Expression and possible role of fibroblast growth factor family members in porcine antral follicles during final maturation

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Abstract

The aim of this study was to investigate the possible participation of fibroblast growth factor (FGF) family members (FGF1, FGF2 and FGF7 and their receptors) in porcine follicles (polyovulatory species) under special consideration for FGF2 during final growth. A classification of follicles was done by size and follicular fluid content of oestradiol-17 β , progesterone and prostaglandin F2 α . The mRNA expression of examined factors was analysed by real-time PCR. The hormone concentration was estimated by enzyme immunoassay, protein characterisation by western blotting and localisation by immunohistochemistry. Follicle tissue separated in theca interna and granulosa cells was extracted and tested for mRNA of *FGF1*, *FGF2*, *FGF7* and receptors (*FGFR1IIIc*, *FGFRIIIb* and *FGFR2IIIc*). Additionally, the mRNA expression of *FSHR*, *LHR* and aromatase cytochrome P450 for further characterisation of follicles was analysed. Significantly, higher FGF2 protein levels were measured in stroma when compared with total follicle or corpus luteum tissue. This result was confirmed by western blot with two strong bands. Immunological localisation of FGF2 only in stroma (fibroblasts) confirms the protein measurements. The results show a clear difference for FGF2 protein expression during final growth of follicles if monovulatory (bovine) and polyovulatory (porcine) species are compared. FGF2 protein in porcine ovary may be (due to localisation and concentration in stroma) important for support of angiogenesis of more follicles (polyovulatory species) and not of a single follicle like in cows.

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Introduction

It is now well established in many species that locally produced growth factors have important modulatory and stimulatory roles in final follicular growth. The ovarian cycle is characterised by repeating patterns of cellular proliferation, differentiation and transformation that accompany follicular and luteal development. Tissue growth in the ovary (follicle or corpus luteum (CL)) depends upon the growth of new blood vessels and establishment of a functional blood supply (angiogenesis). Angiogenesis may play an important role in the selection process of follicles. Zeleznik *et al.* (1981) suggested that the selected follicles possess more elaborate microvasculature than other follicles. We concluded from studies in cow that vascular endothelial growth factor (VEGFA) and fibroblast growth factor 2 (FGF2) are involved in the proliferation of capillaries that accompany the selection of the preovulatory follicle resulting in an increased supply of nutrients, precursors and hormones, and therefore supporting the growth of the dominant follicle (Berisha *et al.* 2000a, Yamashita *et al.* 2008).

The importance and possible role of FGFs in ovary is not so well documented. The FGFs belonging to a big family have a wide range of tissue and cell distribution

(Gospodarowicz *et al.* 1987). FGF1, FGF2 and FGF7 were demonstrated in the bovine ovary (Gospodarowicz *et al.* 1985, Schams *et al.* 1994, Parrott & Skinner 1998, Sinowatz *et al.* 2006). The FGF receptor family (FGFR) includes four identified genes and numerous subtypes of alternatively spliced isoforms, particularly within the well-characterised *FGFR1* and *FGFR2* types. Differential responses follow from this diversity (Johnson & Williams 1993, Stauber *et al.* 2000). FGF2 has been identified as a potential regulator of ovarian function (Gospodarowicz *et al.* 1985, Schams *et al.* 1994). FGF2 promotes twice as potent angiogenesis in three-dimensional *in vitro* models compared with VEGF (Pepper *et al.* 1992). Results suggest that FGF2 and VEGFA have a potent synergistic effect on the induction of angiogenesis *in vitro*. In bovine endothelial cell culture, FGF2 in the medium upregulated the expression of both VEGF receptors (FLT1 and KDR; Gabler *et al.* 2004).

The distinct immunohistochemical localisation of FGF2 in selected bovine dominant follicles in cytoplasm of capillary endothelial cells (Berisha *et al.* 2000a, 2006a) suggests an important role of FGF2 for the selection process in monovulatory bovine species. If this hypothesis is correct, we should see differences in FGF2 expression and localisation in polyovulatory species. The aim of the

present study was therefore to evaluate the possible participation of FGF family members (FGF1, FGF2 and FGF7) under special consideration of FGF2 and their receptor variants (FGFR1IIIc, FGFR2IIIb and FGFR2IIIc) during final follicle maturation in porcine ovary as a polyovulatory species. The results should be compared and discussed with those in the monovulatory bovine species.

Results

Characterisation of tested follicular groups

The average concentrations of oestradiol-17 β (E₂), progesterone (P4) and prostaglandin F2 α (PGF2 α) in follicular fluid (FF) for the different groups are shown in Table 1. Concentration levels for both steroids in FF (E₂ and P4) increased significantly in large follicles (>7 mm), followed by sharp decrease of E₂ in periovulatory follicles or remaining high levels of P4 in periovulatory follicles. By contrast, PGF2 α values are very low in antral follicles followed by a dramatic increase in periovulatory follicles.

The mRNA expression for *FSHR*, *LHR* and aromatase cytochrome P450 (*CYP19A1*) in separated theca interna (TI) and granulosa cells (GC) are presented in Fig. 1. *FSHR* showed in GC a relatively constant expression. By contrast, the *LHR* mRNA expression in GC and TI increased significantly from small to large follicles followed by a sharp decrease in the periovulatory group. The *CYP19A1* expression profile in GC and TI was comparable with that of the *LHR*.

Expression of mRNA for FGF1, FGF2 and FGF7 and FGF receptors

The mRNA expression in separated follicle tissue (Fig. 2) for *FGF1*, *FGF2* and *FGF7* was relatively similar in TI and GC tissue with few significant changes for *FGF1* in TI and *FGF2* in TI and GC. The expression intensity was generally higher for *FGF2*, if compared with *FGF1* and *FGF7*. The mRNA expression for the receptors *FGFR1IIIc*, *FGFR2IIIb* and *FGFR2IIIc* in separated follicular tissue is given in Fig. 3. The *FGFR1IIIc* expression profiles in TI and GC were comparable and showed a decrease in large follicles followed by an increase in the periovulatory group (Fig. 3).

The *FGFR2IIIc* expression in TI and GC was relative constant. Only *FGFR2IIIb* showed an upregulation in TI for large and periovulatory groups (Fig. 3).

FGF2 protein concentration and characterisation

Follicle tissue (after removal of FF), stroma and CL tissue were homogenised, extracted and the supernatant was tested for FGF2 by a commercial enzyme immunoassay (EIA). The results are shown in Fig. 4a. Significantly, higher FGF2 protein concentrations were measured in stroma if compared with total follicle or CL tissue. This result was confirmed by semi-quantitative western blot as demonstrated in Fig. 4b. A weak 18 kDa band was seen in follicle tissue after extraction comparable with the bovine FGF2 standard. Two much stronger bands were seen in stroma tissue in all three pools tested corresponding to 18 and 22 kDa.

FGF2 protein localisation

The quantitative and semi-quantitative differences between FGF2 content in ovarian tissue were further confirmed by immunohistological localisation of FGF2 in tissue (see Fig. 5). In paraffin sections of porcine ovaries, immunohistochemical procedure revealed cell-type-specific staining of FGF2. Strong immunoreaction for FGF2 is selectively found in numerous stromal cells around and between different follicle categories obtained from different follicular phases (a and c). At the cellular level, the strong immunostaining is restricted to the nucleus of fibroblasts (b and d) with weak staining of cytoplasm. No staining can be observed in theca or granulosa cells of large (a) and small (c and d) follicles, or in endothelial cells of capillaries or larger blood vessels in porcine ovarian tissue (e). The negative control section on which the primary antiserum was not applied does not show any immunoreactivity (f).

Discussion

The collection of our follicles from slaughterhouse material does not allow determination of the exact stage of follicular development. The classification of our follicles according to size, and the P4, E₂ and PGF2 α content in FF and expression of mRNA for *FSHR*,

Table 1 Follicle diameter (mm) and follicle fluid (FF) concentration of oestradiol-17 β (E₂), progesterone and PGF2 α (mean $\pm$ S.E.M.) for the examined porcine antral follicle groups ($n=6-10$ follicle/group) from $n=5$ sows.

Follicle group Diameter (mm)	Follicular fluid concentration (ng/ml)		
	E ₂	Progesterone	PGF2 α
2-3 mm	12.1 $\pm$ 1.6 ^a	108.0 $\pm$ 8.9 ^a	121.4 $\pm$ 22.1 ^a
4-6 mm	44.8 $\pm$ 7.9 ^a	88.1 $\pm$ 11.6 ^a	234.6 $\pm$ 107.4 ^a
>7 mm	220.2 $\pm$ 29.9 ^b	364.0 $\pm$ 67.8 ^b	425.5 $\pm$ 124.1 ^a
Periovulatory	27.1 $\pm$ 5.3 ^a	281.6 $\pm$ 2.8 ^b	13 782.0 $\pm$ 2851.6 ^b

Different superscripts denote statistically different values ($P<0.05$).

Figure 1 Expression of mRNA for (a) *FSHR* in GC, (b) *LHR* in GC, (c) *LHR* in TI, (d) *CYP19A1* in GC and (e) *CYP19A1* in TI, in porcine antral follicle groups. The changes in mRNA expression of examined factors were assayed by normalisation to the *UBQ* internal control. In order to obtain the CP (crossing point) difference, the data were analysed using the $\Delta\Delta CP$ method described previously by Livak & Schmittgen (2001). Thereby, ΔCP was not subtracted from a control group, but from the value 40, so that a high 40- ΔCP value indicated a high-gene expression level and vice versa. Results are presented as mean $\pm$ S.E.M. ($n=5-6$ follicles/class). Different superscripts denote statistically different values ($P<0.05$).

LHR and *CYP19A1* support our classification system and allow some conclusions in comparison with data from the literature. Our follicle diameter and steroid levels in FF agree quite well with recently published data with well-defined porcine material (Corbin *et al.* 2003) or earlier data (Ainsworth *et al.* 1980, Babalola & Shapiro 1988, Grant *et al.* 1989). The periovulatory group is defined and selected by the very high ascent of *PGF2 α* level in FF. The level increases in such a way a few hours before ovulation,

Figure 2 Expression of mRNA in TI for (a) *FGF1*, (b) *FGF2*, (c) *FGF7* or in GC for (d) *FGF1*, (e) *FGF2*, (f) *FGF7*. The changes in mRNA expression of examined factors were assayed by normalisation to the *UBQ* internal control. Results are presented as mean $\pm$ S.E.M. ($n=5-6$ follicles/class). Different superscripts denote statistically different values ($P<0.05$).

as shown for the pig (Hunter & Poyser 1985) and cow (Berisha *et al.* 2006a). The typical descent of *CYP19A1* and *LHR* mRNA expression confirms further our classification.

In contrast with the cow (Berisha *et al.* 2000a), the *FSHR* mRNA expression trended to decrease during porcine follicle maturation. This agrees with findings by Cardenas & Pope (2002). The *LHR* mRNA shows already high expression in small follicles in both compartments

Figure 3 Expression of mRNA in porcine follicle tissue (TI or GC): in TI, (a) *FGFR1IIIc*, (b) *FGFR2IIIc* and (c) *FGFR2IIIB*; in GC, (d) *FGFR1IIIc*, (e) *FGFR2IIIc* and (f) *FGFR2IIIB*. The changes in mRNA expression of examined factors were assayed by normalisation to the UBQ internal control. Results are presented as mean $\pm$ S.E.M. ($n=5-6$ follicles/class). Different superscripts denote statistically different values ($P<0.05$).

(TI and GC), increases in large follicles and decreases after LH surge. An increase of *LHR* in GC in large follicles was also observed in pigs (Liu *et al.* 1998) and cows (Berisha *et al.* 2000a). The mRNA expression pattern for *CYP19A1* also agrees with reports in the literature (Ainsworth *et al.* 1990, Guthrie *et al.* 1994). Atretic follicles show a low *CYP19A1* mRNA expression (Garrett & Guthrie 1997). Our data for *CYP19A1*

expression and E_2 concentration suggest no atretic follicles in our groups. The clear mRNA expression and localisation of protein for FGF1, FGF2 and FGF7 in the bovine ovary suggest an important role for local ovarian regulation (Parrott & Skinner 1998, Berisha *et al.* 2004, Schams & Berisha 2004). In the present study, we also demonstrated mRNA expression for these factors (ligand and receptors) in the porcine ovary. The possible role of FGF1 in the ovary is still not clear. Significant regulatory changes of FGF1 expression during final follicle growth were observed in porcine (Fig. 2) or bovine tissue (Berisha *et al.* 2004, 2006b). The immunoreactive localisation of FGF1 in smooth muscles of the blood vessels and GCs in cows (Berisha *et al.* 2004) suggests survival function. Due to the mRNA expression in porcine TI and GC, comparable actions can be assumed.

The unregulated mRNA expression for *FGF7* in TI and GC in all groups agrees with data from cows (Berisha *et al.* 2004). In this species, a significant increase in expression was seen only during LH surge and in early CL. The protein is marked localised in the theca, the basal layers of the GC and the smooth muscle cells of blood vessels. FGF7 is distinguished from most other FGF family members by its strict paracrine mode of action. As suggested by Parrott *et al.* (1994) and Parrott & Skinner (1998), FGF7 is expressed and protein secreted to the GC and may

Figure 4 (a) Tissue concentration of FGF2 protein (ng/g wet weight) in different porcine ovary tissue (follicle, stroma and CL) as measured by EIA. Follicle pools, stroma and CL tissue ($n=5$) were obtained from five sows per group. Results are presented as mean $\pm$ S.E.M. Different superscripts denote statistically different values ($P<0.05$). (b) Western-blot protein characterisation of FGF2 in different ovary tissue (stroma and follicle). Stroma tissue (S) and follicle tissue (F) from early (S1 and F1), mid (S2 and F2) and late (S3 and F3) follicle phases ($n=3$ tissue samples/different animals). The standards of rbFGF2 protein are 2, 4 and 6 ng. The position of molecular mass markers is indicated on the left.

Figure 5 Immunohistochemical localisation of FGF2 in porcine ovary. (a) Stroma tissue shows a strong immunolocalisation of FGF2. Very weak staining or no signal of FGF2 was detected in theca externa, TI and GC of the porcine ovary. (b) Nuclei of fibroblasts in stroma show strong staining and weaker staining in cytoplasm. (c and d) Again shows only clear staining in stroma but not in primary follicles. (e) Endothelial cells of blood vessels are also negative for FGF2. (f) Negative control.

stimulate proliferation of GC. We have not tested the localisation in the pig. But the significant higher expression of *FGFR2IIIB* in porcine TI of large follicles and periovulatory follicles may suggest also direct effects on theca cells.

But our main interest for this study was the *FGF2* mRNA expression and protein profile in porcine follicles (polyovulatory species) and the comparison with bovine (monovulatory) species. FGF2 is a heparin-binding growth factor, which occurs in several isoforms resulting from alternative initiations of translation: a 18 kDa cytoplasmic isoform and four larger molecular mass nuclear isoforms (22, 22.5, 24, and 34 kDa respectively). Although devoid of a signal peptide, it could be secreted under certain circumstances. FGF2 upregulates the mRNA expression of *FLT4* (Pepper & Mandriota 1998), *FLT1* and *KDR* (Gabler *et al.* 2004), and amplifies herewith the angiogenic property of VEGFA. The mRNA expression for *FGF2* is comparably expressed in TI and GC. This is in contrast with bovine follicles (Berisha *et al.* 2000a), which show a strong upregulation of mRNA for ligand and receptor in TI of dominant follicles. The GC compartment shows only a weak (unregulated) expression.

Very exciting is the FGF2 protein concentration and localisation in porcine ovary demonstrating clear differences between mRNA expression and protein translation. The negative immunostaining for FGF2 in porcine follicles does not exclude some protein in follicles due to the less sensitive technique of immunohistochemistry. A weak band for FGF2 is seen in follicle by western-blot technique.

But nevertheless, the strong staining for FGF2 in fibroblasts of stroma tissue and higher stroma tissue concentrations when compared with follicle extracts suggest different regulatory mechanisms of porcine FGF2 on follicle growth and maturation if compared with the bovine. FGF2 mainly localised in stroma may act in different ways: i) autocrine–intracrine regulation of fibroblast by stimulating unknown factors acting on follicular growth, ii) paracrine effects on theca tissue (endothelial cells) of follicles and iii) FGF2 is constitutively required for stromal function or general ovarian angiogenesis. FGF2 secreted from fibroblasts may affect more follicles at the same time and stimulate angiogenesis, in contrast to the single bovine-dominant follicle. In contrast to the bovine (Seghezzi *et al.* 1988) FGF2 may stimulate VEGFA and therefore angiogenesis in porcine follicles by a paracrine mechanism. Very interesting is the demonstration of a strong second band (22 kDa). This protein may be responsible for autocrine–intracrine function. Nuclear translocation of the growth factor is required for the induction of DNA synthesis (Imamura *et al.* 1990, Wiedlocha *et al.* 1996).

In conclusion, we have shown a distinct localisation of FGF2 between the single bovine-dominant follicle and the group of preovulatory porcine follicles. FGF2 in porcine ovary may be important for the growth of more follicles due to the localisation and concentration difference between stroma and follicle tissue.

Materials and Methods

Collection, classification and preparation of follicles and CL

Entire reproductive tracts from sows were collected at a local slaughterhouse within 10–20 min after slaughter and were transported on ice to the laboratory. The stage of the oestrous cycle was defined by macroscopic observation of the ovaries (colour, consistency, CL stage, number and size of follicles) and the uterus (colour, consistency and mucus; Leiser *et al.* 1988). Only follicles that appeared healthy (i.e. well vascularised and having transparent follicular wall and fluid) were used. For the periovulatory group, follicles were collected only after CL regression with signs of mucus production in the uterus and cervix.

Follicles were dissected from the ovary for RNA and protein extraction. The surrounding tissue (theca externa) was removed with forceps under a stereomicroscope. After aspiration of FF, the follicles were bisected and their inside wall was scraped

intensively and flushed with Ringer's solution several times (Fresenius, Wendel, Germany) to remove the GC. The GC in the FF as well as in the flushing solution were centrifuged at 2000 *g* for 10 min at 4 °C. The TI tissue and GC pellet were snap frozen in liquid nitrogen and stored at –80 °C until RNA isolation. For follicle FGF2 tissue extraction, after aspiration of FF, total follicle tissue was stored at –80 °C. The FF was stored at –20 °C until determination of P4, E₂ and PGF2 α .

Experiment 1

Stroma tissue from different pieces of the ovary (follicle free), whole antral follicle and CL tissue was collected for comparison of FGF2 protein determination. The follicles were classified according to the E₂, P4 and PGF2 α content in FF. The corresponding size of follicles was in the range of 2–3 mm (early follicle phase), 4–6 mm (mid follicle phase) and >7 mm (late follicle phase).

Experiment 2

Antral follicles were divided into four groups according to the E₂, P4 and PGF2 α FF content and follicle diameter (Table 1). Separation of TI and GC compartments was obtained from following follicle groups: 2–3 mm, 4–6 mm, >7 mm and periovulatory. For further characterisation of the follicle classes, mRNA expression was determined for the *FSHR* in GC and *CYP19A1* and *LHR* in TI and GC.

Hormone determinations in FF

The concentrations of P4 and E₂ were determined directly in the FF with an EIA using the second antibody technique (Meyer *et al.* 1990, Acosta *et al.* 1999). The effective dose for 50% inhibition (ED50) of the assay was 6 ng/ml for P4 and 3.5 pg/ml for E₂. The FF was diluted accordingly. The intra-assay coefficient of variation (CV) values was 4.5% (P4) and 6.6% (E₂) and the inter-assay CV values 8.7% (P4) and 9.4% (E₂) respectively.

The EIA for PGF2 α (Acosta *et al.* 1999) was evaluated in different dilutions directly in FF without extraction. The standard curve for PGF2 α ranged from 0.6 to 700 pg/ml, and the ED50 of the assay was 300–400 pg/ml. The intra and inter-assay CV values were 8 and 14% respectively.

Tissue extraction

One hundred milligrams of follicle (pool from 6 follicles), CL and stroma tissue was transferred into ten volumes of PBS (1 ml) containing one complete mini tablet of BSA (Boehringer, Mannheim, Germany). This tablet contains both reversible and irreversible protease inhibitors, and inhibits a broad spectrum of serine-, cysteine- and metallo-proteases. The tissue samples were homogenised using the FastPrep-System (BIO101, Carlsbad, CA, USA) with 0.8 g of Matrix Green ceramic beads at 6 m/s for 40 s until the material was pulverised. After centrifugation for 10 min at 3500 *g*, the total protein content in the supernatant was determined by a BCA test (Sigma–Aldrich Company).

Isolation of RNA

Total RNA was prepared from follicle and CL tissue according to Chomczynski & Sacchi (1987) with TriPure isolation reagent (Roche Diagnostics) as described earlier in detail (Berisha *et al.* 2000b). Possible DNA contaminations were eliminated by an additional DNase digestion according to the manufacturer's protocol (Promega). Total RNA was finally purified using Nucleospin RNA II (Macherey & Nagel, Düren, Germany) with the concentration and purity being determined spectroscopically at an absorbance of 260 nm using a Biophotometer (Eppendorf, Hamburg, Germany). Aliquots were subjected to 1% denaturing agarose gel electrophoresis and ethidium bromide staining to verify the quantity and quality of total RNA.

RNA RT and real-time PCR

Constant amounts of 1 μ g of total RNA were reverse transcribed to cDNA using the following master mix: 26 μ l RNase-free water, 12 μ l 5 $\times$ buffer (Promega), 3 μ l Random Primers (50 μ M; Invitrogen), 3 μ l dNTPs (10 mM; Fermentas, St Leon-Rot, Germany) and 200 U of M-MLV Reverse Transcriptase (Promega) according to the manufacturer's instructions. Primer sequences are given in Table 2.

A master mix of the following reaction components was prepared: 6.4 μ l water, 1.2 μ l MgCl₂ (4 mM), 0.2 μ l forward primer (0.2 μ M), 0.2 μ l reverse primer (0.2 μ M) and 1.0 μ l LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics). The master mix (9 μ l) was added to the strip tubes and 1 μ l PCR template containing 16.66 ng reverse-transcribed total RNA was added.

The following general real-time PCR protocol was employed for all investigated factors: denaturation for 10 min at 95 °C, 40 cycles of a three-segmented amplification and quantification program (denaturation for 10 s at 95 °C, annealing for 10 s at 60 °C, elongation for 15 s at 72 °C), a melting step by slow heating from 60 °C to 99 °C with a rate of 0.5 °C/s and continuous fluorescence measurement, and a final cooling down to 40 °C. Data were analysed using Rotor-Gene 3000 software (version 5.03). The relative expressions of each target gene were calculated using the 'comparative quantification' method ('take-off' points). The increase in fluorescence signal recorded during the assay is proportional to the amount of DNA synthesised during each amplification cycle. Individual reactions are characterised by the cycle fraction at which fluorescence first rises above a defined background fluorescence, a parameter known as the threshold cycle or crossing point (CP). In order to obtain the CP difference, the data were analysed using the $\Delta\Delta$ (delta, delta) CP method described previously by Livak & Schmittgen (2001). Thereby, Δ CP was not subtracted from a control group, but from the value 40, so that a high '40- Δ CP' value indicated a high gene expression level and vice versa (Kliem *et al.* 2009). The changes in mRNA expression of examined factors were assayed by normalisation to the UBC internal control. The specificity of the desired products in bovine CL was documented using a high-resolution gel electrophoresis and analysis of the melting temperature, which was product specific.

Table 2 Primer sequences of *FGF1*, *FGF2*, *FGF7*, *FGFR1IIIc*, *FGFR2IIIc*, *FGFR2IIIB*, *FSHR*, *LHR*, *CYP19A1* and housekeeping gene ubiquitin (*UBQ*), RT-PCR product length and reference of the investigated factors or of the according accession number in the EMBL database.

Target	Sequence of nucleotide ^a	Fragment size (bp)	EMBL ^b
<i>FGF1</i>	For: 5'-CCAGGAAATTACAAGAAGCCC-3' Rev: 5'-GTTTCCTCCAACCTTCCA-3'	264	X60317
<i>FGF2</i>	For: 5'-TCAAAGGAGTGTGTGCGAAC-3' Rev: 5'-CAGGGCCACATACCAACTG-3'	161	AJ577089
<i>FGF7</i>	For: 5'-CTGCCAAGTTTGCTCTACAG-3' Rev: 5'-TCCAAGTCCAGGGTCTGAT-3'	291	AJ245447
<i>FGFR1IIIc</i>	For: 5'-ACTGCTGGAGTTAATACCACCG-3' Rev: 5'-GCAGAGTGATGGGAGAGTCC-3'	125	AJ577088
<i>FGFR2IIIc</i>	For: 5'-CACCACGCCAAAGAGATTG-3' Rev: 5'-ATGGAGAGTGAAAGGATATCCC-3'	113	AJ439896
<i>FGFR2IIIB</i>	For: 5'-TGGAGAATGAATACGGGTCC-3' Rev: 5'-TCGGTCACAATTGAACAGAGC-3'	289	AJ439895
<i>LHR</i>	For: 5'-GGAAATGGATTGAAAGAAATACAA-3' Rev: 5'-GTGCTTTCACATTGTTGGAAAAG-3'	376	M29525
<i>FSHR</i>	For: 5'-AAGCTTGTGCGCCCTCATG-3' Rev: 5'-CATTGTCTTCTGCCAGAGAGG-3'	178	AF025377
<i>CYP19A1</i>	For: 5'-CATCATGCTGGACACCTCTAAC-3' Rev: 5'-ATGTCTCTTTCACCAACAACAGTC-3'	476	U92245
<i>UBQ</i>	For: 5'-AGATCCAGGATAAGGAAGGCAT-3' Rev: 5'-GCTCCACCTCCAGGGTGAT-3'	198	Z118245

^aFor, forwards; Rev, reverse. ^bEMBL accession number of sequence.

EIA for FGF2 determination in follicle, CL and stroma tissue

After extraction of tissue, the FGF2 concentration was tested by a commercial provided ELISA kit (Duo Set human FGF2, R&D Systems, Inc., Minneapolis, MN, USA) according to the instructions of the manufacturer. The assay cross reacts with recombinant bovine FGF2. The range of the standard curve was 10–640 pg/ml.

Immunohistochemistry of FGF2

Ovarian tissue was fixed via immersion with methanol/glacial acid 2/1 v/v and Bouin's solution for FGF2, dehydrated after fixation in a series of graded ethanols, cleared in xylene and embedded in paraffin wax using conventional procedures as described previously (Steffl *et al.* 2004). Serial sections of 5 µm thickness were cut from paraffin blocks on a Leitz microtome and collected on gelatine chromalaun-coated slides. Following deparaffinisation, the presence of FGF2 was demonstrated immunohistochemically by a streptavidin–biotin–HRP complex (ABC) method (Hsu *et al.* 1981). Potentially, endogenous peroxidase activity was eliminated by incubation with 0.5% (v/v) hydrogen peroxide solution in absolute methanol for 15 min, and non-specific protein binding was blocked by incubation with 10% normal goat serum in PBS for 1 h at room temperature. Sections were incubated overnight at 4 °C with 1:1200 dilution of rabbit anti-FGF2 (polyclonal antibody #14, prepared in our laboratory and raised against recombinant bovine FGF2 (rbFGF2)). After washing in PBS, the sections were incubated for 30 min with biotinylated goat anti-rabbit IgG. The sections were then treated with ABC reagent from a commercial kit (Vector Laboratories, Burlingame, CA, USA). The bound complex was visualised by incubation with 0.05% 3,3'-diaminobenzidine tetrahydrochloride and

0.0006% hydrogen peroxide in 0.1 mol/l PBS. Between each step, sections were washed thrice in PBS. All incubations were carried out in humidified chambers to prevent drying out. Sections were counterstained with Mayer's haematoxylin, dehydrated, cleared and mounted.

The specificity of the immunohistochemical reactions was assessed by: i) replacement of the primary antibody with buffer, ii) its substitution with non-immune rabbit IgG (1:10 diluted), iii) incubation with diaminobenzidine reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity and iv) an absorption test involving the respective antigen (15 mg/ml). Lack of detected staining of tissue elements in the controls demonstrated the specificity of the reactions.

Western blot

Western blotting of FGF2 SDS-PAGE was performed according to the method described by Laemmli (1970). Samples (50 µg total protein in 20 ml buffer) were boiled in NuPAGE SDS sample buffer (Novex, San Diego, CA, USA) for 5 min at 95 °C and separated on a commercially available 12% Bis–Tris gel (Invitrogen) under reducing conditions (for 1 h at 190 V), and transferred to nitrocellulose membranes (Millipore PVDF, 0.45 mm; Millipore, Bedford, MA, USA). For blocking, membranes were incubated in PBS with 0.05% Tween-20 (PBS–T) with 1% non-fat dry milk overnight. The membranes were then incubated in PBS–T and incubated for 75 min with the respective primary antibody for FGF2. The same antibody was utilised as used for immunohistochemistry. For immunoblot analysis, the antiserum was diluted 1:40 000 and detected by using goat anti-rabbit IgG peroxidase conjugate in combination with an ECL system (Amersham-Pharmacia). Negative controls were set up to replace the primary antibody with PBS. Protein samples from bovine CL or rbFGF2 (a kind

gift from Dr D Gospodarowicz, Chiron Corp., Berkeley, CA, USA) was used as a positive control. Specificity of bands was tested by using preabsorbed antiserum involving the respective antigen. The See Blue Plus2 Pre-Stained standard (Novex) was used as a molecular size marker.

Statistical analyses

The statistical significance of differences in mRNA expression of examined factors and the concentrations of PGF2 α and steroids (E₂ and P4) were analysed by ANOVA followed by the Holm Sidak as a multiple comparison test. Data that failed the normality or equal variance test were tested by one-way ANOVA on ranks followed by the Kruskal–Wallis test (Sigma Stat 3.0). All experimental data were shown as mean $\pm$ S.E.M. Differences were considered significant if $P < 0.05$.

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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