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# OBESITY, METABOLIC HORMONE SIGNALING, AND GRANULOSA CELL GENE EXPRESSION

by

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# OBESITY, METABOLIC HORMONE SIGNALING, AND GRANULOSA CELL GENE EXPRESSION

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University of Nebraska, 2010

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It has become increasingly clear that female obesity is associated with a myriad of adverse side effects including abnormal female reproduction due, in part, to amenorrhea and anovulatory infertility. The lethal yellow (LY) mouse possesses a deletion mutation which results in ectopic expression of agouti and adult-onset obesity. Furthermore, LY mice exhibit premature loss of fertility, which has been associated with progressive obesity making the LY mouse line an excellent model to study the effects of obesity-dependent factors on ovarian function. In the current study blood serum and granulosa cells were obtained from LY ($A^y$/a) and age-matched B6 controls (C57BL/6J) to identify changes in metabolic hormone profiles and gene expression, respectively. As expected, LY females exhibited higher circulating levels of insulin and leptin compared to age-matched B6 controls. For the first time, we identified a significant increase in circulating insulin like-growth factor-1 (IGF-1) levels in the LY compared to B6 at 6 weeks of age. Despite these differences in circulating hormone levels, there was little evidence that gene expression is altered in age-matched granulosa cells from LY and B6 females. However, age-dependent changes in the expression of several genes involved in follicular growth in both LY and B6 females were detected.

Given that IGF-1 exhibited increased levels in LY compared to B6 mice at 6 weeks of age, the objective of our in vitro study was to determine the role of IGF-1 on granulosa cell gene expression. To this end, short-term granulosa cell cultures were treated with cAMP (the second messenger of both FSH and LH signaling), IGF-1, or a combination of both. IGF-1 had an additive effect on cAMP-dependent regulation of a subset of genes involved in follicular growth, bi-directional communication, steroidogenesis, and ovulation. Western blot analyses provided evidence that the additive effect of IGF-1 on cAMP regulation of gene expression is mediated by stimulation of Akt phosphorylation. Thus, the cooperative effect of IGF-1 on FSH- and LH-dependent signaling may enhance the expression of genes which are crucial for optimal follicular growth and ovulation. Furthermore, these collective data provide a plausible mechanism for age and obesity-dependent anovulatory infertility.

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# CHAPTER 1

## Literature Review

### Nutrition and Female Reproductive Health

Obesity is undoubtedly a growing problem in the United States and around the world. Since 1980 obesity has increased worldwide more than 75% with over one billion adults considered to be overweight or obese (1). In general, obesity results from a chronic disruption of energy balance. Specifically, when energy intake exceeds energy expenditure, lipid storage and carbohydrate oxidation increases and ultimately results in the expansion of existing fat cells and an increased number of fat cells (1). This increase in mature fat cell numbers is due to the differentiation of pre-adipocytes to adipocytes resulting from the enhanced deposit of trigylcerides into adipocytes (2). According to the American Obesity Association 62% of women aged 20-74 are overweight [body mass index (BMI) $\geq$ 25] whereas 34% of those women are obese (BMI $\geq$ 30). Furthermore, studies report that between 18.5 - 38.3% of pregnant US women are obese. Obesity, either directly or indirectly, has a negative impact on several physiological systems including the cardiovascular, renal, and metabolic systems.

Furthermore, it is becoming increasingly clear that female obesity is associated with a myriad of adverse side effects linked to reproduction. For example, prior to pregnancy, obese women have higher rates of amenorrhea and anovulatory infertility. In a Nurses Health Study, obese women had a 2.7 times higher risk of infertility problems compared to normal weight women (3).

*Complications Associated with Obesity:* Obesity during pregnancy is also associated with clinical complications, which affect both the mother and the fetus. Obese women have a 25-37% higher risk of miscarriage compared to normal weight women (4). Furthermore, overweight women have a 1.8 to 6.5 times greater risk while obese women have a 1 to 20 times greater risk of developing gestational diabetes mellitus (GDM) compared to normal weight women. Other maternal complications linked to obesity include higher arterial blood pressure and altered cardiac function (3). Obese mothers also have 2.2 to 21.4 times greater prevalence of hypertensive disorders and 1.2 to 9.7 times higher occurrence of preeclampsia (5). The frequency of induced labor and caesarean sections is increased in obese women with 50% of deliveries being by caesarean in severely obese women (BMI $\geq$ 40) (6). The most common indications for caesarean delivery in this subset of women included failed cervical dilation, fetal distress, and risk of shoulder dystocia (6).

Along with the maternal complications, the infants of obese mothers can have potentially serious problems as well. Obese mothers deliver large-for-gestational-age infants 1 to 18 times more often than normal weight mothers. This increase in birth weight has been linked to additional skinfold thickness suggesting that the elevation in birth weight is due to a larger fat mass (7). Macrosomia, in turn, can result in other complications including increased risk of shoulder dystocia, birth injury, and perinatal death (6). Infants of overweight and obese mothers also face a considerable increased risk for congenital abnormalities [35% and 37.5%, respectively] (8). However, one of the most substantial problems is the increased risk for perinatal death. For overweight mothers, this incidence is increased by 1.1 to 2.5 fold and for obese women by 2.5 to 3.4

fold compared to normal weight women. Interestingly, in domestic livestock, neonatal mortality is also increased in cattle and sheep that are overweight [body condition score (BCS) ≥ 4] (9).

*Complications Associated with Decreased Energy Balance:* Women as well as female domestic livestock also have reproductive health issues when underweight. Nearly all studies regarding underweight women (BMI < 18.5) and pregnancy complications involve women who have eating disorders, specifically anorexia and bulimia. While several studies have been carried out, there are conflicting results between them. Bulik *et al*. reported a higher instance of miscarriage, lower birth weights, more premature births, and more cesarean sections when anorexic patients were compared to normal weight women (10). Using a similar comparison, Stewart *et al*. (11) also demonstrated lower Apgar scores and lower birth weights. Conversely, Franko *et al.* did not find any differences in Apgar scores and birth weights between underweight and normal weight mothers (12). Despite the discrepancies between these studies, conclusive results regarding underweight women include an increase in the rates/risk of anovulatory infertility, miscarriage, caesarian sections, and postpartum depression (10, 12-14). In domestic livestock species, females that are underweight have similar reproductive health issues. Specifically, cattle, upon severe reduction in their body weight, exhibit cessation of estrous cycles, quiescent ovaries (i.e. lack of follicular development), a higher incidence of embryo mortality, and decreased birth weights of viable offspring (8). Likewise, underweight ewes produce lambs with reduced birth weights compared to normal-weight ewes (8).

*Overeight and Underweight women Experience Increased Costs:* Not only does increased or decreased maternal weight produce these adverse side affects during a women's reproductive lifespan, the cost for gynecologic, obstetric, and neonatal care is also greater for overweight and underweight mothers compared to their normal weight counterparts. A study conducted by Galtier *et al* followed 435 women during and after their pregnancy and the duration of nighttime and daytime hospitalizations were recorded. Overall, the total cost for women who were overweight prior to pregnancy was 5 times higher than normal weight controls. The increase in total cost was primarily due to increased night and daytime hospitalizations, increased cost of pre and post-natal care, and an increased number of infants requiring admission into the neonatal intensive care unit (5). In addition to these costs associated with an established pregnancy, the increased incidence of anovulatory infertility in overweight or underweight females may result in an increased need for assisted reproductive technologies, which also has a significant associated cost. This problem is not unique in human medicine but also impacts the economics of the domestic livestock industry. Specifically, if the reproductive performance of female animals is reduced due to fluctuations in energy balance, they will not be producing profit-generating offspring. The producer will also have to bear the cost of replacing the animal within the herd.

Given all of the complications and costs associated with fertility and pregnancy that overweight and obese women face; the most obvious question is what is the underlying cause and how can it be reversed? While pregnancy loss and infertility is complex and the result of multiple factors, this review will focus on the process of

folliculogenesis and the development of an oocyte with high developmental competence, which plays a crucial role in reproductive success.

## Folliculogenesis

Before evaluating what factors reduce oocyte quality in obese individuals, we must first understand how a mature and developmentally competent oocyte is ovulated. Growth and development of the oocyte takes place in intimate contact with somatic cells of the ovary, which collectively represent the follicle. The process by which a follicle is recruited, matured, and subsequently ovulated is known as folliculogenesis. The major steps involved in the process of ovarian folliculogenesis (Figure 1.1) include: (1) the formation of the quiescent pool of primordial follicles; (2) the recruitment and selection of primordial follicles for growth and development resulting in the progression of follicles through the primary, secondary, antral, and preovulatory stages; and (3) ovulation and the formation of a corpus luteum (CL) from residual somatic cells of the follicle (15).

*Establishment of the Primordial Follicle Pool and Initiation of Follicular Growth:* Current dogma indicates that at birth, a female's primordial pool contains all the oocytes that will ever be produced. However, it should be noted that Johnson *et al*. have recently challenged this dogma by providing evidence that mouse ovaries contain proliferative germ cells that sustain oocyte and follicle production in the postnatal mammalian ovary (16). Initially, oocytes are present in the ovary as germ cell clusters.

These clusters subsequently undergo breakdown and individual germ cells are surrounded by squamous pre-granulosa cells. Primordial follicles form 1-2 days after birth in mice and *in utero* in humans (15). In domestic livestock, there are species-dependent differences with the development of the primordial pool, occurring *in utero* (cattle, sheep) in some animals and during the neonatal period (horses, pigs) in other animals (17). Primordial follicles remain essentially quiescent with a small number of primordial follicles cyclically selected to grow (follicle activation) (18) and enter into the growing pool from puberty through menopause (19). The transition of primordial to primary follicles is characterized by the morphological change in granulosa cells from squamous to cubodial. The follicle has now entered the stage of preantral folliculogenesis, which is characterized by oocyte growth, granulosa cell proliferation, and the addition of a theca somatic cell layer (20). The growth of preantral follicles is primarily intraovarian which means it is dependent on autocrine and paracrine regulatory factors and is largely independent of gonadotropin (i.e. follicle stimulating hormone (FSH) and luteinizing hormone (LH) signaling. This is confirmed by studies in mice deficient in the FSH receptor which have normal preantral follicle growth (21). Once follicles have achieved two layers of granulosa cells, the theca then differentiates into the outermost layer of the follicle (20). The follicle now consists of the oocyte surrounded by multiple layers of cubodial granulosa cells, a theca interna, which is located just outside the basement membrane surrounding the granulosa cells, and the theca externa.

*Antral Follicle Growth and Ovulation:* The antral stage of folliculogenesis begins once the regulation of follicular growth becomes extraovarian (i.e. gonadotropin-driven)

as opposed to intraovarian (i.e. paracrine factor driven). Many changes take place during this stage including the formation of a single antral cavity that separates two distinct granulosa cell populations. Mural granulosa cells line the wall of the follicle and are vital for steroidogenesis and ovulation, while cumulus granulosa cells remain in intimate contact with the oocyte and facilitate oocyte growth and acquisition of developmental competence (15). Antral to preovulatory folliculogenesis is primarily dependent on FSH and LH signaling. Specifically FSH prevents granulosa cell apoptosis and follicular atresia (22), and promotes granulosa cell proliferation, estradiol production, and LH receptor expression (23). Luteinizing hormone regulation is coordinated with FSH for antrum formation while the LH surge promotes ovulation of a fully grown and mature oocyte (15).

The majority of preantral and antral follicles will undergo atresia, whereas a small number will reach the preovulatory stage. Follicles that escape atresia likely have higher responsiveness to FSH due to increased follicle stimulating hormone receptor (FSHR) expression in the granulosa cells of that follicle (15). As follicles are progressing to the pre-ovulatory stage, rising estradiol levels suppress pituitary FSH release. Conversely, as estradiol levels reach a threshold level, pituitary LH secretion is enhanced, resulting in the LH surge, which is critical for the conclusion of folliculogenesis. Specifically, the LH surge results in oocyte meiotic resumption, cumulus expansion, and ovulation of a mature, developmentally competent oocyte (24). The granulosa and theca cells retained in the follicle then undergo differentiation to form the corpus luteum (CL), which is responsible for the production of progesterone, a hormone that is crucial for a viable pregnancy (15).

## Hormone and Paracrine Factor Regulation of Folliculogenesis

### Paracrine Factors

The processes of folliculogenesis and ovulation of a developmentally competent oocyte are complex and require the expression and interplay of many genes and signaling pathways. The expression and activity of these genes and pathways are regulated by several endocrine hormones and paracrine factors. As stated earlier, the regulation of folliculogenesis can essentially be split into two categories, (1) intraovarian regulation during preantral follicle growth and (2) gonadotropin-dependent regulation during antral follicle growth. The major paracrine factors which regulate preantral follicle growth include kit ligand (*Kitl*), anti-müllerian hormone (*Amh*), and growth differentiation factor-9 (*Gdf9*).

*Kit Ligand: Kitl* is expressed in pre-granulosa and granulosa cells throughout folliculogenesis, but the interactions between KITL and KIT, which is a tyrosine kinase receptor expressed by the oocyte, appear to be most crucial during early folliculogenesis (15). This observation is based on the results of several studies conducted using the Steel Panda ($Sl^{pan}$) and Steel Contrasted ($Sl^{con}$) mouse lines. The $Sl^{pan}$ and $Sl^{con}$ mutations result in the reduced expression of *Kitl* transcripts in both male and female gonads. This decrease in *Kitl* expression leads to early arrest or increased atresia of ovarian follicles (15, 25). Additional i*n vivo* and *in vitro* studies support the idea that KIT-KITL signaling is important for early follicular growth. For example, when newborn mice are injected with ACK2 (an antibody to KIT, which blocks its interaction with KITL), follicular

growth is blocked resulting in an ovary populated with only primordial stage follicles. Conversely, when neonatal rat ovaries were treated in whole organ culture with recombinant KITL, there is an acceleration of the primordial to primary follicle transition, resulting in an increased number of growing follicles (26). Collectively, these studies indicate the *Kitl* is crucial for the transition from primordial to primary follicles and the initiation of follicle development (15).

*Anti-Mullerian Hormone: Amh* expression in granulosa cells is detected in primary follicles, with the highest expression occurring in the granulosa cells of preantral and early antral stage follicles (27). Amh is known to inhibit two major steps in folliculogenesis, initial follicle recruitment and cyclic selection of dominant follicles (19, 27). Mice lacking Amh ($Amh^{-/-}$) are fertile, but by 4 months of age, they have an increased number of growing follicles and a reduced number of primordial follicles compared to wild-type controls. At 13 months of age, there is a depletion of primordial follicles in $Amh^{-/-}$ females and there are very few growing follicles, reminiscent of premature ovarian failure (15). There is also evidence presented by Durlinger *et al*. indicating that Amh is an inhibitor of FSH-dependent follicle growth. This *in vivo* study demonstrated that Amh null mice have more growing follicles than wild-type mice in the presence of both low and high serum FSH concentrations (28). This inhibitory effect of Amh on FSH sensitivity in follicles could play a role in dominant follicle selection, as it has been speculated that each follicle exerts its own threshold FSH concentration that has to be exceeded for selection (19). Despite this evidence that Amh inhibits primordial

follicle growth and dominant follicle selection, the exact mechanism of Amh-dependent repression of follicle growth is not fully understood (15).

*Growth Differentiation Factor 9:* Like Amh, *Gdf9* expression first appears in the oocytes of primary follicles, and is sustained until ovulation (29, 30). *Gdf9* $^{-/-}$ female mice form primordial and primary follicles, but experience a block in follicular development at the primary stage of folliculogenesis, indicating a role for Gdf9 during preantral follicle growth (31). The granulosa cells of *Gdf9*$^{-/-}$ mice also have reduced proliferation and defects in differentiation, as well as absence of theca layer development (31, 32). In addition, few granulosa cells undergo apoptosis, and *Kitl*, as well as the peptide inhibin, are dramatically increased in *Gdf9*$^{-/-}$ granulosa cells compared to controls, suggesting that *Gdf9* inhibits granulosa cell production of these growth factors (32).

*Inhibin and Activin:* Inhibin is a gonadal peptide that is present in follicular fluid and granulosa cells. Structurally, inhibin is made up of two subunits ($\alpha$ and $\beta$) that are linked by disulfide bonds. Dimers of the $\beta$ subunits of inhibin ($\beta_A\beta_B$, $\beta_A\beta_A$, $\beta_B\beta_B$) can also form and these dimers, which are collectively called activins, stimulate FSH actions (33). Inhibin is considered the antagonist of activin and both are reportedly important for regulating the formation and development of ovarian follicles (34). Recently, Woodruff *et al*. provided evidence that activin stimulates estrogen receptor (ER) expression in granulosa cells and helps maintain ER levels in the mouse ovary (34). However, further research needs to be conducted to determine inhibin and activin's specific effects during preantral folliculogenesis.

*Wnt Genes:* In humans and mice, the Wnt family encodes a group of 19 highly conserved, secreted signaling molecules that are critical regulators of cell fate, growth, and differentiation, as well as cell-cell interactions (35). This family of genes is related to the Drosophila segment polarity gene, wingless (wg). In the prepubertal mouse, the majority of Wnt ligands, receptors and antagonists are expressed in the ovary suggesting a functional role for this pathway in folliculogenesis and oocyte maturation (36). Furthermore, Wnt2, Wnt4, and Wnt5A are expressed in the fully grown, but not ovulated oocyte, whereas Wnt7A is expressed in both the fully grown and ovulated oocyte. Hsieh *et al*. demonstrated that Wnt4 and the receptor Fzd-1 are expressed in granulosa cells of the adult ovary and the expression of these genes is increased by eCG and hCG stimulation (37). Furthermore, females lacking Wnt4 expression in granulosa cells exhibit decreased ovary size, reduced litter sizes, reduced numbers of antral follicles, and decreased expression of genes involved in steroidogenesis (38).

*Early Growth Response 1:* An additional gene important in follicle growth and ovulation is early growth response factor-1 (Egr-1). It is an inducible zinc finger transcription factor that binds specific GC-rich enhancer elements. Female mice null for Egr-1 are infertile (39). Furthermore, multiple groups have demonstrated that FSH or forskolin, which activates adenylate cyclase, rapidly induces Egr-1 expression in granulosa cells (40) Early growth response-1, in turn, regulates the expression of the LH receptor as well as components of the prostaglandin biosynthesis pathway (40). Research on the role of Egr-1 is limited and further studies are required to fully understand the

importance of this transcription factor for follicular growth, oocyte maturation, and ovulation.

## Gonadotropin-Dependent Regulation of Folliculogenesis

Once folliculogenesis has progressed to the antral follicle stage, regulation by gonadotropins (i.e. FSH and LH) is critical. This is evident in FSH-deficient female mice. Specifically, FSH-deficient females are infertile due to a block in antral follicle formation. However, when the ovaries of 6 week old animals are examined, they contain all earlier stages of folliculogenesis including primordial, primary, and multilayered preantral follicles (21, 41). Likewise, knockout of LH in female mice causes several abnormalities with regard to ovarian function. The mice are hypogonadal and show thin uterine horns as well as impaired estrous cycles evident by the fact that there was an absence of healthy antral and preovulatory follicles and CLs. Primary and secondary follicles appeared normal, whereas many antral follicles were abnormal containing degenerating oocytes (42). For both FSH and LH null mice, exogenous gonadotropin administration rescues the follicular defects and restores fertility (15). Taken together, these studies indicate that early folliculogenesis is indeed gonadotropin-independent, but that gonadotropins are crucial for antral follicle development and ovulation (15).

*Gonadotropin Releasing Hormone:* The gonadotropin dependent regulation of folliculogenesis is initiated by gonadotropin-releasing hormone (GnRH), which is a 10 amino acid peptide hormone secreted from neurons in the hypothalamus (43). Synthesis

occurs in neurons found in the ventral portion of the hypothalamus, specifically in the arcuate nucleus, as well as in the preoptic nucleus of the anterior portion of the hypothalamus. The secretion of GnRH is regulated by several neurotransmitters and neuropeptides (33). Once released, GnRH is delivered into the portal vasculature and travels to the anterior pituitary. Gonadotropin releasing hormone binds to the GnRH receptor, type I, which is a $G_s$-coupled protein receptor, in gonadotroph cells of the anterior pituitary and stimulates the synthesis and release of both FSH and LH. The release of these gonadotropins is differentially regulated by GnRH and is dependent on the area of the hypothalamus (i.e. arcuate nucleus, preoptic nucleus) that is stimulated by neural depolarization to release GnRH. Upon stimulation of the arcuate nucleus, the hypothalamus mediates tonic or basal secretion of LH. When the preoptic nucleus is stimulated, there is a surge release of both FSH and LH. Finally, if there is a surge release of GnRH prior to ovulation, this mediates a secretory surge of LH, with less FSH (43).

*Follicle Stimulating Hormone and Luteinizing Hormone:* Follicle stimulating hormone and LH are both glycoproteins which travel through the systemic circulation to the ovaries. Structurally, FSH and LH are each composed of a common alpha subunit and a unique beta subunit. As previously stated, FSH prevents granulosa cell apoptosis and follicular atresia (22), and promotes granulosa cell proliferation, estradiol production, and LH receptor expression (23). Luteinizing hormone regulates thecal cell steroidogenesis, antrum formation (in coordination with FSH), and ovulation (15).

Follicle stimulating hormone works through a $G_s$-coupled protein receptor (FSHR), and upon binding, activates adenylyl cyclase (AC) resulting in increased

synthesis of the second messenger cyclic adenosine monophosphate (cAMP) and activation of protein kinase A (PKA) (33). Protein kinase A subsequently activates multiple downstream signaling factors including extracellular regulated kinase (Erk1/2), p38 MAPK, and PI3K (44). The primary acitvator of the PKB/Akt signaling pathway is P13K and therefore, cAMP indirectly regulates Akt signaling (45). The activation of the Akt pathway is correlated to granulosa cell differentiation whereas the activation of the Erk1/2 pathway is associated with proliferation and cell survival (44, 46). Follicle stimulating hormone stimulation of the PI3K/Akt pathway is also associated with follicle maturation, granulosa cell proliferation, and cell survival (44, 46).

The actions of LH are also mediated through a $G_s$-coupled protein receptor activating the cAMP/PKA signaling pathway, and subsequently stimulating the Erk1/2 pathway, as well as cAMP-activated guanine nucleotide exchange factors (cAMP-GEFs) and phospholipase C (24). Figure 1.2 depicts the signaling cascades induced by the binding of either FSH or LH to their respective receptors.

The FSH receptor is expressed in granulosa cells of preantral follicles as well as mural and cumulus granulosa cells of antral follicles. Follicle stimulating hormone drives the proliferation, growth and differentiation of granulosa cells, characterized by the formation of a fluid-filled antrum within the maturing follicle, as well as the development of the two distinct populations of granulosa cells (i.e. mural and cumulus) (44). For example, *in vitro* studies in rat granulosa cells suggest that FSH signaling is required to remove the forkhead box-containing proteins in the O subfamily-1 (FOXO1) repression of cyclin D2 (Ccnd2) (47), which is a gene critical for granulosa cell proliferation. Studies have also shown that FSH alone promotes transcription of several

"differentiation" target genes including aromatase, inhibin-α, epiregulin, and LH receptor (44).

Mural granulosa cells adjacent to the basement membrane of the antral follicle express high levels of the LH receptor (LHCGR). Upon binding of LH, these mural granulosa cells stimulate expansion of the cumulus granulosa cells. Conti *et al*. have shown that LH stimulates the expression of the epidermal growth factor (EGF)-like family members amphiregulin (*Areg*), epiregulin (*Ereg*), and betacellulin (*Btc*), which are rapidly expressed after the LH surge and stimulate cumulus expansion and oocyte maturation *in vitro* (48). These findings were confirmed when the U0126 inhibitor of Erk1/2 signaling prevented gonadotropin, EGF, and cAMP analog stimulation of cumulus expansion (49). It should be noted that the oocyte itself is also required for cumulus expansion as demonstrated by several oocytectomized studies (15). The LHCGR is also expressed in thecal cells of preantral and antral follicles. Interactions between LH-LHCGR in thecal cells stimulates several enzymes involved in steroid biosynthesis, resulting in thecal cell synthesis of androgens. These androgens are subsequently aromatized to estrogens under the regulation of FSH in the granulosa cells. This coordinated effort to produce estradiol is known as the *two-cell two-gonadotropin model* (43).

*Ovarian Steroids:* Both acute and chronic regulation of steroidogenesis is predominately controlled by LH and FSH as described above (50). The acute response is initiated by the mobilization and delivery of cholesterol, the substrate required for all steroid hormone biosynthesis, from the outer to the inner mitochondrial membrane. The

protein that facilitates this transfer of cholesterol is steroidogenic acute regulatory (Star) protein. Mutations characterized by loss-of-function in the Star gene are lethal and cause congenital lipoid adrenal hyperplasia, which results in the almost complete loss of steroid synthesis (51). Another important steroidogenic gene is aromatase (Cyp19a1), which is a member of the P450 cytochrome superfamily of enzymes and catalyzes the conversion of androgens to estrogens. Therefore, Cyp19a1 null mice are unable to produce estradiol (15). Ovaries from 12 to 14 week old Cyp19a1 null mice contain follicles of all types, however the mice were infertile and CLs were absent, suggesting impaired ovulation. Furthermore, many antral follicles were histologically abnormal with uneven granulosa cell layers and increased apoptosis (52). Serum FSH and LH were also elevated in Cyp19a1 null mice. An additional member of the P450 cytochrome family important in ovarian steroidogenesis is cholesterol side chain cleavage enzyme (Cyp11a1), which catalyses the conversion of cholesterol to pregnenolone (46). This conversion is the first step in steroid biosynthesis and is therefore critical in the process of steriodogenesis.

Increasing ovarian estrogen synthesis has an initial negative feedback effect on the hypothalamus and anterior pituitary, which depresses FSH and LH release. However, upon reaching a threshold level, estrogen stimulates the surge release of GnRH, which in turn will activate gonadotrophs in the anterior pituitary to release a surge of LH and thereby induce ovulation. During this time, the granulosa cells will also be secreting inhibin, which has a negative feedback effect on FSH but not LH release. Estrogen interacts with inhibin to maximize this effect on FSH. After ovulation, a functional corpus luteum (CL) produces progesterone, which inhibits GnRH and subsequently, LH

release (33). Increasing progesterone levels have a profound negative effect on GnRH secretion as well as FSH and LH secretion. This feedback loop between GnRH, FSH and LH, and the ovarian steroids (estrogen and progesterone) is collectively known as hypothalamic-pituitary-gonadal (HPG) axis, and is crucial for normal ovarian follicle development, as well as ovulation and subsequent fertilization and maintenance of a viable embryo.

## Additional Endocrine Regulators of Follicular Development

Although FSH and LH are the main regulators of antral follicle growth, steroidogenesis, and ovulation, there are several other endocrine hormones that contribute directly or indirectly to these processes. This includes hormones which fluctuate with changes in adipose tissue mass (e.g. insulin, insulin-like growth factor-1 (IGF-1), and leptin).

*Insulin:* Insulin is a polypeptide secreted by the β-cells of the pancreatic islets of Langerhans, and is released when blood glucose levels are high (33). Circulating insulin levels in the peripheral blood of normal women is approximately 10 μ/U/ml in the fasting state and up to 50 μ/U/ml within one hour after oral ingestion of glucose. In obese women, these levels are elevated to 15 μ/U/ml and 60 μ/U/ml respectively (53). Insulin's receptor is a heterotetramer consisting of two α and two β subunits and belongs to the tyrosine kinase family of receptors (33). Upon insulin binding, the activated receptor recruits the insulin receptor substrate-1 complex (IRS-1), which contains several

phosphorylation sites. Once phosphorylated, IRS-1 activates downstream signal transduction cascades including the PI3K/Akt and Erk1/2 pathways (Figure 1.3). Insulin binding to its receptor can stimulate either mitogenic or metabolic effects including stimulation of DNA and protein synthesis, lipogenesis, transmembrane electrolyte transport, and transmembrane glucose transport (53).

Insulin receptors are expressed throughout the ovary, including granulosa, thecal, and stromal tissues across multiple mammalian species (53). However, the effect(s) that insulin has on the ovary is/are not yet fully understood. Several *in vivo* and *in vitro* studies have examined insulin's possible contribution to ovarian processes, including its role in ovarian steroidogenesis and ovulation. At present, there is conflicting evidence regarding insulin-dependent regulation of steroidogenesis. In fact there are opposing results regarding the role of insulin on ovarian steroidogenesis with some studies indicating insulin-dependent increases in steroid production and other studies showing no effect of insulin on steroid synthesis (53). For example, a stimulatory effect of insulin on aromatase has been suggested by some *in vitro* studies of animal and human ovarian cells (54-57), while McGee *et al*. failed to confirm this finding (58). There are even more discrepancies in *in vivo* studies as Poretsky *et al*. provided evidence in rats that insulin has a stimulatory effect on ovarian and peripheral aromatase (59), whereas *in vivo* studies by Stuart *et al*. suggest that insulin may inhibit aromatase (60). There is also evidence that insulin is required for the normal function of the HPG axis and therefore, it is likely that a threshold level of insulin is required for ovulation. However, it is unclear if insulin directly regulates axis function or if insulin stimulates leptin secretion which has been

shown to activate the HPG axis at the onset of puberty (53). Thus, it is clear that further studies are required to fully define insulin's effects on the ovary.

*Insulin-like Growth Factor 1 (IGF-1):* A close relative to insulin, IGF-1, has also been widely studied with regard to ovarian function. The structure of IGF-1 exhibits significant homology to proinsulin, which is the precursor to insulin. Insulin like growth factor-1 is a 70 amino-acid, single-chain polypeptide which is widely expressed in most tissues, although the major source of circulating IGF-1 is the liver (53). Growth hormone (GH) is the primary activator of IGF-1 gene transcription (61), while other activators include estradiol and angiotensin II (53). Insulin like growth factor-1's main effect on its target tissues is cell proliferation. IGF-1's receptor, like the insulin receptor, is a heterotetramer consisting of two $\alpha$ and two $\beta$ subunits and belongs to the tyrosine kinase family of receptors. Upon binding of IGF-1 to its receptor, again, the IRS-1 complex is recruited, which contains several phosphorylation sites, and once phosphorylated will cause several signal transduction cascades to be activated including the PI3K pathway, which in turn activates the Akt pathway. Insulin and IGF-1 can bind to each other's receptors but with less affinity (Figure 1.3) (33).

In the human ovary, the IGF-1 receptor is expressed in granulosa cells and oocytes with higher expression levels in dominant compared to small antral follicles (62, 63). In the rodent ovary, both IGF-1 and the IGF-1 receptor are abundantly and specifically expressed in granulosa cells. Insulin like growth factor-1 null mice are infertile due to diminished sex drives, as well as decreased estradiol synthesis, ovarian weight, uterine size, and FSH receptor expression (64). There is growing evidence that in

the ovary IGF-1 has an additive or synergistic effect on FSH- and LH-dependent regulation of granulosa and thecal cell steroidogenesis and therefore represents a co-gonadotropin. While Mani *et al*. found that IGF-1 treatment alone significantly increased *Cyp19a1* expression, several reports show a synergistic effect of FSH and IGF-1 on *Cyp19a1* expression including experiments using cultured bovine (65, 66) and rat granulosa cells (67). *Star* is also a very important steroidogenic gene, as it facilitates the transfer of cholesterol into mitochondria. A synergistic effect of FSH and IGF-1 on *Star* expression has been shown in porcine granulosa cells (68). Studies have also looked at what pathways are involved in this synergistic effect with an emphasis on the Akt and Erk1/2 pathways due to the role of these pathways on cell survival and proliferation. Insulin like growth factor-1 alone increased Akt phosphorlyation in bovine granulosa cells (46), and there is evidence of significant increases in Akt phosphorylation when cells are co-treated with FSH and IGF-1 (66). In contrast, Mani *et al*. demonstrated that IGF-1 alone and in combination with FSH had no effect on Erk1/2 phosphorylation (46). Therefore, the current data suggests that the Akt pathway mediates IGF-1-dependent expression of granulosa cell genes. However, additional studies are required to confirm the specific pathways involved in the synergism of IGF-1 and gonadotropin regulation of ovarian steroidogenesis. Insulin like growth factor-1 is also implicated to synergize with gonadotropins to enhance their actions on cell cycle progression, apoptosis, and structural maintenance of the follicle during its development (64). Based on evidence collected so far, it is obvious that IGF-1 plays an important role in female reproduction, but further studies need to be completed in order to define the exact function of IGF-1 with regards to ovarian function.

*Leptin:* Leptin is a polypeptide hormone product of the obese gene (*ob*) that consists of 146 amino acids. Leptin is produced by adipocytes, and the amount of leptin in the body is directly proportional to the amount of fat in the body (69). Because it is a protein hormone, leptin can travel through the blood in a free state and acts at the plasma membrane of cells. The leptin receptor is the product of the *db* gene and belongs to the class I cytokine superfamily of receptors (70). The receptor has two isoforms. The long form is the primary form found in the hypothalamus. The short form is generally located in the peripheral tissue and at the blood brain barrier. Leptin receptors can be found in the liver, kidney, heart, skeletal muscles, and the pancreas (69). In the ovary, granulosa and theca cells express leptin receptor mRNA (70) as well as luteal tissue, microvascular endothelial cells, and oocytes (71). Leptin signaling occurs through the JAK/STAT pathway. Once the receptor (Ob-Rb) is bound, the recruitment and activation of JAK2 takes place, which involves dimerization of STATs through interaction with a conserved SH2 domain, which leads to the subsequent phosphorylation of STAT3 (Figure 1.3). STAT3 is a member of the STAT family of proteins, which binds to a DNA response element resulting in increased transcription and therefore regulation of gene expression (70).

The main function of leptin is to regulate appetite by binding to specific neurons in the hypothalamus and producing a signal resulting in decreased food intake. It is also apparent that leptin plays a role in female reproduction given that the *ob/ob* mutant female mouse, which does not produce an active form of leptin, is acyclic and sterile. The sterility is reversed by treatment with recombinant leptin, but not by food restriction, indicating that leptin is required for normal reproductive function (70). Studies

demonstrate that leptin increases plasma concentrations of LH, as well as LH pulse frequency and amplitude (72) and FSH (73, 74) indicating that it is a potential regulator of the HPG axis. Genetic studies have also shown that leptin is required for the initiation of puberty, with higher circulating leptin levels associated with a younger age at menarche (53). Menstrual abnormalities in young, healthy women are notably related to decreased adiposity (<15%) and leptin (<3 ng/ml).

It has also been suggested that leptin is involved in ovarian steriodogenesis. In human granulosa cells, leptin inhibits LH's stimulatory effect on the production of oestradiol, whereas there was no effect on estradiol production when LH is not present (75). Several studies have indicated that leptin has an inhibitory effect on IGF-1 in the ovary. Zachow and Magoffin reported that leptin could directly inhibit IGF-1 action in rat ovarian granulosa cells. Furthermore, leptin impaired IGF-1 dependent increase of FSH stimulation of estradiol synthesis in rat granulosa cells (76). Taken together, these studies indicate an important role for leptin in female reproduction and provide a link between energy balance and reproduction..

## Contribution of Granulosa Cells to Oocyte Growth and Maturation

It is well established that the oocyte and cumulus granulosa cells form a regulatory loop which guarantees coordinated growth of the oocyte and proliferation of the granulosa cells (77). Moreover, granulosa cells also participate in maintenance of oocyte meiotic arrest, global suppression of oocyte transcriptional activity, and the induction of oocyte meiotic and cytoplasmic maturation (78). This coordinated growth is

dependent on bi-directional communication between cumulus cells and the oocyte, which is mediated by both gap junctional and paracrine signaling pathways. Paracrine regulation of folliculogenesis has been discussed in another section of this chapter. Thus, the focus of this section will be gap junctional communication.

*Gap junctions:* Gap junctions are small transmembrane pores that allow the transfer of ions, metabolites, and small molecules between neighboring cells. The core proteins that make up gap junctions are called connexins and several studies have examined the importance of ovarian expression of connexins (15). Two specific connexins, connexin 43 (Cx43) and connexin 37 (Cx37) have essential and distinct roles during folliculogenesis. Connexin 43 forms gap junctions between granulosa cells which are found at all stages of folliculogenesis (79). Connexin 43 knockout mice display a block at the primary follicle stage with impaired granulosa cell proliferation and oocyte growth. Furthermore, the oocytes are morphologically abnormal with defects in meiotic maturation (15). This phenotype persists when ovaries from fetal and newborn Cx43 knockout mice are cultured *in vitro* or transplanted under the kidney capsule of wild-type mice.

Connexin 37 is localized to the interface of the oocyte and granulosa cell and forms gap junctions between the two cells during the primary follicle stage (80). Ovary-specific knock-out of Cx37 also exhibits abnormalities in folliculogenesis. Oocytes in mice lacking Cx37 have defects in the ability to resume meiosis and fail to grow to a normal size, although follicular development progresses to the late preantral stage (80). Connexin 37 deficient ovaries also have numerous small CL-like structures, suggesting

that communication via gap junctions is a major mechanism regulating CL formation. From these studies, it can be concluded that Cx43 gap junctions are required for granulosa cell proliferation earlier in folliculogenesis to form multilayered follicles, whereas Cx37 gap junctions are critical for the preantral to antral follicle transition, with both types of junctions playing an important role in the support of normal oocyte development (15).

Recent data suggests two important functions of gap junction dependent communication between the oocyte and granulosa cells. Mammalian oocytes reach prophase of first meiosis around the time of birth, and remain at this stage for months or years, depending on the species. Only after puberty will the fully grown oocytes begin to resume meiosis, which is stimulated by the LH surge (81). Gap junction communication is required to maintain this meiotic resumption as it mediates two factors that prevent it, cAMP and guanosine 3',5'-cyclic monophosphate (cGMP). When rat follicle-enclosed oocytes were incubated with carbenoxolone, a known blocker of gap junctions, meiotic resumption in the oocyte was inhibited, with a drop in intraoocyte cAMP concentrations indicating that gap junctions facilitate cAMP transfer into the oocyte (82). Furthermore, Norris *et al*. found that cGMP passes through gap junctions into the oocyte, where it inhibits hydrolysis of cAMP by the phosphodiesterase, PDE3A (83). This inhibition maintains a high concentration of intra-oocyte cAMP and thus blocks meiotic progression. Second, gap junctions play an important role in oocyte metabolism and energy production. Specifically, oocytes are deficient in their ability to utilize glucose as an energy substrate and rely on cumulus cells to metabolize glucose (78). The cumulus cells metabolize glucose into pyruvate by glycolysis, which is then transported to the

oocyte via facilitated diffusion using gap junctions (84). Oocytes subsequently metabolize pyruvate through oxidative phosphorylation to produce energy for growth and maturation.

*Transzonal projections:* Both paracrine and gap junctional communication is facilitated by the establishment of transzonal projections (TZP) by the granulosa cell. The TZPs are composed of microtubules or actin which transverse the zona peluicida allowing intimate contact between the oocyte and surrounding granulosa cells (85). Microtubule-derived TZPs originate from the centrosome and associated microtubule organizing center (MTOC). There are a handful of genes that regulate centrosome and MTOC function. One gene encodes for the transforming acidic coiled coil (TACC) proteins which play a role in normal development and tumorigenesis. The TACC proteins are a subfamily of coiled-coil domain containing proteins characterized by a specific 200 amino acid C-terminal coiled coil domain. Transforming acidic coiled coil-1 is the founding member of the transforming acidic coiled coil genes and is expressed in the surface epithelium (86) and granulosa cells (Mack, unpublished data) of the ovary. In addition to its role in microtubule formation, TACC1 interacts with transcriptional and translational machinery.

Another family of genes that participate in centrosome function and interacts with the MTOC is the Nima-related kinase (Nek) genes. Specifically, Nek2 regulates centrosome splitting and is associated with condensing chromosomes during prophase (87). Nima-related kinase 2 is expressed in growing oocytes, granulosa cells, and ovarian surface epithelium. However, the function of Nek2 and its family member, Nek4, during

folliculogenesis, oocyte-granulosa cell communication, and oocyte maturation remains unclear.

TZPs have been reported to be most abundant in preantral follicles, and appear to be crucial for the transport of paracrine factors between the oocyte and granulosa cells. For example, Antczak and Van Blerkom have reported evidence of the delivery of follicle cell-derived leptin and STAT3 to the oocyte by TZPs (88). Likewise, Albertini *et al*. have proposed a model that TZPs support uptake of oocyte products, like GDF-9, by granulosa cells, which is then processed by transcytosis and presented to more distal granulosa cells and even surrounding theca. Central to this model are key properties of TZPs: (i) they possess the appropriate subcellular machinery for orienting the trafficking of paracrine factors; and (ii) their dynamics are controlled by other factors (for example FSH) such that localized delivery and uptake is regulated (85). An *in vivo* study conducted by Combelles *et al*. provided evidence that FSH treatment results in a retraction of TZPs (89). Furthermore, recent studies indicate that a loss of oocyte-granulosa cell contact reduces (90, 91) whereas increased TZP density enhances (92) the development potential of *in vitro* matured oocytes. Based on these collective data, it is obvious that granulosa cells play a pivotal role in folliculogenesis and subsequently the development of a normal, developmentally competent oocyte

## Lethal Yellow Mouse Model

There are several mouse models of obesity that can be used to study the effect of increased adiposity on reproduction including the *ob/ob,* lethal yellow (LY), and diet-

induced lines. Lethal yellow ($A^y$/a) (C57BL/6J $A^y$/a) mice exhibit progressive adult-onset obesity, yellow coat color, embryonic lethality in $A^y$ homozygotes, premature loss of fertility (93), and high circulating insulin, glucose, and leptin levels, which leads to insulin resistance, hyperleptinemia, and central leptin resistance (71, 94).

The LY mouse possesses a gene deletion in the promoter and first exon region of the agouti gene locus, which brings an upstream promoter into place resulting in the constitutive expression of the agouti gene in numerous, if not all tissues (94, 95). The over-expressed agouti protein acts as an antagonist of melanocortin-4 receptors (MCR4) and melanocortin-1 receptors (MCR1). In the hypothalamus, the MCR4 plays a crucial role in central appetite and metabolism regulation. Located in the hypothalamus are the anorexigenic and orexigenic feeding centers of the brain. The anorexigenic center stimulates satiety upon activation of neurons by leptin and insulin. These neurons produce α-melanocyte-stimulating hormone (α-MSH) derived from pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), which control satiety. The orexigenic center stimulates feeding due to ghrelin activation of neurons which produce neuropeptide Y (NPY) and agouti-related peptide (AGRP) (33). In the LY mouse, the excess production of agouti competes with α-MSH in the hypothalamus for binding to MCR4 interfering with normal satiety control (Figure 1.4). As a consequence, the LY mice exhibits uncontrolled eating resulting in adult-onset obesity, insulin resistance, hyperleptinemia, and central leptin resistance (96).

As to why the LY displays a unique fur color, normally α-MSH binding will activate MCR1 initiating a signal transduction pathway for eumelanin (black) synthesis. As in the hypothalamus, agouti competes with α-MSH for MCR1 binding resulting in the

inhibition of the eumelanin pathway and stimulation of only the pathway of phaeomelanin (yellow) synthesis (94). Thus, the coat color of LY heterozygotes is yellow. As mentioned previously, LY also exhibit embryonic lethality in $A^y$ homozygotes. It has been determined by Duhl *et al*. that this lethality characteristic is directly related to the LY gene mutation. The deletion removes most of the coding sequence for a gene that encodes an RNA-binding protein resulting in embryonic lethality of the $A^y$ homozygotes (97).

Several studies have been conducted on LY mice to examine obesity's effect on reproduction. Granholm *et al*. found that LY mice over 120 days old exhibited abnormal estrous cyclicity and decreased mating success compared to control, age-matched black (C56BL/6J a/a) mice. The normal estrous cycle of a control mouse is 4-5 days in length and lasts until 200-250 days of age. However, the LY estrous cycle is lengthened and ceases prematurely (98). To determine if LY impaired fertility is due to intrinsic ovarian defects or to extraovarian factors, Granholm *et al*. performed reciprocal ovarian transplantation between 70-90 day old LY and black mice. Black mice who received ovaries from LY mice exhibited normal fertility. Conversely, LY mice with transplanted ovaries from black mice experienced diminished reproductive function similar to intact LY mice (99). The results of this study indicated that impaired fertility must result from either abnormal hypothalamic-pituitary control or from extraovarian factors that altered the function of ovarian cells. Swier *et al*. then concluded that the loss of reproductive function in LY mice is directly related to obesity. They demonstrated that LY mice maintained on a fat-restricted diet that kept their body weight under 30 g continued to cycle normally as they aged. Furthermore, 270 day old LY mice fed a low-fat diet had

similar ovarian histology and equivalent numbers of antral follicles at proestrus as age-matched black mice (100). Brannian *et al*. also provided *in vitro* evidence that blastocyst development of embryos from 180 day old LY mice was impaired compared to the embryos of age-matched black mice (71). The collective results of these studies indicate that impaired fertility must result from either abnormal hypothalamic-pituitary control or from extraovarian factors that altered the function of ovarian cells, but the evidence regarding LY female reproduction have not distinguished between these two mechanisms. These studies also indicate that the early loss of fertility in LY mice is the result of progressive obesity making the LY mouse line an excellent model to study the effects of obesity on reproduction, specifically ovarian function.

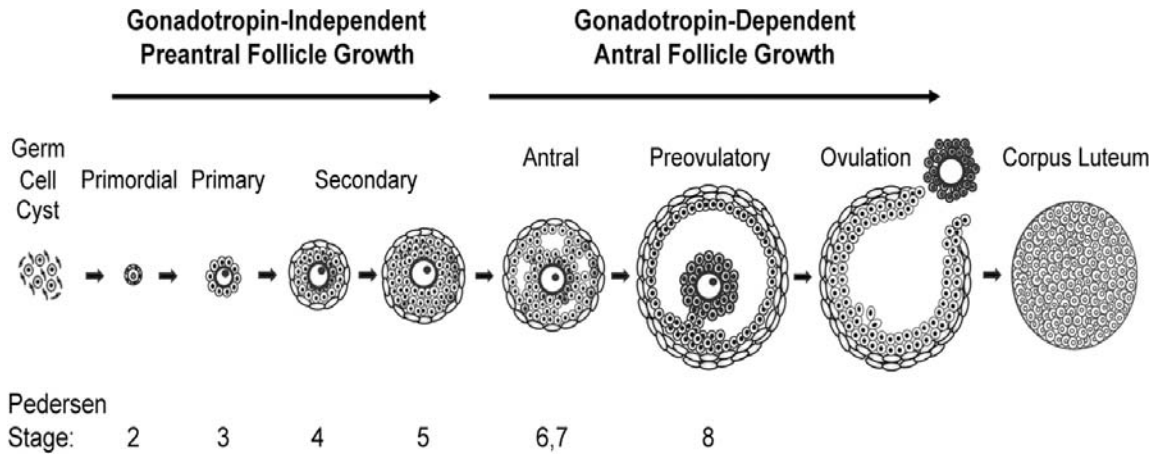


***Figure 1.1.*** **Classification of the major stages of mammalian folliculogenesis.** Schematic representation of the major stages of mammalian folliculogeneis. These stages are divided into two separate groups. (i) Intraovarian regulation of folliculogenesis. This includes the primordial, primary, and secondary stages. (ii) Gonadotropin-dependent regulation of folliculogenesis. This includes the antral, preovulatory, and ovulation of the follicle. Adapted from (15).

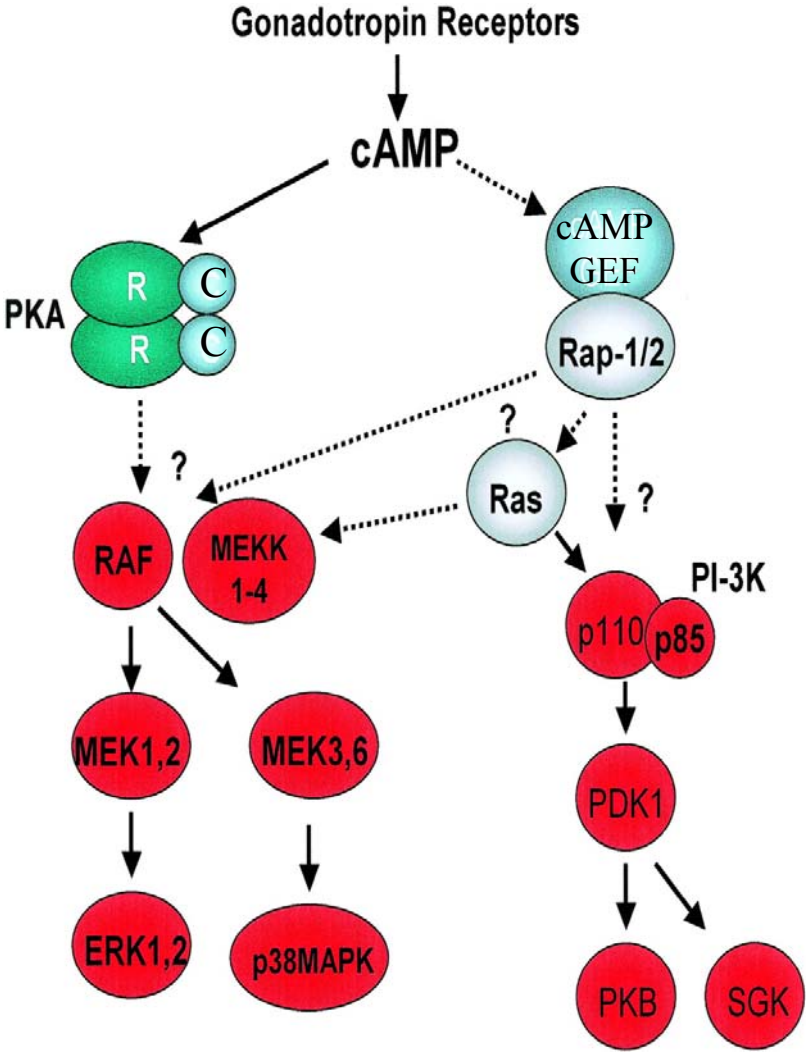


***Figure 1.2.*** **Branching of the cAMP signaling and kinase cascades in granulosa cells.** Schematic representation of the gonadotropin (i.e. FSH and LH) signaling cascade. The binding of either FSH or LH will increase cAMP/PKA activity activating multiple signal transduction cascades including Erk1/2 and PKB (Akt). Adapted from Conti M Biol Reprod 2002;67:1653-1661.

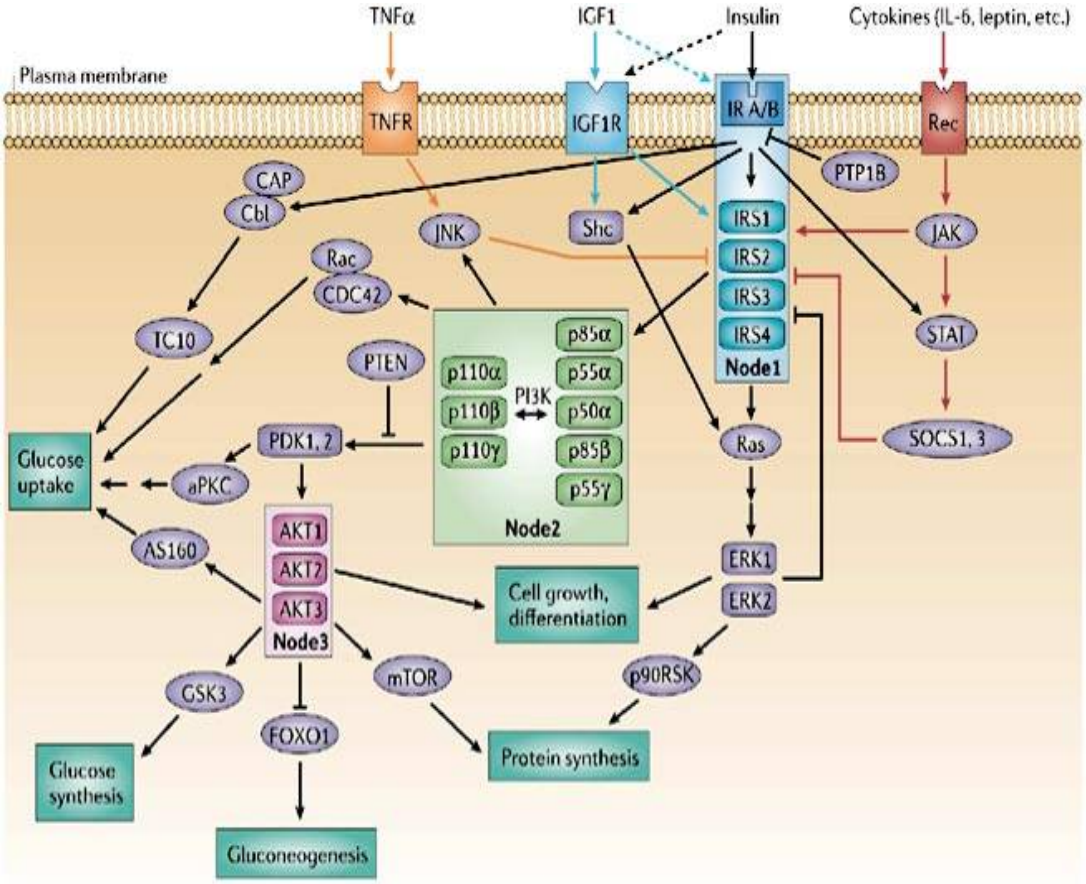


***Figure 1.3.*** **Insulin, IGF-1, and leptin signal transduction pathways.** Upon binding to their receptors, both insulin and IGF-1 recruit the IRS-1 complex, which contains several phosphorylation sites, and once phosphorylated will cause several signal transduction cascades to be activated including Erk1/2 and the PI3K pathway, which in turn activates the Akt pathway. Leptin can also activate signal transduction cascades through the IRS-1 complex via the activation of JAK/STAT pathway. Adapted from (101).

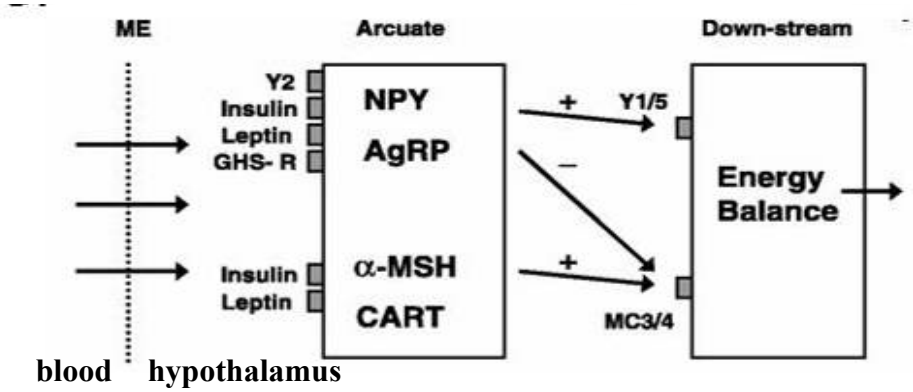


***Figure 1.4.*** **Ectopic expression of agouti interrupts normal satiety control in the lethal yellow (LY) mouse.** Schematic representation of the regulating of the feeding centers (anorexigenic and orexigenic ) in the hypothalamus of the brain. The anorexigenic center stimulates satiety upon activation of neurons by leptin and insulin. These neurons produce α-melanocyte-stimulating hormone (α-MSH) derived from pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), which control satiety. The orexigenic center stimulates feeding due to ghrelin activation of neurons which produce neuropeptide Y (NPY) and agouti-related peptide (AgRP). In the LY mouse, the excess production of agouti competes with α-MSH in the hypothalamus for binding to MCR4 interfering with normal satiety control. As a consequence, the LY mice exhibits uncontrolled eating resulting in adult-onset obesity

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# CHAPTER 2

## Progressive Obesity Alters the Metabolic Hormone Profile and Ovarian Molecular Phenotype of the Lethal Yellow Mouse


### Abstract

Maternal obesity has been correlated with anovulatory infertility and embryonic loss suggesting a negative impact of excess adipose tissue on folliculogenesis and oocyte maturation. The lethal yellow (LY) mouse possesses a deletion mutation which results in ectopic expression of agouti and adult-onset obesity. Furthermore, LY mice exhibit premature loss of fertility, which has been associated with progressive obesity making the LY mouse line an excellent model to study the effects of obesity-dependent factors on ovarian function. In the current study blood serum and granulosa cells were obtained from LY ($A^y$/a) and age-matched C57BL/6J controls (B6) at 3, 6, 12, and 24 weeks of age to identify changes in metabolic hormone profiles and gene expression, respectively. As expected, LY females exhibited higher circulating levels of insulin and leptin compared to age-matched B6 controls. For the first time, we also identified a significant increase in circulating IGF-1 levels in the LY compared to B6 at 6 weeks of age. Recent studies suggest that hormones including leptin, insulin, and IGF-1 regulate the molecular and cellular phenotype of the female reproductive tract. Thus, to determine the impact of the altered hormone profile on the molecular phenotype of the ovary, qPCR analysis of gene expression was carried out using granulosa cell cDNA. Candidate genes selected for this study play a crucial role in steroidogenesis, follicular growth, bi-directional communication, or ovulation. *Star, Egr-1*, and *Tacc1* were differentially expressed in LY

compared to B6 granulosa cells. Interestingly, age-dependent changes in the expression of several genes in both LY and B6 females were also detected. Collectively, these data demonstrate for the first time that adipose tissue mass and age has a specific impact on granulosa cell gene expression. These genes play an important role in follicular growth and development. Therefore, alterations in their expression suggest a possible mechanism for obesity and age-dependent declines in fertility.

## Introduction

Obesity is growing at epidemic rates in the United States and around the world. Since 1980, obesity has increased more than 75% worldwide with over one billion adults considered to be overweight or obese (1). It is also increasingly clear that female obesity is associated with a myriad of adverse side-effects associated with reproduction. For example, prior to pregnancy, obese women (BMI > 30kg/$m^2$) have higher rates of amenorrhea and anovulatory infertility. In a Nurses Health Study, obese women had a 2.7 times higher risk of infertility problems compared to normal weight women (2). Obese women also experience a 1.2-, 3.5-, and 2.6-fold increased risk of early embryonic loss (6-12 weeks of gestation), recurrent (greater than 3) miscarriages, and late fetal pregnancy loss, respectively, compared to normal-weight woman (3, 4). These studies suggest a correlation between excess adipose tissue, infertility, and abnormal embryonic development.

Several mouse models of obesity have been developed including the *ob/ob*, which lacks bioactive leptin and the *db/db*, which carries a dysfunctional leptin receptor (5). However, these mouse models do not mimic adult-onset obesity in humans which is a result of excess calorie intake. Diet-induced models increase fat content and therefore, caloric density which causes progressive obesity. The lethal yellow (LY, $A^y$/a) mouse line also develops adult-onset obesity through increased consumption of normal rodent

chow. Thus, these two mouse models more closely reflect the mechanism of obesity development in humans.

The LY mouse possesses a gene deletion in the promoter and first exon region of the agouti protein gene locus on the C57BL/6 background, which results in the ubiquitous expression of the agouti gene (6, 7). In the hypothalamus, over-expressed agouti acts as an antagonist of α-MSH by binding to melanocortin-4 receptors (MCR4); and therefore, interfering with normal satiety control. As a consequence, the LY mice overeat resulting in the development of obesity. The LY mice also develop high circulating insulin, glucose, and leptin levels, which leads to insulin resistance, hyperleptinemia, and central leptin resistance (7, 8). Furthermore, LY mice exhibit a yellow coat color, embryonic lethality in $A^y$ homozygotes, and early loss of fertility (9).

Early loss of fertility is also a distinctive feature of the LY mouse line. Granholm *et al*. found that LY mice over 120 days old exhibited abnormal estrous cyclicity and decreased mating success compared to control, age-matched C57BL/6J a/a (B6) mice (10). Reciprocal ovarian transplants demonstrate that the impaired fertility is due to abnormal hypothalamic-pituitary function or extraovarian factors that alter ovarian function rather than an intrinsic ovarian defect in the LY mouse (11). Furthermore, Swier *et al*. showed that LY mice maintained on a fat-restricted diet which kept their body weight under 30 g had normal cyclicity, ovarian histology, and antral follicle counts indicating that the loss of reproductive function in LY mice is directly related to obesity (12).

Collectively, these studies indicated that the early loss of fertility in LY mice is the result of progressive obesity mediated by altered ovarian function via extraovarian

factors. Brannian *et al*. has previously looked at ovarian gene expression in 90 and 180 day old female LY mice, and found some statistical differences in gene expression of the LY compared to age matched controls including increased expression of cholesterol synthetic genes, and genes involved in steroid synthesis and metabolism (13). In the current study, the affect of increased caloric intake on metabolic hormone profiles and the expression of genes involved in steroidogenesis and follicular growth in granulosa cells was examined in the LY female mouse.

## MATERIALS AND METHODS

*Animals*: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln. All experiments were performed using C57BL/6 (B6; C57BL/6 a/a) and Lethal Yellow (LY; C57BL/6 $A^y$/a) female mice. Founder mice were originally obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice were fed standard rodent chow and fresh water *ad libitum*, and housed in groups of no more than 5 mice per cage on a 12/12 hour light/dark cycle.

*Granulosa Cell Collection:* Female B6 and LY mice (n=5) were euthanized at 3, 6, 12, and 24 weeks of age following vaginal smears to confirm that animals were in proestrus. Following euthanasia, blood was collected by heart puncture, serum separated by centrifugation, and stored at -20°C for later analysis. Body weight and abdominal fat mass weights were recorded for all mice. Both ovaries were removed, cleaned, and

placed in collection medium (1X Leibovitz (Sigma-Aldrich, St Louis, MO, USA), 10% heat- inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and 1% penicillin /streptomycin (Invitrogen, Carlsbad, CA, USA). Cleaned ovaries were subsequently placed in collection medium containing 6 mM EGTA (Sigma) and incubated for 15 minutes at 37°C. Ovaries were then placed in collection medium containing 0.5 M sucrose (Sigma) for 15 minutes at 37°C. Ovarian follicles were punctured with a 27-gauge needle in collection medium and cells were applied to 40μm filters (BD Falcon, Franklin Lakes, NJ, USA) to remove cumulus-oocyte complexes. Granulosa cells were then collected in a 2mL centrifuge tube and spun down via centrifuge. Collection media was removed from cell pellets which were subsequently re-suspended in TRI reagent (Ambion Inc., Austin, TX, USA) and stored at -80°C.

*RNA extraction:* RNA was extracted and purified from granulosa cells using the Ambion Ribopure Kit (Austin, TX, USA) according to the manufacurer's directions. RNA concentration was determined using the Beckman Coulter DU 730 Life Science UV/Vis Spectrophotometer. Purified total RNA was stored at -80°C.

*Reverse Transcription:* To obtain cDNA for qPCR analysis of gene expression, total RNA (5 μg) from each granulosa cell sample described above was combined with 5 units of RQ1 RNase free DNase (Promega, Madison, WI) and incubated at 37°C for 30 minutes to remove genomic DNA contaminants. The RNA was subsequently combined with 400 units of Moloney Murine Leukemia Virus reverse transcriptase (Promega), 500 μM dNTPs (Promega), and 100 ng of random primers (Roche Applied Science,

Indianapolis, IN) and incubated at 37°C for 2 hours. The resultant cDNA was stored at -20°C.

*Quantitative, Real-Time PCR (qPCR) Analysis:* To carry out qPCR analysis of gene expression, forward and reverse primers (Table 2.1) for genes associated with steroidogenesis, folliculogenesis, or ovulation were designed (Primer Express, Applied Biosystems, Foster City, CA) and synthesized (Integrated DNA Technologies, Coralville, IA). Each set of gene-specific primers was tested empirically to determine the maximal concentration of primers that could be used to produce specific amplification of the target sequence in the absence of primer dimer amplification. Quantitative PCR (qPCR) reactions were carried out using equivalent dilutions of each cDNA sample, *Power* SYBR Green PCR Master Mix (Applied Biosystems), the empirically determined concentration of each primer, and the 7900HT Fast Real-Time PCR system (Applied Biosystems). To account for differences in starting material, qPCR reactions were also carried out for each cDNA sample using TaqMan rodent GAPDH control reagents (Applied Biosystems). The relative abundance of specific gene products and GAPDH in each cDNA sample was determined using serial dilutions of whole ovary cDNA. The relative abundance of each specific gene product was divided by the relative abundance of GAPDH in each sample to generate a normalized abundance for each gene interrogated. The normalized abundance of each gene in granulosa cells from B6 and LY animals at 3, 6, 12, and 24 weeks of age were compared to gene expression in 3 week-old B6 granulosa cells and expressed as a fold-change.

*ELISA Analysis of Metabolic Hormones:* The levels of insulin, insulin-like growth factor-1 (IGF-1), and leptin were measured in blood serum using ELISA kits according to the manufacturers' directions. Specifically, IGF-1 levels were measured using the Quantikine MG100 Mouse/Rat IGF-1 ELISA (R&D Systems, Minneapolis, MN). Insulin and leptin were levels were measured using kits designed to detect mouse/rat insulin (EZRMI - 13K) and mouse leptin (EZML-82K), respectively (Millipore, Billerica, MA). Hormone levels were measured in all samples within a single ELISA assay using standards included in the kit.

*Statistical Analyses:* All statistical analyses were carried out using GraphPad Prism 4.0 (Graphpad Software, La Jolla, CA). QPCR, hormone levels, and weight data were analyzed using 2-way ANOVA with animal and age as the variables. Pairwise comparisons were carried out using the Bonferroni post-test. Differences were considered significant at $P < 0.05$.

## Results

*Progressive obesity in the LY female mouse.*

Previous studies indicated that LY mice develop progressive obesity as they age (8, 9, 13, 14). To confirm this phenotype in our population, both B6 and LY animals in our colony were fed normal rodent chow *ad libitum*. Female animals were euthanized at 3, 6, 12, or 24 weeks of age and body weight and abdominal fat mass were measured for both the B6 and LY mice. Average body weight (Figure 2.1) was significantly different

in LY compared to B6 females ($P < 0.01$) at 12 weeks of age and this difference persisted and became greater at 24 weeks of age. Likewise, average abdominal fat mass (Figure 2.2) was significantly different between LY and B6 females at 12 weeks ($P < 0.01$), and continued to increase in difference as animals aged to 24 weeks. These studies confirm that LY mice become obese as they age and that the increase in body weight is due, in large part, to an increase in visceral fat mass.

*Increased levels of circulating hormones leptin, insulin, and IGF-1 in the LY female mouse.*

Given the differences in the amount of adipose tissue in LY compared to B6 females, circulating levels of leptin, insulin, and IGF-1 (Figure 2.3) were analyzed in blood serum collected from B6 and LY females at 3, 6, 12, and 24 weeks of age. When compared to age-matched B6 controls, LY females had significantly increased circulating levels of leptin ($P < 0.001$) at 12 and 24 weeks of age and increased insulin levels ($P < 0.001$) at 24 weeks of age. Interestingly, at the age of 6 weeks, circulating levels of IGF-1 were significantly increased ($P < 0.01$) between the LY and age-matched B6 control, before returning to comparable levels by the age of 12 weeks.

*Significant gene expression differences between LY and age matched B6 controls.*

Previous studies indicated that LY females develop anovulatory infertility as a result of the obese phenotype (8, 12, 15). Furthermore, several *in vitro* studies indicate that insulin, leptin, and IGF-1 regulate granulosa cell gene expression (16-23). Therefore, in the present study, the expression profiles of a number of candidate genes which

contribute to follicle growth (Amh, Ccnd2, Egr1, Gdf9, Kitl, p27kip, Wnt4, Wnt5A, and Wnt5B), steroidogenesis (Cyp11a1, Cyp19a1, and Star), oocyte-granulosa cell communication (Cx37, Cx43, Tacc1, Nek2, and Nek4), and ovulation (Areg, Btc, Ereg, Il6) were determined using QPCR (Table 2.1). To carry out this study, granulosa cells were collected from females during proestrus since this is when follicular growth is most active. To confirm that animals were in proestrus, vaginal smears were performed prior to euthanasia. Furthermore, the mRNA for several candidate genes associated with ovulation including *Areg, Ereg, Btc*, and LH receptor were not detected (data not shown) in granulosa cells from B6 or LY females.

Most of the genes analyzed did not exhibit differences in mRNA abundance between LY and B6 females at any age. The only significant differences in gene expression between LY and age matched B6 controls were *Egr1*, *Tacc1*, and *Star* (Figure 2.4). The mRNA abundance of *Egr1* was significantly decreased in the LY at age 3 weeks when compared to B6 controls. Similarly, *Star* mRNA expression was also significantly decreased between LY and B6 age matched animals, but at the age of 24 weeks. Conversely, *Tacc1* mRNA abundance was significantly increased between LY and B6 age matched controls at the age of 6 weeks.

*Significant gene expression differences associated with age.*

After analyzing genes specifically examining for gene expression differences between LY and age matched B6 controls, we interestingly discovered age-dependent differences in mRNA abundance for several genes. The first set of candidate genes displayed significant differences associated with age increased during early time points

analyzed (i.e. 3 and 6 weeks) (Figure 2.5). Both *Gdf9* and *Cx37* gene expression were significantly increased at 3 weeks of age compared to all other age groups, whereas *Amh* mRNA abundance is significantly increased at 6 weeks of age compared to all other age groups. The second group of candidate genes displaying significant differences associated with age were increased during the latest time point analyzed (i.e. 24 weeks) (Figure 2.6). Specifically, *Wnt5A*, *Tacc1*, and *Cyp11a1* gene expression were all significantly increased at 24 weeks of age compared to all other age groups.

## Discussion

This study used the LY mouse model to examine potential mechanisms of obesity-dependent female infertility. As expected, LY mice exhibit progressive adult onset obesity with a significant difference in body weight (BW) between LY and B6 females detected at 12 weeks of age. This difference in body weight persisted when animals were compared at 24 weeks of age. Although Brannian *et al*. did not see a difference in BW between LY and age-matched B6 controls at 90 days (i.e. 12 weeks) of age (13), our findings for differences in BW at the age of 24 weeks were consistent with several other studies (8, 9, 13, 14). In addition, LY mice displayed significant differences in abdominal fat (AF) compared to age-matched B6 controls at 12 weeks of age, and this significant difference continued through the age of 24 weeks. Our findings at 24 weeks of age are consistent with that of Czyzyk *et al*. who displayed significant differences in white adipose tissue between 20-23 week old LY and B6 controls (14). Previous studies have only compared LY and wild type animals at older ages (i.e. 90-180 days). In this

study, we measured BW and AF at 3 and 6 weeks of age, and found at trend in increases in both BW and AF at 6 weeks of age. These data suggest that over eating has a deleterious effect prior to clinical signs of obesity.

In previous studies, the circulating levels of leptin and insulin, both of which play an important role in obesity and reproduction, were compared between LY and B6 males and females (16, 24). We demonstrated that insulin and leptin levels were significantly increased in LY female mice compared to age-matched controls at both 12 and 24 weeks of age, consistent with previous findings (7, 8, 13, 14). One important hormone that had not been examined in the LY mouse is insulin like growth factor-1 (IGF-1). The structure of IGF-1 exhibits significant homology to proinsulin, which is the precursor to insulin, and furthermore insulin and IGF-1 can bind to each other's receptors but with reduced affinity (25). While a handful of studies have detected increased circulating IGF-1 levels in obese compared to normal-weight individuals, others have reported decreased circulating IGF-1 levels or no difference in IGF-1 levels between obese and normal-weight individuals. In this study, circulating levels of IGF-1 were significantly increased in the LY compared to B6 controls at 6 weeks. However, this difference in IGF-1 levels dissipated at 12 weeks and was undetectable at 24 weeks of age, suggesting correction of IGF-1 via a negative feedback mechanism during the development of progressive obesity.

Previous studies have indicated a role for insulin, IGF-1, and leptin signaling on ovarian function (24). Brannian *et al*. identified significant changes in ovarian gene expression in LY compared to B6 females at 180 days of age including genes involved in cholesterol metabolism and steroidogenesis (13). While these data provide evidence that the obese phenotype alters ovarian gene expression, it is not clear how it impacts the

function of individual ovarian cells. In the present study, gene expression in purified granulosa cells was compared between LY and B6 females across different ages (i.e. 3, 6, 12, and 24 weeks). Despite the fact that a large number of candidate genes involved in follicular growth were examined, only three genes displayed significant differences in age-matched granulosa cells between LY and B6 controls. Early growth response factor-1 (*Egr-1*) is a gene important in follicle growth and ovulation (26, 27) and was down regulated in LY compared to age-matched B6 controls at three weeks of age. Transforming acidic coiled coil-1 (*Tacc1*), which plays a role in microtubule formation, was upregulated in LY compared to B6 controls at 6 weeks of age. Finally, the steroidogenic acute regulatory (Star) protein, which facilitates the transfer of cholesterol to the mitochondria for steroid hormone biosynthesis, was down regulated in the LY compared to age matched B6 controls at 24 weeks. Brannian *et al*. found that StAR expression was increased in the LY compared to age-matched B6 controls at 180 days of age (i.e. 24 weeks) (13). However, this study was carried out using whole ovary RNA. These data provide two interesting observations. First, it appears that gene expression in proliferating granulosa cells is in some way protected from the obese phenotype during proestrus. Second, differential gene expression occurred, in some cases, prior to changes in body weight or abdominal fat mass suggesting an impact of over-eating on gene expression before clinical presentation of obesity.

Interestingly, this study also identified differences in granulosa cell gene expression associated with age. Aging in the gonads has direct implications for longevity, as increased fertility is associated with decreased longevity in several species (28). There are a limited number of studies involving age and gene expression differences within the

ovary (29), although no studies have specifically looked at age-associated gene expression differences in the granulosa cells. Growth differentiation factor-9 (Gdf9) anti-mullerian hormone (Amh), and connexin 37 (Cx37) in both the LY and B6 controls were significantly increased early in age compared to all other age groups. *Gdf9* expression first appears in the oocytes of primary follicles, and is sustained until ovulation (30, 31). GDF9 null female mice form primordial and primary follicles, but experience a block in follicular development at the primary stage of folliculogenesis indicating a role for Gdf9 during preantral follicle growth (32). Amh is known to inhibit two major steps in folliculogenesis, initial follicle recruitment and cyclic selection of dominant follicles (33, 34). This study provided evidence that *Gdf9* and *Amh* were significantly upregulated in both the LY and B6 controls at 3 and 6 weeks respectively compared to all other age groups. This finding is consistent with a whole ovary gene expression study conducted by Sharov *et al*. (29). Connexin 37 is localized to the interface of the oocyte and granulosa cell and forms gap junctions between the two cells beginning in the primary follicle stage (35). Ovary-specific knock-out of Cx37 also exhibits abnormalities in folliculogenesis. Oocytes in mice lacking Cx37 have defects in the ability to resume meiosis and fail to grow to a normal size, although follicular development progresses to the late preantral stage (35). *Cx37* expression was upregulated in both the LY and B6 controls at 3 weeks of age compared to all other age groups. There were also three genes that displayed an age-associated difference, but at the latest time point measured, 24 weeks. TACC1, as well as cholesterol side chain cleavage enzyme (Cyp11a1), which catalyses the conversion of cholesterol to pregnenolone (17), and Wnt5A. Wnt5A is part of the Wnt family, which encodes a group of 19 highly conserved secreted signaling molecules that

are critical regulators of cell fate, growth, and differentiation, as well as cell-cell interactions (36).

The studies discussed herein indicate that the LY female is a good model of progressive obesity and may serve as a model of obesity-induced infertility in humans. Furthermore, these animals exhibit changes in metabolic hormone signaling and therefore represent a good model to understand how these hormones regulate ovarian function. While we have identified a handful of genes with altered expression due to obesity or age, future experiments must be carried out to determine the significance of these changes in granulosa cell gene expression on fertility.

Table 2.1. Primer sequences used for QPCR analysis

| Gene | Accession # | Primer | Sequence |
|---|---|---|---|
| *Amh* | NM_007445 | Forward | 5'-TCC TAC ATC TGG CTG AAG TGA TAT G-3' |
| | | Reverse | 5'-CAG GTG GAG GCT CTT GGA ACT-3' |
| *Areg* | NM_009704 | Forward | 5'-CAG CTG CTT TGG AGC TCA ATG-3' |
| | | Reverse | 5'-GTG GTC CCC AGA AAG CGA-3' |
| *Btc* | NM_007568 | Forward | 5'-TGC CCC AAG CAG TAC AAG C-3' |
| | | Reverse | 5'-TTT GCT CGT CCA CCA CGA-3' |
| *Cx37* | NM_008120 | Forward | 5'-CGA GAG AGG CCC TGG AAA C-3' |
| | | Reverse | 5'-CCA CCA CGG TCG AGT GTT C-3' |
| *Cx43* | NM_010288 | Forward | 5'-TGA AAG AGA GGT GCC CAG ACA T-3' |
| | | Reverse | 5'-GTG GAG TAG GCT TGG ACC TTG T-3' |
| *Ccnd2* | NM_009829 | Forward | 5'-AGC AGG ATG ATG AAG TGA ACA CA-3' |
| | | Reverse | 5'-GGC TTT GAG ACA ATC CAC ATC AG-3' |
| *Cyp11a1* | NM_019779 | Forward | 5'-TGA ATG ACC TGG TGC TTC GTA AT-3' |
| | | Reverse | 5'-TCG ACC CAT GGC AAA GCT-3' |
| *Cyp19a1* | NM_007810 | Forward | 5'-GGC CCT GGT CTT GTT CGA-3' |
| | | Reverse | 5'-GCC GGT CCA AAT GCT GC-3' |
| *Egr1* | NM_007913 | Forward | 5'-GGG AGC CGA GCG AAC AA-3' |
| | | Reverse | 5'-TCA GAG CGA TGT CAG AAA AGG A-3' |
| *Ereg* | NM_007950 | Forward | 5'-GCA CTC CGC AAG CTG CA-3' |
| | | Reverse | 5'-AGC AGC GTC AAG ACC CAA GA-3' |
| *Gdf9* | NM_008110 | Forward | 5'-GCC GGG CAA GTA CAG CC-3' |
| | | Reverse | 5'-TTT GTA AGC GAT GGA GCC G-3' |
| *Il6* | NM_031168 | Forward | 5'-AGT CGG AGG CTT AAT TAC ACA TGT T-3' |
| | | Reverse | 5'-TGC CAT TGC ACA ACT CTT TTC T-3' |
| *Kitl* | NM_013598 | Forward | 5'-CGC ACA GTG GCT GGT AAC AG-3' |
| | | Reverse | 5'-GGT AGC AAG AAC AGG TAA GGA TGA G-3' |
| *Nek2a* | NM_010892 | Forward | 5'-CTG TGG GCA GGA ACC TTT GT-3' |
| | | Reverse | 5'-CAC ACA TCC ATT TGC AGA CCA-3' |
| *Nek4* | NM_011849 | Forward | 5'-AGA AGG AGA GGC TAC AGG GCA ATA-3' |
| | | Reverse | 5'-CAG GTC CAC CTT GGT TTC CAT CAT-3' |
| *p27kip* | NM_009875 | Forward | 5'-GAA GCC CGG CCT TCG A-3' |
| | | Reverse | 5'-CAT GTA TAT CTT CCT TGC TTC ATA AAG C-3' |
| *Star* | NM_011485 | Forward | 5'-GTG TGC CTT CGA CCC CC-3' |
| | | Reverse | 5'-AAA GTG CTT GCT GCC TAC CC-3' |
| *Tacc1* | NM_199323 | Forward | 5'-CTA GGC AAA CAG TCC TTT TCT TAG AAA-3' |
| | | Reverse | 5'-TGG CCC TCG TAT CCT CAG C-3' |
| *Wnt4* | NM_009523 | Forward | 5'-ACC GGC GCT GGA ACT GT-3' |
| | | Reverse | 5'-TCC CGG GTC CCT TGT GT-3' |
| *Wnt5a* | NM_009524 | Forward | 5'-GCA TCC TCA TGA ACT TAC ACA ACA A-3' |
| | | Reverse | 5'-CTC CAT GAC ACT TAC AGG CTA CAT CT-3' |
| *Wnt5b* | NM_009525 | Forward | 5'-CCA AGA CGG GCA TCA GAG A-3' |
| | | Reverse | 5'-GCG CTC ACT GCA TAC GTG AA-3' |

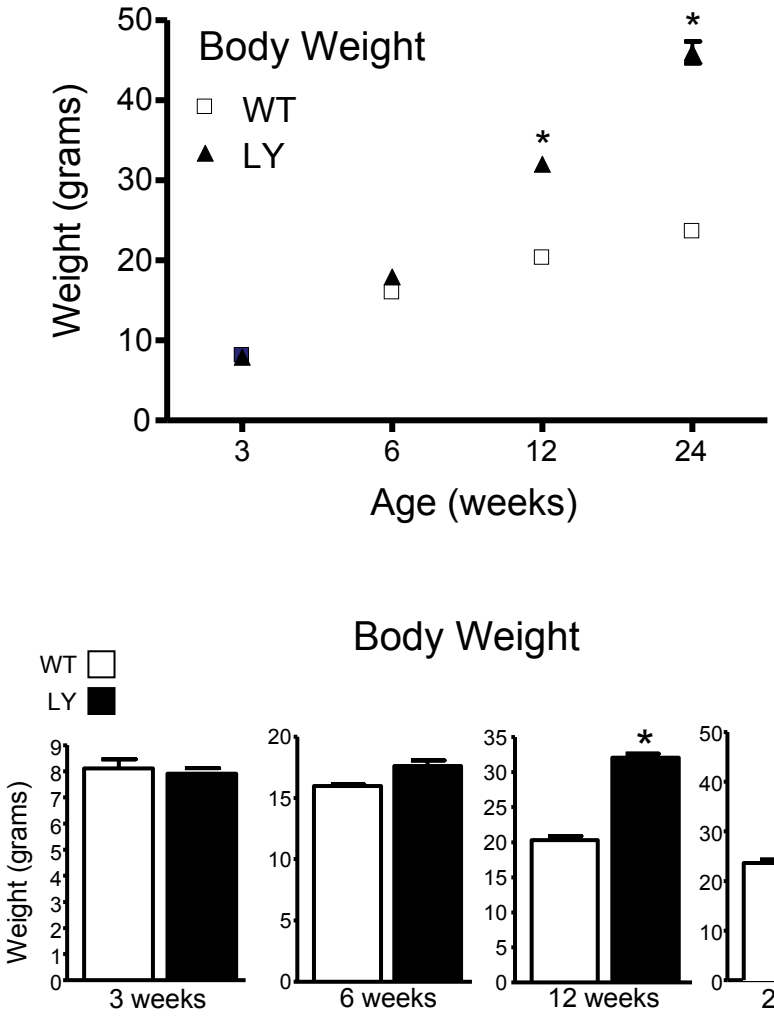


***Figure 2.1*** **Body weight (g) of 3, 6, 12, and 24 week old female B6 and LY mice.** Total body weight (g) of 3, 6, 12, and 24 week old B6 (white square, white bar) and LY mice (black triangle, black bar) are shown. Significant differences between average body weights (n=5) of B6 and LY animals were determined using two-way ANOVA and Bonferonii post-test. **, significant difference ($P < 0.01$) between B6 and LY within each age. *Error bars* represent mean ± SE.

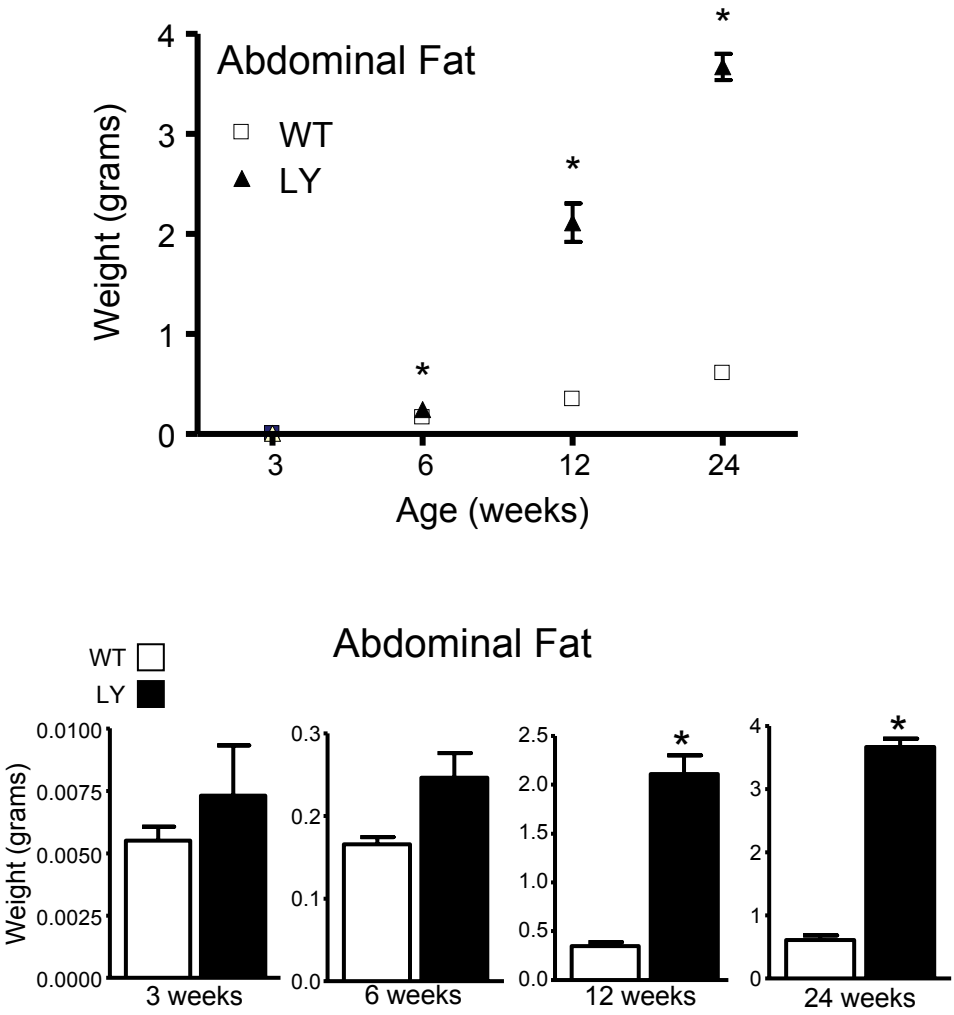


***Figure 2.2*** **Abdominal fat weight (g) of 3, 6, 12, and 24 week old female B6 and LY mice.** Abdominal fat mass (g) of 3, 6, 12, and 24 week old B6 (white square, white bar) and LY mice (black triangle, black bar) are shown. Significant differences between average abdominal fat mass (n=5) of B6 and LY animals were determined using two-way ANOVA and Bonferonii post-test. **, significant difference ($P < 0.01$) between B6 and LY within each age. *Error bars* represent mean ± SE.

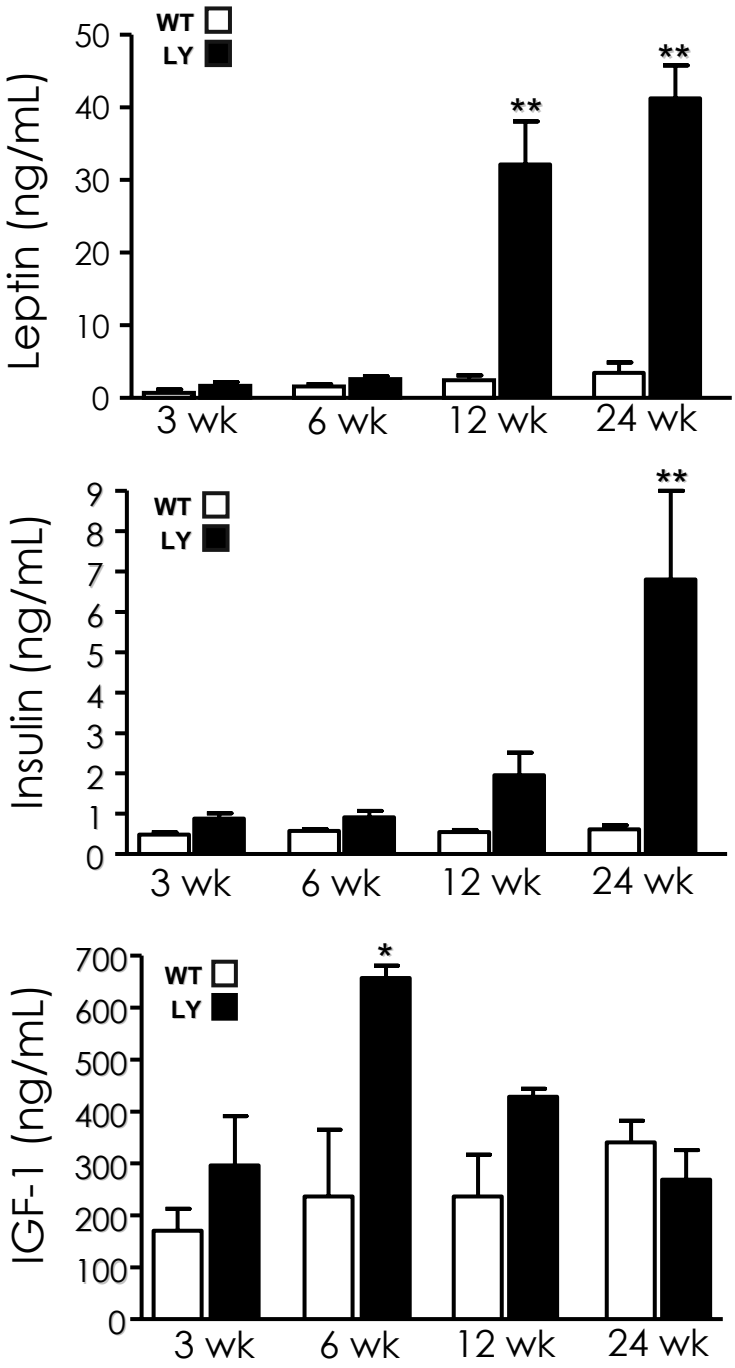


***Figure 2.3*** **Circulating levels of leptin (ng/ml), insulin (ng/ml), and IGF-1 (ng/ml) in 3, 6, 12, and 24 week old female B6 and LY mice.** Circulating levels of leptin (ng/ml), insulin (ng/ml), and IGF-1 (ng/ml) were measured by ELISA using blood serum collected from 3, 6, 12, and 24 week old B6 (white bar) and LY mice (black bar). Significant differences between average circulating hormone levels (n=5) of B6 and LY animals were determined using two-way ANOVA and Bonferonii post-test. *, significant difference ($P < 0.01$); **, significant difference ($P < 0.001$) between B6 and LY within each age. *Error bars* represent mean ± SE.

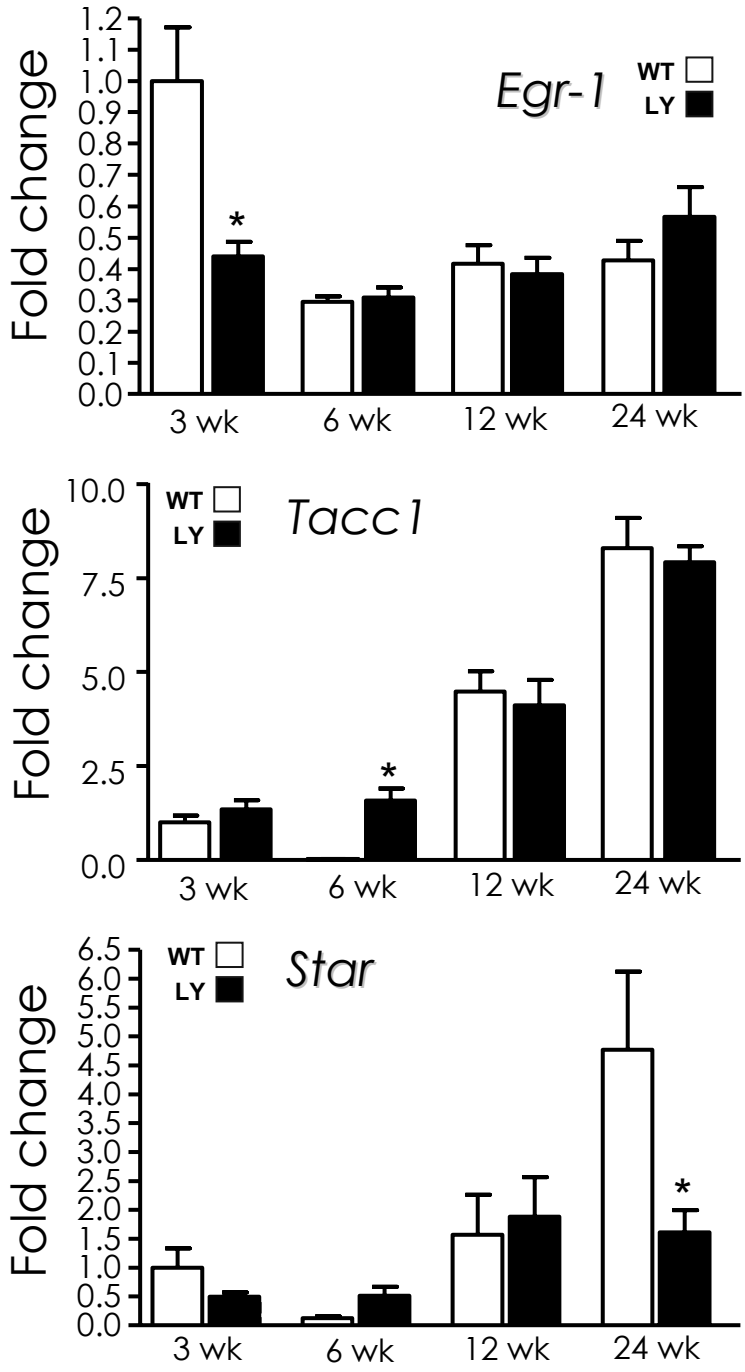


***Figure 2.4*** **Significant differences in *Egr1*, *Tacc1*, and *Star* granulosa cell gene expression between B6 and LY mice.** Granulosa cells were isolated from whole ovaries of B6 (n = 5; white bar) and LY (n = 5; black bar) mice. The mRNA abundance of each candidate gene from each sample group was normalized for GAPDH mRNA abundance. Candidate gene expression from LY and B6 mice across all ages was subsequently compared to expression in 3 week old B6 mice and expressed as a fold change. All QPCR data was tested for significant differences in mRNA abundance using two-way ANOVA and Bonferonii post-test. *, significant fold change ($P < 0.05$) between B6 and LY within each age. *Error bars* represent mean ± SE.

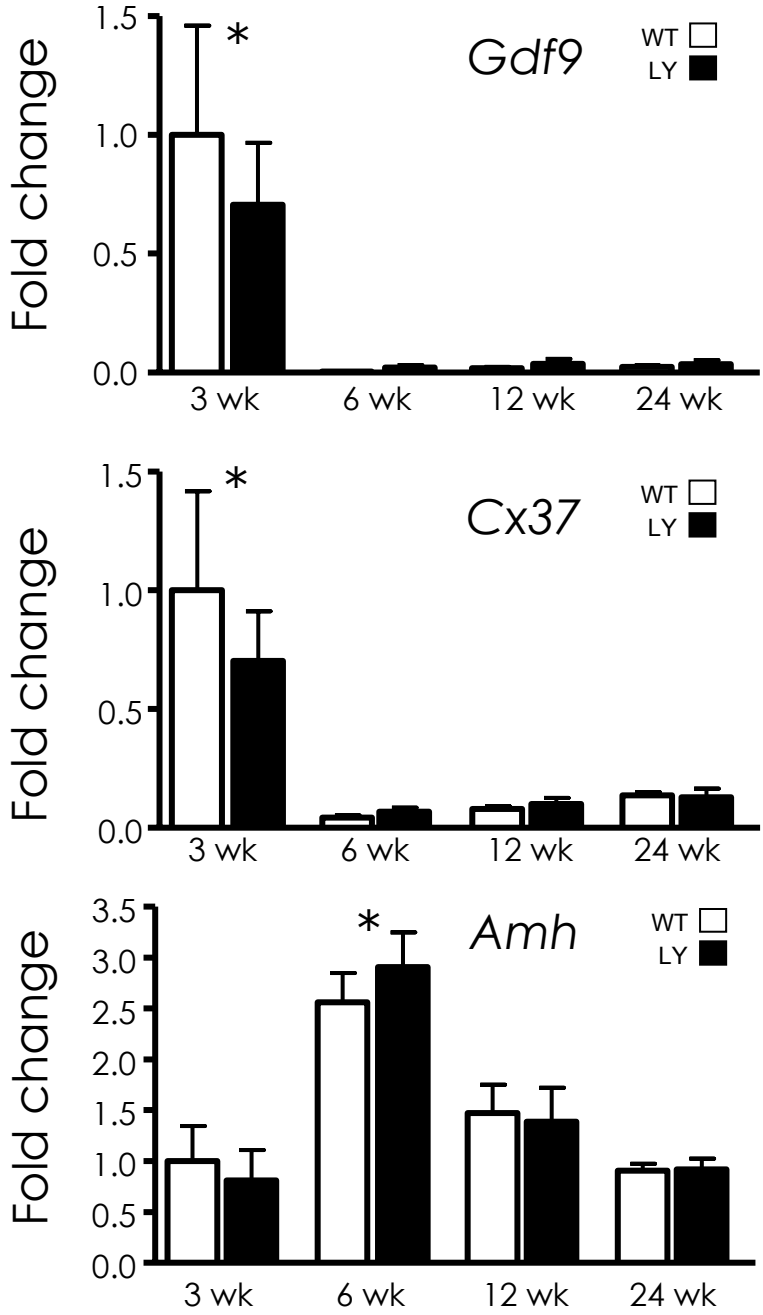


***Figure 2.5*** **Significant differences in *Gdf9*, *Cx37*, and *Amh* gene expression at an early time point compared to all other age groups.** Granulosa cells were isolated from whole ovaries of B6 (n = 5; white bar) and LY (n = 5; black bar) mice. The mRNA abundance of each candidate gene from each sample group was normalized for GAPDH mRNA abundance. Candidate gene expression from LY and B6 mice across all ages was subsequently compared to expression in 3 week old B6 mice and expressed as a fold change. All QPCR data was tested for significant differences in mRNA abundance using two-way ANOVA and Bonferonii post-test. *, significant fold change ($P < 0.05$) between that age group compared to all other age groups. *Error bars* represent mean ± SE.

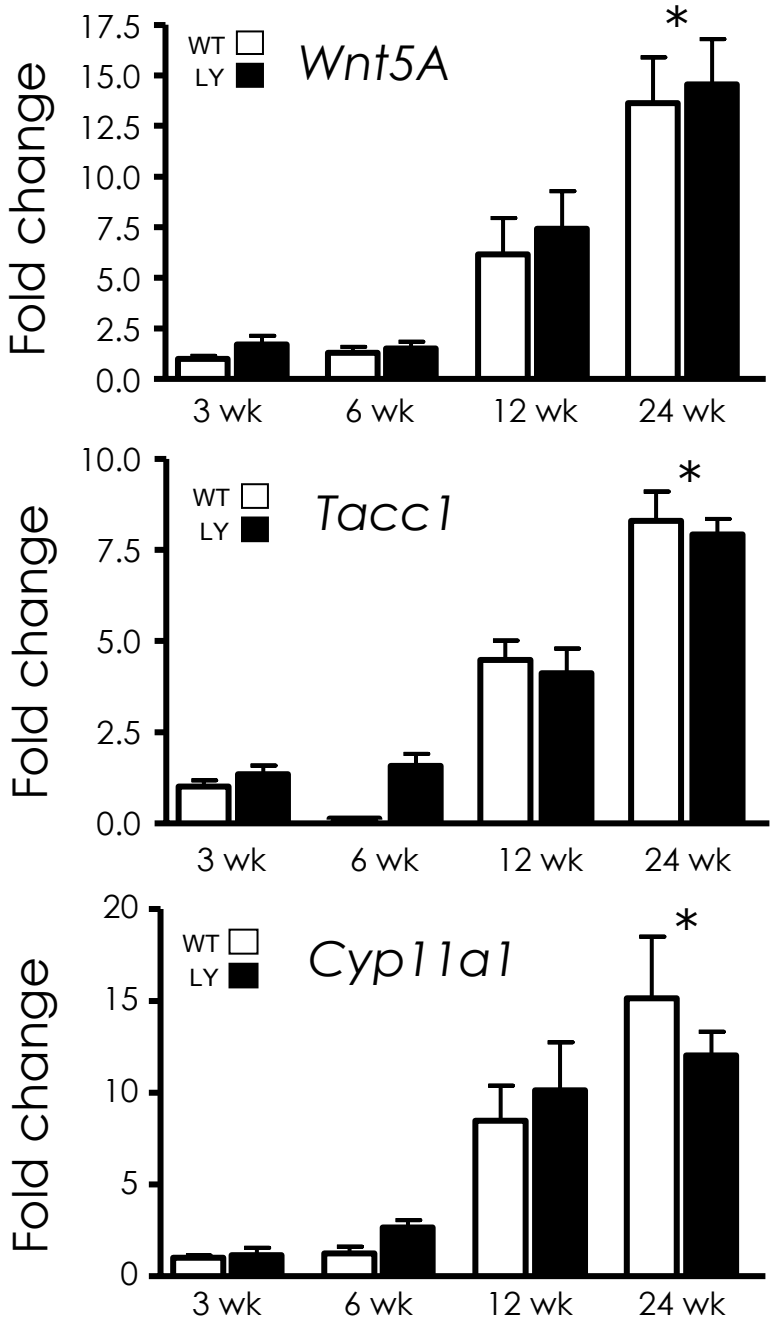


***Figure 2.6*** **Significant differences in *Wnt5a*, *Tacc1*, and *Cyp11a1* gene expression at the later time point compared to all other age groups.** Granulosa cells were isolated from whole ovaries of B6 (n = 5; white bar) and LY (n = 5; black bar) mice. The mRNA abundance of each candidate gene from each sample group was normalized for GAPDH mRNA abundance. Candidate gene expression from LY and B6 mice across all ages was subsequently compared to expression in 3 week old B6 mice and expressed as a fold change. All QPCR data was tested for significant differences in mRNA abundance using two-way ANOVA and Bonferonii post-test. *, significant fold change ($P < 0.05$) between that age group compared to all other age groups. *Error bars* represent mean ± SE.

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# CHAPTER 3

## IGF-1 and cAMP Cooperatively Regulate Granulosa Cell Gene Expression through Akt- but not Erk1/2-Dependent Signaling

### Abstract

Folliculogenesis and granulosa cell function are regulated, in part, by gonadotropin stimulation of cAMP-dependent signal transduction. IGF-1 also contributes to follicular development through its interaction with the IGF-1 receptor, and in some cases, requires simultaneous gonadotropin-dependent signaling. The objective of this study was to identify mechanisms by which IGF-1 regulates the expression of genes in granulosa cells, which are crucial for follicle growth, steroidogenesis, bi-directional communication and ovulation. To achieve this objective, short-term cultures of murine granulosa cells were exposed to no treatment, 1mM cAMP, 100ng/mL IGF-1, or a combination of cAMP and IGF-1. Quantitative, real-time PCR demonstrated that cAMP alone regulated all of the genes tested and IGF-1 alone had no effect on mRNA abundance. However, IGF-1 exhibited an additive effect on a subset of cAMP-regulated genes. IGF-1 and cAMP alter gene expression through activation of the downstream signaling proteins Akt and Erk1/2. To assess the activity of these proteins, Western blot analyses were carried out using untreated, cAMP-treated, IGF1-treated, or cAMP plus IGF-1 treated granulosa cell protein extracts. These Western blots demonstrated that cAMP alone or in combination with IGF-1 increased Erk1/2 phosphorylation compared to untreated cells 30 and 60 minutes post-treatment. IGF-1 or cAMP treatment alone also increased Akt phosphorylation in the granulosa cells. Interestingly, the combined

treatment of IGF-1 and cAMP resulted in an additive increase in Akt phosphorylation indicating cross-talk between the cAMP and IGF-1 signaling pathways. Taken together, these data suggest that IGF-1 and cAMP may have an additive effect on paracrine factor gene expression due to the cooperative activation of Akt. Taken together, these data suggest that IGF-1 enhances the actions of FSH and LH, which may be important for optimal follicular growth and ovulation.

## Introduction

In the primary follicle, granulosa cells are arranged in a single layer surrounding the oocyte. As the follicle grows, the granulosa cells differentiate into a mural granulosa cell layer which is associated with the basement membrane and a cumulus granulosa cell layer which retains intimate contact with the oocyte (1). These granulosa cells play an important role in follicular development. For example, it has been clearly demonstrated that bi-directional communication between the cumulus granulosa cells and the oocyte is essential for oocyte growth and maturation (2). This communication is carried out by both gap junctional and paracrine factor signaling which are mediated by transzonal projections that transverse the zona pelucida (3). The mural granulosa cells are critical for steroidogenesis and ovulation. Thus, the mural and cumulus granulosa cells are necessary for the development and survival of the oocyte (4).

Several hormones are involved in the regulation of granulosa cell function including the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH is essential in preventing granulosa cell apoptosis and follicular atresia, as well as for granulosa cell proliferation, estradiol production, and LH receptor expression while LH regulates steroidogenesis and ovulation (4). FSH and LH work through $G_s$-coupled protein receptors, and upon binding activate adenylyl cyclase (AC) which stimulates cyclic adenosine monophosphate (cAMP) synthesis and activates protein kinase A (PKA). PKA subsequently activates multiple downstream signaling factors including extracellular regulated kinase (Erk1/2) and phosphatidylinositol-3 kinase (PI3K)/Akt (5). These signaling pathways, in turn, regulate the transcription of genes

that contribute to granulosa cell proliferation, cell survival, and differentiation during follicular growth (6, 7).

In addition to the gonadotropins, other endocrine hormones and paracrine factors also modify granulosa cell function. Among these factors, insulin-like growth factor 1 (IGF-1) stimulates DNA synthesis, cell proliferation and survival (i.e. anti-apoptosis), and LH receptor expression in the granulosa and theca cells (8). IGF-1 acts through the IGF-1 receptor, which belongs to the tyrosine kinase family of receptors. Upon IGF-1 binding, the activated receptor recruits the insulin receptor substrate complex-1 (IRS-1), which contains several phosphorylation sites, and once phosphorylated will activate multiple signal transduction cascades including PI3K/Akt pathway (1). Recently Mani *et al.* presented evidence that IGF-1 alone causes activation of the Akt pathway in granulosa cells (7). There is also evidence that FSH and IGF-1 have an additive effect on granulosa cell function including regulation of granulosa cell steroidogenesis, cell cycle progression, follicle development, and apoptosis (1).

While studies have examined the regulation of steroidogenic enzymes by the gonadotropins and IGF-1 (7, 9-11), there is little information about the co-regulation of genes involved in follicular growth or ovulation by these hormones. Therefore, the objectives of this study were to determine genes regulated by cAMP and IGF-1 in murine granulosa cell cultures. Furthermore, the signaling pathways activated by cAMP and IGF-1 that regulate changes in granulosa cell gene expression were also examined. The working hypothesis for this study is that IGF-1 either alone or in combination with cAMP-dependent signaling regulates the expression of genes in granulosa cells, which

are crucial for folliculogenesis and the ovulation of a mature and developmentally-competent oocyte.

## MATERIALS AND METHODS

*Animals:* All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln. All experiments were performed using CF-1 female mice purchased from Charles River Laboratory (Wilmington, MA, USA).

*Granulosa Cell Collection and Culture:* To establish short-term granulosa cell cultures, ovaries (n=20) were isolated from 24-day-old, CF-1 mice and placed in collection medium (1X Leibovitz (Sigma-Aldrich, St Louis, MO, USA), 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and 1% penicillin /streptomycin (Invitrogen, Carlsbad, CA, USA)). Cleaned ovaries were subsequently placed in collection medium containing 6 mM EGTA (Sigma) and incubated for 15 minutes at 37°C. Ovaries were then placed in collection medium containing 0.5 M sucrose (Sigma) for 15 minutes at 37°C. Ovarian follicles were punctured with a 27-gauge needle in collection medium and cells were applied to 40μm filters (BD Falcon, Franklin Lakes, NJ, USA) to remove cumulus-oocyte complexes. Cells were pelleted, counted, and subsequently cultured on fibronectin-coated tissue culture plates in complete medium (DMEM/F12 (Sigma), 10% heat-inactivated FBS (Hyclone), and 1% penicillin/streptomycin (Invitrogen). Prior to culture, tissue culture plates were pre-

treated with 0.25% human fibronectin (Sigma) for one hour at 37°C. Cells were maintained in complete medium for 72 hours followed by culture in serum-free medium (DMEM/F12 (Sigma) and 1% penicillin/streptomycin (Invitrogen). After 16-24 hours in serum-free medium, cells were incubated in the absence or presence of 1mM 8-bromoadenosine 3',5'-cAMP (8-br-cAMP) (Sigma), 100ng/mL IGF-1 (Cell Signaling Technology, Danvers, MA), or a combination of 8-br-cAMP and IGF-1 for 30 min, 60 min, 2 hrs, 4 hrs, or 8 hrs. After treatment, cells were collected in TRI reagent (Ambion Inc., Austin, TX, USA) (2, 4, and 8 hour time-points) or protein extraction buffer (30 and 60 minute time-points).

*RNA extraction:* RNA was extracted and purified from granulosa cells collected in Tri reagent using the Ambion Ribopure Kit (Austin, TX, USA) according to the manufacurer's directions. RNA concentration was determined using the Beckman Coulter DU 730 Life Science UV/Vis Spectrophotometer. Purified total RNA was stored at -80°C.

*Reverse Transcription:* To obtain cDNA for qPCR analysis of gene expression, total RNA (5 mg) from each treated and untreated-control sample was combined with 5 units of RQ1 RNase free DNase (Promega, Madison, WI) and incubated at 37°C for 30 minutes to remove genomic DNA contaminants. The RNA was subsequently combined with 400 units of Moloney Murine Leukemia Virus reverse transcriptase (Promega), 500 mM dNTPs (Promega), and 100 ng of random primers (Roche Applied Science,

Indianapolis, IN) and incubated at 37°C for 2 hours. The resultant cDNA was stored at -20°C.

*Quantitative, Real-Time PCR (qPCR) Analysis:* To carry out qPCR analysis of gene expression, forward and reverse primers (Table 3.1) for genes associated with steroidogenesis, folliculogenesis, or ovulation were designed (Primer Express, Applied Biosystems, Foster City, CA) and synthesized (Integrated DNA Technologies, Coralville, IA). Each set of gene-specific primers was tested empirically to determine the maximal concentration of primers that could be used to produce specific amplification of the target sequence in the absence of primer dimer amplification. Quantitative PCR (qPCR) reactions were carried out using equivalent dilutions of each cDNA sample, *Power* SYBR Green PCR Master Mix (Applied Biosystems), the empirically determined concentration of each primer, and the 7900HT Fast Real-Time PCR system (Applied Biosystems). To account for differences in starting material, qPCR reactions were also carried out for each cDNA sample using TaqMan rodent GAPDH control reagents (Applied Biosystems). The relative abundance of specific gene products and GAPDH in each cDNA sample was determined using serial dilutions of whole ovary cDNA. The relative abundance of each specific gene product was divided by the relative abundance of GAPDH in each sample to generate a normalized abundance for each gene interrogated. The normalized abundance of each gene in treated cells was compared to untreated control cells and expressed as a fold-change.

*Whole Cell Protein Extracts:* To extract total soluble protein from granulosa cell cultures, cells were collected into modified RIPA buffer (50mM Tris-HCl pH7,4, 1% NP-

40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) containing phosphatase inhibitors (1 mM $Na_3VO_4$, and 1 mM NaF) and Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics). Cells in the RIPA buffer were sonicated 10 seconds and the cell supernatant was subsequently separated from cell debris by centrifugation at 12,000 x g. The supernatant was collected, aliquoted, and stored at -80°C. Protein concentrations were determined using the Pierce BCA (bicinchoninic acid) Protein Assay (Rockford, IL, USA).

*Western Blot Analyses:* Whole cell extracts were resolved by SDS-PAGE **(**4% stacking; 10% separating gel) and separated protein transferred to Immobilon PVDF (Millipore, Billerica, MA). Following transfer, the membranes were blocked with 5% nonfat dry milk in 1X TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 01% (v/v) Tween 20). The membrane was then incubated with primary antibody against phospho-Akt (Cell Signaling Technology), or phospho-Erk1/2 (Cell Signaling Technology) overnight at 4°C. Blots were washed with 1X TBST and incubated with HRP-conjugated secondary antibody. Proteins were visualized using West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and exposure of blots to X-ray film. Following visualization of phosphorylated protein, blots were stripped with Restore Plus Western Blot Stripping Buffer (Pierce), blocked with 5% milk in 1XTBST, and incubated with primary antibody against total Akt or total Erk1/2 (Cell Signaling Technology). Total protein was visualized as described for the phosphorylated protein. The visualized total protein served as a loading control for each experiment. To quantify the relative amount of phosphorylated and total protein expressed in each sample, the density of each protein

band in each sample was determined as described by Nahant (http://lukemiller.org/journal/2007/08/quantifying-western-blots-without.html). Briefly, X-ray images of the Western blots were scanned and the density of the protein bands determined in Photoshop. The density of the phosphorylated band in each sample was normalized using the density of the total protein band in each sample. The normalized abundance of phosphorylated Akt or phosphorylated Erk1/2 in treated cells was subsequently compared to untreated control cells and expressed as a fold-change.

*Statistical Analyses:* All statistical analyses were carried out using GraphPad Prism 4.0 (Graphpad Software, La Jolla, CA). QPCR and Western blot data were analyzed using 2-way ANOVA with treatment and time as the variants. Pairwise comparisons were carried out using the Bonferroni post-test. Differences were considered significant at $P < 0.05$.

## Results

*cAMP regulates Granulosa Cell Steroidogenic Gene Expression.*

To identify genes regulated by gonadotropins and IGF-1, isolated granulosa cells were collected from 24-day-old CF-1 mice, placed in short-term culture, and treated with either 1mM of 8-bromoadenosine 3',5'-cAMP (cAMP) which is the second messenger activated by FSH and LH in granulosa cells, 100ng/mL of IGF-1, or a combination of both cAMP and IGF-1. Control cells were maintained in the absence of any treatment. Treatments were maintained for 2, 4, or 8 hours in order to identify acute changes in gene

expression upon stimulation of the granulosa cells with cAMP and/or IGF-1. The regulation of steroidogenic enzyme genes by cAMP and IGF-1, alone or in combination, was examined first using quantitative, real-time RT-PCR (qPCR). When the genes for side-chain cleavage (*Cyp11a1*), P450 aromatase (*Cyp19a1*), and steroidogenic acute regulatory protein (*Star*) were analyzed (Figure 3.1) cAMP increased the expression of all genes at all time points with the exception of *Cyp11a1* at the 2-hour time point. Furthermore, IGF-1 alone had no effect on the expression of any of these genes at any time-point measured. However, when cells were treated with a combination of cAMP and IGF-1, *Star* expression exhibited a modest but significant increase over cAMP alone at 8 hours while *Cyp11a1* expression was blunted at both the 4 and 8-hour time point. These data indicate co-regulation of *Cyp11a1* and *Star* expression by cAMP and IGF-1 at the later time points.

*Genes Associated with Follicular Growth are Co-Regulated by cAMP and IGF-1.*

To determine if cAMP and/or IGF-1 regulate the expression of paracrine factors associated with follicular growth, the mRNA abundance of *Gdf9, Amh, Kitl, Egr-1, Wnt4, Wnt5A,* and *Wnt5B* (Figure 3.2) was examined in the short-term granulosa cell cultures. Like the steroidogenic enzymes, the expression of growth differentiation factor-9 (*Gdf9*), early growth response (*Egr-1*), and *Wnt4* was increased by cAMP while *Wnt5B* expression was modestly decreased by cAMP. Conversely there was no change in anti-Mullerian hormone (*Amh),* Kit ligand *(Kitl),* or *Wnt5A* (data not shown) expression upon cAMP alone treatment. Furthermore, IGF-1 alone had no effect on the expression of any of the genes tested. When cells were treated with a combination of cAMP and IGF-1

several genes exhibited co-regulation. There was an additive effect of the combination treatment on *Egr-1* levels at the 2 hour time point and *Wnt5B* mRNA abundance at the 8 hour time point. Interestingly, *Kitl* showed a significant increase 4 and 8 hours post-treatment only when cells were treated with both cAMP and IGF-1. Likewise, there was a trend with a P-value of less than 0.06 for decreased *Amh* expression 2 hours after cells were co-treated with cAMP and IGF-1. Taken together, these data suggest that the combined actions of cAMP and IGF-1 play an important role in the stimulation of follicular growth.

*Regulation of Gap Junction and MTOC-Associated Genes by cAMP*.

Connexin 37 (*Cx37*) and connexin 43 (*Cx43*) encode proteins that form crucial gap junctions between granulosa cells and between the oocyte and granulosa cells (12). Transforming acidic coiled coil gene 1 (*Tacc1*) interacts with the microtubule organizing center (MTOC) which may contribute to the establishment and maintenance of transzonal projections (13). Given that these genes play an important role in bi-directional communication between the oocyte and granulosa cells, the expression profile of each gene in cAMP and/or IGF-1 treated cells was examined (Figure 3.3). cAMP significantly decreased the expression of both *Cx37* and *Cx43* at the 8 hour time. Conversely, cAMP significantly increased the expression of *Tacc1* at both the 2 and 4-hour time points. However, IGF-1 alone did not alter the mRNA abundance of these 3 genes. Furthermore, there was no additive effect on the expression of any of these genes when cells were co-treated with cAMP and IGF-1.

*Genes Associated with Ovulation are Co-Regulated by cAMP and IGF-1.*

The last group of genes analyzed in the cAMP and IGF-1 treated granulosa cells play an important role during ovulation and include the EGF-like factors amphiregulin (*Areg*), epiregulin (*Ereg*), and betacellulin (*Btc*) (Figure 3.4). Richards *et al*. has compared ovulation to an inflammatory process (5); and therefore, the expression profile of interleukin-6 (Il6) was also measured. As expected, cAMP stimulated the expression of *Areg*, *Epi*, and *Btc* at all tested time points. Likewise, *Il-6* showed a significant fold increase 2 hours post cAMP treatment. Similar to the other genes examined in this study, IGF-1 alone did not change the expression of any of these genes compared to the untreated control. However, the combined treatment of cAMP and IGF-1 had a modest but significant additive effect on the expression of *Areg* 2 and 4 hours post-treatment, *Btc* 4 hours post-treatment and *Il-6* 2 and 8 hours post-treatment.

*cAMP and IGF-1 have an Additive Effect on Akt but not Erk1/2 phosphorylation.*

The QPCR data suggests an additive effect of IGF-1 on cAMP-dependent gene expression. To determine what signaling factors may be involved in this additive effect, Western blot analysis was carried out. Briefly, short-term granulosa cell cultures were treated with 1 mM cAMP, 100 ng/mL IGF-1, or a combination of cAMP and IGF-1 for 30 or 60 minutes. Control cells were maintained in culture an additional 30 or 60 minutes in the absence of any treatment. Protein extracts from the untreated and treated cells were separated by SDS-gel electrophoresis and Western blot carried out using antibodies against phosphorylated Akt or phosphorylated Erk1/2 (Figure 3.5). Blots were subsequently probed for total Akt or total Erk1/2, which served as loading controls.

Insulin like growth factor-1 alone significantly increased phospho-Akt levels compared to the untreated control 30 and 60 minutes post-treatment. Furthermore, there was a significant additive effect on Akt phosphorylation at the 30 and 60-minute time points when cells were co-treated with cAMP and IGF-1. While cAMP alone increased Erk1/2 phosphorylation 30 and 60 minutes post-treatment, IGF-1 alone did not alter Erk1/2 phosphorylation levels compared to untreated controls. Furthermore, combined treatment of cells with cAMP and IGF-1 did not increase Erk1/2 phosphorylation compared to cAMP treatment alone indicating no additive effect of IGF-1 on cAMP-dependent Erk1/2 phosphorylation.

## Discussion

It is well known that FSH- and LH-dependent regulation of steroid biosynthesis is mediated by the cAMP/PKA signaling pathway (14). The increase in steroid synthesis is due, in part, to increased expression of steroidogenic enzymes including *Star, Cyp11a1* (P450 side chain cleavage), and *Cyp19a1* (P450 aromatase). Results from the present study confirm that cAMP increases the mRNA abundance of the steroidogenic genes *Cyp11a1*, *Cyp19a1*, and *Star*. However, the role of IGF-1 dependent signaling on steroidogenic enzyme gene expression is less clear. Eimerl and Orly showed that IGF-1 alone had no effect on *Cyp11a1* or *Star* mRNA abundance in rat granulosa cells (15). Conversely, Mani *et al*. found that IGF-1 alone significantly enhanced mRNA expression of *Cyp11a1* and *Cyp19a1* in bovine granulosa cells (7). Several reports have shown that the effect of IGF-1 on the expression of these steroidogenic enzyme genes is dependent

on FSH. Specifically, a synergistic effect of FSH and IGF-1 on *Cyp19a1* expression has been reported in cultured bovine (10, 11) and rat granulosa cells (16), while a synergistic effect of FSH and IGF-1 on *Star* expression has been shown in porcine granulosa cells (9). In the current study, the combination treatment of cAMP and IGF-1 blunted the expression of *Cyp11a1* 4 and 8 hours post-treatment compared to cAMP alone. IGF-1 also had an additive effect on cAMP-dependent expression of *Star* 8 hours post-treatment. However, there was no additive effect of IGF-1 on cAMP regulation of *Cyp19a1*. Inconsistencies between these studies could be the result of differences in the length of treatment with our study focussing on acute regulation of IGF-1 up to 8 hours and other studies focussing on later time points. Also one should consider that species, stage of estrus, and/or experimental treatment protocols could influence *Cyp11a1*, *Cyp19a1,* and *Star* expression through mechanisms not yet fully understood.

Previous studies examining the effect of IGF-1 and its possible synergistic effect on gonadotropin-dependent regulation of granulosa cell function have generally focused on steroidogenic genes. Therefore, to our knowledge, the co-regulation of genes involved in follicular growth and ovulation by cAMP and IGF-1 has not been examined in granulosa cells. In this study, we demonstrated a significant cooperative effect of cAMP and IGF-1 on the expression profile of genes involved in follicular growth including *Kitl* and *Amh*. *Kitl* is crucial for the transition from primordial to primary follicles, follicle development, and preventing atresia (17), whereas Amh inhibits initial follicle recruitment and cyclic selection of a dominant follicle (18, 19). Co-treatment of granulosa cells with cAMP and IGF-1 was required to increase *Kitl* expression with no effect on *Kitl* mRNA abundance when cells were treated with either cAMP or IGF-1

alone. On the other hand, there was a trend for decreased *Amh* expression at the earliest time point when cells were treated with a combination of cAMP and IGF-1. Together, these findings suggest that IGF-1 may sensitize the granulosa cells to FSH-dependent signaling in order to increase follicular growth and prevent follicular atresia.

This study also demonstrated for the first time an additive effect of IGF-1 on cAMP regulation of genes involved in ovulation including *Areg* and *Btc*. *Areg* and *Btc* are part of the epidermal growth factor (EGF)-1 family, which stimulate cumulus expansion and oocyte maturation. Conti *et al*. have shown that after the LH surge, there is a rapid increase in the expression of epidermal growth factor (EGF)-like family members (20). Although the additive effect of IGF-1 on *Areg* and *Btc* expression was modest, the increased mRNA levels produced when cells were treated with both cAMP and IGF-1 may be very important for reaching a threshold level of these factors which is essential for appropriate ovulation and meiotic resumption in the oocyte. It should be noted that none of the genes regulating follicle growth or ovulation exhibited changes in mRNA abundance when cells were treated with IGF-1 alone, indicating the IGF-1 must work in coordination with gonadotropins to exert its effects on these genes.

The QPCR data demonstrates that IGF-1 has an additive effect on the expression of certain genes, but the pathways that are involved in this regulation have not been clearly defined. This study focused on the Akt and Erk1/2 pathways due to the role of these pathways on cell survival and proliferation. IGF-1 alone increased Akt phosphorlyation in granulosa cells, which has also been demonstrated in bovine granulosa cells (7). In addition, there was a significant increase in Akt phosphorylation when cells were co-treated with cAMP and IGF-1, consistent with findings presented by

Ryan *et al*. (11). In contrast, we found that IGF-1 alone and in combination with cAMP had no effect on Erk1/2 phosphorylation, which is in agreement with studies carried out by Mani *et al*. (7). Therefore, it is our conclusion that the additive effect of IGF-1 on granulosa cell gene expression is mediated by the Akt pathway.

Based on the results of this study, we have proposed a model of cAMP and IGF-1 dependent regulation of granulosa cell gene expression through either the Erk or Akt signaling pathways (Figure 3.6). cAMP-dependent regulation of gene expression which is independent of IGF-1 signaling is likely mediated by the Erk1/2 signaling factor. Conversely, cAMP-dependent regulation of gene expression that is enhanced by IGF-1 is likely mediated through activation of Akt. There are a handful of genes (e.g. *Star*, *Wnt5b*, *Egr1* and *Btc*), which may be regulated by both pathways in a temporal or cooperative manner. Future studies will be carried out using inhibitors of the Akt or Erk signaling pathway to further define the cooperative nature of cAMP and IGF-1 on granulosa cell gene expression.

Together, this study provides evidence that cAMP regulates granulosa cell gene expression through both the Akt and Erk1/2 pathways and that the additive effect of IGF-1 on cAMP-dependent regulation of a subset of genes may be due to the increased stimulation of Akt phosphorylation. We have also shown that IGF-1 alone does not alter gene expression in granulosa cells maintained in short term culture.

Table 3.1. Primer sequences used for QPCR analysis

| Gene | Accession # | Primer | Sequence |
|---|---|---|---|
| *Amh* | NM_007445 | Forward | 5'-TCC TAC ATC TGG CTG AAG TGA TAT G-3' |
| | | Reverse | 5'-CAG GTG GAG GCT CTT GGA ACT-3' |
| *Areg* | NM_009704 | Forward | 5'-CAG CTG CTT TGG AGC TCA ATG-3' |
| | | Reverse | 5'-GTG GTC CCC AGA AAG CGA-3' |
| *Btc* | NM_007568 | Forward | 5'-TGC CCC AAG CAG TAC AAG C-3' |
| | | Reverse | 5'-TTT GCT CGT CCA CCA CGA-3' |
| *Cx37* | NM_008120 | Forward | 5'-CGA GAG AGG CCC TGG AAA C-3' |
| | | Reverse | 5'-CCA CCA CGG TCG AGT GTT C-3' |
| *Cx43* | NM_010288 | Forward | 5'-TGA AAG AGA GGT GCC CAG ACA T-3' |
| | | Reverse | 5'-GTG GAG TAG GCT TGG ACC TTG T-3' |
| *Ccnd2* | NM_009829 | Forward | 5'-AGC AGG ATG ATG AAG TGA ACA CA-3' |
| | | Reverse | 5'-GGC TTT GAG ACA ATC CAC ATC AG-3' |
| *Cyp11a1* | NM_019779 | Forward | 5'-TGA ATG ACC TGG TGC TTC GTA AT-3' |
| | | Reverse | 5'-TCG ACC CAT GGC AAA GCT-3' |
| *Cyp19a1* | NM_007810 | Forward | 5'-GGC CCT GGT CTT GTT CGA-3' |
| | | Reverse | 5'-GCC GGT CCA AAT GCT GC-3' |
| *Egr1* | NM_007913 | Forward | 5'-GGG AGC CGA GCG AAC AA-3' |
| | | Reverse | 5'-TCA GAG CGA TGT CAG AAA AGG A-3' |
| *Ereg* | NM_007950 | Forward | 5'-GCA CTC CGC AAG CTG CA-3' |
| | | Reverse | 5'-AGC AGC GTC AAG ACC CAA GA-3' |
| *Gdf9* | NM_008110 | Forward | 5'-GCC GGG CAA GTA CAG CC-3' |
| | | Reverse | 5'-TTT GTA AGC GAT GGA GCC G-3' |
| *Il6* | NM_031168 | Forward | 5'-AGT CGG AGG CTT AAT TAC ACA TGT T-3' |
| | | Reverse | 5'-TGC CAT TGC ACA ACT CTT TTC T-3' |
| *Kitl* | NM_013598 | Forward | 5'-CGC ACA GTG GCT GGT AAC AG-3' |
| | | Reverse | 5'-GGT AGC AAG AAC AGG TAA GGA TGA G-3' |
| *Nek2a* | NM_010892 | Forward | 5'-CTG TGG GCA GGA ACC TTT GT-3' |
| | | Reverse | 5'-CAC ACA TCC ATT TGC AGA CCA-3' |
| *Nek4* | NM_011849 | Forward | 5'-AGA AGG AGA GGC TAC AGG GCA ATA-3' |
| | | Reverse | 5'-CAG GTC CAC CTT GGT TTC CAT CAT-3' |
| *p27kip* | NM_009875 | Forward | 5'-GAA GCC CGG CCT TCG A-3' |
| | | Reverse | 5'-CAT GTA TAT CTT CCT TGC TTC ATA AAG C-3' |
| *Star* | NM_011485 | Forward | 5'-GTG TGC CTT CGA CCC CC-3' |
| | | Reverse | 5'-AAA GTG CTT GCT GCC TAC CC-3' |
| *Tacc1* | NM_199323 | Forward | 5'-CTA GGC AAA CAG TCC TTT TCT TAG AAA-3' |
| | | Reverse | 5'-TGG CCC TCG TAT CCT CAG C-3' |
| *Wnt4* | NM_009523 | Forward | 5'-ACC GGC GCT GGA ACT GT-3' |
| | | Reverse | 5'-TCC CGG GTC CCT TGT GT-3' |
| *Wnt5a* | NM_009524 | Forward | 5'-GCA TCC TCA TGA ACT TAC ACA ACA A-3' |
| | | Reverse | 5'-CTC CAT GAC ACT TAC AGG CTA CAT CT-3' |
| *Wnt5b* | NM_009525 | Forward | 5'-CCA AGA CGG GCA TCA GAG A-3' |
| | | Reverse | 5'-GCG CTC ACT GCA TAC GTG AA-3' |

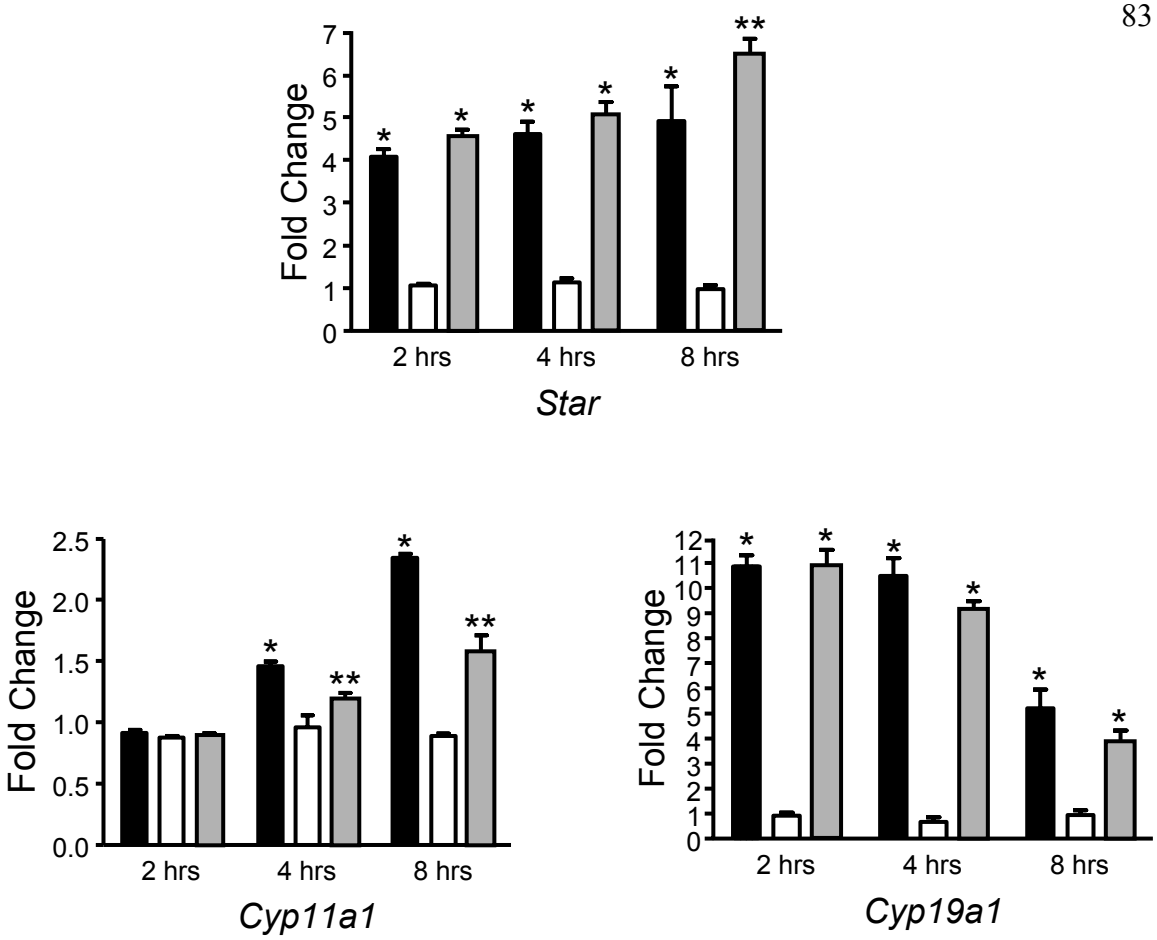


***Figure 3.1.*** **cAMP and IGF-1 stimulate significant changes in the expression of steroidogenic genes in cultured granulosa cells.** Murine granulosa cells were isolated and maintained in short-term culture. After 24h serum starvation, cells were treated with 1mM cAMP (black bar), 100ng/mL IGF-1 (white bar), or a combination of cAMP and IGF-1 (gray bar) for 2, 4, or 8 hours. The mRNA abundance of each candidate gene (*StAR, Cyp11a1,* and *Cyp19a1*) in each sample was normalized for GAPDH mRNA abundance. Candidate gene expression in treated cells was subsequently compared to expression in untreated cells and expressed as a fold change. All QPCR data was tested for significant differences in mRNA abundance using two-way ANOVA and Bonferonii post-test. *, significant fold change ($P < 0.05$) compared to untreated control within that treatment time only. **, significant fold change ($P < 0.05$) between cAMP alone and cAMP + IGF-1 within that treatment time only. *Error bars* represent mean ± SE.

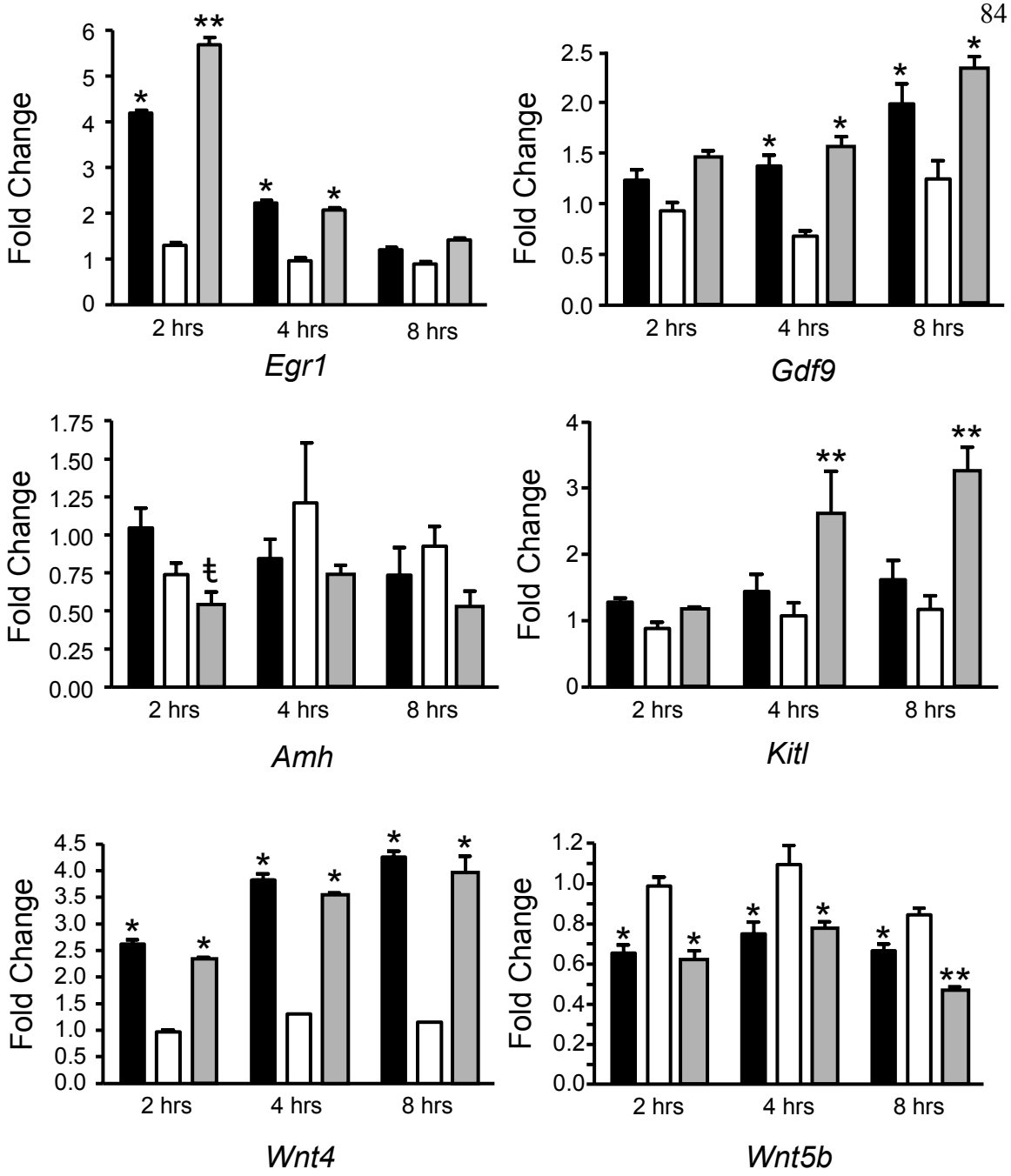


***Figure 3.2.*** **cAMP and IGF-1 stimulate significant changes in the expression of follicular growth genes in cultured granulosa cells.** Murine granulosa cells were treated with cAMP (black bar), IGF-1 (white bar), or a combination of cAMP and IGF-1 (gray bar) and the expression of *Kitl, Gdf9, Amh, Egr1, Wnt4*, and *Wnt5b* analyzed by QPCR as described in Figure 3.1. *, significant fold change ($P < 0.05$) compared to untreated control within that treatment time only. **, significant fold change ($P < 0.05$) between cAMP alone and cAMP + IGF-1 within that treatment time only. ŧ, trend ($P < 0.06$) between cAMP alone and cAMP + IGF-1 within that treatment time only. *Error bars* represent mean ± SE.

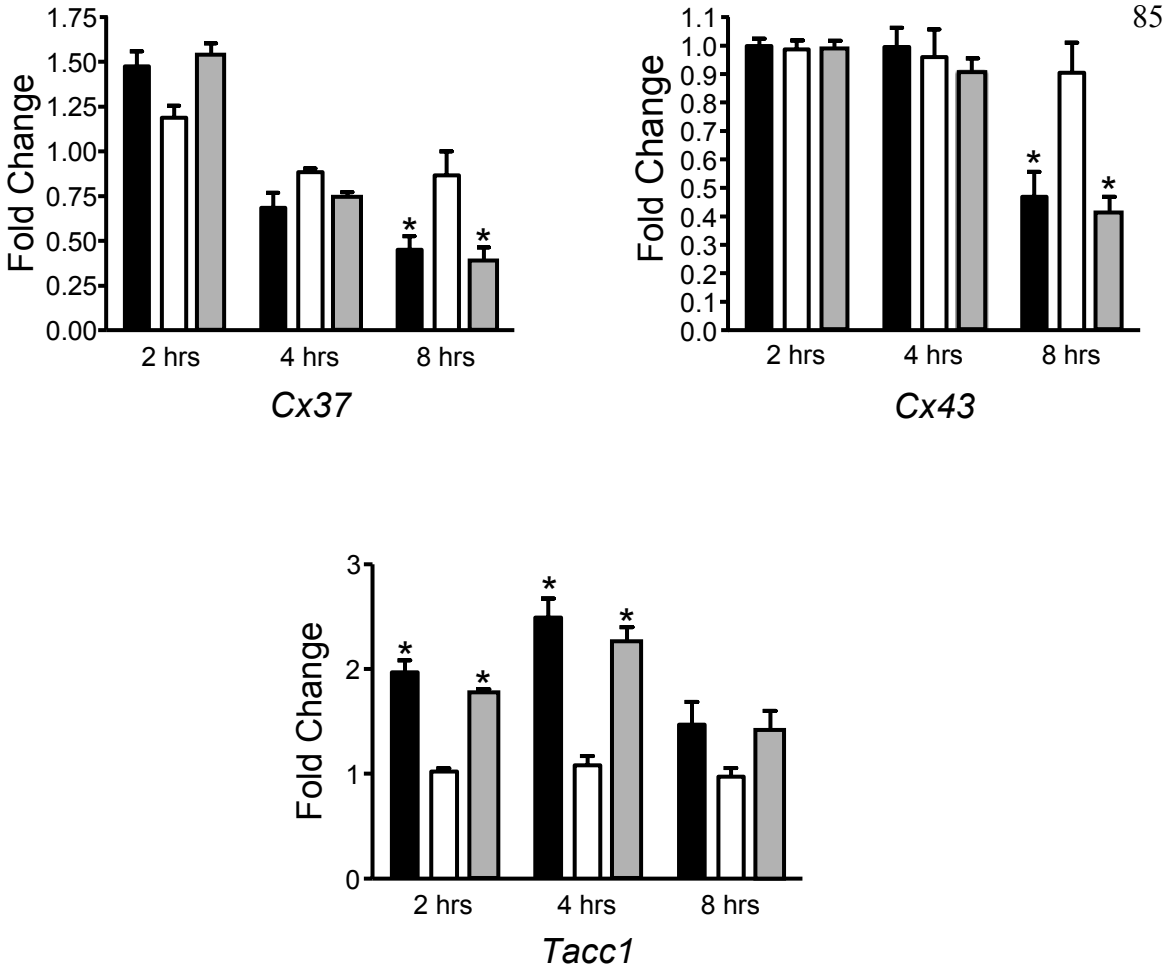


***Figure 3.3.*** **cAMP and IGF-1 stimulate significant changes in the expression of gap junction and MTOC genes in cultured granulosa cells.** Murine granulosa cells were treated with cAMP (black bar), IGF-1 (white bar), or a combination of cAMP and IGF-1 (gray bar) and the expression of *Cx37, Cx43*, and *Tacc1* analyzed by QPCR as described in Figure 3.1. *, significant fold change ($P < 0.05$) compared to untreated control within that treatment time only. **, significant fold change ($P < 0.05$) between cAMP alone and cAMP + IGF-1 within that treatment time only. *Error bars* represent mean ± SE.

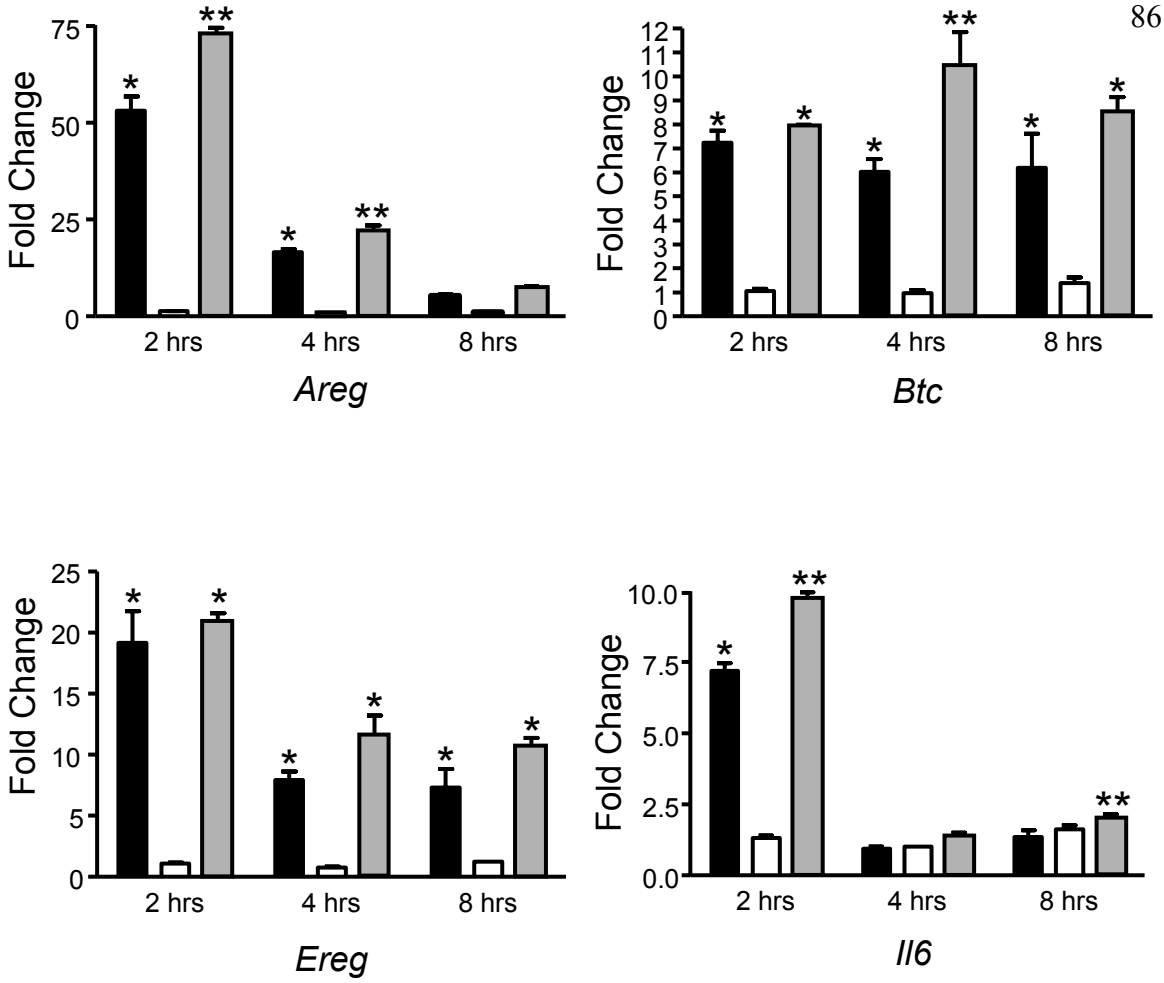


***Figure 3.4.*** **cAMP and IGF-1 stimulate significant changes in the expression of genes associated with ovulation in cultured granulosa cells.** Murine granulosa cells were treated with cAMP (black bar), IGF-1 (white bar), or a combination of cAMP and IGF-1 (gray bar) and the expression of *Areg, Ereg, Btc,* and *Il6* analyzed by QPCR as described in Figure 3.1. *, significant fold change ($P < 0.05$) compared to untreated control within that treatment time only. **, significant fold change ($P < 0.05$) between cAMP alone and cAMP + IGF-1 within that treatment time only. *Error bars* represent mean ± SE.

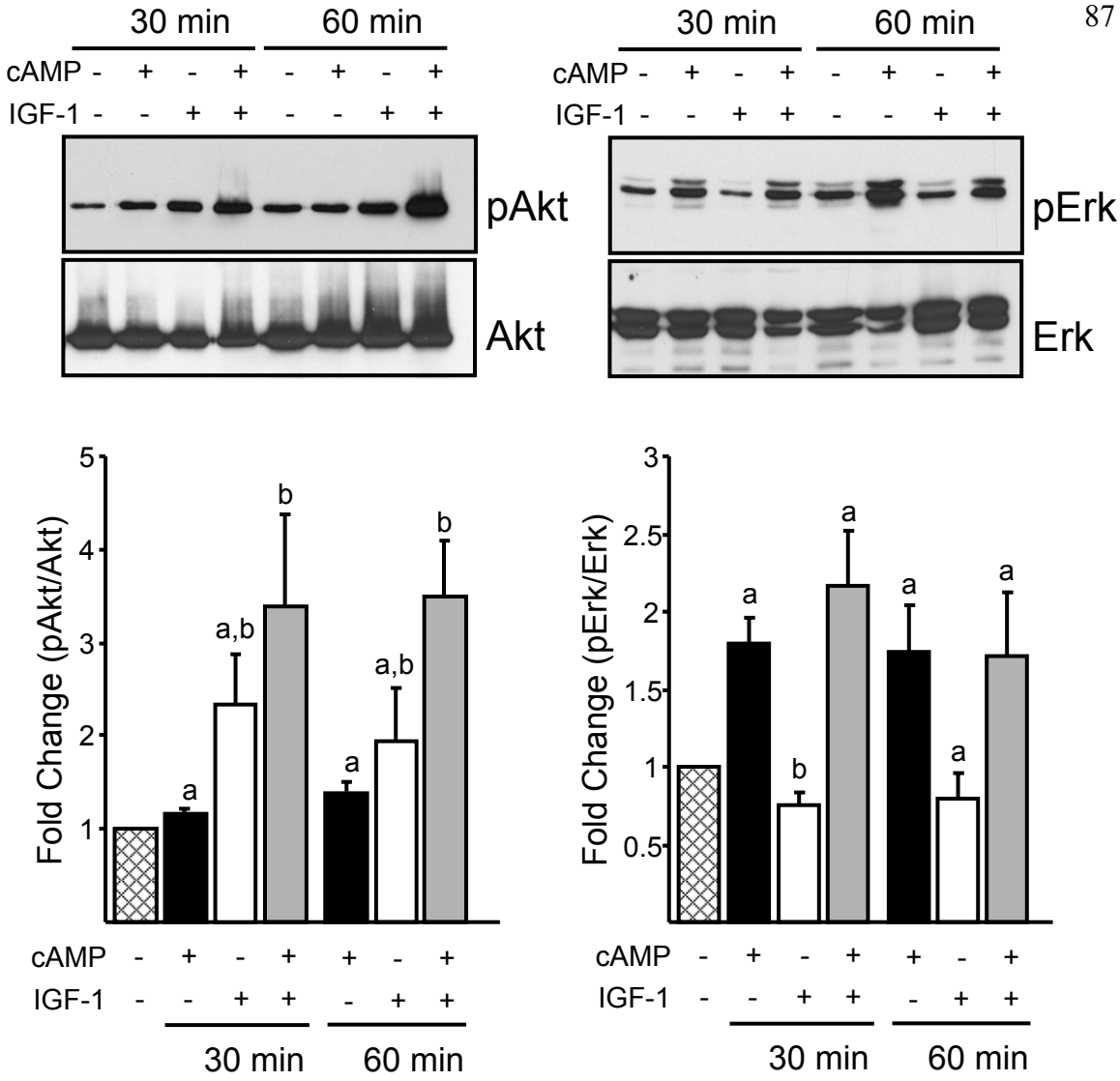


***Figure 3.5.*** **cAMP and IGF-1 activation of Akt phosphorylation and Erk1/2 phosphorylation.** Murine granulosa cells were isolated and maintained in short-term culture. After 24h serum starvation, cells were treated with 1mM cAMP (black bar), 100ng/mL IGF-1 (white bar), or a combination of cAMP and IGF-1 (gray bar) for 30 and 60 minutes. Protein extracts from untreated and treated cells were separated and Western blot analysis carried out using phosphorylated Akt or phosphorylated Erk1/2 antibodies (A). Blots were subsequently probed for total Akt or total Erk1/2, which served as loading controls. Semi-quantitative analysis of band density was tested for statistically significant differences between treatment groups within each time point using two-way ANOVA and Bonferroni post-test analysis. Significant differences ($P < 0.05$) in phospho-protein/total protein ratio between treatments is indicated by different letters *Error bars* represent mean ± SE.

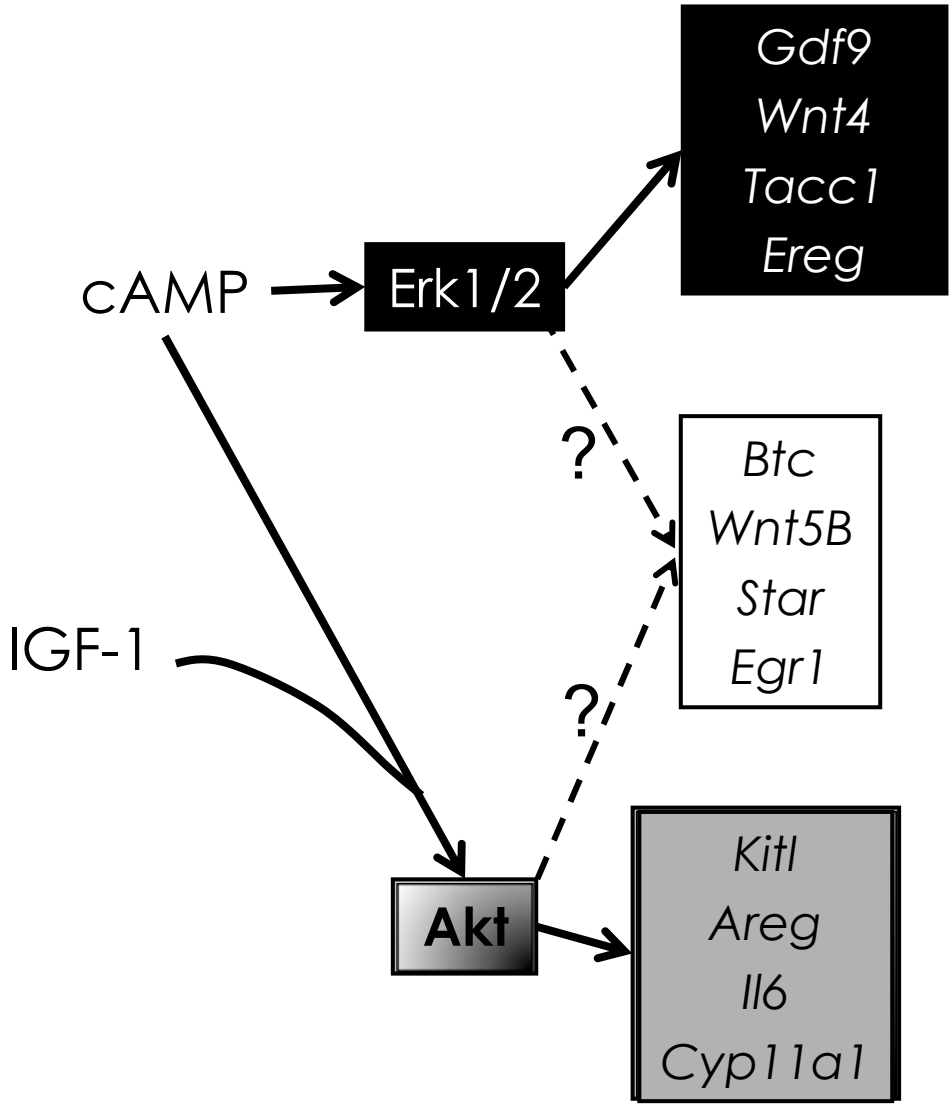


***Figure 3.6.*** **Proposed model for cAMP and IGF-1 regulation of granulosa cell gene expression.** Genes in the black box are regulated solely by cAMP activation of the Erk1/2 pathways, while the genes in the gray box are regulated by the additive activity of both cAMP and IGF-1 on Akt phosphorylation. A subset of genes (white box) may be regulated by both the Akt and Erk1/2 pathways in a temporal or cooperative manner. Future experiments using inhibitors to PI3K (LY294002) or MEK (U0126) will be carried out to confirm the mechanism of cAMP and IGF-1 dependent regulation of granulosa cell gene expression.

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