Regulation and action of fibroblast growth factor 17 in bovine follicles

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Abstract

Fibroblast growth factor 17 (FGF17) is a member of the FGF8 subfamily that appears to be relevant to folliculogenesis and oogenesis, as the prototype member FGF8 is an oocyte-derived protein that signals to cumulus cells. FGF8 has structural and receptor-binding similarities to FGF17, whose expression in the ovary has not been reported. In this study, we demonstrate localization of FGF17 protein to the oocyte of preantral follicles, and to the oocyte and granulosa cells of antral follicles. Real-time PCR demonstrated the presence of mRNA in oocytes and, to a lesser extent, in granulosa and theca cells. FGF17 mRNA abundance was low in granulosa and theca cells from healthy follicles and increased significantly in atretic follicles. Addition of FSH or IGF-I to granulosa cells in vitro decreased FGF17 mRNA abundance, and treatment with FGF17 inhibited estradiol and progesterone secretion from granulosa cells in relation to control cultures without these additives. We conclude that FGF17 is a potential mediator of granulosa cell differentiation.


Introduction

Follicle development is under the control of gonadotropins, steroids, and a variety of locally produced peptides including fibroblast growth factors (FGFs; Webb et al. 2003). Several FGFs and their receptors (FGFR) have been detected in ovarian follicles, suggesting roles in the regulation of folliculogenesis (van Wezel et al. 1995, Berisha et al. 2004, Buratini et al. 2005a,b, 2007). The 22 known FGFs have been grouped into seven subfamilies with distinct receptor-binding properties. The FGFR proteins are encoded by five different genes, three of which, FGFR1, 2, and 3, undergo alternative splicing to produce two functional variants (B and C; Ornitz et al. 1996, Itoh & Ornitz 2004). Paracrine roles within the follicle have been explored for FGF2 and for the FGF7 subfamily, containing also FGF10. FGF2, which is predominantly expressed by theca cells (Berisha et al. 2000), stimulates proliferation and inhibits steroidogenesis in both theca and granulosa cells (Lavranos et al. 1994, Vernon & Spicer 1994, Spicer & Stewart 1996, Nilsson et al. 2001). FGF7 and FGF10 are expressed in the theca cell layer but not in granulosa cells, and the receptor FGFR2B is predominantly expressed in granulosa cells (Parrott & Skinner 1998, Berisha et al. 2004, Buratini et al. 2007). Both FGFs inhibit estradiol (E2) secretion from cultured granulosa cells (Parrott & Skinner 1998, Buratini et al. 2007).

Another subfamily that may be of interest for potential paracrine signaling is the FGF8 subfamily. In adult rodents, Fg8 gene expression is largely confined to the oocyte (Valve et al. 1997), and was reported in oocytes as well as somatic follicle cells in cattle (Buratini et al. 2005b). FGF8 activates FGFR3C, which was found in theca and granulosa cells in cattle, and FGFR4, which was localized only to theca cells (Ornitz et al. 1996, Buratini et al. 2005b). Although the roles of the FGF8 subfamily in the control of the ovarian activity are still poorly understood, it has been recently shown that oocyte-derived FGF8 cooperates with bone morphogenetic protein 15 (BMP15) to promote glycolytic activity in cumulus cells in mice (Sugiura et al. 2007).

The FGF8 subfamily also contains FGF17 (Itoh & Ornitz 2004), which also preferentially activates FGFR3C and FGFR4 (Ford-Perriss et al. 2001, Zhang et al. 2006). FGF17 gene expression was first detected in the embryonic brain, and is most associated with neurogenesis (O’Leary et al. 2007) and skeletal development (Krejci et al. 2007). Very little is known about the pattern of expression of FGF17 in the reproductive system. mRNA encoding FGF17 was detected in human prostatic epithelial cells (Polnasek et al. 2004), human placenta, and in mouse oocytes and embryos (Zhong et al. 2006).

The objective of the present work was to test the hypothesis that FGF17 is a candidate for paracrine signaling within the follicle. Specifically, we sought to localize FGF17 mRNA in follicular cell types, to determine whether mRNA
expression is under the control of the major regulators of follicle development, FSH and insulin-like growth factor-I (IGF-I), and to gain insight into the potential role of FGF17 in the regulation of follicle steroidogenesis.

Materials and Methods

Tissues

Follicles of diameter ≥ 5 mm were dissected from the ovaries of adult cows (predominantly Nellore, *Bos indicus*) obtained in an abattoir local to the Sao Paulo State University campus in Botucatu and transported to the laboratory in saline on ice. Follicular fluid was aspirated, centrifuged, and frozen for future use. The antral cavity was flushed repeatedly with cold saline, and granulosa cells were recovered by centrifugation at 1200 g for 1 min and pooled with the follicular fluid pellet. The remaining granulosa cells adhering to the follicle wall were removed by gently scraping with a blunt Pasteur pipette, and the theca layer was removed with forceps and washed in saline by repeated passages through a 1 ml syringe. Samples were collected in Trizol (Invitrogen Life Technologies), homogenized with a Polytron, and submitted immediately to total RNA extraction according to the manufacturer’s protocol.

Follicles were classed according to E2:P4 ratios of >1, 1–0.01, and <0.01 (Ireland et al. 1994), which we defined as healthy, transitional, and highly atretic respectively (Grimes & Ireland 1986). Mean follicle fluid steroid concentrations and follicle diameters for each follicle class are given in Table 1. Cross-contamination of theca and granulosa cells was tested by the detection of mRNA encoding cytochromes P450 aromatase (*CYP19A1*) and 17α-hydroxylase (*CYP17A1*) in each sample by PCR as described (Buratini et al. 2005b). The detection of *CYP19A1* amplicons in theca samples or of *CYP17A1* amplicons in granulosa samples indicated cross-contamination, and such samples were discarded.

Cumulus–oocyte complexes (grades 1 and 2; Leibfried & First 1979) were aspirated from antral follicles (2–8 mm) collected at an abattoir, and oocytes were mechanically isolated by careful and repeated pipetting until no adhering cumulus cells could be observed under a stereomicroscope. Total RNA was extracted from pools of 20 oocytes with the RNeasy kit (Qiagen).

Cell culture

Granulosa cell culturing was performed as described (Gutiérrez et al. 1997) with modifications (Manuel Silva & Price 2000). All materials were obtained from Invitrogen, except where otherwise stated. Follicles with a ≤5 mm diameter were dissected from ovaries of *Bos taurus* cows obtained at an abattoir local to the University of Montreal in St Hyacinthe, and transported to the laboratory in PBS at 35 °C containing penicillin (100 IU/ml) and streptomycin (100 μg/ml). Follicles with obvious signs of atresia (vascular theca, debris in 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 each, and suspended in DMEM/F12 containing Hepes (20 mM), sodium selenite (4 ng/ml), BSA (0.1%; Sigma–Aldrich), penicillin (100 IU/ml), streptomycin (100 μg/ml), transferrin (2.5 μg/ml), non-essential amino acid mix (1·1 mM), androstenedione (10−7 M at the start of culture and 10−6 M at each medium change), and insulin (10 ng/ml). 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 105/well in 1 ml medium. Cultures were maintained at 37 °C in 5% CO2 in air for 6 days, with 700 μl medium being replaced every 2 days.

To determine the regulation of *FGF17* mRNA expression, cells were stimulated with graded doses of FSH (AFP-5332B, NIDDK, Bethesda, MD, USA; 0, 0·1, 1, 10, and 100 ng/ml) or IGF-I analog (LR3; Sigma–Aldrich; 0, 5, 10, 50, and 100 ng/ml) starting on day 2 of culture. At the end of the culture period, cells were collected in Trizol and stored at 70 °C until RNA extraction. Data were derived from three independent cultures performed at different times.

To determine the potential role of FGF17, granulosa cells were treated with FSH (10 ng/ml) to stimulate FGFR3: mRNA levels (Buratini et al. 2005b) and with graded doses of FGF17 (PeproTech, Rocky Hill, NJ, USA) starting on day 2 of culture. To measure steroid secretion, the medium was removed for steroid assay on day 6 and stored at −20 °C. The cells were lysed with 200 μl of 1 M NaOH for 2 h followed by neutralization with 200 μl of 1 M HCl for total cell protein measurement with the Bradford protein assay (Bio-Rad). These cultures were performed on three independent cultures performed at different times, and were performed several months after the FSH/IGF dose–response cultures.

Reverse transcription (RT)-PCR

Theca and granulosa cell RNA (1 μg) samples were incubated with DNase I (1 U/μg RNA; Invitrogen), and then reverse transcribed with SuperScript III (200 U/μl; Invitrogen) and oligo-d(T) primer. The RNA yield from pools of twenty oocytes was too low to be accurately quantified by

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**Table 1** Mean (± s.e.m.) follicular fluid estradiol (E2) and progesterone (P4) concentrations and follicle diameters of follicles classed as healthy, transitional, and atretic based on E2:P4 ratio

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Healthy (n=16)</th>
<th>Transitional (n=17)</th>
<th>Atretic (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol (ng/ml)</td>
<td>540±249*</td>
<td>16±3*</td>
<td>0-3±0-1*</td>
</tr>
<tr>
<td>Progesterone (ng/ml)</td>
<td>19±3*</td>
<td>44±7*</td>
<td>290±59*</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>9±1</td>
<td>9±1</td>
<td>8±1</td>
</tr>
<tr>
<td>Diameter range (mm)</td>
<td>5–13</td>
<td>6–11</td>
<td>5–11</td>
</tr>
</tbody>
</table>

Within rows, means with different superscripts are significantly different (P<0.05).
shown in Table 2. Reactions were optimized to provide fragment size, and annealing temperature for each gene are and housekeeping genes were similar. The primer sequences, for housekeeping genes. Amplification efficiencies for target Power SYBR Green PCR Master Mix (Applied Biosystems) F: 5 GAPDH Target Journal of Endocrinology www.endocrinology-journals.org F, forward primer; R, reverse primer. FGF17 F: 5 GAGDH H2AFZ Target F: 5 F: 5 H2AFZ (Applied Biosystems, Sao Paulo, Brazil) for the target gene predicted bovine sequences, and amplicons were sequenced to confirm identity. Relative real-time RT-PCR analysis was performed with an ABI 7500 using TaqMan Assay by Design (Applied Biosystems, Sao Paulo, Brazil) for the target gene (FGF17), as it provided a higher amplification efficiency in comparison with SYBR Green. As previous studies have shown that these two detection systems produce similar results comparison with SYBR Green. As previous studies have shown that these two detection systems produce similar results for high abundance messages (Jeong et al. 2005), we used Power SYBR Green PCR Master Mix (Applied Biosystems) for housekeeping genes. Amplification efficiencies for target and housekeeping genes were similar. The primer sequences, fragment size, and annealing temperature for each gene are shown in Table 2. Reactions were optimized to provide maximum amplification efficiency for each gene. PCR was performed on 0.5 μl cDNA in 25 μl reaction volumes in duplicate, and the specificity of each PCR product was determined by melting curve analysis (for housekeeping genes) and confirmation of the amplicon size using electrophoresis with 2% agarose gels (for FGF17 and housekeeping genes). 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 control was a cDNA sample from each cell type analyzed. An initial analysis of FGF17 mRNA across follicle cell types was performed with gyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene, as this gene is expressed at similar levels in theca and granulosa cells (Buratini et al. 2005b). To select the most stable housekeeping gene for detailed analyses of each cell type, peptidylprolyl isomerase A (PPIA), GAPDH, and histone H2AFZ (H2AFZ) amplification profiles were compared using the geNorm applet for Microsoft Excel (medgen.ugent.be/genorm; Ramakers et al. 2003); the most stable housekeeping genes were PPIA for theca cells and oocytes, GAPDH for granulosa, and H2AFZ for cultured granulosa cells.

Table 2 Details of primers used for real-time PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>Fragment size (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
</table>
| GAPDH       | F: 5’ gcg tgt cac gag aag tat aa 3’
R: 5’ ccc tcc acq atg cca aag t 3’ | 119 | 62 |
| PPIA        | F: 5’ gcg atg gag ccg tgt gc 3’
R: 5’ cca cag tca gca atg gtt atc t 3’ | 65 | 60 |
| H2AFZ       | F: 5’ gag gag ctc aac aag ctc tgg 3’
R: 5’ tgt tgg tgt ctc tca gtc ttc 3’ | 74 | 60 |
| FGF17       | F: 5’ ccc ggt tct ctg cag tca aga t 3’
R: 5’ gct tgc ccc tct tat tca cag ac aat ac 3’ | 62 | 60 |

F, forward primer; R, reverse primer.

Immunohistochemistry

Bovine ovaries were collected from an abattoir, bisected, and fixed in paraformaldehyde. Fixed tissues were embedded in paraffin, and 3 μm sections were placed on poly-L-lysine-coated slides. Sections were deparaffinized in xylene twice for 20 min, and hydrated in successive 3 min washes in 95 and 85% ethanol. Antigen retrieval was achieved by incubating in 0.5 mM Tris–EDTA pH 9.0 at 96°C for 30 min. Endogenous peroxidase was quenched by incubation in methanol with 5% hydrogen peroxide for 10 min, and then rinsed ten times in distilled water and twice for 5 min in 0.5 M Tris pH 7.4. Slides were then incubated with polyclonal FGF17 antibody (1.25 μg/ml; 500-P152; PeproTech) for 2 h at room temperature in a humidified chamber. Slides were washed in 0.5 M Tris pH 7.4, then incubated with HRP-conjugated secondary antibody for 40 min (EnVision Dual Link System, Dako, Carpinteria, CA, USA). Immunostaining was revealed with liquid DAB (3,3’ diaminobenzidine; Dako), and sections were counterstained with Harris hematoxylin. Six to seven sections were examined from each of three ovaries. Negative controls were performed by preincubating FGF17 antibody with twice the concentration of recombinant FGF17 protein for 2 h at room temperature (2.5 μg/ml; 100-27; PeproTech). The immunogen used for antibody production has at most 67% homology with other human FGFs. No bovine FGF8 family proteins are available, so it is not possible to test antibody specificity experimentally.

Steroid assays

E2 and P4 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., Webster, TX, USA) respectively with a revised protocol (Buratini et al. 2005b). The standard curves and samples were diluted in PBS–gelatin. The intra- and inter-assay coefficients of variation (CV) were 7-4 and 13-5% respectively for E2, and 6-8 and 7-0% respectively for P4. The sensitivities of the assays were 0.3 ng/ml for E2 and 0.2 ng/ml for P4. E2 was measured in conditioned medium in
duplicate as described (Bélanger et al. 1990) without solvent extraction. Intra- and inter-assay CV were 6 and 9% respectively. P₄ was measured in conditioned medium in duplicate as described (Lafrance & Goff 1985) with mean intra- and inter-assay CV were 7.2 and 18% respectively. The sensitivity of these assays was 10 and 4 pg per tube for E₂ and P₄, equivalent to 0.3 and 20 ng/µg protein respectively. Steroid concentrations in culture medium were corrected for cell number by expressing per unit mass of total cell protein.

**Statistical analysis**

The data were transformed to logarithms if not normally distributed. ANOVA was used to compare follicle size and intrafollicular steroid concentrations across follicle classes, to test the effect of follicle class on FGF17 mRNA abundance in granulosa cells, to test the effects of FSH and IGF-I on FGF17 mRNA levels in cultured granulosa cells, and to test the effect of FGF17 on steroid secretion from cultured granulosa cells. Means comparisons were performed with the Tukey–Kramer HSD test. Non-parametric ANOVA was used to test the effect of follicle class on FGF17 mRNA abundance in theca cells, and to compare FGF17 mRNA abundance across follicle cell types as data were not normally distributed even after transformation to logarithms. Means comparisons were performed with the Kruskal–Wallis test. Data are presented as means ± S.E.M. Analyses were performed with JMP software (SAS Institute, Cary, NC, USA).

**Results**

Immunohistochemistry revealed the presence of FGF17 predominantly in oocytes and granulosa cells of preantral and antral follicles (Fig. 1). Staining was predominant in the nucleus of oocytes in preantral follicles, and was more intense in the cytoplasm of oocytes from antral follicles (Fig. 1A–C). Staining was weak in the theca cell layer, detected at background levels in deep ovarian stroma (Fig. 1E), and also clearly observed in the surface epithelium. Atretic follicles with only a few layers of granulosa cells and many pycnotic nuclei also stained strongly for FGF17 (Fig. 1D). No staining was observed when FGF17 antibody was preincubated with excess FGF17 (Fig. 1F).

A survey of FGF17 expression in follicle cells by real-time PCR showed the presence of mRNA in pooled oocytes and at comparatively low levels in both granulosa and theca cells. We initially compared FGF17 mRNA across follicle cell types using GAPDH as housekeeping gene. Relative abundance was higher in oocytes (51 ± 14) compared with granulosa and theca cells (0.025 ± 0.008 and 0.005 ± 0.001 respectively) from healthy and transitional follicles. A detailed assay of FGF17 in granulosa and theca cells collected from healthy, transitional, and atretic follicles showed that mRNA abundance was significantly higher in granulosa and theca cells from atretic follicles than from healthy or transitional follicles (Fig. 2). There was no effect of diameter on FGF17 expression within any of the health status classes.

As FGF17 protein and mRNA were detected in granulosa cells, we determined whether expression can be regulated by FSH and IGF-I. Cells were cultured in serum-free medium and treated for 6 days with graded doses of FSH or IGF-I. Both gonadotropic hormones significantly inhibited FGF17 mRNA abundance while increasing E₂ secretion (Fig. 3). Given this apparent inverse relationship between E₂ and FGF17, we sought to determine whether FGF17 can regulate E₂ secretion from granulosa cells. FGF17 inhibited E₂ and P₄ secretion from cultured granulosa cells in a dose-dependent manner (Fig. 4).

**Discussion**

These data demonstrate for the first time the expression of FGF17 protein and mRNA in the ovary. The most significant findings are the higher abundance of FGF17 mRNA in granulosa and theca cells of atretic follicles compared with healthy follicles, the inhibition of FGF17 mRNA abundance in granulosa cells by FSH and IGF-I, and the inhibitory effect of FGF17 on granulosa cell steroid secretion. These data point to an inhibitory role for FGF17 in follicular steroidogenesis and its involvement in the mechanisms controlling follicle atresia.
In the female reproductive system, FGF17 mRNA has been described only in isolated mouse oocytes and embryos (Zhong et al. 2006). In the present study, we describe FGF17 mRNA predominantly in oocytes, compared with lower expression in granulosa and theca cells. This conclusion is drawn from an analysis of relative expression (calculated as $\Delta\Delta C_T$ value) in all three cell types using GAPDH as housekeeping gene. This is based on the assumption that this housekeeping gene is expressed at similar levels in all three cell types, and our previous data have shown this to be valid for granulosa and theca cells (Buratini et al. 2005b). Analysing the same data with PPLA as housekeeping gene gave the same results; mRNA abundance was at least 100-fold greater in oocytes compared with granulosa and theca cells. This conclusion is consistent with the oocyte localization of the prototype member of this FGF subfamily, FGF8 (Valve et al. 1997, Buratini et al. 2005b, Sugiura et al. 2007). The higher expression of FGF17 in the oocyte does not imply that this cell is the predominant intrafollicular source of FGF17 as the number of somatic cells is greatly superior within the follicle, but rather suggests that oocyte-derived FGF17 may play a role in the mechanisms controlling cumulus cells differentiation. In fact FGF8 was shown to synergize with BMP15 to promote glycolysis in murine cumulus cells (Sugiura et al. 2007).

Other FGFs have been localized to the bovine oocyte, including FGF2 and FGF10 (van Wezel et al. 1995, Buratini et al. 2007), but this is not a FGF-wide phenomenon as FGF7 mRNA was readily detected in theca cells but not in oocytes (Buratini et al. 2007).

Protein was predominantly localized to the nucleus of oocytes in primordial and primary follicles, whereas in follicles at later stages of development, FGF17 protein was also clearly detected in the ooplasm. This suggests translocation of protein to the nucleus in oocytes, which is more apparent in primordial and primary follicles. Nuclear translocation of endogenous FGF has been reported for FGF1, -2, and -3 (Zhan et al. 1992, Kiefer & Dickson 1995, Sheng et al. 2004). FGF17 protein was also present in the granulosa cell layer in healthy follicles, and to a lesser extent in theca cells, which is in agreement with greater mRNA abundance in granulosa cells. Strong staining in the cytoplasm of granulosa cells may also reflect binding of FGF17 to receptors (FGFR2C and FGFR3C; Berisha et al. 2004, Buratini et al. 2005b) and internalization. This is supported by evidence for receptor-mediated internalization of exogenous FGFs in several cell lines (Belleudi et al. 2002, Olsnes et al. 2003, Wesche et al. 2006). Therefore, the staining pattern combined with mRNA data suggests an autocrine action for FGF17 in granulosa cells within the follicle wall. Detection of FGF17 appears to be specific as preincubation of the antibody with FGF17 abolished staining, although we cannot rule out the possibility that the antibody reacts with other bovine FGFs.

Granulosa and theca cell abundance of FGF17 mRNA was significantly increased in atretic follicles compared with

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**Figure 2** Follicular FGF17 mRNA abundance varies with follicle health. Follicles were classified as healthy, transitional, or atretic based on E2:P4 ratios in follicle fluid and RNA measured by real-time PCR in isolated granulosa and theca cells. Data are presented as mean (±S.E.M.) values relative to the housekeeping gene and to a calibrator sample present in all PCR runs, which were calculated using the ΔΔC_T method with efficiency correction. Bars with different letters are significantly different ($P<0.05$). Numbers in parentheses denote the number of samples analyzed.

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**Figure 3** Effects of FSH and IGF-I on FGF17 mRNA abundance and steroid secretion in granulosa cells. Cells from follicles 2 to 5 mm in diameter were placed in serum-free culture, and the stated doses of FSH or IGF-I were added on day 2. Total RNA was collected on day 6, and FGF17 mRNA abundance was measured by real-time PCR. Data are presented as mean (±S.E.M.) values relative to a calibrator sample by the ΔΔC_T method with efficiency correction. Steroid concentrations were measured by RIA on day 6 and expressed relative to total protein at the end of culture. Bars with different letters are significantly different ($P<0.05$). Data were derived from three independent cultures.
Both hormones decreased FGF17. Tropic hormones FSH and IGF-I regulate mRNA levels. In granulosa cells, we determined whether the major gonadotropin, increasing E2 secretion, which is consistent with the low abundance was significantly lower in atretic follicles, whereas that of FGF expression may significantly account for their well-established pro-survival action in antral follicles.

In conclusion, the present study suggests a physiological role for FGF17 in the control of granulosa cell differentiation. FGF17 suppressed steroid secretion from granulosa cells, and mRNA abundance was decreased by FSH and IGF-I. This is consistent with lower FGF17 mRNA levels in healthy compared with atretic follicles. One potential role for FGF17 may be to inhibit E2 secretion and follicle growth in regressing follicles; therefore, it may be critical for FSH and IGF-I to suppress FGF17 expression during growth of the dominant follicle.

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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