Bone morphogenetic protein 2 inhibits FSH responsiveness in hen granulosa cells

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Abstract

Prior to follicle selection into the preovulatory hierarchy, hen granulosa cells from prehierarchical follicles remain undifferentiated, as defined in part by the virtual absence of *LHR* mRNA expression and inability to produce progesterone. It has previously been proposed that prior to follicle selection, granulosa cells are actively maintained in an undifferentiated state by epidermal growth factor receptor ligands (EGFRL) signaling via the MAP kinase/extracellular regulated kinase pathway. Moreover, there is recent evidence that EGFRL/MAP kinase signaling modulates *FSH* receptor (*FSHR*) transcription, in part, via inhibitor of differentiation/DNA-binding (ID) proteins. In the present studies with undifferentiated granulosa, recombinant human (rh) bone morphogenetic protein 2 (BMP2) induced the phosphorylation of SMAD1/5/8, and blocked transforming growth factor β and FSH-induced *FSHR* expression and progesterone production. Significantly, BMP2 rapidly induced mRNAs encoding betacellulin and EGF, plus ID proteins (ID1, ID3, and ID4). Alternatively, the bioactivity of BMPs can be modulated by one or more BMP antagonists, including noggin (NOG). *NOG* mRNA is expressed by both hen granulosa and theca tissues from prehierarchical follicles. Pretreatment of cultured granulosa with rh NOG reversed both the stimulatory effects of BMP2 on *ID1*, *ID3*, and *ID4* expression and the inhibitory effects of BMP2 on *FSHR* mRNA levels and progesterone production. Collectively, these data provide evidence that prior to follicle selection, BMP2 signaling contributes toward maintaining granulosa cells in an undifferentiated state. The actions of BMP2 are, at least in part, mediated indirectly via enhanced EGFRL expression and ERBB receptor-mediated MAP kinase signaling, and can be modulated by the autocrine/paracrine production of NOG.

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Introduction

In the avian ovary, the selection of a single follicle per day into the preovulatory hierarchy occurs from a small cohort of prehierarchical follicles. In the laying hens, follicles within this cohort measure 6–8 mm in diameter, and generally consist of some 6–15 in number. Prior to follicle selection, the hen granulosa cells from prehierarchical follicles remain undifferentiated, as defined by elevated levels of *FSHR* mRNA expression, the virtual absence of *LHR* mRNA expression, and an inability to produce progesterone. It has been previously demonstrated that members of the transforming growth factor β (TGFB) superfamily, TGFB, and activin A, alone and in combination with FSH, promote *FSHR* mRNA expression and initiate FSH-induced progesterone production in undifferentiated granulosa cells from prehierarchical follicles (Woods & Johnson 2005). Moreover, it has been established that epidermal growth factor receptor ligand (EGFRL) signaling via the MAP kinase/extracellular regulated kinase (ERK) pathway can completely block agonist-induced responses to FSH.

Previous studies with hen prehierarchical follicles have determined that the EGFRLs, betacellulin (BTC), and EGF are produced in an autocrine fashion by the granulosa layer, whereas TGFA is produced by the theca layer and acts on the adjacent granulosa in a paracrine fashion (Woods *et al.* 2005, 2007). Conversely, inhibition of MAP kinase/ERK signaling (e.g. using selective MAP kinase pathway inhibitors) promotes *FSHR* expression, and when combined with FSH treatment, potentiates *FSHR* expression plus promotes granulosa cell differentiation (Woods & Johnson 2005). Together, these findings have formed the basis of a working model predicting that active MAP kinase signaling precludes premature differentiation of granulosa cells in all prehierarchical follicles except the follicle destined for selection (Johnson & Woods 2009). Accordingly, granulosa cell differentiation associated with preovulatory follicle growth is proposed to occur only following selective release from inhibitory MAP kinase/ERK signaling. Established characteristics of differentiated hen granulosa cells from follicles subsequent to selection include enhanced capacity for FSH-induced cAMP formation

(Tilly *et al.* 1991), significant up-regulation of LH receptor expression and LH responsiveness (Johnson *et al.* 1996), STAR protein expression (Johnson & Bridgham 2001), and cytochrome P450 side chain cleavage enzyme activity (Li & Johnson 1993), together with a greatly enhanced capacity for progesterone production.

Although several endocrine/paracrine/autocrine factors and associated signaling pathways have been implicated in regulating FSHR levels in the hen granulosa cells, much less is known about transcriptional processes associated with *FSHR* expression. Inhibitor of differentiation/DNA-binding (ID) proteins represent members of the basic helix-loop-helix (bHLH) superfamily of transcription factors (TFs) that lack a DNA-binding domain. Thus, ID proteins can act as negative antagonists of transcription by dimerizing with bHLH TFs to prevent their binding with a promoter E-box element (Ruzinova & Benezra 2003). Importantly, it has been reported that activation of the rat *FSHR* promoter is dependent upon a functional E-box element (Heckert 2001). More recently, levels of ID proteins (ID1, ID3, ID4, and ID2) in the hen granulosa cells were found to be regulated by BTC (acting in a paracrine/autocrine fashion) and TGFA (acting in a paracrine fashion), and reflective of *FSHR* mRNA expression (Johnson *et al.* 2008a). Specifically, elevated levels of ID1, ID3, and ID4 in granulosa from prehierarchical follicles are associated with decreased levels of *FSHR*, whereas enhanced expression of ID2 is associated with enhanced *FSHR* expression and FSH responsiveness.

Within the mammalian ovary, critical roles have been established for several bone morphogenetic protein (BMP) family members in relation to initial follicle recruitment (e.g. GDF9, Dong *et al.* 1996), follicle selection (e.g. BMP15, Galloway *et al.* 2000), and *FSHR* expression and/or FSH responsiveness (e.g. BMP15, Otsuka *et al.* 2001; BMP6, Shi *et al.* 2009). Several BMP family members, together with their known receptors, are expressed in hen preovulatory follicles (Onagbesan *et al.* 2003, Al-Musawi *et al.* 2007). These published findings have been confirmed during the course of our studies, and have been extended to include prehierarchical follicles (1–12 mm diameter). In particular, *BMP2* mRNA together with mRNAs encoding BMPR1 (subtypes ALK2, ALK3, and ALK6) and BMPR2 is expressed in the hen granulosa and theca tissues from prehierarchical follicles.

There is currently little information regarding mechanisms that regulate the tissue-specific expression of BMPs or their receptors within the ovary. It is, however, generally recognized that the extracellular activity of TGFB superfamily proteins can be modified by one or more antagonists. These secreted proteins selectively bind various TGFB family members and prevent their interaction with BMP receptors. Among the mammalian BMP antagonists expressed in various ovarian tissues are included gremlin, protein related to DAN and cerberus (PRDC), chordin, follistatin, and

noggin (NOG; Sudo *et al.* 2004, Knight & Glister 2006). Expression profiles and proposed functions for follistatin in the hen follicles have previously been discussed (Davis *et al.* 2001, Lovell *et al.* 2003). To our knowledge, however, there have been no reports to date regarding the expression or activity of the BMP-specific antagonist, NOG, in the avian ovary.

To date, published studies designed to elucidate a role for BMPs in the avian ovary have been limited to cultures of differentiated granulosa cells collected from the three to four largest preovulatory (e.g. post-selection) follicles (Onagbesan *et al.* 2003, Al-Musawi *et al.* 2007). In our laboratory, work has been initiated to establish a role for BMPs in the organization and maintenance of the prehierarchical cohort of avian follicles from which follicle selection reoccurs on a daily basis. Unlike in mammals where subdominant (non-selected) follicles undergo atresia each cycle, the maintenance and viability of this prehierarchical cohort in birds are critical to enable daily follicle selection and ovulation for the duration of a clutch. The present study focused on a role for BMP2 and its associated signaling mechanisms in modulating *FSHR* mRNA expression and FSH responsiveness in granulosa cells from this cohort of prehierarchical follicles.

Results

BMP2 mRNA expression in ovarian follicles

BMP2 cDNA was amplified from the granulosa and theca tissues from prehierarchical follicles (6–8 mm diameter) and the second largest preovulatory (F2) follicles (Fig. 1). These results provide evidence that BMP2 may act as an autocrine or paracrine factor in the hen ovary.

BMP2 inhibition of *FSHR* expression and FSH responsiveness (progesterone production)

Treatment of undifferentiated granulosa cells with BMP2 at doses of 10 and 50 ng/ml for 30 min enhanced the phosphorylation of SMAD1/5/8 (Fig. 2, inset). As previously established and confirmed herein, a 20-min treatment with BTC (10 ng/ml) promoted significant

Figure 1 *BMP2* cDNA amplified from granulosa (Gr) and theca (Th) tissues from prehierarchical (Prehier) follicles of 6–8 mm diameter and preovulatory (Preovul) follicles. +, reverse transcriptase; –, without reverse transcriptase; and H₂O, water blank.

phosphorylation of ERK (Woods *et al.* 2005). By contrast, BMP2 (10 ng/ml) failed to promote any significant increase in levels of phosphorylated ERK within this time frame (data not shown). These results provide evidence for initial BMP2 signaling via the SMAD1/5/8 pathway. Whereas a 24-h culture of undifferentiated granulosa cells with TGFB alone or in combination with FSH promoted *FSHR* expression (Fig. 2A) and a very modest quantity of progesterone production (Fig. 2B), a 3-h pretreatment with BMP2 (10 ng/ml) completely blocked these stimulatory effects.

BMP2-induced EGFR and ID protein isoform mRNA expression

Treatment of undifferentiated granulosa cells from prehierarchal follicles with BMP2 (10 ng/ml) for 20 min and for 3 h increased both *EGF* and *BTC* mRNA expression (Fig. 3). In addition, BMP2 treatment promoted expression of *ID1*, *ID3*, and *ID4* mRNA after a 3-h treatment, but not after a 20-min treatment (Fig. 4).

Figure 2 *FSHR* mRNA expression (A) and progesterone levels (B) from undifferentiated granulosa cells precultured for 3 h in the absence or presence of BMP2 (10 ng/ml), and then cultured with TGFB1 (10 ng/ml) and FSH (100 ng/ml) for an additional 21 h. *FSHR* is expressed as fold difference compared to control cultured (T24) cells. Data represent the mean $\pm$ S.E.M. from four replicate experiments. A, B, and C; $P < 0.01$. Inset represents SMAD1/5/8 phosphorylation (p-SMAD) in undifferentiated granulosa cells after treatment with 2, 10, or 50 ng BMP2/ml for 30 min. This experiment was repeated once with similar results.

Figure 3 BMP2 treatment (+; 10 ng/ml) for 20 min or for 3 h increased betacellulin (*BTC*) and epidermal growth factor (*EGF*) mRNA expression levels compared with control cultured (-) granulosa cells from prehierarchal follicles. Data are expressed as mean fold difference $\pm$ S.E.M. compared to uncultured (T0) cells (not shown). $N = 3$ replicate experiments. A, B and d, e, f; $P < 0.05$.

ID2 mRNA levels were significantly decreased by a 3-h treatment with BMP2. These findings indicate that under these culture conditions, BMP2-induced *BTC* and *EGF* expression precedes changes in *ID* mRNA expression.

BMP2 actions are dependent upon ERBB receptor tyrosine kinase activity

In light of the finding that BMP2 induced autocrine *EGF* and *BTC* mRNA expression, it was hypothesized that the inhibitory effects of BMP2 on *FSHR* expression could be indirectly mediated by EGFR via ERBB/MAP kinase signaling. Accordingly, the ability of the selective ERBB1/ERBB4 receptor tyrosine kinase inhibitor, AG1478, to block the inhibitory effects of BMP2 on *FSHR* mRNA expression was evaluated. In fact, pretreatment of cultured cells with AG1478 prevented the ability of BMP2 to inhibit TGFB+FSH-induced *FSHR* (Fig. 5). These results suggest that the differentiation-inhibiting effects of BMP2 in the hen granulosa cells occur, at least in part, via inhibitory MAP kinase signaling.

BMP2 activity is antagonized by NOG

NOG mRNA was readily amplified from the granulosa and theca tissues collected from the prehierarchal follicles of 3–5 and 6–8 mm diameter, as well as from the most recently selected (9–12 mm) follicle (Fig. 6A). The potential regulatory activity of NOG is indicated by the ability of recombinant human (rh) NOG pretreatment to prevent BMP2-mediated induction of *ID1*, *ID3*, and *ID4* mRNA expression in a dose-dependent fashion (Fig. 6B). Significantly, a 25 ng/ml dose of BMP2 (compared with 10 ng BMP2/ml; Fig. 1) decreased both basal expression of *FSHR* and basal (unstimulated) levels of progesterone production (Fig. 7). Preculture with increasing doses of NOG reversed the BMP2-mediated reduction in *FSHR* mRNA expression, and restored basal levels of progesterone production.

Figure 4 BMP2 treatment (+; 10 ng/ml) for 3 h induced *ID1*, *ID3*, *ID4*, and *ID2* mRNA expression compared with control cultured (-) granulosa cells. Data are expressed as mean fold difference $\pm$ S.E.M. compared to uncultured (T0) cells (not shown). $N=3$ replicate experiments. A, B; d, e; and G, H: $P<0.05$.

Discussion

Results demonstrate that BMP2 treatment, *in vitro*, blocks TGF β - and TGF β plus FSH-induced *FSHR* mRNA expression in undifferentiated granulosa cells from prehierarchical follicles (Fig. 2A). Based upon the data presented herein, a working model to describe the autocrine/paracrine effects of BMP2 on *FSHR* expression is described in Fig. 8.

The ability of BMP2-induced signaling to block *FSHR* expression in undifferentiated granulosa cells is similar to previous results related to EGFR (e.g. BTC, EGF, and TGFA)-mediated inhibitory signaling via MAP kinase/ERK (Woods & Johnson 2005, Woods *et al.* 2005). It is well established that BMP2 receptor signaling occurs via SMAD1/5/8, and there are various reports that BMP2 can also activate MAP kinase/ERK signaling in a latent and prolonged fashion (Lou *et al.* 2000, Lai & Cheng 2002). Although we found no evidence for this in the present study, it is premature to dismiss the possibility for BMP-induced MAP kinase/ERK signaling in the hen granulosa cells. It is, however, notable that BMP2 treatment rapidly induced *BTC* and *EGF* mRNA expression (Fig. 3). This is noteworthy because treatment with BTC or TGFA was reported to increase *ID1*, *ID3*, and *ID4* expression (and decrease levels of *ID2*) in undifferentiated granulosa cells (Johnson *et al.* 2008a). These effects on *ID* expression are completely blocked by treatment with

the selective MAP kinase/ERK inhibitor, U0126. We have recently provided evidence that changes in *ID* protein expression can modulate *FSHR* mRNA expression in undifferentiated granulosa cells (Johnson *et al.* 2008a, Johnson & Woods 2009). In the present studies, BMP2 treatment was found to increase *ID1*, *ID3*, and *ID4* expression (Fig. 4), and this response (at 3 h) occurred after the induced increase in *BTC* and *EGF* expression (at 20 min). Accordingly, BMP2 up-regulation of *ID1*, *ID3*, and *ID4* may be mediated by EGFR/MAP kinase/ERK signaling. This is supported by the finding that pretreatment with the selective ERBB tyrosine kinase inhibitor, AG1478, reversed the inhibitory effects of BMP2 on *FSHR* expression (Fig. 5).

It is also significant that BMP2 pretreatment also negatively impacted FSH-induced progesterone production (Fig. 2B). Although it has not yet been established whether this loss of FSH responsiveness occurs prior and/or subsequent to cAMP formation, there is a recent report that BMP2 (and BMP4) inhibits both FSH- and forskolin-induced progesterone production and cAMP synthesis in rat granulosa cells (Inagaki *et al.* 2009). Importantly, highest levels of *FSHR* mRNA during follicle development occur in the hen granulosa cells from this cohort of prehierarchical follicles of 6–8 mm diameter (You *et al.* 1996, Woods & Johnson 2005). Accordingly, it is evident that elevated *FSHR* mRNA (and presumably *FSHR* protein) by itself is not sufficient to initiate follicle selection and granulosa cell differentiation. It has previously been demonstrated that significant FSH-induced cAMP production in granulosa cells occurs only subsequent to follicle selection (e.g. in 9–12 mm follicles; Tilly *et al.* 1991). Nevertheless, granulosa cells from follicles at all stages

Figure 5 Inhibition of ERBB receptor kinase activity by pretreatment (20 min) with the selective inhibitor, AG1478 (AG; 10 μ M), prevents the inhibitory effects of BMP2 (10 ng/ml) on TGF β (10 ng/ml)- and FSH (100 ng/ml)-induced *FSHR* expression. Cells were precultured for 1 h with AG1478, and then treated with BMP2 for 3 h prior to TGF β plus FSH treatment. Data are expressed as mean fold difference $\pm$ S.E.M. compared to control (T24) cultured cells. $N=3$ replicate experiments. A, B, and C: $P<0.05$.

Figure 6 (A) *NOG* mRNA expression in granulosa and theca tissues collected from prehierarchical follicles of 3–5 and 6–8 mm diameter and the most recently selected (9–12 mm) follicle from three ovaries. Data are expressed relative to levels in ovarian stromal tissue (not shown). A and B: $P < 0.05$. (B) BMP2 (+; 25 ng/ml)-induced *ID1*, *ID3*, and *ID4* mRNA expression is bionutralized by a 20-min pretreatment with recombinant human *NOG*. Data represent the mean $\pm$ S.E.M. from three replicate experiments. A, B; c, d, e; and G, H, J, K: $P < 0.05$.

of development (beginning with follicle 1–2 mm in diameter) are competent to respond, *in vitro*, to the cAMP analog, 8-bromo-cAMP, with a rapid and robust increase in STAR expression and cytochrome P450 side chain cleavage enzyme activity (Li & Johnson 1993, Woods & Johnson 2005). Collectively, these results observed from the hens provide evidence that prerequisite for the daily selection of a single follicle into the preovulatory hierarchy is: 1) a release from tonic inhibitory signaling within the granulosa layer of the selected follicle; 2) a consequent increase in *FSHR* mRNA expression mediated by enhanced responsiveness to TGFB and FSH; combined with 3) a fully potentiated coupling of the *FSHR* protein to the protein kinase A/cAMP signaling pathway. Additional studies are required to understand the timing and mechanisms underlying *FSHR* coupling to cell signaling pathways in granulosa cells during early follicle development, together with the ability of EGFRs and BMPs to modify this signaling.

In mammalian follicles, *NOG* is reported to bind several BMP family members produced by the theca, granulosa, and/or oocyte, including BMP2, -4, -5, -6, and -7, but not by the oocyte-derived BMP15 or GDF9 (Gazzerro & Canalis 2006). For instance, treatment with rh *NOG* effectively bionutralized the inhibitory effects of BMP2 treatment on progesterone production by

cultured ovine granulosa cells (Pierre *et al.* 2005). Similarly, in the present studies, *NOG* effectively reversed both the stimulatory effects of BMP2 on *ID1*, *ID3*, and *ID4* expression (Fig. 6B) and the inhibitory actions of BMP2 on *FSHR* expression and progesterone production (Fig. 7). Each of these processes affected by *NOG* treatment reflects an endpoint associated with the differentiation in the hen granulosa cells. Thus, it is possible that the coordinated expression and secretion of *NOG* (or some other BMP2 antagonist) protein could play a role in follicle selection and/or the regulation of initial steroid production by the granulosa layer. For completeness, it is observed that there have been two novel *NOG* homologs reportedly encoded within the chicken genome (*NOG2* and *NOG4*; Eroshkin *et al.* 2006). To date, however, there is apparently no information regarding patterns of expression or BMP-binding partners for these, or any other, BMP-specific antagonist in the hen granulosa cells.

Whereas a functional role of BMP15 in granulosa from preovulatory follicles has previously been proposed (Elis *et al.* 2007), the studies described herein provide some of the first functional data from the avian ovary to implicate BMP family members in the regulation of granulosa cell function during early follicle development. Significantly, the expression of, and a functional role for, another BMP-related protein, anti-Müllerian hormone (AMH), has also been reported in the hen granulosa cells from prehierarchical follicles (Johnson *et al.* 2008b). In addition to its initially described role in the regression of the right Müllerian duct in females during embryonic development, AMH was shown to regulate granulosa cell proliferation and may be implicated in the rate of follicle growth and/or selection

Figure 7 Bionutralization of BMP2 (25 ng/ml) by a 20-min preculture with recombinant human *NOG* facilitates *FSHR* expression and progesterone accumulation in the medium over a 24-h culture period. *FSHR* data are expressed as fold difference compared to control (T24) cultured cells (not shown). $N = 3$ replicate experiments. A, B, and C: $P < 0.05$.

Figure 8 Working model for BMP2 inhibitory effects on *FSHR* mRNA expression in undifferentiated hen granulosa cells from prehierarchal follicles. BMP2 produced in a paracrine and/or autocrine fashion promotes phosphorylation (p-) of SMAD1,5,8 and rapidly up-regulates expression of epidermal growth factor receptor ligand mRNAs (including *EGF* and *BTC*) and presumably proteins (EGF and BTC). In turn, EGF and BTC signal via ERBB-associated tyrosine kinase activation (p-ERK) to up-regulate expression of the inhibitor of differentiation/DNA-binding proteins, ID1, ID3, and ID4. A requirement for ERBB/ERK signaling is indicated by the ability of AG1478 to block the inhibitory actions of BMP2. We have previously provided evidence that endogenous up-regulation of ID1, ID3, or ID4 protein is associated with inhibition of *FSHR* transcription in the hen granulosa cells (Johnson *et al.* 2008b), presumably via the disruption of transcription factor (TF) binding to one or more E-box element within the FSH receptor promoter region. The BMP antagonist, NOG, is also expressed within follicle tissues, and may serve as a paracrine and/or autocrine regulator of BMP2 activity during follicle development.

(Johnson *et al.* 2009). Additional functional studies that specifically focus on the role of these and related BMP family members in regulating FSH responsiveness and mediating follicle selection are warranted.

Materials and Methods

Animals and reagents

Single-comb White Leghorn hens 25–40 weeks of age and laying sequences of six or more eggs on a regular basis were used in all studies described. Hens were housed individually in laying batteries, with free access to feed (Purina Layena Mash; Purina Mills, St Louis, MO, USA) and water, under a controlled photoperiod of 15 h light:9 h darkness, (lights on at midnight). The lay patterns were monitored daily. Hens were killed by cervical dislocation 14–18 h prior to a mid-sequence ovulation, and the ovary was 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 University of Notre Dame and 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.

Rh FSH was provided by the National Hormone and Pituitary Program (Torrance, CA, USA), and rh BMP2, BTC, TGFB, and NOG were purchased from PeproTech (Rocky Hill, NJ, USA). The rh BMP2 peptide shares 96% identity with *Gallus* BMP2.

Gallus NOG protein is predicted to share 85% identity with the rh NOG, and the ability for rh NOG to inhibit endogenous BMP signaling in the chicken has previously been demonstrated (Schlueter *et al.* 2006). The selective ERBB1/ERBB4 receptor tyrosine kinase inhibitor, AG1478, was from Calbiochem (San Diego, CA, USA). The duration of culture experiments and doses of FSH, TGFB, and AG1478 utilized were based upon previously published studies (Woods *et al.* 2005, 2007, Johnson *et al.* 2008b).

Granulosa cell cultures

Prehierarchal follicles (3–5 and 6–8 mm diameter; prior to follicle selection) and the single, most recently selected follicle (9–12 mm) were utilized for the present studies. Granulosa cell layers were collected and grouped by follicle size, then dispersed for culture as described previously (Woods & Johnson 2005). In some instances, an aliquot of cells was immediately frozen at -70°C (T0 control). 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 $\sim 1 \times 10^5$ cells per well in 1 ml DMEM plus 2.5% FBS containing 0.1 mM non-essential amino acids plus 1% antibiotic–antimycotic mixture (Invitrogen; DMEM). In addition, 20-min incubations were conducted with granulosa from follicles of 6–8 mm diameter where 5×10^5 cells in 1 ml DMEM were placed in 12×75 mm polypropylene tubes (Fisher Scientific, Pittsburgh, PA, USA; Woods & Johnson 2007). Where appropriate, cells were pretreated with $10 \mu\text{M}$ AG1478 and/or BMP2, or with rh NOG, as described for each experiment.

PCR amplification of BMP2 and NOG cDNA

BMP2 and NOG mRNA were amplified from follicle tissues using reverse-transcribed RNA (Promega RT System, Promega). Specific primers designed for *Gallus* BMP2 (GenBank NM_204358) were as follows: sense, 5'-AGCGAAACACAAA-CAGCGTAAGCG-3' and antisense, 5'-AGTTTAGGTGATCT-GCCAGCGGAA-3'. *Gallus* NOG (GenBank NM_204123) primers were as follows: sense, 5'-TGCCAGCACTACCTGCA-CATCC-3' and antisense, 5'-AACTCCAGCCCCTTGATTTCGCCG-3'. Amplification conditions included denaturing at 94°C , annealing at 56°C (BMP2) or 55°C (NOG), and extension at 72°C for 35–40 cycles using *Taq* DNA polymerase (Eppendorf, Westbury, NY, USA). The PCR products were subsequently sequenced for verification of nucleic acid identity.

Real-time PCR

Forward and reverse primers specific for *Gallus* *EGF*, *BTC*, *FSHR*, ID protein isoforms (*ID1*, *ID2*, *ID3*, and *ID4*), and 18S rRNA have previously been described (Woods & Johnson 2007, Johnson *et al.* 2008a). Random-primed, reverse-transcribed cDNA synthesis reactions were performed using the Promega RT System (Promega), according to the conditions described by the manufacturer. For real-time PCR, primers were added to 10 μl total reaction volume using the reagents provided in

the ABgene QPCR SYBR Green Mix (ABgene, Rochester, NY, USA). 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 StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Reactions were conducted with the following conditions: 15 min at 94 °C, followed by 40 cycles each for 15 s at 95 °C, 1 min at either 55 °C or 60 °C depending upon primer pair, and 1 min at 72 °C. The C_t (the cycle number at which the fluorescence exceeds a threshold level) was determined for each reaction (run in triplicate) using the StepOne software (v.2.0), and quantification was accomplished by standardizing reactions to 18S rRNA, then using the $\Delta\Delta C_t$ method (Livak & Schmittgen 2001). Results were expressed as fold difference compared to an appropriate control tissue or treatment.

Progesterone RIA

Progesterone in media samples was quantified by RIA as described previously (Tilly & Johnson 1988). Data are expressed as mean nanogram progesterone/ml media $\pm$ S.E.M. for the combined replicate experiments.

Immunoblot analysis

Western blot analysis was conducted as described previously (Johnson *et al.* 2008a). Briefly, cells were homogenized in a protein lysis buffer containing a cocktail of enzyme (including phosphatase) inhibitors (Sigma–Aldrich). 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, and a rabbit polyclonal anti-SMAD1 (Santa Cruz, Santa Cruz, CA, USA; diluted 1:1000) was used for standardization. Incubations for the primary antibodies were conducted overnight at 4 °C. The HRP-conjugated anti-rabbit IgG secondary antibody (Pierce, Rockford, IL, USA) was incubated for 2 h at room temperature. Blots were incubated with ECL western blotting agent (Thermo Scientific, Rockford, IL, USA) for 5 min and exposed to X-ray film for 1–5 min. Antibody binding was quantified by densitometry (Storm Imaging system, Molecular Dynamics, Sunnyvale, CA, USA) and analyzed with ImageJ software (NIH).

Data analysis

Experiments were independently replicated a minimum of three times unless otherwise stated. Data for mRNA were expressed as a fold difference compared to uncultured control (T0) or control cultured (T24) cells. Real-time PCR data from replicate experiments were analyzed by one-way ANOVA without including values from the control group (arbitrarily set to 1.0) and by the Fisher's protected least significant difference multiple range test. Progesterone in media samples from the combined replicate experiments, including T24 controls, 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.

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