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Expression of fibroblast growth factor-8 and regulation of cognate receptors, fibroblast growth factor receptor-3c and -4, in bovine antral follicles

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Abstract

Paracrine cell signaling is believed to be important for ovarian follicle development, and a role for some members of the fibroblast growth factor (FGF) family has been suggested. In the present study, we tested the hypothesis that FGF-8 and its cognate receptors (FGFR3c and FGFR4) are expressed in bovine antral follicles. RT-PCR was used to analyze bovine *Fgf8*, *Fgfr3c* and *Fgfr4* mRNA levels in oocytes, and granulosa and theca cells. *Fgf8* expression was detected in oocytes and in granulosa and theca cells; this expression pattern differs from that reported in rodents. Granulosa and theca cells, but not oocytes, expressed *Fgfr3c*, and expression in granulosa cells increased significantly with follicle estradiol content, a major indicator of follicle health. *Fgfr4* expression was restricted to theca cells in the follicle, and decreased significantly with increasing follicle size. To investigate the potential regulation of *Fgfr3c* expression in the bovine granulosa, cells were cultured in serum-free medium with FSH or IGF-I; gene expression was upregulated by FSH but not by IGF-I. The FSH-responsive and developmentally regulated patterns of *Fgfr3c* mRNA expression suggest that this receptor is a potential mediator of paracrine signaling to granulosa cells during antral follicle growth in cattle.

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Introduction

Antral ovarian follicle growth in monovular species is regulated by a number of factors, the most well known of which are the gonadotropins. Follicles are considered to be follicle-stimulating hormone (FSH)-dependent until dominance occurs, after which they become luteinizing hormone-dependent (reviewed by Fortune *et al.* 2001, Ginther *et al.* 2001). It has also become clear that growth factors are key stimulatory/regulatory molecules. Several lines of evidence point to a critical role for members of the transforming growth factor-β (TGF-β) superfamily, especially growth/differentiation factor 9 and bone morphogenetic protein 15 (reviewed by Gilchrist *et al.* 2004, Juengel *et al.* 2004, Shimasaki *et al.* 2004).

The fibroblast growth factor (FGF) family is emerging as a group of factors that are potentially important for follicle growth. For example, FGF-7 is expressed in theca cells, its receptor is expressed in granulosa cells (Parrott & Skinner 1998, Berisha *et al.* 2004), and FGF-7 stimulated bovine granulosa cell proliferation and inhibited steroidogenesis (Parrott & Skinner 1998). Another potentially interesting member of this family is FGF-8. Widely expressed in fetal tissues, this factor is predominantly expressed in the gonads of adult rodents and ruminants (MacArthur *et al.* 1995*a*, Buratini *et al.* 2005). Within the ovary, *Fgf8* gene expression occurs only in the oocyte in adult mice (Valve *et al.* 1997), which suggests a potential role in signaling of follicular cells by the oocyte.

There are five known FGF receptor (FGFR) genes (Kim *et al.* 2001, Sleeman *et al.* 2001), of which FGF-8 preferentially activates FGFR4 and the 'c' splice form of FGFR3 (Ornitz *et al.* 1996). mRNAs encoding *Fgfr4* or *Fgfr3c* were not consistently detected in the rodent ovary (Asakai *et al.* 1994, Puscheck *et al.* 1997), but were detected in bovine testis and ovary (Buratini *et al.* 2005).

In view of the very discrete expression pattern of the *Fgf8* gene (i.e. in oocytes), and its role in tissue

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differentiation, it is of interest to determine if this growth factor may play a role in follicle development. The objectives of the present study were to determine if *Fgf8* expression is restricted to oocytes in cattle, and to determine if the expression of *Fgfr3c* and *Fgfr4* genes may be developmentally regulated in follicular somatic cells.

Materials and Methods

Tissues

Ovaries were obtained from an abattoir local to the São Paulo State University campus in Botucatu, and transported to the laboratory in saline on ice. Follicles greater than 5 mm in diameter were dissected from the ovaries, and follicular fluid was aspirated, centrifuged and frozen for steroid assay. The antral cavity was flushed repeatedly with cold saline and granulosa cells 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 removed with forceps and washed in saline by passing repeatedly through a 1 ml syringe. The samples were collected into Trizol (Invitrogen, São Paulo, Brazil) and homogenized with a Polytron. Total RNA was extracted immediately according to the Trizol protocol.

Follicles were classified according to estradiol and progesterone content, and by size (5–7, 7–10 and >10 mm diameter; $n = 12$, 19 & 11 respectively). Follicles containing less than 100 ng/ml progesterone were considered to be healthy and were grouped according to estradiol content into <5 ng/ml ($n = 16$), 5–20 ($n = 10$), >20–100 ($n = 10$) and >100 ng/ml ($n = 6$) (based on Berisha *et al.* 2000). Follicles containing ≥100 ng/ml progesterone were classed as atretic, and contained mean estradiol concentrations of 0.06 ± 0.02 ng/ml. Cross-contamination of theca and granulosa cells was tested by detection of mRNA encoding cytochromes P450 aromatase (*Cyp19*) and 17β-hydroxylase (*Cyp17*) in each sample by PCR.

Cumulus–oocyte complexes were aspirated from antral follicles and cumulus cells removed from the oocyte by vortexing. Eleven pools of 50 oocytes and ten pools of 100 were collected, and RNA extracted with the RNeasy kit (Qiagen, São Paulo, Brazil).

Cell culture

The cell culture system was based on that described by Gutiérrez *et al.* (1997), with slight modifications (Silva & Price 2000). All materials were obtained from Invitrogen except where otherwise stated. Briefly, bovine ovaries were collected from adult cows, irrespectively of stage of the estrous cycle, at an abattoir local to the University of Montreal Faculty of Veterinary Medicine and were transported to the laboratory in PBS at 35 °C containing penicillin (100 IU/ml), streptomycin (100 μg/ml) and Fungizone (1 μg/ml). Follicles (2–5 mm diameter) were dissected from the ovaries, and those with obvious signs of atresia (avascular 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 α-MEM, containing HEPES (20 mM), sodium bicarbonate (10 mM), sodium selenite (4 ng/ml), BSA (0.1%; Sigma-Aldrich Canada, Oakville, ON, 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 Glass Works, Corning, NY, USA) at a density of 10^6 cells/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. Cells were stimulated with FSH (AFP-5332B; National Institute of Diabetes, Digestive and Kidney Diseases, Bethesda, MD, USA) or IGF-I analogue (Long R3; Sigma-Aldrich), at the doses given in Results. Medium samples were stored at −20 °C until the assay, and cells were collected in Trizol and stored at −70 °C until RNA extraction. Experiments were performed on three independent cultures.

RT-PCR

Primers for *Fgf8* were designed based on alignment of published human and rodent sequences, and bovine-specific *Fgfr3c* and *Fgfr4* primers were based on bovine sequences (Buratini *et al.* 2005). For the receptors, the sense primers were located in the third Ig-like domain, and the antisense primers were located in the second intracellular kinase domain (Table 1), thus spanning several exons. The *Fgf8* sense and antisense primers were located in exons 1D and 3 respectively, allowing amplification of all known splice variants (MacArthur *et al.* 1995*b*).

FGFR expression was measured by semi-quantitative RT-PCR. For theca and granulosa cells, total RNA (1 μg) was incubated with DNAse I (Invitrogen, São Paulo, Brazil) and reverse transcribed with SuperScript II (Invitrogen) and oligo-d(T) primer. The RNA yield from oocytes was too low to be accurately quantified by spectrometry, so 8 μl aliquots of RNA were used in RT reactions.

For *Fgfr4*, target and glyceraldehyde-3-phosphate dehydrogenase (*Gapd*) genes were amplified in the same reaction to minimize errors due to pipetting and to simplify gel handling and documentation. PCR was performed on 0.5 μl cDNA in PCR mastermix containing 1.6 U Taq DNA polymerase (Invitrogen), 0.4 μM *Fgfr4* and 0.06 μM *Gapd* primers (Table 1), 0.2 mM dNTPs and 1.5 mM $MgCl_2$ in a total volume of 25 μl. Samples were denatured for 3 min at 94 °C, followed by 28 cycles of denaturing at 94 °C for 45 s, annealing at 60 °C for 45 s and extension at 70 °C for 1 min.

Table 1 PCR primer data.

Target	Sequence	Fragment size (bp)	Reference
Fgf8	F 5′-ACC AAC TCT ACA GCC GCA CCA G-3′ R 5′-GTA GTT GAG GAA CTC GAA GCG CAG-3′	427	AY183660
Fgfr3c	F 5′-GGT GGT GCT GCC AGC T-3′ R 5′-TAA GTC CTT ATC CGT GGC GT-3′	496	Buratini *et al.* (2005)
Fgfr4	F 5′-AAG GCA GGT ACA CGG ACA TC-3′ R 5′-TAA GCA TCT TGA CAG CCA CG-3′	426	Buratini *et al.* (2005)
Gapd	F 5′-TGT TCC AGT ATG ATT CCA CC-3′ R 5′-TCC ACC ACC CTG TTG CTG-3′	850	Tsai *et al.* (1996)
Cyp19	F 5′-TGC ATG GCA AGC TCT CC-3′ R 5′-TCC TTA TGT CTC TTT CAC C-3′	373	Z32741
Cyp17	F 5′-GAA TGC CTT TGC CCT GTT CA-3′ R 5′-CGC GTT TGA ACA CAA CCC TT-3′	330	Lehmann *et al.* (2000)
H2a	F 5′-GTC GTG GCA AGC AAG GAG-3′ R 5′-GAT CTC GGC CGT TAG GTA CTC-3′	182	Robert *et al.* (2002)

F = forward primer; R = reverse primer.

For *Fgfr3c*, PCR was performed separately for target and housekeeping genes, as multiplexing failed to provide linear amplification of discrete bands for this primer combination. The reaction contained 0.5 μl (for *Gapd*) or 1 μl (for *Fgfr3c*) cDNA in PCR mastermix containing 1.6 U Taq DNA polymerase (Invitrogen), 0.4 μM *Fgfr3c* primers (Table 1) or 0.16 μM *Gapd* primers, 0.2 mM dNTPs and 1.5 mM $MgCl_2$ in a total volume of 25 μl. Samples were denatured for 3 min at 94 °C, followed by 24 (*Gapd*) or 33 (*Fgfr3c*) cycles of denaturing at 94 °C for 30 s, annealing for 45 s and extension at 70 °C for 1 min. Annealing temperatures were 65 °C for *Fgfr3c* and 60 °C for *Gapd*.

To determine if *Fgf8* expression is specific to oocytes in cattle, PCR was performed as described above on 1 μl cDNA at 69.5 °C annealing temperature for 34 (granulosa and theca) or 40 (oocytes) cycles.

All PCR reactions were performed with positive (fetal bovine brain, liver and ovary for *Fgfr3c*, *Fgfr4* and *Fgf8* respectively) and negative (water) controls. 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.).

Cycling conditions for semi-quantitative RT-PCR were optimized in preliminary experiments. The linear range of PCR for each target gene was first determined for 1 μg RNA (Fig. 1), and the linearity of input amount of RNA was verified by performing RT-PCR with 0.5, 1 and 2 μg RNA at the optimized cycle number for each gene (Fig. 1). To verify authenticity of the amplified DNA, amplicons from oocytes and theca and granulosa cell samples were excised from gels and sequenced (Department of Pharmacology, Federal University of São Paulo, São Paulo School of Medicine, São Paulo, Brazil).

To determine cross-contamination of theca and granulosa cells, PCR was performed on 0.5 μl cDNA in the PCR mastermix described above but containing 0.4 μM *Cyp19* or *Cyp17* primers (Table 1). Samples were amplified as above for 30 cycles at an annealing temperature of 58 °C for *Cyp19* and 60 °C for *Cyp17*. The presence of *Cyp19* amplicons in theca samples or of *Cyp17* in granulosa samples indicated cross-contamination, and such samples were discarded. Contamination of theca cells with granulosa cells is most likely as incomplete scraping of the follicle wall may leave adherent granulosa cells, and as 30 cycles of amplification are well beyond the linear range for granulosa cell RNA (Sahmi *et al.* 2004) we believe that we detected most cases of major contamination. Examples of non-contaminated samples are shown in Fig. 1A.

For analysis of gene expression in cultured granulosa cells, semi-quantitative RT-PCR was performed as described above, except that histone H2a (*H2a*) was used as the housekeeping gene. PCR was performed for 30 cycles at an annealing temperature of 55 °C.

Steroid assays

Estradiol and progesterone were assayed in follicular fluid using iodinated tracers and antibodies furnished in the 3rd Generation Estradiol RIA (DSL 39100) and the DSL-3400 Progesterone RIA kit (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). The standard curves were prepared from crystalline steroids (Sigma Chemical Co.) in PBS–gelatin (0.02 M sodium phosphate, 0.15 M sodium chloride, 0.1% gelatin, 0.01% sodium azide, pH 7.5). The assay protocols were as described in the kits, except that the estradiol antibody and tracer were each diluted 1:1 with PBS–gelatin before use, and the progesterone antibody and tracer were diluted 3:2 and 7:3 respectively. Follicular fluid samples were diluted in PBS–gelatin before assay. Intra- and inter-assay coefficients of variation were 7.4 and 13.5% respectively for estradiol, and 6.8 and 7% respectively for progesterone. The sensitivities of the assays were 0.05 ng/ml for estradiol (at 1:25 dilution of follicular fluid) and 0.2 ng/ml for progesterone (at 1:10 dilution).

Statistics

Target gene mRNA abundance was expressed relative to *Gapd* (for follicles) or *H2a* (granulosa cell cultures) mRNA (ratio of optical densities), and the data were transformed

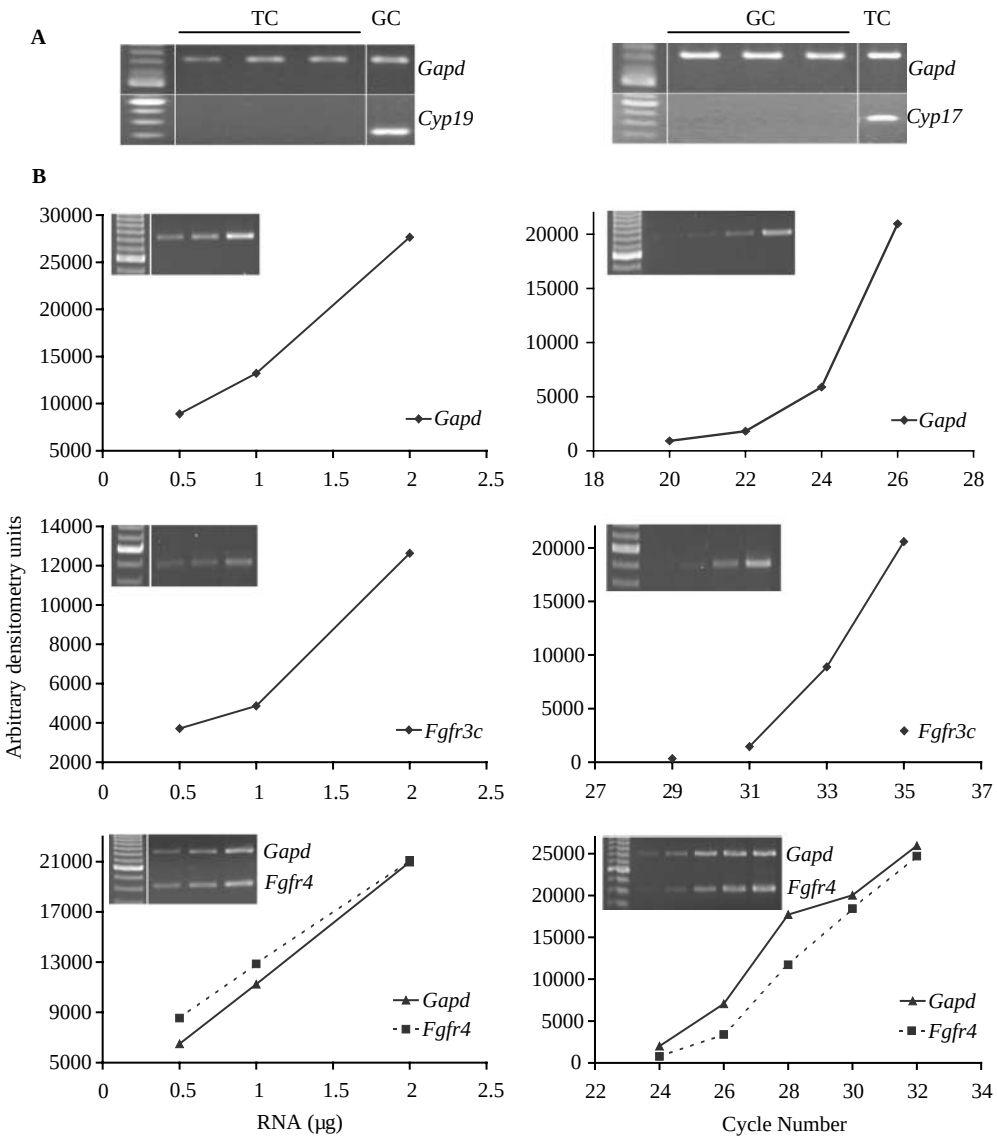

Figure 1 Validation of experimental techniques. (A) Agarose gel demonstrating purity of follicular cell types. *Cyp19* expression was evident in a granulosa cell sample (GC) but not in three representative theca cells (TC), and *Cyp17* was demonstrated in a theca sample but not in three representative granulosa samples. PCR was performed for 30 cycles. (B) Optimization of *Fgfr3c* and *Fgfr4* semi-quantitative PCR, showing that amplified DNA is dependent on RNA amount (at optimized cycle number for each gene) and cycle number (with 1 μg RNA). For analysis of *Fgfr3c* expression, target and housekeeping genes (*Gapd*) were amplified in separate reactions, whereas for *Fgfr4* the reaction with *Gapd* was performed in duplex. See Materials and Methods for cycling conditions.

to logarithms where not normally distributed. ANOVA was used to test effects of follicle size and estradiol concentration on *Fgfr3c* and *Fgfr4* mRNA levels, and the effect of FSH or IGF-I on granulosa cell *Fgfr3c* expression *in vitro*. For cell culture experiments, culture replicate was included as a random effect in the *F*-test. Means comparisons were performed by orthogonal contrasts. Linear correlations between gene expression and follicle estradiol content and size were assessed on untransformed data with Spearman's *r* coefficient. Data are presented as means ± S.E.M. Analyses were performed with JMP software (SAS Institute, Cary, NC, USA).

Results

Fgf8 mRNA was detected in pooled oocytes, and in granulosa and theca cells from individual follicles (Fig. 2A). Amplified DNA was sequenced from oocyte samples and confirmed to be authentic *Fgf8*.

Fgfr3c mRNA was detected in both granulosa and theca cell layers (Fig. 3) but not in oocytes (Fig. 2B). *Fgfr3c* expression in granulosa cells from healthy follicles increased significantly with follicle estradiol content (Fig. 3; $P < 0.05$), and was significantly correlated with estradiol content ($r = 0.66$, $n = 47$, $P < 0.001$). When healthy follicles were classified by diameter, granulosa cell *Fgfr3c* expression increased with size (Fig. 3). Granulosa cell recovery from atretic follicles was very low, and gene expression was not measured. Thecal *Fgfr3c* mRNA levels were not significantly affected by estradiol content or diameter (atretic and healthy follicles).

Fgfr4 mRNA was detected in theca cells but not in granulosa cells (Fig. 4) or oocytes (Fig. 2B). *Fgfr4* expression did not change with estradiol content of healthy follicles, but decreased significantly with increasing follicle size

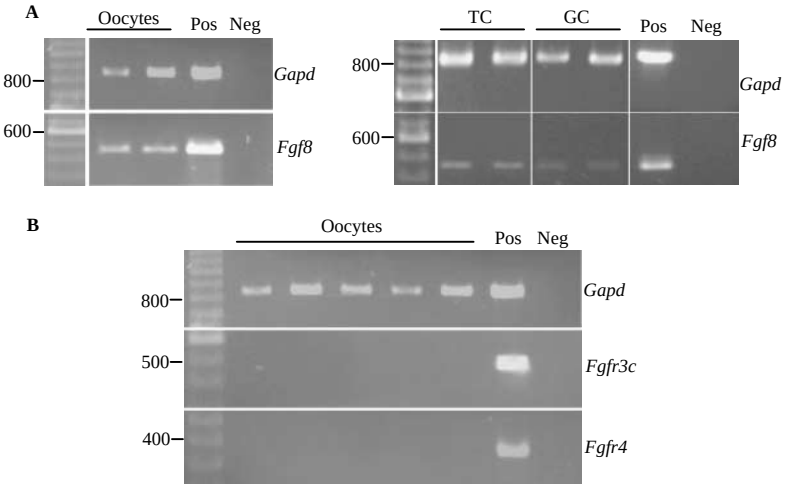

Figure 2 (A) *Fgf8* mRNA is expressed in bovine oocytes, granulosa and theca cells. These representative gels show PCR products for *Fgf8* and *Gapd* amplified from two representative pools of 50 oocytes, and from granulosa (GC) and theca cell (TC) samples from two representative individual follicles. (B) Bovine oocytes did not test positive for *Fgfr3c* or *FGFR4* mRNA. Data shown are of five pools of 50 oocytes each. Similar data were generated from pools of 100 oocytes (not shown). Positive (Pos; fetal ovary) and negative (Neg; water) PCR controls are also shown.

($r = -0.56$, $n = 47$, $P < 0.001$). Follicles smaller than 8 mm expressed more ($P < 0.05$) *Fgfr4* in theca cells compared with follicles 8–10 mm, which in turn expressed more than follicles greater than 10 mm diameter (Fig. 4). In addition, no or barely detectable *Fgfr4* gene expression was observed in theca cells from follicles containing more than 100 ng/ml progesterone.

As granulosa cell *Fgfr3c* mRNA abundance was affected by both follicle size and estradiol content, we determined if expression of this gene was under hormonal control. Granulosa cells were cultured for 6 days in non-luteinizing conditions (Sahmi *et al.* 2004). The expression of *Fgfr3c* increased ($P < 0.05$) upon addition of 1 ng/ml FSH, and did not increase further with higher doses of FSH (Fig. 5). In contrast, IGF-I resulted in a weak stimulation of *Fgfr3c* mRNA levels only at the highest dose used (Fig. 5).

Discussion

In rodents, the oocyte-specific expression of *Fgf8* (Valve *et al.* 1997) suggests that this is a potential paracrine

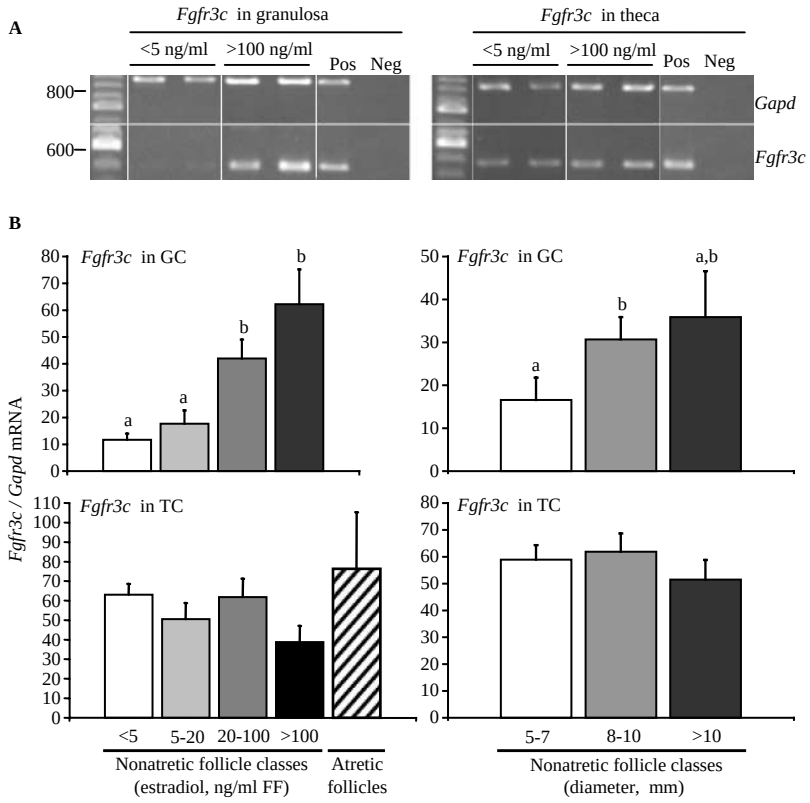

Figure 3 Developmental regulation of *Fgfr3c* expression in bovine antral follicles. (A) Gels showing target and housekeeping (*Gapd*) PCR products in granulosa and in theca cells of two representative follicles with low (<5 ng/ml) and high (>100 ng/ml) estradiol concentrations in follicular fluid (FF). Positive (Pos; fetal brain) and negative (Neg; water) PCR controls are also shown. (B) Mean ± S.E.M. relative *Fgfr3c* mRNA levels in granulosa (GC) and theca (TC) cells from non-atretic follicles classed according to FF estradiol content (n = 16, 10 and 6 follicles, respectively) and from atretic follicles (n = X), and from non-atretic follicles grouped by diameter (n = 12, 19 and 11 follicles, respectively). Means with different letters are significantly different ($P < 0.05$). Data for atretic follicles were derived from theca samples only, as insufficient granulosa cell RNA was recovered from atretic follicles.

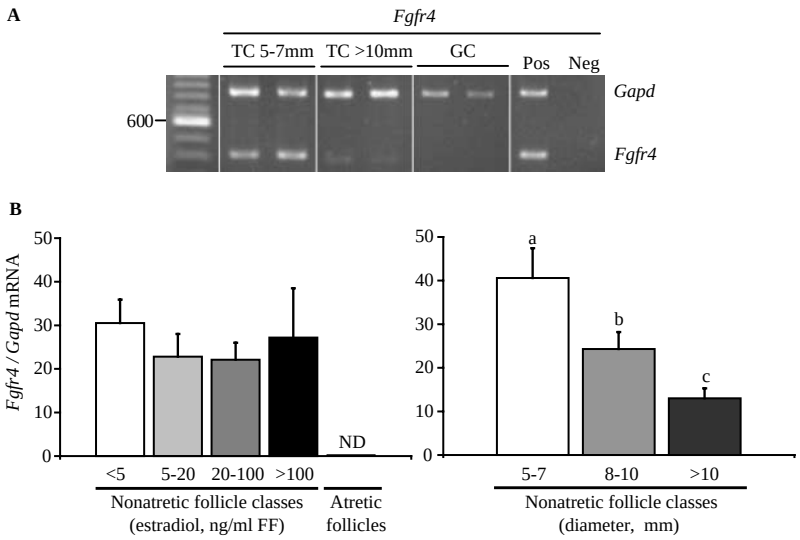

Figure 4 Effect of follicle size but not estradiol content on expression of *Fgfr4* in thecal cells of bovine antral follicles. (A) Gels showing target and housekeeping (*Gapd*) PCR products in theca cells (TC) of two representative medium sized (5–7 mm diameter) and two large (>10 mm diameter) follicles. *Fgfr4* was not amplified from granulosa cells (GC). Positive (Pos; fetal liver) and negative (Neg; water) PCR controls are also shown. (B) Mean ± S.E.M. relative *Fgfr4* mRNA levels in theca cells from non-atretic follicles classed according to follicular fluid (FF) estradiol content (n = 16, 10 and 6 follicles, respectively) and from atretic follicles (n = X), and from non-atretic follicles grouped by diameter (n = 12, 19 and 11 follicles, respectively). Means with different letters are significantly different ($P < 0.05$).

molecule signaling between the oocyte and follicle somatic cells. In this report we provide evidence that *Fgf8* is expressed in theca and granulosa cells as well as in oocytes in cattle. Owing to the low amount of RNA recovered from the oocyte pools used here, we could not determine the amount of RNA used in the reverse transcription. Accordingly, we increased PCR cycle number for *Fgf8* and the housekeeping gene *Gapd*, to produce a *Gapd* PCR product of intensity roughly equivalent to that observed in granulosa and theca cells. Assuming that *Gapd* mRNA levels are not dramatically different between the cell types, we conclude that *Fgf8* expression is not higher in oocytes compared with granulosa and theca cells in cattle, and therefore may not be an important oocyte-specific signaling molecule in ruminants. There are other discrepancies in *Fgf8* localization between species, as expression was not detected in human ovarian cortex by PCR (Valve *et al.* 2000), but the protein was found in human corpus luteum by immunohistochemistry (Zammit *et al.* 2002).

On the assumption of *Fgf8* expression in oocytes, we hypothesized that the principal FGF-8 receptors (FGFR3c and FGFR4) would be expressed in granulosa and/or theca cells. Very little is known about *Fgfr3c* gene expression in the ovary. A pan-*Fgfr3* riboprobe that did not distinguish the 'b' and 'c' splice variants did not detect *Fgfr3* mRNA in mouse ovary by *in situ* hybridization (Puscheck *et al.* 1997), and *Fgfr3c* expression was not detected in the human ovary by PCR (Valve *et al.* 2000). A pan-FGFR3 antibody localized protein to the granulosa layer in mice

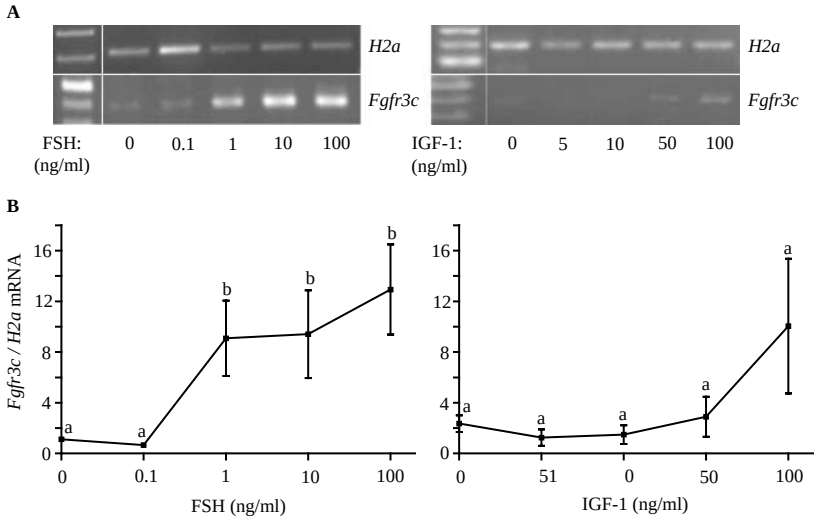

Figure 5 Regulation of *Fgfr3c* expression in granulosa cells by FSH *in vitro*. (A) Representative gels and (B) mean± S.E.M. relative *Fgfr3c* mRNA levels in granulosa cells cultured for 6 days in serum-free medium with the stated doses of FSH or IGF-I. Expression of *H2a* was used as an internal control in RT-PCR. Means with different letters are significantly different ($P < 0.05$). Data are derived from three independent experiments (n = 3).

(Amsterdam *et al.* 2001). In the present study, we demonstrate *Fgfr3c* expression in granulosa and theca cells of bovine antral follicles. Whereas the expression of *Fgfr3c* in theca cells was relatively stable, expression increased during follicle development (based on follicle size and estradiol content) in granulosa cells, consistent with a role for FGFR3c signaling during antral follicle growth. Functional studies were performed to determine if *Fgfr3c* expression is regulated, and the data show that *Fgfr3c* expression is clearly increased by FSH. It is of relevance that near maximal levels of *Fgfr3c* expression were observed at physiological doses of FSH, as observed also for *Cyp19* and 17β-hydroxysteroid dehydrogenase genes (Sahmi *et al.* 2004). Certain FSH-responsive endpoints are also stimulated by IGF-I, including steroid and inhibin-A secretion and *Cyp19* expression (Gutiérrez *et al.* 1997, Glister *et al.* 2001, Spicer *et al.* 2002); however, the present data clearly demonstrate that *Fgfr3c* expression is not under IGF-I control. The physiological importance of *Fgfr3c* regulation by FSH in granulosa cells remains to be clarified, but as regulation of *Fgfr3c* expression by estradiol has been demonstrated in the human endometrium (Wing *et al.* 2003), changes in receptor expression may play a role in regulating signal transduction.

Fgfr4 gene expression has previously been detected in mouse follicles and human ovaries (Puscheck *et al.* 1997, Valve *et al.* 2000). In the present study, *Fgfr4* expression was localized specifically to the theca cell layer, which is in contrast to data from the mouse showing expression only in granulosa cells (Puscheck *et al.* 1997). *Fgfr4* gene expression was highest in small follicles and decreased as follicles reached the size of dominant follicles, suggesting a role for FGFR4 signaling in theca cells during early growth of antral follicles. It is interesting to note that *Fgfr4* expression was reduced to very low or undetectable levels in the theca of atretic follicles, suggesting that this gene is downregulated during atresia. The present studies are unavoidably limited by the lack of antibodies available for protein detection. No isoform-specific antibodies are available for FGFR3, and commercial antibodies against FGFR4 do not adequately recognize protein in bovine tissues (Buratini *et al.* 2005).

Which ligand(s) might activate FGFR3c and FGFR4 in follicles remains to be clarified. FGF-1, -2, -4, -8, -9 and -13 efficiently activate FGFR3c and -4 (Ornitz *et al.* 1996, Greene *et al.* 1998). In cattle, *Fgf1* and *Fgf2* expression was predominantly localized to theca cells (Berisha *et al.* 2004), and *Fgf2* expression increased with estradiol content (Berisha *et al.* 2004) as did the expression of *Fgfr3c* in granulosa cells in the present study. FGF2 binding is not very specific, in the sense that it activates receptors 1, 2c, 3c and 4 (Ornitz *et al.* 1996). *Fgfr1* and *Fgfr2c* appear not to be developmentally regulated in bovine follicles (Berisha *et al.* 2004), whereas *Fgfr3c* is (present report). The actions of FGFs at different stages of follicle growth clearly warrant further study.

Taken together, the present data suggest that signaling pathways activated by FGFR3c and FGFR4 play roles during follicle growth. Specific signaling may be modulated by changes in ligand or receptor levels. The present data clearly demonstrate that *Fgfr3c* gene expression increases in healthy follicles as follicle estradiol content increases, and *Fgfr3c* expression is upregulated by FSH. This important observation suggests that FSH may sensitize granulosa cells to one or more FGFs during early growth of the antral follicle, and may permit continued growth of the follicle in the low-FSH environment that follows follicle deviation (see Fortune *et al.* 2001, Ginther *et al.* 2001).

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