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Modulatory effects of TGF-β1 and BMP6 on thecal angiogenesis and steroidogenesis in the bovine ovary

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Abstract

Angiogenesis plays an integral role in follicular and luteal development and is positively regulated by several intra-ovarian factors including vascular endothelial growth factor A (VEGFA) and fibroblast growth factor 2 (FGF2). Various transforming growth factor-β (TGF-β) superfamily members function as intra-ovarian regulators of follicle and luteal function but their potential roles in modulating ovarian angiogenesis have received little attention. In this study, we used a bovine theca interna culture model (exhibiting characteristics of luteinization) to examine the effects of TGF-β1 and bone morphogenetic protein 6 (BMP6) on angiogenesis and steroidogenesis. VEGFA/FGF2 treatment promoted endothelial cell network formation but had little or no effect on progesterone and androstenedione secretion or expression of key steroidogenesis-related genes. TGF-β1 suppressed basal and VEGFA/FGF2-induced endothelial cell network formation and progesterone secretion, effects that were reversed by an activin receptor-like kinase 5 (ALK5) inhibitor (SB-431542). The ALK5 inhibitor alone raised androstenedione secretion and expression of several transcripts including *CYP17A1*. BMP6 also suppressed endothelial cell network formation under VEGFA/FGF2-stimulated conditions and inhibited progesterone secretion and expression of several steroidogenesis-related genes under basal and VEGFA/FGF2-stimulated conditions. These effects were reversed by an ALK1/2 inhibitor (K02288). Moreover, the ALK1/2 inhibitor alone augmented endothelial network formation, progesterone secretion, androstenedione secretion and expression of several steroidogenesis-related genes. The results indicate dual suppressive actions of both TGF-β1 and BMP6 on follicular angiogenesis and steroidogenesis. Further experiments are needed to unravel the complex interactions between TGF-β superfamily signalling and other regulatory factors controlling ovarian angiogenesis and steroidogenesis.

244 words

Introduction

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

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

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

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

Various transforming growth factor-β (TGF-β) superfamily members, including TGF-β itself and several bone morphogenetic proteins (BMP) are expressed in the ovary and have been firmly implicated as autocrine/paracrine factors regulating different aspects of follicle and CL development, including cell proliferation/survival, differentiation and steroidogenesis (Erickson & Shimasaki 2003, Shimasaki *et al.* 2004, Knight & Glister 2006). Thus far, their potential involvement in the regulation of angiogenesis in the ovary has received little attention with only one report, to our knowledge, documenting an inhibitory action of TGF-β1 on bovine luteal endothelial cell function and capillary morphogenesis (Maroni & Davis 2011). Since the uterine luteolytic signal PGF2α upregulates luteal expression of TGF-β the authors propose a role for TGF-β in the luteolytic mechanism in ruminants (Maroni & Davis 2011). In contrast, a stimulatory role for TGF-β1 in follicular angiogenesis was indicated by its ability to enhance secretion of pro-angiogenic factors, including VEGFA, by rat granulosa cells (Kuo *et al.* 2011). Also, BMP7 was found to upregulate VEGFA expression by human granulosa-lutein cells suggesting a positive role in follicular/luteal angiogenesis (Akiyama *et al.* 2014)

TGF-β1 and TGF-β2 mRNAs are expressed by sheep ovarian thecal, stromal and vascular cells; vascular cells also expressed TGF-β3 mRNA (Juengel *et al.* 2004). Signalling receptors for TGF-β (TGFBR1 and TGFBR2) are expressed by the above cell-types as well as by granulosa cells (Juengel & McNatty 2005) indicative of intrafollicular autocrine/paracrine signalling. Likewise, several BMPs are expressed at the intraovarian level, together with their signallling receptors and extracellular binding proteins (Erickson & Shimasaki 2003, Glister *et al.* 2010). Apart from the aforementioned report of BMP7-induced upregulation of granulosal VEGFA

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

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

Given the paucity of information on the involvement of TGF-β superfamily signalling in follicular angiogenesis, in the present study we utlilized a bovine theca interna culture model to investigate the effects of two TGF-β superfamily ligands, TGF-β1 and BMP6, alone and in combination with selective ALK5 and ALK1/2 inhibitors respectively, on follicular angiogenesis and steroidogenesis. The effects of the ALK inhibitors alone were also examined to seek evidence that endogenous TGFβ/BMP ligands modulate angiogenesis and steroidogenesis. Angiogenesis was evaluated by immunohistological analysis of endothelial cell network formation while steroidogenesis was evaluated by measuring steroid secretion (progesterone and androstenedione) and mRNA expression of key steroidogenesis-related genes.

Material and methods

Bovine ovaries and collection of theca interna layers

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

Follicular angiogenesis cell culture model

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

Briefly, sterile coverslips (circular, 19mm diameter x 0.25mm thick; Thermo Scientific, Rochester, NY) were transferred to wells of a 24-well plate (Nunclon, Life Technologies Ltd, Paisley, UK). One ml of gelatin-based Attachment Factor 1X (Thermo Fisher S006100) was added to each well and incubated at 38.5℃ with saturating humidity in 5% CO_2 in air until used. Theca interna cells were seeded onto the coated coverslips at a density of 1 x 10^5/ml and cultured for 7 days. The medium used for the first day of culture was supplemented with 2% (v/v) fetal calf serum. This medium consisted of EBM-2 endothelial cell basal medium (500ml; Lonza, CC-4176), supplemented with undefined (proprietary) concentration of hydrocortisone (Lonza, CC-4112A), R3-insulin like growth factor-1 (Lonza, CC-4115A), ascorbic acid (Lonza CC-4116A), human epidermal growth factor (Lonza CC-4317A), antibiotics (GA-1000) (Lonza CC-4381) and heparin (CC-4396A). In-house supplements including apo-transferrin 5µg/ml (Sigma, T-2036) and sodium selenite 5ng/ml (Sigma,

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

Cell culture treatments

Recombinant bovine FGF2 and VEGFA (R&D systems) were initially dissolved in sterile PBS containing 0.1% bovine serum albumin and 4 mM HCl. Cells were treated with/without these established angiogenic factors at final concentrations of 1 or 10 ng/ml as used previously in a bovine luteal cell angiogenesis model (Robinson et al., 2008). Recombinant human TGF-β1 (R&D systems) was dissolved in 4mM HCl to give a stock concentration of 10μg/ml. Further dilutions were made in sterile culture medium to achieve final concentrations of 0, 0.1, 1 and 10ng/ml in an initial dose-response experiment. Thereafter, 5ng/ml TGF-β1 was selected as an optimal effective dose in further experiments. SB-431542 (Tocris Biosciences), a potent and selective inhibitor of TGF-β type I receptors ALK4, ALK5, and ALK7 (Vogt *et al.* 2011), was dissolved in ethanol to give a stock concentration of 10mM. Cells were treated with SB-431542 at final concentrations of 2μM and 10μM. Recombinant human BMP6 (R&D Systems) was dissolved in sterile 4 mM HCl containing 0.1% bovine serum albumin to give a stock concentration of 20μg/ml. Further dilutions were made in sterile culture medium to achieve final BMP6 concentrations of 0, 1 and 5ng/ml. The selective inhibitor of BMP-responsive type 1 receptors (ALK1/2/6), K02288 (Tocris), was dissolved in ethanol to give a stock concentration of 10mM. K02288 specifically inhibits the BMP-induced Smad pathway without affecting TGF-β signaling (Sanvitale *et al.* 2013). Cells were treated with K02288 at final concentrations of 2μM and 10μM.

von Willebrand factor (vWF) immunostaining to identify endothelial cells

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

Image analysis of vWF immunostaining

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

Real-time PCR analysis

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

Hormone immunoassays

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

Statistical analysis

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

Results

Effects of VEGFA and FGF2 on endothelial network formation

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

Effect of TGF-β1 and ALK5 inhibitor (SB-431542) on endothelial network formation

As above, treatment of cells with V/F alone enhanced endothelial network formation by ~4-fold compared to basal level ($P<0.02$) **(Fig. 2).** TGF-β1 dose-dependently reduced endothelial cell network formation by up to ~90% under both basal and V/F-induced conditions ($P=0.004$) (Figure 2). As shown in **fig. 3**, treatment with TGF-β1 and the ALK5 inhibitor (SB-431542), alone and in combination, promoted marked

differences in the extent of endothelial cell network formation. As observed in the previous experiment, TGF-β1 (5ng/ml) reduced network formation by ~90% under both basal and V/F-induced conditions. Furthermore, the ALK5 inhibitor at 2 and 10μM significantly reversed the inhibitory effect of TGF-β1 on network formation under both basal and V/F induced conditions.

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

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

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

A significant ($P<0.05$) TGF-β1-induced decrease in progesterone production was observed under both basal and V/F-induced conditions. This suppressive action of TGF-β1was reversed by the TGF-β inhibitor (**Fig. 6A**). Under basal conditions androstenedione concentrations in cell-conditioned media were very low, ~1000-fold lower than progesterone concentrations and less than the assay detection limit in many samples. Treatment with TGF-β inhibitor alone induced a substantial (10 to100-fold; $P<0.001$) increase in androstenedione production under both basal and V/F induced conditions. This increase was reversed in cells co-treated with TGF-β1 (**Fig. 6B**). Two-way ANOVA showed that, overall, V/F treatment did not significantly affect secretion of either progesterone ($P=0.33$) or androstenedione ($P=0.15$).

Effects of TGF-β1 and ALK5 inhibitor on expression of steroidogenesis-related transcripts

Fig. 7 shows the effects of TGF-β1 and its inhibitor on the relative expression of seven steroidogenesis-related transcripts by theca interna cells cultured under basal and V/F-stimulated conditions. Two-way ANOVA (not shown) indicated significant responses to TGF-β1 and its inhibitor for all seven transcripts examined (*NR5A1, STAR, CYP11A1, HSD3B1, CYP17A1, INSL3, LHR*) whereas V/F treatment had only a marginal effect on *NR5A1* and *INSL3* transcript abundance. Under basal conditions (without V/F) TGF-β1alone did not significantly affect levels of any transcript. However, the TGF-β inhibitor significantly ($P<0.05$) increased expression of all transcripts with the exception of *NR5A1*. These increases were reversed by TGF-β1 co-treatment, significantly for all transcripts except *CYP11A1* and *LHR*. Under V/F-stimulated conditions, TGF-β1treatment alone significantly ($P<0.05$) decreased levels of *CYP11A1, HSD3B1, CYP17A1* and *LHR* while co-treatment with TGF-β inhibitor reversed these effects. Treatment with the TGF-β inhibitor alone increased expression of *NR5A1, STAR, HSD3B1, CYP17A1* and *INSL3* ($P<0.05$).

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

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

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

Discussion

During ovarian follicle development *in vivo,* follicular angiogenesis takes place concurrently with steroidogenesis. After ovulation, both processes resume in an intensive manner during follicle luteinization and CL formation (Wulff *et al.* 2001, Fraser *et al.* 2004, Berisha *et al.* 2016). During subsequent CL regression, initiated by the luteolytic action of uterine prostaglandin F2α (PGF2α) in ruminants, degeneration of the vascular bed is accompanied by a sharp decline in progesterone secretion.

This study utilized a primary bovine theca interna cell culture model to generate novel information on the modulatory actions on angiogenesis and steroidogenesis of two TGF-β superfamily ligands known to be expressed at the intrafollicular level (TGF-β1, BMP6). Both ligands were shown to suppress 'basal' and/or VEGFA/FGF2-induced angiogenesis and steroidogenesis while pharmacological inhibitors of TGF-β signaling via ALK5 and BMP signaling via ALK1/2 reversed these effects. Both inhibitors also upregulated androstenedione secretion and expression of key 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-β1 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-β1 on bovine luteal
409 endothelial cell function and capillary morphogenesis has also been reported (Maroni
410 & Davis 2011). Since the uterine luteolytic signal PGF2α 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

present study. TGF-β has also been shown to inhibit progesterone secretion by sheep granulosa cells (Juengel *et al.* 2004). Our findings also concur with a recent study showing that TGF-β1 dose dependently inhibited endothelial cell network formation in a BAEC culture model (Jarad *et al.* 2017). Additionally, the latter study showed that the inhibitory effect of TGF-β1 was accompanied by upregulation of the TGF-β accessory receptor endoglin, and Smad2 phosphorylation, but without affecting Smad1/5 phosphorylation. Moreover, TGF-β down regulated VEGFR2 level on the cell surface with a concomitant increase in secreted VEGFR2 level in endothelial cell-conditioned medium, suggesting that the inhibitory action of TGF-β may involve a reduction in VEGFA signalling (Jarad *et al.* 2017). Further work would be required to determine if these considerations apply to the current theca interna culture model. It is also known that TGF-β family members can function in a paracrine manner to activate the production of pro-angiogenic cytokines, including VEGFA, TGF-α and monocyte chemo-attractant protein-1 (MCP1) (Vinals & Pouyssegur 2001, Deckers *et al.* 2002, Ma *et al.* 2007, Kuo *et al.* 2011, Guerrero & McCarty 2017). Additionally, TGF-β family members may modulate the function of other factors such as switching VEGFA from a pro-survival factor into a pro-apoptotic factor for endothelial cells (Ferrari *et al.* 2006, ten Dijke & Arthur 2007).

Various BMPs, including BMP6 studied here, are expressed in the ovary and are recognised as autocrine/paracrine regulators of follicular and luteal cell proliferation and steroidogenesis (Elvin *et al.* 1999, Shimasaki *et al.* 2004, Knight & Glister 2006, Kayani *et al.* 2009). To our knowledge, the potential intraovarian role of BMPs on follicular or luteal angiogenesis has received little attention. However, BMP7 was reported to enhance VEGFA expression by human granulosa-lutein cells (Akiyama *et al.* 2014). Moreover, BMP6 and other related family members are expressed by vascular system cells including endothelial cells and smooth muscle cells suggesting autocrine or paracrine actions on the endothelium (Valdimarsdottir *et al.* 2002). Indeed, BMP6 was suggested to stimulate migration and tube formation of BAECs (Valdimarsdottir *et al.* 2002). In addition, BMP6 induced the proliferation and migration of mouse embryonic endothelial cells, as well as network formation and micro-vessel development in aortic rings (Ren *et al.* 2007, David *et al.* 2009). BMP2 and BMP4 have also been shown to promote angiogenesis by stimulating the 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-β1, 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 elicites 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*

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

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

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

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

In conclusion, the present results indicate that both TGF-β1 and BMP6 exert inhibitory actions on ovarian angiogenesis and steroidogenesis, likely mediated by ALK5 and ALK1/2 signalling pathways. Further experiments, beyond the scope of the present study, are needed to unravel the complex interactions between multiple TGF-β superfamily ligands and other regulatory factors implicated in the dual control of ovarian angiogenesis and steroidogenesis at different stages of follicular and luteal development.

Declaration of Interest

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

Author contributions

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

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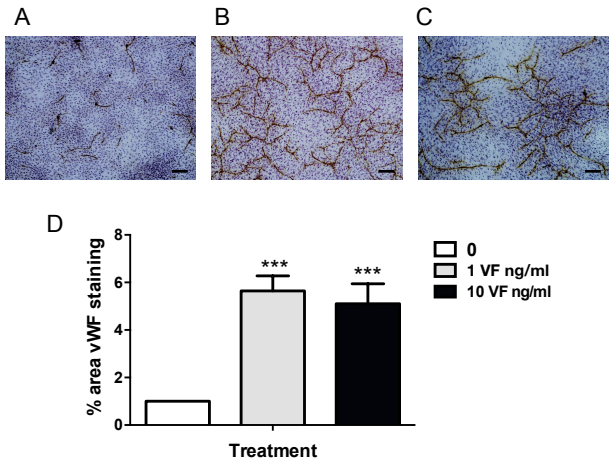
539 **Figure Legends**

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 μm.

Fig. 1



548

549 **Fig. 2** The effect of TGF-β1 alone and in combination with VEGFA and FGF2 (V/F)
550 on network formation by cultured theca interna cells. Values are means and bars

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

Fig. 2

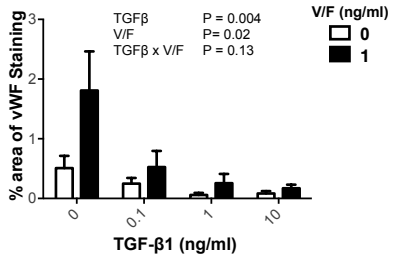
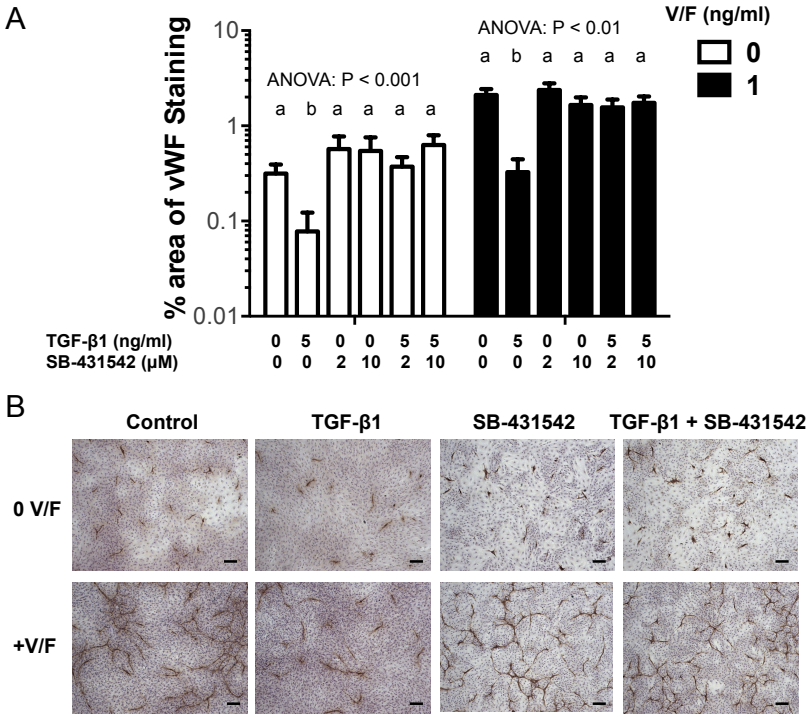


Fig. 3A Effect of TGF-β1 and the ALK5 inhibitor (SB-431542), alone and in combination, on basal and VEGFA/FGF (V/F)-induced network formation in cultured theca interna cells. Values are means and error bars indicate SEM (n=5 independent batches of cells). Separate one-way ANOVA and *post-hoc* pairwise comparisons were made for cells cultured with and without V/F; means without a common letter are significantly different ($P < 0.05$). **B** shows representative images of cells treated with vehicle, TGF-β1and SB-431542 ($2\mu M$) in the presence and absence of V/F. Scale bars = 100 μm.

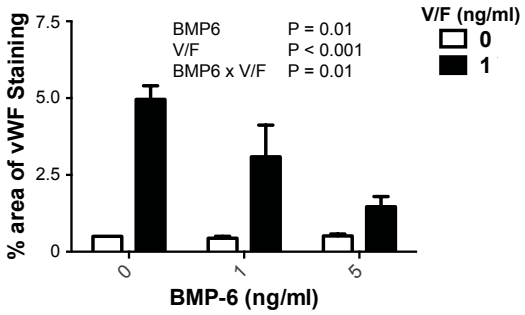
Fig. 3



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.

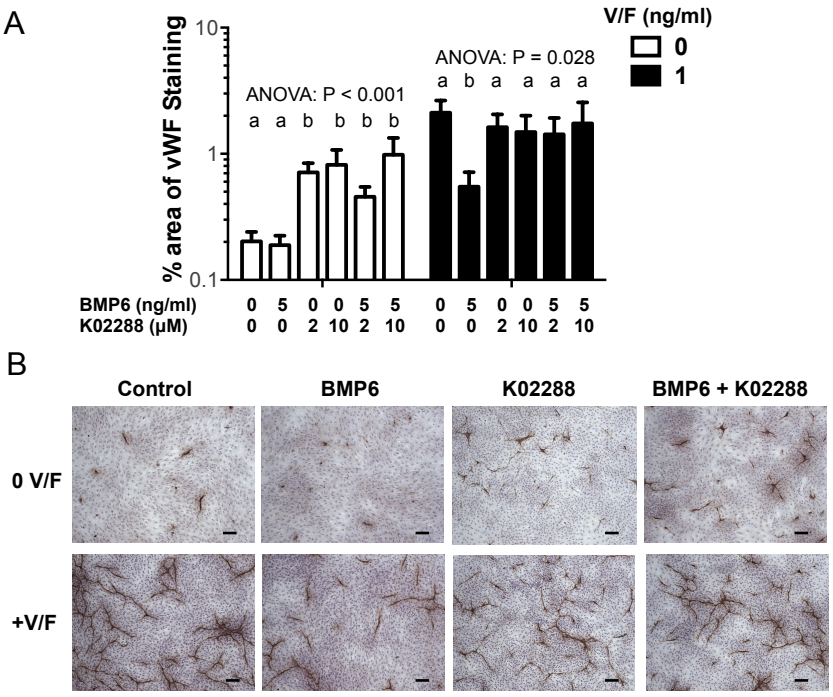
Fig. 4



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 ($2\mu M$) in the presence and absence of V/F. Scale bars = 100 μm.

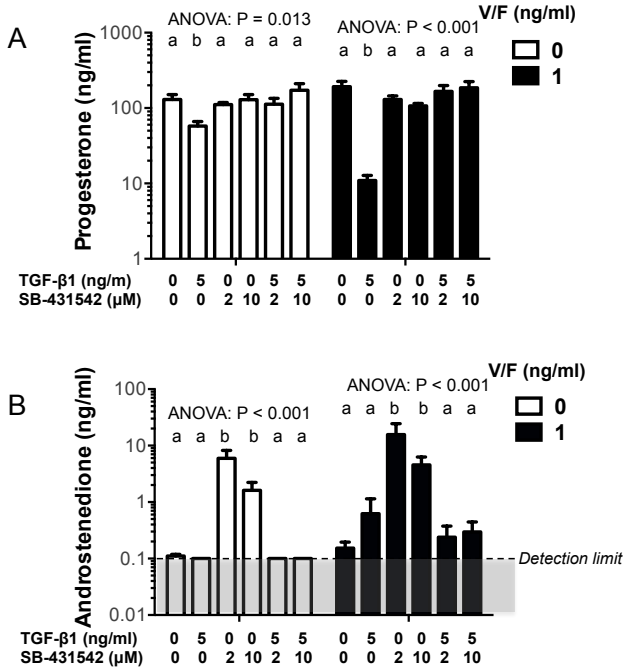
Fig. 5



575

576 **Fig. 6** The effect of TGF-β1 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 ($P < 0.05$).

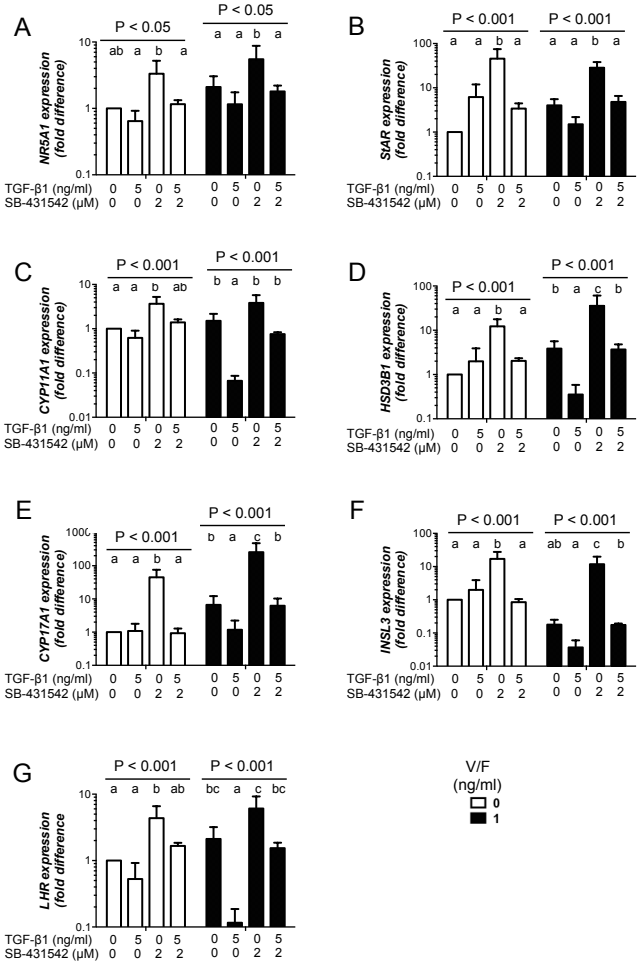
Fig. 6



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 ($P < 0.05$).

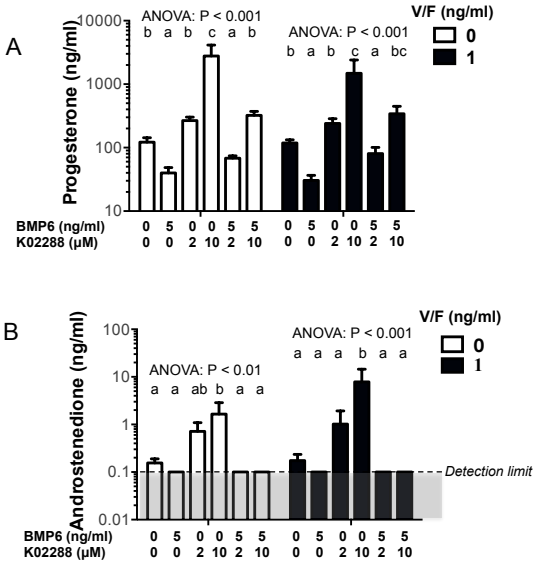
Fig. 7



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 ($P < 0.05$).

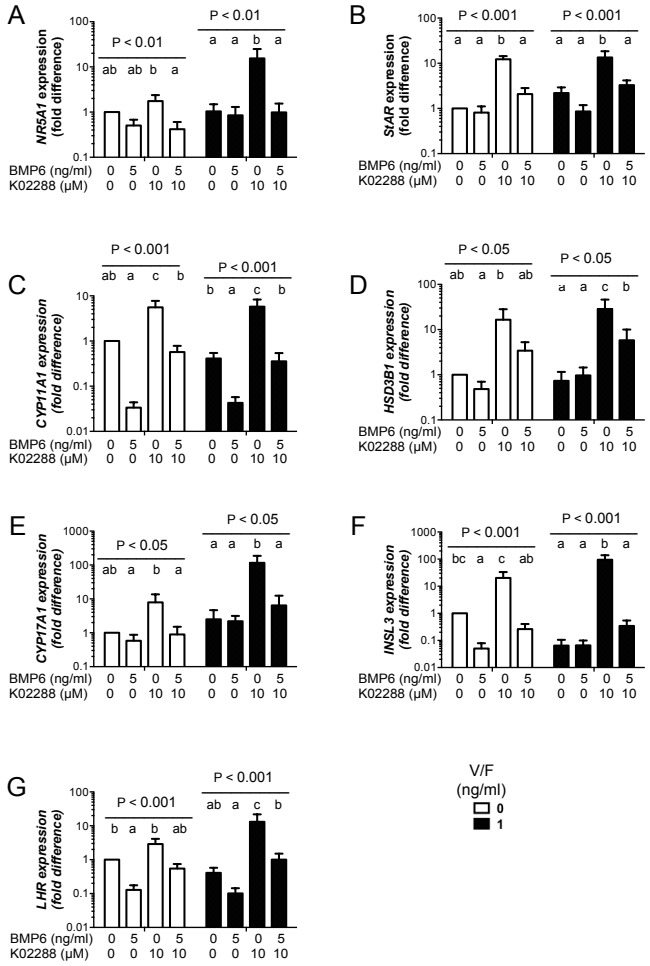
Fig. 8



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



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