Regulation and action of fibroblast growth factor 17 in bovine follicles

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SHORT TITLE: FGF17 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 IGF1 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 (Berisha et al. 2004; Buratini et al. 2005a; Buratini et al. 2007; Buratini et al. 2005b; van Wezel et al. 1995). The twenty-two known FGFs have been grouped into 7 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) (Itoh & Ornitz 2004; Ornitz et al. 1996). 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; Nilsson et al. 2001; Spicer & Stewart 1996; Vernon & Spicer 1994). 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 (Berisha et al. 2004; Buratini et al. 2007; Parrott & Skinner 1998). Both FGFs inhibit estradiol secretion from cultured granulosa cells (Buratini et al. 2007; Parrott & Skinner 1998).

Another subfamily that may be of interest for potential paracrine signalling is the FGF8 subfamily. In adult rodents, Fgf8 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 (Buratini et al. 2005b; Ornitz et al. 1996). Although the roles of the FGF8 subfamily in the control of the ovarian activity are still poorly understood, it was 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. Messenger RNA encoding FGF17 was detected in human prostatic epithelial cells (Polnaszek 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 signalling within the follicle. Specifically, we sought to localize FGF17 mRNA in follicular cell types, to determine if mRNA expression is under the control of the major regulators of follicle development, FSH and IGF1, and to gain insight into the potential role of FGF17 in the regulation of follicle steroidogenesis.

MATERIALS AND METHODS

Tissues

Follicles of diameter $\geq 5$ 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 progesterone and estradiol assays. The antral cavity was flushed repeatedly with cold saline, and granulosa cells were recovered by centrifugation at 1200 X 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, Sao Paulo, Brazil), homogenized with a Polytron and submitted immediately to total RNA extraction according to the manufacturer’s protocol.

Follicles were classed according to estradiol:progesterone (E:P) 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 detection of mRNA encoding cytochromes P450.
aromatase (CYP19A1) and 17α-hydroxylase (CYP17A1) mRNA 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 to 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, Sao Paulo, Brazil).

**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 (Burlington, Canada), except where otherwise stated. Follicles ≤ 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 (avascular theca, debris in antrum) were discarded. Cells were collected by repeated 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; Oakville, Canada), 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 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 (Corning) at a density of \(10^6\)/well in 1 ml medium. Cultures were maintained at 37°C in 5% CO\(_2\) 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, USA; 0, 0.1, 1, 10 and 100 ng/ml) or IGF1 analog (LR3; Sigma-Aldrich; Oakville, Canada; 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 70ºC 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 \(FGFR3c\) mRNA levels (Buratini et al. 2005b) and with graded doses of FGF17 (PeproTech, Rocky Hill, 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 N NaOH for 2 h followed by neutralization with 200 µl of 1 N HCl for total cell protein measurement with the Bradford protein assay (Bio-Rad, Mississauga, Ontario, Canada). 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-Polymerase Chain Reaction (RT-PCR)**

Theca and granulosa cell RNA (1 µg) samples were incubated with DNAse I (1U/µg RNA; Invitrogen) then reverse transcribed with SuperScript III (200U/µL; Invitrogen) and oligo-d(T) primer. The RNA yield from pools of twenty oocytes was too
low to be accurately quantified by spectrometry, so 8µl aliquots of RNA, corresponding
approximately to 5 oocytes, were incubated with DNAse I (1U/sample; Invitrogen) to
produce 10µl of RNA solution, which is the maximum volume for the RNA sample in the
reverse transcription protocol (SuperScript III, Invitrogen).

Primers for **FGF17** mRNA were designed based on the 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 SybrGreen. As previous studies have shown that these two
detection systems produce similar results for high abundance messages (Jeong *et al.* 2005),
we used Power SybrGreen PCR Master Mix (Applied Biosystems, Sao Paulo, Brazil) 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 0.5µl 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 glyceraldehyde-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.

172 **Immunohistochemistry**

173 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 M mM Tris-EDTA pH 9.0 at 96ºC for 30 min. Endogenous peroxidase was quenched by incubation in methanol with 5% hydrogen peroxide for 10 min, 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 (1.25µg/mL; 500-P152; PeproTech, Rocky Hill, USA) for 2 hours at room temperature in a humidified chamber. Slides were washed in 0.5 M Tris pH 7.4, then incubated with horseradish peroxidase-conjugated secondary antibody for 40 min (EnVision Dual Link System, Dako, Carpinteria, USA). Immunostaining was revealed with liquid DAB (Dako, Carpinteria, USA) 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 two hours at room temperature (2.5µg/mL; 100-27; PeproTech, Rocky Hill, USA). 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**

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., Webster, USA), respectively, with a revised protocol (Buratini *et al.* 2005b). The standard curves and samples were diluted in PBSgelatin. 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 conditioned medium in duplicate as described (Bélanger *et al.* 1990), without solvent extraction. Intra- and inter-assay coefficients of variation were 6% and 9%, respectively. Progesterone was measured in conditioned medium in duplicate as described (Lafrance & Goff 1985) with mean intra- and inter-assay coefficients of variation were 7.2% and 18%, respectively. The sensitivity of these assays was 10 pg and 4 pg per tube for estradiol and progesterone, 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.

**Statistics**

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 of IGF1 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 ± SEM. Analyses were performed with JMP software (SAS Institute, Cary, 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, 1B and 1C). 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 to 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 IGF1. Cells were cultured in serum-free medium and treated for 6 days with graded doses of FSH or IGF1. Both gonadotrophic hormones significantly inhibited FGF17 mRNA abundance while increasing estradiol secretion (Fig 3). Given this apparent inverse relationship between estradiol and FGF17, we sought to determine whether FGF17 can regulate estradiol secretion from granulosa cells. FGF17 inhibited estradiol and progesterone 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 to healthy follicles, the inhibition of FGF17 mRNA abundance in granulosa cells by FSH and IGF1, 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 ∆∆Ct 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 PPIA as housekeeping gene gave the same results; mRNA abundance was at least 100-fold greater in oocytes compared to granulosa and theca cells. This conclusion is consistent with the oocyte localization of the prototype member of this FGF subfamily, FGF8 (Buratini et al. 2005b; Sugiura et al. 2007; Valve et al. 1997). 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 (Buratini et al. 2007; van Wezel et al. 1995), 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 (Kiefer & Dickson
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 to healthy follicles. FGF17 protein staining was also strong in the granulosa layer in atretic follicles. It is not known whether this is part of an apoptotic pathway or disregulation of gene expression during cell death. There was no effect of size on FGF17 expression in atretic follicles ranging from 5 to 11 mm, suggesting that FGF17 expression is enhanced as non-ovulatory dominant follicles and also non-selected recruited follicles enter atresia. This pattern of expression of FGF17 differs from that of FGF7 and FGF10: FGF10 mRNA abundance was significantly lower in atretic follicles compared to healthy follicles whereas that of FGF7 did not change (Buratini et al. 2007). There is no information on the expression of other FGFs in atretic follicles.

As FGF17 mRNA and protein were both detected in granulosa cells, we determined if the major gonadotrophic hormones FSH and IGF1 regulate mRNA levels. Both hormones decreased FGF17 mRNA abundance while increasing estradiol secretion, which is consistent with the low mRNA levels observed in healthy, estrogenic follicles.
We are not aware of other reports describing the regulation of FGF expression by gonadotrophins in granulosa cells, but microarray studies identified FGF2 and FGF5 as genes downregulated by FSH in the ovarian surface epithelium (Ji et al. 2004). It has been proposed that genes downregulated by FSH in granulosa cells are those primarily involved in cell cycle control and apoptosis (Sasson et al. 2003), again suggesting a role for FGF17 in atresia. Therefore, the ability of FSH and IGF-1 to suppress FGF expression may significantly account for their well established pro-survival action in antral follicles.

To explore the function of FGF17, we cultured bovine granulosa cells in serum-free medium with recombinant FGF17. In this estrogenic cell model, FGF2 and 10 have been demonstrated to inhibit estradiol secretion (Buratini et al. 2007; Cao et al. 2006), and we show here that FGF17 has a similar effect. Interestingly, FGF2 was more potent at inhibiting estradiol secretion than progesterone secretion (Vernon & Spicer 1994; Cao et al. 2006), whereas in the present study FGF17 was more potent at suppressing progesterone secretion; estradiol secretion was inhibited only at higher doses of FGF17. This suggests that while all FGFs studied to date inhibit steroidogenesis in bovine granulosa cells, they display different specificities for estrogenic and progestagenic pathways. Potential mechanisms of this differential action have not been investigated, but are likely related to mitogen-activated protein kinase (MAPK) activity; FGFRs activate the MAPK pathway (Powers et al. 2000), and MAPK activity suppresses aromatase (CYP19A1) mRNA expression (Manuel Silva & Price 2000; Fan et al. 2009). As it would be critical to keep FGF17 levels low to allow estradiol secretion and continued follicle growth, it is possible that FSH and IGF1 may stimulate estradiol production in part through the inhibition of FGF expression.
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 IGF1. This is consistent with lower FGF17 mRNA levels in healthy compared to atretic follicles. One potential role for FGF17 may be to inhibit estradiol secretion and follicle growth in regressing follicles, therefore it may be critical for FSH and IGF1 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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**FIGURE LEGENDS**

**Figure 1** Immunohistological detection of FGF17 in bovine ovaries. Staining is observed in the oocyte of primordial (A, arrow), primary (B) and antral (C) follicles. The granulosa cell layer was well stained in healthy (C, E) and atretic (D) antral follicles, whereas the theca layer was weakly stained. No staining was observed in the presence of excess FGF17 protein (F). Bars = 20µm (A, B and D), 30 µm (C), 50 µm (E and F). g, granulosa; t, theca; s, stroma.

**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 (± SEM) values relative to the housekeeping gene and to a calibrator sample present in all PCR runs, which were calculated using the ΔΔCt method with efficiency correction. Bars with different letters are significantly different (*P*<0.05). Numbers in parentheses denote the number of samples analyzed.

**Figure 3** Effects of FSH and IGF1 on *FGF17* mRNA abundance and steroid secretion in granulosa cells. Cells from follicles 2-5 mm diameter were placed in serum-free culture and the stated doses of FSH or IGF1 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 (± SEM) values relative to a calibrator sample by the ΔΔCt 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.

**Figure 4** Effects of FGF17 on steroid secretion from granulosa cells. Cells from follicles 2-5 mm diameter were placed in serum-free culture and the stated doses of FGF17 were added on day 2. Media were collected on day 6 for measurement of steroid concentrations by RIA. Data are presented as mean (± SEM) values and expressed relative to total cell protein. Bars with different letters are significantly different (*P*<0.05). Data were derived from three independent cultures.
Fig 1

A

B

C

g t s

D

s t g

E

g t s

F

g t s

95x135mm (250 x 250 DPI)
Fig 2

Granulosa cells

FGF17 mRNA abundance (relative units)

P<0.05

Theca cells

P<0.05

Follicle health status

Healthy  Transitional  Atretic

ab (16)  a (16)  b (10)

89x156mm (300 x 300 DPI)
Fig 4

![Graph showing progesterone and estradiol levels with FGF17 concentrations](image)
Table 1 Mean (± SEM) follicular fluid estradiol and progesterone concentrations, and follicle diameters of follicles classed as healthy, transitional and atretic based on estradiol:progesterone ratio.

<table>
<thead>
<tr>
<th></th>
<th>Healthy (n=16)</th>
<th>Transitional (n=17)</th>
<th>Atretic (n=10)</th>
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</thead>
<tbody>
<tr>
<td>Estradiol (ng/ml)</td>
<td>540±249 (^a)</td>
<td>16±3 (^b)</td>
<td>0.3±0.1 (^c)</td>
</tr>
<tr>
<td>Progesterone (ng/ml)</td>
<td>19±3 (^a)</td>
<td>44±7 (^b)</td>
<td>290±59 (^c)</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).
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>
<tbody>
<tr>
<td>GAPDH</td>
<td>F 5’ ggc gtg aac cac gag aag tat aa 3’</td>
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<td>62</td>
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<td>R 5’ ccc tcc acg atg cca aag t 3’</td>
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<td>PPIA</td>
<td>F 5’ gcc atg gag cgc ttt gg 3’</td>
<td>65</td>
<td>60</td>
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<tr>
<td></td>
<td>R 5’ cca cag tca gca atg gtg atc t 3’</td>
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<tr>
<td>H2AFZ</td>
<td>F 5’ gag gag ctg aac aag ctt tgg 3’</td>
<td>74</td>
<td>60</td>
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<td>R 5’ ttt tgg tgg ctc tca gtc ttc 3’</td>
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<td>F 5’ ccc ggt ggt gct cca g 3’</td>
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<td>R 5’ gct tgc ccc tct tat tca tac aga t 3’</td>
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<tr>
<td></td>
<td>Probe FAM 5’ ctt aga gtt aga aat ac 3’</td>
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</tbody>
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F = forward primer; R = reverse primer; bp = base pairs