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The Role of Estrogen Receptor Beta in Neonatal Oocyte Development

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INTRODUCTION

In the mammalian ovary, oocytes develop from precursors called primordial germ cells. The total number of oocytes available for reproduction is determined at birth. Establishing this pool is essential for fertility (Pepling, 2006). The process of human germ cell development is similar to that of other mammals (Pepling et al., 1999). Studying this progression in *Mus musculus* has provided an approximate timeline of events that help better understand germ cell development (Figure 1).

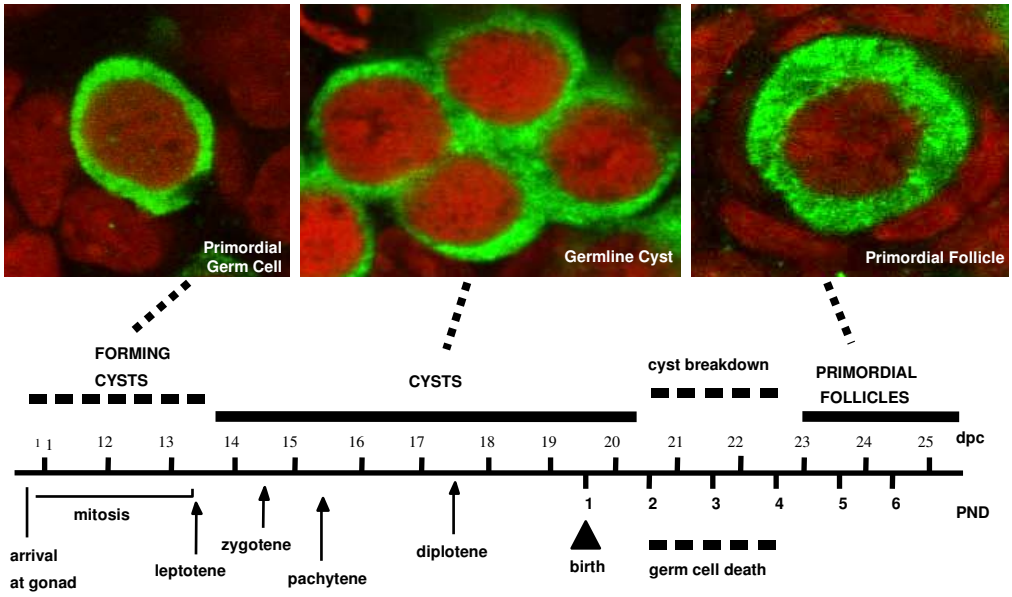

Figure 1. Timeline of Germ Cell Development in the Mouse (Pepling and Spradling, 2001). Green staining is for vasa (oocyte specific marker) to show oocyte cytoplasm and red staining is propidium iodide to show nuclei of both somatic cells and oocytes.

In the developing mouse embryo, primordial germ cells (precursors to oocytes) migrate to the gonad. At approximately 10.5 days post coitum (dpc), the germ cells divide by mitosis and form clusters of cells called germ line cysts. Incomplete cytokinesis results in oocytes connected by intercellular bridges (Pepling and Spradling, 1998). At 13.5 dpc, the germ cells begin to enter meiosis at which point they are referred to as oocytes. The cells remain in cysts until birth. At this point, a process termed cyst breakdown begins and the clusters of cells break apart (Pepling and Spradling, 2001). This results in individual oocytes surrounded by granulosa cells, forming primordial follicles.

At the same time as cyst breakdown, germ cell death occurs and approximately two thirds of the oocytes do not survive (Pepling and Spradling, 2001). The function of this massive loss of oocytes is unknown, although it is hypothesized that the dying cells serve as nurse cells to the surviving oocytes. It is also unknown which cells undergo this programmed death and how their fate is determined. Since cyst breakdown and germ cell death occur simultaneously in a short time frame, these processes may be correlated and part of regulated mammalian development (Pepling and Spradling, 2001).

Throughout the course of the adult ovarian cycle, a group of follicles begins to mature (Dean and Epifano, 2002). Follicles can be found in any of several stages of development at any time during the cycle (Figure 2). In the adult ovary, most oocytes are found in primordial follicles. A primordial follicle is an oocyte surrounded by a layer of granulosa cells and a layer of thecal cells. Each cycle, primordial follicles develop into primary follicles, observable by an

increase in oocyte volume and the number of surrounding granulosa cells. The shape of the granulosa cells is altered; the nuclei become cuboidal. Primary follicles then become secondary follicles which are surrounded by more than one layer of granulosa cells. At this stage only some follicles are selected for further growth and the follicles not selected become atretic and die. A mature vesicular follicle is significantly larger and contains several components. The oocyte is surrounded by the zona pellucida, a thick extracellular matrix. Multiple layers of granulosa cells also enclose the oocyte. During this growth, an antrum forms, a cavity containing proteins, hormones, and other molecules. The oocyte develops from this mature follicle and is released as an egg which may later become fertilized. Following ovulation, the remains of the ruptured follicle become the corpus luteum.

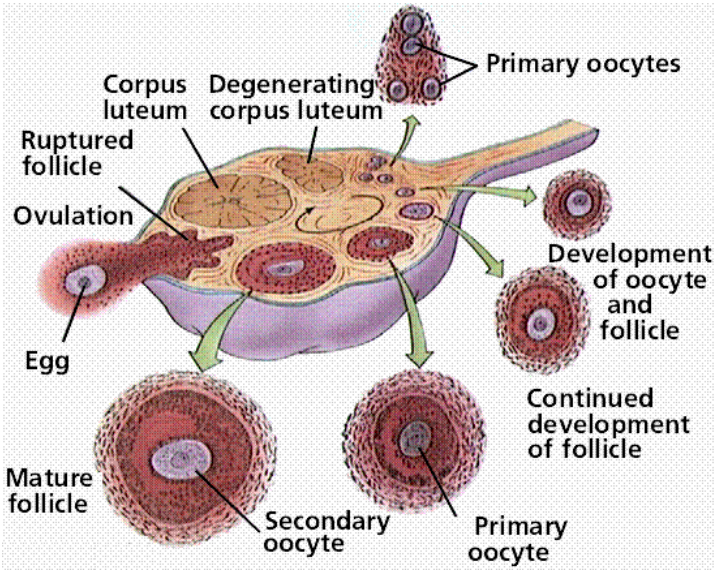

Figure 2. Structure of an Ovary (Farabee, 2007). A primordial follicle goes through several stages of growth and development until it reaches maturity and is released, leaving behind remaining cells and forming the corpus luteum.

If the released egg (now referred to as the ovum) does not become fertilized, the corpus luteum degenerates. If fertilization does occur, the corpus luteum is responsible for secreting hormones which support pregnancy.

Steroid hormones have been found to regulate growth and differentiation as well as cell death in certain target tissues (Mangelsdorf et al., 1995). Estrogen is a hormone which acts primarily on the reproductive organs of the body. It is present in both males and females, but in significantly higher levels in females. The three types of naturally occurring estrogens in females are estradiol, estriol, and estrone. Estrogens regulate growth and differentiation through receptor-mediated pathways. Two types of receptors exist: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (Krege et al., 1998). ERα is expressed over a broad range in the body. Its expression in the adult ovary is in the theca cells. The neonatal ovarian localization, however, is still unknown. ERβ expression in the body is more focused in the ovary, prostate, epididymis, lung, and hyptholamus (Krege et al., 1998). In the ovary, ERβ expression is highest in the granulosa cells and can be detected as early as postnatal day one (PND1) by RNase protection (Jefferson et al., 2000). ERβ levels in the ovary increase with age due to follicular maturation and thus an increase in the number of granulosa cells (Jefferson et al., 2000). Both ERα and ERβ are members of the nuclear hormone receptor family. Estrogen crosses the cell membrane, perhaps by diffusion, and enters the nucleus of the targeted cell where it binds to the receptor. The receptor then homodimerizes, binds to estrogen response elements (EREs) on target genes and initiates transcription (Figure 3**).**

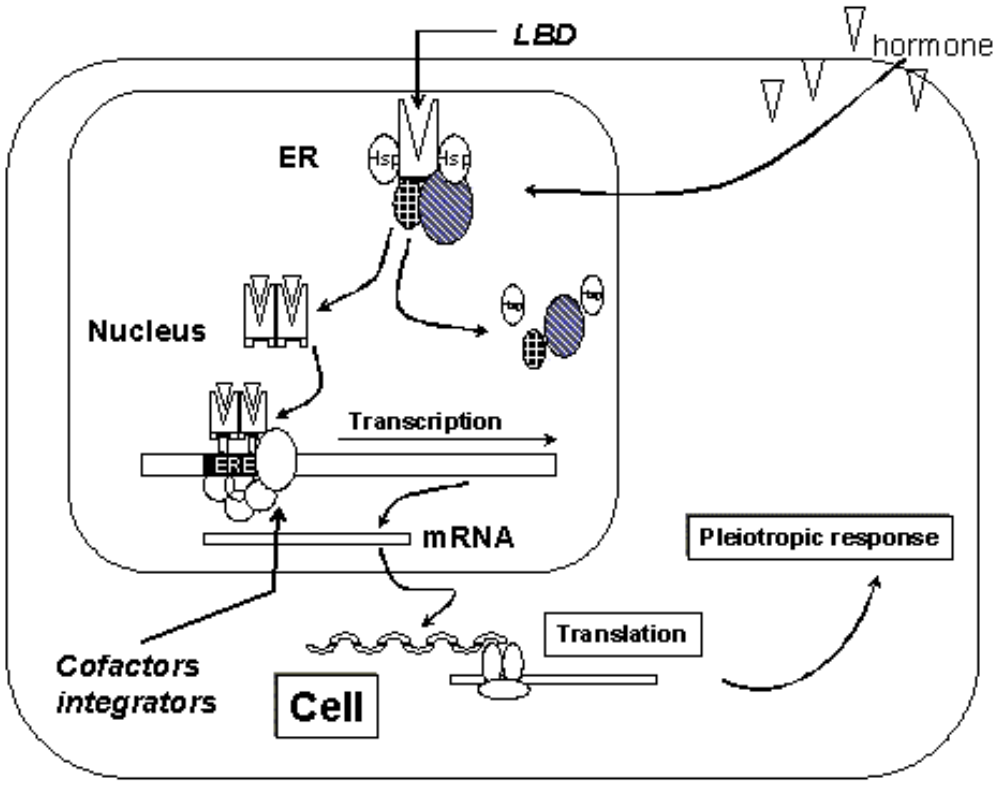

Figure 3. Mechanism of Estrogen Response Signaling (Adapted from the Center for Medical and Urological Andrology). Estrogen signal binds to Ligand Binding Doman (LBD) of receptor protein. Receptors homodimerize and bind to Estrogen Response Element (EREs) on target genes.

Both ERα and ERβ exhibit similar protein domain organization. The A/B domain at the N-terminal contains a ligand-independent transactivation function, AF1(Hewitt and Korach, 2002). AF1 is present in both ERα and ERβ A/B domains. The C domain, where the DNA binds to the protein, is highly conserved and is responsible for the specific binding to estrogen response elements. The C domain is also important for receptor dimerization. The E domain, where the

ligand binds to the protein, is less conserved but also contributes to receptor dimerization (Dupont and Krust, 2000). (Figure 4)

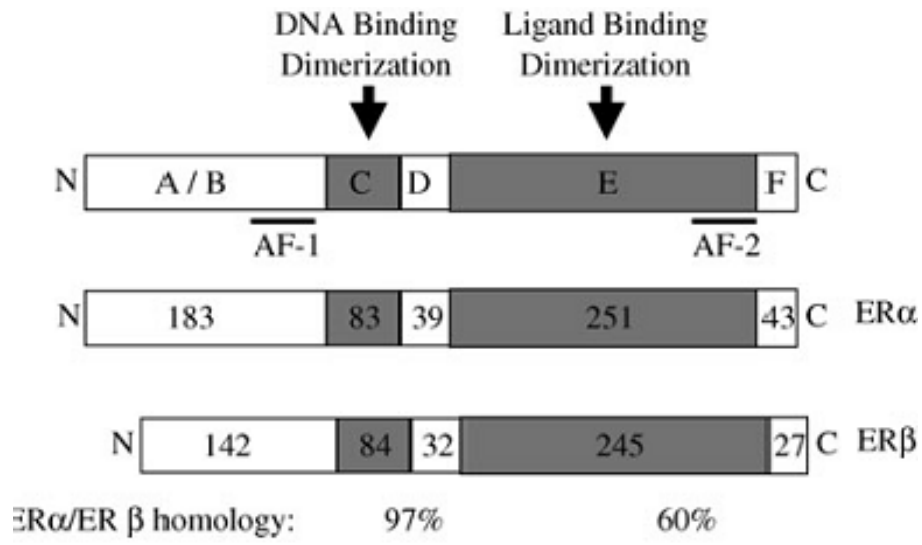

Figure 4. ERα and ERβ Protein Domain Organization (Adapted from Hewitt and Korach, 2002). Shaded domains exhibit regions of high conservation. The number of amino acids is indicated in each domain. Domain C and E have high protein homology (97% and 60% respectively).

Mutant mice lacking these receptors were generated by knocking out expression of the genes that encode each receptor. Available strains lack expression of ERα ($ER\alpha^{-/-}$) or ERβ ($ER\beta^{-/-}$). (Dupont and Krust, 2000). These targeted disruptions caused observable phenotypic effects on the adult mouse body as well as in the ovary (Dupont and Krust, 2000). Female $ER\alpha^{-/-}$ mice exhibited a lack of breast tissue development, gonadal abnormalities, and sterility. Ovaries from adult $ER\alpha^{-/-}$ mice had large hemorrhagic cysts and an absence of

corpora lutea. Atretic follicles were present in excess. The ovaries were anovulatory and hence infertile (Krege et al., 1998).

In contrast, adult ERβ$^{-/-}$ mice exhibited reduced fertility, producing fewer and smaller litters. The ovaries appeared normal and had normal antral follicles, but had a reduction in the number of or absence of corpora lutea (Dupont and Krust, 2000). More immaturely atretic follicles were detected compared to wildtype. Partial arrest of follicular development and less frequent follicular maturation may be responsible for these results. Even when treated with exogenous gonadotropins, ERβ$^{-/-}$ mice failed to exhibit efficient ovulation, suggesting that ERβ has a more critical role in the developing ovary (especially in the granulosa cells) than ERα (Krege et al., 1998).

Estrogen signaling has been implicated in neonatal oocyte development. Genistein is a soy phytoestrogen (a natural plant product) with estrogenic activity. When neonatal mice were treated with genistein, multiple oocyte follicles (MOFs) were observed (Jefferson et al., 2002) (Figure 5). The MOFs could be oocyte cysts that did not break apart. Alternatively, they might result from oocytes that were improperly enclosed by granulosa cells or from the fusion of two individual follicles. Genistein treatment inhibited cyst breakdown, supporting the idea that MOFs are cysts that did not break apart (Jefferson et al., 2006).

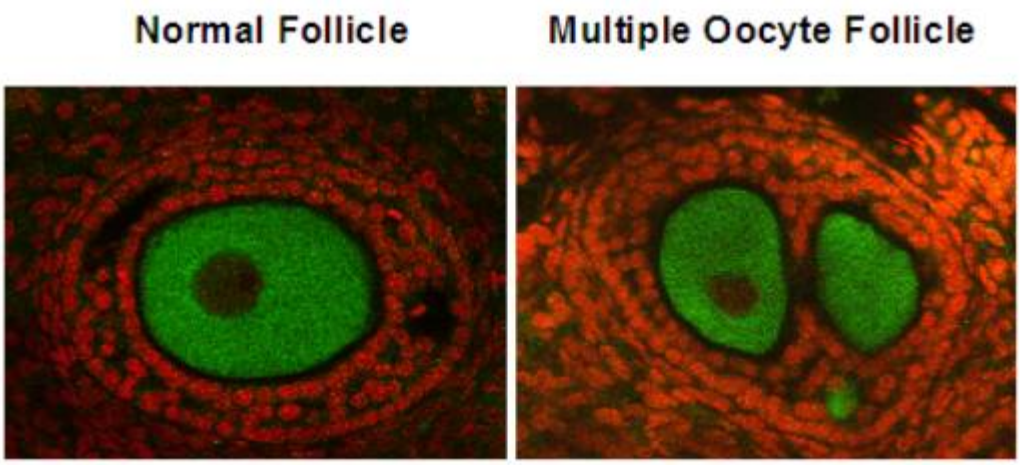

Figure 5. The image on the left is a single developed oocyte (green) surrounded by several layers of somatic cells. The image on the right is of more than one oocyte enclosed within the same follicle, illustrating a multiple oocyte follicle (Pepling, 2006).

When ERβ$^{-/-}$ mice were treated with genistein, no MOFs were formed (Jefferson et al., 2002). This leads to the hypothesis that genistein is acting as an estrogen through ERβ. The role of estrogen signaling in cyst breakdown was further investigated using the endogenous estrogen, estradiol, in an ovary organ culture system. Results demonstrated that estradiol treatment inhibited cyst breakdown, but did not change oocyte survival (Chen et al., in press). ERβ$^{-/-}$ mice have not been examined for defects in neonatal oocyte development. Previous studies have focused on expression of ERβ adult ovaries. The focus of my research is to examine ERβ$^{-/-}$ mice for defects in neonatal cyst breakdown, oocyte survival, and primordial follicle formation. In addition, the expression of ERβ in neonatal ovaries was also investigated.

MATERIALS AND METHODS

Mice

The ERβ$^{-/-}$ strain used in my studies contained a targeted disruption of the ERβ gene, where a neomycin gene had been inserted in exon 3 using homologous recombination. ERβ$^{-/-}$ mice were obtained from Jackson Laboratories. Matings were set up between two heterozygous ERβ mice. Vaginal plugs were checked for four consecutive days post mating. If a plug was present, the pregnant female was isolated. This day marked 0.5 (dpc). Birth occurred at 19.5 dpc. This is designated PND1. Neonatal ovaries were harvested from the litter at PND1, PND4, and PND7. Ovaries were also collected from CD-1 (mice obtained from Charles River Laboratories) at various ages.

Purification of DNA from Mouse Tails

A metal numerical tag was assigned to each mouse for identification purposes. A piece of the tail was collected and placed in a centrifuge tube with the respective number. To isolate the DNA from the mouse tails, the DNAeasy kit (QIAGEN) was used. Pipetted into each tube were 180 microliters (µl) of Buffer AL and 20 µl of the enzyme proteinase K. The reaction 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 made, and 400 µl was added to each sample and vortexed. The samples were transferred into spin columns with collection tubes attached, and 500 µl of AW1 buffer plus ethanol was added to each. The

tubes were centrifuged for 8,000 rpm for one minute. The collection tubes were discarded and replaced. 500 µl of AW2 buffer plus ethanol was added to each tube and centrifuged at 14,000 rpm for three minutes. Again, the collection tubes were discarded and replaced with new Eppendorf centrifuge tubes. 100 µl of AE buffer was added to each tube and centrifuged at 8,000 rpm for one minute at which point the DNA was eluted into the collection tube. The DNA was stored at -20°C.

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a technique used to amplify specific DNA sequences. It is useful because only a small initial amount of DNA is necessary, and multiple copies can be produced. Pipetted into each PCR tube was 1µl each of primer (3145, 3146, 3147), 4 µl of 1.25 mM dNTP mixture (1.25 mM each of dATP, dTTP, dCTP, and dGTP), 10 µl of distilled water, 2.5 µl of 10X Buffer A, 5 µl of mouse DNA, and 0.5 µl of Taq polymerase (Promega). The sequences of the primers are as follows:

Primer 3145: 5'- GTT GTG CCA GCC CTG TTA CT- 3'

Primer 3146: 5'- TCA CAG GAC CAG ACA CCG TA- 3'

Primer 3147: 5'- GCA GCC TCT GTT CCA CAT ACA C- 3'

Primers 3145 and 3146 anneal to Exon 3 of the ERβ gene and amplify a 106 base pair DNA fragment from the wildtype allele (Figure 5). Primer 3147

anneals to a region in the neomycin insertion. Primer 3146 and 3147 amplify a 160 base pair DNA fragment from the targeted allele. In a normal mouse containing two wildtype alleles, only the 106 base pair band was observed. In a mouse heterozygous for the ERβ mutation both a 106 and 160 base pair band was detected. In a mouse homozygous for the ERβ mutation only the 160 base pair band was observed.

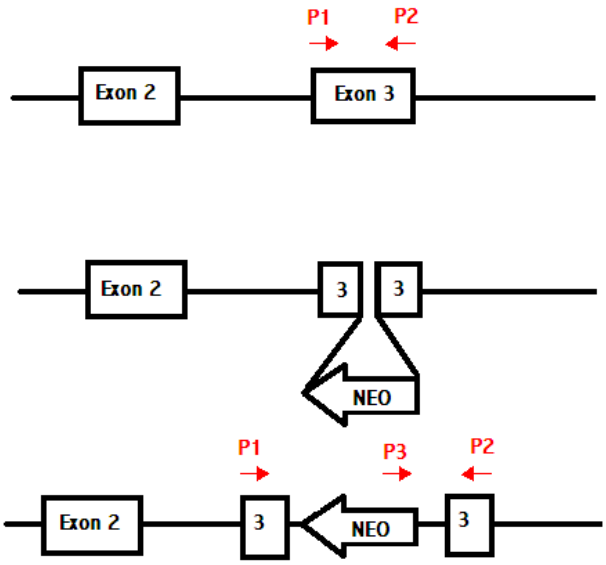

Figure 6. Targeted Disruption of the ERβ gene (Krege et al., 1998). Positions of primers (P1 represents Primer 3145, P2 represents 3146, and P3 represents 3147) are indicated by small directional arrows.

The total volume in each PCR reaction tube was 25 µl. The reactions were placed into the thermocycler on the following ERβ program:

Stage: 1 Step: 1 3 minutes at 94°C

Stage: 2 Step: 1 30 seconds at 94°C

Step: 2 30 seconds at 60°C

Step: 3 30 seconds at 72°C

(Stage 2 repeated for 35 cycles)

Stage: 3 Step: 1 2 minutes at 72°C

(Hold at 4°C)

Gel Electrophoresis

To determine the genotype of each mouse from the PCR products, gel electrophoresis was performed. 2% agarose gels were prepared. 1g of agarose was placed in 50 mL of 1X TBE buffer and dissolved by microwaving for 2 minutes. 2.5 µl of 10 mg/ml ethidium bromide was added to the hot agarose solution and swirled for efficient distribution. The mixture was poured into a gel tray which had a comb with individual wells. After the gel solidified, the gel tray was placed into a gel box and 1X TBE buffer was poured so as to submerge the gel. The comb was removed. Loaded into each well was 10 µl of each PCR reaction product plus 2 µl of 6X loading dye. A mixture of 2 µl of 6X loading dye, 5 µl of 1X TBE, and 5 µl of 100 bp marker was loaded into a well to serve as a reference ladder for band size. The gel was run at approximately 100 volts for one hour. An image of the gel was taken and the bands were analyzed using Kodak Image Station software with UV detection light.

Dissection

Ovaries were harvested in 1X phosphate buffered saline (PBS) from neonates PND1, PND4, and PND7 using a dissecting microscope. Segments of tails from each specimen were collected in a separate tube for genotyping purposes.

Whole Mount Antibody Staining of Neonatal Ovaries

The ovaries were separated into three tubes based on their ERβ genotype: wildtype ($ER\beta^{+/+}$), heterozygous mutant ($ER\beta^{+/-}$), or homozygous mutant ($ER\beta^{-/-}$). 600 µl of fix consisting of 400 µl of 1X PBS and 200 µl of 16% formaldehyde (Ted Pella Inc.) was pipetted into each tube and nutated for one hour at room temperature. The ovaries were washed two times in 1 ml of PT quickly and then once in 1ml of PT for 30 minutes at room temperature. They were nutated for 30 minutes to one hour in PT + 5% BSA at room temperature. Then the ovaries were incubated with 1 µl of primary antibody Stat3 (C20) (Santa Cruz Biotech) in 500 µl of PT + 5% overnight at 4°C on a nutator. They were washed in 1 ml of PT + 1% BSA for 30 minutes at room temperature on a nutator. The ovaries were then incubated in 10 µl of 10 mg/mL RNase A in 1 ml PT + 1% BSA for 30 minutes. Each tube was wrapped in aluminum foil to prevent light from minimizing the fluorescent capacity. The ovaries were incubated in 10 µl of 5µg/ml propidium iodide in 1 ml PT + 1% BSA for 20 minutes at room temperature. They were washed in PT + 1% BSA for 30 minutes and then incubated with the pre-absorbed secondary antibody anti-rabbit Alexa 488 (Molecular Probes) for two hours at

room temperature. To pre-absorb the secondary antibody, a 1:200 dilution was made with 2.5 µl of the antibody in 500 µl of PT + 5% BSA and a small portion of embryo powder. The tube was wrapped in aluminum foil and placed on a nutator at 4°C overnight for use the following day. The ovaries were washed in 1 ml PT + 1% BSA three times at room temperature on a nutator for a total of an hour and a half. The ovaries were rinsed one final time with 1X PBS. The PBS was pipetted out of the tubes and 100 µl of Vectashield was added to each tube for 15 minutes. The ovaries were mounted on a slide. The slides were stored at -20°C.

Confocal Microscopy

Indirect immunofluorescence of PND1, PND4, and PND7 ovaries was observed using a Zeiss Pascal confocal microscope. Cyst breakdown was assessed by counting the number of single oocytes versus connected oocytes. The nuclear marker was propidium iodide and stained the nuclei red. The oocyte marker was Stat3 (C20) and stained the oocytes green (Murphy et al., 2005). Eight optical images were taken per ovary. These regions were obtained by examining two areas of the ovary and taking four confocal sections at least 20 µm apart. A single confocal section was examined for each region. To determine if oocytes in the examined section were connected in cysts out of the plane of focus, a stack of ten sections was taken. Five images, 1 µm apart, were taken above and below the section to be examined (Figure 7). If granulosa cells did not completely surround an oocyte or if Stat3 antibody labeling showed a cellular

connection, oocytes were considered unassembled. Single oocytes were classified as primordial, primary, or secondary. Primordial oocytes were surrounded by several granulosa cells with flattened nuclei. One layer of granulosa cells with cuboidal nuclei surrounded primary oocytes. Secondary oocytes were significantly larger and surrounded by more than one layer of granulosa cells.

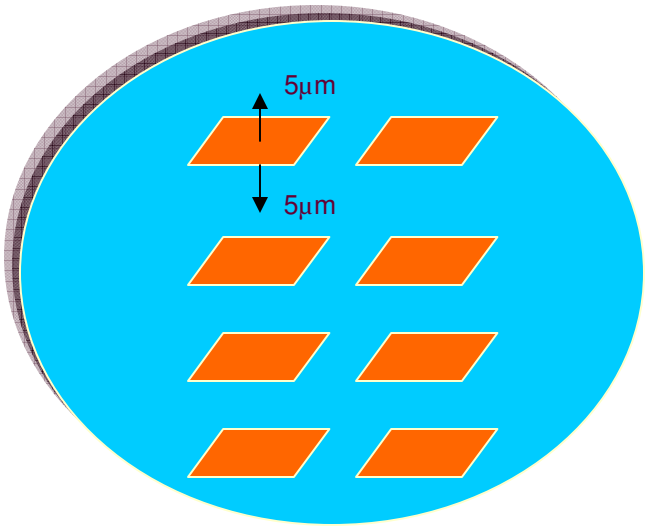

Figure 7. Representative Image of Confocal Sections. The shaded blue area represents the microscopic field of view. Each slanted square represents a single confocal section.

Western Blotting

In order to determine if the ERβ protein is expressed in different tissue samples, western blotting was performed. Ovaries and testes were dissected at different ages (PND1, PND4, PND7, and PND 42) in 1X PBS and immediately stored on ice. Gonad extracts were made by homogenization of the ovaries or testes, using 10 µl of sample buffer per ovary and 20 µl of sample buffer/testes. Sample buffer contains 4 ml of 10% SDS, 2 ml of glycerol, 1 ml of 0.1% bromophenol blue, 2.5 ml of 0.5 M tris (pH 6.8), and 0.5 ml of 2-

mercaptoethanol. 2 µl of 2-mercaptoethanol was then added to each tube. A hole was poked at the top of each tube and the samples were then boiled for 3 minutes, cooled on ice, and centrifuged at 6,000 rpm for 1 minute.

A 4-20% polyacrylamide gel was placed in a Western Blot gel box and the box was filled approximately 1/3 with 1X SDS Running buffer. The running buffer contains 29 g of Tris base, 144 g of glycine, 10 g of SDS, dissolved in of 1 liter of distilled water. 20 µl of each sample was loaded into a well after the wells had been cleaned with a syringe. 10 µl of Precision Plus Standard Protein marker was loaded into the first well to serve as a reference for band size. Gel electrophoresis was performed at 100 volts for one hour.

A 6 cm x 8 cm Polyvilnidene fluoride membrane (PVDF) was soaked in methanol for one minute and then washed in distilled water for 5 minutes on a shaker. It was then soaked in Transfer Buffer for 5 minutes. Transfer buffer contains 3.0 g of Tris base, 14.4 g of glycine, and 200 ml of methanol in distilled water for a total volume of 1liter. The Transfer buffer is stored in a dark bottle at 4°C. The gel was then transferred to the membrane. Two pieces of 6 cm x 8 cm filter paper and two fiber pads were soaked in transfer buffer for 5 minutes. The materials were placed into a transfer cassette dark side facing downward in the following order: fiber pad, filter paper, gel, membrane, filter paper, fiber pad. The transfer cassette was placed into a transfer casket and the whole unit was filled with Transfer buffer. The unit was placed in a 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 and washed with destain solution overnight. It

was then dried on a glass frame using gel wrap. The membrane was placed in Blocking buffer for 30 minutes on a shaker at room temperature. Blocking buffer contains 100 ml of 10X PBS, 50 g of nonfat dry milk, 0.5 ml of 0.05% Tween 20 dissolved in distilled water for a total volume of 1 liter. The blocking buffer was changed and the membrane could be stored overnight at 4°C.

The membrane was then incubated in a primary antibody diluted in blocking buffer, and placed on a nutator for one hour at room temperature. The membrane was washed in blocking buffer for 10 minutes three times on the shaker at room temperature. It was then incubated in a secondary antibody for one hour at room temperature. The primary antibodies were used at several concentrations. Stat3 C-20 (Santa Cruz) was used at a 1:1,000 dilution. ERβ 311 rabbit (Affinity Bioreagents), ERβ Y19 (Santa Cruz), and ERβ H150 (Santa Cruz) were used in a series of dilutions ranging from 1:100 to 1: 5,000. The secondary antibodies used were goat anti-rabbit, goat anti-mouse, and rabbit anti-goat conjugated to HRP (horse radish peroxidase) (Jackson Immunolabs). The secondaries were used in a series of dilutions ranging from 1:500 to 1:40,000.

The membrane was washed in Blocking Buffer for 10 minutes two times. It was then placed in Wash buffer for 10 minutes on the shaker at room temperature. Wash Buffer contains 0.05% Tween in 1X PBS. 1X PBS was used to rinse the membrane, and placed in a 1:1 detection:enhancer solution for five minutes on the shaker. It was given a final rinse in distilled water and placed in a clear plastic cover. The cover was taped to a cardboard mount and exposed to autoradiography film to detect the ERβ protein.

Dot Blotting

Dot Blots were performed with ERβ Long control protein (Affinity Bioreagents) to test the effectiveness of the primary antibodies. A 1:50 dilution was made with 1 µl of ERβL in 49 µl of 1X PBS. 5 µl of this was blotted on a section of nitrocellulose membrane designated by circle A. A 1:500 dilution was made in a new centrifuge tube with 1 µl of the 1:50 dilution in 10 µl of 1X PBS. 5 µl of this dilution was blotted on circle B. A 1:5,000 dilution was made in a new centrifuge tube with 1 µl of the 1:500 dilution in 10 µl of 1X PBS. 5 µl of this was blotted on circle C. This procedure was followed for circle D (1:50,000 dilution) and circle E (1:500,000). The membrane was incubated in primary antibody overnight at 4°. The antibodies were used at the following concentrations: ERβ H150 1:200; ERβY19 1:100; ERβ 311 1:500. The membrane was washed in blocking buffer for 10 minutes three times on the shaker at room temperature. It was incubated in a secondary antibody for one hour at room temperature. The antibodies were used at the following concentrations: Goat anti-rabbit 1:35,000; Rabbit anti-goat 1:25,000; Goat anti-rabbit 1:25,000. Detection of the protein proceeded using the Western Blotting protocol as described above.

RESULTS

Cyst Breakdown in ERβ Mutant Mice

Estrogen signaling has been implicated in neonatal oocyte development (Chen et al., in press). Cyst breakdown is disrupted in neonates treated with estrogen. To test if ERβ is important for this effect, $ER\beta^{-/-}$ mice were examined for defects in cyst breakdown. Cyst breakdown was determined by assessing the number of oocytes still in cysts versus the number of single oocytes in $ER\beta^{+/+}$, $ER\beta^{+/-}$, and $ER\beta^{-/-}$ mice. As expected, the average percentage of single oocytes for wildtype animals increased from PND1 to PND7 as the oocytes in cysts became separated (Figure 8). The number of single oocytes in $ER\beta^{+/-}$ and $ER\beta^{-/-}$ animals also increased from PND1 to PND7. No significant difference in cyst breakdown was found between $ER\beta^{+/+}$, $ER\beta^{+/-}$, and $ER\beta^{-/-}$ animals (Figure 8). Single oocyte percentage remained stable in each genotype at PND1, PND4, and PND7 (Figures 9-11). Thus, cyst breakdown occured similarly in wildtype, ERβ heterozygous, and ERβ homozygous mutants.

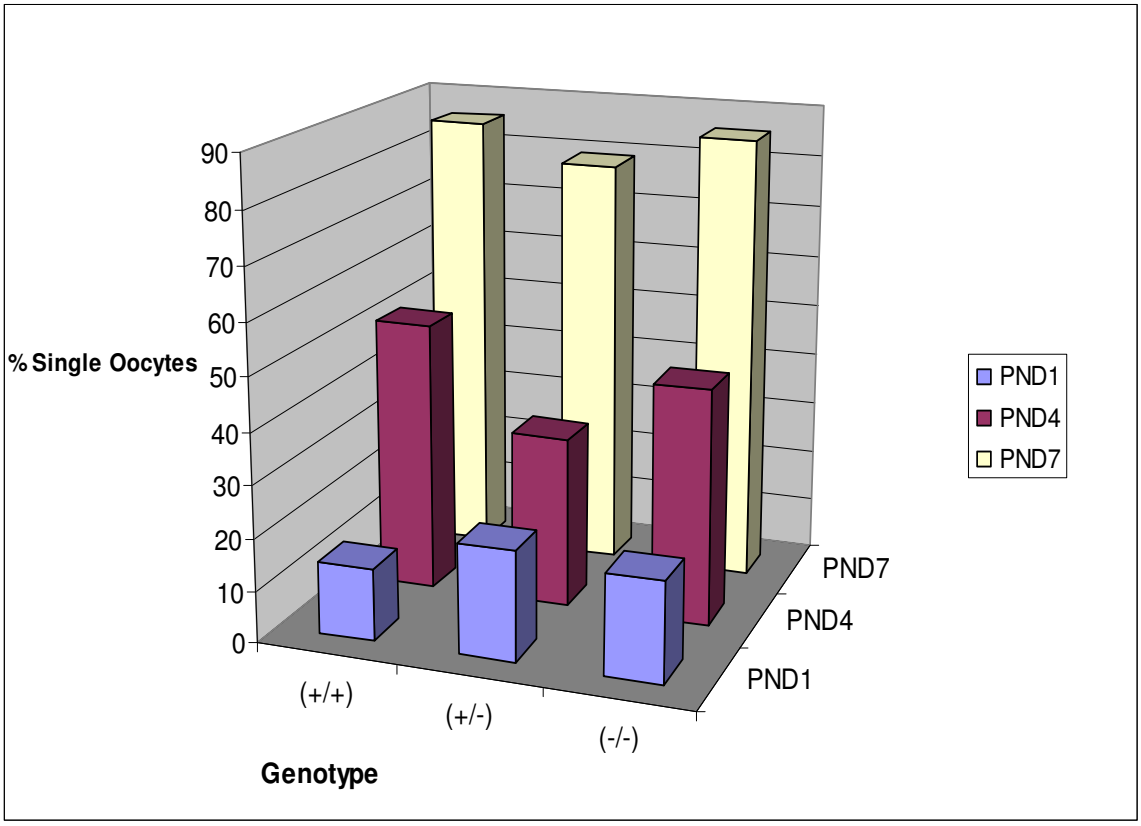

Figure 8. Cyst Breakdown at PND1, PND4, and PND7 in ERβ$^{+/+}$, ERβ$^{+/-}$, and ERβ$^{-/-}$ mice. An increase in single oocyte percentage from PND1 to PND7 indicates cyst breakdown. Data are presented as the mean. N = 22-93 sections in 3-13 ovaries.

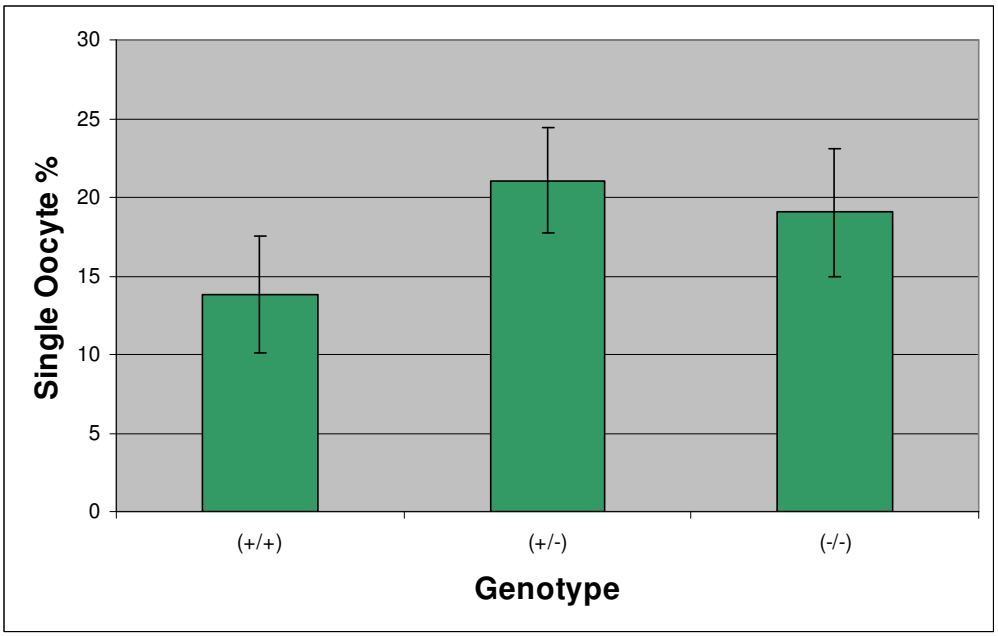

Figure 9. Cyst Breakdown at PND1 ERβ$^{+/+}$, ERβ$^{+/-}$, and ERβ$^{-/-}$ mice. Data are presented as the mean ± SEM. ERβ mutants exhibit no significant difference of single oocyte percentage. (P-value > 0.05) N= 22-30 sections in 7-13 ovaries.

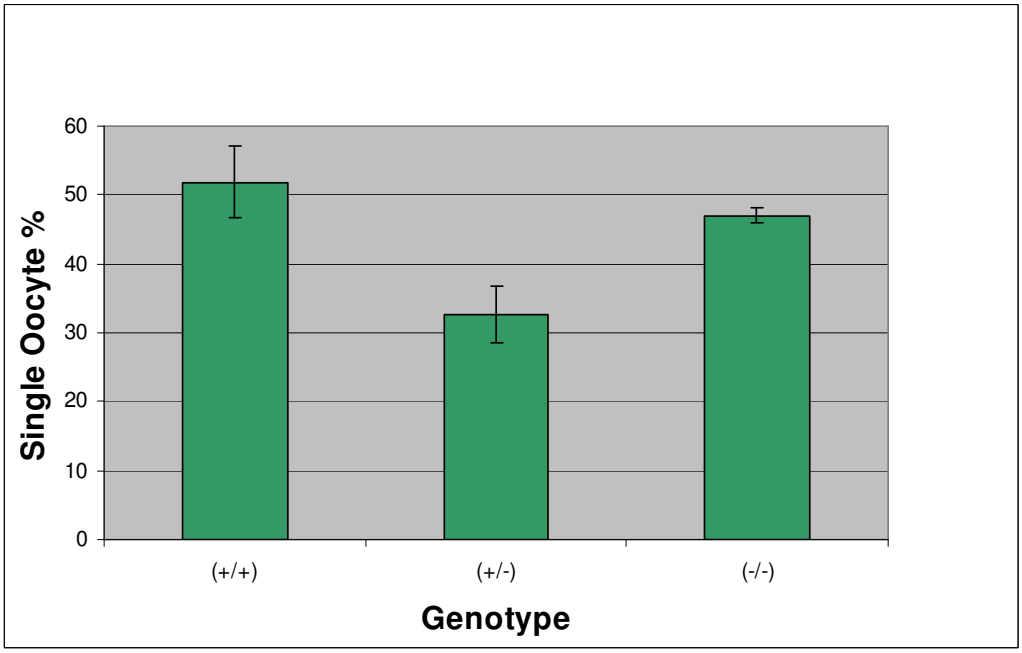

Figure 10. Cyst Breakdown at PND4 ERβ$^{+/+}$, ERβ$^{+/-}$, and ERβ$^{-/-}$ mice. Data are presented as the mean ± SEM. ERβ mutants exhibit no significant difference of single oocyte percentage. (P-value > 0.05) N= 32-58 sections in 5-8 ovaries.

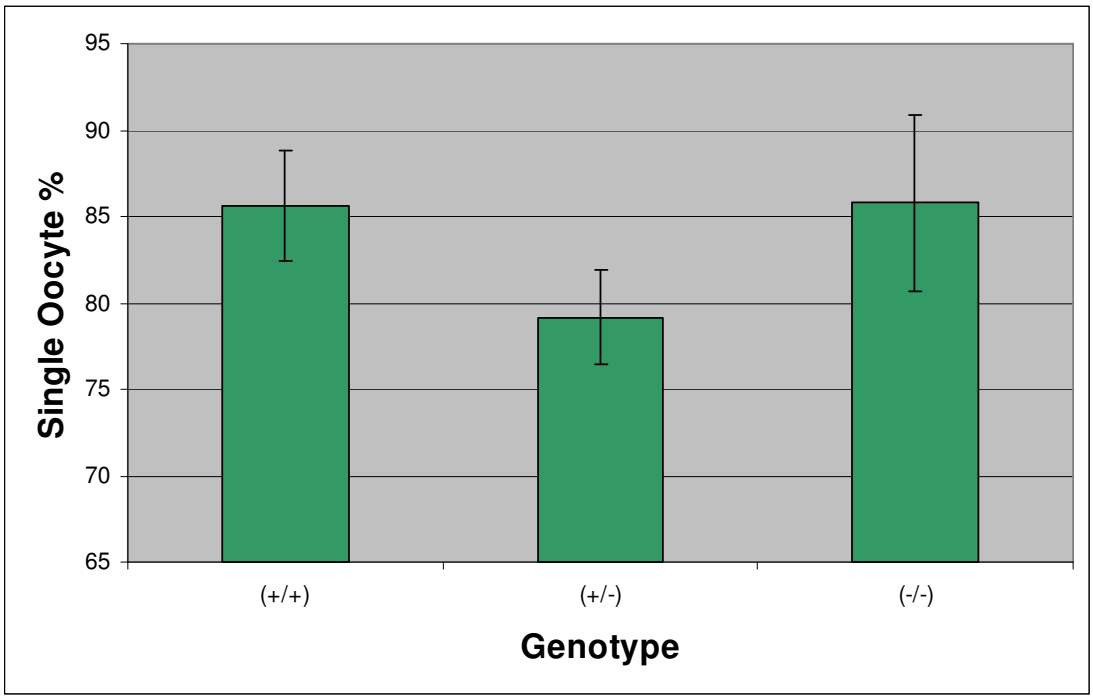

Figure 11. Cyst Breakdown at PND7 ERβ$^{+/+}$, ERβ$^{+/-}$, and ERβ$^{-/-}$ mice. Data are presented as the mean ± SEM. ERβ mutants exhibit no significant difference of single oocyte percentage. (P-value > 0.05) N= 24-93 sections in 3-10 ovaries.

Total Oocyte Numbers in ERβ Mutants

To determine if ERβ played a role in oocyte survival, the total number of oocytes per confocal section was determined for ERβ$^{+/+}$, ERβ$^{+/-}$, and ERβ$^{-/-}$at PND1, PND4, and PND7. At PND1, average number total oocytes for all three genotypes was higher than PND4 and PND7 (Figure 12). PND7 had the lowest average total number of oocytes, indicating a decline in oocyte number throughout the seven day growth period. The difference in oocyte number

between the genotypes was not significantly different for PND1, PND4, or PND7. Thus, the ERβ mutant mice did not suffer a significant difference in oocyte loss.

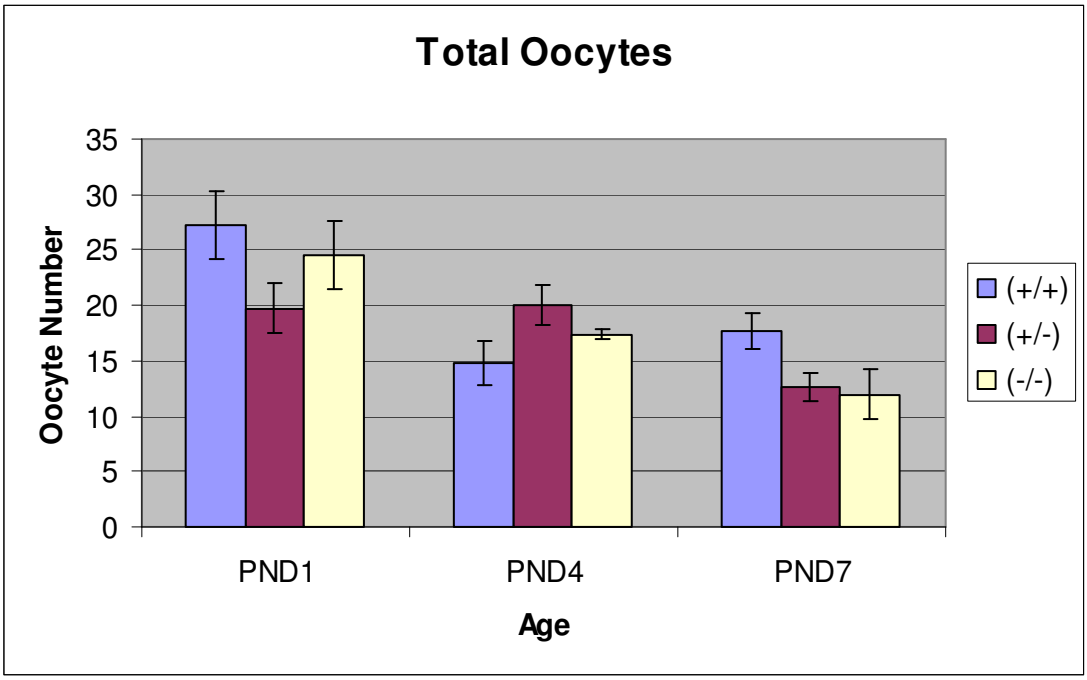

Figure 12. Total Number of Oocytes at PND1, PND4, and PND7 in $ER\beta^{+/+}$, $ER\beta^{+/-}$, and $ER\beta^{-/-}$ mice. Data are presented as the mean ± SEM. Knocking out ERβ did not significantly effect oocyte loss. (P-values >0.05) N = 22-93 in 3-13 ovaries.

Follicle Development in ERβ Mutants

To see if ERβ was important for development of follicles, oocytes were classified as within primordial, primary, or secondary follicles for each confocal section (Figure 13). At PND1, most oocytes in follicles were in primordial follicles, and there was no significant difference for PND1 across genotypes (P-value >0.05) (Figure 14). Both PND4 and PND7 exhibited significant differences in primordial and primary development across genotypes.

PND4 $ER\beta^{-/-}$ animals exhibited significantly more primordial follicles than $ER\beta^{+/+}$ and $ER\beta^{+/-}$ animals. $ER\beta^{+/+}$ mice had the most primordial oocytes while $ER\beta^{+/-}$ had the fewest. $ER\beta^{-/-}$ mice displayed an intermediate effect.

PND7 $ER\beta^{+/+}$ had the most oocytes in primordial stage, while the $ER\beta^{+/-}$ and $ER\beta^{-/-}$ were grouped together and exhibited significantly fewer primordial oocytes (Figures 15 and 16). These differences are currently under investigation.

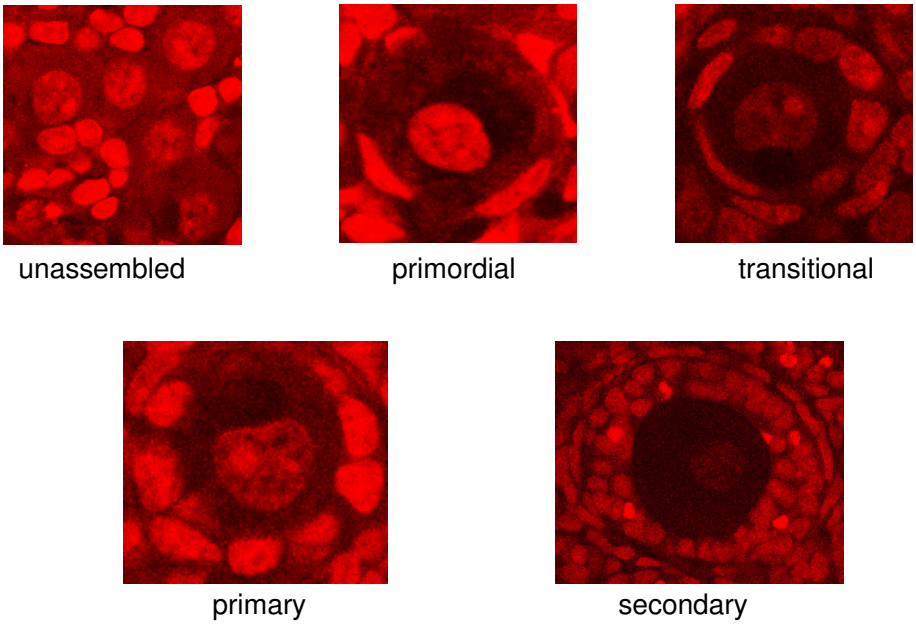

Figure 13. Stages of Follicle Development. Oocytes with connecting cytoplasm are classified as unassembled (cysts). Primordial follicles consist of one oocyte surrounded by a layer of granulosa cells and a layer of thecal cells. An increase in oocyte volume and the number of surrounding granulosa cells designate primary follicles. The shape of the granulosa cells is altered; the nuclei become cuboidal. The primary follicle then becomes a secondary follicle which is surrounded by more than one layer of granulosa cells.

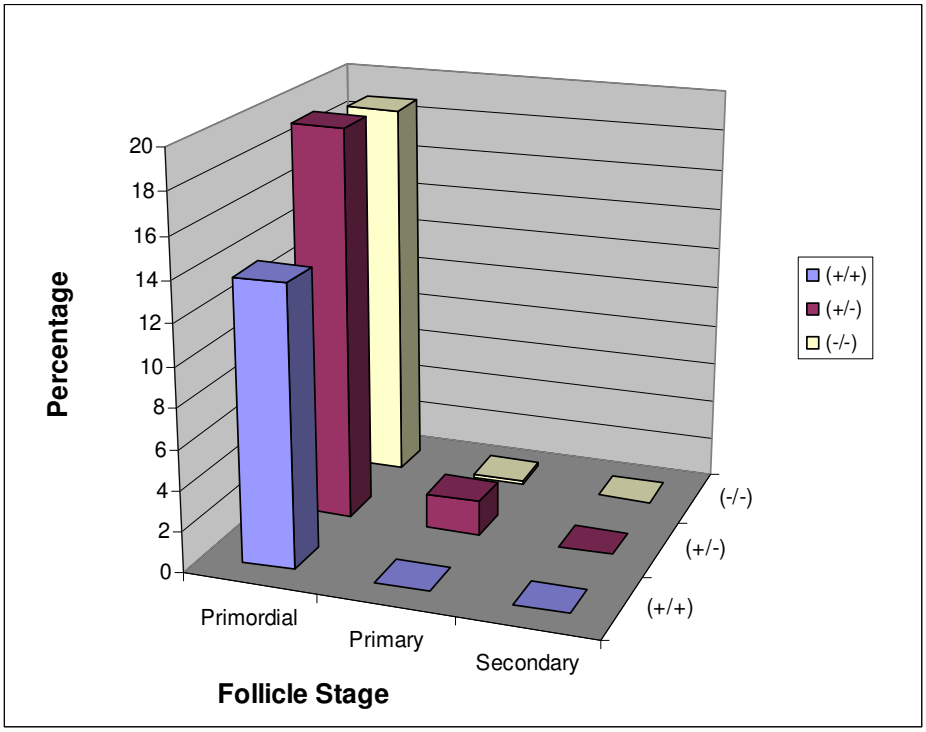

Figure 14. Follicle Development at PND1 in ERβ$^{+/+}$, ERβ$^{+/-}$, and ERβ$^{-/-}$ mice. Data are presented as the mean. There was no significant difference across genotypes (P-value > 0.05) N= 22-30 sections in 7-13 ovaries.

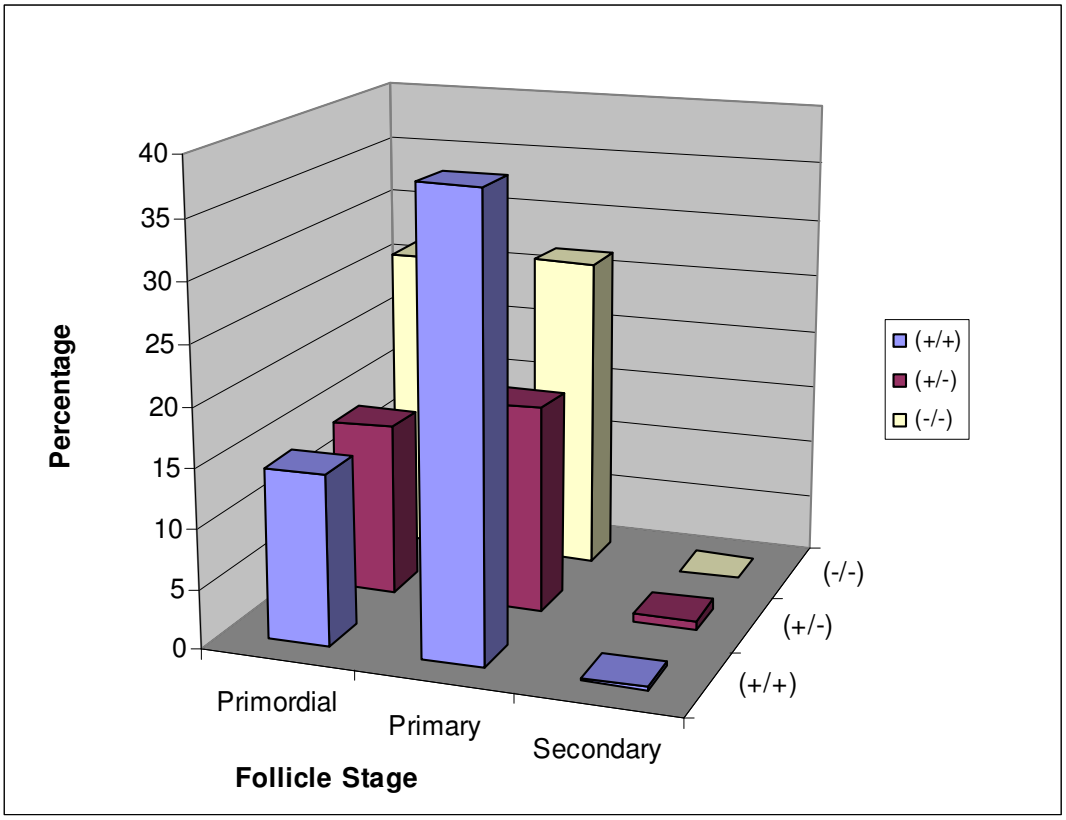

Figure 15. Follicle Development at PND4 in ERβ$^{+/+}$, ERβ$^{+/-}$, and ERβ$^{-/-}$ mice. Data are presented as the mean. Significant differences between percentage of follicles in primordial and primary stages were seen between the three genotypes (one way ANOVA, $p < 0.05$). N= 32-58 sections in 5-8 ovaries.

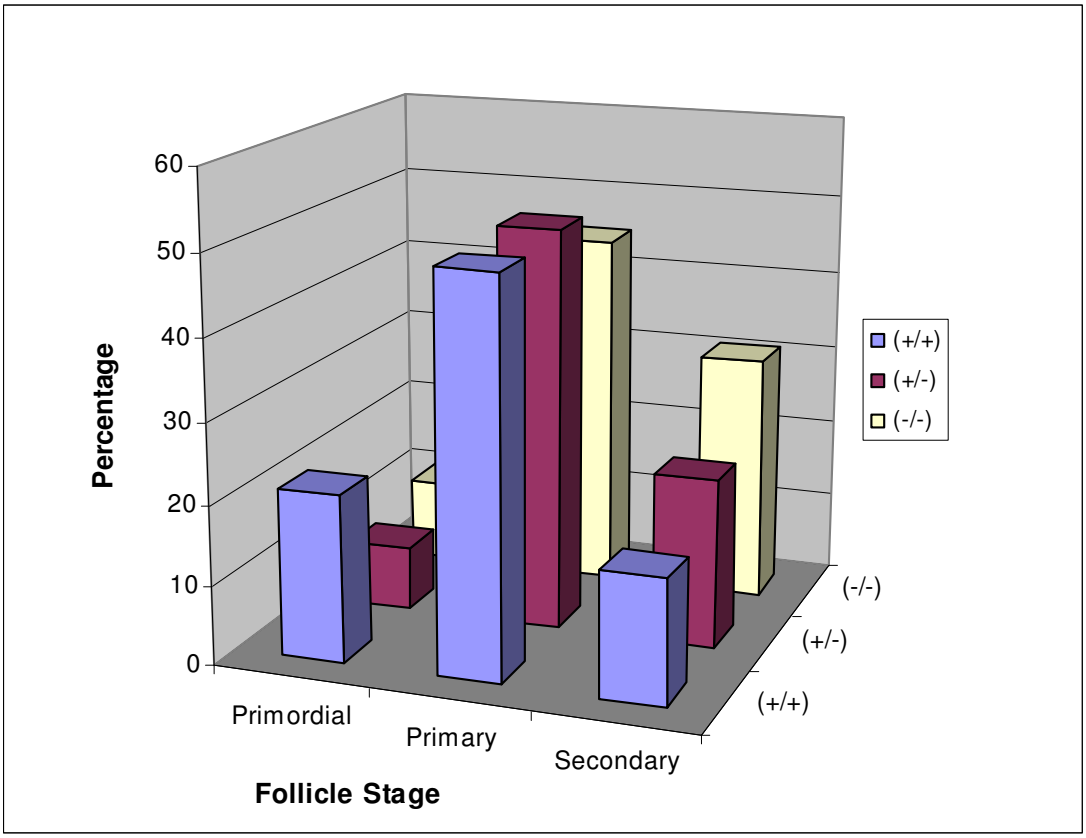

Figure 16. Follicle Development at PND7 in ERβ$^{+/+}$, ERβ$^{+/-}$, and ERβ$^{-/-}$ mice. Data are presented as the mean. Significant differences between percentages of follicles in primordial stages were seen between the three genotypes (one way ANOVA, $p < 0.05$). N= 24-93 sections in 3-13 ovaries.

Testing efficiency ERβ Antibodies

To test the efficiency of the ERβ antibodies, dot blots were performed which used several dilutions of ERβ control protein in 1X PBS. Results from ERβ H150 antibody indicated that the antibody is effective when ERβ control was used at 1:50 or 1:500 dilutions. ERβ H150 was used at a dilution in blocking buffer of 1:200 (Figure17). Results from ERβ Y19 antibody illustrated its effectiveness when ERβ control was used at 1:50 dilution. ERβ Y19 was used at a dilution of 1:100 (Figure 18). Results from ERβ 311 antibody indicated that the antibody is effective when ERβ control was used at a 1:50 dilution. ERβ 311 was used at a dilution of 1:500 (Figure 19).

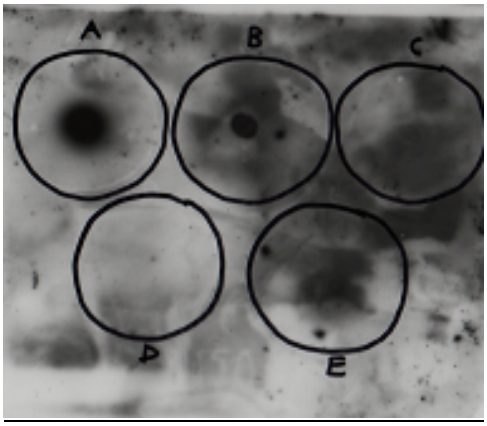

Figure 17. Detection of ERβ Protein Using ERβ H150 Antibody. Primary antibody was used at a 1:200 dilution to detect ERβ Long protein. ERβ L was used at a 1:50 dilution in 1X PBS in circle A, 1:500 dilution in circle B, 1:5,000 in circle C, 1:50,000 in circle D and 1:500,000 in circle E.

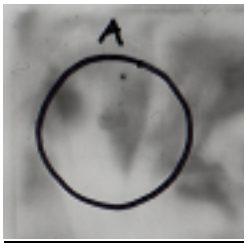

Figure 18. Detection of ERβ Protein Using ERβ Y19 Antibody. Primary antibody was used at a 1:100 dilution to detect ERβ Long protein. ERβ Lwas used at a 1:50 dilution in 1X PBS in circle A.

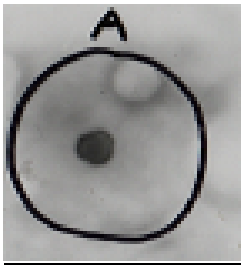

Figure 19. Detection of ERβ Protein Using ERβ 311 Antibody. Primary antibody was used at a 1:500 dilution to detect ERβ Long protein. ERβL was used at a 1:50 dilution in 1X PBS in circle A.

Expression of ERβ in Neonatal Mouse Ovaries

Western blots were performed to detect the ERβ protein in ovaries and testes at several different ages (Figure 20). Human ERβ protein was used as a positive control. A band at 67 kd corresponding to the human ERβ protein was detected. Results from the Western Blot indicate a band at 30kd for PND83 female, one band at approximately 30kd and one band at approximately 23kd for PND1 male. The PND4 and PND53 males had a large, blurred band which could potentially be unseparated doublet bands at approximately 30kd. The PND53 male also had a second, lower band at approximately 18kd. No band was detected at PND1 in females.

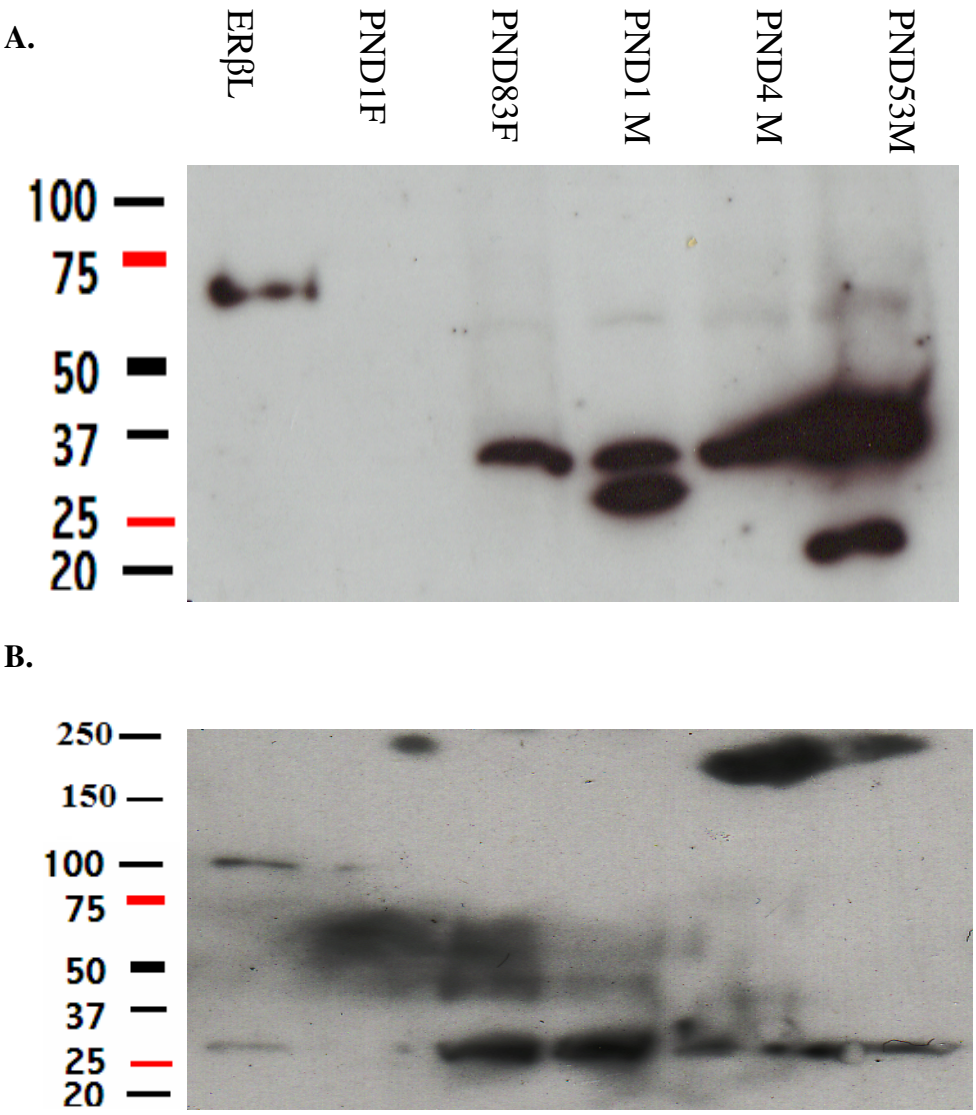

Figure 20. Western Blot Analysis of ERβ Protein. **A.** The control protein used was ERβ Long which is human purified ERβ. Mouse tissue extracts from PND1 and PND83 ovaries and PND1, PND4, and PND53 testes were probed with the ERβ 311 antibody. **B**. Blots were reprobed with GAPDH as a loading control.

Subsequent western blots were run using the same tissue samples. Two gels were probed with two different antibodies (ERβ 311 and an ERα specific antibody). As previously observed, similar protein degradation occurred in the ERβ western blot, while the ERα protein ran at the expected band size (66 kD) (Figures 21 and 22).

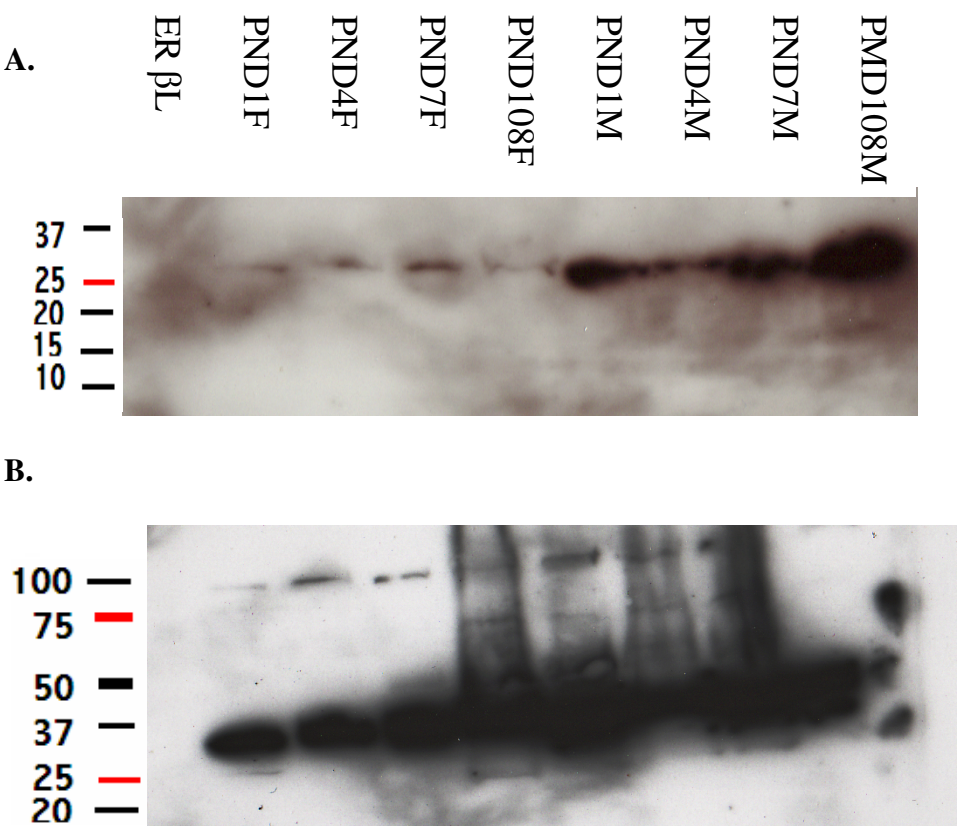

Figure 21. Western Blot Analysis of ERβ Protein. **A.** The control protein used was ERβ Long which is human purified ERβ. Mouse tissue extracts from PND1, PND4, PND7, and PND108 ovaries and PND1, PND4, PND7, and PND108 testes were probed with the ERβ 311 antibody. **B.** Blots were reprobed with GAPDH as a loading control.

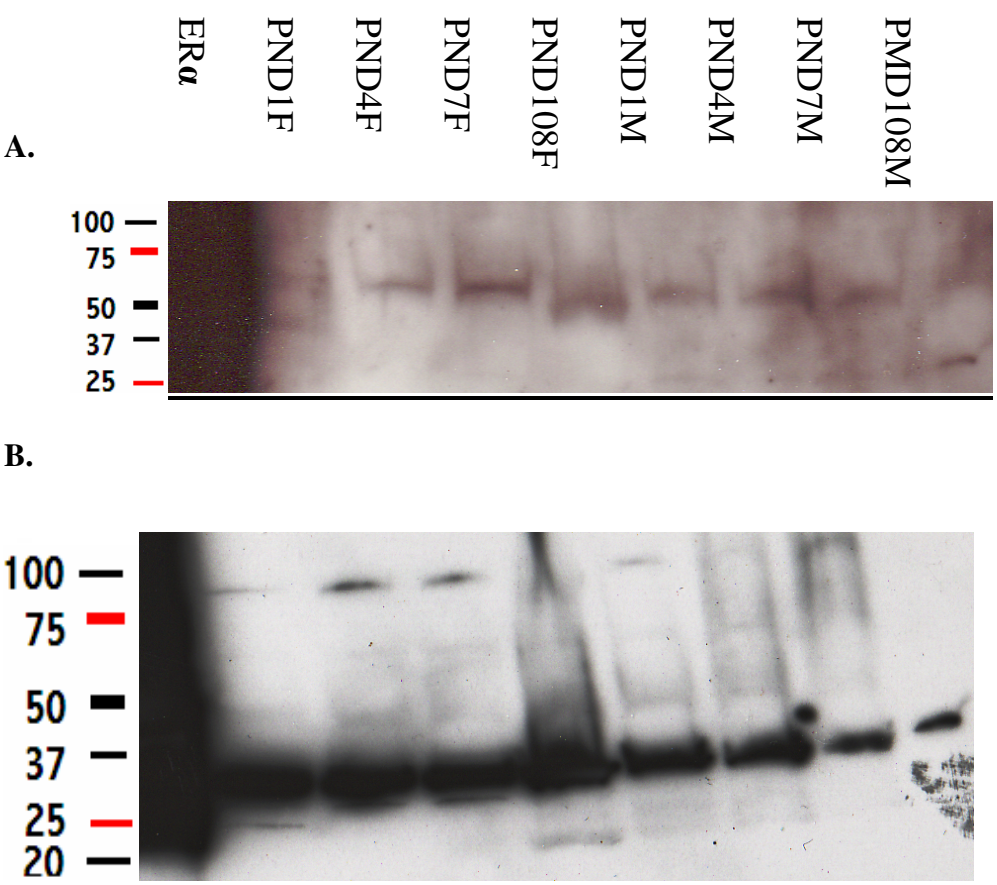

Figure 22. Western Blot Analysis of ERα Protein. **A.** The control protein used was human purified ERα. Mouse tissue extracts from PND1, PND4, PND7, and PND108 ovaries and PND1, PND4, PND7, and PND108 testes were probed with the ERα H184 antibody. **B.** Blots were reprobed with GAPDH as a loading control.

DISCUSSION

The processes important for germ cell development are unknown. The steroid hormone estrogen has been implicated in playing a role in the neonatal ovary throughout this time period (Chen et al., in press). This thesis research involved four main objectives: to determine the expression of ERβ in tissue samples, and to examine ERβ homozygous mutant mice for defects in neonatal cyst breakdown, oocyte survival, and primordial follicle formation at PND1, PND4, and PND7.

ERβ protein was detected in neonatal ovaries using Western Blotting. The expected size of ERβ is 67 kD. Instead a smaller band at approximately 30 kD was detected suggesting degradation of the ERβ protein. Possible explanations for degraded proteins include improper exposure of the tissue samples to heat or adding an insufficient amount of protease inhibitors. To determine if the inaccurate results were due to technical problems with the tissue samples or with the antibody, additional western blots were performed. Two protein gels were run simultaneously using extracts from the same tissue eamples and probed with two different antibodies (ERβ 311 and an antibody specific for ERα). Degraded ERβ proteins at approximately 30 kD were again detected while ERα proteins ran at the expected size of 66 kD. These results indicate that the problem does not arise from the tissue samples. A purified human ERβ protein was used as a control. The control ran at the expected size of the protein (67 kD). Thus, the antibody was effective in adhering to the control protein. This was evident in the results of

the dot blot, which was performed to test the effectiveness of three antibodies: ERβ Y19, ERβ H150, and ERβ 311. Both ERβ H150 and ERβ 311 detected the protein, especially at the concentrated 1:50 ERβ control protein dilution.

Since the tissue samples were undamaged and the antibodies proved effective, there must be a problem with the specific interaction of ERβ antibodies with the protein. Perhaps it is a highly sensitive interaction and only adheres in certain conditions. Additionally, the ERβ protein could be extra sensitive to degradation which results in specifically sized degraded bands. Further analysis of ERβ will help better understand its properties, and successful western blots demonstrating ERβ expression in tissue samples can be successfully performed.

When the total number of oocytes per confocal section was examined, an overall loss of oocytes was observed across all three genotypes. These results indicate that germ cell loss is a regulated process which occurs in $ER\beta^{+/+}$, $ER\beta^{+/-}$, and $ER\beta^{-/-}$ as growth occurs. The total oocyte number in $ER\beta^{-/-}$ and $ER\beta^{+/-}$ animals at each age showed no significant difference from $ER\beta^{+/+}$, therefore the loss of ERβ does not affect total oocyte number.

Examination of the single oocyte percentage showed that PND7 ovaries had significantly higher percentages of single oocytes as compared to PND4 and PND1. These results confirm that cyst breakdown did occur, and by PND7, most of the oocytes were single. A significant difference was not detected in $ER\beta^{+/-}$ or $ER\beta^{-/-}$ animals at each age, therefore the loss of ERβ did not affect cyst breakdown.

Neither the total oocyte number nor the single oocyte percentages were affected in ERβ mutant mice. The original hypothesis was that cyst breakdown would be accelerated in ERβ mutants. This theory was based on the genistein and estradiol experiments. When mice were treated with excess amounts of estrogen, cyst breakdown was inhibited (Chen et al., in press; Jefferson et al., 2005). The estrogen signal somehow prevented or delayed oocyte maturation. If the receptor which receives that signal is no longer available, it was hypothesized that initiation of cyst breakdown would occur sooner. These results were not obtained, and there was in fact no significant difference in total oocyte number or single oocyte percentages between $ER\beta^{+/+}$ and $ER\beta^{-/-}$. Thus, cyst breakdown and germ cell death were not affected by the loss of ERβ. A new hypothesis arose that attempts to explain this observation. Since both ERα and ERβ are present in the ovary, perhaps the cells that are defective for ERβ compensate by utilizing ERα. Therefore, the signal is still transduced.

When the $ER\beta^{+/-}$ mice were analyzed for single oocyte percentage, both PND4 and PND7 mice exhibited a decrease in single oocytes as compared to the $ER\beta^{+/+}$ or $ER\beta^{-/-}$. Perhaps with only one copy of ERβ, this receptor is still used and an alternate receptor is not yet utilized. We termed this the semi-defective theory. But when ERβ is completely removed, the ovary utilizes ERα and the wildtype phenotype is rescued. This semi-defective theory is not completely supported by experimental data. PND1 mice did not show a decrease in heterozygote single oocytes. Perhaps this is because at day one after birth, cyst breakdown has yet to officially begin. It is under debate as to when the cysts

begin to break apart. Some data support single oocytes entering follicle development before birth (Pepling M., unpublished). Further analysis is necessary to prove or disprove the semi-defective theory.

Analysis of follicle development at PND1 mice demonstrated no significant difference in wildtype versus ERβ homozygous mutant mice. Most of the oocytes at this age were still in cysts. Oocytes that were selected to mature after cyst breakdown are just beginning to develop at PND1. Therefore, approximately 15-20% of the total oocytes are in primordial stage for all three genotypes. Follicle development at PND4 exhibited significant difference in the primordial and primary oocyte numbers. While ERβ wildtype and heterozygote had almost the exact same percentage of primordial oocytes, ERβ mutant mice had significantly more. These results could indicate that follicle development in the mutant mice was accelerated so that primordial oocyte formation began earlier. $ER\beta^{-/-}$ had a different effect on percentages of primary oocytes, however. $ER\beta^{+/+}$ mice had the most primordial oocytes while $ER\beta^{+/-}$ had the fewest. $ER\beta^{-/-}$ mice displayed an intermediate effect. These data support the semi-defective theory in which the heterozygotes suffered while the homozygous mutants regained the wildtype phenotype. The significant decline in PND4 heterozygote primary oocytes could imply a delay in oocyte development, but these results were not cohesive across other ages or developmental stages.

PND7 follicle development showed a significant difference in primordial development between $ER\beta^{+/+}$, $ER\beta^{+/-}$, and $ER\beta^{-/-}$ mice. The wildtype mice again had the most oocytes in primordial stage, while the heterozygotes and

homozygous mutants were grouped together and exhibited significantly fewer. Perhaps more oocytes were accelerated to primary and secondary development in the $ER\beta^{+/-}$ and $ER\beta^{-/-}$ animals.

No definite trend resulted from the follicle development data and therefore it is difficult to draw significant conclusions. Several factors may have contributed to the inconsistent follicle development data. Difficulties in determining the stage of oocyte development arose. It is often unclear as to what stage an oocyte may be in. The decision is somewhat subjective in that the researcher must choose one stage or the other if an oocyte is transitioning from one stage to the next. The establishment of a universal counting mechanism would help clarify these discrepancies.

Several other issues contributed to problems in data collection. Although multiple ovaries were obtained for heterozygote and wildtype genotypes, there was a lack of obtainable mutant ovaries for all three ages. There was difficulty collecting data from PND1 ovaries for all three genotypes. Usually only one core of four images could be taken per ovary due to their small size. Also the staining left many oocytes indistinguishable, and it was difficult to ascertain their developmental stage. To obtain more accurate results, more ovaries would need to be collected.

Cyst breakdown and germ cell death are regulated developmental processes that occur in all mammals, including humans (Pepling, 2006). By studying these processes in mice, we hope to better understand the unknown underlying mechanisms. The total number of available oocytes for an

individual's lifetime is determined at birth. Problems involved in human fertility will hopefully be better understood and potentially cured by studying the signal pathways that are involved. Estrogen signaling plays a major role in the development of germ cells. Further research involving estrogen signaling is essential and crucial for understanding germ cell development.

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