Changes in fibroblast growth factor 2 and its receptors in bovine follicles before and after GnRH application and after ovulation

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Abstract

The aim of this study was to evaluate the expression pattern of fibroblast growth factor 2 (FGF2), its receptor variants (FGFR1IIIc, FGFR2IIIc) and nucleolin in time-defined follicle classes before and after GnRH application and after ovulation in the cow. Ovaries containing preovulatory follicles or new corpora lutea (CL) were collected at approximately 0, 4, 10, 20 and 25 h (follicles) and 60 h (new CL) relative to injection of GnRH to induce an LH surge ($n = 5$ animals per group).
The expressions of FGF2 and FGFR1IIIc mRNA were significantly up-regulated only in the follicle group 4 h after GnRH (during the LH surge) with a significant down-regulation immediately afterwards. Western blot analyses showed two protein bands with at 22 and 18 kDa with apparent up-regulation beginning with the LH surge (4 h) and maximum levels 20 h after GnRH. FGF2 protein in follicles collected at 0 h (before LH surge) was localised in theca tissue (endothelial and pericytes of blood vessels) but not in granulosa cells (GCs). The FGF2 staining (by immunohistochemistry) pattern changed dramatically after the LH surge for a short period (about 2 days) and FGF2 protein was localised dominantly in the nucleus of many GCs, while most capillary endothelial cells were FGF2 immunonegative. In conclusion, the novel observation of FGF2 up-regulation and the distinct change in FGF2 localisation from theca (cytoplasm of endothelial cells) to the nucleus of GCs after the LH surge may be important for survival of GCs or for the transition of the GCs to luteal cells.

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Introduction

Ovulation occurs as a result of a dynamic interaction between the luteinising hormone (LH) surge and local follicular factors including steroid hormones, prostaglandins (PGs), vasoactive peptides, growth factors and matrix proteins in a time-dependent manner (Fortune 1994, Berisha & Schams 2005, Richards 2005). The LH surge triggers a biochemical cascade that leads to the rupture of the Graafian follicle, resulting in the expulsion of the oocyte and consequent development of the corpus luteum (CL) (Schams & Berisha 2004).

Several growth factors have been identified as acting locally within the ovary to regulate the follicle maturation and ovulation. The fibroblast growth factor (FGF) family consists of at least 23 different members of signalling polypeptides, which is characterised by a core region of highly conserved sequence and structure (Ornitz & Itoh 2001). FGF2 is the most prominent factor of this family that stimulates cellular functions, cell proliferation, migration and protease secretion. This provides the basis for angiogenesis,

which is an important physiological function of this peptide. During the past decade, there has been an increasing amount of evidence that FGF1 and FGF2 can be present in either the cytoplasm or the nucleus depending upon the physiological situation. Moreover, the nuclear translocation of FGF is required for the induction of DNA synthesis (Imamura *et al.* 1990, Wiedlocha *et al.* 1996). Both signalling through FGF receptors (FGFRs) and nuclear localisation may be required for the stimulation of cell proliferation (Imamura *et al.* 1990). FGF2 can activate ribosomal DNA (rDNA) transcription, and its nuclear accumulation has been associated with cellular proliferation (Bouche *et al.* 1987, Amalric *et al.* 1994). Nucleolin has been implicated in chromatin structure, rDNA transcription, rRNA maturation, ribosome assembly and nucleo–cytoplasmic transport (Ginisty *et al.* 1999). Nucleolin may also be involved in the activities of FGF2, which has been shown to bind to nucleolin in nuclear extracts (Bonnet *et al.* 1996). Thus, FGF2 might be possibly internalised by a nucleolin-dependent mechanism.

The FGF family members mediate their biological activities through high-affinity tyrosine kinase receptors (FGFR1 to FGFR4) and heparan sulphate proteoglycan low-affinity receptors (Powers *et al.* 2000). The FGFRs are characterised by the presence of two or three immunoglobulin-like domains in the extracellular region and a tyrosine kinase domain in the intracellular region of the receptor. Alternative splicing in the extracellular region of FGFR1 to FGFR3 generates receptor variants (IIIb and IIIc) with different ligand-binding affinities and tissue-specific profiles of expression (Powers *et al.* 2000, Berisha *et al.* 2004). Although all the different splice variants of the four FGFRs can be activated by FGF1, most of the FGFR variants have narrower specificity for the different FGF ligands. In particular FGF2 activates the splice variant FGFR1IIIc and FGFR2IIIc but not FGFR2IIIb (Ornitz *et al.* 1996).

FGF2 and its receptors have been identified and localised in the ovarian follicles and CL as a potential regulator of ovarian function in different species (Gospodarowicz *et al.* 1985, Schams *et al.* 1994, Parrott & Skinner 1998, Salli *et al.* 1998, Berisha 2001, Nilsson *et al.* 2001). Recently, it has been shown that vascular endothelial growth factor and FGF families are involved in the proliferation of capillaries that accompanies the selection of the preovulatory follicle. This results in an increased supply of nutrients and precursors, therefore supporting the growth of the dominant follicle in bovine ovaries (Berisha *et al.* 2000*a*). In addition, FGF2 may play a significant role in bovine granulosa cell steroidogenesis (Vernon & Spicer 1994), anti-apoptotic action (Peluso *et al.* 2001) and differentiation of granulosa cells (GCs) into luteal cells during the process of luteinisation (Lavranos *et al.* 1994).

The hypothesis tested was that the preovulatory LH surge may affect FGF2 and its receptors, which may have early effects on GCs. Therefore the aim of the present study was to evaluate the expression pattern of mRNA for FGF2, its receptor variants (FGFR1IIIc, FGFR2IIIc) and nucleolin, different FGF2 molecular isoforms (by Western blotting), and the localisation of FGF2 (by immunohistochemistry) in different time-defined follicle classes between the LH surge and ovulation in the cow. The expression and localisation of FGF2 was compared with dominant follicles before the LH surge and new CL (days 2–3).

Materials and Methods

Animals and superovulation

The experimental protocol was approved by the Institutional Care (AZ 211-2531.3-33/96) and Use Committee. The study was conducted on 30 non-lactating German Fleckvieh cows. The cows were stimulated with follicle-stimulating hormone (FSH) for experimental reasons to gain more follicles per animal for different analytical purposes (e.g. mRNA extraction, immunohistochemistry, Western blot). FSH (Ovagen; Immunochemical Products Ltd, Auckland, New Zealand) injections (in total seven) were given i.m. at 12 h intervals in gradually decreasing doses for 3.5 days, starting between days 8 and 11 of the oestrous cycle after previous oestrus observation. After the sixth FSH injection, a luteolytic dose of 500 μg $PGF_{2\alpha}$ analogue (cloprostenol, Estrumate; BERNA Veterinärprodukte AG, Bern, Switzerland) was injected i.m., and then 40 h after $PGF_{2\alpha}$ injection, 100 μg gonadotrophin-releasing hormone (GnRH) (Receptal; BERNA Veterinärprodukte AG, Bern, Switzerland) were injected to induce the LH surge. For confirmation of LH surge, blood samples were collected from the jugular vein at -24, -12, -1 and 0 h before and 3 and 12 h after GnRH application. Lack of an endogenous LH surge prior to GnRH was confirmed by LH determination in blood plasma (range 0.8–1.0 ng/ml); 4 h after GnRH the mean LH level (induced LH surge) was 11.50 ng/ml (range 8.5–14.1 ng/ml) and at 12 h 0.73 ng/ml (range 0.2–1.0 ng/ml). The ovaries were collected by transvaginal ovariectomy ($n = 5$ cows/group) as described by Schams *et al.* (2003). For each parameter and group of treatment one follicle was selected randomly. The schematic time schedule of the superovulatory treatment and ovary collection is shown in Fig. 1.
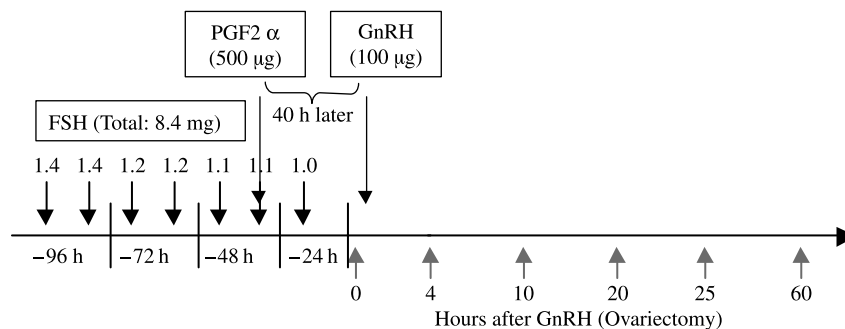
Figure 1 Time schedule of the treatment for multiple ovulation and ovary collection in cows. Ovaries containing preovulatory follicles or new CL were collected at approximately 0, 4, 10, 20 and 25 h (follicles) and 60 h (new CL) relative to injection of GnRH to induce an LH surge ($n = 5$ animals per group).

To confirm that the phenotype of the follicles collected from the superovulated model is comparable with that of the single preovulatory follicle harvested from a normal wave, follicular fluid (FF) was collected by ultrasound-guided FF aspiration by a similar model but without FSH stimulation.

Collection, classification and preparation of follicles and CL

Ovaries containing preovulatory follicles or new CL were collected at approximately 0, 4, 10, 20 and 25 h (follicles) and 60 h (new CL) relative to injection of GnRH to induce an LH surge ($n = 5$ animals per group). An LH surge was induced approximately 4 h after the GnRH administration.

Only follicles which appeared healthy (i.e. well vascularised and having transparent follicular wall and fluid) and whose diameters were >10 mm were collected. The number of follicles per ovary varied between 8 and 20. For the RNA extraction, immunohistochemistry and Western blot the follicles were dissected from the ovary. The surrounding tissue (theca externa) was removed with forceps under a stereomicroscope. Follicles were aliquoted, quickly frozen in liquid nitrogen and stored at $-80°C$ until extraction of RNA and protein. For immunohistochemistry, follicles (after aspiration of FF, and injection of fixative) and pieces of CL tissue were fixed via immersion in methanol/glacial acid (2:1) for 24 h. The FF was aspirated from follicles and stored at $-20°C$ until assayed. Progesterone, oestradiol-17β (E_2), $PGF_{2\alpha}$ and PGE_2 were measured for confirmation of follicle classes and for comparison with spontaneous growing preovulatory follicles.

Hormone determinations

The concentrations of progesterone and E_2 were determined directly in the FF with an enzyme immunoassay (EIA) using the second antibody technique (Meyer *et al.* 1990, Acosta *et al.* 1999). The effective dose for 50% inhibition (ED_{50}) of the assay was 6 ng/ml for progesterone and 3.5 pg/ml for E_2. The FF was diluted accordingly. The intra-assay coefficient of variation (CV) values were 4.5% (progesterone) and 6.6% (E_2) and the inter-assay CV values 8.7% (progesterone) and 9.4% (E_2).

The EIA for $PGF_{2\alpha}$ (Acosta *et al.* 1999) was evaluated in different dilutions directly in FF without extraction. The standard curve for $PGF_{2\alpha}$ ranged from 0.6 to 700 ng/ml, and the ED_{50} of the assay was 300–400 pg/ml. The intra- and inter-assay CV values were 8 and 14% respectively.

The EIA for PGE_2 was a commercial competitive EIA kit (Assay Design, Inc., Ann Arbor, MI, USA). PGE_2 was evaluated in different dilutions directly in FF without extraction. The standard curve for PGE_2 ranged from 40 to 5000 ng/ml, and the ED_{50} of the assay was 200–300 pg/ml. The intra- and inter-assay CV values were 8 and 12% respectively. The plasma LH concentration was determined directly in plasma by a sensitive RIA for bovine LH (Schams *et al.* 1987). The standard curve for LH ranged from 0.06 to 10 ng/ml, and the ED_{50} of the assay was 2.8 ng/ml. The intra- and inter-assay CV values were below 8.5 and 13.5% respectively.

Isolation of RNA

Total RNA was prepared from follicle and CL tissue according to Chomczynski & Sacchi (1987) with TriPure isolation reagent (Roche Diagnostics, Mannheim, Germany) as described earlier in detail (Berisha *et al.* 2000b). Possible DNA contaminations were eliminated by an additional DNase digestion according to the manufacture's protocol (Promega, Madison, WI, USA). 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 (1 μg) were subjected to 1% denaturing agarose gel electrophoresis and ethidium bromide staining to verify the quantity and quality of total RNA.

Reverse transcription

Total cellular RNA was reverse transcribed to cDNA in a volume of 60 μl containing 1 μg RNA, 2.5 μmol/l random hexamers (Gibco BRL, Grand Island, NY, USA) and M-MLV reverse transcriptase (200 U/μl) (Promega, Madison, WI, USA) according to Pfaffl *et al.* (2003). A minus-RT reaction (RT enzyme was replaced by water) was performed to detect residual DNA contamination.

Real-time PCR

Primers for FGF2, FGFR1IIIc, FGFR2IIIc, nucleolin, ubiquitin (UBQ) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using the EMBL database or used according to the literature (Table 1). Optimal conditions for RT-PCR were evaluated in a gradient thermocycler (Eppendorf, Hamburg, Germany). Quantitative fluorescence real-time RT-PCR analysis was performed with a Rotor-Gene 3000 system (Corbett Research, Sydney, Australia). Online PCR reactions were carried out using a LightCycler DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) with 1 μl of each cDNA (16.66 ng) in a 10 μl reaction mixture (3 mmol/l $MgCl_2$, 0.4 μmol/l of each forward and reverse primer, 1 × LightCycler DNA Master SYBR Green I). After initial incubation at 95°C for 10 min to activate Taq DNA polymerase, templates of all specific transcripts were amplified for 40 cycles at 95°C for 10 s followed by annealing temperatures of 60°C for all primers used, each 10 s, and elongation at 72°C for 15 s. Fluorescence data were acquired after each elongation step by SYBR Green binding to the amplified dsDNA at 72°C for 5 s. The specificity of each PCR product was determined by melting curve analysis (Rotor-Gene 3000 software, version 5.03)

Table 1 Primer sequences of FGF2, FGFRIIIIc, FGFR2IIIc, nucleolin, UBQ and GAPDH, RT-PCR product length, and reference of the investigated factors or of the according accession number in the EMBL database.

Target	Sequence of nucleotide*	Fragment size (bp)	EMBL/reference**
FGF2	For 5′-AGCCTTGCAACTCTGCTTTGT-3′ Rev 5′-CCAATTCAGATCCCTCCTGA-3′	210	M13440
FGFRIIIIc	For 5′-ACTGCTGGAGTTAATACCACCG-3′ Rev 5′-GCAGAGTGATGGGAGAGTCC-3′	125	AJ577088
FGFR2IIIc	For 5′ -CACCACGGACAAAGAAATTG-3′ Rev 5′-ATGCAGAGTGAAAGGATATCCC-3′	113	AJ413268
Nucleolin	For 5′-AGCTGTTCCTGTGAAGGCTAAG-3′ Rev 5′ -TTCTTTGACAGGCTCTTCTTCC-3′	154	AF244991
UBQ	For 5′ -ATGCAGATCTTTGTGAAGAC-3′ Rev 5′-CTTCTGGATGTTGTAGTC-3′	189	Berisha *et al.* (2000a)
GAPDH	For 5′-GTCTTCACTACCATGGAGAAGG-3′ Rev 5′-TCATGGATGACCTTGGCCAG-3′	197	Berisha *et al.* (2002)

* For, forwards; Rev, reverse.
** EMBL accession number or reference of published sequence.

immediately after PCR completion by heating at 95°C for 5 s, followed by cooling to 65°C for 5 s and continuous heating to 99°C at 0.5°C/s under permanent fluorescence detection. Confirmation of PCR product identity was obtained through melting curve analysis (Rotor-Gene 3000) and subsequent gel electrophoresis separation.

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 ('takeoff' points). The changes in mRNA expression of examined factors were assayed by normalisation to the UBQ internal control. In order to obtain the CT (cycle threshold) difference the data were analysed using the ΔΔCT method described previously by Livak & Schmittgen (2001), where $\Delta CT = CT_{target} - CT_{UBQ}$ and where $\Delta\Delta CT = \Delta CT_{(group\,I,\,as\,control)} - \Delta CT_{(group\,II-VI)}$. The statistic analyses were determined from ΔCT values ($n = 5$ samples/group). Expression changes (ΔΔCT) for the different groups were defined as the relative expression, compared with the follicle group collected at 0 h (before GnRH application as control). As the PCR amplification is a process with exponential character, a ΔΔCT difference denotes a shift in regulation by a factor of 2 ($2^{-\Delta\Delta CT}$) (Livak & Schmittgen 2001).

Immunohistochemistry of FGF2

Follicles were 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 horseradish peroxidase 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 tetra-hydrochloride and 0.0006% hydrogen peroxide in 0.1 mol/l PBS. Between each step sections were washed three times 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 μg/ml). Lack of detected staining of tissue elements in the controls demonstrated the specificity of the reactions.

Western blot

Tissue extraction

One gram of tissue was transferred into ten volumes of PBS 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 mixture was homogenised in an ice bath with Ultra Turrax equipment (Jahnke and Kunkel, Staufen, Germany). The tissue was homogenised for 1 min and kept in iced water for 1 h. After centrifugation for 10 min at 3500 *g* the total protein content in the supernatant was determined by a BCA test (Sigma-Aldrich).

Western blotting of FGF2

SDS-PAGE was performed according to the method described by Laemmli (1970). Samples (50 μg total protein in 20 μl buffer) were boiled in NuPAGE SDS sample buffer (Novex, Santiago, CA, USA) for 5 min at 95 °C and separated on a commercially available 12% Bis–Tris gel (Invitrogen, Karlsruhe LMA, Germany) under reducing conditions (for 1 h at 190 V), and transferred to

nitrocellulose membranes (Millipore PVDF, 0.45 µm; 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 enhanced chemiluminescence system (Amersham-Pharmacia, Freiburg, Germany). Negative controls were set up to replace the primary antibody with PBS. Protein samples from bovine CL or rbFGF2 (a kind gift from 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. See Blue Plus2 Pre-Stained standard (Novex, San Diego, CA, USA) was used as molecular size marker.

Statistical analyses

The statistical significance of differences in mRNA expression of examined factors and the concentrations of

$PGF_{2\alpha}$ and PGE_2 and steroids (E_2 and progesterone) were analysed by ANOVA followed by Fisher's protected least significant difference test. Because the raw data of $PGF_{2\alpha}$, PGE_2, E_2 and progesterone were heterogeneous they were ln-transformed. Ln-transformed data were homogenous and used for final statistical evaluation by ANOVA. All experimental data are shown as means ± S.E.M. Follicles and CL ($n = 5$) were obtained from five cows per group.

Results

Characterisation of follicle classes

For a better understanding and characterisation of follicle classes used, E_2, progesterone, $PGF_{2\alpha}$ and PGE_2 were determined in FF. The results of E_2, progesterone, $PGF_{2\alpha}$ and PGE_2 concentrations are shown in Fig. 2.

The concentration of E_2 was high in FF of follicle classes 0 h and 4 h, followed by a significant decrease afterwards. The concentration of progesterone in FF increased significantly from 4 h onwards and again in follicles collected at 25 h. The concentrations of $PGF_{2\alpha}$ and PGE_2 in FF were very low prior to the LH surge (0 h) but than increased continuously to maximum levels in follicles collected at
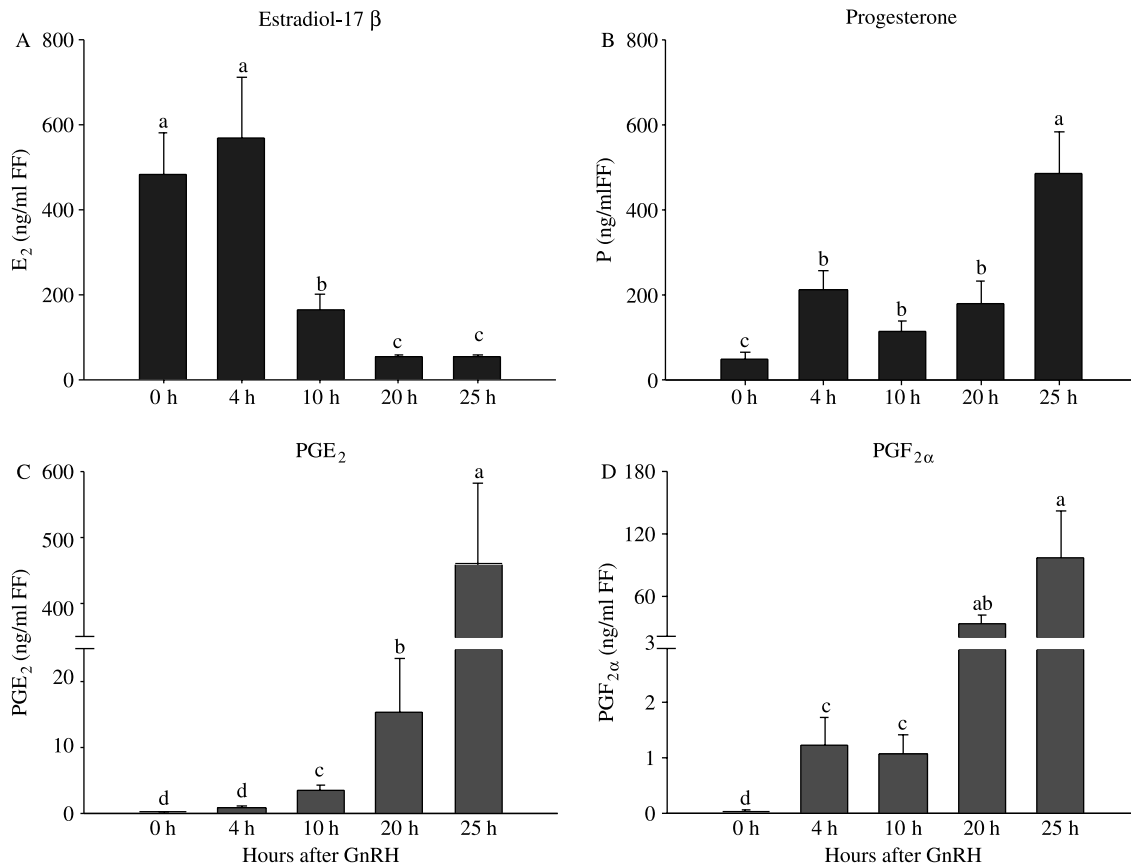
Figure 2 Concentration of (A) oestradiol-17β, (B) progesterone ('P'), (C) PGE_2 and (D) $PGF_{2\alpha}$ in the FF of preovulatory follicles collected at approximately 0, 4, 10, 20 and 25 h relative to injection of GnRH to induce an LH surge. Results are the means± S.E.M. from five follicles (five animals) per group. Different superscripts denote statistically different values ($P < 0.05$).

25 h ($P < 0.001$). In general much higher levels were always found for PGE_2 than for $PGF_{2\alpha}$.

The results of E_2, progesterone, $PGF_{2\alpha}$ and PGE_2 determined in FF of single preovulatory follicles harvested from a normal wave collected by a similar model but without FSH stimulation are shown in Fig. 3. The trends of hormone concentration in this model agree with our experimental data and confirm the validity of our method of follicle classification used after superovulation stimulation.

Confirmation of primer specificity and sequence analysis

The mRNA expression was analysed by block (conventional) and real-time RT-PCR (Rotor Gene 3000). Initial RT-PCR experiments verified specific transcripts for all factors in bovine follicles and CL. For exact length verification, RT-PCR products were separated on 2% high-resolution agarose gel electrophoresis. PCR products were verified by subcloning the cDNA into a transcription vector (pCR-Script; Stratagene, La Jolla, CA, USA), followed by commercial DNA sequencing (TopLab, Munich, Germany). Each PCR product (Table 1) showed 100% homology to the known bovine genes after sequencing.
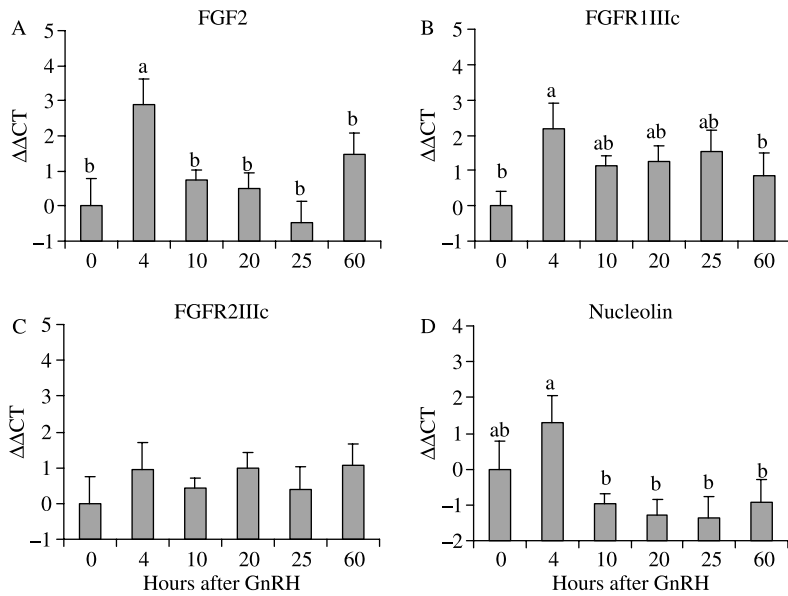
Gene expression

To confirm the integrity of the mRNA templates and RT-PCR protocol, the housekeeping genes UBQ and GAPDH were examined in all samples. The mRNA expression of these housekeeping genes in all samples resulted in constant expression levels.

The results of mRNA expression of examined factors (Fig. 4) are presented as changes ($\Delta\Delta CT \pm$ s.e.m. from five follicles or CL per group) in the target gene expression, normalised to UBQ and relative to follicles collected at 0 h (control). FGF2 mRNA expression was up-regulated in follicles collected at 4 h (Fig. 4A) but was then down-regulated shortly afterwards and with a further tendency for up-regulation after ovulation (60 h). FGFR1IIIc mRNA expression was highest in follicles collected at 4 h (Fig. 4B) and was significantly down-regulated afterwards. There were no significant differences of FGFR2IIIc mRNA transcripts in follicles and in new CL (Fig. 4C). The expression of follicular nucleolin mRNA was higher in follicle group 4 h (during the LH surge) followed by significant down-regulation afterwards in all follicle groups and in the new CL (Fig. 4D).
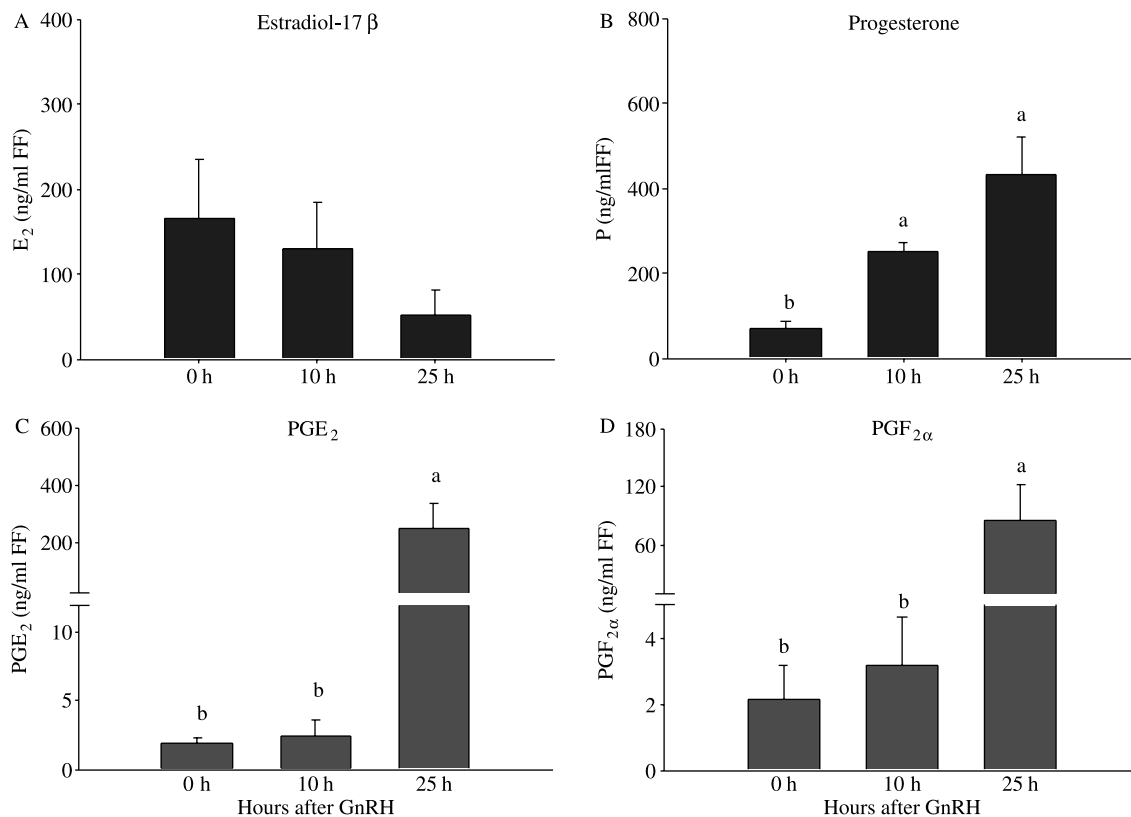
Figure 3 Concentration of (A) oestradiol-17β, (B) progesterone ('P'), (C) PGE_2 and (D) $PGF_{2\alpha}$ in the FF of non-FSH-stimulated animals collected at approximately 0, 10 and 25 h relative to injection of GnRH to induce an LH surge. Results are the means± s.e.m. from five follicles (five cows) per group. Different superscripts denote statistically different values ($P < 0.05$).
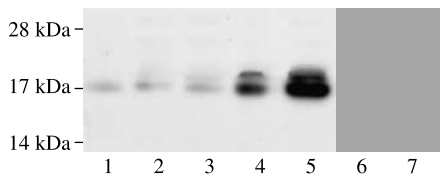
Figure 4 Expression of mRNA for FGF2 (A), FGFR1IIIc (B), FGFR2IIIc (C) and nucleolin (D) in preovulatory follicles or new CL collected at approximately 0, 4, 10, 20 and 25 h (follicles) and 60 h (new CL) relative to injection of GnRH to induce an LH surge ($n = 5$ animals per group). The changes in mRNA expression for the different groups were calculated by normalisation to UBQ and relative to the follicle group 0 h as previously described by Livak & Schmittgen (2001), where $\Delta CT = CT_{target} - CT_{UBQ}$ and where $\Delta\Delta CT = \Delta CT_{(group\,I,\,as\,control)} - \Delta CT_{(group\,II-VI)}$. The statistic analyses were determined from ΔCT values ($n = 5$). Results are presented as expression changes ($\Delta\Delta CT \pm$ S.E.M. from five follicles or CL per group) in the target gene expression, normalised to UBQ and relative to 0 h (control). Different superscripts denote statistically different values ($P < 0.05$).

Western blot analysis of FGF2

The results of FGF2 Western immunoblot analysis of follicles and CL pools (three different samples/pool) are shown in Fig. 5. rbFGF2 in new CL (60 h) and follicle class 0 h showed only one band at 18 kDa. Follicles collected at 4 and 20 h showed two bands at 18 and 22 kDa with apparent up-regulation. Preabsorption of the specific antiserum with rbFGF2 blocked both bands (lane 6 standard 0.1 ng, lane 7 follicle class 20 h after GnRH).

Immunohistochemistry of FGF2

Immunostaining with anti-FGF2 provided specific staining results on paraffin sections of bovine follicles and early CL ($n = 5$ samples from five animals/group). Positive staining for FGF2 varied during the experimental protocol with spatially different staining being observed during and after the LH surge as well as during CL formation. In general, in follicles collected at 0 h (before the LH surge) FGF2 was found to be expressed selectively in endothelial cells and pericytes of capillaries (Fig. 6A, insert) and in endothelial c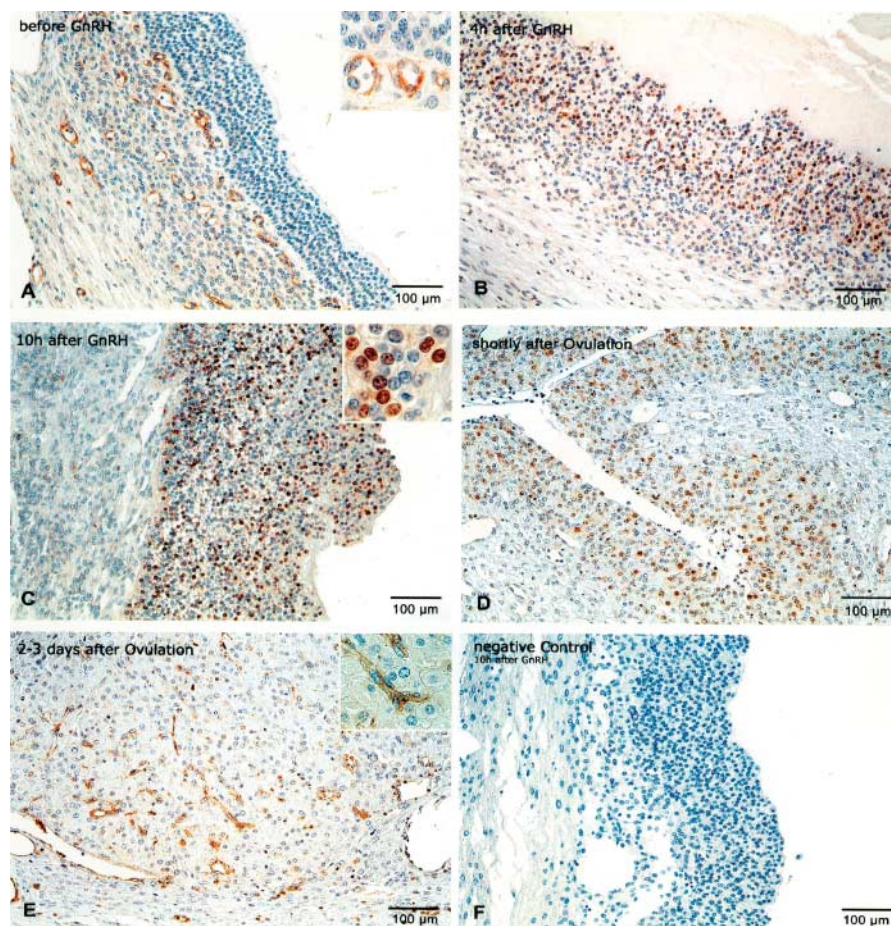ells of larger blood vessels, which are found in both theca layers (Fig. 6A). At the subcellular level, FGF2 was localised exclusively in the cytoplasm and no nuclear staining was observed at this stage. Afterwards (4, 10, 20, 25 h) the immunolocalisation of FGF2 dramatically changed and the protein was found to be expressed in the GC compartment (Fig. 6B and C). It is of particular interest at this stage that not only the cell type but also the subcellular localisation of FGF2 was changed, with an intense immunostaining found in the nucleus and nucleolus of GCs (Fig. 6C, insert). A comparable nuclear staining was also found during or shortly after ovulation (Fig. 6D) in luteal cells and a faint staining for FGF2 was seen in growing blood vessels. During the development of the new CL the immunolocalisation changed dramatically again to non-evident nuclear localisation of FGF2 in luteal cells. The FGF2 protein was now observed again in cytoplasm of endothelial cells of luteal capillaries and larger blood vessels (Fig. 6E). A negative control (primary antibody replaced with preimmun rabbit serum) showed no staining (Fig. 6F).

Discussion

In this study we have shown that the novel observation of FGF2 up-regulation and the distinct change in FGF2 localisation from theca (cytoplasm of endothelial cells) to the nucleus of GCs after the LH surge may be important for survival of GCs or for the transition of the GCs to luteal cells.

Follicle characterisation

Our results of follicle characterisation are in accord with the literature (Ireland & Roche 1983, Fortune & Hansel 1985, Martin *et al.* 1991) and confirm the validity of our method of follicle classification used after superovulation

Figure 5 Western blot analysis of FGF2 in bovine follicles and CL tissue. The position of molecular mass markers is indicated on the left. Lane 1, 0.1 ng rbFGF2 protein; lane 2, CL (pool of three CL at 60 h); lane 3, follicle tissue (pool of three follicles, 0 h); lane 4, follicle tissue (pool of three follicles, 4 h); lane 5, follicle tissue (pool of three follicles, 20 h); lane 6, 0.1 ng standard FGF2, blocking of antibody with FGF2 protein; lane 7, follicle tissue (pool of five follicles, 20 h), blocking of antibody with FGF2 protein.

Figure 6 Immunohistochemical micrographs showing localisation of FGF2 immunoreactivity in the bovine ovary. Before GnRH (follicle collected at 0 h) a strong immunoreaction for FGF2 was selectively found in capillaries and larger vessels of both theca cell layers (A). Higher magnification (insert) demonstrates a strict cytoplasmic staining in endothelial cells and in pericytes. No staining for FGF2 can be found in theca cells or GCs. Already at 4 h (B) localisation of immunoreactivity for FGF2 has dramatically changed. FGF2 was expressed in the GC compartment. Ten hours after GnRH (C) the immunostaining increases further, and a particularly intense immunoreaction for FGF2 was found in the nucleoplasm/nucleolus of GCs (insert in C). A comparable nuclear immunostaining was seen in luteal cells during or shortly after ovulation (D). A faint staining for FGF2 was demonstrated additionally in some blood vessels. During further development of the new CL (60 h) the immunolocalisation for FGF2 changed again and the nuclear staining of luteal cells was no longer visible. A strong expression of FGF2 was now observed again in endothelial cells of blood vessels (E and insert). The FGF2 protein was found again in endothelial cells of luteal capillaries and larger blood vessels. Immunostaining of FGF2 was absent when the primary antibody was replaced by non-immune serum (F).

stimulation. All preovulatory follicles collected prior to GnRH application were oestrogen-active with a E_2:progesterone ratio far greater than 1. PGs increased in our experiment dramatically around ovulation. In general much higher concentrations were always found for PGE_2 than for $PGF_{2\alpha}$. Injection of an ovulatory dose of human chorionic gonadotrophin (hCG) has been reported to induce the expression of cyclooxygenase 2 (COX-2) in the GCs of bovine preovulatory follicles (Sirois 1994). This increase in COX-2 expression was followed by a drastic increase of PG concentrations in the FF starting 20 h after the hCG injection.

This study used follicles collected after FSH stimulation, which means that more follicles were collected for experimental purposes, but this raises the question of whether these follicles are comparable with normal preovulatory

follicles. In a separate experiment (Fig. 3) comparable profiles in FF for steroids and PGs were obtained after ultrasound-guided follicle puncture of non-FSH-stimulated preovulatory follicles, but a GnRH-induced LH surge. This confirms further the usefulness of our follicle classes. Therefore we believe that results reported are likely to be reflective of what one would see in follicles collected during a normal oestrous cycle.

Expression of mRNA

The FGF2 mRNA expression was up-regulated in follicles only during the LH surge (4 h) followed by down-regulation afterwards. Receptors for FGF have been reported in rat and bovine follicles (Shikone *et al.* 1992, Wandji *et al.* 1992, Parrott & Skinner 1998, Berisha 2001). In our

study the mRNA expression pattern of FGFR1IIIc was similar to FGF2 (Fig. 4). These results support the hypothesis that the LH surge may play an important role in the up-regulation of FGF2 and FGFR1IIIc. The FGF2 mRNA up-regulation in follicles collected at 4 h (during the LH surge) correlates with the already high nucleolin mRNA expression in follicle tissue. The main functions of nucleolin relate to rRNA maturation and ribosome assembly (Ginisty *et al.* 1999). Although nucleolin was originally described as a nuclear and cytoplasmic protein, a number of studies show that it can also be expressed at the cell surface (Said *et al.* 2002). Recent results also attributed additional functions to nucleolin as a shuttle protein between the cytoplasm and the nucleus (Yu *et al.* 1998, Shibata *et al.* 2002) and between the cell surface and the nucleus (Said *et al.* 2002, Christian *et al.* 2003).

FGF2 protein

The FGF2 is a heparin-binding growth factor, which occurs in several isoforms resulting from alternative initiations of translation: an 18 kDa cytoplasmic isoform and four larger molecular mass nuclear isoforms (22, 22.5, 24 and 34 kDa). In our previous study (Berisha *et al.* 2004) and again in the present study we found only a weak FGF2 mRNA signal and no localisation of protein in GCs of mature follicles (before the LH surge). This agrees with earlier observations in the rat (Koos & Olson 1989) that FGF2 is not expressed at significant levels in GCs of rat preovulatory follicles before the LH surge, but that the mRNA is abundant in theca/interstitial tissue. Stirling *et al.* (1991) found no FGF2 mRNA in bovine GCs *in vivo*. But FGF2 was produced by bovine GCs from the preantral and antral follicles in cell culture (Neufeld *et al.* 1987). Yamamoto *et al.* (1997) suggested that localisation of FGF2 in human theca and GCs are associated with luteinisation.

The immunohistochemical analysis showed that before the LH surge (Fig. 6A) follicular FGF2 protein was localised to the theca tissue (cytoplasm of endothelial and muscle cells of blood vessels) but not in GCs. The FGF2 staining pattern changed dramatically after the LH surge, with FGF2 now being localised to the nucleus of many GCs while the majority of the capillary endothelial cells were no longer positive (Fig. 6B). With the change to nuclear localisation of FGF2 in GCs, our Western blot analysis proved apparent up-regulation of FGF2 protein in follicle classes before ovulation (Fig. 5). Western blotting showed two bands for FGF2 at 18 and 22 kDa. Both isoforms were up-regulated; however, stronger signals were observed for the 18 kDa isoform. We may speculate that both isoforms are localised in the nucleus due to the strong nuclear staining for FGF2. But this assumption needs further experimental investigation by appropriate cell fractionation procedures to separate nuclear vs cytoplasmic fractions with confirmation by Western blot experiments. Most of the reported functions for FGF2 are described for 18 kDa isoform, which is widely distributed

in many tissues. This smallest FGF2 isoform occurs predominantly in the cytosol and functions in an autocrine manner, whereas the higher molecular mass forms were associated with the nucleus and exert activities through an intracrine pathway (Arese *et al.* 1999). During the past decade, studies revealed that FGF1 and FGF2 are present in cytoplasm as well as in the nucleus and that nuclear translocation of the growth factor is required for the induction of DNA synthesis (Imamura *et al.* 1990, Wiedlocha *et al.* 1996).

Possible function of nuclear FGF2

The dramatic shift in the localisation of FGF2 to the nucleus after the LH surge suggests an important role for FGF2 in GCs. We may only speculate on important effects of FGF2 in the present experiment.

Inhibitor of apoptosis

Previous *in vitro* studies in rat GCs (Tilly *et al.* 1992, Aharoni *et al.* 1995, Trolice *et al.* 1997) have shown that FGF2 inhibits GCs from undergoing apoptosis. The mechanism through which FGF2 mediates GCs survival is unknown. More recent studies demonstrate that FGF2 activates protein kinase delta, which in turn stimulates calcium efflux, accounting in part for the ability of FGF2 to maintain calcium homeostasis and ultimately GC viability (Peluso *et al.* 2001). Comparable studies are not available for large domestic animals.

Factor for transition of GCs to luteal cells

The nuclear localisation and protein up-regulation of FGF2 after the LH surge may indicate an important step for transition of GCs to luteal cells by stimulation of transcription of ribosomal genes. The direct action of FGF2 on the level of ribosomal gene transcription (Imamura *et al.* 1990, Bouche *et al.* 1994, Wiedlocha *et al.* 1996) could correspond to an additional growth-signalling pathway, mediated by this growth factor. The LH-stimulated up-regulation of FGF2 mRNA and translocation to the nucleus of GCs lasts only for a period of 1-2 days. Afterwards the protein is localised again in the cytoplasm of endothelial cells. Therefore the observed FGF2 mRNA up-regulation and nuclear localisation may be an important step for granulosa lutein cell differentiation and growth during early luteinisation after the LH surge. The different localisation of FGF2 suggests different functions of this factor during follicle maturation, the periovulation period, CL formation and CL function.

In conclusion, we have shown for the first time a distinct change in localisation of FGF2 in the bovine follicle from the theca cell compartment (cytoplasm of endothelial cells) to the nucleus of GCs initiated by the LH surge. Nuclear FGF2 may be important for GC survival until ovulation or for the transition of GCs to luteal cells. These assumptions need further experimental clarification.

Acknowledgements

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