TGFB1 disrupts angiogenesis during the follicular-luteal transition through the Smad-SERPINE1/SERPINB5 signaling pathway in the cow

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Abstract

Intense angiogenesis is critical for the development of the corpus luteum and is tightly regulated by numerous factors. However, the exact role transforming growth factor beta 1 (TGFB1) plays during this follicular-luteal transition remains unclear. This study hypothesized that TGFB1 acting through TGFBR1 and Smad2/3 signaling would suppress angiogenesis during the follicular-luteal transition. Using a serum-free luteinizing follicular angiogenesis culture system, TGFB1 (1 and 10ng.mL^{-1}) markedly disrupted the formation of capillary-like structures, reducing endothelial cell network area and number of branch points ($P<0.001$). Furthermore, TGFB1 activated canonical Smad signaling and inhibited endothelial nitric oxide synthase (*NOS3*) mRNA expression, but up-regulated latent TGF-beta binding protein, type I TGFB receptor (*TGFBR1*), *SERPINE1* and *SERPINB5* mRNA expression. TGFBR1 inhibitor, SB431542, reversed the *SERPINE1* and *SERPINB5* up-regulation by TGFB1. Additionally, TGFB1 reduced progesterone synthesis through decreasing *STAR*, *CYP11A1* and *HSD3B1* expression. These results show that TGFB1 regulated *NOS3*, *SERPINE1*, and *SERPINB5* expression via TGFBR1 and Smad2/3 signaling and could be the mechanism by which TGFB1 suppresses endothelial networks. Thereby, TGFB1 may provide a critical homeostatic control of angiogenesis during the follicular-luteal transition. Our findings reveal the molecular mechanisms underlying the actions of TGFB1 in early luteinization which may lead to novel therapeutic strategies to reverse luteal inadequacy.

Keywords: TGFB1, Angiogenesis, Luteinization, Smad, SERPINE1, SERPINB5

39 **1. Introduction**

40 Angiogenesis is an essential process associated with corpus luteum (CL)
41 development and is critical to both luteal structure and progesterone production
42 (Woad and Robinson 2016). The CL requires an extensive vascular supply to support
43 its rapid growth and steroidogenic function, and inadequate progesterone production
44 is associated with poor embryo development and increased pregnancy failure in cows
45 (Robinson *et al.* 2009). The molecular regulation of angiogenesis in the CL is
46 complex and involves numerous regulators (Stocco *et al.* 2007). These include
47 vascular endothelial growth factor A (VEGFA), fibroblast growth factor 2 (FGF2),
48 endocrine gland-derived VEGF, and angiopoietins (Reynolds *et al.* 2000; LeCouter *et*
49 *al.* 2002; Woad and Robinson 2016). Identifying the angiogenic factors involved in
50 angiogenesis is necessary to improve the understanding of luteal development and
51 function. It is becoming evident that transforming growth factor B (TGFB) can
52 stimulate or inhibit angiogenesis *in vivo* and *in vitro* in a highly context- and
53 concentration-dependent manner (Goumans *et al.* 2009). For example TGFB1 has
54 pro-angiogenic roles during tissue remodeling in wound healing (Pepper 1997).
55 However, TGFB1 induced apoptosis in bovine aortic and human umbilical vein
56 endothelial cells (EC) (Ferrari *et al.* 2006). There is further evidence that TGFB1 is
57 involved in the disassembly of capillaries during bovine luteal regression (Maroni and
58 Davis 2011; Farberov and Meidan 2016).

59 TGFB1 complexes with latent TGFB binding protein (LTBP)/ latency associated
60 peptide (LAP) but under certain physiological conditions active TGFB1 is liberated

61 (Robertson *et al.* 2015). TGFB then mediates its effects through coordinated binding
62 of two TGF serine/threonine kinase receptors, TGFBR1 (also known as activin
63 receptor-like kinase 5, ALK5) and TGFBR2 (Heldin *et al.* 1997; Jakobsson and van
64 Meeteren 2013; Goumans and Ten Dijke 2018). Upon ligand binding, TGFBR2
65 phosphorylates TGFBR1 leading to activation and phosphorylation of Smad (i.e.
66 Smad2/3) proteins. Subsequently, Smad2/3 complexes with Smad4 which translocates
67 to the nucleus and regulates expression of target genes, such as endothelial nitric
68 oxide synthase (eNOS; NOS3), serpin family E member 1 (SERPINE1) (Pepper *et al.*
69 1990; Wongnoppavich *et al.* 2017; McCann *et al.* 2019; Wang *et al.* 2019), and serpin
70 family B member 5 (SERPINB5) (Boehm *et al.* 1999; Farberov and Meidan 2016;
71 Wongnoppavich *et al.* 2017). It is likely that TGFB1 regulates angiogenesis by acting
72 through this canonical Smad-dependent pathway (Goumans *et al.* 2003). In the bovine
73 ovary, TGFB1 and the TGFBR1 are expressed at many different timepoints. For
74 example, *TGFB1* mRNA expression increased while its protein levels declined during
75 antral follicle development (Nilsson *et al.* 2003). *TGFBR1* mRNA is also expressed at
76 this time (Roelen *et al.* 1998) with TGFBR1 expression greater in dominant than in
77 pre-selection follicles (Jayawardana *et al.* 2006). Conversely, a luteolytic dose of
78 PGF2α induced *TGFB1* mRNA and protein expression in the bovine CL (Hou *et al.*
79 2008; Farberov and Meidan 2016). It is known that TGFB1 is abundantly secreted by
80 bovine theca cells, luteal cells, and luteinizing granulosa cells *in vitro* (Skinner *et al.*
81 1987; Gangrade *et al.* 1993; Joseph *et al.* 2012). However, the precise
82 temporal-spatial expression of TGFB1 in the different cell types during the

83 follicular-luteal transition is less clear.

84 TGFB1-regulated genes are expressed during the follicular-luteal transition and
85 several are likely to regulate the degeneration of the follicular basement membrane
86 following ovulation (Smith *et al.* 1999). For example, SERPINE1 is a primary
87 negative regulator of plasmin-driven proteolysis, and excessive expression contributes
88 to accumulation of collagen and other extracellular matrix proteins. Furthermore,
89 SERPINE1 expression increased in the newly-formed CL before declining once the
90 CL reached its mature size (Kliem *et al.* 2007). It was also more abundantly expressed
91 in atretic compared with healthy follicles (Hayashi *et al.* 2011) suggesting that
92 SERPINE1 might be upregulated during periods of extensive tissue remodeling.

93 It is known that TGFB1 can modulate the function of steroidogenic cells.
94 However, the actions of TGFB1 on steroidogenesis can be contradictory. TGFB1
95 stimulated proliferation, differentiation and progesterone synthesis in bovine mature
96 granulosa cells (Fazzini *et al.* 2006), which is supported by similar observations in rat
97 granulosa cells (Dodson and Schomberg 1987). Conversely, there are several reports
98 that TGFB1 reduced progesterone secretion from bovine luteal cells (Hou *et al.* 2008)
99 and porcine granulosa cells (Kubota *et al.* 1994). In contrast, Sriperumbudur *et al.*
100 (2010) observed that TGFB1 played a role in the luteinization of pig follicles but did
101 not influence steroidogenesis.

102 To date, the actions of TGFB1 have largely focused on its effects during
103 follicular development and luteal regression, with its mechanism of action in the
104 developing CL less clear. However, based on the observations that the TGFB system

105 is present in the follicular-luteal transition and that it can have potent effects on
106 angiogenesis and steroidogenesis, we hypothesized that TGFB1 plays key roles in
107 regulating these processes during luteinization in the cow. It is likely that these
108 processes require precise control for optimal luteal function. A particular intrigue is
109 that TGFB1 appears to be anti-angiogenic to bovine luteal endothelial cells from
110 mature CL, while during the luteinization process, intense angiogenesis is dominant.
111 Therefore, we have addressed this apparent contradiction by determining the effect of
112 different concentrations of TGFB1 on angiogenesis, progesterone production and its
113 signaling components using a serum-free angiogenic culture system (Laird *et al.* 2013).
114 This system contains multiple follicular cell types (e.g. steroidogenic, EC and
115 pericytes) and is stimulated to undergo luteinization concurrent with angiogenesis, thus
116 maintaining physiological relevance and providing an *in vitro* system whereby the
117 complex interactions between cells types can be investigated.

118 ## 2. Materials and methods

119 *Reagents and antibodies*

120 Unless stated otherwise, cell culture reagents were purchased from
121 Sigma-Aldrich Corp (St Louis, MO, USA). The recombinant human TGFB1 protein
122 and SB431542 were obtained from R&D Systems (Minneapolis, MN, USA) and
123 Sigma-Aldrich, respectively. Anti-Smad2, anti-phospho-Smad2, anti-Smad3,
124 anti-phospho-Smad3, anti-ACTB and anti-GAPDH (glyceraldehyde-3-phosphate
125 dehydrogenase) antibodies were obtained from Cell Signaling Technology (Beverly,
126 MA, USA). Anti-TUBB antibody was from TransGen Biotechnology (Beijing, China).

127 Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgGs were
128 obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

129 *Preparation of coverslips and medium*

130 Fibronectin coated glass coverslips were prepared as detailed previously
131 (Robinson *et al.* 2008; Laird *et al.* 2013). MCDB131 medium (Sigma-Aldrich) is a
132 specialized endothelial basal medium which was supplemented with 100 units.mL^{-1}
133 penicillin, 100 units.mL^{-1} streptomycin, 18.2 μg.mL^{-1} hydrocortisone, 1 μg.mL^{-1}
134 ascorbic acid, 50 ng.mL^{-1} amphotericin-B, 50 μg.mL^{-1} gentamicin, 20 ng.mL^{-1}
135 LR^{3}-IGF1, 10 IU.mL^{-1} heparin, 10 μg.mL^{-1} insulin, 5.5 μg.mL^{-1} transferrin, 5 ng.mL^{-1}
136 selenium and 5 ng.mL^{-1} LH (Prospec-Tany Technogene Ltd, Ness-Ziona, Israel). Fetal
137 bovine serum (FBS, 1%) was added to this medium for the first 18-24 h of culture
138 only and was then replaced with 1 mg.mL^{-1} bovine serum albumin (BSA) for the
139 remainder of the culture period.

140 *Tissue collection and luteinizing follicular angiogenesis culture system*

141 Bovine ovaries were collected from a local slaughterhouse and transported back
142 to the laboratory in phosphate-buffered saline (PBS) at room temperature (RT).
143 Healthy-looking large antral follicles were selected, and follicular cells were collected.
144 These were then cultured in a validated luteinizing follicular angiogenesis culture
145 system, whereby cells from both granulosa and theca cell layers (including endothelial
146 cells) are co-cultured in an environment that supports both luteinization and
147 angiogenesis (Laird *et al.* 2013). In brief, ovaries were washed three times with sterile
148 physiological saline at 37°C, then large follicles (>10 mm) with good vascularization

149 were dissected out, hemisected and granulosa cells were dispersed manually into
150 medium. Clumps of granulosa cells were removed by filtration through a 70μm mesh
151 filter. In the meantime, the theca shells were dispersed by 100 $mg.mL^{-1}$ collagenase
152 type IA, 50 $mg.mL^{-1}$ hyaluronidase type IS and 0.2 $units.mL^{-1}$ DNase type IV
153 digestion in a shaking water bath at 37°C. After 30 min, the digestion was stopped by
154 adding FBS and cell clumps removed by filtration (70μm mesh). The dispersed cells
155 from the theca layer were then centrifuged for 5 min at 300g at 20°C and resuspended
156 in MCDB131 medium. Any red blood cells were lysed by adding 3 volumes of lysis
157 buffer (Solarbio Life Science, Beijing, China) for 5 min. Following a second
158 centrifugation (300g, 10 min), theca cells were resuspended in fresh MCDB131
159 medium. Cell number and viability were determined by trypan blue exclusion before
160 mixing cells derived from both the granulosa and theca cell layers (granulosa: theca
161 ratio 3:1) together. These cells were then plated at a density of $4x10^5$ cells/well in
162 12-well culture plates (Corning Life Sciences, MA, USA). Cells were incubated in a
163 humidified incubator in 5% CO_2 in air at 39°C and thereafter the medium was
164 replaced with fresh supplemented MDCB131 medium after 1, 3, and 5 days of
165 culture.

166 In the initial experiment, the cells were treated with 0, 1, or 10 $ng.mL^{-1}$ TGFB1
167 in the absence or presence of 10 μM SB431542, which was added as a pre-treatment
168 for 1h before the TGFB1 treatment. Treatments started on Day 1 of culture. In
169 subsequent experiments only 10 $ng.mL^{-1}$ TGFB1 was utilised. SB431542 is a
170 selective and potent inhibitor of the related ALK4, ALK5, and ALK7 receptors and

171 has been used extensively to block TGFBR1 activation (Inman *et al.* 2002) and
172 SB431542 was added at 10 μM as previously reported (Maroni and Davis 2011).
173 SB431542 was dissolved in DMSO, to a final DMSO concentration of 0.1% (v/v),
174 with an equivalent concentration of DMSO added to control wells. At least three
175 separate cultures were conducted for each experiment. At the end of culture, the
176 medium was collected and stored at -20°C until analysis, and luteinized follicular
177 cells were fixed in acetone:methanol (1:1) at 4°C for 5 min for immunohistochemical
178 analysis or collected for RNA and protein extraction.

179 *Immunocytochemistry for VWF and image analysis*

180 Endothelial cells (present as part of the heterogeneous cell mix) were
181 immunostained with von Willebrand factor (VWF) as previously validated and
182 described (Robinson *et al.* 2008; Woad *et al.* 2012; Laird *et al.* 2013). VWF is an
183 established endothelial cell marker and was used to assess the formation of tubule-like
184 structures over time (Sadler 1991; Martelli *et al.* 2006). In brief, after fixation and
185 blocking with 20% (v/v) normal goat serum (Sigma-Aldrich Co. Ltd.), coverslips
186 were incubated with 5 $\mu g.mL^{-1}$ rabbit anti-human VWF primary antibody (Abcam
187 Ltd., MA, USA) overnight in a humidified chamber, followed by a biotinylated goat
188 anti-rabbit secondary antibody and detected using the Rabbit specific HRP/DAB
189 (ABC) detection IHC Kit (Abcam Ltd., MA, USA). All image analysis was performed
190 using Image ProPlus 6.3 (Media Cybernetics, Wokingham, UK), with sections
191 visualized under a ×5 objective. The areas of brown (positive) staining were
192 highlighted, with only network-like areas included (>250 μm^2). For each field of view,

193 the number and area of EC networks was recorded; this was repeated across the whole
194 well. The number of branch points present in the EC islands was determined using an
195 automated measure of branching points (Branch/End feature; Image Pro-Plus 6.3)
196 (Woad *et al.* 2012).

197 *Progesterone assay*

198 On Day 3 of culture, the spent culture medium was assayed immediately or
199 stored at -20°C until assayed. Progesterone concentrations in the spent media were
200 determined by a competitive enzyme immunoassay kit (North Institute of Biological
201 Technology., Beijing, China) according to manufacturer's instructions and as
202 previously validated (Qu *et al.* 2019). Collected samples were diluted 50-500-fold in
203 PBS, as appropriate. The intra- and inter-assay coefficients of variation (CV) for all
204 assays were <15%. The r-values for the standard curves were greater than 0.99 for this
205 assay.

206 *Cell viability assay*

207 Cell viability was assessed using the Cell Counting Kit-8 (Beyotime Co. Ltd.,
208 Shanghai, China) according to the manufacturer's instructions and as previously
209 validated (Ma *et al.* 2020). Briefly, bovine granulosa and theca-derived cells (in a 3:1
210 ratio) were cultured in 96-well plates (2×10^3 cells/well) for 24h, and then treated
211 with 0 or 10 ng.mL^{-1} TGFB1 in the presence or absence of pre-treatment with
212 SB431542 (10 μM) for 48h. CCK-8 solution was added to each well (10μL per well),
213 and the plates were incubated for 1-4h before optical density was measured at 490nm
214 on a microtiter plate reader (BioTek., Winooski, VT, USA). In addition to the

215 treatment wells, blank wells with no cells and control wells treated with vehicle
216 treatment alone were also included. Cell viability was calculated as: =
217 $(OD_{sample}-OD_{blank}) / (OD_{control}-OD_{blank}) \times 100$. Three separate cultures were performed
218 with each treatment conducted in triplicate.

219 *Quantification of gene mRNA expression by qPCR*

220 On Day 3 of culture, RNA was isolated from luteinizing follicular cells using the
221 RNAprep Pure Cell kit (Tiangen Biotech; Beijing, China). The RNA concentration
222 was determined using a Nanodrop spectrophotometer (NanoDrop 1000 3.7.1;
223 Nanodrop Technologies). Only samples with an absorbance ratio at 260 nm to 280 nm
224 between 1.8 and 2.0 were used for subsequent analyses. One microgram of total RNA
225 from each sample was transcribed into cDNA using the PrimeScript™ RT reagent kit
226 (Perfect Real Time) (TaKaRa., Dalian, China) in a reaction volume of 20 μL, primed
227 with random hexamers and oligo dTs and with DNase I treatment according to the
228 manufacturer's instructions. Real-time quantitative polymerase chain reaction
229 (qRT-PCR) was performed to quantify the mRNA expression levels of *ACTB*,
230 *GAPDH*, beta-tubulin (*TUBB*), *TBP* (TATA box binding protein), *VWF, LTBP1,*
231 *TGFBR1, NOS3, SERPINE1, SERPINB5*, steroidogenic acute regulatory protein
232 (*STAR*), *CYP11A1*, and 3β-hydroxysteroid dehydrogenase (*HSD3B1*) in luteinizing
233 follicular cells (Table 1 shows primer information). The qRT-PCR was performed in a
234 reaction volume of 20 μL which included 2 × SYBR Green® Premix Ex Taq
235 (TaKaRa., Dalian, China), forward and reverse primers (10 μM) and cDNA template.
236 An ABI 7500 system (Applied Biosystems; Foster City, CA, USA) was used to detect

237 the amplification products, with initial denaturation at 95°C for 30 secs, followed by
238 40 cycles of 95°C for 5 sec and 60°C for 30 seconds. Melting curve analysis was
239 performed for each probe to confirm specificity. Upon completion of the real-time
240 qPCR, threshold cycle (Ct) values were calculated by the ABI 7500 software V.2.0.6
241 (Applied Biosystems; Foster City, CA, USA). mRNA expression levels were
242 expressed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) and were normalized
243 to the expression levels of *ACTB*, which was selected from a panel of housekeeping
244 genes (*ACTB, GAPDH, TUBB* and *TBP*) as the most stable across treatments using
245 NormFinder (Andersen *et al.* 2004). Three separate cultures were performed with
246 each treatment conducted in triplicate. No template controls were included in each
247 analysis.

248 *Western blotting*

249 On Day 3 of culture, the luteinizing follicular cells were washed with ice-cold
250 PBS and removed by scraping. After lysis in RIPA lysis buffer containing a protease
251 and phosphatase inhibitor cocktail (Abcam), the extracts were centrifuged at 20000 g
252 for 20 min at 4°C and stored at -80°C until analysis. Protein concentrations were
253 quantified using a DC Protein Assay (Bio-Rad Laboratories Inc., Hercules, California,
254 USA). Equal amounts (25 μg) of protein were separated by 12% SDS-PAGE and
255 transferred onto polyvinylidene difluoride membranes (Merck Millipore, MA, USA).
256 The membranes were then blocked with 5% (w/v) nonfat dry milk in Tris-buffered
257 saline with 0.05% Tween (TBST) for 2h, and then incubated overnight at 4°C with
258 primary antibodies against phosphorylated forms of Smad2 (1:1000) and Smad3

259 (1:1000) (p-Smad2 and p-Smad3), Smad2 (1:1000), Smad3 (1:1000), ACTB (1:1000),
260 GAPDH (1:1000) and TUBB (1:1000). After washing with TBST thrice, the
261 membranes were incubated with the appropriate horseradish peroxidase-conjugated
262 secondary antibody diluted 1:5000 in 5% (w/v) fat-free dry milk/TBST for 1h at room
263 temperature. Immunoreactive bands were visualized using SuperSignal West Femto
264 Maximum Sensitivity Substrate (Thermo Fisher Scientific; Waltham, MA, USA) with
265 ImageQuant LAS4000 system (GE, MA, USA).

266 *Statistical analysis*

267 All data were checked for normality and heterogeneity of variance and were log
268 transformed where appropriate. Statistical analyses were performed using Genstat 20th
269 Edition (VSNi, Hemel Hempstead, UK). The degree of EC network formation (area,
270 number of branch points) and the production of progesterone was analyzed by
271 randomized block one-way ANOVA, with TGFB treatment as the factor followed by
272 Tukey's multiple comparison tests to determine where differences lay. The changes in
273 mRNA expression levels, cell viability and progesterone production were determined
274 by randomized-block two-way ANOVA with TGFB and SB431542 treatment as
275 factors. For all experiments, $P<0.05$ was considered significant and all data are
276 presented as the mean+SEM.

277 3. Results

278 *3.1 TGFB1 inhibited bovine luteinizing follicular angiogenesis and von Willebrand*
279 *factor mRNA expression*

280 Extensive formation of EC networks was observed in the control wells after 5

281 days of culture. Von Willebrand factor (VWF)-positive cells developed into
282 tubule-like structures and formed highly organized, intricate networks that
283 superficially resembled a capillary bed (Fig. 1A). There was a clear visual reduction
284 in EC network formation in luteinizing follicular cells treated with 1 or 10 ng.mL^{-1}
285 TGFB1 (Fig. 1B,C) with tubule-like formation almost completely inhibited with 10
286 ng.mL^{-1} TGFB1 (Fig. 1C). As controls, cells were immunostained with rabbit IgG and
287 these were blank (Fig. 1D).

288 Quantification of EC networks showed that the total EC area was markedly
289 reduced by TGFB1 (P<0.001, Fig. 1E), with a 5.5-fold reduction with 10 ng.mL^{-1}
290 TGFB1. The degree of endothelial cell sprouting was determined by branch point
291 analysis, which showed 1 and 10 ng.mL^{-1} TGFB1 reduced the total number of
292 endothelial branch points by 3.3-fold (P<0.001) and 6.7-fold (P<0.001), respectively
293 compared with controls (Fig. 1F). These observations were further confirmed by the
294 down-regulation of *VWF* mRNA levels with 10 ng.mL^{-1} TGFB1 treatment for 96h
295 (P<0.001, Fig. 1G). There was an interaction between TGFB1 and treatment with the
296 TGFBR1 inhibitor, SB431542 (P<0.001). This showed that the inhibitory effect of
297 TGFB1 on *VWF* mRNA expression was abolished by treatment with SB431542.

298

299 *3.2 TGFB1 disrupted progesterone secretion in bovine luteinizing follicular cells*

300 Total progesterone production by luteinizing follicular cells from day 1 to 3 of
301 culture was reduced by approximately 2-fold by TGFB1 at both doses (P<0.01, Fig
302 2A). The blockade of TGFBR1 signaling with SB431542 had no effect on the total

303 progesterone output in the control wells (Fig. 2B). In contrast, co-treatment with
304 SB431542 abrogated TGFB1-induced down-regulation of progesterone production by
305 luteinizing follicular cells (P<0.01).
306
307 *3.3 Effect of TGFB1 on viability of bovine luteinizing follicular cells*
308 Treatment with TGFB1 (10 ng.mL^{-1}) did not alter the proliferation or viability of
309 luteinizing follicular cells at 48h (P>0.05, Fig. 3A), but there was a tendency for
310 TGFB1 to reduce cell proliferation at 96h (P=0.08, Fig. 3B). There was no effect on
311 cell viability of SB431542 when added alone. These results indicate that the
312 inhibitory effect of TGFB1 on luteinizing follicular angiogenesis and progesterone
313 production did not occur due to differences in overall cell proliferation.
314
315 *3.4 TGFB1 induced phosphorylation of Smad2 and Smad3 in bovine luteinizing*
316 *follicular cells*
317 To examine the involvement of Smad2/3 signaling in the TGFB1-induced inhibition
318 of EC network formation and progesterone production, the phosphorylation of Smad2
319 and Smad3 after TGFB1 treatment was investigated. Western blotting showed that
320 treatment with 10 ng.mL^{-1} TGFB1 for 30 min induced phosphorylation of Smad2 and
321 Smad3 (Fig. 4), whereas total Smad2 and Smad3 protein levels were unchanged. To
322 clarify whether TGFB1-induced effects were dependent on TGFBR1 activity, cells
323 were treated with SB431542 (10 μM) for 1h prior to treatment with 10 ng.mL^{-1}
324 TGFB1 for 30 min. SB431542 prevented TGFB1-induced Smad2 and Smad3

325 phosphorylation (Fig. 4). The housekeeping proteins GAPDH, ACTB and TUBB were
326 stably expressed across treatments (Fig. 4).
327
328 *3.5 TGFB1 up-regulated LTBP1 and TGFBR1 but down-regulated NOS3 mRNA*
329 *expression in bovine luteinizing follicular cells.*

330 Next, the expression of *LTBP1*, *TGFBR1* and *NOS3* was evaluated as a potential
331 mechanism by which TGFB1 disrupted bovine luteinizing follicular angiogenesis.
332 TGFB1 (10 ng.mL^{-1}) increased levels of *LTBP1* ($P<0.001$) and *TGFBR1* ($P<0.01$)
333 mRNA (Fig. 5). The up-regulation of *LTBP1* and *TGFBR1* mRNA by TGFB1 was
334 reduced by co-treatment with SB431542 as indicated by a significant SB431542 ×
335 TGFB1 interaction ($P<0.05$; Fig. 5A,B). Interestingly, SB431542 treatment alone
336 down-regulated *TGFBR1* mRNA expression ($P<0.05$), indicating that TGFB1 or an
337 alternative TGFBR1 ligand was endogenously secreted by bovine luteinizing
338 follicular cells. In contrast, TGFB1 inhibited *NOS3* expression by 2-fold ($P<0.05$) and
339 there was a trend for this inhibition to be reversed by co-treatment with SB431542
340 ($P=0.06$; Fig. 5C).
341
342 *3.6 TGFB1 induced SERPINE1 and SERPINB5 mRNA expression in bovine*
343 *luteinizing follicular cells*

344 Next, the downstream targets, *SERPINE1* and *SERPINB5* were examined to
345 determine how their expression was affected by TGFB1 in the follicular-luteal
346 transition. *SERPINE1* mRNA levels were increased by 10 ng.mL^{-1} TGFB1 treatment

versus control cells ($P<0.01$, Fig. 5D). In contrast, the effect of TGFB1 on the *SERPINE1* mRNA expression was prevented by co-treatment with SB431542 ($P<0.05$; Fig. 5D). In a similar manner to SERPINE1, the levels of *SERPINB5* mRNA were elevated (2.5-fold) after 24h of treatment with TGFB1 ($P<0.001$). In contrast, SB431542 decreased *SERPINB5* mRNA levels ($P<0.001$). There was a SB431542 × TGFB1 interaction ($P<0.05$) which indicated the effect of TGFB1 was reversed by SB431542 (Fig. 5E).

3.7 TGFB1 downregulates STAR, CYP11A1 and HSD3B1 expression in bovine luteinizing follicular cells

Finally, we investigated whether the TGFB1-induced down-regulation of progesterone production was due to reduced expression of key progesterone synthesis-associated genes: *STAR, CYP11A1*, and *HSD3B1*. TGFB1 (10 $ng.mL^{-1}$) decreased *STAR* (1.6-fold, $P<0.05$, Fig. 5F), *CYP11A1* (2.1-fold, $P<0.001$, Fig. 5G) and *HSD3B1* (2.9-fold, $P<0.001$, Fig. 5H) mRNA levels in bovine luteinizing follicular cells. Conversely, SB431542 increased *STAR* ($P<0.001$), *CYP11A* ($P<0.001$) and *HSD3B1* ($P<0.001$) mRNA levels compared with controls ($P<0.001$). While there was no TGFB1×SB431542 interaction ($P>0.05$) on *STAR* expression, co-treatment with S431542 reversed the TGFB1-induced downregulation of *CYP11A1* ($P<0.001$) and tended to reverse the TGFB effect on *HSD3B1* ($P=0.08$) mRNA expression This suggests that TGFB1 decreased progesterone production by decreasing the expression of progesterone synthesis-associated genes rather than affecting cell proliferation and

369 that effect was mediated through TGFBR1.

370

371 **4. Discussion**

372 This paper describes that TGFB1 markedly limited the development of
373 vasculature *in vitro* as well as reducing progesterone production during the transition
374 from the follicle to the CL. These effects were mediated through TGFBR1 and
375 involved the phosphorylation/activation of Smad2/3 signal pathways. This is the
376 likely pathway by which TGFB1 down-regulated *NOS3* expression and upregulated
377 *SERPINE1* and *SERPINB5* expression. Additionally, the inhibitory effect of TGFB1
378 on progesterone production is explained by the TGFB-induced reduction in *STAR*,
379 *CYP11A1* and *HSD3B1* mRNA expression.

380 Ovarian angiogenesis is absolutely essential in the transition from the follicle to
381 CL and maintaining the luteal vasculature is critical for preserving its steroidogenic
382 capacity (Fraser *et al.* 2005; Henkes *et al.* 2008). There is strong evidence that
383 reduced luteal vasculature is linked to luteal inadequacy in women and livestock
384 (Woad and Robinson 2016). Thus, enhancing luteal vasculature is a therapeutic target
385 to improve subfertility. There are reports that TGFB1 may contribute to CL formation
386 (Knight and Glister 2006) with TGFB1 expressed in luteal cells from cattle (Gangrade
387 *et al.* 1993; Hou *et al.* 2008) and mice (Ghiglieri *et al.* 1995). However, the actions of
388 TGFB1, a known regulator of angiogenesis (Lebrin *et al.* 2005; ten Dijke and Arthur
389 2007) during the follicular-luteal transition period remain less clear. This study has
390 addressed this by utilizing a physiologically-relevant culture system in which multiple

391 follicular cell types are present (including endothelial cells) with the steroidogenic
392 cells stimulated to luteinize (Laird *et al.* 2013).

393 The present study clearly demonstrated that TGFB1 almost entirely suppressed
394 the formation of endothelial cell networks *in vitro* as determined by reduced total EC
395 area and number of endothelial branch points. Notably, this also occurred in the
396 absence of any significant effect of TGFB1 on cell viability. The action of TGFB1
397 was predominately mediated via TGFBR1 receptor, since SB431542 reversed the
398 effect of TGFB1. It is unclear whether TGFB1 was acting directly on the endothelial
399 cells or via other cell types, however TGFBR1 has been located on endothelial cells
400 (Goumans *et al.* 2003; Goumans *et al.* 2009). The suppression of EC networks by
401 TGFB1 was further evidenced by the TGFB1-induced downregulation of *VWF*
402 mRNA. This is further supported by observations conducted on bovine luteinizing
403 follicular cells (Mattar *et al.* 2020), CLENDO cells (ECs isolated from the CL), and
404 bovine luteal endothelial cells (Farberov and Meidan 2016) where TGFB1 limited EC
405 growth and disrupted the formation of capillary-like structures (Maroni and Davis
406 2011; Farberov and Meidan 2016). Similarly, in bovine aortic endothelial cells,
407 TGFB1 dose-dependently inhibited endothelial cord formation and modulation of
408 endothelial angiogenic receptor expression in a TGFBR1 (ALK5) dependent manner
409 (Jarad *et al.* 2017). In contrast, there are conflicting reports where TGFB1 promoted
410 capillary morphogenesis in skin microvascular endothelial cells (Serratì *et al.* 2009).
411 Also, TGFB induced the formation of endothelial capillary-like structures by human
412 umbilical vein endothelial cells (Peshavariya *et al.* 2014).

413 This collectively emphasizes that the action of TGFB1 is often tissue and
414 concentration-dependent (Goumans *et al.* 2009). The current evidence though
415 suggests the anti-angiogenic properties of TGFB1 predominate in luteinized tissue.
416 The inhibitory effect of TGFB1 is unlikely due to cytotoxicity since TGFB1 did not
417 alter luteinizing follicular cell viability. It is though feasible that the different
418 subpopulations of luteinizing follicular cells may have responded to TGFB1
419 differently. For example, TGFB might have suppressed the growth of endothelial and
420 steroidogenic cells but stimulated other cell types (e.g. pericytes). Indeed, TGFB1
421 inhibited CLENDO cell proliferation without reducing cell viability (Maroni and
422 Davis 2011). Thus, TGFB1 might participate in the disruption of follicular capillaries
423 which is the first step in angiogenesis, which then enables endothelial cells to form tip
424 cells and migrate along a VEGFA concentration gradient (Woad and Robinson 2016).
425 A similar process of vascular degeneration may well occur during the initial stages of
426 luteolysis. Thus, the decision between growth or degeneration of the vasculature in
427 dynamic structures, such as the corpus luteum, requires the carefully regulated
428 balance between pro- and anti-angiogenic factors to maintain homeostasis and luteal
429 function. This concept is further supported by previous observations in which
430 inhibition of a negative regulator of angiogenesis, DLL4, led to uncontrolled luteal
431 hypervascularization (Fraser *et al.* 2012).

432 The extracellular matrix-associated protein, LTBP plays a critical role in
433 maintaining the latency of TGFB, and is required for the secretion, matrix association,
434 and activation of latent TGFB complex (Oklü and Hesketh 2000; Kwak *et al.* 2005).

435 However, there is limited information regarding the regulation of LTBP expression
436 during the follicle-luteal transition. The present study showed that *LTBP1* mRNA was
437 upregulated by TGFB1 in bovine luteinizing follicular cells which agrees with
438 observations in cell lines (Weikkolainen *et al.* 2003; Kwak *et al.* 2005). Thus, it is
439 feasible that LTBP1 is involved in the regulation of TGFB1 storage and activation. In
440 this way, it serves to prevent the accumulation of large amounts of TGFB1 in bovine
441 luteinizing follicular cells.

442 TGFB1 activates several signaling pathways, including Smad2/3, MAP kinase
443 pathways, and phosphatidylinositol-3-kinase (PI3K)/AKT and the precise pathway(s)
444 depends on the particular cell types (Zhang 2017). In the present study, TGFB1
445 activated Smad2/3 and this is likely to be the main intracellular effector by which
446 TGFB1 suppressed angiogenesis. Additionally, TGFB1 upregulated *TGFBR1* mRNA
447 expression, whereas SB431542 inhibited *TGFBR1* mRNA expression in the absence
448 of exogenously-added ligand. This suggests that endogenous TGFB1 or an alternative
449 TGFBR1 ligand was synthesized by the bovine luteinizing follicular cells and this is
450 supported by previous studies where TGFB1 protein was produced by bovine theca
451 cells (Skinner *et al.* 1987), luteal cells (Gangrade *et al.* 1993), and luteinizing
452 granulosa cells (Fazzini *et al.* 2006; Joseph *et al.* 2012).

453 The present results suggest that TGFB1 may act to inhibit angiogenesis by
454 down-regulating mRNA levels of *NOS3*, which is a known critical regulator of
455 vascular remodeling, and angiogenesis (Sessa 2004). Conversely, in EC cell lines,
456 TGFB1 stimulated *NOS3* expression (Inoue *et al.* 1995; Santibanez *et al.* 2007;

457 Vásquez *et al.* 2007), and this was mediated by Smad2 signaling pathway (Saura *et al.*
458 2002). This discrepancy may be related to differences in the effects of TGFB1 on
459 endothelial cells in different tissue environments or due to concentration-dependent
460 differences. However, additional studies are required to explore the underlying
461 molecular mechanisms.

462 In the present study, TGFB1 acting through TGFBR1 increased *SERPINE1*
463 expression in bovine luteinizing follicular cells in agreement with previous
464 observations in isolated bovine luteal endothelial cells (Farberov and Meidan 2016).
465 Similarly, follicular *SERPINE1* expression was increased 4h after GnRH
466 administration and at ovulation (Berisha *et al.* 2008). SERPINE1 inhibits plasminogen
467 activation and thus controls plasminogen action and ultimately extracellular matrix
468 remodeling that is associated with ovulation and early corpus luteum formation.
469 Similar proteolytic pathways are involved in the disassembly of endothelial cell
470 networks as part of the angiogenic process and thus TGFB1 may be suppressing this
471 process by increasing *SERPINE1* expression. However, how SERPINE1 acts during
472 luteinization is not completely understood and this requires further analysis.

473 SERPINB5 is a potent angiogenesis inhibitor that controls EC adhesion,
474 migration, and the adhesion-mediated cell signaling pathway (Qin and Zhang 2010;
475 Bodenstine *et al.* 2012). Indeed, SERPINB5 acted directly on cultured vascular
476 endothelial cells to block VEGFA/FGF2-induced migration and attenuate tube
477 formation (Zhang *et al.* 2000). Such suppressive effects of SERPINB5 on
478 angiogenesis were also confirmed in cancer models (Bodenstine *et al.* 2012; Qiu *et al.*

479 2018). In addition, TGFB1 markedly increased *SERPINB5* mRNA expression via the
480 Smad signaling pathways (Wang *et al.* 2007; Wongnoppavich *et al.* 2017). Thus, this
481 is a likely mechanism by which TGFB suppressed angiogenesis in the luteinizing
482 follicular cells and thereby providing the angiogenic balance necessary for optimal
483 vascularization.

484 The primary function of the newly formed CL is to synthesize and secrete
485 increasing amounts of progesterone that are required for establishing pregnancy. The
486 rate-limiting step in progesterone synthesis is the STAR-mediated transport of
487 cholesterol to the inner mitochondrial membrane. Consequently, STAR is often the
488 key regulatory protein in the production of progesterone (Payne and Hales 2004;
489 Stocco *et al.* 2007). In the present study, TGFB1 downregulated *STAR, HSD3B1* and
490 *CYP11A1* expression which agrees with previous studies where TGFB1 also
491 decreased progesterone secretion as well as inhibiting *HSD3B1* and *CYP11A1*
492 expression in bovine luteinizing follicular cells (Mattar *et al.* 2020). In contrast, in
493 human granulosa cells, TGFB1 decreased progesterone production via
494 downregulating *STAR* expression but did not alter *CYP11A1* or *HSD3B1* expression
495 (Fang *et al.* 2014). These discrepancies indicate that TGFB1 might have differential
496 effects on luteinized and non-luteinized granulosa cells or that the presence of theca
497 cells modulates the action of TGB1. An unexpected observation was that SB431542
498 treatment increased *STAR*, *CYP11A* and *HSD3B1* mRNA expression by 1.5-3-fold,
499 whilst SB431542 had no effect on progesterone production by the luteinizing
500 follicular cells. The reasons underlying this are unclear, but it is feasible that mRNA

501 levels were not reflective of protein levels or were negated by some additional
502 post-translational event.
503

504 Conclusion

505 In summary, TGFB1 disrupted the formation of capillary-like structures and
506 reduced progesterone synthesis during the follicular-luteal transition *in vitro*. During
507 this process, TGFB1 activated Smad-2 and Smad-3 and subsequently inhibited *NOS3*,
508 *STAR*, *CYP11A1*, and *HSD3B1* expression but up-regulated *LTBP*, *TGFBR1*,
509 *SERPINE1*, and *SERPINB5* in bovine luteinizing follicular cells. These effects were
510 principally mediated via TGFBR1 since SB431542 reversed the action of TGFB. In
511 summary, our findings clearly indicate that TGFB1 provides a key role in balancing
512 the angiogenic potential necessary for optimal luteal formation and function.
513

514 Declaration of competing interest

515 The authors declare no conflicts of interest with regard to the study.
516

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Figure legends

Fig. 1. TGFB1 reduced the endothelial cell (EC) area, number of branch points and *VWF* expression in bovine luteinizing follicular cells. Representative images of bovine luteinizing follicular cells (including granulosa, theca and endothelial cells) treated with (A) control medium, (B) 1 ng.mL^{-1} TGFB1, (C) 10 ng.mL^{-1} TGFB1. The EC networks were identified by von Willebrand Factor immunohistochemistry with the immunohistochemistry control shown in (D). (E) shows TGFB1 decreased the total area of EC networks (P<0.01). (F) shows the number of branch points in the EC islands was decreased by TGFB1 (P<0.001). (G) shows TGFB1 downregulated *VWF* mRNA expression in bovine luteinizing follicular cells, cells were treated with 10 ng.mL^{-1} TGFB1 for 96 h in the presence of vehicle control (DMSO), or 10 μM SB431542, and mRNA levels of VWF were examined using RT-qPCR. Data are presented as mean+SEM (n= 4 cultures). (E, F) ***P<0.001 vs. control; (G) differences between groups, a<b (P<0.05).

Fig. 2. TGFB1 disrupted progesterone secretion in bovine luteinizing follicular cells *in vitro*. Bovine luteinizing follicular cells (including granulosa, theca and endothelial cells) were treated with 1 or 10 ng.mL^{-1} TGFB1 in the presence or absence of 10 μM SB431542 co-treatment. Progesterone production into spent media over a 2-day window was calculated and expressed as a percentage of control. Data are mean+SEM (n=5 cultures); (A) ***P<0.001 vs. control; (B) differences between groups, a<b (P<0.05).

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817 **Fig. 3. Effect of TGFB1 on the viability of bovine luteinizing follicular cells *in* 818 *vitro*.** Bovine luteinizing follicular cells (including granulosa, theca and endothelial 819 cells) were cultured in 96-well plates and treated with 0 or 10 ng.mL^{-1} TGFB1 in the 820 presence or absence of 10 μM SB431542 (TGFBR1 inhibitor; pre-treatment). The % 821 cell viability was determined at (A) 48h and (B) 96h by measuring the optical density 822 at 490nm by Cell Counting Kit-8.

823

824 **Fig. 4. Smad2/3 signaling pathways are activated by TGFB1 acting through** 825 **TGFBR1 in bovine luteinizing follicular cells *in vitro*.** Bovine luteinizing follicular 826 cells (including granulosa, theca and endothelial cells) were treated with 0 or 10 827 ng.mL^{-1} TGFB1 for 30 minutes with or without pre-treatment with SB431542 for 60 828 minutes. Phosphorylation level of Smad2 and Smad3 were determined by Western 829 blotting using specific antibodies for phosphorylated (activated) forms of Smad2 830 (p-Smad2) and Smad3 (p-Smad3). Membranes were stripped and re-probed with 831 antibodies to total Smad2 and Smad3. Representative housekeeping protein blots for 832 GAPDH and ACTB are also shown.

833

834 **Fig. 5. TGFB1 up-regulated *LTBP1*, *TGFBR1, SERPINE1* and *SERPINB5* but** 835 **downregulated *NOS3, STAR*, *CYP11A1* and *HSD3B1* mRNA expression in bovine** 836 **luteinizing follicular cells *in vitro*.** Bovine luteinizing follicular cells (including 837 granulosa, theca and endothelial cells) were treated with 10 ng.mL^{-1} TGFB1 for 24h

838 (with vehicle control, DMSO) in the presence or absence of pretreatment with

839 SB431542 for 1h then treated with 10 ng.mL^{-1} TGFB1. The mRNA levels of *LTBP1*

840 (A), *TGFBR1* (B), *NOS3* (C), *SERPINE1* (D), *SERPINB5* (E), *STAR* (F), *CYP11A1* (G)

841 and *HSD3B1* (H) were quantified using RT-qPCR. Data are presented as mean+SEM

842 (n= 4 cultures). Significant differences between groups: a<b<c ($P<0.05$).

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847 **Table 1 Details of primers used for quantitative real-time RT-PCR**

Genes	Primer sequence (5'-3')	Amplicon size (bp)	Accession number
ACTB	GTCGACACCGCAACCAGTTC	181	NM_173979.3
	TACGAGTCCTTCTGGCCCAT		
GAPDH	CCTGCCCGTTCGACAGATA	150	NM_001034034.2
	GGCGACGATGTCCACTTTG		
TUBB	TGTCCCTCGTGCTATCTTGGT	180	NM_001046549.2
	CACATCCAGGACCGAGTCAA		
VWF	AGTTCTCCTCCTGGATGGCT	152	NM_001205308.2
	CGTCAACGTTGGTGTTGCTT		
NOS3	TGCGGCGATGTCACTATGG	128	NM_181037.3
	TGATAGCGTTGCTGATCCCG		
LTBP1	GATTTGGGCCAGATCCTACCT	79	NM_001103091.1
	CGGTAACACGGCCCTTTCT		
TGFBR1	TTCTTTCCCAGGACAGTTACAA	151	NM_174621.2
	CTCAGCCATCCAGCTCCTTT		
SERPINE1	CAGAAGGTGAAGATTGAGGTG	154	NM_174137.2
	GGCCCATGAACAGGACAGTTCC		
SERPINB5	CAACTCAGAGACGCTCCTGC	170	XM_005224214.4
	TCCCAGAGAAATCAGAGGTATCC		
STAR	CAAGGTGGTGGCACGTTTTC	85	NM_174189.3
	GAGCCTTGTCCGCATTCTCT		
HSD3B1	ACCAGCACCATAGAAGTGGC	99	NM_174343.3
	GTATGGAGAGGACCATGCCG		
CYP11A1	GGAGGAGGTTCTGAATGCCC	132	NM_176644.2
	TATCTCTGCAGGGTCACGGA		

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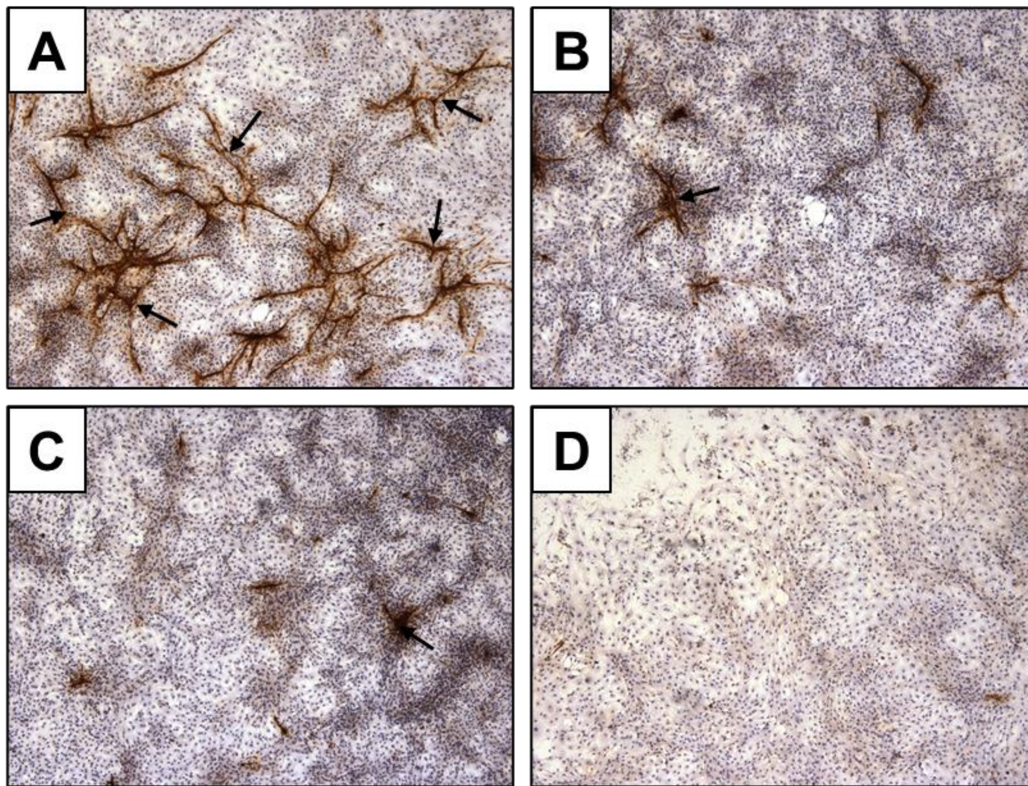
849 **Fig. 1 TGFB1 reduced the endothelial cell (EC) area, number of branch points**

850 **and VWF expression in bovine luteinizing follicular cells.**



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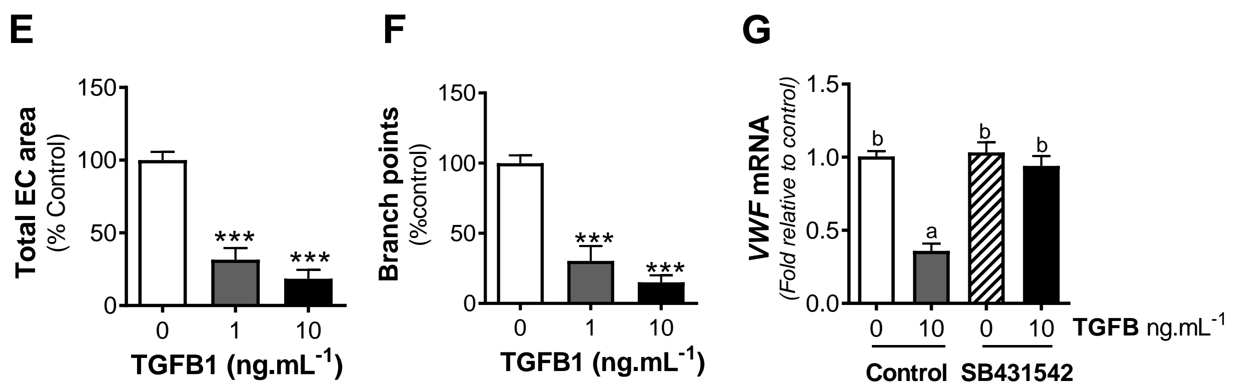

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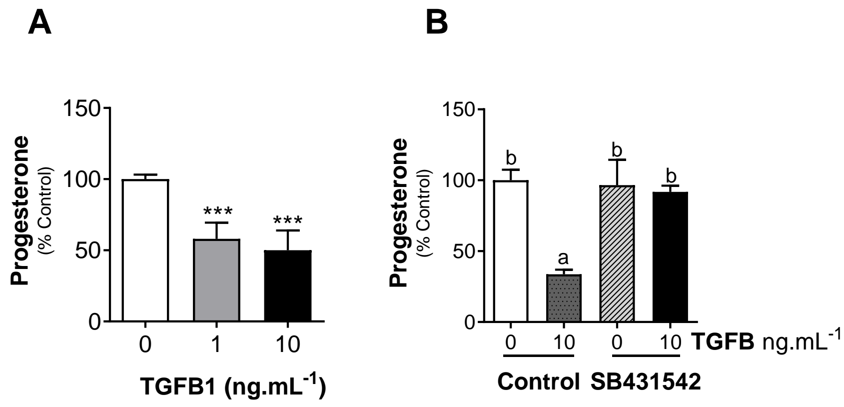
854 **Fig. 2. TGFB1 disrupted the progesterone secretion in bovine luteinizing**

855 **follicular cells *in vitro*.**



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857 **Fig. 3. Effect of TGFB1 on the viability of bovine luteinizing follicular cells *in***

858 ***vitro***

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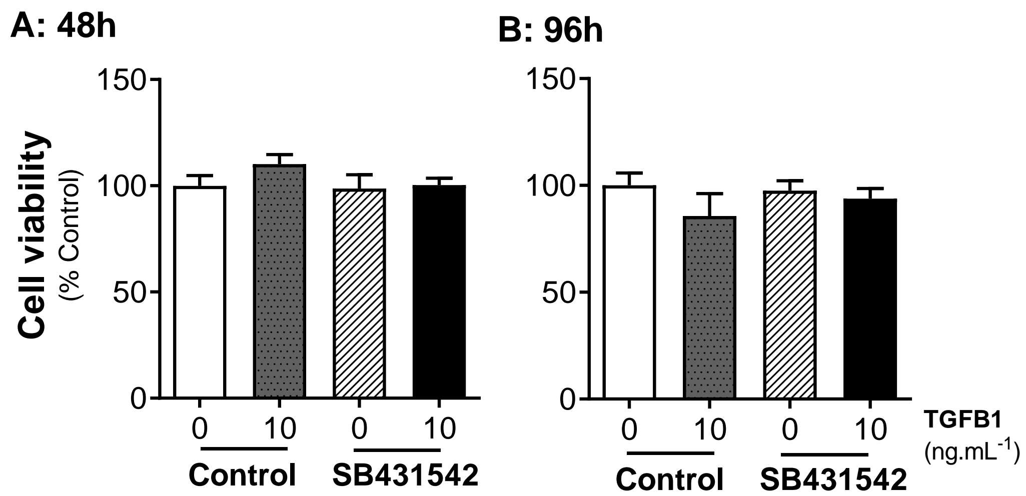

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863 **Fig. 4. Smad2/3 signaling pathways are activated by TGFB1 acting through**

864 **TGFBR1 in bovine luteinizing follicular cells *in vitro*.**

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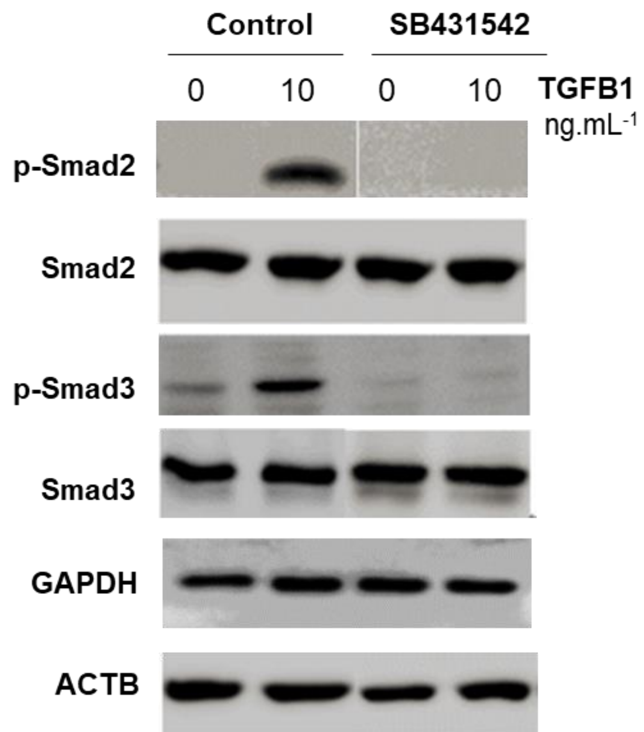
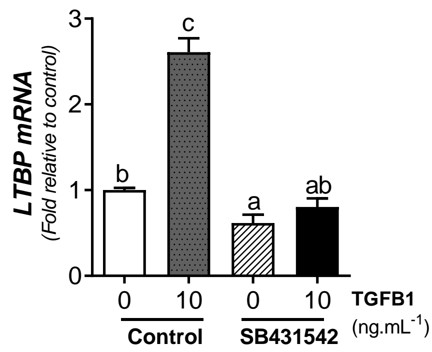


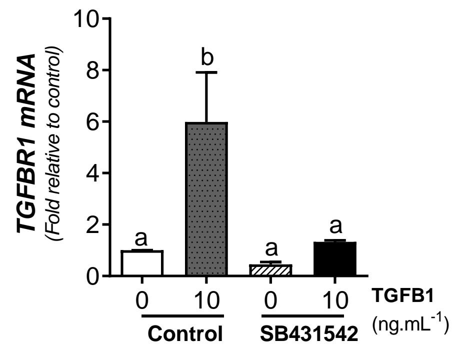
866

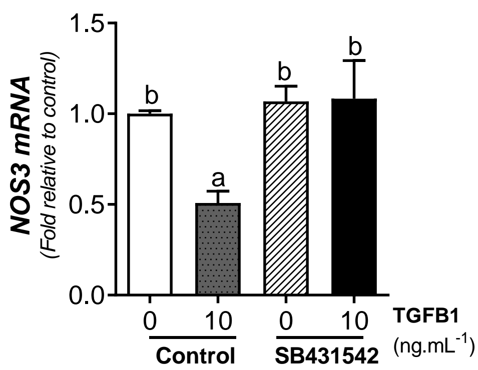
867 **Fig. 5. TGFB1 up-regulated *LTBP1*, *TGFBR1, SERPINE1* and *SERPINB5* but**

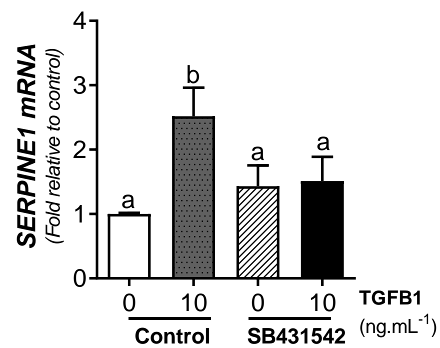
868 **downregulated *NOS3, STAR*, *CYP11A1* and *HSD3B1* mRNA expression in bovine**

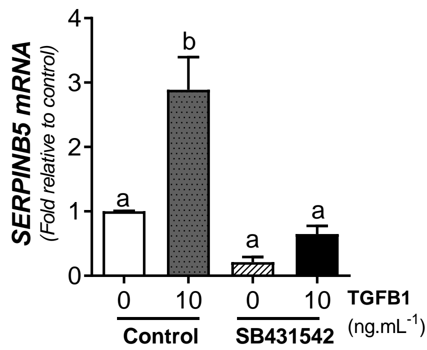
869 **luteinizing follicular cells *in vitro***

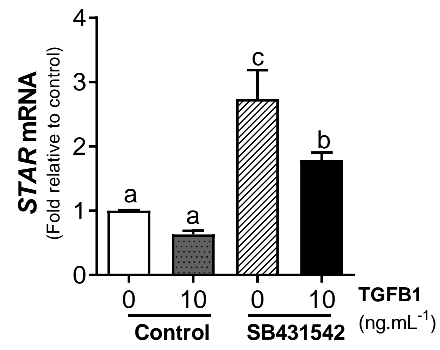


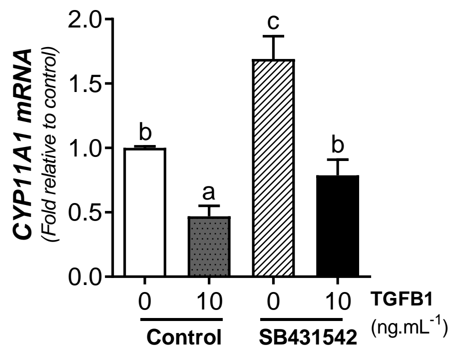


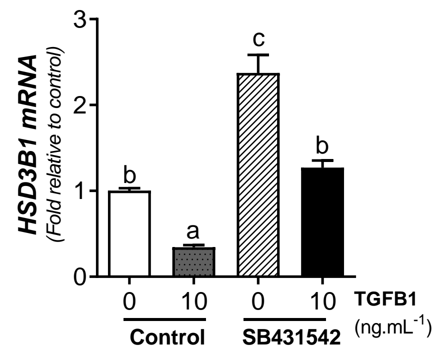












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