The Effects of Exogenous Hormones on the Progression of Oocytes through Prophase I of Meiosis

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ABSTRACT

The process of germ cell cyst breakdown and primordial follicle formation that begins during embryonic development in mammalian ovaries is vital for future reproductive success because it establishes the total pool of viable egg cells the female will have for the rest of her life. Germ cells arrive at the gonad in the mouse at approximately 10.5 days post coitum and begin to form germline cysts through incomplete mitosis. At 13.5 days post coitum, the germ cells enter meiosis and begin to progress through prophase I of meiosis I. Germ cells progress through a series of sub-phases of prophase I starting at pre-meiotic interphase and then moving through leptotene, zygotene, pachytene, and eventually arresting at the diplotene stage. The germ cells enter diplotene arrest beginning at 17.5 days post coitum, which corresponds with the start of cyst breakdown. As more germ cells arrive at diplotene and cysts begin to break down to form primordial follicles, some oocytes experience programmed cell death. The protein MSY2 is believed to be upregulated at the diplotene stage and therefore could be used as a marker for diplotene. Through analysis of the MSY2 protein from 15.5 days post coitum through postnatal day 5 using immunohistochemistry, we were able to see an increase in expression over time. The increase of expression was expected as our current model suggests that more cells should be arriving at diplotene as development progresses. Synaptonemal complex protein 3 (SYCP3) has been shown to be expressed during the pachytene stage of meiotic prophase I and therefore can be used as a marker for this developmental stage. Analysis of SYCP3 starting at 13.5 days post coitum through postnatal day 5 using immunohistochemistry showed the greatest percentage of oocytes in the pachytene stage at postnatal day 1. Exogenous hormone exposure on developing ovaries can disrupt cyst breakdown and follicle formation, but the process by which the hormone exposure disrupts the cyst breakdown is not well understood. It is also unclear how hormones
affect progression through prophase I of meiosis. Recreating environments that mimic exogenous hormone exposure that a female might experience can lead to a better understanding of how early such exposure can lead to further issues with fertility. To examine the effect or effects of hormones on meiosis, ovaries were dissected at 17.5 days post coitum and grown in culture for four days, reaching postnatal day 3, in the presence of $10^{-6}$ M estradiol, $10^{-6}$ M progesterone, both $10^{-6}$ M estradiol and $10^{-6}$ M progesterone, or neither hormone. Ovaries were fixed and double labeled with MSY2 and oocyte marker TRA98 or SYCP3 and oocyte marker VASA. We found a decrease in MSY2 expression and an increase in the number of oocytes in the pachytene stage following hormone treatment, suggesting that estradiol and progesterone can block progression to the diplotene stage of meiosis. This work will contribute to our understanding of the effects of hormones on oocyte development by determining if there are any disruptions in development following the exposure. Information regarding the timing of hormone exposure with regards to the extent of developmental disruptions will allow for a clearer timeline of when an individual could experience fertility issues through such exposure.
The Effects of Exogenous Hormones on the Progression of Oocytes through Prophase I of Meiosis

By

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THESIS

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system. Without her help and support, the journey to this point would have been a lot more difficult.

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TABLE OF CONTENTS

Abstract ................................................................................................................................. i
Title ...................................................................................................................................... iii
Copyright ............................................................................................................................. iv
Acknowledgments ............................................................................................................... v
Table of Contents ................................................................................................................ vii
List of Figures ....................................................................................................................... ix

Chapter 1: Introduction

Significance ........................................................................................................................... 1
Germ Cell Development in Mammalian Ovaries ............................................................... 2
Follicle Formation and Cyst Breakdown ........................................................................... 4
Effects of Hormones on Meiotic Progression and Cyst Breakdown ............................... 4
MSY2 Expression in the Ovary ......................................................................................... 7
SYCP3 Expression in the Ovary ....................................................................................... 7
Specific Aims ...................................................................................................................... 9

Chapter 2: Materials and Methods

Animal Model ..................................................................................................................... 10
Checking Vaginal Plugs and Staging Mouse Development ............................................. 10
Ovary Harvesting ............................................................................................................... 11
Whole Mount Immunohistochemistry ........................................................................... 11
Fluorescent Microscopy ................................................................................................. 13
Analysis of Meiotic Progression .................................................................................... 14
In vitro Ovary Organ Culture ........................................................................................... 15
Analysis of Oocyte Development and Cyst Breakdown ................................................. 16
Statistical Analysis .......................................................................................................... 17
Chapter 3: Results

MSY2 Expression Correlates with Diplotene Arrest ......................................................... 18
SYCP3 Expression Correlates with the Pachytene Stage of Meiotic Prophase I ............. 20
Examining the Effects of Steroid Hormones on Meiotic Progression by Analysis of a Diplotene Arrest Marker ...................................................................................... 21
Examining the Effects of Steroid Hormones on Meiotic Progression by Analysis of Pachytene Oocytes ........................................................................................................... 25
No Variance Observed Between Pairs of Ovaries from Litter Mates ............................. 29
Estradiol Confirmed to Disrupt Cyst Breakdown in Later Stage Ovaries ........................ 31
Organ Culture System has a Negative Effect on Ovarian Health ................................... 33

Chapter 4: Discussion and Future Directions ................................................................. 35

Appendix 1: Arrest at the diplotene stage of meiotic prophase I is delayed by
progesterone but is not required for primordial follicle formation in mice .................. 42

References .......................................................................................................................... 43

CV ..................................................................................................................................... 47
<table>
<thead>
<tr>
<th>Figure #</th>
<th>Figure Title</th>
<th>Page #</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Timeline of mouse germ cell development</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Model for cyst breakdown and estrogen signaling</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Schematic showing method in which ovaries were analyzed through confocal microscopy</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>Asymmetric expression of MSY2 protein in wildtype ovaries</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>MSY2 expression correlates with the progression of oocyte development to the diplotene stage of meiotic prophase I</td>
<td>19</td>
</tr>
<tr>
<td>6</td>
<td>Confocal microscope section showing expression pattern of SYCP3 in PND1 wildtype ovary</td>
<td>20</td>
</tr>
<tr>
<td>7</td>
<td>Percent of oocytes in the pachytene stage of development in ovaries from 13.5 dpc through PND5</td>
<td>21</td>
</tr>
<tr>
<td>8</td>
<td>Organ culture with hormone treatment analyzed by MSY2 expression experimental set up</td>
<td>22</td>
</tr>
<tr>
<td>9</td>
<td>Effects of estradiol and progesterone on oocyte development in organ culture examining diplotene arrest from first series of organ culture experiments</td>
<td>23</td>
</tr>
<tr>
<td>10</td>
<td>Progesterone but not estradiol reduces MSY2 expression</td>
<td>24</td>
</tr>
<tr>
<td>11</td>
<td>Organ culture with hormone treatment analyzed by SYCP3 expression experimental set up</td>
<td>25</td>
</tr>
<tr>
<td>12</td>
<td>Effects of estradiol and progesterone on oocyte development in organ culture examining germ cell progression to the pachytene stage from second series of organ culture experiments</td>
<td>27</td>
</tr>
<tr>
<td>13</td>
<td>Estradiol and progesterone have a combined effect on the number of oocytes at pachytene</td>
<td>28</td>
</tr>
<tr>
<td>14</td>
<td>No significant variance is observed between pairs of ovaries from individuals within the same litter</td>
<td>30</td>
</tr>
<tr>
<td>15</td>
<td>Estradiol disrupts cyst breakdown in PND1 ovaries cultured for 5 days</td>
<td>31</td>
</tr>
<tr>
<td>16</td>
<td>Estradiol reduces the number of oocytes, disrupts cyst breakdown, and delays meiotic progression in PND1 ovaries cultured for 5 days</td>
<td>32</td>
</tr>
<tr>
<td>17</td>
<td>DMSO does not have a negative effect on ovaries, but organ culture system shows a negative effect on ovarian health</td>
<td>34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table #</th>
<th>Table Title</th>
<th>Page #</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Primary antibodies used for immunohistochemistry</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>Secondary antibodies used for immunohistochemistry</td>
<td>13</td>
</tr>
</tbody>
</table>
Chapter 1: INTRODUCTION

Significance

Infertility in humans is defined as the inability to reproduce by natural means. The ability of a female to reproduce is essential to the survival of a species. It is vital to understand the processes that underlie a female’s fertility in order to better the chances of survival for the species. Not only is it important to comprehend the normal process of reproduction, but it is crucial to understand why many individuals, both male and female, experience infertility. Creating this knowledge base can then assure a more fruitful future for that individual. In a recent poll of the United States, it was found that 7.5 million individual females, between the ages of 15 – 44, had impaired fecundity (Chandra et al., 2013). Impaired fecundity can mean that the female may not be able to conceive a child or that she may be able to conceive a child, but not be able to carry the child full term. There are many potential causes for why a female might experience this issue including: DNA damage in the oocyte, predisposed genetic issues, or external environmental factors. Along with these possible fertility complications, females are also born with a finite number of germ cells that decreases as the female matures and eventually reaches the period of reproductive senescence (Kim and Tilly, 2004). The number of females experiencing infertility issues is staggering and the available treatments, such as medication, surgery, and in vitro fertilization, come with their own difficulties including a high price tag and no guarantee of improving the fertility of the individual. It is essential that this issue is further investigated and understood in order to create better treatments that may not only be more cost effective, but also much less invasive.
For a female to reproduce, proper development of germ cells within the ovary must occur. This process is tightly regulated to create viable eggs that will have the opportunity to become fertilized. Starting around 6.5 days post coitum (dpc), or days after mating in mice, a subset of cells are committed to become germ cells (Saffman and Lasko, 1999). Around 10.5 dpc, the germ cells arrive at the gonad and begin to undergo mitosis and divide rapidly to increase germ cell numbers (Figure 1) (Peters, 1970). During these divisions, the germ cells are found in large clusters that synchronously divide and are known as germline cysts (Pepling and Spradling, 1998). Each germline cyst arises from a single germ cell (Pepling et al., 1999). As these cells divide, they do not complete cytokinesis and therefore remain connected by intercellular bridges that may allow for the exchange of important developmental signaling molecules (Pepling and Spradling, 1998). This division takes place until around 13.5 dpc when the cells then begin to enter the first of two meiotic divisions.

Starting at 13.5 dpc, the germ cells enter prophase I of meiosis I. The cells complete pre-meiotic interphase and progress through leptotene, zygotene, pachytene, and diplotene stages (Pepling, 2006). This process occurs in a wave starting from the anterior end of the ovary to the posterior end (Borum, 1961; Menke et al., 2003). Once the diplotene stage is reached, the germ cells arrest and remain arrested until further signals are produced, typically after puberty, that allow the cell to be released and developed into a mature egg cell that is ready to be fertilized. Oocytes begin to arrest at diplotene around 17.5 dpc and the process continues as more germ cells progress through meiosis. At about PND5 almost all oocytes are arrested at the diplotene stage.
Figure 1
Timeline of mouse germ cell development. Germ cells are shown in green and somatic cells are shown in red. At 10.5 dpc, germ cells migrate to the gonad and begin rapidly dividing through mitosis, forming cysts. Beginning at 13.5 dpc, mitosis stops and germ cells begin to enter meiotic prophase I. Oocytes continue through meiosis progressing through leptotene, zygotene, pachytene, and diplotene stages. The oocytes arrive and begin to arrest at the diplotene stage at 17.5 dpc. At this time, germline cysts begin to breakdown and germ cells that are not lost due to apoptosis are surrounded by somatic cells forming primordial follicles. (Adapted from Pepling, 2006)
**Follicle Formation and Cyst Breakdown**

For primordial follicles to form within the ovary, germline cyst breakdown must occur. A primordial follicle is a single germ cell surrounded by a single layer of somatic cells, called granulosa cells. The somatic cells move between germ cells within cysts and are believed to play a role in separating the cells (Pepling and Spradling, 2001). Along with the somatic cells moving between germ cells and breaking apart the germline cysts, apoptosis of germ cells also plays a crucial role in the cyst breakdown and follicle formation process (Coucouvanis et al., 1993; Pepling and Spradling, 2001). The loss of germ cells through programmed cell death correlates with primordial follicle formation (Greenfeld et al., 2007; Pepling and Spradling, 2001). The surviving germ cells that progress into primordial follicles then establish the total pool of germ cells the female will have for their whole reproductive lifespan.

**Effects of Hormones on Meiotic Progression and Cyst Breakdown**

The process of germ cells dividing through mitosis and forming germline cysts prior to entering meiosis has been extensively studied and well documented in the field. From that knowledge base, further investigation into meiotic progression and cyst breakdown was conducted and it was hypothesized that germ cells remain in cysts due to hormone exposure from the mother and the cysts begin to breakdown at birth once the hormone levels significantly drop. Thus, contexts where sustained elevated hormones prevail may lead to serious defects in ovarian development.

Studies to investigate the effects of estrogens on cyst breakdown and follicle formation have been done both *in vivo* and *in vitro* using varying concentrations of estradiol, one of the mammalian estrogens (*Figure 2*) (Chen et al., 2007). Ovaries from neonatal pups injected with
estradiol and ovaries grown in an organ culture system with the addition of estradiol both showed a significant disruption in cyst breakdown and significantly fewer follicles that were also developmentally delayed (Chen et al., 2007). This agrees with previous studies that investigated the role of estrogens through the exposure of genistein, a soybean phytoestrogen, which has estrogenic activity that disrupted cyst breakdown and follicle formation (Jefferson et al., 2006). Along with the disruption of cyst breakdown, estrogen has also been shown to induce multiple oocyte follicles (MOFs) in adult ovaries which are mature follicles containing more than one germ cell, thought to be the result of cysts that never broke down (Iguchi et al., 2001). The genistein exposure also produced the same result, confirming the estrogen effect of promoting the production of MOFs (Jefferson et al., 2002). These MOFs are less viable than single oocyte follicles due to improper breakdown of the cyst. The method by which estrogen can cause these effects has been further investigated, and it has been shown that there are multiple pathways by which estrogen can signal to the oocyte through the use of multiple estrogen receptors (Chen et al., 2009).

Progesterone has also been shown to have a negative effect on ovarian development through the disruption of cyst breakdown and follicle formation (Chen et al., 2007). Ovaries grown in an organ culture system with the addition of progesterone or estradiol + progesterone also had a disruption of cyst breakdown and a delay in follicle development (Chen et al., 2007). Recently, the receptor by which progesterone acts, progesterone receptor membrane component 1 (PGRMC1), to disrupt cyst breakdown and follicle formation was discovered (Guo et al., 2016). It was also shown that progesterone acts via PGRMC1 to suppress oocyte progression through meiotic prophase I.
Further investigation showed that there is actually a decrease in maternal progesterone levels prior to birth, which initiates cyst breakdown at an earlier time point, but the maternal estrogen levels remain high, which maintains some cysts (Dutta et al., 2014). It was also shown that the fetal ovary has its own source of hormones that assists in the regulation of cyst breakdown and follicle formation (Dutta et al., 2014). In either case, it is clear that the hormone exposure on the developing germ cells has an effect and is causing the oocytes to remain in cysts.

Figure 2
Model for cyst breakdown and estrogen signaling. Timeline shows normal cyst breakdown on top and cyst breakdown being affected by estrogen treatment on the bottom. After birth, estrogen levels fall and cyst breakdown can occur. When additional estrogen is introduced, cyst breakdown is disrupted and follicle formation is delayed. (Adapted from Jefferson et al., 2006)
MSY2 Expression in the Ovary

The Y-Box protein MSY2 is a member of the Y box multigene family of nucleic acid binding proteins (Gu et al., 1998). MSY2 is specifically expressed in both male and female germ cells and has also been shown to be maternally inherited (Gu et al., 1998). Expression of MSY2 is localized to the cytoplasm of germ cells and has a temporal expression pattern which suggests a function of storage and translation of mRNAs (Gu et al., 1998). Also MSY2 is very abundant within the oocyte and makes up about 2% of total oocyte protein (Yu et al., 2001). The protein is believed to be involved in regulating global stability of mRNA and when the gene is deleted, females become sterile (Yang et al., 2005). It has been shown in several studies that when there is a loss of MSY2 expression within an organism, the individual becomes infertile and different phenotypic changes are observed, such as decreased folliculogenesis and a reduction in oocyte number (Medvedev et al., 2011).

Within the field of reproduction, MSY2 has been widely used as a marker for diplotene arrest of oocytes progressing through meiotic prophase I (Paredes et al., 2005). Therefore, characterizing MSY2 using immunohistochemistry could provide insight into the regulation of early oocyte development. Also, questions such as which pathways regulate meiotic prophase I progression and how environmental factors might disrupt this process can then be addressed.

SYCP3 Expression in the Ovary

Synaptonemal complexes (SCs) are structures formed between homologous chromosomes during the pachytene stage of meiotic prophase and are involved with chromosome pairing and recombination (Meuwissen et al., 1992). There have been three isoforms of SC proteins (SYCPs), SYCP1, SYCP2, and SYCP3 (also known as SCP1, SCP2,
and SCP3) that have been identified in mammals (Lammers et al., 1994; Meuwissen et al., 1992; Offenberg et al., 1998). These SCs contain two proteinaceous axes, lateral elements (LEs) found along each homologue and transverse filaments (TFs) that connect LEs (Lammers et al., 1994; Meuwissen et al., 1997). Mutant forms of SC components can lead to chromosome instabilities which, in turn, can produce a number of different problems, including cancer. A mutation in SYCP3 specifically was shown to lead to reproductive problems in people (Lopez-Carrasco et al., 2013).

SYCP3, in particular, has been observed to be located in lateral elements of SCs specifically (Ollinger et al., 2005). The expression of SYCP3 mRNA has been detected in primordial germ cells (PCGs), pre-meiotic germ cells and meiotic prophase I oocytes (Zheng et al., 2009). The expression pattern of SYCP3 protein is distinct within meiotic prophase I oocytes during the pachytene stage and is therefore used as a marker for pachytene within the field of reproduction (Grive et al., 2016). However, until now, the majority of research conducted investigating SYCP3 has used chromosome spreads to visualize individual cells. Chromosome spreads involve the destruction of an ovary and the compression of cells in order to isolate single germ cells. The characterization of SYCP3 in whole mount ovaries through immunohistochemistry can provide a larger scale representation of the protein throughout the entire ovary and can then be used as a tool by creating an expression profile in a normal ovary that can be used for baseline comparisons. Therefore, further investigation of a variety of factors that may influence germ cell development either negatively, such as exogenous hormones that disrupt cyst breakdown, or positively, such as the addition of KIT ligand to promote cyst breakdown, can be performed.
Specific Aims

Current methods used to stage and analyze female germ cell development are tedious, time consuming, and often do not allow researchers to gain an understanding of the ovary as a whole. Present methods include hematoxylin and eosin (H&E) staining that requires the mounting, sectioning (through slicing), and staining of the ovary and chromosome spreads, which we described above. Both of these methods deconstruct the ovary and require extensive protocols in order to complete. Given the importance of female infertility research, it is vital to help improve research techniques that will provide more information at a more time effective pace. The goals of my research are to use MSY2 expression levels and SYCP3 expression pattern in whole mount immunohistochemistry as tools to analyze meiotic progression and to determine the effects of estrogen and progesterone on meiotic progression analyzed by MSY2 protein levels and SYCP3 expression.

The specific aims for this thesis are:

1. Use MSY2 expression levels and SYCP3 expression pattern in whole mount immunohistochemistry as tools to analyze meiotic progression.

2. Determine the effects of estrogen and progesterone on meiotic progression analyzed by MSY2 protein levels and SYCP3 expression.
Chapter 2: MATERIALS AND METHODS

Animal Model

The mouse model used in this research is the inbreed strain C57BL/6J. Males and females are caged separately with two to five individual animals per cage until ready to breed. The animals are kept on a 14 hour light/10 hour dark cycle. The cages are changed once a week to provide fresh food, water, and bedding. All work done has been approved by the Institutional Animal Care and Use Committee (IACUC).

Checking Vaginal Plugs and Staging Mouse Development

After male and female mice are placed together in a cage, females are checked for vaginal plugs for the following four days. To check for a vaginal plug, females are raised from the tail end and, using a pair of forceps, the vagina is slightly opened and inspected. If a female has a small white bump consisting of coagulated secretions from the coagulating and vesicular glands of the male inside of the vaginal opening, she is considered plugged (pregnant), separated into her own cage, and is expected to give birth 19 days later. If on day five the female is still not plugged, she is considered a no plug female and separated into her own cage and observed for the next 19 days to make sure she is not pregnant.

Animals are staged by their plug date. Therefore, when a female is found to be plugged, she is considered to be 0.5 dpc. A total of 19 days are counted out following the plug date and that is considered the expected delivery date (19.5 dpc or PND1). Using this timeline, the age of the pups can be determined.
**Ovary Harvesting**

Ovaries were harvested from wildtype C57BL/6J pups at multiple time points during development. Harvested ovaries ranged in development from 13.5 dpc to PND5. In order to harvest ovaries from embryos (13.5 dpc to 18.5 dpc), mothers were sacrificed via CO2 asphyxiation and embryos were removed from the mother. Postnatal pups (PND1 to PND5) were collected from animal cages housed with mothers. All pups were sacrificed via decapitation as stated in the lab’s animal protocol. Once pups were sacrificed, ovaries were harvested from female pups, excess tissue surrounding the ovaries was removed, and the ovaries were placed into the proper solution depending on the protocol. When sacrificing mothers for the collection of embryos, a maximum of two adult females were sacrificed at a time in order to avoid the degradation of essential proteins before ovaries could be collected. When sacrificing postnatal pups, a maximum of four pups were sacrificed at a time, also to avoid the degradation of essential proteins.

**Whole Mount Immunohistochemistry**

Once ovaries were harvested, they were placed into 5.3% formaldehyde to fix the tissue (16% EM grade formaldehyde diluted in 1X PBS). Ovaries were fixed overnight at 4°C on a nutator. After fixation, ovaries went through a series of washes at room temperature in 0.1% Triton X-100 in 1X PBS (PT) and PT + 5% bovine serum albumin (BSA). Following the washes, the ovaries were incubated overnight with primary antibodies at varying concentrations in PT + 5% BSA at 4°C. Primary antibodies are listed in Table 1.
Table 1. Primary Antibodies Used for Immunohistochemistry

<table>
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<tr>
<th>Primary Antibody</th>
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<th>Dilution</th>
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<td>MSY2 (C-15)</td>
<td>Santa Cruz Biotechnology</td>
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<td>Goat</td>
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<td>SYCP3 (D-1)</td>
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<td>ab-13840 Lot #: GR172688-1</td>
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After overnight incubation in primary antibody, ovaries went through another series of washes including: PT + 1% BSA for 30 minutes, RNase A (10 µl of 10 mg/ml RNase A in 1 ml PT + 1% BSA) for 30 minutes, propidium iodide (10 µl of 0.5 mg/ml in 1 ml PT + 1% BSA) or TOTO3 (Molecular Probes; 1:2000 dilution in PT + 1% BSA) for 20 minutes in the dark (samples remained in dark environment after this wash), and one more wash in PT + 1% BSA for 30 minutes. RNase A was used to degrade any residual single stranded RNA present in the sample in order to eliminate potential background staining in the cytoplasm. Propidium iodide or TOTO3 was used to label the nuclei of all cells present in the sample. Ovaries were then incubated with pre-absorbed secondary antibodies overnight at 4°C in order to eliminate any nonspecific staining. Negative controls using only the secondary antibody were previously tested in the lab for all antibodies used. Secondary antibodies were pre-absorbed using a small amount of embryo powder overnight at 4°C. Secondary antibodies used are listed in Table 2. The embryo powder was prepared from 14.5 dpc embryos by taking whole embryos, dehydrating them, and grinding them down to a powder.
Table 2. Secondary Antibodies Used for Immunohistochemistry

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<td>Molecular Probes/Invitrogen</td>
<td>A11055</td>
<td>Donkey</td>
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<td>A21202</td>
<td>Donkey</td>
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<tr>
<td>Alexa Fluor 488 Donkey Anti-Rabbit</td>
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<td>A21206</td>
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<tr>
<td>Alexa Fluor 488 Donkey Anti-Rat</td>
<td>Molecular Probes/Invitrogen</td>
<td>A21208</td>
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Secondary antibodies were used at a 1:200 dilution in PT + 5% BSA. Following overnight incubation, ovaries were washed in PT + 1% BSA at room temperature three times for 30 minutes each. Ovaries were then quickly rinsed in PBS, placed in vectashield to prevent fading, and mounted onto large cover slips.

**Fluorescent Microscopy**

Ovaries were fixed and stained through immunohistochemistry as described in the previous section. After ovaries were stained, they were imaged through fluorescent microscopy on the confocal microscope (Zeiss LSM 710 System with an Axio Observer). In order to create an MSY2 expression pattern profile, ovaries were viewed under 63X magnification and the
“field of most oocytes” was located. A single image for each ovary was captured and scored to create the profile. For each ovary, an expression pattern profile was also created for SYCP3 by viewing the ovaries under 63X magnification and taking two cores of four images each, with the images separated by 20 microns. In order to score ovaries treated in organ culture, z-stacks were created under 63X magnification as well. Stacks consisted of 11 images, each one micron apart, surrounding each center image (Figure 3).

Figure 3
Schematic showing method in which ovaries were analyzed through confocal microscopy. Each ovary was analyzed by taking 2 cores of 4 images each 20 µm apart. Around each image, a stack of 11 images (5 above and 5 below) each 1 µm apart was taken.

Analysis of Meiotic Progression

Ovaries at multiple stages of development were imaged on the confocal microscope and then manually counted. A series of images were taken for each ovary (as explained in previous section) in order to gain a complete representation of the protein expression in the ovary (Figure 3). The images were then processed by examining all of the oocytes present in each section and
analyzing each ovary in several categories. For distinguishing oocytes reaching and arresting at the diplotene stage, the following categories were scored: oocyte number, number of oocytes strongly expressing MSY2 protein, oocytes weakly expressing MSY2 protein, and oocytes not expressing MSY2 protein. To distinguish oocytes reaching the pachytene stage, the expression pattern of SYCP3 was analyzed over a series of developmental stages and the number of oocytes displaying the pattern of expression congruent with pachytene were counted.

**In vitro Ovary Organ Culture**

Ovaries for *in vitro* ovarian organ culture were harvested from 17.5 dpc embryos. The ovaries were harvested in petri dishes filled with Hank’s Balanced Salt Solution. Once the ovaries were dissected from the pups and all excess tissue was removed, the ovaries were transferred onto a filter located in a six-well tissue culture plate kept on ice. Directly before adding the ovaries to the filter, 1.0 ml of cold Hank’s Balanced Salt Solution was added to the well. A total of two ovaries were added to each filter in each well, leaving a maximum of 12 ovaries possible per plate. After all ovaries were harvested and transferred onto their filter, the filters were then transferred to a new sterile six-well tissue culture plate under a sterile hood.

After transferring filters to a new sterile plate, 1.2 ml of warm stock culture media was added to each well under a sterile hood. The media was warmed in a water bath set to 37°C. The stock culture media was prepared using the following solutions: L-ascorbic acid (0.05 mg/ml in DMEM), 10% Albumax stock, 7.5% BSA, penicillin/streptomycin, and DMEM complete media (Invitrogen). The tissue culture plate was then placed in a sterile incubator pre-set at 37°C and 5% CO₂ for one hour to acclimate the ovaries. Following the hour acclimation period, the media was removed from each well and 1.2 ml of fresh media is added. At this point, the stock culture
media was replaced with treatment media. Treatments included: estradiol (β-estradiol ≥98%; Sigma E8875) (10^-6 M in DMSO), progesterone (progesterone ≥99%; Sigma P0130) (10^-6 M in DMSO), and estradiol & progesterone (10^-6 M in DMSO for both). Vehicle controls using DMSO were also included in the treatments (0.001% DMSO and 0.002% DMSO). Once the treatment media was added, tissue culture plates were placed back into the incubator for 22 to 26 hours. Treatment was replaced in the same manner for the following three days and then terminated on the fifth day.

In order to terminate the organ culture, media was removed from each well and replaced and washed with warm Hank’s Balanced Salt Solution. Once rinsed, ovaries were removed from the filters and placed into fixative (5.3% formaldehyde in 1X PBS) overnight at 4°C. Ovaries were then stained with MSY2, SYCP3, TRA98, VASA, TOTO3, or PI (or combination of multiple antibodies) as described earlier in the immunohistochemistry section.

**Analysis of Oocyte Development and Cyst Breakdown**

After ovary samples were collected, treated in organ culture, stained through immunohistochemistry, and imaged through confocal microscopy, as described in previous sections, the ovaries were scored for the following categories: oocyte number, cyst breakdown, follicle development, and meiotic progression. A total of eight images per ovary and a total of eight ovaries per developmental stage were scored and averaged together. Oocyte number was collected by counting the total number of visible oocytes that were present within the field of the image. More than 50% of the nucleus had to be within the field of view in order to be scored. Cyst breakdown was analyzed by scoring the number of single oocytes versus the number of oocytes still in cysts. The numbers were converted into percentages and averaged. In order to
distinguish the different stages of follicle development, follicles were observed and scored based on the developmental status. Follicles were either scored as primordial, germ cell surrounded by flattened granulosa cells, primary, germ cell surrounded by a layer of cuboidal granulosa cells, or secondary, germ cell surround by multiple layers of cuboidal granulosa cells. Finally, the oocytes were scored for meiotic progression through the analysis of either MSY2 (diplotene) or SYCP3 (pachytene). Expression of either MSY2 or SYCP3 was analyzed and counted to determine the meiotic staging of each oocyte.

**Statistical Analysis**

Unpaired, two-tailed t-tests were performed to analyze the effects of hormone treatments through organ culture on oocyte number, cyst breakdown, follicle development, and meiotic progression. T-tests were performed through Microsoft Excel 2013 with a p<0.05 considered as significant.

One-way ANOVA’s were performed to analyze the difference in expression of meiotic markers MSY2 and SYCP3 across the developmental timeline, variance between pairs of ovaries of littermates, and the difference between *in vivo* and *in vitro* ovarian samples. GraphPad PRISM statistical software was used to perform ANOVA’s with Bonferroni’s (<5 groups) and Newman-Keuls (≥5 groups) Multiple Comparison posttests indicating p<0.05 as significant.
Chapter 3: RESULTS

MSY2 Expression Correlates with Diplotene Arrest

In order to follow and stage meiotic progression, specific meiotic markers were chosen to create expression profiles. First, MSY2 expression was used to identify oocytes at the diplotene stage of meiotic prophase I. MSY2 expression was analyzed within the female germ cells over the developmental period starting from 15.5 dpc through PND 5. Each oocyte was counted and marked as either strongly expressing or weakly expressing MSY2 (Figure 4). Strong expression of MSY2 within an oocyte indicates that the cell has reached and arrested at the diplotene stage of meiotic prophase I. Asymmetric expression of MSY2 was observed within the same ovarian cysts in multiple confocal sections.

Figure 4
Asymmetric expression of MSY2 protein in wildtype ovaries. Image shown is a confocal microscope section from a PND1 wildtype ovary. (A) MSY2 expression labeled in green. (B) Nuclei of all cells labeled with propidium iodide in red. (C) Overlay of both MSY2 (green) and propidium iodide (red) channels. Asterisk indicates an oocyte strongly expressing MSY2. Square indicates an oocyte with weak MSY2 expression. The arrow and arrowhead are showing an example of asymmetric expression of MSY2 within the same cyst. The arrow indicates an oocyte with weak MSY2 expression within the cyst and the arrowhead indicates an oocyte with strong MSY2 expression within that same cyst. Scale bar = 20 µm.
Once all sections were counted and analyzed for MSY2 expression, an expression profile was created for the developmental stages of 15.5 dpc through PND5 (Figure 5). Strong expression of MSY2 was seen to be at ~40% as early as 15.5 dpc and the percent of germ cells strongly expressing MSY2 increased over the developmental time points. At PND5, almost all (~94%) oocytes were strongly expressing MSY2. The increase in oocytes strongly expressing MSY2 correlates with expected increase in oocytes arriving at and arresting at the diplotene stage of meiotic prophase I.

Figure 5
MSY2 expression correlates with the progression of oocyte development to the diplotene stage of meiotic prophase I. Percent of oocytes with strong MSY2 labeling in ovaries (+/-SEM) from 15.5 dpc through PND5. Different letters indicate a significant difference between groups (P<0.05; n = 8 ovaries per developmental stage) as determined by a one-way ANOVA followed by Newman-Keuls multiple comparisons test.
SYCP3 Expression Correlates with the Pachytene Stage of Meiotic Prophase I

A second expression profile, using SYCP3, was created to follow and stage meiotic progression. SYCP3 expression was analyzed within the female germ cells over the developmental period starting from 13.5 dpc through PND 5. Each oocyte was analyzed through the SYCP3 expression pattern to be either in the pachytene stage of meiotic prophase I or not (Figure 6). Expression of SYCP3 in the pachytene stage is found as condensed “strings” between homologous chromosomes.

**Figure 6**
Confocal microscope section showing expression pattern of SYCP3 in PND1 wildtype ovary. (A) SYCP3 expression labeled in green. (B) Nuclei of all cells labeled with propidium iodide in red. (C) Overlay of both SYCP3 (green) and propidium iodide (red) channels. Circled oocytes show expression pattern of SYCP3 in pre/post pachytene stage. Arrowhead is showing expression pattern of SYCP3 in an oocyte in the pachytene stage. Scale bar = 20 µm.

After the number of pachytene staged oocytes was counted, a profile was created reporting the percent of oocytes observed in pachytene (Figure 7). Oocytes in the pachytene stage were not seen until 16.5 dpc. After 16.5 dpc, an increase in the number of pachytene labeled oocytes increased until PND1 where a peak of ~55% was seen. Starting at PND2, the number of oocytes at the pachytene stage significantly decreased and no cells were observed to be in pachytene after PND3.
Examining the Effects of Steroid Hormones on Meiotic Progression by Analysis of a Diplotene Arrest Marker

The effects of exogenous hormone exposure on cyst breakdown, follicle formation, and meiotic progression of developing ovaries was tested through treatment of estradiol and/or progesterone. 17.5 dpc ovaries were harvested and grown in vitro using an organ culture system. Ovaries were grown for five days (four 24 hour periods) in various treatment groups (0.001% DMSO vehicle, 0.002% DMSO, $10^{-6}$ M estradiol, $10^{-6}$ M progesterone, and $10^{-6}$ M estradiol + progesterone) until they reached PND3 (Figure 8A). Once at PND3, ovaries were fixed and

Figure 7
Percent of oocytes in the pachytene stage of development in ovaries from 13.5 dpc through PND5. Staging of oocytes determined through SYCP3 expression pattern (+/-SEM). Different letters indicate a significant difference between groups (P<0.05; n = 8 ovaries per developmental stage) as determined by a one-way ANOVA followed by Newman-Keuls multiple comparisons test.
stained with MSY2 and TRA98 (germ cell marker) through immunohistochemistry and then analyzed through confocal microscopy (Figure 8B). Analysis was done by taking two cores of four images as explained in the previous section (Figure 3).

Once imaged through confocal microscopy, ovaries were analyzed for oocyte number, cyst breakdown, follicle development, and meiotic progression. Estradiol and progesterone appeared to have no effect on oocyte number or follicle development (Figure 9A and 9C). However, the treatments of estradiol alone and estradiol with progesterone did significantly disrupt cyst breakdown and decrease the percent of single oocytes in each section (Figure 9B).
Figure 9
Effects of estradiol and progesterone on oocyte development in organ culture examining diplotene arrest from first series of organ culture experiments. (A) Number of oocytes per confocal section after 5 days in culture with varying treatments (+/-SEM). (B) Percent single oocytes after 5 days in culture with varying treatments (+/-SEM). Asterisk indicates significant difference from control (P<0.05) as determined by a two-tailed T-test. (C) Percent of follicles at different stages of development after 5 days in culture with varying treatments. n = 8 ovaries per treatment group. Three replicates were performed.
Following the *in vitro* organ culture experiment, oocytes were marked as either expressing MSY2 strongly, weakly, or not at all in order to analyze the effects of hormone exposure on meiotic progression. Progesterone significantly lowered the percent of oocytes strongly expressing MSY2 and significantly increased the percent of oocytes with no MSY2 expression (*Figure 10*). Estradiol as well as both hormones together did not have a significant impact on MSY2 expression.

*Figure 10*
Progesterone but not estradiol reduces MSY2 expression. Graph showing the percentage of oocytes expressing MSY2 strongly, weakly or not at all (+/-SEM). Asterisk indicates significant difference from control (P<0.05; n = 8 ovaries per treatment group) as determined by a two-tailed T-test.
Examining the Effects of Steroid Hormones on Meiotic Progression by Analysis of Pachytene Oocytes

Another set of in vitro organ culture experiments were conducted using the same protocol as previously described in order to analyze the effects of exogenous hormone exposure on developing ovaries using a marker for the pachytene stage. Again, 17.5 dpc ovaries were harvested and grown in vitro using an organ culture system. Ovaries were grown for five days (four 24 hour periods) in various treatment groups (0.001% DMSO vehicle, 0.002% DMSO, $10^{-6}$ M estradiol, $10^{-6}$ M progesterone, and $10^{-6}$ M estradiol and progesterone) until they reached PND3 (Figure 11A). Once at PND3, ovaries were fixed and stained with SYCP3 and VASA (germ cell marker) through immunohistochemistry and then analyzed through confocal microscopy (Figure 11B). Ovaries were analyzed with the same protocol (Figure 3).

Figure 11
Organ culture with hormone treatment analyzed by SYCP3 expression experimental set up. (A) Timeline of organ culture experiment procedure. (B) Representative confocal section labeled with VASA, oocyte marker (red), SYCP3 (green), and TOTO3, nuclear marker (blue) showing pachytene expression pattern. Scale bar = 20 µm.
Neither estradiol nor progesterone were seen to have an effect on cyst breakdown or follicle development in the second series of organ culture experiments, but treatment with both estradiol and progesterone did significantly decrease the number of oocytes per section (Figure 12). When results are compared to in vivo data of the same developmental stage, there appears to be a promotion of development in ovaries in vitro. Number of oocytes per section is reduced and the percent of single oocytes is increased in vitro when compared to in vivo.
Figure 12
Effects of estradiol and progesterone on oocyte development in organ culture examining germ cell progression to the pachytene stage from second series of organ culture experiments. (A) Number of oocytes per confocal section after 5 days in culture with varying treatments (+/-SEM). Asterisk indicates significant difference from control (P<0.05) as determined by a two-tailed T-test. (B) Percent single oocytes after 5 days in culture with varying treatments (+/-SEM). (C) Percent of follicles at different stages of development after 5 days in culture with varying treatments. n = 8 ovaries per treatment group. Three replicates were performed.
Following the second set of *in vitro* organ culture experiments, oocytes were labeled with SYCP3 and marked as either in pachytene or pre/post pachytene in order to analyze the effects of hormone exposure on an earlier stage of meiotic progression. Ovaries treated with estradiol and progesterone together had a significant increase in the percent of oocytes in the pachytene stage suggesting that progression through meiotic prophase I was delayed (Figure 13).

**Figure 13**
Estradiol and progesterone have a combined effect on the number of oocytes at pachytene. (A) Graph showing the percentage of oocytes in the pachytene stage as determined by SYCP3 expression pattern (+/-SEM). Asterisk indicates significant difference from control (P<0.05; n = 8 ovaries per treatment group) as determined by a two-tailed T-test.
No Variance Observed Between Pairs of Ovaries from Litter Mates

In order to further justify the results achieved and check for any potential error in experimental procedure, variance between pairs of ovaries from PND1 litter mates was analyzed. Ovaries are normally pooled together from litter mates when conducting experiments creating one set of data. There was no variance observed in the number of oocytes per section, cyst breakdown, or follicle development between pairs of ovaries from litter mates. No significant difference between pairs was observed in the average number of oocytes per section, the average percent of single oocytes, or the average percent of follicles at different developmental stages (Figure 14).
No significant variance is observed between pairs of ovaries from individuals within the same litter. (A) Number of oocytes per confocal section in separate pairs of ovaries (+/-SEM). (B) Percent single oocytes in separate pairs of ovaries (+/-SEM). (C) Percent of follicles at different stages of development in separate pairs of ovaries (+/-SEM).
**Estradiol Confirmed to Disrupt Cyst Breakdown in Later Stage Ovaries**

Previous studies found estradiol to disrupt cyst breakdown and follicle formation to a more substantial degree when conducting organ culture experiments starting with PND1 ovaries compared to current experiments starting at 17.5 dpc (Chen et al., 2007). Verification of previous results was needed in order to determine if the lesser effect was due to a change in experimental design or because of researcher error in performing experiments. Confirmation of results was achieved through an organ culture experiment starting with PND1 ovaries and growing them *in vitro* for five days to reach PND5 (**Figure 15A**). Treatment with estradiol ($10^{-6}$ M in DMSO) disrupted cyst breakdown and follicle formation (**Figure 15B**).

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**Figure 15**
Estradiol disrupts cyst breakdown in PND1 ovaries cultured for 5 days. (A) Timeline showing treatment process of organ culture experiment. (B) Representative confocal sections of PND5 vehicle control and estradiol treated ovaries. Ovaries were fixed and stained with MSY2 (red), TRA98, oocyte marker (green), and TOTO3, nuclear marker (blue). Scale bars = 20 µm.
When analyzed through confocal microscopy, there was a significant decrease in the number of oocytes per section and in the percent of single oocytes, but no significant change in follicle development (Figure 16A-C). When looking at the effect on meiotic progression through MSY2 analysis, a significant increase in the number of oocytes not expressing MSY2 at all was observed suggesting a potential delay in meiotic progression (Figure 16D).
**Organ Culture System has a Negative Effect on Ovarian Health**

To determine any potential negative effects the organ culture system or the vehicle control of DMSO may have had on ovarian health, comparisons of treatment groups and *in vivo* ovaries of the same age were observed. When comparing *in vivo* control, *in vitro* control, and *in vitro* DMSO treated ovaries, there is only a significant difference observed between the *in vivo* and *in vitro* groups. Ovaries grown in an organ culture system have a significantly lower number of oocytes per section and higher percent of single oocytes with no change in follicle development (*Figure 17A-C*). Analysis of meiotic progression using MSY2 expression only showed a difference in the *in vitro* control with a significant decrease in the percent of oocytes strongly expressing MSY2 and a significant increase in the percent of oocytes not expressing MSY2 at all, suggesting the organ culture system significantly delays the progression of oocytes through meiosis (*Figure 17D*).
DMSO does not have a negative effect on ovaries, but organ culture system shows a negative effect on ovarian health. (A) Number of oocytes per confocal section both in vivo and after 5 days in culture with varying treatments (+/-SEM). (B) Percent single oocytes both in vivo and after 5 days in culture with varying treatments (+/-SEM). (C) Percent of follicles at different developmental stages both in vivo and after 5 days in culture with varying treatments. (D) Percent of oocytes expressing MSY2 strongly, weakly or not at all both in vivo and after 5 days in culture with varying treatments (+/-SEM). Different letters indicate a significant difference between groups (P<0.05; n = 8 ovaries per treatment group) as determined by a one-way ANOVA followed by Bonferroni’s multiple comparisons test.
Chapter 4: DISCUSSION

Whole mount immunohistochemistry of ovarian samples was successfully used to characterize progression of female germ cells through meiotic prophase I. MSY2, a germ cell specific maternal mRNA binding protein, has been shown to be upregulated in oocytes reaching and arresting at the diplotene stage (Gu et al., 1998). The use of MSY2 as a marker for diplotene arrest was shown through the analysis of YBX2 mRNA (bovine MSY2), which showed an increase of mRNA expression in primordial follicles advancing to primary follicles with oocytes at the diplotene stage (Yang and Fortune, 2008). It was also originally found and then further confirmed that oocytes begin to enter diplotene arrest at 17.5 dpc. In order to link MSY2 expression with diplotene arrest, MSY2 mRNA levels were analyzed across the developmental period from 13.5 dpc through PND5 and it was shown that MSY2 expression does not significantly increase until 18.5 dpc which correlates with the timing of diplotene arrest (Dutta and Pepling, unpublished). However, in the present experiments, when looking at the MSY2 characterization through whole mount antibody staining, it was shown that at 15.5 dpc, 41% of oocytes were already strongly expressing MSY2. This level of expression is much higher than expected given that diplotene arrested oocytes were not even present until 17.5 dpc and MSY2 mRNA expression did not increase until 18.5 dpc. The expression of MSY2 did increase over development correlating with the idea of MSY2 upregulation during diplotene arrest beginning with the first significant increase at PND1, and almost all oocytes strongly expressing MSY2 by PND5 (94%). This finding suggests that MSY2 is not actually a marker for diplotene arrest, but rather its increase in expression over the developmental time period correlates with progression of oocytes to the diplotene stage. SYCP3, a protein found in SCs, is involved with chromosome pairing and recombination (Meuwissen et al., 1992). This protein has been widely used in the
field of reproduction as a marker for meiotic prophase due to its distinct expression pattern during the pachytene stage (Grive et al., 2016). However, most work looks at the expression of SYCP3 at the cellular level and not in whole mount. Through whole mount antibody staining of ovarian samples, I established that the highest percentage of oocytes found in pachytene was 54% at PND1, which then significantly decreased to 12% at PND2 (Figure 7). This result correlates with previous work showing the temporal expression pattern of the pachytene stage (Borum, 1961).

It should be noted that when imaging ovaries that were stained through immunohistochemistry through confocal microscopy, the intensity of the laser did not remain constant for each image taken. When moving deeper into the ovary, the intensity of the laser needed to be increased in order to visualize the staining of the protein. Laser intensities were observed and there was no significant change of laser intensity variance from one developmental age to the next.

Previous research has shown that estrogens and progesterone have negative effects on the development process of female germ cells. Progesterone and estradiol, phytoestrogen genistein, as well as synthetic estrogens all disrupt cyst breakdown and follicle formation (Chen et al., 2007; Jefferson et al., 2006; Karavan and Pepling, 2012) thereby leading to a potential decrease in viable egg cells later in life. In some instances, the number of oocytes present was also affected, but not as consistently. In the work presented in this thesis, two series of organ cultures were completed, each treating ovaries with either estradiol (10^{-6} M), progesterone (10^{-6} M), or estradiol and progesterone (10^{-6} M) with little to no effect on cyst breakdown and follicle formation. In the first series of organ cultures, no effect was seen on oocyte number or follicle development, but there was a small but significant decrease in the percent of single oocytes in
both the estradiol alone and estradiol + progesterone groups indicating a disruption of cyst breakdown. In the second series, there was no significant effect on the percent of single oocytes or follicle development, but there was a significant decrease in the number of oocytes in the estradiol + progesterone treatment group. Similar results were expected for each set of experiments and the difference in results could be due to a lower average number of oocytes present in the second series of organ cultures. Also, even though there was a significant disruption of cyst breakdown in some of the treatment groups of the first series, the result was much less severe than previously reported. The studies conducted prior to these organ culture experiments were initiated on PND1 ovaries, whereas the current studies were started earlier with 17.5 dpc ovary samples. This earlier starting point may be out of the window of sensitivity, which would lead to little or no visible effects (Dutta et al., 2016). Also, when comparing in vivo ovaries similar in developmental age to ovaries treated through organ culture, there does appear to be a noticeable decrease in the number of oocytes and increase in the percent of single oocytes suggesting either an unfavorable organ culture system environment or a potential acceleration of development caused by the organ culture system.

Another aspect of the organ culture experiments that was analyzed was the effects of estrogen and progesterone on meiotic progression. Again, two series of organ cultures were completed with the first series being labeled for MSY2, marker for diplotene, and the second series being labeled with SYCP3, marker for pachytene. Previous research has shown that when pregnant female mice were exposed to Bisphenol A (BPA), an estrogenic chemical, meiotic progression was disrupted by way of a disturbance of the synapsis and recombination of chromosome homologs (Susiarjo et al., 2007). When PND1 ovaries of rats were treated with estradiol in an organ culture system, primordial follicle assembly was slightly delayed and
transition from primordial to primary follicles was inhibited showing a disruption in development (Kezele and Skinner, 2003). However, the major finding was actually that progesterone showed a much stronger effect on follicle assembly. Another study showed that progesterone acts through the progesterone receptor membrane component 1 (PGRMC1) to significantly delay or completely disrupt meiotic progression and therefore disrupt primordial follicle assembly (Guo et al., 2016). The results of my organ culture series agreed with these findings. In the first series of cultures, strong MSY2 expression (marking cells arrested at diplotene) was significantly decreased in only the progesterone treatment and the oocytes not expressing MSY2 at all was significantly increased in both the estradiol treatment and the progesterone treatment indicating a delay in meiotic progression. In the second series, the number of oocytes found in the pachytene stage significantly increased only in the estradiol + progesterone treatment group indicating that treatment with both hormones delayed meiotic progression.

Given some of the unexpected results discovered during the organ culture experiments, it was vital to check a number of different potential causes of error including the use of different littermates across litters and the impact of comparing developmental processes using explanted ovaries into organ cultures and ovaries harvested from whole animals.

Data collected for organ culture experiments came from pools of ovaries that were combined from multiple different litters. Checking for any variance between ovary pairs of different individuals from the same litter would eliminate the possibility of skewed data if no variance was found. Three pairs of ovaries were compared against each other with no variance found justifying the pooling of ovaries from multiple individuals.
Moreover, the effects of estradiol treatments at $10^{-6}$ M on ovaries through an organ culture system had been shown to significantly lower the percent of single oocytes and delay the progression of follicle development while having no effect on the number of oocytes (Chen et al., 2007). Since the effect seen in organ culture here was less severe and the only difference from previous work was the age of the ovary when it entered culture (PND1 vs 17.5 dpc), we repeated the organ culture to confirm that estradiol would block cyst breakdown if started at PND1. Results were only partially replicated with a significant decrease in the percent of single oocytes (though much less severe than the published studies from our lab). The number of oocytes present was significantly decreased in my experiment and also the follicle development was not significantly decreased. We went a step further and looked at the effect of estradiol on meiotic progression through the analysis of MSY2 expression and found that there was a significant increase in the number of oocytes not expressing MSY2 at all which would indicate a delay in meiotic progression. The result combined with the positive result of cyst breakdown disruption indicates that there is no issue with the estradiol. However, the significant decrease in number of oocytes present within the ovary leads to the assumption that there may be an issue with the organ culture system and its ability to maintain the health of the ovaries during culture experiments. Previous studies have shown that when working with a slightly different organ culture system, ovaries treated for seven days with estradiol and progesterone had no visible decline in oocyte number (Chen et al., 2007) and ovaries treated with genistein even had an increase in oocyte number (Jefferson et al., 2006), further confirming this assumption. Finally, the addition of DMSO to the media (solvent for estradiol) could have potential negative effects on ovarian health. Comparing DMSO vehicle controls to an organ culture control and an in vivo control would indicate any potential harmful effects of the DMSO. When looking at oocyte
number, percent single oocytes, and follicle development, there was no significant difference between DMSO vehicle controls and the organ culture control indicating that the DMSO did not have any detrimental effects. However, all three organ culture controls (two DMSO vehicle controls and organ culture control) were significantly different from the in vivo control in all three categories. This again indicates a potential problem with the organ culture system itself and its maintenance of ovarian health. Also, when looking at the effects on meiotic progression through analysis of MSY2 expression, the only group to be significantly different was the organ culture control (significant decrease in percent of oocytes strongly expressing MSY2 and significant increase in the number of oocytes not expressing MSY2 at all).

In summary, protein characterization of female germ cells through whole mount immunohistochemistry is a powerful tool for the field of reproduction and allows researchers to gain a complete picture of the ovary and its development. Researchers can visualize the expression of the protein of interest while also getting a clear picture of ovarian development through analysis of cyst breakdown, follicle formation, follicular development, and meiotic progression. Using this technique, characterization of two meiotic markers, MSY2 and SYCP3, was completed and can now be used to analyze the effects of other pathways involved with germ cell development, such as KIT signaling, or environmental factors, such as BPA and other endocrine disrupters. Here these two protein profiles were used to analyze the effects of exogenous hormone exposure and it was determined that estradiol and progesterone disrupt cyst breakdown and that progesterone significantly delays/disrupts meiotic progression of female germ cells.

In the future, work needs to be done investigating the organ culture system used in order to further optimize growing conditions and improve ovarian health while in the system. Once the
system is optimized, treatments of estradiol and progesterone should be repeated to confirm findings. Also, further investigation into the pathway that progesterone is acting on is required in order to better understand the effects of progesterone specifically on meiotic progression of female germ cells. Finally, with the use of the protein characterization profiles created here, further investigation into other pathways and environmental factors that may affect cyst breakdown and follicle formation can be completed through the use of the organ culture system and then analyzed with the profiles.
APPENDIX 1:

Arrest at the diplotene stage of meiotic prophase I is delayed by progesterone but is not required for primordial follicle formation in mice

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In order to assist in the research investigating diplotene arrest and the effects of steroid hormones on meiotic progression, I imaged and analyzed hematoxylin and eosin (H&E) stained ovarian sections and counted them for the number of oocytes found to be in the diplotene stage, the number oocytes found to be in pre-diplotene stages, and the percent of oocytes found to be in follicles for PND3, 4, and 5. Images of H&E sections taken by myself were used for Figure 3 (see attached article). Data collected for PND3, 4, and 5 were used in graphs for Figures 1 and 2 (see attached article).
REFERENCES


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Syracuse University; Syracuse, NY
2013 – Present

• The Effects of Exogenous Hormones on the Progression of Oocytes through Prophase I of Meiosis

Undergraduate Researcher: Department of Biology
University of Wisconsin Whitewater; Whitewater, WI
2011 – 2013

• Effects of the Ultraviolet Filter Benzophenone on the Growth of Soy Bean and Corn

Undergraduate Researcher: Department of Mathematics and Computer Science
University of Wisconsin Whitewater; Whitewater, WI
2010 – 2011

• Exploring the common ground between high school and college mathematics
• Investigating new and improved methods to improve success rates in entry level college mathematics
TEACHING HISTORY

Teaching Assistant, Syracuse University, Syracuse, NY 2014 – 2016
- Biology 425: Cell and Developmental Biology Laboratory Spring 2015, 2016
- Biology 327: Cell Biology Fall 2015
- Biology 121: Introduction to Biology (Semester 1) Fall 2014

PUBLICATIONS


PROFESSIONAL PRESENTATIONS

Cornell University; Ithaca, NY 2016
Seminar: The Effects of Exogenous Hormones on the Progression of Oocytes through Prophase I of Meiosis

Society for the Study of Reproduction Conference; San Diego, CA 2016
Poster: The Effects of Exogenous Hormones on the Progression of Oocytes through Prophase I of Meiosis