

Modulatory effects of TGF- β 1 and BMP6 on thecal angiogenesis and steroidogenesis in the bovine ovary

Article

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1 **Modulatory effects of TGF- β 1 and BMP6 on thecal**
2 **angiogenesis and steroidogenesis in the bovine ovary**

3

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19 *MS: 5391 words (excluding fig. legends and references)*

20 **Abstract**

21

22 Angiogenesis plays an integral role in follicular and luteal development and is
23 positively regulated by several intra-ovarian factors including vascular endothelial
24 growth factor A (VEGFA) and fibroblast growth factor 2 (FGF2). Various
25 transforming growth factor- β (TGF- β) superfamily members function as intra-ovarian
26 regulators of follicle and luteal function but their potential roles in modulating ovarian
27 angiogenesis have received little attention. In this study, we used a bovine theca
28 interna culture model (exhibiting characteristics of luteinization) to examine the
29 effects of TGF- β 1 and bone morphogenetic protein 6 (BMP6) on angiogenesis and
30 steroidogenesis. VEGFA/FGF2 treatment promoted endothelial cell network
31 formation but had little or no effect on progesterone and androstenedione secretion or
32 expression of key steroidogenesis-related genes. TGF- β 1 suppressed basal and
33 VEGFA/FGF2-induced endothelial cell network formation and progesterone secretion,
34 effects that were reversed by an activin receptor-like kinase 5 (ALK5) inhibitor (SB-
35 431542). The ALK5 inhibitor alone raised androstenedione secretion and expression
36 of several transcripts including *CYP17A1*. BMP6 also suppressed endothelial cell
37 network formation under VEGFA/FGF2-stimulated conditions and inhibited
38 progesterone secretion and expression of several steroidogenesis-related genes under
39 basal and VEGFA/FGF2-stimulated conditions. These effects were reversed by an
40 ALK1/2 inhibitor (K02288). Moreover, the ALK1/2 inhibitor alone augmented
41 endothelial network formation, progesterone secretion, androstenedione secretion and
42 expression of several steroidogenesis-related genes. The results indicate dual
43 suppressive actions of both TGF- β 1 and BMP6 on follicular angiogenesis and
44 steroidogenesis. Further experiments are needed to unravel the complex interactions
45 between TGF- β superfamily signalling and other regulatory factors controlling
46 ovarian angiogenesis and steroidogenesis.

47 *244 words*

48

49 **Introduction**

50 In contrast to most tissues and organs in adult organisms, the ovary is a highly
51 dynamic organ displaying considerable tissue turnover and remodelling associated
52 with recurrent growth and regression of follicles and corpora lutea (CL) throughout
53 the reproductive lifespan of the female (Smith *et al.* 1999, Curry & Osteen 2003).
54 Coordinated endocrine, paracrine and autocrine signals contribute to the regulation of
55 follicle and CL turnover. These signals influence a number of physiological processes
56 in the ovary including somatic cell proliferation, cyto-differentiation and apoptosis,
57 oocyte maturation, steroidogenesis and angiogenesis.

58 Angiogenesis plays an indispensable role in follicle and CL development and is a
59 highly regulated process under the influence of both pro- and anti-angiogenic factors
60 (Gerhardt & Betsholtz 2003, Robinson *et al.* 2009). Key pro-angiogenic factors
61 expressed in the ovary include vascular endothelial growth factor A (VEGFA) and
62 fibroblast growth factor 2, while anti-angiogenic factors include thrombospondins and
63 angiostatin (Berisha *et al.* 2000, Berisha *et al.* 2004, Abramovich *et al.* 2009,
64 Robinson *et al.* 2009, Woad & Robinson 2016). Follicular angiogenesis commences
65 at the preantral stage with the theca layer acquiring a sheath of capillaries by the late
66 secondary follicle stage; these capillaries do not penetrate the basal lamina and so the
67 inner granulosa layer remains avascular until the peri-ovulatory period (Wulff *et al.*
68 2001). Continued follicular growth up to the pre-ovulatory stage is accompanied by
69 further development of the thecal capillary network whereas a decrease in vascularity
70 occurs in atretic follicles (Jiang *et al.* 2003). After ovulation, the remnants of the
71 ruptured follicle undergo transformation into the CL and this is accompanied by a
72 further highly intense phase of angiogenesis, particularly in the early luteal phase
73 when a high proportion of the proliferating cells in the CL are of vascular origin
74 (Jiang *et al.* 2003). The follicular basal lamina breaks down and capillaries from the
75 theca interna layer penetrate the previously avascular granulosa layer.

76 Expression of the pro-angiogenic factors VEGFA and FGF2 is evident in granulosa
77 and theca interna layers of bovine follicles from the secondary stage onwards with
78 expression increasing through antral follicle stages (Berisha *et al.* 2000, Yang &
79 Fortune 2007, Berisha *et al.* 2016). FGF2, FGF receptor (FGFR) and VEGF receptor
80 (VEGFR1/2) mRNA and protein are more abundant in the theca interna than

81 granulosa layer of large bovine antral follicles (Berisha *et al.* 2000, Berisha *et al.*
82 2016). The crucial role of VEGFA in driving ovarian angiogenesis is evidenced by the
83 profound inhibition of both follicular (thecal) and luteal angiogenesis observed in
84 marmosets treated with a ‘decoy receptor’ VEGF antagonist (Wulff *et al.* 2001, Wulff
85 *et al.* 2002). Immunoneutralization of VEGF and FGF2 have also been shown to
86 compromise bovine CL function (Yamashita *et al.* 2008, Woad *et al.* 2012). VEGF
87 and FGF2 promote endothelial cell migration and proliferation, acting in a synergistic
88 manner.

89 Various transforming growth factor- β (TGF- β) superfamily members, including TGF-
90 β itself and several bone morphogenetic proteins (BMP) are expressed in the ovary
91 and have been firmly implicated as autocrine/paracrine factors regulating different
92 aspects of follicle and CL development, including cell proliferation/survival,
93 differentiation and steroidogenesis (Erickson & Shimasaki 2003, Shimasaki *et al.*
94 2004, Knight & Glister 2006). Thus far, their potential involvement in the regulation
95 of angiogenesis in the ovary has received little attention with only one report, to our
96 knowledge, documenting an inhibitory action of TGF- β 1 on bovine luteal endothelial
97 cell function and capillary morphogenesis (Maroni & Davis 2011). Since the uterine
98 luteolytic signal PGF2 α upregulates luteal expression of TGF- β the authors propose a
99 role for TGF- β in the luteolytic mechanism in ruminants (Maroni & Davis 2011). In
100 contrast, a stimulatory role for TGF- β 1 in follicular angiogenesis was indicated by its
101 ability to enhance secretion of pro-angiogenic factors, including VEGFA, by rat
102 granulosa cells (Kuo *et al.* 2011). Also, BMP7 was found to upregulate VEGFA
103 expression by human granulosa-lutein cells suggesting a positive role in
104 follicular/luteal angiogenesis (Akiyama *et al.* 2014)

105 TGF- β 1 and TGF- β 2 mRNAs are expressed by sheep ovarian thecal, stromal and
106 vascular cells; vascular cells also expressed TGF- β 3 mRNA (Juengel *et al.* 2004).
107 Signalling receptors for TGF- β (TGFBR1 and TGFBR2) are expressed by the above
108 cell-types as well as by granulosa cells (Juengel & McNatty 2005) indicative of
109 intrafollicular autocrine/paracrine signalling. Likewise, several BMPs are expressed at
110 the intraovarian level, together with their signalling receptors and extracellular
111 binding proteins (Erickson & Shimasaki 2003, Glister *et al.* 2010). Apart from the
112 aforementioned report of BMP7-induced upregulation of granulosal VEGFA

113 expression (Akiyama *et al.* 2014) we are not aware of any other studies examining the
114 involvement of BMPs in follicular or luteal angiogenesis.

115 Despite this, TGF- β and BMPs have been implicated in the regulation of endothelial
116 cell function and angiogenesis in other tissues during normal development and in
117 pathological conditions such as cardiovascular disease and cancer (Cai *et al.* 2012,
118 Peshavariya *et al.* 2014, Guerrero & McCarty 2017). For instance, TGF- β has been
119 shown to exert both pro- and anti-angiogenic actions, in a concentration and cell
120 context-related manner (Orlova *et al.* 2011). Targeted deletion of TGF- β pathway
121 components in mice is embryonically lethal due to disrupted angiogenesis and
122 vasculogenesis (Goumans *et al.* 2009). Microvascular defects associated with
123 hereditary hemorrhagic telangiectasia and pulmonary arterial hypertension are linked
124 to perturbations in TGF- β /BMP signalling (Cai *et al.* 2012, Guerrero & McCarty
125 2017). Likewise, BMP2, BMP4, BMP6, BMP7 and BMP9 have been shown to induce
126 angiogenesis in various in vitro models such as human or bovine aortic endothelial
127 cells (BAEC) or human umbilical vein endothelial cells (HUVEC) (David *et al.*
128 2009). Several BMPs have been shown to enhance angiogenesis by upregulating
129 VEGF expression (Deckers *et al.* 2002, He & Chen 2005). On the other hand, BMP9
130 was shown to inhibit VEGF-induced angiogenesis in BAECs (Scharpfenecker *et al.*
131 2007) while in a HUVEC culture model BMP4 exerted an anti-angiogenic action that
132 was blocked by the BMP antagonist, chordin-like 1 (Kane *et al.* 2008).

133 Given the paucity of information on the involvement of TGF- β superfamily signalling
134 in follicular angiogenesis, in the present study we utilized a bovine theca interna
135 culture model to investigate the effects of two TGF- β superfamily ligands, TGF- β 1
136 and BMP6, alone and in combination with selective ALK5 and ALK1/2 inhibitors
137 respectively, on follicular angiogenesis and steroidogenesis. The effects of the ALK
138 inhibitors alone were also examined to seek evidence that endogenous TGF β /BMP
139 ligands modulate angiogenesis and steroidogenesis. Angiogenesis was evaluated by
140 immunohistological analysis of endothelial cell network formation while
141 steroidogenesis was evaluated by measuring steroid secretion (progesterone and
142 androstenedione) and mRNA expression of key steroidogenesis-related genes.

143

144

145 **Material and methods**

146

147 **Bovine ovaries and collection of theca interna layers**

148 Ovaries from randomly cycling cattle were obtained from a local abattoir and
149 transported to the laboratory in medium-199 supplemented with 1% (v/v) antibiotic
150 antimycotic solution. Theca interna layers were recovered from 4-8mm diameter
151 antral follicles and dissociated into single cells using collagenase digestion as
152 described in detail elsewhere (Glister *et al.* 2005).

153

154 **Follicular angiogenesis cell culture model**

155 An *in vitro* follicular angiogenesis system, which utilizes primary cells derived from
156 the theca interna, was adapted from the method developed by Robinson et al
157 (Robinson *et al.* 2008) for bovine early CL tissue. In this system, tubule-like
158 structures are produced and after seven days in culture, a network of endothelial cells
159 has developed, which resembles a capillary bed.

160 Briefly, sterile coverslips (circular, 19mm diameter x 0.25mm thick; Thermo
161 Scientific, Rochester, NY) were transferred to wells of a 24-well plate (Nunclon, Life
162 Technologies Ltd, Paisley, UK). One ml of gelatin-based Attachment Factor 1X
163 (Thermo Fisher S006100) was added to each well and incubated at 38.5°C with
164 saturating humidity in 5% CO₂ in air until used. Theca interna cells were seeded onto
165 the coated coverslips at a density of 1 x 10⁵/ml and cultured for 7 days. The medium
166 used for the first day of culture was supplemented with 2% (v/v) fetal calf serum. This
167 medium consisted of EBM-2 endothelial cell basal medium (500ml; Lonza, CC-4176),
168 supplemented with undefined (proprietary) concentration of hydrocortisone (Lonza,
169 CC-4112A), R3-insulin like growth factor-1 (Lonza, CC-4115A), ascorbic acid
170 (Lonza CC-4116A), human epidermal growth factor (Lonza CC-4317A), antibiotics
171 (GA-1000) (Lonza CC-4381) and heparin (CC-4396A). In-house supplements
172 including apo-transferrin 5µg/ml (Sigma, T-2036) and sodium selenite 5ng/ml (Sigma,

173 S-9133), insulin 10ng/ml (bovine pancreas, Sigma, I-1882) and BSA 0.1% (Sigma, A-
174 9418) were also added. After the first day of culture, medium was removed and cells
175 were washed with 1ml PBS. Thereafter cells were maintained in serum-free medium
176 for the remainder of the culture period. Medium was changed and treatments applied
177 on day 1, 3 and 5. On day 7 media were either discarded or retained for hormone
178 assay; coverslips with adherent cells were either washed and fixed for subsequent
179 immuno-staining of endothelial cells or lysed using RNeasy lysis buffer (Qiagen) for
180 subsequent isolation of total RNA.

181

182 **Cell culture treatments**

183 Recombinant bovine FGF2 and VEGFA (R&D systems) were initially dissolved in
184 sterile PBS containing 0.1% bovine serum albumin and 4 mM HCl. Cells were treated
185 with/without these established angiogenic factors at final concentrations of 1 or 10
186 ng/ml as used previously in a bovine luteal cell angiogenesis model (Robinson et al.,
187 2008). Recombinant human TGF- β 1 (R&D systems) was dissolved in 4mM HCl to
188 give a stock concentration of 10 μ g/ml. Further dilutions were made in sterile culture
189 medium to achieve final concentrations of 0, 0.1, 1 and 10ng/ml in an initial dose-
190 response experiment. Thereafter, 5ng/ml TGF- β 1 was selected as an optimal effective
191 dose in further experiments. SB-431542 (Tocris Biosciences), a potent and selective
192 inhibitor of TGF- β type I receptors ALK4, ALK5, and ALK7 (Vogt *et al.* 2011), was
193 dissolved in ethanol to give a stock concentration of 10mM. Cells were treated with
194 SB-431542 at final concentrations of 2 μ M and 10 μ M. Recombinant human BMP6
195 (R&D Systems) was dissolved in sterile 4 mM HCl containing 0.1% bovine serum
196 albumin to give a stock concentration of 20 μ g/ml. Further dilutions were made in
197 sterile culture medium to achieve final BMP6 concentrations of 0, 1 and 5ng/ml. The
198 selective inhibitor of BMP-responsive type 1 receptors (ALK1/2/6), K02288 (Tocris),
199 was dissolved in ethanol to give a stock concentration of 10mM. K02288 specifically
200 inhibits the BMP-induced Smad pathway without affecting TGF- β signaling
201 (Sanvitale *et al.* 2013). Cells were treated with K02288 at final concentrations of 2 μ M
202 and 10 μ M.

203

204 **von Willebrand factor (vWF) immunostaining to identify endothelial cells**

205 At the end of culture, cells were fixed immediately and permeabilized in
206 acetone:methanol (1:1) at 4°C for 5 minutes then washed with PBS (3 x 5 minutes).
207 To block endogenous peroxidase 3% (v/v) hydrogen peroxide in methanol was
208 applied for 10 minutes at room temperature. Plates were washed in PBS buffer (3 x 5
209 minutes), followed by serum blocking with 20% (v/v) normal goat serum for 30
210 minutes at room temperature. Polyclonal rabbit anti-human vWF antibody (Dako,
211 High Wycombe, UK) was used at 5µg/ml diluted in 2% (v/v) normal goat serum in
212 PBS. A 200µl of the antibody solution was applied to each well and then incubated in
213 a humidifier box for overnight at 4°C. On the second day, plates were washed in PBS
214 (3 x 5 minutes). The primary antibodies were detected using the ABC Elite (Vector
215 Laboratories, Peterborough, UK) method as follows: biotinylated secondary goat ant-
216 rabbit antibody was diluted 1:250 with 2% (v/v) normal goat serum in PBS and
217 incubated for 30 minutes at room temperature. Plates were then washed in PBS (3 x 5
218 minutes). The avidin-biotin complex was then prepared according to manufacturer's
219 instructions and applied to each well. After that, plates were incubated for 30 minutes
220 at room temperature followed by further washes in PBS (3 x 5 minutes). Visualisation
221 of bound antibodies was determined using 3,3'-diaminobenzidine tetrahydrochloride
222 (DAB). The DAB solution was prepared according to the manufacturer's instructions
223 and incubated for 2 minutes, after which, the reaction was stopped by washing the
224 wells using distilled water. Plates were counterstained with haematoxylin for 20
225 seconds, washed in tap water before being dehydrated through a series of alcohols
226 (70% ethanol (v/v) 1 x 5 minutes), (90% ethanol (v/v) 1 x 5 minutes and 100%
227 ethanol (v/v) 2 x 5 minutes. Coverslips were placed in histoclear for (2 x 20 seconds),
228 removed (with cells attached) from the 24-well plates and then mounted on slides
229 using DPX mounting medium. Images of all sections were visualised under 5x
230 objective lens and then captured using an inverted microscope (Zeiss A1 Inverted
231 Epifluorescent Microscope) fitted with a digital camera (Nikon NIS Elements).

232

233 **Image analysis of vWF immunostaining**

234 A quantification method was developed, based on a protocol previously used to
235 quantify area of vWF staining in a luteal endothelial cell culture (Robinson *et al.*
236 2008). All image analysis was performed using ImageJ 2.0.0. The areas of brown
237 staining (vWF) were highlighted and only areas stained positively for vWF within
238 endothelial cell clusters were recorded. This was repeated for a total of 25 fields of
239 view across the whole coverslip. In each independent experiment two coverslips were
240 examined for each treatment and from this the mean % area of vWF staining was
241 recorded.

242

243 **Real-time PCR analysis**

244 Cultured cells were processed for total RNA isolation using Qiagen RNeasy mini kits
245 and cDNA was synthesized from 0.5µg RNA using the AB high capacity cDNA
246 synthesis kit (Fisher Scientific, UK) according to the manufacturer's instructions.
247 cDNA samples were used for real-time PCR analysis of the expression of *NR5A1*,
248 *STAR*, *CYP11A1*, *HSD3B1*, *CYP17A1*, *INSL3* and *LHR*, using the primers listed
249 elsewhere (Glister *et al.* 2013). β -actin (*ACTB*) was used for normalization of gene
250 expression. qPCR reactions were carried out as described previously (Glister *et al.*
251 2010) using QuantiTect SYBR Green mastermix (Qiagen) and an AB StepOne plus
252 thermal cycler (Applied Biosystems). Relative transcript abundance was evaluated
253 using the $\Delta\Delta C_t$ method (Livak & Schmittgen 2001), with *ACTB* as the initial
254 normalization control. *ACTB* showed uniform expression levels (Ct value) amongst
255 the different treatment groups. Expression levels for each transcript were re-
256 normalized to corresponding values in vehicle-treated control cells.

257

258 **Hormone immunoassays**

259 Androstenedione and progesterone concentrations in cell-conditioned media were
260 determined by competitive ELISA as described previously (Glister *et al.* 2005, Glister
261 *et al.* 2013). Within and between-assay CVs were <10 and 12%, respectively.

262

263 **Statistical analysis**

264 The effects of the various treatments on endothelial network formation, hormone
265 secretion and gene expression were evaluated by one- and/or two-way analysis of
266 variance (ANOVA) as indicated in results. After one-way ANOVA, *post-hoc* pairwise
267 comparisons amongst different TGF- β -related treatments were made by Fisher's
268 PLSD test. In order to reduce heterogeneity of variance, data were log-transformed
269 prior to statistical analysis. qPCR results were analysed as $\Delta\Delta C_t$ values (i.e. \log^2)
270 before being converted to fold difference values for graphical presentation using the
271 formula $2^{(\Delta\Delta C_t)}$. Results are presented as means \pm SEM of ≥ 3 independent batches of
272 cultured cells, as specified in each figure legend.

273

274 **Results**

275 **Effects of VEGFA and FGF2 on endothelial network formation**

276 Immuno-staining (brown) of endothelial cells using vWF as a marker, revealed that a
277 number of networks had formed in each culture (**Fig. 1**). Each network had a central
278 body of endothelial cells from which a number of branches had developed. These
279 networks appeared to be at different stages of development, with varying size and
280 degree of branching. Statistical analysis showed that there was enhanced formation of
281 endothelial networks in response to co-treatment with VEGFA and FGF2 (hereafter
282 referred to as V/F) at both 1 and 10ng/ml, as indicated by a ~ 5 -fold increase in % area
283 of vWF immuno-staining when comparing to basal level ($P < 0.0001$) (see **Fig. 1d**).

284

285 **Effect of TGF- β 1 and ALK5 inhibitor (SB-431542) on endothelial network** 286 **formation**

287 As above, treatment of cells with V/F alone enhanced endothelial network formation
288 by ~ 4 -fold compared to basal level ($P < 0.02$) (**Fig. 2**). TGF- β 1 dose-dependently
289 reduced endothelial cell network formation by up to $\sim 90\%$ under both basal and V/F-
290 induced conditions ($P = 0.004$) (Figure 2). As shown in **fig. 3**, treatment with TGF- β 1
291 and the ALK5 inhibitor (SB-431542), alone and in combination, promoted marked

292 differences in the extent of endothelial cell network formation. As observed in the
293 previous experiment, TGF- β 1 (5ng/ml) reduced network formation by ~90% under
294 both basal and V/F-induced conditions. Furthermore, the ALK5 inhibitor at 2 and
295 10 μ M significantly reversed the inhibitory effect of TGF- β 1 on network formation
296 under both basal and V/F induced conditions.

297

298 **Effect of BMP6 and ALK1/2 inhibitor (K02288) on endothelial network** 299 **formation**

300 As in previous experiments, V/F significantly increased endothelial network
301 formation in comparison to basal level ($P<0.001$) (**Fig. 4**). Treatment with BMP6
302 decreased V/F-induced endothelial cell network formation by up to ~70% ($P<0.01$)
303 but did not affect network formation under basal conditions. **Fig. 5** shows that Co-
304 treatment with the BMP inhibitor (K02288) reversed the suppressive action of BMP6
305 observed under V/F-induced conditions. Moreover, under basal conditions, treatment
306 with the BMP inhibitor alone, or in combination with BMP6, enhanced network
307 formation ~4-fold.

308 **Effect of TGF- β 1 and ALK5 inhibitor (SB-431542) on progesterone and** 309 **androstenedione secretion**

310 A significant ($P<0.05$) TGF- β 1-induced decrease in progesterone production was
311 observed under both basal and V/F-induced conditions. This suppressive action of
312 TGF- β 1 was reversed by the TGF- β inhibitor (**Fig. 6A**). Under basal conditions
313 androstenedione concentrations in cell-conditioned media were very low, ~1000-fold
314 lower than progesterone concentrations and less than the assay detection limit in many
315 samples. Treatment with TGF- β inhibitor alone induced a substantial (10 to 100-fold;
316 $P<0.001$) increase in androstenedione production under both basal and V/F induced
317 conditions. This increase was reversed in cells co-treated with TGF- β 1 (**Fig. 6B**).
318 Two-way ANOVA showed that, overall, V/F treatment did not significantly affect
319 secretion of either progesterone ($P=0.33$) or androstenedione ($P=0.15$).

320

321 **Effects of TGF- β 1 and ALK5 inhibitor on expression of steroidogenesis-related**
322 **transcripts**

323 **Fig. 7** shows the effects of TGF- β 1 and its inhibitor on the relative expression of
324 seven steroidogenesis-related transcripts by theca interna cells cultured under basal
325 and V/F-stimulated conditions. Two-way ANOVA (not shown) indicated significant
326 responses to TGF- β 1 and its inhibitor for all seven transcripts examined (*NR5A1*,
327 *STAR*, *CYP11A1*, *HSD3B1*, *CYP17A1*, *INSL3*, *LHR*) whereas V/F treatment had only
328 a marginal effect on *NR5A1* and *INSL3* transcript abundance. Under basal conditions
329 (without V/F) TGF- β 1 alone did not significantly affect levels of any transcript.
330 However, the TGF- β inhibitor significantly ($P < 0.05$) increased expression of all
331 transcripts with the exception of *NR5A1*. These increases were reversed by TGF- β 1
332 co-treatment, significantly for all transcripts except *CYP11A1* and *LHR*. Under V/F-
333 stimulated conditions, TGF- β 1 treatment alone significantly ($P < 0.05$) decreased levels
334 of *CYP11A1*, *HSD3B1*, *CYP17A1* and *LHR* while co-treatment with TGF- β inhibitor
335 reversed these effects. Treatment with the TGF- β inhibitor alone increased expression
336 of *NR5A1*, *STAR*, *HSD3B1*, *CYP17A1* and *INSL3* ($P < 0.05$).

337

338 **Effect of BMP6 and ALK1/2 inhibitor (K02288) on progesterone and**
339 **androstenedione secretion**

340 **Fig. 8A** shows that under both basal and V/F induced conditions BMP6 reduced
341 progesterone secretion by ~3-fold. Treatment with 10 μ M BMP6 inhibitor alone
342 greatly increased (20 to 50-fold) progesterone secretion while co-treatment with
343 BMP6 abolished this increase. **Fig. 8B** shows a substantial (~100-fold) increase in
344 androstenedione production in cells treated with 10 μ M of BMP6 inhibitor alone. This
345 increase was reversed in cells co-treated with BMP6, under both basal and V/F-
346 induced conditions. BMP6 alone tended to reduce androstenedione secretion but the
347 effect was not significant. Two-way ANOVA showed that, overall, V/F treatment did
348 not significantly affect secretion of either progesterone ($P = 0.43$) or androstenedione
349 ($P = 0.34$).

350

351 **Fig. 9** shows the effects of BMP6 and its inhibitor on the relative expression of seven
352 steroidogenesis-related transcripts by theca interna cells cultured under basal and V/F-
353 stimulated conditions. Two-way ANOVA (not shown) indicated that V/F treatment
354 had no overall effect on expression levels of any of the seven transcripts ($P > 0.3$).
355 Under basal conditions (without V/F) BMP6 significantly reduced the abundance of
356 INSL3 and LHR mRNA. However, the BMP inhibitor significantly ($P < 0.05$)
357 increased expression of STAR and CYP11A1 and tended to increase levels of the
358 other five transcripts. In all cases except LHR these numerical increases were
359 reversed ($P < 0.05$) by BMP6 co-treatment. Under V/F-stimulated conditions, BMP6
360 treatment alone significantly ($P < 0.05$) decreased CYP11A1 expression, an effect
361 reversed by co-treatment with the BMP inhibitor. In addition, treatment with the BMP
362 inhibitor alone increased ($P < 0.05$) the abundance of all seven transcripts and each
363 increase was reversed ($P < 0.05$) by co-treatment with BMP6.

364
365

366 **Discussion**

367

368 During ovarian follicle development *in vivo*, follicular angiogenesis takes place
369 concurrently with steroidogenesis. After ovulation, both processes resume in an
370 intensive manner during follicle luteinization and CL formation (Wulff *et al.* 2001,
371 Fraser *et al.* 2004, Berisha *et al.* 2016). During subsequent CL regression, initiated by
372 the luteolytic action of uterine prostaglandin F2 α (PGF2 α) in ruminants, degeneration
373 of the vascular bed is accompanied by a sharp decline in progesterone secretion.

374 This study utilized a primary bovine theca interna cell culture model to generate novel
375 information on the modulatory actions on angiogenesis and steroidogenesis of two
376 TGF- β superfamily ligands known to be expressed at the intrafollicular level (TGF- β 1,
377 BMP6). Both ligands were shown to suppress 'basal' and/or VEGFA/FGF2-induced
378 angiogenesis and steroidogenesis while pharmacological inhibitors of TGF- β
379 signaling via ALK5 and BMP signaling via ALK1/2 reversed these effects. Both
380 inhibitors also upregulated androstenedione secretion and expression of key
381 steroidogenesis-related genes, including *CYP17A1*.

382 The ability of endothelial cells from the theca interna layer of follicles to re-assemble,
383 proliferate and form capillary-like networks *in vitro* was demonstrated using a model
384 system in which collagenase-digested theca interna tissue, containing both
385 steroidogenic cells and vascular endothelial cells, was seeded on to gelatin-coated
386 coverslips. To promote endothelial cell network formation, a commercial endothelial
387 cell growth medium supplemented with various proprietary factors was utilized. In
388 agreement with previous findings based on early bovine CL (Robinson *et al.* 2008,
389 Woad *et al.* 2009) we demonstrated a robust increase in the formation of capillary-like
390 networks in response to co-treatment with two well established angiogenic growth
391 factors, VEGFA and FGF2. Despite this marked angiogenic response, VEGFA/FGF2
392 co-treatment had little or no effect on steroidogenesis in this model, as reflected by
393 secretion of progesterone and androstenedione or expression levels of key genes
394 involved in the steroidogenic pathway. This suggests that the steroidogenic cells of
395 the theca interna layer may lack responsiveness to VEGFA and/or FGF2, at least
396 under the culture conditions used here. Endothelial cells from bovine CL express
397 FGFR, VEGFR1 and VEGFR2 (Gabler *et al.* 2004) but whether steroidogenic cells of
398 the follicular theca interna also express these receptors remains to be established. Co-
399 localization studies using immunohistochemistry and/or *in situ* hybridization could
400 address this issue.

401 Whilst it is recognised that TGF- β can exert a dual role to either enhance or suppress
402 different aspects of vasculogenesis and angiogenesis (Pepper *et al.* 1993, Orlova *et al.*
403 2011, Mustafa *et al.* 2012, Guerrero & McCarty 2017), our data for bovine theca
404 interna clearly showed that TGF- β 1 induced a dose dependant inhibition of basal and
405 VEGFA/FGF2-induced endothelial network formation. This action was reversed by a
406 selective ALK5 inhibitor, indicating the likely pathway through which TGF- β signals
407 in this context.

408 In agreement with our findings, an inhibitory effect of TGF- β 1 on bovine luteal
409 endothelial cell function and capillary morphogenesis has also been reported (Maroni
410 & Davis 2011). Since the uterine luteolytic signal PGF2 α upregulates luteal
411 expression of TGF- β the authors proposed a role for TGF- β in the luteolytic
412 mechanism in ruminants (Maroni & Davis 2011). Indeed, this would be consistent
413 with the TGF- β -induced reduction in thecal progesterone secretion observed in the

414 present study. TGF- β has also been shown to inhibit progesterone secretion by sheep
415 granulosa cells (Juengel *et al.* 2004). Our findings also concur with a recent study
416 showing that TGF- β 1 dose dependently inhibited endothelial cell network formation
417 in a BAEC culture model (Jarad *et al.* 2017). Additionally, the latter study showed
418 that the inhibitory effect of TGF- β 1 was accompanied by upregulation of the TGF- β
419 accessory receptor endoglin, and Smad2 phosphorylation, but without affecting
420 Smad1/5 phosphorylation. Moreover, TGF- β down regulated VEGFR2 level on the
421 cell surface with a concomitant increase in secreted VEGFR2 level in endothelial cell-
422 conditioned medium, suggesting that the inhibitory action of TGF- β may involve a
423 reduction in VEGFA signalling (Jarad *et al.* 2017). Further work would be required to
424 determine if these considerations apply to the current theca interna culture model. It is
425 also known that TGF- β family members can function in a paracrine manner to
426 activate the production of pro-angiogenic cytokines, including VEGFA, TGF- α and
427 monocyte chemo-attractant protein-1 (MCP1) (Vinals & Pouyssegur 2001, Deckers *et*
428 *al.* 2002, Ma *et al.* 2007, Kuo *et al.* 2011, Guerrero & McCarty 2017). Additionally,
429 TGF- β family members may modulate the function of other factors such as switching
430 VEGFA from a pro-survival factor into a pro-apoptotic factor for endothelial cells
431 (Ferrari *et al.* 2006, ten Dijke & Arthur 2007).

432 Various BMPs, including BMP6 studied here, are expressed in the ovary and are
433 recognised as autocrine/paracrine regulators of follicular and luteal cell proliferation
434 and steroidogenesis (Elvin *et al.* 1999, Shimasaki *et al.* 2004, Knight & Glister 2006,
435 Kayani *et al.* 2009). To our knowledge, the potential intraovarian role of BMPs on
436 follicular or luteal angiogenesis has received little attention. However, BMP7 was
437 reported to enhance VEGFA expression by human granulosa-lutein cells (Akiyama *et*
438 *al.* 2014). Moreover, BMP6 and other related family members are expressed by
439 vascular system cells including endothelial cells and smooth muscle cells suggesting
440 autocrine or paracrine actions on the endothelium (Valdimarsdottir *et al.* 2002).
441 Indeed, BMP6 was suggested to stimulate migration and tube formation of BAECs
442 (Valdimarsdottir *et al.* 2002). In addition, BMP6 induced the proliferation and
443 migration of mouse embryonic endothelial cells, as well as network formation and
444 micro-vessel development in aortic rings (Ren *et al.* 2007, David *et al.* 2009). BMP2
445 and BMP4 have also been shown to promote angiogenesis by stimulating the
446 secretion of pro-angiogenic growth factors, including VEGFA (Kozawa *et al.* 2001,

447 Deckers *et al.* 2002).

448 At variance with these reports, we found that BMP6 reduced VEGFA/FGF2-induced
449 endothelial network formation in our bovine theca interna model, while the selective
450 ALK1/2 inhibitor (K02288) reversed this effect. Moreover, K02288 alone enhanced
451 network formation suggesting blockade of an inhibitory effect of endogenous BMP(s)
452 signaling via ALK1/2. In contrast, we found that the ALK5 inhibitor alone did not
453 enhance network formation above control levels, suggesting an absence of
454 endogenous TGF- β 'tone' suppressing angiogenesis in this model. Since we have
455 found endogenous expression of TGF- β 1, 2 and 3 mRNA in this culture model (data
456 not shown), this is somewhat surprising. It is possible that TGF- β mRNA is not
457 translated or that post-translational processing does not generate bioactive ligand.
458 Alternatively, binding protein(s) and/or coreceptors (betaglycan, endoglin) may
459 modulate binding to signalling receptors (Castonguay *et al.* 2011). Another possible
460 explanation is that the anti-angiogenic effect of endogenous TGF- β is mediated, at
461 least in part, via a different ALK-Smad pathway in endothelial cells. In this context,
462 evidence suggests that TGF- β can also signal via the ALK1/2-Smad1/5 pathway in
463 endothelial cells (Goumans *et al.* 2002, Goumans *et al.* 2003). However, whilst TGF-
464 β signalling via ALK5 elicits an anti-angiogenic response, consistent with our
465 findings in bovine theca interna cells, TGF- β signalling via ALK1/2 evidently
466 enhances angiogenesis in other models (Oh *et al.* 2000, Shao *et al.* 2009, Orlova *et al.*
467 2011). Evidence for 'cross talk' between ALK5 and ALK1/2-mediated signalling
468 pathways has also been presented for other endothelial cell models, highlighting the
469 complexity of potential regulatory mechanisms governing TGF- β signalling
470 (Goumans *et al.* 2003, Orlova *et al.* 2011).

471 Our finding that TGF- β and BMP6 elicited similar inhibitory effects on endothelial
472 cell network formation under VEGFA-FGF2-stimulated conditions was unexpected
473 given that they are purported to signal via different type1 receptor-Smad pathways,
474 ALK5-Smad2/3 and ALK1/2-Smad1/5, respectively. However, this was reinforced by
475 the observed ability of the ALK5 and ALK1/2 inhibitors to reverse, respectively, the
476 anti-angiogenic actions of TGF- β and BMP6. Moreover, BMP6 clearly suppressed
477 basal and/or VEGFA/FGF2-induced progesterone secretion and expression of several
478 key steroidogenesis-related genes. This observation is consistent with a previous *in*

479 *in vitro* study on bovine theca-lutein cells (Kayani *et al.* 2009). The ability of the
480 ALK1/2 inhibitor to reverse the inhibitory effect of BMP6 on progesterone production
481 indicates that the response is likely mediated by the ALK1/2 pathway. However, as
482 observed for the angiogenic response, treatment with the ALK1/2 inhibitor alone
483 promoted substantial increases in secretion of progesterone and androstenedione,
484 accompanied by increased expression of most of the steroidogenesis-related genes
485 examined. As such, these observations reinforce the view that endogenous BMPs
486 expressed by the cultured cells exert a dual suppressive action on both angiogenesis
487 and steroidogenesis. Interestingly, luteal expression of several BMPs, including
488 BMP6, increases during the late luteal phase in bovine (Kayani *et al.* 2009) and
489 human (Nio-Kobayashi *et al.* 2015) consistent with their involvement in luteolysis.
490 Moreover, BMP expression by human granulosa-lutein cells was downregulated by
491 human chorionic gonadotrophin, reinforcing this concept (Nio-Kobayashi *et al.* 2015).

492 Regarding the gene expression analyses, neither total RNA yield, nor expression
493 levels of the normalization control gene (*ACTB*) were affected by any of the
494 treatments (data not shown). However, it is possible that the observed changes in
495 relative expression levels of steroidogenesis-related genes in our culture model could
496 be due, at least in part, to treatment-induced changes in relative numbers of different
497 cell-types contributing to the total RNA extracted from cell lysates at the end of
498 culture.

499 It should be noted that culture conditions influence the extent to which follicular theca
500 interna cells undergo luteinisation *in vitro*, as reflected by their morphological
501 phenotype, transcriptional profile and steroid secretory profile (i.e. progesterone to
502 androstenedione ratio) (Campbell *et al.* 1998, Glister *et al.* 2005, Kayani *et al.* 2009).
503 In general, exposure to serum-supplemented media and/or high concentrations of LH,
504 forskolin or insulin promotes luteinisation, accompanied by a substantial increase in
505 progesterone to androstenedione ratio. The culture conditions used in the present
506 endothelial cell culture model (including use of serum-supplemented medium for first
507 day of culture) would be expected to induce some degree of cellular luteinisation.
508 Indeed, the cells formed an adherent monolayer and the progesterone to
509 androstenedione ratio in cell-conditioned medium was very high (>100:1) under all
510 treatment conditions. This contrasts with the progesterone to androstenedione ratio of

511 ~2:1 exhibited by ‘non-luteinised’ bovine theca interna cells cultured under defined,
512 serum-free conditions (Glister *et al.* 2005). The challenge remains to devise a
513 follicular theca interna angiogenesis culture model that mimics more closely the
514 physiological status of a healthy growing follicle, rather than a luteinizing follicle.

515 In conclusion, the present results indicate that both TGF- β 1 and BMP6 exert
516 inhibitory actions on ovarian angiogenesis and steroidogenesis, likely mediated by
517 ALK5 and ALK1/2 signalling pathways. Further experiments, beyond the scope of
518 the present study, are needed to unravel the complex interactions between multiple
519 TGF- β superfamily ligands and other regulatory factors implicated in the dual control
520 of ovarian angiogenesis and steroidogenesis at different stages of follicular and luteal
521 development.

522

523 **Declaration of Interest**

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

526

527 **Author contributions**

528 PGK, ML and DM conceived and planned the research; DM, ML and MS performed
529 the experiments and contributed to data analysis and interpretation; PGK drafted the
530 manuscript with input from DM and ML.

531

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537

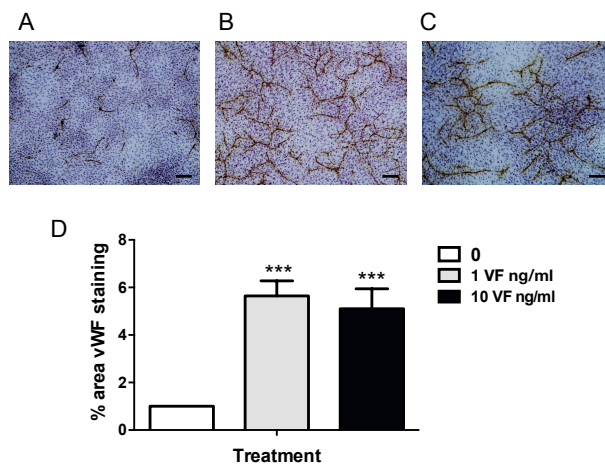
538

539 **Figure Legends**

540

541 **Fig. 1** Development of endothelial cell network in theca interna culture system in
542 response to co-treatment with VEGFA and FGF2 (V/F). Endothelial cells were
543 immuno-stained brown with vWF antibody as shown in representative images of (A)
544 control cells; (B) cells treated with 1ng/ml V/F; (C) cells treated with 10ng/ml V/F;
545 (D) % area of vWF immunostaining based on quantitative analysis of images from 5
546 independent cultures. Values are means and bars indicate SEM. ***P<0.001 versus
547 controls. Scale bars indicate 100 μ m.

Fig. 1

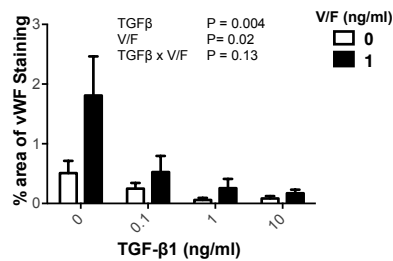


548

549 **Fig. 2** The effect of TGF- β 1 alone and in combination with VEGFA and FGF2 (V/F)
550 on network formation by cultured theca interna cells. Values are means and bars

551 indicate SEM (n=3 independent batches of cells); two-way ANOVA results are
552 shown.

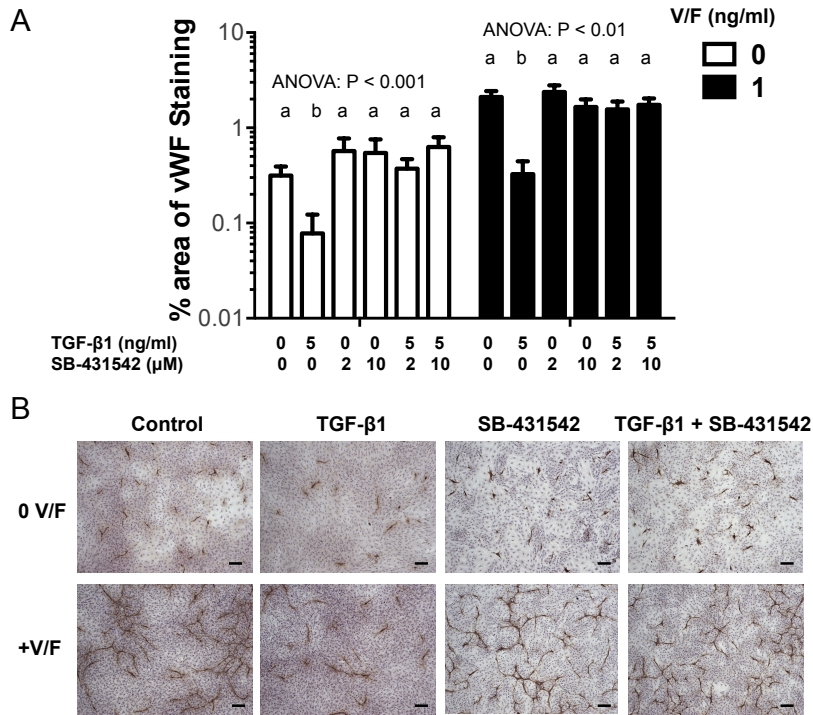
Fig. 2



553

554 **Fig. 3A** Effect of TGF-β1 and the ALK5 inhibitor (SB-431542), alone and in
555 combination, on basal and VEGFA/FGF (V/F)-induced network formation in cultured
556 theca interna cells. Values are means and error bars indicate SEM (n=5 independent
557 batches of cells). Separate one-way ANOVA and *post-hoc* pairwise comparisons were
558 made for cells cultured with and without V/F; means without a common letter are
559 significantly different (P < 0.05). **B** shows representative images of cells treated with
560 vehicle, TGF-β1 and SB-431542 (2μM) in the presence and absence of V/F. Scale
561 bars = 100 μm.

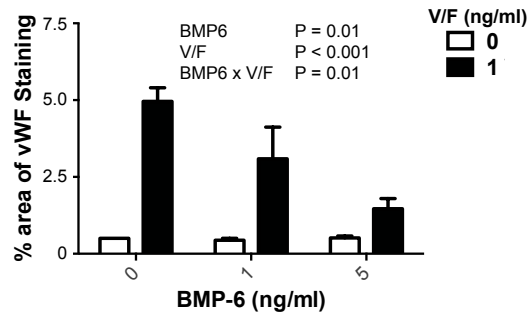
Fig. 3



562

563 **Fig. 4** Effect of BMP6 in the presence/absence of VEGFA and FGF2 (V/F) on
 564 network formation by cultured theca interna cells. Values are means and error bars
 565 indicate SEM (n=3 independent batches of cells); two-way ANOVA results are
 566 shown.

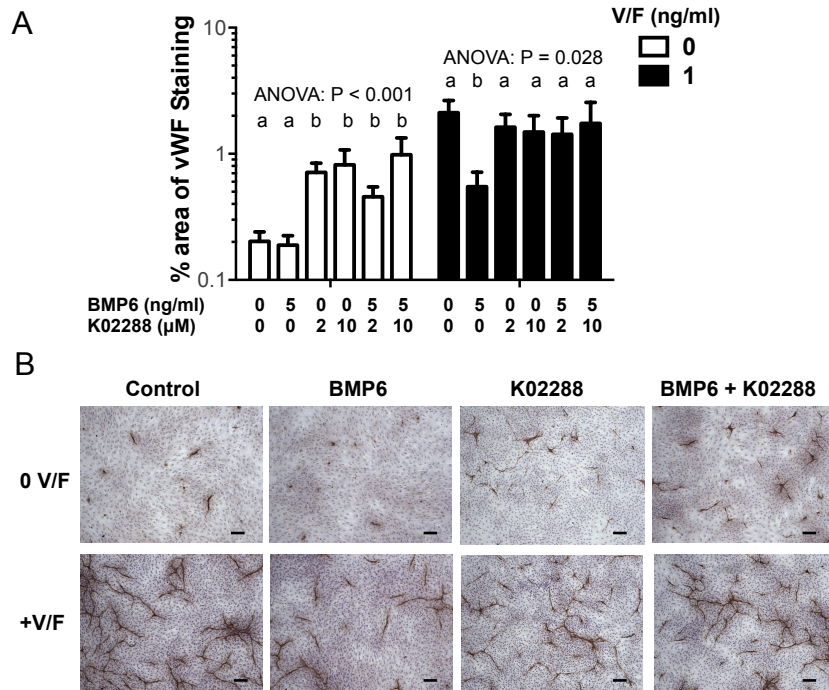
Fig. 4



567

568 **Fig. 5A** Effect of BMP6 and BMP inhibitor (K02288), alone and in combination, on
569 basal and VEGFA/FGF2 (V/F)-induced network formation in cultured theca interna
570 cells. Values are means and error bars indicate SEM (n=5 independent batches of
571 cells). Separate one-way ANOVA and *post-hoc* pairwise comparisons were made for
572 cells cultured with and without V/F; means without a common letter are significantly
573 different (P < 0.05). **B** shows representative images of cells treated with vehicle,
574 BMP6 and K02288 (2 μ M) in the presence and absence of V/F. Scale bars = 100 μ m.

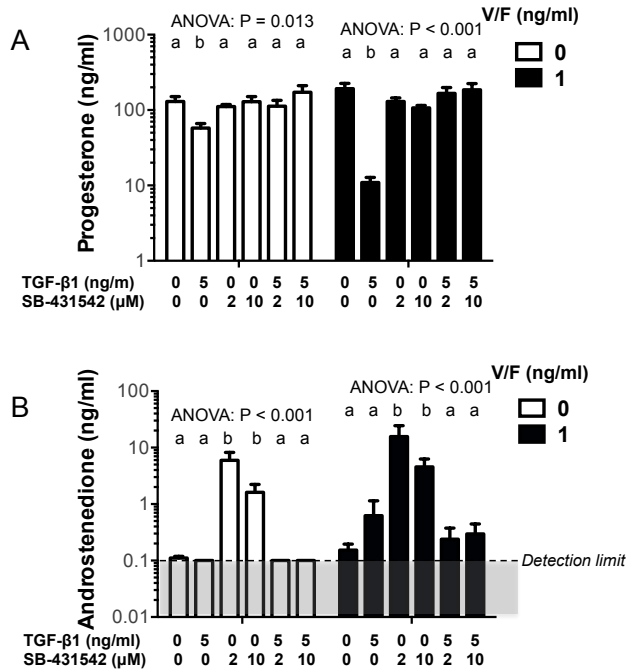
Fig. 5



575

576 **Fig. 6** The effect of TGF- β 1 and its antagonist alone or in combination on basal and
577 VEGFA/FGF2 (V/F)-induced production of (A) progesterone and (B)
578 androstenedione by cultured bovine theca interna cells. Values are means and bars
579 indicate SEM (n=5 independent batches of cells). Separate one-way ANOVA and
580 *post-hoc* pairwise comparisons were made for cells cultured with and without V/F;
581 means without a common letter are significantly different ($P < 0.05$).

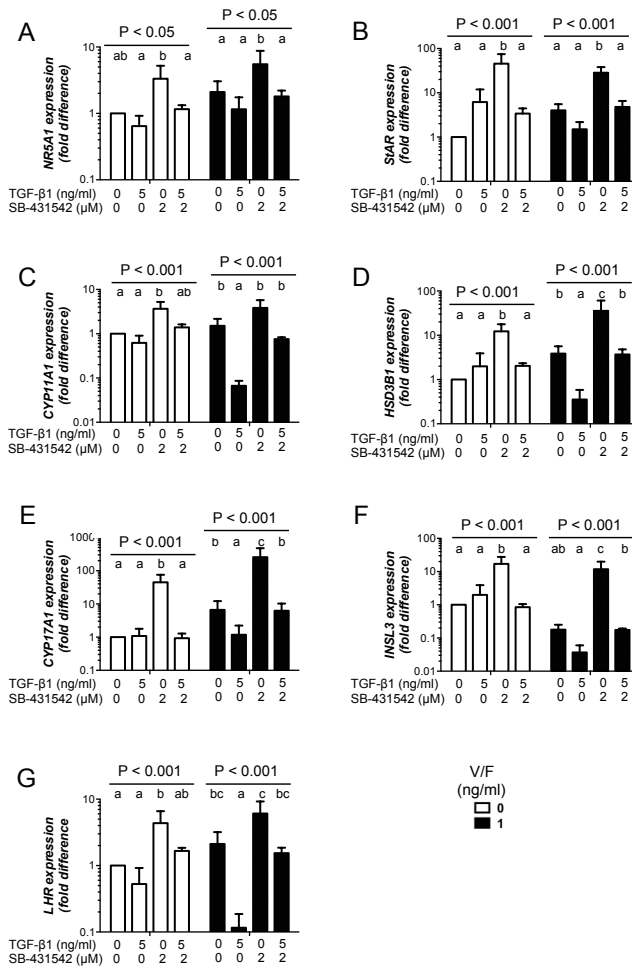
Fig. 6



582

583 **Fig. 7** Changes in relative expression of steroidogenesis-related mRNA transcripts in
 584 cultured theca interna cells treated with TGF-β1 and its inhibitor (SB-431542) alone
 585 and in combination, under ‘basal’ (open bars) and V/F-stimulated (filled bars)
 586 conditions: (A) *NR5A1*; (B) *STAR*; (C) *CYP11A1*; (D) *HSD3B1*; (E) *CYP17A1*; (F)
 587 *INSL3*; (G) *LHR*. Values are means and bars indicate SEM (n=5 independent batches
 588 of cells) Results of one-way ANOVA and *post-hoc* pairwise comparisons are
 589 indicated; means without a common letter are significantly different (P < 0.05).

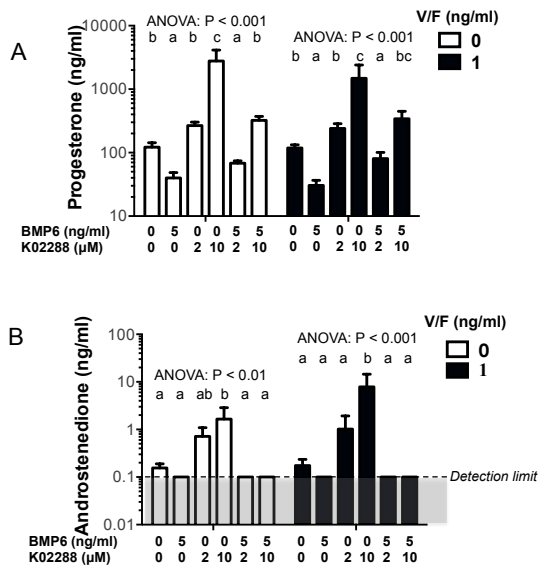
Fig. 7



590

591 **Fig. 8** Effect of BMP6 and its antagonist alone or in combination in the
 592 presence/absence of VEGFA and FGF2 (V/F), on the production of (A) progesterone
 593 and (B) androstenedione by bovine theca layer cultured cells. Values are means and
 594 bars indicate SEM (n=5 independent batches of cells). Separate one-way ANOVA
 595 and post-hoc pairwise comparisons were made for cells cultured with and without
 596 V/F; means without a common letter are significantly different (P < 0.05).

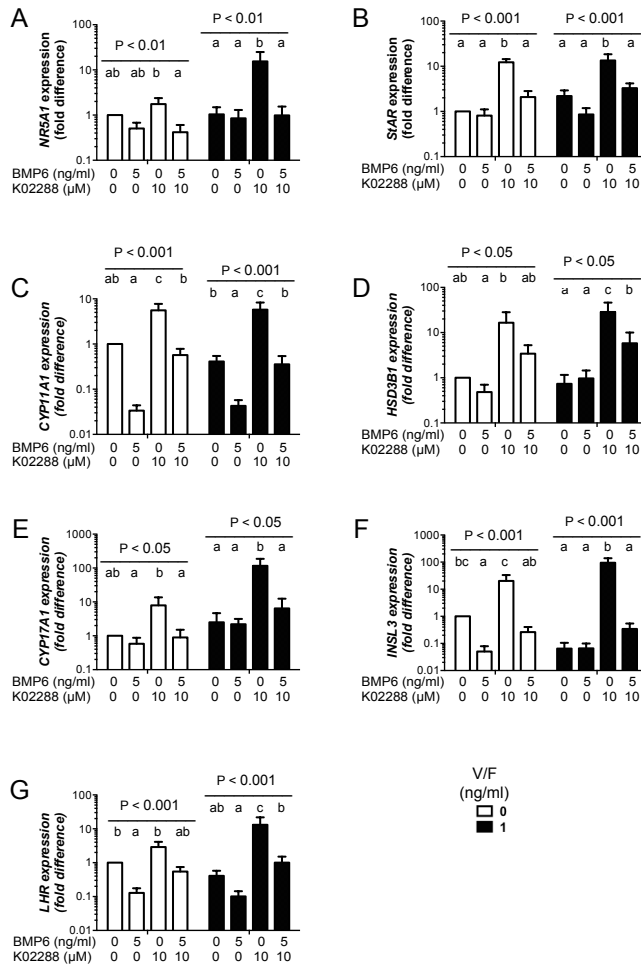
Fig. 8



597

598 **Fig. 9** Changes in relative expression of steroidogenesis-related mRNA transcripts in
 599 cultured theca interna cells treated with BMP6 and the ALK1/2 inhibitor (K02288)
 600 alone and in combination, under ‘basal’ (open bars) and V/F-stimulated (filled bars)
 601 conditions. (A) *NR5A1*; (B) *STAR*; (C) *CYP11A1*; (D) *HSD3B1*; (E) *CYP17A1*; (F)
 602 *INSL3*; (G) *LHR*. Values are means and bars indicate SEM (n=5 independent batches
 603 of cells) Results of one-way ANOVA and *post-hoc* pairwise comparisons are
 604 indicated; means without a common letter are significantly different ($P < 0.05$).

Fig. 9



605

606

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608

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