Evidence for an inhibitory role of bone morphogenetic protein(s) in the follicular–luteal transition in cattle

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Abstract

Bone morphogenetic proteins (BMPs) and their receptors are expressed in ovarian theca cells (TC) and granulosa cells (GC) and BMPs have been implicated in the regulation of several aspects of follicle development including thecal androgen production and granulosa oestrogen production. Their potential involvement in luteal function has received less attention. In this study, we first compared relative abundance of mRNA transcripts for BMPs, activin- β A and BMP/activin receptors in bovine corpus luteum (CL) and follicular theca and granulosa layers before undertaking functional *in vitro* experiments to test the effect of selected ligands (BMP6 and activin A) on luteinizing bovine TC and GC. Relative to β -actin transcript abundance, CL tissue contained more *BMP4* and *-6* mRNA than granulosa, more *BMP2* mRNA than theca but much less activin- β A mRNA than both granulosa and theca. Transcripts for all seven BMP/activin receptors were readily detected in each tissue and two transcripts (*BMPRII*, *ActRIIA*) were more abundant in CL than either theca or granulosa, consistent with tissue responsiveness. *In vitro* luteinization of TC and GC from antral follicles (4–6 mm) was achieved by culturing with 5% serum for 6 days. Treatment with BMP6 (0, 2, 10, and 50 ng/ml) and activin A (0, 2, 10 and 50 ng/ml) under these conditions dose-dependently suppressed forskolin-induced progesterone (P4) secretion from both cell types without affecting cell number. BMP6 reduced forskolin-stimulated upregulation of *STAR* mRNA and raised 'basal' *CYP17A1* mRNA level in theca-lutein cells without affecting expression of *CYP11A1* or hydroxy- Δ -5-steroid dehydrogenase, 3 β - and steroid Δ -isomerase 1 (*HSD3B1*). In granulosa-lutein cells, *STAR* transcript abundance was not affected by BMP6, whereas forskolin-induced expression of *CYP11A1*, *HSD3B1*, *CYP19A1* and oxytocin transcripts was reduced. In both cell types, follistatin attenuated the suppressive effect of activin A and BMP6 on forskolin-induced P4 secretion but had no effect alone. Granulosa-lutein cells secreted low levels of endogenous activin A (~ 1 ng/ml); BMP6 reduced this, while raising follistatin secretion thus decreasing activin A:follistatin ratio. Collectively, these findings support inhibitory roles for BMP/activin signalling in luteinization and steroidogenesis in both TC and GC.

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Introduction

Little is known about the early stages of luteinization, the process by which both granulosa cells (GC) and theca cells (TC) of the post-ovulatory follicle develop into the corpus luteum (CL). Also, the local regulation of the development and regression of the CL, marked by the rise and decline of progesterone (P4) levels, has yet to be fully delineated. Luteinization leads to an oestrogenic to progestagenic shift in follicular steroidogenesis, through upregulation of various components of the steroidogenic pathway in both theca-lutein and granulosa-lutein cells of the CL. These include *STAR*, hydroxy- Δ -5-steroid dehydrogenase, 3 β - and steroid Δ -isomerase 1 (*HSD3B1*) and *CYP11A1* (Ireland *et al.* 1980, Rodgers *et al.* 1987, Couet *et al.* 1990, Stocco 2000). Conversely, expression of cytochrome P450, family 17, subfamily A, polypeptide 1 (*CYP17A1*), required for androgen synthesis by TC, and P450 aromatase (*CYP19A1*) required for conversion of androgens to oestrogens by GC, decrease sharply after the preovulatory LH surge

(Rodgers *et al.* 1987). Other changes in sheep and cattle include decreased secretion of inhibin (Martin *et al.* 1991) and increased secretion of oxytocin (Schams 1987, Luck *et al.* 1990) by GC.

It is well established that intraovarian factors belonging to the transforming growth factor β (TGF β) superfamily, including activins, inhibins and bone morphogenetic proteins (BMPs), are synthesized by follicular GC and TC cells and these proteins have been assigned roles as local autocrine/paracrine regulators of follicle growth and development (Shimasaki *et al.* 2004, Juengel & McNatty 2005, Knight & Glister 2006). However, relatively little is known regarding the expression and role of BMPs in luteinization and CL function.

The biological effects of BMPs are mediated by specific cell-surface receptors, which exist as two subtypes: type I and type II (Massague 1996), both with intrinsic serine/threonine kinase activity. BMP signalling requires binding to and formation of heteromeric complexes with the type I and type II receptors on the

cell surface (Massague & Chen 2000, Miyazono *et al.* 2000, Miyazawa *et al.* 2002). Once the BMPR–ligand complex is formed, the type II receptor phosphorylates and activates the type I receptor, which in turn activates transcriptional regulators called Smads. BMPs can bind to one of three type II receptors (BMPRII, ActRIIA or ActRIIB) and one of three type I receptor (BMPRIA, BMPRIIB or ActRIA). BMP4 and -7 are expressed in rat (Shimasaki *et al.* 1999, Lee *et al.* 2001) and bovine (Glister *et al.* 2004) TC while BMP6 expression has been reported in mouse (Elvin *et al.* 2000) and bovine (Glister *et al.* 2004) oocytes. Ovine GC have been reported to express BMP2 (Souza *et al.* 2002) while BMP6 immunoreactivity was detected in bovine GC (Glister *et al.* 2004). Treatment of non-luteinized bovine GC with BMP4, -6 and -7 enhanced basal and IGF-induced secretion of oestradiol, inhibin A, activin A and follistatin but inhibited basal and IGF-induced secretion of (Glister *et al.* 2004). Treatment of non-luteinized bovine TC with the same three ligands potently suppresses basal and LH-induced androgen secretion and *CYP17A1* mRNA and protein expression (Glister *et al.* 2005).

Such evidence from functional studies on non-luteinized GC and TC suggest a role of these locally derived BMPs in follicular steroidogenesis. With this in mind, the aim of the present study was to extend these observations to the follicular–luteal transition: 1) by using real-time quantitative PCR to compare *ex vivo* expression of mRNA transcripts encoding selected BMP ligands and receptors in bovine CL, granulosa and theca

tissue and 2) by examining the effect of selected ligands (BMP6, activin A) and an associated-binding protein (follistatin) on steroid production and steroidogenic gene expression by bovine GC and TC undergoing serum-induced luteinization *in vitro*.

Results

Comparison of mRNA transcript abundance in *ex vivo* samples of CL, theca and granulosa tissue

mRNA transcripts for a range of BMP-related ligands, receptors and steroidogenic pathway components were readily detectable in freshly harvested samples of CL and follicular granulosa and theca tissue (Table 1). The pattern of expression of steroidogenic pathway components in the three tissues was as expected: relative expression of STAR, CYP11A1 and HSD3B1 was substantially greater in CL tissue than in either GC or TC. In addition, expression of CYP19A1 was much greater in GC than either CL or TC while expression of CYP17A1 was much greater in TC than either CL or GC. The relative amounts of BMP2, -4 and -6 transcript in CL were intermediate between values detected in GC and TC while there was less *BMP7* mRNA and much less activin β A mRNA in CL than in either GC or TC. The relative abundance of BMPRII and ActRIIA transcripts was greater in CL tissue than in either GC or TC while transcript abundance for the other five receptors examined was lower in CL than in follicular GC or TC.

Table 1 Comparison of relative^a fold-differences in mRNA transcript levels in *ex vivo* samples of bovine corpus luteum and follicular granulosa and theca interna tissue.

mRNA transcript	Granulosa (n = 9)	Theca interna (n = 8)	Corpus luteum			Significance (ANOVA)	
			Early (n = 4)	Mid (n = 5)	Late (n = 3)	F ratio	P value
Ligands							
BMP2	6.28 ± 1.81*	0.19 ± 0.04 [†]	0.49 ± 0.13 [†]	0.75 ± 0.17 [†]	0.93 ± 0.31 [†]	5.54	< 0.01
BMP4	0.44 ± 0.10*	1.17 ± 0.01 [†]	1.12 ± 0.15 [†]	0.68 ± 0.15 ^{*,†}	1.07 ± 0.35 [†]	3.62	< 0.02
BMP6	0.32 ± 0.10*	2.70 ± 0.70 [†]	0.34 ± 0.06*	0.53 ± 0.11*	0.75 ± 0.15*	7.64	< 0.001
BMP7	0.32 ± 0.07*	3.94 ± 0.80 [†]	0.12 ± 0.04*	0.14 ± 0.08*	0.84 ± 0.47*	14.96	< 0.0001
Activin βA	1.45 ± 0.23*	0.33 ± 0.07 [†]	0.02 ± 0.00 [†]	0.02 ± 0.01 [†]	0.11 ± 0.06 [†]	17.79	< 0.0001
Receptors							
BMPRIA	1.01 ± 0.18*	1.03 ± 0.07*	0.19 ± 0.01 [†]	0.20 ± 0.03 [†]	0.13 ± 0.02 [†]	11.57	< 0.0001
BMPRIIB	1.15 ± 0.20*	0.75 ± 0.07*	0.16 ± 0.05 [†]	0.24 ± 0.08 [†]	0.19 ± 0.09 [†]	6.77	< 0.001
BMPRII	0.66 ± 0.05*	1.10 ± 0.11*	1.45 ± 0.34 [†]	2.18 ± 0.36 [†]	1.75 ± 0.11 [†]	8.96	< 0.0001
ActRIA	0.60 ± 0.11*	1.58 ± 0.08*	0.47 ± 0.04*	0.62 ± 0.05*	0.50 ± 0.09*	1.99	0.13
ActRIIB	2.03 ± 0.38*	0.63 ± 0.05*	0.56 ± 0.04*	0.55 ± 0.04*	0.55 ± 0.10*	2.62	0.06
ActRIIA	0.84 ± 0.10*	1.09 ± 0.14*	1.29 ± 0.15 ^{*,†}	1.47 ± 0.16 [†]	0.86 ± 0.15*	2.85	< 0.05
ActRIIB	1.28 ± 0.15*	0.83 ± 0.11 ^{*,†}	0.43 ± 0.04 ^{†,‡}	0.62 ± 0.09 ^{†,‡}	0.86 ± 0.16 ^{*,†}	4.08	< 0.01
Steroidogenesis							
STAR	0.39 ± 0.12*	1.80 ± 0.38*	15.4 ± 1.82 [†]	29.6 ± 4.01 [‡]	1.02 ± 0.79*	47.2	< 0.0001
CYP11A1	0.35 ± 0.06*	2.04 ± 0.29 [†]	3.15 ± 0.19 [†]	4.62 ± 0.64 [§]	0.35 ± 0.06*	35.3	< 0.0001
HSD3B1	0.63 ± 0.17*	1.60 ± 0.21*	6.43 ± 0.74 [†]	9.41 ± 1.43 [†]	0.27 ± 0.17*	37.4	< 0.0001
CYP17A1	0.04 ± 0.01*	12.03 ± 2.97 [†]	0.15 ± 0.08*	0.05 ± 0.03*	0.14 ± 0.08*	12.3	< 0.0001
CYP19A1	1.76 ± 0.50*	0.23 ± 0.02 [†]	0.16 ± 0.05 [†]	0.30 ± 0.13 [†]	0.01 ± 0.00 [†]	5.45	< 0.01

ΔC_t values for each transcript in a given sample were re-normalized to the mean ΔC_t value for that transcript in all tissue samples. Within rows, means without a common superscript are significantly different ($P<0.05$). It is not possible to make 'between-row' statistical comparisons (i.e. between levels of expression of different transcripts).

^aRelative levels of each transcript were determined using the $\Delta\Delta C_t$ method with β -actin as the control 'housekeeping' gene.

Evidence for serum-induced in vitro luteinization of GC and TC

Figure 1A shows that after 6 days of culture in serum-supplemented medium, relative abundance of *CYP19A1* mRNA (normalized to β -actin transcript abundance) in GC was 50-fold lower than in GC cultured in serum-free medium ($P < 0.01$). Conversely, expression of *HSD3B1* was 7-fold higher in serum-treated GC ($P < 0.01$). Relative amounts of *STAR* and *CYP11A1* transcript were similar in serum-treated and serum-free GC cultures. Oestradiol concentration in GC-conditioned medium was threefold lower in serum-treated cultures while P4 concentration was fourfold higher. Viable cell number was ~ 10 -fold higher in serum-treated cultures than in serum-free cultures (Fig. 1A). Treatment with an optimal dose-level of FSH (0.33 ng/ml) promoted a marked (~ 20 -fold) rise in oestradiol secretion by serum-free GC cultures but had no effect on oestradiol secretion from serum-treated GC cultures (data not shown).

Figure 1B shows that the relative amount of *CYP17A1* mRNA in serum-treated TC was about 1000-fold lower than in TC cultured in serum-free medium ($P < 0.0001$). Relative amounts of *STAR* (~ 11 -fold; $P < 0.01$), *CYP11A1* (~ 2 -fold; $P > 0.05$) and *HSD3B1* (5-fold; $P < 0.05$) mRNA were also reduced in serum-treated TC but to much lesser extents than *CYP17A1* mRNA. Androstenedione concentration in TC-conditioned medium was > 100 -fold lower in serum-treated cultures (reduced below assay detection limit) while P4 concentration was ~ 3 -fold higher than in serum-free cultures. Viable cell number was ~ 6 -fold higher in serum-treated TC cultures than in serum-free cultures.

In vitro-luteinized GC and TC: effects of BMP6 and activin A on progesterone secretion

Treatment with BMP6 dose-dependently reduced forskolin-induced P4 secretion from luteinized GC ($P < 0.001$) and TC ($P < 0.0001$) without affecting viable cell number at the end of culture (Fig. 2). BMP6 did not significantly affect 'basal' P4 secretion in the absence of forskolin stimulation. As shown in Fig. 3, activin A also reduced forskolin-induced P4 secretion from both GC ($P < 0.0001$) and TC ($P < 0.001$) without affecting cell number. 'Basal' P4 secretion by GC, but not TC, was also suppressed by activin A.

In vitro-luteinized GC and TC: effect of BMP6 on abundance of mRNA transcripts

Neither BMP6 nor forskolin treatment, alone or in combination, had any effect on β -actin mRNA abundance, justifying the use of this transcript as a normalization control in both luteinized GC and TC (data not shown). Treatment of GC with BMP6 decreased forskolin-induced expression of *CYP11A1* by 40% ($P < 0.05$) but had no significant effects on transcript levels for *STAR* and *HSD3B1* either in the presence or absence of forskolin

Figure 1 Comparison of *STAR*, *CYP11A1*, *HSD3B1* and *CYP19A1* or *CYP17A1* transcript abundance (normalized to β -actin), steroid secretion and viable cell number in (A) GC and (B) TC after 6-day culture in serum-supplemented (black bars) or serum-free (white bars) medium. Steroid secretion values are for the final (96–144 h) culture period. Transcript abundance has been re-normalized to mean values in serum-treated cells. Values are means $\pm$ S.E.M. ($n = 4$ independent cultures). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with corresponding bar.

Figure 2 Effect of BMP6 on basal and forskolin-induced secretion of progesterone by luteinizing bovine (A) granulosa cells and (B) theca interna cells. The lower panel shows the viable cell number at the end of the culture period. Results presented are for the final (96–144 h) culture period. Values are $\pm$ S.E.M. ($n=3$ independent cultures).

(Fig. 4A). Forskolin treatment greatly (>100 -fold) enhanced levels of oxytocin and *CYP19A1* mRNA in GC and BMP6 reduced these responses by 75% and 62% respectively ($P < 0.05$). As shown in Fig. 4B treatment of TC

with BMP6 abolished forskolin-induced up-regulation of *STAR* mRNA ($P < 0.05$) but had no effect on *CYP11A1* or *HSD3B1* mRNA levels. BMP6 also increased 'basal' expression of *CYP17A1* mRNA about 5-fold ($P < 0.05$).

Figure 3 Effect of activin A on basal and forskolin-induced secretion of progesterone by luteinizing bovine (A) granulosa cells and (B) theca interna cells. The lower panel shows the viable cell number at the end of the culture period. Results presented are for the final (96–144 h) culture period. Values are $\pm$ S.E.M. ($n=3$ independent cultures).

Figure 4 Effect of BMP6 (20 ng/ml) treatment, in the presence and absence of forskolin, on relative abundance of mRNA transcripts in luteinizing bovine (A) granulosa cells and (B) theca interna cells. Values are $\pm$ S.E.M. ($n=4$ independent cultures). * $P<0.05$ compared with corresponding bar in control cells without BMP6 treatment.

Can follistatin inhibit effects of activin A and BMP6 on luteinizing GC and TC?

Follistatin reversed ($P<0.05$) the suppressive effect of activin A on forskolin-induced P4 secretion from GC (Fig. 5). At the highest follistatin dose-level tested (500 ng/ml) follistatin also attenuated the activin A-induced decline in P4 production by TC. Follistatin alone did not affect P4 secretion by either GC or TC and did not affect viable cell number at the end of culture. As shown in Fig. 6 follistatin partially reversed ($P<0.05$) the suppressive effect of BMP6 on P4 secretion by both cell types, but only at the highest dose-level tested (500 ng/ml).

Does BMP6 affect secretion of endogenous activin A and follistatin by luteinizing GC?

Figure 7 shows effect of BMP6 on activin A and follistatin secretion by GC in the presence and absence of forskolin. BMP6 ($P<0.05$) decreased activin A secretion in the presence of forskolin with no effect in the absence

of forskolin. However, follistatin secretion was increased significantly both in the presence ($P<0.05$) and absence ($P<0.005$) of forskolin resulting in a significant decline ($P<0.05$) in activin A:follistatin mass ratio.

Discussion

This study provides several lines of evidence to support a functional involvement of BMP signalling in the follicular-luteal transition in cattle. First, *ex vivo* analysis of gene expression showed that mRNA transcripts for several BMPs (BMP2, -4, -6) and BMP-responsive receptors (ActRIA, ActRIIA, BMPRII) are present in bovine CL tissue in amounts broadly comparable with those in follicular GC and/or TC compartments. By contrast, expression of activin β A mRNA, a major transcript in follicular GC, was extremely low in CL (~75-fold lower than in GC), indicating that little activin A (or inhibin-A) is synthesized by bovine CL and that the endogenous ligand(s) which bind to the type I and -II receptors shown to be expressed in this tissue is more likely to be BMP(s). This finding of greatly reduced activin β A expression in CL agrees with previous reports of a marked fall in GC expression of inhibin/activin α and β A subunits after the LH surge in cattle (Rodgers *et al.* 1989, Ireland & Ireland 1994) but, to our knowledge, this is the first comparison of relative expression of BMPs and their receptors in bovine follicles and CL. In contrast to the bovine ovary, expression of inhibin/activin α and β A subunits by granulosa-lutein cells is maintained in humans and primates (Fraser *et al.* 1993, Roberts *et al.* 1993). Indeed, circulating levels of inhibin-A are maximal during the luteal phase of the human menstrual cycle (Muttukrishna *et al.* 1994). Low levels of activin A were detectable by ELISA in bovine luteinized GC-conditioned media but, in the absence of suitable BMP immunoassays, the extent to which different BMPs are secreted by the cultured cells is unknown at this stage.

Recent studies utilizing chemically-defined, serum-free culture systems for bovine and ovine GC (Campbell *et al.* 1996, 2006, Gutiérrez *et al.* 1997, Glister *et al.* 2001, 2004) and TC (Glister *et al.* 2005, Campbell *et al.* 2006) have provided useful insights into the 'follicular' phenotype in which GC express *CYP19A1* and are responsive to FSH in terms of upregulation of *CYP19A1* expression and oestrogen production; correspondingly TC expression of *CYP17A1* is maintained and the cells are responsive to LH in terms of upregulation of *CYP17A1* expression and androgen production. In the present study, aimed at exploring the potential actions of BMPs in the follicular-luteal transition and CL function in the bovine, we utilized a serum-supplemented culture model (Channing & Ledwitz-Rigby 1975, Skinner & Osteen 1988, Luck *et al.* 1990, Engelhardt *et al.* 1991, Wrathall & Knight 1993) in which both GC and TC undergo phenotypic changes *in vitro* that, in many respects, mimic those associated with luteinization *in vivo*. Foetal bovine serum (FBS) was

Figure 5 Effect of follistatin on activin A induced suppression of progesterone secretion by (A) granulosa cells and (B) theca interna cells in the presence of 10 μ M forskolin. The lower panel shows the viable cell number at the end of the culture period. Results presented are for the final (96–144 h) culture period. Values are $\pm$ S.E.M. ($n=3$ independent cultures). * $P<0.05$, ** $P<0.01$ *** $P<0.001$ versus corresponding value.

able to induce such changes characteristic of luteinization in both cell-types as evidenced by increased P4 secretion and cell proliferation (GC and TC), greatly diminished 'basal' expression of *CYP17A1* and androgen secretion by

TC, and reduced expression of *CYP19A1* and oestrogen secretion by GC. FBS-treated GC also displayed a sevenfold increase in 'basal' *HSD3B1* expression consistent with an increased production of P4. In the case of TC,

Figure 6 Effect of follistatin on BMP6 induced suppression of progesterone secretion by (A) bovine granulosa cells and (B) theca interna cells in the presence of 10 μ M forskolin. The lower panel shows the viable cell number at the end of the culture period. Results presented are for the final (96–144 h) culture period. Values are $\pm$ S.E.M. ($n=4$ independent cultures). ** $P<0.01$ *** $P<0.001$ versus corresponding value.

Figure 7 Effect of BMP6 on secretion of activin A and follistatin by bovine granulosa cells cultured in the (A) presence and (B) absence of 10 μ M forskolin. The lower panels show activin A:follistatin mass ratio. Values are $\pm$ S.E.M. ($n=4$ independent cultures). P values from one-way ANOVA are shown.

however, 'basal' expression levels (normalized to β -actin) of several components of the steroidogenic pathway (*STAR*, *CYP11A1*, *HSD3B1*) necessary for P4 synthesis were lower in FBS-treated cultures than in serum-free cultures, albeit not to the extent observed for *CYP19A1* expression which was reduced by three orders of magnitude. To our knowledge the relative contributions of granulosa-lutein cells ('large luteal cells') and theca-lutein cells ('small luteal cells') to CL output of P4 *in vivo* has not been established and so it is difficult to ascertain the significance of this observation in relation to the validity of our *in vitro* luteinized TC model.

Based on viable cell number at the end of the 6-day culture period, it is clear that proliferation and/or survival of both GC and TC is much greater in the presence of FBS. Whilst intense cellular proliferation accompanies the rapid growth of ruminant CL tissue *in vivo* much of this is believed to involve endothelial cells and fibroblasts. However, both TC-derived 'small luteal cells' and, to a lesser degree, GC-derived 'large luteal cells' also proliferate, particularly in the early stages of CL formation (Jablonka-Shariff *et al.* 1993, Zheng *et al.* 1994). It is possible that FBS-stimulated proliferation of 'contaminating' endothelial cells and/or fibroblasts during the 6-day period of TC culture could account,

at least in part, for the apparently lower relative expression of *STAR*, *CYP11A1* and *HSD3B1*, compared with expression in serum-free TC cultures.

Of several BMP members found to be expressed in bovine CL we selected BMP6 for testing in our luteinized GC and TC model. Since activin A has been used in previous studies on human/primate granulosa-lutein cells (see below), we also included activin A in many of our experiments. The observation that both BMP6 and activin A inhibit forskolin-induced P4 secretion by luteinizing TC and GC suggests a negative autocrine/paracrine action of these, or related, TGF β superfamily ligands on luteal steroidogenesis in cattle. Previously, activin A has been shown to reduce basal and hCG-induced P4 secretion by human (Rabinovici *et al.* 1990, Di Simone *et al.* 1994) and macaque (Brannian *et al.* 1992) granulosa-lutein cells in a follistatin-reversible manner (Cataldo *et al.* 1994) but, to our knowledge, effects on theca-lutein cells have not been reported previously.

Consistent with its negative effect on P4 secretion by luteinizing GC, BMP6 also reduced forskolin-induced upregulation of *CYP11A1* and *HSD3B1* mRNA expression. Likewise, forskolin-induced upregulation of *CYP19A1* and oxytocin expression were reduced. Previously activin A was shown to reduce oxytocin secretion by bovine GC *in vitro* (Shukovski & Findlay 1990). Although we did not measure oestradiol secretion in this experiment, the observation that luteinizing GC express low but detectable amounts of *CYP19A1* mRNA and that forskolin treatment augments *CYP19A1* expression is consistent with reported ability of bovine CL to produce small amounts of oestradiol (Okuda *et al.* 2001). In the case of luteinizing TC, the inhibition of forskolin-induced P4 production by BMP6 appeared to operate through a different mechanism from that in luteinizing GC; forskolin-induced upregulation of *STAR* mRNA was abolished but neither *CYP11A1* nor *HSD3B1* transcript levels were affected. In addition, BMP6 upregulated basal expression of *CYP17A1*, consistent with an anti-luteinization role, since thecal androgen synthesis is known to fall sharply during luteinization (Meidan *et al.* 1990, Mamluk *et al.* 1998) and androstenedione levels were undetectable in 'basal' conditioned media from these cells.

The proposed role for activin in delaying the onset of follicle atresia and/or luteinization referred to above was based on the finding that activin enhanced *CYP19A1* activity and oestradiol production, while inhibiting P4 secretion by non-luteinized GC (Hutchinson *et al.* 1987, Shukovski *et al.* 1991). In a similar manner BMP4 and -7 enhanced FSH-stimulated steroidogenesis in cultured rat GC (Shimasaki *et al.* 1999) and BMP2 enhanced oestradiol production in cultured sheep GCs (Souza *et al.* 2002). Likewise, BMP2, -4, -6 or -7 increased basal and IGF-induced oestradiol production by non-luteinized bovine (Glister *et al.* 2004) and ovine

(Campbell *et al.* 2006) GC while suppressing P4 production (Glister *et al.* 2004).

Experiments on non-luteinized human, rat and bovine TC have shown reduced LH- and/or forskolin-induced androgen production following treatment with activin (Hsueh *et al.* 1987, Hillier & Miro 1993, Wrathall & Knight 1995). More recently several BMPs were also shown to suppress androgen production by non-luteinized TC in a manner similar to activin A (human: Dooley *et al.* 2000, bovine: Glister *et al.* 2005; ovine: Campbell *et al.* 2006). In two of these reports (Dooley *et al.* 2000, Glister *et al.* 2005) decreased androgen production was associated with an increase in P4 production, evidently due to a profound reduction in *CYP17A1* expression and 17 α hydroxylase activity.

The ability of BMP6 to reduce P4 secretion by luteinized bovine GC accords with studies showing anti-P4 effects of several BMPs (including BMP6) on non-luteinized bovine GC (Glister *et al.* 2004). However, the negative effect of BMP6 on P4 output by luteinized TC contrasts with the positive effect of BMP4, -6 and -7 on P4 output by non-luteinized TC (Glister *et al.* 2005). The likely explanation for this is the divergent effect on expression of *CYP17A1* that was upregulated by BMP6 in luteinized TC (present study) but downregulated by BMPs in non-luteinized TC (Glister *et al.* 2005). By blocking conversion to androgen, an acute loss of *CYP17A1* could lead to a net increase in P4 despite a partial reduction in *STAR*, *CYP11A1* and *HSD3B1*. In contrast to the positive effect of BMP6 on cell number in non-luteinized bovine GC and TC cultures (Glister *et al.* 2004, 2005), no effect of BMP6 on cell number was observed in this study on luteinized GC and TC. Consistent with the activin A effects on luteinized GC and TC cells similar inhibitory effects of activin have been reported on P4 secretion by cultured monkey luteal cells (Brannian *et al.* 1992) and on basal and hCG-induced P4 secretion from human granulosa-lutein cells (Rabinovici *et al.* 1990, Di Simone *et al.* 1994).

It is well established that follistatin, through its activin-binding ability, can oppose the effects of activin; more recent evidence indicates that follistatin can also bind to and neutralize the effects of several BMPs (see Shimasaki *et al.* (2004), Knight & Glister (2006)). The present finding that follistatin antagonized the suppressive effect of activin A and, to a lesser degree, BMP6 on P4 secretion by both cell-types supports this. It should be noted, however, that addition of follistatin alone did not enhance either 'basal' or forskolin-induced P4 secretion by luteinizing GC or TC. While this seems to contradict the earlier suggestion that follistatin has a positive role in promoting follicular atresia and/or luteinization (Shukovski *et al.* 1991, Findlay 1993, Knight & Glister 2003) it is possible that endogenous levels of follistatin were already sufficiently high to neutralize *endogenous* activin A (or related ligand?) produced by these *in vitro* luteinized cells. Direct measurement of immunoreactive

activin A and follistatin concentrations in luteinized GC-conditioned media indicated a 50% excess of follistatin over activin A lending some support to this explanation.

The ability of follistatin to reduce the activin A-induced decline in P4 production by luteinizing bovine GC and TC concurs with findings in human granulosa-lutein cells (Cataldo *et al.* 1994) and with the ability of follistatin to block activin A-induced phosphorylation of Smad-2 in non-luteinized bovine GC (Glister *et al.* 2004). Taken together with the abundant expression of follistatin transcript in both luteinized GC and bovine CL, this suggests a role of this activin-binding protein in promoting P4 production.

BMP6 increased follistatin production from luteinized GC resulting in a decreased activin A:follistatin mass ratio. This is in accordance with a previous study on non-luteinized bovine GC showing that BMPs enhanced basal and IGF-induced secretion of follistatin while inhibiting basal and IGF-induced P4 secretion (Glister *et al.* 2004). Previously Tuuri *et al.* (1994) reported that the expression of follistatin was up-regulated by hCG in human granulosa-lutein cells, known to express considerable amounts of activin β A, and presumably activin protein. In future studies, it would be of interest to examine whether expression of other binding proteins (such as noggin, chordin and gremlin) is regulated by LH/forskolin and BMP ligands in bovine granulosa-lutein and theca-lutein cells since these are likely to play a more prominent role than follistatin in modulating BMP action in these relatively activin-deficient cells. It would also be of interest to corroborate the present findings from the *in vitro* luteinization model used here, by evaluating the actions and interactions of BMPs, activins and their binding proteins on primary luteal cell cultures (i.e. derived from tissue in which luteinization has occurred *in vivo*).

In conclusion, these findings provide evidence to support inhibitory roles for BMP/activin signalling in the follicular-luteal transition in cattle. Both BMP6 and activin A inhibited P4 production by luteinizing TC and GC but BMP(s) are likely to play a more prominent role as their expression is maintained in bovine CL tissue whereas expression of activin β A subunit is greatly diminished relative to that in follicular GC.

Materials and Methods

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

Ovaries and isolation of GC and TC

Ovaries from cattle slaughtered at random stages of the oestrous cycle were collected from an abattoir and transported to the laboratory in medium-199 supplemented with 1% (v/v)

antibiotic antimycotic solution. Samples of CL tissue (early, mid- and late-luteal phase based on criteria of Ireland *et al.* 1980) were removed and snap frozen for subsequent RNA isolation. Follicles, 4–6 mm in diameter, were dissected out, hemisected and GC and theca interna layers were recovered as described previously (Glistner *et al.* 2005). For *ex vivo* analysis of mRNA transcripts, individual samples of granulosa and theca interna were snap frozen for subsequent RNA purification. For cell culture experiments, GC and theca interna layers pooled from ~50 follicles per culture were further processed as described by Glistner *et al.* (2001, 2005) to obtain individual cell suspensions.

Cell culture

Culture medium used 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 µg/ml apo-transferrin, 5 ng/ml sodium selenite, 0.1% (w/v) BSA and 5% (v/v) FBS. Culture medium used for GC was also supplemented with 10^{-7} M androstenedione. GC and TC were routinely seeded at a density of 10^4 viable cells/50 µl culture medium, into 96-well tissue culture plates (Nunc, Life Technologies Ltd) containing 200 µl/well pre-equilibrated culture medium. Culture plates were incubated in a water-saturated atmosphere of 5% CO₂ and 95% air at 38.5 °C for a period of six days. Cell-conditioned medium was removed every 48 h and wells replenished with fresh medium containing treatments (see below). Conditioned media was stored at –20 °C for hormone immunoassays. At the end of the 144 h culture period viable cell number was determined using neutral red assay (Campbell *et al.* 1996, Glistner *et al.* 2001). To provide a comparison between 'luteinized' (serum-treated) and 'non-luteinized' (serum-free) phenotypes, in several experiments GC and TC were split into two batches, one of which was cultured as described above, while the other was cultured in the same medium without FBS.

RNA isolation from cultured cells

In culture experiments in which total RNA was to be extracted for PCR analysis, cells were seeded into 24-well plates (10^5 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

Forskolin (10 mM stock solution) was prepared in DMSO and further diluted in sterile culture medium (without serum and androstenedione) to a final concentration of 10 µM. Control wells received an appropriately matched volume of DMSO. Other treatments included recombinant human (rh) BMP6 (0, 2, 10 and 50 ng/ml; R&D Systems, Abingdon, Oxfordshire, UK), rh activin A (0, 2, 10 and 50 ng/ml; R&D Systems), and rh follistatin-288 (0, 20, 100 and 500 ng/ml; National Hormone and Pituitary Program, Torrance, CA, USA). Follistatin was tested in the presence and absence of 50 ng/ml activin A and

20 ng/ml BMP6. All treatments, except forskolin, 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 P4 in luteinized GC- 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 coefficients of variation (CV) were 8% and 10% respectively. Concentrations of oestradiol-17β and androstenedione in selected cell-conditioned media samples were determined by direct RIA as described previously (Glistner *et al.* 2001, 2005). Detection limits were 2 and 50 pg/ml respectively and intra- and inter-assay CV were less than 10%. Activin A concentrations in selected GC-conditioned media samples were measured using the two-site ELISA (Knight *et al.* 1996). The detection limit of the assay was 4 pg/ml and intra- and inter-plate CV were less than 10%. Follistatin concentrations in selected GC conditioned media samples were determined using two-site ELISA (Tannetta *et al.* 1998). The detection limit of the assay was 60 pg/ml and intra- and inter-plate CV were less than 11%.

Purification of RNA, cDNA synthesis and real-time PCR

Total RNA was isolated from cultured cells and tissue samples using a standard acid guanidium thiocyanate–phenol–chloroform extraction method. Briefly, cell monolayers were directly lysed in 0.5 ml/well Tri Reagent (Sigma UK Ltd) while frozen tissue samples were homogenized (Ultra-Turrax T8;) for 15–20 s in 20 volumes of Tri-Reagent. After aqueous phase separation, RNA was precipitated in isopropanol, 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 with an RNase-free DNase kit (RQ1; Promega UK Ltd). The Tri Reagent extraction process was repeated and the final RNA pellet 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 RNA template using the Reverse-iT RT kit (used according to manufacturers protocol; Abgene, Epsom, Surrey, UK) in a 20 µl reaction primed with random hexamers.

Primers were designed to amplify target sequences based on criteria set by the ABI PRISM primer express software (version 1.5). Primer sequences and Entrez accession numbers are shown in Table 2. 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 log-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× Master Mix (Qiagen). Samples were processed for 40 cycles on an ABI PRISM 7700 Sequence Detection System (Perkin–

Table 2 List of primers used for RT-qPCR.

Target	Accession number	Forward primer 5' to 3'	Reverse primer 5' to 3'	Amplicon size (bp)
BMP2	XM_866011.1	CCAGAGGCGATGCGGATTAGCA	TCCTTCCCATCGTGGCCAAAGT	101
BMP4	NM_001045877.1	TTTATGAGGTATGAAGCCCGCG	AGTTTCCACCGCTCACATTGTG	104
BMP6	XM_600972.2	GGCCCGTTAATCTGACTGTGACAA	TTGAGACCGCCGAACAAAGGA	108
BMP7	XM_612246.2	TGCAAGATAGCCACTTCTCACCAG	GGGATCTTGGAGAGATCAACCGGA	130
Act-βA	NM_174363.1	GAAGAGCCCGATGTCACCCAGC	TGCTGCTCTATCTCCACGTACCCG	113
BMPRIA	NM_001076800	TGGATTGCCCTTACTGGTTACGCA	CCAGCCATTACCCATCCACA	105
BMPRIB	XM_612088.2	AAAGTGGCTGGCGTGGCGAAAGGTAGCT	CCCTGCTTTGATCTGCAGCA	147
BMPRII	XM_617592.2	CCCACCTTTCGACCCCTGG	CCCGCAGTATTTCCTCCG	87
ActRIA	NM_176663.2	CATGCCCCCGAAAGTCTTGTATGA	GCACCTCCCAAGACAAAGTCAAA	102
ActRIIB	XM_586402.2	CATCAGCGTGTCTATCACACCGCC	CACCTGCGCTGGACAAAAGGG	156
ActRIIA	NM_174227.2	GCCAACAACCCGCAATCTCACA	TGCCAGCTCAAACTTAAAGCCAA	114
ActRIIB	NM_174495.2	ACAAAGCATCTATTGCCACAGGGA	CTCAACCCGAACAGCCAGGCCAA	104
STAR	NM_174189	TTTTTCTGGGTCTGACAGCGTC	ACAACCTGATCTGGTCTGCACC	103
CYP11A1	NM_176644	CAGTGCCCTCTGCTCAACGTCC	TTATTGAAATGTGTCCTCCGCG	99
3βHSD	NM_174343.2	GCCACTAGTACTCTTTCCAAACGCG	TGGTTTCTGCTGGCTTCTCTCC	111
CYP17A1	NM_174304	GACAAAGGCACAGCGTTGTGGTCA	TGATCTGCAAGACAGACTGGCATG	301
CYP19A1	NM_174305.1	CGCCACTGAGTTGATTTTGTCTGAGA	TAAGCTTTGCGGATGACCAAGTC	301
Oxytocin	NM_176855.1	GCAGCTCTGCACCATGGC	GGCAGTTCTGAATGTAGCAGCGG	89
β-actin	BC102948.1	ATCCACCATCGGCAATGAGCGGTT	CGGATGTCCAGCTCACACACTTCATG	128

Elmer-Applied Biosystems, Warrington, UK) 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.

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 experimental all 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 (GC, TC, CL) 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 = $2^{(-\Delta\Delta C_t)}$.

Statistical analysis

To reduce heterogeneity of variance, hormone data were log-transformed prior to statistical analysis. QPCR data were analysed as $\Delta\Delta C_t$ values before conversion to fold-difference values. Combined results from three or four independent culture experiments were analysed using ANOVA and provided a significant F ratio was obtained, *post hoc* pairwise comparisons were made using Fisher's protected least significant difference test. Unless otherwise stated, results are presented as arithmetic means $\pm$ S.E.M.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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