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Expression and Regulation of Anti-Mullerian Hormone in an Oviparous Species, the Hen[1]

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ABSTRACT

Anti-mullerian hormone (AMH) has a critical role in regression of the mullerian duct system during development in male mammalian and avian species and in regression of the right oviduct in female avian species. AMH in adult female birds has not been investigated. Chicken-specific cDNA primers were used to isolate *Amh* by RT-PCR. This probe was used in Northern blot analysis to identify a 2.8-kb band with expression in total ovarian RNA and in granulosa cell RNA. Quantitative real-time PCR was used to assess *Amh* expression in follicles of different maturity (1, 3, 5, and 6–12 mm and the largest F1 follicle; n = 4–6 of each size). There was an increased amount of *Amh* mRNA in the granulosa layer of the smaller follicles and a lower amount in the granulosa layer of the larger follicles ($P < 0.01$). There was no difference in granulosa *Amh* expression between the germinal disc and non-germinal disc region of 6- to 12-mm follicles, although expression differed with follicle size ($P < 0.01$). To examine hormone regulation of *Amh*, granulosa cells (from 6- to 8-mm follicles) were cultured with various concentrations of estradiol (E_2) and progesterone (P_4), and *Amh* mRNA was assessed. Neither E_2 nor P_4 influenced *Amh* mRNA accumulation. Granulosa cells were also cultured in the presence of oocyte-conditioned medium (OCM), which decreased *Amh* mRNA expression in a dose-related manner ($P < 0.05$); FSH receptor expression was not affected. Heat treatment of OCM abolished the effect, but growth differentiation factor 9 antiserum did not block the suppression. Immunohistochemistry confirmed that the granulosa layer was the predominant source of AMH in the small follicles of the hen and indicated that AMH was present early in follicle development, with expression in very small follicles (approximately 150 μm).

Amh, *AMH, anti-mullerian hormone, granulosa cells, hen, MIS, mullerian, ovary*

INTRODUCTION

In mammals, anti-mullerian hormone (AMH or mullerian inhibiting substance [MIS]) has the well-described role of regressing the mullerian duct system in males. Assessment of AMH in females has shown that immunoreactive AMH is detected only postnatally in female mammals [1, 2]. In most female birds, only the left ovary and oviduct develop. As in mammals, both male and female avian embryos possess undifferentiated mullerian and wolffian ducts early in embryonic development. By Day 12 of incubation in the male chick, the mullerian ducts have involuted and disappeared [3]. The right mullerian duct of the female disappears by Day 14 while the left mullerian duct continues to develop into the left oviduct [3]. When AMH was implicated in the regression of mullerian ducts in male mammals [4], it was hypothesized and later established that AMH had a role in regression of the mullerian ducts in male birds and of the right mullerian duct in female birds [5–7]. In birds, both embryonic gonads express AMH [7, 8]; however, ovarian estrogen (from the left ovary) protects the left mullerian duct of the chick from AMH action [9, 10] and, therefore, permits development of the left oviduct.

In postnatal mammals, AMH is localized to the granulosa cells with strongest expression in the preantral class of follicles. No immunohistochemical staining is observed in the primordial follicles [1, 2]. In addition, AMH is expressed most strongly in granulosa cells adjacent to the oocyte, and less expression is present in peripheral granulosa cells [1, 11]. Although a variety of functions have been proposed for AMH [12], the AMH knockout mouse provided additional insight into possible ovarian function. Female mice that lack AMH are fertile [13], but subsequent analysis of the ovaries indicated that follicular growth is accelerated in the absence of AMH [14]. As a consequence, young, cycling mice of the AMH-null genotype have a significantly greater number of small, growing follicles, which ultimately results in a significant depletion of primordial follicles in older mice.

There have been many studies examining the regulation of expression of AMH in males, but fewer studies of the regulatory factors involved in AMH expression in the ovary. A variety of transcription factors have been proposed to influence expression in both males and females [15, 16], and testosterone has been inversely correlated with AMH expression in the male [17]. The oocyte has been shown to increase AMH expression from granulosa cells in a dose-related manner, and it has been shown that the effect is influenced by the stage of follicle development [18], although the mechanism by which oocytes exert this effect has not been determined [16].

The cDNA for chicken *Amh* has been cloned and sequenced [19, 20] and the expression pattern of mRNA in embryonic chick gonads determined [19]. The predicted amino acid sequence for chick AMH is approximately 52% identical to mammalian forms of this hormone in the biologically active C-terminal region [19]. In oviparous species, such as the hen, yolk accumulation by the rapidly growing oocyte is the most obvious aspect associated with follicle development. In this case, no follicular fluid accumulates, and all granulosa cells are in contact with the oocyte [21]. From a variety of studies, we know a great deal about follicle development in the hen [22] and have accumulated significant information on the mRNA [23] and protein [24] of TGFβ family members expressed in the hen ovary. Comparative biology would suggest that the hen

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follicle, although actively accumulating yolk, expresses AMH and that this hormone may be expressed in a similar manner and have a role in follicle development, as has been shown in mammals. To our knowledge, there has been no report on the role or regulation of AMH in an adult oviparous species. Therefore, we investigated the occurrence, relative abundance, and localization of AMH in the hen. In addition to these studies, we examined the regulation of *Amh* mRNA in cultured hen granulosa cells.

MATERIALS AND METHODS

Animals

Single-comb White Leghorn hens of the Babcock B300 strain were individually caged, with egg records maintained at 2-h intervals during daylight. The hens had free access to water and feed and were maintained on a lighting schedule of 15L:9D (lights on at 0600 h). Hens aged 22 to 74 wk and laying regular sequences were selected and killed at 1.5–2 h after oviposition for collection of follicles. All animal procedures were approved by the Institutional Animal Care and Use Committee of Cornell University.

RNA Extraction

For Northern blot analysis (n = 5 replicates), the total ovary (TO; without the large yolky follicles), granulosa cell layer from large follicles (F4+5), small yellow follicles (SYF; 6–8 mm and 9–12 mm), and large white follicles (LWF; 3–5 mm) were removed from birds after they were killed, immediately placed in a guanidine isothiocyanate solution, and frozen at −80°C until RNA extraction. Tissues were homogenized and RNA was extracted as previously described by Chomczynski and Sacchi [25]. For quantitative real-time PCR (n = 6 replicates), ovaries were removed from birds after they were killed and immediately placed into ice-cold Krebs-Ringer bicarbonate buffer. The liver, complete ovary, granulosa layer from SYF (6–12 mm), LWF (5 mm, 3 mm), and small intact follicles (approximately 1 mm in diameter) were collected in Buffer RLT (supplied in Qiagen RNeasy Mini Kit; Qiagen). For the more detailed analysis of expression in 6- to 12-mm follicles, one healthy-appearing follicle from each size class of 6, 8, 10, and 12 mm was collected from a hen (n = 4–6 replicates), and the granulosa layer was bisected so that the germinal disc (GD) was in one half and the opposite pole (NGD; non-germinal disc) in the other. Tissue was homogenized and extracted using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions, including optional on-column DNase digestion with RNase-free DNase (Qiagen). For experiments involving cultured granulosa cells, cultures (as described below) were terminated by removing medium and adding Buffer RLT to culture wells. Cells were then scraped gently from the plate and homogenized. RNA was extracted from the homogenates using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions.

Amh *cDNA Probe*

A chicken *Amh* mRNA sequence was identified in the National Center for Biotechnology Information database (GenBank U61754). Primers were designed (forward: 5′CGTCTACGCGGCCAACAACT3′; reverse: 5′GCCGACACGCTGATGATGAG3′) on the basis of this sequence and used in RT-PCR, with total RNA isolated from the hen ovary to generate a 180-bp chicken cDNA for *Amh*. This fragment was then subcloned in pGEM-TEasy Vector (Promega, Madison, WI) and XL1 Blue competent cells (Stratagene, LaJolla, CA), labeled, and used in Northern blot analysis.

Northern Blot Analysis

Approximately 25 μg of total RNA were prepared, separated on a 1.5% denaturing formaldehyde gel, blotted by capillary transfer, and ultraviolet cross-linked to GeneScreen Plus nylon membrane (NEN, Boston, MA). Northern blot analysis was done with the chicken *Amh* cDNA probe (180-bp piece) labeled with 185 kBq ^{32}P-dCTP (DuPont, Boston, MA) by a random primer kit (Prime-It II; Stratagene) to a specific activity of approximately 1.0×10^9 cpm/μg. The membranes were hybridized with the chicken *Amh* cDNA probe at 42°C overnight, stringently washed, and exposed to film (Kodak BioMax MS; Eastman Kodak, Rochester, NY), which was then developed. The membranes were then hybridized to a *Gapdg* probe and processed, and all films were subsequently scanned and the intensity calculated as previously described [23].

Real-Time PCR

Probes and primers for quantitative PCR were designed using Primer Express Software v2.0 from Applied Biosystems, Foster City, CA. The program identified primers for chicken *Amh*, defining a product of 71 bp (forward: 5′CCCCTCTGTCCCTCATGGA3′; reverse: 5′CGTCATCCTGGTGAAACACTTC3′) and a probe (6FAMAGCTCCTCTTTGGCTCA MGBNFQ). For the FSH receptor (*Fshr*; GenBank U51097), the primers defined a product of 70 bp (forward: 5′GCACCTTCCAAGCCTCAGATAT3′; reverse: CCCTATGGACGACGGGTAAA3′), and the probe was 6FAMTGTTAATATCAAACACAGGCCTMGBNFQ. Control reagents were TaqMan ribosomal RNA (18S) primers and probe, and a control reaction was run for each sample. Preliminary Northern blot analysis had shown that *Amh* was abundantly expressed in RNA from the small follicles of the ovary. Therefore, RNA was extracted from the granulosa layer of 3- to 8-mm follicles of two hen ovaries, pooled, subjected to reverse transcription to cDNA, and used to establish a standard curve for the assay. Serial dilutions were made to create seven points on the curve, which was run in duplicate. Samples of RNA (as described above) from the various tissues and treatments were also subjected to reverse transcription to cDNA. The quantitative PCR reactions were performed (using an ABI 7000 Sequence Detection System) in a 25-μl volume of reaction buffer containing 1× TaqMan Universal PCR Master Mix (Applied Biosystems) and 900 nM of the *Amh* and *Fshr* primer pairs or 50 nM of the 18S primer pair and each respective TaqMan probe (*Amh* and *Fshr* at 250 nM and 18S at 200 nM). Control reactions containing no template and reactions lacking reverse transcriptase were also used. All PCR amplifications were carried out in duplicate for each sample and the mean value was calculated relative to 18S reactions (also run in duplicate). The relative amount of mRNA in a particular sample was determined by the comparative threshold cycle (C_T) method using Sequence Detection System Software (Applied Biosystems).

Granulosa Cell Culture

Granulosa cells were isolated from SYF follicles (n = 8–10 follicles; 6–8 mm in size) from one hen for each replicate experiment. Granulosa cells from 6- to 8-mm follicles were chosen because they express relatively high levels of *Amh* (Fig. 1), they are easy to isolate, and they plate well. In contrast, granulosa cells from 3- to 5-mm follicles are less plentiful and more difficult to culture. The granulosa layers were isolated from each follicle, pooled, and dispersed as previously described [26]. The number of cells and viability (trypan blue exclusion) were estimated using a hemocytometer. Cell viability was 95% or greater at the start of an experiment. Cells were plated in six-well plates (in M199 plus 5% fetal bovine serum, Gibco, Carlsbad, CA) at a density of 3×10^6 cells per well and incubated for 24 h as per conditions that have been previously described [27], except that they were cultured in a 1.5-ml volume. After 24 h, the medium (which contained serum) was removed and replaced with M199 plus 0.1% bovine serum albumin (BSA) and either estradiol (E_2), or progesterone (P_4) (n = 6 and n = 4 replicates, respectively) at 0, 0.1, 1, 10, or 50 ng/ml (Sigma-Aldrich, St. Louis, MO). The plates were then returned to the incubator and cultured for an additional 24 h.

In a separate series of experiments, isolated granulosa cells (n = 6 replicates) from 6- to 8-mm follicles (after 24 h of attachment as previously described) were cultured for 24 h in the presence of control medium or oocyte-conditioned medium (OCM) at two concentrations (25% and 50%). In another trial, OCM at 25% or OCM that had been heat treated (65°C, 30 min) was tested. Finally, granulosa cells were treated with control medium, OCM, or OCM plus growth differentiation factor 9 (GDF9) antibody (JH131; n = 6 replicates; obtained from Dr. S.-J. Lee at Johns Hopkins University). At the termination of all granulosa cell cultures, the cells were scraped from the plates, RNA was extracted, and quantitative PCR was performed. Our use of OCM has been previously described [27]; in brief, the large pedunculated follicles were removed from the ovary of a laying hen and discarded. Follicles less than or equal to 1 mm in size were dissected from the ovarian stroma, pooled, and incubated in 3 ml of M199 plus 0.1% BSA at 37°C (in a humidified atmosphere containing 5% CO_2) for 2–3 days. At the end of the incubation period, the tubes were gently centrifuged (for 5 min at 2000 rpm) and the OCM was removed, filter sterilized (0.2-μm membrane), and used immediately or frozen for subsequent use in cell culture. After the granulosa cells were plated (as above), the medium (which contained serum) was removed and replaced with M199 containing 0.1% BSA and various doses (0%, 25%, or 50% of the total volume) of OCM. The plates were then returned to the incubator and cultured for an additional 24 h. To determine specificity of any observed OCM effect, the OCM was preincubated with GDF9 antiserum (1:100 dilution of JH131 antiserum diluted in M199 + 0.1% BSA; the antiserum was incubated 1:1 with OCM for 30 min at room temperature) to neutralize the effect of GDF9 in the OCM before adding to the cultures. Our previous study [27] showed that this

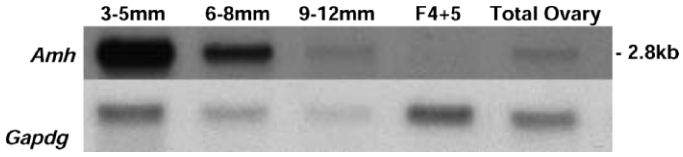


FIG. 1. Representative Northern blot analysis of chicken *Amh* in the granulosa layer of various-sized follicles and in RNA from total ovary. One main band of hybridization is seen at 2.8 kb. *Gapdg* expression is shown for comparison (n = 5 replicates). F4+5, granulosa layer from F4 and F5 follicles.

antiserum effectively blocked stimulation of granulosa cell proliferation induced by OCM.

Immunohistochemistry

Pieces of ovaries (with the large follicles removed) as well as intact follicles (3–5, 6–8, and 12 mm) were isolated from hens and fixed in 10% buffered formalin. The tissue was then embedded in paraffin and sectioned at 4–5 μm by the Histology Laboratory at the Cornell Veterinary School. Prior to immunohistochemistry, the slides were deparaffinized and rehydrated through a series of xylene and ethanol treatments. Sections were incubated in 0.05% pronase at 37°C

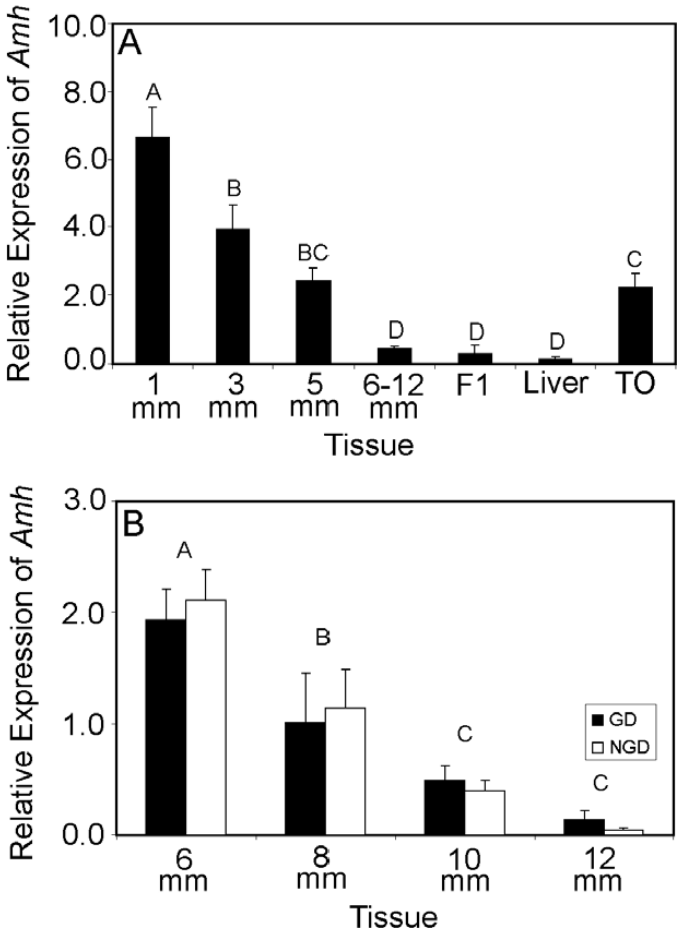
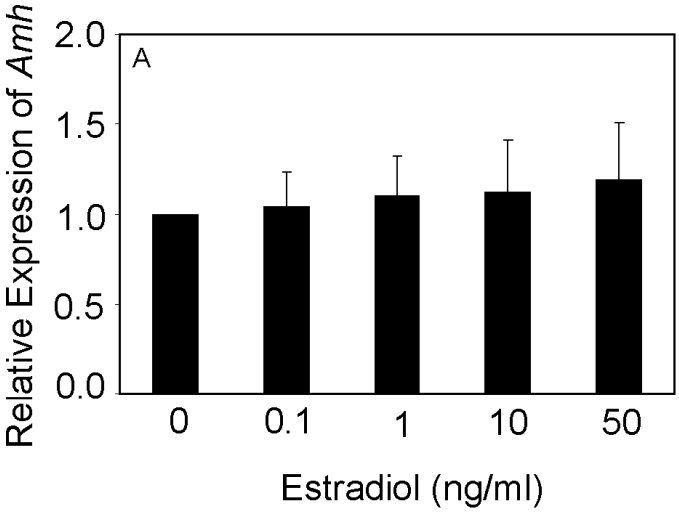


FIG. 2. **A**) Quantitative analysis of expression of *Amh* mRNA in ~1-mm follicles, TO, liver, and granulosa layer from various-sized follicles (3, 5, 6–12 mm, F1). Bars with different letters above them differ significantly ($P < 0.05$) in the amount of *Amh* mRNA expression (n = 6 replicates). **B**) Quantitative expression of *Amh* mRNA in the GD and NGD of small follicles (6–12 mm; n = 4–6 per group). No significant difference was found between GD and NGD for any sized follicle. Error bars represent mean ± SEM. Different letters indicate significant differences in total expression (including GD and NGD for a particular follicle size) among follicle sizes ($P < 0.05$). F1, granulosa layer from the largest follicle.



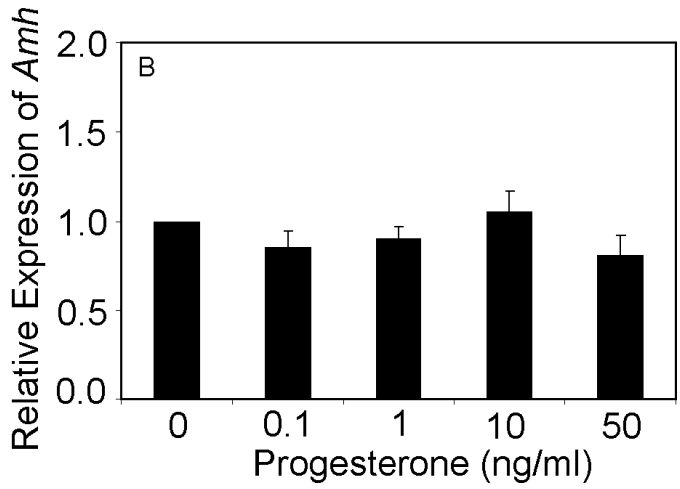


FIG. 3. Quantitative analysis of *Amh* mRNA expression in granulosa cells from 6- to 8-mm follicles cultured with steroids at different doses. **A**) E_2 (n = 6 replicates); **B**) P_4 (n = 4 replicates). Error bars represent mean ± SEM. There was no significant effect among treatments.

and then blocked with 10% goat serum in PBS for 30 min at 37°C and treated with primary antibody for 1 h at 37°C. The primary antiserum was made in a rabbit against the C-terminal portion of human AMH (Genex Bioscience Inc., Hayward, CA) and was used at a dilution of 1:25. Control slides were treated with normal rabbit IgG (Upstate Self Signaling Solutions, Charlottesville, VA). The second antibody was Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR) used at a dilution of 1:2000 for 1 h at 37°C. To identify nuclei, sections were incubated with propidium iodide (PI; 1μg/ml) in PBS for 15 min at room temperature following secondary antibody incubation. Slides were examined with a Nikon Eclipse E600 microscope with fluorescence capability, and the images were recorded with a Spot RT Slider camera.

Statistics

Data were analyzed with SAS (SAS Institute Inc., Cary, NC) using Proc GLM with protected least-significant difference. The significance level was $P < 0.05$.

RESULTS

Figure 1 shows a representative Northern blot using the cDNA probe for chicken *Amh*. One main band of hybridization is observed at approximately 2.8 kb. This band is seen at the

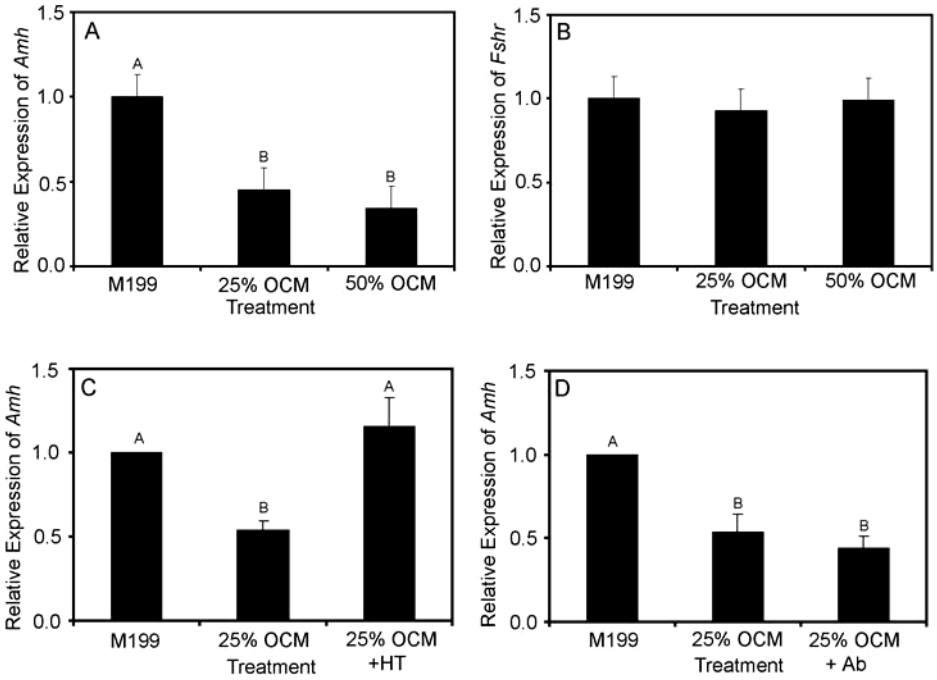


FIG. 4. Regulation of *Amh* expression in cultured granulosa cells (n = 6 replicates for each group). **A**) *Amh* expression with 25 and 50% OCM; **B**) FSH receptor (*Fshr*) expression with 25% and 50% OCM; **C**) *Amh* expression when OCM was heat-treated (HT) prior to culture; **D**) *Amh* expression with 25% OCM and when OCM was preincubated with GDF9 antiserum (labeled Ab). Error bars represent mean ± SEM. Bars with different letters within each panel differ significantly ($P < 0.05$). M199, control.

greatest intensity in the RNA of granulosa cells from LWF (3–5 mm), with decreasing intensity with increased follicle development. *Gapdh* expression is shown for comparison. Results obtained after scanning all five films and correcting for RNA loading with *Gapdh* showed an overall difference in expression of *Amh* with respect to follicle development ($P < 0.01$). Relative expression values (means) for granulosa from <5 mm, 6- to 8-mm, 9- to 12-mm, and F4+F5 follicles and TO were: 0.72^{A}, 0.59^{A}, 0.27^{B}, 0.11^{BC}, 0.11^{BC}, respectively (different letters indicate differences between follicle sizes; $P < 0.05$). Expression was significantly greater in follicles less than 8 mm in size as compared to larger follicles.

Quantitative real-time PCR was performed to assess expression of *Amh* mRNA with respect to follicle development (Fig. 2). After normalization to 18S expression, real-time PCR analysis indicated that *Amh* mRNA abundance differed with respect to the source of the tissue ($P < 0.01$). *Amh* mRNA was most abundant in the approximately 1-mm follicles, with significant amounts in the granulosa layer of 3- and 5-mm follicles and TO. The expression of *Amh* in the granulosa layer of 6- to 12-mm follicles did not differ significantly from that in the granulosa layer of F1 follicles or the liver. Analysis of *Amh* expression between the GD and NGD regions of the granulosa layer from the 6-, 8-, 10-, and 12-mm follicles showed no significant difference. There was an overall significant difference in expression among follicle sizes (Fig. 2B; $P < 0.01$).

Expression data of *Amh* mRNA (as determined from quantitative real-time PCR) from granulosa cells of 6- to 8-mm follicles cultured with various doses of steroid hormones are presented in Fig. 3. There was no significant effect of E_2 (Fig. 3A) or P_4 (Fig. 3B) on *Amh* mRNA expression at any dose tested.

In Figure 4A, we show the relative expression of *Amh* mRNA from granulosa cells from 6- to 8-mm follicles after culture with OCM. Culture with 25% OCM as well as 50% OCM significantly reduced expression of *Amh*, although there was no effect on *Fshr* expression (Fig. 4B). Heat treatment of the OCM prior to culture blocked the inhibitory effect ($P < 0.05$; Fig. 4C). In Figure 4D, the inhibition of *Amh* expression due to 25% OCM was not reversed when GDF9 bioactivity was immunoneutralized by pretreatment of the OCM with specific antiserum ($P < 0.05$).

Representative immunohistochemistry results are shown in Figure 5. Figure 5A shows a hematoxylin and eosin (H&E)-stained section of intraovarian hen follicles. Figure 5B shows results with the AMH antiserum, and Figure 5C also indicates nuclear material stained with PI. Specific AMH staining is particularly obvious in the granulosa layer surrounding the centrally located follicle (approximately 150 μm) and appears cytoplasmic (Fig. 5, B and C). The PI-stained nuclei of the granulosa cells are located at the apical portion of the cell. Figure 5D is an adjacent ovarian section that is the negative control stained only with rabbit IgG; Figure 5E represents the PI staining of the negative control. Immunohistochemical staining of sections from a 12-mm (follicle wall on the left of each panel) and a 6- to 8-mm follicle (follicle wall on the right) is shown in Figure 5, F–J. AMH staining appears to be more intense in the granulosa layer of the 6- to 8-mm follicle as compared to the 12-mm follicle (arrows in Fig. 5, G and H), supporting the mRNA data in Figure 2B. Specific immuno-positive material is also observed in the theca layer of both follicle sizes (Fig. 5, G and H).

DISCUSSION

To our knowledge, this is the first demonstration of AMH presence and regulation in adult females of an oviparous species. Previous data on sexual differentiation of the asymmetric reproductive tract in birds have shown embryonic ovarian AMH production, but have not reported patterns of expression in the adult [7, 19]. AMH may have a role in regulating the progression of follicles through the highly organized hierarchy of the hen. This is suggested by the high level of expression in the small follicles and lower level of expression in the selected follicles. Data in the mouse have indicated the importance of AMH in the rate of follicle recruitment in mammals [14]. Our quantitative PCR analysis showed that the mRNA for *Amh* was highest in the granulosa layer of the very small follicles and was significantly decreased by the 6- to 12-mm stage (Fig. 2). Quantitative analysis of

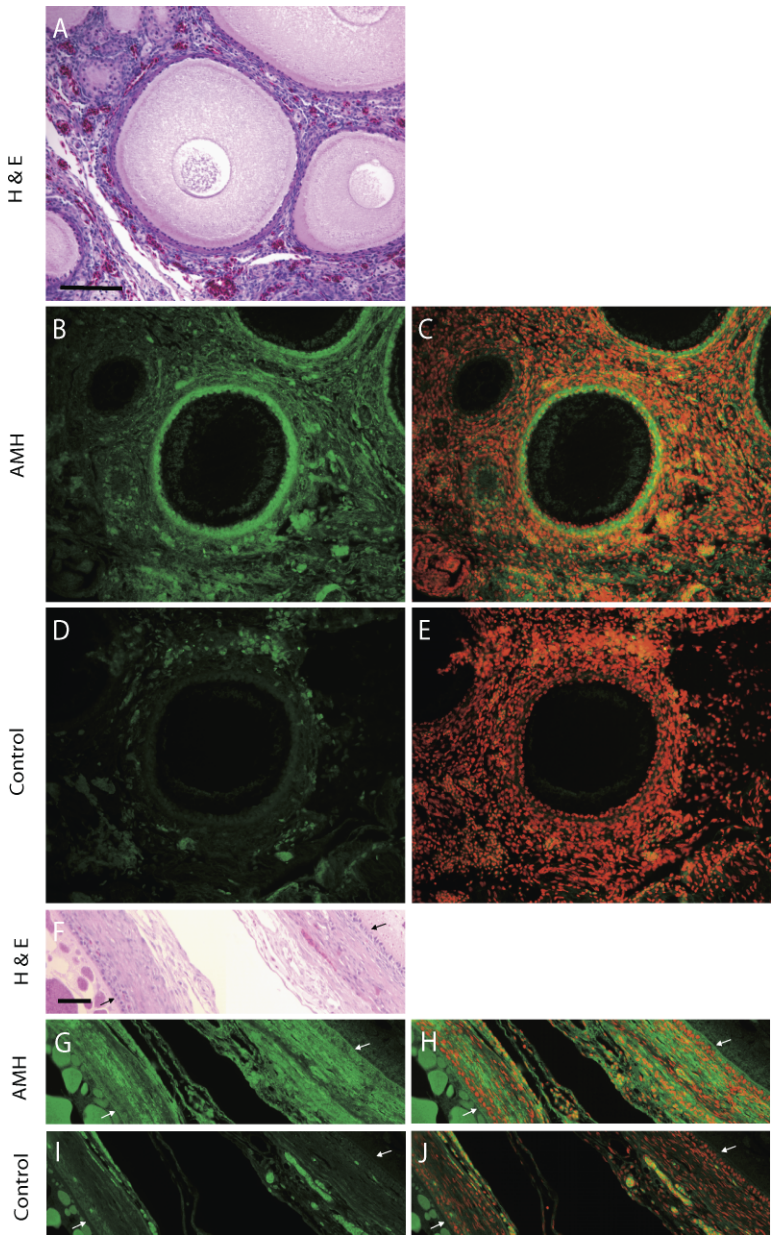


FIG. 5. Immunohistochemistry of *Amh* expression in the hen ovary. **A**) H&E-stained section (labeled H&E) through the ovary containing various-sized follicles. Bar = 50 μm. **B**) Section stained with AMH antiserum (labeled AMH) showing predominant cytoplasmic staining in the granulosa layer. **C**) Section stained with AMH antiserum as well as PI, to indicate nuclei. **D**) Negative control with rabbit IgG used in place of the primary antiserum. Some autofluorescence due to red blood cells is seen in this panel. **E**) Negative control stained with PI. **F**) H&E-stained section of follicle wall from a 12-mm follicle (on left of panel) and a 6- to 8-mm follicle (on right of panel). Bar = 50 μm. **G**) AMH-stained section and **H**) AMH staining with PI. **I**) IgG control and **J**) IgG control with PI. Arrows in **F**, **G**, **H**, **I**, and **J** indicate the granulosa layer of each follicle.

Northern blots indicates that, within the 6- to 12-mm group, there is a big difference in *Amh* expression between granulosa cells from the 6- to 8-mm follicles and those from the 9- to 12-mm follicles. This is also verified by data in Figure 2B. The 6- to 12-mm range encompasses a period of marked transition in hen ovarian follicles (for review, see Johnson [22]). Recent data have shown that follicle selection in the hen most likely occurs at the 6- to 8-mm stage, with one follicle in this group showing a significant increase in *Fshr* mRNA [28]. As seen in Figures 1 and 2B, expression of *Amh* drops off after selection from the 6- to 8-mm pool [28], suggesting an inverse correlation between *Amh* expression and follicle selection.

In mammals, there is a restricted pattern of AMH expression. AMH was found to be highest in preantral to small antral follicles of the rat and markedly reduced in preovulatory follicles [29]. In mice, primordial follicles do not express AMH, but it is first expressed in the primary follicles after recruitment. Moreover, AMH continues to be expressed until the follicles are selected for dominance by FSH [30]. In this regard, AMH expression in mammals is fairly restricted to the interval between primary and antral staged follicles [31]. In the hen, *Amh* mRNA is very low in the granulosa layer of the F1 follicle (Fig. 2A) and not significantly different from the liver. It is difficult to compare follicle stages directly between avian and mammalian species because of the difference in follicle growth (yolk vs. follicular fluid). However, our observation that follicles approximately 50 μm and smaller express little or no AMH (follicle seen in Fig. 5, B and C [top left]; other data not shown) as well as the striking decrease in *Amh* mRNA in granulosa cells from preovulatory follicles (Fig. 2A) suggest a similar dynamic regulation in oviparous species. Immunohistochemical evidence for AMH in the theca layer has been observed in humans [32] and hypothesized to be related to binding of the protein in the theca layer. Studies of mRNA in mammals have not indicated any production in the theca layer, although we have not examined *Amh* mRNA expression in the theca layer of the hen.

AMH expression has been reported to be greater in cumulus granulosa cells as compared to mural granulosa cells [29], suggesting functional differences in the two compartments or

oocyte regulation of AMH in a gradient manner. We studied the equivalent of these regions in the hen (germinal disk and non-germinal disk locations) and found no obvious difference in mRNA expression levels. Based on previous reports using the mouse [18, 16], we examined whether the oocyte may affect the expression of *Amh* mRNA. In earlier work, we showed that oocytes from follicles $\geq$1 mm were a rich source of *Gdf9* mRNA and that OCM stimulated granulosa cell proliferation from 3- to 8-mm follicles [27]. In the current study, we found inhibition of *Amh* expression by OCM in granulosa cells from 6- to 8-mm follicles. Other investigators, however, have shown a positive effect of the oocyte on *Amh* expression in mice [18, 16]. Differences could be due to species, and results obtained from stage-matched oocytes and granulosa cells may have been different, although this is technically very difficult. To our knowledge, it has not been possible to isolate the intact oocyte from the surrounding granulosa cells of growing, yolky follicles in the hen nor to independently culture the granulosa cells from approximately 1-mm follicles. Although there are differences between regulation of AMH in the testis and ovary, there is a precedent for germ cells negatively regulating AMH because TNFα secretion by germ cells in the testis suppresses AMH expression from Sertoli cells [33].

Estrogen has been reported to affect AMH expression in mammals, although perhaps not directly, with increased AMH expression associated with E_2-mediated inhibition of follicle growth [34]. The lack of effect of E_2 on AMH expression in hen granulosa cells is consistent with the effect of estrogen treatment on AMH in the chick embryo [7]. Treatment of chick embryos on various days of incubation with diethylstilbestrol had no significant effect on gonadal AMH secretion [7]. We examined P_4 because of the well-documented increase in P_4 production associated with progression of follicle development. It was possible, although disproved, that P_4 decreased *Amh* mRNA expression.

Mechanisms regulating ovarian *Amh* expression remain unknown, although a variety of transcription factors have been examined (as reviewed in Lasala et al. [15]). Oocyte regulation may be important, as suggested by Salmon et al. [18, 16], and could provide a mechanism for intraovarian control of follicle recruitment [15]. Our results indicate that GDF9 is not likely to be the factor responsible for inhibition of *Amh*, although GDF9 in OCM has previously shown bioactivity when cultured with similar-sized hen granulosa cells [27]. Other defined oocyte factors in the hen, such as EGF [35], or factors yet uncharacterized in the hen oocyte, such as BMP15 or TNFα [33], may be responsible for the effect. EGF receptor activation has been shown to increase mitogen-activated protein kinase activity in hen granulosa cells, and this is associated with inhibition of follicle development [28]. It is important to point out that culture of the approximately 1-mm follicles to produce the OCM includes both the surrounding granulosa cells as well as the oocyte. *Amh* mRNA is abundant in approximately 1-mm follicles of the hen, and AMH likely contributes to the OCM. In the rat, the AMH receptor (AMHRII) appears to be restricted to the granulosa layer, suggesting an autocrine effect [29]. Therefore, it is possible that AMH may inhibit its own production, particularly if the oocyte and granulosa cells are at different stages. *Amh* expression in granulosa cells from 6- to 8-mm follicles may be more sensitive (i.e., have developed receptors) to inhibition by an oocyte factor. As previously mentioned and seen in Figures 1 and 2B, *Amh* is reduced just after this time, soon after follicle selection. When follicles initially start to grow (>150 μm), the granulosa cells express abundant AMH, perhaps decreasing FSH responsiveness [14] and preventing premature maturation. Future experiments examining AMHRII and addressing the effect of AMH on FSH responsiveness are warranted.

Results from these studies show that *Amh* is expressed in the hen ovary in a highly regulated pattern. Quantitative PCR analysis indicates that *Amh* mRNA is highest in the small follicles, prior to selection into the growing hierarchy. Neither E_2 nor P_4 affected granulosa cell expression of *Amh* mRNA. A heat-labile factor present in OCM suppressed *Amh* expression, but incubation with a blocking antibody indicated that this was not likely due to GDF9. Cytoplasmic staining of AMH protein was evident in the granulosa layer of intraovarian follicles, and a decrease in AMH protein was observed between 6- to 8-mm and 12-mm follicles. These experiments will contribute to a more complete understanding of the role of AMH in maintenance of the follicular hierarchy in adult oviparous species.

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