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Accepted Version

Accepted Manuscript published as JME-18-0198.R1. Accepted for publication: 25-Oct-2018

Gremlin, Noggin, Chordin and follistatin differentially modulate BMP-induced suppression of androgen secretion by bovine ovarian theca cells

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Abstract

Bone morphogenetic proteins (BMP) are firmly implicated as intra-ovarian regulators of follicle function and steroidogenesis but information is lacking regarding the regulation of BMP signalling by extracellular binding proteins co-expressed in the ovary. In this study we compared the abilities of four BMP binding proteins (gremlin, noggin, chordin, follistatin) to antagonize the action of four different BMPs (BMP2 BMP4, BMP6, BMP7) on LH-induced androstenedione secretion by bovine theca cells in primary culture. Expression of the four BMP binding proteins and BMPs investigated here has previously been documented in bovine follicles. All four BMPs suppressed androstenedione secretion by >85%. Co-treatment with gremlin antagonized BMP2- and, less potently, BMP4-induced suppression of androgen secretion but did not affect responses to BMP6 and BMP7. Noggin antagonized the effects of three BMPs (rank order: BMP4 > BMP2 > BMP7) but did not affect the response to BMP6. Follistatin partially reversed the suppressive effects of BMP6 on androgen secretion but did not affect BMP2, BMP4 and BMP7 action. Chordin had no effect on the response to any of the four BMPs. BMP6 treatment upregulated thecal expression of *GREM1, NOG, CHRD* and *SMAD6* mRNA whilst inhibiting expression of the four BMPs. Taken together with previous work documenting the intra-ovarian expression of different BMPs, BMP binding proteins and signalling receptors, these observations reinforce the conclusion that extracellular binding proteins selectively modulate BMP-dependent alterations in thecal steroidogenesis. As such they likely constitute an important regulatory component of this, and other intra-ovarian actions of BMPs.

Introduction

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Various ligands belonging to the TGFβ superfamily, including members of the bone morphogenetic protein (BMP) subfamily, are firmly implicated as intra-ovarian regulators of follicle development, steroidogenesis, cell proliferation/survival, ovulation and luteal function (Knight and Glister 2006; Regan, et al. 2018; Shimasaki, et al. 2004). Different ovarian cell-types (theca cells, granulosa cells, oocyte) exhibit selective expression of individual TGFβ superfamily ligands, signalling receptors, pseudo-receptors and secreted binding proteins consistent with operational autocrine/paracrine signalling pathways within and between different intrafollicular compartments. For example, activin, BMP2, BMP4, BMP6 and BMP7 have been shown to exert an anti-luteinization effect on granulosa cells (GC) by enhancing basal, FSH-induced and/or IGF-induced estradiol secretion whilst suppressing progesterone secretion (Glister, et al. 2004; Juengel, et al. 2006; Lee, et al. 2004; Otsuka, et al. 2001b; Souza, et al. 2002). The same TGFβ superfamily ligands have been shown to attenuate basal and LH-induced androgen secretion by cultured theca cells (TC) suggesting a role in preventing a premature increase in androgen production by developing antral follicles (Campbell, et al. 2006; Glister, et al. 2005; Hillier 1991; Wrathall and Knight 1995). As well as providing substrate for GC estrogen synthesis, TC-derived androgens enhance GC FSH receptor expression and FSH-dependent follicle development (Rice, et al. 2007; Sen, et al. 2014).

BMPs and activins exert their effects on target cells in the ovary and elsewhere by forming hetero-oligomeric complexes with two types of signalling receptor (type 1, type 2) on the cell surface. Type 1 receptors include BMPR1A (ALK3), ACVR1B (ALK4) and BMPR1B (ALK6); type 2 receptors include BMPR2, ACVR2A and ACVR2B) (Chen, et al. 2004). At the extracellular level, access of activins/BMPs to signalling receptors on the cell surface can be modulated by a range of secreted binding proteins including gremlin, noggin, chordin and

follistatin (Gazzerro and Canalis 2006; Mulloy and Rider 2015; Walsh, et al. 2010) or by secreted antagonists such as inhibin (Wiater and Vale 2003). At the intracellular level, additional regulatory mechanisms serve to enhance or attenuate BMP-activated signal transduction (Canalis, et al. 2003; Itoh and ten Dijke 2007; Miyazono 2000).

Despite their well-established role in the establishment of morphogen signalling gradients during embryonic and foetal development (Canalis et al. 2003; Chen et al. 2004; Mulloy and Rider 2015; Walsh et al. 2010), within the context of intra-follicular BMP signalling, there have been relatively few studies to examine the functional significance of extracellular binding proteins other than follistatin (Glister et al. 2004; Glister, et al. 2015; Nakamura, et al. 1992; Pierre, et al. 2005; Xiao, et al. 1990). However, gremlin 1 and 2 have been shown to antagonize BMP4-induced inhibition of FSH-induced progesterone production by rat GC (Sudo, et al. 2004) and to reverse BMP4-induced activation of primordial follicles in a rat ovary explant model (Nilsson, et al. 2014). Gremlin 1 was also shown to block BMP4-induced prostaglandin secretion by mouse GC (Pangas, et al. 2004) and to enhance androgen secretion by cultured bovine TC (Glister et al. 2005). The latter observation suggests neutralization of an endogenous ligand (BMP4?) that suppresses thecal androgen secretion in an autocrine/paracrine manner. Noggin was shown to reverse the suppressive effect of BMP2 and BMP4 on progesterone secretion by sheep GC (Pierre, et al. 2004).

Previous reports have documented the spatiotemporal patterns of expression of a range of BMPs (Erickson and Shimasaki 2003; Fatehi, et al. 2005; Glister, et al. 2010; Juengel et al. 2006), signalling receptors (Erickson and Shimasaki 2003; Fatehi et al. 2005; Glister et al. 2010; Regan, et al. 2016) and BMP-binding proteins (Glister, et al. 2011; Pangas et al. 2004) during follicle development in several species including cattle. In bovine follicles, gremlin (*GREM1*), noggin

Accepted Manuscript published as JME-18-0198.R1. Accepted for publication: 25-Oct-2018

(*NOG*), follistatin (*FST*) and chordin (*CHRD*) mRNA expression levels were much higher in the granulosal layer than in the theca interna layer (Glister et al. 2011) indicating they are the principle intrafollicular source of these binding proteins. Moreover, differential binding protein expression patterns in each cell type accompanied antral follicle development, suggesting regulated rather than constitutive expression, and implying functional roles (Glister et al. 2011). For instance, *GREM1* expression was maximal in GC of small antral follicles (1-2mm) declining to a low level in GC of large (11-18mm) estrogen-active follicles. *NOG* expression was also lowest in GC of large estrogen-active follicles while *FST* and *CHRD* expression was greatest in this follicle category (Glister et al. 2011).

Information is lacking regarding the potential regulation of BMP signalling by extracellular binding proteins co-expressed in the ovary, particularly with respect to regulation of follicular theca cell function. To test the hypothesis that extracellular binding proteins differentially regulate the actions of BMPs on theca cells, this study compared the relative abilities of four different extracellular binding proteins (gremlin, noggin, follistatin, chordin) to antagonise to suppressive action of four BMPs (BMP2, BMP4, BMP6, BMP7) on androgen secretion by bovine TC in primary culture. To explore additional autoregulatory mechanisms that may serve to limit BMP action, we also examined the effect of one of these BMPs (BMP6) on thecal expression of each of the above-mentioned BMPs and BMP-binding proteins, and also on expression of the inhibitory Smad, *SMAD6*.

Materials and Methods

Bovine ovaries and theca cell culture

Bovine theca interna cells (TC) were isolated from the ovaries of randomly cycling cattle obtained from the slaughterhouse as described in detail elsewhere (Glister et al. 2005). Briefly, antral follicles (4-6mm diameter) of healthy morphological appearance were hemisected and granulosa cell layers dislodged using a plastic inoculation loop. After vigorous shaking and washing (x3) to remove remaining adherent granulosa cells, follicle halves were examined under the dissecting microscope. Theca interna layers were peeled away from the basement membrane and pooled theca interna layers from approximately 50 follicles were dissociated into single cells by incubating (30 min) with collagenase (type IV, 1 mg/ml; Sigma Ltd., Poole, UK) and trypsin inhibitor (0.1mg/ml; Sigma) in a shaking water bath at 37 C (see (Glister et al. 2005) for further details). Cells were washed and counted using a hemocytometer and viability (>90%) assessed using trypan blue. The resultant theca interna cell preparations obtained using this method were judged to have < 5% contamination with granulosa cells based on a previous RT-qPCR analysis of relative abundance of thecal (*CYP17A1, INSL3*) and granulosal (*CYP19A1, FSHR*) 'marker' transcripts (Glister et al. 2010). Moreover, estradiol levels in TC-conditioned culture media were undetectable (data not shown).

For each experiment cells were seeded into 96-well tissue culture plates (Nunclon, Life Technologies Ltd, Paisley, UK) at 75,000 viable cells/well and cultured for 6 days (144h) under defined serum-free conditions. For experiments in which RNA extraction was planned, cells were seeded into 24-well tissue culture plates at 250,000 viable cells/well. The culture medium was McCoy's 5A modified medium supplemented with 1% (v/v) antibiotic-antimycotic solution, 10 ng/ml bovine insulin, 2 mM L-glutamine, 10mM HEPES, 5 µg/ml apo-transferrin, 5 ng/ml sodium selenite and 0.1% (w/v) BSA (all purchased from Sigma UK Ltd). Cells were cultured without treatments for the first 48h. Medium was removed after 48h and 96h and replaced with

Accepted Manuscript published as JME-18-0198.R1. Accepted for publication: 25-Oct-2018

fresh medium containing treatments (see below). At the end of culture (144h) conditioned media were stored at -20C for subsequent steroid immunoassays. Viable cell number at the end of culture was determined by neutral red dye uptake assay (Glister, et al. 2001) to provide an assessment of cell proliferation/survival.

Treatments

Ovine LH (NIADDK oLH-S-16) was obtained from NHPP, Torrance, CA, USA. Recombinant human BMP2, BMP4, BMP6, BMP7, gremlin, noggin, follistatin-288 and recombinant mouse chordin were purchased from R&D Systems (Abingdon, Oxon, UK). Treatments were prepared in Hank's balanced salt solution containing 0.1% (w/v) BSA and sterile stock solutions prepared using 0.2μm membrane filters before further dilution in sterile culture medium. The concentrations of LH (150 pg/ml) and BMP2, BMP4, BMP6 and BMP7 (10 ng/ml) selected for these experiments were considered optimal based on their modulatory effects on androstenedione secretion observed in our previous studies on bovine TC (Glister et al. 2005; Glister et al. 2010, 2011). Each BMP binding protein was tested at three different concentrations (50, 250, 1250 ng/ml) for its ability to antagonize BMP-induced suppression of androstenedione secretion by LH-stimulated cells. Co- treatments were prepared 30-40 min before addition to cells by mixing appropriate concentrations of BMP and BMP binding protein. A further experiment examined the effect of 24, 48 and 96h exposure to BMP6 (10 ng/ml) alone on the relative abundance of *CHRD, GREM1, NOG, FST, BMP2, BMP4, BMP6, BMP7* and *SMAD6* mRNA.

Steroid assays

Concentrations of androstenedione in TC-conditioned media were determined by ELISA as reported previously (Glister, et al. 2013). The detection limit was 0.1ng/ml and average intra- and inter-assay CVs were 7% and 10% respectively. Progesterone concentrations were determined by ELISA (Satchell, et al. 2013). The detection limit was 0.1ng/ml and average intra- and inter-assay CVs were 8% and 11% respectively.

Real-time PCR analysis

Total RNA was isolated using Tri-reagent as described previously (Glister et al. 2010). cDNA was synthesized from 1µg of RNA using the AB High Capacity cDNA synthesis kit (Thermo Fisher Scientific; used according to manufacturers protocol) with random hexamers. PCR primers (see table 1) were designed using the online primer designing tool 'Primer-BLAST' (http://www.ncbi.nlm.nih.gov/tools/primer-blast) with BLAST specificity checking against all known bovine (Bos Taurus) transcripts to exclude potential amplification of off-target sequences. PCR assays were carried out in a volume of 14µl containing 5µl cDNA template, 1µl each forward and reverse primers (final concentration 0.36µM) and 7µl QuantiTect SYBR Green QPCR 2x Master Mix (Qiagen, Crawley, W. Sussex, UK). Samples were processed on a StepOne Plus thermal cycler (Applied Biosystems) with cycling conditions: 15min at 95^{o}C (one cycle only) followed by 15s at 95^{o}C and 1min at 60^{o}C for 40 cycles. The ΔΔCt method (Livak and Schmittgen 2001) was used to compare the relative abundance of each mRNA transcript. Ct values for each transcript in a given sample were first normalized to the corresponding ß-actin (*ACTB*) Ct value (i.e. ΔCt value). *ACTB* expression level was uniform across experimental treatments. ΔCt values for each transcript in a given sample were then normalized to the corresponding ΔCt value for that transcript untreated control (time zero) samples. For graphical

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presentation ΔΔCt values were converted to fold-differences using the formula: fold-difference = $2^{(-\Delta\Delta Ct)}$.

Statistical analysis

Hormone secretion data were log-transformed prior to statistical analysis to reduce heterogeneity of variance. Effects of treatments (LH, BMP, BMP binding protein) on hormone secretion (for final 96-144h period of culture) and viable cell number at the end of culture were evaluated by one- and two-way analysis of variance (ANOVA). Post hoc pairwise comparisons were made using Fisher's PLSD test. Gene expression results were analysed by one-way ANOVA as ΔΔCt values before conversion to fold-differences. Results are presented as arithmetic means ± SEM based on 3-4 independent culture experiments using different batches of theca cells.

Results

Treatment of cells with LH alone elicited a ~ 4-fold increase in androstenedione secretion ($p<0.001$) but did not affect progesterone secretion, or viable cell number at the end of culture (144h) (**Fig. 1a**). Treatment of cells with BMP2, BMP4, BMP6 and BMP7 promoted a marked suppression of LH-stimulated androstenedione secretion (>85%; $p<0.001$) whilst promoting a ~2-fold increase in progesterone secretion ($p<0.001$). Viable cell number at the end of culture was not affected by BMP treatment (**Fig. 1b**).

Fig. 2 shows the effects of the four BMPs alone and in combination with gremlin. Treatment of cells with BMP2, BMP4, BMP6 or BMP7 promoted a marked (>6-fold) suppression of androstenedione secretion ($P<0.0001$) accompanied by a modest increase in progesterone secretion ($P<0.001$). Treatment with gremlin alone raised mean androstenedione secretion ~2-fold but the effect was not significant. Two-way ANOVA showed a highly significant effect of BMP type and gremlin dose-level on androstenedione secretion, as well as a BMP x gremlin dose-level interaction. Co-treatment with 250 ng/ml gremlin reversed the suppression in androstenedione secretion induced by BMP2 ($P<0.05$) while a higher gremlin concentration (1250 ng/ml) was required to reverse the suppressive effect of BMP4 ($P<0.05$). At the dose-levels tested gremlin did not reverse the effects of BMP6 or BMP7. Regarding progesterone secretion, two-way ANOVA showed a non-significant BMP x gremlin interaction ($P=0.09$).

With respect to noggin treatment (**Fig. 3**), two-way ANOVA indicated a highly significant effect of BMP type ($P<0.0001$) and noggin dose-level ($P<0.0001$) on androstenedione secretion, as well as a BMP x noggin dose-level interaction ($P<0.0001$). Closer examination of the results showed that treatment with noggin alone had no effect on androstenedione secretion but effectively reversed the suppressive actions of BMP2, BMP4 and BMP7. The lowest concentrations of noggin required to promote a significant ($P<0.05$) reversal of BMP-induced suppression of androstenedione secretion were 50 ng/ml for BMP4, 250 ng/ml for BMP2 and 1250 ng/ml for BMP7. At the dose-levels tested noggin did not reverse the effects of BMP6. Regarding progesterone secretion, two-way ANOVA showed a non-significant BMP x noggin interaction ($P=0.02$).

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Fig. 4 shows the effects of BMPs alone and in combination with follistatin. Again, there was a highly significant effect of BMP type ($P<0.0001$) and follistatin dose-level ($P<0.0001$) on androstenedione secretion, as well as a BMP x follistatin dose-level interaction ($P<0.02$). Treatment with follistatin alone had no effect on basal androstenedione secretion but androstenedione secretion in the presence of BMP6 was increased ($P<0.05$) by the addition of follistatin, indicating a partial reversal of the response to BMP6. Follistatin did not affect androstenedione secretion in the presence of BMP2, BMP4 or BMP7. With respect to progesterone secretion, two-way ANOVA showed a non-significant BMP x follistatin interaction ($P=0.3$).

As shown in **fig. 5** chordin had no effect on basal androstenedione secretion and did not reverse the suppressive effects of BMP2, BMP4, BMP6 or BMP7 on androstenedione secretion. Likewise chordin did not affect progesterone secretion and two-way ANOVA showed a non-significant BMP x chordin interaction ($P=0.72$).

Fig. 6 shows that treatment of cells with BMP6 for 96h promoted a marked, time-dependent increase in relative abundance of mRNA for *GREM1* (~25-fold; $p<0.001$), *NOG* (~25-fold; $p<0.001$) and *CHRD* (~10-fold; $p<0.001$) but did not affect *FST* mRNA expression. Only marginal increases in binding protein expression levels were observed after shorter exposure periods (24 and 48h). Treatment with BMP6 promoted a time-dependent reduction in *BMP2, BMP4* and *BMP6* mRNA transcript abundance ($p<0.001$). *BMP7* transcript abundance was also

reduced at 24 and 48h but not at 96h. In addition, BMP6 treatment promoted a marked (~45-fold; $p<0.001$) and time-dependent increase in *SMAD6* transcript abundance.

Discussion

The present study sought to clarify the functional significance of potential interactions between different BMPs and BMP-binding proteins at the intra-follicular level. Since ovarian androgens play key roles in follicle development and function (Hillier 1987; Rice et al. 2007; Sen et al. 2014) we used a bovine primary TC culture model as a bioassay to evaluate, in a combinatorial manner, the abilities of four different binding proteins to counteract the inhibitory action of four different BMPs on androgen secretion. Progesterone secretion was also evaluated but since BMPs only elicit a modest change in progesterone secretion, this provided a much less robust end-point for comparing relative bio-potencies of the different binding proteins. Each of the binding proteins (*CHRD, GREM1, NOG, FST*) and BMPs (*BMP2, BMP4, BMP6, BMP7*) selected for the study has been shown previously to be expressed within bovine antral follicles in a cell-type and follicle stage-dependent manner (Glister et al. 2010, 2011). As anticipated from earlier studies (Glister et al. 2005; Glister et al. 2013) all four BMPs elicited a robust suppression of thecal androgen secretion. Moreover, evidence supporting differential effects of binding proteins was obtained, consistent with selective modulation of autocrine/paracrine BMP signalling in the ovarian follicle. Since GC, rather than TC, appear to be the predominant source of chordin, gremlin, noggin and follistatin in bovine antral follicles (Glister et al. 2011), it is likely that GC-derived binding proteins have a key role in regulating access of BMPs to their

Accepted Manuscript published as JME-18-0198.R1. Accepted for publication: 25-Oct-2018

signalling receptors on TC, regardless of whether the BMPs are secreted by TC, GC or oocyte. In this context, bovine GC were found to express high levels of *BMP2* mRNA and protein while TC express higher levels of *BMP4, BMP6* and *BMP7* mRNA (Glister et al. 2010). BMP6 immunoreactivity was also detected in bovine oocytes and cultured GC while BMP4 and BMP7 immunoreactivity was more prevalent in cultured TC (Glister et al. 2004).

The present results show that gremlin and noggin were the most effective antagonists of BMP2-induced suppression of thecal androgen secretion, whilst follistatin and chordin had no effect. Previous studies have shown that gremlin reverses BMP2-induced suppression of progesterone secretion by rat GC (Sudo et al. 2004) and that noggin, but not follistatin, reverses the BMP2-induced suppression of progesterone secretion by sheep GC (Pierre et al. 2005). Noggin was also shown to reverse BMP2-induced suppression of FSHR expression and progesterone production by hen GC (Haugen and Johnson 2010). As mentioned above BMPs had little effect on progesterone secretion in our bovine TC model and so direct comparison with studies on granulosa cell progesterone production is difficult. To our knowledge there are no reports from other groups examining effects of BMP-BMP binding protein interactions on thecal androgen production in any species. In the bovine ovary BMP2, gremlin and noggin are predominantly of GC origin and showed their lowest expression levels in large estrogen-active follicles (Glister et al. 2010, 2011), in contrast to follistatin and chordin which showed maximal expression in this follicle category (Glister et al. 2011). This leads to speculation that low BMP2 may contribute to the increased output of thecal androgen required for heightened estrogen synthesis by the dominant estrogen-active follicle.

Our data showed that noggin was the most potent antagonist of BMP4-induced suppression of thecal androgen secretion whilst gremlin was only effective at a 25-fold higher concentration and

follistatin and chordin had no effect. Previously, noggin was found to reverse BMP-4-induced inhibition of progesterone secretion by sheep GC while follistatin was without effect (Pierre et al. 2005). Noggin has also been shown to be a potent antagonist of BMP4 action on other non-endocrine cell-types (Canalis et al. 2003; Zimmerman, et al. 1996). As mentioned above BMP4 is predominantly expressed by TC and so the implication for intrafollicular signalling is that GC-derived noggin may diffuse through the basement membrane to modulate the autocrine/paracrine action of BMP4 on TC and thus contribute to the regulation of androgen output. Given the previous observation (Glister et al. 2011) that GC *NOG* expression is minimal in large estrogen-active follicles, this would imply reduced antagonism of thecal BMP4 signalling at this follicle stage. Interestingly, *NOG* expression by cultured GC was inhibited by IGF analogue treatment perhaps accounting for low expression in large estrogen-active follicles (Glister et al. 2005).

In contrast to *NOG, FST* expression is maximal in GC of large estrogen-active bovine follicles (Glister et al. 2011) and is upregulated by both FSH and IGF1 in cultured GC (Glister et al. 2011; Glister et al. 2001). As well as binding to activin with high affinity (Nakamura et al. 1992), follistatin also binds with lower affinity to other TGFβ family members including BMP4, BMP6 and BMP7 (Glister et al. 2004), BMP-15 (Otsuka, et al. 2001a) and myostatin (Amthor, et al. 2004). Moreover, follistatin was shown to reverse BMP4- and BMP6-induced increases in phospho-Smad1 accumulation in bovine GC, but did not affect the response to BMP7 (Glister et al. 2004). Despite these previous findings, in this study follistatin only promoted a weak and partial reversal of BMP6-induced suppression of thecal androgen and did not affect the response to BMP2, BMP4 or BMP7. Similarly, follistatin did not antagonise the suppressive action of BMP2 or BMP4 on progesterone secretion by sheep GC but had a slight modulatory effect on the response to BMP6 (Pierre et al. 2005). As such, it seems questionable whether follistatin,

Accepted Manuscript published as JME-18-0198.R1. Accepted for publication: 25-Oct-2018

306 primarily of GC origin, exerts a significant modulatory effect on intrafollicular BMP2, BMP4,
307 BMP6 and BMP7 signalling although further investigation is needed to clarify this issue.

308 As observed for follistatin, GC of large estrogen-active bovine follicles were found to express the
309 highest level of *CHRD* mRNA (Glister et al. 2011). However, in contrast to follistatin, expression
310 of *CHRD* by cultured GC was not modulated by either FSH or IGF1 (Glister et al. 2011).
311 Furthermore, in this study we found no modulatory effects of chordin on the TC response to any
312 of the four BMPs examined. Whilst we are not aware of any other studies involving ovarian cells,
313 chordin has been shown to bind to and antagonise the effects of several BMPs including BMP2,
314 BMP4 and BMP7 on various development events including early dorsal patterning in chick and
315 mouse (Gazzerro and Canalis 2006; Piccolo, et al. 1997). The lack of effect we observed was
316 therefore unexpected, given the reported biological activity of the recombinant binding protein as
317 stated by the suppliers. Since cleavage by the metalloproteinase, mammalian (m-) tolloid (aka
318 BMP1), renders chordin unable to antagonize BMP activity (Ge and Greenspan 2006; Piccolo et
319 al. 1997), it is tentatively suggested that m-tolloid produced by the cultured TC could account for
320 the lack of effect of chordin. In this regard, co-expression of *BMP1*, *CHRD* and *BMP4* mRNA
321 has been reported in sheep ovarian follicles (Canty-Laird, et al. 2010). Whilst m-tolloid
322 immunoreactivity was mainly localised in the granulosa layer it was also evident in the theca
323 layer of sheep antral follicles, lending some support to this possibility.

324 In a further experiment to explore other potential regulatory mechanisms governing intrafollicular
325 BMP signalling, we examined the ability of one of the BMPs (BMP6) to modulate thecal
326 expression of each of the four BMP-binding proteins and BMPs, as well as expression of the
327 inhibitory Smad, *SMAD6*. Despite the failure of gremlin, noggin and chordin to antagonise the
328 suppressive effect of BMP6 on thecal androgen secretion, BMP6 treatment was found to

upregulate thecal expression of these three binding proteins in a time-dependent manner. This is consistent with previous findings (Glister et al. 2011) and suggests an additional autoregulatory feedback loop at the target cell level to restrict or attenuate signalling by other intra-follicular BMPs, to which the cells are exposed. BMP-induced upregulation of BMP binding protein expression has been observed in other model systems. For example, *GREM1* expression by mouse GC (Pangas et al. 2004) and rat osteoblasts (Pereira, et al. 2000a) was upregulated by BMP2 and BMP4. Likewise, *NOG* expression by osteoblasts was upregulated by BMP2, BMP4 and BMP6 (Gazzerro, et al. 1998).

The finding that BMP6 down regulated its own mRNA expression, as well as expression of *BMP2, BMP4* and *BMP7*, suggests a direct ligand-dependent autoregulatory negative feedback effect operating in ovarian theca cells. Similar effects have been reported for BMP4 and BMP2 which were both found to downregulate their own expression by cultured osteoblasts (Pereira, et al. 2000b).

Inhibitory Smads (SMAD6, SMAD7) attenuate TGFβ family signaling by blocking interaction of type 1 receptors with receptor-regulated (R) Smads and by preventing the association of R-Smads with co-Smad (SMAD4) (Itoh and ten Dijke 2007; Miyazono 2000). Since SMAD6 preferentially inhibits Smad signaling initiated by BMPs (Miyazono 2000), our finding of a marked, BMP6-induced upregulation of *SMAD6* expression provides evidence for a further intracellular negative feedback loop operating at the theca cell level to limit the duration and/or intensity of BMP signaling, akin to that observed in other cell types including lung cancer cell lines and chondrocytes (Afrakhte, et al. 1998; Li, et al. 2003).

In conclusion, these findings underscore the complexity of the intra-ovarian BMP system comprising multiple ligands, extracellular binding proteins and signalling receptors. Thecal

Accepted Manuscript published as JME-18-0198.R1. Accepted for publication: 25-Oct-2018

androgen production is negatively regulated by locally-produced BMPs, the actions of which are modulated by various negative feedback loops. It remains a daunting challenge to evaluate the functional significance of individual BMPs, against a backdrop of multiple interacting autocrine and/or paracrine pathways some of which may be redundant whilst others may play essential physiological roles to regulate different aspects of follicle function. Although suitable assays for BMPs and BMP-binding proteins (other than follistatin) are currently lacking, future studies to determine their respective intrafollicular concentrations would be a useful step towards defining their relative physiological significance.

Acknowledgements

We thank D Butlin and AD Simmonds for technical assistance. This work was supported by the Biotechnology and Biological Sciences Research Council (award BB/M001369/1 and BB/GO17174/1 to PGK). The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this scientific work.

Table 1 Primers used for real-time PCR

Figures

Fig. 1 Effects of (a) LH and (b) BMP2, BMP4, BMP6 and BMP7 on secretion of androstenedione and progesterone by bovine theca interna cells and on viable cell number at the end of culture. In (b) cells were cultured in the presence of LH. Values are means and bars indicate SEM (n = 3 independent cultures). ***$p<0.001$ versus control.

374

375 **Fig. 2** Effects of gremlin on secretion of (a) androstenedione and (b) progesterone by bovine
376 theca interna cells treated with BMP2, BMP4, BMP6 or BMP7 under LH-stimulated conditions.
377 Values are means and bars indicate SEM (n = 3 independent experiments). Results of 2-way
378 ANOVA are indicated. Within each BMP treatment group, means without a common letter are
379 significantly ($p<0.05$) different.

380

381 **Fig. 3** Effects of noggin on secretion of (a) androstenedione and (b) progesterone by bovine theca
382 interna cells treated with BMP2, BMP4, BMP6 or BMP7 under LH-stimulated conditions.
383 Values are means and bars indicate SEM (n = 3 independent experiments). Results of 2-way
384 ANOVA are indicated. Within each BMP treatment group, means without a common letter are
385 significantly ($p<0.05$) different.

386

387 **Fig. 4** Effects of follistatin on secretion of (a) androstenedione and (b) progesterone by bovine
388 theca interna cells treated with BMP2, BMP4, BMP6 or BMP7 under LH-stimulated conditions.
389 Values are means and bars indicate SEM (n = 3 independent experiments). Results of 2-way
390 ANOVA are indicated. Within each BMP treatment group, means without a common letter are
391 significantly ($p<0.05$) different.

392

393 **Fig. 5** Effects of chordin on secretion of (a) androstenedione and (b) progesterone by bovine
394 theca interna cells treated with BMP2, BMP4, BMP6 or BMP7 under LH-stimulated conditions.

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Values are means and bars indicate SEM (n = 3 independent experiments). Results of 2-way ANOVA are indicated. Within each BMP treatment group, means without a common letter are significantly ($p<0.05$) different.

Fig. 6 Time-dependent effect of BMP6 treatment on relative abundance of transcripts for (a) *GREM1*, (b) *CHRD*, (c) *NOG*, (d) *FST*, (e) *BMP2*, (f) *BMP4*, (g) *BMP6*, (h) *BMP7* and (i) *SMAD6* in cultured bovine theca interna cells. Values are means and bars indicate SEM (n = 4 independent experiments). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus control.

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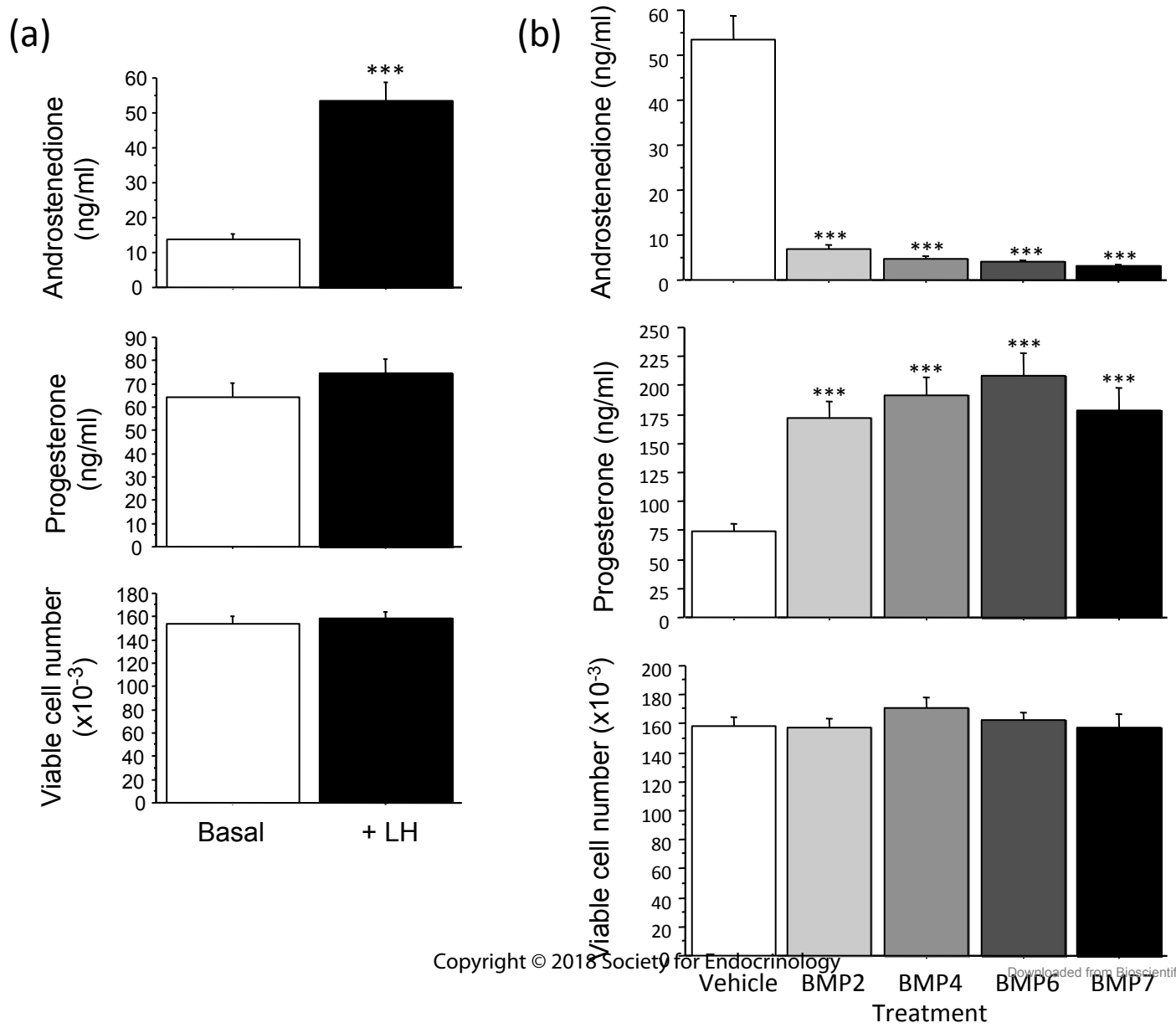
Accepted Manuscript published as JME-18-0198.R1. Accepted for publication: 25-Oct-2018

Target	Accession number	Forward primer 5' to 3'	Reverse primer 5' to 3'	Amplicon size (bp)
BMP2	XM_866011.1	CCAAGAGGCATGTGCGGATTAGCA	TCCTTTCCCATCGTGGCCAAAAGT	101
BMP4	NM_001045877.1	TTTATGAGGTTATGAAGCCCCCGGC	AGTTTCCCACCGCGTCACATTGTG	104
BMP6	XM_600972.2	GGCCCCGTTAACTCGACTGTGACAAA	TTGAGGACGCCGAACAAAACAGGA	108
BMP7	XM_612246.2	TGCAAGATAGCCACTTCCTCACCGA	GGGATCTTGGAGAGATCAAACCGGA	130
Chordin	XM_001788437.1	CCTACCCGAATCCGCTTCTCTGACTCC	GACAACCGAGGCACTGCCCGC	113
Gremlin	NM_001082450.1	GAAGCGAGACTGGTGCAAAACCCA	TATGCAACGGCACTGCTTGACACG	271
Noggin	XM_582573.4	CAAGAAGCAGCGCCTGAGCAAGA	GAAACAGCTGCCCACCTTCACGTAG	142
Follistatin	NM_175801.2 B	TGAGCAAGGAGGAGTGTTGCAGCA	CATCTGGCCTTGAGGAGTGCACATTC	301
Smad6	NM_001206145.1	CGCCACCGCCCTACTCTCGG	GCTGTGATGAGGGAGTTGGCGGC	112
ACTB	NM_173979.3	ATCACCATCGGCAATGAGCGGTTC	CGGATGTCGACGTCACACTTCATGA	128

Table 1: List of primers used for quantitative RT-PCR

Fig 1



 Accepted Manuscript published as JME-18-0198.R1. Accepted for publication: 25-Oct-2018

Fig 2

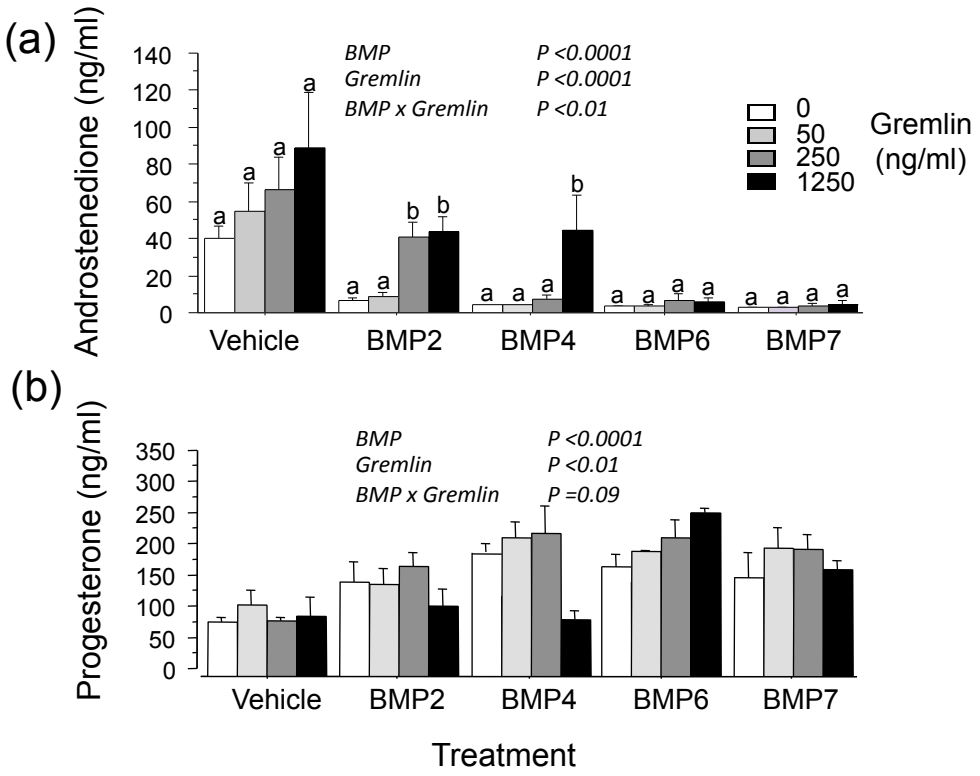


Fig 3

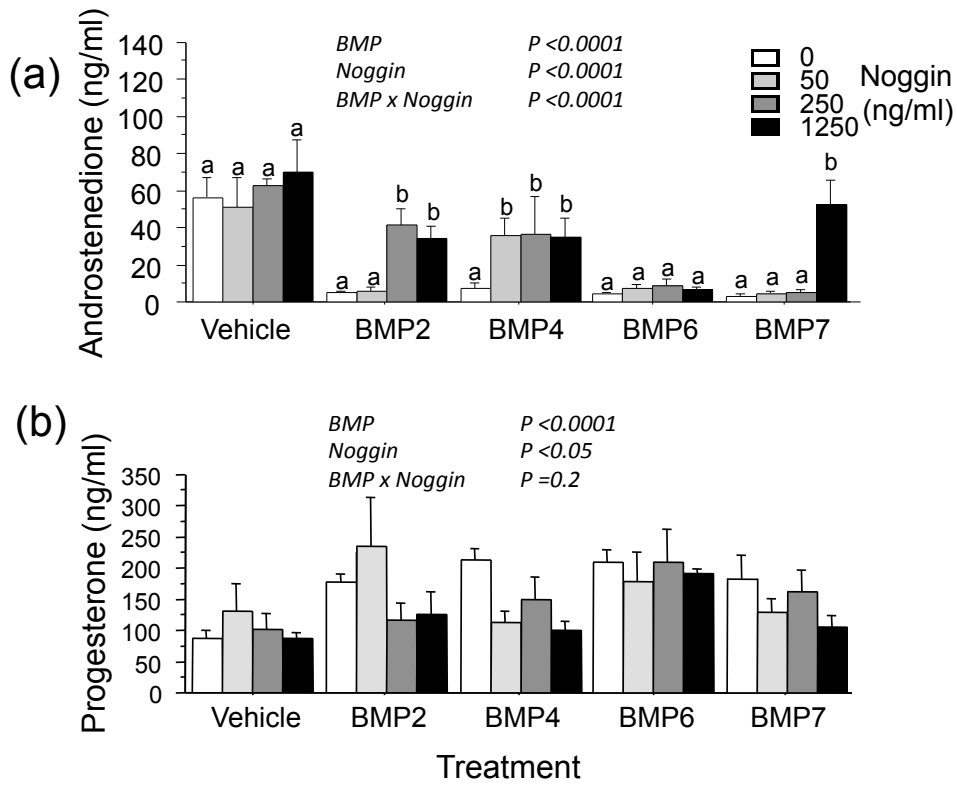


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Fig 4

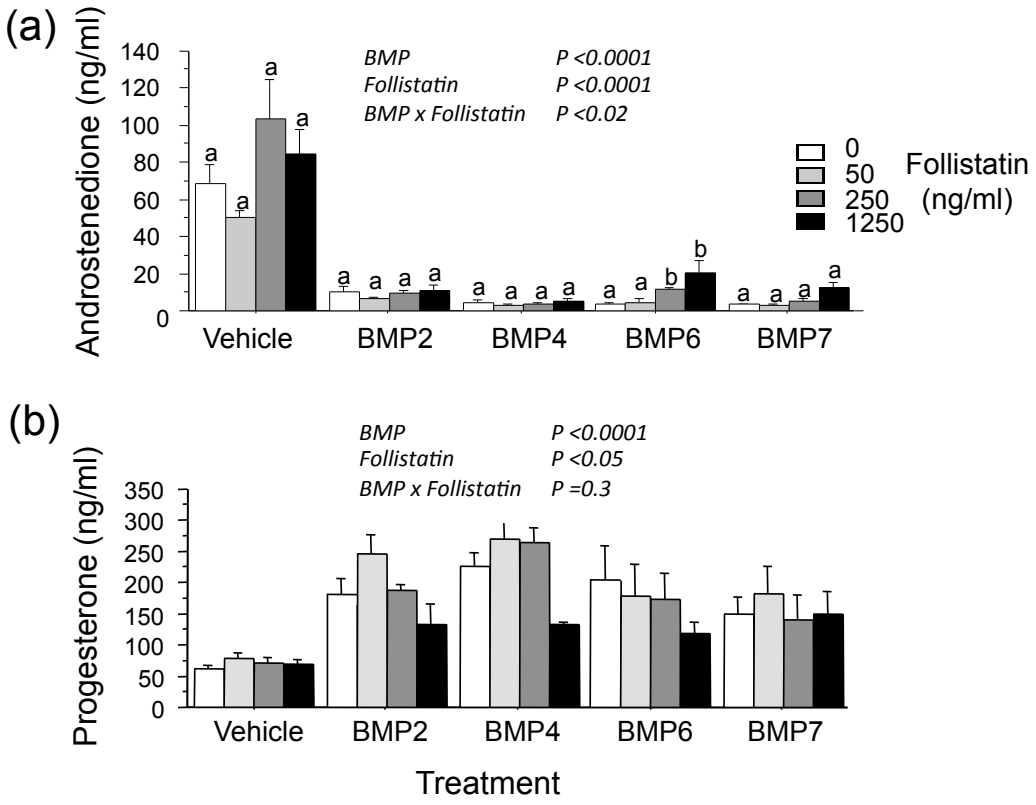
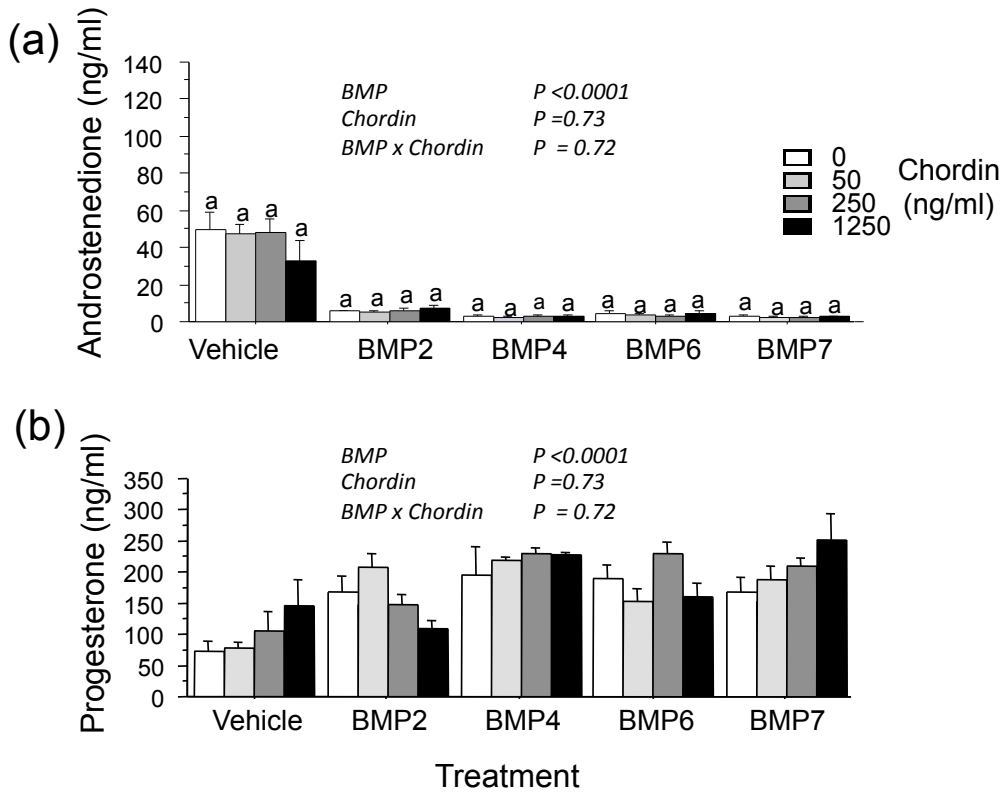


Fig 5



Accepted Manuscript published as JME-18-0198.R1. Accepted for publication: 25-Oct-2018

Fig 6