Granulosal and thecal expression of bone morphogenetic protein- and activin-binding protein mRNA transcripts during bovine follicle development and factors modulating their expression in vitro

Claire Glister, Leanne Satchell and Phil G Knight

School of Biological Sciences, University of Reading, Hopkins Building, Whiteknights, Reading RG6 6UB, UK
Correspondence should be addressed to P G Knight; Email: p.g.knight@reading.ac.uk

Abstract

Evidence supports local roles for transforming growth factor β superfamily members including activins and bone morphogenetic proteins (BMP) in follicle development. Access of these ligands to signalling receptors is likely modulated by extracellular binding proteins (BP). In this study, we compared ex vivo expression of four BPs (chordin, gremlin, noggin and follistatin) in granulosal (GC) and theca interna (TC) compartments of developing bovine antral follicles (1–18 mm). Effects of FSH and IGF on BMP and BP expression by cultured GC, and effects of LH and BMPs on BP expression by cultured TC were also examined. Follicular expression of all four BP transcripts was higher in GC than TC compartments (P < 0.001) a finding confirmed by immunohistochemistry. Follicle category affected (P < 0.01) gremlin and follistatin mRNA abundance, with a significant cell-type × follicle category interaction for chordin, follistatin and noggin. Noggin transcript abundance was lower (P < 0.05) in GC of large 'E-active' than 'E-inactive' follicles while follistatin mRNA level was higher (P < 0.01). FSH enhanced CYP19, FSHR, INHBA and follistatin by GC without affecting BMP or BMP–BP expression. IGF increased CYP19 and follistatin, reduced BMP4, noggin and gremlin but did not affect chordin or FSHR mRNA levels. LH increased TC androgen secretion but had no effect on BMP or BP expression. BMPs uniformly suppressed TC androgen production whilst increasing chordin, noggin and gremlin mRNA levels up to 20-fold (P < 0.01). These findings support the hypothesis that extracellular BP, mostly from GC, contribute to the regulation of intrafollicular BMP/activin signalling. Enhancement of thecal BP expression by BMP implies an autoregulatory feedback role to prevent excessive signalling.

Introduction

During recent years a wealth of investigation has been directed at transforming growth factor β (TGFβ) superfamily members, in particular the largest subset within the family, the bone morphogenetic proteins (BMP). More than 20 BMPs have been identified in vertebrates and non-vertebrate species and these multifunctional proteins contribute to the regulation of cellular proliferation, differentiation and apoptosis in a variety of tissues, including the ovary (Miyazono et al. 2001, Chen et al. 2004, Shimasaki et al. 2004, Knight & Glister 2006). A number of BMPs are expressed in theca cells (TC), granulosa cells (GC) and oocytes in a cell- and follicle stage-specific manner and evidence supports their involvement in the regulation of folliculogenesis, ovolation and corpus luteum function (reviews: Shimasaki et al. 2004, Juengel & McNatty 2005, Knight & Glister 2006). Recently, Glister et al. (2010) reported a survey of BMP2, BMP4, BMP6 and BMP7 expression in TC and GC throughout bovine antral follicular development and related this to the pattern of expression of type 1 and type 2 signalling receptors. Significant cell-type and follicle stage-dependent changes in expression of individual ligands and receptors were revealed but, in general, changes in receptor transcript abundance were relatively modest, supporting the notion that additional levels of control may be operational to modulate BMP signalling at the local level.

Despite the likely functional importance of BMP signalling within the ovary, few studies have addressed the potential role of extracellular BMP-binding proteins (BP) in modulating BMP access to their signalling receptors on different ovarian cell-types. Much of the research on BMP-BPs has been in the developmental biology field where their crucial roles have been identified in establishing BMP morphogen gradients responsible for embryonic patterning (Thomsen 1997, Balemans & Van Hul 2002, Chen et al. 2004, Kishigami & Mishina 2005). To our knowledge no comprehensive surveys of BMP-BP expression throughout ovarian follicle growth have yet been carried out in any species.
Similarly, information is lacking on the factors (systemic or intraovarian) that potentially modulate expression of BMP-BPs by follicle cells. As such, with the exception of follistatin (predominantly an activin-BP), the significance of secreted BMP-BPs as intrafollicular modulators of BMP action has received little attention.

Follistatin was first isolated by Robertson et al. (1987) from bovine follicular fluid based on its ability to suppress FSH secretion from pituitary cells and was subsequently shown to function by binding activin with high affinity, thus neutralising its bioactivity (Nakamura et al. 1990). Follistatin has also been shown to bind directly to several other TGFβ superfamily members, including inhibin (Shimonaka et al. 1991) and BMP2, BMP4, BMP6, BMP7 and BMP15 (Otsuka et al. 2001, Anmor et al. 2002, Glister et al. 2004) albeit with lower affinity. Noggin, a homodimeric glycoprotein, was first isolated from Xenopus embryonic tissues where it blocks BMP action in the dorsal organising centre (Lamb et al. 1993, Smith et al. 1993). Noggin displays a high-affinity association with BMP2 and BMP4, but less so with BMP7 and BMP6; binding directly prevents interaction of ligand with type 1 receptor (Zimmerman et al. 1996). Chordin, a cysteine-rich secreted protein, was also first identified in Xenopus; located in the dorsal organising centre (Sasai et al. 1994, 1995). It also binds directly to BMP2, BMP4 and BMP7 to prevent BMP–receptor interaction. However, the affinity of chordin for BMP4 is some tenfold less than its counterpart noggin (Piccolo et al. 1996). Gremlin belongs to a subfamily of BMP antagonists all of whom contain a characteristic cysteine-rich domain (CAN domain) containing a particular consensus sequence (Avsian-Kretchmer & Hsueh 2004). As with chordin and noggin, gremlin was first identified in a Xenopus screen for genes with dorsalising activity (Hsu et al. 1998) and has since been shown to regulate murine limb, kidney and lung development (Zuniga et al. 1999, Khokha et al. 2003, Michos et al. 2004). Gremlin can bind to and inhibit BMP2 and BMP4, but does not interact with activin (Hsu et al. 1998). Interestingly, expression of noggin and gremlin is enhanced by the very BMPs they act to inhibit, indicative of negative feedback loops in operation (Kameda et al. 1999, Pereira et al. 2000). In addition, certain isoforms of follistatin, noggin and chordin bind to cell-surface associated heparin-sulphate proteoglycans. This association may serve to limit diffusion and ‘action-range’ of either antagonist and/or ligand. It may also promote endocytosis and lysosomal degradation of the target ligand (Sugino et al. 1993, 1997, Jasuja et al. 2004).

Given the current lack of information on potential roles of extracellular BMP-BPs in the ovary, the aims of this study were to: i) use RT-qPCR to quantify chordin, noggin, gremlin and follistatin mRNA expression in thecal and granulosa compartments of developing bovine antral follicles; ii) investigate whether in vitro expression of the above BP transcripts by cultured TC and GC is modulated by gonadotrophins or insulin-like growth factor 1 (IGF1) and iii) examine the effect of exogenous BMP2, BMP4, BMP6 and BMP7 on expression of BP transcripts by cultured TC.

Results

More detailed characteristics of the follicle sample set analysed in this study, including follicular fluid steroid concentrations and relative expression of GC and TC ‘marker’ transcripts including FSHR, CYP19, LHCGR and CYP17, are documented in our recent companion paper focusing on follicular expression of BMP ligands and receptors (Glister et al. 2010).

mRNA expression profiles for gremlin, chordin, follistatin and noggin in developing follicles

mRNA transcripts for each of the four BPs were readily detectable in all GC and TC samples with average qPCR Ct values of 26, 28, 26 and 21 for noggin, chordin, gremlin and follistatin respectively. In Fig. 1, the relative abundance of mRNA transcripts for all four BPs examined was greater in GC than TC (two-way ANOVA: effect of cell-type: P<0.0001). Gremlin expression was highest in GC of 1–2 mm follicles and declined progressively as follicles developed to 11–18 mm (approximately fivefold reduction; P<0.002); no difference in GC gremlin expression was evident between large oestrogen-active (LEA) and large oestrogen-inactive (LEI) follicles.

Although there was no overall effect of follicle category on the relative abundance of chordin mRNA, a highly significant follicle category×cell-type interaction (P<0.0001) justified a series of post hoc pairwise comparisons within cell-type. In GC, chordin mRNA abundance fell (approximately sixfold; P<0.05) as follicles grew from 1–2 to 7–8 mm but then increased again (approximately sixfold; P<0.01) between the 9–10 and 11–18 mm (LEA) follicle categories; GC chordin expression did not differ significantly between LEA and LEI follicles although the mean value was 50% lower in LEI category. In contrast to GC, chordin expression in the TC layer increased from 1–2 to 9–10 mm follicles (approximately twofold; P<0.05), before falling (approximately threefold; P<0.01) to its lowest level in LEA follicles; TC chordin expression was approximately fivefold higher (P<0.01) in LEI than in LEA follicles.

Follistatin expression patterns in GC and TC were similar to those for chordin: in GC a progressive decrease between 1–2 and 9–10 mm was followed by a large increase (~14-fold; P<0.001) in LEA follicles. LEI follicles had approximately sevenfold less follistatin mRNA than LEA follicles (P<0.01). In contrast to GC, follistatin expression in TC increased between 1–2 and 9–10 mm follicles, reaching a relative abundance comparable with that in GC of 9–10 mm follicles. Transcript level then fell (P<0.05) to its lowest level in
Correlation between different transcripts and follicle development

The corresponding correlation matrix for TC (Fig. 2B) showed fewer significant relationships than observed for GC. However, there were strong positive correlations between CYP17 and LHCGR (r = 0.65; P < 0.0001), between follistatin and chordin (r = 0.59; P < 0.0001) and between noggin and gremlin expression (r = 0.72; P < 0.0001). Conversely, a weak negative correlation was evident between LHCGR and noggin expression in TC (r = −0.35; P < 0.05).

Immunohistochemical localisation of BPs in ovary sections

To reinforce the above mRNA expression data, ovarian immunohistochemical staining was carried out using antibodies against each of the four BPs. As shown in Fig. 3, immunoreactivity corresponding to all four BPs was evident in follicles. In each case the intensity of staining was much greater in the GC compartment although weak immunoreactivity was also present in TC and stromal compartments.

Effects of FSH and IGF on expression of CYP19, FSHR, INHBA, BMP and BMP-BP transcripts by cultured GC

Treatment of cultured bovine GC with an optimal dose level of FSH (0.33 ng/ml) increased oestradiol (E2) secretion ~40-fold (22.4 ± 6.7 vs 0.50 ± 0.12 ng/ml in controls; P < 0.001) and promoted a marked upregulation of CYP19 expression (~35-fold; P < 0.01) that was accompanied by increases in FSHR (approximately fivefold; P < 0.05), follistatin (approximately sixfold; P < 0.01) and INHBA (~11-fold; P < 0.01) mRNA abundance (see Fig. 4). However, FSH did not affect abundance of BMP2, BMP4, BMP6, BMP7, chordin, gremlin or noggin transcripts. Treatment of GC with IGF increased E2 secretion approximately sixfold (3.27 ± 1.08 vs 0.50 ± 0.12 ng/ml in controls; P < 0.01) and, as shown in Fig. 4, increased the abundance of transcripts for CYP19 (~12-fold; P < 0.05) and follistatin (approximately threefold; P < 0.05) while decreasing expression of BMP4 (approximately tenfold; P < 0.01), noggin (approximately threefold; P < 0.05) and gremlin (approximately threefold; P < 0.05). IGF did not affect the abundance of FSHR, INHBA, BMP2, BMP6, BMP7 or chordin mRNA.

Effect of LH on expression of BMP and BMP-BP transcripts by cultured TC

LH treatment resulted in a fourfold increase in A4 secretion by cultured TC (124.1 ± 26.8 vs 29.5 ± 7.0 ng/ml in
controls; \( P<0.01 \) but did not affect the abundance of transcripts for BMP2, BMP4, BMP6, BMP7, chordin, follistatin, noggin or gremlin (data not shown).

**Effects of BMPs on expression of steroidogenesis-related transcripts by cultured TC**

Figure 5 shows that treatment of bovine TC with BMP2, BMP4, BMP6 and BMP7 all promoted a profound suppression of CYP17 expression under both basal and LH-stimulated conditions (\( \sim 200 \)-fold reduction; \( P<0.0001 \)). Likewise, BMPs reduced expression of other key transcripts involved in the steroidogenic pathway (\( \text{LHCGR}, \text{STAR}, \text{CYP11A1} \) and \( \text{HSD3B8} \)) although to much lesser extents than the suppression of CYP17.

**Effects of BMPs on expression of BMP-BP transcripts by cultured TC**

Figure 6 shows that BMP2, BMP4, BMP6 and BMP7 had divergent effects on expression of chordin, noggin and gremlin but did not modify follistatin mRNA abundance. Under both basal and LH-stimulated condition levels of chordin and gremlin mRNA were increased (2- to 11-fold; \( P<0.05 \)) by BMP2, BMP4 and BMP6 but not by BMP7. Under LH-stimulated conditions each BMP also increased the abundance of noggin mRNA with the greatest increase being elicited by BMP6 (\( \sim 20 \)-fold; \( P<0.01 \)).

**Discussion**

This study has provided novel information on the mRNA expression pattern of four different activin/BMP-BPs in granulosal and thecal compartments of developing bovine antral follicles. To our knowledge, such information has not yet been documented for any species. Interestingly, the relative abundance of all four BP transcripts was greater in GC compared with TC layers throughout antral follicle development. Immuno-histochemistry confirmed that the corresponding proteins were also more abundant in GC than TC compartments. In contrast, BMP4, BMP6 and BMP7 transcripts were shown to be more abundant in TC than GC (Glister et al. 2010). We have previously examined the effect of exogenous BMP4, BMP6 and BMP7 on bovine GC and TC cultured \( \text{in vitro} \) (Glister et al. 2004, 2005) and found TC to be much more sensitive to BMP action (\( \text{IC}_{50} \sim 1 \text{ ng/ml} \)), exhibiting a >100-fold decrease in LH-induced androgen secretion at the greatest dose tested (50 ng/ml), compared with a more modest threefold maximal BMP-induced increase in IGF-stimulated oestrogen secretion by GC treated with 50 ng/ml BMP (\( \text{EC}_{50} \sim 5 \text{ ng/ml} \)). From the present findings, this relative lack of sensitivity of GC versus TC to exogenous BMPs could be explained, at least in part, by the differential expression patterns of BMP-BPs found between the two cell-types – the greater levels of BPs expressed by GC antagonising the signalling activity of exogenous BMPs to a greater extent. Thus, in an \( \text{in vivo} \) ‘whole follicle’ context it is conceivable that the GC compartment is relatively resistant to local BMP signalling (BMP4, BMP6 and BMP7 from TC layer; BMP2 mostly from GC layer) due to the presence of higher levels of extracellular BPs. As such, this preliminary evidence supports the concept of BMP signalling gradients across the follicle wall, akin to the morphogen gradients clearly implicated in key aspects of embryonic patterning such as dorsalisation, segmentation and limb bud formation (Thomsen 1997, Balemans & Van Hul 2002, Chen et al. 2004, Kishigami & Mishina 2005).

Figure 2 Correlation matrices for (A) granulosal layer and (B) theca interna layer of developing follicles (\( n=47^* \)) showing associations between follicle diameter, follicular fluid \( \text{E}_{2}:\text{P}_{4} \) ratio and relative abundance of selected mRNA transcripts including the four BMP/activin-binding proteins. Statistically significant \( r \) values are in bold with negative correlations in parenthesis. \( *P<0.05, **P<0.01 \) and \( ***P<0.001 \). Note that the same sample set was used in our previous study (Glister et al. 2010) to analyse expression of other transcripts including the four follicular ‘markers’ included in this correlation matrix (\( \text{LHCGR}, \text{FSHR}, \text{CYP19} \) and \( \text{CYP17} \)).
The finding of differential expression patterns of the four extracellular BPs throughout antral follicle progression suggests selectively regulated expression, as well as different biological roles. For instance, GC expression of follistatin was much greater in LEA than LEI follicles with the inverse seen for noggin. Notably, the previously reported GC expression patterns of INHBA (encoding activin A homodimer) and BMP2 in LEA versus LEI follicles (Glister et al. 2010) mirrored these changes. This supports the notion that activin induces follistatin expression (Tano et al. 1995, Zhang et al. 1997, Silva & Knight 1998) while BMP2 induces noggin expression (Gazzarero & Minetti 2007), both acting in an autoregulatory feedback manner to limit signalling by the respective ligands.

Intriguingly, gremlin mRNA level was maximal in GC of the smallest antral follicle stage examined (1–2 mm) and then declined progressively as follicle size increased. In contrast, the other three BPs showed more complex expression patterns but were all relatively low in 1–2 mm follicles. This raises the suggestion that gremlin has a specific role in these small, pre-selection antral follicles. Alternatively, local factors inductive for GC gremlin expression might be more abundant in small antral follicles. In this regard BMP2, BMP4 and GDF9 were found to increase gremlin expression by mouse GC in vitro (Pangas et al. 2004) and this study shows that BMP2, BMP4 and BMP6, but not BMP7, increase gremlin expression by cultured TC. However, as we report elsewhere (Glister et al. 2010) expression of BMP2, BMP4 and BMP7 showed little variation in bovine follicles from 1 to 8 mm in diameter. Moreover, expression of these BPs by cultured TC and GC was unaffected by LH and FSH, respectively, even though IGF selectively reduced GC expression of BMP4. Whilst expression of all four BPs was considerably lower in TC than GC, when comparing LEA versus LEI follicles specifically, some differences are apparent in TC. Gremlin, chordin and follistatin were more highly expressed in the TC of LEI follicles compared with LEA, whilst noggin remained unchanged. This might imply a role in neutralising factors involved in maintenance of the dominant follicle, a proposal that should be amenable to in vitro testing using an RNAi ‘knockdown’ approach.

To extend the above findings of spatiotemporal changes in follicular BMP-BP expression during antral follicle development, we conducted in vitro studies on isolated GC and TC to ascertain whether key trophic factors influenced expression of any of the four BPs, along with several key GC or TC markers. As anticipated, and consistent with other studies (Campbell et al. 1996, Gutierrez et al. 1997, Glister et al. 2001, Marsters et al. 2003), treatment of GC with FSH enhanced E2 secretion and promoted expression of CYP19, its own receptor (FSHR) and INHBA (encoding activin A subunit), while IGF raised E2 secretion and CYP19 without effecting FSHR or INHBA expression. Follistatin mRNA expression was also raised by both treatments, consistent with previous follistatin secretion data for FSH and IGF-treated bovine GC (Glister et al. 2001, 2003, 2006) and with findings in other species (see Findlay 1993). However, FSH had no effect on expression of the four BMPs examined or their antagonists – chordin, noggin and gremlin. Previously, in vivo PMSG treatment in mice was found to increase GC gremlin expression (Pangas et al. 2004). In contrast to the lack of effect on FSH on GC, treatment with IGF selectively reduced GC expression of BMP4, noggin and gremlin perhaps indicating a functional link between BMP4 and these two BPs.

As anticipated, treatment of cultured TC with their key trophic factor, LH, promoted a significant (sixfold) increase in androgen secretion. However, LH had no discernable effect on expression levels of any of the four BMPs or BPs analysed. Therefore, we infer that expression of BMPs and BMP-BPs by GC and TC from antral follicles is largely unaffected by gonadotrophin
stimulation, at least under the defined culture conditions used for this study. Further studies would be useful to ascertain whether gonadotrophins and other factors modulate TC/GC expression of BMP-BPs at other follicle stages, including preantral and luteinisation stages in cattle, and indeed other species.

Given the high responsiveness of cultured bovine TC to exogenous BMPs in terms of suppression of androgen secretion (Glister et al. 2005), we reasoned that this would be a robust model to investigate whether the different BMPs also modulated expression of any of the four extracellular BPs. We first confirmed that BMP2, BMP4, BMP6 and BMP7 drastically reduced expression of the key enzyme involved in androgen biosynthesis CYP17 (>100-fold), with lesser effects on other components of the thecal steroidogenic pathway, including LHCR (approximately sixfold), STAR (approximately twofold), CYP11A (approximately}
sixfold) and HSD3B (approximately twofold). Whilst all four BMPs caused a modest increase in expression of chordin and gremlin, (independent of LH treatment) only one, BMP6, promoted a marked (greater than tenfold) increase in noggin expression. None of the four BMPs tested altered follistatin expression by TC, a finding at variance with previous observations in bovine GC that show a clear BMP-induced upregulation of follistatin secretion (Glister et al. 2004).

Previous studies on various cell-types have reported the ability of BMPs to induce expression of their own antagonists. For instance, BMP2, BMP4 and BMP6 have each been shown to induce noggin expression by osteoblasts, at the mRNA and protein level (Gazzerro & Minetti 2007) even though noggin appears to bind to BMP2 and BMP4 with much greater affinity than to BMP6 (Pierre et al. 2005). Interestingly, the present data indicate that, of the four ligands we tested, only BMP6 has the ability to induce noggin expression by bovine TC. In a similar manner BMP2 and BMP4, which are antagonised by gremlin, have been shown to induce gremlin expression by mouse GC (Pangas et al. 2004) and rat osteoblasts (Pereira et al. 2000). In agreement, we report here that BMP2 and BMP4 can also increase gremlin expression by bovine TC in vitro. BMP6 was similarly effective at promoting gremlin expression whereas BMP7 had little or no effect. Chordin expression has previously been reported to be non-inducible by BMPs (Canalis et al. 2003), despite binding directly to and inhibiting BMP2, BMP4 and BMP7. However, in our study chordin expression was raised by all four BMPs tested. Previously, BMPs were shown to enhance secretion of follistatin by both non-luteinised (Glister et al. 2004) and luteinised (Kayani et al. 2009) bovine GC. In contrast, this study found no effect of BMPs on TC expression of follistatin mRNA. Since the binding affinity of follistatin for activin is much greater than that for BMPs (Otsuka et al. 2001, Amthor et al. 2002, Glister et al. 2004) it would be anticipated that activin is a more effective inducer of follistatin expression and, indeed, activin-induced follistatin expression has been documented for various cell-types including rat GC (Tano et al. 1995), hepatocytes (Zhang et al. 1997) and bovine cumulus–oocyte complexes (Silva & Knight 1998), but not human granulosa–lutein cells (Tuuri & Ritovs 1995).

Apparent differences in the ability of individual BMPs to modulate expression of different BPs, evident from the studies referred to above, can partially be attributed to the different cell-types and species under investigation, and to differences in experimental design and culture conditions. Taken together, however, it is increasingly evident that local autoregulatory loops operate at the target tissue/cell level with BMPs capable of inducing expression of their own extracellular antagonists. In turn, these act primarily as ‘ligand traps’ to sequester BMPs and limit their signalling range by blocking interaction with signalling receptors (Reddi 2001, Gazzerro & Canalis 2006, Walsh et al. 2010).

Whilst it is well established that follistatin has an intraovarian role in antagonising the effects of activin on TC, GC, luteal cells and oocytes (reviews: Findlay 1993, Welt et al. 2002, Knight & Glister 2006) what evidence is there to support a functional role of BMP-BPs in the modulation of follicular function? Noggin has been shown to block the suppressive effect of BMP2 and BMP4, but not BMP6, on progesterone secretion by sheep GC in vitro (Pierre et al. 2004). Likewise, gremlin and gremlin 2 (aka PRDC; protein related to DAN and cerberus) reversed the BMP2/BMP4-induced suppression of progesterone secretion by rat GC (Sudo et al. 2004). Gremlin and chordin also antagonised the suppressive action of BMP4 and BMP7, respectively, on androgen secretion by bovine TC in vitro (Glister et al. 2005). Moreover, in the absence of BMP treatments, both BPs promoted TC androgen secretion

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**Figure 6** Effects of BMP2, BMP4, BMP6 and BMP7, alone and in combination with LH, on the relative abundance of mRNA transcripts for chordin, gremlin, noggin and follistatin in primary cultures of bovine theca interna cells. Values are means and bars indicate S.E.M. (n=4 independent cultures). Results of two-way ANOVA are summarised.
indicating that endogenous BMPs negatively regulate androgen production in an autocrine/paracrine manner.

Given the marked inhibitory effect of activins and BMPs on thecal androgen production, it has also been suggested (Glister et al. 2005) that compromised intraovarian activin and/or BMP signalling, perhaps due to overexpression or activity of extracellular BPs could by a contributory factor in polycystic ovarian syndrome (PCOS), a condition associated with ovarian androgen excess and impaired preovulatory follicle development (Mason 2000). Consistent with this notion, there have been several reports of raised circulating follistatin levels in women with PCOS (Norman et al. 2001, Chen et al. 2009). However, we are not aware of any evidence implicating altered expression of other extracellular BMP-BPs in the aetiology of PCOS.

In summary, this study has revealed that four different secreted BMP/activin-BPs (gremlin, noggin, chordin and follistatin) show divergent expression patterns in GC and TC compartments during bovine antral follicle development. With the exception of FSH-induced follistatin expression by GC, gonadotrophins do not appear to modulate GC and TC expression of either BMPs or BMP-BPs. In contrast, IGF induces follistatin expression whilst suppressing BMP4, noggin and gremlin expression by GC. In addition, we have shown that treatment of TC with BMPs can differentially regulate expression of their own BPs, consistent with the notion of local autoregulatory feedback loop(s) controlling BMP signalling. Taken together, these findings support the hypothesis that extracellular BPs play important roles in regulating BMP signalling in ovarian follicles. Further studies are needed to delineate the relative contributions of individual intraovarian BMP ligands and BPs in this complex and dynamic organ.

Materials and Methods

Unless stated otherwise, all media and reagents were purchased from Sigma UK Ltd or Fisher Scientific Ltd (Loughborough, UK).

Ovaries and isolation of GC and TC for gene expression analysis

As described in our recent companion paper (Glister et al. 2010) ovaries from non-pregnant cattle slaughtered at random stages of the oestrous cycle were collected from an abattoir and oestrous cycle stage (I–IV) was estimated by morphological appearance of the corpus luteum (Ireland et al. 1980). Only ovaries judged to be from cattle in the mid- to late-luteal phase (stages II and III; days 5–17) were selected for follicle dissection. Follicles ranging in diameter from 1 to 18 mm were dissected out, sorted by size and their GC, TC layers and follicular fluid recovered for analysis. Individual follicles in the 1–2 mm (ten follicles per pool, n=6 pools collected, n=4 pools analysed), 3–4 mm (six follicles per pool; n=7 pools collected, n=5 pools analysed) and 5–6 mm (six follicles per pool; n=6 pools collected, n=5 pools analysed) size categories were combined for further analysis while all follicles >7 mm in diameter were processed and analysed individually (n=7–9 per category). The number of follicle samples included in the analysis was lower than the number collected because only 80% of samples passed our quality control criteria based on comparison of GC/TC-specific ‘marker’ transcript levels (see Glister et al. (2010) for further details). Isolated GC and TC were homogenised in 0.5 ml of Tri-reagent (Sigma UK Ltd) and stored at −80 °C for subsequent RNA purification. The size range of antral follicles investigated spanned key points in bovine follicular development, including ‘cyclic recruitment’ at 3–5 mm, selection/deviation at ~6 to 8 mm, LH/CGR acquisition by GC at 9–10 mm and dominance at 11–18 mm (Ireland et al. 2000, Mihm & Austin 2002, Gintther et al. 2003). The final follicle size category was further subdivided based on follicular fluid E:P ratio reflecting presumptive healthy, LEA dominant follicles (E:P ratio >1) and LEI, most likely undergoing regression (E:P ratio <1).

GC and TC culture

For in vitro experiments, GC and 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 was McCoy’s 5A supplemented with 1% (v/v) antibiotic–antimycotic solution, 10 ng/ml insulin (bovine pancreas), 2 mM L-glutamine, 10 mM HEPES, 5 μg/ml apotransferrin, 5 ng/ml sodium selenite and 0.1% (w/v) BSA. For GC cultures medium was supplemented with androstenedione (10−7 M). Cells were plated at 75 000 viable cells/well in 96-well plates (Nunclon, Life Technologies Ltd) and cultured for 6 days at 38.5 °C. Media were removed every 48 h and replaced with fresh media containing treatments (see below). Conditioned media were stored at −20 °C for steroid immunoassays. At the end of culture viable cell number was determined using neutral red assay (Campbell et al. 1996, Glister et al. 2001).

RNA isolation from cultured cells

In culture experiments in which total RNA was to be extracted for qRT-PCR analysis, cells were seeded into 24-well plates (0.5 × 10⁶ cells/ml) with three replicate wells per treatment. At the end of culture lysates were prepared using Tri-reagent and pooled lysates from replicate wells stored at −80 °C.

Culture treatments

Ovine FSH (oFSH-19SIAPP) and LH (oLH-S-16) were provided by the NHPP (Torrance, CA, USA), LR3-IGF-1 was purchased from Sigma and recombinant human (rh) BMP2, BMP4, BMP6 and BMP7 were purchased from R&D Systems (Abingdon, UK). Treatments were sterilised using 0.2 μm filters before further dilution in sterile culture medium. Treatments were added at 25 μl/well and an equal volume of blank medium added to control wells.
Steroid assays
Concentrations of P_4 were determined by ELISA (Sauer et al. 1986, Bleach et al. 2001). The detection limit was 20 pg/ml and intra- and inter-assay coefficient of variation (CV) were 8 and 10% respectively. Concentrations of E_2 were determined by RIA (Tannetta et al. 1997) with a detection limit of 2 pg/ml and intra- and inter-assay CV of 6 and 9% respectively. Concentrations of A4 were determined by RIA (Tannetta et al. 1997) with a detection limit of 100 pg/ml and intra- and inter-assay CV of 7 and 10% respectively.

RNA isolation, cDNA synthesis and real-time PCR
Total RNA was isolated from tissue samples and cultured cells using Tri-reagent as described previously (Glisier et al. 2010). Potential genomic DNA contamination was removed with an RNase-free DNase kit (RQ1; Promega UK Ltd). RNA quantity and quality were evaluated by spectrophotometry at 260/280 nm. cDNA was synthesised from 1 μg of RNA using the Reverse-iT RT kit (Abgene, Epsom, UK; used according to manufacturer’s protocol) in a 20 μl reaction primed with random hexamers.

Primers (see Table 1) were designed using ABI PRISM primer express software (version 1.5; Perkin Elmer-Applied Biosystems, Warrington, UK) and validated using melt-curve analysis and agarose gel electrophoresis to verify that each primer pair generated a single amplicon of the predicted size. cDNA template log-dilution curves were used to demonstrate satisfactory PCR efficiency and linearity. PCR assays were carried out in a volume of 24 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 Quantitec SYBR Green QPCR 2X Master Mix (Qiagen). Samples were processed on an ABI PRISM 7700 thermal cycler (Perkin Elmer-Applied Biosystems) with cycling conditions: 15 min at 95°C (one cycle only) followed by 15 s at 95°C and 1 min at 60°C for 40 cycles.

The ΔΔCt method (Livak & Schmittgen 2001) was used to compare the relative abundance of each mRNA transcript. Ct values for each transcript in a given sample were first normalised to β-actin Ct value (uniform across experimental all groups: ANOVA P>0.2). For cell culture experiments the resultant ΔCt values for each treatment replicate were then normalised to the mean ΔCt value of the respective vehicle-treated control group. For tissue samples (GC and TC) ΔCt values for each transcript in a given sample were normalised to the mean ΔCt value for that transcript in all tissue samples. For graphical presentation ΔΔCt values were converted to fold-differences using the formula: fold-difference = 2^-ΔΔCt.

Immunohistochemistry
Immunohistochemical staining was performed on 6 μm sections of formalin-fixed, paraffin-embedded ovaries as described previously (Glisier et al. 2010). Ovaries from four cattle were processed and each antibody was used to stain at least two sections from each of four different ovaries. After microwave antigen retrieval using citrate buffer (10 mM, pH 6), sections were blocked for 1 h using either 2.5% (v/v) horse serum (supplied with anti-mouse/rabbit ImmPRESS Kit, Vector Laboratories, Peterborough, UK) or 10% (v/v) bovine serum (used with biotinylated anti-goat IgG and Vectastain ABC Elite kit; Vector Laboratories). 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: goat anti-mouse gremlin (R&D Systems) at 5 μg/ml, goat anti-mouse chordin (R&D Systems) at 5 μg/ml, rabbit anti-human noggin (Abcam Ltd, Cambridge, UK) at 5 μg/ml and rabbit anti-human follistatin (FP22; supplied by NIDDK, Baltimore, MD, USA) at 1:500 dilution. For immunodetection of rabbit primary antibodies, the ImmPRESS Universal anti-mouse/rabbit IgG HRP polymer-based detection system was used with diaminobenzidine substrate according to the manufacturer’s instructions. For immunodetection of goat primary antibodies, sections were incubated for 30 min with biotinylated bovine anti-goat IgG (5 μg/ml; Santa Cruz, Insight Biotechnology, Wembley, UK) before labelling with avidin–biotin HRP complex and diaminobenzidine substrate according to the supplier’s protocol. Slides were counterstained with haematoxylin, dehydrated and mounted.

<table>
<thead>
<tr>
<th>Target</th>
<th>Accession number</th>
<th>Forward primer 5’−3’</th>
<th>Reverse primer 5’−3’</th>
<th>Amplicon size (bp)</th>
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<tr>
<td>FSHR</td>
<td>NM_174061.1</td>
<td>GCCAGGCCTCAGCTACCACGC</td>
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<td>LHCR</td>
<td>NM_174381.1</td>
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<tr>
<td>STAR</td>
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<td>BMP2</td>
<td>NM_174343.2</td>
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<tr>
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<tr>
<td>BMP2</td>
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<td>BMP4</td>
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<td>BMP7</td>
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<td>Gremlin</td>
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<td>Noggin</td>
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<td>Follistatin</td>
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mounted under glass cover slips. Sections were viewed on a Zeiss Axiostar 2 microscope (20x objective) and images acquired using a Zeiss Axioimager with Axiovision software.

**Statistical analysis**

To reduce heterogeneity of variance immunoassay data were log-transformed before statistical analysis and QPCR data were analysed as $\Delta C_T$ values before conversion to fold-difference values. Results were evaluated using one- and/or two-way ANOVA and, where indicated, post hoc comparisons made by Fisher's protected least significant difference (PLSD) test. Post hoc tests were only made when ANOVA yielded a significant F-ratio. In vitro results presented are based on combined data from four independent culture experiments.

**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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