2024

EVALUATION OF THE EFFECTS OF O-GLCNACYLATION AND HYPOXIA ON ESTRADIOL PRODUCTION BY BOVINE GRANULOSA CELLS

Lauren DeCastro
University of Rhode Island, laurendecastro03@gmail.com

EVALUATION OF THE EFFECTS OF O-GLCNACYLATION AND HYPOXIA ON ESTRADIOL PRODUCTION BY BOVINE GRANULOSA CELLS

BY

LAUREN DECASTRO

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

BIOLOGICAL AND ENVIRONMENTAL SCIENCES

UNIVERSITY OF RHODE ISLAND

2024

MASTER OF SCIENCE

OF

LAUREN DECASTRO

APPROVED:

Thesis Committee:

Major Professor David Townson

Maria Peterson

Arnob Dutta

Brenton DeBoef
DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND
2024

ABSTRACT

The global population is on the rise putting pressure on the agricultural industry to increase production and increase the global food supply. The dairy industry has responded to this pressure by genetically selecting for higher milk production. This increase in production has coincided with a decline in fertility among dairy cows, due in part to a loss of reproductive cyclicity. Ovarian cyclicity and, in turn, reproductive cyclicity, is dependent upon proper development of ovarian follicles and the function of the cells that comprise the follicle and support the oocyte. One of these cell types is the granulosa cell. Granulosa cells support the growth and maturation of the oocyte of the follicle while producing estradiol to regulate reproductive cyclicity and behavior of the dairy cow (bovine). The functional capacity of bovine granulosa cells has been linked to a post-translational protein modification called O-linked-N-acetylglucosaminylation, or O-GlcANcylation. O-GlcNAcylation influences the proliferation of bovine granulosa cells, and possibly their metabolic activity. However, a connection between O-GlcNAcylation and estradiol production has yet to be explored. Additionally, considering the lack of a vascular supply within the ovarian follicle during its development, it is unclear whether the optimal metabolic environment for granulosa cells is low oxygen tension conditions (hypoxia). In this study granulosa cells from bovine small antral follicles were cultured to evaluate the effects of O-GlcNAcylation and hypoxia on estradiol production. Briefly, the cells were cultured in the absence or presence of small molecule inhibitors (OSMI-1; 50μM and Thiamet-G; 2.5μM) to manipulate O-GlcNAcylation, and in a separate set of experiments, cultured under normoxic (20% O_2). or hypoxic (2% O_2) conditions. The

conditioned medium was collected at the end of the 144hr culture period to evaluate estradiol production. Conversely, the cells were collected and lysed for qPCR analysis of transcripts of the estradiol synthesis pathway (i.e., *FSHR*, *STAR*, and *CYP19A1)* and for immunodetection of hypoxia (i.e., HIF1α). Hormonal stimulation of the granulosa cells stimulated estradiol production ($P<0.05$) as expected. However, manipulation of O-GlcNAcylation under these circumstances had no effect ($P>0.05$). The only detectable effect of O-GlcNAcylation occurred with transcript expression of *STAR*, which decreased ($P<0.05$) as O-GlcNAcylation was inhibited. Hypoxia increased estradiol production ($P<0.05$) by the granulosa cells, but only under basal culture conditions, and there was no effect of hypoxia on any of the evaluated transcripts of estradiol synthesis ($P>0.05$). Surprisingly, HIF1α was undetectable in the cell lysates, so induction of hypoxia remained unverifiable. The results indicate O-GlcNAcylation has minimal effects on hormone-stimulated estradiol synthesis and secretion by bovine granulosa cells, and that hypoxia may be the optimal environment for granulosa cell metabolism.

ACKNOWLEDGMENTS

First, I would like to thank Dr. David Townson for all of his support and guidance throughout my master's. Your continual patience and belief in me made this project happen and I truly could not have done it without your support. I have learned so much throughout this process and I feel prepared to take on the next step of my academic career. I am so deeply appreciative of this opportunity to grow and learn with an advisor who made my growth as a scientist a priority throughout my experience. Thank you.

I would also like to acknowledge my committee members Dr. Maria Peterson and Dr. Arnob Dutta. I am grateful for your input and support throughout this process. It has been so great to learn from you throughout my time at the University of Rhode Island.

I would like to thank my lab-mate and friend Abby Maucieri. Moving institutions at the beginning of my program was nerve-wracking, but it was made less so knowing that you would be here with me to help me get settled in the lab and acclimated to life as a grad student. Thank you for your guidance, your patience, and your encouragement through this process.

To the rest of the Biological and Environmental grad students and faculty that have been a part of this adventure, thank you for making me feel welcomed into this program when I moved here, and thank you for helping this school feel more like a home.

Finally, I would like to thank my family. Your unending faith in me has meant more to me than words could describe, and I hope that I make you all proud.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

As global population inevitably increases, greater demands to keep pace with world food supply have been placed upon the agricultural industry. Global population is estimated to reach 9.15 billion by 2050, an increase of approximately 2.25 billion people since 2010 (Alexandratos & Bruinsma - FAO, 2012). Additionally, global food demand is being driven by a shift in consumer preferences toward animal-derived protein, especially in developing countries. This has resulted in an increased demand for meat and dairy products on an international scale (Alexandratos & Bruinsma - FAO, 2012). Consequently, the dairy industry is addressing this increased demand through efforts to increase milk production and reproductive efficiency in dairy herds. Historically, genetic selection of high producing dairy cows, however, has simultaneously resulted in decreased cow fertility (Pryce et al., 2004). Moreover, dairy herds with low fertility have lower profitability than herds in which reproductive health is prioritized. Improved reproductive performance in a dairy herd increases economic gains through higher milk and calf receipts, lower animal replacement costs, and lower costs associated with reproductive management (Cabrera, 2014). On a per cow basis, these positive economic impacts of improving reproductive performance are still observed. Cows having poor fertility result in losses to farm income through their inability to become pregnant, and even cows with average fertility contribute to these losses because of diminished milk yield. Poor fertility is also associated with longer intervals between calvings (Inchaisri et al., 2010). Notably, higher milk yield and poor fertility in dairy cows is often attributed to metabolic challenges associated

with both high milk production and pregnancy (Wathes et al., 2007). For instance, in high-producing dairy cows during early lactation, feed consumption is unable to meet the nutrient and energy demands to sustain both lactation and resumption of reproductive cyclicity, and the cows enter a state of negative energy balance, or NEB (Coppock = et al., n.d.). These cows tend to remain in NEB until they reach peak lactation, and those that remain in NEB require more time to return to reproductive cyclicity (Butler et al., 1981). The period of negative energy balance in dairy cows is metabolically characterized by mobilization of body fat to provide needed energy, which then leads to increased non-esterified fatty acids (NEFA) in the general circulation. The mobilization of body fat results in a decrease in overall body condition of the cows. Both the decrease in body condition during the post-partum period and the increase in blood NEFA are associated with delays in reproductive cyclicity (Manríquez et al., 2021; Wathes et al., 2007). While nutritional considerations of the postpartum, lactating dairy cow might offer one avenue to manage this fertility problem in dairy herds, the mechanisms of these impacts must be understood to fully address reproductive dysfunction in the high producing dairy cow.

ESTROUS CYCLE

The reproductive cycle of the cow is the estrous cycle. This cycle is subdivided into four distinct stages, generally characterized by hormonal profiles, ovarian activities, and behavioral traits in the cow. These stages are proestrus, estrus, metestrus, and diestrus. The duration of the estrous cycle in the cow averages 21 days (Senger, 2012) .

Estrus is designated as day 0 of the estrous cycle in the cow and is characterized behaviorally as when the cow is receptive to mounting (i.e., so called "standing heat") to the male or other females in the vicinity (Senger, 2012). This stage is also characterized by the ovulation of a dominant ovarian follicle (Swanson et al., 1972). Ovulation coincides with the above behavior in the cow to optimize the likelihood of fertilization. Cows also increase their locomotive activity during estrus (Lewis & Newman, 1984). This increase in locomotive activity is a visual cue to the bull that the cows is in heat. These behaviors are influenced by the concentrations of certain hormones in circulation throughout the estrous cycle. For instance, estradiol increases before estrus and peaks at estrus (Wettemann et al., 1972). The increase and subsequent peak in estradiol coincides with the surge in the gonadotropin, luteinizing hormone or LH, from the anterior pituitary gland and is known as the ovulatory LH surge (Swanson et al., 1972; Wettemann et al., 1972). The ovulatory LH surge is, in turn, triggered by the hypothalamic hormone, gonadotropin releasing hormone or GnRH (Estes et al., 1977). Together, these hormones ensure the timing of ovulation occurs concomitantly with standing estrus and the timing of mating in the cow. Another pituitary gonadotropin, known as follicle stimulating hormone or FSH, is secreted during the estrous cycle to stimulate the growth and development of ovarian follicles. FSH surges have been recorded with the emergence of follicular waves in the cow (Adams et al., 1992). The final major hormone typically characteristic of the estrous cycle is progesterone. Progesterone secretion is low during estrus (Lewis & Newman, 1984; Wettemann et al., 1972). Progesterone is produced by a functional corpus luteum and is low at estrus because no functional corpus luteum exists on the

ovary at that time (Shrestha et al., 2019).This structure develops immediately following ovulation when the cells of the ovulated follicle undergo a process called luteinization (Berisha & Schams, 2005).

Following estrus, the next stages of the estrous cycle in the cow are metestrus and diestrus. During these periods, the numbers of growing follicles increase and then decrease immediately following ovulation (Swanson et al., 1972). Similarly, estradiol concentration decreases once the ovulatory follicle has ovulated, and remains low throughout early metestrus (Wettemann et al., 1972). Progesterone production increases during metestrus and remains high during diestrus (Wettemann et al., 1972). The increase in progesterone is attributed to the development and maintenance of the corpus luteum.

The final stage of the estrous cycle in the cow is known as proestrus. This stage directly precedes estrus and ovulation. During this period, estradiol secretion increases as selection and dominance of the ovulatory follicle occurs (Ginther et al., 2000). Concurrently, progesterone secretion by the corpus luteum decreases as it undergoes regression in response to pulses of prostaglandin F2 alpha from the non-gravid (i.e., non-pregnant) uterus (Wettemann et al., 1972). During this stage, FSH secretion by the pituitary gland is suppressed by the high concentrations of estradiol and inhibin produced by the dominant, ovulatory follicle (Ginther et al., 2000; Good et al., 1995), whereas LH exerts its ovulatory surge in response to the positive feedback effect of high circulating concentrations of estradiol (Swanson et al., 1972).

FOLLICULAR DEVELOPMENT

In the 1960s, understanding of follicular structure and growth dynamics began when a model of two-wave folliculogenesis (i.e., follicular growth) was proposed (RAJAKOSKI, 1960). Initial studies utilized slaughterhouse specimens, in which follicles were observed at specific timepoints of the estrous cycle. As technology improved, ultrasonography became the method of choice for observing growth patterns of follicles in vivo and in real time. This approach confirmed the existence of the two-wave phenomenon, but also pointed to the fact that patterns of follicular growth in cattle and other species can vary from two waves to four waves (Fortune, 1994; Ginther & Kot, 1994). Follicular wave patterns have now been described in a variety of species, including humans, cattle, horses, and goats (Baerwald et al., 2003; Ginther, 2000; Ginther & Kot, 1994; Sirois & Fortune, 1988) and provide a comprehensive view about follicular dynamics during the estrous cycle (Ginther, Kastelic, et al., 1989).

The characterization of follicular waves has led to insight about the dynamics by which one follicle becomes ovulatory in monovulatory species like cows. Each wave begins with a cohort of small antral follicles (<5 mm) (Matton et al., 1981). As these follicles grow in synchrony, one follicle begins to outpace the others in the cohort, growing slightly faster and impairing the growth of the others by secreting inhibin and estradiol as negative feedback mechanisms to the hypothalamus (GnRH secretion) and anterior pituitary gland (FSH secretion) (Gregory & Kaiser, 2004; Ying, 1988). Suppressed gonadotropin secretion in the midst of this process still enables the one follicle to be “selected” and become the dominant follicle, whereas the other

follicles of the cohort become subordinate, eventually destined to undergo atresia (Turzillo & Fortune, 1993). The dominant follicle will either regress and a new follicular wave will emerge, or the dominant follicle will ovulate while the cow exhibits estrus (Swanson et al., 1972). The determining factor of whether or not the dominant follicle ovulates is the absence or presence, respectively, of a functional corpus luteum and the stage of the estrous cycle, as described previously. The presence of a functional corpus luteum prevents ovulation through the negative feedback effect of progesterone. The dominant ovulatory follicle is often larger than the dominant nonovulatory follicle of previous follicular waves and it produces more estradiol (Noseir, 2003). This higher concentration of estradiol stimulates the ovulatory LH surge.

The hormonal changes described above occur in tandem with visual changes in the follicle that can be observed via ultrasonography. Among the first studies to follow individual follicles throughout the estrous cycle, heifers were observed to typically have 2-3 waves of follicular growth with each wave having its own dominant follicle. The dominant follicles of the first waves, however, regress. The dominant follicle of the final wave continues its growth and eventually ovulates (Ginther, Knopf, et al., 1989; Sirois & Fortune, 1988). The three-wave pattern of follicular development is characterized by a new wave emerging every seven days during ~21 day estrous cycle (Sirois & Fortune, 1988). The above studies are important because they showed that antral follicles could individually be tracked over the course of the cycle, and they showed that each follicular wave had its own dominant follicle, rather than the

historical thinking that there was one continuous dominant follicle that grows over the course of the estrous cycle (Marion & Gier, 1971).

While ultrasonography offers insight about growth changes in ovarian antral follicles, slaughterhouse specimens provided the framework concerning the cellular composition of follicles. Follicles are classified based upon size and histological characterizations (Marion et al., 1968). As follicles develop, the cellular layer surrounding the oocyte thickens. This thickening is attributable to proliferation of cells (i.e., called granulosa cells) until the follicle reaches ~10mm. Further growth of the follicle occurs as fluid accumulates within the follicle (i.e., the antrum), and other layers of cells become distinguishable including the theca externa, theca interna, membrana granulosa and the cumulus oophorus (Marion et al., 1968). Morphological development of the follicle has been further described since this study. In preantral primordial follicles, there is a layer of flat, inactive granulosa cells lining the length of the follicle with cuboidal granulosa cells localized to either end, giving the primordial follicles an oblong shape (Van Wezel & Rodgers, 1996). Follicles that have transitioned from primordial to primary are characterized by a layer of granulosa cells separated from the stroma by either a single basal lamina or more than one layer of lamina with loops that extend towards the surface of the granulosa cells (R. J. Rodgers & Irving-Rodgers, 2010). During the preantral stages of development, the stromal cells are recruited to form the theca-cell layer (Fortune, 2003). As the follicle grows, granulosa cells continue to proliferate and the antrum forms. Antrum formation and granulosa cell proliferation are differentially regulated during follicular development (R. J. Rodgers & Irving-Rodgers, 2010), and both are important to follicular

development. The follicular fluid formation commences with the vascularization of the theca cell layer and expansion of the antrum occurs (Raymond J. Rodgers & Irving-Rodgers, 2010). All of these morphological changes within the follicle are tightly regulated to influence follicular development.

Complete development of the differentiated cell layers of the follicle are necessary to ensure proper steroidogenic function. The theca cells and the granulosa cells work together to maintain steroidogenic activity of the follicle. This is the basis for the "two-cell, two-gonadotropin" model of steroidogenesis proposed in 1979 (Midgley et al., 1979). In brief, the model proposes that granulosa cells express FSH receptors, theca cells express LH receptors, LH mediates androgen synthesis by the theca cells, and FSH stimulates aromatase conversion of androgen to estrogen (Midgley et al., 1979). Estrogen production by the follicle is important to estrous cyclicity as described previously, and as healthy follicles develop, aromatase activity of the granulosa cells increases (McNatty et al., 1984).

OVERVIEW OF O-GLCNACYLATION

O-linked-N-acetylglucosaminylation, or O-GlcNAcylation, is a post translational modification of cellular proteins first identified in rat lymphocytes in 1984 (Torres & Hart, 1984). Originally it was thought that any sugar modifications to proteins existed only on the outside of the cell, but this type of modification also occurs on nuclear and cytoplasmic proteins. Further investigation revealed that proteins modified in this manner are located in the nucleus of the cell, but they are also

localized elsewhere (Holts & Hart, 1986). In addition, the modification occurs solely on serine and threonine residues of proteins (Holts & Hart, 1986).

Following the discovery of O-GlcNAcylation, work began to determine how it is regulated. Interestingly, regulation of O-GlcNAcylation is controlled solely by two enzymes, one that adds the sugar and one that removes the sugar from proteins. The first of these was identified in 1989, named O-GlcNAc Transferase or OGT, and is located in the cytoplasm of the cell (Haltiwangers et al., 1989). The second enzyme was named O-GlcNAcase or OGA, and like OGT, is located primarily within the cytoplasm of the cell (Dong & Hart, 1994). These two enzymes work in tandem to regulate the amount of O-GlcNAc modification within the cell.

The biochemical pathway responsible for generating O-GlcNAcylation in cells is the hexosamine biosynthesis pathway or HBP. The HBP utilizes glucose that would otherwise be used to generate energy through glycolysis. It is estimated that between 2 to 5% of all glucose taken up by the cell is utilized by the HBP (Hu et al., 2010). The product generated by the HBP and then added to proteins by OGT is UDP-GlcNAc. The availability of UDP-GlcNAc within the cell directly influences OGT affinity for the targeted protein undergoing modification (Bond & Hanover, 2015). Along with this nutrient sensing mechanism of OGT activity, there is also evidence of other ways in which substrate affinity and specificity of the enzyme is regulated. Structurally the enzyme consists of an N-terminal tetratricopeptide repeat (TPR) domain and a catalytic C-terminal domain (Stephen et al., 2021). Although no consensus sequence has been identified to determine if a protein will be O-GlcNAcylated, the TPR domain of OGT is responsible for interaction between the enzyme and the protein it modifies

(Stephen et al., 2021). A similar understanding about the specificity of the OGA enzyme is known. In an initial study evaluating OGA substrate specificity, a peptide binding groove was identified in the structure of bacterial OGA and was found to be conserved in human OGA (Schimpl et al., 2010). When this domain is altered, catalytic activity is altered, suggesting that OGA interacts with the protein it is altering and not just the O-GlcNAc sugar. Bacterial OGA was used as the starting structure because at the time of the study no eukaryotic OGA crystal structure had been determined (Schimpl et al., 2010). More recently, now that the structure of human OGA has been determined, it is evident the enzyme interacts with target peptides directly at multiple sites (Li et al., 2017). While it is still unclear what dictates the specificity of the enzymes regulating O-GlcNAcylation, as glucose availability is diminished for conversion to UDP-GlcNAc, OGT is less active and global O-GlcNAcylation is decreased. This connection between glucose availability and O-GlcNAc levels translates to O-GlcNAcylation providing a nutrient-sensing and nutrient-dependent mechanism of regulation within the cell (Hu et al., 2010).

Acknowledging that regulation occurs primarily from glucose availability, O-GlcNAcylation of cellular proteins is also reciprocally regulated by another post-translational modification, phosphorylation. Similar to O-GlcNAcylation, phosphorylation modifies proteins at serine and threonine residues, with specificity determined by localization of the kinase, depth of the catalytic site on the kinase, and amino acids surrounding the phosphorylation site on the protein (Johnson et al., 2023; Ubersax & Ferrell, 2007). An example of these modifications working together is that of AMP-activated protein kinase, or AMPK, which is O-GlcNAyclated, and AMPK is

responsible for the phosphorylation of OGT (Bullen et al., 2014). Such reciprocity between O-GlcNAcylation and phosphorylation tightens the regulation of these two post-translational modifications and, in turn, more tightly regulates the function of the proteins they modify.

O-GlcNAcylation and its relationship to phosphorylation likely plays many different roles in a variety of organisms and cell types. One ubiquitous role of O-GlcNAcylation is that of modification of transcription factors. For example, one such transcription factor is Specificity Protein 1, or Sp1 (Jackson & Tjian, 1988). O-GlcNAcylation of Sp1 blocks the transcription of the housekeeping genes within all mammalian cell types (Jackson & Tjian, 1988; O'Connor et al., 2016). This regulatory control of housekeeping genes suggests O-GlcNAcylation is important for cell function and survival.

GRANULOSA CELLS, METABOLISM, AND O-GLCNACYLATION

Granulosa cells surround the oocyte within the ovarian follicle and support the growth and development of the oocyte. Oocytes metabolize pyruvate for growth and maturation, and the granulosa cells are the primary source of this substrate through their glycolytic activity (Biggers et al., 1967). Pyruvate is provided to the oocyte via gap junctions between the cumulus granulosa cells and the oocyte (Heller et al., 1981). Transfer of pyruvate from the granulosa cells to the oocyte, however, is only one half of the bidirectional communication that occurs between these two cell types (Alam & Miyano, 2020). The oocyte augments glycolytic activity by the granulosa cells (Sugiura et al., 2005), and does so via oocyte secreted factors, or OSFs (Gilchrist et

al., 2008). These OSFs also stimulate granulosa cell proliferation and prevent luteinization, ultimately maintaining an optimum environment for oocyte development (Gilchrist et al., 2008). *Thus, the granulosa cells play a vital role in supporting the oocyte metabolically, while the oocyte concurrently plays a functional role in sustaining granulosa cell function and phenotype*.

Current thinking about the metabolic needs of the ovarian follicle is somewhat controversial. Historically, ovarian follicles were thought to rely primarily upon glycolysis for energy production (Boland et al., 1994a, 1994b). More recently, others have demonstrated glycolysis alone is insufficient to support follicular growth; energy derived from the TCA cycle and oxidative phosphorylation are required to support continued follicle growth (Wycherley et al., 2005). Yet others have shown that when glycolysis is disrupted, the function and proliferation of the granulosa cells and, in turn, the development of the follicle is adversely impacted (Mazloomi et al., 2023). These uncertainties aside, any disruption of granulosa cell function specifically, and follicular development generally, likely has negative impacts on the development of the oocyte, and fertility on the whole.

As discussed previously, O-GlcNAcylation is a post-translational protein modification that plays a role in the regulation of multiple cellular processes. For instance, altering O-GlcNAcylation in bovine granulosa cells decreases the glycolytic activity of the cells (Wang et al., 2022). Conversely, our laboratory has recently found that alteration of O-GlcNAcylation in bovine granulosa cells has no effect on metabolism (Maucieri & Townson, 2023), but impairs proliferation (Maucieri & Townson, 2021). Obviously, there is some discrepancy that has yet to be resolved

about the potential impact of O-GlcNAcylation on bovine granulosa cells, but all three studies clearly indicate a functional role for this type of modification in this cell type.

Additional studies have identified that not only does O-GlcNAcylation impact granulosa cell function, but it affects cumulus oocyte complexes as well. For example, when bovine cumulus oocyte complexes, or COCs, are cultured with glucosamine, O-GlcNAcylation within the oocyte is increased, and results in lower rates of blastocyst development following fertilization than COCs cultured under control conditions (Sutton-McDowall et al., 2006). When the increase in O-GlcNAcylation is inhibited, blastocyst development progresses at typical rates (Sutton-McDowall et al., 2006). Evaluation of the COCs revealed that the proteins primarily O-GlcNAcylated were in both the cumulus granulosa cells and the oocytes (Zhou et al., 2019). Together, these observations indicate that the regulation of O-GlcNAcylation is important to the physiology of the granulosa cells and the maturation and development of the post-fertilized oocyte. However, given the relative infancy of these findings, there is much yet to be determined about the impact of O-GlcNAcylation in granulosa cells, including effects on hormone responsiveness, steroidogenic activity, and other similar functions which serve to support the development of the follicle and the oocyte contained therein.

HYPOXIA AND GRANULOSA CELLS

Besides the phenomenon of O-GlcNAcylation, another relatively unexplored area of granulosa cell function is oxygen requirements as they relate to metabolism. Prevailing thought is that granulosa cells within the follicle exist in a low oxygen

tension or hypoxic environment. Most follicles within the ovary are not particularly well vascularized, and the observation that granulosa cells of the follicle exist in an avascular compartment (Tamanini & De Ambrogi, 2004), separated from a well-vascularized theca layer by a basement membrane (Tamanini & De Ambrogi, 2004), suggests granulosa cell metabolism operates in a relatively low oxygen environment. However, oxygen concentration is challenging to measure in the follicle without inadvertently introducing extrinsic oxygen. Instead, investigators have resorted to estimating oxygen concentrations inside antral follicles using mathematical modeling (Redding et al., 2008). The models estimate oxygen concentrations within the follicle to be between 1.5%-6.7% (Redding et al., 2008). If accurate, this is a much lower concentration of oxygen than atmospheric and/or physiologic conditions, which are typically 20% oxygen, the same conditions under which granulosa cells are typically incubated in cell culture experiments.

Since this revelation, several laboratories have tested and suggested that granulosa cells of antral follicles are functionally better suited to a hypoxic environment. Our laboratory recently reported that granulosa cells preferentially metabolize glucose via anaerobic glycolysis rather than oxidative phosphorylation (Maucieri & Townson, 2023). This finding is consistent with the idea that granulosa cells might thrive in relatively low oxygen conditions. Indeed, in another study investigating metabolism in granulosa cells, genes associated with glycolysis and the TCA cycle were upregulated only by 5% oxygen conditions compared to 20% oxygen conditions (Shiratsuki et al., 2016). In tandem with the increase in glycolytic activity, the granulosa cells were also more proliferative in 5% oxygen (Shiratsuki et al., 2016).

All of these studies support the concept that granulosa cells are more metabolically suited to a hypoxic environment than a normoxic one, but again like O-GlcNAcylation, there has been little to no investigation of the effects of hypoxia on granulosa cell function in terms of hormone responsiveness or steroidogenic capacity.

Although it is evident that granulosa cells can function under hypoxic conditions, it remains unclear if hypoxic conditions exist *in situ.* Although granulosa cells of follicles exist in an avascular environment histologically, it has been argued that a variety of factors within the follicle operate together to maintain a non-hypoxic environment (Thompson et al., 2015). These include gonadotropin signaling and hemoglobin activity to provide the oocyte with the oxygen it needs to avoid a hypoxic stress response (Thompson et al., 2015).

While the concept of whether ovarian antral follicles exist in a normoxic or hypoxic environment physiologically remains uncertain, there is clear evidence that extreme hypoxia is detrimental to the follicle. In at least one study, for instance, intrafollicular oxygen concentrations in human antral follicles measured between 1.5% and 5.5% revealed that as oxygen concentration decreased, the developmental competence of the oocyte also decreased (Van Blerkom et al., 1997a). Follicles in which the dissolved oxygen content was less than or equal to 1% had a greater proportion of oocytes with chromosomal abnormalities, problems with spindle organization, and issues with cytoplasmic structure (Van Blerkom et al., 1997a). The detrimental effects of extremely low oxygen concentration affected oocyte maturation, yet another study determined that less severe hypoxia is beneficial to oocyte development (Hashimoto et al., 2000). Specifically, oocytes at 5% oxygen developed

into blastocysts at a higher rate than those cultured at 20% oxygen, and those cultured at 5% oxygen also contained less reactive oxygen species (Hashimoto et al., 2000). Additionally, development of the oocytes improved under hypoxic conditions when supplemented with glucose because the TCA cycle and oxidative phosphorylation become inoperable in these conditions (Hashimoto et al., 2000). Overall, it is evident that developmental capacity of the oocyte in conditions that promote glycolysis is increased, and when combined with the results supporting granulosa cell preference for glycolytic metabolism, the concept that follicle growth occurs in a hypoxic environment becomes plausible.

There is compelling evidence that bovine antral follicles might be a good model to investigate hypoxia as part of ovarian function. In bovine antral follicles granulosa cells express the alpha subunit of Hypoxia Inducible Factor, or HIF1α, readily (Berisha et al., 2017). HIF1 is a transcription factor upregulated under hypoxic conditions (Semenza et al., 1997). The alpha subunit activates transcription of genes associated with glycolysis and angiogenesis, specifically (Semenza et al., 1997). These processes are thought to help the tissues adapt to the hypoxic environment. In the work by Berisha and coworkers (2017), mRNA expression of *HIF1α* by the granulosa cells increased as the follicle grew larger and ovulation became imminent (Berisha et al., 2017). Thus, as the follicle and its antral cavity increases in diameter, oxygen concentration decreases, and the granulosa cells respond to the hypoxic environment through HIF1 expression. Both mRNA and protein expression for HIF1α were evaluated to determine the extent and distribution of HIF1α within the follicle (Berisha et al., 2017). Critically, the HIF1α protein was localized to the granulosa

cells, rather than the oocyte or theca cells, indicating that the granulosa cells are exposed to the hypoxia (Berisha et al., 2017). This study provided the first *in vivo* evidence that granulosa cells exist in a hypoxic environment within the follicle.

OBJECTIVES AND HYPOTHESES

The first objective of this thesis was to determine the effects of O-GlcNAcylation on estradiol production by bovine granulosa cells. We hypothesized that impairment of O-GlcNAcylation in the granulosa cells will have a negative effect on estradiol production. The second objective of this thesis was to determine, in a similar manner, the impact of hypoxia on estradiol production by bovine granulosa cells. We hypothesized that under hypoxic conditions, estradiol production by granulosa cells will be augmented.

CHAPTER 2

INTRODUCTION

The first goal of this study was to evaluate the effect of O-GlcNAcylation on estradiol production by bovine granulosa cells. O-GlcNAcylation impacts metabolism and proliferative capacity of bovine granulosa cells (Maucieri & Townson, 2021; Wang et al., 2022). Metabolism and cell proliferation of granulosa cells are vital to the overall growth and development of the follicle as well as the oocyte contained therein. Another important function of granulosa cells is the ability to produce estradiol. Estradiol influences behavioral, reproductive cyclicity of the cow and coordinates the timing of ovulation to optimize fertilization. All of these factors converge to influence fertility. Since O-GlcNAcylation is clearly implicated in some of these important functions of the granulosa cell, we reasoned that O-GlcNAcylation might also influence estradiol synthesis and secretion.

Another goal of this study was to evaluate the effects of hypoxia on estradiol production by bovine granulosa cells. The evidence in support or against the existence of granulosa cells in a hypoxic environment within antral follicles is unclear. There is more compelling evidence that hypoxia aids the developmental capacity of the oocyte. However, the granulosa cells and their supportive role in the development of the oocyte is clear, and it stands to reason that the environment suitable for one cell type is suitable for the other. For this reason, we postulated that hypoxia may be the more suitable environment for granulosa cells and in doing so augments the ability of granulosa cells to synthesize and secrete estradiol.

MATERIALS AND METHODS: O-GLCNAC

Cell Culture and Treatment

Bovine granulosa cells were obtained by aspiration of small antral follicles (5-6 mm) of slaughterhouse ovaries and shipped overnight on ice in 50ml centrifuge tubes containing culture medium from the Pennsylvania State University. Upon arrival, the cells were washed by centrifugation at 4°C at 500rpm for 10min and then resuspended in DMEM/F12 medium prior to seeding and culture in T-25 flasks (Corning Costar). The cells were cultured in DMEM/F12 medium (Gibco, New York, NY) containing 10% fetal bovine serum (Fisherbrand, Waltham, MA) and antibiotic-antimycotic (10,000 units/mL of penicillin, 10,000 ug/mL of streptomycin, 25 ug/mL Amphotericin, Gibco). The cells were incubated at 37°C at 5% CO_2 and 95% air until confluent, approximately 3-5 days. Once confluent, the cells were subcultured to 24-well plates at a density of 50k cells per well. After allowing 48 hours for attachment, the cells were switched to serum-free conditions, DMEM/F12 media without serum, but containing antibiotic-antimycotic and ITS (insulin-10 ng/mL, transferrin-5.5 ng/mL, and sodium-selenite-0.67 pg/mL). All cells were provided androstenedione (10^{-7}M) as a substrate for estradiol production. Wells of control cells received androstenedione only. Remaining wells of cells were exposed to either IGF-1 (100 ng/mL), FSH (2.5 ng/mL), the combination of IGF-1 and FSH, or dibutyril-cyclic-AMP (10^{-3} M), as a positive control for hormone/gonadotropin responsiveness. The cells were cultured under these conditions for a total of 144hrs, with the culture medium and treatments exchanged every 48hrs. In certain experiments, the cells were also exposed to the small molecule inhibitors to inhibit or augment O-GlcNAcylation,

OSMI-1 (50 μM) and Thiamet-G (2.5 μM), respectively, during the last 24hrs of culture. At the end of 144hrs of culture, the culture medium was collected and stored at -80°C until assayed for estradiol. Half of the cells from each experiment were lysed for RNA extraction and subsequent qPCR analysis by adding 350uL of RLT lysis buffer (Qiagen, Hilden, Germany) to each well. The cells were stored in the RLT buffer at -80°C until RNA extraction could be performed. The other half of cells from each experiment were kept on ice and washed with PBS, and then lysed using 75μL of radioimmunoprecipitation assay (RIPA) lysis buffer (20mM Tris HCl, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% TritonX 100) with HALT protease and phosphatase inhibitors (Thermo Scientific, Waltham, MA) added to each well. The plates were kept on ice for 10min to allow for complete cell lysis and then stored at -80°C until further sample preparation. Lysis was completed by thawing the plates on ice and scraping each well with a cell scraper. The lysates were aspirated into clean Eppendorf tubes and placed on ice. The samples were further lysed by aspiration through a 27G needle and syringe, and then vortexed for 15 seconds. Finally, the samples were centrifuged at 12,000 RPM for 10min at 4°C. The supernatant containing the protein fraction was transferred to a new Eppendorf tube and stored at -80°C, whereas the pelleted membrane fraction was discarded This set of experiments was repeated 4 times over the course of 2 months (n= 4 independent experiments).

RNA Extraction and qPCR Analysis

RNA was extracted from the granulosa cells using the RNA Mini Kit from Qiagen (Hilden, Germany). Samples were exposed to DNase according to Qiagen protocols to remove genomic DNA. Following RNA extraction, the concentration and

purity (A260/A280, A260/A230) of each sample was measured using the Thermo Scientific NanoDrop 8000 spectrophotometer. From each RNA sample ~200ng of RNA was reverse transcribed to cDNA using the Azura cDNA synthesis kit. For each set of cDNA reactions run, RNA was pooled, and a non-RT reaction was included to serve as the negative control for the qPCR reaction. This last measure was implemented to ensure that genomic DNA was not amplified by the primers. The final cDNA product was diluted 1:5 using nuclease-free, sterile water.

The use of qPCR analysis was run to evaluate the relative mRNA expression of bovine *STAR, FSHR,* and *CYP19A1* with *RPL4* run as a reference gene. Primers were manufactured by Integrated DNA Technologies (Coralville, IA) and purified through standard desalting. Endpoint PCR was run for each primer pair (Table 1) to synthesize standards for the subsequent qPCR reactions and validate the primer pairs. To validate the primer pairs, each PCR product was run on a gel, purified, and sent in for sequencing. Results were compared to the gene sequences on NCBI BLAST to ensure that they were amplifying the correct gene. For endpoint PCR the Promega GoTaq® Green Master Mix was used. For qPCR, the AzuraQuant™ Green Fast qPCR Mix LoRox was used. The qPCR reactions were run on a Roche Lightcycler 96. For all qPCR, the general protocol was to include a preincubation step, a 3-step amplification, and a melt curve. Preincubation consisted of one cycle at 95°C for 600s. For amplification of *RPL4, STAR,* and *FSHR* the protocol consisted of 40 cycles of 95°C for 10s, 58°C for 10s, and 72°C for 10s. For *CYP19A1,* amplification was 95°C for 10s, followed by 60°C for 10s, and 72°C for 10s. For all genes evaluated, the melting curve consisted of one cycle of 95°C for 10s, 65°C for 60s, and 97°C for 1s. The

difference in amplification conditions was determined based upon the Tm values for each primer pair. The temperatures used were optimized such that each primer pair could efficiently amplify the gene it was designed to target. All efficiencies for each primer pair were between 90% and 110%.

Gene	Fwd Sequence	Rvs Sequence	Product Size
RPL4	5'-TCC TTT GGT GGT GGA AGA TAA A-3'	5'-CTC ATT CGC TGA GAG GCA TAG-3'	120 bp
STAR	5'-CTG CCG AAG ACC ATC ATC AA-3'	5'-GCC TTC AAC ACC TAG CTT CA-3'	112 bp
FSHR	5'-CAT GCT CAT CTT CAC CGA CTT-3'	5'-GAC CAG GAG GAT CTT TGA CTT-3'	112 bp
CYP19A1	5'- CAC CCA TCT TTG CCA GGT AGT C-3'	5'- ACC CAC AGG AGG TAA GCC TAT AAA-3'	78 bp

Table 1: qPCR Primer Pairs

Estradiol Analysis

Estradiol analysis of the conditioned culture medium was performed using an estradiol ELISA assay kit (Cayman Chemical, Ann Arbor, Michigan). The assay was performed according to the manufacturer's protocols. Briefly, a standard curve was generated on each assay plate with each sample of unknown run in duplicate. Assay values for the unknowns were extrapolated based upon the standard curve. The inter-assay coefficient of variation was 23.62%.

Statistical Analysis

All statistical analysis was run using GraphPad Prism 10 statistical software. To evaluate the effects of O-GlcNAcylation status and hormone exposure on estradiol production, the data were log transformed and a QQ plot was generated to evaluate the variance of the data. Next a mixed-effects analysis was performed, followed by a Tukey's multiple comparisons test. Effects of O-GlcNAcylation status and hormone

stimulation on *FSHR, STAR,* and *CYP19A1* mRNA expression were evaluated using non-transformed data, expressed as a ratio of the gene of interest to the reference gene *RPL4*. These ratios were then log transformed and evaluated for heteroscedasticity. A two-way analysis of variance was run followed by posttests based upon the sources of variation identified in the ANOVA (i.e., main effects and/or interaction). Statistical significance was declared at $P<0.05$.

MATERIALS AND METHODS: HYPOXIA

Cell Culture and Treatment

Bovine granulosa cells were obtained and prepared for culture as described above (see MATERIALS AND METHODS: O-GlcNAc). Once confluent, the cells were again subcultured and seeded at 50K cells per well in 24-well plates. Similarly, the cells were cultured for a 144hr period in serum-free conditions with complete medium and treatment exchanges every 48hrs. For the final 24hrs of culture, the cells were also exposed to either OSMI-1 or Thiamet-G to influence O-GlcNAcylation status. Additionally, for each experiment, half of the cultures were exposed to normoxic conditions (20% atmospheric oxygen) for the final 24hrs of culture; while the other half of the cultures were placed in a hypoxia chamber initially flushed with 2% oxygen for ten minutes at 2L/min to induce hypoxia. After an hour of incubation, the hypoxia chamber was again flushed to reduce any residual oxygen that may have been introduced into the culture medium and placed back into the incubator. At the end of each experiment, the conditioned culture medium was collected and stored at -80°C until estradiol analysis. RNA and protein lysates were prepared as described

above (see MATERIALS AND METHODS: O-GlcNAc). This set of experiments was repeated 4 times over the course of 1 month (n= 4 independent experiments).

RNA Extraction and qPCR Analysis

RNA extraction and qPCR analysis were performed as described above (see MATERIALS AND METHODS: O-GlcNAc).

Estradiol Analysis

Estradiol analysis was performed as described above (see MATERIALS AND METHODS: O-GlcNAc). The inter-assay coefficient of variance is 10.24%.

Immunodetection of HIF1α

To verify that the above-described hypoxic culture conditions induced hypoxia, protein lysates from the granulosa cells were obtained and then evaluated for HIF1α expression. Protein concentrations of the samples were analyzed using the Pierce™ BCA Protein Assay Kit according to the standard microplate procedure outlined by the manufacturer. Following the BCA assay, samples were prepared for electrophoresis using 10% precast polyacrylamide gels (Mini-PROTEAN TGX Stain-Free Gel, Bio-Rad, Hercules, CA). The sample lysates were diluted with RIPA buffer to the lowest common concentration across samples determined by the BCA assay. Following dilution, 5X SDS sample buffer (5% SDS, 30% Glycerol, 20μM DTT, 2.5mg BPB) was added to each sample in a 1:5 ratio and the samples were boiled at 100°C for 2 to 3min. The samples were then vortexed prior to loading on the gel. Approximately 7μg of total protein was loaded per well, with certain lanes including a standard protein ladder as well as positive and negative control cell extracts for hypoxia (Cell Signaling, Boston, MA). The gel was run at 200V until the dye front

reached the bottom of the gel. The gel was then imaged for total protein using the Image Lab imaging software (Bio-Rad, Hercules, CA). Electrophoretically separated proteins were transferred from the gel to a PVDF membrane using the TransBlot® Turbo™ Transfer System according to the manufacturer's protocols. Following transfer, the gel was imaged again to make ensure complete transfer of the proteins to the PVDF membrane, and the membrane was incubated overnight in 5% BSA-TBST blocking buffer at 4°C on a platform rocker to block non-specific binding. The membrane was then incubated overnight in primary antibody (HIF-1α (D5F3M) Mouse mAb, Cell Signaling) at a 1:1,000 dilution in 5% BSA-TBST at 4°C on the platform rocker. Following primary incubation, the PVDF membrane was washed in TBST twice rapidly, once for 20min, and then four times for 5mins with agitation throughout prior to transfer to the secondary antibody. The secondary antibody was a goat anti-mouse antibody conjugated with HRP at a 1:5,000 dilution in which the membrane was incubated with agitation at room temperature for 1hr. The membrane was also kept in the dark during the incubation with the secondary antibody. Following this, the membrane was washed in TBST as previously described and then incubated in Clarity ECL substrate according to manufacturer's protocol (Bio-Rad). Detection of HIF-1α on the membrane was imaged using Image Lab imaging software (Bio-Rad).

Statistical Analysis

All statistical analysis was performed using GraphPad Prism 10 statistical software. To evaluate the impact of oxygen availability on estradiol production by the granulosa cells, the data were logarithmically transformed and a QQ plot was

generated to evaluate heteroscedasticity of the data. A two-way analysis of variance test was performed to evaluate effects of O-GlcNAcylation and hormone stimulation on estradiol production. A second two-way ANOVA was performed to evaluate effects of oxygen concentration and hormone stimulation on estradiol production. Statistical evaluation of potential changes in gene expression of *FSHR*, *CYP19A1*, and *STAR* was conducted as described previously (see MATERIALS AND METHODS: O-GLCNAC). Statistical significance was declared at $P<0.05$.

RESULTS: O-GLCNAC

Effects of O-GlcNAcylation on Bovine Granulosa Cells

Effects of manipulating O-GlcNAcylation in bovine granulosa cells have been verified previously in the Townson laboratory (Maucieri & Townson, 2021). Briefly, exposure of the cells to OSMI-1 between 4-8hrs impaired expression of globally O-GlcNAcylated proteins (see Figure 1). Conversely, bovine granulosa cells exposed to Thiamet-G for 24hrs augments global expression of O-GlcNAcylated proteins (see Figure 2).

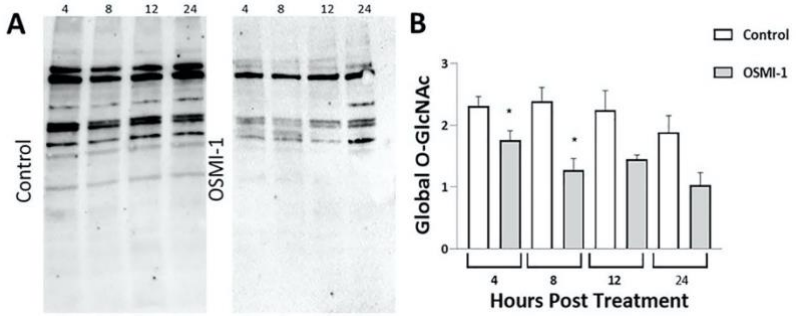

Figure 1: Inhibition of O-GlcNAcylation in Bovine Granulosa Cells by OSMI-1. *OSMI-1 inhibited global O-GlcNAcylation in bovine granulosa cells following 4-8hrs of exposure as depicted by immunodetection (A) and quantified by densitometry (B; $P<0.05$). The effect was not sustained, however, at 12-24hrs post treatment ($P>0.05$).*

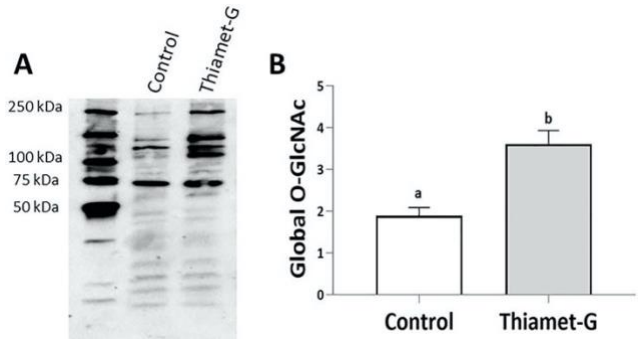

Figure 2: Augmentation of O-GlcNAcylation in Bovine Granulosa Cells by Thiamet-G. *Thiamet-G augmented global O-GlcNAcylation in bovine granulosa cells following 24hrs of exposure as depicted by immunodetection (A) and quantified by densitometry (B; P<0.05).*

Given this information, subsequent experiments were set up similarly in this thesis to manipulate and evaluate the effects of O-GlcNAcylation on estradiol synthesis (qPCR) and secretion (ELISA) by bovine granulosa cells.

qPCR Analysis

Assessment of *FSHR*, *STAR*, and *CYP19A1* transcripts was strategic in that these molecules are critical to the ability of the granulosa cell to respond to gonadotropin stimulation (in this instance, FSH) and synthesize estradiol. The *FSHR* gene encodes the FSH receptor and is directly responsible for the downstream stimulation of estradiol synthesis genes. In the current experiments, and as seen in Figure 3, *FSHR* transcript expression was not affected by O-GlcNAcylation status ($P>0.05$), nor by growth factor (IGF-1) or gonadotropic ligand (FSH) exposure, alone or in combination ($P>0.05$). Direct upregulation of the cAMP signaling pathway (via dbcAMP) had no effect ($P>0.05$), and there was no O-GlcNAc by hormone interaction observed ($P>0.05$).

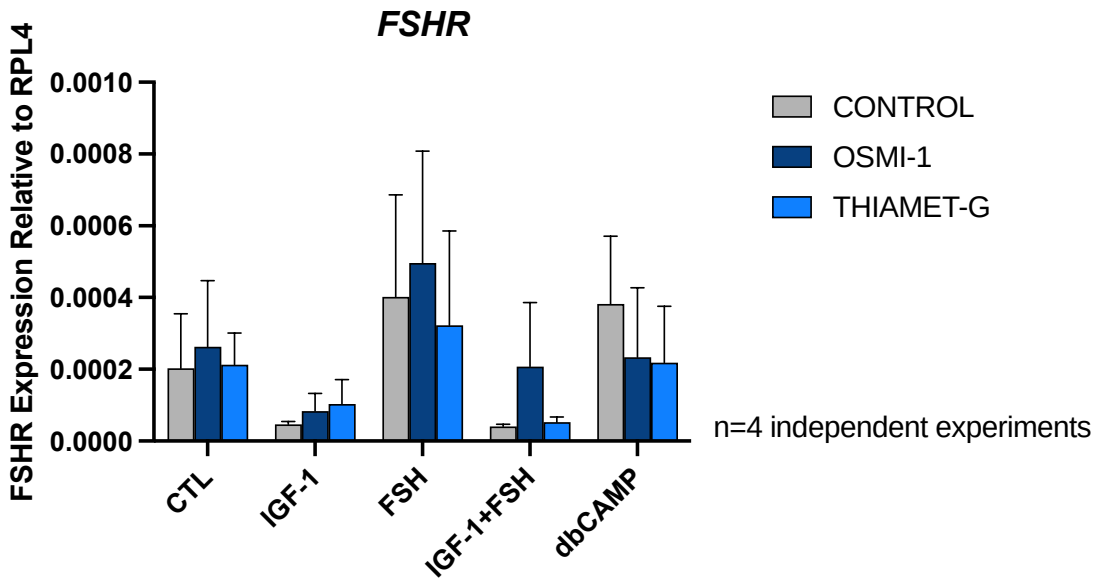

Figure 3: FSHR mRNA Expression in Bovine Granulosa Cells. *No effects of O-GlcNAcylation or hormone stimulation were observed (P>0.05). Similarly, there was no O-GlcNAc by hormone interaction observed (P>0.05).*

The gene, *STAR*, encodes for the steroidogenic acute regulatory protein. This protein transports cholesterol (the precursor to all steroids) from the outer leaflet to the inner leaflet of the mitochondria membrane. It is considered the rate-limiting enzyme in the steroidogenic pathway. As is evident in Figure 4, an increase in O-GlcNAcylation in granulosa cells was associated with a decrease in *STAR* mRNA expression ($P<0.05$). Interestingly, a decrease in O-GlcNAcylation was associated with a further decrease in *STAR* expression in all groups ($P<0.05$). Beyond the effects of O-GlcNAcylation, there were no effects of hormone ($P>0.05$) or O-GlcNAc by hormone interaction ($P>0.05$) on *STAR* expression observed in these experiments.

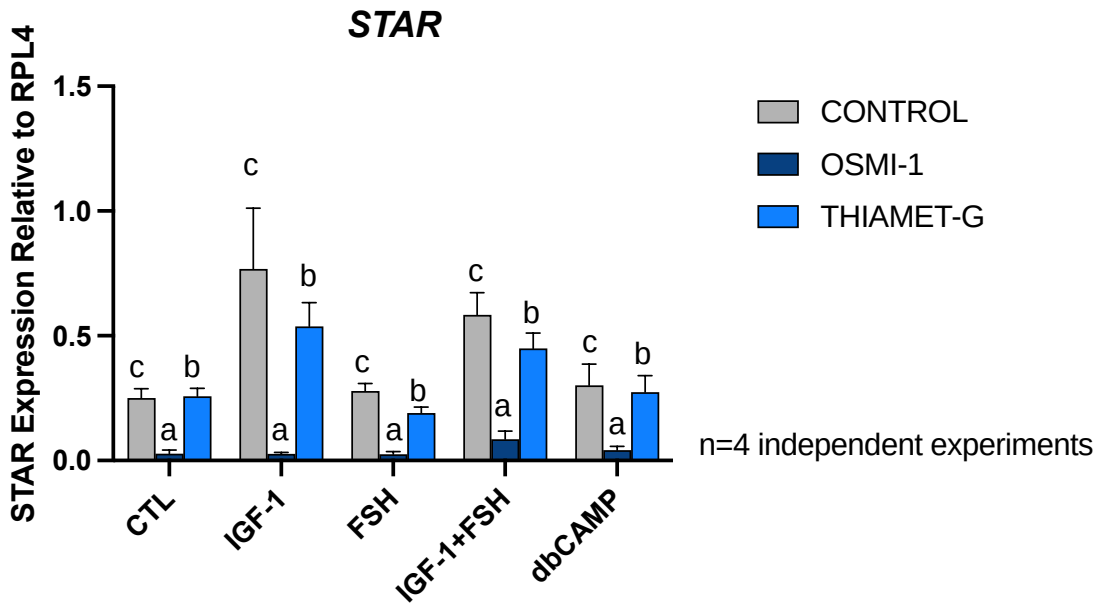

***Figure 4**: **STAR mRNA Expression in Bovine Granulosa Cells.** An effect of O-GlcNAcylation was observed (P<0.05), but no effects of hormone (P>0.05) or O-GlcNAc by hormone interaction observed (P>0.05).*

The final transcript evaluated was *CYP19A1*. This gene, also known as the aromatase enzyme, converts androgens to estrogens. In this instance, the conversion was androstenedione (exogenously provided) to estradiol. As depicted in Figure 5, there was no effect of O-GlcNAc on *CYP19A1* expression (P>0.05), but there was an effect of hormone exposure. Specifically, dbcAMP increased expression of *CYP19A1* (P<0.05), whereas all other hormones, alone or in combination had no effect (P>0.05). Similarly, there was no O-GlcNAc by hormone interaction (P>0.05).

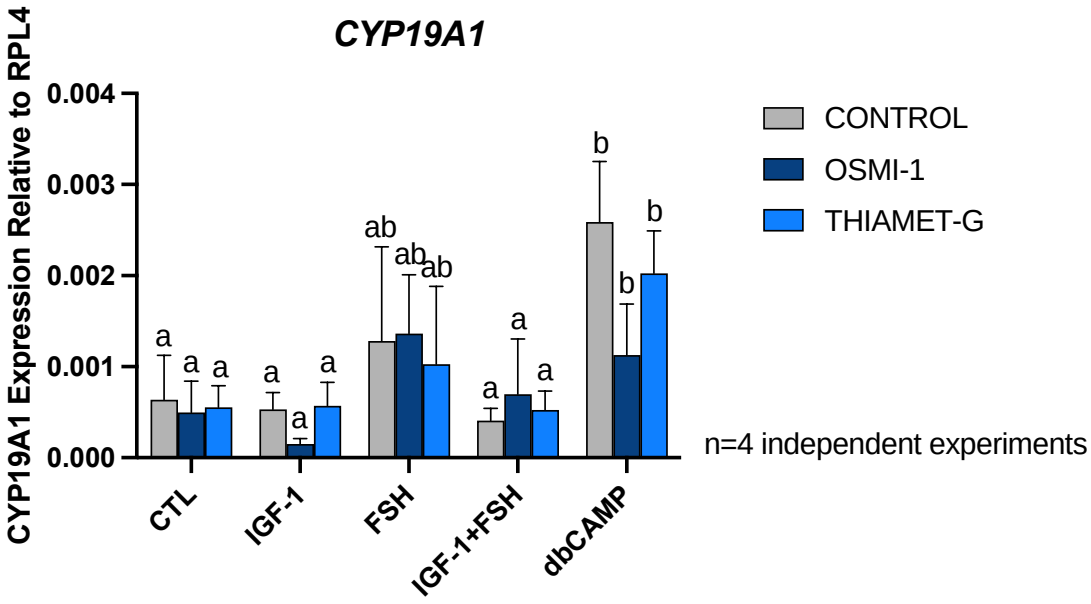

Figure 5: CYP19A1 mRNA Expression in Bovine Granulosa Cells. *No effect of O-GlcNAcylation was observed (P>0.05), but an effect of hormone (i.e., dbCAMP) was observed(P<0.05). There was no O-GlcNAc by hormone interaction observed (P>0.05).*

Estradiol Secretion

Estradiol secretion by bovine granulosa cells was not affected by the manipulation of O-GlcNAcylation. As shown in Figure 6, there was no effect of O-GlcNAcylation status on estradiol secretion by the granulosa cells (P>0.05). Conversely, there were effects of hormone exposure on estradiol secretion (P<0.05). Specifically, IGF-1, IGF-1 plus FSH in combination, and dbcAMP all stimulated estradiol secretion compared to control cultures (P<0.05).

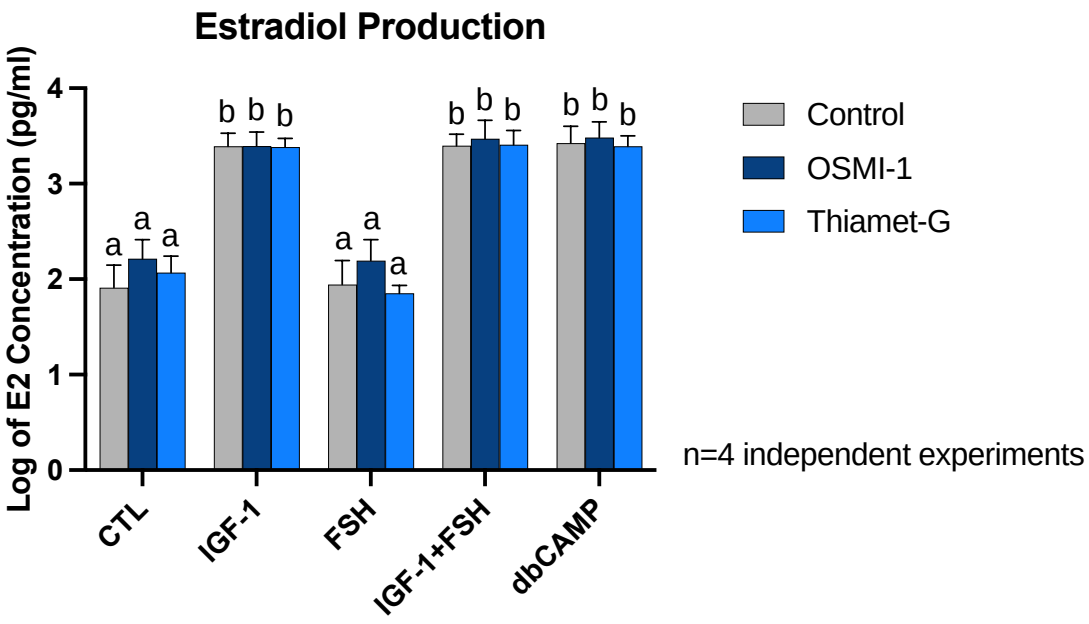

Figure 6: Estradiol Secretion by Bovine Granulosa Cells*-No effect of O-GlcNAcylation was observed (P>0.05), but effects of hormones were evident for IGF-1 alone, IGF-1+FSH, and dbcAMP (P<0.05). There was no O-GlcNAc by hormone interaction observed (P>0.05).*

RESULTS: HYPOXIA

qPCR Analysis

Similar to the above-described experiments, qPCR analysis used to evaluate estradiol synthesis of the granulosa cells by targeting *FSHR*, *STAR*, and *CYP19A1* transcripts following culture in normoxic and hypoxic conditions. For *FSHR*, as depicted Figure 7, hypoxia inhibited *FSHR* expression (P<0.05), that was negated by hormone exposure to either IGF-1+FSH or dbcAMP (P>0.05). Moreover, dbcAMP stimulated *FSHR* expression under hypoxic conditions compared to normoxic conditions (P<0.05).

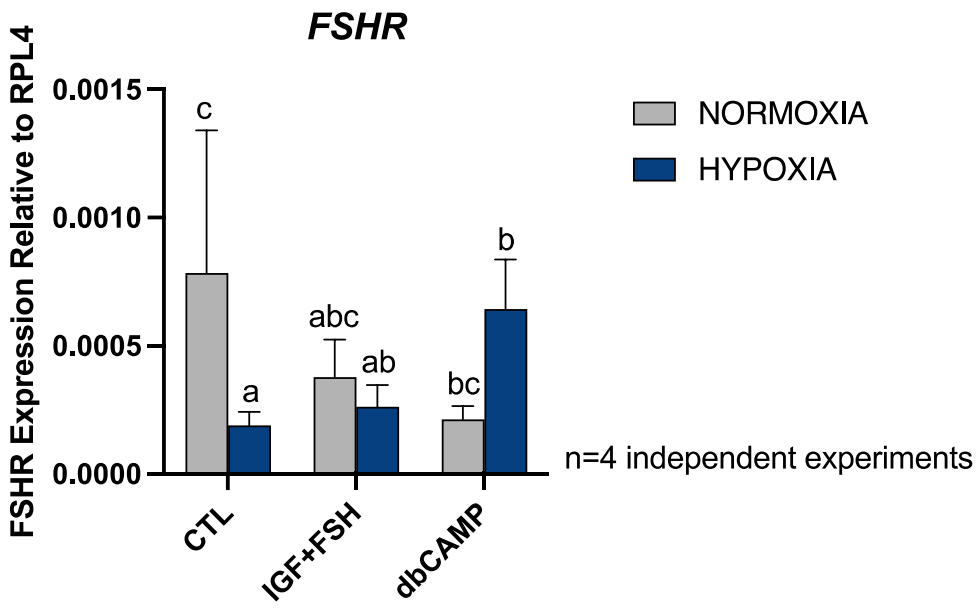

Figure 7: FSHR mRNA Expression in Bovine Granulosa Cells cultured under Normoxic and Hypoxic Conditions*-There were no main effects of oxygen concentration or hormone exposure on FSHR mRNA expression (P>0.05), but there was an oxygen concentration by hormone interaction observed (P<0.05).*

As shown in Figure 8, there was no effect of oxygen concentration on *STAR* expression observed (P>0.05), but the hormone combination of IGF-1+FSH stimulated the expression of *STAR* under both normoxic and hypoxic conditions (P<0.05). Surprisingly, there was no effect of dbcAMP under these culture conditions (P>0.05).

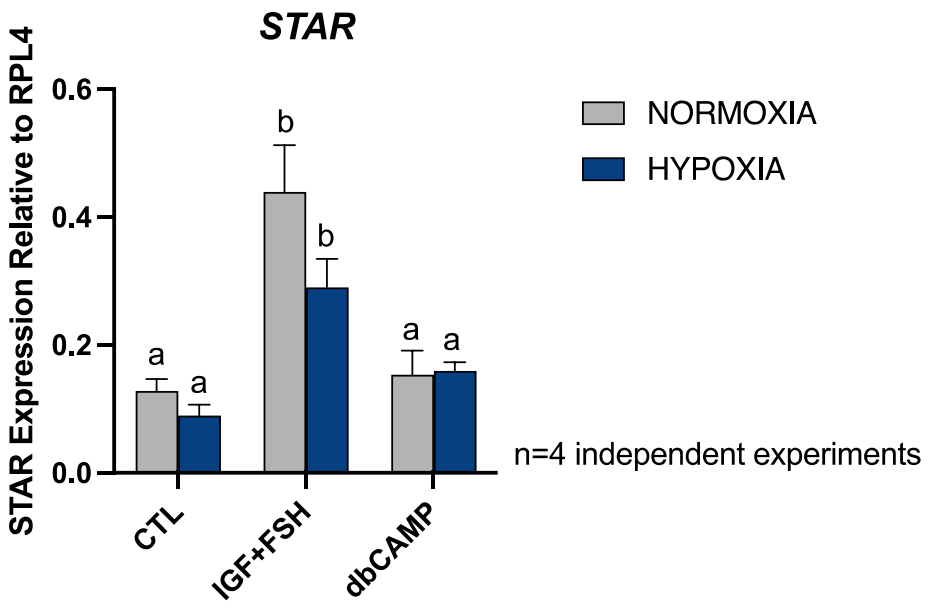

Figure 8: STAR mRNA Expression in Bovine Granulosa cells cultured under Normoxic and Hypoxic Conditions. *No effect of oxygen concentration on STAR expression was observed (P>0.05), but effects of hormone exposure were observed (P<0.05). There was no oxygen concentration by hormone interaction observed (P>0.05).*

Lastly, as depicted in Figure 9, and similar to the results observed for *FSHR*, hypoxia inhibited *CYP19A1* expression (P<0.05), but the effect was negated by hormone exposure to either IGF-1+FSH or dbcAMP (P>0.05). Also similar to *FSHR*, dbcAMP stimulated *CYP19A1* expression under hypoxic conditions compared to normoxic conditions (P<0.05).

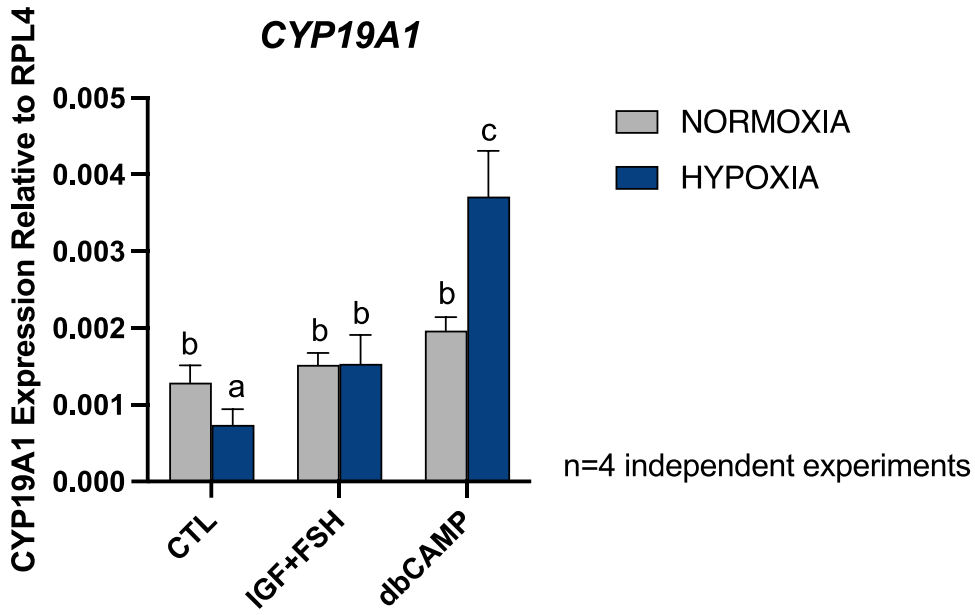

Figure 9: CYP19A1 mRNA Expression in Bovine Granulosa Cells cultured under Normoxic and Hypoxic Conditions-*There were no main effects of oxygen concentration or hormone exposure on CYP19A1 mRNA expression (P>0.05), but there was an oxygen concentration by hormone interaction observed (P<0.05).*

Estradiol Secretion

Estradiol secretion was evaluated based upon O-GlcNAcylation status, hormone exposure, and oxygen concentration during culture conditions. As shown in Figures 10 and 11, there was no effect of O-GlcNAcylation on estradiol secretion by the granulosa cells cultured under either normoxic or hypoxic conditions (P>0.05). However, the hormones IGF-1+FSH and dbcAMP both stimulated estradiol secretion by the granulosa cells cultured under either normoxic or hypoxic conditions (P<0.05).

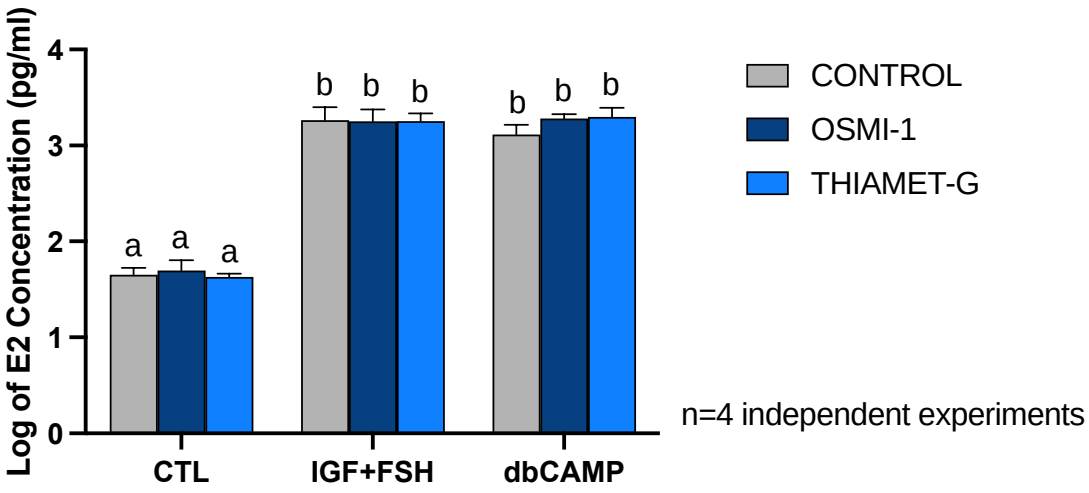

Figure 10: Estradiol Secretion by Bovine Granulosa Cells cultured under Normoxic Conditions. *There was no effect of O-GlcNAcylation (P>0.05), but there was an effect of hormone exposure (P<0.05). There was no O-GlcNAc by hormone interaction observed (P>0.05).*

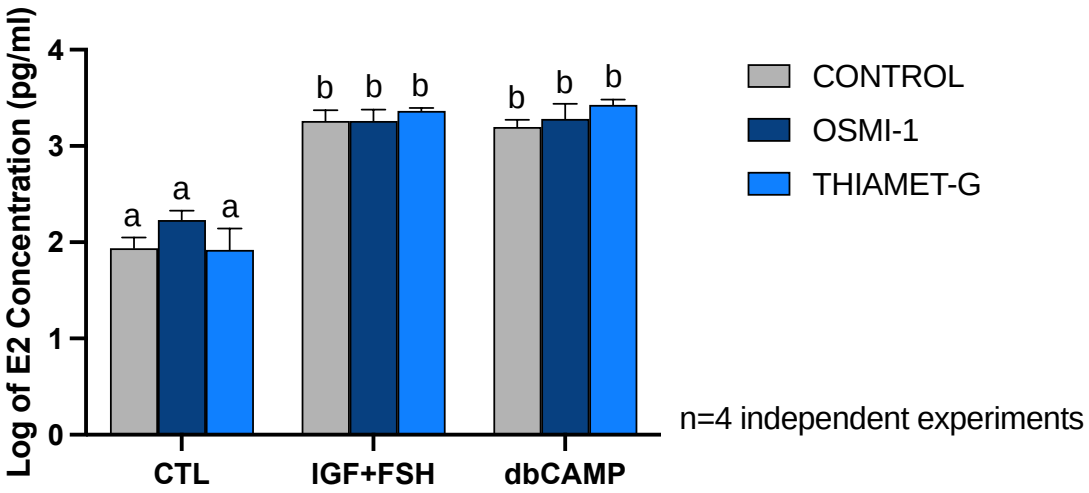

Figure 11: Estradiol Secretion by Bovine Granulosa Cells cultured under Hypoxic Conditions. *There was no effect of O-GlcNAcylation (P>0.05), but there was an effect of hormone exposure (P<0.05). There was no O-GlcNAc by hormone interaction observed (P>0.05).*

As depicted in Figure 12, bovine granulosa cells cultured without manipulating O-GlcNAcylation exhibited greater estradiol secretion under hypoxic conditions than normoxic conditions ($P<0.05$), but this effect was negated by exposure to either IGF-1+FSH or dbcAMP ($P>0.05$).

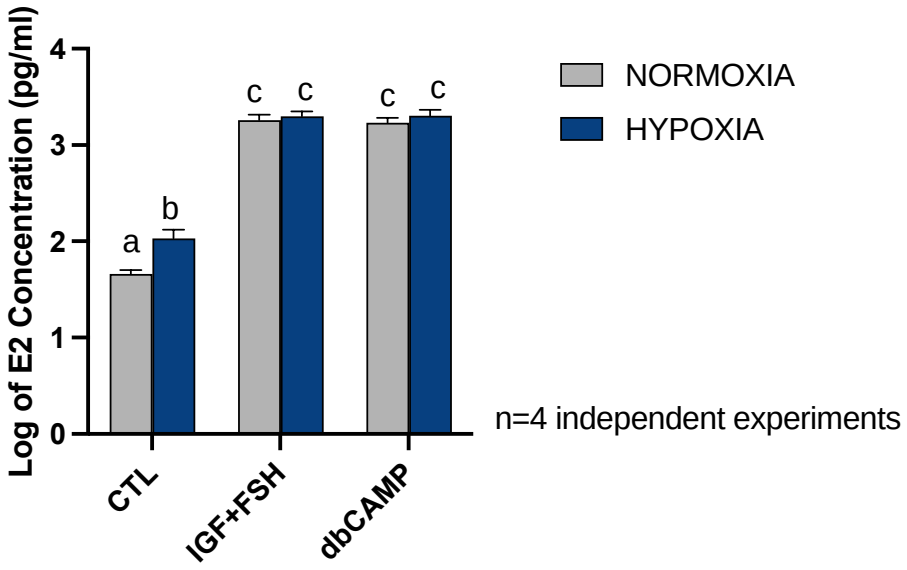

Figure 12: Estradiol Secretion by Bovine Granulosa Cells cultured under Normoxic and Hypoxic Conditions. *Effects of oxygen concentration ($P<0.05$), hormone exposure ($P<0.05$), and oxygen concentration by hormone interaction ($P<0.05$) were observed.*

Immunoblotting for HIF1α

The induction of hypoxic culture conditions was evaluated by immunodetection of HIF1α. As seen in Figure 13, expression of HIF1α was not detectable in bovine granulosa cell samples obtained from normoxic and hypoxic cultures despite evidence of its differential expression in cell lysates of HepG2 cells (an immortal cell line of hepatocellular carcinoma) used as controls.

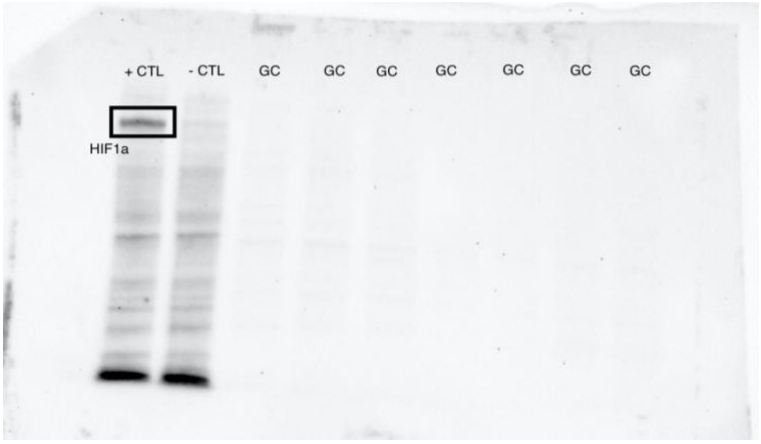

Figure 13: Immunodetection of HIF1α in Positive and Negative Control Lysates

DISCUSSION: O-GLCNAC

O-GlcNAcylation is a dynamic post-translational modification of proteins that has a role in many vital cellular processes. It is described as a nutrient-sensing mechanism (Hu et al., 2010), making it a good indicator of cellular health and nutrient status. It also modulates the transcription of different housekeeping genes through the transcription factor, Sp1 (Jackson & Tjian, 1988). More recently, our laboratory has discovered this modification influences proliferation of bovine granulosa cells; inhibition of O-GlcNAcylation impairs proliferation of the granulosa cells (Maucieri & Townson, 2021). Proliferation of granulosa cells is important to the overall health and function of ovarian follicles and to reproductive cyclicity, implicating O-GlcNAcylation as vital to the reproductive performance of the dairy cow. Another important attribute of granulosa cells is the ability to produce estradiol to regulate behavioral and reproductive cyclicity in the dairy cow. With the knowledge that O-GlcNAcylation affects one of the essential functions of cells (i.e., proliferation), one

goal of this study was to evaluate the impact of O-GlcNAcylation on hormone responsiveness, and estradiol synthesis and secretion by bovine granulosa cells.

There were no overt effects of O-GlcNAcylation on hormone responsiveness or estradiol production by bovine granulosa cells as evidenced by expression of transcripts to gonadotropin receptor (i.e., *FSHR*) or an enzyme of estradiol production (i.e., *CYP19A1*). However, manipulation of O-GlcNAcylation in the cells did influence transcription of *STAR*. Inhibition of O-GlcNAcylation reduced the transcription of *STAR*, and in FSH-treated cells, an increase in O-GlcNAcylation also reduced *STAR* expression. The implications of these effects are unclear, especially because no previous research literature exists about the consequences of O-GlcNAcylation on steroidogenesis in any cell type. However, what is known is that *STAR* is transcriptionally regulated by Sp1 (Reinhart et al., 1999), a transcription factor that is also O-GlcNAcylated (Jackson & Tjian, 1988). Unfortunately, the binding site for Sp1 in the *STAR* gene is not conserved among humans and cattle (Reinhart et al., 1999), suggesting this is not the mechanism of influence of O-GlcNAcylation in the current situation. It is conceivable that another transcription factor is involved in the transcription of bovine *STAR*, and is O-GlcNAcylated, but this would require further investigation to resolve. The decline in transcription of *STAR* is also discordant with the observation that O-GlcNAcylation in the current study did not affect estradiol production by the granulosa cells. This may in part be explained by the fact that transcription of mRNA does not always directly correlate to translation of the protein or its biological activity. That is, even though mRNA transcription of the gene is altered, the abundance of the STAR enzyme and/or its activity might be unchanged.

Another explanation for the disconnect between transcription of *STAR* and the production of estradiol is, experimentally, the granulosa cells were supplied with exogenous androstenedione. Androstenedione is the direct precursor to estradiol, bypassing the need for STAR activity. Physiologically, the STAR enzyme transports cholesterol from the cytoplasm into the mitochondria of the cell (Stocco, 1997). The cholesterol is then converted to pregnenolone, which is then converted to progesterone, androstenedione, and finally estradiol (Payne & Hales, 2004). Thus, by providing the granulosa cells with exogenous androstenedione during cell culture, the biological need for *STAR* and the impact of any alterations in its transcription might have been negated when measuring estradiol production as the experimental endpoint.

The observation that hormone stimulation enhanced estradiol production by bovine granulosa cells but did not influence the transcription of genes supportive of this was surprising. The lack of upregulation of the *FSHR* transcript coincides with the result that FSH did not stimulate estradiol production. The lack of upregulation of receptor transcript suggests the receptor itself is either poorly expressed or not being expressed on the cells, which explains the lack of response to FSH stimulation observed in the current study. These results are consistent, however, with that of another study in which the researchers found that FSH alone does not stimulate *FSHR* expression in granulosa cells (Luo & Wiltbank, 2006). Similarly, previous work in our laboratory determined that FSH treatment alone does not stimulate *FSHR* expression cells or estradiol production by the granulosa cells (Maucieri & Townson, 2021). Collectively, whether these results stem from a deficiency in the culture conditions imposed or some other reason is unknown. Importantly, they bring into question

whether or not FSH alone is suitable to stimulate estradiol production in granulosa cell *in vitro*, as it is recognized to do in ovarian follicles *in vivo* (Ball & Peters, 2007).

Another transcript of interest in this study was *CYP19A1*. Although manipulation of O-GlcNAcylation failed to change the expression of *CYP19A1*, exposure of the granulosa cells to dbcAMP stimulated expression of this transcript. This outcome was anticipated, in part because aromatase activity (i.e., the protein product of *CYP19A1)* is stimulated by cAMP-mediated pathways (Parakh et al., 2006). The molecule dbcAMP is a soluble form of cAMP, so it would be expected to upregulate expression of aromatase, and possibly other factors regulating estradiol synthesis. Having said this, the results for *CYP19A1* in this study do differ from previously published work. For example, previous experiments in our laboratory utilizing similar culture conditions found that hormonal exposure to IGF-1 and FSH together stimulates *CYP19A1* expression (Maucieri & Townson, 2021). The reason for this incongruity is uncertain but may be characteristic of differences of time during which the granulosa cells spent in culture prior to treatments for the two studies. In the previous study, cells were in culture for up to 72hrs prior to treatment, whereas in this instance, the cells spent 3-5 days in culture prior to treatment. For the current study, the transcription of *CYP19A1* was possibly not upregulated to the same extent as the previous study because the cells spent an additional 3-5 days in culture prior to hormone exposure. The granulosa cells of the current study might have reached a state of quiescence and downregulated transcriptional machinery at the time the experiment was terminated, and samples collected. The results of the two studies also differ from other reports in the literature. For instance, in one study evaluating the effects of

hormone stimulation on steroidogenic pathways, FSH stimulated *CYP19A1* transcript expression, but not *FSHR* expression, in bovine granulosa cells (Luo & Wiltbank, 2006). The effect here might be due to differences in cell culture conditions as well. In the latter study, granulosa cells were supplemented with 1% FBS throughout hormonal (i.e., FSH) exposure; whereas the cells cultured in our laboratory are switched to serum-free conditions during hormonal treatments. It has been shown that in cultures containing serum, the hormone responsiveness of the cells is decreased (Orly et al., 1980; Pate & Condon, 1982). Conversely, it has also been demonstrated that cells cultured without serum produce less steroid hormone, most likely due to the lack of cholesterol as a precursor (Pate & Condon, 1982).

All of the above observations point to the idea that a discrepancy may exist between transcript results and the translation and/or activity of the enzymes being transcribed. For example, hormone stimulation (i.e., IGF-1, IGF-1+FSH) and upregulation of cAMP signaling (i.e., via dbcAMP) both increased the production of estradiol by bovine granulosa cells, but only dbcAMP stimulated the expression of *CYP19A1*. Considering the aromatase enzyme is required for the conversion of androstenedione to estradiol, we have evidence the aromatase enzyme (CYP19A1) is biologically active within the cells. Similar results were seen in previous work by our laboratory wherein both IGF-1 and IGF-1+FSH stimulated estradiol production, but IGF-1 failed to stimulate *CYP19A1* expression (Maucieri & Townson, 2021).

The results of the current study offer insight about future directions in which effects of O-GlcNAcylation on follicular function can be focused. Firstly, expression of FSH receptor, STAR, and aromatase proteins within bovine granulosa cells

undergoing O-GlcNAc manipulation should be evaluated. This would provide additional information about what proteins are actively involved in the estradiol synthesis pathway under these culture conditions. Secondly, transcriptomic and proteomic analyses would provide valuable insight about the entire steroidogenic pathway following manipulation of O-GlcNAcylation. This approach would likely elucidate previously unforeseen ways in which the granulosa cells compensate for changes in O-GlcANcylation that might impact estradiol production. Given the observed changes in *STAR* expression following inhibition of O-GlcNAcylation, it would be relevant to measure progesterone production by these cells. The change in *STAR* would be expected to have an impact on total steroidogenesis, more so than estradiol production, by bovine granulosa cells, and this would be evident in progesterone production—the first readily measurable steroid of the steroidogenic pathway. Changes in *STAR* would be relevant to progesterone production by bovine granulosa cells, but also bovine luteal cells. Considering the importance of luteal cells to the bovine corpus luteum (Hanse1 et al., 1991), and the importance of progesterone to maintain pregnancy (Pohler et al., 2012), evaluation of O-GlcNAcylation in bovine luteal cells and how it impacts steroidogenic activity of these cells has merit.

DISCUSSION: HYPOXIA

Among the many contested ideas in the study of ovarian physiology is the question of whether or not the ovarian follicle is a hypoxic environment. Although this idea has been debated for a number of years, there is mounting evidence to suggest granulosa cells are optimally suited to a hypoxic environment. For example, granulosa

cells are more proliferative in 5% O_2 than 20% O_2 (Hashimoto et al., 2000). In our laboratory we discovered bovine granulosa cells preferentially metabolize glucose through aerobic glycolysis rather than the TCA cycle and oxidative phosphorylation (Maucieri & Townson, 2023). These observations support the concept that granulosa cells may be better suited to a hypoxic environment and prompted us to evaluate this idea further in the current study in the context of hormone responsiveness and estradiol production.

We learned that hypoxia (2% O_2) increased estradiol production in bovine granulosa cells when cultured under basal conditions, without any hormonal stimulation. The increase in estradiol production is consistent with studies in which the suggestion of hypoxia in bovine antral follicles is evident (Berisha et al., 2017; Redding et al., 2008), and previous reports suggesting glycolysis is the favored avenue of glucose metabolism in granulosa cells (Maucieri & Townson, 2023). Other studies have also suggested that the granulosa cells are preferential to a hypoxic environment and may exist in such a state *in situ*. In one study investigating the effects of hypoxia on mouse granulosa cells, it was found that hypoxia (1% O_2) increased estradiol synthesis by the granulosa cells in comparison to the cells cultured under normoxia (21% O_2) (Wu et al., 2022). It was also demonstrated in this study that when treated with FSH, the cells under hypoxia increase their estradiol production, and when HIF1α expression is blocked, this increase in estradiol production is blocked (Wu et al., 2022). These results suggest that granulosa cells are more steroidogenically active under hypoxia and that this steroidogenesis may be mediated by the expression of HIF1α (Wu et al., 2022). In another study involving porcine ovaries, the expression of

STC1 (a glycoprotein implicated in angiogenesis and steroidogenesis) was evaluated. In this study it was found that STC1 expression within the follicle increased as the follicle grew larger (G. Basini et al., 2010). It was also demonstrated in this study that granulosa cells cultured under hypoxic conditions (1% and 5% O_2) increased their production of STC1 (G. Basini et al., 2010), suggesting that the cells in the follicle that had been increasing their expression of STC1 were under hypoxia. In another study done in porcine ovaries, follicular oxygen content was measured and it was found to be decreasing as follicular size increased (Giuseppina Basini et al., 2004), but when granulosa cells were cultured under hypoxia (1% and 5% O_2), no effect on steroidogenesis or proliferation was detected (Giuseppina Basini et al., 2004). While this still suggests that the follicle decreases in oxygen content as it grows, this does not suggest that the granulosa cells are better suited to a low oxygen environment. It has also been suggested that even if hypoxia plays a role in follicular function, it may be more so following ovulation and not during follicular development. In bovine follicles, endothelin-2, a protein involved in vascular formation is increased in granulosa cells treated with $CoCl_2$ (a hypoxia mimic) or cultured at 1% O_2, and when measured in the follicle, this protein is at its highest concentration following ovulation (Klipper et al., 2010). This suggests that hypoxia may not play a role in granulosa cell function until ovulation has occurred and luteal formation begins. This role of hypoxia in luteal function was further explored in another study where luteinized and non-luteinized granulosa cells were treated with $CoCl_2$ (Fadhillah et al., 2017). When luteinized granulosa cells were treated with $CoCl_2$ they increased their production of progesterone, but this increase was not observed in non-luteinized granulosa cells

(Fadhillah et al., 2017). This suggests that hypoxia may be more beneficial to the steroidogenic activity of luteal cells rather than the granulosa cells of the antral follicle. Overall, the role that hypoxia may play in granulosa cell and follicular function remains to be fully elucidated.

The results of the transcriptional data obtained in the current study indicated there was no effect of oxygen concentration on any of the transcripts evaluated (i.e., *FSHR*, *CYP19A1*, or *STAR*). While this indicates hypoxia has little to no impact on the transcription of these genes, it could also mean the severity of the hypoxia is too excessive to have a stimulatory effect. In a previous study conducted using mouse granulosa cells, the authors determined 1% and 5% O_2 decreased or did not affect, respectively, *STAR* transcription in dbcAMP stimulated cells, but 10% O_2 increased *STAR* expression (Kowalewski et al., 2015). While 10% O_2 is much greater than what has been estimated or modeled as the oxygen content within follicles (Redding et al., 2008), severe hypoxia negatively affects oocyte development (Van Blerkom et al., 1997b), and likely negatively impacts cumulus-oocyte interactions. As a first attempt, the results of the current study support hypoxia as potentially beneficial to estradiol production by bovine granulosa cells, but further investigation of this idea is required to fully understand the range of optimal oxygen concentrations and the downstream effects on steroidogenic enzymes and their transcripts.

Effects on transcript abundance were observed in the current study when considering interactions between oxygen concentration and hormone exposure. Specifically, for *FSHR*, transcript abundance decreased when bovine granulosa cells were exposed to hypoxia and did not receive any hormonal stimulation. Conversely, in

granulosa cells exposed to hypoxia and treated with dbcAMP, expression of *FSHR* expression increased. A similar pattern was observed with the expression of *CYP19A1*; transcript expression decreased when cells were exposed to hypoxia and basal conditions, whereas transcript abundance increased following hypoxia and dbcAMP exposure. The increase in *CYP19A1* expression may be due to the fact that *CYP19A1* expression is mediated by HIF1α (Baddela et al., 2020). Here, HIF1α knockout cells expressed lower amounts of the *CYP19A1* transcript than control, non-HIF1α knockouts (Baddela et al., 2020). The cells were also stimulated with high concentrations of FSH and IGF-1 (20ng/ml and 50ng/ml respectively), which conceivably stimulated the cAMP pathway in a manner similar to the dbcAMP utilized in current study. Thus, there may be interconnectedness among these pathways (i.e., hypoxia, cAMP signaling, steroidogenesis) to influence transcript expression in steroidogenic cells, including granulosa cells. This idea merits further exploration. Additionally, it may be worthwhile to evaluate granulosa cell proliferation under both hypoxic and normoxic conditions because proliferation is generally a good indication of overall cell health. It would certainly be relevant to current understanding about glucose metabolism in granulosa cells because presumably the cells are metabolizing glucose to provide energy for both mitotic activity and steroidogenesis.

As with all studies, we acknowledge there are limitations to the above described experiments. Beginning with the cell culture period, the granulosa cells were cultured in T-25 flasks for a period of 3-5 days until confluent. Previous work in the Townson laboratory had indicated 3 days was sufficient for the cells to reach

confluency (Maucieri & Townson, 2021), but this was not the case with the current work. The cells remained in culture for up to 5 days to attain confluency, and since this method had not been previously validated, it calls into question whether some aspects of cell responsiveness to hormones might be lost (i.e., receptor expression downregulated, senescent cells). Additionally, the cells were provided 48hrs for attachment following subculture compared to the typical 24hrs. This additional time in culture, in the absence of hormonal support, might have led to an overall downregulation of cell function, including metabolic and steroidogenic activities. Another methodological concern realized after the conclusion of the experiments is that the agent used to dissolve the small molecule inhibitors (OSMI-1 and Thiamet-G), which was DMSO, was not added to the control cultures. Given the concentration of this agent is extremely low in culture (<0.01%), the presence or absence of it in the culture most likely had little to no impact. Nevertheless, from a strictly interpretation standpoint, it implies that any changes associated with OSMI-1 and Thiamet-G could also be due to the DMSO alone rather than the actual treatment (i.e., changes in *STAR* expression). Additionally, although OSMI-1 and Thiamet-G have been used on numerous occasions in our laboratory as well as others as agents to alter O-GlcNAcylation at the concentrations specified, these effects have yet to be validated for the experimental conditions stipulated in the current study. For the hypoxia experiments, the biggest limitation was the inability to verify the induction of hypoxia by immunodetection of HIF1α. Whether the problem stems from a methodological approach (i.e., detectability of bovine HIF1α) or an actual lack of induction of hypoxia has yet to be resolved.

In conclusion, the collective observations of this study are that O-GlcNAcylation status does not impact steroidogenesis or hormone responsiveness in bovine granulosa cells. This means that we must reject the hypothesis that impairment of O-GlcNAcylation decreases estradiol production. It was also determined in this study that while hypoxia increased basal production of estradiol, there was no change in hormone responsiveness under hypoxic conditions. This is in line with the hypothesis that hypoxia increases estradiol production in granulosa cells. Overall, this study gives new insight into the roles that O-GlcNAcylation and hypoxia play in steroidogenesis by bovine granulosa cells.

BIBLIOGRAPHY

Adams, G. P., Matteri, R. L., Kastelic, J. P., Ko, J. C. H., & Ginther, O. J. (1992). Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. *Reproduction*, *94*(1), 177–188. https://doi.org/10.1530/JRF.0.0940177

Alam, M. H., & Miyano, T. (2020). Interaction between growing oocytes and granulosa cells in vitro. *Reproductive Medicine and Biology*, *19*(1), 13–23. https://doi.org/10.1002/RMB2.12292

Alexandratos, N., & Bruinsma - FAO, J. (2012). *World agriculture towards 2030/2050: the 2012 revision*. https://doi.org/10.22004/AG.ECON.288998

Baddela, V. S., Sharma, A., Michaelis, M., & Vanselow, J. (2020). HIF1 driven transcriptional activity regulates steroidogenesis and proliferation of bovine granulosa cells. *Scientific Reports 2020 10:1*, *10*(1), 1–12. https://doi.org/10.1038/s41598-020-60935-1

Baerwald, A. R., Adams, G. P., & Pierson, R. A. (2003). Characterization of Ovarian Follicular Wave Dynamics in Women. *Biology of Reproduction*, *69*(3), 1023–1031. https://doi.org/10.1095/BIOLREPROD.103.017772

Ball, P. J. H., & Peters, A. R. (2007). Reproduction in Cattle: Third Edition. *Reproduction in Cattle: Third Edition*, 1–242. https://doi.org/10.1002/9780470751091

Basini, G., Baioni, L., Bussolati, S., Grolli, S., Kramer, L. H., Wagner, G. F., & Grasselli, F. (2010). Expression and localization of stanniocalcin 1 in swine ovary. *General and Comparative Endocrinology*, *166*(2), 404–408.

https://doi.org/10.1016/J.YGCEN.2009.12.013

Basini, Giuseppina, Bianco, F., Grasselli, F., Tirelli, M., Bussolati, S., & Tamanini, C. (2004). The effects of reduced oxygen tension on swine granulosa cell. *Regulatory Peptides*, *120*(1–3), 69–75. https://doi.org/10.1016/J.REGPEP.2004.02.013

Berisha, B., & Schams, D. (2005). Ovarian function in ruminants. *Domestic Animal Endocrinology*, *29*(2), 305–317. https://doi.org/10.1016/J.DOMANIEND.2005.02.035

Berisha, B., Schams, D., Rodler, D., Sinowatz, F., & Pfaffl, M. W. (2017). Expression pattern of HIF1alpha and vasohibins during follicle maturation and corpus luteum function in the bovine ovary. *Reproduction in Domestic Animals*, *52*(1), 130–139. https://doi.org/10.1111/RDA.12867

Biggers, J. D., Whittingham, D. G., & Donahue, R. P. (1967). The pattern of energy metabolism in the mouse oöcyte and zygote. *Proceedings of the National Academy of Sciences of the United States of America*, *58*(2), 560–567. https://doi.org/10.1073/PNAS.58.2.560

Boland, N. I., Humpherson, P. G., Leese, H. J., & Gosden, R. G. (1994a). Characterization of follicular energy metabolism. *Human Reproduction*, *9*(4), 604–609. https://doi.org/10.1093/OXFORDJOURNALS.HUMREP.A138557

Boland, N. I., Humpherson, P. G., Leese, H. J., & Gosden, R. G. (1994b). The effect of glucose metabolism on murine follicle development and steroidogenesis in vitro. *Human Reproduction*, *9*(4), 617–623. https://doi.org/10.1093/OXFORDJOURNALS.HUMREP.A138559

Bond, M. R., & Hanover, J. A. (2015). A little sugar goes a long way: The cell biology of O-GlcNAc. *Journal of Cell Biology*, *208*(7), 869–880. https://doi.org/10.1083/JCB.201501101

Bullen, J. W., Balsbaugh, J. L., Chanda, D., Shabanowitz, J., Hunt, D. F., Neumann, D., & Hart, G. W. (2014). Cross-talk between Two Essential Nutrient-sensitive Enzymes. *Journal of Biological Chemistry*, *289*(15), 10592–10606. https://doi.org/10.1074/jbc.m113.523068

Butler, W. R., Everett, R. W., & Coppock, C. E. (1981). The relationships between energy balance, milk production and ovulation in postpartum Holstein cows. *Journal of Animal Science*, *53*(3), 742–748. https://doi.org/10.2527/JAS1981.533742X

Cabrera, V. E. (2014). Economics of fertility in high-yielding dairy cows on confined TMR systems. *Animal*, *8*(s1), 211–221. https://doi.org/10.1017/S1751731114000512

Coppock =, C. E., Noller, C. H., & Wolfe, S. A. (n.d.). *Effect of Forage-Concentrate Ratio in Complete Feeds Fed Ad Libitum On Energy Intake in Relation to Requirements by Dairy Cows1*. https://doi.org/10.3168/jds.S0022-0302(74)85069-1

Dong, D. L. Y., & Hart, G. W. (1994). Purification and characterization of an O-GlcNAc selective N-acetyl-β-D-glucosaminidase from rat spleen cytosol. *Journal of Biological Chemistry*, *269*(30), 19321–19330. https://doi.org/10.1016/s0021-9258(17)32170-1

Estes, K. S., Padmanabhan, V., & Convey, E. M. (1977). Localization of

Gonadotropin Releasing Hormone (GnRH) Within the Bovine Hypothalamus. *Biology of Reproduction*, *17*(5), 706–711. https://doi.org/10.1095/BIOLREPROD17.5.706

Fadhillah, Yoshioka, S., Nishimura, R., Yamamoto, Y., Kimura, K., & Okuda, K. (2017). Hypoxia-inducible factor 1 mediates hypoxia-enhanced synthesis of progesterone during luteinization of granulosa cells. *Journal of Reproduction and Development*, *63*(1), 75–85. https://doi.org/10.1262/jrd.2016-068

Fortune, J. E. (1994). Ovarian Follicular Growth and Development in Mammals. *Biology of Reproduction*, *50*(2), 225–232. https://doi.org/10.1095/BIOLREPROD50.2.225

Fortune, J. E. (2003). The early stages of follicular development: activation of primordial follicles and growth of preantral follicles. *Animal Reproduction Science*, *78*(3–4), 135–163. https://doi.org/10.1016/S0378-4320(03)00088-5

Gilchrist, R. B., Lane, M., & Thompson, J. G. (2008). Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Human Reproduction Update*, *14*(2), 159–177. https://doi.org/10.1093/HUMUPD/DMM040

Ginther, O. J. (2000). Selection of the dominant follicle in cattle and horses. *Animal Reproduction Science*, *60–61*, 61–79. https://doi.org/10.1016/S0378-4320(00)00083-X

Ginther, O. J., Bergfelt, D. R., Kulick, L. J., & Kot, K. (2000). Selection of the Dominant Follicle in Cattle: Role of Estradiol. *Biology of Reproduction*, *63*(2), 383–389. https://doi.org/10.1095/BIOLREPROD63.2.383

Ginther, O. J., Kastelic, J. P., & Knopf, L. (1989). Composition and characteristics of

follicular waves during the bovine estrous cycle. *Animal Reproduction Science*, *20*(3), 187–200. https://doi.org/10.1016/0378-4320(89)90084-5

Ginther, O. J., Knopf, L., & Kastelic, J. P. (1989). Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves. *Reproduction*, *87*(1), 223–230. https://doi.org/10.1530/JRF.0.0870223

Ginther, O. J., & Kot, K. (1994). Follicular dynamics during the ovulatory season in goats. *Theriogenology*, *42*(6), 987–1001. https://doi.org/10.1016/0093-691X(94)90121-X

Good, T. E. M., Weber, P. S. D., Ireland, J. L. H., Pulaski, J., Padmanabhan, V., Schneyer, A. L., Lambert-Messerlian, G., Ghosh, B. R., Miller, W. L., Groome, N., & Ireland, J. J. (1995). Isolation of Nine Different Biologically and Immunologically Active Molecular Variants of Bovine Follicular Inhibin. *Biology of Reproduction*, *53*(6), 1478–1488. https://doi.org/10.1095/BIOLREPROD53.6.1478

Gregory, S. J., & Kaiser, U. B. (2004). Regulation of gonadotropins by inhibin and activin. *Seminars in Reproductive Medicine*, *22*(3), 253–267. https://doi.org/10.1055/S-2004-831901/ID/28/BIB

Haltiwangers, R. S., Holtq, G. D., & Hartll, G. W. (1989). Enzymatic Addition of 0-GlcNAc to Nuclear and Cytoplasmic Proteins IDENTIFICATION OF A URIDINE DIPHOSPHO-N-ACETYLGLUCOSAMINE:PEPTIDE p-N-ACETYLGLUCOSAMINYLTRANSFERASE*. *THE JOURNAL. OF B~OUXXCAI. CHEMWKW*, *265*(5), 2563–2568.

Hanse1, W., Mho-, H. W., Dowd, J. P., & Milvaet, R. A. (1991). Differential origin

and control mechanisms in small andd large bovine luteal cells. *J. Reprod. Fert*, *43*, 77–89.

Hashimoto, S., Minami, N., Takakura, R., Yamada, M., Imai, H., & Kashima, N. (2000). Low Oxygen Tension During In Vitro Maturation is Beneficial for Supporting the Subsequent Development of Bovine Cumulus±Oocyte Complexes. *MOLECULAR REPRODUCTION AND DEVELOPMENT*, *57*, 353–360. https://doi.org/10.1002/1098-2795

Heller, D. T., Cahill, D. M., & Schultz, R. M. (1981). Biochemical studies of mammalian oogenesis: Metabolic cooperativity between granulosa cells and growing mouse oocytes. *Developmental Biology*, *84*(2), 455–464. https://doi.org/10.1016/0012-1606(81)90415-2

Holts, G. D., & Hart, G. W. (1986). THE JOURNAL OF BIOLOGICAL CHEMISTRY The Subcellular Distribution of Terminal N-Acetylglucosamine Moieties LOCALIZATION OF A NOVEL PROTEIN-SACCHARIDE LINKAGE, 0-LINKED GlcNAc*. *Journal of Biological Chemistry*, *261*(17), 8049–8057. https://doi.org/10.1016/S0021-9258(19)57510-X

Hu, P., Shimoji, S., & Hart, G. W. (2010). Site-specific interplay between O-GlcNAcylation and phosphorylation in cellular regulation. *FEBS Letters*, *584*(12), 2526–2538. https://doi.org/10.1016/J.FEBSLET.2010.04.044

Inchaisri, C., Jorritsma, R., Vos, P. L. A. M., van der Weijden, G. C., & Hogeveen, H. (2010). Economic consequences of reproductive performance in dairy cattle. *Theriogenology*, *74*(5), 835–846. https://doi.org/10.1016/j.theriogenology.2010.04.008

Jackson, S. P., & Tjian, R. (1988). O-Glycosylation of Eukaryotic Transcription Factors: Implications for Mechanisms of Transcriptional Regulation. *Cell*, *55*, 125–133.

Johnson, J. L., Yaron, T. M., Huntsman, E. M., Kerelsky, A., Song, J., Regev, A., Lin, T. Y., Liberatore, K., Cizin, D. M., Cohen, B. M., Vasan, N., Ma, Y., Krismer, K., Robles, J. T., van de Kooij, B., van Vlimmeren, A. E., Andrée-Busch, N., Käufer, N. F., Dorovkov, M. V., … Cantley, L. C. (2023). An atlas of substrate specificities for the human serine/threonine kinome. *Nature 2023 613:7945*, *613*(7945), 759–766. https://doi.org/10.1038/s41586-022-05575-3

Klipper, E., Levit, A., Mastich, Y., Berisha, B., Schams, D., & Meidan, R. (2010). Induction of Endothelin-2 Expression by Luteinizing Hormone and Hypoxia: Possible Role in Bovine Corpus Luteum Formation. *Endocrinology*, *151*(4), 1914–1922. https://doi.org/10.1210/EN.2009-0767

Kowalewski, M. P., Gram, A., & Boos, A. (2015). The role of hypoxia and HIF1α in the regulation of STAR-mediated steroidogenesis in granulosa cells. *Molecular and Cellular Endocrinology*, *401*, 35–44. https://doi.org/10.1016/J.MCE.2014.11.023

Lewis, G. S., & Newman, S. K. (1984). Changes Throughout Estrous Cycles of Variables That Might Indicate Estrus in Dairy Cows. *Journal of Dairy Science*, *67*(1), 146–152. https://doi.org/10.3168/JDS.S0022-0302(84)81278-3

Li, B., Li, H., Lu, L., & Jiang, J. (2017). Structures of human O-GlcNAcase and its complexes reveal a new substrate recognition mode. *Nature Structural & Molecular Biology*, *24*(4), 362. https://doi.org/10.1038/NSMB.3390

Luo, W., & Wiltbank, M. C. (2006). Distinct Regulation by Steroids of Messenger RNAs for FSHR and CYP19A1 in Bovine Granulosa Cells. *Biology of Reproduction*, *75*(2), 217–225. https://doi.org/10.1095/BIOLREPROD.105.047407

Manríquez, D., Thatcher, W. W., Santos, J. E. P., Chebel, R. C., Galvão, K. N., Schuenemann, G. M., Bicalho, R. C., Gilbert, R. O., Rodriguez-Zas, S., Seabury, C. M., Rosa, G. J. M., & Pinedo, P. (2021). Effect of body condition change and health status during early lactation on performance and survival of Holstein cows. *Journal of Dairy Science*, *104*(12), 12785–12799. https://doi.org/10.3168/JDS.2020-20091

Marion, G. B., & Gier, H. T. (1971). Ovarian and Uterine Embryogenesis and Morphology of the Non-Pregnant Female Mammal. *Journal of Animal Science*, *32*(suppl_1), 24–47. https://doi.org/10.2527/ANIMALSCI1971.32SUPPLEMENT_124X

Marion, G. B., Gier, H. T., & Choudary, J. B. (1968). *MICROMORPHOLOGY OF THE BOVINE OVARIAN FOLLICULAR SYSTEM*. https://academic.oup.com/jas/article/27/2/451/4702457

Matton, P., Adelakoun, V., Couture, Y., & Dufour, J. J. (1981). Growth and Replacement of the Bovine Ovarian Follicles during the Estrous Cycle. *Journal of Animal Science*, *52*(4), 813–820. https://doi.org/10.2527/JAS1981.524813X

Maucieri, A. M., & Townson, D. H. (2021). Evidence and manipulation of O-GlcNAcylation in granulosa cells of bovine antral follicles. *Biology of Reproduction*, *104*(4), 914–923. https://doi.org/10.1093/BIOLRE/IOAB013

Maucieri, A. M., & Townson, D. H. (2023). Evaluating the impact of the hexosamine biosynthesis pathway and O-GlcNAcylation on glucose metabolism in bovine granulosa cells. *Molecular and Cellular Endocrinology*, *564*, 111863. https://doi.org/10.1016/J.MCE.2023.111863

Mazloomi, S., Farimani, M. S., Tavilani, H., Karimi, J., Amiri, I., Abbasi, E., & Khodadadi, I. (2023). Granulosa cells from immature follicles exhibit restricted glycolysis and reduced energy production: a dominant problem in polycystic ovary syndrome. *Journal of Assisted Reproduction and Genetics*, *40*(2), 343–359. https://doi.org/10.1007/S10815-022-02676-W/TABLES/5

McNatty, K. P., Heath, D. A., Henderson, K. M., Lun, S., Hurst, P. R., Ellis, L. M., Montgomery, G. W., Morrison, L., & Thurley, D. C. (1984). Some aspects of thecal and granulosa cell function during follicular development in the bovine ovary. *Reproduction*, *72*(1), 39–53. https://doi.org/10.1530/JRF.0.0720039

Midgley, A. R., Sadler, W. A., & Center for Population Research (National Institute of Child Health and Human Development (U.S.)). (1979). *Ovarian follicular development and function*. 414. https://books.google.com/books/about/Ovarian_Follicular_Development_and_Funct.html?id=me1qAAAAMAAJ

Noseir, W. M. B. (2003). Ovarian follicular activity and hormonal profile during estrous cycle in cows: The development of 2 versus 3 waves. *Reproductive Biology and Endocrinology*, *1*(1), 1–6. https://doi.org/10.1186/1477-7827-1-50/FIGURES/3

O'Connor, L., Gilmour, J., & Bonifer, C. (2016). Focus: Epigenetics: The Role of the

Ubiquitously Expressed Transcription Factor Sp1 in Tissue-specific Transcriptional Regulation and in Disease. *The Yale Journal of Biology and Medicine, 89*(4), 513. /pmc/articles/PMC5168829/

Orly, J., Sato, G., & Erickson, G. F. (1980). Serum suppresses the expression of hormonally induced functions in cultured granulosa cells. *Cell*, *20*(3), 817–827. https://doi.org/10.1016/0092-8674(80)90328-1

Parakh, T. N., Hernandez, J. A., Grammer, J. C., Weck, J., Hunzicker-Dunn, M., Zeleznik, A. J., & Nilson, J. H. (2006). Follicle-stimulating hormone/cAMP regulation of aromatase gene expression requires β-catenin. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(33), 12435–12440. https://doi.org/10.1073/PNAS.0603006103/ASSET/43721A89-E9F3-4AEB-949B-31AF33D58F2F/ASSETS/GRAPHIC/ZPQ0320631180006.JPEG

Pate, J. L., & Condon, W. A. (1982). Effects of serum and lipoproteins on steroidogenesis in cultured bovine luteal cells. *Molecular and Cellular Endocrinology*, *28*(3), 551–562. https://doi.org/10.1016/0303-7207(82)90146-0

Payne, A. H., & Hales, D. B. (2004). Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. In *Endocrine Reviews* (Vol. 25, Issue 6). https://doi.org/10.1210/er.2003-0030

Pohler, K. G., Geary, T. W., Atkins, J. A., Perry, G. A., Jinks, E. M., & Smith, M. F. (2012). Follicular determinants of pregnancy establishment and maintenance. *Cell and Tissue Research*, *349*(3), 649–664. https://doi.org/10.1007/S00441-012-1386-8/TABLES/2

Pryce, J. E., Royal, M. D., Garnsworthy, P. C., & Mao, I. L. (2004). Fertility in the

high-producing dairy cow. *Livestock Production Science*, *86*(1–3), 125–135. https://doi.org/10.1016/S0301-6226(03)00145-3

RAJAKOSKI, E. (1960). The Ovarian Follicular System in Sexually Mature Heifers with Special Reference to Seasonal, Cyclical, and Left-Right Variations. *Acta Endocrinologica*, *34*(3_Supplement), S7–S68. https://doi.org/10.1530/ACTA.0.XXXIVS0007

Redding, G. P., Bronlund, J. E., & Hart, A. L. (2008). Theoretical investigation into the dissolved oxygen levels in follicular fluid of the developing human follicle using mathematical modelling. *Reproduction, Fertility and Development*, *20*(3), 408–417. https://doi.org/10.1071/RD07190

Reinhart, A. J., Williams, S. C., & Stocco, D. M. (1999). Transcriptional regulation of the StAR gene. *Molecular and Cellular Endocrinology*, *151*(1–2), 161–169. https://doi.org/10.1016/S0303-7207(98)00257-3

Rodgers, R. J., & Irving-Rodgers, H. F. (2010). Morphological classification of bovine ovarian follicles. *Reproduction (Cambridge, England)*, *139*(2), 309–318. https://doi.org/10.1530/REP-09-0177

Rodgers, Raymond J., & Irving-Rodgers, H. F. (2010). Formation of the Ovarian Follicular Antrum and Follicular Fluid. *Biology of Reproduction*, *82*(6), 1021–1029. https://doi.org/10.1095/BIOLREPROD.109.082941

Schimpl, M., Schüttelkopf, A. W., Borodkin, V. S., & Van Aalten, D. M. F. (2010). Human OGA binds substrates in a conserved peptide recognition groove. *Biochemical Journal*, *432*(1), 1–12. https://doi.org/10.1042/BJ20101338

Semenza, G. L., Agani, F., Booth, G., Forsythe, J., Iyer, N., Jiang, B. H., Leung, S.,

Roe, R., Wiener, C., & Yu, A. (1997). Structural and functional analysis of hypoxia-inducible factor 1. *Kidney International*, *51*(2), 553–555. https://doi.org/10.1038/KI.1997.77

Senger, P. L. (2012). *Pathways to Pregnancy and Parturiton* (3rd ed.). Current Conceptions.

Shiratsuki, S., Hara, T., Munakata, Y., Shirasuna, K., Kuwayama, T., & Iwata, H. (2016). Low oxygen level increases proliferation and metabolic changes in bovine granulosa cells. *Molecular and Cellular Endocrinology*, *437*, 75–85. https://doi.org/10.1016/J.MCE.2016.08.010

Shrestha, K., Rodler, D., Sinowatz, F., & Meidan, R. (2019). Corpus Luteum Formation. *The Ovary*, 255–267. https://doi.org/10.1016/B978-0-12-813209-8.00016-9

Sirois, J., & Fortune, J. E. (1988). Ovarian Follicular Dynamics during the Estrous Cycle in Heifers Monitored by Real-Time UItrasonograph. *Biology of Reproduction*, *39*(2), 308–317. https://doi.org/10.1095/BIOLREPROD39.2.308

Stephen, H. M., Adams, T. M., & Wells, L. (2021). Regulating the Regulators: Mechanisms of Substrate Selection of the O-GlcNAc Cycling Enzymes OGT and OGA. *Glycobiology*, *31*(7), 724–733. https://doi.org/10.1093/GLYCOB/CWAB005

Stocco, D. M. (1997). A StAR Search: Implications in Controlling Steroidogenesis'. *BIOLOGY OF REPRODUCTION*, *56*, 328–336. https://academic.oup.com/biolreprod/article/56/2/328/2760686

Sugiura, K., Pendola, F. L., & Eppig, J. J. (2005). Oocyte control of metabolic

cooperativity between oocytes and companion granulosa cells: energy metabolism. *Developmental Biology*, *279*(1), 20–30. https://doi.org/10.1016/J.YDBIO.2004.11.027

Sutton-McDowall, M. L., Mitchell, M., Cetica, P., Dalvit, G., Pantaleon, M., Lane, M., Gilchrist, R. B., & Thompson, J. G. (2006). Glucosamine supplementation during in vitro maturation inhibits subsequent embryo development: possible role of the hexosamine pathway as a regulator of developmental competence. *Biology of Reproduction*, *74*(5), 881–888. https://doi.org/10.1095/BIOLREPROD.105.048553

Swanson, L. V., Hafs, H. D., & Morrow, D. A. (1972). Ovarian Characteristics and Serum LH, Prolactin, Progesterone and Glucocorticoid from First Estrus to Breeding Size in Holstein Heifers. *Journal of Animal Science*, *34*(2), 284–293. https://doi.org/10.2527/JAS1972.342284X

Tamanini, C., & De Ambrogi, M. (2004). Angiogenesis in Developing Follicle and Corpus Luteum. *Reproduction in Domestic Animals*, *39*(4), 206–216. https://doi.org/10.1111/J.1439-0531.2004.00505.X

Thompson, J. G., Brown, H. M., Kind, K. L., & Russell, D. L. (2015). The ovarian antral follicle: Living on the edge of hypoxia or not? *Biology of Reproduction*, *92*(6), 153–154. https://doi.org/10.1095/BIOLREPROD.115.128660/2434168

Torres, C.-R., & Hart5, G. W. (1984). Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for O-linked GlcNAc. *Journal of Biological Chemistry*, *259*(5), 3308–3317. https://doi.org/10.1016/S0021-9258(17)43295-9

Turzillo, A. M., & Fortune, J. E. (1993). Effects of suppressing plasma FSH on ovarian follicular dominance in cattle. *Reproduction*, *98*(1), 113–119. https://doi.org/10.1530/JRF.0.0980113

Ubersax, J. A., & Ferrell, J. E. (2007). Mechanisms of specificity in protein phosphorylation. *Nature Reviews Molecular Cell Biology 2007 8:7*, *8*(7), 530–541. https://doi.org/10.1038/nrm2203

Van Blerkom, J., Antczak, M., & Schrader, R. (1997a). The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. *Human Reproduction*, *12*(5), 1047–1055. https://doi.org/10.1093/HUMREP/12.5.1047

Van Blerkom, J., Antczak, M., & Schrader, R. (1997b). The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. *Human Reproduction*, *12*(5), 1047–1055. https://doi.org/10.1093/HUMREP/12.5.1047

Van Wezel, I. L., & Rodgers, R. J. (1996). Morphological Characterization of Bovine Primordial Follicles and their Environment in Vivo. *Biology of Reproduction*, *55*(5), 1003–1011. https://doi.org/10.1095/BIOLREPROD55.5.1003

Wang, T. F., Feng, Z. Q., Sun, Y. W., Zhao, S. J., Zou, H. Y., Hao, H. S., Du, W. H., Zhao, X. M., Zhu, H. Bin, & Pang, Y. W. (2022). Disruption of O-GlcNAcylation Homeostasis Induced Ovarian Granulosa Cell Injury in Bovine. *International Journal of Molecular Sciences*, *23*(14), 7815.

https://doi.org/10.3390/IJMS23147815/S1

Wathes, D. C., Bourne, N., Cheng, Z., Mann, G. E., Taylor, V. J., & Coffey, M. P. (2007). Multiple Correlation Analyses of Metabolic and Endocrine Profiles with Fertility in Primiparous and Multiparous Cows. *Journal of Dairy Science*, *90*(3), 1310–1325. https://doi.org/10.3168/JDS.S0022-0302(07)71619-3

Wettemann, R. P., Hafs, H. D., Edgerton, L. A., & Swanson, L. V. (1972). Estradiol and Progesterone in Blood Serum during the Bovine Estrous Cycle. *Journal of Animal Science*, *34*(6), 1020–1024. https://doi.org/10.2527/JAS1972.3461020X

Wu, G., Li, C., Tao, J., Liu, Z., Li, X., Zang, Z., Fu, C., Wei, J., Yang, Y., Zhu, Q., Zhang, J. Q., Shen, M., & Liu, H. (2022). FSH mediates estradiol synthesis in hypoxic granulosa cells by activating glycolytic metabolism through the HIF-1α–AMPK–GLUT1 signaling pathway. *Journal of Biological Chemistry*, *298*(5), 101830. https://doi.org/10.1016/j.jbc.2022.101830

Wycherley, G., Kane, M. T., & Hynes, A. C. (2005). Oxidative phosphorylation and the tricarboxylic acid cycle are essential for normal development of mouse ovarian follicles. *Human Reproduction*, *20*(10), 2757–2763. https://doi.org/10.1093/HUMREP/DEI132

Ying, S. Y. (1988). Inhibins, activins, and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone. *Endocrine Reviews*, *9*(2), 267–293. https://doi.org/10.1210/EDRV-9-2-267

Zhou, L. T., Romar, R., Pavone, M. E., Soriano-Úbeda, C., Zhang, J., Slawson, C., & Duncan, F. E. (2019). Disruption of O-GlcNAc homeostasis during mammalian oocyte meiotic maturation impacts fertilization. *Molecular Reproduction and*

Development, *86*(5), 543–557. https://doi.org/10.1002/MRD.23131