Evidence that fibroblast growth factor 10 plays a role in follicle selection in cattle

Article *in* Reproduction Fertility and Development · July 2015

DOI: 10.1071/RD15017 · Source: PubMed

9 authors, including:

Anthony C S Castilho
Universidade do Oeste Paulista
58 PUBLICATIONS **358** CITATIONS
SEE PROFILE

Christopher A Price
Université de Montréal
127 PUBLICATIONS **2,281** CITATIONS
SEE PROFILE

Felipe Dalanezi
São Paulo State University
20 PUBLICATIONS **3** CITATIONS
SEE PROFILE

Ronaldo L Ereno
São Paulo State University
37 PUBLICATIONS **180** CITATIONS
SEE PROFILE

CSIRO PUBLISHING

Reproduction, Fertility and Development
http://dx.doi.org/10.1071/RD15017

Evidence that fibroblast growth factor 10 plays a role in follicle selection in cattle

A. C. S. Castilho[A,E], *C. A. Price*[B], *F. Dalanezi*[A], *R. L. Ereno*[A], *M. F. Machado*[A], *C. M. Barros*[A], *B. G. Gasperin*[C], *P. B. D. Gonçalves*[C] *and J. Buratini*[D]

[A]Departamento de Farmacologia, Instituto de Biociências, Universidade Estadual Paulista, Botucatu, Distrito de Rubião Junior, Botucatu, São Paulo 18618-970, Brasil.
[B]Centre de recherche en reproduction animale, Faculté de médecine vétérinaire, Université de Montréal, 3200 Sicotte, Saint-Hyacinthe, Québec J2S 7C6, Canada.
[C]Laboratório de Biotecnologia e Reprodução Animal, Universidade Federal de Santa Maria, Avenida Roraima 1000, Santa Maria, Rio Grande do Sul 97105-900, Brasil.
[D]Departamento de Fisiologia, Instituto de Biociências, Universidade Estadual Paulista, Distrito de Rubião Junior, Botucatu, São Paulo 18618-970, Brasil.
[E]Corresponding author. Email: buratini@ibb.unesp.br

Abstract. There is evidence that regulation of follicle selection in cattle involves locally produced growth factors. In the present study, we investigated the expression of members of the fibroblast growth factor (FGF) 7 family during follicle deviation. The largest and second largest follicles were recovered during the second day of a synchronised follicle wave and the future dominant and future subordinate follicles were identified based on diameter and cytochrome P450, family 19, subfamily A, polypeptide 1 (*CYP19A1*) mRNA levels in granulosa cells. Theca cells of the future dominant follicle contained less mRNA encoding *FGF7* and *FGF10* compared with those from the future subordinate follicle 2.5 days after ovulation, before a significant difference between the diameters of the future dominant and future subordinate follicles could be observed, but *FGF22* mRNA levels did not change. Levels of mRNA encoding FGF receptors *FGFR1B* and *FGFR2B* in theca and granulosa cells, respectively, were lower in the future dominant follicle compared with the future subordinate follicle. Addition of FGF10 to granulosa cells *in vitro* significantly decreased oestradiol secretion, as well as *CYP19A1*, FSH receptor (*FSHR*) and insulin-like growth factor 1 receptor (*IGF1R*) mRNA abundance, whereas FGF22 had no effect. We conclude that *FGF10* and *FGFR2B* expression is increased in the future subordinate follicle before morphological deviation, which may contribute to follicle selection.

Additional keywords: antral follicles, bovine, deviation, ovary.

Received 14 January 2015, accepted 16 June 2015, published online 21 July 2015

Introduction

Ovarian follicle growth occurs in waves, during which there is synchronous growth of a cohort of follicles induced by a transient rise in circulating FSH levels. In monovular species, a single dominant follicle arises from the cohort and continues to grow while the others regress in a process known as follicle deviation (for a review, see Fortune *et al.* 2001). The mechanisms by which only one follicle continues to grow are not clear, but the regression of subordinate follicles is likely linked to a decline in circulating FSH concentrations that follows wave emergence, and the survival of the dominant follicle may be facilitated by increased bioavailability of intrafollicular insulin-like growth factor (IGF) 1 (Fortune *et al.* 2004; Scaramuzzi *et al.* 2011). Secreted IGF1 is sequestered by IGF binding proteins (IGFBP) and liberated following proteolytic degradation of IGFBP by pregnancy-associated plasma protein-A (PAPPA). Granulosa cells of the early growing dominant follicle contain greater levels of *PAPPA* mRNA than those from the subordinate follicle (Luo *et al.* 2011) and intrafollicular injection of IGF1 into the second largest follicle at the beginning of deviation increases oestradiol production and follicle growth (Ginther *et al.* 2004). Although granulosa cells express the IGF1 receptor (IGF1R), it is unclear whether it is regulated during follicle growth in cattle; some reports suggest that IGF1R numbers and *IGF1R* mRNA levels do not change with follicle development (Stewart *et al.* 1996; Armstrong *et al.* 2000), whereas others report higher receptor numbers and mRNA levels in larger oestrogenic follicles (Spicer *et al.* 1994; Sudo *et al.* 2007).

In addition to IGF1, several other growth factors have been identified as acting locally within the ovary to regulate follicular development, including members of the transforming growth factor (TGF)-β and fibroblast growth factor (FGF) families. For

example, theca-derived bone morphogenetic protein (BMP) 4 and BMP7 stimulate bovine granulosa cell steroidogenesis and proliferation (Glister *et al.* 2004) and, in rodents, *Bmp4* and *Bmp7* mRNA levels decrease during follicle atresia (Erickson and Shimasaki 2003). Further, in cattle, levels of mRNA encoding the main Type I and Type II BMP receptors are lower in future dominant follicles compared with subordinate follicles around the time of follicle deviation (Gasperin *et al.* 2014). One potentially important FGF is FGF10, which is produced by theca cells and inhibits granulosa cell steroidogenesis *in vitro* (Buratini *et al.* 2007). In cattle, thecal *FGF10* mRNA levels were higher in subordinate follicles than dominant follicles around the time of deviation, and injection of FGF10 into a future dominant follicle around the time of deviation *in vivo* caused regression of the follicle (Gasperin *et al.* 2012). The FGF subfamily to which FGF10 belongs also contains FGF7 and FGF22 (Itoh and Ornitz 2004). The pattern of expression and actions of FGF7 appear similar to those of FGF10 (Berisha *et al.* 2004; Buratini *et al.* 2007; Parrott *et al.* 1994), whereas nothing is known about the expression or actions of FGF22 in the ovary.

Collectively, the aforementioned studies point to a role for FGF7 family members in the local regulation of follicle development, but whether they are involved in the regulation of follicle selection is not clear. The aim of the present study was to determine the levels of mRNA encoding *FGF7*, *FGF10* and *FGF22* in theca cells, as well as mRNA levels of the receptors *FGFR1B* and *FGFR2B*, in granulosa and theca cells from future dominant and future subordinate follicles during follicle deviation in *Bos taurus indicus* heifers. We then assessed the effects of FGF10 and FGF22 on steroidogenesis of cultured granulosa cells to gain an insight into the mechanisms by which these FGFs may inhibit follicle development.

Materials and methods

Animals and follicle samples

The experiment was conducted with Nelore heifers in a farm located in Ribeirão do Sul, São Paulo, Brazil (latitude 22°47′S, longitude 49°56′W). Purebred heifers ranged in age and bodyweight from 20 to 24 months and from 420 to 470 kg, respectively. Heifers were kept on pasture (*Cynodon* sp.) and received a maintenance diet supplemented with concentrates (16% crude protein and 60% total digestible nutrients) twice a day. Water and mineral were available *ad libitum*.

Ovulation was synchronised in a group of heifers with an intravaginal progesterone release device (1.0 g, DIB; Syntex, Luis Guillón, Argentina) inserted for 7 days, combined with oestradiol benzoate (EB; 2.5 mg, i.m.; Estrogin; Farmavet, São Paulo, Brazil). At DIB removal, heifers were injected with a prostaglandin (PG) $F_{2\alpha}$ analogue (D-cloprostenol; 150 µg, i.m.; Prolise; ARSA SRL, Buenos Aires, Argentina) and 1 mg EB 24 h later. Ultrasonography (SSD 900 with a 7.5–9.0-MHz transrectal probe; Aloka, Tokyo, Japan) was used to detect ovulation and monitor subsequent follicle development. Animals were slaughtered in a local abattoir 2 and 2.5 days after ovulation ($n = 4$ and 5, respectively), and ovaries were collected. The interval between ovulation and time of death was designed to precede (Day 2) or coincide (Day 2.5) with the expected time of follicle deviation in Nelore heifers (Sartorelli *et al.* 2005). The present study was approved by the Local Committee on Ethics in Animal Use, Institute of Biosciences, São Paulo State University (Protocol no. 379).

The ovaries were transported to the laboratory in saline solution (0.9%) at 4°C, and the two largest growing follicles from each heifer were dissected and measured using calipers. The abundance of cytochrome P450, family 19, subfamily A, polypeptide 1 (*CYP19A1*) mRNA was assessed in granulosa cells to confirm the identity of the future dominant and largest subordinate follicle (Ferreira Gasperin *et al.* 2011). Granulosa and theca cells were collected and homogenised as described previously (Buratini *et al.* 2007) and total RNA was extracted with TRIzol (Invitrogen, São Paulo, Brazil) and stored at −80°C. Cross-contamination between granulosa cells and theca cells was tested by assessing the presence of *CYP19A1* mRNA in theca cells and *CYP17A1* in granulosa cells (Buratini *et al.* 2005). Because of cross-contamination, three heifers killed on Day 2 and four heifers killed on Day 2.5 were used for gene expression analysis in granulosa cells.

Granulosa cell culture

Granulosa cell culture was performed as described previously (Gutiérrez *et al.* 1997) with some modifications (Silva and Price 2000). Unless noted otherwise, all materials were obtained from Invitrogen (São Paulo, Brazil). Follicles with a diameter of <5 mm were dissected from ovaries of *Bos indicus* cows obtained at a local abattoir and transported to the laboratory at 35°C in phosphate-buffered saline (PBS) containing penicillin (100 IU mL^{-1}) and streptomycin (100 mg mL^{-1}). Follicles with obvious signs of atresia (avascular theca, debris in the antrum) were discarded. Cells were collected by repeatedly passing the follicle wall through a pipette, washed twice by centrifugation at 980*g* for 20 min at room temperature, and suspended in Dulbecco's modified Eagle's medium (DMEM)/F12 containing HEPES (20 mM), sodium selenite (4 ng mL^{-1}), bovine serum albumin (BSA; 0.1%; Sigma-Aldrich, São Paulo, Brazil), penicillin (100 IU mL^{-1}), streptomycin (100 mg mL^{-1}), transferrin (2.5 mg mL^{-1}), non-essential amino acid mix (1.1 mM), androstenedione (10^{-7} M at the beginning of culture and 10^{-6} M at each medium change) and insulin (10 ng mL^{-1}). Cell viability was estimated with 0.4% Trypan blue stain. Cells were seeded into 24-well tissue culture plates (Sarstedt, Newton, NC, USA) at a density of 1×10^6 per well in 1 mL medium. Cultures were performed at 37°C in 5% CO_2 in air for 6 days, with 700 µL medium replaced every 2 days. To determine the effects of FGF10 and FGF22 on granulosa cell mRNA expression and oestradiol production, graded doses of human recombinant FGF10 (0, 1, 10 and 100 ng mL^{-1}; R&D Systems, Minneapolis, MN, USA; 92% homology between human and bovine nucelotide sequences) or FGF22 (0, 1, 10, 100 and 200 ng mL^{-1}; R&D Systems; 90% homology between human and bovine nucelotide sequences) were added to the culture medium from Day 2 of culture. At the end of culture, cells were collected in TRIzol and stored at −80°C until RNA extraction, and the medium was recovered to measure oestradiol. Data were derived from four independent cultures performed at different times.

Real-time reverse transcription–polymerase chain reaction

Total RNA (1 μg) from follicle cell types and cultured granulosa cell samples was incubated with DNAse I (1 U mg^{-1} RNA; Invitrogen) and then reverse transcribed with SuperScript III (200 U mL^{-1}; Invitrogen) and oligo-d(T) primer. Primers for target genes were designed based on the bovine sequences. Real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis was performed with an ABI 7500 thermocycler using Power SYBR Green PCR Master Mix (Applied Biosystems, São Paulo, Brazil). Amplification efficiencies for target and housekeeping genes were similar. Primer sequences, amplicon sizes and annealing temperatures for each gene are given in Table 1. Reactions were optimised to provide maximum amplification efficiency for each gene. PCR was performed on 0.5–1.0 μL cDNA in 25 μL reaction volumes in duplicate, and the specificity of each PCR product was determined by melting curve analysis and confirmation of the amplicon size using electrophoresis in 1.5% agarose gels. Negative controls (water replacing cDNA) were run in every plate. The relative expression of each target gene was calculated using the ΔΔCt method with efficiency correction (Pfaffl 2001); the calibrator control was a cDNA sample from each cell type analysed. Three housekeeping genes were measured, namely peptidylprolyl isomerase A (*PPIA*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and histone H2AFZ (*H2AFZ*); the geNorm applet for Microsoft Excel (http://www.medgen.ugent.be/genorm; Ramakers *et al.* 2003) identified *PPIA* as the most stable housekeeping gene for cultured granulosa cells and *ex vivo* theca cells, whereas *GAPDH* was the most stable gene for *ex vivo* granulosa cell samples.

Oestradiol assay

Oestradiol concentrations in the culture medium were determined by ELISA using the E_2-EASIA kit (KAP0621; Diasource, Louvain-la-Neuve, Belgium) in a single assay. The sensitivity of

Table 1. Sequence of primers for real-time PCR

FGF, fibroblast growth factor; *FGFR1B*, FGF receptor 1B; *FGFR2B*, FGF receptor 2B; *CYP19A1*, cytochrome P450, family 19, subfamily A, polypeptide 1; *FSHR*, FSH receptor; *HSD3B1*, 3-β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase; *STAR*, steroidogenic acute regulatory protein; *CYP11A1*, cytochrome P450, family 11, subfamily A, polypeptide 1; *IGF1R*, insulin-like growth factor 1 receptor; *IGF2R*, insulin-like growth factor 2 receptor; *PAPPA*, pregnancy-associated plasma protein-A; PPIA, peptidylprolyl isomerase A; *H2AFZ*, histone H2AFZ; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase

Target	Sequence	Amplification efficiency (%)	Fragment size (bp)	Annealing temperature (°C)	Reference
FGF7	Forward: 5′-TGG CAT TCT CAG GTT CTG GCC ATT-3′ Reverse: 5′-TGG CAT TCT CAG GTT CTG GCC ATT-3′	96	294	60	Berisha *et al.* (2004)
FGF10	Forward: 5′-CCA CCA ACT CCT CTT CTT CTT CCT-3′ Reverse: 5′-ATA CTG TAC GGG CAG TTC TCC TTC-3′	94	202	61	Castilho *et al.* (2014)
FGF22	Forward: 5′-GAC TCT ATG GGT CGC GGT TCT-3′ Reverse: 5′-GTT GTA GCC GTT CTC CTC GAT T-3′	97	71	59	NM_001205861.1
FGFR1B	Forward: 5′-ACG TCC TGG TGA CGG AGG-3′ Reverse: 5′- CCG GTG CCA TCC ATT TGA-3′	95	104	60	Castilho *et al.* (2014)
FGFR2B	Forward: 5′-TGT GGT TGG AGG TGA TGT-3′ Reverse: 5′-CGA GTG CTT CAG AAC CTT G-3′	95	242	58	Cho *et al.* (2008)
CYP19A1	Forward: 5′-TGA CCA GAT CCA AAC CAG ACA CCA-3′ Reverse: 5′-ATG AGG TTG CTA AGA GTC GGC ACA-3′	96	182	62	NM_174305.1
FSHR	Forward: 5′-AGC CCC TTG TCA CAA CTC TAT GTC-3′ Reverse: 5′-GTT CCT CAC CGT GAG GTA GAT GT-3′	92	105	60	Caixeta *et al.* (2009)
HSD3B1	Forward: 5′-GCC CAA CTC CTA CAG GGA GAT-3′ Reverse: 5′-TTC AGA GCC CAC CCA TTA GCT-3′	96	135	59	NM_174343.3
STAR	Forward: 5′-CCC AGC AGA AGG GTC TCA TC-3′ Reverse: 5′-TGC GAG AGG ACC TGG TTG AT-3′	93	157	62	NM_174189.2
CYP11A1	Forward: 5′-AGT CCA CAC CTC TTG CAC CTT TCT-3′ Reverse: 5′-CGC CCA TCC CAT GAA GGC AAT AAA-3′	93	140	59	NM_176644.2
IGF1R	Forward: 5′-TTG CAA GAA CCA TGC CTG CAG AAG-3′ Reverse: 5′-TGG CAT TCT CAG GTT CTG GCC ATT-3′	95	101	60	Satrapa *et al.* (2013)
IGF2R	Forward: 5′-TGCGGTGGTGGCCAGAAGATAATA-3′ Reverse: 5′-TCAAACTCGTAGAAGCAGCCGTCA-3′	91	103	62	Satrapa *et al.* (2013)
PAPPA	Forward: 5′-TCC AGA TGT TGA GCA GCC CTG TAA-3′ Reverse: 5′-ACC CAA ACG GTC AAA GAC TCA GGA-3′	90	111	60	Satrapa *et al.* (2013)
PPIA	Forward: 5′-GCC ATG GAG CGC TTT GG-3′ Reverse: 5′-CCA CAG TCA GCA ATG GTG ATC T-3′	100	65	60	Machado *et al.* (2009)
H2AFZ	Forward: 5′-GAG GAG CTG AAC AAG CTG TTG-3′ Reverse: 5′-TTG TGG TGG CTC TCA GTC TTC-3′	97	74	60	Machado *et al.* (2009)
GAPDH	Forward: 5′-GGC GTG AAC CAC GAG AAG TAT AA-3′ Reverse: 5′-CCC TCC ACG ATG CCA AAG T-3′	103	119	62	Machado *et al.* (2009)

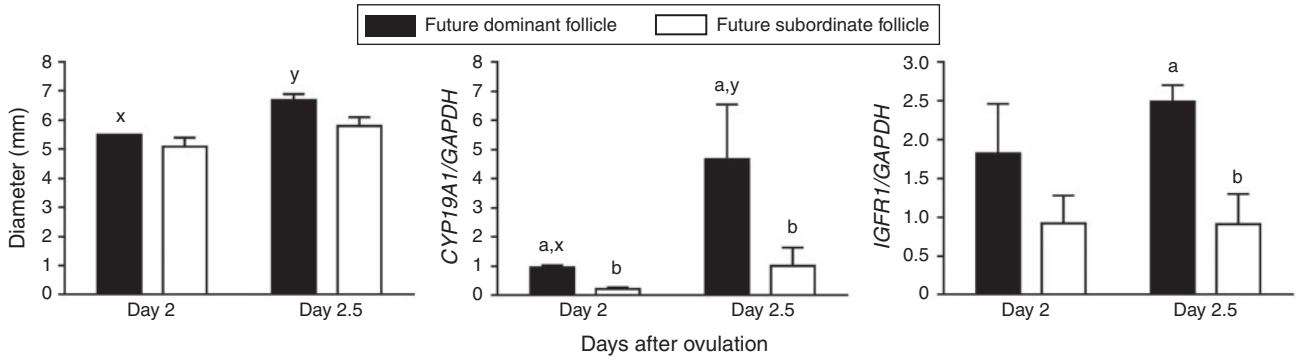

Fig. 1. Effects of day and follicle status on follicle diameter and levels of mRNA encoding cytochrome P450, family 19, subfamily A, polypeptide 1 (*CYP19A1*) and insulin-like growth factor 1 receptor (*IGF1R*) in granulosa cells during follicle deviation. mRNA abundance was measured by real-time polymerase chain reaction. Data are presented as the mean ± s.e.m. relative to a calibrator sample by the ΔΔCt method with efficiency correction. Different letters indicate significant effects of day (x, y; $P < 0.05$) or follicle status (a, b; $P < 0.05$). Data on follicle diameter are derived from follicle pairs (future dominant and future subordinate) obtained from four heifers on Day 2 and five heifers on Day 2.5 after ovulation; mRNA expression data are derived from three heifers on Day 2 and four heifers on Day 2.5 after ovulation.

the assay was 8 pg mL^{-1} and the intra-assay CV was 3.5%. Oestradiol concentration (pg mL^{-1}) was corrected by total protein concentration (μg well^{-1}), as determined by the Bradford assay.

Immunohistochemistry

Bovine adult and fetal ovaries were collected from an abattoir, bisected and fixed in paraformaldehyde. Fixed tissues were embedded in paraffin and 4-μm sections were placed on poly-L-lysine coated slides. Sections were deparaffinised in xylene twice for 20 min and hydrated in successive 3-min washes in 95% and 85% ethanol. Antigen retrieval was achieved by incubating samples in 0.5 mM citrate, pH 6.0, at 125°C for 10 min. Endogenous peroxidase was quenched by incubation in methanol with 8% hydrogen peroxide for 20 min, followed by rinsing 10 times in distilled water and twice for 5 min in 0.5 M Tris, pH 7.4. Slides were then incubated with polyclonal anti-human FGF22 antibody (2 ng mL^{-1}; Catalogue no. ab74860; Abcam, Cambridge, MA, USA) for 18 h at 4°C in a humidified chamber, washed in 0.5 M Tris, pH 7.4, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h (ADVANCE; Dako, Carpinteria CA, USA). Immunostaining was revealed with liquid diaminobenzidine (DAB; Dako) for 3 min and sections were counterstained with Harris haematoxylin. Three ovaries were examined and negative controls were performed by pre-incubating FGF22 antibody with twice the concentration of human recombinant FGF22 protein (Catalogue no. ab51793; Abcam) for 18 h at 4°C.

Statistical analysis

Data that were not normally distributed were transformed to base-10 logarithms before analysis. Levels of mRNA abundance in the future dominant and future subordinate follicles were compared on each day by paired *t*-tests, and differences between days were assessed by *t*-tests. ANOVA was used to test for effects of FGF10 and FGF22 on oestradiol production and mRNA abundance in cultured bovine granulosa cells, and the significance of differences between means was determined with the Tukey–Kramer test. Analyses were performed using JMP software (SAS Institute, Cary, NC, USA). Data are presented as the mean ± s.e.m. and were considered significant at $P < 0.05$ (two-sided).

Results

The mean diameters and *CYP19A1* mRNA levels of the future dominant follicle and future subordinate follicle during the first follicle wave are shown in Fig. 1. In all heifers, one follicle contained higher levels of *CYP19A1* mRNA than the other, and this was used to identify which was the future dominant follicle. The diameter of the future dominant and future subordinate follicle did not differ significantly on Days 2 and 2.5 ($P > 0.05$; Fig. 1), but there was a tendency for the future dominant to be larger on Day 2.5 ($P < 0.06$). The future dominant follicle also contained higher levels of *IGF1R* mRNA than the future subordinate follicle on Day 2.5 (Fig. 1).

Abundance of mRNA encoding *FGF7* and *FGF10* in theca cells was significantly affected by follicle fate. On Day 2, *FGF10* mRNA abundance tended to be greater in the future subordinate follicle than the future dominant follicle ($P = 0.06$), whereas on Day 2.5 mRNA levels of both genes were higher in the future subordinate follicle than future dominant follicle (Fig. 2). mRNA encoding *FGF22* was detected in theca cells (Ct <35 cycles) but weakly in granulosa cells (Ct >38 cycles), and did not differ between future dominant and subordinate follicles. mRNA encoding the two main receptors for the FGF7 family members, namely *FGFR1B* and *FGFR2B*, was detected in granulosa cells (Ct ~29 and 27 PCR cycles, respectively), and abundance of *FGFR2B* mRNA was significantly higher in future subordinate follicles than future dominant follicles on Day 2, whereas *FGFR1B* mRNA levels did not change (Fig. 2). mRNA encoding *FGFR1B* was also detected in theca cells (Ct ~26 PCR cycles), and abundance was higher in subordinate follicles compared with dominant follicles on Day 2.5 (Fig. 2).

Because FGF10 altered *CYP19A1* mRNA levels in granulosa cells of post-deviation follicles *in vivo*, we assessed the effects of FGF10 on granulosa cells from follicles <5 mm in diameter *in*

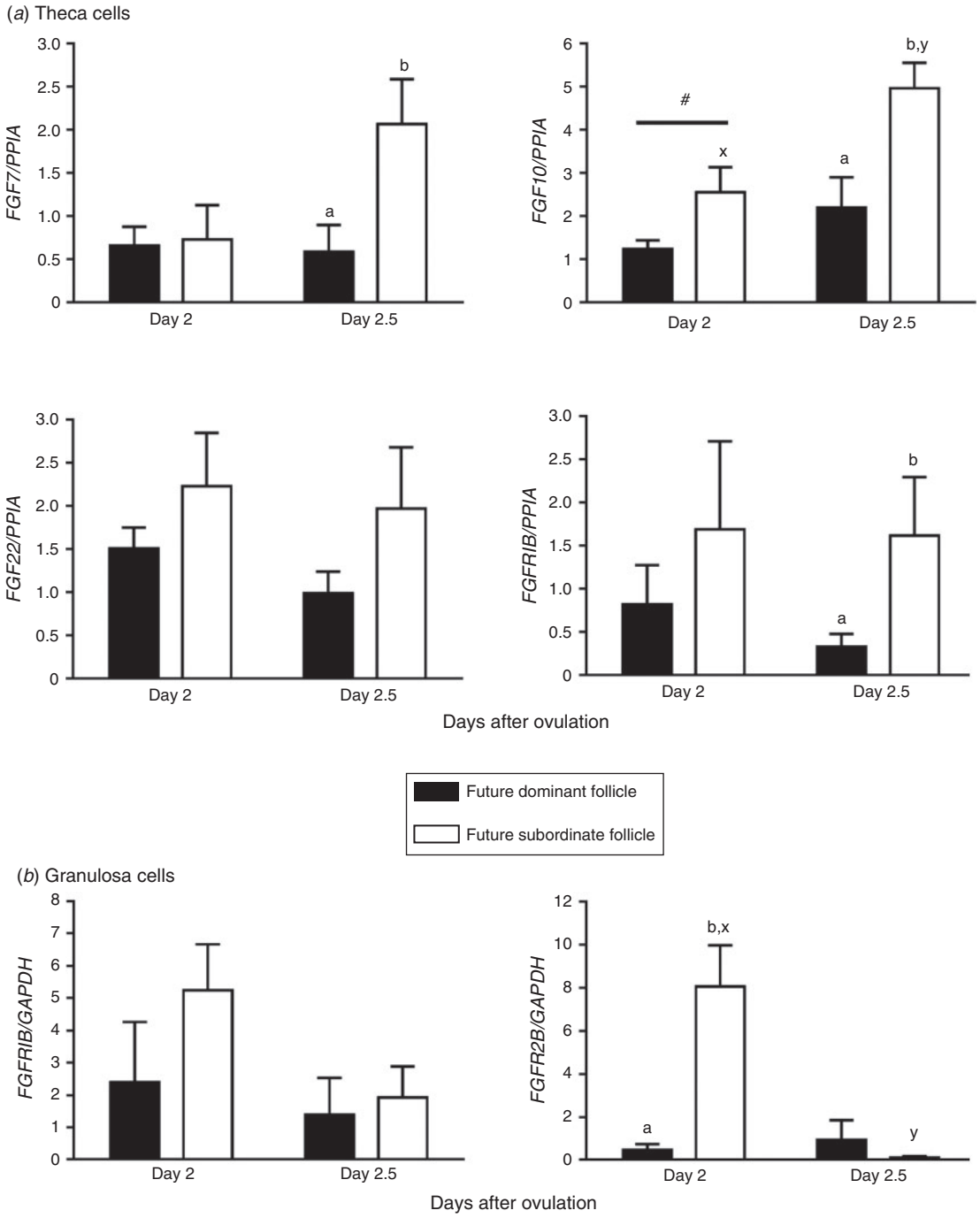

Fig. 2. Effects of time and follicle status on levels of mRNA encoding fibroblast growth factor (FGF) 7 subfamily members and their receptors (*FGFR1B* and *FGFR2B*) in (*a*) theca and (*b*) granulosa cells during follicle deviation. mRNA abundance was measured by real-time polymerase chain reaction. Data are presented as the mean ± s.e.m. relative to a calibrator sample by the ΔΔCt method with efficiency correction. Different letters indicate significant effects of day (x, y; $P < 0.05$) or follicle status (a, b; $P < 0.05$). [#]There was a tendency for a difference between groups ($P = 0.06$). Granulosa cell data are derived from follicle pairs obtained from three heifers on Day 2 and four heifers on Day 2.5 after ovulation. Theca cell data are derived from follicle pairs obtained from four heifers on Day 2 and five heifers on Day 2.5 after ovulation. *PPIA*, peptidylprolyl isomerase A.

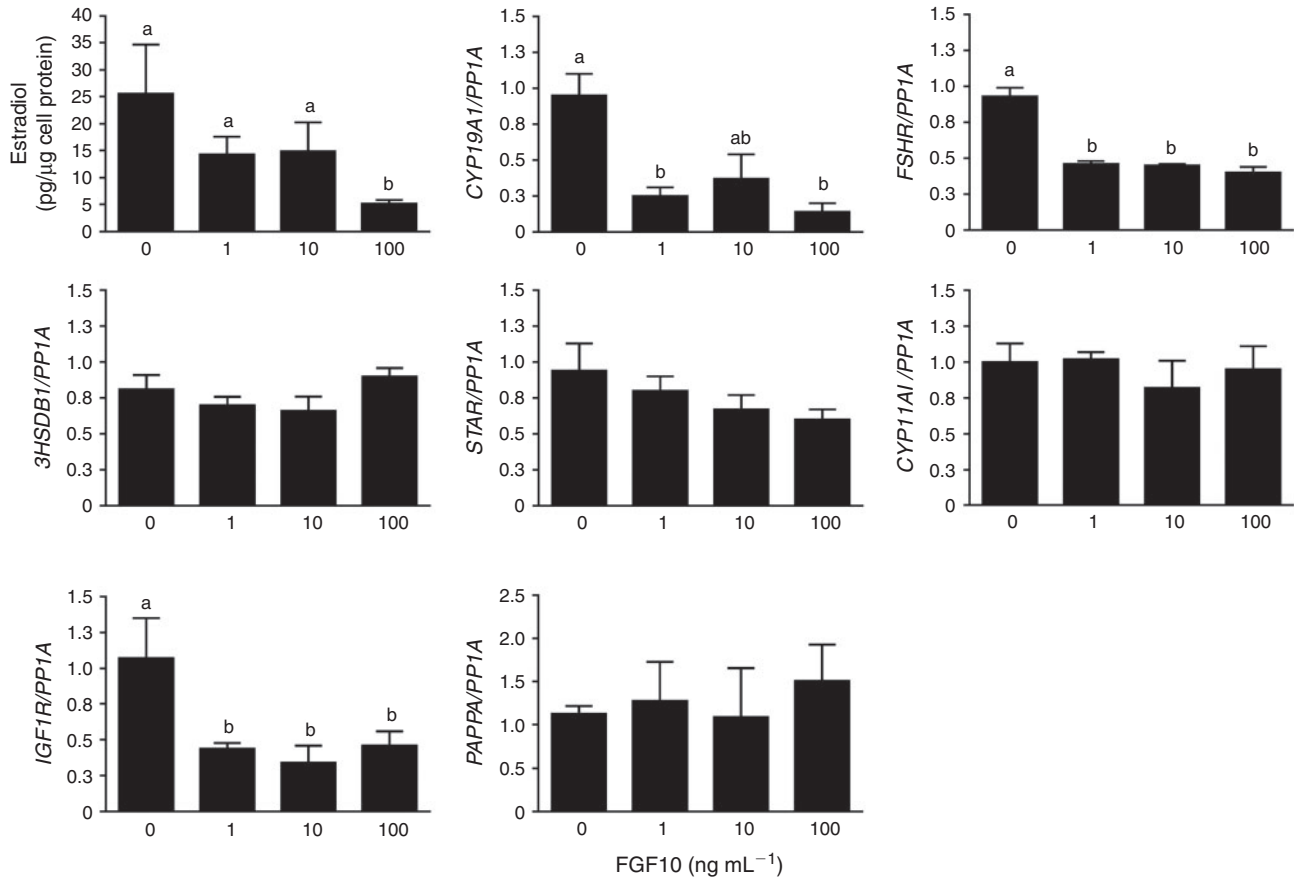

Fig. 3. Effects of graded doses of fibroblast growth factor (FGF) 10 on oestradiol production and levels of mRNA encoding steroidogenic enzymes, FSH receptor (*FSHR*), insulin-like growth factor 1 receptor (*IGF1R*) and pregnancy-associated plasma protein-A (*PAPPA*) in cultured bovine granulosa cells. Data are presented as means (±s.e.m.). Messenger RNA abundance was measured by real-time PCR. Expression values are relative to a calibrator sample and were calculated with the ΔΔCt method with efficiency correction. Different letters indicate significant differences ($P < 0.05$). Data are derived from four independent cultures. *CYP19A1*, cytochrome P450, family 19, subfamily A, polypeptide 1; *PPIA*, peptidylprolyl isomerase A; *3HSDB*, 3-β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase; *STAR*, steroidogenic acute regulatory protein; *CYP11A1*, cytochrome P450, family 11, subfamily A, polypeptide 1.

vitro. Addition of FGF10 significantly inhibited the secretion of oestradiol and abundance of mRNA encoding *CYP19A1* and the FSH receptor (*FSHR*), but not steroidogenic acute regulatory protein (*STAR*), 3-β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase (*HSD3B1*) or *CYP11A1* (Fig. 3). Addition of FGF10 suppressed *IGF1R* mRNA levels (Fig. 3), but did not alter the abundance of mRNA encoding the other IGF receptor expressed in granulosa cells (i.e. *IGF2R*; data not shown).

FGF22 protein was localised to granulosa, cumulus and theca cells from antral follicles, and preantral follicles from fetal ovaries. FGF22 staining was also observed in the oocyte and blood vessels, but not in the ovarian stroma (Fig. 4). To assess the potential function of FGF22, recombinant protein was added to granulosa cells in culture; FGF22 did not significantly alter oestradiol production (Fig. 4).

Discussion

FGF7 and FGF10 may be potential theca-derived factors that inhibit oestradiol secretion from granulosa cells and induce follicle regression (Gasperin *et al.* 2012). In the present study, we investigated whether FGF7, FGF10 or FGF22 may play a role in the process of follicle deviation. Our data demonstrate that *FGF7*, *FGF10* and *FGFR2B* mRNA levels are higher in the future subordinate follicle than in the future dominant follicle, and that FGF action may contribute to the decrease in oestradiol production in the future subordinate follicle during follicle selection in cattle.

The follicles in the present study were collected at specific days of a synchronised first follicle wave and thus represent a well-defined physiological model. Future dominant follicles were characterised by higher *CYP19A1* levels, as expected (Fortune *et al.* 2001) but were not larger than the future subordinate follicles, demonstrating that the follicles were collected before morphological deviation. In these predeviation follicles, *FGF10* and *FGF7* mRNA levels were lower in theca cells from the future dominant than future subordinate follicles. These data are consistent with our previous study of *FGF10* mRNA in post-deviation dominant and subordinate follicles *in vivo* (Gasperin *et al.* 2012); however, *FGF7* mRNA levels did not vary significantly with follicle health (Berisha *et al.* 2004;

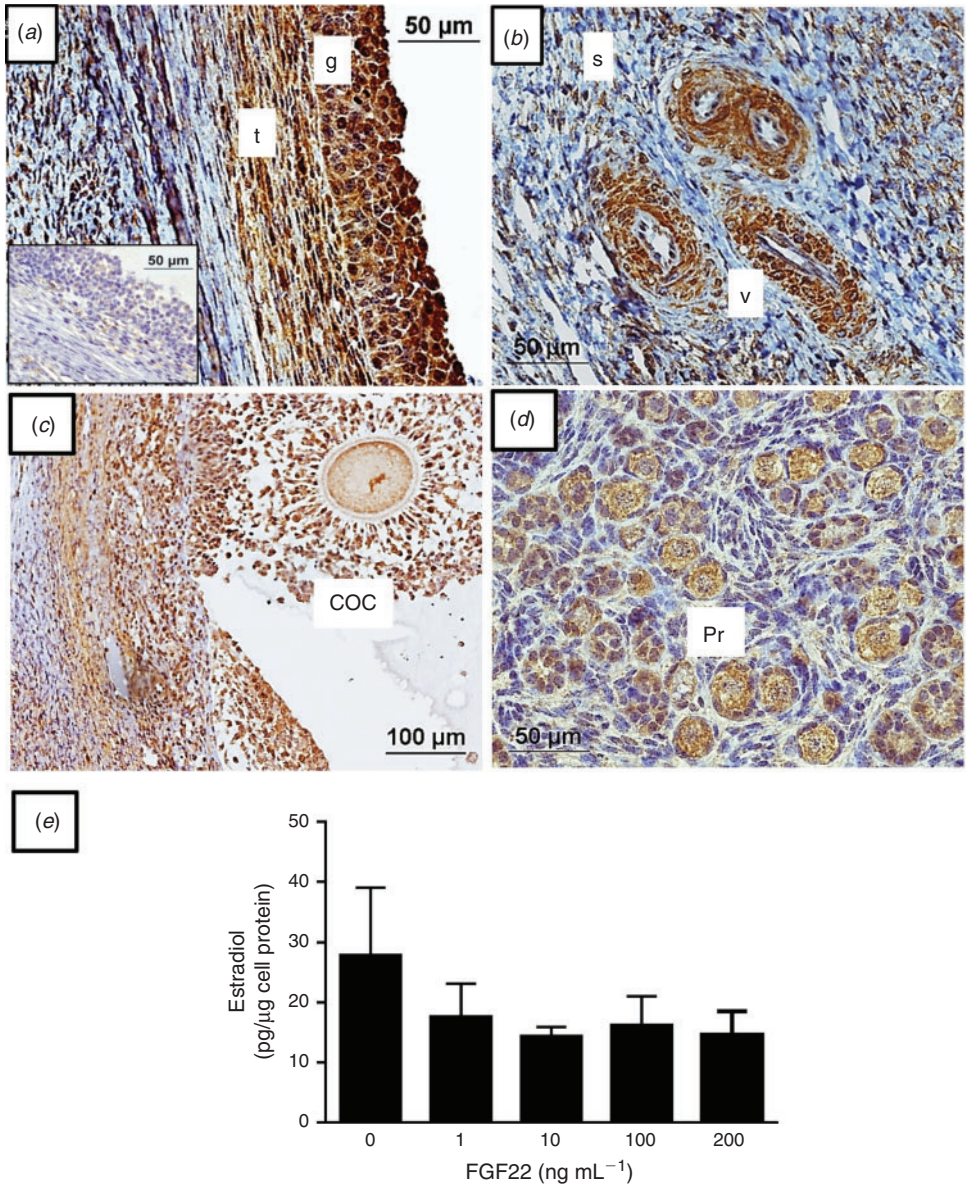

Fig. 4. Expression of fibroblast growth factor (FGF) 22 and its effects on oestradiol secretion from bovine granulosa cells *in vitro*. (*a*) Immunohistological detection of FGF22 in granulosa (g) and theca cell layers (t) of an antral follicle, (*b*) in blood vessels (v) and (*c*) in cumulus cells of adult ovaries and (*d*) in preantral follicles from fetal ovaries. Staining was weak in stromal cells (s) and not observed in the presence of excess FGF22 protein (*a*, inset). Pr, preantral follicles; COC, cumulus–oocyte complex. (*e*) Effect of human recombinant FGF22 on oestradiol secretion from bovine granulosa cells *in vitro*. Data are derived from four independent cultures and are presented as the mean ± s.e.m. Different letters indicate significant differences ($P < 0.05$).

Buratini *et al.* 2007). The discrepancy between the present and previous data regarding *FGF7* expression is likely due to different experimental designs: synchronised dominant and subordinate follicles were assessed in the present study, but abattoir follicles classified according to size and intrafollicular steroid concentrations were used in the previous studies. We also demonstrated, for the first time, the presence of *FGF22* mRNA and protein in the ovary, although mRNA levels were not different between future dominant and future subordinate follicles. Thus, the present data suggest differential expression of *FGF10* and *FGF7* but not *FGF22* mRNA before divergence in follicle diameter occurs.

The main receptors activated by FGF7 subfamily members are FGFR1B and FGFR2B (Zhang *et al.* 2006). mRNA for *FGFR2B* is found almost exclusively in granulosa cells in cattle (Berisha *et al.* 2004) and, in the present study, *FGFR2B* mRNA abundance was higher in granulosa cells from subordinate follicles than dominant follicles on Day 2, after which mRNA

levels decreased abruptly in subordinate follicles. This decrease occurred at a time when plasma FSH levels are declining (Ginther *et al.* 2002), which is consistent with our previous observation that a reduction of FSH decreases *FGFR2B* mRNA levels in granulosa cells *in vitro* (Buratini *et al.* 2007). A previous study reported increasing levels of *FGFR2B* mRNA in granulosa cells from large dominant follicles (Berisha *et al.* 2004), which represents a later stage of follicle growth than that examined here. In contrast with *FGFR2B*, *FGFR1B* mRNA abundance did not change in granulosa cells around follicle deviation. It has not been reported whether *FGFR1B* is expressed in theca cells, therefore we measured mRNA levels in the theca in the present study. *FGFR1B* mRNA levels were relatively abundant and were regulated during follicle deviation, suggesting increased FGF action on theca cells from subordinate compared with dominant follicles. These data suggest that developmental regulation of FGF receptors during follicle growth accounts for enhanced action of FGF7 family members in granulosa (via FGFR2B) and theca cells (via FGFR1B) of subordinate follicles, and thus underscore the potential importance of FGF signalling for follicle selection and dominance.

One caveat to the current model is the choice of the first-wave follicle. It is known that the first- and third-wave dominant follicles grow at similar rates and reach similar maximum sizes, but that the second-wave follicle grows more slowly and attains a smaller maximum diameter (Sirois and Fortune 1988). The second-wave dominant follicle also secretes less oestradiol than the first-wave dominant follicle (Wolfenson *et al.* 1999; Miura *et al.* 2014). Whether the expression of growth factors within the follicle, such as FGFs, also differs between first- and second-wave follicles remains to be determined.

Increased levels of FGF10 and FGF7 in subordinate follicles may be of functional relevance because both these proteins inhibit granulosa cell oestradiol production *in vitro* (Parrott and Skinner 1998; Buratini *et al.* 2007) and FGF10 inhibited granulosa cell *CYP19A1* mRNA levels *in vivo* (Gasperin *et al.* 2012). We further explored the actions of FGF10 on granulosa cells *in vitro* and confirmed that FGF10 decreased oestradiol secretion and *CYP19A1* mRNA without altering levels of mRNA encoding progestagenic enzymes. We found that FGF10 inhibited granulosa cell *FSHR* mRNA levels *in vitro* and, because oestradiol secretion and *CYP19A1* mRNA levels are regulated by FSH (Gutiérrez *et al.* 1997; Silva and Price 2000), these data suggest that FGF10 may decrease oestradiol secretion in part through inhibition of FSH signalling. An alternative explanation is that FGF10 inhibits oestradiol secretion, which, in turn, leads to a decrease in *FSHR* mRNA levels. However, there is good evidence that oestradiol does not regulate *FSHR* mRNA levels in bovine granulosa cells (Luo and Wiltbank 2006). In contrast with FGF10, FGF22 did not significantly inhibit oestradiol production in cultured granulosa. This is an intriguing observation because FGF10 and FGF22 activate FGFR1B and FGFR2B with similar affinities (Zhang *et al.* 2006). However, there are other examples of FGFs with similar receptor activation properties that result in different biological effects. Most relevant to the present study are the divergent effects of FGF7 and FGF10 on salivary gland and lung morphogenesis (Makarenkova *et al.* 2009; Francavilla *et al.* 2013), which may be a result of differential phosphorylation of the receptor (Francavilla *et al.* 2013).

Oestradiol secretion and *CYP19A1* mRNA levels are also regulated by IGF1 (Gutiérrez *et al.* 1997; Spicer and Aad 2007), and we observed higher levels of *IGF1R* mRNA in granulosa cells from future dominant compared with future subordinate follicles. Previous studies have compared follicles of abattoir origin classified by size or oestrogen : progesterone ratios in follicular fluid, and *IGF1R* mRNA levels are reported to be lower in atretic (oestrogen inactive) follicles (Armstrong *et al.* 2000; Sudo *et al.* 2007). In the present study, we demonstrated that *IGF1R* mRNA abundance differs between oestrogen-active and oestrogen-inactive follicles as early as the time of follicle deviation, and that *IGF1R* mRNA abundance was reduced by the addition of FGF10 *in vitro*, suggesting that FGF10 may affect oestradiol synthesis and secretion not only through FSHR, but also via IGF1R signalling.

In summary, the results of the present study suggest a role for FGF10 signalling during follicle selection. Increased levels of mRNA encoding *FGF10* and *FGFR2B* in future subordinate follicles preceding morphological deviation may contribute to the decreased oestradiol content and granulosa *CYP19A1* mRNA levels observed in the future subordinate follicle. Moreover, we provide novel evidence that the mechanism by which FGF10 decreases oestradiol secretion may involve inhibition of *FSHR* and *IGF1R* expression.

Acknowledgements

The authors are grateful to FAPESP (Sao Paulo, Brazil) for financial support (2008/57365-2) and providing a PhD studentship to ACSC. The authors also thank Dr Reneé Laufer Amorin for access to immunohistochemistry tools and technical advise and Fernanda Fagali Franchi for technical assistance.

References

Armstrong, D. G., Gutierrez, C. G., Baxter, G., Glazyrin, A. L., Mann, G. E., Woad, K. J., Hogg, C. O., and Webb, R. (2000). Expression of mRNA encoding IGF-I, IGF-II and type 1 IGF receptor in bovine ovarian follicles. *J. Endocrinol.* **165**, 101–113. doi:10.1677/JOE.0.1650101

Berisha, B., Sinowatz, F., and Schams, D. (2004). Expression and localization of fibroblast growth factor (FGF) family members during the final growth of bovine ovarian follicles. *Mol. Reprod. Dev.* **67**, 162–171. doi:10.1002/MRD.10386

Buratini, J., Jr, Teixeira, A. B., Costa, I. B., Glapinski, V. F., Pinto, M. G. L., Giometti, I. C., Barros, C. M., Cao, M., Nicola, E. S., and Price, C. A. (2005). Expression of fibroblast growth factor-8 and regulation of cognate receptors, fibroblast growth factor receptor (FGFR)-3c and -4, in bovine antral follicles. *Reproduction* **130**, 343–350. doi:10.1530/REP.1.00642

Buratini, J., Jr, Pinto, M. G. L., Castilho, A. C., Amorim, R. L., Giometti, I. C., Portela, V. M., Nicola, E. S., and Price, C. A. (2007). Expression and function of fibroblast growth factor 10 and its receptor, fibroblast growth factor receptor 2b, in bovine follicles. *Biol. Reprod.* **77**, 743–750. doi:10.1095/BIOLREPROD.107.062273

Caixeta, E. S., Ripamonte, P., Franco, M. M., Junior, J. B., and Dode, M. A. N. (2009). Effect of follicle size on mRNA expression in cumulus cells and oocytes of *Bos indicus*: an approach to identify marker genes for developmental competence. *Reprod. Fertil. Dev.* **21**, 655–664. doi:10.1071/RD08201

Castilho, A. C. S., Bueno da Silva, R., Price, C. A., Machado, M. F., Amorim, R. L., and Buratini, J. (2014). Expression of fibroblast growth factor 10

and cognate receptors in the developing bovine ovary. *Theriogenology* **81**, 1268–1274. doi:10.1016/J.THERIOGENOLOGY.2014.02.008

Cho, J. H., Itoh, T., Sendai, Y., and Hoshi, H. (2008). Fibroblast growth factor 7 stimulates *in vitro* growth of oocytes originating from bovine early antral follicles. *Mol. Reprod. Dev.* **75**, 1736–1743. doi:10.1002/MRD.20912

Erickson, G. F., and Shimasaki, S. (2003). The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during the estrous cycle. *Reprod. Biol. Endocrinol.* **1**, 9. doi:10.1186/1477-7827-1-9

Ferreira, R., Gasperin, B., Rovani, M., Santos, J., Barreta, M., Bohrer, R., Price, C., and Gonçalves, P. B. D. (2011). Angiotensin II signaling promotes follicle growth and dominance in cattle. *Endocrinology* **152**, 4957–4965. doi:10.1210/EN.2011-1146

Fortune, J. E., Rivera, G. M., Evans, A. C., and Turzillo, A. M. (2001). Differentiation of dominant versus subordinate follicles in cattle. *Biol. Reprod.* **65**, 648–654. doi:10.1095/BIOLREPROD65.3.648

Fortune, J. E., Rivera, G. M., and Yang, M. Y. (2004). Follicular development: the role of the follicular microenvironment in selection of the dominant follicle. *Anim. Reprod. Sci.* **82–83**, 109–126. doi:10.1016/J.ANIREPROSCI.2004.04.031

Francavilla, C., Rigbolt, K. T. G., Emdal, K. B., Carraro, G., Vernet, E., Bekker-Jensen, D. B., Streicher, W., Wikström, M., Sundström, M., Bellusci, S., Cavallaro, U., Blagoev, B., and Olsen, J. V. (2013). Functional proteomics defines the molecular switch underlying FGF receptor trafficking and cellular outputs. *Mol. Cell* **51**, 707–722. doi:10.1016/J.MOLCEL.2013.08.002

Gasperin, B. G., Ferreira, R., Rovani, M. T., Santos, J. T., Buratini, J., Price, C. A., and Gonçalves, P. B. D. (2012). FGF10 inhibits dominant follicle growth and estradiol secretion *in vivo* in cattle. *Reproduction* **143**, 815–823. doi:10.1530/REP-11-0483

Gasperin, B. G., Ferreira, R., Rovani, M. T., Bordignon, V., Duggavathi, R., Buratini, J., Oliveira, J. F. C., and Gonçalves, P. B. D. (2014). Expression of receptors for BMP15 is differentially regulated in dominant and subordinate follicles during follicle deviation in cattle. *Anim. Reprod. Sci.* **144**, 72–78. doi:10.1016/J.ANIREPROSCI.2013.12.002

Ginther, O. J., Bergfelt, D. R., Beg, M. A., and Kot, K. (2002). Role of low circulating FSH concentrations in controlling the interval to emergence of the subsequent follicular wave in cattle. *Reproduction* **124**, 475–482. doi:10.1530/REP.0.1240475

Ginther, O. J., Bergfelt, D. R., Beg, M. A., Meira, C., and Kot, K. (2004). *In vivo* effects of an intrafollicular injection of insulin-like growth factor 1 on the mechanism of follicle deviation in heifers and mares. *Biol. Reprod.* **70**, 99–105. doi:10.1095/BIOLREPROD.103.021949

Glister, C., Kemp, C. F., and Knight, P. G. (2004). Bone morphogenetic protein (BMP) ligands and receptors in bovine ovarian follicle cells: actions of BMP-4, -6 and -7 on granulosa cells and differential modulation of Smad-1 phosphorylation by follistatin. *Reproduction* **127**, 239–254. doi:10.1530/REP.1.00090

Gutiérrez, C. G., Campbell, B. K., and Webb, R. (1997). Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to follicle-stimulating hormone, and morphological characteristics. *Biol. Reprod.* **56**, 608–616. doi:10.1095/BIOLREPROD56.3.608

Itoh, N., and Ornitz, D. M. (2004). Evolution of the Fgf and Fgfr gene families. *Trends Genet.* **20**, 563–569. doi:10.1016/J.TIG.2004.08.007

Luo, W., and Wiltbank, M. C. (2006). Distinct regulation by steroids of messenger RNAs for FSHR and CYP19A1 in bovine granulosa cells. *Biol. Reprod.* **75**, 217–225. doi:10.1095/BIOLREPROD.105.047407

Luo, W., Gumen, A., Haughian, J. M., and Wiltbank, M. C. (2011). The role of luteinizing hormone in regulating gene expression during selection of a dominant follicle in cattle. *Biol. Reprod.* **84**, 369–378. doi:10.1095/BIOLREPROD.110.085274

Machado, M. F., Portela, V. M., Price, C. A., da Costa, I. B., Ripamonte, P., Amorim, R. L., and Buratini, J., Jr. (2009). Regulation and action of fibroblast growth factor 17 in bovine follicles. *J. Endocrinol.* **202**, 347–353. doi:10.1677/JOE-09-0145

Makarenkova, H. P., Hoffman, M. P., Beenken, A., Eliseenkova, A. V., Meech, R., Tsau, C., Patel, V. N., Lang, R. A., and Mohammadi, M. (2009). Differential interactions of FGFs with heparan sulfate control gradient formation and branching morphogenesis. *Sci. Signal.* **2**, ra55. doi:10.1126/SCISIGNAL.2000304

Miura, R., Haneda, S., Lee, H.-H., Miyamoto, A., Shimizu, T., Miyahara, K., Miyake, Y.-I., and Matsui, M. (2014). Evidence that the dominant follicle of the first wave is more active than that of the second wave in terms of its growth rate, blood flow supply and steroidogenic capacity in cows. *Anim. Reprod. Sci.* **145**, 114–122. doi:10.1016/J.ANIREPROSCI.2014.01.003

Parrott, J. A., and Skinner, M. K. (1998). Developmental and hormonal regulation of hepatocyte growth factor expression and action in the bovine ovarian follicle. *Biol. Reprod.* **59**, 553–560. doi:10.1095/BIOLREPROD59.3.553

Parrott, J. A., Vigne, J. L., Chu, B. Z., and Skinner, M. K. (1994). Mesenchymal–epithelial interactions in the ovarian follicle involve keratinocyte and hepatocyte growth factor production by thecal cells and their action on granulosa cells. *Endocrinology* **135**, 569–575.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45. doi:10.1093/NAR/29.9.E45

Ramakers, C., Ruijter, J. M., Deprez, R. H., and Moorman, A. F. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* **339**, 62–66. doi:10.1016/S0304-3940(02)01423-4

Sartorelli, E. S., Carvalho, L. M., Bergfelt, D. R., Ginther, O. J., and Barros, C. M. (2005). Morphological characterization of follicle deviation in Nelore (*Bos indicus*) heifers and cows. *Theriogenology* **63**, 2382–2394. doi:10.1016/J.THERIOGENOLOGY.2004.08.017

Satrapa, R. A., Razza, E. M., Castilho, A. C. S., Simões, R. A. L., Silva, C. F., Nabhan, T., Pegorer, M. F., and Barros, C. M. (2013). Differential expression of IGF family members in heat-stressed embryos produced in vitro from OPU-derived oocytes of Nelore (*Bos indicus*) and Holstein (*Bos taurus*) cows. *Reprod. Domest. Anim.* **48**, 1043–1048. doi:10.1111/RDA.12211

Scaramuzzi, R. J., Baird, D. T., Campbell, B. K., Driancourt, M.-A., Dupont, J., Fortune, J. E., Gilchrist, R. B., Martin, G. B., McNatty, K. P., McNeilly, A. S., Monget, P., Monniaux, D., Viñoles, C., and Webb, R. (2011). Regulation of folliculogenesis and the determination of ovulation rate in ruminants. *Reprod. Fertil. Dev.* **23**, 444–467. doi:10.1071/RD09161

Silva, J. M., and Price, C. A. (2000). Effect of follicle-stimulating hormone on steroid secretion and messenger ribonucleic acids encoding cytochromes P450 aromatase and cholesterol side-chain cleavage in bovine granulosa cells *in vitro*. *Biol. Reprod.* **62**, 186–191. doi:10.1095/BIOLREPROD62.1.186

Sirois, J., and Fortune, J. E. (1988). Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. *Biol. Reprod.* **39**, 308–317. doi:10.1095/BIOLREPROD39.2.308

Spicer, L. J., and Aad, P. Y. (2007). Insulin-like growth factor (IGF) 2 stimulates steroidogenesis and mitosis of bovine granulosa cells through the IGF1 receptor: role of follicle-stimulating hormone and IGF2 receptor. *Biol. Reprod.* **77**, 18–27. doi:10.1095/BIOLREPROD.106.058230

Spicer, L. J., Alpizar, E., and Vernon, R. K. (1994). Insulin-like growth factor-I receptors in ovarian granulosa cells: effect of follicle size and hormones. *Mol. Cell. Endocrinol.* **102**, 69–76. doi:10.1016/0303-7207(94)90099-X

Stewart, R. E., Spicer, L. J., Hamilton, T. D., Keefer, B. E., Dawson, L. J., Morgan, G. L., and Echternkamp, S. E. (1996). Levels of insulin-like

growth factor (IGF) binding proteins, luteinizing hormone and IGF-I receptors, and steroids in dominant follicles during the first follicular wave in cattle exhibiting regular estrous cycles. *Endocrinology* **137**, 2842–2850.

Sudo, N., Shimizu, T., Kawashima, C., Kaneko, E., Tetsuka, M., and Miyamoto, A. (2007). Insulin-like growth factor-I (IGF-I) system during follicle development in the bovine ovary: relationship among IGF-I, type 1 IGF receptor (IGFR-1) and pregnancy-associated plasma protein-A (PAPP-A). *Mol. Cell. Endocrinol.* **264**, 197–203. doi:10.1016/J.MCE.2006.10.011

Wolfenson, D., Sonego, H., Shaham-Albalancy, A., Shpirer, Y., and Meidan, R. (1999). Comparison of the steroidogenic capacity of bovine follicular and luteal cells, and corpora lutea originating from dominant follicles of the first or second follicular wave. *J. Reprod. Fertil.* **117**, 241–247. doi:10.1530/JRF.0.1170241

Zhang, X., Ibrahimi, O. A., Olsen, S. K., Umemori, H., Mohammadi, M., and Ornitz, D. M. (2006). Receptor specificity of the fibroblast growth factor family. The complete mammalian FGF family. *J. Biol. Chem.* **281**, 15 694–15 700. doi:10.1074/JBC.M601252200