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Bone morphogenetic protein 6 promotes FSH receptor and anti-Müllerian hormone mRNA expression in granulosa cells from hen prehierarchal follicles

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Abstract

A growing body of literature provides evidence of a prominent role for bone morphogenetic proteins (BMPs) in regulating various stages of ovarian follicle development. Several actions for BMP6 have been previously reported in the hen ovary, yet only within postselection (preovulatory) follicles. The initial hypothesis tested herein is that BMP6 increases FSH receptor (*FSHR*) mRNA expression within the granulosa layer of prehierarchal (6–8 mm) follicles (6–8 GC). *BMP6* mRNA is expressed at higher levels within undifferentiated (1–8 mm) follicles compared with selected (≥9 mm) follicles. Recombinant human (rh) BMP6 initiates SMAD1, 5, 8 signaling in cultured 6–8 GC and promotes *FSHR* mRNA expression in a dose-related fashion. In addition, a 21 h preculture with rhBMP6 followed by a 3 h challenge with FSH increases cAMP accumulation, *STAR* (*StAR*) expression, and progesterone production. Interestingly, rhBMP6 also increases expression of anti-Müllerian hormone (*AMH*) mRNA in cultured 6–8 GC. This related BMP family member has previously been implicated in negatively regulating FSH responsiveness during follicle development. Considering these data, we propose that among the paracrine and/or autocrine actions of BMP6 within prehierarchal follicles is the maintenance of both *FSHR* and *AMH* mRNA expression. We predict that before follicle selection, one action of AMH within granulosa cells from 6 to 8 mm follicles is to help suppress FSHR signaling and prevent premature granulosa cell differentiation. At the time of selection, we speculate that the yet undefined signal directly responsible for selection initiates FSH responsiveness. As a result, FSH signaling suppresses *AMH* expression and initiates the differentiation of granulosa within the selected follicle.

Reproduction (2012) **143** 825–833

Introduction

During early growth of hen ovarian follicles, there are at least two stages at which a process of selection normally 'optimizes' the number of follicles that undergo development. The first is initial recruitment when populations of growth-arrested primary follicles embedded within the ovarian cortex are activated by as yet undefined signals. Eventually, such follicles emerge from the cortex to begin a slow growth phase suspended from a well-vascularized pedicle. The second stage, termed cyclic recruitment (or follicle selection), represents the stage at which a single follicle is selected per day to initiate differentiation and begin rapid growth before ovulation. Selection into the laying hen preovulatory hierarchy occurs from a cohort of eight to 12 6–8 mm diameter prehierarchal follicles. While granulosa cells from all prehierarchal follicles within this cohort express comparatively high levels of mRNA encoding the FSH receptor (*FSHR*; Woods & Johnson 2005), follicle selection is limited to the single follicle that has acquired optimal responsiveness to FSH. To date, the proximate signal(s) responsible for initiating FSH responsiveness with the selected follicle has yet to be identified.

Several members of the transforming growth factor β (TGFβ) family have previously been implicated in promoting *FSHR* expression within hen prehierarchal follicles. For instance, *FSHR* mRNA levels in cultured granulosa cells are increased by treatment with TGFβ1 and activin A (Woods & Johnson 2005). Both TGFβ1 and activin A signal by complexing with its own membranes type I and type II serine/threonine kinase receptor and activating SMAD2/3 intracellular signaling. Bone morphogenetic proteins (BMPs) and anti-Müllerian hormone (AMH) represent two additional growth and differentiation factors belonging to the TGFβ superfamily. A previous report has identified mRNA expression for both BMP type I (BMPR1A and BMPR1B) and type II (BMPR2) membrane receptors in hen granulosa cells (Onagbesan *et al*. 2003). In turn, BMP-activated receptor complexes signal via SMAD1/5/8 (Haugen & Johnson 2010). By comparison,

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-11-0271
Online version via www.reproduction-online.org

mammalian AMH signals via a BMP type I receptor (including ACVR1B (ACTR1A)/ACVR1 (ALK2) and BMPR1B/ALK6) that complexes with an AMH-specific type II receptor (Visser 2003). It is noted that thus far the avian ortholog to the mammalian AMH receptor type II (AMHR2) has yet to be identified.

A growing body of literature provides evidence of prominent roles for several BMPs in regulating various stages of follicle development. We recently reported that BMP2 contributes to maintaining hen granulosa cells from prehierarchal follicles in an undifferentiated state by suppressing TGFβ-induced *FSHR* expression (Haugen & Johnson 2010). Based upon published reports, there is general consensus that BMP6 represents an important local regulator of follicle development, both before and after gonadotropin responsiveness. However, there are conflicting reports from mammalian species as to whether BMP6 promotes or inhibits processes related to follicle differentiation. For instance, *BMP6* expression in sheep follicles is localized to the oocyte of primary, preantral, and antral follicles and its actions are reported to inhibit granulosa cell differentiation (Juengel *et al.* 2006). In the rat ovary, BMP6 inhibits FSH responsiveness by suppressing adenylyl cyclase activity (Miyoshi *et al.* 2007). Similar inhibitory effects of BMP6 on FSH responsiveness have been reported in cattle (Glister *et al.* 2004) and pigs (Brankin *et al.* 2005). By comparison, BMP6 treatment was determined to increase *FSHR* mRNA expression in cultured human granulosa cells (Shi *et al.* 2009). The discrepancies in reported actions may result from species differences, or perhaps more likely differences in the stage of follicle development used for investigation. Nevertheless, it is apparent that BMP6 in mammals has the capacity to modulate follicle sensitivity to gonadotropins, and by implication, directly or indirectly regulate follicle selection and ovulation rate.

Several putative BMP6 actions have already been reported for hen ovarian follicles, yet only within postselection (preovulatory) follicles (Onagbesan *et al.* 2003, Al-Musawi *et al.* 2007). In particular, BMP6 treatment of granulosa cells from preovulatory follicles was found to increase levels of *FSHR* and LH receptor (*LHR*) mRNA expression and, as a result, enhance gonadotropin-induced progesterone production (Al-Musawi *et al.* 2007). The related BMP family gene, *AMH*, is also expressed by hen granulosa cells, and levels of *AMH* mRNA dramatically decrease as a follicle grows from 1 to 6–12 mm in diameter (Johnson *et al.* 2008*a*, 2008*b*). Prominent functions predicted for AMH within the mammalian ovary include regulating the rate of initial follicle recruitment and suppressing FSH responsiveness in early antral and small antral follicles before selection (Durlinger *et al.* 1999, 2001, Visser & Themmen 2005). Similarly, a recent report provides the first evidence from birds implicating AMH as a potential factor regulating the rate of follicle selection (Johnson *et al.* 2009).

These studies were directed toward identifying the role of BMP6 in granulosa cells collected from the cohort of prehierarchal follicles. In light of data from a previously published study of preovulatory follicles (Al-Musawi *et al.* 2007), we initially hypothesized that BMP6 functions to enhance FSH responsiveness within the granulosa layer from 6 to 8 mm (prehierarchal) follicles. Subsequently, related studies were conducted to assess a role for BMP6 in regulating *AMH* transcription.

Results

BMP6 *mRNA expression during follicle development*

In this study, *BMP6* mRNA was amplified from granulosa and theca tissues were collected from prehierarchal (6–8 mm) and the largest preovulatory (F1) follicles (Fig. 1, top panel). Both *BMP6* and *AMH* mRNA are expressed at highest levels in follicles during the slow

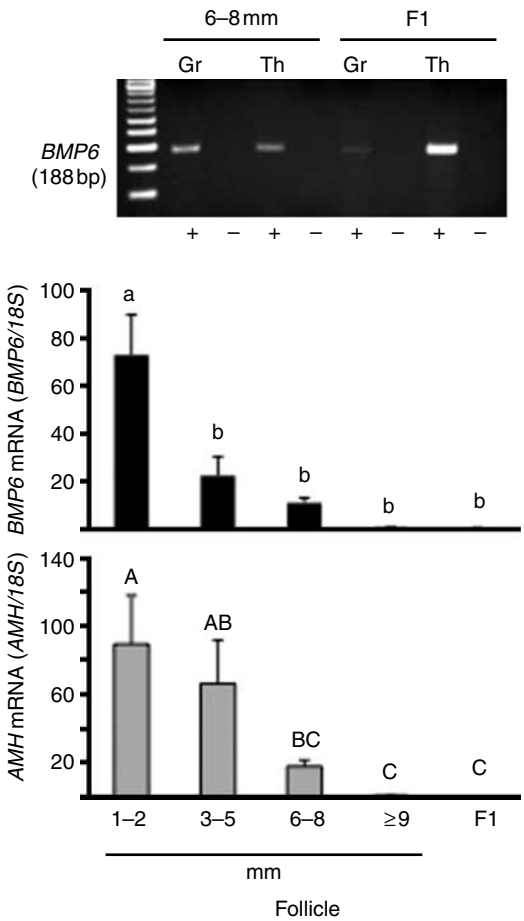

Figure 1 (Top panel) RT-PCR for BMP6 (+) in granulosa and theca tissues from prehierarchal (6–8 mm) and the largest preovulatory (F1) follicle. −, negative control. (Bottom panels) Expression of *BMP6* and *AMH* mRNA in granulosa cells from slow-growing (1–2 and 3–5 mm), prehierarchal (6–8 mm), the most recently selected follicle (≥9 mm), and largest preovulatory (F1) follicle. Mean±S.E.M.; $^{a,b;\ A,B,C}P<0.05$; $n=4$.

growth phase of development (1–2 mm diameter) and are at lowest levels at or immediately after follicle selection (in follicles ≥ 9 mm) (Fig. 1, bottom panels). The expression of BMP6 by both the granulosa and the theca layer of prehierarchal follicles provides evidence that this factor may act as an autocrine and/or paracrine factor before and at the time of follicle selection.

BMP6-induced SMAD1/5/8 signaling and ID2 *and* FSHR *mRNA expression in granulosa cells from prehierarchal follicles*

Treatment of undifferentiated granulosa cells from 6 to 8 mm (prehierarchal) follicles with 10 or 25 ng recombinant human (rh) BMP6/ml for 30 min promoted phosphorylation of SMAD1/5/8 (Fig. 2, top panel). Incubation of cells for 3 h with 25 ng rhBMP6/ml increased mRNA levels encoding the inhibitor of differentiation/DNA-binding (ID) protein, ID2 (Fig. 2, middle panel). Expression of this basic helix–loop–helix transcription factor has previously been associated with facilitating the process of granulosa cell differentiation

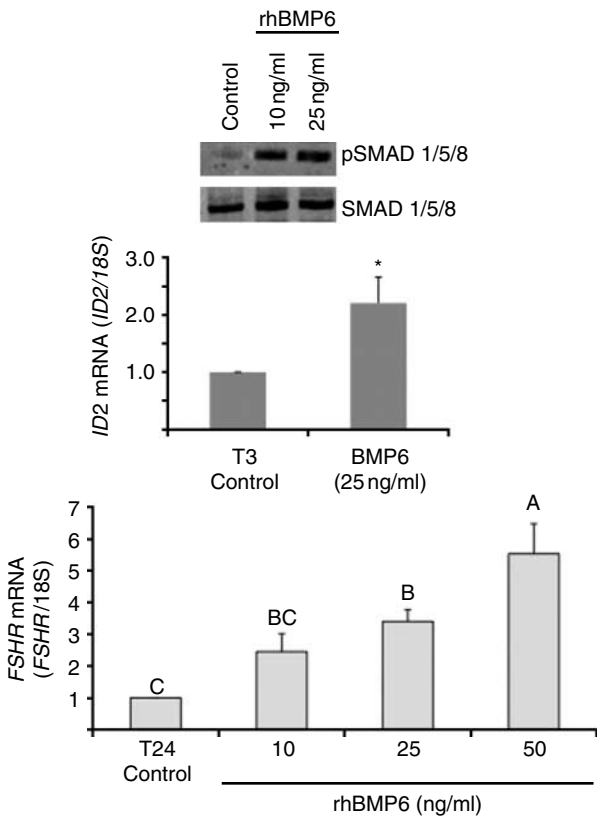

Figure 2 (Top panel) SMAD1, 5, 8 phosphorylation in granulosa cells from 6 to 8 mm (prehierarchal) follicles following treatment with recombinant human (rh) BMP6. Replicated once with similar results. (Middle panel) Expression of *ID2* mRNA following a 3 h incubation with rhBMP6. *$P<0.03$ by *t*-test; $n=5$ replicate experiments. (Bottom panel) *FSHR* mRNA expression following a 24 h culture with rhBMP6. $^{A,B,C}P<0.05$; $n=4$ (10 ng/ml) or 8 (25 and 50 ng/ml) replicate experiments.

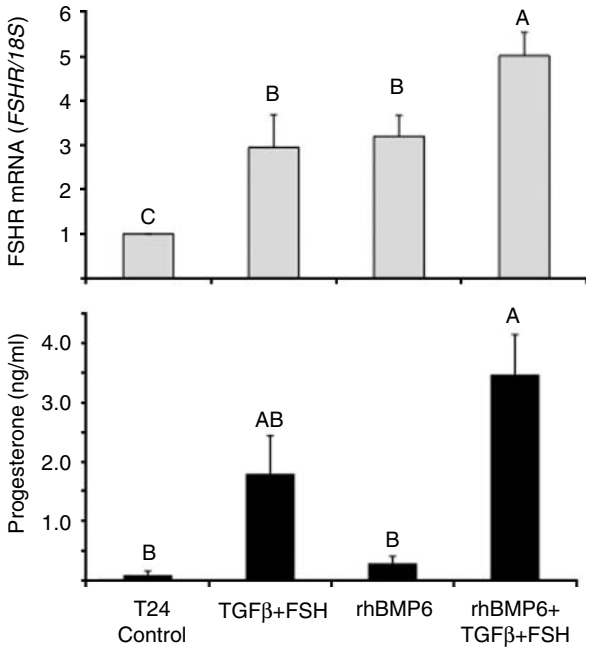

Figure 3 (Top panel) *FSHR* mRNA expression induced in granulosa from 6 to 8 mm follicles following a 24 h culture with rhTGFβ1 (10 ng/ml) and rhFSH (100 ng/ml) and/or rhBMP6 (25 ng/ml). $^{A,B,C}P<0.05$; $n=6$. (Bottom panel) Media progesterone following culture for 24 h with rhBMP6 in the absence and presence of rhTGFβ1 (10 ng/ml) and rhFSH (100 ng/ml). $^{A,B,C}P<0.05$; $n=6$.

(Johnson *et al*. 2008*a*, 2008*b*). Furthermore, BMP6 induced the expression of *FSHR* mRNA in a dose-related fashion following a 24 h culture interval (Fig. 2, bottom panel). Culture of undifferentiated granulosa cells with rhTGFβ1 (10 ng/ml) combined with a maximally effective dose of rhFSH (100 ng/ml) for 24 h increased *FSHR* mRNA expression (Fig. 3, top panel) and initiated significant progesterone production (Fig. 3, bottom panel). Culture with rhBMP6 induced *FSHR* mRNA, yet rhBMP6 alone failed to induce a significant accumulation of media progesterone compared with control cultured cells. Treatment with rhBMP6 combined with rhTGFβ1 and rhFSH resulted in a numerical, but nonsignificant, increase in progesterone production compared with rhTGFβ1 and rhFSH alone.

BMP6 helps to initiate FSH responsiveness in granulosa cells from prehierarchal follicles

Granulosa cells were cultured for 21 h in the absence or presence of rhBMP6 (25 ng/ml) and then challenged with rhFSH during a final 3 h of culture. Levels of cAMP were increased in cultured cells primed with BMP6 and challenged with FSH (Fig. 4, top panel). STAR mRNA and protein expression (Fig. 4, middle panel) was significantly increased only following a challenge with rhFSH, and the response to rhFSH was enhanced when cells were pretreated with rhBMP6. Similarly, progesterone production was greatest when cells were

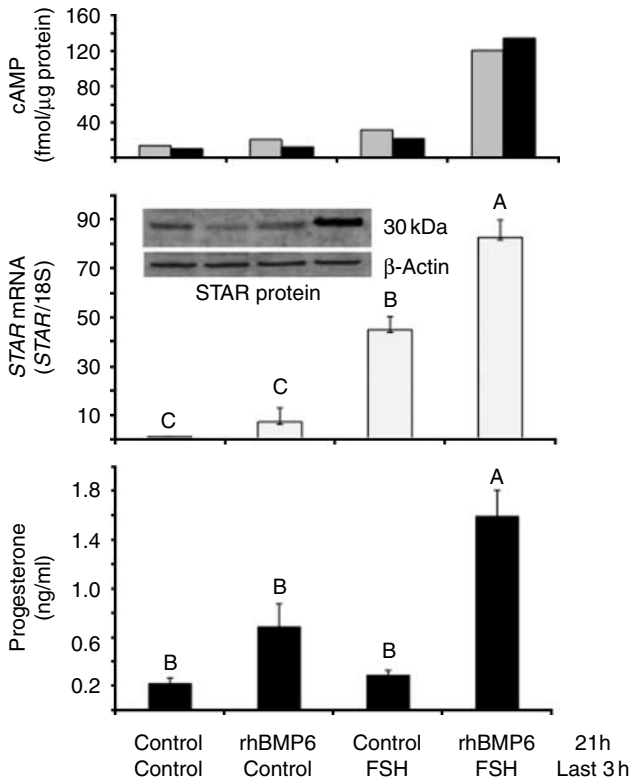

Figure 4 Preculture with rhBMP6 for 21 h enhances FSH responsiveness following a 3 h challenge with rhFSH as measured by increased concentrations of intracellular cAMP (data represent results from replicate experiments; top panel), *STAR* mRNA and STAR protein expression (middle panel), and progesterone production (bottom panel). Cells were cultured in the absence or presence of rhBMP6 (25 ng/ml) for a total of 24 h and in the absence or presence of rhFSH (10 ng/ml) during the last 3 h. $^{A,B,C}P<0.05$; $n=3$. The STAR western blot was replicated once with similar results.

precultured with rhBMP6 and challenged with rhFSH (Fig. 4, bottom panel). Combined with the results from Fig. 3, these findings demonstrate that rhBMP6-induced *FSHR* expression eventually leads to FSH responsiveness in undifferentiated granulosa cells.

Regulation of AMH *expression by BMP6 and FSH and TGFβ1*

Culture with rhBMP6 for 24 h (but not 3 h; data not shown) induced *AMH* expression in a dose-related fashion (Fig. 5, bottom panel). Increased *AMH* expression after 24 h is preceded by enhanced expression of mRNAs encoding the transcription factors, steroidogenic factor 1 (*SF1*), Wilms tumor protein (*WT1*), and *GATA4* (Fig. 5, top panel). In contrast to the ability of rhFSH and rhTGFβ1 treatment to promote *FSHR* expression (Fig. 6, top panel), the differentiating effects of this treatment (see Fig. 3) result in decreased levels of *AMH* mRNA levels after a 24 h culture (Fig. 6, bottom panel).

Discussion

In this study, *BMP6* mRNA is expressed in granulosa cells collected from 6 to 8 mm follicles (the prehierarchal cohort), but levels are relatively low compared with slow-growing 1–2 mm follicles (Fig. 1). Inexplicably, the expression of *BMP6* by the granulosa layer apparently differs from a report by Al-Musawi *et al.* (2007) in which low but detectable levels with 6–8 mm follicles were amplified from theca, but apparently not granulosa, tissue. We note that these findings do not exclude the possibility of *BMP6* expression by the oocyte. A decrease in the relative expression of both *BMP6* and *AMH* as small follicles progressively increase in size is consistent with the previous reports (Johnson *et al.* 2008*a*, 2008*b*, Diaz *et al.* 2011).

During hen follicle development, *FSHR* mRNA in the granulosa layer is expressed at highest levels within the cohort of 6–8 mm follicles (You *et al.* 1996). Moreover, it has been reported that within this prehierarchal cohort, a single follicle typically exhibits a higher level of *FSHR* expression compared with the others, and we have hypothesized that this represents the most recently selected follicle (Woods & Johnson 2005). Although the identity of the proximal factor(s) initiating the selection of a single hen follicle per ovulatory cycle remains unknown, perhaps the most immediate marker of selection is the ability of FSH to enhance cAMP formation in granulosa cells (e.g. initiate FSH responsiveness). For instance, granulosa cells collected from

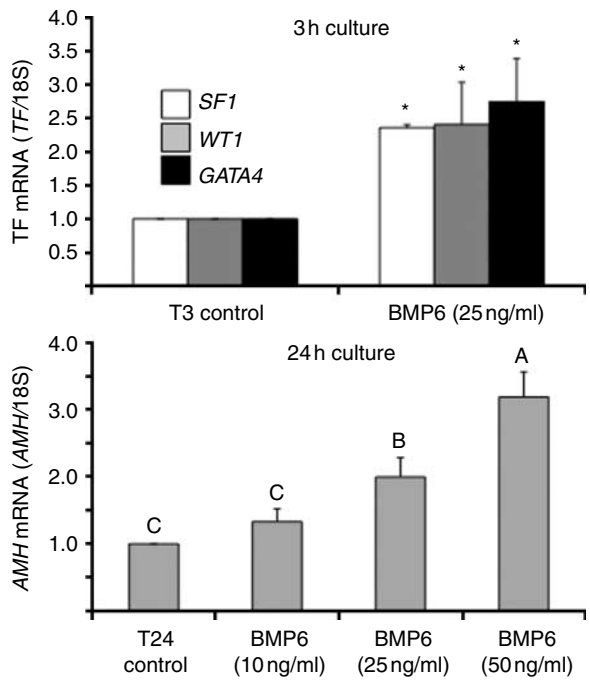

Figure 5 (Top panel) Induced expression of the transcription factors (TF), *SF1*, *WT1*, and *GATA4* following a 3 h incubation with rhBMP6. $^{*}P<0.05$ by *t*-test; $n=3$. (Bottom panel) BMP6-induced *AMH* mRNA following a 24 h culture. $^{A,B,C}P<0.05$; $n=4$ (for 10 ng) or 8 (25 and 50 ng) replicate experiments.

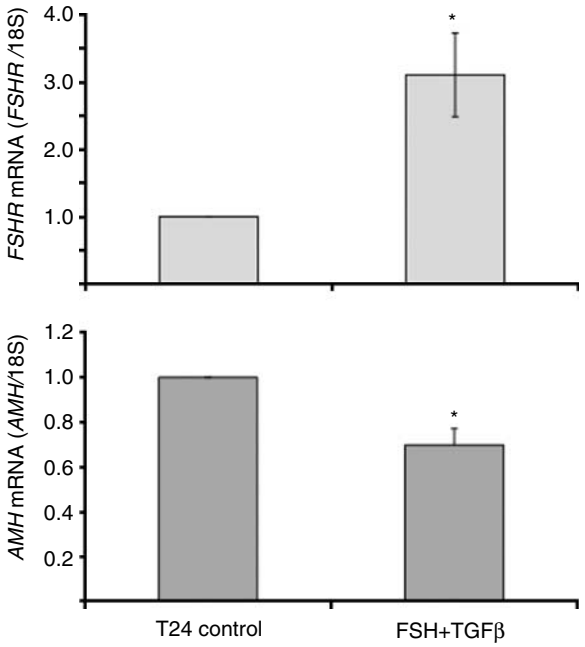

Figure 6 Enhanced *FSHR* expression induced by rhFSH (25 ng/ml) and rhTGFβ1 (10 ng/ml) (top panel) is associated with decreased expression of *AMH* (bottom panel). *$P<0.05$ by paired *t*-test; $n=6$ replicate experiments.

prehierarchal follicles fail to generate the accumulation of cAMP or produce progesterone in response to a FSH challenge. FSH responsiveness is initially detected within granulosa from 9 to 12 (postselection) follicles (Tilly *et al*. 1991). Associated with the detection of FSH responsiveness in 9–12 mm follicles is enhanced expression of *LHR* mRNA and eventual LH responsiveness within the granulosa layer. Results from these studies demonstrate that BMP6 can act in an autocrine and/or paracrine fashion to promote *FSHR* mRNA expression, *in vitro* (Fig. 2). Nevertheless, BMP6 by itself does not initiate FSH responsiveness as measured by the absence of significant cAMP accumulation, *STAR* expression, and progesterone production (Fig. 4). The latter findings are not unexpected, as STAR protein expression, P450 side chain cleavage enzyme (CYP11A1) expression, and the initiation of progesterone production are each dependent upon signaling via cAMP (Li & Johnson 1993, Johnson *et al*. 2002).

This absence of FSH responsiveness has been attributed to inhibitory mitogen-activated protein kinase (MAPK) signaling induced by epidermal growth factor receptor ligands (EGFRL; for example, see Johnson *et al*. (2004), Woods & Johnson (2005) and Woods *et al*. (2007)). Accordingly, while BMP6 promotes and maintains *FSHR* mRNA expression in prehierarchal follicles, FSH responsiveness is attained only at follicle selection. *In vivo*, we predict that the proximal signal that initiates follicle selection is associated with the removal from inhibitory MAPK signaling. It is important to note, however, that granulosa cells cultured overnight in the absence of exogenous EGFRL fail to maintain elevated levels of active MAPK signaling, thus some level of FSH responsiveness is gradually attained. This can explain why a 24 h culture of undifferentiated granulosa with BMP6 followed by FSH challenge will induce cAMP production, *STAR* expression, and progesterone production (Fig. 4).

As previously reported (Johnson *et al*. 2004), culture with rhTGFβ1 and rhFSH for 24 h induced *FSHR* expression and initiated progesterone production in undifferentiated granulosa cells (Fig. 3). The novel finding reported herein is that rhBMP6 induced *FSHR* expression in a dose-dependent fashion (Fig. 2, bottom panel) and provided an additive effect on expression levels when combined with rhTGFβ1 and rhFSH (Fig. 3, top panel). Interestingly, rhBMP6 treatment was also reported to enhance levels of *FSHR* in granulosa cells collected from preovulatory follicles (Al-Musawi *et al*. 2007). The biological significance of this latter observation is less clear as granulosa cells from preovulatory follicles are LHR dominant and highly responsive to LH, but not FSH, treatment (Wells *et al*. 1985, Johnson *et al*. 1996).

We recently reported that FSH treatment induces expression of the early response gene, *ID2*, in undifferentiated hen granulosa cells and that elevated ID2 protein expression in granulosa cells is associated with a differentiated phenotype (Johnson *et al*. 2008*a*, 2008*b*). In addition, overexpression of ID2 protein in undifferentiated granulosa cells promotes increased *FSHR* expression and initiates FSH responsiveness. Recent studies on mammals have determined that *ID* genes (*ID1–4*) are frequent targets of BMP/SMAD signaling (Hogg *et al*. 2010, Nakahiroa *et al*. 2010). Results from this study indicate that the stimulatory effects of BMP6 on *FSHR* expression may also involve enhanced *ID2* expression (Fig. 2).

Consistent with the reports from human (Shi *et al*. 2009) and bovine (Rico *et al*. 2011) granulosa cells, BMP6 treatment also promotes *AMH* mRNA expression in granulosa cells from hen prehierarchal follicles (Fig. 5, bottom panel). In this regard, the actions of AMH have been linked to restricting initial follicle recruitment together with influencing the daily selection of an ovulatory follicle (Durlinger *et al*. 2001, Visser & Themmen 2005). Similar to the mouse ovary (Salmon *et al*. 2005), the stimulatory effects of BMP6 in undifferentiated granulosa cells are correlated with increased expression of the transcription factors, *SF1*, *WT1*, and *GATA4* (Fig. 5, top panel). The expression of AMH has previously been associated with suppressing both the rate of initial follicle recruitment and the granulosa cell differentiation in preantral follicles from mammals (Durlinger *et al*. 1999, 2001, Visser *et al*. 2006). Specifically, elevated levels of AMH have been proposed to suppress *FSHR* mRNA expression and/or downstream signaling of FSHR in the mouse ovary. As noted earlier, previous studies on hen ovary demonstrate that *FSHR* mRNA expression and FSH responsiveness within prehierarchal follicles are suppressed, at least in part, by inhibitory EGFRL-mediated

MAPK signaling (Johnson & Woods 2009). Together, the actions of EGFRL/MAPK signaling with the proposed inhibitory actions of AMH signaling on FSH responsiveness in hen granulosa cells may serve to regulate the process of follicle selection. More recent studies on laying and broiler breeder hens failed to reveal a negative relationship between elevated *AMH* expression and *FSHR* levels in granulosa from prehierarchal follicles (Johnson *et al*. 2009), yet the authors note that these findings do not necessarily preclude suppressive effects on either FSHR protein expression or FSH responsiveness.

The acquisition of FSH responsiveness and signaling via cAMP is associated with reduced levels of *AMH* mRNA (Fig. 6). It has been reported from the rat ovary that FSH inhibits expression of AMH and the AMH type II receptor (*AMHR2*) mRNA at a time coincident with the transition of small antral follicles to differentiated, large antral follicles (Baarends *et al*. 1995). These results suggest that before follicle selection, elevated *AMH* expression can help to maintain an undifferentiated status. Subsequently, at the time of selection and with the acquisition of FSH responsiveness within the granulosa layer, *AMH* expression becomes actively suppressed. We propose that an early action of BMP6 within hen undifferentiated follicles is to maintain elevated *AMH* expression. We speculate that elevated levels of AMH protein can act in a paracrine fashion to regulate the rate of primary follicles entering the slow growth phase of development (e.g. initial recruitment). Subsequently, and before follicle selection, we predict that a second action of AMH within granulosa cells from prehierarchal follicles is to help suppress *FSH* responsiveness and prevent premature granulosa cell differentiation. The ability to directly test this prediction will require a highly purified preparation of biologically active chicken AMH and a characterization of the AMHR2.

In summary, paracrine and/or autocrine signaling by BMP6, *in vivo*, induces *AMH* expression, which presumably serves to regulate the rate of follicle growth and inhibit premature differentiation. BMP6 also promotes and maintains *FSHR* expression in granulosa cells from unselected follicles, yet the capacity for FSH/FSHR signaling via the protein kinase A pathway is attained only at follicle selection. Both the cellular mechanisms responsible for regulating FSHR signaling (e.g. desensitization) in prehierarchal follicles and the proximal signals to initiate FSH responsiveness at follicle selection are currently under investigation.

Materials and Methods

Animals and reagents

Single-comb White Leghorn hens 34–55 weeks of age and laying sequences of five or more eggs were used in the studies described. Hens were housed individually in laying batteries, with free access to feed and water, under a controlled photoperiod of 15 h light:9 h darkness (lights on at 0200 h). Hens were killed by cervical dislocation 14–19 h before a mid-sequence ovulation and the ovary was immediately removed and placed in ice-cold sterile 1% NaCl solution until granulosa cells were collected. All procedures described herein were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committees and were performed in accordance with The Guiding Principles for the Care and Use of Laboratory Animals.

RhBMP6 was purchased from PeproTech (Rocky Hill, NJ, USA) and consists of a biologically active 117 amino acid homodimer corresponding to residues 397–513 of the full-length human BMP6 precursor. The corresponding amino acid sequence for *Gallus* BMP6 is predicted to be 96% identical to the rhBMP6 peptide and contains all seven Cys residues found within rhBMP6. rhTGFβ1 was purchased from PeproTech while rhFSH was provided by the National Hormone and Pituitary Program (Torrance, CA, USA).

Granulosa cell cultures

Ovarian follicles were grouped by size, and granulosa cell layers were collected and dispersed for culture as described previously (Woods & Johnson 2005). In some instances, an aliquot of cells was immediately frozen at −70 °C. The remaining cells were cultured for 3 or 24 h at 40 °C in an atmosphere of 95% air: 5% CO_2 in 12-well polystyrene culture plates (Beckton Dickinson, Franklin Lakes, NJ, USA) with a density of $\sim 1\times10^5$/well in 1 ml DMEM and 2.5% FBS containing 0.1 mM nonessential amino acids and 1% antibiotic–antimycotic mixture (Invitrogen). In addition, 3 h incubations were conducted with granulosa cells from 6 to 8 mm follicles where 5×10^5 cells in 1 ml DMEM were placed in 12×75 mm polypropylene tubes and incubated in a shaking water bath at 40 °C in room air (Fisher Scientific, Pittsburgh, PA, USA; Woods & Johnson 2007). Preliminary studies established that a 24 h culture with BMP6 (25 ng/ml) did not alter cell number compared with control cultured cells ($P>0.20$).

PCR

Forward and reverse primers directed toward *Gallus BMP6*, *FSHR*, *AMH*, *STAR*, *GATA4*, *SF1*, *WT1*, *ID2* mRNA, and *18S* rRNA are described in Table 1. To insure against genomic contamination, BMP6 forward and reverse primers were designed to span a single intron, and all primer pairs were validated for target specificity. Random-primed, reverse-transcribed cDNA synthesis reactions were performed using the Promega RT System (Promega), according to the conditions described by the manufacturer. For negative RT samples, all components of cDNA synthesis were used, but lacked the reverse transcriptase enzyme, to ensure the lack of genomic DNA contamination. For water control samples, all components of the RT-PCR or real-time PCRs were added, but water was substituted for the template to ensure the lack of primer contamination. For real-time PCR, primers and 50 ng cDNA template were added to 10 µl total reaction volume using the reagents provided in the PerfeCTa Sybr Green FastMix Low Rox (Quanta Biosciences, Inc., Gaithersburg, MD, USA). Final concentrations of the sense and antisense primers were

Table 1 Primers directed towards *Gallus gallus* mRNA targets and validated for use with quantitative PCR analysis.

Target	GenBank Accession #	Primer
BMP6	XM_418956	5′-CCAAGTGCTGCAGGAACATCCAAA-3′ Fwd 5′-TTACACTGAAACCGTCGTGGGTCA-3′ Rev
FSHR	NM_205079	5′-TTAATTCCTGTGCTAACCCTTTCC-3′ Fwd 5′-CCAAACTTGCTCAACAGAATGAAG-3′ Rev
AMH	U61754	5′-CCCCTCTGTCCCTCATGGA-3′ Fwd 5′-CGTCATCCTGGTGAAACACTTC-3′ Rev
STAR	NM_204686	5′-TGCCTGAGCAGCAGGGATTTATCA-3′ Fwd 5′-TGGTTGATGATGGTCTTTGGCAGC-3′ Rev
GATA4	U11887	5′-TTTCTGCTAACGGGAGGGAGCAAT-3′ Fwd 5′-AAGTCCAAGTGGTGGCCATTTCAG-3′ Rev
SF1	NM_205077	5′-GCTCAGTACCTTTGGCCTCA-3′ Fwd 5′-GCAGCAGCTTCATCTGGTCT-3′ Rev
WT1	NM_205216	5′-TCTGAAGACTCATACCAGGACTCA-3′ Fwd 5′-CATGTTCCTCTGGTGCATGT-3′ Rev
ID2	AF068831	5′-TGAACGACTGCTACTCCAAGCTGA-3′ Fwd 5′-TAGTCGATGACGTGCTGCAGGATT-3′ Rev
18S rRNA	AF173612	5′-TTAAGTCCCTGCCCTTTGTACAC-3′ Fwd 5′ CGATCCGAGGAACCTCACTAAAC-3′ Rev

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). Reactions were conducted with the following conditions: 30 s at 95 °C, followed by 40 cycles each for 3 s at 95 °C, 1 min at 56 °C, and 30 s at 72 °C. Melting curves were generated for each run. The C_t (the cycle number at which the fluorescence exceeds a threshold level) was determined for each reaction (run in triplicate) using the 7500 software (v.2.0.4) and quantification was accomplished by standardizing reactions to *18S* rRNA and then using the $\Delta\Delta C_t$ method (Livak & Schmittgen 2001). Results were expressed as fold-difference compared with an appropriate control tissue or treatment. Finally, for RT-PCR, primers, dNTP mix (Promega), Taq polymerase (NEB), and 100 ng template cDNA were added to 30 μl total reaction. Final concentrations of the sense and antisense primers were determined for each primer pair based upon optimal amplification efficiency. Reactions were completed on the AB 7800 Fast Thermocycler (Applied Biosystems). Reactions were conducted with the following conditions: 2 min at 94 °C, followed by 40 cycles each for 15 s at 94 °C, 30 s at 60 °C, and 60 s at 72 °C, followed by 7 min at 72 °C. The amplified product was visualized by ethidium bromide after running PCR products on a 1.5% agarose (Invitrogen) gel and the identity was verified by sequencing.

cAMP measurement

Intracellular cAMP accumulation was measured by EIA (Cayman Chemical Co., Ann Arbor, MI, USA). Briefly, cells in the presence of rhBMP6 (25 ng/ml) for 21 h were preincubated with 10 μM of 3-isobutyl-1-methylxanthine (Sigma–Aldrich) for 15 min and then treated with rhFSH (10 ng/ml) for 3 h. The cells were lysed in 0.1 M HCl by repeated pipetting and incubated for 20 min at room temperature. After centrifuged at 1000 ***g*** for 10 min, the supernatant was used for this assay directly in a single plate. The B/B_O for all samples fell between 20 and 80%, while the mean sample intra-assay coefficient of variation was 16.1%.

Progesterone EIA

Progesterone in media samples was quantified by EIA (cat# 582601, Cayman Chemical Co.). The progesterone antiserum is reported to cross-react with 5β-pregnan-3α-ol-20-one (6.7%), pregnenolone (2.5%), 17-hydroxy progesterone (0.5%) and <0.5% for other progestins, androgens, or estrogens. Serial dilutions of media samples over a tenfold range produced a line parallel to the standard curve. After adjustment for dilution, the mean assayed value for the sample used for validation had a 10.9% coefficient of variation. All samples within an experiment were assayed using a single plate. Data are expressed as mean nanogram progesterone/ml media ± S.E.M. for the combined replicate experiments. The mean within-assay coefficient of variation for samples in all experiments was <15%.

Immunoblot analysis

Western blot analysis for STAR, phospho-SMAD, and total SMAD proteins was conducted essentially as described previously (Haugen & Johnson 2010). Briefly, cells were homogenized in a protein lysis buffer (RIPA, Santa Cruz Biotechnology, Santa Cruz, CA, USA) containing a cocktail of enzyme (including phosphatase) inhibitors (Sigma–Aldrich). The STAR antiserum was generously provided by Dr Buck Hales and was used at a dilution of 1:5000 (Hales *et al.* 2000). The rabbit phospho-SMAD1 (Ser463/465)/SMAD5 (Ser463/465)/SMAD8 (Ser426/428) polyclonal antibody (Cell Signaling, Danvers, MA, USA) was used at a dilution of 1:1000, while a rabbit polyclonal anti-SMAD1/5/8 antibody (Pierce, Rockford, IL, USA; 1:6000 dilution) was used for standardization. Incubations for the primary antibodies were conducted overnight at 4 °C with gentle agitation. The HRP-conjugated antirabbit IgG secondary antibody (diluted 1:10 000; Pierce) was incubated for 1 h at room temperature. Blots were incubated with ECL Plus Chemiluminescence Detection Reagent (Amersham Biosciences). Chemiluminescent signals were detected using the Storm 860 optical scanner (Amersham Biosciences), and the signals were analyzed using Image Quant

TL software (Amersham Biosciences). The chemiluminescence signal intensity of each band was calculated using a local average background correction.

Statistical analysis

Experiments were independently replicated a minimum of three times unless otherwise stated. Data for mRNA were expressed as a fold-difference compared with control cultured cells. Real-time PCR data from replicate experiments were analyzed by *t*-test or by one-way ANOVA followed by a Fisher's protected least significant difference multiple range test. Media progesterone from the combined replicate experiments was analyzed by one-way ANOVA.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Walther H Ott Endowment and NSF IOS 0968784 (to A L Johnson).

Acknowledgements

The authors thank Ms Shanequa Smith for her contributions to these studies, Mr Dongwon Kim for conducting the cAMP assay, and Dr Buck Hales, Southern Illinois University School of Medicine, for the anti-STAR serum.

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Received 28 July 2011
First decision 12 September 2011
Revised manuscript received 22 February 2012
Accepted 10 April 2012