Bone morphogenetic protein-6 enhances gonadotrophin-dependent progesterone and inhibin secretion and expression of mRNA transcripts encoding gonadotrophin receptors and inhibin/activin subunits in chicken granulosa cells

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Abstract

The aims were to examine ovarian expression of bone morphogenetic protein (BMP) ligands/receptor mRNAs in the chicken and to test the hypothesis that theca-derived BMP(s) modulates granulosa cell function in a paracrine manner. RT-PCR revealed expression of multiple BMPs in granulosa and theca cells from prehierarchical and preovulatory follicles with greater expression in theca cells; both cell types expressed BMP receptors-IA, -IB and -II consistent with tissue responsiveness. Preovulatory granulosa cells (F1, F2 and F3/4) were cultured with BMP-6 (expressed by theca but not granulosa) in the presence/absence of LH, FSH or 8-Br-cAMP. BMP-6 increased 'basal' and gonadotrophin-induced inhibin-A and progesterone secretion by each cell type but did not enhance the effect of 8-Br-cAMP. This indicates that the observed synergism between BMP-6 and gonadotrophin might involve BMP-induced up-regulation of gonadotrophin receptors. In support of this, BMP-6 alone increased LH-receptor (LHR) mRNA in F1 cells and FSH-receptor (FSHR) mRNA in F1, F2 and F3/4 cells. BMP-6 also enhanced LH/FSH-induced LHR transcript amount in each cell type but did not raise FSHR transcript amounts above those induced by BMP-6 alone. To further explore BMP-6 action on inhibin-A secretion, we quantified inhibin/activin subunits (α , β_A , β_B) mRNAs. Consistent with its effect on inhibin-A secretion, BMP-6 enhanced 'basal' expression of α - and β_A -subunit mRNA in F1, F2 and F3/4 cells, and β_B -subunit mRNA in F3/4 cells. BMP-6 markedly enhanced FSH/LH-induced expression of α -subunit in all follicles and FSH-induced β_A -subunit in F2 and F3/4 follicles but not in F1 follicles. Neither BMP-6 alone, nor FSH/LH alone, affected 'basal' β_B mRNA abundance. However, co-treatment with gonadotrophin and BMP-6 greatly increased β_B -subunit expression, the response being lowest in F1 follicles and greatest in F3/4 follicles. Collectively, these results support the hypothesis that intra-ovarian BMPs of thecal origin have a paracrine role in modulating granulosa cell function in the chicken in a preovulatory stage-dependent manner.

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Introduction

The functional left ovary of the laying hen typically contains six to eight large yolk-filled follicles (designated F1–Fn) that have been recruited to a well ordered and rapidly growing preovulatory hierarchy and are destined to ovulate successively at intervals of 24–26 h. Following ovulation of the largest F1 follicle (~40 mm in diameter), the succeeding F2 follicle will advance to the dominant position and so on (Gilbert *et al.* 1983, Etches & Schoch 1984). The ordered arrangement of follicles throughout the hierarchy along with the short, well-defined ovulatory cycle permits identification of developmental stages with a considerable degree of precision, conferring a distinct advantage over comparable follicle maturation studies in mammals.

In the hen ovary, the granulosa layer is the principal site of progesterone synthesis (Etches & Duke 1984); progesterone serves as a precursor for the production of androgens and oestrogens by the neighbouring theca interna and externa cells respectively (Armstrong 1984, Kato *et al.* 1995). In avian follicles, only the theca layer contains the aromatase enzyme required for oestradiol synthesis (Bahr *et al.* 1983, Armstrong 1984). The structure of avian follicles is such that granulosa and theca cell layers are easily separated. Moreover, both cell types are amenable to cell culture providing an excellent model system for functional *in vitro* studies.

In birds as in mammals ovarian folliculogenesis requires the coordinate actions of intra-ovarian and systemic factors. Upon interaction with cognate

receptors on ovarian granulosa and theca cells, the pituitary gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) facilitate the production of ovarian steroid and peptide (e.g. inhibin and activin) hormones (Huang & Nalbandov 1979, Huang *et al.* 1979, Bahr *et al.* 1983, Marrone & Hertelendy 1983, Lee & Bahr 1989, Lovell *et al.* 2002a, 2002b). Gonadotrophins activate adenylyl cyclase, generating cAMP and activation of cAMP-dependent protein kinase A (PKA) pathways (Calvo & Bahr 1982, 1983). Concomitantly, the hydrolysis of phosphoinositides produces other second messengers including inositol triphosphate and 1,2-diacyl-*sn*-glycerol (DAG) leading to intracellular calcium mobilization and calcium-dependent protein kinase C signal transduction (Marsh 1975, Streb *et al.* 1983).

Locally produced proteins/peptides implicated in follicle regulation include members of the extensive transforming growth factor (TGF)- β superfamily. In vertebrates, the TGF- β superfamily comprises over 35 structurally related extracellular growth factors. Members include the inhibins, activins, bone morphogenetic proteins (BMPs), growth differentiation factors, Müllerian inhibiting substance and TGF- β . Collectively they regulate a multitude of cellular responses including differentiation, proliferation, morphogenesis, chemotaxis and apoptosis. TGF- β superfamily members generally exert their biological response by binding to and forming hetero-oligomeric complexes with two types (I and II) of cell-surface receptors, which exhibit integral serine/threonine kinase activity (for review, see de Caestecker 2004). The type-II receptor transphosphorylates a regulatory GS segment (SGSGSG sequence; Wrana *et al.* 1994) located in the cytoplasmic domain upstream of the serine/threonine kinase domain of the type-I receptor (Huse *et al.* 1999). This, in turn, phosphorylates and activates downstream intracellular effectors termed receptor-regulated Smad (R-Smads). The activated receptor complex associates with common partner Smads (Co-Smads) and translocates to the nucleus to bind DNA. The recruitment and interaction with various co-activator or co-repressor factors orchestrates the transcription of target gene expression (for review, see Derynck & Zhang 2003).

Recent studies in mammals have revealed the existence of a functional BMP system in the ovary that appears to have a number of critical roles in the regulation of folliculogenesis and hence female fertility (for reviews, see Knight & Glister 2003, Shimasaki *et al.* 2003). Various BMP ligands, receptors, binding proteins and signalling intermediaries are expressed in a cell-specific manner and *in vitro* studies in several species have demonstrated BMP-induced alterations in steroidogenesis, gonadotrophin responsiveness and cell proliferation in both granulosa and theca cells (rodent: Shimasaki *et al.* 1999, Otsuka *et al.* 2001, Erickson & Shimasaki 2003; porcine: Quinn *et al.* 2004, Brankin *et al.* 2005; ovine: Bodensteiner *et al.* 1999,

McNatty *et al.* 2003; bovine: Bodensteiner *et al.* 1999, Glister *et al.* 2004, 2005). There is also one recent study (Onagbesan *et al.* 2003) reporting the expression of several BMP ligands and receptors in hen follicles. This study also showed that BMP-4 and -7 enhanced granulosa cell proliferation and gonadotrophin-induced progesterone production.

Given the paucity of information available on BMPs in the avian ovary in the present study, we first used semi-quantitative RT-PCR to compare the pattern of expression of a range of BMP ligands and receptors in chicken granulosa and theca cells recovered from prehierarchical and preovulatory follicles. Having shown that several BMPs (including BMP-6) were expressed by theca but not granulosa cells and that both type-I and type-II BMP receptor (BMPR) transcripts are expressed by granulosa cells, we carried out functional *in vitro* studies on isolated granulosa cells to test the hypothesis that theca cell-derived BMPs modulate granulosa cell function in a paracrine manner. Secretory responses of the cultured cells to BMP-6, in terms of inhibin-A, inhibin-B and progesterone secretion and cell proliferation/survival were monitored. Real-time quantitative PCR was used to examine the effect of BMP-6 on basal, gonadotrophin- and 8-Br-cAMP-induced expression of mRNA transcripts for gonadotrophin receptors, cytochrome P450 side chain cleavage (P450_{SCC}) and the inhibin/activin subunits (α , β_A , β_B).

Materials and Methods

Experimental animals

Laying hens (Highline), towards the end of their first year of lay, which were producing at least five eggs in regular sequences, were used. They were individually caged and maintained under a standard long photo schedule of 16 h light:(lights on at midnight) 8 h darkness, at an ambient temperature of 21–23 °C. Food and water were provided *ad libitum*. The time of ovulation for each hen was predicted (Warren & Scott 1935) and subsequently confirmed from daily recordings of ovipositions, monitored by time-lapse video recording. Hens were killed by cervical dislocation ~10–12 h after the predicted ovulation of a mid-sequence egg. Ovarian follicles were confirmed to be of a mid-sequence laying cycle by the presence of an additional egg in the oviduct shell gland, thus identified as the consecutive egg to be laid in that clutch. The four largest follicles (F1–F4) of the preovulatory ovarian hierarchy were excised and immediately bathed in sterile Dulbecco's PBS (Sigma UK Ltd). For experiment 1, the prehierarchical follicles of 6–8 mm in diameter were also collected.

Isolation of granulosa and theca cells

Granulosa and theca tissue layers were separated in a laminar flow hood under aseptic conditions (Gilbert *et al.* 1977). In brief, layers derived from a given follicle position in three to four different hens were combined for each culture experiment. Pooled tissues were enzymatically dispersed as described in Lovell *et al.* (2002a) except that thecal tissue was dispersed for 45 min rather than 10 min. Cell viability, estimated by trypan blue exclusion (Sigma UK Ltd), was always >90% for both cell types. Isolated cells were diluted in incubation medium (medium 199 with 25 mM HEPES, 0.01% (v/v) L-glutamine and 1% (v/v) antibiotic solution) containing 2% (v/v) charcoal-treated foetal bovine serum (Sigma UK Ltd) and plated out in 24-well plates at 0.5×10^6 cells/0.5 ml/well. After culturing for 24 h at 39 °C, unattached cells were removed by aspiration and the adherent cells were washed thrice with 1 ml serum-free incubation medium. All further incubations were done in serum-free conditions. Incubation buffer (0.5 ml) and test treatments (see below) were added to the appropriate wells. The cells were generally incubated for 3×24 h treatment periods and conditioned media were replenished with the appropriate test treatments after 24 h. At termination of the culture the media were removed and stored at –20 °C and the cell monolayers were washed thrice with PBS prior to RNA purification.

Preparation of treatments

Ovine (o)LH (NIDDK-oLH-25), oFSH (NIDDK-oFSH-19-SIAPP) and recombinant human (rh)BMP-6 (R&D Systems Europe Ltd, Abingdon, UK) were prepared as stock solutions in PBS containing 0.3% (w/v) BSA (fraction V). In water, 8-bromoadenosine cAMP (8-Br-cAMP; Sigma UK Ltd) was prepared as a 0.2 M stock solution. Solutions were filter sterilized by passing through a 0.2 µm membrane filter (Minisart; Sartorius AG, Gottingen, Germany) and diluted to appropriate concentrations in sterile incubation medium. Test substances were diluted in incubation medium and added as 50 µl aliquots to duplicate wells (final volume 500 µl) with control wells receiving an equal volume of blank medium.

Experiment 1

Granulosa and theca cells derived from prehierarchical 6–8 mm and preovulatory F1 follicles were plated for 24 h and incubated under serum-free conditions for a further 48 h. Total RNA was extracted for semi-quantitative RT-PCR analysis as described below.

Experiment 2

Granulosa cells derived from preovulatory follicles (F1, F2 and F3/4) were cultured with increasing doses of BMP-6 (0, 2, 10 and 50 ng/ml). Cell-conditioned media was collected after 48 h to be assayed for inhibin-A and progesterone content. At the end of the culture, cells were sonicated in 200 µl of ice-cold PBS containing 1% (w/v) BSA (protease-free; Sigma UK Ltd), 1% (v/v) Triton X-100 (Sigma UK Ltd) and 0.1% (w/v) sodium azide (Fisher Scientific UK Ltd, Loughborough, UK). The suspensions were stored at –20 °C for future analysis of total cellular DNA content.

Experiment 3

Granulosa cells were cultured with either purified oFSH (10 and 100 ng/ml), oLH (10 and 100 ng/ml) or 8-Br-cAMP (0.5 mM) individually, or in the presence of rhBMP-6 (50 ng/ml; dose established from experiment 2). The doses of the remaining treatments were previously found to be maximally effective in stimulating inhibin-A and progesterone from preovulatory follicles using the same culture system in our laboratory (Lovell *et al.* 2002a). Upon termination of the culture at 48 h, cells were analysed for total DNA content and media was harvested for hormone assay. This experiment was replicated six times.

For RNA extraction the experiment was scaled-up with eight replicate wells of 24-well plates (0.5×10^6 cells/0.5 ml/well) per treatment. Cultures were terminated at 48 h and cells retrieved for total RNA isolation. After removing conditioned media, cell monolayers were incubated in PBS containing 0.05 M EDTA (100 µl/well) at 38.5 °C for 10 min. Cells were then collected by gentle scraping and transferred into 1.5 ml non-stick microtubes (Anachem UK Ltd, Luton, Beds, UK) and retrieved by centrifugation (100 g for 5 min). The scaled-up experiment was replicated thrice.

Purification of RNA and synthesis of cDNA

A standard acid guanidium thiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi 1987) was employed for the purification of total RNA. Briefly, cells were lysed in Tri Reagent (Sigma UK Ltd). Upon aqueous phase separation RNA was precipitated in isopropanol (Sigma UK Ltd) and washed in 75% (vol/vol) ethanol. The RNA pellet was resuspended in 50 µl nuclease-free water treated with RNA Secure (Ambion, Huntingdon, Cambridgeshire, UK). Potential Genomic DNA contamination was removed with an RNase-free DNase kit (RQ1; Promega UK Ltd). The Tri Reagent extraction process was repeated and the final RNA pellet resuspended in 50 µl nuclease-free water; RNA quantity and quality were evaluated by spectrophotometry at

260/280 nm (GeneQuant RNA/DNA calculator, Amersham Pharmacia Biotech UK Ltd).

First strand cDNA was synthesized from 1 µg RNA using the Improm-II Reverse Transcriptase system (using buffers and protocol provided; Promega UK Ltd) in a 20 µl reaction. Random hexamer primers (0.5 µg; Sigma UK Ltd) and RNA template (1 µg) were mixed and denatured (5 min at 70 °C) before rapid chilling in an ice bath for 10 min. MgCl₂ (3 mM final), dNTP mix (0.5 mM final), SUPERase In (20 U/µl; Ambion) and Reverse Transcriptase were added (5 min at 25 °C) followed by 40 °C incubation for 60 min. The reaction was terminated by heat inactivation (15 min at 70 °C). To specifically degrade RNA in RNA:DNA hybrids, 1 µl RNase cocktail (0.5 U/µl RNase A and 20 U/µl RNase T1; Ambion) and 0.5 µl RNase H (40 U/µl; Ambion) was added to each cDNA sample and incubated (37 °C for 30 min and 75 °C for 15 min).

Semi-quantitative RT-PCR analysis

Each reaction consisted of 0.75 µl diluted (10-fold in TE buffer) cDNA template in a total reaction volume of 12.5 µl containing 11.25 µl, 1.1 × Hot Start PCR Master Mix (Abgene, Epsom, Surrey, UK) and 0.25 µl each forward and reverse primers (10 µmol each; see Table 1). For negative controls, cDNA template was substituted with nuclease-free water. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was determined using cDNA template diluted 100-fold. Thermocycling incorporated an initial enzyme activation step at 94 °C for 15 min followed by 35 cycles of amplification comprising denaturation at 94 °C for 20 s, annealing at 68 °C for 1 min and extension at 60 °C for 1 min. Primer sequences were designed to target mRNA sequences based on criteria set by the ABI PRISM primer express software (version 1.5; Perkin-Elmer Applied Biosystems, Warrington, UK). Primer sequences and Entrez accession numbers are outlined in Table 1. Ten microlitres of each PCR product was electrophoresed using a 2% agarose gel containing 10 µg/ml ethidium bromide.

Samples were run with 20% (v/v) Blue-Orange Loading dye (Promega), alongside a 100 bp DNA ladder (Promega UK Ltd). Gels were subsequently viewed and photographed under a u.v. transilluminator (u.v. Products Inc., Upland, CA, USA). Representative PCR products for 11 out of the 12 target mRNA sequences (BMP-10 primers did not generate an amplicon) were purified from agarose gels and cloned into pGEM-T Easy Vector (Promega) for sequencing (Cogenics, Takeley, Essex, UK). Sequences of cloned PCR products corresponded to those expected, confirming the specificity of each PCR.

Quantitative real-time RT-PCR analysis

Reactions were carried out in duplicate and consisted of either 1 µl cDNA or 1 µl synthetic oligonucleotide standard (200–0.195 amol/µl) in a total volume of 25 µl containing 13.5 µl Absolute QPCR master mix (Abgene), 2 µl each forward and reverse primers (final concentration: 50–900 nM), 1 µl TaqMan probe (final concentration: 100–200 nM) and 5.5 µl nuclease-free water. Controls (no template and no probe) were used throughout. Samples were processed for 40 cycles on an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer-Applied Biosystems) with the following thermal cycling conditions: 2 min at 50 °C, 15 min at 95 °C, 15 s at 95 °C and 1 min at 60 °C. Probes were 5'-modified with 6-carboxyfluorescein and 3'-modified with carboxytetramethylrhodamine. Primer and probe sequences with corresponding Entrez accession numbers are outlined in Table 2.

Immunoassays

Concentrations of inhibin-A in granulosa cell-conditioned media were determined by a specific two-site ELISA previously validated for chicken in this laboratory (Lovell *et al.* 1998, 2000, 2003). The assay employs monoclonal antibodies raised against synthetic peptide fragments of the human inhibin- α - and - β -subunits (Muttukrishna *et al.* 1994). Recombinant human 32 kDa rh inhibin-A (NIBSC, Potters Bar, Herts, UK)

Table 1 Primer sequences used for conventional RT-PCR in this study.

Target	Accession number	Forward primer 5' to 3'	Reverse primer 5' to 3'	Amplicon size (bp)
BMP-2	NM_204358	ttggacaccagggttggtgcatcat	ggcgtctacgctgtttgtgttcg	301
BMP-3	NM_001034819	aaacgtccacacacgtcctgttg	atccactcgtccagccaatgtca	302
BMP-4	NM_205237	ctgccaaagccatgaactcttgc	cacgctcgtgaggtgaagacgaa	302
BMP-5	NM_205148	gcaagtgaagtgtctttccgctctg	ccagtgctgaacaatggcgtggg	301
BMP-6	BM440114	acgttcccaaacatgctgtgcac	agctctttgctcatgccacgcgt	301
BMP-7	AF205877	aaggattacatccgcgaacgctcg	ccgtgaagggtgctgtttgttg	301
BMP-10	AJ581667	ggcatccagacagatctatggcaca	ttccagggtctccaagtcaggagct	301
BMP-15	AY729025	atcgctacaaccgcgctactgtaag	tactcctgttagaggatcgtccgic	205
BMPR-IA	NM_205357	tttgatggcagcattcgttgatg	caccatctgtcccttccaacttgc	301
BMPR-IB	NM_205132	gtttttaccacggaggaggccagct	atcacggtgggcaatagccggtt	301
BMPR-II	NM_001001465	acggaatcagcgagagccgaatct	ttgtaaggcgtcgtatcggttggg	301
GAPDH	NM_204305	tgccaaccccaatgtctctgttg	tccttgatgccatgtggacat	301

Table 2 TaqMan primer and probe sequences used for quantitative PCR in this study.

Target	Accession number	Forward primer (5' to 3')	Reverse primer (5' to 3')	TaqMan probe 5' [6-FAM] to 3' [TAMRA]
FSH receptor	NM_205079	cgggacattcccacca	tcccttcggaatgactctcatc	tgccacagaactgagatttgcctcacca
LH receptor	AB009283	ttctcaacggcactggagtc	cattgcattgaggacctgga	ctgcctagctatgggctggaggcc
Inhibin α subunit	U48438	gggctgggacaattggat	actactgccacgggaactgtg	tgaccccagcagcttcgttttc
Inhibin β_A -subunit	U26946	caccatccgcctgtttcaac	cctccatatcttctgctccttca	cagcggcagccgaaaggca
Inhibin β_B -subunit	AF055478	tttagtggtgcaagccg	aagaggcctggagtgccgat	ctcgtgataacaacacaggatccgg
P450scc	D49803	cacaggctgacaaatgtatcca	gtgctctcgtgctcttg	acgtttaccgggacatccggctg
GAPDH	K01458	ggagtcaacggattggcc	tttgccagagaggacggc	tattggccgctggtcaccagg

was used as assay standard and the detection limit was 2 pg/ml.

Inhibin-B concentrations were determined using a specific two-site ELISA previously validated for chicken (Lovell *et al.* 2000, 2003). The assay employs monoclonal antibodies raised against synthetic peptide fragments of the human α - and β_B -subunits (Knight & Muttukrishna 1994, Groome *et al.* 1996). An in-house standard preparation of pooled porcine follicular fluid was used as the working assay standard. It was previously standardized against rh inhibin-B (Genentech Inc., San Francisco, CA, USA) and parallelism between both dilution curves confirmed by linear regression analysis. The assay detection limit was equivalent to 200 pg rh inhibin-B/ml.

Progesterone concentrations were determined by a competitive ELISA as described by Sauer *et al.* (1986); the detection limit was 20 pg/ml. Total cellular DNA content was measured by a fluorometric assay as described by Labarca & Paigen (1980), to determine the effects of treatment on cell proliferation/survival.

In each of the above assays intra- and inter-assay coefficients of variation were below 10 and 12% respectively.

Statistical analysis

Data were analysed by two-way ANOVA and provided a significant *F* ratio ($P < 0.05$) was obtained, *post hoc* Fisher's protected least significant difference tests were used for pair-wise comparisons of hormone secretion and mRNA expression amongst treatments and amongst cells from follicles of different hierarchical position. Expression of each mRNA was normalized to GAPDH mRNA. There were no significant differences (ANOVA: $P > 0.05$) in GAPDH mRNA expression between cells derived from different follicle classes (F1, F2 and F3/4) under any of the treatments conditions (data not shown). Inhibin-A, inhibin-B and progesterone concentrations in media were normalized to total cellular DNA content at the end of culture. To reduce heterogeneity of variance, data were log-transformed prior to statistical analysis. Unless otherwise stated, numerical values are presented as arithmetic means $\pm$ S.E.M. of three replicate experiments.

Results

Cells were cultured for a maximum of 3 days (24-h plating followed by 48-h treatment). Results are presented for treatment day 2 during which observed responses to treatments were greatest.

RT-PCR analysis of BMP ligand/receptor expression in granulosa and theca cells from preovulatory F1 and prehierarchal 6–8 mm follicles

BMP-2, -3, -4 and -5 mRNAs were co-expressed in cultured granulosa and theca cells from preovulatory F1 follicles, albeit at different levels (Fig. 1a). Similarly,

Figure 1 Amplicon bands representing the expression of mRNA transcripts encoding the chicken BMP ligands (BMP-2 to -7, -10 and -15), receptors (BMPR-IA, -IB and -II) and GAPDH in cultured chicken granulosa (G) and theca (T) cells isolated from (a) F1 preovulatory and (b) 6–8 mm prehierarchal follicles.

BMPR-IA, -IB and -II were co-expressed in both cell types. Expression of BMP-6, -7, -10 and -15 was not detected in F1 granulosa cells but BMP-6 and -7 were detected in F1 theca cells. Figure 1b shows that only BMP-2 and BMPR-IA and -II were detectable in granulosa cells from prehierarchal 6–8 mm follicles. Correspondingly, theca cells expressed BMP-3, -4, -5, -6, -7 and -15, along with BMPR-IA, -IB and -II, but BMP-2 and -10 expressions were absent. Relative to GAPDH expression, granulosa cells from F1 follicles appeared to express several BMP ligands (BMP-2, -3 and -4) and all three BMPR forms at much higher amounts than corresponding cells from prehierarchal 6–8 mm follicles.

Effect of BMP-6 on 'basal' inhibin-A and progesterone secretion by granulosa cells from F1, F2 and F3/4 follicles

Treatment with BMP-6 elicited significant dose-dependent increases ($P < 0.0001$) in inhibin-A secretion from all three cell populations (Fig. 2a). There was a

significant ($P < 0.01$ by two-way ANOVA) interaction between the effects of treatment and follicle status: BMP-6 induced inhibin-A secretion was greatest from the F3/4 (75-fold), intermediate from the F2 (45-fold) and lowest from the F1 cells (20-fold). BMP-6 treatment increased progesterone secretion ($\sim$ fourfold; $P < 0.0001$) in all cell cultures (Fig. 2b). However, there was no interaction between the effects of treatment and follicle status ($P > 0.05$).

Effect of BMP-6 alone and in combination with LH, FSH or 8-Br-cAMP, on hormone secretion by granulosa cells from F1, F2 and F3/4 follicles

Treatment with BMP-6 alone, or either FSH, LH or 8-Br-cAMP alone, increased ($P < 0.05$) inhibin-A secretion by F1, F2 and F3/4 granulosa cells (Fig. 3a–c). Co-treatment with BMP-6 further enhanced FSH-induced inhibin-A secretion (above that induced by BMP-6 alone) by F1, F2 and F3/4 granulosa cells but only enhanced LH-induced inhibin-A secretion in F1

Figure 2 The dose-dependent effect of BMP-6 (0, 2, 10 and 50 ng/ml) on the secretion of inhibin-A (a) and progesterone (b) by granulosa cells derived from F1, F2 and F3/4 follicles. Values are expressed as means $\pm$ S.E.M. ($n = 6$ independent cultures). Statistical comparisons were made within each follicle class; means without a common letter are significantly different ($P < 0.05$).

Figure 3 Effect of BMP-6 (50 ng/ml) with or without FSH/LH (100 ng/ml) or 8-Br-cAMP (0.5 mM) on inhibin-A (a–c; log scale), inhibin-B (d–f) and progesterone (g–i; log scale) secretion by cultured granulosa cells derived from F1, F2 and F3/4 follicles. Values are means $\pm$ S.E.M. ($n=6$ independent cultures); within each panel, means without a common letter are significantly different ($P<0.05$). The hashed line in d–f indicates the effective detection limit of the inhibin B assay.

granulosa cells. BMP-6 did not further enhance 8-Br-cAMP-induced inhibin-A secretion by granulosa cells from any follicle position (Fig. 3a–c).

Inhibin-B secretion by granulosa cells cultured under basal, FSH, LH or 8-Br-cAMP conditions was below the detection limit of the assay (Fig. 3d–f). Treatment with BMP-6 alone increased inhibin-B secretion by all cells (at least 2.3-fold above detection limit; $P<0.05$). BMP-6 enhanced FSH-induced inhibin-B secretion by F1 (2.5-fold), F2 (3.5-fold) and F3/4 (3.5-fold) cells ($P<0.05$). BMP-6 also enhanced LH-induced inhibin-B secretion with maximum stimulation by F3/4 cells (5.6-fold; $P<0.05$). BMP-6 increased 8-Br-cAMP-induced inhibin-B secretion in the F3/4 cells (1.4-fold; $P<0.05$) with no or possibly an inhibitory effect in the F1 and F2 cells.

BMP-6 enhanced ($P<0.05$) both FSH-induced (up to 12-fold) and LH-induced (up to 100-fold) progesterone secretion by all three cell populations but did not further enhance the marked response to 8-Br-cAMP (Fig. 3g, f and i). The fold enhancement by BMP-6 of FSH-induced progesterone secretion was greatest in F3/4 cells (~ 12 -fold) and lowest in F1 cells (~ 4 -fold). The fold enhancement by BMP-6 of

LH-induced progesterone secretion was also greatest in F3/4 cells (~ 100 -fold) and lowest in F1 cells (~ 18 -fold).

Effect of treatments on granulosa cells proliferation/survival

Treatment with BMP-6 alone promoted a significant increase in total cellular DNA content ($\sim$ twofold; $P<0.01$), which was unaffected by follicle hierarchical position (Fig. 4). LH significantly enhanced the response to BMP-6 in F3/4 follicles ($\sim$ twofold; $P<0.05$) but not in F1 or F2 follicles. There were no significant differences ($P>0.05$) in DNA content after treatment with either LH, FSH or 8-Br-cAMP alone.

Effects of BMP-6 alone and in combination with FSH, LH or 8-Br-cAMP on steady-state levels of mRNAs encoding the gonadotrophin receptors

Treatment with BMP-6 alone raised LHR mRNA level in F1 cells (1.5-fold; $P<0.01$) but not in F2 or F3/4 cells (Fig. 5a–c). FSH alone raised LHR transcript level in F2 (1.5-fold; $P<0.01$) but not in F1 or F3/4

Figure 4 Effect of BMP-6 (50 ng/ml) with or without FSH/LH (100 ng/ml) or 8-Br-cAMP (0.5 mM) on the total cellular DNA content of cultured granulosa cells derived from the F1 (a), F2 (b) and F3/4 (c) follicles. Values are means $\pm$ S.E.M. ($n=6$ independent cultures); within each panel, means without a common letter are significantly different ($P<0.05$).

cells. LH alone raised LHR transcript level 2.5-fold ($P<0.01$) in F1 cells and 1.6-fold ($P<0.01$) in F2 cells with no effect on F3/4 cells. Treatment with 8-Br-cAMP alone increased LHR transcript level ~two-, three- and fourfold in F1, F2 and F3/4 cells respectively ($P<0.001$). For each follicle position co-treatment with BMP-6 and FSH up-regulated LHR transcript levels two- to fourfold relative to those induced by either treatment alone. Similarly, co-treatment with BMP-6 and LH further enhanced LHR transcript levels in all three cell populations ($P<0.001$). Co-treatment with BMP-6 and 8-Br-cAMP resulted in a further twofold increase in LHR expression in F1 cells ($P<0.01$) but there was no corresponding increase in F2 or F3/4 cells.

Figure 5 d–f shows that treatment with BMP-6 alone raised FSHR transcript abundance by approximately twofold in F1 and F2 cells ($P<0.01$) and approximately fivefold in F3/4 cells ($P<0.001$). Neither FSH nor LH alone raised FSHR mRNA

transcript abundance in any granulosa cell population; however, 8-Br-cAMP increased FSHR transcript approximately fourfold in F3/4 cells ($P<0.001$). Co-treatment of cells with BMP-6 and either FSH, LH or 8-Br-cAMP did not raise FSHR transcript abundance above the corresponding value induced by BMP-6 alone.

Effects of BMP-6 alone and in combination with LH, FSH or 8-Br-cAMP on expression of mRNA encoding cytochrome P450_{sc}

Basal expression of cytochrome P450_{sc} transcript was uniformly low amongst cells from F1, F2 and F3/4 follicles. Treatment with BMP-6 alone increased P450_{sc} mRNA in F1 (25-fold), F2 (5.5-fold) and F3/4 (3.6-fold) cells. FSH alone increased transcript abundance in F1 (9.4-fold), F2 (4.4-fold) and F3/4 (3.3-fold) cells. LH alone also increased P450_{sc} expression in a follicle dependent manner (31-fold in F1;

Figure 5 Effect of BMP-6 (50 ng/ml) with or without FSH/LH (100 ng/ml) or 8-Br-cAMP (0.5 mM) on relative amounts of mRNA transcript encoding the LH (a–c) and FSH (d–f) receptors in the F1, F2 and F3/4 preovulatory granulosa cells. Values are means $\pm$ S.E.M. ($n=3$ independent cultures); within each panel, means without a common letter are significantly different ($P<0.05$).

Figure 6 Effect of BMP-6 (50 ng/ml) with or without FSH/LH (100 ng/ml) or 8-Br-cAMP (0.5 mM) on relative amounts of mRNA transcript encoding cytochrome P450_{SCC} in F1 (a), F2 (b) and F3/4 (c) granulosa cells. Values are means $\pm$ S.E.M. ($n=3$ independent cultures; within each panel, means without a common letter are significantly different ($P<0.05$)).

24-fold in F2; 19-fold in F3/4). Treatment with 8-Br-cAMP alone greatly enhanced P450_{SCC} mRNA expression in the F1 (241-fold), F2 (53-fold) and F3/4 (48-fold) cells (Fig. 6).

In cells co-treated with BMP-6 and FSH, P450_{SCC} mRNA showed a further increase over that induced by BMP-6 alone (F1 2.8-fold; F2 7.4-fold; F3/4 11.8-fold). Values were similarly raised in cells co-treated with BMP-6 and LH (F1 6.1-fold; F2 9.8-fold; F3/4 14.1-fold). In both cases, the enhancement was greatest in F3/4 follicles. Co-treatment with BMP-6 and 8-Br-cAMP did

not raise P450_{SCC} transcript abundance over that in cells given 8-Br-cAMP alone (Fig. 6).

Effects of BMP-6 alone and in combination with LH, FSH or 8-Br-cAMP on steady-state levels of mRNAs encoding the inhibin/activin subunits

Effects of treatments on granulosa cell expression of mRNAs encoding the inhibin/activin α -, β_A - and β_B -subunits are presented in Fig. 7. BMP-6 alone greatly increased amounts of the inhibin α -subunit transcript in

Figure 7 Effect of BMP-6 (50 ng/ml) with or without FSH/LH (100 ng/ml) or 8-Br-cAMP (0.5 mM) on relative amounts of mRNA transcript encoding the inhibin/activin α (a-c; log scale), β_A (d-f) and β_B (g-i) subunits in the F1, F2 and F3/4 granulosa cells. Values are means $\pm$ S.E.M. ($n=3$ independent cultures; within each panel, means without a common letter are significantly different ($P<0.05$)).

the F1 (10.5-fold), F2 (8.5 fold) and F3/4 cells (5.5-fold). To a lesser extent, BMP-6 treatment raised β_A -subunit mRNA in the F1 (3-fold), F2 (2.5-fold) and F3/4 (2.5-fold) follicles. In contrast, BMP-6 reduced β_B -subunit mRNA amount in F1 cells (approximately threefold; $P < 0.05$) and F2 cells (approximately twofold; NS), while enhancing β_B expression in F3/4 cells (twofold; $P < 0.05$). Addition of FSH alone raised inhibin α -subunit mRNA amount in the F2 (5-fold) and F3/4 (3.5-fold) cells but not in F1 cells. In contrast, FSH increased the amount of the β_A -subunit transcript in F1 cells (sixfold) without affecting β_A in F2 or F3/4 cells. FSH alone reduced β_B -subunit mRNA abundance in F1 and F2 cells ($P < 0.05$) but not in F3/4 cells. Treatment with LH alone greatly enhanced the amount of inhibin α -subunit mRNA transcript in F1 (13.5-fold) F2 (22-fold) and F3/4 cells (30-fold), but did not affect β_A or β_B expression. Treatment with 8-Br-cAMP alone greatly increased the amount of inhibin α -subunit transcript with a similar response (~ 75 -fold) in all the three cell populations. Like FSH, 8-Br-cAMP only stimulated β_A expression in F1 cells (fivefold) and had little or no effect on β_B transcript in any cell population.

BMP-6 co-treatment up-regulated ($P < 0.001$) FSH-induced inhibin α - (up to 65-fold), β_A - (up to 10-fold) and β_B - (up to 40-fold) subunit mRNA expression in all cell populations but with F1 cells showing a diminished response relative to F3/4 cells; BMP-6 co-treatment did not raise the amount of β_A transcript in F1 cells above that induced by FSH alone.

BMP-6 also enhanced ($P < 0.001$) LH-induced inhibin α mRNA expression in F1 (12-fold), F2 (8-fold) and F3/4 cells (7-fold). In contrast to its effect on FSH-induced β_A mRNA, BMP-6 did not influence LH-induced β_A mRNA level. However, BMP-6 greatly amplified LH-induced β_B -subunit expression in the F1 (17-fold), F2 (18-fold) and F3/4 cells (20-fold) cells. As with FSH and LH, there was a marked synergistic effect of 8-Br-cAMP and BMP-6 co-treatment on β_B -subunit transcript abundance, the response being greatest in F1 cells (23-fold enhancement).

Discussion

The present study provides novel information on the effects of BMP-6 on basal and gonadotrophin-induced hormone secretion (inhibin-A, inhibin-B and progesterone), steady-state mRNA expression (LH/FSH receptors, P450_{sc}, inhibin/activin subunits) and cell proliferation/survival by chicken granulosa cells. As such, these findings constitute direct evidence to support the existence of a functional BMP system in the avian ovary that has a major influence on granulosa cell function.

Our initial RT-PCR survey of BMP ligand/receptor mRNA expression in cultured granulosa and theca cells from prehierarchical and preovulatory follicles indicated

that cells of preovulatory F1 follicles show abundant mRNA expression for all three BMPR forms (BMPR-IA, -IB, -II), consistent with the finding of [Onagbesan et al. \(2003\)](#), who examined mRNA expression in freshly dissected granulosa and theca layers from F1–F3 preovulatory chicken follicles. Theca cells from prehierarchical 6–8 mm follicles also expressed the three BMPRs but, in contrast, we found only weak expression of two of these receptors (BMPR-IA and -II) in granulosa cells from prehierarchical follicles. This supports the notion that hen granulosa cells acquire increased responsiveness to BMP ligands as follicles progress to the preovulatory stage.

With regard to potential, locally produced BMP ligand(s) that might serve to regulate granulosa cell function in the avian follicle, we found that hen granulosa cells express several BMP ligands; BMP-2 transcript was detected in prehierarchical cells, while BMP-2 and -4 (plus very weak BMP-3 and -5) transcripts were detected in preovulatory cells. Similarly, expression of BMP-2, -4, -6 and -7 has been reported in freshly isolated chicken granulosa cells from F1–F3 preovulatory follicles ([Onagbesan et al. 2003](#)). Thus, an autocrine/paracrine action of granulosa cell-derived BMPs in preovulatory follicle function is a distinct possibility. In comparison with granulosa cells, however, chicken theca cells showed abundant expression of a more extensive range of BMP ligands at both prehierarchical (BMP-3, -4, -5, -6, -7 and -15) and preovulatory (BMP-2, -3, -4, -5, -6 and -7) stages. At both stages theca cells also expressed the three BMPR forms, compatible with an autocrine/paracrine role of BMPs in modulating theca cell function from the prehierarchical stage through to ovulation. This finding is in agreement with [Onagbesan et al. \(2003\)](#), who reported that chicken theca layers from F1 to F3 preovulatory follicles express several BMP ligands (BMP-2, -4, -6 and -7) as well as BMPR-IA, -IB and -II. To our knowledge, this potential action on theca cell has yet to be explored in the chicken but recent studies in cattle ([Glister et al. 2005](#)) and sheep ([Campbell et al. 2006](#)) have demonstrated BMP-induced suppression of both basal and LH-induced androgen secretion by theca cells.

Taken together with accumulating evidence supporting the existence of a functional BMP system in the mammalian ovary (for reviews, see [Shimasaki et al. 2003](#), [Juengel & McNatty 2005](#), [Knight & Glister 2006](#)) the above evidence of BMP ligand/receptor expression in avian follicles led to the principle hypothesis tested in this study that theca-derived BMPs exert a paracrine action on neighbouring granulosa cells to modulate hormone secretion and cell proliferation in a follicle stage-related manner. To test this hypothesis, we carried out functional *in vitro* studies on granulosa cells isolated from preovulatory (F1, F2 and F3/4) follicles. We decided to focus on BMP-6 here, as we found its mRNA to be expressed by chicken theca but not granulosa cells.

Moreover, BMP-6 (albeit human recombinant material) was readily available in a pure bioactive form and, to our knowledge, there have been no previous studies investigating the potential action(s) of this BMP in the avian ovary.

We first examined the effects of BMP-6 on basal and gonadotrophin-induced secretion of two granulosa cell-derived hormones that are produced in increasing amounts by chicken follicles as they progress through the preovulatory hierarchy, namely inhibin-A and progesterone (Etches & Duke 1984, Robinson & Etches 1986, Lovell *et al.* 1998). In the absence of gonadotrophin stimulation, BMP-6 promoted a marked dose-dependent increase in granulosa cell secretion of both inhibin-A (up to 75-fold) and progesterone (4-fold) with the inhibin-A response being greatest in F3/4 follicles and the progesterone response being greatest in F1 follicles. The magnitude of the BMP-6-induced rise in inhibin-A secretion was substantially greater than that elicited by either FSH or LH alone suggesting an important physiological role for this potential theca-derived protein. BMP-6 was also found to synergise with LH and FSH, raising inhibin-A and progesterone secretion by up to 100-fold over basal values. Previous studies have shown that other TGF β superfamily members, namely activin-A (Lovell *et al.* 2002b) and BMP-4 and -7 (Onagbesan *et al.* 2003), can enhance basal and gonadotrophin-dependent progesterone secretion and cell proliferation/survival by chicken granulosa cell from preovulatory follicles, but this is the first study to examine BMP-dependent inhibin secretion in birds.

In contrast, the stimulatory effect of BMP-6 on chicken granulosa cell hormone secretion and expression of mRNA for P450_{sc} and inhibin subunits reported here, studies on rat granulosa cells have shown that BMP-6 inhibits FSH-induced progesterone secretion, an action associated with a decrease in StAR and P450_{sc} mRNA expression (Otsuka *et al.* 2001). Inhibitory effects of BMP-6 on basal and/or FSH-dependent progesterone secretion by granulosa cells have been reported for other mammals including cattle (Glister *et al.* 2004), sheep (Pierre *et al.* 2005) and pigs (Brankin *et al.* 2005).

As the receptor-mediated actions of both FSH and LH are transduced, at least in part, by a cAMP/PKA-dependent signalling pathway (Ascoli *et al.* 2002, Conti 2002), we also compared the effects of BMP-6 on granulosa cell responses induced by the membrane-permeable cAMP analogue, 8-Br-cAMP. This experiment showed that, in contrast to its synergistic action on gonadotrophin-induced hormone secretion, BMP-6 did not further enhance 8-Br-cAMP-induced inhibin-A or progesterone secretion. This observation lends support to the view that BMP-6 potentiates LH/FSH action by interacting with their signalling pathways at a level upstream of cAMP generation, perhaps involving

up-regulation of LH and/or FSH receptor mRNA expression.

To test this, we used real-time Q-PCR (TaqMan) assays to quantify steady-state levels of mRNAs encoding FSH and LH receptors in F1, F2 and F3/4 granulosa cells exposed to the same treatment combinations. Treatment of cells with BMP-6 alone did indeed up-regulate expression of FSH receptor mRNA up to fivefold (greatest increase in F3/4 cells) supporting this hypothesis. However, BMP-6 alone only promoted a small (twofold) increase in LH receptor transcript level, a response that was confined to F1 cells and thus unlikely to account for the synergistic action of BMP-6 and LH/FSH on progesterone secretion that was also observed in F2 and F3/4 granulosa cells. Examination of gonadotrophin receptor mRNA in cells co-treated with BMP-6 and gonadotrophin largely resolved this inconsistency. We found that LH receptor transcript level in all three cell populations was augmented (up to sixfold) by co-treatment with BMP-6 and either FSH or LH. In contrast, co-treatment with BMP-6 and gonadotrophin had little or no effect on FSH receptor expression above that seen in cells treated with BMP-6 alone. In rat granulosa cells, BMP-6 alone had no effect on FSH or LH receptor mRNA expression, but suppressed FSH-induced and forskolin-induced expression of both transcripts (Otsuka *et al.* 2001). Again, this differs markedly from the present finding in the chicken that BMP-6 enhances basal and gonadotrophin-dependent mRNA expression for both FSH and LH receptors.

Thus, through an intracellular mechanism(s) that has yet to be defined, BMP-6 (presumably acting via type I and type II BMPRs on the cell surface) can up-regulate expression of both FSH and LH receptors. Furthermore, it appears that BMP-6 can up-regulate FSH receptor mRNA expression in the absence of gonadotrophin, while BMP-6 dependent up-regulation of LH receptor mRNA requires co-stimulation with gonadotrophin (at least in F2 and F3/4 follicles). The possibility cannot be excluded that the observed increases in steady-state mRNA transcript levels reflect a BMP-6-induced increase in mRNA stability, rather than an up-regulation of gene transcription *per se*. Either way, it seems highly likely from the functional end points monitored here (i.e. gonadotrophin-induced inhibin-A and progesterone secretion) that the increased gonadotrophin receptor mRNA transcript levels are indeed associated with increased expression of functional LH and FSH receptor proteins on the granulosa cell surface. Further studies are needed to elucidate the intracellular signalling mechanisms by which the Smad pathway activated by BMP-6 interacts with the gonadotrophin-induced signalling pathway(s) to alter granulosa cell gonadotrophin receptor mRNA expression and/or stability.

Of course, the observed synergism between BMP-6 and gonadotrophin could be interpreted as a gonadotrophin-dependent up-regulation of BMP-signalling

rather than *vice versa*. Indeed, a recent report (Miyoshi *et al.* 2006) that FSH can enhance BMP-induced Smad 1/5/8 phosphorylation in a human ovarian granulosa-like tumour cell line supports this concept. It should be pointed out that different BMPs can also bind to and form signalling complexes with certain activin receptor subtypes (for reviews, see Knight & Glister 2003, Miyazono *et al.* 2005) and in a recent study (Lovell *et al.* in press), we showed that both LH and FSH augmented ActR-I and ActR-IIB mRNA levels in chicken granulosa cells from F1 follicles; further studies are warranted to investigate whether gonadotrophins modulate granulosa cell expression of BMPR subtypes and their associated downstream signal transduction components.

To gain further insights into the mechanism by which BMP-6 enhanced inhibin-A protein secretion, mRNA transcripts encoding the inhibin/activin subunits (α , β_A and β_B) were also quantified. Consistent with the BMP-6-induced increase in granulosa cell secretion of inhibin-A (α - β_A dimer), treatment with BMP-6 alone was associated with a marked increase in inhibin α - and β_A -subunit mRNA abundance but did not affect β_B -subunit mRNA. Moreover, co-treatment with BMP-6 and either FSH, LH or cAMP analogue further enhanced α subunit mRNA expression to a similar extent in cells from F1, F2 and F3/4 follicles. However, there was an interesting difference between the β_A responses to the different BMP-6 co-treatments: a further rise in β_A mRNA level was observed with FSH co-treatment but not with LH or cAMP analogue co-treatment, despite the fact that all three co-treatments raised inhibin-A protein secretion. This casts some doubt on the generally held view that the level of β_A -subunit mRNA is rate-limiting for inhibin-A synthesis (Ying 1988, Meunier *et al.* 1988, Chen & Johnson 1996, 1997, Fu *et al.* 2001) and suggests that other posttranslational events may be limiting, at least in some circumstances.

Perhaps the most striking and unexpected finding to emerge from the Q-PCR analysis of inhibin/activin subunit expression was the dramatic increase in the amount of β_B mRNA transcript observed in granulosa cells co-treated with BMP-6 and either FSH, LH or cAMP analogue. No such increase was observed in cells given either BMP-6 alone or gonadotrophin/cAMP analogue alone indicating a marked synergism between BMP-6 and gonadotrophin-dependent/cAMP-dependent signalling pathways with respect to β_B expression. It is also notable that the magnitude of the β_B response to FSH or LH co-treatment was greatest in F3/4 cells and lowest in F1 cells. Using the present inhibin-B assay, we observed a modest increase in inhibin-B protein secretion by cells treated with BMP-6 alone or BMP-6 with either FSH or LH. Due to the relatively poor assay sensitivity, we cannot assign fold increases to these responses as basal levels were below our detection limit. Nonetheless,

secretion was greatest in the F3/4 granulosa cells co-treated with BMP-6 and LH.

Interestingly, treatment of human granulosa lutein cells with activin-A (Eramaa *et al.* 1995) and BMP-2 or -6 (Jaatinen *et al.* 2002) was reported to selectively enhance β_B -subunit expression and inhibin-B protein secretion without affecting α or β_A -subunit expression. Furthermore treatment with human chorionic gonadotrophin alone had no effect on β_B expression but suppressed the activin or BMP-induced induction of β_B -subunit expression and inhibin-B secretion. We cannot rule out the possibility that increased expression of β_B -subunit mRNA leads to an increased synthesis of activin B (β_B - β_B dimer) or activin AB (β_A - β_B dimer). While we do not have immunoassays for chicken activin B or AB, a recent immunoblotting study on chicken granulosa cell-conditioned culture medium was unable to detect secretion of any activin isoform (Johnson *et al.* 2005). Previous attempts to measure activin-A secretion by cultured hen granulosa cells in this laboratory have also been unsuccessful (Lovell *et al.* 2002b) despite the presence of low, but detectable amounts of activin-A in granulosa cell extracts from hierarchical follicles (Lovell *et al.* 1998, 2003).

In conclusion these findings constitute direct evidence that intra-ovarian BMPs of thecal origin have a key paracrine role in modulating granulosa cell function in a preovulatory follicle stage-dependent manner in the avian ovary. We speculate that developmental changes in intra-follicular BMP signalling might account for differential expression of gonadotrophin receptors, steroidogenic enzymes and inhibin subunits leading to the dramatic shift in the pattern of steroid output and inhibin-A/inhibin-B production (Lovell *et al.* 2003, Johnson *et al.* 2005) that accompanies follicle development.

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