THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF ANIMAL SCIENCE

THE EFFECT OF ANDROGEN-MEDIATED ZIP9 SIGNALING IN THE CUMULUS OOCYTE COMPLEX

SAMANTHA STRONG
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Reviewed and approved* by the following:

Francisco Diaz
Associate Professor of Reproductive Biology
Thesis Supervisor

Troy Ott
Professor of Reproductive Physiology
Honors Adviser

* Electronic approvals are on file.

ABSTRACT

The ZIP9 protein has recently shown to bind androgens, but its effects on function of the cumulus-oocyte complex (COC) is not yet known. This research intends to gain a better understanding of the effects of androgens on ZIP9 protein and how it impacts the development and maturation of oocytes and cumulus cells. This was done by collecting cumulus-oocyte complexes and locating the presence of the ZIP9 protein in both cumulus cells and the oocyte. We then utilized immunofluorescence to identify the location of ZIP9 and the binding sites of testosterone. The complexes were then subjected to treatment with testosterone, a testosterone antagonist (Bicalutamide), and both the testosterone and the antagonist and imaged with light microscopy to evaluate the effects of treatments on morphology of the cumulus cells. Results showed the presence of ZIP9 and testosterone binding on the cell membrane of the oocyte and cumulus cells. Additionally, ZIP9 was observed on the mitotic spindle following maturation. When the COCs were exposed to testosterone and allowed to undergo cumulus expansion, we observed no obvious change from the cumulus expansion of COC's in the control group. When the COC's were exposed to a testosterone antagonist, the cumulus expansion was not as successful and when exposed to both testosterone and the antagonist, complete cumulus expansion failure was observed. After viewing the expansion, the oocytes were observed. Oocytes that were exposed to testosterone and the antagonist appeared to have an uneven and irregular surface, however all oocytes were alive, suggesting that there was an effect on the cytoskeleton or plasma membrane. This study demonstrated that there are several effects that testosterone or other androgens have on the development and maturation of cumulus-oocyte complexes.

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I would like to thank the other students that work in the Diaz lab. Through my time, I learned that research and academia is a team effort and everyone I ever met at the lab was incredibly nice and helpful. Each of them made this experience positive and productive. I would not have learned half of what I know about research without all of you.

Finally, I want to thank my incredible friends and family for being such a strong support system for me to lean on. Thank you for listening to me ramble about my research, even though you may not have known what I was talking about, and supporting me when I was struggling. I would not be where I am without you.

Chapter 1

Introduction

Oocyte maturation and development is an intricate process that can affect an organism throughout its' entire life. Proper maturation and development are dependent on factors that must be present at specific times in the process of oogenesis. Prior research has supported the importance of several hormones and minerals to oocyte maturation. These include androgens and zinc, which can both be associated to the function of the ZIP9 protein [1]. This is a recently discovered protein that binds to androgen and has several functions including regulating zinc content in follicular cells.

The role of androgens in cumulus-oocyte complexes is not well known. This research intends to further the understanding of testosterone's role in cumulus expansion, which is necessary for oocyte maturation and subsequently embryonic development. Additionally, this study should lead to a better understanding of how ZIP9 fits into these processes and how this knowledge can be used to improve reproductive health of all species.

Chapter 2

Literature Review

Oogenesis, the process of developing eggs that can be fertilized, begins prenatally and is accompanied by the initiation of meiosis, which undergoes arrest at prophase of meiosis I before birth. This process begins when the primordial germ cells move to the developing ovaries and divide into multiple “germ cell cysts” or “nests.” The germ cells in each cyst are interconnected and divide at the same rate [2]. These cells are referred to as oogonia until they divide via mitosis, forming oocytes. They then enter meiosis necessary to become haploid and arrest at prophase I. This is the extent of oocyte and follicle development prior to birth.

After birth, the germ cell cysts undergo programmed break down and cause the formation of primordial follicles [3]. During this process, multiple oocytes undergo apoptosis and the number of primordial follicles left comprise all of the germ cells a female will produce in her lifetime. Not all of these primordial follicles will undergo the process of activation, which is a complex process that is not completely understood. It has been shown to be regulated by a variety of pathways, with both stimulatory and inhibitory effects [4,5]. Activation of the follicles results in division of the granulosa cells and growth of the oocyte. Once activation has occurred, preantral development continues and is independent of FSH concentrations [6]. Within the follicles, granulosa and thecal cells differentiate and develop. Once the follicle forms a fluid-filled cavity, it is referred to as an antral follicle. The development of this follicle is dependent on FSH. the follicle can undergo follicle selection [7]. This process requires adequate quantities of gonadotropins and paracrine factors from the oocyte [8,9]. During this process, the granulosa cells further differentiate into mural and cumulus cells. As the follicle approaches ovulation, estrogen induces an LH surge which causes changes to occur to the oocyte and follicle, allowing

the oogonia to complete the first meiotic division and cumulus expansion, and the follicle to ovulate.

Ovulation involves key changes to the cumulus-oocyte complex. A critical part of COC maturation is cumulus expansion. Oocytes are surrounded by tightly packed cumulus cells. These cells are critical to oocyte survival and fertilization. Studies have shown that these cumulus cells have a variety of functions including immune like inflammation response and some level of interaction with sperm to ensure fertilization [10,11]. Cumulus expansion occurs before fertilization and requires synthesis of extracellular matrix by the cumulus cells. This process requires activation of multiple signaling pathways, including the EGF and SMAD pathways [13]. The SMAD pathway requires zinc in order to activate and function properly [13]. Proper and successful cumulus expansion is necessary for meiotic maturation of the oocyte and the ability of the oocyte to mature further [14]. For this reason, cumulus expansion is a good measure of the success of fertilization. During ovulation, the oocyte undergoes a series of critical changes that result in maturation and the ability to be fertilized. These include cytoplasmic rearrangements and synthesis of specific polypeptides [15]. As the oocyte ovulates, the first meiotic division completes, creating the first polar body. After ovulation, the cell is referred to as a mature egg or ootid and continues to mature.

When the sperm penetrates the zona pellucida, the acrosomal reaction occurs and the sperm bound to the vitelline membrane is internalized. The sperm induces calcium oscillations and causes the resumption of meiosis. The sperm the undergoes decondensation while the ootid undergoes the second meiotic division. This causes the formation of the female pronuclei and second polar body. The haploid male and female pronuclei fuse in a process termed syngamy and form a single diploid nucleus, now called a zygote. The zygote undergoes further mitotic

cleavage into daughter cells called blastomeres. After the eight-cell stage, this is referred to as the morula.

The morula continues to divide and develops into the blastocyst, with an inner cell mass and a cavity called a blastocoele. Additionally, the blastocyst has a single layer of trophoblast cells. The inner cell mass will give rise to the embryo while the trophoblast cells will eventually develop into the chorion, which is the fetal portion of the placenta. This blastocyst will eventually "hatch" out of the zona pellucida and continue development into fetus and placenta.

This research investigates a specific transporter protein, ZIP9, and its role in these processes. This protein is a member of the ZRT-and Irt-like Protein family and it functions to regulate the concentration of zinc within cells [16]. It is a novel androgen binding protein and was discovered in croaker fish in 2014 [17]. Previous research in the Diaz laboratory has shown the impact that decreased zinc concentration in the diet of adult mice for 10 days blocked ovulation and oocyte maturation and also limited cumulus expansion [18]. When the mice were fed zinc deficient diets for 3 days oocyte maturation was reduced and defects were observed in the oocyte spindle with arrest at metaphase and/or telophase of meiosis I. The 3-day zinc deficiency also increased the number of oocytes that did not exit the luteinizing follicles. Further research explored the importance of zinc to proper development of the placenta and growing fetus [19]. Zinc deficient animals had less developed and thinner placentas, both of which have negative effects for the pregnancy and health of the embryo. The fetus's development decreased in all stages when the mother is deficient in dietary zinc [20]. This further supports the importance of zinc and zinc transport-mediating proteins like ZIP9. It was recently shown that ZIP9 is partially regulated by androgens [17]. When testosterone bound to ZIP9 it caused an increase in transport of free zinc into the cell [16]. Additionally, ZIP9 was shown to play a role

in activating g-protein receptor signaling, resulting in apoptosis of primary follicular granulosa cells as well as prostate and mammary cell lines [19,21].

The objective of this research was to investigate the role of androgens acting through the ZIP9 transporter on development and function of cumulus-oocyte complexes. The discovery that androgens bind and activate ZIP9 has implications for oocyte development and subsequent embryonic health and the mechanisms are not yet known. This research could be used to improve the health and well-being of future pregnancies in both animals and humans. To address this objective, ZIP9 was localized in oocyte and cumulus cell membranes, treated with androgen, and the activated signaling pathways were analyzed. In addition, zinc-signaling was monitored.

Chapter 3

Experimental

Animals

The animal care and use was consistent with the Guide for the Care and Use of Laboratory Animals [Institute for Learning and Animal Research]. All animal use was approved by the Pennsylvania State University IACUC Committee (protocol #44472) Female mice were sourced from the research colony of the investigators. The mice were injected on day 18-20 after birth with 5IU of pregnant mare serum gonadotrophin (PMSG) to cause controlled ovarian hyperstimulation. The mice were then euthanized 2 days after injection and the ovaries were removed.

COC Collection

Cumulus-oocyte complexes (COCs) were harvested 2 days after PMSG injection. The ovaries were removed from the body cavity and placed into Minimum Essential Media (MEM) alpha media (Thermofisher, Waltham, MA) with 3mg/mL bovine serum albumin (Sigma-Aldrich, St. Louis, MO) and 10 uM milrinone (Sigma-Aldrich, St. Louis, MO) to maintain oocytes in meiotic arrest. The COCs were removed from the ovarian follicles through puncture with a needle and syringe. Cumulus-oocyte complexes were induced to undergo cumulus expansion by adding Epidermal Growth Factor (EGF) at a final concentration of 10 ng/mL. Some COCs underwent further processing to create fully denuded oocytes. This was done through gentle pipetting to remove the surrounding cumulus cells.

Binding of testosterone to oocyte and cumulus cell plasma membrane

To determine if testosterone can bind to oocyte plasma membrane, oocytes were collected from antral follicles and cultured with testosterone (100 nM, Sigma-Aldrich, St. Louis, MO) conjugated to Bovine Serum Albumin (BSA) and labelled with FITC fluorophore for 30 minutes. Conjugation to BSA prevents testosterone from entering the cell [22]. Other oocytes were cultured alone or with Testosterone-BSA-FITC and the androgen antagonist bicalutamide (1 uM; Sigma-Aldrich, St. Louis, MO) for 30 minutes. Cells were then washed extensively in Phosphate-Buffered Saline (PBS) and fixed in methanol for 15 minutes and imaged on an AxioScope 2 Plus (Nikon, Melville, NY). To determine if testosterone conjugated to BSA binds to cumulus cells, COC were collected and the cumulus cells were stripped from the oocyte and plated on a glass chambered slide with M199 media plus 5% fetal bovine serum (FBS; Thermofisher, Waltham, MA). The next day cells were washed with medium without serum and incubated with medium alone, testosterone-BSA-FITC (100 nM), or testosterone plus bicalutamide (1 uM; Sigma-Aldrich, St. Louis, MO) for 30 minutes. After treatment cells were washed with PBS three times and fixed with ice-cold methanol for 15 minutes followed by washing and imaging on an AxioScope 2 Plus epi-fluorescent microscope.

Effect of testosterone and bicalutamide on cumulus expansion

COC were collected as described above and treated with MEM-alpha media containing EGF (10ng/mL) and 5% FBS for 16 hours in medium only or medium containing testosterone (200 nM), bicalutamide (10 uM) or both testosterone and bicalutamide. After culture, complexes were imaged on a Nikon TE2000 inverted microscope (Nikon, Melville, NY) and a DP20

Olympus digital color camera with DP software (Olympus, Center Valley, PA). Oocytes were also imaged after gentle pipetting of the COC.

Immunofluorescence

Complexes and denuded oocytes were fixed in a microtubule stabilizing buffer made in the laboratory for 30 minutes. After this fixation, oocytes were washed in Phosphate Buffered Saline with Tween 20 (PBST) and 1% BSA three times. The cells then were blocked using a goat serum blocking buffer (PBS, 2% goat serum (Thermofisher, Waltham, MA), 1% BSA, 0.1% Triton-X and 0.05% Tween 20) for 50 minutes. This was followed with incubation in anti-ZIP9 antibody (1:100, SAB3500599; Sigma-Aldrich, St. Louis, MO) for an additional 50 minutes. Oocytes and COCs were then washed three times in PBST, and incubated in goat anti-mouse IgG Alexa Fluor 488 (1:1000; Thermofisher, Waltham, MA) and phalloidin-TRITC (0.5 ug/ml) for 30 minutes. They were then washed three more times with PBST. COCs and denuded oocytes were then mounted with DAPI anti-fade gold (Thermofisher, Waltham, MA) on glass slides with etched rings to prevent oocyte rupture when applying a cover slip. Slides were imaged using the AxioScope 2 Plus.

Results

The first objective of this project was to identify and localize ZIP9 in the COCs. This was done using antibody specific immunofluorescent staining. Results are shown below in Figure 1. The cells were stained using DAPI to identify the location of DNA within both the oocyte and surrounding cumulus cells. Those same cells were then stained with a ZIP9 antibodies that were

localized with a secondary antibody labelled with a fluorophore that fluoresces green, showing the location of the ZIP9 protein. The DNA was heavily concentrated in the cells' nuclei, as expected. Also, ZIP9 was detected in the plasma membrane of both the oocyte before maturation (Germinal Vesicle (GV) stage) and cumulus cells. However, ZIP9 was on the meiotic spindle in the mature egg after maturation. To our knowledge, this is the first observation of ZIP9 localizing to the spindle during oocyte maturation.

The second objective of the project was to understand whether androgens bind on the surface of oocyte and cumulus cells. To evaluate this, immunofluorescence was utilized. Denuded GV oocytes and groups of cultured cumulus cells were stained with DAPI to show DNA binding and T4-BSA-FITC, which binds to androgen receptors but prevents the testosterone from crossing the plasma membrane, and bicalutamide. Bicalutamide serves as an androgen antagonist, blocking testosterone from binding to the membrane receptors. The images shown in Figure 2 demonstrate that testosterone binds on the surface of both cumulus cells and oocytes (GV stage). Whether this is through interaction with ZIP9 remains unknown, but treatment with bicalutamide reduced binding compared to the testosterone alone indicating that it is likely through ZIP9 because bicalutamide is known to block testosterone binding to ZIP9.

The third experiment of this project addressed the effects that androgen alone or in the presence of bicalutamide had on cumulus expansion (Figure 3). The EGF treatment alone served as the control and exhibited normal cumulus expansion. When the COCs were exposed to testosterone, there was a similar level of successful cumulus expansion compared to EGF alone. When the COCs were treated with the testosterone antagonist, bicalutamide, cumulus expansion was not as successful and resulted in odd clumping of cumulus cells. Finally, when the COCs were exposed to both the testosterone and the antagonist, cumulus expansion did not occur.

Additionally, after expansion, the oocyte from the testosterone treatment appeared normal, while the bicalutamide oocyte appeared slightly grainy. Finally, the oocyte from the testosterone and bicalutamide test appeared grainy and rough, however all oocytes were alive as evidenced by the intact nucleus and nucleolus and absence of fragmentation.

Conclusions

Overall, our results showed that testosterone does bind to the plasma membrane of both cumulus and oocytes. Additionally, this testosterone binding was blocked by bicalutamide. This was an expected result because bicalutamide binds to the same receptors as testosterone, as it is a nonsteroidal antiandrogenic molecule [23]. After testing for testosterone binding and blocking by bicalutamide, ZIP9 protein was localized to determine its' location within cumulus and oocytes. This was to determine if the testosterone bound to the ZIP9 protein, as we hypothesized. The protein was detected on the plasma membrane of the GV oocytes and the cumulus cells. Interestingly, we showed that ZIP9 protein was localized to the spindle in mature oocytes. This was a surprising finding but could be due to the need for zinc during meiosis I and II [24]. Our final experiment determined the effects of testosterone and antagonist binding on the process of cumulus expansion. Results showed that cumulus expansion continued normally when exposed to testosterone. When the COCs were exposed to the bicalutamide, the cumulus expansion was partially blocked, resulting in clumped cells. When exposed to the testosterone and bicalutamide together, complete failure of expansion was observed. This was completely unexpected and a novel result. Additionally, the oocyte of this experiment appeared different from those during normal expansion, with a very grainy appearance. While the oocytes surfaces appeared irregular

and uneven, the GV was maintained indicating that these oocytes and all of the other oocytes in this portion of the experiment were alive. The rough appearance suggests that there was an effect on the cytoskeleton or plasma membrane. When the testosterone and bicalutamide compete for binding sites it causes failure of expansion. This could be due to what is comparable to mixed signals, which causes the complex to abort the expansion and caused the grainy appearance in the oocytes. The mixture of bicalutamide and testosterone could cause activation of several second messengers and pathways which when all activated at once result in the failure of cumulus expansion. This grainy, rough appearance indicates that there may be abnormal changes in the cytoskeleton, specifically in the actin filaments or microtubules. This hypothesis would need further experimentation and analysis, which was not feasible for the timeline of this experiment.

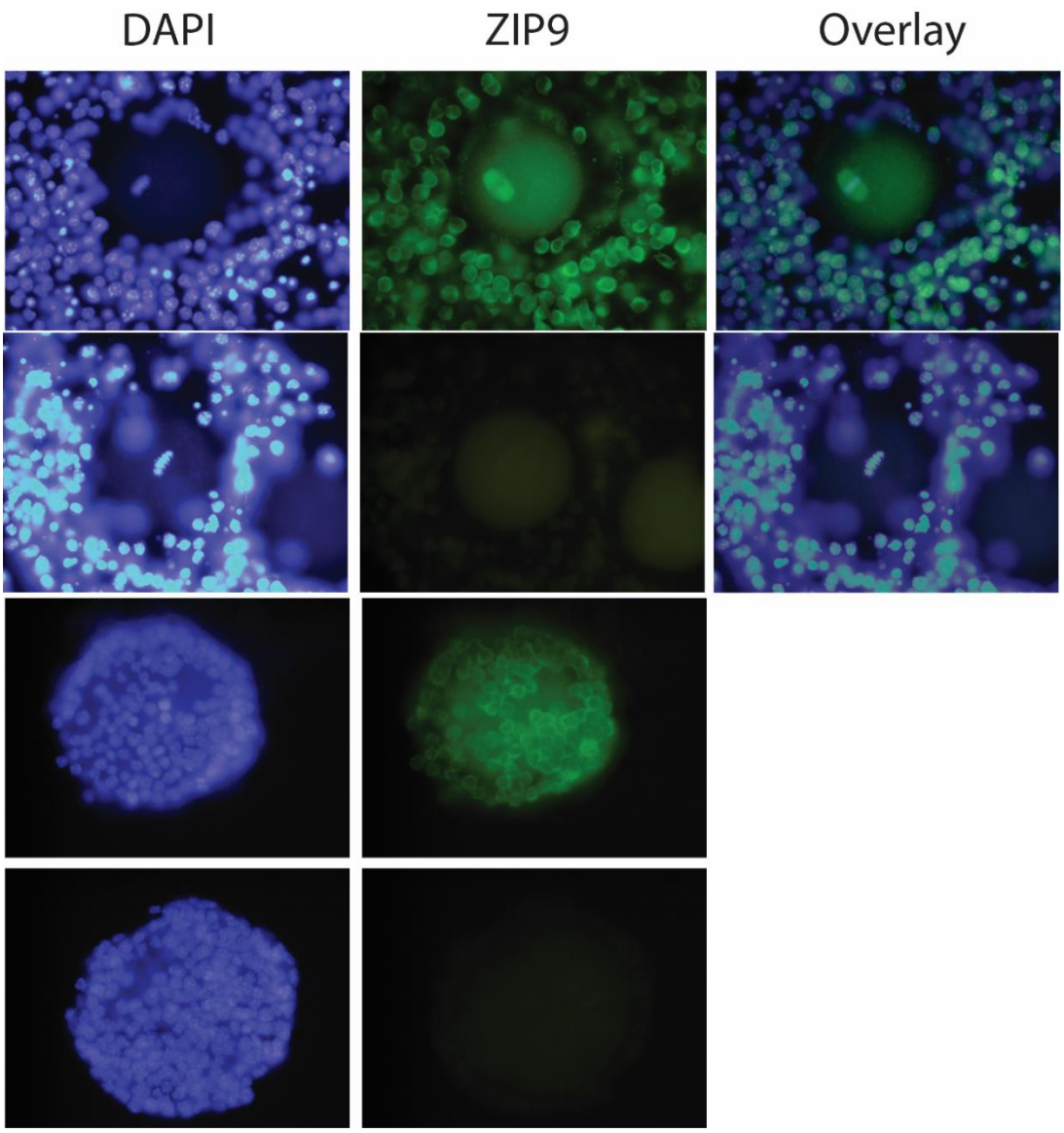

Figure 1: Cumulus oocyte complexes stained with immunofluroescent compounds to show DNA (blue) and the location of the ZIP9 protein (green). The top row of images shows the COCs after expansion and the bottom row shows the COCs before cumulus expansion.

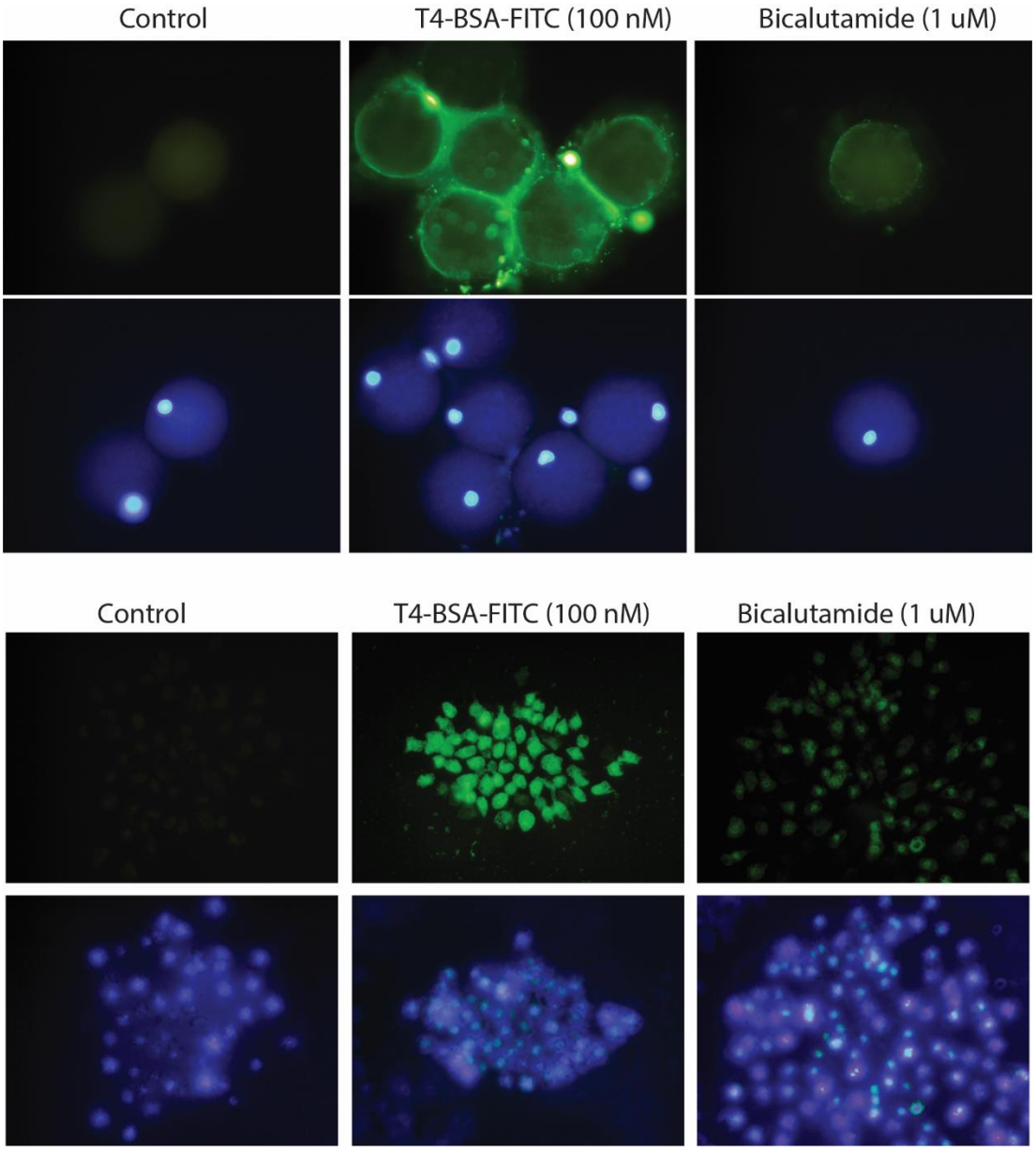

Figure 2: Binding of testosterone (bound to BSA to prevent crossing of the cell membrane) and a testosterone antagonist (bicalutamide) in oocytes (top row) and cumulus cells (bottom row).

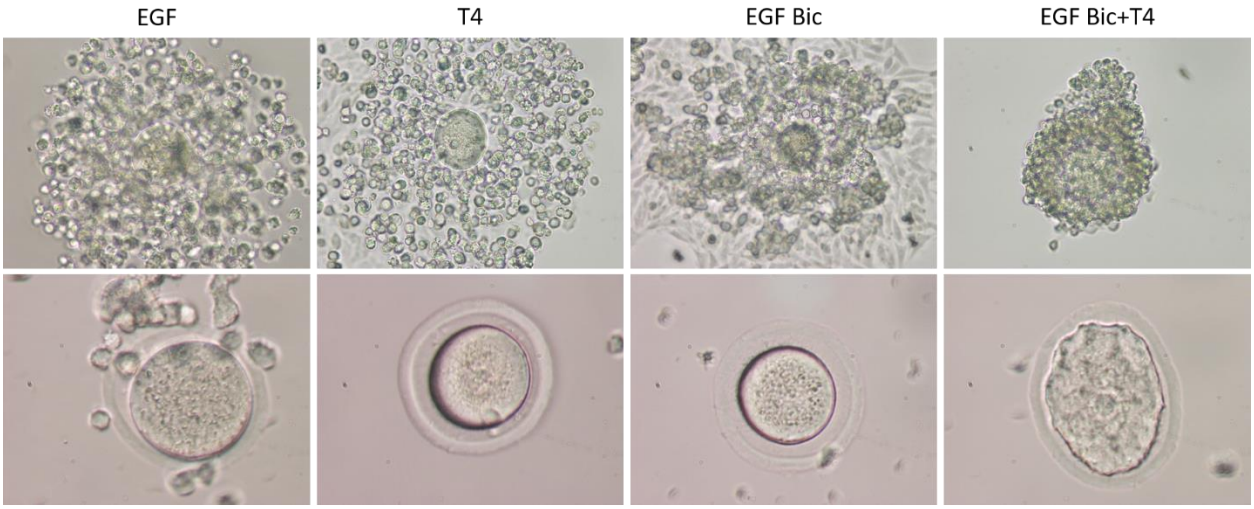

Figure 3: Images that show the effect of testosterone (T4) and a testosterone antagonist (Bic) on cumulus expansion.

Chapter 4

Discussion

Here we showed that testosterone, binds to the plasma membrane of the COC. This binding could be blocked by the androgen antagonist bicalutamide. This supports the concept that the binding is specific. Additionally, we confirmed that ZIP9 is also localized to the plasma membrane of the COCs, and also on the meiotic spindle. This indicates that ZIP9 might be regulating zinc content in the COC through the binding of androgens. Finally, treating the COCs with testosterone and bicalutamide resulted in failure of cumulus expansion indicating that a pathway exists for cumulus expansion to be controlled by androgens.

Results from these experiments raise several questions, First, the localization of ZIP9 on the meiotic spindle was unexpected and not something we had seen in previous literature. Additionally, the complete failure of cumulus expansion when exposed to testosterone and bicalutamide was unexpected. This research was qualitative because not enough samples were collected for statistical analysis. However, the response occurred in all of the COC treated with testosterone and bicalutamide. We expected cumulus expansion to be relatively normal in this instance, due to the binding of testosterone and bicalutamide we observed in the plasma membrane. We know that cumulus expansion requires both EGF and SMAD signaling [12]. We know that SMAD signaling is zinc dependent. The research from this experiment shows that when testosterone is partially blocked, cumulus expansion fails. It is still unknown whether testosterone signaling through ZIP9 modulates either the EGF or SMAD pathways, or if it blocks cumulus expansion through a different mechanism. This is a question that needs further research to answer.

Future research should address the mechanism of how androgens affect cumulus expansion and oocyte maturation. Additional research about gene expression is needed to determine how this mechanism functions. There could also be more research into the role of ZIP9 in meiosis and why it is located on the spindle. Finally, more research could be conducted to determine the pathway by which the combination of testosterone and bicalutamide block cumulus expansion, possibly through blocking activation of both EGF and SMAD signaling pathways.

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ACADEMIC VITA

Samantha Strong
sjs6127@psu.edu

Education

Pennsylvania State University August 2016- May 2020
Major: Animal Science
Schreyer Honors College

Scholarships, Awards, and Achievements

Academic Excellence Scholarship, The Pennsylvania State University- Schreyer Honors College, Fall 2016-Spring 2020
Gamma Sigma Delta Honors Society- Spring 2020
American Society of Animal Science Undergraduate Scholarship Recognition Award- Spring 2020

Work/Professional Experience

Penn State College of Medicine- Animal Caretaker in the Comparative Medicine Department, Summer 2017

- Prepared animal enclosures
- Completed general health checks on mice, rats, and rabbits
- Trained in various laboratory animal procedures, such as handling and anesthesia

Penn State University- Lab Assistant in the Animal Science Department, Spring 2018- Spring 2020

- Completing reproductive biology experiments
- Performing general biology lab tasks
- Compiling and presenting my own research

Penn State University- Part-time Barn Worker at the Dairy Barns, Summer 2019

- Completing general farm work, such as cleaning barns and preparing facilities
- General dairy cattle husbandry, including caring for calves and calving cattle
- Milking cattle

Extra-Curricular Activities

Block and Bridle, Spring 2019-Spring 2020

Positions held: Webpage Chair

Pennsylvania State University Marching Blue Band, Fall 2016- Fall 2019

Positions held: Squad Leader

Penn State Reproduction Research Team, Summer 2018

Positions held: Summer Heat Checking Volunteer