

Changes in expression of bone morphogenetic proteins (BMPs), their receptors and inhibin co-receptor betaglycan during bovine antral follicle development: inhibin can antagonize the suppressive effect of BMPs on thecal androgen production

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Abstract

We reported previously that bone morphogenetic proteins (BMPs) potently suppress CYP17 expression and androgen production by bovine theca interna cells (TC) *in vitro*. In this study, real-time PCR was used to analyse gene expression in TC and granulosa cell (GC) layers from developing bovine antral follicles (1–18 mm). Abundance of mRNA transcripts for four BMPs (*BMP2*, *BMP4*, *BMP6*, and *BMP7*) and associated type I (*BMPRI1A*, *BMPRI1B*, *ACVR1* and *ACVR1B*) and type II (*BMPRII2*, *ACVR2A* and *ACVR2B*) receptors showed relatively modest, though significant, changes during follicle development. *BMP2* was selectively expressed in GC, while *BMP6*, *BMP7* and betaglycan (*TGFBR3*) were more abundant in TC. Abundance of betaglycan mRNA (inhibin co-receptor) in TC increased progressively (fivefold; $P < 0.001$) as follicles grew from 1–2 to 9–10 mm. This suggests a shift in thecal responsiveness to GC-derived inhibin, produced in increasing amounts as follicles achieve dominance. This prompted us to investigate whether inhibin can function as a physiological antagonist of BMP action on bovine TC *in vitro*, in a manner comparable to that for activin signalling. *BMP4*, *BMP6* and *BMP7* abolished LH-induced androstenedione secretion and suppressed CYP17 mRNA >200-fold ($P < 0.001$), while co-treatment with inhibin-A reversed the suppressive action of BMP in each case ($P < 0.001$). Results support a physiological role for granulosa-derived inhibin as an antagonist of BMP action on thecal androgen synthesis. A shift in intrafollicular balance between thecal BMP signalling (inhibitory for androgen synthesis) and betaglycan-dependent inhibin signalling (stimulatory for androgen synthesis) accords with the physiological requirement to deliver an adequate supply of aromatase substrate to GC of developing follicles.

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Introduction

The mechanisms controlling the recruitment, selection, dominance, ovulation and/or regression of follicles in the mammalian ovary have yet to be fully delineated. A multitude of intraovarian factors engage in auto-crine/paracrine signalling between theca interna cells (TC), granulosa cells (GC) and oocytes, and contribute to a coordinated programme of follicle cell proliferation and differentiation. Particularly in the later stages of follicle development, intraovarian factors also modulate the sensitivity of follicular cells to gonadotrophins and other extraovarian factors (Mihm *et al.* 2002, Webb *et al.* 2003). Among this growing list of locally produced regulatory factors are various members of the transforming growth factor- β (TGFB) superfamily, including inhibins, activins and bone morphogenetic proteins (BMPs; reviews: Ying 1988, Knight & Glister 2001, 2006, Welt *et al.* 2002, Shimasaki *et al.* 2004).

Numerous studies have documented the spatio-temporal patterns of mRNA and/or protein expression

for inhibin/activin α/β subunits (*INH1A*, *INH1B* and *INH2B*) during follicle development in several species (reviews: De Jong 1988, Ying 1988, Knight & Glister 2001). To a lesser extent, follicular expression patterns of several BMPs have also been reported (Erickson & Shimasaki 2003, Glister *et al.* 2004, Shimasaki *et al.* 2004, Fatehi *et al.* 2005, Juengel *et al.* 2006). In addition, functional studies have provided clear evidence that inhibins, activins and BMPs exert local intraovarian roles. For instance, activin and several BMPs can suppress androgen production by cultured TC (Hillier *et al.* 1991, Dooley *et al.* 2000, Glister *et al.* 2005), while inhibin can enhance LH-induced androgen secretion (Hsueh *et al.* 1987, Hillier *et al.* 1991, Wrathall & Knight 1995). Activins and BMPs may promote GC proliferation, upregulate FSH receptor expression and enhance oestradiol (E_2) production while inhibiting progesterone (P_4) production (Hasegawa *et al.* 1988, Xiao *et al.* 1992, Findlay & Drummond 1999, Glister *et al.* 2004, Juengel *et al.* 2006).

In common with other TGF β family ligands, BMP signalling involves ligand-induced formation of a heteromeric complex with two types of serine/threonine kinase receptors referred to as type I and type II (Massague & Chen 2000, Miyazono *et al.* 2000, Miyazawa *et al.* 2002). In a combinatorial manner, different BMP ligands bind to one of four different type I receptors such as ACVR1 (ALK2), BMPR1A (ALK3), ACVR1B (ALK4) and BMPR1B (ALK6), before recruiting one of three type II receptors (BMPR2, ACVR2A and ACVR2B) to form an active signalling complex. With regard to type I receptors, evidence suggests that BMP2 and BMP4 preferentially bind BMPR1A and/or BMPR1B, while BMP6 and BMP7 tend to bind ACVR1 and/or BMPR1B (ten Dijke *et al.* 1994, Ebisawa *et al.* 1999, Aoki *et al.* 2001). With regard to type II receptors, BMP6, BMP7 and activin preferentially recruit ACVR2A, while BMP2, BMP4, BMP6, BMP7 and BMP15, but not activin, recruit BMPR2 (Liu *et al.* 1995, Nohno *et al.* 1995, Yamashita *et al.* 1995, Ebisawa *et al.* 1999, Moore *et al.* 2003). Signalling complex formation leads to the type II receptor transphosphorylating (activating) the intracellular kinase domain of the type I receptor, which, in turn, phosphorylates BMP-responsive transcription factors SMADs 1/5/8. These associate with a common partner SMAD (SMAD4) and translocate to the nucleus to modify target gene expression (Miyazono *et al.* 2005, 2010).

Inhibins antagonize the biological effects of activins by binding to activin type II receptors thus inhibiting the formation of activin-induced signalling complexes and subsequent activation of activin-responsive SMADs 2/3 (Harrison *et al.* 2004). For effective presentation of inhibin to type II activin receptors' co-expression of another cell surface molecule, betaglycan (also known as TGFBR3) is essential. Thus, betaglycan functions as an important co-receptor for inhibin as well as TGF β , greatly enhancing the efficacy of both ligands. Betaglycan is abundantly expressed on the surface of many cell types, and a soluble form may also be generated by proteolytic cleavage of the membrane-bound receptor (Cook *et al.* 2004).

In addition to its classical role as an activin antagonist, inhibin may also function as a competitive antagonist of BMPs through the same betaglycan-dependent mechanism, involving competition between inhibin and BMPs for type II receptor(s) utilized by BMPs (Wiater & Vale 2003, Farnworth *et al.* 2006). Thus, betaglycan and inhibin have emerged as potentially important players in the regulation of BMP signalling, although the extent to which this may apply in the ovarian follicle has not been addressed.

Immunocytochemical studies on bovine ovarian cells in primary culture have documented the expression of multiple BMP ligands and BMP-responsive type I and type II receptors (Glister *et al.* 2004). However, information is lacking on the extent to which cellular expression of different BMP ligands and their receptors

varies during bovine follicle development. With the above points in mind, the initial objective of this study was to use real-time PCR for a detailed analysis of *ex vivo* gene expression profiles for a range of BMPs, inhibin/activin subunits, their type I and type II signalling receptors and the inhibin co-receptor betaglycan in GC and TC layers isolated from developing bovine antral follicles. The changes we observed in betaglycan expression in TC, together with the aforementioned reports from studies using immortalized testicular, adrenocortical and gonadotroph cell lines (Wiater & Vale 2003, Farnworth *et al.* 2006), prompted us to use our bovine TC culture model to test the hypothesis that inhibin antagonizes the suppressive effect of BMPs on follicular androgen production.

Results

Pre-screening of follicle samples included in the analysis

Overall, 24/118 (20%) of samples (from pooled or individual follicles) prepared for gene expression analysis were rejected and excluded from the study because they did not meet our quality control criterion of <5% GC/TC cross-contamination as evidenced by relative expression levels of cell-specific 'marker' transcripts (TC marker: CYP17; GC markers: FSHR and CYP19). Following this screening process, respective *n* values were 4, 5 and 5 for 1–2, 3–4 and 5–6 mm follicle pools and 8, 7, 9 and 9 for individual follicles in the 7–8 mm, 9–10 mm, large (11–18 mm) oestrogen-active (LEA) and large (11–18 mm) oestrogen-inactive (LEI) categories (see Materials and Methods section for further details).

Follicular fluid steroid concentrations in developing antral follicles

Figure 1 shows the follicular fluid concentrations (ng/ml protein) of P₄, androstenedione (A₄) and E₂ in the antral follicle set analysed. A progressive increase (*P*<0.001) in E₂ concentration accompanied follicle development to the LEA stage, and this was associated with a concomitant decline in A₄ (*P*<0.001), reflecting increased aromatization by follicular GC. In comparison with LEA follicles, LEI follicles had approximately eightfold lower E₂ (*P*<0.01), approximately fourfold higher P₄ (*P*<0.05) and approximately twofold higher A₄ (*P*>0.05; NS) concentrations.

mRNA expression profiles for gonadotrophin receptors, key steroidogenic enzymes and INHBA subunit in developing antral follicles

The observed patterns of mRNA expression for *FSHR*, *LHCGR*, *CYP19*, *CYP17* and *INHBA* in GC and TC of developing antral follicles accorded with expectations (Fig. 2). *FSHR* mRNA was abundant in GC throughout

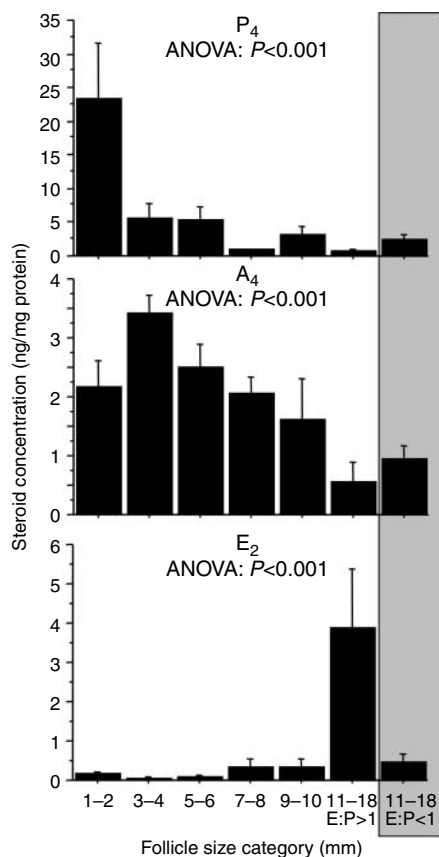


Figure 1 Concentrations (ng/mg protein) of progesterone (P₄), androstenedione (A₄) and oestradiol (E₂) in follicular fluid samples from developing bovine antral follicles. Follicles in the 11–18 mm size class have been subdivided on the basis of oestrogen to progesterone ratio (E:P ratio) as ‘oestrogen-active’ (E:P ratio > 1) or ‘oestrogen-inactive’ (E:P ratio < 1). Values are means, and bars indicate s.e.m. (n=4–9). P values from ANOVA are indicated.

antral follicle development, while GC of follicles > 9 mm also expressed *LHCGR*. Moreover, LEA follicles had much higher levels of *LHCGR*, *FSHR* and *INHBA* mRNA than LEI follicles ($P < 0.001$). *LHCGR* expression was abundant in TC at all stages examined, but transcript levels were lowest in LEA follicles. Expression of *CYP17* mRNA was specific for TC, being abundant at all stages, while *CYP19* and *INHBA* mRNAs were largely confined to GC of large follicles, being approximately sixfold greater in LEA follicles than in LEI follicles ($P < 0.001$).

Expression profiles for BMP2, BMP4, BMP6 and BMP7 mRNAs in developing antral follicles

Developmental changes in the relative abundance of mRNA transcripts for *BMP2*, *BMP4*, *BMP6* and *BMP7* in antral follicles are shown in Fig. 3. Overall, *BMP2* mRNA was selectively expressed in GC ($P < 0.0001$), while *BMP4*, *BMP6* and *BMP7* transcripts were more abundant in TC than in GC ($P < 0.0001$). In GC, *BMP2* mRNA

abundance was much lower in LEA follicles than in other follicle categories ($P < 0.05$ compared with 3–4, 5–6 and 7–8 mm follicles; NS compared with 1–2 mm, 9–10 mm and LEI follicles), while in TC, *BMP2* mRNA was maximal in LEA follicles ($P < 0.05$ compared with 1–2 mm, 2–3 mm, 5–6 mm, 7–8 mm and LEI follicles). In GC, *BMP4* mRNA level was highest in large (11–20 mm) follicles regardless of E:P ratio; in TC, *BMP4* mRNA level was approximately twofold higher in larger (7–18 mm) follicles than in 1–6 mm follicles ($P < 0.05$). The relative abundance of *BMP6* mRNA in GC or in TC did not vary across follicle development. Likewise, *BMP7* transcript abundance in TC was uniform. However, *BMP7* expression in GC was lowest in 7–8 mm follicles ($P < 0.05$ compared with 1–2 and 11–18 mm follicles).

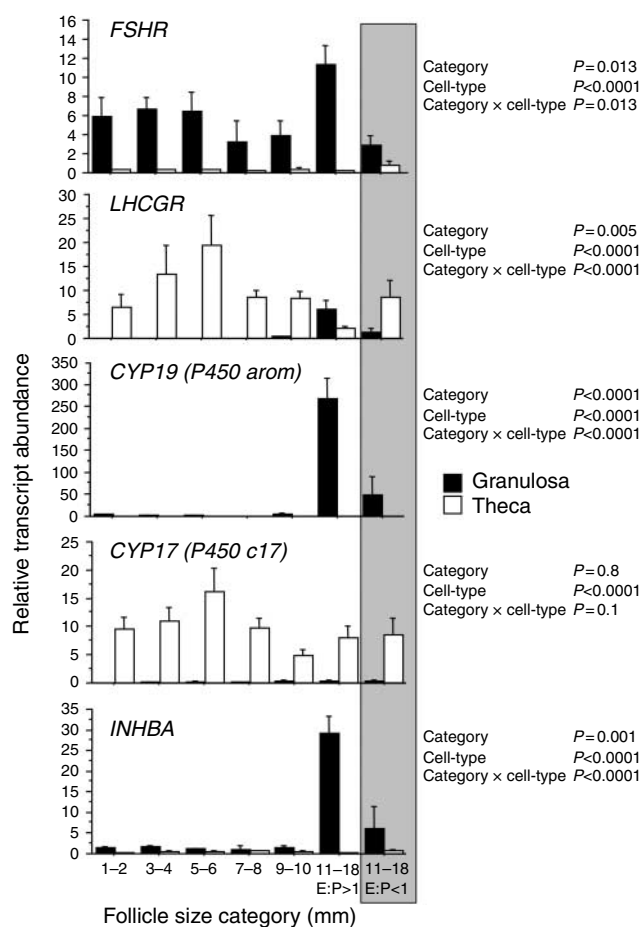


Figure 2 Changes in relative abundance of mRNA transcripts for gonadotrophin receptors (*FSHR* and *LHCGR*), steroidogenic enzymes (*CYP17* and *CYP19*) and inhibin/activin βA subunit (*INHBA*) in thecal and granulosa compartments of developing bovine antral follicles. Follicles in the 11–18 mm size class have been subdivided on the basis of oestrogen to progesterone ratio (E:P ratio) as ‘oestrogen-active’ (E:P ratio > 1) or ‘oestrogen-inactive’ (E:P ratio < 1). Values are means, and bars indicate s.e.m. (n=4–9). Results of two-way ANOVA are summarized.

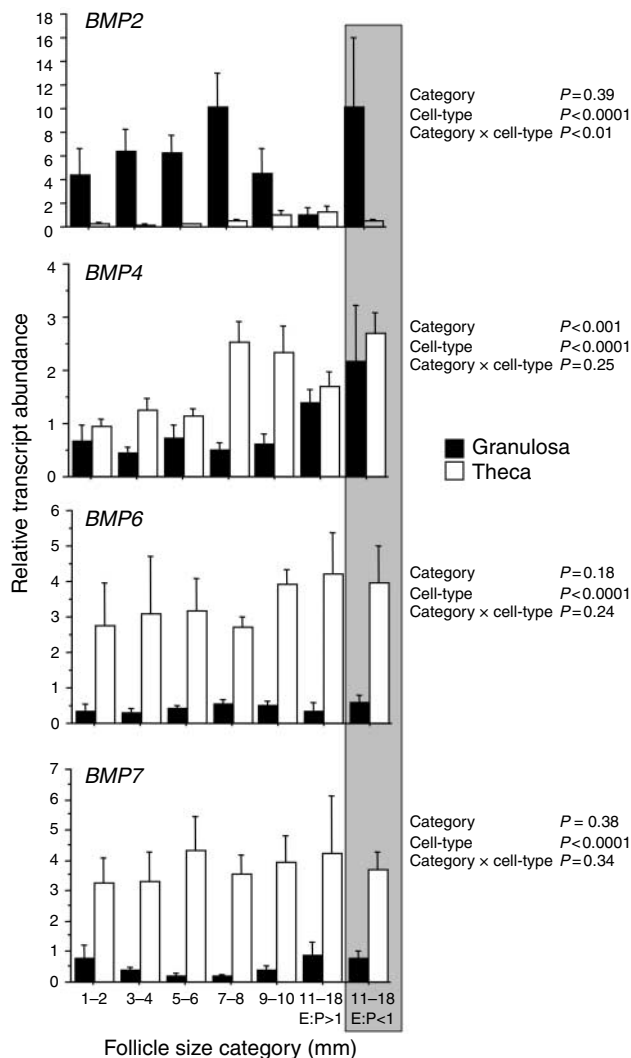


Figure 3 Changes in relative abundance of mRNA transcripts for *BMP2*, *BMP4*, *BMP6* and *BMP7* in thecal and granulosa compartments of developing bovine antral follicles. Follicles in the 11–18 mm size class have been subdivided on the basis of oestrogen to progesterone ratio (E:P ratio) as ‘oestrogen-active’ (E:P ratio > 1) or ‘oestrogen-inactive’ (E:P ratio < 1). Values are mean, and bars indicate s.e.m. ($n=4-9$). Results of two-way ANOVA are summarized.

Expression profiles for BMP receptor mRNAs in developing antral follicles

Overall, the abundance of transcripts for the four type I (*BMPR1A*, *BMPR1B*, *ACVR1* and *ACVR1B*) and three type II (*BMPR2*, *ACVR2A* and *ACVR2B*) signalling receptors examined showed relatively little variation during follicle development (Fig. 4), and all seven receptors were expressed by both cell types at all follicle stages examined. However, with respect to type I receptors, *BMPR1A* mRNA level in GC was highest in LEA follicles, while *BMPR1A* mRNA level in TC was lowest in LEA follicles ($P<0.05$). *BMPR1B* expression in GC increased with follicle size

($P<0.05$; 1–2 vs 9–10 mm follicles), whereas expression in TC decreased with follicle size ($P<0.05$; 1–2 vs 11–18 mm follicles). *ACVR1* expression in GC also increased with follicle size ($P<0.05$; 1–2 vs 11–18 mm follicles), while expression in TC fell in LEA follicles ($P<0.05$ compared with all follicles <10 mm). There were no significant differences in *ACVR1B* expression among different follicle categories. With respect to type II receptors, *BMPR2* mRNA level in GC increased progressively from 1–2 to 11–18 mm follicle categories ($P<0.01$) but did not differ between LEA and LEI follicles. Conversely, *BMPR2* mRNA level in TC fell from 5–6 mm to LEA follicles ($P<0.05$) and was lower in LEA follicles than in LEI follicles ($P<0.05$). GC abundance of *ACVR2A* mRNA increased from 1–2 to 9–10 mm follicles (approximately twofold; $P<0.05$) before falling again in LEA follicles (~50%; $P<0.05$) but not LEI follicles. Expression of *ACVR2B* mRNA did not vary significantly across follicle development for either cell type.

mRNA expression profile for betaglycan (*TGFBR3*) in developing follicles

As shown in Fig. 4, betaglycan mRNA was much more abundant in TC than in GC at all follicle stages examined ($P<0.0001$). In both cell types, betaglycan mRNA level increased progressively (approximately fivefold; $P<0.001$) as follicles grow from 1–2 to 9–10 mm. Levels in TC then fell ($P<0.05$) by about 50% in 11–18 mm follicles, whereas levels in GC remained high. There was no significant difference in betaglycan transcript abundance between LEA and LEI follicles.

Immunostaining of BMP ligands, receptors and betaglycan in ovary sections

To reinforce the above mRNA expression data, bovine ovary sections were immunostained with antibodies against each of the BMP ligands and receptors analysed by real-time PCR. Figure 5 shows the distribution of immunoreactive BMP ligands, BMP/activin receptors and betaglycan in the granulosa and theca layers of small antral follicles (~3–5 mm diameter). *BMP2* and *BMP4* immunoreactivity appeared more abundant in the GC layer, while *BMP6* and *BMP7* immunoreactivity was evident in both GC and TC layers. Similarly, immunoreactivity for each of the seven signalling receptors and betaglycan was evident in both GC and TC layers.

Correlations among expression levels of different transcripts in GC of developing follicles

Figure 6 is a correlation matrix showing the pairwise relationships between follicle diameter, E₂:P₄ ratio and relative abundance of each transcript in GC.

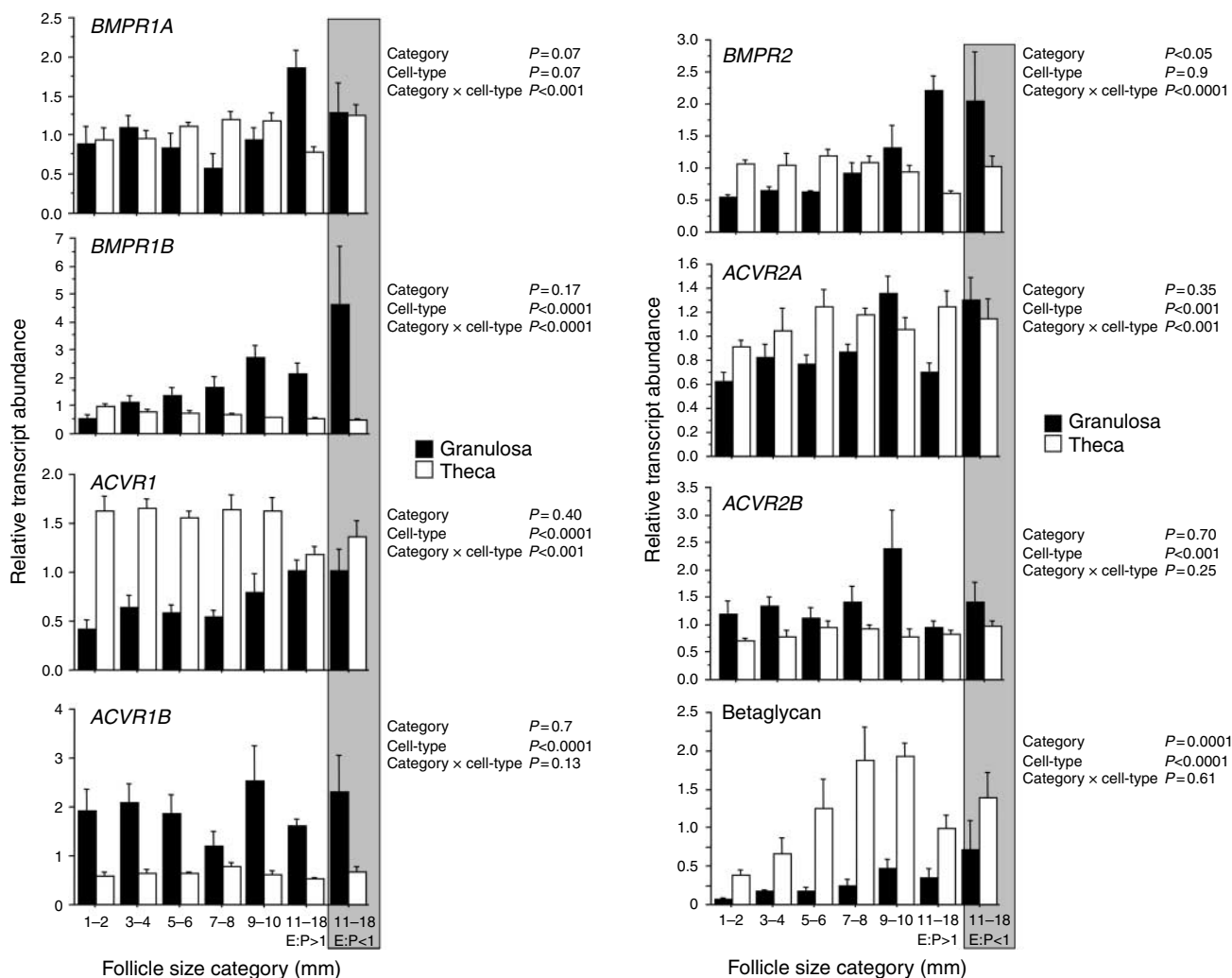


Figure 4 Changes in relative abundance of mRNA transcripts for BMP-responsive type I (*BMPR1A*, *BMPR1B*, *ACVR1* and *ACVR1B*) and type II (*BMPR2*, *ACVR2A* and *ACVR2B*) signalling receptors and betaglycan in thecal and granulosa compartments of developing bovine antral follicles. Follicles in the 11–18 mm size class have been subdivided on the basis of oestrogen to progesterone ratio (E:P ratio) as ‘oestrogen-active’ (E:P ratio >1) or ‘oestrogen-inactive’ (E:P ratio <1). Values are means, and bars indicate s.e.m. ($n=4-9$). Results of two-way ANOVA are summarized.

Follicle diameter was positively correlated with $E_2:P_4$ ratio and with GC abundance of mRNAs for *CYP19*, *LHCGR*, *BMP4*, *INHA*, *INHBA*, *INHBB*, betaglycan (*TGFBR3*) and five of seven BMP/activin receptors. Of the BMP ligand transcripts examined, *BMP2* expression was positively associated with *BMP4* and *BMP6* expression but negatively associated with expression of several established markers of healthy dominant follicles (*CYP19*, *FSHR*, *INHA*, *INHBA* and *INHBB*) and with *BMPR1A*, *ACVR1B*, *BMPR2*, *ACVR2B* and betaglycan expression. There were many positive associations between expression levels of the seven BMP/activin receptors and betaglycan. Notably, there was a very high correlation between *BMPR1B* and betaglycan mRNA abundance in GC ($r=0.91$; $P<0.0001$).

Correlations among expression levels of different transcripts in TC of developing follicles

Figure 7 shows the corresponding pairwise relationships between follicle diameter, $E_2:P_4$ ratio and relative transcript abundance of 17 different transcripts in TC. There were far fewer significant correlations than in the corresponding GC samples. Nonetheless, follicle diameter was positively correlated with *BMP2* transcript abundance and negatively correlated with *LHCGR*, *BMPR1B*, *ACVR1*, *ACVR1B* and *BMPR2* transcript abundance. Follicular $E_2:P_4$ ratio was negatively correlated with *BMPR1A*, *ACVR1*, *ACVR1B* and *BMPR2* transcript abundance in TC. *LHCGR* mRNA was positively associated with *CYP17*, *BMP6*, *ACVR1* and *BMPR2* mRNA. Among the BMP ligands examined,

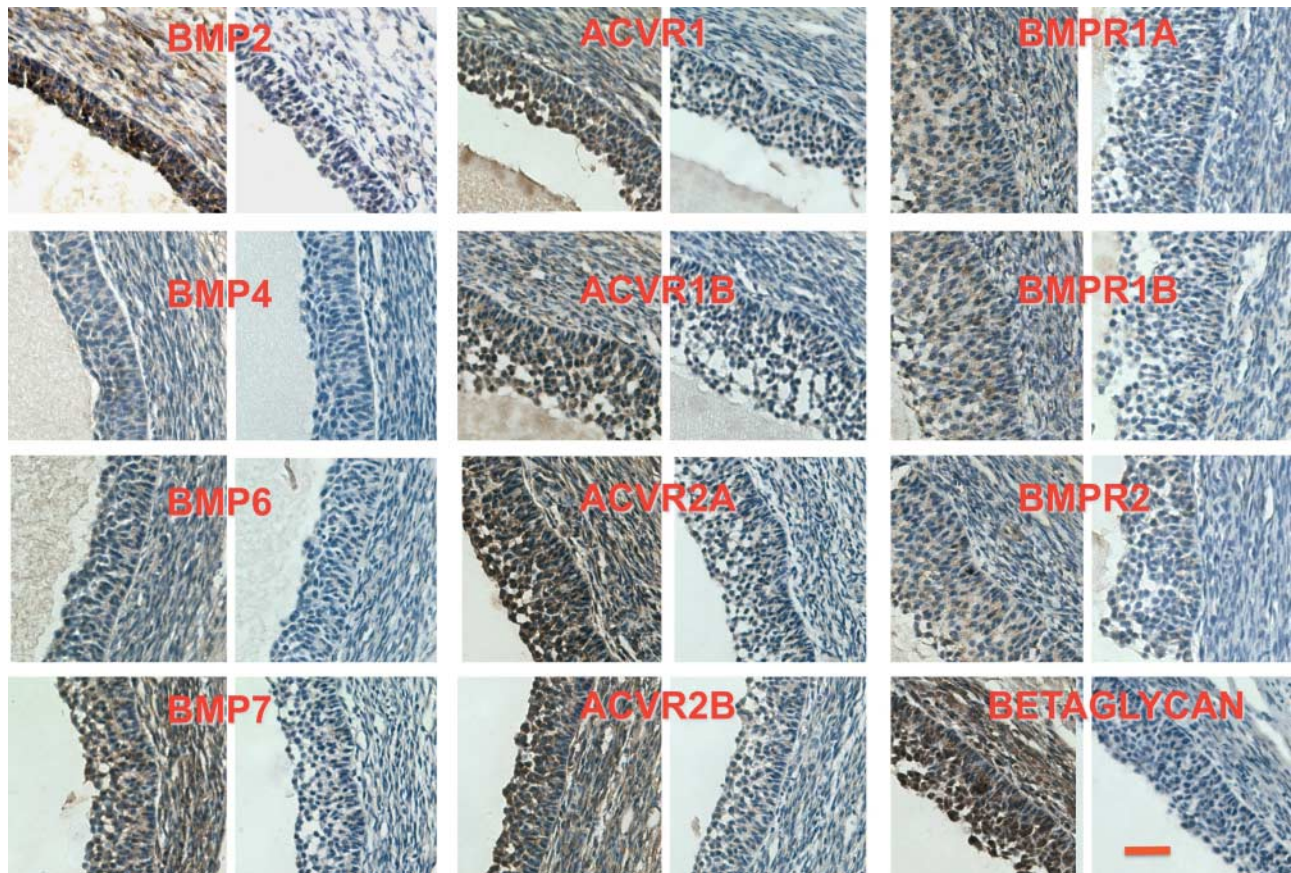


Figure 5 Immunohistochemical staining of bovine ovary sections showing the distribution of immunoreactive BMP ligands, BMP/activin receptors and betaglycan in the granulosa and theca layers of small antral follicles (~3–5 mm diameter). In each panel, the left hand image shows an antibody-treated section, while the right hand image shows an adjacent section treated with the appropriate non-immune serum or purified IgG. All images were captured at the same magnification, and the scale bar in the lower right panel represents 50 μ m.

there was a strong correlation between *BMP2* and *BMP4* mRNA abundance ($r=0.88$; $P<0.0001$). Among the seven signalling receptors examined, 10/28 pairwise comparisons yielded significant positive correlations. For instance, *BMPR1A* was positively correlated with *ACVR1*, *ACVR1B* and *BMPR2*, while *BMPR1B* was positively correlated with *ACVR1B* and *BMPR2* transcript abundance. Although betaglycan expression was not correlated with follicle diameter when the whole set of follicle samples was considered (see Fig. 7), a significant positive correlation was evident when 11–18 mm follicles were excluded from the analysis ($r=0.50$; $P<0.001$, $n=33$).

Can inhibin reverse the suppressive effect of BMP on thecal androgen production?

The selective expression of betaglycan (inhibin co-receptor) in TC combined with the progressive fivefold increase observed during follicle development from 1–2 to 9–10 mm prompted us to evaluate the possibility that inhibin functions as a physiological antagonist of BMP action on bovine TC, in a manner comparable to that

documented for activin signalling. As shown in Fig. 8, *BMP4*, *BMP6* and *BMP7* abolished LH-induced A_4 secretion ($P<0.01$) and suppressed *CYP17* transcript abundance >200-fold ($P<0.001$). Co-treatment with inhibin-A reversed the suppressive action of all three BMPs ($P<0.001$). BMPs also reduced, but to a much lesser degree (two- to ten-fold; $P<0.01$), the levels of three other TC transcripts examined: *STAR*, *CYP11A1* and *LHCGR* (Fig. 9). In each case, the suppressive effect of BMP was reversed by inhibin ($P<0.01$).

Which signalling receptor subtype(s) mediate the suppressive effect of BMPs on thecal androgen production?

In an attempt to identify at least some of the receptor subtype(s) which mediate the suppressive effect of *BMP4*, *BMP6* and *BMP7* on thecal androgen secretion, LH-treated TC were exposed to each BMP in the presence and absence of one of three soluble 'decoy' receptors (ectodomains (ECD) of *BMPR1A*, *BMPR1B* or *ACVR2B*). As shown in Fig. 10, the *BMPR1A* 'decoy' receptor selectively blocked the suppressive effect of

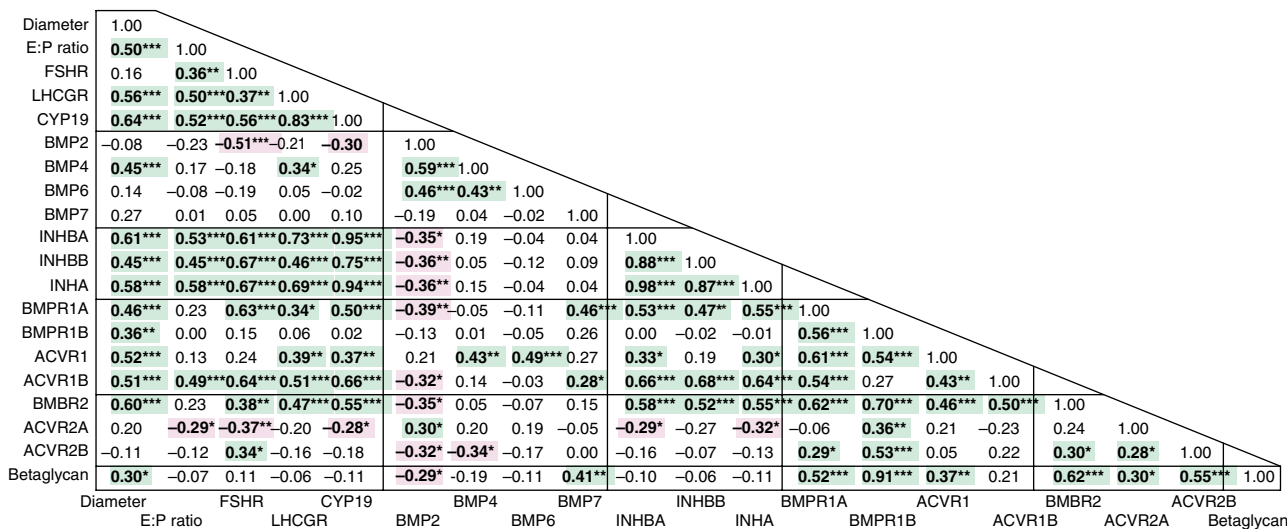


Figure 6 Correlation matrix showing the pairwise linear relationships between follicle diameter, follicular fluid E₂:P₄ ratio and relative abundance of the different GC transcripts quantified in *ex vivo* follicle samples (*n*=47). Statistically significant *r* values are in bold with green highlights indicating positive correlations and pink highlights indicating negative correlations. **P*<0.05, ***P*<0.01, ****P*<0.001.

BMP4 (*P*<0.01) without reversing the effect of BMP6 or BMP7. The BMPR1B ‘decoy’ receptor blocked the suppressive effect of both BMP4 and BMP7 (*P*<0.01) and partially reversed the effect of BMP6 (*P*<0.1). In contrast, the ACVR2B ‘decoy’ receptor did not affect the response to any of the three BMPs tested, suggesting a low affinity for these ligands.

Discussion

The present study provides novel information on the spatio-temporal mRNA expression profiles for four BMPs (BMP2, BMP4, BMP6 and BMP7), associated type I (BMPR1A, BMPR1B, ACVR1 and ACVR1B) and type II

(BMPR2, ACVR2A and ACVR2B) signalling receptors and the inhibin co-receptor betaglycan during bovine antral follicle development. The relative abundance of mRNA transcripts for each of these targets was quantified in GC and TC layers isolated from follicles ranging in diameter from 1 to 18 mm. This size range spans a number of key points in bovine follicle development including ‘cyclic recruitment’ of small antral follicles at ~3–5 mm, dominant follicle ‘selection’ and ‘deviation’ at ~6–8 mm, GC acquisition of LH receptors at ~9–10 mm and dominant oestrogen-active status at 11–18 mm (Ireland *et al.* 2000, Mihm *et al.* 2002, Ginther *et al.* 2003). As expected, follicles in the largest (11–18 mm) size category showed considerable

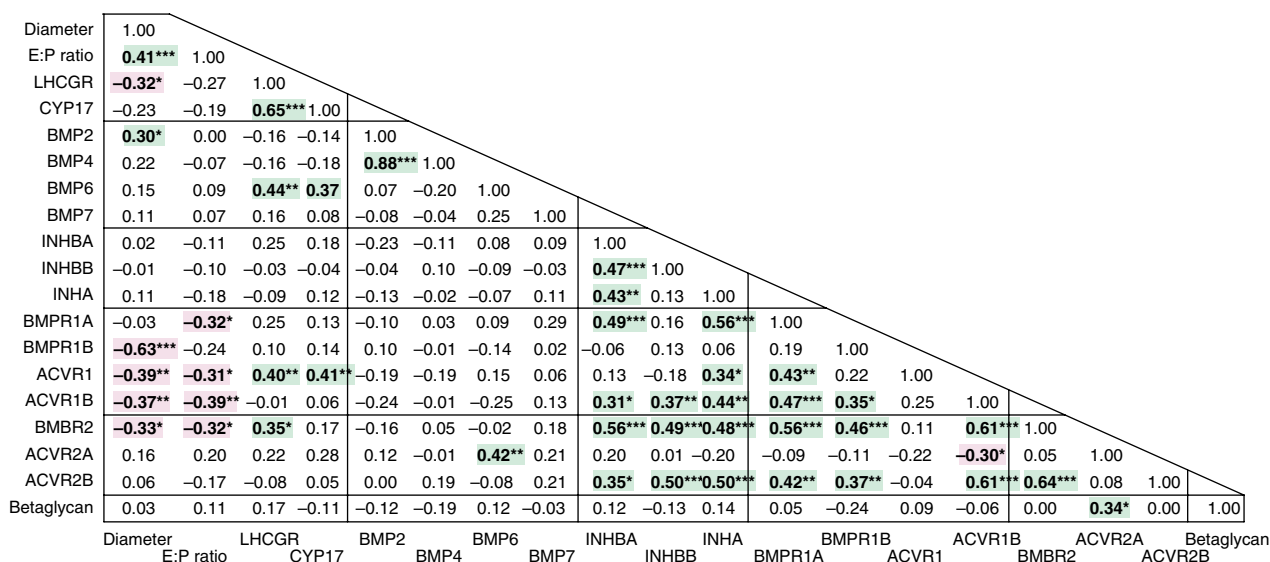


Figure 7 Correlation matrix showing the pairwise linear relationships between follicle diameter, follicular fluid E₂:P₄ ratio and relative abundance of the different TC transcripts quantified in *ex vivo* follicle samples (*n*=47). Statistically significant *r* values are in bold with green highlights indicating positive correlations and pink highlights indicating negative correlations. **P*<0.05, ***P*<0.01, ****P*<0.001.

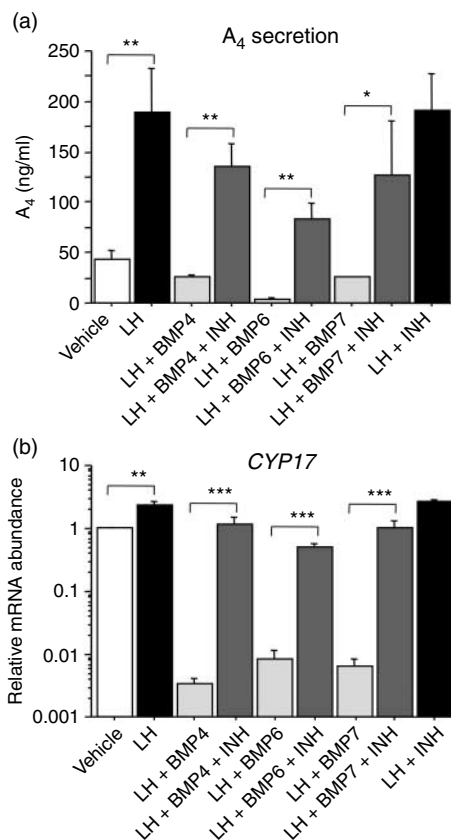


Figure 8 Effects of BMP4, BMP6 and BMP7, alone and in combination with inhibin, on (a) androstenedione secretion and (b) *CYP17* mRNA expression by bovine TC (note use of log scale on y-axis). Values are means, and bars indicate S.E.M. ($n=4$ independent cultures). * $P<0.05$ ** $P<0.01$ and *** $P<0.001$ versus corresponding value.

variation in follicular fluid E:P ratio, reflecting their differing 'health' status. For this reason, we subdivided these follicles into LEA (presumptive 'healthy') and LEI (presumptive 'regressing') groups on the basis of their E:P ratio, a widely used means of classifying bovine follicles (Ireland *et al.* 2000). No attempt was made to subdivide smaller follicles into presumptive 'healthy' or 'regressing' categories since E:P ratios in FF were well below 1 in all except two follicles (one in the 7–8 mm size class and one in the 9–10 mm size class). Furthermore, in the case of 1–2, 3–4 and 5–6 mm follicles, this was precluded by the pooling of individual follicles for analysis. It should also be noted that since the follicles analysed in this study were from cattle in the mid to late luteal phase of the oestrous cycle, most would belong to the first and/or second post-ovulatory wave of follicle development in which the likely fate of the selected oestrogen-active dominant follicle is regression rather than ovulation (Ireland *et al.* 2000, Mihm *et al.* 2002, 2006, Rodgers & Irving-Rodgers 2010).

Analysis of key 'markers' of follicle status in the sample set included in the analysis (including follicular fluid E:P ratio and expression of mRNA transcripts for

gonadotrophin receptors, steroidogenic enzymes and *INHBA* subunit) revealed a pattern of expression consistent with previous work (Bao & Garverick 1998, Webb *et al.* 2004), and confirmed that there was minimal cross-contamination of TC with GC, and vice versa. *FSHR* mRNA was abundant in GC throughout antral follicle development, consistent with the role of FSH, acting exclusively on GC, in driving antral follicle development (Mihm *et al.* 2002, Webb *et al.* 2003). GC expression of *LHCGR* was only detected in follicles >9 mm in diameter, consistent with acquisition of LH dependency as they achieve dominance (Bao & Garverick 1998, Webb *et al.* 2004), which is in agreement with the previous observations. Furthermore, expression levels of *LHCGR*, *CYP19* and *INHBA* were all maximal in GC of LEA (presumptive 'healthy') follicles, being considerably lower in LEI (presumptive 'regressing') follicles. Together, the observed cell type and developmental stage-specific expression profiles of key gonadotrophin receptors, steroidogenic enzymes and *INHBA* subunit support the use of this follicle set to examine the expression pattern of BMP-related transcripts.

BMPs and other TGF β family members have emerged as potentially important regulators of follicle development (Shimasaki *et al.* 2004, Knight & Glister 2006). We showed previously that BMP4, BMP6 and BMP7 can act upon both TC and GC *in vitro*, suppressing LH-induced androgen production by TC (Glister *et al.* 2005) and enhancing E_2 , inhibin-A, activin-A and follistatin secretion by GC (Glister *et al.* 2004). Furthermore, BMP treatment promoted rapid accumulation of phosphorylated SMAD1 in the nuclei of both GC and TC (Glister *et al.* 2004, 2005) confirming the activation of a functional BMP signalling pathway in these cells.

While previous immunocytochemical evidence for the bovine suggested that BMP4 and BMP7 are confined to TC and BMP6 to GC (Glister *et al.* 2004), in the present and previous (Kayani *et al.* 2009) studies, mRNA expression and immunoreactive protein were detected for all three BMPs in both TC and GC layers. *BMP4* and *BMP7* transcripts were more abundant in TC than in GC in agreement with our earlier immunocytochemical evidence (Glister *et al.* 2004). However, at the mRNA level, *BMP6* expression was much greater in TC than in GC. The explanation for this discrepancy is not known but may reflect a lack of specificity of the antibodies used in the previous study or, possibly, sequestration of BMP6 by TC-derived BMP-binding proteins rendering their epitopes undetectable by the BMP6 antibody used for immunocytochemistry. In any event, mRNA expression levels for *BMP6* in each cell type were remarkably uniform across the different follicle stages analysed, perhaps indicating minimal involvement in follicle progression in this species.

Here, we also examined *BMP2* expression and showed that mRNA abundance and *BMP2* immunoreactivity were much higher in GC than in TC in

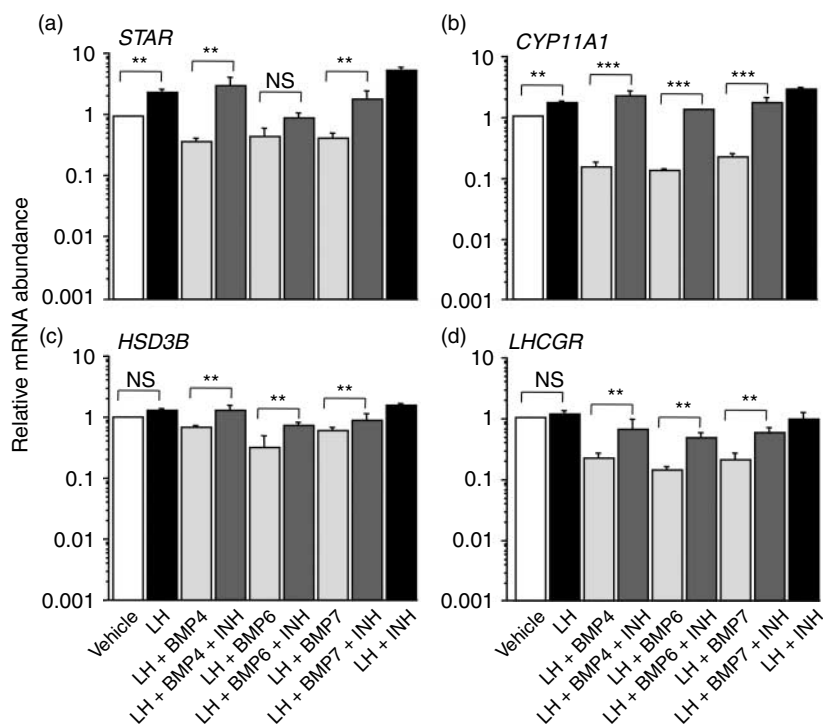


Figure 9 Effects of BMP4, BMP6 and BMP7, alone and in combination with inhibin, on the relative abundance of mRNA transcripts for (a) *STAR*, (b) *CYP11A1*, (c) *HSD3B* and (d) *LHCGR* (note use of log scale on y-axis). Values are means, and bars indicate s.e.m. ($n=4$ independent cultures). ** $P<0.01$ and *** $P<0.001$ versus corresponding value.

agreement with our recent study (Kayani *et al.* 2009), but contradicting an earlier report (Fatehi *et al.* 2005) indicating *BMP2* expression in TC but not in GC of bovine antral follicles. The reason for this inconsistency is not known but Juengel *et al.* (2006) also detected *BMP2* mRNA by PCR in ovine GC. Moreover, using *in situ* hybridization, they were able to detect *BMP2* mRNA in GC of large regressing follicles but not in GC of large healthy follicles. Interestingly, we found that *BMP2* expression in GC fell about tenfold between the 7–8 mm and LEA stage, but was high again in LEI follicles that are presumed to be regressing, as evidenced by their loss of E_2 - and inhibin-synthesizing capacity. In TC, *BMP2* transcript abundance followed an inverse pattern to that seen in GC, although overall expression levels were much lower. Thus, low GC expression of *BMP2* (but not *BMP4*, *BMP6* or *BMP7*) appears to be an additional ‘marker’ of a healthy dominant follicle. This is further supported by the negative correlation between *BMP2* and other key GC transcripts up-regulated in LEA follicles including *CYP19*, *INHA*, *INHBA* and *INHBB*. Within the context of intrafollicular paracrine signalling between GC and TC, we speculate that diminished production of *BMP2* by GC of large E_2 -active follicles might facilitate thecal androgen production.

With regard to BMP/activin receptors, mRNA transcripts and positive immunoreactivity for each of the seven receptors were detected in both GC and TC layers at all follicle stages. Previously, we examined expression of five of these receptors using immunocytochemistry and confirmed that all five were present in both cultured bovine TC and GC (Glister *et al.* 2004). Our data are also

consistent with the findings of Fatehi *et al.* (2005) who detected mRNA transcripts for *BMPR1A*, *BMPR1B*, *ACVR1*, *ACVR1B* and *BMPR2* in both GC and TC layers of bovine antral follicles but did not examine *ACVR2A* or *ACVR2B* expression. Similarly, Souza *et al.* (2002) detected immunoreactive *BMPR1A*, *BMPR1B* and *BMPR2* in both GC and TC layers of sheep antral follicles. Overall, among the type I receptors examined by QPCR in the present study, *BMPR1B* and *ACVR1B* transcripts were more abundant in GC, while *ACVR1* was more abundant in TC. Among type II receptors, *ACVR2A* was higher in GC, while *ACVR2B* was higher in TC.

With respect to follicle developmental stage, observed changes in receptor transcript abundance were generally

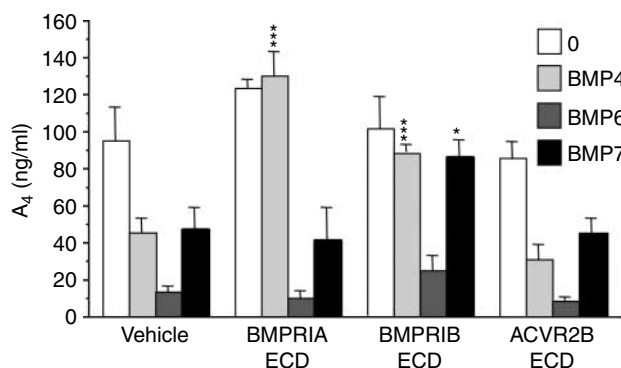


Figure 10 Effects of co-treatment with one of three soluble ‘decoy’ receptors (*BMPR1A*, *BMPR1B* and *ACVR2B*) on BMP-induced suppression of androstenedione secretion by bovine TC. Values are means, and bars indicate s.e.m. ($n=4$ independent cultures). * $P<0.05$ ** $P<0.01$ and *** $P<0.001$ versus corresponding value.

modest. However, two-way ANOVA indicated that TC and GC displayed quite different follicle stage-dependent expression patterns for five of the receptors (*BMPR1A*, *BMPR1B*, *ACVR1*, *BMPR2* and *ACVR2A*). Indeed, the abundance of three of these transcripts in TC (*BMPR1B*, *ACVR1* and *BMPR2*) as well as *ACVR1B* was negatively correlated with follicle diameter, while in GC, abundance of four of the above transcripts (*BMPR1B*, *ACVR1*, *ACVR1B* and *BMPR2*) as well as *BMPR1A* was positively correlated with follicle diameter. In particular, both *BMPR1B* and *BMPR2* expression increased progressively in GC while decreasing slightly in TC. This suggests a progressive shift in the balance of GC/TC responsiveness to an intrafollicular BMP ligand(s) that signals via this particular combination of type I and type II receptors, with GC becoming more responsive as follicles increase in size while TC become less responsive. Ligands known to recruit this combination of type I and type II signalling receptors include BMP2, BMP4, BMP6, BMP7 and BMP15 (ten Dijke *et al.* 1994, Liu *et al.* 1995, Nohno *et al.* 1995, Yamashita *et al.* 1995, Ebisawa *et al.* 1999, Aoki *et al.* 2001, Moore *et al.* 2003).

As a step towards identifying which particular signalling receptors mediate the BMP-induced suppression of androgen production by bovine TC (Glister *et al.* 2005), we attempted to use a soluble 'decoy' receptor approach. Our finding that a soluble ECD of *BMPR1A* could selectively abolish the effect of BMP4, while soluble ECD of *BMPR1B* could block the effects of BMP4 and BMP7 (while partially reversing the effect of BMP6), supports the involvement of both of these type I receptors. Evidently, BMP4 binds strongly to both *BMPR1A* and *BMPR1B*, while BMP7 binds to *BMPR1B* but not to *BMPR1A*. A comprehensive analysis of the effects of different combinations of soluble ECD for all seven potential signalling receptors was beyond the scope of this study, but would be a potentially valuable approach for future work. A complementary *in vitro* strategy would be to use RNA interference to down-regulate the expression of selected combinations of type I and type II receptors before treating the cells with different BMPs.

In contrast to the relatively modest follicle stage-dependent changes in BMP ligands and their type I and type II signalling receptors, we found that expression of betaglycan (*TGFBR3*) mRNA followed a distinct pattern during follicle development. Not only was betaglycan transcript abundance much greater in TC than in GC but its expression increased approximately fivefold in a stepwise fashion as follicles grew, reaching a peak in TC of 9–10 mm follicles before falling about 50% in the largest size class (11–18 mm), irrespective of oestrogenic status. We confirmed that betaglycan immunoreactivity was present in both the TC and GC layers of bovine antral follicles and in cultured bovine TC. Since betaglycan is considered an essential co-receptor required to facilitate the presentation of inhibin to type II signalling receptors and hence disrupt activin signalling (Lewis *et al.* 2000,

Chapman *et al.* 2002, Makanji *et al.* 2008), this observation prompted us to use our bovine TC culture model to evaluate the possibility that GC-derived inhibin functions as a physiological antagonist of BMP signalling in the ovary, in a manner comparable to its well-documented role as an activin antagonist.

BMP4, BMP6 and BMP7 each promoted a marked reduction in LH-induced androgen secretion by TC, which is in agreement with previous work (Glister *et al.* 2005). Real-time PCR revealed that all three BMPs greatly reduced CYP17 expression with lesser reductions in other key TC transcripts (*STAR*, *CYP11A1*, *LHCGR* and *HSD3B*). When co-treated with inhibin, a dramatic reversal of the BMP-induced reduction in androgen secretion and CYP17 expression was observed. Moreover, the BMP-induced reductions in expression of other TC transcripts (*STAR*, *CYP11A1*, *LHCGR* and *HSD3B*) were also reversed by inhibin. To our knowledge, this is the first study to show that inhibin can function as a BMP antagonist in an ovarian model. Our evidence concurs with other reports showing that inhibin can antagonize BMP effects on immortalized testicular, adrenocortical and gonadotroph cells lines (Wiater & Vale 2003, Farnworth *et al.* 2006).

In addition to being secreted into the peripheral circulation to negatively regulate pituitary FSH secretion (De Jong 1988, Bleach *et al.* 2001, Mihm *et al.* 2002), GC-derived inhibin may act on TC in a paracrine fashion (Hillier *et al.* 1991, Wrathall & Knight 1995). As shown previously, inhibin-A levels in bovine follicular fluid increase in parallel with follicle growth (Glister *et al.* 2006). Here, we show that betaglycan mRNA expression in TC increases progressively during follicle growth from 1 to 10 mm. This suggests that the ability of GC-derived inhibin to interact with neighbouring TC depends on this up-regulation of betaglycan expression in growing follicles. Since BMPs reduce LH-induced androgen production, the action of inhibin would facilitate TC androgen output, required for the subsequent conversion to E₂ by GC as follicles progress towards functional dominance.

In summary, we have shown that TC and GC expression of *BMP2*, *BMP4*, *BMP6* and *BMP7* and their associated signalling receptors show relatively modest changes during antral follicle growth in cattle. However, the inhibin co-receptor betaglycan undergoes dynamic changes in mRNA expression levels in TC throughout follicle growth. Inhibin, presumably acting via betaglycan, can reverse the suppressive action of BMPs on androgen production and expression of key steroidogenic genes. Further experiments are required to explore these putative interactions of inhibin, betaglycan and BMPs in the ovarian follicle. In addition, information is required on the follicular expression patterns of extracellular BMP binding proteins and on the endocrine and/or local factors that modulate the expression of these potentially important regulators of BMP signalling.

Materials and Methods

All media and reagents were purchased from Sigma UK Ltd (Pool, Dorset, UK) or Fisher Scientific Ltd (Loughborough, Leicestershire, UK) unless stated otherwise.

Ovaries and isolation of granulosa and theca cells for ex vivo analysis of gene expression

Ovaries from non-pregnant cattle slaughtered at random stages of the oestrous cycle were collected from an abattoir and transported to the laboratory on ice in medium-199 supplemented with 1% (v/v) antibiotic–antimycotic solution. The stage of the oestrous cycle (I–IV) was estimated by the morphological appearance of the corpus luteum (Ireland *et al.* 1980), and only ovaries judged to be from cattle in the mid to late luteal phase of the cycle (stage II–III; days 5–17) were selected for follicle dissection. Follicles ranging in diameter from 1 to 18 mm were dissected out from ovaries and sorted according to size. Any follicles showing obvious signs of degeneration were discarded at this stage. Individual follicles in the 1–2 mm (ten follicles per pool, $n=6$ pools collected), 3–4 mm (six follicles per pool; $n=7$ pools collected) and 5–6 mm (six follicles per pool; $n=6$ pools collected) categories were combined for further analysis, while all follicles >7 mm in diameter were processed and analysed individually. Smaller follicles, pooled as above, were hemisectioned into 1 ml of DPBS, and the interior of the follicle was gently scraped to remove the GC layer. After removing any cumulus–oocyte complexes from the Petri dish, the resulting suspensions were transferred to microcentrifuge tubes and briefly centrifuged (2 min, 2300 *g*) to pellet the cells. Supernatants (follicular fluid and DPBS) were removed and stored for subsequent analysis of steroids and total protein content. With larger follicles (>7 mm), a sample of follicular fluid was aspirated using a needle and syringe before hemisection and recovery of GC as above. The remaining follicle halves (devoid of most of their GC) were then shaken vigorously in DPBS, and the media were changed three times to remove any remaining GC. Theca interna cell (TC) layers were then peeled away from the follicle halves with the aid of a dissecting microscope as described previously (Glister *et al.* 2005). Peeled TC layers were briefly centrifuged to remove any dissecting medium. All GC and TC pellets were homogenized in 0.5 ml of TRI Reagent before snap freezing and storage at -80°C for subsequent RNA purification and cDNA synthesis.

Theca cell culture

For *in vitro* experiments, TC layers pooled from ~ 50 follicles (4–6 mm diameter) per culture were collected as above and further processed as described by Glister *et al.* (2005) to obtain individual cell suspensions. The serum-free culture medium used throughout was McCoy's 5A modified medium supplemented with 1% (v/v) antibiotic–antimycotic solution, 10 ng/ml insulin (bovine pancreas), 2 mM L-glutamine, 10 mM HEPES, 5 $\mu\text{g/ml}$ apotransferrin, 5 ng/ml sodium selenite and 0.1% (w/v) BSA. Cells were seeded at a density of 75 000 viable cells/50 μl culture medium into 96-well tissue culture

plates (Nunc, Life Technologies Ltd, Paisley, UK) containing 200 $\mu\text{l/well}$ pre-equilibrated culture medium. Culture plates were incubated in a water-saturated atmosphere of 5% CO_2 and 95% air at 38.5°C for a period of 6 days. Cell-conditioned medium was removed every 48 h, and wells were replenished with fresh medium containing treatments (see below). Conditioned media were stored at -20°C for steroid immunoassays. At the end of the 144 h culture period, viable cell number was determined using neutral red uptake assay (Campbell *et al.* 1996, Glister *et al.* 2001).

RNA isolation from cultured TC

In culture experiments in which total RNA was to be extracted for PCR analysis, cells were seeded into 24-well plates (0.5×10^6 cells/ml) with three replicate wells per treatment. At the end of culture, cell lysates were prepared using TRI Reagent, and pooled lysates from replicate wells were stored at -80°C until total RNA isolation.

Preparation and addition of treatments

Ovine LH (NIADDK oLH-S-16) was provided by the National Hormone and Pituitary Program (Torrance, CA, USA). Recombinant human (rh) BMP4, BMP6 and BMP7 and three soluble BMP receptor ECD-Fc fusion protein constructs (BMPR1A, BMPR1B and ACVR2B) were purchased from R&D Systems (Abingdon, Oxon, UK). Bovine 32 kDa inhibin-A was purified from bovine follicular fluid in this laboratory as described previously (Knight *et al.* 1990). Treatments were sterilized by passing through 0.2 μm filters before further dilution in sterile culture medium. Each treatment was added at 25 μl per culture well, and an equal volume of culture medium alone was added to the control wells.

Hormone assays

Concentrations of A_4 in TC-conditioned media and follicular fluid samples were determined by RIA as reported previously (Wrathall & Knight 1995, Glister *et al.* 2005). The detection limit was 100 pg/ml, and intra- and inter-assay coefficients of variation (CV) were 7 and 10% respectively. Concentrations of P_4 in follicular fluid samples and TC-conditioned media were determined by competitive ELISA (Sauer *et al.* 1986, Bleach *et al.* 2001). The detection limit was 20 pg/ml, and intra- and inter-assay CV values were 8 and 10% respectively. Concentrations of E_2 in follicular fluid samples were determined by RIA (Tannetta *et al.* 1998) with a detection limit of 2 pg/ml and intra- and inter-assay CV values of 6 and 9% respectively.

Purification of RNA, cDNA synthesis and real-time PCR

Total RNA was isolated from tissue samples and cultured TC using TRI Reagent (Sigma UK Ltd) according to the supplier's instructions. After aqueous phase separation, RNA was precipitated in isopropanol and washed in 75% (v/v) ethanol, and the RNA pellet was re-suspended in 50 μl nuclease-free water. Potential genomic DNA contamination was removed

Table 1 List of primers used for real-time PCR.

Target	Accession number	Forward primer 5'–3'	Reverse primer 5'–3'	Amplicon size (bp)
<i>BMP2</i>	XM_866011.1	CCAAGAGGCATGTGCCGATTAGCA	TCCTTTCCCATCGTGGCCAAAAGT	101
<i>BMP4</i>	NM_001045877.1	TTTATGAGGTATGAAGCCCCGGC	AGTTTCCACCGCGTCACATTGTG	104
<i>BMP6</i>	XM_600972.2	GGCCCCGTTAACTCGACTGTGACAAA	TTGAGGACGCCGAACAAAACAGGA	108
<i>BMP7</i>	XM_612246.2	TGCAAGATAGCCACTTCTCACCGA	GGGATCTTGGAGAGATCAAACCGGA	130
<i>INHBA</i>	NM_174363.1	GAAGAGACCCGATGTCACCCAGC	TGTCGTCTCTATCTCCACGTACCCG	113
<i>INHBB</i>	NM_176852.1	ATGGCCTGGCCCTCTCCCG	CTTCAGGTAGAGCCAGCGCTGGC	101
<i>INHA</i>	NM_174094.3	GAGCCCCGAGGACCAAGATGTCTCC	CCTCAGCCTCTCCAGCATCTGGC	91
<i>BMPR1A</i>	NM_001076800	TGGATTGCCCTTACTGGTTCAGCGA	CCACGCCATTTACCCATCCACA	105
<i>BMPR1B</i>	XM_612088.2	AAAGTGGCGTGGCGTGGCGAAAAGGTAGCT	CCCGTCCCTTTGATATCTGCAGCAA	147
<i>BMPR2</i>	XM_617592.2	CCCCTCTTCGCGCCCTCCG	CCCCGAGTTATTTCCCCCG	87
<i>ACVR1</i>	NM_176663.2	CATGGCCCCGAAAGTTCTTGATGA	GCCACCTCCACAAGACAAGTCCAAA	102
<i>ACVR1B</i>	XM_586402.2	CCTCTCTGCTGCTCTGTTGTGTGCG	CCCGTCCAGTTGAAAATGGAGACC	103
<i>ACVR2A</i>	NM_174227.2	GCCACAAACCCGCCATATCTACA	TGCCAGCCTCAAACCTTAAACGCCAA	114
<i>ACVR2B</i>	NM_174495.2	ACAAGCCATCTATTGCCACAGGGA	CTCAAACCGAACAGCCAGGCCAAA	104
<i>TGFBR3</i>	XM_001253071.2	TGCACTTCTATCCACAAGCCG	CCAGATCATTGAGGCATCCAGCG	197
<i>LHCGR</i>	NM_174381.1	ATTGCCTCAGTCGATGCCAGACC	AAAAAGCCAGCCGCGCTGC	92
<i>FSHR</i>	NM_174061.1	GCCAGCCTCACCTACCCGAGC	AATTGGATGAAGGTCAGAGGTTTGCC	75
<i>CYP17</i>	NM_174304	GACAAAGGCACAGACTTGTGTCAC	TGATCTGCAAGACGAGACTGGCATG	301
<i>CYP19</i>	NM_174305.1	CGCCACTGAGTTGATTTTTGCTGAGA	TAAGGCTTTGCGCATGACCAGGTC	301
<i>ACTB</i>	BC102948.1	ATCCACCATCGCAATGAGCGGTT	CGGATGTCGACGTCACACACTTCATG	128

with an RNase-free DNase kit (RQ1; Promega UK Ltd). The TRI Reagent extraction process was repeated, and the final RNA pellet was re-suspended in 20 µl nuclease-free water; RNA quantity and quality were evaluated by spectrophotometry at 260/280 nm. First strand cDNA was synthesized from 1 µg of RNA template using the Reverse-iT RT kit (used according to the manufacturer's protocol; ABgene, Epsom, Surrey, UK) in a 20 µl reaction primed with random hexamers. Primers were designed using Primer Express software (version 1.5; Perkin-Elmer Applied Biosystems, Warrington, Lancs, UK). Primer sequences and accession numbers are shown in Table 1. In primer validation experiments, dissociation curve analysis and agarose gel electrophoresis were used to verify that each selected primer pair generated a single amplicon of the predicted size. cDNA template dilution curves were used to demonstrate satisfactory PCR efficiency (>85%) and linearity. PCR assays were carried out in a volume of 25 µl, comprising 10 µl cDNA template (equivalent to 20 ng reverse-transcribed RNA), 1 µl each forward and reverse primers (final concentration 0.4 µM) and 12 µl QuantiTect SYBR Green QPCR 2× 'hot start' Master Mix (Qiagen). Samples were processed for 40 cycles on an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems) with the following thermal cycling conditions: 15 min at 95 °C (one cycle only) then 15 s at 95 °C and 1 min at 60 °C (40 cycles). The $\Delta\Delta C_t$ method was used for semi-quantitative comparison of the abundance of each mRNA transcript. C_t values for each transcript in a given sample were first normalized to β -actin C_t value (which was uniform across all experimental groups; ANOVA $P > 0.1$). For cell culture experiments, the resultant ΔC_t values for each treatment were then normalized to the ΔC_t value of the respective vehicle-treated control group. For *ex vivo* tissue samples, ΔC_t values for each transcript in a given sample were normalized to the mean ΔC_t value for that transcript in all tissue samples. For graphical presentation, $\Delta\Delta C_t$ values were finally converted to fold differences using the formula: fold difference = $2^{(-\Delta\Delta C_t)}$.

Immunohistochemistry

Ovaries were fixed for 24 h in neutral-buffered formalin, dehydrated and embedded in paraffin wax. Pairs of adjacent sections (6 µm) were mounted onto poly-L-lysine-coated slides (Polysene, Fisher), dewaxed and rehydrated. Endogenous peroxidase activity was quenched by incubation for 30 min in 0.3% v/v hydrogen peroxide in methanol. Microwave antigen retrieval was performed using citrate buffer (10 mM, pH 6). After cooling, tissue sections were washed (PBS + 0.1% (v/v) Tween 20) and blocked for 1 h using 2.5% (v/v) horse serum (ImmPRESS Kit, Vector Laboratories, Peterborough, UK). The following primary antibodies (and appropriately matched control serum/purified IgG for adjacent tissue section) were diluted in blocking buffer and incubated with sections overnight at 4 °C: rabbit anti-BMP2 (Peprotech EC Ltd, London, UK) at 5 µg/ml; mouse anti-BMP4 (clone 66119; R&D Systems) at 5 µg/ml; mouse anti-BMP6 (clone 74219, R&D Systems) at 5 µg/ml; rabbit anti-BMP7 (Peprotech) at 20 µg/ml; rabbit anti-betaglycan (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1/50; rabbit anti-BMPR1, BMPR1B and BMPR2 (gifts from Prof. C-H Heldin, Ludwig Institute, Uppsala, Sweden) each at 1/100; rabbit anti-ACVR1, ACVR1B, ACVR2A and ACVR2B (raised 'in-house' against synthetic peptides conjugated to PPD) each at 1/100. Peptide sequences corresponded to residues 81–89, 55–63, 91–100 and 90–99 within the extracellular domains of the respective bovine activin receptors, and were synthesized and conjugated by Prof. N P Groome and Dr M Cranfield (Oxford Brookes University, UK). After incubation with primary antibody, slides were washed thoroughly in PBS/Tween, and the ImmPRESS Universal anti-mouse/rabbit IgG HRP polymer-based detection system (Vector Laboratories) was used with diaminobenzidine substrate according to the manufacturer's instructions. Sections were counterstained with haematoxylin, dehydrated and mounted under glass coverslips. Slides were viewed on a Zeiss Axioskop 2 microscope

(20× objective; Carl Zeiss Ltd, Welwyn Garden City, Herts, UK), and images were acquired using a Zeiss Axiocam with Axiovision software.

Statistical analysis

Hormone data were log-transformed before statistical analysis to reduce heterogeneity of variance. QPCR data were analysed as ΔC_t values before conversion to relative fold difference values. Results were evaluated using one- and two-way ANOVA and, where indicated or referred to in the text, *post hoc* pairwise comparisons were made using Fisher's protected least significant difference (PLSD) test. *In vitro* results are presented as means \pm S.E.M. ($n=4$) based on combined data from four independent culture experiments, each using follicle cells harvested from a different set of ovaries.

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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