1-18-2018

The G Protein-Coupled Receptor, GPR3, Promotes the Acquisition of Oocyte Meiotic Competence

Laura Firmani
University of Connecticut - Storrs, ldangelo@uchc.edu

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The G Protein-Coupled Receptor, GPR3, Promotes the Acquisition of Oocyte Meiotic Competence

Laura D’Angelo Firmani, PhD
University of Connecticut, 2018

Mammalian oocytes are arrested in meiotic prophase from around the time of birth until just before ovulation. Following an extended period of growth, they are stimulated to mature to the metaphase II stage by a preovulatory luteinizing hormone (LH) surge that occurs with each reproductive cycle. Small, growing oocytes are not competent to mature into fertilizable eggs because they do not possess adequate amounts of cell cycle regulatory proteins, particularly cyclin-dependent kinase 1 (CDK1). As oocytes grow, they synthesize CDK1 and acquire the ability to mature. After oocytes achieve meiotic competence, meiotic arrest at the prophase stage is dependent on high levels of cAMP that are generated in the oocyte under the control of the constitutively active \( \text{G}_s \)-coupled receptor, GPR3. This dissertation examines the events occurring during the switch between GPR3-independent and GPR3-dependent meiotic arrest. We found that the ability of oocytes to mature, as well as oocyte CDK1 levels, were dependent on follicle size, but CDK1 expression in oocytes from preantral follicles was not acutely altered by the activity of follicle stimulating hormone (FSH). \( Gpr3 \) was expressed and active in incompetent oocytes within early stage follicles, well before cAMP is required to maintain meiotic arrest. Oocytes from \( Gpr3^{+/} \) mice were less competent to mature than oocytes from \( Gpr3^{+/-} \) mice, as assessed by the time course of germinal vesicle breakdown. Correspondingly, \( Gpr3^{+/} \) oocytes contained significantly lower CDK1 levels than their \( Gpr3^{+/-} \) counterparts that were at the same stage of follicle development. These results demonstrate that cAMP in oocytes promotes meiotic competence, through the activity of GPR3.
The G Protein-Coupled Receptor, GPR3, Promotes the Acquisition of Oocyte Meiotic Competence

Laura D’Angelo Firmani

B.S., University of Saint Joseph, 2013

A Dissertation
Submitted in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy
at the
University of Connecticut

2018
The G Protein-Coupled Receptor, GPR3, Promotes the Acquisition of Oocyte Meiotic Competence

Presented by
Laura D’Angelo Firmani, B.S.

Major Advisor ________________________________________________________________
Lisa M. Mehlmann

Associate Advisor _____________________________________________________________
Laurinda A. Jaffe

Associate Advisor _____________________________________________________________
Bruce A. White

Associate Advisor _____________________________________________________________
Ann Cowan

University of Connecticut
2018
PREFACE

Portions of this dissertation have been published:

ACKNOWLEDGEMENTS

There were so many wonderful people who provided me with support while I embarked on the journey of graduate school and I am beyond grateful for every single one of them.

First, I would like to thank my undergraduate biology professors from the University of Saint Joseph, in particular Dr. Marcucci, Dr. Reed (Guttilla), and Prof. Miller. They are responsible for igniting my passion for biology, as they were all inspiring, brilliant, and compassionate teachers. I thank them for providing me the opportunities to gain research experience at the UConn Health Center and Yale University. Without their guidance and advice, I would never have pursued graduate school.

I would like to thank my advisor, Lisa Mehlmann, who believed in me and gave me the opportunity to achieve my doctorate degree. I am grateful to have such a kind, understanding, and supportive mentor, who shared in many insightful discussions about science and life with me. I will forever cherish the moments we shared, including the SSR conference in Puerto Rico, driving up to Woods Hole for the FIR course, shadowing during a gross anatomy class, and working at the microscope bench together as we dissected countless follicles. I am also grateful for being able to spend quality time with Tiger and Emma. Lastly, I will always cherish my fish and I’m thankful for all the extremely helpful advice about maintaining a fish tank!

I would also like to thank the members of my thesis committee. I thank Rindy Jaffe for being invested in my success as a graduate student. I appreciate all the time she took in reviewing papers and answering all my questions. Her love and passion for science is infectious and inspiring. I thank Bruce White for all the serious and fun discussions we shared. I enjoyed the laughs and I value all the insight about teaching anatomy. Lastly, I would like to thank Ann
Cowan who taught my most favorite lessons during the Cell Biology course. I thank her for all her help in succeeding as a graduate student.

I would like to especially thank Tracy Uliasz for her guidance, input, and company during my graduate studies. I would be clueless in the lab without her, as she always had the answers when I had trouble with an experiment or couldn’t find a reagent. I thank her for being patient with me and for helping dissect out the small follicles, even if it wasn’t her favorite thing to do. Also, I would like to thank the members of the Jaffe, Terasaki, and Peluso labs who were always available to answer my questions about my project.

I have been graced with the opportunity to make such delightful friends during my time at UConn including Yuva, Leia, Denisse, and Valentina. I thank them for all their support and love, as we shared in the journey of graduate school together. Also, I thank them for exposing me to their cultures (especially the food) and sharing many unforgettable memories such as attending the Today show, meeting baby Matías, hiking with dogs, and jumping off a waterfall!

I would also like to thank my best friends from USJ. To Ali, Cait, Kailyn, Liz, Kelly, Ashley, Ambreen, Katelyn, and Jessie; I thank you all for believing in me, loving me, and for being my constant cheerleaders during my graduate studies.

Lastly, I would like to especially thank my big Italian family, who undoubtedly always asked when I would be done! I truly thank my parents, Debbie and Joe, who provided me with a life full of opportunities and encouragement. I thank them for always loving me and being there for me. I would like to also thank my younger sisters, Stephanie and Gina. I thank them for their love and for always being there for a fun time together (only if we don’t play new board games)! I also dearly thank my loving grandparents, Mama and Poppy, who are always so happy and
proud of all their grandchildren. (Sorry Poppy that I never got a chance to call for help!) I also thank my Auntie Diane for all her constant love and encouragement. Also, I would like to thank my newly-gained family, the Firmani’s. To Linda, Dave, and Laura, thank you for all your support and for sitting through those boring and rough practice talks. To Marcia, thank you for being a person to talk to about life as a professor, I value all our long and deep conversations we had. To Gram, even though I am sad that you were not able to see me finish, I will always be thankful for your endless love and support.

Most importantly, I would like to dedicate this work to my loving husband, Michael. I thank him for being my biggest supporter through every step in my graduate studies. I thank him for motivating me, wiping my tears away, and celebrating accomplishments. I also thank him for sticking by my side during all my unpleasant meltdowns and constantly reminding me of what I am capable of.
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I. Regulation of Meiosis in Mammalian Oocytes and Eggs

The female sex cell, the oocyte, proceeds through meiosis to reduce the number of homologous chromosomes to become haploid, allowing it to combine with the haploid genome of sperm at fertilization to form a diploid embryo. In fetal development in humans, around the twenty-fourth week of gestation, oocytes have entered meiosis and become arrested at the diplotene stage of prophase I until reproductive maturity, which can last months in mice and years in humans (Eppig et al. 2004; Gougeon 2004). With the onset of puberty, during the menstrual cycle, oocytes are stimulated by a preovulatory surge of luteinizing hormone (LH) to resume meiosis, mature into eggs, and to be ovulated into the fallopian tubes (Figure 1.1). Oocytes proceed through meiosis and become arrested for a second time at metaphase II (Mehlmann 2005a). Ovulated metaphase II eggs will complete meiosis in response to fertilization.
The Oocyte is Maintained at Prophase I Arrest during Folliculogenesis

In the ovary, the oocyte is surrounded by one or more layers of granulosa cells to form the ovarian follicle, the functional unit of the ovary (Gougeon 1996). Initially, one layer of squamous pre-granulosa cells encircles the oocyte, forming primordial follicles. Generation of primordial follicles occurs between the sixth to ninth month of fetal life (Erickson 2008). Soon after the formation of the primordial follicle, folliculogenesis begins. Once a primordial follicle is activated to grow, the granulosa cells begin to change shape from squamous to cuboidal to form the primary follicle (Gougeon and Chainy 1987). Granulosa cells then begin to proliferate to form several more layers around the oocyte, developing into a secondary, preantral follicle. After the preantral follicle contains multiple layers of granulosa cells, a fluid-filled cavity begins to form, known as the antrum. This is characterized by the accumulation of follicular fluid between the granulosa cells, forming tertiary, early antral follicles. In humans, only one follicle within the population of early antral follicles in the ovary will achieve dominance over all the others and mature into a larger preovulatory, or Graafian, follicle in response to follicle-stimulating hormone (FSH) (Erickson 2008; Gougeon 1996). These follicles consist of about 2-3 layers of inner granulosa cells surrounding the oocyte, called cumulus cells, and about 5-10 layers of outer granulosa cells, called mural granulosa cells (Jaffe and Egbert 2017). The enlarged antrum is found between these two types of granulosa cells.
The development of follicles up to early antral follicles is relatively independent of the pituitary gonadotropins FSH and LH (Gougeon 2004), although preantral follicles possess FSH receptors, but not LH receptors (Abel et al. 2000; Hardy et al. 2017). The responsiveness of early antral follicles to FSH allows them the grow into preovulatory follicles which express LH receptors on their outermost mural granulosa cells (Bortolussi, Marini, Dal Lago 1977). As the follicle progresses through these different developmental stages, the oocyte is continuously held in prophase arrest. Only once LH stimulates preovulatory follicles, do the oocytes resume meiosis and ovulate.

**High Levels of Oocyte cAMP Generated by GPR3 Maintains Prophase I Arrest**

In culture, releasing oocytes from antral follicles results in spontaneous meiotic resumption, indicated by germinal vesicle breakdown (GVBD). It was discovered that elevating 3',5'-cyclic AMP (cAMP) in oocytes from antral follicles by using a membrane-permeant, hydrolysis-resistant form of cAMP, or an inhibitor to cAMP phosphodiesterase, kept oocytes in arrest, revealing that cAMP is necessary to inhibit meiotic resumption (Cho, Stern, Biggers 1974; Magnusson and Hillensjo 1977). Initially, it was thought that within large antral follicles, cAMP was being generated in the granulosa cells and diffusing to the oocyte (Bornslaeger and Schultz 1985; Dekel, Aberdam, Sherizly 1984; Dekel 2005; Edry, Sela-Abramovich, Dekel 2006). However, with studies using microinjection in follicle-enclosed oocytes, it was shown that the oocyte itself can generate cAMP.

Earlier experiments conducted by injecting an inhibitory antibody against Gs, or a dominant-negative form of Gs, into oocytes resulted in meiotic resumption (Kalinowski et al. 2004; Mehlmann, Jones, Jaffe 2002). This indicated that Gs activity within the oocyte is indispensable to keeping the oocyte in arrest. Gs stimulates adenylyl cyclase to covert ATP into
the cAMP needed for arrest. It was also found that Gs is activated by the orphan G protein-coupled receptor, GPR3 (Hinckley et al. 2005; Ledent et al. 2005; Mehlmann et al. 2004). Studies showed oocytes spontaneously resume meiosis, independent of LH, in preovulatory follicles of Gpr3−/− mice (Ledent et al. 2005; Mehlmann et al. 2004). The constitutively active GPR3 produces cAMP by particularly activating adenylyl cyclase type 3 (Figure 1.2) (Horner et al. 2003).
Figure 1.2. Different signaling events occurring during meiotic arrest and resumption in response to LH. The top of the figure is a simplistic view of Figure 1.1 to better understand when these signaling events are occurring in the oocyte respective to oocyte and follicle development.

High cAMP levels, associated with meiotic arrest, bind to and activate protein kinase A (PKA) to ultimately phosphorylate and inactivate the maturation promoting factor (MPF) (Figure 1.2) (Bornslaeger, Mattei, Schultz 1986; Holt, Lane, Jones 2013a). MPF is a heterodimer consisting of cyclin-dependent kinase 1 (CDK1) (also known as p34^cdc2) and Cyclin B (Hunt 1989). Through multiple steps, PKA phosphorylates and activates the kinases WEE1B/MYT1, that then phosphorylates Thr14 and Tyr15 sites of CDK1 to inhibit its activity (Figure 1.2) (Duckworth, Weaver, Ruderman 2002; Han et al. 2005; Mueller et al. 1995). To ensure CDK1 remains in a phosphorylated state, PKA also phosphorylates the phosphatase CDC25B to keep it inactive and unable to dephosphorylate CDK1. The phosphorylated CDC25B associates with the 14-3-3 protein that keeps it sequestered in the cytoplasm and unable to translocate into the nucleus where phosphorylated CDK1 resides (Figure 1.2) (Duckworth, Weaver, Ruderman 2002; Pirino, Wescott, Donovan 2009). Restricting CDK1 activity prevents chromosome condensation and nuclear envelope break-down, also known as germinal vesicle breakdown (GVBD), that occurs during meiotic resumption (Figure 1.2) (Jaffe and Egbert 2017).
The Production of cGMP in the Granulosa Cells Contributes to Prophase I Arrest

The follicle cells have a major role in keeping the oocyte in meiotic arrest. Isolating oocytes from antral follicles results in a decrease in oocyte cAMP and subsequent resumption of meiosis (Schultz, Montgomery, Belanoff 1983; Vivarelli et al. 1983). It has been found that the granulosa cells generate cGMP, which diffuses through gap junctions to the oocyte. In the oocyte, cGMP inhibits the cAMP phosphodiesterase type 3A (PDE3A) to prevent the hydrolysis of cAMP and maintain meiotic arrest (Figure 1.2). Also, knocking out PDE3A, or incubating isolated oocytes with milrinone, a PDE3A inhibitor, prevents meiotic resumption (Masciarelli et al. 2004; Shitsukawa et al. 2001). Therefore, limiting the activation of PDE3A either by cGMP in the follicle, or by milrinone in culture, keeps cAMP levels in the oocyte elevated, preserving prophase arrest.

Luteinizing Hormone Stimulates Meiotic Resumption by Decreasing cGMP Levels

During the estrous cycle in mice, and presumably during the menstrual cycle in women, LH is released from the anterior pituitary to act on the LH receptors present on mural granulosa cells of the preovulatory follicle. Activated LH receptors cause a decrease in cGMP in the outermost granulosa cells, which provides a sink for cGMP to diffuse out of the oocyte (Shuhaibar et al. 2015). PDE3A becomes activated due to low oocyte cGMP levels, and hydrolyzes cAMP, lowering oocyte cAMP levels (Norris et al. 2009; Vaccari et al. 2009). This leads to a decrease in PKA activity and an increase in CDC25B activity; both contribute to dephosphorylating and activating MPF, resulting in oocyte meiotic resumption (Figure 1.2). During this phase of meiotic resumption, the oocyte undergoes meiotic maturation to become a metaphase II arrested egg, which will be ovulated for fertilization. Oocyte maturation is characterized by many events, including GVBD and the production of a polar body.
II. The Acquisition of Oocyte Meiotic Competence: Events Leading Up to the cAMP-dependent Arrest of Meiotic Prophase

Oocyte Growth in the Ovary

In conjunction with follicle growth, the prophase I arrested oocyte also goes through a period of growth accompanied by the synthesis and accumulation of macromolecules and organelles (Sorensen and Wassarman 1976a; Szybek 1972). In mice, as well as other mammals, oocytes within preovulatory antral follicles are fully-grown and reach a diameter of approximately 80 µm. Essentially all oocytes of this size are able to resume meiosis, either in vivo after the LH surge or spontaneously after release from their follicle in vitro. This is indicated by chromosome condensation, GVBD, and progression to metaphase II (Abe et al. 2011). Contrary to fully-grown oocytes, growing oocytes that are less than 60 µm in diameter, found within preantral follicles, fail to undergo GVBD under the same conditions and maintain an intact germinal vesicle (GVI) (Sorensen and Wassarman 1976a). This acquired ability to resume meiosis is called the acquisition of meiotic competence and coincides with the formation of the antrum in the follicle (Figure 1.1) (Erickson and Sorensen 1974; Mehlmann 2005a; Sorensen and Wassarman 1976a; Szybek 1972).

Maturation Promoting Factor (MPF) During Oocyte Growth

It is evident that the preovulatory follicle is vital in keeping the fully-grown oocyte in meiotic arrest by keeping intracellular cAMP levels elevated in the oocyte. However, early follicle development, growing oocytes can maintain prophase I arrest themselves and do not rely on signals from the surrounding follicle or the contribution of cyclic nucleotides. This is due to the oocyte being meiotically incompetent and lacking the possession of cell cycle machinery,
such as CDK1 (Adhikari et al. 2012; Chesnel and Eppig 1995a; de Vantery et al. 1997; Nishimura et al. 2009) (Figure 1.3). Adequate amounts of dephosphorylated CDK1 are needed for the progression from prophase to metaphase in response to LH (Chesnel and Eppig 1995a; de Vantly er et al. 1996; Kanatsu-Shinohara, Schultz, Kopf 2000; Mitra and Schultz 1996). The kinase activity of CDK1 assists in the disassembly of nuclear lamins and nuclear pores contributing to the nuclear envelope breakdown (GVBD) associated with meiotic resumption (Abe et al. 2011; Adhikari et al. 2012; Lenart et al. 2003). Therefore, small growing oocytes do not contain enough CDK1 to carry out these meiotic events.
**Figure 1.3. Cell cycle protein expression and regulation during oocyte growth.** The top of the figure is a simplistic view of Figure 1.1 to better understand when these signaling events are occurring in the oocyte respective to oocyte and follicle development. Bold protein names in purple represent increased protein expression for that particular protein. The nucleus in light gray is shown to demonstrate the localization of proteins. Evaluating if GPR3 is active in growing follicles is one aim for this dissertation, indicated by the question mark. Overall, this figure demonstrates changes in protein expression levels, phosphorylation states, MPF complex assembly, and localization of proteins in relation to the acquisition of meiotic competence.

Several studies have demonstrated that even though the concentration of Cyclin B remains moderately constant, the concentration of CDK1 increases significantly from meiotically incompetent to competent oocytes (Chesnel and Eppig 1995a; de Vant'ery et al. 1996; Kanatsu-Shinohara, Schultz, Kopf 2000; Mitra and Schultz 1996) (**Figure 1.3**). It is proposed the CDK1 could be a limiting factor in meiotically incompetent oocytes and that the accumulation of this protein is critical for the acquisition of meiotic competence. Along with total amounts of CDK1, the ability of CDK1 to dimerize with Cyclin B can occur during oocyte growth and competence (**Figure 1.3**). Early experiments injected CDK1 into incompetent oocytes, and even though CDK1 protein levels increased, they were still unable to resume meiosis (de Vantery et al. 1997). It was found that the newly synthesized CDK1 was dephosphorylated and unable to associate with Cyclin B (de Vantery et al. 1997). This suggests that an accumulation of CDK1 alone is not sufficient to establish oocyte competence and that there has to be a posttranslational modification that must occur during the acquisition of meiotic competence.
Another event that takes place during the acquisition of meiotic competence is changes in the ability of the CDK1-Cyclin B complex, also known as MPF, to localize to the nucleus (Figure 1.3). In incompetent oocytes, both CDK1 and Cyclin B are found in the cytoplasm and move to the nucleus upon gaining meiotic competence (Mitra and Schultz 1996). This change is thought to be due to the phosphorylation state of Cyclin B, as phosphorylated Cyclin B is unable to associate with the nuclear export factor CRM1, keeping it in the nucleus (Yang et al. 1998). In agreement with this study, it was found that there is an increase in phosphorylated Cyclin B during the acquisition of meiotic competence (Kanatsu-Shinohara, Schultz, Kopf 2000).

Studies have also been conducted to analyze regulators of MPF during the acquisition of meiotic competence. In particular, the kinases WEE1 and CDC25, that inactivate and activate MPF, respectively, present in both incompetent and competent oocytes, and are always at a greater concentration than MPF (Figure 1.3). However, WEE1 levels decrease and CDC25 levels increase during the acquisition of meiotic competence, suggesting a shift to priming the oocyte for MPF activation at the end of oocyte growth (Kanatsu-Shinohara, Schultz, Kopf 2000; Nakajo et al. 2000). Overall, several events in relation to MPF occur during the acquisition of meiotic competence such as changes in the concentration, phosphorylation state, complex assembly, localization, and regulation.

**cAMP Participates in the Acquisition of Meiotic Competence**

In addition to contributing to meiotic arrest, cAMP also appears to potentiate the acquisition of meiotic competence in growing oocytes. In these previous studies, denuded incompetent mouse oocytes were incubated in dbcAMP, a cell permeable analog of cAMP, or with forskolin, an activator of adenylyl cyclase, for 3 days. Both dbcAMP and forskolin promoted the acquisition of meiotic competence in a dose dependent manner, suggesting that
elevated levels of cAMP allow a growing oocyte to become more meiotically competent (Carroll, Whittingham, Wood 1991; Chesnel, Wigglesworth, Eppig 1994). In agreement with these findings, meiotic competence is compromised in oocytes that cannot generate cAMP (Ledent et al. 2005). These findings suggest a role of cAMP in early oocyte development.

III. Improving Embryonic Developmental Competence

Proper and sequential events must occur during oocyte growth, acquisition of meiotic competence, resumption of meiosis, cytoplasmic maturation, and fertilization to ensure successful embryo development (Figure 1.1). During the acquisition of meiotic competence, as the oocyte grows, cytoplasmic organelles replicate, proteins are translated, and RNAs needed for development after fertilization are stored, contributing to a more developmentally competent embryo (Sorensen and Wassarman 1976b). Poor oocyte quality, with low developmental competence, impacts embryo survival before and after implantation, resulting in loss of pregnancy (Reader, Stanton, Juengel 2017). Even though sperm is an important component to fertilization, many of the cellular and molecular mechanisms required for early embryo development are inherent to the oocyte and egg (Swain and Pool 2008). Overall, insufficient oocyte growth and maturation lead to a less developmentally competent embryo.

Assisted Reproductive Technologies (ART)

By researching and defining the molecular and cellular aspects between high and low-quality oocytes and eggs, it can be better understood what factors have an impact on reproductive health, such as genetic mutations, cancer treatments, aging, and other environmental factors. Also, knowing markers for high oocyte and egg quality can assist in establishing better in vitro
culture systems to improve assisted reproductive technologies (ART). Even though ART is widely used to overcome infertility, the success rates are still relatively low. According to the Society for Assisted Reproductive Technologies (SART), in 2015, the live birth rate from an average of 1.17 embryos transferred per women under the age of 35 years was 48.2% per egg-retrieval cycle (www.sart.org). Therefore, researching the components of a healthy, high-quality, developmentally competent oocyte due to proper acquisition of meiotic competence will allow for potential improvements in the success of both natural fertility and ART.

**Dissertation Aims**

It is evident that the progression of a small growing oocyte into an MII egg encompasses many meiotic events essential for proper embryo development. I set out to determine the acquisition of oocyte meiotic competence within the same ovary, in respect to follicle size. The follicle is a functional structure with the oocyte directly coupled to the granulosa cells, and the granulosa cells coupled to each other by gap junctions. Connections between granulosa cells and the oocyte start during the very early stages of follicle formation and continues through folliculogenesis (Mitchell and Burghardt 1986). These gap junctions are vital for the transport of amino acids, glucose metabolites, nucleotides, and signaling molecules to the growing oocyte to ensure proper development (Bruzzone, White, Paul 1996). Also, prior to being responsive to extraovarian factors, such as pituitary gonadotropins, preantral follicle growth is dependent on gap junction communication, in addition to autocrine and paracrine factors (Halpin et al. 1986). Studies have demonstrated the role of connexins, as components of gap junctions, in regulating both follicle growth and oocyte meiotic competence. Knockout mouse models for both connexin-37 and connexin-43 showed an impaired ability to form follicles beyond the preantral stage and the follicles contain many meiotically incompetent oocytes (Ackert et al. 2001; Carabatsos et al.
This demonstrates that communication between oocytes and granulosa cells during folliculogenesis needs to be intact for appropriate follicle and oocyte development and that gap junctions are required to coordinate oocyte meiotic competence. Investigating oocyte meiotic competence, in relation to follicle size, will show if there is a correlation between different stages in folliculogenesis and oocyte meiotic competence.

Previous studies were conducted to analyze the acquisition of meiotic competence; however, mice of different ages were used and the follicle size was not carefully distinguished (Hirao, Miyano, Kato 1993; Szybek 1972; Wassarman and Josefowicz 1978). As previously mentioned, CDK1 activity has many meiotic functions, as such the breakdown of the nuclear envelope, suggesting it may be a critical factor in the acquisition of meiotic competence. Studies have established that CDK1 contributes to meiotic competence in mouse oocytes; however, they never systematically looked at CDK1 expression in relation to follicle development (Chesnel and Eppig 1995a; de Vant'ery et al. 1996; Kanatsu-Shinohara, Schultz, Kopf 2000). It remains to be known if the expression of oocyte CDK1 can be coordinated with a particular follicle stage. This would establish a better understanding of how follicle development can relate to a differential expression of cell cycle proteins within the oocyte.

By developing a systematic approach to carefully distinguish follicles of different developmental stages, it can be further analyzed how oocytes within small, preantral follicles respond to external stimuli. Even though it is well established that FSH promotes follicle growth, it is uncertain if it can contribute to oocyte-specific molecular changes associated with the acquisition of meiotic competence. Also, even though it has been well demonstrated that the oocyte can intrinsically generate cAMP by GPR3, some studies suggest that FSH causes an
increase in cAMP that diffuses through gap junctions to the oocyte (Webb et al. 2002a). It is unknown if this increase of cAMP has any effect on meiotic competence.

Understanding the molecular basis of the acquisition of meiotic competence, in respect to follicle size, can provide insight into the role of GPR3 early in oocyte growth. Even though GPR3 is a vital factor in keeping the fully-grown oocyte in a cAMP-dependent arrest, it is still uncertain if GPR3 has a role in oocytes earlier in development. Studies have suggested that GPR3 may be active in oocytes of preantral follicles (Freudzon et al. 2005; Hinckley et al. 2005; Vaccari et al. 2008); however, it is still unknown exactly when the GPR3 system becomes active. Also, it has been shown that cAMP may potentiate the acquisition of meiotic competence (Carroll, Whittingham, Wood 1991; Chesnel, Wigglesworth, Eppig 1994); however, the source of cAMP and its role in small, incompetent oocytes has yet to be established.

The overall goals of this dissertation are to correlate oocyte meiotic competence with follicle development to better understand meiotic events in relation to folliculogenesis. In particular, to analyze the cell cycle protein, CDK1, in relation to follicle size. Secondly, this dissertation will investigate the role of GPR3 in early oocyte development and in the acquisition of oocyte meiotic competence.
- CHAPTER 2 -

Materials and Methods
**Media and Reagents**

Unless otherwise noted, all reagents were from Sigma Aldrich (St. Louis, MO). In most cases, the medium used for oocyte and follicle isolation was MEMα (Life Technologies, Carlsbad, CA) supplemented with 20 mM HEPES, 75 μg/ml penicillin G, 50 μg/ml streptomycin, 0.1% polyvinyl alcohol (PVA), and 10 μM milrinone. For experiments in which we cultured follicles with follicle stimulating hormone (FSH), we used bicarbonate-buffered MEMα (Mehlmann 2005b) supplemented with 5% fetal bovine serum (FBS; see below). For experiments in which we treated oocytes with dbcAMP, follicles were isolated in HEPES-buffered MEMα containing 10 μM milrinone, and oocytes were removed and cultured overnight in CZB medium (Chatot et al. 1989) supplemented with 5% FBS, 10 μM milrinone, and 250 μM dbcAMP.

**Oocyte and Follicle Isolation and Culture**

23-26-day-old mice were used for most experiments. In a few cases, we also used 2-, 4-, 8-, and 11-day-old mice. Gpr3−/− mice were generated as described previously (Mehlmann et al. 2004) and the colony was maintained by breeding Gpr3+/− mice to generate Gpr3+/− and Gpr3+/- mice for littermate controls. If littermates were not available, age-matched wild type C57Bl/6J mice were obtained from Jackson Labs (Bar Harbor, ME). Mice were genotyped as previously described (Mehlmann et al. 2004). Follicles were manually dissected from ovaries using 30-gauge needles. Oocytes were removed from all isolated follicles by follicle puncture. Cumulus cells were removed by repeated pipetting through a small-bore pipet.
For experiments in which follicles of different sizes were used, follicles were measured using a calibrated ocular reticle. Oocytes were released from the follicles by tearing a slit on one side. For the time course experiments, follicles were isolated from ovaries in medium containing 10 μM milrinone to prevent germinal vesicle breakdown (GVBD), were grouped according to size, and the oocytes were removed from the follicles. The cumulus cells were removed and the isolated oocytes were then washed into fresh medium without milrinone to initiate meiotic resumption. Oocytes were scored hourly using a Zeiss Discovery V8 stereomicroscope for GVBD. Isolated oocytes were imaged using a 40X 1.2 NA lens and oocyte diameters were measured using ImageJ (NIH).

For experiments in which follicles were cultured with or without FSH, we isolated preantral follicles in bicarbonate-buffered MEMα without milrinone (Mehlmann 2005b) and plated them individually on Millicell membranes (Millipore, Billerica, MA), plating a maximum of 20 follicles per Millicell. Half the follicles were incubated in medium containing 30 ng/ml ovine FSH (from A. F. Parlow, National Hormone and Peptide Program, Torrance, CA). Oocytes were removed from follicles following a 20 hr incubation period. Only GV-intact oocytes within undamaged follicles were utilized for experiments.

**Western Blotting**

Oocytes were obtained from follicles of defined sizes, cumulus cells were removed, and oocytes were transferred to microfuge tubes in a small volume of medium. 15 μl of 1X sample buffer (Laemmli 1970) containing 5% β-mercaptoethanol was added to each tube, samples were boiled for 5 minutes, and the whole volume was loaded into a 4-20% polyacrylamide gel (BioRad). Separated proteins were electrophoretically transferred onto nitrocellulose membranes.
and the membranes were blocked in 1% non-fat dry milk in TBST. Primary antibody (anti-CDK1 from Santa Cruz, catalog #sc-53219) was diluted to 2 μg/ml and was incubated on the blot overnight at 4°C. Peroxidase-conjugated secondary antibody (Santa Cruz) was diluted 1:5,000 in blocking buffer. Blots were developed using either ECL Prime (GE Healthcare, Chicago, IL) or WesternBright Sirius horseradish peroxidase substrate (Advansta, Menlo Park, CA), and imaged using a charge-coupled device camera (G:box Chemi XT4; Syngene). ImageJ software was used to perform the densitometry.

**Salmon-gal Staining**

Whole ovaries were dissected and were either cut into smaller pieces, or follicles were separated manually. In some cases, isolated oocytes were used. Salmon-gal staining was performed as previously described (Sundararajan et al. 2012). Briefly, ovaries, follicles, and isolated oocytes were fixed for 5 min in PBS containing 2% formalin, 0.2% glutaraldehyde, 5 mM EGTA, 2 mM MgCl₂, and 0.1% PVA, then were washed 3 times in rinse solution containing 0.1% sodium deoxycholate, 0.2% Nonidet p40, and 2 mM MgCl₂. After the rinses, follicles were stained using 1 mg/mL salmon-gal and 0.4 mM 4-nitro blue tetrazolium chloride (NBT) for 10-20 minutes at 37°C. Two final washes of PBS with PVA were performed and all tissues were plated on Millicell culture plates (Millipore) and imaged using either a 10X, 0.3 N.A. or a 20X, 0.5 N.A. lens. Photographs were taken using a Canon EOS M digital camera. Stained and unstained follicles, excluding the stroma outside the basement membrane, were measured using ImageJ.
cAMP Measurements

Oocytes obtained from Gpr3+/+ and Gpr3−/− follicles that were ~140-180 μm in diameter were isolated in medium containing milrinone and cleared of all cumulus cells using a small-bore mouth pipet. Oocytes were microinjected with the cAMP sensor protein, Epac2-camps300 (Lowther, Nikolaev, Mehlmann 2011; Nikolaev et al. 2004; Norris et al. 2009), as previously described (Kline 2009; Norris et al. 2009), except that we used a final concentration in the oocyte of 2 μM. Fluorescence was excited at 435 nm and was detected at 535/50 nm (YFP) and 480/40 nm (CFP). Images were collected using a 40X 1.2 NA water immersion objective on a Zeiss Pascal confocal microscope. Following a scan in the presence of milrinone, milrinone was washed out of the medium and scans were performed 30 min after removing milrinone. YFP and CFP intensities were quantified within a circular fluorescent region of interest using the Zeiss Zen software. cAMP concentrations were calculated as described in Norris et al. (2009), using the standard curve that was generated by recording the basal YFP:CFP ratios before and after injecting cAMP to obtain the sensor maximum, and injecting PDE3A (to lower cAMP to a minimum level) to obtain the sensor minimum.

RT-PCR

Total RNA was extracted from isolated oocytes or whole ovaries using the RNeasy Plus Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer’s instructions. RNA was reverse transcribed to cDNA using the iSCRIPT cDNA synthesis kit (Bio-Rad, Hercules, CA), using random primers. The following primer sets were used: Gpr3 forward 5’-TATCCACTCTCCAAGAACATCTGG-3’; Gpr3 reverse 5’-GAATTAAGCCCTGGTGAGCTAAC-3’; Gapdh forward 5’-
TGTTCCTACCCCCCAATGTGT-3’; Gapdh reverse 5’-TGTGAGGGAGATGCTCAGTG-3’; Cdk1 forward 5’-GAACACCTTTCCCAAGTGGA-3’; Cdk1 reverse 5’-CCATTTTGCCAGAGATTCGT-3’; Rpl19 forward 5’-CGGGAATCCAAGAAGATTGA-3’; Rpl19 reverse 5’-TTCAGCTTGTGGATGTGCTC-3’.

Cycling parameters for endpoint PCR (Gpr3 and Gapdh) were an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 45s, 60°C for 45s, 72°C for 90s, and a final extension at 72°C for 7 minutes. PCR products were electrophoresed on a 2% agarose gel and visualized using ethidium bromide (Bio-Rad, Hercules, CA), and imaged using the G:box.

Real-time qPCR (Cdk1 and Rpl19) was performed using a SYBR green kit, SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) on a Bio-Rad CFX96 system (Bio-Rad, Hercules, CA). The cycling parameters included an initial denaturation at 95°C for 3 minutes, followed by 50 cycles of 95°C for 10s and 55°C for 30s. A melt curve was performed, starting at 65°C and increasing by 0.5°C every 5s up to 95°C to determine primer specificity. Cdk1 gene expression was normalized to Rpl19 using the 2^ΔΔCt method.

Statistical Analysis

Statistical tests used for each experiment are described in the figure legends. GraphPad Prism6 was used for all statistical analyses.
-CHAPTER 3-

The Switch from cAMP-independent to cAMP-dependent Arrest of Meiotic Prophase is Associated with Coordinated GPR3 and CDK1 Expression in Mouse Oocyte
INTRODUCTION

Mammalian oocytes are arrested in meiotic prophase for most of a female’s reproductive lifespan and they remain arrested in prophase during a long period of oocyte and follicle growth. When follicles reach the preovulatory stage they become responsive to a surge in luteinizing hormone (LH) that occurs once per reproductive cycle. The LH surge signals the oocyte (via its action on follicle cells) to resume meiosis. Two mechanisms keep oocytes in meiotic arrest and prevent them from spontaneously resuming meiosis while in the ovary, one of which depends on cAMP and one that does not.

Meiotically arrested mouse oocytes progressively increase in size from ~20 to ~75 μm in diameter (Eppig and O'Brien 1996; Picton, Briggs, Gosden 1998). During most of this period of growth, prophase arrest is maintained because oocytes have not yet synthesized and accumulated mRNAs and proteins that are necessary for progression past prophase (Sorensen and Wassarman 1976a). Once the oocyte becomes competent to mature, meiotic arrest becomes dependent on high levels of cAMP, which is generated by the constitutively active, Gs-coupled receptor, GPR3 (Hinckley et al. 2005; Ledent et al. 2005; Mehlmann, Jones, Jaffe 2002; Mehlmann et al. 2004; Mehlmann 2005b). The elevated level of cAMP during meiotic arrest activates protein kinase A (PKA), which leads to the phosphorylation and inactivation of cyclin-dependent kinase 1 (CDK1; also known as p34cdc2); the activation of CDK1 when cAMP levels are decreased is essential for meiotic resumption (Holt, Lane, Jones 2013b; Mehlmann 2005a; Mehlmann 2013). Cyclic AMP levels in the oocyte are kept high by cGMP contributed from the follicle cells; cGMP inhibits the activity of PDE3A, the phosphodiesterase in the oocyte that degrades cAMP when it is active (Mehlmann 2005a). If meiotically competent oocytes are isolated from follicle cells, cAMP levels decrease (Schultz, Montgomery, Belanoff 1983) and oocytes undergo...
spontaneous GVBD (Edwards 1965); thus, both the oocyte and follicle cells contribute to cAMP-dependent meiotic arrest.

Oocytes reportedly achieve meiotic competence when they reach ~80% of their full-grown size (>60 μm) (Hirao, Miyano, Kato 1993; Szybek 1972; Wassarman and Josefowicz 1978). This occurs concomitantly with folliculogenesis and coincides with the antrum formation in the follicle (Szybek 1972). CDK1, as well as its binding partner Cyclin B, are important for this process (Adhikari et al. 2012; Chesnel and Eppig 1995b). The relative and absolute amounts of CDK1 and Cyclin B protein have been measured, and both increase significantly during oocyte growth, whereas the relative amount of Cyclin B is always in excess of CDK1 (Chesnel and Eppig 1995b; de Vant'ery et al. 1996; Kanatsu-Shinohara, Schultz, Kopf 2000; Mitra and Schultz 1996); therefore, CDK1 is a limiting factor in the acquisition of meiotic competence. Thus far, CDK1 levels have only been measured in populations of oocytes removed from 10-12-day-old ovaries compared with fully-grown oocytes removed from 6-week-old mice that had previously been injected with hormones to stimulate follicular growth. Meiotic competence is not solely dependent on CDK1 levels, however, because CDK1 levels increase in cultured small, nongrowing, incompetent oocytes without inducing meiotic maturation (Chesnel and Eppig 1995b). In addition, overexpression of CDK1 in incompetent mouse oocytes is not sufficient to cause meiotic resumption (de Vantery et al. 1997).

Because meiotic arrest begins in a cAMP-independent manner and progresses to GPR3 (and therefore cAMP)-dependent arrest, there must be a switch in maintaining meiotic arrest, at some point, during oogenesis. No information exists as to when the GPR3 system turns on to achieve this switch. In addition to its established role in maintaining meiotic arrest in meiotically competent oocytes, there is evidence that cAMP promotes the acquisition of meiotic competence
in growing oocytes (Carroll, Whittingham, Wood 1991; Chesnel, Wigglesworth, Eppig 1994). This is based on experiments demonstrating that incompetent oocytes can acquire meiotic competence after being cultured in the presence of the cAMP analog, dbcAMP (Chesnel, Wigglesworth, Eppig 1994). However, whether cAMP is produced within the oocyte to contribute to the acquisition of meiotic competence, as well as the source of this cAMP in growing oocytes, has not been investigated.

In this study, we examined the mechanisms of the acquisition of meiotic competence associated with the switch in meiotic regulation from cAMP-independent arrest to cAMP-dependent arrest. In addition, because of the evidence that cAMP contributes to the acquisition of meiotic competence, and given that GPR3 is the receptor that stimulates cAMP production in oocytes, we investigated the potential role of GPR3 in the acquisition of meiotic competence. We found that CDK1 levels increase during the period when an oocyte becomes competent to resume meiosis. In addition, Gpr3 mRNA is expressed in incompetent oocytes very early on in follicle development and cAMP is generated prior to antral follicle development. Furthermore, the expression of Gpr3 appears to potentiate meiotic competence in oocytes that are at the stage where meiotic competence is usually achieved. The data presented here demonstrate that the switch to the GPR3 system occurs before cAMP is required to maintain meiotic arrest, thus ensuring that this system will be in place when it is required.

RESULTS AND DISCUSSION

_Oocyte meiotic competence correlates with follicle size._

To systematically study when oocytes acquire meiotic competence with respect to follicle development, we examined the time course of oocyte maturation, as indicated by GVBD, in
oocytes that were manually dissected from mouse ovaries and grouped according to follicle diameter. Previous studies examining the development of meiotic competence primarily utilized oocytes removed from ovaries of very young (~10-day-old) mice (Hirao, Miyano, Kato 1993; Szybek 1972; Wassarman and Josefowicz 1978). Although one study examined maturation rates in oocytes removed from defined follicle sizes (Erickson and Sorensen 1974), this study utilized follicles from sexually mature, cycling mice irrespective of the stage in the estrous cycle and they did not carefully distinguish early antral from antral follicles, which is the stage at which meiotic competence is thought to be attained (Chesnel, Wigglesworth, Eppig 1994; Mehlmann et al. 2004). Here, we used prepubertal mice (~23-27 days old) that were not yet cycling. We considered follicles ~140-180 μm in diameter to be at the preantral stage, follicles ~220-250 μm in diameter to be at the early antral stage, and follicles ≥290 μm in diameter to be at the antral stage. After follicle isolation, oocytes were removed and scored every hour for GBVD, for up to 5 hrs.

As expected, nearly all of the oocytes retrieved from antral follicles underwent GVBD within 2 hrs after isolation (Fig. 3.1). Similar rates of GVBD were observed in oocytes retrieved from the 220-250 μm follicle group. In contrast, we only observed ~40% GVBD in oocytes obtained from preantral stage follicles after 5 hrs. We found that 30% of oocytes removed from follicles 220-250 μm in diameter had undergone GVBD at the time of follicle isolation, whereas none of the oocytes removed from the ≥290 μm group had undergone GVBD at the time of follicle isolation. This is likely because some of the 220-250 μm follicles were atretic since we did not prime the mice with PMSG before follicle isolation; hormonal priming would be expected to rescue such follicles destined for atresia. Atretic follicles often contain oocytes that have undergone GVBD (Gougeon and Testart 1986).
Figure 3.1. Analyzing meiotic resumption in oocytes from preantral, early antral, and antral follicles. GVBD time course using oocytes isolated from follicles of different size ranges. The graph demonstrates that oocytes become meiotically competent within follicles ~220-250 μm in diameter.

Because the ability to mature has been linked to oocyte size, we measured oocyte diameters in oocytes retrieved from preantral and antral follicles. The average diameter of preantral oocytes was significantly smaller than antral oocytes (71 ± 0.4 μm vs 74 ± 0.3 μm respectively), and the percentage of preantral oocytes that underwent GVBD in our study was lower than that reported by Hirao et al. (1993), who found that 90% of oocytes 65-70 μm in diameter underwent GVBD in culture conditions similar to those used in our study. This discrepancy is likely due to the length of time in culture, as we only monitored GVBD for 5 hrs, whereas Hirao et al. (1993) examined oocytes after they had been cultured for 24 hrs. Previous studies have shown that oocytes within ovaries of ≥15-day old mice can undergo spontaneous
GVBD when removed from ovaries (Sorensen and Wassarman 1976a; Wickramasinghe, Ebert, Albertini 1991a). However, these previous studies retrieved oocytes from ovaries irrespective of follicle size. Our study systematically correlates GVBD competence with follicle size, and the results clearly show that oocytes acquire meiotic competence as follicles reach a diameter of ~220 μm.

**CDK1 levels increase in oocytes from the preantral to the antral follicle stage.**

Several previous studies have identified CDK1 as a key factor that contributes to the acquisition of meiotic competence in mouse oocytes (Chesnel and Eppig 1995b; de Vant'ery et al. 1996; Kanatsu-Shinohara, Schultz, Kopf 2000; Mitra and Schultz 1996). Levels of CDK1 increase during oocyte growth, as determined by measuring proteins using western blots. However, in these previous studies, CDK1 measurements were done using oocytes removed from 10-12-day-old ovaries compared with fully-grown oocytes removed from 6-week-old, hormonally-primed ovaries. To determine if CDK1 levels increase during the period of oocyte growth from the preantral to the antral stage, we isolated follicles of different sizes from the same ovaries, grouped the follicles according to size, removed the oocytes, and performed western blots for CDK1. In an initial experiment, we ran a CDK1 blot using defined numbers of fully-grown oocytes to estimate the number of oocytes to use for subsequent experiments and to confirm the linearity of our western blotting detection method (**Fig. 3.2A**). We found that our CDK1 antibody produced a clean band and the signal increased linearly. We observed a strong band using 25-50 oocytes, so unless noted, we used 30 oocytes per lane in subsequent experiments. The amount of CDK1 increased significantly in oocytes during the period from the preantral to the antral stage, particularly from the preantral to the early antral stage (**Fig. 3.2B,C**).
This increase was not due solely to an increase in oocyte volume which also occurred during the period of follicle growth, because the amount of CDK1 increased approximately 2-fold, whereas the oocyte volume increased only ~1.25-fold (Fig. 3.2D). This result correlates with the increased ability of oocytes within early antral follicles (220-250 μm in diameter) to undergo GVBD, and supports the hypothesis that the level of CDK1 is important for the acquisition of meiotic competence.

**Figure 3.2. CDK1 levels increase in oocytes during the acquisition of meiotic competence.**

A) Defined numbers of full-grown, prophase arrested oocytes were probed for CDK1 with western blot to test the linearity of ECL signal. B) Western blot for CDK1 in oocytes isolated from follicles of defined diameters, from the same ovary. C) CDK1 densitometry, showing that the amount of CDK1 increases significantly in the 220-250 μm group, which corresponds to the
time that oocytes acquire meiotic competence. Data were compiled from 6 independent experiments. Bars with different letters are significantly different (P<0.05). D) Volumes in oocytes retrieved from 140-180 µm diameter follicles compared to ≥290 µm diameter follicles.

*FSH does not acutely alter oocyte CDK1 levels.*

Follicle stimulating hormone (FSH) is essential for follicle growth, as follicle development past the preantral stage does not occur in FSH receptor-deficient mice (Abel et al. 2000). Although follicle cells express RNA encoding FSH receptors in follicles as small as ~75 µm in diameter (Hardy et al. 2017), they generally start becoming responsive to FSH when they are ~130 µm in diameter (Hardy et al. 2017). To investigate if this responsiveness to FSH can be a signal to stimulate CDK1 expression, we isolated oocytes from FSH-responsive, preantral stage follicles (140-180 µm in diameter) after culturing follicles for ~24 hrs with or without FSH. We chose a 24 hr incubation period rather than a longer culture time to determine if FSH acutely regulates CDK1 expression. There was no difference in CDK1 levels in oocytes treated with FSH compared to untreated follicles (*Fig. 3.3A*). This result indicates that FSH does not acutely regulate CDK1 levels, but chronic stimulation probably contributes to CDK1 levels by stimulating follicle growth, which is associated with the acquisition of meiotic competence.

FSH acts through the FSH receptor, a Gs-coupled receptor on the follicle cells, stimulating the production of cAMP (Hunzicker-Dunn and Mayo 2015; Simoni, Gromoll, Nieschlag 1997). A popular hypothesis for many years was that cAMP produced in follicle cells diffuses through gap junctions into the oocyte to elevate cAMP levels and inhibit premature meiotic resumption (Dekel 2005; Edry, Sela-Abramovich, Dekel 2006). Dekel et al. (1984)
demonstrated that elevated cAMP levels in cumulus cells in response to forskolin treatment was required to inhibit spontaneous GVBD in rat oocytes, suggesting that cAMP is produced in follicle cells and diffuses through gap junctions into the oocyte (Dekel, Aberdam, Sherizly 1984). However, they did not measure cAMP levels in isolated oocytes following forskolin treatment. Likewise, inducing gap junction closure with carbenoxolone in follicle-enclosed oocytes causes GVBD in mouse and rat oocytes (Norris et al. 2009; Sela-Abramovich et al. 2006), but no oocyte cAMP measurements were done before 45 minutes after treating follicles with carbenoxolone. Webb et al. (2002) showed that FSH stimulation of the cumulus cells causes a transient increase in cAMP in the oocyte (Webb et al. 2002b). However, this is a delayed response, not consistent with cAMP transfer through gap junctions. Finally, Li et al. (2012) demonstrated a relatively small increase in oocyte cAMP levels in response to treatment of cumulus-enclosed mouse oocytes with FSH, but the cAMP levels did not equilibrate in cumulus cells and oocytes, which is inconsistent with cAMP diffusion through gap junctions (Li, Mao, Xia 2012). In any case, any cAMP increase that might occur in the oocyte in response to FSH stimulation of the cumulus cells did not affect CDK1 expression in the oocyte (Fig. 3.1F). Consistent with this conclusion, treating isolated oocytes obtained from 140-180 µm follicles with dbcAMP for 20 hrs did not stimulate expression of CDK1 (Fig. 3.3B), indicating that cAMP does not acutely regulate CDK1 expression.
Figure 3.3. CDK1 levels are not acutely regulated by FSH or cAMP in oocytes from preantral follicles. For (A), oocytes from 140-180 μm diameter follicles were cultured for 20 hrs in medium with or without 30 ng/ml FSH and the blot was run after removing oocytes from follicles. For (B), isolated oocytes from 140-180 μm diameter follicles were treated for 20 hrs with 250 μM dbcAMP before running western blots. Significance was determined by Student’s t-test; P<0.05 was considered significant. Bars are mean ± SEM.

*The constitutively active G-protein coupled receptor, Gpr3, is expressed in oocytes early in follicle development.*

Once meiotic competence has been achieved, arrest at the prophase stage becomes dependent on cAMP that is produced in oocytes through the constitutive activity of the Gs-coupled receptor, GPR3 (Hinckley et al. 2005; Ledent et al. 2005; Mehlmann et al. 2004; Mehlmann 2005b). There is some evidence that GPR3 may be active in oocytes within preantral follicles (Freudzon et al. 2005; Hinckley et al. 2005; Vaccari et al. 2008), suggesting that the cAMP-producing system is functional before it is needed to maintain meiotic arrest; however, it has not been determined when Gpr3 expression begins. cAMP may also promote the acquisition of meiotic competence in mouse oocytes (Chesnel, Wigglesworth, Eppig 1994). We examined
when the Gpr3 promoter becomes active during follicle development using a Gpr3 knockout mouse in which the single exon of the Gpr3 gene was replaced with a lacZ cassette under the control of the Gpr3 promoter (Mehlmann et al. 2004; Tanaka et al. 2009). When the Gpr3 promoter is active, the lacZ cassette stimulates the production of β-galactosidase, the activity of which can be detected by staining with 6-chloro-3-indoly l-β-D-galactopyranoside (salmon-gal), a more sensitive substrate than the commonly used X-gal (Sundararajan et al. 2012). We stained whole ovaries and isolated follicles from 17-23-day-old Gpr3−/− mice and found that β-galactosidase was present in virtually all oocytes from preantral follicles, indicating active transcription of Gpr3 (Fig. 3.4A). β-galactosidase was expressed only within the oocytes rather than the follicle cells, and staining was restricted to the GV, possibly due to a nuclear localization signal commonly associated with β-galactosidase. As expected, Gpr3+/+ oocytes, which did not contain the lacZ cassette, did not stain when incubated with salmon-gal (Fig. 3.4B).
Figure 3.4. *Gpr3* mRNA is present in oocytes of all follicle sizes in 3-week-old mice. A,B) Salmon-gal staining in whole ovaries or in isolated follicles obtained from 3-week-old *Gpr3*\(^{-/-}\) (A) or *Gpr3*\(^{+/+}\) (B) mice. The inset in (A) shows staining within the GV of an isolated oocyte.

Because essentially all the oocytes in follicles from 3-week-old *Gpr3*\(^{-/-}\) mice stained positive for β-galactosidase, we examined β-galactosidase expression in 11-day-old *Gpr3*\(^{-/-}\) mice to determine when the *Gpr3* promoter becomes active. As in the older mice, almost all oocytes within small, preantral follicles stained, although we occasionally found an oocyte from the smallest follicles that did not stain (Fig. 3.5A). We then tested even younger (8-day-old) *Gpr3*\(^{-/-}\) mice and found that 61% (19/31) of oocytes within follicles from these mice were also positive for β-galactosidase (Fig. 3.5B). We could not detect positive staining in ovaries from newborn (4-day-old) mice using salmon-gal (not shown). To confirm the absence of detectable *Gpr3* mRNA in newborns, we examined *Gpr3* expression in the ovaries of 2-day-old mice using RT-PCR. As shown in Fig. 3.5C, ovaries from four separate mice did not express detectable *Gpr3*, whereas 2 oocyte equivalents from a 25-day-old mouse showed a strong band positive for *Gpr3*. The newborn ovaries had similar or higher amounts of *Gapdh* compared to the oocytes from the 25-day-old mouse. Because newborn ovaries only contain primordial follicles consisting of an oocyte surrounded by a single layer of squamous epithelial cells, the majority of the ovary is composed of oocytes rather than somatic cells. Therefore, the lack of *Gpr3* signal in the newborn ovaries strongly suggests that *Gpr3* is not expressed in mouse oocytes at this age.
Figure 3.5. Oocytes in small follicles and newborn ovaries lack Gpr3 mRNA expression.

A,B) Salmon-gal staining in follicles from 11-day-old (A) and 8-day-old (B) Gpr3<sup>-/-</sup> mice. C) PCR amplifying Gpr3 in ovaries from four different 2-day-old mice (lanes 1-4). 2 oocytes from a 25-day-old mouse (+) were used as a positive control for Gpr3. Gapdh was used a positive control for the newborn ovary cDNA.

The average diameter of Gpr3-positive oocytes from 8-day-old mice was significantly greater than that of unstained oocytes (36 ± 1 μm S.E.M., vs 27 ± 1 μm). Likewise, follicles containing Gpr3-positive oocytes had significantly larger diameters than Gpr3-negative follicles (53 ± 1 μm vs 45 ± 3 μm). We did not observe any Gpr3-positive oocytes smaller than 31 μm, while 75% (n=12) of the Gpr3-negative oocytes we measured were 30 μm or smaller. Therefore, Gpr3 RNA starts to be expressed in oocytes when they are ~30 μm in diameter. Due to the
unavailability of antibodies that can effectively detect endogenous GPR3 in mouse oocytes, we were unable to examine when GPR3 protein becomes expressed.

This result demonstrates that oocytes express the machinery necessary to generate cAMP within small, growing oocytes. In newborn mouse ovaries, cAMP has been linked to meiotic progression to diplotene as well as the formation of primordial follicles (Wang et al. 2015). This study showed that cAMP levels increase significantly in mouse ovaries just before birth; cAMP controls the disassembly and degradation of synaptonemal complex protein 1 (SYCP1) in the oocyte, thereby permitting the oocyte to reach the dictyate stage of prophase. Inhibiting cAMP production in newborn ovaries causes an increase in SYCP1 and leads to a delay in meiotic prophase as well as a disruption in germline cyst breakdown and primordial follicle formation (Wang et al. 2015). The effects of cAMP at these early stages appear to be due to the activity of adenylate cyclase type 2 in the oocyte, whereas meiotic arrest in meiotically competent oocytes is due to the activity of adenylate cyclase type 3 (Horner et al. 2003). Because we could not detect *Gpr3* in newborn ovaries using salmon-gal staining and PCR, it is unlikely that GPR3 provides the source of cAMP in newborn ovaries. In addition, oocytes from *Gpr3*−/− mice clearly reach the diplotene stage and follicles form normally (this study; (Mehlmann et al. 2004), so it is likely that newborn oocytes produce cAMP through a different system. Our results demonstrate that *Gpr3* is expressed in small, growing oocytes, suggesting that cAMP could be produced before the fully-grown stage, in which meiotic arrest at prophase depends on cAMP.

**GPR3 is active in oocytes from preantral follicles (140-180 μm diameter)**

To confirm that GPR3 is functional in the oocytes within preantral follicles, we used a FRET-based assay to measure free cAMP in oocytes removed from follicles that were 140-180
µm in diameter. We included milrinone in the culture medium to prevent phosphodiesterase 3A (PDE3A) activation during oocyte isolation, thus preventing cAMP hydrolysis. Isolated oocytes from \(Gpr3^{+/+}\) and \(Gpr3^{-/-}\) mice were injected with a sensor (Epac2-camps300) consisting of the cAMP binding domain of EPAC2, fused to CFP and YFP (Lowther, Nikolaev, Mehlmann 2011; Nikolaev et al. 2004; Norris et al. 2009). FRET measurements showed significantly higher CFP/YFP fluorescence ratios in \(Gpr3^{+/+}\) oocytes compared to \(Gpr3^{-/-}\) oocytes (Fig. 3.6A). These ratios corresponded to cAMP concentrations of ~700 nM vs ~300 nM, respectively (Fig. 3.6B). 700 nM cAMP in \(Gpr3^{+/+}\) oocytes is similar to the ~660 nM that was reported in full-grown oocytes within antral follicles (Norris et al. 2009), using the same FRET sensor that we used here. When milrinone was washed out of the culture medium, the CFP/YFP fluorescence ratios decreased and cAMP levels in the \(Gpr3^{+/+}\) oocytes fell to ~100 nM within 30 min (Fig. 3.6). Cyclic AMP levels also fell to ~100 nM in the \(Gpr3^{-/-}\) oocytes (Fig. 3.6B), although this observation was not statistically significant (\(p = 0.05\)). It was somewhat surprising that there was measurable cAMP in the \(Gpr3^{-/-}\) oocytes. This suggests that another mechanism other than GPR3 can contribute to cAMP production in the oocyte. This small amount of cAMP, however, is clearly not sufficient to maintain meiotic arrest, as the depletion of \(Gpr3^{-/-}\) in oocytes renders them incapable of maintaining meiotic arrest (Mehlmann et al. 2004; Mehlmann 2005b). Overall, this result demonstrates that GPR3 is active in oocytes within small follicles, and shows that PDE3A is also present and capable of being activated in these oocytes.
Figure 3.6. GPR3 is active and stimulates cAMP production in oocytes from follicles that are 140-180 μm in diameter. Measurement of cAMP concentrations in Gpr3<sup>−/−</sup> and Gpr3<sup>+/+</sup> oocytes was performed using the Epac2-camps300 FRET sensor. cAMP concentrations were first measured in oocytes from follicles 140-180 μm in diameter in the presence of the PDE3A inhibitor, milrinone, then milrinone was washed out, stimulating cAMP degradation, and cAMP was measured 30 min later. A) Data presented as ratios of CFP/YFP fluorescence. B) Data
presented as cAMP concentration. Bars are mean ± SEM and significance was determined by one-way ANOVA; P<0.05. Bars with different letters are significantly different.

*Fewer oocytes from Gpr3−/− mice are competent to mature than Gpr3+/+ oocytes.*

Two earlier studies showed that cAMP promotes the acquisition of meiotic competence. Both isolated as well as follicle-enclosed small, growing, incompetent oocytes become competent when cultured in the presence of dbcAMP (Carroll, Whittingham, Wood 1991; Chesnel, Wigglesworth, Eppig 1994). As it has been found that oocytes within preantral follicles express *Gpr3* and produce cAMP, our data are consistent with the possibility that GPR3 may contribute to the acquisition of meiotic competence. To examine this, we analyzed the time course of GVBD using *Gpr3*+/+ and *Gpr3*−/− oocytes removed from follicles of different sizes, as in the experiments described for Fig. 3.1. Fig. 3.7 shows that *Gpr3*−/− oocytes took longer to undergo GVBD, and fewer completed GVBD. The difference was particularly striking in oocytes retrieved from early antral follicles, the stage at which oocytes are beginning to acquire meiotic competence (Chesnel, Wigglesworth, Eppig 1994; Mehlmann et al. 2004). This result shows that, although *Gpr3*−/− oocytes eventually acquire the ability to undergo GVBD, they do so at a slower rate than their *Gpr3*+/+ counterparts.
Figure 3.7. Oocytes deficient in Gpr3 are slower to achieve meiotic competence than Gpr3+/+ oocytes. Time course of GVBD in Gpr3−/− and Gpr3+/+ oocytes removed from follicles of defined sizes. Gpr3+/+ data are the same as in Figure 3.1. Significance was determined using two-way analysis of variance after arc-sin transformation to normalize data. (P<0.05).

That Gpr3−/− oocytes undergo GVBD at a slower rate than Gpr3+/+ oocytes is in agreement with results published by Ledent et al. (2005), who showed that fewer oocytes from 3.5-week-old and 6-month old Gpr3−/− mice undergo GVBD and first polar body formation than Gpr3+/+ oocytes. The higher percentage of Gpr3−/− oocytes from ≥290 µm follicles that underwent GVBD within 5 hrs in our study is likely due to the fact that Ledent et al. (2005) did not distinguish antral follicle sizes; rather, they obtained oocytes from all follicles ≥190 µm in diameter (Ledent et al. 2005). It should also be noted that we saw ~20% GVBD at the time of isolation of Gpr3−/− oocytes from antral follicles. This value is much lower than the ~85-90% GVBD rate observed previously in Gpr3−/− antral follicles (Mehlmann et al. 2004), and also lower than the ~50% GVBD observed by Ledent et al. (2005). The difference could be due to hormonal priming; Mehlmann et al. (2004) and Ledent et al. (2005) primed mice with PMSG prior to collecting ovaries or isolating follicles, which yielded follicles ≥350 µm in diameter, whereas here we used follicles from unprimed prepubertal mice that generally did not exceed 320 µm in diameter, so follicle size could be a factor in our results. It is also possible that priming could cause the synthesis of other proteins that are important for meiotic competence. In addition, previous studies counted all antral follicles, including follicles that may have been atretic, whereas here we isolated only follicles that were clear and presumably not atretic. It might be expected that oocytes within atretic follicles are either GVBD or degenerated, which could be
reflected in the higher percentage of GVBD observed in the previous studies. Our results demonstrate that $Gpr3^{-/-}$ oocytes undergo GVBD at a slower rate even within follicles that are apparently healthy and viable.

CDK1 levels increase in oocytes from $Gpr3^{-/-}$ oocytes but the levels are lower than in $Gpr3^{+/+}$ oocytes.

To examine if GPR3 activity could be associated with CDK1 levels, we isolated follicles of various sizes from ovaries of $Gpr3^{-/-}$ mice and grouped them according to size, as above. We removed oocytes from these follicles and compared the amount of CDK1 in oocytes using western blot. We found that, as in $Gpr3^{+/+}$ oocytes, the amount of CDK1 increased during oocyte growth in $Gpr3^{-/-}$ oocytes (Fig. 3.8A,B). However, the amount of CDK1 was significantly lower in $Gpr3^{-/-}$ oocytes in the 140-180 μm and 220-250 μm groups. This difference was not due to differences in total protein among the $Gpr3^{+/+}$ and $Gpr3^{-/-}$ groups, as probing blots for the highly expressed IP₃ receptor showed no differences (Fig. 3.4C).
Figure 3.8. CDK1 protein expression is altered in Gpr3<sup>−/−</sup> oocytes of preantral and early antral follicles. A) Western blot for CDK1 in Gpr3<sup>−/−</sup> oocytes removed from follicles of defined diameters. B) The amount of CDK1 is significantly lower in oocytes retrieved from preantral and early antral follicles of Gpr3<sup>−/−</sup> mice compared with Gpr3<sup>+/+</sup> mice. The Gpr3<sup>+/+</sup> graph shown here is the same as in Figure 3.2. CDK1 amounts for Gpr3<sup>+/+</sup> vs Gpr3<sup>−/−</sup> were compared by <i>t</i>-tests with the Holm-Sidak correction for multiple comparisons. *=P<0.03. C) IP<sub>3</sub> receptor expression, as determined by western blot, in oocytes from both genotypes and removed from follicles of defined diameters was used as a loading control.
It is not clear how the expression of GPR3 leads to a higher amount of CDK1 in $Gpr^{3+/+}$ oocytes compared to $Gp3^{-/-}$ oocytes. The simplest explanation is that $Gpr^{3+/+}$ oocytes contain more mRNA encoding CDK1 than $Gpr^{3/-}$ oocytes. However, real-time PCR using oocytes retrieved from follicles of different sizes and from both genotypes showed that the levels of $Cdk1$ RNA were similar in all groups (Fig. 3.9), indicating that increased transcription is unlikely to account for higher amounts of CDK1 protein in the $Gpr^{3+/+}$ oocytes. Our results suggest that the increase in the amount of CDK1 protein during oocyte maturation occurs at the translational level. There is at least one example of cAMP increasing translation independently of stimulating gene transcription. Synthesis of tyrosine hydroxylase (TH) protein is stimulated by cAMP in midbrain dopaminergic neurons without an increase in mRNA levels (Chen et al. 2008). In these cells, cAMP treatment increases the association of TH mRNA with polysomes through proteins that bind to cis-acting sequences within the 3’UTR of the TH RNA (Chen et al. 2008; Xu, Sterling, Tank 2009). It is possible that cAMP has a similar stimulatory effect in oocytes, but this needs to be further examined. It is also possible that cAMP influences the expression or activity of an intermediate protein that can regulate CDK1 translation.
Figure 3.9. *Cdk1* mRNA levels do not change between *Gpr3*+/+ and *Gpr3*−/− oocytes removed from follicles of defined diameters. RT-PCR was performed and all data was normalized to *Rpl19*.

**Conclusions**

This study shows that CDK1 levels increase in mouse oocytes as they progress from meiotically incompetent to meiotically competent stages, as follicles transition between preantral (140-180 µm in diameter) and early antral (220-250 µm in diameter) stages. *Gpr3* is expressed and active early in oocyte development, prior to the acquisition of meiotic competence and subsequent dependence on cAMP for meiotic arrest. The presence and activity of GPR3 protein are likely to precede the synthesis of CDK1, but are not required for it. Our results suggest that the production of cAMP through GPR3 signaling early on in development potentiates the acquisition of meiotic competence in oocytes. In support of this, fewer *Gpr3*−/− oocytes within 140-180 µm diameter follicles underwent GVBD than oocytes containing an intact GPR3 system. Lower amounts of CDK1 in *Gpr3*−/− oocytes could contribute to this decrease in meiotic competence observed in *Gpr3*−/− oocytes. Overall, these results indicate that GPR3 has a role in the acquisition of oocyte meiotic competence, but the mechanism through which it affects meiotic competence needs further study.

**Acknowledgments**

We thank Viacheslav Nikolaev for providing the EPAC2-camps300 protein, and Laurinda Jaffe for helpful discussions. This work was supported by the University of Connecticut Health Center Research Advisory Council and The Fund for Science.
-CHAPTER 4-

Concluding Remarks and Future Directions
The research presented in this dissertation highlights key meiotic events that lead to proper acquisition of meiotic competence in relation to follicle development.

**The Switch from cAMP-independent to cAMP-dependent Arrest of Meiotic Prophase is Associated with Coordinated GPR3 and CDK1 Expression in Mouse Oocytes**

**Conclusions**

Prior to being fully-grown, the incompetent growing oocyte is synthesizing and accumulating the necessary macromolecules and organelles termed the acquisition of meiotic competence (Sorensen and Wassarman 1976a). Proper coordination and presence of different factors during oocyte growth ensures that a high-quality oocyte will develop into a healthy embryo. This study set out to systematically analyze the when oocytes become meiotically competent, which was found to be around the time the mouse follicle is approximately 220 μm in diameter which coincides with the beginning stages of the antrum forming. Furthermore, it was shown that oocyte CDK1 levels significantly increased as the follicle developed from a preantral to antral follicle, suggesting this cell cycle protein to be a key component in the acquisition of meiotic competence.

Since preantral follicles could be responsive to FSH (Hardy et al. 2017), and since it was suggested that FSH can cause an increase in cAMP levels in the oocyte via gap junctions (Webb et al. 2002b), both FSH and dbcAMP were incubated with oocytes from preantral follicles to see if these components could induce the expression CDK1 resulting in a more competent oocyte. Neither FSH or dbcAMP caused any changes in CDK1 expression, indicating that these signaling molecules do not acutely regulate oocyte CDK1 levels.
Previous studies showed that GPR3 may be expressed in oocytes from preantral follicles (Freudzon et al. 2005; Hinckley et al. 2005; Vaccari et al. 2008). However, exactly when GPR3 becomes expressed and if it is active in small incompetent oocytes remains to be determined. By using the β-gal staining method, we showed that Gpr3 becomes expressed in mice between 4- to 8-days old, as no staining was detected in 4-day old ovaries. PCR of ovaries from 2-day-old mice indicated that Gpr3 is not expressed in newborn ovaries, despite evidence that there is cAMP in the oocyte early in development. There may be other mediators present in the oocyte, besides GPR3, that generate cAMP through adenylate cyclase type 2 activation in newborn mice (Wang et al. 2015). Analyzing β-gal staining in 8-day-old mice revealed that Gpr3 transcription starts to become active when the oocyte reaches approximately 30μm in diameter. This demonstrates that the machinery to generate cAMP is expressed early in oocyte development and is present prior to the cAMP-dependent meiotic arrest in fully-grown oocytes.

Knowing that Gpr3 is expressed early in oocyte development, we then investigated if the GPR3 protein was active in oocytes from preantral follicles. Using the FRET-based assay, we showed that Gpr3+/+ oocytes had normal cAMP levels around 700nM that fell to 100nM when milrinone was washed out of culture causing PDE3A activation. The conclusions from this are two-fold: it shows first that GPR3 is present and active in small oocytes; and second, that PDE3A can be activated during this oocyte stage as well. Additionally, we showed that Gpr3−/− had significantly lower cAMP levels, around 300nM, compared to Gpr3+/+, indicating that GPR3 is the receptor responsible for generating cAMP at this oocyte developmental stage.

Earlier studies showed that cAMP can promote the acquisition of meiotic competence (Carroll, Whittingham, Wood 1991; Chesnel, Wigglesworth, Eppig 1994). Knowing that GPR3 is a receptor responsible for generating cAMP in incompetent oocytes, we looked for a possible
correlation between GPR3 expression and meiotic competence. We found that \( Gpr3^{-/-} \) oocytes were less competent than \( Gpr3^{+/+} \) oocytes, especially at the follicle developmental stage when oocytes start to become meiotically competent. Further, CDK1 levels were significantly decreased in \( Gpr3^{-/-} \) oocytes compared to \( Gpr3^{+/+} \); however, there was still CDK1 present, indicating that the mechanisms stimulating CDK1 expression may be delayed in \( Gpr3^{-/-} \) oocytes. It can be observed that the \( Gpr3^{-/-} \) oocytes from the 220-250\( \mu \text{m} \) follicles had similar CDK1 levels compared to \( Gpr3^{+/+} \) oocytes from the 140-180\( \mu \text{m} \) follicles. Along with this finding, the GVBD time course data is very similar between \( Gpr3^{-/-} \) oocytes from the 220-250\( \mu \text{m} \) follicles and \( Gpr3^{+/+} \) oocytes from the 140-180\( \mu \text{m} \) follicles. This indicates that the amount of CDK1 strongly correlates with the ability to resume meiosis. In all other follicle sizes, the RT-PCR results suggests that this difference in CDK1 expression between \( Gpr3^{+/+} \) and \( Gpr3^{-/-} \) oocytes is regulated at the translational level.

Lastly, during this study, \( Gpr3^{+/+} \) and \( Gpr3^{-/-} \) oocytes of defined follicle sizes were analyzed for \( G_{s} \) expression by western blot. As reported previously, \( G_{s} \) levels were significantly higher in the \( Gpr3^{-/-} \) oocytes from all follicle size groups (Fig. 4.1) (Ledent et al. 2005). Higher \( G_{s} \) levels in \( Gpr3^{-/-} \) oocytes could possibly explain the lower meiotic competence in these oocytes. However, this is unlikely to be the case, because in the absence of GPR3, all \( G_{s} \) resides in the plasma membrane, indicating that it is inactive (Ledent et al. 2005). When \( G_{s} \) is activated by GPR3 it translocates into the cytoplasm and is likely to be degraded, reflecting lower \( G_{s} \) levels in the \( Gpr3^{+/+} \) oocytes (Ledent et al. 2005). \( G_{s} \) levels did not increase in the oocytes from follicles of increasing size (Fig 4.1). This indicates that only genotype seems to alter \( G_{s} \) expression, and \( G_{s} \) is not a marker of the acquisition of oocyte meiotic competence.
Figure 4.1. $G_s$ increases in expression in $Gpr3^{-/-}$ oocytes compared to $Gpr3^{+/+}$ oocytes at all follicle sizes. Western blot for $G_s$ in oocytes isolated from follicles of defined diameters, from the same ovary.

**Future Directions**

*What additional markers can indicate $Gpr3^{-/-}$ oocytes are less meiotically competent compared to $Gpr3^{+/+}$ oocytes?*

The germinal vesicle break down (GVBD) time course is one way to analyze the ability of the oocyte to be able to resume meiosis and to be able to make conclusions about oocyte meiotic competence. However, there are other ways to test and support the conclusion that $Gpr3$-deficient oocytes are less competent compared to $Gpr3^{+/+}$. Staining oocytes with Hoechst or labeling for anti-tubulin can give insight about the structure and localization of chromatin and microtubules. It was found that incompetent oocytes have more diffused nuclear chromatin, unlike competent oocytes with condensed chromatin around the nucleous (Wickramasinghe, Ebert, Albertini 1991b). Also, microtubules are diminished in the cytoplasm of competent oocytes (Wickramasinghe, Ebert, Albertini 1991b). Lastly, higher levels of phosphorylation on centrosomes are in competent oocytes compared to incompetent oocytes (Wickramasinghe and
Albertini 1992). These nuclear and cytoplasmic markers can be applied to Gpr3⁻/⁻ oocytes to analyze their state of meiotic competence.

Are other cell cycle proteins, in addition to CDK1, differentially expressed in Gpr3⁻/⁻ oocytes?

By specifically using the previous approach of systematically dissecting follicles of determined sizes, we can further study oocytes from these follicles for other cell cycle proteins between Gpr3⁺/⁺ and Gpr3⁻/⁻ oocytes. As previously mentioned, prior studies have indicated a change in expression of WEE1B and CDC25B; however, they used mice of different ages and didn’t correlate this expression to the follicle size, making these proteins ideal to analyze in regards to follicle development. Also, phosphorylation status of CDK1 and Cyclin B have been indicated to change with regard to achieving meiotic competence, and can be further studied to better understand when these events are taking place with respect to follicle development, as well as to understand when kinases become active to phosphorylate these proteins.

How are reduced CDK1 levels in Gpr3⁻/⁻ oocytes resulting in a less competent oocyte?

From the data, it is evident that CDK1 is significantly reduced in Gpr3⁻/⁻ oocytes, leading it to be hypothesized that this is the cause for the slower rate of meiotic resumption compared to Gpr⁺/⁺. It has been shown that an oocyte-specific deletion of Cdk1 results in fully-grown oocytes permanently arrested and unable to resume meiosis, contributing to mouse infertility (Adhikari et al. 2012). However, there are several downstream targets of CDK1 that can also possibly be differentially regulated in the absence of GPR3, contributing to an incompetent oocyte. For instance, CDK1 phosphorylates A-type lamins (lamin A/C) on Ser22 and Ser392 to promote the disassembly of the nuclear lamina during meiotic resumption (Heald and McKeon 1990;
In conjunction to phosphorylating lamins, CDK1 also phosphorylates phosphatase PP1 at Thr320 causing it to be inactive (Kwon et al. 1997; Wu et al. 2009). Inactivated PP1 is unable to dephosphorylate lamin A/C, further contributing to a net phosphorylation of nuclear lamins initiated by CDK1 in meiotically competent oocytes (Figure 4.3) (Ceulemans and Bollen 2004).

In the Gpr3−/− oocytes, it is not clear if the lower levels of CDK1 result in lamins not becoming phosphorylated, or perhaps there is not a sufficient amount of CDK1 to phosphorylate and inactivate PP1. Clearly CDK1 can’t sufficiently phosphorylate both lamins and PP1, as this results in GVBD, which is delayed in Gpr3−/− oocytes. To test the effect of reduced CDK1 expression in Gpr3−/− oocytes, we can utilize the newly developed technique involving posttranslational protein degradation called Trim-Away (Clift et al. 2017). This method uses the antibody receptor and ubiquitin ligase, TRIM21 (Figure 4.2) (Clift et al. 2017).

In overview, Gpr3−/− oocytes from early antral follicles will be used, as these had the most striking delay in GVBD compared to Gpr3+/+. The follicle-enclosed oocytes will be microinjected with RNA encoding the TRIM21 protein, which contains a GFP tag to ensure the RNA is translated (Figure 4.2) (Clift et al. 2017). After TRIM21 protein is expressed in the oocytes, antibody against PP1 will be microinjected into the same oocyte allowed for time to be translated. TRIM21 will recognize the antibody to PP1, ubiquitinate PP1, and tag it for degradation through the proteasome pathway (Figure 4.2). These now PP1-deficient Gpr3−/− oocytes will be released from their follicle and analyzed for a change in the rate of GVBD.
Figure 4.2. Schematic of the Trim-Away Technique. TRIM21 is drawn with an antibody receptor component conjugated with a ubiquitin ligase and GFP. Once an antibody against a protein of interest is introduced, TRIM21 will bind to the antibody-protein complex, ubiquitinate the protein, causing it to be endogenously degraded (Clift et al. 2017).

If the rate of GVBD increased, this would suggest that CDK1 was able to phosphorylate lamins, but unable to phosphorylate PP1, resulting in it being active. Degrading PP1 in this case would remove the active PP1, allowing lamins to stay phosphorylated resulting in more GVBD (Figure 4.3). Conversely, if there is a similar rate of GVBD with and without PP1 degradation, this would indicate that CDK1 is unable to phosphorylate lamins, resulting in an oocyte that is less competent to resume meiosis (Figure 4.3).
Figure 4.3. Using Trim-Away to Degrade PP1 in incompetent Gpr3<sup>−/−</sup> Oocytes. It has been shown that CDK1 is downregulated in incompetent Gpr3<sup>−/−</sup> oocytes. By degrading PP1 it can be determined if the CDK1 present in these oocytes is sufficient enough to phosphorylate nuclear lamins, as indicated by GVBD.

We have tested the Trim-Away approach in oocytes to degrade the highly expressed IP<sub>3</sub> receptor. Along with TRIM21 RNA, we microinjected an affinity purified antibody specific for the IP<sub>3</sub> receptor (Runft, Watras, Jaffe 1999), or normal rabbit IgG. As shown in Figure 4.4, the protein was completely degraded by 2 hours after antibody microinjection. This leads us to believe this approach will work to degrade PP1 in oocytes.

Figure 4.4. Depletion of IP<sub>3</sub>R in oocytes using Trim-Away. Oocytes were injected with RNA encoding TRIM21 and were subsequently injected with IgG or IP<sub>3</sub> receptor antibody, and were
collected 2-2.5 hrs after antibody injection. 12 oocyte lysates were run per lane on this Western blot, which was probed with an anti-IP3 receptor antibody. U = uninjected control oocytes; IgG = IgG-injected oocytes; IP3R = IP3 receptor antibody-injected oocytes.

Which genes are upregulated during the acquisition of meiotic competence?

Even though there are a few cell cycle proteins that can promote the acquisition of meiotic competence, it is not completely known if other genes can also be involved in this process. To identify the differential expression of genes during the acquisition of meiotic competence, RNAseq data can be analyzed between incompetent Gpr3+/+ oocytes from preantral follicles and competent Gpr3+/+ oocytes from antral follicles. The difference in gene expression from these two groups of oocytes can indicate those genes that are needed during oocyte growth to become competent. Also, RNAseq can be performed on Gpr3−/− oocytes from the preantral and antral follicle sizes and be compared with the data from Gpr3+/+ oocytes. This would indicate genes that are differential expression dependent on cAMP presence.
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