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Abstract

Increasing the efficiency of farm animal reproduction is necessary to reduce the environmental impact of food production systems. One approach is to increase the number of healthy eggs (oocytes) produced per female for fertilization, thus it is important to understand factors that decrease oocyte health. One paracrine factor that decreases ovarian follicle growth is fibroblast growth factor 18 (FGF18) secreted by cells in the theca layer of the ovarian follicle, however the factors that regulate FGF18 secretion are unknown. In this study we hypothesized that FGF18 secretion is controled by intrafollicular factors and is linked to fertility, which we tested by using cell culture and sheep genetic models in vivo. Separation of theca cell populations revealed that *FGF18* mRNA is located mainly in thecal endothelial rather than endocrine cells, and immunohistochemistry localized FGF18 protein to microvessels in the theca layer in situ. Culture of ovine theca-derived endothelial cells was used to demonstrate stimulation of FGF18 mRNA and protein abundance by bone morphogenetic protein-4 (BMP4), a growth factor derived from theca endocrine cells. Taking advantage of a sheep genetic model, we demonstrate reduced ovarian and peripheral FGF18 concentrations in the hyperprolific Booroola ewe harboring the $FecB^{B}$ mutation in BMPR1B. These data suggest a novel control of fertility by follicular endothelial cells, in which theca endocrine cells secrete BMP4 that stimulates the secretion of FGF18 from thecal endothelial cells, which in turn diffuses into the granulosa cell layer and promotes apoptosis.

1. Introduction

Modern agriculture contributes to climate change, and animal production produces significant amounts of greenhouse gasses (Davis & White, 2020). Approaches to mitigate the negative impacts of animal production are multifaceted, but include improving reproductive efficiency by increasing the number of healthy oocytes available for natural fertilization or assisted reproductive technologies, particularly in monovulatory ruminants. The health of the oocyte is dependent on the health of the ovarian follicle, and follicle growth is driven by hormones and growth factors including follicle stimulating hormone (FSH) and insulin-like growth factor (IGF) (Webb et al., 2016); maintaining granulosa cell health is critical for follicle development (Hughes & Gorospe, 1991).

The microenvironment within the follicle also affects follicle development, and factors secreted locally by either granulosa or theca cells can have stimulatory or inhibitory effects on granulosa cell proliferation and follicle function (Juengel et al., 2021). Stimulatory factors include bone morphogenetic protein 4 (BMP4) secreted from theca cells and BMP15 from the oocyte that stimulate granulosa cell proliferation and suppress apoptosis in cattle (Glister et al., 2004; Hussein et al., 2005; McNatty et al., 2005; Shimizu et al., 2012). The signaling pathway of BMPs involves forming a complex at the cell surface with a type I receptor and a type II receptor, such as BMPR1B and BMPR2 for BMP4. Activated receptor complexes then phosphorylate SMAD1/5/8 or SMAD2/8 intracellular messengers (Mazerbourg & Hsueh, 2006), which then associate with SMAD4 in order to bind to SMAD-response elements in gene promoters. The importance of BMP signaling for fertility is illustrated by the loss-of-function mutation in the *BMPR1B* gene (*FecB*) that leads to

hyperprolificacy in sheep (McNatty et al., 1986; Mulsant et al., 2001; Wilson et al., 2001)

A potential inhibitory factor is theca-derived fibroblast growth factor-18 (FGF18) that increases bovine granulosa cell apoptosis in vitro and causes follicle regression when injected directly into a growing follicle in vivo in cattle (Portela et al., 2015; Portela et al., 2010). The theca layer of atretic follicles contains greater amounts of *FGF18* mRNA than the theca layer of healthy growing follicles, but it is unclear how the production of FGF18 secretion is controled as mRNA abundance in cultured bovine theca cells is not altered by physiological concentrations of luteinizing hormone (LH) (Portela et al., 2010). It is also unclear which cell types within the theca express FGF18 as this cell layer is complex, consisting of endocrine cells, immune cells, fibroblasts and endothelial cells, (Richards et al., 2017).

Rodent and human endothelial cells contain *FGF18* mRNA (Antoine et al., 2006; Antoine et al., 2005), therefore we hypothesized that FGF18 is derived from thecal endothelial cells and is a novel endothelial-to-granulosa cell signaling molecule that contributes to the modulation of fertility. The objectives of the present study were to determine which cell types of the ovine theca layer express FGF18 and to identify which paracrine factors regulate FGF18 mRNA/protein. In addition, taking advantage of genetic models of fecundity, we reveal a unique link between thecal endothelial cells and prolificacy in sheep.

2. Materials and methods

2.1 Experimental design

Localization of FGF18 protein was determined with immunohistochemistry performed on mixed-breed sheep ovaries obtained from an abattoir, and which were

fixed in Bouin's fixative and embedded in paraffin. Immunohistofluorescence was used on different ovaries to examine in more detail the localization of FGF18 in small blood vessels. Ovaries were fixed in formalin and embedded in paraffin for FGF18 detection, and endothelial cells were identified by localization with the endothelial cell marker, von Willebrand factor. To assess which cell types within the theca layer express *FGF18* mRNA, a cell-enrichment approach was taken to enrich or deplete endothelial cells from a theca layer digest using CD29-coated beads as described below, followed by qPCR for *FGF18* and the endothelial cell marker, coagulation factor VIII (*F8*).

The regulation of *FGF18* mRNA and protein abundance was measured in vitro by culturing thecal endothelial cells from abattoir-derived ovaries. Passaged cells were serum-starved and then treated with Transforming Growth Factor-β (TGFβ) family members as detailed below. The intracellular pathways employed by BMPs to regulate *FGF18* expression were determined in endothelial cells after short-term incubation with BMPs, and cell protein was harvested for the measurement of phosphorylated second messengers. Involvement of the SMAD signaling pathway was investigated by knocking-down SMAD4 through siRNA before the addition of BMP.

The role FGF18 may play in follicle development *in vivo* was then assessed using a genetic sheep model of partial loss-of-function of BMP signaling. First, cycling Lacaune ewes were used to measure plasma FGF18 concentrations during an estrous cycle. Then, Mérinos d'Arles ewes with or without the $FecB^{B}$ mutation in the *BMPR1B* receptor were used to collect blood samples and ovaries, to measure plasma FGF18 concentrations by ELISA and tissue FGF18 protein abundance by immunohistochemistry, respectively.

2.2 Immunohistochemistry and Immunohistofluorescence

For immunohistochemistry, ovaries were serially sectioned at a thickness of 7 μm with a microtome and sections were deparaffinized in toluene, rehydrated, and incubated in antigen unmasking solution (Vector Laboratories, Burlingame, VT) for 4 min in a microwave and then left for 2 h at room temperature. Sections were washed three times in PBS with 0.1% saponin and then treated with 0.3% H_2O_2 for 30 min at 4°C in the dark to remove endogenous peroxidase. Then sections were washed three times in PBS with 0.1% saponin again and then treated with PBS with 0.1% saponin and mare's serum diluted at 1/15 for 30 min at 4°C in the dark to block nonspecific sites. After sections were washed three times, they were incubated with mouse monoclonal anti-FGF18 (Biorbyt # orb1778; 1:2000 in PBS, 0.1% saponin & 5% mare serum) in a humidified chamber overnight at 4°C. Sections were washed three times and then incubated with donkey anti-mouse peroxidase-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) diluted 1:800 in PBS, saponin 0.1% and BSA 0.1% at room temperature for 4 h.

Immunostaining was developed by incubating sections in 50 mM Tris-HCl (pH 7.8) containing 0.4 mg/ml 3,3′-diaminobenzidine tetrahydrochloride dehydrate (Sigma) and 0.012% H_2O_2 for 10 min at room temperature. Negative control sections involved omitting the primary antibody.

Immunohistofluorescence was performed on 7 μm sections of ovary that were deparaffinized in toluene, rehydrated, boiled in 10 mM sodium citrate (pH 6) for 30 minutes, and then cooled to room temperature. Non-specific sites were blocked for 1 h at room temperature in 5% normal goat serum (NGS) (Jackson ImmunoResearch; 005-020-121, FGF18) or 5% mare serum, followed by incubation with anti-FGF18

147 goat polyclonal antibody (Bioss; 1:200) or donkey polyclonal antibody raised against
148 von Willebrand factor (VWF; Abcam #ab6994; 1:400) diluted in 5% goat or mare
149 serum respectively, in PBS plus 0.1% Tween20 overnight at 4°C. Negative control
150 sections were incubated with 5% NGS only. Sections were washed and incubated with
151 cyanine 3 (Cy3)-conjugated anti-goat or anti-donkey IgG (Jackson ImmunoResearch)
152 diluted 1:300 in PBS with 5% bovine serum albumin (BSA) (A3294; Sigma) for 1 h
153 at room temperature. After incubation, sections were washed and counterstained with
154 4,6-diamidino-2-phenylindole (DAPI) (Sigma) diluted 1:1000 in PBS for 5 min, then
155 slides were mounted in Permafluor (Thermo Fisher Scientific).
156
157 2.3 Cell isolation and culture
158 All materials were obtained from Life Technologies Inc. (Burlington, ON,
159 Canada) unless otherwise stated. Mixed-breed sheep ovaries were obtained from
160 cycling ewes, irrespective of stage of the estrous cycle, at a slaughterhouse near the
161 University of Montréal Faculty of Veterinary Medicine. Theca layers were peeled
162 from the stroma of follicles 2-6 mm diameter, granulosa cells were scraped off, and
163 the theca shell incubated in a solution of 1mg/ml collagenase type IV (Sigma–Aldrich,
164 Oakville ON, Canada) and 100µg/ml trypsin inhibitor (Sigma–Aldrich) at 37ºC for 45
165 minutes. The supernatant was centrifuged for 10 minutes at 800g, and the cells
166 resuspended. An endothelial cell-enriched fraction and an endothelial-depleted
167 fraction of theca-derived cells was prepared with the sheep CD29 pluriBead kit
168 according to the manufacturer's instructions (pluriSelect, El Cajon CA). The optimal
169 recovery of endothelial cells was obtained by incubation of 4 x 10^6 theca-derived cells
170 with 80µl pluriBeads in 2ml incubation buffer. Cells from both fractions were pelleted
171 by centrifugation and frozen for RNA extraction and RT-PCR (n=7).

Cells from the endothelial-rich fraction were seeded in 15 cm^2 gelatin-coated flasks in endothelial cell culture medium (M199 with L-glutamine, 25 mM HEPES and Hanks salts; Invitrogen), supplemented with antibiotics and 10% FBS and 100 μg/ml endothelial cell growth supplement (ECGS; Sigma) until confluence, and then passaged up to three times. Aliquots were frozen in culture medium:DMSO (1:1 vol:vol) before experimental treatments.

Cells were thawed and cultured in gelatin-coated 24-well plates at a density of 0.5 x 10^6 cells/well in 1 ml supplemented endothelial cell culture medium until 60% confluence (24-48 h) at 37 °C and under 5% CO_2 atmosphere. Cells were then 'starved' for 9 h in endothelial cell culture medium without FBS or ECGS, after which cells were challenged with the growth factors Transforming Growth Factor β (TGFβ), BMP4, BMP5, BMP15, Growth Differentiation Factor 9 (GDF9) or anti-Müllerian hormone (AMH) (all from R&D Systems) for times and concentrations shown in the legends to figures 2 and 3 before harvesting for RNA or protein extraction. Experiments were performed with 4 wells per treatment that were pooled to obtain a single RNA or protein sample per treatment. Cultures were performed on 4-5 independent cell cultures.

To measure abundance of FGF18 protein, thecal endothelial cells were cultured in Lab-Tek chamber slides (ThermoFisher) at 1 x 10^5 cells/well in 0.5 ml supplemented endothelial cell culture medium as above. After treatment with 50 ng/ml BMP4 for 48 h, cells were fixed in 10% formalin and stained with anti-FGF18 (Biorbyt; 1:2000) as described above. All images were obtained using Axio Imager M1 (Zeiss Microscopy, Toronto, ON, Canada) and immunostaining was quantified using ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA,

196 http://imagej.nih.gov/ij/, 1997–2015) on photomicrographs of Lab-Tek chamber
197 slides.
198
199 2.4 RNA interference
200 Endothelial cells were cultured in supplemented endothelial cell culture
201 medium as above until 60% confluence, after which 100µl Opti-MEM premixed with
202 3µl Lipofectamine RNAiMAX and siRNA (10 nM final concentration) were added
203 for 48 h. Culture medium was then replaced with medium without FBS or ECGS,
204 after which cells were challenged with BMP4 (50 ng/ml) for 24 h. Cells were then
205 harvested for RNA or protein extraction. Four wells per treatment were combined to
206 form a single sample, and experiments were performed on 5 independent culture
207 replicates.
208
209 2.5 Animals, blood collection and FGF18 assay
210 Lacaune ewes (n=9, 2 years-old, primiparous, cycling) were housed at INRAE
211 experimental facilities in Toulouse, and estrus was synchronized during the breeding
212 season by insertion of intravaginal progestagen sponges (fluogestone acetate FGA, 20
213 mg; Intervet) for 14 days. Blood (4 ml into heparinized tubes) was sampled at 12-h
214 intervals from intravaginal sponge removal (Day 0) to Day 4 (96h) along the follicular
215 and periovulatory phases of the estrus cycle, and plasma was used to measure FGF18
216 by ELISA.
217 Mérinos d'Arles (n=28, 6-8 years-old, multiparous, cycling) were housed at
218 INRAE experimental facilities in Tours, and 14 homozygous carrier of the $FecB^{B}$
219 mutation in *BMPR1B* ($FecB^{B/B}$) and 14 non-carrier ($FecB^{+/+}$) ewes were used. Estrous
220 cycles were synchronized as above, and a blood sample was collected 36 h after

sponge removal. One week after sponge removal, animals were sacrificed and the mean number of corpora lutea observed in the ovaries were 1.75 ± 0.13 and 5.92 ± 0.34 for $FecB^{+/+}$ and $FecB^{B/B}$ ewes, respectively (Estienne et al., 2015). Ovaries from 3 $FecB^{+/+}$ and 3 $FecB^{B/B}$ ewes were fixed in Bouin's fixative and embedded in paraffin for immunohistochemistry as described above.

All experiments were performed in accordance with French guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching (approval numbers C37-175-2 and D31-429-01), and jugular vein puncture procedure for blood sampling was approved by the French Ministry of Teaching and Scientific Research and local ethical committee (approval number 01171.02).

For all blood samples, plasma was stored at -20°C until assay for FGF18 with a commercial sheep FGF18 ELISA (MBS027703; MyBioSource, San Diego, CA, USA); preliminary experiments determined that samples were parallel with the standard curve between dilutions of 1:1 and 1:4 in assay diluent. All samples were analysed on a single plate with a coefficient of variation of 2%.

2.6 Total RNA extraction and real-time RT-PCR

Total RNA from cell cultures was extracted using PureLink RNA mini extraction kit according to the manufacturer's instructions (12183025, Ambion by Life Technologies). Reverse transcription was performed on 1 µg RNA with SuperScript Vilo cDNA synthesis kit (Thermo Fisher Scientific). Real-time PCR was performed with 10µl SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Mississauga ON), 6µl of cDNA, 10 pmol primers (1.5 µl) and 1µl water in a CFX96 Touch thermocycler (Bio-Rad). Primers were designed to measure mRNA encoding ovine F8, luteinizing hormone receptor (LHCGR), FGF18, endothelin-1 (EDN1), SMAD4

and SMAD6 (Table 1). Common thermal cycling parameters (3 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C) were used to amplify each transcript. Melting curve analyses were performed to verify production of single amplicons, and amplification efficiency of primer pairs was between 2.0 and 2.15 measured as described (Monniaux et al., 2008). Samples were run in duplicate, and were expressed relative to the geometric mean of *RPL19, SDHA* and *YWHAZ* as housekeeping genes and expressed as the ratio $R = [E_{Ref}{}^{Ct\ (Ref)}/E_{target}{}^{Ct\ (target)}]$ (Estienne et al., 2015).

2.7 Immunoblotting

Cells were lysed in 100 µl/well cold RIPA buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, phosphatase inhibitor cocktail, and protease inhibitor cocktail) and protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL). Proteins were resolved on 10% SDS–PAGE gels (3.5 µg total protein per lane) and transferred onto PVDF membrane in a Bio-Rad wet Blot Transfer Cell apparatus (transfer buffer: 39 mM glycine, 48 mM Tris-base, 20% methanol, pH 8.3). After 2 hours of transfer, the membranes were blocked in 5% non-fat dry milk in TTBS (10 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 hour. Membranes were cut horizontally between molecular mass markers according to the size of the target proteins, and strips incubated for 1 h with rabbit polyclonal antibodies raised against phospho-SMAD1/5/8 (1:1000, Cell Signaling, 13820), phospho-SMAD2/3 (1:1000, Cell Signaling, 3108), total-SMAD1/5/8 (0.625µg/ml, Abcam, ab66737) and total-SMAD2/3 (0.625µg/ml, Abcam, ab47083). After three washes in TTBS, membranes were incubated for 1 hour at room temperature with 1:10 000 HRP-conjugated anti-

rabbit IgG (GE Healthcare Canada). Protein bands were revealed by chemiluminescence (ECL, Bio-Rad Laboratories Ltd, CA, USA). Semi-quantitative analysis was performed with Image Lab software (Bio-Rad).

2.8 Statistical analysis

Data were tested for homogeneity of variance by Bartlett's test, and for normal distribution by Shapiro-Wilk test, and log transformations were performed as appropriate. One-way ANOVA were performed with Tukey-Kramer multiple comparisons tests (effects of TGFB ligands on *FGF18* mRNA), Dunnett's test to compare each treatment with control (signaling pathway analyses), t-tests to compare gene expression in cell-enriched and depleted fractions, and Mann-Whitney test to compare FGF18 protein abundance between sheep genotypes. Culture data included culture replicate as a random variable. Data are presented as means ± SEM with $P < 0.05$ considered significant.

3. Results

3.1 FGF18 is expressed in ovarian endothelial cells

After fractionation of the cells from the theca cell layer, the endothelial-enriched fraction contained significantly more mRNA encoding the endothelial marker F8 and significantly less mRNA encoding the LH receptor gene (LHCGR) compared with the endothelial-depleted fraction (Fig 1A). Abundance of *FGF18* mRNA was significantly higher in the endothelial-enriched fraction, suggesting that endothelial cells rather than endocrine cells are a major source of thecal FGF18. This was supported by immunohistochemical analysis, which demonstrated FGF18 staining in the endothelium and smooth muscle layer of blood vessels of the ovarian

296 medulla and cortex with little to no staining in the oocyte or granulosa cells (Fig 1B).
297 The endothelium of blood venules in the stroma and theca cell layer stained for
298 FGF18 as detected by immunofluorescence, and cell identity was confirmed by
299 immunofluorescence for VWF (Fig 1C).
300
301 3.2 Theca-derived endothelial cell FGF18 expression is stimulated by BMPs
302 The regulation of FGF18 expression by thecal endothelial cells was then
303 assessed *in vitro*. Cells responded to TGFB1 and to BMP4 with dose-dependent
304 increases in *FGF18* mRNA levels after 24 h culture (Fig 2). Abundance of mRNA
305 encoding a known TGF-responsive endothelial protein, endothelin-1 (EDN1), was
306 significantly stimulated by BMP4 (Fig 2) but not by TGFB1. Treatment with other
307 members of the TGF superfamily, BMP5, BMP15 or BMP15+GDF9 for 24 h
308 significantly increased the abundance of *FGF18* mRNA, whereas AMH or GDF9
309 alone were without effect (Fig 3A). Immunocytochemistry demonstrated that of the
310 TGFB family members tested, only BMP4 increased the abundance of FGF18 protein
311 in cultured cells (Fig 3B).
312 The intracellular pathway used by BMPs to increase FGF18 expression was
313 investigated by Western blotting. Challenge of thecal endothelial cells with BMP4,
314 BMP5 or BMP15 for 30 min significantly increased phosphorylation of SMAD1/5/8
315 (Fig 3C), as did combinations of BMP15 with GDF9 or AMH, but neither GDF9 nor
316 AMH alone significantly stimulated SMAD1/5/8 phosphorylation although a
317 numerical increase was observed. The activation of the SMAD1/5/8 pathway was
318 corroborated by measuring abundance of *SMAD6* mRNA after 24 h of treatment,
319 which was significantly increased by BMP4, BMP5, BMP15 and the combinations of
320 BMP15 with GDF9 or AMH, but not by GDF9 or AMH alone (Fig 3D). Treatment

321 with GDF9 or AMH increased the levels of phospho-SMAD2/3 protein, whereas
322 neither BMP4, BMP5 nor BMP15 had any effect (data not shown).

323 To demonstrate that the SMAD pathway was necessary for BMP regulation of
324 endothelial *FGF18* mRNA levels, *SMAD4* mRNA was knocked-down to 22% of
325 control levels with an siRNA approach. Addition of BMP4 increased the abundance
326 of *FGF18* and *SMAD6* mRNA in scrambled RNA-transfected cells as expected, and
327 did not alter *SMAD4* mRNA levels compared with controls (Fig 4). Knockdown of
328 *SMAD4* abolished the ability of BMP4 to stimulate *FGF18* and *SMAD6* mRNA levels
329 (Fig 4). As a negative control, cells were also treated with GDF9 that did not increase
330 *FGF18* mRNA levels compared with controls, and the siRNA had no effect on
331 *FGF18* mRNA abundance in the presence of GDF9 compared with GDF9 alone or
332 controls (Fig 4).

333

334 3.3 Ovarian and plasma FGF18 levels are reduced in hyperprolific sheep harboring a
335 mutation in the BMP receptor gene.

336 As BMP4 appeared to be a major regulator of FGF18 protein production, we
337 used a genetic sheep model ($FecB^{B/B}$) of partial loss-of-function of BMP signaling to
338 assess the role of FGF18 in ovarian function. Plasma concentrations of FGF18 in
339 wild-type Lacaune ewes ($FecB^{+/+}$) did not change during the follicular phase of the
340 estrus cycle and the peri-ovulatory period (Fig 5A). Immunohistochemistry revealed
341 significantly less staining for FGF18 protein in $FecB^{B/B}$ ovaries compared with
342 $FecB^{+/+}$ ovaries in medullary blood vessels (Fig 5B, C), and this was mirrored by
343 significantly lower plasma FGF18 concentrations in $FecB^{B/B}$ compared to $FecB^{+/+}$
344 ewes (Fig 5D).

345

4. Discussion

Paracrine signaling is likely involved in the fine tuning of follicle development and fertility, and the atypical growth factor FGF18 has been shown to cause apoptosis in granulosa cells. In the present work, we present three lines of evidence that FGF18 is part of a novel link between endothelial cells and fertility in sheep. Firstly, FGF18 is produced mainly in the endothelial cells rather than in the endocrine cells of the theca layer. Secondly, FGF18 mRNA and protein abundance in thecal endothelial cells are stimulated mainly by BMP4, and as BMP4 is secreted by theca endocrine cells, this suggests a novel cell-cell communication within the theca cell layer. Thirdly, ovarian and circulating FGF18 protein concentrations are reduced in hyperprolific ewes carrying a loss-of-function mutation in BMP signaling, which suggests a novel control of fertility by endothelial cells.

Within the ovarian follicle, FGF18 has previously been localized to the theca cell layer in cattle, although mRNA levels were not regulated by gonadotropins (LH) in thecal endocrine cells *in vitro* (Portela et al., 2010). This can be explained by the present data showing that endothelial cells and not endocrine cells are the primary source of FGF18. Localization of the protein demonstrated clear presence in the endothelium and smooth muscle layer in blood vessels in the ovarian cortex and medulla, and immunofluorescence detected FGF18 protein in the endothelium of thecal venules. This is consistent with data showing FGF18 expression in human and rat vascular endothelial cells (Antoine et al., 2006; Antoine et al., 2005).

Endothelial cells play a vital role in folliculogenesis, as reduced vascularization of the theca is associated with follicular atresia (reviewed in (Robinson et al., 2009), and follicular capillary formation is stimulated by granulosa and theca cells through the secretion of VEGF and FGF2 (Mattar et al., 2020;

371 Reynolds & Redmer, 1998; Robinson et al., 2009). A major regulator of endothelial
372 cell function is TGFB1, which controls the expression and secretion of endothelial-
373 specific molecules including endothelin-1 (EDN1) (Castañares et al., 2007; Star et al.,
374 2009). Although *EDN1* mRNA abundance was not significantly increased by TGFB1
375 in endothelial cells in the present study, the cells responded to BMP4 with a 4-fold
376 increase in *EDN1* mRNA levels. This stands in contrast to data with human lung
377 microvessel or aortic endothelial cells in which BMP4 had no effect on EDN1
378 secretion (Park et al., 2012; Star et al., 2010).

379 In addition to EDN1, the present data show that FGF18 is also a target of
380 TGFB family signalling in endothelial cells. Although TGFB1, BMP4, BMP5 and
381 BMP15 all increased the abundance of *FGF18* mRNA in thecal endothelial cells,
382 BMP4 was the most potent and the only BMP that also increased FGF18 protein
383 abundance. The main ovarian source of BMP4 is generally considered to be theca
384 endocrine cells (Knight & Glister, 2006), suggesting that endocrine cells signal to
385 endothelial cells within the theca layer, however as BMP4 is also expressed in
386 endothelial cells (Sorescu et al., 2003), an autocrine component to BMP4 - FGF18
387 signaling is also possible. This signaling pathway involves phosphorylation of
388 SMAD1/5/8, and knock-down of *SMAD4* indicated that the SMAD4 pathway is
389 necessary for BMP4 regulation of *FGF18* expression. Although TGFB1 has been
390 shown to increase *Fgf18* mRNA in a rat chondrocyte cell line (Reinhold et al., 2004),
391 no such effect was noted in a microarray study with human microvessel endothelial
392 cells (Wu et al., 2006). Neither GDF9 nor AMH, primarily activators of the Smad2/3
393 pathway, altered *FGF18* mRNA levels in the present study, whereas activation of the
394 SMAD2/3 pathway increased *FGF18* mRNA levels in human microvessel endothelial
395 cells (Wu et al., 2006). Collectively, the present data on EDN1 and FGF18 regulation

396 suggest that theca endothelial cells have specialized functional properties that differ
397 from some other endothelial cells, as has been suggested for other organ systems
398 (Rafii et al., 2016).

399 As FGF18 has been shown to promote apoptosis in granulosa (Portela et al.,
400 2010) but not in theca cells (Han et al., 2018), the present data suggest a three-cell
401 signaling network in the follicle in which endocrine theca cells act on endothelial cells
402 through BMP4, and endothelial cells then act on granulosa cells though FGF18 to
403 increase the rate of apoptosis. Although most coculture studies have focused on the
404 role of theca, granulosa or luteal cells on endothelial cell function (Mattar et al., 2020;
405 Woad et al., 2009), a few previous investigated the role of endothelial cell-derived
406 (angiocrine) factors ; endothelial cells had a positive effect on bovine granulosa cell
407 proliferation in coculture (Spanel-Borowski et al., 1994) and ovarian endothelial cells
408 expressing constitutively active AKT promoted primary follicle growth in mice
409 (Kedem et al., 2017). Together, these observations indicate a complex role of
410 endothelial cells in follicular development, likely involving the secretion of both
411 promoting and suppressive factors.

412 Altered BMP signaling may play a role in fertility, particularly in sheep where
413 the *FecB*^{B} loss-of-function mutation in BMPR1B increases ovulation rate (McNatty et
414 al., 1986; Mulsant et al., 2001; Wilson et al., 2001). The current model for the
415 mechanism of the *FecB*^{B} mutation is that BMP4 signaling is defective leading to
416 increased sensitivity to FSH and to decreased secretion of AMH, which in turn
417 increase follicle activation and survival (Estienne et al., 2015; Fabre et al., 2006). The
418 present study shows that abundance of FGF18 protein is also reduced in the ovarian
419 blood vessels of *FecB*^{B} ewes, indicating that it likely contributes to the hyperprolific
420 phenotype. As FGF18 has been shown to promote apoptosis in granulosa cells

(Portela et al., 2010), a reduction in ovarian FGF18 content is entirely consistent with the reduced incidence of apoptosis observed in *FecB*^{B} follicles (Driancourt et al., 1985; Mandiki et al., 2000; Sheena et al., 2015).

The reduction in follicular FGF18 protein in *FecB*^{B} ewes was associated with a reduction in FGF18 concentrations in peripheral blood, as is the case for AMH (Estienne et al., 2015). It is unlikely that decreased FGF18 concentrations are a result of decreased AMH concentrations as AMH did not alter endothelial FGF18 mRNA or protein abundance in the present study. This would imply a potential effect of the mutation on other organ systems, however the only other tissues known to be affected by this mutation are the fetal heart and the adult adrenal gland, both of which are smaller but not functionally compromised in *FecB*^{B} carriers (McNatty et al., 1995; Souza & Baird, 2004). Peripheral plasma FGF18 concentrations did not change during the follicular and peri-ovulatory phases of the estrus cycle, which again is similar to the situation with AMH (Rico et al., 2011).

In summary, this study demonstrates that follicular FGF18 originates from endothelial cells within the ovary and may play a role in controlling fertility. The present data suggest a three-cell signaling network in which theca endocrine cells secrete BMP4 that stimulates the secretion of FGF18 from thecal endothelial cells, which in turn diffuses into the granulosa cell layer and promotes apoptosis. Reduced BMP signaling, as seen in hyperprolific sheep harboring a mutation in BMPR1B, could attenuate FGF18 secretion leading to a reduced granulosa cell apoptosis rate, thus allowing increased follicle development.

Declaration of interest.

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Data sharing statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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628

629 **Figure legends**

Fig 1. Localization of FGF18 mRNA and protein in the ovary. **A**, cells of the theca layer were dispersed and endothelial cell enriched (Enr) and depleted (Depl) fractions were obtained with anti-CD29 coated beads. Enrichment was assessed by qPCR for the endothelial marker Factor VIII (F8) and the steroidogenic marker LH receptor (LHCGR). Data are means ± SEM of 7 replicates, and asterisks identify means that are significantly different (Student's t test). **B**, immunohistochemistry for FGF18 demonstrated staining in small blood vessels around follicles (left panel, arrows) but not in oocytes (o) or granulosa (gc) cells, and in larger vessels in the medulla (right panel). Inset shows a negative control without primary antibody. **C**, immunohistofluorescence for VWF and FGF18 in the endothelium of venules in the ovarian medulla and in the theca layer ; nuclei are stained with DAPI, and specific antigens are stained red.

Fig 2. TGFB1 and BMP4 stimulate *FGF18* mRNA levels in a dose-dependent manner. Bovine thecal endothelial cells were cultured with the given doses of human recombinant TGFB1 or BMP4 for 24 h and mRNA extracted for qPCR analysis of *FGF18* and *EDN1* mRNA levels. Data are means ± SEM of 4 (TGFB1) and 5 (BMP4) replicates. Bars with different letters are significantly different (Tukey-Kramer HSD test); asterisk, orthogonal contrast showed a significant difference between control and cells treated with 1, 10 and 100 ng/ml TGFB1.

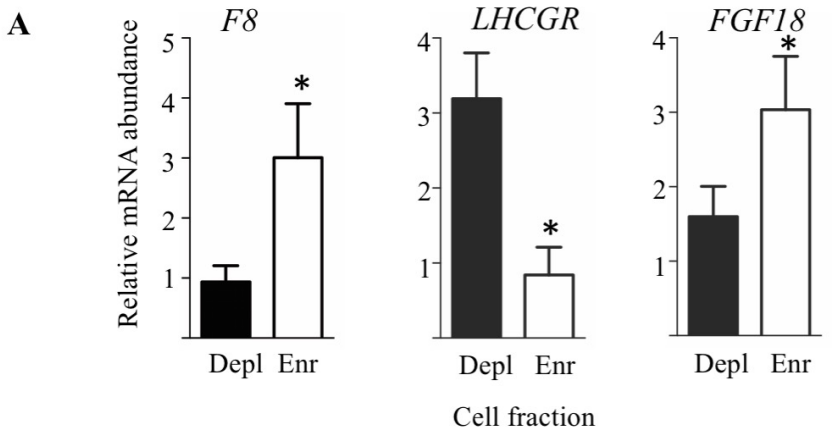
Fig 3. Effect of different TGFB family ligands on FGF18 expression and signaling pathways in thecal endothelial cells. Cells were cultured with 50 ng/ml of human recombinant BMP4, BMP5, BMP15, AMH or GDF9 alone, or a combination of BMP15 and AMH (B15+A) or BMP15 and GDF9 (B15+G9). Cells were recovered

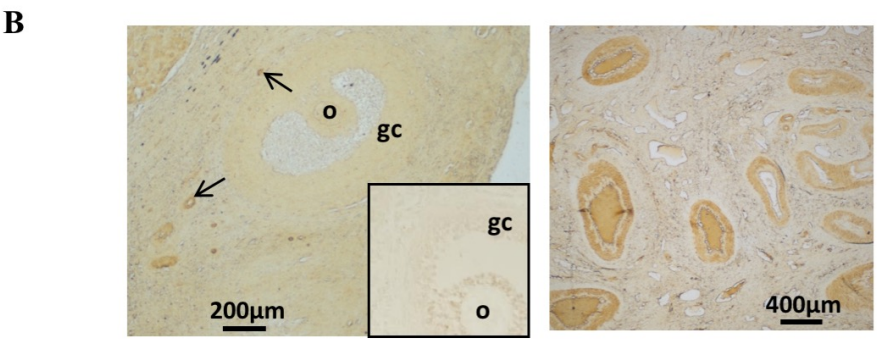
after 24 h for mRNA extraction (panels A, D), after 48 h to measure FGF18 protein by immunocytochemistry (B) or after 30 min for assay of SMAD1/5/8 phosphorylation (C). Inset in B shows staining in representative control and BMP4-treated cells. Inset in C shows representative immunoblots of one replicate culture. Data are means ± SEM of 5 replicates, and asterisks identify means that are significantly different from control (Dunnett's test).

Fig 4. The SMAD signaling pathway is essential for BMP4 regulation of *FGF18* mRNA abundance in thecal endothelial cells. Cells were transfected with siRNA targeting SMAD4 or scrambled siRNA, and then treated with BMP4 (50 ng/ml; grey bars, B4), GDF9 (50 ng/ml; black bars, G9) or medium controls (hollow bars) for 24 h. Data are means ± SEM of 5 replicates. Asterisks identify significant effects of siRNA within ligand treatment, or effect of ligand compared to control (Dunnett's test).

Fig 5. Abundance of FGF18 protein is lower in hyperprolific ewes carrying a mutation in *BMPR1B*. **A**, plasma FGF18 concentration in wild-type Lacaune ewes (n=9) at times (h) after FGA sponge removal. **B**, immunohistochemisty showing staining for FGF18 in ovarian blood vessels in non-carriers ($FecB^{+/+}$) and homozygous carriers ($FecB^{B/B}$) of a mutation in *BMPR1B*. **C**, quantification of immunostaining in the blood vessel wall of 3 $FecB^{+/+}$ and 3 $FecB^{B/B}$ ewes. **D**, measurement by ELISA of blood FGF18 concentrations 36h after FGA sponge removal in carrier and non-carrier *FecB* ewes (n=14/genotype). Data are means ± SEM and asterisks identify means that are significantly different (Mann-Whitney).

Fig 1


A
F8
LHCGR
FGF18
Relative mRNA abundance
Depl
Enr
Cell fraction


B
o
gc
200µm
400µm

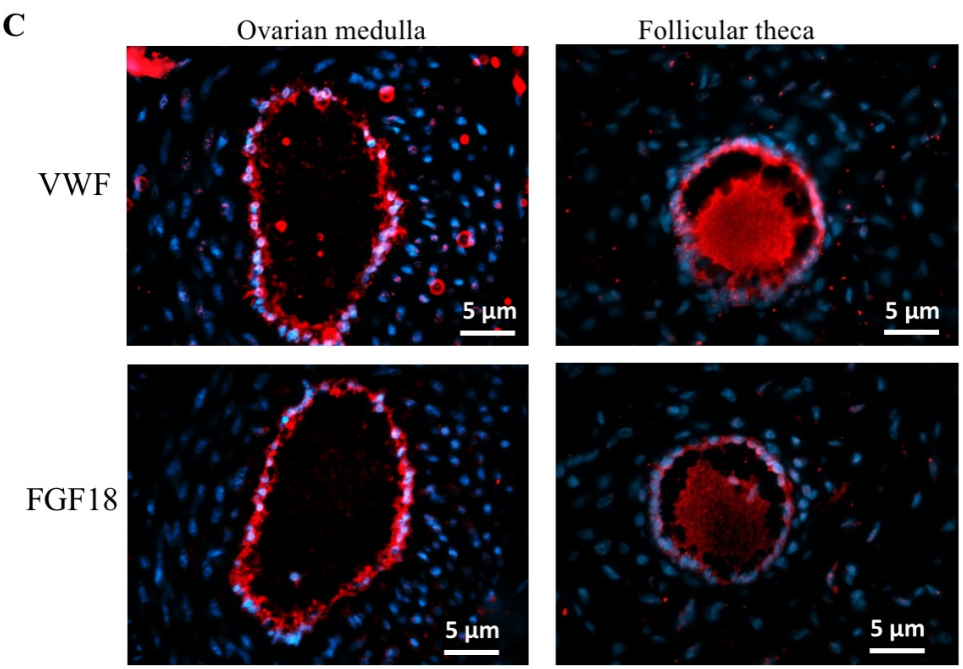

C
Ovarian medulla
Follicular theca
VWF
FGF18
5 µm

Fig 2

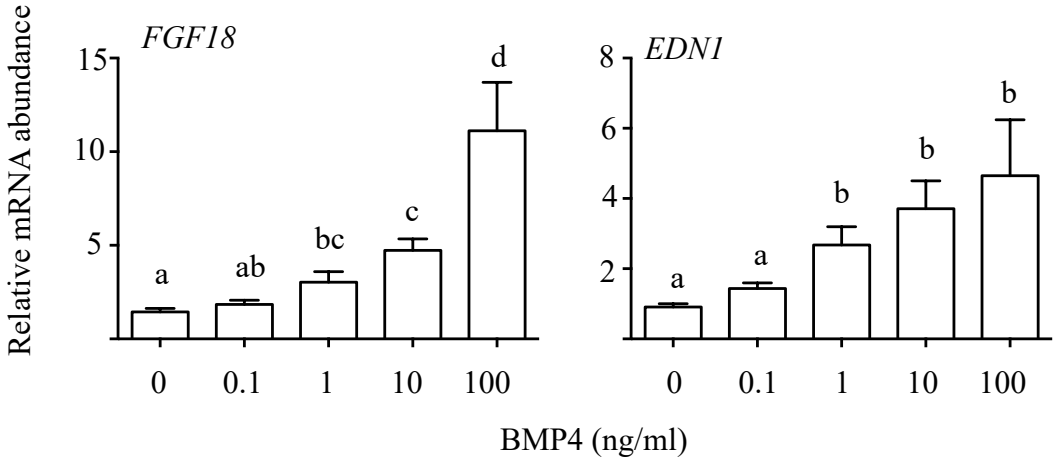

Relative mRNA abundance
FGF18
15
10
5
a
ab
bc
c
d
0
0.1
1
10
100
EDN1
8
6
4
2
a
a
b
b
b
0
0.1
1
10
100
BMP4 (ng/ml)

Fig 3

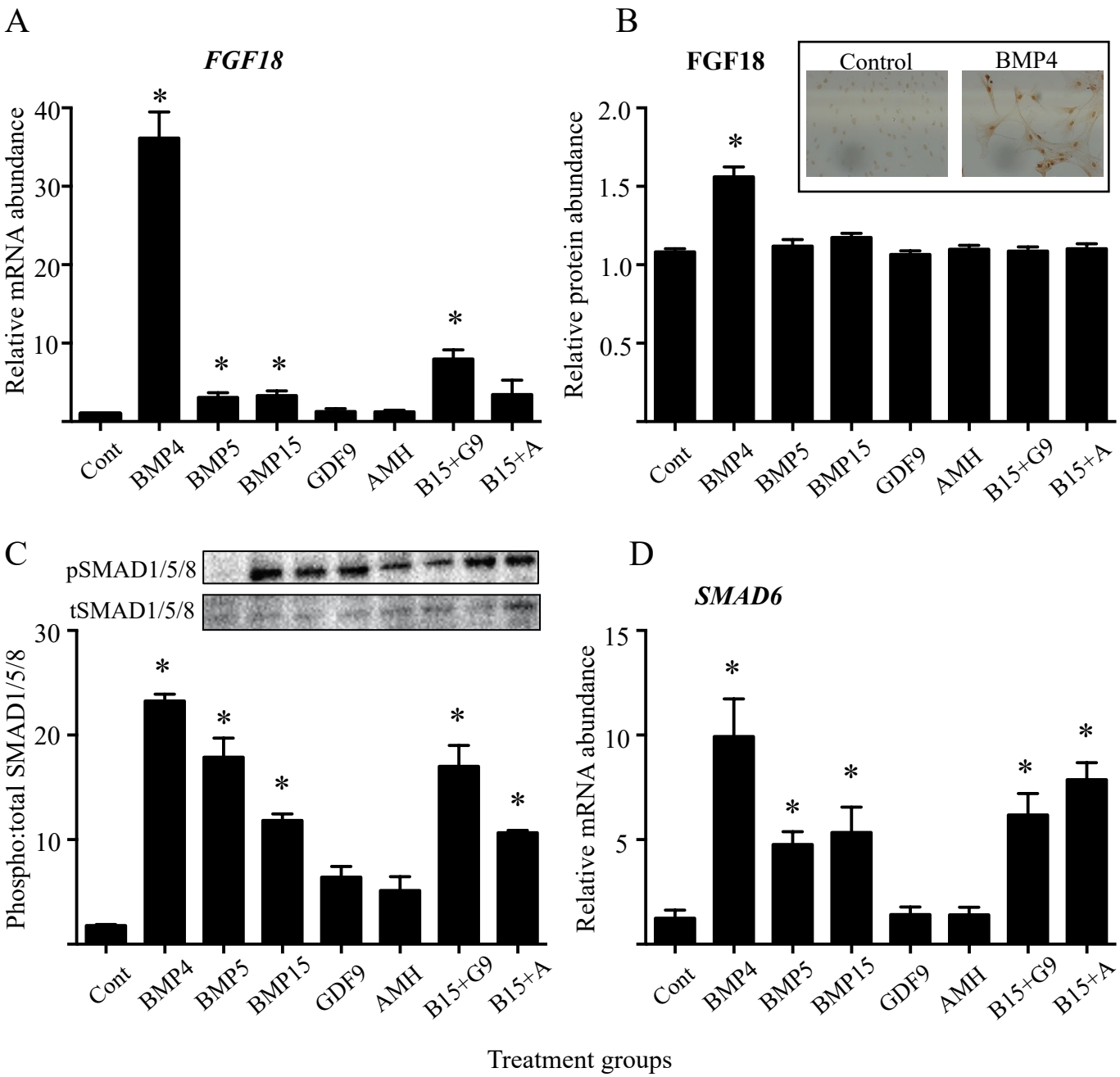

A
FGF18
Relative mRNA abundance
40
30
20
10
Cont
BMP4
BMP5
BMP15
GDF9
AMH
B15+G9
B15+A
B
FGF18
Control
BMP4
Relative protein abundance
2.0
1.5
1.0
0.5
C
pSMAD1/5/8
tSMAD1/5/8
Phospho:total SMAD1/5/8
30
20
10
D
SMAD6
Relative mRNA abundance
15
10
5
Treatment groups

Fig 4

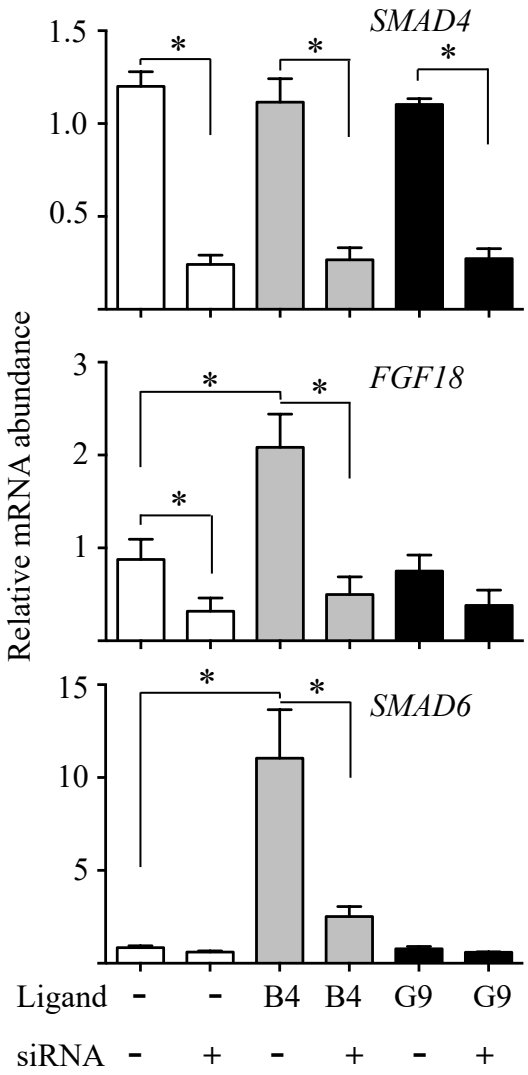
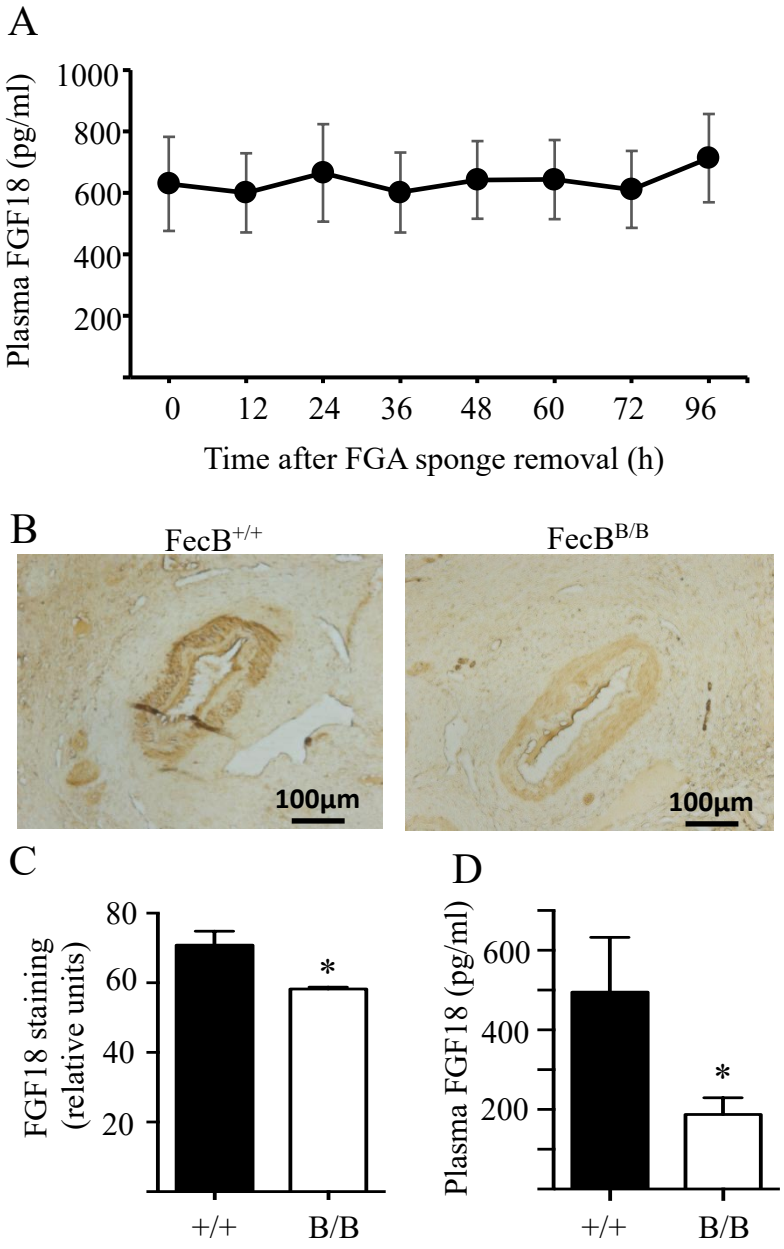

SMAD4
FGF18
SMAD6
Relative mRNA abundance
1.5
1.0
0.5
3
2
1
15
10
5
*
Ligand - - B4 B4 G9 G9
siRNA - + - + - +

Fig 5


A
Plasma FGF18 (pg/ml)
1000
800
600
400
200
0
12
24
36
48
60
72
96
Time after FGA sponge removal (h)
B
FecB+/+
FecB B/B
100µm
100µm
C
FGF18 staining (relative units)
80
60
40
20
*
+/+
B/B
D
Plasma FGF18 (pg/ml)
600
400
200
*
+/+
B/B

Gene	Primer forward	Primer reverse	Amplicon lenght	Efficiency
Factor 8	AAGAGGATGACTGGGACTAT	AAGTCTGTATCTGAACCACG	78	2,02
LHCGR	GGAGACCAAATAATGAAACACTT	GATTATGACTATGGTTTCTGCTC	85	2,01
FGF18	CCTTCGGTAGTCAAGTCC	GTGTTCATTGAGAAGGTTCTG	128	2,05
EDN1				
SMAD4	TGTCGACGGATATGTAGACCC	GGTCAGGTGCCTTAGTGACC	167	2,11
SMAD6	TCTGATTCCACATTGTCTTA	TTCTACGACCTACCTCAG	207	2,13
YWHAZ	ACCAACACATCCTATCAGAC	GCTAATAATGCAGTTACTGAGAG	185	2,04
SDHA	GAATGGTCTGGAACACTGA	GGTTGACGAGTACGATTACT	156	2,1
RPL19	TATGGGTATAGGTAAGCGAAAG	CTAAGAAGATTGACCGCCA	129	2,15