BIOLOGY OF REPRODUCTION **77**, 743–750 (2007)
Published online before print 20 June 2007.
DOI 10.1095/biolreprod.107.062273

Expression and Function of Fibroblast Growth Factor 10 and Its Receptor, Fibroblast Growth Factor Receptor 2B, in Bovine Follicles[1]

J. Buratini Jr.,[2,3] M.G.L. Pinto,[4] A.C. Castilho,[3] R.L. Amorim,[5] I.C. Giometti,[4] V.M. Portela,[6] E.S. Nicola,[6] and C.A. Price[6]

Departamento de Fisiologia[3], Instituto de Biociências, Universidade Estadual Paulista, Botucatu, São Paulo, CEP 18618-000, Brazil
Departamento de Reprodução Animal[4] and Departamento de Clínica Veterinária,[5] Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Botucatu, São Paulo, CEP 18618-000, Brazil
Centre de Recherche en Reproduction Animale,[6] Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, Québec, Canada J2S 7C6

Downloaded from https://academic.oup.com/biolreprod/article/77/4/743/2629786 by guest on 21 August 2022

ABSTRACT

Some fibroblast growth factors (FGFs) affect ovarian follicle cell growth and/or differentiation. Whereas many FGFs activate several FGF receptors, FGF7 and FGF10 primarily activate only one, FGFR2B. As FGF7 is produced by bovine theca cells and acts on granulosa cells, we tested the hypothesis that FGF10 may also play a role in folliculogenesis in cattle. Reverse transcription-polymerase chain reaction demonstrated the presence of *FGF10* mRNA in the oocytes and theca cells of the antral follicles, as well as in the preantral follicles. FGF10 protein was detected by immunohistochemistry in the oocytes of the preantral and antral follicles, and in the granulosa and theca cells of the antral follicles. *FGF10* expression in theca cells changed during follicle development; mRNA abundance decreased with increasing follicular estradiol concentration in healthy follicles, and was lowest in highly atretic follicles. Culturing of granulosa cells in serum-free medium revealed FSH regulation of FGF10 receptor expression. The addition of FGF10 to cultured granulosa cells decreased the level of estradiol but did not alter cell proliferation. These data support a role for FGF10 in signaling to granulosa cells from theca cells and/or the oocyte.

fibroblast growth factors, follicle, granulosa cells, growth factors, theca cells

INTRODUCTION

Ovarian follicle growth is orchestrated by complex interactions between the three main cell types within the follicle: theca cells, granulosa cells, and the oocyte. There is considerable evidence to indicate that members of the transforming growth factor β superfamily are mediators of paracrine signaling within the follicle [1]. For example, growth/differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are produced by oocytes and affect bovine granulosa cell function [2–6], while BMP4 and BMP7 are theca proteins that alter bovine granulosa cell function [7, 8]. Fibroblast growth factors (FGFs) are also potential paracrine signaling molecules. In the bovine ovary, the *FGF7* gene is expressed principally or exclusively in theca cells, although expression appears not be regulated during development of nonatretic follicles [9, 10]. The receptor for FGF7 is the ‘b’ splice variant of the *FGFR2* gene [11], referred to herein as *FGFR2B*, which is expressed predominantly in granulosa cells and is upregulated during follicle development [9, 10].

Another FGF that activates FGFR2B is FGF10, which is structurally similar to FGF7 [12, 13]. In mice, FGF10 is essential for normal development of lungs, limbs, teeth, mammary buds, stomach and heart [14–18]. We hypothesized that FGF10 signaling to FGFR2B plays a role in follicle development, although nothing is known about the expression of *FGF10* in the ovary or the biological role of the protein. The first objective of the present study was to examine the developmental and spatial patterns of *FGF10* expression in the bovine follicle. We also measured *FGF7* mRNA to determine if there were any differences in the expression patterns of these two genes. As *FGFR2B* gene expression changes during follicle development, the second objective was to determine whether *FGFR2B* expression in granulosa cells is regulated by FSH or IGF1. Lastly, we investigated whether FGF10 acts upon nonluteinizing granulosa cells in serum-free culture.

[1]Supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil, and the Natural Sciences and Engineering Research Council of Canada.
[2]Correspondence: FAX: 55 14 38116251;
e-mail: buratini@ibb.unesp.br

Received: 15 April 2007.
First decision: 5 May 2007.
Accepted: 7 June 2007.
© 2007 by the Society for the Study of Reproduction, Inc.
ISSN: 0006-3363. http://www.biolreprod.org

MATERIALS AND METHODS

Tissues

A variety of tissues was collected from fetal and adult cows. Adult tissues and whole fetuses were obtained from an abattoir located near the Sao Paulo State University campus in Botucatu and transported to the laboratory in saline on ice. Samples were homogenized in TRIzol (Invitrogen), and stored at −70°C until RNA extraction.

Preantral follicles were harvested from pairs of ovaries obtained from individual fetuses using a Tissue Chopper (Mickle Laboratory Engineering Co.), as described previously [19]. The resulting follicle suspension was examined under an inverted microscope and primordial, primary, and secondary follicles without obvious stroma investment were separated with a capillary pipette [20]. Once isolated, the preantral follicles were pooled within each class (primordial, primary, and secondary) for each fetus, homogenized in 500 μl TRIzol with a Polytron, and stored at −70°C. Total RNA was extracted according to the TRIzol protocol.

Antral follicles were obtained from the ovaries of adult cows. Follicles of diameter ≥5 mm were dissected from the ovaries, and 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 × *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 a forceps and washed in saline by repeated passages through a 1-ml syringe. The samples were collected in TRIzol and homogenized with a

Polytron. Total RNA was extracted immediately according to the TRIzol protocol.

Follicles were classed according to estradiol:progesterone (E:P) ratios of >1, 1–0.01, and <0.01 [21], which we defined as healthy, transitional, and highly atretic, respectively [22]. Follicles were subgrouped by size (5–7 mm, 8–10 mm, and >10 mm in diameter) and by follicular fluid estradiol concentration (<5, 5–20, >20–100, and >100 ng/ml [23]). Cross-contamination of theca and granulosa cells was tested by PCR detection of the mRNAs that encode cytochrome P450 aromatase (CYP19A1) and 17α-hydroxylase (CYP17A1) in each sample, as described previously [24]. The detection of *CYP19A1* amplicons in theca samples or of *CYP17A1* amplicons in granulosa samples indicated cross-contamination, and these samples were discarded.

Cumulus-oocyte-complexes were aspirated from antral follicles, and cumulus cells were removed from the oocyte by vortexing. Pools of 50 oocytes were collected, and RNA was extracted with the RNeasy kit (Qiagen).

Cell Culture

Granulosa cell culturing was performed as described [25], with modifications [26]. All materials were obtained from Invitrogen Life Technologies, except where otherwise stated. Follicles (≤5 mm in diameter) were dissected from slaughterhouse ovaries, and those with obvious signs of atresia (i.e., avascular theca, debris in the antrum) were discarded. Cells were collected by repeatedly passing the follicle wall through a pipette, washing twice by centrifugation at 980 × *g* for 20 min each, and suspension in α-MEM that contained Hepes (20 mM), sodium bicarbonate (10 mM), sodium selenite (4 ng/ml), BSA (0.1%; Sigma-Aldrich), penicillin (100 IU/ml), streptomycin (100 μg/ml), transferrin (2.5 μg/ml), nonessential 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 by 0.4% Trypan Blue staining. Cells were seeded into 24-well tissue culture plates (Corning) at a density of 10^6/well in 1 ml of medium. The cultures were maintained at 37°C in 5% CO_2 in air for 6 days, with 700 μl of medium being replaced every 2 days.

To determine the regulation of *FGFR2B* expression, cells were stimulated with FSH (AFP-5332B, NIDDK) or IGF1 analog (LR3; Sigma-Aldrich) at the doses listed in *Results*. At the end of the culture period, cells were collected in TRIzol and stored at −70°C until RNA extraction. To determine the effect of FGF10 on granulosa cells, cells were cultured with FSH (1 ng/ml) and graded doses of recombinant human FGF10 (PeproTech). On Day 6 of culture, the medium was removed for steroid assay and the cells were lyzed with 100 μl of 1 N NaOH for 2 h followed by neutralization with 100 μl of 1 N HCl for total cell protein measurement with the Bradford protein assay (Bio-Rad). Cell proliferation was measured with the MTT assay (R&D Systems). Experiments were performed on three independent culture replicates.

Reverse Transcription-Polymerase Chain Reaction

The primer sequences for *FGF7* and *FGFR2B* were as published and validated for bovine granulosa and theca cells [10, 11]. The primer sequences for *FGF10* were those used previously in sheep [27]. The primers for housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; for follicle samples) and histone 2 H2aa1 (*HIST2H2AA1*; for cell culture samples) have been described previously [24]. All of the primers spanned at least one intron and all of the PCR reactions produced amplicons of the expected size, demonstrating the absence of contamination by genomic DNA.

Expression of *FGF7* and *FGF10* was surveyed in fetal and adult bovine tissues by RT-PCR. Total RNA (1 μg) was incubated with DNase I (Invitrogen), and then reverse-transcribed with SuperScript II (Invitrogen) and oligo(dT) primer. PCR was performed with 1 μl cDNA in a PCR master mix that contained 1.6 U Taq DNA polymerase (Invitrogen), 0.4 μM 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 40 cycles of denaturing at 94°C for 45 sec, annealing at 60°C (for *FGF10* and *GAPDH*) or 65°C (for *FGF7*) for 45 sec, and extension at 70°C for 1 min.

Expression of *FGF10* in isolated granulosa and theca cells was measured by semiquantitative RT-PCR, as described above but in a duplex reaction with *GAPDH* primers (0.06 μM) for 30 cycles. *FGF7* mRNA was measured in simplex reactions with 32 cycles for *FGF7* and 24 cycles for *GAPDH*. Semiquantitative RT-PCR was validated by choosing the number of PCR cycles and amount of RNA within the linear range of the amplification curve.

All PCR reactions were performed with positive (fetal lung) and negative (water replacing cDNA) controls. PCR products were separated on 1.5% agarose gels and stained with ethidium bromide, and the bands were quantified by densitometry (Image Gauge; Fuji Photo Film Co.). The authenticity of the amplified *FGF10* cDNA was verified by sequencing the amplicons from bovine oocyte and fetal lung samples; the bovine sequence (GenBank accession no. AY183659) is 98% homologous with the published sheep sequence (NM_001009230).

The RNA yield from oocytes was too low to be quantified accurately by spectrometry, so 8-μl aliquots of RNA were reverse-transcribed with SuperScript III (Invitrogen). PCR was performed with 1 μl cDNA and 1 U Platinum Taq (Invitrogen) for 40 cycles (for *FGF7* and *FGF10*) or 30 cycles (for *GAPDH*).

For the analysis of *FGFR2B* expression in cultured granulosa cells, RNA was reverse-transcribed with Omniscript (Qiagen), and semiquantitative RT-PCR was performed as described for freshly harvested granulosa cells, with the exceptions that the PCR was performed with Platinum Taq for 38 cycles and that *HIST2H2AA1* was used as the housekeeping gene (30 cycles).

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 30 min, and hydrated in successive 5-min washes in 95% and 85% ethanol. Formaldehyde was washed from the sections by incubation with 10% ammonium hydroxide in ethanol for 10 min, followed by washing in distilled water. Antigen retrieval was achieved by incubating the sections in 10 mM EDTA (pH 8.0) at 96°C for 25 min. Endogenous peroxidase was quenched by incubation in hydrogen peroxide for 20 min, followed by two rinses for 5 min in distilled water and one rinse in 0.5 M Tris (pH 7.4).

Tissue sections were blocked in 5% BSA in Tris for 1 h at room temperature in a humidified chamber, and then incubated with polyclonal anti-FGF10 antibody (H-121, 1:200 or 1 μg/ml; Santa Cruz Biotechnology) overnight at 4°C. Slides were washed twice for 5 min in Tris, and then incubated with horseradish peroxidase-conjugated secondary antibody (EnVision Dual Link System; DakoCytomation). Immunostaining was developed with liquid DAB (DakoCytomation). Negative controls involved omission of the primary antibody from the procedure or preadsorption of the primary antibody (1 μg/ml) with human recombinant FGF10 (2 μg/ml) overnight at 4°C before application to the slides.

Steroid Assays

Estradiol and progesterone were assayed in follicular fluids using iodinated tracers and the antibodies furnished in the Third Generation Estradiol RIA and Progesterone RIA kit (Diagnostic Systems Laboratories Inc.), respectively, with a revised protocol [24]. The standard curves and samples were diluted in PBS-gelatin. The intraassay and interassay coefficients of variation were 7.4% and 13.5%, respectively, for estradiol, and 6.8% and 7.0%, respectively, for progesterone. The sensitivities of the assays were 0.3 ng/ml for estradiol and 0.2 ng/ml for progesterone.

Estradiol was measured in culture medium with the RIA as described but without solvent extraction [28], and progesterone was measured as described [29]. All samples were run in a single assay for each steroid, with intra-assay coefficients of variation of 8.5% and 7.2% for estradiol and progesterone, respectively. The sensitivities of these assays were 10 pg and 4 pg per tube for estradiol and progesterone, which are equivalent to 0.3 and 20 ng/μg protein, respectively.

Statistics

Target gene mRNA abundance is expressed relative to the level of *GAPDH* (for follicles) or *HIST2H2AA1* (granulosa cell cultures) mRNA. Estradiol secretion in vitro was corrected for cell number by expression per unit mass of total cell protein. The data were transformed to logarithms if not normally distributed. ANOVA was used to test for the main effects of follicle class, size, and estradiol concentration on *FGF7* and *FGF10* mRNA levels in theca cells, the effects of FSH and IGF1 on *FGFR2B* mRNA levels in granulosa cells in vitro, and the effect of FGF10 protein on granulosa cell estradiol secretion and proliferation in vitro. For the cell culture experiments, culture replicate was included as a random effect in the F-test; owing to the significant effect of replicate on estradiol secretion, the data are expressed relative to the control (no FGF10). Means comparisons were performed with the Tukey-Kramer HSD test. Data are presented as means ± SEM. Analyses were performed with the JMP software (SAS Institute, Cary, NC).

RESULTS

FGF7 and *FGF10* were widely expressed in two bovine fetuses (Fig. 1). The only tissue examined that lacked expression of *FGF10* in both fetuses was the liver, although

Downloaded from https://academic.oup.com/biolreprod/article/77/4/743/2629786 by guest on 21 August 2022

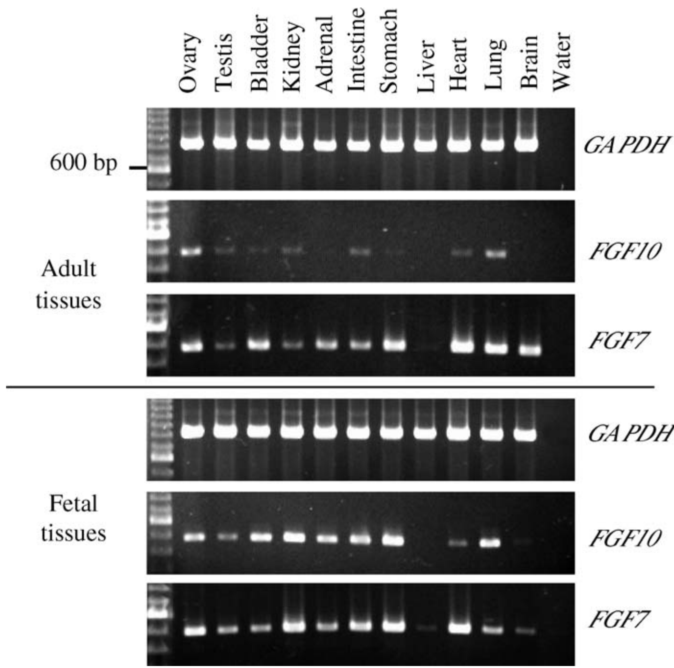

FIG. 1. Expression of *FGF7* and *FGF10* genes in a variety of fetal and adult bovine tissues. The tissues were obtained from an abattoir and mRNA was subjected to RT-PCR for 40 cycles (qualitative PCR). The gel shows one of two adults and one of two fetuses examined. The housekeeping gene *GAPDH* was also amplified, to verify the integrity of the RNA, and water was used to replace the cDNA template as a negative control for PCR.

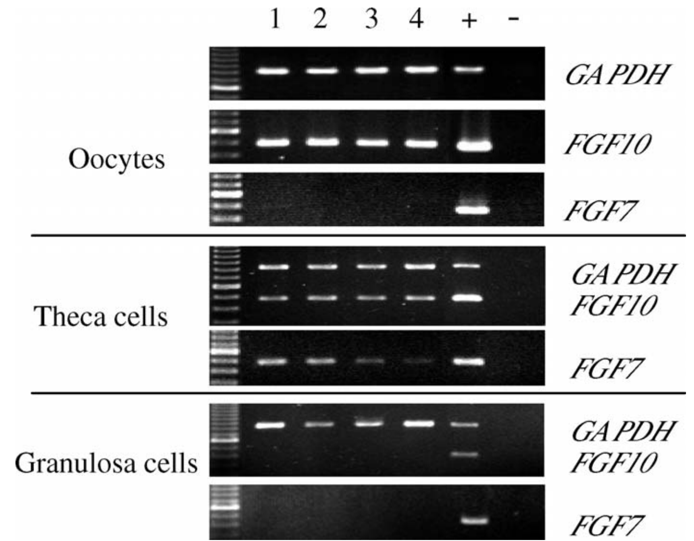

FIG. 2. Expression of *FGF7* and *FGF10* genes in bovine oocytes, theca, and granulosa cells. These representative gels show the RT-PCR products for *FGF7*, *FGF10*, and *GAPDH* mRNAs in pools of 50 oocytes, and in granulosa (GC) and theca cells (TC) from four individual follicles (lanes 1–4). PCR amplification of the theca and granulosa cell cDNAs was performed under semiquantitative conditions (30 cycles for *FGF10* and 32 for *FGF7*), whereas oocyte cDNA was amplified for 40 cycles. Positive (+; fetal lung) and negative (–; water) PCR controls are also shown.

one fetus also failed to show expression in the brain (Fig. 1). Expression in adult tissues followed a similar distribution but was less readily detectable (Fig. 1). In the ovary, *FGF7* mRNA was evident in the theca cells but not in the granulosa cells or oocytes. *FGF10* mRNA was detected in theca cells and oocytes but not in granulosa cells (Fig. 2). *FGF10* and *FGF7* mRNAs were expressed in primordial, primary, and secondary follicles, all of which showed robust *GAPDH* mRNA expression (Fig. 3).

Immunohistochemistry revealed the presence of FGF10 protein in the oocytes of the preantral and antral follicles, as well as in the theca and granulosa cell layers of the antral follicles (Fig. 4). A low level of staining was observed in stromal tissues, including blood vessels. No staining was observed in the absence of primary antibody or with primary antibody that was preadsorbed with FGF10 (Fig. 4).

Developmental regulation of *FGF7* and *FGF10* expression was assessed by measuring the mRNA levels in the theca cells of follicles classified according to size and follicular fluid steroid concentration. Estradiol concentrations were higher in healthy follicles compared to transitional follicles ($P < 0.001$), which in turn had higher levels than the highly atretic follicles (Table 1; $P < 0.001$). Although the progesterone concentrations were not different between healthy and transitional follicles, they were significantly higher in highly atretic follicles ($P < 0.001$). Mean follicle diameter did not differ between the groups (Table 1). ANOVA indicated that *FGF7* expression was not significantly affected by the E:P ratio (Table 1), estradiol concentration or diameter. However, *FGF10* expression was significantly affected by the E:P ratio and estradiol concentration. Consequently, follicles were classified by estradiol concentration within the E:P groups. *FGF10* mRNA abundance was highest in the transitional follicles that contained <5 ng/ml estradiol and lower in the follicles that contained 5–20 ng/ml or 20–100 ng/ml estradiol. No transitional follicles contained >100 ng/ml estradiol. Healthy follicles that contained 20–100 ng/ml estradiol expressed levels of *FGF10* mRNA that were similar to those of the transitional follicles in the same estradiol group, and expressed more *FGF10* than healthy follicles that contained >100 ng/ml estradiol. No healthy follicles contained <20 ng/ml estradiol. The lowest *FGF10* expression was observed in highly atretic follicles (Table 1, Fig. 5A), none of which contained >5ng/ml estradiol. Within a follicle group, there was no effect of diameter on *FGF10* expression (Fig. 5B).

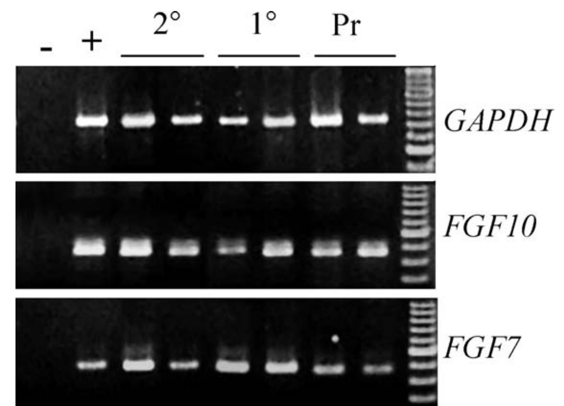

FIG. 3. Detection of *FGF7* and *FGF10* mRNAs in preantral bovine follicles. The follicles were mechanically dispersed from fetal ovaries, and pools of primordial (Pr), primary (1°) and secondary (2°) follicles were prepared for each fetus. The entire RNA sample from each pool was subjected to RT-PCR for 40 cycles. These representative gels show the results for two fetuses, with positive (+; fetal lung) and negative (–; water) PCR controls.

Downloaded from https://academic.oup.com/biolreprod/article/77/4/743/2629786 by guest on 21 August 2022

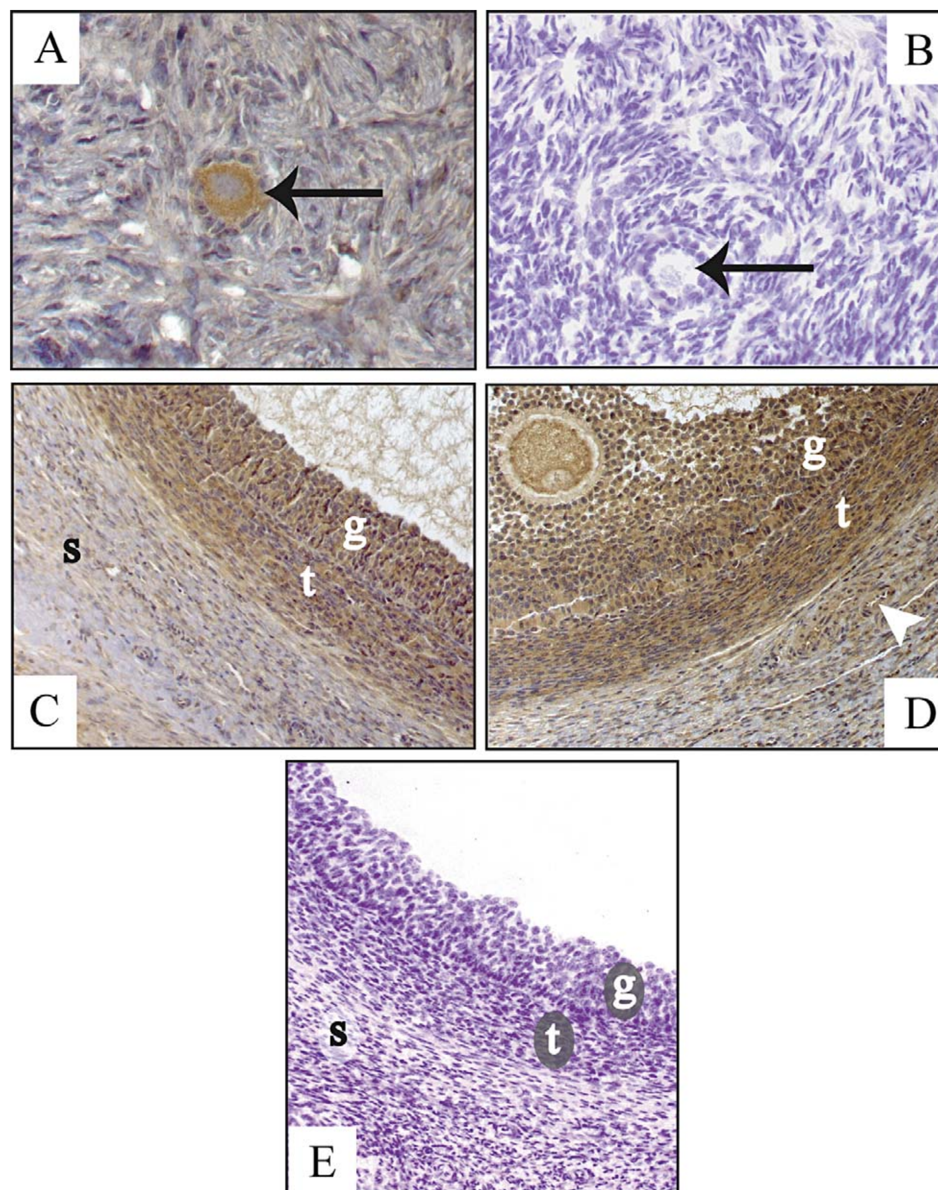

FIG. 4. Immunohistochemical localization of FGF10 protein in the bovine ovary. FGF10 was detected in the oocytes of preantral and antral follicles (**A**, **D**), as well as in the theca (t) and granulosa (g) cell layers of the antral follicles (**C**, **D**). Staining was weak in the ovarian stroma (s) and absent in sections stained with primary antibody that was preadsorbed with FGF10 (**B**, **E**). Original magnification ×640 (**A**, **B**) or ×200 (**C**, **D**, **E**). Arrows indicate oocytes in preantral follicles; white arrowhead indicates blood vessels.

The gene that encodes FGFR2B, which is the major receptor for FGF7 and FGF10, was expressed in cultured granulosa cells. There were significant and opposing effects of FSH and IGF1 on *FGFR2B* expression, i.e., FSH increased ($P < 0.01$) and IGF1 decreased *FGFR2B* mRNA abundance in cells that were cultured for 6 days in serum-free medium ($P < 0.05$) (Fig. 6).

To assess the role of FGF10 signaling, FSH-stimulated granulosa cells were cultured in the presence of graded doses of FGF10. Estradiol secretion was inhibited ($P < 0.05$; Fig. 7) by FGF10 in a dose-dependent manner, whereas cell proliferation was not affected (Fig. 7).

DISCUSSION

There is increasing evidence for FGF participation in follicle development, including roles for FGF2 [30] and FGF7 [9]. In the present report, we present four lines of evidence that FGF10 is also involved in follicle growth. First, both the mRNA and protein encoded by the *FGF10* gene were detected in oocytes and theca cells. Second, theca expression of *FGF10* changed with follicle health. Third, granulosa cell expression of *FGFR2B*, which is the major receptor for FGF10, was regulated by FSH, and fourth, FGF10 inhibited estradiol secretion from cultured granulosa cells.

TABLE 1. *FGF7* and *FGF10* gene expression in healthy and atretic bovine follicles.[a]

Follicle class (n)[b]	Estradiol (ng/ml)	Progesterone (ng/ml)	Diameter (mm)	*FGF10* mRNA	*FGF7* mRNA
Healthy (16)	198 ± 61[c]	26 ± 6[c]	9.8 ± 0.7[c]	68 ± 11[c]	46 ± 9[c]
Transitional (43)	11 ± 2[d]	41 ± 7[c]	8.6 ± 0.4[c]	143 ± 14[d]	84 ± 15[c]
Highly atretic (6)	0.05 ± 0.02[e]	404 ± 68[d]	10.3 ± 0.5[c]	5 ± 1[e]	92 ± 41[c]

[a] Values are mean ± SEM.
[b] n = Number of follicles.
[c–e] Means with different superscripts are significantly different ($P < 0.05$).

Downloaded from https://academic.oup.com/biolreprod/article/77/4/743/2629786 by guest on 21 August 2022

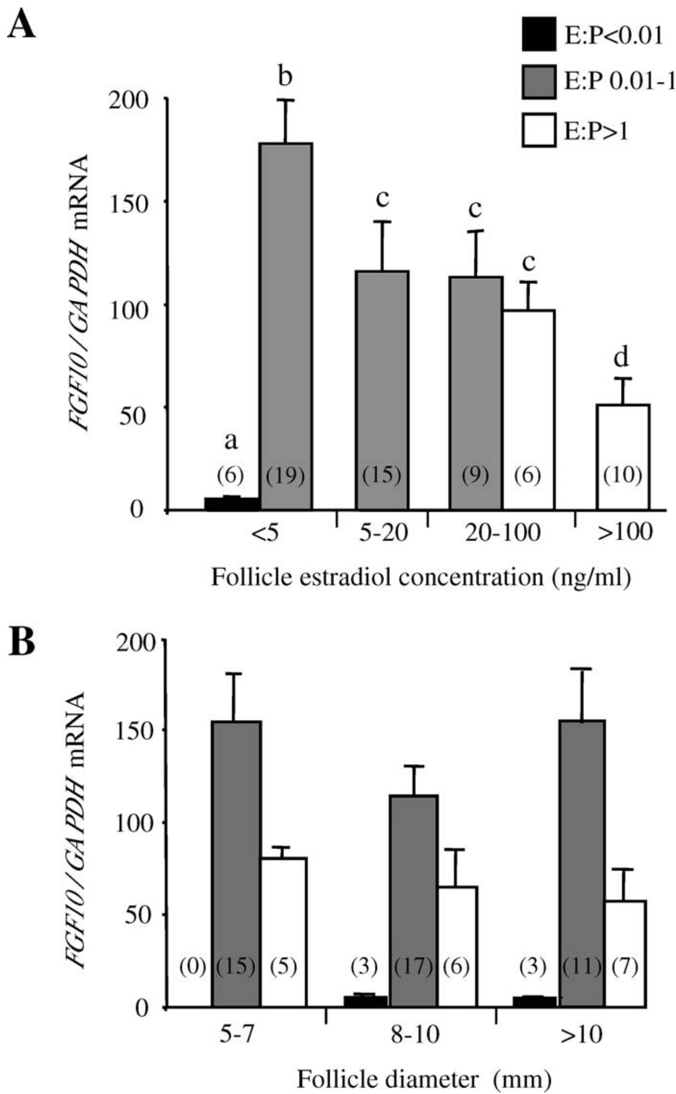

FIG. 5. Developmental regulation of *FGF10* expression in bovine theca cells. Follicles obtained from an abattoir were grouped according to the follicular estradiol:progesterone (E:P) ratio, and the effects of estradiol concentration in the follicular fluid (**A**) and follicle size (**B**) were tested by ANOVA. Gene expression was measured by semiquantitative RT-PCR (30 cycles). Data shown are mean (± SEM) of the *FGF10* mRNA levels expressed relative to the levels of the housekeeping gene *GAPDH*. The number of follicles analyzed in each group is given in parentheses. Means with different letters are significantly different ($P < 0.05$).

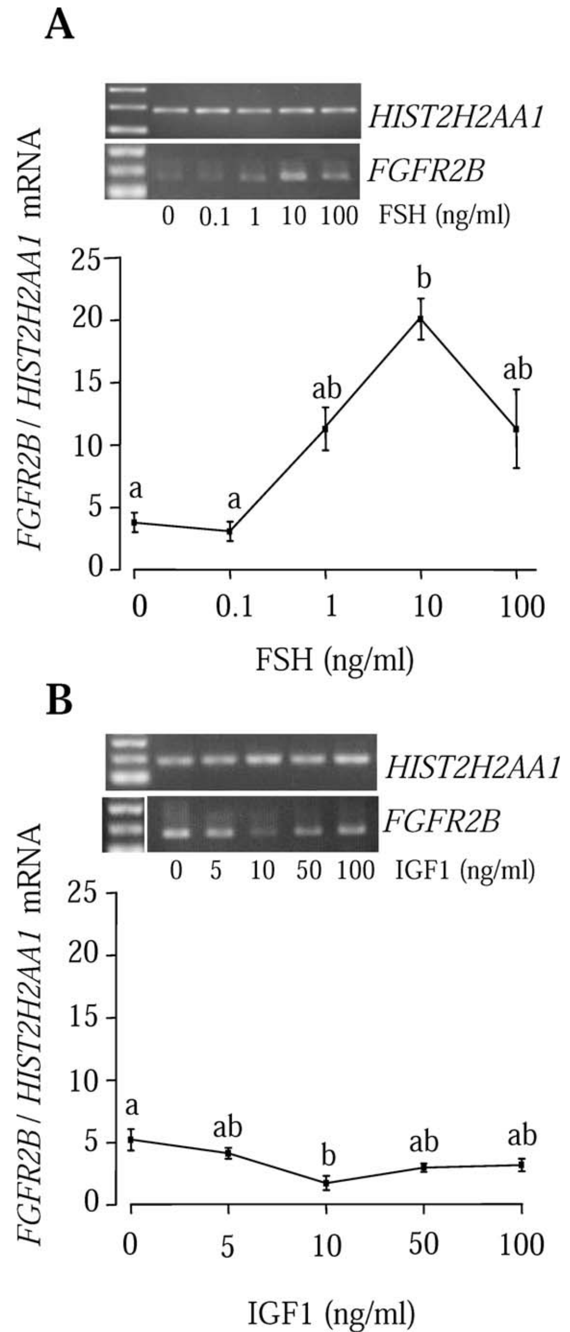

FIG. 6. In vitro regulation of *FGFR2B* expression in granulosa cells by FSH (**A**) or IGF1 (**B**). Representative gels and the mean (± SEM) *FGFR2B* mRNA levels (relative to the housekeeping gene *HIST2H2AA1*) in granulosa cells from small follicles (2–5 mm) cultured for 6 days in serum-free medium with the stated doses of FSH or IGF1 are shown. RT-PCR was performed under semiquantitative conditions (38 cycles for *FGFR2B* and 30 cycles for *HIST2H2AA1*). The molecular weight standard of the gel *FGFR2B* in panel B was spliced to position it adjacent to the representative samples in the gel. Means with different letters are significantly different ($P < 0.05$, n = 3).

We have demonstrated for the first time that the *FGF10* gene is expressed in the ovary. *FGF7* and *FGF10* mRNAs were detected in theca cells, consistent with the mesenchymal origin of these growth factors [12, 31]. The expression of *FGF10* was confirmed by localizing FGF10 protein to the theca layer. This protein was also detected in the granulosa layer, whereas the associated mRNA was not. This discrepancy may be explained by the internalization of FGF10 after binding to its receptor, which is expressed by granulosa cells ([9, 10] and present study). This is supported by evidence for receptor-mediated internalization of exogenous FGF1 and FGF2 in several cell lines [32, 33], and of most relevance to the present work is the internalization of FGF7 after binding to FGFR2B in NIH3T3 fibroblasts [34, 35]. FGF10 detection in the granulosa appears to be specific, as preincubation of the anti-FGF10 antibody with FGF10 abolished staining, although we cannot rule out the possibility that this antibody cross-reacts with other bovine FGFs.

Of great interest is the presence of FGF10 protein in the oocytes of preantral and antral follicles and the detection of *FGF10* mRNA in isolated oocyte pools and preantral follicles. Thus, this member of the FGF family joins FGF2 [36] and FGF8 [24, 37] in the group of potential oocyte-derived signaling molecules. In contrast, we show in the present study that *FGF7* is expressed in bovine preantral follicles but not in

Downloaded from https://academic.oup.com/biolreprod/article/77/4/743/2629786 by guest on 21 August 2022

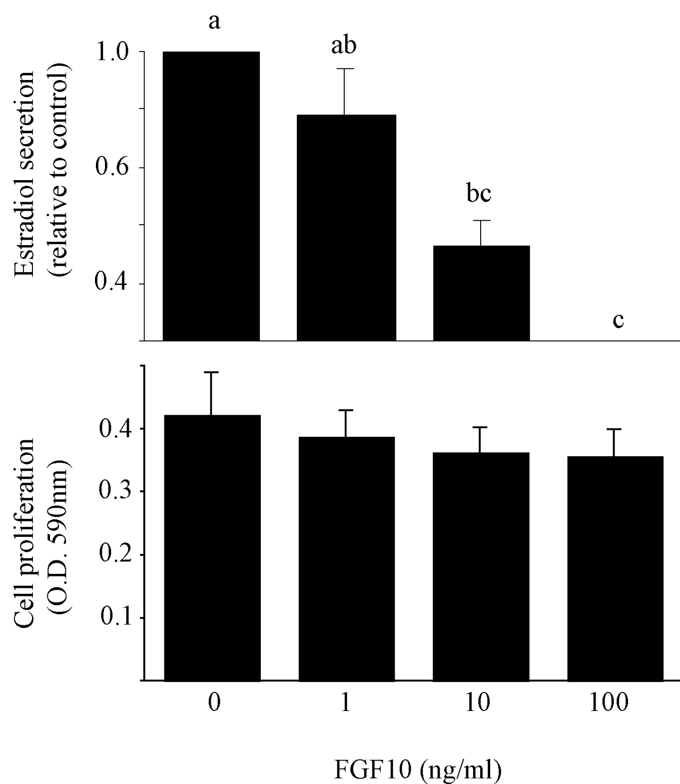

FIG. 7. Effect of FGF10 on granulosa cell estradiol secretion (upper panel) and proliferation (lower panel). Cells from small follicles (2–5 mm in diameter) were cultured in serum-free medium for 6 days with 1 ng/ml FSH and with graded doses of FGF10. Estradiol secretion was corrected for cell number (total protein) and expressed relative to the control values (1.0). Cell proliferation was assessed with the MTT assay. Mean (± SEM) values were derived from three independent replicates. Means with different letters are significantly different ($P < 0.05$, n = 3).

oocytes. Although the preantral follicles used in the present study were carefully selected [20], we cannot discount the possibility that *FGF7* mRNA arises from contamination with stromal cells, as suggested by the presence of FGF7 protein in rodent ovarian stroma but not in oocytes [38]. It has been proposed that stromal cell FGF7 signals via FGFR2B to promote the activation of primordial follicles in rats [38]. In light of the considerable FGF10 immunostaining detected in the oocytes of the preantral follicles, we propose that oocyte-derived FGF10 promotes follicle activation in cattle. This is difficult to test directly, as a high percentage of bovine primordial follicles spontaneously activate in vitro [39, 40], which precludes the study of factors that stimulate primordial follicle transition.

The expression of *FGF10* in theca cells was developmentally regulated. We classified the follicles with E:P ratios >1 as healthy and those with E:P ratios <0.01 as highly atretic, which are widely recognized criteria [21]. We defined those follicles with E:P ratios of 0.01–1.0 as transitional, based on the report of Grimes and Ireland [22], who described these follicles as progressing from the nonatretic to atretic stage. However, since smaller growing follicles also have E:P ratios of 0.01–1.0 [41, 42], the transitional follicle group probably includes both growing and early atretic follicles. For both transitional and healthy follicles, *FGF10* mRNA abundance decreased as the estradiol concentration increased, which suggests that *FGF10* plays a role during growth of the smaller, less-estrogenic follicles. Highly atretic follicles displayed a marked reduction in *FGF10* expression, which suggests that this gene is downregulated in theca cells during atresia. This is in contrast to the expression of *FGF7*, which was not altered by the E:P ratio or estradiol concentration in the follicles ([10], and present study), although it has been reported that *FGF7* expression is higher in follicles of >10 mm in diameter than in smaller follicles [9]. The pattern of *FGF10* expression in theca cells also differed from the expression patterns observed for *FGF1*, which did not change with estradiol concentration in bovine antral follicles, and *FGF2*, which increased with estradiol concentration [10]. Although somewhat speculative, these data suggest that different FGFs act at different times during follicle development, with FGF10 having a greater role during the early-mid phase of antral follicle growth.

FGF receptor expression is also regulated, as granulosa cell *FGFR2B* expression increased with increasing estradiol concentrations in bovine follicles [10]. In the present study, we show that *FGFR2B* mRNA in granulosa cells is upregulated by FSH in a manner similar to that described for granulosa *FGFR3C* [24]. Neither of these receptors were upregulated by IGF1 ([24] and present study), and indeed we show that IGF1 suppresses *FGFR2B* expression. The progressive increase of *FGFR2B* expression with follicle growth in vivo [10] is similar to the increases in other FSH-responsive genes, such *CYP19A1* [43, 44], suggesting an important role for this receptor during follicle growth.

To determine the potential biological importance of FGF10 signaling for granulosa cells, we tested the effect of FGF10 on estradiol secretion and cell proliferation in nonluteinizing bovine granulosa cells in serum-free culture. This culture system represents the most physiologically relevant model of granulosa cell differentiation available in ruminants, as steroidogenic enzymes that are upregulated or induced during granulosa cell differentiation in vivo are also upregulated or induced in vitro, and there is no upregulation of the luteinization marker, steroidogenic acute regulatory protein [45]. In this cell model, FGF10 significantly inhibited estradiol secretion without affecting cell proliferation. This is consistent with FGF7 inhibition of aromatase activity in bovine granulosa cells [9]. Therefore, paracrine control of estradiol secretion appears to be a balance between inhibitory (GDF9 [5]; FGF10, present study) and stimulatory (BMP4 and BMP7 [7, 8]) factors.

FGF10 did not affect cell proliferation in the present study, although FGF7 has been reported to stimulate granulosa cell proliferation [46]. The effects of some growth factors on ruminant granulosa cell proliferation remain controversial; whereas McNatty et al. [6] have reported no effect of ovine GDF9 on bovine granulosa cell proliferation, Spicer et al. [5] have reported that rat GDF9 stimulates bovine granulosa cell proliferation. These discrepancies may be related to the source (species) of the peptide hormones or the method used to measure cell proliferation (thymidine incorporation vs. MTT assay).

In summary, the present report provides new evidence for paracrine FGF10 signaling in bovine follicles. Based on the patterns of gene expression and actions of FGF10 discussed above, a possible model of FGF10 action can be formulated as follows. Theca cells from early antral follicles express the *FGF10* gene. The rise in FSH that precedes a wave of follicle growth [47, 48] increases *FGFR2B* expression in granulosa cells, which enhances the response to secreted FGF10. The action of FGF10 at this stage is to inhibit the differentiation of granulosa cells, as reflected by limited estradiol secretion, which is a major marker of differentiation [43, 44]. As follicle development proceeds, there is a progressive decrease in theca FGF10 secretion, which allows increased estradiol secretion and granulosa cell differentiation.

Downloaded from https://academic.oup.com/biolreprod/article/77/4/743/2629786 by guest on 21 August 2022

ACKNOWLEDGMENTS

We thank Dr. M.C.W. Avellar (Universidade Federal de São Paulo, Escola Paulista de Medicina) for sequencing the FGF10 PCR product, Dr. P.R. Ramos (Universidade Estadual Paulista, Botucatu) for access to gel documentation equipment, and Drs. A.B. Teixeira, I.B. Costa, and V.F. Glapinski for technical assistance.

REFERENCES

1. Knight PG, Glister C. TGF-beta superfamily members and ovarian follicle development. Reproduction 2006; 132:191–206.
2. Gilchrist RB, Ritter LJ, Armstrong DT. Oocyte-somatic cell interactions during follicle development in mammals. Anim Reprod Sci 2004; 82–83: 431–446.
3. Juengel JL, Bodensteiner KJ, Heath DA, Hudson NL, Moeller CL, Smith P, Galloway SM, Davis GH, Sawyer HR, McNatty KP. Physiology of GDF9 and BMP15 signalling molecules. Anim Reprod Sci 2004; 82–83: 447–460.
4. Shimasaki S, Moore RK, Otsuka F, Erickson GF. The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 2004; 25:72–101.
5. Spicer LJ, Aad PY, Allen D, Mazerbourg S, Hsueh AJ. Growth differentiation factor-9 has divergent effects on proliferation and steroidogenesis of bovine granulosa cells. J Endocrinol 2006; 189:329–339.
6. McNatty KP, Juengel JL, Reader KL, Lun S, Myllymaa S, Lawrence SB, Western A, Meerasahib MF, Mottershead DG, Groome NP, Ritvos O, Laitinen MP. Bone morphogenetic protein 15 and growth differentiation factor 9 co-operate to regulate granulosa cell function in ruminants. Reproduction 2005; 129:481–487.
7. Cao M, Nicola E, Portela VM, Price CA. Regulation of serine protease inhibitor-E2 and plasminogen activator expression and secretion by follicle stimulating hormone and growth factors in non-luteinizing bovine granulosa cells in vitro. Matrix Biol 2006; 25:342–354.
8. Glister C, Kemp CF, Knight PG. Bone morphogenetic protein (BMP) ligands and receptors in bovine ovarian follicle cells: actions of BMP-4, –6 and –7 on granulosa cells and differential modulation of Smad-1 phosphorylation by follistatin. Reproduction 2004; 127:239–254.
9. Parrott JA, Skinner MK. Developmental and hormonal regulation of keratinocyte growth factor expression and action in the ovarian follicle. Endocrinology 1998; 139:228–235.
10. Berisha B, Sinowatz F, Schams D. Expression and localization of fibroblast growth factor (FGF) family members during the final growth of bovine ovarian follicles. Mol Reprod Dev 2004; 67:162–171.
11. Itoh N, Ornitz DM. Evolution of the Fgf and Fgfr gene families. Trends Genet 2004; 20:563–569.
12. Lu W, Luo Y, Kan M, McKeehan WL. Fibroblast Growth Factor-10. A second candidate stromal to epithelial cell andromedin in prostate. J Biol Chem 1999; 274:12827–12834.
13. Igarashi M, Finch PW, Aaronson SA. Characterization of recombinant human fibroblast growth factor (FGF)-10 reveals functional similarities with keratinocyte growth factor (FGF-7). J Biol Chem 1998; 273:13230–13235.
14. Marguerie A, Bajolle F, Zaffran S, Brown NA, Dickson C, Buckingham ME, Kelly RG. Congenital heart defects in Fgfr2-IIIb and Fgf10 mutant mice. Cardiovasc Res 2006; 71:50–60.
15. Sekine K, Ohuchi H, Fujiwara M, Yamasaki M, Yoshizawa T, Sato T, Yagishita N, Matsui D, Koga Y, Itoh N, Kato S. Fgf10 is essential for limb and lung formation. Nat Genet 1999; 21:138–141.
16. Spencer-Dene B, Sala FG, Bellusci S, Gschmeissner S, Stamp G, Dickson C. Stomach development is dependent on fibroblast growth factor 10/fibroblast growth factor receptor 2b-mediated signaling. Gastroenterology 2006; 130:1233–1244.
17. Veltmaat JM, Relaix F, Le LT, Kratochwil K, Sala FG, van Veelen W, Rice R, Spencer-Dene B, Mailleux AA, Rice DP, Thiery JP, Bellusci S. Gli3-mediated somitic Fgf10 expression gradients are required for the induction and patterning of mammary epithelium along the embryonic axes. Development 2006; 133:2325–2335.
18. Yokohama-Tamaki T, Ohshima H, Fujiwara N, Takada Y, Ichimori Y, Wakisaka S, Ohuchi H, Harada H. Cessation of Fgf10 signaling, resulting in a defective dental epithelial stem cell compartment, leads to the transition from crown to root formation. Development 2006; 133:1359–1366.
19. Figueiredo JR, Hulshof SC, Van den Hurk R, Ectors FJ, Fontes RS, Nusgens B, Bevers MM, Beckers JF. Development of a combined new mechanical and enzymatic method for the isolation of intact preantral follicles from fetal, calf and adult bovine ovaries. Theriogenology 1993; 40:789–799.
20. Buratini J Jr, Glapinski VF, Giometti IC, Teixeira AB, Costa IB, Avellar MC, Barros CM, Price CA. Expression of fibroblast growth factor-8 and its cognate receptors, fibroblast growth factor receptor (FGFR)-3c and-4, in fetal bovine preantral follicles. Mol Reprod Dev 2005; 70:255–261.
21. Ireland J, Good T, Knight P, Ireland J. Alterations in amounts of different forms of inhibin during follicular atresia. Biol Reprod 1994; 50:1265–1276.
22. Grimes RW, Ireland JJ. Relationship of macroscopic appearance of the surface of bovine ovarian follicles concentrations of steroids in follicular fluid, and maturation of oocytes in vitro. Biol Reprod 1986; 35:725–732.
23. Berisha B, Schams D, Kosmann M, Amselgruber W, Einspanier R. Expression and localisation of vascular endothelial growth factor and basic fibroblast growth factor during the final growth of bovine ovarian follicles. J Endocrinol 2000; 167:371–382.
24. Buratini J Jr, Teixeira AB, Costa IB, Glapinski VF, Pinto MGL, Giometti IC, Barros CM, Cao M, Nicola ES, Price CA. Expression of fibroblast growth factor-8 and regulation of cognate receptors, fibroblast growth factor receptor (FGFR)-3c and -4, in bovine antral follicles. Reproduction 2005; 130:343–350.
25. Gutiérrez CG, Campbell BK, Webb R. Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to follicle-stimulating hormone, and morphological characteristics. Biol Reprod 1997; 56:608–616.
26. Silva JM, Price CA. Effect of follicle-stimulating hormone on steroid secretion and messenger ribonucleic acids encoding cytochromes P450 aromatase and cholesterol side-chain cleavage in bovine granulosa cells in vitro. Biol Reprod 2000; 62:186–191.
27. Chen C, Spencer TE, Bazer FW. Fibroblast growth factor-10: a stromal mediator of epithelial function in the ovine uterus. Biol Reprod 2000; 63: 959–966.
28. Bélanger A, Couture J, Caron S, Roy R. Determination of nonconjugated and conjugated steroid levels in plasma and prostate after separation on C-18 columns. Ann N Y Acad Sci 1990; 595:251–259.
29. Lafrance M, Goff AK. Effect of pregnancy on oxytocin-induced release of prostaglandin F2 alpha in heifers. Biol Reprod 1985; 33:1113–1119.
30. Nilsson E, Parrott JA, Skinner MK. Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. Mol Cell Endocrinol 2001; 175:123–130.
31. Yamasaki M, Miyake A, Tagashira S, Itoh N. Structure and expression of the rat mRNA encoding a novel member of the fibroblast growth factor family. J Biol Chem 1996; 271:15918–15921.
32. Olsnes S, Klingenberg O, Wiedlocha A. Transport of exogenous growth factors and cytokines to the cytosol and to the nucleus. Physiol Rev 2003; 83:163–182.
33. Wesche J, Malecki J, Wiedlocha A, Skjerpen CS, Claus P, Olsnes S. FGF-1 and FGF-2 require the cytosolic chaperone Hsp90 for translocation into the cytosol and the cell nucleus. J Biol Chem 2006; 281:11405–11412.
34. Marchese C, Mancini P, Belleudi F, Felici A, Gradini R, Sansolini T, Frati L, Torrisi M. Receptor-mediated endocytosis of keratinocyte growth factor. J Cell Sci 1998; 111:3517–3527.
35. Belleudi F, Ceridono M, Capone A, Serafino A, Marchese C, Picardo M, Frati L, Torrisi M. The endocytic pathway followed by the keratinocyte growth factor receptor. Histochem Cell Biol 2002; 118:1–10.
36. van Wezel IL, Umapathysivam K, Tilley WD, Rodgers RJ. Immunohistochemical localization of basic fibroblast growth factor in bovine ovarian follicles. Mol Cell Endocrinol 1995; 115:133–140.
37. Valve E, Penttila TL, Paranko J, Harkonen P. FGF-8 is expressed during specific phases of rodent oocyte and spermatogonium development. Biochem Biophys Res Commun 1997; 232:173–177.
38. Kezele P, Nilsson EE, Skinner MK. Keratinocyte growth factor acts as a mesenchymal factor that promotes ovarian primordial to primary follicle transition. Biol Reprod 2005; 73:967–973.
39. Derrar N, Price CA, Sirard MA. Effect of growth factors and co-culture with ovarian medulla on the activation of primordial follicles in explants of bovine ovarian cortex. Theriogenology 2000; 54:587–598.
40. Wandji SA, Srsen V, Voss AK, Eppig JJ, Fortune JE. Initiation in vitro of growth of bovine primordial follicles. Biol Reprod 1996; 55:942–948.
41. Price CA, Carrière PD, Bhatia B, Groome NP. Comparison of hormonal and histological changes during follicular growth, as measured by ultrasonography, in cattle. J Reprod Fertil 1995; 103:63–68.
42. Smith LC, Olivera-Angel M, Groome NP, Bhatia B, Price CA. Oocyte quality in small antral follicles in the presence or absence of a large dominant follicle in cattle. J Reprod Fertil 1996; 106:193–199.
43. Bao B, Garverick HA, Smith GW, Smith MF, Salfen BE, Youngquist RS. Changes in messenger ribonucleic acid encoding luteinizing hormone

Downloaded from https://academic.oup.com/biolreprod/article/77/4/743/2629786 by guest on 21 August 2022

receptor, cytochrome P450-side chain cleavage, and aromatase are associated with recruitment and selection of bovine ovarian follicles. Biol Reprod 1997; 56:1158–1168.
44. Xu Z, Garverick HA, Smith GW, Smith MF, Hamilton SA, Youngquist RS. Expression of messenger ribonucleic acid encoding cytochrome P450 side-chain cleavage, cytochrome p450 17 alpha-hydroxylase, and cytochrome P450 aromatase in bovine follicles during the first follicular wave. Endocrinology 1995; 136:981–989.
45. Sahmi M, Nicola ES, Silva JM, Price CA. Expression of 17beta- and 3beta-hydroxysteroid dehydrogenases and steroidogenic acute regulatory protein in non-luteinizing bovine granulosa cells in vitro. Mol Cell Endocrinol 2004; 223:43–54.
46. Parrott JA, Vigne JL, Chu BZ, Skinner MK. Mesenchymal-epithelial interactions in the ovarian follicle involve keratinocyte and hepatocyte growth factor production by theca cells and their action on granulosa cells. Endocrinology 1994; 135:569–575.
47. Fortune JE, Rivera GM, Yang MY. Follicular development: the role of the follicular microenvironment in selection of the dominant follicle. Anim Reprod Sci 2004; 82–83:109–126.
48. Ginther OJ, Beg MA, Bergfelt DR, Donadeu FX, Kot K. Follicle selection in monovular species. Biol Reprod 2001; 65:638–647.

Downloaded from https://academic.oup.com/biolreprod/article/77/4/743/2629786 by guest on 21 August 2022