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2 **Running head: *AMHR2 in bovine granulosa cells***

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4 **Anti-Müllerian Hormone (AMH) Receptor Type II Expression and AMH Activity in**
5 **Bovine Granulosa Cells**

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ABSTRACT

Anti-Müllerian hormone (AMH) produced by granulosa cells has previously been proposed to play a role in regulating granulosa cell differentiation and follicle selection. While AMH receptor type II (AMHR2) dimerizes with a type I receptor to initiate AMH signaling, little is known about the regulation of AMHR2 expression in bovine granulosa cells and the role of AMH in follicle development. The primary objectives of this study were to: 1) characterize *AMHR2* expression in granulosa cells during follicle development; 2) identify factors that regulate *AMHR2* mRNA expression in granulosa cells; and 3) examine the role of AMH signaling in granulosa cell differentiation and proliferation. Bovine granulosa cells were isolated from 5-8 mm follicles prior to selection and deviation, as well as from 9-12 mm and 13-24 mm follicles after selection. Analyses revealed that expression of *AMHR2* was greater in 5-8 mm follicles compared to 13-24 mm follicles ($p<0.05$). Granulosa cells treated with bone morphogenetic protein 6 (BMP6) or BMP15, but not BMP2, significantly increased *AMHR2* expression when compared to control cultured cells ($p<0.05$). In addition, expression of *AMH* was greater in granulosa cells cultured with BMP2, BMP6, or BMP15 when compared to controls ($p<0.05$). Finally, treatment with recombinant human AMH, *in vitro*, inhibited *CYP19A1* expression in a dose-related (10-100 ng/ml) fashion, and reduced granulosa cell proliferation at 48 and 72 h ($p<0.05$). Results from these studies indicate that AMH signaling plays a role in both regulating granulosa cell proliferation and preventing granulosa cells from 5-8 mm follicles from undergoing premature differentiation prior to follicle selection.

KEYWORDS: Follicle development, granulosa cell proliferation, bone morphogenetic proteins

1. Introduction

The mechanisms by which a single bovine follicle becomes selected and deviates in size from the rest of the cohort to attain dominance while the remaining, non-selected follicles undergo atresia have not been completely elucidated. Several intrafollicular factors belonging to the transforming growth factor (TGF)- β superfamily have been shown to regulate granulosa cell growth and differentiation, and accordingly, impact follicle selection [1]. Anti-Müllerian hormone (AMH), a member of the TGF β superfamily, is highly expressed by granulosa cells in preantral and small antral follicles in rat and mouse ovaries, but not primordial or atretic follicles [2-5]. Intense staining for AMH has been identified in both mural granulosa cells lining the antrum and in cumulus granulosa cells surrounding the oocyte of the mouse ovary, but was not found in theca cells, oocytes or interstitial cells [3-6]. Previous reports have demonstrated that AMH treatment decreased aromatase activity in granulosa cells and inhibited FSH-induced follicular development in rodent and porcine ovaries [7, 8]. Thus, AMH signaling via has a potential role to regulate granulosa cell function during the period of follicle selection [9, 10].

Similar to other members of the TGF β superfamily, AMH requires a ligand-specific type II receptor that dimerizes with a type I receptor to initiate downstream signaling through activation of SMAD1, -5 and -8 [9]. Expression of AMHR2 is reportedly restricted to mesenchymal cells surrounding the Müllerian duct during embryogenesis, tubular and follicular structures of fetal gonads, Sertoli and Leydig cells of adult testis, and granulosa cells isolated from different sized follicles from mouse and goat ovaries [9-13]. Anti-Müllerian hormone signaling via its receptor, AMHR2, has been well characterized in Müllerian duct regression in rodents and in humans with persistent Müllerian duct syndrome [14, 15]. The expression of *AMHR2* mRNA has recently been evaluated in bovine granulosa cells at times surrounding

follicle deviation [9, 10]. Nevertheless, the regulation of *AMHR2* mRNA expression during early follicle development, potentially mediated through the transcription factors, nuclear receptor subfamily 5, group A, member 1 (NR5A1; formerly known as steroidogenic factor 1) and Wilms tumor protein (WT1) [16, 17], has yet to be evaluated. Finally, there is evidence from various tissues, including bovine granulosa cells, that AMH signaling is influenced by bone morphogenetic proteins (BMPs) [18], yet a role for BMPs in regulating *AMHR2* expression in bovine granulosa cells remains to be established.

Thus, the central hypothesis of this study is that in bovine granulosa cells several BMPs regulate both *AMH* and *AMHR2* expression, which in turn can mediate inhibitory signaling to suppress premature follicle selection. The present studies were directed towards characterizing *AMHR2* expression in bovine granulosa cells prior and subsequent to follicle selection, elucidating paracrine/autocrine factors capable of modulating *AMHR2* expression in bovine granulosa cells from 5-8 mm antral follicles, and evaluating the ability of AMH to suppress premature granulosa cell differentiation and proliferation. We predict that a better understanding of the mechanisms regulating AMH / *AMHR2* signaling in granulosa cells from follicles prior to selection and deviation will contribute to improving assisted reproductive technologies in cattle.

2. Material and methods

2.1 Animals and Tissue Collection

Ovaries from cycling, non-pregnant dairy cows were collected within 10 minutes following exsanguination at a local abattoir. Ovaries were placed into ice-cold phosphate buffered saline (PBS) containing 2 mM EDTA (Sigma Chemical Co., St. Louis, MO, USA) and transported to the lab. All ovaries were collected as pairs and visually examined to classify the

93 stage of estrous cycle (Stage I, day 1 to 4; Stage II, day 5 to 10; Stage III, day 11 to 17; Stage IV,
94 day 18 to 20) based on morphological parameters of the corpus luteum (CL), as previously
95 described by Ireland *et al.* [19]. In brief, CL on ovaries were examined and classified based on
96 external appearance, diameter and presence of vasculature on the luteal surface. Ovary pairs that
97 possessed a 1.6 to 2.5 cm CL with a tan to orange appearance, together with peripheral blood
98 vessels (representing animals in stages II and III of the estrous cycle) were used to obtain
99 granulosa cells for these experiments. According to these criteria, granulosa cells were collected
100 from follicles at a time when a functional corpus luteum was present and progesterone
101 concentrations were elevated. The diameter of the antral cavity of individual follicles was
102 measured via trans-illumination with a fiber optic single light microscope illuminator containing
103 a 150 Watt halogen light. Follicles were classified according to size as 5-8 mm, 9-12 mm or 13-
104 24 mm follicles. Follicular fluid was gently aspirated with an 18-gauge needle and 10 ml Norm-
105 Ject luer lock syringe (Henke Sass Wolf GMBH, Tuttlingen, Germany). To collect granulosa
106 cells, each follicle was flushed within 1 h of harvest with PBS containing 2 mM EDTA in an
107 amount of equal to the volume of follicular fluid aspirated. Using this procedure, both cumulus-
108 oocyte complexes and mural granulosa cells were obtained. Cumulus-oocyte complexes were
109 removed using a Stripper-CC[®] pipettor (Mid-Atlantic Diagnostics, Marlton, NJ, USA) under a
110 dissecting microscope. Following removal of the cumulus-oocyte complexes, flushings and
111 follicular fluid were centrifuged for 5 min at 200 x g three times with ice-cold MEM α
112 supplemented with 1% non-essential amino acids (AA) and 1% AA (Invitrogen Corp., Carlsbad,
113 CA, USA) to obtain the granulosa cells. Granulosa cells from 5-8 mm undifferentiated follicles
114 were either immediately frozen, or pooled and placed into culture. Granulosa from these 5-8 mm
115 follicles are considered undifferentiated due to elevated *FSHR* expression plus the absence of

detectable *LHCGR* [20]. Mural granulosa cells from individual 9-12 and 13-24 mm preovulatory follicles were immediately frozen until further analysis.

2.2 Granulosa Cell Cultures

Freshly isolated mural bovine granulosa cells from undifferentiated 5-8 mm follicles were placed into culture for 48 h prior to treatment to monitor bovine granulosa cell purity and cell viability, as described by Lerner *et al.* [21]. Due to the limited number of granulosa cells isolated from a single 5-8 mm follicle, granulosa cells from all 5-8 mm follicles per animal were pooled prior to being placed in culture. After 48 h, bovine granulosa cells were trypsinized, counted and re-plated at a seeding density of 5.0×10^5 cells per ml. For all *in vitro* experiments, bovine granulosa cells were cultured in MEM α plus 2.5% FBS (Atlanta Biologicals) containing 0.1 mM non-essential amino acids plus 1% AA. To evaluate *AMH*, *AMHR2* and *CYP19A1* expression following AMH or BMP treatment, bovine granulosa cells were cultured for 24 h at 37 C in an atmosphere of 95% air: 5% CO₂ in 12-well polystyrene culture plates (Beckton Dickinson, Franklin Lakes, NJ, USA) in 1 ml total volume. Granulosa cells were subsequently cultured in the absence or presence of recombinant human (rh) BMP2 (50 ng/ml, Peprotech, Rocky Hill, NJ, USA), rhBMP6 (25 or 50 ng/ml, Peprotech, Rocky Hill, NJ, USA), or rhBMP15 (10 ng/ml, R&D Systems, Minneapolis, MN, USA) to evaluate effects on expression of *AMH*, *AMHR2* or associated transcription factor genes. Comparable concentrations of BMPs have been shown to exert significant effects on granulosa cell function, *in vitro* [22-24]. The transcription factors, NR5A1 and WT1, also were monitored as each was previously associated with regulating *AMHR2* expression [16, 17]. To examine transcription factor gene expression, freshly collected bovine granulosa cells from 5-8 mm follicles were placed in 12 x 75 mm polypropylene tubes (Fisher Scientific, Pittsburgh, PA, USA) and incubated for 3 h in a 37 C

circulating water bath at normal atmospheric pressure. Untreated granulosa cells served as controls for these experiments.

Finally, to confirm that cultured granulosa cells from undifferentiated, 5-8 mm antral follicles did not undergo spontaneous luteinization *in vitro*, expression of *CYP19A1*, *LHCGR*, and *FSHR* mRNA together with media progesterone (P4) concentrations were analyzed.

2.3 Purification of RNA, cDNA synthesis and real-time PCR

Total RNA was extracted and cDNA synthesis performed as previously described by Ocón-Grove *et al.* [23]. Forward and reverse primers directed towards *Bos taurus AMH*, *AMHR2*, *CYP19A1*, *FSHR*, *LHCGR*, *RPL19*, *NR5A1*, *WT1* mRNA are described in Table 1. Each primer pair was validated for specificity. Random-primed, reverse-transcribed cDNA synthesis reactions were performed using the Promega RT System, according to the conditions described by the manufacturer (Promega, Madison, WI, USA). For quantitative real-time PCR (qPCR), primers (Integrated DNA Technologies, Coralville, IA, USA) were added to 10 µl total reaction volume using the reagents provided in the PerfeCTa Sybr Green FastMix Low Rox (Quanta BioSciences, Gaithersburg, MD, USA). Final concentrations (300 nM) of the sense and antisense primers were determined for each primer pair based upon optimal amplification efficiency. Reactions were completed on the AB 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and conducted with the following conditions: 30 sec at 95° C, followed by 40 cycles each for 3 s at 95 C, 1 min at 56 C and 30 sec at 72 C. Melting curves were generated and evaluated for each run. The qPCR amplification products were electrophoretically separated on 1.5% agarose gels and visualized with ethidium bromide under UV light to ensure a specific band corresponded to the size of the expected cDNA fragment. Specific bands were extracted and purified using QIAquick gel extraction kit (Qiagen Sciences, Valencia, CA, USA).

Following extraction, qPCR products were sequenced to confirm product specificity. The Ct (the cycle number at which the fluorescence exceeds a threshold level) was determined for each reaction (conducted in triplicate) using the Applied Biosystems 7500 software (v.2.0.4). Target gene expression was standardized to ribosomal protein L19 (*RPL19*) RNA using the $\Delta\Delta C_t$ method [25]. The stable expression of *RPL19* was confirmed across each stage of follicle development and growth factor treatment utilized (data not shown). Results were expressed as the relative transcript abundance compared to an appropriate control tissue or treatment (set to 1.0).

2.4 Cell Proliferation Assay

Validation experiments were conducted to determine if carboxyfluorescein succinimidyl ester (CFSE, Fisher Scientific, Pittsburgh, PA, USA) would provide an efficient, non-radioactive means of measuring cell proliferation in bovine granulosa cells. Granulosa cells from 5-8 mm follicles were cultured as described above. Bovine granulosa cells were labeled with 2.5 μ M CFSE according to the manufacturer's protocol and as previously described [26]. Briefly, cells were incubated with CFSE at 37 C for 15 min in serum-free culture medium, followed by 10 min incubation at 25 C in medium containing 10% calf serum. The cells were washed for 3 times for 10 min in medium containing 10% calf serum to remove excess CFSE. Following the last wash, bovine granulosa cells were counted then re-plated at a density of 2.0×10^5 cells per ml and were cultured for an additional 24, 48 or 72 h in MEM α plus 2.5% FBS containing 0.1 mM non-essential amino acids plus 1% AA (Invitrogen Corp., Carlsbad, CA, USA) at 37 C in an atmosphere of 95% air: 5% CO₂ in 96-well polystyrene culture plates (Beckton Dickinson, Franklin Lakes, NJ, USA). To determine the effect of AMH on granulosa cells proliferation, bovine granulosa cells were labeled with CFSE and were placed in culture with increasing

concentrations of rhAMH (0, 10, 25, or 100 ng/ml, R&D Systems, Minneapolis, MN, USA). At 24, 48 or 72 h post treatment, bovine granulosa cells were washed 3 times with warm PBS containing 2 mM EDTA, then trypsinized and re-suspended in PBS containing 2 mM EDTA. Propidium iodide (1 μ M, Invitrogen Corp. Carlsbad, CA, USA) was added to assess cell viability and the percentage of proliferating bovine granulosa cells was evaluated using the Guava EasyCyte™ Plus (Milipore, Billerica, MA, USA). Unlabeled granulosa cells were cultured under the same conditions, and served as an internal calibration control to determine autofluorescence (Fig. 4A, i). Betacellulin (BTC, Peprotech, Rocky Hill, NJ, USA), a promoter of granulosa cell proliferation [27], served as a positive control (Fig. 4A, ii.). To evaluate the effectiveness of CFSE incorporation, bovine granulosa cells were analyzed immediately (T0), 48 h and 72 h after CFSE incorporation to evaluate the loss of green intensity, an indication of cell division (Fig. 4A, iii-v). Data were collected by first analyzing bovine granulosa cells for cell size (forward scatter) and cell density (side scatter) dot plots to clearly identify live and dead cells. Unlabeled cells were analysed to determine the level of bovine granulosa cells autofluorescence (Fig. 4A, i). Labelled, unstimulated cells were then analysed to determine the maximal level of CFSE fluorescence (Fig. 4A iii). To determine the population of proliferating and non-proliferating granulosa cells, acquisition gates were set based on these controls as recommended by the manufacturer's protocol. During acquisition, dead cells and debris were gated and excluded from plots and a total of 10,000 granulosa cells were analyzed for each sample. Granulosa cells cultured in the presence of 3% FBS continued to proliferate, *in vitro*, therefore these data were normalized to control cells and presented as a fold-change in proliferation.

2.5 Statistical Analysis

All statistical analyses were performed using the mixed model of SAS (Statistical Analysis System Institute, Cary, NC, USA). Gene expression data are presented as a fold-difference compared to control tissue or cultured cells. Covariate analysis with *RPL19* as the covariate was utilized to analyze gene expression data. Cell proliferation data were normalized and expressed as a fold-difference compared to control cells. Two-way ANOVA followed by the Student-Newman-Keuls test was performed to determine differences among treatment groups for granulosa cell proliferation. Experiments were independently replicated with cells from different animals a minimum of four times unless otherwise stated. Differences were considered significant at $p < 0.05$.

3. Results

The undifferentiated status of 5-8 mm follicles prior and subsequent to culture was confirmed by the finding of elevated *FSHR* levels (expression standardized to the *RPL19* reference gene was 15.7 ± 4.0 -fold and 9.8 ± 4.0 -fold, respectively; $P > 0.05$) together with the absence of detectable *LHCGR* (relative expression standardized to the *RPL19* was 0.32 ± 0.4 and 1.12 ± 0.4 , respectively; $P > 0.05$). Moreover, concentrations of P4 increased slightly in culture media over the 72 h culture period (77.4 ± 2.7 ng/500,000 cells at 12 h to 240.5 ± 45.6 ng/500,000 cells at 72 h) but were markedly lower than media samples collected from luteal cells cultured under similar conditions (118.1 ± 22.0 ng/500,000 granulosa cells vs. 2433.7 ± 122.2 ng/500,000 luteal cells after 24 h, *in vitro*; $P > 0.05$).

3.1 Expression and regulation of AMH, AMHR2 and associated transcription factors in bovine granulosa cells

Expression of *AMHR2* mRNA in bovine granulosa cells was greatest in 5-8 mm undifferentiated follicles and decreased in bovine granulosa cells isolated from 13-24 mm follicles ($P<0.05$; Fig. 1). Culture of granulosa cells from 5-8 mm follicles with 50 ng recombinant human (rh) BMP6 per ml or 10 ng rhBMP15 per ml for 24 h (but not 3 h; data not shown) increased mRNA levels encoding the *AMHR2* (Fig. 2A; black bars). Culture with rhBMP6 (25 or 50 ng/ml) increased *AMH* expression in bovine granulosa cells, *in vitro* (Fig. 2A; white bars). Furthermore, *AMH* mRNA levels were increased when granulosa cells from 5-8 mm follicles were cultured for 24 h in the presence of rhBMP2 (50 ng/ml), or rhBMP15 (10 ng/ml; Fig. 2A; white bars). Increased *AMHR2* expression after 24 h in the presence of 10 ng/ml rhBMP15 is preceded by enhanced expression of mRNA encoding Wilms Tumor 1 (*WT1*), but not *NR5A1*, after 3 h (Fig. 2B). In these experiments, addition of 50 ng/ml of rhBMP2 or rhBMP6 failed to induce expression of either *WT1* or *NR5A1* in bovine granulosa cells after 3h, *in vitro* ($P>0.05$; Fig. 2B).

3.2 *AMH* inhibits differentiation and proliferation of bovine granulosa cells

Bovine granulosa cells from 5-8 mm follicles were cultured for 24 h with increasing concentrations of rhAMH (10, 25 or 100 ng/ml) to evaluate the effects of AMH on granulosa cell function. Treatment of bovine granulosa cells with 100 ng rhAMH/ml reduced *CYP19A1* expression ($P<0.05$; Fig. 3A), without a significant decrease in cell viability (data not shown). In a separate experiment, bovine granulosa cells were treated with rhBMP2 (50 ng/ml), rhBMP6 (50 ng/ml) or rhBMP15 (10 ng/ml) for 24 h to evaluate *CYP19A1* expression (Fig. 3B). Treatment of bovine granulosa cells with 10 ng rhBMP15/ml significantly reduced *CYP19A1* expression compared to controls ($P<0.05$; Fig. 3B). Interestingly, there was no significant change in *CYP19A1* expression in bovine granulosa cells cultured with rhBMP2 or rhBMP6 compared to

controls (Fig. 3B). To evaluate AMH signaling on bovine granulosa cell proliferation, granulosa cells from 5-8 mm follicles were labeled with carboxyfluorescein diacetate (CFSE) and placed in culture with increasing concentrations of rhAMH (10, 25 or 100 ng/ml) for 48 and 72 h post treatment. Proliferation was inhibited in bovine granulosa cells treated with rhAMH (100 ng/ml) after 48 and 72 h *in vitro* when compared to controls ($P<0.05$; Fig. 4). Interestingly, there was a significant reduction in bovine granulosa cell proliferation after 72 h in the presence of the lowest concentration of rhAMH (10 ng/ml). As a positive control, treatment with the known mitogen, betacellulin (10 ng/ml), significantly increased granulosa cells proliferation after 48 and 72 h of culture ($P<0.05$).

4. Discussion

The focus of the present study was directed towards identifying *AMHR2* expression in bovine granulosa cells during follicular recruitment and selection, elucidating paracrine/autocrine factors capable of modulating *AMHR2* expression in granulosa cells from preselected follicles, and evaluating effects of AMH signaling on granulosa cell differentiation and proliferation. Anti-Müllerian hormone receptor type II is expressed in the pre-granulosa cells of rodent and porcine primordial follicles and in granulosa cells of mainly non-atretic pre-antral and small antral follicles [5, 7, 9]. In addition, Monniaux *et al.* [13] recently characterized *AMHR2* expression in granulosa cells isolated from different sized follicles in goats. In the present study, expression of *AMHR2* mRNA in bovine granulosa cells was greatest in undifferentiated 5-8 mm follicles and decreased in bovine granulosa cells isolated from recently deviated 13-24 mm follicles. While it was not possible to determine which follicle would eventually deviate *versus* those that are destined to become atretic, a decrease in *AMHR2* expression within granulosa cells

as a follicle continues to grow may be directly related to the process of selection. Thus, understanding the potential mechanisms regulating AMHR2 in granulosa cells may provide insight to the most proximal factor(s) controlling follicle selection.

Several BMPs have been shown to increase AMH expression in granulosa cells of various species (BMP2 [23]; BMP6 [28]; BMP4 and BMP6 [29, 30]). In 3-5 mm bovine follicles, theca-derived BMP4 increased *AMH* mRNA, whereas both BMP4 and BMP6 increased *AMH* mRNA in 5-10 mm follicles [29]. Consequently, the greatest intensity of immunostaining for AMH in 3-5 mm bovine follicles was greater in granulosa cells adjacent to the theca cell layer compare to those lining the antrum [29]. In the present studies, granulosa cells from 5-8 mm follicles cultured for 24 h with rhBMP2, rhBMP6 or rhBMP15 resulted in increased expression of *AMH*. Additionally, *AMHR2* expression was increased in granulosa cells from 5-8 mm antral follicles when cultured in the presence of rhBMP15 (10 ng/ml) and rhBMP6 (50 ng/ml). Based on these findings, it is concluded that oocyte-specific BMPs can regulate expression of *AMH* and *AMHR2* in granulosa from 5-8 mm follicles.

The stimulatory effects of BMPs in granulosa cells may be mediated through AMHR2 associated transcription factors, in particular, NR5A1 and WT1 [16, 17]. A recent report from our laboratory demonstrated an increase in *WT1* expression in chicken granulosa cells following 3 h incubation with rhBMP6 [23]. Enhanced expression of WT1 transcription factor preceded an increase in *AMH* expression in this same system, however it has yet to be evaluated whether this factor regulates *AMHR2* expression. Based on published data from avian granulosa cells, NR5A1 and WT1 were analyzed in bovine granulosa cells to determine if the increase expression of *AMH* and *AMHR2* is mediated through these transcription factors. In the present study, WT1, but not NR5A1, expression was increased in bovine granulosa cells treated with rhBMP15 whereas

treatment with rhBMP2 or rhBMP6 had no effect on WT1 or NR5A1 mRNA in short term (3 h) culture system.

Follicle stimulating hormone (FSH) has been showed to inhibit expression of both *AMH* and *AMHR2* mRNA in the rat ovary at a time coincident with the transition of small antral follicles to differentiated, large antral follicles [5]. In addition, FSH signalling reduced *AMH* expression in hen granulosa cells [23] and circulating AMH concentrations in women [31]. In contrast, low concentrations (0.01 to 0.5 ng/ml) of FSH stimulated *AMH* mRNA expression and subsequent AMH production in a dose dependent manner, whereas concentrations (1 to 25 ng/ml) of FSH reduced both *AMH* mRNA and protein expression in long-term bovine granulosa cells cultures [32]. The different actions of FSH on AMH production among these studies may be attributed to the source of the granulosa cells, stage of follicle maturation and/or length of culture period. Furthermore, elevated concentrations of AMH have been proposed to suppress *FSHR* mRNA expression and/or FSHR cell signaling in the mouse ovary [33]. Chang *et al.* [34] demonstrated that AMH signaling inhibits FSH-induced *CYP19A1* expression and subsequent estrogen production in human granulosa-lutein cells. In addition, Durlinger *et al.* [8] showed that AMH decreased aromatase activity in murine granulosa cells and inhibited FSH-induced follicular development. Consistent with previous reports in other species (ovine, [11]; rat and swine, [7]; mouse, [8]) rhAMH reduced expression of *CYP19A1* in cultured bovine granulosa cells. The intrafollicular concentrations of AMH in cattle ranged from 400-800 ng/ml in 3-5 mm follicles and was much less in 7-10 mm follicles (approximately 200 ng/ml) [8, 29, 35]. Therefore, the inhibition of aromatase observed in this study was achieved at a concentration predicted to be lower than that found in follicular fluid.

Oocyte-derived factors, such as BMP15, stimulate granulosa cell mitosis of pre-antral follicles and inhibits FSH-induced differentiation *in vitro* [36]. In the present study, rhBMP15 increased both *AMH* and *AMHR2* expression in bovine granulosa cells and decreased *CYP19A1* expression, which potentially modifies bovine granulosa cell function through increased AMH signaling. Granulosa cells from 5-8 mm follicles cultured in the presence of rhBMP2 or rhBMP6 increased AMH expression, but there was no change in *CYP19A1* expression when compared to controls. Others have reported that BMP6 reduced forskolin-induced CYP19A1 expression, however no difference was observed in the absence of forskolin [37]. Conversely, as AMH expression decreases with follicle development, granulosa cells proliferate and acquire the capacity for estradiol production. Finally, Durlinger et al. [8] speculated that the decrease in follicle size in the mouse ovary cultured in the presence of AMH was due to a decrease in the rate of granulosa cell proliferation. Data from the present studies in which AMH inhibited bovine granulosa cells proliferation after 48 and 72 h, *in vitro* would support this speculation. It is possible that these BMPs also regulate follicular inhibitors, such as AMH, and that the inhibitory effects, *in vivo*, are to inhibit granulosa cell proliferation and *CYP19A1*, the latter of which would reduce aromatase activity.

In summary, these data demonstrate changes in the *AMHR2* expression during follicle development in a monovular species and elucidate potential mechanisms regulating *AMHR2* expression in bovine granulosa cells. Factors produced by the oocyte, such as BMP15, increase expression of *AMH*, *AMHR2* and the transcription factor, *WT1*, in bovine granulosa cells from 5-8 mm follicles, *in vitro*. Up-regulation of these factors can initiate AMH signaling which in turn can suppress *CYP19A1* expression and granulosa cell proliferation. Collectively, these actions may serve to maintain follicles in an undifferentiated state prior to follicle selection.

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FIG. 1: Expression of *AMHR2* mRNA in bovine granulosa cells is highest in undifferentiated 5-8 mm follicles and decreases as a follicle grows and undergoes selection (13-24 mm follicles). *AMHR2* data were standardized to the *RPL19* reference gene and expressed as a fold-difference +/- S.E.M. compared to 5-8mm follicles. Different letters indicate significant differences ($p < 0.05$, $n=6$ per follicle size group).

FIG. 2: Treatment of bovine granulosa cells from 5-8 mm follicles with rhBMP2 (50 ng/ml), rhBMP6 (25 or 50 ng/ml), or rhBMP15 (10 ng/ml) for 24 h significantly increased *AMH* expression (A, open bars). Expression of *AMHR2* mRNA was greater when bovine granulosa cells were cultured for 24 h with rhBMP6 (50 ng/ml) and rhBMP15 (10 ng/ml) when compared to untreated controls (A, solid bars).

Dan, we need to be consistent here and elsewhere with the treatment of rhBMP as compared to BMP.
e.g., see Fig 3

Treatment of bovine granulosa cells with rhBMP15 (10 ng/ml) for 3 h significantly increased *WT1* expression, whereas rhBMP2 (50 ng/ml) and rhBMP6 (50 ng/ml) had no effect (B, solid bars). In contrast, these treatments did not change *NR5A1* expression in bovine granulosa cells (B, open bars). Gene of interest data were standardized to the *RPL19* reference gene and are expressed as a fold-difference +/- S.E.M. compared to untreated controls. Different letters indicate significant differences ($p < 0.05$, $n=4$ independent cultures).

FIG. 3: A) Treatment of bovine granulosa cells from 5-8 mm follicles with increasing concentrations of rhAMH (10, 25, 100 ng/ml) for 24 h significantly reduced *CYP19A1* expression. B) Treatment of bovine granulosa cells with 10 ng/ml of rhBMP15, but not rhBMP2 (50 ng/ml) or rhBMP6 (25 or 50 ng/ml), significantly reduced *CYP19A1* expression compared to

controls. *CYP19A1* data were standardized to the *RPL19* reference gene and are expressed as a fold-difference $\pm$ S.E.M. compared to untreated controls. Different letters indicate significant differences ($p < 0.05$, $n=4$ independent cultures).

FIG. 4: A) Representative histograms of carboxyfluorescein succinimidyl ester (CFSE) labeled bovine granulosa cells (GC) displaying proliferating and non-proliferating cells. i) Granulosa cells not labeled with CFSE served as an internal calibration control; betacellulin (BTC; a known inducer of granulosa cells proliferation) served as a positive control (ii.). Bovine granulosa cells from 5-8 mm follicles were analyzed immediately (T0, iii.), 48 h (iv.) and 72 h (v.) after CFSE incorporation to quantify proliferation. B) Proliferation was inhibited in bovine granulosa cells treated with 100 ng rhAMH/ml after 48 and 72 h, *in vitro*, while cell proliferation was induced in BTC treated granulosa cells after 48 and 72 h. Bovine GC proliferation is expressed as a fold-difference $\pm$ S.E.M. compared to untreated controls. Different letters indicate significant differences ($p < 0.05$, $n=5$ independent cultures).

508 Table 1: Primers directed towards *Bos taurus* mRNA targets and validated for use with
509 quantitative PCR analysis.

Target	GenBank Accession #	Primer Sequences
<i>AMH</i>	NM_173890	5'-CAGGGAAGAAGTCTTCAGCA-3' Fwd 5'-AAGGTGGTCAAGTCACTCAG-3' Rev
<i>AMHR2</i>	XM_002687239	5'-AGCTGTGCTTCTCCCAGGTCATA-3' Fwd 5'- AAAGCGGACAATGTGGTCATGCTG-3' Rev
<i>CYP19A1</i>	NM_174305	5'- CACCCATCTTTGCCAGGTAGTC-3' Fwd 5'- ACCCACAGGAGGTAAGCCTATAAA-3' Rev
<i>FSHR</i>	NM_174061	5'- ATGTTTTCCAGGGAGCCTCT-3' Fwd 5'- TGCAAATTGGATGAAGGTCA-3' Rev
<i>LHCGR</i>	NM_174381	5'- CAGTGTGCTCCTGAACCAGA-3' Fwd 5'- GTCTGCAAAGGAGAGGTTGC-3' Rev
<i>NR5A1</i>	NM_001081614	5'-TTGAATGGGACCCTTCGGGAAGAT-3' Fwd 5'- CCAGGCCTTTGGAAGTTGCAATCA-3' Rev
<i>RPL19</i>	NM_001040516	5'- ATCGATCGCCACATGTATCA-3' Fwd 5'-GCGTGCTTCCTTGGTCTTAG-3' Rev
<i>WT1</i>	XM_866138	5'-TGTGTGCTTACCCAGGCTGCAATA-3' Fwd 5'-TCACACCTGTGTGTCTCCTTTGGT-3' Rev

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513 Fig. 1

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Fig. 4