FGF10 inhibits dominant follicle growth and estradiol secretion *in vivo* in cattle

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Abstract

Fibroblast growth factors (FGFs) are involved in paracrine control of follicle development. It was previously demonstrated that FGF10 decreases estradiol (E₂) secretion in granulosa cell culture and that theca cell *FGF10* mRNA expression is decreased in healthy follicles from abattoir ovaries. The main objectives of this study were to evaluate *FGF10* and *FGFR2b* mRNA expression during follicular development *in vivo*, to evaluate the effect of FGF10 on follicle growth using *Bos taurus taurus* cows as a model, and to gain more insight into the mechanisms through which FGF10 inhibits steroidogenesis. Messenger RNA encoding both *FGF10* and *FGFR2b* (main FGF10 receptor) was significantly more expressed in subordinate follicles (SFs) than in dominant follicles (DFs). The intrafollicular injection of FGF10 into the largest growing follicle at 7–8 mm in diameter interrupted the DF growth in a dose-dependent manner (11 ± 0.4 , 8.3 ± 1 and 5.9 ± 0.3 mm for 0, 0.1, and 1 µg/ml FGF10, respectively, at 72 h after treatment; $P < 0.05$). In a third experiment, follicles were obtained 24 h after FGF10 (1 µg/ml) or PBS treatment through ovariectomy. In theca cells, FGF10 treatment did not affect mRNA encoding steroidogenic enzymes, *LHCGR* and *IGFBPs*, but significantly upregulated *FGF10* mRNA expression. The expression of *CYP19A1* mRNA in granulosa cells was downregulated by FGF10 treatment, which was accompanied by a 50-fold decrease in E₂ production, and decreased cyclin D2 mRNA. These results have shown that *FGF10* and its receptor *FGFR2b* are more expressed in SFs and provide solid *in vivo* evidence that FGF10 acts as an important regulator of follicular growth in cattle.

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Introduction

Follicular development is mainly orchestrated by gonadotropins (FSH and LH), their receptors, and ovarian steroids. During the bovine estrous cycle, an initial rise in FSH stimulates the growth of a cohort of small antral follicles (Adams *et al.* 1992, Ginther *et al.* 1996). From this group of growing follicles, only one is selected (dominant follicle (DF)) for continued growth even during the nadir of FSH secretion, while all other follicles (subordinates) enter atresia in an event known as follicle deviation (Ginther *et al.* 1996). As all follicles are under the same endocrine environment, the process of follicle development and atresia involves many locally differentially produced factors (Fortune *et al.* 2004). There is a complex autocrine and paracrine system that is not well understood, the insulin-like growth factor (IGF) system being the most characterized during follicle development (Stewart *et al.* 1996, Ginther *et al.* 2004, Sudo *et al.* 2007).

Several fibroblast growth factors (FGFs) and their receptors (FGFRs) have been detected in ovarian follicles, suggesting roles in the paracrine control of follicle development (Parrott & Skinner 1998, Buratini *et al.* 2007, Portela *et al.* 2010). The pattern of *FGF2* expression suggests that this factor is involved in vascular proliferation during bovine DF growth (Berisha *et al.* 2000). Moreover, some FGFs are involved in steroidogenesis control. Treatment with FGF7 reduced basal and FSH-stimulated levels of granulosa cell CYP19A1 activity *in vitro* (Parrott & Skinner 1998). A similar antisteroidogenic effect was attributed to FGF17, which increases significantly in granulosa and theca cells from atretic follicles (Machado *et al.* 2009) and is negatively regulated by FSH and IGF1. Recently, Portela *et al.* (2010) demonstrated that FGF18 from theca cells is involved in follicle atresia.

FGF10 was first described in rat embryos and lungs from adult rats (Yamasaki *et al.* 1996). Mice lacking the *Fgf10* gene showed total absence of lung formation,

indicating that FGF10 is crucial during organogenesis (Min *et al.* 1998). In the ovary, the expression of *FGF10* was detected in human theca and stromal cells (Taniguchi *et al.* 2008) and in bovine oocytes, theca cells from antral follicles, and luteal cells (Buratini *et al.* 2007, Castilho *et al.* 2008). In bovine theca cells, *FGF10* mRNA abundance decreases in healthy follicles (Buratini *et al.* 2007), and recombinant FGF10 inhibits estradiol (E_2) secretion from cultured bovine granulosa cells (Buratini *et al.* 2007, Portela *et al.* 2008). The main FGF10 receptor (FGFR2b) is expressed in bovine cumulus cells (Cho *et al.* 2008), oocytes (Zhang *et al.* 2010), granulosa cells, and theca interna cells (Berisha *et al.* 2004). Its expression in granulosa cells is positively and negatively regulated by FSH and IGF1 respectively (Buratini *et al.* 2007). Collectively, these data suggest a role for FGF10 in the regulation of antral folliculogenesis in cattle.

The fact that some FGFs regulate steroidogenesis has long been recognized (Baird & Hsueh 1986). However, the participation of more recently described FGFs in ovarian reproductive events needs further clarification. The addition of FGF10 to bovine granulosa cell culture decreases E_2 production in a dose-dependent manner (Buratini *et al.* 2007, Portela *et al.* 2008). Nevertheless, the mechanism through which FGF10 decreases E_2 secretion was not assessed in the culture systems. The aforementioned studies were predominantly *in vitro*; therefore, *in vivo* functional studies are necessary to establish the physiological role of FGFs in follicle development. The main objectives of this study were to compare *FGF10* and *FGFR2b* mRNA expression in the two largest follicles (LFs) before and after follicular deviation during the first follicle wave in *Bos taurus* cows, and to test the effect of an intrafollicular injection of FGF10 in the DF on follicular growth, steroidogenesis, and cell differentiation *in vivo*.

Results

FGF10 and FGFR2b mRNA expression near follicle deviation

Abundance of mRNA encoding *FGF10* and its receptor, *FGFR2b*, was measured in the LF and second largest follicle (SLF) of cows before (LF <8.5 mm) and after (LF >8.5 mm) deviation during the first follicle wave. A total of 24 follicles were collected from 12 cows (out of 18 synchronized cows). The LF and SLF before expected time of deviation were 7.4 ± 0.2 and 6.6 ± 0.3 mm diameter respectively ($P < 0.05$) and those after deviation were 10.1 ± 0.7 and 6.8 ± 0.5 ($P < 0.05$; Fig. 1A). Follicles obtained before deviation were classified as LF or SLF and those obtained after deviation were classified as DF or subordinate follicle (SF) based on follicular diameter. E_2 levels were significantly higher ($P < 0.05$) in the LF before (190.1 ± 54.9 vs

80.3 ± 48.4 ng/ml for LF and SLF respectively) and after deviation (249.4 ± 39.7 vs 3.98 ± 3.1 ng/ml for DF and SF, respectively; Fig. 1B).

For the gene expression analyses in theca cells, samples from four cows were discarded due to contamination by granulosa cells in one of the samples from the pair, leaving four pairs of follicles collected before and four pairs collected after deviation. *FGF10* mRNA abundance did not differ between LF and SLF before deviation, but was more abundant in theca cells from SF compared with DF after deviation ($P < 0.01$; Fig. 1C).

From a total of 24 granulosa samples obtained from 12 pairs of follicles, four samples were positive for *CYP17A1* mRNA expression, indicating the presence of theca cells in the granulosa sample. After removing these four cows from the analysis, cells from three pairs of follicles collected before and five pairs collected after deviation were used to evaluate *FGFR2b* mRNA expression. The expression of *FGFR2b* was significantly higher in granulosa cells from SF compared with DF after deviation ($P < 0.01$; Fig. 1D). The abundance of mRNA encoding *FGF10* in theca cells and *FGFR2b* in granulosa cells was negatively correlated with E_2 concentration in follicular fluid ($P < 0.01$; Fig. 1E and F respectively).

Follicular growth after intrafollicular injection of FGF10

To test the hypothesis that FGF10 acts as an inhibitor of follicular growth, follicles were injected with PBS (control group) or FGF10 at a final intrafollicular concentration of 0.1 or 1 μ g/ml, and follicular growth was monitored daily. The intrafollicular injection of FGF10 interrupted DF growth compared with control group and a dose-response effect was observed (Fig. 2). All follicles from control group continued to grow and spontaneously ovulated 96–120 h after PBS intrafollicular injection, validating the follicular wave synchronization and intrafollicular injection protocols. When treated with 0.1 μ g/ml FGF10, two follicles regressed while the other two follicles ovulated 96–120 h after FGF10 treatment. All follicles that received an intrafollicular injection of FGF10 in a final concentration of 1 μ g/ml regressed 24 h after injection (decreased in comparison with diameter at treatment) and were significantly smaller than control follicles from 48 to 72 h after intrafollicular injection and failed to ovulate.

Changes in follicular environment after *in vivo* FGF10 treatment

To gain insight into the mechanisms through which FGF10 prevents follicular growth, we assessed the changes in mRNA expression of key genes known to be involved in steroidogenesis and follicular cell differentiation. Growth of follicles treated with FGF10

Figure 1 Regulation of *FGF10* and *FGFR2b* mRNA abundance in first follicular wave. After estrus detection, follicular dynamics, and ovariectomy, the two largest follicles from 12 cows were collected before or after the expected time of follicular deviation. Panels A and B show follicular diameter and estradiol levels, respectively, from 12 pairs of follicles collected before (largest follicle (LF) and second largest follicle (SLF); $n=6$ pairs) or after deviation (dominant follicle (DF) and subordinate follicle (SF); $n=6$ pairs). Cross-contamination of theca and granulosa cells was assessed, and when one of the granulosa or theca samples in a pair of follicles was contaminated, the pair was removed from the analysis. In panel C, four pairs of follicles collected before and four collected after deviation were used to assess *FGF10* mRNA expression in theca cells. Panel D shows *FGFR2b* mRNA expression in granulosa cells from three pairs of follicles collected before and five collected after deviation. Correlations between follicular fluid estradiol levels and theca cell *FGF10* mRNA (including the 16 follicles used in panel C) and granulosa cell *FGFR2b* mRNA (including the 16 follicles used in panel D) are shown in panels E and F respectively. Asterisks indicate significant differences between pairs of follicles accessed by paired Student's *t*-test using cow as subject ($P < 0.05$).

(in a final intrafollicular concentration of $1 \mu\text{g/ml}$) was blocked at 24 h post-injection (-0.2 ± 0.1 vs $1.1 \pm 0.4 \text{ mm/24 h}$ in FGF10 and control groups, respectively; Fig. 3). Follicular fluid E_2 concentrations were lower in FGF10-treated follicles (5.6 ± 3.8 vs $264.9 \pm 115.5 \text{ ng/ml}$ E_2 in FGF10 and PBS groups, respectively; $P < 0.01$; Fig. 3). Treatment with FGF10 significantly decreased abundance of mRNA encoding *CYP19A1* and *cyclin D2* in granulosa cells ($P < 0.05$; Table 1), while *FGFR2b* mRNA tended to be upregulated after FGF10 treatment. Expression of *STAR*, *HSD17B1*, *HSD3B1*, *FSHR*, *LHCGR*, *IGFBP2* and 5, and X-linked inhibitor of apoptosis protein (*XIAP*) was not affected by FGF10 ($P > 0.05$; Table 1).

In theca cells, there was no difference between control and FGF10 treated follicles in mRNA encoding steroidogenic enzymes (*STAR*, *CYP11A1*, *CYP17A1*, and *HSD3B1*), *LHCGR*, *cyclin D2*, and *IGFBP2* and 3 ($P > 0.05$; Table 2). However, theca cell *FGF10* mRNA expression was upregulated after FGF10 treatment ($P < 0.05$).

Discussion

The involvement of theca cell-derived factors in the regulation of follicle growth and steroidogenesis is still poorly understood. Our significant findings are 1) both *FGF10* and *FGFR2b* are upregulated in the SF; 2) a single intrafollicular injection of FGF10 interrupts DF growth in a dose-dependent manner, and 3) FGF10 negatively regulates granulosa *CYP19A1* and *cyclin D2* mRNA expression and decreases E_2 concentration in follicular fluid.

In bovine granulosa cells obtained from abattoir ovaries, Berisha *et al.* (2004) observed that *FGFR2b* mRNA expression was positively associated with follicular fluid E_2 level. However, in our *in vivo* model, the abundance of *FGFR2b* mRNA was consistently higher in the smaller follicles in all the pairs of samples. These discrepant results may be related to follicular health and differentiation status, as Berisha *et al.* (2004) did not include atretic follicles (as assessed by progesterone follicular fluid levels) in their study. In our follicular deviation model, we accessed the difference between

Figure 2 *In vivo* effect of FGF10 treatment on bovine follicular growth. A new follicular wave was induced, and when the largest follicle reached a diameter between 7 and 8 mm, PBS (control; $n=4$) or FGF10 in doses of 0.1 ($n=4$) or 1 µg/ml ($n=3$) was intrafollicularly injected in a single follicle per cow. Main effects of treatment group, day, and their interaction were determined using the MIXED procedure with a repeated measure statement. Differences between follicular sizes at a specific time point were compared between groups using estimates and different letters indicate statistical significance ($P<0.05$).

DF and non DF (healthy vs atretic follicles) near follicular deviation. Additionally, it was previously demonstrated that IGF1 negatively regulates *FGFR2b* mRNA expression in granulosa cells (Buratini *et al.* 2007). It is well established that an important difference between DF and SF is the intrafollicular level of free IGF1 (Ginther *et al.* 2004, Sudo *et al.* 2007). Thus, the increased free IGF1 levels may be involved in the downregulation of *FGFR2b* mRNA in DFs granulosa cells.

FGF7 (also known as KGF) is structurally similar to FGF10 and also binds to *FGFR2b*. When added to bovine granulosa cell culture, FGF7 and FGF10 have negative effects on *CYP19A1* activity (Parrott & Skinner 1998) and E_2 synthesis (Buratini *et al.* 2007) respectively. *FGF10* mRNA is not readily detectable (after 30 PCR cycles) in granulosa cells (Buratini *et al.* 2007), indicating that FGF10 synthesized in theca cells and/or oocyte is regulating granulosa cell functions during follicle deviation.

Recently, it has been shown that intrafollicular injection of cocaine- and amphetamine-regulated transcript, a granulosa cell-derived factor, significantly reduced E_2 synthesis in bovine preovulatory follicles (Lv *et al.* 2009). In this study, we addressed the function of a theca cell and oocyte-derived factor during antral follicle growth. All the follicles that received an intrafollicular injection of FGF10 at a final intrafollicular concentration of 1 µg/ml ceased growing 24 h after injection and 'lost' the dominant status. This is unlikely to be a cytotoxic effect, as abundance of mRNA encoding a number of genes was not affected in either granulosa or theca cells by this treatment. Similar

concentrations of FGF10 have been previously used *in vitro* without affecting cell viability (Steinberg *et al.* 2005, Benjamin *et al.* 2007, Buratini *et al.* 2007). The *in vivo* model was suitable to study the role of FGF10 in follicular growth as follicles injected with PBS maintained their growth, reached ovulatory size, and spontaneously ovulated.

To investigate the action of FGF10 on follicular cell mRNA expression, we chose the dose of 1 µg/ml, as it was effective in suppressing follicular growth. The ovariectomy was performed 24 h after intrafollicular injection to avoid the collection of follicles in advanced atresia. In granulosa cells, *CYP19A1* mRNA expression was downregulated by FGF10 treatment, which was accompanied by a decrease in E_2 production. These results are in agreement with the previous reports of decreased E_2 production in cultured granulosa cells after FGF10 treatment (Buratini *et al.* 2007, Portela *et al.* 2008). The intracellular pathway responsible for the negative effect of FGF10 on *CYP19A1* expression and E_2 synthesis remains unknown.

The lack of significant effect on the expression of gonadotropin receptors indicates that FGF10 function is downstream of FSH and LH signaling and is not related to granulosa cell differentiation. Markers of follicular atresia such as *IGFBP2* and 5 (Stewart *et al.* 1996) and the suppressor of apoptosis *XIAP*, an important survival factor in the control of follicular atresia (Wang *et al.* 2003), were not affected by FGF10. This is interesting and implies a specific role for FGF10 in inhibiting E_2 secretion. Recently, Portela *et al.* (2010) demonstrated that FGF18 from theca cells is involved in follicle atresia. Nevertheless, FGF18 seems to have a broad range of antisteroidogenic effects on granulosa cells, as it down-regulates *CYP19A1*, *HSD3B1*, *FSHR*, *STAR*, and *HSD17B1* *in vitro* (Portela *et al.* 2010). In this study, progesterone concentration in follicular fluid was not assessed, but we did not identify significant effects of FGF10 on *HSD3B1* or *CYP11A1* mRNA in either

Figure 3 Follicular growth (mm/24 h) and follicular fluid estradiol levels after FGF10 treatment. A new follicular wave was induced and when the largest follicle reached a diameter between 7 and 8 mm, PBS (control; $n=4$) or FGF10 (1 µg/ml; $n=4$) was intrafollicularly injected in a single follicle per cow. Cows were ovariectomized 24 h after intrafollicular injection. Different letters indicate statistical significance.

Table 1 Effect of fibroblast growth factor 10 (FGF10) treatment on granulosa cell mRNA expression.

Gene	PBS	FGF10	Fold change in FGF10 group	P value
<i>HSD17B1</i>	0.42±0.07	0.34±0.33	0.8	0.81
<i>STAR</i>	0.04±0.01	0.21±0.13	4.86	0.46
<i>HSD3B1</i>	0.58±0.22	0.49±0.3	0.84	0.81
<i>CYP19A1</i>	79.08±50.08	1.18±0.71	0.01	0.02
<i>FGFR2b</i>	6.48±3.07	33.78±19.89	5.21	0.07
Cyclin D2	14.56±0.97	3.13±2.17	0.21	0.01
<i>FSHR</i>	0.42±0.11	0.45±0.28	1.08	0.91
<i>LHCGR</i>	5.18±4.8	0.3±0.06	0.06	0.41
<i>IGFBP2</i>	25.72±22.15	29.1±2.65	1.13	0.88
<i>IGFBP5</i>	1.18±0.65	2.87±1.78	2.43	0.36
<i>XIAP</i>	7.76±4.13	29.77±15.35	3.84	0.23

A single 7–8 mm follicle was injected with PBS ($n=4$) or FGF10 (1 µg/ml; $n=4$) and the cows were ovariectomized 24 h after intrafollicular injection. Data are presented as the average gene expression (arbitrary units) ± S.E.M. in PBS- and FGF10-treated follicles. Fold change was calculated by dividing average relative gene expression of both groups by expression in PBS (control) group.

granulosa or theca cells. The different actions of FGF10 and FGF18 are likely related to the activation of different receptors; while FGF10 preferably activates FGFR1b and FGFR2b, FGF18 activates FGFR3c and FGFR4 (Zhang *et al.* 2006).

The intrafollicular FGF10 treatment also decreased cyclin D2 mRNA abundance in granulosa cells. Cyclin D2 regulates granulosa cell proliferation (Robker & Richards 1998) by controlling G1 to S transition and is regulated in part by E_2 (Quirk *et al.* 2006). Thus, suppression of follicle growth by FGF10 may be a result of its effect on *CYP19A1* and E_2 synthesis and cell proliferation. In the absence of E_2 stimulus to cell cycle progression, granulosa cells become very susceptible to apoptosis (Quirk *et al.* 2006) and follicles enter atresia. Nevertheless, FGF10 *in vitro* did not affect cell proliferation (Buratini *et al.* 2007) in contrast to the downregulation of cyclin D2 expression promoted by intrafollicular injection of FGF10.

Theca cells are not the only source of FGF10 (Buratini *et al.* 2007). We cannot rule out that oocyte-derived FGF10 is also involved in regulation of follicle growth. Also, cumulus cells and oocytes express *FGFR1b* and *2b* (Cho *et al.* 2008, Zhang *et al.* 2010). Thus, FGF10 intrafollicular injection may interfere with oocyte–granulosa cell interactions, culminating in follicle atresia. However, the pattern of *FGF10* and *FGFR* mRNA expression in oocytes during follicle growth is unknown, and it is very difficult to get enough samples to assess *in vivo* FGF10 effects in cumulus–oocyte complexes.

In theca cells, there was no difference between control and FGF10-treated follicles in mRNA encoding steroidogenic enzymes (*CYP17A1*, *CYP11A1*, *STAR*, and *HSD3B1*), cyclin D2, *LHCGR*, and *IGFBP2* and 3, binding proteins known as negative modulators of

IGF1 actions in theca cell proliferation and androstenedione and progesterone production (Spicer *et al.* 1997). These results suggest that theca cells from FGF10-treated follicles were still producing precursors for E_2 synthesis and further indicate that granulosa cell *CYP19A1* is the main target of FGF10. Other evidence that theca cell factors regulate follicle development is the fact that intrafollicular treatment with FGF10 significantly increased theca cell *FGF10* mRNA expression, suggesting an interesting autocrine-positive feedback loop. The fact that granulosa cell *FGFR2b* mRNA expression tended to be upregulated after FGF10 treatment is consistent with this hypothesis. We also evaluated *FGFR2b* mRNA expression in theca cells in control and treated follicles. However, this receptor is weakly expressed in theca cell (data not shown).

In conclusion, results presented in this manuscript provide the first *in vivo* evidence that FGF10 acts as an important regulator of follicular growth, being differentially expressed in DF and SF from cows. Moreover, FGF10 treatment selectively downregulates *CYP19A1* and E_2 synthesis, indicating that FGF10 inhibits SF development. Taken together, the present results suggest that reduced FGF10 signaling in DFs accounts for continued follicle growth, whereas enhanced FGF10 signaling in SF favors atresia through the inhibition of E_2 production and cyclin D2 expression.

Materials and Methods

FGF10 and FGFR2b mRNA expression near follicle deviation

All experimental procedures using cattle were reviewed and approved by the Federal University of Santa Maria Animal Care and Use Committee (ACUC no. 23081.009594/2007-41). Eighteen cyclic adult (5–9 years old) beef cows (Hereford and Aberdeen-Angus), with an average body condition score of 3

Table 2 Effect of fibroblast growth factor 10 (FGF10) treatment on theca cells mRNA expression.

Gene	PBS	FGF10	Fold change in FGF10 group	P' value
<i>STAR</i>	0.26±0.25	0.05±0.03	0.2	0.98
<i>CYP11A1</i>	1.96±1.75	0.94±0.52	0.48	0.82
<i>CYP17A1</i>	820.3±731.32	58.06±25.67	0.07	0.22
<i>HSD3B1</i>	1.94±0.98	2.73±1.86	1.41	0.89
<i>IGFBP2</i>	1.63±0.44	1.63±0.26	1.0	0.99
<i>IGFBP3</i>	0.5±0.13	0.46±0.11	0.91	0.8
<i>FGF10</i>	1.24±0.47	10.36±6.12	8.34	0.04
<i>LHCGR</i>	0.9±0.38	4.14±3.8	4.62	0.7
Cyclin D2	1.41±0.53	1.69±0.45	1.2	0.7

A single 7–8 mm follicle was injected with PBS ($n=4$) or FGF10 (1 µg/ml; $n=4$) and the cows were ovariectomized 24 h after intrafollicular injection. Data are presented as the average gene expression (arbitrary units) ± S.E.M. in PBS- and FGF10-treated follicles. Fold change was calculated by dividing average relative gene expression of both groups by expression in PBS (control) group.

(1–5, emaciated to obese), were synchronized with two i.m. injections of 125 µg sodium cloprostenol (PGF2 α analogue; Schering-Plough, Cotia, SP, Brazil), 12 h apart. Twelve cows were observed in estrus within 3–5 days after PGF2 α and were used in the experiment. Ovaries were then examined once a day by transrectal ultrasonography, using an 8 MHz linear-array transducer (Pie Medical Aquila Vet, Maastricht, The Netherlands), and all follicles larger than 5 mm were drawn using three virtual slices of the ovary allowing a three-dimensional localization and monitoring of individual follicles during the first follicular wave after ovulation. The cows were ovariectomized at days 2, 3, or 4 of the follicular wave to recover the two LFs from each cow. Follicle deviation occurs when the LF reaches 8.5 mm (Ginther *et al.* 1996, 2000, Ferreira *et al.* 2011a, 2011b); therefore, follicle waves were classed as before or after deviation when the LF was smaller or larger than 8.5 mm, respectively.

The ovariectomy was performed via colpotomy in the standing position (Drost *et al.* 1992) under caudal epidural anesthesia using 80 mg lidocaine chlorhydrate (4 ml lidocaine 2%). Ovaries were washed with saline and follicular fluid samples were recovered for E₂ assay. Granulosa cells were harvested from follicles through repeated flushing with PBS and theca cells were obtained through dissection of the follicle wall. Samples were frozen until RNA extraction for PCR analysis.

Intrafollicular injection

This experiment was performed to assess the effects of FGF10 on growth and ovulation of the follicular-phase follicle, using an intrafollicular injection method validated in our laboratory (Ferreira *et al.* 2007, 2011b). Follicle waves of *Bos taurus taurus* cyclic adult (5–9 years old) beef cows (Hereford and Aberdeen-Angus), with an average body condition score of 3, were synchronized by the placement of a progesterone-releasing intravaginal device (1 g progesterone, DIB; Intervet Schering Plough), an i.m. injection of 2 mg E₂ benzoate (Genix, Anápolis, Brazil) to induce follicular regression and a new follicular wave. Two i.m. injections of 250 µg sodium cloprostenol (12 h apart; Intervet Schering Plough) were also administered. Four days later, the progesterone devices were removed and ovaries were monitored daily for at least 3 days before treatment to ensure that new follicles were growing and that no aged follicles were present in the ovaries. Only cows without a corpus luteum in the ultrasound image were included in the study to avoid progesterone inhibitory effect during final follicular growth and ovulation. When the LF of the growing cohort reaches a diameter between 7 and 8 mm, it is reliably identifiable as the future DF (Ferreira *et al.* 2011a, 2011b) and was injected with PBS (control group; $n=4$) or human recombinant FGF10 (Peprotech, Rocky Hill, NJ, USA) diluted in PBS. A total of 11 out of 18 cows responded to the

Table 3 Primers used in the expression analysis of *Bos taurus taurus* genes.

Gene	Sequence	Conc. (nM)	Reference/accession no.
<i>GAPDH</i>	F: GATTGTCAGCAATGCCTCCT R: GGTCATAAGTCCCTCCACGA	200 200	NM_001034034.1
<i>CYP19A1</i>	F: GTGTCCGAAGTTGTGCCTATT R: GGAACCTGCAAGTGGGAAATGA	300 300	Luo & Wiltbank (2006)
<i>CYP17A1</i>	F: CCATCAGAGAAGTGCTCCGAAT R: GCCAATGCTGGAGTCAATGA	200 200	Lagaly <i>et al.</i> (2008)
<i>LHCGR</i>	F: GCACAGCAAGGAGACCAATAA R: TTGGGTAAGCAGAAACCATAGTCA	200 200	NM_174381.1
<i>HSD17B1</i>	F: TGTGGTACTCATTACCGCTGTT R: CAGCGTGGCATACACTTTGAA	200 200	NM_001102365.1
<i>HSD3B1</i>	F: GCCCAACTCCTACAGGGAGAT R: TTCAGAGCCCACTTAGCT	200 200	Orisaka <i>et al.</i> (2006)
<i>CYP11A1</i>	F: CTTGCACCTTTCTGGCTAGG R: AAGGGGAAGAGGTAGGGTGA	200 200	Orisaka <i>et al.</i> (2006)
<i>STAR</i>	F: CCCAGCAGAAGGGTGTCTATC R: TGCGAGAGGACCTGGTTGAT	200 200	Buratini <i>et al.</i> (2005)
<i>FSHR</i>	F: AGCCCTTGTCACAACTCTATGTC R: GTTCCTCACCCTGAGGTAGATGT	200 200	Luo & Wiltbank (2006)
<i>XIAP</i>	F: GAAGCACGGATCATTACATTTGG R: CCTTCACCTAAAGCATAAAATCCAG	200 200	Boelhaue <i>et al.</i> (2005)
<i>Cyclin D2</i>	F: TGCCCCAGTGCTCCTACTTC R: CGGGTACATGGCAAACCTTGA	200 200	Mihm <i>et al.</i> (2008)
<i>IGFBP2</i>	F: GACGGGAACGTGAACCTGATG R: TCCTTCATGCCGGACTTGA	200 200	Voge <i>et al.</i> (2004)
<i>IGFBP3</i>	F: AAAGAGATGTTTGAAATGCCTAGTTT R: TCAAACCTCGGTTTCACTGACTACTG	200 200	Voge <i>et al.</i> (2004)
<i>IGFBP5</i>	F: GTTTGCTGAACGAAAAGAAGCTA R: CGAGTAGGTCTCCTCTGCCATCT	200 200	Voge <i>et al.</i> (2004)
<i>FGF10</i>	F: AAGGAGATGTCCGCTGGAGAAAGCTA R: ACTGTACGGGCAGTTCTCCTTCTT	300 300	NM_001206326.1
<i>FGFR2b</i>	F: TGTGGTTGGAGGTGATGT R: CGAGTGCTTCAGAACCTTG	300 300	Cho <i>et al.</i> (2008)

F, forward primer; R, reverse primer; Conc., primer concentration used for gene amplification.

synchronization protocol and were successfully injected. The injection volume was calculated based on the volume of follicular fluid estimated by the linear regression equation $V = -685.1 + 120.7D$, where V corresponds to the estimated follicular volume and D to the diameter of the follicle to be injected (Ferreira *et al.* 2007). The administered volume corresponded to approximately one-tenth of total follicular fluid volume and the concentration of FGF10 was tenfold higher than the desired final intrafollicular concentration, i.e., 0.1 µg/ml ($n=4$) or 1 µg/ml ($n=3$). The average follicular diameter ($\pm$ s.e.m.) and diameter range at treatment were 7.5 ± 0.1 , ranging from 7.1 to 8.1 mm in PBS group, 7.2 ± 0.1 , ranging from 7.0 to 7.4 mm in FGF10 0.1 µg/ml group, and 7.6 ± 0.1 , ranging from 7.1 to 7.9 mm in FGF10 1 µg/ml group. Two hours after the injections, follicles were evaluated to ensure that no follicle damage occurred during procedure (a reduction in diameter larger than 1 mm within 2 h of injection is evidence of follicle leakage). Animals were monitored daily by ultrasound to evaluate the effects on follicular dynamics and ovulation.

Based on follicular dynamics, the third experiment was performed with cows synchronized as described above. In this experiment, nine out of 14 cows responded to the protocol and were intrafollicularly injected and one cow was discarded due to follicular rupture after injection. The average of follicular diameter ($\pm$ s.e.m.) and diameter range at the moment of follicular injection were 7.8 ± 0.2 ranging from 7.1 to 8.2 mm in PBS group and 7.9 ± 0.2 ranging from 7.8 to 8.2 mm in FGF10 1 µg/ml group. PBS-treated ($n=4$) and FGF10 (1 µg/ml; $n=4$)-treated follicles were obtained through ovariectomy via colpotomy 24 h after intrafollicular injection.

RNA extraction, RT, and real-time PCR

All materials were obtained from Invitrogen Life Technologies unless otherwise stated. For theca cells, total RNA was extracted with Trizol reagent according to the manufacturer's instructions. Total RNA was extracted from granulosa cells using the RNeasy Mini Kit (Qiagen Biotechnology). Quantitation and estimation of RNA purity was performed using NanoDrop (Thermo Scientific, Waltham, MA, USA; Abs 260/280 nm ratio) spectrophotometer. Ratios above 1.8 were considered pure, and samples below this threshold were discarded. To generate the cDNA, 1 µg RNA was first treated with 0.1 U DNase and then incubated in a final volume of 20 µl with dNTP (0.5 mM final each; Omniscript kit, Qiagen), 1 µM oligo-dT, RNase out inhibitor (10 U; Invitrogen), Omniscript reverse transcriptase (RT; 4 U; Qiagen), and 1×buffer RT (2 µl; Qiagen). The reaction was performed in four steps: step 1: 37 °C – 5 min; step 2: 65 °C – 10 min (DNA digestion); step 3: 37 °C – 60 min; and step 4: 93 °C – 3 min (RT). The cDNA generated was stored at –20 °C.

To test cross-contamination, PCR detection of the mRNAs that encode *CYP19A1* in theca and *CYP17A1* in granulosa cells was performed in each sample. All granulosa cell samples expressing *CYP17A1* mRNA and theca cells expressing *CYP19A1* after 30 PCR cycles were considered to be contaminated as described previously by Buratini *et al.* (2005). In the first experiment, when one of the granulosa or theca samples in a pair of follicles was contaminated, the pair

was removed from the analysis; this approach of keeping the samples in pairs was adopted to allow including the 'cow' effect in the statistical model.

Real-time PCR was conducted in a Step One Plus instrument (Applied Biosystems, Foster City, CA, USA) with Platinum SYBR Green qPCR SuperMix and bovine-specific primers (Table 3) taken from the literature or designed using Primer Express Software (Applied Biosystems). The thermal cycling parameters were 3 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The product identity was verified through melting curve analyses. Samples were run in duplicate and were expressed relative to *GAPDH* as housekeeping gene. The relative quantification of gene expression across treatments was evaluated using the $\Delta\Delta C_T$ method (Livak & Schmittgen 2001). The fold change in relative mRNA concentrations was calculated using the formula $2^{-\Delta\Delta C_T}$.

E₂ assay

Follicular fluid samples from follicles were collected and individually stored at –196 °C. E₂ concentration was determined using the multispecies E₂ ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). The dilution of follicular fluid samples ranged between 1:10 and 1:1000. The intra- and inter-assay coefficients of variation were 9.3 and 12.9% respectively.

Statistical analysis

The regression analysis and correlation between follicular fluid E₂ levels and *FGF10* or *FGFR2b* mRNA expression were calculated using JMP Software. The assessment of treatment effects on follicular dynamics was performed as repeated measures data and analyzed using the MIXED procedure with a repeated measure statement and using the compound symmetry as covariance structure. Main effects of treatment group, day, and their interaction were determined. Differences between follicular sizes at a specific time point were compared between groups using estimates. All analyses from follicular dynamics were performed using SAS Software package (SAS Institute, Inc., Cary, NC, USA). Continuous data were tested for normal distribution using Shapiro–Wilk test and normalized when necessary. The differences between the two LFs were accessed by paired Student's *t*-test using cow as subject. Other continuous data were submitted to ANOVA using JMP Software (SAS Institute, Inc.). Results are presented as means $\pm$ s.e.m. A $P < 0.05$ was considered statistically significant.

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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