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Progesterone Signaling in Mouse Oocyte Development via the PGRMC1 and PGRMC2 Receptors and the PAIRBP1 Protein

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Abstract

There are thought to be many different causes that can lead to the development of infertility in females. Many of the causes for this condition present themselves before birth, which can predetermine a woman's fertility capability before she is even born. The complex process of germ cell development possesses possibilities for error and malfunction that can have permanent, lasting effects on a developing fetus. The process of oocyte development is initiated when primordial germ cells migrate to the embryotic gonad and begin dividing mitotically. As mitosis proceeds, the process of cytokinesis does not go to completion, which maintains the cells in cysts. The fundamental process of cyst breakdown occurs, in conjunction with apoptosis, in order to separate cysts into individual oocytes surrounded by a number of granulosa cells, known as primordial follicles. The success of the process of cyst breakdown is thought to be a valuable determinant of the overall pool of viable primordial follicles that could be used by a female for fertilization in the future. This developmental process is well conserved across mammalian species, such as mice and humans.

Hormones are known to play a role in the process of germ cell development via various signaling pathways. Abundant research has already been conducted to investigate the role of estrogen in this developmental process, specifically exploring the hormone's ability to inhibit cyst breakdown. Similar research has demonstrated progesterone's ability to inhibit cyst breakdown, as well, but much more is yet to be investigated about this steroid hormone. Although specific progesterone signaling receptors, such as progesterone receptor membrane component 1 (PGRMC1) and PGRMC2, and proteins, such as the plasminogen activator inhibitor 1 RNA-binding protein (PAIRBP1), have been identified, their regulatory functions and interactions within their prospective signaling pathways have yet to be well researched. In this study, we investigated where PGRMC1, PGRMC2 and PAIRBP1 were expressed in the fetal and neonatal mouse ovary via immunocytochemistry. We also investigated when PGRMC1, PGRMC2 and PAIRBP1 were most strongly expressed in the mouse ovary across germ cell development via western blotting.

The results demonstrate that the expression of PGRMC1 and PAIRBP1 localize to the same cells within the mouse ovary, but exhibit different levels of expression across fetal and neonatal mouse development. In regards to PGRMC2, the results reveal a complete lack of expression of this membrane receptor in the mouse ovary at any time throughout the process of germ cell development. In order to validate and expand on these results future studies will need to be conducted to gain more information about the independent and interactive functions of PGRMC1, PGRMC2 and PAIRBP1 in germ cell development.

Table of Contents

Executive Summary

It may be difficult to believe, but reproductive development in mammals begins while the developing fetus is still in utero. At this time, a female will go through a complex process in which all of her oocytes will be produced and maintained in her ovaries. This is a critical mammalian characteristic for females, considering that they are not capable of producing any more oocytes beyond this developmental stage. Once the process of oocyte development is complete, the pool of oocytes will remain dormant until a female reaches reproductive maturity and is capable of releasing a viable egg for fertilization. However, the process of fertilization is not always successful and if a woman experiences difficulty conceiving a child for an extended period of time she could be considered infertile. There are various factors that can contribute to or cause infertility, with one of the main considerations being that a dysfunction in the process of oocyte development while in utero could have a prolonged effect on a woman's fertility.

The process of oocyte development, also known as germ cell development, is very complex in nature, involving a wide variety of mechanisms and signaling pathways in order to proceed normally. It is known that several steroid hormones play an integral role in mediating this process, by binding to specific receptors that will activate corresponding pathways that will carry the signal until it reaches its destination and the intended response is elicited. Estrogen is a hormone that has been thoroughly researched and is known to be involved in the process of oocyte development. However, research has also

demonstrated that exposure to excess levels of this hormone can cause a glitch in the process, as well. Excess levels of estrogen in the developing ovary can lead to a deficit in the final number of viable oocytes that a female fetus can possess for future reproductive purposes.

Progesterone is another hormone that is known to play a role in the process of oocyte development, but the information that is available regarding the mechanisms and pathways through which progesterone is transmitted is staggeringly less than what is available on estrogen. Some previous research has shown that exposure to excess levels of progesterone can have a similar effect that estrogen has on the developing oocytes. Still, considering how much less is known about progesterone than estrogen, this research focuses on learning more about the role of progesterone throughout oocyte development, specifically looking at three components of progesterone signaling pathways. We wanted to concentrate on the progesterone receptor membrane component 1 (PGRMC1) receptor, PGRMC2 receptor and the plasminogen activator inhibitor 1 RNA-binding protein (PAIRBP1), which all play a role in the progesterone signaling pathway, but are in desperate need of further investigation.

Since the process of oocyte development is reasonably similar in mice and humans, mice were used as the model organism for this research. Male and female mice were mated each the beginning of each week and female mice were checked for vaginal plugs each day prior in order to determine the exact date on which gestation began. Fetal mice at 15.5 days post coitum (dpc) and 17.5 dpc, also known as days after conception, and neonatal pups at post-natal day (PND) 1,

PND 3 and PND 5 were dissected in order to obtain their ovaries for various experiments.

The fetal and neonatal mouse ovaries were used for two main experimental techniques, one of which was immunocytochemistry, or ovary staining. This process involved treating the dissected ovaries with a primary antibody that would bind to either the receptor or protein of interest that is thought to be expressed in the tissue. The ovaries were then treated with a secondary antibody, containing a fluorescent tag, which would bind to the primary antibody. The ovaries were also treated with propidium iodide, which would stain the nuclei of all of the cells in the tissue. Once the staining procedure was complete, the ovaries were transferred to a slide and were viewed using the confocal microscope. A confocal microscope is a type of microscope that uses lasers that are specific to the wavelength at which the tag on a secondary antibody fluoresces to infiltrate material on a slide. Since the primary-secondary antibody complex is bound to the receptor or protein of interest, it is possible to view the expression of the given molecule within the mouse ovary using the confocal microscope and to take images in order to compare expression and localization patterns across different stages of oocyte development. Another experimental technique used for this research was western blotting, which serves to identify specific proteins within a sample. Dissected ovaries were homogenized in solution, where they were called ovary extracts. The extracts from different fetal and neonatal ages were loaded into wells on a gel in chronological order. The molecular components of the extracts were then separated by molecular size as they were pushed down

3

the gel by an electric current; a process called gel electrophoresis. The results from the gel were then transferred to a membrane, which was incubated with a primary and secondary antibody that bind in the same manner as discussed with immunocytochemistry. The primary-secondary antibody complex allowed the receptor/protein of interest to be detected as bands on the membrane. Whereas any insignificant molecules found in the extracts were suppressed using a specific solution that functions to block irrelevant bands.

The results obtained from the immunocytochemistry and western blot experiments were relatively variable in nature. The images coinciding with the immunocytochemistry showed that the PGRMC1 receptor and the PAIRBP1 protein were detected in the surface cells of the fetal and neonatal mouse ovary from 15.5 dpc to PND 5, but not within the oocytes themselves. The immunocytochemistry images also showed no detection of PGRMC2 anywhere within the ovary during that same timeframe. The results from the western blots showed that PGRMC1 was expressed at consistent levels in the mouse ovary from 15.5 dpc to PND 5, but PAIRBP1 showed increased expression as oocyte development proceeds. Similar to the immunocytochemistry results obtained for PGRMC2, there didn't appear to be any expression of PGRMC2 from 15.5 dpc to PND 5.

Given the inconclusive results, future studies will need to be conducted in order to learn more about PGRMC1, PGRMC2 and PAIRBP1 and their dynamic function in the process of oocyte development. The experiments completed here will need to be repeated in order to obtain more conclusive data. Other types of

experiments, such as organ cultures, could be extremely beneficial in investigating the function of these progesterone signaling components and their long-term effect on oocyte development. Organ culture experiments would involve growing ovaries in a completely sterile environment for several days in a special solution that will block the function of the corresponding receptor/protein as the ovaries develop. Once the organ culture is complete, the ovaries can be stained using the immunocytochemistry protocol in order to determine how the function-blocking mechanism affects the overall number of individual oocytes that are present at the end of oocyte development.

The field of research that is dedicated to exploring the role of progesterone in oocyte development and the hormone's corresponding signaling pathways is expanding and will hopefully continue to grow in the coming years. Recent research has focused more on how progesterone receptors, such as PGRMC1 and PGRMC2, play a role in cancer, which is of crucial importance. However, continued research revolving around progesterone signaling receptors and proteins could assist in a better understanding of the complex process of oocyte development and the potential infertility conditions that could arise if a malfunction occurs.

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Introduction

  The act of reproducing is an innate human function that is necessary for the survival of our species. The process of reproduction is quite diverse across species, but the overall goal is unanimous; to procreate in order to maintain our existence. In terms of human reproduction, the process is extremely complex and has evolved to signify more than strictly procreation. There are so many minute details and functions that must simultaneously work in unison in order to successfully conceive a child that it is no surprise that infertility is a prominent issue that people are continuing to struggle with.

  Roughly 12% of women in the United States, who are at reproductive age, face difficulties with fertility (Chandra et al., 2006). In retrospect, this is a significant proportion of women that are experiencing fertility issues, often times not knowing exactly what the cause is or how to go about fixing it. A major concern to consider is the fact that women produce their complete supply of viable eggs long before they even consider reproducing. With no ability to produce any more eggs after that crucial point in development, it is impossible to realize the reproductive outcome until it is too late (Skinner, 2005). During this process of oocyte development, which is complete and irreversible upon birth, a woman loses a significant percentage of potential oocytes. Even though this rapid decrease in oocyte numbers is observable, it is far from being completely understood or explained. If we had a clearer and more concise understanding of the various processes involved in oocyte development, specifically focusing on

sporadic oocyte loss, then we could potentially gain a better understanding of possible infertility triggers and how to better aid individuals being affected by difficulties with infertility.

Although, ideally, we would prefer to learn more about this complex process in humans, it is more realistic and feasible to study the process of oocyte development in a model organism that is most similar to humans in terms of reproductive qualities. In the Pepling Lab we use the species *Mus musculus* as a model organism for research purposes. Being one of the most closely related model organisms to humans, mice share a number of commonalities with humans in this aspect of development, which makes the results obtained from research somewhat transferable across species. The mouse genome is entirely sequenced, which makes it a reliable organism to use for research purposes since a great deal of background information has already been collected. This fully sequenced genome is also relatively easy to manipulate in order to create a variety of genetically modified, or transgenic, strains of mice to be used for research. The ability to quickly and easily produce transgenic mice is a predominant quality that makes the species so marketable for biological research.

In terms of laboratory use, mice are a relatively easy model organism to contain in a laboratory setting. They are small in size and easily maintainable. They are not a difficult species to breed and their embryonic development is relatively short, lasting 19.5 days on average, with a relatively larger number of pups being produced. The short gestation period, coupled with the larger number of pups being born in relation to other mammals, provides opportunities to

acquire a large amount of data across a short period of time. This turnover rate

would not be possible using other mammalian model organisms that possess

relatively the same similarity to humans, but require a longer gestation period and

give birth to a smaller number of offspring.

As in human females, female mice begin the reproductive process long

before birth. Initially, precursor cells, known as primordial germ cells migrate to

the developing gonad of the fetus at approximately 10.5 days after conception,

also known as days post coitum (dpc) (Pepling, 2006). Upon arrival at the gonad,

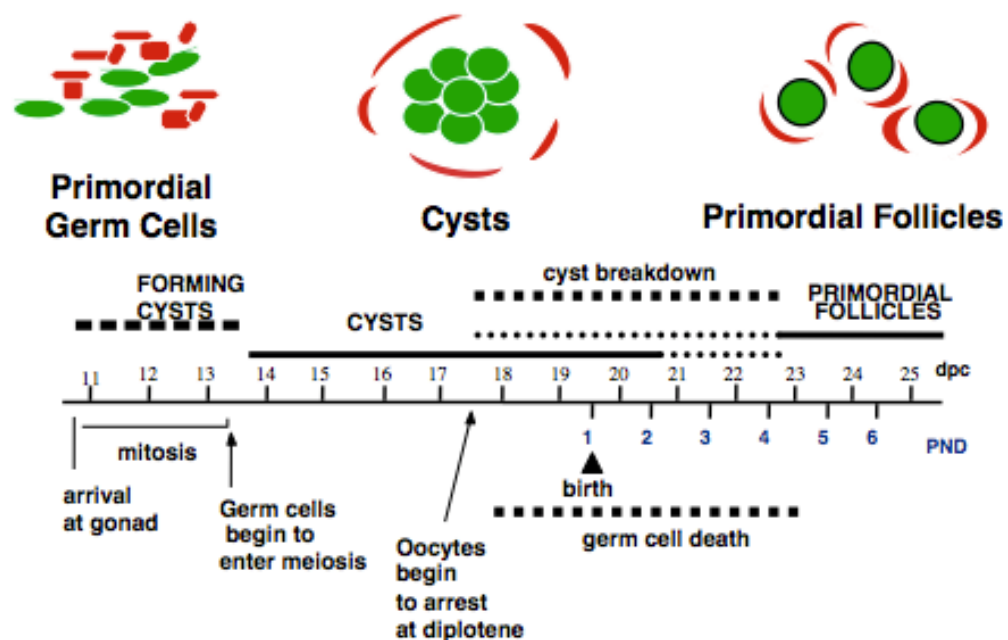the primordial germ cells begin to divide via mitosis, forming clusters

Figure 1: A timeline of mouse germ cell development ranging from 11 dpc to PND 6. Significant developmental stages are labeled, with the green structures representing germ cells and the red structures representing somatic granulosa cells (adapted from Pepling, 2006).

known as germ cell cysts (Pepling and Spradling, 1998). At this stage, the primordial germ cells divide mitotically, where cytokinesis does not go to completion and the cells of the cyst remain connected by intercellular bridges (Pepling et al., 1999). At this point the cells are known as oogonia. Once the process of mitosis is complete, the oogonia begin the process of meiosis at roughly 13.5 dpc, as shown in Figure 1, and become oocytes. However, the meiotic process is then arrested at the diplotene stage of meiosis I. It was thought that the process of meiosis and the consecutive meiotic arrest occur at precisely the same time, across all oocytes, but it has been determined that the meiotic process often does not occur collectively for all oocytes (Pepling, 2006). The oocytes remain in cysts, surrounded by somatic cells, for several consecutive days of development. As shown in Figure 1, at roughly 18 dpc, cyst breakdown is triggered and the compacted germ cell cysts begin to separate. This process of cyst breakdown signals the maturation of the germ cells into individual oocytes. Following the days after birth, also known as post-natal days (PND), cyst breakdown progresses. By PND 5, the majority of the germ cells have matured and formed individual primordial follicles, containing a single oocyte surrounded by a layer of granulosa cells, as shown in Figure 1 (Pepling and Spradling, 2001).

The timing of cyst breakdown coincides with another fundamental process that occurs during oocyte development, germ cell death. As shown in Figure 1, only slightly after the beginning of cyst breakdown is randomized germ cell death, known as apoptosis, initiated. In an older scientific model it is thought that the ongoing process of cyst breakdown is facilitated by continued apoptosis of
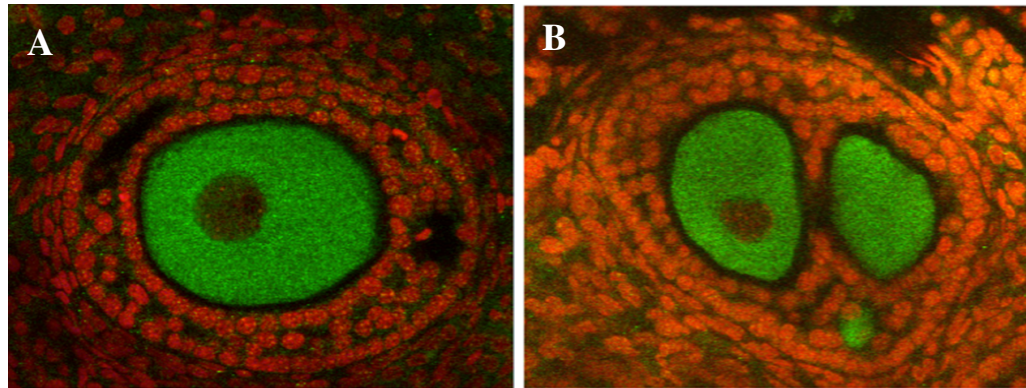
Figure 2: Images depicting the difference between a normal follicle (A) versus a multiple oocyte follicle (B) in the adult ovary. The green staining signifies STAT 3 antibody staining the cytoplasm of the oocytes and the red staining signifies propidium iodide staining the nuclei of the oocytes, as well as the nuclei of the granulosa cells (Pepling, 2006).

cells within the cysts, demonstrating that cell death is essential in order for this process to occur and, in turn, the formation of primordial follicles (Pepling and Spradling, 2001). However, sometimes cyst breakdown does not go to completion and an irregular follicle, known as a multiple oocyte follicle (MOF), forms. Multiple oocyte follicles consist of several oocytes enclosed within a single follicle, surrounded by a layer of granulosa cells, as depicted in Figure 2. These MOFs are thought to contain inviable eggs for future reproduction and, therefore, could potentially contribute to causes of infertility.
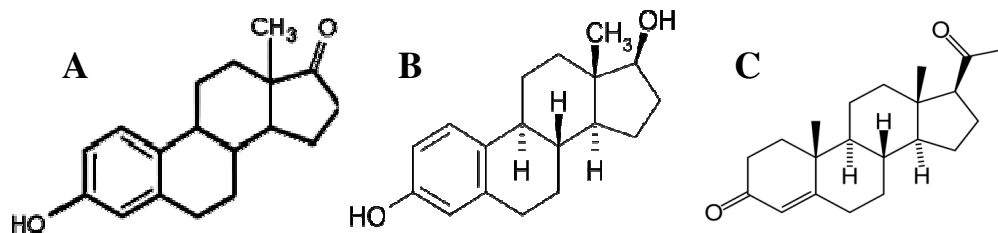

Figure 3: Structures of the hormones discussed, including estrogen (A), estradiol (B) and progesterone (C).

The process of cyst breakdown, coupled with apoptosis, is a critical stage in oocyte development that we are consistently attempting to gain a better understanding of. Prior research has demonstrated that steroid hormones such as estrogen, specifically in the form of estradiol, as shown in Figure 3, possess the ability to inhibit cyst breakdown. This inhibition of cyst breakdown leads to a decreased number of functional oocyte follicles in the ovary, which could, in turn, lead to an increased chance for infertility (Chen et al., 2007; Jefferson et al., 2006). Additional hormones, such as progesterone, have been shown to block cyst breakdown in the neonatal mouse ovary when expressed at high levels, as well. Even though the function of estrogen, in the form of estradiol, on cyst breakdown in female mice has received vast attention, progesterone is also a hormone that produces roughly the same effects (Chen et al., 2007). Estrogen and progesterone individually have the ability to alter the process of cyst breakdown, but these hormones are also known to additively block cyst breakdown when their effects are combined.

Progesterone, as shown in Figure 3, is a naturally occurring hormone that plays a crucial role in a number of different functions throughout the body. Progesterone is known to be involved in hypothalamus and pituitary activity, as well as mammary gland and uterine development. The most significant application of progesterone for research in the Pepling Lab focuses on the role of progesterone in intra-ovarian activity, specifically follicular development. Progesterone continues to play a consistent role in future female reproductive processes, ranging from menstruation to menopause. This demonstrates how

crucial of a contributor progesterone is throughout a woman's lifetime, starting

before birth. In order for progesterone to serve these various functions, a number

of different signaling pathways must be utilized in order to distinguish between

particular functions in which progesterone is necessary at different points in time.
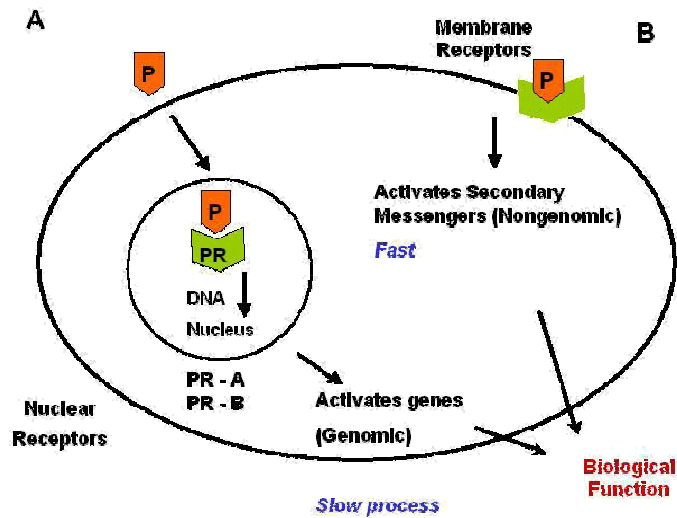

Figure 4: Diagram depicting the potential steps involved in the inhibition of cyst breakdown upon the binding of progesterone to either a nuclear receptor or a membrane receptor. P represents the progesterone signal and the biological function produced in this particular case would be the inhibition of cyst breakdown (Dzadyk, unpublished).

The signaling pathways of steroid hormones vary, but, as with any

signaling pathway, the receptors play a crucial role in the progression of the

signal, whether it is an estrogen or progesterone signaling pathway. As shown in

Figure 4, progesterone signaling can occur in a variety of ways through either

nuclear or membrane receptors that could potentially signal to inhibit cyst

breakdown in the mouse ovary (Peluso, 2006). Nuclear receptors such as

progesterone receptor-A (PR-A) and progesterone receptor-B (PR-B) originate

from the same gene and are translocated to the nucleus upon binding progesterone in the cytoplasm (Pepling, 2012). The translocated receptor/hormone complex activates regions of target genes that will elicit the intended progesterone response via transcriptional and translational mechanisms (Leonhardt et al., 2003). This signaling pathway is thought to take relatively longer than alternative progesterone signaling pathways, seeing as transcriptional and translational mechanisms proceed over a longer period of time.

Another known progesterone signaling pathway involves the use of cellular membrane receptors, known as progesterone receptor membrane components (PGRMCs). Known cellular membrane receptors that play a role in progesterone signaling are PGRMC1 and PGRMC2. Progesterone binds to these receptors, a secondary messenger is activated and a signaling cascade proceeds until the desired biological function is produced. Not nearly as much is known about these cellular membrane receptors as is known about the nuclear receptors, which is why they are of particular interest in this research. However, previous studies have demonstrated that the term cellular membrane receptor is somewhat of a misnomer, because these receptors are not strictly limited to the cellular membrane. Even though they were first discovered in the cellular membrane, they have since been found to be expressed in the nucleus, as well as a number of other intercellular membranes, such as the membranes of endoplasmic reticulum, depending on the type of cell they are localizing in (Wendler and Wehling, 2013). These observations further intrigue researchers to learn more about PGRMC1 and PGRMC2, their origins and applications in mouse oocyte development.

Slightly more information is known about PGRMC1 and more current research is being conducted in order to learn more about the function of this receptor. Although PGRMC1 has been shown to be involved in a number of functions outside of the ovary, it does play a significant role in later stages of oocyte development, leaving the question of whether or not it affects earlier developmental processes, such as cyst breakdown and apoptosis, as well. PGRMC1 acts to mediate progesterone activity and these receptors are a prime candidate due to the fact that they possess such a strong binding affinity for progesterone (Peluso, 2013). PGRMC1 serves as a mediator for the anti-apoptotic function that progesterone expression induces, either enhancing the effect and reducing the number of germ cells lost or reducing the effect if the receptor is inhibited, leading to a spike in apoptotic activity (Peluso et al., 2008). This function of PGRMC1 is important since the coupled processes of apoptosis and cyst breakdown are both influenced by progesterone, with progesterone having an anti-apoptotic effect on the process of germ cell death that coincides with inhibited cyst breakdown. Learning more about the use of the PGRMC1 signaling pathway for the anti-apoptotic effect of progesterone will only help us gain more information regarding progesterone's synchronous effect on cyst breakdown.

Unlike other estrogen and progesterone receptors, research studies on PGRMC1 have found the receptor to localize to a number of different areas, including the nucleus, the cytoplasm and other intracellular membranes in the ovary (Lösel et al., 2008). This localization pattern is conserved within mice and humans, and, specifically in granulosa cells within the ovary, PGRMC1 is

expressed in the plasma membrane, the cytoplasm and sometime the nucleus (Peluso, 2006). Granulosa cells act as protective, nursing cells surrounding the individual oocytes, and since progesterone manipulates granulosa cell function via the PGRMC1 receptor, it allows for the consequential manipulation of oocyte development. Alterations in these PGRMC1-progesterone interactions have been shown to have effects on future female reproductive processes, and, in turn, fertility capabilities.

Even less information is known about PGRMC2, with minimal recent research having been conducted in order to gain more knowledge about the receptor and its functions. The majority of current information available on this receptor has been obtained from studies conducted using rats as the model organism, and the researched presence of PGRMC2 in humans does not explicate that the receptor is strictly present in the female ovary (Cahill, 2007; Chen et al., 2010). Limited information that is known states that PGRMC2 is quite homologous to PGRMC1 (Albrecht et al., 2012). Some research has been conducted studying the effects of PGRMC2 on ovarian cancer cells, and its potential to act as a tumor suppressor (Albrecht et al., 2012). This potential PGRMC2 function differs drastically from PGRMC1 seeing as PGRMC1 is thought to be a tumor promoter when studying cancer cells. Even though some research has investigated the role of PGRMC2 in cancer cells, little information is known regarding the role of PGRMC2 in oocyte development. Previous studies in the Pepling Lab, using immunocytochemistry, have demonstrated that the progesterone receptor, PGRMC2, is present in the fetal mouse ovary at 15.5 dpc

(Dzyadyk and Pepling, unpublished). However, further research looking into the expression of PGRMC2 throughout the entirety of mouse oocyte development will be discussed later on in the paper.

There are several other proteins that are thought to interact with either PGRMC1 or PGRMC2, or both receptors, and researchers are attempting to gain more information about these proteins in order to learn more about the receptors that they work together with. The plasminogen activator inhibitor 1 RNA-binding protein (PAIRBP1) is thought to interact with PGRMC1 in the PGRMC1-progesterone signaling pathway. Since PAIRBP1 does not appear to possess any type of transmembrane domain that could allow the protein to function as a receptor in a signal transduction pathway, PAIRBP1 binds to and uses PGRMC1 as a means to affect the progesterone signaling pathway (Peluso, 2006).

Even though progesterone's effect on cyst breakdown in mouse germ cell development has been discussed, progesterone is also known to have an inhibitory effect on the process of apoptosis. Although the signal mechanism of PAIRBP1 is unknown, the interaction between PAIRBP1 and PGRMC1 is thought to be necessary in order for progesterone to successfully inhibit apoptosis (Peluso et al., 2013). This anti-apoptotic function has a prominent effect on granulosa cells and spontaneously immortalized granulosa cells, specifically in the rat. The fact that PGRMC1 is known to be expressed in numerous areas throughout the granulosa cell, whereas PAIRBP1 has been found to be expressed only in the plasma membrane and cytoplasm of these cells, could be signifying the limitations of PAIRBP1 activity as a component of the progesterone-PGRMC1 signal

transduction pathway that does not affect the cell at a nuclear level. It is believed that there is a particular amino acid sequence in PGRMC1 that is responsible for its binding with PAIRBP1, and studies have been conducted using this amino acid sequence in order to understand its implications on the PGRMC1-PAIRBP interaction and how manipulations of this sequence can affect biological processes on a larger scale (Peluso et al., 2013).

Again, not nearly as much information is known about the potential interaction of the PAIRBP1 protein with PGRMC2. However, since PGRMC2 is a member of the PGRMC family and shares a number of similarities with PGRMC1, it is thought that a similar anti-apoptotic effect is dependent upon an interaction between PAIRBP1 and PGRMC2. The crucial amino acid sequence found in PGRMC1 is known to be roughly 80% identical in PGRMC2, demonstrating that the effects of this sequence could be transferable across either member of the PGRMC family (Peluso et al., 2013).

The goal of this research is to gain a more in depth understanding of PGRMC1 and PGRMC2 as individual contributors, as well as the PAIRBP1 protein, to the intricate processes involved in oocyte development within the mouse ovary. If we can acquire more detailed knowledge regarding the timeframes in which these membrane receptors and corresponding protein are expressed throughout oocyte development and the level of expression at each crucial stage, then maybe we could gain a more clear and concise understanding of each stage of oocyte development, specifically cyst breakdown and apoptosis, which could have such direct effects on future fertility concerns.

Materials and Methods

Animals

Research in the Pepling Lab was conducted using the species, *Mus musculus*, as the model organism. The specific transgenic strain of mice that I used for my research was the inbred strain, C57BL/6J (B6). Mice were originally supplied from companies, such as Jackson Labs, but breeding also took place within the laboratory animal resource facility located on campus at Syracuse University. All of the mice used for experiments were maintained and cared for in the laboratory animal resource facility, as well, with collaborative efforts between members of the Pepling Lab and the laboratory animal facility staff.

Matings between male and female B6 mice were set up every Monday afternoon at approximately 2PM, caging 2 females with 1 male per cage. Females were then checked for a vaginal plug each morning before 10AM for the remainder of the week as a sign of conception. Females that appeared to have a vaginal plug were isolated and their plug date was recorded in order to keep track of expected birth dates for litters of mice. On the final day of the mating week, regardless of whether or not a female mouse had conceived, the females were isolated. Pregnant females would then give birth approximately 19.5 days after conception, also known as days post coitum (dpc). The day of birth was known as post-natal day 1 (PND 1) and every day after the pups' birth is referenced in terms of post-natal days. Occasionally, litters would not necessarily be born on the

expected due date, but the age of litters was always determined strictly by plug date, not by birth date, when considering ages of litters for research purposes.

Dissection

For my particular project, I obtained ovaries from B6 litters ranging from 15.5 dpc through PND 5. This required performing fetal dissections and post-natal dissections. Fetal dissections required euthanizing the pregnant female by CO_2 asphyxiation using a Euthenex machine in order to dissect the fetal pups. Fetal pups were removed from the amniotic sac and separated from the placenta in order to dissect them individually. Pups taken at post-natal day ages were simply removed from the mother's cage for dissection and were sacrificed by decapitation. A dissecting microscope was used in order to magnify the animals that were being dissected and dissections took place in phosphate buffered saline (PBS) solution, which served as a solution similar to where the ovaries were naturally maintained. Once dissection of the ovaries was complete, ovaries were transferred to a solution known as fix, which consists of 200 µL of 16% formaldehyde and 400 µL of 1x PBS. The ovaries were placed in fix in order to preserve the tissue at the stage at which it was dissected and to prevent the ovaries from deteriorating. Ovaries were then incubated in this solution overnight at 4°C on a nutator in a tube labeled with the age at which the ovaries were dissected, the dissection date and the number of ovaries collected. The stored ovaries could then be used for future experiments, such as whole mount antibody staining.

Whole Mount Antibody Staining

After being incubated overnight in the solution of fix, a detailed protocol was followed to perform an antibody staining procedure using indirect immunofluorescence. Typically, I focused on collecting data from antibody stainings performed at 15.5 dpc, since the various antibodies that I use are thought to be highly expressed at that stage in mouse oocyte development. Therefore, the specific protocol that I followed was designed specifically for antibody staining of fetal mouse ovaries. Following incubation in fix, ovaries were quickly washed two times in 1 mL of PT, which is a solution consisting of 1x PBS and 0.1% of Triton X-100. Then, they were incubated in 1mL of PT for approximately 30 minutes at room temperature on a nutator. Next, the ovaries were incubated in 1 mL of PT + 5% BSA. The incubation time in PT + 5% BSA could be modified in several different ways. The ovaries could either be incubated in the solution for 1 hour at room temperature on a nutator or the ovaries could be incubated in the solution overnight on a nutator at 4˚C. Either method yielded identical results and did not affect the end product.

After incubation in the PT + 5% BSA solution, the ovaries were incubated with the primary antibody being used in the staining procedure. Different antibodies have certain dilutions at which they will optimally label the tissue in immunofluorescence procedures, so I used several different primary antibody dilutions throughout the various antibody stainings that I performed. As

Table 1: Primary antibodies used in whole mount antibody staining procedure

Primary antibody	Company	Lot Number	Antibody function	Dilution amount used in 500 µL of PT+5% BSA
PGRMC1	Santa Cruz Biotechnology	C1510	labels PGRMC1 receptor	5 µL
PGRMC1	Sigma Aldrich	C41005	labels PGRMC1 receptor	5 µL
PGRMC2	Santa Cruz Biotechnology	L0312	labels PGRMC2 receptor	5 µL
PGRMC2	Sigma Aldrich	R38704	labels PGRMC2 receptor	5 µL
PAIRBP1	Abcam	GR151078-1	labels PAIRBP1 protein	5 µL
STAT 3	Santa Cruz Biotechnology	I0613	oocyte marker	1 µL

shown in Table 1, when staining for the expression of STAT 3, a dilution factor of 1:500 was used; whereas staining for the expression of PGRMC1, PGRMC2 or PAIRBP1 required a larger concentration of antibody with a dilution factor of 1:100 in order to adequately express the proteins of interest. However, the nature of the solution in which the primary antibody was diluted, and the exact volume of that solution used, was consistently 500 µL of PT + 5% BSA. After adding the PT + 5% BSA and the appropriate primary antibody, the ovaries were incubated overnight on a nutator at 4˚C. At this time, the solution containing the secondary antibody was made in order to allow the secondary antibody to appropriately pre-

absorb. A very small pinch of embryo powder, 500 µL of PT + 5% BSA and 2.5 µL of the corresponding secondary antibody are all added together in a tube and incubated overnight on a nutator at 4˚C alongside the ovaries being incubated in the primary antibody. The different secondary antibodies used depending on the species in which the primary antibody originated are listed in Table 2. The secondary antibody solution was maintained in foil the entire time, since it is fluorescent and light sensitive, which are characteristics that make it possible to visualize the stained ovaries at the end of the procedure.

Table 2: Secondary antibodies used in whole mount antibody staining procedure

Secondary antibody	Company	Wavelength (nm)	Antibody function	Dilution used in 500µL PT+5% BSA (µL)
donkey anti-goat	Molecular Probes	488 nm	indirectly labels PGRMC2	2.5 µL
donkey anti-mouse	Molecular Probes	488 nm	indirectly labels PAIRBP1	2.5 µL
donkey anti-rabbit	Molecular Probes	488 nm	indirectly labels PGRMC1and PGRMC2	2.5 µL
goat anti-mouse	Molecular Probes	488 nm	indirectly labels PAIRBP1	2.5 µL
goat anti-rabbit	Molecular Probes	488 nm	Indirectly labels PGRMC1 and Stat 3	2.5 µL
rabbit anti-goat	Molecular Probes	488 nm	indirectly labels PGRMC2	2.5 µL

The following day, the ovaries were washed in 1 mL of PT + 1% BSA for no less than 30 minutes on a nutator at room temperature. Then, the ovaries were incubated in RNase A solution, containing 10 µL of 10 mg/mL RNase A and 1 mL of PT + 1% BSA, for exactly 30 minutes on a nutator at room temperature. Next, the ovaries were incubated in a solution containing 10 µL of 0.5 mg/mL propidium iodide and 1 mL of PT + 1% BSA for exactly 20 minutes on a nutator at room temperature, in complete darkness in order to preserve the effect of the propidium iodide. From this point on, all the steps in the staining process were completed in foil-wrapped tubes in order to preserve the fluorescent staining reagents. After incubation in propidium iodide, the ovaries were washed in 1 mL of PT + 1% BSA for approximately 30 minutes on a nutator at room temperature. The ovaries were then incubated in a solution containing 2.5 µL of the pre-absorbed secondary antibody solution with 500 µL of PT + 5% BSA, as shown in Table 2, for either 2-4 hours on a nutator at room temperature or overnight on a nutator at 4˚C.

On the final day, the ovaries were washed three separate times in 1 mL of PT + 1% BSA, for 30 minutes each time, on a nutator at room temperature. The ovaries were then washed quickly in 1 mL of 1x PBS before mounting the ovaries on a new slide. In order to transfer the ovaries to the slide, roughly 700 µL of 1x PBS was removed from the tube containing the ovaries after the wash was complete. A new pipette tip was then cut at an angle to create a larger opening in which the ovaries would freely be able to be pipetted out on the tube, into the tip and back onto the slide. Once the ovaries were transferred to the slide and any

excess 1x PBS was removed from the slide, 2 drops of VectaShield were added to the slide. A coverslip was carefully placed on the top of the slide and the ovaries sat in the VectaShield in the dark for approximately 15 minutes, because the VectaShield served to further preserve the fluorescent character of both the propidium iodide and the secondary antibody for visualization later on. Nail polish was then spread around the exterior of the coverslip in order to ensure that the coverslip was secure and the slide was labeled with sex of gonads, the age at which the gonads were dissected, the primary and secondary antibodies used for the staining, the current date and my personal initials. After the nail polish had completely dried, the completed slide was placed in a folder to prevent exposure to light and the folder was stored in the freezer at -20˚C.

Confocal Microscopy

Confocal microscopy is an effective method used to visualize the staining patterns on a specimen. The microscope uses point illumination to pinpoint a discrete region of the specimen in which the fluorescent reagent that the specimen was stained with can be made visible. Ovaries that were stained using the whole mount antibody staining protocol were visualized using the Zeiss LSM 710 confocal microscope located in the imaging room at the Life Sciences Complex at Syracuse University. When observing the slides under the microscope, the ovaries were first looked at using a 10x objective lens, in order to locate and focus on individual ovaries on the slides. Once an ovary was clearly visible under the 10x

lens, a drop of oil was added and the magnification was increased to a 63x objective in order to observe various stages of oocyte development occurring within the individual ovaries. The confocal microscope was used to analyze the appropriately labeled proteins that coincided with the antibodies that were used to stain for their expression during the antibody staining procedure. In this case, in order to visualize the appropriate fluorescent wavelength, the confocal reflector used was always alexa-fluoro-488, which would allow the fluorescing secondary antibody reagent to be made visible under the confocal microscope. Images of the stained ovaries would be taken on a computer linked to the Zeiss confocal microscope, using Zen 2010 software, and could then be transferred to a disc for future reference outside of the imaging room.

Western Blotting

Western blotting is a technique that can be used to test for the presence of certain proteins at various stages in development. Western blot analysis also provides subsequent information about the magnitude of protein expression across developmental stages. In order to determine protein expression throughout oocyte development, I dissected ovaries at several days of fetal and neonatal development, specifically 15.5, 17.5 dpc and PND 1, 3 and 5. The dissection protocol was slightly altered when preparing ovaries for western blotting. Ovaries were still dissected in 1x PBS, but were then transferred to an eppendorf tube containing approximately 500 µL of ice-cold 1x PBS. After all of the ovaries

were dissected and transferred to the tube, the 1x PBS was removed and a proportionate supply of 1x Sample Buffer plus protease inhibitors was added. 1x Sample Buffer consists of 500 µL of 2x Sample Buffer, 100 µL of 10x protease inhibitors and 400 µL of distilled water. For each ovary dissected, 10 µL of 1x Sample Buffer was added, being sure that only 50 µL of 1x Sample Buffer was added at a time. Once the first 50 µL of Buffer was added, the ovaries were homogenized using a pestle and then any additional required Buffer was added to finish the process. From the moment the ovaries were dissected until the moment that they were homogenized, it was critical that the ovaries were kept on ice. Once homogenization was complete, the tube containing the ovary extracts was labeled with the age of the ovaries, the date of the dissections, the number of ovaries homogenized and my personal initials. The homogenized ovary extracts were then stored in the freezer at -20˚C, until they were needed to run a western blot.

Once enough ovary extracts were collected from a variety of fetal and neonatal ages, the electrophoresis step of the western blot was initiated. In order to prepare the samples, 20 µL of each extract and 2 µL of 2-mercaptoethanol were added to an eppendorf tube. The samples were then boiled for 3 minutes and centrifuged for 1 minute at 6000 rpm, being returned to ice after each step. The gel apparatus was assembled, the BioRad Mini Protein Gel was inserted into the gel holder and the appropriate amount of 1x Running Buffer, consisting of 100 mL of 10x SDS Glycine Running Buffer diluted in 900 mL of milli-Q water, was added. The wells of the gel were cleaned out to avoid bubbling, and then the samples were loaded into the individual wells. 10 µL of Precision Plus Standard

Protein marker were loaded into the first well to serve as a reference ladder. Positive control lysates were also used as a means of regulating the efficiency of the protocol being used and to ensure that the antibodies being used were functioning properly. A Hep G2 cell lysate was used as a positive control for the PGRMC1 antibody used and a HeLa whole cell lysate was used as a positive control for the PGRMC2 and the PAIRBP1 antibodies used. 20 µL of each positive control lysate were loaded into the appropriate wells on the gel. 20 µL of the boiled and spun ovary samples were then loaded into the wells in order of increasing age. The gel was then run at approximately 100 volts for 1 hour.

Once electrophoresis was complete, the resulting bands present on the gel were transferred to a polyvinylidene fluoride transfer membrane for manipulation with the desired antibodies. Before the transfer procedure occurs, the transfer membrane was soaked in methanol for 1 minute then milli-Q water for 5 minutes. The membrane, BioRad gel, 2 pieces of filter paper and 2 filter pads were then individually soaked in transfer buffer, consisting of 3.0 grams of Tris base, 14.4 grams of glycine and 200 mL of methanol diluted in milli-Q water to a total volume of 1 liter, for 5 minutes. After being soaked in transfer buffer, a transfer sandwich was assembled within a transfer cassette, laying one fiber pad, one piece of filter paper, the gel, the transfer membrane, the second piece of filter paper and the second filter pad down on the cassette. The closed cassette was then placed in the transfer apparatus, an ice-cube was added, a small stir bar was placed in the bottom and the apparatus was filled with transfer buffer. The entire apparatus was relocated to the 4°C refrigerator and the transfer took place at 4°C at 100 volts for

1 hour. Once the transfer was complete, the membrane was placed in blocking buffer on a shaker at room temperature for 30 minutes to block any future non-specific binding of proteins once the detection antibodies were added. After 30 minutes, the blocking buffer was changed and the membrane was incubated in fresh blocking buffer overnight on the nutator at 4˚C. The blocking buffer used depended upon the particular detection antibody. When working with a PGRMC1 antibody, a PGRMC2 antibody made in rabbit, or a PAIRBP1 antibody, standard blocking buffer was used throughout the protocol, which consisted of 100 mL of 10x PBS, 50 g of non-fat dry milk and 0.5 mL Tween 20 diluted in milli-Q water to a total volume of 1 liter. When working with a PGRMC2 antibody made in goat, the ingredients of the blocking buffer had to be altered due to the origin of the antibody. The PGRMC2 antibody used was produced in goat and the milk used in standard blocking buffer is known to cross-react with antibodies made in goat, leading to non-specific protein binding. The 5% non-fat dry milk in blocking buffer was substituted with 50 µL of goat serum (Molecular Probes, Lot # 1114869A) in order to avoid error.

The following day, a primary antibody and its corresponding secondary antibody were applied to the membrane in order to test for the presence of a protein of interest. The membrane was first incubated in a solution of the primary antibody diluted in blocking buffer. The antibody dilution is typically 1:1000, but varies depending on the particular antibody. As shown in Table 3, the antibodies used were often diluted much less than normal. The membrane incubation in

Table 3: Primary antibodies / lysates used for western blotting

Primary antibody/lysate	Company	Lot Number	Antibody/lysate function	Dilution in blocking buffer (BB)
GAPDH	Santa Cruz Biotechnology	E0912	loading control antibody	1:5000 (1 µL:5mL)
HeLa whole cell lysate	Santa Cruz Biotechnology	I0612	positive control lysate for several antibodies	N/A
Hep G2 cell lysate	Santa Cruz Biotechnology	E1012	positive control lysate for an antibody	N/A
PAIRBP1	Abcam	GR151078-1	detect expression of PAIRBP1 protein	1:100 (30 µL: 3 mL
PGRMC1	Sigma Aldrich	C41005	detect expression of PGRMC1 receptor	1:500 (6 µL: 3 mL)
PGRMC2	Santa Cruz	L0312	detect expression of PGRMC2 receptor	1:1000 (3 µL: 3 mL)
PGRMC2	Sigma Aldrich	R38704	detect expression of PGRMC2 receptor	1:100 (30 µL: 3 mL)

primary antibody solution varied in time and temperature depending on the nature

of the primary antibody. When the membrane was incubated using PGRMC1 as

the primary antibody, incubation occurred for 1 hour on a nutator at room

temperature. When the membrane was incubated using PGRMC2 or PAIRBP1 as

the primary antibody, the membrane was incubated in solution overnight on a

nutator at 4˚C. The membrane was then washed in blocking buffer 3 separate

times, for 10 minutes each, on a shaker at room temperature.

Table 4: Secondary HRP-conjugated antibodies used for western blotting

Secondary antibody	Company	Lot Number	Antibody function	Dilution in BB
goat anti-mouse	Pierce Immuno Pure Antibodies	FI814529	Indirectly detects expression of PAIRBP1	1:10,000 (1 µL:10 mL)
goat anti-rabbit	Thermo Scientific Pierce Antibodies	NL181270	indirectly detects expression of PGRMC1	1:10,000 (1 µL:10 mL)
rabbit anti-goat	Thermo Scientific Pierce Antibodies	OE1711053	indirectly detects expression of PGRMC2	1:10,000 (1 µL:10 mL)
rabbit anti-mouse	Pierce Immuno Pure Antibodies	BC631905	indirectly detects expression of GAPDH and PAIRBP1	1:10,000 (1µL:10 mL)

Next, the membrane was incubated in a solution of the secondary Horseradish Peroxidase (HRP) antibody diluted in blocking buffer. As shown in Table 4, the antibody dilution used for every secondary antibody was 1:10,000, so 1 µL of secondary antibody was diluted in 10 mL of blocking buffer. The origin of the secondary antibody depended on the origin of the primary antibody being used, because in order to bind to the primary antibody, it was essential that the secondary antibody recognized the primary antibody as an antigen. The incubation in the secondary antibody dilution took place on a nutator, at room temperature, for 1 hour. Then, the membrane was washed in blocking buffer 2 additional times, for 10 minutes each, on a shaker at room temperature. The membrane was then washed once in wash buffer, consisting of 50 µL of Tween diluted in 1 L of 1x PBS, for 10 minutes on a shaker at room temperature. The membrane was then quickly rinsed in 1x PBS and incubated in 5 mL of a

detection buffer-enhancer solution for 5 minutes on a shaker at room temperature. The ratio of detection buffer to enhancer was 1:1, so 2.5 mL of each liquid was used in the solution. The membrane was then thoroughly rinsed in milli-Q water, wrapped in a clear plastic cover and placed in an autoradiography cassette. The membrane was then imaged using a BioRad Molecular Imaging ChemiDoc XRS+ Imaging System specialized for western blot detection. Protein bands of interest and the intensity of protein expression were detected across different stages of mouse development based on the size, shade and location of the bands. Images were captured on a program connected to the imaging system and were stored for future reference. Once imaging was complete, the membrane was rinsed in 1x PBS and blocked in blocking buffer for 30 minutes on a shaker at room temperature. Then, the blocking buffer was changed and the membrane was incubated in blocking buffer overnight at 4˚C.

The following day, the detection protocol was repeated, except different antibodies were substituted as the primary and secondary antibodies. The membrane was reprobed using the loading control protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH was used as the primary antibody; diluted 1:5000 in blocking buffer. The secondary antibody used was an anti-mouse HRP antibody, since GAPDH is made in mouse, and was diluted 1:10,000 in blocking buffer. The reprobing procedure was used to ensure that equal amounts of sample were loaded into each well to confirm that resulting differences in signal strength were not simply due to over-loading or under-loading of a well. GAPDH is an efficient loading control protein to use because it

is stable across various conditions and typically possesses dependable signal strength.

Results

PGRMC1 appears to be expressed in the surface epithelium of the fetal and neonatal mouse ovary

The technique of immunocytochemistry was performed to determine if PGRMC1 was expressed in the fetal mouse ovary at 15.5 dpc and 17.5 dpc and in the neonatal mouse ovary at PND 1, 3 and 5.

The expression of PGRMC1 appears to be limited to the epithelium of the fetal and neonatal mouse ovaries, as shown in Figure 5A, C and E. The staining of the epithelial cells with the PGRMC1 antibody is relatively strong and appears to localize to the cytoplasm, as well as the nuclei of these cells. The rate at which the antibody detection diffuses as you progress towards the ovary interior is inconsistent and varies from one ovary to another. PGRMC1 was not detected in the ovary interiors of these 3 stages, as shown in Figure 5B, D and F.
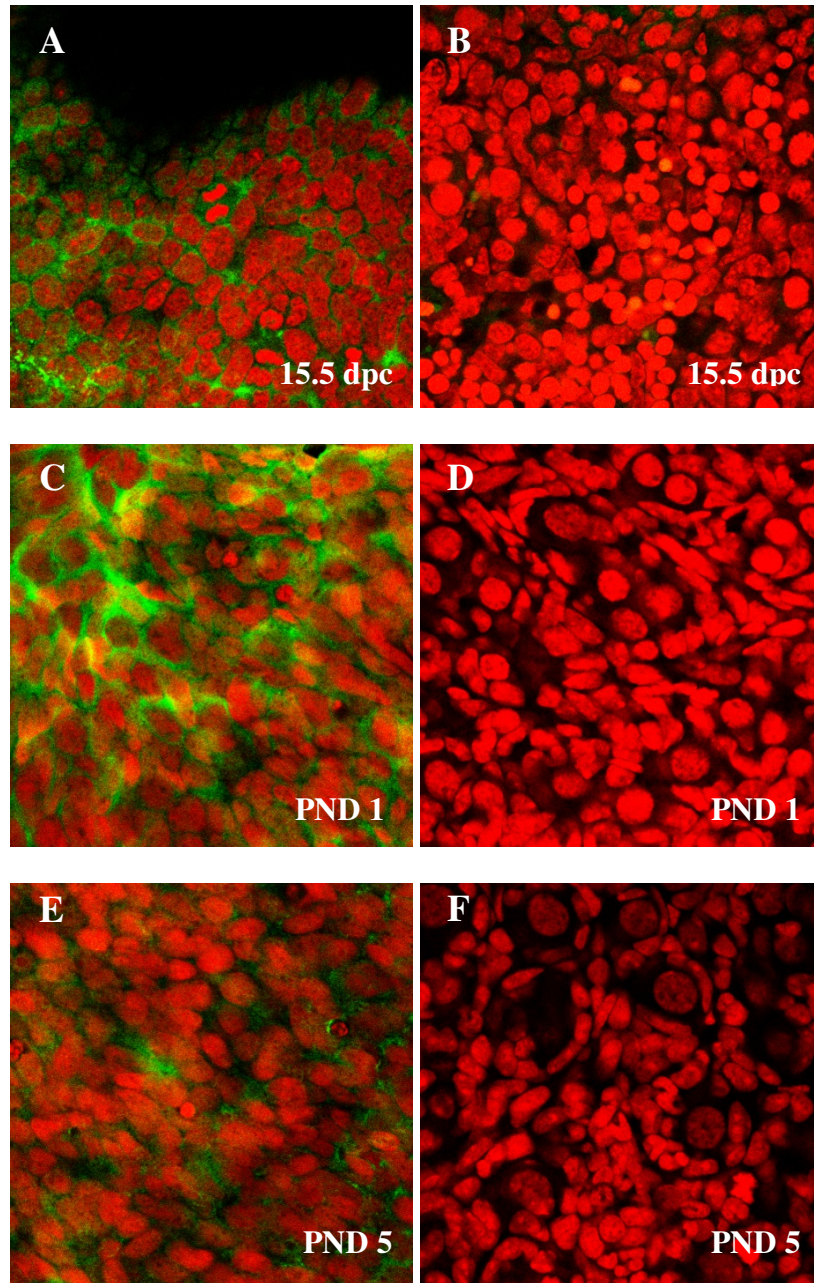
Figure 5: Confocal images of fetal and neonatal mouse ovary sections demonstrating expression of PGRMC1 via the PGRMC1 antibody staining (green) at the epithelial layer of the ovary. The propidium iodide (red) is staining the nuclei of the oocytes, surrounding pre-granulosa and epithelial cells. Images of the epithelial cells at 15.5 dpc (A), PND 1 (C) and PND 5 (E) are shown in comparison to images of the ovary interior at 15.5 dpc (B), PND 1 (D) and PND 5 (F).

PGRMC2 does not appear to be expressed at all throughout fetal and neonatal mouse ovary development

The technique of immunocytochemistry was performed to determine if PGRMC2 was expressed in the fetal mouse ovary at 15.5 dpc and 17.5 dpc and the neonatal mouse ovary at PND 1, 3 and 5.
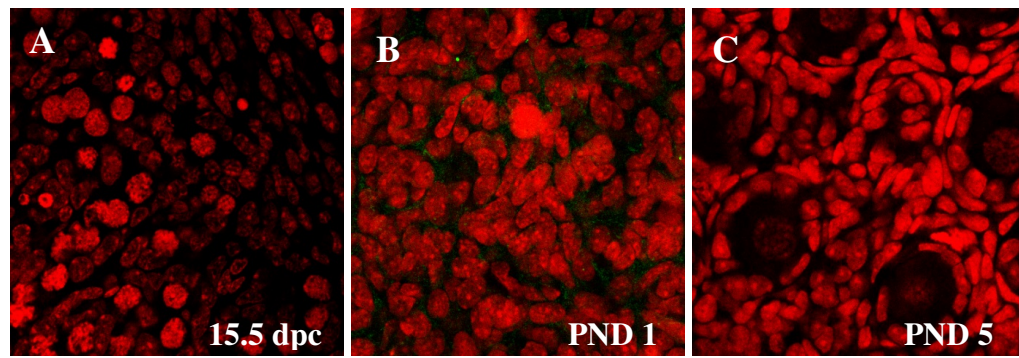

Figure 6: Confocal images of fetal and neonatal mouse ovaries showing no expression of PGRMC2 via the PGRMC2 antibody (green). The propidium iodide (red) is staining the nuclei of the oocytes and the somatic, or granulosa, cells. Ovaries at 15.5 dpc (A), PND 1 (B) and PND 5 (C) only show appropriate staining of nuclei within various ovary components.

There does not appear to be any significant expression of PGRMC 2 in the mouse ovary at these stages of fetal and neonatal mouse development, as shown in Figure 6. There was no expression in the cytoplasm or nuclei of the oocytes or the surrounding somatic cells at 15.5 dpc or PND 5. In several ovaries at PND 1, the ovaries appear to have weak background staining with no specific localization pattern; therefore expression of PGRMC2 in the neonatal mouse ovary is not significant at PND 1 either.

PAIRBP1 appears to be expressed in the epithelium of the fetal and neonatal mouse ovary

The technique of immunocytochemistry was performed to determine if PAIRBP1 was expressed in the fetal mouse ovary at 15.5 dpc and 17.5 dpc and the neonatal mouse ovary at PND 1, 3 and 5.
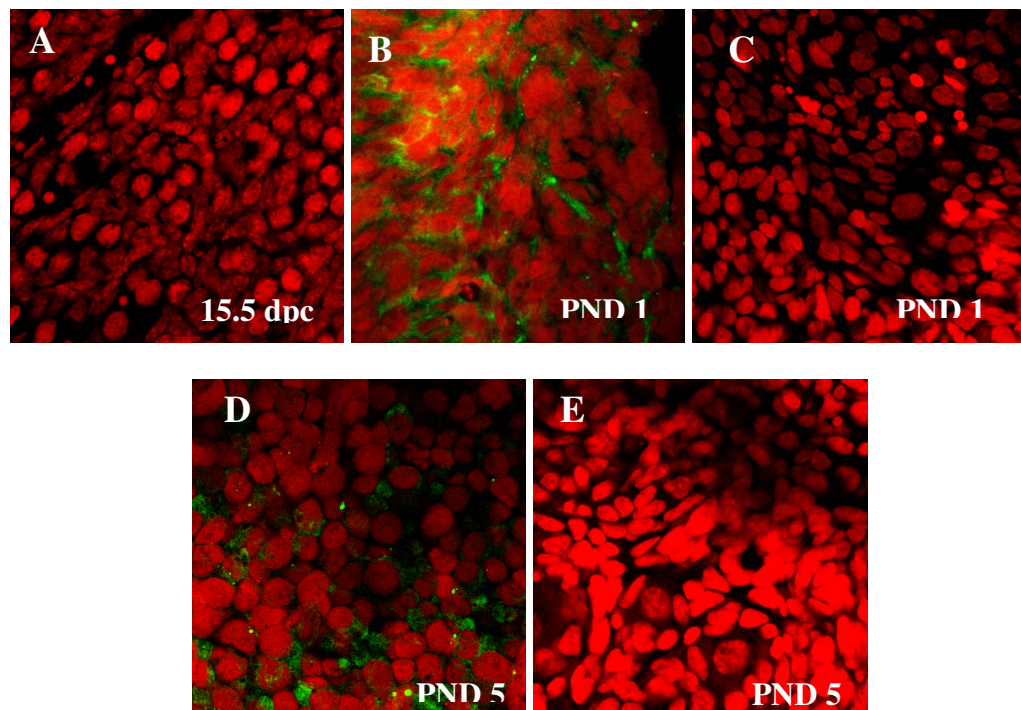

Figure 7: Confocal images of fetal and neonatal mouse ovary sections demonstrating expression of PAIRBP1 via the PAIRBP1 antibody staining (green) at the epithelial layer of the neonatal ovary. The propidium iodide (red) is staining the nuclei of the oocytes, surrounding pre-granulosa and epithelial cells. An image of the ovary at 15.5 dpc (A) is shown. Images of the epithelial cells in the neonatal ovary at PND 1 (B) and PND 5 (D) are shown in comparison to images of the ovary interior at PND 1 (C) and PND 5 (E).

The expression of PAIRBP1 appears to be limited to the epithelium of the fetal and neonatal mouse ovaries, as shown in Figure 7. The staining of the epithelial cells with the PAIRBP1 antibody is relatively strong and appears to localize to the cytoplasm, as well as the nuclei of these cells. The rate at which the antibody expression diffuses as you progress towards the ovary interior is inconsistent and varies from one ovary to another. PAIRBP1 was not detected in the ovary interiors of these 3 stages, as seen in Figure 7. This correlation between PAIRBP1 expression and PGRMC1 expression could potentially support the hypothesis that PGRMC1 and PAIRBP1 interact in order for the PGRMC1-progesterone signaling pathway to proceed normally.

PGRMC1 appears to be present throughout fetal and neonatal mouse development

The western blotting procedure was performed to confirm that PGRMC1 is present in the fetal and neonatal mouse ovary and to determine if the levels of this membrane receptor change throughout the process of germ line development. The expected size of PGRMC1 is 28 kilo Daltons (kD). Ovary extracts at 15.5 dpc, 17.5 dpc, PND 1, PND 3 and PND 5 were tested using western blot.
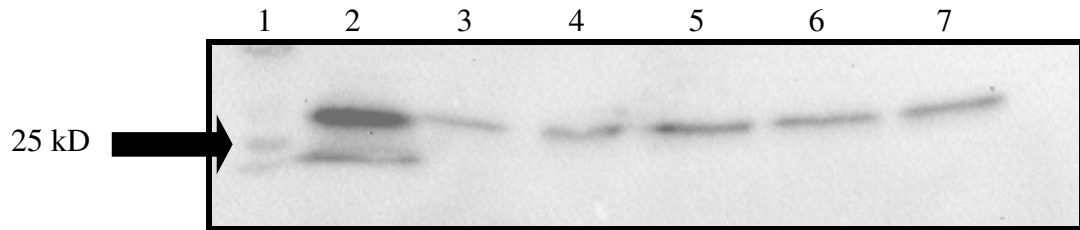
Figure 8: Image taken from the BioRad Molecular Imaging ChemiDoc XRS+ Imaging System showing the PGRMC1 western blot results. The prominently detected protein is PGRMC1. Lane 1 consists of the Precision Plus Standard Protein marker used as a reference to determine band size. The arrow shows a 25 kD size marker. Lane 2 consists of the HepG2 whole cell lysate positive control. Lane 3 consists of ovary extracts at 15.5 dpc. Lane 4 consists of ovary extracts at 17.5 dpc. Lane 5 consists of ovary extracts at PND 1. Lane 6 consists of ovary extracts at PND 3. Lane 7 consists of ovary extracts at PND 5.

The western blot membrane detection demonstrates that PGRMC1 is present in the fetal and neonatal mouse ovary from 15.5 dpc to PND 5, as seen in Figure 8. The protein detected appears to be of the appropriate size expected for PGRMC1. The protein appears to be slightly larger than the 25 kD reference protein size marker, while still being significantly smaller than the 37 kD reference protein. The HepG2 whole cell lysate that was used as a positive control appeared to have protein detected at the same size as the PGRMC1 protein (28 kD), which is to be expected considering that the positive control lysate sample contains PGRMC1. An additional protein in the HepG2 whole cell lysate was also detected at approximately 20 kD. The darkness, or intensity, of the protein from 15.5 dpc to PND 5 appears to be consistent. This demonstrates that the level of PGRMC1 in the mouse ovary across germ line development is relatively stable and does not fluctuate significantly. An additional protein was detected at each of the ages, located slightly above the 100 kD reference size marker. It is not

included in Figure 8, because it is not the primary protein signifying the presence of PGRMC1 in the fetal and neonatal mouse ovary.

The western blotting detection procedure was repeated using the loading control protein, GAPDH. The same membrane was re-probed using this antibody to determine if an equal amount of sample was loaded into each of the wells in order to ensure that any change in the protein signal intensity was inherently due to a change in expression of the PGRMC1 receptor at that stage of development. The expected size of GAPDH is 42 kD, and the same ovary extract ages in Figure 8 were used here.

Figure 9: Image taken from the BioRad Molecular Imaging ChemiDoc XRS+ Imaging System showing the GAPDH reprobing results for PGRMC1. The prominently detected protein is GAPDH. Lane 1 consists of the Precision Plus Standard Protein marker used as a reference to determine protein size. The arrow shows a 37 kD size marker. Lane 2 consists of the HepG2 whole cell lysate. Lane 3 consists of ovary extracts at 15.5 dpc. Lane 4 consists of ovary extracts at 17.5 dpc. Lane 5 consists of ovary extracts at PND 1. Lane 6 consists of ovary extracts at PND 3. Lane 7 consists of ovary extracts at PND 5.

The results from the western blot membrane re-probing procedure, as shown in Figure 9, demonstrating that the results from the PGRMC1 detection are relatively accurate. The protein detected appears to be of the appropriate size for GAPDH. The protein appears to be located slightly above the 37 kD reference band, while still being a moderate distance from the 50 kD reference band, which demonstrates that it was detected at the expected location on the membrane. An

additional protein was detected at approximately 37 kD; the size was difficult to

determine given the non-linear shape that progressed across the lanes on the

membrane. The darkness, or intensity, of the bands appears to change slightly

across the 5 developmental ages. The band detected at 17.5 dpc appears to be

slightly lighter than the other bands detected, especially in comparison to the

bands detected at PND 1 and PND 3. The slight fluctuation in protein signal

intensity of GAPDH could demonstrate that some volumes of sample loaded were

slightly less than or slightly more than the instructed 20 µL. Given the slight

fluctuation in the intensity of the GAPDH protein signal, the PGRMC1 levels

shown in Figure 8 might require re-evaluation. Faint protein signals were also

detected at approximately 28 kD and 100 kD, but are not shown above. These

proteins are not shown in Figure 9, because they are remnants from the PGRMC1

detection.

**PGRMC2 does not appear to be present throughout fetal and neonatal
mouse development**

The western blotting procedure was performed to confirm that PGRMC2

is not present in the fetal and neonatal mouse ovary and to determine if the levels

of this membrane receptor change throughout the process of germ line

development. The expected size of PGRMC2 is 24 kD. Ovary extracts at 15.5

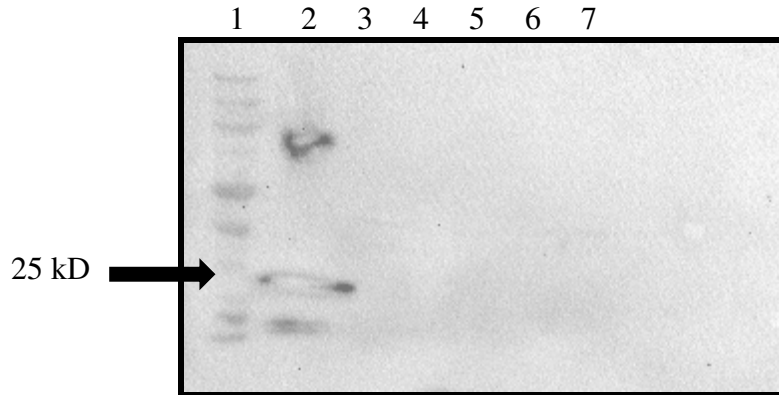dpc, 17.5 dpc, PND 1, PND 3 and PND 5 were tested using western blot.

Figure 10: Image taken from the BioRad Molecular Imaging ChemiDoc XRS+ Imaging System showing the PGRMC2 western blot results. There were no prominently detected proteins for PGRMC2. Lane 1 consists of the Precision Plus Standard Protein size marker. The arrow shows a 25 kD size marker. Lane 2 consists of the HeLa whole cell lysate. Lane 3 consists of ovary extracts at 15.5 dpc. Lane 4 consists of ovary extracts at 17.5 dpc. Lane 5 consists of ovary extracts at PND 1. Lane 6 consists of ovary extracts at PND 3. Lane 7 consists of ovary extracts at PND 5.

The western blot experiment did not detect PGRMC2 in the fetal and neonatal mouse ovary extracts, as shown in Figure 10. If PGRMC2 were expressed in the fetal and neonatal mouse ovary, a protein of approximately 24 kD would be expected to be detected in the blot. However, there were no proteins detected at the appropriate location expected for PGRMC2. A protein of the expected size for PGRMC2 was detected in the HeLa whole cell lysate that was used as a positive control. This demonstrates that it was possible for PGRMC2 to be detected in at least one sample on the blot. An additional protein was detected at approximately 15 kD, as well as an un-identifiable spot slightly below the 100 kD reference band. Since no protein signals were detected upon imaging the membrane, the darkness, or intensity, of PGRMC2 was unable to be determined.

The western blotting detection procedure was repeated using the loading control protein, GAPDH. Typically, this procedure would be used to determine if

an equal amount of sample was loaded into each of the wells in order to ensure

that any change in protein signal intensity was inherently due to a change in

expression of the PGRMC2 receptor at that stage of development. In this case, the

membrane was re-probed in order to determine if the original lack of PGRMC2

expression was due to an error in how the PGRMC2 western blot was conducted,

or if there was an error with the antibody itself. The expected size of GAPDH is

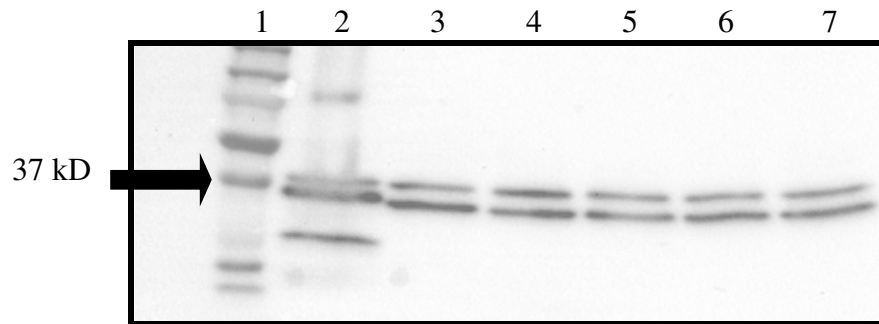42 kD, and the same ovary extract ages in Figure 10 were used here.

Figure 11: Image taken from the BioRad Molecular Imaging ChemiDoc XRS+
Imaging System showing the GAPDH reprobing results for PGRMC2. The
prominently detected protein is GAPDH. Lane 1 consists of the Precision Plus
Standard Protein size marker. The arrow shows a 37 kD size marker. Lane 2
consists of the HeLa whole cell lysate. Lane 3 consists of ovary extracts at 15.5
dpc. Lane 4 consists of ovary extracts at 17.5 dpc. Lane 5 consists of ovary
extracts at PND 1. Lane 6 consists of ovary extracts at PND 3. Lane 7 consists of
ovary extracts at PND 5.

The results from the western blot re-probing procedure demonstrated that

GAPDH was detected from 15.5 dpc to PND 5, as shown in Figure 11. As shown

by the 37 kD reference protein, the protein signal detected appears to be at the

appropriate size for GAPDH, only having a slightly different mobility than

expected. The consistent darkness of the detected protein signal demonstrates that

an equal amount of sample was loaded into each of the wells upon the original

running of the gel; therefore the lack of PGRMC2 detection in Figure 10 was not a result of a severely decreased amount of ovary extract present during gel electrophoresis. The proteins in Lane 2 of Figure 11, representing the HeLa whole cell lysate positive control, appear to be slightly smeared, which might indicate that too much of the positive control was used. Definitive protein signals were detected from 15.5 dpc to PND 5, demonstrating that the lack of PGRMC2 expression in Figure 10 is most likely not a result of an error in the western blot procedure. These results help narrow down the possible reasons as to why PGRMC2 was consistently not detected at all in the mouse ovary throughout fetal and neonatal development across a number of western blot experiments.

PAIRBP1 expression appears to increase across fetal and neonatal mouse development

The western blotting procedure was performed to confirm that PAIRBP1 is present in the fetal and neonatal mouse ovary and to determine if the levels of this protein change throughout the process of germ line development. The expected size of PAIRBP1 is 45 kD. Ovary extracts at 15.5 dpc, 17.5 dpc, PND 1, PND 3 and PND 5 were tested using western blot.
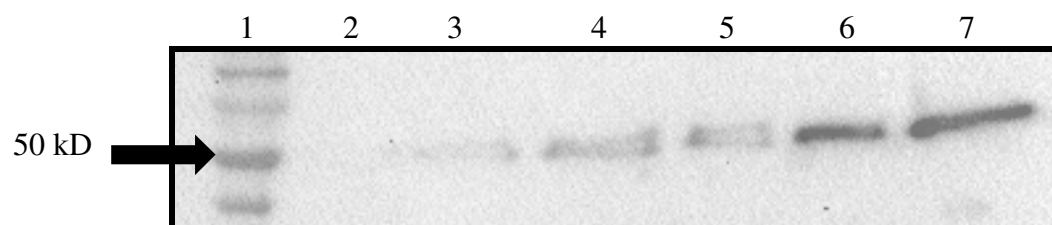
Figure 12: Image taken from the BioRad Molecular Imaging ChemiDoc XRS+ Imaging System showing the PAIRBP1 western blot results. The prominently detected protein is PAIRBP1. Lane 1 consists of the Precision Plus Standard Protein size marker. The arrow shows a 50 kD size marker. Lane 2 consists of the HeLa whole cell lysate. Lane 3 consists of ovary extracts at 15.5 dpc. Lane 4 consists of ovary extracts at 17.5 dpc. Lane 5 consists of ovary extracts at PND 1. Lane 6 consists of ovary extracts at PND 3. Lane 7 consists of ovary extracts at PND 5.

The western blot membrane detection demonstrates that PAIRBP1 is present in the fetal and neonatal mouse ovary at various levels across germ line development, as shown in Figure 12. The protein detected appears to be located at approximately the appropriate location correlating with the expected size of PAIRBP1. The protein appears to be in line with the 50 kD reference band, which is slightly above the expected 45 kD PAIRBP1 size. However, the expected size for any given protein is simply an estimate that can fluctuate based on a number of different occurrences during the western blot procedure. The PAIRBP1 protein detected only slightly deviated from the estimate; therefore these results maintain their validity. The HeLa whole cell lysate that was used a positive control did not appear to express PAIRBP1 upon treatment with the PAIRBP1 antibody, which was unexpected. The darkness, or intensity, of each protein signal from 15.5 dpc to PND 5 appears to increase as development progresses. According to the protein signal intensities, the expression of PAIRBP1 in the mouse ovary steadily increases as germ line development proceeds.

The western blotting detection procedure was repeated using the loading control protein, GAPDH. The same membrane was re-probed using this antibody to determine if an equal amount of sample was loaded into each of the wells in order to ensure that any change in protein signal intensity was inherently due to a change in expression of the PAIRBP1 receptor at that stage of development. The expected size of GAPDH is 42 kD, and the same ovary extract ages in Figure 12 were used here.
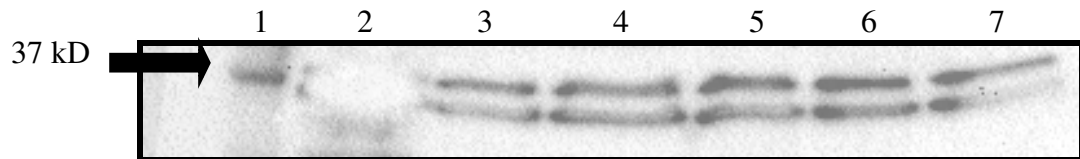

Figure 13: Image taken from the BioRad Molecular Imaging ChemiDoc XRS+ Imaging System showing the GAPDH reprobing results for PAIRBP1. The prominently detected protein is GAPDH. Lane 1 consists of the Precision Plus Standard Protein marker. The arrow shows a 37 kD size marker. Lane 2 consists of the HeLa whole cell lysate. Lane 3 consists of ovary extracts at 15.5 dpc. Lane 4 consists of ovary extracts at 17.5 dpc. Lane 5 consists of ovary extracts at PND 1. Lane 6 consists of ovary extracts at PND 3. Lane 7 consists of ovary extracts at PND 5.

The results from the western blot re-probing procedure demonstrated that GAPDH was detected from 15.5 dpc to PND 5, as seen in Figure 13. As shown by the 37 kD reference size marker, GAPDH is detected at the appropriate size, showing only a slightly different mobility on the membrane than expected in comparison to the protein size marker. Figure 13 also demonstrates that the results from the PAIRBP1 detection were relatively accurate. It appears as though a relatively consistent amount of sample was loaded into each of the wells based on the similar protein signal intensities seen from 15.5 dpc to PND 5. The HeLa

whole cell lysate in Lane 2 of Figure 13 appears to be completely bleached out upon detection, which means that an excess amount of positive control lysate could have been used, causing the GAPDH protein signal to be overexpressed to such an extreme. The results of the PAIRBP1 re-probing demonstrate that the PAIRBP1 expression pattern shown in Figure 12 is relevant and could be due to the intrinsic variation in expression of the protein across fetal and neonatal mouse development. The results in Figure 13 ensure that the expression of PAIRBP1 in the neonatal mouse ovary at PND 3 and PND 5 is significantly greater than at earlier fetal and neonatal ages.

Discussion

Although issues with infertility might not entail life-threatening scenarios, there is still a great deal of relevance in conducting research in order to learn more about the potential processes and mechanisms that could contribute to this dysfunction. A major issue that correlates with infertility is the fact that once women establish their finite supply of viable ovaries, approximately around their time of birth, there is no action that can be taken to increase their supply or reverse permanent damage beyond that crucial developmental stage. It is for this reason that a great deal of research is currently being conducted in order to gain an in-depth understanding of the complex processes and pathways that are utilized throughout germ line development. If we can learn more about the intricate, interdependent processes that are occurring during this critical timeframe, then

perhaps we can gain a better understanding of how fertility dysfunctions occur and how we could eradicate or compensate for those dysfunctions in the future.

Looking specifically at the role of hormone function throughout the process of germ line development, estrogen and progesterone are two hormones that are considered foundational contributors and are known to affect various aspects of development. Changes in levels of these hormones across development are known to be correlated with certain phases in the process of germ line development, acting as essential triggers for time-sensitive processes. However, excess amounts of these same hormones are known to have an equally negative effect on certain phases in this process, which, in turn, have a significant lasting effect on the overall developmental outcome.

Previous research has demonstrated that increased levels of estrogen and progesterone in the fetal and neonatal mouse ovary have an inhibitory effect on the process of cyst breakdown (Chen et al., 2007). By inhibiting the breakdown of cysts into single primordial follicles, the overall percent of viable individual oocytes is drastically decreased. Instead of containing a large percentage of viable oocytes for future fertilization, the neonatal ovary is more likely to contain a more prominent number of MOF's, which are thought to be unviable for future reproductive use. It is scenarios such as this that could contribute to increased chances for future infertility issues in females that were exposed to amplified levels of estrogen or progesterone at a critical time in reproductive development.

A great deal of research has been conducted revolving around the roles of estrogen and the negative effects that excess treatment of the hormone can have

on the process of germ line development. In comparison, the role of progesterone and the effects that excess levels of the hormone can have on developmental processes is less researched and less understood. We are less knowledgeable about the specific signaling pathways, receptors and affiliated proteins involved in progesterone signaling, which is why the research discussed here was conducted. We hoped to focus on specific progesterone signaling pathway receptors and proteins utilized across germ line development in mice in an attempt to gain information about how the overall role of progesterone might be dynamic, changing in function at different stages in reproductive development.

Our research yielded a number of different results through experiments in immunocytochemistry and western blotting. Our results demonstrated that the PGRMC1 receptor and the interacting PAIRBP1 protein are both present in some capacity across germ line development in the fetal and neonatal mouse ovary, ranging from 15.5 dpc to PND 5. However, the expression of both of these progesterone signaling components were limited to the epithelial layer of the ovary. As constituents that are both thought to be actively involved in the progression and development of individual oocytes, we would have expected to see PGRMC1 and PAIRBP1 expressed, in one capacity or another, within the actual developing oocytes themselves. The spatial limitation of the receptor and protein could present a localization pattern found within developing fetal and neonatal mouse ovaries or the limitation could signify an error in the staining ability of the given antibodies. The lack of penetration of the antibody signal beyond the epithelial layer of the mouse ovary could be cognizant of a penetration

error of the appropriate antibody or an error in the whole mount antibody staining protocol that was utilized. Since the propidium iodide that was used to stain the nuclei of the cells within the ovary was capable of penetrating the entire ovary, it is most likely an issue with the anti-PGRMC1 and anti-PAIRBP1 antibodies that were utilized for this research. These experiments need to be repeated again, possibly altering the concentrations of the current primary antibodies or ordering a completely new lot of each antibody in order to continue testing the expression of PGRMC1 and PAIRBP1 in the mouse ovary across these crucial developmental stages.

Unfortunately, the experiment conducted to test the expression of PGRMC2 in the mouse ovary from 15.5 dpc to PND 5 yielded no conclusive results. Unlike the epithelial expression characteristic of the experiments conducted with PGRMC1 and PAIRBP1, PGRMC2 was not detected in the fetal or neonatal mouse ovary. Previous studies in the Pepling Lab detected PGRMC2 at 15.5 dpc, using the same antibody and technique that was used here, so these results were extremely unexpected and unexplainable. According to the results obtained here, PGRMC2 is not expressed in the developing ovary and therefore could not play a role in progesterone signaling pathways in the mouse ovary. If PGRMC2 were not expressed in the developing mouse ovary, then all progesterone signals entering the cell at the membrane level would have to be utilizing the PGRMC1 signaling pathway, which seems questionable. However, if certain progesterone signaling pathways, such as the PGRCM2 pathway, were not active during hormone treatments in which the ovaries are exposed to excess

amounts of progesterone, would that affect the amount of progesterone that is able to signal cells to inhibit cyst breakdown?

In terms of the PGRMC2 immunocytochemistry experiment, it is more likely that there was some error in the experimental method used. More knowledge needs to be obtained to learn how the previous experiment that successfully demonstrated the expression of PGRMC2 in the fetal and neonatal mouse ovary was conducted. This experiment needs to be repeated after obtaining that knowledge and certain aspects of the protocol will need to be altered. Perhaps a change in the corresponding anti-PGRMC2 primary or secondary antibody concentration needs to be altered in order for the antibody to penetrate the entire mouse ovary.

Overall, the results of the immunocytochemistry experiments did not provide any conclusive data. More experiments similar in nature to the experiments conducted in this research study will need to be altered and repeated in the hopes that more conclusive results can be obtained. The only result that could potentially support a current theory is the overlapping localization of both PGRMC1 and PAIRBP1 in the fetal and neonatal mouse ovary. The interaction between this membrane receptor and protein is not well understood, but thought to be necessary in order for the PGRMC1-progesterone signaling pathway to function properly, so it is logical to assume that these two progesterone signaling pathway components are co-localizing to the same region of the mouse ovary.

The results obtained from western blotting experiments yielded variable data, as well. Our results demonstrated that PGRMC1 is expressed at relatively

constant levels in the mouse ovary across germ line development. According to the role that PGRMC1 plays in the progesterone signaling pathway, we would not expect the membrane receptor to be present at consistent levels from 15.5 dpc to PND 5. Given progesterone's inhibitory function in cyst breakdown, perhaps we would expect progesterone, and therefore PGRMC1, to be expressed at decreased levels during the timeframe in germ line development when cyst breakdown is thought to occur, from approximately 17.5 dpc to PND 5. However, according to the western blot results, PGRMC1 is still strongly expressed in ovaries at these crucial ages. These results could raise further questions about the role of PGRMC1 in the progesterone signaling pathway in the ovary across fetal and neonatal mouse development. Perhaps more research needs to be conducted in order to determine the limitations and functions of this membrane receptor, which might fulfill additional functions that we are not yet aware of. The constant expression of PGRMC1 in the mouse ovary from 15.5 dpc to PND 5 could also be a result of several possible errors in the western blot protocol and technique. For example, perhaps more ovary extract sample was loaded into the wells at several of the ages, such as PND 1 and PND 3, which could affect the intensity of the bands upon detection and, in turn, alter the interpretation of the results. This is why re-probing with GAPDH is often performed in order to account for discrepancies such as this. Re-probing with the GAPDH loading control informed us that further tests need to be conducted using PGRMC1 in order to validate the expression of this receptor across germ line development.

Results from the western blot testing for the presence and intensity of PGRMC2 across germ line development were also unexpected. Even though a variety of antibody dilutions were used and several different incubation methods were tested, we did not find any expression of PGRMC2 in the fetal and neonatal mouse ovary from 15.5 dpc to PND 5. No bands of interest were detected at any of the developmental ages tested. Several different PGRMC2 antibodies were tested and a number of alterations were made to the western blot protocol in an attempt to detect PGRMC2, but PGRMC2 did not appear to be expressed across any of the experimental trials. These results also align with the results obtained from the immunocytochemistry experiments, which showed no detection of PGRMC2 via antibody staining. The complete lack of PGRMC2 detection in both experiments strengthens the argument that PGRMC2 is not functioning in the process of germ line development in the capacity that we had anticipated. Perhaps PGRMC2 is not playing a role in germ line development directly within the fetal and neonatal mouse ovary, but the membrane receptor is instead playing an indirect role. This could explain why PGRMC2 is not directly expressed in the mouse ovary across this critical developmental time period.

There could be several errors in the western blot protocol and technique used, though, which could explain the unexpected results for PGRMC2 expression in the fetal and neonatal mouse ovary. Two different PGRMC2 antibodies, obtained from two different biotechnology companies, were tested, which leads us to believe that the lack of detectable protein signal is not a result of dysfunctional antibodies However, there could have been errors in the method

that was used to block insignificant bands from detection on the transfer membrane. One of the primary PGRMC2 antibodies that was used for a number of western blot experiments was made in goat. Since this antibody was made in goat and goats are closely related to cows, the chances of a cross-reaction between a blocking buffer containing milk and a secondary anti-goat antibody are thought to be increased. In order to ensure that any insignificant bands would be properly blocked, it was recommended that we substitute the 5% non-fat dry milk with goat serum. However, this alteration in the protocol led to several errors in the procedure. An appropriate amount of goat serum was not substituted into the blocking buffer solution, with only 50 µL of goat serum being added to each liter of blocking buffer instead of the 5 mL of goat serum that would be necessary in order to equal 5% of the overall blocking buffer solution volume. Adding such a small amount of goat serum in comparison to the remainder of the solution components could easily affect the results of each western blot that the blocking buffer was used in. Also, we recently learned about a contradicting theory recommending that when using primary antibodies made in goat for a western blot, a serum corresponding to the secondary anti-goat antibody should be used instead. For example, instead of substituting the 5% non-fat dry milk with goat serum each time blocking buffer solution was made, we should have used rabbit serum as a substitute instead, seeing as the secondary anti-goat antibody was made in rabbit. Upon first considering these discrepancies, we thought that perhaps altering these steps in the western blot protocol would yield different, or even more conclusive, results. However, the discrepancies discussed above would

be thought to cause insufficient blocking of insignificant proteins, but the major issue in this research was the complete lack of detectable PGRMC2 signal. For this reason, more thought needs to be given to these technical discrepancies and further experimental tests need to be conducted in order to determine their effects.

The results obtained from the western blots investigating PAIRBP1 expression in the mouse ovary across germ line development were quite interesting. Given the significant interaction that is thought to be present between PGRMC1 and PAIRBP1 in the PGRMC1-progesterone signaling pathway, we would have expected to see a similar detection pattern for PAIRBP1 bands of interest from 15.5 dpc to PND 5. However, PAIRBP1 appeared to be only faintly detected at 15.5 dpc, and then continually gained band intensity as germ line development proceeded. The western blot results suggested that the protein exhibits a definitive expression trend, where PAIRBP1 expression directly increases as the age of the mouse ovaries increase.

Perhaps this difference in detection between PGRMC1 and PAIRBP1 could have something to do with the anti-apoptotic function that PAIRBP1 is thought to be involved in in the PGRMC1-progesterone signaling pathway. Levels of PAIRBP1 expression could be lower in the days of fetal development leading up to the commencement of germ cell death, or apoptosis, which is thought to begin at approximately 18 dpc. Lower levels of this protein could act as some sort of trigger that initiates the apoptotic process. After the initiation of germ cell death, PAIRBP1 expression could strengthen. This could happen in order to ensure that the PGRMC1-progesterone signaling pathways anti-apoptotic

function is serving to terminate the apoptotic phase of germ line development, which ends at approximately PND 4.5. In order for this PAIRBP1 expression pattern to be valid, it would require some type of delay between the time at which PAIRBP1 levels change and the subsequent germ line development phase is initiated. A wider range of fetal and neonatal mouse ages would need to be tested in order to further explore this idea.

There are several technical errors that could account for the interesting PAIRBP1 detection results that were obtained. Unfortunately, not a great deal of time was able to be dedicated to investigating this particular protein so, in order to validate the results shown in Figure 12, a number of western blot experiments would need to be repeated using the same antibody dilution and incubation procedures. Also, the differences in the band intensities across each of the 5 ages tested could be a result of unequal loading of samples into each well upon the initial running of the gel. If a smaller amount of sample from 15.5 dpc or 17.5 dpc was loaded than sample from several of the postnatal ages, then that could explain the weaker band detection at those fetal ages.

Overall, the results obtained from the western blot experiments yielded a number of different unexpected, but intriguing results. The complete lack of detection of PGRMC2 expression in the fetal and neonatal mouse ovary across germ line development aligns with the results obtained in the immunocytochemistry experiment, but will obviously require further research since the complete non-existence of PGRMC2 in the mouse ovary at any point in germ line development is not likely. The protein blot detection results for

PGRMC1 and PAIRBP1 do not correlate in the same manner as was seen in the immunocytochemistry results for this membrane receptor and protein. Perhaps PGRMC1 is required for a number of other functions that occur throughout the process of germ-line development, whereas PAIRBP1 is limited to playing a role in the PGRMC1-progesterone signaling pathway's anti-apoptotic function. Even so, the PAIRBP1 expression pattern that was detected by changes in band intensity from 15.5 dpc to PND 5 does not precisely align with the germ cell death timeframe that is shown in Figure 1, so a great deal more research will need to be conducted in order to learn more about this protein individually, as well as its interaction with PGRMC1.

Future Work

Given the variability of the results, as well as the unsuccessful completion of several of the experiments, there is a great deal of future work that can be conducted on this topic and the individual components of this research. Each of the experiments conducted in this research study could be repeated, or altered and repeated, to gain more conclusive and valid results. The overall age range used for the immunocytochemistry and western blot experiments could be expanded to include fetal ovaries at 13.5 dpc, as well as post natal ovaries at PND 7. Even though the crucial timeframe during which cyst breakdown and apoptosis is accounted for throughout this research study, it could always be beneficial to obtain more results and gain more information across a wider age range

Since some of the experimental methods used, such as the western blot, required a variety of alterations in antibody dilutions, perhaps a different experimental technique called a dot blot could be used. Dot blots could be used as a simplification of the western blot technique, where ranges of different primary antibody dilutions are tested on the sample of interest. This could be beneficial to determine which antibody dilution will yield the best detectable band before running an entire western blot with a number of different samples. Completing dot blots prior to future western blots could help ensure that the antibody of interest is functioning properly and at an optimal level before proceeding on to future experimental tests.

The incorporation of organ culture experiments into further research on this topic could be extremely beneficial in terms of investigating how changes in the functioning of PGRMC1, PGRMC2 and/or PAIRBP1 have a lasting effect on the progression of germ line development in the mouse ovary. Having the ability to manipulate these signaling pathways via function-blocking antibodies would allow us to analyze the direct effects that are had on the formation of primordial follicles. Organ cultures could be conducted using ovaries at 13.5 dpc, 15.5 dpc and PND 1, which would each be grown in culture for 5 days. Upon completion of each function-blocking organ culture the ovaries, now 17.5 dpc, PND 1 and PND 5, could be analyzed via immunocytochemistry using the same whole mount antibody staining procedure that was used in this research study. Once the treated ovaries were stained it would be possible to count and calculate the number of individual oocytes in each ovary as a determinant of overall follicle development.

The ability to calculate the approximate overall success of cyst breakdown and, in turn follicle development, via oocyte counting would be extremely valuable in quantifying the importance of progesterone signaling pathway components, such as PGRMC1, PGRMC2 and PAIRBP1. Incorporating these types of experiments into future research would bring an entirely new and significant dimension to this topic of study.

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Appendices

Figures

Tables