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## The Role of Estrogen Receptor Alpha in Neonatal Oocyte Development

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## **INTRODUCTION**

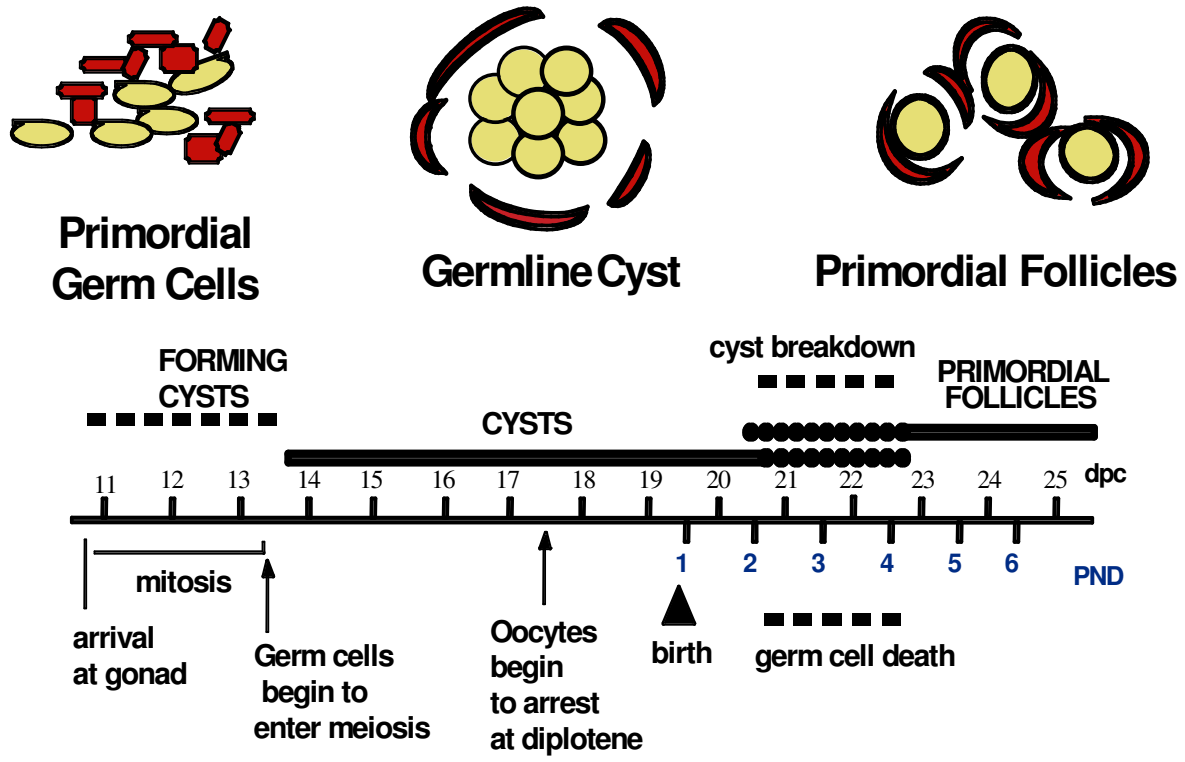
As it stands, science has not yet come to fully understand the mechanisms of mammalian reproduction. However, what has become quite evident, as a result of research, is that certain components of female reproduction are very important. The production of female germ cells, known as oocytes in *Mus musculus* (mice), through the formation of a pool of primordial follicles, before birth, is one such integral component (Pepling and Spradling, 1998). The pool of primordial follicles is essential to reproduction in that it is the complete amount of oocytes that a female will have available for fertilization for the rest of her life. Thus, it is important to understand how primordial follicles are assembled and what intrinsic and extrinsic influences affect their formation. Developing a better understanding of the formation of these follicles gives scientists further insight into female sexual maturation and the problems that may arise within it.

The importance of the primordial follicle is also evident as it is the first step in the ovarian cycle. This cycle is a process involving the enlargement and maturation of a group of follicles until they are ready to be ovulated (Dean and Epifano, 2002). It begins with a primordial follicle, which is one oocyte surrounded by a single layer of flattened granulosa cells that support its growth. The follicle continues to develop until it becomes a primary follicle. Primary follicles are denoted by a larger oocyte encased in an increased number of granulosa cells that have become more cuboidal in shape. The primary follicle then matures into the secondary follicle. Likewise, the secondary follicle is much larger than its precursor, but is now surrounded by more than one layer of

granulosa cells. The oocyte is then surrounded by an extra-cellular matrix called the zona pellucida that contains proteins that will later allow penetration of sperm. The granulosa cells then become surrounded by a layer of stromal-like theca cells that differentiate into the theca externa and interna. A network of capillaries becomes embedded between these two layers. Few oocytes survive beyond this point in the cycle. Most oocytes become atretic and die, due to either oocyte or granulosa cell apoptosis (Hsu and Hsueh 2000). The survivors continue to grow into tertiary follicles, also known as antral or Graafian follicles. These pre-ovulatory follicles contain a fluid-filled space called an antrum containing proteins, hormones and other molecules that support the follicle's continued growth. This follicle continues to mature until expelled from the ovary as an egg during ovulation, to possibly be fertilized. The remains of the follicle become the corpus luteum, which secretes hormones that support pregnancy, but if the egg is not fertilized within 14 days this turns into the corpus albicans.

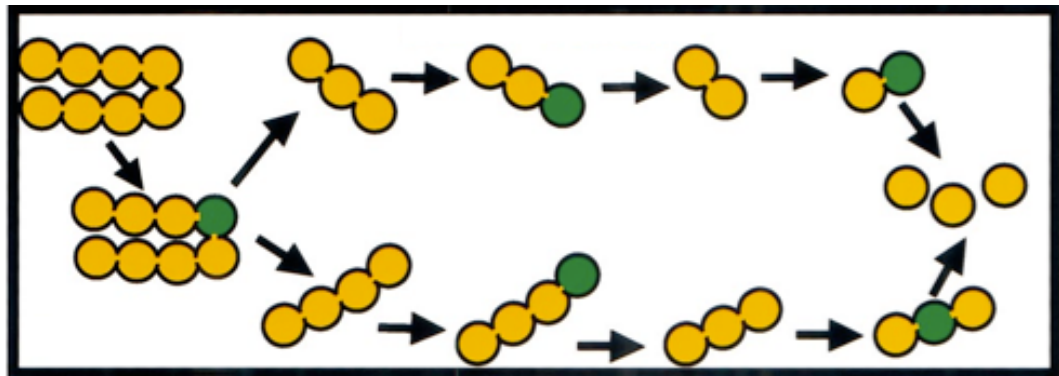
The pool of primordial follicles is formed from the oocyte precursor cells called primordial germ cells (Figure 1). Initially, within the developing mouse embryo, primordial germ cells migrate to the gonads. By approximately 10.5 days post coitum (dpc) the primordial germ cells have arrived at the gonad, divided mitotically, and between 10.5 dpc and 13.5 dpc clusters of cells called germ line cysts form. The oocytes in these cysts are connected via intercellular bridges that formed as a result of incomplete cytokinesis (Pepling and Spradling 1998). The germ cells enter meiosis at 13.5 dpc and are now considered oocytes. The oocyte cysts persist until birth. Following birth, these clusters begin to break

apart in a process coined cyst breakdown (Pepling and Spradling 2001). After the disassembly of the cysts, individual oocytes eventually become primordial follicles.



**Figure 1.** Timeline of Germ Cell development in Mice (adapted from Pepling, Spradling, 2001). Yellow cells are oocytes and the red cells are granulosa cells.

Although the process of cyst breakdown is not yet fully understood, it is clear that apoptosis, or programmed cell death occurs simultaneously and may be integral to mammalian reproduction (Pepling and Spradling, 2001). As a result of germ cell death, approximately two thirds of the oocytes present before birth do not survive (Pepling and Spradling 2001). According to the model of cyst breakdown, dying cells disrupt the intercellular bridges (Figure 2). These breaks diminish the size of the cyst until only a few oocytes remain. It is not known why these cells die or which cells will undergo apoptosis. However, it has been postulated that the cells that die serve as nurse cells for the oocytes that survive. Nurse cells are cells that provide nutrients to the developing oocyte (Mahajan-Miklos and Cooley, 1994). For example, the *Drosophila* ovary contains 16-cell cysts, only one cell in each cyst becomes an oocyte and the other 15 become nurse cells. These cysts are formed from stem cells that create cystoblasts that divide exactly four times mitotically and form interconnected cellular bridges by incomplete cytokinesis. Within these cysts, the nurse cells eventually die by apoptosis. Overall, it seems apparent that for the primordial follicle formation in mice requires cyst breakdown and simultaneous apoptosis is imperative.



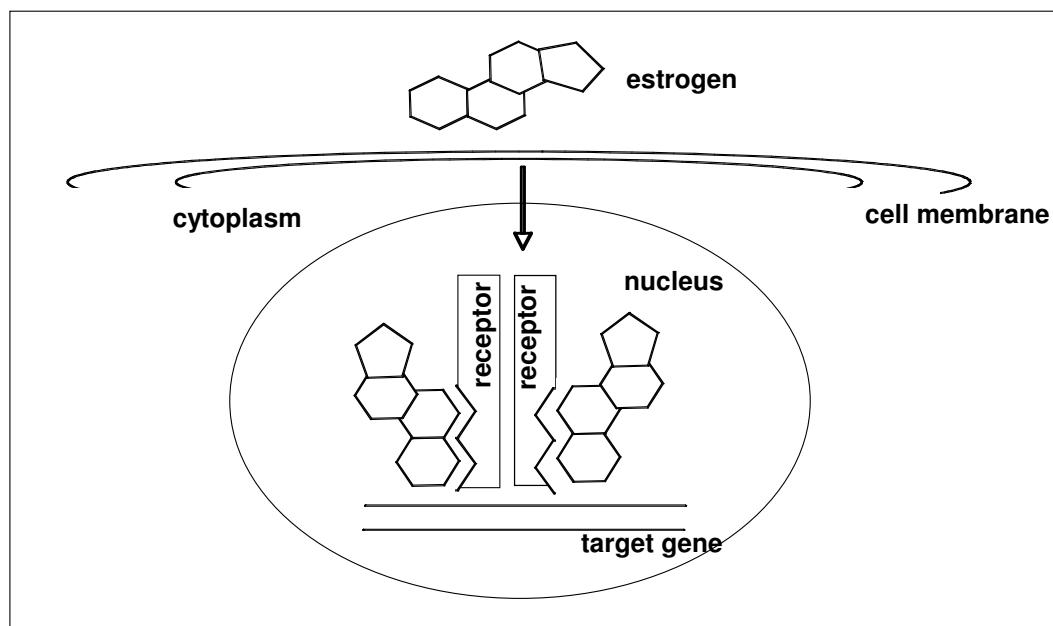
**Figure 2.** Model of Cyst Breakdown (Pepling and Spradling, 2001). The breakdown of an 8 cell cyst. The green cells are apoptotic (dying) and their death result in the disassembly of the cyst. The process ends in the formation of single oocytes that will later become surrounded by granulosa cells to become primordial follicles.

Cell death and follicular development have been found to be regulated by steroid hormones such as estrogen (Manglesford et al., 1995, Findlay 1993). Estrogen is of particular interest in this case because of its extensive impact on female sexual development. This hormone is known to have an effect on female sex determination, reproduction, and pregnancy (Kos et al., 2002). Thus, it would not seem improbable for it to also have a role in cyst breakdown and the subsequent maintenance of follicular maturation.

Estrogen is a steroid hormone. Though it is most active in females, it is also known to play critical roles in male sexual development. It is also active in other bodily functions, such as bone metabolism, and cardiovascular and neuronal activity (Kos et al., 2002). The hormone is produced by the ovaries, adrenal glands, and testes. There are three major forms of estrogen active in females called estradiol, estrone, and estriol. Within the ovary, estrogen production is initiated by developing follicles and the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH). The process begins with the formation of androstenedione from cholesterol in the theca cells, which are cells that line the outside of the granulosa cells. From here this precursor hormone is transported to the granulosa cell where it is later converted to some form of estrogen, either estradiol or estrone, via the enzyme aromatase (Kato et al., 1995).

The estrogen hormone signals cells through receptor-mediated pathways. Research has shown that aberrations in this signaling during development can lead to multiple and long-term abnormalities in the reproductive system (Jefferson et al., 2002). Estrogen first maneuvers through the cell membranes, probably by

diffusion, and comes into contact with an estrogen receptor in the nucleus of the cell (Figure 3). Estrogen has two receptors, estrogen receptor alpha ( $ER\alpha$ ) and estrogen receptor beta ( $ER\beta$ ) (Krege et al., 1998). These receptors are part of the steroid nuclear receptor superfamily.  $ER\alpha$  and  $ER\beta$  are classified as ligand-dependent transcription factors whose ligands are either estrogen or estrogen-like compounds (Mathews and Gustafsson, 2003). These receptors are known to homodimerize once activated. Once estrogen binds to these receptors, the ligand-receptor complex interacts with a special portion of the DNA present in target genes called an estrogen response element (ERE). After recruiting co-regulators and other transcription factors, the target genes are then activated, producing proteins that go on to elicit pleiotropic effects within the organism.

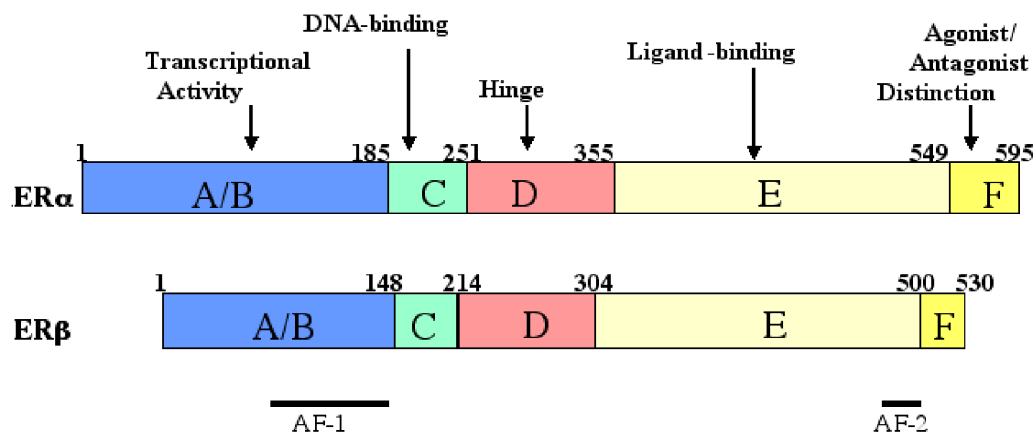


**Figure 3.** Estrogen Signaling Pathway. Estrogen binds to the receptor protein. The receptor protein then homodimerizes and bind to estrogen response elements on target genes.

scientists believed that estrogen signaling was only carried out through  $ER\alpha$ , but

the discovery of ER $\beta$  and the divergence of the two receptors' expression patterns implies that they may affect reproductive development quite differently (Jefferson et al., 2002). ER $\alpha$  is expressed in many parts of the body including the liver, kidney, heart, and ovary (Matthews and Gustafsson, 2003). Within the adult ovary ER $\alpha$  is expressed in the theca and interstitial cells (Couse et al., 1999). However, its expression in the neonatal ovary is unknown. ER $\beta$  is mainly expressed in the ovary, prostate, epididymis, lung, and hypothalamus (Krege et al., 1998). ER $\beta$  is expressed in the granulosa cells of the ovary and has been detected in neonatal ovaries by RNase protection (Jefferson et al., 2000). Though ER $\alpha$  is slightly bigger than ER $\beta$ , they both have six protein domains (A-F) and share similar protein domain composition (Figure 4). In both receptors, a ligand-independent transactivation function called AF-1 is present in the A/B domain of the N-terminus (Hewitt and Korach, 2002). AF-1 along with AF-2, a C-terminal ligand-dependent activation function found in the E domain, assists with estrogen-mediated transcription and cell- and promoter-specificity (Matthews and Gustafsson, 2003). Domain C is highly conserved; it is responsible for specific binding to estrogen response elements on the DNA and for receptor dimerization. The ligand binds to the receptor protein at the E domain, which is less conserved and partially responsible for dimerization (Dupont and Krust, 2000).





**Figure 4.** ER $\alpha$  and ER $\beta$  Functional Protein Domain Composition (adapted from Klinge, 2000). The amino acid length is indicated at the beginning and end of each domain. Domains C and E are the most homologous (97% and 55% respectively).

Mutations within both the ER $\alpha$  and ER $\beta$  receptors can lead to severe dysfunction in the adult mouse ovary. Each receptor has been knocked out by insertion of the neomycin gene into important coding exons (Kos et. al., 2002). Adult female ER $\beta$  knock out (ER $\beta$ KO or ER $\beta^{-/-}$ ) mice display smaller litters as a result of reduced fertility. In these knockout mice there was a decreased number or absence of corpora lutea, though the ovaries looked fairly normal with normal antral follicles (Dupont and Krust, 2000).

In adult female ER $\alpha^{-/-}$  mice the phenotypic abnormalities are more severe. These mice display abnormalities in the reproductive tract, gonads, skeletal and cardiovascular systems. ER $\alpha^{-/-}$  females have normal follicles until the antral stage. However, adults later lack corpora lutea and have cystic and hemorrhagic follicles (Emmen et. al., 2007). Many follicles are atretic. ER $\alpha^{-/-}$  mice do not ovulate and are thus sterile. There is also an increase in the amount of LH circulating in ER $\alpha^{-/-}$  females (Rosenfeld et al., 2000). Increased amounts of

hormones can alter follicle development. Even after being treated with exogenous gonadotropins, the anovulation phenotype could not be reversed in adult mice (Couse and Korach, 1999). This suggests that the hormonal imbalance, coupled with the defective ER $\alpha$  receptor has irreversible and more severe effects on the adult ovary than ER $\beta$ <sup>-/-</sup>.

Although research on both receptors has yielded important information, further inquiry into ER $\alpha$  is still necessary when it comes to neonatal mice. In a study done using neonatal mouse ovaries grown in organ culture, estradiol treatment inhibited cyst breakdown and primordial follicle formation, but did not influence oocyte survival (Chen et. al., 2007). In addition, ER $\alpha$  and ER $\beta$  receptor specific agonists cause delays in cyst breakdown (Chen and Pepling, in preparation). In adult mice, immunohistochemistry has revealed that ER $\alpha$  is expressed in the interstitial and theca cells of rats, mice, cows, and humans (Couse et. al., 1999). ER $\alpha$  has also been detected in the human ovary (Couse and Korach, 1999). However, it is not known where or if ER $\alpha$  is present in the neonatal mouse ovary. The focus of my research is thus to explore the expression of ER $\alpha$  in neonatal mice. This study has two goals: 1) determining where ER $\alpha$  is normally expressed in neonatal ovaries; and 2) discovering what happens to oocyte development when ER $\alpha$  protein expression is disrupted by examining mice for defects in primordial follicle formation, cyst breakdown, and oocyte survival. The results of this research will add substance to what is already known about estrogen signaling and possibly shed some light on sterility in mice that may be applied to humans in later studies.

## **MATERIALS AND METHODS**

### **Animals**

The mouse strain lacking ER $\alpha$  used in this study was obtained from Jackson Laboratories. In this strain, homologous recombination had been used to insert a neomycin resistance gene into exon 2 of the ER $\alpha$  gene, disrupting its function (Lubahn et al., 1993). The mice are maintained as ER $\alpha^{+/-}$  heterozygotes. To obtain neonatal mice for analysis, matings were set up between two heterozygous ER $\alpha^{+/-}$  mice. For four days following mating, mice were checked for vaginal plugs, which would indicate pregnancy. On the day that a female displays a plug she is isolated and the day is denoted as 0.5 days post coitum (dpc). Birth usually occurs at 19.5 dpc. This day is then denoted post natal day 1 (PND1). Neonatal ovaries were harvested from PND1, PND4 and PND7 offspring. Ovaries were also collected from the CD-1 strain of mice (obtained from Charles River Laboratories) at various ages for Western blotting and immunocytochemistry using an antibody against the ER $\alpha$  protein.

### **Purification of DNA from Mouse Tails**

Each mouse was given a metal numerical tag. A piece of tail was then collected and placed in a centrifuge tube marked with the mouse's identification number. The DNA was isolated from tissue via the DNAeasy kit (QIAGEN). 180 microliters ( $\mu$ l) of Buffer AL and 20  $\mu$ l of the enzyme proteinase K were then pipetted into each tube. This solution was placed in a 55°C water bath for at least

six hours and then vortexed. A one to one mixture of Buffer ATL and 100% ethanol was prepared. 400  $\mu$ l of the mixture was added to each sample and then vortexed. The samples were then transferred into spin columns with collection tubes attached, to which 500  $\mu$ l of AW1 buffer plus ethanol was added. The tubes were centrifuged at 8,000 rpm for one minute. The collection tubes were discarded and replaced. Then 500  $\mu$ l of AW2 buffer plus ethanol was added to each tube and centrifuged at 14,000 rpm for three minutes. The collection tubes were discarded and replaced, this time with new centrifuge tubes. To elute the DNA, 100  $\mu$ l of AE buffer was added to each of these tubes and centrifuged at 8,000 rpm for one minute. Lastly, the DNA was stored at  $-20^{\circ}\text{C}$  to keep it from degrading.

### **Polymerase Chain Reaction (PCR)**

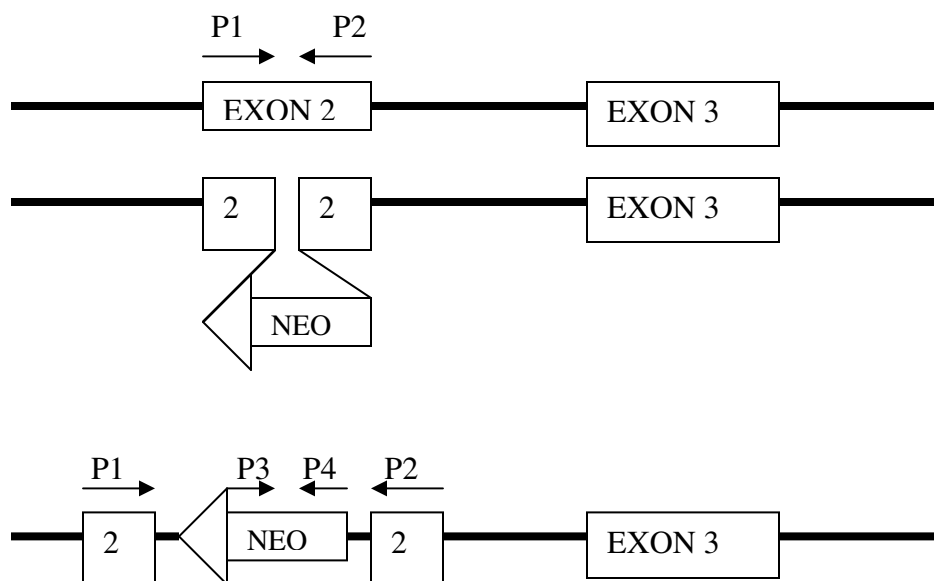
PCR amplification was used to determine the genotypes of offspring obtained from crosses of  $\text{ER}\alpha^{+/-}$  mice. To do this two reactions were used; an A reaction to amplify the wildtype allele and a B reaction to amplify the mutant allele. The A reaction mixture consisted of 4.5  $\mu$ l, 1  $\mu$ l of 2/FOR primer, 2.5  $\mu$ l of 2/REVII primer and, 7  $\mu$ l of Red Taq (Sigma) polymerase. The B reaction mixture consists of 6  $\mu$ l of distilled water, 4  $\mu$ l of 1.25 mM dNTP mixture (1.25 mM of each of dATP, dTTP, dCTP, and dGTP), 2.5  $\mu$ l of 10X Buffer A, 1  $\mu$ l each of primers 13 and 14, and 0.5  $\mu$ l of Taq polymerase (Fisher). The sequences of the primers are as follows:

Primer 2/FOR: 5'-CTGTGTTCAACTACCCCGAGG

Primer 2/REV II: 5'-GGCGCGGGTACCTGTAGAA

Primer 13: 5' CTT GGG TGG AGA GGC TAT TC-3'

Primer 14: 5'AGG TGA GAT GAC AGG AGA TC-3'



**Figure 5.** Primers 1 and 2 anneal to Exon 2 of the ER $\alpha$  gene. A neomycin insertion is placed into Exon 2 to create the mutant allele (Jackson Laboratories) and primers 3 and 4 amplify the insert.

In the A reaction, primers 2 FOR and 2 REV II amplify a 327 base pair DNA fragment from the wildtype allele. In the B reaction, primers 13 and 14 amplify a 290 base pair DNA fragment from the targeted allele, amplifying the neomycin insertion. Using wildtype DNA, only the 327 base pair fragment is amplified. Using heterozygous (ER $\alpha$ <sup>+/-</sup>) mice, both the 327 and 290 base pair fragments are amplified. Using a (ER $\alpha$ <sup>-/-</sup>) mouse only the 290 base pair fragment is amplified.

The total volume in each reaction tube was 15 $\mu$ l. Both tubes were then placed in the thermocycler on the following ER $\alpha$  programs:

**A Reaction:**

Stage: 1	Step: 1	2 minutes at 95°C
Stage: 2	Step: 1	45 seconds at 54°C
	Step: 2	45 seconds at 72°C
	Step: 3	45 seconds at 72°C
		(Stage 2 repeated for 35 cycles)
Stage: 3	Step: 1	7 minutes at 72°C
		(Hold at 4°C)

**B Reaction:**

Stage: 1	Step: 1	3 minutes at 95°C
Stage: 2	Step: 1	30 seconds at 94°C
	Step: 2	1 minute at 64°C
	Step: 3	90 seconds at 72°C
		(Stage 2 repeated for 35 cycles)
Stage: 3	Step: 1	2 minutes at 72°C
		(Hold at 10°C)

**Gel Electrophoresis**

Gel electrophoresis was used to determine the size of each PCR product. To do this, a 2% agarose gel was prepared. To make the gel, 50 ml of 1X TBE

buffer and 1 gram of agarose were placed into a 125 ml Erlenmeyer flask and microwaved for 2 minutes. The flask was mixed every 40s so the agarose would dissolve properly. 2.5  $\mu$ l of 10 mg/ml ethidium bromide was added to the hot mixture and swirled for even distribution. The gel was then poured into a gel tray, equipped with a comb to create individual wells. The gel was then left to cool for 10-15 minutes. After the gel had solidified, the gel tray was placed into a gel box and 1X TBE buffer was poured over it until the gel was completely submerged. The comb was removed. A mixture of 2  $\mu$ l of 6X loading dye and 10  $\mu$ l of each PCR product was loaded into each well. 2  $\mu$ l of 6X loading dye, 5  $\mu$ l of 100 bp marker, and 5  $\mu$ l of 1X TBE buffer were loaded into a well to serve as a base pair marker, for band size reference. The gel box was then covered and run at approximately 100 volts for one hour. Kodak Image Station software and UV detection were then used to take an image of the gel and to analyze the amplification products.

### **Dissection**

Neonatal ovaries were harvested from both CD1 and ER $\alpha$  mice at PND1, PND4, and PND7. The ovaries were harvested in 1X phosphate buffered saline (PBS) using a dissecting microscope. For analysis of ER $\alpha$  mice, a portion of the tail of each mouse was also collected and placed in a separate tube to be genotyped.

### **Whole Mount Antibody Staining of Neonatal Ovaries**

For analysis of ER $\alpha$  mutant mice, ovaries were dissected and placed into tubes containing 600  $\mu$ l of fixative, which consisted of 400  $\mu$ l of 1XPBS and 200  $\mu$ l of 16% formaldehyde (Ted Pella Inc.), for a final concentration of about 5.3% formaldehyde. Each of these tubes was labeled according to genotype: wildtype (ER $\alpha^{+/+}$ ), heterozygous mutant (ER $\alpha^{+/-}$ ), and homozygous mutant (ER $\alpha^{-/-}$ ). Each tube was then nutated overnight at 4°C. The ovaries were quickly washed two times in 1 ml of PT and then a third time in 1 ml (PBS/Triton) PT for at least 30 minutes at room temperature. The ovaries were incubated in PT + 5% bovine serum albumin (BSA) for 30- 60 minutes on a room temperature nutator. The ovaries were then incubated with 2.5  $\mu$ l of primary antibody against the oocyte marker Stat3 (C20) (Santa Cruz Biotechnology), in 500  $\mu$ l of PT + 5% BSA overnight at 4°C on a nutator (Murphy et al., 2005). They were washed in 1 ml PT + 1% BSA for 30 minutes at room temperature. Then the ovaries were incubated in 10  $\mu$ l of 10 mg/mL RNase A in 1 ml PT +1% BSA for 30 minutes at room temperature. The ovaries were incubated in 10  $\mu$ l of 5  $\mu$ g/ml propidium iodide in 1 ml of PT + 1% BSA for 20 minutes at room temperature. Propidium iodide was used as a nuclear marker. At this point each tube was wrapped in aluminum foil to preserve fluorescence. The ovaries were washed in 1 ml PT +1% BSA for 30 minutes at room temperature.

The secondary antibody anti-rabbit Alexa 488 (Molecular Probes) was prepared a day in advance. It was first pre-absorbed by making a 1:200 dilution of 2.5  $\mu$ l of antibody in 500  $\mu$ l of PT + 5% BSA and a small amount of embryo



powder. Embryo powder is a dried mixture of homogenized mouse embryos at ages 12.5-14.5 dpc. This mixture was then wrapped in aluminum foil and placed on a 4°C nutator overnight. The ovaries were then incubated in this pre-absorbed secondary antibody for 2-4 hours on a room temperature nutator. Next, the ovaries were washed three times in 1 ml of PT + 1% BSA for 30 minutes each time on the nutator at room temperature. They were washed once quickly in 1X PBS. The PBS was then removed, 100 µl of Vectashield (Vector Laboratories) was added to each tube and incubated for 15 minutes. The ovaries were then mounted on a slide, with a cover slip and stored at -20°C.

Antibody staining of neonatal CD1 mice at PND1, PND4, and PND7 was also done to determine the localization and expression of the ER $\alpha$  protein. The anti-ER $\alpha$  MC-20 (Santa Cruz Biotechnology) antibody was used as a primary antibody at 1:100 dilution in this portion of the study. The same Alexa Flour® 488 Goat Anti-rabbit IgG (H+L) (Molecular Probes) described above was used as a secondary antibody at a 1:200 dilution.

### **Confocal Microscopy**

The collected and stained PND1, PND 4, and PND7 ovaries were observed via indirect immunofluorescence with the Zeiss Pascal confocal microscope. The images obtained were used to analyze cyst breakdown, follicle development and oocyte number. PND7 ER $\alpha$ <sup>+/+</sup> images were obtained from the work of a previous undergraduate in our lab. Eight optical sections were taken per ovary. Images were obtained from four optical planes at sites at least 5 µm apart

(8 sections in all). Oocyte number was determined by adding the total number of oocytes in each optical section. Cyst breakdown was assessed by counting the number of single oocytes and comparing that the number to the number of oocytes still in cysts. For this, a stack of ten slices was taken for each of the eight sections to determine if oocytes were connected in cysts outside of the plane of focus. Each stack consisted of five images above and below the section being examined; each image was 1  $\mu\text{m}$  apart from the next. Follicle development was determined by counting how many oocytes were at each stage of development in each of the optical sections. If there were no oocytes or follicles in a sample it was not counted in the average. Oocytes were considered unassembled if granulosa cells did not completely surround the oocyte. Single oocytes, completely surrounded by granulosa cells, were classified as primordial, primary, or secondary. Primordial oocytes were surrounded by a single layer of flattened granulosa cells. Primary oocytes were surrounded by one layer of granulosa cells that are cuboidal in shape. Secondary oocytes, considerably larger, were surrounded by more than one layer of cuboidal granulosa cells.

One-way ANOVA was conducted to look at differences between wildtype and mutant ovaries for oocyte number, cyst breakdown, and follicle development. PROC GUM of SAS 9.1 (SAS Institute Inc., Cary, NC) was used to calculate the least square means and test specific hypotheses for effects. A p value  $< 0.05$  was considered significant.

### **Western Blotting**

Western Blotting was utilized to determine if the ER $\alpha$  protein was expressed in different tissue samples. Ovaries at PND1, PND4, PND7, PND42, and PND 108 were dissected in 1X PBS and immediately stored on ice. Ovary extracts were made by homogenizing the ovaries in 10  $\mu$ l of 1X sample buffer per ovary. 1X sample buffer is a mixture of 500  $\mu$ l of 2X sample buffer, 100  $\mu$ l of 10X stock of protein inhibitor, and 400  $\mu$ l of distilled water. 2X sample buffer consists of 4 ml of 10% SDS, 2 ml of glycerol, 1ml of 0.1% bromophenol blue, 2.5 ml of 0.5 M tris (pH 6.8), and 0.5 ml of 2-mercaptoethanol. The proteinase inhibitor stock was made by dissolving one tablet of mini complete protease inhibitor (Roche Diagnostics) into 1 ml of distilled water. 20  $\mu$ l of each ovary extract is placed into a boil-proof tube and 2  $\mu$ l of 2-mercaptoethanol was then added to each. 20  $\mu$ l of MCF-7 whole cell lysate (Santa Cruz) was used as a positive control. MCF-7 cells are taken from human breast adenocarcinoma cell lines and are positive for estrogen receptors (Mairesse et al. 1980). The tubes were then boiled for three minutes, cooled on ice, and then centrifuged at 6,000 rpm for 1 minute.

A 4-20% polyacrylamide gel was placed in a western blot gel box that was approximately one-third full of with 1X SDS Running buffer. The running buffer is made from 100 ml of a 10X stock plus distilled water to bring volume to 1 liter. The 10X Running Buffer contains 29g of Tris base, 144 g of glycine, 10 g of SDS, dissolved in 1 liter of distilled water. 20 $\mu$ l of each ovary extract sample was loaded into a well after the wells had been cleaned. 10  $\mu$ l of Precision Plus Standard Protein marker (Bio-Rad) plus 10  $\mu$ l of 2X sample buffer was added into

the first well as a reference for band size. Gel electrophoresis was performed at 100 volts for approximately 1 hour.

Protein was transferred to a Polyvinylidene fluoride (PVDF) membrane (Millipore) as follows. A 6 x 8 cm piece of PVDF membrane was soaked in methanol for 1 minute and then washed in distilled water for 5 minutes. The PVDF membrane and gel were then soaked in Transfer buffer for 5 minutes (in separate trays). Simultaneously, two pieces of filter paper cut to the size of the gel and two fiber pads were also soaked in Transfer buffer for 5 minutes. The Transfer buffer contains 3.0 g of Tris base, 14.4 g of glycine, and 200 ml of methanol in distilled water brought to a total volume of 1 liter and stored in a dark bottle at 4°C. All of the materials were placed in a transfer cassette, dark side (of the transfer cassette) down, in the following order: fiber pad, filter paper, gel, membrane, filter paper, fiber pad. The transfer cassette was then inserted into the transfer casket with the black side of the cassette facing the black side of the casket. A magnetic stir bar was then placed into the transfer unit. The transfer casket was placed into the transfer unit, with the black side facing a pre-made ice cube. The transfer unit was filled with Transfer buffer. The unit was placed at 4°C fridge and the transfer was performed at 100 volts for one hour. The gel was then stained with Coomassie Blue for 30 minutes to visualize whether the protein had run and for preservation. The gel was then washed with destain solution overnight. The destain solution consisted of 450 ml of distilled water, 450 ml of methanol, and 90 ml of acetic acid. The gel was dried on a glass frame and gel wrap. The membrane was put into Blocking buffer in a tray on a shaker for 30

minutes at room temperature, the buffer was then changed and the membrane was stored at 4°C overnight. Blocking buffer contains 100 ml of 10X PBS 50 g of 5% nonfat dry milk, and 0.5 ml of 0.05% Tween 20 in distilled water brought to a volume of 1 liter.

The membrane was then incubated in primary antibody diluted in Blocking buffer and placed on a nutator at room temperature for one hour. ER $\alpha$  MC-20 (Santa Cruz) was used as the primary antibody at a concentration of 1:500. The membrane was washed in Blocking buffer for three 10 minute intervals on a shaker at room temperature. It was then incubated in secondary antibody diluted in blocking buffer for one hour on a room temperature nutator. The secondary antibody used was goat anti-rabbit conjugated to horseradish peroxidase (HRP) (Jackson Immunolabs) in dilutions ranging from 1:10,000 to 1:15,000.

Following antibody treatment the membrane was then placed in Wash buffer for 10 minutes on a shaker at room temperature. The Wash buffer contains 0.05% Tween in 1X PBS. The membrane was washed in 1X PBS, placed in a 1:1 mixture of detection and enhancer solutions (Pierce), 5 ml per membrane, for 5 minutes on the shaker, and then rinsed in distilled water. A plastic pouch cut slightly larger than the PVDF membrane was then prepared and the membrane placed inside. The plastic pouch was taped to a cardboard mount and exposed to autoradiography film to detect the ER $\alpha$  protein.

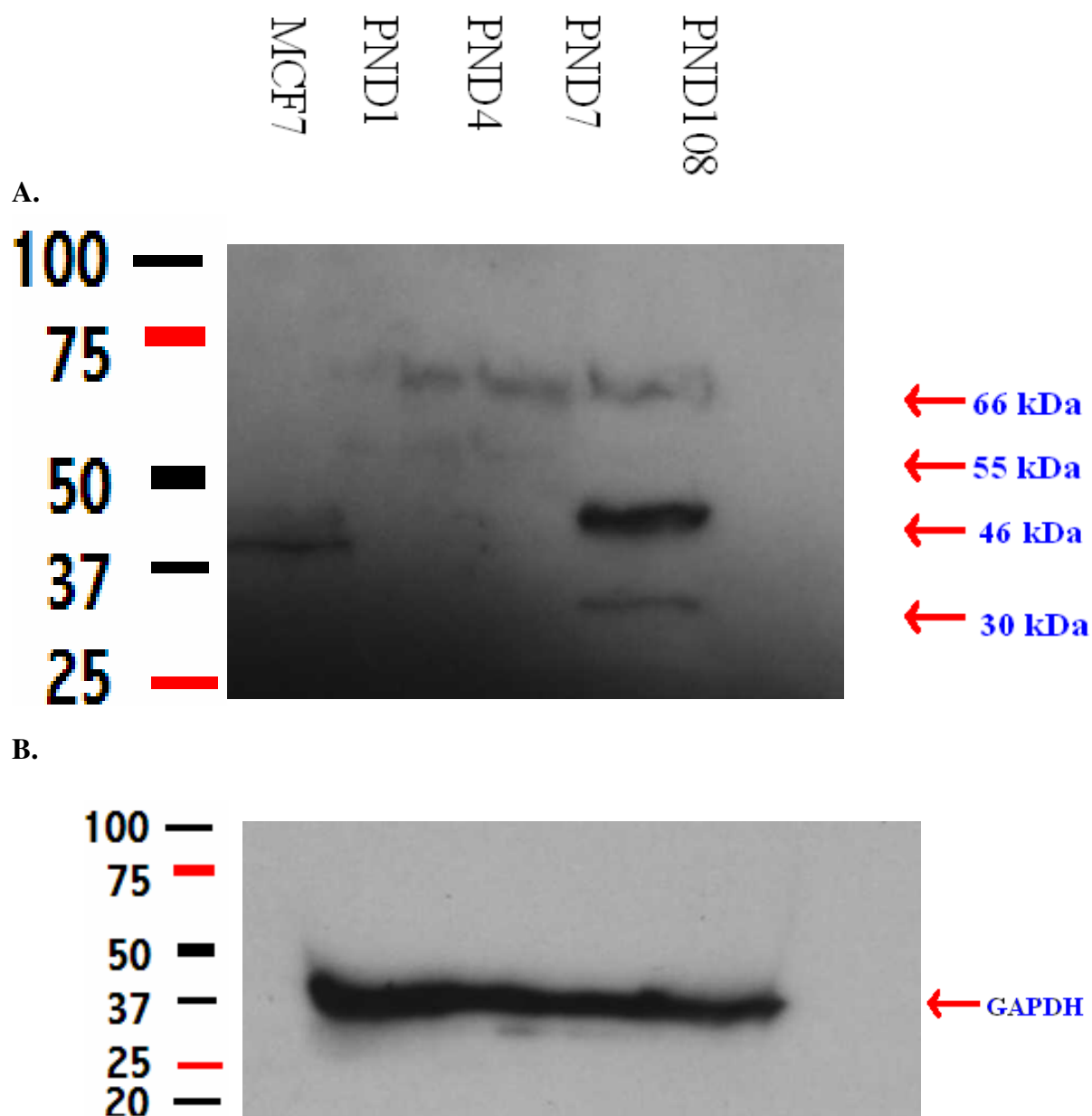
The PVDF membrane was reprobbed with the antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (EnCor Biotechnology

Inc.) as a loading control. The membrane was first rinsed in 1X PBS. It was then placed in a Blocking buffer for one hour on shaker at room temperature. The same detection procedure was then followed using GAPDH at 1:500 as a primary antibody and anti-mouse-HRP (Santa Cruz) at 1:10,000 as the secondary antibody.

## **RESULTS**

### Expression of ER $\alpha$ in Neonatal Mouse Ovaries

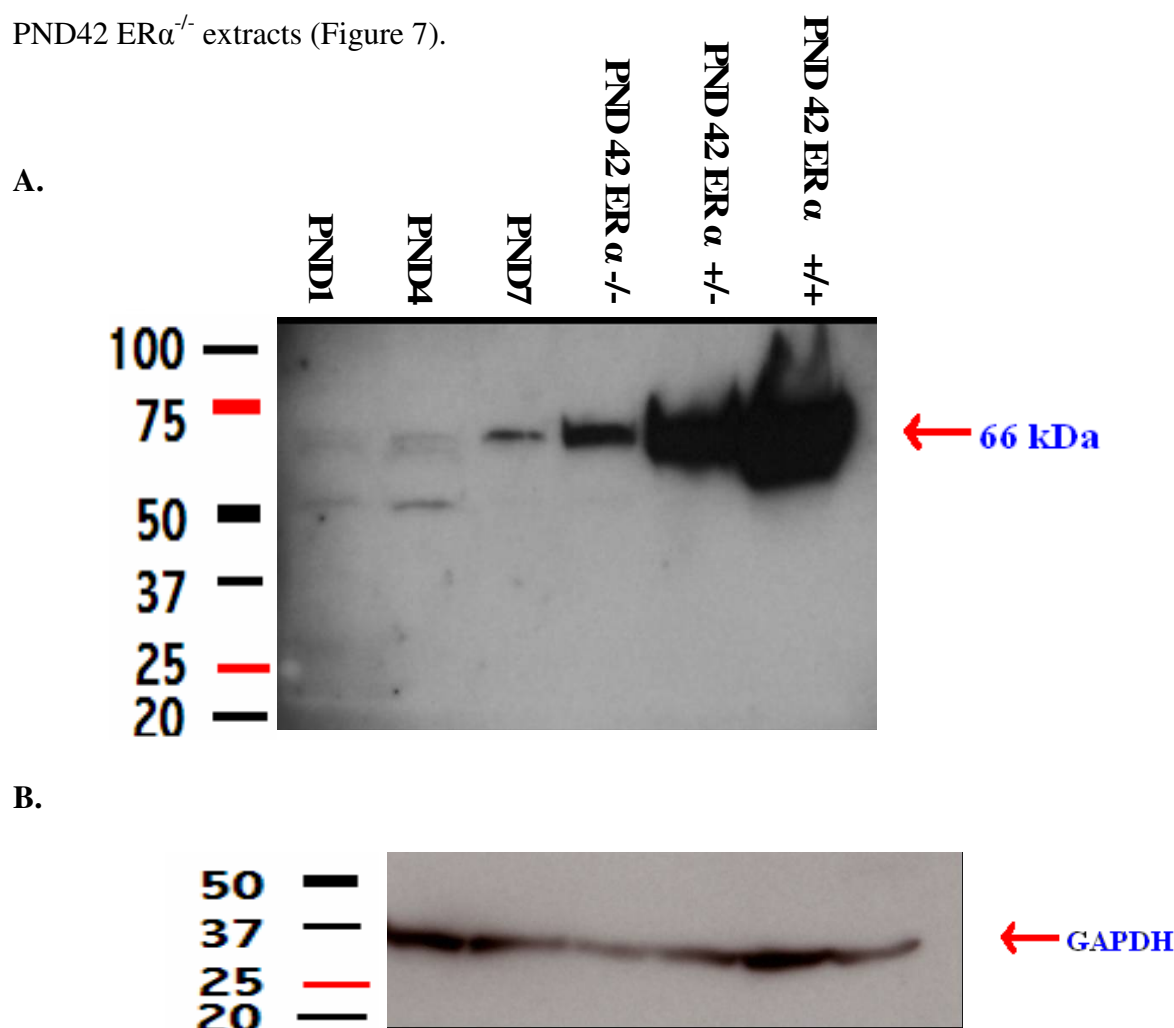
Western blot analyses were carried out to detect the ER $\alpha$  protein in ovaries at several different ages (Figure 6). Anti-ER $\alpha$  antibody, MC-20, was used at a 1:500 dilution and the goat anti-rabbit was used as at 1:10,000 dilution. ER $\alpha$  is known to have at least three splice variants corresponding to proteins of 66, 55, and 46 kDa (Pendaries et al., 2002). MCF7 cell lysate, containing a human ER $\alpha$  protein taken from human breast adenocarcinoma lines was used as a positive control. A band at 46 kDa corresponding to ER $\alpha$  protein was detected in the positive control. Bands at 66 kDa and 55 kDa were detected for PND1, 4, and 7 wildtype mice. PND 108 displayed bands at 66 kDa, 46 kDa, and 30 kDa. The ER $\alpha$  MC-20 antibody is directed toward the C-terminal portion and will detect all three ER $\alpha$  isoforms (66 kDa, 55 kDa, and 46 kDa). The blot was reprobbed with GAPDH as a loading control.



**Figure 6.** Western Blot Analysis of ER $\alpha$  Protein. **A.** MCF7 whole cell lysate purified from human breast adenocarcinoma lines was used as a positive control. Tissue extracts from wildtype CD-1 mice at PND1, PND4, PND7, PND108 were probed with ER $\alpha$  MC-20 antibody. **B.** Blots were reprobbed with GAPDH.

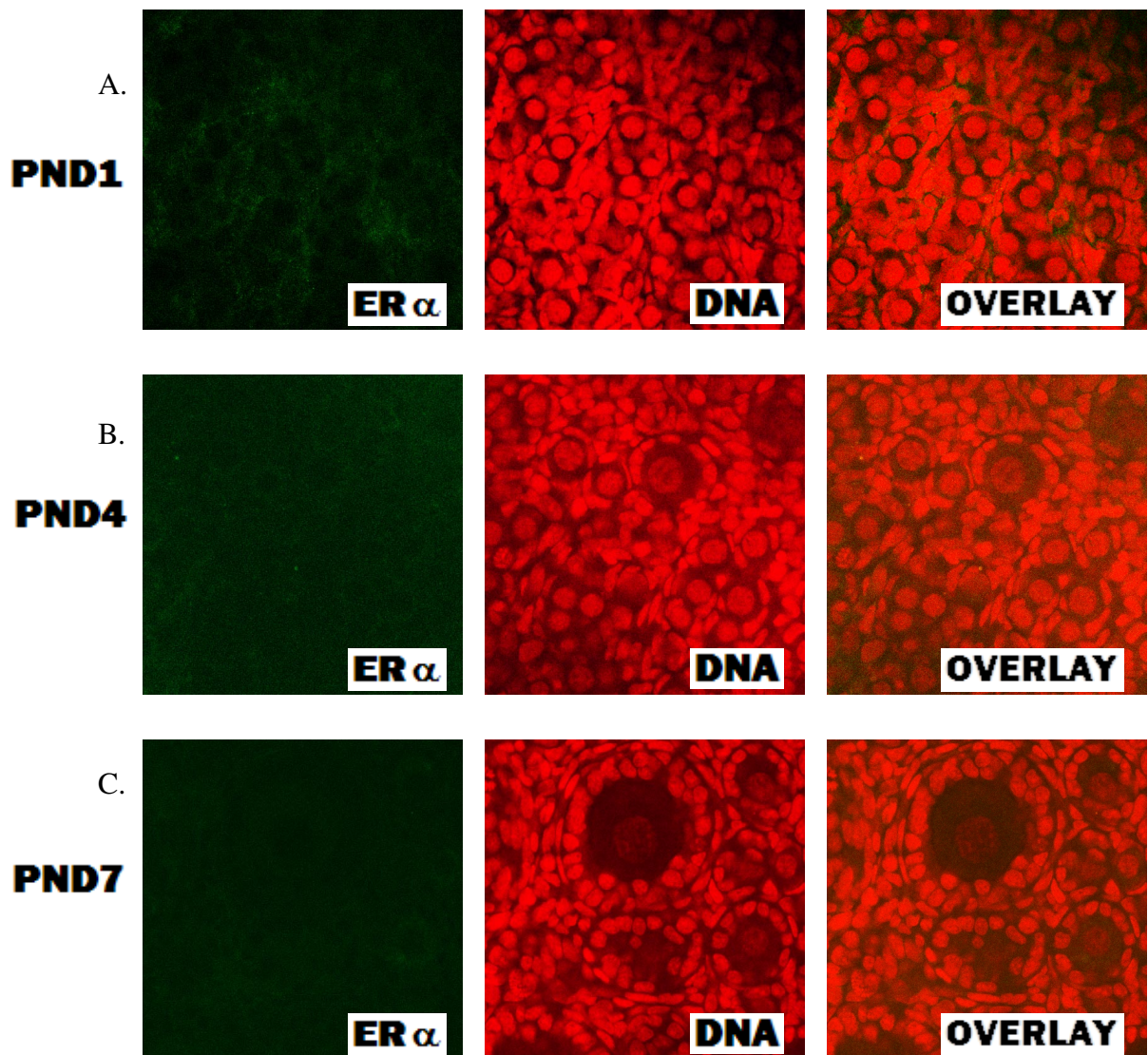


An additional Western blot was run with PND1, PND4, PND7, and PND42 ovary extracts from wildtype CD-1 mice. ER $\alpha$  MC-20 was kept at the same concentration as the previous blot, but the goat anti-rabbit secondary concentration was adjusted to 1:15,000 in an effort to reduce background. Three PND42 extracts were used to detect the difference in expression of the ER $\alpha$  protein in ER $\alpha^{+/+}$ , ER $\alpha^{+/-}$ , and ER $\alpha^{-/-}$  mice. Results from the Western blot indicate 66 kDa and 55 kDa isoforms are present at PND1 and PND4 (Figure 7). The 66 kDa isoform was detected at PND7. All three of the PND42 extracts contain the 66 kDa isoform; the level is highest in PND42 ER $\alpha^{+/+}$  extracts and lowest in PND42 ER $\alpha^{-/-}$  extracts (Figure 7).



**Figure 7.** Western Blot Analysis of ER $\alpha$  Protein. **A.** PND1, PND4, PND7, and PND42 ER $\alpha$  wildtype (+/+), heterozygote (+/-), and homozygous mutant (-/-) were probed with ER $\alpha$  MC-20 antibody. **B.** Blots were reprobed with GAPDH as a loading control.

Whole mount indirect immunofluorescence was carried out to determine the localization of the ER $\alpha$  protein in wildtype CD1 strain neonatal ovaries. The anti-ER $\alpha$  MC-20 antibody was used to detect ER $\alpha$  in CD-1 mouse ovaries at PND1, PND4, and PND7. Ovaries were also labeled with propidium iodide to visualize DNA (nuclei). The ER $\alpha$  protein is not detected in oocytes or somatic cells at PND1, PND4 or PND7 (Figure 8).

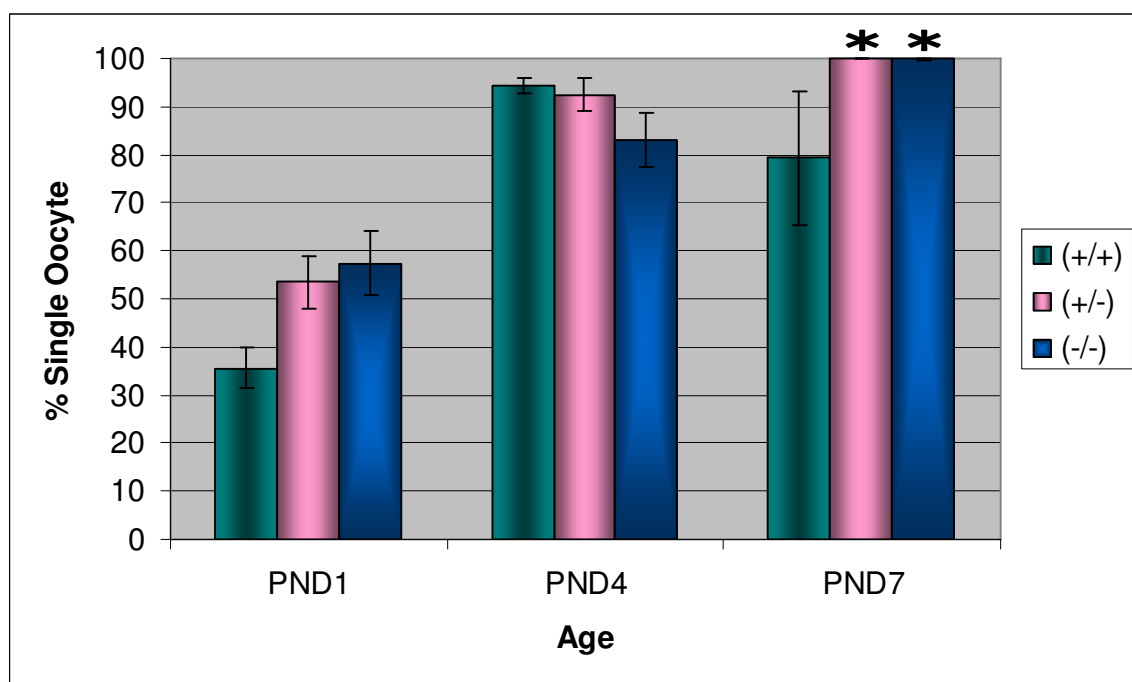


**Figure 8.** Detection of ER $\alpha$  Protein Using MC-20 Antibody. **A.** In staining of PND1 ovaries, ER $\alpha$  exhibits non-specific staining. **B.** PND4 exhibits non-specific staining. **C.** PND7 exhibits non-specific staining.

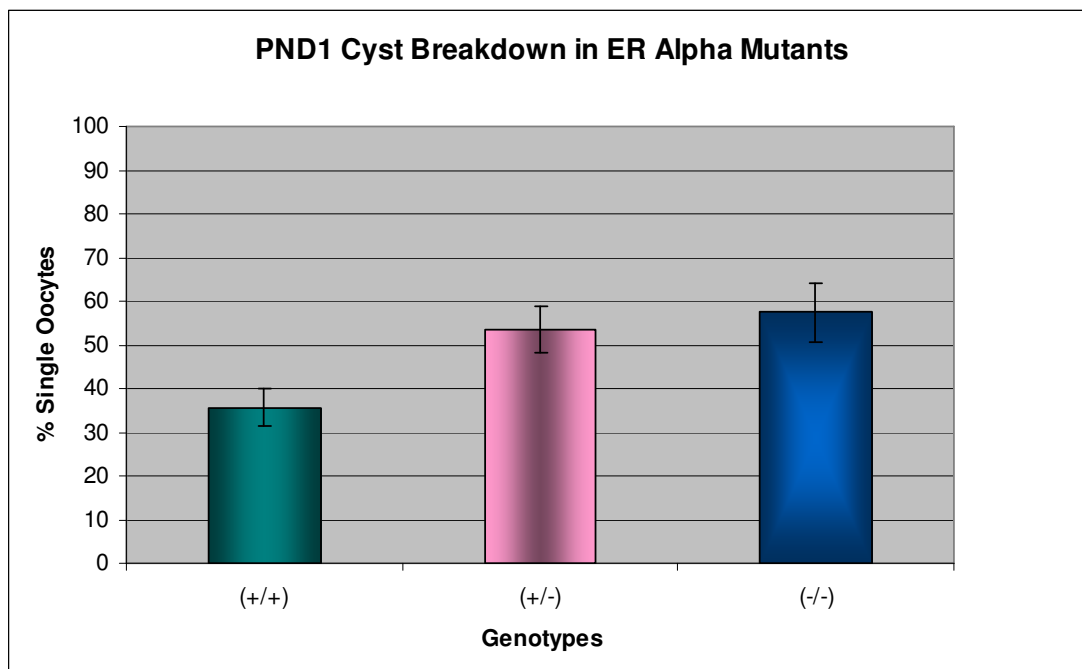
### Cyst Breakdown in ER $\alpha$ Mutant Mice

Estrogen treatment has been implicated in aberrant cyst breakdown in neonatal mice, suggesting that estrogen signaling has a role in cyst breakdown (Chen et al., 2007). Neonatal mice treated with genistein, an estrogen-like compound found in soybeans, exhibit multiple oocyte follicles (MOFs) in ovaries as adults (Jefferson et al., 2002). ER $\alpha$  knockout mice treated with genistein exhibit MOFs, while ER $\beta$  knockout mice do not (Jefferson et al., 2002). This result implies that genistein signals through ER $\beta$ . However, both ER $\alpha$  and ER $\beta$  agonists can inhibit cyst breakdown (Chen and Pepling, in preparation). This implies that ER $\alpha$  as well as ER $\beta$  may be involved in cyst breakdown. To determine the role of ER $\alpha$  in cyst breakdown and oocyte development, ER $\alpha^{+/+}$ , ER $\alpha^{+/-}$ , and ER $\alpha^{-/-}$  mice were examined at PND1, PND4, and PND7. Cyst breakdown was determined by comparing the number of oocytes in cysts to the number of single oocytes. The average percentage of single oocytes increased during the seven days of development as expected in all genotypes, indicating that the process of cyst breakdown was occurring (Figure 9). The single oocyte percentage was elevated in ER $\alpha^{+/-}$ , and ER $\alpha^{-/-}$  mice as compared to ER $\alpha^{+/+}$  at PND1, however, it was determined that this difference was not statistically significant (Figures 10). There is no significant difference in cyst breakdown across the genotypes at PND4 (Figure 11). However, there is a slight decrease in single oocyte percentage in ER $\alpha^{-/-}$  ovaries, but this is not statistically significant. Thus, cyst breakdown occurred similarly across genotypes at PND4. At PND7, ER $\alpha^{+/-}$  and ER $\alpha^{-/-}$  ovaries had an increased percentage of single oocytes and this

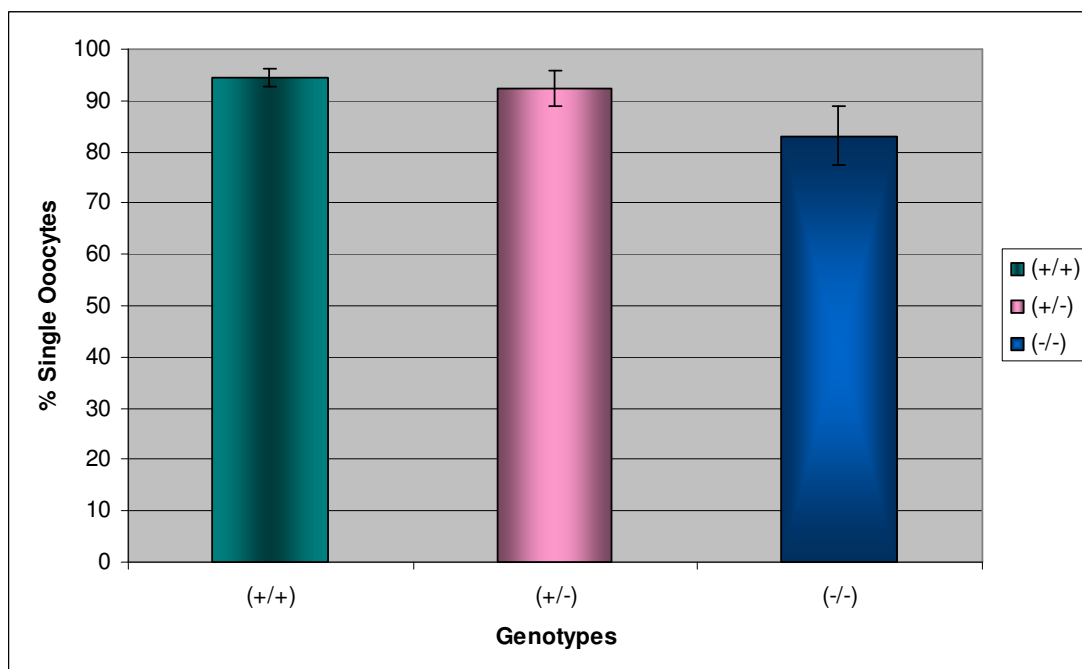
difference was statistically significant (Figure 12). Thus, cyst breakdown appears to be accelerated at PND7.



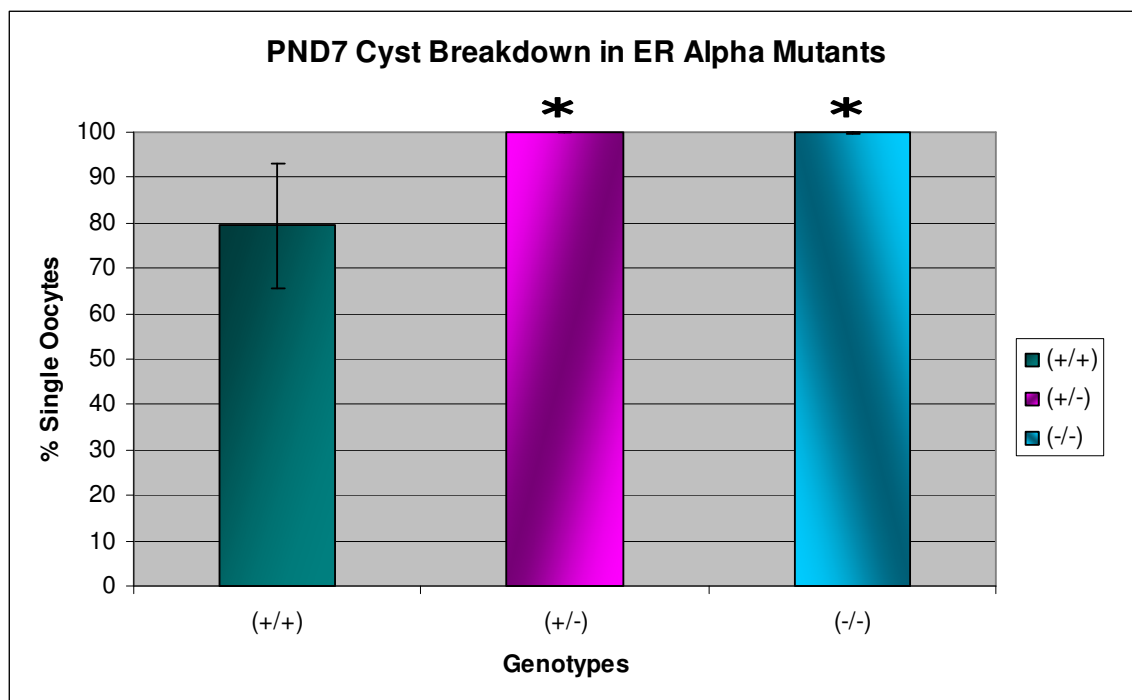
**Figure 9.** Summary of Cyst Breakdown at PND1, PND4, and PND7 in  $ER\alpha^{+/+}$ ,  $ER\alpha^{+/-}$ , and  $ER\alpha^{-/-}$  mice. A rise in single oocyte percentage is indicative of cyst breakdown from PND1 to PND7. \* indicates a statistically significant difference between the genotypes with  $p < 0.05$  considered statistically significant. N= 7-40 sections in 1-5 ovaries.



**Figure 10.** Cyst Breakdown at PND1 in  $ER\alpha^{+/+}$ ,  $ER\alpha^{+/-}$ , and  $ER\alpha^{-/-}$  mice. There is more cyst breakdown in  $ER\alpha^{+/-}$  and  $ER\alpha^{-/-}$  at PND1 as compared with to  $ER\alpha^{+/+}$ , but this is not statistically significant. N= 72 sections in



**Figure 11.** Cyst Breakdown at PND4 in  $ER\alpha^{+/+}$ ,  $ER\alpha^{+/-}$ , and  $ER\alpha^{-/-}$  mice. There is no statistical significance difference between cyst breakdown between these three genotypes at PND4. N= 12-40 sections in 2-5

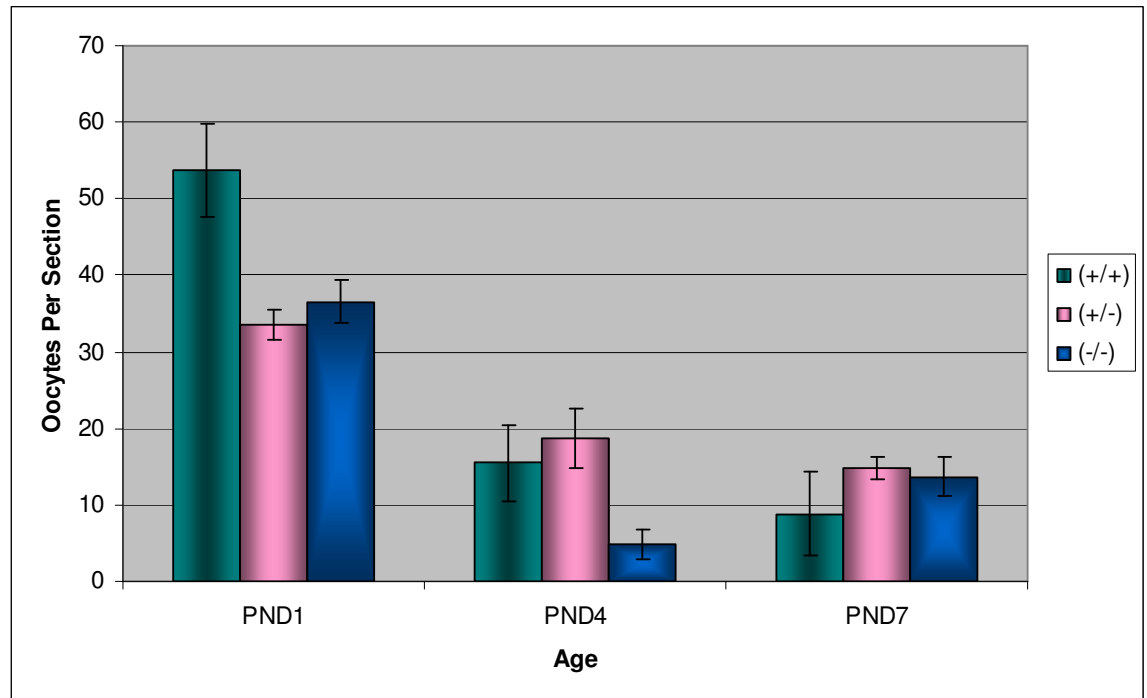


**Figure 12.** Cyst Breakdown at PND7 in  $ER\alpha^{+/+}$ ,  $ER\alpha^{+/-}$ , and  $ER\alpha^{-/-}$  mice. Cyst breakdown is elevated in PND7 in  $ER\alpha^{+/-}$  and  $ER\alpha^{-/-}$  resulting in a higher single oocyte percentage. Differences between genotypes were statistically significant. \* indicates statistical significance where  $p < 0.05$ . N= 7-40 sections in 1-5 ovaries.

### Total Oocyte Numbers in $ER\alpha$ Mutants

The total number of oocytes per confocal section was determined for  $ER\alpha^{+/+}$ ,  $ER\alpha^{+/-}$ , and  $ER\alpha^{-/-}$  at PND1, PND4, and PND7 to establish whether  $ER\alpha$  has a role in oocyte survival. At PND1, there was higher average total oocyte number in all three genotypes, than at PND4 and PND7 (Figure 13). PND4 and PND7 had lower average total oocyte numbers, showing a decline of oocyte number during the seven days of development, which normally occurs at this

time. Although there were variations in oocyte number at PND1, PND4, and PND7, no differences were determined to be statistically significant.



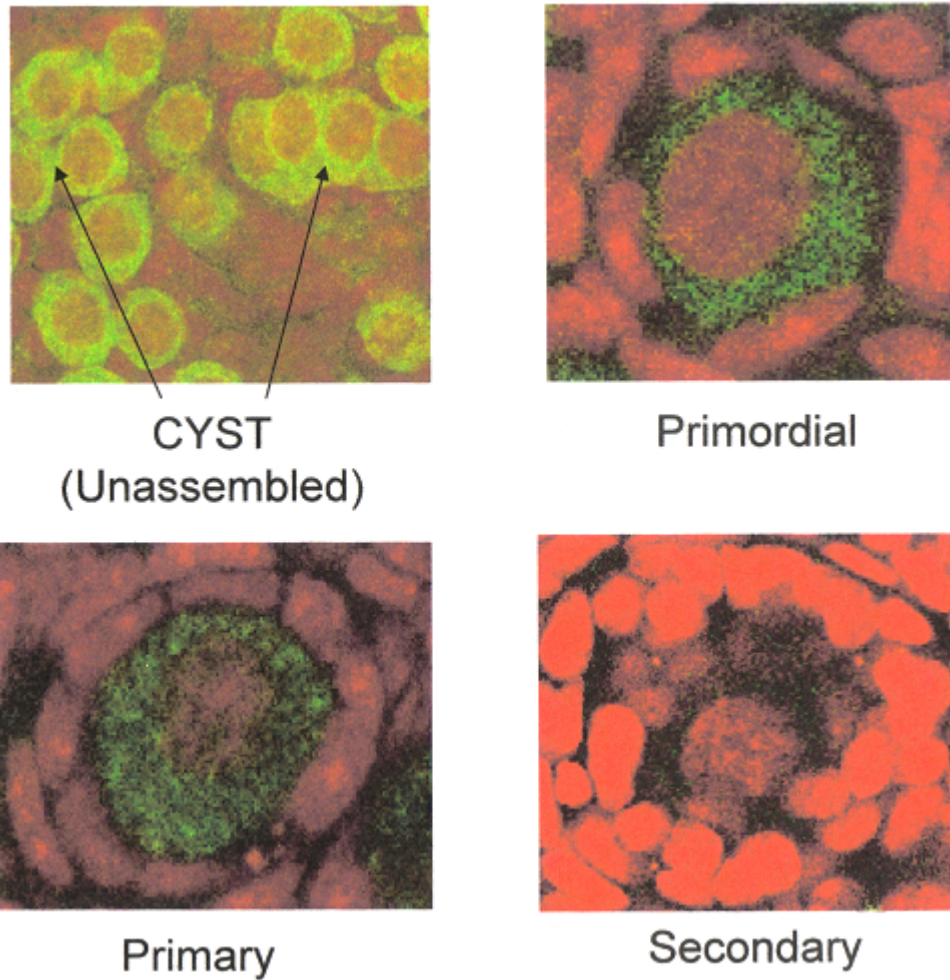
**Figure 13.** Total Number of Oocytes at PND1, PND4, and PND7 in ERα<sup>+/+</sup>, ERα<sup>+/-</sup>, and ERα<sup>-/-</sup> mice. Differences between genotypes were not statistically significant. N= 7-40 sections in 1-5 ovaries.

### Follicle Development in ER $\alpha$ Mutants

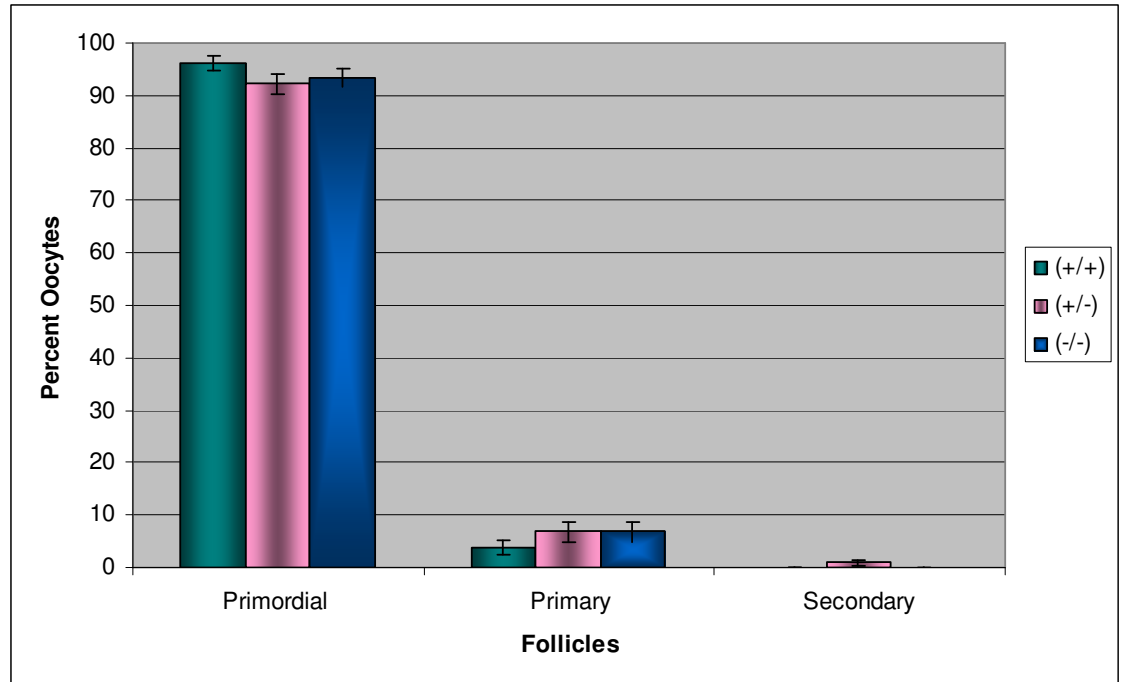
To determine if ER $\alpha$  had a significant role in follicle development, oocytes collected at PND1, PND4, and PND7 were assessed using confocal microscopy. For each confocal section, oocytes were classified as primordial, primary, or secondary follicles (Figure 14). At PND1, most oocytes were unassembled, and the oocytes that were enclosed by granulosa cells were in primordial follicles. There was no significant difference in development across all stages and genotypes at PND1 (Figure 15). Likewise, there was no significant difference in development across all stages and genotypes at PND7 (Figure 17).

In contrast, ER $\alpha^{+/-}$  and ER $\alpha^{-/-}$  primordial follicle development was different from ER $\alpha^{+/+}$  at PND4 (Figure 16). ER $\alpha^{+/-}$  has a slightly lower number of oocytes in primordial follicles than did wildtype, but this is not statistically significant. However, ER $\alpha^{-/-}$  had considerably more oocytes in primordial follicles than did the other genotypes. Also, oocytes from ER $\alpha^{-/-}$  mice did not develop past the primordial follicle stage, suggesting that they arrested in this phase of development and this difference is statistically significant. Primary and secondary follicle development at PND1 and PND7 exhibited no significant difference between the genotypes.

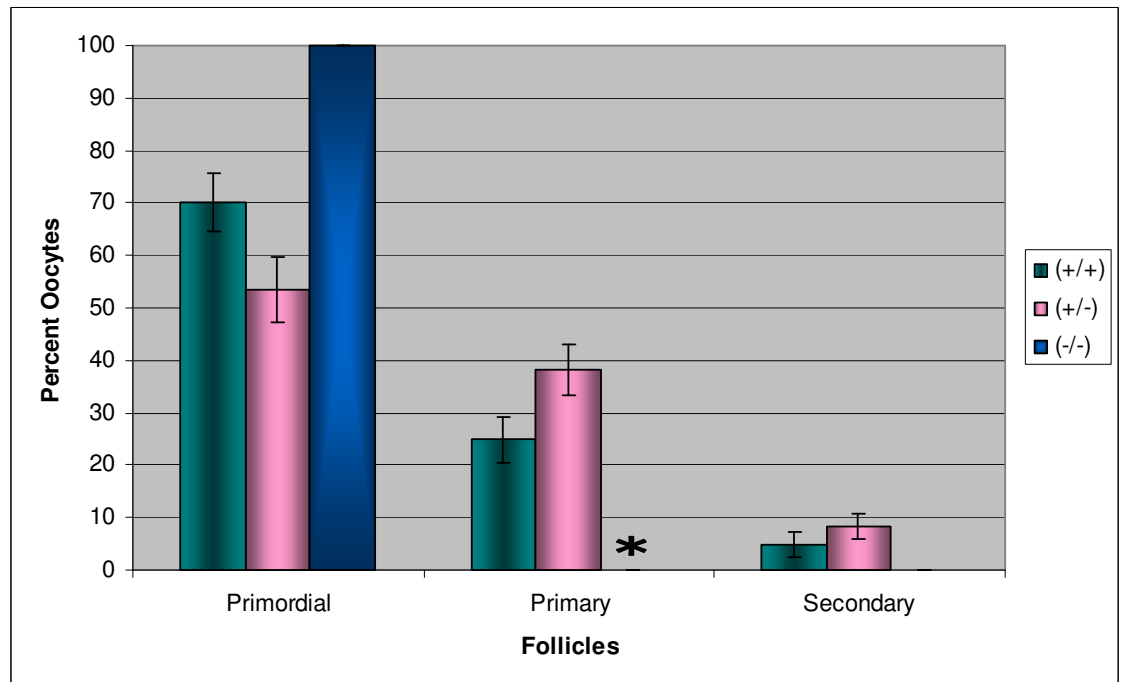




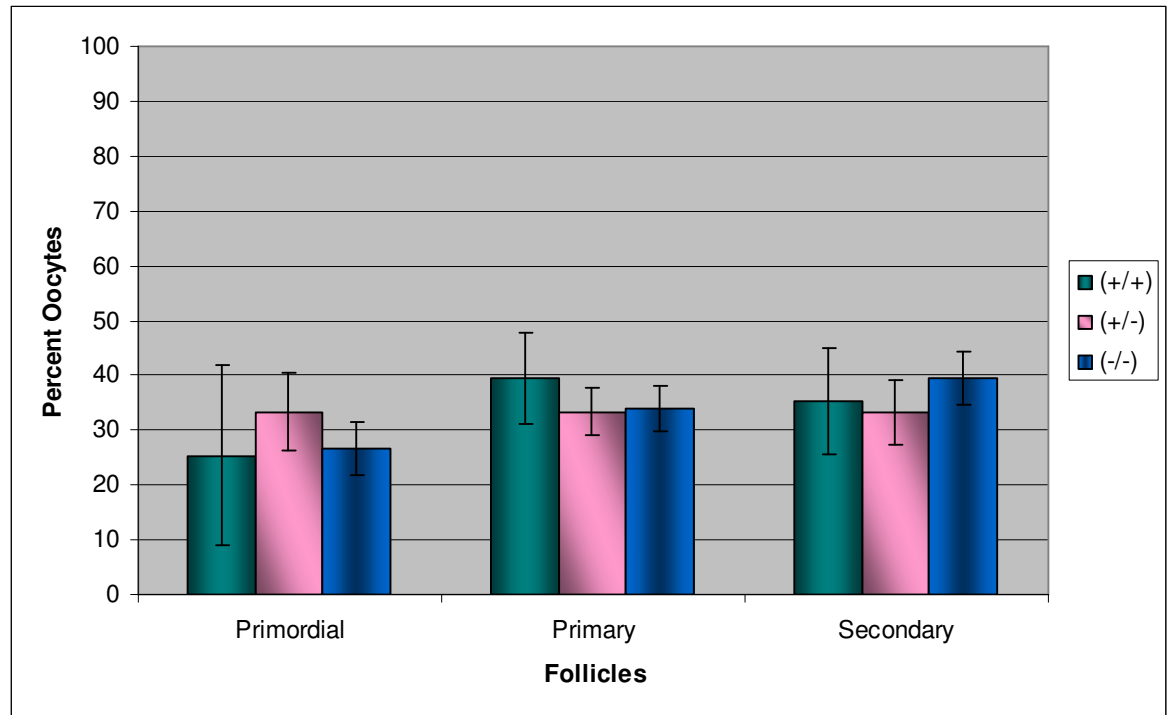
**Figure 14.** Stages of Follicle Development. Oocytes with connecting cytoplasm are classified as unassembled (cysts). Primordial follicles consist of one oocyte and a single layer of flattened granulosa cells. As these follicles mature the oocyte increases in volume and its surrounding layer of granulosa cells become more cuboidal in shape. Granulosa cells increase in number. At this point, the follicle is considered primary. The oocyte continues to develop into a secondary follicle, denoted by more than one layer of granulosa cells.



**Figure 15.** Follicle Development at PND1 in ER $\alpha^{+/+}$ , ER $\alpha^{+/-}$ , and ER $\alpha^{-/-}$  mice. Difference between genotypes was not statistically significant. N= 72 sections in 9 ovaries



**Figure 16.** Follicle Development at PND4 in ER $\alpha^{+/+}$ , ER $\alpha^{+/-}$ , and ER $\alpha^{-/-}$  mice. \* indicates statistically significant difference between the genotypes with p<0.05. N= 12-40 sections in 2-5 ovaries.



**Figure 17.** Follicle Development at PND7 in ER $\alpha$ <sup>+/+</sup>, ER $\alpha$ <sup>+/-</sup>, and ER $\alpha$ <sup>-/-</sup> mice. Differences between the genotypes were not statistically significant. N= 7-40 sections in 1-5 ovaries.

## **DISCUSSION**

Estrogen signals through receptor-mediated pathways. Disruption of pathways results in abnormalities in the adult (Jefferson et al., 2002) and neonatal reproductive systems (Chen et al., 2007). However, it is not known whether ER $\alpha$  plays an integral role in normal oocyte development or if the occurrence of these abnormalities in mice may be an indirect effect of a disruption in this receptor. Thus, the main objectives of this research were to establish whether ER $\alpha$  was expressed in neonatal ovaries and if so where, and to examine ER $\alpha$  homozygous mutant mice for defects in neonatal cyst breakdown, oocyte survival and follicle development at PND1, PND4, and PND7.

The ER $\alpha$  protein was detected in neonatal ovaries using protein Western blot analyses. The expected size of the “classical” full-length ER $\alpha$  is 66 kDa (Pendaries et al., 2002). A protein of this size was detected in PND1, PND4, PND7, and PND108 tissue samples by Western blotting (Figure 6). However, there were also polypeptides detected at 55kDa and 46kDa in PND1, PND4, and PND7. ER $\alpha$  is known to have three isoforms, the 55 kDa and 46 kDa isoforms being ER $\alpha$  mRNA splice variants (Pendaries et al., 2002). They are all detected by the ER $\alpha$  MC-20 antibody, which explains why these other isoforms were detected (Pendaries et a., 2002). Likewise, the MCF7 human ER $\alpha$  positive control exhibited a band at 46 kDa. Therefore, all of the mouse protein extracts contained ER $\alpha$ , indicating that the protein was in fact expressed at the

developmental stages assayed. There was also protein detected at approximately 30 kDa at PND108, which may have been due to protein degradation.

A second Western blot was run to determine whether the ER $\alpha$  protein level was reduced in ER $\alpha^{+/-}$  ovaries and absent from ER $\alpha^{-/-}$  ovaries. Tissue samples from PND42 (adult) ER $\alpha^{+/+}$ , ER $\alpha^{+/-}$ , and ER $\alpha^{-/-}$  mice were collected and run on the western with the original tissue samples from PND1, PND4, and PND7 (Figure 7). As was expected, the ER $\alpha^{+/+}$  tissue sample showed the strongest band and ER $\alpha^{+/-}$  showed a weaker band. It was initially hypothesized that the ER $\alpha$  protein would not be detected in the ER $\alpha^{-/-}$  tissue sample due to the fact that the gene had been knocked out. However, the ER $\alpha$  protein was detected, though very weakly. This may have occurred because the ER $\alpha^{-/-}$  mouse may not have been a full knockout and thus may have retained some of its protein activity.

In the second Western blot the concentration of the secondary antibody goat anti-rabbit was increased from 1:10,000 to 1:15,000. This was done in efforts to minimize the appearance of background or nonspecific labeling. Based on the results of the second Western blot, the concentration change did not have the desired effects because portions of the Western were still blurry. Further analysis of the ER $\alpha$  protein will have to be carried out to alleviate issues with concentrations, full ER $\alpha$  knockout protein expression, and to increase overall understanding of the ER $\alpha$  protein.

ER $\alpha$  localization was also investigated in this study. ER $\alpha$  is expressed by theca cells in the adult ovary. The ER $\alpha$  protein is detected as early as PND1 by Western blotting. However, the results of immunocytochemistry only show non-

specific staining in the PND1, PND4, and PND7 ovary. Due to the confirmed expression via Western blot, it was assumed that the concentration of the MC-20 antibody needed to be increased to exhibit results that mirror that of the Western blot analysis, or that the protein is expressed at very low levels.

ER $\alpha$  protein activity was further analyzed in the assessment of oocyte development using mutants lacking ER $\alpha$ . The total number of oocytes per confocal section was examined to determine if there were significant differences in oocyte survival across all three genotypes at each age of development. There was an overall loss of oocytes across all genotypes, indicating that germ cell loss is a regulated process that occurs in ER $\alpha^{+/+}$ , ER $\alpha^{+/-}$ , and ER $\alpha^{-/-}$  over the seven days of development normally (Pepling and Spradling, 2001). However, there was a significantly lower amount of oocytes in ER $\alpha^{+/-}$  and ER $\alpha^{-/-}$  than there was in ER $\alpha^{+/+}$  animals, in all three days of development. This would suggest that reduction in the level of ER $\alpha$  may accelerate oocyte loss.

Single oocyte percentage was examined to determine if ER $\alpha$  had a role in cyst breakdown. PND4 and PND7 showed the highest percentages of single oocytes. This confirms that cyst breakdown did occur and that by PND7 the majority of oocytes were single. There did not seem to be a significant difference in single oocyte percentage between the genotypes at PND4. However, there was an elevated percentage of single oocytes in ER $\alpha^{+/-}$  and ER $\alpha^{-/-}$  relative to ER $\alpha^{+/+}$  animals at PND1 and PND7. This suggests that at PND1 and PND7 cyst breakdown is elevated. However, the results at PND4 are not consistent with this interpretation.

Past research using estradiol treatment on neonatal mice suggested that estrogen signaling had a major role in preventing or delaying cyst breakdown (Chen et al., 2007). Thus it was originally postulated that ER $\alpha$  was necessary for the maintenance of cysts, and mice lacking this receptor would have accelerated cyst breakdown. The results presented here are inconclusive. One possible interpretation is as follows: perhaps ER $\alpha$  activity is important for the maintenance of cysts at PND1, thus its absence serves to speed up cyst breakdown. It is possible that by PND4, ER $\beta$  compensates for the lack of ER $\alpha$  activity and cyst breakdown rates begin to normalize, but by PND7 ER $\beta$  is no longer able to compensate and the increase in cyst breakdown returns as a result. Ultimately the results of this research are not conclusive enough to provide true evidence for these hypotheses, and further research will need to be done to determine whether ER $\alpha$  truly plays a role in cyst breakdown.

In the examination of ER $\alpha$ 's role in follicle development, there was no difference in follicle development across all genotypes at PND1 and PND7. At PND4 there seemed to be a lower number of primordial follicles in the ER $\alpha$ <sup>+/-</sup> heterozygote than the ER $\alpha$ <sup>+/+</sup> wildtype and ER $\alpha$ <sup>-/-</sup> mutant mice. There is also a considerably higher percentage of oocytes in primordial follicles in ER $\alpha$ <sup>-/-</sup> mice than ER $\alpha$ <sup>+/-</sup> and ER $\alpha$ <sup>+/+</sup> mice. Strikingly, there are no primary or secondary follicles in ER $\alpha$ <sup>-/-</sup>. This indicates that there is an inhibition of primordial follicle development and the follicles can not advance to the primary stage as a result in ER $\alpha$ <sup>-/-</sup> at PND4. Again, further research will be needed to support this idea.

There seemed to be the most noticeable differences in oocyte development at PND4. However, since these trends were not continuous throughout PND1 and PND7, it is difficult to derive concrete conclusions. A number of factors may have contributed to this. Determining the stage of oocyte development was often an issue when it came to distinguishing a definite stage for oocytes that were transitioning between phases of follicle development. The creation of a consistent and universal method of making this determination and counting oocytes according to it would probably help to alleviate this problem.

Technical difficulties may have played a role in discrepancies in data collection. An inability to accurately genotype collected ovaries via PCR analysis delayed the research process, leaving less time to analyze those ovaries initially collected once the PCR problem was solved. This also left less time to collect more ovaries for a consistent sample size across genotypes. In particular, it was difficult to acquire as many homozygous mutant ovaries for analysis as heterozygous ovaries at PND1 and PND4. It was also difficult to acquire wildtype ovaries for all three ages. Thus, smaller sample sizes for homozygous mutant and wildtype ovaries may have contributed to inconclusive results. There were also technical difficulties with antibody staining, due to a lack of germ cell staining, it was difficult to distinguish developmental stages. More accurate results will require the collection of a larger and consistent number of ovaries from each genotype and developmental stage.

The purpose of this research was to further investigate how cyst breakdown, cell death, and follicle development are regulated in the neonatal



ovary. It is known that these processes occur in all mammals, including humans (Pepling, 2006). It is our hope that research in mice will help us to further uncover the mechanisms involved in these processes. Since the pool of oocytes that one will have throughout her entire life is designated at birth, it is our hope that this research sheds more light on how this pool is established. Likewise, estrogen has a major role in the development of these germ cells. With the discovery of how endocrine disruption can have a deleterious effect on reproductive ability, coupled with the appearance of hormones in just about every product we ingest, it is imperative that these type of studies be carried out so scientists have a better understanding of how to combat their possible negative effects. Thus, further research is necessary to create preventative and corrective methods for disorders in estrogen signaling and ultimately establishing an increased understanding of germ cell development as a whole.

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## Capstone Summary

Reproductive ability is an issue that has maintained importance in the world. Thus, as with most issues, scientists are working diligently to study and form a better understanding of how the reproductive system operates. In females, this study is mainly based around the capacity to produce viable oocytes or eggs. In the mouse model system, the production of these eggs begins well before birth, where precursor cells called primordial germ cells divide a number of times by mitosis. Mitosis is the process by which cells in the body divide to create duplicates of themselves. After these primordial germ cells, or egg precursors, divide they form cysts, or clusters of cells that are still connected because of incomplete severance after divisions. Eventually, after birth in mice, a number of cells begin to die via programmed cell death (also known as apoptosis) and these cysts begin to break down. The end result is what is known as a primordial follicle, or one oocyte/egg surrounded by a number of flattened supporting cells called granulosa cells. This step is of extreme importance and interest because the pool of primordial follicles a female has after birth is all she will have for the rest of her life. This research has looked into the mechanisms involved in the cyst breakdown that leads to the formation of this primordial follicle pool, the ability of oocytes to survive despite programmed cell death, and how follicle development continues after primordial follicle formation.

It is well-known that estrogen has many roles in sexual development in females. Therefore, it is not difficult to assume that it may have a role in the

individual stages of egg development. Estrogen is a steroid hormone. There are three types of estrogen that circulate in the body called estradiol, estriol, and estrone. Estradiol is the most prevalent form. Estrogen operates through two types of protein called estrogen receptors located in the nucleus of a cell. These receptors are specifically called estrogen receptor alpha ( $ER\alpha$ ) and estrogen receptor beta ( $ER\beta$ ). In order for estrogen to carry out its effects, it must first bind to these receptors. The estrogen-bound receptors then homodimerize, meaning that two of the same type of receptor (i.e. two  $ER\alpha$ s) bind to each other, and then the homodimer binds to a specific part of the DNA that recognizes this complex called an estrogen response element (ERE). After binding to the DNA, the receptor-estrogen complex recruits helpers called co-regulators to transcribe, or read, the DNA and produce RNA. Ultimately, the RNA is translated into proteins that will carry out estrogen's effects throughout the body.

Previous research has shown that estrogen's mechanism of action can be disrupted and lead to malfunctions in normal cyst breakdown, egg production, and development. When neonatal (baby) mice were treated with genistein, an estrogen-like compound (behaves like estrogen in the cell) found in soy plants, they developed multiple oocyte follicles (MOFs) in adulthood. Throughout the female life, eggs go through a number of stages of development where the egg is encased in supporting cells and called a follicle. Follicle development, prior to ovulation, normally proceeds from primordial follicle, to primary, and then secondary follicle, where the shape of the granulosa cells becomes more cuboidal and their number increases, and the oocyte itself gets larger with each stage.

MOFs are thought to be the result of incomplete cyst breakdown that leads to more than one egg per follicle. Another study was done treating neonatal (baby) mouse ovaries with estradiol (excess estrogen that operates through both receptors). Researchers found that cyst breakdown was inhibited; the oocytes remain in cysts instead of separating breaking down into single oocytes in primordial follicles. All of this tells us that estrogen has a definite effect on cyst breakdown and since it works through ER $\alpha$  and ER $\beta$ , they too have a role in this process.

Both ER $\alpha$  and ER $\beta$  have been shown to be important in the body. Initially, scientists thought that ER $\alpha$  was the only estrogen receptor, but recent research has shown that ER $\beta$  is also an estrogen receptor. ER $\beta$  is found in the ovary, epididymis, prostate, lung, and hypothalamus. ER $\alpha$  is found throughout the body in many areas including the ovary. Our research is particularly focused on these receptors' effects in the ovary when they are working normally and also on the consequences of loss of ER $\alpha$  activity. ER $\beta$  activity in the ovary is found in the granulosa cells that surround the oocyte (egg). Research has shown that when this receptor's activity is disrupted, meaning that it no longer works or is knocked out in mice, the mice develop normally, but have smaller litters. However, my research is focused on the role of ER $\alpha$ . ER $\alpha$  is expressed in other supporting cells called theca cells, which line the granulosa cells. If the ER $\alpha$  receptor is disrupted, past research showed that adult mice are infertile, lack corpus lutea (which secretes hormones that support implantation of the egg into the uterus after fertilization), and have hemorrhagic (bleeding) and cystic (follicles that fail to

ovulate) follicles. Although studies tell us a great deal about ER $\alpha$  in adult and treated neonatal mice ovaries, there has been no research done on the role of ER $\alpha$  in normal neonatal and the mutant phenotypes of mice lacking working ER $\alpha$ .

As a result of the lack of information on neonatal mice lacking ER $\alpha$ , my research is focused on the role of ER $\alpha$  in oocyte development in these mice. Since lacking this receptor is shown to have such severe effects in adult mice, it was my hope to establish whether these effects could be detected earlier on in reproductive development. This research attempted to answer two questions: 1) is ER $\alpha$  expressed (present) in normal neonatal ovaries and if so where; and 2) what are the effects on cyst breakdown, oocyte number (survival), and follicle development when ER $\alpha$  is absent.

A number of scientific methods were used to answer these questions. Mice from a normal strain (no abnormalities in ER $\alpha$  activity) called CD-1 was used to answer the first question. A technique called Western blot analysis was used to test whether the ER $\alpha$  protein was present in the neonatal ovary in these mice. Ovaries were collected from mice at 1, 4, and 7 days after birth (called PND1, PND4, and PND7 from now on), which is the time when cyst breakdown, cell apoptosis, and primordial follicle development occurs. To find out if ER $\alpha$  was localized to the theca cells as it is in adult mice ovaries, were treated with an antibody against the ER $\alpha$  protein called MC-20 and a secondary antibody equipped with a fluorescent tag that would label MC-20.

Mice that were purchased as ER $\alpha$  heterozygotes (having one normal copy and one defective copy) and bred to produce mice with two normal copies of

ER $\alpha$ , one normal/one defective, and two defective copies were also used to answer the second question. Ovaries were collected from these mice at PND1, PND4, and PND7 as well. Polymerase chain reactions (PCRs), which amplify specific regions of DNA (the ER $\alpha$  gene in this case) were used to detect normal (working) or mutant (disrupted) copies of the gene. The amplification product was then used visualized using gel electrophoresis, a technique which uses UV light to detect fragments of DNA. This method was used to determine the genotype of each mouse. The genotype is the genetic profile, indicating the presence of genes. In this case, if a mouse has two normal copies of the ER $\alpha$  gene it is known as ER $\alpha$  wildtype (normal), if it has one normal and one defective copy it is ER $\alpha$  heterozygous, if it has two defective copies the mouse is considered ER $\alpha$  mutant. Once it was known which type of mouse the ovaries came from, a fluorescent marker was used to label the oocytes/eggs in these ovaries. Pictures were then taken of the ovaries using a fluorescent microscope called a confocal microscope. Oocytes in these pictures were counted and analyzed to see if there were differences in cyst breakdown, oocyte survival, and follicle development in the wildtype, heterozygote, and mutant ER $\alpha$  mice.

The results of this study were as follows. A technique called Western blot analysis indicated that ER $\alpha$  was present in neonatal ovaries. However, labeling ovaries with the fluorescent tag did not show any ER $\alpha$  present, suggesting that the expression was very low at PND1, PND4, and PND7. By analyzing the pictures that were taken of the ovaries from the ER $\alpha$  wildtype (normal), ER $\alpha$  heterozygous, and ER $\alpha$  mutant mice, we were able to see limited differences. Cyst



breakdown increased over the seven days of development as it should have for all three genotypes. However, the rate that the cysts were breaking down was faster in the heterozygous (having partial ER $\alpha$  activity) and mutant (having no ER $\alpha$  activity) mice, than it was in normal mice (having fully active ER $\alpha$ ) at the PND7. This indicates that ER $\alpha$  may have a role in the normal rate of cyst breakdown. There was no significant difference in oocyte survival between the different types of mice in all seven days of development, suggesting that ER $\alpha$  does not have a role in oocyte survival or programmed cell death. In this study, follicle development was found to proceed normally in all types of mice at PND1 and PND7. However, at PND4, the ER $\alpha$  mutant mice were all at the primordial follicle stage. Normally by this time some oocytes have moved into the primary and secondary follicle stage. However, this is not the case in the ER $\alpha$  mutants. This indicates that at PND4, mutant mice were arrested in their development and could not develop past the primordial follicle stage. Thus, ER $\alpha$  may have a role in follicle development as well.

Overall, this research is important for a better understanding of how reproductive development works in the ovary. Many of the conclusions made will need further research to support them because of small sample sizes (small numbers of ovaries analyzed) and inconsistent oocyte counting methods. However, this research does contribute information about the role of ER $\alpha$ , which was not previously known. Hopefully, this will be able to be used by future researchers to fully explain how estrogen operates in the body. Ultimately,

scientists hope to utilize this and other research in the field of reproductive development to properly diagnose and treat human infertility.