



Gremlin, noggin, chordin and follistatin differentially modulate BMP induced suppression of androgen secretion by bovine ovarian theca cells

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

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15

16 **Abstract**

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

36

37 **Introduction**

38 Various ligands belonging to the TGF β superfamily, including members of the bone
39 morphogenetic protein (BMP) subfamily, are firmly implicated as intra-ovarian regulators of
40 follicle development, steroidogenesis, cell proliferation/survival, ovulation and luteal function
41 (Knight and Glister 2006; Regan, et al. 2018; Shimasaki, et al. 2004). Different ovarian cell-types
42 (theca cells, granulosa cells, oocyte) exhibit selective expression of individual TGF β superfamily
43 ligands, signalling receptors, pseudo-receptors and secreted binding proteins consistent with
44 operational autocrine/paracrine signalling pathways within and between different intrafollicular
45 compartments. For example, activin, BMP2, BMP4, BMP6 and BMP7 have been shown to exert
46 an anti-luteinization effect on granulosa cells (GC) by enhancing basal, FSH-induced and/or IGF-
47 induced estradiol secretion whilst suppressing progesterone secretion (Glister, et al. 2004;
48 Juengel, et al. 2006; Lee, et al. 2004; Otsuka, et al. 2001b; Souza, et al. 2002). The same TGF β
49 superfamily ligands have been shown to attenuate basal and LH-induced androgen secretion by
50 cultured theca cells (TC) suggesting a role in preventing a premature increase in androgen
51 production by developing antral follicles (Campbell, et al. 2006; Glister, et al. 2005; Hillier
52 1991; Wrathall and Knight 1995). As well as providing substrate for GC estrogen synthesis, TC-
53 derived androgens enhance GC FSH receptor expression and FSH-dependent follicle
54 development (Rice, et al. 2007; Sen, et al. 2014).

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

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

65 Despite their well-established role in the establishment of morphogen signalling gradients during
66 embryonic and foetal development (Canalis et al. 2003; Chen et al. 2004; Mulloy and Rider
67 2015; Walsh et al. 2010), within the context of intra-follicular BMP signalling, there have been
68 relatively few studies to examine the functional significance of extracellular binding proteins
69 other than follistatin (Glister et al. 2004; Glister, et al. 2015; Nakamura, et al. 1992; Pierre, et al.
70 2005; Xiao, et al. 1990). However, gremlin 1 and 2 have been shown to antagonize BMP4-
71 induced inhibition of FSH-induced progesterone production by rat GC (Sudo, et al. 2004) and to
72 reverse BMP4-induced activation of primordial follicles in a rat ovary explant model (Nilsson, et
73 al. 2014). Gremlin 1 was also shown to block BMP4-induced prostaglandin secretion by mouse
74 GC (Pangas, et al. 2004) and to enhance androgen secretion by cultured bovine TC (Glister et al.
75 2005). The latter observation suggests neutralization of an endogenous ligand (BMP4?) that
76 suppresses thecal androgen secretion in an autocrine/paracrine manner. Noggin was shown to
77 reverse the suppressive effect of BMP2 and BMP4 on progesterone secretion by sheep GC
78 (Pierre, et al. 2004).

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

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

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

103

104 **Materials and Methods**

105 *Bovine ovaries and theca cell culture*

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

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

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

133

134 *Treatments*

135 Ovine LH (NIADDK oLH-S-16) was obtained from NHPP, Torrance, CA, USA. Recombinant
136 human BMP2, BMP4, BMP6, BMP7, gremlin, noggin, follistatin-288 and recombinant mouse
137 chordin were purchased from R&D Systems (Abingdon, Oxon, UK). Treatments were prepared
138 in Hank's balanced salt solution containing 0.1% (w/v) BSA and sterile stock solutions prepared
139 using 0.2µm membrane filters before further dilution in sterile culture medium. The
140 concentrations of LH (150 pg/ml) and BMP2, BMP4, BMP6 and BMP7 (10 ng/ml) selected for
141 these experiments were considered optimal based on their modulatory effects on androstenedione
142 secretion observed in our previous studies on bovine TC (Glister et al. 2005; Glister et al. 2010,
143 2011). Each BMP binding protein was tested at three different concentrations (50, 250, 1250
144 ng/ml) for its ability to antagonize BMP-induced suppression of androstenedione secretion by
145 LH-stimulated cells. Co- treatments were prepared 30-40 min before addition to cells by mixing
146 appropriate concentrations of BMP and BMP binding protein. A further experiment examined the
147 effect of 24, 48 and 96h exposure to BMP6 (10 ng/ml) alone on the relative abundance of *CHRD*,
148 *GREM1*, *NOG*, *FST*, *BMP2*, *BMP4*, *BMP6*, *BMP7* and *SMAD6* mRNA.

149

150 *Steroid assays*

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

156

157 *Real-time PCR analysis*

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

174 presentation $\Delta\Delta C_t$ values were converted to fold-differences using the formula: fold-difference =
175 $2^{(-\Delta\Delta C_t)}$.

176

177 *Statistical analysis*

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

185

186

187 **Results**

188

189 Treatment of cells with LH alone elicited a ~ 4-fold increase in androstenedione secretion
190 ($p < 0.001$) but did not affect progesterone secretion, or viable cell number at the end of culture
191 (144h) (**Fig. 1a**). Treatment of cells with BMP2, BMP4, BMP6 and BMP7 promoted a marked
192 suppression of LH-stimulated androstenedione secretion ($>85\%$; $p < 0.001$) whilst promoting a ~2-
193 fold increase in progesterone secretion ($p < 0.001$). Viable cell number at the end of culture was
194 not affected by BMP treatment (**Fig. 1b**).

195 **Fig. 2** shows the effects of the four BMPs alone and in combination with gremlin. Treatment of
196 cells with BMP2, BMP4, BMP6 or BMP7 promoted a marked (>6-fold) suppression of
197 androstenedione secretion ($P<0.0001$) accompanied by a modest increase in progesterone
198 secretion ($P<0.001$). Treatment with gremlin alone raised mean androstenedione secretion ~2-
199 fold but the effect was not significant. Two-way ANOVA showed a highly significant effect of
200 BMP type and gremlin dose-level on androstenedione secretion, as well as a BMP x gremlin
201 dose-level interaction. Co-treatment with 250 ng/ml gremlin reversed the suppression in
202 androstenedione secretion induced by BMP2 ($P<0.05$) while a higher gremlin concentration
203 (1250 ng/ml) was required to reverse the suppressive effect of BMP4 ($P<0.05$). At the dose-levels
204 tested gremlin did not reverse the effects of BMP6 or BMP7. Regarding progesterone secretion,
205 two-way ANOVA showed a non-significant BMP x gremlin interaction ($P=0.09$).

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

216

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

226

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

231

232 **Fig. 6** shows that treatment of cells with BMP6 for 96h promoted a marked, time-dependent
233 increase in relative abundance of mRNA for *GREM1* (~25-fold; $p<0.001$), *NOG* (~25-fold;
234 $p<0.001$) and *CHRD* (~10-fold; $p<0.001$) but did not affect *FST* mRNA expression. Only
235 marginal increases in binding protein expression levels were observed after shorter exposure
236 periods (24 and 48h). Treatment with BMP6 promoted a time-dependent reduction in *BMP2*,
237 *BMP4* and *BMP6* mRNA transcript abundance ($p<0.001$). *BMP7* transcript abundance was also

238 reduced at 24 and 48h but not at 96h. In addition, BMP6 treatment promoted a marked (~45-fold;
239 $p < 0.001$) and time-dependent increase in *SMAD6* transcript abundance.

240

241 **Discussion**

242

243 The present study sought to clarify the functional significance of potential interactions between
244 different BMPs and BMP-binding proteins at the intra-follicular level. Since ovarian androgens
245 play key roles in follicle development and function (Hillier 1987; Rice et al. 2007; Sen et al.
246 2014) we used a bovine primary TC culture model as a bioassay to evaluate, in a combinatorial
247 manner, the abilities of four different binding proteins to counteract the inhibitory action of four
248 different BMPs on androgen secretion. Progesterone secretion was also evaluated but since
249 BMPs only elicit a modest change in progesterone secretion, this provided a much less robust
250 end-point for comparing relative bio-potencies of the different binding proteins. Each of the
251 binding proteins (*CHRD*, *GREM1*, *NOG*, *FST*) and BMPs (*BMP2*, *BMP4*, *BMP6*, *BMP7*)
252 selected for the study has been shown previously to be expressed within bovine antral follicles in
253 a cell-type and follicle stage-dependent manner (Glister et al. 2010, 2011). As anticipated from
254 earlier studies (Glister et al. 2005; Glister et al. 2013) all four BMPs elicited a robust suppression
255 of thecal androgen secretion. Moreover, evidence supporting differential effects of binding
256 proteins was obtained, consistent with selective modulation of autocrine/paracrine BMP
257 signalling in the ovarian follicle. Since GC, rather than TC, appear to be the predominant source
258 of chordin, gremlin, noggin and follistatin in bovine antral follicles (Glister et al. 2011), it is
259 likely that GC-derived binding proteins have a key role in regulating access of BMPs to their

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

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

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

283 follistatin and chordin had no effect. Previously, noggin was found to reverse BMP-4-induced
284 inhibition of progesterone secretion by sheep GC while follistatin was without effect (Pierre et al.
285 2005). Noggin has also been shown to be a potent antagonist of BMP4 action on other non-
286 endocrine cell-types (Canalis et al. 2003; Zimmerman, et al. 1996). As mentioned above BMP4 is
287 predominantly expressed by TC and so the implication for intrafollicular signalling is that GC-
288 derived noggin may diffuse through the basement membrane to modulate the autocrine/paracrine
289 action of BMP4 on TC and thus contribute to the regulation of androgen output. Given the
290 previous observation (Glister et al. 2011) that GC *NOG* expression is minimal in large estrogen-
291 active follicles, this would imply reduced antagonism of thecal BMP4 signalling at this follicle
292 stage. Interestingly, *NOG* expression by cultured GC was inhibited by IGF analogue treatment
293 perhaps accounting for low expression in large estrogen-active follicles (Glister et al. 2005).

294 In contrast to *NOG*, *FST* expression is maximal in GC of large estrogen-active bovine follicles
295 (Glister et al. 2011) and is upregulated by both FSH and IGF1 in cultured GC (Glister et al. 2011;
296 Glister et al. 2001). As well as binding to activin with high affinity (Nakamura et al. 1992),
297 follistatin also binds with lower affinity to other TGF β family members including BMP4, BMP6
298 and BMP7 (Glister et al. 2004), BMP-15 (Otsuka, et al. 2001a) and myostatin (Amthor, et al.
299 2004). Moreover, follistatin was shown to reverse BMP4- and BMP6-induced increases in
300 phospho-Smad1 accumulation in bovine GC, but did not affect the response to BMP7 (Glister et
301 al. 2004). Despite these previous findings, in this study follistatin only promoted a weak and
302 partial reversal of BMP6-induced suppression of thecal androgen and did not affect the response
303 to BMP2, BMP4 or BMP7. Similarly, follistatin did not antagonise the suppressive action of
304 BMP2 or BMP4 on progesterone secretion by sheep GC but had a slight modulatory effect on the
305 response to BMP6 (Pierre et al. 2005). As such, it seems questionable whether follistatin,

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

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

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

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

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

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

360

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365 perceived as prejudicing the impartiality of this scientific work.

366

367 **Table 1** Primers used for real-time PCR

368

369 **Figures**

370 **Fig. 1** Effects of (a) LH and (b) BMP2, BMP4, BMP6 and BMP7 on secretion of
371 androstenedione and progesterone by bovine theca interna cells and on viable cell number at the
372 end of culture. In (b) cells were cultured in the presence of LH. Values are means and bars
373 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.

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

398

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

403

404

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406

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552

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	GGCCCCGTAACTCGACTGTGACAAA	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	GAAACAGCTGCCACCTTCACGTAG	142
Follistatin	NM_175801.2 B	TGAGCAAGGAGGAGTGTTCAGCA	CATCTGGCCTTGAGGAGTGCACATTC	301
Smad6	NM_001206145.1	CGCCACCGCCCTACTCTCGG	GCTGTGATGAGGGAGTTGGCGGC	112
ACTB	NM_173979.3	ATCACCATCGGCAATGAGCGGTTC	CGGATGTGACGTCACACTTCATGA	128

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

Fig 1

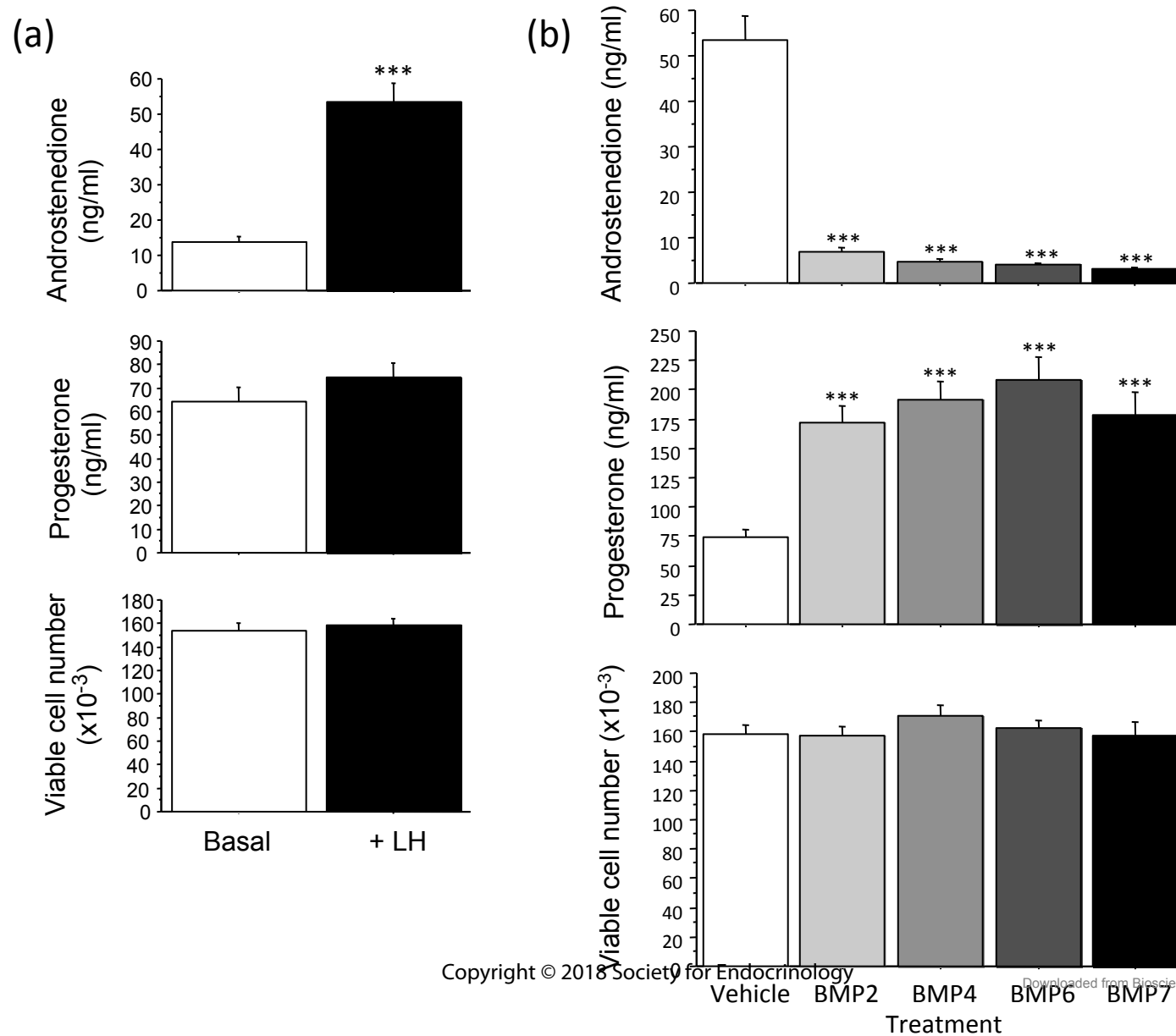


Fig 2

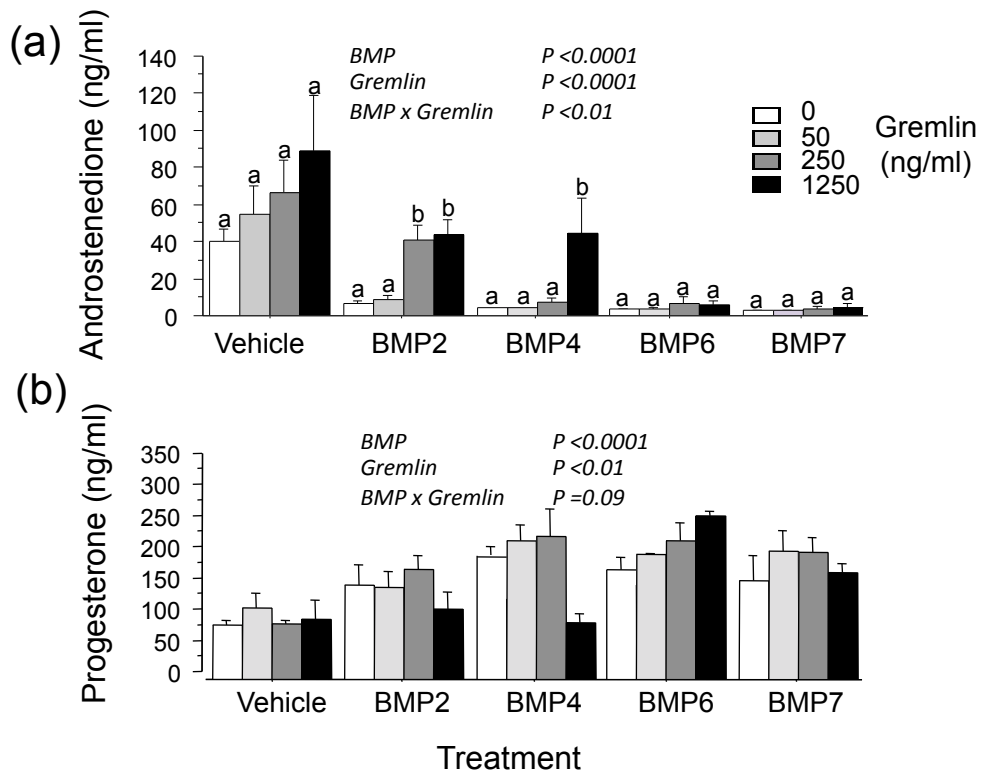


Fig 3

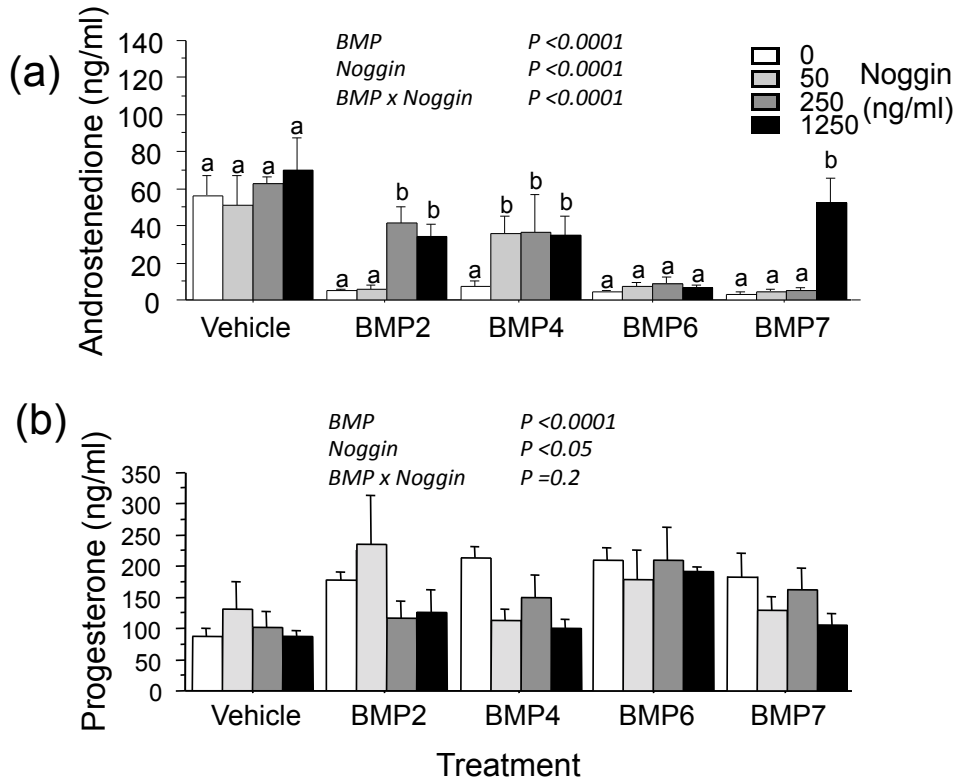


Fig 4

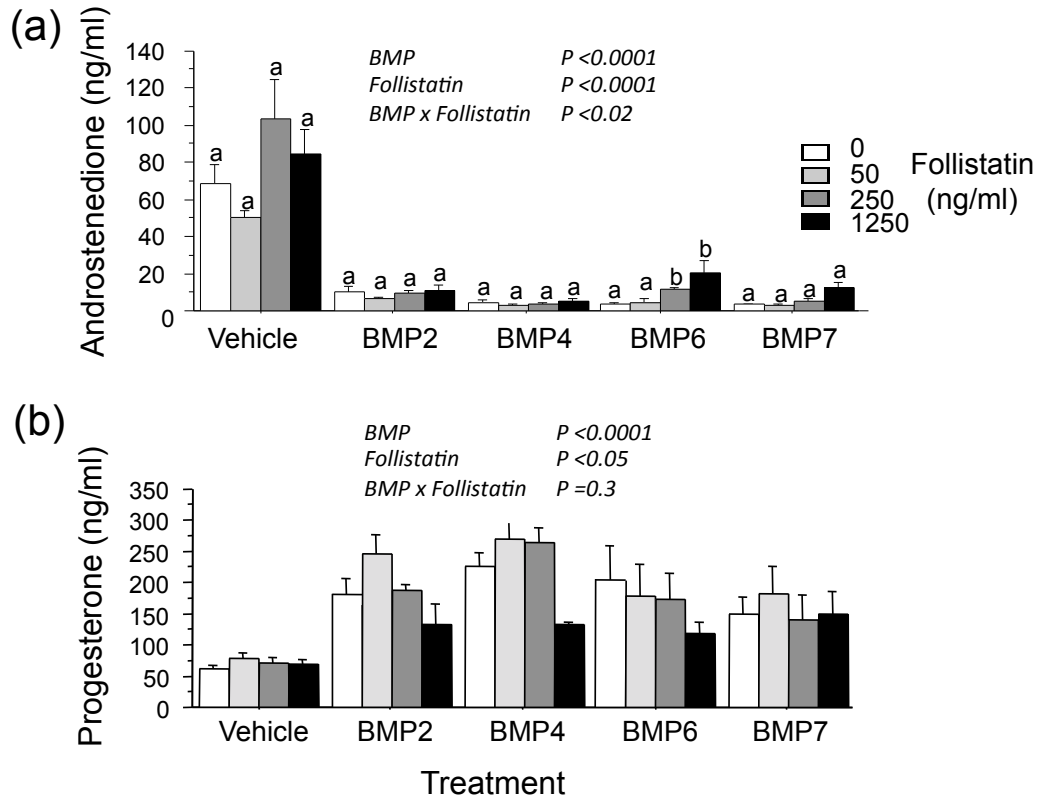


Fig 5

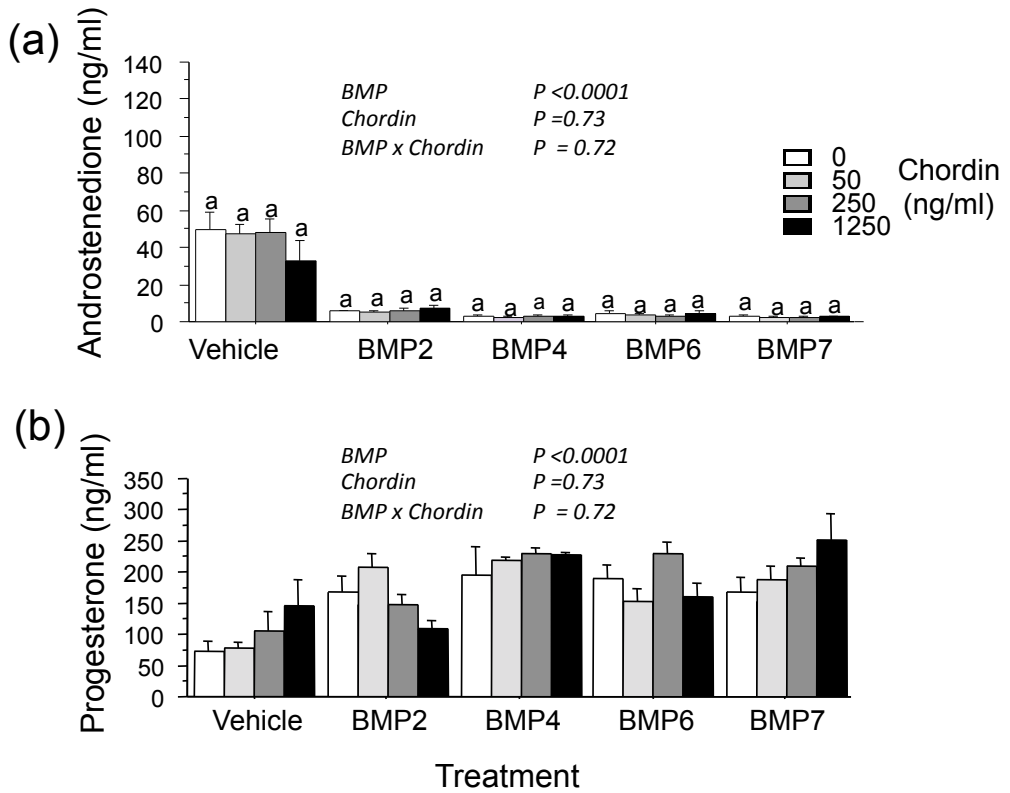
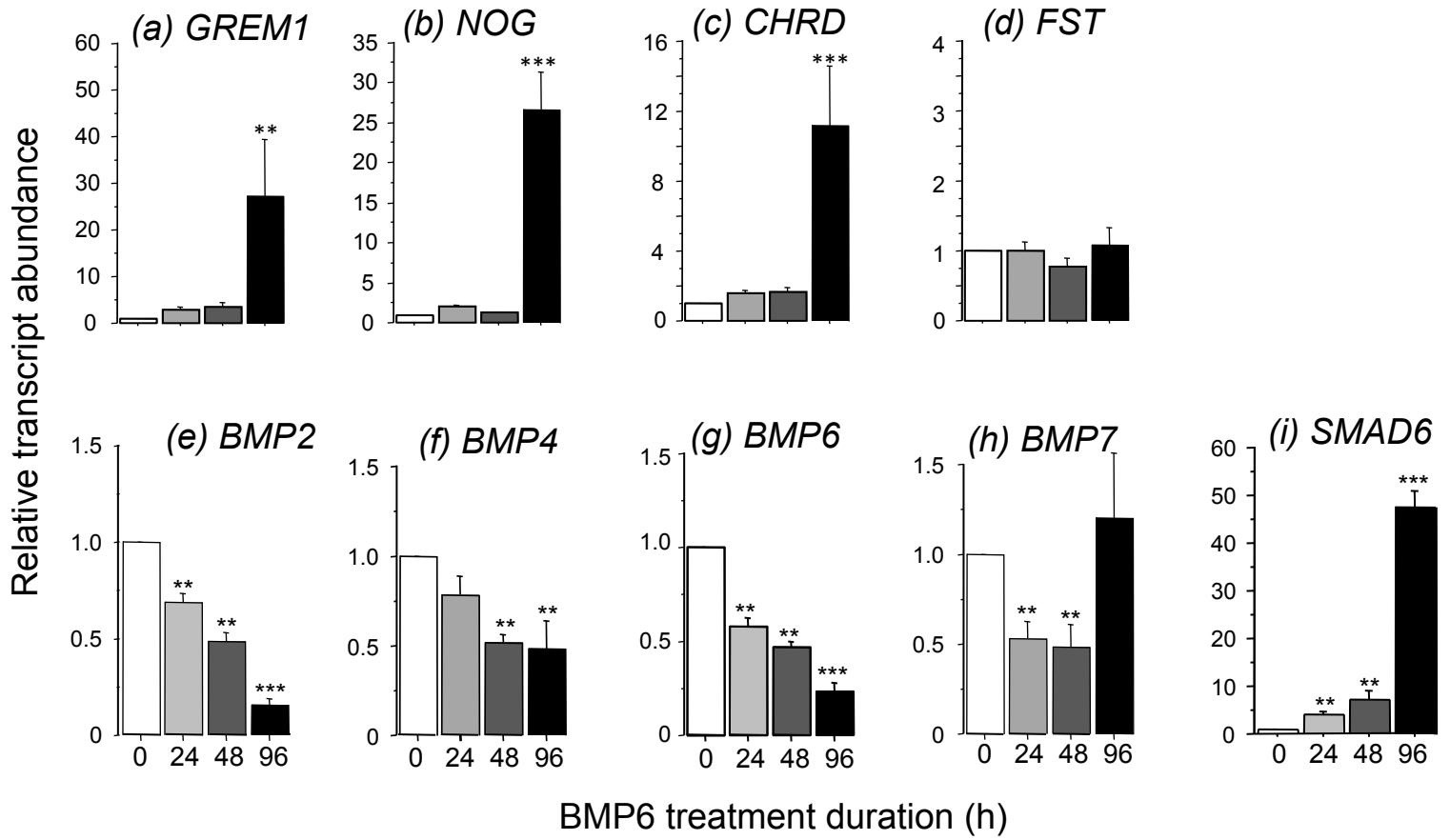


Fig 6



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