Assessment of VEGF-receptor system expression in the porcine endometrial stromal cells in response to insulin-like growth factor-I, relaxin, oxytocin and prostaglandin E

Monika M. Kaczmarek, Agnieszka Blitek, Katarzyna Kaminska, Gabriel Bodek, Marek Zygmunt, Dieter Schams, Adam J. Ziecik

► To cite this version:

Monika M. Kaczmarek, Agnieszka Blitek, Katarzyna Kaminska, Gabriel Bodek, Marek Zygmunt, et al.. Assessment of VEGF-receptor system expression in the porcine endometrial stromal cells in response to insulin-like growth factor-I, relaxin, oxytocin and prostaglandin E. *Molecular and Cellular Endocrinology*, 2008, 291 (1-2), pp.33. 10.1016/j.mce.2008.04.020 . hal-00532024

HAL Id: hal-00532024

<https://hal.science/hal-00532024>

Submitted on 4 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Assessment of VEGF-receptor system expression in the porcine endometrial stromal cells in response to insulin-like growth factor-I, relaxin, oxytocin and prostaglandin E₂

Authors: Monika M. Kaczmarek, Agnieszka Blitek, Katarzyna Kaminska, Gabriel Bodek, Marek Zygmunt, Dieter Schams, Adam J. Ziecik

PII: S0303-7207(08)00157-3
DOI: doi:10.1016/j.mce.2008.04.020
Reference: MCE 6870

To appear in: *Molecular and Cellular Endocrinology*

Received date: 28-9-2007
Revised date: 20-4-2008
Accepted date: 28-4-2008

Please cite this article as: Kaczmarek, M.M., Blitek, A., Kaminska, K., Bodek, G., Zygmunt, M., Schams, D., Ziecik, A.J., Assessment of VEGF-receptor system expression in the porcine endometrial stromal cells in response to insulin-like growth factor-I, relaxin, oxytocin and prostaglandin E₂, *Molecular and Cellular Endocrinology* (2007), doi:10.1016/j.mce.2008.04.020

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Assessment of VEGF-receptor system expression in the porcine endometrial stromal cells in response to insulin-like growth factor-I, relaxin, oxytocin and prostaglandin E₂

Monika M. Kaczmarek^{a,*}, Agnieszka Blitek^a, Katarzyna Kaminska^a, Gabriel Bodek^a, Marek Zygmunt^b, Dieter Schams^c, Adam J. Ziecik^a.

^aDivision of Reproductive Endocrinology and Pathophysiology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland

^bDepartment of Obstetrics and Gynecology, University of Greifswald, Greifswald, Germany

^cPhysiology Weihenstephan, Technical University Munich, Freising, Germany

*Corresponding author at: Institute of Animal Reproduction and Food Research Polish Academy of Sciences (IARFR PAS), Tuwima 10, 10-747 Olsztyn, Poland. Tel.: +48 89 5357422; fax: +48 89 5357421.
E-mail: moka@pan.olsztyn.pl (M.M. Kaczmarek)

Keywords: angiogenesis; VEGF-receptor system; endometrium; early pregnancy

Abstract

Several factors participate in regulation of growth and development as well as angiogenesis of the uterus during pregnancy, and hence little is known about the role of hormonal regulation of vascular endothelial growth factor (VEGF)-receptor system expression. This study has examined the effect of insulin-like growth factor-I (IGF-I), relaxin (RLX), oxytocin (OT) and prostaglandin (PG) E₂, on VEGF secretion and VEGF-receptor system mRNA expression in the porcine endometrial stromal cells. IGF-I and RLX were identified as the most effective inducers of VEGF secretion and mRNA expression. Although PGE₂ stimulated VEGF secretion and VEGF164 mRNA expression, OT inhibited both secretion and mRNA expression of VEGF. When tested for VEGF receptors (R), all factors failed to affect their mRNA expression. Media conditioned by stromal cells collected after IGF-I and RLX treatment significantly increased endothelial cell proliferation and this effect was blocked by soluble VEGFR-1. These data suggest that during early pregnancy IGF-I, RLX and PGE₂ can affect VEGF expression in the endometrium and therefore may support uterine and embryo development, implantation and pregnancy.

1. Introduction

Periimplantation is a critical period during early pregnancy, when synchronization between developing embryo and maternal uterus must occur to ensure proper initiation of embryo attachment and implantation. More specifically, the embryo must reach the blastocyst stage and gain 'implantation competence'; meanwhile, the uterus must reach the receptive phase (Dey et al., 2004). In pigs, as in other species, the embryo-maternal crosstalk is essential to gain such synchronization. This dialogue is regulated by several biological molecules, including growth factors, cytokines, prostaglandins and hormones (for a review see Ziecik et al., 2006).

Early pregnancy is associated with morphological and functional changes in the uterus, accompanied by vascular remodeling. One of the first reactions on the embryo-derived estrogenic signal is increased uterine blood supply. In pigs, increased uterine arterial blood flow was observed on days 11-13 of pregnancy (Ford and Christenson, 1979; Ford et al., 1982) and is thought to support prostaglandin production in the endometrium and corpus luteum (CL) functions. Moreover, early stages of embryo implantation are characterized by increased endometrial vascular permeability in many species, including primates (Enders et al., 1983), rodents (Psychoyos, 1973), sheep (Boshier, 1970) and pigs (Keys et al., 1986; Keys and King, 1988). In general, increased permeability is thought to play an essential role in the induction and direction of tissue growth and remodeling wherever it occurs (Dvorak et al., 1995). Thus it may be a basic requirement for the rapid growth and differentiation of endometrium to reach the receptive phase. In addition, increased permeability facilitates the angiogenesis associated with the maternal component of placenta formation (Dvorak et al., 1995).

The most potent inducer of angiogenesis as well as microvascular permeability yet identified is vascular endothelial growth factor (VEGF). The various forms of VEGF are

generated by alternative splicing of single gene. Four different isoforms of VEGF were initially reported in humans VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, VEGF₂₀₆ (Ferrara et al., 1991; Tischer et al., 1991), however, to date several less frequent and tissue specific splice variants have also been reported, for example VEGF₁₄₅ and VEGF₁₈₃ (Anthony et al., 1994; Lei et al., 1998). VEGF activity is mainly mediated by two high affinity tyrosine kinase receptors (R); VEGFR-1 (fms-like tyrosine kinase, Flt-1) and VEGFR-2 (fetal liver kinase-1/kinase insert domain-containing receptor, Flk-1/KDR; Ferrara et al., 2003).

It is believed that VEGF play important roles during implantation. Experiments in rodents have indicated that VEGF participates in vascular events before implantation, regulating periimplantation vascular permeability (Rabbani and Rogers, 2001). Rockwell et al. (2002) showed that VEGF is the major mediator of estrogen-induced uterine microvascular permeability and is absolutely essential for implantation in mice. In pigs, vessel permeability appeared in conjunction with blastocyst elongation at day 12 (Keys et al., 1986). Recently, increased VEGF₁₆₄ protein expression was found in porcine endometrium on days 9-15 of pregnancy (Kaczmarek et al., 2008), suggesting its possible participation in implantation-associated vascular events.

The endometrial growth and remodeling require the coordinated expression of many genes, and for many of these the estrogen receptor (ER) is an essential component of transcriptional complex. In pigs, during maternal recognition of pregnancy estrogenic signal of blastocyst occurs between days 10-12 of gestation (Geisert et al., 1982) and regulates expression of numerous genes by binding to and activating ER. Among the genes identified as targets for regulation by the estradiol/ER complex in the uterus is that encoding *VEGF* (Hyder et al., 1997) and insulin-like growth factor-I (*IGF-I*; Klotz et al., 2002), both increased in the uterus during early pregnancy in the pig (Letcher et al., 1989; Kaczmarek et al., 2008). Furthermore, relaxin (RLX), like estrogens, promotes the growth of the uterus and other

estrogen target tissues, since they share the ability to activate ER (Pillai et al., 1999). Although, serum RLX levels during early pregnancy in pigs are maintained low (Anderson et al., 1983), its mRNA and protein are present in the endometrium between days 10-20 of gestation (Knox et al., 1994).

It has been suggested that, besides estrogens, uterine synthesis of prostaglandins (PG) is a critical element of successful implantation in many domestic animals, including pigs (for a review see Ziecik et al., 2006). A dramatic increase in the $\text{PGE}_2/\text{PGF}_{2\alpha}$ ratio is observed in the uterine lumen and utero-ovarian vein of pregnant animals on days 11-12 (Davis and Blair, 1993; Christenson et al., 1994), suggesting that PGE_2 can overcome the luteolytic effect of $\text{PGF}_{2\alpha}$ and maintain the function of CL. Furthermore, it was suggested that maintenance of pregnancy in pigs is also mediated by oxytocin (OT), a potent inducer of uterine $\text{PGF}_{2\alpha}$ secretion (Carnahan et al., 1996; Edgerton et al., 1996), since a low concentration of OT receptors (OTR) in the endometrium of early pregnant pigs was found (Okano et al., 1996). However, several other results suggest that the OT-receptor system may not be mandatory in the establishment of pregnancy in pigs (for a review see Ziecik et al., 2006).

Knowledge about the regulators of endometrial VEGF-receptor system expression is rather limited. Therefore, to better identify the role of VEGF and its receptors in the endometrium during the periimplantation period, we investigated whether VEGF-receptor system expression in porcine endometrial stromal cells could be affected by IGF-I, RLX, OT and PGE_2 .

2. Materials and methods

2.1 Materials

Chemicals were obtained from Sigma (St Louis, MO, USA), unless otherwise indicated. The recombinant bovine VEGF164 was a kind gift from Dr. D. Gospodarowicz

(Chiron Corp., Barkeley, CA, USA). Purified porcine RLX was a generous gift from Dr. O.D. Sherwood (University of Illinois at Urbana-Champaign, IL, USA).

2.2 Primary cell culture and treatment

The endometrial tissue was collected on days 10-12 of pregnancy from 17 crossbred gilts of known breeding date. Pregnancy was confirmed by the presence of at least four conceptuses in the uterine horns. The concepti were flushed from uterine horns with 20 ml of phosphate-buffered saline (PBS; pH 7.4) at 37°C. Porcine endometrial stromal cells were isolated from the middle portion of the uterine horn using a procedure described earlier (Blitek and Ziecik, 2004). The cell viability was higher than 90% as assessed by 0.5% trypan blue dye exclusion. Cells were cultured in Medium 199 containing 2% BSA (ICN, Biomedicals, Inc., Costa Mesa, CA, USA), 10% NCS and 20 µg/ml gentamycin at 37°C in a humidified atmosphere of 95% air/5% CO₂. After 24 h of seeding (2.5×10^5 for secretion studies or 1.5×10^6 for gene expression studies) on culture plates (24- or 6-well plates), stromal cells were washed gently with PBS to remove contaminating epithelial cells. Afterwards, cells were additionally cultured for 24 h to allow complete cell adhesion before initiation of the experiment, until approximately 80-90% confluency. The purity of stromal cell culture was 95-98% as assessed by immunofluorescent staining for the presence or absence of vimentin and cytokeratin (Blitek and Ziecik, 2004).

Forty eight hours after plating, the medium was changed for fresh Medium 199 containing 0 (no treatment, control); 25-100 ng/ml RLX; 10-100 ng/ml IGF-I; 1-100 nM OT or 0.001-1 µM PGE₂ (Cayman Chemical, MI, USA). For dose and time-dependent studies, liquid medium was collected after 6, 12, 24 or 48 h of treatment and stored at -40°C. Cells were then washed with PBS, lysed with 100 mmol/l NaOH and total cellular protein content was measured (Bradford, 1976). For gene expression studies, treatments were performed for

24 h, cells were washed with PBS and total RNA was extracted. All experiments were performed in duplicates.

2.3 Immunofluorescence for VEGF

For immunofluorescent staining, isolated porcine endometrial stromal cells were grown on sterile plastic coverslips (Nunc, Rochester, NY, USA) inserted into 24-well plates. After 24 h of culture stromal cells were washed gently with PBS to remove contaminating cells and further cultured for 24 h to 90% confluency. Then, cells were washed gently with PBS, fixed in 4% paraformaldehyde in PBS for 10 min at room temperature and rinsed three times for 10 min with PBS. After 1.5 h at room temperature in blocking solution (1% BSA, 10% NDS in PBS), cells were incubated overnight at 4°C with antiserum for VEGF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1:100. In control staining, primary antibodies were omitted. Next day, cells were washed three times for 10 min with PBS and secondary antibodies (CY3- anti-rabbit; Jackson ImmunoResearch, West Grove, PA, USA) at a dilution of 1:300 were added for 2 h at room temperature. At the end of incubation coverslips were rinsed with PBS, mounted on slides with a mixture of glycerol and PBS (1:1), viewed with Olympus microscope (Olympus BX 40; Olympus Optical Co Ltd, Tokyo, Japan) equipped with a filter set for CY3 and photographed with a Olympus Digital Camera.

2.4 Radioimmunoassay (RIA) for VEGF

Concentrations of VEGF in media were measured by RIA according to the method described earlier (Berisha et al., 2000). Briefly, the recombinant bovine VEGF₁₆₄ was used for preparation of rabbit antiserum and iodination. The incubation buffer for RIA was 3 M NaCl containing 1 % BSA, 0.1% Triton X-100 (pH 7.5). The antiserum was used at a final dilution of 1:400 000. Separation of bound and free VEGF was completed using the double

antibody technique and 6% polyethylene glycol (Serva, Heidelberg, Germany). The intra-assay variations were below 6% and the inter-assay variations below 14%. The ED 50 of the assay was 0.6 ng/ml. Dilution of samples containing VEGF from blood plasma, follicular fluid and tissue extracts ran parallel to the standard curve. The average recovery of exogenous VEGF was 93-95%.

2.5 Reverse transcription and Real-Time PCR

Total RNA was prepared using Total RNA Prep Plus kit (A&A Biotechnology, Gdansk, Poland) according to the manufacturer's instructions. Constant amounts of 2 µg of total RNA were treated with DNase I (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) as described in supplier's protocol. The RT reaction was carried out according to the protocol detailed previously (Kaczmarek et al., 2007). RT products were diluted fourfold in sterile water and stored at -20°C until Real time PCR amplification.

Real time PCR was performed with an ABI Prism 7300 sequence detection system using SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) as described elsewhere (Kaczmarek et al., 2007, Kowalczyk et al., 2008). Table 1 shows the oligonucleotide primers used for PCR amplification of VEGF, VEGF164, VEGF120, VEGFR-1, VEGFR-2 and β-actin genes and expected product sizes. Serial dilutions of the appropriate DNA were used as standard curves. The following general PCR conditions were used: denaturation for 10 min at 95°C, followed by 35 cycles of 15 sec at 95°C and 1 min at 59°C (for VEGFR-1) or 60°C (for VEGF, VEGF 164, VEGF 120, VEGFR-2, and β-actin). Melting curves were obtained after each PCR reaction. The reactions were also run either on blank-only buffer samples or in absence of the reverse transcriptase. The specificity of RT/PCR products was confirmed by gel electrophoresis and sequencing. Data obtained from the Real

time PCR for VEGF, VEGF164, VEGF120, VEGFR-1 and VEGFR-2 were normalized on the basis of β -actin mRNA content.

2.6 Endothelial cell proliferation assay

A colorimetric assay was used for quantification of cell proliferation, based on chemical reduction of growth indicator alamarBlue™ (Serotec Ltd., Oxford, UK). Human umbilical vein endothelial cells (HUVEC) at passage one to four were allowed to adhere and spread on 96-well dishes for 18 h (1×10^4 cells/well) in 10% NCS MCDB 131 supplemented with antibiotics (100 IU/ml penicillin, 100 μ g/ml streptomycin). Cells were first pretreated with or without soluble VEGFR-1 or VEGFR-2 (100 ng/ml; sVEGFR-1, sVEGFR-2; Abcam, Cambridge, UK) in MCDB 131 for 40 min. Afterwards, 35% of conditioned medium collected during VEGF secretion studies (24 or 48 h treatment with IGF or RLX, selected in preliminary experiments) in 2% NCS MCDB 131, alone or in combination with 100 ng/ml sVEGFR-1 or sVEGFR-2 was added to each well in duplicates and incubated for 3 days. VEGF (10 ng/ml; PeproTech EC Ltd., London, UK) was used as positive control. Conditioned media were then replaced by fresh MCDB 131 containing 10% of alamarBlue™ reagent and incubated for 7 h. Absorbance was measured at 570 and 595 nm and cell proliferation equal to reduction of alamarBlue™ reagent was calculated according to the supplier's manual. The plating density for HUVEC and length of incubation time with alamarBlue™ was determined in preliminary experiments, as suggested by the supplier.

2.7 Statistical analysis

The data are expressed as mean $\pm$ S.E.M. of values obtained in three to seven experiments (pigs), each performed in duplicate. Levels of VEGF were standardized on protein concentration per well (pg/ μ g protein). Statistical analyses were conducted using one-

way ANOVA for repeated measurements followed by Dunnett's or Bonferroni's multiple comparison post hoc tests (GraphPad PRISM v. 4.0, GraphPad Software, Inc., San Diego, CA). Differences were considered to be statistically significant at $P < 0.05$.

3. Results

3.1 Immunofluorescent localization of VEGF in stromal cell culture

Immunostaining for VEGF was detectable in stromal cells, with characteristic morphology, after 48 h of culture and was localized in the cytosol (Fig. 1A, B). Negative controls were consistently free from staining (Fig. 1C).

3.2 Effect of IGF-I and RLX on VEGF secretion

Both, IGF-I and RLX stimulated VEGF secretion from endometrial stromal cells in dose- and time-dependent manner (Fig. 2). Basal secretion of VEGF (pg/ μ g protein) increased from 2.5 ± 0.4 (12 h) to 5.3 ± 0.6 (48 h). IGF-I-stimulated secretion of VEGF was detected during all studied time-periods (12-48 h). Although, after 12 h only the highest dose of IGF-I was effective (100 ng/ml; $P < 0.05$), longer incubation resulted in a significant enhance of VEGF secretion from stromal cells at all tested doses ($P < 0.01$). The most effective action of IGF-I was found after 48 h of incubation, when VEGF concentration in medium increased 1.8-fold for doses 10 and 100 ng/ml (8.1 ± 0.5 and 8.2 ± 0.6 vs 4.6 ± 0.4 , respectively; $P < 0.01$). Effect of RLX on VEGF release was observed only after 24 and 48 h of incubation, when two or all used doses significantly stimulated secretion of VEGF, respectively ($P < 0.01$). Long-term exposure (48 h) of stromal cells to 100 ng/ml RLX led to maximal 1.8-fold increase of VEGF release (11.0 ± 1.7 vs 6.0 ± 1.1 ; $P < 0.01$).

3.3 Effect of OT and PGE₂ on VEGF secretion

Release of VEGF from cultured stromal cells was affected by both dose of OT or PGE₂ and time of incubation (Fig. 3). Basal secretion of VEGF (pg/μg protein) increased from 1.9 ± 0.4 (6 h) to 4.6 ± 0.2 (48 h). Although, OT seemed to inhibit VEGF secretion starting from 6 h of incubation, significant effect was detected only after 24 and 48 h, when 100 nM OT was used (3.4 ± 0.6 vs 4.0 ± 0.7 , $P < 0.05$ and 3.4 ± 0.7 vs 4.9 ± 0.2 , $P < 0.01$; respectively). An augmentation of VEGF secretion by PGE₂ in stromal cells was detected as early as 6 h, but exclusively for 1 μM PGE₂ (1.4-fold, $P < 0.05$). However, the most effective action of PGE₂ was observed after 48 h of incubation, when amounts of VEGF released into medium were induced approximately 1.3-fold for doses 0.1 and 1 μM (5.4 ± 0.5 and 5.8 ± 0.3 vs 4.4 ± 0.3 , $P < 0.05$, $P < 0.01$; respectively).

3.4 Effect of IGF-I and RLX on VEGF gene expression

The dose-dependent effect of IGF-I and RLX (for both 0-100 ng/ml) on VEGF, VEGF164 and VEGF120 mRNA expression in stromal cells after 24 h of treatment is shown on Figure 4. The expression of VEGF, VEGF164 and VEGF120 mRNA was affected only by 50 and 100 ng/ml IGF-I ($P < 0.05$). The maximal, almost 2-fold increase of VEGF, VEGF164 and VEGF120 mRNA expression was observed when the highest dose of IGF-I was used (100 ng/ml; $P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively). Although VEGF and VEGF164 expression was induced by 50 and 100 ng/ml RLX ($P < 0.05$ and $P < 0.01$, respectively), VEGF120 expression was significantly increased when the highest dose was used ($P < 0.05$). The maximal stimulation of VEGF, VEGF164 and VEGF120 mRNA expression was found in cells stimulated with 100 ng/ml RLX (1.6-, 1.8- and 1.5-fold; $P < 0.01$, $P < 0.01$, $P < 0.05$, respectively).

3.5 Effect of OT and PGE₂ on VEGF gene expression

Figure 5 shows the dose-dependent effect of OT (0-100 nM) and PGE₂ (0-1 µM) on VEGF, VEGF164 and VEGF120 mRNA expression in endometrial stromal cells after 24 h of incubation. In OT-treated cells tendency to decreased expression of VEGF, VEGF164 and VEGF120 mRNA was observed, however significant inhibition was only detected for dose 100 nM ($P < 0.05$). Although, stimulation of VEGF, VEGF164 mRNA expression by PGE₂ was dose-dependent, VEGF120 levels were maintained constant. The maximal induction (1.8-fold) was obtained with 1 µM PGE₂ for VEGF and 0.1 and 1 µM PGE₂ for VEGF164 ($P < 0.05$).

3.6 Effect of IGF-I, RLX, OT and PGE₂ on VEGFRs gene expression

Table 2 shows the effect of IGF-I (0-100 ng/ml), RLX (0-100 ng/ml), OT (0-100 nM) and PGE₂ (0-1 µM) on VEGFR-1 and VEGFR-2 expression in endometrial stromal cells after 24 h of incubation. Both receptors mRNA expression in cells incubated with various concentrations of IGF-I, RLX, OT or PGE₂ was not affected by the treatment.

3.7 Endothelial cell proliferation assay

The ability of media conditioned by porcine endometrial stromal cells after IGF-I or RLX treatment to affect HUVEC proliferation is shown on Figure 6. Both IGF-I and RLX conditioned media increased HUVEC proliferation ($P < 0.001$ and $P < 0.01$; respectively). The effect was comparable to the level of proliferation observed for the positive control (45.6 ± 8.3 and 40.4 ± 6.3 vs 45.7 ± 7.2 , respectively). sVEGFR-1 effectively neutralized the effect of IGF-I and RLX conditioned media on endothelial cell proliferation (45.6 ± 8.3 vs 38.0 ± 5.7 ; $P < 0.05$ and 40.4 ± 6.3 vs 31.4 ± 5.2 ; $P < 0.01$, respectively), whereas sVEGFR-2 had no significant effect on HUVEC proliferation stimulated by IGF-I and RLX conditioned media.

Both soluble receptors had no affect on cell proliferation in controls, however sVEGFR-2 slightly increased HUVEC proliferation (31.2 ± 4.7 vs 36.2 ± 6.4 ; $P > 0.05$).

4. Discussion

Several studies, using different animal models, have shown that periimplantation period is critical for embryo implantation, pregnancy success as well as the future growth and developmental potential of embryos. Since VEGF regulates periimplantation vascular permeability in rodents (Rabbani and Rogers, 2001) and female mice treated with VEGF antiserum on day 4 after mating (the normal day of implantation) fail to deliver any pups (Rockwell et al., 2002) it is tempting to believe that VEGF is one of the factors of uterine periimplantation environment being crucial for pregnancy success. Recent results have indicated upregulation of VEGF164 protein in endometrium in the periimplantation period in pigs that agrees with patterns of estradiol secretion by the conceptus (Kaczmarek et al, 2008). Thus, we have investigated whether factors creating a unique microenvironment for successful embryo implantation in pigs may affect VEGF-receptor system expression in the porcine endometrium.

Our study demonstrated, for the first time, the effect of IGF-I, RLX, OT and PGE₂ on VEGF secretion and VEGF-receptor system mRNA expression in porcine endometrial stromal cells from tissues collected during early gestation (days 10-12). Stromal cell cultures were selected for *in vitro* experiments, since this cell population is present in the uterus in a much higher number than luminal epithelial cells (Blackwell et al., 2003) and thus they are expected to contribute to the synthesis of the major portion of biological molecules produced by the endometrium. For example, Northern blot analysis revealed that porcine stromal cells are the main source of IGF-I (Rodriguez-Sallaberry et al., 2001) and *in vitro* experiments showed that stroma population collected on days 11-13 of pregnancy may produce more

1 PGE₂ than PGF_{2α} (Zhang and Davis, 1991). The close proximity of stromal cells to the uterine
 2 vascular bed also predestinates them to be the main source of factors regulating
 3 vascularization and vessel permeability, such as VEGF. Recently, we have demonstrated that
 4 uterine stroma may be an important source of VEGF during early pregnancy in pigs
 5 (Kaczmarek et al, 2008). Furthermore, results of the present immunofluorescent staining
 6 showed that after 48 h-long culture stromal cells were not deprived of VEGF expression.

7 During the periimplantation period, the porcine uterine endometrium and conceptuses
 8 exhibit dramatic biochemical, structural and morphological changes (Geisert et al, 1982).
 9 Prior to complete attachment to the endometrial epithelium, blastocysts are dependent upon
 10 hormones and nutrients secreted by the uterine endometrium. The presence of the IGF system
 11 in porcine endometrium as well as blastocyst suggests important paracrine/autocrine modes of
 12 IGF-I action in the uterine environment. In addition to the positive action on uterine growth in
 13 early pregnancy, uterine IGF-I is thought to be a critical component for the implantation
 14 process (for a review see Simmen et al., 1995). The endometrial IGF-I concentration between
 15 days 8-14 of pregnancy is maintained in constant levels (21.1 ± 1.9 ng/g tissue), but its
 16 content in the uterine lumen increases and peaks at day 12 of pregnancy (approximately 2.5
 17 µg of total recoverable IGF-I; Letcher et al., 1989). IGF-I has been proposed to increase
 18 estrogen synthesis through stimulation of porcine conceptus aromatase gene expression
 19 (Green et al., 1995). Although IGF-I was shown to influence VEGF expression in several cell
 20 types under physiological conditions, endometrial expression of this factor was studied only
 21 in human adenocarcinoma cells (Bermont et al., 2000). Therefore, the present results are the
 22 first available data showing IGF-I as the potent stimulator of VEGF mRNA expression as
 23 well as VEGF secretion in the normal endometrium, during early pregnancy in the
 24 mammalian female. It seems that IGF-I, together with estrogens of embryonic origin, may be

the main factor involved in regulation of VEGF, but not VEGFR, synthesis in endometrial stroma during periimplantation period in pigs.

It is well established that the CL is the main source of circulating RLX throughout pregnancy in several species, including pigs (for a review see Sherwood, 2004). Although serum RLX levels rise gradually from about 0.15 ng/ml on day 6 to about 10 ng/ml on day 110 of pregnancy (Anderson et al., 1983; Eldridge-White et al., 1989), it seems unlikely that this hormone exerts important effects during early gestation in pigs. However, it is tempting to believe that small amounts of RLX produced locally by the endometrium may support implantation through autocrine/paracrine mechanisms. Interestingly, RLX immunoactivity and mRNA was localized between days 10-20 of pregnancy in pigs (Knox et al., 1994). On the other hand, in humans, having a placentation type different to the porcine, circulating RLX is highest during the first trimester (Stewart et al., 1990). Nevertheless, clinical observation makes it seem unlikely that circulating RLX is an important factor during implantation in humans. Further experiments demonstrating local synthesis of RLX in human endometrial cells and its positive effect on VEGF expression (Unemori et al., 1999; Palejwala et al., 2002) led to the hypothesis that this hormone supports uterine vascular events needed for implantation and pregnancy maintenance in humans. Similarly, we showed that porcine stromal cells secrete increased amounts of VEGF in response to RLX treatment. The present results suggest that, also in pigs, endometrial RLX may be involved in regulation of vasculature function within the endometrium during implantation through the control of VEGF expression. However, definitive studies are needed to verify that hypothesis.

The uterotrophic actions of exogenous RLX are pronounced in ovariectomized gilts receiving estrogen as well as progesterone (Hall et al., 1992). The important synergism is thought to exist between conceptus-derived estrogen and uterine-derived RLX that stimulate uterine growth during the early stages of conceptus attachment and placentation (Knox et al.,

1994). Since RLX was more effective in stimulation of VEGF164 than VEGF120 mRNA expression, it seems likely that previously established specific pattern of VEGF164 expression during early pregnancy in the pig (Kaczmarek et al., 2008) is a result of the synergistic action of estrogen of embryonic origin and endometrial RLX.

In addition to direct action through specific receptor and initiation of a signal transduction cascade, RLX may exert its biological effects indirectly by influencing production of growth factors and/or their availability. The involvement of the uterine IGF system in the local action of RLX was demonstrated by coincidence of increased uterine weight and protein synthesis with a significant enhancement of uterine luminal content of IGFs and IGF-binding proteins (IGFBP), but not IGF-I-receptor expression in the uterus (Ohleth et al., 1997). Thus, induction of mRNA expression and secretion of VEGF in cultured porcine endometrial stromal cells by RLX may be additionally mediated via stimulation of IGF-I expression. Collectively, the evidence for the local production of RLX by the pig endometrium around the time of implantation (Knox et al., 1994) together with the RLX-induced increase in IGF-I secretion by the endometrium (Ohleth et al., 1997) and presently observed stimulation of VEGF expression in stromal cells by both, IGF-I and RLX support an important role of these factors in preparation of a suitable environment for pregnancy success in the pig.

Moreover, PGs are thought to be pivotal for establishment of pregnancy in the pig, since inhibition of its synthesis results in pregnancy failure (Kraeling et al., 1985). On days 11-12 of gestation, embryo-derived estrogens provide a signal for maternal recognition of pregnancy and increase $PGE_2/PGF_{2\alpha}$ ratio due to augmented production of luteoprotective PGE_2 (Davis and Blair, 1993; Christenson et al., 1994). In addition, during implantation prostaglandins are thought to participate in important vascular events: modulation of blood flow and increase of vascular permeability (Ford et al., 1982; Keys et al., 1986; Keys and

King, 1988). PGE₂ has been shown to upregulate VEGF in a number of tissues; however there is only one report demonstrating transcriptional regulation of *VEGF* gene expression in the uterus (Lopes et al., 2006). Interestingly, we found PGE₂ to significantly increase VEGF164 mRNA levels in porcine endometrial stromal cells, whereas VEGF120 and VEGFR mRNA was not affected by the treatment. Halder et al. (2000) showed that VEGF164 is one of the primary mediators of VEGF signaling in the uterine vascular changes during implantation in mice. Thus, it appears that PGE₂ may be involved in induction of vascular permeability during implantation time in the pig, by improving VEGF164 secretion from endometrial stroma.

The presence of a low concentration of OTR in the endometrium during early pregnancy (Okano et al., 1996) at first suggested that OT, known inducer of uterine PGF_{2α} secretion, is involved in the process of maternal recognition of pregnancy in pigs (Carnahan et al., 1996; Edgerton et al., 1996). Further demonstration that endometrial sensitivity to OT is not regulated primarily through changes in OTR population density (Ludwig et al., 1998) and blocking of OTR did not prevent luteolysis in pigs (Kotwica et al., 1999) deflated mandatory role OT in establishment of pregnancy in pigs. This study provides the first demonstration of OT-mediated inhibition of VEGF secretion as well as mRNA expression in endometrial stromal cells from early pregnancy. In our experiments, only the highest dose of OT was effective in inhibition of VEGF secretion and mRNA expression. This concentration of OT (100 nM) is substantially higher than was reported in the peripheral circulation during luteolysis in pigs (Kotwica et al., 1990). However, as it was reported for RLX, porcine endometrium could be a source of OT during pregnancy (Boulton et al., 1996). Although it was suggested that OT is a rather redundant component of the uterine environment during maternal recognition of pregnancy, our present results suggest that high doses of OT, apart

1 from stimulation of luteolytic PGF_{2α} secretion from uterus, are able to affect the process of
 2 uterine vascularization and vascular permeability during early pregnancy in pigs.

3 Although all tested factors affected secretion and mRNA expression of VEGF,
 4 VEGFR-1 and VEGFR-2 mRNA levels were maintained at a very low and constant level,
 5 without significant stimulatory or inhibitory effects. The result is not surprising since the
 6 dominant localization VEGFRs was found not in stromal, but in uterine capillary endothelial
 7 cells in pigs (Charnock-Jones et al., 2001; Kaczmarek et al., 2008). The examined factors may
 8 have also some effects on receptors in intact tissue if they are produced in stromal cells.
 9 VEGF may act therefore directly on endothelial cells and also as a chemokine in attracting
 10 endothelial cell migration during the outgrowth of new vessels in the endometrium.

11 In the present study, proliferation of HUVEC *in vitro* was induced directly by media
 12 conditioned by stromal cells collected after IGF-I and RLX treatment. These effects were
 13 blocked by sVEGFR-1, suggesting that IGF-I- and RLX-stimulated VEGF is able to induce
 14 endothelial cells proliferation. More significant inhibition of HUVEC proliferation by media
 15 conditioned by stromal cells collected after RLX treatment, may have an explanation in our
 16 gene expression studies, where levels of highly mitogenic VEGF₁₆₄ mRNA were induced by
 17 RLX in a grater manner comparing to IGF-I. In contrast, sVEGFR-2 (no naturally occurring
 18 splice variant) was not able to significantly inhibit HUVEC proliferation; however such a
 19 tendency was observed. Like other growth factor transmembrane tyrosine kinase receptors,
 20 VEGF receptors presumably undergo ligand-induced dimerization to activate transduction
 21 pathways. Thus, dimers between sVEGFR and full-length VEGF receptor block signal
 22 pathways dependent on intracellular tyrosine kinase dimerization. Both soluble receptors used
 23 in the experiment has the same ED₅₀ for VEGF-dependent proliferation of HUVEC provided
 24 by the supplier (10-30 ng/ml), therefore the reason of such observation in a present study may
 25 rather not lay in a dose of the sVEGFR used in a culture. However, previous results showed

1 that VEGFR-1 and its soluble form bind VEGF with higher affinity (approximately K_d 10-20
2 pM; de Vries et al., 1992; Kendall and Thomas, 1993) than VEGFR-1 (approximately K_d 75-
3 125 pM; Terman et al., 1992). On the other hand, sVEGFR-2 seemed to increase the cell
4 proliferation *in vitro* itself, thus it is possible that its action on VEGF-stimulated proliferation
5 of HUVEC was masked by this effect in our experiment.

6 In summary, the present study is the first to demonstrate that VEGF-receptor system
7 expression in porcine endometrial stromal cells is controlled by factors creating a unique
8 microenvironment for successful embryo implantation in pigs. IGF-I and RLX are the most
9 potent inducers of VEGF secretion and mRNA expression, acting together with estrogens of
10 embryonic origin. Luteoprotective PGE_2 was effective in stimulation of VEGF secretion as
11 well as VEGF164 mRNA expression suggesting its participation in vascular events during
12 implantation. In contrast, high doses of OT seem to inhibit VEGF-mediated actions in
13 endometrial stroma during early pregnancy in pigs. These results suggest that during the
14 periimplantation period essential crosstalk exists between examined factors that ensures
15 uterine and embryo development, implantation and pregnancy success by affecting uterine
16 vasculature function.

1 **Acknowledgements**

2 This research was supported by the State Committee for Scientific Research in Poland grant
3 No PBZ-KBN-084/P06/2002. We thank Mrs. K. Gromadzka-Hliwa, Mr. J. Klos for their
4 excellent technical assistance in the laboratory and Mr. M. Blitek for help in care and
5 handling of animals. We greatly acknowledge the expert technical assistance of Mrs. Gabi
6 Schwentker in radioimmunoassay. The research collaboration in this study was supported by
7 Roman Herzog Research Fellowship and Return Fellowship from Hertie and Alexander von
8 Humboldt Foundations (to M.M.K.). M.M. Kaczmarek was awarded the Domestic Grant for
9 Young Scientist from the Foundation for Polish Science. This work was partly presented on
10 the International Conference on Pig Reproduction, the Netherlands, 12-15 June 2005 and the
11 Annual Meeting of the ESDAR, Germany, 20-22 September 2007.

References

- Anderson, L.L., Adair, V., Stromer, M.H., McDonald, W.G., 1983. Relaxin production and release after hysterectomy in the pig. *Endocrinology* 113, 677-686.
- Anthony, F.W., Wheeler, T., Elcock, C.L., Pickett, M., Thomas, E.J., 1994. Short report: identification of a specific pattern of vascular endothelial growth factor mRNA expression in human placenta and cultured placental fibroblasts. *Placenta* 15, 55-561.
- Berisha, B., Schams, D., Kosmann, M., Amselgruber, W., Einspanier, R., 2000. Expression and tissue concentration of vascular endothelial growth factor, its receptors, and localization in the bovine corpus luteum during estrous cycle and pregnancy. *Biol. Reprod.* 63, 1106-1114.
- Bermont, L., Lamielle, F., Fauconnet, S., Esumi, H., Weisz, A., Adessi, G.L., 2000. Regulation of vascular endothelial growth factor expression by insulin-like growth factor-I in endometrial adenocarcinoma cells. *Int. J. Cancer* 85, 117-123.
- Blackwell, D.M., Speth, R.C., Mirando, M.A., 2003. Morphometric analysis of the uterine endometrium of swine on days 12 and 16 postestrus. *Anat. Rec.* 270, 59-66.
- Blitek, A., Ziecik, A.J., 2004. Prostaglandins F and E secretion by porcine epithelial and stromal endometrial cells on different days of the oestrous cycle. *Reprod. Domest. Anim.* 39, 340-346.
- Boshier, D.P., 1970. The pontamine blue reaction in pregnant sheep uteri. *J. Reprod. Fertil.* 22, 595-596.
- Boulton, M.I., McGrath, T.J., Goode, J.A., Broad, K.D., Gilbert, C.L., 1996. Changes in content of mRNA encoding oxytocin in the pig uterus during the oestrous cycle, pregnancy, at parturition and in lactational anoestrus. *J. Reprod. Fertil.* 108, 219-227.

- 1 Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram
2 quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72,
3 248-254.
- 4 Carnahan, K.G., Prince, B.C., Mirando, M.A., 1996. Exogenous oxytocin stimulates uterine
5 secretion of prostaglandin F2 alpha in cyclic and early pregnant swine. *Biol. Reprod.* 55,
6 838-843.
- 7 Charnock-Jones, D.S., Clark, D.E., Licence, D., Day, K., Wooding, F.B., Smith, S.K., 2001
8 Distribution of vascular endothelial growth factor (VEGF) and its binding sites at the
9 maternal-fetal interface during gestation in pigs. *Reproduction* 122, 753-760.
- 10 Christenson, R.K., Farley, D.B., Anderson, L.H., Ford, S.P., 1994. Luteal maintenance during
11 early pregnancy in the pig: role for prostaglandin E2. *Prostaglandins* 47, 61-75.
- 12 Davis, D.L., Blair, R.M., 1993. Studies of uterine secretions and products of primary cultures
13 of endometrial cells in pigs. *J. Reprod. Fertil. Suppl.* 48, 143-155.
- 14 de Vries, C., Escobedo, J.A., Ueno, H., Houck, K., Ferrara, N., Williams, L.T., 1992. The
15 fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 255,
16 989-991.
- 17 Dey, S.K., Lim, H., Das, S.K., Reese, J., Paria, B.C., Daikoku, T., Wang, H., 2004. Molecular
18 cues of implantation. *Endocrine Rev.* 25, 341-373.
- 19 Dvorak, H.F., Brown, L.F., Detmar, M., Dvorak, A.M., 1995. Vascular permeability
20 factor/vascular endothelial growth factor, microvascular hyperpermeability, and
21 angiogenesis. *Am. J. Pathol.* 146, 1029-1039.
- 22 Edgerton, L.A., Kaminski, M.A., Silvia, W.J., 1996. Changes in uterine secretion of
23 prostaglandin F2 alpha in response to oxytocin during the estrous cycle, early pregnancy,
24 and estrogen-induced pseudopregnancy in swine. *Biol. Reprod.* 55, 657-662.

- 1 Eldridge-White, R., Easter, R.A., Heaton, D.M., O'Day, M.B., Petersen, G.C., Shanks, R.D.,
2 Tarbell, M.K., Sherwood, O.D., 1989. Hormonal control of the cervix in pregnant gilts. I.
3 Changes in the physical properties of the cervix correlate temporally with elevated serum
4 levels of estrogen and relaxin. *Endocrinology* 125, 2996-3003.
- 5 Enders, A.C., Hendrickx, A.G., Schlafke, S., 1983. Implantation in the rhesus monkey: initial
6 penetration of endometrium. *Am. J. Anat.* 167, 275-298.
- 7 Ferrara N, Gerber HP, LeCouter J. 2003. The biology of VEGF and its receptors. *Nat. Med.* 9,
8 669-676.
- 9 Ferrara, N., Houck, K.A., Jakeman, L.B., Winer, J., Leung, D.W., 1991. The vascular
10 endothelial growth factor family of polypeptides. *J. Cell. Biochem.* 47, 211-218.
- 11 Ford, S.P., Christenson, R.K., 1979. Blood flow to uteri of sows during the estrous cycle and
12 early pregnancy: local effect of the conceptus on the uterine blood supply. *Biol. Reprod.*
13 21, 617-624.
- 14 Ford, S.P., Reynolds, L.P., Magness, R.R., 1982. Blood flow to the uterine and ovarian
15 vascular beds of gilts during the estrous cycle and early pregnancy. *Biol. Reprod.* 27, 878-
16 885.
- 17 Geisert, R.D., Renegar, R.H., Thatcher, W.W., Roberts, R.M., Bazer, F.W., 1982.
18 Establishment of pregnancy in the pig: I. Interrelationships between preimplantation
19 development of the pig blastocyst and uterine endometrial secretions. *Biol. Reprod.* 27,
20 925-939.
- 21 Green, M.L., Simmen, R.C., Simmen, F.A., 1995. Developmental regulation of steroidogenic
22 enzyme gene expression in the periimplantation porcine conceptus: a paracrine role for
23 insulin-like growth factor-I. *Endocrinology* 136, 3961-3970.
- 24 Halder, J.B., Zhao, X., Soker, S., Paria, B.C., Klagsbrun, M., Das, S.K., Dey, S.K., 2000.
25 Differential expression of VEGF isoforms and VEGF(164)-specific receptor neuropilin-1

- 1 in the mouse uterus suggests a role for VEGF(164) in vascular permeability and
- 2 angiogenesis during implantation. *Genesis* 26, 213-224.
- 3 Hall, J.A., Cantley, T.C., Galvin, J.M., Day, B.N., Anthony, R.V., 1992. Influence of ovarian
- 4 steroids on relaxin-induced uterine growth in ovariectomized gilts. *Endocrinology* 130,
- 5 3159-3166.
- 6 Hyder, S.M., Chiappetta, C., Murthy, L., Stancel, G.M., 1997. Selective inhibition of
- 7 estrogen-regulated gene expression in vivo by the pure antiestrogen ICI 182,780. *Cancer*
- 8 *Res.* 57, 2547-2549.
- 9 Kaczmarek, M.M., Kowalczyk, A.E., Waclawik, A., Schams, D., Ziecik, A.J., 2007.
- 10 Expression of vascular endothelial growth factor and its receptors in the porcine corpus
- 11 luteum during the estrous cycle and early pregnancy. *Mol. Reprod. Dev.* 74, 730-739.
- 12 Kaczmarek, M.M., Waclawik, A., Blitek, A., Kowalczyk, A.E., Schams, D., Ziecik, A.J.,
- 13 2008. Expression of the vascular endothelial growth factor-receptor system in the porcine
- 14 endometrium throughout the estrous cycle and early pregnancy. *Mol. Reprod. Dev.* 75,
- 15 362-372.
- 16 Kendall, R.L., Thomas, K.A., 1993. Inhibition of vascular endothelial cell growth factor
- 17 activity by an endogenously encoded soluble receptor. *Proc. Natl. Acad. Sci. U.S.A.* 90,
- 18 10705-10709.
- 19 Keys, J.L., King, G.J., 1988. Morphological evidence for increased uterine vascular
- 20 permeability at the time of embryonic attachment in the pig. *Biol. Reprod.* 39, 473-487.
- 21 Keys, J.L., King, G.J., Kennedy, T.G., 1986. Increased uterine vascular permeability at the
- 22 time of embryonic attachment in the pig. *Biol. Reprod.* 34, 405-411.
- 23 Klotz, D.M., Hewitt, S.C., Ciana, P., Raviscioni, M., Lindzey, J.K., Foley, J., Maggi, A.,
- 24 DiAugustine, R.P., Korach, K.S., 2002. Requirement of estrogen receptor-alpha in

- 1 insulin-like growth factor-1 (IGF-1)-induced uterine responses and in vivo evidence for
- 2 IGF-1/estrogen receptor cross-talk. *J. Biol. Chem.* 277, 8531-8537.
- 3 Knox, R.V., Zhang, Z., Day, B.N., Anthony, R.V., 1994. Identification of relaxin gene
- 4 expression and protein localization in the uterine endometrium during early pregnancy in
- 5 the pig. *Endocrinology* 135, 2517-2525.
- 6 Kotwica, G., Dusza, L., Ciereszko, R., Okrasa, S., Schams, D., 1990. Oxitocin plasma levels
- 7 during spontaneous and cloprostenol-induced luteolysis in sows. *Anim. Repro. Sci.* 22,
- 8 109-119.
- 9 Kotwica, G., Franczak, A., Okrasa, S., Kotwica, J., 1999. Effect of an oxytocin antagonist on
- 10 prostaglandin F2 alpha secretion and the course of luteolysis in sows. *Acta Vet. Hung.* 47,
- 11 249-262.
- 12 Kowalczyk, A.E., Kaczmarek, M.M., Schams, D., Ziecik, A.J., 2008. Effect of prostaglandyn
- 13 E2 and tumor necrosis factor α on the VEGF-receptor system expression in cultured
- 14 porcine luteal cells. *Mol. Reprod. Dev.* (DOI: 10.1002/mrd.20897).
- 15 Kraeling, R.R., Rampacek, G.B., Fiorello, N.A., 1985. Inhibition of pregnancy with
- 16 indomethacin in mature gilts and prepuberal gilts induced to ovulate. *Biol. Reprod.* 32,
- 17 105-110.
- 18 Lei, J., Jiang, A., Pei, D., 1998. Identification and characterization of a new splicing variant of
- 19 vascular endothelial growth factor: VEGF183. *Biochim. Biophys. Acta* 1443:400-406.
- 20 Letcher, R., Simmen, R.C., Bazer, F.W., Simmen, F.A., 1989. Insulin-like growth factor-I
- 21 expression during early conceptus development in the pig. *Biol. Reprod.* 41, 1143-1151.
- 22 Lopes, F.L., Desmarais, J., Ledoux, S., Gevry, N.Y., Lefevre, P., Murphy, B.D., 2006.
- 23 Transcriptional regulation of uterine vascular endothelial growth factor during early
- 24 gestation in a carnivore model, *Mustela vison*. *J. Biol. Chem.* 281, 24602-24611.

- 1 Ludwig, T.E., Sun, B.C., Carnahan, .KG., Uzumcu, M., Yelich, J.V., Geisert, R.D., Mirando,
2 M.A., 1998. Endometrial responsiveness to oxytocin during diestrus and early pregnancy
3 in pigs is not controlled solely by changes in oxytocin receptor population density. *Biol.*
4 *Reprod.* 58, 769-777.
- 5 Ohleth, K.M., Lenhart, J.A., Ryan, P.L., Radecki, S.V., Bagnell, C.A., 1997. Relaxin
6 increases insulin-like growth factors (IGFs) and IGF-binding proteins of the pig uterus in
7 vivo. *Endocrinology* 138, 3652-3658.
- 8 Okano, A., Okuda, K., Takahashi, M., Schams, D., 1996. Oxitocin receptors in the porcine
9 endometrium during the estrous cycle and early pregnancy. *Anim. Reprod. Sci.* 41, 61-70.
- 10 Palejwala, S., Tseng, L., Wojtczuk, A., Weiss, G., Goldsmith, L.T., 2002. Relaxin gene and
11 protein expression and its regulation of procollagenase and vascular endothelial growth
12 factor in human endometrial cells. *Biol. Reprod.* 66, 1743-1748.
- 13 Pillai, S.B., Rockwell, L.C., Sherwood, O.D., Koos, R.D., 1999. Relaxin stimulates uterine
14 edema via activation of estrogen receptors: blockade of its effects using ICI 182,780, a
15 specific estrogen receptor antagonist. *Endocrinology* 140, 2426-2429.
- 16 Psychoyos, A., 1973. Endocrine control of egg implantation. In: Greep, R.O., Astwood, E.G.,
17 Geiger, S.R. (ed.), *Handbook of physiology*. American Physiology Society, Washington,
18 pp. 187-215.
- 19 Rabbani, M.L., Rogers, P.A., 2001. Role of vascular endothelial growth factor in endometrial
20 vascular events before implantation in rats. *Reproduction* 122, 85-90.
- 21 Rockwell, L.C., Pillai, S., Olson, C.E., Koos, R.D., 2002. Inhibition of vascular endothelial
22 growth factor/vascular permeability factor action blocks estrogen-induced uterine edema
23 and implantation in rodents. *Biol. Reprod.* 67, 1804-1810.
- 24 Rodriguez-Sallaberry, C., Simmen, F.A., Simmen, R.C., 2001. Polyamine- and insulin-like
25 growth factor-I-mediated proliferation of porcine uterine endometrial cells: a potential

- 1 role for spermidine/spermine N(1)-acetyltransferase during peri-implantation. *Biol.*
- 2 *Reprod.* 65, 587-594.
- 3 Sherwood, O.D., 2004 Relaxin's physiological roles and other diverse actions. *Endocr. Rev.*
- 4 25, 205-234.
- 5 Simmen, R.C.M., Greek, M.L., Simmen, F.A., 1995. IGF system in periimplantation uterus
- 6 and embryonic development. In: Dey, S.K. (ed.), *Molecular and Cellular aspects of*
- 7 *Periimplantation Process*. Springer-Verlag, New York, pp. 185-204.
- 8 Stewart, D.R., Celniker, A.C., Taylor Jr, C.A., Cragun, J.R., Overstreet, J.W., Lasley, B.L.,
- 9 1990. Relaxin in the peri-implantation period. *J. Clin. Endocrinol. Metab.* 70, 1771-1773.
- 10 Terman, B.I., Dougher-Vermazen, M., Carrion, M.E., Dimitrov, D., Armellino, D.C.,
- 11 Gospodarowicz, D., Böhlen, P., 1992. Identification of the KDR tyrosine kinase as a
- 12 receptor for vascular endothelial cell growth factor. *Biochem. Biophys. Res. Commun.*
- 13 187, 1579-1586.
- 14 Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J.C., Abraham,
- 15 J.A., 1991. The human gene for vascular endothelial growth factor. Multiple protein forms
- 16 are encoded through alternative exon splicing. *J. Biol. Chem.* 266, 11947-11954.
- 17 Unemori, E.N., Erikson, M.E., Rocco, S.E., Sutherland, K.M., Parsell, D.A., Mak, J., Grove,
- 18 B.H., 1999. Relaxin stimulates expression of vascular endothelial growth factor in normal
- 19 human endometrial cells in vitro and is associated with menometrorrhagia in women.
- 20 *Hum. Reprod.* 14, 800-806.
- 21 Zhang, Z., Davis, D.L., 1991. Prostaglandin E and E2 alpha secretion by glandular and
- 22 stromal cells of the pig endometrium in vitro: effects of estradiol-17 beta, progesterone,
- 23 and day of pregnancy. *Prostaglandins* 42, 151-162.

- 1 Ziecik, A.J., Blitek, A., Kaczmarek, M.M., Wacławik, A., Bogacki, M., 2006. Inhibition of
2 luteolysis and embryo-uterine interactions during the peri-implantation period in pigs.
3 Soc. Reprod. Fertil. Suppl. 62, 147-161.
4

Figure legends

Fig. 1. Porcine endometrial stromal cells in culture. (A) Light microscopy of stromal cells after 48 h after plating. (B) Immunofluorescent staining of porcine stromal cells for VEGF protein expression. (C) Negative control. Magnification, x 400.

Fig. 2. IGF-I- and RLX-induced changes in VEGF secretion (pg/μg protein) by cultured porcine endometrial stromal cells obtained on days 10-12 of pregnancy. Cells were incubated with IGF-I or RLX (for both 0-100 ng/ml) for 12 h (n=3), 24 h (n=7) and 48 h (n=4). Data are presented as means ± S.E.M. * $P < 0.05$, ** $P < 0.01$ compared to controls.

Fig. 3. OT- and PGE₂-induced changes in VEGF secretion (pg/μg protein) by cultured porcine endometrial stromal cells obtained on days 10-12 of pregnancy. Cells were incubated with OT (0-100 nM) or PGE₂ (0-1 μM) for 6 h (n=4), 12 h (n=3), 24 h (n=7) and 48 h (n=7). Data are presented as means ± S.E.M. * $P < 0.05$, ** $P < 0.01$ compared to controls.

Fig. 4. Dose-dependent effect of IGF-I and RLX on VEGF, VEGF₁₆₄ and VEGF₁₂₀ expression in cultured porcine endometrial stromal cells obtained on days 10-12 of pregnancy. Cells were incubated with IGF-I or RLX (for both 0-100 ng/ml) for 24 h (n=4). Data are presented as means ± S.E.M. * $P < 0.05$, ** $P < 0.01$ compared to controls.

Fig. 5. Dose-dependent effect of OT and PGE₂ on VEGF, VEGF₁₆₄ and VEGF₁₂₀ expression in cultured porcine endometrial stromal cells obtained on days 10-12 of pregnancy. Cells were incubated with OT (0-100 nM) or PGE₂ (0-1 μM) for 24 h (n=3). Data are presented as means ± S.E.M. * $P < 0.05$, ** $P < 0.01$ compared to controls.

1 Fig. 6. Induction of endothelial cell proliferation by 35% of conditioned medium collected
2 after IGF-I (A; n=5) or RLX (B; n=4) treatment of porcine endometrial stromal cells. HUVEC
3 were cultured in control or conditioned medium in the absence or presence of sVEGFR-1 and
4 sVEGFR-2 (100 ng/ml). VEGF (10 ng/ml) was used as a positive control. Data are presented
5 as means $\pm$ S.E.M.* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
6

Kaczmarek et al.
(Fig. 1)

Kaczmarek et al.
(Fig. 2)

Kaczmarek et al.
(Fig. 3)

Kaczmarek et al.
(Fig. 4)

Kaczmarek et al.
(Fig. 5)

Kaczmarek et al.
(Fig. 6)

Table 1

Gene specific primers used for Real Time PCR

Genes ^a	Primers (5'-3')	Product size
<u>VEGF</u>	Forward: CCTGATGCGGTGCGGGGGCT Reverse: TGGTGGTGGCGGCGGCTATG	VEGF ₁₈₈ :510 VEGF ₁₆₄ :438 VEGF ₁₂₀ :306
<u>VEGF164</u>	Forward: GAGGCAAGAAAATCCCTGTG Reverse: TCACATCTGCAAGTACGTTTCG	150
<u>VEGF120</u>	Forward: AAGGCCAGCACATAGGAGAG Reverse: CCTCGGCTTGTCACATTTT	101
<u>VEGFR-1</u>	Forward: CACCCCGGAAATCTATCAGATC Reverse: GAGTACGTGAAGCCGCTGTTG	180
<u>VEGFR-2</u>	Forward: GATGCTCGCCTCCCTTTGA Reverse: AGTTCCTTCTTTCAGTCGCCTACA	180
<u>β-actin</u>	Forward: ACATCAAGGAGAAGCTCTGCTACG Reverse: GAGGGGCGATGATCTTGATCTTCA	366

^aSee text for definitions.

Table 2

Relative expression of VEGFR-1 and VEGFR-2 in porcine endometrial stromal cells after treatment

Treatment		Relative expression ^a	
		VEGFR-1 vs. β -actin	VEGFR-2 vs. β -actin
IGF I (ng/ml) (n=4)	0	0.017 $\pm$ 0.004	0.002 $\pm$ 0.0007
	10	0.017 $\pm$ 0.005	0.003 $\pm$ 0.0010
	50	0.014 $\pm$ 0.003	0.003 $\pm$ 0.0010
	100	0.015 $\pm$ 0.003	0.003 $\pm$ 0.0007
RLX (ng/ml) (n=4)	0	0.018 $\pm$ 0.005	0.002 $\pm$ 0.0010
	25	0.020 $\pm$ 0.005	0.003 $\pm$ 0.0013
	50	0.016 $\pm$ 0.004	0.002 $\pm$ 0.0009
	100	0.019 $\pm$ 0.004	0.003 $\pm$ 0.0009
OT (nM) (n=3)	0	0.020 $\pm$ 0.005	0.007 $\pm$ 0.0029
	1	0.021 $\pm$ 0.007	0.006 $\pm$ 0.0015
	10	0.022 $\pm$ 0.008	0.006 $\pm$ 0.0016
	100	0.024 $\pm$ 0.007	0.006 $\pm$ 0.0026
PGE2 (μ M) (n=3)	0	0.018 $\pm$ 0.004	0.006 $\pm$ 0.0017
	0.001	0.020 $\pm$ 0.006	0.007 $\pm$ 0.0015
	0.1	0.021 $\pm$ 0.006	0.006 $\pm$ 0.0021
	1	0.020 $\pm$ 0.007	0.005 $\pm$ 0.0013

^aData are presented as means $\pm$ S.E.M.