta. *Science* 286, 2328-2331.

Couse, J.F., Korach, K.S., 1999. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocrine reviews* 20, 358-417.

Couse, J.F., Yates, M.M., Deroo, B.J., Korach, K.S., 2005. Estrogen receptor-beta is critical to granulosa cell differentiation and the ovulatory response to gonadotropins. *Endocrinology* 146, 3247-3262.

Couse, J.F., Yates, M.M., Walker, V.R., Korach, K.S., 2003. Characterization of the hypothalamic-pituitary-gonadal axis in estrogen receptor (ER) Null mice reveals hypergonadism and endocrine sex reversal in females lacking ERalpha but not ERbeta. *Molecular endocrinology* 17, 1039-1053.

- de Cuevas, M., Lee, J.K., Spradling, A.C., 1996. alpha-spectrin is required for germline cell division and differentiation in the *Drosophila* ovary. *Development* 122, 3959-3968.
- de Vries, F.A., de Boer, E., van den Bosch, M., Baarends, W.M., Ooms, M., Yuan, L., Liu, J.G., van Zeeland, A.A., Heyting, C., Pastink, A., 2005. Mouse Sycp1 functions in synaptonemal complex assembly, meiotic recombination, and XY body formation. *Genes & development* 19, 1376-1389.
- Deng, W., Lin, H., 1997. Spectrosomes and fusomes anchor mitotic spindles during asymmetric germ cell divisions and facilitate the formation of a polarized microtubule array for oocyte specification in *Drosophila*. *Developmental biology* 189, 79-94.
- Di Carlo, A.D., Travia, G., De Felici, M., 2000. The meiotic specific synaptonemal complex protein SCP3 is expressed by female and male primordial germ cells of the mouse embryo. *The International journal of developmental biology* 44, 241-244.
- Dokshin, G.A., Baltus, A.E., Eppig, J.J., Page, D.C., 2013. Oocyte differentiation is genetically dissociable from meiosis in mice. *Nature genetics* 45, 877-883.
- Dupont, S., Krust, A., Gansmuller, A., Dierich, A., Chambon, P., Mark, M., 2000. Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development* 127, 4277-4291.
- Edwards, R.G., Fowler, R.E., Gore-Langton, R.E., Gosden, R.G., Jones, E.C., Readhead, C., Steptoe, P.C., 1977. Normal and abnormal follicular growth in mouse, rat and human ovaries. *Journal of reproduction and fertility* 51, 237-263.
- Elmore, S., 2007. Apoptosis: a review of programmed cell death. *Toxicologic pathology* 35, 495-516.

Eppig, J.J., Vivieros, M.M., Marin-Bivens, C., Fuente, R.D.L., 2004. Regulation of mammalian oocyte maturation. Amsterdam: Elsevier Academic Press.

Evans, C.W., Robb, D.I., Tuckett, F., Challoner, S., 1982. Regulation of meiosis in the foetal mouse gonad. *Journal of embryology and experimental morphology* 68, 59-67.

Fisher, C.R., Graves, K.H., Parlow, A.F., Simpson, E.R., 1998. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the *cyp19* gene.

Proceedings of the National Academy of Sciences of the United States of America 95, 6965-6970.

Fortune, J.E., Eppig, J.J., 1979. Effects of gonadotropins on steroid secretion by infantile and juvenile mouse ovaries in vitro. *Endocrinology* 105, 760-768.

Fowler, P.A., Anderson, R.A., Saunders, P.T., Kinnell, H., Mason, J.I., Evans, D.B., Bhattacharya, S., Flannigan, S., Franks, S., Monteiro, A., O'Shaughnessy, P.J., 2011. Development of steroid signaling pathways during primordial follicle formation in the human fetal ovary. *The Journal of clinical endocrinology and metabolism* 96, 1754-1762.

Franchi, L., Baker, T., 1973. Oogenesis and follicular growth. In: *Human Reproduction, Conception and Contraception*. Eds: E. SE Hafez and TN Evans, Harper & Row, Maryland. 53-83.

Franchi, L.L., Mandl, A.M., 1964. The Ultrastructure of Germ Cells in Foetal and Neonatal Male Rats. *Journal of embryology and experimental morphology* 12, 289-308.

Freeman, S., 2005. *Biological Science*. Upper Saddle River, NJ: Pearson Prentice Hall. (3rd ed.), 249-250.

- Gava, N., Clarke, C.L., Byth, K., Arnett-Mansfield, R.L., deFazio, A., 2004. Expression of progesterone receptors A and B in the mouse ovary during the estrous cycle. *Endocrinology* 145, 3487-3494.
- George, F.W., Ojeda, S.R., 1987. Vasoactive intestinal peptide enhances aromatase activity in the neonatal rat ovary before development of primary follicles or responsiveness to follicle-stimulating hormone. *Proceedings of the National Academy of Sciences of the United States of America* 84, 5803-5807.
- George, F.W., Wilson, J.D., 1978. Conversion of androgen to estrogen by the human fetal ovary. *The Journal of clinical endocrinology and metabolism* 47, 550-555.
- Gomperts, M., Garcia-Castro, M., Wylie, C., Heasman, J., 1994. Interactions between primordial germ cells play a role in their migration in mouse embryos. *Development* 120, 135-141.
- Gondos, B., 1973. Germ cell degeneration and intercellular bridges in the human fetal ovary. *Zeitschrift fur Zellforschung und mikroskopische Anatomie* 138, 23-30.
- Gondos, B., 1987. Comparative studies of normal and neoplastic ovarian germ cells: 1. Ultrastructure of oogonia and intercellular bridges in the fetal ovary. *International journal of gynecological pathology : official journal of the International Society of Gynecological Pathologists* 6, 114-123.
- Gondos, B., Bhiraleus, P., Hobel, C.J., 1971. Ultrastructural observations on germ cells in human fetal ovaries. *Am J Obstet Gynecol* 110, 644-652.
- Gondos, B., Zamboni, L., 1969. Ovarian development: the functional importance of germ cell interconnections. *Fertil Steril* 20, 176-189.

Gougeon, A., 1981. Frequent occurrence of multiovular follicles and multinuclear oocytes in the adult human ovary. *Fertil Steril* 35, 417-422.

Greco, T.L., Payne, A.H., 1994. Ontogeny of expression of the genes for steroidogenic enzymes P450 side-chain cleavage, 3 beta-hydroxysteroid dehydrogenase, P450 17 alpha-hydroxylase/C17-20 lyase, and P450 aromatase in fetal mouse gonads. *Endocrinology* 135, 262-268.

Gu, W., Tekur, S., Reinbold, R., Eppig, J.J., Choi, Y.C., Zheng, J.Z., Murray, M.T., Hecht, N.B., 1998. Mammalian male and female germ cells express a germ cell-specific Y-Box protein, MSY2. *Biol Reprod* 59, 1266-1274.

Guigon, C.J., Mazaud, S., Forest, M.G., Brailly-Tabard, S., Coudouel, N., Magre, S., 2003. Unaltered development of the initial follicular waves and normal pubertal onset in female rats after neonatal deletion of the follicular reserve. *Endocrinology* 144, 3651-3662.

Gumienny, T.L., Lambie, E., Hartweg, E., Horvitz, H.R., Hengartner, M.O., 1999. Genetic control of programmed cell death in the *Caenorhabditis elegans* hermaphrodite germline. *Development* 126, 1011-1022.

Gutzeit, H.O., 1986. Transport of molecules and organelles in meriostic ovarioles of insects. *Differentiation; research in biological diversity* 31, 155-165.

Gutzeit, H.O., Koppa, r., 1982. Time-lapse film analysis of cytoplasmic streaming during late oogenesis of *Drosophila*. *J.Embryol.Exp.Morphol.* 67, 101-111.

Hasbi, A., O'Dowd, B.F., George, S.R., 2005. A G protein-coupled receptor for estrogen: the end of the search? *Molecular interventions* 5, 158-161.

- Heinlein, C.A., Chang, C., 2002. The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. *Molecular endocrinology* 16, 2181-2187.
- Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., Tujague, M., Strom, A., Treuter, E., Warner, M., Gustafsson, J.A., 2007. Estrogen receptors: how do they signal and what are their targets. *Physiological reviews* 87, 905-931.
- Hernandez-Gonzalez, I., Gonzalez-Robayna, I., Shimada, M., Wayne, C.M., Ochsner, S.A., White, L., Richards, J.S., 2006. Gene expression profiles of cumulus cell oocyte complexes during ovulation reveal cumulus cells express neuronal and immune-related genes: does this expand their role in the ovulation process? *Molecular endocrinology* 20, 1300-1321.
- Hirshfield, A.N., 1991. Development of follicles in the mammalian ovary. *Int Rev Cytol* 124, 43-101.
- Hiura, M., Fujita, H., 1977. Electron microscopic observations on the elimination of the oocyte through the peritoneal epithelium in the neonatal mouse ovary. *Cell and tissue research* 182, 73-79.
- Iguchi, T., Fukazawa, Y., Uesugi, Y., Takasugi, N., 1990. Polyovular follicles in mouse ovaries exposed neonatally to diethylstilbestrol in vivo and in vitro. *Biology of reproduction* 43, 478-484.
- Iguchi, T., Takasugi, N., 1986. Polyovular follicles in the ovary of immature mice exposed prenatally to diethylstilbestrol. *Anatomy and embryology* 175, 53-55.
- Iguchi, T., Takasugi, N., Bern, H.A., Mills, K.T., 1986. Frequent occurrence of polyovular follicles in ovaries of mice exposed neonatally to diethylstilbestrol. *Teratology* 34, 29-35.

Iguchi, T., Todoroki, R., Takasugi, N., Petrow, V., 1988. The effects of an aromatase inhibitor and a 5 alpha-reductase inhibitor upon the occurrence of polyovular follicles, persistent anovulation, and permanent vaginal stratification in mice treated neonatally with testosterone. *Biology of reproduction* 39, 689-697.

Jefferson, W.N., Couse, J.F., Padilla-Banks, E., Korach, K.S., Newbold, R.R., 2002. Neonatal exposure to genistein induces estrogen receptor (ER)alpha expression and multiocyte follicles in the maturing mouse ovary: evidence for ERbeta-mediated and nonestrogenic actions. *Biology of reproduction* 67, 1285-1296.

Jones, R.L., Pepling, M.E., 2013. Role of the antiapoptotic proteins BCL2 and MCL1 in the neonatal mouse ovary. *Biology of reproduction* 88, 46.

Jost, A., 1988. In "Development and Function of the Reproductive Organs". M.Parvinen, I. Huhtaniemi, and L.J. Pelliniemi, eds. Aeres-Serono, Rome, 1-9.

Karavan, J.R., Pepling, M.E., 2012. Effects of estrogenic compounds on neonatal oocyte development. *Reprod Toxicol* 34, 51-56.

Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H., Chambon, P., 1990. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. *The EMBO journal* 9, 1603-1614.

Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., Chambon, P., 1995. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* 270, 1491-1494.

- Kavlock, R.J., Ankley, G.T., 1996. A perspective on the risk assessment process for endocrine-disruptive effects on wildlife and human health. *Risk analysis : an official publication of the Society for Risk Analysis* 16, 731-739.
- Kezele, P., Nilsson, E., Skinner, M.K., 2002. Cell-cell interactions in primordial follicle assembly and development. *Frontiers in bioscience : a journal and virtual library* 7, d1990-1996.
- Kezele, P., Skinner, M.K., 2003. Regulation of ovarian primordial follicle assembly and development by estrogen and progesterone: endocrine model of follicle assembly. *Endocrinology* 144, 3329-3337.
- Kono, T., Obata, Y., Yoshimzu, T., Nakahara, T., Carroll, J., 1996. Epigenetic modifications during oocyte growth correlates with extended parthenogenetic development in the mouse. *Nature genetics* 13, 91-94.
- Krege, J.H., Hodgin, J.B., Couse, J.F., Enmark, E., Warner, M., Mahler, J.F., Sar, M., Korach, K.S., Gustafsson, J.A., Smithies, O., 1998. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proceedings of the National Academy of Sciences of the United States of America* 95, 15677-15682.
- Kushner, P.J., Agard, D.A., Greene, G.L., Scanlan, T.S., Shiau, A.K., Uht, R.M., Webb, P., 2000. Estrogen receptor pathways to AP-1. *The Journal of steroid biochemistry and molecular biology* 74, 311-317.
- Lawson, K.A., Hage, W.J., 1994. Clonal analysis of the origin of primordial germ cells in the mouse. *Ciba Foundation symposium* 182, 68-84; discussion 84-91.

- Lin, H., Spradling, A.C., 1997. A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the *Drosophila* ovary. *Development* 124, 2463-2476.
- Lin, H., Yue, L., Spradling, A.C., 1994. The *Drosophila* fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. *Development* 120, 947-956.
- Lubahn, D.B., Moyer, J.S., Golding, T.S., Couse, J.F., Korach, K.S., Smithies, O., 1993. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proceedings of the National Academy of Sciences of the United States of America* 90, 11162-11166.
- Lydon, J.P., DeMayo, F.J., Funk, C.R., Mani, S.K., Hughes, A.R., Montgomery, C.A., Jr., Shyamala, G., Conneely, O.M., O'Malley, B.W., 1995. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes & development* 9, 2266-2278.
- McGee, E.A., Hsueh, A.J., 2000. Initial and cyclic recruitment of ovarian follicles. *Endocrine reviews* 21, 200-214.
- McLaren, A., 2000a. Establishment of the germ cell lineage in mammals. *J Cell Physiol* 182, 141-143.
- McLaren, A., 2000b. Germ and somatic cell lineages in the developing gonad. *Mol Cell Endocrinol* 163, 3-9.
- McLaren, A., Monk, M., 1981. X-chromosome activity in the germ cells of sex-reversed mouse embryos. *Journal of reproduction and fertility* 63, 533-537.

- Mendelson, C.R., Kamat, A., 2007. Mechanisms in the regulation of aromatase in developing ovary and placenta. *The Journal of steroid biochemistry and molecular biology* 106, 62-70.
- Milewich, L., George, F.W., Wilson, J.D., 1977. Estrogen formation by the ovary of the rabbit embryo. *Endocrinology* 100, 187-196.
- Miller, W.L., 1995. Mitochondrial specificity of the early steps in steroidogenesis. *The Journal of steroid biochemistry and molecular biology* 55, 607-616.
- Miller, W.L., 2013. Steroid hormone synthesis in mitochondria. *Molecular and cellular endocrinology* 379, 62-73.
- Miller, W.L., Auchus, R.J., 2011. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocrine reviews* 32, 81-151.
- Mork, L., Tang, H., Batchvarov, I., Capel, B., 2012. Mouse germ cell clusters form by aggregation as well as clonal divisions. *Mechanisms of development* 128, 591-596.
- Mulac-Jericevic, B., Lydon, J.P., DeMayo, F.J., Conneely, O.M., 2003. Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. *Proceedings of the National Academy of Sciences of the United States of America* 100, 9744-9749.
- Mulac-Jericevic, B., Mullinax, R.A., DeMayo, F.J., Lydon, J.P., Conneely, O.M., 2000. Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. *Science* 289, 1751-1754.
- Murphy, K., Carvajal, L., Medico, L., Pepling, M., 2005. Expression of Stat3 in germ cells of developing and adult mouse ovaries and testes. *Gene Expr Patterns* 5, 475-482.

- Nilsson, E.E., Skinner, M.K., 2009. Progesterone regulation of primordial follicle assembly in bovine fetal ovaries. *Molecular and cellular endocrinology* 313, 9-16.
- Nilsson, E.E., Stanfield, J., Skinner, M.K., 2006. Interactions between progesterone and tumor necrosis factor-alpha in the regulation of primordial follicle assembly. *Reproduction* 132, 877-886.
- Niswender, G.D., 2002. Molecular control of luteal secretion of progesterone. *Reproduction* 123, 333-339.
- Ohno, S., Smith, J.B., 1964. Role of Fetal Follicular Cells in Meiosis of Mammalian Oocytes. *Cytogenetics* 3, 324-333.
- Oktay, K., Briggs, D., Gosden, R.G., 1997. Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles. *The Journal of clinical endocrinology and metabolism* 82, 3748-3751.
- Opferman, J.T., 2008. Apoptosis in the development of the immune system. *Cell death and differentiation* 15, 234-242.
- Otto, C., Fuchs, I., Kauselmann, G., Kern, H., Zevnik, B., Andreasen, P., Schwarz, G., Altmann, H., Klewer, M., Schoor, M., Vonk, R., Fritzemeier, K.H., 2009. GPR30 does not mediate estrogenic responses in reproductive organs in mice. *Biology of reproduction* 80, 34-41.
- Paredes, A., Garcia-Rudaz, C., Kerr, B., Tapia, V., Dissen, G.A., Costa, M.E., Cornea, A., Ojeda, S.R., 2005. Loss of synaptonemal complex protein-1, a synaptonemal complex protein, contributes to the initiation of follicular assembly in the developing rat ovary. *Endocrinology* 146, 5267-5277.

- Payne, A.H., Abbaszade, I.G., Clarke, T.R., Bain, P.A., Park, C.H., 1997. The multiple murine 3 beta-hydroxysteroid dehydrogenase isoforms: structure, function, and tissue- and developmentally specific expression. *Steroids* 62, 169-175.
- Pedram, A., Razandi, M., Levin, E.R., 2006. Nature of functional estrogen receptors at the plasma membrane. *Molecular endocrinology* 20, 1996-2009.
- Pelletier, G., Li, S., Luu-The, V., Tremblay, Y., Belanger, A., Labrie, F., 2001. Immunoelectron microscopic localization of three key steroidogenic enzymes (cytochrome P450(scc), 3 beta-hydroxysteroid dehydrogenase and cytochrome P450(c17)) in rat adrenal cortex and gonads. *The Journal of endocrinology* 171, 373-383.
- Peluso, J.J., 2006. Multiplicity of progesterone's actions and receptors in the mammalian ovary. *Biology of reproduction* 75, 2-8.
- Pepling, M.E., 2006. From primordial germ cell to primordial follicle: mammalian female germ cell development. *Genesis* 44, 622-632.
- Pepling, M.E., 2012. Follicular assembly: mechanisms of action. *Reproduction* 143, 139-149.
- Pepling, M.E., 2013. Oocyte Development before and after Folliculogenesis. *Oocyte Physiology and Development in Domestic Animals Wiley-Blackwell*, 10.
- Pepling, M.E., Spradling, A.C., 2001. Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. *Developmental biology* 234, 339-351.
- Pepling, M.E., Sundman, E.A., Patterson, N.L., Gephardt, G.W., Medico, L., Jr., Wilson, K.I., 2010. Differences in oocyte development and estradiol sensitivity among mouse strains. *Reproduction* 139, 349-357.

Peters, H., 1970. Migration of gonocytes into the mammalian gonad and their differentiation. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* 259, 91-101.

Peters, H., 1976. Intrauterine gonadal development. *Fertil Steril* 27, 493-500

Pettersson, K., Gustafsson, J.A., 2001. Role of estrogen receptor beta in estrogen action. *Annual review of physiology* 63, 165-192.

Ratts, V.S., Flaws, J.A, Klop, R., Sorenson, C.M., Tilly, J.L. , 1995. Ablation of bcl-2 gene expression decreases the number of oocytes and primordial follicles established in the postnatal female mouse gonad. *Endocrinology*

136, 3665–3668.

Revankar, C.M., Cimino, D.F., Sklar, L.A., Arterburn, J.B., Prossnitz, E.R., 2005. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* 307, 1625-1630.

Rosenbusch, B., 2006. The contradictory information on the distribution of non-disjunction and pre-division in female gametes. *Human reproduction* 21, 2739-2742.

Roy, A.K., Lavrovsky, Y., Song, C.S., Chen, S., Jung, M.H., Velu, N.K., Bi, B.Y., Chatterjee, B., 1999. Regulation of androgen action. *Vitamins and hormones* 55, 309-352.

Ruby, J.R., Dyer, R.F., Skalko, R.G., 1969. The occurrence of intercellular bridges during oogenesis in the mouse. *Journal of morphology* 127, 307-339.

Russe, I., 1983. Oogenesis in cattle and sheep. *Bibl Anat* 24, 77-92.

Saville, B., Wormke, M., Wang, F., Nguyen, T., Enmark, E., Kuiper, G., Gustafsson, J.A., Safe, S., 2000. Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements. *The Journal of biological chemistry* 275, 5379-5387.

Sawyer, H.R., Smith, P., Heath, D.A., Juengel, J.L., Wakefield, S.J., McNatty, K.P., 2002. Formation of ovarian follicles during fetal development in sheep. *Biology of reproduction* 66, 1134-1150.

Sawyer HR, S.P., Heath DA, Juengel JL, Wakefield SJ, and KP McNatty, 2002.

Formation of ovarian follicles during fetal development in sheep. *Biol Reprodu* 66.

Schomberg, D.W., Couse, J.F., Mukherjee, A., Lubahn, D.B., Sar, M., Mayo, K.E., Korach, K.S., 1999. Targeted disruption of the estrogen receptor-alpha gene in female mice: characterization of ovarian responses and phenotype in the adult. *Endocrinology* 140, 2733-2744.

Sebastian, S., Bulun, S.E., 2001. A highly complex organization of the regulatory region of the human CYP19 (aromatase) gene revealed by the Human Genome Project. *The Journal of clinical endocrinology and metabolism* 86, 4600-4602.

Senger, P.L., 2003. *Pathways to Pregnancy and Parturition*, Pullman, WA. Current Conceptions, Inc.

Shutt, D.A., Smith, I.D., Shearman, R.P., 1974. Oestrone, oestradiol-17beta and oestriol levels in human foetal plasma during gestation and at term. *The Journal of endocrinology* 60, 333-341.

Simpson, E.R., Mahendroo, M.S., Means, G.D., Kilgore, M.W., Hinshelwood, M.M., Graham-Lorence, S., Amarneh, B., Ito, Y., Fisher, C.R., Michael, M.D., et al., 1994.

- Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocrine reviews* 15, 342-355.
- Simpson, E.R., Zhao, Y., Agarwal, V.R., Michael, M.D., Bulun, S.E., Hinshelwood, M.M., Graham-Lorence, S., Sun, T., Fisher, C.R., Qin, K., Mendelson, C.R., 1997. Aromatase expression in health and disease. *Recent progress in hormone research* 52, 185-213; discussion 213-184.
- Smith, O. W., Smith, G.V. , 1949. Use of diethylstilbestrol to prevent fetal loss from complications of late pregnancy. . *The New England journal of medicine* 241, 562-568.
- Snow, M., Monk, M., 1983. Emergence and Migration of mouse germ cells In: *Current problems in germ cell differentiation*. Eds:A. McLaren and C.C. Wylie, Cambridge University Press, NY 115-135.
- Sorensen, R.A., Wassarman, P.M., 1976. Relationship between growth and meiotic maturation of the mouse oocyte. *Developmental biology* 50, 531-536.
- Spradling, A.C., 1993. Developmental genetics of oogenesis. In: Bate M, Arias AM, editors. . *The development of Drosophila melanogaster*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. , 1-70.
- Stocco, C., 2008. Aromatase expression in the ovary: hormonal and molecular regulation. *Steroids* 73, 473-487.
- Stocco, C., 2012. Tissue physiology and pathology of aromatase. *Steroids* 77, 27-35.
- Susiarjo, M., Hassold, T.J., Freeman, E., Hunt, P.A., 2007. Bisphenol A exposure in utero disrupts early oogenesis in the mouse. *PLoS genetics* 3, e5.
- Suzuki, A., Sugihara, A., Uchida, K., Sato, T., Ohta, Y., Katsu, Y., Watanabe, H., Iguchi, T., 2002. Developmental effects of perinatal exposure to bisphenol-A and

diethylstilbestrol on reproductive organs in female mice. *Reproductive toxicology* 16, 107-116.

Tam, P.P.L., and Snow, M.H.L., 1981. *J.Embryol.Exp.Morphol.* 64, 133-147.

Toda, K., Takeda, K., Okada, T., Akira, S., Saibara, T., Kaname, T., Yamamura, K., Onishi, S., Shizuta, Y., 2001. Targeted disruption of the aromatase P450 gene (Cyp19) in mice and their ovarian and uterine responses to 17beta-oestradiol. *The Journal of endocrinology* 170, 99-111.

Trubo, R., 2005. Endocrine-disrupting chemicals probed as potential pathways to illness. *JAMA : the journal of the American Medical Association* 294, 291-293.

Vaskivuo, T.E., Anttonen, M., Herva, R., Billig, H., Dorland, M., te Velde, E.R., Stenback, F., Heikinheimo, M., Tapanainen, J.S., 2001. Survival of human ovarian follicles from fetal to adult life: apoptosis, apoptosis-related proteins, and transcription factor GATA-4. *The Journal of clinical endocrinology and metabolism* 86, 3421-3429.

Vegetti, W., Alagna, F., 2006. FSH and folliculogenesis: from physiology to ovarian stimulation. *Reproductive biomedicine online* 12, 684-694.

Vigier, B., Forest, M.G., Eychenne, B., Bezard, J., Garrigou, O., Robel, P., Josso, N., 1989. Anti-Mullerian hormone produces endocrine sex reversal of fetal ovaries. *Proceedings of the National Academy of Sciences of the United States of America* 86, 3684-3688.

Villeneuve, A.M., Hillers, K.J., 2001. Whence meiosis? *Cell* 106, 647-650.

Wang, C., Prossnitz, E.R., Roy, S.K., 2008. G protein-coupled receptor 30 expression is required for estrogen stimulation of primordial follicle formation in the hamster ovary. *Endocrinology* 149, 4452-4461.

- Wang, C., Roy, S.K., 2007. Development of primordial follicles in the hamster: role of estradiol-17beta. *Endocrinology* 148, 1707-1716.
- Weakly, B.S., 1967. Light and electron microscopy of developing germ cells and follicle cells in the ovary of the golden hamster: twenty-four hours before birth to eight days postpartum. *J. Anat.* 101, 435-459.
- Wenzel, J., Odend'hal, S., 1985. The mammalian rete ovarii; a literature review. *Cornell Vet* 75.
- Woodruff, T.K., Mayo, K.E., 2005. To beta or not to beta: estrogen receptors and ovarian function. *Endocrinology* 146, 3244-3246.
- Wylie, C., 1999. Germ cells. *Cell* 96, 165-174.
- Yang, M.Y., Fortune, J.E., 2008. The capacity of primordial follicles in fetal bovine ovaries to initiate growth in vitro develops during mid-gestation and is associated with meiotic arrest of oocytes. *Biology of reproduction* 78, 1153-1161.
- Yen, S.S.C., 1991. Chronic anovulation caused by peripheral endocrine disorders. In: Yen SSC, Jaffe RB, editors. *Reproductive endocrinology* Philadelphia: WB Saunders. pp 631-689.
- Yoshinaga, K., Hess, D.L., Hendrickx, A.G., Zamboni, L., 1988. The development of the sexually indifferent gonad in the prosimian, *Galago crassicaudatus crassicaudatus*. *Am J Anat* 181, 89-105.
- Yuan, J., Yankner, B.A., 2000. Apoptosis in the nervous system. *Nature* 407, 802-809.
- Yuan, L., Liu, J.G., Hoja, M.R., Wilbertz, J., Nordqvist, K., Hoog, C., 2002. Female germ cell aneuploidy and embryo death in mice lacking the meiosis-specific protein SCP3. *Science* 296, 1115-1118.

Yue, L., Spradling, A.C., 1992. hu-li tai shao, a gene required for ring canal formation during *Drosophila* oogenesis, encodes a homolog of adducin. *Genes & development* 6, 2443-2454.

Zachos, N.C., Billiar, R.B., Albrecht, E.D., Pepe, G.J., 2002. Developmental regulation of baboon fetal ovarian maturation by estrogen. *Biology of reproduction* 67, 1148-1156.

Zamboni, L., Gondos, B., 1968. Intercellular bridges and synchronization of germ cell differentiation during oogenesis in the rabbit. *The Journal of cell biology* 36, 276-282.

BIOGRAPHICAL SKETCH

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