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Estrous Cycle Specific Immunolocalization of Different Domains of the Epidermal Growth Factor Receptor in the Porcine Oviduct

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Although porcine uterus is known to contain active and inactive forms of epidermal growth factor receptor (EGF-R), the latter consist of the extracellular domain only; it is currently unknown whether different EGF-R isoforms are expressed in the porcine oviduct during estrous cycle. Therefore, we used two different monoclonal antibodies, one against the extracellular and the other against the cytoplasmic domain of the EGF-R, to investigate cycle-dependent and cell-type-specific expression of full-size and truncated receptor forms. At metestrus, the majority of epithelial cells of the oviduct were strongly immunopositive for both antibodies, indicating the presence of the full-size receptor. In diestrous and proestrous stages, we found a low level of cytoplasmic but no extracellular EGF-R staining in epithelial cells. While the staining intensity of cytoplasmic domain of the EGF-R was only faint or absent in muscular tissue and blood vessels throughout the estrous cycle, extracellular domain of the EGF-R exhibited a strong immunostaining of smooth muscle cells and vascular smooth muscle cells, especially in diestrous and proestrous stages. There was no significant difference between the oviductal ampulla and isthmus in either the intensity or the pattern of both cytoplasmic and extracellular EGF-R immunostaining. We conclude that the restricted presence of the functional full-size receptor to the epithelial layer indicates a specific role during early embryonic development, whereas truncated EGF-R forms may potentially regulate contractions and blood flow in the oviduct.

Key Words: Epidermal growth factor receptor; extracellular domain; cytoplasmic domain; oviduct; pig; immunohistochemistry.

Introduction

Different growth factors and their receptors were detected in porcine oviductal and endometrial tissue during the estrous cycle and the time of implantation, respectively *(1)*. In general, growth factors seem to be involved in the development, growth, and differentiation of reproductive tissues, and in establishing and maintaining of pregnancy. In porcine reproductive tissues the epidermal growth factor (EGF) receptor system is frequently characterized *(2–5)*. The biological effects of EGF-related proteins, such as EGF, transforming growth factor α (TGFα), heparin-binding EGF-like growth factor (HB-EGF), and amphiregulin (AR), are mediated through binding to the EGF receptor (EGF-R). The full-size EGF-R is a 170-kDa integral membrane protein consisting of three distinct parts: the ligand-binding extracellular domain, the transmembrane domain, and the cytoplasmic receptor tyrosine kinase domain. Previous studies have demonstrated that concentration and bioactivity of the EGF-R protein in the pig oviduct and uterus was dependent on the stage of the estrous cycle. Higher concentrations of this receptor were found under estrogen dominance *(5,6)*. Furthermore, in the porcine oviduct the mitogenic effect of estradiol in the proliferative phase of the estrous cycle was shown to be mediated by the EGF-R system *(5,7)*.

In the last decade, alternative splicing of transcripts arising from the human EGF-R gene was an area of active research. In addition to the full-size 170-kDa membrane-bound form of the EGF-R, tumor cell lines and normal cells are known to produce a shortened transcript of the EGF-R gene that encodes the extracellular ligand-binding domain but lacks the transmembrane and tyrosine kinase domains *(8,9)*. Previously, it was demonstrated that a truncated 100-kDa isoform of EGF-R (trEGF-R) is present in the porcine endometrium *(10)*. These authors showed that trEGF-R is secreted into the uterine lumen of pregnant pigs. The function of the trEGF-R is presently unknown, particularly because the truncated form is not able to transmit signals into the cell. It is suggested that the truncated form of the EGF-R binds to the EGF-related growth factors, thereby regulating the local ligand concentration *(9)*. Moreover, a soluble truncated receptor form has been demonstrated to inhibit

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tyrosine kinase activity of the transmembrane EGF-R through direct interreceptor interaction *(11)*. It is currently not known whether the porcine oviduct may also represent a target tissue for active and inactive forms of the EGF-R that could be involved in complex modulation of growth hormone responsiveness. Therefore, the aim of our study was to analyze the EGF-R status and cell-type-specific localization in porcine oviduct by immunohistochemistry with two different monoclonal antibodies. One against the extracellular (E-2760) and the other against the cytoplasmic (E-3138) domain of the EGF-R. E-2760 antibody is not able to distinguish between the full-size and the trEGF-R form, whereas E-3138 antibody recognizes only the full-size protein and an oncogenic 68-kDa form (v-erb) lacking the extracellular domain. The results of our study clearly demonstrate the estrous cycle and cell-type-specific immunolocalization of different EGF-R domains indicating that different EGF-R isoforms are present in the porcine oviduct and may be important regulators of EGF-mediated cell growth and differentiation.

Results

Immunohistochemical analyses indicated that both domains of the EGF-R are expressed in porcine oviduct during the estrous cycle. However, remarkable temporal and spatial differences were evident in the pattern of expression of the distinct receptor domains.

Using the antibody against the cytoplasmic domain of the EGF-R (E-3138), staining was most intense in the oviductal epithelium at metestrus (Fig. 1A). A significant reduction in the number of epithelial cells with positive staining and the intensity of staining was detected at diestrous (Fig. 1C) and proestrous (data not shown), respectively. Immunostaining of cytoplasmic domain of the EGF-R was only faint or absent in muscular tissue and blood vessels throughout the estrous cycle (Fig. 1A). At the subcellular level, intense localization of cytoplasmic domain was found in the supranuclear region of oviductal epithelial cells at metestrus (Fig. 1A, inset). In diestrous and proestrous stages, respectively, this domain of the EGF-R was located predominantly close to the apical plasma membrane (Fig. 1C). There was no apparent difference between the oviductal ampulla and isthmus in either the intensity or the pattern of cytoplasmic EGF-R immunostaining (Figs. 2A–F).

With the antibody E-2760 directed against the extracellular domain, intense immunostaining of epithelial cells was equally found at metestrus (Fig. 1B). Contrary to the polarized distribution of cytoplasmic EGF-R in the supranuclear region, staining of extracellular EGF-R domain was most intense at the apical cell surface of epithelial cells with particular staining of apical cell membranes and cilia, respectively. Additional staining of the cytoplasm and supranuclear region was detected in numerous epithelial cells (Fig. 1B, inset). In all other cycle stages examined, epithelial cells were uniformly not stained with the antibody E-2760 (Figs. 1D,E,F). Moreover, the extracellular domain of the EGF-R was specifically localized in the muscular layer and blood vessels, respectively (Figs. 1E,F). The cytoplasm of smooth muscle cells (Fig. 1E, inset) and vascular smooth muscle cells (Fig. 1F, inset) exhibited a strong staining, especially in diestrous and proestrous stages. There were no obvious differences in extracellular EGF-R immunostaining between ampullar and isthmic regions of the oviduct (Figs. 2A–F).

With both monoclonal antibodies, no specific immunohistochemical labeling of stromal cells was found throughout the estrous cycle.

The absence of detectable staining of tissue elements in the control sections verified the specificity of the applied antibodies (data not shown). Additionally, sections of porcine uterine tissues were stained in parallel as positive controls. As clearly demonstrated in Fig. 1G, cytoplasmic EGF-R immunoreactivity was exclusively found in luminal epithelial cells, whereas specific and strong immunolabeling of extracellular EGF-R was detected in the luminal and glandular epithelium (Fig. 1H).

Discussion

The results we have presented in this article indicate that different forms of the EGF-R are present in the porcine oviduct during the estrous cycle. Exclusively at metestrus, evidence was found for the presence of the functional full-size receptor due to co-expression of cytoplasmic and extracellular domains in the oviductal epithelium. However, in all other tissue parts and cycle stages examined, there was a remarkable difference in either the staining intensity or the distribution pattern of cytoplasmic and extracellular domains. In particular, the antibody directed against the extracellular domain of the EGF-R, which cannot distinguish between the full-size and the truncated form, was specifically localized in smooth muscle cells and vascular smooth muscle cells where the cytoplasmic domain was only weakly stained or absent. These immunohistochemical findings provide further evidence for the existence of truncated receptor forms in the porcine oviduct.

The EGF-R system was intensely studied in porcine reproductive tissues but in almost all cases only the presence and function of the 170-kDa full-size receptor was examined *(4,6)*. In porcine oviduct epithelial cells higher concentrations and significantly higher endogenous protein tyrosine kinase activity of EGF-R have been demonstrated on d 1 compared to d 6 and 12 of the cycle *(6)*. Even if d 1 was determined for the second day after human chorion gonadotropin (hCG) application, our immunohistochemical data are largely comparable. We found strong immunostaining of cytoplasmic and extracellular domain in epithelial cells at metestrus (d 1–3) indicating a functional status of EGF-R in the peri- and postovulatory phase of the cycle. Immunoreactivity of both domains decreased markedly in the epithelium of following cycle stages, whereas extracellular ligand-binding domain of the EGF-R was completely absent. Therefore, our results suggest little growth-regulatory influence of EGF-

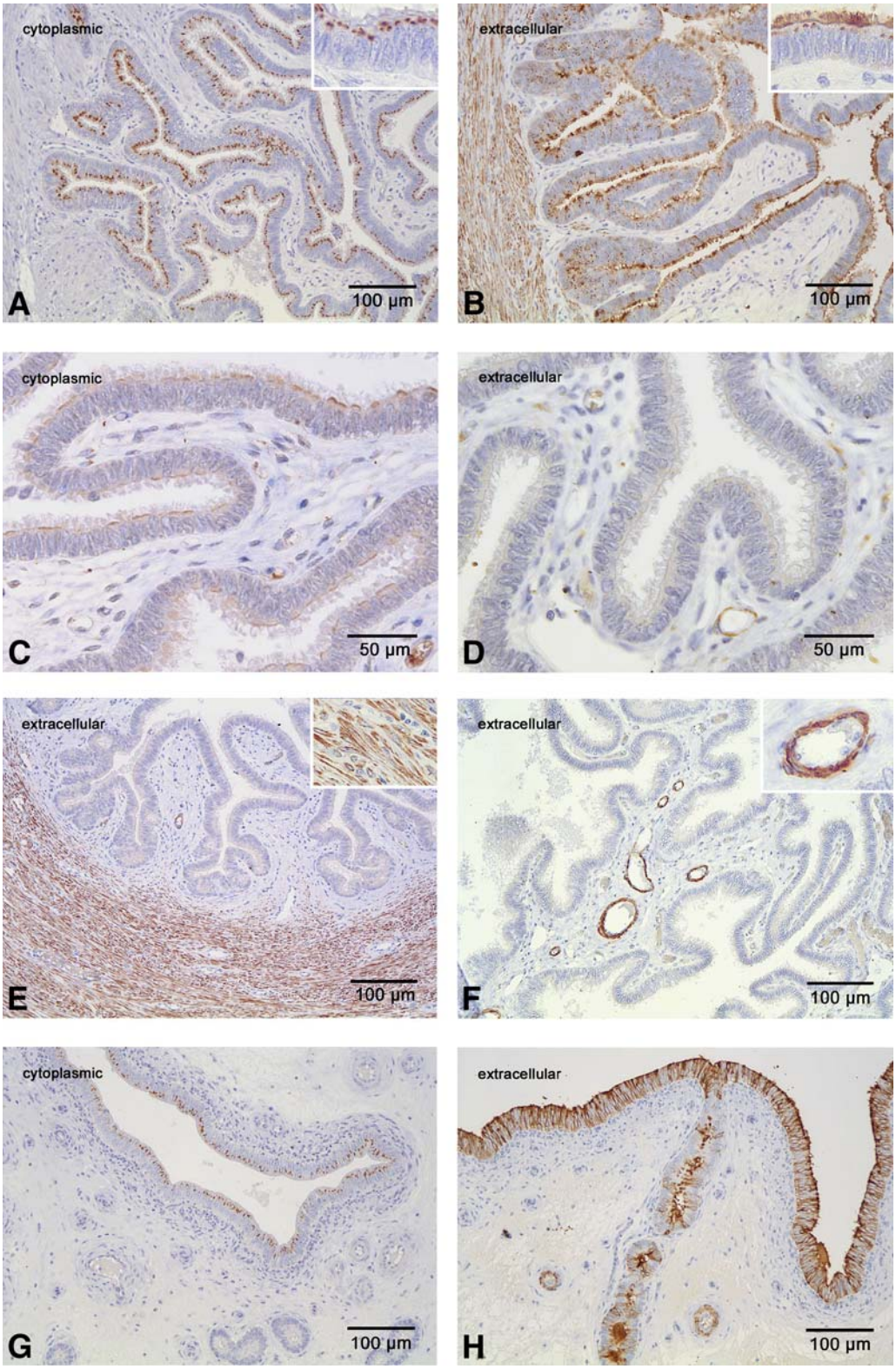

Fig. 1. Immunohistochemical localization of cytoplasmic (**A,C,G**) and extracellular (**B,D,E,F,H**) domain of EGF-R in porcine oviduct (**A–F**) and uterine (**G,H**) tissues. Strong immunostaining for both EGF-R types was found in supranuclear (**A**) and cell surface (**B**) region of the oviduct epithelium at metestrus. Faint apical (**C**) or absent staining (**D**) intensity was found at diestrous and following stages. Additionally, intense immunoreaction of extracellular domain was detected in smooth muscle cells (**E**) and vascular smooth muscle cells (**F**). In positive control sections cytoplasmic EGF-R was exclusively located in luminal epithelial cells (**G**), whereas extracellular domain of EGF-R intensely stained glandular and luminal epithelial cells (**H**). Estrous cycle stages shown are metestrus (**A,B,G,H**), diestrous (**C,D,E**), and proestrous (**F**).

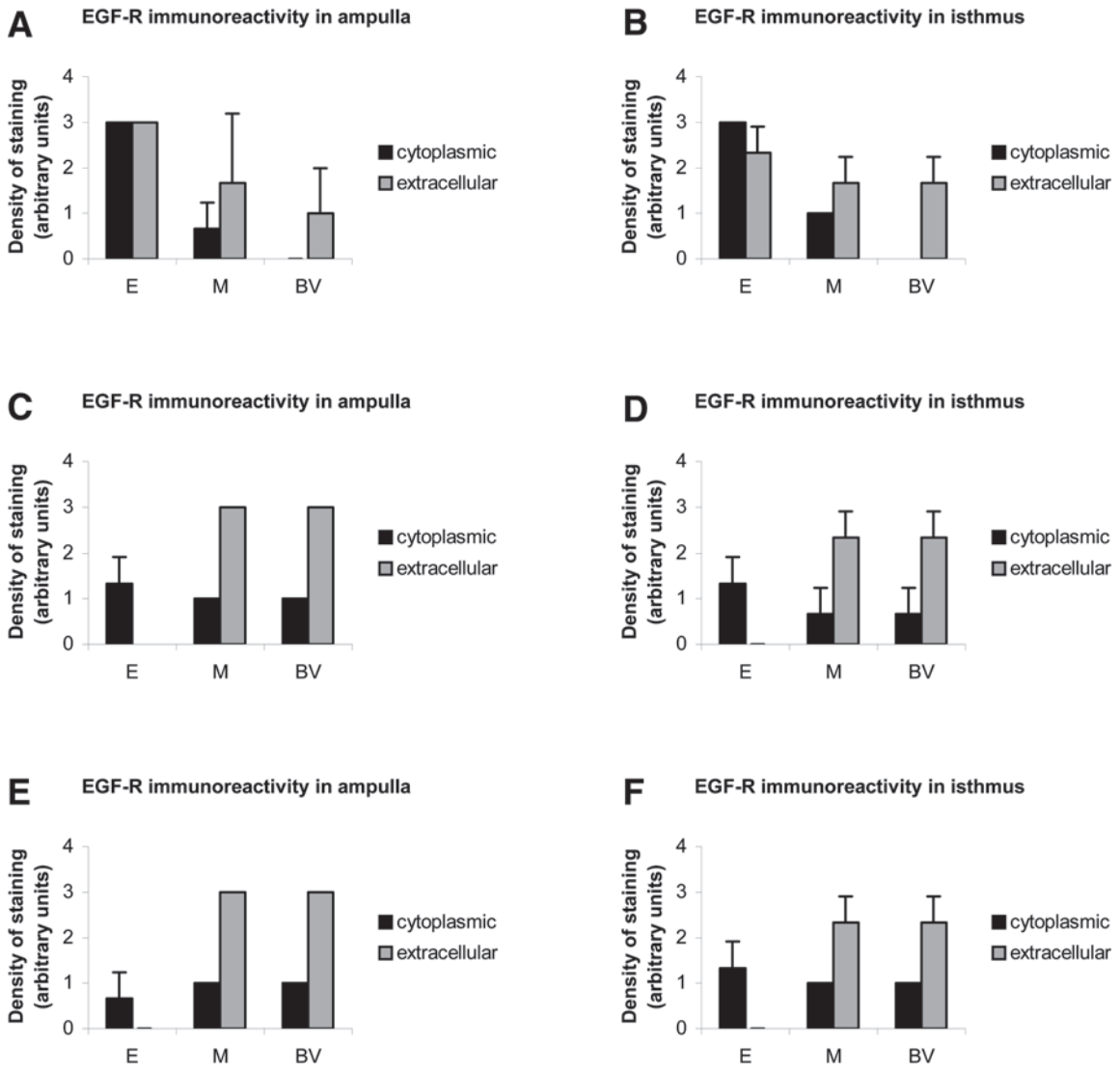

Fig. 2. Intensity of cytoplasmic and extracellular EGF-R immunoreactivity in ampullar (**A,C,E**) and isthmic (**B,D,F**) tissue of the pig oviduct at metestrus (**A,B**; $n = 3$), diestrous (**C,D**; $n = 3$), and proestrous (**E,F**; $n = 3$); Mean scores ± SEM of arbitrary units of immunoreactivity are given. Each part of the tissue showing immunoreactivity (E = epithelium, M = muscularis, BV = blood vessels) was assigned a score from 0 to 3, where 0 represented no detectable staining, 1 faint staining, 2 moderate staining, and 3 intense staining.

related growth factors on the epithelium at these time points after ovulation. This is strongly in contrast with results reported by others that higher concentrations of EGF-R are found under estrogen dominance *(5,6)*. One possible explanation for this is that our oviduct specimens in proestrous stage were predominantely taken at the early time point of this estrous phase. It is most likely that differences in the expression of the EGF-R exist between early and late proestrous stages and periovulatory phase, respectively. The results of Chegini et al. *(12)* in human fallopian tubes that EGF-R immunostaining was considerably higher during late proliferative than during early proliferative phase of the menstrual cycle confirm our observations. In this context it appears to be absolutely necessary for comparability of future EGF-R studies to choose not only exactly defined cycle stages but also well-defined oviduct regions. Regarding the experimental design of the above-described investigators *(5–7)*, it becomes evident that different oviduct tissue regions, e.g., the region of ampullary–isthmic junction, and methods used for EGF-R determination (e.g., the whole oviduct for EGF-R binding assays) are probably another reason for differing results compared with our immunohistochemical data.

In the present study, staining levels of the extracellular domain of the EGF-R showed changes in relation to the stage of the estrous cycle and cellular localization, respectively. The strong immunostaining of epithelial cells at metestrus was followed by strong immunoreaction in muscular tissues and blood vessels during diestrous and proestrous stages. Owing to very low staining levels of the cytoplasmic domain at the same time and in the same cells, we suggest that the antibody against the extracellular domain recog-

nizes the truncated form of the EGF-R (trEGF-R), which is not always co-regulated with full-size receptor expression.

Only one report in the porcine endometrium demonstrated the presence of trEGF-R in luminal and glandular epithelial cells as well as secretion of truncated form into the uterine lumen of the pig *(10)*. On the other hand, several studies in uterine tissues of different animals and humans have shown the presence of truncated receptor isoforms *(9, 13,14)*. To our knowledge, this is the first report indicating that a truncated receptor form is also present in the porcine oviduct. The function of the trEGF-R in the oviduct is presently unknown, but, in general, a specific growth-inhibitory potential of this receptor due to ligand-binding domain was suggested *(15)*. Consequentially, we suggest that oversupply with growth factors like EGF and TGFα may be critical for proliferative and differentiation-associated events in reproductive tissues, especially during pregnancy. Therefore, truncated receptor isoforms could be seen as part of a regulatory system that restricts the growth factor–mediated effects to the physiological requirement. Such a similar function has been ascribed to progesterone that inhibits the bioavailability of insulin-like growth factors to the embryo during the early development within the fallopian tube *(16)*. In this study, we have demonstrated that truncated receptor forms might be specifically present in smooth muscle cells and vascular smooth muscle cells of the oviduct. These tissue parts are also known to be influenced by growth factors. Specific localization of EGF, TGFα, and its receptors was already presented in smooth muscle cell layers and vascular smooth muscle cells, which suggests that these two growth factors may potentially regulate contractions and blood flow in the oviduct *(12,17)*. Interestingly, the presence of trEGF-R was already noted in mouse myometrium and a similar regulation in terms of fine-tuning the ligand-receptor signaling is suggested *(17)*.

In conclusion, in the porcine oviduct different domains of EGF-R were localized in a cycle-dependent and cell-type-specific manner. The presence of the functional full-size receptor was restricted to the epithelium at metestrus indicating a specific role of the EGF-R during early embryonic development. In diestrous and proestrous stages truncated forms of the EGF-R are dominantly expressed in smooth muscle cells and could possess important growth-regulatory functions. Our data could be useful to understand the role that growth factors and their receptors play in normal and pathological biological processes as similarly shown for smooth muscle hypertrophy *(18)*.

Materials and Methods

Animals and Preparation of Oviductal Tissue

A total of nine gilts of the German Landrace ranking of 8–16 mo in age with a body weight of 114–160 kg were obtained from the experimental station of animal husbandry, animal breeding, and small animal breeding of the University of Hohenheim. Estrous detection was performed twice daily in the presence of a boar. The gilts were slaughtered in metestrus (d 1–3; $n = 3$), diestrous (d 13–14; $n = 3$), and proestrous (d 18–20; $n = 3$) phases, repectively. The day the female pig accepts the boar for mating was determined as d 0 of the estrous cycle *(19)*. Immediately after slaughter, the genital tracts were removed and explored for confirmation of ovarian status and normality. For immunohistochemistry, tissue pieces from oviductal ampulla and isthmus were fixed in methanol/glacial acid 2:1 for 24 h and paraffin-embedded according to standard procedures *(20)*. For immunohistochemical stainings, tissues were cut into 5-µm-thick serial sections, and mounted on Superfrost® glass slides.

Immunohistochemistry

An avidin–biotin–peroxidase complex (ABC) method was used as previously described *(21)*. To analyze the EGF-R status, we used two different monoclonal mouse anti-human antibodies (Sigma, Deisenhofen, Germany), one against the extracellular (E-2760) and the other against the cytoplasmic (E-3138) domain of the EGF-R. The primary antibodies were diluted 1:50 in phosphate-buffered saline (PBS, pH 7.4).

Briefly, after deparaffinization and antigen retrieval in a microwave oven in 10 m*M* sodium citrate buffer at pH 6.0 (4 × 5 min at 600 W), endogenous peroxidase activity and nonspecific protein binding of the sections were blocked by 1% hydrogen peroxide in double-distilled water for 10 min at room temperature (RT) and 10% normal rabbit serum (Dako, Hamburg, Germany) for 30 min at RT, respectively, before incubation with the primary antibodies at 5°C overnight in a humid chamber. The next day, sections were treated with the biotinylated rabbit–anti-mouse second antibodies (diluted 1:300 in PBS; Dako) for 30 min at RT and incubated with Strept-ABC kit (Dako). Each incubation step was followed by 3 × 5 min rinsing with PBS. The reaction product was visualized with 3,3'-diaminobenzidine–hydrogen–peroxide reagent (Biotrend Chemicals, Köln, Germany). Finally, sections were counterstained with Mayer's hematoxylin, dehydrated, cleared with xylene, and mounted in DePeX (Boehringer, Ingelheim, Germany).

Using a brightfield light microscope (DMRBE, Leica, Bensheim, Germany) and a video camera (ProgRes, Kontron Instruments, Watford, UK) coupled to a Pentium PC, the intensity of immunohistochemical staining was ranked between 0 (absent) and 3 (most intense). For analyses of staining intensity, three sections from each animal (nine sections per cycle stage) were evaluated in different tissue parts (epithelium, muscular tissue, blood vessels). Results are summarized as mean scores ± SEM of arbitrary units of immunoreactivity.

Controls were performed by (i) replacing of the primary antibodies with non-immune serum; (ii) their substitution with buffer; and (iii) incubation with diaminobenzidine

reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity. The control sections were next to those used for the immunostaining of the EGF-R proteins. Lack of detectable staining of tissue elements in the controls demonstrated the specificity of the reactions (data not shown). Additionally, sections of porcine uterus were stained in parallel as positive controls.

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