Prostaglandin F2 α promotes angiogenesis and embryo–maternal interactions during implantation

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Abstract

Implantation in humans and other mammals is a critical period during which high embryonic mortality rates occur. Prostaglandins (PGs) are key mediators regulating interactions between the reproductive tract and the conceptus (embryo with extraembryonic membranes). Although the significance of PGF2 α as a regulator of corpus luteum regression is well established, the role of its high amounts in the uterine lumen in most mammals, regardless of placentation type, during the implantation period remains unresolved. We hypothesized that PGF2 α acting as an embryonic signal mediator contributes to pregnancy establishment. Using a porcine model, we demonstrated that the conceptus and its signal (estradiol-17 β) elevated endometrial expression of PGF2 α receptor (PTGFR) *in vivo* and *in vitro*. PTGFR protein was expressed mainly in luminal epithelial (LE) and glandular epithelial cells and blood vessels in the endometrium. PGF2 α stimulated the MAPK1/3 pathway in endometrial LE cells that coincided with elevated gene expression and secretion of endometrial vascular endothelial growth factor A (VEGFA) protein. PGF2 α –PTGFR and adenylyl cyclase signaling were involved in this process. PGF2 α -induced VEGFA acting through its receptors stimulated proliferation of endometrial endothelial cells. Moreover, PGF2 α elevated gene expression of biglycan, matrix metalloproteinase 9, transforming growth factor β 3, and interleukin 1 α in the endometrium. In summary, our study indicates that PGF2 α participates in pregnancy establishment by promoting angiogenesis and expression of genes involved in tissue remodeling and conceptus–maternal interactions in porcine endometrium during early pregnancy.

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Introduction

Implantation in humans and other mammals is a critical period during which embryo mortality rates are high (reviewed in [Wilmut et al. \(1986\)](#)). Prostaglandins (PGs) are key factors in the development and maintenance of pregnancy because inhibition of their synthesis leads to termination of gestation before implantation in many species including pigs ([Waclawik 2011](#)). PGF2 α is the main luteolytic factor in the female reproductive tract of most mammals ([McCracken et al. 1999](#)). Acting on its receptor (PTGFR) localized in luteal cells, it invokes a process of regression in the corpus luteum (CL). PTGFR is coupled to G $_q$ -protein, and its activation results in elevation of the intracellular Ca²⁺ concentration mediated by PKC phosphorylation ([Bos et al. 2004](#)). PGF2 α acting via PTGFR may also stimulate the MAPK1/3 pathway, resulting in elevated expression of angiogenic factors in endometrial pathologies ([Sales et al. 2005](#)).

Owing to the luteolytic effect of PGF2 α on the CL, exogenous administration of this PG decreases

pregnancy rates or even terminates early pregnancy in many species such as humans, cattle, pigs, and dogs ([Podany et al. 1982](#), [Pajor & Zsolnai 1984](#), [Romagnoli et al. 1993](#), [Seals et al. 1998](#), [Lemaster et al. 1999](#)). However, elevated amounts of PGF2 α in the uterine lumen and/or its increased endometrial synthesis are observed during the implantation period in various mammals with different types of placentation: humans ([Wang et al. 2010](#), [Vilella et al. 2013](#)), pigs ([Zavy et al. 1980](#), [Waclawik et al. 2006](#)), cattle ([Ulbrich et al. 2009](#)), dogs ([Kowalewski et al. 2014](#)), and rats ([Kennedy et al. 2007](#)). Interestingly, our studies revealed that during the implantation and early placentation period, expression of PGF2 α synthase increases in both the porcine endometrium and conceptus (embryo with extraembryonic membranes; [Waclawik et al. 2006, 2007](#)). The phenomenon of increased PGF2 α amounts in the uterine lumen can be partially explained by the concept of maternal recognition of pregnancy, indicating that the porcine conceptus signal (estradiol-17 β (E₂)) changes PGF2 α secretion from an endocrine into an

exocrine manner (Bazer *et al.* 1977, Zavy *et al.* 1980). Intriguingly, our recent study demonstrated elevated expression of PTGFR protein in the endometrium during the implantation period (Kaczynski & Waclawik 2013). Apparently, contradictory reports on beneficial (Dorniak *et al.* 2012) vs detrimental (Podany *et al.* 1982, Pajor & Zsolnai 1984, Romagnoli *et al.* 1993, Seals *et al.* 1998, Lemaster *et al.* 1999) roles of PGF2 α on pregnancy establishment prompted us to further investigate its actions on the uterus during the implantation period in a porcine model. Key questions are as follows: Is PGF2 α need for the endometrium during the implantation period? What role does it exert when it is secreted in an autocrine and/or paracrine manner by the conceptus and uterus?

As in pathological conditions PGF2 α signaling mediates the expression of angiogenic factors in human endometrial cells (Sales *et al.* 2005), we propose that an increased level of PGF2 α in the uterine lumen during implantation could be involved in angiogenesis. Herein, we hypothesize that the conceptus by secreting its signals modulates endometrial expression of PTGFRs and that PGF2 α acting in an autocrine/paracrine manner through its receptors in the endometrium promotes establishment of the proper environment for development and maintenance of pregnancy. The main aim of this study was to determine the role of PGF2 α in early pregnancy using the porcine model. The objectives of this research were: (i) to determine the effect of the conceptus and its signal (E₂) on endometrial PTGFR gene and protein expression using *in vitro* and *in vivo* models; (ii) to characterize localization of the PTGFR within the uterus; (iii) to identify intercellular signaling pathways activated by PGF2 α in endometrial cells and to determine the effects of PGF2 α on synthesis and secretion of vascular endothelial growth factor A (VEGFA) by the porcine endometrium *in vitro*; (iv) to investigate the influence of PGF2 α on endometrial endothelial cell proliferation; and (v) to determine the effect of PGF2 α on the expression of factors involved in extracellular matrix (ECM) and tissue remodeling and embryo–maternal communication in the endometrium *in vitro*.

Materials and methods

All procedures involving the use of animals were conducted in accordance with the national guidelines for agricultural animal care and were approved by the Animal Ethics Committee, University of Warmia and Mazury in Olsztyn, Poland, permission no. 17/2008, 32/2008, and 36/2012.

Experiment 1: effect of conceptus on PTGFR gene and protein expression in endometrium *in vivo*

Local and systemic effects of the conceptus on PTGFR gene and protein expression were studied using a porcine *in vivo* model of unilateral pregnancy. Although unilateral pregnancy cannot be maintained in pigs, it can be used at the early stage of pregnancy

to study the direct and systemic effects of conceptuses on the uterus (Christenson *et al.* 1994, Wasielak *et al.* 2009). In our experiment, prepubertal crossbred gilts ($n=24$) of similar age (6–6.5 months), weight (100–110 kg), and genetic background were subjected, under general anesthesia, to a surgical procedure in order to isolate one uterine horn from the cervix. After 6–9 days of recovery, gilts were treated hormonally with 750 IU equine chorionic gonadotropin (eCG) (Folligon; Intervet, Boxmeer, The Netherlands) and 500 IU human chorionic gonadotropin (hCG) (Chorulon; Intervet) given 72 h later to induce estrus. Between days 12 and 14 of the estrous cycle gilts were injected with 10 mg PGF2 α (Dinolytic; Pfizer). After 16 h later, 10 mg of PGF2 α were injected simultaneously with 750 IU eCG pregnant mares serum gonadotropin (Folligon; Intervet). After 72 h, 500 IU hCG (Chorulon; Intervet) were given intramuscularly. Animals assigned to pregnant groups were artificially inseminated 24 and 48 h after hCG injection. The day of second insemination was defined as the first day of pregnancy. After insemination, conceptuses were developing only in the intact horn. Animals were then killed on day 11 ($n=6$) or day 14 ($n=6$) of pregnancy because these are the periods that correspond with the maternal recognition of pregnancy and the initial stage of implantation in pigs respectively. The control group consisted of gilts that underwent the same procedures, except of insemination (gilts on day 11 ($n=6$) and day 14 ($n=6$) for the estrous cycle). After killing, uterine horns of all gilts were washed with 20 mL PBS. Pregnancy was confirmed by the presence of conceptuses, which were flushed only from the connected uterine horn of pregnant animals. The ovulation rates, in relation to the number of corpora lutea, did not differ between analyzed groups: gilts on day 11 of the estrous cycle, day 11 of pregnancy, day 14 of the estrous cycle, and day 14 of pregnancy (Supplementary Table 1, see section on supplementary data given at the end of this article). Fertilization rate was presented for pigs on day 11 of pregnancy (Supplementary Table 1). In day 14 of pregnancy group, conceptuses possessed filamentous forms that are very fragile and not possible to flush in one piece. For this group, the number of conceptuses was not counted and therefore fertilization rates were not possible to present.

Endometrial samples were dissected from myometrium and snap-frozen in liquid nitrogen and then stored at -80°C until analyzed for gene and protein expression. Local effects of the conceptus on PTGFR expression were studied by comparison of PTGFR mRNA and protein levels in each gilt in endometrial samples from the horn in which conceptuses were present (intact horn) to its expression in endometrial samples from the isolated horn. Systemic effects of the conceptus on the expression of PTGFR were studied by comparison of PTGFR mRNA and protein levels in endometrial samples of intact and isolated horns from the pregnant group to the respective endometrial expression in the control cyclic group on day 11 or 14 after estrus.

Experiment 2: effect of E2 on endometrial PTGFR expression *in vitro*

Incubation of luminal epithelial and stromal cells of endometrium with E2

Luminal epithelial (LE) and stromal (ST) cells were isolated as described previously (Kaczynski & Waclawik 2013) and

Table 1 List of antibodies used in western blot and immunolocalization experiments.

Ptide/protein target	Antigen sequence	Name of antibody	Manufacturer, catalog no., or name of source	Species raised in monoclonal or polyclonal	Dilution used
PTGFR	QRFRQKSKASFLLLASGL-VITDFFGHLINGAIAVFVYAS-DKEWIRFDQSNVLCIS	PGF2aR antibody (H-55)	Santa Cruz Biotechnology, sc-67029	Rabbit, polyclonal	1:50
MAPK1/3	NA	p44/42 MAPK (Erk1/2) antibody	Cell Signaling, #9102S	Rabbit, polyclonal	1:300
Phospho-MAPK1/3	NA	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody	Cell Signaling, #9101	Rabbit, polyclonal	1:300
HIF1A	NA	HIF1α antibody (H167)	Santa Cruz Biotechnology, sc-53546	Mouse, monoclonal	1:100
GAPDH	NA	Anti-GAPDH antibody	Abcam, ab9485	Rabbit, polyclonal	1:100
ACTB	NA	Anti-β-actin antibody	Abcam, ab8227	Rabbit, polyclonal	1:2000
PTGFR	SMNSSKQPVSPAAGL	FP receptor antibody	Cayman Chemical, 101802	Rabbit, polyclonal	1:1000
CDH5	NA	VE-cadherin antibody (C-19)	Santa Cruz Biotechnology, sc-6458	Goat, polyclonal	1:50
VWF	NA	Polyclonal rabbit anti-human von Willebrand factor	Dako, A-0082	Rabbit, polyclonal	1:100
ESR1	NA	ERα antibody (HC-20)	Santa Cruz Biotechnology, sc-543	Rabbit, polyclonal	1:150
PGR	NA	PR antibody (C-19)	Santa Cruz Biotechnology, sc-538	Rabbit, polyclonal	1:200

grown in Medium 199 with antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin) supplemented with 1% (w/v) BSA, 10% (v/v) NCS, 10 nM E_2 , and 100 nM progesterone. After reaching 70–90% confluency, cells were treated with E_2 (100 nM) or control (vehicle) and incubated for 24 h at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 . After incubation, cells were lysed with Fenzol buffer (A&A Biotechnology, Gdansk, Poland), harvested, and stored at –80°C until *PTGFR* gene expression analysis.

Incubation of endometrial explants in vitro with E_2

Endometrial explants collected from gilts ($n=7$) on day 12 of the estrous cycle were prepared as described previously (Waclawik *et al.* 2009). After 2 h of preincubation, explants were treated in duplicates as follows: E_2 (1, 10, and 100 nM) or control (vehicle – M199 with 0.1% v/v ethanol) for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air. Then, explants were snap-frozen in liquid nitrogen and stored at –80°C until protein and gene expression analyses.

Experiment 3: effect of E_2 and PGE2 on *PTGFR* mRNA and protein expression in endometrium in vivo

Effects of E_2 and/or PGE2 administered alone or simultaneously were studied using a porcine *in vivo* model. Crossbred gilts at the same age and genetic background, after second natural estrous cycle underwent estrus synchronization according to the procedure described in Experiment 1. On days 8–9 of the third estrous cycle, animals underwent surgery in which both uterine horns were cannulated. The intrauterine cannula was perforated along its length in an attempt to simulate hormone delivery by conceptuses as reported in (Ford *et al.* 1982) with some modifications. The cannula was introduced into the uterine lumen at a distance of 10–15 cm from the isthmus and

it did not cover whole uterine horn. Mixing between uterine horns was excluded. In the control group, each horn received intrauterine vehicle (5 mL of 1% v/v ethanol saline) infusions. In the experimental group, doses of hormones used were similar to those previously published (Ford *et al.* 1982, Akinlosotu *et al.* 1986). Randomly selected horns within each gilt received hormonal infusions: E_2 (833 ng or 33.3 µg per infusion), PGE2 (200 µg per infusion), or E_2 (833 ng or 33.3 µg per infusion) simultaneously with PGE2 (200 µg per infusion) whereas the contralateral horn received only vehicle infusions. Treatments were administered every 4 h for 24 h on days 11–12 after the onset of estrus. Endometrial samples were dissected from myometrium and snap-frozen in liquid nitrogen and then stored at –80°C until gene and protein expression analyses. Uteri with inflammatory changes and/or with fluid accumulation were not included in experiments. Local effects of E_2 , PGE2, or E_2 together with PGE2 on *PTGFR* expression in endometrium were studied by comparison of *PTGFR* mRNA and protein levels in the endometrial samples from the hormone-treated horn to its expression in the samples from the vehicle-treated horn of each gilt. Systemic effects of E_2 and PGE2 on the expression of *PTGFR* were studied by comparison of *PTGFR* mRNA and protein levels in endometrial samples from the experimental group to the levels in the control group (vehicle infusions into both horns).

Experiment 4: immunolocalization of *PTGFR* protein in uterus

Immunohistochemical localization of *PTGFR* protein in porcine endometrium on days 12 and 15 of pregnancy and on day 12 of the estrous cycle was performed as described previously with some modifications (Kowalewski *et al.* 2006). Incubations with primary antibody (Table 1) were performed overnight at 4°C. Antiserum-specific isotype controls (rabbit IgG) at the same dilution and protein concentration as the primary

antibody as well as samples with omitted primary antibodies were used as negative controls to confirm the immunostaining specificity. Subsequently, sections were incubated with biotinylated goat anti-rabbit IgG (1:200; Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA). After development procedure, slides were observed under a light microscope (Olympus BX-60, Tokyo, Japan) and photographed.

Experiment 5: effect of PGF2 α in endometrium *in vitro*

Incubation of LE cells of endometrium with PGF2 α : phosphorylation studies

LE cells isolated as described in Experiment 2 were pretreated for 1 h with 25 μ M PD098059 (a pharmacological inhibitor of MAPK-kinases; Sigma–Aldrich) or vehicle. Subsequently, cells were incubated with PGF2 α (10 nM–1 μ M; Sigma–Aldrich) or control (vehicle) for 10 min. Cells were lysed in RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% v/v Triton X-100, 1% w/v SDS, 0.5% w/v sodium deoxycholate, and 1 mM EDTA) containing protease and phosphatase inhibitor cocktail (Sigma–Aldrich) and stored at –80°C until western blot analysis.

Incubation of endometrial explants with PGF2 α and fluprostenol

To study the effect of PGF2 α and its analog – fluprostenol – on VEGFA secretion *in vitro*, endometrial explants prepared as described in Experiment 2 were incubated for 24 h at humidified atmosphere of 5% CO₂ in air with the following treatments: control (vehicle), M199 with PGF2 α (100 nM and 1 μ M), and fluprostenol (100 nM and 1 μ M; Sigma–Aldrich) in the presence/absence of 50 μ M AL8810, 50 μ M LY294002, 50 μ M PD98059, and 50 μ M SQ 22,536 (PTGFR inhibitor, phosphatidylinositol-3-kinase inhibitor, MAPK1/3 kinases inhibitor and adenylyl cyclase inhibitor respectively; Sigma–Aldrich). After incubation, collected culture media (CM) were frozen for further analyses (RIA and proliferation assay). Conditioned media from incubation of endometrial explants treated with vehicle were defined as C-CM, whereas conditioned media from incubation of endometrial explants treated with PGF2 α were defined as PGF-CM. Endometrial explants were rinsed twice with sterile PBS and snap-frozen in liquid nitrogen and then stored at –80°C until gene expression analyses. Expression of VEGFA was analyzed by real-time RT-PCR. Our preliminary studies (A Waclawik and P Kaczynski, unpublished observations) indicate that another PG (PGE2), which is secreted by the endometrium and the conceptus, regulates the expression of biglycan (BGN), interleukin 1 α (IL1A), IL6, matrix metalloproteinase 9 (MMP9), and transforming growth factor β 3 (TGFB3) genes. In this study, we determined by real-time RT-PCR whether endometrial expression of these genes can be regulated by PGF2 α .

Experiment 6: effect of PGF2 α on endometrial endothelial cell proliferation

Isolation of endothelial cells from porcine endometrium

Uteri were collected from gilts ($n=6$) on day 12 of the estrous cycle and killed at the local abattoir. Uterine horns were cut into 10 cm length and rinsed in ice-cold, sterile PBS containing antibiotics (100 IU/mL penicillin and 100 μ g/mL

streptomycin). Endometrial tissue was separated from the myometrium and digested in 0.48% Dispase (Sigma–Aldrich) in Hanks' balanced salt solution (Ca²⁺, Mg²⁺, and phenol red-free, pH 7.4; Sigma–Aldrich) at 37°C for 60 min with gentle stirring. Released cells were pelleted by centrifugation at 200 g for 10 min and washed twice with fresh MCDB-131 (Sigma–Aldrich) supplemented with 1% (w/v) BSA and 5% (v/v) NCS. After washing, cells were suspended in CM (MCDB-131, 1% w/v BSA, 10% v/v NCS, 50 μ g/mL (w/v) ECGS, and antibiotics) and plated on petri dish. Undigested tissue was minced with lancet and digested in 0.06% (w/v) collagenase (Sigma–Aldrich) in MCDB-131 supplemented with 1% BSA. The cell suspension was centrifuged at 200 g for 10 min and washed twice with fresh MCDB-131 supplemented with 1% (w/v) BSA and 5% (v/v) NCS. Washed cells were then suspended in CM and plated on petri dish. On the next day, plated cells were washed three times with PBS and CM was replaced. After reaching 80% confluency, cells were dislodged with 0.025% Trypsin in EDTA (Life Technologies). Endothelial cells were then reselected by Dynabeads CD31⁺ (Life Technologies) according to the manufacturers' protocol. Selected porcine endometrial endothelial (PEE) cells were suspended in MCDB-131 supplemented with 10% NCS (v/v) and 50 μ g/mL ECGS (Sigma–Aldrich).

Characterization of primary endometrial endothelial cells by immunolocalization of VE-cadherin and von Willebrand factor

As VE-cadherin (CDH5) and von Willebrand factor (VWF) are markers of endothelial cells, their expression was examined to confirm homogeneity of the isolated cells. Primary endothelial cells were grown to confluency on 13 mm sterile plastic coverslips (Thermo Fisher Scientific) in 24-well culture plate. Cells were then fixed in ice-cold methanol at –20°C for 10 min. Immunocytochemistry was performed as described previously (Chrusciel *et al.* 2011) with some modifications. Detailed information including the source of the antibodies and their concentration is presented in Table 1. In control staining, primary antibodies were replaced with normal goat IgG (for CDH5) and normal rabbit IgG (for VWF) diluted, respectively, to the antibody concentrations.

Proliferation assay

PEE cells and the immortalized swine umbilical vein endothelial cell line (G1410; Chrusciel *et al.* 2011) were assigned for proliferation assay. Cells (4×10^4 PEE or 2×10^4 G1410 cells/well) were seeded in 96-well culture plates. Following attachment, at the 45–50% confluency, cells were pretreated with 5 μ M AAL993 (inhibitor of VEGF receptors: FLT1, KDR, and FLT4) or vehicle. After pretreatment, cells were treated with conditioned media from Experiment 5 (C-CM, PGF-CM, PGF-CM+AL8810, or PGF-CM+SQ22,536) diluted three-fold with M199, in the presence/absence of 5 μ M AAL993. VEGFA (50 ng/mL) in M199 and 20% (v/v) of NCS in C-CM were used as positive control treatments. As we observed the nonspecific effects of PGF-CM and LY294002 or PGF-CM and PD98059 on endothelial cells, they were excluded from further experiments. In the separate experiment, we examined the direct effect of PGF2 α and fluprostenol on endothelial cell proliferation. Cells were treated with PGF2 α (100 nM and 1 μ M) and fluprostenol

in the presence/absence of AL8810 or control (vehicle). The effects of AAL993, AL8810, and SQ22,536 alone, present in the conditioned media, were also tested. After 48 h of treatment, proliferation was determined using the Cell Titer 96 Aqueous One Solution Proliferation Reagent (Promega) according to the manufacturer's instructions.

Experiments were repeated six times (PEE) or five times (G1410) in duplicate. Fold difference was determined by dividing the absorbance obtained by PGF-CM-treated cells by the absorbance obtained by C-CM treated cells.

Effect of AAL993 on HIF1A protein expression in G1410 cells

As AAL993 (inhibitor of VEGF receptors: FLT1, KDR, and FLT4) may suppress HIF1A *in vitro* and HIF1A can exert its effects also under normoxia, we decided to determine the expression of HIF1A protein in order to assess the basic capability of AAL993 to affect our results. G1410 cells were cultured on six-well plates (Thermo Fisher Scientific). After reaching 80–90% confluency, they were incubated with control, C-CM, or PGF-CM without/with AAL993 (5 μ M) for 48 h in a humidified atmosphere containing 5% CO₂ and 95% air. After incubation, cells were lysed in RIPA buffer. Experiments were repeated three times. Expression of HIF1A was analyzed by western blot.

Western blot

Western blot analysis for protein expression in samples from *in vivo* models and *in vitro* experiments was performed as described previously (Kaczynski & Wacławik 2013, Wacławik *et al.* 2013). Briefly, equal amounts of protein samples (40 μ g for PTGFR, 18 μ g for MAPK1/3, or 35 μ g for HIF1A) from endometrial tissue homogenates or cell lysates were separated on 10% (PTGFR and MAPK1/3) or 8% (HIF1A) SDS–PAGE. Proteins were electroblotted onto 0.45 μ m PVDF membrane. After blocking, blots were incubated overnight at 4°C with primary antibodies (Table 1) and afterward with 1:20,000 dilution of secondary anti-rabbit HRP antibodies (Bio-Rad Laboratories) or 1:20,000 dilution (HIF1A) of secondary polyclonal anti-mouse alkaline phosphatase-conjugated antibodies (Sigma–Aldrich) for 1.5 h at 25.6°C. Immune complexes were visualized using Clarity

Western ECL Substrate (Bio-Rad) or using alkaline phosphatase visualization procedure (HIF1A). Sample loading was standardized to expression of GAPDH (for PTGFR and MAPK1/3 quantification) or to expression of ACTB (for HIF1A quantification).

Real-time RT-PCR

RNA isolation and reverse transcription as well as analysis of gene expression in samples from *in vivo* and *in vitro* experiments were performed as described previously (Kaczynski & Wacławik 2013). *PTGFR* and *VEGFA* gene expressions were quantified using Power SYBR Green (Life Technologies) with specific primers (1 μ M; Table 2), whereas *BGN*, *IL1A*, *IL6*, *MMP9*, and *TGFB3* genes were analyzed using TaqMan Master Mix with TaqMan probes (Life Technologies) according to the manufacturers' protocol. The PCR program for *PTGFR* and *VEGFA* genes was performed as follows: initial denaturation (95°C, 10 min) followed by 36 cycles of denaturation (95°C, 15 s), annealing and elongation (60°C, 1 min). For *ACTB*, *PPIA*, and *GAPDH* amplification, the PCR program was as follows: initial denaturation (95°C, 10 min) followed by 36 cycles of denaturation (95°C, 15 s), annealing (55°C, 30 s), and elongation (72°C, 1 min). Gene expression was estimated using real-time PCR Miner (<http://ewindup.info/miner/>) Software (Zhao & Fernald 2005). Expression of genes analyzed in endometrial samples was normalized against the expression of *ACTB* (Kaczynski & Wacławik 2013).

RIA for VEGFA

Concentrations of VEGFA secreted by endometrial explants to CM were measured as described previously (Berisha *et al.* 2000). The intra-assay variation was 5.97%. The ED50 was 3.121 ng/ μ L. Dilution of samples containing endogenous VEGFA ran parallel to the standard curve. The average recovery of exogenous VEGFA was 95%.

Statistical analyses

Two-way ANOVA followed by Bonferroni post-tests was used to analyze data obtained from *in vivo* experiments.

Table 2 Primer sequences and assays used in real-time RT-PCR analyses.

Gene	Primer sequence/TaqMan Assay ID	GenBank accession no.	References
<i>PTGFR</i>	Sense: 5'-TCAGCAGCACAGACAAGG-3' Antisense: 5'-TTCACAGGCATCCAGATAATC-3'	NM_214059	Kaczynski & Wacławik (2013)
<i>VEGFA</i>	Sense: 5'-GAGGCAAGAAATCCCTGTG-3' Antisense: 5'-TCACATCTGCAAGTACGTTCCG-3'	NM_214084	Wacławik <i>et al.</i> (2013)
<i>ACTB</i>	Sense: 5'-ACATCAAGGAGAAGCTCTGCTACG-3' Antisense: 5'-GAGGGGCGATGATCTTGATCTTCA-3'	U07786	Wacławik <i>et al.</i> (2006)
<i>GAPDH</i>	Sense: 5'-CAGCAATGCCTCCTGTACCA-3' Antisense: 5'-GATGCCGAAGTTGTCATGGA-3'	AF017079	Wacławik <i>et al.</i> (2013)
<i>PPIA</i>	Sense: 5'-TAACCCACCGTCTTCTT-3' Antisense: 5'-TGCCATCCAACCACTCAG-3'	AY266299.1	Kaczynski & Wacławik (2013)
<i>BGN</i>	Ss03375454_u1	AF159382.1	–
<i>IL1A</i>	Ss03391335_m1	X52731.1	–
<i>IL6</i>	Ss03384604_u1	AF309651.1	–
<i>MMP9</i>	Ss03392100_m1	DQ132879.1	–
<i>TGFB3</i>	Ss03394352_m1	X14150.1	–
<i>ACTB</i>	Ss03376081_u1	AK237086.1	–
<i>PPIA</i>	Ss03394782_g1	AY266299.1	–
<i>GAPDH</i>	Ss03375435_u1	NM_001206359.1	–

Results obtained from all *in vitro* experiments were analyzed by one-way ANOVA followed by Tukey's test. Differences were considered statistically significant at 95% CI level ($P < 0.05$). All statistical analyses were conducted using GraphPad Prism v. 5.02 Software (GraphPad Software, Inc.).

Results

The conceptus up-regulates PTGFR protein expression in porcine endometrium *in vivo* (Experiment 1)

Porcine conceptuses regulated PTGFR gene and protein expression *in vivo*. Endometrial PTGFR protein expression was approximately three-fold greater in the intact and isolated uterine horns of gilts on day 14 of pregnancy compared with its expression in the endometrium of intact and isolated horns of control gilts on day 14 of the estrous cycle (Fig. 1D). The increase in PTGFR protein expression was observed both in intact and isolated horns and was not dependent on the direct presence of conceptuses (local action), suggesting a systemic effect of conceptuses on this receptor. In pigs on day 14 of the estrous cycle, mRNA expression of *PTGFR* in both uterine horns was higher ($P < 0.05$) compared with its expression in endometrium from intact and isolated horns in pregnant gilts (Fig. 1B). No effect of conceptus ($P > 0.05$) was observed on endometrial PTGFR expression on day 11 of the estrous cycle and pregnancy (Fig. 1A and C).

Figure 1 Effect of conceptus on prostaglandin F $_{2\alpha}$ receptor (PTGFR) mRNA (A and B) and protein (C and D) expression in endometrium from the porcine *in vivo* unilateral model of pregnancy on days 11 and 14 after estrus. The control group consisted of gilts surgically operated but not inseminated (gilts on days 11 and 14 of the estrous cycle). Different letters (small letters for intact uterine horn and capital letters for isolated uterine horn) indicate statistically significant differences between cyclic and pregnant pigs ($P < 0.05$). Data are expressed as the mean $\pm$ S.E.M. PTGFR protein expression values were normalized vs GAPDH protein expression. PTGFR mRNA expression values were normalized vs *ACTB*.

E₂ stimulates PTGFR mRNA and protein expression in endometrium *in vitro* (Experiment 2)

Effect of *E₂* on PTGFR mRNA expression in LE and ST cells of endometrium

As *E₂* is the conceptus signal for maternal pregnancy recognition in pigs, we further studied whether the

Figure 2 Effect of estradiol-17 β (*E₂*, 1–100 nM) on prostaglandin F $_{2\alpha}$ receptor (PTGFR) mRNA (A) and protein expression (B) in porcine endometrial explants *in vitro*. Different letters (a and b) indicate statistically significant differences ($P < 0.05$). Effects of *E₂* (833 ng or 33.3 μ g per infusion *E₂*), prostaglandin E $_2$ (PGE $_2$; 200 μ g per infusion) and PGE $_2$ together with *E₂* administered *in vivo* on endometrial expression of PTGFR protein (C). Hormones were infused by cannulas into the uterine horns six times every 4 h from day 11 until 12 after onset of estrus. Data are expressed as the mean $\pm$ S.E.M. Different letters (small letters for uterine horns receiving vehicle-only infusions and capital letters for horns receiving hormonal infusions) indicate statistically significant differences between control and hormone-treated gilts ($P < 0.05$). PTGFR mRNA expression values were normalized vs expression of *ACTB*. PTGFR protein expression values were normalized vs GAPDH protein expression.

Figure 3 Immunolocalization of prostaglandin $\text{F}_2\alpha$ receptor (PTGFR) protein in porcine uterus on day 12 of the estrous cycle (A and B) and days 12 (C and D) and day 15 (E and F) of pregnancy. Positive signals were detected in the luminal epithelial cells (LE) and superficial (A, C and E), and deep (B, D and F) glandular epithelial cells (GE). Moreover, PTGFR protein was localized in tunica intima and media of arteries (marked as 'a' in B and D) and in tunica intima only of larger veins ('v' in C and F) and venules ('ve' in E). ST, stromal cells. A negative control for anti-PTGFR (normal rabbit IgG) is presented in the upper-right corner of section B.

conceptus regulates PTGFR expression in porcine endometrium via E_2 . No effect ($P > 0.05$) of E_2 on PTGFR mRNA expression was observed in endometrial LE cells or ST cells cultured separately (Supplementary Fig. 1A and B, see section on supplementary data given at the end of this article).

Effect of E_2 on PTGFR mRNA and protein expression in endometrial explants

It seemed likely that the *in vitro* model, which included only LE and ST cells cultured separately, could be distinct from physiological conditions. Therefore, we used endometrial tissue explants as a more appropriate model to study the effect of E_2 on PTGFR gene and protein expression *in vitro*. E_2 stimulated PTGFR mRNA and protein expression in porcine endometrial explants *in vitro*. The content of PTGFR mRNA increased 1.8-fold, whereas its protein content was elevated 1.6-fold after treatment with 1 and 100 nM E_2 ($P < 0.05$; Fig. 2A and B).

Figure 4 Effect of prostaglandin $\text{F}_2\alpha$ ($\text{PGF}_2\alpha$; 10 nM–1 μM) on phosphorylation of MAPK1/3 in luminal epithelial cells of endometrium (A). Cells were preincubated with control (vehicle) or MAPK1/3 inhibitor (50 μM PD98059) for 30 min. Subsequently, cells were treated with different doses (10 nM–1 μM) of $\text{PGF}_2\alpha$ for 15 min. Phosphorylation of MAPK1/3 was analyzed by western blot analysis. MAPK1/3 and pMAPK1/3 expression values were normalized against expression of GAPDH protein. Effects of $\text{PGF}_2\alpha$ (100 nM and 1 μM) and its stable analog – fluprostenol (100 nM and 1 μM) on gene expression (B) and secretion of vascular endothelial growth factor A (VEGFA) (C) by porcine endometrial explants *in vitro*. Data are expressed as the mean $\pm$ s.e.m. Different letters (a, b and c) indicate statistically significant differences ($P < 0.05$).

C-CM	+	-	-	-	-	+	-
PGF-CM	-	+	+	+	+	-	-
AAL993	-	-	+	-	-	-	-
PGF-CM+AL8810	-	-	-	+	-	-	-
PGF-CM+SQ22,536	-	-	-	-	+	-	-
20% NCS	-	-	-	-	-	+	-
VEGFA	-	-	-	-	-	-	+

Figure 5 Effect of conditioned medium from endometrial explants treated with PGF2 α in the presence or absence of inhibitors (50 μ M AL8810: PTGFR inhibitor, and 50 μ M SQ 22,536: adenylyl cyclase inhibitor) on primary endometrial endothelial (A) and G1410 cell proliferation (B). AAL993 (inhibitor of VEGF receptors: FLT1, KDR, and FLT4, 5 μ M) was used to assess the VEGFA–VEGFR involvement in proliferation of conditioned medium-stimulated endothelial cells. As positive control treatments, VEGFA (50 ng/mL) and 20% of NCS in C-CM were used. Data are presented as means $\pm$ S.E.M. of fold-change vs control (C-CM). Different letters (a, b and c) indicate statistically significant differences ($P < 0.05$). C-CM, conditioned medium from endometrial explants treated with vehicle; PGF-CM, conditioned medium from endometrial explants treated with PGF2 α .

E₂ elevates PTGFR protein expression in endometrium in vivo (Experiment 3)

As we determined that E₂ can increase PTGFR gene and protein content in endometrium, we further studied whether E₂ and/or PGE₂, which is also secreted by the conceptus, regulate PTGFR expression in porcine endometrium *in vivo*. Greater levels of PTGFR protein were observed in endometrial samples collected from both horns of gilts receiving infusions of E₂ (833 ng/infusion) or vehicle compared with their expression in samples from vehicle-treated only horns in the control group (Fig. 2C). PGE₂ or a higher dose

of E₂ administered alone did not exert statistically different effects. Simultaneous administration of E₂ together with PGE₂ had no influence on PTGFR protein expression. Neither PGE₂ nor E₂ administered alone or simultaneously had any effect ($P > 0.05$) on PTGFR gene expression in the samples examined (Supplementary Fig. 2).

Immunolocalization of PTGFR protein in uterus (Experiment 4)

Localization of PTGFR protein was detected in LE cells and superficial glandular epithelial (GE) cells (Fig. 3A, C and E) in the deep GE cells (Fig. 3B, D and F) and in the blood vessels (Fig. 3). PTGFR protein was localized in tunica intima and media of arteries (marked in Fig. 3B and D) and in tunica intima only of larger veins and venules (marked in Fig. 3C, E and F). A weak signal for PTGFR expression was detected in ST cells and myometrium. The isotype control (negative control) confirmed the specificity of antibodies and is shown in the upper-right part of Fig. 3B.

PGF2 α increases MAPK1/3 kinase phosphorylation in LE cells (Experiment 5)

As we showed PTGFR protein up-regulation by conceptuses and E₂ in porcine endometrium *in vitro* and *in vivo*, we decided to determine whether PGF2 α acting on its receptor can activate the MAPK1/3 pathway. PGF2 α (10 and 100 nM) increased ($P < 0.05$) phosphorylation of MAPK1/3 proteins two-fold in endometrial LE cells (Fig. 4A). PGF2 α -induced phosphorylation

Figure 6 Effect of PGF2 α (100 nM and 1 μ M) on biglycan (BGN; A), interleukin 1 α (IL1A; B), matrix metalloproteinase 9 (MMP9; C), and transforming growth factor beta isoform 3 (TGFβ3; D) gene expression in porcine endometrium *in vitro*. Data are presented as means $\pm$ S.E.M. of fold-change vs control (vehicle). Different letters (a and b) indicate statistically significant differences ($P < 0.05$).

Figure 7 Proposed mechanism of auto-/paracrine actions of PGF2 α in porcine endometrium and conceptus during the implantation period. Embryonic signal for pregnancy (estrogens) up-regulates the expression of PGF2 α receptor (PTGFR) protein in endometrium. PGF2 α secreted by both endometrium and conceptus acting on PTGFR in the endometrium stimulates phosphorylation of MAPK1/3 resulting in enhanced synthesis and expression of vascular endothelial growth factor A (VEGFA). This in turn, by involvement of VEGF receptors (VEGFR: FLT1, KDR, and FLT4), promotes endometrial angiogenesis by increasing proliferation of endometrial endothelial cells. A diminishing effect of AL8810 (PTGFR inhibitor) and SQ22,536 (adenylyl cyclase inhibitor) on endothelial cell proliferation suggests the involvement of PTGFR receptor and adenylyl cyclase (CA) in endometrial VEGFA secretion. Moreover, PGF2 α acting on its receptor in endometrium regulates expression of genes involved in tissue remodeling and embryo-maternal communication (*BGN*, *IL1A*, *MMP9*, and *TGFB3*).

was abolished by addition of the MAPK1/3 inhibitor (PD098059).

PGF2 α and its analog stimulate VEGFA synthesis and secretion by endometrial explants *in vitro* (Experiment 5)

As in human endometrial adenocarcinoma cells, PGF2 α by activation of MAPK1/3 pathway enhanced expression of VEGFA (Sales *et al.* 2005), we studied whether PGF2 α present in the uterine lumen can stimulate synthesis and secretion of VEGFA *in vitro*. PGF2 α (100 nM and 1 μ M) and fluprostenol (100 nM) stimulated VEGFA gene expression ($P < 0.05$) in porcine endometrial explants *in vitro* (Fig. 4B). RIA measurement of VEGFA concentration in conditioned CM revealed that both PGF2 α (1 μ M) and fluprostenol (100 nM and 1 μ M) elevated secretion ($P < 0.05$) of VEGFA by porcine endometrial explants (Fig. 4C).

PGF2 α promotes endometrial endothelial cell proliferation (Experiment 6)

We further studied whether PGF2 α -induced VEGFA synthesis and secretion by endometrium and conceptus cells can regulate endothelial cell proliferation. Primary endothelial cells isolated from PEE were characterized (Supplementary Fig. 3). Similar to the immortalized swine umbilical vein endothelial cell line (G1410; Chrusciel *et al.* 2011), PEE exhibited cobblestone morphology, and at 40–50% confluency, they started forming capillary-like tubules typical of endothelial cells (Supplementary Fig. 3A and B). Moreover, we confirmed the presence of key markers of endothelial cells, VE-cadherin (CDH5), and VWF in PEE. CDH5 was observed only in cell

membranes, whereas VWF was localized in cytoplasm (Supplementary Fig. 3C, D, E and F). Using PEE isolated from porcine endometrium and the G1410 cell line, we found that conditioned media collected from endometrial explants treated with 1 μ M PGF2 α stimulated ($P < 0.001$) proliferation of these cells (Fig. 5A and B). Stimulation of both PEE and G1410 cells by PGF-CM was abolished using the selective, cell-permeable inhibitor of VEGF receptors (AAL993; $P < 0.001$). Co-treatment of endometrial explants with PGF2 α and PTGFR inhibitors (AL8810) or adenylyl cyclase inhibitor (SQ22,536) abolished PGF2 α -mediated effects on proliferation of PEE and G1410 cells ($P < 0.05$). VEGFA (50 ng/mL) and 20% NCS, used as positive controls, significantly elevated proliferation of PEE and G1410 cells to a similar extent as PGF-CM. We did not observe any direct effect of PGF2 α or inhibitors alone on PEE and G1410 proliferation (Supplementary Fig. 4A, B and C). AAL993 had no effect on HIF1A protein expression in the G1410 cell line (Supplementary Fig. 4D).

PGF2 α regulates expression of genes potentially important for embryo-maternal interactions (Experiments 5)

Additionally, we examined whether PGF2 α can regulate the expression of genes potentially involved in other processes important for implantation, such as ECM and tissue remodeling, cell adhesion, or immune response. In porcine endometrial explants, 1 μ M PGF2 α elevated gene expression of *BGN* (Fig. 6A), *MMP9* (Fig. 6B), *TGFB3* (Fig. 6C), and *IL1 α* (*IL1A*; Fig. 6D). A lower dose of PGF2 α (100 nM) elevated expression of *BGN* and *IL1A*

($P < 0.05$). There was no effect of $\text{PGF2}\alpha$ (100 nM and 1 μM) on *IL6* mRNA expression in porcine endometrial explants (Supplementary Fig. 5).

Discussion

PGs are involved in the implantation of embryos as well as the proper development of early pregnancy in humans and other mammals (Geisert *et al.* 1982, Weems *et al.* 2006, Kennedy *et al.* 2007, Ulbrich *et al.* 2009, Waclawik *et al.* 2009, Vilella *et al.* 2013, Waclawik *et al.* 2013). Disrupted synthesis of PGs results in pregnancy loss in humans (Achache *et al.* 2010) and other animals such as pigs (Kraeling *et al.* 1985), ruminants (Erdem & Guzeloglu 2010, Dorniak *et al.* 2011), and rodents (Kennedy *et al.* 2007). However, the types of PGs involved have not been definitely established in most studies, as general inhibitors of PG synthesis were used (Kennedy *et al.* 2007). Mainly, PGE2 and/or PGI2 are believed to promote processes such as adhesion of human and porcine conceptus cells to ECM (Waclawik *et al.* 2013), decidualization in mice (Lim *et al.* 1999), blastocyst hatching, and implantation (Huang *et al.* 2004, Kennedy *et al.* 2007). However, the possible role of high amounts of $\text{PGF2}\alpha$ secreted into the uterine lumen during early pregnancy in pigs and other species remains unresolved (Zavy *et al.* 1980, Waclawik *et al.* 2006, Kennedy *et al.* 2007, Ulbrich *et al.* 2009, Wang *et al.* 2010, Vilella *et al.* 2013, Kowalewski *et al.* 2014). However, there are reports suggesting a detrimental role of this PG on blastocyst growth (Seals *et al.* 1998) and generally on the establishment of pregnancy because of the luteolytic action of exogenous $\text{PGF2}\alpha$ on the CL (Podany *et al.* 1982, Pajor & Zsolnai 1984, Romagnoli *et al.* 1993, Seals *et al.* 1998, Lemaster *et al.* 1999).

Using a model of unilateral pregnancy, we demonstrated that the presence of conceptuses up-regulated the expression of PTGFR protein in the endometrium *in vivo*. PTGFR protein content was greater in the endometrium of pregnant gilts on day 14 in both the intact (gravid) and isolated (non-gravid) horns compared with the endometrium of the intact and isolated horns of control cyclic gilts. This result indicates that conceptus products may be transported by local blood and/or lymph circulation or systemic circulation to isolated uterine horn. Our finding implies a systemic manner of conceptus action and is in agreement with our findings showing up-regulation of PTGFR protein expression in porcine endometrium on day 15 of pregnancy (Kaczynski & Waclawik 2013). It is also consistent with our recent results demonstrating the stimulatory effect of conceptus secretions on endometrial PTGFR gene expression *in vitro* (Kaczynski & Waclawik 2013). Moreover, higher expression of PTGFR protein coincides with elevated expression of $\text{PGF2}\alpha$ synthase in porcine endometrium and conceptuses as well as

greater concentrations of $\text{PGF2}\alpha$ in the uterine lumen of pregnant gilts during this period (Zavy *et al.* 1980, Waclawik *et al.* 2006, 2007).

In studies on the mechanisms of maternal recognition of pregnancy in pigs, usually *in vivo* models of pseudopregnancy are used in which 5 mg of E_2 benzoate is administered systemically on days 11–15 of the estrous cycle, resulting in pharmacological concentrations of E_2 (Frank *et al.* 1977, Geisert *et al.* 1982, Pusateri *et al.* 1996, Kawarasaki *et al.* 2012). The novel approach applied in our study allowed us to examine both local and systemic effects of E_2 in concentrations close to the physiological levels secreted by embryos into the uterine lumen. This is the first report showing that intra-uterine E_2 administration increased the abundance of PTGFR protein in porcine endometrium *in vivo*. Interestingly, PTGFR protein expression was up-regulated in both E_2 -treated and vehicle-treated uterine horns compared with the endometrium of vehicle-treated horns of control cyclic gilts. These findings correspond with the results obtained in this study using the unilateral model of pregnancy *in vivo* that indicate a systemic effect of E_2 administered into the uterine lumen. Moreover, this is in agreement with the finding that intrauterine infusion of E_2 can exert not only a local effect in the uterine horn into which it was administered but also a systemic effect, i.e., on the contralateral uterine horn or ovaries (Ford *et al.* 1982). Accordingly, the *in vitro* experiment performed in this study demonstrated that E_2 stimulated PTGFR gene and protein expression in endometrial explants. These results support our previous findings indicating that the porcine conceptus can mediate its effects on PTGFR expression by estrogen receptor (Kaczynski & Waclawik 2013). Similarly, up-regulation of *PTGFR* mRNA content by E_2 was observed in the uteri of rats (Yallampalli & Dong 2000). In cattle, higher levels of *PTGFR* mRNA were detected in caruncles of placentomes, i.e., uterine tissue in direct contact with cotyledons of the placenta (Fortier *et al.* 2004).

Immunolocalization of PTGFR protein in porcine uterus revealed its expression mainly in LE and GE and blood vessels, as well as in ST cells of porcine endometrium when examined on days 12 and 15 of pregnancy and day 12 of the estrous cycle. A similar distribution of PTGFR was observed in the ovine uterus (Dorniak *et al.* 2011). Previously, we demonstrated the presence of *PTGFR* mRNA in LE and ST cells of the endometrium (Kaczynski & Waclawik 2013). The present results suggest that LE and GE cells have a greater potential to respond to $\text{PGF2}\alpha$ action than ST cells. Moreover, PTGFR expression in the tunica media of endometrial arteries suggests a vasoconstructive function of $\text{PGF2}\alpha$. Differences in distribution of PTGFR protein in arteries and veins suggest possible differential function of $\text{PGF2}\alpha$ -PTGFR signaling in these vessels.

Our present results strongly support the hypothesis that $\text{PGF2}\alpha$ is a mediator of E_2 , the porcine conceptus signal

for recognition of pregnancy. In these *in vivo* and/or *in vitro* studies, we demonstrated that the conceptus and its signal, E₂, elevated the expression of PTGFR protein in the endometrium. Our *in vitro* studies revealed that both PGF2 α and its analog (fluprostenol) increased VEGFA gene expression and VEGFA protein secretion by porcine endometrium. Therefore, we suggest that PGF2 α acting on via PTGFR in an autocrine/paracrine manner in porcine endometrium enhanced gene expression, protein synthesis, and secretion of VEGFA. Accordingly, elevated expression of VEGFA, FLT1, and KDR was reported in porcine endometrium during the implantation period compared with earlier stages of pregnancy and the estrous cycle (Kaczmarek *et al.* 2008).

Until now, there were no data concerning the effect of PGF2 α on the MAPKs signaling in endometrial cells under normal physiological conditions. In this study, we demonstrated that PGF2 α treatment activated the MAPK1/3 signaling pathway in porcine LE cells of the endometrium, which is probably involved in elevated synthesis and secretion of VEGFA by endometrial cells. Likewise, treatment of human adenocarcinoma (Ishikawa FPS) cells with PGF2 α rapidly induced phosphorylation of MAPK1/3 via the PTGFR. This, in turn, resulted in an increase in VEGFA promoter activity, expression of VEGFA mRNA, and secretion of VEGFA protein (Sales *et al.* 2005).

VEGFA can be involved in early placentation by stimulating endometrial and conceptus angiogenesis and regulation of vascular permeability in pigs. Endothelial cell proliferation is one of the processes required for angiogenesis. We suggest that PGF2 α present in the uterine lumen during the implantation period mediates angiogenesis by indirect effects on the function of endometrial endothelial cells. The this study indicates that PGF2 α stimulated endometrial secretion of VEGFA, which can act on blood vessels in a paracrine manner to promote proliferation of endothelial cells in endometrium. PGF-CM enhanced proliferation of PEE cells and the immortalized swine umbilical vein endothelial cell line (G1410). Using specific inhibitors of PTGFR and adenylyl cyclase, we demonstrated that the stimulatory effects of conditioned medium on proliferation of these endothelial cells were dependent on PGF2 α –PTGFR and adenylyl cyclase signaling in endometrium. It was reported that in some cases, PGF2 α could be a stimulator of cAMP production (Yamaguchi *et al.* 1988, Tachado *et al.* 1993). In turn, cAMP is also involved in VEGFA synthesis in human endometrium (Popovici *et al.* 1999). In this study, co-treatment of endothelial cells with AAL993 and conditioned medium from PGF2 α -treated endometrial explants abolished the stimulatory effect of PGF-CM on PEE and G1410 cell proliferation, which indicates involvement of VEGFA and its receptors in PGF2 α -mediated proliferation of endothelial cells. Concordantly, an indirect effect of PGF2 α in the promotion of angiogenic events was observed for human

endometrial adenocarcinoma cell lines (Keightley *et al.* 2010). Intriguingly, it can be suggested that PGF2 α -stimulated synthesis of VEGFA by endometrial cells may not only be involved in angiogenic changes but also in stimulating proliferation and migration of porcine trophoblast cells (Jeong *et al.* 2014).

Angiogenesis is not the only process regulated by PGF2 α in the endometrium. We found that PGF2 α up-regulated expression of *BGN*, *IL1A*, *MMP9*, and *TGFB3* genes in porcine endometrium. Processes accompanying the implantation period are controlled by a broad range of molecules. One of them is BGN, a protein belonging to the family of small leucine-rich proteoglycans (Iozzo & Murdoch 1996). It is involved in endometrial ECM remodeling during decidualization in mice (San Martin *et al.* 2003), cellular migration and adhesion, and regulation of growth factor availability and activity (Couchman *et al.* 1990, Tufvesson & Westergren-Thorsson 2003, Nastase *et al.* 2012). Moreover, BGN can modulate inflammatory processes and induce secretion of IL1B (Nastase *et al.* 2012). Up-regulation of *BGN* gene in endometrium by PGF2 α suggests its involvement in ECM remodeling and immune interactions at the maternal–embryo interface.

Cell invasion is a result of active biochemical processes in which, among other enzymes, MMPs have been implicated (Bischof & Campana 2000). In mouse endometrium, administration of MMPs inhibitors delays decidual remodeling and growth of ST cells (Alexander *et al.* 1996). In tissues, expression of MMPs is usually low and is induced when ECM remodeling is required. It has also been reported that MMP9 by interaction with integrin α V β 8 can activate latent TGFB (Mu *et al.* 2002). In human adenocarcinoma cells, MMPs are associated with EGFR transphosphorylation, which results in elevated VEGFA expression (Sales *et al.* 2005). Our findings suggest a regulation of MMP9 activity by PGF2 α at the maternal–embryo interface. Elevated expression of MMP9 after PGF2 α treatment in porcine endometrium may promote ECM remodeling, as well as augmenting stimulation of VEGFA synthesis by PGF2 α .

TGFB is thought to mediate many key processes in reproduction, such as steroidogenesis, regulation of immunotolerance, embryogenesis, and tissue remodeling (Guzeloglu-Kayisli *et al.* 2009). It is also an important cytokine regulating conceptus development and implantation in pigs (Blitek *et al.* 2013). TGFB stimulates fibronectin synthesis by porcine trophoblast as well as trophoblast cell adhesion to this protein (Jaeger *et al.* 2005). Moreover, TGFB together with tumor necrosis factor α stimulates MMP9 expression (Stuelten *et al.* 2005). We determined that PGF2 α up-regulated *TGFB3* mRNA in porcine endometrium *in vitro*, which supports previous findings indicating the up-regulation of *TGFB3* expression in porcine endometrium during early stage (days 10, 12, and 14) of gestation (Gupta *et al.* 1998). Our results suggest that PGF2 α can act as a conceptus

signal mediator due to its ability to trigger TGF β 3 signaling in the endometrium.

IL1A is a cytokine belonging to the IL1 superfamily. It is synthesized mostly by macrophages and is mainly known as a pro-inflammatory factor (Dinarello & Savage 1989). It also promotes angiogenesis by activating the VEGF–KDR pathway (Salven *et al.* 2002). In humans, IL1A is involved in changing endometrial structure during implantation and menstrual cycle (Singer *et al.* 1997, Huang *et al.* 1998). This cytokine regulates PG secretion in several organs and tissues (Takács *et al.* 1988). In cattle, IL1A stimulates PGF2 α synthesis and secretion by endometrium *in vitro* and *in vivo* (Tanikawa *et al.* 2005, Majewska *et al.* 2010). Our results indicate that PGF2 α increased *IL1A* mRNA expression in porcine endometrium. Thus, it may be hypothesized that PGF2 α self-regulates its synthesis by increasing *IL1A* mRNA expression in porcine endometrium.

In conclusion, this study presents the first comprehensive evidence for a role for PGF2 α acting in an autocrine and/or paracrine manner through its receptor in the porcine endometrium during the peri-implantation period (Fig. 7). Our results strongly support the hypothesis that PGF2 α is a mediator of the embryonic signal for recognition of pregnancy in endometrium. In this study, the conceptus and its signal (E₂) elevated expression of endometrial PTGFR protein in endometrium both *in vivo* and *in vitro*. PGF2 α , acting on PTGFR in an autocrine/paracrine manner in the porcine endometrium, enhanced synthesis and secretion of VEGFA. In turn, secreted VEGFA stimulated endometrial endothelial cell proliferation, which can promote angiogenesis at the maternal–conceptus interface during the implantation period in pigs. Moreover, regulation of *BCN*, *MMP9*, *TGFB3*, and *IL1A* gene expressions by PGF2 α in the endometrium may promote endometrial remodeling and embryo–maternal communication during early pregnancy.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-15-0496>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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