ウシ成熟卵胞内におけるGnRH投与による排卵時期のFibroblast Growth Factor 1（FGF1）およびFGF7遺伝子発現の変化

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—Research Note—

Expression of Fibroblast Growth Factor 1 (FGF1) and FGF7 in Mature Follicles during the Periovulatory Period after GnRH in the Cow

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Abstract. The aim of this study was to evaluate the expression pattern of mRNA for fibroblast growth factor 1 (FGF1), FGF7, and their receptor variants (FGFR2IIIb) in time-defined follicle classes before LH surge, between LH surge and ovulation, and in the early corpus luteum (CL) in the cow. The ovaries were collected by transvaginal ovariectomy (n=5 cows/group), and the follicles (n=5, one follicle/cow) were classified into the following groups: before GnRH administration (before LH surge); 3–5 h after GnRH (during LH surge); 10 h after GnRH; 20 h after GnRH; 25 h after GnRH (periovulation), and early CL (Days 2–3). The mRNA expression was analyzed by quantitative real-time PCR (RotorGene 3000). The mRNA expression of FGF1 showed no significant differences in the follicle groups examined, but increased significantly at the early CL phase. A transient increase in FGF7 mRNA expression was observed 3–5 h after GnRH and again in the early CL phase. In contrast, the expression of FGFR2IIIb was constant throughout the period from the final growth of the follicle to early CL formation. The results of this study suggest that FGF1 and FGF7 may be involved differently in the process of follicle maturation and CL formation, which is strongly dependent on angiogenesis.
Key words: Corpus luteum, Cow, FGF1, FGF7, Follicle, LH surge

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The fibroblast growth factor (FGF) family consists of at least 23 different signalling polypeptide members, which are characterized by a core region with a highly conserved sequence and structure [1]. FGFs are necessary for many biological processes and induce mitogenic, chemotactic, and angiogenic activity in a wide variety of cell and tissue types. Acidic FGF (FGF1), basic FGF (FGF2), and FGF7 (keratinocyte growth factor) are prototypic members of this large family that regulate ovarian function as a potential angiogenic factor [2–8]. FGF1 mRNA and protein have been found in the rat [9], ovine [10], and bovine [8, 11] ovary. FGF7 is mainly produced and secreted by theca cells, and granulosa cells express its receptor [8]. Therefore, it is possible that FGF7 is a paracrine hormone within the ovarian follicle.

FGF family members mediate their biological activities through high affinity tyrosine kinase receptors (FGFR1 to FGFR4) [12]. FGF receptors are characterised by the presence of two or three

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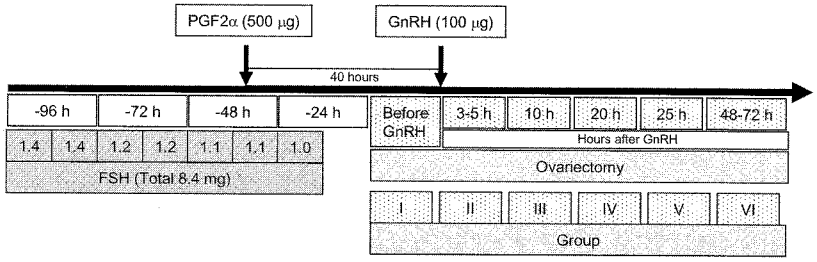

Fig. 1. Time schedule of treatment for multiple ovulation and ovariectomy in cows. Groups: (I) follicle before GnRH administration (before LH surge); (II) 3–5 h after GnRH administration (during LH surge); (III) 10 h after GnRH; (IV) 20 h after GnRH, (V) 25 h after GnRH (peri-ovulation); and (VI) early phase CLs (Days 2–3).

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 [8, 12]. Although all the different splice variants of the four FGFRs are activated by FGF1, most of the FGFR variants have narrower specificity for the different FGF ligands. In particular, FGF7 is primarily localized on epithelial cells [13, 14], and activates the splice variant FGFR2IIIb [15].

Recently, it has been shown that FGF family members and their receptors are involved in the formation of capillaries that accompany selection of the pre-ovulatory follicle [8]. However, the expression pattern of FGF1, FGF7 and FGFR2IIIb in the process of follicular maturation and corpus luteum (CL) formation in the bovine ovary is still unknown. It would be valuable, in terms of a better understanding of angiogenic regulation in dominant follicles and the CL, to investigate the mRNA expression of FGF1, FGF7, and FGFR2IIIb. Therefore, we investigated the mRNA expression of these factors in relation to periovulatory phase dominant follicles (before and after LH surge) and of early stage CLs.

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 induced to have multiple follicles (for different experimental purposes) by administration of a reduced dose (8.4 mg in total) of follicle stimulating hormone (FSH; Ovagen, ImmunoChemical Products Ltd., Auckland, New Zealand). The time schedule scheme for the superovulatory treatment and ovariectomy is shown in Fig. 1. FSH 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 the sixth FSH injection, a luteolytic dose of 500 μg of $PGF_{2\alpha}$ analogue, Estrumate (Cloprostenol; BERNA Veterinärprodukte AG, Bern, Switzerland) was injected i.m., and then 40 h after injection, 100 μg of GnRH (Receptal; BERNA Veterinärprodukte AG, Bern, Switzerland), was injected to induce LH surge. The ovaries were collected by transvaginal ovariectomy (n=5 cows/group).

Collection, classification and preparation of follicles and CL

Follicles (n=5, one follicle/cow) collected by transvaginal ovariectomy (5 cows/group) were classified into the following groups: (I) before GnRH administration (control, before LH surge); (II) 3–5 h after GnRH administration (during LH surge); (III) 10 h after GnRH; (IV) 20 h after GnRH; (V) 25 h after GnRH (peri-ovulation); and (VI) early CL (Days 2–3). An LH surge was induced 3–5 h after GnRH administration.

Only follicles that appeared to be healthy (i.e. well vascularised and having transparent follicular wall and fluid) and that had a diameter of >10 mm were collected. The number of follicles per ovary varied between 8–20. For RNA extraction, the

Table 1. Primer sequences and product size (bp) of FGF1, FGF7, FGFR2IIIb, ubiquitin (UBQ), and glycerolaldehyde-3-phosphate-dehydrogenase (GAPDH)

Genes	Nucleotide of sequence*	Size (bp)	References**
FGF1	For 5'-GCTGAAGGAGAAACCACGAC-3' Rev 5'-GTTTTCCTCCAACCTTTCCA -3'	317	[8]
FGF7	For 5'-CTGCCAAGTTTGCTCTACAG-3' Rev 5'-TCCAACTGCCAGGGTCCTGAT-3'	294	[8]
FGFR2IIIb	For 5'-TGGAGAATGAATACGGGT CC-3' Rev 5'-TCGGTCACATTGAACAGAGC-3'	298	[8]
UBQ	For 5'-ATGCAGATCTTTGTGAAGAC-3' Rev 5'-CTTCTGGATGTTGTAGTC-3'	189	[8]
GAPDH	For 5'-GTCTTCACTACCATGGAGAAGG-3' Rev 5'-TCATGGATGACCTTGGCCAG-3'	197	[25]

*: For, forward; Rev, reverse. **: Reference of the published sequence.

follicles were dissected from the ovary. The surrounding tissue (theca externa) was removed with forceps under a stereomicroscope. All follicles were aliquoted, quickly frozen in liquid nitrogen, and stored at –80 C until RNA extraction. Follicular fluid (FF) was aspirated from the follicles and stored at –20 C until assayed. Progesterone (P), oestradiol–17β (E_2), $PGF_{2\alpha}$, and PGE_2 were measured for confirmation of follicle classes and comparison with spontaneous growing preovulatory follicles.

Isolation of RNA

Total RNA was prepared from follicular and CL tissue according to the method of Chomczynski & Sacchi [16] with TriPure® isolation reagent (Roche Diagnostics, Mannheim, Germany) as described previously in detail [17]. Possible DNA contaminations were eliminated by an additional DNase digestion, according to the manufacture's protocol (Promega, Madison, WI, USA). Total RNA was 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 on 1% denaturing agarose gel electrophoresis and ethidium bromide staining to verify the quantity and quality of the total RNA.

Reverse transcription

Total RNA was reverse transcribed to cDNA in a volume of 60 μl containing 1μg RNA, 2.5 μmol/l random hexamers (Gibco BRL, Grand Island, NY), and M-MLV reverse transcriptase (200 U/μl, Promega, Madison, WI, USA) according to Pfaffl *et al.* [18]. A minus RT-reaction (the RT-enzyme was replaced by water) was performed to detect residual DNA-contamination.

Conventional PCR (Polymerase Chain Reaction)

Primers 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) and amplification was carried out in a 25 μl reaction mixture containing 1.5 μl cDNA (25 ng) as described previously [8]. PCR products were separated by 2% high-resolution NuSieve agarose (FMC BioProducts, Rockland, USA) gel electrophoresis, and band intensities (optical intensity) were analysed using an Image Master system (Amersham-Pharmacia, Freiburg, Germany) as described previously [17]. In order to validate conventional PCR results, the mRNA expression of all factors were also evaluated by real-time RT-PCR.

Real-time RT-PCR (Rotor-Gene 3000 ™)

Quantitative fluorescence real-time RT-PCR analysis was performed [18] using 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 the specific transcripts were amplified for 40 cycles at 95 C for 10 s followed by annealing at a temperature of 60 C for all primers used, each 10 s, and elongation at 72 C for 15 s. Fluorescence data used for quantitation was acquired at the end of each 72 C elongation step for 5 s by SYBR Green binding to the amplified dsDNA. Melting curve analysis (Rotor-Gene 3000™), by heating at 95 C for 5 s, followed by cooling to 65 C for 5 s, and then continuous heating to 99 C at 0.5/s under permanent fluorescence detection, and agarose gel analyses were conducted immediately after completion of PCR to verify single product formation.

Relative quantification analysis was performed with the Rotor-Gene software (version 5.03) using a dynamic amplification efficiency determination for each amplification run as provided in the comparative quantification method. The number of cycles for each sample was determined using the take-off point, which is automatically calculated by the Rotor-Gene software. The data was analyzed using the Rotor-Gene 3000® software (version 5.03). The changes in mRNA expression of the examined factors were assayed by normalization to the ubiquitin internal control. In order to obtain the CT (cycle threshold) difference, the data was analysed using the ΔΔCT method described previously [19], with $\Delta CT = CT_{target} - CT_{ubiquitin}$ and $\Delta\Delta CT = \Delta CT_{(group\ I,\ as\ control)} - \Delta CT_{(group\ II\text{-}VI)}$. The statistical analyses were based upon ΔCT values (n=5). Expression changes (ΔΔCT) for the different groups were defined as the relative expression (CT) compared with follicle group I (as control). As PCR amplification is a process with exponential characteristics, a ΔΔCT difference denotes a shift in regulation by a factor of two ($2^{\Delta\Delta CT}$).

Statistical analyses

The statistical significance of differences in examined factors were analyzed by ANOVA followed by Fisher's protected least significant difference test. All experimental data are shown as the mean ± SEM. Follicles and CLs (n=5) were obtained from 5 cows per group.

Results

Confirmation of primer specificity and sequence analysis

The mRNA expression was analysed by block (conventional) and real-time RT-PCR (RotorGene 3000). Initial RT-PCR experiments verified specific transcripts for all examined factors in the bovine follicles and CLs. For exact length verification, RT-PCR products were separated by 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.

Expression of mRNA

To confirm the integrity of the mRNA templates and RT-PCR protocol, the housekeeping genes ubiquitin (UBQ) and glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) were examined in all samples. The mRNA expression of these housekeeping genes in all samples resulted in constant expression levels.

The conventional and real-time PCR (RotorGene 3000) experiments revealed no differences in the mRNA expression patterns. Therefore, in this study, we present only mRNA expression data from real-time PCR. The results of mRNA expression for the examined factors (Fig. 2) are presented as changes (ΔΔCT ± SEM from 5 follicles or CLs per group) in the target gene expression, normalized to UBQ and relative to group I (control). The mRNA signal for FGF1 showed no significant differences in the follicle groups examined. The FGF1 mRNA expression only increased significantly (Fig. 2a) after ovulation (in the early CL phase). FGF7 mRNA expression was significantly upregulated during the LH surge and down-regulated again shortly afterwards. A further significant up-regulation occurred after ovulation (Fig. 2b). In contrast, mRNA levels of FGFR2IIIb did not change significantly in either follicles between the LH surge and ovulation or in early phase CLs (Fig. 2c).

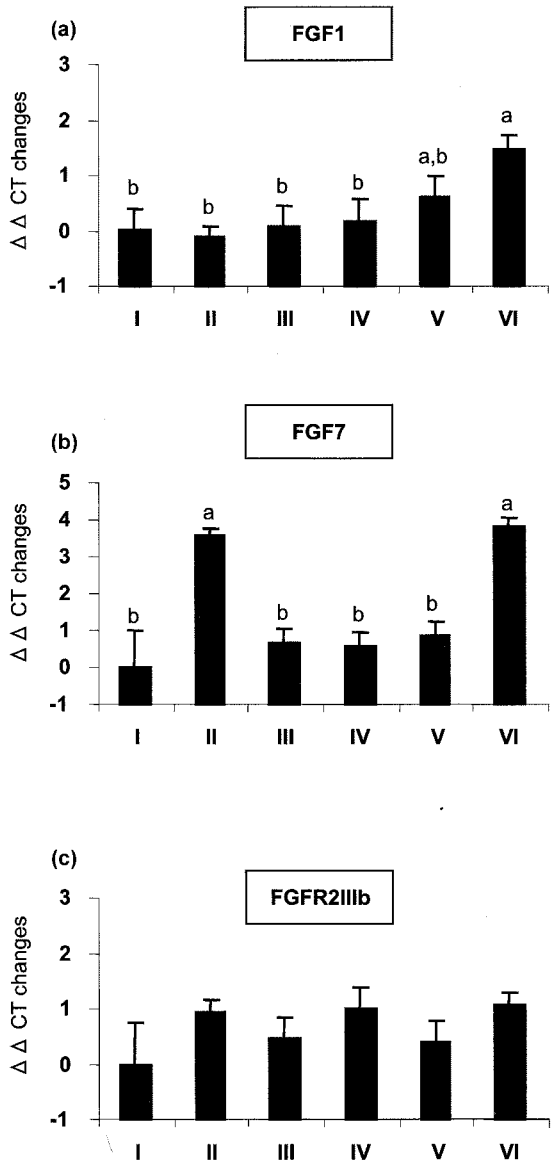

Fig. 2. Expression of mRNA for (a) FGF1, (b) FGF7 and (c) FGFR2IIIb in bovine follicle tissue and early phase CLs. (I) Before GnRH administration (before LH surge); (II) 3–5 h after GnRH administration (during LH surge); (III) 10 h after GnRH; (IV) 20 h after GnRH; (V) 25 h after GnRH (peri-ovulation); and (VI) early phase CLs (Days 2–3). Changes in mRNA expression for the different groups were calculated relative to the follicle group I (before GnRH application, as control) as the previously described by Livak and Schmittgen (2001), with $\Delta CT = CT_{target} - CT_{ubiquitin}$ and $\Delta\Delta CT = \Delta CT_{(group\ I=control)} - \Delta CT_{(group\ II\text{-}VI)}$. The statistic analyses were based upon ΔCT values (n=5). Results are presented as expression changes (ΔΔCT ± SEM from 5 follicles or CLs per group) in the target gene expression, normalized to UBQ and relative to group I (control). Different superscripts denote statistically different values ($P < 0.05$).

Discussion

The present study indicated that the expression of FGF1 mRNA increased only at the early CL phase, whereas FGF7 expression transiently increased 3–5 h after GnRH and again in the early phase CL. Since FGF7 expression is observed in the theca interna but not granulosa cells [8], the results of the present study indicates that expression of FGF7 mRNA is of theca interna cell origin. It is generally accepted that FGF1 and FGF7 are important factors for angiogenesis [8, 20]. After ovulation, definitive structural and functional changes dramatically occur, with the resultant growth and vascularization of the ovulated follicles transforming them into CL [21]. Therefore, our data suggest that FGF1 and FGF7 may be associated with angiogenesis for CL formation as well as follicular maturation toward ovulation. The results of the present study showed a transient increase in FGF7 3–5 h (during the LH surge) after GnRH treatment. Vascular endothelial growth factor (VEGF) and angiopoietins are known to be associated with follicular development and perifollicular capillary network changes during the LH surge. A recent study demonstrated that expression of angiopoietin-2 mRNA increased during the preovulatory period and ovulation in eCG/hCG-treated rats [22]. Since angiopoietin-2 acts on the destabilization of blood vessels, FGF7 may be associated with initiation of vascular formation to form the early phase CL.

The LH receptor mRNA is expressed in the theca interna cells of antral follicles in the bovine ovary. During follicular growth, the level of LH receptor mRNA expression increases with follicle size [23]. The present study demonstrated that expression of FGF7 mRNA increased 3–5 h after GnRH treatment, indicating that expression of FGF7 may be regulated by LH. Future studies are needed to examine the effect of LH on FGF7 expression in bovine theca interna cells using a cell culture system.

In this study, the expression of FGF1 and FGF7 mRNA increased in the early phase CL. At the time of ovulation, as the follicle is converted into a CL, the changes in the vascular network surrounding the mature follicle are remarkable. The capillary plexus adjacent to the basement membrane expands by 'sprouting' into the avascular granulosa layer to form a dense network of

sinusoidal capillaries. The outer capillary plexus delays sprouting until about 24 hours after ovulation, but becomes interconnected in the CL with the first network capillaries [24]. Therefore, the results of the present study suggest that FGF1 and FGF7 are associated with vascular formation in the early phase CL.

Our present data showed that expression of FGFR2IIIb is constant throughout the period from final maturation of the follicle to early phase CL formation. Constant expression of FGFR2IIIb implies that this gene is not affected by LH surge or LH surge-induced factors.

In conclusion, the present study suggests that the different mRNA levels of FGF1 and FGF7 may be implicated in different functions in relation to angiogenesis during follicle maturation, ovulation, and CL formation.

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