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The Role of Cell Adhesion Molecules in Cyst Breakdown and Follicle Formation in the Mouse Neonatal Ovary

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The Role of Cell Adhesion Molecules in Cyst Breakdown and Follicle Formation in the Mouse Neonatal Ovary

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Abstract

The total number of oocytes that an individual will have available for reproduction is defined at birth. This established oocyte pool is essential to fertility, as a female loses oocytes throughout her life and never makes more. In the mouse, germ cells form in the fetus and migrate to the gonad, where they undergo mitosis and are referred to as oogonia. These cells are in germ line cysts, which are clusters of germ cells connected by intercellular bridges that form by incomplete cytokinesis. Germ cells in the human also develop in clusters with characteristics of cysts. The cells within the cysts soon begin to enter meiosis, and are now referred to as oocytes. Around post natal day (PND) 2, the cysts undergo a process called cyst breakdown, in which the oocytes break apart and become surrounded individually by granulosa cells. The mechanism of cyst breakdown is currently under investigation, as cysts that fail to properly break down often result in multiple oocyte follicles, which develop abnormally. The regulation of cell adhesion molecules (CAMs) likely plays a role in the process of cyst breakdown, and a hamster model has found two specific CAMs expressed in the ovary during egg cell development, E-Cadherin and N-Cadherin. The presence and location of these CAMs, as well as their role in cyst breakdown and follicle formation in the mouse, is being investigated in this study.

Three different experimental techniques were used in order to determine the role of E-Cadherin and N-Cadherin in cyst breakdown and follicle development in the mouse. First, immunicytochemistry was used in order to visualize the proteins in the mouse ovary. Then, organ culture experiments were performed in order to block these proteins during PND1-5 to determine their function. Finally, western blots were performed in order to determine protein expression levels of E-Cadherin and N-Cadherin during development.

From these experiments, it can be concluded that both E-Cadherin and N-Cadherin are expressed during development from 17.5 days post coitum (dpc) to PND5. E-Cadherin expression is higher in the cytoplasm of oocytes than in the granulosa cells. Starting at PND2, E-Cadherin is also expressed in the cell membrane of the oocytes, and is localized to certain spots within the membrane. The location of N-Cadherin expression within the ovary was unable to be determined from the immunocytochemistry experiments. Overall, N-Cadherin expression is higher in the neonatal ovary than E-Cadherin expression. Also, E-Cadherin expression increases from PND1 to PND3 and N-Cadherin expression increases from 17.5dpc to PND5.

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Introduction

An organism is said to be biologically successful if it can reproduce and produce viable offspring. The process of egg cell development, called oogenesis, is necessary for a female to reproduce. As a result, defects in this process may interfere with the proper development of a female's eggs and cause her to be infertile, or unable to conceive. Close to seven million women in the U.S. have an impaired ability to conceive (CDC, 2012), which can cause emotional and psychological impacts such as depression and anxiety. While there are several different potential causes of infertility, defects in oogenesis during development likely cause infertility in some cases. Understanding how egg cell development works, as well as factors that influence the process of egg cell development, can help to prevent and/or treat cases of infertility. While various aspects of oogenesis have been thoroughly studied, there are several mechanisms of this developmental process that are still poorly understood.

The total number of oocytes that an individual will have available for reproduction is defined at birth (Pepling, 2006). These egg cells are essential to fertility, as a female loses oocytes throughout her life and never makes more. In the mouse, which has a very similar genome to the human, primordial germ cells form in the embryo and migrate to the genital ridge, where they undergo mitosis and are referred to as oogonia. Because these cell divisions undergo incomplete cytokinesis, all cells remain connected by intercellular bridges. These clusters of oocytes are known as germ line cysts (Pepling and Spradling, 1998). The cells within the cysts divide by mitosis until about 13.5 days post coitum (dpc), when

they enter meiosis. At this time, the cells are referred to as oocytes, and they arrest from cell division when they reach the diplotene stage of prophase I.

The timeline and mechanisms of oogenesis have also been extensively researched in *Drosophila*. Germ cell cysts in *Drosophila* contain sixteen interconnected cells that form as a result of incomplete cell divisions as well as the formation of intercellular bridges known in the fly as ring canals in between all of the cells in the cyst (Pepling *et al.*, 1999). One germ cell in the cluster will go on to become the oocyte, while the rest are called nurse cells and transport mitochondria and mRNA to the developing oocyte via microtubules that pass through the ring canals (deCuevas *et al.* 1997). It is speculated that a similar process occurs in the mouse, because endoplasmic reticulum and mitochondria were identified in the intercellular bridges of cysts in the mouse ovary (Pepling and Spradling, 2001).

There has been speculation as to the purpose of cyst formation in egg cell development, as cysts have been conserved through evolution in both invertebrates and vertebrates, including additional organisms to the mouse and *Drosophila* (Pepling *et al*., 1999). Perhaps cysts provide the benefit of synchronizing development of a small group of germ line cells (Pepling and Spradling, 1998). The cyst also provides proximity to allow the cells to use certain gene products from their neighboring cells. Another possible reason for cyst formation is to allow for early specialization, as certain cells within the cyst may acquire necessary materials from other cells, which may later enter apoptosis (Pepling and Spradling, 1998).

Starting around 17.5 days post coitum (dpc), the cysts begin to undergo a process called cyst breakdown, in which the oocytes within the cysts break apart and become surrounded individually by granulosa cells (Pepling & Spradling, 2001; Pepling *et al*., 2010). This breakdown process is thought to occur as a result of apoptosis of a few cells within the cyst, causing the cyst to break apart (Figure 1).

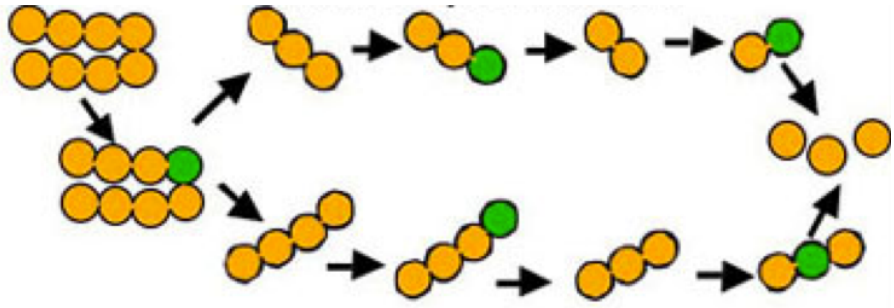

Figure 1. Model for Cyst Breakdown. Surviving oocytes are shown in yellow, dying oocytes are shown in green. Adapted from Pepling and Spradling, 2001.

In fact, a correlation has been found between germ cell loss and cyst break down (Figure 2), and also between germ cell loss and apoptosis (Pepling and Spradling,

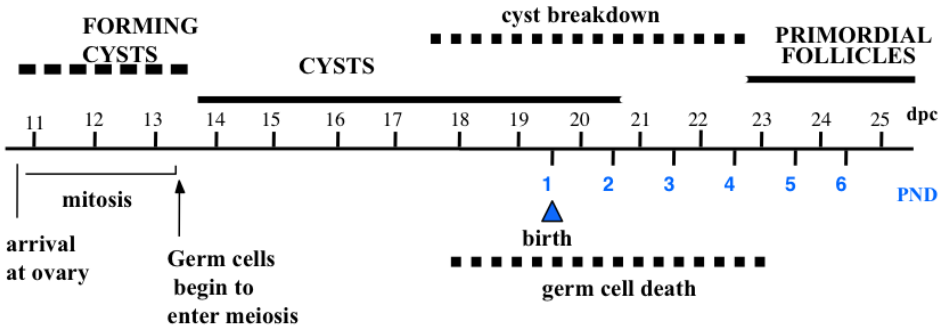

Figure 2. Timeline for Germ Cell Development in the Mouse. Adapted from Pepling, 2006.

2001). The timing of primordial follicle formation was also found to coincide with these processes. In mice, the stepwise cyst breakdown process occurs most substantially from postnatal day (PND) 2 to PND4, but this process is not 100% complete by PND4. In fact, small cysts of two to four cells were found in the ovaries of mice as old as PND8. It is evident that cyst breakdown is a programmed step in oogenesis. And while many aspects of cyst breakdown have been extensively studied, there are several mechanisms of the process that are not yet completely understood.

Once a single oocyte leaves the cyst, it becomes surrounded by granulosa cells, and this whole group of cells is referred to as a primordial follicle. The primordial follicle develops into a primary, secondary, and tertiary follicle, which are distinguished based on changes in the shape and number of layers of granulosa cells surrounding the oocyte in the follicle (Matzuk *et al*., 2002). In a primordial follicle, the granulosa cells surrounding the oocyte are flat in shape. In a primary follicle, the granulosa cells have taken on a cuboidal shape. When multiple layers of cuboidal-shaped granulosa cells surround the oocyte, it is known as a secondary follicle, and the oocyte itself has grown in size. Additional stages of development prepare the mature follicle to be released during ovulation, when the time comes.

In a variety of mammals, a decrease in germ cell number at a point in development has been evidenced. In the human, about seven million oocytes form during initial development, but by birth, only about two million have survived (Baker, 1963). In the mouse, the oocyte number has been found to drop

from about 6,000 to about 2,000 over a two-day period during development. While this germ cell loss has been shown to correlate with apoptosis resulting in cyst breakdown in the mouse, there have been additional hypotheses attempting to explain the decrease in germ cell number that has been found in other mammals as well. Perhaps the surviving oocyte depends on the other germ cells in the cyst to transport materials via microtubules that are necessary for survival and viability (Pepling and Spradling, 1998). In *Drosophila,* determination of the oocyte within the cyst occurs because the surrounding cells, called nurse cells, transport materials to that one surviving oocyte (Pepling and Spradling, 2001). Another hypothesis suggests that the decrease in germ cell number is necessary to eliminate germ cells containing chromosome abnormalities and to ensure that all cells have high quality mitochondria.

Failure of a cyst to properly breakdown can lead to multiple oocyte follicles, which develop abnormally and may lead to infertility. While a normal, viable follicle consists of a single oocyte surrounded by several granulosa cells (Figure 3 left), a multiple oocyte follicle consists of more than one oocyte within one follicle (Figure 3 right), both or all oocytes surrounded by several granulosa cells (Pepling, 2006). The oocytes in multiple oocyte follicles may develop with abnormal shape and size, and are not viable for reproduction.

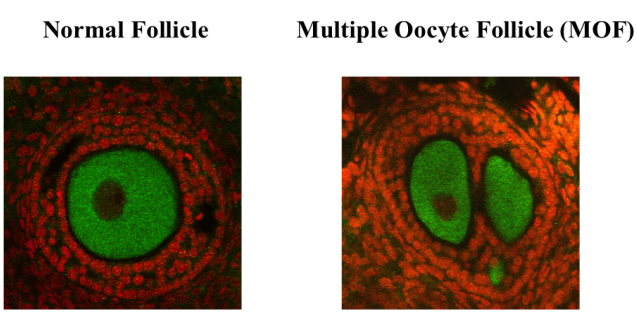

Figure 3. Normal (left) and Abnormal (right) Follicle Formation in the Mouse. All nuclei stained red with propidium iodide, cytoplasm of oocytes stained green with oocyte marker, STAT3. Adapted from Pepling, 2006.

Among studies on cyst formation and breakdown, hormones have been found to influence these processes. More specifically, steroid hormones have been found to play a role in the process of cyst breakdown to form individual single oocyte follicles (Chen *et al.*, 2007). Multiple oocyte follicles were present in the adult ovaries of mice that were treated with estrogens as neonates (Jefferson *et al*., 2002). In addition, estradiol, progesterone, and genistein were found to inhibit cyst breakdown and primordial follicle formation, but treatment with these hormones had no effect on oocyte number (Chen *et al.*, 2007).

Since cyst formation and break down play such important roles in the production of viable egg cells, it is important to understand all mechanisms involved in these processes in order to better understand the molecular basis of infertility. It is understood that cysts form as a result of cell division without cytokinesis, and that the cells within the cysts are connected by intercellular bridges. But researchers have found an additional player that may help hold together the cells within the cysts: cell adhesion molecules (CAMs). CAMs are proteins that play a role in cell adhesion. When these proteins are expressed, they migrate to the cell membrane where they anchor so that they can bind to a CAM on another cell, holding the cells together. Cell adhesion molecules play a large role in organ formation during embryogenesis (Cell Adhesion Molecules, 1997). They are made of an intracellular domain, which attaches to the cytoskeleton inside the cell with the help of other proteins, a transmembrane domain, which sits within the membrane, and an extracellular domain, which acts as a receptor for other CAMs to bind to in order to adhere the two cells together. Cytoplasmic

actin and intermediate filaments play a role in cell adhesion by providing the CAMs with a base to anchor onto in addition to the membrane of the cell (http://www.vivo.colostate.edu/hbooks/cmb/cells/pmemb/adhesion.html).

There are many different types of CAMs, but the specific CAMs that I have focused on in my project are cadherins, which are calcium-binding cell adhesion molecules. Cadherins depend on calcium to function, as they contain calcium-binding sites in their extracellular domain. Cadherins have been found in a variety of organisms throughout evolution, including vertebrates, insects, nematodes, and a few unicellular organisms (Halbleib and Nelson, 2006). Over 100 members of the cadherin family have been identified, all containing the characteristic extracellular cadherin repeats. Cadherins are important in a wide variety of biological functions within organisms, and therefore their absence or defective expression can be correlated with a variety of diseases. A hamster model has suggested the presence of two specific cadherins, E-Cadherin and N-Cadherin, in egg cell development. The role of these two cadherins in mouse oogenesis is the focus of my research project, as the cDNA and amino acid sequences for both E-Cadherin and N-Cadherin in the hamster are more than ninety percent similar to those in the mouse and the human (Roy and Wang, 2010).

E-Cadherin and N-Cadherin are categorized into a group called type 1 classical cadherins, and their full names are epithelial cadherin and neuronal cadherin, respectively (Halbleib and Nelson, 2006). The names of the genes for these proteins are *cdh1* (E-Cadherin) and *cdh2* (N-Cadherin). The extracellular

domains of these proteins contain five calcium binding extracellular cadherin repeats, which usually participate in homophilic adhesion, meaning they bind to other cadherins.

The current speculated model for the role of cell adhesion molecules in cyst formation and break down is that certain CAMs play a role in holding together the cells within cysts and that these CAMs must be down regulated in order for the cyst to break apart. In addition, it is thought that another set of CAMs, perhaps the same ones, play a role in holding together oocytes and granulosa cells in follicles. In the hamster, E-Cadherin was found in oocytes, and its expression was found to decrease coinciding with primordial follicle formation, suggesting a role in the maintenance of cells in cysts and the regulation of cyst breakdown (Roy and Wang, 2010). Results of neutralization experiments in the hamster lead to a conclusion that a decrease in E-Cadherin expression is necessary for cysts to break down. N-Cadherin mRNA expression was proportionately higher than E-Cadherin mRNA expression in the hamster. N-Cadherin expression was found in the oocytes as well, but expression moved to the granulosa cells adjacent to the oocytes as development progressed, suggesting a role in somatic cell congregation with the oocyte during cyst breakdown and follicle formation and growth. Further, neutralization of N-Cadherin lead to significantly reduced follicle formation. My research on these cadherins was performed in order to determine the specific roles of these proteins in mouse neonatal oocyte formation (Figure 4).

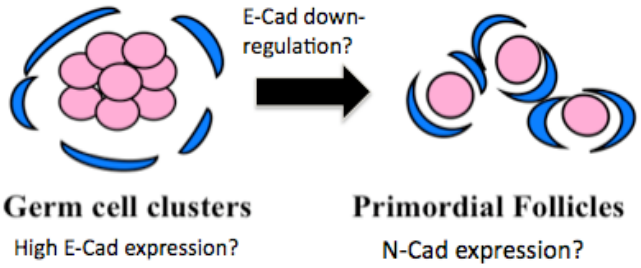

Figure 4. Model for the role of E-Cadherin and N-Cadherin in holding cells together in cysts, allowing cysts to breakdown, and holding cells together in follicles. Pink cells are oocytes, blue cells are granulosa cells. Adapted from Pepling, 2006.

E-Cadherin is thought to play a role in germ cell aggregation in the fetal mouse gonad, and was found to be expressed early in development at ages 11.5dpc, 12.5dpc, and 13.5dpc (Mork *et al*, 2012). My project researches the role of E-Cadherin in mouse neonatal oogenesis more extensively, and also looks at the role of N-Cadherin in this process in the mouse. I focus on the timeframe of cyst breakdown (about 17.5dpc to PND4) to determine if the two cadherins play a role in the breakdown of cysts to form follicles.

My first aim is to determine the presence and location of E-Cadherin and N-Cadherin in the mouse ovary during cyst breakdown. More specifically, to determine which cadherin(s) is/are expressed in oocytes in cysts and which is/are expressed in oocytes and granulosa cells during follicle formation. I am also looking at the location within the cell of the expression of these two proteins. My second aim is to determine the effects of blocking E-Cadherin and N-Cadherin on cyst breakdown. My third aim is to determine protein expression levels of E-Cadherin and N-Cadherin during cyst breakdown.

Materials and Methods

Animals

The mice used in all of these experiments were the C57BL/6 inbred strain, also known as the B6 strain. The mice were maintained by the animal resource facility at Syracuse University, with the help of the members of the Pepling lab. Each Monday after 2:00pm, up to two females were placed in a cage with one male for mating. Only females from the same litter were mated with the same male. Each morning prior 10:00am on Tuesday through Friday, the females were checked to determine if mating had occurred since the previous day. This was done by checking for the presence of a vaginal plug. If the plug was present, the female was separated from the male and the "plug date" was recorded. Litters were only used for experiments if the plug date was recorded. In other words, if a female that was not found to have a plug still ended up having a litter, the litter could not be used because the exact time of conception was unknown. The female then remained in her own cage during pregnancy, and 19.5dpc was marked as the day of birth, or PND1.

Dissection

Dissection of the ovaries from the neonatal or fetal mice was the first step in all experiments. The mice that were used ranged in age from 17.5dpc to PND5. The neonates were removed from the cage with their mother on the appropriate day for dissection. A Nikon SMZ1500 dissecting microscope was used to help locate and remove the ovaries. Dissection was done in a petri dish containing 1x phosphate buffered saline (PBS) solution, and the dissected ovaries were then

transferred to a 1.5mL ependorf tube containing room temperature fix, which is made of 400µL of PBS and 200µL of 16% formaldehyde. The ovaries remained incubating in the fix solution overnight on a nutator at 4°C.

For fetal dissection, the mother was first euthanized in a carbon dioxide chamber. The fetuses were then removed from her uterine horns and separated from the surrounding embryonic tissue. All other steps remained the same as the neonatal dissections.

For organ culture experiments, the dissections were done in Hank's Balanced Salt Solution instead of PBS. The dissected ovaries were transferred into wells filled with Hank's Balanced Salt Solution on ice instead of ependorf tubes filled with fix.

For western blot experiments, the ovaries were dissected in PBS and transferred to an ependorf tube filled in PBS on ice.

Whole Mount Antibody Staining of Fetal and Neonatal Mouse Ovaries

The staining procedure was done following a protocol developed by the Pepling lab based on protocols from the Spradling lab, N. Patel, H. Lin, B. Capel, and the CSH mouse course. After dissecting out the ovaries and incubating them in fix overnight, two quick washes followed by a 30 minute wash (on nutator) were done using 1mL PT (1X PBS/0.1% Triton X-100). The ovaries were then incubated in 1mL PT + 5% BSA (bovine serum albumin; made using 50mL PT and 2.5g BSA) for 30 minutes to one hour at room temperature or overnight at 4°C on the nutator. The next step was to incubate the ovaries with the primary antibody overnight at 4°C on the nutator. The primary antibody was first diluted

in 500µL PT + 5% BSA. The dilution factor depends on the specific antibody. When using STAT3 (oocyte marker) as the primary antibody, 1µL was used in 500µL PT + 5% BSA (1:500 dilution). When using N-Cadherin or E-Cadherin as the primary antibody**,** 5µL were used in 500µL PT + 5% BSA (1:100 dilution). The first antibodies purchased from BD Transduction Laboratories for N-Cadherin (610920) and E-Cadherin (610181) did not stain well, so new stainings were done using antibodies from Invitrogen. The monoclonal antibodies used from Invitrogen were mouse anti-N-Cadherin (333900) and rat anti-E-Cadherin (131900), so the fluorescent secondary antibodies used against these primary antibodies were anti-mouse alexa 488 (green) and anti-rat alexa 488 (green), respectively. These secondary antibodies from Molecular Probes were pre-absorbed overnight at 4°C on the nutator while the ovaries were incubating in the primary antibodies. The secondary antibodies (2.5µL) were pre-absorbed with a pinch of embryo powder in 500µL PT + 5% BSA. The tubes were wrapped in foil to prevent damage from light. The following day, the primary antibody solution was removed and the ovaries are washed in 1mL PT + 1% BSA (made using 50mL PT and 0.5g BSA) for 30 minutes at room temperature on the nutator. They were then incubated in RNase A (10µL of 10 mg/mL RNase A in 1mL PT + 1% BSA) for 30 minutes on the nutator. The next step was to incubate in propidium iodide (10µL of 0.5 mg/mL PI in 1mL PT + 1% BSA) for 20 minutes at room temperature on the nutator in the dark. From this point on, the tube was always covered in foil. Following another 30 minute wash in PT + 1% BSA, the ovaries were incubated in the pre-absorbed secondary antibody overnight at 4°C or for 2-

4 hours at room temperature on the nutator. The next day, three washes were done at room temperature on the nutator for 30 minutes each using 1mL PT + 1% BSA. For the final steps, the ovaries were washed with 1mL PBS and transferred to a slide, sitting in Vectashield under the cover slip. The slides were always labeled with sex, age, antibodies used, date and my initials. Nail polish was used to seal the cover slip, and the slides were put in the dark to dry for 10 minutes. They were then stored at -20°C.

Confocal

The slides were analyzed using the LSM 710 Confocal Microscope. This microscope, connected to a computer, uses a laser to take images of different layers of the ovary. The images from the microscope displayed on the computer show propidium iodide (red dye) staining all of the nuclei in the ovary. The images also light up green (because of the fluorescent secondary antibody) where the protein of interest is present in the cells. The 10x magnification was used to focus on the ovary, but the images were taken at 63x magnification using oil. The Zen program on the computer was used to capture the images, and they were then saved onto a disc for further analysis and use of the images. A special tool called Z-stack was used for organ culture analysis on the confocal, explained below.

Organ Culture

The organ culture experiments were performed in order to test the function of E-Cadherin and N-Cadherin in cyst breakdown and follicle formation. During all steps of the organ culture, the tools and surfaces used must be extremely sterile

in order to prevent contamination. When making solutions and during dissection, 70% ethanol was used to clean the lab bench, all tools, and gloved hands. All surfaces were also sprayed down in the organ culture room, in addition to the microscope and incubator (inside and out). A hood with UV light was also used to sterilize tools for 30 minutes prior to touching the organ culture plates, but the treatment media should not be exposed to the UV light, as it can damage the antibodies.

The first step in the organ culture was to make the stock media, which should be made no more than one day before the start of the culture. One component of the media was L-ascorbic acid, which was made by dissolving 0.0025g of 0.05mg/mL L-ascorbic acid in 10mL DMEM (pre-made, ordered from Invitrogen). The next component was 0.5mL of 10% Albumax stock solution, which was made my dissolving 1g of Albumax in 10mL of autoclaved Millipore water. The next three components in the stock media were 667µL of 7.5% BSA, 2.5mL of 100x ITS-X (insulin, sodium transferring, sodium selenite, ethanolamine), and 32.5µL of penicillin/streptomycin antibiotics. DMEM media was then used to bring the solution up to 50mL.

Once the stock media was made, the treatment media was made by transferring 5mL of the stock media to a 15mL tube and adding 50µL of the blocking antibody for a 1:100 dilution (either anti-E-Cadherin or anti-N-Cadherin). These blocking antibodies were purchased from Takara Bio Inc. (anti-E-Cadherin AC01520C and anti-N-Cadherin AA11505Z). All media was stored at 4ºC.

The next step was to prepare the incubator and plates for the organ culture. The incubator was set to 37ºC and 5% CO_2 at least three hours before the start of the culture. The inside and outside of the incubator were sprayed with alcohol, and all of the removable parts were placed under the hood and exposed to UV light for 60 minutes. All tools to be used in the organ culture (plates, filters, stock media, pipettes and tips, forceps, etc.) were placed under the UV light for sterilization as well. Once sterilized, the parts were returned to the incubator and the water pan was filled with autoclaved Millipore water (just enough for the entire surface of the pan to be covered in water). To prepare the organ culture plate, Millicell floating filters were first cut into quarters using a scalpel. Then, 400µL of control media were pipetted into the two left wells (labeled "C") and 400µL of treatment media were pipetted into the two right wells (labeled "E-Cad or N-Cad"). One cut filter was then placed on top of the media in each well, and the microscope was used to ensure the filters were floating. The plate was then placed into the incubator to warm up while the dissection occurred.

The dissection was done in the same way as for the stainings, with a few changes. The area was sterilized with alcohol and the ovaries were dissected in Hank's Balanced Salt Solution and transferred to wells filled with Hank's solution on ice. It is important to remove all tissue from around the ovaries so they do not stick to the forceps during transfer.

The ovaries were then transferred from the Hank's solution to the filters in the organ culture plate wells. Two pairs of forceps were used for the transfer. The first pair was used to scoop up the ovary out of the Hank's solution. The ovary

was then transferred to the second pair of forceps and the first pair was wiped until dry using a kimwipe. The ovary was then transferred back to the dry forceps and placed onto a filter in the plate. Finally, the forceps were used to place a small drop of media on top of each ovary. Between 2 and 4 ovaries could be placed on each filter (mine never had more than 3 each). The plate was then placed back into the incubator to sit overnight.

The following three days around the same time, the media was replaced by simply drawing out the old media and adding new media to the wells. On the fifth day, the ovaries were transferred from the plate to two different ependorf tubes, one for control ovaries and one for treatment ovaries, both filled with 600µL of fix.

The normal staining procedure was then followed. The ovaries were stained with STAT3, an oocyte marker, in order to be analyzed for differences in cyst breakdown and follicle formation, including total oocyte count, number of oocytes in cysts, and number of primordial, primary, and secondary follicles formed.

Organ Culture Analysis (Counting Oocytes)

The confocal microscope was used to obtain images of different layers of the ovary in order to determine if blocking E-Cadherin and N-Cadherin had an effect on oogenesis. The z-stack function on the microscope aided in taking images of different sections of the ovary. In each ovary, two cores were imaged from opposite ends of the ovary. Within each core, four sections were imaged, each going deeper into the ovary. The microscope then took images above and

below each of the four sections. This was helpful in determining whether or not oocytes in the section are in cysts, as they could be touching in one plane but not in another.

Once the images were taken, the number of oocytes in each section were counted. It was also documented whether each oocyte was in a cyst or a single oocyte, and whether the single oocytes were in primordial, primary, or secondary follicles. In order to distinguish between different follicle types, the shape and orientation of the granlosa cells surrounding the oocyte were used. Primordial oocytes contain granulosa cells that are just starting to form around the oocyte. As a result, they appear flat in shape and are not in a perfect circle around the oocyte; rather, there are spaces between neighboring granulosa cells. In primary follicles, the granulosa cells are cuboidal in shape and starting to move closer to one another. In secondary follciles, the granulosa cells form more than one layer around the oocyte.

This data was then used to calculate the total single oocytes, total oocytes in cysts, and total oocytes, in addition to percentages of primordial, primary, and secondary follicles, as well as percentage of single oocytes. Once caluculating the average number of oocytes and the average percentages of oocytes, graphs were made and standard error was used to compare control versus treatment for the significance of the results.

Western Blot

Following dissection, the 1xPBS was removed form the ependorf tube containing the ovaries. The ovaries were then homogenized in 1x sample buffer

plus protease inhibitors on ice, using 10µL per ovary. The 1x sample buffer was made using 500µL of 2x sample buffer (10% SDS, glycerol, 0.1% bromophenol blue, 0.5M Tris, and 2-mercaptoethanol), 100µL of 10x protease inhibitors (one tablet of mini complete protease in 1mL of dH_20), and 400µL of dH_20. Once the ovaries were crushed, they were stored at -20ºC until ready for the western blot.

To prepare the samples for the gel, 20µL of each sample were thawed and 2µL of 2-mercaptoethanol were added. The tubes were then boiled for 3 minutes and moved to the centrifuge machine on ice, where they were spun for 1 minute at 600rpm.

To set up the gel, the 10% BIORAD gel was removed from the plastic and inserted into the gel holder. The gel holder was inserted into the gel box and 1x running buffer (made of Tris base, glycine, and SDS) was used to fill the space in between the gels. Once it was confirmed that the gel holder was not leaking, the 1x running buffer was used to fill the gel box and the combs were removed from the gels. The samples were then loaded into the wells (20µL of sample, 10µL of precision plus standard protein marker). Any empty wells were loaded with 20µL of 2x sample buffer to prevent the sample from running sideways. The gel was the run at 100 volts for 1 hour.

The next step was to transfer the proteins from the gel to a membrane so that it could be visualized and imaged. The membrane was soaked in methanol, dH_20, and transfer buffer (made of Tris base, glycine, and methanol). In addition, the gel, filter papers (2), and fiber pads (2) were soaked in transfer buffer for 5 minutes. The transfer sandwich was then assembled with the fiber pads, filters,

gel and membrane, and inserted into the transfer cassette, which was placed into the transfer unit up against an ice cube. The unit was filled with transfer buffer and run at 4ºC at 100 volts for 1 hour.

The next step was to block the membrane using blocking buffer for 30 minutes at room temperature, and then to change the blocking buffer and soak at 4ºC overnight.

The following day, the detection procedure was performed. First, the membrane was incubated in primary antibody diluted 1:1000 in blocking buffer for 1 hour on the nutator. The antibodies used in my project were mouse anti-N-Cadherin and mouse anti-E-Cadherin (BD Transduction Laboratories). For the dilution, 3µL of the primary antibody were diluted in 3mL of blocking buffer. The membrane was then washed 3x for 10 minutes each in blocking buffer in a small tray on the shaker. Next, the membrane was incubated in secondary antibody diluted 1:10,000 in blocking buffer for 1 hour on the nutator. The secondary antibody used was horseradish peroxidase (HRP) rabbit anti-mouse (ImmunoPure). For the dilution, 1µL of the secondary antibody was diluted in 10mL of blocking buffer. The membrane was then washed 2x for 10 minutes each in blocking buffer in a small tray on the shaker, and then 1x for 10 minutes in wash buffer (0.05% Tween in 1x PBS) on the shaker. Then, the membrane was rinsed in PBS and incubated in detection buffer + enhancer (2.5mL of each) for 5 minutes in a small tray on the shaker. Finally, the membrane was rinsed in dH_20 and carried to the imaging room in a clear plastic cover and inside an autoradiography cassette.

Images of the membrane were taken using the BioRad Molecular Imaging ChemiDoc XRS+ Imaging System. After taking images, the membrane was rinsed in PBS and stored overnight in blocking buffer at 4ºC.

The following day, the same detection procedure was followed using the primary antibody GAPDH as a loading control. The dilution for GAPDH was 1:5000, so 1µL of GAPDH was diluted in 5mL of blocking buffer. The HRP secondary antibody used was against the animal that the GAPDH was made in (either mouse or rabbit). The membrane was then imaged again as a control, and the imaging system was used to compare the intensity of the bands on the treatment and control membranes.

Results

Expression of E-Cadherin in the mouse neonatal ovary

Immunocytochemistry was performed using neonatal ovaries of ages 17.5dpc and PND1-5 in order to determine if E-Cadherin is expressed during cyst breakdown and follicle development, and if so, to determine its location within the ovary (figure 5).

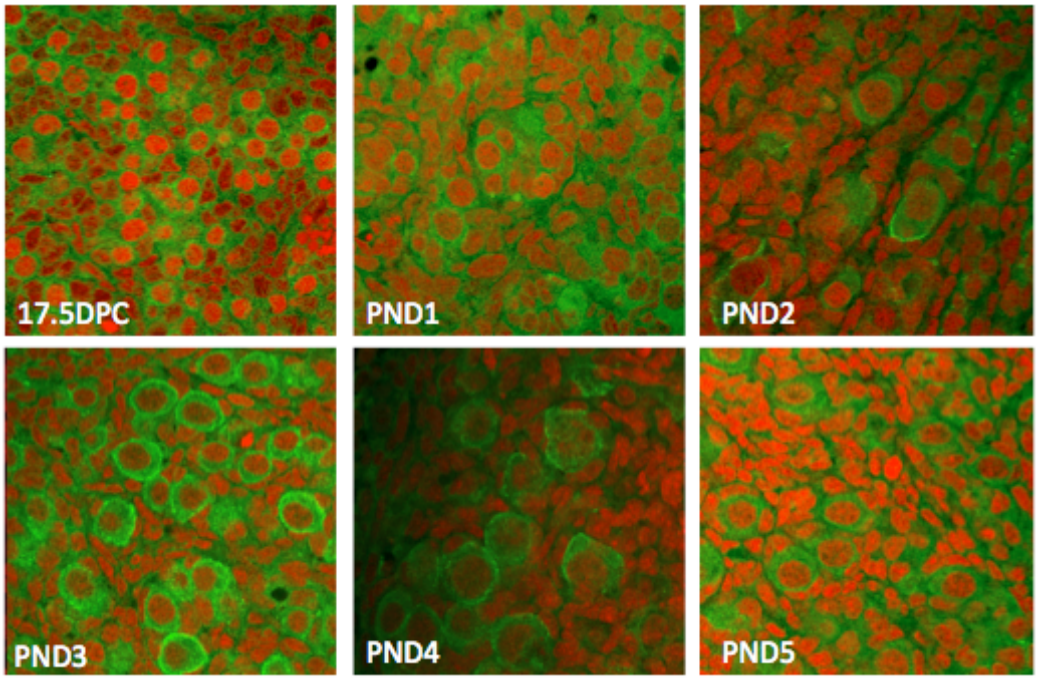

Figure 5. Immunocytochemistry results using anti-E-Cadherin antibody (green) at various ages of the neonatal mouse. All cell nuclei are stained using propidium iodide (red). A) 17.5dpc, B) PND1, C) PND2, D) PND3, E) PND4, F) PND5.

E-Cadherin was found to be expressed at least in moderate amounts at all ages from 17.5dpc to PND5. At 17.5dpc and PND1, E-Cadherin is moderately expressed in the cytoplasm of all oocytes and lightly expressed in granulosa cells. During all ages shown, E-Cadherin expression is stronger in the oocytes than in the granulosa cells. At PND2, E-Cadherin is expressed in the cytoplasm of all

oocytes and also in certain parts of the cell membrane of some oocytes. This same expression pattern is amplified at PND3, where E-Cadherin expression is substantial in both the cytoplasm of all and cell membrane of most oocytes. The E-Cadherin expression does not seem to differ between oocytes that are in cysts and single oocytes. Many of the oocytes containing E-Cadherin in the cell membrane at PND3 show strong expression around the entire membrane, but some oocytes show localized expression within the cell membrane. Additional images showing E-Cadherin expression at PND 3 show the cell adhesion molecule in the cytoplasm of all oocytes, including those in cysts as well as those in developing follicles (figure 6). These images also show how E-Cadherin is localized to regions of the cell membrane of the oocytes, whether they are in cysts or in developing follicles.

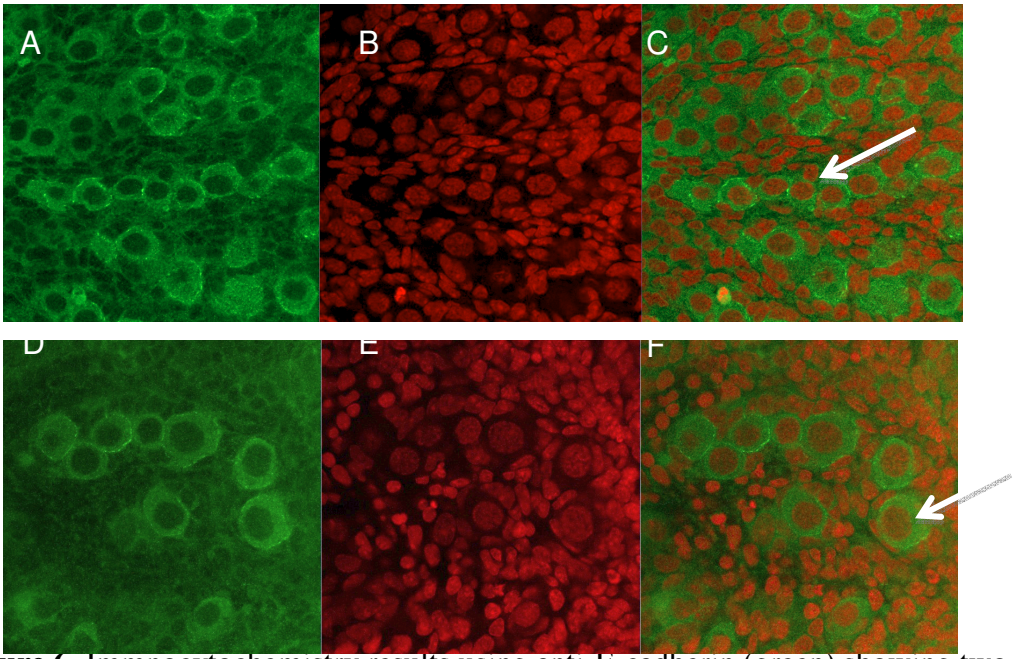

Figure 6. Immnocytochemistry results using anti-E-cadherin (green) showing two more examples at PND3. Nuclei of all cells stained with propidium iodide (red). Boxes A and D show just anti-E-cadherin, boxes B and E show just propidium iodide, and boxes C and F show the overlay. Arrow in box C shows oocytes in a cyst. Arrow in box F shows a single oocyte primordial follicle.

Similar expression of E-Cadherin is shown at PND4, as all oocytes stained with anti-E-Cadherin in the cytoplasm and at certain regions of the cell membranes. At PND5, E-Cadherin remains in the cytoplasm of the oocytes but it does not appear in the cell membranes.

In summary, during the ages that were tested, E-Cadherin expression starts in the cytoplasm of all cells and moves to both the cytoplasm and cell membrane of just the oocytes at PND2. E-Cadherin is continuously expressed in the cytoplasm of all oocytes as well as in the cell membrane of most oocytes during PND3 and PND4. Membrane expression is localized in most oocytes and continuous in some oocytes. Expression at PND5 remains in the oocytes but only in the cytoplasm.

As a control, the neonatal mouse ovary was stained with just the anti-rat secondary antibody to see if it would show significant staining without the anti-E-Cadherin primary antibody. While faint staining appeared in the control (figure 7), it only appeared when the channels on the microscope were turned all the way up, and the ovaries stained with the primary antibody as well were significantly brighter, so the data are valid.

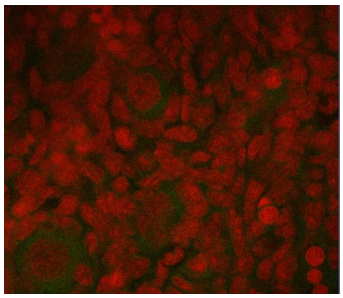

Figure 7. Immunocytochemistry showing a PND2 ovary treated with the anti-rat antibody (faint green). The nuclei of all cells are stained with propidium iodide. Because no primary antibody was used, this staining is a control to show that the

secondary antibody is actually sticking to the primary antibody in the previous experiments.

Expression of N-Cadherin in the mouse neonatal ovary

Immunocytochemistry was performed using neonatal ovaries of ages 17.5dpc and PND1-5 in order to determine if N-Cadherin is expressed during cyst breakdown and follicle development, and if so, to determine its location within the ovary (figure 8).

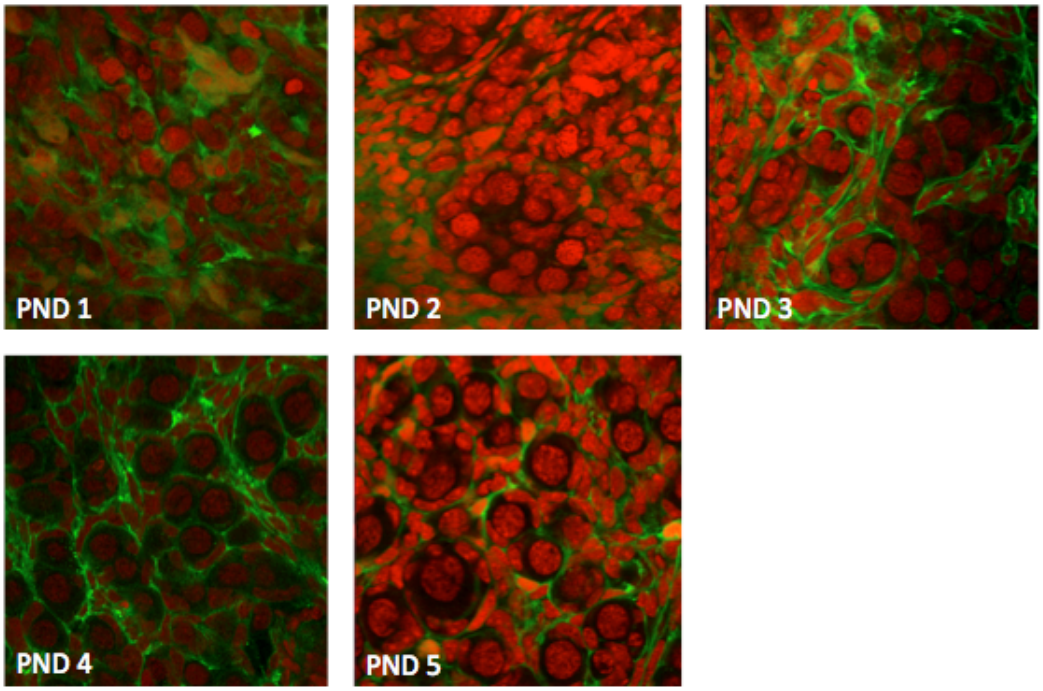

Figure 8. Immunocytochemistry results using anti-N-Cadherin antibody (green) at various ages of the neonatal mouse. All cell nuclei are stained using propidium iodide (red). A) PND1, B) PND2, C) PND3, D) PND4, E) PND5.

Because of the results of the control (figure 9) using just the secondary antibody (anti-mouse), these data cannot be used.

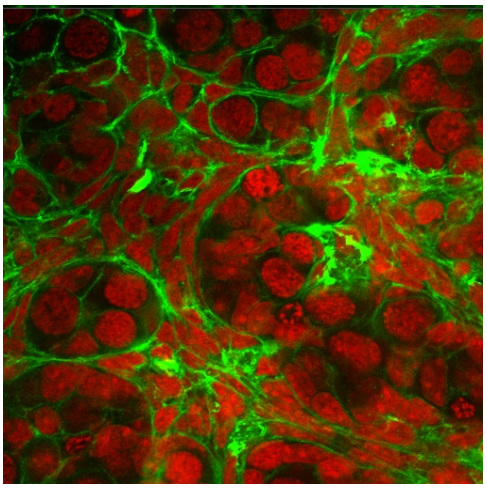

Figure 9. Immunocytochemistry showing a PND2 ovary treated with the anti-mouse antibody (green). The nuclei of all cells are stained with propidium iodide. Because no primary antibody was used, this staining is a control. This control shows that the secondary antibody is sticking to the tissue even in the absence of the primary antibody. This control makes the N-Cadherin stainings above invalid.

Effects of blocking E-Cadherin on oocyte development

Now that E-Cadherin was found to be expressed in the neonatal mouse ovary, and its location within the ovary was tested, organ cultures were performed in order to test the function of this cell adhesion molecule. For the organ culture analysis, seven ovaries grown from PND1 to PND 5 were counted for both the control group and the anti-E-Cadherin treatment group. The graphs below reflect the results after counting total oocytes, percentages of oocytes in different types follicles, and percentage of single oocytes for both control and treatment ovaries (figures 10-12).

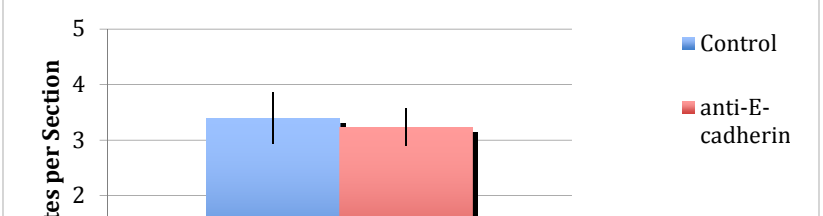

Figure 10. Graph of the average number of total oocytes per section. The control ovaries had an average of 3.4 oocytes per section (blue bar) and the anti-E-Cadherin treatment ovaries had an average of 3.2 oocytes per section (pink bar).

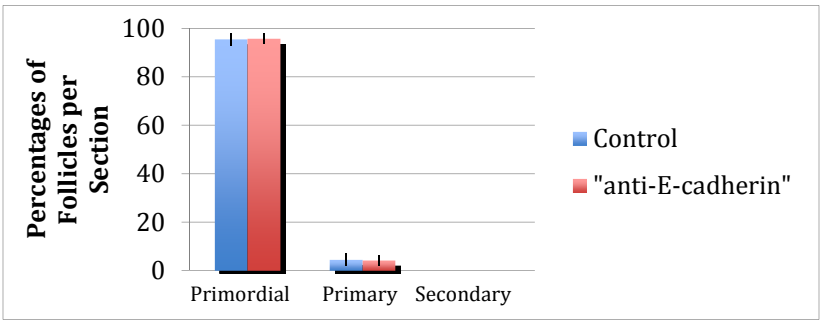

Figure 11. Graph of the average percentage of primordial, primary, and secondary follicles per section. The control ovaries, on average, had 95.6% primordial follicles, 4.4% primary follicles, and 0% secondary follicles per section (blue bars). The anti-E-Cadherin treatment ovaries, on average, had 95.8% primordial follicles, 4.2% primary follicles, and 0% secondary follicles per section (pink bars).

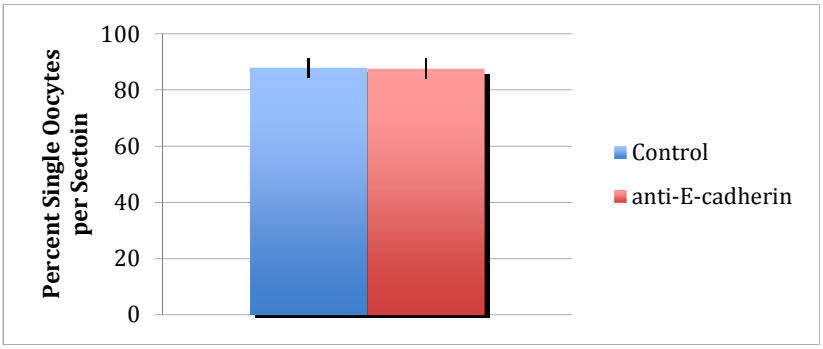

Figure 12. Graph of the average percent of single oocytes per section. The control ovaries had an average 88.0% oocytes as single oocytes (blue bar). The anti-E-Cadherin treatment ovaries had an average of 87.6% oocytes as single oocytes (pink bar).

Statistically, there is no significant difference between the control and treatment ovaries in terms of number of total oocytes, follicles, or single oocytes. These data therefore show that under these conditions, blocking E-Cadherin from PND 1-5 does not affect cyst breakdown, oocyte death or follicle development

Another organ culture was performed using ovaries at age 17.5dpc and grown until PND3, but the plate was contaminated and the data could not be used.

Effects of blocking N-Cadherin on oocyte development

The same experiments were performed using anti-N-Cadherin. For the organ culture analysis, nine ovaries were counted for the control group and eight ovaries were counted for the treatment group. Similar to the E-Cadherin organ culture experiments, the results did not show a statistical difference between control and treatment groups; therefore, these data show that under these conditions, blocking N-Cadherin from PND 1-5 does not affect cyst breakdown, oocyte death or follicle development (figures 13-15).

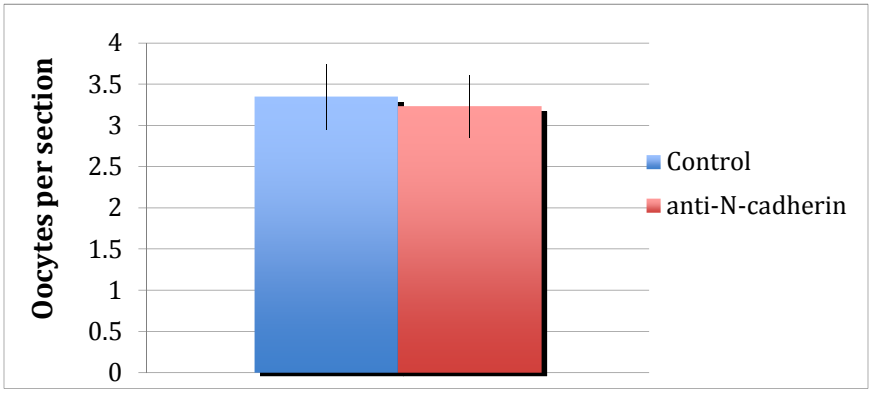

Figure 13. Graph of the average number of total oocytes per section. The control ovaries had an average of 3.4 oocytes per section (blue bar) and the anti-N-Cadherin treatment ovaries had an average of 3.2 oocytes per section (pink bar).

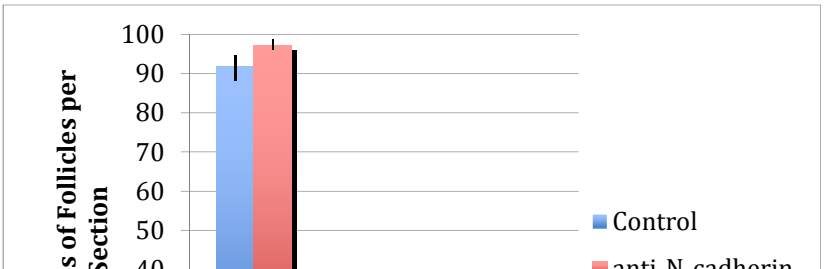

Figure 14. Graph of the average percentage of primordial, primary, and secondary follicles per section. The control ovaries, on average, had 91.6% primordial follicles, 8.4% primary follicles, and 0% secondary follicles per section (blue bars). The anti-N-Cadherin treatment ovaries, on average, had 97.3% primordial follicles, 2.7% primary follicles, and 0% secondary follicles per section (pink bars).

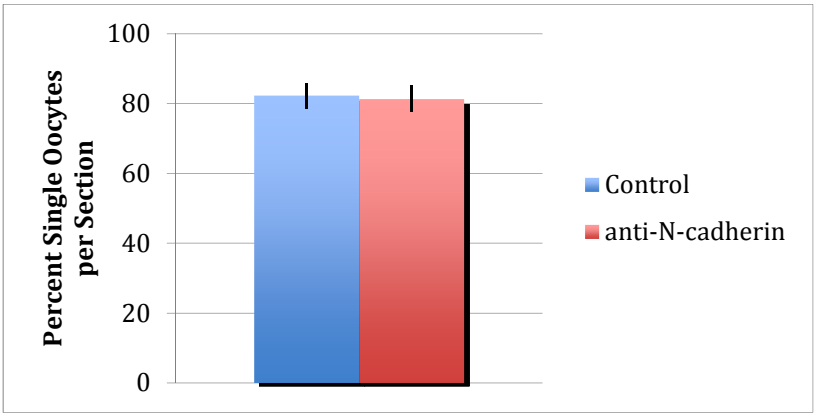

Figure 15. Graph of the average percent of single oocytes per section. The control ovaries had an average 82.3% oocytes as single oocytes (blue bar). The anti-E-Cadherin treatment ovaries had an average of 81.3% oocytes as single oocytes (pink bar).

Another organ culture was performed using ovaries at age 17.5dpc and grown until PND3, but there were not enough samples to do an analysis and there was not enough time to run more organ cultures to create a large enough sample size.

Protein expression levels of E-Cadherin

In order to test protein expression levels of E-Cadherin in the mouse, four western blots were run using samples from ovaries of neonates of ages PND1, PND3, and PND5. Two of the four blots also included age 17.5dpc, but only one of these was also run with a loading control. While all of the western blots that were run did not turn out to be completely consistent with one another, some conclusions can be drawn from the similarities in expression trends among the western blot results obtained (Table 1, Figure 16).

For all three blots analyzed, there was an increase in E-Cadherin expression from PND1 to PND 3. Even on blots 2 and 3, while the loading control decreased (less protein was loaded), the amount of E-Cadherin still increased. There was not enough protein in the samples loaded for age 17.5dpc to make any conclusions. The results for the relative expression at PND5 are inconclusive as well, as one blot showed drastically increasing expression, one showed decreasing expression, and one showed constant expression. As shown in figure 16, it can be concluded that E-Cadherin is present at ages PND1, PND3, and PND5, and that expression increases from PND1 to PND3.

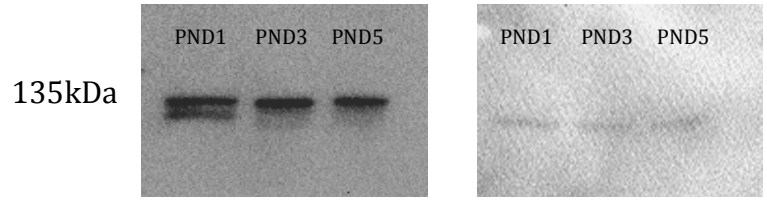

Figure 16. Western Blot results for probing with anti-E-Cadherin (left) and with GAPDH as a loading control (right). These results represent E-Cadherin 1 (left) and E-Cadherin 1 GAPDH (right) (Table 1). E-Cadherin is 135kDa in size, but the mature protein is only 120kDa, which explains the presence of two bands.

Western Blot	Band	Relative Intensity

E-Cadherin 1	PND1	**1.0**
	PND3	1.22 (1.17)
	PND5	1.0 (0.29)
E-Cadherin 1 GAPDH	PND1	**1.0**
	PND3	1.04
	PND5	4.2
E-Cadherin 2	PND1	**1.0**
	PND3	1.25 (1.69)
	PND5	1.37 (1.10)
E-Cadherin 2 GAPDH	PND1	**1.0**
	PND3	0.74
	PND5	1.25
E-Cadherin 3	17.5dpc	0.57 (1.90)
	PND1	**1.0**
	PND3	4.14 (6.18)
	PND5	5.12 (7.88)
E-Cadherin 3 GAPDH	17.5dpc	0.30
	PND1	**1.0**
	PND3	0.67
	PND5	0.65

Table 1. Relative intensities of bands on three different Western Blots probed with anti-E-Cadherin and then GAPDH as a loading control. The bolded values are the reference band; all other values show intensities relative to the bolded value.

Protein expression levels of N-Cadherin

In order to test protein expression levels of N-Cadherin in the mouse, four western blots were run using samples from ovaries of fetuses/neonates at ages 17.5dpc, PND1, PND3, and PND5. Only three of the western blots were used for analysis because one of the blots does not have a matching loading control blot. While all of the western blots that were run did not turn out to be completely consistent with one another, some conclusions can be drawn from the similarities in expression trends among the western blot results obtained (Table 2, Figure 17).

N-Cadherin expression seems to stay relatively constant (or *slightly* increase) from 17.5dpc to PND1. Expression at PND3 seems to increase a little more, and expression at PND5 seems to be the highest. It can be concluded that N-Cadherin is expressed during all of these ages, and that expression increases slowly from 17.5dpc to PND5.

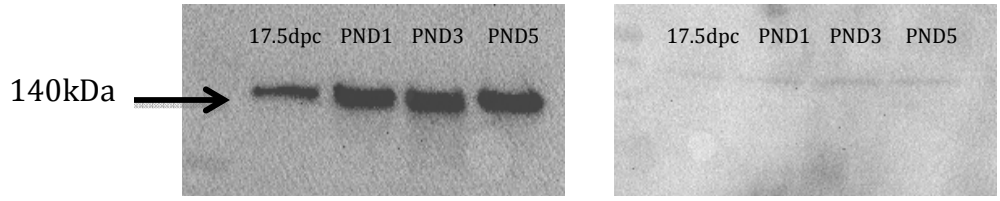

Figure 17. Western Blot results for probing with anti-N-Cadherin (left) and with GAPDH as a loading control (right). These results represent N-Cadherin 2 (left) and N-Cadherin 2 GAPDH (right) (Table 1). N-Cadherin is 140kDa in size.

Western Blot	Band	Relative Intensity
N-Cadherin 1	17.5dpc	**1.0**
	PND1	0.9 (1.14)
	PND3	2.21 (1.19)
	PND5	3.65 (2.19)
N-Cadherin 1 GAPDH	17.5dpc	**1.0**
	PND1	0.79
	PND3	1.85
	PND5	1.67
N-Cadherin 2	17.5dpc	**1.0**
	PND1	2.84 (1.19)
	PND3	2.58 (1.25)
	PND5	3.26 (1.88)
N-Cadherin 2 GAPDH	17.5dpc	**1.0**
	PND1	2.39
	PND3	2.06
	PND5	1.73
N-Cadherin 3	17.5dpc	**1.0**
	PND1	4.01
	PND3	3.29
	PND5	1.21
N-Cadherin 3 GAPDH	17.5dpc	0
	PND1	**1.0**
	PND3	1.22
	PND5	0.60

Table 2. Relative intensities of bands on three different Western Blots probed with anti-N-Cadherin and then GAPDH as a loading control. The bolded values are the reference band; all other values show intensities relative to the bolded value.

Discussion

Oogenesis is a crucial process in the mouse and human, as without the successful production of egg cells, reproduction cannot occur. Egg cell development, which occurs in the fetus and continues for a few days after birth, is a highly regulated process involving a variety of hormones and proteins. More specifically, the process of cyst formation and cyst breakdown to form primordial follicles is regulated to avoid the formation of multiple oocyte follicles, which are not viable for reproduction. There is speculation that cell adhesion molecules play a role in holding the cysts intact and in allowing the cysts to breakdown, but this exact mechanism has not yet been scientifically determined.

The results of my E-Cadherin experiments show this cell adhesion molecule to be expressed in the neonatal ovary from age 17.5dpc to PND5. The stainings using anti-E-Cadherin show the protein to be expressed in all oocytes and granulosa cells at 17.5dpc and PND1, but only in oocytes at PND2-5. During PND2-4, expression is also found in the cell membrane of oocytes, whether they are in cysts or in follicles. But the E-Cadherin cell membrane expression was localized to certain spots in the membrane. These results differ from the hamster model for E-Cadherin expression, which was found exclusively in the oocytes throughout development (Roy and Wang, 2010).

The organ culture experiments showed that under these conditions, blocking E-Cadherin during PND1-5 does not cause a significant change in cyst breakdown, follicle formation, or oocyte number. However, it is possible that the ovaries did not grow to their full potential in the cultures, as there were only 3-4

oocytes per section (normal, healthy ovaries have 6-8 oocytes per section). In addition, cyst breakdown begins prior to PND1, so it is also possible that the oocytes in cysts already started to break down and form follicles prior to blocking E-Cadherin.

The western blot experiments showed E-Cadherin expression increasing from PND1 to PND3, which matches the images from the tissue antibody labelings. This opposes what was found in the hamster, however, as E-Cadherin was found to decrease throughout development (Roy and Wang, 2010).

While the hamster model suggests that E-Cadherin plays a role in cyst breakdown by deregulating and allow cells within cysts to break apart (Roy and Wang, 2010), this does not seem to be the case in the mouse. E-Cadherin expression remains prominently expressed throughout the major timeframe of cyst breakdown, and is also expressed in the membranes of oocytes in follicles.

During the timeframe that I looked at (17.5dpc-PND5), both cyst breakdown and follicle formation are occurring. Because E-Cadherin is expressed at all of these ages, it is likely that this cell adhesion molecule plays a role in these two processes. Further, E-Cadherin may aid in holding oocytes together in cysts and also in holding oocytes to granulosa cells in follicles. Although E-Cadherin was not expressed in granulosa cells later in development, it is possible that E-Cadherin could bind to a different cadherin on a neighboring cell (heterotypic binding). While these experiments show that E-Cadherin likely plays a role in cyst breakdown and follicle formation in the mouse, the exact role and the mechanism of function as still not completely clear.

The N-Cadherin antibody labelings cannot be used to draw conclusions because of the prominent staining that appeared in the control. Similar to E-Cadherin, the organ culture experiments showed that under these conditions, blocking N-Cadherin during PND1-5 does not cause a significant change in cyst breakdown, follicle formation, or oocyte number. Again, it is possible that the ovaries did not grow to their full potential in the cultures. It is also possible that the oocytes in cysts already started to break down and form follicles prior to blocking N-Cadherin.

But the N-Cadherin western blots show the protein to be present at ages 17.5dpc, PND1, PND3, and PND5. It also appears that N-Cadherin expression increases from 17.5dpc to PND5. The hamster model also showed N-Cadherin expression shifting from oocytes to somatic cells as development progressed, but this cannot be concluded for sure in the mouse due to the invalidity of the N-Cadherin stainings. The hamster model further suggests that N-Cadherin plays a role in assembly of somatic cells around the oocyte during follicle formation, due to both antibody labeling experiments as well as blocking experiments that show a decrease in follicle formation in the absence of N-Cadherin. It is possible that N-Cadherin plays a similar role in the mouse ovary because its expression increases throughout the timeframe analyzed, throughout which more and more follicles are developing.

While the exact role of E-Cadherin and N-Cadherin was not determined from these experiments, it was determined that the two cell adhesion molecules are expressed in the mouse ovary from 17.5dpc to PND5. It was also determined

that E-Cadherin is expressed in both the cytoplasm and cell membrane (localized) of oocytes, and that E-Cadherin expression increases form PND1 to PND 3, suggesting a role in cyst breakdown and follicle formation. N-Cadherin expression was found to increase throughout the time period studied, suggesting a role in proper follicle formation.

Future Directions

There are numerous other experiments that could be performed to continue testing the role of N-Cadherin and E-Cadherin in cyst breakdown and follicle formation in the neonatal mouse ovary. The N-Cadherin immunocytochemistry images were invalid because they looked similar to the control; therefore, these experiments could be repeated using an antibody made in an animal other than the mouse in order to determine the location of N-Cadherin expression within the ovary, now that it has been shown to be expressed. Triple stainings could also be done to try to determine the localization of E-Cadherin within the cell membrane of oocytes and to determine if E-Cadherin is binding to any other cadherins during development. The organ culture experiments could also be repeated in order to try to get results that show statistical significance. It is likely that the ovaries did not thrive in the organ culture, as there were only 3-4 oocytes per section (healthy ovaries should have 6-8 oocytes per section). These cultures could be run at 17.5dpc to PND3 to try to block the CAMs earlier in development. In addition, the E-Cadherin western blots could be re-run, especially at age 17.5dpc, as the samples used in my western blots at this age were insufficient (did not contain enough protein).

In addition to repeating the experiments that I have completed, there are other experiments that can be done to test the role of E-Cadherin and N-Cadherin in cyst breakdown and follicle development. In particular, estrogen upregulation has been shown to hold cells in cysts, preventing them from breaking down and forming viable follicles. Perhaps the mechanism of this has to do with cell

adhesion molecules. Organ cultures can be done using an antibody that blocks estrogen during development, and the ovaries could then be stained with E-Cadherin and N-Cadherin (separately) to see if there are any differences in expression in the presence and absence of estrogen.

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Capstone Summary

In order for a woman to reproduce, she must contain egg cells that are healthy and properly developed. A female's body makes all of the egg cells that she will contain for the rest of her life around the time of birth. In fact, this essential pool of egg cells that is established early on slowly gets depleted throughout the female's life. For this reason, when a woman is infertile, the cause of her inability to conceive often stems back to around the time of birth when her ovaries were forming.

When a man and a woman conceive, and the man's sperm fertilizes the woman's egg, an embryo forms. This embryo is comprised of numerous cells starting to specialize into different cell types to ultimately form a fetus, and then a baby. Throughout a woman's pregnancy, her fetus' ovaries are undergoing a process called Egg Cell Development (oogenesis). This process is very similar in the human and the mouse, making the mouse an appropriate organism to use for research pertaining to oogensis.

In the developing mouse embryo, the germ cells (precursors to egg cells) migrate to the genital ridge (a specific location within the embryo). These germ cells then begin a process called mitosis, in which they divide and multiply to create more cells. In normal mitosis (which occurs throughout the entire embryo and human body), the two daughter cells split apart from each other during the final step of division. But in the case of oogenesis, the cells remain connected to one another, forming what is known as a cyst, or a ball of interconnected cells. Soon these cells start dividing my meiosis, which is another type of cell division

that only occurs in germ cells (eggs and sperm). At a very specific stage of meiosis called “diplotene of prophase I”, the cells stop dividing until later in the life of the female. Around 17.5 days after conception, a process called cyst breakdown begins, during which the egg cells (oocytes) within the cysts start to separate from one another. Once a single oocyte separates from the rest of the cyst, it starts to develop into a follicle, consisting of somatic cells (non-germ cells) surrounding the oocyte. Around the same time as cyst breakdown and follicle formation, germ cells are also dying. There is a theory that it is necessary for certain cells within a cyst to die in order for the cyst to break apart and for single oocyte follicles to form. This germ cell death explains the decrease in oocyte number during development that occurs in both mice and humans.

When a cyst breaks down properly, single oocyte follicles form, which go on to become viable for reproduction later in the baby’s life. But when cyst breakdown does not occur properly, multiple oocyte follicles form as a result of the granulosa cells (specific somatic cells) surrounding multiple connected oocytes, rather than one single oocyte. These multiple oocyte follicles do not develop into viable eggs for reproduction, and are therefore one of the factors contributing towards infertility. This concept is the basis of my research, as I am studying the mechanisms involved in cyst breakdown and follicle formation in order to determine the causes of, and hopefully one day the treatments for, infertility.

While the cells within the cyst are held together by intracellular canals, it is thought that cell adhesion molecules (proteins on the surface of cells that bind

to one another and hold cells together) play a role as well. In fact, research using the hamster has found two specific cell adhesion molecules (CAMs) present in the ovary during oogenesis. These two CAMs are called E-Cadherin and N-Cadherin and are the two proteins that I studied in my research.

I first tested to see if E-Cadherin and N-Cadherin are present in the neonatal mouse ovary, and if so, where within the ovary they are located (expressed). I did this using a method called immunocytochemistry, which involves dissecting the ovaries out of the neonatal mice and staining them with fluorescent tags. The fluorescent tags are attached to proteins called antibodies, which bind to very specific molecules. In this case, I used anti-E-Cadherin and anti-N-Cadherin in order to create images of the ovaries that fluoresce in all areas expressing the proteins E-Cadherin or N-Cadherin, respectively.

Because I was able to see fluorescence on the E-Cadherin images, I know that the E-Cadherin protein is present in the ovary. More specifically, the fluorescence was visualized in the cytoplasm (inside of the cell) and on the cell membrane (on the surface of the cell) of all oocytes (single oocytes and oocytes in cysts). E-Cadherin expression was also found in the granulosa cells, but only earlier in development.

For N-Cadherin, the staining experiments were invalid because the antibody used ended up just sticking to the tissue in the ovary and staining the entire thing.

Now that the presence and location of these CAMs was determined (at least for E-Cadherin), the next test was to determine the *function* of E-Cadherin

and N-Cadherin in cyst breakdown and follicle formation. This was done by first dissecting the ovaries out of the neonatal mice, and then growing them in organ cultures in the presence and absence of E-Cadherin and N-Cadherin. The ovaries were then stained with oocyte markers in order to determine any changes in oocyte numbers in the control versus treatment ovaries. The results showed no statistical difference, but there were also a lower number of total oocytes than in a normal healthy ovary, so it is possible that the ovaries did not thrive in the organ culture.

The last experiment was western blot, which was performed to determine the amount of protein expression for E-Cadherin and N-Cadherin in the mouse ovary at different ages during development. For a western blot, the dissected ovaries are crushed and a procedure is following to isolate the proteins from the ovaries, which are then run on a gel and stained with antibodies to detect the protein of interest. The proteins show up on the gel as bands, and the bands are transferred to a membrane for analysis.

For both sets of western blots, the proteins appeared on the gels, once again showing that E-Cadherin and N-Cadherin are expressed during oogenesis. N-Cadherin expression was more prominent than E-Cadherin expression, which matches the current hamster model for these two proteins. Further, E-Cadherin expression was found to increase from PND1 (the day of birth) to PND3 (two days after birth). N-Cadherin expression was also found to increase with age during the timeframe studied.

The results of all completed experiments show that both E-Cadherin and N-Cadherin are expressed in the neonatal mouse ovary during the timeframe of oogenesis that was looked at (17.5 days after conception to PND5, or four days after birth). Because E-Cadherin appeared in the cytoplasm of all and the cell membrane of most oocytes, it is likely that this cell adhesion molecule plays a role in both cyst breakdown and follicle development. But the exact role of E-Cadherin in oogensis is still unknown at this time. N-Cadherin expression was found to increase during the timeframe studied, coinciding with increasing follicle formation. As a result, it is likely that N-Cadherin plays a role in ensuring proper follicle formation.

While this research was successful in drawing conclusions pertaining to the role of E-Cadherin and N-Cadherin in neonatal mouse oogenesis, there are more experiments that can be done. The N-Cadherin stainings can be re-done using different antibodies to try to obtain valid images. The organ cultures could also be re-done to try to obtain healthier ovaries. These cultures could be run starting at an earlier age to try to block the function of E-Cadherin and N-Cadherin before they have a chance to impact oogenesis. In addition, estrogen has been found to hold oocytes in cysts. Therefore, organ cultures could also be run in which estrogen is blocked in order to determine if estrogen has any impact of the expression of cell adhesion molecules as a mechanism to prevent cysts from breaking down.

My research on E-Cadherin and N-Cadherin shows that these two proteins play a role in oogenesis in the mouse. It is therefore advantageous to continue studying these two cell adhesion molecules and their potential impact on fertility.