ABSTRACT

In mammals, formation of the primordial follicle is a complex process involving the breakdown of germ cell cysts, where oocytes must separate from each other and subsequently become surrounded by somatic cells. As cysts separate, a large number of germ cells are lost by apoptosis, however the mechanisms by which cyst breakdown and germ cell death occur are not well understood. We first hypothesized that two anti-apoptotic regulators from the BCL2 family of proteins, BCL2 and MCL1, may be responsible for regulating neonatal oocyte survival. To elucidate the effects of BCL2 in the neonatal ovary, we examined ovaries of both *Bcl2* overexpressing and knockout transgenic mice. When compared to wild-type mice, neither *Bcl2* overexpression nor abrogation significantly altered ovarian histology. Another BCL2 family protein, MCL1, is expressed in human oocytes during ovarian development, suggesting a role for MCL1 in oocyte survival. We found that MCL1 was localized to both oocytes and somatic cells during cyst breakdown. Subsequently, we used an *in vitro* organ culture system to identify a role for MCL1 in oocyte survival. We found that inhibition of MCL1 with an antibody to MCL1 in culture resulted altered germ cell numbers and oocyte cyst breakdown. Our data demonstrate that while BCL2 is not likely involved in perinatal oocyte survival, MCL1 may be an important regulator of the ovarian primordial follicle reserve. Next, we hypothesized that the KIT signaling pathway may be important for oocyte survival and cyst breakdown in the neonatal ovary. The tyrosine kinase receptor, KIT, and its ligand, KITL, have been implicated in oocyte survival and follicle development in both fetal and adult ovaries but have not been well studied at the perinatal time point. To elucidate the functional role of KIT signaling in the neonatal ovary, we began by using immunohistochemistry to test the expression of KIT and KITL. We found both proteins to be expressed in the developing ovary from 17.5 dpc to PND 3, suggesting an

important role for this protein in cyst breakdown or oocyte survival. To test this hypothesis, ovaries from 17.5 dpc fetal mice were cultured for 5 days in control media, or in media with the KIT blocking antibody, ACK2, or recombinant KITL. Our data demonstrated a role for KIT signaling in cyst breakdown, as inhibition and activation of the pathway altered ovarian histology. Using cell proliferation and TUNEL assays at the conclusion of culture, we identified a reduction in somatic cell proliferation when KIT signaling was inhibited and likewise, a decrease in cell death. Finally, we investigated which pathway downstream of KIT affects cyst breakdown and the effect of KIT signaling on MCL1 protein expression. After 3 days in culture with KITL supplemented media, Western blotting was used to analyze the total and phosphorylated forms of proteins from the PI3K, MAPK and JAK-STAT pathways as well as the BCL2 family protein, MCL1. We found that there was an increase in the phosphorylated forms of p44/p42 in the MAPK pathway and a downregulation of MCL1 on KIT activation. Overall our data have shown that while BCL2 may not contribute to oocyte survival during cyst breakdown, both MCL1 and KIT play important roles in formation of the primordial follicle pool.

Molecular Mechanisms Regulating Neonatal Oocyte Survival and Primordial Follicle Formation in the Mouse Ovary

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TABLE OF CONTENTS

Acknowledgements……v

List of Figures……viii

Chapter 1

Introduction……1

Chapter 2

Role of the Anti-Apoptotic Proteins BCL2 and MCL1 in the Neonatal Mouse Ovary

Abstract……26

Introduction……27

Materials and Methods……30

Results……35

Discussion……47

Chapter 3

KIT Signaling Regulates Primordial Follicle Formation in the Neonatal Mouse Ovary

Abstract……51

Introduction……52

Materials and Methods……56

Results……62

Discussion……82

Chapter 4

Conclusions and Future Directions…….....……...……89

References.....98

Curriculum Vitae……..…….108

LIST OF FIGURES

Figure	Page
1.1 Timeline of mouse germ cell development	3
1.2 Aberrant cyst breakdown may lead to multiple oocyte follicles	5
1.3 Ovarian folliculogenesis	9
1.4 BCL2 family mediated apoptosis and survival	15
2.1 Expression of BCL2 in the fetal and neonatal ovary	36
2.2 Ovarian BCL2 overexpression does not alter neonatal ovarian histology	37
2.3 Ovarian BCL2 knockout does not alter neonatal ovarian histology	39
2.4 Expression of MCL1 in the fetal and neonatal ovary	41
2.5 MCL1 antibody treatment decreases oocyte numbers and increases cyst breakdown in organ culture	43
2.6 Apoptosis increases when PND 1 ovaries are cultured with an antibody to MCL1	44
2.7 MCL1 antibody is taken up by live oocytes and somatic cells	46
3.1 Expression of KIT in the fetal and neonatal ovary	63
3.2 KIT labels oocytes at PND 1	64
3.3 Expression of KITL in the fetal and neonatal ovary	66
3.4 Expression of KIT and cleaved PARP at PND 1	67

3.5 Ovarian follicle formation is affected by KIT signaling	69
3.6 KIT signaling is important for follicle development from 17.5 dpc to PND 1	71
3.7 KIT signaling is important for follicle formation and development from 17.5 dpc to PND 1	73
3.8 KIT signaling is important for follicle formation in ovaries from PND 1-4	75
3.9 Inhibition of KIT signaling decreases cell death	76
3.10 Granulosa cell proliferation is reduced when KIT is inhibited	78
3.11 KIT ligand activates the MAPK pathway	80
3.12 KIT signaling alters expression of MCL1	81

CHAPTER 1:

INTRODUCTION

Significance

The number of germ cells available to a female mammal at birth is a finite number that decreases as the female matures and eventually reaches the period of reproductive senescence (Kim & Tilly 2004). Not only is she initially limited in the number of oocytes allotted to her at birth, but any abnormalities in the development of these cells can further reduce numbers, leading to infertility. Infertility has been associated with various reproductive disorders, including premature ovarian failure, polycystic ovary syndrome, and primary amennorhea; nevertheless the etiology of many infertility issues remains unknown. In addition to disorders of conception, ovarian cancers are common in the human population. One specific type of cancer, ovarian dysgerminoma, involves germ cell tumors. The exact pathophysiology of how these tumors arise remains a mystery. Procuring a basic understanding of early follicle development, regulation of follicle numbers and oocyte differentiation may aid in the pursuit of treatments for infertility and germ cell tumors of both known and unknown etiologies.

Fetal germ cell development

In mouse, between 6.5 and 7 days post-coitum (dpc), a small subset of cells in the developing embryo become committed to a germ cell fate, and are first identifiable at 7 dpc by alkaline phophatase positivity in the posterior primitive streak (Saffman & Lasko 1999). These primordial germ cells migrate to the genital ridge at about 10.5 dpc and begin dividing by mitosis to dramatically increase the number of germ cells (Fig. 1.1; Peters 1970). The now large number of oogonia are found at the prospective ovary in clusters of synchronously dividing cells known

as germline cysts (Pepling & Spradling 1998). Cysts are groups of germ cells that remain connected due to incomplete cytokinesis, and intercellular bridges are found between neighboring cells within a cyst (Pepling & Spradling 1998). At 13.5 dpc, the oogonia cease mitosis and begin to enter meiosis progressing through the stages of prophase I from 13.5 dpc until their arrest in the diplotene stage, beginning at 17.5 dpc. Meiosis appears to occur in waves, initiated in oocytes populating the anterior ovary at 13.5 dpc and arresting in the diplotene stage of meiosis at 17.5 dpc, while oocytes in the posterior of the ovary may not arrest until five days after birth (Borum 1961, Menke *et al.* 2003).

Around the time of diplotene arrest, germ cell cysts begin to break down. This begins in the medulla, or innermost portion of the ovary, at 17.5 dpc and occurs in the cortical, or outer region of the ovary between 19.5 dpc and PND 4 in the mouse (Pepling *et al.* 2010). Coincidentally, as the cysts break down, two-thirds of oocytes are lost by apoptosis (Pepling & Spradling 2001).

Cyst formation, cyst breakdown and primordial follicle formation

Germline cyst formation is highly conserved. Many species, including rat, mouse, hamster, rabbit, human, frog, fruit fly, sheep, cattle and pig display the characteristics of germline cysts, such as synchronous division or intercellular bridge connections (Russe. 1983, Smith *et al.* 1993, Pepling *et al.* 1999, Bielanska-Osuchowska 2006). As oocyte cysts form, they become arranged into structures called

Figure 1.1

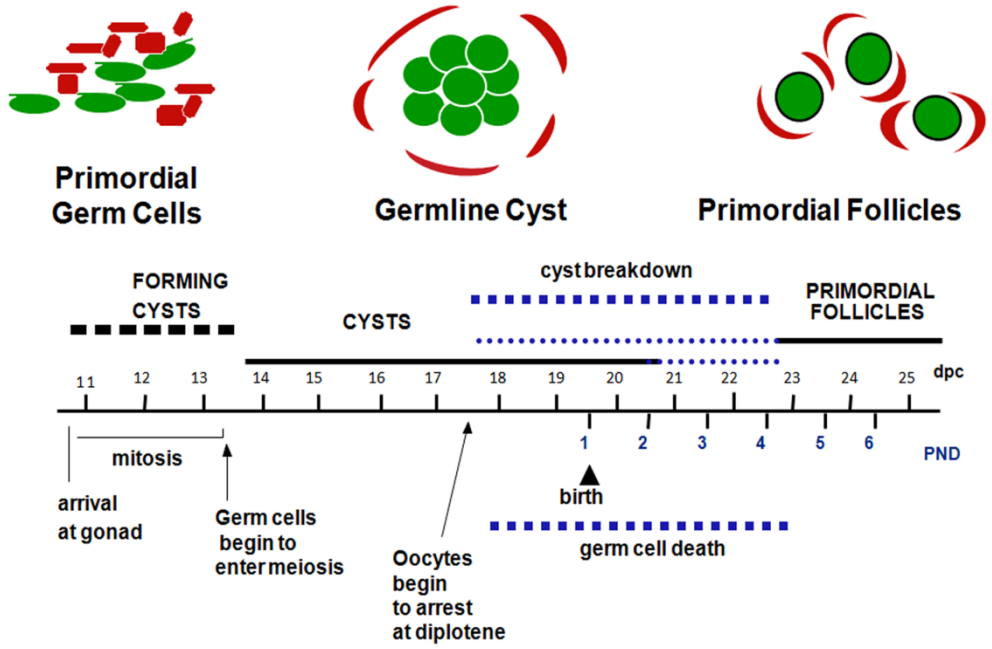


Figure 1.1. Timeline of mouse germ cell development. Germ cells are depicted in green and somatic granulosa cells depicted in red. After migration to the genital ridge at 10.5 dpc, germ cells begin dividing, forming cysts. At 13.5 dpc mitosis stops, and germ cells begin to enter meiosis and progress through the beginning of prophase I. During this time oocytes are found in ovigerous cords, where large cysts are surrounded by somatic cells. Germ cells begin to arrest in the diplotene stage of meiosis at 17.5 dpc. Coincidentally, cysts start to break down and oocytes that are not lost through apoptosis become surrounded by granulosa cells. (Adapted from Pepling 2006)

ovigerous cords which consist of layers of somatic cells, called pregranulosa cells, surrounding oocyte cysts (Mazaud *et al.* 2005). These structures are evident by 13.5 dpc (Pepling & Spradling 2001). In order for primordial follicle formation to occur, these ovigerous cords must break apart in the process of cyst breakdown. Cyst breakdown results in the formation of primordial follicles, which consist of an oocyte surrounded by a single layer of somatic granulosa cells. As part of the cyst breakdown process, surrounding granulosa cells begin to intrude between oocytes, and it is thought that this contributes the formation of primordial follicles (Pepling & Spradling 2001). In some instances, the adult ovary will contain follicles with more than one oocyte enclosed within the granulosa cells, and these structures are known as multiple oocyte follicles (MOFs; Fig. 1.2). MOFs are thought to be oocyte cysts that did not properly break down (Jefferson *et al.* 2006).

Although not much is known about oocyte cyst breakdown and the formation of primordial follicles, research is beginning to uncover various molecules that play a role in these processes. The Notch family of proteins have increasingly been implicated in controlling cyst breakdown and primordial follicle formation. Johnson *et al.* (2001) first examined the expression of components of this pathway in the 6 week old mouse ovary and found the Notch receptors NOTCH 2 and NOTCH 3 to be expressed in granulosa cells along with the ligand JAGGED1. The Notch ligand, JAGGED 2, was localized to oocytes, suggesting a possible role for these proteins in communication between germ cells and granulosa cells in folliculogenesis. Lunatic fringe, a protein involved in regulation of Notch signaling, is expressed in the granulosa cells of mouse ovaries (Hahn *et al.* 2005). Knockout of this protein results in MOFs

Figure 1.2

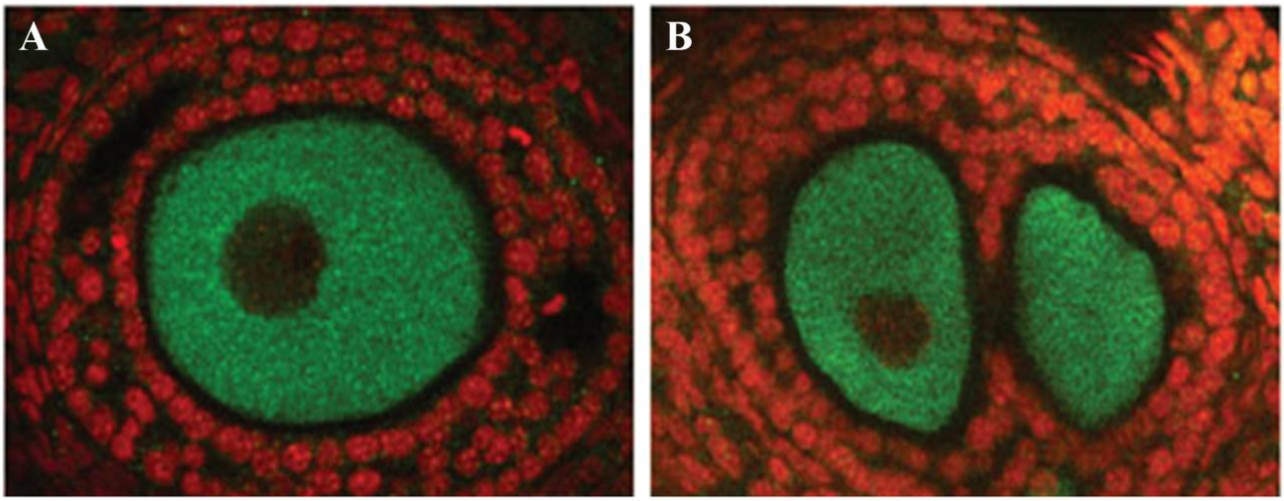


Figure 1.2. Aberrant cyst breakdown may lead to multiple oocyte follicles (MOFs). Representative confocal sections of an ovary containing either A) a normal follicle or B) a MOF labeled for STAT3 (green) to visualize oocytes and propidium iodide (red) to visualize nuclei. (Adapted from Pepling 2006)

so these mice may have defects in cyst breakdown, although this has not been specifically examined. Another study examining the effects of Notch signaling on ovarian development identified the importance of this signaling pathway specifically in cyst breakdown and primordial follicle formation. Trombly and colleagues (2009), found the ligand JAGGED 1 expressed in the oocytes of newborn mouse ovaries and the NOTCH 2 receptor expressed in granulosa cells. In the same study, when Notch signaling was blocked in an ovary organ culture, cyst breakdown and primordial follicle formation were inhibited.

Experiments have also demonstrated a role for Neurotrophin signaling in primordial follicle formation. In the ovary, Neurotrophin signaling either occurs through the binding of the ligand, Nerve Growth Factor (NGF) with its cognate receptor, Tropomyosin-Related Kinase A (TRKA) or through the binding of either Brain Derived Neurotrophic Factor (BDNF) or Neurotrophic Factor 4 (NT4) with their cognate receptor, TRKB (Dissen *et al.* 2002). Spears *et al.* (2003), found that blocking PND 1 ovaries in culture with both BDNF and NT4 antibodies resulted in a marked reduction in the number of germ cells and an increase in the percent of dying cells in treated ovaries. In the same study, TRKB knockout ovaries contained fewer oocytes than wild type controls at PND 4. More recently, it was shown that both TRKA and TRKB knockout mice contained ovaries with fewer primordial follicles and more oocytes in cysts at PND 2. Using a Terminal Deoxynucleotidyl Nick End Labeling (TUNEL) analysis the authors also examined cell death in these ovaries and found no differences in the number of dying cells between control and TRKA or TRKB ovaries, demonstrating a role for both receptors in cyst breakdown and primordial follicle formation (Kerr *et al.* 2009). Likewise, NGF mutants show a defect in primordial follicle assembly at PND 2. NGF knockout ovaries have a decrease in the number of primordial and primary follicles, but an increase in oocytes found in cysts. In

addition, there was a decrease in somatic cell proliferation, indicating a need for NGF in granulosa cell mitosis that may lead to primordial follicle formation (Dissen *et al.* 2001) .

There is evidence that several of the Transforming Growth Factor Beta (TGFB) family of proteins may be involved in cyst breakdown and follicle formation. Activin, expressed in the germ cells of newborn mice, increased primordial follicle formation and the number of primordial follicles in mice treated with recombinant Activin (Bristol-Gould *et al.* 2006). The effect appeared to be through an increase in granulosa cell proliferation, indicating an importance for Activin signaling in somatic cell mitosis during follicle formation. Further evidence that Activin regulates follicle formation comes from mice mutant for the protein Follistatin, a negative regulator of Activin. These mice also have increased numbers of primordial follicles, however cyst breakdown occurs over a longer period of time (Kimura *et al.* 2011). Mice mutant for two other TGFB family members, Growth and Differentiation Factor 9 (GDF9) and Bone Morphogenetic Protein 15 (BMP15), have ovaries that contain MOFs when both proteins are concurrently deleted (Yan *et al.* 2001). Further, hamster ovaries exposed to GDF9 siRNA in culture show a reduction in primordial follicle formation, suggesting that the MOFs seen in double knockout mice may be due to aberrant cyst breakdown (Wang & Roy 2006). Another TGFB protein, TGFB2, also affects formation of primordial follicles. Knockout of this protein resulted in accelerated formation of the primordial follicle pool at PND 1, implying that TGFB2 is a negative regulator of cyst breakdown (Memon *et al.* 2008). Finally, experimental data on the Bone Morphogenetic Protein (BMP) antagonist, Gremlin (GREM1), may indirectly shed light on the role of BMPs in ovarian development. Upon observing the ovaries of *Grem1* knockout mice, Myers and colleagues (2011) discovered that lack of this protein led to a reduction in the number of oocytes but an increase in the number found in germ cell cysts. This indicates a role for one or

more BMPs in regulating both germ cell numbers and primordial follicle formation, however the direct role of BMPs in these processes remains to be examined.

Folliculogenesis

At the conclusion of cyst breakdown, the ovary is populated with a pool of primordial follicles arrested in the diplotene stage of prophase I. This is considered the resting pool of follicles from which a few be selected to grow and ovulate, however many will die before activation (Faddy *et al.* 1976). Once primordial follicles are formed, some are activated, or selected to develop further (Fig. 1.3). Activated primordial follicles will first transition into primary follicles as the flattened granulosa cells surrounding the oocyte divide and become cuboidal (Zamboni 1974). Next, oocytes will grow and continue to accumulate more layers of somatic cells around them. Primary follicles that have increased in size and have gained multiple layers of granulosa cells are called secondary, or preantral follicles (Hirshfield 1991). In the secondary follicle phase, oocytes undergo significant growth, gain more granulosa cells and a new layer of theca cells that are derived from the stromal cells. These follicles will eventually gain a fluid filled space, or antrum, and become antral follicles (Hirshfield 1991). Antral follicle survival is Follicle Stimulating Hormone (FSH) dependent, and those that are selected for ovulation will resume meiosis upon a surge of Luteinizing Hormone (LH)

Figure 1.3

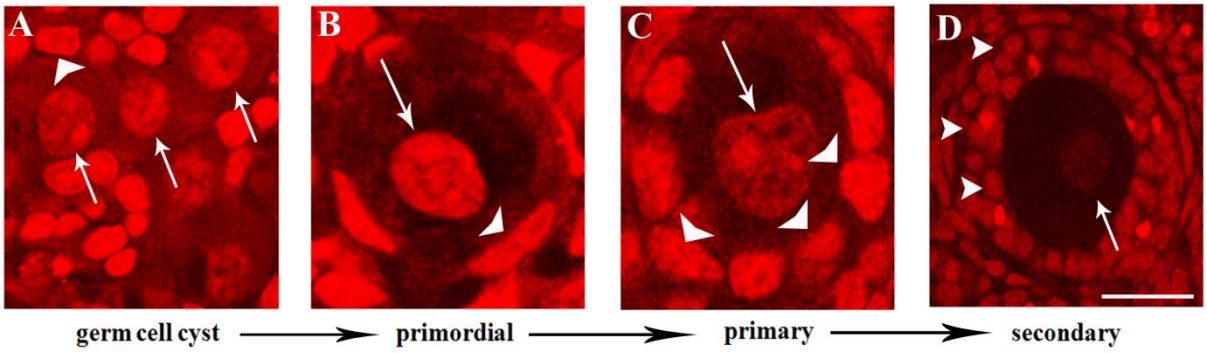


Figure 1.3. Ovarian folliculogenesis. Representative confocal sections of different stage ovarian follicles labeled with propidium iodide (red) to visualize nuclei. A) several oocytes within a cyst. B) primordial follicle with one oocyte and a layer of flattened granulosa cells. C) primary follicle with one oocyte and a layer of cuboidal granulosa cells. D) secondary/preantral follicle with one oocyte and several layers of granulosa cells. White arrows denote oocytes and arrowheads show granulosa cells. Black arrows indicate the progression of follicle development. Scale bar, 20μm. (Images courtesy of Chen and Pepling, unpublished)

and subsequently arrest in metaphase II until fertilization, a process called oocyte maturation. Just prior to ovulation, the oocyte nucleus, termed the germinal vesicle, will break down and meiosis will proceed from the diplotene stage of prophase I to metaphase of meiosis II (Wiersma *et al.* 1998). Antral follicles that are not selected for maturation will not be ovulated and die by apoptosis (Hsueh *et al.* 2000).

Germ cell death

Loss of oocytes occurs at several different time periods in germ cell development, and much of this death is thought to be due to apoptosis. In support of this, Coucouvanis (1993) found that classic apoptotic germ cell shrinkage and nuclear chromatin condensation were present in the murine gonad between 13.5 dpc and PND 1. Further, Ratts and colleagues (1995) showed that between 15.5 dpc and PND 1, dying oocytes from mouse ovaries demonstrated the characteristic DNA laddering pattern of apoptosis.

Oocyte programmed cell death is not unique to mice and in fact, appears to be conserved throughout the animal kingdom. In hermaphroditic nematodes, oocytes arrested in pachytene undergo apoptosis, resulting in a 50% loss of germ cells (Gumienny *et al.* 1999). Furthermore, *Drosophila* produce prospective germ cells in cysts, known as cystocytes, that serve a supportive nurse cell function and die just after dumping their cytoplasmic contents into the oocyte. Moreover, as in mouse and human, *Drosophila* germ cells undergo programmed cell death if they do not reach the developing gonad (Pritchett *et al.* 2009).

Neither the function, nor mechanism, by which mammalian germ cells die is understood. Apoptosis occurs in many systems throughout the course of development; however clues to germ

cell death mechanisms and functions may come from the nervous and immune systems. In both systems, cells are overproduced during early neuro- and lymphogenesis and are subject to at least two different waves of apoptosis both in utero and postnatally (Elmore 2007). Numbers of developing neurons are limited by the availability of neurotrophic factors during the proliferative period and post-mitotically. Not only does this process determine the number of neurons, but it also establishes appropriate, functional synaptic connections between neurons (Yuan & Yankner 2000). Similarly, young lymphocytes are overproduced and removed by limited cytokine availability. Once initial cell numbers are reduced, further apoptosis occurs to generate lymphocytes with functional antigen receptors possessing the exact level of responsiveness needed for a working immune system (Opferman 2008). As in immune and nervous systems where cell numbers rapidly decline and modules of functionality are assembled, oocyte numbers are sharply reduced and placed into functional units, the ovarian follicles.

Although the mechanisms in these other systems are still uncertain, involvement of various growth factors/cytokines, members of the B-cell Leukemia (BCL2) family and death receptor pathways have been established. One model for the role of perinatal oocyte apoptosis is to break down cysts, consequently allowing the remaining germ cells to form primordial follicles (Pepling & Spradling 2001). It is unknown at this time which mechanisms are controlling the apoptosis of germ cells, but it is our hypothesis that like neurons and lymphocytes, death is controlled by growth factor signaling and its subsequent regulation of intrinsic and extrinsic apoptotic pathways.

Extrinsic regulation of oocyte apoptosis

Programmed cell death occurs through two distinct mechanisms, the extrinsic and intrinsic pathways. Extrinsically, death receptors can initiate the death program in cells. Death receptors belong to the Tumor Necrosis Factor(TNF)/Nerve Growth Factor (NGF) receptor superfamily, which consists of 26 known members. Some of the more well-studied members include: TNFR1, TRAIL-R1, TRAIL-R2, DR-3 and FAS. Each of these receptors is characterized by a unique cytoplasmic region consisting of approximately 80 residues, known as the death domain (DD) (Lavrik *et al.* 2005). Once a receptor has been activated by its ligand, it homotrimerizes, and recruits an adaptor protein and Caspase-8 to the DD. The recruitment of the Death-Inducing Signaling Complex (DISC) to the DD results in cleavage of Caspase 8 and the initiation of two possible death signaling pathways (Schmitz *et al.* 2000), either direct amplification of a caspase cascade, or the cleavage of BID (a BCL2 family member) and activation of the apoptosome through the mitochondrial pathway, demonstrating cross-talk between the pathways (Scaffidi *et al.* 1998).

Several studies have examined the expression (Guo *et al.* 1994) and role of FAS receptor signaling in spermatogenesis and oogenesis (Sakata *et al.* 2003, Moniruzzaman *et al.* 2007, Kim *et al.* 2009). Mice express FAS at five weeks of age in oocytes, but not granulosa cells, and the receptor has been found to be functional in both male and female gametes (Guo *et al.* 1994, Sakata *et al.* 2003). In *Kit/Fas* double knockout mice, the loss of the FAS receptor rescues the loss of germ cells seen in the *Kit* knockout alone, implicating the FAS pathway in germ cell apoptosis in *Kit* deficient mice (Moniruzzaman *et al.* 2007). Recently, FAS knockout ovaries were analyzed for oocyte death, and the percent of oocytes staining positive for TUNEL was significantly lower in the knockout mouse (Kim *et al.* 2009). However, the number of oocytes

per section was not different between the control and FAS deficient ovaries. This could be attributed to the timing of analysis; PND2 may be too early a time point to see a significant difference in cell numbers since PND2 and PND3 are the days of peak ooocyte death (Pepling & Spradling 2001).

Another death receptor pathway involved in perinatal oocyte death may be Tumor Necrosis Factor. When PND 1 rat ovaries were treated with an antibody to TNF in organ culture, there was an increase in germ cells (Morrison & Marcinkiewicz 2002). In the same study, when PND 1 ovaries were exposed to exogenous TNF in culture, there was a reduction in oocyte numbers. Supporting this, Greenfeld *et al.* (2007) found that treating newborn mouse ovaries with exogenous TNF in culture resulted in a marked reduction in the number of oocytes present. However, a deletion of either TNFR1 or TNFR2 in vivo did not result in any differences in germ cell numbers, making it unclear how important TNF signaling is to perinatal in vivo ovarian development.

Intrinsic regulation of oocyte apoptosis and the BCL2 family

Intrinsically, apoptosis is regulated by factors that ultimately change the permeability of the outer mitochondrial membrane. This can occur because of a negative signaling event such as withdrawal or absence of growth factors, cytokines or hormones, or through a positive signaling event like exposure to radiation, hypoxia, toxins or viruses. Once the mitochondrial membrane has been compromised, the apoptotic factor Cytochrome C can be released into the cytoplasm. Cytochrome C then joins with Procaspase 9 and Apaf-1 to form the apoptosome. At this point the caspase cascade commences and cell death quickly ensues.

Proteins known to regulate the mitochondrial cell death pathway are members of the BCL2 superfamily. This family can be divided into two discrete groups of molecules, those that are pro-apoptotic (BAX, BAK, BAD, BID) and those that are anti-apoptotic (BCL2, BCLX, MCL1) (Kim and Tilly 2004). BCL2 molecules share some degree of protein homology as each protein contains at least one BCL2 homology (BH) domain, BH3. Anti-apoptotic BCL2 family members share 3-4 BCL2 homology (BH) domains, while the pro-apoptotic members either share only domains 1-3 or are part of the BH3-only group of proteins. These domains are thought to be crucial for protein-protein interactions between family members (Chao & Korsmeyer 1998). Generally, members such as BAX reside either in the cytoplasm and translocate to the mitochondria on activation or reside in the mitochondrial membrane itself and form pores in the outer mitochondrial membrane, releasing Cytochrome C. This can be prevented by heterodimerization with anti-apoptotic members like BCL2, which typically reside in the cytoplasm until activated. Figure 1.4 represents general BCL2 family mediated apoptosis and survival. Overall, it is thought that BH3-only proteins "sense" the death signal (i.e. withdrawal of growth factor) and either activate pro-apoptotic BCL2 proteins directly, or inhibit the ability of anti-apoptotic BCL2 to heterodimerize with the death-inducing members, resulting in apoptosis (Walensky 2006). Ultimately, the balance of anti-apoptotic members with pro-apoptotic members determines cell death or survival (Danial 2007).

Figure 1.4

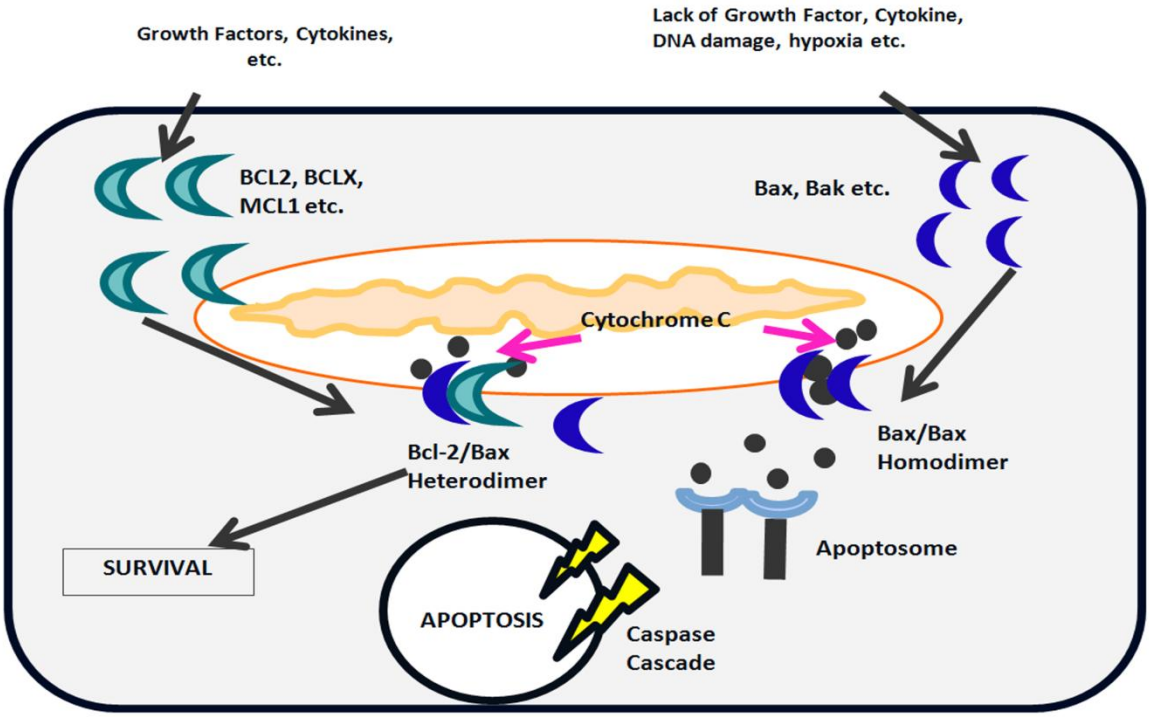


Figure 1.4. BCL2 family mediated apoptosis and survival. Upon cell stress, BCL2 proapoptotic proteins such as BAX or BAK (blue half moons) are activated and translocate to the outer mitochondrial membrane where they homodimerize and create a pore that allows release of Cytochrome C. As Cytochrome C escapes, it aids in apoptosome formation which in turn activates the caspase cascade, resulting in apoptosis. In the presence of survival cues such as positive growth factor signaling, BCL2 antiapoptotic proteins such as BCL2, BCLX or MCL1 (green half moons) are activated and heterodimerize with proapoptotic BCL2 proteins at the mitochondria. This prevents Cytochrome C release from the mitochondria, resulting in cell survival.

The BCL2 family member BCL2 –associated X protein, BAX, has been implicated in the establishment of the primordial follicle pool. At PND 42, deletion of *Bax* in the murine ovary tripled the number of primordial follicles present when compared to wild type mice (Perez *et al.* 1999). However, no differences in the number of primordial or primary follicles were observed at PND 4 in the *Bax* null strain. Oocytes found in cysts were not included in the total germ cell count at PND 4 and this may be why no differences were noted. In a later study, it was determined that mutation of the *bax* gene resulted in an increased number of oocytes, and significantly more oocytes were found in germ cell cysts at 13.5 dpc, 15.5 dpc, PND 4, and PND 7 (Greenfeld *et al.* 2007). Both studies implicate BAX as being important in determining oocyte number in the ovary, but the role of BAX in the perinatal wave of apoptosis is still unsolved. One puzzling finding in the Greenfeld study was that although the pro-death protein, BAX, was absent from ovaries, oocytes continued to stain positively for TUNEL between 15.5 dpc and PND 4. This may indicate a redundancy with another Bcl2 family member, such as BCL2 homologous antagonist killer (BAK). *Bak* knockout mice do not display a phenotype, however when combined with a *Bax* deletion, multiple tissues are affected, resulting in perinatal death (Lindsten *et al.* 2000). This effect is more severe than either BAK or BAX elimination alone. Unfortunately, although female mice were reported to have imperforate vaginal canals, no observations or reports have been made on the ovaries of *Bax/Bak* double mutants. No information is available on how double deletion of these pro-apoptotic proteins affects perinatal oocyte survival.

Anti-apoptotic BCL2 family members may also regulate oocyte apoptosis. Recently, the BCL2 protein has been identified as a possible regulator of germ cell death in female mice. Both overexpression and knockout of the *Bcl2* gene alter ovarian histology. Overexpression of BCL2

in the mouse ovary resulted in increased numbers of primordial, primary and secondary follicles at PND 9 and PND 27, however this difference was resolved by PND 60 (Flaws *et al.* 2001). Additionally, in a *Bcl2* knockout model, PND 42 murine ovaries contained follicles devoid of oocytes and had fewer primordial follicles (Ratts *et al.* 1995). While these studies do indicate a role for the BCL2 protein in follicle development and germ cell death, further research needs to be done to determine if BCL2 exerts its effect specifically during the perinatal wave of apoptosis. Another anti-apoptotic member of the BCL2 family, BCL2-like Protein 1 (BCLX), may play a role in ovarian germ cell death. Transgenic *Bcl-x* knockdown mice display 94% fewer primordial follicles at PND 9 than controls (Rucker *et al.* 2000). At 13.5 dpc, the number of oocytes was markedly reduced, indicating a role for BCLX in embryonic germ cell death. In contrast, utilizing the same transgenic mice, Riedlinger and colleagues (2002) found no differences in ovarian follicle numbers in mice at 5 weeks or 3 months of age. Finally, the anti-apoptotic protein Myeloid Cell Leukemia Sequence 1 (MCL1) is expressed in human ovary during the time of primordial follicle formation, but no work has been done to demonstrate a role for this protein in oocyte survival at that time (Hartley *et al.* 2002).

KIT signaling in the ovary

Apoptosis can be controlled by molecules that may up- or down-regulate BCL2 family members, or molecules that activate BCL2 independent pathways. For example, the processes of erythropoiesis, lymphopoeisis, mast cell generation, gametogenesis and melanogenesis all depend of the binding of KIT ligand (KITL), also known as Stem Cell Factor (SCF) to its receptor, KIT, for regulation of cell survival (Roskoski 2005). As a Receptor Tyrosine Kinase (RTK), KIT has the ability to activate at least three different pathways that are known be

involved in cell survival and proliferation: RAS/MAP kinase, Phosphoinositide 3 Kinase (PI3K) and Janus Kinase-Signal Transducer and Activator of Transcription JAK-STAT (Schlessinger 2000).

Much of what is known about KIT and KITL function in the ovary comes from mice mutant for the *W* and *Steel* loci, respectively. Mutations at either locus are responsible for both male and female sterility, as well as severe anemia and white coat color (Little & Cloudman 1937; Huang *et al.* 1992). Numerous studies using various gene deletions, knockdowns and organ culture systems have attempted to dissect when and where KIT signaling is important in the ovary.

Studies first discovered that KIT was imperative for oocyte survival at various stages. Early on, KIT signaling is important in proliferation and survival of the primordial germ cells as they migrate to the genital ridge (Huang *et al.* 1993). Expression of KITL was found in the somatic cells lining the migratory pathway from hindgut to potential gonad, and the expression increased nearer the genital ridge, while the KIT receptor was expressed on germ cells (Matsui *et al.* 1990, Keshet *et al.* 1991). KIT signaling is also important to later follicle development after birth. Using an injection of the KIT antibody ACK2, Yoshida *et al.*(1997) found that both primordial and antral follicle survival was attenuated when KIT signaling was blocked. Reynaud and colleagues (2001) demonstrated that KITL and KIT were also essential for follicular survival, protecting pre-antral follicles from apoptosis. The exact contribution of KIT signaling to the survival of oocytes in cysts in later fetal and early neonatal development is unclear. Yoshida and colleagues (1997) failed to find that KIT plays any role in the survival of oocytes before or during primordial follicle formation. In contrast, oocyte death was rescued by three days of KITL treatment in culture between 16.5 and 19.5 dpc (Lobascio *et al.* 2007). Further,

KITL has been shown to prevent oocyte apoptosis in the neonatal rat ovary (Jin *et al.* 2005). In this study, the authors used both KITL and ACK2 to demonstrate the necessity of this signaling pathway to the survival of both unassembled oocytes and oocytes found in primordial follicles.

Processes such as proliferation and differentiation can also be controlled by the KIT system. Matusi and colleagues found that culturing 11.5 dpc PGCs with recombinant KITL greatly increased their proliferation, especially when in the presence of Leukemia Inhibitory Factor (Matsui *et al.* 1991). There is evidence demonstrating that KIT signaling may both enhance and halt meiotic progression. When 13, 14 and 17 dpc ovaries were cultured with a combination of three different growth factors including KITL, they progressed to the pachytene stage of meiosis more quickly, suggesting the KITL/KIT system may play a role in regulating meiotic progression (Lyrakou *et al.* 2002). However, because KIT expression is reported to cease by 14.5 dpc and because the authors used a three growth factor cocktail in this experiment, it is unclear whether KITL would actually regulate meiosis after 13.5 dpc (Manova *et al.* 1990). It has also been reported that KITL actually maintains diplotene arrest in developing follicles. Ismail (1996), cultured 28 day old rat oocytes primed with gonadotropins in media containing KITL and found that progression to metaphase II of meiosis was transiently blocked. Further, injecting oocytes within oocyte-cumulus cell (OCC) complexes with antisense oligonucleotides to the KIT receptor significantly reduced germinal vesicle breakdown, one of the key signs of meiotic resumption (Ismail *et al.* 1997). In the same study, when OCCs were exposed to Luteinizing hormone the number of KITL transcripts decreased specifically in the cumulus cells, indicating that a reduction in KITL in the cells closest to the oocyte may be responsible for the progression of meiosis.

KIT signaling has also been shown to be important to developing follicle transitions. *Steel*^{panda} mutant mice, a KITL hypomorph, secrete only a small amount of KITL and have a complete block on follicle development at the primordial follicle stage (Huang *et al.* 1993). Follicles in these ovaries remain as primordial follicles that never transition to primary follicles, resulting in sterility. Supporting this, primordial follicle development is blocked when ACK2 antibody is injected into newborn mice (Yoshida *et al.* 1997). Likewise, when KIT signaling is activated by adding recombinant KITL to rat ovary organ culture, the primordial to primary follicle transition is significantly enhanced (Parrott & Skinner 1999). More recent evidence has begun to demonstrate reciprocal signaling between granulosa cells and oocytes during the primordial follicle transition involving KIT signaling. Otsuka and Shimasaki (2002) found that in the presence of KITL, granulosa cell mitosis increased, indicating an indirect regulation of granulosa cell division via the oocyte KIT signal. In the same study, when an antibody to KIT was added to culture media, granulosa cell division decreased.

Several factors have been suggested to either regulate the expression of KITL or act in concert with KIT signaling to induce primordial follicle activation. BMP15 secretion from the oocyte increases somatic cell mitosis, as well as expression of KITL in these cells (Otsuka & Shimasaki 2002). In another study, both granulosa cell derived KITL and oocyte secreted Basic Fibroblast Growth Factor (bFGF) were shown to act in synergy to increase the primordial to primary follicle transition. Ovaries grown in culture with recombinant KITL in conjunction with an antibody to bFGF were unable to promote the primordial to primary transition seen when KITL was added alone. In addition, when exogenous bFGF was added to culture in combination with ACK2, the activation of primordial follicle development was again blocked. Analysis of mRNA expression at the conclusion of ovary culture with either exogenous KITL or bFGF alone

found that addition of bFGF resulted in an increase in KITL transcripts, suggesting bFGF regulates expression of KITL during this phase (Nilsson & Skinner 2004). Knockout of another growth factor, GDF9, increases ovarian KITL mRNA, implying that this protein may directly regulate KITL expression (Elvin *et al.* 1999). Finally, Leukemia Inhibitory Factor (LIF) was found to be expressed in the granulosa cells of newborn rat primordial follicles and when added to culture, promoted the primordial to primary transition. Further, granulosa cells cultured with LIF expressed mRNA for KITL more highly, suggesting that LIF and KITL may synergistically regulate primordial follicle activation (Nilsson *et al.* 2002). It is possible that some of these KITL regulatory pathways are also working during primordial follicle formation.

Later, the KIT/KITL system is involved in the transition from pre-antral to antral follicle. By blocking KIT signaling specifically during the time of antrum formation, Yoshida and colleagues (1997) were able to disrupt antrum formation. Further, granulosa cell mitosis was greatly reduced, again supporting the notion that KIT signaling at the oocyte regulates downstream molecules that communicate directly with surrounding somatic cells (Yoshida *et al.* 1997). Similarly, when rat ovaries were subjected to the ACK2 antibody in culture for 14 days, gonadotropin induced transition of pre-antral to antral follicles was abolished (Parrott & Skinner 1999).

In addition to examining the function of KIT signaling in oocyte apoptosis, proliferation, meiotic progression and folliculogenesis, studies have begun focusing on the mechanisms by which KIT is working. KIT can signal through several different signaling cascades including JAK-STAT, PI3K and MAPK (Schlessinger 2000). Of these, PI3K seems to be the likely candidate pathway activated downstream of KIT during cyst breakdown. The PI3K pathway acts to transform PIP2 to PIP3, which then activates AKT. AKT can then phosphorylate several

different molecules including: TSC1 and 2, mTOR, GSK3, FOXO3, p27 or BCL2 proteins. *Akt1* knockout mouse ovaries contain multiple oocyte follicles at PND 25, indicating a role for the PI3K pathway in cyst breakdown (Brown *et al.* 2009). Further, investigations using neonatal rat ovaries in culture with an AKT inhibitor downstream of the KIT signal resulted in a decrease in phosphorylated FOXO3A and total p27 which was associated with a decrease in oocyte apoptosis (Liu *et al.* 2009). It is not likely however, that FOXO3A is involved in mouse cyst breakdown as *Foxo3a* knockouts demonstrate normal primordial follicle formation (John *et al.* 2009). Additionally, although *p27* knockout mice show modulation of primordial follicle formation, expression of p27 was found to be in the somatic cells surrounding cysts and so is not likely activated downstream of AKT in the oocyte during cyst breakdown in mice (Rajareddy *et al.* 2007). Regulation of TSC1/2 and mTOR may be the key PI3K downstream pathway activated during cyst breakdown within oocytes. Few studies have examined the role of this pathway in follicle development, but it has recently been shown to be important in primordial follicle activation in *Tsc1* knockout mice (Adhikari *et al.* 2010). Although conditional knockout lines of *Tsc1* or *PTEN* (a negative regulator of PI3K signaling) proteins demonstrate normal primordial follicle formation (Adhikari *et al.* 2010, Reddy *et al.* 2008), in each study the GDF9 promoter was used to drive Cre recombinase expression in the oocyte, and this promoter is not active early enough to affect cyst breakdown (McGrath *et al.* 1995). The p85 subunit of PI3K binds directly to tyrosine 719 on KIT, activating the PI3K pathway. Mice with a point mutation at KIT Y719 demonstrate normal cyst breakdown and even PGC migration during fetal life (Kissel *et al.* 2000; John *et al.* 2009). However, Farini and colleagues (2007) showed that PGC migration was dependent on PI3K activity downstream of KIT, contradicting the results of the genetic studies. Recently, the scaffolding protein, GAB2 has been shown to bind directly to KIT and activate

PI3K signaling, bypassing the need for a direct p85 binding site (Sun *et al.* 2008). Sun (2008) also showed that a mutation at Y719 downregulated PI3K signaling but did not abolish it. Therefore, more work needs to be done to determine the affects of the PI3K pathway in cyst breakdown and primordial follicle formation.

In addition to examining the function of KIT signaling in follicle formation and development, studies have begun focusing on the mechanisms by which KIT affects oocyte apoptosis. In the ovary, several BCL2 family members have been implicated in the regulation of oocyte survival (Ratts *et al.* 1995; Perez *et al.* 1999; Flaws *et al.* 2001; Hartley *et al.* 2002). In addition, compelling evidence from several studies implicates the PI3K signaling pathway, downstream of KIT activation, in regulating BCL2 family members BCL2, BCLX, BAD and BAX to promote rat oocyte survival (Jin *et al.* 2005, Liu *et al.* 2009). In human, anti-apoptotic MCL1 is expressed during primordial follicle formation (Hartley *et al.* 2002). This may be a more likely candidate BCL2 protein regulated by the PI3K pathway in mouse during follicle formation.

Both MAPK and JAK-STAT can be activated by KIT, but very few studies have examined these pathways during cyst breakdown and perinatal oocyte loss. One study found that when newborn rat ovaries were exposed to exogenous KITL in culture, both MAPK and AKT were upregulated (Jin *et al.* 2005) as were anti-apoptotic members of the BCL2 family. Using an inhibitor of the MAPK pathway did not have any effect on BCL2 family member expression or oocyte survival. However, primordial follicle assembly was not evaluated in this study and therefore the effects of MAPK phosphorylation are unclear. In other cell types the JAK-STAT pathway regulates BCL2 family members (Alvarez & Frank 2004), but no evidence to date has shown that this is functional in the oocyte during the perinatal wave of apoptosis. Although the

work on the PI3K pathway downstream of KIT in the ovary has been extensive, clear evidence of a role for this pathway in cyst breakdown and germ cell survival during the perinatal apoptotic wave does not exist. Further, few studies have examined the effects of KIT mediated MAPK or JAK-STAT phosphorylation during cyst breakdown.

Conclusion

Cyst breakdown and oocyte death have become more extensively studied in recent years. However, there has not been a clear understanding of the factors that control these processes, as no study to date has isolated a mechanism that results in a complete block on cyst breakdown or germ cell apoptosis. Many growth factors and a few death pathways have been identified as playing a partial role in the perinatal wave of follicle formation and oocyte survival, but more work needs to be done to determine which molecules will have the largest impact on these processes. Here, we describe two different investigations involving BCL2, MCL1 and KIT and their role in both neonatal oocyte survival and primordial follicle formation.

Very little work has been done on the BCL2 family and oocyte survival during the perinatal wave of apoptosis, and we sought to elucidate a role for an anti-apoptotic BCL2 family member in the neonatal ovary. Given that overexpression of BCL2 has been shown to increase the ovarian reserve and that BCL2 knockout results in a reduction of primordial follicles in adult ovaries, we hypothesized that this protein may be responsible for determining oocyte numbers at the time of birth (Ratts *et al.* 1995, Flaws *et al.* 2001). Additionally, because MCL1 is expressed in the human ovary during cyst breakdown and oocyte loss, we postulated that this protein may also have a role in oocyte survival (Hartley *et al.* 2002). While we found BCL2 to be dispensible for early oocyte survival, using an ovary organ culture system, we found MCL1 to be an

important regulator of germ cell survival at the time of birth. This work will be presented in Chapter 2.

Although much literature exists on KIT and later follicle formation, not much has been done to examine its role specifically at the time of cyst breakdown. Yoshida and colleagues (1997) did not observe any effect of injecting an antibody to KIT on primordial follicle formation. Conversely, when growing hamster ovaries in culture with KITL, Wang and Roy (2004) found that KIT signaling promoted the formation of primordial follicles from cysts. To clarify the requirement for KIT signaling during cyst breakdown and oocyte death, we used an organ culture system to directly inhibit and activate KIT signaling during these processes. Our results show that the KITL/KIT system is instrumental in promoting the formation of the primordial follicle pool. This work will be described in Chapter 3.

CHAPTER 2:

Role of the Anti-Apoptotic Proteins BCL2 and MCL1 in the Neonatal Mouse Ovary

2.1 ABSTRACT

The mammalian ovarian lifespan is determined at the time of birth through a delicate balance of oocyte survival and apoptosis as primordial follicles form, and the mechanism by which germ cells die is not understood. We hypothesized that two BCL2 family proteins, BCL2 and MCL1, may be responsible for regulating neonatal oocyte survival. Previous work has shown that BCL2 is important for germ cell survival in adult mouse ovaries, but no work has been done to examine its role at the time of birth. To elucidate the effects of BCL2 in the neonatal ovary, we examined ovaries of both *Bcl2* overexpressing and knockout transgenic mice. When compared to wild-type mice, neither *Bcl2* overexpression nor abrogation significantly altered ovarian histology. Another BCL2 family protein, MCL1, is expressed in human oocytes during ovarian development, suggesting a role for MCL1 in oocyte survival. To test this, we first examined the expression of MCL1 in the newborn mouse ovary. MCL1 was localized to both oocytes and somatic cells during primordial follicle formation. Subsequently, we used an in vitro organ culture system to identify a role for MCL1 in oocyte survival. We found that inhibition of MCL1 with an antibody to MCL1 in culture resulted in a reduced number of germ cells and an increase in cyst breakdown. Our data demonstrate that while BCL2 is not likely involved in perinatal oocyte survival, MCL1 may be an important regulator of the ovarian primordial follicle reserve.

2.2 INTRODUCTION

The endowment of germ cells in a mammalian female is determined at the time of her birth, and the process that establishes this reserve is complex. In mouse, germ cells arrive at the potential gonad at approximately 10.5 days post coitum (dpc) and begin to divide rapidly by mitosis until 13.5 dpc (Peters 1970). Due to incomplete cytokinesis, the oogonia remain connected by intercellular bridges, forming germ cell cysts (Pepling & Spradling 1998). Once mitosis has ended, oogonia enter meiosis, proceed through the stages of prophase I and subsequently arrest in the diplotene stage beginning at 17.5 dpc. Germ cells, now known as oocytes, become separated from each other as cysts begin to break down and individual oocytes become surrounded by granulosa cells, forming primordial follicles. Cyst breakdown and follicle formation are gradual, lasting until postnatal day (PND) 5 (Borum 1961, Menke *et al.* 2003). Coincident with cyst breakdown is a large loss of germ cells, and at the end of this process only about one-third of the initial endowment of oocytes remains (Pepling & Spradling 2001). How and why oocytes are selected for either death or survival remains a mystery.

Loss of oocytes occurs at three different time periods in germ cell development, but in all cases, this death is thought to be due to apoptosis (Pepling & Spradling 2001, Coucouvanis *et al.* 1993, Ratts *et al.* 1995). Members of the B-cell Lymphoma 2 (BCL2) family of proteins are known regulators of apoptosis and can be divided into two discrete groups of molecules, those that are pro-apoptotic (BAX, BAK, BAD, BID) and those that are anti-apoptotic (BCL2, BCL-X, MCL1) (Danial 2007). BCL2 molecules share some degree of protein homology as each protein contains at least one BCL2 homology (BH) domain, BH3. These domains are thought to be crucial for protein-protein interactions between family members and it is the interactions and balance of family members that determine cell fate. BCL2 and the other anti-apoptotic family

members have 3 to 4 BH domains as well as a transmembrane domain. Some pro-apoptotic members such as BAX share this structure while others contain only the BH3 domain.

BCL2 and other anti-apoptotic BCL2 family proteins bind to pro-apoptotic BCL2 family members such as BAX at the mitochondrial membrane, keeping them inactive (Boumela *et al.* 2011). When activated, BAX homodimers form serving as pores allowing mitochondrial Cytochrome C to enter the cytoplasm. Cytochrome C associates with Caspase 9 and Apoptotic Peptidase Activating Factor 1 (APAF1) forming the apoptosome and activating downstream effector caspases which in turn activate apoptosis (Fuchs & Steller 2011).

A role for several BCL2 family proteins has been established in germ cell survival. Knockout of the pro-apoptotic *Bax* gene in the murine ovary resulted in an increased number of oocytes in fetal, neonatal, and adult mouse ovaries (Perez *et al.* 1999, Greenfeld *et al.* 2007). The anti-apoptotic protein, BCL2, has been identified as a possible regulator of germ cell death in female mice, as both overexpression and knockout of the *Bcl2* gene alter ovarian germ cell numbers (Ratts *et al.* 1995, Flaws *et al.* 2001). Additionally, Hartley and colleagues (2002) found that the anti-apoptotic Myeloid Cell Leukemia Sequence 1 (MCL1) protein was expressed in human oocytes during the time when primordial follicles are forming. MCL1 has a structure similar to BCL2 with 3 BH domains and a transmembrane domain (Danial 2007). MCL1 is also believed to function in a manner similar to BCL2 blocking function of proapoptotic family members.

Although studies on *Bax* have determined a role for pro-apoptotic BCL2 family members, no research to date has elucidated which anti-apoptotic members are important for oocyte survival in the perinatal ovary. Based on observations that BCL2 manipulation alters adult ovarian histology and that MCL1 protein is expressed in human fetal ovary, we

hypothesize that either BCL2, MCL1 or both proteins may play a role in determining final oocyte numbers. Here, we show that neither knockout nor over-expression of BCL2 results in a change in germ cell numbers, while inhibition of MCL1 significantly decreases the number of oocytes in newborn ovaries. Our findings establish MCL1 as a likely regulator of female fecundity.

2.3 MATERIALS AND METHODS

Animals

For *Bcl2* deletion studies, *Bcl2* heterozygous mice were obtained from Jackson laboratories. Heterozygotes were bred using timed matings to produce homozygous *Bcl2* knockout mice. The morning after mating, female mice were examined for vaginal plugs and the presence of a plug was denoted 0.5 dpc. Pregnant mice delivered at 19.5 dpc (PND 1) and pups were sacrificed at PND 1, 4 and 7 and genotyped; ovaries were examined for oocyte number, follicle formation, and progression. BCL2 overexpressing mice in an FVB background were obtained from Jodi Flaws (Flaws *et al.* 2001). These mice were generated in the Flaws lab by fusing the *Bcl2* gene to the *Kit* promoter, allowing overexpression of the BCL2 protein in oocytes. KIT receptor is expressed in dividing primordial germ cells and oogonia but is downregulated as the germ cells enter meiosis (Manova & Bachvarova 1991). KIT is upregulated in oocytes when they reach the diplotene state of prophase I of meiosis which occurs coincident with primordial follicle formation (Manova *et al.* 1990). Adults were mated using timed matings; pups were sacrificed at PND 1, 4 and 7 and ovaries were examined for oocyte number, follicle formation and progression. For BCL2 overexpression controls, adult FVB mice were also obtained from Jodi Flaws, mated and pups' ovaries were analyzed at PND 1, 4 and 7.

For the MCL1 studies adult male and female C57BL/6 mice were purchased from Jackson Laboratories. Adults were bred using timed matings to produce offspring for experiments. Pregnant mice delivered at 19.5 dpc (PND 1) and pups were euthanized for either in vitro ovary organ culture at PND 1 or immunohistochemistry on 15.5 dpc, 17.5 dpc, PND 1, 3 or 5.

All mice were housed under 12 hour light/dark cycles, temperatures of 21-22 °C, and had free access to chow and water. All animal protocols were approved by the Syracuse University Institutional Animal Care and Use Committee.

Genotyping tissue for Bcl2 knockout studies

Tail biopsies were collected from pups generated by mating *Bcl2* heterozygotes and lysed in 20 µl proteinase K and 180 µl tissue lysis buffer overnight at 65 °C. DNA was extracted using the Qiagen DNeasy kit. PCR was run on a program of 30 cycles of 94 °C for 1 minute, 57 °C for 1 minute and 72 °C for 1 minute. To amplify the wild type allele forward and reverse primers (Eurofins MWG/OPERON) 5'-CTT TGT GGA ACT GTA CGG CCC CAG CAT GCG-3' and 5'-ACA GCC TGC AGC TTT GTT TTC ATG GTA CAT C-3' were used. To amplify the mutant allele forward and reverse primers 5'-CCG GTT CTT TTT GTC AAG ACC G-3' and 5'-CGG CAG GAG CAA GGT GAGAT-3' were used.

Antibodies

STAT3 (C20) (Santa Cruz Biotechnology) and VASA (Abcam) antibodies were each used at a dilution of 1:500 for immunohistochemistry. MCL1 and BCL2 antibodies (Santa Cruz Biotechnology) were used at a dilution of 1:100 for immunohistochemistry. TUNEL labeling (Intergen) was used to visualize dying cells by immunohistochemistry. Secondary antibodies, goat anti- rabbit Alexa 488 and goat anti- rabbit Alexa 568 (Invitrogen), were at a dilution of 1:200.

In vitro Ovary Organ Culture

Ovaries from PND 1 female mice were harvested and placed on 0.4 μm floating filters (Millicell-CM; Millipore Corp) in 0.4 ml culture media consisting of D-MEM/HAM'S F12 Media (Invitrogen), 0.1% Albumax (Invitrogen), 0.1% BSA (Fisher), 5X ITS-X (Life Technologies), 0.05 mg/ml L-ascorbic acid (Sigma) and penicillin-streptomycin (Life Technologies) in 4-well dishes (Nunc) as previously described (Chen *et al.* 2007). A single drop of media was placed on each of the ovaries to keep them hydrated.

To assess MCL1 function in the ovary, ovaries were cultured for 3 days in either media alone, media supplemented with a rabbit polyclonal antibody to MCL1 (S-19, Santa Cruz) in a 1:100 dilution or media supplemented with IgG (Santa Cruz) in a 1:100 dilution. (n=8 ovaries per group). For cell death studies, a second group of ovaries were cultured in control or anti-MCL1 supplemented media for 2 days (n=8 ovaries per group). At the conclusion of culture, all ovaries were prepared for whole-mount immunohistochemistry.

Immunohistochemistry

Ovaries were fixed in 5% EM-grade paraformaldehyde (Electron Microscopy Sciences) overnight at 4 °C and stained as previously described (Pepling & Spradling 1998). Whole ovaries were labeled with primary antibody overnight, then with anti-rabbit secondary antibody. Nuclei were labeled with TOTO-3 or propidium iodide (Invitrogen). A Zeiss LSM 710 Confocal Microscope was used to image ovaries. As a control, some ovaries were labeled with only anti-rabbit secondary antibody.

To detect the uptake of MCL1 antibody by live ovarian cells, ovaries were first cultured either in control media or in media containing the anti-MCL1 antibody overnight. Ovaries were then

fixed in 5% paraformaldehyde overnight and labeled with anti-rabbit secondary antibody. Nuclei were labeled with propidium iodide and ovaries were then imaged.

Analysis of germ cell cyst breakdown and follicle development

For both BCL2 and MCL1 studies, oocytes were labeled with STAT3 antibody, a known marker of germ cells and imaged with confocal microscopy (Murphy *et al.* 2005). For each ovary, 2 cores were visualized and counted. A core is a region 135 x 135 µm consisting of optical sections at 4 different depths in the ovary each 15-20 µm apart. Thus, for each ovary, 2 cores were obtained consisting of 4 optical sections per core for a total of 8 optical sections per ovary. The number of oocytes found in cysts relative to the total number of oocytes was determined for each ovary by analyzing each section and reported as percent single oocytes. In order to determine whether oocytes were in cysts or not, for each of the 4 optical sections in a core, a z-stack of images each 1 µm apart was obtained with 5 images above the section and 5 images below the section being analyzed. This allowed us to determine whether an oocyte was part of a germ cell cyst above or below the plane of focus. Follicle development was determined by counting the number of primordial, primary and secondary follicles present in relation to the total number of follicles found and reported as percent primordial, primary or secondary follicles.

Analysis of oocyte numbers

Oocyte numbers were determined by counting the number of germ cells found within each optical section used for analyzing cyst breakdown and follicle development. The numbers were averaged and reported as number of oocytes per section. The number of oocytes per section

is used as a proxy for the total oocytes per ovary, therefore, only ovaries that appeared to be of similar size and depth were used for counting oocyte numbers.

Analysis of cell death

To visualize dying germ cells, ovaries were first labeled for cell death using the Terminal Deoxynucleotidyl Transferase dUTP nick end labeling (TUNEL) kit (Intergen) according to manufacturer's specifications and subsequently labeled for the germ cell marker VASA. Ovaries were then imaged with confocal microscopy. In each ovary, 4 optical sections were visualized and analyzed by counting the number of TUNEL positive cells relative to the total number of cells present. This measure was reported as % TUNEL positive cells per ovary.

Statistical Analysis

A Student's t-test was used to analyze the effects of BCL2 overexpression in the ovary. Effects of blocking MCL1 activity in the ovary and *Bcl2* knockout results were analyzed with a one-way ANOVA using SPSS statistical software. For all results, p values less than 0.05 were significant.

2.4 RESULTS

Expression of BCL2 Protein in Neonatal Ovaries

To test the expression of BCL2 protein in neonatal ovaries, ovaries from 15.5 dpc, 17.5 dpc, PND 1, 3 and 5 mice were harvested, fixed and labeled with an antibody to the BCL2 protein. Figure 2.1 shows that BCL2 protein is expressed primarily in the cytoplasm of oocytes at all ages and appears more strongly expressed in the oocyte at PND 3. Additionally, the protein appears to be weakly expressed in the somatic cells.

BCL2 Overexpression Does Not Alter Oocyte Survival or Cyst Breakdown

BCL2 overexpression was previously shown to increase the number of oocytes and primordial follicles in mouse ovaries at PND 12 (Flaws *et al.* 2001), therefore we questioned whether this difference originated during the neonatal period. To answer this, we examined neonatal ovaries from mice overexpressing BCL2. Ovaries were collected from both BCL2 overexpressing mice and FVB control neonatal mice at PND 1, 4 and 7, fixed, and labeled with the oocyte marker STAT3. Oocyte numbers, cyst breakdown and follicle progression were examined. Control and overexpressing mice had an equal number of oocytes per section at PND 1 and oocyte number decreased at the same rate by PND 4 and 7 (Fig. 2.2A). The drop in oocytes/section was statistically significant in both the wild type and overexpressing mice. Cyst breakdown was also similar between BCL2 overexpressing and FVB control mice at PND 1, 4 and 7. Overexpressing mice had 65.0% single oocytes and control mice had 72.1% single oocytes at PND 7 (Fig. 2.2B). Again, there was no effect of BCL2 overexpression on follicle progression as the percentage of follicles per optical section was similar in

Figure 2.1

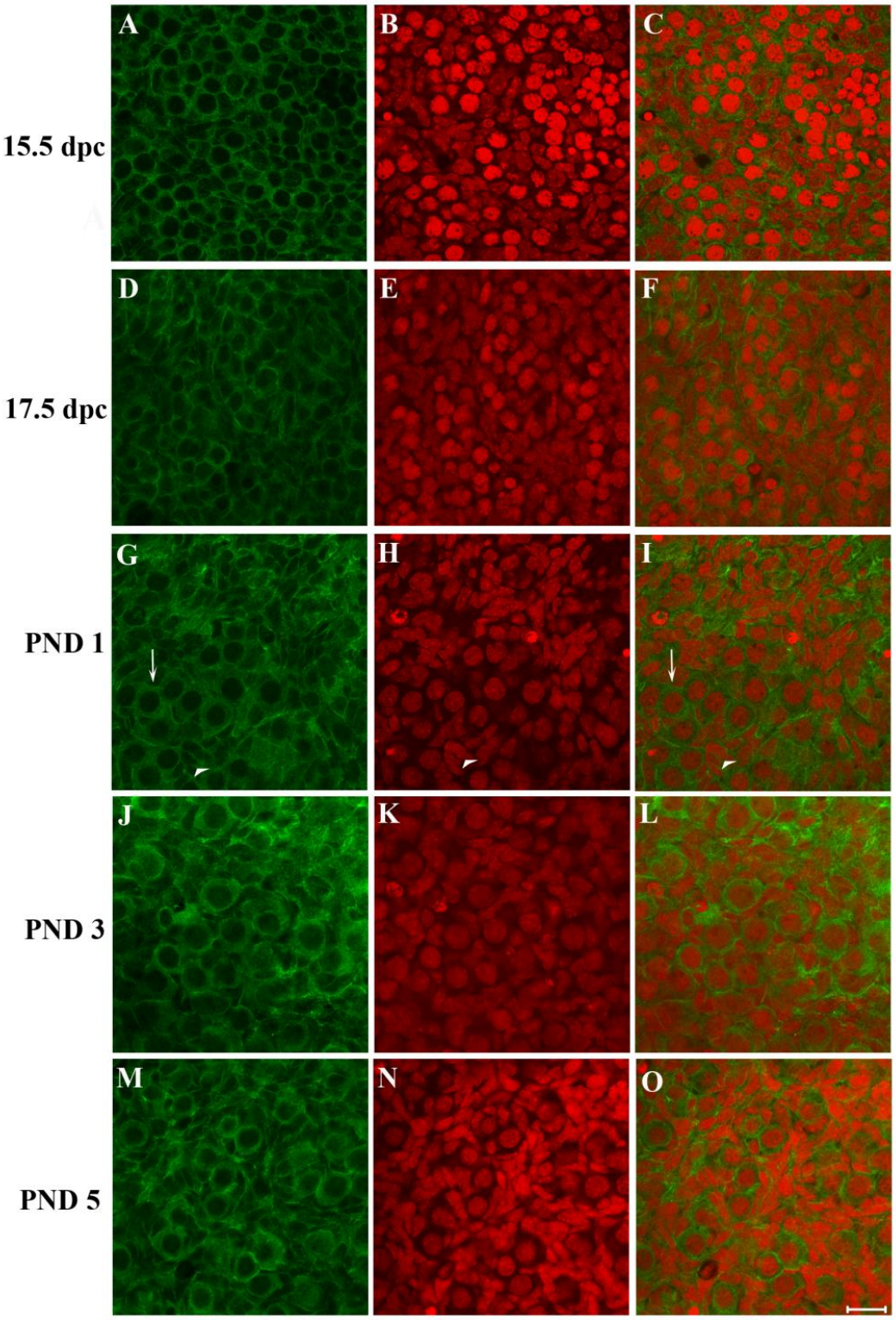


Figure 2.1. Expression of BCL2 in the fetal and neonatal ovary. Confocal sections from 15.5 dpc (A-C), 17.5 dpc (D-F), PND 1 (G-I), PND 3 (J-L) and PND 5 (M-O) ovaries labeled for BCL2 (green) (A, D, G, J and M) and the nuclear marker, propidium iodide (red) (B, E, H, K and N) with overlay shown in C, F, I, L and O. Arrows in G-I indicate oocytes and arrowheads indicate somatic cells. Scale bar, 20 μm.

Figure 2.2

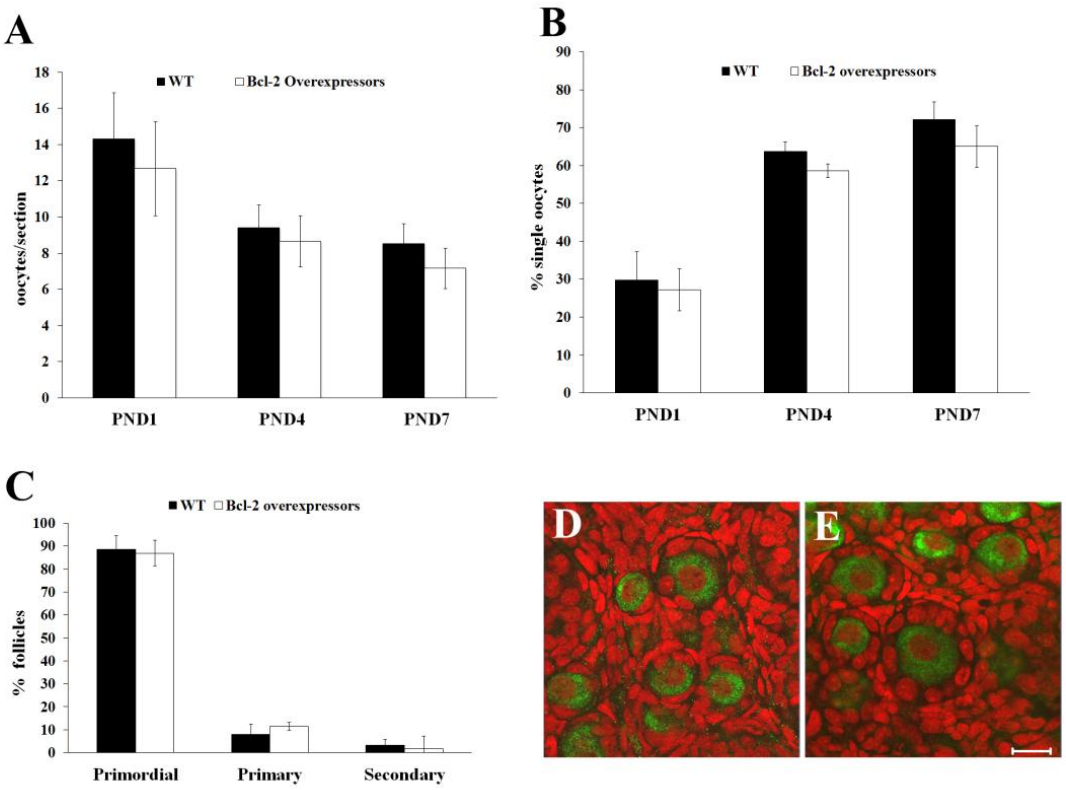


Figure 2.2. Ovarian *Bcl2* overexpression does not alter neonatal ovarian histology. A) Number of oocytes per confocal section at PND 1, 4 and 7 in both WT and *Bcl2* overexpressing mice. B) Percent single oocytes at PND 1, 4 and 7 in both WT and *Bcl2* overexpressing mice. C) Percent of follicles found at either the primordial, primary or secondary stage at PND 7 in both WT and *Bcl2* overexpressing mice. Data are presented as the mean ± SEM. D) Confocal section of a PND 7 WT ovary and E) a PND 7 *Bcl2* overexpressing ovary labeled for STAT3 (green) to visualize oocytes and propidium iodide (red) to visualize nuclei. Scale bar, 20 μm.

control and overexpressing mice (Fig. 2.2C). Figures 2.2D and E show representative confocal sections from control and BCL2 overexpressing ovaries. Therefore, it does not appear that increasing BCL2 protein in the ovary alters the number of oocytes during the neonatal period.

Targeted Disruption of Bcl2 Does Not Affect Germ Cell Survival or Oocyte Cyst Breakdown

To examine whether deletion of ovarian *Bcl2* would result in a decrease in the number of oocytes in the perinatal period we harvested ovaries from *Bcl2* knockout mice and determined oocyte numbers, cyst breakdown and follicle progression. No significant differences were seen in oocyte numbers at any age among $Bcl2^{+/+}$, $Bcl2^{+/-}$ and $Bcl2^{-/-}$ ovaries (Fig. 2.3A). Similarly, *Bcl2* knockout had no effect on cyst breakdown (Fig. 2.3B). Likewise, deletion of *Bcl2* had no effect on follicle progression as the percentage of follicles per optical section was similar in control and knockout mice (Fig. 2.3C). Again, representative confocal sections from WT and *Bcl2* knockout ovaries are shown (Fig. 2.3D and E). From these results, BCL2 protein does not seem to play a role in determining oocyte survival during the perinatal wave of apoptosis and follicle formation.

MCL1 is Expressed in Oocytes

MCL1 has been shown to be expressed in the human ovary during primordial follicle formation, the time when oocyte loss is highest (Hartley *et al.* 2002), and we questioned whether it was also expressed in the neonatal mouse ovary during this

Figure 2.3

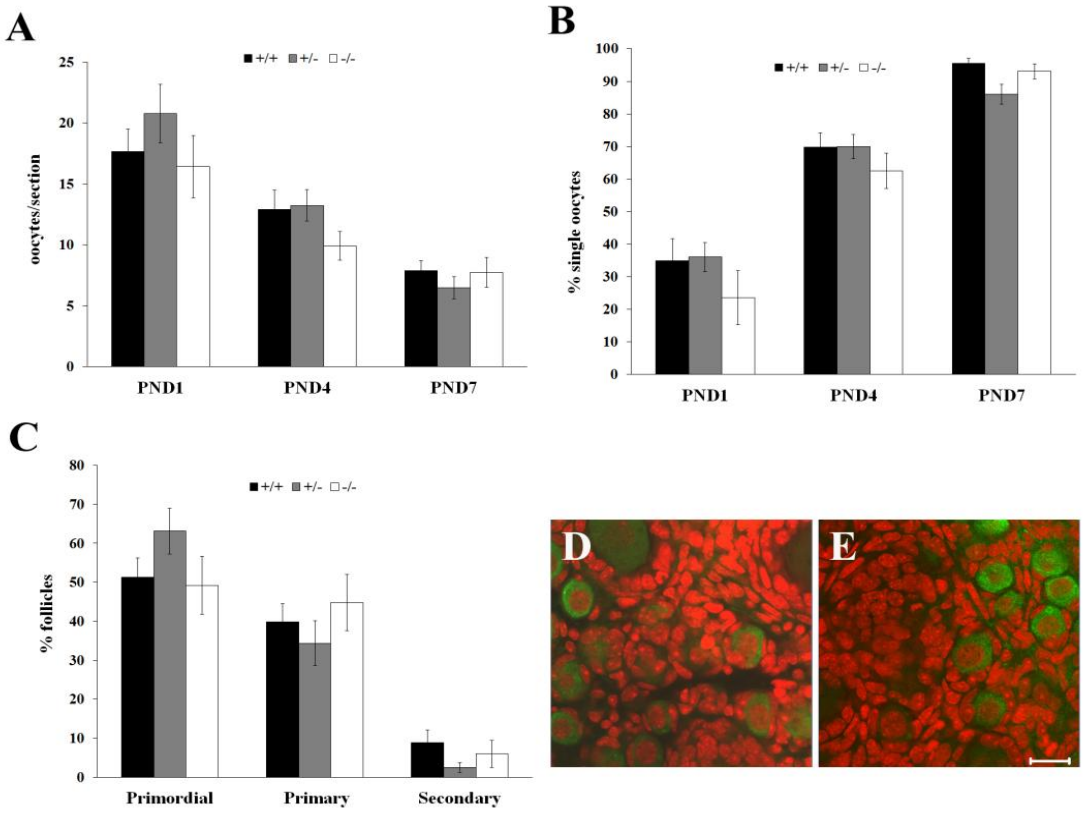


Figure 2.3. Ovarian *Bcl2* knockout does not alter neonatal ovarian histology. A) Number of oocytes per confocal section at PND 1, 4 and 7 in WT, heterozygous and homozygous *Bcl2* knockout mice. B) Percent single oocytes at PND 1, 4 and 7 in WT, heterozygous and homozygous *Bcl2* knockout mice. C) Percent of follicles found at the primordial, primary or secondary stage at PND 7 in WT, heterozygous and homozygous *Bcl2* knockout mice. Data are presented as the mean ± SEM. D) Confocal section of a PND 7 WT ovary and E) a PND 7 *Bcl2* knockout ovary labeled for STAT3 (green) to visualize oocytes and propidium iodide (red) to visualize nuclei. Scale bar, 20 μm.

same process. We harvested fetal mouse ovaries at 15.5 and 17.5 dpc and neonatal ovaries at PND 1, 3 and 5 and fixed and labeled them with an antibody to the MCL1 protein. Figure 2.4A-C shows the lack of expression of MCL1 at 15.5 dpc. At 17.5 dpc MCL1 expression is detected and appears to increase in oocytes between 17.5 dpc and PND 3 (Fig. 2.4D-L). MCL1 expression appears to decrease but remains in the oocyte at PND 5 (Fig. 2.4M-O). From 17.5 dpc onward, MCL1 expression can also be detected in the somatic cells so it is possible that this protein is involved in oocyte or somatic cell survival during the fetal or neonatal time period.

Inhibition of MCL1 in Organ Culture Reduces Oocyte Survival and Promotes Cyst Breakdown in a Dose Dependent Manner

Since MCL1 protein was present at PND 1, we wanted to test the effects of blocking the protein on germ cell survival. We harvested ovaries at PND 1 and placed them in organ culture for 3 days either in control media alone, media supplemented with IgG, or in media supplemented with a function-blocking antibody to MCL1 at a dilution of 1:200, 1:100 or 1:50. After culture, ovaries were then fixed and labeled for the oocyte marker STAT3 to examine oocyte survival, cyst breakdown and follicle progression. Ovaries treated with antibody at 1:200 show a slight but nonsignificant decrease in the number of germ cells present (8.5 oocytes) from control and IgG ovaries (11.8 and 10.7 oocytes; Fig. 2.5A). However, ovaries treated with anti-MCL1 antibody at 1:100 showed a large decrease in the number of oocytes present after 3 days in culture (4.2 oocytes) when compared with both control and IgG treated ovaries (Fig. 2.5A). Further loss of oocytes was observed when the MCL1 antibody was used at a dilution of 1:50 in culture

Figure 2.4

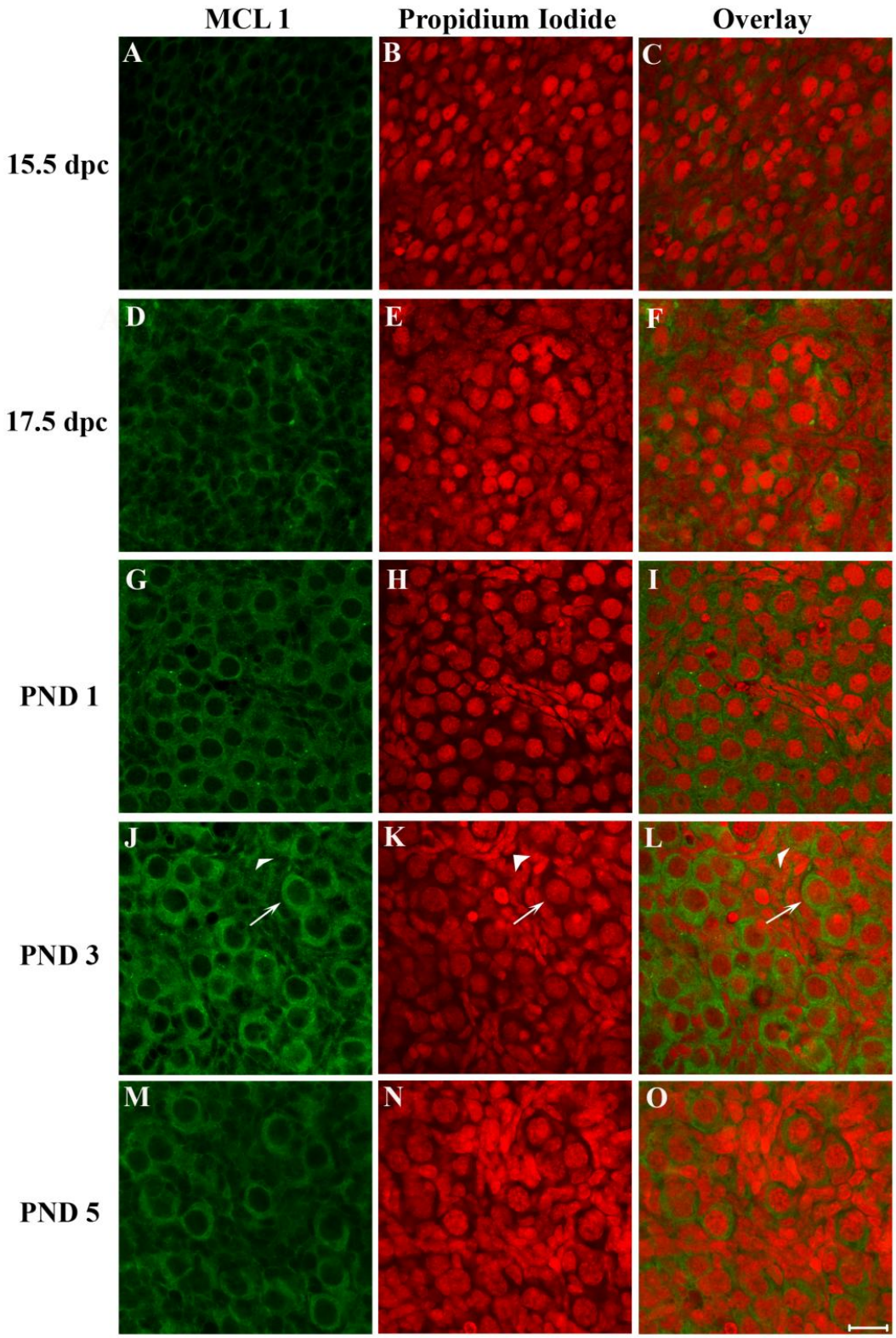


Figure 2.4. Expression of MCL1 in the fetal and neonatal ovary. Confocal sections of ovaries from 15.5 dpc (A-C), 17.5 dpc (D-F), PND 1 (G-I), PND 3 (J-L) and PND 5 (M-O) mice labeled for MCL1 (green) (A, D, G, J and M) and the nuclear marker, propidium iodide (red) (B, E, H, K and N) with overlay shown in C, F, I, L and O. Arrows in J-L indicate oocytes and arrowheads indicate somatic cells. Scale bar, 20 µm.

with treated ovaries containing only 1.1 oocytes (Fig 2.5A). Interestingly, the decrease in the number of oocytes was statistically significant between treatment groups as well, showing a dose response effect of the antibody on germ cell survival. After 3 days in culture, cyst breakdown was also affected by MCL1 inhibition at both the 1:100 and 1:50 dilutions. Treatment with 1:100 MCL1 antibody caused a slight increase in cyst breakdown with treated ovaries containing 85.3% single oocytes. Treatment with 1:50 MCL1 antibody resulted in a statistically significant increase with treated ovaries containing 92.8% single oocytes versus 76.7% in IgG treated ovaries and 70.7% single oocytes in control ovaries (Fig. 2.5B). Follicle progression, however, was unaffected by MCL1 antibody treatment at all three dilutions with control, IgG and anti-MCL1 treated ovaries demonstrating similar percentages of primordial, primary and secondary follicles (Fig. 2.5C). Figures 2.5D and E are representative confocal sections of both control and anti-MCL1 treated ovaries.

Cell Death is Increased When Treated with an Antibody to MCL1

To confirm that inhibition of MCL1 indeed resulted in increased oocyte death, we grew PND 1 ovaries overnight in organ culture. Ovaries treated with the antibody to MCL1 show a statistically significant increase in apoptosis with 14% TUNEL positive cells compared with only 4.9% in controls (Fig. 2.6A). Representative confocal sections showed a larger number of cells labeling with TUNEL in MCL1 treated ovaries when compared with controls (Fig. 2.6B and C). These results demonstrate a role for MCL1 in the survival of oocytes and primordial follicle formation in the neonatal mouse ovary.

Figure 2.5

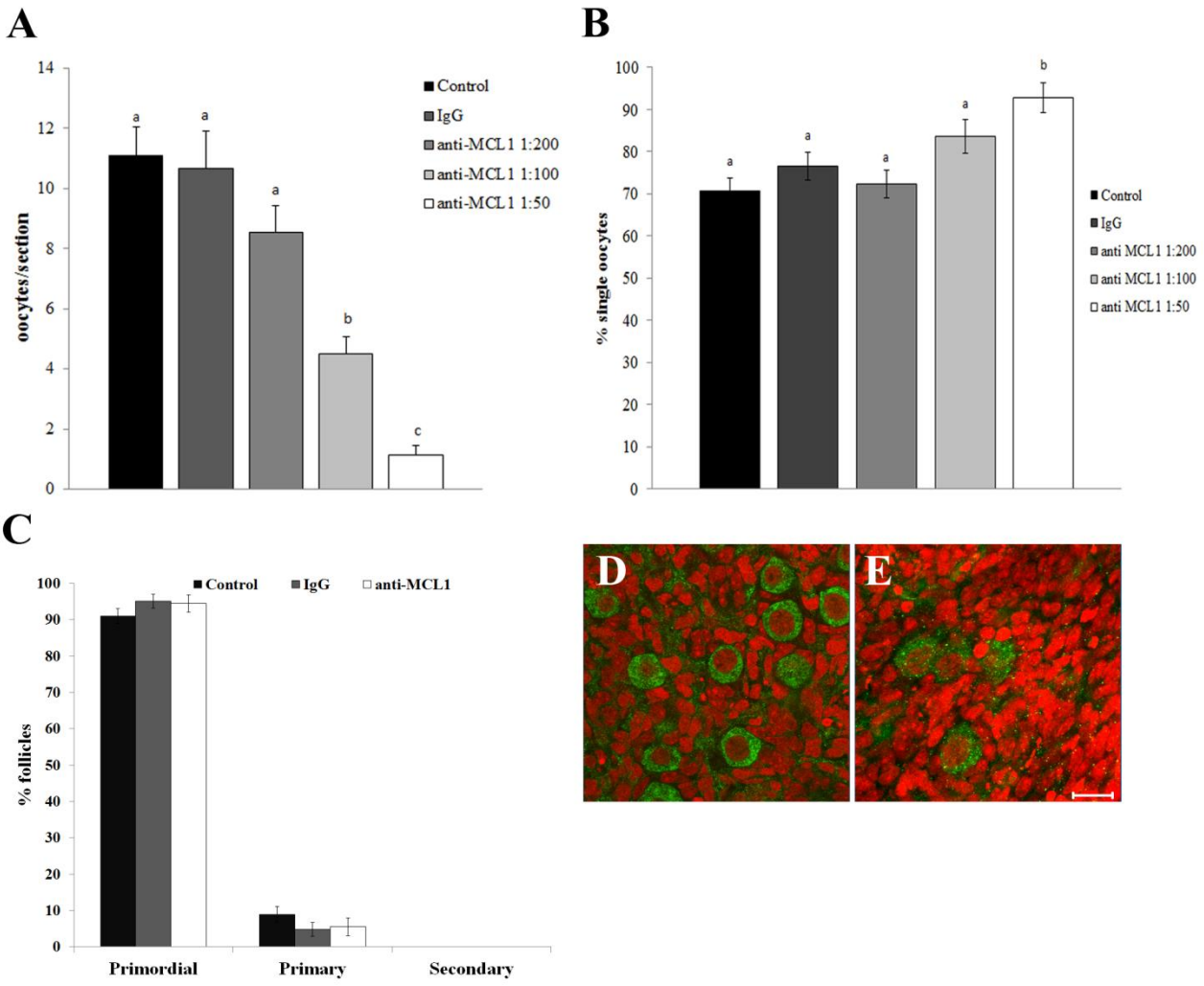


Figure 2.5. MCL1 antibody treatment decreases oocyte numbers and increases cyst breakdown in organ culture. A) Number of oocytes, B) percent single oocytes and C) percent of follicles found at the primordial, primary or secondary stage per confocal section in newborn ovaries after 3 days in culture with an antibody to MCL1 at 1:200, 1:100 and 1:50 dilutions. Data are presented as the mean ± SEM. Different letters indicate a significant difference between groups (ANOVA, $p < 0.05$). D) Confocal section of an ovary cultured for 3 days in control media or E) with an anti-MCL1 antibody labeled for STAT3 (green) to visualize oocytes and propidium iodide (red) to visualize nuclei. Scale bar, 20 µm.

Figure 2.6

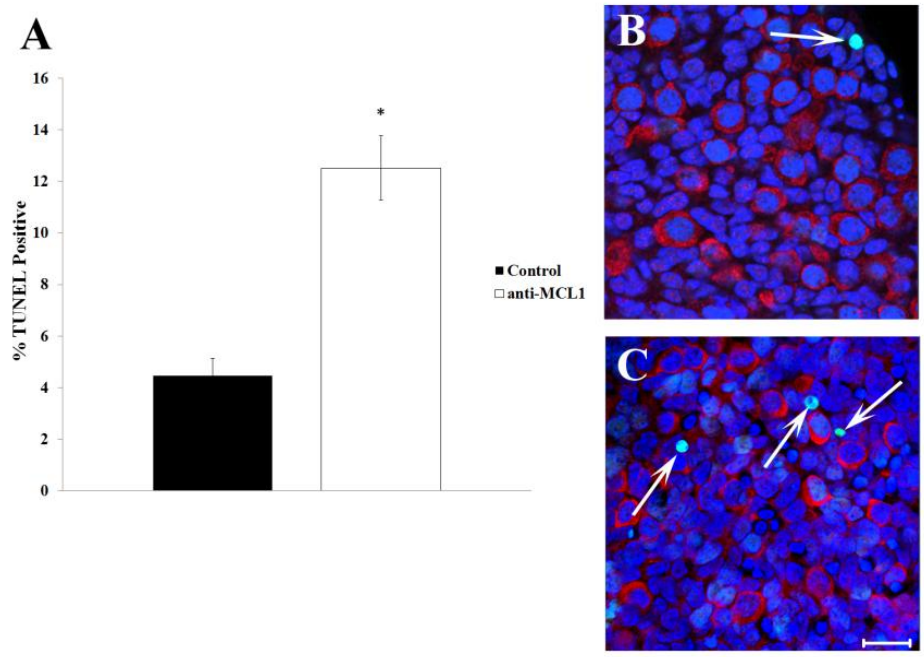


Figure 2.6. Apoptosis increases when PND 1 ovaries are cultured with an antibody to MCL1. A) Percentage of TUNEL positive cells in newborn ovaries after 1 day in culture with MCL1 antibody. Data are presented as the mean ± SEM. * indicates a significant difference between control and treated ovaries (ANOVA, $p < 0.05$). B) Confocal section of an ovary cultured for 1 day in control media or C) with an anti-MCL1 antibody labeled for the oocyte marker VASA (red), death marker TUNEL (green) and TOTO-3 (blue) to visualize nuclei. Arrows indicate dying cells. Scale bar, 20 μm.

Live Cells Are Able to Take Up MCL1 Antibody in Culture

To determine whether the MCL1 antibody can access the cytoplasm of live cells, we first cultured PND 1 ovaries in either control media alone or in media supplemented with the MCL1 antibody. After 24 hours, ovaries were fixed and incubated with the secondary antibody to detect the presence or absence of the MCL1 antibody within oocyte or somatic cells. As expected, no MCL1 antibody is detected in control ovaries (Fig. 2.7A). Figures 2.7B and C show the presence of the MCL1 antibody in the cytoplasm of both oocytes and somatic cells. Overall, a total of 12.8% of oocytes showed presence of MCL1 antibody within the cytoplasm after 24 hours in culture. The antibody was also found to be present in several granulosa cells, however this data was not quantified because the total number of granulosa cells was not determined. This demonstrates the ability of living ovarian cells to take up the MCL1 antibody while in culture.

Figure 2.7

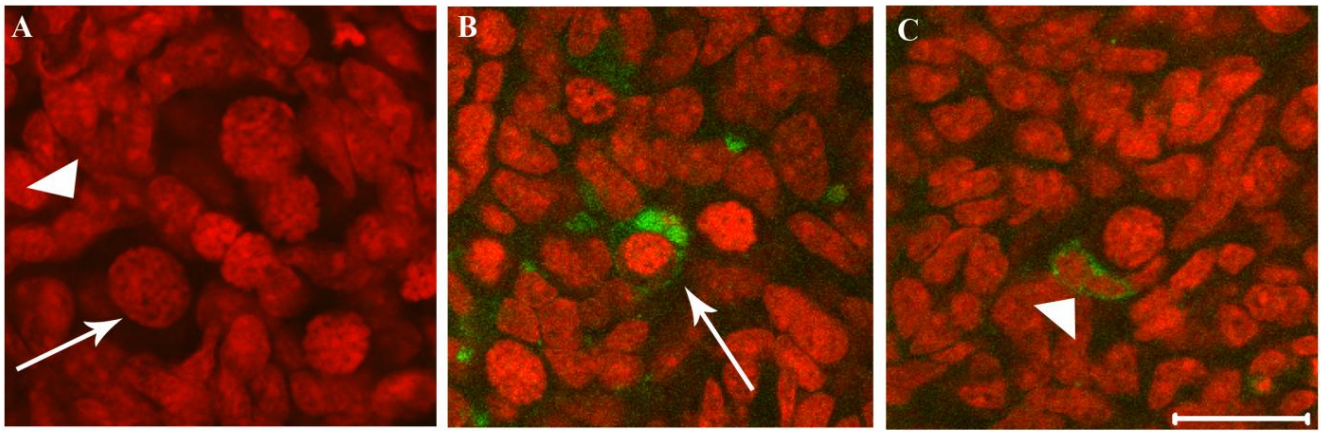


Figure 2.7. MCL1 antibody is taken up by live oocytes and somatic cells. Confocal section of an ovary cultured for 1 day in A) control media or B and C) with an anti-MCL1 antibody. Localization of MCL1 antibody was detected by labeling with a secondary antibody (green) and nuclei were labeled with propidium iodide (red). Arrow in A shows an example of lack of MCL1 labeling in oocytes of a control ovary and arrowheads show somatic cells without labeling. Arrow in B demonstrates the ability of live oocytes to take up the MCL1 antibody (green) and arrowhead in C shows MCL1 antibody (green) taken up by somatic cells. Scale bar, 20 μm.

2.5 DISCUSSION

Previous studies have demonstrated a role for BCL2 in establishing and maintaining the ovarian reserve (Ratts *et al.* 1995, Flaws *et al.* 2001). Flaws *et al.*(2001) reported an increase in the number of primordial follicles when BCL2 was overexpressed in the mouse ovary . At PND 8 overexpressors had more primordial follicles than control animals. In addition, by PND 60, the *Bcl2* transgenic mice have the same number of oocytes as control mice indicating that these extra germ cells are not maintained in the adult. Timepoints earlier than PND 8 were not examined so it was not known when the difference in oocyte numbers occurred. We hypothesized that this difference may be due to a reduction in perinatal oocyte apoptosis. To test this, we analyzed newborn ovaries from *Bcl2* transgenic mice. Contrary to our prediction, we did not see an increase in the number of oocytes at any time point, indicating that BCL2 protein is not important for germ cell survival in early neonatal life. Using *Bcl2* knockout mice, Ratts and colleagues (1995) found a reduction in the number of primordial follicles at PND 42 but earlier timepoints were not examined. However based on the results from our overexpression studies, we hypothesized that the reduction found in the previous study would not be a result of increased oocyte death during perinatal oocyte apoptosis. To confirm our findings that BCL2 was not necessary for oocyte survival in the newborn ovary, we examined neonatal *Bcl2* knockout mouse ovaries. Again, no significant differences were noted in the number of germ cells between wild type and *Bcl2* transgenic ovaries. Therefore, we conclude that *Bcl2* is dispensable for neonatal oocyte survival.

Although we found no role for BCL2 in perinatal germ cell survival, previous work demonstrated that manipulation of BCL2 protein levels altered ovarian histology, specifically in the endowment of primordial follicles (Ratts *et al.* 1995, Flaws *et al.* 2001). Recently, Tingen *et*

al.(2009) showed that there is a large loss of pre-pubertal primordial follicles in the mouse ovary beginning at PND 6. It is possible that the BCL2 protein is responsible primarily for the survival of primordial follicles during this time period. Further studies examining the effects of ablating the *Bcl2* gene on primordial follicle numbers after PND 6 would shed more light on the importance of the protein in pre-pubertal follicle survival.

We found that the MCL1 protein was increasingly expressed in murine oocytes from 17.5 dpc through PND 3, the window of time when primordial follicles form and the greatest amount of oocyte death is occurring in the ovary (Pepling & Spradling 2001). Similarly, Hartley *et al.* (2002) reported that MCL1 was expressed in human oocytes during the time of primordial follicle formation and oocyte death, suggesting that MCL1 may be conserved across mammalian species as an important pro-survival factor for germ cells during the massive germ cell apoptosis that accompanies primordial follicle formation. More importantly, it implicates a role for MCL1 in germ cell survival during cyst breakdown and primordial follicle formation.

To test whether the MCL1 protein is important for oocyte survival, we used an in vitro organ culture system, because knockout of *Mcl1* results in embryonic lethality (Rinkenberger *et al.* 2000). Ovaries cultured in the presence of an antibody to MCL1 demonstrated a decrease in the number of oocytes present. This reduction in germ cell numbers was also dose dependent, indicating that there is a threshold in the level of MCL1 protein required for survival within oocytes. Since MCL1 is a known anti-apoptotic protein (Kozopas *et al.* 1993), we suspected that the reduction in the number of oocytes was due to an increase in apoptosis when MCL1 was inhibited in culture. This hypothesis was supported by the increase in TUNEL labeling found in ovaries treated with the antibody to MCL1 overnight in culture. We cannot confirm that the TUNEL labeled cells are oocytes because they are not labeled with the oocyte marker. We

believe that antibody reactivity is lost in dying oocytes and have observed this previously (Pepling & Spradling 2001). MCL1 is expressed in both the oocytes and somatic cells during the time of our culture, thus we cannot conclude that the blocking antibody is acting directly on the oocytes. The effect could be indirect with the antibody acting on the somatic cells. In any case, MCL1 appears to be not only expressed in the murine ovary, but also a key regulator of germ cell survival during the time of germ cell loss, cyst breakdown and primordial follicle formation.

It is thought that oocyte cyst breakdown may be reliant on germ cell death, as these two processes are temporally correlated in the ovary (Pepling & Spradling 2001). Support for this idea comes from several studies demonstrating that a delay in cyst breakdown is associated with an increase in germ cell number (Chen *et al.* 2007, Jefferson *et al.* 2006, Trombly *et al.* 2009, Karavan & Pepling 2012). Greenfeld and colleagues (2007) found that lack of the pro-apoptotic BCL2 family protein, BAX, led to a decrease in oocyte death and a reduction in cyst breakdown. Likewise, we found that when MCL1 was inhibited and germ cell death increased, the rate of cyst breakdown and the percentage of single oocytes also increased. These findings suggest that oocyte death may be involved in regulation of cyst breakdown. Alternatively, it is possible that the mass germ cell apoptosis caused by MCL1 inhibition left only a few, single oocytes remaining.

To our knowledge, we are the first to use an antibody in ovarian culture that targets an internal cellular protein. Guo and colleagues successfully utilized antibodies targeted to the phosphatase of regenerating liver proteins, proteins localized inside the cell, to ablate cancer cells (Guo *et al.* 1994). Within their study, they were able to demonstrate the ability of both live CHO and cancer cell lines to take up the PRL antibodies using a cell culture system. By showing the presence of the MCL1 antibody in the cytoplasm of both oocytes and somatic cells

after culture, we have confirmed the findings by Guo and colleagues that live cells can, in fact, allow antibodies access to internal portions of the cell *in vitro*. This is an exciting finding as it may lead to the ability to examine more ovarian cell signaling pathways inside of oocytes and somatic cells utilizing antibodies that target intracellular proteins. However, it is still possible that the MCL1 antibody is accessing the cytoplasm after fixation due to antibody that may not be completely washed out of the tissue prior to membrane permeabilization. Further experiments using immunoprecipitation to detect an MCL1 antibody-MCL1 protein complex would add strength to our findings that the antibody appears to cross the cell membrane.

The experiments presented here are the first to examine the involvement of BCL2 anti-apoptotic family members in perinatal oocyte survival and cyst breakdown. We have shown that while BCL2 itself does not play a role in early germ cell survival, MCL1 is important for preventing oocyte death during cyst breakdown and primordial follicle formation. A conditional knockout model of MCL1 should be developed and examined in the future to determine if our in vitro organ culture studies reflect what is truly happening in the animal. In addition, how MCL1 works to affect cell death remains a mystery and should be a focus of future studies. Previous work on BCL2 family members has demonstrated that these molecules may heterodimerize with pro-apoptotic members such as BOK, BOD, BAK and BAX at the mitochondria (Hsu &Hsueh 2000, Boumela *et al.* 2011) or can regulate cellular calcium levels at the endoplasmic reticulum to inhibit apoptosis (Bassik *et al.* 2004). It would be interesting to discover where and how MCL1 prevents oocyte death in the ovary during mass germ cell apoptosis. Finally, future work needs to identify the molecules that regulate the action of MCL1 in the oocyte to prevent germ cell death. New studies revealing the mechanisms by which MCL1 regulates the size of the primordial follicle pool would give insight into the establishment and maintenance of the ovarian

lifespan and may lead to new discoveries in the regulation of primordial follicle formation and fertility.

CHAPTER 3:

KIT signaling regulates primordial follicle formation in the neonatal mouse ovary

3.1 ABSTRACT

The primordial pool of follicles determines the reproductive lifespan of the mammalian female, and its establishment is highly dependent upon proper oocyte cyst breakdown and regulation of germ cell numbers. The mechanisms controlling these processes remain a mystery. We hypothesized that KIT signaling might play a role in perinatal oocyte cyst breakdown, determination of oocyte numbers and the assembly of primordial follicles. We began by examining the expression of both KIT and KIT ligand in fetal and neonatal ovaries. KIT was expressed only in oocytes during cyst breakdown, but KIT ligand was present in both germ and somatic cells as primordial follicles formed. To test whether KIT signaling plays a role in cyst breakdown and primordial follicle formation, we used ovary organ culture to inhibit and activate KIT signaling during the time when these processes occur in the ovary. We found that when KIT was inhibited, there was a reduction in cyst breakdown and an increase in germ cell numbers. Subsequent studies using TUNEL and proliferation analyses showed that when KIT was inhibited, cell death and somatic cell proliferation were both reduced. Conversely, when KIT was activated, cyst breakdown was promoted and oocyte numbers decreased. Using Western blotting, we found increased levels of phosphorylated MAP Kinase and that the survival protein, MCL1 was lost when KIT ligand was added to culture. Taken together, these results demonstrate a role for KIT signaling in perinatal oocyte cyst breakdown that may be mediated by MAP Kinase downstream of KIT.

3.2 INTRODUCTION

Female fecundity is determined at the time of birth through the establishment of the primordial follicle pool, and any aberration in the formation of primordial follicles can result in infertility. Follicular formation is tightly regulated and begins during fetal life. After migration to the gonad, primordial germ cells (PGCs) divide rapidly but do not complete cytokinesis and are connected by intercellular bridges, forming germ cell cysts (Peters 1970, Pepling & Spradling 1998). As the germ cells enter meiosis, they cease mitotic division and remain connected until they begin to arrest in the diplotene stage of meiosis. At that time, the germ cells, now called oocytes, begin to separate from each other and become surrounded by somatic granulosa cells, forming primordial follicles. The process of cyst breakdown and primordial follicle formation typically lasts from 17.5 days post coitum (dpc) to post natal day (PND) 5 (Borum 1961, Menke *et al.* 2003). Concomitant with cyst breakdown is a large loss of germ cells, beginning at approximately 17.5 dpc and peaking between PND 2 and 3 of development (Pepling & Spradling 2001, Pepling *et al.* 2010). The mechanisms regulating both perinatal cyst breakdown and oocyte survival remain a mystery. One pathway of interest may be the KIT signaling pathway, as it has been previously shown to be important in oocyte survival and follicle development at other timepoints.

KIT signaling is widely known for its ability to promote cell survival, proliferation and differentiation. The receptor, KIT, and its ligand, KIT ligand (KITL) also known as Stem Cell Factor (SCF), are encoded by the *White Spotting (W)* and *Steel* loci, respectively. Mutations at either locus have been studied extensively and result in an array of developmental defects in melanogenesis, hematopoiesis and gametogenesis (Roskoski 2005). Signaling depends on the binding of KITL to KIT and when bound, the KIT receptor homodimerizes and

autophosphorylates at tyrosine residues, attracting and binding downstream signaling molecules containing phosphotyrosine binding sites (Roskoski 2005). KIT autophosphorylation is capable of activating several downstream cascades, including the JAK-STAT, PI3K, and MAPK pathways (Schlessinger 2000).

KITL exists in both a soluble isoform and a membrane bound isoform (Roskoski 2005). The soluble form (KITL1) is a result of cleavage of the transmembrane portion of the protein, allowing the protein to dislodge from the cell membrane. The membrane bound isoform (KITL2) actually lacks exon 6 and therefore, the transmembrane cleavage site, forcing the protein to remain anchored at the cell membrane (Ashman 1999). The exact function of each isoform is not well understood, however it has been reported that KITL2 binding results in more sustained KIT signaling (Miyazawa *et al.* 1995).

The KIT pathway has been shown to be important for many ovarian functions including germ cell survival and migration. Early studies examining *Steel* mutant mice demonstrated the importance of this signaling pathway in the proper migration of PGCs to the genital ridge. A decrease in KITL expression results in PGC migration to ectopic sites and a reduction in germ cell proliferation and survival (McCoshen & McCallion 1975, Huang *et al.* 1992, Huang *et al.* 1993). Reynaud and colleagues demonstrated that KITL and KIT were also imperative to later follicular survival, protecting pre-antral follicles from apoptosis (Reynaud *et al.* 2001). Finally, ovaries of newborn rats exposed to recombinant KITL in culture had a lower percentage of dying oocytes than control ovaries (Jin *et al.* 2005).

Postnatal development of follicles is also dependent on the KIT ligand/KIT system. After birth, the flattened granulosa cells of primordial follicles become cuboidal and proliferate, forming the primary follicle. This change from primordial to primary follicle has been shown to

be dependent on the KIT pathway in several studies. *Steel*^{panda} mutant mice, a KITL hypomorph which produces only a small amount of KITL, show a nearly complete block on the primordial to primary follicle transition, leaving a large pool of primordial follicles that cannot develop. These mice are sterile (Huang *et al.* 1993). In another experiment, when neonatal mice were injected with the KIT neutralizing antibody, ACK2, the transition from primordial to primary follicle was severely blocked (Yoshida *et al.* 1997). Conversely, when exogenous KITL was added to 4 day old rat ovaries in culture, there was a significant increase in developing primordial follicles (Parrott & Skinner 1999). These experiments demonstrate the importance of KIT signaling in primordial follicle development, and studies have begun to describe an involvement of the KIT signaling pathway in an oocyte-granulosa cell feedback loop. During the primordial to primary transition, KIT activation at the oocyte regulates other molecules downstream that affect granulosa cell division. Otsuka and Shimasaki (2002) found that in the presence of KITL, granulosa cell mitosis increased, indicating an indirect regulation of granulosa cell division via the oocyte KIT signal. In the same study, when an antibody to KIT was added to culture media, granulosa cell division decreased.

KIT signaling is also important in a subsequent stage of follicle development, the transition from preantral to antral follicle. As the granulosa cells of primary follicles divide to produce multiple layers, a secondary or preantral follicle is formed. These preantral follicles eventually gain a fluid filled space, and are then classified as antral follicles. Yoshida and colleagues (1997) were able to block the transition from the preantral to the antral follicle stage by ACK2 antibody injection. Similarly, when ACK2 was used to block KIT signaling in rat ovary culture, a block on gonadotropin induced preantral follicle development was seen (Parrott & Skinner 1999).

Although much evidence exists for the role of KIT signaling in ovarian development, the contribution of KIT signaling to primordial follicle formation in later fetal and early neonatal development is unclear. In hamster, KIT signaling has been shown to promote cyst breakdown and primordial follicle formation in vitro (Wang & Roy 2004). However, using an injection of a KIT antibody, Yoshida *et al.* (1997), failed to find any role for KIT in primordial follicle formation in newborn mouse ovaries. Since KIT is directly involved in, and important to, early survival of oocytes and subsequent postnatal development of follicles, we hypothesized that this pathway might also regulate the formation of primordial follicles from oocyte cysts. In the current study we took advantage of an organ culture system to directly inhibit and activate KIT signaling in fetal ovaries, when cyst breakdown and follicle formation begin. Our results illustrate the importance of this pathway in the establishment of the primordial follicle pool.

3.3 MATERIALS AND METHODS

Animals

All C57BL/6 mice were purchased from Jackson Laboratories. Adult male and female mice were mated utilizing timed matings, and females were checked for the presence of a vaginal plug the following morning. The presence of a vaginal plug was denoted 0.5 dpc of pregnancy. Pregnant mice were euthanized on either 17.5 dpc for organ culture experiments or 16.5, 17.5 and 18.5 dpc for immunohistochemistry. Otherwise, pregnant dams gave birth at 19.5 dpc (PND 1) and pups were euthanized at PND 1 and 3 for immunohistochemistry.

All mice were housed under 12 hour light/dark cycles, temperatures of 21-22 °C and had free access to chow and water. All animal protocols were approved by the Syracuse University Institutional Animal Care and Use Committee.

Antibodies

For immunohistochemistry, STAT3 (C-20) antibody (Santa Cruz Biotechnology) was used at a dilution of 1:500, KIT (C-19) and Stem cell factor (G-19) antibodies (Santa Cruz Biotechnology) were used at a dilution of 1:100, VASA antibody (Abcam) was used at a dilution of 1:250 and cleaved PARP antibody (Abcam) was used at a dilution of 1:100. The secondary antibodies, goat anti-rabbit Alexa 488, rabbit anti-goat Alexa 488 and goat anti-rabbit Alexa 568 (Invitrogen), were at a dilution of 1:200. TUNEL labeling kit (Intergen) was used to visualize dying oocytes and the Ki-67 (Ab-4) antibody (NeoMarkers) was used to assess cell proliferation. For Western blotting, AKT 1/2/3 (H-136), phospho-AKT 1/2/3 (Ser473), STAT3 (C-20) and phopho-STAT3 (Tyr 705) antibodies (Santa Cruz Biotechnology) were used at a dilution of 1:1000. MAPK p44/42 ERK1/2 and Phospho-p44/42 (T202/Y204) antibodies (Cell Signaling)

were used at a dilution of 1:1000 for Western blotting. Additionally, MCL1 antibody (S-19; Santa Cruz Biotechnology) was used at a dilution of 1:50 for Western blotting.

In vitro ovary organ culture

As previously described ovaries from PND 1 female mice were harvested and placed on 0.4 µm floating filters (Millicell-CM; Millipore Corp) in 4 well culture dishes (Nunc) with 0.4 ml culture media consisting of D-MEM/HAM'S F12 Media (Invitrogen), 0.1% Albumax (Invitrogen), 0.1% Fraction V BSA (Invitrogen), 5X ITS-X (Life Technologies),0.05 mg/ml L-ascorbic acid (Sigma) and penicillin-streptomycin (Life Technologies) (Chen *et al.* 2007). A single drop of media was placed on each of the ovaries to keep them from drying out.

To assess the role of KIT signaling in the ovary, 17.5 dpc ovaries were cultured for 5 days in either media alone, media supplemented with a function blocking antibody to KIT, ACK2 (e-biosciences) at a 1:100 dilution, media supplemented with recombinant Stem cell factor (R&D Systems) at 100 ng/ml or media supplemented with IgG (Santa Cruz) at a 1:100 dilution. (n=8 ovaries per group). To determine the role of KIT signaling before PND 1, ovaries (n=8 ovaries per group) were cultured for 3 days either in control media alone, or in media supplemented with ACK2 at 17.5 dpc then switched to control media after 2 days in culture. To determine the importance of KIT signaling at birth, ovaries (n=8) were grown in culture for 5 days either in control media alone, or in control media at 17.5 dpc then switched to media supplemented with ACK2 after 2 days in culture. For cell death and proliferation studies, ovaries were cultured in control or ACK2 supplemented media for 3 days (n=8 ovaries per group). At the conclusion of culture, all ovaries were prepared for whole-mount immunohistochemistry.

To determine pathways regulated by KIT signaling, 17.5 dpc ovaries were grown in culture for 3 days in either control media or media supplemented with SCF (n=8). At the conclusion of culture, ovaries were prepared for Western blotting.

Immunohistochemistry

Ovaries were fixed in 5% EM-grade paraformaldehyde (Electron Microscopy Sciences) overnight at 4 °C and stained as previously described (Pepling & Spradling 1998). Whole ovaries were labeled with primary antibody overnight, then with anti-rabbit or anti-goat secondary antibody. Nuclei were labeled with TOTO-3 or propidium iodide. A Zeiss LSM 710 Confocal Microscope was used to image ovaries. As a control, some ovaries were labeled with only anti-rabbit secondary antibody.

Analysis of germ cell cyst breakdown and follicle development

At the conclusion of cultures, ovaries prepared for immunohistochemistry were labeled with STAT3 antibody, a known oocyte marker and imaged with confocal microscopy (Murphy *et al.* 2005). In each ovary, two cores were visualized and counted. A core is a region 512 x 512 µm made up of optical sections at 4 separate depths in the ovary 15-20 µm apart. For each ovary, 2 cores with 4 optical sections were analyzed for a total of 8 sections examined. The number of oocytes found in cysts relative to the total number of oocytes was determined for each ovary by analyzing each section and reported as percent single oocytes. In order to determine whether oocytes were in cysts or not, for each of the 4 optical sections in a core, a z-stack of images each 1 µm apart was obtained with 5 images above the section and 5 images below the section being analyzed. This allowed us to determine whether an oocyte was part of a germ cell cyst above or

below the plane of focus. Follicle development was determined by counting the number of primordial, primary and secondary follicles present in relation to the total number of follicles found and reported as percent primordial, primary or secondary follicles.

Analysis of oocyte numbers

To determine oocyte numbers, we counted the number of oocytes found within the 8 optical sections used for analyzing cyst breakdown and follicle development. The numbers were averaged and reported as number of oocytes per section. Only ovaries that appeared to be of similar size and depth were used for counting oocyte numbers because the number of oocytes per section is an estimate of the total number of oocytes within an ovary.

Analysis of cell death and proliferation

To analyze dying cells, ovaries were first labeled with the TUNEL labeling kit (Intergen) according to the manufacturers recommendations and then with the oocyte marker antibody, VASA. Ovaries were imaged with confocal microscopy. In each ovary, 4 optical sections were visualized and analyzed by counting the number of TUNEL positive cells relative to the total number of cells present. This number was reported as % TUNEL positive cells per ovary. For proliferation studies, ovaries were first labeled with an antibody to Ki-67, a marker of cell division, and subsequently labeled with VASA. Again, ovaries were imaged with confocal microscopy and in each ovary, 4 optical sections were visualized. Each section was analyzed by counting the number of Ki-67 positive cells relative to the total number of cells present. This number was reported as % Ki-67 positive cells per ovary.

Western blot hybridization

17.5 dpc ovaries were grown in culture for 3 days and then homogenized in 10µl of 2X sample buffer (10% SDS, Glycerol, 0.1% Bromophenol Blue, 0.5M Tris pH 6.8, and 2-mercaptoethanol) plus mini-complete protease inhibitors (Roche) per ovary. 2-mercaptoethanol was added to extracts at 1/10 of the volume of extract. Samples were then heated to 95 °C and centrifuged at 6000 rpm for 1 minute, separated on a 10% SDS-polyacrylamide gel (BioRad) and then electroblotted onto either Immobilon P PVDF (Millipore) or nitrocellulose (BioRad) membranes. Membranes were blocked in either PBST (0.05% Tween/PBS) containing 5% nonfat milk for AKT, phospho-AKT, MAPK p44/42, STAT3, MCL1 and BAX antibodies or TBST (0.05% Tween/TBS) with 5% BSA for phospho-p44/42 and phospho-STAT3 antibodies for 1 hour at room temperature. Blots were then incubated with primary antibody diluted in the appropriate blocking solution for 1 hour at room temperature. Membranes were washed 3-6 times in either PBST or TBST then incubated with secondary antibody diluted in 5% milk blocking solution for 1 hour at room temperature. Goat anti-rabbit horseradish peroxidase conjugated secondary (Thermoscientific) antibody for the AKT, pAKT, MAPK p44/42, STAT3, MCL1 and BAX hybridized blots was used at a dilution of 1:10,000 and at a dilution of 1:15,000 for phospho-p44/42 MAPK and phospho-STAT3 hybridized blots. Again, blots were washed 3-6 times in PBST or TBST and bands were visualized using the Supersignal kit (Pierce) on films. Blots were reprobed with anti-mouse GAPDH (1:5000; Santa Cruz Biotechnology) as a control.

Statistical analysis

A Student's t-test was used to analyze cell death and proliferation and studies analyzing the effect of blocking KIT in the ovary at different time points. Effects of ACK2 and

recombinant SCF on ovaries cultured for five days beginning on 17.5 dpc were analyzed with a one-way ANOVA using SPSS statistical software. For all results, p values less than 0.05 were considered significant.

3.4 RESULTS

Expression of KIT and KITL in the ovary

We first tested the expression of both KIT and KITL in the fetal and neonatal ovary to determine if KIT signaling could be involved in oocyte survival and follicle formation at the time of birth. To evaluate expression, we harvested ovaries from 16.5 dpc, 17.5 dpc, 18.5 dpc, PND 1 and PND 3 mice, fixed and labeled them with an antibody to either KIT or KITL. Figure 3.1 shows the expression of KIT weakly in the cytoplasm of germ cells at 16.5 (A-C) and 17.5 (D-F) dpc, and expression at the cell membrane appears to begin in one oocyte at 17.5 dpc. At 18.5 dpc (Fig. 3.1G-I) KIT is expressed in a subset of the oocytes in either the cytoplasm or cell membrane but becomes pronounced at the cell membrane of oocytes by PND 1 (Fig. 3.1J-L). Interestingly, at PND 1 a small subset of cells within a cyst do not express KIT (inset Fig. 3.1L). KIT protein appears to be similarly expressed at PND 3 although it may be less pronounced at the membrane (Fig. 3.1M-O). To demonstrate that KIT labels germ cells, we used immunohistochemistry on PND 1 ovaries and labeled them with an antibody to KIT and to the germ cell marker, VASA. Figure 3.2 shows that KIT labels only germ cells at PND 1 and that some oocytes within a cyst do not express KIT.

Figure 3.1

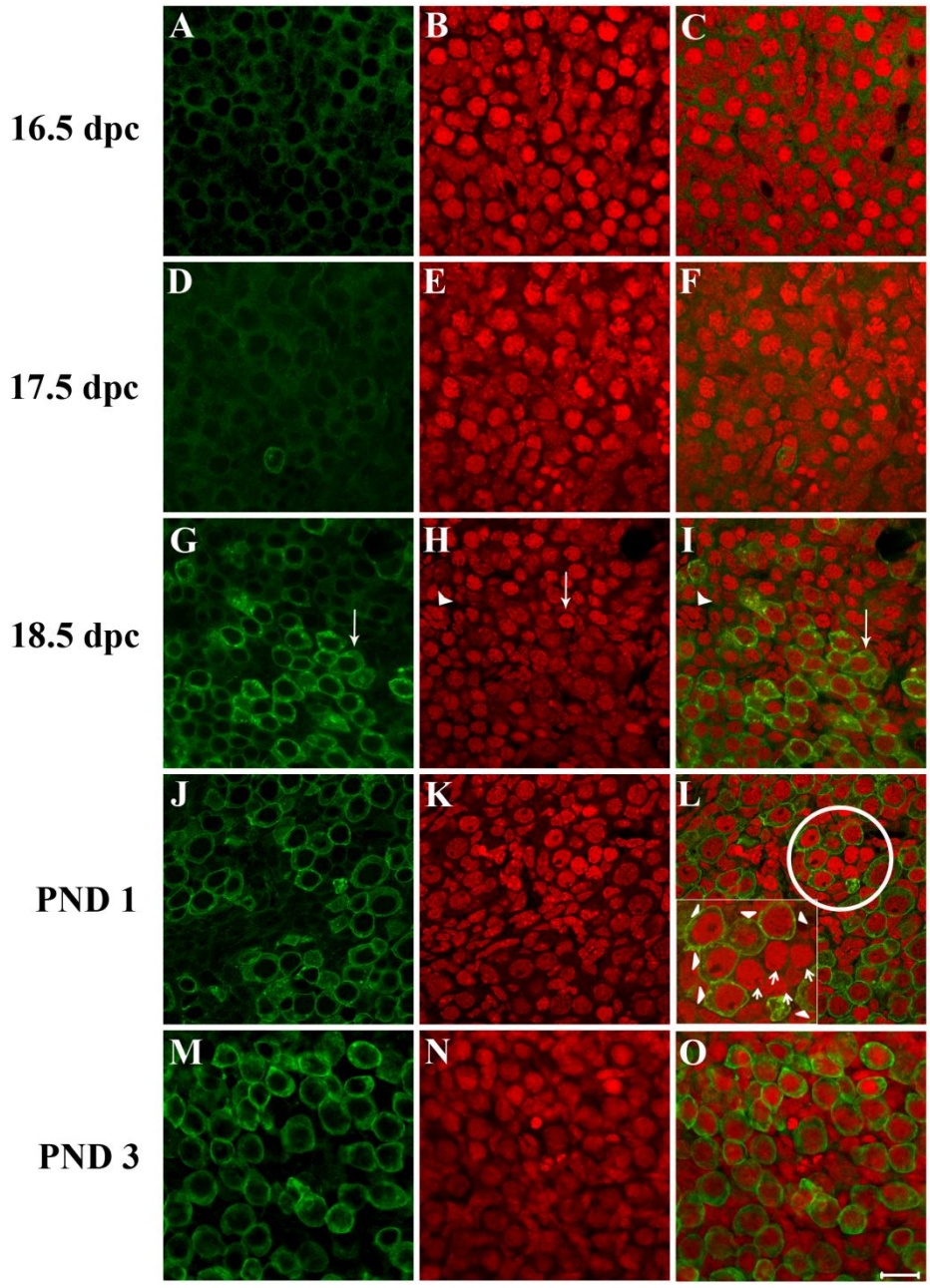


Figure 3.1. Expression of KIT in the fetal and neonatal ovary. Confocal sections from 16.5 dpc (A-C), 17.5 dpc (D-F), 18.5 dpc (G-I), PND 1 (J-L), and PND 3 (M-O) ovaries labeled for KIT (green) (A, D, G, J and M) and the nuclear marker, propidium iodide (red) (B, E, H, K and N) with overlay shown in C, F, I, L and O. Inset in L is an enlarged image of the area within the white circle. Arrows indicate oocytes without KIT labeling and arrowheads denote oocytes with KIT labeling. Arrows in G-I indicate oocytes and arrowheads indicate somatic cells. Scale bar, 20 μm.

Figure 3.2

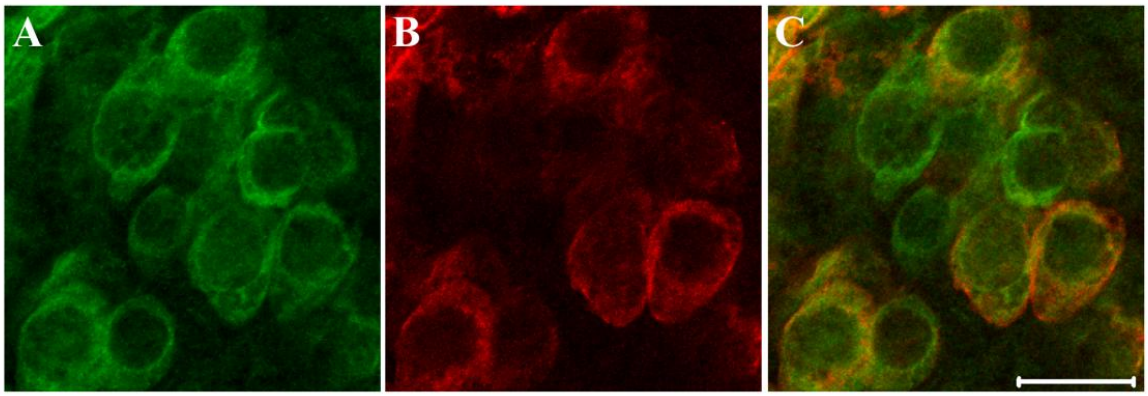


Figure 3.2. KIT labels a subset of oocytes at PND 1. Confocal section of a PND 1 ovary labeled with A) the germ cell marker, VASA (green) antibody, B) KIT (red) antibody and C) overlay.

We also tested for the presence of KITL in fetal and neonatal ovaries. Figure 3.3A-C shows weak expression of KITL in the cytoplasm of germ and somatic cells at 16.5 dpc. By 17.5 dpc, KITL appears to accumulate in small areas of some oocytes, which may represent expression of the membrane bound isoform of KITL (Fig 3.3D-F). From 18.5 dpc onward, KITL expression remains in the cytoplasm of oocytes and somatic cells (Fig. 3.3G-O) and expression appears to increase from PND 1 to 3.

Based on previous work showing that KIT signaling was responsible for oocyte survival, we hypothesized that the germ cells within a cyst not expressing KIT at PND 1 would be marked for apoptosis. To test this, we double labeled PND 1 ovaries with an antibody to the apoptosis marker, cleaved PARP, and the germ cell marker, VASA. Figure 3.4A-F shows representative confocal sections demonstrating that some oocytes expressing KIT and some oocytes not expressing KIT are both positive for cleaved PARP. To quantify that data, we calculated the percentage of oocytes in each confocal section labeling with both the KIT and PARP antibodies and those that do not label with KIT but are positive for PARP (Fig. 3.4G). There was not a statistically significant difference between these groups. Thus there does not appear to be a correlation between KIT signaling and oocyte survival.

KIT signaling regulates oocyte numbers, primordial follicle formation and cyst breakdown

We first hypothesized that KIT signaling might regulate perinatal germ cell numbers by increasing oocyte survival for those expressing KIT. To test this, we harvested 17.5 dpc ovaries and placed them in organ culture for 5 days in either control media alone, media supplemented with a 1:100 dilution of an antibody to KIT, ACK2, media supplemented with 100 ng/ml of recombinant KITL or media supplemented

Figure 3.3

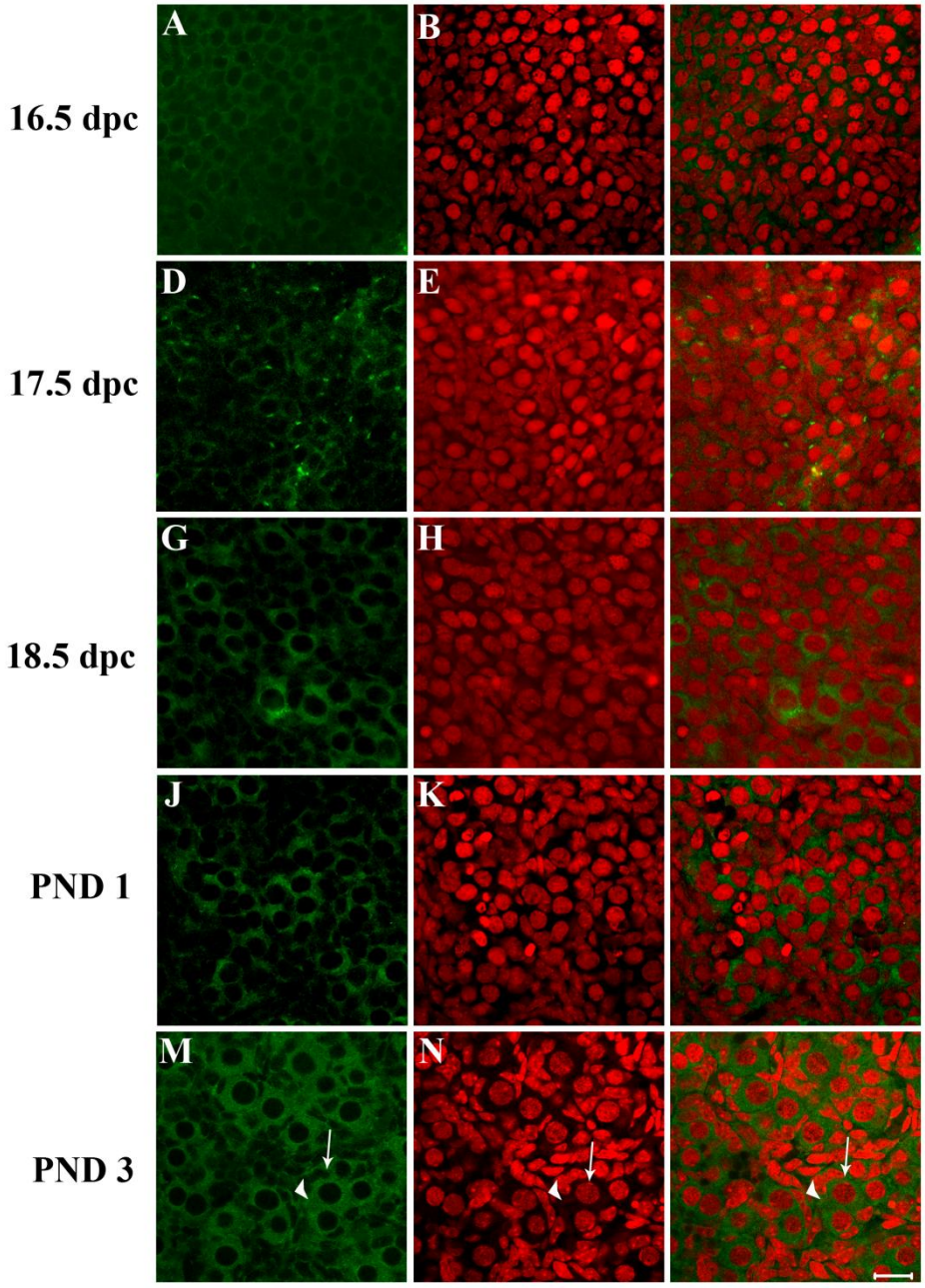
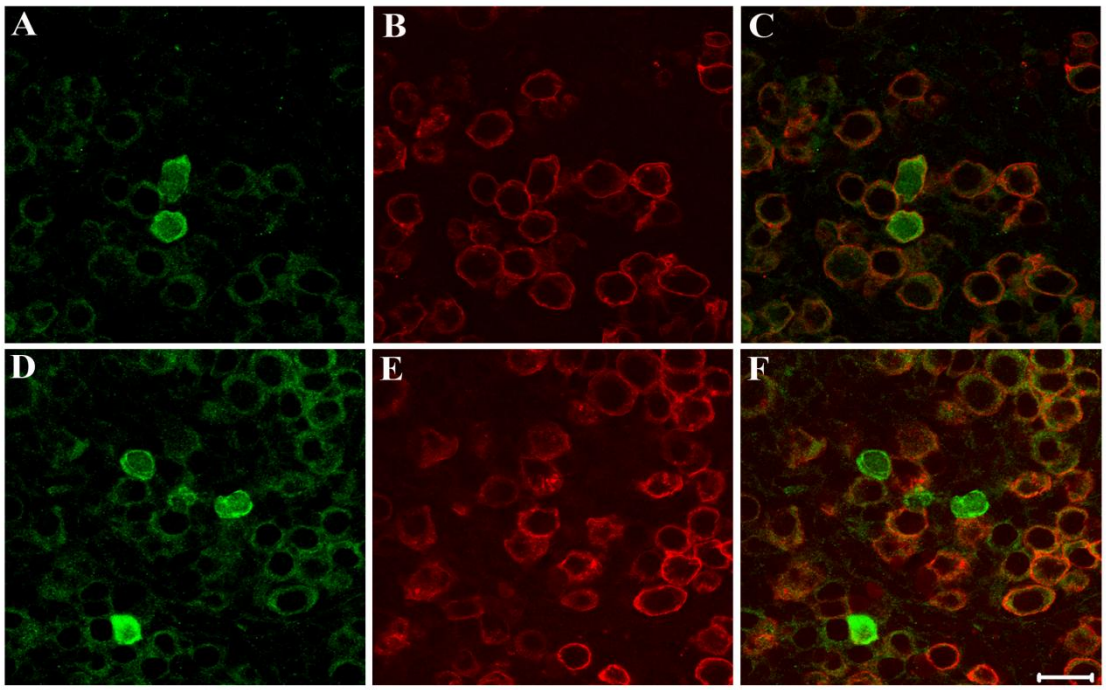


Figure 3.3. Expression of KITL in the fetal and neonatal ovary. Confocal sections from 16.5 dpc (A-C), 17.5 dpc (D-F), 18.5 dpc (G-I), PND 1 (J-L), and PND 3 (M-O) ovaries labeled for KITL (green) (A, D, G, J and M) and the nuclear marker, propidium iodide (red) (B, E, H, K and N) with overlay shown in C, F, I, L and O. Arrows in M-O indicate oocytes and arrowheads indicate somatic cells. Scale bar, 20 μm.

Figure 3.4



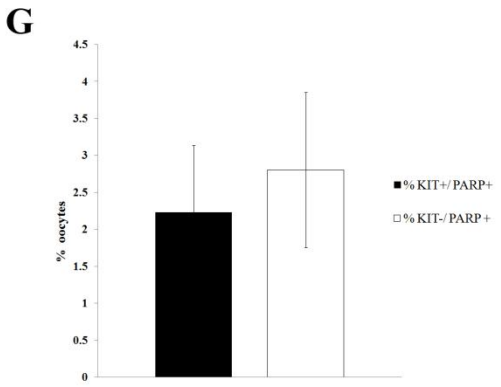


Figure 3.4. Expression of KIT and cleaved PARP at PND 1. Confocal sections of a PND 1 ovary labeled with A and D) the cell death marker, cleaved PARP (green) antibody, B and E) KIT (red) antibody and C and F) overlay. G) Percent of oocytes positive for both KIT and cleaved PARP proteins or negative for KIT but positive for cleaved PARP protein. Data are presented as the mean ± SEM.

with 1:100 dilution of IgG as a negative control. Surprisingly, we found an increase in the number of germ cells present in the ACK2 inhibited ovaries (14.5 oocytes) and a reduction in the number of oocytes in KITL treated ovaries (5.8 oocytes) when compared with both control and IgG treated ovaries (8.7 oocytes and 8.1 oocytes; Fig. 3.5A).

In addition, we found that when KIT signaling was blocked with ACK2, a large reduction in cyst breakdown occurred with only 47% single oocytes found, while an increase in cyst breakdown was present in KITL treated ovaries which contained 84% single oocytes (Fig. 3.5B). These were both statistically different from the control and IgG groups which had 71.1% single oocytes and 77.4% single oocytes, respectively (Fig. 3.5B). Finally, both blocking and activating the KIT signaling pathway resulted in changes in follicle development. The ACK2 inhibited ovaries showed a complete block on follicle progression with 100% of follicles found in the primordial stage and 0% found in the primary or secondary stages (Fig. 3.5C).

Follicle development was facilitated by the addition of KITL to culture and resulted in 78% of follicles found at the primordial stage, 21% at the primary stage and 1% at the secondary stage (Fig. 3.5C). Normal follicle development, as demonstrated by the control ovaries, showed an average of 87% primordial follicles, 13% primary follicles and 0% secondary follicles (Fig. 3.5C). Figures 3.5 D and E are representative confocal sections of both control and ACK2 treated ovaries demonstrating the increase in oocytes and reduction in cyst breakdown found in KIT inhibited ovaries.

Figure 3.5

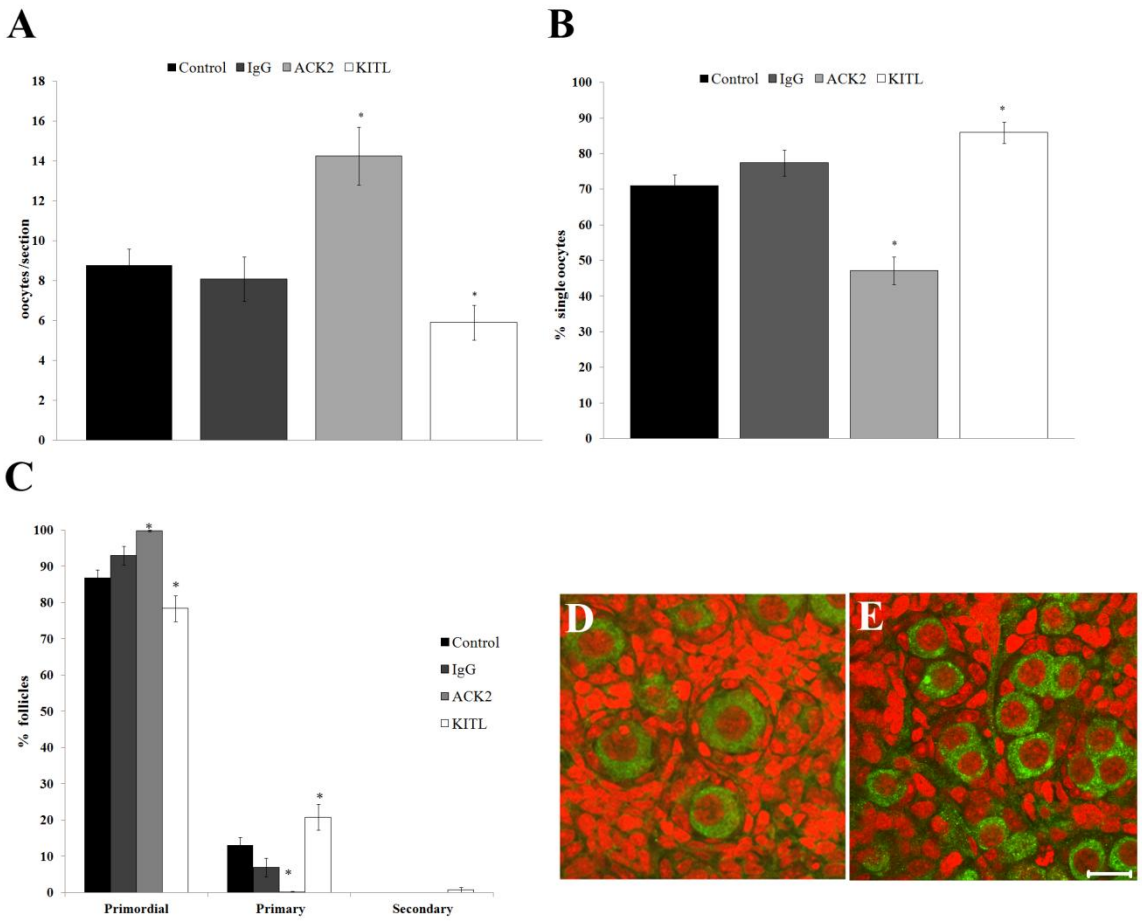


Figure 3.5. Ovarian follicle formation is affected by KIT signaling. A) Number of oocytes, B) percent single oocytes and C) percent of follicles found at the primordial, primary or secondary stage per confocal section cultured for 5 days either in control media alone or media supplemented with IgG at 1:100, ACK2 at 1:100, or 100ng/mL KITL. Data are presented as the mean ± SEM. Asterisk (*) indicates a significant difference between groups (ANOVA, $p < 0.05$). D) Confocal section of an ovary cultured for 5 days in control media or E) with ACK2 antibody labeled for STAT3 (green) to visualize oocytes and propidium iodide (red) to visualize nuclei. Scale bar, 20 μm.

Kit signaling is required for follicle formation before and after birth

Yoshida and colleagues (1997) injected female neonatal mice with ACK2 every other day beginning on PND1 and found no effect on germ cell numbers prior to PND 5. Additionally, they did not evaluate oocyte cyst breakdown and follicle formation, although they found normal numbers of primordial follicles in the ovary at PND 5. We wondered if the discrepancy between our results and those findings was a result of the timing of the ACK2 treatment, as cyst breakdown has been shown to begin prior to PND 1 (Pepling *et al.* 2010). Therefore, we wanted to test the effects of inhibiting KIT signaling on cyst breakdown and oocyte number specifically before birth to determine if the signal was important for follicle formation at that time. We began by culturing 17.5 dpc ovaries for 5 days either in media alone, or in media supplemented with ACK2 antibody for the first two days of culture then in media alone for the remaining 3 days of culture to test the effects of inhibiting KIT signaling only before birth. Figures 3.6 A and B show a slight but insignificant increase in oocyte numbers when inhibiting KIT signaling and an insignificant decrease in cyst breakdown when ovaries were cultured with ACK2 for only the first two days of culture. There was, however, a significant decrease in follicle development when KIT signaling was inhibited only for the first two days of culture, with ACK2 treated ovaries containing 96% primordial and 4% primary follicles compared to 87% primordial and 13% primary in control ovaries (Fig. 3.6C).

Figure 3.6

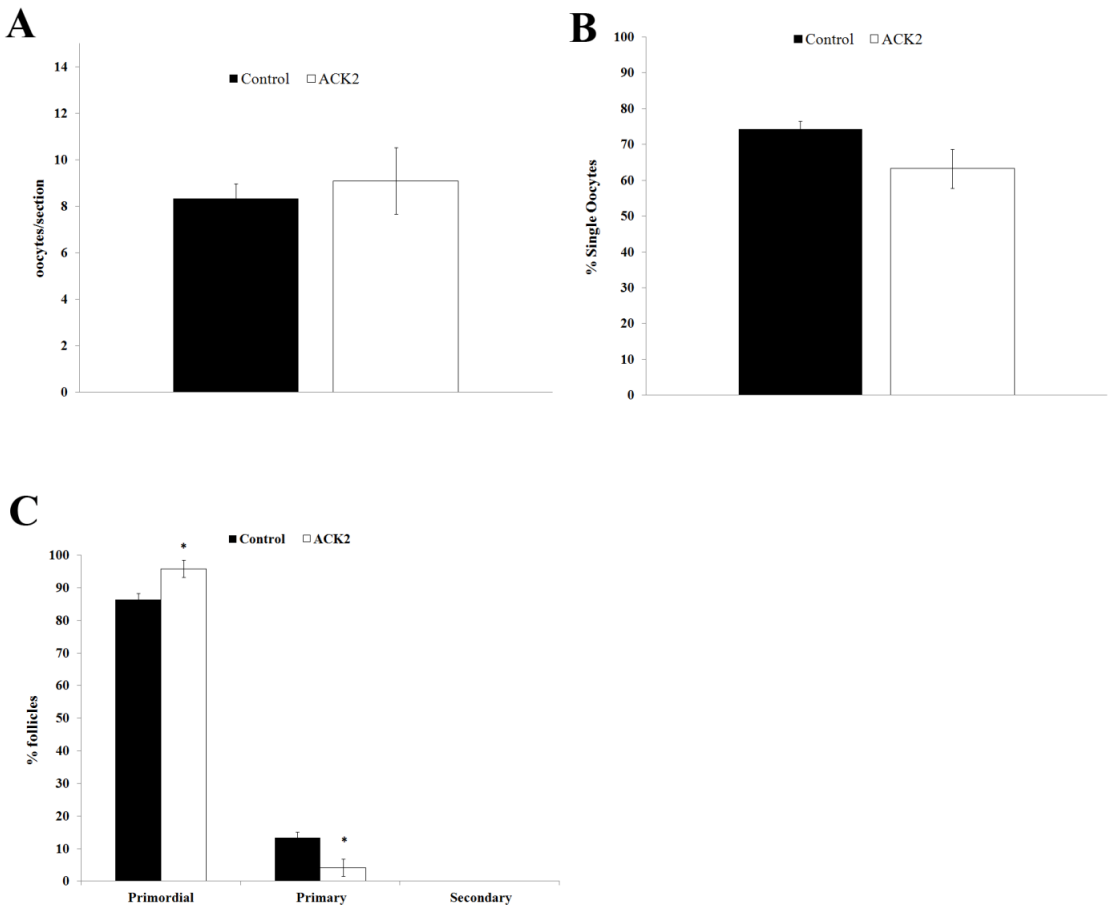


Figure 3.6. KIT signaling is important for follicle development from 17.5 dpc to PND 1. A) Number of oocytes, B) percent single oocytes and C) percent of follicles found at the primordial, primary or secondary stage per confocal section cultured for 5 days either in control media alone (days 1-5) or media supplemented with ACK2 at 1:100 (days 1 and 2) and subsequently in control media (days 3-5). Data are presented as the mean ± SEM. * indicates a significant difference between groups (t-test, $p < 0.05$).

These data suggest that KIT signaling has either no effect on cyst breakdown or oocyte numbers before birth, or that both were affected but rescued by removing the KIT antibody from culture and allowing treated ovaries to grow in plain media for the remaining 3 days of culture.

To better clarify the affect of blocking KIT signaling before birth, we decided to place 17.5 dpc ovaries in culture and treat with ACK2 antibody again for the first two days, but this time culture them only an additional day in plain media. A length of three days of culture was chosen because the final day would correspond to PND 2, the time when germ cell death peaks and cyst breakdown is well underway (Pepling & Spradling 2001). As shown in Figure 3.7A, there was a slight but insignificant increase in the number of germ cells present in treated ovaries. However, cyst breakdown was greatly reduced with ACK2 treated ovaries containing only 49% single oocytes while control ovaries had 69% single oocytes (Fig. 3.7B). Finally, follicle development was inhibited when KIT signaling was inhibited. Figure 3.7C shows that ACK2 treated ovaries had 99% primordial and 1% primary follicles and control ovaries had 94% primordial and 6% primary follicles.

Kit signaling is important for follicle formation after birth

Since cyst breakdown is a continuous process lasting from approximately 17.5 dpc to PND 5, we wanted to determine whether KIT signaling was still important for follicle formation after birth (Pepling *et al.* 2010). To test this, we cultured 17.5 dpc ovaries for 5 days in either control media alone, or control media for the first two days of culture and then in ACK2 supplemented media for the remaining 3 days.

Figure 3.7

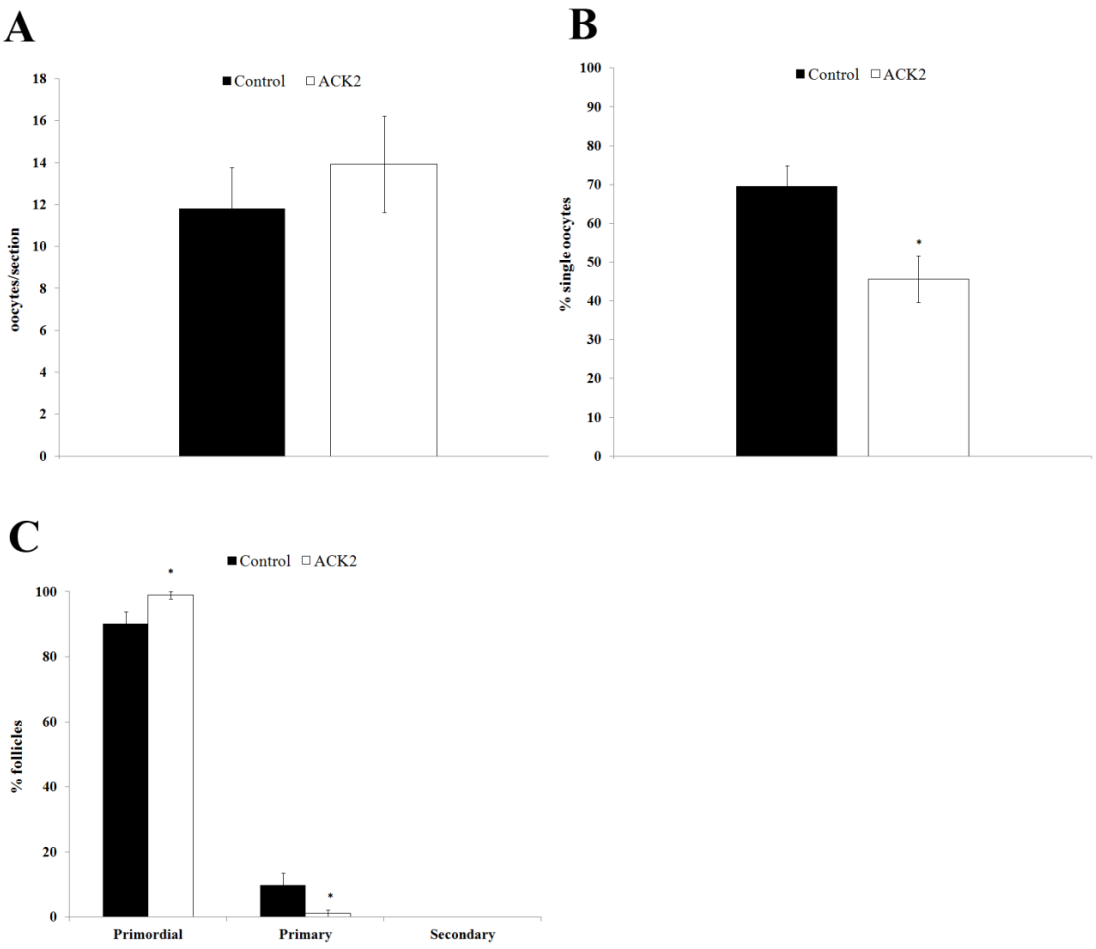


Figure 3.7. KIT signaling is important for follicle formation and development from 17.5 dpc to PND 1. A) Number of oocytes, B) percent single oocytes and C) percent of follicles found at the primordial, primary or secondary stage per confocal section cultured for 3 days either in control media alone (days 1-3) or media supplemented with ACK2 at 1:100 (days 1 and 2) and subsequently in control media (day 3). Data are presented as the mean ± SEM. * indicates a significant difference between groups (t-test, $p < 0.05$).

While there was a only a slight but insignificant increase in the number of oocytes in the ACK2 treated ovaries (9.8 oocytes) when compared with controls (8.2 oocytes; Fig. 3.8A), cyst breakdown was significantly reduced with ACK2 treatment (Fig. 3.8B) with inhibited ovaries having 59.1% single oocytes and control ovaries containing 77% single oocytes.

Cell death and proliferation are modulated by KIT inhibition

Our results showed that KIT signaling is important for regulating cyst breakdown and germ cell numbers. Upon KIT inhibition in the ovary, we found a substantial increase in the number of oocytes present (Fig. 3.5A). It is possible that the increase in the number of germ cells was either due to an increase in oocyte proliferation or a decrease in cell death. It is generally thought that oocytes do not proliferate after fetal life, but this is controversial, and manipulation of Activin in neonatal mouse ovaries has been show to increase germ cell proliferation (Johnson *et al.* 2005, Bristol-Gould *et al.* 2006). To examine the origin of this finding, we placed 17.5 dpc ovaries in organ culture for 3 days, ending on the day of peak germ cell death (Pepling & Spradling 2001), to assess both cell death and proliferation in the ovary when KIT signaling was inhibited. Ovaries were either grown in control media alone, or in the presence of the ACK2 antibody for 3 days. At the conclusion of culture, ovaries were fixed and assessed for cell death using a TUNEL assay or assessed for cell proliferation using a Ki-67 assay. Ki-67 is a protein that expressed only in dividing cells, and is absent in cells that are quiescent. Ovaries that were treated with the KIT inhibitor consisted of fewer dying cells and had only 2.3% TUNEL positive cells compared to 8.0% dying in controls (Fig. 3.9A). Figures 3.9B and 3.9C are

Figure 3.8

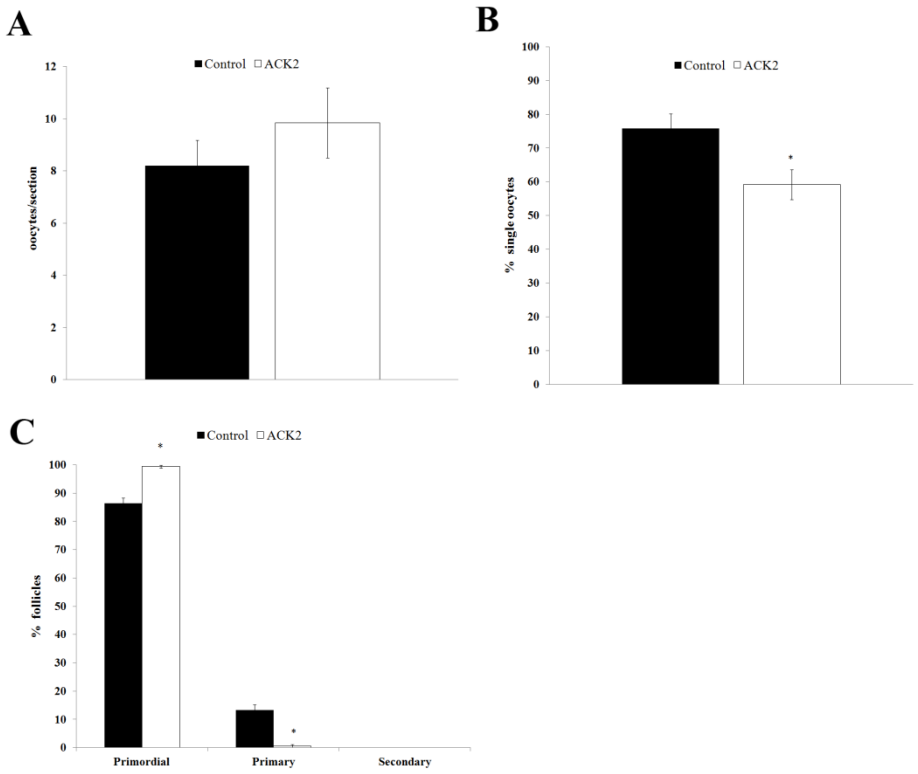


Figure 3.8. KIT signaling is important for follicle formation in ovaries from PND 1-4. A) Number of oocytes, B) percent single oocytes and C) percent of follicles found at the primordial, primary or secondary stage per confocal section cultured for 5 days either in control media alone (days 1-5) or control media (days 1 and 2) and subsequently in media supplemented with ACK2 at 1:100 (days 3-5). Data are presented as the mean ± SEM. * indicates a significant difference between groups (t-test, $p < 0.05$).

Figure 3.9

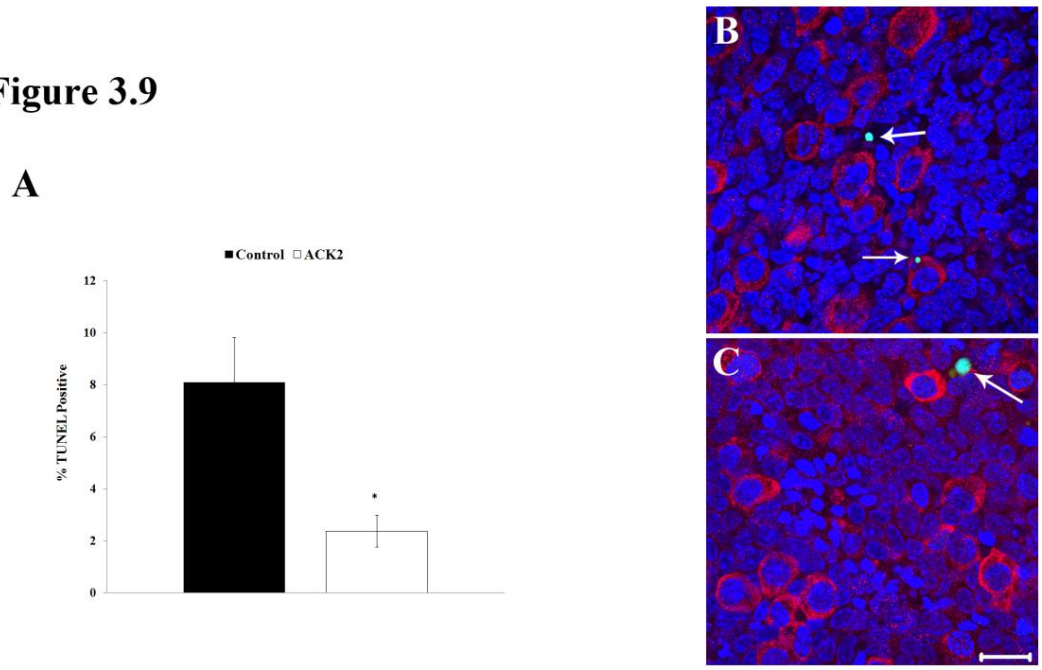


Figure 3.9 Inhibition of KIT signaling decreases cell death. 17.5 dpc ovaries were cultured for 3 days either in control media alone or in the presence of ACK2 at 1:100. A) Percent of cells in 17.5 dpc ovaries cultured for 3 days with ACK2 that are positive for TUNEL. B) Confocal section of a control ovary showing apoptotic TUNEL positive cells (green), oocytes labeled for the oocyte specific protein VASA (red) and nuclei labeled with TOTO-3(blue). Data are presented as the mean ± SEM. * indicates a significant difference between control and treated ovaries (t-test, $p < 0.05$). C) Confocal section of an ACK2 treated ovary showing apoptotic TUNEL cells (green), oocytes labeled for the oocyte specific protein VASA (red) and nuclei labeled with TOTO-3 (blue). Scale bar, 20 μm. Arrows indicate dying cells.

representative confocal sections of a control and ACK2 inhibited ovary showing fewer TUNEL positive cells in ACK2 treated ovaries.

Figure 3.10A shows the effect of ACK2 on cell proliferation in the ovary. We hypothesized that either oocyte numbers increased when KIT signaling was inhibited due to increased germ cell proliferation, or that KIT may be regulating cyst breakdown by controlling somatic cell proliferation as demonstrated in rat ovary by Otsuka and Shimisaki (2002). Interestingly, we found that when KIT signaling was inhibited granulosa cell proliferation decreased. The representative confocal sections in 3.10B and C are of a control and ACK2 treated ovary labeled with Ki-67, showing the KIT inhibited ovary containing fewer proliferating granulosa cells. These data point to the regulation of a molecule downstream of KIT in the oocyte that is involved in communication with granulosa cells.

KITL activates the MAP Kinase pathway

Kit signaling can activate several different pathways downstream of the receptor, and we wondered which of these pathways might be involved in cyst breakdown. To evaluate the downstream signaling pathway activated by KIT signaling during cyst breakdown, ovaries were cultured for 3 days in either control media alone, or in media supplemented with recombinant KITL to activate KIT signaling. At the conclusion of culture, ovaries were prepared for Western blotting and extracts were probed for total AKT, MAPK and STAT 3 as well as the phosphorylated forms of these proteins. Figure 3.11 shows that both control and KITL treated ovaries contain similar levels of total AKT, STAT3 and MAPK proteins. Phosphorylated AKT and STAT3 levels were also

Figure 3.10

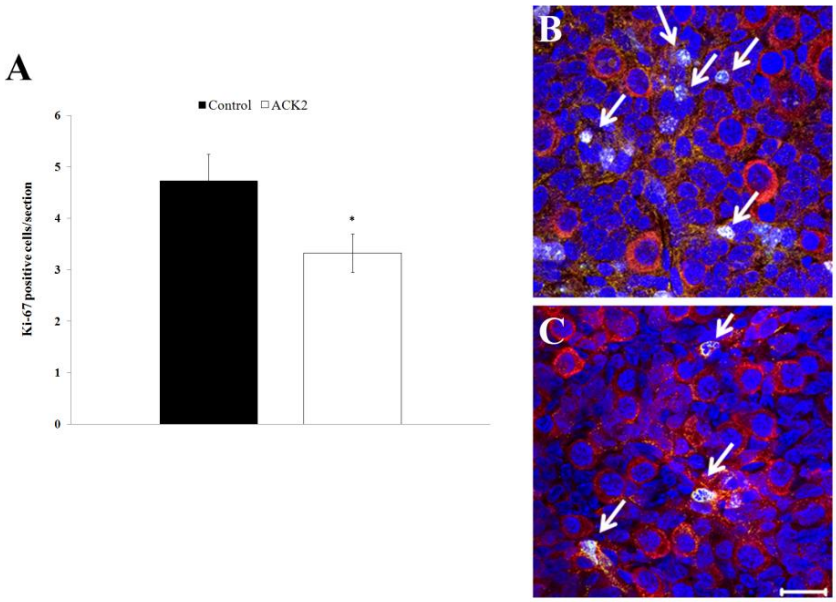


Figure 3.10. Granulosa cell proliferation is reduced when KIT is inhibited. 17.5dpc ovaries were grown in either control media alone or in the presence ACK2 at 1:100 for 3 days. A) Percent of granulosa cells in control or ACK2 treated ovaries positive for the proliferation marker Ki-67. Data are presented as the mean ± SEM. * indicates a significant difference between control and treated ovaries (t-test, $p < 0.05$). B) Confocal section of a control ovary showing proliferating Ki-67 positive cells (green), oocytes labeled for the oocyte specific protein, VASA (red) and nuclei labeled with TOTO3 (blue). C) Confocal section of an ACK2 treated ovary showing proliferating Ki-67 positive cells (green), oocytes labeled for the oocyte specific protein,VASA (red) and nuclei labeled with TOTO3 (blue). Scale bar, 20 μm. Arrows indicate proliferating cells.

comparable between control and KITL ovaries. KITL treatment did, however, increase phosphorylation of MAPK, indicating that KITL activates the MAPK pathway downstream of the KIT receptor.

MCL1 protein is downregulated by KIT signaling

The BCL2 family of proteins represents a group of molecules that regulate cell survival. Family members such as BCL2 and MCL1 are known to be anti-apoptotic (Kim & Tilly 2004). Jin *et al.* (2005) found that when KIT signaling was activated in newborn rat ovaries, BCL2 was increased in response to adding KITL in culture. However, work from our lab has shown that BCL2 is not likely involved in oocyte survival during primordial follicle formation, and that MCL1 may play a role in regulating germ cell survival instead (Jones & Pepling, in review). To determine whether MCL1 expression changes in response to KIT signaling, we cultured 17.5 dpc ovaries for 3 days in either control media alone, or in media supplemented with recombinant KITL. Ovaries were then prepared for Western blotting and extracts were probed for the protein MCL1. Figure 3.12 shows that when KIT signaling was activated, MCL1 was downregulated, indicating that KIT signaling may decrease survival proteins such as MCL1 during cyst breakdown.

Figure 3.11

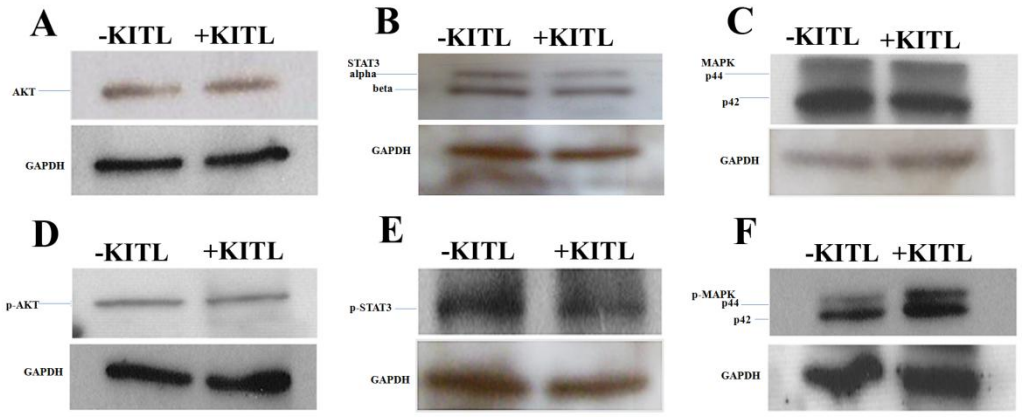


Figure 3.11. Kit ligand activates the MAPK pathway. (A-F) 17.5dpc ovaries were grown in culture with (+) or without (-) KITL for 3 days and prepared for Western blotting analysis. Ovary extracts were probed for A) total AKT (56 kDa), B) total STAT3 (92 kDa), C) total MAPK (44 and 42 kDa), D) phosphorylated AKT (56kDa), E) phosphorylated STAT3 (92 kDa), and phosphorylated MAPK (44 and 42 kDa). All extracts were probed for the loading control GAPDH.

Figure 3.12

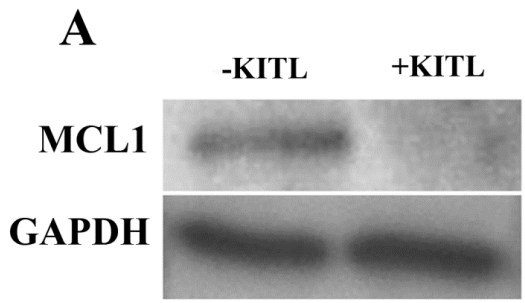


Figure 3.12. Kit signaling alters expression of MCL1. 17.5 dpc ovaries were grown in culture for three days with (+) or without (-) the addition of 100ng/ml SCF. Ovary extracts were probed for (A) MCL1 protein and the loading control, GAPDH.

3.5 DISCUSSION

Although many studies have shown a role for KIT signaling in ovarian development, there is sparse evidence examining its importance in oocyte cyst breakdown and primordial follicle formation. Several labs have examined mice mutant for either the *Steel* or *W* loci, and have found dramatic defects in PGC migration and proliferation that result in a complete loss of oocytes by birth (Reith *et al.*, 1990; Huang *et al.*, 1993). These mice are not optimal for observing the effects of KIT signaling on cyst breakdown, as cysts do not appear to form in these mutant ovaries and there are few oocytes remaining. Other *Steel* mutants show a decreased number of germ cells, but normal primordial follicle formation (Bedell *et al.*, 1995). However, these mice continue to secrete low amounts of KITL which may allow for normal cyst breakdown and follicle formation. The present study examines the role of KIT signaling in cyst breakdown and primordial follicle assembly using an organ culture system which permits the study of KIT signaling specifically at the time these processes are occurring. Using this in vitro system, we have elucidated a role for KIT signaling in the fetal and neonatal mouse ovary.

We found KIT to be expressed at the oocyte membrane from 17.5 dpc to PND 3. This supports the findings of Manova *et al.*, where *Kit* RNA was localized to the oocyte beginning in just a few cells at 17.5 dpc then increasingly throughout the life of the oocyte (Manova *et al.* 1990). Interestingly, we found that at PND 1 a subset of germ cells within some cysts did not express the KIT receptor. This may be due to expression of Activin in those cells. Using immunohistochemistry, Coutts and colleagues (2008) found human fetal ovaries expressed KIT incongruously in oocyte cysts and those that lacked expression stained positively for Activin. We did not test the expression of Activin in our experiments, and therefore cannot say whether this same expression pattern is true in mouse ovary. It would be interesting to test the co-

expression patterns of KIT and Activin in the mouse ovary to see if this is a conserved pattern in mouse. Finally, we found that KITL was expressed in the mouse ovary from 17.5 dpc through PND 3 in both oocytes and somatic cells, however the signal for KITL was strongest in oocytes at 17.5, but then becomes more evenly distributed between germ cells and granulosa cells by PND 3. It is possible that there is a switch from the KITL membrane bound isoform at 17.5 dpc in the oocyte to the soluble form by PND 1, and this switch may take part in regulation of cyst breakdown. This could indicate either an autocrine or paracrine signaling mechanism for the KIT/KITL system in the developing mouse ovary particularly during follicle formation as expression of both components was detected during this time period.

Given the positive expression of both KIT and KITL in the mouse ovary, we hypothesized that this system would play a role in cyst breakdown and primordial follicle formation. To test this, we used in vitro ovary organ culture to both inhibit and activate the KIT signaling pathway. When exposed to the KIT inhibiting antibody ACK2, cyst breakdown was greatly reduced, and upon activation with recombinant KITL, cyst breakdown was accelerated. Our data supports that of Wang and Roy in hamster ovary, where addition of KITL to culture promoted the formation of primordial follicles (Wang & Roy 2004). Alternatively, our results contradict earlier findings that suggest that KIT signaling has no effect on follicle formation in mouse ovary (Yoshida *et al.* 1997). Using injections of ACK2 every other day beginning on the day of birth, Yoshida and colleagues failed to find that KIT played any role in primordial follicle formation, but instead blocked the primordial to primary follicle transition. This study utilized newborn mice at PND 1 and, based on work from our lab showing that cyst breakdown begins as early as 17.5 dpc, injecting at PND 1 may have been too late a time to affect cyst breakdown and follicle formation (Pepling *et al.* 2010). Additionally, the every other day injection schedule of

ACK2 may have allowed a rescue of cyst breakdown, such that on the days when antibody was not given, normal signaling was permitted and primordial follicle formation occurred.

In an effort to clarify the differences between our results and those of Yoshida *et al.*, we first wanted to test the effect of blocking KIT prior to PND 1 to determine its importance in inhibiting cyst breakdown before birth. To accomplish this, we cultured 17.5 dpc ovaries in the presence of ACK2 for 2 days and then allowed the ovaries to continue in culture in control media for the remaining 3 days. To our surprise, ovaries that were treated only on the first two days had only a slight decrease in cyst breakdown, but a delay in follicle development remained, suggesting a possible rescue of cyst breakdown and follicle formation on the 3 days ovaries were not in the presence of the antibody. To test the idea that follicle formation had been rescued, we again cultured 17.5 dpc ovaries in the presence of ACK2 for 2 days but this time only allowed them to be cultured for 1 more day before examining them. When removed from culture at the earlier time point, ovaries that were treated with ACK2 demonstrated a significant decrease in cyst breakdown and primordial follicle formation. This suggests that follicle formation can be rescued when ovaries are allowed to resume normal KIT signaling and in addition, the KIT signal is important before birth in cyst breakdown.

We also postulated that the KIT signal would continue to be important for cyst breakdown at birth, as this process continues from 17.5 dpc to PND 5. To examine this, we cultured 17.5 dpc ovaries first in control media for 2 days and then in ACK2 supplemented media for the remaining 3 days. In support of our hypothesis, we found that cyst breakdown was again inhibited, though not as severely as when inhibition was started at 17.5 dpc. The KIT signal appears to be important for cyst breakdown on or after PND 1 in the neonatal ovary.

In our initial experiment, inhibiting KIT signaling in culture for 5 days from 17.5 dpc onward also resulted in an increase in the number of oocytes present in the ovary. We suspected that this increase may be due to a decrease in cell death. Using a TUNEL analysis, we were able to quantify the percentage of dying cells in both control and ACK2 treated ovaries and found that the inhibited ovaries contained a lower percentage of dying cells. Although we used an oocyte marker to label germ cells, we cannot say for sure that the dying cells are actually oocytes because the germ cell marker antibody may lose reactivity in dying cells, therefore dying oocytes may not label for Vasa. (Pepling & Spradling 2001). However, since KIT is expressed only at the germ cell membrane and we see an increase in oocyte numbers, it is likely that the decreased TUNEL labeling upon KIT inhibition is due to a reduction in oocyte specific death.

Alternatively, the increase in germ cell numbers could be due to an alteration in oocyte proliferation, although the idea of postnatal germ cell proliferation is controversial. It has generally been accepted that oocytes stop dividing during fetal life and do not resume mitosis, however more recent studies have shown oocytes to be proliferating in the newborn mouse ovary, challenging the classic dogma (Johnson *et al.* 2005, Peters, 1969). In addition, we also hypothesized that blocking KIT at the oocyte could interrupt communication with the surrounding granulosa cells, thus decreasing granulosa cell invasion and/or proliferation. We utilized a Ki-67 assay to examine the effects of KIT inhibition on both oocyte and somatic cell proliferation. Although there was no evidence for proliferation of oocytes in any ovaries analyzed, there was a significant decrease in the proliferation of granulosa cells when KIT signaling was blocked. This supports the idea that interrupting KIT at the oocyte interrupts some downstream molecule that may be involved in communication with the surrounding somatic cells, similar to the findings of Otsuka and Shimisaki (2002). In their study, when soluble KITL

was administered to cultured oocyte-granulosa cell complexes, synthesis of DNA was increased in the granulosa cells, indicating that activated KIT signaling at the oocyte upregulated granulosa cell mitosis through some downstream effector.

Much of the work in the ovary has focused on the activation of the PI3K pathway downstream of KIT in the oocyte. Upon autophosphorylation of KIT, tyrosine 719 becomes phosphorylated and is the primary binding site for the p85 subunit of PI3K and its subsequent activation. Mice with a point mutation in the KIT receptor at Y719 are not able to bind p85 and therefore cannot activate PI3K downstream of KIT (John *et al.* 2009). These mice undergo normal cyst breakdown and primordial follicle formation, suggesting the PI3K pathway is not involved in primordial follicle formation upon KIT activation (Sun *et al.* 2008). In our study, we have found that MAPK is activated downstream of KIT upon KITL binding. We used whole ovary protein extracts for Western blot to identify the pathways activated by KIT, therefore we cannot conclude that the upregulation of MAPK by KITL is actually occurring in the oocyte. Future studies will need to examine the cell types in which MAPK becomes phosphorylated when KITL is added to culture, using immunohistochemsitry. Presumably MAPK phosphorylation would be within the oocyte since the KIT receptor is found at the oocyte membrane. However KIT signaling appears to increase granulosa cell divison, so the phosphorylation of MAPK could be found in those cells instead. Also, future experiments should include using recombinant KITL in conjunction with an inhibitor to MAPK signaling to determine whether this pathway is truly regulating cyst breakdown as a result of activated KIT signaling.

Two BCL2 family proteins, BAX and MCL1, have been shown to be important to perinatal oocyte survival during cyst breakdown (Greenfeld *et al.* 2007, Jones & Pepling, in

review). The BCL2 family is a group of proteins primarily responsible for cell death and survival and can be divided into two main groups, those that are pro-apoptotic and those that are anti-apoptotic. When BAX, a pro-apoptotic protein, is knocked out, neonatal ovaries contain an increase in oocytes, suggesting a decrease in cell death when this death protein is eliminated (Greenfeld *et al.* 2007). Likewise, when the anti-apoptotic protein MCL1 is inhibited in culture, ovaries contain fewer oocytes, indicating a need for MCL1 in perinatal oocyte survival. We hypothesized that MCL1 protein may be regulated by KIT signaling to alter germ cell survival, as we saw an increase in oocytes when KIT was blocked and a reduction in oocyte numbers when KIT signaling was activated. Using Western blot on whole ovary protein extract, we found that KITL activated ovaries showed a complete absence of MCL1 protein when compared to control ovaries. The lack of MCL1 in the KITL treated ovaries may explain the loss of germ cells that accompanied treatment with exogenous KITL. These results contrast with those of Jin *et al.*(2005) who found that in cultured newborn rat ovaries, KITL increased cell survival through upregulation of two BCL2 anti-apoptotic proteins, BCL2 and BCLX. The difference in action of KIT signaling between rat and mouse may be a species-specific difference in ovarian development. One example of this is the contrasting action of GDF-9 in primordial follicle activation. In hamster, GDF-9 is responsible for the transition of primordial to primary follicle in vitro, but similar experiments in rat have shown GDF-9 to be important only in later follicle development, demonstrating a species-specific requirement of GDF-9 in primordial follicle activation (Nilsson & Skinner 2002, Wang & Roy 2006).

In summary, we have found a role for KIT in the formation of primordial follicles using an in vitro ovary culture system to block KIT signaling specifically at the time when cyst breakdown and primordial follicle formation begin. While the in vitro system is a reliable

method for testing the effects of hormones and signaling molecules on ovarian development, they may not accurately reflect what is happening in vivo. Developing and evaluating a tissue specific knockout of the KIT receptor would shed light on the importance of this pathway during cyst breakdown in vivo. Additionally, studying the genes regulated downstream of KIT is essential to understanding its role in primordial follicle formation and further, in its effect on somatic cell division. Trombly *et al.*(2009), found that blocking Notch signaling in vitro resulted in a defect in primordial follicle formation in the mouse ovary. Interestingly, the Notch receptor (NOTCH2) is expressed on the granulosa cells and its ligand, Jagged 1, is present in oocytes. This may be a pathway involved in an oocyte granulosa cell feedback system with the KITL/KIT system, where activation of KIT in the oocyte might upregulate JAGGED1 expression in the oocyte, resulting in communication with notch at the granulosa cells. Testing the expression of both NOTCH2 and JAGGED1 by quantitative RT-PCR or Western blot following organ culture with exogenous KITL would be a valuable follow up study. Likewise, culture with recombinant KITL could be followed by a microarray analysis to determine the global genes being regulated by KIT signaling in the ovary during cyst breakdown. Finally, it would be interesting to determine if KIT signaling might act synergistically or redundantly with other pathways that have been identified as regulators of cyst breakdown and primordial follicle formation. Studies examining connective tissue growth factor (CTGF) (Schindler *et al.* 2010) and nerve growth factor (NGF) (Dissen *et al.* 2001) have demonstrated a role for both of these signaling pathways in primordial follicle assembly and cyst breakdown, so experiments that explore the effects of inhibiting and activating multiple signaling pathways in conjunction with KIT could prove worthwhile.

CHAPTER 4:

CONCLUSIONS AND FUTURE DIRECTIONS

Female fertility is established by the time of birth. Fecundity is determined by the proper establishment of the primordial pool of follicles which requires precise control of cyst breakdown and regulation of oocyte numbers through apoptosis. Improper control of these processes can lead to a number of fertility disorders. It is believed that improper regulation of cyst breakdown and primordial follicle formation can lead to premature ovarian failure or primary amenorrhea, where an early depletion of oocytes leads to infertility (Yen & Jaffe 1991, Kezele & Skinner 2003). In addition, aberrant regulation of oocyte death as primordial follicles form may be the underlying cause of ovarian dysgerminoma, or germ cell tumors (Berek & Novak 2007). Little is known about what molecules regulate cyst breakdown, primordial follicle formation and oocyte death. The present research has illuminated the roles that BCL2, MCL1 and KIT signaling play in these processes.

By evaluating transgenic mice, we have shown that the BCL2 protein is not primarily responsible for perinatal oocyte survival. Interestingly, by PND 9, Flaws and colleagues (Flaws *et al.* 2001) found a marked increase in the number of primordial follicles present when BCL2 was overexpressed. This may mean that BCL2 is important during a recently discovered period of primordial follicle atresia, which begins after PND 6 (Tingen *et al.* 2009). Alternatively, BCL2 may play a role in oocyte death during cyst breakdown, but its role may be redundant with other BCL2 antiapoptotic proteins. For example, Chao *et al.* (1995) found that in T cells BCL2 and BCLX controlled a common pathway, such that T cell apoptosis in *bcl2* knockout mice was rescued by expression of a *bclx* transgene in thymocytes. In the same study it was found that the two molecules regulate the other's expression, so as BCLX expression increased, BCL2 protein

was reduced. If this were to be the case in the neonatal mouse ovary then expression of another BCL2 protein such as MCL1 may keep BCL2 downregulated enough so that knockout of BCL2 may not cause an effect on oocyte survival. Likewise, if BCL2 were to be eliminated, another BCL2 family protein's expression may increase and substitute for its function in the oocyte.

BCL2 protein was shown to be expressed in oocytes but also in somatic cells within the ovary, and because of this, we cannot be sure that BCL2 exerts its effects in the oocyte rather than the granulosa cells. We did not quantify the number of granulosa cells within the ovary, so we cannot say whether BCL2 alters their survival. Somatic cells are important to the formation of primordial follicles and lower numbers may reduce cyst breakdown. Therefore, granulosa cell numbers should be measured in neonatal BCL2 knockout and overexpressor ovaries by flow cytometry. Additionally, the expression pattern of BCL2 demonstrated stronger staining at PND 3 than at earlier ages suggesting BCL2 may be important to later primordial follicle survival. A further study should examine germ cell numbers in a BCL2 knockout ovary after PND 7.

MCL1 is expressed in the mouse ovary from 17.5 dpc to PND 5, but its expression increased between 17.5 dpc and PND 3 indicating a possible role for this molecule during the peak time of oocyte loss and cyst breakdown. To test that hypothesis, we cultured PND 1 ovaries with an antibody to MCL1. We found that MCL1 is very likely an important regulator of neonatal oocyte apoptosis. Upon inhibition in culture, oocyte numbers in MCL1 inhibited ovaries (1:100 dilution) were reduced to 35% of controls. This was a substantial loss of germ cells, however some oocytes survived despite MCL1 inhibition, even at the highest dilution. Several possible explanations may exist. First, we began culture at PND 1, but evidence has shown that oocyte death and cyst breakdown begin earlier at 17.5 dpc (Pepling *et al.* 2010). A future experiment may be to test oocyte survival upon MCL1 inhibition in culture beginning at

17.5 dpc. This may result in loss of all the germ cells in the ovary. Another possible explanation is again the idea of BCL2 family redundancy. It is entirely logical to hypothesize that in the absence of MCL1, the presence of another BCL2 protein may rescue some oocytes. Unpublished data from our lab showed that two antiapoptotic BCL2 family proteins, DIVA/BOO and A1 were expressed strongly in the oocyte at PND 1. No research has been done on the role of either of these proteins in oocyte survival at any time point, so further experiments may investigate DIVA or A1 in conjunction with MCL1 in the ovary.

Using ovary organ culture, we were able to block MCL1 at different dosage levels and found that the loss of oocytes was dose dependent. This is an interesting finding, as it may lend support for the idea that a BCL2 family "rheostat" exists in cells where the balance of pro- and anti- apoptotic proteins determines cell survival or death (Chao & Korsmeyer 1998). In one study, when BAX was overexpressed in lymphocytes, cell death quickly ensued. However, when BCL2 was overexpressed, it heterodimerized with BAX and cells survived (Oltvai *et al.* 1993). Our dose dependent results may be indicating a similar phenomenon. It is possible that at the lower dilution (1:200), not enough free BAX exists in cells to increase apoptosis to a significant level, but as we double and quadruple MCL1 inhibition, more BAX is free to translocate to the mitochondria and homodimerize, releasing Cytochrome c and inducing apoptosis.

Our MCL1 experiments used in vitro methods to determine the role of MCL1 in oocyte survival because MCL1 knockout is embryonic lethal. Contruction of a transgenic conditional oocyte-specific deletion of MCL1 would be very useful in determining the in vivo role of MCL1 protein. Use of the Vasa-Cre line of mice in conjunction with the floxed MCL1 mouse line

would ideally result in MCL1 deletion specifically in the oocyte beginning around 15.5 dpc when Vasa-Cre becomes active (Gallardo *et al.* 2007, Opferman *et al.* 2003).

KIT signaling has been important to many follicle transitions as well as fetal oocyte survival and development. Several labs have examined mice mutant for either the *steel* or *W* loci, and have found dramatic defects in PGC migration and proliferation that result in a complete loss of oocytes by birth (Reith *et al.* 1990, Huang *et al.* 1993). These mice are not optimal for observing the effects of KIT signaling on cyst breakdown as there are few oocytes, and cysts do not appear to form in these mutant ovaries. Other *steel* mutants show a decreased number of germ cells but normal primordial follicle formation (Bedell *et al.* 1995). However, these mice continue to secrete low amounts of KITL which may allow for normal cyst breakdown and follicle formation. Due to the limitations of these genetic mutants, it was unclear whether KIT signaling played a role in perinatal cyst breakdown or oocyte survival. Therefore, we set out to determine if the KITL/KIT system was important in early ovarian development.

KIT was expressed in only a few oocytes at 17.5 dpc but became expressed in a majority of germ cells beginning at 18.5 dpc. By PND 1, expression in the oocyte cell membrane was strong except in a few oocytes within a cyst. Likewise, KITL expression increased in the oocyte from 17.5 dpc onward and remains expressed through PND 5. These data imply that the components of KIT signaling are present during the time of cyst breakdown and oocyte death.

Interestingly, we found a few oocytes within a cyst did not express KIT. We first thought that this may indicate cells that were destined for apoptosis. However, co-labeling with an antibody to cleaved PARP demonstrated that cells absent for KIT were not necessarily set for death and that some cells staining positively for KIT were about to undergo apoptosis. It has been demonstrated that the KIT receptor ceases expression around 13.5-14.5 dpc, when germ

cells enter meiosis, and resumes expression at 17.5 dpc, when oocytes begin to arrest in the diplotene stage (Manova *et al.* 1990, Manova & Bachvarova 1991). It is possible that oocytes that are negative for the KIT label are cells that have not yet reached diplotene. In support of this idea, KIT appears to play a role in maintaining diplotene arrest later in follicle development, so KIT receptor may not be expressed until the oocytes have reached diplotene (Ismail *et al.* 1996, Ismail *et al.* 1997). Premature expression may force germ cells to arrest in an earlier stage of prophase I, but this would need to be determined experimentally as there are no studies examining the relationship between KIT and meiosis in the fetal/neonatal ovary. A more recent study examining expression of the KIT receptor found that protein expression correlated with zygotene/ pachytene stage oocytes at 16.5 dpc, although meiotic stage was not directly analyzed. Further investigation into the relationship between meiotic stage, KIT expression, meiotic progression and KIT signaling is warranted.

To test the hypothesis that KIT signaling might play a role in ovarian differentiation we employed an ovary organ culture system to specifically inhibit and activate KIT signaling during the period of cyst breakdown, primordial follicle formation and oocyte death. We found that altering KIT signaling changed the ovarian histology, such that inhibition reduced primordial follicle formation and oocyte loss while activation promoted the formation of follicles and oocyte death. These data support the findings of Wang and Roy (2004) that showed that exposing hamster ovaries to KITL in culture increased primordial follicle formation, however their work did not provide any information on the number of follicles present, so we do not know if adding KITL in culture accelerated death in hamster ovaries.

Our experiments showed an accelerated loss of oocytes on KIT activation and a decrease in oocyte death when KIT was blocked. This seems to be in contrast to most of the other studies

that demonstrate that KIT is an important survival factor in the ovary. Only two of these, Jin *et al.* (2005) and Yoshida *et al.* (1997) have considered whole neonatal ovaries. The first study considered neonatal rat ovaries and found that KIT signaling rescued oocyte loss when KITL was administered to ovaries in culture. Likewise, oocyte death increased upon ACK2 exposure (Jin *et al.* 2005). One important idea to consider that was mentioned in Chapter 3 is the notion of species specific differences. It is entirely possible that rat and mouse ovaries have different responses to KIT signaling. In addition, Jin and colleagues did not quantify or characterize cyst breakdown or primordial follicle formation. Therefore, it may be that in rat there is an effect of KIT signaling on oocyte survival, but in mouse and hamster the effect is primarily on cyst breakdown. Yoshida and colleagues (1997) used an injection of ACK2 every other day beginning on PND 1 to determine whether KIT signaling was important for follicle formation and oocyte survival in mouse ovaries. This has been well addressed in Chapter 3, however one further point to mention is that in our data we also did not see an effect on oocyte numbers when culture with ACK2 began at PND 1. This supports our argument that starting KIT inhibition at PND 1 may have been too late a time point to find an effect of KIT on oocyte numbers. Finally, Lobascio *et al.* (2007) found that oocyte death was rescued by KITL when oocytes were cultured alone at 16.5 dpc, disaggregated from the rest of the ovarian cells. While this does demonstrate that KIT signaling at the oocyte can increase its survival, the study disregards any contribution the granulosa cells may have in regulating KIT signaling and/or the existence of a granulosa cell-oocyte feedback loop. We showed that when KIT was blocked, granulosa cell mitosis decreased, implying that active KIT in the oocyte regulates a downstream molecule involved in granulosa cell communication. Due to this interruption in communication and decrease in formation of

primordial follicles, germ cell death actually decreased, stressing the importance of the somatic cells in regulating oocyte survival and cyst breakdown.

Activin has been one of the few molecules shown to regulate KIT during cyst breakdown. Using fetal human ovary tissue samples, Coutts and colleagues (2008) were able to demonstrate that oocytes in cysts express KIT non-ubiquitously and those that are not expressing KIT express Activin. Interestingly, as Activin decreased in the oocyte, KIT was upregulated just prior to the time when cyst breakdown would begin. However when cultured with recombinant Activin, ovarian expression of KITL was reduced to two-thirds of control levels, suggesting that Activin's actions are on repressing KITL and that when KITL becomes expressed, KIT is then expressed. One study has demonstrated that KITL is able to upregulate KIT (Thomas *et al.* 2008). All of this suggests a system in the ovary where Activin expression must be reduced in order for KIT evidence from Bristol-Gould *et al.* (2006) showed that ovaries from mice injected with recombinant Activin had an increased number of primordial follicles, which indicates Activin may accelerate cyst breakdown. In contrast, Kimura *et al.* (2011) found that although there was an increase in primordial follicles when Follistatin, the negative regulator of Activin, was deleted from ovaries, cyst breakdown was delayed and occurred over a longer period of time, suggesting Activin may increase oocyte survival but delay follicle formation. Our data may support a negative feedback loop where KIT signaling keeps Activin repressed, and when this happens too early, oocyte survival is markedly reduced. For example, when KIT signaling is activated at 17.5 dpc, Activin could be downregulated too early and its survival signals lost so that as primordial follicles form, more oocytes die. When KIT is inhibited at 17.5 dpc, Activin may be allowed to continue to signal for a longer period of time, increasing survival of oocytes, but inhibiting follicle formation. Additionally, Activin has been shown to upregulate expression of MCL1 in

cells, so perhaps if Activin is downregulated too early, expression of MCL1 is not increased in time to rescue oocytes from apoptosis (Fukuchi *et al.* 2001). In our study, we showed that when recombinant KITL was added to culture at 17.5 dpc, ovaries lacked MCL1 protein. This may support a model for a KITL-Activin negative feedback loop that should be investigated further.

Increasingly, growth factor pathways are being identified that affect the perinatal survival of germ cells and the formation of primordial follicles, however none have been identified that have complete control of either cyst breakdown or oocyte survival. This is likely due to regulation by a complex network of signaling molecules in these vital processes. Even in our study, when ACK2 was used at a dilution of 1:100 to block KIT signaling, there were still over 40% single oocytes in the KIT inhibited ovaries. It is probable that other growth factor signaling is responsible for co-regulating cyst breakdown with KIT signaling, so that in the absence of one molecular signal another can still maintain primordial follicle formation, albeit in a reduced fashion. Several possibilities exist for molecules that may work in concert with KIT to promote primordial follicle formation, and co-inhibition of KIT and other growth factors would be necessary to determine all of the factors that work together to carry out primordial follicle formation. For instance, inhibition of KIT signaling in NGF knockout ovaries in culture may reveal a complete block on cyst breakdown, as NGF knockout ovaries have a large reduction in cyst breakdown (Dissen *et al.* 2001). Other candidates for double inhibition with KIT might be GDF9 or BMP15, as adult knockout ovaries contain more MOFs (Yan *et al.* 2001).

Finally, as with the MCL1 studies, it is important to note that these experiments relied on in vitro culture and may not reflect what is happening in vivo. If it is possible for ACK2 antibody to cross the placenta, injecting pregnant dams at 17.5 dpc may be a way to evaluate in vivo effects of KIT inhibition. Otherwise, a conditional knockout mouse would need to be made

in order to delete KIT protein during the time of primordial follicle formation. Currently, no floxed KIT allele mice exist, so this type of mouse would first need to be constructed in order to generate conditional knockouts.

We have been the first to demonstrate the importance of MCL1 and KIT signaling in oocyte survival and primordial follicle formation in the mouse ovary. This is an important step in the elucidation of mechanisms regulating cyst breakdown, oocyte numbers and primordial follicle formation which can lead to the development of early screens and interventions for infertility and germ cell cancers. Once mechanisms regulating these processes become known, it may be possible to screen for early genetic abnormalities or develop pharmacologic interventions for aberrant signaling in infertility or germ cell cancers. In addition, this research may be of significance in increasing fertility in endangered and agricultural species.

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Curriculum Vitae

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EDUCATION

Ph.D. 2012 Cell and Molecular Biology, Syracuse University. Expected graduation Fall 2012.
Dissertation: ***Roles of kit signaling, Bcl2 and Mcl1 in perinatal oocyte cyst breakdown and survival.***

M.S. 2002 Science Education, Syracuse University

B.S. 1998 Respiratory Care, SUNY Upstate Medical University

RESEARCH EXPERIENCE

Mechanisms of Perinatal Germ Cell Death and Oocyte Cyst Breakdown
2007-2012 LAB: Dr. Melissa Pepling, Syracuse University

Project: Identify members of the Bcl-2 family involved in oocyte survival as primordial follicles form.

- Analyzed effects of overexpression and deletion of Bcl2 protein on follicle development in female mice.
- Characterized expression of Mcl1 protein in the oocyte and its role in survival in organ culture

Project: Characterize the role of kit signaling in oocyte cyst breakdown, survival, and follicle formation.

- Tested the expression of kit receptor and ligand using immunohistochemistry
- Identified the role of kit signaling in cyst breakdown and oocyte survival using organ culture
- Clarified the effect of kit signaling on cell death and division using immunohistochemistry
- Elucidated the downstream pathways used to effect cyst breakdown and survival using Western Blotting

LABORATORY SKILLS

- Immunohistochemistry/Whole mount ovary antibody staining
- Western Blotting
- Organ culture
- Confocal microscopy
- PCR

- DNA isolation and purification
- Gel electrophoresis
- Cell death analysis using immunohistochemistry
- Mouse colony management
- Mouse genotyping including ear tagging and tail snips
- Mouse dissection including fetal pup dissection
- Proficient with SPSS, Microsoft Office and Adobe Photoshop software

PUBLICATIONS

Accepted with revisions

Jones, RL and Pepling, ME. *Role of the anti-apoptotic proteins Bcl2 and Mcl1 in the neonatal mouse ovary*. Biology of Reproduction.

Manuscripts in progress

Jones, RL and Pepling, ME. *Kit signaling regulates oocyte cyst breakdown in the neonatal mouse ovary.*

INVITED PRESENTATIONS

2010 Jones, RL and Pepling, ME. *Evidence that Bcl2 is dispensable for neonatal ovarian oocyte survival.*
Society for the Study of Reproduction Annual Meeting, Milwaukee, WI.

2011 Jones, RL and Pepling, ME. *Mechanisms of ovarian germ cell apoptosis and follicle formation: The role of Bcl2 and kit signaling.* Central New York Developmental Biology Interest Group, Syracuse, NY.

2011 Jones, RL and Pepling, ME. *Role of Bcl2 in Oocyte Survival.* 1st Annual Life Sciences Symposium, Syracuse, NY.

2011 Jones, RL and Pepling, ME. *Kit signaling promotes oocyte cyst breakdown and primordial follicle formation in the mouse ovary.* Society for the Study of Reproduction Annual Meeting, Portland, OR.

2012 Jones, RL and Pepling, ME. *Mouse ovarian development and the role of kit signaling.* Syracuse University Graduate Recruitment Day, Syracuse, NY.

2012 Jones, RL and Pepling, ME. *Role of the anti-apoptotic proteins Bcl2 and Mcl1 in perinatal oocyte survival.* 2nd Annual Life Sciences Symposium, Syracuse, NY.

TEACHING EXPERIENCE

Spring 2009	Introduction to Biology	Syracuse University
Fall 2009, 2010	Genetics Laboratory	Syracuse University

Spring 2010, 2012	Cell and Developmental Biology Laboratory	Syracuse University
Spring 2011	Molecular Biology Laboratory	Syracuse University
Spring 2007	Current Research in Science Education	Syracuse University
Fall 2006	Science Teaching Methods	Syracuse University
2004-2006	Biology Teacher	Liverpool High School
2003-2004	Science Teacher	Bloomfield Middle School
Fall 2001, 2002	Elementary Science Education Methods	Syracuse University

UNIVERSITY SERVICE

- Member, Institutional Animal Care and Use Committee, Syracuse University
- Graduate Student Member, Graduate Education and Recruitment Committee, Syracuse University
- Member, Tenure Committee, Biology Department, Syracuse University
- Listserv Manager, Graduate Student Organization, Biology Department, Syracuse University

AWARDS AND HONORS

- Member of Phi Kappa Phi
- Academic fellowship Syracuse University
- Society for Study of Reproduction trainee travel grant award
- Syracuse University Graduate Student travel award
- Syracuse University Biology travel award
- Clinical excellence award SUNY Health Science Center
- Magna Cum Laude SUNY Health Science Center

PROFESSIONAL AFFILIATIONS

2010- PRESENT	Society for the Study of Reproduction
2011- PRESENT	American Association for the Advancement of Science

REFERENCES

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