Prostaglandin F2 α promotes embryo implantation and development in the pig

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Abstract

Successful establishment and development of pregnancy requires proper communication between developing conceptuses and the maternal reproductive tract. Prostaglandins are key players involved in the regulation of reproductive processes in mammals including pigs. Due to its luteolytic action, prostaglandin F2- α (PGF2 α) is mainly considered as an undesirable factor during early pregnancy. However, its content in the uterine lumen is elevated in pigs and other mammals. Recently, we reported an important role of PGF2 α in the endometrium during early pregnancy in the pig. Thus, the aim of the present study was to determine whether PGF2 α can act on porcine trophoblast and if so, to elucidate what effect it could exert. We detected increased expression of PGF2 α receptor during the implantation period (from day 14 until day 19 of pregnancy). Global gene expression profiling using microarrays and quantitative PCR studies revealed that PGF2 α acting on porcine trophoblast cells *in vitro* alters expression of genes potentially involved in processes related to implantation, such as cell proliferation, focal adhesion, extracellular matrix binding, cell migration, cytoskeleton organization, immune interactions, ion homeostasis and lipid metabolism. Using primary porcine trophoblast cells, we demonstrated that PGF2 α stimulated trophoblast cell proliferation and adhesion to extracellular matrix protein. This was likely mediated by mitogen-activated protein kinases (MAPK1/3) and focal adhesion kinase (FAK) since we observed increased phosphorylation of MAPK1/3 and FAK in trophoblast cells treated with PGF2 α . To conclude, the present report indicates a novel role for PGF2 α in the porcine conceptus as a paracrine and autocrine factor supporting pregnancy establishment.

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Introduction

The establishment and development of pregnancy in the pig, as in any mammalian species, requires proper communication between developing conceptuses and the intrauterine environment. This embryo–maternal dialog includes hormonal and immune interactions precisely controlled by hormones, cytokines, growth factors and other molecules interacting in endo-, para- and autocrine manners.

During the maternal recognition of pregnancy period, which occurs in pigs on days 11–12 of pregnancy, conceptuses secrete increased levels of estrogens that are one of the major conceptus signals for the establishment of pregnancy. This leads to the redirection of luteolytic prostaglandin F2 α (PGF2 α) secretion from endocrine to exocrine (to the uterine lumen) mode and also affects the hormonal environment in the reproductive tract, resulting in prolonged lifespan of the corpus luteum (CL) and, thereby, maintained progesterone (P4) synthesis (Bazer & Thatcher 1977). The second phase

of increased estrogen secretion occurs in pigs on day 15 of pregnancy and lasts until day 25 after fertilization (Geisert *et al.* 1990).

Key factors involved in the control of reproductive processes in mammals are prostaglandins (PGs). Their importance for pregnancy establishment and development is shown by the fact that lack of PG synthesis leads to termination of pregnancy just before the implantation process in mammals such as pigs (Kraeling *et al.* 1985), cattle (Erdem & Guzeloglu 2010) and rodents (Kennedy *et al.* 2007). Interestingly, intrauterine administration of prostaglandin synthase inhibitors resulted in improper conceptus elongation in sheep (Dorniak *et al.* 2011), which has a much different programming for conceptus growth compared to rapid trophoblast elongation in the pig. In contrast, PG synthesis inhibition does not affect elongation of porcine conceptuses (Geisert *et al.* 1986), but it does affect their survival during the attachment period (Kraeling *et al.* 1985). Prostaglandin E2 (PGE2) and PGF2 α are the major prostaglandins involved in reproductive functions in mammalian reproductive

tracts. They act through their receptors: PTGER1–4 (PGE2 receptors isoforms 1–4) and PTGFR (PGF2 α receptor). The expression profiles of PTGER2 and PTGER4 were determined in porcine trophoblasts at different stages of development (Waclawik *et al.* 2013). However, the expression profile of PTGFR in porcine conceptuses during early pregnancy has not been studied yet. Interestingly, the expression of PGE2 and PGF2 α synthases is elevated both in porcine endometrium and in conceptuses during the maternal recognition of pregnancy and/or implantation (Waclawik *et al.* 2006, Waclawik & Ziecik 2007). PGE2 has been described as playing a luteoprotective/luteotrophic role (Waclawik *et al.* 2008, 2009, Waclawik 2011) whereas PGF2 α has been mainly perceived as a luteolytic factor in sheep, cattle and pigs, as well as in rats, rabbit and hamsters (reviewed in McCracken *et al.* 1999). Thus, it could also have a detrimental effect in pregnancy development in mammals, including human, cattle, pigs and dogs (reviewed in Kaczyński *et al.* 2016). However, it should not be regarded as undesirable factor in pregnancy because elevated amounts of PGF2 α in the uterine lumen and/or its increased endometrial synthesis occur not only in pigs (Zavy *et al.* 1980, Waclawik *et al.* 2006) but also in other mammals including rodents, cattle, dogs and humans (Kennedy *et al.* 2007, Ulbrich *et al.* 2009, Wang *et al.* 2010, Vilella *et al.* 2013, Kowalewski *et al.* 2014). Moreover, a novel role for PGF2 α in the porcine endometrium during the peri-implantation period has been recently reported by our group, indicating that PGF2 α participates in pregnancy establishment by promoting angiogenesis and expression of genes involved in endometrial remodeling and embryo–maternal communication (Kaczyński *et al.* 2016).

Previously we indicated that synthesis of PGF2 α during the implantation period is elevated both in the porcine endometrium and the conceptus (Waclawik *et al.* 2006, Waclawik & Ziecik 2007). Recently, we also elucidated the mechanism of PGF2 α action in the endometrium (Kaczynski & Waclawik 2013, Kaczyński *et al.* 2016). Hence, the question arose whether PGF2 α can act through its receptor in the conceptus and if so, what could be the role of PGF2 α in porcine trophoblast cells? During the period when the endometrium becomes receptive for embryo implantation, both the uterine luminal epithelium and conceptus cells simultaneously initiate the adhesion cascade (Geisert *et al.* 2015). Attachment of trophoblast cells to luminal epithelium is supported by interactions between integrins and extracellular matrix (ECM) proteins. Integrin receptors (AVB1, AVB3, AVB5 and A4B1) are able to bind to the RGD (Arg-Gly-Asp) sequence present in osteopontin, fibronectin and vitronectin proteins (Bowen *et al.* 1996, Johnson *et al.* 2003). Our studies indicate the important role of PGE2 in activation of intracellular pathways in porcine trophoblast cells related to adhesion

(mitogen-activated protein kinases 1/3 – MAPK1/3); FAK and intracellular adhesion molecule 1 (ICAM1) and promotion of trophoblast adhesion to ECM proteins (Waclawik *et al.* 2013). Moreover, we demonstrated a stimulatory effect of PGE2 on trophoblast cell estradiol-17 β synthesis (Waclawik *et al.* 2013). It has been shown that lack of PGs (PGE2 and PGF2 α) decreased adhesion of mouse blastocysts and JEG-3 spheroids to human luminal epithelial cells (Vilella *et al.* 2013). However, the mechanisms by which PGF2 α may stimulate trophoblast cell adhesion to ECM proteins remain unknown. Furthermore, despite elevated conceptus and endometrial synthesis of PGF2 α , its role in the proliferative capacities of trophoblast cells and activation of intracellular pathways is still unresolved.

Based on data in the literature including results from our previous studies, we hypothesized that during early pregnancy in the pig, PGF2 α acting through its receptor in trophoblast cells activates intracellular pathways important for the implantation process. Thus, the objectives of the present study were (i) to determine the expression profile of the PTGFR gene and protein in porcine conceptuses/trophoblasts at different stages of development; (ii) to investigate the effect of PGF2 α on global gene expression profiles in porcine primary trophoblast cells *in vitro*; (iii) to study the effect of PGF2 α on trophoblast cell adhesion and proliferation and (iv) to evaluate whether PGF2 α may activate MAPK1/3 and FAK in porcine trophoblast cells, which could be involved in adhesion and/or proliferation processes.

Materials and methods

All procedures involving 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. 36/2012.

Experiment 1: PTGFR mRNA and protein expression in the porcine conceptus/trophoblast

Gilts after two natural estrous cycles were bred at the onset of estrus (day 0) and then 12 h and 24 h later. Pregnant gilts were slaughtered at the local abattoir. Conceptuses at the preimplantation stage were flushed from the uterine horns with 20 mL of sterile PBS (137 mM NaCl, 27 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄, pH 7.4). Conceptuses/trophoblasts at the implantation and early placentation stage were dissected from the endometrium. Considering the days of pregnancy and morphology of trophoblasts, the conceptus samples derived from the same animal were pooled and classified into the following groups: days 10–12 (preimplantation stage; spherical and tubular forms, $n=5$), days 14–15 (beginning of attachment/implantation; filamentous forms, $n=5$), days 16–19 (filamentous forms; implantation stage, $n=7$) and days

20–25 (chorion which originates from trophoblast; the onset of placenta development; $n=10$). Dissections of trophoblast tissues from conceptuses were performed since day 18 post mating (Waclawik *et al.* 2013). Therefore, expression of PTGFR was analyzed in 10- to 17-day conceptuses and 18- to 25-day trophoblast tissues. Collected conceptuses/trophoblasts were snap-frozen in liquid nitrogen and stored at -80°C until gene and protein expression analyses.

RNA isolation and cDNA synthesis

Collected tissues were homogenized in 800 μL of FenoZol buffer (A&A Biotechnology, Gdansk, Poland) using Lysing Matrix D beads (MP Biomedicals, Santa Ana, USA) and Fast Prep homogenizer (MP Biomedicals). RNA was isolated from tissues with RNA Mini Kit (A&A Biotechnology), according to the manufacturer's protocol. Integrity and concentration of isolated RNA was analyzed by using Agilent 2100 Bioanalyzer (Agilent Technologies). Additionally, concentration of RNA was measured with NanoDrop spectrophotometer (Thermo Fisher Scientific). To generate cDNA for qPCR reaction, total RNA sample (1 μg) was reverse-transcribed with MultiScribe Reverse Transcriptase kit (Thermo Fisher Scientific) according to the manufacturer's protocol. cDNA samples were stored in -80°C for further quantitative PCR (qPCR) analyses.

Quantitative PCR

Analysis of *PTGFR* gene expression in conceptuses was performed as described previously (Kaczynski & Waclawik 2013). Reverse-transcribed cDNA (3.5 μL) was added to the reaction mixture: 12.5 μL Power SYBR Green master mix (Thermo Fisher Scientific), 2.5 μL of each sense and antisense primer (1 μM ; Supplementary Table 1, see section on supplementary data given at the end of this article) and 4 μL of nuclease-free water. Specific primers used for real-time RT-PCR are listed in Table 1. The PCR program for *PTGFR* gene 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 actin beta (*ACTB*), cyclophilin A (*PPIA*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) amplification, the PCR program was 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). After PCR, melting curves were acquired by gradual increases in the temperature from 60 to 95°C to ensure that a single product was amplified in the PCR reaction. All real-time PCR reactions were performed with Applied Biosystems 7900HT Real-Time PCR system (Life Technologies). Gene expression was estimated using real-time PCR Miner software (Zhao & Fernald 2005). Stability of the reference genes in the porcine conceptuses was assessed using the statistical algorithms Normfinder 2.0 (Andersen *et al.* 2004). Three of the following reference genes were analyzed: *GAPDH*, *PPIA* and *ACTB*. The most stable combination of reference genes was the geometrical mean of *ACTB* and *GAPDH* expression values.

Protein isolation

Conceptuses (days 10–17)/trophoblasts (days 18–25) collected from pregnant gilts were homogenized on ice in 150 μL of homogenization buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl, 1 mM EDTA) supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich). Homogenates were then centrifuged for 15 min at $800\times g$ at 8°C and stored at -80°C for further analysis. The protein concentration was determined with Bradford Assay. PTGFR protein abundance in conceptus homogenates was assessed by Western blot analysis.

Western blot

Western blot analysis for PTGFR protein expression in conceptuses was performed as described previously (Kaczynski & Waclawik 2013). Briefly, equal amounts of protein samples (40 μg) were separated on 10% SDS PAGE. Proteins were electroblotted onto 0.2 mm PVDF membrane. After blocking in 5% nonfat dry milk, the blots were incubated overnight at 4°C with primary antibodies (Table 2) and afterward with 1:20,000 dilution of secondary anti-rabbit horseradish peroxidase antibodies (Bio-Rad Laboratories) for 90 min at room temperature. Immune complexes were visualized using Clarity Western ECL Substrate (Bio-Rad). Sample loading was standardized to expression of GAPDH. Signal was quantitated using Versa-Doc 4000M imaging system (Bio-Rad Laboratories).

Experiment 2: The effect of PGF2 α on global gene expression profile in porcine trophoblast cells

Incubation of the porcine primary trophoblast cells with PGF2 α

Porcine trophoblast cells were isolated as described previously (Waclawik *et al.* 2013). Crossbred gilts ($n=5$) of similar age and genetic background were inseminated and slaughtered on day 15 of pregnancy at the local abattoir. Conceptuses were recovered from uteri by gentle flushing of each uterine horn with medium M199 (Sigma-Aldrich) containing 0.1% (wt/vol) bovine serum albumin (BSA), penicillin (100 IU/mL, Sigma-Aldrich) and streptomycin (100 $\mu\text{g}/\text{mL}$, Sigma-Aldrich), warmed up to 37°C . Conceptuses were separated from flushing medium by centrifugation for 10 min at $150\times g$ at 8°C . Trophoblast tissue was dissected from the embryonic disk region. Trophoblast cells were then non-enzymatically dispersed by pipetting. After dispersion, the cells were washed three times with sterile medium M199 with antibiotics and 0.1% BSA, re-suspended in sterile M199 containing antibiotics and 5% newborn calf serum (NCS, Sigma-Aldrich) and seeded on human collagen I-coated 6-well plates (BioCoat 356400; BD Biosciences). Adherent cells were washed with PBS 48 h after plating to remove contaminating aggregates. Additionally, to assess the purity of cell cultures, isolated cells were stained for the presence of the trophoblast marker – cytokeratin as described previously (Blitek *et al.* 2012). After reaching 70–80% confluence, the cells were washed with sterile PBS. The following treatments were performed: PGF2 α (100 nM; 1 μM ; Sigma-Aldrich) and control (medium M199

Table 1 Primer sequences and assays used in qPCR analyses.

Gene	Primer sequence/TaqMan Assay ID	RefSeq ID/GenBank accession no.	Reference
<i>PTGFR</i>	Sense: 5'-TCAGCAGCACAGACAAGG-3' Antisense: 5'-TTCACAGGCATCCAGATAATC-3'	NM_214059	Kaczynski & Waclawik (2013)
<i>ACTB</i>	Sense: 5'-ACATCAAGGAGAAGCTCTGCTACG-3' Antisense: 5'-GAGGGGCGATGATCTTGATCTTCA-3'	U07786	Kaczynski & Waclawik (2013)
<i>GAPDH</i>	Sense: 5'-CAGCAATGCCTCCTGTACCA-3' Antisense: 5'-GATGCCGAAGTTGTCATGGA-3'	AF017079	Kaczynski & Waclawik (2013)
<i>PPIA</i>	Sense: 5'-TAACCCACCGTCTTCTT-3' Antisense: 5'-TGCCATCCAACCACTCAG-3'	AY266299.1	Kaczynski & Waclawik (2013)
<i>ADAM9</i>	Ss03373282_m1	AF069646.1	—
<i>AGER</i>	Ss03390846_g1	EU282357.1	—
<i>AMBP</i>	Ss03374771_m1	AK232748.1	—
<i>BGN</i>	Ss03375454_u1	AF159382.1	Kaczyński et al. (2016)
<i>BMP2</i>	Ss03373798_g1	AY669080	—
<i>DECR1</i>	Ss01051816_m1	AK230726.1	—
<i>IL1A</i>	Ss03391335_m1	X52731.1	Kaczyński et al. (2016)
<i>IL6</i>	Ss03384604_u1	AF309651.1	Kaczyński et al. (2016)
<i>IL6R</i>	Ss03394904_g1		
<i>KNG1</i>	Ss03373690_m1	AY321363.1	—
<i>LPAR1</i>	Ss03377225_u1	AK344044.1	—
<i>MASP1</i>	Ss04246770_m1	GU810082.1	—
<i>MFG8</i>	Ss03390111_m1	AK233926.1	—
<i>MMP2</i>	Ss03394318_m1	AF295805.1	—
<i>MMP9</i>	Ss03392100_m1	DQ132879.1	Kaczyński et al. (2016)
<i>PROX1</i>	Ss04246028_m1	EF486324.1	—
<i>S100G</i>	Ss03382848_u1	L13068.1	—
<i>SLCO2A1</i>	Ss03390713_m1	NM_001123195	—
<i>STC2</i>	Ss03389624_m1	AK349078.1	—
<i>TGFB3</i>	Ss03394352_m1	X14150.1	Kaczyński et al. (2016)
<i>THBS1</i>	Ss03373620_m1	AM238691.1	—
<i>TLR3</i>	Ss03388861_m1	AB111939.1	—
<i>VTN</i>	Ss03382603_u1	AK232304.1	—
<i>ACTB</i>	Ss03376081_u1	AK237086.1	Kaczyński et al. (2016)
<i>PPIA</i>	Ss03394782_g1	AY266299.1	Kaczyński et al. (2016)
<i>GAPDH</i>	Ss03375435_u1	NM_001206359.1	Kaczyński et al. (2016)

with vehicle). Treated cells were incubated for 24 h at 37°C in humidified atmosphere containing 95% air and 5% CO₂. After incubation, culture media were collected and frozen. Cells were lysed with FenoZol buffer (A&A Biotechnology), harvested and stored at –80°C until isolation of RNA.

Global gene expression profiling by using expression microarrays

Total RNA was isolated from trophoblast cell lysates as described in Experiment 1 and destined for downstream analyses: global gene expression profiling (expression microarray analysis) and qPCR. Microarray analysis was performed starting from 100 ng total RNA isolated from trophoblast cells after *in vitro* experiment (Experiment 2). Cy3-labeled cRNA was produced with the Low-Input Quick Amp Labeling Kit, one-color (Agilent Technologies) and hybridized to the Agilent 4x44k Porcine Gene Expression microarrays (G2519F-026440) according to the manufacturer's instructions. Hybridized and washed slides were scanned at 2 µm resolution with an Agilent DNA Microarray Scanner (G2505C; Agilent Technologies). Image processing was performed with Feature Extraction Software 10.5.1.1 (Agilent Technologies). Signals were filtered based on 'well above background' flags (detection in three of four samples) and normalized with the

BioConductor package VSN (Huber et al. 2002). For quality control, normalized data were analyzed with a distance matrix and a heatmap based on pairwise distances (BioConductor Package Genefilter). Differentially expressed genes (DEGs) were identified in the contrast (pairwise comparison) 'PGF2α-treated cells' vs 'vehicle-treated cells' using the BioConductor package LIMMA (Linear Model for Microarray Analysis) (Smyth 2004). Low number of genes showing expression differences leads to a too strong correction of nominal *P*-values and a very low power to detect true DEGs (Hackstadt & Hess 2009). Regarding the sensitivity problem of the FDR in case of a low number of DEGs, the cut-off values were set as follows: ≥1.2-fold difference in expression values with a *P*-value less than 0.05. Significant probes were annotated based on mapping of the probe sequences (60 nt sequences) to the porcine genome (SusScrofa 11.1). Known and potential human ortholog or homolog genes were assigned using a custom ortholog annotation database (Mammalian Ortholog and Annotation Database, MOAdb; J T Bick, S E Ulbrich and S Bauersachs, unpublished data). If a gene in the list was represented by more than one probe sequence, the mean fold-change and *P*-value were calculated. To visualize the distribution of expression signals of DEGs among samples, hierarchical clustering using Pearson correlation distance was performed using Multiexperiment Viewer software (MeV) (Saeed et al. 2003).

Table 2 List of antibodies used in Western blot analyses and immunolocalization experiments.

Peptide/Protein target	Antigen sequence	Name of antibody	Manufacturer, catalog no., or name of source	Species raised in monoclonal or polyclonal	Dilution used
PTGFR	QRFQRKSKASFLLLASGLVITDFFGHLI NGAIAVFVYASDKIEWIRFDQSNVLCIS	PGF2 α R antibody (H-55)	Santa Cruz Biotechnology, sc-67029	Rabbit, polyclonal	1:50
MAPK1/3	N/A	p44/42 MAPK (Erk1/2) antibody	Cell Signaling, #91025	Rabbit, polyclonal	1:300
Phospho-MAPK1/3	N/A	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody	Cell Signaling, #9101	Rabbit, polyclonal	1:300
FAK	N/A	FAK antibody	Cell Signaling, #3285S	Rabbit, polyclonal	1:300
Phospho-FAK	N/A	Phospho-FAK (Tyr397) antibody	Cell Signaling, #3283	Rabbit, polyclonal	1:300
Cytokeratin	N/A	Anti-Pan Cytokeratin antibody	Sigma-Aldrich, C 9687	Mouse monoclonal	1:50
Anti-rabbit HRP	N/A	Immun-Star Goat Anti-Rabbit (GAR)-HRP Conjugate	Bio-Rad Laboratories, #170-5046	Goat, polyclonal	1:20,000
Anti-mouse	N/A	Cy3 AffiniPure Donkey Anti-Mouse IgG	Jackson ImmunoResearch, #715-165-150	Donkey, polyclonal	1:1000

Functional annotation of microarray data

Lists of DEGs generated by LIMMA analysis were used as input data for functional annotation. To study the effect of PGF2 α on the global gene expression profile in trophoblast cells, open-source and commercial software was applied. The Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang *et al.* 2009a,b) was used to calculate the fold enrichment of identified gene ontology (GO) terms using following databases: Functional Categories (UP_KEYWORDS); gene ontology Biological Process (GOTERM_BP_FAT), Cellular Component (GOTERM_CC_FAT), Molecular Function (GOTERM_MF_FAT); General Annotations (SP_COMMENT); Pathways (BIOCARTA; KEGG PATHWAY); Protein Domains (INTERPRO) and Protein Interactions (UCSC_TFBS). Results generated by DAVID were summarized in tabular format. Topp Cluster software (Kaimal *et al.* 2010) was used to identify gene ontologies enriched by DEGs and to generate a network showing the shared and list-specific functional features. Results were summarized in tabular format and visualized by interaction network. To identify signaling pathways, molecular networks and biological functions for DEGs Ingenuity Pathway Analysis (IPA; v. 01.12; Qiagen) were used. Results were summarized in tabular and in graphic formats.

Validation of microarray results

To verify results obtained in microarray analysis we validated the expression of *ADAM9*, *AGER*, *AMBP*, *BMP2*, *DECR1*, *IL1A*, *KNG1*, *LPAR1*, *MASP1*, *MFGE8*, *MMP2*, *PROX1*, *S100G*, *SLCO2A1*, *STC2*, *THBS1*, *TLR3* and *VTN* genes using real-time RT-PCR method. Additionally, based on our previous studies (Kaczyński *et al.* 2016) and literature data, we studied whether PGF2 α affected the expression of *BGN*, *IL6*, *IL6R*, *MMP9*, *TGFB3*, *TIMP1* and *TIMP2* genes. Based on microarray results, we also decided to evaluate the gene expression profile of *MMP9* in porcine conceptuses (days 10–17)/trophoblasts (days 18–25) throughout pregnancy development. cDNA was generated from RNA isolated from trophoblast cells as described in Experiment 1. Real-time RT-PCR was performed using TaqMan assays (Thermo Fisher Scientific; Table 1) according to the manufacturer's protocol. Results from qPCR were analyzed as described in Experiment 1. Stability of the reference genes in the porcine trophoblast cells was assessed using the statistical algorithm Normfinder 2.0 (Andersen *et al.*

2004). Three reference genes were analyzed namely *GAPDH*, *PPIA* and *ACTB*. The most stable combination of reference genes was the geometrical mean of *ACTB* and *GAPDH* expression values.

Experiment 3: The effect of PGF2 α on trophoblast cell adhesion and proliferation

Porcine primary trophoblast cells were isolated as described previously (Waclawik *et al.* 2013) with some modifications. Briefly, recovered conceptuses were washed three times with medium M199 supplemented with 5% (v/v) NCS and 0.1% (m/v) BSA. Subsequently, trophoblast tissue underwent mild digestion in 0.25% trypsin solution (Biomed, Lublin, Poland) for 30 min at 37°C. After digestion, solution was neutralized with M199 supplemented with 10% (v/v) NCS. Cells were then centrifuged at 150 $\times$ g for 10 min and washed three times with medium M199 supplemented with 5% (v/v) NCS. After washing, the cells were re-suspended in medium M199 with 1% (v/v) NCS. Viability and cell number was assessed with trypan blue dying.

For adhesion assay, cells (1 $\times$ 10⁶ cells/mL) were pre-incubated with medium M199 containing vehicle (0.01% ethanol) or PGF2 α (100 nM, 1 μ M) with/without 50 μ M of PTGFR antagonist (AL8810, Sigma-Aldrich) for 30 min at 37°C. Subsequently, 100 μ L of each cell suspension was transferred onto Millicore Cell Adhesion Strips coated with human fibronectin (ECM101; Millipore) and incubated for 3 h at 37°C. All treatments were performed in triplicates. After incubation, cell adhesion was measured using colorimetric method accordingly to the manufacturer's protocol.

Cells used in proliferation assay were re-suspended in culture medium (M199 supplemented with 5% NCS) in concentration 5 $\times$ 10⁴ cells/mL. After attachment, the cells were incubated for 48 h at 37°C in humidified atmosphere (95% air, 5% CO₂) with medium M199 containing vehicle (0.01% ethanol) or PGF2 α (100 nM, 1 μ M) or stable PGF2 α analog (fluprostenol; 100 nM, 1 μ M) with/without 50 μ M of PTGFR antagonist (AL8810). Based on our previous results (Kaczyński *et al.* 2016), 20% NCS was used as a positive control. 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 in triplicates. Fold difference was determined by dividing

the absorbance obtained by PGF2 α -treated cells (with/without inhibitors) by the absorbance obtained by vehicle-treated cells.

Experiment 4: Effect of PGF2 α on downstream targets potentially involved in porcine trophoblast cells adhesion and proliferation

To determine the protein expression of downstream targets of PGF2 α involved in porcine trophoblast cells adhesion and proliferation, we used previously described (Waclawik et al. 2013) *in vitro* model in which primary trophoblast cells were incubated for 24 h with either vehicle (0.01% ethanol) or PGF2 α (100 nM, 1 μ M) in the presence/absence of 50 μ M AL8810. After incubation, cells were lysed with RIPA buffer (PBS; pH 7.4; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate; 1 mM EDTA) containing Protease Inhibitor Cocktail (Sigma-Aldrich) and Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich). Content of MAPK1/3, pMAPK1/3, FAK and pFAK in cell homogenates was determined using Western blot and was normalized against GAPDH expression. Phosphorylation of MAPK and FAK protein was calculated by dividing the relative content of phosphorylated to non-phosphorylated protein.

Western blot

MAPK1/3 and FAK protein phosphorylation was evaluated using Western blot analysis as described previously (Waclawik et al. 2013). Briefly, equal amounts of protein samples (18 μ g) from conceptus cell lysates were separated on 10% SDS PAGE. Proteins were electroblotted onto 0.2 mm PVDF membrane. After blocking in 5% BSA, blots were incubated overnight at 4°C with primary antibodies (Table 2) and afterwards with 1:20,000 dilution of secondary anti-rabbit horseradish peroxidase antibodies (Bio-Rad Laboratories) for 90 min at room temperature. Immune complexes were visualized using Clarity Western ECL Substrate (Bio-Rad). Sample loading was standardized to expression of GAPDH. Signal was quantitated using Versa-Doc 4000M imaging system (Bio-Rad Laboratories).

Statistical analysis

Results from *in vitro* experiments (cell adhesion and proliferation assays), Western blot and qPCR studies were analyzed by one-way ANOVA followed by Tukey's test. All statistical analyses were conducted using GraphPad PRISM v. 6.01 software (GraphPad Software Inc.).

Results

Experiment 1: Expression of PTGFR gene and protein increases in the conceptus/trophoblast during the implantation period

Expression of PTGFR mRNA was elevated in conceptuses/trophoblasts from day 14 until day 19 of pregnancy (the implantation period; $P < 0.05$) compared with conceptuses/trophoblasts on days 10–12 (the preimplantation period). The highest expression of PTGFR mRNA was observed in conceptuses/trophoblasts from

days 16 to 19 of pregnancy (Fig. 1A) and was 48-fold greater than that in conceptuses/trophoblasts from days 10 to 12 of pregnancy ($P < 0.05$). PTGFR protein expression was elevated at the implantation period (days 14–15, 16–19 of pregnancy) when compared to later stage (days 20–25 of pregnancy; $P < 0.05$; Fig. 1B). Expression of PTGFR protein in day 10–12 conceptuses was not evaluated due to insufficient amount of material for both gene and protein analyses. Thus, in collected material only expression of PTGFR gene was possible to analyze.

Experiment 2: Effect of PGF2 α on global gene expression profile in porcine trophoblast cells

Characterization of trophoblast cells

The purity of the isolated cells was assessed by the evaluation of the presence of the trophoblast marker - cytokeratin and cell morphology. Isolated trophoblast cells presented uninuclear morphology (Supplementary Fig. 1A). Positive immunoreaction for cytokeratin confirmed the epithelial phenotype of trophoblast cells (Supplementary Fig. 1B). Antiserum-specific isotype control (negative control) used at the same dilution and protein concentration as the primary antibody is shown in Supplementary Fig. 1B (lower-left panel). Purity of cell cultures was assessed at 98%.

Global gene expression profiling by using expression microarrays

The porcine trophoblast cells' response toward the PGF2 α treatment was characterized using a porcine Agilent microarray assay. Differential gene expression between the two experimental groups ('PGF2 α -treated cells' vs 'vehicle-treated cells') was evaluated. The number of detected probes passing the filters was 27045. Pairwise distance analysis based on all detectable probes revealed grouping replicates, but differences between PGF2 α -treated and vehicle-treated samples are also indicated in the heat map (Supplementary Fig. 2). Using LIMMA package, 810 probes were found as differentially regulated (fold-change ≥ 1.2 ; $P < 0.05$; Supplementary Table 1). Annotation of probe sequences revealed 576 DEGs (216 downregulated, 360 upregulated) affected by PGF2 α . The five DEGs showing highest upregulation in the pairwise comparison 'PGF2 α -treated cells' vs 'vehicle-treated cells' were obtained for the following genes: *S100G* (3.9-fold), *NPEPPS* (2.6-fold), *PDE1B* (2.5-fold), *KNG1* (2.3-fold), *AMBP* (2.0-fold), whereas the five most downregulated genes (suppressed by PGF2 α) were *SELENBP1* (−1.8-fold), *DIO1* (−1.8-fold), *ENTPD8* (−1.8-fold), *LYZ* (−1.7-fold), *FMO1* (−1.6-fold). Hierarchical clustering of the obtained DEGs based on Pearson correlation revealed treatment grouping of analyzed samples with two main distinct clusters of genes with uniform distribution of expression signals (Fig. 2).

Figure 1 Expression of PTGFR mRNA (A) and protein (B) in the porcine conceptuses/trophoblasts at different stages of development. The representative samples of Western blots are shown in the upper panels. Data are represented as the mean $\pm$ S.E.M. Bars with different letter differ significantly ($P < 0.05$).

Functional annotation of microarray data

Functional terms enriched for upregulated and downregulated DEGs from both experimental groups were identified by DAVID analysis. For upregulated DEGs, DAVID identified 83 functional annotation clusters with enrichment scores from 1.04 up to 3.96 (Supplementary Table 2), whereas for downregulated DEGs, 62 functional annotation clusters were identified with enrichment scores from 1.06 up to 6.79 (Supplementary Table 3). The most interesting functional terms of overrepresented annotation clusters enriched for upregulated genes were related to focal adhesion, cell migration, hypoxia, response to fibroblast growth factor (FGF2) and transforming growth factor beta (TGFB) and regulation of cell proliferation and MAPK cascade (Table 3). Terms overrepresented by downregulated genes were mainly related to fatty acid biosynthetic process, cell migration, anion transport, response to interferon gamma and placenta development (Table 3). Complete results from functional annotation clustering were summarized in Supplementary Tables 2 and 3.

Using ToppCluster software, GO terms (biological process, cellular component and molecular function) and pathways (KEGG) enriched by DEGs were identified and summarized in tabular format (Supplementary Table 4). Similarly, as in DAVID analysis, overrepresented terms were related to processes potentially important for embryo implantation and development of early pregnancy such as adhesion, immune response, placenta development, morphogenesis, cytoskeleton organization and others. The most interesting enriched terms from PGF2 α -upregulated and PGF2 α -downregulated clusters were visualized as a network (Fig. 3).

Using IPA on 577 DEGs, the most interesting overrepresented canonical pathways were associated with regulation of lipid metabolism, inflammation and cholesterol catabolism (LXR/RXR; $P = 3.47E-06$), as well as with integrin ($P = 2.2E-04$) and mTOR ($P = 1.701E-02$) signaling (Supplementary Table 5). The main functional terms derived from IPA analysis showed similar results, compared to DAVID analysis, with highly involved functional terms such as embryonic development, inflammatory response, cellular growth and proliferation and lipid metabolism. (Supplementary Fig. 3 and Supplementary Table 6). Upstream analysis revealed that identified DEGs can be also regulated by factors other than PGF2 α factors (Supplementary Table 7). The most interesting factors potentially stimulating DEGs were estradiol-17 β , HOXA10 and TGFB1 and 2.

Effect of PGF2 α on the selected gene expression in porcine conceptuses

Validation of microarray results using qPCR confirmed expression of 14 out of 18 selected genes (*IL1A*, *MFGE8*, *MMP2*, *SLCO2A1*, *STC2*, *ADAM9*, *AGER*, *AMBP*, *BMP2*, *LPAR1*, *MASP1*, *S100G*, *THBS1* and *VTN*), whereas four of them (*KNG1*, *PROX1*, *DECR1* and *TLR3*) showed diverse expression in the qPCR to that detected by microarray analysis (Supplementary Table 8). Results from qPCR revealed that PGF2 α (100 nM) acting on trophoblast cells stimulated expression of *ADAM9*, *VTN*, *LPAR1*, *BMP2*, *DECR1* and *THBS1* genes ($P < 0.05$). Treatment of the trophoblast cells with 1 μ M of PGF2 α resulted in increased expression of *AGER*, *AMBP*, *BGN*, *IL6R*, *S100G*, *TLR3* and *MASP1* genes ($P < 0.05$) and in downregulation of *MFGE8*, *SLCO2A1*, *TIMP1*, *TIMP2*, *KNG1*, *MMP9*, *SCT2* and *TGFB3* gene expression ($P < 0.05$). Expression of *IL1A*, *MMP2* and *PROX1* genes was decreased in the trophoblast cells treated with both doses (100 nM; 1 μ M) of PGF2 α ($P < 0.05$). Results from qPCR analyses are shown in the Fig. 4. Additionally, we evaluated the expression profile of *MMP9* in conceptuses (days 10–17)/trophoblasts (days 18–25) at different stages of development. We found that the abundance of *MMP9* mRNA was lowest at the period corresponding to maternal recognition of pregnancy (days 10–12 of pregnancy) and at the beginning of implantation

Figure 2 Heatmap showing microarray analysis of DEGs in PGF2 α -treated porcine trophoblast cells (PGF2 α 1–4), and corresponding vehicle-treated controls (Control 1–4). Normalized expression data was clustered based on pairwise Pearson correlation using identified DEGs (red: correlation = 1; blue: correlation = -1). 576 genes were differentially expressed between the two groups. A total of 360 genes were up-regulated and 216 genes were downregulated ($P < 0.05$). The full list of DEGs is provided in Supplementary Table 1.

(days 14–15). Expression of *MMP9* was intermediate during implantation (days 16–19 of pregnancy) and markedly increased during placenta development (days 20–25 of pregnancy; $P < 0.05$; Fig. 4E).

Experiment 3: PGF2 α stimulates adhesion and proliferation of the porcine trophoblast cells

We demonstrated that PGF2 α (100 nM; 1 μ M) acting through its receptor increased adhesion of trophoblast cells to fibronectin ($P < 0.05$; Fig. 5A). Stimulating effect of PGF2 α (100 nM and 1 μ M) was blocked by using PTGFR antagonist (AL8810). AL8810 used alone did not have any effect on cell adhesion.

During early pregnancy in mammals, rapid transformation of conceptus morphology and conceptus cell differentiation should be accompanied by increased cell proliferation. In the present study, we evaluated the influence of PGF2 α on conceptus cell proliferation during

implantation period. We found that PGF2 α (100 nM) as well as its stable analog – fluprostenol (100 nM), acting through PTGFR-stimulated proliferation of porcine trophoblast cells ($P < 0.05$; Fig. 5B). The effects of PGF2 α (100 nM) and fluprostenol (100 nM) were abolished by using AL8810. AL8810 used alone had effect similar to the control.

Experiment 4: PGF2 α acting on trophoblast cells increases phosphorylation of factors potentially involved in adhesion and proliferation processes

Since we previously indicated the involvement of FAK and MAPK (Waclawik *et al.* 2013) in adhesion of trophoblast cells and as MAPK signaling is also involved in cell proliferation, we determined whether PGF2 α may stimulate phosphorylation of these proteins in the trophoblast cells. In the present study, we demonstrated for the first time that PGF2 α stimulated phosphorylation of MAPK1/3 (Fig. 5C) and FAK (Fig. 5D) proteins ($P < 0.05$) in porcine trophoblast cells and that these effects were mediated through PGF2 α receptor (Fig. 5C and D).

Discussion

In the present study, we demonstrated for the first time the para- and/or autocrine mechanisms of prostaglandin F2 α action on conceptus/trophoblast cells during the implantation period. We also described the expression profile of PGF2 α receptor (PTGFR) in porcine conceptuses at different stages of their development.

Results from our experiments discussed below provide a new perspective on the role of PGF2 α in embryo–maternal communication in the pig. We postulate that PGF2 α , which is highly abundant in the uterine lumen during early pregnancy, is not an undesirable factor but instead is an important hormone supporting processes related to proper embryo development and implantation. In the present study, we examined, for the first time, the expression profiles of PTGFR mRNA and protein in porcine conceptuses/trophoblasts during early pregnancy. Maximal expression of the PTGFR gene in conceptuses/trophoblasts occurred during the implantation period. Similarly, increased abundance of PTGFR protein was observed on days 14–19 of pregnancy. These results concur with our previous findings that PGF2 α synthase protein expression is increased in conceptuses/trophoblasts from days 14 to 25 compared to days 10–12 of pregnancy (Waclawik & Ziecik 2007). Strong signals of PTGFR protein expression are observed in the ovine trophectoderm on days 16, 18 and 20 of pregnancy (Dorniak *et al.* 2011). PGs secreted together with IFNT by ovine conceptuses regulate endometrial gene expression and functions that are important for conceptus growth and development (Dorniak *et al.* 2012). Our results are also consistent with transcriptomic data, in which expression of the PTGFR

Table 3 Selected results of DAVID functional annotation clustering for differentially expressed genes in trophoblast cells treated with PGF2 α .

Selected functional terms of overrepresented annotation clusters	Enrichment score
Upregulated	
Focal adhesion (30; 3.67); cell adhesion (50; 1.48); protein binding involved in cell adhesion (14; 2.29)	3.96
Cell migration (42; 1.79); regulation of cell motility (28; 1.91)	2.91
Response to hypoxia (17; 3.01)	2.84
Response to fibroblast growth factor (10; 3.71); fibroblast growth factor receptor signaling pathway (8; 4.01)	2.53
Response to growth factor (28; 2.23); response to TGFB (13; 3.05); transmembrane receptor protein serine/threonine kinase signaling pathway (14; 2.19)	2.43
Regulation of cell proliferation (19; 1.50)	2.32
Response to steroid hormone (16; 2.11)	2.26
Anatomical structure formation involved in morphogenesis (38; 1.67); angiogenesis (17; 2.07)	2.01
Regulation of MAP kinase activity (18; 2.78); protein phosphorylation (50; 1.36)	1.95
Regulation of epithelial cell proliferation (14; 2.35)	1.68
Organ morphogenesis (36; 1.83); epithelium development (32; 1.52)	1.52
ECM binding (5; 4.72)	1.35
Downregulated	
Fatty acid biosynthetic process (6; 3.38)	2.71
Regulation of intracellular signal transduction (34; 1.62)	2.04
Steroid biosynthetic process (7; 3.91)	1.89
Cell migration (28; 1.93)	1.85
Anion transport (14; 2.44)	1.79
Regulation of cell proliferation (31; 1.63)	1.78
ECM organization (11; 2.69)	1.74
Female pregnancy (8; 3.49)	1.65
Cellular response to interferon gamma (6; 3.63)	1.34
Placenta development (6; 3.38); reproductive structure development (11; 2.15)	1.35

gene in day 14 filamentous conceptuses was 14-fold greater than in day 10 (spherical) and 12 (filamentous) conceptuses (Ross *et al.* 2009). Interestingly, we indicated the upregulation of endometrial PTGFR protein during the implantation period (Kaczynski & Wacławik 2013). These results strongly support the hypothesis that PGF2 α secreted by the endometrium and conceptuses into the uterine lumen can act on trophoblasts in an auto- and/or paracrine manner.

In the present study, we evaluated the effect of PGF2 α on global gene expression profiles in porcine conceptuses at the onset of the implantation period. Using different bioinformatics software, we were able to demonstrate that PGF2 α potentially affects a broad range of processes and functions related mainly with focal adhesion, ECM binding, cytoskeleton organization, cell migration and proliferation, but also with immune processes, metabolism, response to hypoxia, growth factor signaling and ion homeostasis. Intriguingly, similar processes (e.g., protein kinase regulator activity, cell motility, transport) identified in conceptuses undergoing the transition from day 12 to day 14 filamentous forms (Ross *et al.* 2009) were also observed in trophoblast cells treated with PGF2 α . Moreover, it is interesting that analysis of potential upstream regulators of genes which were differentially regulated by PGF2 α revealed that these DEGs may also be regulated by other factors such as estradiol-17 β , HOXA10 or TGFB, which are important players in the embryo–maternal dialog during early pregnancy (reviewed in Wacławik *et al.* 2017).

Due to the relatively low fold-change threshold for detection of DEGs, it is recommended to validate these results for corresponding DEGs if more in-depth studies are projected based on our transcriptomic analyses results. In the present study, we validated part of the microarray results using qPCR and functional *in vitro* experiments. Validation of expression of particular genes of interest (*AGER*, *AMBP*, *MASP1*, *MFGE8*, *PROX1*,

Figure 3 Functional map showing shared and treatment-specific functional annotation terms generated in multi-cluster gene functional enrichment analysis for DEGs identified in porcine primary trophoblast cells treated with PGF2 α or vehicle (control). Analysis was performed in ToppCluster software, functional map was edited and adjusted using Cytoscape. The redundant and non-informative terms were removed. Complete results are presented in tabular format in Supplementary Table 4.

Figure 4 Expression of selected target genes determined by quantitative (real-time) RT-PCR in porcine primary trophoblast cells treated with PGF2α (100 nM, 1 μM) or vehicle (control). (A, B, C, D, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V, W, X and Y). ADAM9, ADAM metalloproteinase domain 9; AGER, advanced glycosylation end-product specific receptor; AMBP, alpha-1-microglobulin/bikunin precursor; BGN, biglycan; BMP2, bone morphogenetic protein 2; DECR1, 2,4-dienoyl-CoA reductase 1; IL1A, interleukin 1 alpha; IL6R, interleukin 6 receptor; LPAR1, lysophosphatidic acid receptor 1; MASP1, mannan binding lectin serine peptidase 1; MFGE8, milk fat globule-EGF factor 8 protein; MMP2, matrix metalloproteinase 2; MMP9, matrix metalloproteinase 9; PROX1, prospero homeobox 1; S100G, S100 calcium binding protein G; SLCO2A1, solute carrier organic anion transporter family member 2A1; STC2, stanniocalcin 2; TGFB3, transforming growth factor beta 3; THBS1, thrombospondin 1; TIMP1, metalloproteinase inhibitor 1; TIMP2, metalloproteinase inhibitor 2; TLR3, toll-like receptor 3; VTN, vitronectin. Expression of MMP9 mRNA in the porcine conceptuses/trophoblasts at different stages of development (E). Data on panels A, B, C, D, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V, W, X and Y are presented as means ± S.E.M. of fold-change vs control (vehicle). Data on panel E is presented as mean ± S.E.M. Bars with different letter differ significantly ($P < 0.05$).

SLCO2A1, VTN, ADAM9, LPAR1, BMP2, DECR1, THBS, S100G, STC2, IL1A and TLR3) confirmed overall the results from the microarray analyses. Additionally, we analyzed the PGF2α-mediated expression of genes that are potentially important in embryo-maternal interactions during the implantation period (BGN,

MMP2, MMP9, TIMP1, TIMP2, KNG1, TGFB3, IL6, IL6R and TGFB3). Results of the microarray and qPCR analyses revealed that the PGF2α affected the expression of genes related to processes crucial for successful implantation, such as ECM organization, ECM protein binding, focal adhesion, inflammatory response, cell

Figure 5 PGF2 α increased adhesion to ECM protein – fibronectin (A) and proliferation (B) of primary porcine trophoblast cells. Effect of PGF2 α on MAPK1/3 (C) and FAK (D) phosphorylation in porcine primary trophoblast cells *in vitro*. NCS-newborn calf serum (used as a positive control), AL8810, PTGFR antagonist; FLP, fluprostenol (stable prostaglandin F2 α analog). Data are presented as means $\pm$ S.E.M. of fold-change vs control (vehicle). Different letters (a, b and c) indicate statistically significant differences ($P < 0.05$).

proliferation, migration and motility or activation of intracellular protein kinases. Here, we briefly discuss the importance of validated genes in the most important processes accompanying the implantation process.

In all mammalian species, during the peri-implantation period, formation of the placenta is preceded by adhesion of trophoblast cells to luminal epithelium of the endometrium (Geisert *et al.* 2015). This requires a profound rearrangement of endometrial tissue that is essential for successful implantation of embryos. Reorganization of tissue structures is related to processes such as ECM remodeling and vasculature development and is tightly controlled by a number of factors. Metalloproteinases are factors regulating ECM remodeling. MMPs together with their associated inhibitors (TIMPs) act concomitantly regulating aspects of reproductive function (reviewed in Curry & Osteen 2003). MMP2 and MMP9 were reported to mediate human trophoblast cell invasion into matrigel *in vitro* (Bischof *et al.* 1995). Recently, we reported PGF2 α -stimulated expression of the MMP9 gene in porcine endometrium, suggesting its involvement in ECM remodeling (Kaczyński *et al.* 2016). In this study, we observed a decrease in *MMP2* and *MMP9* mRNA content in porcine trophoblast cells in response to PGF2 α treatment. Simultaneously, we noticed that PGF2 α upregulated expression of the *TIMP2* gene, which is an inhibitor of MMP2 and MMP9. These results correspond to the *MMP9* gene expression profile in porcine conceptuses at different stages of development that was evaluated in the present study. Intriguingly, we found that PGF2 α acting on human trophoblast cells has an opposite effect, i.e. it increases *MMP9* gene expression that is probably related to invasive type of implantation of human trophoblast (Baryla *et al.* 2017). We suggest that the downregulation of *MMP2* and 9 and upregulation of *TIMP2* mRNA expression in porcine conceptuses could be an effect of action of endometrium, which by secreting PGF2 α may protect the endometrial tissue against excessive degradation of its ECM, and this may be related to the non-invasive type of implantation specific for the pig. Moreover, we demonstrated that PGF2 α differentially regulated other genes (*BGN*, *THBS*, *VTN*, *MFG8*, *ADAM9*, *KNG1*, *AMBP* and *PROX1*) that were reported to be involved in ECM remodeling, cell adhesion and proliferation in different mammalian species (Couchman *et al.* 1990, Bowen *et al.* 1996, Taylor *et al.* 1997, Hettinger *et al.* 2001, Bloor *et al.* 2002, Vonnahme *et al.* 2004, Kim *et al.* 2006, 2013, Kaczyński *et al.* 2016, Barua *et al.* 2017, Kolakowska *et al.* 2017). Results from functional *in vitro* experiments performed herein confirmed results from gene expression studies and support the conclusion that PGF2 α acting through its receptor in trophoblast cells stimulates adhesion of trophoblast cells to ECM proteins. Interestingly, we recently demonstrated that the role of PGE2 in increasing trophoblast adhesion is not

species-specific because the mechanism is PTGER2 and integrin dependent in both porcine and human trophoblast cells (Waclawik *et al.* 2013). Moreover, iloprost (prostacyclin analog) acting through prostacyclin membrane receptors (PTGIR) stimulated porcine day 14 trophoblast cells to adhere to fibronectin (Morawska-Pucinska *et al.* 2014). Thus, the present report emphasizes the role of prostaglandins in proper control of the adhesion cascade during the implantation period in the pig.

During the peri-implantation period, porcine conceptuses undergo rapid transformation. On day 12 of pregnancy, trophoblasts elongate to more than 100 mm in length (Anderson 1978, Geisert *et al.* 1982). It has been stated that the conceptus elongation process is more dependent on cell outgrowth than cell division (Perry 1981). However, elongation is not the only process occurring during conceptus development. Cellular differentiation is also very important for embryonic development in all mammalian species. This process requires tight control over cell proliferation, migration and cell–cell interaction. The potential role of TGF β , LPA1, PROX1, AMBP and BMP2 in pregnancy establishment was reported in a number of mammals (McKeehan *et al.* 1986, Lee *et al.* 2007, Blitek *et al.* 2013, Kim *et al.* 2013, Jeong *et al.* 2016). Interestingly, IL6 which is mostly related to immunological processes has been reported to stimulate trophoblast cell proliferation and promote their adhesion to fibronectin (Blitek *et al.* 2012). In the present study, we found that PGF2 α did not affect IL6 mRNA; however, it increased IL6 receptor (IL6R) mRNA content in porcine day 15 trophoblast cells. The present study is the first demonstrating the role of PGF2 α in promoting trophoblast cell proliferation in any mammalian species. We recently reported an indirect influence of PGF2 α on uterine endothelial cell proliferation (Kaczyński *et al.* 2016). Moreover, fluprostenol (PGF2 α analog) was reported to stimulate the expression of proteins related to proliferation of bovine endometrial cells (Zhang *et al.* 2017).

During the implantation period in some mammals, including pigs both pro- and anti-inflammatory processes are activated at the embryo–maternal interface and the proper balance between these two kinds of immunological responses is crucial for successful development of pregnancy (reviewed in Waclawik 2011). In the present study, we found that PGF2 α differentially regulated the expression of *KNG1*, *MFGE8*, *AMBP*, *IL1*, *IL6R*, *MASP1*, *AGER* and *TLR3* genes that are involved in modulation of immunological processes in many species (Tuo *et al.* 1996, Vonnahme *et al.* 1999, Allen *et al.* 2002, Wang *et al.* 2007, Zhang *et al.* 2007, Berbaum *et al.* 2008, Prins *et al.* 2012, Tan *et al.* 2015). Thus, we infer that PGF2 α by differentially regulating genes in trophoblast cells may contribute to the control of immune interactions between developing conceptuses and the endometrium.

The importance and function of calcium during early embryo development and implantation have been well studied (reviewed in Whitaker 2006). Binding of endometrial epithelial cells to trophoblast cells results in calcium influx in endometrial epithelial cells in humans (Tinel *et al.* 2000). In the porcine uterine lumen, concentration of calcium ions increases during the peri-implantation period (Geisert *et al.* 1982). Bikunin, one of the products of the *AMBP* gene, has been reported to inhibit the release of intracellular calcium ions induced by lipopolysaccharide (Kanayama *et al.* 1995). Stanniocalcin (STC), a glycoprotein regulating calcium homeostasis and has been suggested to be a luminal epithelial marker for implantation in pigs (Song *et al.* 2009). Another factor related to calcium ion homeostasis in endometrium during pregnancy is *S100G* protein also known as calbindin-d9k (Choi *et al.* 2009). Our data indicate that PGF2 α by regulating *AMBP*, *STC2* and *S100G* gene expression in porcine conceptuses may be an upstream regulator of factors involved in calcium homeostasis. Our data suggest that in porcine conceptuses PGF2 α regulates *MFGE8* and *DECRL1* which are involved in fatty acid metabolism, fatty acid resorption and placental transport (Kunau & Dommes 1978, Marques *et al.* 2009, Khalifeh-Soltani *et al.* 2014).

Prostaglandin signaling is crucial for proper conceptus development (Kennedy *et al.* 2007, Dorniak *et al.* 2011). *SLCO2A1* was suggested to play a critical role in supporting the establishment and maintenance of pregnancy by regulating PG transport at the maternal–fetal interface in pigs. Expression of *SLCO2A1* mRNA in day 15 conceptuses is decreased compared to day 12 conceptuses (Seo *et al.* 2014). Accordingly, we found down-regulation of *SLCO2A1* gene expression in porcine trophoblast cells in response to PGF2 α treatment. This may suggest that PGF2 α by regulating its own transporter expression in trophoblast cells may autoregulate its secretion by conceptuses during implantation period.

Processes accompanying the implantation process such as cell adhesion and proliferation are triggered by activation of particular intracellular signaling pathways. Results from gene expression studies indicated that PGF2 α -enriched terms related to activation of protein kinases, such as MAP kinase activity and protein phosphorylation. In the present study, we demonstrated that PGF2 α stimulated MAPK1/3 and FAK protein phosphorylation in trophoblast cells. Interestingly, PGF2 α has been reported to stimulate tyrosine phosphorylation of p125 FAK in NIH-3T3 cells (Watanabe *et al.* 1994) and in 293-EBNA cells stably expressing isoform A of the PTGFR prostanoid receptor (Pierce *et al.* 1999). PTGFR is a G-protein-coupled receptor activating Gq11 protein (Bos *et al.* 2004), and it has been demonstrated that genes upregulated at the end of gestation in a G α q/11-dependent manner in ovarian (granulosa/luteal) cells included genes involved in focal adhesion and ECM interactions (Waite *et al.* 2016). Moreover,

in human adenocarcinoma cells (Ishikawa cell line), PGF2 α was able to induce MAPK1/3 phosphorylation (Sales *et al.* 2005). We also found that PGE2 induced phosphorylation of MAPK1/3 and FAK. Taking all the above facts together, we speculate that PGF2 α acting through its receptor in trophoblast cells may activate Gq11 protein that leads to increased MAPK1/3 and FAK phosphorylation. This may result in enhanced adhesion of porcine trophoblast cells to ECM proteins.

In summary, the present study indicates a new role for PGF2 α secreted both by the conceptuses and endometrium during the implantation period in pigs. Results from global gene expression profiling indicate that PGF2 α acting in an auto- and/or paracrine manner is involved in processes important for conceptus development and attachment to the uterine epithelium (cell proliferation and focal adhesion, ECM binding, cell migration, cytoskeleton organization, immune interactions, ion homeostasis, metabolism, activation of TGF β and FGF signaling pathways, response to hypoxia). Functional *in vitro* experiments confirmed insights from transcriptomic studies, allowing us to conclude that PGF2 α by inducing transcriptomic changes in porcine trophoblast cells affects a broad range of processes potentially involved in implantation and development of the placenta, particularly activation of intracellular protein kinases (MAPK, FAK) and stimulation of trophoblast cell adhesion and proliferation.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0225>.

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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