Effects of Follicle Stimulating Hormone and Insulin-Like Growth Factor 1 on Anti-Mullerian Hormone mRNA Expression in Granulosa Cells of Small Follicles in the Hen

Honors Thesis
Presented to the College of Agriculture and Life Sciences, Animal Science
of Cornell University
in Partial Fulfillment of the Requirements for the
Research Honors Program

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May 2020

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ABBREVIATIONS

AMH- Anti-Mullerian Hormone

$AMH_{N,C}$- Noncovalent Complex of N- and C-Terminus Units of Anti-Mullerian Hormone

AMHRII- Anti-Mullerian Hormone Receptor II

cAMP- Cyclic Adenosine 5'-Monophosphate (AMP)

cDNA- Complementary DNA

CYP19A1- Cytochrome P450, Family 19, Subfamily A, Member 1

CYP11A1- Cytochrome P450, Family 11, Subfamily A, Member 1

FSH- Follicle Stimulating Hormone

FSHR- Follicle Stimulating Hormone Receptor

IGF-1-Insulin-like Growth Factor 1

mRNA- Messenger Ribonucleic Acid

proAMH- Precursor Anti-Mullerian Hormone

StAR- Steroidogenic Acute Regulatory Protein

TGF- β- Transforming Growth Factor-β

ABSTRACT

The laying hen *(Gallas gallus)* has a high reproductive efficiency, laying approximately one egg per day. To maintain this level of productivity, ovarian follicle development must be regulated through a series of coordinated events. Follicle stimulating hormone (FSH) and insulin-like growth factor 1 (IGF-1) are important regulators of follicle development. IGF-1 has been determined to act synergistically with gonadotropins to increase cell proliferation and FSH receptor (FSHR) in mammalian granulosa cells (GC). Anti-mullerian hormone (AMH) inhibits follicle recruitment in both mammalian and avian species. In the chicken, AMH is mainly expressed in GCs of smaller follicles and expression decreases with follicle size. We hypothesized that FSH inhibits AMH expression and that IGF-1 potentiates this effect. GCs from 3-5 mm and 6-8 mm follicles from commercial Single-comb White Leghorn hens (n=7 cultures) were removed and pooled according to follicle size. In Experiment 1, we tested the effect of ovine FSH (0, 10, 25, 100 ng/ml) on AMH and FSHR expression. FSH at all doses decreased AMH expression in GCs from 3-5 mm ($p=0.0004$) and 6-8 mm follicles ($p=0.0022$). In Experiment 2, we cultured GCs similarly, using the optimal dose of FSH (0, 10 ng/ml) and cultured cells with and without recombinant human IGF-1 (0, 10, 100 ng/ml). In conclusion, we observed that FSH decreased AMH expression in GCs from 3-5 mm follicles ($p<0.0001$), and IGF-1 treatment showed no additional effect on AMH expression in 3-5 mm or 6-8 mm follicles.

ACKNOWLEDGEMENTS

First and foremost, I would like to sincerely thank Dr. Pat Johnson for her guidance and mentorship throughout this project and my time spent in the lab.

I would also like to thank Laurie Francoeur for spending hours in the lab with me and always being willing to give me a helping hand. This project would not be possible without her. She has been a great mentor and friend and someone I look up to. She has made my time spent in the lab one of the greatest highlights of my undergraduate career.

Thank you to Dr. Susan Quirk for being a guide through the honors process, particularly through the virtual transition.

Finally, I would like to thank my parents, Michael and Virginia Scoville, my brother Alex, and my friends for their endless support and encouragement through this project and my time at Cornell.

INTRODUCTION

Follicle development and selection is a highly efficient process in the laying hen *(Gallas gallus)*. In the laying hen, follicles are recruited into a growing pool and later selected into an organized hierarchy, resulting in daily ovulation. In order to maintain this level of productivity throughout her reproductive lifetime, ovarian follicle development is regulated through a series of coordinated events. Broiler breeder hens, on the other hand have been selected for efficient feed conversion and growth. One consequence to this extreme selection is a low reproductive efficiency. One strategy to improve reproductive efficiency in broiler breeder hens is restricted feeding. While this improves egg production, it is not sufficient to increase egg production to the level of the laying hen. Laying hens are able to produce 264 eggs by 60 weeks of age, while broiler breeder hens will only produce 154 eggs in the same amount of time (Hy-Line International2020, cobb-vantress.com).

Anti-Mullerian hormone (AMH) is an important ovarian regulator of follicle development in both mammalian and avian species. AMH functions in mammals to maintain the ovarian reserve by inhibiting premature recruitment of primordial follicles in the ovary (Durlinger et al. 1999). Similar to what occurs in mammals, AMH expression is highest in granulosa cells of small follicles and decreases with follicle size (Johnson et al. 2008). Not much is known about the regulation of AMH in laying hens, but its similar pattern of expression suggests that it plays a similar role as in mammals. Broiler breeder hens fed ad libitum have increased AMH mRNA expression in the ovary compared to laying hens (Johnson et al. 2009). AMH mRNA expression in ovaries of broiler breeder hens is decreased by restricted feeding but not to the level of laying hens (Johnson et al. 2009).

Follicle stimulating hormone (FSH) plays an important role in follicle development by increasing differentiation in granulosa cells (Nitta et al. 1991). FSH acts by binding to follicle stimulating hormone receptor (FSHR) in granulosa cells and activates second messenger cyclic adenosine monophosphate (cAMP) (Johnson & Woods 2009). FSHR is expressed in granulosa cells of 3-5 mm and 6-8 mm follicles and is highest in the largest 6-8 mm follicle, which is the next one to be selected into the pre-ovulatory hierarchy (Woods & Johnson 2005). AMH has been shown to decrease FSH sensitivity in the follicles of mice (Durlinger et al. 2001). Selection and viability of pre-hierarchal follicles are dependent on FSH responsiveness and expression of FSHR (Woods & Johnson, 2005). Another important follicle regulator is insulin-like growth factor 1 (IGF-1). IGF-1 was identified by RNA sequencing as an upregulated gene in livers of broiler breeder hens fed ad libitum compared to restricted-fed hens (unpublished data from Johnson lab). IGF-1 stimulates cell proliferation, differentiation and development, and regulates granulosa cell steroidogenesis in follicle development (Mani et al. 2010). IGF-1 has also been determined to act synergistically with FSH in bovine suggesting that these together play an important role in follicle development (Mani et al. 2010). The goal of this study is to investigate the effect of FSH and IGF-1 on AMH expression in granulosa cells from 3-5 mm and 6-8 mm follicles of laying hens. We hypothesized that FSH inhibits AMH expression and that IGF-1 potentiates this effect. Our underlying interest (although not directly tested in these experiments) was that excessive IGF-1 in full fed broiler hens may synergize with FSH to promote excessive follicle selection.

LITERATURE REVIEW

Ovarian function

Folliculogenesis

Folliculogenesis is the process of follicle maturation and selection which ultimately leads to ovulation. In vertebrates this may result in selection of several or only one dominant follicle per ovulatory cycle (Johnson 2012). A follicle produces a mature oocyte and gonadal hormones that play a role in the regulation of folliculogenesis. Several tissue layers surround and support the developing oocyte within the follicle of the hen. The vitelline membrane, surrounds the oocyte, serves as an osmotic barrier (Trziszka and Smolinska 1982). It is composed of three unique layers; two fibrous layers surrounding an inner granular layer (Back et al. 1982, Mann 2008). The outer layer is partly made of lysozyme, making it a potential antibacterial barrier (Mann 2008). Finally, the vitelline membrane provides structural support and separation of the yolk-filled oocyte from the next layer (Back et al. 1982, Mann 2008). The next layer of the functional follicle is the granulosa cell layer. The organization of the cells within the granulosa layer depends on the stage of follicle development. In small follicles, granulosa cells are arranged in a pseudostratified layer and this transitions to one thick wall of cuboidal granulosa cells surrounding the vitelline layer in the preovulatory stage (Perry et al. 1979). Granulosa cells produce hormones and growth factors throughout follicle development, influencing follicle development and ovulation. Surrounding the granulosa layer is a thin acellular barrier known as the basal lamina which affects the morphology of granulosa cells (Asem et al. 2000). The final layer of the follicle surrounding the basal lamina is the theca layer. It is made up of collagen, fibroblast and steroidogenic cells. Theca consists of a theca interna and theca externa. The theca

layer and the outer connective tissue of the follicle are well vascularized (Dahl 1970), and are vital for the transport of extra-ovarian derived yolk material, growth factors and hormones.

The laying hen ovulates one follicle per cycle from a very efficient and well-organized preovulatory follicular hierarchy. The chicken ovary contains follicles of various development stages and sizes including: primordial follicles, prehierarchal follicles, and the large yellow preovulatory follicles (Gilbert et al. 1983). Follicles begin as primordial follicles that enter the growing pool upon activation. Once activated, follicles enter a stage of slow growth with a size range from 0.5-8 mm and make up the prehierarchal stage (Gilbert et al. 1983). Within the prehierarchal stage, follicles can be further divided into large white follicles (LWF) which range from 3-5 mm and small yellow follicles (SYF) which range from 6-8 mm. It is from the 6-8 mm follicles stage that a follicle is selected to join the preovulatory stage. Small follicles less than 5 mm in diameter appear white due to the lack of yolk material, while 6-8 mm follicles slowly accumulate yellow yolk until a single follicle is selected into the pre-ovulatory hierarchy (Perry & Gilbert 1979, Marza & Marza 1935). The number of preovulatory follicles (>8 mm) varies between 4 and 7 (Gilbert et al. 1983). The total time for the growth and development of a follicle from 1 mm to ovulation (40 mm) is around seventeen days (Perry et al. 1983). Development from 3 mm to 5 mm takes 3 days, from 5 mm to 8 mm 2 days, and from 8 mm to ovulation 6 days (Gilbert et al. 1983). In mammals, follicles that are not selected at each step undergo degeneration or atresia at some point during their development. In hens and other bird species, there is little atresia as the follicles advance toward ovulation. It is believed that this is partly due to the high energy demand of yolk accumulation and the time required for growth to pre-ovulatory size (Johnson 2012). The largest follicle labeled F1, will be the next follicle to

ovulate; upon ovulation, the second largest follicle (F2) is promoted to the F1 position and will ovulate 24-26 hours later (Gilbert et al. 1983).

Prehierarchal follicles are considered to have undifferentiated granulosa cells that are steroidogenically incompetent. As follicles transition from the prehierarchal to the preovulatory stage, they begin to produce steroid hormone in response to gonadotropins (Woods et al. 2007). The gonadotropin FSH initiates the differentiation of the granulosa cell layer, and as the follicle increases in size, the responsiveness of the granulosa cell layer to FSH increases (Ghanem & Johnson 2019). FSH binding to FSH receptor (FSHR) is highest in the 6-8 mm follicular pool, the stage in which selection into the preovulatory hierarchy is believed to occur (You et al. 1996).

Anti-mullerian hormone (AMH)

Structure and Pathway

AMH is a disulfide-linked glycoprotein homodimer and is a member of the transforming growth factor-β (TGF-β) superfamily (Lambeth et al 2015). In the chicken, the gene is located on chromosome 28, five exons long with 61% GC content across introns and exons. The chicken AMH gene encodes 644 amino acids with molecular mass of 74 kDa (Teng et al. 1987). Western blots have approximated molecular weights of 12.5, 72, and 94 kDa (Neeper et al. 1996), with a mature size of ~70 kDa (Johnson et al. 2009).

AMH signals through a heterodimer complex of its nonspecific type 1 (ALK2/3/6) and its specific type II (AMHRII) serine/threonine kinase receptors. When the AMH ligand binds to the type II receptor, the type I receptor is recruited to the receptor complex where it is brought into the receptor complex and becomes phosphorylated and activated. As a result, downstream

Smad second messenger proteins are phosphorylated where receptor specific Smads interact with the common Smad4. This complex moves to the nucleus where it regulates gene expression directly or through other DNA-binding proteins (reviewed in Visser 2003). The type II receptor (AMHRII) has been described in the chicken (Lemcke et al. 2018).

When AMH is first synthesized, it is synthesized as a large homodimeric precursor that is made up of two polypeptide chains with a N-terminal pro-region and a C-terminal mature domain that must be cleaved through proteolytic processing (di Clemente et al. 2010). Research on human AMH has observed that 140-kDa proAMH is cleaved 109 amino acids upstream from the carboxyl terminus, forming a 25 kDa C-terminal dimer and 70 kDa N-terminal dimer (Pepinsky et al. 1988). After cleavage, pro-regions of TGF-β ligands often remain noncovalently associated to the mature domains preventing binding of the ligands to the receptor (di Clemente et al. 2010). Like other TGF-β ligands, AMH must be cleaved before it can bind to its receptor. Unlike other TGF-β ligands, proAMH does not have to dissociate from the mature C-terminal dimer to bind to AMHRII. The 70 kDa N-terminus (pro-region) of AMH connects to the 25 kDa C-terminus dimer, forming a noncovalent complex ($AMH_{N.C}$). When the complex binds the receptor, the pro-region dissociates and phosphorylation and receptor activation occurs (di Clemente et al. 2010).

Bioactivity of mammalian AMH varies based on cleavage locations and dimerization. Research has reported that $AMH_{N,C}$ has the greatest bioactivity. ProAMH (full length AMH) had low activity while N-terminal pro-region alone and noncleavable full-length AMH were completely inactive (di Clemente et al. 2010). Sertoli and granulosa cells express varying levels of enzymes that cleave proAMH that depend on testicular and ovarian development, and the stage of ovarian follicular development (Nachtigal & Ingraham 1996, Bae et al. 2008). ProAMH

is not easily cleaved by proteases in the blood and is predominantly found in the ovary while $AMH_{N,C}$ is the predominate form in circulation (Pankhurst et al. 2016). However, it is possible that proAMH is cleaved to $AMH_{N,C}$ while in circulation because proAMH cleaving enzymes are found in vascular tissue (Stawowy et al. 2001).

AMH in embryo development

In mammalian and avian species, the primary role of AMH during embryonic development is the regression of Mullerian ducts in males (Teng 1987). AMH is synthesized and secreted by Sertoli cells in the testis and acts directly to regress the male Mullerian ducts (Sasanami 2017). Without AMH, the Mullerian ducts would develop into fallopian tubes, oviducts, and upper vagina, as in females (Lambeth et al. 2015).

In the female chicken embryo, only the left ovary develops into a functional organ. AMH regresses the right reproductive Mullerian duct of birds. At day 7 of embryonic growth, or at Hamburg and Hamilton (HH) stage 31, the secondary sex cords form (Hamburger & Hamilton 1951). At this stage in development, asymmetry between the left and right gonad in the female embryo is initiated by the expression of a paired-like homeodomain transcription factor, pair-like homeodomain 2 (PITX2). The surface epithelial layer of the left gonad begins to thicken and develop compared to the right (Guioli & Lovell-Badge 2007). Estrogen synthesis is essential for the growth and organization of the left ovary. Aromatase, an estrogen-synthesizing enzyme, is expressed (CYP19A1) and activated by the transcription factor, forkhead box L2 (FOXL2) by embryonic day 10 or stage 26 of HH (Smith et al. 1997, Hamburger & Hamilton 1951). Both the left and right ovary in the hen begins to express aromatase and produce estrogens. However, the right epithelium lacks estrogen receptor alpha (ERα), inhibiting the right gonad from further

differentiating (Nakabayashi et al 1998). The left gonad expresses ERα, enabling development of the left ovary and protecting the left oviduct from regression by AMH (González-Morán 2014, Sasanami 2017, Johnson 2014).

AMH in folliculogenesis

In mammals, AMH plays an important role in follicle development. The AMH knock out (KO) mouse has been influential in understanding the role of AMH in the ovary. AMH is believed to help maintain the ovarian reserve by suppressing primordial follicle activation (Durlinger et al. 1999). In female KO mice, the absence of AMH accelerates follicular growth and recruitment and leads to premature follicle depletion (Durlinger et al 1999). In avian and mammalian females, AMH is most highly expressed in small follicles. In the adult hen ovary, the granulosa layer of growing follicles in the ovary produces AMH (Johnson et al. 2008). Highest levels of AMH mRNA are detected in 1 mm whole follicles and decreased as the follicle size increases, with very low levels in the F1 (pre-ovulatory follicle) (Johnson et al. 2008).

In chickens, AMH mRNA expression in follicles varies according to the strain of hen and metabolic status. Broiler breeder hens that have excessive follicular growth and poor ovulation rates have high AMH expression compared to laying hens (Johnson et al. 2009). The poultry industry utilizes restricting feed intake as a technique to improve egg production in broiler breeder hens. As a result, ovulation becomes more constant and follicular hierarchies are similar in appearance to those of laying hens. Restricted feeding (87% of full fed) decreases AMH mRNA expression in broiler breeder hens, yet when compared to laying hens, AMH levels are still high in the ovary (Johnson et al. 2009). It is unknown why increased feed intake results in

elevated AMH levels. Understanding the regulation of AMH could provide insight into the role of AMH in follicle selection.

Factors involved in AMH regulation

In hens, it was observed that estradiol and progesterone had no effect on expression of AMH mRNA expression in cultured granulosa cells (Johnson et al. 2008) whereas, vitamin D decreased the expression of AMH in granulosa cells of small follicles *in vitro* (Wojtusik & Johnson 2012). Oocyte-conditioned medium (OCM) caused a dose-related decrease in AMH mRNA expressionn in hen granulosa cells (Johnson et al. 2008) suggesting regulation of AMH by a secreted factor from the oocyte. It was subsequently determined that the oocyte hormone BMP15 decreases AMH mRNA expression in 3-5 mm and 6-8 mm granulosa cells (Stephens & Johnson 2016). In a mouse model, co-treatment with BMP15 and GDF9 induced AMH expression *in vivo* and *in vitro,* suggesting they are more potent together than individually (Roy et al. 2018). Another important regulator of AMH in mammals is FSH (Baarends et al. 1995, Devillers et al. 2019, Taieb et al. 2011). FSH's role in regulating AMH in the hen has not yet been evaluated.

Follicle stimulating hormone (FSH)

Another critical regulator of reproductive physiology is follicle stimulating hormone (FSH). FSH is heterodimeric glycoprotein that is made up of an α- and β-subunit and it is expressed by gonadotrophic cells in the anterior pituitary. The α-subunit is shared by all pituitary glycoprotein hormones, while the β-subunit is unique to each hormone (Das & Kumar 2018). FSH binds and activates FSH receptor (FSHR) expressed in granulosa cells. The FSH

receptor is characterized by an intracellular and an extracellular domain and it belongs to the family of G protein-coupled receptors (Gudermann et al. 1995). The intracellular domain couples to G_s protein and increases the cAMP signal in the granulosa cell (Means et al. 1980).

Responsiveness of the granulosa cell layer to FSH is a critical marker of cyclic recruitment of a pre-recruitment follicle to the pre-ovulatory stage (Johnson 2015). FSH-responsiveness initiates differentiation of the granulosa cell layer, which results in the initial capacity for steroidogenesis (Nitta et al. 1991, Tilly et a. 1991). FSH receptor (FSHR) mRNA and protein have been reported to be expressed as early as the 1-2 mm stage of development (Woods & Johnson 2005). Prior to recruitment, freshly collected granulosa cells from 1 to 8 mm follicles do not differentiate and do not initiate cyclic adenosine monophosphate (cAMP) production when they were treated with recombinant human (rh) FSH *in vitro* compared to GC from the most recently recruited (9-12 mm) follicle (Johnson & Lee 2016, Tilly et al. 1991). Granulosa cells from pre-recruitment follicles essentially lack cAMP-dependent steroidogenic acute regulatory protein mRNA (StAR) expression and cytochrome p450 side-chain cleavage enzyme (CYP11A1) activity (Bauer et al., 2000, Nitta et al, 1991**,** Tilly et al. 1991). There is evidence that the inability of granulosa to generate cAMP in response to FSH, *in vitro* is mediated through tonic inhibitory effect of the mitogen-activated protein kinase (MAPK) and/ or protein kinase C pathways (Johnson et al. 2002, Woods et al. 2007, Woods & Johnson 2005). There is also evidence that β-arrestin (βARR) inhibits FSHR signaling therefore inhibiting cAMP formation-dependent StAR protein and CYP11A1 expression (Kim & Johnson 2018).

Interactions between AMH and FSH

AMH is known to affect FSH sensitivity in mammalian follicles. In an *in vitro* mouse follicle culture system, it was found that AMH reduces FSH stimulated preantral follicle growth, suggesting that AMH plays a role in regulating follicle selection (Durlinger et al. 2001). *In vitro* studies using mouse granulosa cells have shown that AMH has inhibitory effects on FSH-induced processes including FSH-induced follicular growth (Hayes et al. 2016). This study also reported that AMH treatment decreases expression of StAR and aromatase, and FSHR (Hayes et al. 2016). It is not known if AMH affects FSH sensitivity in hens.

Effect of FSH on AMH expression has been studied in several models, yet findings depend on the species studied. Several studies observed that FSH decrease AMH. There was a decrease in AMH and AMHR mRNA expression in granulosa cells and an increase in follicle growth (Baarends et al. 1995) when rat follicles were treated with gonadotropin releasing hormone (GnRH) antagonist and human recombinant FSH. A more recent study in mice revealed that a high concentration of FSH decreased AMH expression (Devillers et al. 2019). Another study showed that FSH and cAMP enhanced AMH transcription in human granulosa cells (Taieb et al. 2011), while a study in bovine granulosa cells determined that FSH inhibited the production of AMH (Rico et al. 2011).

Insulin-like growth factor-1 (IGF-1)

The insulin-like growth factor (IGF)-system is made up of IGF-I, IGF-II, IGF receptors, and IGF-binding proteins (IGFBP) and plays an important role in the reproductive system in mammals (Onagbesan et al. 1999). Chicken IGF-1 is a 7 kDa polypeptide protein that contains

70 amino acids, and differs from human, bovine, and porcine IGF-1 protein by having 8 amino acid substitutions (Ballard et al. 1990).

Function of IGF-1 is mediated by the cell surface receptor, insulin like growth factor type 1 receptor, IGF-1R. IGF-1R is a tyrosine kinase receptor, which is activated upon ligand binding (Dupont & LeRoith 2001). Chickens do not have a type II IGF-2/cation-independent mannose-6-phosphate receptor (IGF-R2) like mammals. Both IGF-1 and IGF-2 functions are mediated by binding to the IGF-1R. In the chicken ovary, receptor mRNA and protein expression of IGF-1 have been observed to be most abundant in granulosa cells in pre-ovulatory follicles. As follicle development increases, the receptor abundance also increases and is most abundant in the largest F2 and F1 follicles (Tosca et al. 2008). IGF-1 is expressed in both the theca and granulosa cells of large pre-ovulatory follicles (Roberts et al. 1994). However, IGF-1 expression is more abundant in the theca layer (Armstrong et al. 1996). Granulosa cells of large follicles produce both IGF-1 and IGF-2, and significantly more IGF-2 than IGF-1 *in vitro* (Onagbesan et al. 1999).

Function of IGF-1 in small prehierarchal follicles is not well understood. In hen pre-ovulatory follicles, IGF-1 stimulates granulosa cell proliferation and differentiation (Armstrong et al. 1996, Onagbesan et al. 1999, Onagbesan & Peddie 1995) and progesterone production (Onagbesan & Peddie 1995). The mechanism of IGF-1 stimulation of progesterone production was studied in pre-ovulatory follicles; the treatment of granulosa cells from F3, F4, and F1 follicles with IGF-1 decreased Thr172 phosphorylation of AMP activated protein kinase (AMPK), a protein kinase involved in steroidogenesis in chicken granulosa cells (Tosca et al. 2008). Using an AMPK activator AICAR (5- aminoimidazole-4-carboxamide ribonucleoside), researchers increased IGF-1-stimulated progesterone production and increased StAR protein levels in the F1 follicle (Tosca et al. 2008); the opposite effect appeared in the granulosa cells

from F3 and F4 follicles (Tosca et al. 2008). This could indicate that energy balance affects granulosa cell steroidogenesis and viability differentially at different follicle stages. This signaling pathway in the granulosa of prehierarchal follicles is not known, but this could give insight into mechanisms of follicle selection.

IGF-1 acts synergistically with gonadotropins in mammals. IGFs act with FSH and luteinizing hormone (LH) to stimulate the expression of gonadotropic receptors, which enhances follicle maturation and steroidogenesis (Kwintkiewicz & Giudice 2009). In bovine granulosa cells, IGF-1 alone significantly increased cell number and mRNA expression of CYP11A1, CYP19A1, FSHR and 17beta-estradiol (OE(2)) production while FSH alone had no effects on granulosa cell expression or proliferation. IGF-1and FSH acted synergistically to increase the number of granulosa cells and, increased the expression of IGF-1R and CYP19A1 (Mani et al. 2010). IGF-1 synergistically acting with FSH may be important for the development of follicles and may act together to decrease AMH expression.

Comparison of the liver transcriptomes of broiler breeder hens fed ad libitum compared to restricted fed showed IGF-1 to be upregulated in hens fed ad libitum. The effect of IGF-1 on AMH has not yet been investigated in hens. Furthermore, it is not known if FSH and IGF-1 synergize in hens to affect certain downstream target such as AMH.

Conclusion

AMH is an important reproductive hormone in both mammalian and avian species that plays a critical role in embryonic development, specifically sex differentiation. AMH also plays a role in ovarian folliculogenesis. Regulation of AMH and the role of AMH in follicle development in the hen is not well understood. Preliminary data in our lab show that there is an

interaction between FSH and AMH. In this study we examined the regulation of AMH by FSH in hens and investigated whether IGF-1 and FSH act synergistically to decrease AMH expression.

MATERIALS AND METHODS

Animals

Single-comb White Leghorn laying hens were housed in individual laying batteries and were exposed to 15 h light: 9 h darkness per day. They received *ad libitum* access to feed and water and individual egg laying was recorded daily. Selected hens were within their first year of lay had a consistent laying pattern and had recently laid a mid-sequence egg. Hens were euthanized via CO_2 and ovaries were immediately removed and placed in ice-cold Krebs-Ringer Bicarbonate buffer. All animal procedures and techniques were approved by the institutional Animal Care and Use Committee of Cornell University.

Granulosa cell culture

For each replicate (n=5-7), granulosa cells from 3-5 mm and 6-8 mm follicles were isolated from 2-3 laying hens and pooled in 4° C Krebs buffer and medium 199. Granulosa layers were digested in 0.2% collagenase solution for 10-15 min at 37° C. After the time, they were inverted, and then placed back into the water bath. This process was done three times. Cells were centrifuged, resuspended and washed in medium 199 and 1% antibiotic antimycotic (ABAM). Following washing, viability and cell number were determined using trypan blue exclusion and hemocytometer. Granulosa cells were plated in 6-well culture dishes at a density of 2×10^6 cells per well with 1.5 ml M199 + 5% FBS +1% ABAM and were cultured for 22.5 h until approximately 70% confluent at 37° C with 5% CO_2. The medium was replaced with serum free media and treated for 24 hours. In experiment 1, granulosa cells were treated with ovine FSH at 0, 10, 25, or 100 ng/ml. In experiment 2, granulosa cells were treated with ovine FSH at 0 or 10 ng/ml in the presence or absence of rh IGF-1 (BioVision, Milpitas, CA) at 0, 10,

or 100 ng/ml. Following 24-hour culture, cells were scraped from wells and stored in RLT buffer at -80℃ until processing.

RNA isolation and cDNA synthesis

RNA extraction was conducted using an RNAeasy Mini Kit with an optional on-column DNase treatment (Qiagen Inc., Valencia CA). Reverse transcriptase reactions were performed using 1 µg of total mRNA in a 20 µl volume using the high capacity cDNA RT kit (Applied Biosystems).

PCR Analysis

Primer pairs for FSHR (forward: 5' -GCACCTTCCAAGCCTCAGATAT-3'; reverse: 5' -CCCTATGGACGACGGGTAAA-3'), 70 bp product size and AMH (forward 5' -CCCCTCTGTCCCTCATGGA-3'; reverse: 5' -CGTCATCCTGGTGAAACACTTC-3'), 71 bp product size were used. Primers were designed (Stephens & Johnson 2016) with Primer Express Software 2.0 (Applied Biosystems, Foster City, CA). Primer efficiencies were within 90.4-103.2%, determined by the slope of the standard curve (Stephens & Johnson 2016). A standard curve was created using serial dilutions of cDNA prepared from a pool of granulosa cell RNA.

Quantitative real time PCR reactions were set up in a 25 µl volume in duplicate with a final concentration of 1X Power SYBER GREEN (Applied Biosystem) and 300 nM of primers. All reactions were normalized to 18S rRNA (Applied Biosystems) and all samples were run in duplicate. Control reactions containing no template and reactions lacking reverse transcriptase were also run. The unknown sample expression was determined from the standard curve and normalized to 18S.

Statistical Analyses

Messenger RNA expression from qRT-PCR data was analyzed by the SAS version 9.4 packaged PROC MIXED. Culture number was used as a random variable. Data were log transformed to improve normality before running an analysis of variance in PROC MIXED. Results are presented as back transformed estimates. Tukey HSD post hoc test was conducted for group assignment.

RESULTS

FSH Effect on AMH and FSHR expression

In Experiment 1, the effect of FSH on AMH mRNA was evaluated. Granulosa cells from 3-5 mm and 6-8 mm follicles were treated with different doses of FSH. FSH treatment decreased AMH mRNA expression in granulosa cells from both 3-5 mm ($p=0.0004$) and 6-8 mm follicles ($p=0.0022$) after 24 hours (Fig. 1). A dose of 10 ng/ml was sufficient to decrease AMH expression, while higher doses did not further affect AMH expression. FSHR mRNA expression was not affected by FSH treatment at any dose in granulosa cells from 3-5 mm follicles ($p=0.8462$). FSH decreased FSHR mRNA expression at 25 ng/ml in 6-8 mm follicles ($p=0.057$) (Fig. 2).

Effect of FSH and IGF-1 on AMH and FSHR Expression

To determine whether IGF-1 acted synergistically with FSH to decrease AMH expression, granulosa cells were treated with 10 ng/ml FSH in the presence and absence of IGF-1 at different doses. We observed that FSH decreased AMH mRNA expression in granulosa cells from 3-5 mm follicles ($p<0.001$) as seen in Experiment 1. The addition of IGF-1 did not increase the effect of FSH on AMH levels in 3-5mm follicles (Fig. 3). In 6-8 mm follicles, we noted that treatment of IGF-1 of 10 ng/ml without the 10 ng/ml of FSH was different from both 100 ng/ml treatments of IGF-1 with and without FSH ($p=0.0033$); however, neither of these treatments was different from controls (Fig. 3). AMH mRNA expression was not different from the control with treatment of FSH and IGF-1 in granulosa cells from 6-8 mm follicles (Fig. 3). FSH and IGF-1 had no effect on FSHR expression in granulosa cells from 3-5 mm ($p=0.8942$) and 6-8 mm follicles ($p=0.1162$) (Fig. 4).

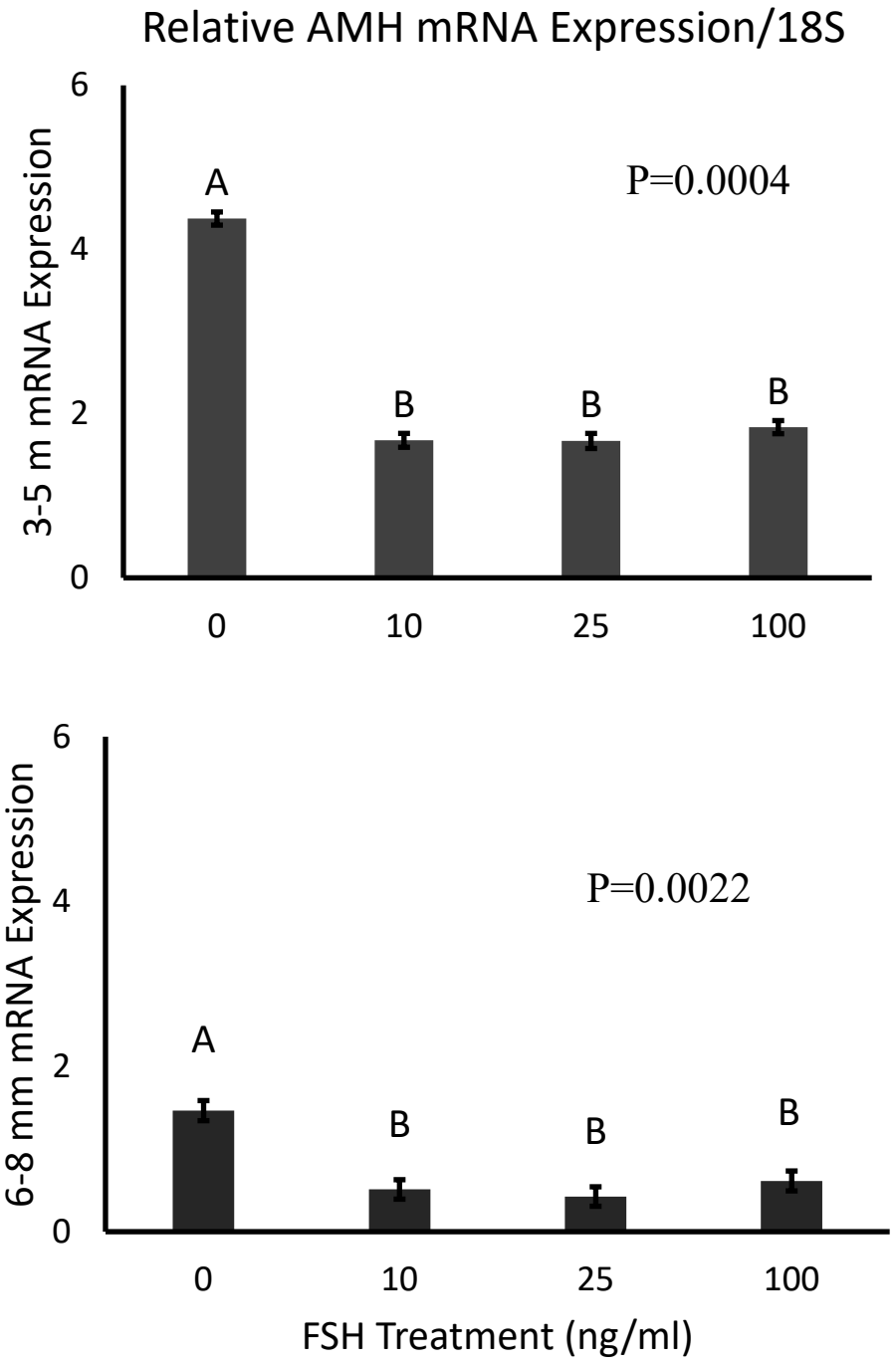

Figure 1. Relative AMH mRNA expression in granulosa cells treated with FSH in 3-5 mm (top graph) and 6-8 mm (bottom graph) follicles (n=5-7 replicate experiments). P value in the graph refers to overall treatment effect. Bars represent the mean ± SEM and bars with different letters differ significantly (P<0.01).

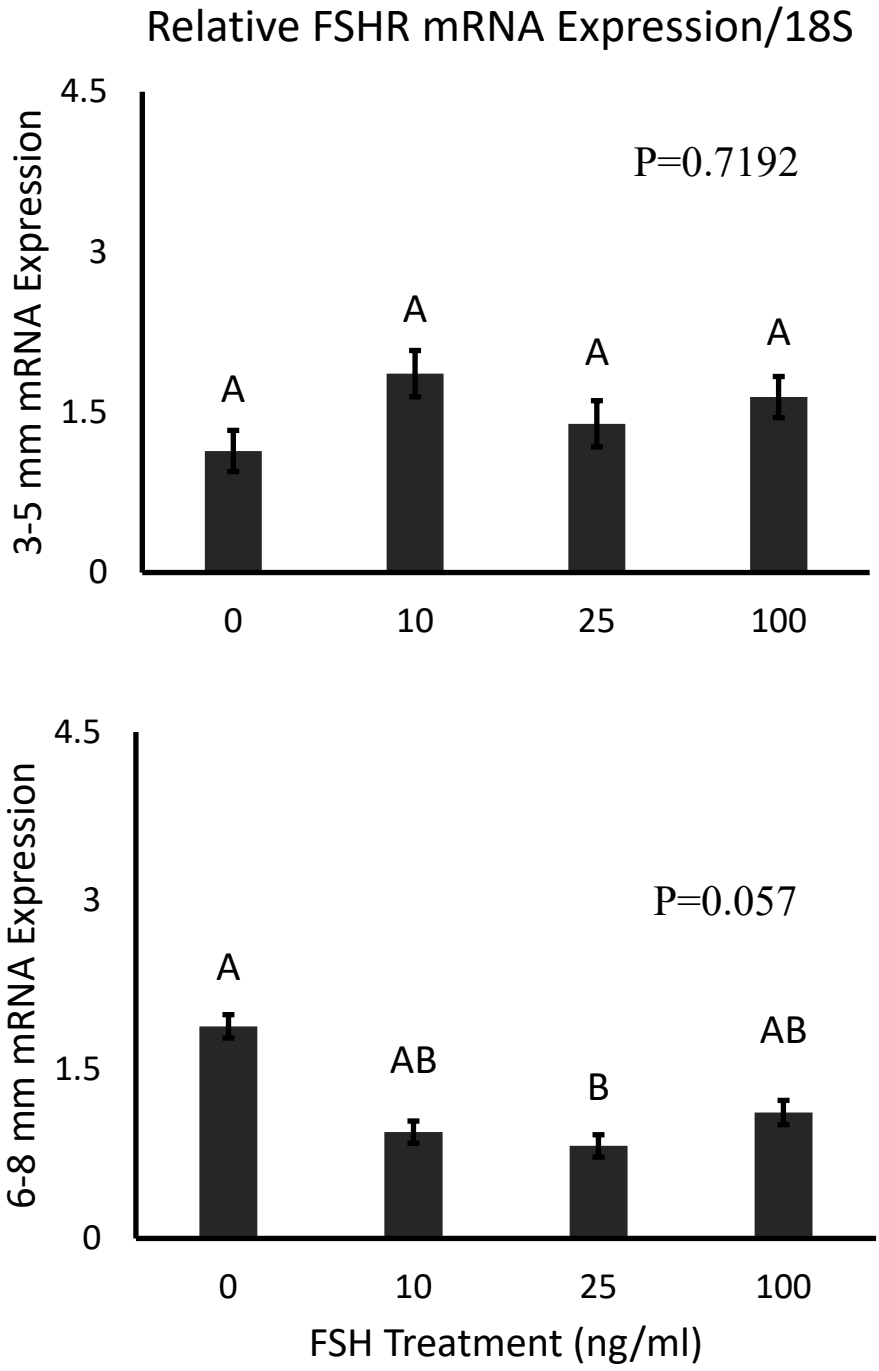

Figure 2. Relative FSHR mRNA expression in granulosa cells treated with FSH in 3-5 mm (top graph) and 6-8 mm (bottom graph) follicles (n=5-7 replicate experiments). P value in the graph refers to overall treatment effect. Bars represent the mean ± SEM and bars with different letters differ significantly ($P<0.01$).

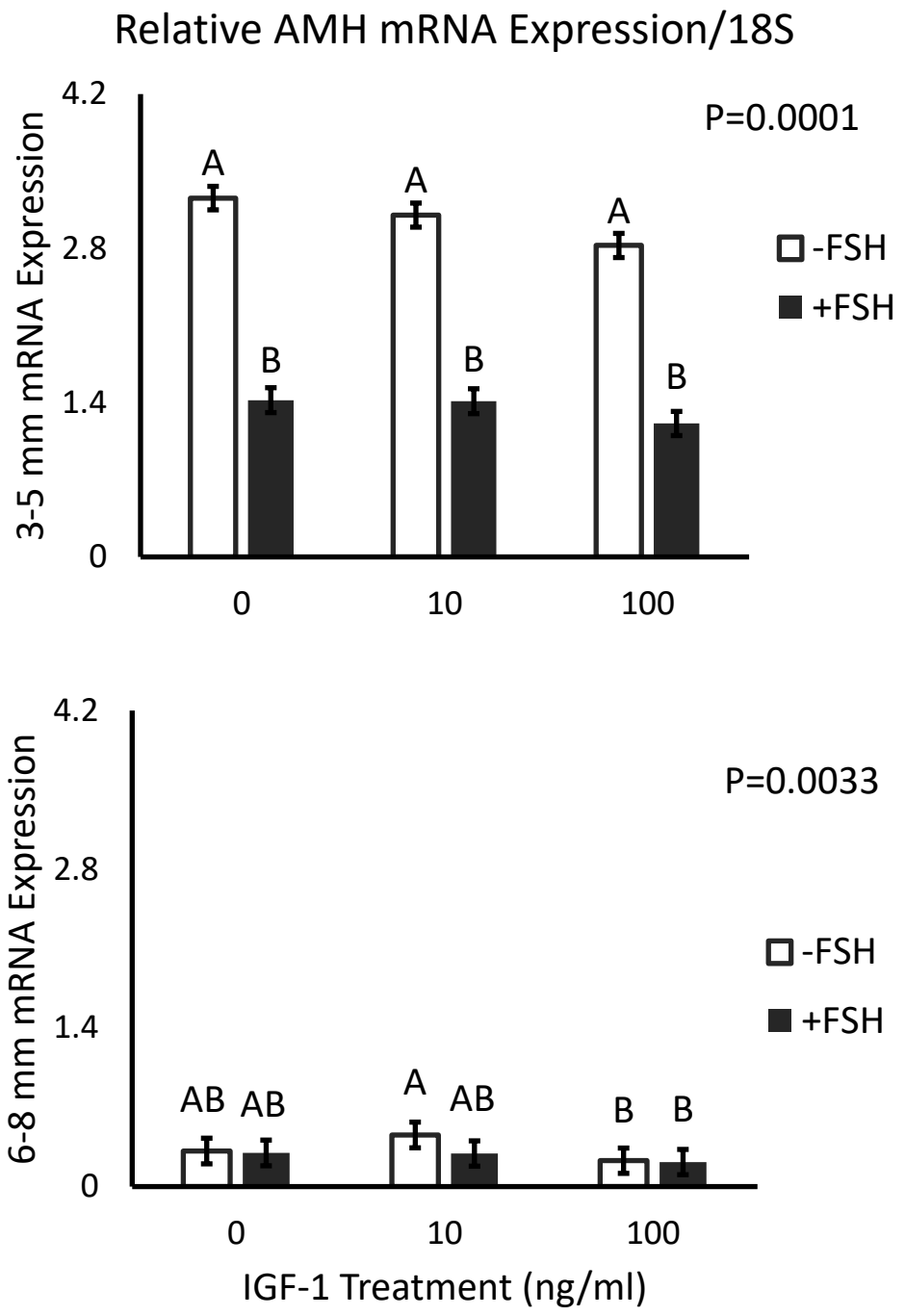

Figure 3. Relative AMH mRNA expression in granulosa cells treated with FSH and IGF-1 in 3-5 mm (top graph) and 6-8 mm (bottom graph) follicles (n=5-7 replicate experiments). P value in the graph refers to overall treatment effect. Bars represent the mean ± SEM and bars with different letters differ significantly (P<0.01).

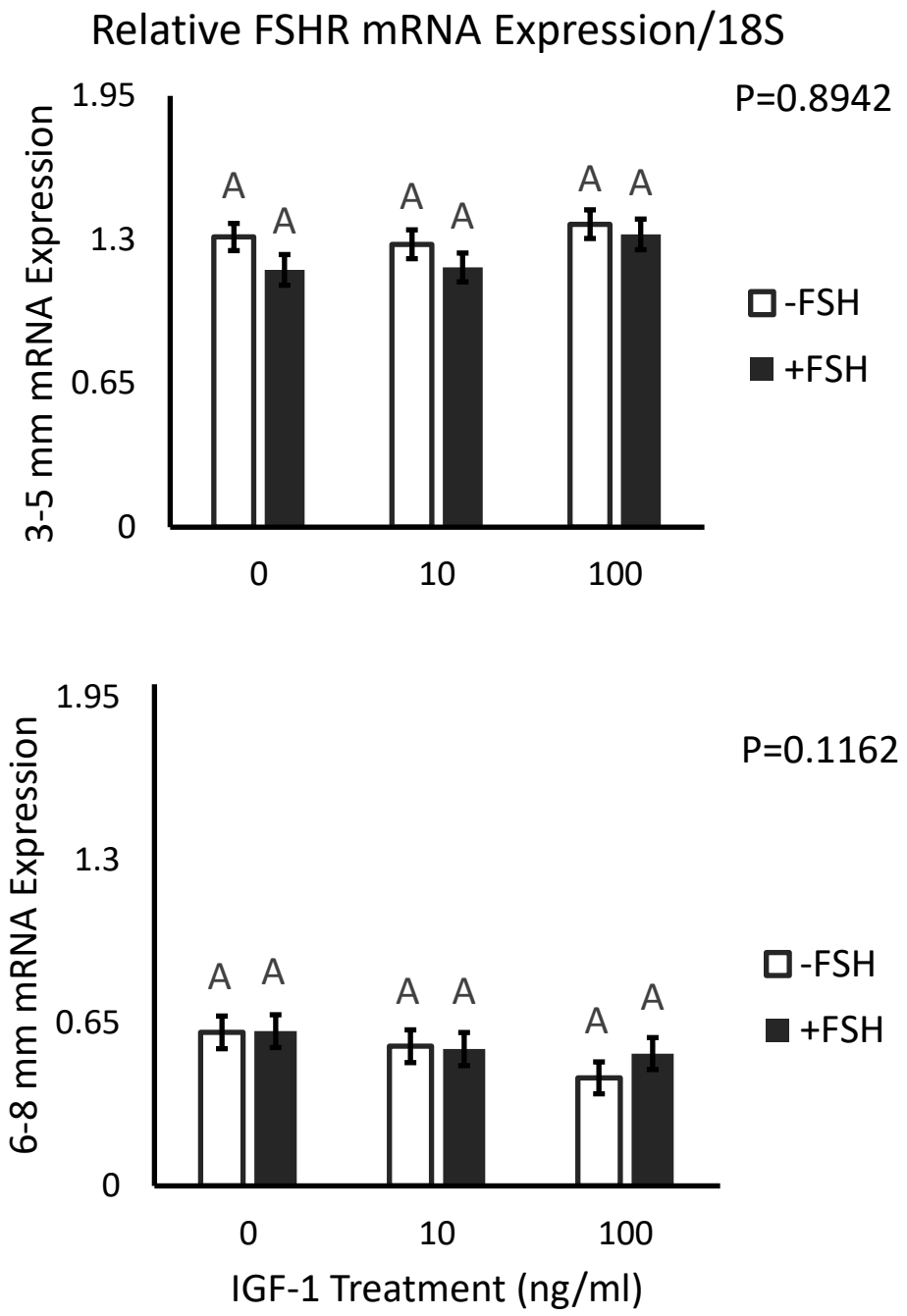

Figure 4. Relative FSHR mRNA expression in granulosa cells treated with FSH and IGF-1 in 3-5 mm (top graph) and 6-8 mm (bottom graph) follicles (n=5-7 replicate experiments). P value in the graph refers to overall treatment effect. Bars represent the mean ± SEM and bars with different letters differ significantly (P<0.01).

DISCUSSION

The purpose of this study was to investigate the effect of FSH and IGF-1 AMH and FSHR mRNA expression in granulosa cells from laying hens. The laying hen has a very organized follicle hierarchy. Understanding how AMH is regulated in a reproductively efficient model can help elucidate factors that may be associated with the disruption of the follicle hierarchy in broiler breeder hens that have high levels of AMH.

In Experiment 1, granulosa cells from, 3-5 mm and 6-8 mm follicles were treated with FSH to investigate the role of FSH on AMH and FSR mRNA expression. Consistent with mammalian studies, FSH decreased AMH mRNA expression (Baarends et al. 1995, Devillers et al. 2019). FSH decreased AMH expression in granulosa cells from both 3-5 mm and 6-8 mm follicles, with a maximum effect observed at a dose of 10 ng/ml (Fig. 1). AMH mRNA expression was lower in granulosa cells from 6-8 mm follicles than from granulosa cells from 3-5 mm follicles (Fig. 1 & 2). This is consistent with earlier research reporting that AMH mRNA expression is more highly expressed in granulosa cells of smaller follicles (Johnson et al. 2008). A previous study evaluated AMH expression in hen granulosa cells and reported that a decrease in AMH mRNA expression with increasing follicle size is associated with an increase in expression of FSHR (Wojtusik & Johnson 2012). The initial responsiveness of the granulosa cell layer to FSH is a critical marker of cyclic recruitment (Johnson 2015). FSH-responsiveness initiates differentiation of the granulosa cell layer, which results in the initial capacity for steroidogenesis (Nitta et al. 1991, Tilly et a. 1991). It is possible that FSH may directly decrease AMH at this stage so that cyclic recruitment and steroidogenesis can be initiated. In this study, FSH did not have an effect on FSHR expression in 3-5 mm follicles although, FSH at the 25

ng/ml dose decreased FSHR expression relative to the control in granulosa cells from 6-8 mm follicles (Fig. 2).

In Experiment 2 of this study, granulosa cells were treated with FSH and IGF-1 to investigate whether IGF-1 and FSH might act synergistically to regulate AMH as seen in mammalian studies (Kwintkiewicz & Giudice 2009, Mani et al. 2010). Similar to Experiment 1, we showed that FSH decreased AMH expression. However, IGF-1 did not significantly affect AMH mRNA expression in granulosa cells from 3-5 mm follicles (Fig. 3). Treatment of IGF-1 at 10 ng/ml without FSH was different from both treatments of IGF-1 at 100 ng/ml, however neither of these treatments were different from the controls (Fig. 3). Cotreatment of FSH and IGF-1 did not have an effect on AMH expression, suggesting that these hormones do not act synergistically to affect AMH expression. It is possible that AMH levels in 6-8 mm follicle granulosa cells may have been too low to detect a difference.

FSH and IGF-1 treatment had no effect on FSHR expression in granulosa cells of either 3-5 mm or 6-8 mm follicles. An *in vitro* study in secondary follicles of goats reported that IGF-1 and FSH together resulted in a greater increase in FSHR mRNA expression than either hormone independently (Brito et al. 2012). Although we did not observe that IGF-1 enhanced the effect of FSH on AMH mRNA expression, IGF-1 and FSH may have other effects that promote follicle development.

The decrease in AMH expression following FSH treatment is intriguing. The mechanism by which FSH decreases AMH has not yet been investigated. Future studies will investigate pathways by which FSH may be decreasing AMH. Although we did not see any effect on AMH expression with co-treatment of FSH and IGF-1, FSH and IGF-1 may synergize to affect other markers of follicle development. Recent unpublished work in our lab has shown that liver IGF-

1mRNA and protein is upregulated in broiler breeder hens fed ad libitum compared to those on restricted feed (unpublished). IGF-1 may influence other follicle markers to promote excessive follicular growth and this may be amplified by FSH treatment. We are also interested in a possible regulatory role for FSH on AMH expression in vivo. Level of dietary intake in broiler breeder hens influenced gonadotropin levels both at the mRNA level and in plasma concentrations (Ciccone et al. 2007).

Alteration of FSH secretion may affect AMH levels in the ovary and may provide an explanation for the high AMH levels seen in broiler breeder hens (Johnson et al. 2009). Full fed broiler breeder hens have reduced plasma levels of FSH mRNA relative to restricted fed broiler breeder hens (Ciccone et al. 2006).

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