

Expression of fibroblast growth factor 13 (*Fgf13*) mRNA in bovine antral follicles and corpora lutea

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Abstract

Recent studies have suggested a paracrine role for several fibroblast growth factors (FGFs) in the regulation of follicle and luteal development. *Fgf13* is a non-secreted FGF that has been previously localized to the developing gonads, but it is not known if it is expressed in the adult ovary. The objective of the present study was to determine the expression pattern of *Fgf13* mRNA in the bovine ovary. *Fgf13* mRNA expression was examined by semiquantitative RT-PCR using *Gapdh* as the internal control gene in theca and granulosa cells, corpora lutea (CL) and oocytes collected from abattoir ovaries. Follicles were grouped according to estradiol content (<5, 5-20, >20-100 and >100 ng/ml) and size (5-7, 8-10 and >10 mm diameter). CL samples were morphologically classified into four developmental stages. *Fgf13* mRNA expression was assessed in pools containing 50 oocytes aspirated from follicles larger than 4 mm in diameter. ANOVA was used to test for the main effects of follicle size group, and estradiol concentration group in granulosa and theca cells, and to test the effect of CL developmental stage on *Fgf13* mRNA abundance. *Fgf13* mRNA was detected in the CL and in somatic follicle cells, but not in oocytes. Thecal *Fgf13* expression increased with increasing follicle diameter but did not change with intrafollicular estradiol concentrations. No evidence of developmental regulation of *Fgf13* mRNA expression was observed in granulosa cells and CL. The present data demonstrate for the first time the expression of an intracellular FGF in the bovine ovary and suggests that *Fgf13* mRNA is upregulated in bovine theca cells during antral follicle growth.

Keywords: antral follicle, cattle, corpus luteum, fibroblast growth factor.

Introduction

The control of follicle and corpus luteum (CL) growth and regression involves complex interactions between gonadotropins and numerous endocrine and

paracrine factors (Schams and Berisha, 2004; Webb and Campbell, 2007). The fibroblast growth factor (FGF) family is emerging as a group of factors that are involved in the regulation of ovarian function. This family consists of 22 peptides, which have been grouped into 7 subfamilies with distinct receptor-binding properties (Ornitz and Itoh, 2001; Itoh and Ornitz, 2004). FGF 1, 2, 7, and 10 were localized to somatic follicle cells (predominantly to the theca layer; Parrot and Skinner, 1998; Berisha *et al.*, 2004; Buratini *et al.*, 2007b) and CL (Zheng *et al.*, 1993; Van Wezel *et al.*, 1995; Castilho *et al.*, 2008) in cattle. In addition, *Fgf8* and *Fgf10* expression was also localized to bovine oocytes (Buratini *et al.*, 2005, 2007b). Consistent with a paracrine role for FGFs, FGF receptors (FGFR) have been also detected in theca and granulosa cells and in the CL (Berisha *et al.*, 2004, Buratini *et al.*, 2005, Castilho *et al.*, 2008, Guerra *et al.*, 2008) in cattle. Functional data corroborates with expression data to suggest paracrine roles for FGFs in the regulation of follicle and CL development. In the bovine follicle, FGF2 and FGF7 stimulate somatic cell proliferation (Lavranos *et al.*, 1994; Parrot *et al.*, 1994; Nilsson *et al.*, 2001), and together with FGF10 inhibit steroidogenesis (Vernon and Spicer, 1994; Spicer and Stewart, 1996; Parrot and Skinner, 1998; Buratini *et al.*, 2007b). In the ruminant CL, FGF2 stimulates cell proliferation, progesterone secretion and angiogenesis (Miyamoto *et al.*, 1992; Grazul-Bilska *et al.*, 1995; Liebermann *et al.*, 1996; Robinson *et al.*, 2008).

Whilst most FGFs bind to the extracellular domain of cell surface receptor tyrosine kinases, members of the FGF11 subfamily (FGFs 11, 12, 13, and 14) are unique in that they do not possess a signal sequence for secretion and are incapable of activating FGFRs (Smallwood *et al.*, 1996; Olsen *et al.*, 2003); these FGFs are termed FGF homologous factors or intracellular FGFs (iFGF; Powers *et al.*, 2000; Itoh and Ornitz, 2008). Intracellular FGFs are mostly expressed in the developing and adult nervous system, where they bind to mitogen-activated protein kinase (MAPK) scaffolding proteins and to voltage-gated sodium channels (Goldfarb, 2005). However, the roles of iFGFs in different

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physiological processes still remain to be clarified.

Although it is not known if iFGFs are expressed in the mature reproductive system, *Fgf13* expression has been recently localized to the mesonephros of both sexes in mice (Cory *et al.*, 2007). Since *Fgf13* is expressed in the developing gonad, we hypothesized that it would be also expressed in the adult ovary, and therefore might be a candidate for intracellular regulation of follicle and luteal development. Specifically, we aimed to determine the expression pattern of *Fgf13* mRNA in bovine antral follicles and CL at different developmental stages.

Materials and Methods

Tissues

A variety of tissues were collected from fetal and adult cows (predominantly Nelore - *Bos indicus*). Adult tissues and whole fetuses were obtained from an abattoir near the Sao Paulo State University campus in Botucatu and transported to the laboratory in saline on ice. Fetuses were dissected and the different organs were collected on ice. Adult and fetal tissue samples weighing 50 to 100 mg were homogenized in Trizol (Invitrogen) with a Polytron, and stored at -70°C until RNA extraction.

Antral follicles of 5 mm in diameter and greater were dissected from the ovaries of adult cows. Follicles smaller than 5 mm in diameter were not used as they did not provide sufficient RNA yield for individual cell types. The follicular fluid was centrifuged at 1200 g for 1 min to separate the granulosa cells and was then frozen for progesterone and estradiol assays. To harvest mural granulosa cells, the antral cavity was vigorously and repeatedly flushed with cold saline using a 1 ml syringe with a 25 gauge needle. The resulting solution was centrifuged at 1200 g for 1 min and the pellet of granulosa cells was added to the pellet obtained from the follicular fluid. The follicle was cut in 2 halves and remaining granulosa cells adhering to the follicle wall were removed by gently scraping with a blunt Pasteur pipette to prevent contamination of theca cells with granulosa cells. The granulosa cells obtained by scraping were discarded to avoid contamination with theca cells. The theca layer was detached from the follicle wall with a small forceps and washed in saline by repeated passages through a 1 ml syringe to remove adhering granulosa cells. Granulosa and theca samples were collected in Trizol (Invitrogen) and homogenized with a Polytron. Total RNA was extracted immediately according to the Trizol protocol. Follicles were classified according to estradiol content into <5, 5-20, >20-100 and >100 ng/ml (based on Berisha *et al.*, 2000), and by size (5-7, 8-10 and >10 mm diameter). Size categories were designed to represent FSH-dependent recruited follicles, LH-responsive dominant follicles, and preovulatory follicles in Nelore cattle, respectively (Figueiredo *et al.* 1997; Nogueira *et al.* 2007). Follicles with progesterone concentrations ≥ 100 ng/ml were classified as atretic, and were excluded from the analysis. These follicles also

presented estradiol:progesterone ratios lower than 0.01 as previously described for atretic follicles (Grimes and Ireland, 1986). Cross-contamination of theca and granulosa cells was tested by PCR detection of the mRNAs that encode cytochrome P450 aromatase (*Cyp19a1*) and 17 α -hydroxylase (*Cyp17a1*) in each sample (Buratini *et al.*, 2005). The detection of *Cyp19a1* amplicons in theca samples or of *Cyp17a1* amplicons in granulosa samples indicated cross-contamination, and these samples were discarded.

Cumulus-oocyte-complexes were aspirated from antral follicles larger than 4 mm in diameter, and cumulus cells were removed from the oocyte by vortexing. Fifty oocytes from follicles at variable sizes were pooled (n = 4), and total RNA was extracted separately from these four pools with the RNeasy kit (Qiagen).

CL at different stages of development were also dissected from abattoir ovaries (n = 10 per developmental stage). Tissue samples weighing 50-100 mg were homogenized in Trizol and total RNA was extracted. CL were morphologically classified into four developmental stages as previously described (Ireland *et al.*, 1980): stage I were corpora hemorrhagica with a dark red color, incomplete epithelial cover of the rupture point and measured from 0.5 to 1.5 cm diameter, corresponding to days 1 to 4 after ovulation; stage II CL featured complete epithelial cover of the rupture point and visible vasculature in its periphery, were red or brown at the apex and orange below it, and measured from 1.6 to 2.0 cm, corresponding to days 5 to 10 after ovulation; stage III mid-cycle CL showed well developed vasculature often visible at the apex, were completely orange or yellow and measured from 1.6 to 2.0 cm, corresponding to days 11 to 17 of CL after ovulation; and stage IV regressed CL showed no visible vasculature at the surface, were pale yellow to white in color and measured less than 1 cm diameter, corresponding to days 18 to 20 after ovulation.

Reverse transcription-polymerase chain reaction (RT-PCR)

Expression of *Fgf13* in isolated granulosa and theca cells was measured by semiquantitative RT-PCR. The same RT-PCR protocol was used to assess *Fgf13* expression in a variety of bovine fetal and adult tissues. Total RNA (1 μ g) was incubated with DNase I (Invitrogen), and then reverse-transcribed with SuperScript III (Invitrogen) and oligo (dT) primer. PCR was performed with 1 μ l cDNA in a PCR master mix that contained 1.6 U Taq DNA polymerase (Invitrogen), 0.4 μ M primers, 0.2 mM dNTPs, and 1.5 mM MgCl₂ in a total volume of 25 μ l. Samples were denatured for 3 min at 94°C, followed by 34 (for granulosa and theca), 31 (for CL and fetal and adult tissues) or 40 (for oocytes) cycles of denaturing at 94°C for 45 sec, annealing at 55°C (for oocytes, granulosa, and theca) or 60°C (for CL and fetal and adult tissues) for 45 sec, and extension at 72°C for 40 sec. As follicle samples and

CL/tissue samples were analyzed several months apart, PCR conditions were optimized twice immediately before each of these analyses, which determined different annealing temperatures for follicle samples and CL/tissue samples. Primers for *Fgf13* were designed based on alignment of human and rodent sequences; forward and reverse primers were 5' tgt gct gta ctt tgt gcg tag a 3' and 5' ttc ttc cat gca aca gat atg aa 3', respectively. The identity of the *Fgf13* amplicon was confirmed by sequencing. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as the internal control gene, and was amplified for 24 cycles using primers validated for bovine follicles (forward 5' tgt tcc agt atg att cca cc 3', reverse 5'tcc acc acc ctg ttg ctg 3'; Buratini *et al.*, 2005) and remaining conditions as described above except for the annealing temperature that was 60°C.

Semiquantitative RT-PCR was validated by choosing the number of PCR cycles and amount of RNA within the linear range of the amplification curve. PCR products were separated on 1.5% agarose gels and stained with ethidium bromide, and specific bands quantified by densitometry (Image Gauge, Fuji Photo Film Co.). All PCR reactions were performed with fetal brain as positive control and water replacing cDNA as negative control.

Steroid assays

Estradiol and progesterone were assayed in follicular fluids using iodinated tracers and the antibodies furnished in the Third Generation Estradiol RIA and Progesterone RIA kit (Diagnostic Systems Laboratories Inc.), respectively, with a revised protocol (Buratini *et al.*, 2005). The standard curves and samples were diluted in PBS-gelatin. The intraassay and

interassay coefficients of variation were 7.4% and 13.5%, respectively, for estradiol, and 6.8% and 7.0%, respectively, for progesterone. The sensitivities of the assays were 0.3 ng/ml for estradiol and 0.2 ng/ml for progesterone. Estradiol was measured in culture medium with the RIA as described but without solvent extraction (Bélanger *et al.*, 1990), and progesterone was measured as described (Lafrance and Goff, 1985). All samples were run in a single assay for each steroid, with intra-assay coefficients of variation of 8.5% and 7.2% for estradiol and progesterone, respectively. The sensitivities of these assays were 10 pg and 4 pg per tube for estradiol and progesterone, which are equivalent to 0.3 and 20 ng/lg protein, respectively.

Statistical analyses

Target gene mRNA abundance is expressed relative to the level of *Gapdh* mRNA. The data were transformed to logarithms if not normally distributed. ANOVA was used to test for the main effects of follicle size group, and estradiol concentration group in granulosa and theca cells, and to test the effect of CL developmental stage on *Fgf13* mRNA abundance. Means comparisons were performed with the Tukey-Kramer HSD test. Data are presented as means ± SEM. Analyses were performed with the JMP software (SAS Institute, Cary, NC).

Results

Fgf13 mRNA was widely detected in bovine fetal and adult tissues including the testis and the ovary (Fig. 1 and Fig. 2). The only tissue that failed to express *Fgf13* at 31 PCR cycles was the adult liver.

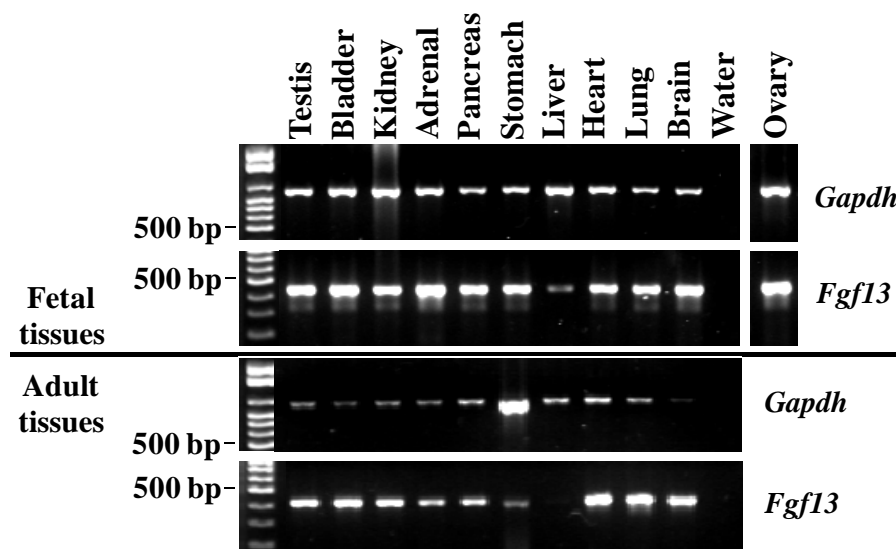


Figure 1. Expression of *Fgf13* gene in a variety of fetal and adult bovine tissues. The tissues were obtained from an abattoir and mRNA was subjected to RT-PCR for 31 cycles. The housekeeping gene GAPDH was also amplified (24 cycles), to verify the integrity of the RNA, and water was used to replace the cDNA template as a negative control for PCR.

Fgf13 mRNA was detected in the CL, and in theca and granulosa cells from antral follicles, but not in pools of oocytes (Fig. 2). Thecal *Fgf13* mRNA abundance increased with follicle diameter (21 ± 5 , 41 ± 10 , 57 ± 10 relative units for 5-7, 8-10, and >10mm follicle size groups, respectively; $P < 0.05$), but did

not vary with intrafollicular estradiol concentrations (Fig. 3). *Fgf13* mRNA abundance did not vary in granulosa cells with either diameter or estradiol concentrations (Fig. 3). Expression of *Fgf13* mRNA was detected in all four stages of CL lifespan and did not differ between them (Fig. 4).

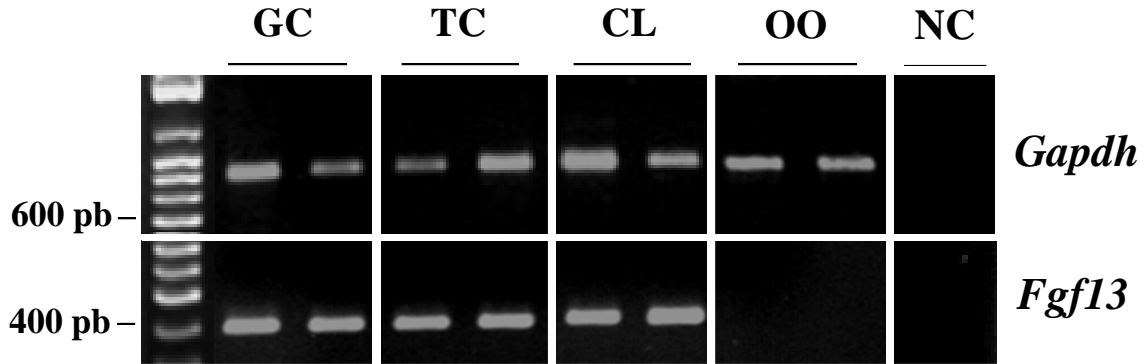


Figure 2. *Fgf13* mRNA expression in the bovine ovary. Representative gels show RT-PCR products for *Fgf13* and GAPDH mRNA in granulosa cells (GC), theca cells (TC), corpus luteum (CL) and pools of 50 oocytes (OO). GAPDH was amplified for 24 PCR cycles and *Fgf13* for 34 cycles (GC, TC and CL) or 40 cycles (OO). NC = negative control (water replacing cDNA).

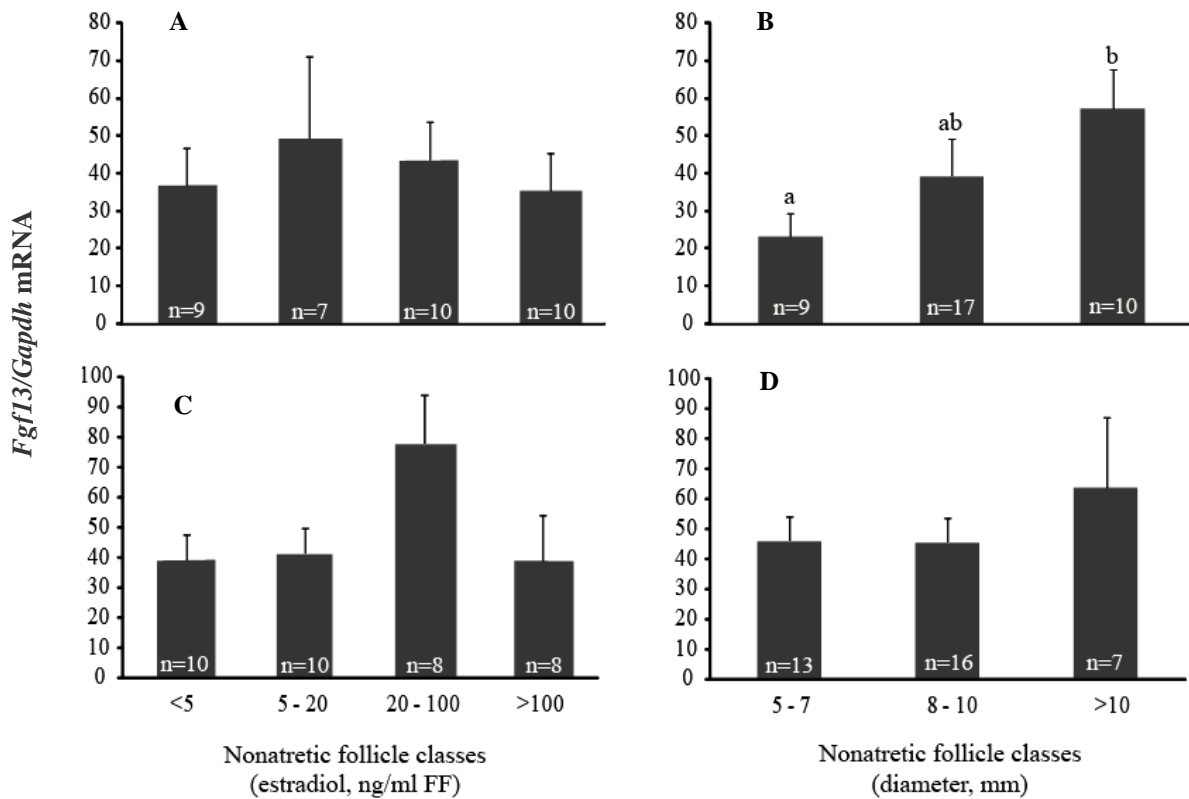


Figure 3. Semiquantitative analysis of *Fgf13* mRNA abundance in theca (A and B) and granulosa cells (C and D) from bovine antral follicles classed according with diameter and estradiol concentration in the follicular fluid (FF).

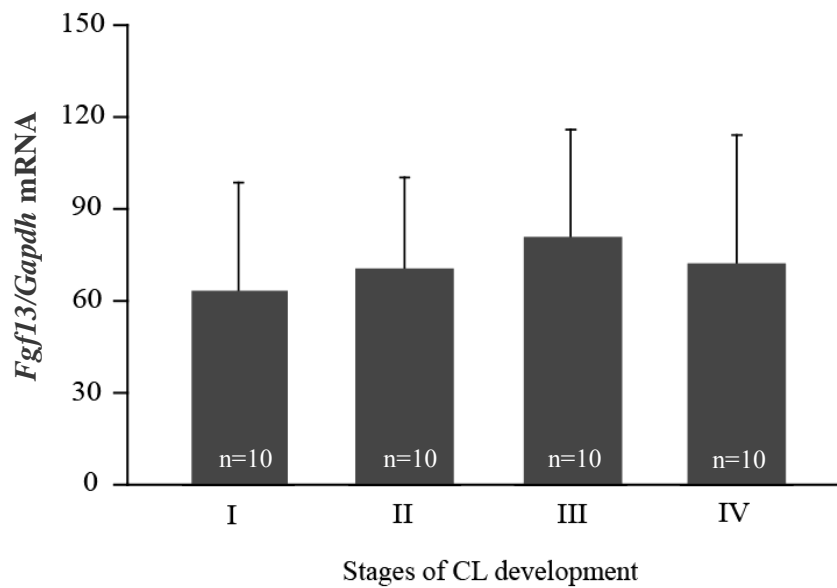


Figure 4. Semiquantitative analysis of *Fgf13* mRNA abundance in bovine CL at different stages of development.

Discussion

Several studies support the participation of fibroblast growth factors (FGFs) and their receptors (FGFRs) in the mechanisms controlling ovarian function in cattle (Liebermann *et al.*, 1996; Parrot and Skinner, 1998; Buratini *et al.*, 2005, 2007b; Castilho *et al.*, 2008; Guerra *et al.*, 2008; Robinson *et al.*, 2008). A subfamily of FGFs joins the intracellular FGFs (iFGF), which are non-secreted proteins and thus expected to play different roles in relation to FGFs at the intracellular level (Smallwood *et al.*, 1996; Powers *et al.*, 2000; Itoh and Ornitz, 2008). The present study reports for the first time the expression of *Fgf13*, a gene that encodes an iFGF, in antral follicles and CL.

To our knowledge, this is also the first report of *Fgf13* mRNA expression in fetal and adult tissues other than the developing gonads and the nervous system (Goldfarb, 2005; Cory *et al.*, 2007). *Fgf13* expression was previously detected in the mesonephros of both sexes in mice (Cory *et al.*, 2007). This, together with the detection of *Fgf13* mRNA in the fetal and adult gonads (present study), suggests that *Fgf13* may be a regulator of gonad development as well as of mature gonad function.

Fgf13 mRNA expression was assessed in non-atretic antral follicles and detected in granulosa cells with no changes with either follicle diameter or intrafollicular estradiol concentration, which suggests stable expression in granulosa cells before follicles undergo atresia. Differently, thecal *Fgf13* mRNA expression increased with follicle diameter which suggests a possible involvement in the control of theca cell differentiation during antral follicle growth. Since iFGFs can bind to MAPK scaffolding proteins (Goldfarb, 2005), and secreted FGFs bind to tyrosine

kinase receptors activating MAPK pathways (Creuzet *et al.*, 1995), iFGFs could modulate at the intracellular level actions of secreted FGFs. As *Fgfr2c* and *Fgfr3c* expression have been localized to bovine theca cells (Berisha *et al.*, 2004; Buratini *et al.*, 2005), their signaling pathways are candidates for modulation by *Fgf13*. FGFR4 signaling also seems to occur in bovine theca cells, but is unlikely to be specifically modulated by *Fgf13* as expression patterns do not match; *Fgfr4* expression was higher in small antral follicles (Buratini *et al.*, 2005), whereas *Fgf13* expression was higher in large antral follicles (present study).

In the CL, *Fgf13* mRNA abundance did not vary across developmental stages, indicating steady gene expression throughout the CL lifespan. As CL stages 3 and 4 contain samples undergoing functional and structural luteolysis, respectively, the luteal expression pattern suggests that *Fgf13* might be involved in the mechanisms controlling luteal growth and also luteolysis. Luteal regression requires regulation of apoptosis (Taniguchi *et al.*, 2002) and tissue remodeling (Casey *et al.*, 2005), and the participation of FGF10/FGFR2B signaling in these mechanisms has been suggested (Castilho *et al.*, 2007). As iFGFs bind to MAPK scaffolding proteins (Goldfarb, 2005), *Fgf13* could modulate at the intracellular level the actions of FGF10 and other factors capable of activating MAPK pathways in the CL.

The spatial pattern of *Fgf13* mRNA expression described in the present study agrees with preliminary immunohistochemical studies in which *Fgf13* protein was localized to granulosa and theca cells in antral follicles, and to large and small luteal cells in the CL in cattle (Buratini *et al.*, 2007a). These preliminary data indicate that *Fgf13* is translated in all these ovarian cell types, reinforcing the hypothesis that *Fgf13* may be an



intracellular regulator of the ovarian activity.

The functions of *Fgf13* in the control of follicle and luteal development remain to be determined. iFGFs were shown to control voltage gated sodium channels (Goldfarb, 2005), but these ion channel types have not been reported in the ovary yet. Nevertheless, iFGFs were also shown to bind to MAPK scaffolding proteins (Goldfarb, 2005), through which they could modulate various intracellular pathways such as those activated by other FGFs (as discussed above) and epidermal growth factor (EGF; Creuzet *et al.*, 1995; Umata, 2004). In addition to ligands of tyrosine kinase receptors, FSH also activates MAPK through protein kinase A in granulosa cells (Hunzicker-Dunn and Maizels, 2006), which leads to the speculation that intracellular signaling triggered by FSH could also be modulated by iFGFs.

In conclusion, the present data demonstrate for the first time that *Fgf13* mRNA is expressed in bovine antral follicles and CL. In addition, the increase in *Fgf13* expression with follicle diameter in theca cells suggests that *Fgf13* transcription is developmentally regulated in bovine antral follicles. The present data suggest *Fgf13* as an intracrine regulator of folliculogenesis and luteal development and regression in cattle.

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