INVESTIGATING THE REPRODUCTIVE ROLE OF ANTI-MÜLLERIAN HORMONE VIA ANTI-MÜLLERIAN HORMONE RECEPTOR, TYPE II IN THE HEN

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ABSTRACT

In the mammalian ovary, anti-müllerian hormone (AMH) helps maintain the ovarian reserve by regulating primordial follicle activation and follicular selection, although its role within the avian ovary is unknown. In adult mammals, AMH is primarily produced in granulosa cells of preantral and early antral follicles. Similarly, in the hen, the granulosa cells of smaller follicles are the predominant source of AMH. AMH importance in mammalian ovarian dynamics suggests AMH may have conserved, protective functions within developing follicles in the hen. To better understand the role of AMH in avian follicle development, we studied the expression pattern of the specific AMH receptor. AMH utilizes the TGF-β/SMAD signaling pathway, including the specific Type II receptor, AMHRII. qRT-PCR results indicate that AMHRII mRNA expression, as well as that of AMH, are highest ($p<0.01$) in small follicles (1 mm) and decrease as follicle size increases. Dissection of 3-5 mm follicles into ooplasm and granulosa components shows that AMHRII mRNA levels are significantly greater in ooplasm than granulosa cells. Immunohistochemistry revealed AMHRII staining in the oocyte and granulosa cells. In mammals, AMH expression is elevated during periods of reproductive dormancy, possibly protecting the ovarian reserve. To assess the expression of AMHRII and AMH during a similar state in chickens, we evaluated mRNA expression in a broiler strain and an egg-laying strain of hens in different reproductive states. AMH and AMHRII mRNA were significantly higher ($p<0.05$) in non-laying ovaries of broiler hens. In molting (with documented feather loss) and non-molting layer hens, AMHRII mRNA was significantly greater ($p<0.05$) in molting hen ovaries. These results suggest AMH may contribute to the intra-follicular bidirectional communication between oocyte and granulosa cells, and support a potential role of AMH in limiting follicle recruitment in hens.

BIOGRAPHICAL SKETCH

Rachel A. Lemcke attended secondary school in north metro Atlanta, Georgia, and received a B.S. in Animal Science and a Minor in Business from Berry College in northwest Georgia. While at Berry College, she studied under Dr. Jay Daniel and Dr. Kyle Caires, and developed her interests in scientific research, ranging from quantitative genetics and population structure to reproductive physiology, spermatogenesis and immunohistochemistry. She presented work on the population structure and genetic diversity of American Angus cattle at the 2009 American Society of Animal Science, Southern Section meeting. In August 2013, she accepted a graduate student position at Cornell University in Ithaca, NY, and joined Dr. Patricia Johnson's laboratory in the spring of 2014 to begin work on identifying a hormone receptor in poultry. Outside of research, her interests include dressage, equine coat color genetics, and equine biomechanics.

DEDICATION

The author would like to dedicate this Thesis to her best friend and supportive boyfriend, Mark, her academic and personal mentors--especially Dr. Kyle C. Caires, and devoted family and loved ones who help reaffirm and encourage her and make life colorful. This thesis is also dedicated in part to the author's maternal grandparents, Dr. Paul D. Drechsel and Mrs. Ruth Anne Drechsel, both of whom were Cornell University graduates, Class of 1951 and 1950, respectively, and members of Phi Beta Kappa Society. She guesses neither much enjoyed orchid petal soup. Finally, the author dedicates this thesis to the late, brave Eva C. Conant, DVM, with whom the author enjoyed a friendship and shared a mutual love of horses.

The Woman Who Thinks She Can

If you think you are beaten, you are;
If you think you dare not, you don't.
If you'd like to win, but you think you can't,
It is almost a cinch that you won't.

If you think you'll lose, you're lost;
For out of the world we find
Success begins with a fellow's will
It's all in the state of mind.

If you think you're outclassed, you are;
You've got to think high to rise.
You've got to be sure of yourself before
You can ever win the prize.

Life's battles don't always go
To the stronger or faster woman;
But sooner or later the woman who wins
Is the one who thinks she can!

- Adapted from Walter D. Wintle

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LIST OF ABBREVIATIONS

AMH	Anti-Müllerian Hormone; MIS; MIF
$AMH_{N,C}$	Noncovalent Complex of N- and C-Terminus Units of Anti-Müllerian Hormone
AMHRII	Anti-Müllerian Hormone, Receptor Type II
BMP	Bone Morphogenetic Protein
bp	Base Pair
cAMP	Cyclic Andosine 5'-Monophosphate (AMP)
cDNA	Complementary DNA
CYP19A1	Cytochrome P450, Family 19, Subfamily A, Polypeptide 1
CYP11A	Cytochrome P450, Family 11, Subfamily A, Polypeptide 1
DAX1	Dosage-sensitive Sex Reversal, Adrenal Hypoplasia Critical Region on Chr. X gene 1
DNA	Deoxyribonucleic Acid
dpc	Days Post Coitum
DMRT1	Double sex and Mab-3-related transcription factor 1
En	Embryonic day n
FF	Full-fed Broiler Breeder Hens (*ad libitum* food intake*)*
FSH	Follicle Stimulating Hormone
FSHR	Follicle Stimulating Hormone Receptor
FOG2	Friend of GATA 2
GATA	Transcription Factor Family That Binds the Regulatory Element "GATA"
GDF9	Growth and Differentiation Factor 9
GH	Growth Hormone
GnRH	Gonadotropin Releasing Hormone
IEX-1S	Radiation-inducible Immediate-early Gene IEX-1
ID3	Inhibitor of DNA-binding Protein 3
IGF1	Insulin-like Growth Factor 1
IHC	Immunohistochemistry
ISH	*In Situ* Hybridization
IVF	*In Vitro* Fertilization
LH	Luteinizing Hormone
MIS, MIF	Müllerian Inhibiting Substance/Factor

LIST OF ABBREVIATIONS *(continued)*

MMP2	Matrix Metalloproteinase-2; Type IV Collagenase
mRNA	Messenger RNA
OCM	Oocyte-Conditioned Medium
PMDS	Persistent Müllerian Duct Syndrome
proAMH	Precursor Anti-Müllerian Hormone
RF	Restricted-fed Broiler Breeder Hens
rhAMH	Recombinant Human Anti-Müllerian Hormone
RNA	Ribonucleic Acid
RNA-seq	RNA Sequencing
SF1	Steroidogenic Factor 1; Nuclear Receptor Subfamily 5, Group A, Member 1; NR5A1
shRNA	Short Hairpin RNA
SOX8	SRY-related High Mobility Group (HMG) box 8
SOX9	SRY-related High Mobility Group (HMG) box 9
SP600125	c-Jun N-terminal kinase inhibitor II (SP600125); JNK II Inhibitor
SRY	Sex-Determining Region on Y
StAR	Steroidogenic Acute Regulatory Protein
TCF-4	Transcription Factor 4; Immunoglobulin Transcription Factor 2 (ITF-2)
TCM	Testis-Conditioned Medium
TGF-β	Transforming Growth Factor-β
WIF1	WNT Inhibitory Factor 1
WNT	Glycoprotein Family Widely Involved In Cell Structure and Cell-Protein Signaling
WT1	Wilms' Tumor 1

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CHAPTER I

REVIEW OF LITERATURE

I. Introduction

Successful, efficient reproduction is critical to sustained profitability of animal production systems. Although extensive research into reproductive pathways has yielded significant insight into the mechanics of this complex system, the cryptic regulation behind this intricate network is not fully elucidated. Over the last several decades, extensive research has increasingly recognized Anti-Müllerian Hormone (AMH) as an essential regulator of prepubertal and adult gonadal growth and function, in addition to its previously known role in sexual differentiation during embryonic development.

Alfred Jost discovered AMH in the late 1940's, calling it "*l'hormone inhibitrice*" as it was originally suspected of inhibiting Müllerian duct development in embryonic males. It was subsequently named Müllerian Inhibiting Substance or Factor (MIS, MIF) after 19th century comparative anatomist Johannes Peter Müller[1,2]. In addition to müllerian duct regression, AMH helps maintain the ovarian reserve, regulates folliculogenesis, and modulates steroidogenesis within both gonadal environments. Current research points to AMH action extending beyond the local gonads, as several studies reveal hormone action or hormone-receptor colocalization in the pituitary[3], brain[4,5,6], placenta[7], uterus[8], prostate[9], and lung[10]. Additionally, AMH has recently grown in importance in regards to human and animal health, due to its diagnostic uses for *in vitro* fertilization techniques[11,12,13,14,15], granulosa cell neoplasia[16,17,18,19,20] and evaluating the ovarian reserve[21,22,23,24], as well as for its suspected roles in endometriosis[8,25] and polycystic ovary syndrome[26,27,28,29].

II. Mammalian AMH

Location and Structure of AMH Gene in Mammals

First sourced from newborn calf testes[30], a 2.1-kb cDNA fragment encoding AMH was cloned and purified. The gene structure and sequence of AMH resembled that of other growth factors or inhibitors[31], leading researchers to incorporate AMH into the large Transforming Growth Factor-β (TGF-β) family[31,32]. Early analysis revealed AMH to be a disulfide-bonded[32] homodimeric glycoprotein with lectin affinity[33]. Evidence suggested multiple forms of the hormone existed, as molecular weights ranged from 12.5-57 kDa[31] to 70-74 kDa[30] to 120-300 kDa[33] depending on fractionation technique[34,35]. The human AMH precursor gene is located on chromosome 19 (NCBI: NC_000019.10 (2249114..2252073)) and is five exons long with 71% GC content across introns and exons. The first 25 nucleotides encode a signal peptide that is not well-conserved across species. The human and mouse genes encoding the AMH precursor form are 560 and 555 amino acids, respectively, and are relatively conserved (72%) across the entire peptide. In non-mammals, such as the zebrafish and the hen, AMH precursor sequence homology drops to 41% and 47%, respectively, compared to human. To date, AMH or an AMH homologue has not yet been identified in organisms outside of the phylum *Chordata*.

AMH Expression and Involvement During Embryogenesis

During mammalian embryogenesis, fetal testes produce large quantities of AMH, which helps facilitate sexual differentiation in males by regressing the müllerian or paramesonephric duct, the precursor tissue that forms the uterus, oviducts, and cranial vagina in females[32,36]. AMH RNA and protein have been detected within the rough endoplasmic reticulum and golgi apparatus[37] in cytoplasm of immature Sertoli cells[38] in fetal or early prepubertal calves[30,32,37,39], sheep, goats, pigs, humans, rats[36,40,41], rabbits, cats, and chicks. *In situ* hybridization (ISH) also

showed positive AMH signal in spermatocytes[41]. AMH mRNA expression has been identified in rat and mouse tissue along the urogenital ridges and within testes as early as embryonic day 15[42], and 12.5 dpc[40], respectively. On embryonic day 16 in the male rat, AMH mRNA visualized using ISH was present surrounding the müllerian duct[42], which undergoes subsequent regression through embryonic day 19[43]. During regression, lysosomes enter the targeted cells, followed by macrophagic activity. Subsequent characteristic whorled patterns arise within the degenerating müllerian duct as the extracellular matrix degrades[44,45]. Similar timing was observed *in vivo* during regression within Chinese hamster ovary (CHO) cells transfected with recombinant-human AMH (rhAMH)[30]. AMH transplantation via renal capsules into nude mice also successfully regressed the müllerian duct[46]. AMH testicular production decreases postnatally throughout the prepubertal period[40,47] with low but discernible levels found in adult testes[48,49,50].

In the mammalian female, biological activity of AMH is generally accepted as absent at the embryonic stage in most species evaluated[32,36,40,51]. Exogenous AMH introduced *in vivo* and *in vitro* to embryonic females resulted in several reproductive abnormalities. Freemartinism, a physiological condition that occurs within females when male and female fetuses connect via anastomosis[52,53], is typically characterized by masculinized, smaller ovaries and potential seminiferous tubule or sex-cord development[47,52,54,55]. The predominant cause of freemartinism is considered to be vascular introduction of AMH produced by the male fetal testes[56]. Serum from freemartins also revealed higher AMH levels[57] than normal females, and freemartin gonads produced much less AMH than their male counterparts[56]. Additionally, the freemartin female becomes chimeric, and some chimeric cells within freemartin gonads express AMH protein[58,59]. Physiologically normal male and female fetuses have been observed if joint vascularization fails to occur, providing additional support that AMH is the causative factor[60,61].

Seemingly related to placentation type, a freemartin female can occur if a chorion is vascularly shared with a male fetus between 50 and 80 days gestation (in bovine)[56,60]. Freemartins have been primarily reported in bovine[59,61] and ovine[62] fetuses, and can exhibit a wide scale of reproductive anomalies[59,63]. Some freemartin females form intermediate or intersex[58] gonads, while others develop smaller, masculinized ovaries or small, under-developed testes[62]. Steroidogenesis (i.e. estrogen production, etc.) in the freemartin ovary is also affected accordingly[61,64].

In addition to those effects witnessed in natural and induced[64] freemartins, germ cell depletion occurred *in vitro* when fetal rat ovaries were cultured with bovine AMH[54]. However, naturally-occurring AMH with observable bioactivity during female mammalian embryogenic development has been reported. Researchers detected AMH protein expression within embryonic ovine ovaries beginning at 120 days gestation, concomitant with observed follicular development[65]. In sheep, production of AMH late in gestation is unlikely to interfere with sexual differentiation, which occurs between days 30 and 85 gestation in fetal sheep[66]. Granulosa cells from preantral follicles displayed faint AMH immunoreactivity, parallel to results observed in granulosa cells of prepubertal and adult females.

AMH Expression in Adult Female Mammals

The postnatal bioactivity of AMH in female mammals is well documented. The presence and production of AMH coincides with follicular growth. Observations suggest AMH production within granulosa cells corresponds with increased follicular development, as strong ISH signal was seen from growing small preantral and antral follicles of mice[40], sheep[65] and cows[67], and within primordial follicles in primates[68]. AMH RNA or protein expression has been repeatedly detected in cytoplasm of granulosa cells in prepubertal and adult females[42,33,36], as early as three

and six days postpartum in the rat[36] and mouse[40], respectively. It should be noted that AMH has been localized via *in situ* hybridization and IHC within rat[69] and caprine[209] follicles, respectively. A signal gradient has occasionally been observed within the granulosa cell layer, with intensity highest in cells nearest the antrum[40,65,70] of larger follicles, while homogenous AMH distribution[36] throughout the granulosa cell layer has also been reported. This potential gradient towards the oocyte occurred alongside AMH secretion into follicular fluid within the antrum, suggesting AMH could have biological functions within developing follicles[71,72]. In adult females, AMH production continues during folliculogenesis into the pre-ovulatory phase[73,74], and steadily declines overall as the ovarian reserve diminishes until menopause[21,24].

AMH-null Mouse Model

AMH-deficient mice were developed to better understand the specific role of AMH *in vivo*. A neomycin resistance expression cassette was inserted via electroporation into embryonic mouse stem cells, replacing 0.6-kb of the mouse AMH gene (part of Exon 1, and all of Intron 1 and Exon 2)[75]. These mutant stem cells were then injected into C57BL/6J mice wild-type, which were then bred to B6/129 wild-type mice to generate chimeras[75]. To evaluate dosage response of AMH, AMH heterozygous ($AMH^{+/-}$) mice were bred in addition to AMH null ($AMH^{-/-}$) mice. Southern blot DNA analysis validated the successful transfection, showing a band corresponding with AMH present in wild-type and $AMH^{+/-}$ mice, and a band for the inserted neomycin cassette in both the $AMH^{+/-}$ and $AMH^{-/-}$ mice.

While $AMH^{+/-}$ males were morphologically normal and capable of fathering pups[75], $AMH^{-/-}$ males expectedly developed components of the female reproductive tract: the müllerian duct developed into a partial vagina, cervix, uterus and oviducts[21]. This morphology, termed Persistent Müllerian Duct Syndrome (PMDS), characterized by retained müllerian-derived

tissues that develop alongside normal male tissues, has been infrequently reported in human males. Additionally, Leydig hyperplasia was occasionally observed in $AMH^{-/-}$ males, with one observation of Leydig neoplasia.

A dosage effect observed in AMH transgenic males revealed $AMH^{-/-}$ males had azoospermia and therefore reduced fertility when compared to $AMH^{+/-}$ and wild-type males. However, spermatogenesis did not appear to be affected, as sperm collected from the epididymides of $AMH^{-/-}$ males were able to successfully fertilize wild-type embryos via IVF and produce pups[75]. Researchers concluded that AMH was not necessary for male germ cell development, theorizing successful fertilization was physically blocked due to the presence of additional female organs and structures. Infertility was reversed when a transgene chronically overexpressing human AMH was inserted into $AMH^{-/-}$ males. Additionally, the majority of these $AMH^{-/-}$ males exhibited no müllerian duct-derived tissue following the procedure[76].

Female AMH transgenic mice were also affected. $AMH^{+/-}$ females were viable, fertile and phenotypically normal[75]. $AMH^{-/-}$ females displayed morphologically normal reproductive tracts, with granulosa cells of comparable quantity and histological properties relative to $AMH^{+/-}$ and wild-type females. However, folliculogenesis in AMH transgenic mice was significantly altered. $AMH^{+/-}$ and $AMH^{-/-}$ female mice were analyzed relative to age-matched wild-type mice[21]. In prepubertal females, no significant difference was found between primordial follicle populations of $AMH^{-/-}$ and $AMH^{+/+}$ mice, but 4- and 13-month old $AMH^{-/-}$ mice had significantly fewer remaining primordial follicles than their wild-type counterparts[21]. Females exhibited an AMH dosage effect, and $AMH^{+/-}$ females had an ovarian reserve of intermediate size among the genotypes[21]. Serum inhibin and FSH levels corresponded with progressive follicular development in $AMH^{-/-}$ females relative to wild-type females: inhibin was two-fold higher and

lower in 4- and 13-month old $AMH^{-/-}$ females, respectively, and FSH was lower in 4-month old $AMH^{-/-}$ females. All three groups exhibited regular estrous cycles of equivalent length and comparable numbers of corpora lutea[21]. Uterine weight was also similar among genotypes. Further analysis to explain the relatively equal number of corpora lutea revealed increased atresia and oocyte degeneration in non-wild-type females[77].

Mouse Model Overexpressing AMH

A mouse model chronically overexpressing AMH was designed to help characterize the *in vivo* role of AMH during embryonic sexual development. The metallothionein-1 promoter joined to the human AMH gene was introduced via pronuclear injection[76]. Male and female transgenic mice showed reproductive abnormalities of varying severity, attributed to germ-line mosaics producing varying AMH serum concentrations. Two sterile founder females lacked ovaries, oviducts and uteri, but founder males were fertile[76]. However, female offspring from those founder males continued to exhibit morphological deformities similar to the founding females. Ovaries were present in females with lower AMH serum concentrations. Female offspring from the mouse line with the highest AMH serum concentration were evaluated throughout different developmental stages to temporally chronicle changes in reproductive tissues. Two days after birth, no uteri were observed, and ovaries, although present, contained fewer germ cells than controls. By nine days postpartum, no follicles were present and zero germ cells remained. While the gonads had by this time developed structures analogous to seminiferous tubules, no gonadal structures remained by adulthood in female transgenic mice[76].

Some of the male offspring chronically expressing high AMH serum concentrations displayed reproductive abnormalities as well. Several males exhibited broad signs of feminization, including mammary gland development and a vaginal opening[76], as well as

undescended testes lacking a germ cell population, and poorly developed accessory male tissues. Based on these results, researchers hypothesized AMH might help regulate Leydig cell development and function by reducing androgen production.

AMH transgenic male mice exhibit physiological anomalies apart from changes within reproductive tissues. In addition to its direct effects on the müllerian duct, AMH is believed to contribute to systemic sexual differentiation beyond the local testes[5,78]. Researchers hypothesized AMH exerts sex-dependent, neuronal influences to varying degrees based on systemic AMH circulation during embryonic and early prepubertal development[5,79,80]. Circulating AMH levels can be sexually dimorphic: AMH serum levels in young boys have reported as highly variable[81,82], yet were quite consistent in young girls[81]. Androgen-dependent, neuronal masculinization during development has already been established across multiple species, though the contributions of AMH during this process are still cryptic[66,83,84].

AMH Involvement in Non-reproductive Mammalian Tissues

AMH protein has also be identified in neurons, and has since been shown to be potent survival factor *in vitro* for cultured lateral motor column (LMC) neurons[4,78,85]. A gender-based phenomenon has been observed in mouse neurons: $AMH^{-/-}$ male mice develop less LMC neurons than wild-type males[5], while comparable neuron counts were found between $AMH^{-/-}$ males and wild-type females. Evaluations of newborn (D 0) AMH transgenic mice from the original AMH transgenic mouse colony revealed LMC neuron quantity decreased significantly per AMH copy removed. $AMH^{+/-}$ and $AMH^{-/-}$ males contained intermediate and feminized numbers of motor neurons, respectively[5]. Wild-type females had significantly fewer LMC neurons than wild-type males[5]. Similarly, sex-biased expression was observed in calbindin-positive (CP) neurons in prepubertal wild-type mice with increased CP neuron quantities in males, but the sex-bias was

absent within $AMH^{-/-}$ prepubertal mice[85]. However, adult $AMH^{-/-}$ males developed additional CP neurons as they aged, though wild-type quantities were not restored.

In addition to local and systemic sexual differentiation, AMH may have roles within several other tissues, including the prostate[9], placenta[7] and uterus[8]. A more well understood role of AMH is within the immature embryonic lung, where AMH has been shown to negatively affect embryonic lung maturation in males[86,87]. Mouse embryonic lung tissues were investigated *in vitro*, due to their spatial proximity to the urogenital ridge from 13.5 dpc through 15 dpc[10]. Embryonic lung tissues (13.5 dpc) cultured with two different rhAMH concentrations both exhibited significantly less lung development compared to control and vehicle buffer culture mediums[10]. Observations included fewer lung bud counts, decreased explanted lung perimeter length (i.e. branching) and increased apoptosis. These *in vivo* effects provide morphological and histological details supporting sex-dependent embryonic lung development and maturation.

AMH Biochemistry and Signaling Pathway

While AMH affects multiple tissues across the body, its employs a consistent biochemical mechanism of action to exert biological influence. Akin to other TGF-β family members, AMH elicits cellular changes via a SMAD signaling pathway. Secreted as a 140 kDA[30] homodimeric glycoprotein, specific proteolytic cleavage must first occur to ensure efficient bioactivity and receptor reactivity[88]. In humans, cleavage of the AMH precursor (proAMH) occurs at 109 amino acids upstream from the carboxyl terminus, forming two 25 kDA C-terminus dimers[89]. Subtilisin/kexin-type proprotein convertases (PCSKs) or serine proteases can facilitate initial proteolytic cleavage of proAMH[89,90]. However, AMH undergoes a variation[88,91] of cleavage different than TGF-β: the 70 kDA N-terminus (pro-region) of AMH remains connected to the 25 kDA C-terminus dimer, forming a noncovalent complex ($AMH_{N,C}$)

that subsequently binds a receptor then dissociates[91]. BMP-7[92] and BMP-9[93] utilize similar dimerization, but other TGF-β family members[88], including TGF-β, GDF-8 and BMP-2, require the noncovalent complex to first dissociate prior to receptor binding[91].

Bioactivity of AMH varies based on cleavage locations and dimerization. While $AMH_{N,C}$ has been shown to have the greatest bioactivity of the AMH forms, proAMH is still bioactive, but to a much lesser extent[91]. In the blood, proAMH is not easily cleaved by proteases[94], but evidence exists demonstrating some enzymes locally accessible or native to the target tissue can and do cleave AMH, increasing $AMH_{N,C}$ concentration at the target site[95,96]. Researchers have shown proAMH is the primary AMH biochemical species within ovine follicular fluid and granulosa cell[97] cytoplasm and nuceli[98]. Both proAMH and $AMH_{N,C}$ are found in adult human serum, but $AMH_{N,C}$ is the predominant form[99]. The half-life of rhAMH, evaluated using rh-proAMH and rh $AMH_{N,C}$ injected into mice, were quantified using a two-phase elimination curve. The assay solely measured the rhAMH, not the mouse AMH native to the animals. In the slow phase, the half-lives for rh-proAMH and $rhAMH_{N,C}$ in mice were 2.4 and 3.8 hours, respectively, while the fast-phase half-lives were 6 minutes and 11 minutes, respectively[94]. The half life of AMH in other species, including calves[100] and women[101], has been reported at approximately two days and just over one day, respectively, with castrations and hysterectomies immediately preceding the half-life evaluations.

Additionally, *in vivo* AMH protein variations have been identified. Three human AMH splice isoforms have been documented, but their biological activity and relevance have not yet been characterized. Several variations or mutations exist within the human AMH promoter region or whole gene that severely limit AMH bioreactivity[102,103], while other mutations do not appear to elicit a change of function[104]. AMH dimerization capabilities or proper transcription

factor signaling are likely reduced in loss-of-function cases. Furthermore, studies focused on determining AMH serum concentrations have been hampered by challenging assays. Precisely analyzing accurate AMH concentrations in serum has been difficult due to inclusion or exclusion of the cleaved-yet-associated forms of AMH. These complications[105] contributed to the impaired sensitivity and repeatability throughout several assay generations and modifications[106,107,108,109,110,111], at times imparting dubious biases[112,113,114], but newly updated assays equipped to handle these technicalities are being incorporated into research protocols. Also, the various cleaved forms of AMH can now be studied separately or in combination to better elucidate its mechanism of action[115].

Once cleaved, AMH utilizes two TGF-β serine-threonine domain receptors[116,117]. First, AMH binds to the extracellular portion of its specific[118] primary receptor, AMH Type II Receptor (AMHRII), which subsequently phosphorylates one of three non-specific Type I receptors: ALK2/ACVR1[119,120], ALK3/BMPR1A[121,122] or ALK6/BMPR1B[123]. Once AMH binds both TGF-β receptors, the AMH-receptor complex triggers the SMAD signaling cascade, phosphorylating three receptor-regulated Smads (R-Smads) within the cellular cytoplasm: Smad1, Smad5 and Smad8[20,123,124,125,126]. The three activated R-Smads then complex with common-mediator Smad4, stimulating active transcription factor formation within the nucleus[127].

Type I receptor recruitment by AMH appears to be target-tissue specific[121]. Regressed müllerian ducts in ALK6 null male mice suggest localized redundant or tissue-specific receptors[120]; indeed, ALK2 has been shown to compensate in ALK6 null males[120,124]. Researchers determined ALK3 is also required for müllerian duct regression[128]. Preferential phosphorylation of candidate secondary receptors also exists within granulosa and Leydig cells, observed during ALK3 knock-down experiments in mice. Granulosa cells from prepubertal

ALK3 knock-down mice were unresponsive to AMH[122], and steroidogenesis decreased within Leydig cells, leading to plasma androgen levels equivalent to control mice[121].

Regulation of AMH in Mammals

AMH regulation in mammals has not been fully elucidated. The human AMH promoter, first thought to be well under 450 bp in length, has since been determined to be considerably longer. Experiments conducted utilizing 202-, 423-, and 3-kb length human AMH promoters within SMAT1 Sertoli cell lines incubated with cAMP revealed significantly greater luciferase activity with the 3-kb promoter, suggesting additional transcription factor binding sites exist farther upstream than originally suspected[129]. Several binding sites identified within the human AMH promoter have been evaluated for their potential roles in transcription and mechanism of action of AMH. SRY, a master sex-specific gene underlying the male sex-determination cascade, binds to a site -145 bp upstream of the AMH transcription start site[130,131,132]. In the embryonic testis, SRY mRNA production begins approximately 10.5 dpc in the mouse[133], declining shortly after 12 dpc until cessation by 13.5 dpc[134]. AMH mRNA transcripts are detectable within a day following the initiation of SRY production, and reach and sustain high levels after 12.5 dpc[133]. Mutagenesis of the SRY binding site did not eliminate AMH transcription, suggesting SRY may influence AMH indirectly[135].

SOX9, a second transcription factor important for AMH bioactivity, binds 141 bp upstream of the AMH transcription start site. In the testis, cAMP and PKA help facilitate the cytoplasmic-to-nuclear translocation of SOX9 protein within Sertoli cells prior to the onset of AMH protein expression; in females, this protein repositioning fails to occur[136,137,138]. Corruption of the SOX binding site inhibits AMH transcription and müllerian duct regression, leading researchers to conclude SOX9 is required for AMH transcription in mammals[103]. However,

evidence exists of SOX8 binding in lieu of SOX9, resulting in attenuated AMH transcription levels[139,140]. Transgenic mice lacking either SOX8 or SOX9 develop spermatogenesis defects, likely caused by the dissolution of the basal lamina[141], and $SOX8^{-/-}$/$SOX9^{-/-}$ double mutant males exhibit signs of feminization, as evidenced by up-regulation of ovarian markers[142]. Within the ovary, SOX8 mRNA expression did not colocalize with AMH in granulosa cells of preantral follicles, but was instead observed within oocytes[143]. In preovulatory follicles, SOX8 mRNA expression was evident in both oocytes and granulosa cells, and was significantly greater in mural granulosa compared to cumulus cells.

Another transcription factor, SF1, acts synergistically alongside SOX8 or SOX9 to upregulate AMH transcription[139,144]. Two functional SF1 binding sites have been identified within the human AMH promoter at -92 and -218 bp, and AMH and SF1 colocalizes at E12.5 in embryonic testes[145,146]. Disruption of one SF1 binding site failed to block müllerian duct regression, but AMH transcription was greatly reduced[103]. When both sites were mutated, AMH transcription was repressed[145]. Additionally, SF1 exhibits sexually dimorphic expression patterns in rats[145,147]. In males, SF1 RNA transcript levels sharply increase parallel to müllerian duct regression, while SF1 transcript levels steadily decline in females until parturition[146].

SF1 also plays a critical role within the postnatal ovary. Produced in granulosa cells[148,149], SF1 regulates steroidogenesis by helping facilitate cellular cholesterol uptake and processing[150,151,152], and induces transcription via binding to the promoters of CYP19A1[152], CYP11A[153], oxytocin[154], prolactin[155] and StAR[156], in addition to AMH. $SF1^{-/-}$ female mice displayed hemorrhagic ovarian cysts along with sterility[157]. When SF1 production was knocked down in granulosa cells, $SF1^{-/-}$ females failed to produce corpora lutea and folliculogenesis was significantly reduced[158]. AMH tRNA expression showed a dose-dependent response to SF1: 48

hours following eCG treatment, $SF1^{-/-}$ females produced only basal AMH levels, and $SF1^{+/-}$ females exhibited intermediate AMH levels relative to wild type females[158]. Additionally, ovaries from $SF1^{-/-}$ females contained significantly fewer numbers of remaining primordial follicles, providing another indication of the importance of SF1 in the AMH pathway.

Bone morphogenetic proteins (BMP), a TGF-β subfamily, can upregulate AMH promoter activity. Several BMP family members, including BMP-2, -6, -7 and -15, have been shown to increase AMH mRNA and protein levels within granulosa cells[159,160]. In the presence of BMP-4, inclusion of the SF1 and SMAD1 binding regions within the AMH promoter have been shown to significantly stimulate AMH transcription versus exclusion of those regions[161]. This evidence suggests members of the BMP family may interact with other transcription factors to regulate AMH, although the mechanism of action remains unknown.

Two additional transcription factors, GATA4 and WT1, are capable of working in collaboration with[162,163,164] or independent of[165,166,167] SF1 to up-regulate AMH transcription. Mutations in SF1 prevent synergistic activity with GATA4[168]. The AMH promoter contains two GATA regulatory elements at -75 and -168 bp that are necessary for full AMH transcription; targeted mutagenesis to either GATA binding site severely impaired or eliminated this process[145,167]. In males, GATA4 protein increased within Sertoli cells during embryonic development, but decreased in females following ovarian differentiation[167,169]. In the adult ovary, GATA4 mRNA, as well as mRNA of family member GATA6, has been located within nuclei of granulosa cells in primary and antral follicles, as well as theca cells and other ovarian tissue[170,171,172,173]. Ovarian dynamics are severely affected when GATA4 and GATA6 are simultaneously silenced, including retained oocyte nests, decreased primordial follicle activation and diminished developmental capacity of oocytes concomitant with poor

folliculogenesis[174,175,176]. Such observations suggest a level of functional redundancy between these two GATA family members; indeed, eliminating either GATA4 or GATA6 did not result in such widespread deleterious effects[175]. However, GATA1 can also bind both GATA regulatory elements within the AMH promoter, and increases during the onset of meiosis while AMH levels decline, suggesting GATA1 may help curtail AMH transcription[177].

FOG2, a GATA4 cofactor, is present in the fetal and postnatal ovary. In the embryonic testis, FOG2 decreases with sex cord development, suggesting FOG2 may play a greater role within the ovary. Indeed, a dosage effect for both FOG2 and GATA4 has been implicated in sex reversal in mice[178]. FOG2 protein expression has been found in granulosa and theca cells[143,173,179]. While the FOG2-GATA4 complex upregulates SRY[180] and SOX9[181] transcription, it has been shown to repress AMH transcription *in vitro* in the presence of GATA4[179]. FOG2 might therefore modulate the effects of GATA4 activation of AMH within the ovary.

In men, mutations within the transcription factor WT1 result in retained müllerian duct tissue, prompting researchers to analyze the role of WT1 within the AMH pathway. WT1 binds to a site within the AMH promoter, increasing AMH transcription[182]. WT1 can coordinate with both GATA4 and SF1 to regulate AMH transcription, but if either the GATA4 or WT1 binding sites within the AMH promoter are mutated, the synergistic activity is lost[183]. This cooperative effort between GATA4 and WT1 also up-regulates SRY[183]. In the ovary, WT1 mRNA colocalizes with AMH within granulosa cells, suggesting WT1 likely also regulates AMH transcription in females[143]. Additionally, while multiple WT1 isoforms have been identified, isoforms lacking the tripeptide KTS yield conflicting effects on AMH transcription[164,165,183]. Furthermore, the synergistic complexes formed with SF1 are disrupted by DAX1, allowing for negative regulation of AMH transcriptional activation. DAX1 interferes with SF1, reducing the

capacity of SF1 to synergize with WT1[164] or GATA4[184]. DAX1 mRNA colocalizes with SF1 and GATA4 within granulosa and Sertoli cells, providing a sex-independent mechanism to modulate AMH transcription[143,184].

In addition to the sex differentiation genetic pathway, endogenous hormonal factors contribute to AMH regulation, although the mechanisms utilized by hormone signaling pathways to modify AMH remain unclear. Estradiol functions through two follicular receptors, ERα and ERβ, which are predominantly found within thecal and granulosa cells, respectively. Estradiol has been shown to influence AMH transcription *in vitro* through an abridged estrogen response element site within the AMH promoter at -1772 bp. When estradiol signals through ERβ, AMH transcription declines; however, if estradiol instead utilizes ERα, AMH promoter activity increases[185]. This evidence suggests follicular estradiol production may promote folliculogenesis by transitioning its effect from boosting to curtailing AMH within developing follicles. Additionally, estradiol may only exert an indirect influence on AMH, as the partial estrogen binding site within the AMH promoter does not appear to share high affinity with ERβ[185]. Furthermore, the addition of FSH accompanying ERβ in the absence of estradiol yielded more than a three-fold increase in AMH promoter activity, yet FSH stifled AMH transcription in the presence of ERβ coupled with estradiol[185].

The interaction between FSH and AMH has been evaluated within testicular and ovarian dynamics. Within the prepubertal testis, FSH stimulates AMH secretion prior to androgen receptor development, suppressing steroidogenesis via reduced aromatase activity by lowering aromatase mRNA levels[186,187,188,189,190]. In this manner, AMH temporarily inhibits testosterone synthesis[191]. Once Sertoli cells gain functional androgen receptors, intratesticular testosterone downregulates AMH, attenuating the stimulatory effect of FSH[192]. Likewise, FSH upregulates

AMH transcription within granulosa cells *in vitro*[193]. To induce these effects, FSH utilizes cAMP and the PKA pathway[194]. The 3-kb AMH promoter fails to contain a classical cAMP regulatory element (CRE), though two cAMP-responsive sites, NF-κB and AP2, have been identified[138,195]. These two sites work in concert with GATA4 and SF1 to facilitate a response to cAMP stimulation (Figure 1.1).

The hormonal components of the hypothalamic-pituitary axis have also been implicated in AMH regulation. GnRH agonists briefly stimulated then reduced AMH levels in primary and antral follicles[196], and decreased AMH concentrations within follicular fluid[197]. While the mechanism GnRH employs to curtail AMH is unknown, GnRH has been shown to decrease AMH receptivity[3]. Pregnant rats immunized against GnRH systemically increased AMH in offspring[198]. Similarly, growth hormone (GH) treatment reduced AMH serum concentration, but the addition of GH-agonist resulted in AMH serum levels comparable with controls[199]. Downregulation of AMH by GH may occur through the JAK2/STAT3 signaling pathway. Additionally, evidence exists of the metabolic pathway affecting AMH production. In rats, over-nutrition during gestation or immediately following birth altered AMH secretion and folliculogenesis within offspring, but the direct changes that modified AMH are unclear[200]. However, leptin is believed to utilize the JAK2/STAT3 pathway to repress levels of AMH mRNA in human granulosa cells[201]. Overall, the combined effects of these hormones on AMH production and function, as well as their mechanisms of action, are not resolved.

Downstream Targets of AMH in Mammals

The downstream targets of AMH remain largely uncharacterized. Evidence indicates AMH-induced müllerian duct regression results from a systematic cascade of events culminating in mesenchymal breakdown and ultimately apoptosis. AMH does not appear to

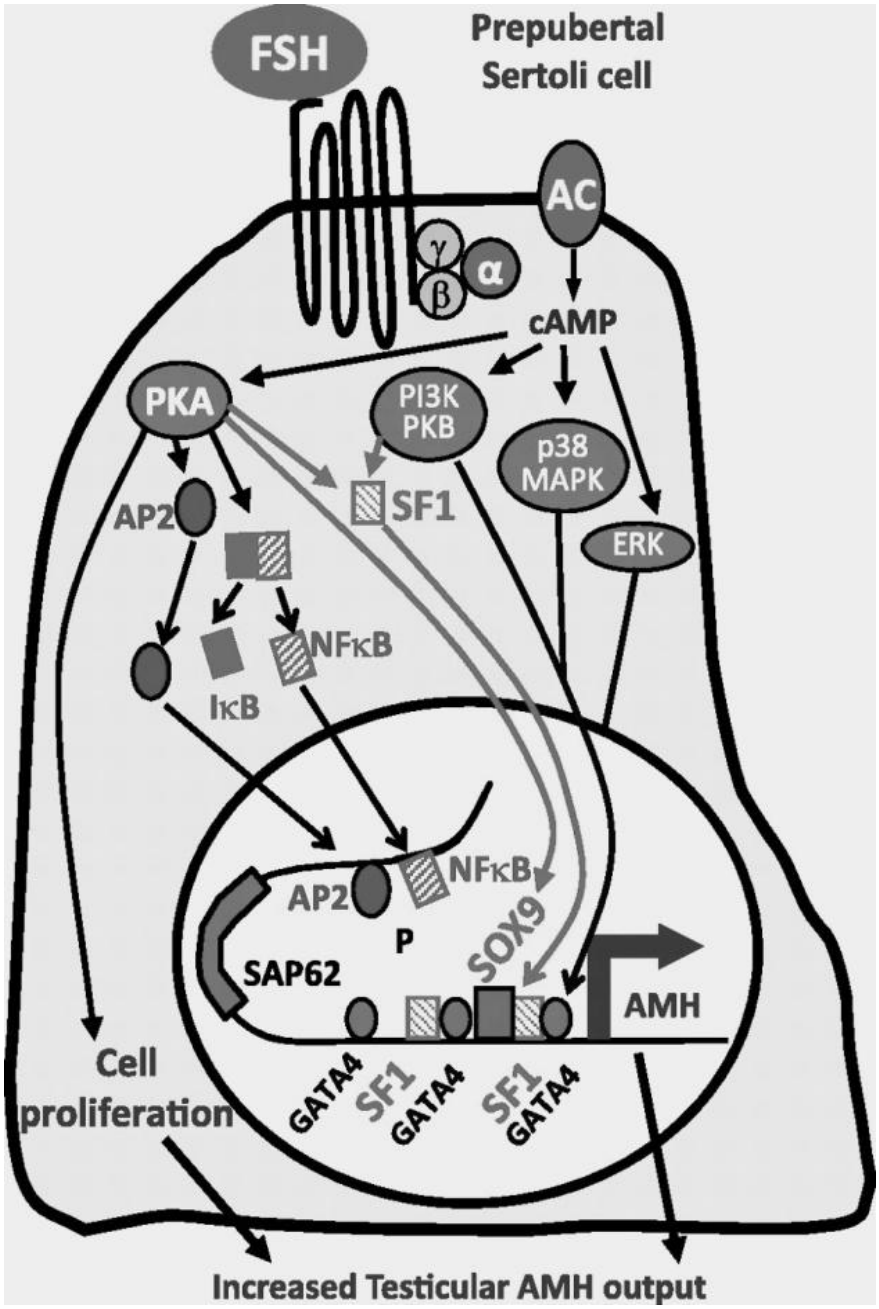

Figure 1.1 Adapted from Lasala, et al. 2011[138]. Schematic model to explain the increase of testicular AMH production following FSH stimulation. Several transcription factors that bind to the AMH promoter are also illustrated.

directly stimulate apoptosis within target cells[202]. However, AMH triggers a spatial relocation of AMHRII from epithelial to mesenchymal cells via ALK2, accompanied by autocrine regulation of the AMH pathway[123]. In mesenchymal cells, AMH suppresses the WNT/β-catenin (CTNNB1) pathway, which promotes proper spatial interaction between cells and cellular adhesion, by stimulating WIF1, a β-catenin inhibitor[203,204]. If the WNT/β-catenin pathway is not suppressed, the müllerian duct fails to regress. Granulosa cell tumors may develop if the WNT/β-catenin pathway is incorrectly regulated[17]. Similarly, AMH also targets another gene, matrix metalloproteinase-2 (MMP2), to degrade the extracellular matrix. MMP2 exhibits temporal- and male-sex-biased expression similar to AMH. Without AMH, male-biased MMP2 expression disappears; MMP2 exclusion impairs müllerian duct regression[205].

Within the testis microenvironment, AMH reduces steroidogenic activities within Leydig cells by blocking mRNA production and transcription of CYP17A1[190,194] and decreasing LH receptor mRNA[206,191]. As a result, AMH reduces testosterone synthesis[191]. AMH also lessens the upregulating effects of cAMP, although the precise interactions between AMH and cAMP in this physiological microenvironment are still undetermined[188,207,208].

The ovary represents another critical target site for AMH. In many species, one of the primary functions of AMH in the ovary is to suppress primordial follicle activation from the ovarian reserve[209,210]. AMH can inhibit meiosis within oocytes of some species[211] but knowledge of the mechanism of action in this context is highly limited. A secondary role of AMH is to help regulate the development of gonadotropin-dependent follicles. AMH has been shown to reduce FSH sensitivity within these follicles[97,212]. Two micro-RNAs, miR-181a and miR-181b, have recently been identified as targets of AMH; AMH upregulates these micro-RNAs, reducing intra-follicular cAMP levels and thus FSH sensitivity[213]. In addition, within granulosa cells,

exogenous AMH significantly upregulates expression of ID3, a gene involved in stem cell differentiation[122]. A third level of AMH involvement in the ovary targets periods of reproductive quiescence. During these times, AMH serum levels often increase, suggesting AMH guards the ovarian reserve during times of restricted fertility, further limiting follicular recruitment and development[72,214]. To date, the mechanism of action underlying this phenomenon has yet to be identified.

Several non-reproductive pathways and genes are recognized as targets of AMH action. Within several cancer cell lines *in vitro* and within the prostate *in vivo*, AMH has been shown to upregulate IEX-1S, a gene within the NF-κB pathway, stunting cell growth and stimulating apoptosis within the cell lines[9,215,216,217]. In the brain, AMH promotes transcription of neuroserpin, which targets and inhibits tissue-type plasminogen activator, a compound with deleterious effects on brain and nervous tissue function and longevity[6]. In immature female rats *in vivo*, AMH promotes FSH transcription via upregulating Fshb in the pituitary[218]. Within the pituitary and neurons, AMH and GnRH interacts in a conflicting manner. While AMH has been shown to increase LH secretion from GnRH neurons in mice[4], exogenous GnRH decreases AMHRII protein in the pituitary of female rats[218]. These findings may indicate the presence of a negative feedback loop involving gonadotropins and AMH between the gonads and pituitary.

III. Mammalian AMHRII

Location and Structure of Mammalian AMHRII

AMH requires a specific receptor AMHRII to facilitate its downstream effects. First identified in rat testicular tissue, a 1.9-kb cDNA encoding AMHRII was sequenced, which revealed several conserved regions characteristic of TGF-β type II receptor proteins, including a well-conserved series of serine-threonine kinase domains and cysteine residues[42,219]. In humans,

AMHRII is found on chromosome 12 (NCBI: NC_000012.12 (53423855..53431534)) and is 13 exons long with GC content of approximately 53% across introns and exons. AMHRII is comprised of 573 and 568 amino acids in the human and the mouse, respectively, with 81% homology between the entire peptides. The first three exons encode a signal peptide and extracellular domain, while the fourth and part of the fifth exons represent the transmembrane domain; the remaining exons form the intracellular C-terminus. The signal peptide and extracellular domains appear to be relatively species-specific, but homology across species increases dramatically within the biologically active C-terminus.

AMHRII Expression in Mammals

To date, AMHRII expression strictly occurs in association with concomitant AMH expression. Thus, AMHRII has not been observed temporally without impending or current utilization by AMH. The aforementioned temporal expression patterns of AMH coincide with AMHRII. However, AMH and AMHRII do not inherently share the same spatial expression. In embryonic males from E14-E16, AMHRII mRNA and protein are found in mesenchymal tissue and surrounds the müllerian duct to facilitate müllerian duct regression[219]. In addition, AMHRII has been observed via ISH in both the embryonic male (urogenital ridges) and female (ovary) rat on E15-16[42]. Postnatally, AMHRII mRNA is present within Leydig and Sertoli cells, remaining even after AMH declines[220,221]. AMH and AMHRII are co-expressed within follicles in granulosa cells in females[222], and also colocalize in GnRH neurons[4].

Regulation of AMHRII in Mammals

The AMH signaling pathway necessitates the mutually inclusive presence of both AMH and AMHRII, so the previously mentioned experiments utilizing AMH knockout mice theoretically represent the effects of inactivating AMHRII. Regulation of AMHRII is also similar

to that of AMH. The AMHRII promoter has been identified as 2252 bp in length, and BMP4 and BMP15 have been shown to upregulate AMHRII in both human and sheep granulosa cells[223]. In combination with exogenous AMH, c-Jun N-terminal kinase inhibitor II (SP600125), a reversible JNK inhibitor, synergistically upregulated AMHRII promoter activity[224].

Within a shorter (1136-bp) AMHRII promoter, regulatory sequences corresponding to two GATA, one SOX, one SF-1 (estrogen receptor half site), and two SP1 sites were identified[225]. The inclusion of binding sites for SF-1, the distal GATA, and SP1 increased AMHRII promoter activity, as observed via a luciferase assay[225]. Additionally, SF1 has been shown to synergistically upregulate activity of an 863-bp AMHRII promoter when coupled with β-catenin and TCF-4 (Transcription Factor 4). Without SF-1, however, β-catenin and TCF-4 still activate the AMHRII promoter. Although TCF-4 utilizes four binding sites, mutagenesis of each site revealed sites one and four to be functionally critical[226]. Furthermore, recent studies have identified a different method of negative regulation of AMHRII. Biological activity of AMHRII can be affected by mutations or SNPs within AMHRII[227], or from significant levels of post-translational processing[228,229]. These alterations can yield truncated or inactive forms of AMHRII with reduced AMH binding capacity, and may contribute to an autocrine manner of regulation of AMH and AMHRII.

IV. Avian AMH and AMHRII

Location and Structure of AMH in the Chicken

Isolated from eight week old chick testes, a 2846-bp cDNA was sequenced and identified as AMH. The chicken AMH gene, located on chromosome 28 (NCBI: NC_006115.4 (2018795..2022986, complement)), is also five exons long but has only a 61% GC content across introns and exons. The chicken AMH gene encodes 644 amino acids with a molecular mass of

70.6 kDa[230] or 74kDa[231]; the first twenty amino acids form a signal peptide. Molecular weights have been observed on Western blots at approximately 12.5, 72 and 94 kDa[232]. While avian AMH is biologically active in mammals, mammalian AMH fails to activate müllerian duct regression in avian species[233], possibly resulting from low homology in the binding region of AMH or extracellular region of AMHRII.

AMH Activity During Avian Embryogenesis

Similar to mammalian embryology, AMH is produced in the avian testes beginning on E4.5-5[230,234,235,236]. Unlike mammals, however, the embryonic chick ovary also produces high levels of AMH at the same age, although AMH expression becomes male-biased by E5.5-6[237]. In muscovy duck embryos, a similar gene expression shift is observed: AMH mRNA expression was significantly higher in males than females on E8-10, and AMH mRNA increased in males between E7-10[238]. While AMH facilitates müllerian duct regression in male chicks during E9-13, AMH also regresses the right reproductive müllerian duct between E11-14 in females of many avian species[239]. In female chick embryos, estrogen binds to estrogen receptors in the cytoplasm of the müllerian duct, selectively protecting the left reproductive tract[240,241,242].

AMH Expression in Adult Birds

AMH production continues in birds after hatching. In the prepubertal rooster, a ten-fold increase in AMH mRNA is observed in 12-week testes compared to E6 testes[243]. However, in sexually mature roosters, only negligible quantities of AMH mRNA were detected[243]. In avian and mammalian females, AMH is most strongly expressed in small follicles. In the adult hen, AMH continues to be produced by the ovary, with the highest levels of AMH mRNA detected in 1 mm whole follicles[244]. AMH mRNA levels decrease as follicle size increases, with very low levels in the F1 (pre-ovulatory follicle) but moderate quantities in the ovarian stroma[244].

AMH mRNA expression was negligible in the liver[244]. Immunohistochemistry studies detected AMH protein localization within granulosa cells of small follicles, and granulosa cells in close or distant proximity to the germinal disc did not reveal a gradient of AMH mRNA expression, as is sometimes observed in mammals[244]. AMH expression patterns in avian species correspond to similar temporal and spatial expression of AMH during mammalian sexual development and follicular development.

AMH mRNA expression in adult hens varies according to production type. AMH mRNA was evaluated in layer and broiler breeder hens, and was found to be greater across follicle sizes and within ovarian stroma in broiler hens[245]. In addition, metabolic status in adult hens also impacts AMH mRNA expression within developing follicles. The poultry industry utilizes a technique to improve egg production in broiler breeder hens. By reducing feed intake, ovulation becomes more consistent and follicular hierarchies are similar in appearance to those of layers. Broiler hens on reduced feed intake are termed Restricted-fed (RF), while hens on an *ad libitum* diet are referred to as Full-fed (FF). The physiological changes involving egg production and ovulation between RF and FF hens suggest modifications to the rate of the follicle development might be affected by metabolic status. AMH mRNA expression is increased in smaller follicles (3-5 mm) and within ovarian stroma of FF broiler breeder hens as compared to RF hens[245]. It is hypothesized that metabolic factors, including GH and IGF1, might contribute either directly or indirectly to these changes observed in AMH and follicular dynamics within the ovary. Indeed, GH-stimulated progesterone production within cultured granulosa cells, and GH mRNA and protein, as well as the GH receptor, are located in follicles[246,247].

Regulation of AMH in Chickens

To date, regulation of AMH in avian physiology is largely uncharacterized. Cloning of the 1050-bp chick AMH promoter revealed moderately conserved regulatory binding sites, including two SOX, one SF1 and one estrogen-responsive site[236]. However, unlike mammalian physiology, gonadal AMH mRNA expression predates SOX9 mRNA expression, which does not appear until E6 in the avian testes. SOX9 mRNA increases substantially shortly before E7, and a parallel temporal increase is observed in AMH mRNA. Additionally, SOX9 and AMH mRNA colocalize within the testes. SOX9 mRNA was absent from the embryonic ovary[236]. Other members of the SOX family, including SOX8, -3, -4, and 11, were identified within the gonads at E6-7, but none exhibited a sex-bias[248].

Other genes that contribute to sex determination were evaluated in chick embryonic gonads. DMRT1, a transcription factor, is present in both sexes, although a male-biased mRNA expression pattern develops between E5.5-7[235,249]. Knockdown of DMRT1 induces feminization of the testes, but over-expression of DMRT1 masculinizes the ovaries and downregulates aromatase[250]. Another transcription factor, SF1, displays a sexually dimorphic shift, as SF1 mRNA is male-biased at E5.5, but reverses to exhibit a female-bias at E7.5-8.5[249]. SF1 has been shown to bind to the avian AMH promoter[251]. This modification in SF1 mRNA expression likely corresponds with increased AMH in the testes around E5.5.-6, as SF1 upregulates AMH expression in mammals. Furthermore, DAX1, a transcription factor that downregulates AMH expression by interfering with SF1 in mammals, becomes female-biased by E6.5-7.5, which may also contribute to the relatively static AMH expression in females[249]. Another transcription factor, GATA4, which is known to upregulate mammalian AMH, did not develop any changes in

mRNA expression between E5.5-8.5[249]. After hatching, AMH, SF1, GATA4 and WT1 mRNA are simultaneously expressed within the ovarian cortex[235].

In the adult hen ovary, granulosa cells collected from 6-8 mm follicles were cultured with increasing dosages of estradiol or progesterone, but failed to induce changes in AMH mRNA expression[244]. Although the avian AMH promoter contains an estrogen responsive element, data from mammalian experiments reveal estradiol acting via ERα and ERβ increases and decreases AMH expression, respectively, in particular stages of follicular development. Granulosa cells from 3-5 and 6-8 mm follicles cultured with vitamin D displayed increased AMH and FSHR mRNA expression accompanied by increased cell proliferation, when compared to controls[252]. In addition, bidirectional communication between ooplasm and granulosa cells is known to exist in mammalian and avian follicles. To better identify the role of the oocyte in AMH regulation, granulosa cells sourced from 6-8 mm follicles were cultured with different dosages of Oocyte-Conditioned Medium (OCM)[244]. AMH mRNA expression was inhibited on a dosage-dependent basis, supporting the hypothesis that the oocyte might help regulate AMH in the surrounding granulosa cells within the follicle. When the OCM was exposed to 65°C for 30 minutes, the inhibiting effect provided by the OCM disappeared, indicating a heat-dependent factor within the oocyte was likely regulating AMH[244]. In an effort to better understand the downstream effects of AMH in the adult ovary, granulosa cells from 3-5 and 6-8 mm follicles were cultured in the presence of different dosages of Testis-Conditioned Medium (TCM) and/or AMH antibody[245]. Proliferation rates increased with TCM dosage, but declined when an AMH antibody was included[245].

Members of the BMP family also contribute to AMH regulation in the chicken, similar to their effects in mammalian signaling pathways. In addition to BMP genes, members of the ID

family and AMH are hypothesized to contribute to an FSH-responsive regulation of follicular differentiation and further development. BMP6 (rhBMP6), primarily located within granulosa cells of smaller follicles (1-5 mm), has been shown to increase both AMH and FSHR mRNA in cultured granulosa cells collected from 6-8 mm follicles[253]. Granulosa cells cultured with BMP4 and FSH also increase AMH and FSHR mRNA levels, in addition to cAMP[254]. BMP2 significantly increases ID3 and decreases FSHR mRNA expression in cultured granulosa cells[255]. ID3, as well as ID1 and -4, have been shown to decrease FSHR mRNA within granulosa cells[256]. This data suggests FSH is regulated via a paracrine and autocrine manner through systematically modified expression of its receptor, FSHR, which is a membrane-bound receptor located on granulosa cells[257,258].

Within avian physiology, the downstream effects of AMH beyond müllerian duct regression are not well known. Suppression of AMH *in vitro* and *in vivo* via RNA interference utilizing short hairpin RNA (shRNA) resulted in stunted gonadal growth, which was observed in both sexes from E4.5-7.5, due to suppressed cellular growth and decreased germ cell populations within testes[259]. Similarly, AMH overexpression using viral vectors led to underdeveloped, moderately masculinized gonads that lacked steroidogenic capabilities in either embryonic sex, as well as proper spermatogenesis functions in males[260]. The effects of AMH overexpression were continued *in vivo* in adult chickens of both sexes (nine of the 812 microinjected eggs hatched; 1.11%): four females and two males. At the time of sexual maturity (<18 wks post-hatching), the birds overexpressing AMH failed to display any sexually dimorphic traits except weight, though they had a small, pale comb and some females exhibited a spur characteristic of males[260]. Serum estradiol levels in control females ranged from 531-2205 pmol/L, compared to

undetectable levels in three of the four treatment hens[260]. Testosterone levels were also lower in the two treatment males versus controls.

AMHRII in the Chicken

While AMH provides critical functions within avian species, little is known about avian AMHRII. Identified in RNA-Seq reads as early as E4.5, AMHRII is estimated to be approximately 547 amino acids long, although the full nucleotide sequence has not yet been available publicly or published[261]. On E4.5-8.5, the müllerian ducts and gonads of both sexes showed AMHRII protein localization via IHC. When treated with an aromatase inhibitor, AMHRII protein increased within the ovarian medulla, which underwent a degree of masculinization[261]. When DMRT1 was knocked down, AMHRII protein expression decreased in male embryos to a feminized expression level.

V. The Modern Hen as a Model to Study the AMH/AMHRII Signaling Pathway

Two main hen types exist in modern commercial poultry production systems: the layer hen--a lighter, physically refined hen selected for efficient and prolific egg production, and the broiler hen--a much larger hen selected for robust growth patterns and relatively extreme muscle development to meet the consumer demand for poultry. The layer hen regularly maintains a full follicular hierarchy, ovulating almost daily with only minor interruptions. Unlike layers, broiler hens selected to produce offspring typically have irregular egg production, which is problematic for broiler producers who desire to replenish their poultry stocks. The full-fed broiler breeder hen produces an over-developed follicular hierarchy, which causes irregular ovulation patterns, resulting in double or missed ovulations[245]. When broiler breeder hens are placed on a restricted diet, however, ovarian function is less excessive and more closely resembles that of a layer hen.AMH, which helps regulate follicle recruitment and development in mammals, has been

hypothesized to contribute to the differences observed between broiler breeder and layer ovaries[245].

During molting, which can be stimulated or spontaneous, the hen enters a hypogonadotropic state and the ovary regresses, with no follicles entering the follicular hierarchy[262,263]. This is often characterized by feather loss and replacement. Commercial chickens are typically maintained on long days for prolonged periods and they ovulate and lay eggs throughout the year. In contrast, wild birds are highly responsive to prevailing photoperiod, limiting follicle development to a very restricted portion of the year. Although the period of molt of anovulation in commercial chickens is of shorter duration than that which occurs in wild species, in both cases, the regressed ovary is accompanied by a hypogonadotropic state. We have hypothesized that these anovulatory periods may mandate protection of the ovarian reserve as observed in short day-reared, hypogonadotropic Siberian hamsters[214]. Evaluation of AMH within the ovary during these different reproductive statuses, including in and out of molt, may provide evidence for AMH involvement in protecting the ovarian reserve, as well as during folliculogenesis and follicle activation.

VI. Conclusion

While extensive research has already been performed, many aspects of AMH and AMHRII have yet to be clarified in both mammalian and avian physiology. The role of AMH as a critical reproductive hormone has been discovered to include processes outside of embryonic development and testicular processes and regulation. AMH action in the ovary, including early and developing follicular dynamics, still needs to be explored. Investigation of AMH apart from strict gonadal functions, such as its contributions in brain and neuronal

processes, is a quickly developing field of interest. In addition, the autocrine and paracrine regulation of AMH and AMHRII remains unclear.

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CHAPTER II

AMHRII IN AVIAN FOLLICLE DEVELOPMENT

I. Abstract

In the mammalian ovary, anti-müllerian hormone (AMH) helps maintain the ovarian reserve by regulating primordial follicle activation and follicular selection, although its role within the avian ovary is unknown. In adult mammals, AMH is primarily produced in granulosa cells of preantral and early antral follicles. Similarly, in the hen, the granulosa cells of smaller follicles are the predominant source of AMH. AMH importance in mammalian ovarian dynamics suggests AMH may have conserved, protective functions within developing follicles in the hen. To better understand the role of AMH in avian follicle development, we studied the expression pattern of the specific AMH receptor. AMH utilizes the TGF-β/SMAD signaling pathway, including the specific Type II receptor, AMHRII. qRT-PCR results indicate that AMHRII mRNA expression, as well as that of AMH, are highest ($p<0.01$) in small follicles (1 mm) and decrease as follicle size increases. Dissection of 3-5 mm follicles into ooplasm and granulosa components shows that AMHRII mRNA levels are significantly greater in ooplasm than granulosa cells. Immunohistochemistry revealed AMHRII staining in the oocyte and granulosa cells. In mammals, AMH expression is elevated during periods of reproductive dormancy, possibly protecting the ovarian reserve. To assess the expression of AMHRII and AMH during a similar state in chickens, we evaluated mRNA expression in a broiler strain and an egg-laying strain of hens in different reproductive states. AMH and AMHRII mRNA were significantly higher ($p<0.05$) in non-laying ovaries of broiler hens. In molting (with documented feather loss) and non-molting layer hens, AMHRII mRNA was significantly greater ($p<0.05$) in molting hen ovaries. These results suggest AMH may contribute to the intra-follicular bidirectional

communication between oocyte and granulosa cells, and support a potential role of AMH in limiting follicle recruitment in hens.

II. Introduction

Much of the success behind the modern commercial laying hen lies in its highly coordinated reproductive proficiency, making it a valuable animal model for studying successful ovarian function. The hallmark of an efficient laying hen is the maintenance of a precise follicular hierarchy, where follicles continuously develop and ovulate according to size, ideally resulting in one daily egg. Current research in the hen attempts to elucidate the delicate hormonal regulation and intercellular communication that masterfully controls this follicular hierarchy. In the mammalian ovary, anti-müllerian hormone (AMH) is critical in helping maintain the ovarian reserve by regulating primordial follicle activation and follicular growth and selection (Durlinger, et al. 2001; Durlinger, et al. 2002). AMH importance in mammalian ovarian dynamics suggests AMH may have conserved, protective functions on the ovarian reserve and within developing, perihierarchal follicles in the hen.

In many mammalian species, a primary function of AMH in the ovary is to regulate primordial follicle activation from the ovarian reserve (Durlinger, et al. 2002; Rocha, et al. 2016). A secondary role of AMH is to help regulate the development of gonadotropin-dependent follicles. AMH has been shown to reduce FSH sensitivity in follicles (Durlinger, et al. 2001; Campbell, et al. 2012). Two micro-RNAs, miR-181a and miR-181b, have recently been identified as targets of AMH. AMH is believed to upregulate these micro-RNAs, reducing intra-follicular cAMP levels and thus FSH sensitivity (Hayes, et al. 2016). A third proposed function of AMH in the ovary relates to periods of reproductive quiescence. During these times, AMH mRNA expression increases, suggesting that AMH limits follicular recruitment and development

during times of restricted fertility (Nilsson, et al. 2007; Kabithe and Place, 2008; Shahed, et al. 2013). To date, the mechanism of action underlying this phenomenon has yet to be identified. Ovarian factors including BMP15 are involved in the regulation of AMH. BMP15 upregulates AMHRII mRNA expression in human and ovine granulosa cells (Pierre, et al. 2016).

In mammals, AMH utilizes two TGF-β type receptors (Grootegoed, et al. 1994; Josso, et al. 2001). AMH first binds to the extracellular portion of its specific (Mishina, et al. 2009) primary receptor, AMH Type II Receptor (AMHRII), which subsequently phosphorylates one of three non-specific Type I receptors: ALK2/ACVR (Visser, et al. 2001; Clarke, et al. 2001), ALK3/BMPR1A (Wu, et al. 2012; Sèdes, et al. 2013) or ALK6/BMPR1B (Zhan, et al. 2006). Once AMH binds both receptors, the AMH-receptor complex triggers the SMAD signaling cascade, phosphorylating three receptor-regulated Smads (R-Smads) within the cellular cytoplasm: Smad1, Smad5 and Smad8 (Hutson, et al. 1984; Dutertre, et al. 2001; Zhan, et al. 2006; Orvis, et al. 2008; Kristensen, et al. 2014). The three activated R-Smads then complex with common-mediator Smad4, stimulating active transcription factor formation within the nucleus (Gouédard, et al. 2000).

In mammalian females, AMH is most strongly expressed in small follicles (Baarends, et al. 1995). In the adult hen, AMH is produced in granulosa cells of follicles, with the highest levels of AMH mRNA detected in 1 mm whole follicles (Johnson, et al. 2008). AMH mRNA levels decrease as follicle size increases, with very low levels in the pre-ovulatory (F1) follicle (Johnson, et al. 2008). Immunohistochemistry studies detected AMH protein localization within granulosa cells of small follicles, and there was no gradient of AMH mRNA expression in granulosa cells relative to the germinal disc (Johnson, et al. 2008), as is sometimes observed in mammals (Kedem, et al. 2014).

Two main types of chickens have been selected in modern commercial poultry production systems: the layer hen, which is a lighter hen selected for efficient and prolific egg production, and the broiler hen, which has been largely selected for robust growth and muscle development for meat. A similar pattern of AMH mRNA expression was found in developing follicles of laying and broiler hens, although the absolute quantity of AMH mRNA was greater in broiler hen ovaries (Johnson, et al. 2009).

While avian AMH is biologically active in mammals, mammalian AMH fails to activate müllerian duct regression in avian species (Josso, et al. 1977), possibly resulting from low homology in the binding region of AMH or extracellular region of AMHRII. It is possible that information about AMHRII expression and regulation may help clarify AMH function in birds. Identified in the chicken in RNA-Seq reads as early as E4.5, AMHRII is estimated to be approximately 547 amino acids long, although the full nucleotide sequence is not yet available (Cutting, et al. 2014). The first objective of this research was to characterize AMHRII mRNA and protein expression in the adult hen ovary to better understand the potential roles of AMH in avian reproductive physiology. The second objective was to evaluate AMH and AMHRII in different reproductive states. Finally, we evaluated regulation of AMH by the oocyte factor BMP15. We hypothesized hen granulosa cells treated with BMP15 would exhibit an increase in AMHRII mRNA expression.

III. Materials and Methods

Animals and Treatment Protocols

Commercial strains of Single-comb White Leghorn laying hens (Babcock B300 and Shaver) were used. The hens were individually caged, with egg records maintained daily. All hens had *ad libitum* food and water, and were maintained on 15 h light: 9 h dark (lights on at

0600 h). Hens between 8-21 mo of age and laying regularly were euthanized via CO_2 1.5-2.5 h post oviposition. Embryos (embryonic day 6-12; E6-12) used in immunofluorescence or protein lysates were a Cornell laying strain.

Fifteen-month old broiler breeder hens on a restricted diet were also used and were housed in floor pens. Non-laying broiler breeder hens (n=5) exhibited small, pale combs, yellow shanks, and fully regressed ovaries. Broiler hens which were laying (n=7) had large, red combs, full follicular hierarchies, and were trapped in a nesting box after laying an egg. Laying hens were induced to molt with dietary supplementation of ZnO_2 (Goodman, et al. 1986) or molted spontaneously. In all molting cases, feather loss was documented and the ovary was regressed. Non-molting hens of the same age were used as controls to the molting hens. All animal care and procedures were approved by the Institutional Animal Care and Use Committee of Cornell University.

Sequencing

The validated *Mus musculus* and predicted *Falco peregrinus* AMHRII sequences were aligned and primers were designed using Primer3 and OligoAnalyzer from the aligned *F. peregrinus* sequence. Based on earlier work on AMH in the hen, cDNA from 1 mm whole follicles from an adult layer hen was chosen as the template for PCR. The resulting PCR product was sequenced and analyzed (see Appendix). Additionally, the PCR product matched unpublished cDNA from Cutting and colleagues (2014).

In a second effort to sequence AMHRII, the predicted chicken AMHRII coding sequence available on NCBI (from *Gallus gallus* 5, uncharacterized LOC101749915, Chr. 33, 829,346-832,058 bp) was used to design overlapping PCR primer pairs spanning the 5' untranslated region (UTR), predicted 1101 bp coding sequence, and 3' UTR. For template, cDNA from 1 and

3 mm whole follicles from an adult layer ovary were used. An additional reagent, preCESII, was made with H_2O in lieu of BSA and used as directed in several PCR reactions (Ralser, et al. 2006). An aliquot of all the PCR products was run with loading dye on a 1.5% agarose gel containing Ethidium Bromide at 106 V for approximately 46-55 min and imaged. If a single band or multiple bands were present, the PCR product or excised band of interest, respectively, was purified according to manufacturer's instructions (Qiagen, Valencia, CA, USA; QIAquick Gel Extraction Kit; Cat. No. 28704). The purified PCR product was then combined with the forward or reverse primer (10 pmol/µL) and diluted to approximately 10 ng total nucleic acid immediately prior to submission for sequencing at the Biotechnology Resource Center at Cornell University. PCR products were sequenced in both directions to increase sequence accuracy (see Appendix).

Tissue Collection and RNA Isolation

Fresh tissues were immediately placed into cold Krebs-Ringer bicarbonate buffer on ice prior to dissection or subsequent storage. Whole follicles (1, 3, 5 mm) and liver samples were used fresh or immediately flash frozen or frozen in RNAlater Stabilization Solution (Thermo Fisher Scientific, Cat. No. AM7021) at -80°C, and homogenized immediately preceding RNA isolation. Granulosa cells and ooplasm were collected from individual 3-5 mm follicles of a hen and pooled (n=8-10 hens), as previously described in Stephens and Johnson (2016) and Wang et al. (2007). Granulosa cells were also collected from 3-5, 6-8, 9-12 mm and F1 follicles (n=5-6 hens) for mRNA expression. For granulosa cell culture, granulosa cells were isolated from 3-5 and 6-8 mm (n=5-6). Cells were dispersed and cultured as previously described (Stephens and Johnson, 2016). Recombinant human BMP15 (rhBMP15) (R&D Systems, Minneapolis, MN) was added to the cultures at doses of 0, 10, and 25 ng/mL.

RNA from follicles (1, 3, 5 mm), liver tissue (n=4-5), ooplasm, granulosa cell layers and cultured granulosa cells was extracted using the RNeasy Mini Kit (Qiagen, Cat. No. 74106) according to manufacturer's instructions, including on-column DNA-digestion with RNase-free DNase (Qiagen, Cat. No. 79254). RNA from total ovarian tissue (cortex tissue without ≥1 mm follicles) from laying hens (n=16, 16) and from broiler breeder hens (n=7,5) was extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Cat. No. 74704) according to manufacturer's instructions.

Reverse Transcription and qRT-PCR

RNA was analyzed for quality and concentration using an Implen NanoPhotometer UV/Vis spectrophotometer before cDNA synthesis was performed according to manufacturer's instructions (Applied Biosystems, High-Capacity cDNA Reverse Transcription Kit, Cat. No. 4368814) and with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA; Cat. No. N8080119). For AMHRII, custom Invitrogen primers were designed from our initial AMHRII sequence information using Primer3 software. For qRT-PCR, 25 µL reactions were run in duplicate using AB StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA; Cat. No. 4376600) containing 1X Power SYBR Green (Applied Biosystems, Cat. No. 4368706) and 300 nM of primer pairs (Table 2.1). All sample reactions were normalized to duplicate *18S* rRNA reactions (Applied Biosystems, QuantumRNA 18S Internal Standards Kit, Cat. No. AM1718) as an endogenous control. Control reactions lacking template or reverse transcriptase were run concomitantly. Mean expression levels of all normalized samples were analyzed using a 9-point standard curve containing serial dilutions of pooled cDNA from 1, 3, and 5 mm whole follicles from an adult laying hen.

Western Blot

Protein lysates were made from pooled E11-12 testes, granulosa cells from 3-5 mm and F1 follicles (n=4 hens), 1, 2, <2, 3, 4, 5, 6 mm whole follicles, and liver. A non-interfering Tissue Lysis Buffer (1 M HEPES, pH 7.5; 5 M NaCl; 20% Triton X-100; 0.5 M EDTA; 100 mM Na. PyroPhos.; 100 mM Na. OrthoVand.; 100 mM PMSF; 1 M NaF; Protease Inhibitor Cocktail (Sigma Aldrich, St. Louis, MO, USA; Cat. No. P8340)) and distilled H_2O were used. To determine protein concentrations, protein lysate samples were run in duplicate using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Cat. No. 23225) according to manufacturer's instructions. Fifty or 75 µg of total, freshly thawed protein (equal total protein loaded per gel) were subjected to SDS-PAGE with β-Mercaptoethanol and run on a 12% polyacrylamide gel (Thermo Fisher Scientific, Precise™ 12% Tris-HEPES Gels, Cat. No. 25202) for approximately 40-54 minutes at 106 V, then transferred onto a nitrocellulose membrane for 77-80 minutes at 400 mA at 4°C. The membrane was then blocked in 5% BSA in 1X phosphate-buffered saline with 0.1% Tween-20 (PBST) for 1 hr, evaluated with Ponceau S Stain to confirm protein transfer from the gel to the membrane, and incubated overnight at 4°C with 1:2000 AMHRII (custom polyclonal rabbit anti-chicken AMHRII antibody generously donated by Dr. Craig Smith (Cutting, et al. 2014, Lambeth, et al 2016), in 1% non-fat dried milk (NFDM) in PBST in a sealed bag. Primary antibody was retained, stored at 4°C and reused in subsequent immunoblotting runs. After incubation with the primary antibody, the membrane was washed in PBST three times for 10 min each and incubated with goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cat. No. SC-2030) at 1:5000 in 5% NFDM in PBST for 1 hr at room temperature, followed by three PBST washes for 10 min each. Immediately before imaging, membranes were incubated with Clarity Western ECL

Substrate (Bio-Rad, Hercules, CA, USA; Cat. No. 1705060) for 5 min at room temperature. Bands were visualized and recorded using a Fluor Chem HD2 Imager (Alpha Innotech, Protein Simple, San Jose, CA, USA). As an endogenous control, membranes were incubated with 1:3000 β-actin mouse anti-mouse primary antibody and 1:5000 goat anti-mouse HRP-conjugated secondary antibody (Thermo Fisher, Cat. No. 31430) and imaged as described above.

Immunofluorescence

The same custom polyclonal rabbit anti-chicken AMHRII antibody was used to localize AMHRII binding in control and sample tissues. Embryonic testes (E11-12) were used as a positive control and to demonstrate the successful replication of the fixation and immunofluorescence procedures described in Cutting, et al. 2014. Fresh tissue, including pooled E11-12 testes and 1, 3, and 5 mm whole follicles (n=3 hens), from adult laying hens were fixed for cryosections according to the procedures detailed in Cutting, et al. (2014). Cryosection blocks were sectioned at 5 µm at the Histology Laboratory at the Cornell University Veterinary School, and sections (hematoxylin and eosin) were examined prior to use. The immunofluorescence protocol previously described (Cutting, et al 2014) was modified for cross sections of ovarian and follicular tissues: AMHRII antibody was increased to 1:750; 10% goat serum IgG in 5% NFDM in PBS was used to block; and a 10 min thaw at room temperature was included before a 3 hr soak in PBS at room temperature. The secondary antibody was 1:750 goat anti-rabbit Alexa Fluor 555 (Thermo Fisher Scientific, Cat. No. A-21429), and slides were incubated with Hoechst 33358 (1:1000 in H_2O) for 15 min at room temperature for nuclear staining. Tissues were imaged using a Nikon Eclipse E600 microscope with light and fluorescent capabilities and a Spot RT Slider camera.

Statistical Analyses

Messenger RNA expression from qRT-PCR data was analyzed using a one-way ANOVA. When significance was detected, means were compared using Tukey's test, and P values of <0.05 were considered significant. All statistical analyses were performed using GraphPad Prism (Version 6.0 for Windows, GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

Gene	Accession No.	Prod. Size (bp)	Primer (5'-3')
AMH	U61754	71	F - CCCCTCTGTCCCTCATGGA
			R - CGTCATCCTGGTGAAACACTTC
AMHRII	N/A	180	F - GCAGGTTCCAGACTCTCATCC
			R - CAGCAGTCAGAACGTGTTGG
BMP15	AY725199	197	F - ACATGCTGGAGCTGTACCAA
			R - GACACGGAGAAGGTGCTCA
GDF9	AY672110	89	F - ACTTTTCACCCCGTGTTCTG
			R - CCAGGTTGAAGAGCAAATCC

Table 2.1. Primers used for qRT-PCR.

IV. Results

A 247-bp sequence of AMHRII was found to be 81% identical to the predicted *F. peregrinus* sequence. Strong conservation in this region of AMHRII suggests the sequence spans the intron between Exons 8 and 9. This sequence was also present within a partial AMHRII cDNA sequence (Cutting et al, unpublished) and later gene annotations in NCBI. A 180-bp sub-sequence was utilized as a probe for qRT-PCR to evaluate mRNA expression. Additional sequence of AMHRII was obtained for a total sequence length of 532 bp. All primer sequences and resulting trace images and raw sequences from successful sequencing reactions are available in the Appendix.

To determine the potential involvement of AMHRII in folliculogenesis, AMHRII mRNA expression levels were evaluated across different follicle sizes, and found to decrease significantly as follicle size increases ($p<0.01$) (Figure 2.1). AMHRII was significantly ($p< 0.05$) greater in 1 mm follicles compared to 5 mm follicles; AMHRII mRNA was undetectable in liver. Within granulosa cells from small (3-5 mm), developing (6-8, 9-12 mm), and the pre-ovulatory (F1) follicles, both AMHRII ($p<0.001$) and AMH ($p<0.01$) mRNA expression were significantly highest in 3-5 mm follicles (Figure 2.2). Messenger RNA expression was examined in ooplasm and granulosa cells collected from 3-5 mm follicles (Figure 2.3). AMHRII mRNA was also significantly higher in ooplasm than in granulosa cells ($p<0.04$). However, similar levels of AMH mRNA expression were found in both ooplasm and granulosa ($p=0.7591$), likely indicating some granulosa cell contamination of the ooplasm. To evaluate separation of the two follicular components, two known oocyte-specific factors, BMP15 ($p<0.01$) and GDF9 ($p<0.05$), were assessed in these follicular compartments and found to be significantly higher in ooplasm and negligible in granulosa cells, as expected.

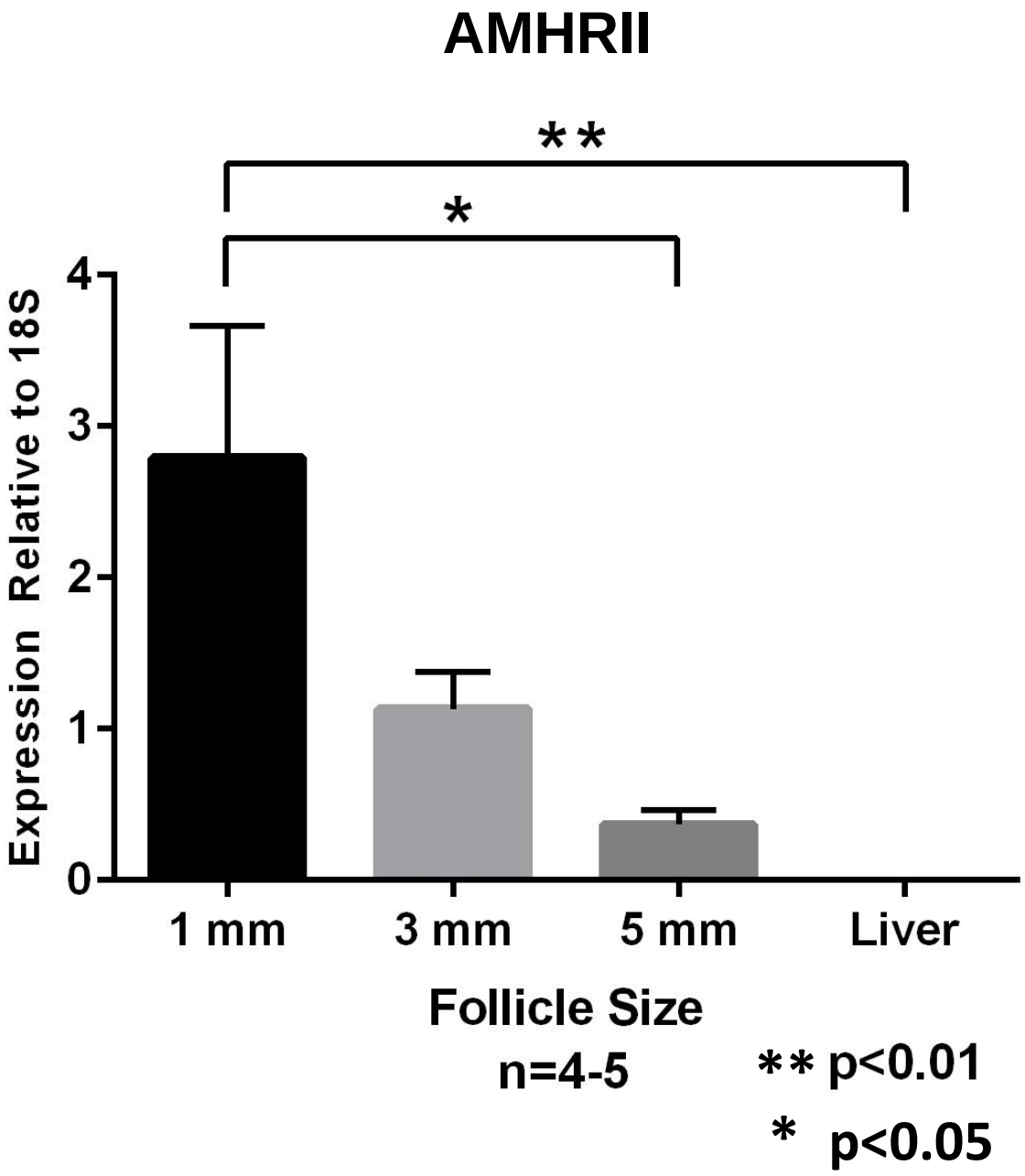

Figure 2.1 AMHRII mRNA expression across different whole follicle sizes and tissues.

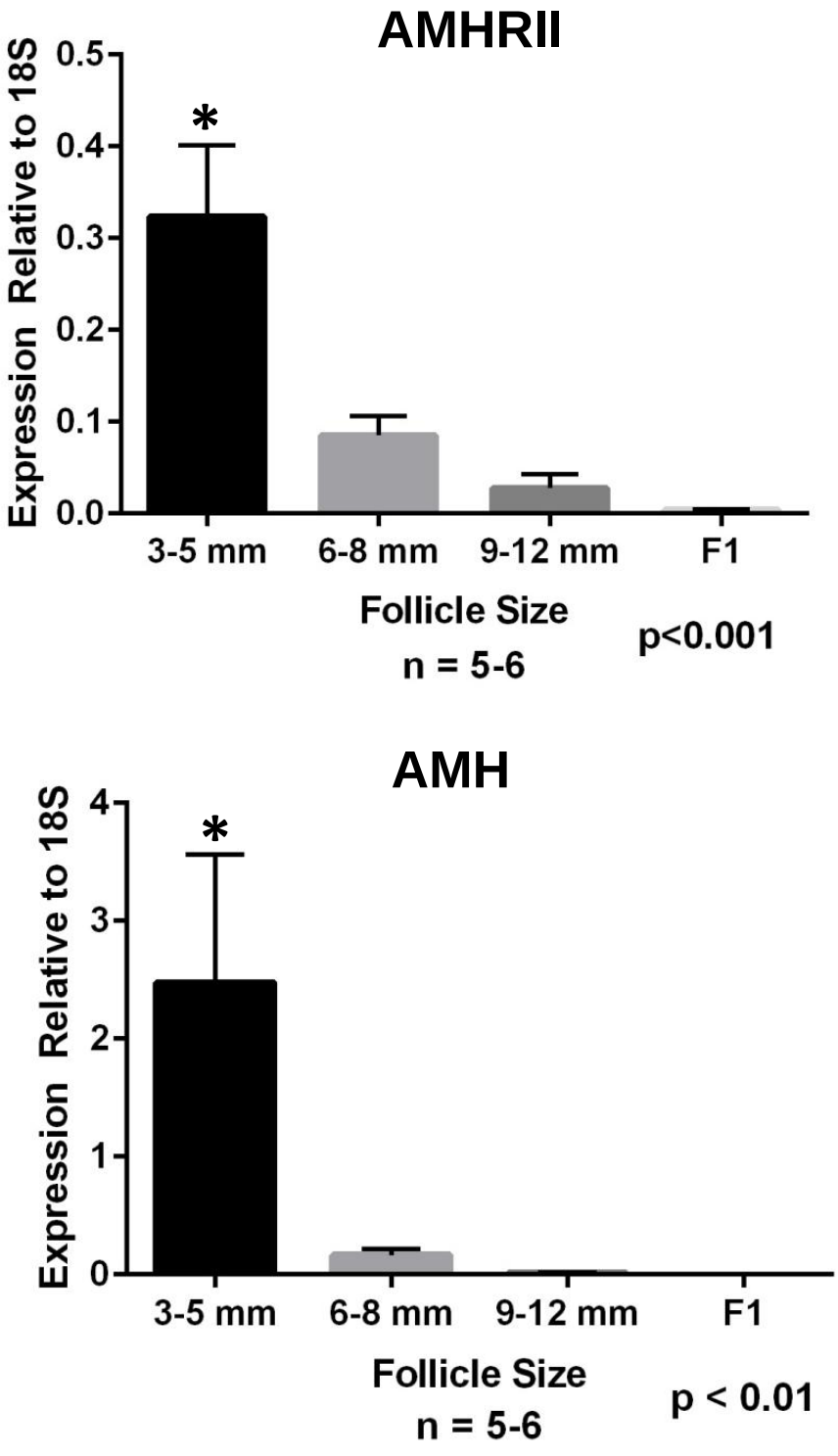

Figure 2.2 mRNA expression from granulosa cells from different follicle sizes.

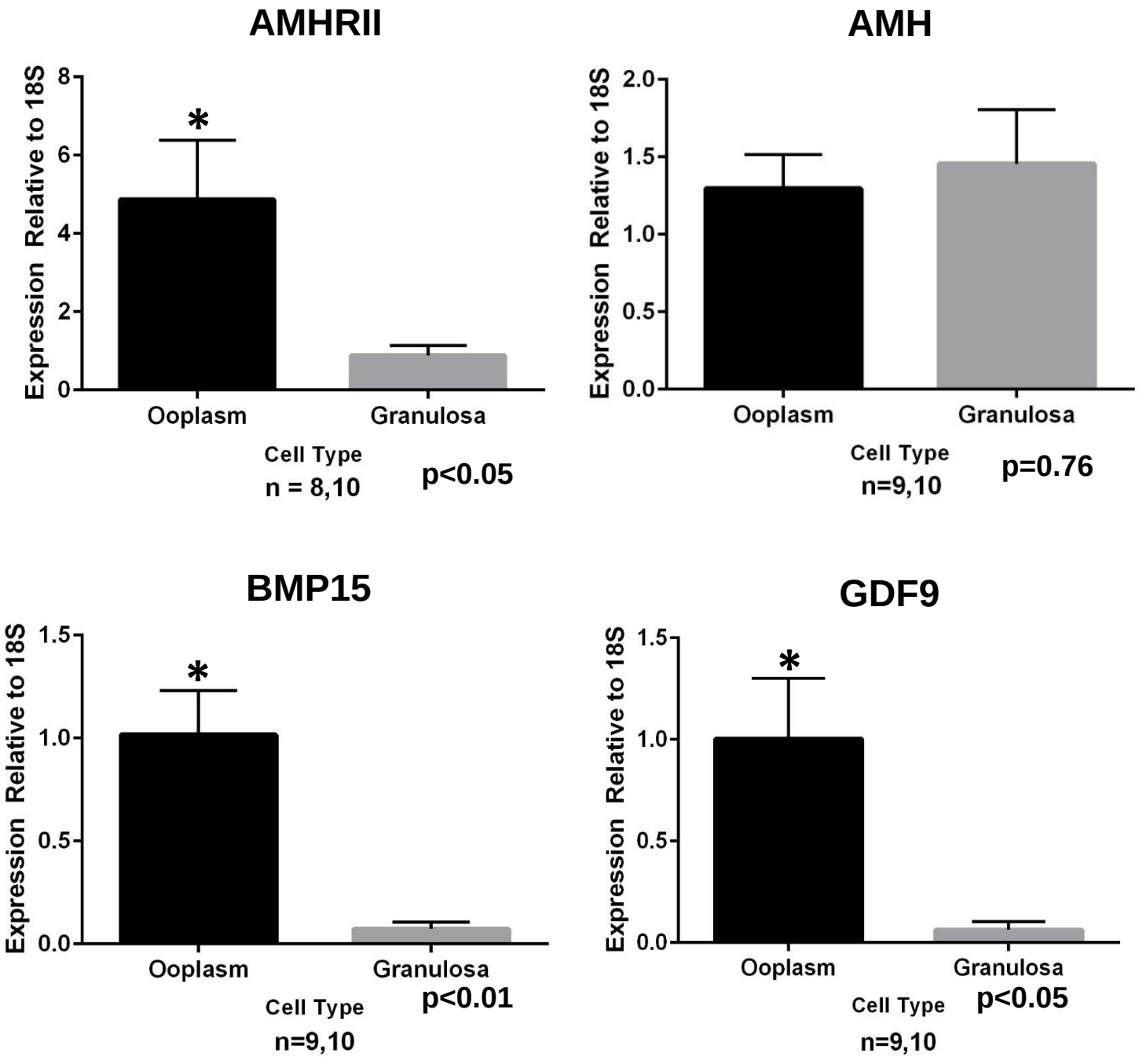

Figure 2.3 mRNA expression in ooplasm and granulosa of 3-5 mm follicles.

In laying and non-laying broiler breeder hens, both AMHRII ($p<0.05$) and AMH ($p<0.05$) mRNA expression were higher in non-laying hens (Figure 2.4). AMHRII mRNA expression was significantly higher in molting hens as compared to non-molting hens ($p<0.03$), while AMH mRNA levels were not different ($p=0.56$) (Figure 2.5). rhBMP15 at 25 ng/mL significantly decreased ($p<0.05$) AMHRII mRNA in 3-5 and 6-8 mm granulosa cells and also decreased AMH mRNA in 6-8 mm granulosa cells (Figure 2.6).

AMHRII western blots yielded a main band between 60-80 kDa in whole follicles (Figure 2.7). As follicle size increases, an additional band between 30-40 kDa strengthens. β-actin, at approximately 42 kDa, was evaluated as an endogenous control. AMHRII protein expression in Western blot using protein from 3-5 mm and F1 granulosa appears at a similar size, although much less intense than that in whole follicles (data not shown).

The results of AMHRII immunofluorescence within Sertoli cells in the E12 testis successfully replicated the results found in Cutting, et al. (2014) (Figure 2.8). Immunohistochemistry showed AMHRII protein localization within the oocyte and granulosa cells of small intra-ovarian follicles (Figure 2.9). Binding in the oocyte displayed a centralized binding pattern. When the primary antibody was replaced with 10% goat serum, the signal was absent in both the testis and follicle.

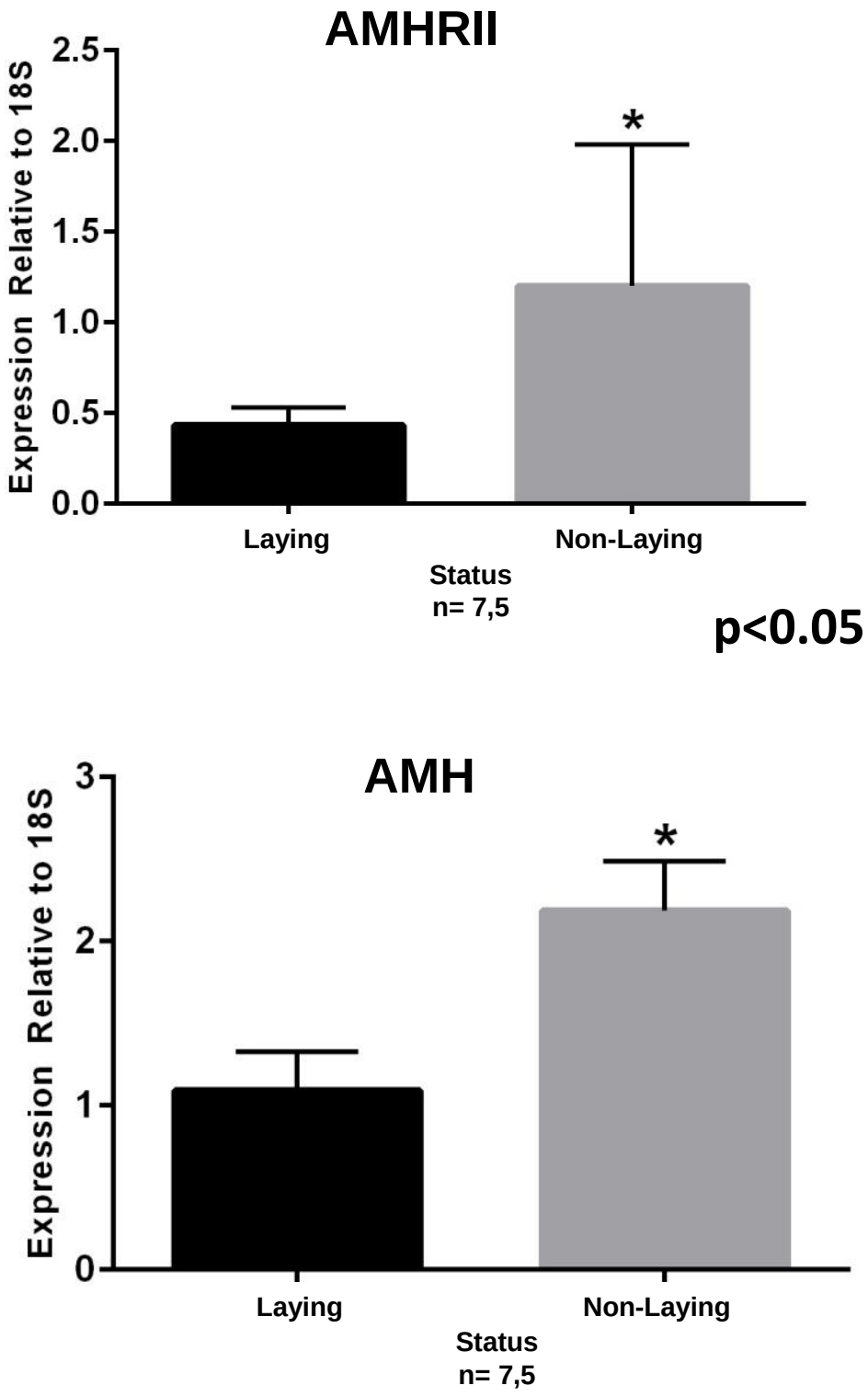

Figure 2.4 mRNA expression in total ovarian tissue from laying and non-laying restricted-fed broiler breeder hens.

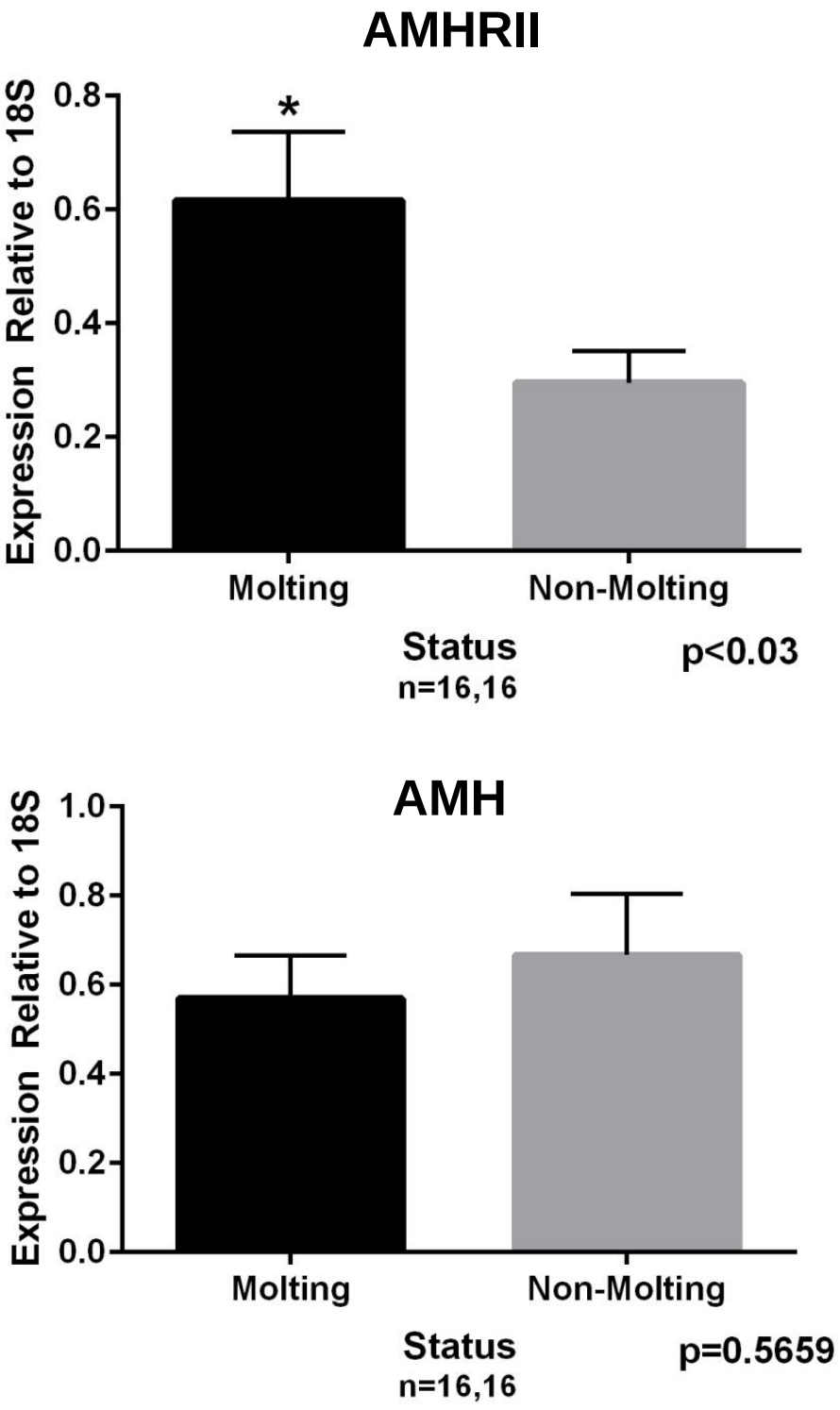

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Figure 2.5 mRNA expression in total ovarian tissue from molting and non-molting layer hens.

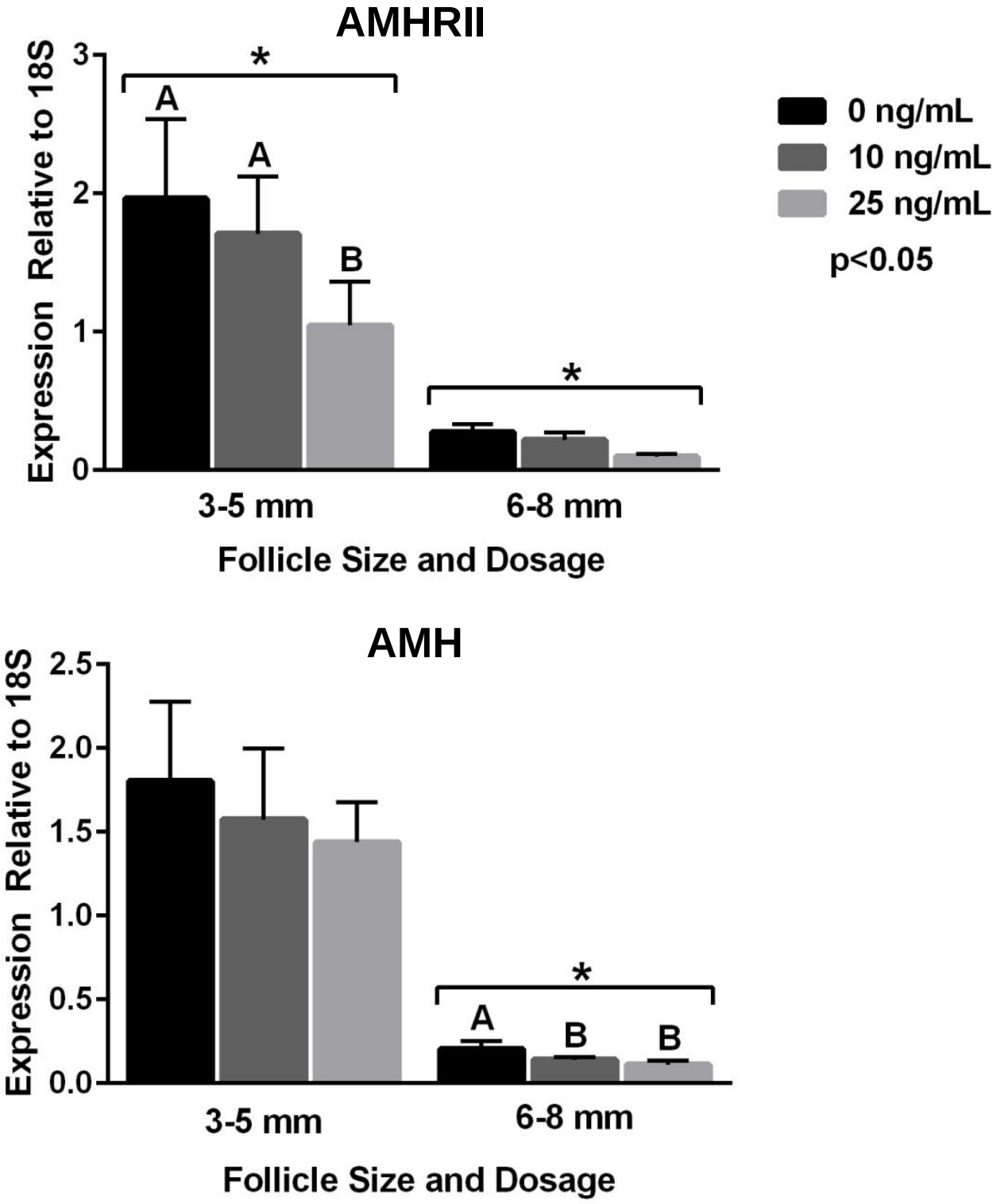

Figure 2.6 AMH and AMHRII mRNA expression in granulosa cells from 3-5 and 6-8 mm follicles treated with different doses of rhBMP15.

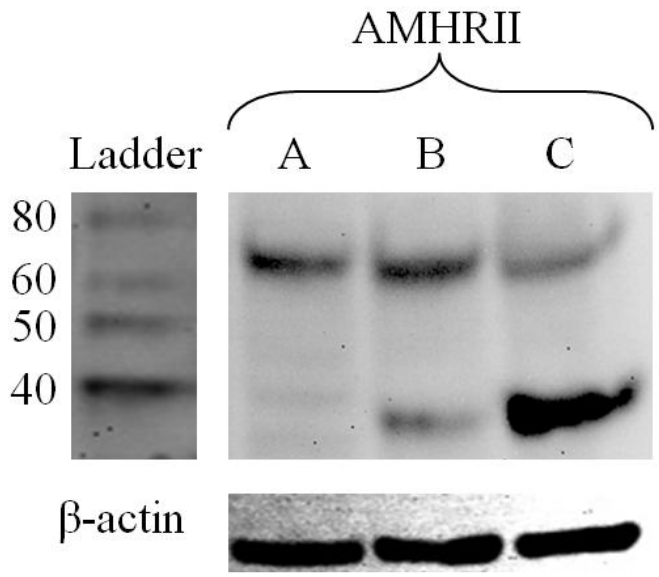

Figure 2.7 Western Blots of AMHRII in various avian tissues: A: 2 mm Whole Follicle (WF); B: 4 mm WF; C: 6 mm WF. β-actin as endogenous control shown below respective lanes.

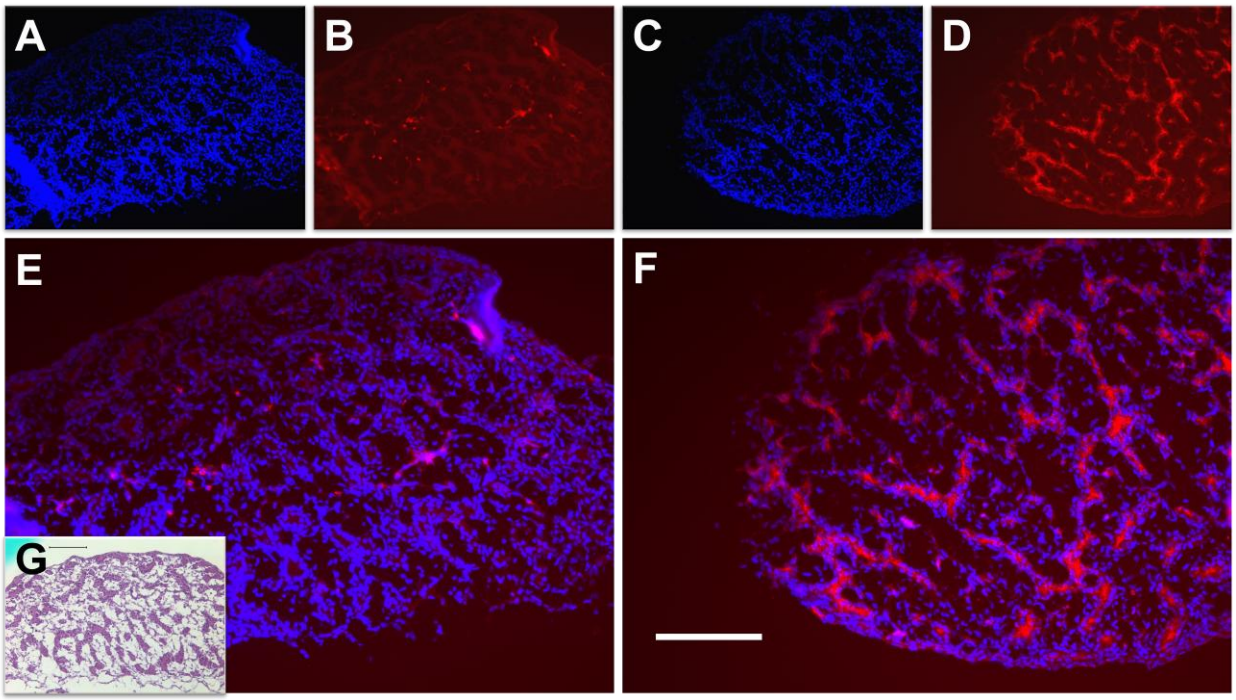

Figure 2.8 Immunofluorescence of rabbit anti-chicken AMHRII antibody in cross sections of E12 rooster testes. Hoechst (blue) as nuclear stain (A,C); 10% goat serum substituted for antibody (B); rabbit anti-chicken AMHRII antibody (D); goat anti-rabbit Alexa Fluor 555 (Red) (B, D); merged images (E,F); H&E (G). Size bar equals 100 µm.

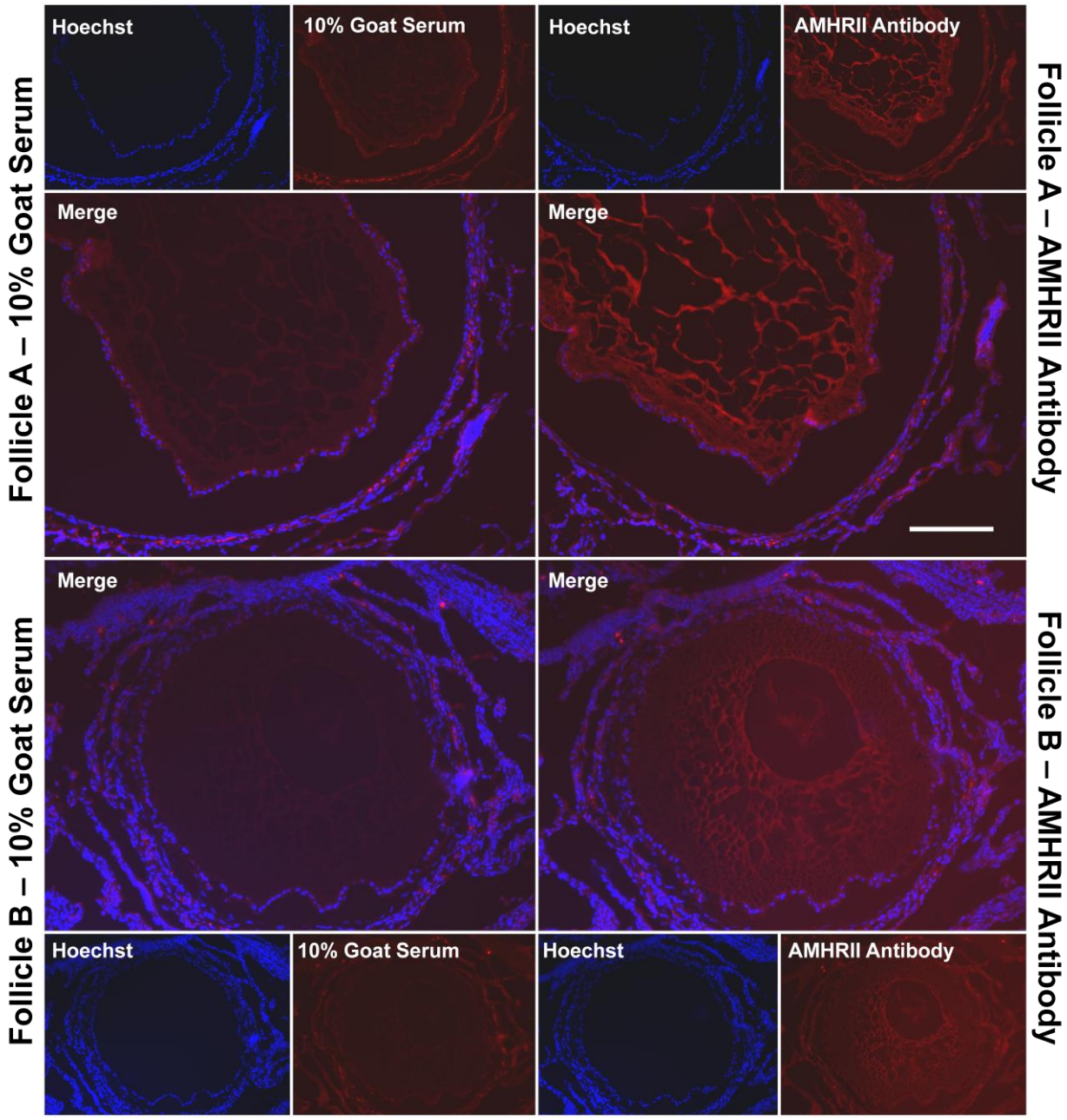

Figure 2.9 Immunofluorescence of rabbit anti-chicken AMHRII antibody in adjacent cross sections of intraovarian follicles (Follicles A and B) within the cortex of adult layer hens. Hoechst (blue) as nuclear stain; 10% goat serum substituted for antibody; goat anti-rabbit Alexa Fluor 555 (Red). Size bar equals 100 µm.

V. Discussion

To our knowledge, this is the first time AMHRII has been characterized in the adult hen and throughout ovarian follicle development. Sequencing of AMHRII in the chicken remains incomplete, although we sequenced 532 bp, likely representing a large portion of the C-terminus (bp 928-1460 of an estimated 1735 (encompassing coding sequence and both UTR)). AMHRII mRNA expression was highest in 1 mm whole follicles and decreased as follicle size increased. This notable decline in both AMH and AMHRII mRNA expression strongly correlates with increasing follicle size, suggesting AMH plays a primary role in early follicle development, prior to yolk accumulation and recruitment into the follicular hierarchy. AMHRII mRNA was also highest in granulosa cells from 3-5 mm follicles, and was almost negligible in F1 granulosa.

Interestingly, AMHRII mRNA expression was significantly higher in ooplasm as compared to granulosa cells. AMHRII has been historically reported to directly colocalize with AMH in granulosa cells within follicles (di Clemente, et al. 1994). However, our results indicated significantly higher levels of AMHRII mRNA in ooplasm than in granulosa cells. Additionally, immunofluorescence results show strong localization of AMHRII protein within the oocyte, though this could be an artifact. These findings are supported by the results of *in situ* hybridization studies of small antral follicles in the rat (Baarends, et al. 1995). Baarends' studies show AMHRII mRNA signal throughout the oocyte and granulosa of some follicles. Subsequent studies have primarily investigated AMHRII mRNA and protein expression within granulosa cells, although AMH was localized in oocytes and granulosa cells within caprine (Rocha, et al. 2016) and Atlantic salmon (von Schalburg, et al. 2013) follicles via IHC.

Surprisingly, AMH mRNA levels were comparable in the ooplasm and granulosa, likely indicating granulosa contamination in the ooplasm samples. This is interesting because our

previous IHC data showed intense staining for AMH in the granulosa layer (Johnson, et al. 2008), and AMH has repeatedly been found only within granulosa cells of a variety of species (Takahashi, et al. 1986; Bézard, et al. 1987; Münsterberg and Lovell-Badge, 1991; Modi, et al. 2006). Oocyte-specific BMP15 and GDF9 mRNA levels show that there is extremely low contamination of ooplasm in granulosa cells. This is likely due washing the granulosa cells prior to RNA extraction. In contrast, it was not feasible to "wash" the aqueous ooplasm before extraction. Future refinement of ooplasm collection techniques, including filtering and centrifugation, could increase the purity of ooplasm samples.

AMHRII western blots results show a strong protein band between 60-80 kDa. The full chicken AMHRII peptide sequence and molecular weight are unknown, although Cutting et al. (2014) estimated the chicken AMHRII sequence to be 514 AA. Human AMHRII is 573 AA, and is observed at approximately 83 kDa on a Western blot, while the European seabass and Fugu pufferfish are only 499 and 514 AA, respectively. A shorter amino acid sequence in the chicken might yield a smaller band than what is observed in mammals. In the mouse, different AMHRII forms have been identified at approximately 58 and 66-68 kDa, depending on glycosylation and disulfide bond cleavage (Hirschhorn, et al. 2015). Finally, a second band at approximately 40 kDa is visible in whole follicles 4 mm and greater; this band from an unidentified protein seems to intensify as follicle size increases. To date, AMHRII has not been reported to yield a protein form similar in weight to the 40 kDa band. AMHRII protein expression in Western blots from 3-5 mm and F1 granulosa appear at a similar size, although much less intense than that in whole follicles, supporting the hypothesis that the AMHRII in the oocyte may be very important in bidirectional communication between the oocyte and the granulosa cells.

BMP15 is an oocyte-specific protein that has been shown to regulate AMHRII. When granulosa cells were treated with rhBMP15, AMHRII and AMH mRNA expression decreased significantly, contrary to the effects of BMP15 on AMHRII in humans (Pierre, et al. 2016). BMP15 increased AMHRII mRNA expression within luteinized human and ovine granulosa cells, as well as increased AMHRII promoter activity within ovine granulosa cells (Pierre, et al. 2016). Perhaps a species or dosage difference, or cellular location of AMHRII, is responsible for the difference between these two studies. Our earlier work using oocyte conditioned medium (OCM) revealed an oocyte factor was capable of decreasing AMH mRNA expression (Johnson, et al. 2008). BMP15 may possibly decrease both AMH and AMHRII in the granulosa cells and oocyte. During follicular development, both AMH and AMHRII mRNA levels decrease at the time of follicle selection and growth. Signals from the oocyte, such as BMP15, might act through an autocrine mechanism or interact with the surrounding granulosa cells to permit the acquisition of FSHR and thus allow the follicle to develop.

Reproductive state also seemed to play a role in AMH and AMHRII expression. AMHRII mRNA expression was up-regulated in both the molting layer hens and the non-laying broiler breeder hens. AMH mRNA was significantly higher in non-laying broiler breeder hens. In mammals, reproductive dormancy correlates with increased AMH protein levels in the ovary (Kabithe and Place, 2008). In the hen, both AMH and AMHRII seemed to be influenced by the reproductive state. If AMH is important in regulating the rate of follicle recruitment, these date support our hypothesis that AMH and AMHRII help protect the hen's ovarian reserve, possibly by inhibiting folliculogenesis.

Further work to evaluate the direct effects of AMH in the hen is warranted. Factors that affect the AMH/AMHRII signaling pathway in mammalian physiology, including members of

the GATA (Beau, et al. 2000) and SOX (De Santa Barbara, et al. 1998; Arango, et al. 1999; Schepers, et al. 2003) families, may perform comparable roles in the chicken. Likewise, downstream targets in mammals include ID3 and the newly identified microRNA-181a and microRNA-181b (Hayes, et al. 2016). Transcription factors and downstream targets of AMH/AMHRII in the chicken should be identified. In addition, manipulating the regulatory elements supporting the AMH/AMHRII signaling pathway might influence oviposition rates by modifiying the rate of folliculogenesis.

VI. Conclusion

In conclusion, AMHRII was characterized in folliculogenesis and ovarian dynamics to better understand the potential reproductive role of AMH in the hen. AMH and AMHRII mRNA and protein are present in small whole follicles. AMH could be exerting an effect on the oocyte, as AMHRII mRNA has been found at higher levels in ooplasm than in granulosa cells. High AMH/AMHRII mRNA expression early in folliculogenesis may indicate AMH plays a role in regulation of follicular activation and development. The increase in AMHRII mRNA during periods of suppressed follicular growth points to a potential inhibitory effect of AMH on the follicular pool. Future work should identify direct transcription factors involved in the AMH/AMHRII signaling pathway in chickens, and continue to investigate the mechanisms of action utilized by AMH/AMHRII regarding bidirectional communication between the ooplasm and granulosa cells.

VII. Acknowledgements

The custom Rabbit Anti-Chicken AMHRII antibody was generously donated by Dr. Craig Smith and colleagues. Dr. Claire Stephens performed the granulosa cell culture work. Dr. Jim Giles assisted in immunofluorescence procedures and troubleshooting. This work was

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APPENDIX

Primers used for sequencing AMHRII. References for Primer Set Regions: 1: 5' UTR/N Terminus, 2: Middle Region, 3: C Terminus/3' UTR. Letters within Primer Set name represent a different primer set targeting the same region.

Primer Set	**Prod. Size**	**Primer (5'-3')**	**Outcome**
F. peregrinus aligned with *M. musculus*	272 bp	F - GATCTCCCAGAGGAGCAGTG	Sequenced - 928-1170 bp AMHRII
		R - TGTACAAGCCCAGTGTGGTG	Not Sequenced
PS1	581	F - CCAACACGTTCTGACTGCTG	Sequenced - Primer Overlap
		R - CATGGGTGCATGGCGATG	Sequenced - Primer Overlap
PS1a	448	F - AAGGAAGTGCTGCAGGGAG	No Band
		R - GGTGGTGCTTGGACCCAT	No Band
PS1b	463	F - CTGGCGAAGGAAGTGCTG	No Band
		R - TAACGGGGTGGTGGTGCTT	No Band
PS2	501	F - GCAGGTCCAGACTCTCATCC	Sequenced - 1002-1460 bp AMHRII
		R - GCCCGGTCGCCATAAAAG	Sequenced - 961-1423 bp AMHRII
PS3	565	F - AGAAACTGCTCCGGAACCTC	Sequenced - Failed
		R - CAGCAGTCAGAACGTGTTGG	Band; Not Sequenced
PS3a	583	F - GGTCCCACATGGCAGAAACT	No Band
		R - GACCTCAGCAGTCAGAACGT	No Band
PS3b	576	F - GGGTCCCACATGGCAGAA	Sequenced - Transmembrane protein 59
		R - CAGTCAGAACGTGTTGGTGC	Sequenced - Transmembrane protein 59
PS3c	538	F - CTTCTTTGCCGCCTTCTTGT	No Band
		R - CACGCAGCGCTATTTGGC	No Band

All trace images and raw sequences from successful sequencing reactions.

F. peregrinus aligned with *M. musculus:*

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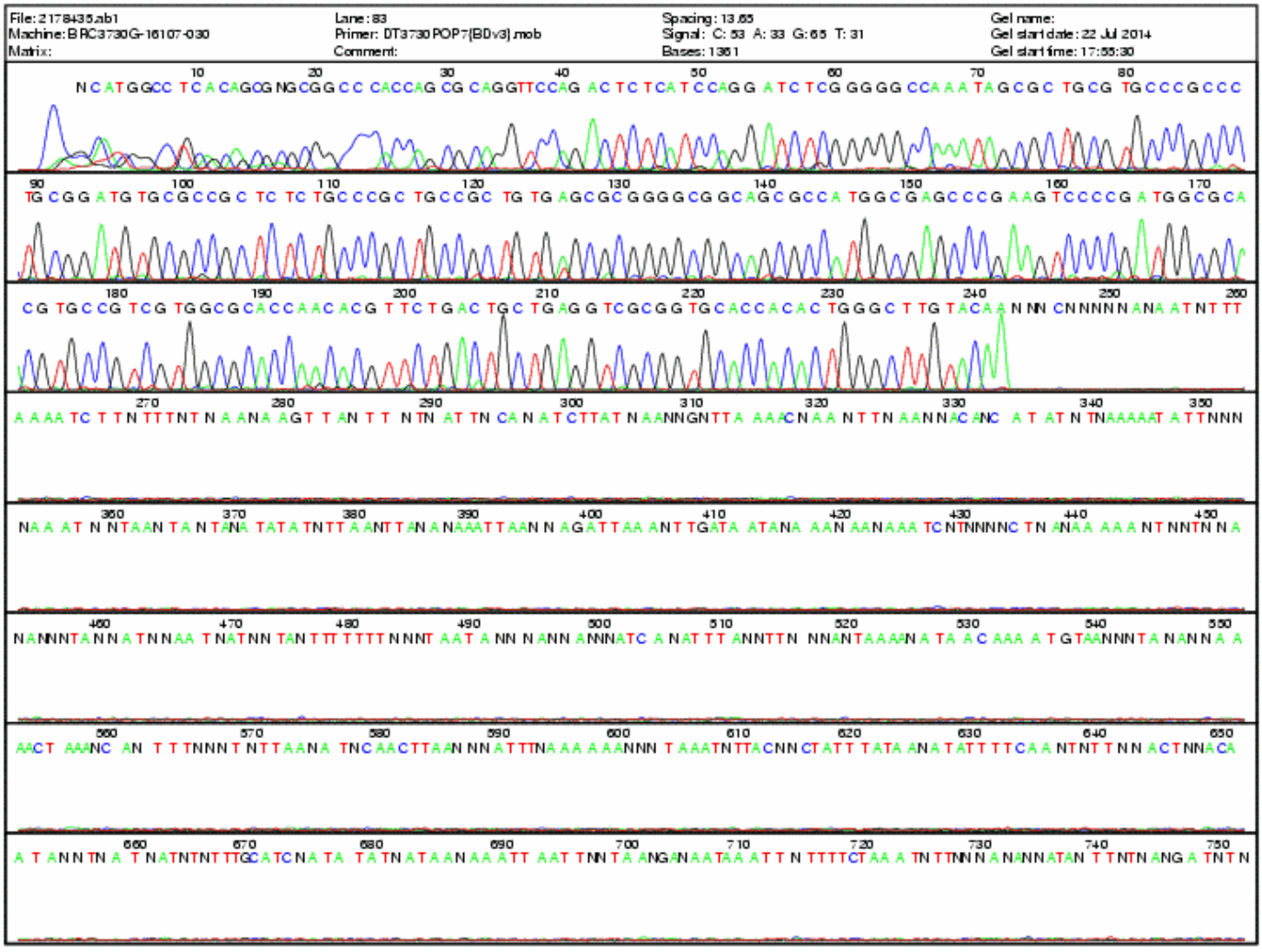

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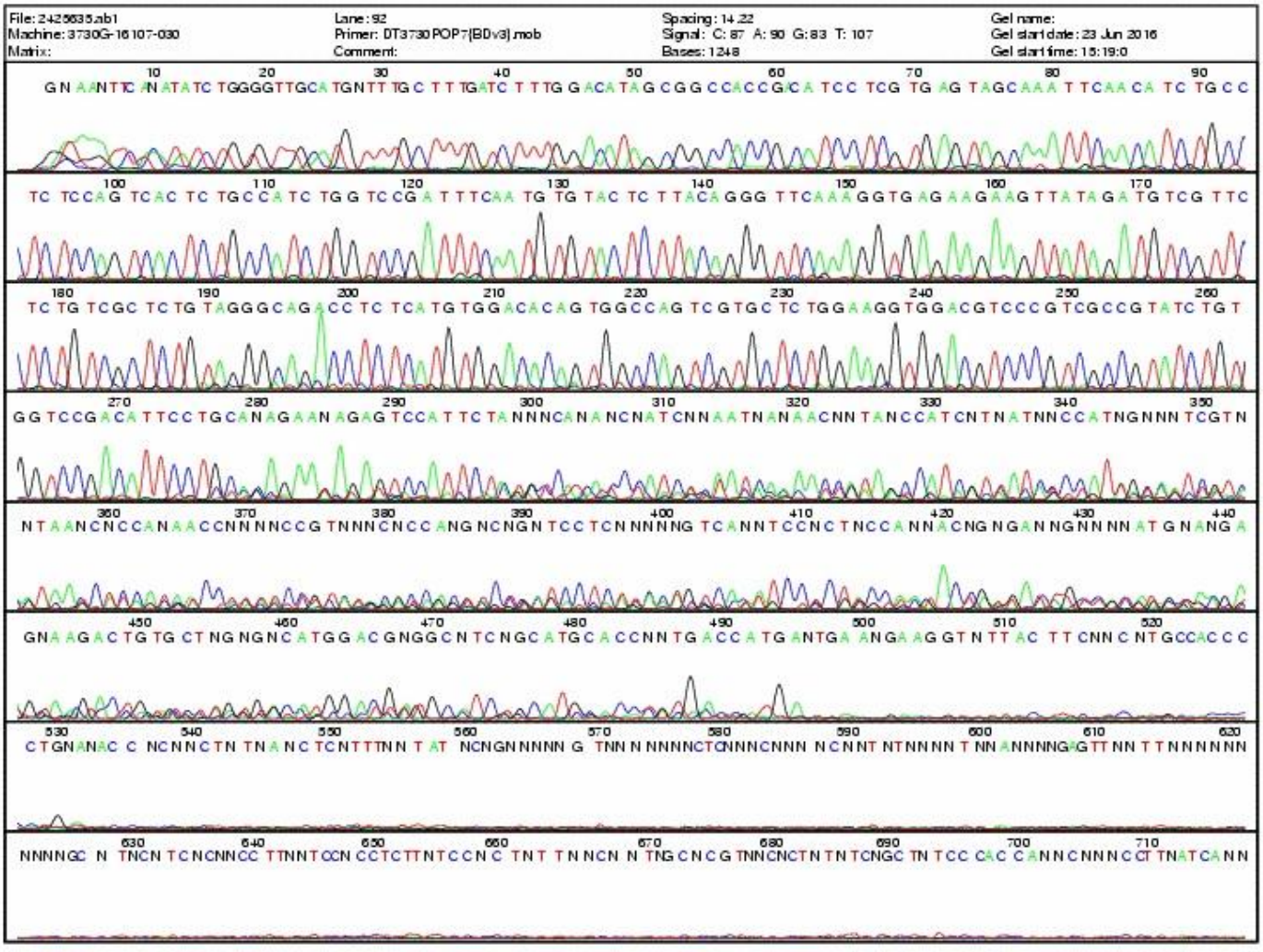

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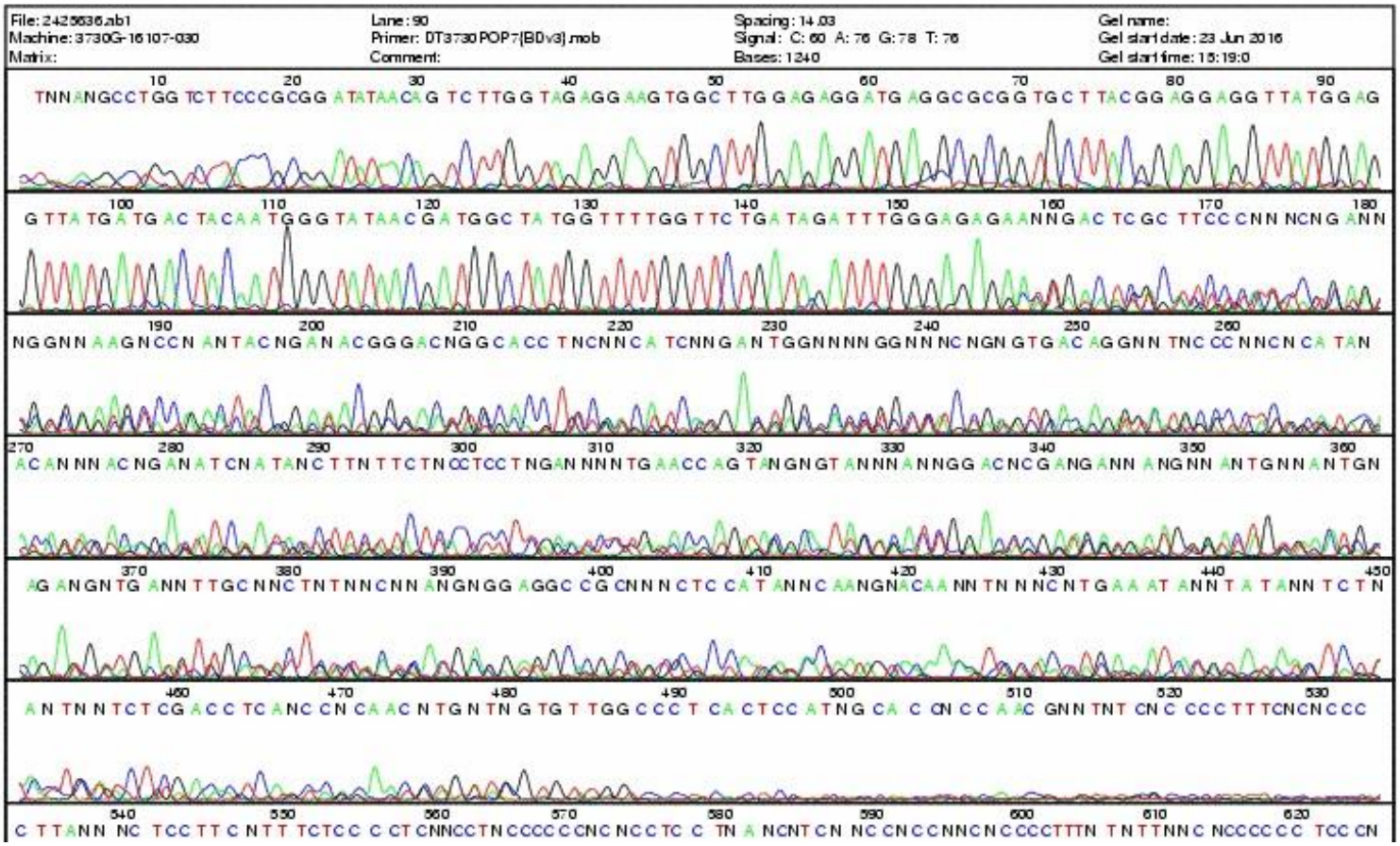

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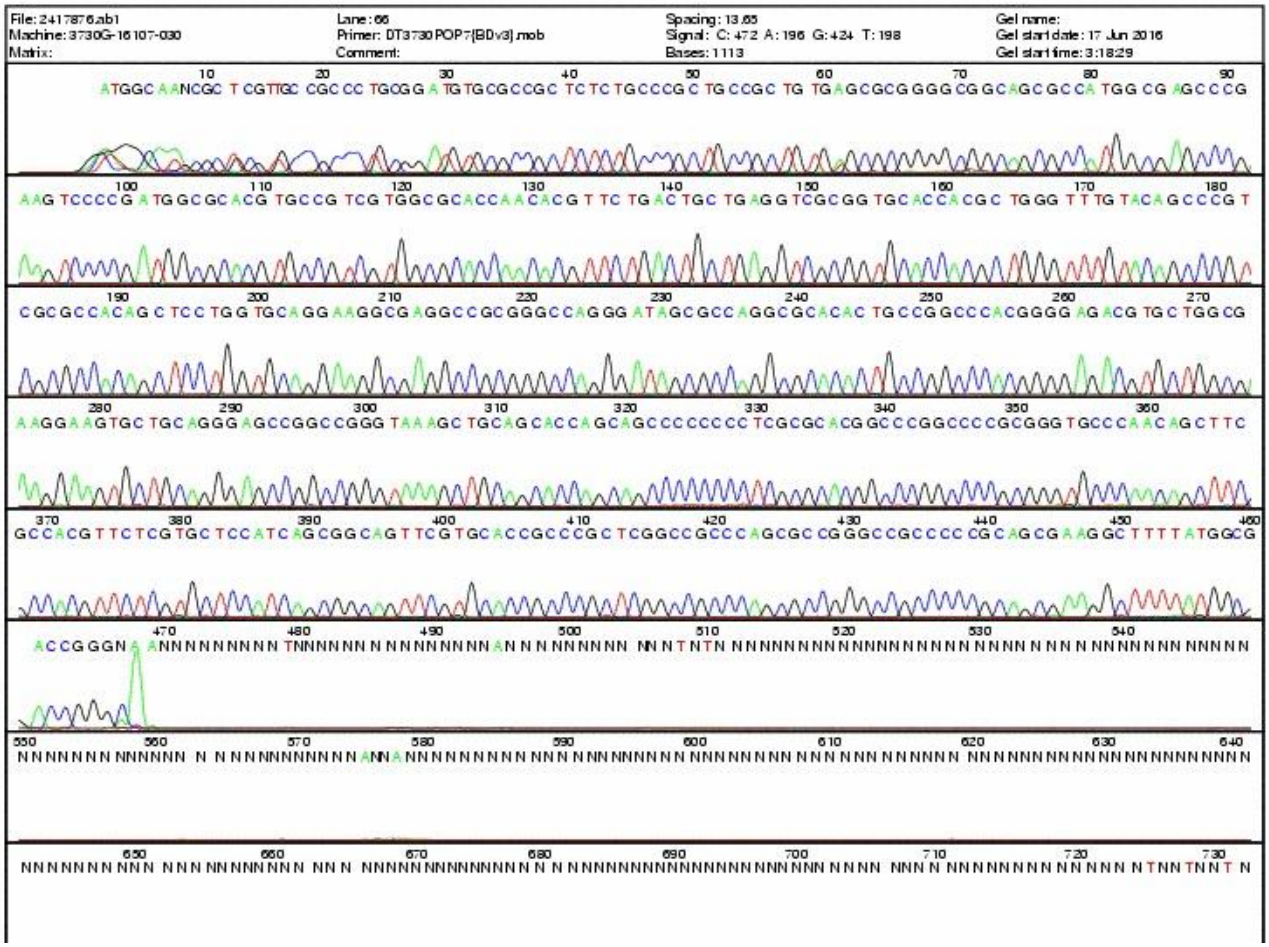

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Order Number: 10361045

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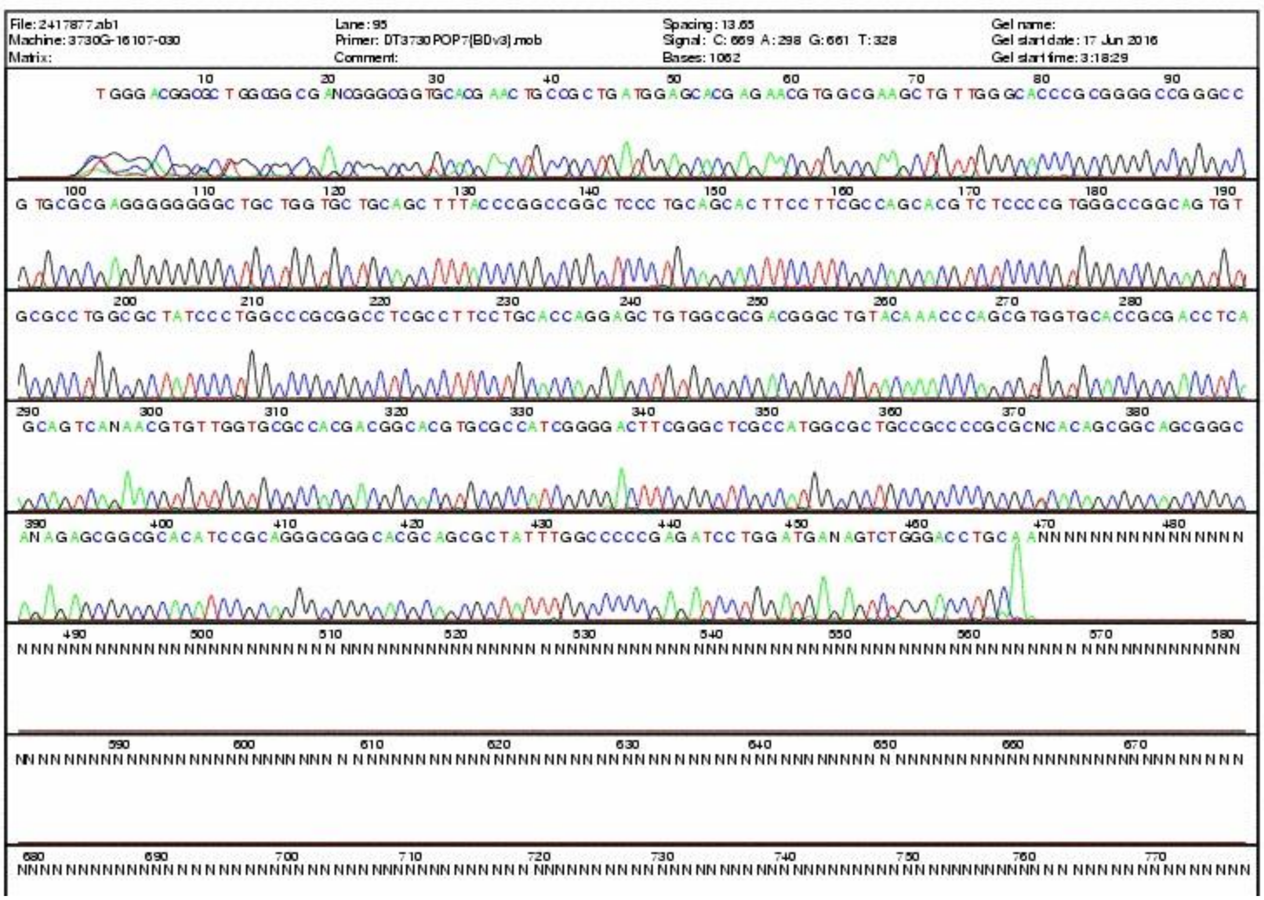

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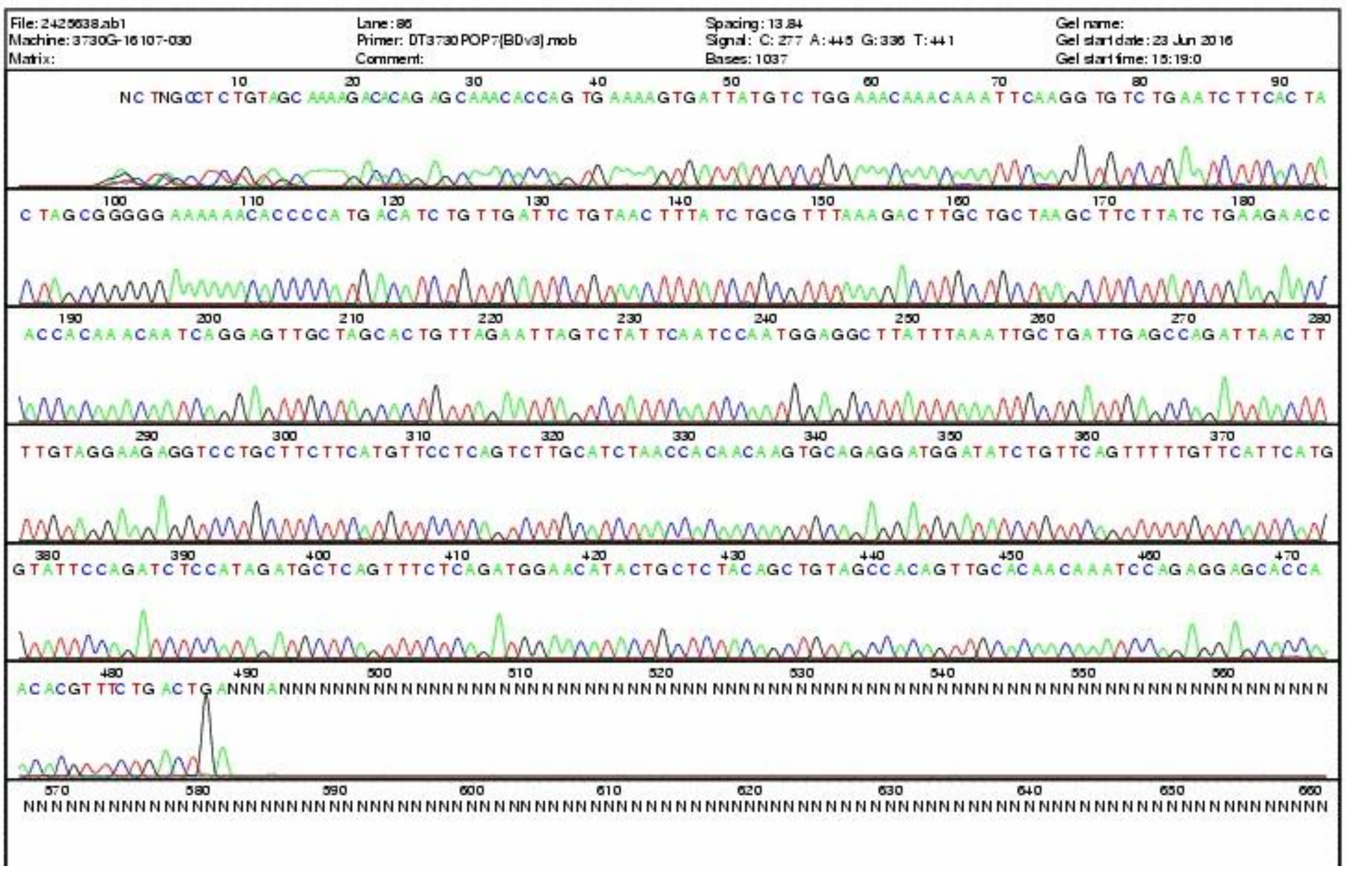

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