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Role of Progesterone Receptors in Neonatal Ovary Development

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Abstract

In female mammals, proper oocyte development is a vital prerequisite for future gamete viability and fertility. This development of oocytes, known as oogenesis, begins with the migration of primordial germ cells to the genital ridge of the early embryo, where multiple rounds of mitotic division occur without complete cytokinesis. The result is temporary cyst morphology. Cyst breakdown is a crucial process in the next developmental stage, resulting in formation of the single oocytes which will grow in follicles surrounded by granulosa cells and eventually develop into eggs. These aspects of embryogenesis are conserved across multiple species, including Drosophila, mice, and humans. Extensive research has already been completed to elucidate the mechanisms through which the steroid hormone estrogen regulates these developmental processes. Some research shows that progesterone, among other steroid hormones, also plays a role in inhibiting cyst breakdown and disrupting proper follicular assembly. However, specific types of progesterone receptors (PRs) and the signaling pathways they mediate have not yet been studied in the fetal stages of ovarian development. Here, the expression of specific PRs has been investigated and assessed via immunocytochemistry. The functional characterization of each individual progesterone receptor using organ culture is now in early stages of investigation. The experimental results confirm the presence of four different types of PRs: PR-A and PR-B, PAQR3, PGRMC1, and PGRMC2. Each receptor type shows evidence of expression at different stages of development as well as localization to different cell types within the ovary. Elucidating the detailed signaling pathways pertaining to each receptor requires additional investigation.

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Introduction

The reproductive success of all organisms is the most critical element of generating off-spring and contributing to the genetic variability of life in general. Oogenesis in females is a vital developmental process which determines the future reproductive ability of animals by generating viable oocytes for the future. Though many fundamental concepts have been studied, this process is not completely understood.

In female mice, oocyte precursor cells known as primordial germ cells migrate from the exterior of the embryo into the genital ridge, which will become the organism's reproductive organs. This migration of the cells into the embryo occurs at around 10.5 days post coitum (dpc). At the genital ridge, the primordial germ cells divide synchronously multiple times by mitosis to form germ-line cysts (Pepling and Spradling, 1998). The process results in cysts due to the lack of cytokinesis following each individual primordial germ cell's mitotic replication, and intercellular bridges connect the various cells within a cyst and hold it together (Fig. 1).

The germ cells within the multiple clusters of cysts within the ovary are known as oogonia at this stage. This process is conserved and also present in invertebrate females such as *D. melanogaster* (De Cuevas *et al*., 1997). At around 13.5 dpc, the oogonia begin to undergo meiotic division and are known as oocytes. The oocytes arrest at prophase I of meiosis, which will allow for homologous recombination of the chromosomes via the formation of the

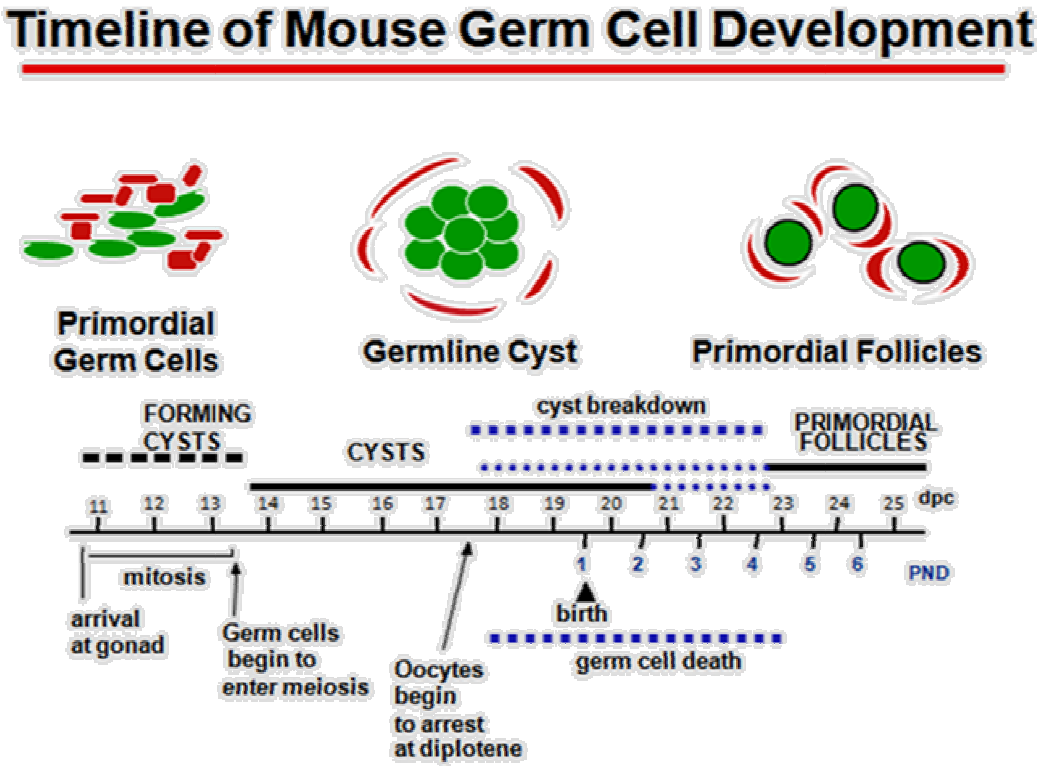

Figure 1. Timeline of germ cell development in the mouse and stages leading to oocyte development and maturation. Precursor germ cells and oocytes are shown in green; granulosa cells are shown in red (adapted from Pepling, 2006).

synaptonemal complex; this is a necessary process which results in increased genetic variability. At 17.5 dpc, the cyst starts to undergo breakdown as the oocytes proceed in maturation and begin the process of becoming enclosed in single follicles. The cyst breakdown process is a combination of cellular processes including cytokinesis, apoptosis, and continuing differentiation of the germ cell into a mature oocyte.

The proposed explanation for the mechanism of cyst breakdown states that cyst breakdown is a cumulative effect of these several processes. Many of the oocytes within the cyst undergo apoptosis and die, which results in a disassembly

of the cyst. In fact, nearly two-thirds of the oocytes die at around this time, leaving only about one third of the original number of oocytes to become enclosed in granulosa cells and then mature into primordial follicles (Pepling and Spradling, 2001). Granulosa cells are a type of somatic cell within the ovary which are usually found surrounding single oocytes. It is possible that the granulosa cells serve a nursing function for the particular oocyte that they enclose (Fig. 2).

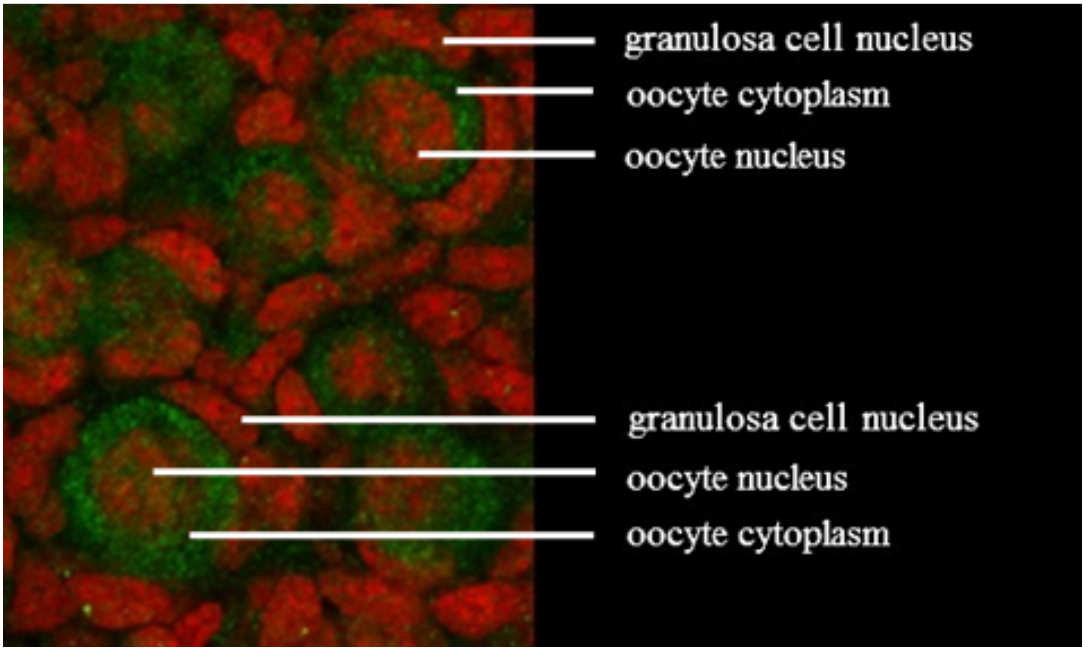

Figure 2. Diagram of the cellular layout within the ovary. Oocytes were labeled with Stat-3 (green), a marker specific for a protein in the oocyte cytoplasm. All cell nuclei were labeled with propidium iodide (red), a DNA intercalating agent.

In mice, the numerical statistic of this process is quiet large. Out of around 6,000 initial germ cells, only around 2,000 germ cells survive following cyst breakdown for assembly into primordial follicles. In human females, the same process takes place and the number of oocytes remaining following cyst

breakdown is equivalent to around 2 million oocytes out of an original 7 million germ cells (Baker, 1963). The number of a female's oocytes steadily decreases during her life span, and after a certain age, the quality of her oocytes also begins to decrease. This occurs for a few decades until the period known as menopause, by which time the oocyte pool is largely depleted.

The process of cyst breakdown has many unanswered questions. The need for such a significant amount of apoptosis is unclear, although current theory suggests that this wide-spread apoptosis in the ovary is actually a critical element for completion of cyst breakdown (Greenfield *et al*., 2007). Based on recent research, it is known that exogenous neonatal treatment of ovaries with steroid hormones such as estrogen and mimics such as estradiol and genistein can prevent proper cyst breakdown; this prevention inhibits the proper assembly of oocytes into single follicles and results in infertility (Chen *et al*., 2007; Jefferson *et al*., 2002). Other remaining questions regarding this process concern the selection of the oocyte's fate for survival or apoptosis. It is not known if there is a specific signal, pathway, or reason to label certain oocytes for apoptosis. Even more so, the exact signals for regulation and coordination of cyst breakdown are not all identified. In the female mouse, germ cell death and cyst breakdown are completed about three to four days after birth; in the human female, this process occurs just before birth. The oocytes which successfully assemble into primordial follicles are single cells which are enclosed and surrounded by a layer of granulosa cells. These remaining germ cells become the limited number of

gametes the female can use for sexual reproduction over the course of her life time. Essentially, this is the source of fertility.

Beyond the presented timeline of neonatal germ cell development, the primordial follicles continue to mature as gametes. At puberty, various hormones stimulate the follicle to undergo folliculogenesis (Matzuk *et al*., 2002). The oocyte within the follicle progresses through a primary, secondary, and tertiary stage of growth and development. The primordial follicle develops into a primary follicle when the granulosa cells surrounding the oocyte take on a cuboidal shape. Continued growth and division of the granulosa cells into multiple layers surrounding the developing oocyte leads into the secondary follicle stage. At this stage, an additional layer of theca cells envelops the exterior of the follicle as a whole, surrounding the granulosa cells. The follicle then proceeds through an extra tertiary stage of growth into the preovulatory stage.

Complete development of the oocyte through all of the stages prepares it to be finally released during ovulation, when the oocyte is officially an ovum (or egg). The ovum is fully functional and competent to be fertilized by sperm. Penetration by sperm finally completes the process of meiosis in the ovum which had begun when it was still only a germ cell in a dividing cyst. Successful fertilization of the ovum is followed by implantation in the female's uterus and generation of a new and unique organism.

The importance of researching the processes of germ cell development and oocyte maturation cannot be underestimated. Infertility is a problem which can result when oocytes are not properly developed and assembled within the ovary.

What is unfortunate is that in humans, follicular assembly occurs a few months before the time of birth; however, infertility may not be diagnosed until two, three, or even four decades later. According to the Centers for Disease Control and Prevention, 7.3 million females of reproductive age are infertile in the United States. This accounts for almost 12% of the female population at reproductive age (Chandra *et al*., 2006). Even more significantly, there has been a notable increase in this statistic over the past few decades, which coincides with an exponential increase in the number of industrial compounds in use (Stephen and Chandra, 1995).

Besides the fact that the population size has obviously increased in the past few decades, there are possibly less obvious factors involved in this increased occurrence of infertility. As mentioned previously, estrogen plays a critical role in the proper development of the oocytes and the ovary as a whole. It is important to notice that the amount of exogenous estrogens in the environment has been increasing, and there appears to be a correlation between this trend and increased infertility rates among female mammals. There are both naturally-occurring as well as synthetic chemical compounds which are very similar to estrogen in structure and mimic its activity via the estrogen receptor.

Exposure to elevated levels of these exogenous estrogenic compounds during the critical stages of neonatal development can result in various defects within the ovary, one of which is the presence of multiple oocyte follicles (MOFs). Rather than follicles forming properly with a single oocyte enclosed within layers of granulosa cells, MOFs contain two or more oocytes enclosed

within a follicle. The MOF appears to be the product of a cyst which did not break down completely. Observation of adult female mouse ovaries that had been treated as neonates with estrogen, estradiol, or other estrogen mimics such as bisphenol-A or diethylstilbestrol shows a higher percentage of MOFs and a lower percentage of single oocytes when compared to control mice (Suzuki *et al*., 2002). Additional compounds such as genistein and progesterone (P4) exhibit similar effects on the developing ovary when present in excessive amounts (Chen *et al*., 2007). Many of these are not uncommon industrial compounds and their presence in manufactured products such as plastics or polycarbonates may be altering the natural hormonal composition of mammals exposed to these products (Fig. 3).

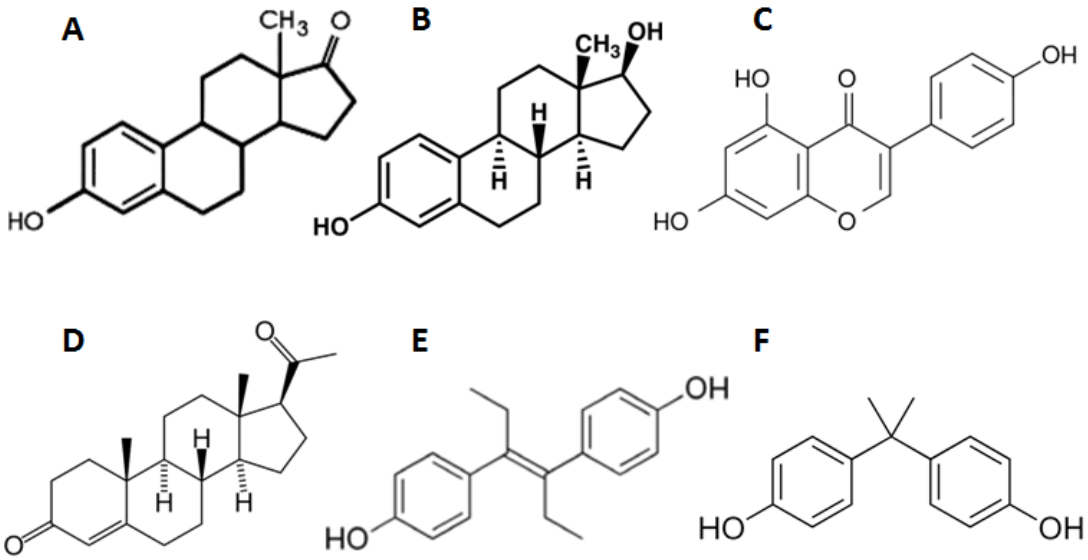

Figure 3. Examples of natural hormones and mimicking synthetic compounds: estrogen (A), estradiol (B), genistein (C), progesterone (D), diethylstilbesterol (E), bisphenol-A (F).

The steroid hormone estrogen and its receptors in particular have been the focus of extensive research in understanding the normal mechanisms of germ cell development and the possible complications or disruptions in the mechanisms involved. Estrogens can signal through two classic nuclear receptors, estrogen receptor (ER) α and ER β (Pettersson and Gustafsson, 2001). These receptors have already been studied meticulously for many years, and their characterization continues to develop today. Double knockout female mice missing both receptors are infertile, as are ER α knockout mice (Lubahn *et al*., 1993). ER β knockout mice exhibit reduced fertility (Krege *et al*., 1998; Hewitt *et al*., 2000).

The fragility of development can be deduced from this research. Estrogen is vital for proper development of an embryo's reproductive organs and for future reproductive viability. Mice lacking estrogen receptors are infertile, but treatment of neonatal female mice with levels of estrogen which are higher than normal also results in reduced fertility due to the formation of MOFs. Because of this effect in mice, it is hypothesized that in the fetal stage, the substantial amount of circulating maternal estrogen (relative to the embryo) inhibits cyst breakdown before birth (Chen *et al*., 2009). The significant decrease in estrogen levels at birth and afterwards allows for cyst breakdown to take place and for oocyte assembly into primordial follicles to take place. Therefore, treatment of ovaries with estrogenic compounds in the lab or exposure of ovaries to these compounds in the environment may continue to inhibit cyst breakdown and oocytes will not form properly.

Estrogen is only one of multiple factors in the developmental processes being described. Research results have provided a glimpse of the great complexity found in estrogen receptors. For example, the ERs can even bind ligands other than estrogen. ER β can also bind and mediate the effects of genistein, a compound normally found in plants (Jefferson *et al*., 2002). Many other hormones and their receptors mediate their own designated pathways and together they all culminate into an integrated network of signaling cascades, transcription regulation, and phenotypic responses.

Progesterone is another steroid hormone which has been considered in more current research, but not nearly as extensively in regards to oocyte development as compared to the efforts of estrogen receptor research. Together with estrogen and other hormones, progesterone regulates and coordinates the continuous cycles of the reproductive system such as menstruation (Conneely *et al*., 2003). Progesterone is a vital hormone involved in processes throughout a female's lifetime: oogenesis, ovulation, pregnancy, and menopause. It is produced mainly in the ovaries, but a few other tissues such as the adrenal cortex produce progesterone as well.

During menstruation, developing follicles in the ovaries secrete estrogen and progesterone to thicken the lining of the uterus and to induce the endometrium to secrete proteins in preparation of possible zygote implantation. After ovulation of an ovum, progesterone levels continue to increase in the reproductive system due to its secretion by the corpus luteum cells, which are formed from the tissue of the remaining follicle after release of the ovum.

Following fertilization and implantation, the placenta takes on the role of producing progesterone to maintain pregnancy, and the elevated levels of progesterone also suppress ovulation during that time.

As a side note, it is important to mention that a synthetic molecule known as progestin is currently of particular interest in research due to its useful function in oral contraceptives, but possibly detrimental role in increasing risk of breast cancer. Progestin was purposefully synthesized to be used as a drug and function as a progesterone mimic (Fig. 4). It is known to bind to some progesterone receptors in the body, but not necessarily all of them, and the effects of progestin are considered to be more potent than those of progesterone.

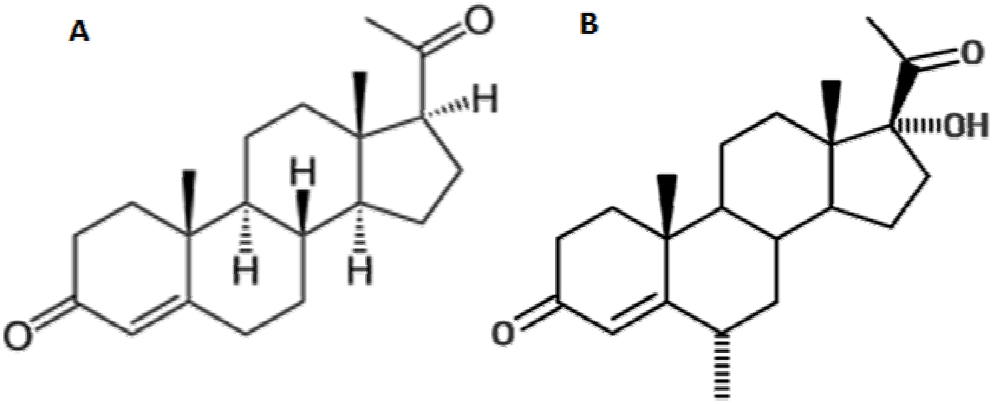

Figure 4. Structure of progesterone steroid hormone (A) and structure of a synthetic progestin molecule (B).

Estrogen has been shown to play a large role in development of breast cancer (Holmberg *et al*., 2008).The roles of progesterone and progestin are less conclusive. Females undergoing estrogen and progestin hormone replacement

therapy have an increased risk of breast cancer, the breast cancer tumors are larger, and the breast cancer overall has more fatal consequences. Progestin, as a ligand bound to a receptor, plays a role by specifically regulating certain microRNA (miRNA) levels in cells (Cochrane *et al*., 2012). It has also been shown to stimulate expression of growth factors, influence cell-cycle control, and induce expression of protooncogenes (Pasqualini *et al*., 1998). While more and more research is being dedicated to studying progestin, much of the data being currently obtained in regards to its overall effects and risk is inconclusive or contradictory. On the other hand, progesterone has been shown to have an inhibitory effect on breast cancer and prevent the proliferation of breast cancer cells (Formby and Wiley, 1998). It appears that these two seemingly similar compounds have opposing functions and it is important to continue research on progesterone and progestin, their receptors, and their signaling pathways to determine their mechanisms of action.

Progesterone itself can signal via multiple types of progesterone receptors (PRs), and its effects during embryogenesis are similar to those of estrogenic compounds. Developing ovaries are naturally exposed to and need specific levels of maternal progesterone, but further treatment of neonatal ovaries by elevating the levels of progesterone blocks cyst breakdown (Chen *et al*., 2007).

Progesterone can be converted to estrogen within the body, so a nonmetabolizable form of progesterone known as promegestone has also been utilized in organ cultures to verify whether the effects of progesterone are direct or indirect. Organ cultures treated with promegestone also resulted in inhibition of

cyst breakdown and follicle assembly, which is evidence that progesterone does have an important and direct effect on oogenesis. Previous research also shows that estrogen might be a slightly more potent inhibitor of cyst breakdown as compared to progesterone; ovaries cultured from PND 1-8 with progesterone showed a decrease in percent single oocytes (68%) when compared to control cultured ovaries, which had 86% single oocytes (Chen *et al*., 2007). Ovaries cultured from PND 1-8 with estrogen had only 53% single oocytes. Significantly, treatment of ovaries with both progesterone and estrogen showed an additive effect resulting in only 34% single oocytes.

Progesterone can signal through two known nuclear receptors: PR-A and PR-B; the use of different promoters results in both receptors being isoforms of the same gene (Conneely *et al*., 1989; Kastner *et al*., 1990). Progesterone signaling can also occur at the membrane; for example, a nuclear isoform may translocate to the membrane. In the family of progestin and AdipoQ receptors (PAQR), PAQR3 is a seven transmembrane spanning receptor which may bind progesterone. Progesterone receptor membrane component 1 (PGRMC1) and PGRMC2 also act as PRs (Peluso, 2006).

A goal of this research is to begin investigating PRs with greater intensity. Due to the scarcity of research information available on PRs and their role in neonatal reproductive organ development, this preliminary research begins with a straight forward approach and basic questions: which specific PRs are present in the neonatal ovary, and which specific cell types are expressing these receptors.

An elementary amount of information is available regarding each of the four PRs mentioned. PR-A and PR-B are nearly identical isoforms of the human progesterone receptors (PR) gene. The two isoforms have 780 identical residues, but PR-B contains an additional 164 residues on the N-terminal polypeptide end (Tung *et al*., 2006). Ligand-bound PR-B is also the stronger regulator of transcription, and functionally, both isoforms regulate the expression of different genes. A PR polyclonal antibody was used to identify both isoforms simultaneously in this project.

While expression of PR-A and PR-B in most normal tissues (generally on breast tissue and reproductive organs) is equimolar, the ratio of the two isoforms is not consistent in tumors, particularly in breast cancer (Sartorius *et al*., 2003). An important factor to consider is that PR-A and PR-B both serve as inhibiting regulators of ER mediated transcription, but they do so via two different mechanisms, due to their different regulatory functions. The PR isoform composition of the tumor may therefore influence hormonal therapy targeting the tumor. Unfortunately, however, clinical assays are not currently capable of distinguishing one specific isoform from the other. These receptors have therefore had significant implications in the study of breast cancer. In this research, an alternate evaluation of PR will begin in regards to its role in development of the reproductive organs. Both of the PR isoforms have already been identified in the granulosa and theca cells of adult ovaries (Gava *et al*., 2004).

The PAQR family of seven transmembrane spanning (7TM) receptors is produced from a total of 11 different genes (Tang *et al*., 2004). The individual

receptors bind a variety of ligands. Even though the entire family has been identified recently, the receptors appear to be highly conserved with homologs found even in eubacteria. The discovery of these seven transmembrane domain G-protein-coupled receptors in 2003 was considered a significant breakthrough (Fernandes *et al*., 2008). This is because progesterone and its derivatives were already in use for treatment of various disorders related to the reproductive organs, and the discovery of these receptors introduced a possibly new therapeutic target in treatment. This family of receptors is currently being evaluated for a possible role in mediating progesterone signaling and exhibiting anti-tumorigenic effects in ovarian cancer. It is thought that pharmacological activation of these types of receptors which mediate anti-tumorigenic responses may be useful in developing cancer treatments.

PAQRs 7, 8, and 5, also known as mPRs α, β, and γ respectively, have already been identified as membrane progestin receptors (mPRs), with the PAQR7 isoform being predominant in reproductive tissues (Tang *et al*., 2004). PAQR7 has also been identified as a membrane progesterone receptor in trout (Charles *et al*., 2010). Initial studies showed that PAQR7 was localized to the oocyte membrane, but later studies concluded that the receptor was in fact localized to the endoplasmic reticulum of oocytes (Fernandes *et al*., 2008). Regarding the breakthrough in 2003 and the discovery of these receptors, it was shown that injection of mPR antisense oligonucleotides into zebra fish inhibited oocyte maturation, which is proof that these receptors are involved in promotion and regulation of proper oocyte maturation (Zhu *et al*., 2003). Very little detail is

known otherwise regarding the functional activity and mechanisms of the different PAQRs, however. PAQR3 does not seem to have been assigned a specific role yet (Tang *et al*., 2004). In this research, PAQR3 is shown to exhibit expression in the ovary localized to the cytoplasm of the oocytes a few days after birth; it is likely that it can mediate the effects of progestin and possible that it can mediate the effects of progesterone as well. Specific localization to the endoplasmic reticulum was not noted in this case. Due to the likelihood that PAQR3 mediates the effects of progesterone, PAQR3 will be considered as a progesterone receptor.

One of the PRs receiving the most attention in current research is PGRMC1. Its expression is localized to intracellular membranes, the cytoplasm, and the nucleus, particularly in the ovary and the liver (Lösel *et al*., 2008). However, its expression shows significant variability and may be due to dependence on factors such as the cell cycle. It is currently undecided whether or not PGRMC1 functions as a dimer, although it may instead be forming a functional complex with another protein; different experiments are contradictory (Falkenstein *et al*., 2001; Lösel *et al*., 2008). The selectivity and affinity of PGRMC1 for progesterone is higher compared to all other possible steroid ligands, and it is has been discovered that PGRMC1 is a receptor that serves an important role in mediating progesterone's anti-apoptotic effect (Peluso *et al*., 2007). Overexpression of the receptor results in enhanced responsiveness to progesterone, whereas an antibody to PGRMC1 inhibits progesterone's anti-apoptotic action (Peluso *et al*., 2008).

PGRMC1 also appears to have a great variety of other properties and functions. For example, PGRMC1 expression is increased in conditions of low oxygen, the mechanism for which is still unknown (Hughes *et al*., 2007). Although an exact function for PGRMC1 in other cells has not yet been elucidated, it is hypothesized that the receptor may play a role in the regulation of cholesterol and steroid synthesis in cells that are steroid generating, such as granulosa cells (Peluso *et al*., 2008).In granulosa cells, PGRMC1 is localized to the plasma membrane, the cytoplasm, and the nucleus. The nuclear localization suggests that PGRMC1 is involved in gene expression regulation. Therefore, it is likely that PGRMC1 is important for proper reproductive organ development and viability.

Interestingly, PGRMC1 is consistently expressed in most cells of ovarian tumors and promotes the survival of the normal cells as well as the tumor cells. PGRMC1-depleted ovarian cancer cells were found to promote apoptosis and form tumors less often when introduced into mice. Any tumors that did form were generally smaller in size as compared to normal tumors, with an average decrease in size being about 75% (Peluso, 2011). This indicates that PGRMC1, like PAQRs, could be a critical target of gene therapy for the treatment of ovarian cancer.

A receptor closely related to PGRMC1 is PGRMC2. However, very few papers have been published in the past few years mentioning this receptor. Most of the currently existing data on PGRMC2 only states that PGRMC2 (along with PGRMC1) exhibits expression in rat ovaries, and some minor structural

characterization of the PGRMC2 receptor has taken place (Cahill, 2007). However, PGRMC2 expression can be evident on many other cell types within humans in an almost ubiquitous manner, excluding a few specific tissue types such as adipose tissue (Chen *et al*., 2010). Whether or not this receptor plays a role in proper oocyte development and follicular assembly is not currently known and will be investigated here.

So far, the obtained results suggest that PGRMC2 is expressed earlier on while the oocytes are still in the cyst stage, at around 13.5 dpc to PND 1. The receptors PGRMC1 and PAQR3 do not appear to be expressed until the latest stages of cyst breakdown and as late as PND 7 in the case of PAQR3. PR-A and PR-B expression occurs in both the early and later stages of development.

Materials and Methods

Animals

The C57BL/6 (B6) inbred strain of mice, supplied by Jackson Labs, was used for the experiments. The animals were maintained by the lab animal resource facility at Syracuse University. B6 females were mated with males weekly and impregnation was evaluated by checking for vaginal plugs in the females daily. Matings were set up on Mondays after 2:00pm and females were checked for vaginal plugs every morning before 10:00am for the following four days. Females with plugs were then isolated and marked as 0.5 dpc, estimating that conception

occurred at around midnight. Pregnant females gave birth at around 19.5 dpc, at which time the litter age was marked post natal day 1 (PND1).

Dissection

Animals collected for experiments ranged in age from 13.5 dpc to PND 7. Fetal animals were obtained by euthanizing the pregnant female with carbon dioxide and dissecting the fetal pups from her abdomen. Pregnant females were euthanized using the Euthenex Equipment manufactured by E-Z Systems. Neonatal litters were collected from the female's cage. The litters were dissected using a Nikon SMZ1500 dissecting microscope in 1x phosphate buffered saline (PBS) solution. The ovaries were removed from the female and incubated in a "fix" solution composed of 200:400µL 16% formaldehyde (from Ted Pella Inc.) to 1x PBS, for a final dilution of 5.3% formaldehyde. This solution was used to preserve the ovaries for 24 hours on the nutator at 4 °C. The incubated tubes were labeled with the tissue collected, age of the organism, date of dissection, and antibody to be used for staining.

Whole Mount Antibody Staining of Neonatal Ovaries

The ovaries were prepared for indirect antibody staining following a protocol specifically developed for whole mount antibody staining of fetal mouse gonads. Following preservation in fix, the ovaries were washed twice in 1 mL of

PT (1X PBS/0.1% Triton X-100) and then incubated in 1 mL of PT on a nutator for at least 30 minutes. The ovaries were then incubated in 1 mL of PT + 5% bovine serum albumin (BSA) on a nutator for at least 30 minutes. For incubation with the primary antibody, the ovaries were incubated with 5 µL of primary antibody diluted in 500 µL of PT + 5% BSA for 24 hours on the nutator at 4 °C (Table 1). At this time, the secondary antibody was also prepared for later use. This required 2.5µL of fluorescent secondary antibody (Alexa Fluor 488) to be pre-absorbed with embryo powder (ground pellets from homogenized PND 12.5 – 14.5 mouse embryos incubated in ice-cold acetone and centrifuged at 10,000 g) and diluted in a tube with 500 µL of PT + 5% BSA, wrapped in aluminum foil, and incubated for 24 hours on the nutator at 4 °C (Table 2).

Table 1

Primary antibodies used in whole mount antibody staining		
Antibody	Description	Amount used (diluted in 500 µL PT+5% BSA)
PR (Santa Cruz Biotechnology)	labels PR-A and PR-B progesterone receptors	5 µL
PAQR3 (Santa Cruz Biotechnology)	labels PAQR3 receptor	5 µL
PGRMC1 (Santa Cruz Biotechnology)	labels PGRMC1 receptor	5 µL
PGRMC2 (Santa Cruz Biotechnology)	labels PGRMC2 receptor	5 µL
Stat-3 (Santa Cruz Biotechnology)	oocyte marker	1 µL
Vasa (Santa Cruz Biotechnology)	germ cell marker	2 µL

The following day, the ovaries were washed in 1 mL of PT + 1% BSA for at least 30 minutes on the nutator at room temperature. Then they were incubated

with 10 µL of RNase A (10 mg/mL RNase A) diluted in 1 mL PT + 1% BSA and placed on the nutator for 30 minutes at room temperature. RNase A is a reagent used to hydrolyze chains of ribonucleotides in tissue. After 30 minutes in RNase A, the ovaries were incubated with 10 µL of propidium iodide (0.5 mg/mL, Molecular Probes) diluted in 1 mL PT + 1% BSA, wrapped in aluminum foil, and placed on the nutator for 20 minutes at room temperature. Propidium iodide is a DNA intercalating agent which fluoresces when excited by light with a wavelength of 488 nm. The tube of ovaries stayed wrapped with aluminum foil from this point on to the end of the staining procedure to prevent loss of reagent fluorescence. After 20 minutes, the ovaries were washed in PT + 1% BSA on the nutator for at least 30 minutes at room temperature. Then, the ovaries were incubated with the secondary antibody solution prepared the previous day. The 1 mL of PT + 1% BSA was pipetted out of the tube of ovaries, and the dilution of secondary antibody was added to the tube of ovaries, after the embryo powder was allowed to sediment out of the secondary antibody preparation. The ovaries were incubated with the pre-absorbed fluorescent secondary antibody on the nutator for 24 hours at 4°C.

Table 2

Secondary antibodies used in whole mount antibody staining

Antibody	Wavelength	Description	Amount used (diluted in 500µL PT+5% BSA)
goat anti-rabbit (Molecular Probes)	Alexa 488	used to label PR-A and PR-B	2.5 µL
donkey anti-goat (Molecular Probes)	Alexa 488	used to label PAQR3, PGRMC1, PGRMC2	2.5 µL
donkey anti-goat (Molecular Probes)	Alexa 568	used to label PGRMC2	2.5 µL

The following day, the ovaries were washed three individual times with 1 mL of PT + 1% BSA, each wash for at least 30 minutes on the nutator at room temperature. They were then washed with 1 mL of PBS. About 500 µL of PBS was then pipetted out of the tube with the ovaries, while the other 500 µL was left as solution for the ovaries. A large pipette tip was cut to widen the opening at the tip, and the altered tip was used to pipette the ovaries from the tube to the glass slide (Fisher Scientific) for preparation. The PBS was completely removed from the ovaries on the slide, and about 2 drops of VectaShield (Vector Laboratories) were added to the ovaries on the slide for preservation of fluorescence in the dye and in the secondary antibody. A coverslip (Corning) was placed over the ovaries and after sealing the slide with nail polish, the slide was labeled with the tissue stained, the age of the litter, the date of dissection, the primary antibody used for staining, and the initials MND. The slides were allowed to dry at room temperature in the dark for about ten minutes and then placed into slide holding booklets (Fisher) for storage at -20 °C.

Confocal Microscopy

Prepared slides were observed on the Zeiss LSM 710 Confocal Microscope in the Blatt Image Center of Syracuse University's Life Sciences Complex. The ovaries were observed under magnifications of 10x, 20x, and 63x with oil. With confocal microscopy, the ovaries may be analyzed for expression of the labeled protein. The oocyte development stages may be monitored as well.

Images were captured on a computer connected to the confocal microscope using Zen software and standard magnification conditions: 63x with oil. The images were then saved to a data disc and transferred to an LSM Image Browser program for editing.

Organ Culture

An additional experiment was performed which allowed for the treatment and manipulation of ovarian growth in vitro before staining to observe the effects of the treatment. Prior to the dissection of the ovaries, the organ culture media was prepared (Chen *et al*., 2007). All equipment and surfaces, including gloves, were sterilized with alcohol before starting the procedure. Materials must also be autoclaved (sanitization through cycles of high temperature and pressure) occasionally as an extra precaution in sterility. Stock (control) media was prepared in a 50 mL falcon tube according to the recipe using 0.5 mL D-MEM (Invitrogen) / HAM's F12 Media with 0.1% BSA, 0.1% Albumax 5X ITS-X, and 10 mg of L-ascorbic acid (0.05 mg/mL). Treatment media could be made using a few microliters the treatment antibody diluted in a few milliliters of transferred stock media, generally 25 µl antibody to 2.5 mL stock media. At least three hours prior to the start of the dissection, the organ culture incubator (VWR) was sprayed with alcohol, turned on, and set to 37 °C and 5% CO_2. All of the equipment and the removable shelves were placed under a UV light hood (The Baker Company) for at least 60 minutes prior to use. After the incubator equilibrated to culture

conditions, a sterilized water pan was filled with autoclaved Millipore water and placed at the bottom of the incubator. Millicell floating filters for the organ culture were cut into culture well-size pieces and stored in a small petri dish under the UV light as well. Immediately preceding the dissection, the well plate (BD) used for the organ culture was prepared with 400 μL of the control media or the treatment media pipetted into each individual, labeled well. One floating filter was placed atop the media in each well. The culture plate was then placed in the incubator to equilibrate while the ovaries were being dissected.

Ovaries were dissected from the female mice in Hank's Balanced Salt Solution and collected in a plate filled with the Hank's Solution. The plate was kept in a covered container of ice. During dissection, extra care was taken to detach all the surrounding mesonephrous tissue from around the ovaries so that the organ was entirely removed from the organism. Once all of the ovaries are collected, they were transferred to the culture wells for incubation. Each ovary was individually scooped up with watchmaker's forceps, transferred to a second pair of forceps while wiping the first set on a kimwipe, and then transferred back to the first pair of forceps to remove all of the Hank's Solution from the ovary. Under a microscope (Zeiss), the ovary was then transferred from the tip of the forceps to the floating filters in the well plates. Two to three ovaries were placed per filter in a well. A drop of media was then placed on each ovary using forceps. This sequence was repeated with each ovary from a dissection. The well plate was then placed in the incubator and incubated at around 37 °C with 5% CO_2 for at least 24 hours up to a few days, depending on the purpose of the experiment.

The culture media was replaced each day. All of the equipment used was sprayed with alcohol and placed under UV light for at least 30 minutes prior to each replacement. The old media was then drawn out from under the filter, and 400 µL of new control or treatment media was added to each well, taking care not to submerge the filters. A fresh drop of media was also placed on each ovary using forceps. Replacements were completed using similar conditions in regularly spaced 24 hour intervals.

Once the ovaries were cultured for a specified number of days, they were removed from the filters and incubated in tubes of fix solution (200:400µL 16% formaldehyde to 1x PBS) for 24 hours on the nutator at 4 °C for preservation. The control ovaries were collected together in one tube, and the treated ovaries were collected together in a separate tube. The incubated tubes were labeled with the tissue collected, age of the organism during the time span of the organ culture, date of organ culture completion, and antibody to be used for staining. The standard whole mount antibody staining protocol was then used to stain the cultured ovaries for observation of treatment results.

Counting Follicle Development

When performing an experiment where both quantitative and qualitative changes are expected, there must be standardized methods of analysis to determine and characterize the results. An expected result when performing organ culture experiments with ovaries is that the number of oocytes present and their

morphology will change. One way to quantify this result is to count follicle development.

Counting follicle development is a dependable and standardized method of interpreting immunocytochemistry results. The numbers of oocytes per section are counted and then the percent of cyst breakdown can be calculated based on the statistics obtained. Therefore, oocytes or follicles can be counted and compared among other samples in a standardized way. Counting follicle development was not performed in the research described here. Because most of the experimental data is from simple stains of tissue which has not undergone treatment, this method has no relevant application. The last two experiments mentioned were performed recently and do include manipulation of the ovary environment. The counting follicle development method will need to be applied in the near future to analyze the results of these two organ cultures as well as future organ culture experiments.

Results

Receptors PR-A and PR-B are expressed in the developing neonatal ovary

To determine which cell types express progesterone receptors PR-A and PR-B in developing neonatal ovaries, immunocytochemistry was performed for 15.5 and 17.5 dpc, as well as PND 1, 3, 5, and 7 (Fig. 5).

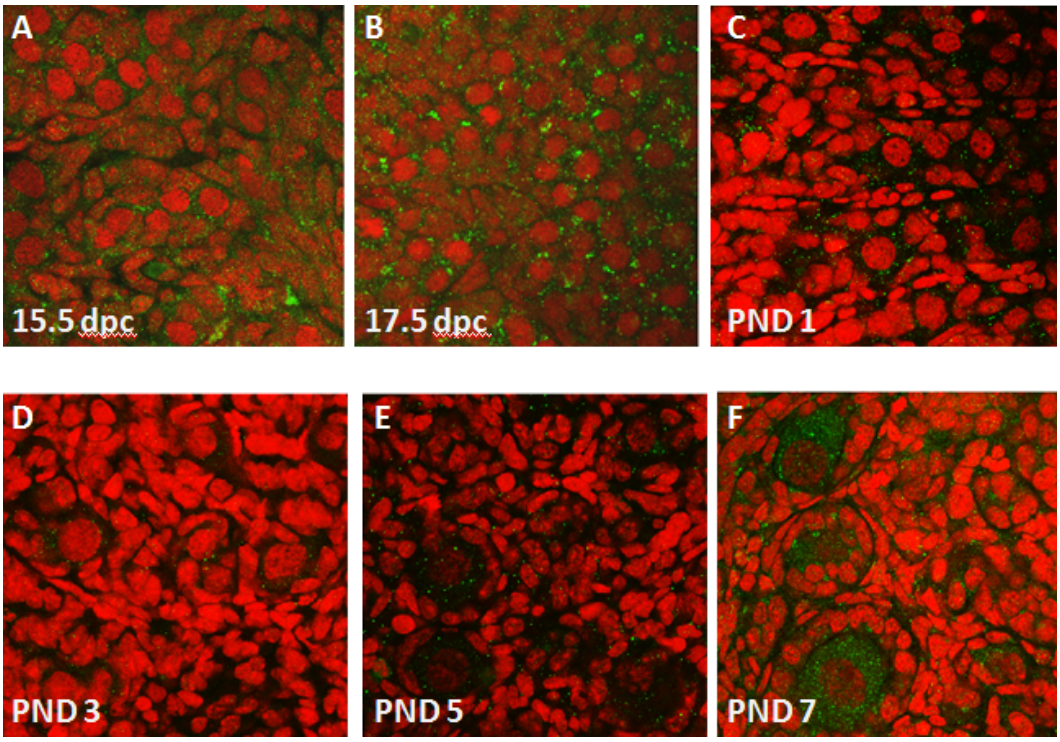

Figure 5. Expression of PR-A and PR-B progesterone receptors in neonatal mouse ovaries. Confocal sections show evidence of labeling with PR antibody (green) at 15.5 dpc (A), 17.5 dpc (B), and PND 7 (F). Cell nuclei were labeled with propidium iodide (red).

Moderate expression of the PR-A and PR-B progesterone receptors was detected in the developing neonatal ovary, as evidenced by immunocytochemistry, at 15.5 – 17.5 dpc, as well as PND 7 (Table 3). Expression during 15.5 – 17.5 dpc was evident in the cytoplasm of multiple cell types in the ovary, including oocytes and granulosa cells. Particularly at 17.5 dpc, expression of PR-A and PR-B seems to indicate an interesting result that the receptor may be localized to certain organelles within the oocyte. This hypothesis is due to the fact that the brightest spots of fluorescence are present in sections bordering almost all of the round oocyte nuclei. It is also possible that PR-A and PR-B function as

nuclear membrane receptors. Weak expression at PND 7 was limited mainly to the cytoplasm of the oocytes.

Table 3

Summary of PR-A and PR-B Expression			
Age	15.5-17.5 dpc	PND 1-5	PND7
Location	cytoplasm of oocytes and granulosa cells, may be localized at 17.5 dpc	———	cytoplasm of oocytes
Level of expression over	moderate expression	insignificant expression	weak expression

Receptor PAQR3 is expressed in the developing neonatal ovary

To determine which cell types express progesterone receptor PAQR3 in developing neonatal ovaries, immunocytochemistry was performed for 15.5 and 17.5dpc, as well as PND 1, 3, 5, and 7 (Fig. 6).

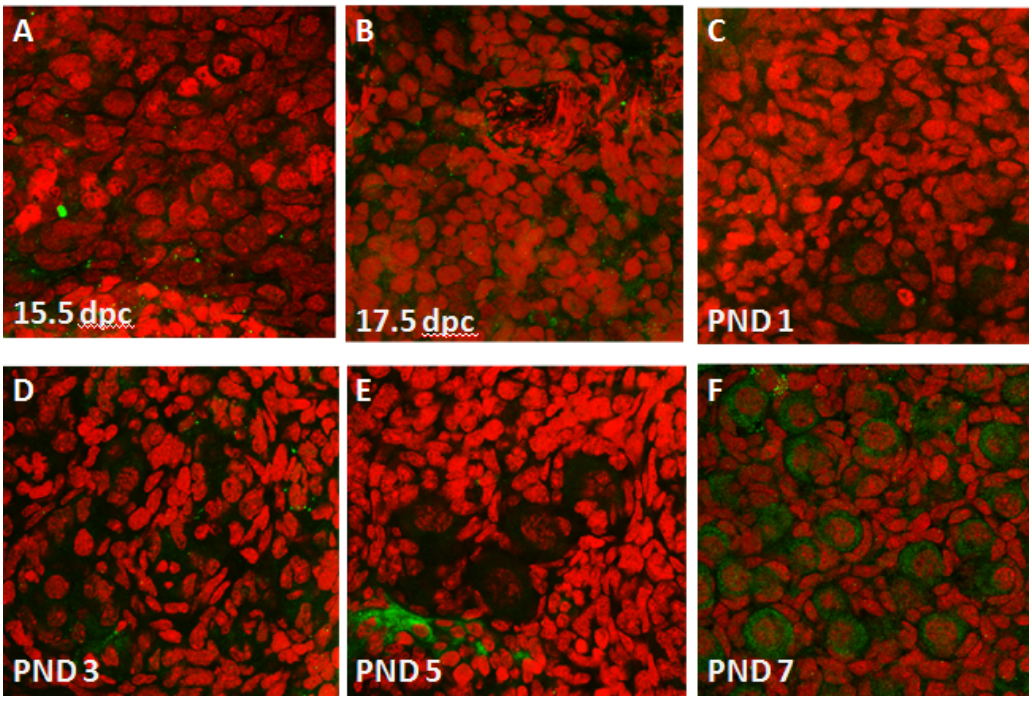

Figure 6. Expression of PAQR3 progesterone receptor in neonatal mouse ovaries. Confocal sections show evidence of labeling with PAQR3 antibody (green) at PND 7 (F). Cell nuclei were labeled with propidium iodide (red).

There does not appear to be significant expression of the PAQR3 receptor in the developing neonatal ovary until PND 7. On PND 7, the PAQR3 receptor shows strong expression specifically localized to the cytoplasm of the oocytes (Table 4).

Table 4

Summary of PAQR3 Expression		
Age	15.5 dpc – PND 5	PND 7
Location	———	cytoplasm of oocytes
Level of expression	insignificant expression	strong expression

Receptor PGRMC1 is expressed in the developing neonatal ovary

To determine which cell types express progesterone receptor PGRMC1 in developing neonatal ovaries, immunocytochemistry was performed for 15.5 and 17.5dpc, as well as PND 1, 3, 5, and 7 (Fig. 7).

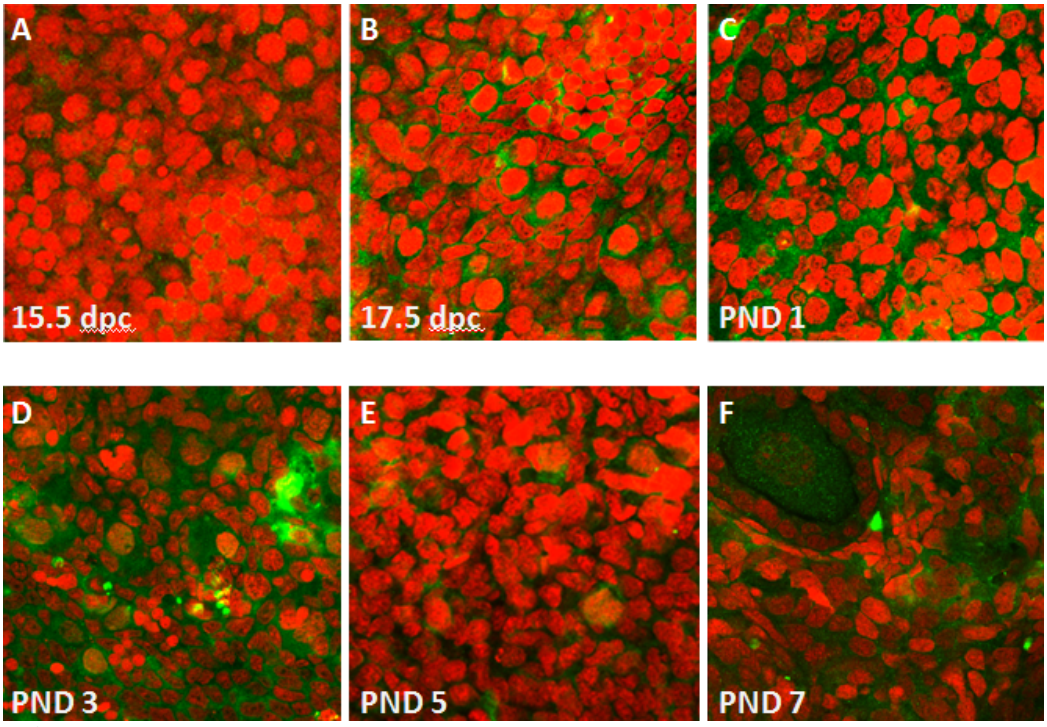

Figure 7. Expression of PGRMC1 progesterone receptor in neonatal mouse ovaries. Confocal sections show evidence of labeling with PGRMC1 antibody (green) at PND 3 (D), PND 5 (E), and PND 7 (F). Cell nuclei were labeled with propidium iodide (red).

Moderate expression of the PGRMC1 progesterone receptor was detected in the developing neonatal ovary, as evidenced by immunocytochemistry, at PND 3 – PND 7 (Table 5). During this age, PGRMC1 is expressed in the cytoplasm of both oocytes and granulosa cells. A significant amount of background staining exists for most of the samples, and ovaries at 17.5 dpc and PND 1 only showed

random background staining with little localization or specificity of the antibody. PGRMC1 antibody labeling at PND 3 appears moderate, but somewhat diffuse. Overall, the evidence of expression appears across multiple cell types. Labeling of oocyte nuclei with PGRMC1 antibody is also apparent.

Table 5

Summary of PGRMC1 Expression		
Age	15.5 dpc – PND 1	PND 3-7
Location	———	cytoplasm of oocytes and granulosa cells, possible labeling of oocyte nuclei
Level of expression	insignificant expression	moderate expression

Receptor PGRMC2 is expressed in the developing neonatal ovary

To determine which cell types express progesterone receptor PGRMC2 in developing neonatal ovaries, immunocytochemistry was performed for 13.5, 15.5, and 17.5dpc, as well as PND 1, 3, 5, and 7 (Fig. 8).

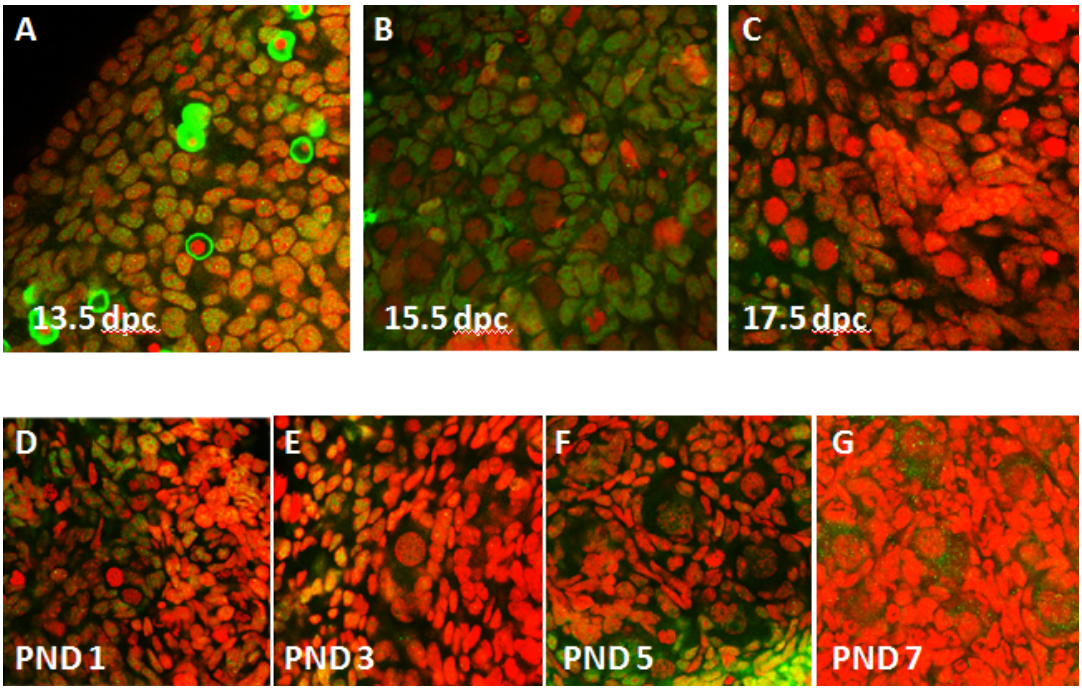

Figure 8. Expression of PGRMC2 progesterone receptor in neonatal mouse ovaries. Confocal sections show evidence of labeling with PGRMC2 antibody (green) at 13.5 dpc (A), 15.5 dpc (B), 17.5 dpc (C), and PND 1 (D). Cell nuclei were labeled with propidium iodide (red).

Strong expression of the PGRMC2 progesterone receptors was detected in the developing neonatal ovary, as evidenced by immunocytochemistry, at 13.5 dpc-PND1 (Table 6). An additional immunocytochemistry experiment was performed for age 13.5 dpc with the PGRMC2 antibody because of the notably strong and specific expression observed at 15.5 dpc. Strong expression of PGRMC2 was localized to the cytoplasm of the granulosa cells at 13.5 and 15.5 dpc with expression levels decreasing slightly approaching PND 1. PGRMC2 labeling at PND 3 – PND 7 was extremely diffuse and did not appear to be significant.

Table 6

Summary of PGRMC2 Expression		
Age	13.5 dpc – PND 1	PND 3-7
Location	cytoplasm of granulosa cells	———
Level of expression	strong and slightly decreasing over time	insignificant expression

PGRMC2 expression at 13.5 dpc also showed a unique expression pattern with strong labeling of the membranes of certain cells characterized by a dense nucleus. This dense nucleus is not uncommon for the gonads of early embryos, but there are currently no explanations for its unique appearance. Immunocytochemistry was then performed with a triple stain to observe PGRMC2 expression in relation to the germ cells at 13.5 dpc more closely (Fig. 9).

A triple stain was performed for a few reasons. First, labeling with the germ cell marker Vasa confirmed that the organ under observation was an ovary and not a testis. It can be difficult to differentiate ovaries from testes at 13.5 dpc in mice because they haven't differentiated significantly and can often look very similar. Also, it confirmed the previous results and showed that expression of PGRMC2 is found on the granulosa cells. The unknown unusual cells were also present and labeled with PGRMC2.

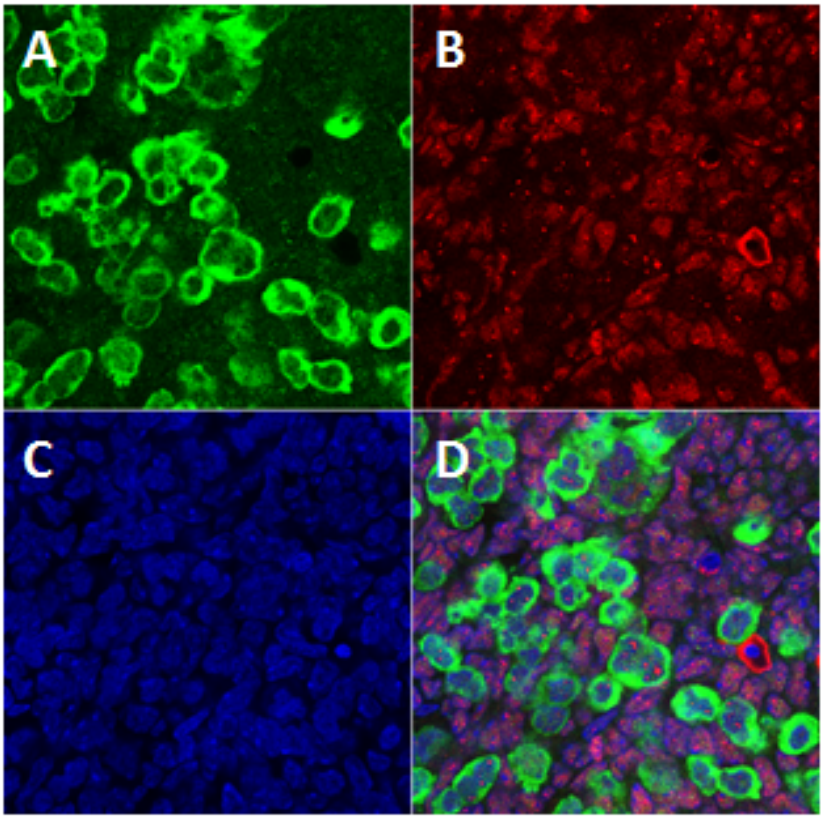

Figure 9. Expression of PGRMC2 progesterone receptor at 13.5 dpc in neonatal mouse ovaries. Confocal sections of ovaries labeled with Vasa (green) (A), PGRMC2 labeled with PGRCM2 antibody (red) (B), cell nuclei labeled with Toto-3 (blue) (C), and overlay (D) show evidence of PGRMC2 expression in the cytoplasm of granulosa cells.

All four receptors of interest: PR-A and PR-B, PAQR3, PGRMC1, and PGRMC2 are therefore expressed within the time frame introduced in Figure 1. A diagram of the cumulative expression of these receptors is illustrated in Figure 10.

Ideally, a more objective approach would be employed in quantifying the expression of these receptors. Although it is not completed here, western blots could be used to verify the dependability of the results already obtained. Real-time polymerase chain reaction (RT-PCR) could also be used as an even more accurate way to determine the expression patterns of these receptors.

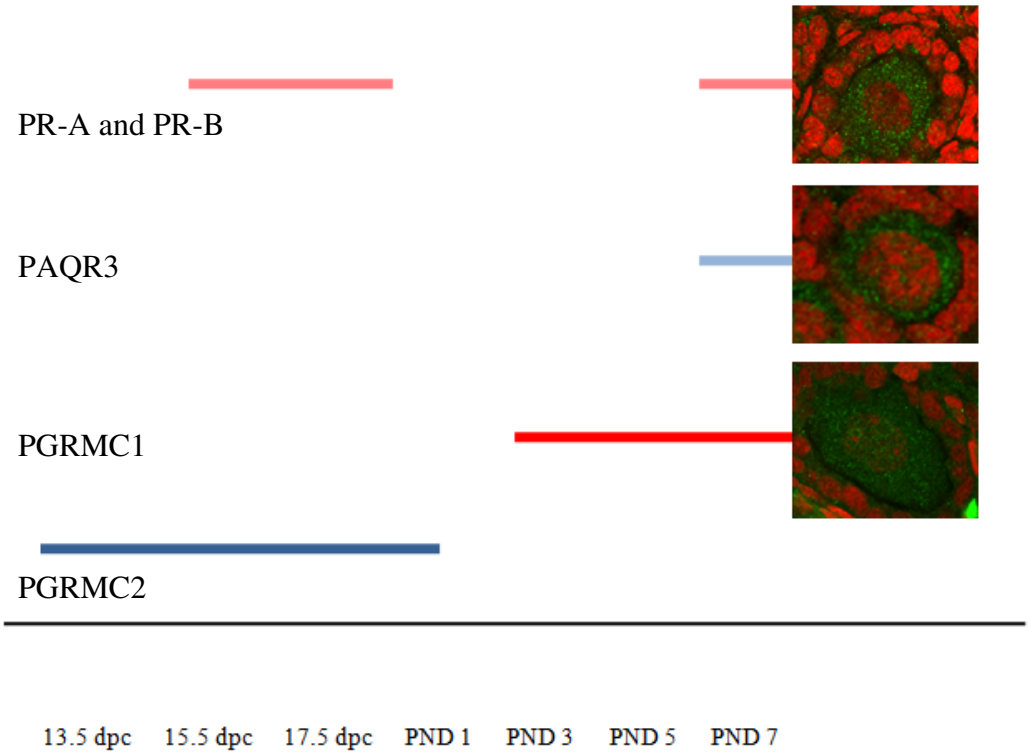

Figure 10. Summary diagram of progesterone receptor expression in the female mouse ovary from age 13.5 dpc to PND 7. Order of lines follows the sequence of the receptors as presented in the text: PR-A and PR-B (pink), PAQR3 (light blue), PGRMC1 (red), and PGRMC2 (dark blue). Pictures are included of the first three receptors to show that expression by PND 7 is localized to the cytoplasm of the oocytes.

It is important to notice the trend as the mouse reaches PND 5 and afterwards. The expression of three of the four receptors is present. Due to the fact that these three receptors are present in the adult ovary as well, it is possible that the expression of these receptors begins as early as PND 5 or PND 7 in the pup and continues throughout the lifetime of the female. Also, it is an interesting result that at PND 7 all of the progesterone receptor expression is localized to the cytoplasm of the oocyte.

Effects of blocking PGRMC2 on oocyte development

The mechanisms of PGRMC2 signaling were investigated further given the receptor's strong immunocytochemical staining results. An organ culture experiment was performed with incubation of neonatal ovaries at PND 1 to PND 5. A function blocking antibody was included in the organ culture media (1: 100 PGRMC2 antibody to media dilution) as a treatment to test the effects of blocking PGRMC2 on oocyte development (Fig. 11).

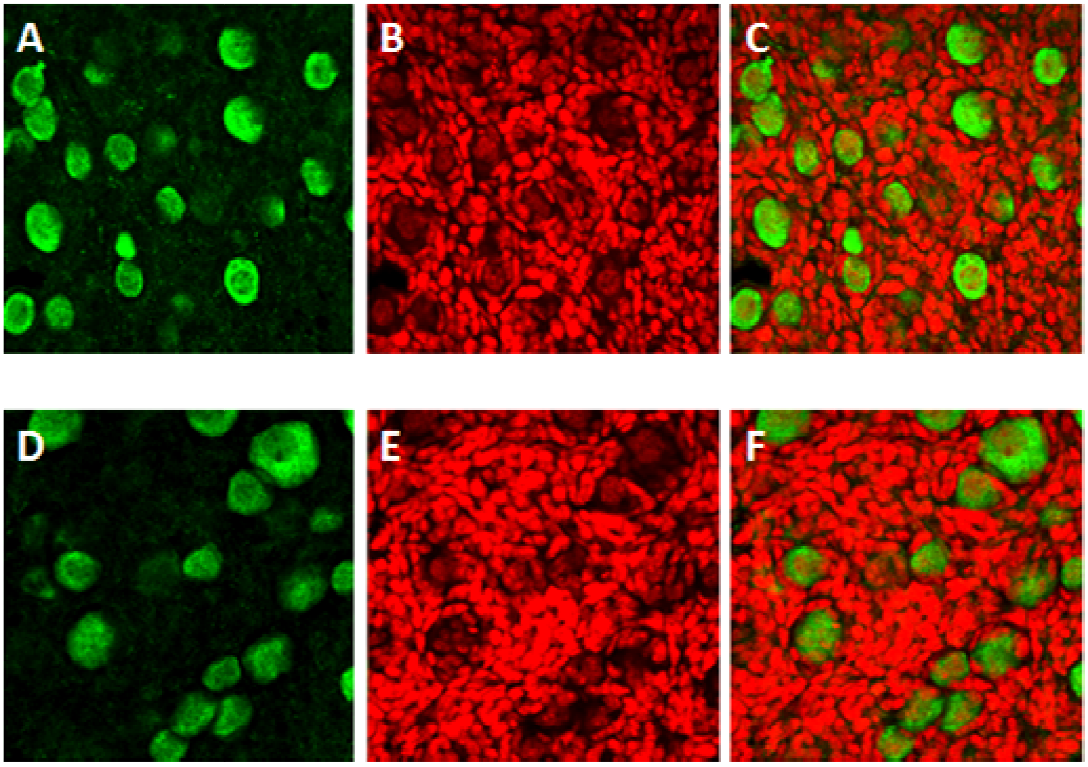

Figure 11. Confocal sections of neonatal mouse ovary cultures from PND 1 to PND 5. PGRMC2 antibody treated ovaries are shown labeled with Stat-3 (A), propidium iodide (B), and overlay (C). Control ovaries are shown labeled with Stat-3 (D), propidium iodide (E), and overlay (F). Oocytes were labeled with Stat-3 (green) and cell nuclei (red) were labeled with propidium iodide.

After organ culture treatment and immunocytochemistry preparation, ovaries were observed for proper cyst breakdown and follicular assembly, as well as oocyte survival. Treatment of PND 1 to PND 5 ovaries with PGRMC2 antibody in the culture media did not appear to have a significant effect on cyst breakdown, follicular assembly, or oocyte survival and number; however, the counting follicle development method needs to be applied for verification.

Another organ culture experiment was performed with incubation of neonatal ovaries, at the earlier age 15.5 dpc to PND 1. The same concentration of PRGRMC2 function blocking antibody was used in the treatment media (Fig. 12).

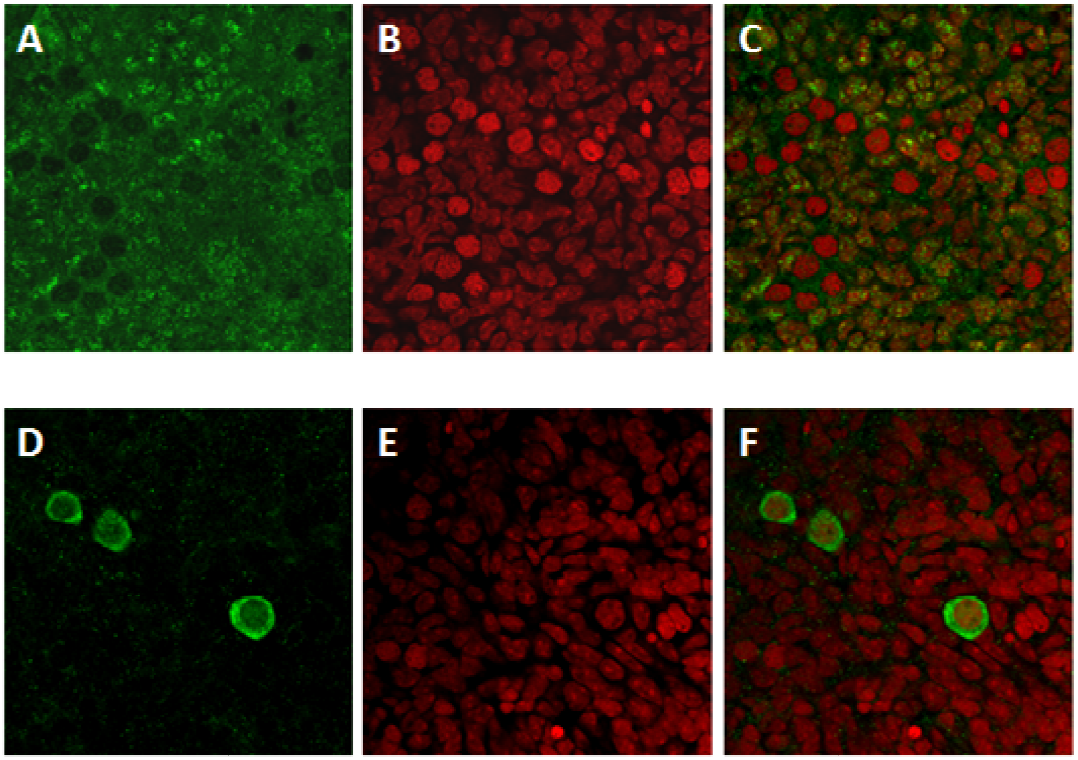

Figure 12. Confocal sections of neonatal mouse ovary cultures from 15.5 dpc to PND1. PGRMC2 antibody treated ovaries are shown labeled with Stat-3 (A), propidium iodide (B), and overlay (C). Control ovaries are shown labeled with Stat-3 (D), propidium iodide (E), and overlay (F). Oocytes were labeled with Stat-3 (green) and cell nuclei (red) were labeled with propidium iodide.

Results for the second organ culture experiment were inconclusive. The first observation was that the Stat-3 antibody did not bind to and label the oocytes in the treated ovary as usually expected. Rather, the Stat-3 antibody appeared to exhibit generalized non-specific binding throughout the ovary, and specific binding was localized to random granulosa cells dispersed throughout the ovary. Nonetheless, the propidium iodide staining of the oocyte nuclei showed an abundance of oocytes within the ovary. Is it extremely unusual for there to be so many oocyte nuclei present without any labeling by Stat-3. The oocytes are easily identified by their characteristic round shape compared to surrounding irregularly shaped cells, and also by their slightly brighter staining than the surrounding cells.

Since the ovary does not appear to have died during organ culture (it appears to be completely intact), a possible explanation for this unusual result could simply be that the organ culture became contaminated during the course of the experiment. The control ovaries, however, do not appear to have been contaminated and exhibit normal Stat-3 binding. Nevertheless, the control ovaries show an abnormality in that there are very few Stat-3 labeled oocytes within the ovary. Another explanation could be that Stat-3 does not label oocytes as early as 13.5 dpc (Murphy *et al*., 2005). If this is the case, PGRMC2 inhibition may have prevented the oocytes from developing any further. This is a complicated result made even less promising by the fact that the control sample size was two ovaries and the treatment sample size was one ovary. This was not an ideal sample size because it does not allow for much comparison to be made between sample ovaries.

Therefore, possible explanations for the results obtained in this organ culture experiment are contamination (of the treatment culture well, at least), inherent abnormalities within the specific litter sample, and possibly but not likely—an actual and severe morphological effect of PGRMC2 receptor inhibition within the ovary. It is necessary to repeat this experiment for verification. A higher number of sample ovaries for both the control and treatment organ culture are required. Additionally, the counting follicle development method would have to be applied in the future to quantify the results of the organ culture experiments more accurately and precisely.

Discussion

Establishing a viable pool of mature oocytes is an essential prerequisite for the fertility of animals. This is a fragile process which begins early on in embryogenesis. Many of the most critical steps in development of the reproductive organs take place while the organism is still a fetus, as is the case in humans. Proper development depends on the reception and transduction of hormones, growth factors, and many other types of signal-pathway inducing ligands.

Two hormones, estrogen and progesterone, are shown to have active roles in the development of the reproductive organs as well as in the function of these organs for the majority of the female's lifetime. Previous research shows that exogenous treatment of ovaries with estrogen and/or progesterone during a critical

time period in development when cyst breakdown occurs significantly decreases the number of percent single oocytes. This treatment inhibits cyst breakdown from occurring properly. The name for the resulting morphological abnormality caused by this exogenous treatment is MOFs. Again, even though estrogen and progesterone are required for the proper development of ovaries, exogenous treatment of ovaries resulting in excessive amounts of these hormones has detrimental effects on the future viability of the gametes and the fertility of the female.

Extensive research has already been dedicated to elucidating the estrogen signaling pathways and characterization of various estrogen receptors. Compared to estrogen, current research results for progesterone receptors regarding these same developmental processes are scarce and insufficient. The research described here sought to address this problem. Here, we began preliminary investigation of progesterone's role in development by beginning with the basic identification of possible receptors for progesterone to signal through to perform its function.

Our research results showed that the progesterone receptors PR-A and PR-B, PAQR3, PGRMC1, and PGRMC2 are all expressed in the ovary at some point during mouse development between the ages of 13.5 dpc to PND 7. PR-A and PR-B show moderate expression in the cytoplasm of oocytes and granulosa cells during 15.5 – 17.5 dpc, and then a lack of expression until they are expressed again at PND 7, although to a slightly weaker degree. Also, staining at 17.5 dpc may exhibit localization of PR-A and PR-B at that exact age to a certain organelle

within the oocyte, possibly the endoplasmic reticulum or nuclear membrane. Further experiments are required for confirmation of this result.

The receptor belonging to the progestin and AdipoQ family of receptors, PAQR3, exhibits strong expression in the ovary starting at PND 7. Expression is distinctly localized to the cytoplasm of the oocytes. Although a specific ligand for PAQR3 has not been identified, it is likely that it can bind progestin and possible that it may bind progesterone as well, since multiple other receptors in the PAQR family can bind progestin.

PGRMC1 has already been researched extensively in effort to elucidate its significance in ovarian cancer. Here, we found that PGRMC1 expression begins at around PND 3 and grows slightly stronger afterwards. It is expressed throughout the ovary in the granulosa cells, in the oocytes, and even within some of the oocyte nuclei. Unfortunately, staining with the PGRMC1 antibody did not present an adequately specific result; results for PND 3, 5, and 7 all appear to have significant background staining. Unless the receptor is actually expressed to such an ubiquitous degree, more stainings with variation of antibody concentration are necessary for confirmation of the results.

Although it is one of the least researched receptors to date, PGRMC2 showed the most interesting staining results. PGRMC2 showed strong expression as early as 13.5 dpc which decreased through PND 1. Expression was localized to the cytoplasm of the granulosa cells throughout the ovary. After PND 1, results were mostly background staining and nonspecific. At 13.5 dpc, there was an unusual expression pattern of PGRMC2 in that some cells showed extremely

dense expression of PGRMC2 on the membrane and within the cytoplasm. These unidentified cells were also characterized by a very dense nucleus. While the presence of these types of cells with dense nuclei is not uncommon at that age in development and has been noted before, the expression pattern of PGRMC2 localized to some of these types of cells is peculiar and unexplained.

After identification of these progesterone receptors, their level of expression, and their location within the ovary, organ culture experiments were performed to elucidate the importance of these receptors and to determine their function. Two organ culture experiments with PGRMC2 function blocking antibody were performed. Ovaries with anti-PGRMC2 treatment during PND 1 – 5 did not appear to show any change in formation of primordial follicles and percent of single oocytes when compared to ovaries in control organ culture. This is an expected result due to the inherent lack of PGRMC2 expression after PND 1, as found with prior immunocytochemistry experiments.

Ovaries with anti-PGRMC2 treatment during 15.5 dpc – PND 1 showed inconclusive results. The control ovaries did contain oocytes; however, there were a small number of oocytes present. The treated ovaries seemed to contain multiple oocytes, based on the presence of many round nuclei which are characteristic of oocyte nuclei. However, there was no specific staining of any oocytes by Stat-3, which is a widely used oocyte marker. It is unlikely that PGRMC2 inhibition is potent enough to induce such an unusual and extreme morphological response in the ovary on its own. Possible explanations for this result may be contamination of media used for organ culture, death of the ovaries at some point during culture,

or a prior defect in the ovaries or dissection technique. Due to the unusual results as well as the fact that a very small sample size was used, repeated experiments are required for confirmation of PGRMC2's role during these early ages.

Based on previous research, there is conclusive evidence that treatment of neonatal ovaries with exogenous estrogens and progesterone will inhibit the proper breakdown of cysts during neonatal development and result in MOFs which are present for the entire lifetime of the organism. Unfortunately, infertility usually cannot be diagnosed until many years after cyst breakdown occurs. Although there is currently no way to detect possible exposure to exogenous compounds in vivo during development, this research is still useful for possible future study and development of treatments.

Firstly, it is naturally essential to understand the fundamental mechanisms of embryogenesis. Understanding the proper mechanisms of development is imperative to identify what is abnormal later on. Consequently, understanding which processes normally occur and how they are disrupted is useful for developing proper prevention strategies. For example, identification of certain industrial compounds as estrogen mimics which bind to receptors in living tissue or as any other disruptors of development that can do so may lead to strict regulation and/or prohibition of their use.

Additionally, identifying the mechanisms of proper oocyte development is crucial for some infertility treatments, known as Assisted Reproductive Technology (ART). A female under the age of 30 has around a 40% chance of becoming impregnated via ART with the use of her own oocytes, rather than a

donor's oocytes (Krisher, 2004). Using a female's own oocytes and maturing them in vitro, known as in-vitro maturation (IVM), is a possible infertility treatment which has had very little success as an ART method, but it does show promise. This method is not commonly applied clinically; however, this in-vitro method has shown success in livestock and other animals (Krisher, 2004). Continuing to elucidate the intricate mechanisms of oocyte maturation will allow for refinement of oocyte maturation techniques, optimization of culturing conditions, and improvement of the rate for successful implantation of the oocytes matured in-vitro (Fasano *et al*., 2012). These ART treatments are dependent on understanding the function of various compounds involved in oocyte maturation, including steroid hormones and gonadotropins. The refinement of these techniques is an active area of research.

Equally important is the fact that hormone imbalance or misregulation has implications in cancer as well. For example, PGRMC1 mediated progesterone signaling has been shown to play a role in ovarian cancer (Peluso, 2011). PGRMC1 promotes the survival of both normal and cancerous cells in the ovary, and its expression is particularly noted on ovarian tumors. The ability of cancer cell lines with PGRMC1 levels depleted by more than 80% to undergo mitosis is impaired and apoptosis of many cells is induced, which is evidence that PGRMC1 promotes the proliferation of ovarian cancer cells (Peluso, 2011). One aspect of ovarian cancer treatment could therefore be targeting PGRMC1 via gene therapy. Clearly, the characterization of PGRMC1 and other progesterone receptors is becoming more important as the research and understanding of these receptors

progresses. Developing a timeline of expression for these receptors is a useful step in determining the mechanisms of development and in identifying the problems associated with multiple pathologies.

Future Directions

Additional experiments are necessary and valuable to verify the results obtained so far. Two sets of organ cultures should be completed with function blocking antibodies targeting PGRMC1 and PGRMC2, with three different ages cultured for each antibody treatment (ages: 13.5 – 17.5 dpc, 15.5 dpc – PND 1, and PND 1 – PND 5). The results obtained from using the antibody treated culture media could provide data on the importance of progesterone during this critical period of gamete development and the role that the progesterone receptors play.

Western blots would be useful experiments to verify the immunocytochemistry results. A western blot for each progesterone receptor type at each of the ages analyzed by immunocytochemistry (15.5 dpc – PND 7) would provide a reliable secondary method for evaluating progesterone receptor expression. Comparison of the western blot results to the staining results and agreement between them would confirm the accuracy and dependability of the results obtained so far.

Completing four immunocytochemistry experiments (as well as four western blots) with adult female mouse ovaries for each type of progesterone receptor would be helpful in comparing the expression of the receptors in the

developing neonate to the expression of the receptors in the fully mature mouse. This would be useful in identifying possible trends as the mouse develops into an adult. Also, while some data regarding expression in adults may have already been published, it is often in reference to pathologies and cancer. It would be useful to personally obtain the experimental results with significant findings.

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Summary of Capstone Project

Not long after fertilization of an egg by sperm, cellular multiplication and division quickly begins to assemble and develop the tissues of a new organism in a process known as embryogenesis. Even more impressively, it is not long before the fetus is already developing its reproductive system for its own reproductive purposes in the future. In females, the two ovaries are small organs that contain cells called oocytes. Oocytes which have been completely differentiated, or matured into a preovulatory stage during menstruation later on in the female's life, may then be released from the female's ovary and fertilized by sperm to create a new organism. This is the essence of fertility. Infertility, therefore, can often be the result of an abnormality in the proper development of the oocytes. In this case, infertility is characterized as a disorder which is based on disrupted oocyte development very early on in embryogenesis, while the new organism is still a fetus.

The development of these reproductive cells known as oocytes is a complex process. It is influenced and regulated by many different hormones and other signaling molecules, particularly because the new developing organism (in the case of most mammals) is still found within its mother. During embryogenesis, therefore, the neonate is directly exposed to the mother's hormones and indirectly exposed to compounds in the environment through its mother. Examples of these hormones are estrogen, estrogenic compounds and mimics, and other steroid hormones. These are molecules which bind receptors on

cells in the ovary and/or receptors within cells in the ovary. The binding of these hormones and molecules can create an immediate effect in the cell by activating signaling cascades where a chain reaction of activation occurs, or they can have prolonged effects by activating transcription and expression of genes on the DNA within the cell nucleus.

Estrogen is a hormone which plays an active role in causing these changes in the cells throughout the ovaries for most of the female's lifetime. It has been studied extensively and information is available regarding both the effects of the hormones as well as the various estrogen receptors which mediate these effects. Another steroid hormone involved that plays an equally important role is progesterone. Although progesterone's effects have been studied to some extent, its role in the development of the oocytes during embryogenesis is not known and it receives little attention in research today.

The aim of this research was to begin preliminary analysis of progesterone's role in the development of the oocytes using a very basic approach. The model organism for this research was the mouse. Male and female mice were mated and the gestation (pregnancy) period of the female was monitored to keep track of the exact age of the offspring, known as a litter of pups. Neonatal mice at ages 13.5 dpc (days post coitum) to PND 7 (post natal day) were used for experiments. The ovaries from the female neonatal mice were dissected and then an experiment called immunocytochemistry, or staining, was performed. The protocol for immunocytochemistry includes a sequence of washings to purify impurities from the ovary and preserve it, and also included

the addition of primary and secondary antibodies. The specific primary antibody would attach to the progesterone receptor of interest, and the secondary antibody would attach to the primary antibody and make it fluoresce when the ovaries were views on a slide using confocal microscopy. The entire procedure from dissection to the completion of immunocytochemistry takes four days.

After immunocytochemistry was complete and the ovaries were on a slide, they were viewed using confocal microscopy. A confocal microscope uses lasers to penetrate preserved tissue and allows for the fluorescent secondary antibodies inside the tissue to be detected. Pictures were taken of the ovaries and then analyzed and compared to see changes in expression of various progesterone receptors and to see how the expression of each changes over time.

Additionally, organ culture experiments were started to manipulate the environment that the ovaries were grown in and see what effect these progesterone receptors may be mediating in the ovary. Organ culture experiments involve dissecting ovaries as had been done previously, but than an incubator is used to keep the ovaries alive for a few days and allow for experimental manipulation. The environment and temperature of the incubator are similar to those that the ovaries would experience if they were within a body. The media, or special fluid that the ovaries are grown on within the incubator, also creates a nutrient environment for the ovaries as if they were within a living body. This media is the variable factor and can be made with the addition of function blocking antibodies, which in this case would prevent the progesterone receptors from functioning properly. After organ culture, the ovaries were then stained

following the same procedure mentioned previously and the ovaries were observed using confocal microscopy to see what changes had occurred as a result of blocking the progesterone receptors from functioning.

These were the experiments performed in this project and they allowed for identification of four progesterone receptors which are present from 13.5 dpc to PND 7 in the neonatal mouse. Our results showed that PGRMC2 is expressed at the earliest time point in development, as early as 13.5 dpc. Receptors PR-A and PR-B are expressed briefly towards the end of gestation, at around 15.5 – 17.5 dpc. Generally, however, the three receptor types PR-A and PR-B, PAQR3, and PGRMC1 begin expression a few days after birth in the mouse, making an appearance at PND 5 or later.

Two organ culture experiments were performed with a PGRMC2 function blocking antibody. PGRMC2 was selected as the target for these experiments due to the unique timing of its expression and the unusually strong staining it exhibited after immunocytochemistry. PGRMC2 function was inhibited first at PND 1 – PND 5. There did not appear to be significant results, or more specifically, any changes in oocyte morphology or number. This can be expected since the immunocytochemistry experiments showed that PGRMC2 is not expressed after PND 1; therefore, it could not mediate any effect on the oocyte. PGRMC2 function was then inhibited at 15.5 dpc – PND1. The results of this experiment are inconclusive due to possible contamination and small sample size.

Future experiments to elucidate the mechanisms of progesterone signaling in the developing ovary will need to include multiple organ cultures to manipulate

each of the four individual receptors mentioned. Additional experiments will need to be performed to confirm the findings presented here. For example, a western blot experiment could be used to supplement each of the immunocytochemistry experiments and would allow for visual quantification of the receptor of interest. Agreement between the immunocytochemistry experiments and the western blots would be very beneficial in determining the dependability of the results obtained to date.

The future for research on progesterone receptors looks promising. Progesterone receptors are receiving more attention due to discoveries of their presence in breast and ovarian cancers. Having a timeline of progesterone receptors and the time frames within which they appear will be useful in determining where developmental errors might have occurred. Also, if future research finds that a common industrial compound might serve as an inhibitor (or possibly an activator) of a certain progesterone receptor, this research will be useful in determining what effects that compound may have on the future fertility of neonates who might be indirectly exposed to it during gestation. It would also result in regulation of that compound's use in industry and manufacturing.

This is just one example of the value of this research. The results which have been obtained so far regarding progesterone receptors are interesting, and future research will surely indicate that these receptors play an important role throughout the entire lifetime of the female. They are involved in proper development of the oocytes in a female during embryogenesis, regulating the cycles of menstruation decades later throughout the reproductive life span, and

also play a role in cancer and tumor growth many more decades later. The discoveries yet to be made will be invaluable in more ways than one.