*Praca oryginalna* *Original paper*

# Differential expression of genes encoding EGF, IGF-I, TGF β1, TGF β2 and TGF β3 in porcine endometrium during estrus cycle at different ages*)

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**Differential expression of genes encoding EGF, IGF-I, TGF β1, TGF β2 and TGF β3 in porcine endometrium during estrus cycle at different ages**

Summary

**The endometrium undergoes several biochemical and morphological changes during estrus and early pregnancy. These changes include expression of genes encoding growth factors that stimulate the endometrium to proliferate, differentiate and develop. All of these factors have been well defined in the porcine endometrium during pregnancy. However, further investigation is necessary into the expression of growth factors during estrus. Gonadotropin-primed (eCG, hCG) prepuberal gilts (n = 20), cycling gilts (n = 20) and multiparous sows (n = 20) were used in this study. After using real-time quantitative PCR (RQ-PCR) reaction methods, the gene expression of genes encoding epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1) and transforming growth factors (TGF) β1, β2, and β3 was determined in the porcine endometrium during estrus in different periods of porcine life. An increased level of all transcripts under investigation was found in prepuberal gilts, cycling gilts and multiparous sows during estrus as compared to non-estrous controls. Moreover, the examinations demonstrated higher levels of EGF and IGF mRNAs in the endometrium isolated from multiparous sows as compared to prepuberal gilts and cycling gilts. Such results suggest that increased levels of these growth factors in estrous females as compared to controls may be associated with biochemical changes and hormone secretion in the endometrium during this period. This may also be associated with the preparation of the endometrium for pregnancy and successful embryo implantation. The results achieved require further investigations involving the analysis of protein levels and the evaluation of hormone concentrations.**

**Keywords: pig, estrus, endometrium, growth factors**

Uterine morphogenesis is regulated by several endocrine, cellular, and molecular mechanisms, many of which still need to be investigated in detail (2, 8). Endometrial morphogenesis requires specific interactions between the epithelium and stroma. To date, several stroma-derived factors, which are responsible for the regulation of epithelial proliferation, differentiation and development have been identified. Generally, growth factors act via their specific receptors, which may change the patterns of membrane protein presentation to target cells. However, growth factors may also act as specific elements on cell surface receptor complexes. Recent findings indicate that both local and systemic growth factors are involved in processes of uterine development (20).

Epidermal growth factor (EGF) is expressed in mammals in a variety of tissue types, including the female reproductive tract. It has been well described that the EGF receptor may bind several ligands, including EGF, transforming growth factor α (TGFα), amphiregulin (Ar) and heparin-binding EGF (HB-EGF) (6, 18). The biological function of EGF and its receptor in the porcine oviduct and endometrium is still under investigation (13). Transforming growth factor (TGF) is a protein that, via binding with its receptor, may regulate cell proliferation, cell differentiation and several other functions

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in many cell types in mammals. The TGF superfamily proteins (TGF β1, TGF β2, TGF β3) are encoded by different genes located on various chromosomes (9). The expression of the TGF gene superfamily in the human endometrium has been investigated intensively (7). However the potential role of the expression of these genes in porcine endometrial tissues has yet to be proven.

The growth factors are potential mediators of several remodeling and repair processes in the uterus (19). Several studies indicate that insulin growth factor (IGF) is one of the most important elements in the correction of wounds of defective tissue repair and in the regeneration and epithelialisation of connective tissue (16).

The knowledge of uterine development and the regulation of changes in secretion in endometrial tissues may be useful for future research on biochemical tools for increasing reproductive efficiency and the production of healthy offspring.

The role of age in the reproductive competence of females has been intensively investigated with respect to the oocyte's developmental, meiotic and maturation potential (1, 4). There have also been studies indicating the role of growth factors during pregnancy in pigs (12). However, the role of the expression of growth factors in the porcine endometrium during estrus in different periods of porcine life requires further investigation. We hypothesized that the expression of the investigated growth factors (1) may change during the estrus stage and (2) may be different at various stages of the pig's life. In order to analyze the influence of estrus, the experiment involved a control group consisting of prepuberal gilts, cycling gilts and multiparous sows not in estrus.

Therefore, the aim of this study was to analyze the expression of genes encoding EGF, IGF, TGF β1, TGF β2 and TGF β3 in the endometrium isolated from prepuberal gilts, cycling gilts and multiparous sows during their estrus stage.

## Material and methods

**Groups of animals, superovulation and estrus induction.** This study was conducted using three groups of crossbred Polish Landrace × Polish Large White prepuberal gilts (n = 40), cycling gilts (n = 40) and multiparous sows (n = 40).

The prepuberal gilts (n = 20, age 140-160 days) were injected with 1500 I.U. equine chorionic gonadotropin (eCG, Folligonan, Intervet International, Netherlands) and after 72 hours with 500 I.U. human chorionic gonadotropin (hCG, Chorulon, Intervet). The cycling gilts (n = 20, age 160-180 days) were treated *per os* with Regumate (20 mg/kg, Hoechst Roussel Vet, Romainville Cedex, France) for 15 days. Twenty-four hours after the last treatment of Regumate, the animals were injected with 1500 I.U. eCG, followed by 500 I.U. hCG 80 hours after the eCG. Twenty-four hours after weaning, the multiparous sows (n = 20, age 2.5-3.5 years) were injected with 1200 I.U. eCG, followed by 500 I.U. hCG 58 hours later. All animals were checked once a day for 15 minutes using fence-line contact with a mature boar. Estrus detection continued for 17 days after the initiation of the experiment, and on day 17 after the onset of estrus all animals were slaughtered and their reproductive tracts were recovered (14).

The control groups consisted of prepuberal gilts (n = 20), cycling gilts (n = 20), and multiparous sows (n = 20) not in estrus and of similar age and weight. The animals were fed an appropriate standard diet. The experiment was approved by the local Ethics Committee.

**RNA extraction from endometrial tissue.** Following slaughter, the reproductive tracts were recovered and transported to the laboratory within 20 minutes at 38°C in 0.9% NaCl. Then 2 ml of TRIzol (Invitrogen, USA) were added to approximately 1 g of the endometrium, which was micro-surgically dissected away from the myometrium. The samples were homogenized using a Virtishear homogenizer (Virtis Company, Inc., Gardiner, NY, USA). Thereafter they were incubated at room temperature for 5 min and 1 ml chloroform was added to the sample, incubated at room temperature for 3 min and then centrifuged at 4°C for 30 min at 5000 *g*. The aqueous phase was transferred into a fresh tube, 2.5 ml isopropyl alcohol was added, and then the samples were placed in a –80°C freezer overnight. The samples were subsequently centrifuged at 4°C for 30 min at 22 500 *g*. The supernatant was discarded and the pellet was washed with 3 ml of 75% ethanol and then air-dried for 5 min. Total RNA was resuspended in 500 µl of diethyl pyrocarbonate (DEPC)-treated water and further purified by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation. Samples were treated with DNase I (Invitrogen) according to the manufacturer's protocol to eliminate possible DNA contamination. Total RNA was quantified with a spectrophotometer at an absorbance of 260 nm and purity was verified based on the ratio of 260 : 280. For reverse transcription PCR reaction (RT-PCR) 1.5 µg of total RNA was used. RNA was treated with DNase I (Promega Co. Madison, USA) and reverse-transcribed into cDNA using random hexamer priming and reverse transcriptase (RT), (Sigma Co. St. Louis, USA).

**Real-time quantitative PCR (RQ-PCR) analysis of EGF, IGF, TGF β1, TGF β2 and TGF β3 in the porcine endometrium.** RQ-PCR was conducted in a LightCycler real-time PCR detection system Roche Diagnostics GmbH, (Mannheim, Germany), using SYBR® Green I as detection dye, and target cDNA was quantified using relative quantification method. For amplification, 2 µl of total (20 µl) cDNA solution was added to 18 µl of QuantiTect® SYBR® Green PCR Master Mix Qiagen GmbH (Hilden, Germany) and primers. One RNA sample of each preparation was processed without RT-reaction to provide a negative control in subsequent PCR. The housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were amplified as references for mRNA quantification.

To quantify specific gene expression in the endometrium, expression levels of particular endometrial mRNAs in each sample were calculated relative to GAPDH and β-actin. To ensure the integrity of these results, an additional housekeeping gene, 18S rRNA, was used as an internal standard to ensure that GAPDH and β-actin mRNA were not regulated in the groups of animals. This gene has been identified as an appropriate housekeeping gene for use in quantitative PCR studies (21). The expression of GAPDH, β-actin and 18S rRNA mRNA was measured in cDNA samples from the endometrium. The expression of GAPDH and β-actin did not vary when normalized against 18S rRNA (results not shown).

**Statistical analysis.** Results were estimated using two-way analysis of variance (ANOVA) with Dunn's *post-hoc* test. Results are presented as mean ± SEM with the level of significance, * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$.

## Results and discussion

Using RQ-PCR, we evaluated the transcript levels encoding EGF, IGF-I, TGF β1, TGF β2 and TGF β3 in the porcine endometrium during the estrus cycle in females of different age groups. We demonstrated significantly increased mRNA levels encoding all investigated growth factors in all estrous animals as compared to controls (figure 1A, 1B, 1C, 1D, 1E). Comparing the transcript levels of growth factors in prepuberal gilts, cycling gilts and multiparous sows, we found higher levels of mRNA encoding all growth factors in multiparous sows as compared to prepuberal gilts ($p < 0.05$, $p < 0.001$). No difference was observed when comparing TGF β2 transcript levels in these two groups of animals ($p = 0.075$). We also demonstrated an increase in the mRNA contents encoding EGF, IGF-I and TGF β3 in cycling gilts as compared to prepuberal gilts ($p < 0.001$, $p < 0.001$, $p < 0.05$), respectively. We detected higher EGF mRNA abundance in multiparous sows as compared to cycling gilts ($p < 0.05$). Expression of the investigated growth factors in control animals (not in estrus) from each of the groups was similar. Therefore, we combined the results from control prepuberal and cycling gilts as well as multiparous sows into a single control group (figure 1A, 1B, 1C, 1D, 1E).

The endometrium undergoes several morphological and biochemical changes during the estrus cycle and pregnancy in mammals (11, 15). The ultrastructural modifications include proliferation, differentiation, tissue breakdown and formation of specific sites of embryo attachment during implantation and further fetus development (17). These modifications also include biochemical changes of gene expression for genes encoding specific polypeptides, which play a central role in building the endometrial environment.

The most recent findings suggest the possible function of growth factors in regulatory processes of endometrial growth and development during early pregnancy in mammals (5, 12). In this study we described, for the first time, the mRNA levels encoding EGF, IGF, TGF β1, TGF β2 and TGF β3 in the porcine endometrium during estrus in animals of different age groups. We demonstrated an increased level of EGF, IGF, TGF β1, TGF β2 and TGF β3 mRNAs in the porcine endometrium at the beginning of estrus as compared to controls. It has also been hypothesized that growth factors stimulate embryonic growth and development (3). Expression of these factors in the porcine endometrium may suggest the mechanism of growth factor secretion into the lumen of the reproductive tract, which may supplement the embryonic development of these factors (22). There have also been suggestions that the EGF receptor and its ligands may be associated with steroid hormone metabolic pathways in the endometrium and reproductive tract (10).

There is also important information on the hormonal regulation of growth factor gene expression during

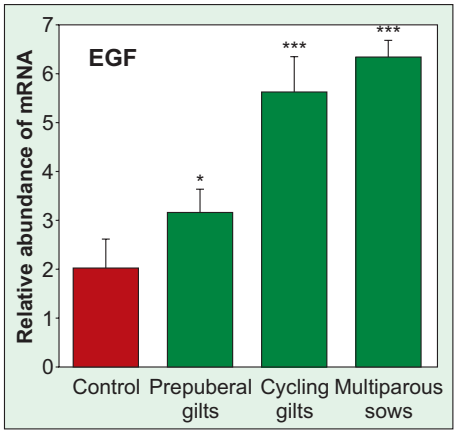


Fig. 1A

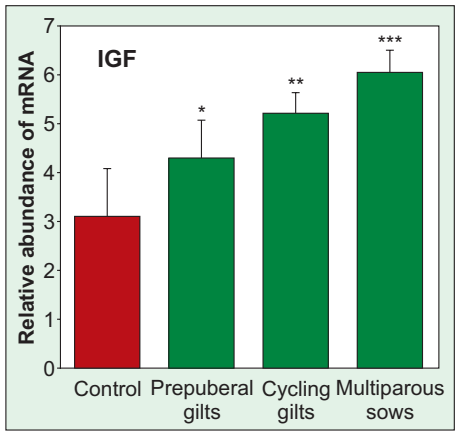


Fig. 1B

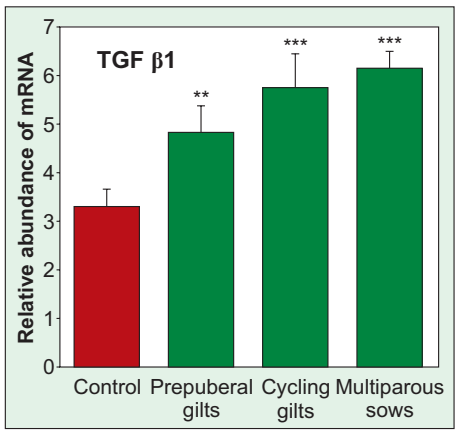


Fig. 1C

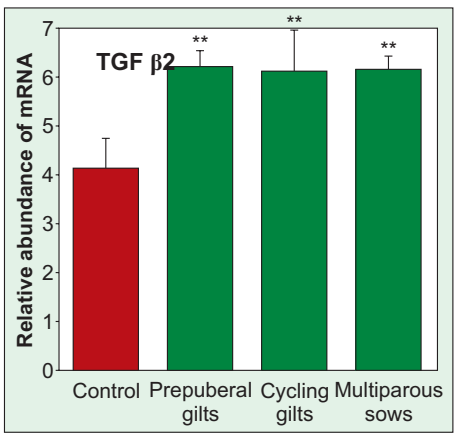


Fig. 1D

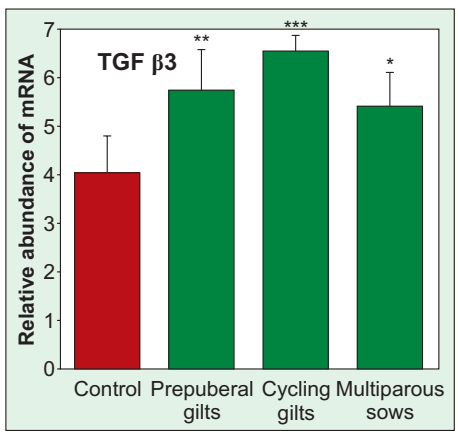


Fig. 1E

**Figures. Transcript levels of EGF, IGF-I, TGFβ1, TGFβ2 and TGFβ3 in endometrium isolated from prepuberal gilts, cycling gilts and multiparous sows.**
**RNA from the porcine endometrium was isolated immediately after recovery of the reproductive tract. The RNA was reverse-transcribed into cDNA. RQ-PCR was used to evaluate the presence and quantity of EGF (fig. 1A), IGF-I (fig. 1B), TGF β1 (fig. 1C), TGF β2 (fig. 1D) and TGF β3 (fig. 1E) transcripts. Each sample was determined in triplicate. Results are presented as mean ± SEM with the level of significance, * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$**

estrus and early pregnancy. It has been demonstrated that the possible regulation of EGF expression by estrogens during various estrus cycle stages is quantitative rather than qualitative (13). In this study we have presented the expression pattern of growth factors in the endometrium. Our results indicate that different expression of growth factors in estrus pigs may be related to hormone-specific regulation of these genes. This was demonstrated by a decrease in the expression of all investigated genes in control non-estrus animals of the same age. Furthermore, we observed differences in the expression of all investigated genes between each age--specific group of females during estrus. However, we did not observe such results in the control groups. This may confirm our hypothesis that the specific distribution of hormones during estrus plays an important role in regulating the expression of these genes.

Increased levels of mRNAs encoding growth factors may suggest a specific manner of endometrium preparation for embryo attachment following successful implantation in mammals. This has been partially confirmed by Kaczmarek et al., who demonstrated a higher level of vascular endothelial growth factor (VEGF) during the periovulatory and peri-implantation period. They also showed an increased concentration of VEGFR-1 on days 16-17 of the estrus cycle (12).

Our examinations demonstrated an increased level of IGF mRNA in the female endometrium during estrus as compared to controls. Simmen et al. (21) showed higher levels of IGF in pregnant gilts as compared to cycling females. They suggested that different expression of IGF during the estrus cycle and gestation may be important in this period for maternal recognition of pregnancy and may be related to a critical role of IGF in embryo attachment and fetal development.

In this study we also compared the mRNA levels of transcripts encoding growth factors in different age groups of females. We showed higher levels of mRNA encoding growth factors in the endometrium isolated from multiparous sows as compared to prepuberal gilts. The differences between multiparous sows and cycling gilts are less significant and depend on which of the genes are compared. We suggest that the transcript levels encoding growth factors are related to the age of donors, especially multiparous sows compared to younger animals, which may be due to biochemical and ultrastructural changes in the endometrium of females after several pregnancies (4, 23).

## Conclusions

The present study demonstrates specific transcript levels encoding growth factors in the porcine estrous endometrium. Moreover, it demonstrates the expression of growth factors in the endometrium of females in different age groups. This could be a new insight into the role of growth factors in possible ultra-changes in the endometrium during estrus. The results obtained in different age groups of animals reveal differences in the reproductive potential of the females, which may be reflected in different embryo attachment abilities. However, the results require further investigation involving the evaluation of protein levels and hormone concentrations in serum during estrus cycle in pigs.

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