Kit Ligand Effect on Growth of Bovine Follicles in Culture and the Effect of Kit Ligand on the Function of Theca Cells

by
Shaina Jachter

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Animal Science
(Honors Associate)

Presented June 3, 2020
Commencement June 2020

AN ABSTRACT OF THE THESIS OF

Shaina Jachter for the degree of Honors Baccalaureate of Science in Animal Science presented on June 3, 2020. Title: Kit Ligand Effect on Growth of Bovine Follicles in Culture and the Effect of Kit Ligand on the Function of Theca Cells.

Abstract approved:_____

Cecily V. Bishop

Ovarian steroidogenesis is heavily controlled by the formation and function of the theca cells. A condition known as polycystic ovarian disease in humans and cystic ovarian disease in cattle is due to abnormal steroidogenesis. Knowing more about theca cell formation and function can aid in gaining further knowledge about polycystic ovarian disease and cystic ovarian disease. Currently very little is known about the role theca cells play in these two diseases. The goal of this thesis research was to look at the effect of kit ligand neutralization on the growth of bovine follicles in culture. Ovariectomies were performed on Holstein and Jersey dairy cows to retrieve follicles. The effects of kit ligand replacement were observed on the growth of bovine follicles in a novel matrix-free 3D culture using round bottom low attachment plates. The effects of kit ligand replacement were also observed on the formation and function of the theca cell layer in those follicles. The results suggest that kit ligand is secreted by multilayer follicles, but only small antral follicles possess the receptor for kit ligand (c-kit). No statistically significant differences were found in all follicle types in survival rate or in the rate of growth when comparing two types of media used for in vitro follicle maturation (α-MEM and T-199). Additionally, no significant differences were observed in the percentage of surviving follicles of each type per media. Statistical significance was observed in the kit ligand treated group as the treatment tended to reduce the number of days it took a follicle to form an antrum. Both fast and slow antrum follicles that were treated with kit ligand developed a significantly larger diameter compared to the controls, but the fast antrum follicles died after two days. While not statistically significant, addition of kit ligand does appear to increase percent antrum formation.

Key Words: Kit ligand, polycystic ovary syndrome, cystic ovarian disease, theca cells

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ACKNOWLEDGEMENTS

I would like to thank Dr. Cecily Bishop for being my mentor and guiding me through this process, and Will Simmons for being my lab partner. I would also like to thank Dr. Michelle Kutzler for being on my thesis committee. The collaborators at the ONPRC (Oregon National Primate Research Center) include Dr. Jing Xu and Maralee Lawson. Thank you to Drs. Charles Estill and Vanessa Peixoto De Souza, our veterinarians, for retrieving ovaries and Dr. Fredrick Stormshak for supplying materials. I would like to thank those who helped heat check, which include Jared Jensen, Juriana Barboza Sagrero, Rosalia Tanori, and Chelsey Naito. I would also like to thank the USDA experiment station for funding.

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued) Page

LIST OF FIGURES

Figure Page

LIST OF TABLES

1. INTRODUCTION

Cystic ovarian disease (COD) in cattle and polycystic ovary syndrome (PCOS) in woman are the leading reproductive disorders in both of these species. The etiology and pathophysiology of both of these diseases are poorly understood. One of the goals of this study is to identify the ovarian processes behind these disorders so we have the understanding to further develop treatment plans for COD in cattle. Scientists agree that both these diseases are closely related and both involve a dysfunction in the hypothalamic-pituitary-ovarian axis (De Silva and Reeves 1988). Symptoms are similar among the two, with irregular cycling, increased androgens in the circulation and, most notably, polycystic ovaries (Lin et al., 2018). COD has made a large impact in the dairy industry with, on average, $137 lost per lactation and with 12.8% of the herd being afflicted with this disease (Bartlett et al. 1986).

A failure of the hypothalamus to respond to an LH surge is thought to be one of the causes of COD in cattle (Silvia et al. 2002). Progesterone and its dysfunction also may be a contributor to the disease (Silvia et al. 2002). The human equivalent of COD, PCOS, is present in 5-10% of women of reproductive age and is responsible for 75% of anovulatory follicles (Lin et al. 2018). Polycystic Ovarian Syndrome can be characterized as having 12 or more follicles with a 2-9 mm diameter and may increase the risk of anovulation, infertility, and pregnancy complications such as preterm delivery and pre-eclampsia (Balen 2004; Legro et al. 2013). This disease can be very debilitating, leaving the affected woman with a profound impact on her reproductive, metabolic, and cardiovascular health. In order to be classified with this disease, two of three following qualifications must be met:

anovulation, hyperandrogenism, and/or polycystic ovaries (Ehrmann 2005). Increased LH secretion, hyperinsulinemia, ovarian insulin resistance, theca cell dysfunction and hyperandrogenism are all associated symptoms of PCOS (Legro et al. 2013; Mikhael et al. 2019). Cystic ovarian disease in cattle and PCOS in women are closely related reproductive diseases with similar symptoms and pathophysiology. Greater understanding of one disease can contribute to the knowledge of pathogenesis of the other.

The function and development of theca cells are of main interest in developing targeted treatments for these diseases. Theca cells serve as one of the main steroid-producing cells of the follicle and they are essential to follicular growth and development. Theca cells indirectly allow for granulosa cell and oocyte communication by creating healthy granulosa cells that can function to communicate with the developing oocyte. They also give structural support to the follicle as it produces a fertilizable oocyte and function as the main steroid producing cell, as reviewed in the article by Young and McNeilly (2010). The theca is separated into the theca interna and the theca externa, with the interna serving as the majority steroid secreting cell layer (Magoffin 2005). The theca interna consists of 3-5 layers of elongated steroid-secreting cells (Magoffin 2005). The theca cell synthesizes androgens which are then converted to estradiol in the granulosa cells using P450 aromatase (Young and McNeilly 2010). In vitro culture models are being developed in order to better study these cells as they are difficult to mechanically isolate from other cells (Gan et al. 2017). Although the origins of theca cells remain a mystery, they are thought to have originated from precursor cells within the stroma (Honda et al. 2007; Young and McNeilly 2010). During development of theca cells, different granulosa cell proteins are secreted, and these proteins drive differentiation of theca cells (Magoffin

2005). Developing targeted treatments to the steroid-producing cells is thought to be one way of relieving symptoms of COD in cattle and PCOS in women.

Kit Ligand (referred to by its abbreviation KITLG) is an important contributor to the health and function of the reproductive system. It is proposed KITLG and its receptor (cKit) may be responsible, in part, for theca cell recruitment, development, and differentiation in the ovarian follicle. Kit ligand is a growth factor produced by granulosa cells that act on stromal cells and differentiated theca cells (Manova et al. 1993; Young and McNeilly 2010). The c-kit receptor is expressed in primordial germ cells, theca cells, and oocytes, while the kit ligand protein is expressed in the granulosa cells and epithelial cells (Hutt et al. 2006a). Kit ligand is thought to directly affect theca cell growth, steroid production, and differentiation of theca cells (Parrott and Skinner 1997). Kit ligand has many functions in the ovary, and contributes to many processes such as folliculogenesis, oogenesis, preventing apoptosis in preantral follicles, oocyte growth, and, most relevant to this study, theca cell growth and differentiation (Driancourt et al. 2000). Activation of Kit ligand with c-kit is necessary to maintain follicle growth and development (Williams et al. 1990).

Follicle culture is used to examine theca cell growth and development under the influence of different hormones, proteins, and growth factors. Very little research has been done on follicle culture of bovine follicles due to the difficulty of isolating follicles from the ovarian cortex (Araújo et al. 2015). Two different culture media (TCM-199 and α-MEM) have been used in previous cultures of bovine follicles (Araújo et al. 2015; Braw-Tal and Yossefi 1997; Katska and Ryńska 1998). Because it was unknown which of these

media would be best for growth of bovine follicles in matrix-free 3D culture, media performance was also compared.

2. LITERATURE REVIEW

2.1 CYSTIC OVARIAN DISEASE

2.1.1 Summary

The main cause of temporary infertility and reproductive failure in cattle can be attributed to cystic ovarian disease (COD) (Hooijer et al. 2001; Ortega et al. 2015). Ovarian cysts in cattle are follicles that are 20 mm or greater that fail to ovulate, persist for 10 days with no luteal tissue present, and interrupt the normal reproductive cycle (Ortega et al. 2015; Silvia et al. 2002). The main cause of COD in cattle is the failure of the hypothalamus to respond to estradiol to produce a luteinizing hormone (LH) surge (Silvia et al. 2002). Progesterone plays the role of ovulation and establishing the dominant follicles during cycling, and its dysfunction is also thought to be a major contributor to the disease (Silvia et al. 2002). Cystic ovarian disease results in significant economic loss for the dairy farmer due to the costs of treatment for those affected and for the prolonged calving period (Vanholder et al. 2006). In a study done on Holstein-Friesian cattle, the average money lost by COD was $137 for one lactation and the incidence of COD was 12.8% (Bartlett et al., 1986).

2.1.2 History

Cystic ovarian disease (COD) is the most prevalent reproductive disorder in high producing dairy cattle and has been of great interest to scientists since the 1940's (as reviewed in Vanholder et al. 2006). Cows were observed with nymphomania and bull-like

behavior (as reviewed in Casida and Chapman, 1951 and in Vanholder et al. 2006). In 1917, Albrechsten hypothesized that the ovarian disease was secondary to a uterine disease, while in 1921, Hess hypothesized it was the uterine disease that was secondary to ovarian disease (as reviewed in Casida and Chapman 1951). In 1915, Pearl and Surface suggested that the disease was due to a dysfunction in the endocrine system (as reviewed in Casida and Chapman 1951). However, in 1944, it was found that the cause of COD in cattle was due to a positive action of the pituitary gonadotropin on cystic ovaries (Casida et al. 1944).

2.1.3 Etiology

Cystic ovarian disease occurs primarily in highly productive dairy cattle. Cattle that are milked 4 times daily have a COD incidence of 10.6% (Casida and Chapman, 1951); cattle originally milked 4 times daily but had milking reduced to twice daily have a COD incidence of 8.5%; cattle that are milked twice daily have a COD incidence at 6.8%; and cattle that are no longer lactating have a COD incidence of 3.4% (Casida and Chapman 1951).

Metabolic disorders can also contribute to COD in cattle. Insulin resistance during early post-partum period is correlated with a higher incidence of cystic ovaries (Vanholder et al. 2006). Two of the bulls who sired just 11.6% of all daughters, also sired Nearly 20% of the daughters sired by two bulls developed COD (Kirk et al. 1982). However, the heritability of this disease is low, being only 0.07 to 0.12 (Hooijer et al. 2001; Vanholder et al. 2006). Some scientists hypothesize that the artificial selection of dairy cattle, due to a genetic correlation between COD and milk yield, will increase COD in cattle (Hooijer et al. 2001).

2.1.4 Pathophysiology

The most widely accepted cause of COD is a dysfunction of the hypothalamic-pituitary-ovarian axis (De Silva and Reeves 1988; Hooijer et al. 2001; Vanholder et al. 2006). The cyst formation is believed to be caused by the preovulatory luteinizing hormone (LH) surge either appearing at the wrong time, in an insufficient supply, or is completely absent during the period of time where the dominant follicle is maturing (Lopez-Diaz and Bosu 1992; Vanholder et al. 2006). In one study, it was shown that a premature gonadotropin releasing hormone (GnRH)/LH surge, when done at the time of no dominant ovarian follicle present, would produce an anovulatory cow that would grow follicles up to a size greater than 25 mm in diameter, producing follicular cysts (Gumen et al. 2002). High-producing dairy cattle would have an estrogen concentration that would rise at a slower rate when compared to low-producing cattle because of the increased steroid metabolism which causes an LH surge at a larger follicular size and a lower estrogen concentration (Gumen and Wiltbank 2005). Testosterone concentrations are the highest in cystic follicles compared to small antral follicles and pre-ovulatory follicles in dairy cattle (Paredes et al. 2011). However, the introduction of progesterone can cause the dairy cow to develop normal hypothalamic responses and return to normal cycling (Gumen and Wiltbank 2005). Because the complete pathophysiology of ovarian cysts is not well understood, a model is necessary in order to better understand the physiology of this disease (Gumen and Wiltbank 2005). However, the pathophysiology of COD in cattle appears to have a strong relationship with the insensitivity that the hypothalamus develops to estradiol during a time that estradiol elevated and this insensitivity is caused by a GnRH/LH surge in which an increase of progesterone did not follow (Gumen and Wiltbank 2005). It is

possible that this hypothalamic-pituitary dysfunction could be a symptom as opposed to the cause of COD in dairy cattle (Vanholder et al. 2006). At the follicular level, alterations of the LH receptor may cause the follicle to not ovulate (Vanholder et al. 2006). LH is essential at the follicular level as if LH is disrupted, then ovulation and luteinization become disrupted, trapping the oocyte within an unruptured corpus luteum (Robker et al. 2000). Additionally, it was noted that apoptosis and cell proliferation were altered in cystic follicles (Isobe and Yoshimura 2000; Vanholder et al. 2006). These alterations could disrupt normal follicle growth and steroidogenesis (Vanholder et al. 2006). Another notable aspect of the pathophysiology of COD in dairy cattle is the abnormal steroidogenesis that occurs. Disrupted steroid receptors are observed in cattle with COD, along with modified coregulatory proteins that affect receptor expression (Ortega et al. 2015). One of the essential coregulators is NCOA3, which is necessary for normal physiology of the cell and this coregulator affects a wide range of different signaling systems (Wu et al. 2002). Additionally, it was found that 66% of dairy cattle with COD had abnormal progesterone concentrations at the time of detection (0.1–1.0 ng/mL) (Silvia et al. 2002). Of the cattle in this abnormal progesterone range, 10% ovulated and of that 10% ovulated 76% of new follicles that developed became cystic (Silvia et al. 2002). Furthermore, cystic cows typically have higher concentrations of LH due to higher pulse frequencies and these frequencies contribute to the development of cystic follicles (Cook et al. 1991; Hamilton et al. 1995). It is thought that, because of this high LH exposure, cystic follicles secrete higher levels of estradiol than non-cystic follicles (Hamilton et al. 1995; Kittok et al. 1974; Silvia et al. 2002).

2.2 POLYCYSTIC OVARY SYNDROME

2.2.1 Summary

The human variation of this disease is commonly known as polycystic ovarian syndrome (PCOS) and is also characterized by polycystic ovaries and ovulatory disfunction (Lin et al. 2018). Polycystic ovary syndrome is a very common reproductive disorder, with 5-10% of women of reproductive age having this syndrome and 75% of anovulation infertility being attributed to this disease (Lin et al. 2018). Unlike COD in cattle, PCOS can be characterized as having 12 or more follicles in the ovary with a 2-9 mm diameter, as opposed to at least one 20 mm follicle (Balen 2004). Women who have PCOS increase their risk of anovulation, infertility, and pregnancy complications such as preterm delivery and pre-eclampsia (Legro et al. 2013). This disease is known to have a significant impact on the reproductive, metabolic, and cardiovascular health of a woman throughout her life (Ehrmann 2005). Women with PCOS have at least two of the following: anovulation, hyperandrogenism, and/or polycystic ovaries (Ehrmann 2005). Clinical, morphological, biochemical, endocrine, and molecular studies have added to the complexity of the understanding of PCOS (Laven et al. 2002). Increased LH secretion and loss of GnRH, hyperinsulinemia, ovarian insulin resistance, theca cell dysfunction and hyperandrogenism are all associated with the pathophysiology of PCOS (Legro et al. 2013; Mikhael et al. 2019). If the etiology of COD is better understood, then this understanding can be applied to offer more treatment options for women who are affected with PCOS.

2.2.2 History

In 1721, an Italian physician named Antonio Vallisneri described the symptoms of what is now known as polycystic ovary syndrome (PCOS) as he performed a post-mortem examination on an infertile woman who had shiny ovaries that appeared white and were the size of pigeon eggs (as reviewed by Insler and Lunenfeld 1990 and Szydlarska et al. 2017). In another report in 1844, the ovary was described as discolored and swollen to the size of a goose egg (as reviewed by Rokitansky et al. 1855). Lawson Tait presented the need for the removal of ovaries in women who presented with PCOS-like symptoms in 1879 (as reviewed by Szydlarska et al. 2017). Cystic ovaries and their pathology were further investigated by von Kahlden in 1902 (as reviewed by Szydlarska et al. 2017). In 1935, Stein and Leventhal found bilateral polycystic ovaries were associated with endometrial hyperplasia, multiple follicle cysts, and the absence of a corpora lutea (Stein and Leventhal 1935; as reviewed by Szydlarska et al. 2017). Eventually, the National Institute of Health (NIH) held a conference on PCOS that established diagnostic criteria for the disease in the 1990s (as reviewed by Szydlarska et al. 2017). These criteria for diagnosis were established in 1990 during a National Institute of Health (NIH) conference in which the symptoms, hyperandrogenism and oligo-anovulation, were associated with PCOS (as reviewed by Dumesic et al. 2015 and Zawadzki and Dunaif 1992).

2.2.3 Etiology

The specific etiological factors of PCOS are not yet understood, and therefore cannot be prevented (Apter 1998). However, the disease is thought to be multifactorial with an additional genetic component (Dennett and Simon 2015). First generation women born from females with PCOS had a 20-40% chance of developing PCOS themselves, as

opposed to the population who contract PCOS at just 4-6% (Dennett and Simon 2015; Goodarzi et al. 2011). A high-fat high-sugar diet led to metabolic dysfunction, irregular cycling, and PCOS in the rat model (Roberts et al. 2017). Weight loss has had many positive impacts on people with this disease as symptoms lessened and health benefits followed (Apter 1998). Weight loss helped to ease symptoms such as infertility, metabolic disorders, and the long-term implications (Apter 1998). Other risk factors for PCOS include non-alcoholic fatty liver disease, type 2 diabetes, hypertension, cardiovascular disease, and dyslipidemia. (Alexander et al. 2009; Baldani et al. 2015; Zhang et al. 2018). A study in prepubertal and pubertal girls who were all daughters of women with PCOS suggested that, when compared to controls, these girls had higher ovarian volume, BMI, insulin, LH, and 17 hydroxyprogesterone (Dumesic et al. 2015; Sir-Petermann et al. 2009). However, a premature increase in androgen and DHEA production was not observed in the daughters of women with PCOS. Therefore, during puberty is when reproductive abnormalities are thought to develop in the daughters of women with PCOS (Dumesic et al. 2015).

2.2.4 Pathophysiology

Similar to cystic ovarian disease (COD) in cattle, PCOS appears to be due to a malfunction in the hypothalamic-pituitary-ovarian access and is associated with metabolic disorders such as hyperinsulinemia (Balen 2004). Most scientists agree that the excess of androgens originate in the ovary, which appears to follow the abnormal steroidogenesis (Balen 2004). Masculinized regulation of androgen forming enzymes in theca cells are hypothesized to originate from excessive LH stimulation or desensitization to LH and give

rise to PCOS (Rosenfield et al. 1990). Androgen synthesis is prevalent in the theca interna layer of the ovary. Enzymes used to form androgens are under the control of LH in the ovary (Balen 2004). An irregular LH level was observed in PCOS patients, with 100% of the non-obese patients with the disease having an elevated LH to follicle stimulating hormone (FSH) ratio (Taylor et al. 1997). Elevated LH level and an increase in the frequency and amplitude of LH pulsations are also associated with an increase in the incidence of PCOS (Taylor et al. 1997). The elevated LH pulse frequency suggests that the dysfunction associated with PCOS is due to rapid GnRH release and this rapid GnRH release is at least somewhat at the hypothalamic level as the hypothalamus is responsible for gonadotropin releasing hormones (Taylor et al. 1997). As seen in cattle, administration of progesterone to the patient can help to lower the GnRH pulse frequency and help the patient return to normal cycling (Ehrmann 2005).

Estradiol in the follicular fluid of woman with PCOS is lower than normal (Mason et al. 1994; Wallach and Goldzieher 1981). However, in a second study where estradiol production from both PCOS patients and normal patients were similar, the PCOS patients exhibited a decrease in progesterone secretion when compared to the normal patients, which is similar to COD findings (Erickson et al. 1992; Mason et al. 1994). This suggests a similarity in the dysfunction of the hypothalamic-pituitary-ovarian axis that is seen in COD. Additionally, insulin could play a key role in the pathophysiology of this disease as insulin has a direct impact on the androgen production of theca cells (Ehrmann 2005). Insulin not only works with LH to increase androgen production, but insulin additionally inhibits the sex hormone-binding globulin, the protein that binds to testosterone (Ehrmann 2005). This leads to more testosterone circulating in an unbound state, leading to an

increase of testosterone concentration in those affected (Ehrmann 2005). However, Schoemaker (1991) reported that the effect of androgens on LH secretion was inconsistent while the effects on androgens on the ovary was similar to other reports of PCOS symptoms (Schoemaker 1991).

Therefore, it is suggested that the causes of this PCOS are multifactorial with a strong relationship to the hypothalamic-pituitary-ovarian axis, and most likely originate in the ovary. These studies aimed to additionally identify the origin of this disease and thus, provide better treatment options.

2.3 THECA CELLS

2.3.1 Summary

The main ovarian process that will be analyzed in this study is the function and development of theca cells. Theca cells are essential to the process of folliculogenesis by playing the role of the endocrine cells for ovarian follicles (Magoffin 2005). Follicles secrete proteins during growth, and these proteins induce differentiation of theca cells in the interstitial stroma (Magoffin 2005). The theca cell plays many roles during the process of folliculogenesis. These cells allow communication between the granulosa cells and oocyte through crosstalk, synthesize essential androgens, and give structural support to the follicle as it develops to produce a fertilizable oocyte (Young and McNeilly 2010). Theca cells are separated into the theca interna and the theca externa. The theca interna is essentially made up of 3-5 layers of elongated endocrine cells and have characteristics essential of a steroid secreting cell (Magoffin 2005). Theca cells are rather difficult to study

as opposed to other ovarian cells because the process of isolating them from granular cells is complicated (Gan et al. 2017). However, because of the important role that these cells play in reproduction and steroidogenesis, it is necessary to find an in vitro culture model to better understand these cells (Gan et al. 2017).

2.3.2 Physiology

Theca cells begin to form when there are two or more layers of granulosa cells during secondary follicle formation when mesenchymal precursor cells adjacent to the follicles in the ovarian stroma begin to differentiate (Edson et al. 2009; Young and McNeilly 2010). The theca externa layer, however, is made up of non-steroidogenic cells between the theca interna layer and the interfollicular stroma (Magoffin 2005). It is known that theca cells differentiate from unspecialized mesenchymal cells in the stroma. The precursors, however, to these cells have not yet been identified (Magoffin 2005). It is believed, however, that these precursors have properties of stem cells due to their expected ability to proliferate and differentiate (Magoffin 2005). Not much is known about theca cell recruitment and growth because of a lack of *in vitro* experimental systems to culture and differentiate these cells (Honda et al. 2007). Once theca cells are established in the follicle, they begin a bi-directional communication with granulosa cells by serving as the rate-limiting step in steroidogenesis in response to insulin-like-growth-factor-1 (IGF1) and kit ligand (KL) (Orisaka et al. 2009). Theca cells additionally communicate with the oocyte. Most notably, growth differentiation facor-9 (GDF-9), a product of the oocyte, plays a key role on theca cell function (Solovyeva et al. 2000). Without the presence of GDF-9, follicle growth stops at the primary stage, with the presence of abnormal granulosa

cells and an absence of the theca cell layer (Dong et al. 1996; Solovyeva et al. 2000). Theca cells, in response to luteinizing hormone (LH), will produce androgens during enzymatic conversion of cholesterol to progestins and androstenedione, which are finally converted to testosterone by the enzyme CYP17A1, and testosterone and androstenedione are further transformed by the enzyme aromatase triggered by FSH in the granulosa cells to produce 17β-estradiol and estrone (Young and McNeilly 2010).

Not much is known about the origins of theca cells (Honda et al. 2007). They appear after the presence of two layers of granulosa cells and are thought to have originated from precursor cells within the stroma (Young and McNeilly 2010). It is assumed that precursor cells originating in the follicle would recruit the cells that form the theca from the stroma (Young and McNeilly 2010). However, these precursors have been poorly studied and therefore it is unclear as to whether the theca is formed from cells surrounding the follicle or from the stroma (Young and McNeilly 2010). It is hypothesized that not just one precursor is responsible for the formation of the theca cell layer, but rather a complex system that forms the steroidogenic follicle layer (Young and McNeilly 2010). In a 2007 study involving theca stem cells originating from mice, when theca cells were stimulated in vitro by up to four stimuli: (1) LH, (2) insulin-like-growth-factor (IGF1), (3) kit ligand, or (4) granulosa cell conditioned media, then theca cells were able to develop into androstenedione producing cells (Honda et al. 2007; Young and McNeilly 2010).

2.3.3 Significance

Theca cells are especially challenging to study due to the difficulty of isolating them, and they can only be effectively isolated mechanically (Gan et al. 2017). No method

is known to separate theca cells from granulosa cells effectively, making this the reason that mechanical isolation is the normal requirement (Tian et al. 2015). In particular, the best marker for theca cells is expression of CYP17A1, but this is a cytoplasmic marker and thus cannot be used in cell-sorting methods without destroying the integrity of the cell. In a study that compared PCOS thecal cells to non-PCOS thecal cells, it was noted that an increase in a steroid enzyme named CYP17 was observed, as well as a higher rate of androgen secretion in the PCOS cells (Cadagan et al. 2016). This suggests that there is a correlation between the high androgen secretion associated with thecal cells and PCOS. The communication and interaction between granulosa and theca cells is necessary for efficient follicle growth and steroid production (Hamilton et al. 1995). Since the abnormal steroidogenesis is linked as a symptom to PCOS and COD, this makes theca cells an important factor to look at in order to develop targeted treatments for these diseases.

Insulin treatment increases the secretion of androgens and progesterone in PCOS theca cells (Cadagan et al. 2016). LH manipulation along with insulin manipulation has been seen to increase androgen production in non-PCOS theca cells in vitro (Cadagan et al. 2016). Theca cells contribute to nearly 50% of all androgen secretion in the body (Adashi 1994). Although the etiology of PCOS in humans and COD in cattle remains a mystery, it is suggested that abnormal steroidogenesis is a major contributor (Franks 2006). There are three theories as to what contributes to this excess of androgen production. One theory is that hyperinsulinemia and insulin resistance is a major factor on why excess androgen production is taking place. However, two other proposed theories include: (1) a primary theca cell defect, and (2) excess thecal cell stimulation due to an excess of LH secretion (Cadagan et al. 2016). In response to human chorionic gonadotropin (hCG), even

after LH suppression, increased thecal steroid production is still observed (Abbott et al. 2000). Cultured theca cells were obtained from PCOS ovaries, and it was noted that steroid production was twenty times more than normal theca cells (Gilling-Smith et al. 1994). In the PCOS theca cell, elevated steroidogenic enzymes were observed. Specifically, 17alpha hydroxylase/17,20 lyase (CYP17A1) and P450 side chain cleavage enzyme (CYP11A1) excess production are thought to increase androgen production in the PCOS theca cell (Wood et al. 2004). Other PCOS theca cell abnormalities are altered mRNA gene expression, altered retinoic acid synthesis, altered WNT signal transduction, and increased GATA 6 (Wood et al. 2004).

Learning more about theca cell function and formation in both the normal process and the abnormal hyper-androgen producing process could help lead to targeted treatments for COD and PCOS. In COD, this can help to improve herd management and milk production in high-production dairy cattle and help improve overall productivity and economic profit (Bartlett et al. 1986).

2.4 KIT LIGAND

2.4.1 Summary

Kit ligand (particularly it's disruption) is thought to be correlated with theca cell dysfunction. Kit ligand is a growth factor that granulosa cells produce in order act on differentiated theca cells and stromal factors (Manova et al. 1993; Young and McNeilly 2010). Bovine granulosa cells were shown to have expressed kit ligand, while their respective theca cells were shown to express the receptor c-kit (Parrott and Skinner 1997).

Kit ligand directly affects theca cell growth and steroid production during development. By stimulating androgen production but not progesterone production suggesting that kit ligand promotes differentiation of the theca cells (Parrott and Skinner 1997).

Kit ligand and its receptor are important regulators of folliculogenesis and oogenesis (Driancourt et al. 2000). Kit ligand functions in the ovary to: grow follicles past the primary stage, prevent apoptosis in preantral follicles, grow oocytes, and, most relevant to this study, grow and differentiate theca cells (Driancourt et al. 2000). Kit ligand is also thought to stimulate androgen output from theca cells (Driancourt et al. 2000). Therefore, kit ligand plays an important role on the health of the oocyte and also follicle survival (Hutt et al. 2006b). Kit ligand is also known as stem cell factor (SCF) and is activated when it is bound to its receptor, c-kit (Williams et al. 1990).

2.4.2 Physiology

The c-kit receptor is a tyrosine kinase-type receptor, and is a part of the PDGF/CSF-1/c-kit receptor subfamily (Besmer et al. 1993). Two spliced mRNA's form the kit ligand that can take two forms, the soluble form or as the cell-associated molecule form (Besmer et al. 1993). Kit ligand is essential for the production of blood cells and platelets, gamete production, and melanin production (Dehbashi et al. 2017). Kit ligand additionally assists in the development of the hippocampus in the brain and the development of interstitial cells in the intestine (Dehbashi et al. 2017; Hutt et al. 2006a).

In the mouse, kit ligand codes for in the white spotting and steel loci, and mutations in these loci cause effects on hematopoiesis, production of red blood cells, gametogenesis, and melanogenesis (Yee et al. 1994). Furthermore, mutations in these same areas affected

oocyte migration and follicle development, effectively causing the mouse to develop infertility (Parrott and Skinner 2000). It was shown that kit ligand had an effect on the cell cycle, being required for the G1 phase and the S phase (Yee et al. 1994). Kit ligand was able to suspend apoptosis, for when cells were deprived of growth factors or exposed to irradiation in the presence of kit ligand, irreversible apoptosis was able to be suspended for up to an hour (Yee et al. 1994).

Kit ligand and its receptor are essential to melanogenesis and melanocyte maintenance and function (Hachiya et al. 2009). After hair follicle growth was induced and cutaneous tyrosinase production was activated, an increased amount of membrane bound kit ligand was observed (Hachiya et al. 2009). Tyrosinase expression was inhibited when an antibody was introduced that acted on neutralized kit ligand, which lead to hair depigmentation in both mice and humans (Hachiya et al. 2009). Hence, the function and development of pigment cells can be greatly affected by the presence or absence of kit ligand (Lee et al. 2010). Kit ligand and its receptor also play an important role in hematopoiesis, the generation of red blood cells (Hoffman et al. 1993). Hematopoietic progenitor cells including burst forming unit megakaryocyte, high proliferative potential colony forming cell, and long-term bone marrow culture-initiating cell, are greatly affected by the presence of kit ligand in regards to the proliferation of kit ligand (Hoffman et al. 1993).

Kit ligand and c-kit additionally play an important role in both male and female gamete production (Figueira et al. 2014). In the Sertoli cells of the testes, kit ligand concentrations were raised (Manova et al. 1993). Sertoli cells help to nourish and sustain developing spermatogonia in the testes (Figueira et al. 2014). The sperm cells that were in

the developing stages in the testes were shown to have expressed the receptor c-kit (Unni et al. 2009), and furthermore, kit ligand in the testes was observed to affect the differentiation of spermatogonia (Manova et al. 1993). The interactions between c-kit and kit ligand are essential for necessary germ cell growth in the testicle and is necessary to prevent apoptosis (Yan et al. 2000). Apoptosis was not only prevented in spermatogonia, but also spermatids and spermatocytes, leaving kit ligand treated tubules much less vulnerable to apoptosis (Yan et al. 2000).

Kit ligand is also notably important for normal reproductive development in the ovary. Kit ligand and its receptor, c-kit, interact with oocytes at every stage in folliculogenesis (Miyoshi et al. 2012). The c-kit receptor is expressed in primordial germ cells, theca cells, and oocytes, while the kit ligand protein is expressed in the granulosa cells and epithelial cells (Hutt et al. 2006a). Granulosa cells additionally associate with kit ligand mRNA expression, and granulosa cells interactions with kit ligand are necessary for healthy fertility (Miyoshi et al. 2012). It is also observed in situ and through immunohistochemistry in infantile mice that high levels of kit ligand are located in central cords, which are essential for the development of the early follicle (Manova et al. 1993). Granulosa cells secrete kit ligand in the growing follicle and are thought to act on theca cells and oocytes (Manova et al. 1993; Parrott and Skinner 2000).

Most relevant to this study, kit ligand is thought to directly influence the development and function of theca cells in the follicle (Parrott and Skinner 2000). In stem cells in the bone marrow, it is observed that kit ligand can induce stem cell development, and this stem cell development therefore suggests that the mechanism is that kit ligand acts on ovarian stromal stem cells, which then become theca cells (de Vries et al. 1991; Parrott

and Skinner 2000). Kit ligand is thought to be important to many processes in the reproductive system. Those processes are: primordial germ cell dominant follicle establishment, primordial to antral follicle development, oocyte development, production of granulosa cells, meiotic arrest, and theca cell recruitment, development, and function (Hutt et al. 2006a). In antral follicles, kit ligand is predicted to influence the androgen production of theca cells in pigs, mice, and cows (Hutt et al. 2006a; Parrott and Skinner 2000). During early development in the follicle, kit ligand is also observed to initiate primordial follicle recruitment. In addition, granulosa cells produced kit ligand, which recruits precursors to theca cells (Hutt et al. 2006a; Kezele et al. 2005). Theca cell recruitment is thought to be induced by the granulosa cells, because granulosa cells release kit ligand, which induces stromal stem cells to differentiate into theca cells (Parrott and Skinner 2000).

2.5 FOLLICLE CULTURE

2.5.1 Summary

Follicle culture in this study was used to examine follicle growth and development in the presence of different hormones and proteins. There is limited research done on the improvement of bovine follicle culture systems, while there is more extensive research done on goats, sheep, and mice (Araújo et al. 2015; Arunakumari et al. 2010; Eppig and O'Brien 1996; Saraiva et al. 2010). Two different culture mediums, α-MEM and TCM-199, have been used in cattle follicle culture and will be used in this study (Araújo et al. 2015; Braw-Tal and Yossefi 1997; Katska and Ryńska 1998). Much remains

unexplored in bovine follicle culture. This includes: the effects of additional media being added periodically to the culture, the effects of the base medium itself, and the medium replacement effect on estradiol levels and steroid production (Araújo et al. 2015). Because the literature did not provide clarity as to which of these media types would perform better, initial studies compared follicles cultured in α-MEM or TCM-199.

2.5.2 History and Significance

Research in other species regarding follicle culture has led to progressive new discoveries, such as growing follicles in culture, achieving antrum formation, in vitro ovulation, and even producing young after IVF was performed (Katska and Ryńska 1998). One reason bovine follicle culture is more difficult to study is the difficulty of isolating the follicle from the ovary (Katska and Ryńska 1998). The bovine ovary is much more fibrous and difficult to mechanically manipulate than the ovary of the rodent (Katska and Ryńska 1998). Additionally, large livestock have longer reproductive cycles and mature much more slowly than the rodent (van den Hurk et al. 1998). For adult bovine ovaries, more common follicle isolation techniques include mechanical isolation by mincing the ovary into small pieces and then loosening the follicles from the ovary via pipetting (Figueiredo et al. 1993; Telfer 1996). Grating cortex of the ovary is also a technique that has been used to release small preantral follicles (Telfer 1996). Enzymatic digestion of ovarian tissue is another method of obtaining follicles for culture (Heiligentag and Eichenlaub-Ritter 2017). In a 1998 study, it was noted that follicle survival rates were significantly reduced when no other additives were supplemented in the medium (Katska and Ryńska 1998). Follicle culture and its development is significant, because with this tool to study follicle culture,

further endocrine factors and their effects on growing follicles can be observed (Roy and Greenwald 1985; van den Hurk et al. 1998).

2.5.3 Culture Medium

Currently, culture systems for bovine follicle growth is limited. However, many improvements are being made currently when it comes to the addition of growth factors and hormones to the culture media. These additions can increase follicle growth and activation (Araújo et al. 2014). Hormones and growth factors added to the media to improve follicle growth and health in other ruminants include EGF, LH, insulin-like growth factor, FSH, thyroxine, and LIF (Araújo et al. 2014; Arunakumari et al. 2010; Luz et al. 2012; Saraiva et al. 2010). EGF can have many profound effects on follicles developing in a culture medium. In a 2004 study performed on goat tissue, an increase in oocyte and follicle diameter was observed, suggesting that FSH and EGF increase follicle size and promote oocyte growth (Silva et al. 2004). Insulin is also an additional valuable resource to add into a follicle culture medium. In a 2006 study, insulin-like growth factor (IGF) and its effects on bovine follicle growth were observed. Follicle diameter and estradiol production were all significantly improved when follicles were put in the treatment group with IGF (Walters et al. 2006). This is due to the fact that IGF works with LH and FSH during follicle growth to initiate differentiation and production of granulosa cells and theca cells (Campbell et al. 1996; Gutiérrez et al. 1997; Walters et al. 2006). The leukemia inhibitory factor (LIF) is thought to increase the kit ligand expression, which in turn increases primordial follicle growth and cellular growth (Nilsson et al. 2002).

Therefore, adding LIF to follicle culture could be beneficial as it is suggested to work with kit ligand to promote follicle growth and development.

Medias used for bovine culture included α-MEM and T-199. The base medium as well as the method of renewing the base medium during follicle culture has a profound effect on follicular development and estradiol concentrations in cattle (Araújo et al. 2015). Both of the media contain essential supplements that can support the health of the follicle, including amino acids, vitamins, and salts (Araújo et al. 2015). The addition of FSH and insulin is essential for proliferation of the cells, greater estradiol concentrations, and increased cell size (Araújo et al. 2015; Hulshof et al. 1995). As a culture medium, TCM-199 has been shown to have greater maintenance of follicle health and more follicles that reached antrum formation in a 2012 study (Rossetto et al. 2013). However, basement membrane, organelles, and oocyte integrity were not altered between the two mediums (Araújo et al. 2015; Rossetto et al. 2013). One noted difference between the two media is that estradiol concentrations were higher in the α-MEM treated groups, while antrum formation was more significant in the TCM-199 group (Araújo et al. 2015). Therefore, both media had the potential to be viable substances for follicle growth and are explored in this study.

3. MATERIALS AND METHODS

3.1 IMMUNOHISTOCHEMISTRY

Deparaffinization of bovine and primate ovarian tissue sections (5 micrometer) were performed (n=2 primate; n=3 bovine; replicates of 3-4 sections/slide). A dip into citric clearing solution occurred for five minutes three times. Two dips into 100% ethanol (EtOH) solutions occurred for three minutes each. A dip into 70% EtOH occurred afterwards for one minute. The section was then dipped in distilled water (diH2O) for five minutes.

Antigen retrieval was then performed after deparaffinization and rehydration. The antigen retrieval buffer was filled to the top of the fill depression in the microwavable vessel. The vessel was then heated in a microwave until boiling. Once 98°C was hit, antigens were then retrieved for 30 seconds.

Primary antibodies, KITLG or c-kit, were prepared by diluting the antibody with Tris-buffered saline (TBS) in 1% bovine serum albumin (BSA) to a final concentration of 1:50 or 1:25, respectively. Secondary antibodies, donkey anti-rabbit IgG and goat anti-rabbit IgG, were diluted in TBS with 1% BSA, to a final concentration of 1:50 or 1:3000, respectively. As a negative control, the primary antibody was omitted from an adjacent tissue section.

The slides were then washed in for five minutes two times in TBS plus 0.025% Triton X-100 with gentle agitation. The slides were then blocked overnight at 4°C in 10% normal donkey serum. The border of the tissue section was outlined with a dry, hydrophobic pen. After blocking, slides were then drained and the area around the sections

were wiped with a chem wipe. The primary antibody was diluted in TBS with 1% BSA and applied to the slides. Slides were then incubated overnight at 4°C. After incubation, slides were then rinsed two times for five minutes in 0.025% Triton with gentle agitation. The fluorophore-conjugated secondary antibody was then applied to slide after being diluted in TBS with 1% BSA. Slides were then incubated for 1 hour in the dark to avoid photo bleaching. Slides were then rinsed three times for five minutes in the dark with TBS. A mounting medium was then applied to the tissue sections, being careful to avoid bubbles that could alter the image. A coverslip was applied on the top and the slides were kept in the dark till the next day. Once kept overnight, nail polish was applied to secure the coverslip to the slide.

Images were then taken at 20x magnification using DAPI and Texas Red imaging to locate where in the tissue sections kit ligand and its receptor were expressed. Images started with DAPI, with lighting adjusted. Exposure was turned down so spots in nuclei could be observed. DAPI was to be the brightest on the image without it overpowering. Texas Red florescence was first imaged in the no-primary antibody controls, and emission spectra were lowered until all non-specific staining was set at background levels, then sections with primary antibody were imaged, and areas of high intensity were considered positive. These settings were adjusted for each slide set. DAPI and Texas Red were captured, and overlay was edited when done. Annotations and scale bars were then added to the images.

3.2 OVARIECTOMIES

A mixture of Holstein and Jersey cows (n=12) were housed at the Oregon State University Hogg Animal Metabolism Building for this study. Cattle were fed hay silage and water ad libitum. PGF2-α was administered two days before surgery to lyse the CL on the ovary and ensure the female is in proestrus (Whittier et al. 1991). Previous studies in other females have shown that follicles collected from females in the early follicular phase have the greatest survival in culture (Xu et al. 2009). All animal procedures were approved by the Oregon State University institutional animal care and use committee.

Bilateral routine ovariectomies were performed via flank laparotomy. Briefly, the hair was shaved over the left paralumbar fossa and skin was aseptically cleaned prior to surgery. Cows received an epidural and were sedated. A skin incision was extended through the muscle layers into the peritoneum. The left ovary and then the right ovary were removed using an ecraseur. The ovaries were transferred to a container with holding media to be transported to the lab in order to retrieve tissue sections for follicle dissection and follicle culture.

3.3 FOLLICLE RETRIEVAL AND CULTURE

Holding media was created using α-MEM media with 3 mg/ml BSA, penicillin, streptomycin and fungizone. The media was then filter sterilized. Small gauge needles, tissue slicer and blades, culture plates, warm bench, and CO_2 incubator were used in this process. Once the ovaries were retrieved, the ovarian cortex is minced into 2 x 2 x 1 mm cortical strips and put into holding media. Follicles were then mechanically isolated using dissecting scopes in the holding media using 27-gauge needles and small, multilayer follicles displayed the following characteristics for culture; an intact basement membrane,

two to three layers of granulosa cells, and a visible healthy oocyte that was round and centrally located within the follicle.

Culture media was made with α-MEM media. For media comparison, T-199 was also used (n=4 cows). This media was supplemented with 3mg/ml bovine serum albumin (BSA), insulin 10µg/ml, taurine 5mM, sodium selenite 6.7 ng/ml; 2mM glutamine, 2mM hypoxanthine, 50 µg/ml ascorbic acid, 50 U/ml aprotinin, 10 µg/ml gentamicin, penicillin, streptomycin and fungizone. Media was then filtered with 50 ml syringes under sterile conditions.

For FSH, the final concentration was 200 ng/ml. EGF was added to 5 µl into 10 ml of α-MEM culture media once chosen for optimal growth (n=8 cows). LH was at a final concentration of 100 ng/ml. The main media component is α-MEM, α-MEM+FSH/EGF are administered from start of culture, and α-MEM+FSH/EGF/LH is introduced on day 3 of culture.

Addition of 100 ng/ml LH on day 3 of culture was performed. This was added to culture media already containing FSH. 0.1 mg/ml stock was made by diluting 4 µl concentrated stock into 96 µl media. 10 µl of this diluted stock was added to 10 ml culture media for dose of 100 ng/ml from start of culture.

One plate was made per cow with 15-22 follicles per plate. Follicles were arranged into rows, with each row being a different treatment group. One row was to be the control follicles with just culture media. Another row is treated with the protein kit Ligand (5µl/10ml) at the time of EGF addition (n=4 cows, or half of the cows receiving EGF) to observe the effects on follicle growth.

Once follicles were retrieved and put into low attachment plates, media was then changed every third day. Media was collected into small aliquots and labeled based on cow, day, and follicle plate. Images were saved the day after follicles were placed into culture and then once a week thereafter. Most bovine follicles needed ~21 days to grow to the small antral stage. Antral follicles were measured and organized based on speed of antral growth (fast and slow) and days until antral formation. Media at the end of follicle culture were pooled by cow, survival, time to antrum formation, and days +/-1 in culture. Follicles were separated by treatment group (Kit Ligand or EGF control).

3.4 STATISTICS

Using a SAS system, version 9.4, statistics for follicle culture was performed. Non-parametric ANOVA function of SAS and Wilcoxon rank-sums are used to statistically analyze the percent of the follicles that survived and the percent that formed an antrum. This is performed on all follicle types, then another analysis is done on follicle type, with the variables being the treatment group or the treatment group by follicle type. Linear Models function of SAS is used to statistically analyze the final diameter of the follicles, and the amount of days it took an antrum to form, with the variable being the treatment group. Repeated Measures function of SAS is used in order to statistically analyze the follicle size by day of culture, with each follicle type having a separate analysis. The variables here are the treatment group, the culture day, and the treatment group by culture day. Least-squared means function of SAS with Turkey-Kramer adjustment for multiple comparisons was used to make a pairwise comparison when a trend was observed ($p<0.09$).

4. RESULTS

4.1 Immunohistochemistry

Bovine and primate paraffinized tissue sections were observed. Tissue sections were sorted based on follicle type (multilayer or small antral). Red fluorescence indicates positive expression, while nuclei are denoted by blue DAPI fluorescence to visualize cellular structure of the tissues. Kit Ligand expression was analyzed by percent observed to have KITLG expression. In primates, 31.6% of multilayer follicles had KITLG expression, while small antral expression was minimal. In the bovine tissue sections, 69.2 % of multilayer follicles had KITLG expression while expression in the small antral follicles was minimal.

The Kit Ligand receptor c-kit was noted as either present or absent in for each follicle type (Figure 1), since all follicles observed displayed the same pattern of expression. Primate multilayer follicles were absent of the KITLG receptor. Kit Ligand receptor in the primate small antral follicles, however, was present. In bovine multilayer follicles, c-kit was absent. Bovine small antral follicles had the presence of c-kit.

4.2 Follicle Culture Media

Bovine follicles that had clear and defined oocytes were mechanically isolated using dissecting scopes and placed into low attachment dishes (Figure 2). Two medias were compared, T-199 or α-MEM. No significant differences were found in the media in % survival (Figure 3) ($p>0.7$). Additionally, rate of growth was not different by media type ($p>0.3$). Days to antrum formation is not shown. Furthermore, no significant differences

were observed in the percentage of surviving follicles of each type per media ($p>0.7$). When separated to fast vs. slow in respect to antrum formation, no significant effects were detected ($p>0.4$). The α-MEM had slightly better performance in culture.

4.3 Experiments with Kit Ligand Addition

Maximum diameter of all follicles at the end of culture for all follicle types combined was compared between EGF and EGF+KITLG (Figure 4) (A). No statistical significance was observed. Antrum formation for the fast forming antrum and the slow forming antrum are shown (Figure 4) (B). A trend was observed where Kit Ligand tended to reduce the number of days it took for a follicle to form an antrum ($p=0.09$). Growth of all of the follicle types by day was observed (Figure 4) (C). No difference was observed for all of the follicle types combined together and their size.

The follicles were classified by three categories by speed of antrum formation: fast antrum (antrum <5 days), slow antrum (antrum in 6-19 days), and no antrum (no antrum in 19+ days). Kit Ligand fast antrum (Figure 5) did have a significant effect ($p=0.0002$) but formed an antrum and then died off. Slow forming antrum (Figure 6) had statistical significance ($p<0.005$) as kit ligand treated follicles had a larger diameter. At the end of culture, the slower forming antral follicles were larger with KITLG than with just EGF alone. No antrum follicles (Figure 7) had no significant growth in culture with no difference between EGF alone and the addition of KITLG.

Survival rate of KITLG follicles and the proportions vs. EGF alone is shown in Table 2. While not statistically significant, addition of KITLG does appear to increase percent antrum formation.

Species	Follicle Type	KITLG Expression	c-kit Expression
Primate	Multilayer	31.6% of follicles	Absent
	Small Antral	minimal	Present
Bovine	Multilayer	69.2% of follicles	Absent
	Small Antral	minimal	Present

Table 1. Expression of kit ligand (KITLG) and its receptor (c-kit) by follicle type.

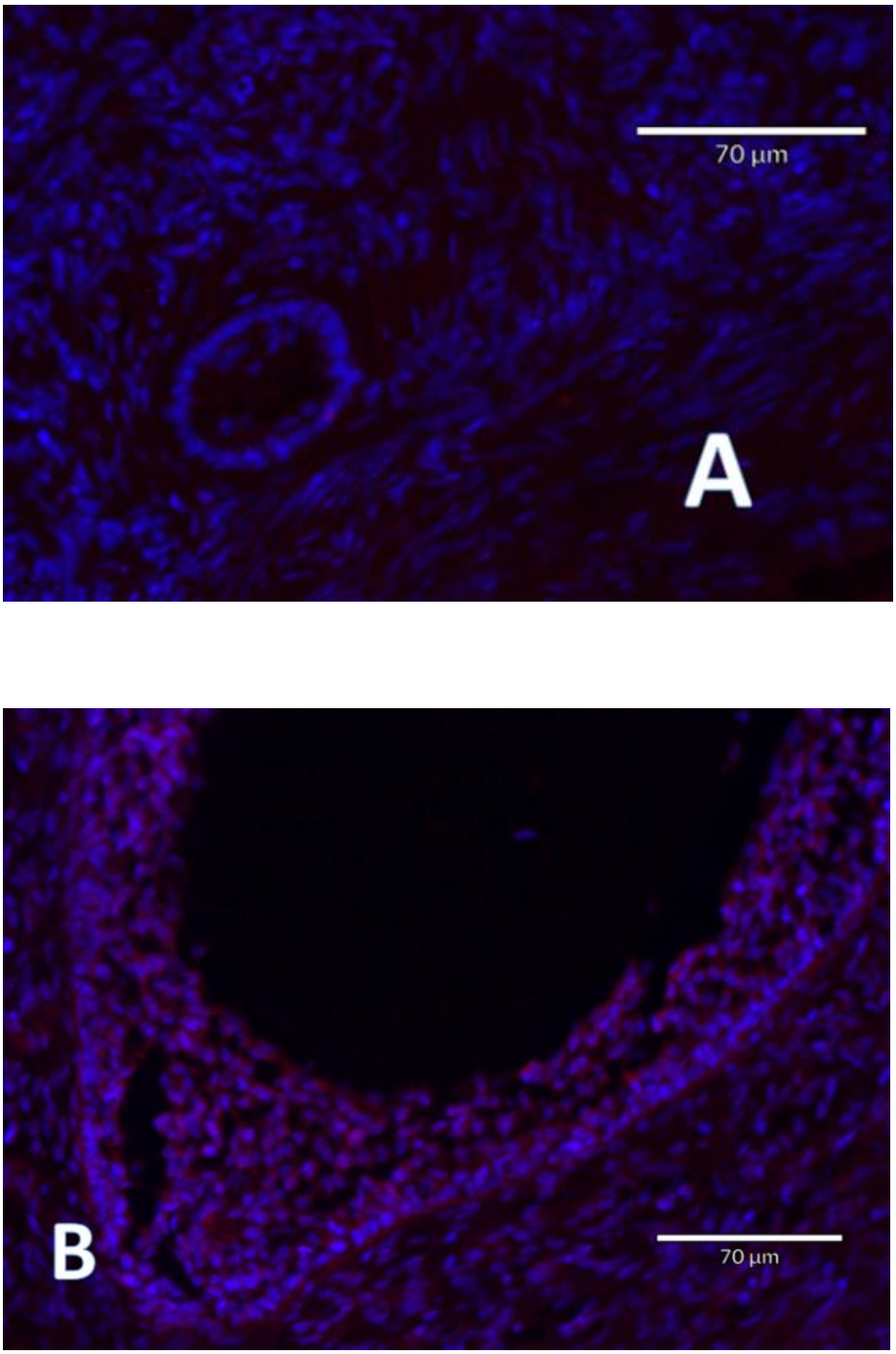

Figure 1. The presence of the KITLG receptor c-kit is shown in multilayer (A) and primary (B) bovine follicles.

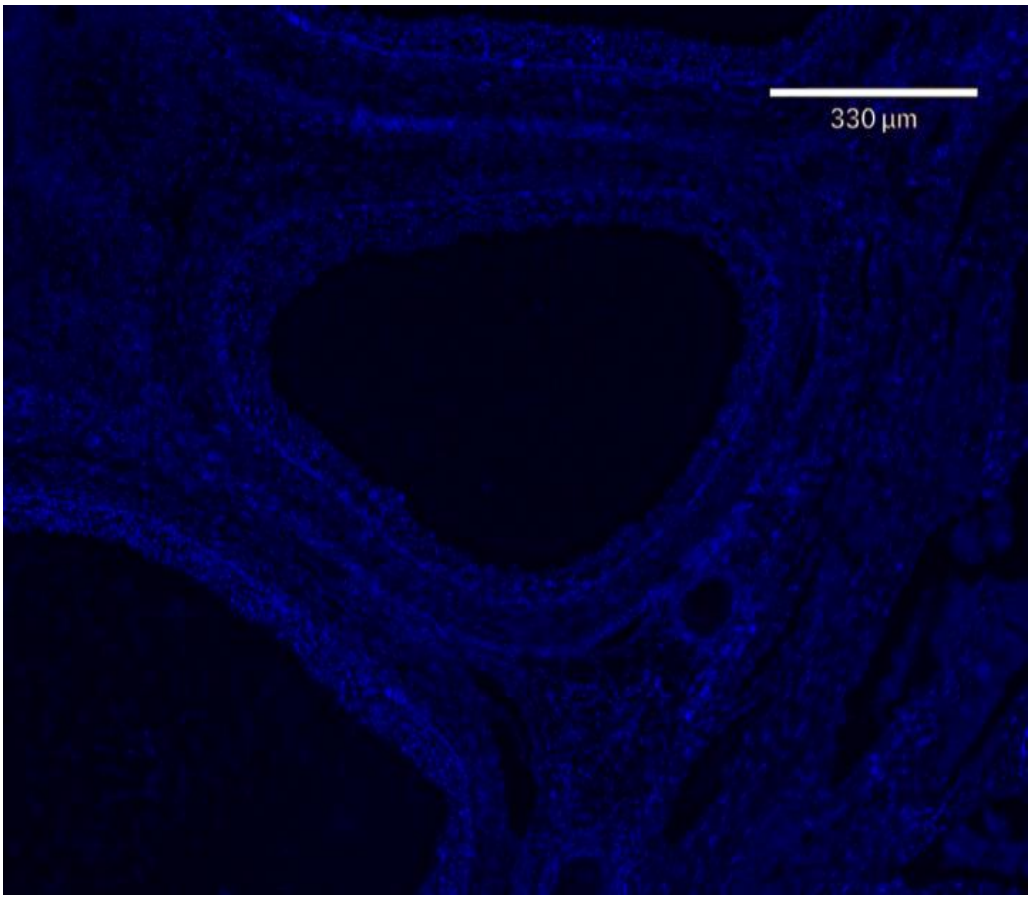

Figure 2. This is a representative image of a multi-layer bovine follicle in which the primary antibodies were omitted (negative control).

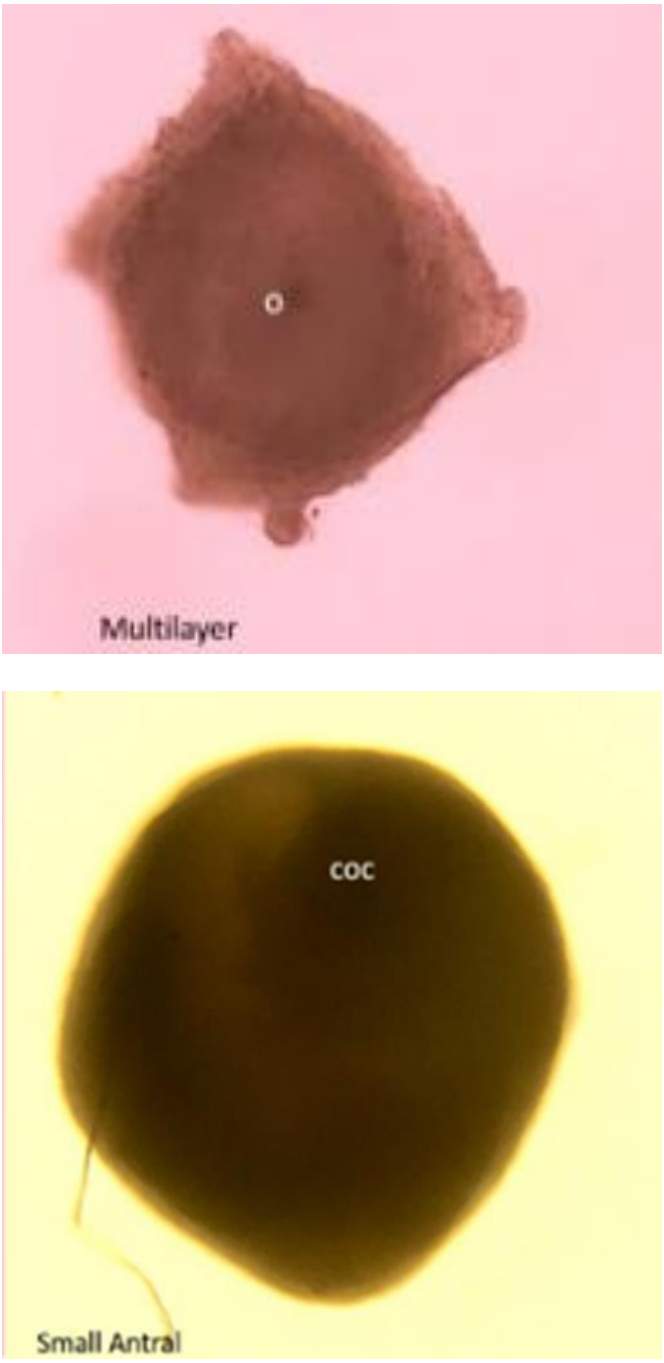

Figure 3. Multilayer follicles with clear and defined oocytes are identified and placed in are placed into low attachment dishes. Based on two different media formulations, we measure time to antral formation.

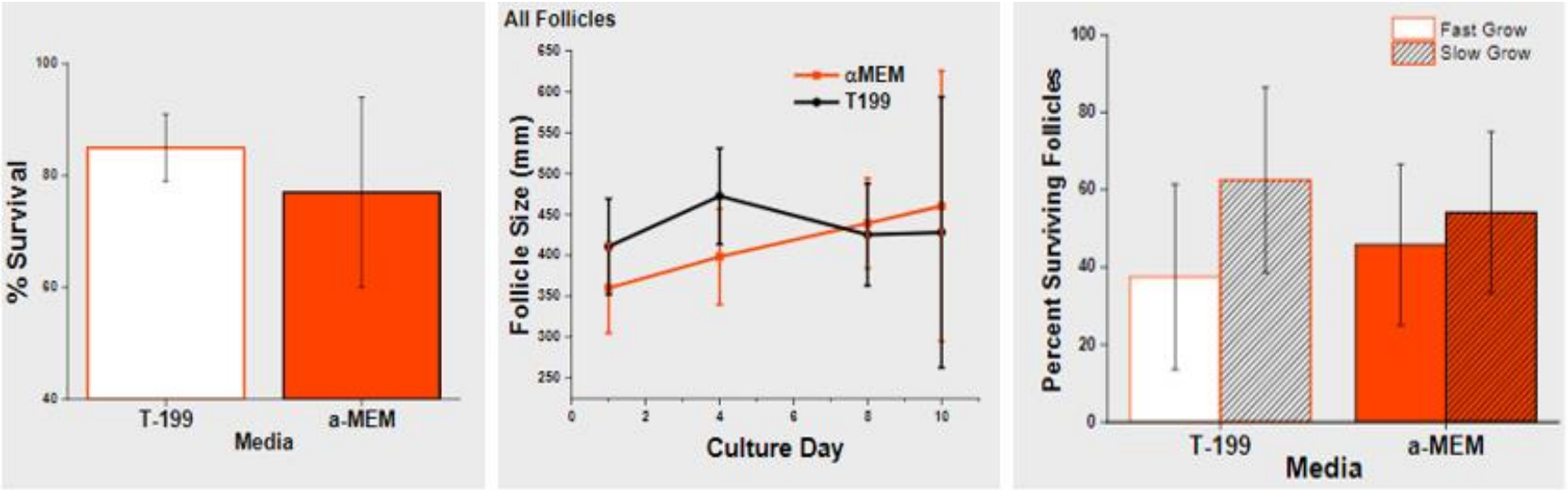

Figure 4. Two media compared, T-199 or α-MEM. No difference detected in % survival ($p>0.7$), rate of growth not different by media type ($p>0.3$). In fast vs. slow media, no significant growth effects were detected ($p>0.4$).

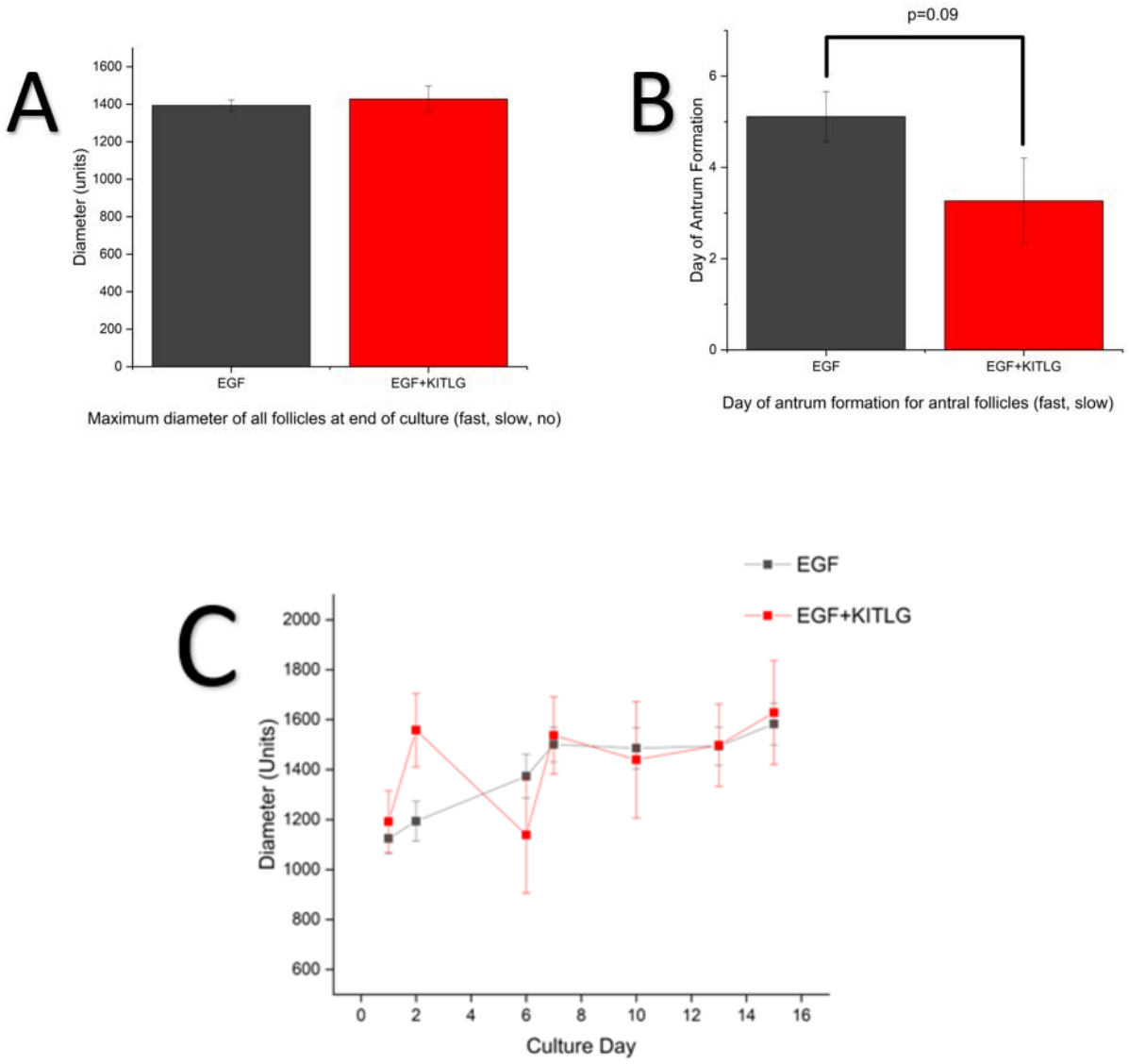

Figure 5. Maximum diameter of all follicles, days to antrum formation for fast and slow follicles, and growth of all follicle types by day was observed

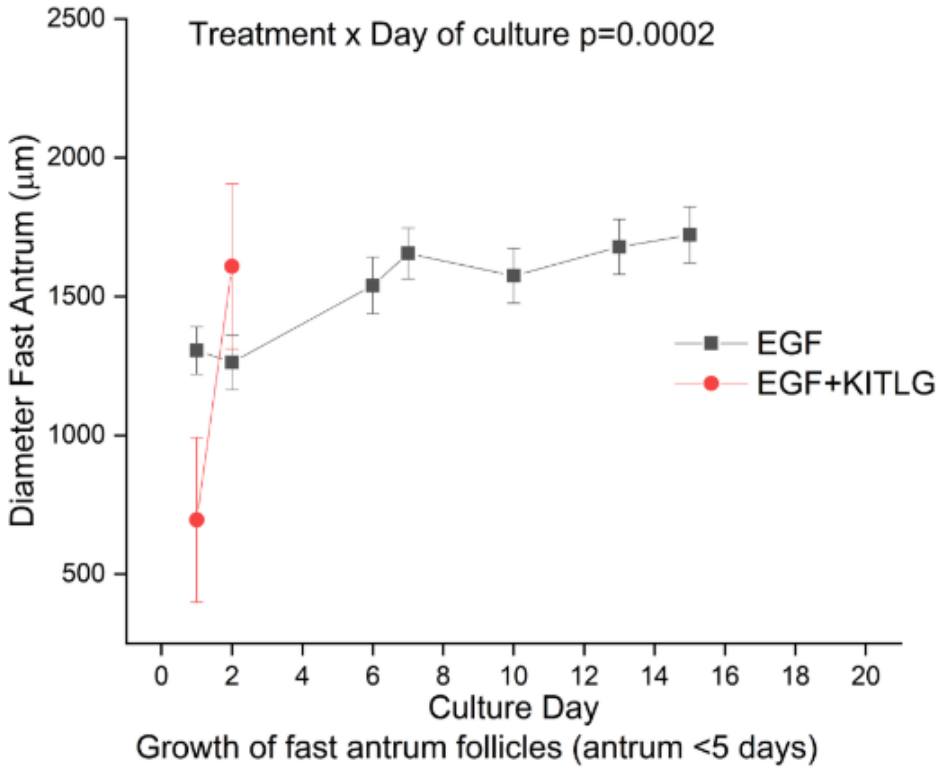

Figure 6. Growth of fast antrum follicles treated with epidermal growth factor (EGF) or EGF with kit ligand (KITLG).

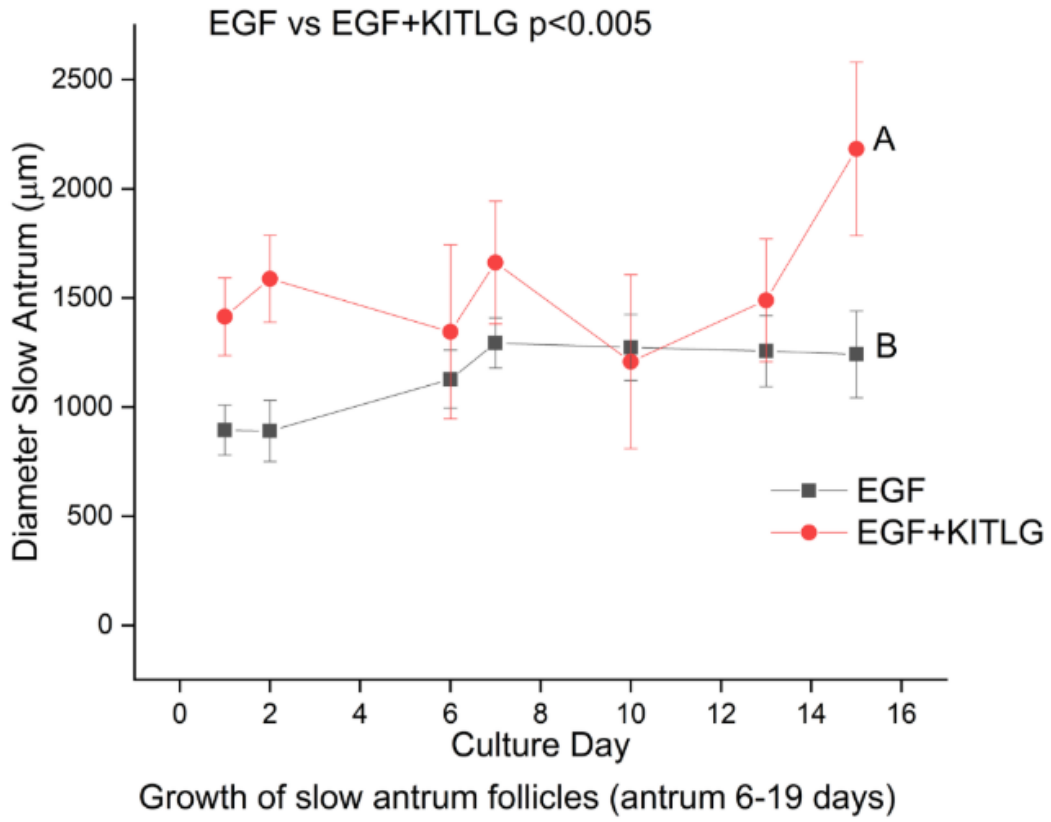

Figure 7. Growth of slow antrum follicles treated with epidermal growth factor (EGF) or EGF with kit ligand (KITLG).

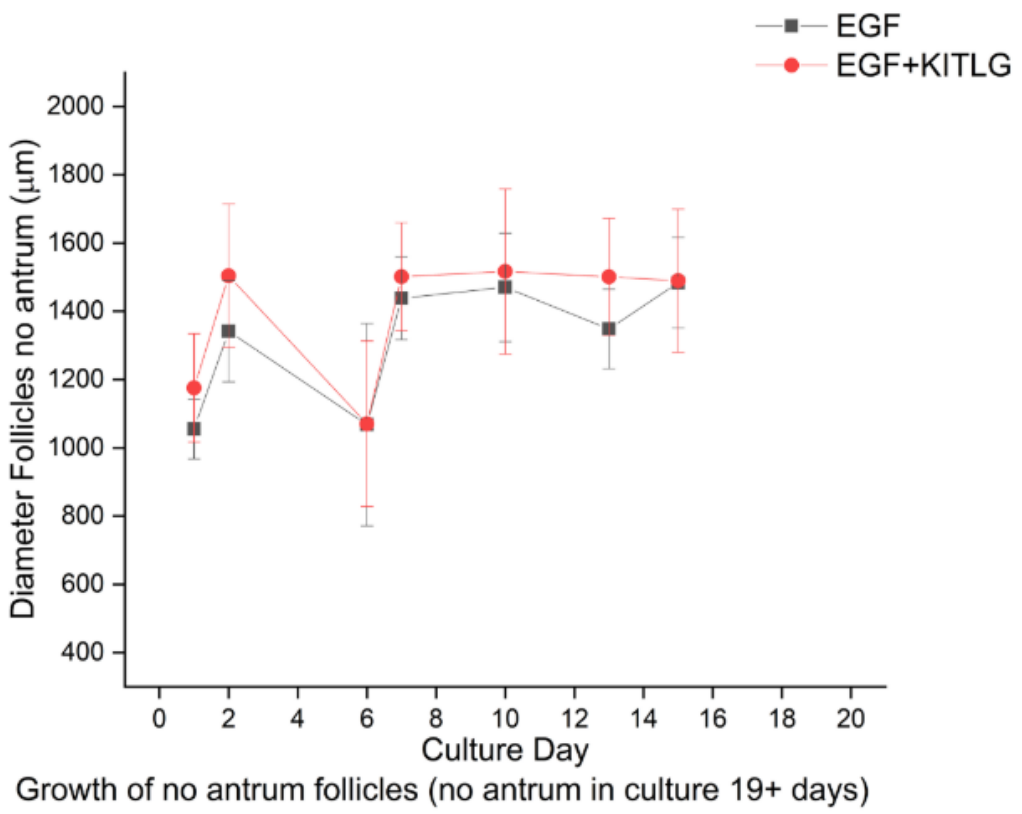

Figure 8. Growth of no antrum follicles treated with epidermal growth factor (EGF) or EGF with kit ligand (KITLG).

Group	Survival	Antrum Formation (Surviving Only)			
	% Yes	% Fast	% Slow	% No	% Total Antrum
EGF	95.7	46.3	19.4	34.3	65.7%
EGF+KITLG	100	45.5	22.7	31.8	77.3%

Table 2. Bovine follicles that survived to antrum formation and/or to the end of the 21-day culture period in the 3D matrix-free culture treated with epidermal growth factor (EGF) or EGF with kit ligand (KITLG).

5. DISCUSSION

In cystic cows, a defect of the theca cell is thought to be one of the major factors that plays a role in their reproductive disorder. However, the cell type is difficult to study in culture. Additionally, there is a lot of cell-to cell contacts including tight junction between the granulosa cells, the theca cells, and the oocyte where hormones and small molecules pass between them to coordinate follicle growth. Interruption of any of these processes could contribute to this disease. Therefore, a 3D follicle culture lets us study all of those cell types together, where other culture methods study them in isolation.

EGF is used as the control in this study, while EGF plus KITLG is used to observe the results of KITLG on the follicle. The role of EGF in follicle culture is being done by another individual in our research group (Wilson Simmons, Master's Thesis, 2020, Oregon State University). Approximately 20 follicles per cow were equally divided between all experimental groups and cultured together in a single plate for these experiments. Therefore, to provide enough follicles for valid comparisons between treatment groups, an individual cow contributed follicles to more than 1 experiment.

In the first experiment, immunohistochemistry was performed in order to determine the presence of the kit ligand protein and its receptor, c-kit. The results suggest that kit ligand is secreted by multilayer follicles, but only small antral follicles possess the receptor for c-kit. Since kit ligand is a secreted protein, the presence of the receptor is what is of interest as this is more specific to the cell and its actions. Kit ligand appears have paracrine actions on the developing follicles due to the variance that the kit ligand protein and its receptor c-kit have in their expression. For ruminants and primates, the kit ligand protein appears to have viable in

vitro utilization in order to target the molecular events that help facilitate the multilayer to antral transition in the follicle.

The second experiment compared two base mediums for follicle culture, α-MEM and TCM-199. The base medium α-MEM is commonly used for keeping the follicle viable and effectively improving in vitro development (Araújo et al. 2015). Additionally, TCM-199 has been suggested to be an effective medium, with high amount of cultured secondary follicles that remain viable throughout the culture process (Araújo et al. 2015). No statistically significant differences were found in all follicle types in survival rate or in the rate of growth. Additionally, no significant differences were observed in the percentage of surviving follicles of each type per media. However, the α-MEM media had slightly better performance in culture. Overall, both base medias had good survival in culture, as previously reported in the literature (Araújo et al. 2014). However, α-MEM is preferable as it does not contain red phenol. TCM-199, however, does contain red phenol. Red phenol is not ideal for culture media as it acts as a weak estrogen and can interfere with some studies (Berthois et al. 1986). However, in order to optimize follicle culture, more studies will need to be done in regards to the addition of other growth factors, and the modulation of oxygen tension.

The third experiment looked at the effects of kit ligand and its role in follicle growth and antrum formation. Statistical significance was observed as EGF+KITLG tended to reduce the number of days it took a follicle to form an antrum. Kit Ligand fast antrum did have a significant effect, however, shortly after antrum formation, the follicle died. Slow forming antral follicles in the kit ligand group statistically performed better and had a larger diameter. At the end of culture, the slower forming antral follicles were larger with KITLG than with just EGF alone. In the slow antrum follicles, the end of the culture period associated with larger

diameter in the KITLG treated group could indicate that KITLG plays an active role in folliculogenesis and maintaining viability of the follicle. It should be noted that a developmentally viable oocyte should reach 3-6 mm, and while the addition of kit ligand significantly increased size, competent oocyte size was not reached (Hendriksen et al. 2000). Therefore, further studies with the addition of other growth factors and the manipulation of oxygen tension in follicle cultures would help to optimize 3D culture systems (Simon et al. 2020).

For cattle, these follicles are larger than the other methods that have been used in cattle [~2000-2500 μm vs 500 μm (Simon et al. 2020)]. In order to obtain enough viable follicles from mature dairy cows in this study we needed to perform ovariectomies, or spay procedures in live cows. These were done by American Veterinary Medical Association approved method for spaying of cows in a feedlot setting. Unfortunately, a very small population of viable secondary follicles were found in initial pilot studies of the ovaries obtained from cattle in a slaughterhouse setting. These were beef cows, and these were corroborated by data from beef cattle ovarian histology (Warren et al. 2015). Kit ligand has been proposed to have many functions in the follicle. It does not appear to be a large survival factor for the growing follicles that we are mostly interested in which are the slower forming follicles. This is because the fast ones were most likely at the early tertiary stage, with an antrum already formed we could not visualize under the dissecting scope, and so would already have fully functional theca cells. The slower forming ones can be used to manipulate different factors (proteins, small molecules) to provide a greater understanding of follicle dynamics in cattle. In those slow forming follicles, kit ligand does appear to promote growth.

In conclusion, follicles appeared to grow well in the novel matrix-free three-dimensional culture system. The addition of KITLG into follicle culture impacted growth, reducing the number of days until antral formation and increasing the diameter of slow antral follicles. However, follicle sizes remained smaller than those previously associated with a fertilizable oocyte (3-6 mm), so further experimentation is needed in order to fully optimize the culture system.

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