FSH AND IGF-I SIGNAL VIA AKT TO REGULATE BETA-CATENIN ACCUMULATION AND ESTRADIOL PRODUCTION IN GRANULOSA CELLS

By

BELINDA IRENE GÓMEZ

Bachelor of Science in Agriculture and Animal Science
West Texas A&M University
Canyon, TX
2010

Master of Science in Animal Science
Oklahoma State University
Stillwater, OK
2012

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May 2016

FSH AND IGF-I SIGNAL VIA AKT TO REGULATE BETA-CATENIN ACCUMULATION AND ESTRADIOL PRODUCTION IN GRANULOSA CELLS

Dissertation Approved:

Dr. Jennifer Hernandez Gifford

Dissertation Adviser

Dr. Udaya DeSilva

Dr. Guolong Zhang

Dr. Penny Thompson

ACKNOWLEDGEMENTS

Above all I thank God. For he is the reason why I am blessed with family, friends, and mentors who helped me accomplish this goal.

I want to first express my sincere appreciation for my mentor Dr. Jennifer Hernandez Gifford. I received valuable advice from her in my research, future, and personal life. Her encouragement and guidance have prepared me for a future in research and teaching. I am grateful for Dr. Craig Gifford who was extremely impactful in the success of my research and has helped me with various experiments. I would also like to thank my remarkable committee members Dr. DeSilva, Dr. Glen Zhang, and Dr. Penny Thompson for their impactful words that helped shape my future.

I have great appreciation of all past and present members of the Gifford Lab and fellow graduate students who helped me in my research. I want to specifically thank my two dear friends, Bahaa Aloquily and Rita Flores who so selflessly helped me with cell culture preparation. Lastly, I am grateful for the undergraduate research scholars: Julia Matera, Alexis Gullic, and Juliet Ellison who made working in the lab more pleasurable.

I would also like to express my gratitude to my amazing family. I thank my role model, my Momma, for her unconditional love and the sacrifices she made to get me to this point. I thank my Dad for his positive thinking and wise words. Finally, I am forever indebted to my husband Rene who cared for me, helped me, and loved me endlessly. He made my dreams his own and embraced everything he could do to get me here. Finally, I want to thank my little man Ian who with just one smile reminds me why it's all worth it.

Name: BELINDA IRENE GÓMEZ

Date of Degree: MAY 2016

Title of Study: FSH AND IGF-I SIGNAL VIA AKT TO REGULATE BETA-CATENIN ACCUMULATION AND ESTRADIOL PRODUCTION IN GRANULOSA CELLS

Major Field: ANIMAL SCIENCE

Abstract: Estradiol is the female sex hormone that profoundly influences the female reproductive system from puberty to fertility. Circulating estradiol in women is synthesized primarily by ovarian granulosa cells in response to the pituitary glycoprotein follicle-stimulating hormone (FSH). In granulosa cells FSH triggers a signaling cascade that subsequently induces expression of aromatase (*Cyp19a1*), a steroidogenic enzyme that catalyzes the aromatization of testosterone into estradiol. While FSH stimulates estradiol production, estradiol concentrations are regulated by intra-ovarian signaling molecules wingless-type mammary tumor virus integration-site (WNT) family molecules and insulin-like growth factor-I (IGF-I). Intracellular signaling cascades elicited by FSH, WNT, and IGF-I eventually overlap at β-catenin, a transcription co-factor. In granulosa cells β-catenin accumulates in response to FSH, and WNT, and is required for *Cyp19a1* expression. Although it is evident that granulosa cells require β-catenin to maintain estradiol production, there is still much to be identified about its role, regulators, and downstream effectors. In this dissertation, evidence is presented that enhances our understanding of the complex intracellular regulation of estradiol biosynthesis. Data demonstrates β-catenin accumulation in response to FSH and IGF-I occur via phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT) pathway as inhibition of PI3K signaling reduced β-catenin and estradiol concentrations. Additionally, IGF-I rescued FSH-mediated *Cyp19a1* expression and estradiol production from the inhibitory effects of WNT3A. To elucidate the mechanism, β-catenin accumulation, phosphorylation status of β-catenin and Forkhead box O protein were analyzed by Western blot and *Axin2* mRNA expression by real-time PCR. Data indicates IGF-I did not modulate expression of the above mentioned target markers and therefore was ruled out as potential mechanisms. A noteworthy discovery was in comparing animal models and identifying bovine but not rat granulosa cells have increased β-catenin accumulation with IGF-I treatment which further adds to the complex nature of estradiol production. In the final study it was confirmed that the crucial mechanism by which β-catenin regulates *Cyp19a1* transcription is through its association with T-cell Factor on the promoter. Together, these data provide a new appreciation and understanding of the complex regulation of β-catenin in estradiol production.

TABLE OF CONTENTS

Chapter Page

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

CHAPTER I

INTRODUCTION

Steroids play an important role in regulating homeostatic and pathological pathways. Estradiol is the female sex steroid produced primarily by the ovarian follicle and is essential in maintenance of the female reproductive system. Estradiol drives onset of puberty and development of secondary sex characteristics. Thereafter, estradiol maintains cyclicity, enhances oocyte fertility and is responsible for female sexual receptivity. This female sex steroid is also associated with various diseases such as breast cancer, uterine cancer, and infertility (Couse et al., 1999; Weiderpass et al., 1999; Yager and Davidson, 2006). Understanding the complex biological regulation of estradiol biosynthesis is necessary to set a foundation for what is normal and identify future target interventions for when estradiol is abnormally present in inappropriate circulatory concentrations.

Follicle-stimulating hormone (FSH) is a pituitary glycoprotein released in response to hypothalamic gonadotropin-releasing hormone. The primary function of FSH is to enhance expression of genes required for maturation of ovarian follicles while simultaneously promoting expression of steroidogenic enzymes necessary for estradiol biosynthesis. Granulosa cells in the follicle are the only cells in the female containing FSH receptors

(Camp et al., 1991) and require testosterone, a precursor derived from surrounding theca cells to synthesize estradiol. Follicle-stimulating hormone signals via a G-protein coupled receptor to target downstream effectors such as cAMP, protein kinase A (PKA) and phosphatidylinositol-3 kinase (PI3K) (Izadyar et al., 1998). Granulosa cells proliferate as follicles develop and produce increasing amounts of estradiol from 3.6 pg/mL in the luteal phase (days 1 to 18 of the estrous cycle) to 9.7 pg/mL in the estrus phase (day 20) (Wettemann et al., 1972). In cattle, the largest dominant follicle produces increasing amounts of estradiol at the start of deviation (Beg et al., 2001), whereas in the human greater follicular estradiol occurs in the dominant follicle when identified by its larger diameter (van Dessel et al., 1996). The elevated estradiol concentration, a hallmark of large antral follicles, causes a decline in FSH to prevent the emergence of new follicles (Evans et al., 1997). Greater amplitude or a surge in luteinizing hormone and is responsible for triggering the release of luteinizing hormone for ovulation (Rahe et al., 1980).

A requirement for the gonadotropins as regulators in estradiol production has been well established and current studies focus on contributions of additional regulatory compounds. A more recently studied signaling factor in the ovary is wingless-type mammary tumor virus integration-site (WNT) family of extracellular signaling molecules. The WNTs are a family of 19 secreted glycoproteins and 18 of them are expressed in the ovary of the human, mouse, rat, pig, and cow (Hernandez Gifford, 2015). These short range signaling molecules can elicit a signal through three pathways including the planar cell polarity, WNT/Ca^{2+}, and the canonical WNT/β-catenin pathway which is the most extensively studied. In the absence of WNT, cytoplasmic β-catenin concentrations are controlled by a multiprotein destruction complex composed of adenomatous polyposis coli,

Axin 1, and glycogen synthase kinase-3β (GSK-3β). Phosphorylation of β-catenin at serine-45 by the priming kinase, casein kinase (CK1α) and at theronine-41, serine-37, and serine-33 by GSK-3β induces its ubiquitination and subsequent degradation by the proteosome (Liu et al., 2002; Kimelman and Xu, 2006). In the canonical WNT signaling cascade, WNT binds to frizzled (FZD) (Janda et al., 2012) and low density lipoprotein 5/6 receptors (LRP 5/6) (He et al., 2004). Dishevelled is then recruited by FZD which promotes its clustering with LRP5/6 (Gao and Chen, 2010). This polymerization by dishevelled provides a platform for Axin 1 to associate with LRP5/6 which subsequently causes disassociation of the degradation complex. This results in loss of GSK-3β activity which subsequently prevents β-catenin from being phosphorylated and targeted for degradation. β-catenin accumulates in the cytoplasm, translocates to the nucleus, and associates with T-cell factor/lymphoid enhancer binding protein (TCF/LEF) to activate transcription of target genes (Gordon and Nusse, 2006).

The requirement for WNTs in ovarian development was first demonstrated in females heterozygous for a disrupted *Wnt4* allele that displayed a partial female to male sex reversal (Vainio et al., 1999). The loss of WNT4 in females resulted in ovaries that contained structures which resembled seminiferous tubules in testes and expressed sertoli cell marker *Desert hedgehog*, and leydig cell markers *3β-hydroxysteroid deyhdrogenase* and *17α hydroxylase* all of which are markers strictly associated with the male testis of neonates. The requirement for WNT in ovarian development raised the possibility of a role for WNTs in the adult ovary. Subsequent studies have identified transcripts for WNT and downstream WNT signaling molecules expressed at specific stages of follicle development (Hsieh et al., 2002; Ricken et al., 2002; Wang et al., 2010). β-catenin, the linchpin molecule in the WNT canonical pathway is required for optimal estradiol production (Hernandez Gifford et al.,

2009). In culture, FSH regulates expression of WNT/β-catenin signaling in granulosa cells as demonstrated by an increase in *WNT2* mRNA expression and total β-catenin accumulation (Castañon et al., 2012). Consistent with the role of FSH in estradiol production, total β-catenin is highly abundant in large estradiol active follicles when compared with large estrogen inactive follicles suggesting, β-catenin accumulates in response to FSH in vivo. Interestingly, co-stimulation of WNT3A and FSH signaling pathways is inhibitory on aromatase (*Cyp19a1*) mRNA expression and estradiol production in granulosa cells (Stapp et al., 2014b). A focus of this dissertation is to investigate if a potential mechanism by which WNT3A is inhibitory on FSH-stimulated estradiol production is through a modification of β-catenin transcriptional activity.

Follicle-stimulating hormone-mediated estradiol and granulosa cell proliferation is enhanced in response to the growth factor, insulin-like growth factor-I (IGF-I) in porcine (Baranao and Hammond, 1984), rodent (Adashi et al., 1985b), and bovine granulosa cells (Spicer and Aad, 2007). Insulin-like growth factor-I signals through phosphatidylinositol-3 kinase to activate protein kinase B (AKT) which enhances expression of genes involved in cell cycle progression and phosphorylates numerous proteins, including GSK-3β (Alessi and Cohen, 1998). In cultured bovine granulosa cells, FSH and IGF-I increases phosphorylated and total AKT (Mani et al., 2010). Additionally, the ability of FSH to increase total β-catenin (Castañon et al., 2012) suggest AKT is required for β-catenin accumulation. Therefore, research presented herein was designed to determine if FSH and IGF-I activation of AKT contributes to estradiol synthesis by modulating β-catenin accumulation.

The ability of FSH, WNT, and IGF-I to regulate estradiol production in granulosa cell relies in their capacity to modulate Cyp19a1 expression, a steroidogenic enzyme required for

the final conversion of C19 androgens to the C18 estrogens. β-catenin is a downstream molecule consistently associated with all three pathways and is required for *Cyp19a1* mRNA expression (Hernandez Gifford et al., 2009). β-catenin increases expression of target genes by associating with multiple nuclear transcription factors on specific promoter regions. Interestingly, expression of the FSH-induced luteinizing hormone receptor (*Lhcgr*), a differentiation factor, is dependent on β-catenin association with steroidogenic factor-1 (SF-1; officially designated NR5A1) a nuclear orphan receptor, and TCF on the *Lhcgr* promoter (Law et al., 2013). Maximal *Cyp19a1* mRNA expression relies on β-catenin association with SF-1 on the *Cyp19a1* promoter to regulate its expression (Parakh et al., 2006). It is suggested that activation of the *Cyp19a1* promoter by FSH additionally requires β-catenin/TCF association. However, this is only demonstrated through an increased expression of a TCF-luciferase reporter plasmid in response to FSH (Fan et al., 2010). Therefore, the final focus is designed to determine if β-catenin is required to associate with TCF for optimal *Cyp19a1* mRNA expression.

Results herein, provide novel insight to the mediators of β-catenin in estradiol biosynthesis. Understanding the normal intracellular molecules that regulate and maintain appropriate circulating estradiol concentrations are critical for identifying targets to enhance reproductive efficiency or designing therapeutic compounds that modulate estradiol biosynthesis. This dissertation advances the knowledge of estradiol biosynthesis in granulosa cells by demonstrating the requirement of key intracellular molecules controlling β-catenin.

Abbreviation	Name of protein
AKT/PKB	protein kinase B
APC	adenomatous polyposis coli
cAMP	cyclic adenosine mono-phosphate
CK	casein kinase
CBP	CREB binding protein
CRE	cAMP response element
CREB	cAMP response element binding protein
CYP19A1	aromatase
ER	estrogen receptor
FRT	female reproductive tract
FZD	frizzled receptor
FSH	follicle-stimulating hormone
FOXO	forkhead box protein O
GnRH	gonadotropin releasing hormone
GSK3β	glycogen kinase synthase
IGF-I	insulin-like growth factor-I
LH	luteinizing hormone
SF-1	steroidogenic factor-1
PI3K	phosphatidylinositol-3 kinase
TCF	T-cell factor
WNT	wingless-type mammary tumor virus integration-site molecule

TABLE 1

CHAPTER II

REVIEW OF LITERATURE

1. Estradiol Maintains Reproductive Homeostasis

In the adult, estradiol plays a prominent role in maintaining homeostasis of the female reproductive system. Estradiol regulates a series of reproductive processes including; development of the reproductive tract in utero, onset of puberty, development of secondary sex characteristics, and folliculogenesis which are required for optimal fertility. Misregulation of these processes causes reduced if not complete infertility in females. Furthermore, unregulated estradiol in humans is associated with various diseases such as breast cancer, polycystic ovaries, and uterine cancer.

1.1 Development of the Female Reproductive Tract

Mammals have a bipotential gonad and differentiation into the ovary or testis is directed by sex chromosomes and expression of sex-determining genes. In mammals, genetic females have two X chromosomes, and males posses a single X and Y chromosome. The Y chromosome contains a gene encoding sex determining region on

Y chromosome (*Sry*), and when expressed in the primitive gonads, testes rather than ovaries develop. This previously suggested that ovarian development was a default event; however recent studies indicate genes directed at development of the female reproductive tract (FRT) precede expression of the SRY protein (Vainio et al., 1999). Homozygous null mutations of R-spondin 1in female fetal and newborn mice had abnormally high concentrations of testosterone and ovaries depleted of oocytes that were surrounded by a coiled duct system that resembled the male epididymis (Tomizuka et al., 2008). The Vainio et al., (1999) study was the first to establish the requirement of wingless-type mammary tumor virus integration-site 4 (WNT) in ovarian development, as females deficient in WNT4 displayed a partial sex reversal that will be discussed in more detail in the following sections. During embryo development of the FRT, the mesonephric duct degenerates and the caudal ends of the paramesonephric ducts fuse to form the cervix and uterine body (Yin and Ma, 2005). A small evagination from the urogenital sinus attaches to the fused paramesonephric ducts to form the caudal end of the vagina. Ultimately, in a cranial to caudal direction the oviducts, uterus, cervix, and cranial vagina, are properly formed. Although estrogens are not essential for differentiating the FRT from that of the male, they are required for reproductive capabilities. Discovery of two estrogen receptors (ER) and gene deletion of one or both designated alpha (ERα) or beta (ERβ) demonstrated the crucial role of estradiol during embryonic development of a functional FRT (Greene et al., 1986; Giguere et al., 1988; Kuiper et al., 1996; Mosselman et al., 1996; Tremblay et al., 1997). The effects of estradiol on the FRT was first identified in ERα knockout (KO) mice which had ovaries that lacked developed follicles and corpora lutea, displayed severe uterine hypoplasia,

and consequently were infertile (Lubahn et al., 1993). However, KO of ERβ in mice results in subfertility as demonstrated by smaller litters than wild-type mice while the FRT is grossly and histologically indistinguishable from their litter mates (Krege et al., 1998). Deletion of both ERα and ERβ (αβERKO) was not lethal but mice did display a phenotype that is similar to αERKO (Couse et al., 1999). As adults, the αβERKO ovary lacked corpora lutea, and possessed structures resembling seminiferous tubules of testis. Development of the FRT is a complex process mediated by several factors, of these is estradiol that is clearly required for fertility.

1.2 Cyclicity and Fertility in the Female

Normal development of the FRT sets the stage for cyclicity which is a process through the fertile life of the female and depending on the species results in the release of one or few oocytes from follicles. The estradiol profile in the follicle microenvironment is essential for maturation and fertility of the oocyte (Hennet and Combelles, 2012). Individual phases in the cycle mediate the concentrations of estradiol, of these is proestrus, a transitional phase where progesterone concentrations decline and estradiol increases. In timed-artificial insemination (AI) estrous synchronization program designed to achieve a short or long proestrus period was determined by the interval from Lutalyse® administration to Cystorelin® injections (Bridges et al., 2010). Cows in both groups had ovulatory follicles of similar diameter but distinct estradiol peak concentrations in the cows with a long proestrus phase (2.25 d) averaged 3.6 pg/mL greater than cows with a short proestrus phase (1.25 d). In the group of animals that were

classified as having a normal luteal phase, cows with a long proestrus had 73% pregnancy rates to AI versus 14.3% in cows having a short proestrus. Therefore, limiting the length of exposure to estradiol and reducing estradiol concentrations prior to ovulation negatively impacts the fertility of the female. Another recognized role for the rise in circulating estradiol is its contribution to sexual receptivity in domestic livestock females. Animals display a variety of behaviors including increased locomotion, vocalization, irritability, and the mounting of other animals. These sexual behaviors allow managers take advantage of peak fertility.

2. Synthesis and Regulation of Estradiol

Steroidogenesis entails a process of enzymatic reactions by which cholesterol is converted to active steroid hormones. Of these is estradiol an important gonadal steroid whose primary circulating source is from ovarian follicles. Regulation of ovarian synthesis and release of estradiol is critical for normal function of the FRT and homeostatic pathways. The following section will examine the endocrine and intracellular signals relevant to this dissertation that regulate ovarian production of estradiol.

2.1 Hypothalamic, Pituitary, and Ovarian Regulation of Estradiol

Stimulation and regulation of estradiol depends on the tightly regulated and coordinated actions of the hypothalamus, anterior pituitary, and the ovary. Estradiol

synthesis is controlled by the tonic center (ventromedial nucleus and arcuate nucleus) and the surge center (preoptic nucleus, anterior hypothalamic area, and suprachiasmatic nucleus), two specialized regions in the ventral portion of the brain (Gorski, 1970). These specialized regions contain neurons specific to controlling the decapeptide, gonadotropin-releasing hormone (GnRH) which acts on the anterior pituitary to stimulate the release of the gonadotropins (Schally et al., 1971).

The anterior pituitary is located below the hypothalamus and connected by the stalk of the pituitary which contains a specialized circulatory network called the hypothalamo-hypophysial portal system which serves as a transport system for hypothalamic neurotransmitters including GnRH (Popa, 1930; Green and Harris, 1947). Specialized cells that secrete hormones in response to hypothalamic stimuli are in the anterior pituitary. The gonadotropes synthesize and secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH) which are essential for normal reproductive function. The binding of GnRH to its evolutionary conserved G-protein coupled receptors (GnRHr) on the gonadotropes results in the synthesis of FSH beta, LH beta, and the common subunit chorionic gonadotropin alpha (Kakar et al., 1993). The pattern of GnRH delivery is episodic and released in pulses to maintain cyclicity and prevent receptor desensitization. Whereas, constant infusion of GnRH in primates with hypothalamic lesions fails to restore release of FSH and LH (Belchetz et al., 1978).

The gonadotropins, FSH and LH, travel by the general circulation system and are recognized by receptors on theca and granulosa cells within follicles of the ovaries. Females have one pair of ovaries that are dense in structure and encapsulated by an outer layer of connective tissue termed the tunica albuginea. The innermost region of the ovary

is the medulla that is rich in blood vessels, lymphatic vessels and nerves. The cortex is immediately beneath the tunica albuginea which contains primordial, primary, secondary, and tertiary follicles and the corpus luteum that are responsible for carrying out the primary functions of the reproductive system. Inside follicles is one oocyte that is enclosed by cumulus granulosa cells. The cumulus oophorus is in immediate contact with the oocyte and keeps it static by adhering to mural granulosa cells that border the basement membrane. Contact between cumulus granulosa cells and the oocyte gap junctions throughout follicle development are crucial for the movement of metabolites between cells and oocyte growth (Brower and Schultz, 1982). In comparison to mural granulosa cells, cumulus granulosa cells replicate ten times faster in response to gonadotropins and growth factors (Khamsi and Roberge, 2001). Finally, theca interna and externa cells surround the outside of the basement membrane. Luteinizing hormone can bind to granulosa, and theca cells, whereas only granulosa cells have FSH receptors, however both cell types coordinate estradiol synthesis by the granulosa cells. The requirement for theca and granulosa cells to work in synchrony is known as the two-cell theory of steroidogenesis as the production of estradiol relies on both cell types. The newly synthesized estradiol circulates systemically and feeds back to the hypothalamus.

Feedback actions of estradiol can be positive and negative to regulate the reproductive cycle. The effect of estradiol as a mediator of the negative feedback loop is demonstrated when the removal of the ovaries causes an increase in the gonadotropins (Yamaji et al., 1972). Thus, estradiol prevents the gonadotropins from continually being secreted. Conversely, positive feedback of estradiol is demonstrated by injecting ovariectomized guinea pigs with estradiol benzoate and eliciting a surge release of LH

(Terasawa et al., 1979). Additionally, a single injection of estradiol benzoate caused high amplitude pulses of GnRH in ovariectomized rhesus monkeys fitted with a push-pull cannula in the median eminence (Levine et al., 1985). In the bovine on day 18 to19 of the estrous cycle when estradiol peaks, a LH surge was generated and detected in the serum (Rahe et al., 1980). Collectively, these data demonstrate that estradiol has a positive effect on the hypothalamus, by causing the pulsatile release of GnRH that subsequently leads to the LH surge which is required for ovulation.

2.2 Folliculogenesis and the Estrous Cycle

The ovary is a critical component of the female because it accommodates a finite number of oocytes or female sex cells. The female gonad is a dynamic organ in which follicles continually develop, become atretic, ovulate, and differentiate into a corpus luteum. Folliculogenesis is the process of follicle development that is crucial for estradiol production and occurs until depletion of the oocyte reserve.

Folliculogenesis begins with the most immature follicle type, the microscopic primordial follicle, that consists of one oocyte surrounded by a single layer of flattened squamous granulosa cells (Rodgers and Irving-Rodgers, 2010). Through an unknown mechanism independent of gonadotropin input, some primordial follicles leave the pool of quiescent non-growing follicles and enter the growth phase to develop into small preantral follicles. The primary follicle is characterized by the differentiation of the surrounding granulosa cells from squamous to cuboidal, and an increase in diameter (Erickson, 1966; Aerts and Bols, 2010). The follicle proceeds through differentiation

independent of gonadotropins into a secondary follicle. The secondary follicle is characterized as having two or more layers of granulosa cells and still lacks fluid filled cavity referred to as an antrum (Fair et al., 1997). During the transition into a secondary follicle, the stroma around the basal lamina organizes to become the theca cells (Young and McNeilly, 2010). When the follicle begins to acquire an antrum it is considered a tertiary follicle and is highly responsive to the gonadotropins to achieve ovulatory capacity. Granulosa cells of the tertiary follicle proliferate and the theca can be divided into interna (hormone production) and externa (transition to stroma). In monovulatory species only one follicle deviates from the rest to be selected as the pre-ovulatory follicle whereas, in the litter bearing species such as pigs, approximately 15 to 20 follicles are destined to ovulate (Ginther et al., 1996; Mihm and Evans, 2008). In larger species the antral follicle can be observed without a microscope and resembles blister-like structures that range in size from 1 to 20 mm (Erickson, 1966). Follicle development is a lengthy process, where the first 300 days primordial follicles grow in the absence of FSH, and mature in response to gonadotropins for the next 50 days. However, folliculogenesis is a process denied by many because over 90% of follicles at any stage of development undergo atresia. Follicle atresia is often as a result of a reduced response to gonadotropins or overall reduced basal concentrations of the gonadotropins (Kaipia and Hsueh, 1997).

Folliculogenesis can occur in waves throughout the estrous cycle depending on the species and breed. The estrous cycle is a period of predictable reproductive events beginning at estrus and ending at the subsequent estrus. Length of the estrous cycle varies in livestock species however, the physiological changes are similar. The estrous

cycle is unequally divided between the follicular phase where follicles are the primary structures on the ovary and the luteal phase where the corpus luteum in the dominant structure. Additionally, these two phases can be distinguished by the hormonal profile from the contributing structures on the ovary. The follicular phase is characterized by the greatest concentrations of circulating estradiol whereas in the luteal phase progesterone secretion by the corpus luteum is greatest. To further characterize the ovarian structures and the hormonal profile, the luteal phase is subdivided into metestrus and diestrus while the follicular phase is divided into proestrus and estrus. A period where concentrations in estradiol rapidly drop and progesterone slowly increases can be attributed to ovulation which is the rupture of a dominant follicle to release the oocyte. In cattle, ovulation occurs at the end of estrus (Peters and Lamming, 1983), whereas in rats it occurs near the end of proestrus (Butcher et al., 1974). The major structure present on the ovary in metestrus is a newly ovulated dominant follicle that has collapsed into folds and is transitioning from a corpus hemorrhagicum to a corpus luteum. Next is diestrus, a period where the corpus luteum is the prominent structure on the ovary and is secreting greatest concentrations of progesterone. Proestrus follows which is characterized by a decline in progesterone due to luteolysis of the corpus luteum and an increase in estradiol by means of developing follicles. Lastly, estrus is recognized by a dominant pre-ovulatory follicle secreting the greatest estradiol concentrations. At ovulation the female will have completed one estrous cycle and will shift back into metestrus initiating the beginning of a new cycle unless pregnancy is established.

2.3 Biosynthesis of Estradiol

Ovarian estradiol production is a process coordinated by the gonadotropins and other intra-ovarian signaling molecules to increase the expression of key steroidogenic enzymes in theca and granulosa cells (Hanukoglu, 1992; Peter and Dubuis, 2000). Two major families of steroidogenic enzymes include 1) hydroxylase enzymes (cytochrome P450) and 2) hydroxysteroid dehydrogenases (Miller and Auchus, 2011). These enzymes perform a series of enzymatic reactions to convert cholesterol, the steroidogenic precursor, into gonad derived hormones. Cholesterol is derived from 1) acetyl-CoA synthesized in the cells, 2) cholesteryl esters from lipid droplets, and 3) low and high-density lipoproteins from the plasma (Hu et al., 2010). Steroid acute regulatory protein (StAR) shuttles cholesterol into the mitochondria so that it is available for steroidogenesis. The following steps required for estradiol production are complex as the series of conversions can occur through the delta-4 or delta-5 pathway (Hu et al., 2010).

The first step to estradiol synthesis is conversion of the 27 carbon cholesterol to pregnenolone by the mitochondrial side-chain cleavage enzyme (P450scc). Pregnenolone can then be converted to progesterone (delta-4 pathway) or 17α-hydroxypregnenolone via 3β-hydroxysteroid dehydrogenase (3β-HSD) or 17α-hydroxylase/17, 20-lyase (P450c17), respectively. Only theca cells can further metabolize 17α-hydroxypregnenolone to androstenedione (delta-5 pathway) through conversions mediated by P450c17 and 3β-HSD which can be secreted or is further metabolized to testosterone by 17β-hydroxylase. Granulosa cells then uptake androstenedione and testosterone and under FSH stimulation express the enzyme aromatase (CYP19A1) that performs the final enzymatic reaction in converting thecal derived testosterone to estradiol (Corner, 1938; Simpson et al., 1994).

3. Signaling Molecules Regulating Estradiol Synthesis

Estradiol synthesis is primarily regulated by the gonadotropins, however the concentrations of estradiol produced by granulosa cells can be altered positively or negatively by intra-ovarian signaling molecules such as insulin-like growth factor-I (IGF-I) (Mani et al., 2010; Zhou et al., 2013) and WNT (Stapp et al., 2014b). This section will focus on FSH, IGF-I, and WNT receptor recognition, activation, and the elicited intracellular signal transduction related to ovarian estradiol production (Fig. 1).

3.1 Follicle-Stimulating Hormone Signaling in Granulosa Cell Estradiol Production

Follicle-stimulating hormone and its receptor (FSHR) are the major physiological regulators of granulosa cell proliferation and estradiol production. The FSHR is a large protein that belongs to the rhodopsin-like subfamily of receptors that sense the environment for a ligand and elicit a cascade of signaling events inside the cell (Simoni et al., 1997; Ulloa-Aguirre et al., 2007). It is a seven-transmembrane domain guanine-protein coupled receptor (GPCR) and consists of an extacellular, transmembrane, and intracellular domain (Pierce and Parsons, 1981; Vassilatis et al., 2003). The extracellular (NH_2-terminus) region is the hormone binding domain and possesses an enigmatic hinge region containing a sulfotyrosine (sTyr) site for receptor specificity (Dohlman et al., 1987). Two highly conserved cysteine residues of the transmembrane domain build disulfide bonds to stabilize the receptor. The intracellular domain or the COOH-terminus contains the region that directly activates the heterotrimeric G-protein (Gudermann et al., 1995). Follicle-stimulating hormone is specifically recognized and associated with FSHR in a two step process: FSH recruitment then sTyr recognition. Follicle-stimulating

hormone binds to the high-affinity binding domain on the NH_2-terminus resulting in a conformation change, thus forming a sTyr pocket where the sTyr of FSH will be inserted to activate the receptor (Costagliola et al., 2002).

Stimulation of FSHR triggers the alpha subunit ($G_{\alpha s}$) of the heterotrimeric G protein to activate adenylate cyclase which catalyzes ATP to increase the amount of the second messenger, cyclic adenosine 3',5'-monophosphate (cAMP) (Izadyar et al., 1998). Then, cAMP will activate protein kinase A (PKA) which will phosphorylate specific serine and threonine amino acids of the target proteins. The tissue specific CYP19A1 proximal promoter II of the ovary contains specific response elements that are activated by transcription factors under FSH stimulation (Mendelson et al., 2005). The transcription factor cAMP response element (CRE)-binding protein (CREB) is activated by PKA and binds the CRE-like sequence (159 bp upstream of the transcription initiation site) (Fitzpatrick and Richards, 1994; Michael et al., 1997). A nuclear receptor response element on the CYP19A1 promoter (130 bp upstream of the transcription initiation site) was identified to bind the orphan nuclear receptor steroidogenic factor 1 (SF-1) (Fitzpatrick and Richards, 1994; Michael et al., 1995). Additionally, PKA can activate kinases that initiate other intracellular signaling cascades such as extracellular regulated kinase (ERK), p83 mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) (Silva and Price, 2000; Zeleznik et al., 2003; Hunzicker-Dunn and Maizels, 2006). In granulosa cells, CYP19A1 expression is regulated by FSH stimulation in the human, rat, and bovine (Steinkampf et al., 1987; Fitzpatrick and Richards, 1991; Silva and Price, 2000), whereas IGF-I alone can increase CYP19A1 expression (Steinkampf et al., 1988; Ryan et al., 2008) and in the rat enhances FSH-mediated

properties (Zhou et al., 2013). The intracellular pathways elicited by FSH and IGF-I are mediated by distinct receptors, however the downstream signals overlap to direct granulosa cell proliferation and estradiol production.

3.2 Insulin-like Growth Factor-I Signaling

Insulin-like growth factor-I belongs to an integrated growth factor system that includes IGF-II, insulin, and six binding proteins (Laviola et al., 2007). Circulating IGF-I is bound to the high affinity binding proteins (IGFBP) produced by granulosa and theca cells to mediate the half-life, and interaction with IGF-I receptor (Armstrong et al., 1996). Insulin-like growth factor-I is locally produced in the ovary and is recognized for promoting granulosa cell differentiation, proliferation, steroid output, and modulating the action of the gonadotropins (Baranao and Hammond, 1984; Adashi et al., 1985b). In reproduction IGF-I is required for fertility, as nullizygous *Igf-I* mutant mice fail to ovulate, and have an infantile uterus that lacks muscle tone (Baker et al., 1996). In cattle IGF-I is often regarded as a major determining factor to selection of the ovulatory follicle (Fortune et al., 2004). At emergence the follicle selected to ovulate has a follicular fluid profile that includes decreased IGFBP-2, -4, -5, greater IGFBP protease activity, and subsequently increased free IGF-I in comparison with the second largest follicle.

Insulin-like growth factor-I is a small single chain peptide of 70 amino acids cross linked by three disulfide bonds (Rinderknecht and Humbel, 1978). The IGF-I receptor is a hetero-tetrameric protein that consist of two identical extracellular α-subunits containing the IGF-I binding site and two transmembrane β-subunits that have ATP-dependent intrinsic tyrosine kinase activity (Adams et al., 2000). The binding of IGF-I to

a cysteine-rich region of the α-subunit activates the tyrosine kinase domain, subsequently catalyzing the phosphorylation of the intrinsic tyrosines on the β-subunits, causing the autophosphorylation of tyrosine phosphorylation substrates, such as insulin receptor substrate (IRS) (Ogawa et al., 1998). Activated IRS recruits and activates PI3K, leading to the synthesis of phosphoinositol (3,4,5)- triphosphate (PIP3) which in turn activates protein kinase B (AKT).

Protein kinase B is a major regulator of the cell cycle and is involved in FSH, IGF-I, and WNT signaling. During follicle selection active/phosphorylated AKT protein expression is greatest in estrogen active follicles and FSH-treated granulosa cells when compared with the subordinate follicles and non-treated cells, respectively (Ryan et al., 2007; Castañon et al., 2012). A major AKT target is glycogen synthase kinase 3-β (GSK-3β), which becomes inactive upon phosphorylation of serine 9 (Cross et al., 1995; Fang et al., 2000), therefore AKT indirectly promotes glycogen synthase. In granulosa cells inactivation of GSK-3β, promotes the accumulation of β-catenin, a key molecule in estradiol production that will be reviewed intensively in the subsequent sections. Additionally, AKT activates the protein kinase mechanistic target of rapamycin (mTOR), which is a key regulator of cell growth, and is a mechanism by which IGF-I increases granulosa cell proliferation (Sirotkin et al., 2015).

3.3 Canonical Wingless-Type Mammary Tumor Virus Integration-Site (WNT) Signaling

Wingless-Type Mammary Tumor Virus Integration-Site molecules are composed of a family of 19 highly conserved secreted glycoproteins with paracrine and autocrine

functions (Logan and Nusse, 2004). They are involved in diverse and often critical roles such as survival, cell polarity, cell fate, cell proliferation, sexual differentiation and more recently in granulosa cell function (Cadigan and Nusse, 1997; Komiya and Habas, 2008; Wang et al., 2010). The initial study establishing the requirement for WNT in the ovary was conducted by Vainio et al. (1999) demonstrating that mice null for *Wnt4* develop ovaries that resembled testes, expressed markers associated with testicle development, and had fewer oocytes at birth. Subsequent studies focused on the requirement for WNT in the adult ovary and concluded WNT ligands and components of the pathway are differentially expressed at specific stages of follicle development and under gonadotropin stimulation in rodents (Hsieh et al., 2002; Ricken et al., 2002; Wang et al., 2010) and cattle (Castañon et al., 2012; Gupta et al., 2014). Immunostaining of whole ovaries demonstrated greatest intensity of WNT2 in granulosa cells of healthy antral follicles (Wang et al., 2010). Overexpression of WNT2 via transfection with a recombinant viral vector in granulosa cells increased expression of proliferating cell nuclear antigen a marker of enhanced DNA synthesis. Conversely, WNT3A is inhibitory on FSH-mediated estradiol production in cultured granulosa cells (Stapp et al., 2014b) and follicular development in cultured secondary follicles (Li et al., 2014) which suggest WNT is negative on FSH signaling. Evidently, normal ovarian function requires WNT signaling and misregulation of the pathway is associated with granulosa cell tumor formation (Boerboom et al., 2005).

The *Wnt* gene, first recognized as integration region-1 (*int-1*) in mice, was identified in 1982 (Nusse and Varmus, 1982) , and the first protein (WNT3A) was purified in 2003 (Willert et al., 2003). The abbreviation WNT is derived from

combination of the two gene names *wingless* (*wg*) in drosophila and *int-1* in the mouse. WNTs are around 350 to 400 amino acids in length, approximately 40 kDa in size and specifically WNT3A is palmitoylated on a conserved cysteine (Cys77) where enzymatic removal results in a loss of signaling activity (Willert et al., 2003; Gao and Hannoush, 2014). Additionally, WNTs have a signal sequence for secretion and a characteristic distribution of 22 cysteine residues for proper folding (Mason et al., 1992). The nature of their posttranslational modifications revealed why WNTs are difficult to purify. To date, the crystal structure of WNT's has not been identified despite advances in characterizations of the protein.

To elicit an intracellular signal, WNTs binds a frizzled (FZD) receptor and low density lipoprotein receptor-related protein co-receptor (LRP5/6). Frizzled is a G-protein coupled seven transmembrane receptor and to date 10 have been identified (Bhanot et al., 1996). The overall genomic organization of all ten *FZD* genes does not appear to be highly conserved, however some individual *FZD* genes can share up to 75% identity with another (Huang and Klein, 2004). The protein can vary in length from about 500 to 700 amino acids and the extracellular NH_2-terminus contains a conserved 120-amino acid cysteine-rich domain that is ligand specific. Lipoprotein receptor-related protein co-receptor 5/6 is a single transmembrane receptor that share 70% identity and made of more than 1,600 amino acids (Tamai et al., 2000). Interaction between specific WNT ligands and FZD receptor activates one of three distinct pathways known as the planar cell polarity, non-canonical WNT/Ca^{2+} and WNT/β-catenin (canonical) pathways (Kohn and Moon, 2005; Karner et al., 2006; Huang and He, 2008). Of these, the most extensively studied is the canonical by which WNT/FZD/LRP5/6 form a trimeric complex whose

overall signaling results in the hypophosphorylation and accumulation of β-catenin (Huang and He, 2008).

In the absence of WNT molecules to stimulate the canonical pathway, a cytoplasmic destruction complex dedicated to the phosphorylation, ubiquitination, and degradation of β-catenin is active. This complex consist of the central scaffold protein Axin 1, adenomatous polyposis coli (APC), and GSK-3β. β-catenin is phosphorylated at serine-45 by the priming kinase, casein kinase 1α (CK1α) then subsequently phosphorylated at theronine-41, serine-37, and serine-33 by GSK-3β in the COOH-terminal to NH_2 direction (Liu et al., 2002; Kimelman and Xu, 2006). These specific phosphorylations on β-catenin act as a tag to promote its interaction F-box containing protein (β-TrCP), a member of the ubiquitin ligase complex. Finally, ubiquitination of β-catenin targets its degradation by the proteosome (Aberle et al., 1997; Hart et al., 1999).

WNT ligands bind FZD and LRP5/6 to activate the canonical WNT signaling pathway which ultimately prevents the degradation of β-catenin. The previously proposed model of canonical signaling suggests in the presence of WNT, Axin 1 is sequestered to membrane by binding LRP5/6 (Mao et al., 2001; Zeng et al., 2005) and dishevelled is recruited to FZD which promotes its clustering with LRP5/6 and subsequent phosphorylation by GSK-3β and CK1γ (Gao and Chen, 2010). This ultimately contributes to the disassembly of the destruction complex, and β-catenin is no longer degraded but instead accumulates in the cytoplasm. However, a recently established model suggest the activity of CK1α and GSK-3β are not inhibited by WNT, only β-TrCP dissociates, therefore β-catenin is no longer ubiquitinated and the degradation complex saturates with phosphorylated β-catenin (Li et al., 2012). Newly

synthesized β-catenin (Staal et al., 2002) becomes stabilized in the cytoplasm because it is no longer degraded and translocates to the nucleus where it regulates transcription by serving as a co-transcription factor with T-call Factor (TCF) (Behrens et al., 1996; Huber et al., 1996).

3.4 Complex Signal Overlap

Regulation of estradiol synthesis in granulosa cells is not linear and as demonstrated in this section is controlled primarily by FSH. Recent discoveries have demonstrated FSH, WNT, and IGF-I elicit signals that often overlap and are directed at mediating β-catenin activity. The ability of β-catenin to be modulated by various signaling molecules and its involvement in estradiol production establishes the motive to fully understand this multifunctional protein which is covered in the subsequent section.

4. β-catenin the Central Reoccurring Theme

β-catenin is involved in two unrelated physiological roles in the cell, the first identified is to maintain cell-cell adhesion and the second is as a signaling molecule. Its role as a signaling molecule is of particular importance as it is the central theme in this dissertation and plays a fundamental role in estradiol synthesis. Importantly, deregulation of β-catenin results in various malignancies including ovarian cancer (Arend et al., 2013). The ability β-catenin to participate in cell adhesion and cell signaling is further explained in its evolutionary conserved structure.

4.1 The Structure of β-catenin

The central region of β-catenin consists of a central stretch of 12 imperfect repeats (R1-R12) that each contains approximately 42 amino acids (Peifer et al., 1994a). This consecutive region of repeats is known as armadillo repeats and the structure it forms is highly conserved. The central region is flanked by 130 amino acids on the NH_2-terminal side and 100 amino acids on the COOH-terminal side. The terminal domains are distinct and are structurally flexible whereas the central region is more rigid to serve as an interaction site with binding partners. The three dimensional structure of β-catenin is a superhelix that features a long positively charged groove and is cylindrical in shape (Huber et al., 1997). Each of the 12 repeats contains three α helices arranged in a triangular shape that form the superhelix. The positively charged groove is what enables β-catenin to associate with other proteins to carry out its function.

4.2 Brief History of β-catenin/armadillo

In this section the nomenclature of the molecule of interest can cause some confusion because as it was being discovered and its functions studied in various species, it adopted different names. In *Drosophila melanogaster* it is recognized as Armadillo and in vertebrates β-catenin is accepted; it is a member of the Armadillo repeat protein superfamily however, to avoid confusion only β-catenin will be used to address the protein.

β-catenin is an evolutionary conserved molecule that was first identified as a component of cell adhesion (Ozawa et al., 1989; McCrea et al., 1991). Three

independent proteins named alpha, beta, and gamma were found to form a complex with uvomorulin (E-cadherin) a membrane adhesion molecule. In its role as a cell adhesion molecule, β-catenin tightly binds the cytoplasmic tail of E-cadherin to link it to alpha-catenin.

In 1980 it was discovered that the *Wg* gene which encodes the WNT protein of *Drosophila melanogaster* was involved in segment polarity during larval development as a mutation in *Wg* caused mirror-image duplications of individual denticle bands (Nusslein-Volhard and Wieschaus, 1980). In the mid to late 1980's it was revealed that a mutation in *armadillo* resulted in a similar phenotype to the *Wg* mutation (Wieschaus et al., 1984). With the knowledge that *Wg* gene encodes a secreted signaling protein (van den Heuvel et al., 1989) and mutations in *Wg* and *armadillo* have phenotypic similarities the next logical step was to establish that these two proteins are components of one pathway involved in pattern formation. The first evidence that both proteins are involved in a signaling pathway was in 1991, when Peifer et al. confirmed that cells of *Drosophila melanogaster* that require the greatest concentrations of β-catenin are the same cells that express *Wg* (Peifer et al., 1991). Then in 1994 it was discovered that both proteins work together when β-catenin accumulated in the cytosol (Peifer et al., 1994c) and nucleus (Funayama et al., 1995) in cells expressing *Wg*. This discovery raised the possibility that β-catenin could be signaling by interacting with other nuclear proteins and modifying the expression of target genes. During this period it became evident that β-catenin is a substrate for serine and threonine phosphorylation and this post-translational modification was dependent on WNT and GSK-3β (Peifer et al., 1994b). By the late 1990's it was well established that WNT4 signaling was required for early embryo

ovarian development (Vainio et al., 1999), and subsequent research would target WNTs and their downstream pathway components such as β-catenin in the adult female gonad.

4.3 Transcriptionally Active β-catenin

β-catenin is tightly regulated by WNT signaling and WNT-independent molecules that bind β-catenin to mediate its location and function. Recent investigations are focused on how these molecules direct β-catenin to function as a co-transcription factor in granulosa cells. Of the molecules identified to associate with β-catenin are TCF, CREB binding protein (CBP), steroidogenic factor 1 (SF-1), and Forkhead box protein O (FOXO) which are crucial in mediating ovarian estradiol production. Current and future studies are exploring the role of β-catenin as a signaling protein and a co-activator of transcription.

For β-catenin to be transcriptionally active it must be present in the nucleus and associate with co-transcription factors. The mechanism of β-catenin nuclear import is unclear as it does not contain a nuclear localization sequence and therefore does not translocate to the nucleus using the traditional import pathways (Fagotto et al., 1998). Others have suggested the type of β-catenin interacting protein will retain its location either within the cytoplasm or nucleus (Krieghoff et al., 2006).

Members of the TCF family are a family of transcription factors that share the same DNA-binding domain more often referred to as a high mobility group. A mutation in the N-terminal region of TCF (the site that interacts with β-catenin) suppressed β-catenin induced axis duplication in *Xenopus* (Molenaar et al., 1996). Later, the NH_2-

terminal region of TCF, specifically amino acids 1-90 was identified to interact with repeat 3-8 of Armadillo (van de Wetering et al., 1997). T-cell factor will bind to a conserved DNA binding sequence (A/T)(A/T)CAA(A/T)G within promoters (van Beest et al., 2000) and association of TCF with β-catenin will regulate transcription of the target gene (Huber et al., 1996). In the absence of β-catenin, TCF binds members of the Groucho family and this complex represses transcription however, WNT stimulation activates β-catenin which displaces Groucho and binds TCF (Daniels and Weis, 2005).

To aid in the expression of target genes, CBP has histone acetyltransferase activity that allows for transcription by loosening chromatin (Ogryzko et al., 1996). It has been demonstrated using a yeast two-hybrid that CBP physically interacts with repeat 10 on the COOH terminus of β-catenin (Takemaru and Moon, 2000). When HeLa cells were co-expressed with stable β-catenin and CBP, stimulation of the TOPflash promoter was significantly enhanced. Conversely, in *drosophila* CBP has a negative impact on TCF through the acetylation of the β-catenin binding domain on TCF and consequently lowering the interaction of β-catenin with TCF (Waltzer and Bienz, 1998).

Steroidogenic factor-1 is a 53 kD orphan receptor member of the steroid hormone nuclear receptor family (Morohashi et al., 1992) and functions as a transcriptional regulator protein that interacts with the promoter sequence (C/A)AGGTCA (Lala et al., 1992). This sequence site is present on the promoters of all P450 steroidogenic enzymes demonstrating it is essential for steroid synthesis (Morohashi et al., 1992). Armadillo repeats 9-12 of β-catenin associates with amino acids 235-238 of SF-1 to activate transcription of target genes and is independent of TCF (Gummow et al., 2003; Mizusaki et al., 2003).

Transcriptional activity of FOXO transcription factors is enhanced by binding β-catenin (Essers et al., 2005). The interaction occurs on the armadillo repeats 1-8 of β-catenin with the COOH-terminal half of FOXO. In granulosa cells, FOXO1 is a negative regulator of FSH induced proliferation and differentiation (Park et al., 2005). Interestingly, oxidative stress induces association of FOXO and β-catenin thus diverting its binding away from TCF (Essers et al., 2005). β-catenin is highly regulated and contributes to expression of target genes by serving as a co-transcription factor and in granulosa cells is required for estradiol production.

4.4 β-catenin in Granulosa Cells

Granulosa cell function depends heavily on β-catenin signaling. Follicle-stimulating hormone regulation of β-catenin was confirmed when knockdown of endogenous β-catenin protein by siRNA in granulosa cells attenuated FSH and forskolin-mediated *Cyp19a1* luciferase activity (Parakh et al., 2006). Additionally, this study reported through chromatin immunoprecipitation assay and specific SF-1 mutations that β-catenin associates with SF-1 and is an essential transcriptional regulator of *Cyp19a1*. In vivo deletion of β-catenin mediated by *Amhr2cre* mice had no effect on ovarian morphology and serum estradiol concentrations were in the normal range likely because CRE-mediated recombination is unstable in proliferating granulosa cells (Hernandez Gifford et al., 2009). In vitro recombination of β-catenin in granulosa cells isolated from mice homozygous for floxed β-catenin alleles (*Ctnnb1*$^{fl/fl}$) was performed and granulosa cells deficient for β-catenin had reduced FSH-mediated *Cyp19a1* expression and media

estradiol concentrations. When β-catenin expression was specifically depleted in only granulosa cells by using *Cyp19cre* mice mated to mice *Ctnnb1*$^{fl/fl}$ alleles again there were no defects in follicular development, ovulation, or corpus luteum formation indicating β-catenin depleted granulosa cell does not cause any overt abnormalities in ovarian morphology (Fan et al., 2010). Cultured granulosa cells from mice in this study exhibited a decreased response to FSH which was demonstrated by a loss of FSH-mediated *Cyp19a1* and *Fshr* mRNA expression and TCF transcriptional activity. Conversely, β-catenin depleted granulosa cells had enhanced *Lhcgr*, *Star*, *Cyp11a1*, and *Sfrp4* in response to the LH mimetic treatment (forskolin/phorbol-12-myristate 13-acetate) (Fan et al., 2010). β-catenin knockdown by siRNA in granulosa cells inhibited DNA synthesis as demonstrated by a reduced proportion of cells in the S phase when compared with negative siRNA-treated cells (Wang et al., 2010). Overexpression of β-catenin specific to granulosa cells was achieved by mating *Ctnnb1*$^{(EX3)fl/fl}$ mice to *Cyp19cre* mice which resulted in stabilized β-catenin as phosphorylation and degradation occurs on the β-catenin exon 3 translated product. Constitutively active β-catenin resulted in increased follicle number and reduced apoptosis (Fan et al., 2010) and in response to FSH (100 ng/mL) in culture. Additionally, granulosa cells had increased *Cyp19a1* and *Fshr* expression compared with vehicle controls. However, FSH did not induce *Lhcgr* expression, suggesting β-catenin functions to enhance FSH signaling and blocks luteinization in granulosa cells. Due to the lack of LH responsiveness these mice had reduced expression of genes for ovulation and luteinization and therefore were subfertile despite a greater number of follicles.

Total β-catenin protein abundance in large follicles is hormone dependent, as granulosa cells of large estrogen active (≥ 25 ng/mL) follicles have more β-catenin compared with low estrogen (< 7.5 ng/mL) large follicles (Castañon et al., 2012). Likewise, FSH-treated granulosa cells had greater accumulation of β-catenin, further demonstrating its requirement for normal granulosa cell function.

The drug to treat bipolar disorder, LiCl has been shown to have undesirable reproductive effects in females which resulted in reduced fertility. Rats injected with LiCl had reduced ovarian active β-catenin (not targeted for degradation) and subsequently increased follicular atresia and decreased serum estradiol concentrations (Mirakhori et al., 2013). These data demonstrate β-catenin is required for healthy follicle development and optimal estradiol production, whereas unregulated β-catenin concentrations can lead to detrimental effects in the ovary (Boerboom et al., 2005).

4.5 β-catenin and Ovarian Granulosa Cell Tumors

In females, ovarian cancer is the fifth leading cause of death from cancer in the United States. In 2015, there was an estimated 21,290 new cases and 14,180 estimated deaths from this disease (Siegel et al., 2015). Reports have described point mutations in the β-catenin gene at GSK-3β phosphorylation sites have been detected in ovarian carcinomas (Palacios and Gamallo, 1998; Sagae et al., 1999). Transgenic mice (*Ctnnb1$^{(EX3)fl/fl}$; Amhr2cre)* expressing stabilized β-catenin in the ovaries developed follicle-like lesions that in 57% of the mice developed into granulosa cell tumors by 7.5 months of age (Boerboom et al., 2005). Additionally, β-catenin was detected by

immunohistochemistry to be localized to the nucleus in the majority of ovarian granulosa cell tumors, suggesting that β-catenin hyperactivity is associated with tumor development. More recently, female mice transgenically altered to obtain constitutively active PI3K/AKT and β-catenin signaling developed aggressive early-onset metastatic granulosa cell tumors (Lague et al., 2008). The evidence supporting the role of β-catenin in granulosa cell tumors is strong yet the molecular mechanisms and signal overlap remain unresolved. By characterizing β-catenin and its downstream effectors in normal granulosa cell we can eventually understand its actions when it is deregulated.

5. Conclusion and Specific Aims

The ovary is the female gonad responsible for reproductive competence. Its ability to allow for propagation of the species lies in the release of one or a few gametes during the reproductive cycle. The cycle is regulated by the ovary itself and its interaction with the hypothalamus and pituitary. Estradiol from the ovary plays a prominent role in reproduction as it stimulates the development of the reproductive tract, initiates the onset of puberty, aids in the development of secondary sex characteristics, and contributes to folliculogenesis. Understanding the complexity of the ovary as it pertains to estradiol production is a crucial component of reproduction. The mediators of β-catenin and its roles in normal granulosa cell function and estradiol synthesis remains to be fully elucidated.

The **first study** (chapter 3) of this dissertation was designed to identify the requirement of AKT as a mediator of β-catenin and subsequent estradiol production in

the bovine. Experiments were carried out to investigate whether AKT regulation of β-catenin was required for FSH-mediated estradiol production. To accomplish this, AKT activity was stimulated or inhibited utilizing specific pathway regulators and β-catenin protein and estradiol medium concentrations were quantified.

Previously, we have demonstrated that WNT3A is inhibitory on FSH-mediated *Cyp19a1*, *Star*, *Cyp11a*, *Lhcgr*, and *Inha* mRNA expression and estradiol medium concentrations in rat granulosa cell cultures (Stapp et al., 2014b). This data suggest WNT3A may be critical in keeping estradiol production from going unregulated. Thus, the **second study** (chapter 4) was two-fold: 1) identify if IGF-I rescues FSH signaling from the inhibitory effects of WNT3A and 2) investigate if the mechanism by which WNT3A is inhibitory is through a modulation of the phosphorylation pattern of β-catenin and FOXO.

It has been demonstrated that reduction of β-catenin in granulosa cells prevents TOPflash luciferase activity and reduces FSH-mediated *Cyp19a1* expression (Fan et al., 2010). However, whether or not β-catenin and TCF directly interact to mediate *Cyp19a1* expression has not been demonstrated. The third and **final study** (chapter 5) utilized novel small-molecule inhibitors that prevent β-catenin/TCF association (Gonsalves et al., 2011) to test the hypothesis that β-catenin associates with TCF to regulate *Cyp19a1*.

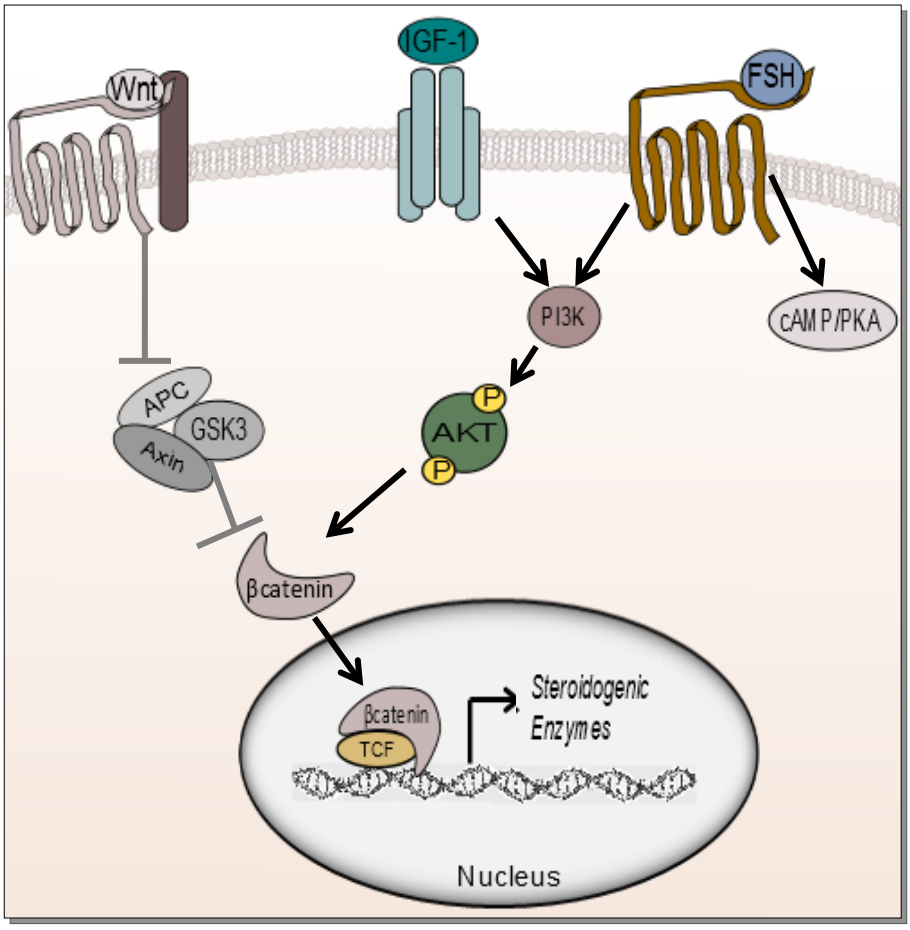

IGF-1
Wnt
FSH
PI3K
cAMP/PKA
APC
GSK3
Axin
AKT
P
P
βcatenin
βcatenin
TCF
Steroidogenic
Enzymes
Nucleus

FIGURE 1

Figure 1: FSH, IGF-I, and WNT Extracellular Signaling Molecules Converge at β-catenin.

Model demonstrates the intracellular signaling overlap of the WNT/β-catenin (Canonical), IGF-I, and FSH pathways. Binding of FSH and IGF-I with their respective receptors activate phosphatidylinositol-3 kinase (PI3K) which phosphorylates protein kinase B (AKT) on Thr-308 and Ser-473. The activation of AKT subsequently leads to β-catenin accumulation which will translocate to the nucleus and initiate transcription of genes required for estradiol biosynthesis by association with T-cell factor (TCF). The intracellular signaling cascade elicited by WNT prevents phosphorylation of β-catenin on Thr-41, Ser-37, and Ser-33 by the degradation complex comprised of Axin 1, adenomatous polyposis coli (APC), and glycogen synthase kinase-3β (GSK-3 β). β-catenin not targeted for degradation will accumulate in the cytoplasm and regulate gene expression of target genes.

CHAPTER III

PROTEIN KINASE B IS REQUIRED FOR FOLLICLE-STIMULATING HORMONE MEDIATED BETA-CATENIN ACCUMULATION AND ESTRADIOL PRODUCTION IN GRANULOSA CELLS OF CATTLE

B. I. Gomez, C. A. Gifford, D. M. Hallford, and J. A. Hernandez Gifford

This chapter is published in the Animal Reproduction Science (2015) 163:97–104 and appears in this dissertation with the permission of the journal. The author was involved in all research and writing aspects of this article.

1. Abstract

Follicle-stimulating hormone regulation of ovarian estradiol production requires involvement of β-catenin, a transcriptional co-factor. In cultured granulosa cells of cattle, FSH treatment increased protein abundance of β-catenin as well as protein kinase B (AKT), a molecule known to regulate components of the β-catenin degradation complex. However, whether FSH induction of β-catenin is through direct modulation of AKT remains to be determined. To investigate specific contributions of AKT to β-catenin accumulation, granulosa cells were treated with insulin-like growth factor-I (IGF-I), a well-established AKT activator, in the presence or absence of FSH. Granulosa cells treated with FSH, IGF-I, and IGF-I plus FSH had increased β-catenin accumulation compared with controls ($P \leq 0.02$; $n = 6$). Estradiol medium concentrations were greater ($P = 0.09$; $n = 4$) in FSH treated cells compared to controls (166 and 100 ± 28 pg/mL, respectively). Treatment with IGF-I and IGF-I plus FSH increased ($P < 0.01$) estradiol to comparable concentrations. Subsequently, granulosa cells treated with lithium chloride (LiCl), a pharmacological activator of AKT, provided a response consistent with IGF-I treated cells, as LiCl, FSH, and FSH plus LiCl increased β-catenin accumulation compared with non-treated controls ($P \leq 0.03$; $n = 3$). In contrast, inhibition of AKT signaling with LY294002 suppressed the ability of FSH and IGF-I to regulate β-catenin. Additionally, LY294002 treatment reduced FSH and IGF-I mediated estradiol medium concentrations ($P \leq 0.004$). These results demonstrate activation of AKT is required for gonadotropin regulation of β-catenin accumulation and subsequent ovarian estradiol production.

2. Introduction

The ovary is a dynamic reproductive organ that undergoes numerous changes during follicular maturation. Ovarian follicles develop in response to endocrine regulation by the hypothalamic-pituitary-gonadal axis, and several intra-ovarian factors. Pituitary derived FSH signals via a G-protein coupled receptor to initiate cAMP/protein kinase A (PKA) activity as well as induce additional intracellular signaling pathways to regulate granulosa cell granulosa cell target genes involved in proliferation, maturation, and estradiol synthesis (Hunzicker-Dunn and Maizels, 2006). In particular, phosphoinoside 3-kinase (PI3K) is an essential component of FSH signaling which leads to phosphorylation and activation of protein kinase B (PKB/AKT) (Richards et al., 2002; Gloaguen et al., 2011). Furthermore, FSH-treated rat granulosa cells transfected with a dominant negative AKT vector fail to induce aromatase (*Cyp19a1*) mRNA and estradiol production (Zeleznik et al., 2003). Additionally, binding of locally secreted insulin-like growth factor I (IGF-I) to tyrosine kinase receptors results in receptor auto-phosphorylation which ultimately leads to PI3K activation. Through a series of signaling cascades resulting from PI3K, AKT is phosphorylated on Thr-308 and Ser-473 which is important for its kinase activity (Alessi et al., 1996; Nicholson and Anderson, 2002).

Recent data indicate that FSH induction of β-catenin may be by a direct effect of increased AKT activity. Castañon et al. (2012) noted FSH-treated granulosa cells of cattle had increased protein accumulation of β-catenin and a tendency for an increase in AKT compared with non-treated controls. Therefore, the objective of the present study was to determine if FSH increased β-catenin accumulation in granulosa cells of cattle via activation of AKT. To accomplish this, AKT signaling was modulated using specific

pathway activators and inhibitors. We report that AKT activity is required for FSH and IGF-I induction of β-catenin accumulation necessary in the regulation of estradiol production from granulosa cells.

3. Materials and methods

3.1. Tissue collection

Cattle ovaries were collected at a local federally inspected abattoir (Creekstone Farms, Arkansas City, KS) from non-pregnant cows and heifers not exposed to growth-promoting implants, ionophores, or antibiotics. For each biological replicate, paired ovaries were collected from 18 females and placed in individually marked whirl packs. A minimum of three biological replicates were performed for each experiment.

Ovaries at random stages of the estrous cycle were rinsed with 0.9% saline, followed by a second rinse with 70% ethanol and placed in an ice cold antibiotic saline solution containing 0.15 M NaCl with 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen, Grand Island, NY) for transport to the laboratory. Upon arrival, ovaries were transferred into chilled fresh antibiotic saline solution.

3.2. Granulosa cell culture

Granulosa cells from small follicles (1 to 5 mm) were isolated by follicular fluid aspiration using a 3-mL syringe fitted with a 20-gauge needle. Follicle size was selected

based on previous observations demonstrating that 1) recruitment occurs when follicle diameter reaches 4 to 6 mm in cattle (Savio et al., 1988; Sirois and Fortune, 1988); 2) granulosa cells acquire FSH receptors prior to recruitment (Xu et al., 1995; Evans and Fortune, 1997); and 3) granulosa cell steroidogenic enzyme mRNA biosynthesis occurs before biosynthesis of LH receptor mRNA in recruited follicles (Bao et al., 1997). Granulosa cells were cultured using methods previously described (Castañon et al., 2012). Briefly, granulosa cells and follicular fluid was centrifuged at 220 x g for 7 min at 4°C, supernatant was removed and cells were washed twice in short-term media (1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 containing 0.12 mM gentamycin, 2.0 mM glutamine, and 38.5 mM sodium bicarbonate obtained from Sigma-Aldrich, St. Louis, MO). Following the final wash, cells were suspended in 5 mL of re-suspension media (serum-free medium with 2.5 mg/mL collagenase and 1 mg/mL DNase, Sigma-Aldrich) to prevent clumping, and cell number and viability was determined by trypan blue exclusion using a hemocytometer. Cells were seeded at a density of 1.2×10^6 per 60 mm dish in complete medium (1:1 DMEM and Ham's F-12 containing 10% fetal bovine serum, 0.12 mM gentamycin, 2.0 mM glutamine, and 38.5 mM sodium bicarbonate) and allowed to reach 60-75% confluence (30-50 h) at 38.5°C and 5% CO_2, 95% air before treatment.

Complete medium was removed and cells were rinsed with PBS prior to the addition of treatments which were conducted in serum-free medium supplemented with 10^{-7} M testosterone propionate (Sigma-Aldrich) for 24 h at 38.5°C and 5% CO_2. To activate the AKT signaling pathway, cells were treated with 50 ng/mL IGF-I (Sigma-Aldrich) or 20 mM of lithium chloride (LiCl; EMD Chemicals, San Diego, CA)

concurrently in the presence or absence of 100 ng/mL of purified human FSH (S1AFP-B-3; National Hormone and Peptide Program, National Institutes of Diabetes, Digestive, and Kidney Diseases, National Institutes of Health, Bethesda, MD). Insulin-like growth factor-I concentration was selected based on a preliminary concentration dependent study (Fig. 7) demonstrating the ability to regulate β-catenin, and literature demonstrating 50 ng/mL of IGF-I was the minimal concentration capable of inducing *CYP19A1* gene expression and estradiol production (Mani et al., 2010), whereas LiCl at 20 mM induces β-catenin in oligodendroglial cells (Ye et al., 2010). Inactivation of AKT signaling was achieved by a 30 min pre-treatment of granulosa cells with 30 μM LY294002 (Sigma-Aldrich), a potent inhibitor of PI3K which subsequently decreases AKT activity (Vlahos et al., 1994), or dimethyl sulfoxide (DMSO) control. Following pre-treatment for AKT inhibition, FSH (100 ng/mL), IGF-I (50 ng/mL), or vehicle control was added to the media.

At the conclusion of the 24 h treatment period, experiments were terminated by aspirating media and rinsing with PBS. Media was collected from individual dishes for steroid analysis and granulosa cells were collected into 200 μL of mammalian-protein extraction reagent (M-PER) lysate buffer (Thermo-Scientific, Rockford, IL) and samples were stored at -20°C until analysis.

3.3. Western blot

Total protein was quantified using the bicinchoninic acid (BCA) protein assay kit according to the manufacturer's protocol (Thermo-Scientific). Protein lysates (5 μg)

were separated by 10% SDS-PAGE Tris-HCL gels and transferred to Hybond-C Extra nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 before antibody incubation at room temperature for 1 h. Membranes were incubated overnight at 4 °C in primary rabbit anti-β-actin (1:10,000; Cell Signaling Technology (CST), Danvers, MA) or rabbit anti-GAPDH (1:3,000; CST) to account for equal loading. Following primary antibody incubation, membranes were rocked at room temperature for 1 h with horseradish peroxides-conjugated (HPR) goat anti-rabbit (Thermo-Scientific). For detection of β-catenin, membranes were incubated at 4 °C overnight in mouse anti-β-catenin (1:10,000; BD Transduction Laboratories, San Diego, CA) followed by a 1 h incubation in HRP conjugated goat anti-mouse at 1:10,000 (Thermo-Scientific). Membranes were incubated at 4 °C overnight in rabbit anti-AKT or anti-phospho-AKT Ser-473 (1:1,000; CST) followed by a 1 h incubation in HRP conjugated goat anti-rabbit at 1:3,000 (Thermo-Scientific) for detection of AKT and phospho-AKT. Antigen-antibody complexes were detected using chemiluminescence with Immobilon detection substrate reagent (Millipore, Billerica, MA) and images were captured using the C-DiGit Blot Scanner (LI-COR, Lincoln, NE). All treatments for a single experiment are represented on the same blot. Band intensity and relative abundance of proteins in relation to loading controls was quantified using Image Studio™ Software (LI-COR). When appropriate, phospho-AKT was normalized to total AKT.

3.4 Radioimmunoassay

Granulosa cell culture media were analyzed for estradiol by solid phase RIA using components of commercial kits manufactured by Siemens Medical Diagnostics Corp. (Los Angeles, CA) as previously described (Castañon et al., 2012). Briefly, 200 μL of culture media was used to determine the estradiol concentration. All tubes were normalized to 0.5 mL using assay buffer and assayed in duplicate. Each tube received 1.0 mL of Siemens tracer, vortexed and incubated at room temperature for 24 h. Tubes were decanted and counted for 1 min in a Packard Cobra II gamma counter. The specific binding was 71%. Detection limit (95% of maximum binding) of the assay was 2 pg/mL and intra-assay CV was 6.8%.

3.5 Statistical analysis

Experiments were conducted at minimum in 3 independent replicates. For estradiol concentrations, generalized linear mixed models methods were used to analyze the data, accounting for non-normal responses and unequal variances where necessary. All statistical analyses were performed using SAS (Version 9.3; SAS Institute, Inc., Cary, NC). The estradiol concentration and protein abundance were analyzed using ANOVA methods and least squares means comparisons between treatments were performed only when the model was significant to determine differences among treatments. For Western blot, quantitative analysis is presented with the protein of interest expressed as a percentage of control and a t-test was performed to compare individual treatments to control by comparing each treatment mean to 100%.

4. Results and discussion

A requirement of β-catenin as a key transcriptional co-factor in gonadotropin regulation of steroidogenesis has brought new perspectives to granulosa cell signal transduction. Previous data suggest β-catenin is hormonally regulated in developing cattle follicles, as relative amounts of β-catenin protein increase in large follicles producing greater amounts of estradiol likely in response to endogenous FSH (Castañon et al., 2012). Likewise, β-catenin increased and total AKT protein abundance tended to increase in primary bovine granulosa cells incubated with FSH further indicating a role for β-catenin in follicle development possibly regulated through the AKT signaling pathway (Castañon et al., 2012). Similarly, granulosa cells of cattle cultured in the presence of FSH have increased amounts of total and active AKT (Ryan et al., 2008). Together, these findings suggest that FSH stimulation of AKT may participate in mediating β-catenin and estradiol biosynthesis in granulosa cells of cattle.

4.1. Activation of AKT induces β-catenin accumulation

To examine the ability of AKT signaling to contribute to β-catenin accumulation in granulosa cells of cattle, known pathway stimulators including FSH, IGF-I and LiCl were utilized. Although FSH signaling occurs primarily through cAMP second messenger systems, additional FSH intracellular pathways including AKT are obligatory for granulosa cell function (Zeleznik et al., 2003). Moreover, IGF-I is a known regulator of AKT in granulosa cells of cattle, as evidenced by increased phosphorylation of AKT (Mani et al., 2010). In the present study (Fig. 2A and B), treatment with all AKT

stimulators increased the amount of β-catenin protein to a similar abundance ($P \geq 0.87$; n = 6). The granulosa cells treated for 24 h with FSH had increased relative amounts of β-catenin protein compared with vehicle controls ($P = 0.02$). Likewise, greater amounts of β-catenin protein were detected in granulosa cells treated with IGF-I alone and in cells co-treated with IGF-I in combination with FSH compared with controls ($P \leq 0.02$).

In a separate experiment, primary granulosa cells of cattle were treated with LiCl, a pharmacological agent capable of activating the AKT signaling pathway (Chalecka-Franaszek and Chuang, 1999), in the presence or absence of FSH (Fig. 2C and D). Specifically, LiCl enhances AKT by disrupting the signaling complex comprised of AKT, beta-arrestin 2, and protein phosphatase 2A (Brown and Tracy, 2013). Similar to the previous experiment, Western blot analysis indicated that β-catenin accumulation was consistently greater in granulosa cells treated with FSH and LiCl ($P \leq 0.03$; n = 3) when compared with controls. Co-incubation of FSH and LiCl also increased ($P = 0.004$) abundance of β-catenin above controls in amounts that paralleled each treatment independently. The ability of AKT to be mediated by both FSH and IGF-I suggests a signaling overlap downstream of FSH and IGF-I receptor, as demonstrated in granulosa cells of cattle where co-treatment with FSH and IGF-I increased relative amounts of (*CYP19A1*) mRNA above FSH or IGF-I alone (Mani et al., 2010). Insulin-like growth factor I and FSH are important for normal ovarian function and reduction in function of either gene or the respective receptors results in infertility (Baker et al., 1996; Abel et al., 2000). Additionally, IGF-I signals through the PI3K/AKT pathway and synergizes with FSH to increase steroidogenic output (Zhou et al., 2013). It is possible that both

pathways function collectively to activate AKT which in turn leads to regulation of β-catenin and subsequent steroid production.

β-catenin is essential in FSH-mediated steroid production through its co-transcriptional properties. Depleting endogenous β-catenin compromises maximal induction of FSH-regulated *Cyp19a1* mRNA biosynthesis (Parakh et al., 2006) and estradiol production (Hernandez Gifford et al., 2009). To determine whether AKT stimulation of β-catenin also contributed to regulation of granulosa cell steroid production, media concentrations of estradiol were evaluated. Following FSH treatment, media concentrations of estradiol tended to increase ($P = 0.09$; n = 4) when compared with non-treated controls (166 ± 28 vs. 100 ± 14 pg/mL respectively) (Fig. 3). The granulosa cells stimulated with IGF-I had increased estradiol concentrations to 380 ± 33 pg/mL; similar to cells co-incubated with IGF-I and FSH which also had increased estradiol to 398 ± 41 pg/mL when compared with vehicle-treated controls ($P < 0.01$). The actions of FSH in inducing increased amounts of estradiol is affected by numerous factors including animal variation in age, breed and FSH sensitivity (Breuel et al., 1991) as well as exposure to exogenous hormones (Stapp et al., 2014). Mani et al. (2010) did not demonstrate an FSH induced increase of estradiol in cultured granulosa cells of cattle; however, other previous research has established FSH-mediated estradiol increases in granulosa cells of cattle and rats (Castañon et al., 2012; Stapp et al., 2014). In agreement with Mani et al. (2010), IGF-I alone or in combination with FSH significantly enhanced estradiol production but a synergistic effect of IGF-I and FSH on estradiol production was not detected. Supporting a role for IGF-I in steroidogenesis, Mani et al. (2010) highlighted the ability of IGF-I to up-regulate steroidogenic activity of genes essential for

promoting the conversion of cholesterol to estradiol via PI3K/AKT. Accumulating evidence indicates circulating FSH and locally produced IGF-I increases granulosa cell estradiol production by enhancing AKT activity. However, this finding is extended in the present research by demonstrating that AKT mediates relative amounts of β-catenin, and the AKT/ β-catenin pathway is important in estradiol production in granulosa cells of cattle.

4.2. Effects of inhibition of AKT activity

To test whether inhibition of AKT activity impacted β-catenin abundance in granulosa cells of cattle, the PI3K-family inhibitor LY294002 was used (n = 3). Inactivation of AKT signaling was assessed by evaluating phospho-AKT/total AKT ratios (Fig. 4A). A reduction in phospho-AKT/AKT was confirmed in granulosa cells treated with LY294002 alone or co-incubated with FSH when compared with DMSO vehicle controls, demonstrating inactivation of the pathway ($P \leq 0.001$). Differences were not detected between AKT ratios of control and FSH treated cells ($P = 0.23$), likely a result of the 24 h time point. Additionally, total amounts AKT did not differ among treatment groups treated with LY294002 in the presence or absence of FSH ($P = 0.18$). Treatment with FSH consistently increased amounts of β-catenin protein ($P < 0.01$) compared with controls as demonstrated in earlier experiments (Fig. 4C and D). However, constraint of AKT signaling by LY294002 also abrogated the ability of FSH to mediate β-catenin abundance. The granulosa cells of cattle co-exposed to the AKT inhibitor and FSH or IGF-I (Fig. 4C and D; 5A) had reduced β-catenin compared with

cells treated with FSH or IGF-I alone. Inactivation of AKT in granulosa cells and consequent reduction in β-catenin suggests AKT activity is an essential component of FSH and IGF-I directed β-catenin protein accumulation. Because both FSH and IGF-I signal via AKT it is probable that these signaling molecules mediate β-catenin abundance in granulosa cells of cattle in part via AKT. LY294002 is inhibitory on PI3K and can also target additional kinases related and unrelated to the PI3K family (Gharbi et al., 2007) therefore, the possibility cannot be excluded that β-catenin accumulation is in part regulated by other kinases in addition to AKT. The likelihood of the indirect inhibitory effects of LY294002 is minimal based on results of the present study where three AKT activators (FSH, IGF-I, and LiCl) increased β-catenin accumulation and LY294002 reduced AKT phosphorylation.

The estradiol medium concentrations followed a similar pattern as demonstrated by β-catenin protein abundance. Treatment of granulosa cells with FSH resulted in increased ($P < 0.001$; n = 3) estradiol accumulation in cell culture media compared with non-treated controls, while cells treated with LY294002 had similar estradiol concentrations as non-treated controls (Fig. 4E). Co-incubation of granulosa cells with LY294002 plus FSH reduced FSH-mediated estradiol biosynthesis ($P = 0.002$). Similarly, LY294002 reduced ($P < 0.001$; n = 6) IGF-I mediated estradiol biosynthesis compared with IGF-I treatment (Fig. 5B). Treatment with LY294002 demonstrates AKT signaling is required for FSH and IGF-I mediated β-catenin accumulation. The ability of the AKT inhibitor to evoke a comparable change in estradiol concentrations in the presence of FSH as occurred with β-catenin protein is similar to reports by Ryan et al. (2007) demonstrating LY294002 reduces FSH and IGF-I induced estradiol biosynthesis

after 144 h in culture. While it is recognized that cyclic-AMP and its major effector, protein kinase A, are responsible for controlling the majority of the actions of FSH (Hunzicker-Dunn and Maizels, 2006) these data highlight the role of AKT activity in β-catenin mediated estradiol biosynthesis.

5. Conclusion

In summary, findings in the present study extend the knowledge regarding how FSH regulates β-catenin protein in granulosa cells of cattle. Previous data suggested canonical wingless-type mouse mammary tumor virus integration site 2 (WNT2) or AKT signaling as two possible mechanisms for β-catenin accumulation, although WNT2 was not directly tested and therefore, cannot be completely discounted; the present studies reveal the important role of AKT-mediated β-catenin regulation. A model is proposed whereby signaling molecules upstream of AKT can regulate β-catenin as depicted in Figure 6. Signaling by FSH and IGF-I activates AKT, a serine/threonine specific kinase, with multifunctional roles such as cell proliferation, protein synthesis, transcription and regulation of the deactivation of glycogen synthase kinase-3 (GSK3β) by Ser-9 phosphorylation (Alessi and Cohen, 1998; Testa and Tsichlis, 2005). Axin2 serves as a scaffold protein and forms a complex with adenomatous polyposis coli (APC) and GSK3β to regulate β-catenin cytoplasmic protein concentrations (Kishida et al., 1998). When associated as a component of a degradation complex, GSK3β will phosphorylate β-catenin at Thr-41, Ser-37, and Ser-33 which ultimately targets β-catenin for ubiquitination and subsequent degradation (MacDonald et al., 2009). Inactivation of

GSK3β allows β-catenin to escape the degradation complex and accumulate in the cytoplasm and translocate to the nucleus where it binds to TCF to regulate gene expression (Behrens et al., 1996; Eastman and Grosschedl, 1999). In the ovary, β-catenin regulates normal developmental and steroidogenic processes including maximal biosynthesis of FSH-stimulated *Cyp19a1* (Parakh et al., 2006) and estradiol production in granulosa cells (Hernandez Gifford et al., 2009).

Data herein support previous studies in which AKT was found to phosphorylate GSK3β and increase free β-catenin (Fukumoto et al., 2001); and where FSH activation of AKT results in GSK3β phosphorylation (Fan et al., 2010). Furthermore, in cultured oligodendrocytes β-catenin is increased following IGF-I induced AKT and GSK3β phosphorylation (Ye et al., 2010). Together these results suggest β-catenin is an important component of the PI3K/AKT/GSK3β pathway in IGF-I and FSH-mediated estradiol biosynthesis.

Acknowledgements

This research was supported by the Oklahoma Agric. Exp. Sta., Stillwater (OKL02789); and Oklahoma Center for the Advancement of Science and Technology (OCAST) grant HR10-030S to JAHG. The authors would like to thank Creekstone Farms (Arkansas City, KS) for generous donation of cattle ovaries. We express the deepest gratitude to Dr. Carla Goad for assisting with statistical evaluation of data. Appreciation is also expressed to the NIDDK's National Hormone and Peptide Program and A. F. Parlow for supplying the FSH reagent.

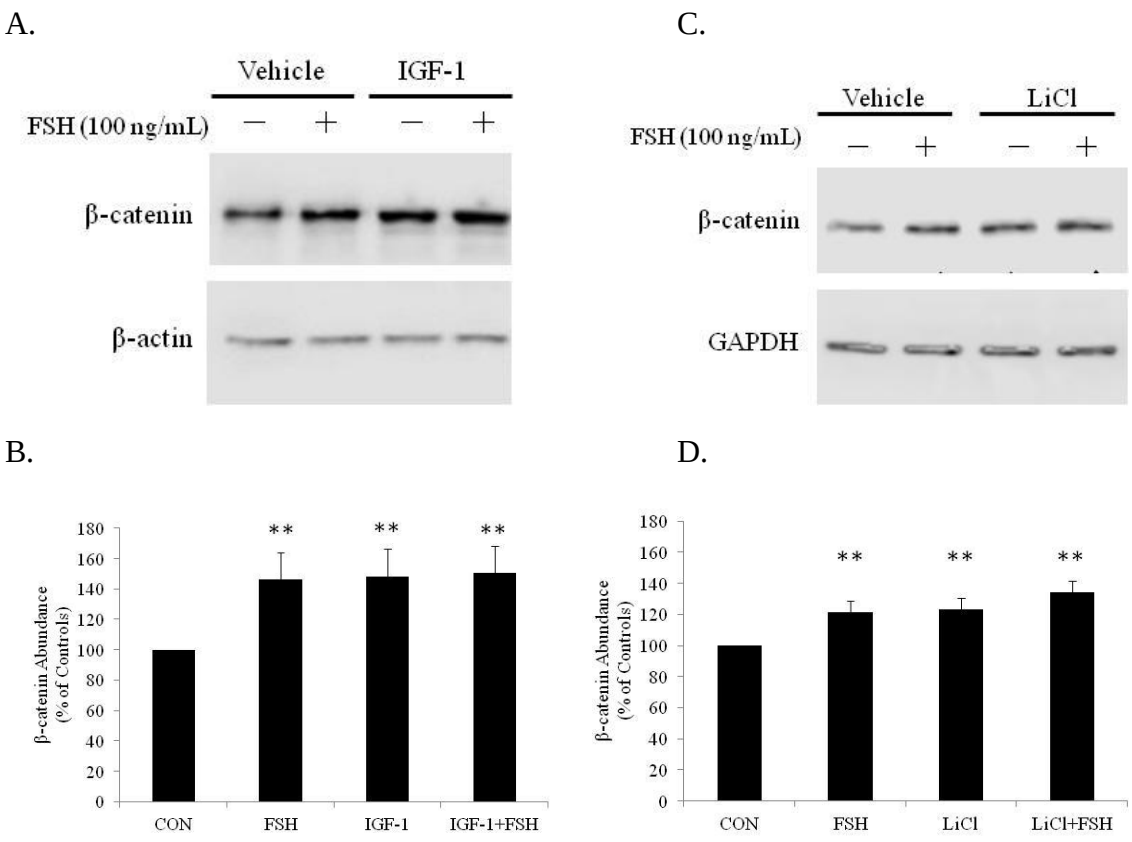

A.
Vehicle
IGF-1
FSH (100 ng/mL)
β-catenin
β-actin
C.
Vehicle
LiCl
FSH (100 ng/mL)
β-catenin
GAPDH
B.
β-catenin Abundance (% of Controls)
CON
FSH
IGF-1
IGF-1+FSH
D.
β-catenin Abundance (% of Controls)
CON
FSH
LiCl
LiCl+FSH

FIGURE 2

Figure 2: β-catenin Protein in Bovine Granulosa Cells Treated with the AKT Activators FSH, IGF-I, and LiCl.

Small follicle (1 to 5 mm) granulosa cells of cattle were isolated from bovine ovaries and cultured with AKT pathway stimulators IGF-I (50 ng/mL) or LiCl (20 mM) in the presence or absence of FSH (100 ng/mL) for 24 h. Effect of treatment on β-catenin accumulation was determined by Western blot analysis with β-actin or GAPDH serving as the loading control. (A and B) Representative Western blot and quantitative analysis of granulosa cells treated with IGF-I (n = 6). (C and D) Representative Western blot and quantitative analysis of bovine granulosa cells treated with LiCl (n = 3). Quantitative analysis of β-catenin abundance is expressed as a percentage of controls. An asterisk denotes level of significance when compared to controls (**$P < 0.05$). Least squares means ± SEM are presented.

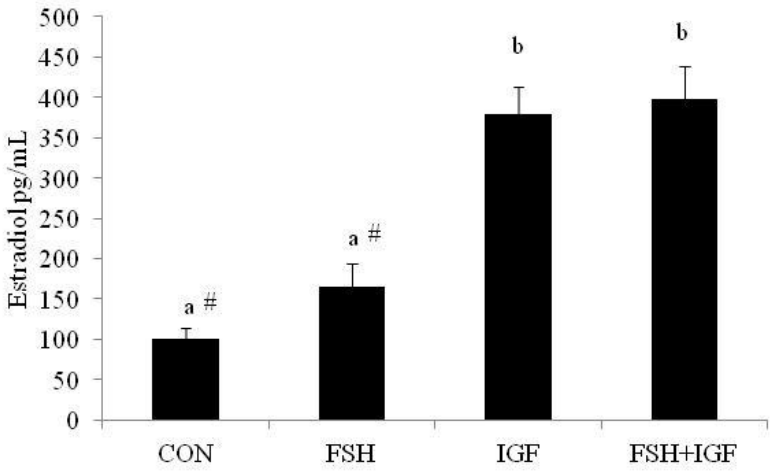
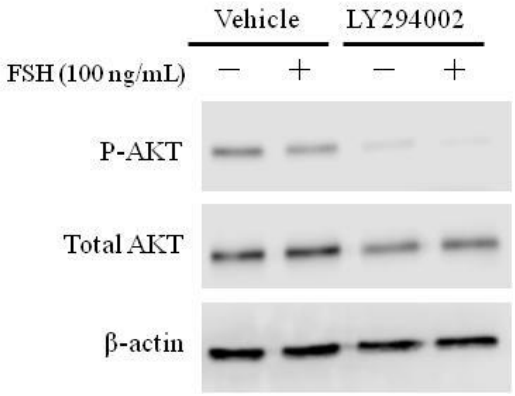

500
450
400
350
300
250
200
150
100
50
0
Estradiol pg/mL
a #
a #
b
b
CON
FSH
IGF
FSH+IGF

FIGURE 3

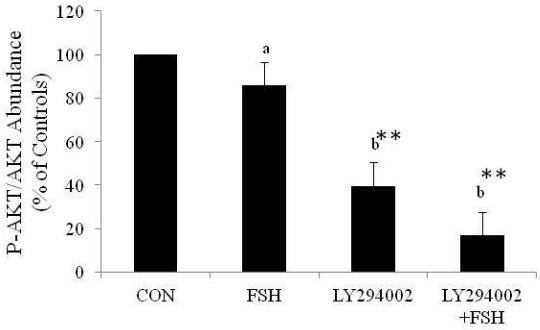
Figure 3: Estradiol Production in Bovine Granulosa Cells in Response to FSH and IGF-I.

Granulosa cells of cattle were collected from small follicles (1 to 5 mm) and cultured with IGF-I (50 ng/mL), FSH (100 ng/mL) or the combination to stimulate AKT for 24 h prior to media collection. Estradiol concentrations were analyzed by radioimmunoassay. Quantitative analysis is presented as least squares means ± SEM (n = 4). Bars without a common superscript differ ($P < 0.05$; n = 4) and a # indicates a tendency ($P = 0.09$) exist between CON and FSH.

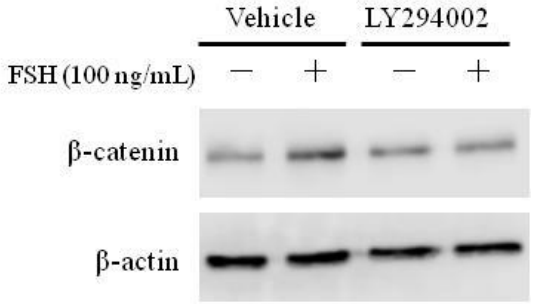
A.



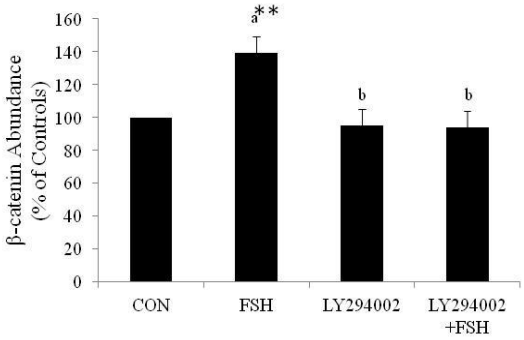
B.



C.



D.



E.

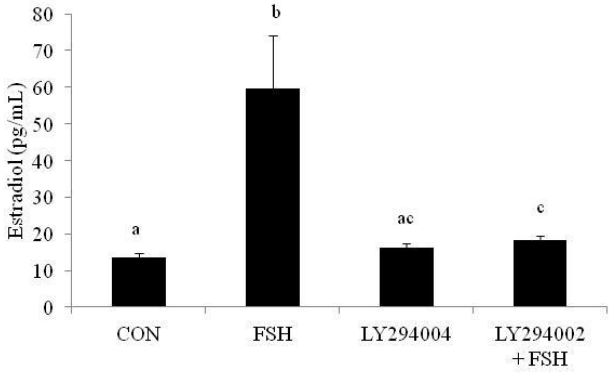
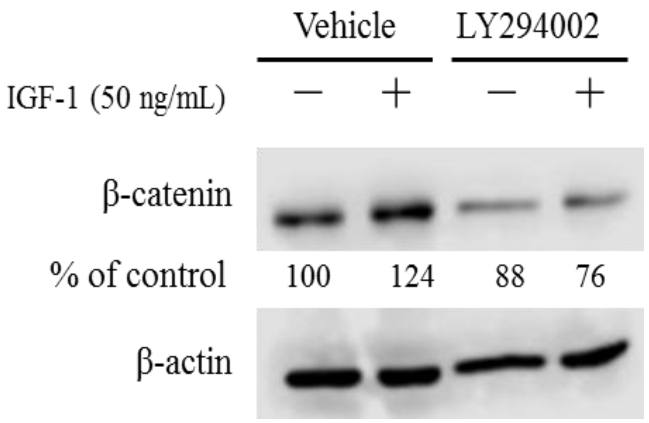


FIGURE 4

Figure 4: AKT Activity, β-catenin Accumulation, and Estradiol Production of Bovine Granulosa Cells Treated with LY294002 Prior to FSH Stimulation.

Granulosa cells of cattle isolated from small follicles (1 to 5 mm) were cultured and treated with AKT inhibitor LY294002 (30 μM) or vehicle for 30 min, prior to incubation in the presence or absence of FSH (100 ng/mL) for 24 h. Total protein was collected for Western blot analysis and β-actin was used as a loading control (n = 3; 2 technical replicates per experiment). Representative Western blot and quantitative analysis for P-AKT, total AKT (A and B), and β-catenin (C and D). Quantitative analysis is presented as least squares means ± SEM with the protein of interest expressed as a percentage of controls. Data are normalized to the expression of β-catenin in vehicle control. Bars without a common superscript differ ($P < 0.05$). An asterisk denotes level of significance when compared to controls (*$P < 0.10$; **$P < 0.05$).

A.


Vehicle
LY294002
IGF-1 (50 ng/mL)
− + − +
β-catenin
% of control
100 124 88 76
β-actin

B.

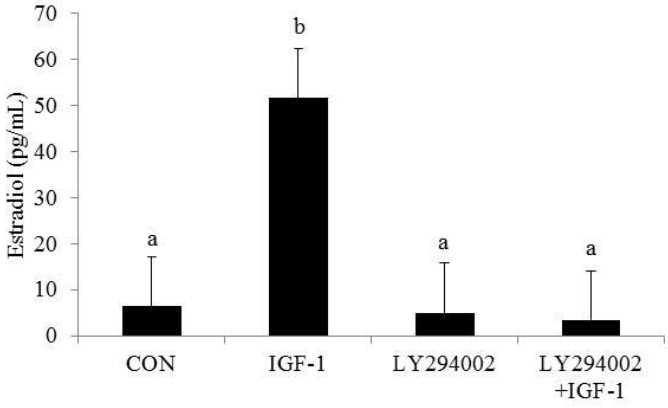

Estradiol (pg/mL)
70
60
50
40
30
20
10
0
a
b
a
a
CON
IGF-1
LY294002
LY294002 +IGF-1

FIGURE 5

Figure 5: Total β-catenin Accumulation in Bovine Granulosa Cells Treated with LY294002 Prior to IGF-I Stimulation.

Granulosa cells of cattle isolated from small follicles (1 to 5 mm) were cultured and treated with AKT inhibitor LY294002 (30 μM) or vehicle for 30 min, prior to incubation in the presence of IGF-I (50 ng/mL) for 24 h. A) Representative Western blot for beta-catenin with β-actin serving as a loading control. Numbers below demonstrate the change as a percent of control. B) Quantitative analysis of estradiol concentrations presented as least squares means ± SEM were analyzed by radioimmunoassay(n = 6). Bars without a common superscript differ ($P < 0.05$).

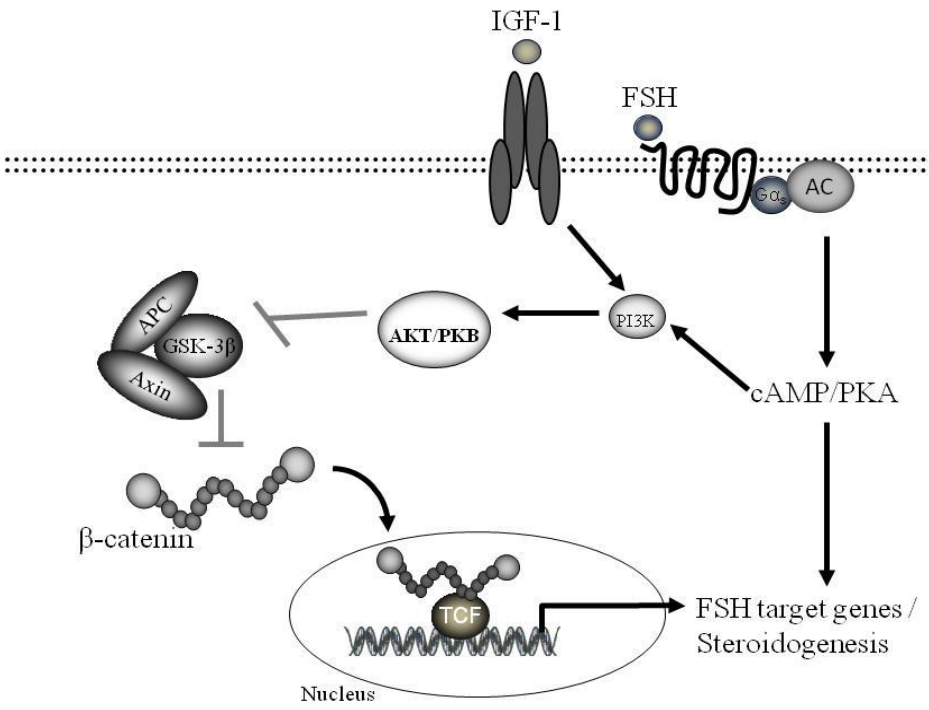

IGF-1
FSH
AC
APC
GSK-3β
Axin
AKT/PKB
PI3K
cAMP/PKA
β-catenin
TCF
FSH target genes /
Steroidogenesis
Nucleus

FIGURE 6

Figure 6: Model for FSH and IGF-I Regulation of β-catenin via AKT Signaling in Granulosa Cells.

Data suggest activation of protein kinase B (AKT) is a physiologically important transduction molecule that is necessary to block glycogen synthase kinase-3β (GSK3β) mediated degradation of β-catenin for subsequent activation of FSH target genes in the bovine. GSK3β is a component in a multi-protein degradation complex embedded along with adenomatous polyposis coli (APC) and the scaffold protein Axin that is responsible for regulating β-catenin accumulation. β-catenin is phosphorylated on Thr-41, Ser-37, and Ser-33 by GSK3β and subsequently degraded by the proteosome (MacDonald et al., 2009). Stimulation by FSH and IGF-I activates phosphoinositide 3-kinase (PI3K) which activates AKT by phosphorylation on Thr-308 and Ser-473 (Alessi et al., 1996; Nicholson and Anderson, 2002). Activate AKT will inhibit GSK3β by phosphorylation on Ser-9, allowing for accumulation of β-catenin in the cytoplasm which translocates to the nucleus (Alessi and Cohen, 1998; Testa and Tsichlis, 2005). Nuclear β-catenin then associates with transcription factor T-cell factor (TCF), to initiate the transcription of FSH target genes involved in steroidogenesis (Parakh et al., 2006; Hernandez Gifford et al., 2009).

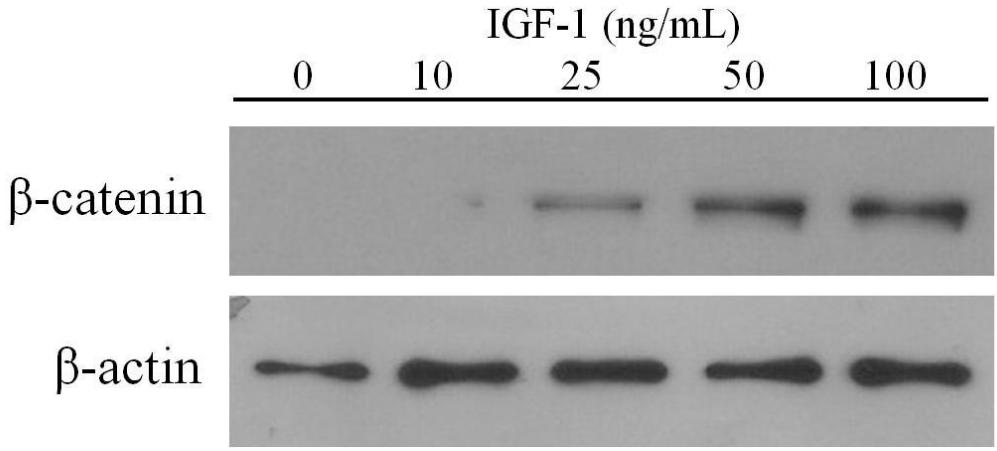


FIGURE 7

Figure 7: β-catenin Response to Increasing Concentrations of IGF-I in Bovine Granulosa Cells.

A preliminary study (n = 1) was performed to confirm that IGF-I was capable of inducing β-catenin at concentrations utilized in the literature. Small follicle bovine granulosa cells were isolated from bovine ovaries and treated with increasing concentrations of IGF-I (0, 10 25, 50, or 100 ng/mL) for 24h. Effect of treatment on β-catenin accumulation was determined by Western blot analysis with β-actin serving as the loading control.

CHAPTER IV

IGF-I ATTENUATES WNT INHIBITION ON FSH TARGET GENES AND ESTRADIOL PRODUCTION IN RODENT GRANULOSA CELLS

B. I. Gomez, C. A. Gifford, D. M. Hallford, and J. A. Hernandez Gifford

This chapter is published in the Western Section, American Society of Animal Science Proceedings, 2015, 66:63-66. Here it is presented with minor modifications and additional data. The author was involved in all research and writing aspects of this article.

1. Abstract

In livestock production, infertility is a major source of economic loss. Preovulatory estradiol biosynthesis relies on coordinated input from pituitary and intraovarian signaling pathways and impacts fertility of the ovulated follicle. In granulosa cells, canonical wingless-type mammary tumor virus integration-site 3A (WNT3A) signaling is inhibitory on FSH target genes and steroid biosynthesis indicating a role in regulation of follicle maturation and differentiation. Additionally, insulin-like growth factor I (IGF-I) contributes to estradiol production and dominant follicles contain greater concentrations of IGF-I and estradiol than their cohorts. The objective of this study was to investigate if IGF-I would overcome the inhibitory effects of WNT3A on FSH-mediated steroidogenesis. To determine the effects of IGF-I in this negative feedback system, primary cultures of rat granulosa cells were exposed to FSH (100 ng/mL) and WNT (50 ng/mL) with or without IGF-I (50 ng/mL) for 24 h (n = 3). Activation of an aromatase (*Cyp19a1)* type II promoter *(PII)*-luciferase reporter was achieved by treatment with FSH with or without IGF-I ($P < 0.001$). Inhibition of WNT3A on FSH-mediated *Cyp19a1 PII* activity was partially attenuated by the addition of IGF-I to the co-treatment paradigm ($P < 0.001$). Granulosa cells treated with FSH+WNT3A had lower estradiol concentrations than cells treated with FSH alone (113 vs 482 ± 92 pg/mL, respectively; $P = 0.01$), while addition of IGF-I in the presence of FSH+WNT3A tended to increase estradiol production (341 ± 92 pg/mL; $P = 0.10$). To identify the mechanism by which IGF-I suppress WNT3A inhibition on FSH activity, β-catenin and forkhead box protein 1 phosphorylation status and mRNA expression of *Axin2*, a negative regulator of WNT signaling, and was evaluated. Compared with

controls, FSH treatment promoted β-catenin phosphorylation at Ser-552 and Ser-675 irrespective of co-treatments ($P < 0.05$). Treatment with IGF-I did not modulate β-catenin phosphorylation at these specific C-terminal sites. Specific WNT pathway activation was demonstrated by up regulation of *Axin2* ($P < 0.05$), and the addition of FSH, or IGF-I alone or in combination with WNT3A regulated *Axin2* expression to similar levels. These data indicate that IGF-I contributes to FSH and WNT signaling in granulosa cells to mediate ovarian follicle maturation and estradiol production. Future studies are necessary to identify the mechanisms by which IGF-I is able to restore estradiol biosynthesis in the presence of WNT negative regulation on FSH signaling.

2. Introduction

Circulating estradiol concentrations prior to ovulation must remain elevated by the dominant follicle to prevent undesirable subordinate follicle maturation. Additionally, estradiol influences fertility as indicated by increased pregnancy rates in cows whose dominant follicle developed under a longer period of proestrus and increased estradiol (Bridges et al., 2010). Follicle maturation and estrogen synthesis is regulated by follicle-stimulating hormone (FSH), as well as intraovarian regulatory molecules including insulin-like growth factor I (IGF-I) (Adashi et al., 1985b) and wingless-type mammary tumor virus integration-site (WNT) (Boyer et al., 2010). In granulosa cells, FSH generates a cAMP-dependent signaling cascade to initiate transcription of cytochrome P450 enzyme aromatase (*Cyp19a1*) to catalyze the conversion of androgens to estrogens.

The estrogenic potential of granulosa cells can be positively modulated by IGF-I through its contributions to cell differentiation and proliferation (Zhou et al., 2013). Additionally, in cattle the largest dominant follicle has elevated IGF-I relative to the second largest (Beg et al., 2001). Recent data indicates that IGF-I and FSH activate protein kinase B (AKT) leading to β-catenin accumulation (Gomez et al., 2015; chapter 3 of dissertation), a transcriptional co-factor required for *Cyp19a1* mRNA accumulation and subsequent estrogen production (Parakh et al., 2006; Hernandez Gifford et al., 2009). Collectively, these data suggest that IGF-I and FSH pathways converge downstream of their receptors to promote normal granulosa cell function.

β-catenin activity is regulated by the canonical WNT signaling pathway in granulosa cells. Treatment with WNT3A inhibits FSH induction of *Cyp19a1* and subsequent estradiol production in granulosa cells (Stapp et al., 2014b). However, the mechanism by which WNT3A negatively regulates FSH signaling remains to be determined. In this study, we tested the hypothesis that IGF-I plays a role in rescuing FSH-mediated *Cyp19a1* activity and estradiol production from the inhibitory effects of WNT3A.

3. Materials and methods

3.1 Cell culture

Female Sprague-Dawley rats were purchased from Charles River Laboratories (Hollister, CA) and housed at Oklahoma State University in accordance with the Oklahoma State University Institutional Animal Care and Use Committee (AG-10-3).

Rat granulosa cells were isolated and cultured as previously described (Stapp et al., 2014) and seeded in 24-well culture plates at a density of 1.6 x 10^5 cells per well (reporter assay) or 5.4 x 10^5 per 60-mm tissue culture dish (mRNA analysis) in Dulbecco's Modified Eagle Medium/Ham's F-12 (Invitrogen, Carlsbad, CA) with 1% (vol/vol) 10,000 IU/mL penicillin/10,000 µg streptomycin/mL penicillin and streptomycin (DMEM/F12/PS) medium supplemented with 10% FBS.

3.2 Transfection and luciferase assay

Granulosa cells were transiently transfected with 10 ng/well p-HRC-B *Renilla* and 200 ng/well of *CYP19A1 PII* or empty luciferase reporter vector using Lipofectamine LTX and Plus (Invitrogen) reagent as previously described (Stapp et al., 2014). The following day, cells were treated with DMEM/F12/PS supplemented with 10^{-7} M testosterone propionate (Sigma-Aldrich, St. Louis, MO). Individual treatments included: 1) vehicle control, 2) 100 ng/mL FSH (S1AFP-B-3; National Hormone and Peptide Program, National Institutes of Diabetes, Digestive, and Kidney Diseases, National Institutes of Health, Bethesda, MD), 3) IGF-I (50 ng/mL; Invitrogen), 4) WNT3A (50 ng/mL; R&D Systems, Minneapolis, MN), 5) FSH+WNT3A, 6) IGF-I+WNT3A, 7) FSH+IGF-I, and 8) FSH+WNT3A+IGF-I. Following a 24 h incubation period in their respective treatments, protein lysates were collected and luciferase values were measured by the Dual-Luciferase Reporter Assay System kit (Promega, Madison, WI) according to manufacturer's protocol. Luciferase values were measured in duplicate by a single tube Modulus Luminometer (Turner BioSystems, Sunnyvale, CA).

3.3 Western blot

Five micrograms of total cell lysate collected from transfection experiments was separated by 10% SDS-PAGE Tris-HCl gels, and transferred to a nitrocellulose membrane (Invitrogen). Membranes were blocked for 1 h in Tris-buffered saline with 5% non-fat dry milk and 0.1% Tween-20 before antibody incubation. Membranes were incubated overnight at 4˚C in primary rabbit anti-β-actin (1:10,000; Cell Signaling Technology (CST), Danvers, MA) to account for equal loading. Membranes were next incubated at room temperature for 1 h with horseradish peroxidase-conjugated (HPR) goat anti-rabbit at a final concentration of 1:10,000 (Thermo-Scientific, Waltham, MA). For detection of phosphorylated β-catenin at Ser-552 or Ser-675, non-phosphorylated (active) β-catenin (Ser-33/37/Thr-41) and phosphorylated forkhead box protein O (FOXO1) at Ser-256 membranes were incubated at 4˚C overnight with primary antibody (1:1,000; CST) followed by a 1 h incubation in HRP conjugated goat anti-rabbit at 1:3,000. For detection of total β-catenin, membranes were incubated at 4 °C overnight in mouse anti-β-catenin (1:10,000; BD Transduction Laboratories, San Diego, CA) followed by a 1 h incubation in HRP conjugated goat anti-mouse at 1:10,000 (Thermo-Scientific). Antigen-antibody complexes were detected using chemiluminescence with Immobilon detection substrate reagent (Millipore, Billerica, MA) and images were captured using the C-DiGit Blot Scanner (LI-COR, Lincoln, NE).

3.4 Quantitative real-time PCR

Total RNA was isolated from granulosa cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Integrity of RNA was assessed by visualization of 18S and 28S ribosomal RNA resolved by agarose gel electrophoresis. RNA purity and quantity was determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Purity was determined by 260/280 nm absorbance ratios, absorbance ratios above 1.8 were considered acceptable. Total RNA (1 µg) was treated with 1 µL DNase I (Invitrogen) to remove genomic DNA contamination following manufacturer's instructions. First-strand mRNA was reversed transcribed into cDNA using oligo (dT) primers and Superscript II Reverse Transcriptase (Invitrogen). Quantitative real-time PCR analysis was performed using methods and primers previously reported (Stapp et al., 2014). Mitochondrial ribosomal protein L19 (*Mrpl19*) was used as an internal housekeeping gene for *Axin*2 gene normalization. Relative fold change for target mRNA was quantified using the ΔΔCq method.

3.5 Radioimmunoassay

Granulosa cell culture media were analyzed for estradiol by solid phase RIA using components of commercial kits manufactured by Siemens Medical Diagnostics Corp. (Los Angeles, CA) as previously described (Castañon et al., 2012). The specific binding was 65% and detection limit (95% of maximum binding) of the assay was 2 pg/mL and intra-assay CV was 8.9%.

3.6 Statistical analysis

Three biological replicates were evaluated for data analysis on luciferase activity, estradiol concentrations, protein abundance, and mRNA expression. All statistical analysis was performed using SAS (Version 9.3; SAS Institute, Inc., Cary, NC). Generalized linear mixed models methods were used to analyze the data, accounting for non-normal responses and unequal variances where necessary. Estradiol concentration and protein abundance were analyzed using ANOVA methods and least squares means comparisons between treatments were performed only when the model was significant to determine differences among treatments. For protein accumulation, quantitative analysis is presented with the protein of interest expressed as a percentage of control and additionally, a t-test was performed to compare individual treatments to control by comparing each treatment level mean to 100%.

4. Results and discussion

4.1 IGF-I attenuates the inhibitory effect of WNT3A on *Cyp19a1 PII* activity and estradiol production

Insulin-like growth factor I contributes to estrogen production by increasing the sensitivity of granulosa cells to FSH in cattle (Spicer et al., 2002) and IGF-I knockout rodents fail to develop follicles past the pre-antral stage indicating a fundamental role in follicle development (Adashi et al., 1985a; Baker et al., 1996). Moreover, in cattle concentrations of IGF-I are greatest in the largest follicle compared with the second largest follicle, suggesting IGF-I contributes to dominant follicle selection (Beg et al.,

2001). The ability of IGF-I and FSH signaling to mediate AKT and subsequent β-catenin accumulation suggest, signaling overlap and a potential mediator of WNT signaling. To examine if IGF-I signaling can alleviate WNT3A inhibition of FSH-mediated *Cyp19a1* mRNA expression and estradiol production, rat granulosa cells were treated with FSH, WNT3A, IGF-I or a combination of the treatments. As expected FSH treatment induced a 36-fold change in luciferase *Cyp19a1 PII* activity above controls ($P < 0.001$; Fig. 8A), and consistent with our previous studies, WNT3A inhibited ($P < 0.001$) FSH-mediated *Cyp19a1 PII* activity (Stapp et al., 2014b). Interestingly, the addition of IGF-I to FSH+WNT3A increased ($P < 0.02$) *Cyp19a1 PII* activity from 36 relative light units (RLU) in FSH+WNT3A treated cells to 55 RLU in FSH+WNT3A+IGF-I treated cells. Media concentrations of estradiol followed a parallel response to *Cyp19a1 PII* activity (Fig. 8B). Following FSH treatment, estradiol concentration increased ($P < 0.01$) when compared with vehicle controls (482 vs. 14 ± 92 pg/mL). Co-treatment of FSH with WNT3A reduced estradiol concentrations, while the addition of IGF-I to the FSH+WNT3A treatment group increased ($P < 0.10$) estradiol in medium from 113 to 341 ± 92 pg/mL, respectively.

4.2 β-catenin phosphorylation is not modulated by IGF-I

Phosphorylation of β-catenin at Ser-552 and Ser-675 are mediated by FSH and found to associate with T-cell factor on FSH target gene promoters (Law et al., 2013). Therefore, these transcriptionally active and inactive forms of β-catenin were measured to evaluate if IGF-I rescues WNT3A inhibition of FSH-target genes by modulating β-

catenin phosphorylation status. Treatment with FSH consistently induced phosphorylation of β-catenin at Ser-552 and Ser-675 accumulation when compared with controls ($P < 0.05$) irrespective of treatment. However, FSH co-treated with IGF-I or WNT3A had no effect on phosphorylation of β-catenin at Ser-552 or Ser-675 (Fig. 9 and 10C and D). In addition to phosphorylated β-catenin, total concentrations were not enhanced with IGF-I treatment when compared with control (Fig 9A). Active β-catenin (non-phosphorylated at Ser-33/37/Thr-41) and total β-catenin between experiments was highly variable and therefore the model was not significant (Fig. 10A and B). However, a common trend was FSH and WNT3A treatment induced active and total β-catenin and IGF-I down regulated FSH-mediated active β-catenin accumulation. Insulin-like growth factor-I induced accumulation of total β-catenin in cattle (Gomez et al., 2015), but not rats demonstrating IGF-I intracellular signaling is species dependent. These results demonstrate IGF-I did not affect FSH's ability to induce total β-catenin or modulate the phosphorylation status.

4.3 *Axin2* is not regulated by IGF-I

A negative regulator of downstream WNT signaling components is Axin2 (Jho et al., 2002; Bernkopf et al., 2014), which was induced in cells treated with exogenous WNT3A ($P < 0.005$; Fig. 11) compared with cells that did not receive WNT3A treatment. To address if a mechanism by which IGF-I partially rescues FSH-mediated estradiol production is by suppressing *Axin2* expression, granulosa cells were stimulated with FSH, WNT3A, IGF-I or the combination. As expected, WNT3A increased *Axin2* expression 11-fold greater than control. However, co-treatment of WNT3A with FSH,

IGF-I or the combination of did not differentially regulate *Axin2* mRNA expression, therefore eliminating this as a possible mechanism in which IGF-I suppresses WNT3A inhibition.

4.4 FOXO1 phosphorylation is not differentially regulated at 24h by IGF-I

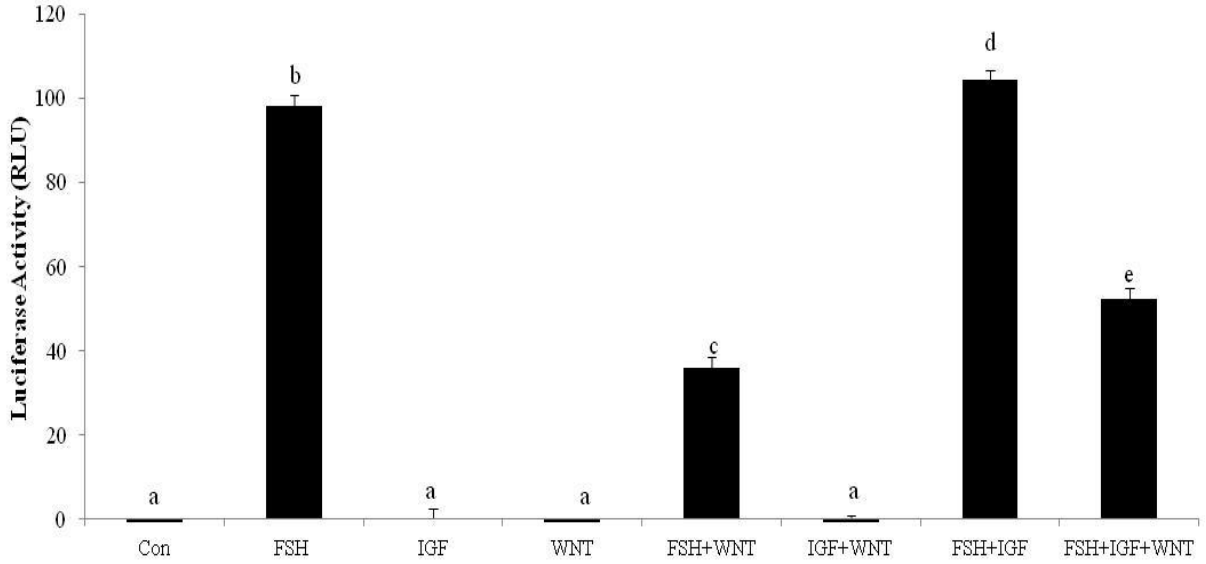
Forkhead box O protein induces expression of genes involved in cell cycle arrest and quiescence. Non-phosphorylated FOXO is primarily nuclear and presumed to be inhibitory on gene transcription. However, the phosphorylation of FOXO by AKT initiates its translocation out of the nucleus and into the cytoplasm (Biggs et al., 1999; Brunet et al., 1999). In granulosa cells FOXO1 is highly expressed and its transcript is hormonally induced by FSH and estradiol (Liu et al., 2009; Gloaguen et al., 2011). Transient transfection of granulosa cells with active FOXO1 mutant suppressed FSH-mediated proliferation and differentiation (Park et al., 2005). Additionally, in cultured granulosa cells IGF-I readily phosphorylates FOXO1 (Richards et al., 2002). These discoveries highlight the influence of FOXO on estradiol synthesis in granulosa cells therefore, phosphorylated FOXO1 was quantified to determine if IGF-I enhanced its exclusion from the nucleus in the presence of WNT. At the 24 h time point there was no difference in phosphorylation status of FOXO1 between IGF-I and co-treatment with FSH, WNT3A, and IGF-I (Fig 12). Data suggest the ability of IGF-I to partially rescue FSH-mediated estradiol production is not through enhancing FOXO1 phosphorylation. However, we cannot completely rule this out as a mechanism as maximal phosphorylation of FOXO by IGF-I occurs 90 min post treatment (Richards et al., 2002).

Data herein support previous studies in which WNT3A is inhibitory on FSH-mediated estradiol biosynthesis (Stapp et al., 2014b). Moreover, the endogenous intraovarian signaling molecule, IGF-I partially rescues *Cyp19a1* promoter activity from WNT3A inhibition resulting in increased estradiol concentrations. However, the mechanism(s) is still under current investigation.

5. Implications

In cattle estrogen concentrations are critical in fertility and dominant follicle selection however, the mechanisms responsible are diverse and remain unclear. These results indicate FSH, IGF-I, and WNT contribute to regulate estrogen in granulosa cells. Data herein demonstrate that IGF-I is capable of overriding a negative feedback system set up by WNT3A on FSH target genes that may be necessary to keep follicle maturation and estrogen production from going unregulated.

A.


Luciferase Activity (RLU)
120
100
80
60
40
20
0
a
b
a
a
c
a
d
e
Con
FSH
IGF
WNT
FSH+WNT
IGF+WNT
FSH+IGF
FSH+IGF+WNT

B.

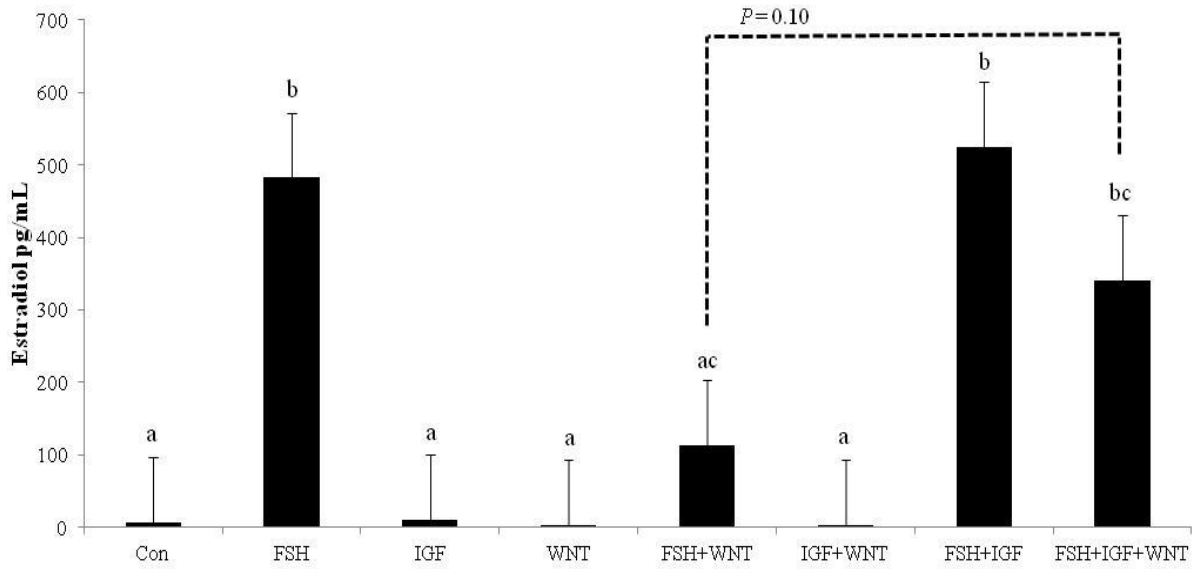

Estradiol pg/mL
700
600
500
400
300
200
100
0
P=0.10
a
b
a
a
ac
a
b
bc
Con
FSH
IGF
WNT
FSH+WNT
IGF+WNT
FSH+IGF
FSH+IGF+WNT

FIGURE 8

Figure 8: *Cyp19a1 PII* Activity and Estradiol Production in Rat Granulosa Cells Treated with FSH, WNT3A, and IGF-I.

Primary rat granulosa cells were transfected with a *Cyp19a1 PII* luciferase reporter plasmid and treated for 24 h with 1) vehicle control, 2) 100 ng/mL highly purified human FSH, 3) IGF-I (50 ng/mL), 4) WNT3A (50 ng/mL,) 5) FSH+WNT3A, 6) IGF-I+WNT3A, 7) FSH+IGF-I, or 8) FSH+WNT3A+IGF-I. Cell lysate was collected for luciferase assay and treatment culture medium for quantification of estradiol concentrations. Least squares means ± SEM are presented and bars without a common superscript differ ($P < 0.05$; n = 3). **A)** *Cyp19a1* promoter activity and **B)** estradiol concentrations (pg/mL).

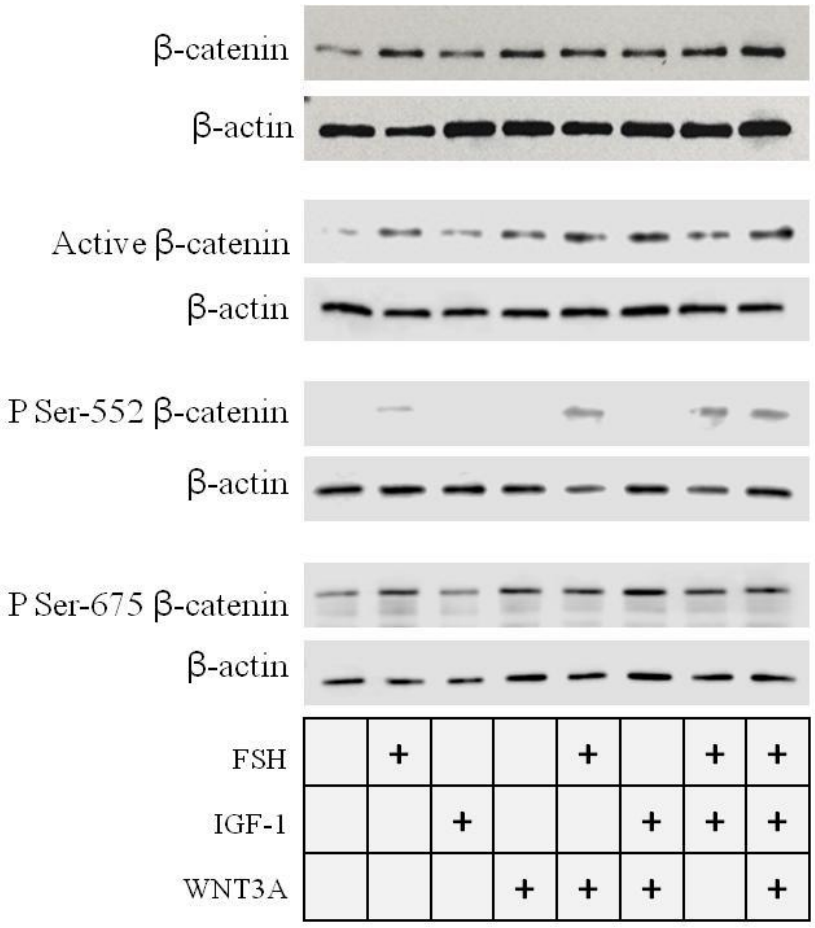
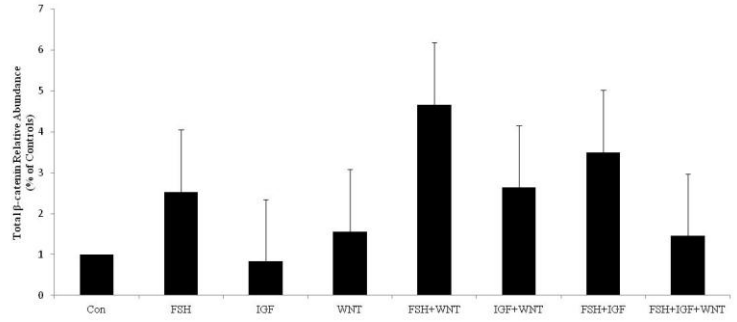

β-catenin
β-actin
Active β-catenin
β-actin
P Ser-552 β-catenin
β-actin
P Ser-675 β-catenin
β-actin
FSH
IGF-1
WNT3A

FIGURE 9

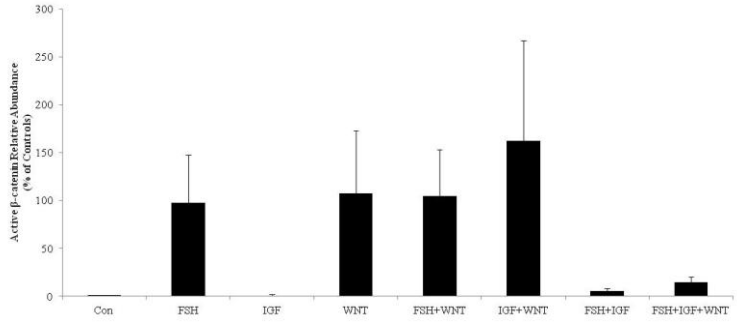
Figure 9: β-catenin Phosphorylation Status in Response to FSH, WNT3A, and IGF-I in Rat Granulosa Cells.

Primary rat granulosa cell lysate from luciferase assay was quantified for Western blot analysis of total β-catenin, active β-catenin, phosphorylated β-catenin at Ser-552, and Ser-675. Representative blots for target proteins are displayed over the loading control β-actin (n = 3).

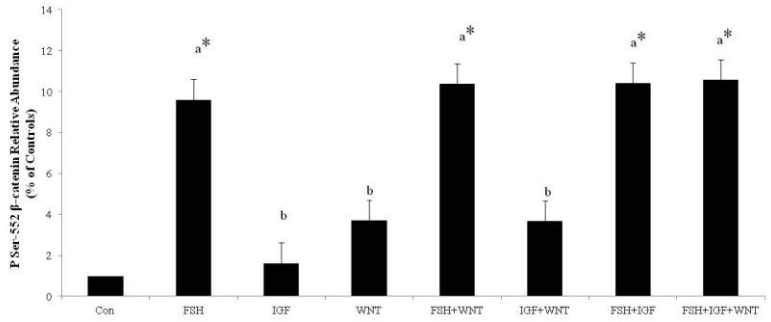
A.



B.



C.



D.

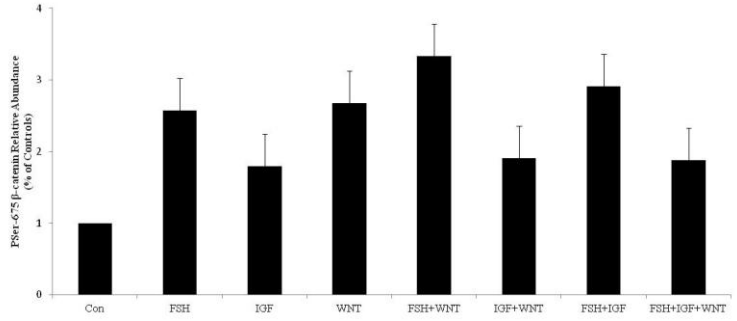


FIGURE 10

Figure 10: Quantification of total and Phosphorylated β-catenin in Response to FSH, WNT3A, and IGF-I in Rat Granulosa Cells.

Band intensity for Western blot analysis was quantified for **A)** total β-catenin, **B)** active β-catenin, **C)** phosphorylated β-catenin at Ser-552, and **D)** Ser-675. Quantitative analysis of target protein abundance is expressed as a percentage of controls. Bars without a common superscript differ ($P < 0.05$) and an asterisk denotes level of significance when compared to control ($^*P < 0.0001$). Least squares means ± SEM are presented.

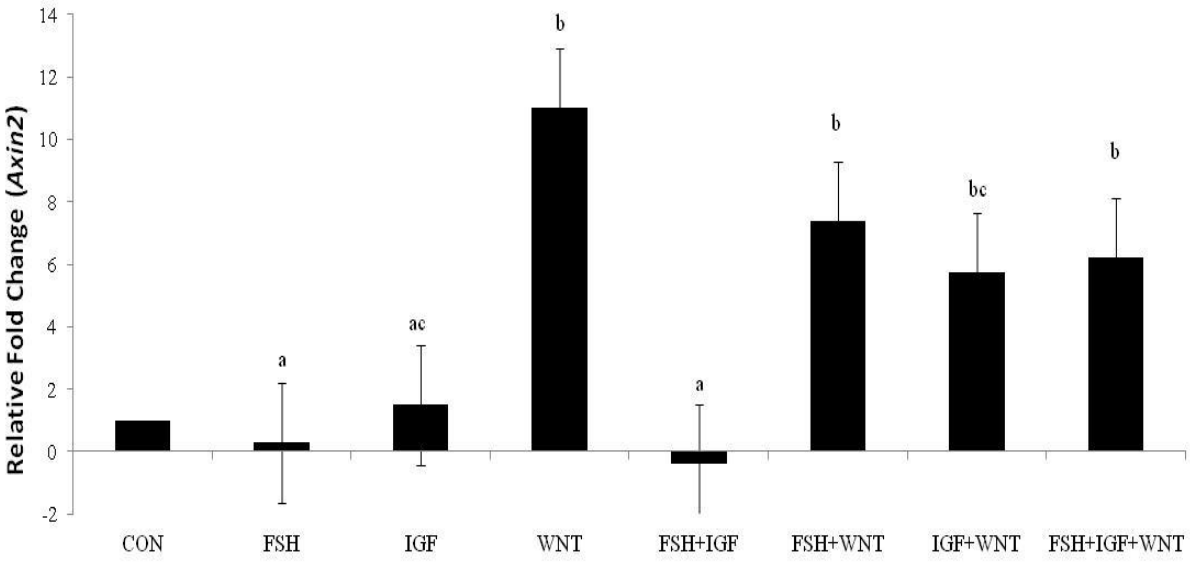
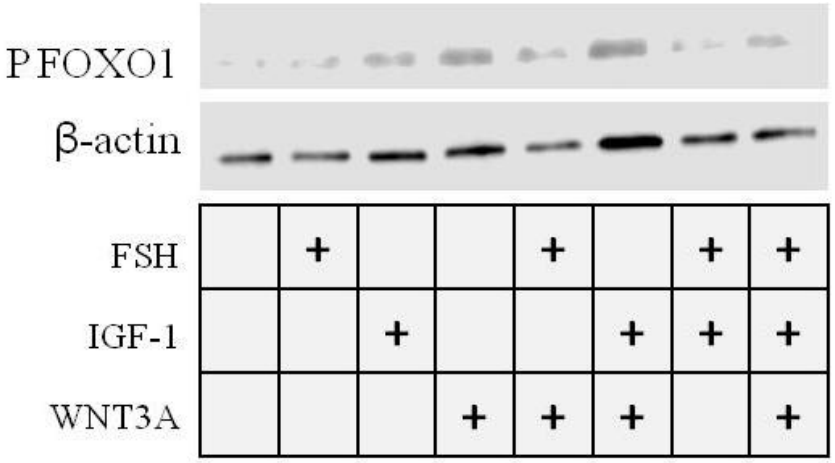

Relative Fold Change (Axin2)
14
12
10
8
6
4
2
0
-2
a
ac
b
a
b
bc
b
CON
FSH
IGF
WNT
FSH+IGF
FSH+WNT
IGF+WNT
FSH+IGF+WNT

FIGURE 11

Figure 11: *Axin2* mRNA Expression in Rat Granulosa Cells Treated with FSH, WNT3A, and IGF-I.

Primary rat granulosa cells were treated for 24 h with 1) vehicle control, 2) 100 ng/mL highly purified human FSH, 3) IGF-I (50 ng/mL), 4) WNT3A (50 ng/mL), 5) FSH+WNT3A, 6) IGF-I+WNT3A, 7) FSH+IGF-I, or 8) FSH+WNT3A+IGF-I. Cells were collected into TRIzol for quantitative PCR analysis of *Axin 2*. *Mrpl19* was used as a reference gene. Bars without a common superscript differ ($P < 0.05$; n = 3) least squares means ± SEM are presented.

A.


PFOXO1
β-actin
FSH
IGF-1
WNT3A

B.

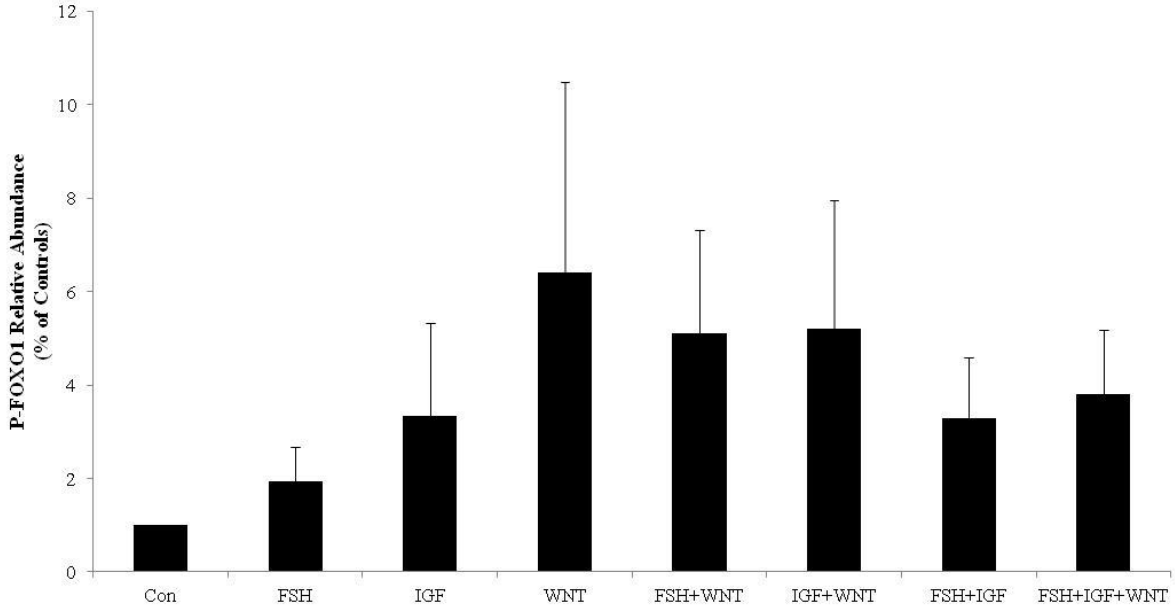

P-FOXO1 Relative Abundance (% of Controls)
12
10
8
6
4
2
0
Con
FSH
IGF
WNT
FSH+WNT
IGF+WNT
FSH+IGF
FSH+IGF+WNT

FIGURE 12

Figure 12. Phosphorylation Status of FOXO1 in Rat Granulosa Cells Treated with FSH, WNT3A, and IGF-I.

Primary rat granulosa cell lysate from luciferase assay were collected for Western blot analysis of phosphorylated FOXO1. β-actin was used as a loading control (n = 3). **A)** A representative Western blot and **B)** Quantitative analysis of FOXO1 abundance is expressed as a percentage of controls. Least squares means ± SEM are presented, no significance was detected.

CHAPTER V

AROMATASE EXPRESSION IN GRANULOSA CELLS REQUIRES BETA-CATENIN/TCF ASSOCIATION

B. I. Gomez, C. A. Gifford, and J. A. Hernandez Gifford

This chapter is a component of an ongoing study. The primary author was involved in all research and writing aspects of this chapter.

1. Abstract

Aromatase (*Cyp19a1*) is expressed in response to the pituitary gonadotropin follicle-stimulating hormone (FSH) and is responsible for the aromatization of androgens into estrogens. Follicle-stimulating hormone induction of *Cyp19a1* is enhanced by β-catenin, a multifunctional protein that serves as a co-transcriptional regulator. Whether the association of β-catenin with the transcription factor T-cell factor (TCF) is required for *Cyp19a1* expression is unknown. To elucidate if β-catenin associates with TCF on the *Cyp19a1* promoter in the presence of FSH, primary granulosa cells were cultured in the presence or absence of FSH (100 ng/mL) and treated with increasing concentrations (5, 10, 25 μM) of iCRT14, an inhibitor of β-catenin /TCF association. To ascertain if iCRT14 is detrimental on primary granulosa cell number, viability was quantified at termination of the treatment period. Concentrations of iCRT14 at 5, 10, and 25 μM had no deleterious effects on cell viability when compared with DMSO control ($P > 0.61$). Successful inhibition of β-catenin and TCF binding was confirmed by real-time PCR quantification of luteinizing hormone receptor (*Lhcgr*), a TCF responsive gene. As expected, treatment with FSH increased *Lhcgr* expression (516-fold) when compared with vehicle control ($P < 0.001$; n = 3). Conversely, FSH-mediated induction of *Lhcgr* was returned to control levels in granulosa cells co-treated with FSH and iCRT14. Expression of *Cyp19a1* mRNA was enhanced with FSH when compared with control ($P < 0.001$) and iCRT14 at 5 and 25 μM abolished FSH-stimulated *Cyp19a1* expression (15,245 vs 1,414 and 51.67 fold, respectively). These data demonstrate that FSH induction of *Cyp19a1* requires β-catenin binding to TCF on the promoter and further emphasizes the requirement for β-catenin in estradiol production.

2. Introduction

Follicle-stimulating hormone (FSH) is a pituitary glycoprotein that enhances ovarian follicle development and estradiol biosynthesis by granulosa cells. Expression of the steroidogenic enzyme aromatase (*Cyp19a1*) is increased in response to FSH and is required for conversion of testosterone to estradiol. β-catenin, a transcriptional co-factor accumulates in the cytoplasm in response to FSH (Castañon et al., 2012; Stapp et al., 2014a) and intra-ovarian signaling molecules such as the wingless-type mammary tumor virus integration-site (WNT). The transcriptional activity of β-catenin was first established in the canonical WNT pathway where in the presence of a WNT ligand, a degradation complex is prevented from phosphorylating the NH_2-terminal of β-catenin and targeting it for degradation (Cadigan and Nusse, 1997). β-catenin then accumulates in the cytoplasm translocates to the nucleus and binds T-cell factor (TCF) to mediate transcription of target genes (Huber et al., 1996). Additionally, nuclear β-catenin mediates gene expression by associating with other transcription factors of these is steroidogenic factor-1 (SF-1), a nuclear orphan receptor. In granulosa cells, association of β-catenin with SF-1 enhances *Cyp19a1* expression (Parakh et al., 2006). Transient transfection of granulosa cells with Δ90 β-catenin, an adenovirus encoding a β-catenin mutant lacking NH_2-terminal residues required for degradation, enhanced FSH stimulation of *Cyp19a1* expression (Parakh et al., 2006). Conversely, knock down of endogenous β-catenin by siRNA reduced forskolin-mediated *Cyp19a1* promoter activity. Additionally, cultured granulosa cells from transgenic mice deficient for β-catenin had reduced FSH-mediated *Cyp19a1* expression and media estradiol concentrations (Hernandez Gifford et al., 2009). These studies emphasize the requirement for β-catenin

in *Cyp19a1* expression and subsequent estradiol production. Currently, there are no reports indicating *Cyp19a1* expression is mediated by β-catenin binding to TCF on the *Cyp19a1* promoter.

Luteinizing hormone receptor (*Lhcgr*), an FSH target gene, promotes differentiation of the follicle. Chromatin immunoprecipitation demonstrated FSH promoted SF-1/β-catenin and TCF3 association with the *Lhcgr* promoter (Law et al., 2013). Transfection with an artificial *Lhcgr* promoter luciferase reporter in which the TCF binding site was mutated abolished FSH stimulation of the reporter. Similarly, mutation of the SF-1 binding site on the *Lhcgr* promoter prevented promoter activity under FSH treatment. Follicle-stimulating hormone induces *Lhcgr* through promoting SF-1 and TCF-1 activity.

Based on the crucial nature of β-catenin to regulate estradiol production, a study was designed to investigate if expression of *Cyp19a1* requires β-catenin/TCF association. Inhibitors of β-catenin responsive transcription (iCRTs) are small-molecules that target β-catenin transcription by docking into TCF-specific pockets of β-catenin and preventing TCF/ β-catenin association. For the purpose of elucidating if *Cyp19a1* expression requires β-catenin/TCF association, we utilized iCRT14 in primary granulosa cell cultures. Results herein provide functional evidence that an interaction between β-catenin and TCF is essential for the FSH cascade that enhances *Cyp19a1* expression.

3. Materials and methods

3.1 Cell culture

Female Sprague-Dawley rats were purchased from Charles River Laboratories (Hollister, CA) and housed at Oklahoma State University and held in accordance with the Oklahoma State University Institutional Animal Care and Use Committee (AG-10-3). Rat ovaries were collected and transported in Dulbecco's Modified Eagle Medium/Ham's F-12 (Invitrogen, Carlsbad, CA) with 1% (vol/vol) 10,000 IU/mL penicillin/10,000 µg streptomycin/mL penicillin and streptomycin (DMEM/F12/PS) medium to laboratory for further processing. Granulosa cells were isolated and cultured as previously described (Stapp et al., 2014) and seeded in 24-well culture plates at a density of 1.6×10^5 cells per well (luciferase assay and cell viability) or 5.4×10^5 cells per 35 mm dish (RNA extraction) in DMEM/F12/PS medium supplemented with 10% FBS (complete media). Cells for luciferase analysis were first transfected with the appropriate plasmid construct before PBS or FSH (100 ng/mL) supplemented with 10^{-7} M testosterone propionate 24 h treatment. In a separate experiment, granulosa cells were treated with DMEM/F12/PS supplemented with 10^{-7} M testosterone propionate (Sigma-Aldrich, St. Louis, MO) and one of the following treatments: 1) vehicle control (DMSO), 2) 100 ng/mL FSH (S1AFP-B-3; National Hormone and Peptide Program, National Institutes of Diabetes, Digestive, and Kidney Diseases, National Institutes of Health, Bethesda, MD), 3) iCRT14 at 5, 10, or 25 µM (CAS: 677331-12-3; Sigma-Aldrich, St. Louis, MO), and 4) the combination of iCRT14 and FSH. Granulosa cells were treated with iCRT14 or DMSO 1 h prior to adding FSH directly to individual wells and incubated for 24 h.

3.2 Plasmid constructs, co-transfection, and luciferase assay

The reporter vectors *Renilla* pHRG-B, 517-promoter fragment *CYP19A1*-wildtype (*CYP19A1*-WT) and *CYP19A1*-mutant (*CYP19A1*-TCF MUT) in pGL3-basic were a donation by Mary Hunzicker-Dunn at Washington State University. A putative TCF binding site in human *CYP19A1 PII* was mutated using site directed mutagenesis. The empty pGL3-basic vector which served as a control was purchased from Promega (Madison, WI).

Cultured granulosa cells were co-transfected with 10 ng/well p-HRG-B *Renilla* and 200 ng/well of *CYP19A1*-WT, *CYP19A1*-TCF MUT or empty luciferase reporter vectors in DMEM/F-12. Cells were transfected using Lipofectamine LTX and Plus reagent (Invitrogen, Carlsbad, CA) according to manufacturers protocol for approximately 5 h. DMEM/F-12 and transfection agents were aspirated and cells were returned to fresh medium plus 10% FBS for 18 hours. Cells then received the appropriate treatment as described earlier. After the final 24 h incubation period in treatment, cell lysates were collected and luciferase values were measured using the Dual-Luciferase Reporter Assay System according to manufacturer's protocol (Promega). A single tube Modulus Luminometer (Turner BioSystems, Madison, WI) was used to measure luciferase activity for all samples.

3.3 Analysis of cell viability

At the completion of the iCRT14 treatment period granulosa cells were rinsed with ice cold PBS and detached using TrypLE (Thermo-Scientific, Waltham, MA). Once

cells detached they were collected and 0.5 mL of complete media was used to rinse the culture dish to collect remaining cells. Cells were centrifuged at 200 x g and the remaining pellet was resuspended in 250 µL of PBS. To assess cell viability and minimize variation, the Bio-Rad TC20 automated cell counter was used to quantify cell viability (Bio-Rad, Hercules, CA).

3.4 Quantitative real-time PCR

Total RNA was isolated from granulosa cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA Integrity was assessed by visualization of 18S and 28S ribosomal RNA resolved by agarose gel electrophoresis. RNA purity and quantity was determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Total RNA (1 µg) was treated with DNase I (Invitrogen) to remove genomic DNA contamination following manufacturer's instructions. First-strand cDNA was reversed transcribed into cDNA using oligo (dT) primers and Superscript II Reverse Transcriptase (Invitrogen). Quantitative real-time PCR analysis was performed using methods and primers previously reported (Stapp et al., 2014). Mitochondrial ribosomal protein L19 (*Mlpl19*) was used as an internal housekeeping gene for *Cyp19a1* and *Lhcgr* gene normalization. Relative fold change for target mRNA was quantified using the ΔΔCq method.

3.5 Statistical analysis

Luciferase activity, cell viability, and relative changes in gene expression for *Lhcgr* and *Cyp19a1* were evaluated on at least three biological replicates and statistical analysis was performed using SAS (Version 9.3; SAS Institute, Inc., Cary, NC). Generalized linear mixed models methods were used to analyze the data. Effects of treatment on cell viability and mRNA expression were analyzed using ANOVA methods and least squares means comparisons between treatments were performed only when the model was significant to determine differences among treatments.

4. Results and discussion

β-catenin, a multifunctional protein, is known to bind TCF and initiate transcription of target genes in the nucleus. Follicle-stimulating hormone stimulates *Cyp19a1* expression and more recently has been shown to increase total β-catenin protein (Castañon et al., 2012). Moreover, the conversion of testosterone to estradiol relies on *Cyp19a1*expression. To identify if FSH-mediated accumulation of β-catenin associates with TCF to regulate *Cyp19a1*, rat granulosa cells were treated with iCRT14 and with a 517 bp *CYP19A1 PII* plasmid in which one putative TCF site was mutated.

4.1 *CYP19A1*-WT and *CYP19A1*-TCF MUT luciferase expression is similarly in response to FSH

A putative TCF site in a 517 bp fragment of the *CYP19A1 PII* was mutated to address if TCF is required for optimal *CYP19A1 PII* luciferase expression in granulosa cells. As expected cells transfected with the *CYP19A1*-WT plasmid and treated with FSH

had increased (P = 0.02; n = 7) luciferase activity when compared with PBS vehicle control (Fig. 13). The TCF site mutation did not affect the ability of FSH to stimulate *CYP19A1 PII* luciferase activity. Cells transfected with the TCF-mutated plasmid and treated with FSH had increased (P = 0.001) luciferase activity (8.92 RLU) compared with controls (1.99 RLU). These results indicate no significant interaction between wild-type and the mutated TCF site in the aromatase promoter.

4.2 β-catenin is required to associate with TCF for maximal *Cyp19a1* mRNA Expression

β-catenin misregulation is associated with several malignancies and therefore recent work is focused on identifying therapeutic molecules that prevent β-catenin transcriptional activity. Of these molecules are iCRT's which are inhibitory compounds that obstruct β-catenin association with TCF (Gonsalves et al., 2011). The iCRT's are successful at antagonizing β-catenin-mediated transcription by docking into TCF-specific pockets in β-catenin and physically blocking TCF binding. Because of its effectiveness and specificity at inhibiting β-catenin and TCF attachment, iCRT14 was utilized to study the requirement of their association on *Cyp19a1* expression.

In breast cancer cell lines iCRT14 effectively inhibits cell proliferation (Bilir et al., 2013) and are cytotoxic to colon cancer cell lines (Gonsalves et al., 2011). To first assess whether the iCRTs at increasing concentrations affect primary granulosa proliferation and cell number, a cell counter was utilized to quantify cell viability. Cell count ranged between 2.8 and 3.4 x 10^5 and there was no difference in cell viability (P >

0.61; Fig. 14) with increasing concentrations of iCRT14. These data suggest that iCRT14 regardless of concentration (5, 10, 25 μM) is not cytotoxic to primary granulosa cells, and therefore will not affect data interpretation.

To further study the requirement for β-catenin/TCF association for FSH-mediated *Cyp19a1* expression in granulosa cells, mRNA for FSH target genes *Cyp19a1* and *Lhcgr* was quantified by real-time PCR. Expression of *Lhcgr* was use as a positive control, as β-catenin is required to associate with SF-1 and TCF to regulate its expression (Law et al., 2013). As expected, co-treatment with FSH and iCRT14 at 5 or 25 μM reduced FSH-mediated *Lhcgr* expression (29.80 and 7.74 vs 516.29 fold, respectively; Fig. 15A). Treatment with FSH drastically induced *Cyp19a1* expression 15,245-fold compared with controls ($P < 0.0001$; Fig. 15B). At 5 or 25 μM iCRT14 had no effect on *Cyp19a1* expression as both were comparable to vehicle controls ($P = 0.99$). Consistent with a necessary role for TCF/β-catenin in *Lhcgr* expression, preventing TCF/β-catenin association with iCRT14 abolished FSH-mediated *Cyp19a1* expression. Fold change expression was 1,417 with 5 μM and 51 with 25 μM iCRT14 co-incubated with FSH, a significant reduction from 15,245 in FSH treated granulosa cells.

5. Conclusion

These results demonstrate iCRT14 is not cytotoxic to primary granulosa cells but is effective at preventing TCF from binding β-catenin. In estradiol biosynthesis *Cyp19a1* encodes a steroidogenic enzyme that catalyzes the conversion of testosterone to estradiol. Depletion of β-catenin in granulosa cells reduces *Cyp19a1* expression and subsequent

estradiol production (Parakh et al., 2006; Hernandez Gifford et al., 2009). It has been established that β-catenin functions as a co-transcription factor by associating with SF-1 (Parakh et al., 2006) and now TCF for FSH-mediated estradiol production. No change in *Cyp19a1* promoter activity was detected between the wild-type and mutant TCF binding site. It is important to note that the *CYP19 PII* plasmid only contains a fragment of the promoter and not the entire sequence therefore, it is probable that additional TCF sites compensate for the mutated one. Whereas, β-catenin/TCF interaction is prevented by iCRT14 therefore inhibiting all β-catenin/TCF transcriptional activity. Expression of *Cyp19a1* mRNA was reduced with iCRT14 demonstrating β-catenin/TCF is required for activation of the *Cyp19a1* promoter.

Follicle-stimulating hormone and additional intra-ovarian signaling molecules such as WNT and insulin-like growth factor-I mediate β-catenin availability and activity. This study highlights the multiple functions and requirements for β-catenin by contributing to the body of literature that *Cyp19a1* requires β-catenin/TCF association. Understanding the mechanisms involved in mediating estradiol production in granulosa cells is of importance to normal function of the female reproductive system.

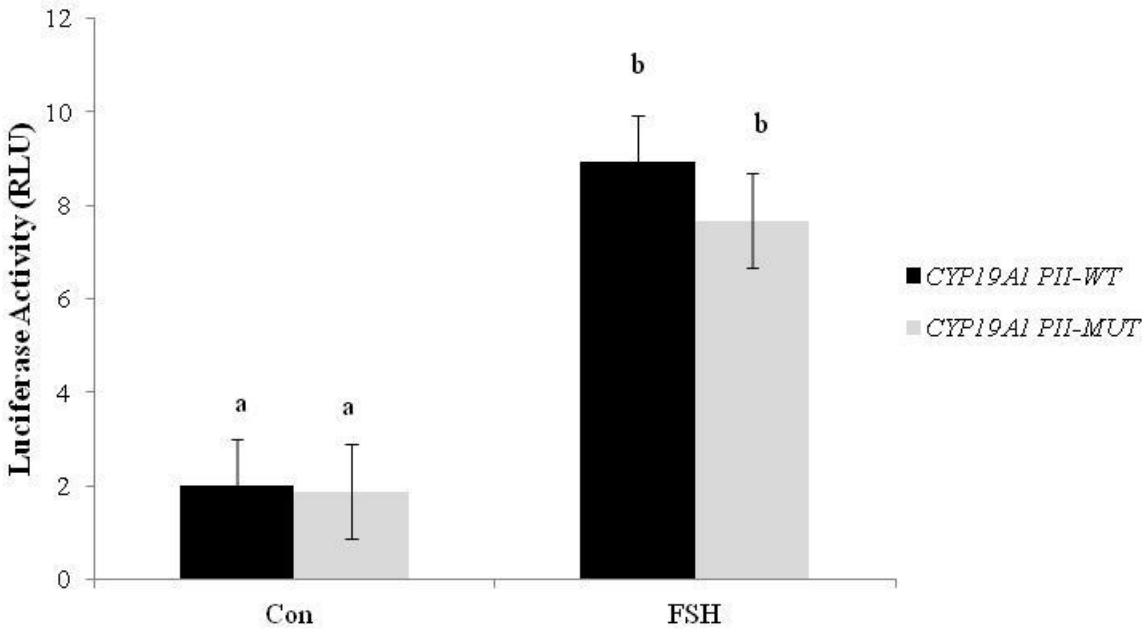

12
10
8
6
4
2
0
Luciferase Activity (RLU)
a
a
b
b
Con
FSH
CYP19A1 PII-WT
CYP19A1 PII-MUT

FIGURE 13

Figure 13: *CYP19A1 PII* Expression in Rat Granulosa Cells Transfected with *CYP19A1*-WT or *CYP19A1*-TCF MUT is similar in response to FSH.

Primary rat granulosa cells were transfected with *CYP19A1 PII*-WT or *CYP19A1 PII*-TCF-MUT prior to treatment with 100 ng/mL highly purified human FSH or PBS vehicle control for 24 h. Cell lysate was collected for luciferase assay and data is presented as least square means ± SEM ($P < 0.05$; n = 7) bars with without a common superscript differ.

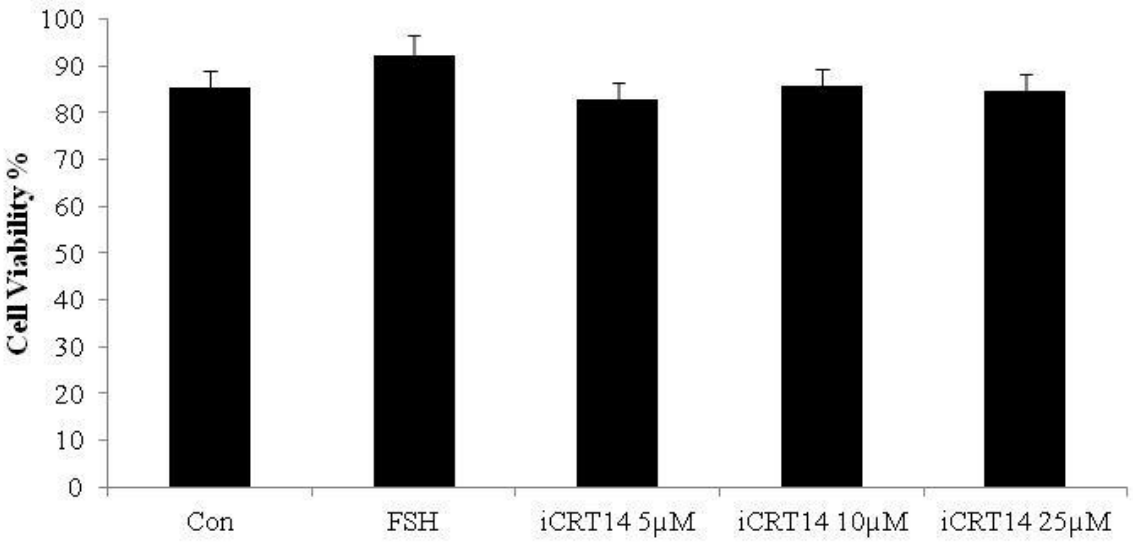
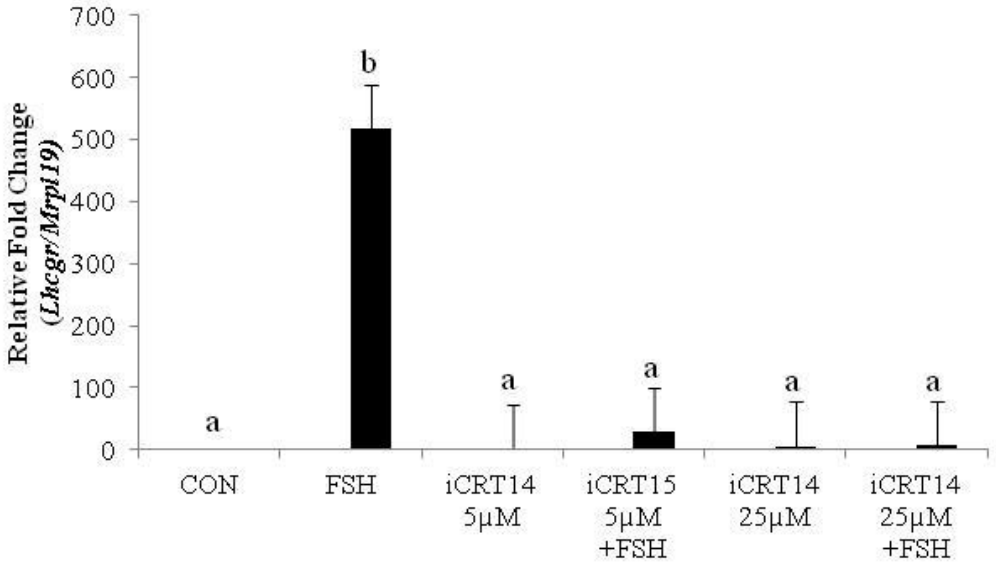

Cell Viability %
100
90
80
70
60
50
40
30
20
10
0
Con
FSH
iCRT14 5μM
iCRT14 10μM
iCRT14 25μM

FIGURE 14

Figure 14: Primary Rat Granulosa Cell Viability in Response to Increasing Concentrations of iCRT14.

Primary rat granulosa cells were treated with 100 ng/mL of highly purified human FSH, or increasing concentrations of iCRT14 (5, 10, 25 μM) for 24 h. Cell viability was quantified using an automated cell counter. Viability is presented as least square means ± SEM. No difference in cell viability was detected between treatments (n = 3).

A.


Relative Fold Change
(Lhcgr/Mrpl19)
700
600
500
400
300
200
100
0
a
b
a
a
a
a
CON
FSH
iCRT14 5μM
iCRT15 5μM +FSH
iCRT14 25μM
iCRT14 25μM +FSH

B.

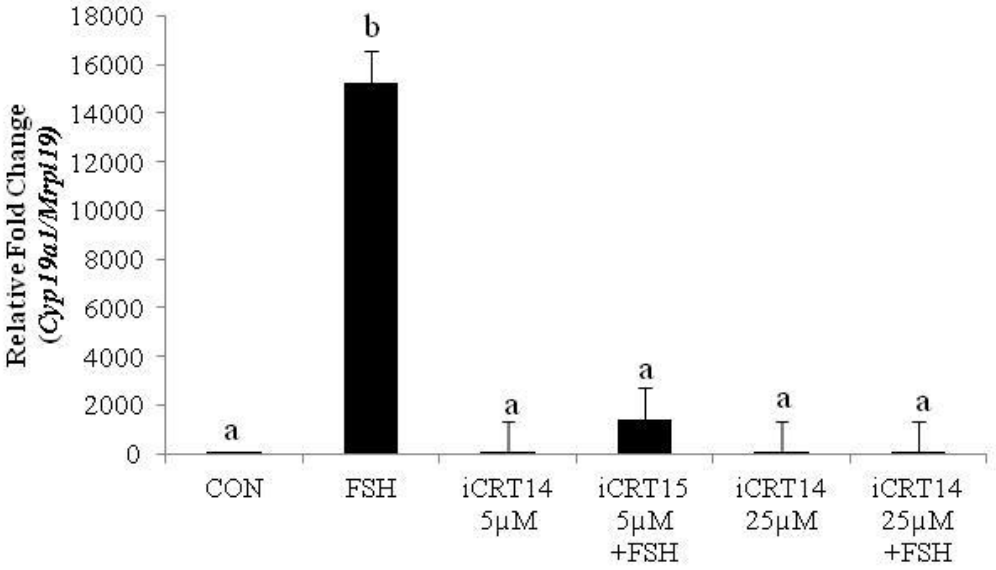

Relative Fold Change
(Cyp19a1/Mrpl19)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
a
b
a
a
a
a
CON
FSH
iCRT14 5μM
iCRT15 5μM +FSH
iCRT14 25μM
iCRT14 25μM +FSH

FIGURE 15

Figure 15. *Cyp19a1* and *Lhcgr* expression in FSH and iCRT14 Treated Rat Granulosa Cells.

Quantitative PCR analysis of *Lhcgr* and *Cyp19a1* mRNA in primary rat granulosa cells were treated with iCRT14 (5 or 25 μM) 1 h prior to the addition of highly purified human FSH (final concentration 100 ng/mL) for 24 h. Least square means ± SEM are presented (n = 3). Bars without a common superscript differ ($P < 0.001$).

CHAPTER VI

CONCLUSION

Estradiol biosynthesis is complex and involves many intracellular signaling cascades. Knowledge of the mechanisms required to regulate estradiol production in ovarian granulosa cells is of importance to normal function of the female reproductive system. Recognizing and comprehending the intricacy of estradiol regulation within granulosa cells will progress techniques targeted at enhancing female fertility.

β-catenin is a transcriptional co-factor mediated by follicle-stimulating hormone (FSH), insulin-like growth factor- I (IGF-I), and wingless-type mammary tumor virus integration-site (WNT) in granulosa cells. Nuclear β-catenin is further regulated by various transcription factors that enhance or suppress expression of target genes. Studies performed herein provide novel insight into the multifaceted roles and regulators of β-catenin in estradiol production.

Accumulated β-catenin in response to IGF-I and FSH suggest this is an AKT dependent event as both are known to signal downstream and activate AKT. It remains to be determined whether or not transcriptionally active (phosphorylated Ser-552, Ser-675) is temporally regulated under FSH and IGF-I conditions. Additionally, the

accumulation of β-catenin in response to IGF-I stimulation is species specific. The first study performed on bovine granulosa cells demonstrates 24 hour treatment with IGF-I increases total β-catenin, whereas in the second study rat granulosa cells under similar conditions had no effect. These data align with capacity of IGF-I treatment in the bovine granulosa cells but not rodent cells to stimulate estradiol production. The marked differences in species could provide an insight for follicular development differences in monovulatory versus polyovulatory species. Additional studies are necessary to identify if β-catenin in is responsive to FSH and IGF-I in human granulosa cells so the appropriate animal model will be used when studying β-catenin regulation in steroid production.

Of interest is the ability of IGF-I to partially attenuate inhibition of WNT3A on FSH-mediated estradiol production. Further investigation is required to identify the intracellular mechanism(s) by which WNT is inhibitory on FSH and the ability of IGF-I to relieve this inhibition. Future studies may involve precipitating β-catenin and characterizing the proteins associated with it under FSH, WNT, and FSH+WNT conditions. Even more interesting would be to identify if the inhibitory nature of WNT3A is only targeted at FSH-mediated estradiol signaling or does it extend to IGF-I in species where IGF-I is sufficient in stimulating estradiol production.

The availability of novel inhibitors of catenin responsive transcription (iCRT) molecules that inhibit β-catenin association with T-cell factor (TCF) offers plentiful research opportunities for applications such as therapeutic agents for granulosa cell cancer. Data presented herein demonstrate iCRTs do not affect primary granulosa cell viability, but are effective in preventing expression of TCF promoter genes.

These data support a proposed model (Fig. 16) for IGF-I as a regulator of estradiol production and possibly follicle deviation by relieving WNT inhibition. Stimulation by FSH on granulosa cells enhances expression of WNT and WNT downstream components (Castañon et al., 2012; Gupta et al., 2014). Treatment with WNT3A or LiCl a WNT agonist suppresses follicle development, inhibits FSH-mediated mRNA expression of the steroidogenic enzymes: *Cyp11a1, Cyp19a1,* and differentiation factor *Lhcgr,* and reduces subsequent estradiol production (Li et al., 2014; Stapp et al., 2014b). This dissertation provided evidence that IGF-I attenuates WNT3A inhibition on *Cyp19a1* promoter activity and estradiol production. The IGF-I system enhances the sensitivity of granulosa cells to FSH. In the bovine the concentrations of IGF-I in large follicles increases, however only the dominant follicle do these levels remain consistent (Ginther et al., 2001). Therefore, it is probable that WNT is expressed in response to FSH as a negative feedback mechanism to keep estradiol from going unregulated. Then when IGF-I concentrations increase in dominant follicles it will relieve the inhibitory properties of WNT on FSH for continual estradiol synthesis.

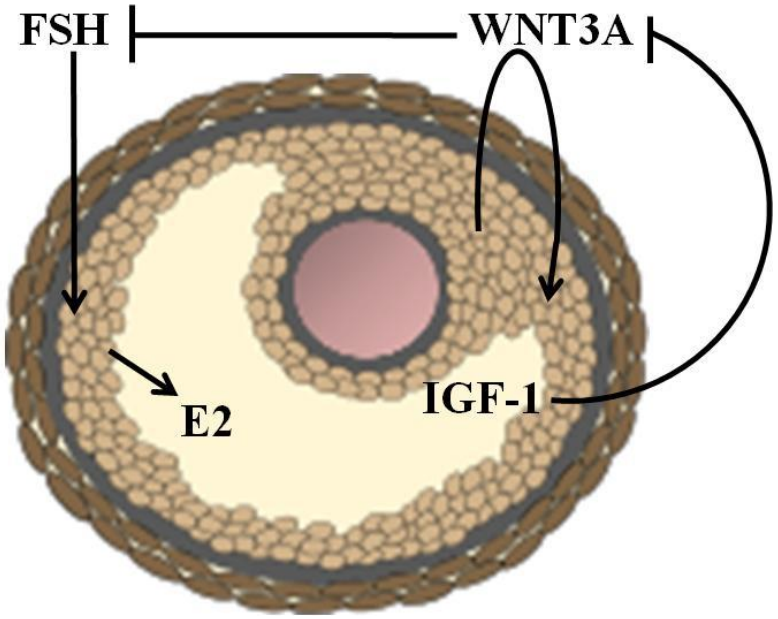


FIGURE 16

Figure 16: Proposed Model for Regulation of Estradiol Biosynthesis by FSH, WNT, and IGF-I in Developing Ovarian Follicles.

Initiation of estradiol production begins with FSH binding its receptor on granulosa cells to increase expression of steroidogenic enzymes. Of these is aromatase which requires β-catenin association with nuclear transcription factors steroidogenic factor-1 and T-cell factor. Stimulation by FSH also increases expression of WNT ligands and WNT signaling components. Canonical WNT signaling molecule, WNT3A, inhibits FSH-mediated estradiol production. Insulin-like growth factor-I expression is greatest in follicular fluid of the preovulatory follicle and partially prevents WNT3A inhibition on FSH-mediated estradiol production through an unknown mechanism.

REFERENCES

Aberle, H., A. Bauer, J. Stappert, A. Kispert, and R. Kemler. 1997. Beta-catenin is a target for the ubiquitin-proteasome pathway. EMBO J. 16:3797-3804.

Adams, T. E., V. C. Epa, T. P. Garrett, and C. W. Ward. 2000. Structure and function of the type 1 insulin-like growth factor receptor. Cell. Mol. Life Sci. 57:1050-1093.

Adashi, E. Y., C. E. Resnick, A. M. Brodie, M. E. Svoboda, and J. J. Van Wyk. 1985a. Somatomedin-C-mediated potentiation of follicle-stimulating hormone-induced aromatase activity of cultured rat granulosa cells. Endocrinology 117:2313-2320.

Adashi, E. Y., C. E. Resnick, A. J. D'Ercole, M. E. Svoboda, and J. J. Van Wyk. 1985b. Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. Endocr. Rev. 6:400-420.

Aerts, J. M., and P. E. Bols. 2010. Ovarian follicular dynamics. A review with emphasis on the bovine species. Part II: Antral development, exogenous influence and future prospects. Reprod. Domest. Anim. 45:180-187.

Alessi, D. R., and P. Cohen. 1998. Mechanism of activation and function of protein kinase B. Curr. Opin. Genet. Dev. 8:55-62.

Arend, R. C., A. I. Londono-Joshi, J. M. Straughn, Jr., and D. J. Buchsbaum. 2013. The Wnt/beta-catenin pathway in ovarian cancer: a review. Gynecol. Oncol. 131:772-779.

Armstrong, D. G., C. O. Hogg, B. K. Campbell, and R. Webb. 1996. Insulin-like growth factor (IGF)-binding protein production by primary cultures of ovine granulosa and theca cells. The effects of IGF-I, gonadotropin, and follicle size. Biol. Reprod. 55:1163-1171.

Baker, J., M. P. Hardy, J. Zhou, C. Bondy, F. Lupu, A. R. Bellve, and A. Efstratiadis. 1996. Effects of an Igf1 gene null mutation on mouse reproduction. Mol. Endocrinol. 10:903-918.

Baranao, J. L., and J. M. Hammond. 1984. Comparative effects of insulin and insulin-like growth factors on DNA synthesis and differentiation of porcine granulosa cells. Biochem. Biophys. Res. Commun. 124:484-490.

Beg, M. A., D. R. Bergfelt, K. Kot, M. C. Wiltbank, and O. J. Ginther. 2001. Follicular-fluid factors and granulosa-cell gene expression associated with follicle deviation in cattle. Biol. Reprod. 64:432-441.

Behrens, J., J. P. von Kries, M. Kuhl, L. Bruhn, D. Wedlich, R. Grosschedl, and W. Birchmeier. 1996. Functional interaction of beta-catenin with the transcription factor LEF-1. Nature 382:638-642.

Belchetz, P. E., T. M. Plant, Y. Nakai, E. J. Keogh, and E. Knobil. 1978. Hypophysial responses to continuous and intermittent delivery of hypopthalamic gonadotropin-releasing hormone. Science (New York, N.Y 202:631-633.

Bernkopf, D. B., M. V. Hadjihannas, and J. Behrens. 2014. Negative feedback regulation of the Wnt pathway by conductin/axin2 involves insensitivity to upstream signalling. J. Cell Sci.

Bhanot, P., M. Brink, C. H. Samos, J. C. Hsieh, Y. Wang, J. P. Macke, D. Andrew, J. Nathans, and R. Nusse. 1996. A new member of the frizzled family from Drosophila functions as a Wingless receptor. Nature 382:225-230.

Biggs, W. H., 3rd, J. Meisenhelder, T. Hunter, W. K. Cavenee, and K. C. Arden. 1999. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. Proc. Natl. Acad. Sci. U. S. A. 96:7421-7426.

Bilir, B., O. Kucuk, and C. S. Moreno. 2013. Wnt signaling blockage inhibits cell proliferation and migration, and induces apoptosis in triple-negative breast cancer cells. J Transl Med 11:280.

Boerboom, D., M. Paquet, M. Hsieh, J. Liu, S. P. Jamin, R. R. Behringer, J. Sirois, M. M. Taketo, and J. S. Richards. 2005. Misregulated Wnt/beta-catenin signaling leads to ovarian granulosa cell tumor development. Cancer Res. 65:9206-9215.

Boyer, A., A. K. Goff, and D. Boerboom. 2010. WNT signaling in ovarian follicle biology and tumorigenesis. Trends. Endocrinol. Metab. 21:25-32.

Bridges, G. A., M. L. Mussard, C. R. Burke, and M. L. Day. 2010. Influence of the length of proestrus on fertility and endocrine function in female cattle. Anim. Reprod. Sci. 117:208-215.

Brower, P. T., and R. M. Schultz. 1982. Intercellular communication between granulosa cells and mouse oocytes: existence and possible nutritional role during oocyte growth. Dev. Biol. 90:144-153.

Brunet, A., A. Bonni, M. J. Zigmond, M. Z. Lin, P. Juo, L. S. Hu, M. J. Anderson, K. C. Arden, J. Blenis, and M. E. Greenberg. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96:857-868.

Butcher, R. L., W. E. Collins, and N. W. Fugo. 1974. Plasma Concentration of LH, FSH, Prolactin, Progesterone and Estradiol-17β Throughout the 4-Day Estrous Cycle of the Rat. Endocrinology 94:1704-1708.

Cadigan, K. M., and R. Nusse. 1997. Wnt signaling: a common theme in animal development. Genes Dev. 11:3286-3305.

Camp, T. A., J. O. Rahal, and K. E. Mayo. 1991. Cellular localization and hormonal regulation of follicle-stimulating hormone and luteinizing hormone receptor messenger RNAs in the rat ovary. Molecular endocrinology (Baltimore, Md 5:1405-1417.

Castañon, B. I., A. D. Stapp, C. A. Gifford, L. J. Spicer, D. M. Hallford, and J. A. Hernandez Gifford. 2012. Follicle-stimulating hormone regulation of estradiol production: possible involvement of WNT2 and beta-catenin in bovine granulosa cells. J. Anim. Sci. 90:3789-3797.

Corner, G. W. 1938. The sites of formation of estrogenic substances in the animal body. Physiol. Rev. 18:154-172.

Costagliola, S., V. Panneels, M. Bonomi, J. Koch, M. C. Many, G. Smits, and G. Vassart. 2002. Tyrosine sulfation is required for agonist recognition by glycoprotein hormone receptors. The EMBO journal 21:504-513.

Couse, J. F., S. C. Hewitt, D. O. Bunch, M. Sar, V. R. Walker, B. J. Davis, and K. S. Korach. 1999. Postnatal sex reversal of the ovaries in mice lacking estrogen receptors alpha and beta. Science 286:2328-2331.

Cross, D. A., D. R. Alessi, P. Cohen, M. Andjelkovich, and B. A. Hemmings. 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378:785-789.

Daniels, D. L., and W. I. Weis. 2005. Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. Nat. Struct. Mol. Biol. 12:364-371.

Dohlman, H. G., M. G. Caron, and R. J. Lefkowitz. 1987. A family of receptors coupled to guanine nucleotide regulatory proteins. Biochemistry (Mosc). 26:2657-2664.

Erickson, B. H. 1966. Development and senescence of the postnatal bovine ovary. J. Anim. Sci. 25:800-805.

Essers, M. A., L. M. de Vries-Smits, N. Barker, P. E. Polderman, B. M. Burgering, and H. C. Korswagen. 2005. Functional interaction between beta-catenin and FOXO in oxidative stress signaling. Science (New York, N.Y 308:1181-1184.
Evans, A. C., C. M. Komar, S. A. Wandji, and J. E. Fortune. 1997. Changes in androgen secretion and luteinizing hormone pulse amplitude are associated with the recruitment and growth of ovarian follicles during the luteal phase of the bovine estrous cycle. Biol. Reprod. 57:394-401.
Fagotto, F., U. Gluck, and B. M. Gumbiner. 1998. Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of beta-catenin. Curr. Biol. 8:181-190.
Fair, T., J. S. C. Hulshof, P. Hyttel, T. Greve, and M. Boland. 1997. Oocyte ultrastructure in bovine primordial to early tertiary follicles. Anat. Embryol. (Berl). 195:327-336.
Fan, H. Y., A. O'Connor, M. Shitanaka, M. Shimada, Z. Liu, and J. S. Richards. 2010. Beta-catenin (CTNNB1) promotes preovulatory follicular development but represses LH-mediated ovulation and luteinization. Mol. Endocrinol. 24:1529-1542.
Fang, X., S. X. Yu, Y. Lu, R. C. Bast, Jr., J. R. Woodgett, and G. B. Mills. 2000. Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. Proc. Natl. Acad. Sci. U. S. A. 97:11960-11965.
Fitzpatrick, S. L., and J. S. Richards. 1991. Regulation of cytochrome P450 aromatase messenger ribonucleic acid and activity by steroids and gonadotropins in rat granulosa cells. Endocrinology 129:1452-1462.
Fitzpatrick, S. L., and J. S. Richards. 1994. Identification of a cyclic adenosine 3',5'-monophosphate-response element in the rat aromatase promoter that is required for transcriptional activation in rat granulosa cells and R2C leydig cells. Mol. Endocrinol. 8:1309-1319.
Fortune, J. E., G. M. Rivera, and M. Y. Yang. 2004. Follicular development: the role of the follicular microenvironment in selection of the dominant follicle. Anim. Reprod. Sci. 82-83:109-126.
Funayama, N., F. Fagotto, P. McCrea, and B. M. Gumbiner. 1995. Embryonic axis induction by the armadillo repeat domain of beta-catenin: evidence for intracellular signaling. The Journal of cell biology 128:959-968.
Gao, C., and Y. G. Chen. 2010. Dishevelled: The hub of Wnt signaling. Cell. Signal. 22:717-727.
Gao, X., and R. N. Hannoush. 2014. Single-cell imaging of Wnt palmitoylation by the acyltransferase porcupine. Nat. Chem. Biol. 10:61-68.
Giguere, V., N. Yang, P. Segui, and R. M. Evans. 1988. Identification of a new class of steroid hormone receptors. Nature 331:91-94.
Ginther, O. J., M. A. Beg, D. R. Bergfelt, F. X. Donadeu, and K. Kot. 2001. Follicle selection in monovular species. Biol. Reprod. 65:638-647.
Ginther, O. J., M. C. Wiltbank, P. M. Fricke, J. R. Gibbons, and K. Kot. 1996. Selection of the dominant follicle in cattle. Biol. Reprod. 55:1187-1194.
Gloaguen, P., P. Crepieux, D. Heitzler, A. Poupon, and E. Reiter. 2011. Mapping the follicle-stimulating hormone-induced signaling networks. Front. Endocrinol. 2:45.
Gonsalves, F. C., K. Klein, B. B. Carson, S. Katz, L. A. Ekas, S. Evans, R. Nagourney, T. Cardozo, A. M. Brown, and R. DasGupta. 2011. An RNAi-based chemical genetic screen identifies three small-molecule inhibitors of the Wnt/wingless signaling pathway. Proc. Natl. Acad. Sci. U. S. A. 108:5954-5963.
Gordon, M. D., and R. Nusse. 2006. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. J. Biol. Chem. 281:22429-22433.
Gorski, R. A. 1970. Localization of hypothalamic regulation of anterior pituitary function. Am. J. Anat. 129:219-222.
Green, J. D., and G. W. Harris. 1947. The neurovascular link between the neurohypophysis and

adenohypophysis. The Journal of endocrinology 5:136-146.
Greene, G. L., P. Gilna, M. Waterfield, A. Baker, Y. Hort, and J. Shine. 1986. Sequence and expression of human estrogen receptor complementary DNA. Science (New York, N.Y 231:1150-1154.
Gudermann, T., B. Nurnberg, and G. Schultz. 1995. Receptors and G proteins as primary components of transmembrane signal transduction. Part 1. G-protein-coupled receptors: structure and function. J. Mol. Med. 73:51-63.
Gummow, B. M., J. N. Winnay, and G. D. Hammer. 2003. Convergence of Wnt signaling and steroidogenic factor-1 (SF-1) on transcription of the rat inhibin alpha gene. J. Biol. Chem. 278:26572-26579.
Gupta, P. S., J. K. Folger, S. K. Rajput, L. Lv, J. Yao, J. J. Ireland, and G. W. Smith. 2014. Regulation and regulatory role of WNT signaling in potentiating FSH action during bovine dominant follicle selection. PLoS ONE 9:e100201.
Hanukoglu, I. 1992. Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone biosynthesis. J. Steroid Biochem. Molec. Biol. 43:779-804.
Hart, M., J. P. Concordet, I. Lassot, I. Albert, R. del los Santos, H. Durand, C. Perret, B. Rubinfeld, F. Margottin, R. Benarous, and P. Polakis. 1999. The F-box protein beta-TrCP associates with phosphorylated beta-catenin and regulates its activity in the cell. Curr. Biol. 9:207-210.
He, X., M. Semenov, K. Tamai, and X. Zeng. 2004. LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. Development (Cambridge, England) 131:1663-1677.
Hennet, M. L., and C. M. Combelles. 2012. The antral follicle: a microenvironment for oocyte differentiation. The International journal of developmental biology 56:819-831.
Hernandez Gifford, J. A. 2015. The role of WNT signaling in adult ovarian folliculogenesis. Reproduction.
Hernandez Gifford, J. A., M. E. Hunzicker-Dunn, and J. H. Nilson. 2009. Conditional deletion of beta-catenin mediated by Amhr2cre in mice causes female infertility. Biol. Reprod. 80:1282-1292.
Hsieh, M., M. A. Johnson, N. M. Greenberg, and J. S. Richards. 2002. Regulated expression of Wnts and Frizzleds at specific stages of follicular development in the rodent ovary. Endocrinology 143:898-908.
Hu, J., Z. Zhang, W. J. Shen, and S. Azhar. 2010. Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. Nutr. Metab. 7:47.
Huang, H., and X. He. 2008. Wnt/beta-catenin signaling: new (and old) players and new insights. Curr. Opin. Cell Biol. 20:119-125.
Huang, H. C., and P. S. Klein. 2004. The Frizzled family: receptors for multiple signal transduction pathways. Genome Biol 5:234.
Huber, A. H., W. J. Nelson, and W. I. Weis. 1997. Three-dimensional structure of the armadillo repeat region of beta-catenin. Cell 90:871-882.
Huber, O., R. Korn, J. McLaughlin, M. Ohsugi, B. G. Herrmann, and R. Kemler. 1996. Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. Mech. Dev. 59:3-10.
Hunzicker-Dunn, M., and E. T. Maizels. 2006. FSH signaling pathways in immature granulosa cells that regulate target gene expression: branching out from protein kinase A. Cell. Signal. 18:1351-1359.
Izadyar, F., E. Zeinstra, and M. M. Bevers. 1998. Follicle-stimulating hormone and growth hormone act differently on nuclear maturation while both enhance developmental competence of in vitro matured bovine oocytes. Mol. Reprod. Dev. 51:339-345.
Janda, C. Y., D. Waghray, A. M. Levin, C. Thomas, and K. C. Garcia. 2012. Structural basis of

Wnt recognition by Frizzled. Science (New York, N.Y 337:59-64.
Jho, E. H., T. Zhang, C. Domon, C. K. Joo, J. N. Freund, and F. Costantini. 2002. Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol. Cell. Biol. 22:1172-1183.
Kaipia, A., and A. J. Hsueh. 1997. Regulation of ovarian follicle atresia. Annu. Rev. Physiol. 59:349-363.
Kakar, S. S., C. H. Rahe, and J. D. Neill. 1993. Molecular cloning, sequencing, and characterizing the bovine receptor for gonadotropin releasing hormone (GnRH). Domest. Anim. Endocrinol. 10:335-342.
Karner, C., K. A. Wharton, Jr., and T. J. Carroll. 2006. Planar cell polarity and vertebrate organogenesis. Semin. Cell Dev. Biol. 17:194-203.
Khamsi, F., and S. Roberge. 2001. Granulosa cells of the cumulus oophorus are different from mural granulosa cells in their response to gonadotrophins and IGF-I. The Journal of Endocrinology 170:565-573.
Kimelman, D., and W. Xu. 2006. beta-catenin destruction complex: insights and questions from a structural perspective. Oncogene 25:7482-7491.
Kohn, A. D., and R. T. Moon. 2005. Wnt and calcium signaling: beta-catenin-independent pathways. Cell Calcium 38:439-446.
Komiya, Y., and R. Habas. 2008. Wnt signal transduction pathways. Organogenesis 4:68-75.
Krege, J. H., J. B. Hodgin, J. F. Couse, E. Enmark, M. Warner, J. F. Mahler, M. Sar, K. S. Korach, J. A. Gustafsson, and O. Smithies. 1998. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc. Natl. Acad. Sci. U. S. A. 95:15677-15682.
Krieghoff, E., J. Behrens, and B. Mayr. 2006. Nucleo-cytoplasmic distribution of beta-catenin is regulated by retention. J. Cell Sci. 119:1453-1463.
Kuiper, G. G., E. Enmark, M. Pelto-Huikko, S. Nilsson, and J. A. Gustafsson. 1996. Cloning of a novel receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. U. S. A. 93:5925-5930.
Lague, M. N., M. Paquet, H. Y. Fan, M. J. Kaartinen, S. Chu, S. P. Jamin, R. R. Behringer, P. J. Fuller, A. Mitchell, M. Dore, L. M. Huneault, J. S. Richards, and D. Boerboom. 2008. Synergistic effects of Pten loss and WNT/CTNNB1 signaling pathway activation in ovarian granulosa cell tumor development and progression. Carcinogenesis 29:2062-2072.
Lala, D. S., D. A. Rice, and K. L. Parker. 1992. Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor I. Molecular endocrinology (Baltimore, Md 6:1249-1258.
Laviola, L., A. Natalicchio, and F. Giorgino. 2007. The IGF-I signaling pathway. Curr. Pharm. Des. 13:663-669.
Law, N. C., J. Weck, B. Kyriss, J. H. Nilson, and M. Hunzicker-Dunn. 2013. Lhcgr expression in granulosa cells: roles for PKA-phosphorylated beta-catenin, TCF3, and FOXO1. Mol. Endocrinol. 27:1295-1310.
Levine, J. E., R. L. Norman, P. M. Gliessman, T. T. Oyama, D. R. Bangsberg, and H. G. Spies. 1985. In vivo gonadotropin-releasing hormone release and serum luteinizing hormone measurements in ovariectomized, estrogen-treated rhesus macaques. Endocrinology 117:711-211.
Li, L., S. Y. Ji, J. L. Yang, X. X. Li, J. Zhang, Y. Zhang, Z. Y. Hu, and Y. X. Liu. 2014. Wnt/beta-catenin signaling regulates follicular development by modulating the expression of Foxo3a signaling components. Mol. Cell. Endocrinol. 382:915-925.
Li, V. S., S. S. Ng, P. J. Boersema, T. Y. Low, W. R. Karthaus, J. P. Gerlach, S. Mohammed, A. J. Heck, M. M. Maurice, T. Mahmoudi, and H. Clevers. 2012. Wnt signaling through

inhibition of beta-catenin degradation in an intact Axin1 complex. Cell 149:1245-1256.
Liu, C., Y. Li, M. Semenov, C. Han, G. H. Baeg, Y. Tan, Z. Zhang, X. Lin, and X. He. 2002. Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell 108:837-847.
Liu, Z., M. D. Rudd, I. Hernandez-Gonzalez, I. Gonzalez-Robayna, H. Y. Fan, A. J. Zeleznik, and J. S. Richards. 2009. FSH and FOXO1 regulate genes in the sterol/steroid and lipid biosynthetic pathways in granulosa cells. Molecular endocrinology (Baltimore, Md 23:649-661.
Logan, C. Y., and R. Nusse. 2004. The Wnt signaling pathway in development and disease. Annu. Rev. Cell. Dev. Biol. 20:781-810.
Lubahn, D. B., J. S. Moyer, T. S. Golding, J. F. Couse, K. S. Korach, and O. Smithies. 1993. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc. Natl. Acad. Sci. U. S. A. 90:11162-11166.
Mani, A. M., M. A. Fenwick, Z. Cheng, M. K. Sharma, D. Singh, and D. C. Wathes. 2010. IGF1 induces up-regulation of steroidogenic and apoptotic regulatory genes via activation of phosphatidylinositol-dependent kinase/AKT in bovine granulosa cells. Reproduction 139:139-151.
Mao, J., J. Wang, B. Liu, W. Pan, G. H. Farr, 3rd, C. Flynn, H. Yuan, S. Takada, D. Kimelman, L. Li, and D. Wu. 2001. Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. Mol. Cell 7:801-809.
Mason, J. O., J. Kitajewski, and H. E. Varmus. 1992. Mutational analysis of mouse Wnt-1 identifies two temperature-sensitive alleles and attributes of Wnt-1 protein essential for transformation of a mammary cell line. Mol. Biol. Cell 3:521-533.
McCrea, P. D., C. W. Turck, and B. Gumbiner. 1991. A homolog of the armadillo protein in Drosophila (plakoglobin) associated with E-cadherin. Science (New York, N.Y 254:1359-1361.
Mendelson, C. R., B. Jiang, J. M. Shelton, J. A. Richardson, and M. M. Hinshelwood. 2005. Transcriptional regulation of aromatase in placenta and ovary. J. Steroid Biochem. Molec. Biol. 95:25-33.
Michael, M. D., M. W. Kilgore, K. Morohashi, and E. R. Simpson. 1995. Ad4BP/SF-1 regulates cyclic AMP-induced transcription from the proximal promoter (PII) of the human aromatase P450 (CYP19) gene in the ovary. J. Biol. Chem. 270:13561-13566.
Michael, M. D., L. F. Michael, and E. R. Simpson. 1997. A CRE-like sequence that binds CREB and contributes to cAMP-dependent regulation of the proximal promoter of the human aromatase P450 (CYP19) gene. Mol. Cell. Endocrinol. 134:147-156.
Mihm, M., and A. C. Evans. 2008. Mechanisms for dominant follicle selection in monovulatory species: a comparison of morphological, endocrine and intraovarian events in cows, mares and women. Reprod. Domest. Anim. 43 Suppl 2:48-56.
Miller, W. L., and R. J. Auchus. 2011. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. Endocr. Rev. 32:81-151.
Mirakhori, F., B. Zeynali, A. P. Tafreshi, and A. Shirmohammadian. 2013. Lithium induces follicular atresia in rat ovary through a GSK-3beta/beta-catenin dependent mechanism. Mol. Reprod. Dev. 80:286-296.
Mizusaki, H., K. Kawabe, T. Mukai, E. Ariyoshi, M. Kasahara, H. Yoshioka, A. Swain, and K. Morohashi. 2003. Dax-1 (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1) gene transcription is regulated by wnt4 in the female developing gonad. Mol. Endocrinol. 17:507-519.
Molenaar, M., M. van de Wetering, M. Oosterwegel, J. Peterson-Maduro, S. Godsave, V. Korinek, J. Roose, O. Destree, and H. Clevers. 1996. XTcf-3 transcription factor

mediates beta-catenin-induced axis formation in Xenopus embryos. Cell 86:391-399.
Morohashi, K., S. Honda, Y. Inomata, H. Handa, and T. Omura. 1992. A common trans-acting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s. J. Biol. Chem. 267:17913-17919.
Mosselman, S., J. Polman, and R. Dijkema. 1996. ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett. 392:49-53.
Nusse, R., and H. E. Varmus. 1982. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell 31:99-109.
Nusslein-Volhard, C., and E. Wieschaus. 1980. Mutations affecting segment number and polarity in Drosophila. Nature 287:795-801.
Ogawa, W., T. Matozaki, and M. Kasuga. 1998. Role of binding proteins to IRS-1 in insulin signalling. Mol. Cell. Biochem. 182:13-22.
Ogryzko, V. V., R. L. Schiltz, V. Russanova, B. H. Howard, and Y. Nakatani. 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 87:953-959.
Ozawa, M., H. Baribault, and R. Kemler. 1989. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. EMBO J. 8:1711-1717.
Palacios, J., and C. Gamallo. 1998. Mutations in the beta-catenin gene (CTNNB1) in endometrioid ovarian carcinomas. Cancer Res. 58:1344-1347.
Parakh, T. N., J. A. Hernandez, J. C. Grammer, J. Weck, M. Hunzicker-Dunn, A. J. Zeleznik, and J. H. Nilson. 2006. Follicle-stimulating hormone/cAMP regulation of aromatase gene expression requires beta-catenin. Proc. Natl. Acad. Sci. U. S. A. 103:12435-12440.
Park, Y., E. T. Maizels, Z. J. Feiger, H. Alam, C. A. Peters, T. K. Woodruff, T. G. Unterman, E. J. Lee, J. L. Jameson, and M. Hunzicker-Dunn. 2005. Induction of cyclin D2 in rat granulosa cells requires FSH-dependent relief from FOXO1 repression coupled with positive signals from Smad. J. Biol. Chem. 280:9135-9148.
Peifer, M., S. Berg, and A. B. Reynolds. 1994a. A repeating amino acid motif shared by proteins with diverse cellular roles. Cell 76:789-791.
Peifer, M., L. M. Pai, and M. Casey. 1994b. Phosphorylation of the Drosophila adherens junction protein Armadillo: roles for wingless signal and zeste-white 3 kinase. Dev. Biol. 166:543-556.
Peifer, M., C. Rauskolb, M. Williams, B. Riggleman, and E. Wieschaus. 1991. The segment polarity gene armadillo interacts with the wingless signaling pathway in both embryonic and adult pattern formation. Development (Cambridge, England) 111:1029-1043.
Peifer, M., D. Sweeton, M. Casey, and E. Wieschaus. 1994c. wingless signal and Zeste-white 3 kinase trigger opposing changes in the intracellular distribution of Armadillo. Development (Cambridge, England) 120:369-380.
Peter, M., and J. M. Dubuis. 2000. Transcription factors as regulators of steroidogenic P-450 enzymes. Eur. J. Clin. Invest. 30 Suppl 3:14-20.
Peters, A., and E. Lamming. 1983. Hormone patterns and reproduction in cattle. In Pract. 5:153-158.
Pierce, J. G., and T. F. Parsons. 1981. Glycoprotein hormones: structure and function. Annu. Rev. Biochem. 50:465-495.
Popa, G. 1930. A Portal Circulation from the Pituitary to the Hypothalamic Region. J. Anat. 65:88-91.
Rahe, C. H., R. E. Owens, J. L. Fleeger, H. J. Newton, and P. G. Harms. 1980. Pattern of plasma luteinizing hormone in the cyclic cow: dependence upon the period of the cycle. Endocrinology 107:498-503.
Richards, J. S., S. C. Sharma, A. E. Falender, and Y. H. Lo. 2002. Expression of FKHR,

FKHRL1, and AFX genes in the rodent ovary: evidence for regulation by IGF-I, estrogen, and the gonadotropins. Mol. Endocrinol. 16:580-599.
Ricken, A., P. Lochhead, M. Kontogiannea, and R. Farookhi. 2002. Wnt signaling in the ovary: identification and compartmentalized expression of wnt-2, wnt-2b, and frizzled-4 mRNAs. Endocrinology 143:2741-2749.
Rinderknecht, E., and R. E. Humbel. 1978. The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. J. Biol. Chem. 253:2769-2776.
Rodgers, R. J., and H. F. Irving-Rodgers. 2010. Morphological classification of bovine ovarian follicles. Reproduction (Cambridge, England) 139:309-318.
Ryan, K. E., S. M. Casey, M. J. Canty, M. A. Crowe, F. Martin, and A. C. Evans. 2007. Akt and Erk signal transduction pathways are early markers of differentiation in dominant and subordinate ovarian follicles in cattle. Reproduction 133:617-626.
Ryan, K. E., C. Glister, P. Lonergan, F. Martin, P. G. Knight, and A. C. Evans. 2008. Functional significance of the signal transduction pathways Akt and Erk in ovarian follicles: in vitro and in vivo studies in cattle and sheep. J. Ovarian. Res. 1:2.
Sagae, S., K. Kobayashi, Y. Nishioka, M. Sugimura, S. Ishioka, M. Nagata, K. Terasawa, T. Tokino, and R. Kudo. 1999. Mutational analysis of beta-catenin gene in Japanese ovarian carcinomas: frequent mutations in endometrioid carcinomas. Jap. J. Cancer Res. 90:510-515.
Schally, A. V., A. Arimura, Y. Baba, R. M. Nair, H. Matsuo, T. W. Redding, and L. Debeljuk. 1971. Isolation and properties of the FSH and LH-releasing hormone. Biochem. Biophys. Res. Commun. 43:393-399.
Siegel, R. L., K. D. Miller, and A. Jemal. 2015. Cancer statistics, 2015. CA. Cancer J. Clin. 65:5-29.
Silva, J. M., and C. A. Price. 2000. Effect of follicle-stimulating hormone on steroid secretion and messenger ribonucleic acids encoding cytochromes P450 aromatase and cholesterol side-chain cleavage in bovine granulosa cells in vitro. Biol. Reprod. 62:186-191.
Simoni, M., J. Gromoll, and E. Nieschlag. 1997. The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. Endocr. Rev. 18:739-773.
Simpson, E. R., M. S. Mahendroo, G. D. Means, M. W. Kilgore, M. M. Hinshelwood, S. Graham-Lorence, B. Amarneh, Y. Ito, C. R. Fisher, M. D. Michael, and et al. 1994. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr. Rev. 15:342-355.
Sirotkin, A. V., R. Alexa, P. Dekanova, A. Kadasi, A. Stochmalova, R. Grossmann, S. H. Alwasel, and A. H. Harrath. 2015. The mTOR System Can Affect Basic Ovarian cell Functions and Mediate the Effect of Ovarian Hormonal Regulators. International Journal of Pharmacology 11:570-578.
Spicer, L. J., and P. Y. Aad. 2007. Insulin-like growth factor (IGF) 2 stimulates steroidogenesis and mitosis of bovine granulosa cells through the IGF1 receptor: role of follicle-stimulating hormone and IGF2 receptor. Biol. Reprod. 77:18-27.
Spicer, L. J., C. S. Chamberlain, and S. M. Maciel. 2002. Influence of gonadotropins on insulin- and insulin-like growth factor-I (IGF-I)-induced steroid production by bovine granulosa cells. Domest. Anim. Endocrinol. 22:237-254.
Staal, F. J., M. Noort Mv, G. J. Strous, and H. C. Clevers. 2002. Wnt signals are transmitted through N-terminally dephosphorylated beta-catenin. EMBO Rep 3:63-68.
Stapp, A. D., C. A. Gifford, D. M. Hallford, and J. A. Gifford. 2014a. Evaluation of steroidogenic capacity after follicle stimulating hormone stimulation in bovine granulosa cells of Revalor 200(R) implanted heifers. J Anim Sci Biotechnol 5:2.

Stapp, A. D., B. I. Gomez, C. A. Gifford, D. M. Hallford, and J. A. Hernandez Gifford. 2014b. Canonical WNT signaling inhibits follicle stimulating hormone mediated steroidogenesis in primary cultures of rat granulosa cells. PLoS ONE 9:e86432.

Steinkampf, M. P., C. R. Mendelson, and E. R. Simpson. 1987. Regulation by follicle-stimulating hormone of the synthesis of aromatase cytochrome P-450 in human granulosa cells. Molecular endocrinology (Baltimore, Md 1:465-471.

Steinkampf, M. P., C. R. Mendelson, and E. R. Simpson. 1988. Effects of epidermal growth factor and insulin-like growth factor I on the levels of mRNA encoding aromatase cytochrome P-450 of human ovarian granulosa cells. Mol. Cell. Endocrinol. 59:93-99.

Takemaru, K. I., and R. T. Moon. 2000. The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression. The Journal of Cell Biology 149:249-254.

Tamai, K., M. Semenov, Y. Kato, R. Spokony, C. Liu, Y. Katsuyama, F. Hess, J. P. Saint-Jeannet, and X. He. 2000. LDL-receptor-related proteins in Wnt signal transduction. Nature 407:530-535.

Terasawa, E., J. S. Rodriguez, W. E. Bridson, and S. J. Wiegand. 1979. Factors influencing the positive feedback action of estrogen upon the luteinizing hormone surge in the ovariectomized guinea pig. Endocrinology 104:680-686.

Tomizuka, K., K. Horikoshi, R. Kitada, Y. Sugawara, Y. Iba, A. Kojima, A. Yoshitome, K. Yamawaki, M. Amagai, A. Inoue, T. Oshima, and M. Kakitani. 2008. R-spondin1 plays an essential role in ovarian development through positively regulating Wnt-4 signaling. Hum. Mol. Genet. 17:1278-1291.

Tremblay, G. B., A. Tremblay, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, F. Labrie, and V. Giguere. 1997. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. Molecular endocrinology (Baltimore, Md 11:353-365.

Ulloa-Aguirre, A., A. Uribe, T. Zarinan, I. Bustos-Jaimes, M. A. Perez-Solis, and J. A. Dias. 2007. Role of the intracellular domains of the human FSH receptor in G(alphaS) protein coupling and receptor expression. Mol. Cell. Endocrinol. 260-262:153-162.

Vainio, S., M. Heikkila, A. Kispert, N. Chin, and A. P. McMahon. 1999. Female development in mammals is regulated by Wnt-4 signalling. Nature 397:405-409.

van Beest, M., D. Dooijes, M. van De Wetering, S. Kjaerulff, A. Bonvin, O. Nielsen, and H. Clevers. 2000. Sequence-specific high mobility group box factors recognize 10-12-base pair minor groove motifs. J. Biol. Chem. 275:27266-27273.

van de Wetering, M., R. Cavallo, D. Dooijes, M. van Beest, J. van Es, J. Loureiro, A. Ypma, D. Hursh, T. Jones, A. Bejsovec, M. Peifer, M. Mortin, and H. Clevers. 1997. Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. Cell 88:789-799.

van den Heuvel, M., R. Nusse, P. Johnston, and P. A. Lawrence. 1989. Distribution of the wingless gene product in Drosophila embryos: a protein involved in cell-cell communication. Cell 59:739-749.

van Dessel, H. J., I. Schipper, T. D. Pache, H. van Geldorp, F. H. de Jong, and B. C. Fauser. 1996. Normal human follicle development: an evaluation of correlations with oestradiol, androstenedione and progesterone levels in individual follicles. Clin. Endocrinol. (Oxf). 44:191-198.

Vassilatis, D. K., J. G. Hohmann, H. Zeng, F. Li, J. E. Ranchalis, M. T. Mortrud, A. Brown, S. S. Rodriguez, J. R. Weller, A. C. Wright, J. E. Bergmann, and G. A. Gaitanaris. 2003. The G protein-coupled receptor repertoires of human and mouse. Proc. Natl. Acad. Sci. U. S. A. 100:4903-4908.

Waltzer, L., and M. Bienz. 1998. Drosophila CBP represses the transcription factor TCF to antagonize Wingless signalling. Nature 395:521-525.

Wang, H. X., T. Y. Li, and G. M. Kidder. 2010. WNT2 regulates DNA synthesis in mouse

granulosa cells through beta-catenin. Biol. Reprod. 82:865-875.
Weiderpass, E., H. O. Adami, J. A. Baron, C. Magnusson, R. Bergstrom, A. Lindgren, N. Correia, and I. Persson. 1999. Risk of endometrial cancer following estrogen replacement with and without progestins. J. Natl. Cancer Inst. 91:1131-1137.
Wettemann, R. P., H. D. Hafs, L. A. Edgerton, and L. V. Swanson. 1972. Estradiol and progesterone in blood serum during the bovine estrous cycle. J. Anim. Sci. 34:1020-1024.
Wieschaus, E., C. Nüsslein-Volhard, and G. Jürgens. 1984. Mutations affecting the pattern of the larval cuticle in Drosophila melanogaster. III. Zygotic loci on the X-chromosome and fourth chromosome. Wilhelm Roux' Archiv 193:296-307.
Willert, K., J. D. Brown, E. Danenberg, A. W. Duncan, I. L. Weissman, T. Reya, J. R. Yates, 3rd, and R. Nusse. 2003. Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature 423:448-452.
Yager, J. D., and N. E. Davidson. 2006. Estrogen carcinogenesis in breast cancer. The New England journal of medicine 354:270-282.
Yamaji, T., D. J. Dierschke, A. N. Bhattacharya, and E. Knobil. 1972. The negative feedback control by estradiol and progesterone of LH secretion in the ovariectomized rhesus monkey. Endocrinology 90:771-777.
Yin, Y., and L. Ma. 2005. Development of the mammalian female reproductive tract. J. Biochem. 137:677-683.
Young, J. M., and A. S. McNeilly. 2010. Theca: the forgotten cell of the ovarian follicle. Reproduction 140:489-504.
Zeleznik, A. J., D. Saxena, and L. Little-Ihrig. 2003. Protein kinase B is obligatory for follicle-stimulating hormone-induced granulosa cell differentiation. Endocrinology 144:3985-3994.
Zeng, X., K. Tamai, B. Doble, S. Li, H. Huang, R. Habas, H. Okamura, J. Woodgett, and X. He. 2005. A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. Nature 438:873-877.
Zhou, P., S. C. Baumgarten, Y. Wu, J. Bennett, N. Winston, J. Hirshfeld-Cytron, and C. Stocco. 2013. IGF-I signaling is essential for FSH stimulation of AKT and steroidogenic genes in granulosa cells. Mol. Endocrinol. 27:511-523.

VITA

Belinda Irene Gómez

Candidate for the Degree of

Doctor of Philosophy

Thesis: FSH AND IGF-1 SIGNAL VIA AKT TO REGULATE BETA-CATENIN ACCUMULATION AND ESTRADIOL PRODUCTION IN GRANULOSA CELLS

Major Field: Animal Science

Biographical:

Education:

Doctor of Philosophy in Animal Science at Oklahoma State University, Stillwater, Oklahoma in May, 2016.

Master of Science in Animal Science at Oklahoma State University, Stillwater, Oklahoma in 2012.

Bachelor of Science in Animal Science at West Texas A&M University, Canyon, Texas in 2010.

Professional Memberships:

American Society of Animal Science, Animal Science Graduate Student Association, Western Section American Society of Animal Science

PhD Funding Source:

USDA National Needs Fellowship 2013-2016

Awards:

OSU Animal Science Outstanding Graduate Student 2016, OSU Distinguished Graduate Fellowship 2013-2016, Honorary Graduate Commencement Marshal Fall 2015, Ned and Essie Raun International Graduate Student Scholarship 2014, 1st place for Joe Whiteman Outstanding Graduate Student Scientific Paper Award 2015, 2nd place for American Society of Animal Science Western Section Graduate Student Competition 2015, OSU Women's Faculty Council Research Award 2012

Conferences Presented:

Western Section of the American Society of Animal Science 2015
OSU CASNR 3 Minute Thesis 2015
OSU 26th Annual Research Symposium, Whiteman Competition 2015
American Society of Animal Science, Joint Annual Meeting 2014