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Prostaglandin $F_{2\alpha}$ stimulates adhesion, migration, invasion and proliferation of the human trophoblast cell line HTR-8/SVneo

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ABSTRACT

Introduction: The amount of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the uterine lumen increases during the window of implantation in many mammals, including humans. We hypothesized that $PGF_{2\alpha}$ regulates processes related to human embryo implantation.

Methods: The effect of $PGF_{2\alpha}$ was studied using an *in vitro* model of human extravillous trophoblast (EVT) cell line (HTR-8/SVneo). Adhesion, proliferation, invasion and migration assays, zymography for metalloproteinases (MMP) activity, and gene/protein expression analyses were applied. Doses of 100 nM and/or 1 μM of $PGF_{2\alpha}$ and fluprostenol were used. $PGF_{2\alpha}$ receptor (PTGFR), MMP9 and MMP2 proteins in the human first trimester placenta were localized by immunohistochemistry and immunofluorescence.

Results: This study is the first reporting the expression of PTGFR protein in the first trimester placenta, as well as in HTR-8/SVneo cells. $PGF_{2\alpha}$ and fluprostenol increased HTR-8/SVneo cell proliferation and adhesion to extracellular matrix protein ($P < 0.05$). This effect was abolished by mitogen activated protein kinases (MAPK) inhibitor. $PGF_{2\alpha}$ induced phosphorylation of focal adhesion kinase and MAPK1/3 ($P < 0.05$). $PGF_{2\alpha}$ increased mRNA content and protein activity of MMP9, and gene and protein expression of interleukin-6 ($P < 0.05$). EVT cell migration and invasiveness were stimulated by $PGF_{2\alpha}$ ($P < 0.05$). The $PGF_{2\alpha}$ effect on cell invasion was reduced by inhibitors of MMP2, MMP9 and mTOR. In all experiments, the stimulatory effects of $PGF_{2\alpha}$ were diminished by using a PTGFR antagonist.

Discussion: Our findings suggest a significant role for $PGF_{2\alpha}$ in mechanisms associated with implantation. $PGF_{2\alpha}$ acting by PTGFR in HTR-8/SVneo cells stimulates their adhesion and proliferation through the MAPK signaling pathway and increases invasiveness inducing MMP proteolytic activity and mTOR signaling.

1. Introduction

Tightly regulated crosstalk between uterine environment and embryo signals is essential for successful implantation. A large number of molecules involved in embryo-maternal communication makes this process complicated and frequently vulnerable to disorders. Increasing evidence indicates prostaglandins (PGs) as key factors during implantation in mammalian species [1–7]. Implantation failure has been linked to impaired PGs synthesis in humans [5]. On the other hand, exogenous administration of $PGF_{2\alpha}$ can be used as a method to induce abortion, because of its luteolytic properties and ability to cause contractions in the myometrium [8–10]. Both observations imply that concentrations of $PGF_{2\alpha}$ in the uterine lumen at the early stage of pregnancy would be rather low. However, during the secretory phase of the menstrual cycle, about 8–9 days after ovulation, a time corresponding to the window of implantation during pregnancy, $PGF_{2\alpha}$ is secreted into the uterine lumen and expression of $PGF_{2\alpha}$ synthases is upregulated in the endometrium [11,12]. The increased amount of $PGF_{2\alpha}$ in uterine fluid and/or endometrial $PGF_{2\alpha}$ synthesis when the endometrium becomes receptive to embryos was also observed in other mammals: pigs [13,14], dogs [15], rats [1], cattle [16] and sheep [2]. Using a porcine model, we observed that $PGF_{2\alpha}$, by acting as a mediator of the embryonic signal, regulates expression of genes involved in tissue remodeling and conceptus-maternal interaction, as well as stimulating angiogenesis in endometrium [17]. Our recent studies revealed that $PGF_{2\alpha}$ is involved in trophoblast proliferation and adhesion to the endometrium during the implantation process in pigs [18].

In humans, implantation consists of three stages: blastocyst

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Table 1
List of antibodies used in Western Blot analyses.

Peptide/Protein Target	Antigen Sequence	Name of antibody	Manufacturer, Catalog No. or Name of Source	Species Raised in, Monoclonal or Polyclonal	Dilution used
PTGFR (WB)	QRFRQKSKASFLLLASGLVITDFFGHLINGAIAVFVYASDKEWIRFDQSNVLCSI	$PGF_{2\alpha}$ Receptor antibody (H-55)	Santa Cruz Biotechnology, sc-67029	Rabbit, polyclonal	1:100
PTGFR (IHC)	SMNSSKQPVSPAAGL	$PGF_{2\alpha}$ Receptor Antibody	Cayman Chemical, 101802	Rabbit, polyclonal	1:1000
HLAG	against amino acids 61–83 of HLA-G of human origin	HLA-G (4H84)	Santa Cruz Biotechnology, sc-21799	Mouse, monoclonal	1:250
MMP9	N/A	MMP-9 (2C3)	Santa Cruz Biotechnology, sc-21733	Mouse, monoclonal	1:200
MMP2	N/A	MMP-2 (4D3)	Santa Cruz Biotechnology, sc-53630	Mouse, monoclonal	1:200
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
ICAM-1	NA	CD54 (ICAM-1) antibody	Cell Signaling, #4915S	Rabbit polyclonal	1:300
FAK	NA	FAK antibody	Cell Signaling, #3285S;	Rabbit, polyclonal	1:300
Phospho-FAK	NA	Phospho-FAK (Tyr397) antibody	Cell Signaling, #3283	Rabbit, polyclonal	1:300
IL6	against full length IL6	IL6 (10E5)	Santa Cruz Biotechnology, sc-57315	Mouse, monoclonal	1:300
GAPDH	NA	Anti-GAPDH antibody	Abcam, ab9485	Rabbit, plyclonal	1:2000
anti-rabbit HRP (WB)	NA	Immun-Star™ Goat Anti-Rabbit (GAR)-HRP Conjugate	Bio-Rad Laboratories, #170-5046	Goat, polyclonal	1:20000
anti-mouse HRP (WB)	NA	Immun-Star™ Goat Anti-Mouse (GAM)-HRP Conjugate	Bio-Rad Laboratories, #170-5047	Goat, polyclonal	1:20000
anti-rabbit (IF)	NA	Cy™3 AffiniPure Donkey Anti-Rabbit IgG (H + L)	Jackson Immuno Research, code: 711-165-152	Donkey, polyclonal	1:3000
anti-mouse (IF)	NA	Donkey anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Invitrogen, #A-21202	Donkey, polyclonal	1:800
anti-rabbit (IHC)	N/A	VectaStain Biotinilated Secondary Antibody	Vector Laboratories (PK-6100)	Goat, polyclonal	1:200
anti-mouse (IHC)	N/A	VectaStain Biotinilated Secondary Antibody	Vector Laboratories (PK-6100)	Horse, polyclonal	1:200

apposition, adhesion to the endometrial epithelium, and trophoblast cell invasion. Invasive multinuclear syncytiotrophoblast cells form anchoring chorionic villi in the first week of fetal development. Subsequently, the villous cytotrophoblast inner cell layer initiates expansion through syncytia into maternal tissue by degradation of the endometrial epithelial extracellular matrix. The trophoblast forms columns and migrates into different parts of the decidua. The site of cell localization determines the subtypes of extravillous trophoblast (EVT), such as interstitial, endovascular, intramural and 'epithelial'. The endovascular phenotype of EVT migrates up the lumina of spiral arterioles to create hemochorial connections with the maternal blood circulation [19]. Impaired properties of EVTs are the reason for insufficient perfusion of the placenta, which carries the risk of pregnancy complications [20].

It is important to investigate molecular mechanisms involved in proper trophoblast implantation. Studies in humans are, however, hindered due to the scarcity of primary cells/tissues and appropriate models. Cell lines are a widely used source of experimental data concerning the control of human trophoblast function. There are three popular cell lines of choriocarcinoma trophoblast origin: BeWo, JAR and JEG-3 [21–23]. Another frequently used cell line is HTR-8/SVneo, which unlike the others, was generated from first trimester placenta by transfection with a plasmid containing the simian virus 40 large T antigen (SV40) [24]. Despite the latest reports indicating heterogeneity in the population of this cell line [25], HTR-8/SVneo for years has been regarded as an appropriate model of human trophoblast for functional studies on adhesion, migration and invasion [26]. Although reports indicate increased synthesis and/or secretion of $PGF_{2\alpha}$ in the uterine environment and its possible role during the peri-implantation period in livestock animals [16,18], the role of $PGF_{2\alpha}$ in pregnancy establishment in humans is still unknown. The critical role of PGs, especially PGE_2, during pregnancy [1], and their effects during implantation processes (e.g., proliferation, migration, adhesion) have been identified and partially explained with trophoblast cell lines [3,4,12]. However, it is still necessary to determine the contribution of $PGF_{2\alpha}$ to trophoblast function and embryo-maternal communication during the peri-implantation period in humans.

Based on the role of EVT during implantation and our previous results obtained with porcine trophoblast primary cells [18], we hypothesized that $PGF_{2\alpha}$ regulates adhesion, proliferation, invasiveness and migration of human trophoblast. Therefore, the aims of the present study were to determine whether $PGF_{2\alpha}$ acting through its receptor (PTGFR) on HTR-8/SVneo cells may be involved in implantation-associated processes, and which intracellular pathways are then activated. Furthermore, we hypothesized that $PGF_{2\alpha}$ regulates expression of genes in tissue remodeling, trophoblast invasiveness and embryo-maternal communication in human trophoblast cells, such as *BGN, IL6, IL1A, TGFB* and *MMP9* [27–37].

2. Materials and methods

2.1. In vitro culture of HTR-8/SVneo cells

Experiments were performed using the HTR-8/SVneo cell line [24]. The cell line was authenticated using Short Tandem Repeat analysis as described in 2012 in ANSI Standard (ASN-0002) Authentication of Human Cell Lines and in Ref. [38] (www.lgcstandards-atcc.org).

2.2. Immunolocalization of PTGFR, MMP2 and MMP9 in first trimester human trophoblast tissue

Human placental tissue samples obtained after surgical termination of pregnancy in the first trimester of pregnancy were kindly gifted by Dr Simon C Riley (MRC Centre for Reproductive Health, University of Edinburgh, UK). These were archived tissues, which were collected with approval of the local ethics committee and with informed consent, as detailed previously [39]. Immunolocalization of PTGFR, human leukocyte antigen G (HLA-G; a marker for EVT cells), matrix metalloproteinases (MMP) MMP2 and MMP9 proteins in human first trimester placenta was performed as described previously [17]. Additionally, colocalization of PTGFR and HLA-G proteins was performed using anti-rabbit antibodies conjugated to Cy3 (for PTGFR detection) and secondary anti-mouse antibodies conjugated to Alexa Fluor 488 (for HLA-G detection). The primary and secondary antibodies used in experiments are listed in Table 1. Antiserum-specific isotype controls (normal rabbit control IgG, normal mouse control IgG) at the same dilution and protein concentration as the primary antibody were used as negative controls. Slides were observed under a light microscope (Olympus BX43) and photographed using CellSens Dimension 1.4.1 software. PTGFR and HLA-G colocalization signals were detected and documented using Axio Imager Z1 microscope with AxioVision software (Zeiss, Germany).

2.3. Adhesion assay

The effect of $PGF_{2\alpha}$ on trophoblast cell adhesion was examined as previously described, with some modifications [4]. Briefly, HTR-8/SVneo cells suspended in medium RPMI 1640 containing 1% FBS were incubated for 45 min in the presence or absence of inhibitors: 50 μM PTGFR antagonist (AL8810; A3846, MERCK) or 50 μM inhibitor of MAPK kinase - MEK1 (PD98059; P215, MERCK). Cells were treated with either of two doses (100 nM; 1 μM) of $PGF_{2\alpha}$ or a stable $PGF_{2\alpha}$ analogue - fluprostenol (FLP) (F8549, MERCK) and incubated for another 15 min. The cells (n = 16 passages) were plated in triplicates (10^5 cells/well) on Millicoat™ Human Fibronectin Coated Strips (ECM101, MERCK, MO, USA) and incubated for 3 h.

2.4. Proliferation assay

To determine the influence of $PGF_{2\alpha}$ on trophoblast cell proliferation, HTR-8/SVneo cells (3×10^4 cells/well) were seeded into 96-well plates in RPMI 1640 medium supplemented with 5% FBS and incubated for 18 h at 37 °C in a humidified atmosphere containing 5% CO_2. After reaching 40% confluence, the cells were treated for 24 h with RPMI 1640 medium containing 1% FBS and 100 nM or 1 μM of $PGF_{2\alpha}$/fluprostenol or vehicle (as a control) with or without inhibitors: 50 μM AL8810 or 50 μM PD98059 (MERCK). Effects of $PGF_{2\alpha}$ on HTR-8/SVneo cell proliferation were determined by using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, WI, USA) according to the manufacturer's protocol and were measured using a Multiscan EX spectrophotometer (Labsystem) at λ = 490 nm. The results were expressed as n-fold change relative to the control. Experiments were repeated eleven times in triplicates.

2.5. Gene expression analyses

Total RNA was isolated from cell lysates and reverse-transcribed as described previously [17]. The Real-time RT-PCR reaction mixture included 6 μl TaqMan MasterMix, 0.5 μl TaqMan Assay (Table 2), and

Table 2
Assays used in qPCR analyses.

Gene	TaqMan Assay ID
ACTB	Hs01060665_g1
GAPDH	Hs02758991_g1
PPIA	Hs04194521_s1
MMP9	Hs00234579_m1
IL6	Hs00985639_m1
TGFB3	Hs01086000_m1
BGN	Hs00959141_g1
IL1A	Hs00174092_m1

3.5 μl of generated cDNA (32 ng). The PCR program was performed as follows: 10 min of initial denaturation (95 °C), 40 cycles of denaturation (15 s, 95 °C) and annealing (1 min, 60 °C). All qPCR reactions were performed using the Applied Biosystems 7900 Real-Time PCR system (Thermo Scientific). Data obtained from qPCR analyses were normalized against the geometric average of *ACTB* and *GAPDH*. The most stable housekeeping gene combination was chosen based on the NormFinder algorithm [40].

2.6. Incubation of HTR- 8/SVneo cells with $PGF_{2\alpha}$

To study the effects of $PGF_{2\alpha}$ on the expression of intracellular adhesion molecule 1 protein (ICAM-1), MAPK1/3 and FAK phosphorylation, on expression of *MMP9*, *IL6*, *BGN*, *TGFB3* and *IL1A* genes in human trophoblast cells, and on matrix metalloproteinases activity, cells were cultured in 6-well plates in RPMI 1640 medium (R6504, MERCK) supplemented with 5% FBS and antibiotics for 48 h. Cells at 60–70% confluence were treated with 100 nM or 1 μM of $PGF_{2\alpha}$ in the presence or absence of 50 μM PTGFR antagonist (AL8810). For the gene and ICAM-1 protein expression analyses, the cells were incubated with treatments for 24 h (n = 7), whereas for MAPK1/3 and FAK phosphorylation studies for only 15 min (n = 7). Conditioned media from 24 h experiments were collected and cells used for the gene expression studies were lysed in Fenozol buffer (A&A Biotechnology, Poland). The cells used in protein studies (ICAM-1 and PTGFR expression, MAPK1/3 and FAK phosphorylation) were lysed in RIPA buffer (50 mM Tris-HCl (pH = 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM EDTA (pH = 8.0)) supplemented with a protease inhibitor cocktail (P8340, MERK), or for the cells used in the phosphorylation studies, with a protease inhibitor cocktail and a phosphatase inhibitor cocktail (04906845001, Roche Applied Science, Germany). The cell lysates were centrifuged at 800 × *g* for 10 min at 4 °C. The supernatant was collected and stored at −35 °C. Total protein concentration was measured [41]. Gene expression was analyzed using real-time RT-PCR. PTGFR and ICAM-1 protein expression and MAPK1/3 and FAK phosphorylation were analyzed using Western blot.

2.7. Protein expression analyses

Western blot analyses were performed as described previously [5,7]. The primary antibodies used in these analyses are presented in Table 1. Immune complexes were visualized using Clarity™ Western ECL Substrate (170–5060, Bio-Rad, Poland) and documented in the ChemiDoc™ MP Imaging System (Bio-Rad). Protein expression was normalized against GAPDH protein expression (Table 1).

2.8. Zymography of gelatinolytic activity of MMP2 and MMP9 proteins

Conditioned media after incubation of HTR-8/SVneo cells with $PGF_{2\alpha}$ were subjected to SDS-PAGE electrophoresis (125 V, 90 min) using gelatin (0.2%) polyacrylamide (10%) gels in the presence of SDS under nonreducing conditions. Gelatin was used as a substrate to detect protease activities of MMP2 and MMP9 secreted by trophoblast cells into the media as described previously [42]. Bands were measured with densitometric analysis software (ImageLab, Bio-Rad). MMP2 and MMP9 bands were identified using standards.

2.9. Invasion assay

The effect of $PGF_{2\alpha}$ on human trophoblast cell invasive properties was performed using the QCM™ 96-Well Cell Invasion Assay kit (ECM555, MERCK) according to the manufacturer's protocol. Briefly, cells were re-suspended (6×10^5 cells/mL) in serum-free RPMI medium and pretreated with the following inhibitors: AL8810 (50 μM), rapamycin (110 nM), MMP9 inhibitor-1 (20 nM; WBC018, R&D System, Canada), MMP2 inhibitor-1 (10 μM; 924-MP, R&D Systems) or vehicle (0.01% ethanol). After pretreatment, cells were treated with $PGF_{2\alpha}$ (1 μM) in the presence or absence of inhibitors or with vehicle (0.01% ethanol). Subsequently, cells were seeded in the upper inserts (6×10^4 cells/well) containing an 8 μm pore size polycarbonate membrane coated with a thin layer of ECMatrix™. RPMI medium containing 10% FBS was added into the bottom chamber. Cells were incubated for 24 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The range of HTR-8/SVneo cell invasion in response to treatments was measured by reading the fluorescence of labeled cells that invaded through the insert using a multi-mode microplate reader with a 480/520 nm filter set (Synergy H1 Hybrid Multi-Mode Reader, BioTek). Experiments were repeated for 8 cell passages in triplicates. Fold difference was determined by dividing the fluorescence obtained from treated cells by the fluorescence obtained from vehicle-treated cells.

2.10. Migration assay

The effect of $PGF_{2\alpha}$ on HTR-8/SVneo cell migration was investigated as follows: cells (6×10^5) were plated in 24-well plates in 750 μL of medium RPMI (5% FBS) and incubated at 37 °C in a humidified atmosphere of air and 5% CO_2 until 60% confluence. Cells were then incubated with the following treatments: vehicle (0.01% ethanol), $PGF_{2\alpha}$ (1 μM), $PGF_{2\alpha}$ (1 μM) + AL8810 (50 μM), and AL8810 (50 μM). After 18 h, cells were scraped off with a sterile pipette tip, rinsed with PBS and incubated again with treatments as mentioned above. Preselected fields were photographed using the Zeiss Axio Observer System with software ZEN Blue 2.3 pro (Zeiss, Germany). The area of the gap crossed by the cells was measured using Fiji software with the MRI Wound Healing Tool [43]. The results were expressed as n-fold change relative to the control.

2.11. Statistical analysis

Two-way ANOVA was used to evaluate the statistical significance of the effect of $PGF_{2\alpha}$ treatment and time of treatment on HTR-8/SVneo cell migration. Other results were analyzed by one-way ANOVA followed by Tukey's test. All numerical data are presented as the mean ± SEM. Differences were considered statistically significant for P-values lower than 0.05 ($P < 0.05$). In graphs, means (bars) with different letters indicate significant differences ($p < 0.05$). If the means/bars share common letters, they do not differ from each other (e.g. if the bar has the indication 'ab' then it does not differ from bar with 'a' and does not differ from bar with 'b'). Analyses were conducted using GraphPad Prism v7.03 (GraphPad Software, Inc., CA, USA).

3. Results

3.1. PTGFR protein is expressed in first trimester extravillous trophoblast and the HTR-8/SVneo cell line

The presence of PTGFR protein was detected in human first trimester placenta. Positive signals of PTGFR immunoreactivity were localized in cytotrophoblast, syncytiotrophoblast, and potentially in extravillous trophoblast, as well as the cells residing in the villous core (Fig. 1A and B). Expression of PTGFR protein was confirmed in EVT cells within the EVT column (Fig. 1C–H) by colocalization with human leukocyte antigen G (HLA-G) which is a specific marker for EVT cells signals. The isotype (negative) control - rabbit IgG (for PTGFR, shown in the panel A of Fig. 1) and mouse IgG (for HLA-G; lower-left panel in Fig. 5E) confirmed the specificity of antibodies used in the experiments. Expression of PTGFR protein was identified in the EVT cell line HTR-8/SVneo (Fig. 1I). As a positive control, the Ishikawa cell line was used. PTGFR protein of about 41 kDa mass weight was detected in both types of cells (Fig. 1I). Human STR Profiling Cell Authentication Service report (SO0265269) confirmed that HTR-8/SVneo cell line used in our experiments matched to the ATCC CRL-3271 (HTR-8/SVneo) human

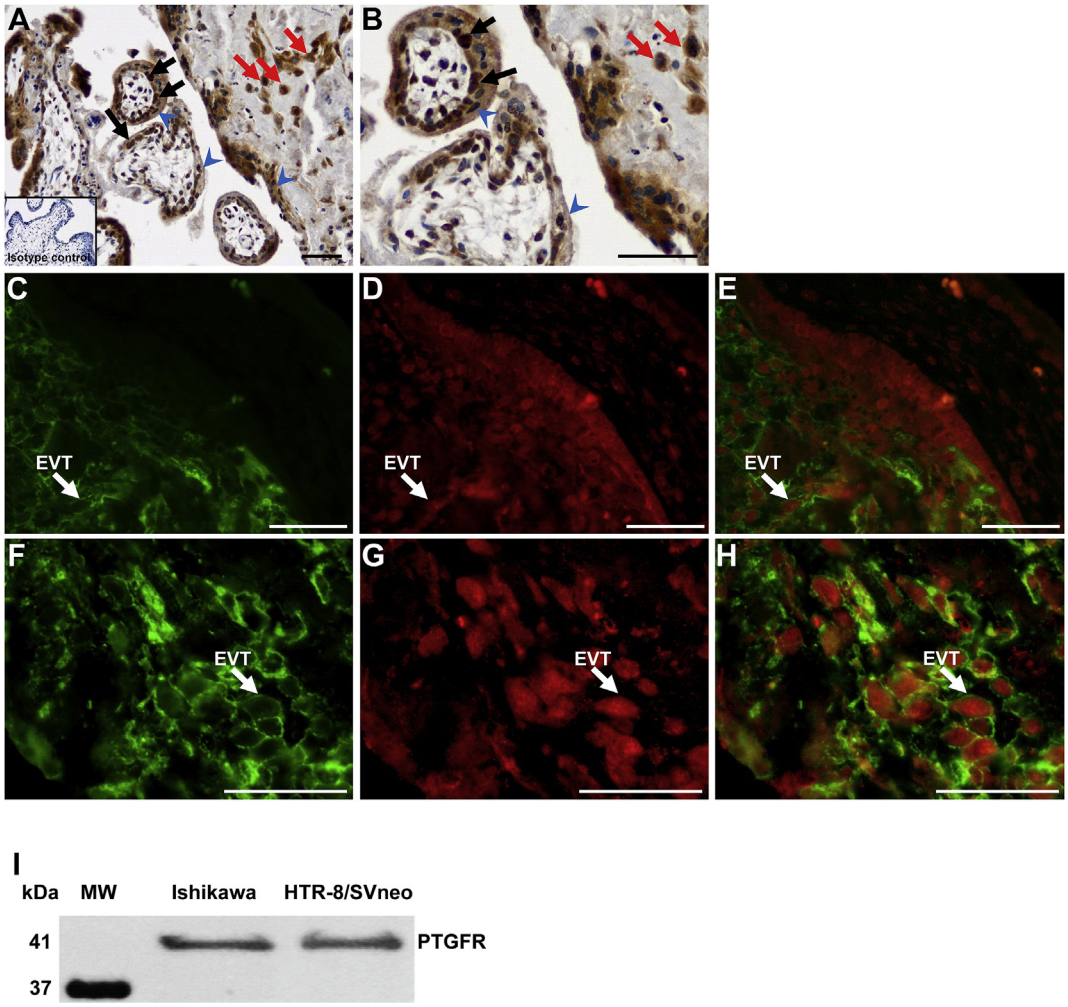

Fig. 1. $PGF_{2\alpha}$ receptor (PTGFR) is expressed in human first trimester placenta and HTR-8/SVneo cells. Representative images showing the immunolocalization of $PGF_{2\alpha}$ receptor (**A-B**) in the human trophoblast: the syncytiotrophoblast (blue arrowheads), cytotrophoblast (black arrows) and potentially in extravillous cytotrophoblast (EVT) cells (red arrows). Colocalization of a marker of EVT cells (human leukocyte antigen G; HLA-G, green signals) and PTGFR protein in EVT column (red signals, C-H) in 5–6 μm slices of human first trimester placenta (n = 4); (**C**) and (**F**) – HLA-G detection; (**D**) and (**G**) – PTGFR detection; (**E**) and (**H**) merged images. The isotype (negative) control rabbit IgG is shown in the lower-left corner of panel **A**. Scale bar represents 50 μm. (**I**) Protein expression of PTGFR in the extravillous trophoblast cell line (HTR-8/SVneo) confirmed by Western blot (41 kDa). The Ishikawa cell line was used as a positive control (**I**).

cell line.

3.2. $PGF_{2\alpha}$ enhances adhesion of HTR-8/SVneo cells

Both doses of $PGF_{2\alpha}$ had a significant effect (for 100 nM, increase to 143% (SEM ± 14%); for 1 μM, increase to 150% (SEM ± 20%), $P < 0.05$) on trophoblast cell adhesion to fibronectin, and this effect was abolished by using a PTGFR antagonist (AL8810). Additionally, we applied the same doses of a more stable analog of $PGF_{2\alpha}$ - fluprostenol (FLP). FLP also (1 μM, $P < 0.05$) increased adhesion of HTR-8/SVneo cells to fibronectin to 147% (SEM ± 9%). The stimulating effect of $PGF_{2\alpha}$ (1 μM) was diminished by using a PTGFR antagonist or MAPK kinase inhibitor (Fig. 2A).

3.3. $PGF_{2\alpha}$ stimulates proliferation of HTR-8/SVneo cells

Cell proliferation was significantly stimulated with 1 μM of $PGF_{2\alpha}$ to 124% (SEM ± 8%) ($P < 0.05$) and with both doses of FLP (100 nM, 1 μM; $P < 0.05$; Fig. 2B) to 124% (SEM ± 9%) and to 126% (SEM ± 7%), respectively. The stimulating effect of all doses of $PGF_{2\alpha}$ and FLP on cell proliferation was blocked by a PTGFR antagonist. The effect of $PGF_{2\alpha}$ (1 μM) was also diminished by using a MAPK kinase inhibitor. The effects of AL8810 or PD98059 used alone were similar to the control (Fig. 2B).

3.4. $PGF_{2\alpha}$ induces phosphorylation of FAK and MAPK1/3

During focal adhesion, extracellular matrix proteins are bonded by cell membrane-related integrin receptors. FAK is a integrin-activated protein tyrosine kinase, which is important in cross-talk with the MAPK1/3 pathway. We examined if $PGF_{2\alpha}$ initiates phosphorylation of kinases involved in adhesion signaling. Moreover, activation of MAPK signaling pathway can be involved in increased cell proliferation. Results of these analyses revealed that $PGF_{2\alpha}$ (1 μM) increased ($P < 0.05$) phosphorylation of FAK to 115% (SEM ± 3%), whereas phosphorylation of MAPK1/3 was stimulated by both doses of $PGF_{2\alpha}$ (100 nM, $P < 0.05$; 1 μM, $P < 0.0001$; Fig. 3A and B) to 125% (SEM ± 3%) and 147% (SEM ± 6%), respectively. The stimulating effects of $PGF_{2\alpha}$ on phosphorylation of both proteins were abolished by using AL8810. The effects of AL8810 used alone were similar to the controls (Fig. 3A and B).

ICAM-1, a protein with ability to bind integrins, could be involved in cell adhesion. We confirmed that ICAM-1 is expressed in the HTR-8/SVneo cell line. $PGF_{2\alpha}$ had no significant effect on ICAM-1 protein

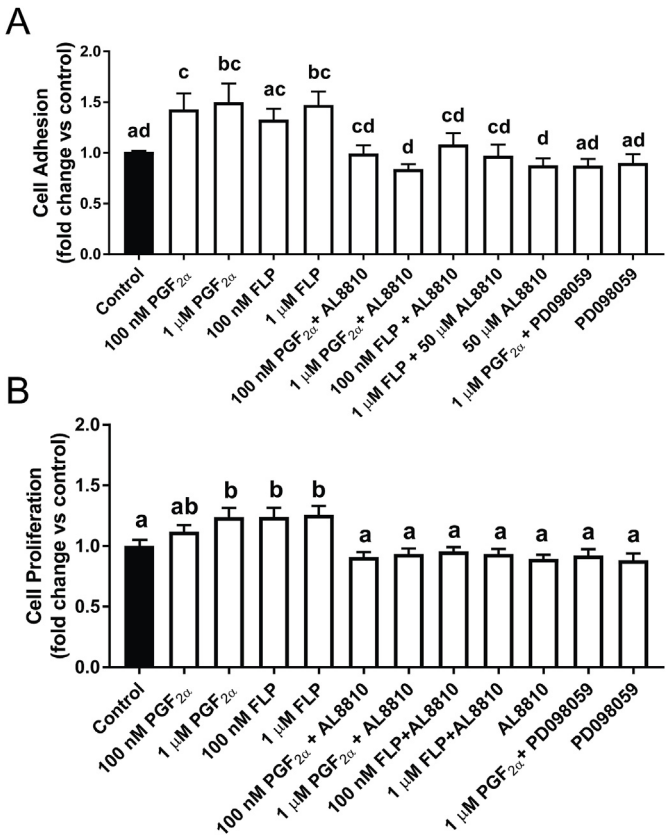

Fig. 2. $PGF_{2\alpha}$ and its analogue fluprostanol (FLP) stimulate cell adhesion (**A**) and proliferation (**B**) in the HTR-8/SVneo cell line. Cells were preincubated with either vehicle or $PGF_{2\alpha}$ antagonist (50 μM AL8810) or inhibitor of MAPK kinase - MEK1 (50 μM PD98059) for 30 min. Afterwards, cells were incubated in the presence of vehicle or 100 nM $PGF_{2\alpha}$/FLP or 1 μM $PGF_{2\alpha}$/FLP for 3 h (adhesion assay) or 24 h (proliferation assay). Each bar represents the mean ± SEM of fold change relative to control in at least 9 experiments. Means (bars) with different letters (a, b, c, d) indicate statistically significant differences ($p < 0.05$). If bars share common letters, they do not differ from each other.

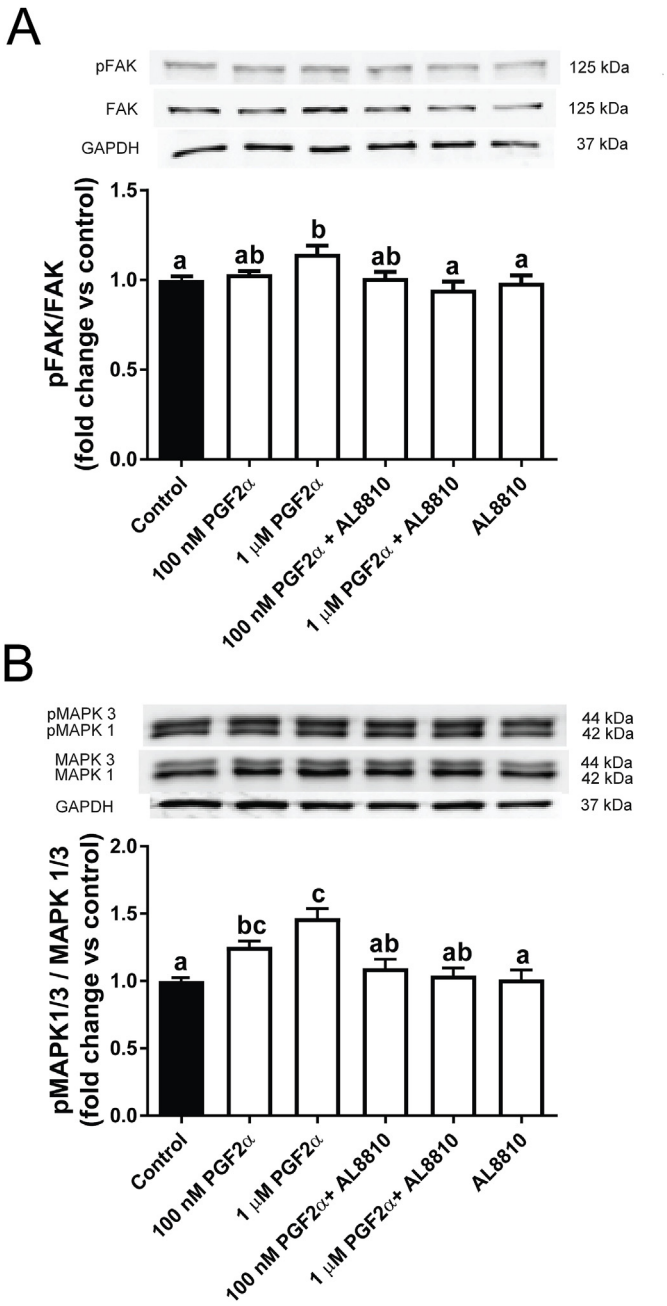

Fig. 3. Phosphorylation of focal adhesion kinase (FAK) (**A**) and mitogen activated protein kinases 1/3 (MAPK1/3) (**B**) in human trophoblast cells is induced by prostaglandin $F_{2\alpha}$ (100 nM, 1 μM). HTR-8 SVneo cells were preincubated with control or PTGFR antagonist (50 μM AL8810) for 30 min. Subsequently, cells were treated with 100 nM or 1 μM of $PGF_{2\alpha}$ for 15 min. Phosphorylation of MAPK1/3 and FAK were analyzed by Western blot. MAPK, pMAPK, FAK, pFAK, expression values were normalized against expression of GAPDH protein. Data are expressed as the mean ± SEM of fold change relative to control in at least 10 experiments. Means (bars) with different letters (a, b, c) indicate statistically significant differences ($p < 0.05$). If bars share common letters, they do not differ from each other.

expression (Suppl. Fig. 1).

3.5. $PGF_{2\alpha}$ increases MMP9 gene expression and IL6 gene and protein expression in the HTR-8/SVneo cells

$PGF_{2\alpha}$ significantly induced gene expression of *MMP9* to 136% (SEM ± 13%) (1 μM, $P < 0.05$; Fig. 4A) and *IL6* to 185% (SEM ± 25%) and to 163% (SEM ± 23%) (100 nM, $P < 0.01$; 1 μM, $P < 0.05$; Fig. 4E in the human trophoblast cell line. The effect of $PGF_{2\alpha}$ was abolished in groups treated with AL8810. No significant effect of $PGF_{2\alpha}$ was observed on *TGFB3, BGN* and *IL1A* gene expression (Fig. 4B, C and D). $PGF_{2\alpha}$ (1 μM) increased IL6 protein expression in HTR-8/SVneo cells ($p < 0.05$) but this was diminished by the presence of PTGFR antagonist (Fig. 4F).

3.6. MMP9 activity is induced by $PGF_{2\alpha}$

Consistent immunostaining for MMP2 and MMP9 proteins was observed in extracellular matrix of surrounding extravillous trophoblast cells in trophoblast cell column (Fig. 5A and B) and in cytotrophoblast (Fig. 5D and E). The identity of EVT cell column was confirmed by HLA-G staining in serial section of placenta tissue (Fig. 5C). The isotype control, mouse IgG, was used to confirm the specificity of antibodies and is shown in the lower-left panel in Fig. 5E. Using zymography, we determined that $PGF_{2\alpha}$ (100 nM, 1 μM) acting on HTR-8/SVneo cells stimulated proteolytic activity of MMP9 to 132% (SEM ± 12%) and to 131% (SEM ± 7%) ($p < 0.05$), respectively. This effect was reduced by adding PTGFR antagonist (Fig. 5G). There was no effect of $PGF_{2\alpha}$ on proteolytic activity of MMP2 in HTR-8/SVneo cells (Fig. 5F).

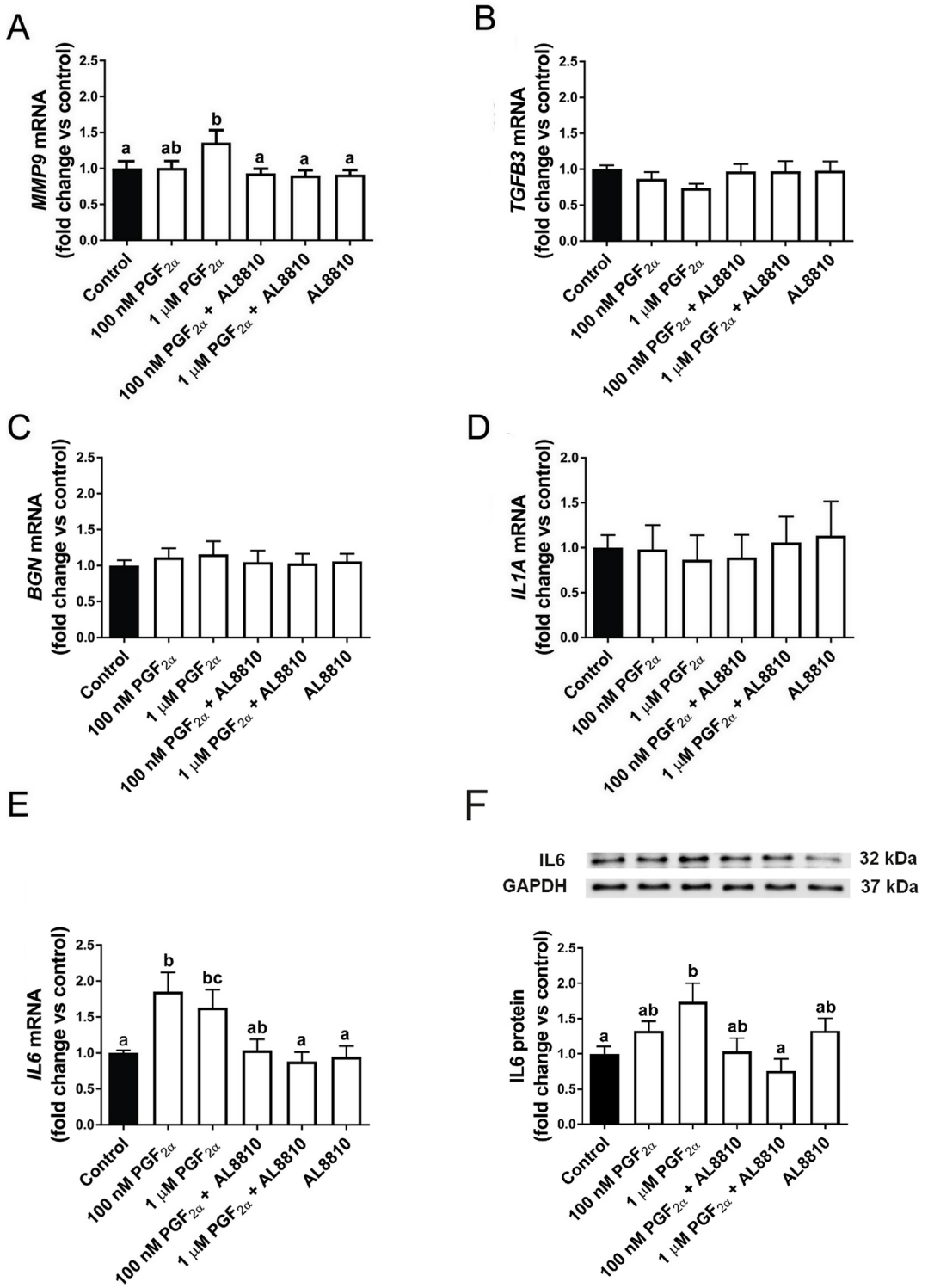

Fig. 4. Effect of $PGF_{2\alpha}$ (100 nM, 1 μM) on metalloproteinase 9 (MMP9) (**A**), transforming growth factor beta isoform 3 (TGF3B) (**B**), biglycan (BGN) (**C**), interleukin 1A (IL1A) (**D**), interleukin 6 (IL6) (**E**) gene expression (studied by Real-Time PCR) and interleukin 6 protein (studied by Western blot) (**F**) in HTR-8/SVneo in the presence or absence of $PGF_{2\alpha}$ receptor antagonist (50 μM AL8810). The representative samples of Western blots are shown in the upper panel in section F. Data are presented as mean ± SEM of fold change versus control (vehicle). Means (bars) with different letters (a, b) indicate statistically significant differences ($p < 0.05$). If bars share common letters, they do not differ from each other.

3.7. $PGF_{2\alpha}$ elevates HTR-8/SVneo cell invasion

We found that $PGF_{2\alpha}$ (1 μM) increased invasion of HTR-8/SVneo cells through ECM coated inserts ($p < 0.01$; Fig. 6) to 149% (SEM ± 10%). Invasive abilities of HTR-8/SVneo cells stimulated by $PGF_{2\alpha}$ were abolished by adding AL8810, mTOR inhibitor (rapamycin) and MMP2- and MMP9-inhibitors (Fig. 6).

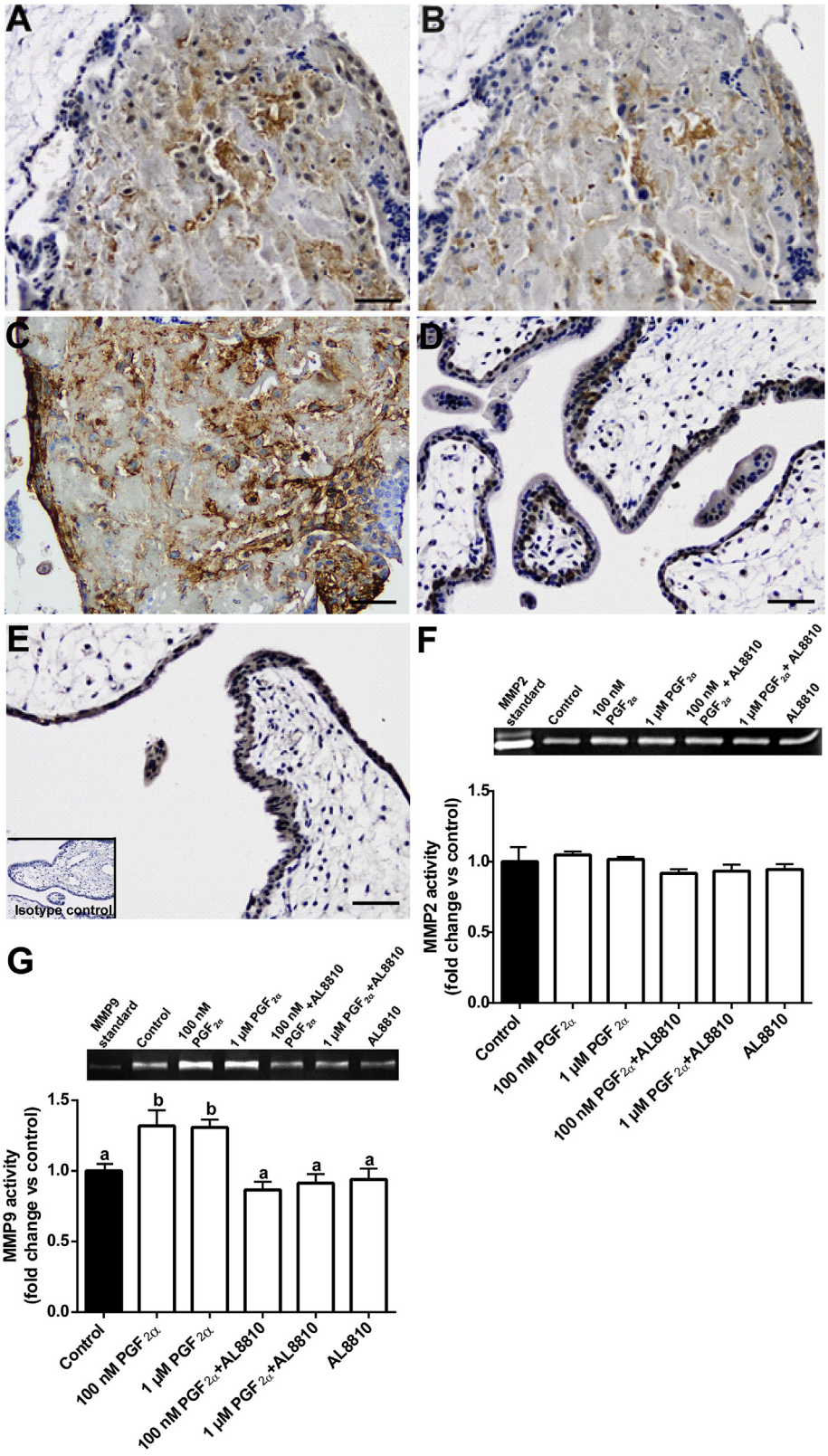

Fig. 5. Representative images showing the immunolocalization of MMP9 (**A, E**) and MMP2 (**B, D**) proteins in an extravillous trophoblast column in 5–6 μm slices of human first trimester placenta explants (n = 4) and cytotrophoblast (**D, E**) (black arrows). (**C**) the identity of EVT cell column was confirmed by human leukocyte antigen G staining (HLA-G; marker for EVT cells) in a serial section of placenta tissue. The isotype (negative) control (mouse IgG) is shown in the lower-left corners of panel **E**. Scale bar represents 50 μm. Gelatinolytic activity of MMP9 (**G**) and MMP2 (**F**) in conditioned media after incubation of HTR-8/SVneo cells with $PGF_{2\alpha}$ (100 nM, 1 μM) in the presence/absence of PTGFR antagonist (50 μM AL8810). The representative zymograms are presented in the upper panels. Data are presented as mean ± SEM of fold change vs. control (vehicle). Means (bars) with different letters (a, b) indicate statistically significant differences ($p < 0.05$).

3.8. $PGF_{2\alpha}$ increases HTR-8/SVneo cell migration

$PGF_{2\alpha}$ stimulated migration of HTR-8/SVneo cells (Fig. 7A). An interaction between treatment and time of treatment was detected ($p < 0.0001$). Upon 4, 8, 12, 24 and 36 h of culture, HTR-8/SVneo cell migration in the presence of $PGF_{2\alpha}$ (1 μM) was significantly increased to 203% (SEM ± 23%), 303% (SEM ± 53%), 264% (SEM ± 33%), 139% (SEM ± 9%) and 142% (SEM ± 9%), respectively, when compared to control ($p < 0.0001$; Fig. 7B). This increase was blocked by using PTGFR antagonist (Fig. 7B).

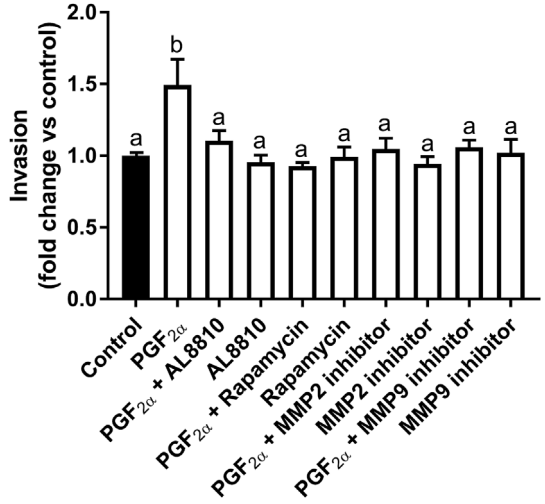

Fig. 6. $PGF_{2\alpha}$ stimulates HTR-8/SVneo cell invasion. Cells were treated with control medium (RPMI 1% FBS, 0.1% ethanol) or medium RPMI with $PGF_{2\alpha}$ (1 μM) in the presence or absence of inhibitors: AL8810 (50 μM), rapamycin (110 nM), MMP2 inhibitor-1 (10 μM), MMP9 inhibitor-1 (20 nM). Data are presented as mean ± SEM of fold change vs. control. Means (bars) with different letters (a, b) indicate statistically significant differences ($p < 0.05$).

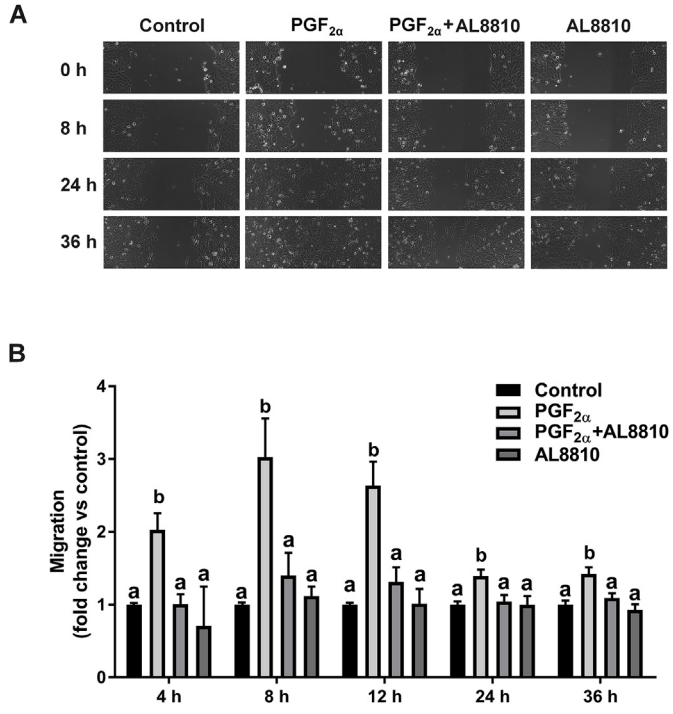

Fig. 7. $PGF_{2\alpha}$ elevates HTR-8/SVneo cell migration. (**A**) representative fields per treatment from one of 8 experiments is shown, at zero time and after 8, 24 and 36 h. (**B**) HTR-8/SVneo cells were incubated with control (vehicle) or $PGF_{2\alpha}$ (1 μM) in the presence/absence of PTGFR inhibitor (AL8810, 50 μM) for 4, 8, 12, 24 and 36 h. The areas of the gaps crossed by the cells were measured. Data are presented as mean ± SEM of fold change vs. control. Means (bars) with different letters (a, b) indicate statistically significant differences ($p < 0.05$). The effect of $PGF_{2\alpha}$ on HTR-8/SVneo cell migration within each time interval was tested using a one-way ANOVA with Tukey's post test.

4. Discussion

The present study is the first reporting the expression of PTGFR protein in the first trimester human placenta (in cytotrophoblast, syncytiotrophoblast and in EVT cells (based on morphology and colocalization with HLA-G)), as well as in EVT cell line - HTR-8/SVneo. PTGFR was reported to be also expressed in mouse, porcine and ovine trophoblasts [1,12,18]. Herein, we investigated the effect of $PGF_{2\alpha}$ on human trophoblast using an *in vitro* model. The HTR-8/SVneo cell line has been proved to be appropriate and is widely used for functional studies on human trophoblast invasion, adhesion and proliferation [26]. In the present study, we determined that $PGF_{2\alpha}$ stimulated adhesion of the trophoblast cells to extracellular matrix protein – fibronectin which is present in human stromal cells and is up-regulated in the first trimester decidua [44–46]. Our results indicated that $PGF_{2\alpha}$ acting through PTGFR also increased proliferation of the human trophoblast-derived cell line HTR-8/SVneo. $PGF_{2\alpha}$-stimulated adhesion and proliferation of human EVT cells was mediated via the MAPK signaling pathway. Interestingly, inhibition of PGE_2 and $PGF_{2\alpha}$ receptors in an *in vitro* model of embryo adhesion in which mouse embryos and JEG-3 spheroids attachment to human epithelial cells both resulted in decreased embryo adhesion rates [12]. Both PGE_2 and $PGF_{2\alpha}$ increase adhesion of mouse embryos, but the effect of $PGF_{2\alpha}$ is approximately two times stronger than with PGE_2 treatment [12]. Previous studies using the HTR-8/SVneo cell line also indicate that PGE_2 increases adhesion [4,12] but it inhibits proliferation of human trophoblast cells [3]. There are contradictory reports on the effect of PGE_2 on HTR-8/SVneo cell migration [3,47]. Based on above mentioned findings, it may be suggested that PGE_2 could have similar or contrary actions to $PGF_{2\alpha}$ depending which processes related to human embryo implantation are considered. Therefore, the PGE_2:$PGF_{2\alpha}$ ratio can determine the final $PGF_{2\alpha}$ effect. To the best of our knowledge there are no data describing the PGE_2:$PGF_{2\alpha}$ ratio in the pregnant human uterine lumen. Nevertheless, higher concentrations of $PGF_{2\alpha}$ than PGE_2 were observed in human endometrial fluid [12] or tissues collected during the implantation window period compared to samples collected from the proliferative phase [48]. Hence, regarding above data in human and our data from *in vivo* experiments in pigs [14], we speculate that similar mechanisms decreasing the PGE_2:$PGF_{2\alpha}$ ratio in the uterine lumen may occur during the implantation window in humans [12,48]. It can be suggested that $PGF_{2\alpha}$ promotes processes related to trophoblast implantation but their extent could be controlled by the opposing action of PGE_2. Furthermore, the difference in the control of cell adhesion and proliferation is likely associated with a second messenger activation. In human endometrium PGE_2 binding with PGE_2 receptors commonly activates the cAMP pathway [3,49], whereas $PGF_{2\alpha}$ and PTGFR receptor mostly mobilize the inositol 3-phosphatase (IP3) signaling pathway [50]. The inositol 3-phosphate system might be activated by the phosphorylated form of FAK after integrin binding to the extracellular matrix [51]. The complex of pFAK with proto-oncogene tyrosine-protein kinase (Src) finally leads to activation of MAPK1/3, which results in transcription regulation that controls cell proliferation and adhesion [52,53]. Our studies confirmed that $PGF_{2\alpha}$ stimulated the phosphorylation of FAK and MAPK1/3. Moreover, results presented herein are also in line with our observations on the effect of $PGF_{2\alpha}$ on porcine trophoblast cells proliferation and adhesion as well as on the activation of FAK and MAPK pathways [18].

In the present study, we also evaluated whether $PGF_{2\alpha}$ affects expression of genes potentially involved in embryo-maternal interactions. Genes chosen for the study were based on our previous findings on the role of $PGF_{2\alpha}$ at the embryo-maternal interface using porcine endometrial [17] and trophoblast cells [18] and based on reports indicating their potential role in human embryo implantation [27–37]. $PGF_{2\alpha}$ increased the content of MMP9 mRNA and its protein activity. This effect was abolished using PTGFR antagonist. MMP9 is a proteinase that degrades type IV collagen in the extracellular matrix (ECM), the main component of the uterine basement membrane [34]. Many studies indicate a crucial role for MMP9 and MMP2 in the mediation of EVT invasion during implantation. Human EVT cells are a potent source of MMPs in the first trimester, however, it is speculative which factor, MMP9 or MMP2, is more important in trophoblast invasion [35,37,54].

Our results confirmed localization of MMP9 and MMP2 in the extracellular matrix of surrounding extravillous trophoblast cells in villous column and in cytotrophoblast [54–56]. These MMPs have a major substrate of collagen IV, a main component of basement membrane, which cytotrophoblasts are located upon. Therefore, MMP9 and MMP2 may have a role in the cell-matrix interactions that regulate the cytotrophoblast stem cell niche and the terminal differentiation process when forming syncytiotrophoblast. In the present study, we demonstrated $PGF_{2\alpha}$ acting through its receptor stimulated migration and invasion of human EVT cells. It can't be excluded that the stimulating effect of $PGF_{2\alpha}$ on HTR-8/SVneo cell proliferation could have partially contributed to increase in invasion of $PGF_{2\alpha}$-treated cells. Using specific MMP9 and MMP2 inhibitors, we indicated $PGF_{2\alpha}$-mediated involvement of both metalloproteinases in trophoblast invasion. Moreover, the stimulating effect of $PGF_{2\alpha}$ on human EVT cell invasion was abolished by rapamycin, which suggests the involvement of $PGF_{2\alpha}$-mediated mTOR signaling in this process. This is in agreement with a report indicating the involvement of mTOR signaling in HTR-8/SVneo cell invasion [57]. Intriguingly, we observed that $PGF_{2\alpha}$ increased MMP9 gene expression in the porcine endometrium, whereas it reduced expression of MMP9 and MMP2 in conceptuses [17,18]. It might suggest that $PGF_{2\alpha}$ controls trophoblast invasiveness by differential regulation of MMP9 depending on the type of implantation: non-invasive in pigs vs. invasive in humans.

Our results indicate that $PGF_{2\alpha}$ acting on human EVT cells significantly increased expression of IL6 mRNA and protein. The most intense expression of IL6 occurs during the mid-secretory phase of the menstrual cycle, which corresponds with the time of implantation and the increased amount of $PGF_{2\alpha}$ in the uterine lumen [11,58]. This proinflammatory cytokine regulates activity (but not expression) of MMPs in *in vitro* studies with primary first-trimester human cytotrophoblastic cells [28]. The stimulating effect of IL6 on MMP9 activation was also observed in HTR-8/SVneo [59]. IL6 stimulates processes like invasiveness, migration and integrin expression in HTR-8/SVneo [60]. We speculate that $PGF_{2\alpha}$ besides direct effect in human EVT cells, by acting indirectly through IL6 may regulate above mentioned implantation-related processes but also immune-related processes such as host defense [61].

To conclude, we demonstrate that $PGF_{2\alpha}$ plays a significant role during EVT implantation in the human. Our results indicate that $PGF_{2\alpha}$ acting through its receptor in human trophoblast cells activates FAK and MAPK1/3 signaling pathways, and stimulates adhesion and proliferation of these cells. Furthermore, $PGF_{2\alpha}$ increased IL6 gene and protein expression, which led us to speculate its involvement in processes implicated in immune-response, such pro- or anti-inflammatory mechanisms during the implantation period. Moreover, $PGF_{2\alpha}$ induced MMP9 gene expression and protein activity, as well as EVT cell migration. Our findings indicate that $PGF_{2\alpha}$ stimulates EVT cells invasiveness which is mediated through $PGF_{2\alpha}$-induced MMP2 and MMP9 proteolytic activity and mTOR signaling.

Authors' roles

MB performed all experiments, participated in the data analysis and interpretation, and wrote the first draft of the manuscript. EG contributed to gene and protein expression analyses and migration assay. PK assisted at *in vitro* experiments and contributed to data interpretation. SR provided expertise in interpretation of immunohistochemistry of human placenta. AW conceptualized the study design, participated in data interpretation, in writing the manuscript and performed the final revision of the article. All authors contributed to critical revision of the paper.

Conflicts of interest

The authors declare that there is no conflict of interest in publishing the content of this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.01.020.

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