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THE IMPACT OF ZINC DEFICIENCY DURING OOGENESIS, FOLLICLE ASSEMBLY, AND GROWTH

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ABSTRACT

The ovarian follicle is the fundamental unit of the ovary and of female reproduction in mammals. Each follicle contains one oocyte enclosed in somatic cells and follicle number is determined during fetal development in humans. Growth and development of ovarian follicles (folliculogenesis) is necessary to produce viable gametes as well as ovarian hormones including estrogen and progesterone. Folliculogenesis begins in the fetal ovary and may span several decades of life. Environmental and nutritional factors that affect folliculogenesis have the potential to impact health and fertility, and may be a source of new biotechnological innovation. One such factor, zinc, has previously been found to impact the final stages of follicle development including meiotic division, ovulation, epigenetic modification, fertilization, and embryo development. However, the role of zinc during the early stages of folliculogenesis have not been evaluated. To investigate the zinc requirement during early follicle development, we have employed 2 model species (*C. elegans* and *Mus musculus*) over the course of three separate studies of zinc deficiency. Study 1: We sought to introduce a new model organism to the study of micronutrient-mediated infertility. The roundworm *C. elegans* contains a unique germline in which oocytes are constantly generated in the adult, rather than only during fetal development as in mammals. To test the zinc requirement of this model, we pretreated nematode growth media with the zinc chelator TPEN. *C. elegans* grown on zinc deficient media showed a severe reduction in fertility (fewer progeny produced per hermaphrodite), defects in oocyte development, and disruption of the pachytene-to-diplotene meiotic transition. All effects were rescued by the addition of exogenous zinc to the TPEN treated media. This is the first time that a zinc requirement for early meiotic development has been shown in any model species. Study 2: To test the effect of zinc deficiency on growing follicles, we collected preantral ovarian follicles from 14-day old mice and cultured them for up to 10 days in control, or zinc deficient media.

Zinc restriction altered follicle growth, particularly proliferation of somatic cells surrounding the oocyte. Zinc-deficient follicles also displayed higher rates of apoptosis in somatic cells, impaired paracrine signaling, and a reduced ability of the oocyte to undergo meiotic division – a prerequisite to fertilization. The detrimental effects of zinc chelation were rescued by the addition of exogenous zinc. Study 3: In mammals, the primordial follicle pool is established in the fetal ovary when mitotically derived germ cells irreversibly enter meiosis and become enclosed in somatic pregranulosa cells. Revealing a zinc requirement during this phase of development would be significant, as many pregnant women worldwide are marginally zinc deficient. To test the effects of zinc deficiency of primordial follicle formation, we collected fetal mouse ovaries on embryonic day 16.5 and cultured them in control, zinc deficient, or rescue media for 5 days to evaluate meiotic progression of the oocytes. We also collected newborn mouse ovaries and cultured them for 4 days under the same treatment conditions to evaluate the breakdown of germ cell nests into individual follicles. Zinc deficiency did not affect development in the fetal ovary. There were no changes in meiotic progression, oocyte survival, or gene expression. Zinc deficiency had a larger impact during newborn ovary culture. Zinc deficiency impaired germ cell nest breakdown leading to fewer primordial follicles. Gene expression and follicle activation were also affected by zinc deficiency. These effects were rescued by exogenous zinc. Taken together, these studies demonstrate a conserved zinc requirement for oocyte development beginning at earlier stages of oogenesis that previously observed. If these results translate to humans, impaired follicle development and impaired preantral follicle growth may contribute to infertility as a result of zinc deficiency.

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For Bridget, who makes it all possible

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Chapter 1

Introduction

The ovarian follicle is the fundamental unit of the mammalian ovary and of female reproduction. Each follicle is composed of a single oocyte surrounded by a complement of somatic granulosa cells. In humans, follicles are formed in the fetal ovary and at birth are arranged into quiescent structures known as primordial follicles. Activation, growth, and eventual ovulation of ovarian follicles is required for gamete production as well as the production of ovarian hormones. Oocytes enter into meiosis during fetal development and do not replicate mitotically after birth, meaning the pool of primordial follicles is a non-renewable resource in mammals. Environmental, metabolic, or genetic insults that disrupt follicle formation or growth can have devastating consequences for fertility. Understanding factors that impact follicle development may hold the key to developing new infertility treatments and artificial reproductive techniques.

One factor shown to impact fertility is the essential micronutrient zinc. Proper zinc homeostasis is required for mammalian reproduction in both males and females. New research has revealed a critical role for zinc in oocyte development, particularly during ovulation and fertilization. Zinc deficiency near the time of ovulation impairs meiotic division, fertilization, and embryonic development. The role of zinc during earlier stages of follicle development, such as follicle assembly or growth, have not investigated. Uncovering a zinc requirement for early follicle stages would be significant as a large portion of the global population, as well as the majority of pregnant women may be marginally zinc deficient.

Hypotheses

Given that zinc deficiency has been shown to disrupt fertility, meiotic division, cell survival, and paracrine signaling in large ovulatory follicles, we propose the following broad hypotheses to be tested:

1. The zinc requirement for oogenesis is conserved between mammals and the invertebrate model species *C. elegans*.
2. Zinc deficiency disrupts the growth of preantral ovarian follicles by disrupting paracrine signaling between the oocyte and surrounding somatic cells.
3. Zinc deficiency disrupts meiotic entry in oocytes as well as primordial follicle assembly during the earliest stages of oogenesis.

These hypotheses will be tested with mouse tissues using established *in vitro* culture protocols and the use of zinc chelators. For the first time, the research model *C. elegans* will also be used to study the effect of zinc deficiency during the earliest stages of oogenesis and meiosis.

Chapter 2

Literature Review

The mammalian ovary serves two important and related physiological functions: the production of haploid gametes, and the production of reproductive hormones including estrogen, progesterone, anti-mullerian hormone, activin, and inhibin. These ovarian-derived hormones regulate fertility by exerting endocrine effects at the hypothalamus and pituitary as well as through paracrine action within the ovary. Besides their reproductive function, ovarian hormones impact key physiological functions including growth (*1*), bone turnover (*2, 3*), and metabolism (*4-6*). Both gamete and hormone production require precise coordination of oocytes and somatic cells arranged in structures known as ovarian follicles. Follicle production and development is termed folliculogenesis. Along with pregnancy, folliculogenesis is the fundamental process affecting female fertility and reproduction.

Follicles are classified according to their size and developmental stage (Figure 1-1). Follicle development is a lengthy process, particularly in humans where the reproductive lifespan lasts several decades. The production of an egg requires both paracrine and endocrine signaling and precise coordination of developmental progression between cells. Understanding the factors that regulate folliculogenesis is key to developing treatments and artificial reproductive techniques that can: 1) Preserve fertility – extending the reproductive span and delaying menopause. 2) Enhance fertility – providing new treatment options for infertility, a condition affecting millions of women world-wide and 12% of women in the U.S. (*7*). 3) Control fertility – developing new contraception methods provides women with more control over their reproductive health. Investigating environmental and metabolic signals that impact oocyte development may hold the key to the next biotechnology breakthrough in fertility research.

This chapter will review the process of folliculogenesis in detail, including formation of primordial follicles, activation of dormant follicles, preantral follicle growth, and maturation/ovulation of dominant antral follicles. Species differences between humans and our model species will be highlighted where relevant. Furthermore, this chapter will review the role of zinc in ovarian function and fertility and demonstrate the need for further studies of zinc deficiency during the early stages of folliculogenesis.

Ovarian Follicle Development

Unlike males, mammalian female germ cells do not replicate mitotically in the adult gonad. Since females are born containing the total number of germ cells available for reproduction, germ cell replication and survival in the fetal ovary is vital and highly regulated. This discussion will begin with a description of fetal ovary development in the laboratory mouse before examining differences in human physiology.

Follicle Stage	Description	Timing/Duration	Follicle Diameter
Germ Cell Nest 	Germ cells joined by cytoplasmic bridges after replication. Unorganized pregranulosa cells are present (red).	Formation: Weeks 4-13 of gestation Breakdown: Weeks 17-20 of gestation	Each PGC: ~25µM
Primordial Follicle 	A single dormant oocyte surrounded by squamous granulosa cells.	May remain dormant >40 years	30-40µM
Primary Follicle 	Active and growing follicle surrounded by a single layer of cuboidal granulosa cells.	Activation: Unknown duration Primary to Secondary: 120d	60-100µM
Secondary Follicle 	Multiple layers of granulosa cells surround oocyte. Theca cells (blue) organize outside the basal lamina.	70d to reach small antral stage	120-400µM
Small Antral Follicle 	Follicles >2mm may be recruited into the cohort of growing antral follicles. The cells are FSH-dependent for growth and survival.	14 days	0.4-5mm
Ovulatory Follicle 	Largest antral follicle. LH surge will trigger ovulation. Mural (green) and cumulus (blue) granulosa cells are present.	Ovulation occurs 36h after the LH surge	16-20mm

Figure 2-1: Classification and Morphology of Human Ovarian Follicles

Ovarian follicles are classified according to their size and morphology. Primordial follicle formation occurs in the fetal ovary. After activation of a primordial follicle, it takes approximately 300 days to reach the ovulatory stage. Precise cell signaling and development is required for folliculogenesis. Only 1% of activated follicles will become large ovulatory follicles. Follicle descriptions from Mcgee and Hsueh (*8*) and Erickson (*9*).

Primordial Follicle Assembly

The gestational period of a mouse is 20-22 days. By convention, the morning after mating is designated 0.5 days post coitum (dpc) or embryonic day 0.5. Primordial germ cells appear in the mouse on embryonic day 7 (E7), derived from epiblast cells at the base of the allantois (*10*). These cells remain outside the epiblast in the extraembryonic membranes until appropriate signals induce migration to the genital ridge and colonization of the nascent gonad on E10.5 followed by mitotic expansion until approximately E13.5 (*11*). At this stage, the germ cells are found in clusters or nests comprised of mitotically derived cells connected by cytoplasmic bridges as a result of incomplete cytokinesis (*12*). Similar germ cell arrangements have been examined in drosophila where "germ cell cysts" contain exactly 16 germ cells. In drosophila, only one germ cell from each cyst will survive, with the remaining 15 acting as nurse cells that furnish cytoplasm and organelles to the surviving cell (*13*). Recent advances in mammalian research have demonstrated that a similar phenomenon occurs in mouse germ cell nests with some oocytes receiving organelles and cytoplasmic factors from connected germ cells. The cells which donate their cellular reserves are more likely to express pro-apoptotic markers, indicating they are slated for cell death (*14*). On embryonic day 13.5, the mouse ovary contains its maximum number of female germ cells, approximately 15,000 (*15*). Only one third of these cells will survive entry into meiosis and germ cell nest breakdown to become true oocytes contained within ovarian follicles (*16*).

A hallmark of mammalian oocytes is the prolonged duration of meiosis. Primordial germ cells cease replication and enter meiosis while still in germ cell nests, and will not complete the process until egg activation at fertilization. Meiosis, or reductive division consists of one round of chromosomal replication, followed by two rounds of division to produce a haploid genetic complement. Meiosis also involves recombination between homologous chromosomes after

replication – yielding a unique genetic sequence for each gamete. Retinoic acid arising from the nearby mesonephros is the signal for female primordial germ cells to enter meiosis, at which point they are referred to as oocytes. On E13.5, retinoic acid signaling leads to induction of the meiotic factor Stra8 (stimulated by retinoic acid 8) (*17*) which initiates premeiotic DNA replication and is required for subsequent chromosomal pairing and recombination (*18*). Genes responsible for meiosis and recombination are upregulated soon after Stra8, including synaptonemal complex proteins (Scp-1 and Scp-3) (*19*) which connect homologous proteins during the pachytene stage, Spo11 which initiates double strand breaks , and DNA meiotic recombinase (Dmc-1) which generates crossing over between chromosomes during DNA repair (*20*). Oocytes progress through the leptotene (chromosomes condense), zygotene (chromosomes align), and pachytene (recombination) stages of meiotic prophase before arresting at diplotene. Meiotic entry proceeds in an anterior-to-posterior wave along the ovary, with the first diplotene oocytes appearing in the fetal ovary on E18.5, and the majority of oocytes reaching diplotene within the next 72 hours (*21*). At diplotene, the oocytes have a replicated chromosomal complement that has undergone recombination. Sister chromatids remain connected at crossing over points. Oocytes will arrest at this stage of meiosis and maintain a suspended cell cycle until ovulation.

In mice, germ cell nests begin to break down at birth. Loss of steroid hormone signaling is most likely responsible for the onset of germ cell nest breakdown (*22*). Separation from the maternal circulation, as well as downregulation of endogenous estrogen production within the fetal ovary (*23*) combine to lower hormone signaling in rodent ovaries. Nest breakdown involves both germ cell loss and organization of the remaining oocytes and somatic cells into primordial ovarian follicles. Primordial follicles consist of one oocyte surrounded by a single layer of flattened squamous somatic cells. The primordial follicle is a dormant phase of oocyte development in which the oocyte can survive for many months in mice and many decades in

humans. The onset of nest breakdown in humans is a key species difference between humans and murine model species. In humans, nest breakdown and meiotic entry occur nearly simultaneously during the second trimester of pregnancy, while circulating maternal estrogen levels are quite elevated (*24-26*). Lowered estrogen signaling may still play a role in nest breakdown in humans, either through lowered estrogen receptor expression, or through a reduction in endogenous production by the fetal ovary. However, pregnant non-human primates treated with an aromatase inhibitor gave birth to offspring with ovaries containing fewer primordial follicles and more oocytes in germ cell nests (*27, 28*). This may indicate that estrogen is required for nest breakdown in primates, contrary to the effect found in rodents. Under normal conditions, human ovaries contain oocytes already arranged into primordial follicles at birth.

Nest breakdown requires extensive changes in cell signaling including cell-death pathways, upregulation of growth factors, and control of other signaling molecules, particularly TGFβ family members (reviewed in (*29*)). The use of mouse knockout strains and *in vitro* culture assays has provided the field with a tremendous amount of information regarding the molecular mechanisms governing nest breakdown. Nest breakdown involves the programmed cell death of two thirds of oocytes in the mouse ovary (*16*), coupled with invasion of the oocyte nests by somatic cells. Germ cell death appears to be regulated by the BCL family of apoptosis regulators. The balance of pro-apoptotic (BCL2) and anti-apoptotic (BAX) family members, as well as regulators of autophagy seem to determine germ cell survival (*30, 31*). *Bcl2* knockout mice have fewer oocytes in the adult ovary, indicating a role in germ cell survival (*32*), however the anti-apoptotic BCL2 family member MCL1 appears to play a more significant role in promoting oocyte survival during nest breakdown (*30*). Conversely, *Bax* knockouts retain more oocytes during the perinatal period and though adulthood but show disruptions in follicle formation (*33*) and ovulatory deficits leading to infertility (*34*). In addition to apoptosis, germ cells regulate

autophagy to promote cell survival. Mouse mutants lacking the *Atg7* gene show a dramatic reduction in germ cells on post-natal day one (*35*).

Three nuclear transcription factors play key regulatory roles in germ cell nest breakdown – FIGLA, NOBOX, and FOXL2. FIGLA or "factors in the germline α" – is a germ-cell specific helix loop transcription factor. FIGLA regulates the transcription of important oocyte development genes including the zona pellucida proteins that form the outer protective layer of the oocyte (*36*), and the NALP family of genes which regulate inflammatory responses (*37*). *Figla* knockout mice do not form primordial follicles and rapidly lose oocytes after birth, leading to total infertility (*36*). NOBOX or "newborn ovary homeobox encoding gene" is another oocyte specific transcription factor which upregulates genes required for folliculogenesis. *Nobox* null mice display delayed nest breakdown, few primordial follicles, and no evidence of follicle progression (*38*). Oocyte numbers also decline rapidly after birth, leading to a depleted ovarian reserve within 14 days (*38*). Unlike *Figla* and *Nobox*, *Foxl2* expression is specific to somatic cells. *Foxl2* mutant ovaries contain germ cell nests that have not broken down, indicating that this transcription factor is necessary for somatic cell invasion of germ cell nests (*39*).

Other signaling molecules and growth factors foster communication between oocytes and somatic cells during primordial follicle formation. Notch signaling promotes primordial follicle formation through the Notch2 receptor on pregranulosa somatic cells. Disruption of Notch signaling promotes incomplete cyst breakdown and fewer primordial follicles in rodents (*40*). Kit-ligand (KITL) originates from the somatic cells and plays a role in almost all aspects of folliculogenesis (*41*). KITL promotes follicle formation *in vitro* (*42*), though its role *in vivo* is unclear. The transforming growth factor β (TGFβ) superfamily of proteins are key components of oocyte-somatic cell interactions (*43*). Four superfamily members are of particular relevance during germ cell nest breakdown: GDF9, BMP15, Activin, and AMH. Growth differentiation factor 9 (GDF9) and Bone Morphogenic Protein 15 (BMP15) are oocyte specific factors which

exert control over somatic cells in the ovary. *Gdf9* and *Bmp15* knockout mutants display an increased number of multiple oocyte follicles (MOFs) – follicles in which nest breakdown was not complete – leading to multiple enclosed oocytes (*44*). Activin is a TGFβ superfamily member expressed by both oocytes and somatic cells (*45*). Mouse ovaries treated with exogenous activin increase their number of primordial follicles (*45*), while overexpression of the activin antagonist Inhibin results in a higher rate of MOFs (*46*). The final TGFβ superfamily member of interest is Anti-Müllerian hormone (AMH) secreted by stromal tissue during nest breakdown, and later by granulosa cells in growing secondary follicles. AMH inhibits nest breakdown during primordial follicle formation. Cultured ovaries in the presence of AMH display fewer assembled follicles and a higher number of total oocytes, indicating that AMH acts to preserve oocytes within germ cell nests (*47*).

Meiotic entry and nest breakdown result in the initial pool of primordial follicles. This reserve contains the total population of gametes available during the female reproductive lifespan (*48*). (The existence of de novo follicle formation in the adult ovary from germline stem cells has been postulated and is hotly debated within the field (*25, 49-51*)) In humans, the fetal ovary contains a maximum of approximately $5x10^6$ germ cells at 20 weeks of gestation, but an average of only $3x10^5$ primordial follicles a few days after birth (*24*). The size of the initial follicle pool, as well as its rate of depletion are the determining factors in reproductive longevity and age at menopause (*8, 24*). Environmental exposure to endocrine disruptors including synthetic estrogens have been shown to disrupt primordial follicle formation and affect later fertility (*52, 53*). Environmental or dietary factors which impact follicle formation are therefore incredibly relevant to human health and fertility.

Primordial Follicle Regulation

After formation of the follicular reserve, three possible fates await primordial follicles: activation, atresia, or continued dormancy (*54*). A large number of primordial follicles (by some estimates, up to 155 per day in the mouse) undergo atresia prior to activation. The mechanism of cell death appears not to be classical apoptotic signaling, and more likely involves autophagy (*55*). Primordial follicles that survive may be "activated" or recruited into the pool of growing follicles that have a chance to be ovulated. The initial stage of primordial follicle activation appears to occur independently of circulating gonadotropins. Two lines of evidence point toward paracrine/autocrine control of follicle activation: primordial follicles activate spontaneously in ovary organ cultures without added gonadotropins (*56*); and primordial follicles do not express the Follicle Stimulating Hormone Receptor (*Fshr*) (*57*). In each primordial follicle, the decision to activate or continue quiescence is determined by the balance of inhibitory and activating signals in the local microenvironment. Activation signaling begins in the primordial follicle granulosa cells, where upregulation of mTORC1 signaling drives the secretion of KIT ligand (*58*). KIT receptor activation in the oocytes induces the phosphatidylinositol 3 kinase (PI3K) signaling pathway which controls the initiation of oocyte growth and survival (*54, 59*). In the absence of KIT signaling, oocyte dormancy is maintained by the phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome ten), a negative regulator of PI3K (*60*). PTEN inhibits the serine/threonine kinase AKT, which in turn phosphorylates the forkhead transcriptional factor FOXO3a. Non-phosphorylated FOXO3a suppresses activation by binding to DNA in the nucleus of dormant primordial follicles, but is relocated to the cytoplasmic compartment during activation as a result of PI3K/AKT signaling and becomes inactive (*58*).

Paracrine factors within the ovary control the rate of follicle activation. Since primordial follicle activation is irreversible (*8*), inhibitory factors that limit the rate of activation are key to

preserving the follicular reserve. One key inhibitory substance is anti-mullerian hormone (*61*), which inhibits both primordial follicle activation and subsequent follicle growth (*62-64*). AMH is produced in the granulosa cells of primary and growing preantral follicles, and acts to inhibit activation of surrounding primordial follicles. AMH thereby serves as to maintain a steady rate of primordial follicle activation, and can be a useful biomarker of ovarian follicle status (*65*).

One final noteworthy aspect of primordial follicle activation is the presence of the initial follicular wave. A subset of primordial follicles activates simultaneously after assembly rather than entering a dormant phase (reviewed in (*66*)). In the mouse, this pool of follicles arises in the medullary region of the ovary and composes the majority of growing follicles prior to the onset of sexual maturity (*67*). The physiological role of these follicles remains under investigation, but endocrine signaling from these follicles likely plays a role in establishing the hypothalamic-pituitary-ovarian feedback axis; while inhibitory paracrine signaling (in the form of AMH) from the growing first-wave follicles prevents premature activation of the dormant follicle pool. The initial wave of activated follicles is conserved in mammals, including humans (*68*), indicating it may play a critical role in fertility. Factors which affect the initial follicular wave, or the activation rate of primordial follicles may have an impact on lifelong fertility.

Preantral Follicle Growth

After activation, follicles begin a long process of growth and development. Activated follicles pass through the primary follicle stage of development, characterized by a single layer of associated granulosa cells, before becoming secondary follicles with multiple layers of surrounding granulosa cells. Preantral follicle growth has the longest duration of any follicular growth phase, and may require up to 300 days in humans (*9*). The longest phase of preantral growth is the primary follicle stage. Human primary follicles require more than 120 days to

advance to secondary follicles, while secondary follicles require only 70 days to reach the antral stage (*8*). Preantral follicle growth is not gonadotropin-dependent, and largely driven by the interaction between the oocyte and somatic cells.

The oocyte plays a dominant role in folliculogenesis after activation through the secretion of paracrine growth factors (*69*). Two of the TGFβ superfamily members involved in primordial follicle formation are also essential for preantral growth. GDF9 and BMP15 are oocyte secreted factors that regulate follicle development by activation of Smad proteins in the surrounding granulosa cells (*70, 71*). Ligand activation of membrane-bound receptor compounds phosphorylates SMAD proteins in the cytoplasm of the cell. pSMADS then form heteromeric complexes with SMAD4 (co-SMAD) and migrate to the nucleus to alter transcription (*72*). Eight Smad proteins have been identified in mammals and can be classified as receptor regulated Smads (R-Smad: Smad-1,-2,-3,-5,-8), common-partner Smad (Co-Smad: Smad4), and inhibitory Smads (I-Smad: Smad-6,-7). GDF9 Phosphorylates Smad2 and Smad3, while BMP15 activates Smad1, Smad5, and Smad8 (*71*); however the most bioactive form of these molecules is the GDF9:BMP15 heterodimer that signals through a BMPR2 receptor (*73*). One crucial role for GDF9 and BMP15 is regulation of KIT-ligand (*Kitl*) expression by the granulosa cells. KITL from the granulosa cells promotes oocyte growth, leading to increased synthesis of GDF9 in enlarged oocytes. GDF9 inhibits *Kitl* expression in granulosa cells – slowing oocyte growth via negative feedback (*74*). Conversely, BMP15 promotes *Kitl* expression, balancing inhibition from GDF9. Through this feedback system, the oocyte regulates its own growth rate through interaction with surrounding granulosa cells (*75*). GDF9 is also a mitogen and necessary for proliferation of granulosa cells, a feature of the primary to secondary follicle transition. *Gdf9* null female mice contain follicles arrested at the primary stage of development, resulting in infertility (*76, 77*).

Preantral follicle development in secondary follicles involves continued oocyte growth and replication of granulosa cells. A new cell type, the theca cells, are recruited from the ovarian stroma and appose the outer layer of granulosa cells, separated by the follicle basement membrane (*78, 79*). Theca cells provide structural integrity to the growing follicle and enhance the rate of follicular growth, possibly by upregulating Insulin-like Growth Factor-1 (*Igf1*) expression in granulosa cells (*80, 81*). Theca cells are the source of androgen production in the ovary, and their addition to the ovarian follicle is necessary for steroid hormone production. According to the two-cell, two-gonadotropin model of steroid synthesis, theca cells express key steroidogenic enzymes in response to luteinizing hormone from the pituitary, resulting in the production of androgens (*82*). These androgens are then aromatized to estrogen by the granulosa cells under the influence of follicle stimulating hormone (*83*). Follicular growth in preantral follicles is not gonadotropin dependent, however it is gonadotropin-*responsive*, in that FSH has been shown to promote the survival and growth of preantral follicles (*84, 85*). The endocrine influence on folliculogenesis becomes vital after the preantral stage.

Antral follicle development

The preantral to antral follicle transition is a key milestone in mammalian follicle development. Follicles develop a fluid filled antrum (*86*) and become dependent on hormonal (FSH) signaling from the pituitary to prevent apoptosis (*87*). *Fsh* knockout mice have ovaries devoid of antral follicles, indicating that FSH is required for development past the preantral stage (*88, 89*). The majority of growing follicles undergo atresia during the preantral to small antral follicle transition (*90*). In the antral follicle, oocytes complete their growth and gain the ability to resume meiosis. Meiotic competence coincides with chromatin condensation around the nucleolar precursor body and the onset of transcriptional silencing (*91-94*). The granulosa cells continue to

proliferate as the antral follicle grows, and also differentiate into two lineages: mural granulosa cells that line the wall of the follicle, or cumulus granulosa cells near the oocyte. Mural granulosa cells have a primarily endocrine function and are the main site of estrogen synthesis as well as the main targets of circulating gonadotropins LH and FSH. As a consequence of their peripheral follicle location, mural granulosa cells are exposed to the highest levels of FSH from the circulating vasculature. FSH-induced gene transcription drives differentiation to the mural cell phenotype (*95*). In contrast, cumulus cells are located farther from the vasculature (and circulating FSH) and are in close contact with the oocyte. Exposure to oocyte secreted factors induces cumulus cells differentiation and antagonizes FSH signaling. GDF9 from the oocyte inhibits the PKA pathway – a downstream target of FSH binding (*96*), while BMP15 reduces expression of FSH-receptor transcripts in surrounding cells (*97*). This inhibition of FSH signaling, combined with continued SMAD protein activation as described earlier, leads to the cumulus cells phenotype in somatic cells proximate to the oocyte (*98, 99*). Cumulus cells support the growth and metabolism of the oocyte by producing KIT-Ligand, energy metabolites such as pyruvate, and even proteins and mRNA transcripts (*100*). During the preantral to antral transition, Cumulus cells also develop the ability to undergo mucification and expansion in response to ovulation (*101*). The ability to undergo cumulus expansion requires TGFβ signaling from the oocyte and is a determining factor in fertility after ovulation (*102*).

In mammals, selection of a dominant ovulatory follicle is accomplished through both endocrine and paracrine signaling. In humans, approximately 10 FSH-responsive small antral follicles (2-5mm) begin to grow at the onset of each menstrual cycle due to high levels of circulating FSH (*103*). One follicle in this cohort will grow faster than the rest, perhaps due to enhanced FSH sensitivity (*104*). As the fast-growing follicle achieves dominance, it secretes high levels of estrogen and inhibin which downregulate FSH production by the pituitary during the midfollicular phase. The loss of circulating FSH leads to atresia of smaller growing follicles in

the cohort (*105*). The largest follicle also secretes paracrine factors which upregulate vascularization and FSH responsiveness in the immediate environment, ensuring continued FSH mediated growth even as circulating FSH levels decline. IGF-1 is one paracrine substance produced by dominant follicles that has a synergistic effect with FSH (*106, 107*). Upregulation of the LH receptor in mural cells of the dominant follicle promotes survival in a low-FSH environment and prepares the follicle to respond to the ovulatory LH surge (*108*). Rodents and other polyovular species display similar features of antral follicle growth, with the exception that more than one follicle reaches the dominant preovulatory phase.

Oocyte-Granulosa Cell Communication

Understanding the intercellular communication between oocytes and cumulus cells is key to exploring the mechanism of oocyte growth, ovulation, and meiotic resumption. The nature of this bidirectional communication – primarily oocyte secreted TGFβ proteins eliciting phospho-SMAD signaling in surrounding granulosa cells- has been discussed. However, the direct cellular connections between oocytes and granulosa cells play an equally vital role in oocyte development during the later stages of oocyte development. Granulosa cells are connected to the growing oocyte via actin-rich projections that traverse the zona pellucida and contact the oocyte cell membrane (the oolemma) (*109*). These projections are called transzonal projections and terminate in gap junctions (*110*). The connexon is the fundamental unit of a gap junction and is formed by 6 integral membrane proteins called connexins (Cx). Cx37 proteins form the gap junctions between oocytes and granulosa cells. Female Cx37 knockout mice are infertile due to a lack of large dominant follicles and inappropriate luteinization of granulosa cells (*111*).

Through these gap junctions, ions, metabolites, amino acids, and intracellular signaling molecules pass between the granulosa cells and the oocyte. Granulosa cells maintain oocyte

growth and survival, while oocytes promote granulosa cell differentiation and replication (*112*). Oocytes cannot efficiently utilize glucose (*113*), and rely on the transfer of pyruvate and other glycolysis energy substrates from cumulus cells (*114*). Oocytes also lack SLC38a transporters for sodium-coupled neutral amino acids, such as L-alanine; making protein transfer from the cumulus cells a necessity (*115, 116*). After fully grown oocytes gain the ability to resume meiosis in the antral follicle, cumulus cells prevent premature meiotic maturation by furnishing the oocyte with cGMP via gap junctions (Figure 1-2a) (*117*). High levels of cGMP in the oocyte maintain PKA signaling and inhibition of the maturation promoting factor in the oocyte cytoplasm. Removal of cumulus cells from a fully grown oocyte results in spontaneous meiotic resumption in the absence of any other stimuli (*118*). The production of cGMP by cumulus cells is driven by natriuretic peptide C secreted by mural cells into the follicular fluid (*119, 120*). Oocyte derived factors (ODF) regulate cumulus cell morphology in a reciprocal manner. ODFs prevent apoptosis in surrounding cells (*121*), and are necessary for upregulation of expansion related transcripts required by the cumulus oophorus after ovulation (*122*). Microsurgical removal of the oocyte from the cumulus-oocyte-complex (COC) leads to premature luteinization of the cumulus cells (*123*), indicating the oocyte inhibits premature cell progression in the somatic cells. Paracrine and gap junction signaling are key for follicle development, particularly in the antral follicle.

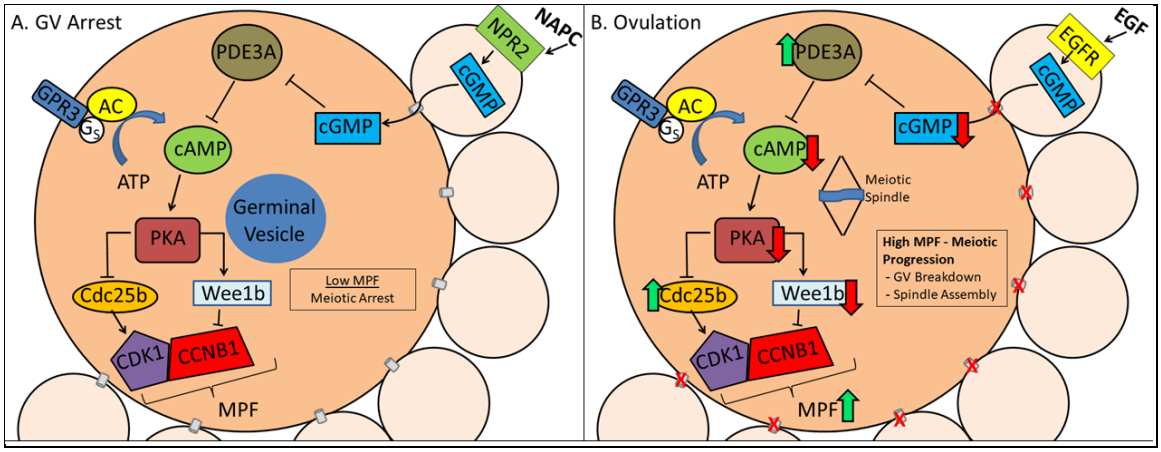

Figure 2-2: Cumulus Cells Inhibit Meiotic Resumption in the Oocyte

In the fully mature oocyte, paracrine communication with cumulus cells is required for control of meiotic progression. (A) Prior to ovulation, secreted Natriuretic Peptide-C (NAPC) from the mural granulosa cells triggers production of cGMP by cumulus cells. cGMP is transferred into the oocyte via gap junctions where it serves to keep cAMP levels high via inhibition of PDE3A. (B) Mural cells release EGF-like peptides in response to the LH surge at ovulation. EGF activation of cumulus cells leads to cumulus cell expansion and closing of gap junctions to the oocyte. Release from meiotic inhibition activate the Maturation Promoting Factor (MPF), leading to germinal vesicle breakdown and meiotic spindle formation.

Ovulation

Just prior to ovulation, the human ovary is characterized by one large dominant antral follicle, which contains a fully mature oocyte. Less than 1% of primordial follicles will survive folliculogenesis to become ovulatory. The oocyte has reached its terminal size (200µM in humans, 85µM in mice) and ceased gene transcription, preparing for meiotic division. The oocyte remains in cell cycle arrest at the diplotene stage of meiosis, inhibited from progression by the inhibitory influence of cGMP from the surrounding cumulus cells (*124*). High levels of estrogen production create a positive feedback loop at both the hypothalamus and the pituitary resulting in a tremendous release of luteinizing hormone (LH) from the pituitary. LH levels are elevated to

10-20 times their basal levels and remain elevated for approximately 36 hours (*125*). The LH receptor is primarily expressed in the stroma, theca and mural granulosa cells in the ovary. The LH surge causes mural cells to release EGF-related peptides. These peptides activate the EGF receptor of cumulus granulosa cells, leading to increased expression and translation of expansion related transcripts *Has2*, *Ptgs2*, *Ptx3*, and *Tnfaip6*. Expansion transcripts are responsible for cumulus cell expansion and the formation of an extra-cellular matrix that encompasses the oocyte (*126, 127*). Simultaneously, gap junctions connecting the oocyte and cumulus cells are disrupted leading to a drop of cGMP levels within the oocyte. The loss of inhibitory regulation by the cumulus cells leads to activation of the maturation promoting factor. High MPF activity signals through multiple pathways including the MOS-MAPK cascade to initiate meiotic resumption (Figure 1-2b).

Meiosis requires two sequential asymmetric divisions which generate a haploid genetic complement while maintaining maternal factors necessary for embryonic development. The first meiotic division is characterized by nuclear membrane (germinal vesicle) break down (GVBD) and formation of the first meiotic spindle. In order to achieve asymmetrical division, the meiotic spindle must migrate to the cell periphery prior to anaphase. Movement of the spindle is achieved by association with a dynamic cytoplasmic actin mesh(*128, 129*). 8-10 hours after GVBD, the oocyte undergoes the first polar body extrusion (PBE). The oocyte immediately enters metaphase of the second meiotic division and forms the M2 meiotic spindle. Oocytes remain arrested at metaphase II until fertilization, and only M2 oocytes can successfully form an embryo.

The LH surge affects not only the oocyte and granulosa cells, but also the structural components of the follicle, eventually leading to follicle rupture. VEGF-mediated vascularization, invasion of leukocytes, basement membrane degradation, and increasing hydrostatic pressure within the follicle, combine to accomplish follicle rupture (*130*). After release of the oocyte and cumulus cells, the remaining theca and granulosa cells form the corpus luteum (CL). High levels

of angiogenesis, hypertrophy, and cellular remodeling are required to form the endocrine structure of the corpus luteum (*131*). The CL produces progesterone under the influence of LH. High progesterone levels are necessary for the uterus to establish and support pregnancy.

If copulation has occurred, ovulated oocytes may encounter sperm in the oviduct. Fertilization generates a transient rise in intracellular Ca2+ levels that initiates "activation" and overcomes the M2 meiotic arrest (*132, 133*). Activation in the oocyte involves the release of zinc containing vesicles from the oocyte, completion of meiosis II, hardening of the zona pellucida to prevent polyspermy, and formation of a haploid pronuclei (*134*). Fusion of the male and female pronuclei leads to formation of the zygote (*135*).

Reproduction in *Caenorhabditis elegans*

The production of female gametes in mammals is a long process, even in rodent models. At the earliest, fully grown cumulus-oocyte complexes can be collected from three-week old mice; and then only by employing an exogenous hormone injection regimen. The study of early folliculogenesis is even more difficult, as nest breakdown and primordial follicle assembly occur in the fetal and newborn ovaries which are difficult tissues to collect and study. A new model species with a short generation time, yet ample access to the early stages of oocyte development would be valuable to the field.

The nematode *C. elegans* is an intriguing candidate for this area of research. *C. elegans* was developed as a research model in the 1960s by Sydney Brenner (*136*), for which he was awarded the Nobel Prize in Physiology in 2002. Dr. Brenner introduced the model with the goal to investigate mechanisms of development and nervous system formation (*137*). The advantages of this research model include a short generation time, genetic tractability, invariable somatic cell number, transparency (allowing microscopy of living specimens), and ease of cultivation. These

features have made *C. elegans* one of the most studied model organisms in the world. The unique reproductive system of the worm first drew our attention to this model (Figure 1-3). The majority of *C. elegans* are hermaphrodites which produce both sperm and oocytes (*138*). Each hermaphrodite contains two U-shaped gonadal arms which connect to a common uterus. During larval development, each gonadal arm produces sperm which are stored in structures called spermatheca at each uterine opening. Each worm produces around 300 sperm before switching to oogenesis during the adult phase of development. Female germ cells divide mitotically at the tip of each gonadal arm before entering meiosis. These meiotic cells pass through the leptotene, zygotene, and pachytene stages of meiosis while developing in a syncytium known as the rachis. At the turn of each gonadal arm, germ cells become membrane enclosed and enter diplotene. High levels of oocyte death are observed during exit from the rachis (*139*). Surviving oocytes then develop in a single-file line through the proximal arm of the gonad before reaching the spermatheca and uterus near midline. The most developed oocyte ovulates through the spermatheca, entering the uterus as a 1-cell diploid zygote. Embryos develop in the uterus before being released at approximately the 24-cell stage.

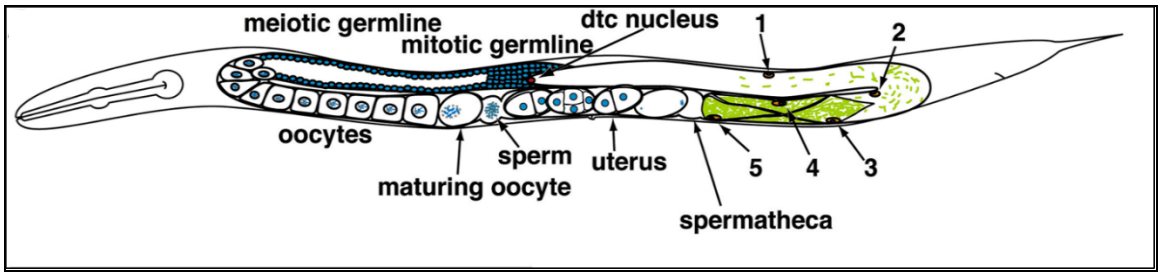

Figure 2-3: *C. elegans* Hermaphroditic Germline

Illustration of the reproductive system in an adult hermaphrodite. The germline is displayed on the left side of the figure (anterior gonadal arm), while somatic sheath cells labelled 1-5 are displayed on the right (posterior gonadal arm). Female germ cells proliferate at the distal tip of each gonadal arm and enter meiosis in the syncytial rachis. Only at the turn of gonad arm do nuclei become membrane enclosed. Oocytes develop in an orderly single-file progression before being fertilized and entering a common uterus near midline. Figure is from Greenstein D. (2005) (*140*); reproduced here under a Creative Commons Attribution License.

The ability to observe female germ cell proliferation and entry into meiosis in a living organism is incredibly valuable to folliculogenesis research. The developmental and signaling parallels between *C. elegans* and mammals are also remarkable, including early development in a syncytium followed by prodigious cell death as oocytes become membrane enclosed (*139*). Homologous chromosomes undergo recombination during meiosis mediated by synaptonemal complex proteins, just as in mammals (*141*). Oocyte maturation in the proximal gonadal arm is also regulated by increases in MAPK activity (*140*), similar to the effect seen in mouse oocytes (*142, 143*). One significant difference in reproductive signaling is hormonal control of oocyte development and ovulation. In *C. elegans* the secreted sperm cytoskeletal protein MSP-1 acts as a hormone on both oocytes and somatic cells in the germline (*144*). The presence of sperm-derived proteins during oogenesis is obviously unique to a hermaphroditic germline, but it is worth noting that MSP-1 signals via the PKA pathway to remove the inhibition of oocyte maturation via Map-kinase (*145*). This signaling network involves MSP-1 action on both oocytes and somatic cells. This control of oocyte cell progression is very similar to the inhibition of MPF in mammalian oocytes, which is removed via PKA signaling during ovulation and involves coordination of signaling between the oocyte and cumulus cells (*146*).

The regulation of zinc transport in *C. elegans* has been investigated (*147, 148*), and zinc deficiency has been shown to elicit dramatic changes in worm physiology and lifespan (*149*). The effect of zinc deficiency on reproduction in *C. elegans* had not been investigated prior to my thesis work, though research in this area has recently gained momentum (*150, 151*). Evaluating the role of zinc in *C. elegans* reproduction may provide novel insights into oogenesis and provide a novel experimental paradigm for studies of nutrient deficiency and fertility.

Zinc in Biology and Reproduction

Nutrition and micronutrient status have been shown to affect folliculogenesis in humans and animals. Starvation interferes with germ cell nest breakdown in mice while upregulating germ cell apoptosis (*152*). Follicle activation is also reduced by nutrient restriction in cell culture – characterized by overexpression of *Foxo3* and fewer primary follicles in cultured ovaries (*153*). Specific micronutrient deficiencies can also play a role in fertility loss. Proper folate metabolism in the oocyte is required for epigenetic modification of the oocyte and embryonic DNA (*154*), and is necessary for proper neural tube closure in the developing embryo (*155*). Vitamin D reduces AMH signaling and reduces pSMAD 1/5/8 in the nucleus of cultured granulosa cells (*156*). High levels of iron are required for granulosa cell proliferation (*157*), yet excess iron in the aged ovary may generate reactive oxygen species that contribute to the age-related fertility decline (*158*). Zinc, the focus of research in our lab, plays a critical role in regulating reproduction and oocyte development. This section will review both the physiological function and control of zinc, before examining the role of zinc in folliculogenesis.

Zinc Biology

Even among essential micronutrients, zinc plays an outsized role in physiology and reproduction. Zinc is the second most abundant trace mineral after iron, and the human body contains approximately 2 grams of total zinc (*159*). A human dietary requirement for zinc was identified in 1961 (*160*). Since that discovery, zinc has been shown to play an integral role in human physiology as a controlling factor for growth, wound healing, immune function, apoptosis, and reproduction among others (reviewed in (*161*)). Zinc can serve a structural, catalytic, or regulatory role within the cell (*162, 163*). Over 300 enzymes require zinc catalysis (*164*), wherein

zinc acts as a Lewis acid to accept a pair of electrons during the catalyzed reaction. Zinc also plays a structural role in the 3-dimensional folding of many proteins. Next generation sequencing and bioinformatic analysis predicts that approximately 3000 proteins contain zinc-binding sites, accounting for ~10% of the human proteome (*165*). The most abundant class of zinc binding proteins are the zinc finger proteins which coordinate the positive zinc ions between negative histidine and cysteine residues in order to achieve their proper form. Zinc finger proteins are involved in gene transcription and DNA replication (*166*). Recently, the regulatory role of zinc in cell fate has received intense scrutiny. Due to zinc's integral role in enzymatic and transcriptional regulation, zinc levels have long been known to play a role in modulating a cells enzymatic activity or protein synthesis. The observation that labile zinc can act as a second messenger similar to calcium flux (*167*) has demonstrated an even larger biological role for zinc in regulating cellular homeostasis.

Due to the critical biological role of zinc ions, zinc concentration and movement within cells is tightly regulated. Labile zinc levels are regulated at the plasma membrane, as well as within subcellular compartments. Two classes of multipass transmembrane protein families regulate this homeostasis: the Zrt, Irt-like Protein (ZIP) family and the zinc transporter (ZnT) family. Both ZIP and ZnT proteins are encoded by two solute-linked carrier (SLC) gene families, SLC39 and SLC30, respectively (*168*). ZIP transporters transfer zinc into the cell cytoplasm from either the extracellular space or intracellular compartment. ZnT family members move zinc in the opposite direction, removing it from the cytosol. Currently, 14 ZIP and 9 ZnT transporters have been identified in mammals (*169*). Mutation of zinc transporter genes are responsible for or contribute to several human pathologies including acrodermatitis enteropathica (ZIP4 (*170*)), transient neonatal zinc deficiency (ZnT2 (*171*)), diabetes mellitus (ZnT8 (*172*)), and breast cancer (Zip10 (*173*)) among many others.

Zinc in Reproduction

Zinc is a requisite for reproduction in both males and females. In men, zinc levels are positively correlated with testosterone production from testicular Leydig cells (*174*). Zinc deficiency impairs spermatogenesis (*175, 176*) and zinc flux is required for sperm capacitation prior to fertilization (*177*). The zinc requirement for sperm capacitation likely explains the high concentration of zinc in seminal plasma. Low zinc concentrations in seminal plasma are associated with male infertility (*178, 179*).

In women, zinc deficiency during pregnancy is associated with increased risk of preterm birth and low-birthweight infants (*180-182*). Zinc's most important reproductive role may be during oogenesis, where zinc levels play a critical role in meiosis and oocyte activation. Rodent studies from our lab and others have shown severe oocyte defects resulting from both *in vitro* and *in vivo* zinc restriction. Total zinc content increases by more than 50% during oocyte maturation (*183*). *In vitro* chelation of zinc in fully grown mouse oocytes has been shown to prematurely initiate germinal vesicle breakdown (*143, 184*), while simultaneously interfering with progression through meiosis (*185*). These oocytes exhibit an abnormal phenotype including enlarged polar bodies, symmetric division, and an inability to reach metaphase II (*183*). Even short periods (3-5 days) of dietary zinc deficiency in female mice reduce the number of ovulations, fertilization rate, number of blastocysts, and litter size (*186, 187*). Oocytes from female mice fed a zinc-deficient diet also have reduced DNA and histone methylation that is associated with subsequent neural tube defects in the developing embryo (*188*). These defects are not rescued by subsequent zinc supplementation *in utero*, indicating that periovulatory zinc deficiency impacts the oocyte and embryo permanently. During oocyte maturation, large quantities of zinc are stored in a labile pool in vesicles near the oocyte plasma membrane (*189*). At fertilization, these zinc-containing vesicles rapidly undergo exocytosis in a process termed the "zinc spark" which has been shown to

be a biomarker of egg activation in many species including humans (*189-192*), and has proven to be a reliable indicator of embryo quality in rodent models (*193*). The role of zinc in folliculogenesis prior to the antral follicle has not been investigated.

Human Zinc Deficiency – A Public Health Concern

Though often overlooked, zinc deficiency threatens a large portion of the global population. Estimates of global dietary zinc deficiency vary according to the survey method employed, but between 17% and 31% of the global population is likely at risk of dietary zinc deficiency (*194, 195*). Animal products are the primary source of zinc in the diet, therefore the most vulnerable populations are those that subsist on a primarily plant-based diet. Compounding the risk of zinc deficiency, many staple plant foods (cereals, corn, rice) contain high levels of phytate which can inhibit zinc absorption in the intestine (*196, 197*). The highest rates of zinc deficiency are predicted in Africa, the Eastern Mediterranean, and South Asia (*195*). Regardless of location and national food supply, zinc deficiency threatens vulnerable populations. Food insecurity, a vegan/vegetarian diet, absorptive disorders of the GI tract, injury, or liver damage can result in zinc deficiency in patients otherwise not at risk of nutrient deficiencies. Clinical studies have shown an increased growth rate in infants and children after zinc supplementation, indicating an undiagnosed marginal deficiency within populations in the United States (*198, 199*).

As zinc status plays an integral role in fertility for both men and women, undiagnosed marginal zinc deficiency may play a role in infertility. Zinc supplementation has been shown to improve sperm morphology in subfertile men (*200, 201*), and women seeking IVF treatment exhibit lower levels of zinc in their follicular fluid than age matched controls (*202*). Zinc supplementation during oocyte culture has shown benefits in a number of livestock and model species (*203-206*). To this point, the effect of zinc deficiency on female fertility has been studied

in large ovulatory antral follicles using *in vitro* fertilization techniques. The role of zinc during preantral growth, a longer developmental period than the antral phase, has not been examined. Even more alarming is the report that 82% of pregnant women worldwide consume less than the recommended dietary allowance (RDA) for zinc (*207*). While this is a gross estimate of zinc status due to the survey methods employed as well as the method of assigning RDAs, it is clear that a huge population of women are zinc deficient during pregnancy. If zinc status impacts germ cell nest breakdown or primordial follicle formation in the fetal ovary, the effects of maternal zinc deficiency may irreparably affect the fertility of the next generation.

Conclusion

Zinc is a key mediator of fertility, but the effect of zinc deficiency on early oocyte and follicle development has not been investigated. Thus, the goal of this research project is to answer the following questions: Is *C. elegans* a useful model organism of zinc-mediated fertility loss? Are the effects of zinc deficiency apparent in developing oocytes distant from the *C. elegans* uterus? Is zinc required for preantral follicle growth in mammals? Does zinc deficiency play a role in germ cell nest breakdown and primordial follicle assembly in mammals? Answering these questions will not only move reproductive science forward, but may provide new insight into nutritional requirements prior to ovulation and uncover new biological roles of zinc in the ovary.

Chapter 3

Zinc deficiency reduces fertility in *C. elegans* hermaphrodites and disrupts oogenesis and meiotic progression*

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Author Contribution: JH, WHR, and FD devised experiments. JH collected and analyzed all data and wrote the manuscript. All authors edited and approved final manuscript.

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Key Words: Zinc, *C. elegans*, oogenesis, fertility

Abstract

Zinc is necessary for successful gametogenesis in mammals; however the role of zinc in the gonad function of non-mammalian species has not been investigated. The genetic tractability, short generation time, and hermaphroditic reproduction of the nematode *C. elegans* offer distinct advantages for the study of impaired gametogenesis as a result of zinc deficiency. However, the phenotypic reproductive effects arising from zinc restriction have not been established in this model. We therefore examined the effect of zinc deficiency on *C. elegans* reproduction by exposing worms to the zinc chelator N,N,N',N'-tetrakis (2-pyridylmethyl)ethane-1,2-diamine (TPEN). Treatment began at the early larval stage and continued until reproductive senescence. TPEN treatment reduced the total number of progeny produced by *C. elegans* hermaphrodites compared with control subjects, with the largest difference in output observed 48 hours after larval stage 4. At this time-point, zinc deficient worms displayed fewer embryos in the uterus and disorganized oocyte development when observed under DIC microscopy. DAPI staining revealed impaired oogenesis and chromosome dynamics with an expanded region of pachytene stage oocytes extending into the proximal arm of the gonad. This phenotype was not seen in control or zinc-rescue subjects. This study demonstrates that reproduction in *C. elegans* is sensitive to environmental perturbations in zinc, indicating that this is a good model for future studies in zinc-mediated subfertility. Aberrant oocyte development and disruption of the pachytene-diplotene transition indicate that oogenesis in particular is affected by zinc deficiency in this model.

Abbreviations: NGM – Nematode Growth Media. TPEN - N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine

Introduction

The essential micronutrient zinc is necessary for successful reproduction in vertebrate species. Adequate zinc bioavailability is required for sperm production (*208*), oocyte maturation (*183, 184, 209-211*), fertilization (*189, 190*), and embryo development (*187, 188*). The periovulatory period surrounding oocyte maturation and ovulation is particularly sensitive to zinc insufficiency (*187, 188, 209*). A better understanding of the reproductive mechanisms that rely on zinc is a vital step in identifying and treating idiopathic infertility and optimizing assisted-reproduction techniques**.** New models are necessary to fully elucidate the mechanistic role of zinc in reproduction.

The invertebrate nematode *C. elegans* is a valuable research model where the role of zinc in reproduction has not yet been established. This genetically tractable model offers a unique life cycle that provides many advantages for the study of gonadal function. The majority of *C. elegans* are self-fertilizing hermaphrodites in which sperm and oocytes develop sequentially from a common gonad (*212*). The reproductive tract of the *C. elegans* hermaphrodite is U-shaped and composed of two gonadal arms joined by a common uterus. Each gonadal arm is independently capable of spermatogenesis, oogenesis, and fertilization (*138*). The ability to study sperm, oocytes, and embryos in the same subject, combined with the short generation time and well-annotated genome allow subtle reproductive phenotypes to be identified (*213*).

The lifecycle of the worm is well characterized (*136*). Worms hatch in the first stage of larval development (L1) and then progress through 3 more larval stages (L2-L4) over the next 52 hours before reaching adulthood with a functional reproductive system. The distal tip of each gonadal arm in *C. elegans* is comprised of a mitotic zone in which germ cells are generated under the influence of NOTCH signaling (*214*). As germ cells migrate away from the mitotic zone, they enter meiosis. Spermatogenesis begins during late L3 resulting in the production of

approximately 300 sperm cells primarily generated during L4 (*215*). These cells are stored in specialized structures known as spermatheca. Subsequently, the gonad begins oogenesis and the development of female germ cells exclusively. Early female germ cells develop in a syncytium, in which nuclei are not completely membrane-enclosed and open to a common cytoplasm known as the rachis. This syncytial arrangement persists through the pachytene stage of nuclear development, with oocyte differentiation and membrane enclosure occurring with the pachytene-diplotene transition at the beginning of the proximal gonad. The oocytes undergo further development including dynamic changes in chromosomal arrangement as they progress toward the spermatheca. Oocytes are ovulated sequentially into the spermatheca where they complete meiosis and are fertilized. The resulting embryos pass into the uterus and undergo mitotic divisions until being released from the adult worm around the 24-cell stage.

Germ cell and embryo development are well characterized in *C. elegans* (*138, 216*), as are the effects of zinc deficiency on lifespan (*149*). Storage and transport of zinc in intestinal cells have also been investigated under zinc deficient conditions (*147, 148*), however the effects of zinc deficiency on *C. elegans* reproduction have not been investigated. We therefore undertook to induce and characterize the phenotypic effects of zinc deficiency on reproduction in *C. elegans*. The well-established zinc requirement for reproduction in mammalian species, as well as highly conserved mechanisms of oogenesis in both *C. elegans* and higher animals, led us to hypothesize that zinc restriction would decrease fertility in *C. elegans* hermaphrodites. Furthermore, given the emerging role of zinc in oocyte development prior to fertilization, we hypothesized that deficits in oogenesis would be evident in a zinc-deficient model.

Materials and Methods

C. elegans culture

Wild-type (strain N2) *C. elegans* were maintained under standard conditions on Nematode Growth Media (NGM)-agar plates seeded with Escherichia coli OP50 as a source of food (*217*). Worms were maintained at 20°C. To create experimental conditions, NGM-agar plates were treated prior to addition of *C. elegans*. Zinc-deficient conditions were created with addition of N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN) in 4% ethanol buffer; final concentration 50 μM/plate. Control plates were treated with ethanol buffer alone. A rescue group was created by treating plates with TPEN and ZnSO4 in ethanol buffer (final concentration 50 μM of both TPEN and Zn/plate). Animals were cultured on treatments for 24 hours prior to choosing L4 stage subjects for further treatment and analysis (Fig. **3-1**). Worms treated with this protocol can be estimated to enter treatment during the late L2/early L3 stage of development. Animals subsequently remained on treatment for the duration of the experiment.

Effect of TPEN exposure on progeny production

L4 Worms were picked 24 hours after exposure to experimental conditions and placed singly on either TPEN-treated (n=4) or control plates (n=5). Worms were moved to a new plate (either TPEN-treated or control) daily and the number of progeny produced during the previous 24 hours was recorded. Progeny numbers were calculated as live worms remaining on each plate after transferring the adult worm (Fig. **3-1**A). Plates were re-examined on multiple days to ensure that all progeny were counted. This procedure was repeated daily until reproductive output reached zero, which required 5 days for both control and TPEN treated groups.

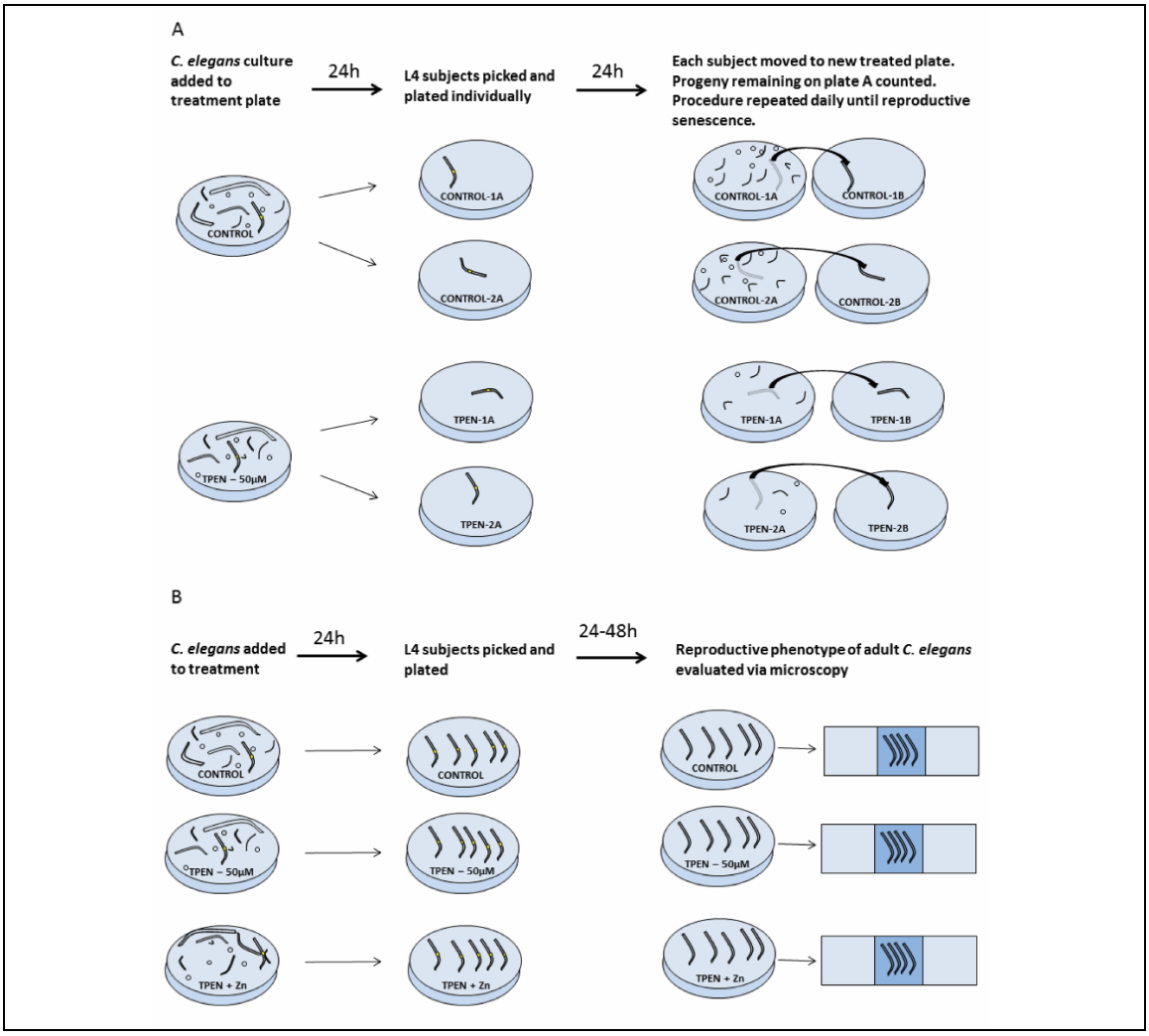

Figure 3-1: **Experimental Protocol**

(A) To test the effect of TPEN on reproduction in *C. elegans*, high numbers of N2 eggs, larvae, and adults were added to NGM plates treated with either TPEN (50µM) or buffer as a control. Twenty-four hours later, L4 animals were identified by size and vulval morphology and transferred individually to new treated plates. Animals were subsequently moved to a new plate every day and progeny released over the previous 24 hours was quantified. This procedure continued until reproductive senescence. (B) Experiments conducted after the timecourse study were performed using a similar protocol. Large numbers of N2 animals were placed onto control, TPEN (50µM), or TPEN+Zn (50µM each) treated plates. After 24 hours L4 subjects were isolated. Subjects remained on treatment for an additional 24 or 48 hours before being processed for DIC or fluorescent microscopy. In all experiments, animals were placed onto treatments 24 hours prior to the L4 stage and remained on treatment until the experimental endpoint.

Embryo number and oocyte development

L4 worms were picked 24 hours after treatment with TPEN (n=38 worms), TPEN+ZN (n=38 worms), or buffer only (n=36 worms). Animals were subsequently maintained on treatment plates for the duration of the experiment (Fig **3-1**B).

Results of the prior progeny counting experiment showed the largest difference in reproductive performance was on day two of egg-laying. Therefore, at this time point worms were placed onto slides and observed via DIC on Zeiss Axioplan2 microscope at 40X magnification. The number of embryos developing in the uterus was recorded for each worm. Defects in oocyte development were also noted and imaged at this time. The stage of embryo development and rate of gonadal deformity was observed but did not differ between treatments.

DAPI staining

Worms from each treatment group – TPEN, control, or TPEN +Zn – were created as before. 24 hours post L4, worms were fixed in Carnoy's fixative (60% ethanol, 30% glacial acetic acid, 10% chloroform) and stained with DAPI (4',6-diamidino-2-phenylindole). Worms were mounted on 15 mm etched ring slides for fluorescent imaging using an Olympus FV10i confocal microscope at 60X magnification. We chose this timepoint in order to test if the reproductive defects in oogenesis noted 48 hours post L4 could be attributed to meiotic defects in the early adult worm.

Bright-field imaging for representative images

Worms were placed onto treatment 24 hours before reaching L4 as described above. Live worms were mounted on slides and imaged at 40X Zeiss axiocam2 DIC microscope. Images were taken for all groups once daily until egg-laying ceased.

Statistics

Daily progeny output data was analyzed using repeated measures ANOVA with an imposed autoregressive covariance structure. Treatment effects within day were compared using Student's t-test. Treatment effects on total progeny released and embryo number were analyzed using one-way ANOVA and post-hoc Tukey pairwise comparisons. The effect of treatment on pachytene germ cells in the proximal gonad was tested using chi-square test for association. P value <0.05 was considered significant for all experiments.

Results

TPEN exposure reduces progeny output in *C. elegans*

C. elegans hermaphrodites were raised individually on either control or TPEN treated plates. The number of progeny released from each subject per day was calculated from the onset of egg-laying until reproductive senescence. The total number of progeny was severely attenuated when worms were treated with TPEN (mean of 42.75±3.8 progeny compared with 171.8±23.8 under control conditions, Fig **3-2**B). The largest deficit was seen on day 2 of egg-laying, 48h post-L4 (Fig. 3-2A). Repeated measures ANOVA showed a significant effect of treatment, day,

and treatment by day interaction ($p<0.0001$). Due to the large treatment-induced effect in progeny output 48 hours post L4, this time point was selected to examine differences in embryo number and oocyte development in subsequent experiments.

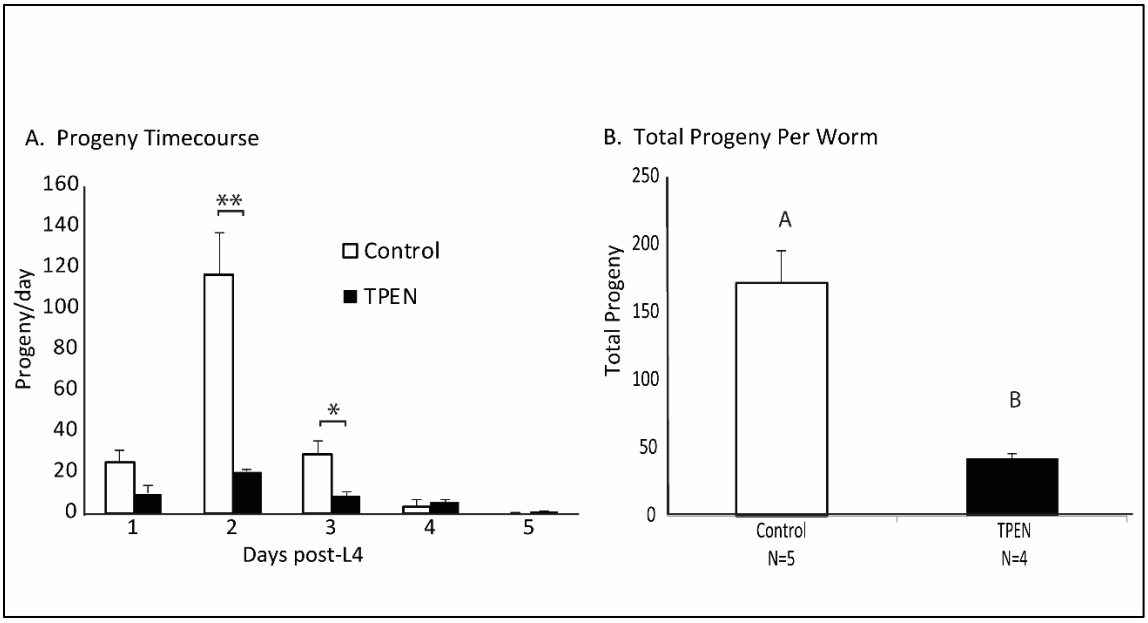

Figure 3-2: TPEN treatment reduces fertility in *C. elegans*

(A) Number of progeny from worms placed on TPEN (50 μM) or control plates as early stage larva. Data was analyzed via repeated measures ANOVA (N=4-5) and showed significant effect of day ($P<0.0001$), treatment ($P=0.0003$) and treatment x day interaction ($P<0.0001$). *Indicated significant difference within day by student's t-test, (*$P<0.05$; **$P<.01$). (B) TPEN treatment significantly reduced the number of total progeny released. Subjects in the control group produced a mean of 171.8±23.8 progeny. TPEN treated subjects produced a mean of 42.75±3.8 progeny ($P<0.01$). Results analyzed by one-way ANOVA and Tukey's post-hoc test.

Zinc restriction lowers the number of developing embryos in the uterus

At 48 hours post-L4, zinc deficient worms had fewer developing embryos *in utero* (7.5±0.4) compared with control (11.4±0.6) or rescue (12.7±0.6) groups (Fig. **3-3** $p<0.001$). Subjects were examined for disruption of embryo development or gonadal formation including: instances of intra-uterine larvae (bagging), gonadal atrophy, disrupted vulva formation, and

malformation of either gonadal arm. These observations were combined and used to compare the rates of gonadal deformation between groups. There was no statistical difference between groups for this comparison. (Con – 22%, TPEN – 29%, Rescue – 27%; P=0.785 Chi square test for association).

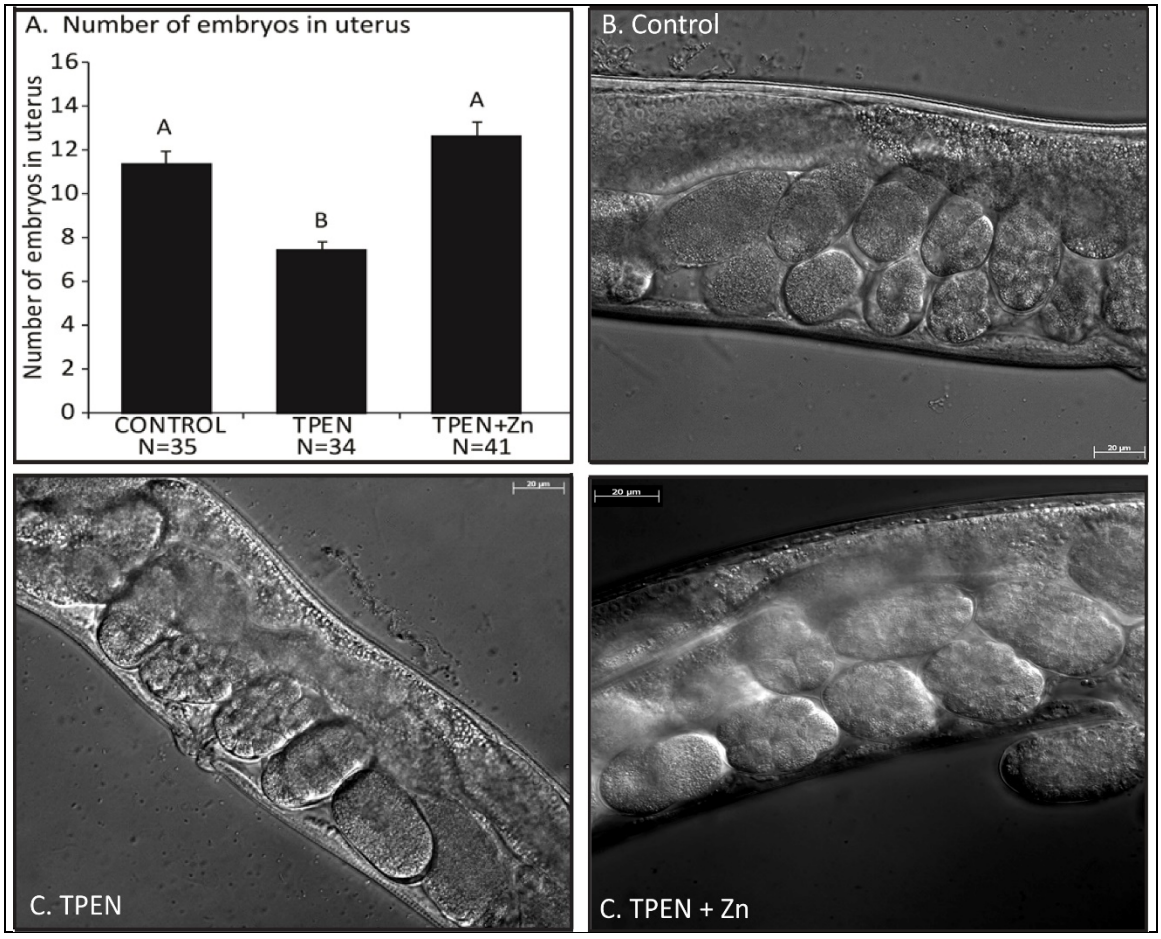

Figure 3-3: Zinc restriction reduces the number of developing embryos.

(A) Number of embryos in the uterus of control (11.4±0.6; n=41), TPEN (7.5±0.4; n=34), and TPEN plus zinc (12.7±0.6; n=41) worms 48 hours post-L4. [abc]Indicates significant difference by one-way ANOVA followed by Tukey's post-hoc test, P<0.05. (B-D) Representative DIC images (40X) from each group. [ab]Indicates significant difference by one-way ANOVA followed by Tukey's post-hoc test. P<0.05.

Zinc restriction disrupts oocyte development

Under control conditions, female germ cells emerged from the rachis and began orderly, single-file oocyte development in the proximal gonadal arms (Fig **3-4**A). Disrupted oogenesis was noted in 45% of TPEN treated subjects. Aberrant phenotypes included oocyte stacking, binucleate oocytes, and small (likely pachytene) oocytes extending past the gonadal turn (Fig **3-4**C-D). Animals from the rescue group did not display disrupted oocyte development and resembled the phenotype of control worms (Fig **3-4**B).

Figure 3-4. Zinc restriction disrupts oocyte development in the proximal gonad

(A) Representative DIC image (40X) of control gonad undergoing orderly meiotic progression. (B) Representative image of rescue group. (C) Image of TPEN-treated gonadal arm. Arrow – stacked and overlapping oocytes. Bracket – Apparent extended region of pachytene cells. (D) TPEN treated gonadal arm. Boxed – binucleate oocyte.

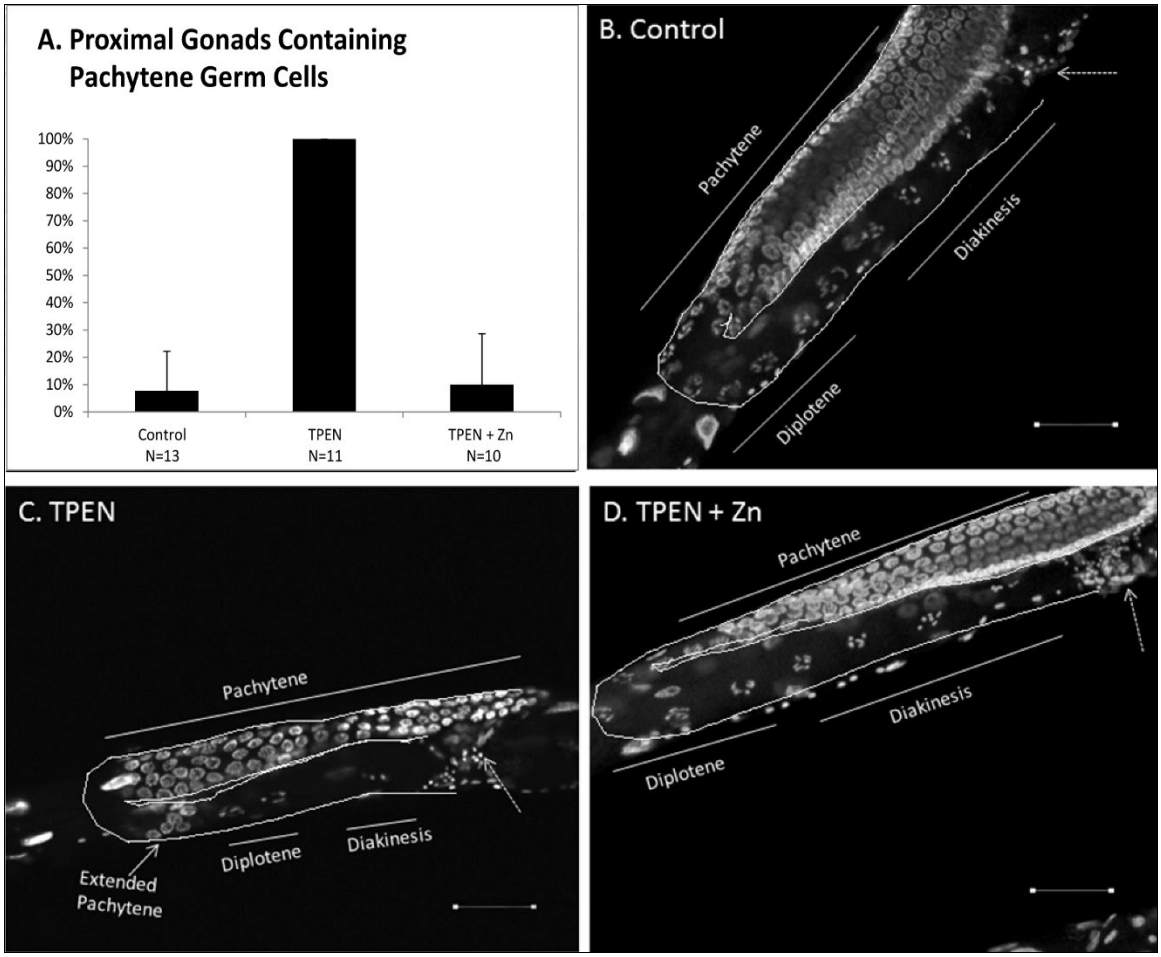

Figure 3-5. Zinc restriction extends the region of pachytene stage oocytes.

(A) Percentage of proximal gonads containing pachytene oocytes. (B-D) Representative DAPI images. Pachytene oocytes visible in proximal gonad in panel C. Dotted arrow points to the Spermatheca. Scale bar equals 20µm. Chi square test for association showed a significant effect of treatment on % extended pachytene phenotype ($P<0.001$). Error bars represent 95% confidence interval for control and rescue groups. Confidence interval cannot be calculated for proportions of 100% (TPEN).

Zinc restriction leads to an expanded region of pachytene germ cells in the proximal gonad

We used DAPI staining to examine meiotic progression and chromosomal dynamics directly and to investigate the suspected presence of pachytene oocytes in the proximal gonadal arm of zinc-deficient animals. When chromosomes were examined under fluorescence, all TPEN

treated worms (100%) displayed an expanded region of pachytene-stage oocytes extending past the gonadal bend and into the proximal gonadal arm 24 hours post-L4. This morphology was rarely seen in control (7.6%) or TPEN+Zn (10%) subjects (Chi-square test for association: $P<0.001$, Fig. **3-5**).

Discussion

These findings clearly show an impaired reproductive phenotype resulting from zinc restriction in *C. elegans*. The ubiquity of zinc's role in reproductive processes poses a challenge in elucidating the exact mechanism(s) responsible for the TPEN-induced reproductive defects. Studies of zinc restriction in other models indicate five critical points in reproduction that are vulnerable to zinc restriction: development of the reproductive tract (*218*), spermatogenesis (*175*), oogenesis (*209*), fertilization (*190*), and embryo development (*188*). The results of this study indicate oogenesis as the most obviously disrupted reproductive process in TPEN-treated *C. elegans*, however effects on other reproductive processes cannot be ruled out.

Animals began treatments as early-stage larvae, prior to reproductive tract development. However, TPEN treated subjects did not show gross reproductive tract deformity or disrupted development at a higher rate than control animals. Indeed, zinc-deficient animals began releasing progeny on the same day and persisted for the same duration as hermaphrodites in other treatment groups. This may indicate that *C. elegans* do not require labile zinc during reproductive tract development as in other species, or more likely that the zinc reserves identified by Roh et al. (*147*) in the digestive tract of *C. elegans* persisted through larval development and facilitated completion of a functional reproductive system. Regardless, the results of this study do not indicate any major changes in the anatomical development of the reproductive tract in TPEN

treated *C. elegans,* and it is more likely that deficits in oogenesis, fertilization, and/or embryo development are responsible for the effects seen in this study.

Zinc plays a key role in fertilization and sperm dynamics. Liu et al (*219*) demonstrated that zinc is sufficient to activate sperm in *C. elegans* and that the spermatheca is enriched in labile zinc. A unique aspect of reproduction in *C. elegans* is the hormonal action of the sperm cytoskeletal protein MSP-1. This protein acts outside of the spermatheca to initiate ovulation. MSP-1 simultaneously signals meiotic resumption in the diakinesis-arrested oocyte adjacent to the spermatheca and causes contraction of the gonadal sheath cells which push the oocyte into the spermatheca (*144*). The MSP-1 ovulation signal depends on the presence of two redundant zinc-finger proteins in the maturing oocyte – OMA-1 and OMA-2 (*220*). The phenotype of OMA-mutant *C. elegans* presents as an increased number of fully grown unfertilized diakinetic oocytes in the proximal gonadal arm. This phenotype is in contrast with the zinc-restriction phenotype described here which is characterized by an enlarged region of small pachytene-stage germ cells; making it unlikely that zinc-deficiency is acting primarily at the site of MSP signaling. Finally, although subjects in all groups were fertile, indicating the presence of sperm; sperm count and morphology were not evaluated. Therefore we cannot discount the possibility that differences in sperm quantity or quality may have affected reproductive output.

The most striking effect of zinc restriction in *C. elegans* hermaphrodites was seen in the female germ cells. The disorganization of oocyte progression in the gonad indicates impaired oogenesis in the zinc-restricted group, undoubtedly a contributing factor to lowered fertility in TPEN-treated subjects. DAPI staining also revealed an increase in the area of pachytene-stage germ cells, extending well past the gonadal turn. This phenotype may indicate meiotic disruption. Meiosis of female germ cells is driven by the MAPK cascade in *C. elegans*, a pathway previously shown to be affected by intracellular zinc concentration in *C. elegans* (*221, 222*). MPK-1 (MAPK ortholog) signaling is required for progression past the pachytene arrest (*223*) and mutation of

mpk-1 or *mek-2* (mapkk ortholog) results in total pachytene arrest of female germ cells (*224*). Interestingly, MPK-1 has been shown to interact with GLA-3, a protein containing two zinc-finger domains (*225*). *gla-3* mutant worms are characterized by increased levels of oocyte apoptosis, however double mutants that lack both *gla-3* and the apoptotic caspase gene *ced-3* display a phenotype very similar to our zinc-restricted hermaphrodites. In both cases the region of pachytene nuclei was extended into the proximal gonadal arm, but diakinetic nuclei were still visible. The comparable germ cell phenotype, as well as the presence of zinc-finger domains make GLA-3 a likely target for disruption by zinc restriction. We will investigate the hypothesis that zinc sequestration interferes with germ cell progression by disrupting GLA-3 (and by extension MAPK) activity in future studies.

Reduced brood size and an extended pachytene region in the proximal gonadal arm are also an established phenotype after CPB-3 knock-down with siRNA (*226*). CPB-3 is an ortholog of cytoplasmic polyadenylation element binding protein (CPEB), a highly conserved RNA-binding protein involved in temporal control of mRNA translation in the developing oocyte (*227*). Oocytes in CPEB knockout mice do not progress beyond the pachytene stage (*228*) and synaptonemal complex formation is disrupted in both developing oocytes and spermatids (*229*). Hake et al (*230*) demonstrated that CPEB requires a zinc cofactor in order to bind RNA, therefore it is possible that zinc deficiency impairs CPEB activity and interferes with the pachytene to diplotene transition. Future studies in our model will seek to confirm the relationship between zinc deficiency and CPEB activity.

Our experimental treatments were applied to NGM-agar plates seeded with E. coli OP50 as a food source. It is conceivable that TPEN treatment affected the density or quality of the bacterial lawn. However, this is unlikely since the bacterial lawn of TPEN –treated plates appeared normal under light microscopy, and a similar TPEN-treatment protocol has been used previously with no noted starvation effects (*147, 149*). Moreover, the worms in our study do not

display the reproductive phenotype associated with starvation described by Seidel and Kimble (*231*); which includes intra-uterine larval hatching (bagging), germline shrinkage, constriction of the gonadal arms, and bulging germ cells. While starvation does disrupt the progression of oocytes in the proximal gonad, the starvation phenotype presents with only one oocyte per gonadal arm developing and ovulating before another oocyte begins to differentiate. This is distinct from our model and indicates that the reproductive effects of our study are a result of zinc-deficiency, rather than total nutrient deprivation.

In conclusion, our group has identified a novel *C. elegans* reproductive phenotype resulting from zinc restriction with the zinc chelator TPEN. Reproductive deficits were obvious and pronounced, indicating that *C. elegans* is a valuable model for zinc-restricted subfertility. The exact mechanism(s) of reduced fertility remains to be elucidated, but defective oogenesis is responsible for at least some of the reduced fertility. The TPEN-induced phenotype is similar to the phenotype seen in *C. elegans* with depleted GLA-3 or CPB-3 levels. Future studies will investigate whether zinc restriction impairs oogenesis though disruption the MAPK pathway via reduced GLA-3 activity, disrupted synaptonemal complex formation mediated by CPB-3, or a combination.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

Chapter 4

Zinc Deficiency Disrupts *In vitro* Maturation of Preantral Ovarian Follicles

Authors: James Hester, Francisco Diaz

Author Contribution: JH and FD devised experiments. JH performed all experiments and analysis. JH wrote the manuscript. Manuscript reviewed and edited by both JH and FD.

Key words: Preantral follicles, Zinc, Oocyte, Granulosa Cells, *In vitro* Maturation

Abstract

Zinc is emerging as a key factor in mammalian oocyte and embryo development. Zinc deficiency has been shown to disrupt fertility in mature ovarian follicles, fully grown GV oocytes, mature MII eggs, and developing zygotes/embryos. The effects of zinc deficiency on oocyte and granulosa cell development in the preantral ovarian follicle have not been tested. Vulnerability to zinc deficiency at this stage would be significant because oocytes spend more time developing in preantral follicles than in mature antral follicles. To establish the role of zinc during preantral follicle and oocyte development, we collected preantral granulosa cell-oocyte complexes from 14 day old mice and cultured them under control, zinc deficient (10μM DTPA), or rescue (10μM DTPA + 50μM ZnSO4) conditions for up to 10 days. Zinc deficient oocytes showed impaired growth, impaired nuclear maturation, and a reduced ability to resume meiosis in response to an ovulatory signal. Zinc deficient granulosa cells underwent apoptosis at a higher rate than control, had fewer physical connections to the oocyte, and did not undergo cumulus cell differentiation in response to paracrine signaling. We have demonstrated that zinc is necessary for proper development of preantral ovarian follicles, particularly for cumulus cell differentiation and survival. The loss of somatic support cells likely explains some or all of the growth and developmental deficits seen in the zinc deficient oocytes.

Introduction

Infertility affects millions of women world-wide; and twelve percent of women in the US have sought infertility treatment (*7*). One contributing factor of reproductive dysfunction is zinc deficiency (*232, 233*), a condition estimated to affect 17% of the global population (*194*). Zinc levels in the follicular fluid of women undergoing IVF are

lower than age-matched controls (*202*), pointing to zinc status as a likely contributor to subfertility in humans. The role of zinc in the oocyte has begun to be elucidated, particularly during meiotic resumption and fertilization. Zinc deficiency has been shown to prematurely initiate germinal vesicle breakdown (*143, 184*), while simultaneously interfering with progression through meiosis (*185*). Preovulatory dietary zinc deficiency in female mice reduces the number of ovulations, fertilization rate, number of blastocysts, and litter size (*186, 187*). Oocytes from females fed a zinc-deficient diet have reduced DNA and histone methylation that is associated with subsequent neural tube defects in the developing embryo (*188*). Total zinc content increases during oocyte maturation (*183*), with much of this increase in zinc stored in a labile pool in vesicles near the plasma membrane (*189*). At fertilization, zinc-containing vesicles rapidly undergo exocytosis in a process termed the "zinc spark" which has been shown to be a biomarker of egg activation in many species including humans (*189-192*), and has proven to be a reliable indicator of embryo quality in rodent models (*193*). A critical role of zinc in the fully-grown competent oocyte has been established in mammals including humans, and recent work from our lab and others (*150, 151*) revealed defects in early oogenesis resulting from zinc deficiency in an invertebrate model. However, the effect of zinc deficiency on oocyte growth and development in preantral follicles has not been examined in mammals.

In humans, development from a primordial follicle to a dominant graafian follicle capable of ovulation takes almost a year, and over 99% of activated follicles undergo atresia at some point in development (*8, 9, 90*). Environmental, dietary, or disease factors that impair follicular development therefore have a severe impact on fertility (*234-236*).

One critical point of follicle development is the preantral to antral follicle transition. Both the oocyte and the surrounding granulosa cells must pass multiple developmental milestones to form a gonadotropin-dependent antral follicle containing a fully-grown oocyte capable of ovulation and fertilization. Rodent studies have shown follicles are most vulnerable to atresia during this transition (*8*).

During the preantral to antral transition, the oocyte reaches its full size and undergoes nuclear maturation, changing the chromatin arrangement to a transcriptionally silent configuration tightly wrapped around a large nucleolus (*93, 237*). Condensed chromatin surrounding the nucleolus (SN), as well as a large oocyte diameter are indicators of viability and meiotic potential (*91, 238*). Changes in the oocyte actin cytoskeleton also occur, preparing the cell for asymmetric division – the hallmark of oocyte meiosis (*128*). Reorganization of the oocyte cortical actin in particular is required to allow the meiotic spindle to migrate to the cell periphery (*239, 240*).

Granulosa cells also undergo a profound transition at the end of preantral development. The preantral granulosa cells differentiate into two distinct lineages – cumulus cells which surround the oocyte, and mural granulosa cells which line the wall of the follicle. Physiological function as well as gene expression patterns are distinct between the two cell types (*95*). In general, the cumulus cells support the metabolism and development of the oocyte while mural cells perform the endocrine function of the antral follicle. Both granulosa cell types cooperate to maintain meiotic arrest (*119, 241*), as well as induce ovulation in response to the LH-surge (*242*). Successful follicle development requires paracrine communication between the oocyte and granulosa cells (*74, 98, 99, 101, 112, 122, 243-245*), as well as endocrine signaling from the hypothalamus and

pituitary (*87, 246, 247*). The balance between oocyte derived factors and endocrine factors transmitted through the circulation appear to drive the differentiation of the granulosa cells during the preantral to antral transition (*95, 99*). Paracrine signaling between the oocyte and surrounding granulosa cells promotes development of the cumulus oophorus, while exposure to follicle stimulating hormone (FSH) antagonizes cumulus cell gene expression and promotes the mural phenotype (*98*). The oocyte derived factors (ODFs) GDF9 and BMP15, members of the TGF-β superfamily, induce differentiation into cumulus cells through pSMAD2/3 and pSMAD1/5/8 pathways; and antagonize FSH signaling in the granulosa cells closest to the oocyte (*248, 249*).

Previous studies have demonstrated that zinc deficiency interferes with pSMAD2/3 signaling (*186*) and reduces GDF9 transcription in oocytes collected from antral follicles (*188*). Our lab has also demonstrated that proper zinc homeostasis in the oocyte requires cumulus cell/oocyte interaction (*250*). We therefore hypothesize that zinc deficiency during preantral growth will compromise both oocyte and granulosa cell viability and interfere with cumulus cell differentiation as a result of impaired pSMAD2 signaling. To test these hypotheses, we collected preantral follicles from 14 day old mice and cultured them for up to 10 days in control, zinc deficient, or rescue media. Zinc deficient media was created by adding 10μM of the membrane impermeable chelator diethylenetriamine pentaacetic Acid (DTPA). DTPA has been used extensively to chelate zinc in cell culture media (*251-254*), and induces apoptosis at a significantly lower rate than membrane-permeable chelators such as (*N*,*N*,*N*′,*N*′-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN) (*255*). We evaluated the effect of zinc deficiency on parameters of oocyte development including size, nuclear maturation, and meiotic potential. To

explore the mechanisms underpinning disrupted development with DTPA treatment, we evaluated changes in zinc accumulation, apoptosis, gene expression, SMAD2 activation, and gap junction formation (Figure 1).

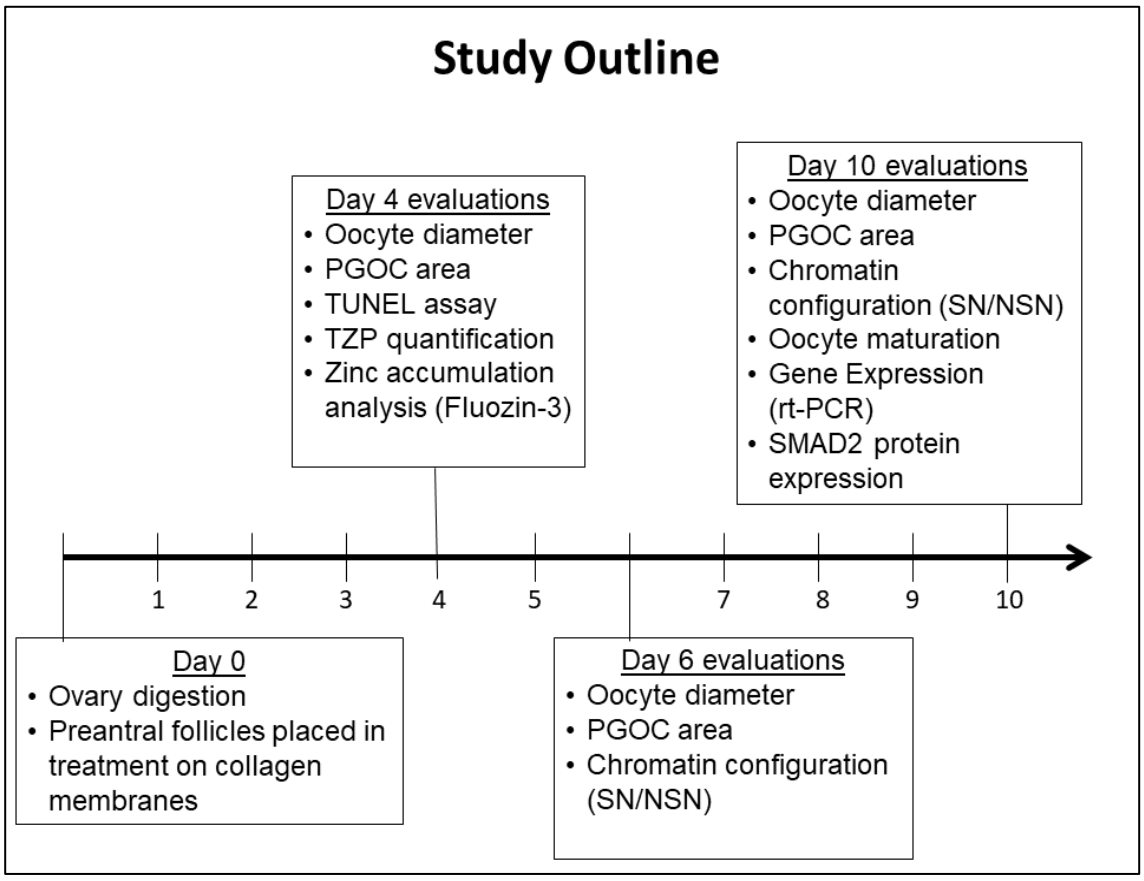


Figure 4-1: **Study outline**

Preantral granulosa cell-oocyte complexes were collected from 14d old mice and cultured in control, zinc deficient (DTPA), or rescue conditions for 4, 6, or 10 days. Oocyte diameter and PGOC area were calculated at all three timepoints to evaluate cell growth. Nuclear staining to evaluate chromosome condensation was conducted at the later timepoints – day 6 and 10. Meiotic potential, gene expression, and protein expression were evaluated in the most developed PGOCs (day 10). TUNEL assay and TZP staining were conducted on day 4, because prolonged DTPA culture resulted in fewer granulosa cells surrounding the oocyte. FLZN-3 staining to confirm changes in zinc trafficking in DTPA treated oocytes was also conducted on day 4 of culture.

Materials and Methods

Preantral follicle collection and culture

CD-1 mice (mus musculus) were commercially obtained from Charles River laboratory (Wilmington, MA). Animal care and handling were reviewed and approved by the Penn State IACUC. 14 day old female mice were euthanized prior to ovary collection and digestion as described previously (37, 38, 57). All reagents were purchased from Sigma-aldrich (St. Louis, MO.) unless otherwise specified. Briefly, ovaries were placed in a digest buffer containing 1mg/ml collagenase II, 1mg/ml collagenase IV, and 0.2% DNAse I. Ovaries were incubated at 37 degrees C in digest buffer and mixed by pipetting every 5 minutes until digestion was complete. This procedure produces preantral granulosa cell-oocyte complexes devoid of theca cells. Preantral granulosa cell-oocyte complexes (PGOCs) consisting of an oocyte and multiple layers of granulosa cells were collected from the digest buffer and washed three times in control culture media: MEMa supplemented with BSA (3mg/ml), ITS (insulin, 5 µg/ml; transferrin, 5 µg/ml; selenium, 5 ng/ml), and epidermal growth factor (1ng/ml). PGOCs were cultured for 4, 6, or 10 days on collagen membranes submerged in either control media, zinc depleted media containing 10µM DTPA or rescue media containing 10µM DTPA and 50µM ZnSO4. Cells were kept in a humidified 37° incubator infused with a gas mix of 5% CO2 and 5% O2. At the end of culture, PGOCs were separated from the collagen membrane by striking the outside of the culture dish sharply with a gloved hand. After dislodging from the collagen membrane, PGOCs were collected from the culture well using a mouth pipet and either flash frozen in liquid nitrogen for RNA/protein analysis, or moved to a fresh dish of media for further analysis.

Oocyte diameter calculation

After 4, 6, or 10 days of culture, PGOCs were dislodged from collagen membranes and collected. Complexes were moved to dishes containing fresh media and immediately imaged using a Nikon eclipse TE200 inverted microscope fitted with an Olympus DP20 brightfield camera. Images were taken through a 4x objective. Oocyte diameter was measured from resulting images using DP2-BSW software from Olympus. Diameter was calculated for a minimum of 125 oocytes per time point and treatment. Diameter was taken of only the oocyte and did not include surrounding somatic cells. In the case that oocytes were not perfectly spherical, the longest axis was taken as the diameter. Datasets were generated by two independent researchers and averaged to calculate results.

PGOC total area calculation

The same brightfield images used to calculate oocyte diameter were used to measure the total area of PGOCs after culture and removal from the collagen membrane. The 2-dimensional area of PGOCs consisting of one oocyte and all attached granulosa cells was measured after 4, 6, or 10 days of culture using ImageJ. Analysis was performed on three biological replicates/timepoint, and a minimum of 75 PGOCs were included in each treatment/timepoint calculation.

Fluozin-3 imaging

To test the effect of treatment on zinc accumulation in the oocyte, PGOCs were cultured in control, DTPA, or rescue media for 4 days. PGOCs were collected as described previously and

placed into fresh drops containing the corresponding treatment. Oocytes were mechanically denuded by gentle pipetting through a narrow-bore glass pipet, a technique we have previously shown induces zinc accumulation within the oocyte (51). Denuded oocytes were placed into culture media containing 4μM of the amyl ester of the fluorophore, fluoZin-3 (Invitrogen, Carlsbad, CA.; excitation 494nm/emission 516nm) which binds labile zinc. After 30 minutes, oocytes were moved to a chamber slide with fresh media containing Prolong Live antifade reagent. After an additional 30 minute culture period, cells were imaged using an inverted fluorescent microscope. To prevent signal bleaching, cells were imaged as a group. Fluorescence was measured in each oocyte with imageJ and reported as corrected total cell fluorescence (58). Experiment was repeated two times and at least 50 cells were measured per treatment. Cells were kept in treatment (Control, DTPA, Rescue) through all steps of the imaging process.

Meiotic progression

To test the meiotic potential of cultured oocytes, PGOCs were cultured for 10 days. PGOCs were washed through three drops of control media and then cultured overnight in media containing 10ng/ml EGF (maturation media) to stimulate cumulus expansion and maturation (37, 38). Maturation media was the same for all groups and did not contain DTPA or exogenous zinc. After 20 hours, oocytes were denuded from the surrounding granulosa cells and placed in a fresh drop of media. Meiotic progression was observed via bright field microscopy. Oocytes containing a germinal vesicle were classified as GV-arrested oocytes, MII oocytes were identified by the presence of a polar body. Oocytes with neither a germinal vesicle nor polar body were classified as MI. Results are combined from 5 biological replicates per treatment. In total, at least 150 PGOCs from each treatment were matured.

Nuclear maturation and chromatin visualization

To observe the effect of treatment on maturation of the oocyte nucleus, PGOCs were collected and immediately fixed for 1 hour in 4% PFA in PBS. Cells were washed in PBST, stained with Phalloidin-488 at 1:1000, and mounted on ring slides with Prolong antifade plus DAPI (Fisher Scientific). Cells were imaged with a Zeiss Axiovert 200 fluorescent microscope through a 40X objective to observe chromatin arrangement (surrounding nucleolus/not surrounding nucleolus) and nucleolar configuration.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL assay)

Apoptotic cells were labelled in 4d cultured PGOCs using the Deadend Fluorometric TUNEL System (Promega) according to manufacturer's instructions. TUNEL positive controls were generated by pretreating cells with DNase-I for 10 minutes. Labelled complexes were mounted on etched ring slides with slowfade anti-fade mounting media plus DAPI. Cells were imaged with a Zeiss Axiovert 200 fluorescent microscope through a 40X objective. The number of apoptotic granulosa cells/complex was calculated from images. Only complexes in which the oocyte was completely enclosed by granulosa cells were used for this analysis. Experiment was repeated three times and at least 30 complexes were included in each treatment group.

Trans-zonal projection and cortical actin quantification

Connections between oocytes and the surrounding granulosa cells were examined in 4d cultured PGOCs according to El-Hayek and Clarke (48). Briefly, oocytes were collected and denuded of granulosa cells by passing through a narrow bore glass pipet. Immediately after

removal of surrounding cells, oocytes were fixed for 1hr in 4% paraformaldehyde at room temperature. Cells were washed three times in PBS with 0.1% tween-20 and stained with Phalloidin-488 (Molecular Probes Cat# A-12379, Eugene, OR) at 1:100 in PBST for one hour to label actin filaments. Cells were washed and mounted on slides with prolong anti-fade mounting media plus DAPI. Cells were imaged with a confocal FV-10i microscope through a 60X objective. Fluorescence was measured in the perivitelline space, directly outside the plasma membrane and cortical actin ring using image J and corrected for background signal (58). To eliminate bias during the analysis, the region of interest was selected automatically by the software based on changes in fluorometric intensity. In this way, it was possible to select only the TZPs, without including the bright cortical actin ring or the empty region outside the perivitelline space. Cortical actin width was also quantified by drawing a linear ROI across the cell membrane (59). The full width at half max (FWHM) from the resulting emission spectra was calculated and used to compare groups. Cortical actin width was averaged from 8 equally spaced measurements/oocyte. Measurements were combined from three biological replicates and a minimum of 20 cells were included per treatment.

Western blot

SMAD2 activation was measured in PGOCs after 10 days of culture via western blot as described previously (9). Briefly, complexes were removed from the collagen membrane and snap frozen in LN2 in groups of 50. Pelleted cells were thawed in laemli buffer (Alfa Aesar) containing beta-mercaptoethanol and boiled 10 minutes. Proteins were separated on an 8-12% bis/tris gel using mini-page blot system and transferred to a PVDF membrane. Proteins were visualized according to standard immunoblotting procedures. Primary antibodies – Rabbit anti-pSMAD2 (Cell Signaling Technology Cat# 3101,Danvers, MA.) 1:1000, Rabbit anti-βactin

(Sigma-Aldrich Cat# A5060, St. Louis, MO.) 1:10,000. Secondary antibody – mouse anti rabbit-IgG HRP (Thermo Fisher Scientific Cat# 31464, Waltham, MA.) 1:15,000. Protein signal was quantified and background corrected with ImageJ.

Total RNA extraction and qPCR

Granulosa cells and oocytes were separated and snap frozen immediately after collection. RNA was isolated separately for each cell type. Total RNA was purified from 25 PGOC/replicate using the RNeasy Plus micro kit (Qiagen, Germantown,MD.) according to manufacturer's instructions. Total RNA was reverse transcribed into cDNA using quantitect RT kit (Qiagen). Gene expression was calculated according to the ddct method normalized to rpl19 gene expression as described (60). Primers used are listed in table 1. Amplification products were sequenced to validate primer specificity.

Digital image manipulation

All fluorescence and densitometry measurements were made on raw, unaltered images. Representative images have been adjusted uniformly across treatment groups according to recommended best practices (61).

Statistics

Meiotic progression after EGF treatment was compared at each meiotic stage using the Kruskal-Wallis one way anova of ranked variables. Post-hoc comparison of treatments at each meiotic stage was performed using the Mann-Whitney test. The rate of multi-nucleolar phenotype

was analyzed via chi-square analysis. All other experimental outcomes were analyzed with ANOVA. When a significant treatment effect was identified by ANOVA, Fisher's LSD method was used for post-hoc comparison. All statistical calculations were performed with minitab version 18. $P<0.05$ was considered significant in all cases. Data is presented as Mean±SEM.

Results

DTPA treatment for up to 10 days does not induce oocyte cell death

PGOC culture for up to 10 days did not lead to cell death in the oocytes, regardless of treatment (Figure 2). The use of the membrane impermeable chelator DTPA (10μM) is appropriate for long-term cell culture.

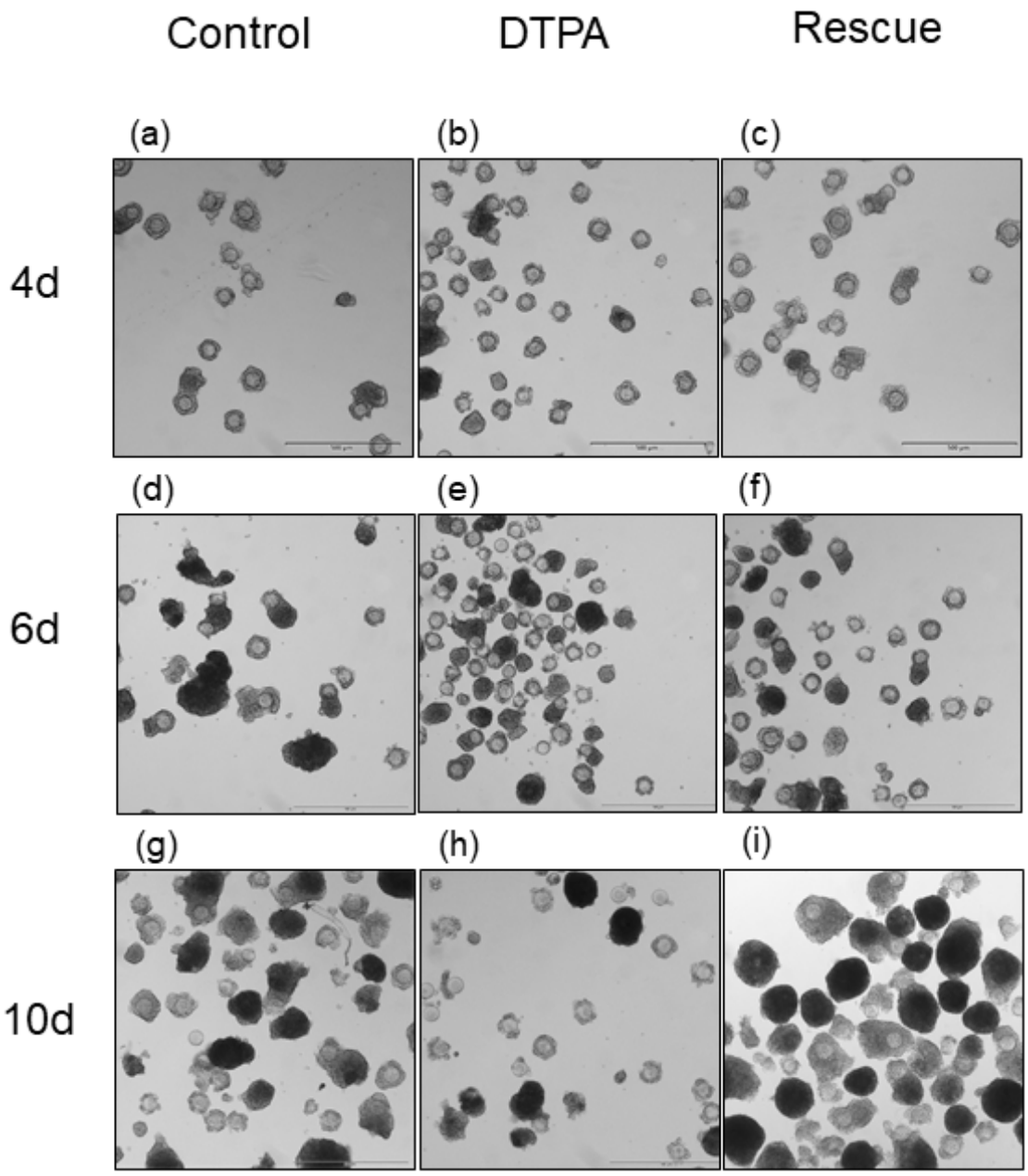


Figure 4-2: **DTPA treatment does not induce cell death in cultured oocytes**.

(a-i): Representative brightfield images of preantral oocyte-granulosa cell complexes after 4, 6, and 10 days of culture. 4X magnification; Scale bar - 500µM.

DTPA lowers oocyte diameter on day 4 and 6 of PGOC culture

Oocytes from PGOCs cultured in DTPA were smaller than control samples on day 4 and 6, but not day 10 of cell culture (Figure 3a). Oocytes cultured in rescue media were not statistically different from control at any time point. The most significant effect on oocyte diameter was seen on day 4 (Control 60.77±0.41μM, DTPA 58.40±0.31 μM, Rescue 60.64±0.40 μM; $P<0.001$). A smaller, though statistically significant difference was seen on day 6 of culture (Control 59.8±0.33μM, DTPA 58.69±0.28μM, Rescue 59.4±0.31 μM; $P=0.028$). There was no effect of treatment on day 10 of culture (Control 64.557±0.24 μM, DTPA 64.286±0.25 μM, Rescue 63.878±0.30 μM; $P=0.186$). Regardless of treatment, *in vitro* matured oocytes were smaller than fresh control oocytes collected from mice primed with 5IU pregnant mare's somatogonadotrophin (Fresh control 72.19±0.44μM).

DTPA culture decreases PGOC size

To further assess the development of cells in culture, we measured the two dimensional area of collected PGOCs on day 4, 6, and 10 of culture. This measurement includes both the oocyte, and attached granulosa cells after removal from the collagen membrane. DTPA treated PGOCs were significantly smaller than control at all timepoints examined (day 4: Control 10,776 ± 599.8 μM^2, DTPA 7,505 ± 306.8 μM^2, Rescue 11,114 ± 605.9 μM^2, $P<0.001$; day 6: Control 7,918 ± 431.2 μM^2, DTPA 6,490 ± 200.5 μM^2, Rescue 7092 ± 308.6 μM^2, $P<0.01$; day 10: Control 10,241 ± 390.2 μM^2, DTPA 7,368 ± 284.0 μM2, Rescue 9,313 ± 321.5 μM2, $P<0.001$). We observed a similar effect of treatment on both oocyte diameter and surface area on day 4 and 6 of culture, but not day 10 (Figure 3). On day 10 of culture, DTPA treated oocytes are not significantly smaller than control or rescue (figure 3a), but the surface area of the PGOCs is

significantly reduced (figure 3b). This indicates that fewer granulosa cells are present on DTPA treated PGOCs after collection on day 10.

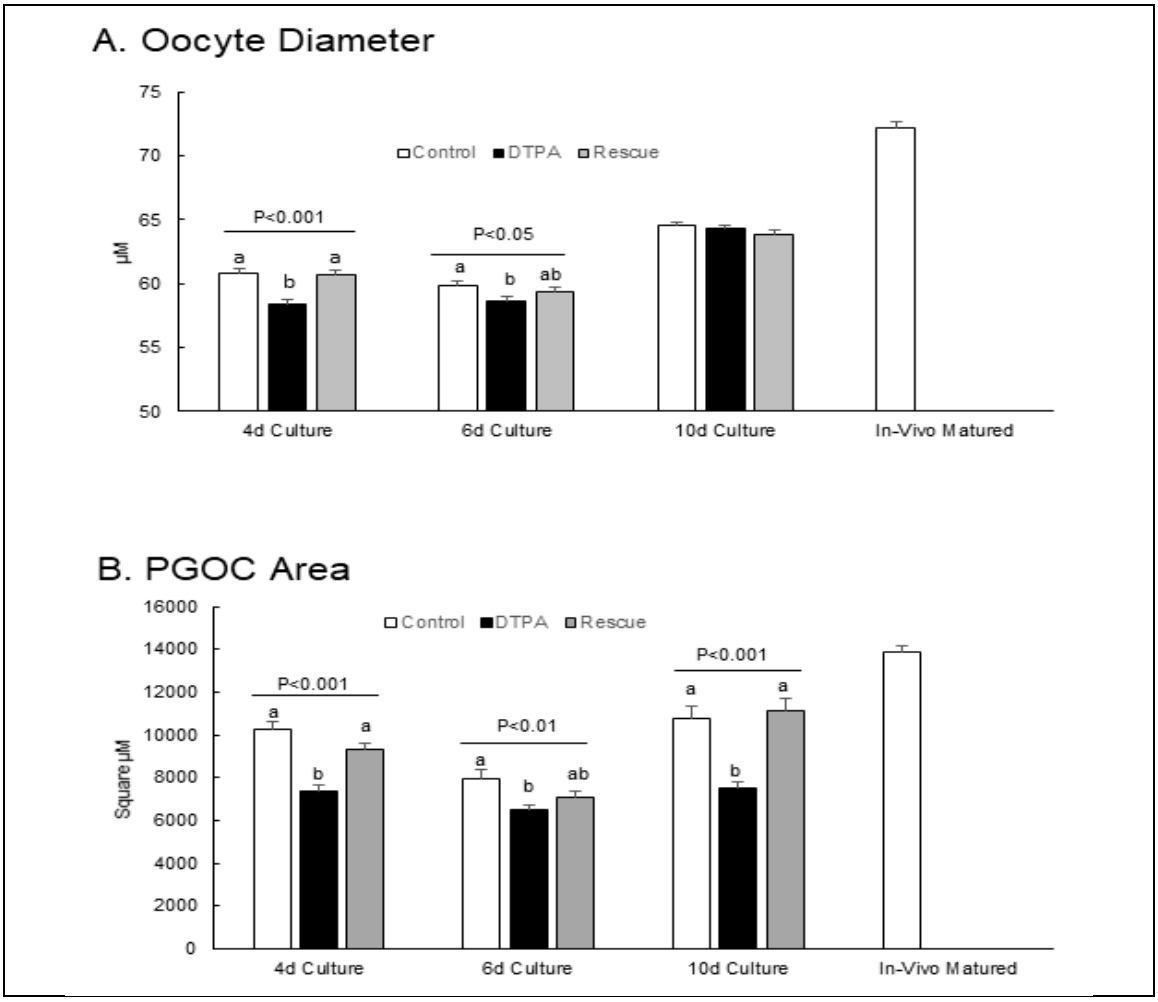


Figure 3. DTPA treatment reduces oocyte diameter and PGOC area.

(a) DTPA treated oocytes were significantly smaller than control on day 4 (Control 62.70±0.4µM, DTPA 60.86±0.36 µM, Rescue 63.26±0.39 µM; P<0.001) and day 6 (Control 59.8±0.33µM, DTPA 58.69±0.28µM, Rescue 59.4±0.33 µM; P=0.028). Zinc supplementation of DTPA treated media rescued this phenotype at both timepoints. No significant difference was observed on culture day 10 (Control 64.56±0.24µM, DTPA 64.29±0.25µM, Rescue 63.88±0.30µM; P=0.186). Cultured oocytes did not reach the diameter of *in vivo* matured fresh oocytes (72.19±0.44µM). (b) DTPA treatment reduced the overall area of PGOCs – a measure of both the oocyte and surrounding granulosa cells - on all days of treatment compared with control (day 4: Control 10,776 ± 599.8 μM^2, DTPA 7,505 ± 306.8 μM^2, Rescue 11,114 ± 605.9 μM^2, P<0.001; day 6: Control 7,918 ± 431.2 μM^2, DTPA 6,490 ± 200.5 μM^2, Rescue 7092 ± 308.6 μM^2, P<0.01; day 10: Control 10,241 ± 390.2 μM^2, DTPA 7,368 ± 284.0 μM^2, Rescue 9,313 ± 321.5 μM^2, P<0.001). The reduced total area on day 10 indicates a loss of granulosa cells, as there was no difference in oocyte dimensions.

DTPA culture disrupts zinc accumulation in the oocyte

To demonstrate that DTPA treatment impacts zinc homeostasis specifically, we examined the ability of treated oocytes to import zinc. After 4 days of culture, PGOCs were collected and mechanically denuded – a process previously shown to result in zinc accumulation within the oocyte (*250*). Denuded oocytes from each treatment group were then loaded with the zinc-specific fluorophore FluoZin-3 to visualize labile zinc within the oocytes. Zinc induced fluorescence was significantly lower in DTPA treated cells than in control or rescue (Figure 4 Control – 1.88a.u., DTPA – 0.79a.u., Rescue – 1.85a.u.; $P<0.001$). DTPA treatment of culture media impacts zinc accumulation in the oocyte.

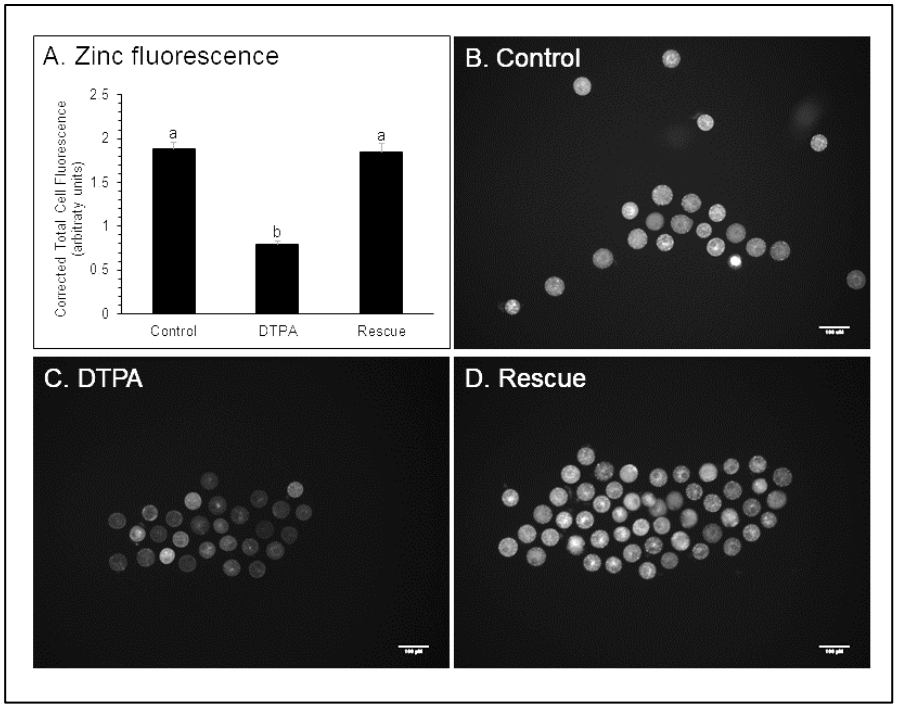
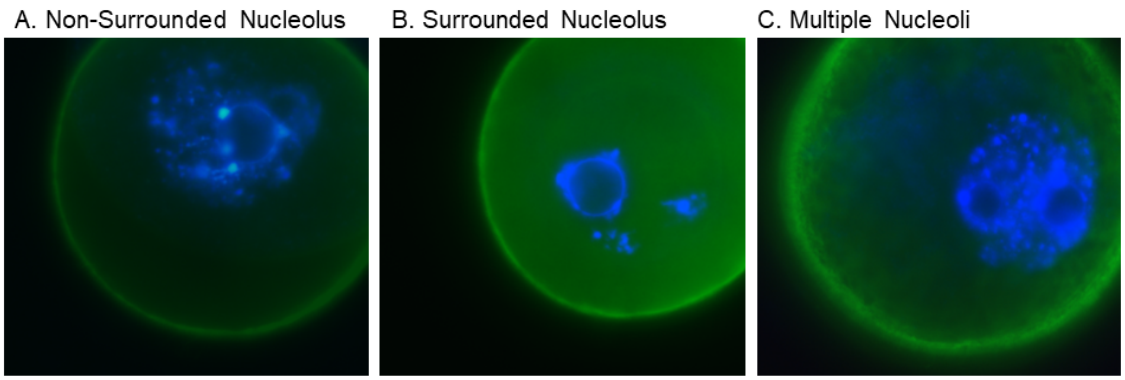


Figure 4-4: **DTPA treatment reduces zinc accumulation in the oocyte**.

To demonstrate that DTPA affects zinc levels in the oocyte, cells were cultured for 4 days in control, DTPA, or rescue media. PGOCs were collected and the oocytes were stripped of granulosa cells by passing through a narrow-bore glass pipet. This procedure has previously been shown to induce zinc accumulation in the oocyte. Cells were then loaded with a zinc fluorophore – Fluozin-3 – to visualize labile zinc accumulation in the oocyte. (a) Cells in DTPA treated media showed significantly lower fluorescence than control or rescue oocytes, indicating that the effects of DTPA are zinc specific. (b-d) Representative images – 10X. Scale bar - 100μM.

DTPA treatment induces a multi-nucleolar phenotype in cultured PGOCs

Nuclear maturation was evaluated on day 6 and 10 of PGOC cell culture using DAPI staining (Figure 5). Cells were evaluated based on chromatin condensation – surrounding nucleolus (SN) or non-surrounding nucleolus (NSN) phenotype – and number of nucleoli. No differences were observed between treatment groups in the ratio of SN:NSN cells. A multinucleolar phenotype was identified in DTPA treated complexes on day 6 of culture. Chi-square analysis revealed a highly significant treatment effect on the rate of multiple nucleoli in GV cells ($P<0.001$).



D. Proportion of SN and NSN oocytes

Days in Culture	Treatment	n	Degraded (%)	GV-Surrounding Nucleolus (%)	GV-Not Surrounding Nucleolus (%)	SN:NSN p value	Multi-nucleate	Multinucleate p value
6	Control	105	8 (7.6)	19 (18.1)	78 (74.3)	0.267	11 (10.5)	<0.001
	DTPA	185	15 (8.1)	23 (12.4)	147 (79.5)		63 (34.1)	
	Rescue	104	11 (10.6)	11 (10.6)	82 (78.9)		26 (25.0)	
10	Control	215	29 (13.5)	52 (24.2)	134 (62.3)	0.184	39 (18.1)	0.256
	DTPA	256	50 (19.5)	55 (21.5)	151 (59.0)		58 (22.7)	
	Rescue	224	28 (12.5)	40 (17.9)	156 (69.6)		48 (21.4)	

Figure 4-5: DTPA treated oocytes display a multinucleolar phenotype after 6 days of Culture.

(a) Representative image of a not-surrounding nucleolus (NSN) chromatin phenotype. (b) Representative image of a surrounding nucleolus (SN) phenotype. (c) Oocyte with two nucleolar bodies. Blue - DAPI staining; Green - actin. (d) – nuclear maturation data. DTPA treatment led to a significantly higher instance of oocytes with multiple nucleolar bodies on day 6 of treatment ($p<0.001$)

Zinc deficiency reduces the meiotic potential of cultured PGOCs

Progression to the M2 phase of meiosis at ovulation is required for successful oocyte fertilization and zygote formation. The ovulatory signal was simulated *in vitro* by transferring 10d cultured PGOCs to media containing 10ng/ml EGF. All oocytes appeared healthy and were GV arrested when placed into maturation media. Treatment had a significant effect on meiotic progression (Figure 6). DTPA treated oocytes were less likely to reach either the M1 (Control – 40.2±6.3%, DTPA – 10.2±11%, Rescue – 54.6±5.1%; $P<0.01$) or M2 (Control – 16.4±5.1%, DTPA – 3.4±1.4%, Rescue – 8.13±1.3%; $P<0.05$) stage of meiosis. Cells cultured in rescue media were not significantly different that control for any meiotic stage. Maturation media was the same for all cells and contained control levels of zinc.

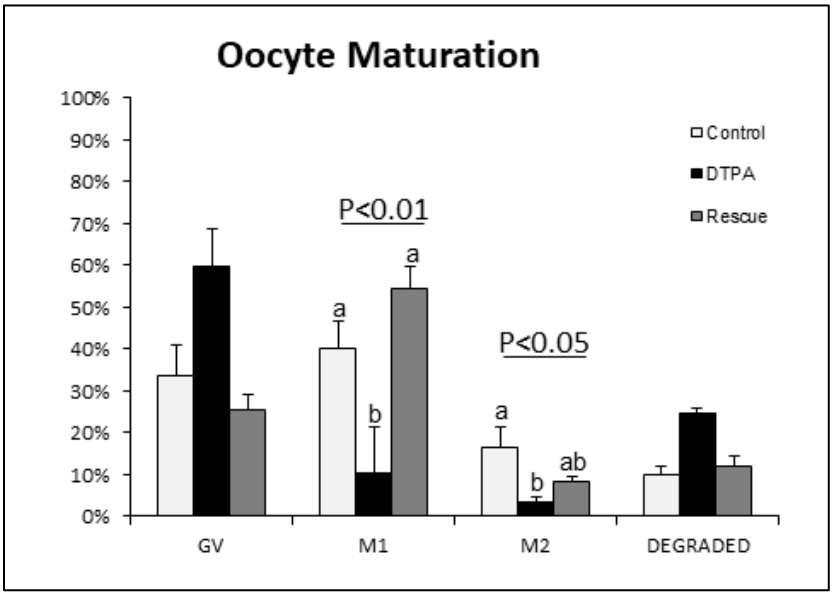


Figure 4-6: **DTPA treatment interferes with meiotic resumption after 10 days**

After 10 days of culture PGOCs were removed from treatment media and placed in maturation media containing 10ng/ml EGF. After 20 hours, oocytes were removed from surrounding granulosa cells and classified by meiotic developmental stage. DTPA treated cells did not progress through meiosis at the same rate as control or rescue cells. DTPA treated oocytes were less likely to reach the M1 or M2 stage of meiosis (Kruskal wallis test $P<0.01$ and $P<0.05$, respectively). The majority of DTPA treated cells did not undergo germinal vesicle breakdown (59.86%), and nearly 25% became degraded. Meiotic progression was not significantly different between control and rescue treated cells.

Zinc deficiency increases apoptosis in granulosa cells

Four days of culture with DTPA increased the number of apoptotic cells per complex compared with control or rescue groups (Control – 13.6±2.05, DTPA - 52.2±4.37, Rescue – 13.8±2.34; P<0.001). Apoptotic signaling was seen exclusively in the granulosa cells, not in oocytes (Figure 7).

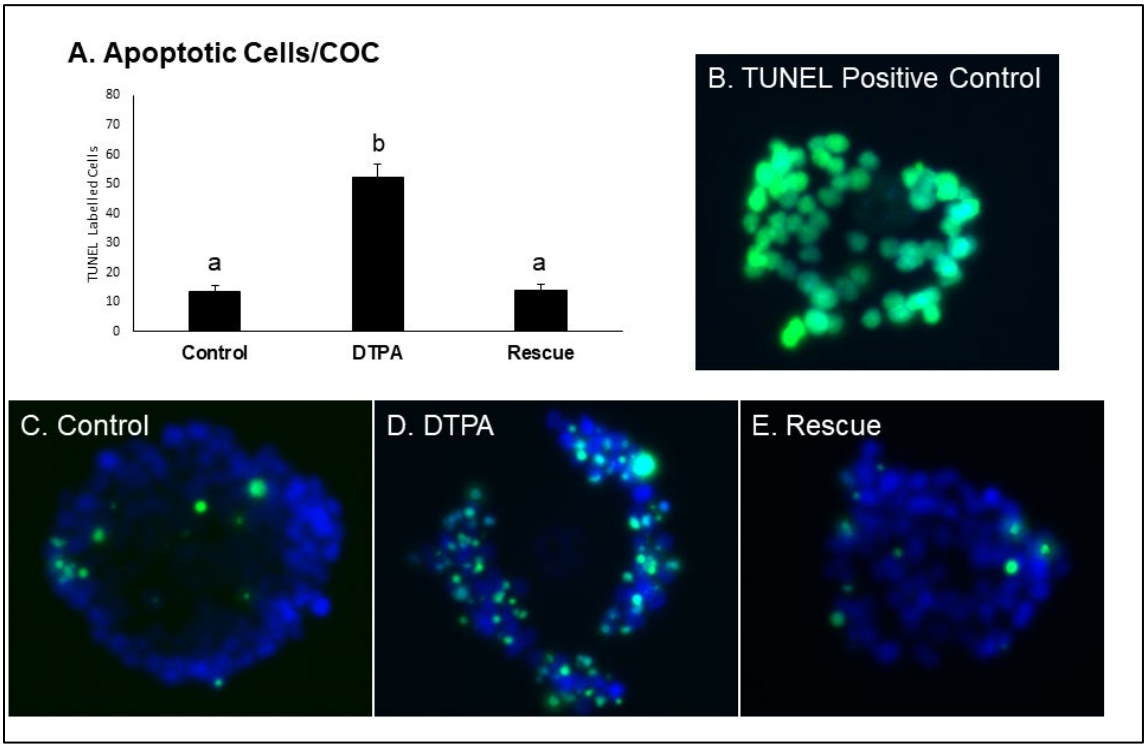


Figure 4-7: **DTPA treatment increases granulosa cell apoptosis after 4 days of culture.**

After 4 days of cell culture, a TUNEL assay was performed to compare apoptosis rates between treatments. (a) DTPA treatment significantly increased granulosa cell apoptosis (P<0.001). (b-e) Representative images -40X. Blue – DAPI staining; Green – TUNEL labelling.

Zinc deficiency reduces trans-zonal projections between oocytes and granulosa cells

Fluorescent labelling of actin filaments in the perivitelline space was used to quantify TZP connections between granulosa cells and the oocyte (Figure 8-G). DTPA treated cells had

lower fluorescent signal that control, indicating fewer connections between the oocyte and surrounding cells (P=0.023). There was no significant difference in TZP fluorescence between control and rescue treatments. Cortical actin width was also quantified but we detected no significant difference between treatments.

Zinc deficiency decreases SMAD2 activation in PGOCs

The oocyte derived factor GDF9 induces phosphorylation of SMAD2 in cumulus granulosa cells surrounding the oocyte. Western blot analysis of 10 day cultured PGOCs showed decreased levels of phosphorylated SMAD2 in DTPA treated granulosa cell-oocyte complexes compared with control ($P<0.05$, Figure 9). The pSMAD2:β-actin ratio demonstrates impaired signaling between the oocyte and surrounding somatic cells.

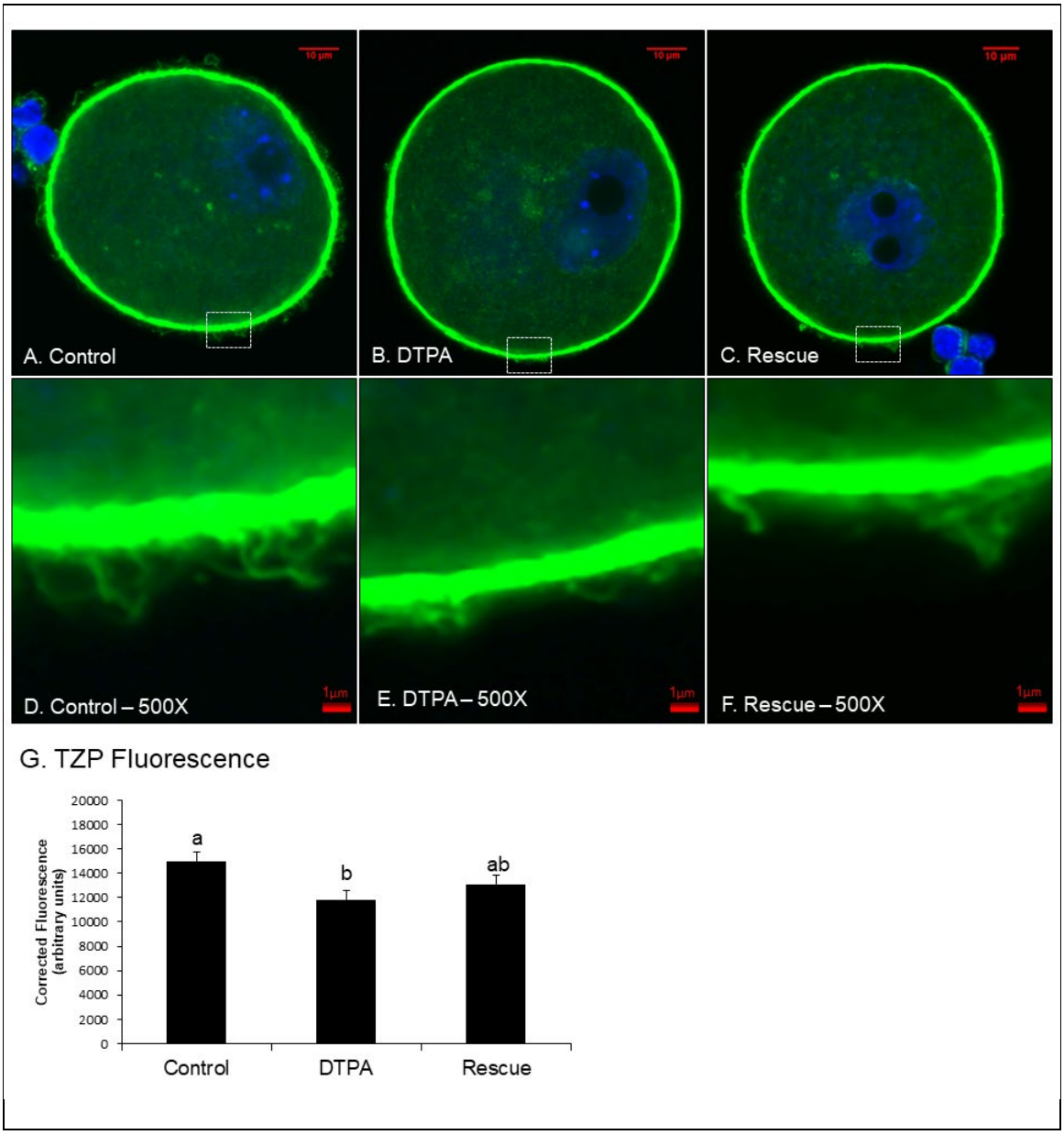


Figure 4-8: DTPA treatment reduces trans-zonal projections after 4 days of culture.

To observe changes in the density of trans-zonal projections in the perivitelline space, denuded oocytes were labelled with Phalloidin-alexa 488 at 1:100. Fluorescent signal was measured between the zona pellucida and the cortical actin ring using a confocal fluorescent microscope. (a-c) Representative confocal images – 100X. Scale bar - 10µM. (d-f) boxed region from a, b, c – 500X. Scale bar - 1µM (Blue – DAPI; Green – Actin.) (g) DTPA treatment significantly reduced fluorescent signal ($P<0.05$), corresponding to a loss of TZPs in the perivitelline space.

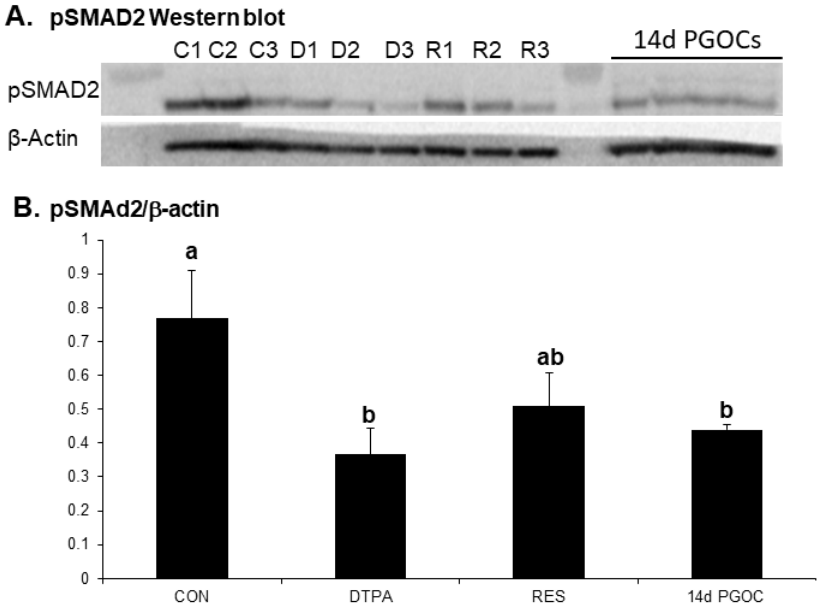


Figure 4-9: **DTPA treatment inhibits SMAD2 signaling in oocyte-granulosa complexes after 10 days of culture.**

Phosphorylated SMAD2 was measured via western blot and normalized to β-actin in PGOCs after 10d of culture. (a) Western blot showing pSMAD2 and actin control. At least three biological replicates from each treatment were run on the same gel. 50 PGOCs/lane (b) Graph of densitometry results. ANOVA showed a significant effect of treatment ($P<0.05$). DTPA treated cells showed no statistical difference in SMAD2 phosphorylation compared with fresh PGOCs that did not undergo *in vitro* maturation (14D PGOCs).

Zinc deficiency alters the gene expression profile of cultured granulosa cells.

Oocytes and somatic cells were separated prior to freezing in order to evaluate gene expression of each PGOC component separately. DTPA treatment lowered expression of the oocyte marker *figla* compared to rescue treatment (Figure 10-B. $P<0.01$). DTPA treatment disrupted gene expression in the granulosa cells which showed higher expression of the mural cell marker *lhcgr,* the cumulus cell markers *ar* and *fosb*, and the undifferentiated granulosa cell marker *pcsk6* when compared with control granulosa cells (Figure 10-A. $P<0.05$ for all). There

was no difference in expansion transcript markers between groups after exposure to 10ng/ml EGF (Figure 10-C).

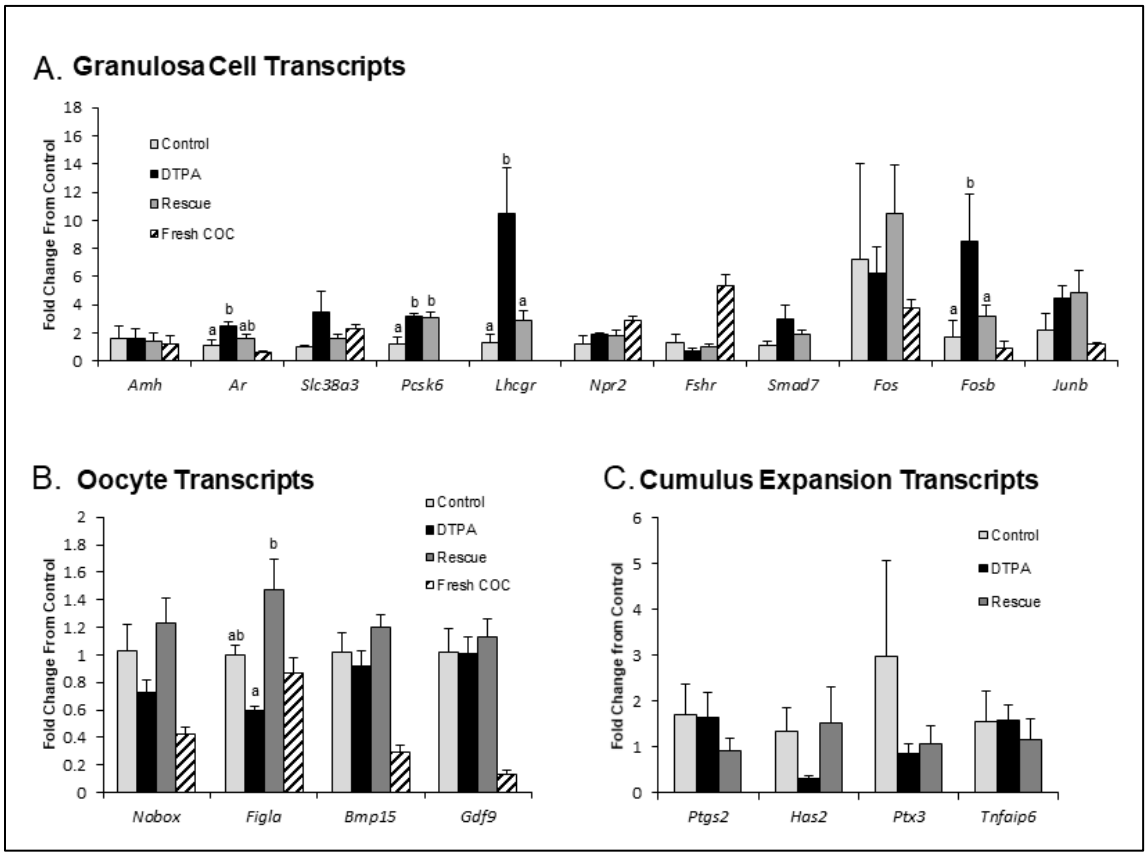


Figure 10-1: **DTPA treatment alters gene expression during *in vitro* maturation.**

(a) DTPA treated granulosa cells expressed higher levels of the mural cell marker *lhcgr*, cumulus cell markers *ar, and fosb,* and the undifferentiated cell marker *pcsk6* ($p<0.05$ for all). Transcript levels from fresh *in vivo* matured cumulus-oocyte complexes are shown for reference but were not included in the ANOVA. (b) DTPA treatment lowered expression of the oocyte marker *figla* compared with the rescue group ($p<0.01$). (c) There was no statistical difference in expression of expansion-related transcripts between the treatment groups. Expression represented as fold change over control. *Rpl19* transcript levels used as internal control.

Discussion

Extracellular zinc chelation with DTPA during prolonged cell culture

In order to investigate the effect of zinc deficiency on oocyte/follicle maturation and granulosa cell development, we have employed an *in vitro* system to grow preantral follicles in the presence of the metal chelator DTPA. Preantral granulosa cell-oocyte complex culture on a collagen membrane has been employed for *in vitro* follicle growth (*56, 256-258*), and has been previously employed to study the interaction of the oocyte and granulosa cells during preantral development (*98, 99, 243, 247, 259*). To our knowledge, this is the first study to combine preantral follicle maturation with a metal chelator to induce micronutrient deficiency.

We chose to employ the extracellular metal chelator DTPA rather than the intracellular zinc-specific chelator TPEN, as TPEN has been shown to induce cell death after prolonged culture (*255*). Extracellular chelation of zinc in the media, rather than intracellular chelation is also likely a more physiologically relevant model as it places the cell into a zinc deficient environment akin to zinc-deficient serum/plasma, rather than immediately disrupting the zinc-dependent processes occurring within the cell by intracellular chelation. DTPA and TPEN both have a much higher affinity for binding zinc (TPEN $K_d = 10^{-15.58}$ M, DTPA $K_d = 10^{-18.75}$ M) and divalent iron (TPEN $K_d = 10^{-14.61}$ M, DTPA $K_d = 10^{-16.55}$ M) compared to their affinity for other metals. DTPA has a relatively higher affinity than TPEN for calcium (TPEN $K_d = 10^{-4.4}$ M, DTPA $K_d = 10^{-10.74}$ M) and magnesium (TPEN $K_d = 10^{-1.7}$ M, DTPA $K_d = 10^{-9.3}$ M), but these are still much lower than DTPA's affinity for zinc. Since DTPA has the ability to chelate metals other than zinc, the use of a rescue treatment which combined DTPA and additional exogenous zinc was key in order to establish the zinc-specific requirement for preantral follicle growth and development. Prior studies have added DTPA to cell culture media in concentrations as high as

600μM (*255*). However, pilot studies with ovarian follicles resulted in oocyte death and follicle disassembly when cells were exposed to concentrations above 25μM for more than 96h (unpublished data). We have therefore selected 10μM as the optimal DTPA concentration to chelate zinc during longterm preantral follicle cell culture. Estimates of zinc levels in human follicular fluid range from 1-11μM (*202, 260-262*). The rescue treatment used in this study (10μM DTPA + 50μM $ZnSO_4$) is within one order of magnitude of the physiological range for zinc. The effect of our treatment on zinc movement into the oocyte was tested using the zinc fluorophore fluozin-3. We have demonstrated a significant reduction of zinc accumulation in the oocyte (Figure 4). Zinc-mediated fluorescence was reduced by approximately 50% in the DTPA treated cells, indicating a reduction of labile zinc inside the DTPA treated oocytes. Rescue treatment returned fluorescence to control levels.

DTPA treatment compromises oocyte and granulosa cell development

DTPA-treated preantral follicles displayed a delayed oocyte growth trajectory, fewer granulosa cells surrounding the oocyte, and nucleolar abnormalities in the oocyte compared with cells cultured under control conditions. Zinc supplementation of DTPA media rescued these defects, demonstrating that zinc is required for proper growth and maturation of oocytes from preantral follicles. Most notably, DTPA treated oocytes were less likely than controls to undergo germinal vesicle breakdown and reach the MI or MII stage when placed into maturation media. All maturation media contained adequate and equal levels of zinc, indicating a zinc requirement during development in addition to the zinc requirement for meiotic resumption shown in previous studies (*184, 188, 209, 263-265*). The inability to resume and complete meiosis indicates a severe loss of fertility in those oocytes, as meiotic resumption and division are a prerequisite for proper embryo development after fertilization.

Oocyte growth in the preantral and early antral stage is a key process in follicle development. While changes in oocyte diameter may be slight, the associated increase in cell volume is substantial. Previous studies have shown that while the diameter of oocytes from small antral follicles increases only about 4% before ovulation, the attendant increase in cell volume is approximately 13% (*74*). Increased cell size is correlated with the ability to reach blastocyst after fertilization (*256*). As we see growth restriction under zinc deficient conditions on days 4 and 6 of culture (Figure 3), it is not surprising that this group also displayed less developmental potential (Figure 6). It is surprising that we observed no treatment effects on oocyte diameter on day 10. This may indicate a compensatory physiological mechanism for oocyte growth under zinc deficient conditions, or that the oocyte growth curve was merely shifted but retained the same maximum potential. Regardless, although zinc deficient oocytes eventually reached the same diameter as control or rescue groups, their ability to undergo germinal vesicle breakdown remained compromised. In humans, oocyte size is often used as a parameter for oocyte selection for IVF (*266*). If confirmed in humans, these results indicate that oocyte selection based on diameter alone may not exclude cells compromised by nutrient deficiency during development.

Chromatin maturation is an accepted marker of oocyte developmental potential. Preantral oocytes exhibit a diffuse DNA arrangement (non-surrounded nucleolus, NSN – Figure 5a) during their growth phase. As oocytes gain the ability to resume meiosis, the nucleus undergoes a dramatic rearrangement characterized by condensation of chromatin around the nucleolus (surrounded nucleolus, SN – Figure 5b). This shift is concurrent with transcriptional silencing in the maturing oocyte (*94, 267*). We hypothesized that zinc deficiency would reduce the number of oocytes displaying the surrounding-nucleolus (SN) phenotype, as this is a marker of oocyte maturity. Instead, while there was no significant treatment effect on the ratio of SN to NSN oocytes, we were surprised to encounter a multi-nucleolar phenotype in zinc-deficient oocytes on day 6 of culture (Figure 5-D).

Multiple nucleoli are common in the nucleus of growing oocytes, but this phenotype decreases as oocytes reach maturity (*237*). Growing oocytes contain nucleoli similar to somatic cells, but as the oocyte reaches maturity, the nucleoli adopt a compact physiological arrangement called the Nucleolar Precursor Body (NPB) (*268*). Advances in nucleolar staining have recently revealed that the nucleoli of mature SN oocytes contain little ribosomal RNA (*269*), indicating ribosome biogenesis is not the primary function of nucleoli at this stage. The oocyte NPB is required for subsequent embryonic development, as removal of the NPB leads to developmental arrest at the two-cell stage. In contrast, removal of nucleoli from developing zygotes has no effect on embryonic development (*268*). Genetic models in which NPB organization is disrupted by knocking out the protein NPM2 display subfertility as a result of unsuccessful mitotic division after fertilization (*270, 271*). These studies indicate that the primary role of the fully grown oocyte nucleolus (NPB) is coordination of DNA segregation during meiosis and early mitosis, rather than simply ribosomal biogenesis as in the somatic cells. One intriguing possibility is that delayed growth under zinc deficient conditions necessitated prolonged ribosome production by the oocyte, thereby disrupting the maturation of the nucleoli to the NPB morphology. Disrupted nucleolar development in our zinc deficient model should be investigated as a potential cause of subfertility, rather than simply an indicator of delayed oocyte maturation.

The temporal gene expression patterns of maturing oocytes and granulosa cells have been well characterized (*95, 272, 273*). Under normal conditions, the maturing preantral oocyte is marked by elevated expression of oocyte markers *nobox, figla, sohlh1, sohlh2, zp3,* and the secretory oocyte derived factors *gdf9* and *bmp15*. GDF9 and BMP15 induce a cumulus cell phenotype in the surrounding preantral granulosa cells through phospho-SMAD signaling (*99, 259, 274*). Cumulus cells surrounding the oocyte are characterized by increased expression of *slc38a3, ar, npr2,* and *amh (99, 116, 119).* Cumulus cells have also been shown to express higher levels of the AP-1 protein transcripts – *fos, fosb, jun, junb (95)*– which are associated with

granulosa cell differentiation (*275*). Preantral granulosa cells farther from the oocyte differentiate into mural granulosa cells under the influence of FSH and upregulate expression of the LH receptor (*Lhcgr)* and C*yp19a1.* Finally, fully developed cumulus cells exposed to an ovulatory stimuli express the expansion related transcripts *has2, ptx3, ptgs2, and tnfaip6.* Both expression and translation of expansion transcripts rise in response to ovulation and are required for cumulus cell expansion (*276-280*), an integral event in normal ovulation and fertility (*102*).

To investigate changes in gene expression resulting from zinc deficiency, we first separated oocytes from somatic cells after 10 days of culture and processed each cell type separately. By separating the cell types, it was possible to compare transcript levels between groups and treatments despite possible differences in granulosa cell number (Figure 10). DTPA treatment decreased expression of *figla* in the oocyte, an effect noted previously during *in vivo* zinc restriction experiments in our lab (*188*). While the levels of GDF9 and BMP15 transcripts in the oocyte were not different between groups, the induction of cumulus cell gene expression was not seen in the zinc-restricted granulosa cells. DTPA treated granulosa cells displayed significantly higher expression of *lhcgr* (mural cell marker), *ar* and *fosb* (cumulus cell markers), and *pcsk6* (undifferentiated granulosa cell marker) compared with controls. These results indicate a dysregulation of granulosa cell differentiation in the zinc deficient preantral follicle. Expansion related transcripts were not different between groups, and no group was able to successfully undergo cumulus cell expansion in response to EGF. This was in contrast to our previous study with hybrid C57BL/6J X SJL F1 mice which did develop the capacity to undergo cumulus expansion (*101*). Strain differences may explain this discrepancy. CD-1, the strain used in our current study, is an outbred strain and may have different requirements for *in vitro* growth. For example, CD-1 preantral follicles may requirement FSH stimulation which we previously showed promotes development of cumulus cell phenotype (*98*) but also caused premature induction of expansion transcripts and excessive cell proliferation, which is why FSH was not used here.

Eliminating FSH from our protocol also enabled focused hypothesis testing on the role of zinc in paracrine signaling within the ovarian follicle.

The oocyte derived TGFβ superfamily members GDF9 and BMP15 induce changes in granulosa cells via SMAD signaling (*274, 281*). Ligands activate a membrane-bound receptor complex which phosphorylates target SMAD proteins in the cytoplasm of the cell. pSMADS then form heteromeric complexes with SMAD4 (co-SMAD) and migrate to the nucleus to alter transcription (*72*). GDF9 promotes SMAD2 phosphorylation in the granulosa cells and has been shown to drive cumulus cell differentiation including CYP19a expression (*282*) and expression of expansion related transcripts (*274*). Unsurprisingly, pSMAD2 levels are therefore highest in the cumulus cells of large preantral follicles (*283*). Despite similar levels of GDF9 and BMP15 expression by oocytes in all treatments, SMAD2 activation was severely impaired by DTPA treatment (Figure 9). Western blotting of oocyte-granulosa complexes on day 10 of culture showed a reduction of phosphorylated SMAD2. Zinc restricted cells displayed pSMAD2 levels similar to freshly collected preantral follicles, likely indicating either an inability of cumulus cells to respond to GDF9 or reduced secretion of GDF9 protein by the oocyte. A loss of granulosa cells during DTPA treatment may also contribute to the reduced pSMAD2 signal. These results, combined with the gene expression profiling described above, indicate that the ability of oocytes to induce cumulus cell differentiation is zinc dependent during *in vitro* maturation.

Zinc restriction impairs oocyte-granulosa cell interactions after four days of treatment

Ten days of culture were necessary before control PGOCs were capable of resuming meiosis and reaching the M2 stage when transferred to maturation media. While oocytes in the zinc deficient medium survived this culture period, they were associated with fewer granulosa cells (Figure 3). Therefore, in order to explore oocyte-granulosa cell interactions and paracrine

signaling more closely, we performed a second round of experiments on PGOCs after 4 days of culture. DTPA treated oocytes at this stage were still surrounded by a complete layer of granulosa cells. We examined rates of apoptosis, and physical connections between the oocyte and granulosa cells (trans-zonal projections, TZPs).

TUNEL assay revealed increased apoptosis in the granulosa cells after 4 days of DTPA treatment (Figure 7), likely explaining the reduced somatic cell numbers in DTPA-treated samples on day 10. Increased apoptosis in the cumulus cells of human IVF patients correlates with negative IVF outcomes (*284*). Increased apoptosis may be further evidence of impaired TGFβ signaling between the oocyte and granulosa cells, as oocyte secreted factors protect the granulosa cells from apoptosis under ordinary conditions (*121*). Apoptosis may also be a direct effect of zinc chelation, as zinc has demonstrated anti-apoptotic effects in a variety of contexts (*285-287*), however the chelator used in these studies (DTPA) does not enter the cell and has not been previously shown to induce apoptosis even when used at significantly higher concentrations (*251, 255*). The presence of apoptotic granulosa cells might also explain the aberrant and varied gene expression seen in DTPA-treated granulosa cells.

Disrupted oocyte-granulosa cell communication was also evident in the reduction of trans-zonal projections (TZPs) connecting DTPA treated oocytes to the surrounding granulosa cells after 4 days of culture (Figure 8). TZPs are actin-rich projections that originate from granulosa cells, pierce the zona pellucida, cross the perivitelline space and contact the oocyte. TZPs terminate in gap junctions, through which cumulus cells provide the oocyte with nutrients and substrates for energy metabolism (*114, 115*), as well as the KIT ligand, a growth factor shown to promote oocyte growth during development (reviewed in (*100*)). In this study, we employed phalloidin staining to label TZPs and measured fluorescence in the perivitelline space to compare TZP connections between treatment groups. The paracrine communication between the oocyte and granulosa cells is critical for proper energy metabolism in both cell types (*244,*

259). Compromised energy production, as well as reduced transfer of KIT ligand to the oocyte due to TZP reduction may explain the growth disruption seen in our DTPA treated samples. Whether DTPA treatment reduced cell-to-cell connections as a result of fewer granulosa cells surrounding the oocyte, or interfered with TZP formation itself is not clear from this study and remains to be investigated.

Conclusion

In this study, we have demonstrated that preantral follicle growth and development is impaired by treatment with the chelator DTPA, and largely rescued by supplementing the chelated media with zinc. While the reduced rate of meiotically competent oocytes is the most direct evidence of compromised fertility resulting from zinc restriction, the granulosa cells surrounding the oocyte appear to have been the primary site of damage from DTPA treatment. Granulosa cells showed an increased rate of apoptosis, fewer trans-zonal projections to the oocyte, and an inability to express cumulus cell transcripts in response to oocyte derived TGFβ signaling. *In vivo* matured cumulus cells contain high levels of labile zinc in the preovulatory cumulus-oocyte complex (*250*). It is not surprising that damage from zinc restriction would therefore be seen first in the granulosa cells. The impacts of zinc restriction on preantral oocyte development are likely mediated in part through a loss of paracrine signaling and metabolic support from the granulosa cells.

During antral follicular development there are severe defects associated with dietary zinc deficiency after just 4-5 days including defects in ovulation and maturation of the oocyte (*187, 188*). The effect of zinc deficiency on preantral follicle development remains to be tested *in vivo*. The results of this study lead us to hypothesize that zinc deficiency not only impacts the ovulatory and developmental potential of oocytes from large antral follicles, but also harms oocyte growth

and development at the preantral stage. Compromised fertility resulting from impaired preantral follicle development will be difficult to evaluate *in vivo* due to the extended developmental period prior to ovulation. More directly, these results underscore the critical need for proper zinc levels during *in vitro* maturation and fertilization. Zinc supplementation during oocyte culture has shown benefits in a number of livestock and model species (*203-206*). These studies, as well as the established reproductive deficits of zinc deficiency, point to zinc optimization as a possible avenue for improved ART protocols in humans and animals.

Table **4-1**: qPCR primer list

Gene Symbol	Forward Primer (5'-3')	Reverse Primer (5'-3')
Amh	AGCTGGACACCATGCCTTT	TTCGAAGCCTGGGTCAGA
Ar	TCACCAAGCTCCTGGATTCT	TGGGCACTTGCACAGAGA
Bmp15	ACACAGTAAGGCCTCCCAGA	GATGAAGTTGATGGCGGTAAA
Figla	ACAGAGCAGGAAGCCCGTA	GTCAGAGGGTCTGCCACTGT
Fos	ATGGGCTCTCCTGTCAACAC	TCAAGTTGATCTGTCTCCGCTT
Fosb	CCCGAGAAGAGACACTTACCC	GAAGCCGTCTTCCTTAGCGG
Fshr	CCAGCCTTACCTACCCCAGT	GATCCCCAGGCTGAGTCATA
Gdf9	CTACAATACCGTCCGGCTCT	CAAGTGTTCCATGGCAGTCA
Has2	CGAGTCTATGAGCAGGAGCTG	GTGATTCCGAGGAGGAGAGACA
Junb	CGCCCGGATGTGCACGAAAATG	GCGCCCCAGGACCCTTGAGACC
Lhcgr	GGATAGAAGCTAATGCCTTTGACAAC	TAAAAGCACCGGGTTCAATGTATAG
Nobox	AGGGACGTTCCTGGCAGT	GCTGCTTGCTTGGTAGTCCT
Npr2	GCTGACCCGGCAAGTTCTGT	ACAATACTCGGTGACAATGCAGAT
Pcsk6	CGAGGGCTCCAGTAGGAACT	CCGATTGGACTTCACCATCT
Ptgs2	TCCATTGACCAGAGCAGAGA	TTCTGCAGCCATTTCCTTCT
Ptx3	TGGCTGAGACCTCGGATGAC	GCGAGTTCTCCAGCATGATGA
Slc38a3	TATCTTCGCCCCCAACATCTT	TGGGCATGATTCGGAAGTAGA
Smad7	CAGGCTGTCCAGATGCTGT	CCTCCCAGTATGCCACCA
Tnfaip6	GAACATGATCCAGGCTGCTT	GGTCATGACATTTCCTGTGCT

Acknowledgements

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Chapter 5

Zinc deficiency disrupts germ cell nest breakdown during *in vitro* ovary culture

Short title: zinc and follicle assembly

Author Contribution Statement: James Hester and Francisco Diaz planned all experiments. JH performed all experiments and analysis with the exception of whole-mount Immunocytochemistry (ICC). JH wrote the manuscript. Manuscript reviewed and edited by JH and FD. Immunocytochemistry performed by Lindsey Banks under the direction of Melissa Pepling – Syracuse University, Dept. of Biology.

Abstract

In mammals, the size of the non-renewable primordial follicle pool is established prior to or soon after birth. Primordial follicles, composed of a single oocyte surrounded by somatic cells, are the only source of gametes during the entire reproductive span of the female. The size of the initial follicle reserve is a key component in determining reproductive longevity; and impaired follicle assembly has severe consequences for fertility. Here, we evaluate the effect of zinc deficiency on two parameters of primordial follicle assembly – meiotic progression and nest breakdown - using *in vitro* organ culture of mouse ovaries. Meiotic progression, apoptosis, germ cell number, and gene expression were not different between treatment groups during fetal ovary culture. Germ cell nest breakdown, which occurs in the newborn mouse ovary, involves invasion of germ cell cysts by somatic cells to form primordial follicles. Nest breakdown was disrupted by zinc deficiency leading to fewer oocytes enclosed in follicles ($P=0.002$). Gene expression of both an oocyte specific factor (*Bmp15*) and granulosa cell specific factor (*Foxl2*) shown to regulate nest breakdown were decreased by zinc deficiency ($P<0.05$). There was also a strong trend toward fewer activated growing follicles after zinc deficient culture of newborn ovaries ($P=0.051$). The initial wave of activated follicles is key for both paracrine and endocrine signaling to control ovarian function. Disruption of this process, as well as impaired primordial follicle formation may impact subsequent fertility.

Introduction

In mammals, female germ cells divide mitotically only during fetal development. Therefore, at birth the ovary contains the lifetime supply of oocytes available for reproduction (*31*). The oocytes are surrounded by somatic pregranulosa cells in structures known as primordial

follicles – a dormant structure that may persist for months in mice or decades in humans. The number of follicles in the ovary at any time is referred to as the ovarian reserve. The initial size and maintenance of the primordial follicle pool is the determining factor in reproductive longevity (288), as the depletion of the ovarian reserve leads to reproductive senescence (25). Factors that affect primordial follicle assembly and activation are therefore intriguing targets for understanding and treating infertility. In particular, this line of research holds promise for treating Premature Ovarian Insufficiency (POI), a disorder in 1% of women characterized by rapid depletion of the ovarian reserve leading to early menopause (289, 290). Prenatal and neonatal nutrition have been shown to affect formation of the follicle reserve, as starvation in rodent models reduces primordial follicle numbers while increasing germ cell apoptosis (152). The role of specific micronutrients during this period is less clear.

The gestation period of the mouse is 20-22 days. Primordial germ cells first appear on embryonic day 8 (E8), reach the developing gonad on E10, and divide mitotically until E13.5 (*11*). At the end of mitosis, the cells are found in germ cell cysts formed from incomplete cytokinesis during division (*12*). Beginning on E13.5, the germ cells enter meiosis under the influence of retinoic acid arising from the mesonephros (*291*). Germ cells pass through the early stages of meiosis, including recombination, before arresting at the diplotene stage of meiotic prophase I. The first diplotene oocytes appear on E17.5, and the % of diplotene oocytes increases over the next 4 days until all oocytes reach diplotene arrest (*21*). Birth initiates the breakdown of germ cell nests into individual follicles composed of one oocyte enclosed by somatic cells. The loss of estrogen signaling is the likely initiator of nest breakdown in rodents (*22, 23*). A disruption of either meiotic progression or nest breakdown may compromise fertility. After assembly, the majority of primordial follicles become dormant until paracrine signals activate the follicle to enter a growth phase (*292*). However, a subset of medullary follicles activates immediately after assembly with no dormant phase (*67*). These follicles are unlikely to ovulate

but produce both endocrine and paracrine factors that are hypothesized to promote development of the hypothalamic-pituitary-ovarian signaling axis, as well as regulate the activation in the dormant follicle pool (*66*).

One factor that plays a role in follicle development is the essential micronutrient zinc. Zinc plays an integral role in oocyte development during follicle growth (unpublished work – manuscript in preparation) and at ovulation (*187, 188, 190, 209*). In the fully developed ovulatory follicle, proper zinc homeostasis is necessary to control meiotic division (*143, 183-185, 293*), but it's role in the early stages of meiosis have not been examined in mammals. Our previous study in the nematode *C. elegans* showed that zinc deficiency can impair early meiotic progression, particularly the pachytene-to-diplotene transition (*151*). A zinc requirement for oocyte and follicle development in the fetal ovary would be a significant finding, as 82% of pregnant women worldwide consume less than the recommended dietary allowance for zinc (*207*).

To evaluate the effects of zinc deficiency on early meiotic progression and germ cell nest breakdown, we have employed an *in vitro* organ culture system using mouse ovaries. Zinc deficient media was created with the addition of the zinc-specific chelator TPEN (N,N,N′,N′-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine). As meiosis and nest breakdown are temporally distinct in the mouse, we conducted two separate studies using either fetal or newborn mouse ovaries (Figure 1). Establishing a zinc requirement for the fetal ovary will provide new insight into female fertility, and may have implications for nutritional recommendations and micronutrient supplementation during pregnancy.

Study 1: Zinc requirement for diplotene progression

Objective: To test the zinc requirement for meiotic progression to diplotene in the mouse.

Method: Fetal ovaries collected on embryonic day 16.5 and cultured for 120 hours to the equivalent of PND1.

Treatments: Control media, zinc deficient (TPEN 1µM), Rescue (zinc deficient media + ZnSO4 1µM)

Study 2: Zinc requirement for nest breakdown

Objective: To test the zinc requirement for germ cell nest breakdown

Method: Newborn ovaries collected and cultured for 96 hours to the equivalent of PND5.

Treatments: Control media, zinc deficient (TPEN 1µM), Rescue (zinc deficient media + ZnSO4 1µM)

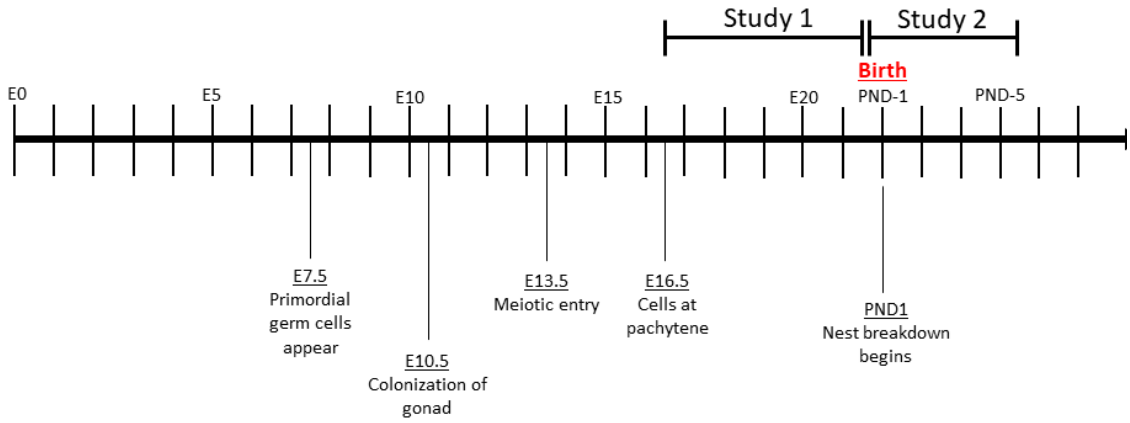

Figure 5-1: Study Outline

Methods

Animals

CD-1 mice (*mus musculus*) were commercially obtained from Charles River Laboratory (Wilmington, MA). Animal care and handling was reviewed and approved by the Institutional Animal Care and Use Committee of Penn State. Animals were maintained on a 14:10 light/dark cycle and fed ad libitum. One adult male and one adult female were housed per cage. Females

were checked for the presence of a vaginal mating plug each morning in order to monitor pregnancy onset. The morning of plug discovery was designated as embryonic day 0.5 (E0.5). To collect fetal ovaries, pregnant dams were euthanized by CO2 asphyxiation followed by cervical dislocation on E16.5. Parturition most often occurred on the equivalent of E21.5 and was designated post-natal day 1 (PND1). To collect newborn ovaries, pups were euthanized by decapitation on PND1 prior to dissection.

Ovary Culture

Ovaries were collected from fetal and newborn mice immediately after euthanasia. Ovaries were stored in Hanks Balanced Salt Solution on ice until collection was complete. Ovaries were cultured on a porous polycarbonate membrane floated on culture media. Culture media was distinct for fetal and newborn ovaries. For fetal ovary culture, ovaries were cultured in DMEM-Ham's F-12 media supplemented with penicillin-streptomycin, ITS, 0.1% BSA, 0.1% albumax, 0.05 mg/ml L-ascorbic acid, 10μM estradiol, and 10μM progesterone (hormones added to prevent premature nest breakdown). For newborn ovary culture, ovaries were cultured in Waymouth 752/1 medium supplemented with 0.23 mM pyruvic acid, penicillin-streptomycin, and 10% fetal bovine serum. Zinc deficient media was created by adding the zinc specific intracellular chelator N,N,N′,N′-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN; sigma) dissolved in DMSO to a concentration of 1μM. Rescue media contained 1μM TPEN and 1μM $ZnSO_4$ (sigma). Fetal ovaries were cultured for 5 days to the equivalent of E21.5. Newborn ovaries were cultured for 4 days to the equivalent of PND5.

Histology

After organ culture, ovaries were fixed in Bouin's fixative for two hours at room temperature, followed by an ethanol dehydration series. Ovaries were paraffin-embedded and serially sectioned at 4μM. Hematoxylin and eosin staining was accomplished using standard procedures. Stained sections were imaged with a Nikon eclipse TE200 inverted microscope fitted with an Olympus DP20 brightfield camera. Every 5th section was analyzed as follows: For cultured newborn ovaries, every 5th section was analyzed by 3 independent researchers, two of whom were blinded to treatment. Total oocyte number, oocytes in nest (two or more oocytes proximal to each other with no intervening somatic cells), primordial follicles (one oocyte completely surrounded by granulosa cells), and activated follicles (identified by cuboidal granulosa cells surrounding the oocyte) were counted per section. An average of 7 sections were analyzed per ovary. Data from all three researchers was averaged for each tissue section. Section counts were combined to provide counts per ovary. 5 ovaries were analyzed per treatment group. To calculate oocyte density (oocytes/area), the surface area of each tissue section was calculated with imageJ. Cultured fetal ovaries were fixed, embedded, sectioned, and stained identically to newborn ovaries. For fetal ovary analysis, total oocyte count, total diplotene oocytes, and total prediplotene oocytes were counted on every 5th tissue section. Researcher was blinded to treatment group prior to analysis. Meiotic phase was judged from chromosomal morphology (*21, 294, 295*). Briefly, diplotene oocytes display areas of condensed chromatin interspersed with clear areas that show no staining. Prediplotene oocytes are characterized by long chromatin threads (leptotene), thick chromatin "bouquets" (zygotene), or thick chromatin strands (pachytene). An average of 9 sections were analyzed per fetal ovary and 2-5 ovaries were evaluated per treatment. To eliminate the possibility of double counting oocytes, only cells displaying nuclear staining were quantified in either the newborn or fetal ovaries.

Total RNA extraction and qPCR

After culture, ovaries were snap frozen and stored at -80C prior to RNA extraction. Total RNA was extracted with the Absolutely RNA Microprep kit (Agilent Technologies) and reverse transcribed into cDNA using quantitect RT kit (Qiagen). cDNA was evaluated for stable expression of three housekeeping genes – *Rpl19*, *Gapdh*, and *βactin* – by qPCR. Only cDNA samples that demonstrated a consistent housekeeping gene expression pattern were used for further analysis. Gene expression was calculated for 3-4 ovaries per treatment according to the ddct method (*296*) normalized to the geometric mean of cT values from the three housekeeping genes (*297*). Primer sequences are listed in table 1. Amplification products were sequenced to validate primer specificity.

TRA98 and Cleaved PARP Immunohistochemistry

Ovaries were fixed in 5% electron microscopy-grade paraformaldehyde (Electron Microscopy Sciences) overnight at 4°C and stained as previously described (*12, 298*). Whole ovaries were incubated with TRA98 antibody (BBridge) diluted 1:100 to label germ cells (*299, 300*) and with cleaved PARP (product no. E51; Abcam) diluted 1:100 to label cells undergoing apoptosis overnight and then with goat anti-rat secondary Alexa 488 and goat anti-rabbit Alexa 568, respectively. Nuclei were labeled with TOTO-3 (Invitrogen). A confocal microscope (model LSM 710; Zeiss) was used to image the ovaries.

Germ Cell Counting

Total oocytes (TRA98-positive) and apoptotic oocytes (cleaved PARP positive) were determined by counting the number of cells found within each of 4 optical sections per ovary. Germ cell counts were combined from each section to give oocyte count per ovary. Data from 5 ovaries was averaged per treatment. Since data from a limited number of sections was used as a proxy for the total oocytes per ovary, only ovaries that appeared to be of similar size and depth were used for this analysis.

Statistics

Treatment effect was analyzed by 1-way ANOVA for all experiments. For significant results, differences between groups were evaluated using Tukey's LSD method. All results are presented as mean±SEM.

Results

Zinc deficiency does not alter oocyte number or rate of apoptosis during fetal ovary culture

Total oocyte number was quantified in two ways. Oocytes were counted during histological analysis of fetal ovaries, and whole ovaries were labelled with an antibody to the germ cell marker TRA98 followed by confocal optical sectioning. Both experimental methods showed that treatment did not affect the number of germ cells in the ovary after 5 days of culture. For histological analysis, oocytes were calculated per square micron. The result from each treatment was multiplied by a factor of 10^5 to simplify presentation (Control - 54.8±23.1, TPEN –

40.5±5.7, Rescue – 57.0±10.1 ooc/sq μM x10^5; P=0.443 Figure 2-A). For whole ovary immunostaining, TRA98 positive germ cell counts were combined from 4 sections per ovary and averaged for 4-5 ovaries per treatment (Control – 65.0±11.5, TPEN –71.2±8.3, Rescue – 90.25±26.6 germ cells/ovary; P=0.537 Figure 2-B). Apoptotic cells were labelled in fetal ovaries after 5 days of culture using an antibody to cleaved-PARP1. As expected from the cell counting data, the rate of apoptosis was not different between treatments (Control – 9.8±5.9%, TPEN – 11.6±5.4%, Rescue – 13.4±1.2%; P=0.887 Figure 2-C).

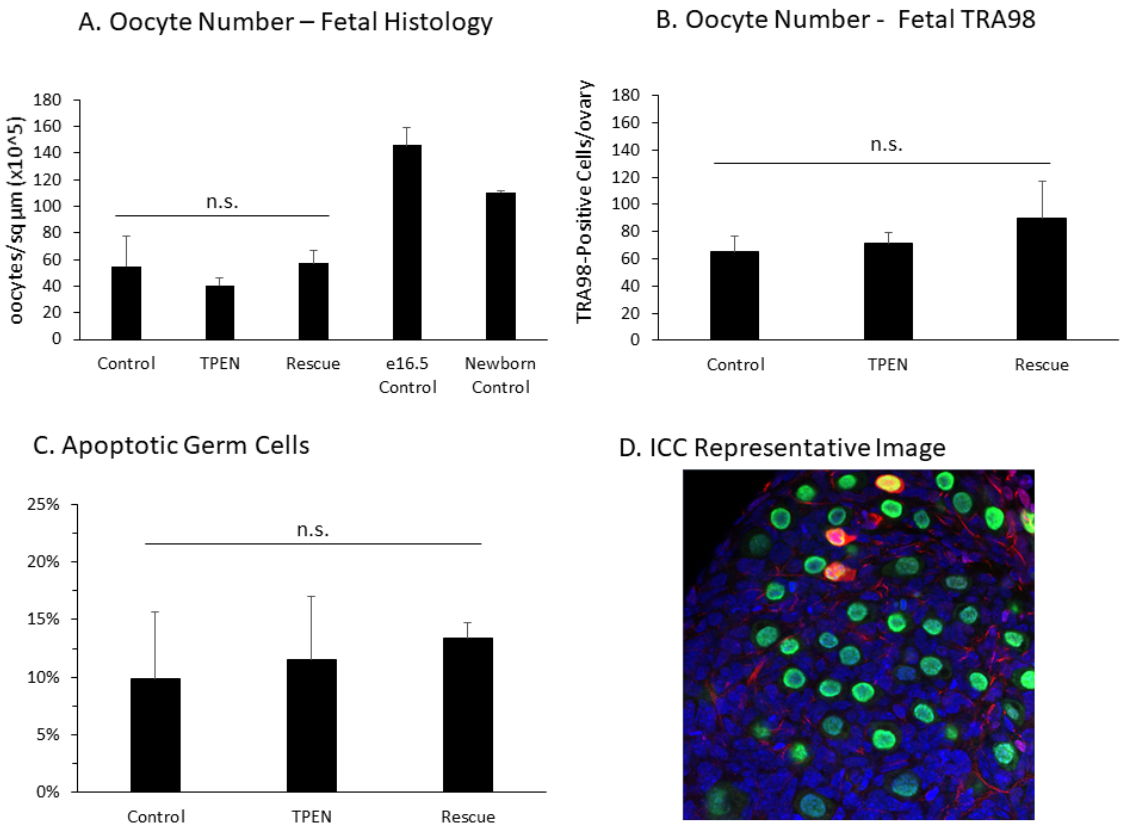

Figure 5-2: Zinc deficiency does not alter oocyte number or apoptosis in the fetal ovary.

Oocyte number was quantified from both H&E stained slides and TRA98 labelling of whole ovaries. (A) Treatment groups showed no significant difference in oocyte number when analyzed by histological analysis (P=0.443). E16.5 and newborn ovary controls are shown for reference, but were not included in the analysis. (B) Whole ovaries were labelled with the germ cell marker TRA98 and optically sectioned with a confocal microscope. Germ cell counts were combined from 4 sections per ovary. No significant difference found between treatments (P=0.537). (C) Whole ovaries were labelled with an antibody to cleaved-PARP1 to label apoptotic cells. % of apoptotic cells was calculated from 4 optical sections per ovary. No significant difference found between treatment groups (P=0.887). (D) Representative image of whole mount ICC staining. 3 apoptotic oocytes are shown. Green – TRA98; Red – Cleaved PARP1; Blue – TOTO-3 nuclear stain. Data for panels B,C,D courtesy of Lindsey Banks and Melissa Pepling – Syracuse University.

Zinc deficiency does not inhibit progress to diplotene in the mammalian ovary

The oocyte meiotic stage was evaluated in cultured fetal ovaries after 5 days of treatment. Meiotic stage was classified according to chromatin morphology viewed after H&E staining. Treatment did not alter the percentage of oocytes at the diplotene stage (Control 25.1±2.2%, TPEN – 30.7±2.9%, Rescue – 36.0±1.9%; P=0.084 Figure 3-A).

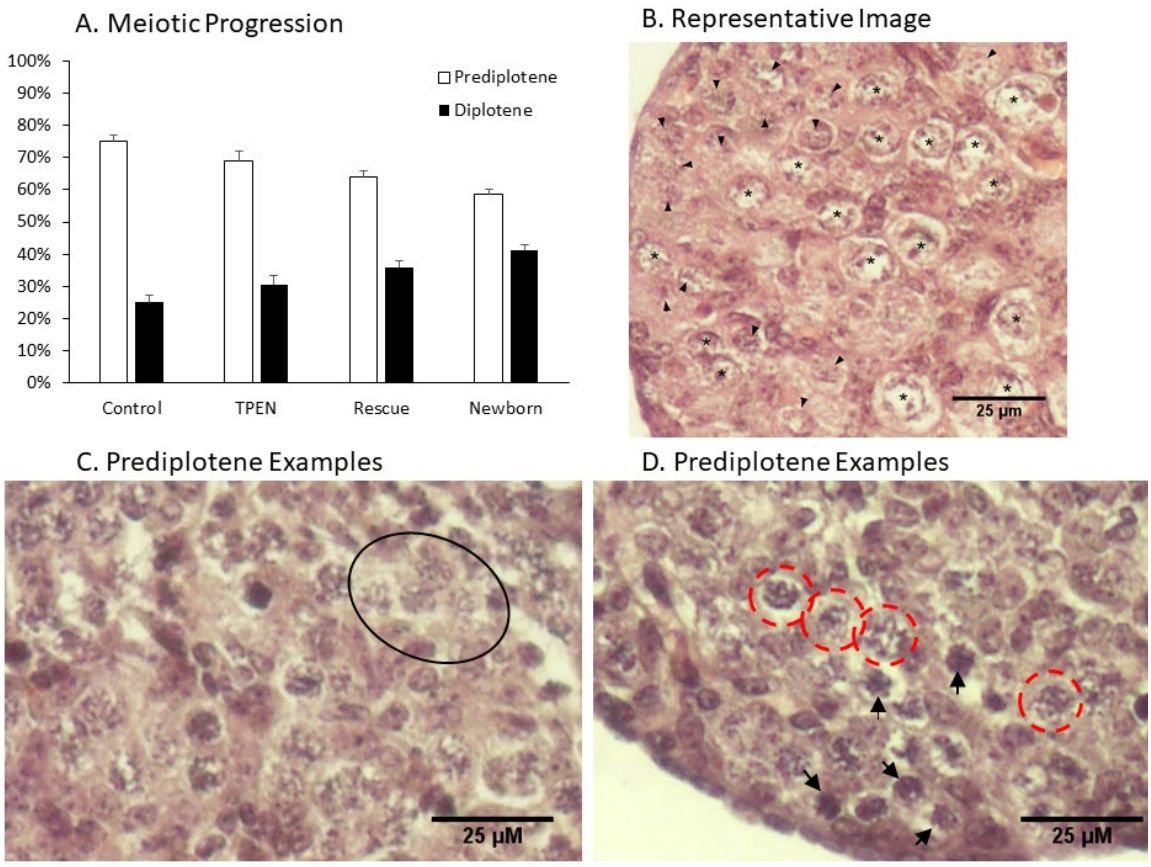

Figure 5-3: Zinc deficiency does not impair progression to diplotene during fetal ovary culture.
Fetal ovary sections were stained with hematoxylin and eosin to observe chromatin configuration. Diplotene and prediplotene oocytes were quantified by nuclear morphology on every 5th section. Diplotene oocytes were identified by condensed peripheral chromatin surrounding clear areas in the nucleus. Earlier meiotic stages were identified by thin chromatin filaments (leptotene), dense chromatin bouquets (zygotene), and thick chromatin strands (pachytene). (A) Meiotic progression. No significant difference between % at diplotene or prediplotene (P=0.084). (B) Representative image from cultured ovary showing diplotene (*) and prediplotene (arrow) oocytes. (C) Prediplotene Example. Circle – four leptotene oocytes. (D) Prediplotene Examples. Zygotene oocytes indicated by arrows. Pachytene oocytes outlined in red.

Zinc deficiency does not alter gene expression in the fetal ovary

Gene expression was compared between groups using qPCR (Figure 4). Zinc status did not affect gene expression of meiotic stage markers (*Scp1, Scp3, Msy2*), genes governing meiotic recombination (*Spo11, Dmc1, Ddx4, Dazl*), or genes governing apoptosis/autophagy (*Bcl2, Bax, Mcl1, Atg7*). Expression was measured as the fold change from control (P>0.05 for all genes).

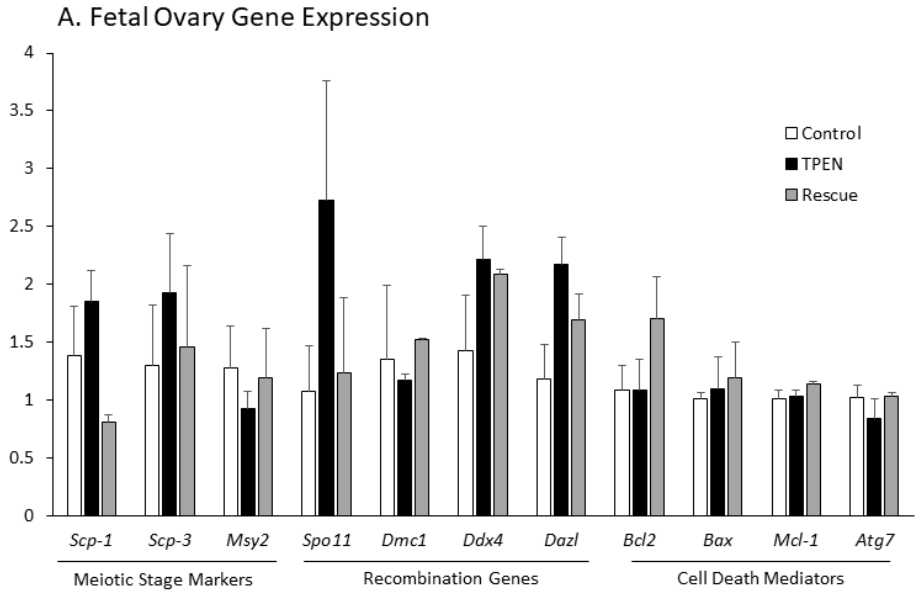

Figure 5-4: Zinc status does not affect gene expression in the fetal ovary.

Gene expression of stage-specific meiotic markers, meiotic recombination genes, and cell death regulators was evaluated by qPCR. No change in gene expression was seen between groups. Expression shown as fold change from control. 3-4 ovaries analyzed per treatment.

Zinc deficiency does not alter oocyte number in cultured newborn ovaries

Oocyte number was evaluated with histology after 4 days of newborn ovary culture in treated media. Oocyte number/area was evaluated according to the same procedures detailed for fetal ovary histology. Treatment did not affect oocyte number (Control – 58.7±5.3, TPEN –

65.9±3.4, Rescue – 63.1±1.8 ooc/sq µM x10^5; P=0.429 Figure 5). All cultured oocytes contained fewer oocytes than freshly collected newborn ovaries collected as a control (110.5±0.9 ooc/sq µM x10^5).

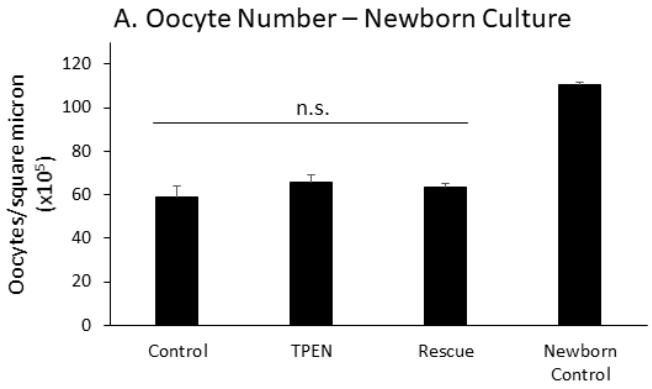

Figure 5-5: Zinc deficiency does not alter oocyte number after newborn ovary culture.

Newborn ovaries were cultured in treatment for 4 days before sectioning and H&E staining. Every 5th section was analyzed independently by 3 researchers. (A) Oocyte number expressed as oocyte/square micron (x10^5). No significant difference was found in oocyte number after treatment (P=0.429).

Zinc deficiency impairs nest breakdown during newborn ovary culture

After 5 days of culture, newborn ovaries were processed for histology and stained with hematoxylin and eosin. Ovaries cultured in TPEN treated media contained fewer enclosed primordial follicles and a higher proportion of oocytes in germ cell nests. (Figure 6. Percent of nested oocytes: Control - 39.2±1.6, TPEN - 47.1±2.1, Rescue – 34.3±2.2; P=0.002).

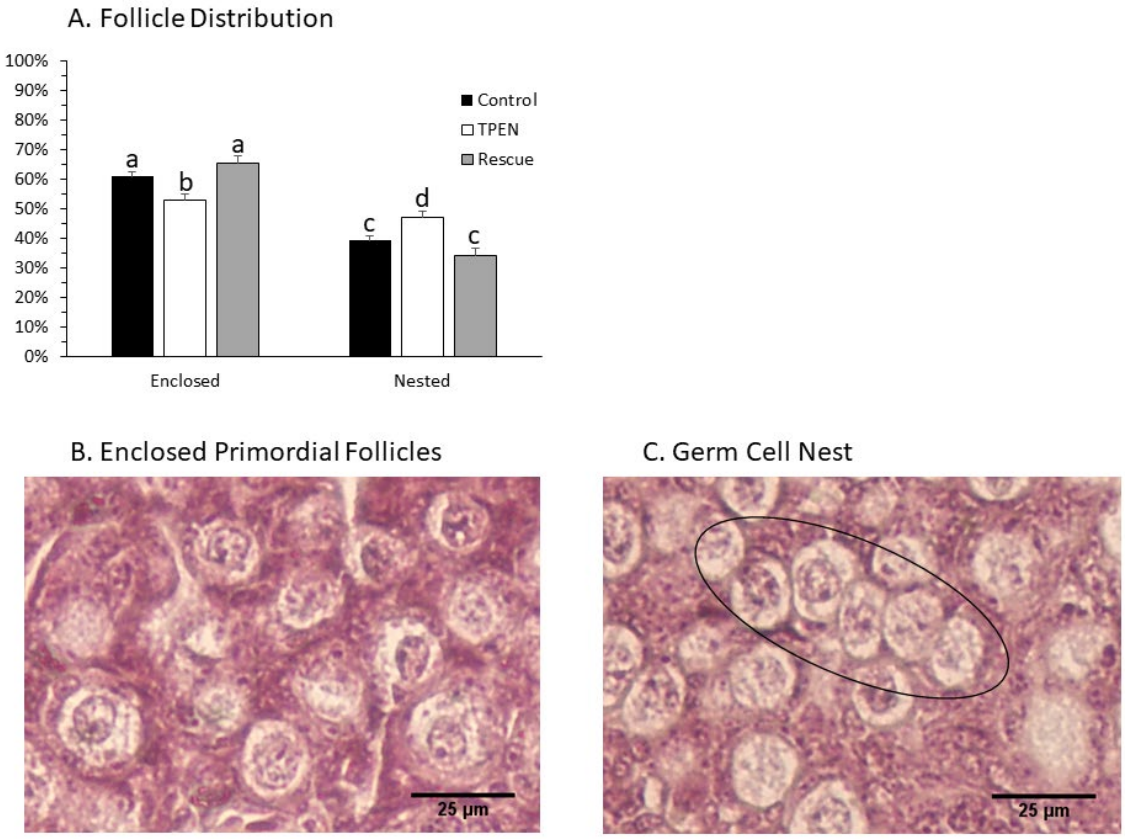

Figure 5-6: Zinc deficiency impairs germ cell nest breakdown in newborn ovary culture.

Oocytes were classified as either enclosed primordial follicles or part of a germ cell nest by 3 independent researchers. (A) Follicle distribution. TPEN treated ovaries contained more oocytes in germ cell nests and fewer enclosed follicles than control or rescue ovaries (P=0.002). (B) Representative image of fully enclosed primordial follicles. (C) Representative image of a germ cell nest (oval).

Zinc deficiency may reduce the size of the initial follicular wave

The number of activated follicles (identified by association with cuboidal granulosa cells) was counted in newborn ovary sections after H&E staining (Figure 7). There was a strong trend (P=0.051) toward a treatment effect on activated follicle %. Tukey pairwise comparisons were used to test differences between treatment groups. TPEN and Rescue treated ovaries displayed a significantly different rate of activation (TPEN - 7.4±1.0%, Rescue – 10.3±0.6%; P<0.05). The

activation rate in control ovaries (9.2±0.6%) was not significantly different from either TPEN or Rescue treatment.

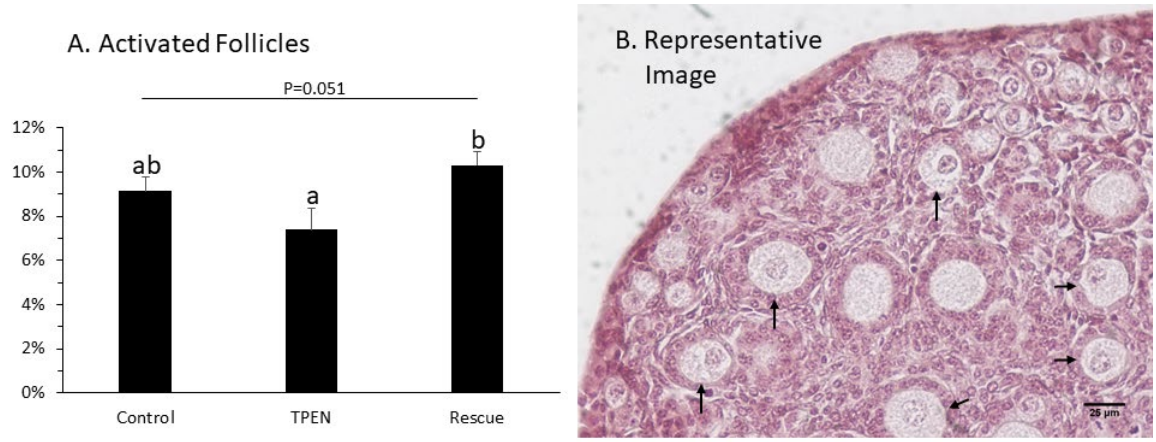

Figure 5-7: Zinc deficiency may reduce the size of the initial follicle wave.

Activated follicles were identified by the presence of cuboidal granulosa cells surrounding the oocyte. To prevent double-counting between sections, only activated follicles with a visible oocyte nucleus were counted in the analysis. (A) TPEN treated ovaries displayed the lowest percentage of activated follicles, though the treatment did not reach significance (P=0.051). Tukey pairwise comparisons show a statistical difference between TPEN and Rescue treatment, indicating a possible zinc-mediated effect. (B). Representative image. Activated follicles with visible oocyte nuclei are indicated (arrows).

TPEN treatment reduces expression of *Bmp15* and *Foxl2* in cultured newborn ovaries

Gene expression was compared between treatment groups by qPCR according to the ddct method. Zinc deficiency reduced the expression of the oocyte secreted TGFβ family member *Bmp15* and the somatic cell transcription factor *Foxl2* ($P<0.01$ for each). Other factors previously shown to regulate nest breakdown were also evaluated, but showed no change between treatments (Figure 8).

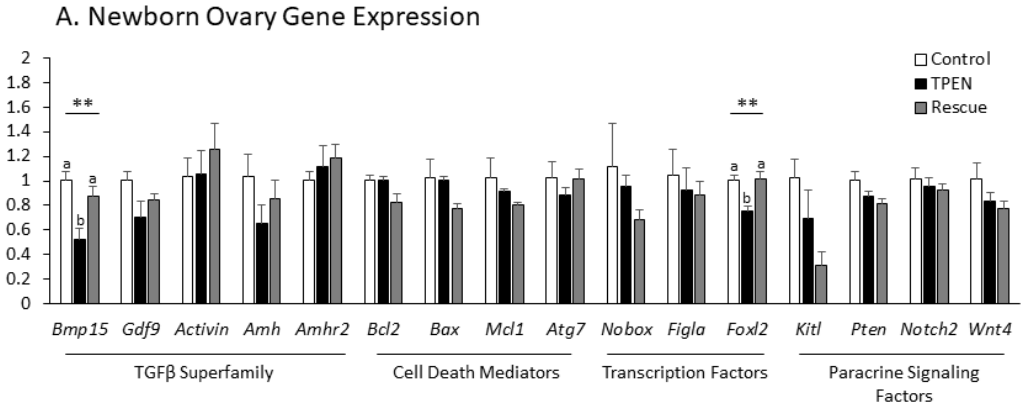

Figure 5-8: Zinc deficiency reduces expression of *Bmp15* and *Foxl2* in cultured newborn ovaries.

Genes previously shown to regulate nest breakdown were evaluated by qPCR. The oocyte secreted factor *Bmp15* and the somatic-cell specific transcription factor *Foxl2* were down regulated by TPEN treatment ($P<0.01$ for each). Expression shown as fold change from control. 3-4 ovaries were analyzed per treatment.

Discussion

Primordial follicle assembly involves meiotic progression of the oocyte as well as germ cell nest breakdown and invasion of somatic cells. These two processes are closely related (*301*), but not dependent processes – meaning neither is a prerequisite for the other (*21*). We tested the effect of zinc deficiency on meiotic progression and germ cell nest breakdown independently as these processes are distinct in the mouse, and both contribute to primordial follicle formation. It is important to note that nest breakdown and meiotic progression happen concurrently in humans during the second trimester (*26*). The results of this study indicate that zinc deficiency impairs germ cell nest breakdown as well as activation of the initial medullary follicular wave. Meiotic progression in the fetal ovary appears to be unaffected by the presence of our zinc chelator at the concentrations used in this study.

Meiotic progression during fetal development in mammals is key to formation of viable haploid gametes. After undergoing duplication, chromosomes condense (leptotene stage), align with

homologous chromosomes (zygotene stage), and complete recombination (pachytene stage), before reaching diplotene and undergoing cell cycle arrest (*302*). Several genes are upregulated during meiosis including Spo11, Dmc1, Ddx4, and Dazl and are required for successful recombination between homologous chromosomes. Our earlier work in *C. elegans* indicated that zinc deficiency impaired meiotic progression, particularly at the pachytene-to-diplotene transition. Zinc deficient *C. elegans* hermaphrodites display an enlarged region of pachytene germ cells in their gonad and fewer diplotene oocytes near the uterus (*151*). In order to test for a similar effect in mammals, we began fetal ovary culture on embryonic day 16.5 when the majority of oocytes are at the pachytene stage. Contrary to our invertebrate model, zinc deficiency did not interfere with progression to diplotene in the fetal ovary. This may indicate that the zinc requirement for early meiosis is not conserved between *C. elegans* and the mouse. It is also possible that zinc deficiency impairs meiosis at the early stages of pachytene development, and that the timing of TPEN treatment in our mouse study was too late to mimic the effect seen in *C. elegans*.

TPEN treatment did not affect the rate of germ cell apoptosis during either fetal or newborn ovary culture. This result is surprising given the already high rate of apoptosis during primordial follicle formation and the established role of zinc in apoptosis. In mice, only one third of germ cells will survive to become primordial follicles (*16*), while the survival rate in humans is even lower (*303*). Oocyte survival appears to be regulated by the Bcl2 family of apoptotic regulators. The balance of proapoptotic (BAX) and antiapoptic (BCL2, MCL1) factors determine cell fate during primordial follicle assembly (*30, 33, 34*) in conjunction with regulation of autophagy (*35*). Zinc impacts apoptosis rates in a variety of cell types, primarily promoting cell survival (*285-287*), however zinc chelation did not affect the apoptosis rate in oocytes. The consistent apoptotic rate across treatments was demonstrated by cell counting in stained histology sections, whole mount ICC, and gene expression assays. These results demonstrate that the apoptotic rate of oocytes is tightly regulated even during environmental perturbations.

TPEN treatment did affect germ cell nest breakdown during newborn ovary culture. TPEN treated ovaries contained a higher percentage of oocytes in nests and fewer primordial follicles after 5 days of treatment. Whether the rate of nest breakdown was merely slowed by TPEN treatment, or permanently disrupted in a subset of oocytes is unclear from this experiment. Equally remarkable is the effect of zinc deficiency on activation of the initial follicular wave.

This unique class of follicles is the first to be assembled and plays a vital role in controlling ovarian function prior to puberty in mammals (*66, 67*). The initial follicle wave is important in providing endocrine feedback to the hypothalamus and pituitary, as well as inhibiting overactivation of dormant primordial follicles via paracrine inhibition. The impact of fewer follicles in the initial follicular wave remains to be investigated. Reduced activation of this class of follicles could be hypothesized to disrupt signaling in the hypothalamic-pituitary-gonadal axis as well as lead to over-recruitment of dormant primordial follicles which may lead to premature ovarian insufficiency.

Nest breakdown requires the coordination of multiple signaling pathways among a variety of cell types. To identify signaling pathways vulnerable to zinc deficiency, we evaluated the effect of zinc deficiency on the expression of 16 genes previously shown to regulate nest breakdown. (*30, 33, 35, 36, 38, 42, 45, 47, 60, 304-309*). TPEN treatment downregulated transcription of the oocyte derived TGFβ superfamily member *Bmp15* (Bone Morphogenic Protein 15) and the somatic cell transcription factor *Foxl2* (Forkhead Box L2). Oocytes secrete BMP15 along with another TGFβ member GDF9 (Growth Differentiation Factor 9). BMP15 and GDF9 activate SMAD proteins in the surrounding somatic cells (*249*), thereby influencing cell differentiation, mitosis, and survival. Previous studies in our lab have shown that zinc deficiency reduces *Gdf9* expression in fully grown oocytes (*188*). The reduction of *Bmp15* expression seen here indicates that zinc deficiency impairs TGFβ signaling at multiple developmental stages. *Bmp15* or *Gdf9* knockout mice display an increased incidence of follicles containing multiple oocytes, indicating impaired nest breakdown (*304*).

Ovarian morphology is even more disrupted in *Foxl2* knockout mice in which entire germ cell nests remain in the adult ovary (*305*). In addition, follicles activate prematurely in the knockout mice due to a lowered inhibitory signaling from the granulosa cells, leading to rapid follicle loss. In humans, mutations in *Foxl2* result in blepharophimosis/ptosis/epicanthus inversus syndrome (BPES), a rare disease characterized by eyelid deformation and premature ovarian insufficiency (*310*). FOXL2 activity is key to controlling granulosa cell proliferation, differentiation, and development (*311, 312*), and contributes to premature ovarian insufficiency in humans.

Study Limitations

This study was designed to evaluate the effect of zinc deficiency on two parameters of primordial follicle assembly: meiotic progression to diplotene, and germ cell nest breakdown. While the results provided new insights into these cellular processes, there are limits to the information we can gather using this design. For both fetal and newborn ovary culture, samples were collected and processed at only one timepoint. This method may mask transient zinc dependent effects during the early stages of culture. In the meiotic progression study, fetal ovaries were collected on E16.5 when the majority of oocytes are in the pachytene stage of meiotic prophase. We therefore did not test the zinc requirement for any earlier meiotic stages or mitosis of the primordial germ cells.

The most severe limitation of this study resulted from a technical error. While processing fetal ovaries for histology, three control ovaries were improperly sectioned leaving only two samples in the control group for analysis. The small sample size of one treatment group limits the statistical power of these experiments. However, several factors lead us to conclude that the results of the fetal histology analysis are still valid. One, no difference was seen between TPEN and Rescue fetal ovaries during histology analysis despite an appropriate sample number for pairwise comparisons (data not shown). Two, the fetal ovary histology cell counts were confirmed by whole mount immunocytochemistry in a separate and appropriately powered experiment. Three, histological analysis, whole mount immunocytochemistry, and qPCR data all showed no effect of treatment at this developmental stage. Most likely, this consistency indicates there was actually no treatment effect of TPEN at this timepoint, rather than a type II error.

Conclusion and Future Directions

These results indicate that the zinc requirement for meiotic progression to diplotene seen in *C. elegans* is not conserved in mice when zinc deficiency begins at the pachytene stage. This study has demonstrated that zinc deficiency impairs germ cell nest breakdown and follicle assembly in the newborn mouse ovary. Two genes which control nest breakdown were downregulated by TPEN treatment. Oocyte apoptosis, a key factor in primordial follicle assembly, was not affected by zinc status in either the fetal or newborn ovary. Interestingly, zinc

deficiency may reduce the size of the initial follicular wave which plays an important role in the prepubertal female. The long-term effects of disrupting the initial follicular wave are unknown.

Future studies should test the effect of *in vivo* zinc deficiency on primordial follicle assembly and the long term impact on fertility. Inducing marginal zinc deficiency during fetal or neonatal development is complicated by homeostatic regulation of zinc status. Zinc absorption is upregulated in the intestine in response to low dietary intake (*197*) or increased physiological need such as lactation (*313*). Zinc transfer from the maternal circulation to the placenta is also highly regulated such that the zinc level in serum collected from the umbilical cord is unchanged over a wide range of circulating maternal zinc levels (*314*). These homeostatic mechanisms make it difficult to induce marginal zinc deficiency in experimental models. Genetic ablation of zinc transporters in the placenta or fetal ovary may allow researchers to induce fetal zinc deficiency without altering maternal zinc status. Regardless of the method, inducing zinc deficiency during *in vivo* primordial follicle assembly will allow subsequent fertility evaluation in the adult animal. This step is required to test if fetal or neonatal zinc deficiency impacts subsequent fertility in mammals.

Table 5-1: qPCR primers

Gene Symbol	Forward Primer (5'-3')	Reverse Primer (5'-3')	Fetal P Value	Newborn P Value
Scp-1	GTCAAGTGTCCGCCGTGAAA	CCTGATAGTGACAACTGCCAGA	0.145	
Scp-3	TCGGGGCCGGACTGTATT	AAGGTGGCTTCCCAGATTTCC	0.71	
Msy2	AAGTCCTGGGCACAGTCAAAT	CTCCCCATCTCCAACACTCC	0.697	
Spo11	AGCATGAAGTGTCTCACTAGCA	CATTAACAGGGCAAGGCACCTA	0.414	
Dmc1	CCCTCTGTGTGACAGCTCAAC	GGTCAGCAATGTCCCGAAG	0.888	
Ddx4	GAAGAAATCCAGAGGTTGGC	GAAGGATCGTCTGCTGAACA	0.288	
Dazl	GATGGACATGAGATCATTGGAC	ATACCAGGGAGCAATCCTGAC	0.081	
Bcl2	GAACTGGGGGAGGATTGTGG	GCATGCTGGGGCCATATAGT	0.268	0.522
Bax	GATCCAAGACCAGGGTGGCTG	TCCCCTTCCCCCATTCATCC	0.868	0.09
Mcl-1	TGTAAGGACGAAACGGGACT	AAAGCCAGCAGCACATTTCT	0.325	0.242
Atg7	CCTGCACAACACCAACACAC	CACCTGACTTTATGGCTTCCC	0.538	0.52
Bmp15	ACACAGTAAGGCCTCCCAGA	GATGAAGTTGATGGCGGTAAA		**<0.01**
Gdf9	CTACAATACCGTCCGGCTCT	CAAGTGTTCCATGGCAGTCA		0.219
Activin	GGGTAAAGTGGGGGAGAACG	ACTTCTGCACGCTCCACTAC		0.65
Amh	AGCTGGACACCATGCCTTT	TTCGAAGCCTGGGTCAGA		0.314
Amhr2	TTACAGCCATCTGCCTCCTT	TCAGCAACAACACGAGAAACA		0.732
Nobox	AGGGACGTTCCTGGCAGT	GCTGCTTGCTTGGTAGTCCT		0.272
Figla	ACAGAGCAGGAAGCCCGTA	GTCAGAGGGTCTGCCACTGT		0.778
Foxl2	AACACCGGAGAAACCAGACC	CGTAGAACGGGAACTTGGCT		**0.008**
Kitl	CAAGGAGATCTGCGGGAAT	CAATGACTAGGCAAAACATCCA		0.067
Pten	TCTGCCATCTCTCTCCTCCTT	TTCTGCAGGAAATCCCATAGCAA		0.22
Notch2	AGCAGGAGCAGGAGGTGATA	TGGGCGTTTCTTGGACTCTC		0.727
Wnt4	CCTGCGACTCCTCGTCTT	TCTGGATCAGGCCTTTGAGT		0.19

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Chapter 6

Summary and Future Studies

Summary

Acute dietary zinc deficiency is prevalent in the developing world (*194*), and marginal zinc deficiency may affect vulnerable populations in developed nations as well (*315, 316*). Zinc deficiency has long been known to affect mammalian fertility in both males and females (*232*), but the mechanisms underlying the zinc requirement for reproduction are only beginning to be elucidated. The role of zinc in the oocyte has long been a research focus of our laboratory and previous work has demonstrated that even short periods of zinc deficiency can compromise the development and fertility of fully-grown oocytes. In the previous work, either dietary or *in vitro* zinc restriction impaired meiotic progression, fertilization, embryo development, implantation, and fetal development (*187, 188, 209*). While the effect of zinc deficiency in the ovulatory follicle is clear, very little was known about the effect of zinc restriction on the early stages of oocyte development prior to this thesis work.

Understanding the impact of zinc deficiency on early stages of oocyte development is an important research goal for two reasons. One, the high prevalence of dietary zinc deficiency worldwide means that any zinc-mediated defects in folliculogenesis are likely to be prevalent as well. Zinc optimization may therefore be an attractive first step in improving fertility as most cases of zinc deficiency can be remedied by changes in the diet. Micronutrient supplementation is less difficult to implement on a global scale than treatments to address genetic or disease contributions to infertility. Two, human ovarian physiology involves careful maintenance of the ovarian reserve for decades prior to reproduction. This means there is a large pool of dormant and

growing follicles which are vulnerable to environmental degradation. The ovarian arrangement also ensures that the pool of dormant and growing follicles will always be larger than the ovulatory pool – meaning that the effect of zinc deficiency on early stages of follicle development may be more critical to lifelong fertility than the brief effects previously shown in ovulatory follicles. To investigate zinc's role in early folliculogenesis three studies were conducted as part of this thesis work.

In study 1 we introduced *C. elegans* as a model organism for the study of zinc and oogenesis. This model organism is particularly suited to our research goals as *C. elegans* hermaphrodites continually generate new oocytes from mitotically dividing germ cells at the distal tip of each gonadal arm (*138*). This physiological arrangement permits examination of early germ cell development and meiotic progression in the adult specimen. In contrast, the mammalian ovary contains mitotic germ cells and oocytes entering meiosis only during fetal development. By treating nematode growth media with the zinc chelator TPEN we were able to demonstrate that zinc restriction impairs fertility in *C. elegans*, indicating that this is a useful model to study the effects of zinc restriction (*151*). Worms exposed to TPEN released fewer progeny throughout their reproductive lifespan. Notably, the effects of zinc deficiency were most clearly seen in oocyte development, similar to our previous studies in mice. TPEN treated worms had fewer oocytes proximal to the uterus, and displayed oogenic defects such as binucleate oocytes and disorganized oocyte progression. Most notably, the region of pachytene stage germ cells was extended, indicating that zinc deficiency impairs early meiotic progression in *C. elegans*. Pachytene germ cells are only found in the fetal ovary of mammals, and regulation of pachytene development is not well studied. This was the first time that a zinc requirement for meiosis prior to the diplotene arrest has been shown in any model system.

In studies two and three, we evaluate the effect of zinc deficiency on early folliculogenesis using the mouse model. In study two, preantral follicles were collected and

matured *in vitro* under control, zinc deficient, or rescue conditions. Zinc deficient culture conditions were created by the addition of the extracellular zinc chelator DTPA which has been shown to reduce labile zinc in the media without inducing apoptosis in cultured cells. The preantral stage of folliculogenesis is marked by oocyte growth and differentiation of granulosa cells into either endocrine mural cells or oocyte-associated cumulus cells. Zinc deficient conditions impaired preantral development including disruption of oocyte growth, impaired meiotic potential of the oocytes, and disrupted paracrine signaling between the oocyte and granulosa cells. The granulosa cells were particularly vulnerable to zinc deprivation during culture. DTPA treatment increased the rate of apoptosis among somatic cells, reduced physical connections between oocytes and granulosa cells, and prevented expression of cumulus cell markers during development. Cumulus cells play a vital role in supporting the oocyte metabolically (*317*), therefore the loss of cumulus cell signaling may play a role in the reduced developmental potential of the oocytes. Preantral follicle development is the longest phase of follicle growth, lasting several months in humans. Impaired preantral follicle development may impact fertility only later as the preantral follicles enter the hormone responsive antral follicle pool. This hypothesis remains to be tested *in vivo*.

In study three we investigated the effect of zinc deficiency on an even earlier stage of follicle development, primordial follicle assembly. The effect of zinc deficiency was evaluated on two parameters of early follicle development, meiotic progression to diplotene and germ cell nest breakdown. We hypothesized that zinc restriction would impair meiotic progression as seen in our invertebrate model. To test this hypothesis, we cultured fetal ovaries in control, zinc deficient, or rescue media for 5 days during the pachytene-to-diplotene transition. In contrast to our *C. elegans* data, zinc deficiency did not impair meiotic progression. Oocyte number and apoptosis were also not affected by treatment during fetal ovary culture. We next evaluated the role of zinc during germ cell nest breakdown and follicle assembly by culturing newborn ovaries for 4 days in

control, TPEN, or rescue media. In this experiment, zinc deficiency significantly delayed germ cell nest breakdown leading to a higher percentage of oocytes remaining in germ cell nests. Two genes which regulate nest breakdown showed reduced transcription under zinc deficient conditions – *Bmp15* and *Foxl2*. Zinc deficient newborn ovaries also contained fewer activated follicles after culture. The presence of growing follicles in the neonatal ovary is conserved between mice and humans and hypothesized to control ovarian paracrine and endocrine function prior to the onset of puberty. Inhibiting the size of this cohort of follicles, the "initial follicular wave," may have an unforeseen impact on ovarian function and fertility. Alternatively, these results may indicate that zinc deficiency inhibits follicle activation in general, rather than in the particular subset of medullary follicles that compose the initial wave of activation. The effect of zinc deficiency on activation of primordial follicles in the mature ovary remains to be tested.

The effect of zinc deficiency on early meiosis was examined in both studies 1 and 3. In study 1, zinc deficiency appears to have delayed the diplotene transition in *C. elegans*. In study 3, mouse germ cells progressed to the diplotene stage at an equivalent rate regardless of treatment. There are several possibilities that could account for the differing effects of zinc deficiency in these studies. First, the zinc requirement for pachytene exit may not be conserved between *C. elegans* and *Mus musculus*. This finding would be surprising as the mechanisms of fertility and the zinc requirement for oogenesis are largely conserved between these models. Second, it is possible that mammals do require zinc during the pachytene-to-diplotene transition, but that our intervention or quantification methods were not sensitive enough in study 3 to show this effect. Third, it is possible that we have misinterpreted the results of study 1 and the *C. elegans* gonadal phenotype elicited by zinc deficiency is not caused by a delay in the diplotene transition. Further studies to test possibilities two and three are outlined in the future studies section below.

In conclusion, these studies have increased our understanding of zinc in reproductive biology and defined new periods of follicle and oocyte development that are vulnerable to zinc

deficiency. We have introduced a new model organism to the field by demonstrating that *C. elegans* reproduction is vulnerable to zinc deficiency. The advantages of this model, particularly the genetic toolbox, can now be applied to uncovering the zinc-dependent mechanisms in the oocyte. Our preantral follicle study (study 2) demonstrated that zinc deficiency impairs follicle growth during development. While it is intuitive that zinc deficiency could impair folliculogenesis at multiple points of development, this study was the first to show preantral follicles require zinc for optimal growth. Our results also show that the somatic cells of the preantral follicle are particularly vulnerable to zinc deficiency, indicating a new target of micronutrient deprivation. These results have implications for prenatal vitamin supplementation as well as optimization of *in vitro* maturation for ART. Finally, the third study demonstrates that primordial follicle assembly in the fetal ovary can be affected by zinc status. Zinc deficiency reduced the percentage of oocytes enclosed in primordial follicles after culture, leaving more oocytes in germ cell nests. If these results translate to humans, it may have dramatic implications for health and fertility. Eighty-two percent of pregnant women world-wide consume less than the RDA for zinc. If marginal zinc deficiency reduces the size of the primordial follicle pool, zinc status *in utero* may be a determining factor of fertility and reproductive longevity.

These studies, combined with previous work from our lab and others, demonstrate that zinc status determines oocyte quality at every stage of development. Identifying a zinc requirement for nest breakdown in the fetal ovary underscores the vital role of micronutrients during gestation for fetal development. Our study of preantral follicle development implies that micronutrient support is vital for fertility months prior to conception, an intriguing idea that must be tested *in vivo*.

By demonstrating the absolute zinc requirement for folliculogenesis, we hope this work also generates continued interest in employing zinc to improve and control fertility in women both *in vivo* and during ART. Finally, zinc status impacts not only fertility, but also immunity,

growth, and disease morbidity (*315*). As we uncover more zinc-dependent physiological processes, it becomes even more apparent that addressing the widespread zinc deficiency among humans could improve not only fertility but health and survival as well. Developing new methods to improve zinc status in humans has the potential to save hundreds of thousands of lives each year.

Future Studies

The studies detailed in this dissertation demonstrate that the nematode *C. elegans* is an appropriate model for testing the effects of zinc deficiency, and that *in vitro* zinc chelation has a detrimental effect on folliculogenesis in the mouse. Notably, a zinc requirement for the pachytene-to-diplotene meiotic transition was not consistently shown across our studies and model organisms. The following studies are proposed to capitalize on knowledge gained from this graduate work and can be divided into three categories: studies to reexamine the pachytene-diplotene transition; studies to take advantage of the new model system *C. elegans*; and studies to confirm our *in vitro* findings in a whole animal model.

Zinc during the pachytene-diplotene transition

Reevaluate the zinc requirement in mice

Our study of zinc deficiency during the diplotene transition in mammals showed no change in the percent of diplotene oocytes after 5 days of *in vitro* culture beginning on embryonic day 16.5 (study 3). This analysis was based on histological scoring of oocytes, a subjective and time-consuming method. Reevaluating the effect of zinc deficiency on diplotene arrest in mice

using the gold standard of meiotic analysis – immunostaining germ cell spreads for meiotic markers – may provide more conclusive results. This more detailed analysis could be conducted after repeating the culture conditions described previously. Briefly, fetal oocytes will be collected on embryonic day 16.5 and cultured in control, zinc deficient (TPEN – 1μM), or rescue (TPEN + $ZnSO_4$ - 1μM) media for 5 days. At the end of culture, ovaries will be dissociated into single cell suspensions and germ cells will be fixed to slides. Immunostaining for SCP-3 (a prediplotene marker) and MSY2 (a diplotene marker) will be used to determine the percentage of diplotene oocytes in each cell spread. A minimum of 5 ovaries per treatment will be evaluated. After mastering the technique, a similar protocol could be employed to test the effects of zinc deficiency earlier in development. By starting treatment on day 16.5 in the previous experiment, any effects of zinc deficiency on the earliest stages of meiosis (leptotene, zygotene, and early pachytene) were not evaluated. Combining the gold standard approach for meiotic measurement with a timecourse study in which zinc deficiency onset begins on embryonic day 13.5, 14.5, 15.5, and 16.5 will fully and unquestionably test the zinc requirement for meiotic progression in the mammalian fetal ovary.

Reevaluate the extended pachytene zone in C. elegans

After completing the full study of early meiosis in mammals, our original findings may be confirmed – that zinc deficiency does not alter the rate of meiotic progression in the fetal mammalian ovary. This may indicate a nonconserved mechanism, or it may indicate that we have misinterpreted the cause of the extended pachytene region found in TPEN treated *C. elegans* in study 1. A subsequent study by another research group using *C. elegans* and TPEN treatment confirmed many of our initial findings (*150*) including that zinc deficiency impairs fertility in *C. elegans* and oogenesis in particular. Although the authors do not discuss a zinc mediated delay in

the pachytene-diplotene transition, the published images in their study clearly show an extended pachytene region in the proximal gonad of TPEN treated subjects. This phenotype is therefore real and appears to be zinc specific. It is plausible that the extension of the pachytene zone is not caused by a disruption of meiosis, but rather disruption of another physiological process which also occurs at the gonadal turn. The most likely candidates (other than meiosis) are a defect in membrane enclosure of the germ cells or in germ cell apoptosis. Experiments to test the effect of zinc deficiency on both of these parameters should be conducted. To visualize membrane enclosure we will use the *C. elegans* hermaphrodites expressing the SYN4:GFP mutation, in which oocyte plasma membrane proteins expresses Green Fluorescent Protein (GFP). The membrane demarcation between developing oocytes is therefore very clear in these mutants (*318*). Exposing this mutant strain to TPEN treatment as described previously will allow exact delineation between the rachis and the membrane enclosed oocytes; and may show defects in membrane enclosure when compared with mutants grown under control conditions. Membrane enclosure in *C. elegans* is analogous to nest breakdown in mice which we have demonstrated is impaired by zinc deficiency in mouse tissues. Impaired apoptosis may also promote an extended pachytene region if nurse cells are unable to undergo cell death and instead cluster in the proximal gonad (*139, 319*). The fluorescent dye acridine orange has been used extensively to identify apoptotic cell bodies in *C. elegans (320)*. We will use this method to identify changes in the rate of apoptosis in the *C. elegans* germline in response to zinc deficiency.

Further Studies in *C. elegans*

One major accomplishment of this work has been the introduction of *C. elegans* to studies of zinc-dependent fertility. Now that we have established this model, the genetic tractability of this species can be put to use in hypothesis generation and testing. We propose to

take advantage of *C. elegans* genetics in two studies: one to perform a genetic screen to identify new targets of zinc deficiency and another to use established mutant strains to define the mechanistic impacts of zinc deficiency.

Forward genetic screen to identify genes involved in zinc-mediated fertility loss

We will employ common genetic screening techniques (*321*) and a protocol developed to identify mutations in zinc metabolism (*322*) to identify *C. elegans* mutants that are resistant to TPEN mediated fertility loss. N2 worms will be mutagenized with ethyl methanesulfonate (EMS) according to Brenner (*137*). Mutagenized hermaphrodites will self-fertilize and give rise to F1 progeny. F1 adult hermaphrodites will be treated with alkaline hypochlorite solution to collect F2 embryos. Unhatched F2 embryos will be placed on nematode growth media containing 50μM TPEN and allowed to develop to the L4 + 48h stage. At this point, hermaphrodite mutants will be screened based on the number of embryos developing in the uterus. Mutants that have more than 10 embryos in the uterus will be selected as being TPEN-resistant. 10 developing embryos represents a 33% increase in the number of developing embryos found with TPEN treatment of wildtype N2 worms. TPEN resistant subjects will be picked and allowed to self-fertilize to establish a population. Single nucleotide polymorphism mapping (*323*) or genome sequencing (*324*) will be carried out according to standard methods to identify mutations conferring TPEN resistance. Analysis of this data is expected to provide tremendous information about the targets of zinc deficiency and regulation of zinc homeostasis within the germline.

Zinc status and MAPK pathway activation during oogenesis

In addition to the genome-wide studies possible with *C. elegans* genetic screens, this model also allows very narrow and focused mechanistic studies at the cellular level. This feature is particularly useful in evaluating zinc-mediated effects since zinc plays a role in multiple signaling pathways, often in the same cell or tissue. The role of zinc in MAPK signaling in the oocyte is one area of research in which mechanistic studies are lacking. MAPK signaling increases during meiotic resumption in mouse oocytes (*325*) and during oogenesis in the proximal gonadal arm of *C. elegans* (*225*). Zinc deficiency in mice affects MAPK signaling in the oocyte, although not uniformly during development (*143, 265*). Under some conditions, zinc deficiency appears to prematurely activate MAPK signaling, while in other contexts zinc deficiency mirrors the phenotype seen in MAPK knockout mouse oocytes (*326*). In order to examine the relationship between zinc deficiency and MAPK signaling, we propose to use the *C. elegans* temperature sensitive mutant strain *let-60(ga89)*. This strain contains a temperature sensitive gain of function mutation in *let-60 ras*, a MAPKKK (*327*). Therefore, culturing this strain at elevated temperature leads to a constitutive activation of MAPK signaling. I propose repeating the protocol for inducing zinc deficiency in *C. elegans* using *let-60(ga89)* mutants cultured at either high or low temperature. Evaluating whether constitutive MAPK signaling compensates for zinc deficiency should determine if this pathway is impaired under ordinary zinc deficient conditions. A pilot study has been completed for this project, although experimental design factors such as treatment onset and proper controls must be refined. The proposed study is only one example of the type of study possible using *C. elegans* mutants. The wide range of mutations available in this model will allow complete investigation of molecular mechanisms that are sensitive to zinc deficiency.

Future studies in mice

We have used *in vitro* culture and zinc chelation to evaluate the role of zinc during early folliculogenesis in the mouse. While these techniques produced a clear phenotype and demonstrated a zinc requirement for nest breakdown and preantral follicle development, studies conducted in whole animals using a dietary intervention are more relevant to human physiology. We therefore propose to confirm the zinc requirement for follicle assembly and preantral follicle growth in the following studies.

Marginal dietary zinc deficiency during preantral follicle growth in mice

Mice will be placed on a marginal deficient zinc diet (5µg Zn/gram chow (*328*)) for 14 days. Ovulation will then be induced by administration of gonadotropins. Parameters of oocyte health and development will be evaluated including the number of ovulated cumulus-oocyte complexes, oocyte size and morphology, cumulus cell number, ability to undergo meiotic resumption, cumulus cell expansion, fertilization rate by IVF, and rate of blastocyst development as described previously (*188*). The prolonged marginal diet will ensure that ovulated oocytes collected at the end of treatment were enclosed in preantral follicles at the beginning of treatment. Induction of marginal zinc deficiency is also likely to be more physiologically relevant to human health than zinc chelation *in vitro* or use of a totally zinc free diet.

Dietary zinc deficiency during nest breakdown in mice

In order to induce zinc deficiency in neonatal pups, we will foster wildtype newborn pups onto *znt4* knockout dams. Z*nt4* knockout mice, known as the lethal milk (lm) mutation (*329,*

330), produce zinc deficient milk due to an inability to transport zinc from the plasma to the mammary gland. Only 40% of pups nursed by *znt4* knockout mice survive to weaning (*331*). Pups will be fostered onto *znt4* knockout dams on postnatal day one (PND1) and nurse until PND5. Ovaries will be collected from surviving pups and processed as before to evaluate germ cell nest breakdown. The rate of primordial follicle formation will be compared to littermates which were not fostered on knockout mice. If the zinc requirement for primordial follicle formation is maintained *in vivo*, pups nursed by knockout dams will display a higher percentage of oocytes in germ cell nests and fewer primordial follicles compared to control animals.

Conclusion

The three studies detailed in this dissertation have built on previous knowledge of zinc deficient fertility reduction and expanded our understanding of zinc's role in ovarian follicle development. In particular these studies have demonstrated that zinc deficiency can impair oocyte and follicle health at earlier periods of development than previously investigated. Just as this research project expanded on previous work done in our lab, it has also laid the groundwork for future studies to address zinc deficiency and fertility. The introduction of *C. elegans* to our work will allow broad genome-wide investigations through genetic screenings, while also allowing elegant investigation of fertility mechanisms. Our work in mice has shown that both follicle assembly and preantral growth are disrupted by zinc deficiency *in vitro*. Confirming these findings in whole-animal models is the next step in establishing zinc as a critical mediator of early oogenesis and follicle development. Optimizing zinc homeostasis at all stages of follicle development may serve to improve female fertility in mammals, including humans.

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Appendix

Zinc requirement for actin nucleation and meiotic spindle migration during meiosis

When oocytes are matured in a zinc deficient environment, meiotic defects such as failure to form a polar body, telophase-I arrest, and symmetric division (figure 1) are common (*1, 2*). Symmetric division in oocytes is caused by a disruption of actin nucleation; a process in which the role of zinc has not been examined. Successful meiosis requires migration of the spindle to the cell cortex prior to division, a process driven by dynamic actin nucleation in the cytoplasm (*3-5*). Briefly, migration of the meiotic spindle is accomplished by an actin mesh organized around vesicles containing the small GTPase RAB11 (*4, 5*). These RAB11 vesicles organize two actin nucleators, SPIRE and FMN2, both of which are necessary for proper spindle migration (*6, 7*). Our research has shown that intracellular zinc is also found in vesicles which appear to co-localize with RAB11 vesicles (figure 2). Activated SPIRE proteins embed in vesicle membranes via a zinc-binding motif, and removal of zinc from culture media lowers SPIRE-vesicle interaction (*8*). We propose that the RAB11 vesicles necessary for actin nucleation contain high levels of labile zinc which are necessary for proper anchoring and activation of the SPIRE-FMN2 protein complex. Under zinc deficient conditions, improper activation of the actin nucleators leads to loss of the cytoplasmic actin mesh and impaired spindle migration (figure 3). Preliminary experiments have already shown changes in cytoplasmic actin nucleation under zinc deficient conditions (figure 4).

To test this hypothesis, three experiments were conducted to explore the interaction between zinc status and cytoplasmic actin nucleation during meiotic resumption. In experiment 1, we tested the hypothesis that zinc chelation would alter the formation of filamentous actin, leading to a change in the F:G actin ratio. In experiment 2, we attempted to microinject mouse oocytes with fluorescent probes to label actin (UTRCH-mCherry probe) or RAB11 vesicles (RAB11-mCherry) in living cells. This process would allow tracking of actin nucleation in real time, as well as colocalization of zinc and actin during

meiosis. In experiment 3, we evaluated the effect of zinc chelation on spindle positioning in the GV and M2 oocyte.

Experiment 1 – Methods and Results

To monitor treatment effects on the ratio of granular to filamentous actin, we used the "G-actin/F-actin *in vivo* assay biochem kit" from cytoskeleton (Denver, Colorado) according to manufacturer's instructions. Briefly, denuded oocytes or cumulus-oocyte complexes from 20 day old mice were cultured in control, zinc chelated (10μM TPEN), or rescue (TPEN + 10μM $ZnSO_4$) media. Cells were disrupted into filament-stabilization buffer. Ultracentrifugation (100,000 x G for 1hr) was used to separate the pools of F and G actin. Western blotting was employed to evaluate the ratio of the actin pools (figure 5).

Three variations of this protocol were attempted. In attempt 1 (9-15-15), denuded oocytes were cultured and results of the actin assay demonstrated a reduction in filamentous actin, even below the levels seen in a cytochalasin b control which should eliminate F-actin formation. Attempt one did not have a rescue treatment included in the protocol. In attempt 2 (9-30-15), whole cumulus-oocyte complexes were cultured under experimental conditions. F-actin was not detectable after centrifugation. Clearly this indicates a technical error, as even treatment with phalloidin (which promotes filament formation) did not affect the actin ratio. In attempt 3 (10-22-15) we also used cumulus-oocyte complexes, and did detect a reduction in F-actin with TPEN treatment. However, this effect was not rescued by the addition of exogenous zinc.

Experiment 2 – Methods and Results

To visualize RAB11 and actin cellular location we obtained plasmids containing the sequence for fluorescent probes to label both actin and RAB11. Plasmid preparation, bacterial digestion, and nucleic acid purification were conducted according to standard methods. Fully grown mouse GV oocytes were collected and microinjected with either probe while being held in media containing the PDE4-inhibitor

milrinone, which prevents meiotic resumption. After 6 hours to allow translation of the probe, cells were imaged using a fluorescent microscope.

Results – All analysis of probe quality (restriction digest, product length, nanodrop) indicated successful probe preparation before microinjection. Very few oocytes survived the microinjection procedure, and even fewer displayed a fluorescence after culture. Most likely this was caused by user error as well as technical inefficiency related to aging microinjection equipment. While we were able to obtain representative images of the UTRCH-mCherry fluorescence (figure 6), we did not generate enough fluorescent oocytes for the planned experiments.

Experiment 3 – Method and Results

Both rounds of meiotic division in oocytes are asymmetric, leading to release of half the genetic complement with only a small contribution of cytoplasmic factors into the polar body. During the first division, this occurs due to spindle migration prior to anaphase. For the second meiotic division, the M2 spindle forms directly after completion of the first division and remains anchored at the cell periphery. We hypothesized that zinc chelation could alter cellular localization of either the GV nucleus or the M2 meiotic spindle if actin nucleation was impaired.

To test this hypothesis we placed GV oocytes in control, 1μM, 5μM, or 10 μM TPEN for up to 6 hours. Cells were fixed and stained with DAPI to identify DNA. The shortest distance from chromosomes to the oocyte cortex was measured. The experiment was repeated using M2 oocytes to evaluate the effect of TPEN of M2 spindle anchoring (figure 7). There was a significant interaction effect between treatment and culture time in GV oocytes ($P<0.001$), however the effects of TPEN treatment were not clear in the GV oocyte. This is best demonstrated by the fact that control oocytes displayed both the smallest distance to the cortex (most asymmetric) and largest distance (most centered) depending on culture time – indicating higher levels of GV movement than we anticipated.

The treatment effects in the M2 oocyte were clearer as there was no treatment effect of culture time, but only of dose ($P<0.05$). M2 oocytes cultured with 10uM TPEN displayed a significantly

increased distance between the spindle and cortex, indicating the asymmetric cortical anchoring of the M2 spindle was disrupted with TPEN (figure 7-C). This implies zinc deficiency may disrupt the M2 division, where the effect of zinc deficiency has been less well studied by our lab.

Conclusion

This project aimed to explore one of the most exciting hypotheses generated during my graduate studies. The proposed mechanism, in which loss of vesicular zinc impairs actin nucleation, is quite elegant and supported by the published observations of both reproductive and cytoskeletal investigators. Bridging the gap between these two research fields, and providing a mechanism for the meiotic disruption observed in zinc deficient oocytes would have been a welcome addition to the scientific cannon. Unfortunately, these experiments were plagued by technical frustrations and inconclusive results.

The F:G actin ratio analysis (experiment 1) was planned as a simple first step in establishing our model of actin disruption; but even when employing an expensive retail assay kit we could not reliably provide an answer to the change in actin ratio. Clearly most of the limitations in this experiment were technical. For instance, the complete disappearance of the F-actin pool in round two indicates a major flaw in sample processing.

The use of fluorescent probes in experiment 2 was envisioned as the backbone of this study. Tracking actin filaments and zinc vesicles in real time would have provided tremendous evidence that zinc is required for actin nucleation and therefore spindle migration. Here again technical impediments halted our progress. Difficulty implementing the microinjection technique derailed our efforts in this experiment. The lengthy process of generating the probes, as well as the high cost for microinjection supplies eventually made further attempts at fluorescent labelling unattractive.

One bright spot in these experiments was the spindle positioning assay in experiment three. We showed here that under zinc-deficient culture conditions, the M2 spindle migrates away from the cell cortex. The effects of zinc deficiency on the second meiotic division have not been studies as closely as the first division, and these results indicate this may be a fertile field for investigation. An effect here may

also have implications for IVF, as this second division occurs in response to fertilization and may be dependent on optimized zinc levels in the IVF medium. One consideration for future studies is that this assay measured only two-dimensional distance between the spindle and cell cortex – which may not accurately reflect the three-dimensional location of the spindle within the cytoplasm.

Since embarking on these experiments, no convincing mechanistic theory to explain the meiotic defects of zinc-deficient oocytes has been put forward. The results of these studies do not disprove our hypothesis, but only demonstrate we were unable to effectively test it. Future studies to revisit this issue should be seriously considered.

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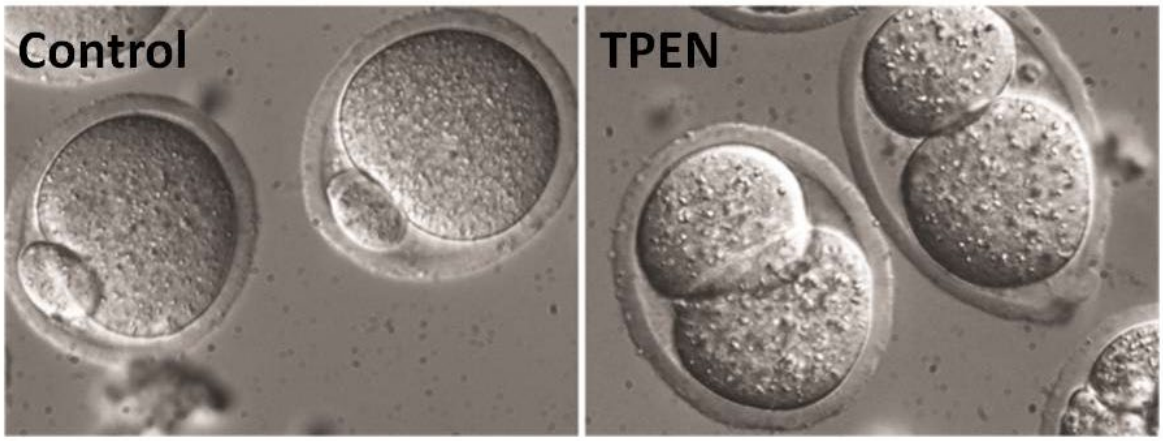

Figure 1. Zinc deficiency during meiosis leads to symmetric division in mouse oocytes.
GV oocytes were collected from antral follicles of mice primed with 5IU of pregnant mare's serum gonadotropin. Oocytes were matured for 16 hours in either control media or media containing 10μM TPEN. Asymmetric meiotic division requires migration of the meiotic spindle to the cell periphery, an actin-dependent process. (Cell culture and imaging by Xi Tian).

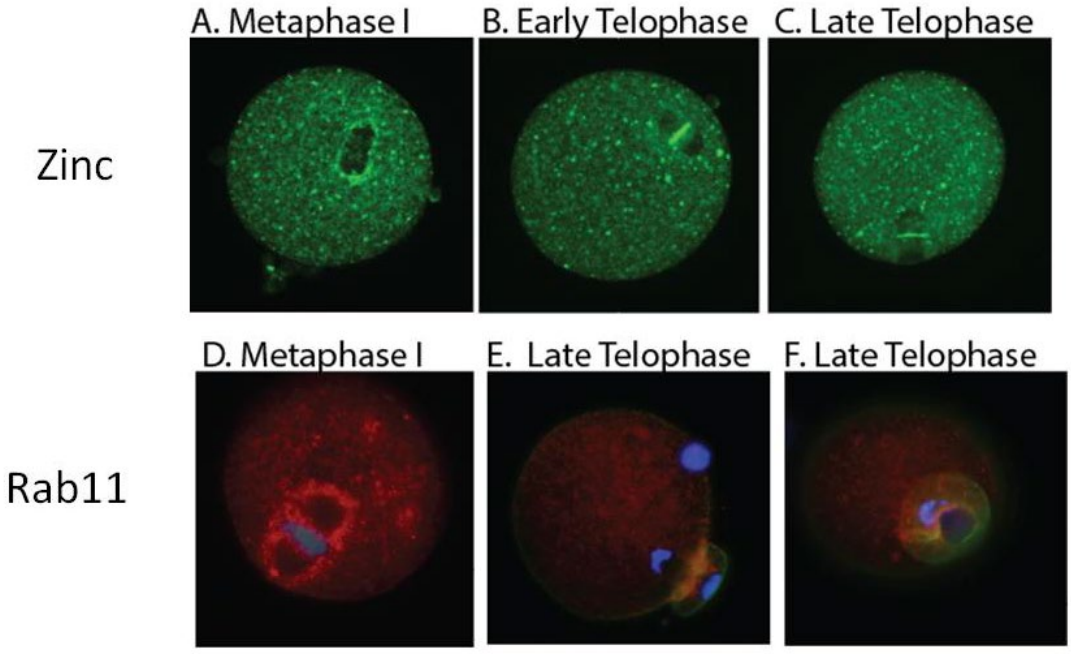

Figure 2. Rab11 vesicles and Zinc vesicles both accumulate around the spindle during meiosis. A-C: Cells were incubated with Fluozin-3 to visualize zinc vesicles in live oocytes during meiotic progression. D-F: Oocytes were fixed in PFA at regular intervals during meiotic progression. Immunostaining shows a similar cellular location of RAB11A-positive vesicles and zinc

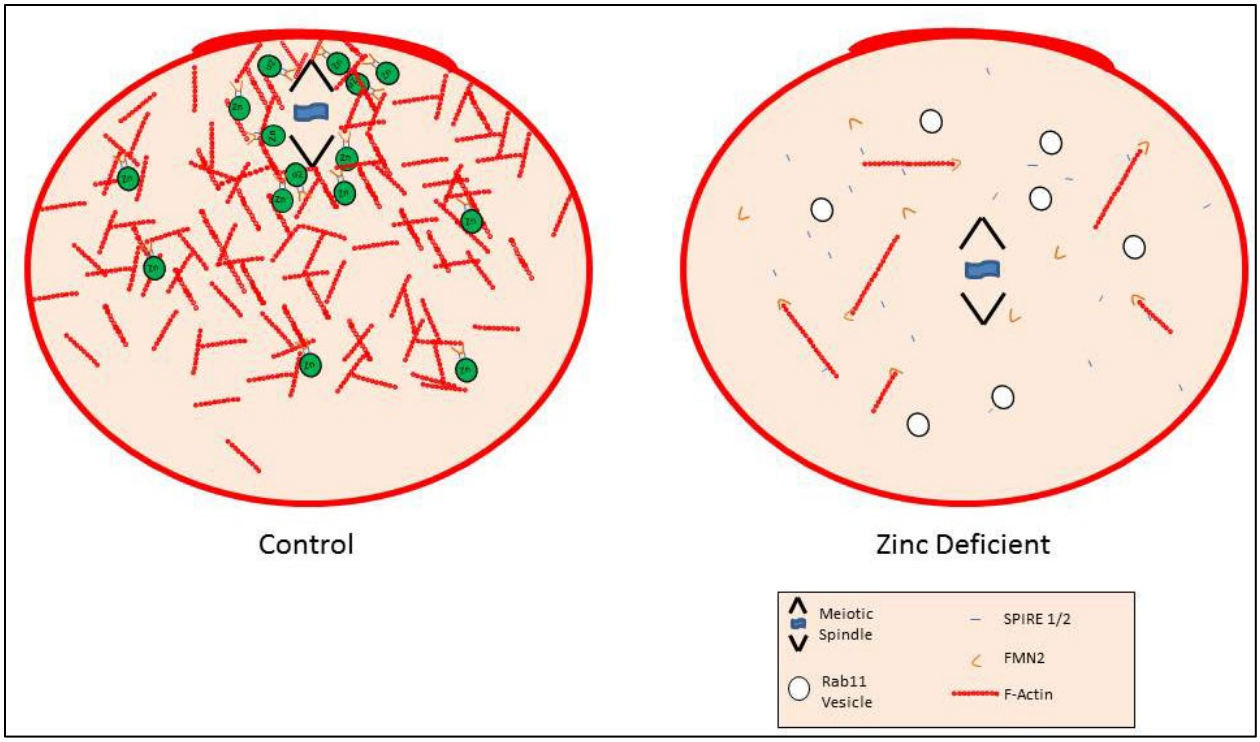

Figure 3. Proposed model for impaired spindle migration in zinc deficient oocytes.
Actin nucleators organize around RAB11 vesicles and create an actin mesh which causes migration of the meiotic spindle to the cortex. We propose that RAB11 vesicles contain zinc which is necessary for proper anchoring of SPIRE proteins (left). Without sufficient zinc, the SPIRE/FMN2 nucleator complex does not form and actin nucleation is impaired (right).

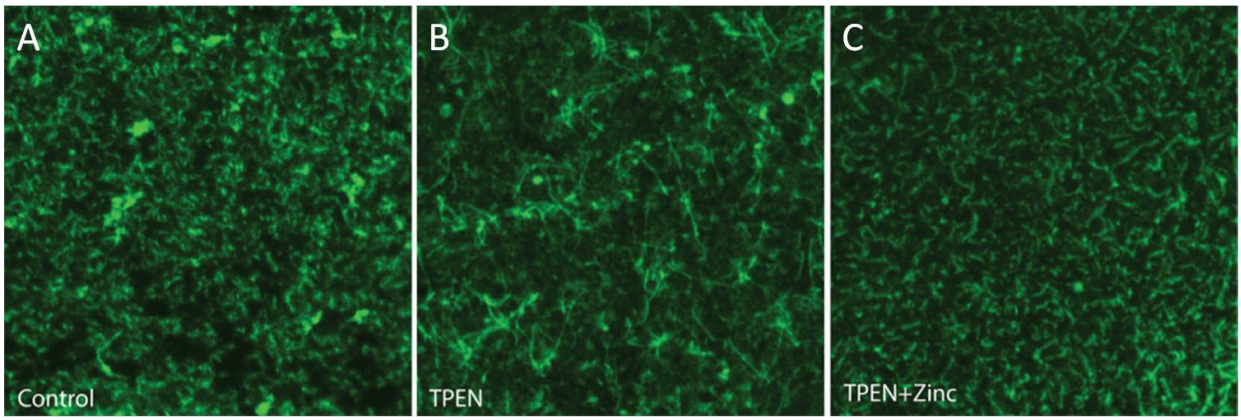

Figure 4. Zinc deficiency affects actin dynamics in maturing oocytes.
GV oocytes were collected and matured for 8 hours under control (A), zinc deficient (B), or rescue (C) conditions. Oocytes were fixed in 4% PFA and immunostained with phalloidin-488 to label actin filaments. Images were taken on a Zeiss FV10i confocal microscope at 300X. Zinc deficiency changes the appearance of the cytoplasmic actin mesh necessary for spindle migration and is characterized by longer actin filaments.

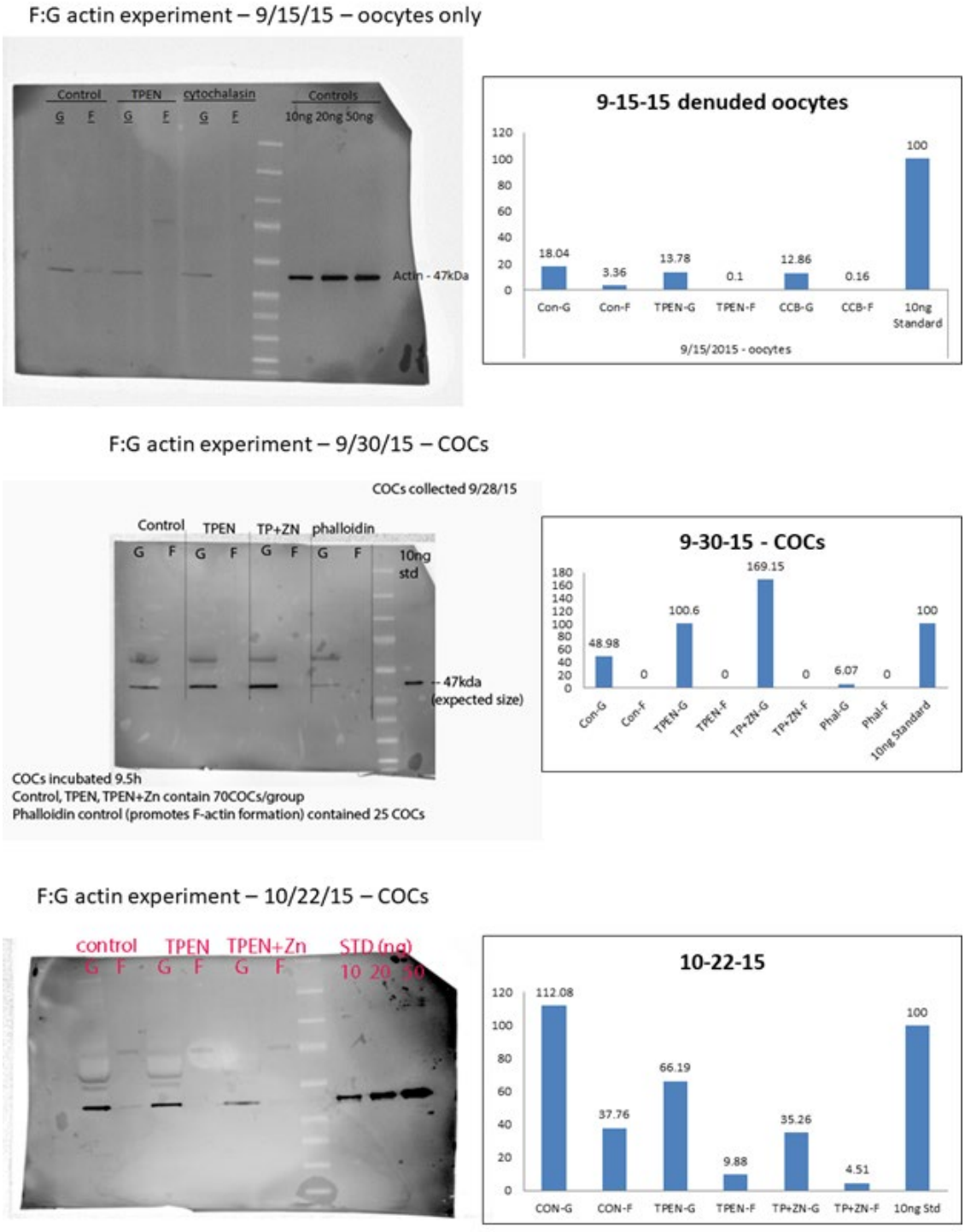

Figure 5. TPEN effect on actin filament formation is unclear
In 2/3 of replicates, TPEN reduced the ratio of F actin. However exogenous zinc was unable to rescue these effects. Cells were cultured for 9.5 hours in treatment before separation and quantification of actin forms. Densitometry data is presented relative to 10ng actin standard set at 100.

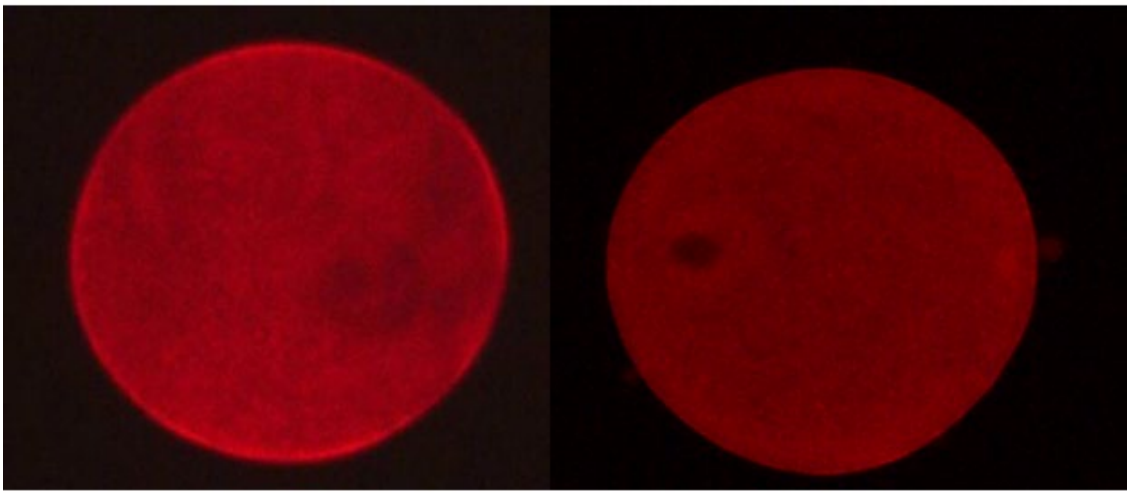

Figure 6. Representative images of oocytes microinjected with UTRCH-mcherry
Oocytes were microinjected with a fluorescent probe to label actin filaments. Actin shown in red. 63X magnification. Success rate of microinjection was too low for use in analyzing the effect of zinc deficiency.

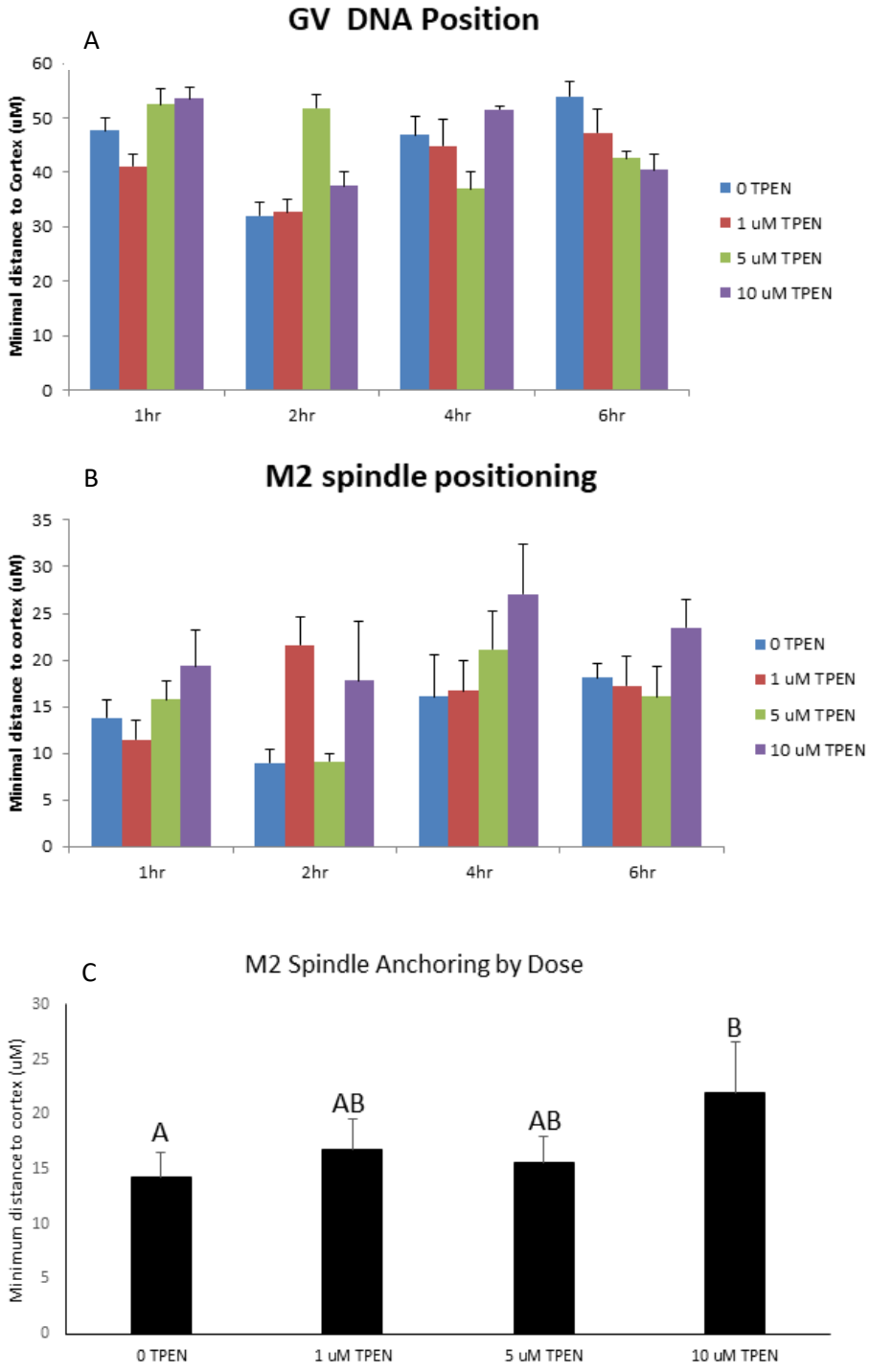

Figure 7. TPEN treatment was evaluated on spindle positioning in both GV and M2 oocytes.
A. There was a significant interaction effect of TPEN dose and culture time in GV oocytes ($P<0.001$). B. Raw M2 Data. C. There was a significant effect of dose on spindle positioning in M2 oocytes ($P<0.05$), indicating that TPEN impairs cortical anchoring of the M2 spindle.

VITA

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Selected Publications

Hester J, Hanna-Rose W, Diaz F. (2017) Zinc deficiency reduces fertility in *C. elegans* hermaphrodites and disrupts oogenesis and meiotic progression. Comp Biochem Physiol C Toxicol Pharmacol. Jan. 191:203-209.

Steele K, **Hester J**, Stone B, Carrico K, Spear B, Fath-Goodin A. (2013) Nonsurgical embryo transfer device compared with surgery for embryo transfer in mice. JAALAS, Volume 52 (1) 17-21.

Hester J, Mullins J, Sa Q, Payne L, Mercier C, Cesbron-Delauw MF, Suzuki Y (2012) Toxoplasma gondii antigens recognized by IgG antibodies differ between mice with and without active proliferation of tachyzoites in the brain during the chronic stage of infection. Infection and Immunity, 80(10): 3611-20.

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Selected Presentations

Hester J, Diaz F. Growing Oocytes Need Zinc: Zinc Deficiency in the Preantral Ovarian Follicle. Poster at Experimental Biology 2018. San Diego, CA.

Hester J, Hanna-Rose W, Diaz F. *Caenorhabditis elegans:* a new model for impaired fertility resulting from zinc deficiency. Oral Presentation and Poster. Society for the Study of Reproduction 2016. San Diego Ca.

Steele K, **Hester J**, Stone B, Spear B, Goodin A. Non-surgical embryo transfer with the NSET™ device is a 3Rs refinement technique that reduces stress in CD-1 mice. Poster presentation at the 12th FELASA SECAL Congress (2013). Barcelona Spain.