Expression of Epidermal Growth Factor Receptor (EGF-R), Vascular Endothelial Growth Factor Receptor (VEGF-R) and Fibroblast Growth Factor Receptor (FGF-R) Systems in Porcine Oviduct and Endometrium during the Time of Implantation

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Abstract. The oviduct and uterus provide the environment for the establishment of pregnancy. Among others, growth factor systems are involved in functional signaling interactions at the pre- and peri-implantation maternal-conceptus interface in pigs. Distinct regulation of epidermal growth factor Receptor (EGF-R), vascular endothelial growth factor receptor (VEGF-R) and fibroblast growth factor receptor (FGF-R) systems and of bioactivation of EGF-R in porcine oviduct and endometrium during the estrous cycle, early pregnancy and during steroid replacement in ovariectomized gilts is summarized. Remarkable influences of ovarian steroids and EGF on the expression of specific markers of transcription and translation in these tissues are discussed. Known biological effects of the EGF, VEGF and FGF are related to cellular differentiation and angiogenesis. This suggests their involvement in the transformation of the endometrium into a decidua subsequently leading towards successful establishment of pregnancy. Peripheral steroids may exert their effects on epithelial cells both in a direct genomic manner or through mediators such as growth factors. The aim of our study was to draw specific attention to the paracrine regulation in the porcine endometrium especially during the implantation window.

Key words: Endometrium, Epidermal growth factor receptor, Fibroblast growth factor receptor, Implantation, Oviduct, Pig, Vascular endothelial growth factor receptor

are thought to play an important role in uterine preparation for implantation [3, 4].

This review will focus on selected growth factor systems involved in functional local interactions at the pre- and peri-implantation maternal-conceptus interface in pigs. Especially, the regulation of the expression and bioactivation of the epidermal growth factor Receptor (EGF-R), vascular endothelial growth factor receptor (VEGF-R) and fibroblast growth factor receptor (FGF-R) systems in porcine oviduct and endometrium during the estrous cycle, early pregnancy and after steroid replacement will be dealt with. Furthermore, first results considering the influence of ovarian steroids and EGF on the expression of specific markers of transcription and translation in these tissues are discussed.

### Expression of the EGF-R System

Oviductal and endometrial tissue proliferate and differentiate during the estrous cycle under the influence of the steroid hormones, 17β-estradiol (E2) and progesterone (P4) to provide an essential environment for the early embryo development. One mechanism by which steroids might exert their mitogenic effects on the oviduct and uterus is by stimulating the EGF-R system [5–7]. By means of histochemistry, cross-linking and [125I]-labelled EGF-receptor binding assay, we have demonstrated qualitative and quantitative changes in EGF-R (ErbB1) in porcine oviduct and endometrium on different days (days 1, 6, 12 and 20) of the estrous cycle [8, 9]. These results revealed that EGF-R is distributed both in glandular and stromal cells of the endometrium. Furthermore it was found in the epithelium of the porcine oviduct
with increased staining at the apical border of the epithelial cells. The specific ligand binding of the EGF-R in the oviduct and endometrium was dependent upon the stage of the estrous cycle (Table 1). We observed that the concentration of EGF-R increased significantly with the periovulatory period (day 20 of the estrous cycle) compared to the concentration at mid-cycle (day 12). Since the estrogen concentration in the circulation is increased at about day 20, it could be suggested that 17β-estradiol (E2) plays an important role in the regulation of the EGF-R system. This is supported by increased EGF-R mRNA and protein concentrations in oviductal membranes of ovariectomized (OVX) pigs, which were treated with estradiol benzoate (EB) (Fig. 1). Results from Salvatori et al. [10] demonstrated a dose-dependent activation of the EGF-R gene transcription by ligand-bound ER-α in HeLa cells, but in the endometrium a down-regulation of EGF-R mRNA as well as protein concentrations were observed after EB treatment. A different regulation was detected under in vivo conditions during the periovulatory period: a required specific relationship between E2 and P4 is assumed. In this context, P4 could be responsible for an increased sensitivity of the porcine endometrium to estrogen. Both steroids may control the magnitude of the combined response by a complex regulation of the EGF-R [7].

In early pregnant pigs (days 1, 6 and 12) the EGF-R protein in oviductal membranes revealed the same changes as those detected in cyclic animals [11]. Obviously, maternal steroid hormone concentrations are responsible for the EGF-R expression in the oviduct. In the endometrium of pregnant pigs, the binding capacity of the EGF-R was significantly higher on days 6 and 12 than detected on day 1, and these data are different to those obtained from cyclic pigs. Since there are no differences in the steroid hormone concentrations between cyclic and pregnant pigs at this time [12], the increase in the receptor concentrations seems not to be coordinated through the maternal plasma steroid hormones, but preimplantation pig conceptuses produce estrogen between day 11 and 12 of gestation [13, 14], which is believed to be a critical component of the signaling mechanism for maternal recognition of pregnancy. Conceptus-derived estrogen could exert effects through specific receptors on endometrial function triggering the EGF-R system during early pregnancy [15]. Since EGF-mRNA was in parallel detectable in blastocysts recovered on days 6 and 12 [11], a specific co-regulation of EGF and steroids from the blastocysts influencing the endometrial EGF-R concentrations seems likely.

Whereas EGF-R expression is related to maternal steroid hormones, no differences could be determined between the mRNA concentrations for the EGF-R ligands, EGF and transforming growth factor (TGF)α, on days 12 and 20 of the estrous cycle, and after ovariectomy and subsequent steroid substitution by real-time reverse transcription-polymerase chain reaction (RT-PCR) [9]. Therefore, especially oviductal mRNA encoding both EGF and TGFα, and endometrial TGFα mRNA seem to be relatively unresponsive to steroids. These results are in agreement with data from Kennedy et al. [16], but contrary to the results of Lei and Rao [17]. Nevertheless, endometrial EGF-specific mRNA was decreased on day 20 of the estrous cycle, which was in contrast to results reported in human and mouse uterus [18]. Such cell specific regulation of EGF expression requires further investigation to better understand its specific function in the pig.

**EGF-R Bioactivation and Signal Transduction**

Signal transduction studies on carcinoma cell lines indicate that in the initial step after binding of EGF, its receptor involves activation of the cytoplasmic receptor tyrosine kinase domain, resulting in autophosphorylation of the receptor protein. The phosphorylated tyrosine residues serve as docking sites for specific SH2-domain-containing proteins, leading to phosphorylation of cellular substrates [19] and modulation of specific signal transduction cascades.

To gather information on the functional state of the receptor, we analyzed the bioactivity of the EGF-R in the oviduct and endometrium determining EGF-dependent autophosphorylation of the receptor protein [9]. These results demonstrated that the activity of the EGF-R protein kinase is well preserved in both oviductal and endometrial tissue of mature pigs, and independent of plasma steroid hormone levels because no obvious differences were found in the endogenous tyrosine phosphorylation status of the
EGF-R protein on days 12 and 20, or after OVX and OVX+EB treatment. Interestingly, the very low plasma E2 and P4 levels after ovariectomy of pigs did not result in irreversible deactivation of EGF-R kinase activity (Fig. 2). Therefore, the increase in the EGF-R level above the threshold value in conjunction with the specific basic phosphorylation could stimulate the EGF-R system-mediated signal transduction cascade. Activation of specific kinases of the transcription and translation system may be required for local cell growth or differentiation.

In carcinoma cell lines, bioactivation of the EGF receptor protein can result in activation of the Ras-Raf-mitogen-activated protein (MAP) kinase as a main signaling pathway inducing transcription factor activation and cell mitogenesis [19, 20–22]. Furthermore, there is evidence to suggest that activation of EGF-R could also be involved in the regulation of cellular translation control. The increase in translation of specific transcripts is an important response to mitogen stimulation. Besides the activation of the cap binding protein (eukaryotic initiation factor 4E: eIF-4E), phosphorylation of a specific repressor protein (eucaryotic initiation factor 4E binding protein-1: 4E-BP1) can control cap-dependent translation of mRNAs with extensive secondary structures. Growth stimuli such as EGF, platelet derived growth factor (PDGF) or insulin, activate phosphorylation of 4E-BP1 [23], which decreases its affinity for eIF-4E and releases the block on cap-dependent translation [21]. It is presumed that these reactions result in a facilitation of the attachment of smaller ribosomal subunits to mRNA, but details of the steroid-dependent EGF-receptor system mediated processes of proliferation and differentiation in oviductal cells remain to be determined. Therefore, in the first few experiments we determined the influence of altered plasma steroid hormone levels during the estrous cycle and after steroid replacement therapy, and the influence of EGF on the abundance and bioactivity of markers of transcription (MAP\(^{42/44}\) kinase) and translation (4E-BP1). The results of our studies [24] revealed that the MAP\(^{42/44}\) kinase activity appears to be stimulated by E2, as demonstrated by increased phosphorylation of myelin basic protein (MBP) in EB treated OVX animals compared with the OVX pigs. The phosphorylation of this protein was also increased in gilts on day 20 compared with pigs on day 12 of the estrous cycle. Furthermore, the phosphorylation of 4E-BP1 in the oviductal tissue also appears dependent on E2. This finding was supported by the appearance of specific signals of 4E-BP1 at approximately 20 kDa in cytosolic preparations of day 20 pigs compared with day 12 pigs (Fig. 3). The stimulating effect of estradiol on the MAP kinase and 4E-BP1 activities might be mediated by the EGF-R system, as higher concentrations of this receptor were found in oviductal tissue under estrogen dominance [8, 9]. This is supported by the observation that both tyrosine phosphorylation of MAP\(^{42/44}\) kinase and the gel mobility retardation of 4E-BP1 are
stimulated by E2 and EGF in cultured oviductal explants of OVX pigs. In previous results we could demonstrate that the EGF-R tyrosine kinase could be also activated in the oviduct of ovariectomized pigs [9].

Expression of the VEGF-R System

VEGF has emerged as an important regulator of angiogenesis. Chakraborty et al. [25] focused on VEGF as a candidate for inducing vascular changes associated with implantation and early pregnancy. VEGF is a homodimeric heparin-binding glycoprotein encoded by a pre mRNA which can be alternatively translated into four different isoforms representing 121 amino acids (soluble form), 165 amino acids (partly bound to extracellular matrix) as well as 189 and 206 amino acids (completely associated with heparin-containing proteoglycans) [26]. VEGF transmits its signal via two tyrosine kinase family receptors: c-fms-like tyrosine kinase (VEGF-R1) and fetal liver kinase-1 (VEGF-R2).

Besides a remarkable abundance of the VEGF isoforms and their receptors in cyclic human and rat uterus [27, 28], around implantation in mice [25], rabbits [29], sheep [30] and rats [31], VEGF is highly expressed in pig deciduas [32] and at the maternal-fetal-interface during pregnancy in pigs [33]. In rat uterus and primate endometrium [28, 34] VEGF seems to be regulated by E2 and P4. In porcine endometrium a higher expression of VEGF mRNA was found after treatment of OVX animals with P4 [35]. In contrast, EB treatment led to a reduction in this specific mRNA in comparison to non-treated OVX pigs whereas for VEGF-R1, none of the steroids increased mRNA expression compared to the OVX-group. Analysis of VEGF-R2 mRNA demonstrated that only after combined EB + P4 treatment mRNA expression was increased. These results indicate that endometrial VEGF expression appears to be sensitive to P4. For the stimulation of the VEGF-R2 expression, however, a specific combination of both progesterone and E2 appears to be required as also supported by immunohistochemical data.

During early pregnancy a significant increase in VEGF mRNA as well as its receptors VEGF-R1 and VEGF-R2 was detected in porcine endometrium at day 12 compared to day 1 (Fig. 4). Since the expression of the VEGF and its receptors correlated temporarily as pregnancy advanced, our observations suggest a participation of the VEGF-R system in vascular development and the permeability of the early pregnant uterus. A similar pattern of coordinated expression of the VEGF-R system was reported during implantation in mice [25], rat endometrium [31] and in rabbit uterus [29]. Nevertheless, the endocrine regulation of such a local VEGF-R system during the time of early pregnancy is not yet completely understood. The increase in the mRNA expression of VEGF and its receptors from day 1 to day 12 of pregnancy presumes that P4 is one of its key regulators. This
observation is supported by the results of steroid replacement therapy. In the mouse system, however, E2 pretreatment is necessary to elicit a P4 effect [36]. This may indicate a different species-specific steroid-dependent regulation of the VEGF-R system, but the local influence of conceptus derived steroids and proteins on the expression of this growth factor system remains to be determined.

**Expression of FGF-2 and its Receptors (FGFR1IIlc and FGFR2IIlc)**

The members of the expanding fibroblast growth factor family are also considered to be involved in uterine stromal cell transformation regulating embryonic growth and invasion. One of the most famous members, FGF-2 (basic FGF: bFGF), expressed in a wide variety of adult and fetal tissues has been shown to mediate the proliferation of fibroblasts and endothelial cells [37]. This growth factor binds with different affinities to four transmembrane tyrosin kinase receptors (FGF-R1, -R2, -R3 and -R4). Alternative splicing in the extracellular domain of the third IgG-like domain of FGF-R1, -R2 and -R3 leads to further receptor variants IIIa, IIIb and IIIc, respectively [38, 39]. These of FGF-R1IIlc and FGF-R2IIlc are the common receptors for FGF-2. FGF-2 mRNA has been identified in endometrial epithelium, stroma and myometrium of gilts during the estrous cycle and early pregnancy. Katsahambas and Hearn [40] detected no changes in the expression of this growth factor during the estrous cycle and early pregnancy, but Gupta et al. [41] reported an increase in FGF-2 expression in luminal epithelium and stroma between days 10 and 14 of pregnancy. In our studies [42] no obvious changes in FGF-2 and FGF-R2IIlc mRNA levels could be found in porcine endometrium from day 1 to day 12 of pregnancy, but we detected a decrease of the FGF-R1IIlc mRNA level at this time (Fig. 5). Otherwise, we could observe an intense immunoreactivity of both FGF-2 and FGFR1 proteins in stromal cells and increased stromal matrix staining at day 12 of pregnancy.
Fig. 6. Immunohistochemical localization of FGF-2 (A and B) and FGF-R1 proteins (C and D) (brownish precipitate) in the endometrium of pregnant gilts on days 1 (A and C) and 12 (B and D).

Fig. 7. (a) Absolute mRNA quantification of FGF-R1IIIc in porcine endometrium by real-time PCR on days 12 and 20 of the estrous cycle. Indicated mRNA-levels were normalized against 18 S and depicted on a linear scale, (n=4). (b) Absolute mRNA quantification of FGF-R2IIIc in porcine endometrium by real-time PCR on days 12 (CD 12) and 20 (CD 20) of the estrous cycle. Indicated mRNA-levels were normalized against 18 S and depicted on a linear scale (n=4). Significant differences (P<0.05) are indicated by different letters, mean ± SEM.
pregnancy compared to day 1 (Fig. 6). These results suggest that FGF-2 and FGFR1 may participate as a stromal expressed growth factor system which mediates a P4-dependent transformation of the endometrium into a decidua. A strong incidence of FGFR1 protein in the luminal epithelium at day 12 of pregnancy may indicate a possible participation of this receptor type in responding to embryonic release of FGF-2. Furthermore, it could be demonstrated that endometrial FGF-R1IIIc mRNA was more strongly expressed during the late follicular phase than in the luteal phase suggesting that this FGFR subtype is mainly regulated by estrogen. In contrast, the FGF-R2IIIc transcripts were most abundant during the luteal phase and a P4-dependent expression may be assumed (Fig. 7). The absolute higher FGF-R2IIIc mRNA content compared to FGF-R1IIIc favors the former to play the more important role in early developmental processes of the porcine endometrium [42].

Conclusions

The growth factor systems EGF and its receptor EGF-R, VEGF188aa and its receptors VEGF-R1 + 2, as well as FGF-2 and its receptors FGF-R1IIIc and FGF-R2IIIc were detected in porcine oviductal and endometrial tissue during the estrous cycle and the time of implantation. Estrous cycle dependent variations in the expression of growth factor systems were found in correlation with specific cell types of the endometrial tissue. Maternal steroids are critical components in the regulation of the expression and bioactivation of these growth factor systems, but not all changes in the expression and bioactivation of the growth factor systems in porcine reproductive tissue appear to be coordinated with the maternal plasma steroid hormone levels. Moreover, trophectoderm cells of the day 12 blastocysts are another source of estrogen and growth factors. Therefore, a specific growth factor controlled regulation of their receptors seems likely, possibly in conjunction with the high local concentrations of estrogen as a consequence of steroid synthesis by the blastocysts. The biological effects of the ligands EGF, VEGF and FGF are mediated through phosphorylation of specific regulators of transcription and translation, cellular differentiation and angiogenesis, suggesting their involvement in the transformation of the endometrium into a decidua. Consequently, these growth factor systems seem to be involved in a supposed paracrine network to successfully establish and maintain pregnancy in pigs.

References


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