Spring 5-1-2011

Germline Cyst Formation and Development in Zebrafish

Tess Cherlin

Germline Cyst Formation and Development in Zebrafish

A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University

Tess Cherlin

Candidate for B.S., Biochemistry
and Renée Crown University Honors
May 2011

Honors Capstone Project in _____Biochemistry_____

Capstone Project Advisor: _____
(Dr. Melissa Pepling)

Honors Reader: _____ _____
(Dr. Craig Albertson)

Honors Director: _____
James Spencer, Interim Director

Date:_____

Abstract

In developing pre-natal mice, germline cysts are clusters or packages of cells consisting of individual oocytes. They are formed after the primordial germ cells move to the ovary during embryogenesis. The primordial germ cells divide, but do not completely separate. The cells within the cysts are linked by intercellular bridges, which end up breaking down leading to the formation of primordial follicles. The primordial follicles are oocytes surrounded by somatic cells called granulosa cells. Only one third of the original oocytes survive cyst breakdown and become the eggs that will be used by the mouse for her reproduction. This developmental process is conserved in many organisms including *Drosophila* and humans. Infertility is a disorder that affects 33 million women in the United States alone. This striking statistic has led to much research on oocyte development. The development of cysts and cyst breakdown are vital to reproductive success as infertility arises when this process is hindered. Many factors can inhibit cyst breakdown, such as fetal exposure to estrogenic compounds found in the environment. By studying model organisms we hope to elucidate the mechanisms of cyst formation and regulation and bring this knowledge to the human scale. Cyst formation has already been well studied in fruit flies and in mice. My research has focused on oocyte development specifically cyst formation in zebrafish and how it compares to that of mice and fruit flies. Germline cysts have not been well studied in zebrafish so this research is relatively uncharted territory. To date there is some evidence to suggest the existence of germline cysts in other teleosts (bony fishes) such as the Medaka. By using the knowledge of what is known about cyst formation in *Drosophila* and mice, as well as literature on oogenesis in a wide range of organisms, I have conducted research to identify germline cysts in zebrafish. Zebrafish were sacrificed at different ages: from two weeks to adult, and their ovaries were stained with oocyte specific antibodies as well as antibodies that have been shown to mark intercellular bridges in *Drosophila* and mice. The ovary tissues were then imaged using confocal microscopy. My findings show preliminary data that supports the existence of germline cysts in zebrafish.

Table of Contents

ACKNOWLEDGMENTS

Thank you Dr. Melissa Pepling for all of your support and guidance over the past two years, especially these past few weeks as I feverishly worked to finish my Capstone. Your knowledge, care, and dedication to my Capstone are only three qualities that represent what an amazing mentor and teacher you are. Thank you so much.

Thank you to everyone in the Pepling Lab. Your support has been most appreciated.

Thank you Dr. Craig Albertson. From introducing me to the zebrafish anatomy, to donating your zebrafish, to reading my Capstone, you have been extremely generous and kind and have always sought to teach me.

Thank you Dr. Katharine Lewis for donating your female zebrafish to my research. They were most appreciated.

Thank you Nicole Jacobs for all of your assistance with zebrafish maintenance, from breaking the chorion to showing me how to tell the males from the females, you have been most helpful to my studies and overall knowledge.

I would like to acknowledge the Ruth Meyer Scholars Fund for funding to support my summer research.

I would like to acknowledge the Renée Crown University Honors Program and their funding which supported my efforts in completing my Capstone with funding as well as moral support.

Finally I would like to thank my family and friends for the constant support and distractions. Thanks for listening to me talk about science and wanting to understand.

Introduction

Oocyte Development

Infertility is a disorder and problem that affects 7.3 million (11.8%) women in the United States alone. In New Zealand, infertility is documented at 26% while in Australia, 29% of women are infertile. In Europe 14% of women in the United Kingdom and 15% of French and German women cannot have children. These striking statistics are increasing. As women wait to have children later and later in life, their egg pool, already reduced, is also less viable. Young eggs are ideal for fertilization as they have been less exposed to mutagenic and environmental factors, but even eggs of young women are not automatic baby-makers. By the age of 42 a woman has a 4% chance of conceiving (Family Health Diary, 2011).

A woman is born with all of the eggs she will ever have and she ovulates on average one egg per month from puberty to menopause. During embryonic development, 7 million eggs are created but by the time a baby girl is born, she only has about 2 million eggs to use for the rest of her reproductive life. The decrease of eggs is a normal occurrence in development (Gondos et al., 1971). However, while the embryo is developing, large amounts of estrogenic compounds can prove detrimental to the embryo's egg population by disrupting an important event known as cyst breakdown (Gondos et al., 1978; Jefferson et al., 2002).

Cysts are clusters of oocytes found in the ovaries of many species of developing embryonic mammals as well as the ovaries of *Drosophila* and some fishes (de Cuevas et al., 1997; Nakamura et al., 2010; Pepling et al., 1999). Across species, progenitor germ cells multiply by mitosis and migrate to the gonad where they will divide with incomplete cytokinesis. The cyst consists of cells connected by intercellular bridges that pass nutrients and signals to one another. At a determined point in the developmental timeline the cysts begin to breakdown by way of apoptosis. This breakdown is necessary for the individual oocytes to separate from the cyst in order to develop into mature egg cells (Pepling and Spradling, 2001). Thus cyst formation and breakdown is vital for normal reproduction leading to fertile females, and its regulation is what the Pepling Lab is studying.

Oocyte Development in *Drosophila*

Drosophila melanogaster, more commonly known as the fruit fly is the most widely studied organism. It is easy to maintain, proliferates frequently, and has huge, easily identifiable ovaries. The ovary is the reproductive organ, where in *Drosophila* oocytes are generated from germline stem cells located in the germarium of the 16-20 ovarioles, which make up the each of the ovaries (Spradling, 1993). The oocytes grow and mature into the egg cells that will be fertilized and lend to the *Drosophila's* progeny. Because *Drosophila* (and other insects, and even some fish and mammals) has germline stem cells, *Drosophila* can reproduce continuously for much of their lives (Spradling, 1993). This is in

contrast to most mammals, which are born with a determined amount of eggs that governs the length of their reproductive lives (Pepling, 2006).

A germline stem cell is a stem cell predestined to become either oocytes or sperm. The first division of a germline stem cell in the germarium leads to two daughter cells. One of these cells is another germline stem cell and the second cell, which contains the fusome (A germ cell specific organelle apparent only in the intercellular bridges between cyst cells (Gilboa et al., 2004), called the cystoblast, will divide again. The cystoblast undergoes four rounds of synchronous mitotic divisions. These divisions have incomplete cytokinesis and give rise to 16 identical cystocytes connected by intercellular bridges, known as cysts. One of the cystocytes in a cyst is destined to become an oocyte while the others will become nurse cells (Spradling, 1993). The nurse cells pass nutrients and signals through the intercellular bridges of the cyst into the oocytes so that it can grow and develop. At a certain point, the nurse cells die through a cell mediated death pathway known as apoptosis and the remaining cystocyte, now oocyte, develops into a mature egg cell surrounded by somatic cells called follicle cells (de Cuevas et al., 1997).

Oocyte Development in Mouse

Mice are the organisms that our lab uses to look at oocyte development, as they are anatomically similar to humans. Cyst formation in mice begins around 10.5 days post coitum (dpc) when the primordial germ cells (PGCs) arrive in the

gonad, what will become the ovary. The germ cells divide mitotically, forming oocytes in cysts by way of synchronous mitotic divisions, until 13.5 dpc, at which point the germ cells begin to enter meiosis. Like in Drosophila, intercellular bridges are present between cells in a cyst. The cells grow in the cysts until 16.5 dpc when the oocytes arrest in the diplotene stage. This is when the homologous chromosomes of each bivalent remains in the position where crossing over occurs and stays there. In humans this arrest occurs until until puberty. In mice this happens until 6 weeks after birth, at which point the mice are sexually mature (Pepling and Spradling, 1998). Cyst breakdown begins at the same time as the cells arrest in the diplotene stage. Cyst breakdown takes place from 17.5-23.5 dpc and continues after birth (19.5 dpc) by way of programmed cell death. After an oocyte breaks away from the cyst, it becomes surrounded by somatic cells called granulosa cells, which provide nutrients and protection to the growing oocytes. The primordial follicle will grow and eventually become a mature oocyte, egg (Pepling and Spradling, 1998). Mice, like humans produce a determined number of mature egg cells that they will use to reproduce (Kezele et al., 2002). At the end of oogenesis, only one third of the original follicles will become eggs viable for the next generation so it is incredibly important that these cells are viable. Extensive studies in our lab have gone into elucidating cyst breakdown, but much is still unknown (Pepling and Spradling, 2001). It has been observed that if a mother mouse is injected with estrogen the developing mice are exposed to the estrogenic molecules, and the cyst breakdown is inhibited. These mice are determined to be infertile (Iguchi and Takasugi, 1986; Jefferson et al., 2002). This

is projected to be similar to human development. With an increase of estrogen-like molecules in the environment and in the products we use, this system may be a mode of infertility in humans.

Zebrafish Oocyte Development

I am using zebrafish (*Danio rerio*) as a model organism to study cyst formation in developing embryos. Zebrafish are easily maintained and can breed all year in captivity. In mixed populations females will spawn eggs every 4-7 days. This differs from zebrafish in the wild, which spawn annually. Zebrafish are exceptional organisms to study because their embryos are transparent, so embryogenesis can be observed with live fish under a microscope. Fertilization is also external so embryos do not need to be harvested from the womb like in mammals. Furthermore, like *Drosophila*, zebrafish have relatively large ovaries and reproduce continuously and rapidly, and it is proposed by Bruce Draper that ooytes are generated from germline stem cells (Draper et al., 2002). Ovarian development in zebrafish begins 10 days after the larvae hatch, and gonads arise bilaterally from the germinal ridges, which are derived from the dorsalateral lining of the peritoneal cavity. The PGCs migrate from the germinal ridges to the germinal epithelium of the developing ovaries, developing into gametes (Connaughton and Aida, 1999). The ovaries are made up of a thin epithelium: oogonia and maturing follicles consist of oocytes that are surrounded by somatic cells, zona radiata (vitelline envelope), granulosa cells, and theca cells (Clelland

and Peng, 2009). There are five stages of ooyctes in an ovary at any given time (stage I being the most underdeveloped and stage V the mature oocyte) and it would seem that oocyte growth and spawning is a continuous process (Leu and Draper, 2010). Early gonads develop into ovaries in both future female and male zebrafish and oocytes can be observed beginning 10 days post hatching (dph) (vonHolfsten and Olsson, 2005; Uchida et al., 2002). Hormones (ie. estrogen), temperature, pH, and rate of development all play a role in the gamete differentiation (Pang and Thomas, 2010; Spence et al., 2008). Because there is no sex-linked chromosome, there is no way to determine if a fish is male or female before apoptosis and testes morphogenesis occurs (vonHolfsten and Olsson, 2005). But beginning at 30 dph, oocytes begin to die in future males and spermatocytes develop (Takanhashi, 1977; Uchida et al., 2002). Extensive studies have gone into determining the mechanisms of oocytic differentiation and it has been observed that oocytes in females undergo apoptosis as well as in males. It is clear that this process, similar to *Drosophila, Xenopus,* and mammals is conserved for a reason and is important for gamete maturation in many species. (Uchida et al., 2002)

Medaka Oocyte Development

Although there have been no germline cysts found in zebrafish ovaries to date, (Nakamura et al., 2010), has shown that germline cysts exist in the teleost Medaka. Nests of oocytes, called germinal cradles Fig. 1C and E, were observed

to “form in interwoven threadlike cords” called ovarian cords (Fig. 1B). The germinal cradles were observed to contain three types of germ cells growing in succession. The first were single isolated germ cells surrounded by somatic cells, the second were cysts, and the third were early stage oocytes. After development through this ovarian cord, it would seem that the oocytes would exit and go into the next stage of oocyte maturation. The germinal cradle of the Medaka fish is very similar in

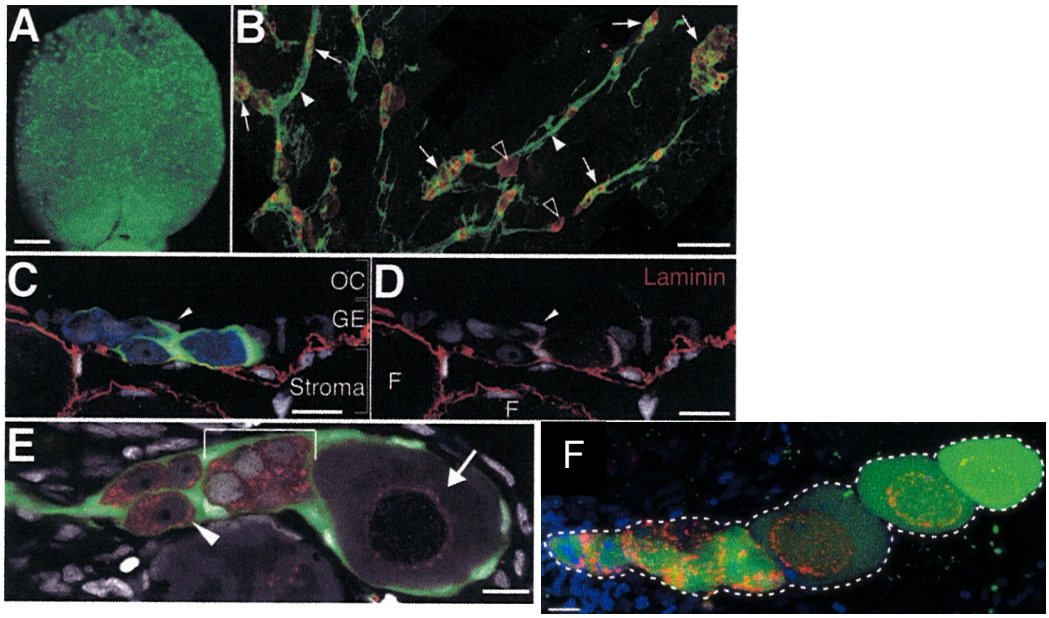


Fig. 1 Medaka ovary A. Low power image of ovary. B. Ovarian cords. C-E. Germinal cradle E. Larger image of individual germ cells (shown with arrowhead), cyst (shown with bracket), and oocyte follicle (shown with arrow) in germinal cradle. F. Single isolated germ cells surrounded by somatic cells. (Nakamura et al., 2010).

physiology to the Drosophila’s germarium, a structure with germline stem cells that produce oocytes to give rise to germline cysts (Nakamura et al., 2010).

Xenopus Oocyte Development

Kloc et al., 2003, were able to find germline cysts in *Xenopus laevis*, a frog. Their studies show that *Xenopus* has an ovary that develops like zebrafish, consisting of oocytes at different stages of development. The germ cells of *Xenopous* develop synchronously and are found in cysts. Post metamorphic froglets' ovaries were studied and numerous germ cell cysts were observed surrounded by somatic cells. Younger cysts were found at the periphery of the ovary while the older cysts are found closer to the middle of the ovary. Like the germ cells in mouse and *Drosophila* the germ cells in *Xenopus* were connected by intercellular bridges, which was brought about by incomplete cytokinesis. Fusomes were also found in the ring canals of the *Xenopus* cysts, a similar structure to that of *Drosophila*. The first two divisions in cyst formation are identical to that in *Drosophila*, and then the development deviates structurally. But overall the process is conserved and is consistent across many species. (Kloc et al., 2003)

Materials and Methods

Animals

The zebrafish strain used in these experiments were a wildtype strain called AB. The zebrafish were obtained from Dr. Craig Albertson's lab and Dr. Katherine Lewish' lab at Syracuse University. The zebrafish span a wide age range. The fish were grown from embryos and research was done at 2 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 4 months, 6 months and over a year old. Female fish from the desired age group were removed from fish tanks and placed into a petri dish filled with water. The zebrafish were euthanized using 1-2 ml of neural inhibitor MS222 per petri dish of fish. After 5-10 minutes the fish were determined to be dead, when their opericula no longer showed signs of movement.

Maintaining Zebrafish

Between conception and age 2 weeks, zebrafish were kept in very low water volumes. The water was cleaned using a plastic pipette to remove dead zebrafish, food, and feces.

The zebrafish were reared in 1 liter, which are part of a system in Dr. Albertson's fish room. Water was continuously cycled through the tanks to keep them clean.

The zebrafish were fed twice a day and the room was regulated to be light for 14 hours and then dark for 10 hours.

Dissection

The petri dish of zebrafish was brought to the dissecting microscope in Dr. Albertson's lab. In order to ensure that the zebrafish are dead we used scissors to dissociate the fish's head from its body, cutting the spinal cord. Next the belly of the fish was cut from the pectoral fin to the lungs and heart, exposing the innards. The heart and intestines were removed from the carcass and the ovaries are identified. They reside on either side of the swim bladder.

Whole Mount Antibody Staining of Zebrafish Ovaries

The exposed zebrafish were transferred into falcon tubes containing a fixative. The fixative is a proportion of 200:400 16% formaldehyde to 1x Phosphate-buffered saline (PBS) for a final concentration of 5.3% formaldehyde. These tubes were labeled with the age of the zebrafish and are incubated on a nutator at 4°C overnight. The next day, the ovaries were dissected from the zebrafish carcasses and transferred into labeled tubes containing 1 ml of PT (1x PBS/.01% Triton X-100). This was a wash for the ovaries and it occurs twice, quickly. Then for 30 minutes, at room temperature the ovaries were incubated with 1 ml of PT. Next the ovaries were incubated on a nutator for an hour in 1 ml of PT + 5%

Bovine serum albumin (BSA) at room temperature. At this point in the experiment, the ovaries continued to the whole mount antibody staining or stored at 4°C in 1 ml of PT + 5% BSA until needed.

After the removal of 1 ml of PT + 5% BSA, a 1 µl : 500 µl mix of primary antibody to PT + 5% BSA mixture was added to the ovary. There was a wide variety of antibodies used for this experiment.

Table 1 Antibodies Used in Staining

PRIMARY ANTIBODY	**DESCRIPTION**
VASA (abcam)	Oocyte Marker
STAT3 (Santa Cruz Biotechnology)	Oocyte Marker
PHOSPHOTYROSINE (BP Biomedicals)	Intercellular bridge/ring canal marker
ACTIN (Santa Cruz Biotechnology)	Ring canal
ORB (Developmental Studies Hybridoma Bank)	Oocyte marker
TUBULIN (Sigma)	Metaphase spindle marker
SPECTRIN (Santa Cruz Biotechnology)	Fusome marker
PHALLOIDIN (Invitrogen)	Actin

In this study I was looking for evidence that oocytes develop in cysts in zebrafish ovaries by using a variety of antibodies, shown in Table 1 that are known to stain for cyst characteristics. Fig. 2 shows examples of what these antibody stainings look like in mouse and *Drosophila*.

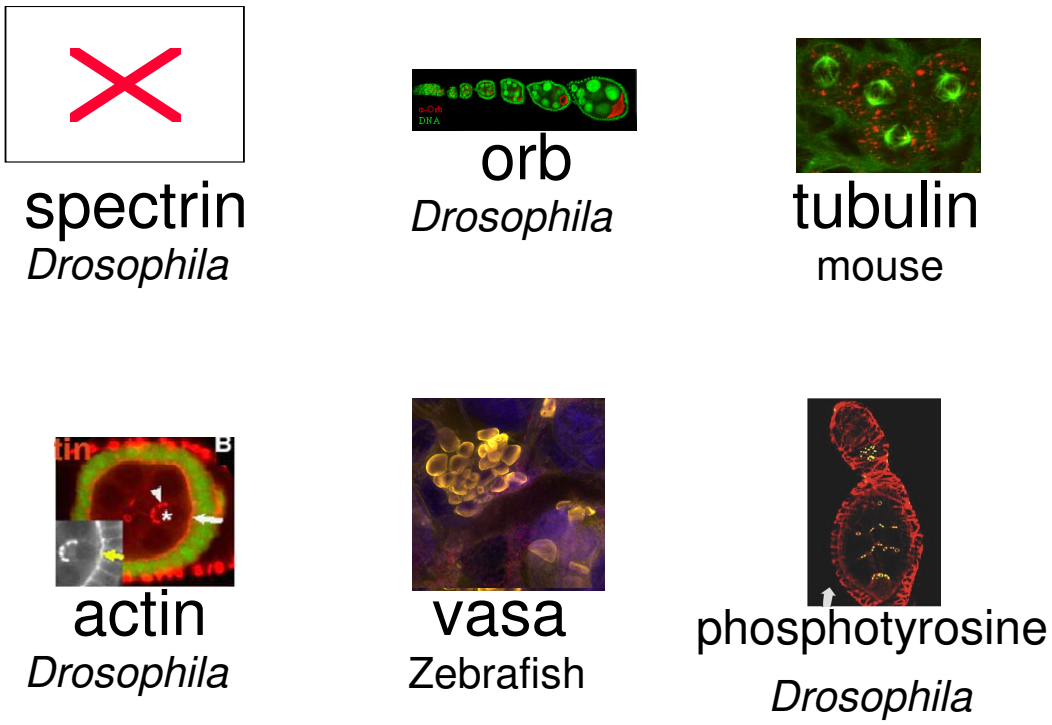


Fig. 2 *Drosophila*, mouse and zebrafish germ cells stained with Spectrin (fusome marker), Orb (oocyte marker), Tubulin (mitotic spindle marker), actin (intercellular bridge marker), Vasa, (germ cell marker) Phosphotyrosine (intercellular bridge marker)

Cyst regulation is an important developmental process conserved across many species, contributing to the fertility of female animals. The different characteristics that helped me determine that cysts exist are synchronous divisions of cyst cells, intercellular bridges and fusomes. These characteristics have been studied extensively in *Drosophila*, mouse, and now in *Xenopus* and have

confirmed the existence of cysts. There have been many antibodies used to identify germ cells. Orb is a maker of oocytes in Drosophila (Spradling et al., 1993), Stat3 is used to label the oocytes of mice (Murphy et al., 2005), and Vasa has been used to label germ cells in zebrafish already (Yoon et al., 1997). I used all three of these markers to see their efficiency in staining oocytes in zebrafish. After identifying the oocytes, I began a process of staining zebrafish ovaries with a variety of antibodies known to mark mitotic spindles (Tubulin), intercellular bridges (Phosphotyrosine), ring canals (Actin, Phosphotyrosine). Phalloidin, a stain derived from mushrooms is not an antibody but it can be used to bind to actin, thus showing the ring canals between oocytes.

Drosophila, *Xenopus*, and other insects, have organelles called fusomes in their germ cells. The fusome divides with the cell and is present in the ring canals of cysts and the fusomes can be identified by using the spectrin antibody, which is a fusome marker in fruit flies and *Xenopus* (Fuller, 1993; Kloc, et al., 2004).

The ovaries were incubated overnight with either one or two of these antibodies overnight at 4°C on the nutator.

The next day, the ovaries in the primary antibody were washed (on nutator) for 30 minutes in 1 ml of PT +1% BSA at room temperature. 10 µl of 10 mg/ml RNase and 1 ml of PT + 1% BSA was then added to the ovaries in order to digest their RNA. This mixture incubated for 30 minutes. Next, 10 µl of 0.5 mg/ml propidium

iodide (PI) (single staining) or DAPI (double staining) and 1 ml of PT + 1% BSA was added to the ovaries and incubated for 20 minutes at room temperature. After PI or DAPI (1 mg/ml diluted to 1:5000) was added, the tubes were wrapped in aluminum foil for the rest of the experiment so that the ovaries would not lose their fluorescense. After the PI or DAPI incubation, the ovaries were washed for 30 minutes at room temperature with PT +1% BSA.

The secondary antibody was prepared using 500 µl PT + 5% BSA and 2.5 µl of a secondary antibody (Table 2).

Table 2 Secondary Antibodies Used to Stain Ovary Tissue

Secondary Antibody	Goat anti-rabbit	Mouse anti-rabbit	Goat anti-Rabbit	Anti-mouse
Wavelength	Alexa 488	Alexa 488	Alexa 568	Alexa 568

The secondary antibody was then added to the ovaries and incubated overnight on the nutator at 4°C. After this incubation, the ovaries were washed three times with 1 ml PT +1%BSA for 30 minutes each time. Lastly, the ovaries were washed quickly with 1 ml of 1x PBS then removed. 100 µl of Vectashield was added to each tube for 15 minutes. After which, the ovaries were pipetted out of the tubes and mounted onto glass slides (Fisher) and covered with a coverslip (Corning). If the ovaries were too thick for the coverslip to easily cover the tissue, Vacuum

Grease was applied to seal the space between the slide and the coverslip. The coverslips were sealed with nail polish and the ovaries were stored at -20 °C.

Confocal Imaging

The stained ovaries were imaged using indirect immunofluorescence, laser scanning confocal microscopy, by way of a Zeiss LSM 710 confocal microscope. The ovaries were located and images are taken of the oocytes at 10x, 20x, 40x, and 63x normal vision.

Breaking the Chorion

Transgenic zebrafish embryos with the *ziwi* promoter driving the expression of GFP in the ovaries were shipped from Dr. Bruce Draper at UC Davis in a dilute bleach solution. The bleach prevents fungus growth but also hardens the zebrafish chorion making it difficult for the zebrafish to hatch on their own. 100 zebrafish embryos were placed in two petri dishes and using size 5 forceps, the chorions were mannualy removed from around the embryos.

GFP Imaging

Zebrafish were transferred into a 4x3 well glass plate. One zebrafish was assigned to each well, which was filled with water. A miniscule amount of MS222 was added to the well based on the size of the zebrafish. The plate was put under the GFP microscope and imaged using ultra violet light. The zebrafish ovaries were seen under the light and pictures were taken.

Results

Location and Identification of Oogonia and Oocytes in Zebrafish

Stat3 Staining

The first antibody I used is a known oocyte marker for mouse called Stat3 (Murphy et al., 2005). It is a transcription factor found in the cytoplasm of oocytes. The Stat3 antibody was successfully used to stain the oocyte in 1 year-old zebrafish and propidium Iodide (PI) was the nucleic marker used. I was able to find many images of the different staged oocytes when looking at the zebrafish ovaries stained with Stat3. In Fig. 3 it appears that two mature oocytes, surrounded by somatic cells, are bordering what looks like a cluster of very small germ cells resembling a cyst.

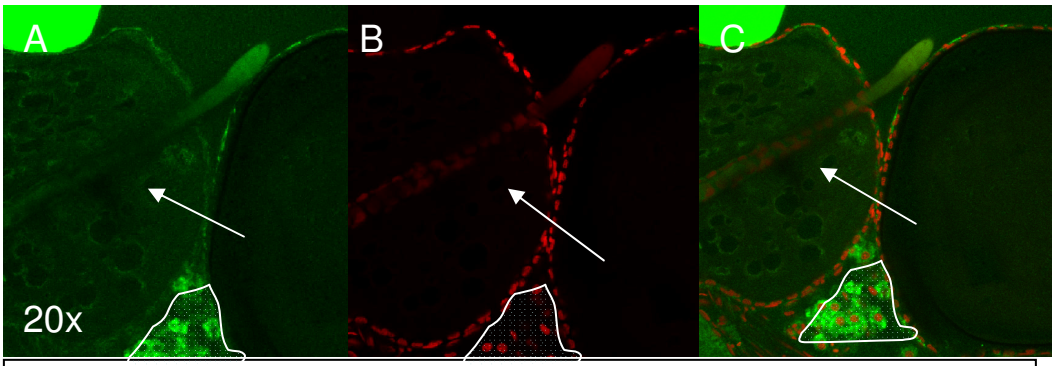


Fig. 3 Adult Zebrafish ovary. A Stat3 (Green) antibody. B Nuclei are stained with PI (red). C. Overlay of A and B. Small green cells are proposed stage I oocytes. Germinal cord shown with arrow. The germ cells are outlined in white.

In Fig. 3 there is also a structure that looks like a green tube, which resembles a germinal cord that was observed in the Medaka fish.

Orb Staining

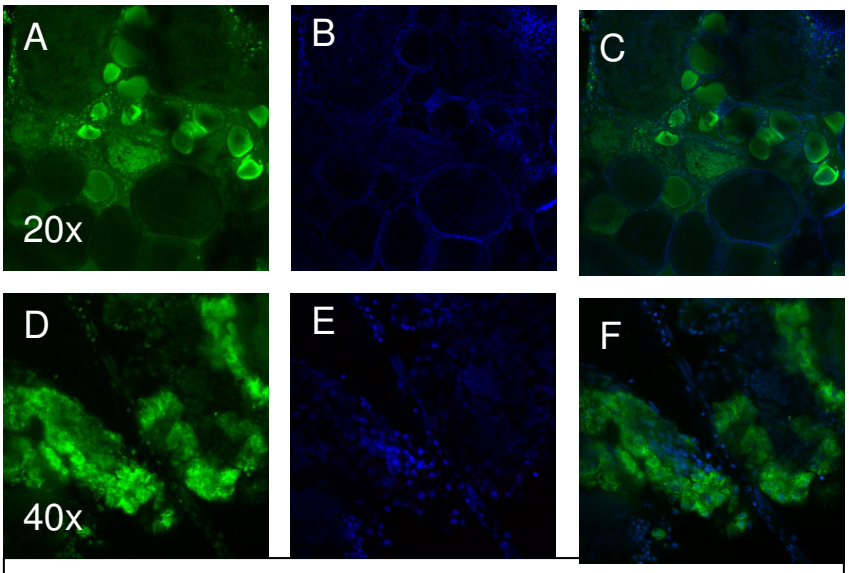


Fig. 4 1 year ovary stained with Orb (green) and DAPI (blue). A and D. Orb B and E. DAPI C and F. Overlay A-C. 20x oocytes at different stages in the ovary, D-F. 40x cluster of oocytes.

Orb was the next antibody used for identifying the oocytes in zebrafish. Orb has mainly been used to label oocytes in *Drosophila* ovarioles. In order to stain with Orb, I used DAPI as the nucleic marker and this proved to be another successful nuclei stain, as seen in Fig. 4. There are some larger more developed oocytes most likely at stage four or five, but there are also less developed oocytes present in the image. This is helpful to show the range of stages in an adult ovary. Fig. 4B shows some less developed oocytes stained in green. They appear to be in clusters as if to suggest that they are in cysts.

Vasa Staining

After the success I had with Stat3 and Orb as oocyte markers, I was interested in looking at another antibody that might stain oocytes in zebrafish. Vasa proved to be a reliable antibody and I ended up using it double stain many other antibodies to try and find cysts in zebrafish ovaries.

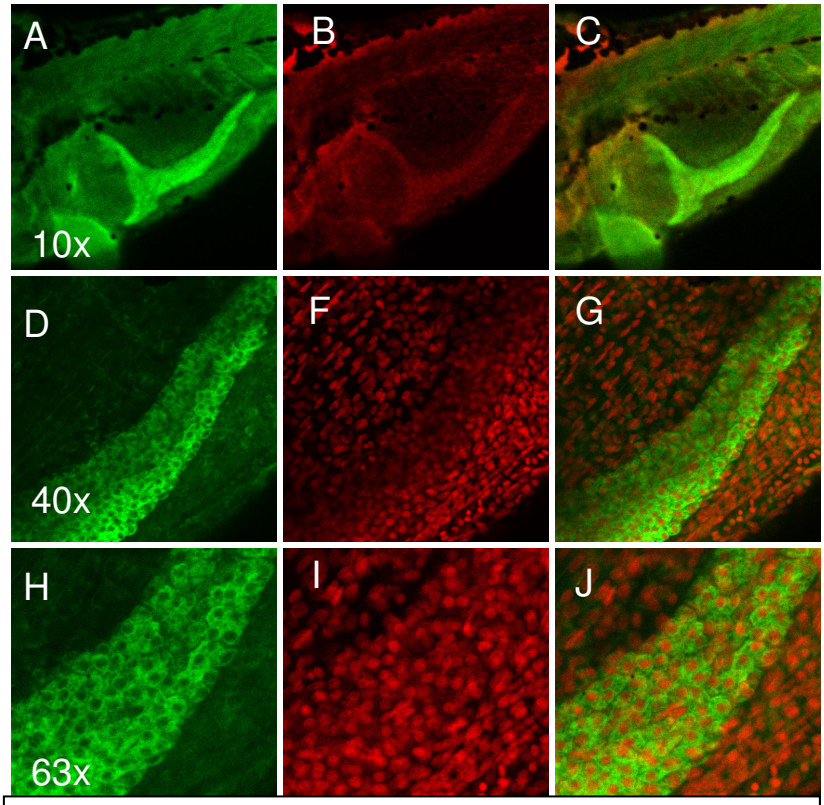
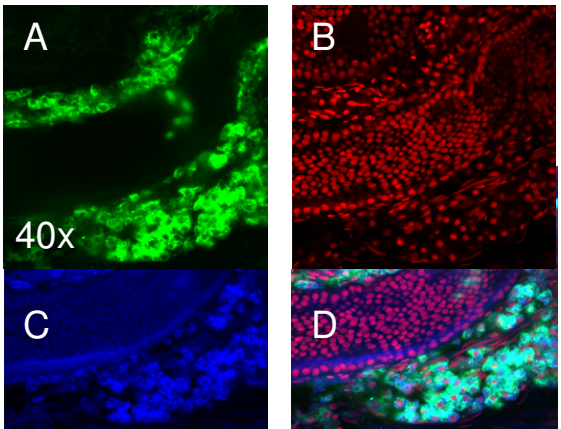


Fig. 5 Two week zebrafish ovaries. A,D,H. Vasa only (green), B,F,I. are PI only (red), C,G,J. are overlays of Vasa and PI.



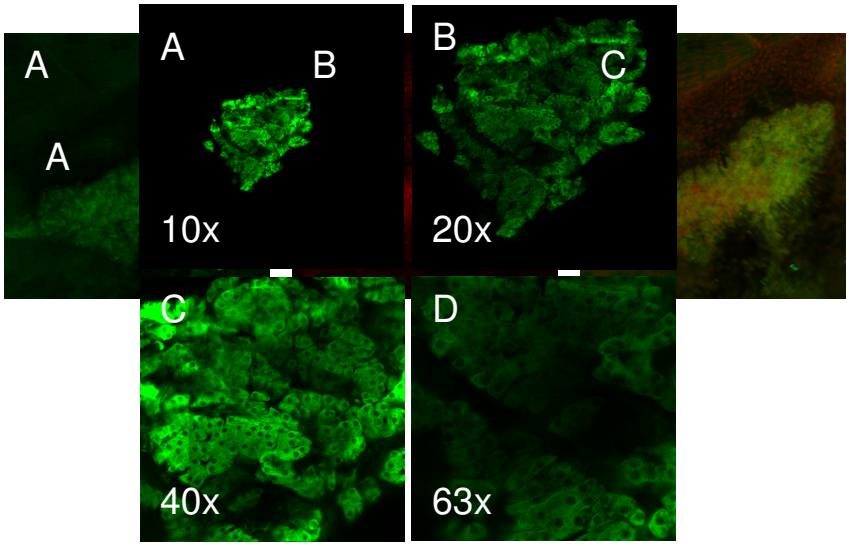


Fig. 7 Five week zebrafish ovary stained with Vasa. A. 10x, B. 20x, C. 40x and D. 63x.

Fig. 8 7.5 week zebrafish ovary. A. Stained with Vasa (green), B. Stained with PI (red) and C. An overlay of A and B.

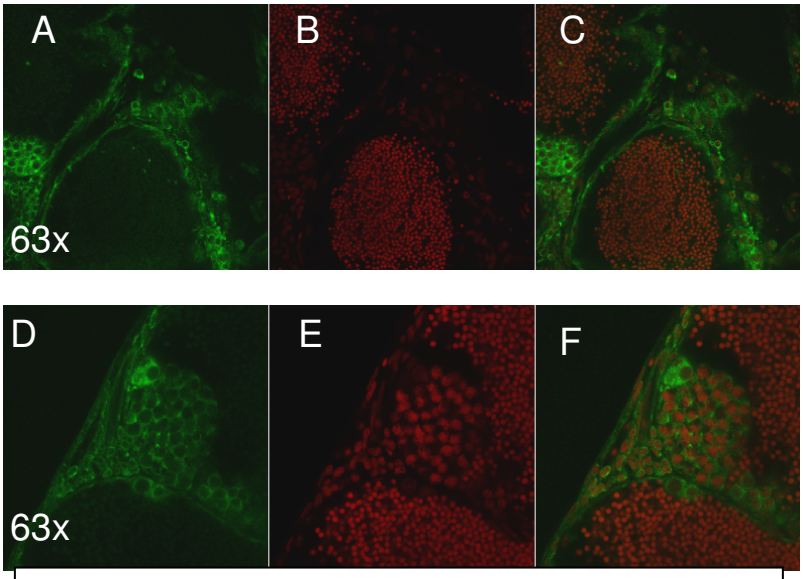


Fig. 9 Four-month zebrafish ovary. A and D. Stained with Vasa (green). B and E. Stained with PI (red). X and F. Overlay of Vasa and PI.

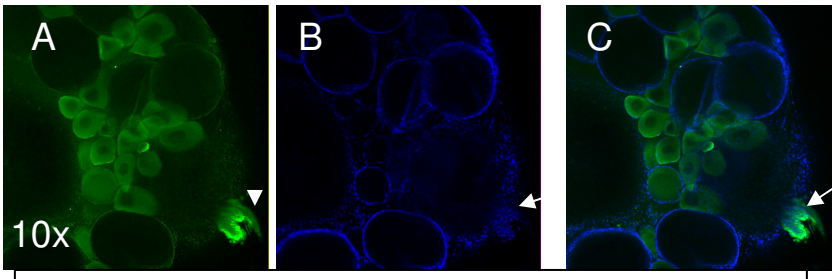


Fig. 10 1 year zebrafish ovary. A. Vasa (green). B. DAPI (blue). C. Overlay of A and B. Larger oocytes surround younger less developed oocytes. Arrows point to epithelial tissue.

Fig 5. is the first image of a zebrafish ovarian time series and shows an entire ovary in a fixed zebrafish body. It would appear that at two weeks, all of the oocytes are at one young stage in the ovary. It is uncertain if this is one large cyst because there is nothing marking the cyst characteristics. At four weeks Fig. 6, the oocytes appear more spread out. This time, the ovary was stained with

Phosphotyrosine in red and it is apparent that although the oocytes all still look to be in the same stage of development, there is some physiological changes in the ovary that was not apparent at 2 weeks, such as the cluster of somatic cells indicated by the red marker between the two sections of oocyte clusters. Fig. 7 is a series magnification of a five-week zebrafish ovary. The figure is only stained with Vasa and the cluster of oocytes is clearly defined. Once again it appears that the oocytes at this age are all connected, but it is unknown if they are interconnected by bridges because the ovaries were not stained with cyst markers. Just under two months, Fig. 8 shows a Vasa-stained ovary. The ovary is still in one large mass of young oocytes. Four months is past the critical point in ovarian development. The zebrafish in Fig. 9 is now sexually mature and as such shows a more diverse ovary. Stained in Vasa and PI, there are noticeable areas where mature oocytes have grown, while there are still clear young oocytes. Fig. 10 shows an end of an adult zebrafish ovary stained with Vasa and DAPI. There are different staged oocytes in this image, and there is even a bit of tissue labeled in green that could show the connection between the ovary and the rest of the zebrafish. After looking at the different aged ovaries using vasa as an oocyte marker, it is evident that while oocytes grow and mature into larger oocytes, some oocytes remain in their premeiotic germ cell form later in development.

GFP Transgenic Zebrafish

I was able to grow up transgenic zebrafish with GFP labeled germ cells from embryos and image them at different ages under a UV microscope. The magnification of the UV microscope was only at 10x so I was unable to see the individual oocytes. The ovaries of the zebrafish were imaged through the semi-transparent ectoderm of the live zebrafish. I began with 100 zebrafish embryos and by 4 weeks I only had 2. This hindered my live transgenic studies. The fish themselves did not grow as large as the wild-type AB zebrafish did at 2, 3 and 4 weeks. The florescence fades when the die, so even when I tried observing the dead zebrafish under immunofluorescence, through antibody staining, the images did not come out clearly. The image in Fig. 11 shows a live transgenic GFP-labeled zebrafish at four weeks old. The ovary is quite large and is located under the swim bladder, a transparent membrane filled with air.

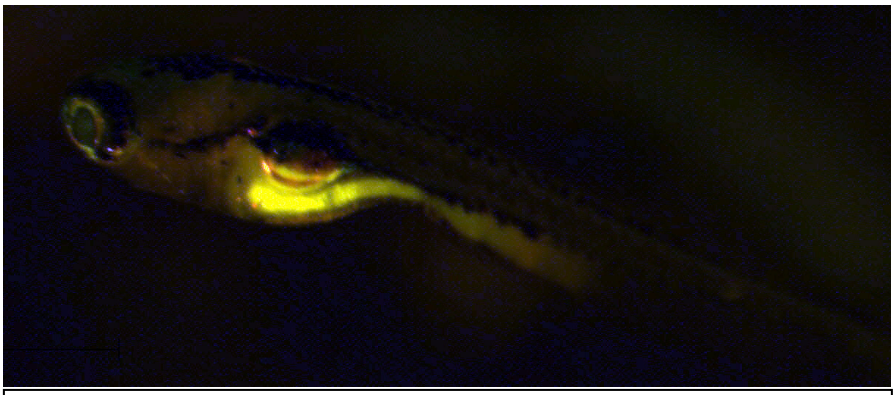


Fig. 11 Four-week old transgenic zebrafish with GFP labeled ovaries.

Do Oocytes Divide Synchronously?

Tubulin Staining

Tubulin is a mitotic spindle marker and has been used to stain cysts in *Drosophila* and mouse because it labels the metaphase spindles in synchronously dividing cells. In Fig. 12, a 1-year adult ovary section can be viewed. By observing the overlay, it is noticeable that there are red-labeled germ cells, shown with white arrows, are enveloped in a tissue that resembles a germinal cord. Because the red is staining Tubulin, these structures may be synchronously dividing cells in cysts. Bu further studies should investigate further. Fig. 13 shows a section of the zebrafish ovary at two different magnifications. Although it is still unclear if the germ cells are dividing synchronously, Fig. 13 A and B are able to reinforce the potential germinal cord structure in the zebrafish ovary.

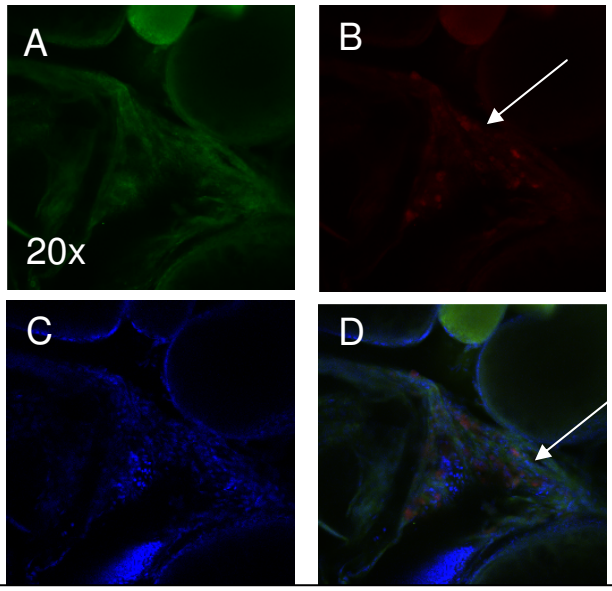


Fig. 12 1 year zebrafish ovary. A. Vasa (green). B. Tubulin (red). C. DAPI (blue). D. Overlay of A-C. Arrows are pointing at Tubulin in the germinal cradle-like epithelial tissue.

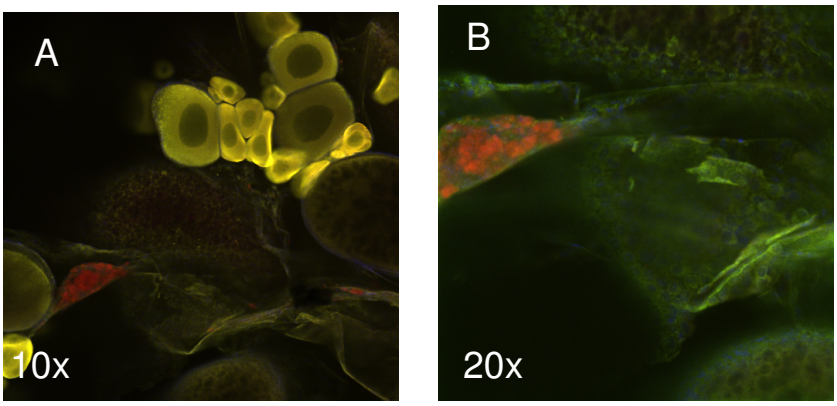


Fig. 13 1 year zebrafish ovary. A and B. double stained with Tubulin (red) and Vasa (green) with DAPI (blue). A is at a 10x magnification and B is at a 20x magnification.

Phosphotyrosine antibody recognizes phosphorylated tryrosine amino acids in cells of all species. A great way to look at intercellular bridges in *Drosophila* is using Phosphotyrosine antibody because Phosphorylated tyrosine changes the activation of proteins. If a germ cell is in a cyst, tyrosine molecules are presumably phosphorylated in the intercellular bridges and the antibody can recognize if tyrosines are present. I was unable to use Phosphotyrosine effectively. But the staining did lead to some valuable images of the ovary. Fig 14 A shows an adult ovary double stained with Vasa (green) and Phosphotyrosine (red) with nuclei stained with DAPI (blue). It would seem that the Vasa marked the younger oocytes, but the older ones are covered in somatic cells as represented by the blue, nuclei dense areas.

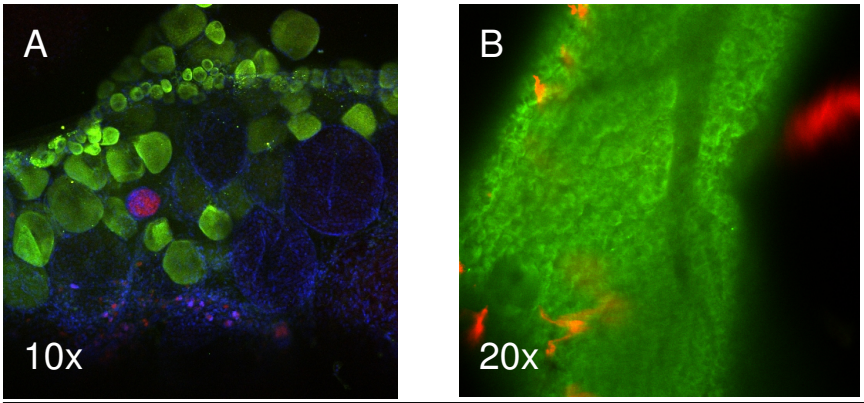


Fig. 14 Zebrafish ovaries labeled with Phosphotyrosine A. 1 year zebrafish ovary stained with phosphotyrosine (red), Vasa (green), and DAPI (blue) 10x B. 5 week zebrafish ovary stained with Phosphotyrosine (red), Vasa (green), and DAPI (blue) 20x.

In the top-most region, there appears to be a linked chain of cells. Draper et al. 2007, proposed this portion of the ovary as that where the regeneration of oocytes oocurs, growing from germline stem cells. There are regions in the lower half of Fig. 14 A that have some red staining. But it is unclear if the Phosphotyrosine has stained oocytes, because the area is not clearly labeled with green Vasa. Fig 14 B is a five-week zebrafish stained with Vasa, Phosphotyrosine, and DAPI. The image appears to be one large cluster of oocytes with some phophotyrosine staining consistent with the young ovaries discussed in the Vasa section. The Phosphotyrosine could be staining the intercellular bridges, but it is still unclear that this antibody works properly in zebrafish. These slides are two of many others that did not stain Phosphotyrosine.

Actin Staining

In order to see if cysts exist, it was necessary to stain the oocytes and somatic tissues with different antibodies that could show interconnected oocytes. The first antibody I used was Actin. I double stained Actin and Vasa, using DAPI as the nuclei marker. Although I was trying to find images that identified intercellular bridges in zebrafish ovaries, the Actin antibody I used was ineffective in marking intercellular bridges. Fig. 15 shows that Actin stains the oocytes, suggesting that Actin is a part of the oocyte, perhaps its membranous portion.

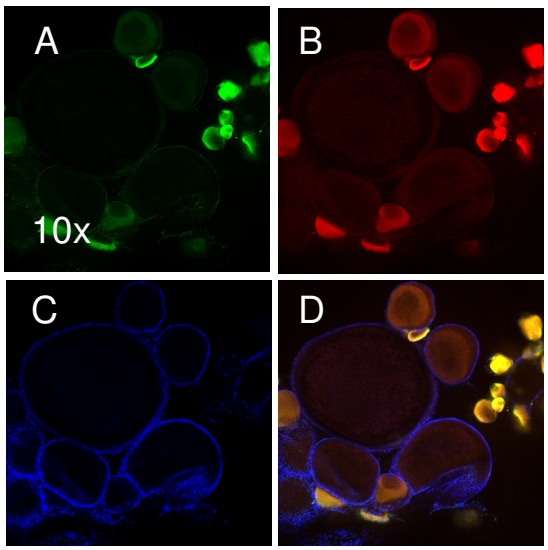
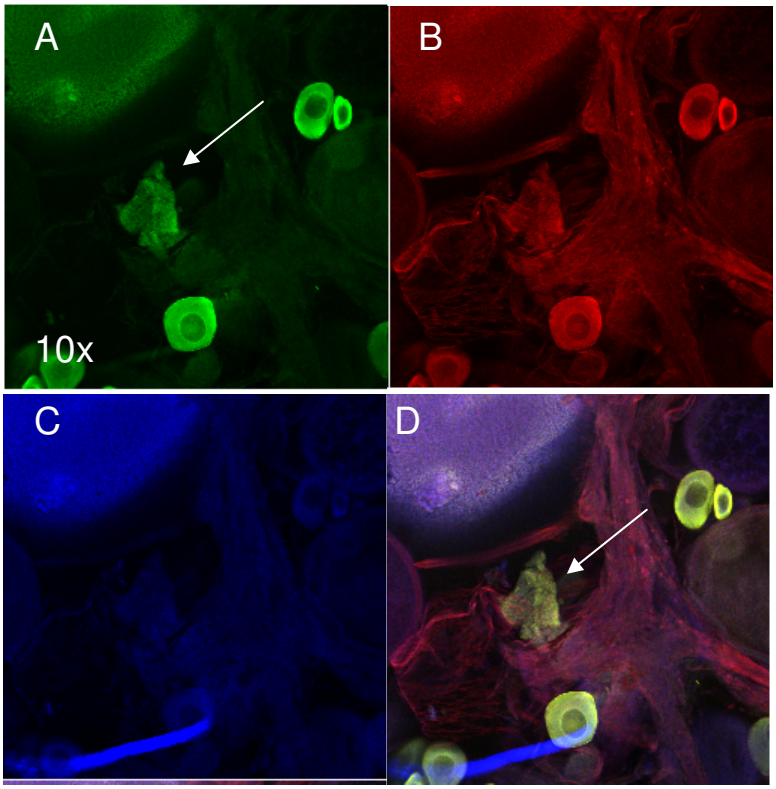


Fig. 15 1 year zebrafish ovary. A. Vasa (green). (B), Actin (red), stains mitotic spindles. C. DAPI (blue). D. Overlay of A-C.

Phalloidin 568 Staining

By staining with phalloidin an F-actin binding protein derived from the mushroom, *Amanita phalloides,* I was hoping to use phalloidin as a tool to see ring canals between oocytes in cyts. I was able to image a wide range of ovarian physiology in zebrafish. Phalloidin stains very clearly and very strongly in many of the ovaries that I stained as seen in Fig. 16, 17 and 18. Fig. Looking at the adult ovary, I was able to locate structures like Fig. 16 A-D, that were clusters of small, potentially stage I oocytes hidden among more developed oocytes and ovarian tissues. After I located this structure, I was able to "zoom in" and look at the physical composition of the oocyte cluster. I was struck by Fig. 16 E-H, which showed two stage II oocytes attached to the end of the stage I cluster. This was an exciting find because it would seem that the two oocytes were growing from the cluster of oocytes. The next observation showed the phalloidin-labeled Actin containing all of the oocytes together in the cluster as seen in Fig. 17 A-D. The arrows in Fig. 17 B and C are pointing to the Actin in red. It would appear that the stage I, or perhaps even PGCs, are connected by the Actin and holds them in place until the appointed time of growth. Fig. 17 E and H shows another structure, clearly labeled in green and blue to show oocytes, in Fig. 17 F, it is clear that the phalloidin stained Actin is present between these oocytes, that are clustered in what has been coined a germinal cord. Phalloidin was used to locate the intercellular bridges in *Xenopus* (Kloc et al., 2004) and it would appear that the zebrafish oocytes are organized in a similar fashion with Actin connecting all of

the oocytes in the clusters. At 63x magnification, Fig 18 A-D, I observed another interesting structure. It would appear that the oocytes in the germinal cord are maturing. Fig. 18 D shows an overlay of the Vasa-labeled oocytes and Actin. The arrows in A and D are pointing to larger oocytes, while the arrows in B and D are pointing to more primordial germ cells.



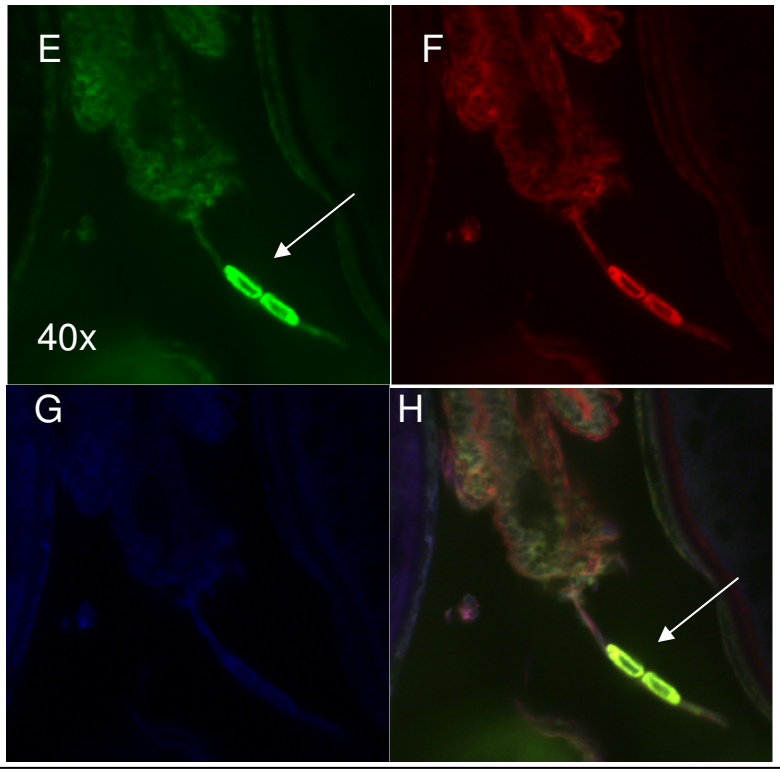
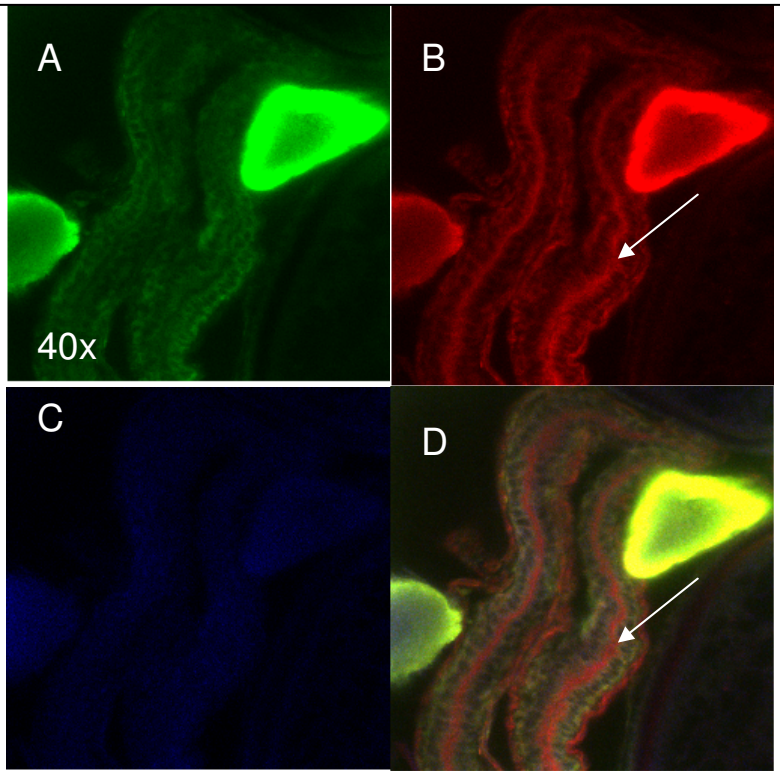


Fig. 16 1 year zebrafish ovary. A. stains Phalloidin , B. Stains Vasa , and C. stains DAPI. A-D is at 10x. The arrows in A and D are pointing to a cluster of young oocytes. The arrows in E and H point to an enlarged image of the A-D cluster with two older staged oocytes attached.



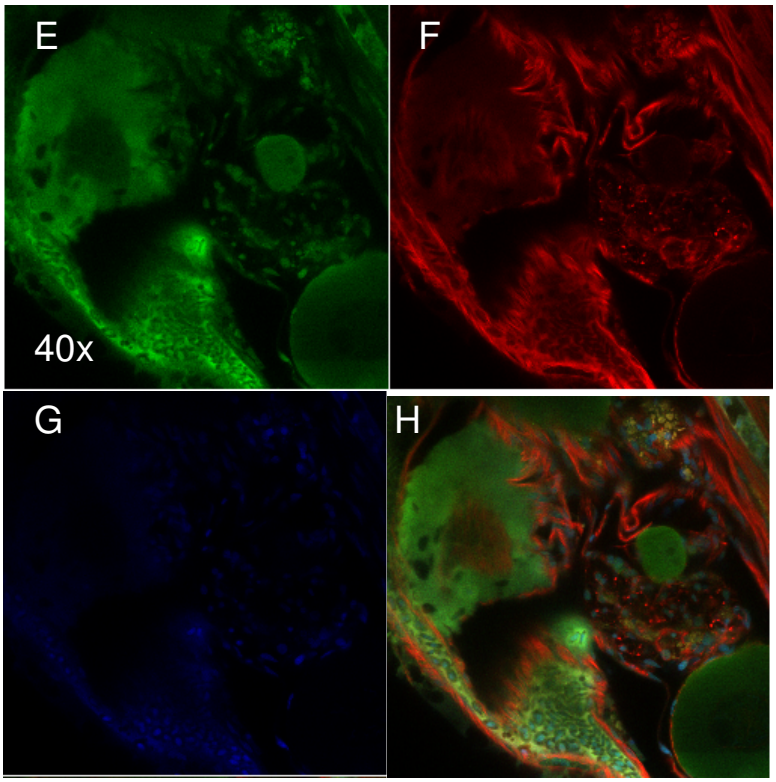


Fig. 17 1 year zebrafish ovary. A. Phalloidin (green). B. Vasa (red). C. DAPI (blue). D. Overlay of A-C. A-D shows structure with oocytes arrow points to a cluster of young oocytes. E-H is an image of clusters of oocytes in green encircled by Actin at 40x.

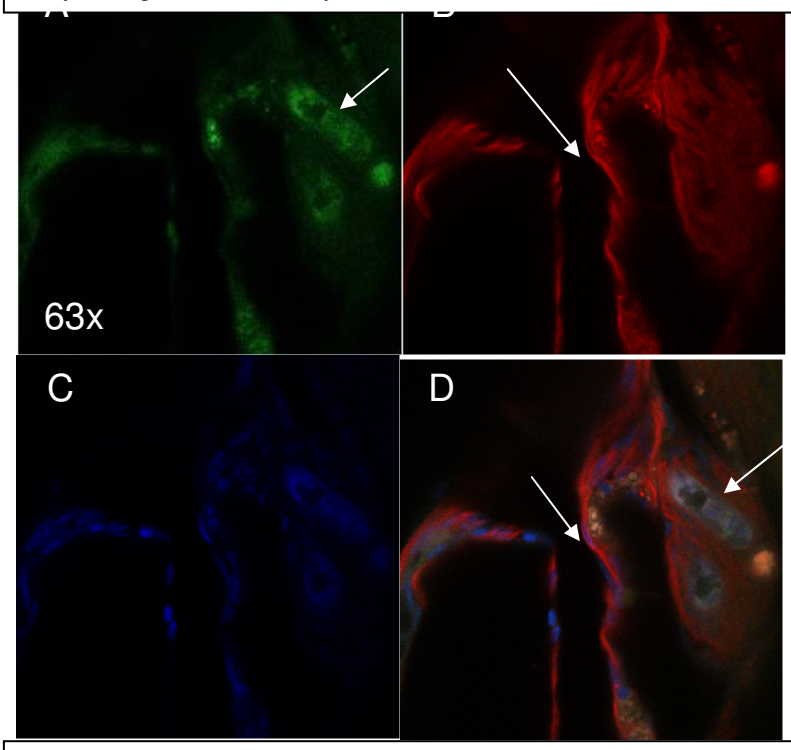


Fig. 18 1 year zebrafish ovary. A. Phalloidin (green). B. Vasa (red). C. DAPI (blue). D. An overlay of A-C. A-D shows oocytes in a actin-rich tissue. The arrows in A and D are pointing to potential oocyte follicles while the arrows in B and D are pointing to the proposed germinal cord.

Can Fusomes Be Detected?

Spectrin Staining

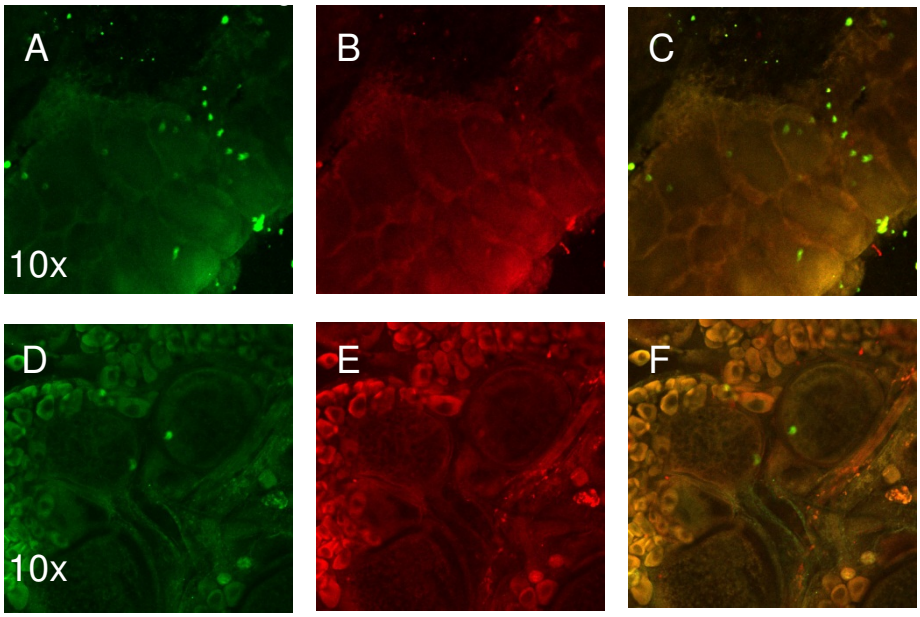


Fig. 19 1-year zebrafish ovary stained with Spectrin (green), Vasa (red). A-C shows a cluster of oocytes with green spectrin specs between the oocytes. D-F show two mature oocytes stained in red, each with a green, Spectrin labeled speck.

Spectrin is an important antibody used to stain fusomes in dividing oocytes of *Drosophila* and *Xenopus*. Although fusomes have not be observed in mouse, it is clear that the fusome is conserved in *Drosophila*, *Xenopus* as well as other insects and vertebrates. I used two different spectrin antibodies Spectrin alpha II (C-11) and Anti-human Spctrin to try and identify fusomes in zebrafish. I was only able to obtain results from Spectrin alpha II (C-11). And although I stained in a wide age range of zebrafish ovaries, staining was only present in the adult ovaries. The green specks observed in Fig. 19 are observed in each of the oocytes. Though in *Drosophila* and *Xenopus* studies the fusome has been found in the intercellular bridges of the cystocytes, not maturing oocytes, I was only able to find these in the individual older oocytes that do not appear to be in cysts. I observed an example of a germinal cradle as described in Nakamura et al., 2010 (Fig. 20). In this image, there are four oocytes stained with Spectrin (green), Vasa (red) and DAPI (blue). The Spectin that should be staining the fusomes, seems to be staining the germinal cord. And in B it is apparent that oocytes are connected by green tissue, perhaps cysts. Also in Fig. 20, are single isolated germ cells as identified in the Medaka by Nakamura et al., 2010.

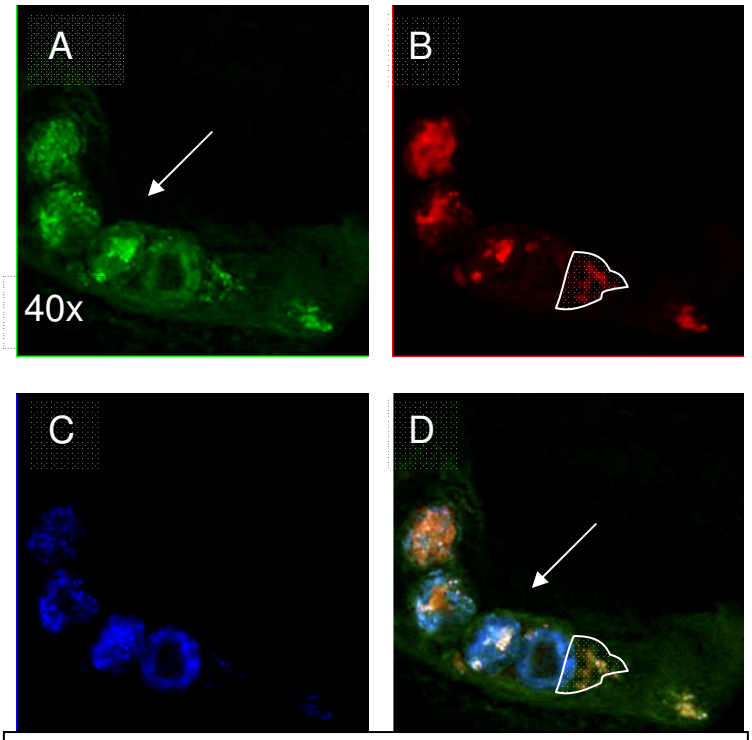


Fig. 20 Germinal cradle-like structure in adult zebrafish ovary stained with Spectrin (green), Vasa (red), and DAPI (blue). A and D. Arrows point to four germ cells in cyst-like structure. B and D. Individual germ cells are shown encircled by the white lines. A-D. shows individual germ cells that appear to be moving up the ovarian cord to the germinal cradle of the germinal cord.

Discussion

The oogenesis of zebrafish is still not well understood. Although zebrafish are a highly studied organism for developmental purposes, only in recent years has research gone into the reproductive process (Draper et al., 2002). Bruce Draper at UC Davis has been the most notable source for staging oocytes in zebrafish, and establishing a comparison for my work on determining cysts in zebrafish ovaries. Draper discusses finding potential germline stem cells in the germinal zone of the zebrafish ovary, but did not continue to discuss any cyst findings (Draper et al.,

2007). While I was unable to stain the germinal zone effectively with intercellular bridge and mitotic spindle markers, tubulin, phosphotyrosine, and actin, my findings suggest that cysts may be located elsewhere in the ovary, possibly in the germinal cords described in the Medaka paper (Nakamura et al., 2010).

I was successful in using the three antibodies, Stat3, Orb, and Vasa to stain oocytes in zebrafish ovaires. Vasa has been used by those studying zebrafish, other teleosts and *Xenopus* already, but I was unable to find any literature that discussed using Stat3 or Orb to study oocytes in zebrafish. These findings may be useful for double staining ovaries, especially because these antibodies are made in different organisms so different secondary antibodies can be used if necessary for double staining. Staining with different oocyte markers as well as nuceli markers (PI, TOTO3, and DAPI) helped me to gain a greater understanding of the physiology of the zebrafish ovary. Beginning this project, the ovary of the zebrafish was completely unknown to me. But by using Stat3, Orb, and Vasa, I was able to distinguish mature oocytes from growing oocytes and growing oocytes from conserved clusters of oocytes that were present from two weeks of age to sexually mature adults. These young premeiotic oocytes were not mentioned in the literature on zebrafish ovaries. In fact, I was unable to confirm some of the claims that show cysts on the surface of the ovary. It was deeper, between larger, more mature oocytes that I observed the structures that show very small oocytes. I first observed a germinal cord using the Stat3 antibody and did not know what it was. There were many images that I obtained that included long

strands of tissue with tiny germ cells inside them. These structures were nothing that I had found to be reported before and did not concur with *Drosophila* or mouse or even *Xenopus* studies. It was not until very recently when a Professor from Yale suggested I read Nakamura's paper on the Medaka fish that I was able to piece together all of my findings with those that have been done in the Medaka fish (Nakamaru et al., 2010). The tube-like tissues I was looking at were ovarian cords that contained individual germ cells, germline cysts, and produced oocyte follicles.

To determine if the germ cells divided synchronously, I first used tubulin, hoping to identify mitotic spindles, a characteristic of cysts. After observing many ovaries stained with tubulin at different ages, I found tubulin staining in the adult ovaries in epithelial tissues interlacing the maturing oocytes. Initially I was looking for cysts in the germinal zone as already mentioned (Draper et al., 2010). The results I obtained were surprising because Tubulin only stained inside pockets of membranous tissues, not between normal oocytes as has been observed. At first I was completely thrown off and did not know the meaning of the red staining. But now it is proposed that oocytes may be undergoing cyst formation in the germinal cords. This finding would be consistent with that in Medaka, and possibly other organisms. Now that I am able to understand the potential meaning of the Tubulin staining in zebrafish ovaries, I would want to stain again with different dilutions of Tubulin to see if the results varied and try and see if Tubulin could stain in younger zebrafish ovaries. Are there cysts

forming in sexually immature oocytes? Or is it not until zebrafish reach sexual maturity that the oocytes go through mitosis with incomplete cytokinesis?

Although Phosphotyrosine was not a useful marker for determining intercellular bridges between oocytes in zebrafish, it is unclear if the antibody should be ruled out completely or if it is telling us that the cells are not undergoing mitosis where we might expect. In a five-week zebrafish the staining is not reliable. I was only able to obtain one image where Phosphotyrosine stained and I do not know if it is staining the oocyetes or some other tissue. The adult image stained with Phosphotyrosine is a little more helpful. If the marker is staining the intercellular bridges, then they are not in the germinal zone (locate on the dorsal side) at all but on the bottom (ventral side) of the ovary. Again, I cannot conclude that Phosphotyrosine stains the intercellular bridges properly, but as of yet no studies have been done using this antibody so I am unable to validate my observations. Similar to Tubulin, future studies should include repeating my experiments with different concentrations of the Phosphotyrosine antibody.

Actin stained the membranes of oocytes as seen in Fig. 8. All of the oocytes were stained with both Vasa and Actin suggesting that like Vasa, Actin is present in oocytes. The Actin staining that I performed was not successful in staining intercellurlar bridges from what I observed, which proposes that Actin is either not an intercellular bridge marker in zebrafish, or there are not intercellular bridges between oocytes of zebrafish. A third hypothesis is that the Actin

antibody I was using was not at the correct dilution or concentration and thus, unable to penetrate into the depths of the ovary where I found germinal cords stained with phalloidin-labeled Actin. I was unable to locate any significant Actin staining in young oocytes with the Actin antibody I used.

I was able to locate Actin staining by using phalloidin, an Actin-binding stain. Phalloidin staining provides great insight into the structure of germinal cords in zebrafish. Like the other mitotic spindle and intercellular bridge markers, I was only able to see clear phalloidin in adult ovaries, which further emphasizes that germ cells may not form cysts until 3 mpf (months post fertilization). The actin that surrounds the oocytes in germinal cords is illuminated by phalloidin, which is expressed stronger than the Actin antibody I used. The structures I found in zebrafish have never been observed before. I cannot confidently say that these structures are indeed germinal cords, or that the germ cells go through a process where they form cysts, and become primordial follicles as stated in other organisms. All I can say with certainty is that the structures that I have observed labeled with phalloidin show preliminary evidence to support that there are clusters of germ cells connected by Actin and from these clusters arise oocyte follicles. The germinal cord-like structures that I observed are consistent with those in the Medaka fish and it was fortunate I had such a comparable opportunity.

Upon looking for fusomes, I can conclude that the Spectrin antibody was useful staining Spectrin proteins in adult zebrafish ovaries (Fig. 19 and 20). I was able to identify similar fusome-like structures to those found in *Drosophila* and *Xenopus,* which suggest that the cells are either in cysts or merely that they are germ cells. If this hypothesis were true, studies would not need to be done to look at embryonic germline development in regard to cyst formation, which is what I attempted to do with GFP transgenic fish.

The most critical image I have is stained in spectrin (Fig. 20) and is the first clue I got into the oogenesis of zebrafish. The oocytes are enveloped by a Spectrin rich structure. It is not necessarily important whether or not the green-labeled structure is a fusome, what is important is that I was able to compare an image of what appears to be four connected germ cells to an image from the paper on the Medaka fish (Nakamura, et al., 2010). This image (Fig. 1B) shows the progression of germ cell development in the germinal cavity of the ovarian cord. My results suggest a similar process. That the individual germ cells outlined in white enter mitosis with incomplete cytokinesis. Some of these cyst-like cells undergo apoptosis and the remainder, develop into follicles that will mature into oocytes that will go through five stages of development until they are mature eggs ready to be spawned and fertilized. My research suggests that there are germline cysts in zebrafish ovaries. The zebrafish is an organism that can be added to the repertoire of cyst-forming animals.

Future Directions

Although I have preliminary data to support the hypothesis that germline cysts exist in adult zebrafish ovaries, I would expect experiments to be done to repeat my studies in order to confirm my claim. Also, it would be valuable to image zebrafish embryogenesis with transgenic GFP-labeled germ cells in order to watch the development of the zebrafish ovary. Initially I postulated that it would be important to image embryonic ovarian development because in mouse, human, and *Xenopus* cysts form before the embryo is hatched. But it would seem based on my data and on other studies that state that zebrafish do not reach sexual maturity until 3 mpf that zebrafish germ cells may not form cysts until later in development, perhaps closer to the time-line of 3 mpf. In the future, studies should be conducted to learn more about the mechanisms behind cyst formation and breakdown as have been done in *Drosophila* and mice.

References

Braat, A. E., Zandbergen, T., Van de Water, S., Goos, H., Zivkovic, D. "Characterization of Zebrafish Primordial Germ Cells: Morphology and Early Distribution of vasa RNA." Developmental Dynamics 216: 153-167 (1999).

Ciruna, B., Weidinger, G., Knaut, H., Thisse, B., Thisse, C., Raz, E., Schier, A. "Production of maternal-zygotic mutant zebrafish germ-line replacement." PNAS, 99: 14919:14924 (2002).

Clelland, E., Peng, C. "Endocrine / Paracrine Control of Zebrafish Ovarian Development." Molecular and Cellular Endocrinology 312: 42-52 (2009).

de Cuevas M, Lilly MA, Spradling AC. "Germline cyst formation in drosophila. Annual Revue Genetics 31: 405-428 (1997).

Draper, D. H., McCallum, C. M., Moens, C. B. “nanos1 is required to maintain oocyte production in adult zebrafish.” Developmental Biology 305: 589-598 (2007).

Doitsidou, M., Reichman-Fried, M., Stebler, J., Köprunner, M. et al. “Guidance of Primordial Germ Cell Migration by the Chemokine SDF-1.” Cell 111: 647-659 (2002).

Fuller, M., “The Development of Drosophila melanogaster.” Development 122: 1125-1135 (1993).

Gilboa, L. and Lehmann, R. “How different is Venus from Mars? The genetics of germ-line cells in Drosophila females and males.” Development. 131: 4895-4905 (2004).

Gondos B. “Oogonia and Oocytes in Mammals”. The Vertebrate Ovary 83-120 (1978).

Hsiao, C. and Tsai, H. “Transgenic zebrafish with fluorescent germ cell: a useful tool to visualize germ cell proliferation and juvenile hermaphroditism in vivo.” Developmental Biology 262: 313-323 (2003).

Iguchi T, Takasugi N. “Poloyovular follicles in the ovary of Immature Mice Exposed to Prenatally to Diethylstilbestrol”. Anatomy Embryology 175: 53-55 (1986).

Jefferson WN, Couse JF, Padilla-Banks E, Korach KS, Newbold RR. “Neonatal Exposure to Genestein Induces Estrogen Receptor (EF) a Expression and Multioocyte Follicles in the Maturing Mouse Ovary: Evidence for ERB-mediated and nonestrogenic actions”. Biology Reproduction 67: 1285-1296 (2002).

Kezele P, Skinner MK. “Cell-cell Interactions in Primordial Follicle Assembly and Development.” Front Biosci 7: d1990-1996 (2002).

Kezele P, Skinner MK. “Regulation of Ovarian Primordial Follicle Assembly and Development by Estrogen and Progesterone: Endorcrine Model of Follicle Assembly. Endocrinology 144: 3329-3337 (2003).

Kimmel, C., Ballard, W., Kimmel, S., Ullmann, B. and Schilling, T. F. “Stages of Ebryonic Development of the Zebrafish.” Developmental Dynamics 203: 253-310 (1995).

Kloc, M., Bilinski, S., Dougherty, M.T., Brey, E. M., and Etkin, L. D. "Formation, architecture and polarity of germline cyst in Xenopus." Developmental Biology 266: 43-61 (2004).

Knaut, H., Werz, C., Geisler, R., The Tüblingen 2000 Sreen Consortium, and Nüsslein-Volhard, C. "A Zebrafish Homologue of the Chemokine Receptor Cxcr4 is a Germ-cell Guidance Receptor." Nature 421: 479-485 (2001).

Leu, D. H., Draper, B. W. "The *ziwi* Promoter Drives Germline-Specific Gene Expression in Zebrafish." Developmental Dynamics 1-8 (2010).

Murphy, D., Carvajal, L., Medico, L., and Pepling, M. "Expressio of Stat3 in germ cells of developming and adult mouse ovaries and testes." Gene Expression Patterns 5: 475-482 (2005).

Nakamura, S., Kobayashi, K., Nishimura, T., Higashijima, S. Tanaka, M., "Identification of Germline Stem Cells in the Ovary of the Teleost Medaka." Science 28: 1561-1563 (2010).

Pang, Y., Thomas, P. "Role of G protein-coupled estrogen receptor 1, GPER, in inhibition of oocyte maturation by endogenous estrogens in zebrafish." Developmental Biology 342: 194-206 (2010).

Pepling, M. E., and Spradling, A. C. "Female mouse germ cells form synchronously dividing cysts." Development 125: 3323-3328 (1998).

Pepling, M. E., de Cuevas, M., Spradling, A. C. "Germline cysts: a conserved phase of germ cell development?" Cell Biology 9: 257-262 (1999).

Pepling ME, Spradling AC. "The Mouse Ovary Contains Germ Cell Cysts that Undergo Programmed Breakdown to Form Follicles". Developmental Biology 234: 339-351 (2001).

Pepling, M. E. "From Primordial Germ Cell to Primordial Follicle: Mammalian Female Germ Cell Development." Genesis 44: 622-632 (2006).

Spradling AC. " Developmental Genetics of Oogenesis". The Development of *Drosophila melanogaster* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press 1-70 (1993).

Slanchev, K., Stebler, J., de la Cueva-Méndez, G., Raz, E. "Development Without Germ Cells: The Role of the Germ Line in Zebrafish Sex Differentiation." PNAS 11: 4074-4079 (2005).

Tilly, J. T. "Apoptosis and ovarian function." Reviews of Reproduction 1: 162-172 (1996).

Uchida, D., Yamashita, M., Kitano, T., Iguchi, T. "Oocyte apoptosis during the transistion from ovary-like tissue to testes during sex differentiation of juvenile zebrafish." The Journal of Developmental Biology 205: 711-718 (2002).

von Hofsten, J. and Olsson, P. "Zebrafish sex determination and differentiation: Involvement of FTZ-FI genes." Reproductive Biology and Endocrinology 3:63 1-11 (2005).

Yoon, C., Kawakami, K., Hopkins, N. "Zebrafish vasa Homologue RNA is localized to Cleavage Planes of 2- and 4- cell-stage embryos and is Expressed in the Primordial Germ Cells." Development 124: 3157-3166 (1997).

Capstone Project Summary

Currently research is being done to treat and cure many of today's diseases, from the flu to cancer. Although infertility is not a life threatening disorder, it is an inhibitor of human progression. Thus, it is vitally important for us to study the reasons why infertility arises so that we can take action and either reverse the effects or take preventative measures to ensure mothers can have children if they

so choose. While males also have infertility problems, the Pepling lab that I work in focuses mainly on studying the female reproductive system. A woman is born with all the eggs she will ever have for reproduction. The fact that many women today are waiting longer and longer to have children may explain why infertility is on the rise. Eggs work best when they are young, before their expiration date, so to speak. The longer a woman waits to have children, the more exposure a woman's eggs have to environmental, and mutagenic factors. There are many components that play a role in female infertility, but there is evidence, which suggests estrogen-like compounds disturb proper egg development. When a woman is pregnant with a baby girl the foods the mother eats and the chemicals she is exposed to affect her baby's reproductive development. BPA, a chemical used in plastics and found in water bottles, is an example of an estrogen-like chemical that has negative effects on the reproductive success of females.

Today there is a relatively conserved developmental process essential for proper egg development. This process is called cyst formation and regulation. Cysts are clusters or nests of germ cells (precursors to eggs) connected by intercellular bridges. There are cysts in fruit fly ovaries, mouse ovaries, frog ovaries, hamster ovaries, fish ovaries and most importantly human ovaries. Cysts are formed when one germ cell, divides, but instead of completely dividing in two, remains connected by intercellular bridges. Then each of those cells divides, again without a complete separation. This process continues until cysts are formed. Depending on the organism cysts grow until reaching a different number of interconnected

cells. At a certain point in developments cells within the cysts are signaled to die, and cyst breakdown begins. Cyst breakdown is vital for cyst regulation because it frees up the egg cells allowing them to mature into normal egg cells for future development and fertilization. When cyst breakdown does not occur, females from many different organisms have been observed to be infertile in their adult lives.

Although the Pepling lab uses mice as a model organism to study cyst formation and regulation, I decided to go in different direction with my Capstone project. I began research on the zebrafish in order to add another organism to the list of those known to have cysts. Starting out, I had to get to know the anatomy of the zebrafish. Because the reproductive development of the zebrafish is not well known, I did a lot of independent investigations. First I practiced dissecting preserved adult zebrafish. Then, once I became comfortable with where the ovaries of the zebrafish are, I began my studies. I was unable to find any literature that definitively found cysts in zebrafish, so I was excited to find out if I could. The Medaka fish has recently been shown to have cysts in its ovary. This is great news because the Medaka and the zebrafish are very similar structurally.

How does one find a cyst? You may ask. Well by adapting protocols from research done with mice and fruit flies, I was able to dissect the ovaries of the zebrafish, fix the ovaries so that the proteins in the cells would not die, stain the ovaries with different antibodies that bind to the proteins in the germ cells and to

cyst characteristic proteins. Cyst characteristics include intercellular bridges, mitotic spindles, actin, and fusomes. All of these organelles are indicators of cysts. By staining the zebrafish ovaries with antibodies that can recognize the proteins that make up these organelles, I hoped to determine if cysts existed. The antibodies are attached to secondary antibodies that are colored with florescent dyes. So when I put a slide with a zebrafish ovary stained with these antibodies under the confocal microscope, which cuts through thick tissues using a laser, I hoped to see colors, showing that I had indeed found cysts.

After using many different antibodies on zebrafish ovaries from a variety of different ages, I came to the personal conclusion that cysts are not apparent in young zebrafish ovaries. I was only able to achieve antibody staining of the cyst markers in adult zebrafish ovaries. I was not able to find cyst structures that compared to fruit flies or to the mouse. But I was able to find cyst-like structures that compared to the Medaka fish. In the Medaka, cysts are found in structures called germinal cradles, housed in long strands of epithelial tissue called ovarian cords. Individual germ cells form cysts in the germinal cradles and then cyst breakdown occurs leading to egg follicles. My studies have revealed structures that are comparative to those in this Medaka fish. Although I was unable to go back and confirm my findings with more antibody staining due to the completion of my Capstone project, I am excited to present preliminary data that supports germline cysts in zebrafish. Please look at the images in the Capstone to add to the summary I have given on my research with the zebrafish ovary.

The studies that I have done will hopefully be continued in future investigations into the germline cysts of zebrafish. Once cysts are confirmed to be present in the zebrafish ovary, then research can continue to study the mechanisms behind cyst formation and regulation. Then I hope zebrafish can be added to the repertoire of model organism used to study infertility. And then! One day, infertility will be a disorder of history.