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Developmental and hormonal regulated gene expression of fibroblast growth factor 2 (FGF-2) and its receptors in porcine endometrium

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Abstract

This study examined the mRNA levels of the fibroblast growth factor 2 (FGF-2) and two of its receptors, FGFR1IIIc and FGFR2IIIc, at days 12 and 20 of the ovarian cycle (DC 12 and DC 20), days 1 and 12 of pregnancy (DP 1 and DP 12) as well as the influence of progesterone (P) and estradiolbenzoate (EB) on their expression in the endometrium of ovariectomized (ovx) gilts by real-time PCR. Proteins of FGF-2 and FGFR1 were immunolocalized. FGF-2 and FGFR2IIIc mRNAs were always found with a 5- to 30-fold higher absolute concentration compared to FGFR1IIIc. The latter transcript significantly declined between DP 1 and DP 12, whereas FGF-2 and FGFR2IIIc showed no significant changes at that time. FGF-2 transcription was greater at DC 20 than at DC 12, but significantly most transcripts were found in ovx gilts. EB induced a significant suppression of FGF-2 mRNA, an effect which was antagonized by P and even prevented by P + EB. FGFR1IIIc mRNA was significantly increased at DC 20, that of FGFR2IIIc at DC 12 displaying a 10 times higher absolute mRNA amount. Suppression of FGFR1IIIc mRNA by P was abolished by EB while P + EB attenuated this effect. FGFR2IIIc transcripts were equally restrained by P or EB while a combination of both slightly reduced such declines. Localization of FGF-2 and FGFR1 proteins in stromal, glandular and vascular compartments was effected by sex steroids. Both proteins were strongly expressed at DP 12 but not at DP 1. Summarized, differential temporal and spatial localization of FGF-2 and FGFR1 after response to sex steroids support a complex regulation of this ligand receptor system important for proliferation and differentiation of uterine cells including angiogenic processes. While FGFR1IIIc is presumed to be promoted by estradiol FGFR2IIIc appears to be dominated by progesterone implicating different biological importance for a functional endometrium.

Keywords: Endometrium; FGF; Gilt; Pregnancy; Real-time PCR; Steroids

1. Introduction

Ovarian-derived estrogens and progesterone are essential prerequisites for endometrial receptivity comprising growth and differentiation of glands and stroma as well as angiogenic processes. Especially in pigs, early pregnancy is characterized by a rapid development and growth of uterus and conceptus [1]. These complex processes are supposed to be supported and regulated by several growth factors [2] mediating their actions in an autocrine/paracrine manner. Among them, the members of the still expanding fibroblast growth factor (FGF) family have emerged as multifunctional regulators of cellular processes implicating differentiation, migration and cell growth [3]. One of its most famous members, FGF-2 (also called bFGF), expressed in a wide variety of adult and fetal tissues is involved in the proliferation of fibroblasts and endothelial cell, migration and differentiation processes [4]. This ligand binds with different affinity to four known transmembrane tyrosine kinase receptors, FGFR1–4. Alternative splicing in the extracellular domain of the third Ig-like domain of FGFR1–3 leads to receptor variants, IIIa–IIIc, [5,6]. FGFR1IIIc and FGFR2IIIc are thought to be the common receptors for FGF-2. Both receptors are considered to be mainly expressed in tissues of mesenchymal origin [7]. Concerning the presence and biological role of the reported FGFs within reproductive tissues, numerous studies have accumulated. FGF-2 was detected by in situ hybridization and immunohistochemistry in primate uterus [8], porcine uterine and conceptus tissues [9] as well as mouse uterus [10]. FGF-2 is expressed during gastrulation in the rabbit [11], involved in blastocyst implantation in mice [12,13] as well as in human endometrial

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neovascularization [14,15]. FGFR1 has been detected in distinct isoforms in different mouse tissues and cell lines [16,17], rat stromal cells and uterine tissues [18–20] as well as in human endometrium [21]. FGFR2 was found in the early mouse embryo [22]. The importance of the isoform FGFR2IIIc has been shown during early developmental processes and organogenesis in the mouse embryo [23,24] as well as in human ovarian tumors [25] and prostate cancer [26]. Selected candidates of the FGF family are regulated by steroid hormones: uterine FGF-2 was upregulated by estrogens in vivo [8,27] as well as in vitro in human endometrial cancer and adenocarcinoma cells [28,29]. On the other hand, it has been shown that FGF-2 control of cultured rat uterine stromal cell proliferation is progesterone-dependent [18]. Though much is known about the ligands of the FGF system, to our knowledge no physiological information is available about a developmental or steroid dependent expression of the specific receptor splice variants FGFR1IIIc and FGFR2IIIc in porcine endometrium. Therefore, one purpose of this study was to elucidate the presence, abundance and regulation of the mRNA of these FGF receptors along with their specific ligand FGF-2 in ovariectomized gilts after independent or combined replacement with P and EB as well as at two specific stages of cycle (days 12 and 20) and pregnancy (days 1 and 12) in porcine endometrium by real-time RT-PCR. As FGFR1 is proposed to be the principal receptor for FGF-2, a second objective was to assess the effect of pregnancy, ovariectomy and subsequent steroid supplementation on the localization of this ligand receptor system in porcine endometrium by immunohistology.

2. Material and methods

2.1. Experiments and treatment

This study was conducted on adult female German Landrace gilts (120 kg body weight (BW)). The experimental design was deduced from a former porcine study [30] with following alterations: endometrial samples from cycling gilts of the secretory phase of day 12 (DC 12, $n = 4$) and the proliferative phase of day 20 (DC 20, $n = 4$) as well as from pregnant gilts of days 1 and 12 (DP 1, $n = 3$; DP 12, $n = 5$) were collected and stored at $-80\,^{\circ}C$ until use. Furthermore, endometrial tissue from sex hormone depleted ovariectomized (ovx) non pregnant gilts were harvested after various treatments with estradiolbenzoate (EB: 3-benzoyloxy-1,3,5(10)-estratrien-17beta-ol) and progesterone (P) as follows: Ovariectomies were performed at day 10 of the ovarian cycle. After a recovery of 5 days, gilts received single dosis of 500 μg EB-in oil or 100 mg P or a combination of both from days 16 to 19 under frequent blood hormone control. After hysterectomy at day 20 of cycle, endometrial biopsies were collected and utilized for RNA-extraction. In addition, an aliquot of the endometrial tissue was freshly fixed in 3.7% phosphate bufferd saline (PBS), pH 7.4, formalin for subsequent immunohistological procedure.

2.2. Hormone determination

Blood samples were collected twice a day at 8.00 a.m. and 2.00 p.m. from days 11 to 19 of the estrous cycle and between days 1 and 9 after ovariectomy. The peripheral contents of estradiol-17β and progesterone were measured in blood plasma as described earlier [31].

2.3. RNA preparation

Total RNA was isolated from endometrial biopsies using a previously described method [32] with TriPure isolation reagent (Roche Diagnostics, Mannheim, Germany) as described earlier in detail [33]. The quantity and quality of the extracted RNA was determined spectroscopically through UV measurement (OD 260 nm) using a Biophotometer (Eppendorf, Hamburg, Germany).

2.4. Reverse transcription and polymerase chain reaction (RT-PCR)

First-strand cDNA synthesis was carried out in a 60 μl reaction mixture using 1 μg total RNA, 2.5 μM random hexamers (Gibco BRL, Grand Island, NY, USA) and M-MLV reverse transcriptase (200 U μl^{-1}, Promega, Madison, WI, USA) as reported earlier [34]. To detect residual DNA-contamination, a minus RT-reaction was performed replacing the RT-enzyme by water. Commercially synthesized primers (MWG, Ebersberg, Munich, Germany; Amersham Pharmacia Biotech, Freiburg, Germany) were deduced from indicated known sequences and used to amplify porcine mRNA-transcripts (predicted amplicon size and EMBL database accession numbers are assigned in parenthesis): FGF-2 (161 bp, AJ577089) forward 5′ TCAAAGGAGTGTGTGCGAAC 3′ and reverse 5′ CAGGGCCACATACCAACTG 3′; FGFR1IIIc (125 bp; AJ577088) forward 5′ ACTGCTGGAGTTAATACCACCG 3′ and reverse 5′ GCAGAGTGATGGGAGAGTCC 3′; FGFR2IIIc (139 bp; AJ439896): forward 5′ GGTGTTAACACCACGGACAA 3′ and reverse 5′ CTGGCAGAACTGTCAACCAT 3′; 18S rRNA (488 bp; QuantumRNA, Ambion, Austin, USA): forward 5′ TCAAGAACGAAAGTCGGAGG 3′ and reverse 5′ GGACATCTAAGGGCATCACA 3′. The annealing temperature of each primer pair was primarily optimized in a gradient thermocycler (Mastercycler gradient, Eppendorf, Hamburg). Subsequent RT-PCR was performed with 1.5 μl of cDNA in a volume of 25 μl as previously described in detail [34] with individual annealing temperatures of 59 °C for FGF-2 and 60 °C for 18S rRNA, FGFR1IIIc and FGFR2IIIc each for 45 s. PCR-products were amplified within 35 cycles for FGF-2 and FGFR2IIIc and 37 cycles for FGFR1IIIc. The housekeeping gene 18S rRNA served as control of the integrity of the RNA and the efficiency

of the RT-PCR including 12 cycles. Correct amplicon size was evaluated by gel electrophoresis of 7 μl aliquots (1.5% (w/v), 1 μg ml^{-1} ethidium bromide, 100 bp Ladder, Bio-Labs, Beverly, MA, USA). As a negative control, water was used instead of cDNA to detect possible contaminations.

2.5. Quantitative real-time PCR (Lightcycler)

Transcript concentrations were quantified introducing external cDNA standards based on specific PCR-products as described earlier [33] by use of a real-time-PCR cycler (LightCycler, Roche Diagnostics, Mannheim, Germany). After serial dilutions of resulting DNA-standards final sensitivity levels between 10 pg and 10 ag of specific transcripts per sample were achieved during real-time PCR as follows: using 1 μl of each cDNA the Master SYBR Green protocol was carried out (Roche Diagnostics, Mannheim, Germany) through a previously standardized Lightcycler program [33] and modified as follows: annealing temperature for 18S rRNA, FGF-2 and FGFR2IIIc 60 °C, FGFR1IIIc 62 °C; individual fluorescence detection at 77 °C for FGF-2, 78 °C for FGFR2IIIc, 81 °C for 18S rRNA and FGFR1IIIc. The efficiency (E) of the RT-PCR defined to the equation: $E = 10^{[-1/\text{slope}]}$ [35], is given by characteristic slopes for each standard curve as follows: −4.055 for 18S rRNA, −3.254 for FGF-2, −3.606 for FGFR1IIIc and −3.318 for FGFR2IIIc indicating similar amplification efficiency. The regression coefficient as control of the suitability of the linear fit was $r = -1$. Confirmation of each amplicon identity was obtained through melting curve analysis and subsequent gelelectrophoretic separation. As negative controls, water instead of cDNA was always used.

2.6. Immunohistochemistry

Immunohistochemical detection of FGF-2 and FGFR1 was performed on paraffin sections (5–7 μm). Except when otherwise mentioned PBS, pH 7.4, containing 0.05% Tween 20 was always used. Quenching of endogenous peroxidase was carried out in 1% H_2O_2 for 30 min at room temperature (RT). Antigen retrieval of FGF-2 and FGFR1 was achieved by a proteolytic pretreatment with 0.06% trypsin for 15 min at RT. Prior to immunolocalization, sections were incubated with normal goat serum (1:10, Dako, Hamburg, Germany) to reduce non-specific background staining for 30 min at RT. Sections were incubated at 4 °C overnight with an anti FGF-2 rabbit polyclonal antiserum (diluted 1:10,000) raised against the recombinant bovine FGF-2 [36] or a rabbit polyclonal antibody to human FGFR1 (diluted 1:200, F 5421 Sigma, Deisenhofen, Germany; this antibody is not able to discriminate between the different isoforms of FGFR1 but shows no crossreactivity with FGFR2 and FGFR3). Primary antibodies of FGF-2 and FGFR1 were linked with a biotinylated anti-rabbit IgG secondary antibody (1:1000, Sigma) for 1 h at RT followed by a streptavidin-immunoperoxidase technique (Sigma) for 15 min. Immunoreactive proteins were visualized via the chromogen diaminobenzidine (DAB, Sigma). Finally, sections were examined under a light microscope (Zeiss, Munich, Germany) and imaged using a digital camera (Carl Zeiss Axio Cam MR) with the axiovision® software, 3.0.6.36 (Zeiss). The specifity of the immunological reaction of FGF-2 was assessed by replacement of the specific antibody with a non immune rabbit serum, of FGFR1 with rabbit IgG, omitting primary antibodies or incubation with DAB alone.

2.7. Statistics

Results are expressed as means + S.E.M. for each group. Differences between cycling and steroid-treated animals were analyzed using one-way analysis of variance, all pair-wise multiple comparison procedures, Fisher LSD method, between pregnant groups using student's t-test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Hormone determination

The peripheral concentrations of estradiol-17β and progesterone were measured in blood plasma during the experiments revealing the expected hormone levels during normal cycle and pregnancy. Exogenous supplementation resulted in hormone concentrations during the whole treatment period indicating a physiological range of about 39–170 pM for EB and 3.18–31.8 nM for P as previously indicated [30].

3.2. Transcript identification

MessengerRNAs for the selected FGF ligand and its receptor splice variants were amplified by specific primers in porcine endometrium from sex hormone depleted, untreated cycling and pregnant gilts using conventional RT-PCR. First screening of the expected amplicons for FGF-2 (161 bp), FGFR1IIIc (125 bp) and FGFR2IIIc (139 bp) after separation by gel electrophoresis provided different staining intensities dependent on the hormonal status of the investigated animals (data not shown). The suitability and initial amount of all mRNA templates were always surveyed by evaluating the housekeeping gene 18S rRNA (488 bp). To reliably determine suspected expression changes, quantitative real-time PCRs (LightCycler) were performed. Subsequent absolute quantifications of mRNA expression in the endometrium of gilts revealed that there was no significant increase of FGF-2 mRNA between DP 1 and DP 12 (Fig. 1). The amount of FGFR1IIIc transcripts significantly declined during the course of gestation to least concentrations while the content of FGFR2IIIc mRNA increased at that time, concomitantly displaying a 7- to 30-fold higher absolute mRNA level compared to FGFR1IIIc. FGF-2 transcripts of DC 12 and DP 12

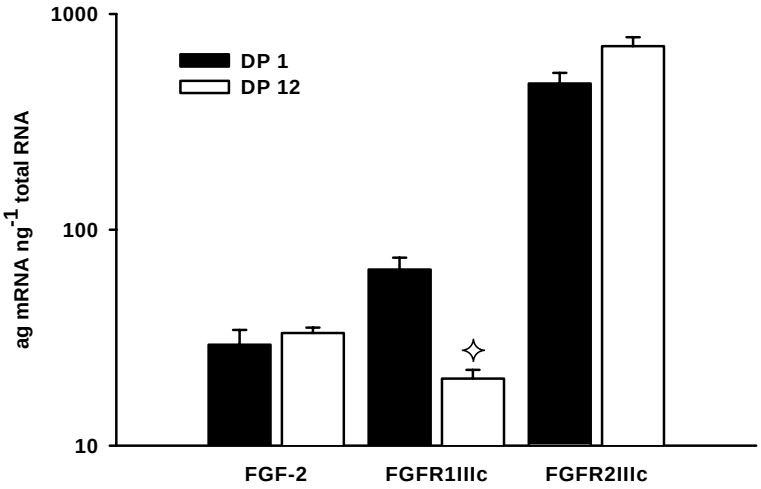

Fig. 1. Absolute mRNA quantification of FGF-2 and the receptor isoforms FGFR1IIIc and FGFR2IIIc in porcine endometrium during early pregnancy by real-time PCR. Indicated mRNA-levels were normalized against 18S rRNA and depicted on a logarithmic scale, DP 1 ($n = 5$) and DP 12 ($n = 3$): days 1 and 12 of pregnancy; t-test, $P = 0.015$ for FGFR1IIIc; (✧) significant to DP 1, mean + S.E.M.

were almost equally expressed whereas FGFR2IIIc mRNA was found to be higher expressed at DC 12 compared to DP 12. Ovariectomy resulted in significantly elevated mRNA concentrations for FGF-2 compared with cycling or steroid-treated gilts (Fig. 2). Transcription of FGF-2 was enhanced at DC 20 compared to DC 12. Though independent treatments with EB and P for 5 days significantly reduced FGF-2 mRNA expression compared to ovx and DC 20 gilts, P clearly antagonized EB induced FGF-2 suppression. A concomitant injection of P + EB reinfored FGF-2 transcription and resulted in concentrations tending to reach those for cycling gilts. FGFR1IIIc mRNA expression (Fig. 3) significantly increased at DC 20 compared to any other experimental treatment or state of cycle. Supplementation of ovx gilts with P declined FGFR1IIIc mRNA to lowest level comparable to DC 12 gilts. EB treatment alone clearly restored the amount of FGFR1IIIc transcripts while a concerted action of P + EB attenuated this effect without significant difference. In contrast to FGFR1IIIc, quantification of FGFR2IIIc mRNA (Fig. 4) resulted in significantly elevated transcripts at DC 12 compared to DC 20, ovx- and steroid-treated animals, concomitantly displaying a 10 times higher basal transcription rate. Administration of single doses of P and EB inhibited FGFR2IIIc transcripts to a greater extent than a synchronous action compared to ovx and intact cycling gilts.

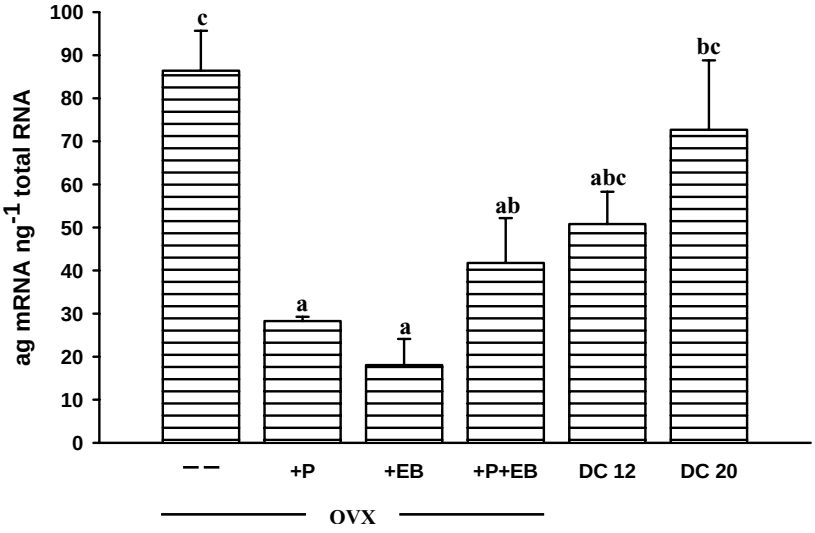

Fig. 2. Absolute mRNA quantification of FGF-2 in porcine endometrium by real-time PCR after ovariectomy (ovx/––), substitution with progesterone (P), estradiolbenzoate (EB), P + EB as well as on days 12 (DC 12) and 20 (DC 20) of the estrous cycle. Indicated mRNA-levels were normalized against 18S rRNA and depicted on a linear scale, $n = 3$ except for OVX + EB, DC 12 and DC 20 ($n = 4$), one-way analysis of variance, all pairwise multiple comparison procedures (Fisher LSD method). Significant differences ($P < 0.05$) are indicated by different letters, mean + S.E.M.

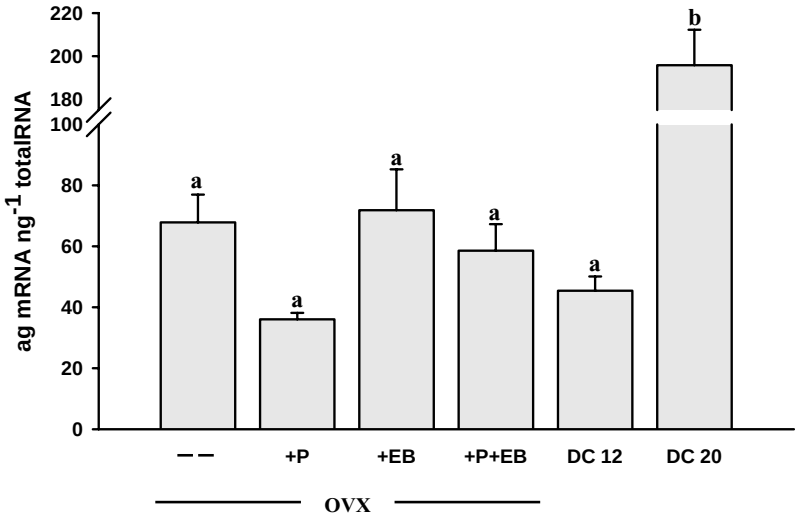

Fig. 3. Absolute mRNA quantification of FGFR1IIIc in porcine endometrium by real-time PCR after ovariectomy (ovx/−−), substitution with progesterone (P), estradiolbenzoate (EB), P + EB as well as on days 12 (DC 12) and 20 (DC 20) of the estrous cycle. Indicated mRNA-levels were normalized against 18S and depicted on a linear scale ($n = 3$) except for OVX + EB, DC 12 and DC 20 ($n = 4$), One-way analysis of variance, all pairwise multiple comparison procedures (Fisher LSD method). Significant differences ($P < 0.05$) are indicated by different letters, mean + S.E.M.

3.3. Immunohistology of FGF-2 and FGFR1

Immunohistology of FGF-2 and FGFR1 proteins was conducted with endometrial sections of pregnant (DP 1 and DP 12), ovariectomized (ovx) and sex steroid supplemented gilts. Localization of FGF-2 during early pregnancy is illustrated in Fig. 5A and B. At day 1 of pregnancy (Fig. 5A), endometrial epithelia were immunonegative whereas stromal cells showed a moderate staining. As gestation advanced to day 12 (Fig. 5B), an intense stromal and endothelial cell labeling for FGF-2 became obvious including a heterogenous pattern of partially moderate or intense stained glandular cells. FGF-2 also became evident in the luminal epithelium and the stromal matrix. When sections were examined for FGFR1 immunoreactivity at day 1 of pregnancy (Fig. 5C), protein was exclusively restricted to blood vessels while stromal and epithelial cells were FGFR1 immunonegative. A strong staining of the luminal epithelium and the stromal compartment emerged as gestation progressed to day 12 (Fig. 5D) whereas endometrial glands showed a faint FGFR1 immunoreactivity. Immunohistological experiments in ovariectomized gilts revealed that the cellular distribution

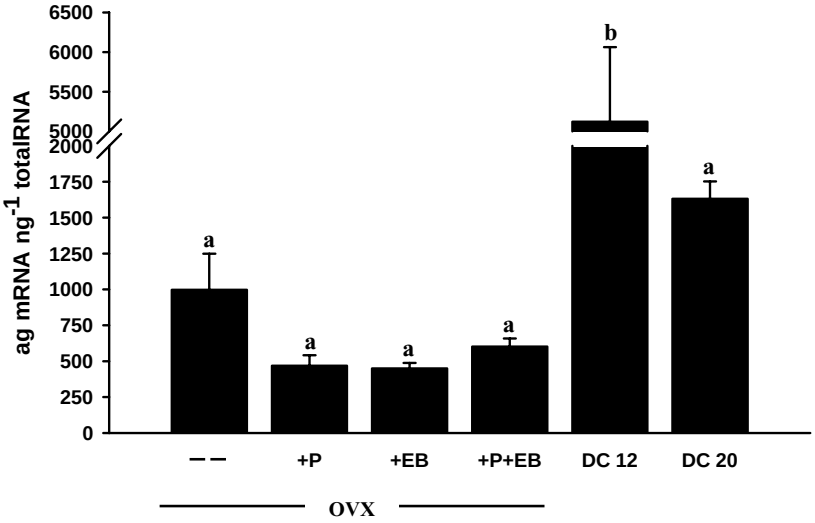

Fig. 4. Absolute mRNA quantification of FGFR1IIIc in porcine endometrium by real-time PCR after ovariectomy (ovx/−−), substitution with progesterone (P), estradiolbenzoate (EB), P + EB as well as on days 12 (DC 12) and 20 (DC 20) of the estrous cycle. Indicated mRNA-levels were normalized against 18S and depicted on a linear scale ($n = 3$) except for OVX + EB, DC 12 and DC 20 ($n = 4$), one-way analysis of variance, all pairwise multiple comparison procedures (Fisher LSD method). Significant differences ($P < 0.05$) are indicated by different letters, mean + S.E.M.

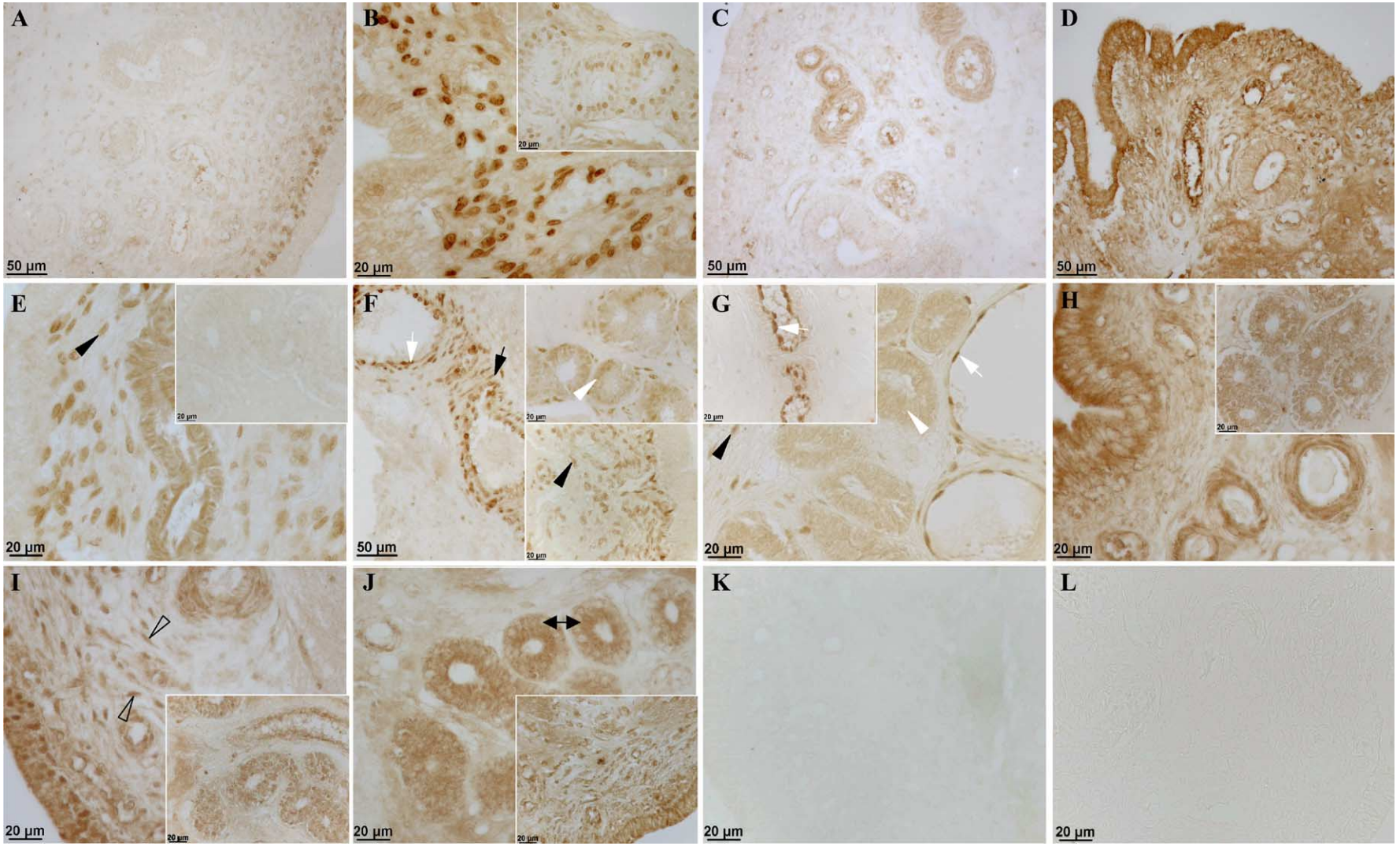

Fig. 5. Immunohistochemical localization of FGF-2 and FGFR1 proteins (brownish precipitate) in porcine endometrium. (A and B) Representative graphics of FGF-2 in pregnant gilts of days 1 and 12. (C and D) FGFR1 in pregnant gilts of days 1 and 12. At day 12, an intense stromal cell immunoreactivity including a heterogenous glandular and stromal matrix staining is seen for FGF-2; for FGFR1 a strong immunologial response of the luminal epithelium and the stromal matrix is evident. (E–G) FGF-2 localization after treatment of ovariectomized (ovx) gilts with progesterone (P) (E), estradiolbenzoate (EB) (F) and P + EB (G). Persistent FGF-2 stromal cell staining (black arrowheads) is seen under the influence of any steroid treatment. After P, glands were FGF-2 negative while vascular smooth muscles cells stained FGF-2 positive. After EB and P + EB, endothelial cells in arteries and veins (white arrows), nuclei of vascular smooth muscle cells (black arrow) as well as endometrial glands (white arrow heads) stained for FGF-2. FGFR1 protein (H–J) in ovx gilts after P (H) was found around the luminal epithelium, the basal laminae and the nuclei and cytoplasm of glands but not in stromal cells. Note the nuclear staining of FGFR1 in stromal cells (open arrowheads) after EB. (I) The loss of nuclear FGFR1 in endometrial epithelia (double arrow) after P + EB (J) is evident. Representative control sections with a rabbit non immune serum instead of FGF-2 (K) and rabbit IgG instead of FGFR1 (L) displayed no immunostaining. Scale bars are indicated in the graphics.

of FGF-2 in the endometrial vasculature and the glandular epithelium changed after different steroid treament (Fig. 5E–H). In contrast to that, stromal cells always remained FGF-2 immunopositive whereas luminal epithelial cells were constantly immunonegative under the influence of any exogenous sex steroid substitution. In detail, after P supplementation (Fig. 5E), endometrial glands of ovx gilts were clearly devoid of immunoreactive FGF-2 and staining was restricted to vascular smooth muscle layer. In contrast to P, injection of EB (Fig. 5F) resulted in a moderate and heterogenous immunoreactivity of partially nuclear and cytoplasmic glandular staining. An intense nuclear labeling of vascular smooth muscle and endothelial cells also became evident. After simultanous treatment with P and EB (Fig. 5G), a defined nuclear endothelial cell staining was observed in veins and arteries while staining of cytoplasm escaped from immunodetection. Cytoplasmic distribution of glandular FGF-2 appeared to prevail and stromal cells were still FGF-2 positive.

FGFR1 staining in sex hormones supplemented gilts (Fig. 5H–J) was present in the luminal and glandular epithelium, stromal cells and arterial vessels. According to FGF-2, cellular localization of FGFR1 was influenced by individual steroid replacement. P application (Fig. H) resulted in cytoplasmic immunoreactivity around the luminal epithelium, the basal laminae as well as the nuclei and cytoplasm of the glandular epithelium. FGFR1 also targeted cytoplasm of vascular smooth muscles while stromal cells were immunonegative. After EB treatment (Fig. 5I), nuclei of stromal and epithelial cells appeared FGFR1 positive. While localization to stromal cells and the vascular smooth muscle layer remained unchanged, nuclei of glands lacked FGFR1 staining after P + EB (Fig. 5J). Specificity of the immunological reaction for FGF-2 was verified by the replacement of the primary antibody by a non-immune serum (Fig. 5K) and for FGFR1 by rabbit IgG (Fig. 5L) as well as omitting primary antibodies (data not shown).

4. Discussion

Distinct details of a hormonal and development-dependent mRNA transcript regulation of members of the FGF family within porcine endometrium were made available in this study. It could be demonstrated that FGF-2 mRNA was higher expressed during the late proliferative (DC 20) compared with the secretory phase (DC 12) of estrous cycle. Like FGF-2, FGFR1IIIc mRNA was highest at day 20 whereas FGFR2IIIc transcripts were most abundant during the secretory phase. Hormone supplementation with P and EB resulted in differential expression patterns of each of the investigated growth factors. Though these growth factors belong of the same polypeptide family, FGFR1IIIc mRNA significantly decreased during early gestation while FGF-2 and FGFR2IIIc displayed no significant changes suggesting different physiological importance at that time. Relatively balanced FGF-2 mRNA levels between days 1 and 12 in our study agree with observations during in situ hybridisation experiments in porcine endometrium throughout the first 36 days of pregnancy [37]. In contrary, the intense labeling of FGF-2 positive stromal and glandular epithelial cells as well as luminal epithelium and the stromal matrix is intriguing as gestation advanced from days 1 to 12. This motif parallels a staining pattern found for FGF-2 protein between days 10 and 14 of pregnancy in porcine endometrium [9].

In addition to FGF-2, FGFR1 also showed an intense immunoreactivity in stromal cells which agrees with a former study in pregnant rats [20]. Taken together, their coordinated increase in stromal matrix staining at day 12 compared to day 1 support a gestation driven event. Earlier studies indicated that treatment of human endometrial stromal cells with progesterone increased FGF-2 synthesis and proliferation [38]. The loss of stromal FGF-2 synthesis, stromal proliferation and a failure of implantation is reported in the rat after withdrawal of progesterone action by the antagonist RU 486 [39]. In consideration with previous reports, immunohistological results from the present study suggest that FGF-2 may participate as a stromal expressed growth factor which mediates the transformation of endometrium into a decidua under the effect of progesterone. In support of that, P clearly antagonized the EB provoked decline of FGF-2 transcripts. Therefore, we suggest progesterone to be a potent stimulus in promoting FGF-2 expression in porcine endometrium. As recently evidenced, formation of a decidua is inevitably dependent on the activation of the progesterone receptor [40]. FGF-2 secretion by stromal cells and their uptake by FGFR1 could contribute for an autocrine mechanism in porcine endometrium. This assumption is supported by in situ hybridization experiments in porcine uterus [37] detecting FGF-2 mRNAs in stromal cells.

Beside maternally produced proteins, conceptus derived secretions of growth factors [41] such as FGF-2 from preimplanting rabbit embryos [11] are supposed to be connected with successful implantation. Corresponding receptors for FGF-2 were immunolocalized in the pregnant rat uterine epithelium during implantation [20]. Results from the present study also document a strong incidence of FGFR1 in the luminal epithelium at day 12 of pregnancy. Therefore, a possible participation of this receptor type in responding to embryonic release of FGF-2 cannot be excluded.

The present investigation indicates that EB negatively modulates porcine FGF-2 expression. From previous studies, it is known that estrogens stimulated FGF-2 expression in human fibroblasts and endometrial cancer cells in vitro [28,42]. An increase in FGF-2 mRNA after estradiol treatment in ovariectomized ewes has also been reported [43]. However, this study clearly demonstrated that EB inhibited FGF-2 mRNA to lowest levels. Presumably, the regulation of FGF-2 is suggested to be a species specific event, restricted to tissue as well as dependent on developmental status and experimental conditions. Apart from mRNA analysis, we did not detect such a variation on the protein

level. Intracellular processing, deposition or anchoring on the extracellular matrix (ECM) with heparin sulfate proteoglucans [44] may account for this observation. In parallel, the additive effect of both steroids was clearly seen for the transcriptional but not for the translational product. The applied steroid doses reached plasma steroid concentration similar to cycling gilts of day 20 [30] implying that a P sensitized porcine endometrium in conjunction with EB seemed to be indispensable to fulfill capacious physiological functions.

Localization of FGF-2 to stromal and glandular epithelial cells appeared to be sensitive to EB while P injection resulted in exclusively stromal but not epithelial FGF-2 expression. FGF-2 was abundant in the cytoplasm of vascular smooth muscle cells after P but clearly targeted their nuclei after EB treatment. Given that, nuclear distribution in these cells may result in a steroid-mediated mitogenic action of FGF-2. Its colocalization with FGFR1 to the nuclei of stromal and epithelial cells is intriguing as the activation of fibroblast growth factors normally involves an intracellular signaling network via tyrosine phosphorylations [45]. Nuclear translocation of FGFR1 in response to FGF-2 is reported in fibroblasts [46]. No definitive function for nuclear growth factor receptors has yet been elucidated; however, nuclear association is connected with proliferation of human glial cell [47]. At the moment, one may speculate that binding of FGF-2 leads to the internalization of the FGF-2/FGFR1 complex which is involved in the control of cell growth and proliferation of endometrial stromal and epithelial cells by regulating gene activity in response to steroids. On the other hand, it must be taken into account that this antibody does not discriminate between different FGFR1 isoforms. Therefore, we cannot rule out that the heterogenous localization to nuclear and cytoplasmic compartments reflects a cell specific targeting to the receptor isoforms FGFR1IIIa, b or c.

Besides a stromal and epithelial presence of FGF-2 and FGFR1 proteins, their widespread localization to endothelial cells and the perivascular tissue is intriguing. An impact of FGF-2 on angiogenic events is well-known [48]. Sustained presence of both FGF-2 and its receptor 1 protein in these cells seems to be essential to initiate and maintain the renewal and growth of endometrial vasculature for successful implantation and conceptus nourishment. In addition to that, the pivotal role of this ligand receptor system in angiogenic and nonangiogenic events in porcine endometrium is brought out by the fact that immunization of rodents with FGF-2 resulted in failure of pregnancy [49].

In the present study, mRNA analysis of porcine endometrial receptor splice variants FGFR1IIIc and FGFR2IIIc revealed a partial opposite regulation by sex hormones. Our results demonstrate a reduction of FGFR1IIIc mRNA after P treatment, at day 12 of cycle and gestation whereas highest concentrations were found at day 1 of gestation and in response to EB suggesting this FGF-receptor subtype is mainly driven by estrogens. Both a higher content of estradiol-17β at day 20 of cycle as well as at day 1 of gestation in the blood plasma compared to P may support this contention [30,50]. In contrast, significantly increased FGFR2IIIc transcripts at day 12 of the secretory phase of the cycle and even slightly elevated transcripts at day 12 compared to day 1 of gestation rather indicates a progesterone-dependent expression.

The putative impact(s) of different FGFR isoform expression and the presence of steroid hormones on reproductive health or disorders have been widely investigated in humans but less is known about farm animals. It is supposed that FGFR1 and FGFR2 were influenced by sex steroids in human uterine leiomyomas and myometrium [51]. FGFR1 is presumed to be critical for endometrial maturation and regeneration in humans [21]. Women with adenomyosis demonstrated increased expression of FGF-2 and FGFR1 compared with autologous endometrium [52]. FGFR1 was much higher expressed in cancer tissues than FGFR2 correlating with higher levels of estrogen receptors [53]. In our study, it is of interest that, despite of comparable amplification efficiencies and though both splice forms are considered to be expressed in mesenchymal tissues [7], absolute higher mRNA concentrations were always found for FGFR2IIIc but not for FGFR1IIIc. Though this report did not explicitly focus on their expression in malignancy, preliminary results from porcine endometrium suggest that both receptor isoforms may involved in very different processes of endometrial regeneration owing to the great difference in their absolute mRNA content and countercurrent behavior during cycle and especially early gestation.

Concerning the latter, most available data arose for FGFR2IIIc suggesting its importance during early embryonic development in mice [23] as well as in other animals where FGFR2IIIc was found to be regulated developmentally in a tissue specific manner [54]. Alternative splicing occurs for several FGF receptors and various transcripts have been detected for FGFR1 in rat tissues [18] as well as in human fetal ovaries and uterus [55]. Thus, the presence and influence of further alternatively spliced transcripts such as the secreted receptor, namely FGFR1IIIa or the membrane bound form FGFR1IIIb in porcine endometrium possibly contributing to physiological functions should be taken into account.

In summary, it could be demonstrated that sex steroids and the developmental status promotes distinct effects on the mRNA transcription of FGF-2 and two of its receptors. Different spatiotemporal localization of FGF-2 and FGFR1 proteins to stromal, epithelial and vascular elements of the endometrium after response to sex steroids supports a complex regulation of this ligand receptor system important for proliferation and differentiation of uterine cells including angiogenic processes. While FGFR1IIIc is presumed to be promoted by estradiol FGFR2IIIc appears to be dominated by progesterone implicating different biological importance for a functional endometrium.

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