CHARACTERIZATION OF TWO FACTORS WITH OPPOSING REGULATORY ACTIONS ON FOLLICLE DEVELOPMENT IN THE HEN OVARY: KIT LIGAND AND ANTI-MÜLLERIAN HORMONE

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ABSTRACT

In two separate studies, the present research investigated two factors noted to have opposing regulatory actions on follicle activation: KL, which is thought to promote follicle activation, and AMH, known to inhibit follicle activation. With respect to regulation and function, there has been little study of either factor in the hen ovary. The domestic hen ovary, with its highly regulated follicle hierarchy, is a useful animal model for study of folliculogenesis. Our goal was to characterize the mRNA expression pattern of kit ligand (KL) throughout follicle development, as well as examine regulation and function of the Kit system in the hen ovary. Additionally, we examined the effects of bioactive chicken AMH on granulosa cell mRNA expression of FSHR and steroid production.

In the first study, the highest levels of steady-state KL mRNA expression were seen in the very small (<1 mm) follicles and 3-5 mm follicle granulosa cells, with lower levels seen in the larger, selected follicles ($p<0.001$). There was no difference seen in KL mRNA expression between granulosa cells from the germinal disc region versus the non-germinal disc region. With regard to regulation, KL mRNA expression was examined following various treatments of granulosa cell culture. KL expression in granulosa cells from 6-8 mm follicles was shown to be increased by treatment with oocyte-conditioned medium (OCM), vitamin D3, and FSH. KL mRNA expression was unaffected by treatment with testosterone, progesterone and estradiol. With respect to function, the Kit system appears to regulate granulosa cell progesterone production from the large follicles, but has no influence on theca estradiol production or on granulosa cell

proliferation. Expression of cKit protein was observed in <1 mm follicles, theca tissue, and ovarian body, but was not seen in granulosa cells.

In the second study, we examined effects of bioactive chicken AMH (in the form of TCM, testis conditioned media) on granulosa cell mRNA expression of FSHR. TCM consistently decreased granulosa cell FSHR mRNA expression in 3-5 mm and 9-16 mm follicles, but we were unable to determine the specificity of this effect using an AMH antibody. TCM increased granulosa cell proliferation, indicating downregulation of FSHR mRNA was not due to adverse effects on the granulosa cells. TCM treatment significantly decreased granulosa cell progesterone production, but this was not blocked by AMH antibody. Our results show that TCM contains a bioactive substance which regulates granulosa cell function, and suggest that this substance is not AMH. However, it is possible that the antibody against AMH did not completely immunoneutralize the AMH present in TCM, so we are hesitant to rule out the possibility that our results are due to bioactive AMH.

BIOGRAPHICAL SKETCH

Mila Christen Kundu was born on February 8, 1984 in Waverly, New York to Mary Lou and Radha Kundu. Mila grew up in Waverly and graduated from Waverly High School in June of 2002. Mila attended college at SUNY Geneseo in Geneseo, NY and studied abroad in Lismore, Australia during her junior year. She received a Bachelor of Science degree in May, 2006 and then worked for a couple of years before resuming her education. In the fall of 2008, she enrolled in the Department of Animal Science at Cornell University in pursuit of a Master of Science degree in Reproductive Physiology in the laboratory of Dr. Patricia Johnson. In the fall of 2010, following completion of the Master of Science degree, Mila will pursue a degree in Veterinary Medicine at Cornell University.

This thesis is dedicated to Mary Lou and Radha Kundu

in appreciation for being the best parents I could have asked for.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ILLUSTRATIONS

LIST OF TABLES

CHAPTER 1
LITERATURE REVIEW

1.1 ACTIONS OF THE KIT SYSTEM IN THE OVARY

Functionality of the Kit system, composed of Kit ligand (KL) and its cognate tyrosine kinase receptor, cKit, has been shown to be essential in a variety of tissues and processes (Hutt *et al.*, 2006). KL is a cytokine that has two primary isoforms (soluble and membrane-spanning), synthesized from two alternatively spliced forms of mRNA that differ in the presence of exon 6. This exon has been shown to encode a cleavage site involved in proteolysis of soluble KL (Huang *et al.*, 1992). KL initiates its biological activity by binding the cKit receptor in its extracellular domain (Lemmon *et al.*, 1997; Roskoski, 2005). There is also evidence of a soluble form of cKit that consists of part of the extracellular domain. This form, detected in human serum, (Wypych *et al.*, 1995) follicular fluid, (Tanikawa *et al.*, 1998) and endothelial cells, (Broudy *et al.*, 1994) binds KL with high affinity and could prevent its binding with the membrane-associated cKit. Functions of the Kit system found in the chicken include proliferation of normal haemopoeitic cells (Hayman *et al.*, 1993; Larsen *et al.,* 1993), proliferation and differentiation of neural crest-derived melanocytes (Lahav *et al.*, 1994), differentiation and functional activation of osteoclasts (Van't Hof *et al.*, 1997), and assisting in the survival of dorsal root ganglia neurons *in vitro* (Carnahan *et al.*, 1994). There has been limited examination of the KL/cKit system in the ovary of chickens.

Kit Ligand Expression

In the chicken, the soluble and longer isoform of KL is a 287 amino acid polypeptide that was cloned from a chicken brain cDNA library. Northern blot hybridization showed the presence of KL RNA in many tissues including the brain, bursa, heart, kidney, spleen, thymus, ovary, and testis. Chicken KL shows 53% identity with mouse and 52% identity with human KL (Zhou *et al.*, 1993; Petitte and Kulik, 1996). Although KL expression throughout follicle development in the chicken ovary is currently unreported, four isoforms of one chicken KL gene have been identified and cloned: KL-1, KL-2, KL-3 and KL-4. KL-1 (the soluble isoform) was reported to be predominantly expressed in the chicken ovary (Wang *et al.*, 2007).

The ovarian expression pattern of KL mRNA and protein has been well studied in mammals. In the mouse, KL protein and mRNA was detected in follicle granulosa cells at all stages of development, with low expression found in primordial follicles and then increasing with follicle growth to the highest levels in preantral follicles. KL expression then decreases to low levels in cumulus cells of antral follicles, while remaining high in mural granulosa cells (Manova *et al.*, 1993; Motro and Bernstein, 1993). KL protein was also detected in mouse oocytes, thought to be an outcome of receptor-mediated endocytosis (Manova *et al.*, 1993; Jahn *et al.*, 2002; Kang *et al.*, 2003). In goats, KL protein and mRNA were expressed in granulosa cells at all stages of development in addition to corpus luteum and epithelium (Silva *et al.*, 2006); the highest level of steady-state KL mRNA expression was found in secondary follicles (Celestino *et al.*, 2010). Similarly, granulosa cell KL mRNA expression has been shown in the rat (Ismail *et al.*,

1996), sheep, (Tisdall *et al.*, 1999) human (Hoyer *et al.,* 2005) and pig (Brankin *et al.*, 2004). Additionally, KL protein has been localized to the granulosa layer in human primordial follicles using *in situ* hybridization (Carlsson *et al.*, 2006).

Regulation of Kit Ligand

The gonadotropins, as well as paracrine and autocrine factors, have been found to play a part in the regulation of KL in mammalian reproduction. In one study, four hours after hCG stimulation of PMSG-treated mice, KL mRNA expression was increased in preovulatory granulosa cells. This increase in KL mRNA occurred in both mural and cumulus granulosa cells, with predominant expression found in mural granulosa cells. Additionally, an increase in KL protein was seen in mural but not cumulus cells at four hours following hCG stimulation (Ye *et al.*, 2009). Similarly, six hours after hCG stimulation in PMSG-primed rats, KL mRNA was elevated in whole ovaries and in granulosa cells (Ismail *et al.*, 1996). The same effect of increased KL mRNA occured after culturing granulosa cells with hCG in the cow (Parrott and Skinner, 1998). FSH also significantly increased KL mRNA expression in bovine granulosa cell cultures, to the same level found after hCG treatment (Parrott and Skinner, 1998). The stimulatory effect of FSH on KL (both isoforms) mRNA expression in preantral granulosa cell culture was shown to be dose responsive in mice (Joyce *et al.*, 1999). This effect was diminished with addition of partly grown oocytes and drastically reduced with fully grown oocytes (Joyce *et al.*, 1999). This study also demonstrated an increase in granulosa cell KL mRNA expression after testosterone treatment, an effect amplified by the addition of FSH (Joyce *et al.*, 1999). Other research using mice found no effect on soluble KL mRNA

with FSH treatments and increased membrane-bound KL mRNA with low (but not high) FSH treatment (Thomas *et al.*, 2005). In contrast, in human granulosa-luteal cell cultures, FSH treatment was shown to significantly decrease KL mRNA expression (Laitinen *et al.*, 1995).

With respect to paracrine regulation of KL, GDF9 and BMP15 are two oocyte-derived factors shown to affect KL in alternate ways. In murine cumulus granulosa cell cultures, fully grown oocytes can inhibit KL mRNA expression. It was suggested that this was due to GDF9, which has been shown to negatively regulate KL when added to preantral granulosa cell cultures (Joyce *et al.*, 2000; Elvin *et al.*, 1999). Although the effects on KL granulosa cell mRNA that can be attributed solely to BMP15 are unknown, granulosa cells co-cultured with oocytes and BMP15 showed significantly increased KL mRNA compared to granulosa cells cultured alone (rat: Otsuka and Shimasaki, 2002; mouse: Thomas *et al.*, 2005). In other studies, addition of partly grown oocytes to mouse preantral granulosa cell culture promotes KL mRNA expression, (Joyce *et al.*, 1999) hypothesized to be due to the presence of BMP15 in the oocytes (Joyce *et al.*, 2000). Treatment with another oocyte-derived factor, bFGF, resulted in elevated KL mRNA expression in cultured whole ovaries in the rat (Nilsson and Skinner, 2004).

Paracrine regulation of KL by factors secreted from the theca cell layer has also been examined. Bovine studies have indicated that treatment of cultured granulosa cells with keratinocyte growth factor (KGF) or hepatocyte growth factor (HGF), two proteins secreted by theca cells, will upregulate KL granulosa cell mRNA expression (Parrott and Skinner, 1998). Autocrine and/or paracrine regulation of KL by Leukemia inhibitory factor (LIF) was studied in the rat. LIF protein is predominantly expressed in the

granulosa cells in primordial and primary follicles, a pattern that changes with growth to the pre-antral and antral follicle stages, when LIF is primarily expressed in the oocyte (Nilsson *et al.*, 2002). Treatments of granulosa cell cultures with LIF resulted in increased KL mRNA expression (Nilsson *et al.*, 2002). A recent study examined the function of fibroblast growth factor 7 (FGF7), found in theca and granulosa cells. When bovine cumulus-oocyte complexes (COCs) were cultured with granulosa cells treated with FGF7 an increase in KL mRNA expression was found (Cho *et al.*, 2008). Although granulosa cell-derived AMH had no direct effect on KL gene expression, the stimulatory effects of KL on rat primordial follicle development were inhibited by AMH added to whole ovary culture. This suggests that AMH could act in an autocrine manner to regulate KL activity (Nilsson *et al.*, 2007).

A single study that investigated regulation of KL in the chicken ovary revealed that heparin binding EGF-like growth factor (HB-EGF) treatment resulted in a dose-dependent decrease of granulosa cell KL mRNA. This inhibitory effect was seen in undifferentiated granulosa cells from 3 mm and 6 mm follicles, and differentiated cells from F4+5 and F1; the maximal effect was found with 20 ng/mL HB-EGF treatments in all cases. Granulosa cell expression of chicken KL-1 and KL-3 were examined in this study; both isoforms showed the same pattern of regulation by HB-EGF, although KL-1 mRNA expression was much greater (Wang *et al.*, 2007).

Regulatory Functions of KL

With regard to reproduction, studies in mammals have shown that the Kit system is involved in signaling between the oocyte and somatic cells during the process of follicle maturation. KL is one of a few factors with an

established role in the continuous and gradual recruitment of follicles from the resting pool (mouse: Yoshida *et al.*, 1997; hamster: Wang and Roy, 2004). It is hypothesized to be sufficient in inducing primordial follicle activation in the cow (Parrott and Skinner, 1997; Muruvi and Fortune, 2009) and goat (Celestino *et al.*, 2010), but recent work in the mouse suggests it is dispensable (John *et al.*, 2009). KL also has the ability to provoke first polar body extrusion in murine preovulatory denuded oocytes, suggesting direct effects on its receptor (Ye *et al.*, 2009).

As a growth factor, KL induces proliferation of mouse preantral granulosa cells (Reynaud *et al.*, 2000), an effect postulated to be mediated by GDF9 or BMP15 (Gilchrist *et al.*, 2004; Otsuka *et al.*, 2000). While BMP15 (plus oocytes) treatment can increase KL as discussed previously, KL subsequently decreases oocyte mRNA expression of BMP15, creating a paracrine negative feedback loop between the oocyte and granulosa cell layer (Otsuka and Shimasaki, 2002; Thomas *et al.*, 2005). Effects of KL on basic FGF (bFGF), another oocyte factor, have also been examined. Results from one study showed that KL was able to stimulate bFGF oocyte expression (Jin *et al.*, 2005), while another study found no difference in whole ovary bFGF expression, but did show that the ability of KL to increase the primordial to primary follicle transition was blocked with antibody to bFGF (Nilsson and Skinner, 2004).

KL also displays a variety of paracrine effects on the theca cell layer. Treatment of intact mouse preantral follicle cultures with KL increased thecal testosterone output (Reynaud *et al.*, 2000). Studies of cow ovaries showed that KL treatment resulted in theca cell proliferation (Parrott and Skinner, 1997), in addition to an increase of KGF and HGF mRNA

expression (Parrott and Skinner, 1998). As previously stated, these factors in turn upregulate KL expression, forming a positive feedback loop (Parrott and Skinner, 1998). Interestingly, KL is also thought to have a regulatory effect on theca cell differentiation. In rats, theca cell precursor differentiation in whole ovary cultures was induced by treatment with follicle-conditioned medium (created from preantral follicles incubated for two days), an effect blocked by the addition of KL antibody (Huang *et al.*, 2001). Further study of KL-treated rat ovaries showed an increase (mRNA and protein) of steroidogenesis-related factors SF-1, StAR, and P450 aromatase, in parallel with an inhibition of FSHR expression (Jin *et al.*, 2005).

cKit Expression

The 960 amino acid sequence of chicken cKit showed 63% identity to mouse cKit. Furthermore, the 5.5-kb chicken mRNA transcript was detected in many organs including the brain, bursa, heart, kidney, spleen, thymus, ovary, and testis (Sasaki *et al.*, 1993). Whereas the ovarian expression pattern of cKit in the chicken has not been established, cKit protein and mRNA expression was shown in oocytes of mice (Motro and Bernstein, 1993; Horie *et al.*, 1991; Ye *et al.*, 2009), sheep (Clark *et al.*, 1996; Tisdall *et al.*, 1999), goats (Silva *et al.*, 2006), pigs (Brankin *et al.*, 2004), and humans (Horie *et al.,* 1991, Horie *et al.,* 1993; Carlsson *et al.,* 2006). Expression was also found in theca of mice (Motro and Bernstein, 1993; Kang *et al.,* 2003), pigs (Brankin *et al.*, 2004), and cattle (Parrott and Skinner, 1997). In humans, expression of cKit is also seen in granulosa cells from primordial (protein: Hoyer *et al.*, 2005), secondary (protein: Carlsson *et al.*, 2006), and preovulatory follicles (mRNA: Tanikawa *et al.*, 1998). As previously stated, a soluble cKit has also been found in human follicular fluid (Tanikawa *et al.*, 1998). Although cKit is predominantly

expressed in murine oocytes, a low level of mRNA expression was discovered in cumulus and mural granulosa cells (Ye *et al.*, 2009).

cKit Function and Regulation

The cKit receptor must bind to its ligand to become biologically activated; therefore functionality of cKit (membrane-bound) is dependent on interactions with KL. With respect to reproduction, binding of granulosa cell KL to its receptor on the oocyte may activate different signaling pathways. Among these, cellular response occurs through the phosphatidylinositol 3 kinase (PI3K) pathway, the pathway thought to control primordial follicle activation (John *et al.*, 2009). This is supported with the discovery that KL treatments of mouse and rat oocyte cultures results in phosphorylation of PI3K pathway components protein kinase B (PKB) and its substrate, forkhead homolog rhabdomyosarcoma-like 1 (FKHRL1). This phosphorylation activates PKB, involved in cell proliferation, and suppresses FKHRL1, involved in apoptosis. Expression of these PI3K components following KL treatment supports the suggestion that the Kit system regulates follicle development and affects secretion of oocyte factors that regulate the primordial to primary follicle transition (Reddy *et al.*, 2005). Recent *in vivo* work using mice with a mutation preventing cKit signaling via PI3K provided evidence that cKit is not a required factor, since primordial follicle activation still occurred in these mice. Nevertheless, abnormalities in these mice, such as primordial follicle depletion, support the importance of cKit in promoting survival of primordial follicles (John *et al.*, 2009).

Many studies have attempted to disrupt functionality of the Kit system by using an antibody to block the binding of cKit to its ligand.

Addition of an antibody to cKit to mouse preantral follicle cultures showed that antrum formation was severely affected and oocyte death occurred after six days, an effect found to be independent of somatic cell survival (Reynaud *et al.*, 2000). Similarly, blocking cKit with an antibody significantly increases follicular atresia in human ovarian cultures (Carlsson *et al.*, 2006), while in cattle, it was able to completely block KL-stimulated follicle activation (Muruvi and Fortune, 2009). A different approach to inhibit cKit activity was used to investigate the role of cKit signaling in FSH-stimulated oocyte growth in the mouse. An inhibitor of cKit tyrosine kinase activity (Gleevec, specific to just three of the known tyrosine kinases), was used in granulosa cell cultures in the presence of FSH plus oocytes. This attenuated FSH-induced inhibition of BMP15, suggesting a cKit-mediated action of FSH. However, the study also showed that FSH treatments had no effect on cKit mRNA (Thomas *et al.*, 2005).

A few factors that have a role in the regulation of cKit have been identified. In one study, mRNA for cKit was increased from cultured bovine COCs after treatment with FGF7. Additional experiments showed that the stimulatory effect of FGF7 on oocyte growth was inhibited by the addition of an antibody to cKit (Cho *et al.*, 2008). Microarray analysis of rat whole ovaries cultured in the presence of AMH indicated suppression of cKit mRNA by AMH (Nilsson *et al.*, 2007). Since AMH is known to inhibit the primordial-to-primary follicle transition (Durlinger *et al.*, 1999; Durlinger *et al.*, 2002), this relationship may be important in the regulation of folliculogenesis.

It has been well documented that the Kit system plays an important role in mammalian reproduction. The numerous interactions that KL and

cKit have with other factors can be autocrine, paracrine or endocrine in nature, and in many cases, it is still not well understood whether effects seen are direct or indirect. It is important to study the Kit system in the reproduction of other non-mammalian vertebrates, such as the chicken. The unique arrangement of the ovarian follicle hierarchy in the domestic hen makes it a fine model to study factors involved in folliculogenesis.

Table 1.1 Factors Regulating KL Expression in Granulosa Cells

Factor	Endogenous Source	Effect	Species
FSH	Anterior pituitary	↑	Mouse (Joyce *et al.*, 1999), Cow (Parrott and Skinner, 1998)
LH/hCG	Anterior pituitary	↓	Cow (Parrott and Skinner, 1998), Mouse (Ye *et al.*, 2009), Rat (Ismail *et al.*, 1996)
HB-EGF	Oocyte	↓	Chicken (Wang *et al.*, 2007)
BMP15	Oocyte	↓	Rat (Otsuka and Shimasaki, 2002), Mouse (Joyce *et al.*, 2000; Thomas *et al.*, 2005)
GDF9	Oocyte	↓	Mouse (Joyce *et al.*, 2000; Elvin *et al.*, 1999)
bFGF	Oocyte	↑	Rat (Nilsson and Skinner, 2004)
LIF	Granulosa or oocyte	↑	Rat (Nilsson *et al.*, 2002)
AMH	Granulosa	↓	Rat (Nilsson *et al.,* 2005)
FGF7	Theca & granulosa	↑	Cow (Cho *et al.*, 2008)
Testosterone	Theca & granulosa	↑	Mouse (Joyce *et al.*, 1999)
KGF	Theca	↑	Cow (Parrott and Skinner, 1998)
HGF	Theca	↑	Cow (Parrott and Skinner, 1998)

Table 1.2 Functions of KL in the Ovary

Factor Regulated	Endogenous Source	Effect	Species
BMP15	Oocyte	↓	Rat (Otsuka and Shimasaki, 2002; Thomas *et al.*, 2005)
bFGF	Oocyte	↑	Rat (Jin *et al.*, 2005)
Aromatase	Ooctye	↑	Rat (Jin *et al.*, 2005)
SF-1	All ovarian cells	↑	Rat (Jin *et al.*, 2005)
StAR	All ovarian cells	↑	Rat (Jin *et al.*, 2005)
FSHR	Granulosa	↓	Rat (Jin *et al.*, 2005)
Testosterone	Theca	↑	Mouse (Reynaud *et al.*, 2000)
KGF	Theca	↑	Cow (Parrott and Skinner, 1998)
HGF	Theca	↑	Cow(Parrott and Skinner, 1998)
Proliferation	Theca, granulosa	↑	Mouse (Reynaud *et al.*, 2000), Cow (Parrott and Skinner, 1997)

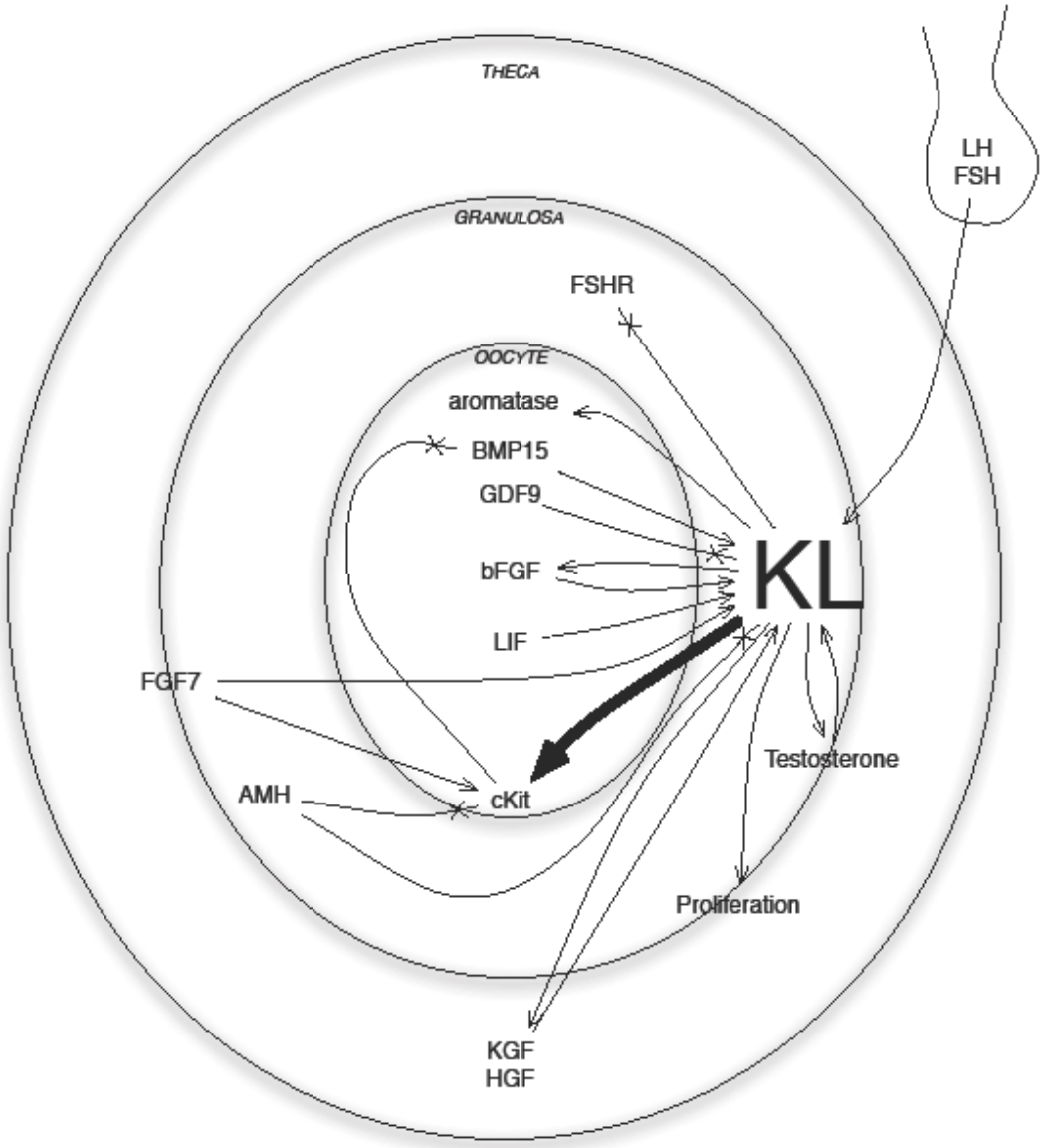

Figure 1.1 Interactions of KL with other intraovarian factors in the mammalian ovary

1.2 ACTIONS OF ANTI-MÜLLERIAN HORMONE IN THE OVARY

Anti-Müllerian hormone (AMH, also known as Müllerian inhibiting substance, MIS), named for its role in regressing the Müllerian duct system in male mammals, has the same function in male birds, in addition to regressing the right Müllerian duct in female birds (Tran and Josso, 1977). Estrogen produced by the left ovary of birds protects the left Müllerian duct from regression, discovered by *in vivo* treatments with DES that prevented apoptotic cell death of the right Müllerian duct (Teng, 2000; Teng, 2001).

Actions of AMH in mammalian reproduction

Although no receptors for AMH have been identified thus far in the chicken, two receptors have been identified in rodents (di Clemente *et al.*, 1994). As a member of the TGFβ superfamily, AMH signals through type I, type II receptors, utilizing the Smad second messenger system. In this system, once a ligand binds its type II receptor, the type I receptor is additionally recruited to bind to this ligand-receptor complex. Activation of these two transmembrane serine-threonine kinase receptors results in subsequent downstream signaling via Smad proteins (Massague and Chen, 2000). AMH signal transduction was shown to be mediated through Smad1 and Smad5, type I BMP downstream signaling molecules, but not mediated by Smad2, a TGFβ-like signaling Smad (Visser *et al.*, 2001). Expression of the AMH type II receptor is found in embryonic Müllerian duct mesenchymal cells and gonads as well as postnatal rodent granulosa cells (Baarends *et al.*, 1994; Baarends *et al.*, 1995) and rat preantral and antral theca cells (Ingraham *et al.*, 2000). The type I receptor (ALK2) is also expressed in fetal and adult mouse ovaries (Visser *et al.*, 2001).

Expression of AMH mRNA or protein is not seen in mouse primordial follicles, oocytes or theca, and can be first seen in granulosa cells of primary follicles (Ueno *et al.*, 1989; Baarends *et al.*, 1995; Durlinger *et al.*, 2002). AMH is highest in preantral and small antral follicles, with greater expression in granulosa cells surrounding the oocyte and antrum, as compared to the mural granulosa cells, possibly reflecting functional differences between the cells (Ueno *et al.*, 1989; Baarends *et al.*, 1995; Durlinger *et al.*, 2002).

AMH knock-out studies indicate the importance of AMH in normal ovarian function. Female knock-out mice appear normal and are fertile, which may indicate some functional redundancies with other factors such as inhibin and activin (Behringer *et al.*, 1994). Further study of ovaries from these null mice demonstrated that AMH is an inhibitor of follicular activation. The number of small, growing follicles was found to be greatly increased, leading to a depletion of primordial follicles in the ovaries of older mice (Durlinger *et al.*, 1999). This function was confirmed by *in vitro* studies showing that murine ovaries cultured with AMH had 40% fewer growing follicles and lower α-inhibin mRNA expression (Durlinger *et al.*, 2002).

AMH has the ability to decrease FSH sensitivity, with a proposed inverse pattern of follicular expression between FSHR and AMH, a relationship which may regulate orderly follicle selection (Visser and Themmen, 2005). By culturing follicles, it was discovered that preantral follicle growth stimulated by FSH treatment was reduced with addition of AMH (Durlinger *et al.*, 2001). Furthermore, AMH-deficient mice given injections of FSH had higher numbers of growing follicles when compared to wild-type mice, demonstrating the inhibitory regulation by AMH on

follicle recruitment (Durlinger *et al.*, 2001). Other examples of AMH attenuation of FSH actions were found in cultured rat granulosa cells treated with FSH ±AMH or cAMP ±AMH. FSH and cAMP both stimulated aromatase activity that was blocked with the addition of AMH. AMH also decreased FSH-stimulated granulosa cell LHR. These results are interesting because LHR and aromatase are two markers of granulosa cell differentiation (di Clemente *et al.*, 1994). Furthermore, microarray analysis of AMH-treated rat ovaries showed that AMH downregulated the TGFβ pathway which signals for processes including cell differentiation, angiogenesis and cell cycle regulation (Nilsson *et al.*, 2007). This study also showed that the stimulatory actions of KL, bFGF and KGF on the transition from primordial to primary follicles in ovary cultures were attenuated by addition of AMH to culture treatments (Nilsson *et al.*, 2007).

Further evidence that AMH suppresses follicle activation was demonstrated in the cow. In this study, pieces of bovine ovarian cortex filled with primordial follicles were grafted beneath the chorioallantoic membranes (CAM) of chick embryos, an approach used because ovaries of large mammals cannot be cultured intact. This experiment showed an inhibition of follicle activation, presumed to be due to the high levels of AMH produced by chick embryonic gonads (Cushman *et al.*, 2002). An additional study repeated this experiment, instead using CAM of gonadectomized chick embryos, and found that follicle activation ensued (Gigli *et al.*, 05). This experiment was also done using mouse ovaries, and showed that follicle activation in wild-type whole ovaries was inhibited, and yet no inhibition occurred when cultured *in vitro* or when using CAM-grafted ovaries of AMH type II receptor knock-out mice (Gigli *et al.*, 05).

Studies in human reproduction have linked AMH to various forms of ovarian dysfunction. Correlations are seen between low serum AMH and premature ovarian failure (POF) (Meduri *et al.*, 2007) in addition to high levels of serum AMH seen in women with polycystic ovary syndrome (PCOS) (Cook *et al.*, 2002). AMH is currently believed to be the best known marker of ovarian reserve; AMH decreases with age, reflecting declines in the oocyte and follicle pool (Van Rooij *et al.*, 2004). AMH is also useful in predicting outcomes of ovarian response to assisted reproductive technology, specifically gonadotropin stimulation (Seifer *et al.*, 2002).

AMH in the domestic hen ovary

The sequence of chicken AMH is 52% similar to mammalian AMH in the biologically active C-terminal region. In the female embryo, expression of AMH in the developing left ovary was found to gradually increase to its highest level at day 14 then decline to close to baseline by the day before hatching (Teng *et al.*, 1987). In the adult hen, AMH mRNA expression in granulosa cells is highest in small, growing follicles and decreases with follicle development (Johnson *et al.*, 2008).

Interestingly, when comparing two breeds of hens with different ovulation rates, high levels of AMH expression were correlated with impaired follicle selection (Johnson *et al.*, 2009). AMH was found to be higher in hens on a diet of free feeding than those on restricted feeding. When allowed to eat *ad libitum* these hens become very fat, display excessive follicle development, and frequently experience erratic ovulations. Further experiments determined that AMH was not regulated by glucose or insulin, factors related to metabolic state (Johnson *et al.*, 2009). The finding that

follicle development is not optimal when AMH is high is similar to the relationship found in women with PCOS (Cook *et al.*, 2002).

Culture of 6-8 mm granulosa cells with various concentrations of estradiol and progesterone showed that neither steroid was able to influence AMH mRNA expression in the hen. In contrast, cultures treated with oocyte-conditioned medium (OCM) revealed decreased AMH mRNA expression. This suppression by one or more oocyte-derived factors was eliminated when the OCM was heat-treated, but was not blocked by the addition of an antibody to GDF9 (Johnson *et al.*, 2008). With regard to AMH actions in the hen ovary, not much is known. This is, in large part, due to the fact that mammalian AMH preparations are biologically inactive in the hen. One experiment treated granulosa cell cultures with bioactive AMH, shown to be present in testis-conditioned medium (TCM) by western blot analysis. Through a paracrine or autocrine mechanism, AMH induced cell proliferation that was blocked when the TCM was preincubated with an antibody to AMH (Johnson *et al.*, 2009). As knowledge of AMH regulation and function in the hen ovary is limited to these few studies, there is a need for further investigation of its actions and regulation in an oviparous species.

1.3 FUNCTION AND REGULATION OF OOCYTE FACTORS IN THE HEN

Folliculogenesis is a dynamic and precise developmental process in all vertebrates. While much is known about the numerous factors that play a role in follicle maturation, there are still vast amounts of research to be done on the interactions of growth factors that come from the follicle, the functional unit of the ovary. Much of the focus has been on the granulosa

and theca cell layers, with few studies on paracrine or autocrine factors expressed by the oocyte. Expanding this knowledge should be a vital goal in gaining a better understanding of ovarian physiology. In particular, the unique hierarchal arrangement of follicles in the hen ovary makes it an ideal model to study these interactions relative to the process of folliculogenesis.

Germinal Disc

Communication between the oocyte and its surrounding cells is important for all systems of reproduction. The avian germinal disc (GD) is a tiny white structure on the surface of the oocyte that contains the nucleus and 99% of the cell's organelles, including the DNA and mitochondria. The germinal disc and its overlying granulosa cells comprise the germinal disc region (GD), said to be the growth center of the follicle (Tischkau *et al.*, 1997). The GD is the structural and functional equivalent of the mammalian oocyte, but in reality the chicken oocyte includes the yolk. In a preovulatory follicle, granulosa cells are defined by their proximity to the GD: GD granulosa cells are found covering the germinal disc and nonGD granulosa cells are on the distal side of the follicle. These subsets have different properties; GD granulosa cells are very proliferative and incorporate more [^{3}H] thymidine, a measure of mitosis. NonGD granulosa cells are more differentiated and produce greater amounts of progesterone (Tischkau *et al.*, 1997; Yao and Bahr, 2001b).

EGF

It was shown that destruction of the preovulatory GD resulted in apoptosis and the absence of ovulation, which means the GD produces one or more growth factors required to sustain follicle development (Yoshimura *et al.*, 1994). One paracrine regulator of preovulatory granulosa cells is

epidermal growth factor (EGF), found in GD-region-conditioned media (Yao and Bahr, 2001b). Culture treatments with this media (plus normal rabbit serum) increased granulosa cell proliferation compared to control treatments (lacking serum), an effect that was blocked by addition of an EGF antibody (Yao and Bahr, 2001b). This finding is supported by other work in the chicken showing that EGF stimulates proliferation, inhibits apoptosis, and decreases progesterone production by granulosa cells (Volentine *et al.*, 1998) and that EGF decreases LHR in preovulatory GD granulosa cells (Yao and Bahr, 2001a).

GDF9

Two related TGFβ superfamily members derived from the oocyte are bone morphogenic protein 15 (BMP15) and growth differentiation factor 9 (GDF9). Biological forms of these proteins share interesting similarities such as their lack of the cystine involved in dimer formation in most other members of the superfamily. However, because both are produced in the oocyte, they may form biologically active homodimers or heterodimers (Juengel *et al.*, 2004). While both factors are known to signal through the type II receptor BMPRII, they signal through different type I receptors, ALK6 for BMP15; ALK5 for GDF9 (Moore *et al.*, 2003; Mazerbourg *et al.*, 2004).

In the hen ovary, GDF9 exhibits highest expression in the oocyte, but was also shown to be in granulosa cell mRNA at low levels in follicles up to 8 mm, with no difference between GD and nonGD granulosa cells. Furthermore, immunocytochemistry showed staining predominantly in the oocyte, highest in the periphery, with limited positive staining in the granulosa cells (Johnson *et al.*, 2005b). GDF9 is more likely to be involved

in follicle differentiation than in early embryo development, as mRNA expression during early embryogenesis was shown to decrease to very low levels following ovulation (Elis *et al.*, 2007).

Chicken GDF9 was shown to be present in oocyte-conditioned media (OCM) via western blot analysis (Johnson *et al.*, 2005b). Treatment of granulosa cells from 3-8 mm follicles with OCM resulted in increased granulosa cell proliferation. This effect is attributed to GDF9, because pre-treatment of OCM with GDF9 antibody returned proliferation levels to baseline (Johnson *et al.*, 2005b). Although we have limited information about the function of GDF9 in the hen, mammalian studies have outlined its importance in folliculogenesis. The GDF9 knock-out mouse model exhibits disrupted follicle development with no progression past the primary stage (Dong *et al.*, 1996). In mammals, GDF9 is also found to regulate steroid production by the theca and granulosa cell layers (Elvin *et al.*, 2000; Solovyeva *et al.*, 2000).

BMP15

In chickens, BMP15 mRNA was shown via *in situ* hybridization to be highly expressed in oocytes from follicles ranging from 50 μm to 6 mm in size. This finding was supported by high expression of BMP15 in the GD of preovulatory follicles via real-time RT-PCR; additionally, low levels of BMP15 mRNA were observed in granulosa cells of these follicles (Elis *et al.*, 2007). Treatment of chicken granulosa cells with rhBMP15 (72% identity with chicken) affected steroid production and signaling in a number of ways. BMP15 was found to inhibit gonadotropin-stimulated granulosa cell progesterone production, an effect correlated with BMP15 suppression of StAR protein. However it had no effect on the steroidogenesis enzymes 3β-

HSD and $P450_{SCC}$. BMP15 was also able to increase Smad1 phosphorylation, activating a signaling pathway in granulosa cells (Elis *et al.*, 2007). Various studies in mammals have shown the importance of BMP15 in reproduction may be species specific. For example, BMP15 knock-out mice are fertile (Yan *et al.*, 2001), yet loss of function in humans results in ovarian failure (Di Pasquale *et al.*, 2004). The loss of BMP15 function in sheep yields non-linear effects: partial loss increases ovulation rates, while complete loss renders sheep infertile (Juengel *et al.*, 2002; Hanrahan *et al.*, 2004).

Study of BMP15 actions in the rat ovary suggested functions similar and different to those described in the hen ovary. Rat granulosa cells, the target cells of BMP15 signaling, were treated with BMP15, which increased cell proliferation (Otsuka *et al.*, 2000), an effect not seen in the hen (Elis *et al.*, 2007). Furthermore, BMP15 was shown to decrease FSH-stimulated progesterone production in both the rat (Otsuka *et al.*, 2000) and the hen (Elis *et al.*, 2007), but did not affect FSH-stimulated estradiol production by rat granulosa cells (Otsuka *et al.*, 2000).

HB-EGF

Another oocyte-derived factor that has been studied in the chicken is HB-EGF, which has low expression in the embryo and high expression in immature and mature ovaries (highest in 10 week old chickens). In a 3 mm follicle, HB-EGF was found to be highly expressed in the oocyte and weakly expressed in granulosa, while its receptor, EGFR, has strong granulosa cell expression. Treatment with various doses of HB-EGF greatly increased granulosa cell proliferation and downregulated KL expression in follicles of all sizes, as discussed previously (Wang *et al.*, 2007).

1.4 REPRODUCTIVE BIOLOGY OF THE DOMESTIC HEN

Reproductive cycle

A major anatomical difference between mammals and birds is the solitary existence of the left ovary and oviduct in birds, compared to two of each found in mammals. As embryos, birds begin with both ovaries and oviducts, but by day four there is a disproportionate balance of primordial germ cells that advances development of the left ovary alone. As previously discussed, regression of the right oviduct begins by day ten (Tran and Josso, 1977).

In the hen's 24-28 hour ovulation-oviposition cycle, a single follicle is selected for ovulation. Ovulation is the release of an ovum from a ruptured follicle, which then travels through the reproductive tract, gaining layers of membrane, albumen and shell. The process of generating a fully formed egg takes 25-26 hours and culminates in oviposition, the laying of an egg (Fraps and Hammond, 1955). The typical laying pattern consists of several days of consecutive ovulations known as a sequence, followed by a pause day (Etches, 1996).

Hen ovaries exhibit a defined follicular hierarchy (**Illustration 1.1**) that permits these successive ovulations, where the follicles range in size representative of the order in which they are selected to ovulate. There are between five and eight large yellow, yolk-filled follicles (LYF), with F1 being the largest and therefore next to ovulate, followed by F2, and so on (Gilbert *et al.*, 1983). Hens have a resting primordial pool from which this preovulatory hierarchy originates. Initially, primary oocytes are enclosed by a vitelline membrane. With the addition of granulosa cells that form the

Illustration 1.1 Photograph of the hen ovary

Photograph depicts the unique anatomy of the hen ovary, emphasizing the highly regulated hierarchy of preovulatory follicles.

perivitelline membrane (similar to the mammalian zona pellucida), a small white follicle is formed (SWF, <2 mm diameter) (Gilbert *et al.*, 1983). As a SWF develops, a theca layer, produced from mesenchymal cells, is formed. The basal lamina separates the theca and granulosa layers (Johnson *et al.*, 2007). Theca differentiation into interna and externa, along with accumulation of white yolk (less lipid content than yellow) classifies the large white follicles (LWF, 2-4 mm diameter). As yellow yolk is taken up by the growing oocyte, the small yellow follicles develop, (SYF, 6-12 mm diameter) a stage approximately 2-3 weeks from ovulation. At this point, the theca layer starts to become vascularized and innervated (Johnson *et al.*, 2007). These SYF make up the pool of prehierarchal follicles, characterized by slow growth, from which they are recruited to enter the preovulatory hierarchy (Etches, 1996). Once a follicle is selected into the hierarchy it is destined to ovulate (Gilbert *et al.*, 1983). Following this selection, follicles grow very quickly up to 40 mm (Johnson *et al.*, 2007). This vast increase in size is due to receptor-mediated uptake of increasing amounts of yellow yolk, a protein mass made by the liver in response to estrogen that circulates in the blood (Barber *et al.*, 1991). Avian follicles lack the follicular fluid and antrum seen in the mammalian follicle and contain the highly specialized germinal disk region (Etches, 1996).

Steroid Production

Accompanying the increase in size with follicle maturation, various other changes occur in the cell layers surrounding the oocyte. Steroid production in preovulatory follicles occurs via the Δ^4 pathway, while the Δ^5 pathway is highly utilized by prehierarchal follicles and stromal tissue (Johnson, 1996). Estradiol, synthesized by theca externa cells with

aromatase activity (Nitta *et al.*, 1991), is the main steroid produced by the small white follicles (Robinson and Etches, 1986). The granulosa cells from these same follicles are not steroidogenic due to their lack of $P450_{SCC}$ enzyme activity (Tilly *et al.*, 1991).

Immediately after selection (9-12 mm follicles), mRNA for the enzyme $P450_{SCC}$ increases, and the granulosa cells become steroidogenically competent, primarily producing progesterone (Johnson, 1996). As progesterone production increases to maximal levels during preovulatory follicle growth from F5 (120 hours before ovulation) to F1, estradiol simultaneously decreases. Testosterone, synthesized by granulosa and theca, increases from F5-F2, then decreases in F1 (Bahr *et al.*, 1983). Similarly, androstenedione, a precursor of androgens and estrogens, is lowest in F1 (Etches and Duke, 1984).

Steroid production is an important factor in the timing of ovulation. Levels of estradiol, believed to prepare the hypothalamus for positive feedback of progesterone, peak 4-6 hours before ovulation. During these hours before ovulation, the F1 follicle is the major source of progesterone. Rising plasma concentrations of progesterone stimulate GnRH secretion which acts on the pituitary to trigger secretion of LH. Acting through its receptor, this LH further stimulates progesterone production primarily by granulosa cells of F1. This positive feedback loop generates the preovulatory surge (Johnson and van Tienhoven, 1980; Johnson, 1996; Etches and Cunningham, 1976). In contrast to the interactions of LH and progesterone seen in the hen, mammalian dominant follicles produce high levels of estradiol, thought to inhibit FSH secretion in order to suppress growth of subordinate follicles (Fortune, 2004). This regulation of follicle

development also occurs in the avian hierarchy, acting primarily through progesterone and inhibin (Johnson, 1996), which will be discussed later in greater detail.

Gonadotropin Involvement

Follicular development is mediated by the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). The primary functions of FSH are in follicle development and the LH surge induces ovulation. In order to achieve these objectives, LH and FSH act on the ovary following their release from the anterior pituitary. An important aspect of their activity is seen in their regulation of steroid synthesis, through actions on $P450_{SCC}$ or StAR, both necessary for steroid production. In one study, western blot analysis showed increased granulosa StAR protein following a four hour incubation with FSH in all follicle sizes (Woods and Johnson, 2005). Another study indicated that LH was also able to increase StAR protein expression in preovulatory follicle granulosa cells, through signaling via the PKC pathway. Interestingly, activation of this pathway in undifferentiated granulosa cells has been shown to suppress FSH-induced StAR protein levels (Woods *et al.*, 2007).

Other work has examined gonadotropin effects on $P450_{SCC}$ enzyme activity. In follicles following selection (9-12 mm), four hour incubations with FSH and VIP were found to initiate $P450_{SCC}$ enzyme activity, resulting in high levels of progesterone synthesis. In contrast, this effect was not found in the F1 follicle after treating with FSH or VIP (Johnson, 1993). Although LHR are detectable in 9-12 mm follicles, they were found to be insufficient for LH-induced progesterone production. Again, granulosa

from the F1 follicle exhibited a different response, showing elevated progesterone synthesis following incubation with LH (Johnson, 1993).

The increase in progesterone production in preovulatory follicles goes along with the finding that granulosa LHR mRNA drastically increases at the F3 follicle stage. Theca cells also express LHR in these follicles, decreasing at F1, but LHR mRNA levels change very little in comparison to the change seen in granulosa cells (Johnson, 1996). As this LH responsiveness increases with follicle maturation, there is a parallel decrease in FSH responsiveness, in the form of decreased FSHR (Johnson, 1993). Expression of FSHR mRNA, lowest in the F1, exhibits high expression in 1-2 and 3-5 mm follicle stages. FSHR reaches its highest levels in 6-8 and 9-12 mm follicles, coinciding with follicle selection. One follicle from the 6-8 mm prehierarchal pool was shown to have much higher FSHR mRNA expression than the rest and therefore presumed to be the next follicle selected (Woods and Johnson, 2005).

Other important factors

As follicles mature they accumulate yolk, taken up by the oocyte as mentioned above (Barber *et al.*, 1991). In smaller follicles, tight junctions in granulosa cells prevent yolk precursors (VLDL and vitellogenin) from reaching receptors on the oocyte. Protein expression of occludin, necessary for these tight junctions, decreases greatly with follicular maturation from SWF to SYF, and is undetectable in isolated F1 granulosa. Granulosa occludin expression was shown to increase by culturing with activin A ±FSH, and decrease with TGFα ±activin A (Schuster *et al.*, 2004).

The inhibin/activin family of hormones is also known to have an important role in the regulation of folliculogenesis. Corresponding with

follicle maturation, changes in what has been described as "activin tone" occur concomitantly with inverse changes in "inhibin tone." Therefore their actions differ with different stages of development (Lovell *et al.*, 2003). Protein and mRNA expression of the three inhibin subunits (α, β_A, β_B) that homo- and hetero-dimerize to form the members of the inhibin/activin family vary with follicle size in the chicken. The majority of circulating inhibin A ($\alpha\beta_A$ subunits) is produced by granulosa cells of hierarchal follicles F1-F4 (Wang and Johnson, 1993; Chen and Johnson, 1996), while 6-10 mm small yellow follicles are the source of inhibin B ($\alpha\beta_B$), maximal in 6-8 mm, suggesting its role in follicle selection (Hecht, 2000). Large white follicles show low levels of inhibin protein expression for all subunits (Johnson, 2005a). Follistatin is a soluble binding protein that can bind inhibins and activins through the β subunit, capable of binding activin with higher affinity due to its two β subunits. Follistatin mRNA and protein expression is found in theca and granulosa and is more highly expressed in prehierarchal than in hierarchal follicles (Lovell *et al.*, 2003; Davis and Johnson, 1998).

Inhibin and activin were initially discovered, and named, for their opposing abilities in endocrine regulation of FSH. Inhibin reduces pituitary secretion of FSH while activin stimulates secretion. In addition to this important role, they provide paracrine regulation in follicle selection and development (reviewed in Mather *et al.*, 1997; Findlay, 1993). A recent study using a neonatal (cancer-free) inhibin knock-out mouse (Myers *et al.*, 2009) revealed that the loss of inhibin disrupted early follicle development, possibly due to increased activin signaling. Ovaries from these mice exhibited unusually small oocytes along with an obvious increase in ovary size. These effects could also be due to the altered gene expression that was

discovered: increased cyclin D2 and GDF9; decreased KL, AMH and BMP15 (Myers *et al.*, 2009).

Regulation of inhibin and activin in the hen has also been studied extensively. These results include increased expression of α and β_A mRNA due to FSH and LH (Davis *et al.*, 1999; Davis *et al.*, 2001; Safi *et al.*, 2003), IGF1 (Safi *et al.*, 2003), and BMP6 (Al-Musawi *et al.*, 2007). BMP6 additionally stimulated β_A mRNA expression (Al-Musawi *et al.*, 2007), while FSH elevated follistatin mRNA, but inhibited β_B mRNA expression, although this result varied with follicle size (Davis *et al.*, 2001). Following treatment of granulosa cell cultures with estradiol, mRNA expression of β_A, β_B and follistatin was elevated (Davis *et al.*, 2000). Altogether, these findings tell us that inhibin and activin are subject to endocrine, paracrine and autocrine regulation, evidence which strongly supports their importance in hen reproduction.

The tightly regulated process of folliculogenesis ultimately culminates in the ovulation of a follicle. Factors important for successful ovulation include steroids and gonadotropins, already discussed, as well as the plasminogen activators (PA), present in both follicular fluid and the follicle wall. The two types of PAs identified in the chicken, urokinase and tissue type, assist in degrading the follicle wall by converting plasminogen to the active plasmin at the time of ovulation (Beers, 1975; Johnson *et al.*, 1997). In the hen, F1 granulosa cell-derived PA activity is regulated by several factors, often in ways not seen in mammals. PA activity was found to be inhibited by testosterone and progesterone treatments and stimulated by EGF, TGFα, TGFβ, PGE_1 and PGE_2 (Tilly and Johnson, 1987; Tilly and Johnson, 1990a; Tilly and Johnson, 1990b; Lafrance *et al.*, 1993a; Lafrance *et al.*, 1993b). PA

is also produced at higher levels by hierarchal follicle GD granulosa (high throughout) than nonGD, which decreased significantly in a stage-dependent manner (Tischkau *et al.*, 1997).

REFERENCES

Al-Musawi S, Gladwell RT, Knight PG. Bone morphogenetic protein-6 enhances gonadotrophin-dependent progesterone and inhibin secretion and expression of mRNA transcripts encoding gonadotrophin receptors and inhibin/activin subunits in chicken granulosa cells. Reproduction. 2007; 134:293-306.

Baarends WM, Uilenbroek JT, Kramer P, Hoogerbrugge JW, van Leeuwen EC, Themmen AP, Grootegoed JA. Anti-Müllerian hormone and anti-Müllerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle and gonadotropin-induced follicle growth. Endocrinology. 1995; 136:4951-4962.

Baarends WM, van Helmond MJL, Post M, van der Schoot PJCM, Hoogerbrugge JW, de Winter JP, Uilenbroek, JTJ, Karels B, Wilming LG, Meijers JHC, Themmen APN, Grootegoed JA. A novel member of the transmembrane serine/threonine kinase receptor family is specifically expressed in the gonads and in mesenchymal cells adjacent to the Müllerian duct. Development. 1994; 120:189-197.

Bahr JM, Wang S-C, Huang MY, Calvo FO. Steroid concentrations in isolated theca and granulosa layers of preovulatory follicles during the ovulatory cycle of the domestic hen. Biol Reprod. 1983; 29:326-334.

Barber DL, Sanders EJ, Aebersold R, Schneider WJ. The receptor for yolk lipoprotein deposition in the chicken oocyte. J Biol Chem. 1991; 266:18761-18770.

Beers WH. Follicular plasminogen and plasminogen activator and the effect of plasmin on ovarian follicle wall. Cell. 1975; 6:379-386.

Behringer RR, Cate RL, Froelick GJ, Palmiter RD, Brinster RL. Abnormal sexual development in transgenic mice chronically expressing

Müllerian-inhibiting substance. Nature. 1990; 345:167-170.

Behringer RR, Feingold MJ, Cate RL. Müllerian inhibiting substance function during mammalian sexual development. Cell. 1994; 79:415-425.

Brankin V, Hunter MG, Horan TL, Armstrong DG, Webb R. The expression patterns of mRNA-encoding stem cell factor, internal stem cell factor and KIT in the prepubertal and adult porcine ovary. J Anatomy. 2004; 205:393-403.

Broudy VC, Kovach NL, Bennett LG, Lin N, Jacobsen FW, Kidd PG. Human umbilical vein endothelial cells display high-affinity c-kit receptors and produce a soluble form of the c-kit receptor. Blood. 1994; 83:2145-2152.

Carlsson IB, Laitinen MPE, Scott JE, Louhio H, Velentzis L, Tuuri T, Aaltonen J, Ritvos O, Winston RML, Hovatta O. Kit ligand and c-Kit are expressed during early human ovarian follicular development and their interaction is required for the survival of follicles in long-term culture. Reproduction. 2006; 131:641-649.

Carnahan JF, Patel DR, Miller JA. Stem cell factor is a neurotrophic factor for neural crest-derived chick sensory neurons. J Neurosci. 1994; 14:1433-1440.

Carre-Eusebe D, di Clemente N, Rey R, Pieau C, Vigier B, Josso N, Picard JY. Cloning and expression of the chick anti-Müllerian hormone gene. J Biol Chem. 1996; 271:4798-4804.

Celestino JHH, Bruno JB, Lima-Verde IB, Matos MHT, Saraiva MVA, Chaves RN, Martins FS, Almeida AP, Cunha RMS, Lima LF, Name KPO, Campello CC, Silva JRV, Báo SN, Figueiredo JR. Steady-state level of kit ligand mRNA in goat ovaries and the role of kit ligand in

preantral follicle survival and growth in vitro. Mol Reprod Dev. 2010;

77:231-240.

Chen CC, Johnson PA. Molecular cloning of inhibin/activin beta A-subunit complementary deoxyribonucleic acid and expression of inhibin/activin alpha- and beta A-subunits in the domestic hen. Biol Reprod. 1996; 54:429-435.

Cho J-H, Itoh T, Sendai Y, Hoshi H. Fibroblast growth factor 7 stimulates in vitro growth of oocytes originating from bovine early antral follicles. Molec Biol Gametes Interactions. 2008; 75:1736-1743.

Clark DE, Tisdall DJ, Fidler AE, McNatty KP. Localization of mRNA encoding c-kit during the initiation of folliculogenesis in ovine fetal ovaries. J Reprod Fertil. 1996; 106:329-335.

Cook CL, Siow Y, Brenner AG, Fallat ME. Relationship between serum Müllerian-inhibiting substance and other reproductive hormones in untreated women with polycystic ovary syndrome and normal women. Fertil Steril. 2002; 77:141-146.

Cushman RA, Wahl CM, Fortune JE. Bovine ovarian cortical pieces grafted to chick embryonic membranes: a model for studies on the activation of primordial follicles. Hum Reprod. 2002; 17:48-54.

Davis AJ, Brooks CF, Johnson PA. Estradiol regulation of follistatin and inhibin alpha- and beta(B)-subunit mRNA in avian granulosa cells. Gen Comp Endocrinol. 2000; 119:308-316.

Davis AJ, Brooks CF, Johnson PA. Follicle-stimulating hormone regulation of inhibin alpha- and beta(B)-subunit and follistatin messenger ribonucleic acid in cultured avian granulosa cells. Biol Reprod. 2001; 64:100-106.

Davis AJ, Brooks CF, Johnson PA. Gonadotropin regulation of inhibin alpha-subunit mRNA and immunoreactive protein in cultured chicken

granulosa cells. Gen Comp Endocrinol. 1999; 116:90-103.

Davis AJ, Johnson PA. Expression pattern of messenger ribonucleic acid for follistatin and the inhibin/activin subunits during follicular and testicular development in gallus domesticus. Biol Reprod. 1998; 59:271-277.

di Clemente N, Goxe B, Remy J-J, Cate R, Josso N, Vigier B, Salesse R. Inhibitory effect of AMH upon the expression of aromatase and LH receptors by cultured granulosa cells of rat and porcine immature ovaries. Endocrine. 1994a; 2:553-558.

di Clemente N, Wilson C, Faure E, Boussin L, Carmillo P, Tizard R, Picard J-Y, Vigier B, Josso N, Cate R. Cloning, expression, and alternative splicing of the receptor for anti-Müllerian hormone. Mol Endocrinol. 1994b; 8:1006-1020.

Di Pasquale E, Beck-Peccoz P, Persani L. Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. Am J Hum Gen. 2004; 75:106-111.

Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM. Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature. 1996; 383:531-535.

Durlinger AL, Gruijters MJ, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong F, Uilenbroek JT, Grootegoed JA, Themmen AP. Anti-Müllerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. Endocrinology. 2001; 142:4891-4899.

Durlinger ALL, Gruijters MJG, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JT, Grootegoed JA, Themmen APN. Anti-Müllerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology. 2002; 143:1076-1084.

Durlinger AL, Kramer P, Karels B, de Jong F, Uilenbroek JT, Grootegoed JA, Themmen AP. Control of primordial follicle recruitment by anti-Müllerian hormone in the mouse ovary. Endocrinology. 1999; 140:5789-5796.

Elis S, Dupont J, Couty I, Persani L, Govoroun M, Blesbois E, Batellier F, Monget P. Expression and biological effects of bone morphogenetic protein-15 in the hen ovary. J Endocrinol. 2007; 194:485-497.

Elvin JA, Clark AT, Wang P, Wolfman NM, Matzuk MM. Paracrine actions of growth differentiation factor-9 in the mammalian ovary. Mol Endocrinol. 1999; 13:1035-1048.

Elvin JA, Yan C, Matzuk MM. Growth differentiation factor-9 stimulates progesterone synthesis in granulosa cells via a prostaglandin E2/EP2 receptor pathway. Proc Natl Acad Sci. 2000; 97:10288-10293.

Etches RJ, Cunningham FJ. The interrelationship between progesterone and luteinizing hormone during the ovulation cycle of the hen (*Gallus domesticus*). J Endocrinol. 1976; 71:51-58.

Etches RJ, Duke CE. Progesterone, androstenedione and oestradiol content of theca and granulosa tissues of the four largest ovarian follicles during the ovulatory cycle of the hen (Gallus domesticus). J Endocrinol. 1984; 103:71-76.

Etches RJ. The ovulatory cycle of the hen. CRC Critical Reviews in Poultry Biology 1990; 2:293-318.

Etches, RJ. The Ovary. In: Etches, RJ. Reproduction in Poultry. University Press, Cambridge. 1996:125-166.

Findlay JK. An update on the role of inhibin, activin, and follistatin as local regulators of folliculogenesis. Biol Reprod. 1993; 48:15-23.

Fortune JE, Rivera GM, Yang MY. Follicular development: the role of the follicular microenvironment in selection of the dominant follicle. Animal Reprod Sci. 2004; 82-83: 109-126.

Fraps RM, Hammond J (ed). Egg production and fertility in poultry. In: Progress in the Physiology of Farm Animals. Butterworths, London 1955; 2:671-740.

Gigli I, Cushman RA, Wahl CM, Fortune JE. Evidence for a role for anti-Müllerian hormone in the suppression of follicle activation in mouse ovaries and bovine ovarian cortex grafted beneath the chick chorioallantoic membrane. Mol Reprod Dev. 2005; 71:480-488.

Gilbert AB, Perry MM, Waddington D, Hardie MA. Role of atresia in establishing the follicular hierarchy in the ovary of the domestic hen (Gallus domesticus). J Reprod Fertil. 1983; 69:221-227.

Gilchrist RB, Ritter LJ, Cranfield M, Jeffery LA, Amato F, Scott SJ, Myllymaa S, Kaivo-Oja N, Lankinen H, Mottershead DG, Groome NP, Ritvos O. Immunoneutralization of growth differentiation factor 9 reveals it partially accounts for mouse oocyte mitogenic activity. Biol Reprod. 2004; 71:732-739

Hanrahan JP, Gregan SM, Mulsant P, Mullen M, Davis GH, Powell R, Galloway SM. Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (Ovis aries). Biol Reprod. 2004; 70:900-909.

Hayman MJ, Meyer S, Martin F, Steinlein P, Beug H. Self-renewal and differentiation of normal avian erythroid progenitor cells: regulatory roles of the TGF-α/*c-ErbB* and SCF/c-*kit* receptors. Cell. 1993; 74:157-169.

Hecht DJ, Davis AJ, Brooks CF, Johnson PA. Molecular cloning and expression analysis of the complementary deoxyribonucleic acid for

chicken inhibin/activin beta (B) subunit. Biol Reprod. 2000; 62:1128-34.

Horie K, Fujita J, Takakura K, Kanzaki H, Suginami H, Iwai M, Nakayama H, Mori T. The expression of c-kit protein in human adult and fetal tissues. Hum Reprod. 1993; 8:1955-1962.

Horie K, Takakura K, Taii S, Narimoto K, Noda Y, Nishikawa S, Nakayama H, Fujita J, Mori T. The expression of c-kit protein during oogenesis and early embryonic development. Biol Reprod. 1991; 45:547-552.

Hoyer PE, Byskov AG, Mollgard K. Stem cell factor and c-Kit in human primordial germ cells and fetal ovaries. Mol Cell Endocrinol. 2005; 234:1-10.

Huang CTF, Weitsman SR, Dykes BN, Magoffin DA. Stem cell factor and insulin-like growth factor-I stimulate luteinizing hormone-independent differentiation of rat ovarian theca cells. Biol Reprod. 2001; 64:451-456.

Huang EJ, Nocka KH, Buck J, Besmer P. Differential expression and processing of two cell associated forms of the kit-ligand: KL-1 and KL-2. Mol Biol Cell. 1992; 3:349-362.

Hutson JM, Donahoe PK, MacLaughlin DT. Steroid modulation of Müllerian duct regression in the chick embryo. Gen Comp Endocrinol. 1985; 57:88-102.

Hutt KJ, McLaughlin EA, Holland MK. Kit ligand and c-Kit have diverse roles during mammalian oogenesis and folliculogenesis. Mol Human Reprod. 2006; 12:61-69.

Ingraham HA, Hirokawa Y, Roberts LM, Mellon SH, McGee E, Nachtigal MW, Visser JA. Autocrine and paracrine Müllerian inhibiting substance hormone signaling in reproduction. Recent Prog Hormone

Res. 2000; 55:53-68.

Ismail RS, Okawara Y, Fryer JN, Vanderhyden BC: Hormonal regulation of the ligand for c-kit in the rat ovary and its effects on spontaneous oocyte meiotic maturation. Mol Reprod Dev. 1996; 43:458-469.

Jahn T, Seipel P, Coutinho S, Urschel S, Schwarz K, Miething C, Serve H, Peschel C, Duyster J. Analysing c-kit internalization using a functional c-kit-EGFP chimera containing the fluorochrome within the extracellular domain. Oncogene. 2002; 21:4508-4520.

Jin X, Han CS, Zhang XS, Yu FQ, Guo SH, Hu Z, Liu YX. Stem cell factor modulates the expression of steroidogenesis related proteins and FSHR during ovarian follicular development. Front Biosci. 2005; 10:573-1580.

John GB, Shidler MJ, Besmer P, Castrillon DH. Kit signaling via PI3K promotes ovarian follicle maturation but is dispensible for primordial follicle activation. Dev Biol. 2009; 331:292-299.

Johnson AL, Bridgham JT, Anthony RV. Expression of avian urokinase and tissue-type plasminogen activator messenger ribonucleic acid during follicle development and atresia. Biol Reprod. 1997; 56:581-588.

Johnson AL, van Tienhoven A. Plasma concentrations of six steroids and LH during the ovulatory cycle of the hen, Gallus domesticus. Biol Reprod. 1980; 23:386-393.

Johnson AL, Woods DC, Jamieson B (ed). Reproductive biology and phylogeny of birds: Phylogeny, morphology, hormones, fertilization. Part A. Science Pub, Inc. 2007.

Johnson AL. Regulation of follicle differentiation by gonadotropins and growth factors. Poult Sci. 1993; 72:867-873.

Johnson AL. The avian ovarian hierarchy: A balance between follicle differentiation and atresia. Poult Avian Biol Rev. 1996; 7:99-110.

Johnson PA, Brooks CF, Davis AJ. Pattern of secretion of immunoreactive inhibin/activin subunits by avian granulosa cells. Gen Comp Endocrinol. 2005a; 141:233-239.

Johnson PA, Dickens MJ, Kent TR, Giles JR. Expression and function of growth differentiation factor-9 in an oviparous species. Gallus domesticus. Biol Reprod. 2005b; 72:1095-1100.

Johnson PA, Kent TR, Urick ME, Giles JR. Expression and regulation of anti-Müllerian hormone in an oviparous species, the hen. Biol Reprod. 2008; 78:13-19.

Johnson PA, Kent TR, Urick ME, Trevino LS, Giles JR. Expression of anti-Müllerian hormone in hens selected for different ovulation rates. Reproduction. 2009; 137:857-863.

Joyce IM, Clark AT, Pendola FL, Eppig JJ. Comparison of recombinant growth differentiation factor-9 and oocyte regulation of KIT ligand messenger ribonucleic acid expression in mouse ovarian follicles. Biol Reprod. 2000; 63:1669-1675.

Joyce IM, Pendola FL, Wigglesworth K, Eppig JJ. Oocyte regulation of kit ligand expression in mouse ovarian follicles. Dev Biol. 1999; 214:342-353.

Juengel JL, Bodensteiner KJ, Heath DA, Hudson NL, Moeller CL, Smith P, Galloway SM, Davis GH, Sawyer HR, McNatty KP. Physiology of GDF9 and BMP15 signalling molecules. Animal Reprod Sci. 2004; 82-83:447-460.

Juengel JL, Hudson NL, Heath DA, Smith P, Reader KL, Lawrence SB, O'Connell AR, Laitinen MP, Cranfield M, Groome NP, Ritvos O, McNatty KP. Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular

development in sheep. Biol Reprod. 2002; 67:1777-1789.

Kang JS, Lee CJ, Lee JM, Rha JY, Song KW, Park MH. Follicular expression of c-Kit/SCF and inhibin-alpha in mouse ovary during development. J Histochem Cytochem. 2003; 51:1447-1458.

Lafrance M, Croze F, Tsang BK. Influence of growth factors on the plasminogen activator activity of avian granulosa cells from follicles at different maturational stages of preovulatory development. J Mol Endocrinol. 1993a; 11:291-304.

Lafrance M, Croze F, Tsang BK. Interactions of transforming growth factor-alpha and -beta and luteinizing hormone in the regulation of plasminogen activator activity in avian granulosa cells during follicular development. Endocrinology. 1993b; 133:720-727.

Lahav R, Lecoin L, Ziller C, Nataf V, Carnahan JF, Martin FH, *et al.* Effect of the Steel gene product on melanogenesis in avian neural crest cell cultures. Differentiation. 1994; 58:133-139.

Larsen J, Meyer S, Steinlein P, Beug H, Hayman, MJ. Transformation of chicken bone marrow cells by the v-*ski* oncogene. Oncogene. 1993; 8:3221-3228.

Laitinen M, Rutanen E, Ritvos O. Expression of c-kit ligand messenger ribonucleic acids in human ovaries and regulation of their steady state levels by gonadotropins in cultured granulosa luteal cells. Endocrinology. 1995; 136:4407-4414.

Lemmon MA, Pinchasi D, Zhou M, Lax I, Schlessinger J. Kit receptor dimerization is driven by bivalent binding of stem cell factor. J Biol Chem. 1997; 272:6311-6317.

Lovell TM, Gladwell RT, Groome NP, Knight PG. Ovarian follicle development in the laying hen is accompanied by divergent changes in inhibin A, inhibin B, activin A and follistatin production in granulosa

and theca layers. J Endocrinol. 2003; 177:45-55.

Manova K, Huang EJ, Angeles M, De Leon V, Sanchez S, Pronovost SM, Besmer P, Bachvarova RF. The expression pattern of the c-kit ligand in gonads of mice supports a role for the c-kit receptor in oocyte growth and in proliferation of spermatogonia. Dev Biol. 1993; 157:85-99.

Massague J, Chen YG. Controlling TGF-ß signaling. Genes Dev. 2000; 14:627-644.

Mather JP, Moore A, Li RH. Activins, inhibins, and follistatins: further thoughts on a growing family of regulators. Proc Soc Exp Biol Med. 1997; 215:209-222.

Mazerbourg S, Klein C, Roh J, Kaivo-Oja N, Mottershead DG, Korchynskyi O, Ritvos O, Hsueh AJ. Growth differentiation factor-9 signaling is mediated by the type I receptor, activin receptor-like kinase 5. Mol Endocrinol. 2004; 18:653-665.

Méduri G, Massin N, Guibourdenche J, Bachelot A, Fiori O, Kuttenn F, Misrahi M, Touraine P. Serum anti-Müllerian hormone expression in women with premature ovarian failure. Hum Reprod. 2007; 22:117-123.

Moore RK, Otsuka F, Shimasaki S. Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. J Biol Chem. 2003; 278:304-310.

Motro B, Bernstein A. Dynamic changes in ovarian c-kit and Steel expression during the estrous reproductive cycle. Dev Dyn. 1993; 197:69-79.

Muruvi W, Fortune JE. Kit ligand-KIT Interaction is required for follicle activation and mediates insulin-stimulated activation of bovine primordial follicles in vitro. Biol Reprod. 2009; 81:200.

Myers M, Middlebrook BS, Matzuk MM, Pangas SA. Loss of inhibin alpha uncouples oocyte-granulosa cell dynamics and disrupts postnatal folliculogenesis. Dev Biol. 2009; 334:458-467.

Nilsson E, Rogers N, Skinner MK. Actions of anti-Müllerian hormone on the ovarian transcriptome to inhibit primordial to primary follicle transition. Reproduction. 2007; 134:209-221.

Nilsson EE, Kezele P, Skinner MK. Leukemia inhibitory factor (LIF) promotes the primordial to primary follicle transition in rat ovaries. Mol Cell Endocrinol. 2002; 188:65-73.

Nilsson EE, Skinner MK. Kit ligand and basic fibroblast growth factor interactions in the induction of ovarian primordial to primary follicle transition. Mol Cell Endocrinol. 2004; 214:19-25.

Nitta H, Osawa Y, Bahr JM. Multiple steroidogenic cell populations in thecal layer of preovulatory follicles of the chicken ovary. Endocrinology. 1991; 129:2033-2040.

Otsuka F, Shimasaki S. A negative feedback system between oocyte bone morphogenetic protein 15 and granulosa cell kit ligand: its role in regulating granulosa cell mitosis. Proc Natl Acad Sci USA. 2002; 99:8060-8065.

Otsuka F, Yao Z, Lee T, Yamamoto S, Erickson GF, Shimasaki S. Bone morphogenetic protein-15. Identification of target cells and biological functions. J Biol Chem. 2000; 275:39523-39528.

Parrott JA, Skinner MK. Direct actions of kit-ligand on theca cell growth and differentiation during follicle development. Endocrinology. 1997; 138:3819-3827.

Parrott JA, Skinner MK. Thecal cell-granulosa cell interactions involve a positive feedback loop among keratinocyte growth factor, hepatocyte growth factor, and kit ligand during ovarian follicular development.

Endocrinology. 1998; 139:2240-2245.

Petitte JN, Kulik MJ. Cloning and characterization of cDNAs encoding two forms of avian stem cell factor. Biochim Biophys Acta. 1996; 1307:149-151.

Reddy P, Shen L, Ren C, Boman K, Lundin E, Ottander U, Lindgren P, Liu YX, Sun QY, Liu K . Activation of Akt (PKB) and suppression of FKHRL1 in mouse and rat oocytes by stem cell factor during follicular activation and development. Dev Biol. 2005; 281:160-170.

Reynaud K, Cortvrindt R, Smitz J,Driancourt MA. Effects of Kit Ligand and anti-Kit antibody on growth of cultured mouse preantral follicles. Mol Reprod Dev. 2000; 56:483-494.

Robinson FE, Etches RJ. Ovarian steroidogenesis during follicular maturation in the domestic fowl (*Gallus domesticus*). Biol Reprod. 1986; 35:1096-1105.

Roskoski R Jr. Signaling by Kit protein-tyrosine kinase-the stem cell factor receptor. Biochem Biophys Res Commun 2005; 337:1–13.

Safi M, Onagbesan OM, Bruggeman V, Vleugels B, Volckaert G, Decuypere E. Regulation of inhibin α- and β_A-subunit messenger ribonucleic acid levels by gonadotropins and IGF-I in cultured chicken granulosa cells. Gen Comp Endocrinol. 2003; 131:159-167.

Sasaki E, Okamura H, Chikamune T, Kanai Y, Watanabe M, Naito M, Sakurai M. Cloning and expression of the chicken c-*kit* proto-oncogene. Gene. 1993; 128:257-261.

Schuster MK, Schmierer B, Shkumatava A, Kuchler K. Activin A and follicle-stimulating hormone control tight junctions in avian granulosa cells by regulating occludin expression. Biol Reprod. 2004; 70:1493-1499.

Seifer DB, MacLaughlin DT, Christian BP, Feng B, Shelden RM. Early follicular serum Müllerian-inhibiting substance levels are associated with ovarian response during assisted reproductive technology cycles. Fertil Steril. 2002; 77:468-471.

Silva JR, van den Hurk R, van Tol HT, Roelen BA, Figueiredo JR. The Kit ligand/c-Kit receptor system in goat ovaries: gene expression and protein localization. Zygote. 2006; 14:317-328.

Solovyeva EV, Hayashi M, Margi K, Barkats C, Klein C, Amsterdam A, Hsueh AJW, Tsafriri A. Growth differentiation factor-9 stimulates rat theca-interstitial cell androgen biosynthesis. Biol Reprod. 2000; 63:1214-1218.

Tanikawa M, Harada T, Mitsunari M, Onohara Y, Iwabe T, Terakawa N. Expression of c-kit messenger ribonucleic acid in human oocyte and presence of soluble c-kit in follicular fluid. J Clin Endocrinol Metab. 1998; 83:1239-1242.

Teng CS, Wang JJ, Teng JIN. Purification of chicken testicular Müllerian inhibiting substance by ion exchange and high-performance liquid chromatography. Dev Biol. 1987; 123:245-254.

Teng CS. Differential activation of MAPK during Müllerian duct growth and apoptosis: JNK and p38 stimulation by DES blocks tissue death. Cell Tissue Res. 2001; 306:27-34.

Teng CS. Differential expression of c-Jun proteins during Müllerian duct growth and apoptosis: caspase-related tissue death blocked by diethylstilbestrol. Cell Tissue Res. 2000; 302:377-385.

Thomas FH, Ethier JF, Shimasaki S, Vanderhyden BC. Follicle-stimulating hormone regulates oocyte growth by modulation of expression of oocyte and granulosa cell factors. Endocrinology. 2005; 146:941-949.

Tilly JL, Johnson AL. Effect of several growth factors on plasminogen activator activity in granulosa and theca cells of the domestic hen. Poult Sci. 1990a; 69:292-299.

Tilly JL, Johnson AL. Modulation of hen granulosa cell steroidogenesis and plasminogen activator activity by transforming growth factor alpha. Growth Factors. 1990b; 3:247-255.

Tilly JL, Johnson AL. Presence and hormonal control of plasminogen activator in granulosa cells of the domestic hen. Biol Reprod. 1987; 37:1156-1164.

Tilly JL, Kowalski KI, Johnson AL. Stage of ovarian follicular development associated with the initiation of steroidogenic competence in avian granulosa cells. Biol Reprod. 1991; 44:305-314.

Tischkau SA, Neitzel LR, Walsh JA, Bahr JM. Characterization of the growth center of the avian preovulatory follicle. Biol Reprod. 1997; 56:469-474.

Tisdall DA, Fidler AE, Smith P, Quirke LD, Stent VC, Heath DA, McNatty KP. Stem cell factor and c-kit gene expression and protein localization in the sheep ovary during fetal development. J Reprod Fertil. 1999; 116:277-291.

Tran D, Josso N. Relationship between avian and mammalian anti-Müllerian hormones. Biol Reprod. 1977; 16:267-273.

Ueno S, Takahashi M, Manganaro TF, Ragin RC, Donahoe PK. Cellular localization of Müllerian inhibiting substance in the developing rat ovary. Endocrinology. 1989; 124:1000-1006.

Van Rooij IA, Tonkelaar I, Broekmans FJ, Looman CW, Scheffer GJ, de Jong FH, Themmen AP, te Velde ER. Anti-Müllerian hormone is a promising predictor for the occurrence of the menopausal transition.

Menopause. 2004; 11:601-606.

Van't Hof RJ, von Lindern M, Nijweide PJ, Beug H. Stem cell factor stimulates chicken osteoclast activity *in vitro*. FASEB J. 1997; 11:287-293.

Visser JA, Olaso R, Verhoef-Post M, Kramer P, Themmen AP, Ingraham HA. The serine/threonine transmembrane receptor alk2 mediates Müllerian inhibiting substance signaling. Mol Endocrinol. 2001; 15:936-945.

Visser JA, Themmen APN. Anti-Müllerian hormone and folliculogenesis. Mol Cell Endocrinol. 2005; 234:81-86.

Volentine KK, Yao HH, Bahr JM. Epidermal growth factor in the germinal disc and its potential role in follicular development in the chicken. Biol Reprod. 1998; 59:522-526.

Wang J, Roy SK. Growth differentiation factor-9 and stem cell factor promote primordial follicle formation in the hamster: modulation by follicle-stimulating hormone. Biol Reprod. 2004; 70:577-585.

Wang SY, Johnson PA. Complementary deoxyribonucleic acid cloning and sequence analysis of the alpha subunit of inhibin from chicken ovarian granulosa cells. Biol Reprod. 1993; 49:453-458.

Wang Y, Li J, Ying Wang C, Yan Kwok AH, Leung FC. Epidermal growth factor (EGF) receptor ligands in the chicken ovary: I. Evidence for heparin-binding EGF-like growth factor (HB-EGF) as a potential oocyte-derived signal to control granulosa cell proliferation and HB-EGF and kit ligand expression. Endocrinology. 2007; 148:3426-3440.

Woods DC, Haugen MJ, Johnson AL. Actions of epidermal growth factor receptor/mitogen-activated protein kinase and protein kinase C signaling in granulosa cells from Gallus gallus are dependent upon

stage of differentiation. Biol Reprod. 2007; 77:61-70.

Woods DC, Johnson AL. Regulation of follicle-stimulating hormone-receptor messenger RNA in hen granulosa cells relative to follicle selection. Biol Reprod. 2005; 72:643-650.

Wypych J, Bennett LG, Schwartz MG, Clogston CL, Lu HS, Broudy VC, Bartley TD, Parker VP, Langley KE. Soluble kit receptor in human serum. Blood. 1995; 85:66-73.

Yan C, Wang P, DeMayo J, DeMayo FJ, Elvin J, Carino C, Prasad SV, Skinner SS, Dunbar BS, Dube JL, Celeste AJ, Matzuk MM. Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Mol Endocrinol. 2001; 15:854-866.

Yao HHC, Bahr JM. Chicken granulosa cells show differential expression of epidermal growth factor (EGF) and luteinizing hormone (LH) receptor messenger RNA and differential responsiveness to EGF and LH dependent upon location of granulosa cells in the germinal disc. Biol Reprod. 2001a; 64:1790-1796.

Yao HHC, Bahr JM. Germinal disc-derived epidermal growth factor: A paracrine factor to stimulate proliferation of granulosa cells. Biol Reprod. 2001b; 64:390-395.

Ye Y, Kawamura K, Sasaki M, Kawamura N, Groenen P, Gelpke MD, Rauch R, Hsueh AJ, Tanaka T. Kit ligand promotes first polar body extrusion of mouse preovulatory oocytes. Reprod Biol Endocrinol. 2009; 7:26-37.

Yoshida H, Takakura N, Kataoka H, Kunisada T, Okamura H, Nishikawa SI. Stepwise requirement of c-kit tyrosine kinase in mouse ovarian follicle development. Dev Biol. 1997; 184:122-137.

Yoshimura Y, Tischkau SA, Bahr JM. Destruction of the germinal disc region of an immature preovulatory follicle suppresses follicular

maturation and ovulation. Biol Reprod. 1994; 51:229-233.

Zhou, JH, Ohtaki, M, Sakurai, M. Sequence of a cDNA encoding chicken stem cell factor. Gene. 1993; 127:269-270.

CHAPTER 2
EXPRESSION AND REGULATION OF KIT LIGAND IN THE OVARY OF THE HEN

2.1 INTRODUCTION

Functionality of the Kit system, composed of Kit ligand (KL) and its cognate tyrosine kinase receptor, cKit, has been shown to be essential in a variety of tissues and processes (Hutt *et al.*, 2006). KL is a cytokine that has two primary isoforms (soluble and membrane-spanning), that initiates its biological activity by binding to the cKit receptor in its extracellular domain (Lemmon *et al.*, 1997). There is also evidence of a soluble form of cKit that consists of part of the extracellular domain. This is proposed to bind KL with high affinity and thus could prevent binding of KL with the membrane-associated cKit (Tanikawa *et al.*, 1998). In the current study, we postulate that a soluble cKit is available in our bioactive media and has the ability to exert effects by binding KL. In the chicken, the Kit system has been implicated in proliferation of normal haemopoeitic cells (Hayman *et al.*, 1993), proliferation and differentiation of neural crest-derived melanocytes (Lahav *et al.*, 1994), differentiation and functional activation of osteoclasts (Van't Hof *et al.*, 1997), and assisting in the survival of dorsal root ganglia neurons *in vitro* (Carnahan *et al.*, 1994). There has been limited examination of the KL/cKit system in the ovary of chickens.

In the chicken, the soluble and longer isoform of KL is a 287 amino acid polypeptide shown to be present in many tissues (Zhou *et al.*, 1993). Although KL expression throughout follicle development in the chicken ovary is currently unreported, four isoforms of one chicken KL gene have

been identified and cloned: KL-1, KL-2, KL-3 and KL-4. Of these, KL-1 was reported to be primarily expressed in the ovary (Wang *et al.*, 2007).

The ovarian expression pattern of KL mRNA and protein has been well studied in mammals. In the mouse, KL protein and mRNA was detected in follicle granulosa cells at all stages of development, with low expression found in primordial follicles and then increasing with follicle growth to the highest levels in preantral follicles. KL expression is decreased to low levels in cumulus cells of antral follicles, while remaining high in mural granulosa cells (Motro and Bernstein, 1993). In goats, KL protein and mRNA were expressed in granulosa cells at all stages of development with highest expression found in secondary follicles (Celestino *et al.*, 2010). Similarly, granulosa cell KL mRNA expression has been shown in the rat (Ismail *et al.*, 1996), sheep, (Tisdall *et al.*, 1999) human (Hoyer *et al.*, 2005) and pig (Brankin *et al.*, 2004).

In mammalian reproduction, regulation of KL occurs through endocrine, paracrine, and autocrine signaling. Treatment with FSH significantly increased KL mRNA expression in bovine (Parrott and Skinner, 1998) and murine (Joyce *et al.*, 1999) granulosa cell cultures. Increased KL mRNA expression in granulosa cultures was also found after testosterone treatment (Joyce *et al.*, 1999). Other research found no effect on soluble KL mRNA with FSH treatments in the mouse and increased membrane-bound KL mRNA with low (but not high) FSH treatment (Thomas *et al.*, 2005). In contrast, in human granulosa-luteal cell cultures, FSH treatment was shown to significantly decrease KL mRNA expression (Laitinen *et al.*, 1995).

With respect to paracrine regulation of KL, oocyte-derived BMP15 treatment of granulosa cells co-cultured with oocytes significantly increased

KL mRNA in rats (Otsuka and Shimasaki, 2002) and in mice (Thomas *et al.*, 2005). Addition of partly grown oocytes to mouse granulosa cell culture also promotes KL mRNA expression, an effect thought to be attributed to the presence of BMP15 in the oocytes (Joyce *et al.*, 1999; Joyce *et al.*, 2000). Although granulosa cell-derived AMH had no direct effect on KL gene expression, the stimulatory effects of KL on rat primordial follicle development were inhibited by AMH added to whole ovary culture. This suggests that AMH could act in an autocrine manner to regulate KL activity (Nilsson *et al.*, 2007).

A single study that investigated autocrine regulation of KL in the chicken ovary revealed that heparin binding EGF-like growth factor (HB-EGF) treatment resulted in a dose-dependent decrease of granulosa cell KL mRNA. This inhibitory effect was seen in granulosa cells from 3 mm, 6 mm, F4+5, and F1 follicles (Wang *et al.*, 2007).

The 960 amino acid sequence of chicken cKit was detected in many organs including the brain, bursa, heart, kidney, spleen, thymus, ovary, and testis (Sasaki *et al.*, 1993). Whereas the ovarian expression pattern of cKit in the chicken has not been established, cKit protein and mRNA expression was shown in oocytes of mice (Motro and Bernstein, 1993; Horie *et al.*, 1991), sheep (Tisdall *et al.*, 1999), goats (Silva *et al.*, 2006), pigs (Brankin *et al.*, 2004), and humans (Horie *et al.,* 1991; Carlsson *et al.,* 2006). Expression was also found in theca of mice (Motro and Bernstein, 1993), pigs (Brankin *et al.*, 2004), and cattle (Parrott and Skinner, 1997). As previously stated, a soluble form of cKit has also been found in human follicular fluid (Tanikawa *et al.*, 1998).

The cKit receptor must bind to its ligand to become biologically activated; therefore functionality of cKit (membrane-bound) is dependent on

interactions with KL. With respect to reproduction, binding of granulosa cell KL to its receptor on the oocyte may activate different signaling pathways. Among these, cellular response occurs through the phosphatidylinositol 3 kinase (PI3K) pathway, the pathway thought to control primordial follicle activation (John *et al.*, 2009). Expression of PI3K components following KL treatment supports the suggestion that the Kit system regulates follicle development and affects secretion of oocyte factors that regulate the primordial to primary follicle transition (Reddy *et al.*, 2005).

The ovary of the laying hen contains five to eight large yolk-filled follicles that are arranged in a hierarchy according to size representative of the order in which they are selected to ovulate, with F1 being largest and therefore next to ovulate, followed by F2 (Gilbert *et al.*, 1983). Development of these follicles is well-regulated, with a 24-26 hour period between ovulations. With each ovulation the follicles progress through the hierarchy and an additional follicle is recruited from the pool of nonhierarchal follicles that consists of small yellow follicles (5-10 mm diameter) and small white follicles (<5 mm diameter) (Gilbert *et al.*, 1983). A single follicle per day is selected from the pool of 6-8 mm follicles to enter the preovulatory hierarchy, making this group very interesting to study. The present studies were designed to characterize KL in the ovary of the hen with a goal to understand the role of the Kit system in follicle development in the hen. These include study of the developmental pattern of KL expression, the effects of oocyte factors, steroids and FSH on KL expression, and functions of the Kit system in steroid regulation. Investigation of FSH and oocyte factors such as GDF9 and BMP15 is based on previous reports that these effect KL expression in the mouse.

2.2 MATERIALS AND METHODS

Animals

Laying hens (SCWL Babcock B300 strain), between 22 and 74 weeks of age were housed individually in laying batteries. They had free access to feed and water and were exposed to 15 h light: 9 h darkness per day, with lights on at 0600 h. Individual egg laying was recorded daily. Hens with regular laying patterns were selected and euthanized at 1-2 h after oviposition in order to collect tissue samples. All procedures were approved by the IACUC of Cornell University.

Oocyte Conditioned Media (OCM)

OCM was produced by dissecting very small follicles from the ovarian stroma. These follicles, which were less than 1 mm in size, were pooled and incubated in M199 plus 0.1% BSA for 3 days in a humidified atmosphere containing 5% CO_2 at 37°C for 3 days. After 3 days the supernatant was collected, filter-sterilized (0.2 μm membrane), and frozen. Many separate preparations of OCM were combined to create a large pool for subsequent use in cell culture (Johnson *et al.*, 2008).

RNA Isolation

Total RNA was extracted from ovarian body, liver, intact follicles (<1 mm), and granulosa cell layers from different sized follicles (3, 5, 6-12 mm and F1) using the Qiagen RNeasy mini kit and following manufacturer instructions. For more detailed analysis of expression, the granulosa layer was split so that one half contained the germinal disc (GD) and the other half consisted of non-germinal disc (NGD) granulosa cells (Johnson *et al.*, 2008). Comparisons were then made between GD and NGD KL mRNA expression.

PCR Analysis

Quantitative real-time PCR with Sybr Green was performed to quantify expression of KL, using 18S expression as an internal control for KL normalization. Chicken-specific primers (GenBank accession #205130) designed to span intron regions were used (Invitrogen software program). These primers predicted a product of 133bp for KL (forward: 5'-CCAGAGTCGCT GTCACAAAA-3'; reverse: 5'-AGGCTGGAGCTGCTAATGAA-3'). Control reagent was Ambion Quantum RNA Universal 18S primers (#1718) and controls were run for each sample. A standard curve was created using serial dilutions of cDNA transcribed from a pool of granulosa cell RNA prepared from 6-8 mm follicles. Control reactions lacking template and reverse transcriptase were also performed in parallel on each plate. Relative RNA concentration was determined using the C_t method as calculated by the ABI Prism 7000 Sequence Detection System Software and the mean value for duplicate PCR amplifications was calculated relative to 18S reactions.

Granulosa Cell Culture

Granulosa layers from 3-5 mm, 6-8 mm, 9-16 mm, F3/F4, and F1 follicles were isolated and dispersed as described previously (Davis *et al.*, 1999). Cell number and viability (>95%) were estimated with a hemocytometer. Cells were plated in 6-well plates (in M199 plus 5% FBS) at a density of $3x10^6$ cells/well in 1.5 ml volume and then incubated as described previously (Johnson *et al.*, 2005). After 24 h, medium was replaced with M199 plus 0.1% BSA as control. Other treatments included OCM (10%, 25% or 50% of volume), FSH (0.1, 0.5, 1, 5, 10, 25 ng/ml; oFSH-19-SIAFP), testosterone (0.1, 1, 10, 50 ng/ml), estradiol (0.1, 1, 10, 50 ng/ml),

progesterone (0.1, 1, 10, 50 ng/ml), Vitamin D_3 (10, 100 nM) and recombinant mouse KL (25, 50, 100, 200 ng/ml; BioLegend, #579702). Pooled OCM was heat inactivated (30 min at 65°C) for characterization of OCM as described previously (Johnson *et al.*, 2008).

Medium was also size-fractionated using Amicon Ultra-15 centrifugal filter devices (Millipore; 35 min at 5000xg, 25°C) to separate OCM into two fractions of molecular weight less than and greater than 30 kDa. In some experiments, OCM was pre-incubated with an antibody for 30 min at room temperature. Antibodies used were against cKit (M14, Santa Cruz Biotechnology), BMP15 (GenScript, against chicken peptide sequence CLPGYLRDAGGDKSD), or GDF9 (JH131; obtained as a gift from Dr. S.-J. Lee, Johns Hopkins University).

Granulosa Cell proliferation

Granulosa cells were plated in 96-well plates at a density of $1.2x10^5$ cells/well in 100 μL of media and cultured as described above. The number of viable cells was determined using the CellTiter 96 Aqueous One Solution Assay (Promega) according to manufacturer instructions. Visual inspection of plate confluence confirmed proliferation.

Theca Cell Culture

The theca cell layer was isolated from 6-8 mm follicles. For each repetition, the theca shells from one hen were pooled and cut up into small pieces. Theca pieces were weighed and approximately equal weights (18-23 mg) were allocated to wells in a 24-well plate with 0.5 ml of media. Control treatments were M199 plus 0.1% BSA; other treatments were 25% or 50% OCM by volume, 25% OCM pre-incubated with an antibody to cKit (M14), cKit antibody alone, or recombinant mouse KL (50, 100, 200 ng/ml;

BioLegend, #579702). After 24 h of incubation at 37°C in a humidified atmosphere of 5% CO_2, medium was collected and frozen.

Western Blot

Protein lysates were made from very small (<1 mm) follicles, F1 granulosa cells, theca tissue from 3-5 mm follicles, and ovarian body. Protein concentrations were determined using a BCA Protein Assay Kit (Pierce) and 40 mg of total protein for each sample was loaded onto a 12% polyacrylamide gel (Pierce) along with a Biotinylated protein ladder (Cell Signaling). Electrophoresis was performed under reducing conditions and the gel was transferred to a nitrocellulose membrane (Thermo Scientific). The membrane was blocked in 1X Tris-buffered saline with 0.1% Tween-20 (TBST) containing 5% milk for 30 minutes at room temperature. The membrane was washed and then incubated with a primary polyclonal antibody against mouse cKit (M14) at a dilution of 1:200 in TBST containing 1% milk for 2 h at room temperature. The membrane was washed and then incubated with a horseradish peroxidase conjugated donkey anti-goat IgG, (Santa Cruz #2020, diluted 1:2500) and an anti-biotin antibody (Cell Signaling, diluted 1:1000) in TBST containing 5% milk for 1h at room temperature. The membrane was incubated with LumiGLO chemiluminescent substrate (KPL, Gaithersburg, MD) for signal detection and examined using a Fluor Chem HD2 Imager (Alpha Innotech).

Radioimmunoassay

Culture media were collected and assayed to determine granulosa cell progesterone production or theca cell estradiol production. Steroid hormone concentrations were estimated using Coat-A-Count radioimmunoassay kits for estradiol and progesterone (Diagnostics Product

Corporation, Los Angeles, CA). The analytical sensitivity of the kits is 8.0 pg/ml for estradiol and 0.02 ng/ml for progesterone. Granulosa culture media was diluted 1:10 prior to assay. For both assays, plasma from immature roosters was used as a low pool and plasma from ovulating hens treated with exogenous progesterone was used as a high pool. Standards provided in the kit were assayed in triplicate and all culture media samples were assayed in duplicate. The coefficient of variation (CV) for the progesterone assay was 6.8% and the mean CV for the estradiol assays was 4.4%.

Statistical Analysis

Data were analyzed by the Statistical Analysis Systems (SAS) General Linear Model Procedure (Proc-GLM) with protected least-significant difference. P values of <0.05 were considered significant, and means were compared using Duncan's multiple range test. Results are presented as means ± SE, percent of control, with different letters indicating significance at $p<0.05$.

2.3 RESULTS

Kit Ligand Expression with Follicle Development

After normalization to 18S expression, real-time PCR analysis (**Figure 2.1**; n=4-5) indicated that KL mRNA expression decreased with increased follicle size ($p=0.001$). KL mRNA was most abundant in preparations from very small (<1 mm) follicles, with high levels also found in the granulosa layer of 3 mm follicles and ovarian body. The lowest levels of KL mRNA expression were found in the granulosa layer from 6-12 mm and F1 follicles, which did not differ from levels found in the liver tissue.

Analysis of KL expression from the GD and NGD regions of the granulosa layer of follicles 6 mm-F1 in size showed no significant difference between GD and NGD regions (**Figure 2.2**; n=2-5).

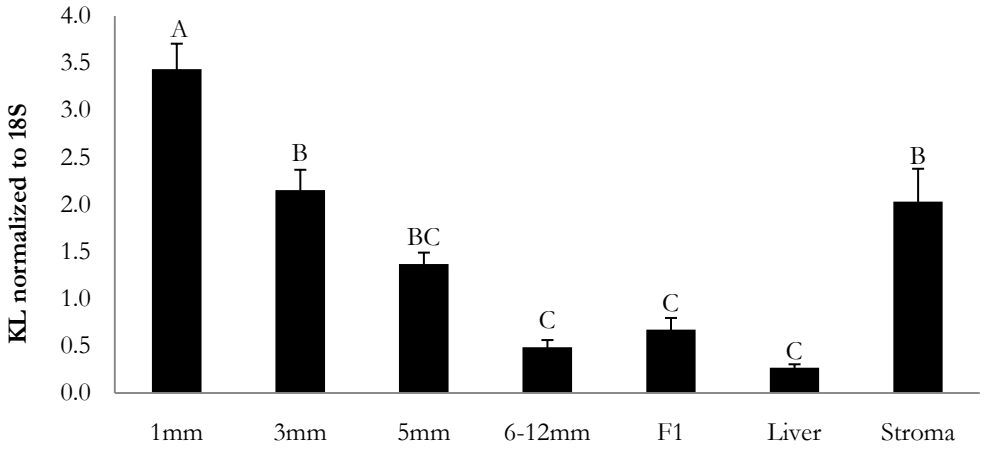

Figure 2.1 KL mRNA expression during follicle development
KL expression is highest in <1 mm follicles and granulosa cells from 3 mm follicles and decreases with follicle development, n=4-5; p<0.001.

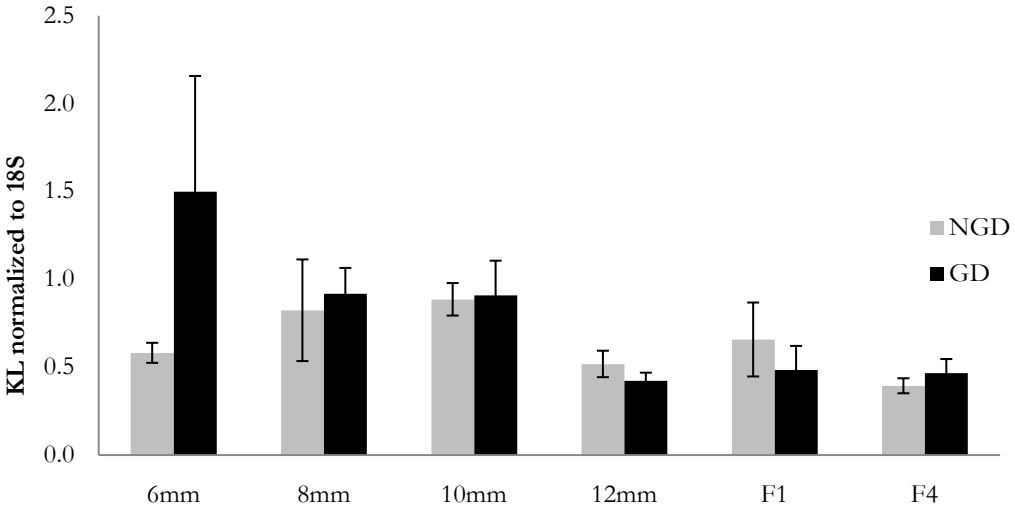

Figure 2.2 KL mRNA expression in GD vs. NGD granulosa cells
KL expression did not differ between GD and NGD granulosa cells, n=2-5.

Regulation of KL by the Oocyte

Culture of granulosa cells from 6-8 mm follicles with 25% and 50% OCM increased KL mRNA expression in a dose-related way (**Figure 2.3**; n=4; $p<0.05$). Pre-incubation of 25% OCM with antibodies against cKit (**Figure 2.4**; n=4), BMP15 (**Figure 2.5**; n=6) or GDF9 (**Figure 2.6**; n=2) did not block the stimulatory effect of OCM upon KL expression. When OCM was separated into fractions by molecular weight, culture treatment with 25% OCM of high molecular weight (MW; >30 kDa) significantly increased KL expression ($p<0.05$) while low molecular weight (<30 kDa) OCM treatments had no effect (**Figure 2.7**; n=3). KL mRNA expression was also examined following OCM treatments of granulosa cells from F1 follicles (**Figure 2.8**). 25% OCM significantly increased KL mRNA expression in F1, an effect that was blocked by heat treatment (HT) of OCM prior to culture (n=3; $p<0.01$).

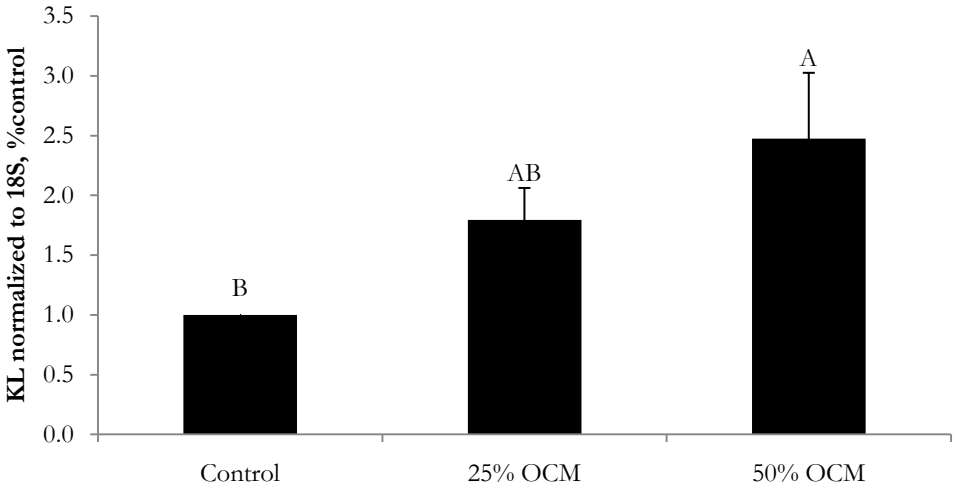

Figure 2.3 KL mRNA expression after OCM treatment in granulosa cells from 6-8 mm follicles

KL expression is significantly increased by treatments with OCM, n=4, $p<0.05$.

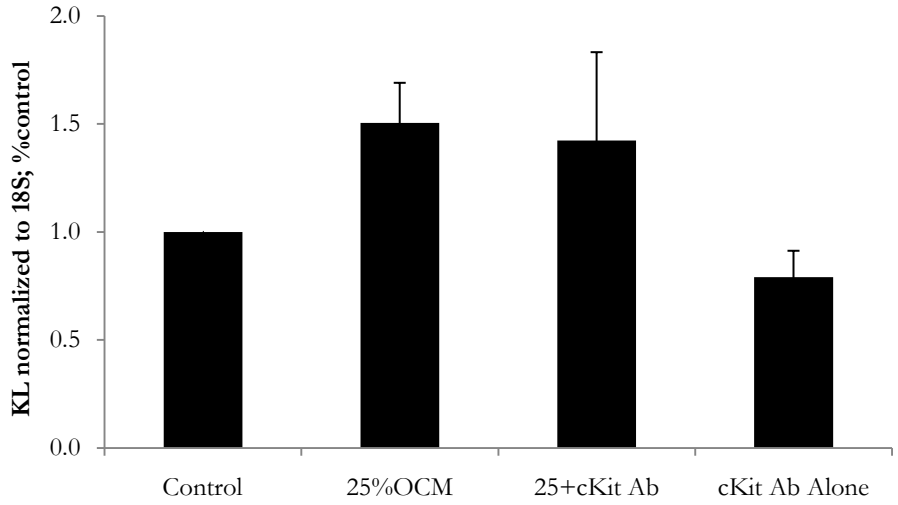

Figure 2.4 KL mRNA expression after OCM +cKit Ab treatment in granulosa cells from 6-8 mm follicles

The antibody to cKit did not block the stimulatory effect of OCM on KL mRNA, n=4.

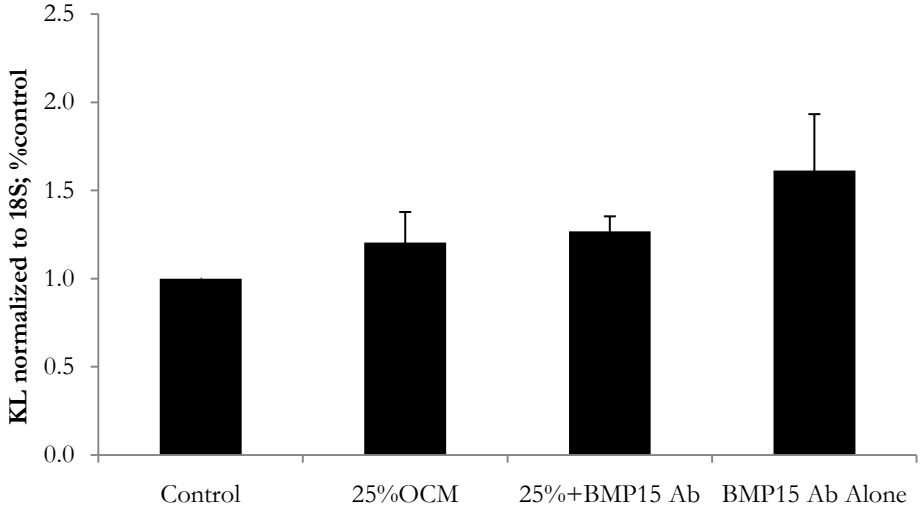

Figure 2.5 KL mRNA expression after OCM +BMP15 Ab treatment in granulosa cells from 6-8 mm follicles

The BMP15 antibody did not block the stimulatory effect of OCM on KL mRNA, n=6.

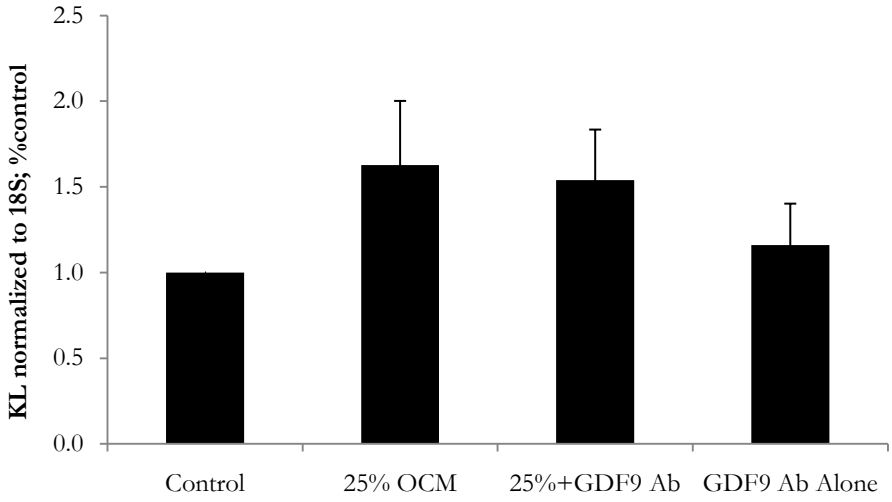

Figure 2.6 KL mRNA expression after OCM +GDF9 Ab treatment in granulosa cells from 6-8 mm follicles
The antibody to GDF9 did not block the stimulatory effect of OCM on KL mRNA, n=2.

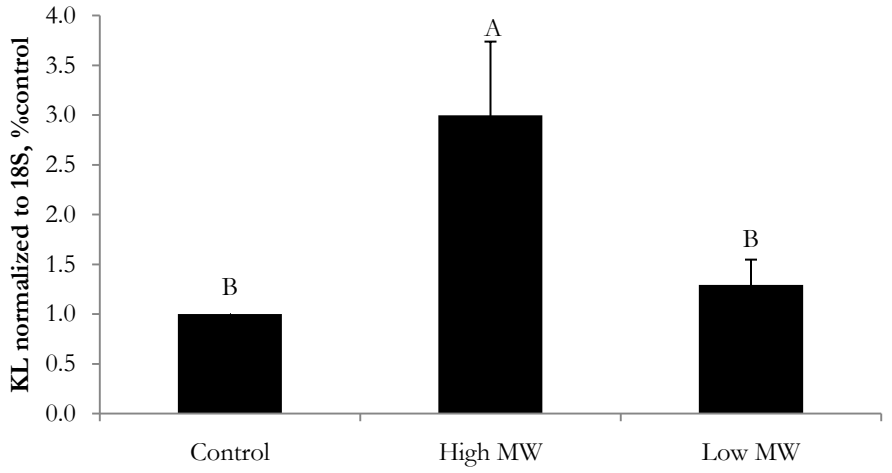

Figure 2.7 KL mRNA expression after OCM treatment in granulosa cells (6-8 mm) treated with high and low MW OCM
KL expression is significantly increased by high MW OCM and unchanged by low MW OCM, n=3, $p<0.05$.

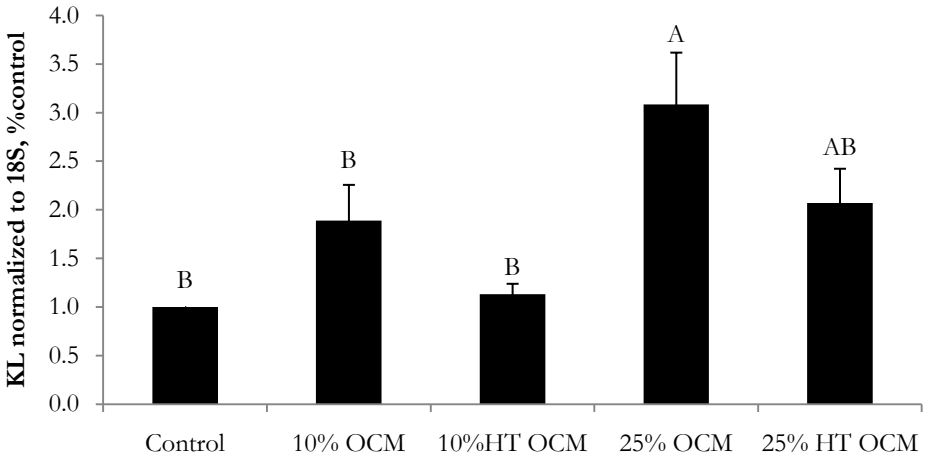

Figure 2.8 KL mRNA expression after OCM treatment in granulosa cells from F1

KL expression increased by OCM ($p<0.01$) is partially reduced by HT of OCM, n=3.

Regulation of KL by Steroids

KL mRNA expression was assessed following granulosa cell treatment with various doses of steroid hormones. Vitamin D3 (VD3) treatment significantly increased KL mRNA expression in granulosa cells from 6-8 mm (**Figure 2.9**; n=5-6; $p<0.05$) and 3-5 mm (**Figure 2.10**; n=4-5; $p<0.01$) follicles at the dose of 100 nM but had no effect at the 10 nM dose. Expression of KL mRNA in granulosa cells from 6-8 mm follicles treated with estradiol, progesterone, or testosterone was not significantly altered (data not shown).

Additional Regulation of KL

Relative expression of KL mRNA from granulosa cells of 6-8 mm follicles was significantly decreased by FSH (**Figure 2.11**; n=4; $p<0.05$). In a separate experiment, lower doses of FSH (0.1, 0.5, 1, 5, 10 ng/ml) had no effect on KL mRNA expression in granulosa cells from 6-8 mm follicles

(data not shown). In contrast, KL mRNA expression in 9-16 mm granulosa cells was increased by FSH (data not shown; n=5; $p<0.05$).

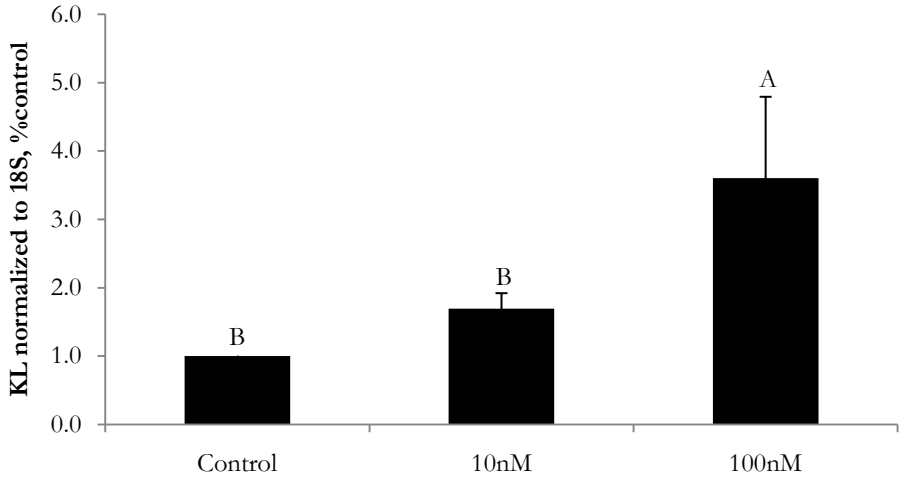

Figure 2.9 KL mRNA expression after VD3 treatment in 6-8 mm granulosa cells

KL expression is significantly increased by treatment with 100 nM VD3, n=5-6, $p<0.05$.

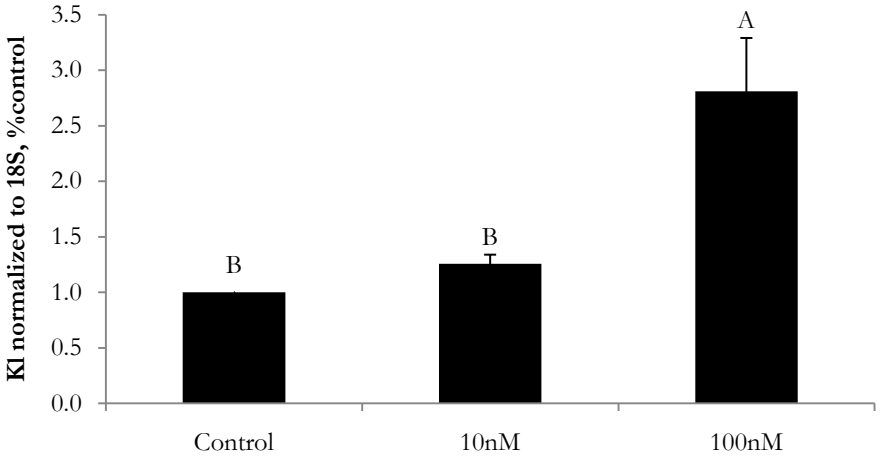

Figure 2.10 KL mRNA expression after VD3 treatment in 3-5 mm granulosa cells

KL expression is significantly increased by treatment with 100 nM VD3, n=4-5, $p<0.01$.

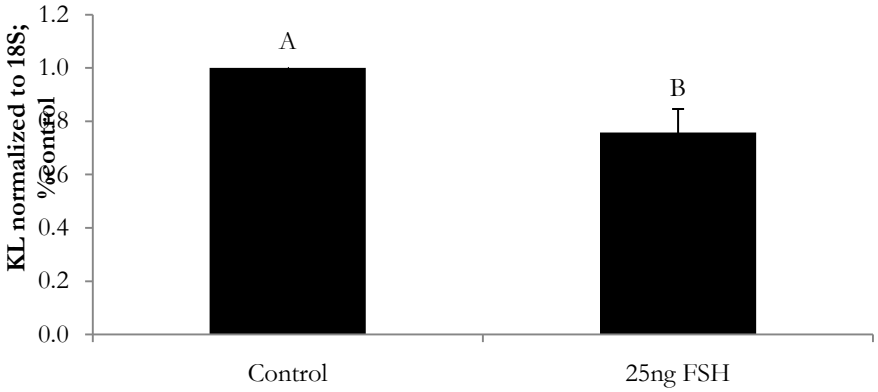

Figure 2.11 KL mRNA expression after FSH treatment in granulosa cells from 6-8 mm follicles

KL expression is significantly decreased by treatment with FSH (25 ng/ml), n=4, $p<0.05$.

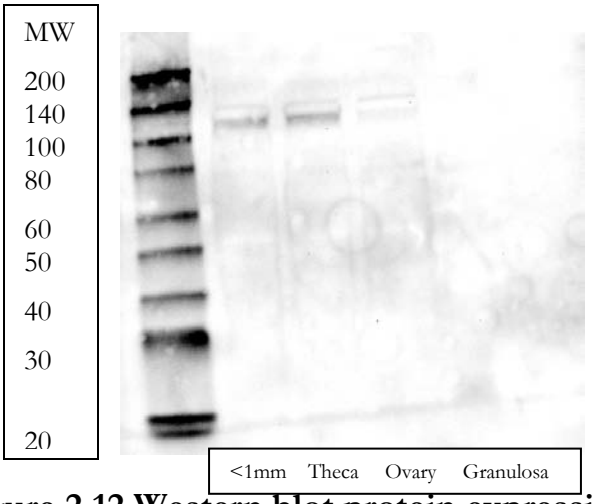

Figure 2.12 Western blot protein expression of cKit

Protein bands are seen at the expected sizes of 125 kDa (precursor) and 140 kDa (mature) in <1 mm follicles, theca (3-5 mm follicles), and ovarian body.

Protein Expression of cKit

A representative western blot shows cKit protein expression in very small (<1 mm) follicles, the theca layer from 3-5 mm follicles, and ovarian body (**Figure 2.12**). Two bands were found at the expected sizes: 125 kDa (precursor) and 140 kDa (mature). cKit protein expression was not found in the granulosa layer from the F1 follicle.

Kit System Regulation of Steroid Synthesis

Progesterone (P4) production by cultured F1 (**Figure 2.13**; n=4; $p<0.01$) and F3/4 (data not shown, n=4; $p<0.05$) granulosa cells was significantly inhibited by treating with 10% OCM. In both experiments, this effect was blocked by pre-incubating the OCM with an antibody to cKit, thought to bind soluble cKit present in the OCM.

Estradiol (E2) production by cultured pieces of theca from 6-8 mm follicles was increased by OCM in a dose-related way (**Figure 2.14**; n=5; $p<0.05$). Addition of cKit antibody in an attempt to block the effect was not successful. Additionally, pre-incubation of the OCM with cKit antibody further increased estradiol production to the level found with 50% OCM.

In another experiment, E2 production from 6-8 mm theca pieces was not affected by treatment with KL at a variety of doses (**Figure 2.15**; n=4).

Proliferation

Various doses of KL had no effect on proliferation of cultured granulosa cells from 6-8 mm follicles (data not shown; n=3).

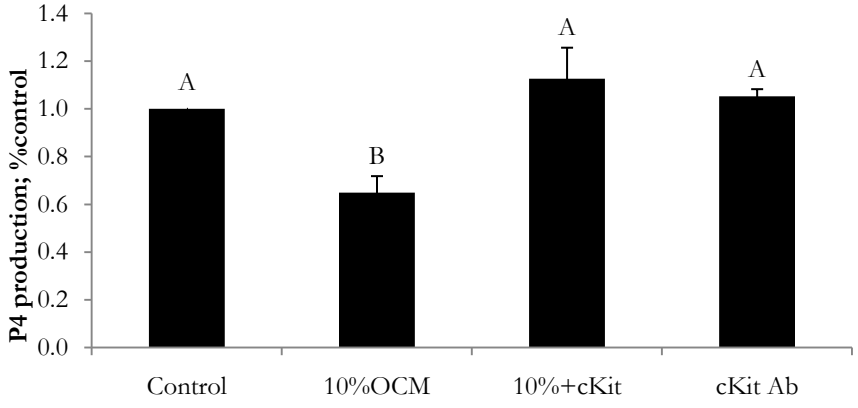

Figure 2.13 P4 production by F1 granulosa cells after OCM treatment
P4 production is significantly decreased by treatment with OCM. Preincubation with cKit antibody blocks the effect, n=4, $p<0.01$.

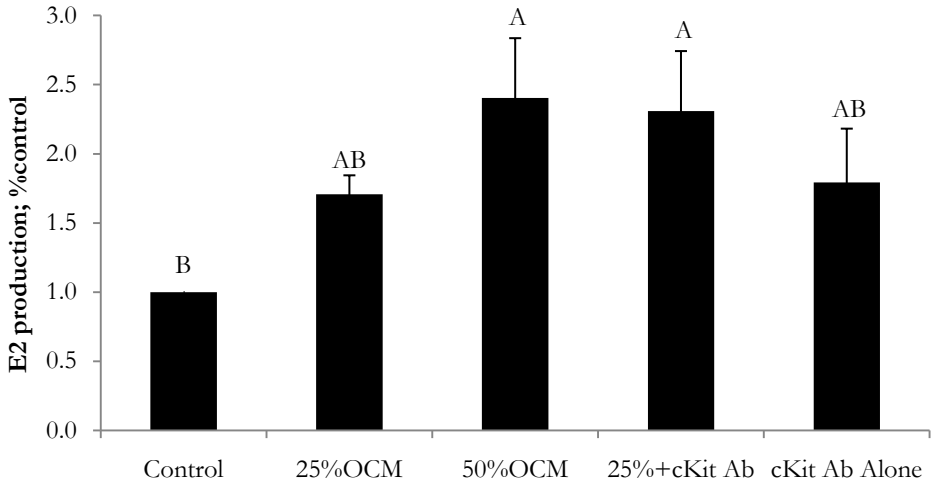

Figure 2.14 E2 production by 6-8 mm theca cells after OCM treatment
E2 production is significantly increased by treatment with 50% OCM, $p<0.05$. Pre-incubation of 25% OCM with cKit Ab increases E2 production to levels seen with 50% OCM treatments, n=5.

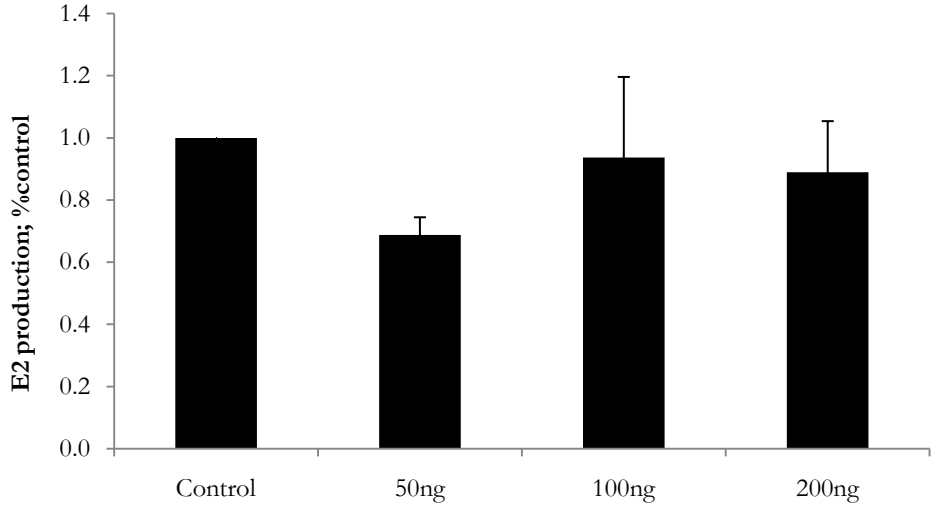

Figure 2.15 E2 production by 6-8 mm theca cells after KL treatment
E2 production is not affected by treatment with KL at any dose, n=4.

1.4 DISCUSSION

To our knowledge, this is the first demonstration of a quantified pattern of KL expression in the hen ovary throughout follicle development.

This is also the first demonstration of cKit protein expression in the ovary of the hen. Previous data have shown downregulation of KL by HB-EGF in the adult hen (Wang, 2007), but have not reported patterns of expression during follicle development or regulation by other factors. KL may have a role in regulating the progression of follicles through the highly organized hierarchy seen in the hen ovary. This is suggested by high levels of expression seen in the small follicles and lower levels seen in the selected follicles. Data from several mammalian studies have indicated the importance of KL in follicle recruitment from the resting primordial follicle pool (mouse: Yoshida *et al.*, 1997; hamster: Wang and Roy, 2004; cow: Parrott and Skinner, 1997; Muruvi and Fortune, 2009; goat: Celestino *et al.*, 2010). Our quantitative PCR analysis showed the highest KL mRNA in very small intact follicles (<1 mm) and the granulosa layer from 3 mm follicles (Figure 1). GD versus NGD granulosa KL expression was investigated based on studies in the mouse that found differences in expression between cumulus (low) and mural (high) granulosa cells in antral follicles. Although the present study showed no difference in KL mRNA expression between GD and NGD granulosa from follicles of size 6 mm and larger, future efforts are needed in order to verify that there is no difference between GD and NGD granulosa cell KL mRNA in smaller follicles.

Protein expression of cKit was shown to be present in the oocytes of very small follicles (<1 mm), in addition to the theca cell layer from 3-5 mm follicles and in ovarian body. No protein bands were detected in the granulosa cell layer isolated from the F1 follicle. This pattern of expression matches what has been found in mammals (mice: Motro and Bernstein, 1993; Horie *et al.*, 1991; sheep: Tisdall *et al.*, 1999; goats: Silva *et al.*, 2006;

pigs: Brankin *et al.*, 2004; humans: Horie *et al.,* 1991; Carlsson *et al.,* 2006; cattle: Parrott and Skinner, 1997) with high levels of expression in theca and oocytes from preantral, growing follicles. In the hen, follicles approximately 8 mm in size or less represent the growing follicles, prior to follicle selection (Woods and Johnson, 2005).

Based on previous reports in the mouse (Joyce *et al.*, 1999; Joyce *et al.*, 2000), we examined whether the oocyte may affect the expression of KL mRNA. Earlier work in the chicken showed that OCM treatments of granulosa cells from 6-8 mm follicles significantly decreased AMH mRNA expression (Johnson *et al.*, 2008) and that GDF9 protein is present in OCM (Johnson *et al.*, 2005). In the current study, we found upregulation of KL mRNA expression by OCM in granulosa cells from 6-8 mm follicles. Treatment of 6-8 mm granulosa cells with high MW OCM increased KL mRNA, which leads us to believe the oocyte-derived factor involved in stimulation of KL has a weight greater than 30 kDa. We also used heat treatment of OCM to verify that the effective component(s) in OCM was/were protein. With this in mind, we performed further granulosa cell culture experiments using OCM pre-incubated with antibodies against cKit (to bind soluble cKit; 145 kDa), BMP15 (38 kDa), and GDF9 (32 kDa); all three antibodies detected chicken proteins at the appropriate sizes by western blot in our lab (GDF9: Johnson *et al.*, 2005). These attempts to block the effect of OCM were unsuccessful; the results are outlined below (**Table 2.1**). The inability of these antibodies to block the effects of OCM on KL mRNA expression could be due to insufficient antibody concentrations, since the concentrations of factors found in OCM are

Table 2.1 Potential Oocyte Factors involved in the Regulation of KL

Oocyte Factor	Experiment	Results: Effect on KL mRNA
BMP15	OCM+BMP15 Ab; BMP15 treatments	No effect
GDF9	OCM+GDF9 Ab	No effect
cKit	OCM+cKit Ab	No effect
EGF	EGF treatments	No effect
Vitamin D3	VD3 treatments	Increased
E2, P4, T	Steroid treatments	No effect

unknown. Alternatively, the bioactivity in the OCM could be due to an untested factor.

VD3 has been shown to significantly decrease anti-Müllerian hormone (AMH) mRNA expression and significantly increase follicle stimulating hormone receptor (FSHR) mRNA expression in hen granulosa cells from 6-8 mm follicles (Wojtusik and Johnson, 2010). This suggested to us that it may modulate other factors involved in follicle selection and development. In the current study, a high dose of VD3 stimulated KL mRNA expression in 3-5 and 6-8 mm granulosa cells. This was the only example we found of steroid regulation of KL expression. Furthermore, mRNA expression for the VD3 receptor (VDR) has been found in very small follicles (<2 mm; Wojtusik and Johnson, 2010), so it is possible that a portion of OCM effects on KL expression can be attributed to regulation by VD3, although this is unlikely since VD3 would have been in the low MW portion of OCM (**Table 2.1**). The influence of VD3 on KL, AMH, and

FSHR indicates its importance in regulating factors involved in follicle development, although the exact mechanism of such actions is currently unknown.

We investigated regulation of KL by FSH based on previous reports in the cow (Parrott and Skinner, 1998) and mouse (Joyce *et al.*, 1999). These studies demonstrated an increase of KL mRNA by FSH, while the current study found decreased KL mRNA expression in 6-8 mm granulosa cells with FSH treatment. However, we did find an increase in KL mRNA in 9-16 mm granulosa cells by FSH. These results suggest a differential effect of FSH with increased follicle differentiation, although this is difficult to reconcile because FSHR mRNA is reported to be at similar levels in follicles of 6-8 and 9-16 mm (Woods and Johnson, 2005).

Granulosa cell progesterone production was examined in large (F1, F3/4) follicles because they produce high levels of progesterone while theca cells from small follicles are the source of estradiol synthesis (Johnson, 1996). The ability of the cKit antibody to block the inhibitory effect of OCM on progesterone production suggests the existence of a soluble cKit isoform in OCM that binds and biologically activates KL. This possibility is backed by the data showing the existence of soluble cKit in human follicular fluid (Tanikawa *et al.*, 1998) and human endothelial cells (Broudy *et al.*, 1994). These results suggest that the Kit system exhibits regulatory actions on steroid production from hierarchal follicles. To our knowledge, this is the first report on the function of the Kit system in the ovary of an oviparous species. This result, along with regulation of KL is depicted more clearly in **Figure 2.16**.

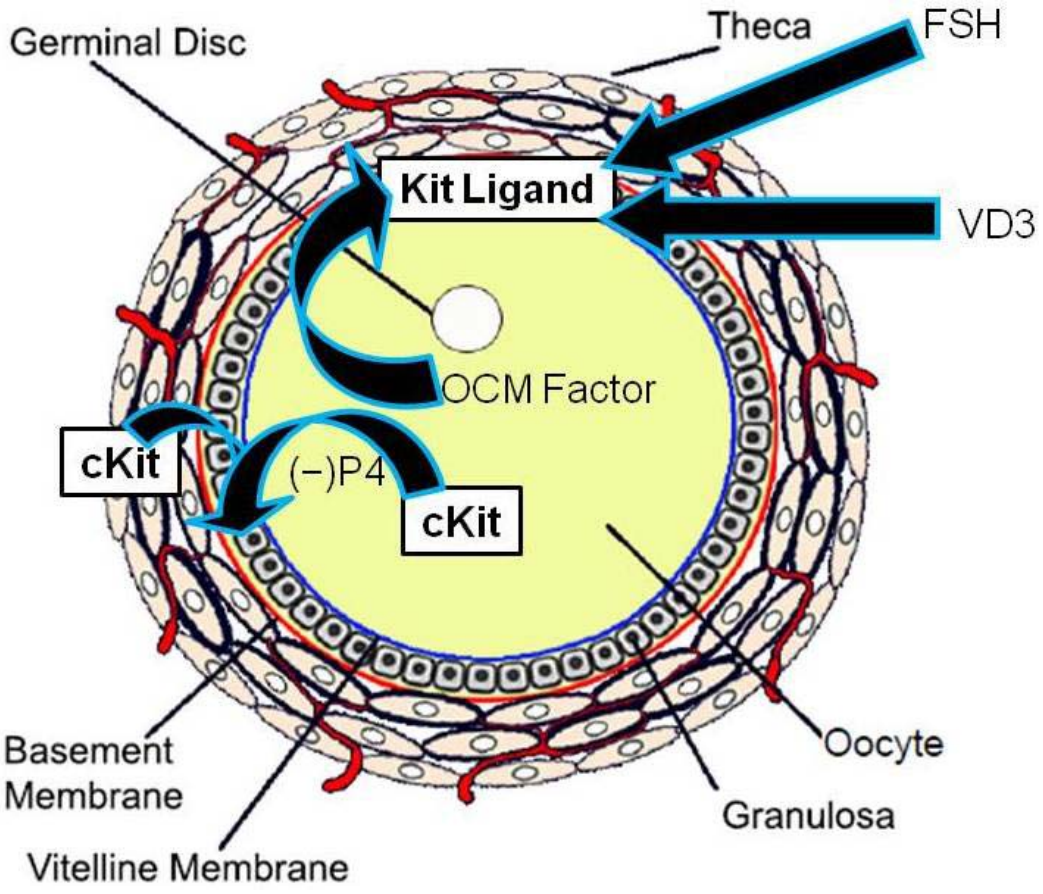

Figure 2.16 Actions of the Kit System in the hen ovary

The capability of the cKit antibody to block the effect of OCM on progesterone suggested cKit may be involved in the stimulatory effect of OCM on estradiol production by the theca layer from 6-8 mm follicles. This hypothesis is backed by literature suggesting regulatory effects of the Kit system in increasing P450 aromatase mRNA and protein expression (for a subsequent increase in E2 production) in the rat (Jin *et al.*, 2005). Instead of blocking the effect, however, pre-incubation of the cKit antibody with 25% OCM resulted in an additional increase in estradiol production. This suggests that the antibody could neutralize the biological activity of soluble and thecal cKit, which might otherwise act to decrease estradiol production.

This could be an indirect effect following Kit system regulation of another factor, such as testosterone or aromatase. In a different approach to examine Kit system regulation of estradiol in the hen ovary, we treated 6-8 mm theca cultures with KL, but estradiol production was unaffected.

KL did not increase proliferation of granulosa cells from 6-8 mm follicles, which contrasts with the proliferative effect of the Kit system on haemopoeitic cells (Hayman *et al.*, 1993) and melanocytes (Lahav *et al.*, 1994). Together with the finding that KL did not affect E2 production, these results may mean that recombinant mouse KL is not bioactive in the chicken ovary.

Unfortunately, we were unable to determine protein expression of KL because there was no available antibody against chicken and other available antibodies were not recommended for use because of low homology with chicken KL. Additionally, despite running various immunohistochemistry protocols and acquiring and using two separate lots of the cKit antibody (M14), we were unable to demonstrate specific localization of cKit protein in the ovarian follicle, as only autofluorescence was detectable.

In interpreting the results from the present study, we can conclude that OCM contains one or more heat-labile, bioactive substances that play a role in regulating follicle development. Although it is likely that this substance is oocyte-derived, it is important to point out that in the culturing of OCM, the entire follicle is present including granulosa and theca cells, so factors derived from these cells may be having effects of their own.

Unfortunately, our study of the Kit system in the hen ovary was impeded by the lack of available bioactive reagents for use in the hen. Perhaps development of recombinant chicken KL as well as development of

specific antibodies against chicken cKit and KL, would alleviate the problems we had. We cannot rule out the possibility that GDF9, BMP15 or cKit have a role in regulating the Kit system, because we cannot be sure if our reagents were able to block the actions of the bioactive proteins found in OCM, due to a mismatch between antibody and OCM concentrations. On the other hand, the factor in OCM having effects on KL and estradiol may not be one of these proteins, and regulatory actions of other untested factors should be considered.

Future experiments examining the function and regulation of cKit and KL in the ovary of the hen are necessary for a more complete understanding of follicle development. The relationship between the Kit system and AMH would be interesting to study, given the contrasting roles they have in follicle recruitment (Yoshida *et al.*, 2007; Durlinger *et al.*, 1999; Durlinger *et al.*, 2002). Additionally, we know that VD3 has opposite effects on mRNA expression of KL and AMH, so it is possible that there could be an interaction. It would also be interesting to determine whether the Kit system is involved in the regulation of other steroids, such as testosterone, or regulation of other ovarian factors including GDF9 and BMP15.

REFERENCES

Brankin V, Hunter MG, Horan TL, Armstrong DG, Webb R. The expression patterns of mRNA-encoding stem cell factor, internal stem cell factor and KIT in the prepubertal and adult porcine ovary. J Anatomy. 2004; 205:393-403.

Broudy VC, Kovach NL, Bennett LG, Lin N, Jacobsen FW, Kidd PG. Human umbilical vein endothelial cells display high-affinity c-kit receptors and produce a soluble form of the c-kit receptor. Blood. 1994; 83:2145-2152.

Carlsson IB, Laitinen MPE, Scott JE, Louhio H, Velentzis L, Tuuri T, Aaltonen J, Ritvos O, Winston RML, Hovatta O. Kit ligand and c-Kit are expressed during early human ovarian follicular development and their interaction is required for the survival of follicles in long-term culture. Reproduction. 2006; 131:641-649.

Carnahan JF, Patel DR, Miller JA. Stem cell factor is a neurotrophic factor for neural crest-derived chick sensory neurons. J Neurosci. 1994; 14:1433-1440.

Celestino JHH, Bruno JB, Lima-Verde IB, Matos MHT, Saraiva MVA, Chaves RN, Martins FS, Almeida AP, Cunha RMS, Lima LF, Name KPO, Campello CC, Silva JRV, Báo SN, Figueiredo JR. Steady-state level of kit ligand mRNA in goat ovaries and the role of kit ligand in preantral follicle survival and growth in vitro. Mol Reprod Dev. 2010; 77:231-240.

Davis AJ, Brooks CF, Johnson PA. Gonadotropin regulation of inhibin alpha-subunit mRNA and immunoreactive protein in cultured chicken granulosa cells. Gen Comp Endocrinol. 1999; 116:90-103.

di Clemente N, Wilson C, Faure E, Boussin L, Carmillo P, Tizard R, Picard J-Y, Vigier B, Josso N, Cate R. Cloning, expression, and alternative splicing of the receptor for anti-Müllerian hormone. Mol Endocrinol.

1994b; 8:1006-1020.

Durlinger ALL, Gruijters MJG, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JT, Grootegoed JA, Themmen APN. Anti-Müllerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology. 2002; 143:1076-1084.

Durlinger AL, Kramer P, Karels B, de Jong F, Uilenbroek JT, Grootegoed JA, Themmen AP. Control of primordial follicle recruitment by anti-Müllerian hormone in the mouse ovary. Endocrinology. 1999; 140:5789-5796.

Gilbert AB, Perry MM, Waddington D, Hardie MA. Role of atresia in establishing the follicular hierarchy in the ovary of the domestic hen (Gallus domesticus). J Reprod Fertil. 1983; 69:221-227.

Hayman MJ, Meyer S, Martin F, Steinlein P, Beug H. Self-renewal and differentiation of normal avian erythroid progenitor cells: regulatory roles of the TGF-α/*c-ErbB* and SCF/c-*kit* receptors. Cell. 1993; 74:157-169.

Horie K, Takakura K, Taii S, Narimoto K, Noda Y, Nishikawa S, Nakayama H, Fujita J, Mori T. The expression of c-kit protein during oogenesis and early embryonic development. Biol Reprod. 1991; 45:547-552.

Hoyer PE, Byskov AG, Mollgard K. Stem cell factor and c-Kit in human primordial germ cells and fetal ovaries. Mol Cell Endocrinol. 2005; 234:1-10.

Huang CTF, Weitsman SR, Dykes BN, Magoffin DA. Stem cell factor and insulin-like growth factor-I stimulate luteinizing hormone-independent differentiation of rat ovarian theca cells. Biol Reprod. 2001; 64:451-456.

Huang EJ, Nocka KH, Buck J, Besmer P. Differential expression and processing of two cell associated forms of the kit-ligand: KL-1 and

KL-2. Mol Biol Cell. 1992; 3:349-362.

Hutt KJ, McLaughlin EA, Holland MK. Kit ligand and c-Kit have diverse roles during mammalian oogenesis and folliculogenesis. Mol Human Reprod. 2006; 12:61-69.

Ismail RS, Okawara Y, Fryer JN, Vanderhyden BC: Hormonal regulation of the ligand for c-kit in the rat ovary and its effects on spontaneous oocyte meiotic maturation. Mol Reprod Dev. 1996; 43:458-469.

Jin X, Han CS, Zhang XS, Yu FQ, Guo SH, Hu Z, Liu YX. Stem cell factor modulates the expression of steroidogenesis related proteins and FSHR during ovarian follicular development. Front Biosci. 2005; 10:573-1580.

John GB, Shidler MJ, Besmer P, Castrillon DH. Kit signaling via PI3K promotes ovarian follicle maturation but is dispensible for primordial follicle activation. Dev Biol. 2009; 331:292-299.

Johnson PA, Dickens MJ, Kent TR, Giles JR. Expression and function of growth differentiation factor-9 in an oviparous species. Gallus domesticus. Biol Reprod. 2005; 72:1095-1100.

Johnson PA, Kent TR, Urick ME, Giles JR. Expression and regulation of anti-Müllerian hormone in an oviparous species, the hen. Biol Reprod. 2008; 78:13-19.

Johnson PA, Kent TR, Urick ME, Trevino LS, Giles JR. Expression of anti-Müllerian hormone in hens selected for different ovulation rates. Reproduction. 2009; 137:857-863.

Joyce IM, Clark AT, Pendola FL, Eppig JJ. Comparison of recombinant growth differentiation factor-9 and oocyte regulation of KIT ligand messenger ribonucleic acid expression in mouse ovarian follicles. Biol Reprod. 2000; 63:1669-1675.

Joyce IM, Pendola FL, Wigglesworth K, Eppig JJ. Oocyte regulation of kit ligand expression in mouse ovarian follicles. Dev Biol. 1999; 214:342-353.

Lahav R, Lecoin L, Ziller C, Nataf V, Carnahan JF, Martin FH, *et al.* Effect of the Steel gene product on melanogenesis in avian neural crest cell cultures. Differentiation. 1994; 58:133-139.

Laitinen M, Rutanen E, Ritvos O. Expression of c-kit ligand messenger ribonucleic acids in human ovaries and regulation of their steady state levels by gonadotropins in cultured granulosa luteal cells. Endocrinology. 1995; 136:4407-4414.

Lemmon MA, Pinchasi D, Zhou M, Lax I, Schlessinger J. Kit receptor dimerization is driven by bivalent binding of stem cell factor. J Biol Chem. 1997; 272:6311-6317.

Muruvi W, Fortune JE. Kit ligand-KIT Interaction is required for follicle activation and mediates insulin-stimulated activation of bovine primordial follicles in vitro. Biol Reprod. 2009; 81:200.

Motro B, Bernstein A. Dynamic changes in ovarian c-kit and Steel expression during the estrous reproductive cycle. Dev Dyn. 1993; 197:69-79.

Nilsson E, Rogers N, Skinner MK. Actions of anti-Müllerian hormone on the ovarian transcriptome to inhibit primordial to primary follicle transition. Reproduction. 2007; 134:209-221.

Otsuka F, Shimasaki S. A negative feedback system between oocyte bone morphogenetic protein 15 and granulosa cell kit ligand: its role in regulating granulosa cell mitosis. Proc Natl Acad Sci USA. 2002; 99:8060-8065.

Parrott JA, Skinner MK. Direct actions of kit-ligand on theca cell growth and differentiation during follicle development. Endocrinology. 1997;

138:3819-3827.

Parrott JA, Skinner MK. Thecal cell-granulosa cell interactions involve a positive feedback loop among keratinocyte growth factor, hepatocyte growth factor, and kit ligand during ovarian follicular development. Endocrinology. 1998; 139:2240-2245.

Reddy P, Shen L, Ren C, Boman K, Lundin E, Ottander U, Lindgren P, Liu YX, Sun QY, Liu K . Activation of Akt (PKB) and suppression of FKHRL1 in mouse and rat oocytes by stem cell factor during follicular activation and development. Dev Biol. 2005; 281:160-170.

Robinson FE, Etches RJ. Ovarian steroidogenesis during follicular maturation in the domestic fowl (*Gallus domesticus*). Biol Reprod. 1986; 35:1096-1105.

Sasaki E, Okamura H, Chikamune T, Kanai Y, Watanabe M, Naito M, Sakurai M. Cloning and expression of the chicken c-*kit* proto-oncogene. Gene. 1993; 128:257-261.

Silva JR, van den Hurk R, van Tol HT, Roelen BA, Figueiredo JR. The Kit ligand/c-Kit receptor system in goat ovaries: gene expression and protein localization. Zygote. 2006; 14:317-328.

Tanikawa M, Harada T, Mitsunari M, Onohara Y, Iwabe T, Terakawa N. Expression of c-kit messenger ribonucleic acid in human oocyte and presence of soluble c-kit in follicular fluid. J Clin Endocrinol Metab. 1998; 83:1239-1242.

Thomas FH, Ethier JF, Shimasaki S, Vanderhyden BC. Follicle-stimulating hormone regulates oocyte growth by modulation of expression of oocyte and granulosa cell factors. Endocrinology. 2005; 146:941-949.

Tisdall DA, Fidler AE, Smith P, Quirke LD, Stent VC, Heath DA, McNatty KP. Stem cell factor and c-kit gene expression and protein localization in the sheep ovary during fetal development. J Reprod Fertil. 1999;

116:277-291.

Van't Hof RJ, von Lindern M, Nijweide PJ, Beug H. Stem cell factor stimulates chicken osteoclast activity *in vitro*. FASEB J. 1997; 11:287-293.

Wang J, Roy SK. Growth differentiation factor-9 and stem cell factor promote primordial follicle formation in the hamster: modulation by follicle-stimulating hormone. Biol Reprod. 2004; 70:577-585.

Wang Y, Li J, Ying Wang C, Yan Kwok AH, Leung FC. Epidermal growth factor (EGF) receptor ligands in the chicken ovary: I. Evidence for heparin-binding EGF-like growth factor (HB-EGF) as a potential oocyte-derived signal to control granulosa cell proliferation and HB-EGF and kit ligand expression. Endocrinology. 2007; 148:3426-3440.

Wojtusik J, Johnson PJ. Vitamin D3 regulates anti-Müllerian hormone in granulosa cells of the hen. Biol Reprod. 2010, 614.

Woods DC, Johnson AL. Regulation of follicle-stimulating hormone-receptor messenger RNA in hen granulosa cells relative to follicle selection. Biol Reprod. 2005; 72:643-650.

Yoshida H, Takakura N, Kataoka H, Kunisada T, Okamura H, Nishikawa SI. Stepwise requirement of c-kit tyrosine kinase in mouse ovarian follicle development. Dev Biol. 1997; 184:122-137.

Zhou, JH, Ohtaki, M, Sakurai, M. Sequence of a cDNA encoding chicken stem cell factor. Gene. 1993; 127:269-270.

CHAPTER 3
ANTI-MÜLLERIAN HORMONE REGULATION OF FOLLICLE STIMULATING HORMONE RECEPTOR IN THE HEN OVARY

3.1 INTRODUCTION

Anti-Müllerian hormone (AMH, also known as Müllerian inhibiting substance, MIS), named for its role in regressing the Müllerian duct system in male mammals, has the same function in male birds, in addition to regressing the right Müllerian duct in female birds (Tran and Josso, 1977). Estrogen produced by the left ovary of birds protects the left Müllerian duct from regression, thereby permitting development of the left oviduct (Teng, 2000; Teng, 2001). Anti-Müllerian hormone has a well-defined function during embryonic development in mammals and birds but the function of this hormone in adult females is less clear.

AMH is produced by the granulosa layer of growing follicles in females and is believed to be important in regulating the rate of recruitment from the primordial follicle pool as well as in regulating follicle stimulating hormone (FSH) sensitivity (Durlinger *et al.*, 1999; Durlinger *et al.*, 2001). In mice, expression of AMH mRNA or protein is not seen in primordial follicles, oocytes or theca, and is first detected in granulosa cells of primary follicles (Ueno *et al.*, 1989; Baarends *et al.*, 1995; Durlinger *et al.*, 2002). AMH is highest in preantral and small antral follicles, with greater expression in granulosa cells surrounding the oocyte and antrum, as compared to the mural granulosa cells, possibly reflecting functional differences between the cells (Ueno *et al.*, 1989; Baarends *et al.*, 1995; Durlinger *et al.*, 2002). AMH knock-out studies indicate the importance of AMH in normal ovarian

function. Female knock-out mice appear normal and are fertile, (Behringer *et al.*, 1994) but their ovaries exhibit greatly increased numbers of small, growing follicles, leading to premature depletion of primordial follicles (Durlinger *et al.*, 1999). These findings were supported by *in vitro* studies showing that murine ovaries cultured with AMH had 40% fewer growing follicles and lower α-inhibin mRNA expression (Durlinger *et al.*, 2002).

The sequence of chicken AMH is 52% similar to mammalian AMH in the biologically active C-terminal region. In the female chick embryo, expression of AMH in the developing left ovary was found to gradually increase to its highest level at day 14 then decline to close to baseline by the day before hatching (Teng *et al.*, 1987). Characterization of the expression pattern of AMH throughout follicle development in the hen showed that granulosa cell mRNA for AMH is expressed at high levels in the small growing follicles but is decreased in the large hierarchical follicles (Johnson *et al.*, 2008; Johnson *et al.*, 2009). Interestingly, the decline in AMH expression with increasing follicle size is associated with an increase in expression of the FSH receptor (FSHR) in the granulosa layer, a relationship which may regulate orderly follicle selection (Visser and Themmen, 2005). FSHR expression increases around the time of selection of a follicle into the preovulatory hierarchy of the hen (Woods and Johnson, 2005).

In the mouse, it was discovered that *in vitro* preantral follicle growth stimulated by FSH treatment was reduced with addition of AMH (Durlinger *et al.*, 2001). Furthermore, AMH-deficient mice given injections of FSH had higher numbers of growing follicles when compared to wild-type mice, demonstrating the inhibitory regulation by AMH on follicle activation (Durlinger *et al.*, 2001). The attenuation of FSH actions by AMH has also

been demonstrated in the rat ovary. FSH-stimulated LHR mRNA and aromatase activity in cultured rat granulosa cells was blocked with the addition of AMH. This is interesting because LHR and aromatase are markers of granulosa cell differentiation (di Clemente *et al.*, 1994). As this is a post-activation role, this suggests that AMH suppresses follicle recruitment in addition to follicle activation. Furthermore, microarray analysis of AMH-treated rat ovaries showed that AMH downregulated the TGFβ pathway which signals for processes including cell differentiation, angiogenesis and cell cycle regulation (Nilsson *et al.*, 2007). This study also showed that the stimulatory actions of kit ligand (KL) on the transition from primordial to primary follicles in whole ovary cultures was attenuated with addition of AMH to the culture treatments (Nilsson *et al.*, 2007).

With regard to the regulation of AMH in the hen ovary, it has been shown that AMH is not regulated *in vitro* by glucose or insulin, factors related to metabolic state (Johnson *et al.*, 2009). Additionally, expression of AMH mRNA was not influenced by estradiol or progesterone (Johnson *et al.*, 2008). Little is known with respect to the function of AMH in the hen ovary. Bioactive AMH, shown to be present in testis-conditioned medium (TCM) by western blot analysis, induced cell proliferation that was blocked when the TCM was preincubated with an antibody to AMH (Johnson *et al.*, 2009). As knowledge of AMH regulation and function in the hen ovary is limited to these few studies, there is a need for further investigation of its actions and regulation in an oviparous species. The objective of the present study was to determine if AMH has a direct effect on mRNA expression of FSHR in follicles before or after selection.

3.2 MATERIALS AND METHODS

Animals

Laying hens (SCWL Babcock B300 strain), aged between 22 and 74 weeks were housed individually. They had free access to feed and water and were exposed to 15 h light: 9 h darkness per day, with lights on at 0600 h. Individual egg laying was recorded daily. Hens with regular laying patterns were selected and euthanized at 1-2 h after oviposition in order to collect tissue samples. All procedures were approved by the IACUC of Cornell University.

Testis Conditioned Media (TCM)

TCM was produced by collecting testes from chick embryos aged between 12 and 14 days (di Clemente *et al.*, 1992). Two testes were cultured in a tube for 3 days in a humidified atmosphere containing 5% CO_2 at 37°C with 0.5 ml Medium 199 plus 0.1% BSA. At termination, the supernatant was collected, filter-sterilized (0.2 μm membrane) and frozen until many preparations were pooled together for use in culture (Johnson *et al.*, 2009). Western blot analysis confirmed the presence of AMH in the medium (shown in Johnson *et al.*, 2008).

Granulosa Cell Culture

Granulosa cells from laying hen follicles (3-5 mm and 9-16 mm) were isolated and dispersed as described previously (Davis *et al.*, 1999). Cell number and viability (>95%) were estimated with a hemocytometer. Cells were plated in 6-well plates (in M199 plus 5% FBS) at a density of $3x10^6$ cells/well in 1.5 ml volume and then incubated as described previously (Johnson *et al.*, 2005). After 24 h, medium was replaced and cells were

cultured for an additional 24 h in the presence of control medium (M199 plus 0.1% BSA), 25% TCM, or 50% TCM by volume. In some experiments, an antibody against AMH (#4055-2, Genex Bioscience Inc., Hayward, CA) in a 1:100 dilution in M199 + 0.1% BSA was incubated 1:1 by volume with TCM for 30 minutes at room temperature before use. To determine cell proliferation, granulosa cells were plated in 96-well plates at a density of $1.2x10^5$ cells/well in 100 μl of medium and cultured as described. The number of viable cells was quantified using the Aqueous One Solution Cell Proliferation Assay (Promega) according to manufacturer instructions. Visual inspection of plate confluence confirmed proliferation. After culture, RNA was extracted from the granulosa cells for evaluation of RNA expression by quantitative PCR (Qiagen).

PCR Analysis

Chicken-specific probes and primers for analysis of FSHR expression (70bp product; GenBank accession #U51097) were derived using ABI Primer Express Software as described previously (Johnson *et al.*, 2008). TaqMan ribosomal RNA 18S primers and probe were the control reagents, and a control reaction for FSHR normalization was run for each sample. A standard curve was created using serial dilutions of cDNA transcribed from a pool of granulosa cell RNA prepared from 6-8 mm follicles. Control reactions lacking template and reverse transcriptase were also performed in parallel on each plate. Relative RNA was determined using the C_t method as calculated by the ABI Prism 7000 Sequence Detection System Software and the mean value for duplicate PCR amplifications was calculated relative to 18S reactions.

Theca Cell Culture

The theca cell layer was isolated from 3-5 mm and 6-8 mm follicles. For each repetition, the theca shells from one hen were pooled and cut up into small pieces. Theca pieces were weighed and approximately equal weights (18-23 mg) were allocated to wells in a 24-well plate with 0.5 ml of media. Control treatments were M199 plus 0.1% BSA; other treatments were 25% or 50% TCM by volume. After 24 h of incubation at 37°C in a humidified atmosphere of 5% CO_2, medium was collected and frozen.

Radioimmunoassay

Culture media were collected and assayed to determine granulosa cell progesterone production or theca cell estradiol production. Steroid hormone concentrations were estimated using Coat-A-Count radioimmunoassay kits for estradiol and progesterone (Diagnostics Product Corporation, Los Angeles, CA). The analytical sensitivity of the kits is 8.0 pg/ml for estradiol and 0.02 ng/ml for progesterone. For both assays, plasma from immature roosters was used as a low pool and plasma from ovulating hens treated with exogenous progesterone was used as a high pool. Standards provided in the kit were assayed in triplicate and all culture media samples were assayed in duplicate. The coefficient of variation (CV) for the progesterone assay was 0.4% and the CV for the estradiol assay was 4.7%.

Immunoneutralization of TCM

An immunoprecipitation (IP) technique was used to precipitate AMH protein out of whole TCM solution. 50 μg of AMH antibody (Genex) was incubated with TCM (volume ranging 75 μl-200 μl) overnight at 4°C with constant agitation. 100 μl of Protein A resin slurry was added to the antigen-antibody complex as the solid substrate for binding of the antibody, and the

mixture was incubated for 2 h at room temperature with constant agitation. IP buffer (TBS) was added, mixture was centrifuged for 3 minutes at 2500xg, and supernatant was removed. This was repeated three times. A reducing sample buffer (SDS, glycerol, Tris-HCL) was added to the complex-bound resin and incubated 5 minutes at 95°C. The mixture was centrifuged for 3 minutes at 2500xg to separate the resin from the immune complex in the eluate. Gel electrophoresis was then performed using whole TCM as well as the initial supernatant and the eluate, followed by transfer to a nitrocellulose membrane. The membrane was probed for AMH as previously described (Johnson *et al.*, 2008).

Statistical Analysis

Data were analyzed by the Statistical Analysis Systems (SAS) General Linear Model Procedure (Proc-GLM) with protected least-significant difference. P values of <0.05 were considered significant, and means were compared using Duncan's multiple range test. Results are presented as means $\pm$ SE, percent of control.

3.3 RESULTS

Presence of AMH in TCM

Western blot analysis confirmed the presence of immunoreactive AMH in TCM (**Figure 2.1**).

FSHR mRNA Expression

In a preliminary experiment, FSHR mRNA expression was decreased in granulosa cells from 3-5 mm follicles with 25% and 50% TCM by volume (**Figure 2.2**; $n=2$; $p<0.05$). In a subsequent series of experiments, pre-incubation of 25% TCM with an antibody against AMH did not block the

inhibition of FSHR expression (**Figure 2.3**; n=8; $p<0.05$). Similarly, using granulosa cells from 9-16 mm follicles, preliminary results demonstrated a decrease in FSHR mRNA expression when treated with 25% TCM (**Figure 2.4**; n=5; $p<0.05$; 50% TCM was not used as a treatment). In following experiments, pre-incubation of 25% TCM with an antibody against AMH did not block the inhibition of FSHR expression (**Figure 2.5**; n=8).

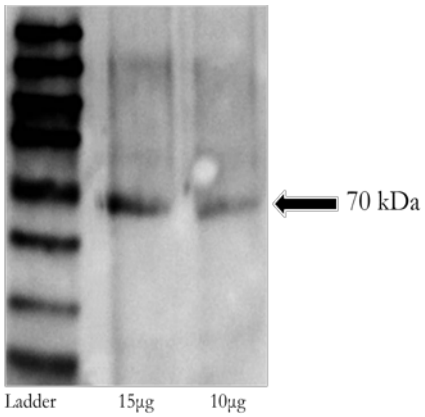

Figure 3.1 Western blot confirming presence of AMH in TCM
Protein from TCM (10 and 15 µg) was run and probed with AMH Ab. Approximate size of 70 kDa is indicated.

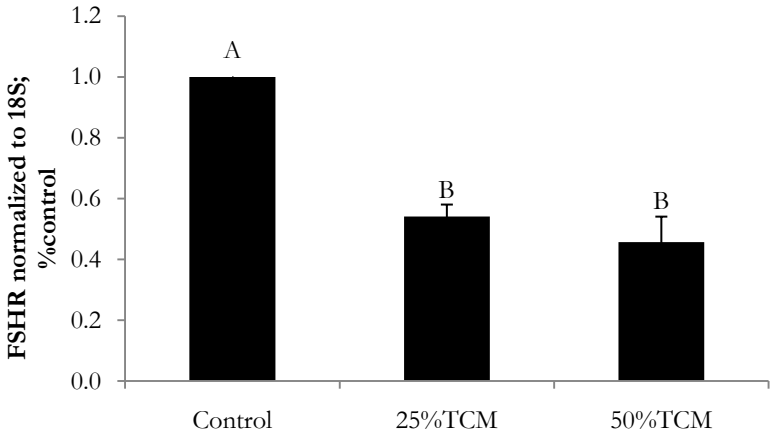

Figure 3.2 FSHR mRNA expression after TCM treatment in granulosa cells from 3-5 mm follicles
FSHR expression is decreased by treatments with TCM, n=2, $p<0.05$.

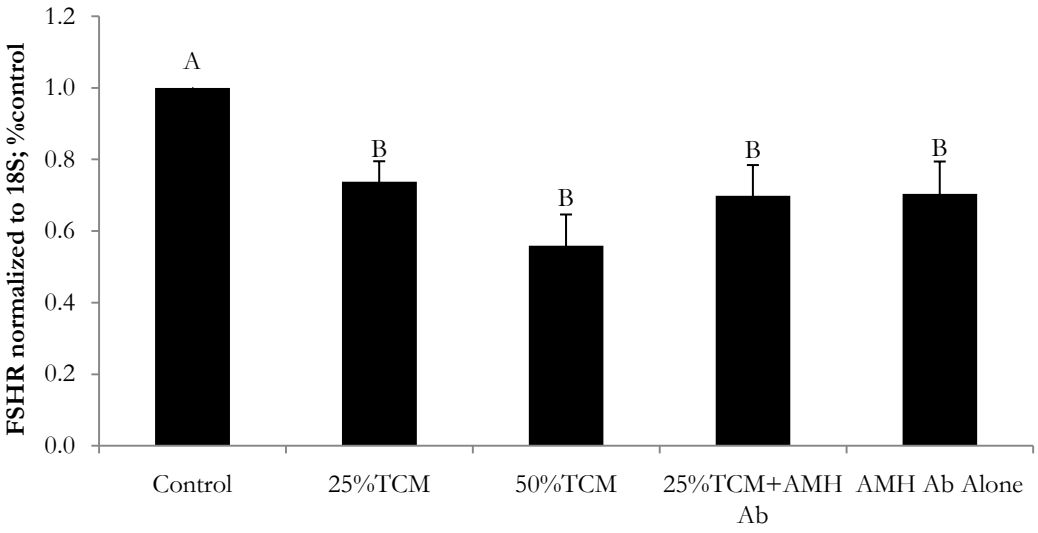

Figure 3.3 FSHR mRNA expression after TCM plus AMH Ab treatment in granulosa cells from 3-5 mm follicles

FSHR expression is significantly decreased by treatments with TCM, effect is not blocked using an AMH Ab, n=8, $p<0.05$.

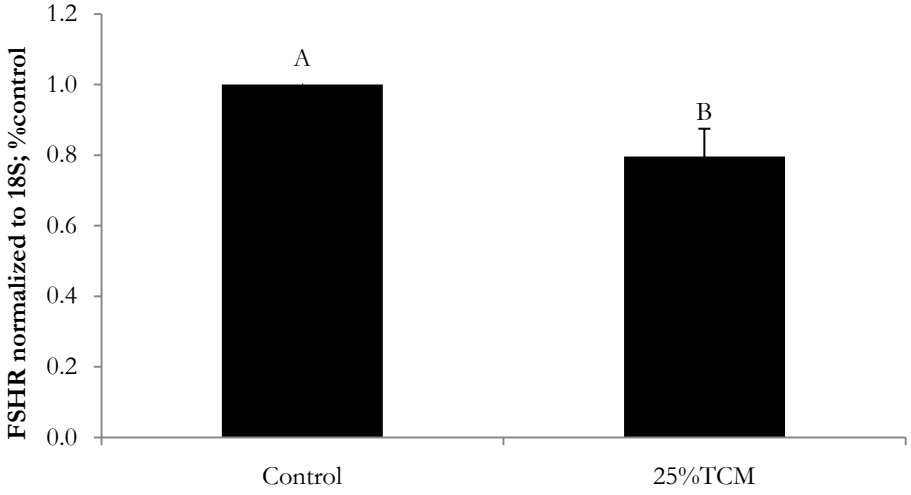

Figure 3.4 FSHR mRNA expression after TCM treatment in granulosa cells from 9-16 mm follicles

FSHR expression is decreased by treatments with TCM, n=5, $p<0.05$.

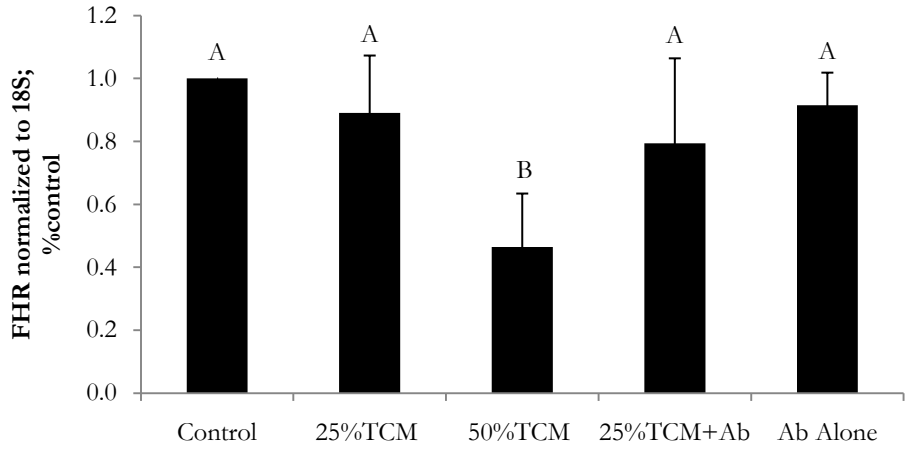

Figure 3.5 FSHR mRNA expression after TCM plus AMH Ab treatment in granulosa cells from 9-16 mm follicles

There is no difference between control, 25%TCM, or 25% TCM plus AMH Ab, n=8, $p<0.05$.

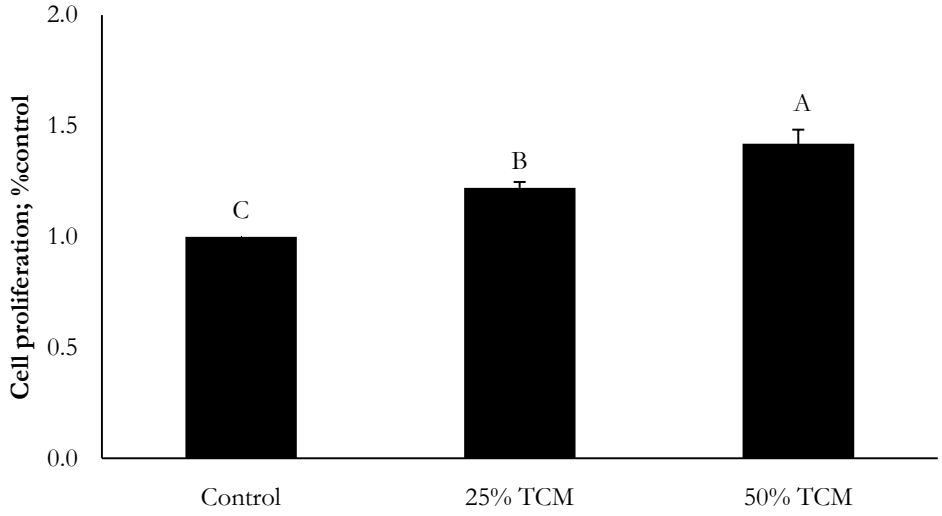

Figure 3.6 Proliferation of 3-5 mm granulosa cells

Proliferation is significantly increased by treatment with 25% and 50% TCM, n=3, $p<0.05$.

Proliferation

There was a dose-related increase in granulosa cell proliferation of 3-5 mm follicles in response to treatments of 25% and 50% TCM (**Figure 2.6**;

n=3; p<0.05). Similarly, granulosa cells from 9-16 mm follicles proliferated in response to 50% TCM treatment (**Figure 2.7**; n=7; p<0.05).

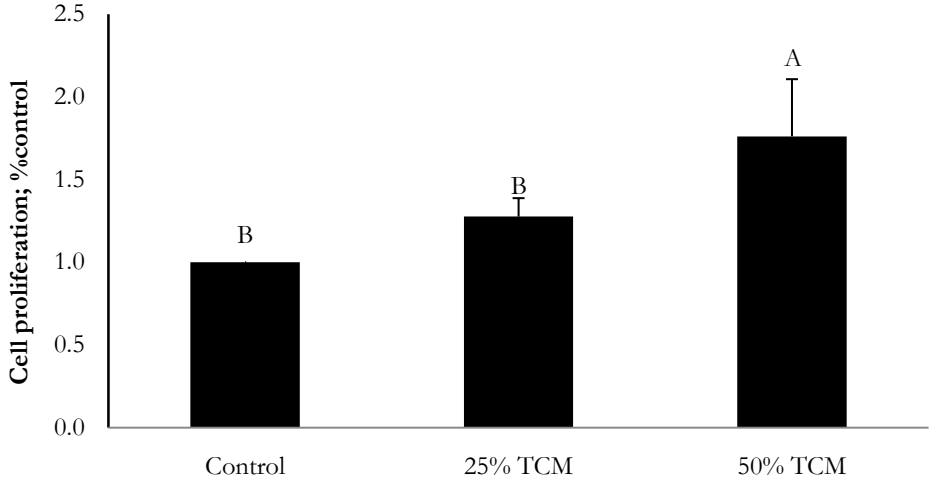

Figure 3.7 Proliferation of 9-16 mm granulosa cells
Proliferation is increased by treatment with 50% TCM, n=7, p<0.05.

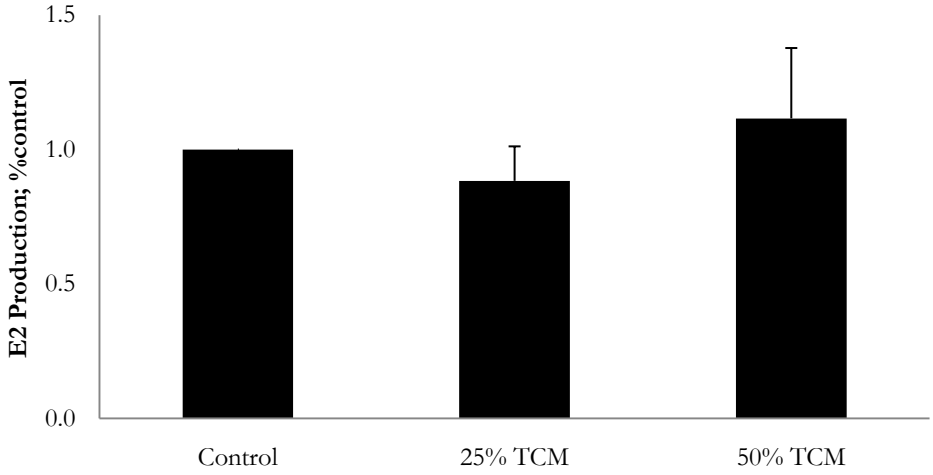

Figure 3.8 Estradiol production by 3-5 mm theca with TCM treatment
Estradiol production was unaffected by treatment with TCM, n=6.

Regulation of Steroid Production

Treatment with TCM had no effect on theca estradiol production by 3-5 mm (**Figure 2.8**, n=6) or 6-8 mm follicles (**Figure 2.9**, n=6) at either

dose. Progesterone (P4) production by granulosa cells from 9-16 mm follicles was significantly decreased by treatment with 25% TCM (50% TCM dose was not used). Preincubation of TCM with an antibody to AMH was unable to block the inhibitory effect of TCM (**Figure 2.10**, n=6).

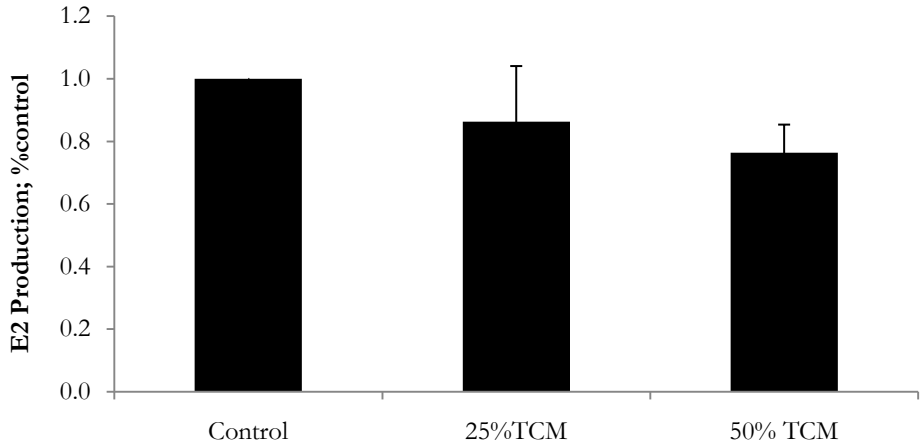

Figure 3.9 Estradiol production by 6-8 mm theca with TCM treatment
Estradiol production was unaffected by treatment with TCM, n=6.

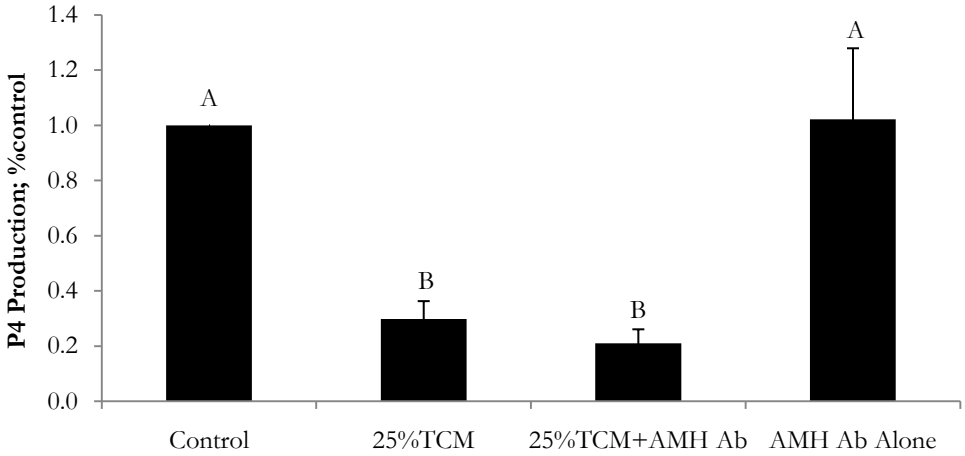

Figure 3.10 P4 production by 9-16 mm granulosa cells with TCM treatment
P4 production is decreased by treatment with 25% TCM, n=6; $p<0.001$.

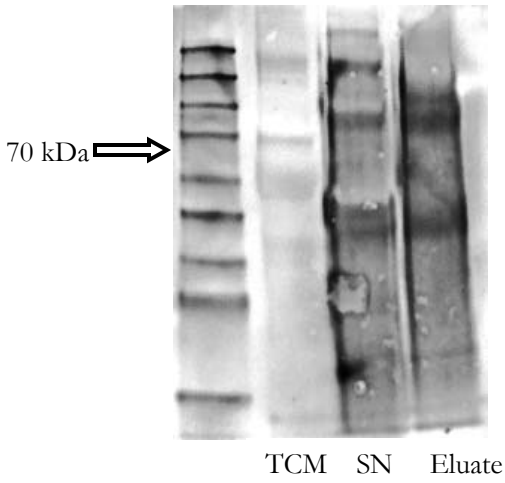

Figure 3.11 Western blot detecting AMH following immunoprecipitation
Protein from whole TCM, IP supernatant (SN) and IP eluate was probed with AMH Ab. Approximate size of 70 kDa is indicated.

Immunoneutralization of TCM

Following immunoprecipitation (IP) of AMH from TCM, a western blot probe was unable to confirm that AMH was removed from the supernatant and present in the eluate. The appropriate band (found in the lane with whole TCM) was not seen in either the supernatant or the eluate (**Figure 2.11**).

3.4 DISCUSSION

It is known that mammalian AMH is not bioactive in avian systems (di Clemente *et al.*, 1992) and avian AMH is not commercially available. For these reasons, we utilize TCM, a source of bioactive AMH for use in cell culture experiments (Johnson *et al.*, 2008). The presence of AMH protein was detected in TCM with western blot analysis, (Johnson *et al.*, 2008) which was confirmed in the present study.

The current study of the regulation of FSHR by AMH was based on previous reports in the mouse (Durlinger *et al.*, 1999; Durlinger *et al.*, 2001). TCM was consistently capable of downregulating granulosa cell FSHR mRNA expression in follicles before selection (3-5 mm) and follicles that were recently selected (9-16 mm). Unfortunately, the current experiments were unable to demonstrate specificity of the results, since preincubation of TCM with an antibody against AMH was unable to attenuate the effect. The stimulatory effect of TCM on granulosa cell proliferation from both groups of follicles provides evidence that the decrease of FSHR mRNA is not due to adverse effects on the granulosa cells.

Granulosa cell progesterone production was examined in differentiated (9-16 mm) follicles because they produce progesterone while theca cells from small follicles are the source of estradiol (Johnson, 1996). It was predicted that the AMH found in TCM would inhibit P4 production, due to its direct and indirect actions in suppressing follicle recruitment in mice (Durlinger *et al.*, 1999; Durlinger *et al.*, 2001), cows (Cushman *et al.*, 2002), and rats (Nilsson *et al.*, 2007), and since granulosa cell steroid production is correlated with follicle development (Johnson, 1996). AMH was expected to inhibit estradiol production by the cultured pieces of theca, based on previous reports that FSH-stimulated aromatase activity in rat granulosa cells was decreased by AMH (di Clemente *et al.*, 1994). AMH inhibited P4 production by 9-16 mm granulosa cells, but had no effect on estradiol production by pieces of theca from 3-5 mm or 6-8 mm follicles, suggesting differential actions on steroidogenesis based on follicle stage or cell layer. This may also provide evidence that the AMH receptor is present

in granulosa cells and not present in theca cells in the hen ovary, since the receptor has not yet been identified in the chicken.

The present data imply that TCM contains a bioactive substance which regulates granulosa cell function in follicles before or after selection. This substance has the ability to consistently decrease FSHR mRNA expression and progesterone production by differentiated granulosa cells. The results we obtained by use of the AMH antibody suggest that this substance is not AMH. However, it is possible that the antibody against AMH did not completely immunoneutralize the AMH present in TCM, as we have no way of quantifying the concentration in TCM in order to use the correct amount of antibody. Another approach we have used to remove AMH from TCM was to incubate various volumes of TCM with immobilized antibody, in an IP technique. Western blot analysis of the "purified" supernatant indicated that AMH may still be present, and showed no difference from the eluate. This suggested that we were unable to estimate the appropriate amount of antibody to neutralize bioactive AMH in the TCM. We intended to repeat this procedure with higher amounts of antibody to be sure all the AMH is bound, but we were restrained by the high cost of this particular antibody. In the future, we may use a different antibody to bind the AMH in TCM and then use the supernatant (lacking free AMH) to treat cell cultures in order to demonstrate that the effect on FSHR mRNA is blocked. If we succeed, this will demonstrate that suppression of FSHR mRNA is due to bioactive AMH in the TCM. Additional future experiments will further investigate the actions of AMH in the hen ovary, as FSHR may not be the only factor important for ovarian function that is regulated by AMH.

REFERENCES

Baarends WM, Uilenbroek JT, Kramer P, Hoogerbrugge JW, van Leeuwen EC, Themmen AP, Grootegoed JA. Anti-Müllerian hormone and anti-Müllerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle and gonadotropin-induced follicle growth. Endocrinology. 1995; 136:4951-4962.

Behringer RR, Feingold MJ, Cate RL. Müllerian inhibiting substance function during mammalian sexual development. Cell. 1994; 79:415-425.

Cushman RA, Wahl CM, Fortune JE. Bovine ovarian cortical pieces grafted to chick embryonic membranes: a model for studies on the activation of primordial follicles. Hum Reprod. 2002; 17:48-54.

Davis AJ, Brooks CF, Johnson PA. Gonadotropin regulation of inhibin alpha-subunit mRNA and immunoreactive protein in cultured chicken granulosa cells. Gen Comp Endocrinol. 1999; 116:90-103.

di Clemente N, Goxe B, Remy J-J, Cate R, Josso N, Vigier B, Salesse R. Inhibitory effect of AMH upon the expression of aromatase and LH receptors by cultured granulosa cells of rat and porcine immature ovaries. Endocrine. 1994a; 2:553-558.

Durlinger AL, Gruijters MJ, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong F, Uilenbroek JT, Grootegoed JA, Themmen AP. Anti-Müllerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. Endocrinology. 2001; 142:4891-4899.

Durlinger ALL, Gruijters MJG, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JT, Grootegoed JA, Themmen APN. Anti-Müllerian hormone inhibits initiation of primordial follicle

growth in the mouse ovary. Endocrinology. 2002; 143:1076-1084.

Durlinger AL, Kramer P, Karels B, de Jong F, Uilenbroek JT, Grootegoed JA, Themmen AP. Control of primordial follicle recruitment by anti-Müllerian hormone in the mouse ovary. Endocrinology. 1999; 140:5789-5796.

Johnson AL. The avian ovarian hierarchy: A balance between follicle differentiation and atresia. Poult Avian Biol Rev. 1996; 7:99-110.

Johnson PA, Dickens MJ, Kent TR, Giles JR. Expression and function of growth differentiation factor-9 in an oviparous species. Gallus domesticus. Biol Reprod. 2005b; 72:1095-1100.

Johnson PA, Kent TR, Urick ME, Giles JR. Expression and regulation of anti-Müllerian hormone in an oviparous species, the hen. Biol Reprod. 2008; 78:13-19.

Johnson PA, Kent TR, Urick ME, Trevino LS, Giles JR. Expression of anti-Müllerian hormone in hens selected for different ovulation rates. Reproduction. 2009; 137:857-863.

Nilsson E, Rogers N, Skinner MK. Actions of anti-Müllerian hormone on the ovarian transcriptome to inhibit primordial to primary follicle transition. Reproduction. 2007; 134:209-221.

Teng CS, Wang JJ, Teng JIN. Purification of chicken testicular Müllerian inhibiting substance by ion exchange and high-performance liquid chromatography. Dev Biol. 1987; 123:245-254.

Tran D, Josso N. Relationship between avian and mammalian anti-Müllerian hormones. Biol Reprod. 1977; 16:267-273.

Ueno S, Takahashi M, Manganaro TF, Ragin RC, Donahoe PK. Cellular localization of Müllerian inhibiting substance in the developing rat

ovary. Endocrinology. 1989; 124:1000-1006.

Visser JA, Themmen APN. Anti-Müllerian hormone and folliculogenesis. Mol Cell Endocrinol. 2005; 234:81-86.

Woods DC, Johnson AL. Regulation of follicle-stimulating hormone-receptor messenger RNA in hen granulosa cells relative to follicle selection. Biol Reprod. 2005; 72:643-650.

CHAPTER 4
SUMMARY AND CONCLUSIONS

The overall goal of these studies was to investigate various factors involved in folliculogenesis in the hen ovary. The two studies investigated two factors noted to have opposing regulatory actions on follicle activation: KL, which is thought to promote follicle activation, and AMH, known to inhibit follicle activation. With respect to regulation and function, there has been little study of either factor in the hen ovary.

The first study examined expression, regulation and function of the Kit system (composed of KL and its receptor, cKit) in the hen ovary. We found a distinct pattern of KL mRNA expression throughout follicle development, with the highest expression seen in very small (<1 mm) follicles and granulosa cells from 3 mm follicles. KL mRNA expression decreased in parallel with follicle development, with the lowest levels seen in 6-12 mm and F1 follicle granulosa cells. To our knowledge, this is the first demonstration of quantified KL mRNA expression during follicle development in the hen ovary. Western blot analysis demonstrated protein expression of cKit in very small follicles, theca tissue from 3-5 mm follicles, and ovarian body. cKit protein was not detected in granulosa cells from the F1 follicle. To our knowledge, this is the first demonstration of cKit expression in the hen ovary.

In our examination of KL regulation in the hen ovary, we found upregulation of KL mRNA expression by OCM in granulosa cells from 6-8 mm follicles. It was then discovered that this upregulation is specific to factors in OCM with molecular weights greater than 30 kDa. Unfortunately,

we were unable to conclusively determine which factor(s) in OCM were having this effect on KL. Our data would suggest that cKit, BMP15 and GDF9 were not involved, but we are not confident of the results, in light of the possibility that we might have failed to use an effective dose of antibody to counteract the factor in OCM.

The first study also demonstrated steroid and gonadotropin regulation of KL mRNA expression. Steroid regulation was found by treating 3-5 mm and 6-8 mm granulosa cultures with vitamin D3. VD3 was shown to significantly increase KL mRNA expression in both sets of follicles. In contrast, granulosa cell treatment with progesterone, estradiol and testosterone exhibited no effect on KL mRNA. With regard to gonadotropin regulation, FSH demonstrated disparate effects. In cultures of 6-8 mm granulosa, FSH significantly decreased KL mRNA expression, yet KL expression was significantly increased in 9-16 mm granulosa cells.

Function of the Kit system was demonstrated with regard to steroid production. A decrease in progesterone secretion by granulosa cells from F1 and F3/4 is hypothesized to be due to actions of soluble cKit found in OCM that binds and activates KL produced by granulosa cells. This was shown when preincubation of OCM with cKit antibody blocked the decrease in progesterone production by treatment with OCM alone.

We also found that KL does not affect estradiol synthesis by theca tissue from 6-8 mm follicles. Additionally, granulosa cell proliferation from these follicles is not altered by treatment with KL. However, these results may suggest that recombinant mouse KL is not bioactive in the chicken.

In the second study, we examined the effects of bioactive chicken AMH (in the form of TCM) on granulosa cell mRNA expression of FSHR.

We found that TCM is consistently capable of downregulating granulosa cell FSHR mRNA expression in follicles before selection (3-5 mm) and follicles that were recently selected (9-16 mm). Unfortunately, preincubation of TCM with AMH antibody did not block the decrease in FSHR mRNA, so we were unable to determine the specificity of this effect. TCM was able to increase granulosa cell proliferation, which indicates that the downregulation of FSHR mRNA was not due to adverse effects of the TCM on the granulosa cells.

The second study also showed a significant decrease in granulosa cell progesterone production with treatment of TCM. Again, this effect was not blocked by the antibody against AMH. Our results show that TCM contains a bioactive substance which regulates granulosa cell function, and suggest that this substance is not AMH. However, it is possible that the antibody against AMH did not completely immunoneutralize the AMH present in TCM, as we have no way of quantifying the concentration in TCM in order to use the correct amount of antibody.

KL and AMH are thought to have essential and opposing roles in regulating the progression of follicles through the highly organized hierarchy seen in the hen ovary. This is suggested by high levels of mRNA expression for both factors seen in the small follicles and lower levels seen in the larger selected follicles. Clearly, additional work is needed to gain a more complete understanding of the function and regulation of both factors in the chicken ovary. It is especially important to determine the interactions between KL and AMH, and whether or not they act to regulate one another.