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RESEARCH

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Yao Ye[1], Aurelia Vattai[1], Nina Ditsch[1], Christina Kuhn[1], Martina Rahmeh[1], Sven Mahner[1], Myriam Ripphahn[2], Roland Immler[2], Markus Sperandio[2], Udo Jeschke[1] and Viktoria von Schönfeldt[1]

[1]Department of Obstetrics and Gynecology, University Hospital, LMU Munich, Munich, Germany
[2]Walter Brendel Centre of Experimental Medicine, Department of Cardiovascular Physiology and Pathophysiology, Biomedical Center, Ludwig-Maximilians University of Munich, Munich, Germany

Correspondence should be addressed to U Jeschke: Udo.Jeschke@med.uni-muenchen.de

Abstract

Although an inflammatory microenvironment is required for successful implantation, an inflammatory overreaction is one of the causes of unexplained recurrent pregnancy losses (uRPL). Prostaglandin E$_2$ (PGE$_2$) plays a pivotal role in regulating immune balance during early pregnancy, and it can stimulate inflammatory reactions via prostaglandin E$_2$ receptor 3 (EP3). However, the role of PGE$_2$ receptor signaling in the uRPL remains unknown. We aimed to investigate whether EP3 signaling is involved in the mechanism of uRPL. Via immunohistochemistry we could show that the expression of cyclooxygenase-2, EP3 and G protein alpha inhibitor 1 (G$_{i1}$) was enhanced in the decidua of the uRPL group in comparison to the control group in first-trimester placentas. *In vitro,* we demonstrated that sulprostone (an EP1/EP3 agonist) inhibited the secretion of beta-hCG and progesterone in JEG-3 cells and the secretion of beta-hCG in HTR-8/SVneo cells while it induced the expression of plasminogen activator inhibitor type 1 in JEG-3 cells. In addition, PGE$_2$/sulprostone was able to stimulate the expression of G$_{i1}$, phosphorylated-extracellular signal-regulated kinases 1/2 (p-ERK1/2) and p53. L-798,106 (an EP3-specific antagonist) suppressed the expression of EP3 and p-ERK1/2 without affecting the secretion of beta-hCG. Elevated activation of EP3 signaling in first-trimester placentas plays an important role in regulating the inflammatory microenvironment, the hormone secretion of extravillous trophoblasts and the remodeling of extracellular matrix in the fetal-maternal interface. L-798,106 might be a 'potential therapeutic candidate' for the treatment of uRPL.

Introduction

Recurrent pregnancy losses (RPL) are defined as two or more consecutive failed pregnancies before the 20th week of pregnancy according to the Practice Committee of the American Society for Reproductive Medicine (1). RPL affects 1% of all women worldwide (2), and only a minority can be explained by particular causative factors, including genetic abnormalities, structural abnormalities, infection, endocrine abnormalities, immune dysfunction and thrombophilic disorders (1). Up to 50% of RPL cases are classified as unexplained RPL (uRPL). Abnormal inflammation in the fetal-maternal interface and unbalanced extracellular matrix (ECM) remodeling are considered as key factors contributing to uRPL (3).

Prostaglandin E_2 (PGE_2) has long been known as a potentiator of inflammation (4) and is the major fraction of prostaglandins produced by the placenta (5). PGE_2 can regulate the balance between Th1 and Th2 cell responses (6), modulate chemokine production (6) and suppress lymphocyte alloreactivity of decidual cells during early gestation (7). Th1/Th2 cell ratios are higher in women with uRPL compared to controls with healthy pregnancies (8). The prostaglandin E_1 analog (Misoprostol) and the PGE_2 analog (Sulprostone) have been proven to be effective drugs for pregnancy termination (9, 10). PGE_2 has been demonstrated to be upregulated in cervical ovulatory mucus (11) and the endometrium (12, 13) of women with uRPL compared to controls with healthy pregnancies during implantation. Therefore, increased expression of PGE_2 might be a disadvantage for implantation, placentation and maintenance of pregnancy.

Arachidonic acid is metabolized by cyclooxygenase-1 and cyclooxygenase-2 (COX-2) into prostaglandin H_2 (PGH_2), which is converted into multiple prostaglandins including PGE_2 (14). As a rate-limiting enzyme that catalyzes the synthesis of PGE_2, COX-2 is localized in the endometrium throughout the menstrual cycle and during early pregnancy (15). However, results of COX-2 expression in uRPL patients are contradictory.

PGE_2 can combine with both nuclear receptors and membrane receptors. Previous studies of our group analyzed the expression of nuclear receptors in the placenta of spontaneous miscarried and RPL patients (16, 17, 18, 19, 20, 21). However, little is known about the expression of membrane receptors of PGE_2 in the placenta of women with uRPL. The effects of PGE_2 are mainly facilitated by specific membrane-bound G protein-coupled EP receptors (EP1–EP4) with various signaling pathways: EP1 is coupled to the G protein alpha q (G_q) to mobilize intracellular Ca^{2+}, EP2 and EP4 are coupled to the G protein alpha stimulator (G_s) to stimulate adenylyl cyclase, and EP3 is mainly coupled to the G protein alpha inhibitor (G_i) to inhibit adenylyl cyclase (22). All four membrane receptors of PGE_2 are expressed by the placenta and fetal membranes, and only EP3 is intensely localized in the membrane of syncytiotrophoblast in the placenta of women in labor (23). This indicates that EP3 may have an influence on trophoblast invasion and placentation. The expression of COX-2 and EP3 has been proven to be elevated in the placenta of a rat model for ischemia-reperfusion injury (24). PGE_2 triggers mast cell activation via an EP3-$G_i/_o$-Ca^{2+} influx/phosphatidyl-3-kinase signaling pathway, which contributes to vascular permeability and consequent edema formation in mice (4).

However, no previous studies exist about the expression of EP3 in the placenta of uRPL patients, as well as the possible signal processes of EP3 in normal pregnancy and uRPL during the first trimester of pregnancy.

In addition, PGE_2 binding with EP1/EP3 receptor can induce the production of plasminogen activator inhibitor type 1 (PAI-1) in cardiac fibroblasts (25). PAI-1 levels in the plasma are increased in RPL patients in comparison to women with healthy pregnancies (26). Among all the thrombophilic genes, *PAI-1-675* 4G/5G polymorphism is one of the most frequently analyzed PAI-1 genetic variants, although the contribution of *PAI-1-675* 4G/5G to uRPL is conflicting (27).

We hypothesized that EP3 signaling may be an essential pathway in the mechanism of uRPL. Our study group aimed to analyze the main proteins' expression of EP3 signaling in the placentas of first-trimester pregnancies with uRPL immunohistochemically. Additionally, we studied the effects of PGE_2, an EP1/EP3 agonist (sulprostone) and an EP3 antagonist (L-798,106) on the function of trophoblast cells by using the choriocarcinoma cell line JEG-3 and the human trophoblast-derived cells HTR-8/SVneo *in vitro*.

Materials and methods

Placental tissue samples

The study was approved by the ethical committee of the Medical Faculty, Ludwig-Maximilian-University of Munich (Number of approval: 337-06) and informed consent was obtained from each patient in written form. Samples and clinical information were anonymized and encoded for statistical workup.

We analyzed placenta tissues of 19 patients with a history of more than two consecutive pregnancy losses of unknown causes (uRPL group) and of 19 healthy controls with legal pregnancy termination (control group) from a private practice in Munich, Germany. Exclusion criteria for the uRPL group, described thoroughly in our previous studies (28), include infectious diseases, uterine anomalies, endocrinological dysfunctions, hyperprolactinemia, hyperandrogenemia, thyroidal dysfunctions, autoimmunologic disorders, deficiencies in coagulation factors, as well as fetal and parental chromosomal disorders. All placentas were obtained within the first 24 h after diagnosis without any prior hormonal pretreatment. Samples were obtained by dilatation and evacuation without any prior pharmaceutical induction. These samples were fixed immediately in 4% buffered

formalin for 20–24 h and embedded in paraffin for further immunohistochemical and double immunofluorescence analysis (29).

Immunohistochemistry

Paraffin-embedded slides were dewaxed in xylol and washed in 100% ethanol. For inhibition of the endogen peroxidases, tissue samples were incubated in methanol with 3% H_2O_2 and rehydrated in a descending alcohol series. Slides were heated in a pressure cooker using sodium citrate buffer (pH = 6.0), containing 0.1 M citric acid and 0.1 M sodium citrate in distilled water. After cooling and washing in PBS, all slides were incubated with blocking solution (Reagent 1, Zytochem-Plus HRP-Polymer-Kit (mouse/rabbit)) for 20 min to avoid non-specific binding of the primary antibodies. The primary antibodies used for the experiment were anti-COX rabbit IgG polyclonal antibody (Sigma, SAB4502491, 1:400 dilution), anti-EP3 rabbit IgG polyclonal antibody (Abcam, ab189131, 1:300 dilution) and anti-G_i1 rabbit IgG polyclonal antibody (Novus Biologicals, Littleton, CO, USA; NBP2-16558, 1:1000 dilution). The slides were incubated with different primary antibodies for 16 h at 4°C. After washing, the secondary antibodies/complexes of the ABC detection kits (Vector Laboratories) were applied following the manufacturer's protocols to detect reactivity. Immunostaining was visualized with the substrate and the chromogen-3, 3′-diaminobenzidine (DAB; Dako) for 1 min in case of COX-2, 2.5 min for EP3 and 45 sec for G_i1.

Third-trimester placenta was used as a positive control for the immunohistochemical staining of COX-2 and G_i1 to test antibody function and to determine an appropriate dilution of the antibody for staining. Colon was used as a positive control for EP3. Positive cells showed a brownish color and the negative control, as well as unstained cells, appeared blue (30). Negative controls were used with the same control tissues and were performed by replacement of a pre-immune serum at the same concentration as the primary antibody (negative control for super sensitive rabbit antibodies, BioGenex, California, USA).

All slides were analyzed under the microscope by two independent observers using a Leitz photomicroscope. For the light microscopy analysis, a semi-quantitative IRS score was used (31), which is calculated via the multiplication of optical staining intensity (grades: 0 = no, 1 = weak, 2 = moderate and 3 = strong staining) and the percentage range of positive stained cells (0 = no staining, 1 = ≤10% of the cells; 2 = 11–50% of the cells; 3 = 51–80%

of the cells and 4 = ≥81% of the cells were stained for the antibody, respectively).

Double immunofluorescence staining

For the characterization of COX-2, EP3 and G_i1-expressing cells in the decidua, we applied the same paraffin-embedded slides with double immunofluorescence staining. The same experimental steps were carried out as for immunohistochemistry until the step of blocking. Slides were blocked with a blocking solution (Ultra V Block, Lab Vision, Fremont, CA, USA) to avoid non-specific staining and then incubated with various primary antibodies overnight at 4°C. Prolactin was used as a specific marker for stromal cells and human leukocyte antigen-G (HLA-G) was used as a specific marker for trophoblast cells. Different primary antibodies were diluted with a diluting medium (Dako) according to the following ratios: 1:400 for rabbit anti-COX-2 polyclonal IgG (Sigma, SAB4502491) and 1:500 for mouse anti-Prolactin polyclonal IgG (Serotec, Kidlington, Oxfordshire, UK; MCA712), 1:300 for rabbit anti-EP3 polyclonal IgG (Abcam, ab189131), 1:300 for rabbit anti-G_i1 polyclonal IgG (Novus Biologicals, NBP2-16558), 1:50 for mouse anti-HLA-G polyclonal IgG (Serotec, MCA2044). After washing, slides were incubated with Cy2-/Cy3-labeled antibodies (Jackson Dianova, Hamburg, Germany) as fluorescent secondary antibodies for 30 min at room temperature in darkness to avoid fluorescence quenching. Cy2-labeled secondary antibodies were at a dilution of 1:100 and Cy3-labeled antibodies were at a dilution of 1:500. Finally, the slides were embedded in mounting buffer containing 4′,6-diamino-2-phenylindole (DAPI, Vectastain, Vector Laboratories) for blue staining of the nucleus after washing and drying. Digital images were obtained with a digital camera system (Axiocam; Zeiss CF20DXC; KAPPA Messtechnik, Gleichen, Germany) and digitally saved.

Cell culture and cell stimulation

JEG-3 cells (ATCC HTB-36) and HTR-8/SVneo cells (ATCC CRL-3271) were used as trophoblast models. Both cell lines were cultured in RPMI 1640 medium + Gluta MAX (Gibco) supplemented by 10% fetal bovine serum (FCS, Gibco). Cells were seeded into 24-well plates for the determination of beta-hCG, progesterone and PAI-1 and 6-well plates for Western blotting. After 6–8 h, cell culture medium was replaced with fresh RPMI1640 with stimulation chemicals for the remaining 48 h, which included dimethyl sulfoxide (DMSO, 0.5%) as vehicle control, PGE_2 (TOCRIS, Bristol,

UK), sulprostone (TOCRIS) and L-798,106 (TOCRIS). Both JEG-3 (50,000 cells/ well) and HTR-8/SVneo cells (60,000 cells/well) were incubated with PGE_2, sulprostone and L-798,106 at different concentrations of 0.1, 1, 10, 100, 1000 nM and 10 µM in 24-well plates.

Cell viability assay

JEG-3 cells were seeded at the density of 1.5×10^4 cells/ well in 96-well plates and were incubated with different concentration of PGE_2 and L-798,106 for 48 h. To each well, 20 µg MTT (Sigma) were added for 1.5 h at 37°C. After removing MTT, 200 µL DMSO were added to each well and mixed thoroughly on the shaker for 5 min at room temperature. The optical density was examined at 595 nm using Elx800 universal Microplate Reader.

ADVIA Centaur assay

Supernatants of JEG-3 and HTR-8/SVneo cells in 24-well plates were collected and centrifuged (13,200 g, 10 min) to remove debris. The concentration of beta-hCG and progesterone in the supernatant was detected according to the manufacturer's instructions on an ADVIA Centaur XP autoanalyzer (Siemens Medical Solution Diagnostics) as described in our previous publications (32, 33). The concentration of total beta-hCG was measured in a two-site sandwich immunoassay (ADVIA Centaur Total hCG Test; Siemens Medical Solutions Diagnostics). The detectable concentration of total beta-hCG ranges from 2.0 IU/mL to 1000 IU/mL. The concentration of progesterone in supernatants was measured by an automated quantitative immunoassay (ADVIA Centaur Progesterone Test; Siemens Medical Solutions Diagnostics) with a sensitivity of 0.2 ng/mL. Intraobserver and interobserver coefficients of variation were 9.8 and 5.8%, respectively.

PAI-1 ELISA

PAI-1 levels in the supernatants of JEG-3 and HTR-8/SVneo were measured utilizing commercially available ELISA kit (R&D system, DSE100). A standard curve was obtained in parallel to each assay and results converted into ng/mL. The intra- and inter-assay variability was respectively 4.6 and 8.7%.

Western blotting

Cell lysates were extracted from JEG-3 and HTR-8/SVneo cells with radioimmunoprecipatation assay buffer (RIPA,

Sigma-Aldrich, R0278-50ML). For Western blotting, 20 µg of cell lysates were first separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane (Bio-Rad). The membrane was blocked in 4% skim milk powder and then incubated with the primary antibodies for 16 h at room temperature. Different primary antibodies were used as the following: rabbit polyclonal anti-EP3 antibody (Abcam, ab94496, 1:500), rabbit polyclonal anti-G_{i1} antibody (Novus Biologicals, NBP2-16558, 1:500), rabbit polyclonal anti-phosphorylated extracellular signal-regulated kinases (p-ERK1/2) antibody (Abcam, ab47339, 1:500), mouse monoclonal anti-p53 antibody (Abcam, ab26, 1:200). Beta-actin was used as a housekeeping gene and mouse monoclonal anti-beta-actin antibody was diluted 1:1000 in 4% milk powder (Sigma, A5441). Afterward, the membrane was incubated with the goat-anti-rabbit/mouse secondary antibody conjugated with alkaline phosphatase (1:1000 dilution, Jackson Immuno Research), and detected with 5-bromo-4-chloro-3′-indolylphosphate/nitro-blue tetrazolium (BCIP/NBT)-chromogen substrate solution (Promega). Western blots were scanned and quantified using the GelScan V6.0 1D Analysis Software (SERVA, Electrophoresis GmbH, Heidelberg, Germany). Band intensities of EP3, G_{i1}, p-ERK1/2 and p53 were normalized with band intensities of beta-actin. The blots were repeated three times.

Statistical analysis

All data were analyzed with SPSS Statistics 23 software (IBM Corporation, Armonk, NY, USA) and are expressed as the mean ± s.d. Independent *t*-test was used to compare the clinical data of uRPL and control group. Mann–Whitney-*U* test was applied for evaluating IRS scores of EP3, COX-2 and G_{i1} expression in placentas of two groups. Spearman's rank correlation analysis was adopted to evaluate the correlation between two monotonic, nonlinear variables. Wilcoxon test was used for the evaluation of beta-hCG, progesterone and PAI-1 expression levels between vehicle and stimulation groups. Wilcoxon test was also used for analyzing the band intensities of EP3, G_{i1}, p-ERK1/2 and p53. *P*-values <0.05 were regarded statistically significant.

Results

Clinical data of the uRPL group and control group

The mean age of the women in the uRPL group was 37.76 ± 4.88 years and of the control group

35.78±5.88 years (P=0.41). Mean gestational age of the uRPL group was 9.09±2.17 weeks and of the control group 9.71±1.88 weeks (P=0.66). The gravidity times were decreased in patients with uRPL (3.11±1.08) compared to healthy controls (3.42±1.90, P=0.002), while the parity times of two groups showed no significant differences (0.94±0.94 vs 1.63±1.12, P=0.35). Demographic and clinical data are illustrated in Table 1.

Expression of COX-2, EP3 and G_{i1} in the placenta of women with uRPL and controls

Expression of COX-2, EP3 and G_{i1} was identified in the cytoplasm of cells in the syncytium and the decidua of first-trimester pregnancies in both the uRPL group and the control group (Fig. 1A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q and R). A significant increase in the expression of COX-2 could be observed in the syncytium of the uRPL group in comparison to the control group (IRS 8.73 vs 5.59; P=0.007, Fig. 1A, B and E). COX-2 expression in the decidua of the uRPL group was also significantly increased in comparison to the control group (IRS 4.29 vs 2.00; P=0.004, Fig. 1C, D and F).

Expression of both EP3 and G_{i1} in the decidua was significantly upregulated in patients with uRPL compared to normal controls (Fig. 1L and R), but no significant changes of EP3 and G_{i1} could be observed in the syncytium (Fig. 1K and Q). In the uRPL group, EP3-staining in the decidua was more intense with a mean IRS of 4.15, which is higher than the mean IRS of the control group (IRS=2.22, P=0.001, Fig. 1I, J and L). EP3-staining in the syncytium of the uRPL group was similar to the control group (IRS 5.75 vs 6.32; P=0.350, Fig. 1G, H and K). G_{i1}-staining was stronger in the decidua of the uRPL group compared to the control group (IRS 4.48 vs 2.89; P=0.008, Fig. 1O, P and R). There was no significant staining difference for G_{i1}-staining in the syncytium between the uRPL group and the control group (IRS 5.27 vs 4.47; P=0.292, Fig. 1M, N and Q). Both positive and negative controls are shown in the Supplementary Fig. 1 (see section on supplementary data given at the end of this article).

Expressions of COX-2, EP3 and G_{i1} were all increased in the decidua of patients with uRPL compared to healthy controls, implying that a correlation could exist between them. We further analyzed the correlation of each two of these three components in the decidua. Unfortunately, there was no statistically significant correlation between COX-2 and EP3 expression (r=−0.059; P=0.788), neither for EP3 and G_{i1} (r=0.251; P=0.261) nor for COX-2 and G_{i1} (r=0.158, P=0.450) in the decidua.

Localization of COX-2, EP3 and G_{i1} in the decidua of first-trimester placentas

As the decidua consists of maternal decidual stroma cells and extravillous trophoblasts, double immunofluorescence was used to identify COX-2, EP3 and G_{i1}-expressing cells. Prolactin was used as a marker for stromal cells and HLA-G as a maker for trophoblasts, respectively. COX-2 was co-expressed with prolactin predominantly in the cytoplasm of stromal cells (Fig. 2A, B and C). Both EP3 (Fig. 2D, E and F) and G_{i1} (Fig. 2H, I and G) were co-expressed with HLA-G in the cytoplasm of extravillous trophoblasts. G_{i1} was expressed especially beneath the cell membrane of extravillous trophoblasts (Fig. 2G). Since co-expression of COX-2, EP3 and G_{i1} was similar in both the uRPL and control group, we only showed pictures of the uRPL group.

Influence of PGE_2, sulprostone and L-798,106 on the expression of EP3 signaling in JEG-3 cells *in vitro*

In order to investigate the mechanism of the EP3 signaling in extravillous trophoblasts of uRPL *in vitro*, JEG-3 and HTR-8/SVneo cells were used and stimulated with different concentrations of PGE_2, EP1/EP3 agonist (sulprostone) and EP3 antagonist (L-798,106) for 48h.

Firstly, the MTT assay was used to assess the viability of JEG-3 cells after 48h of treatment with 0.1, 1, 10, 100, 1000nM and 10μM PGE_2, L-798,106 or the vehicle control (DMSO, 0.1%). 10nM PGE_2 and 10nM L-798,106

**Table 1** Demographic and clinical characteristics of the study population.

Characteristic	Normal pregnancy (n=19)	uRPL (n=19)	*P*-value (*t*-test)
Maternal age (years)	35.78±5.88 (25–46)	37.76±4.88 (30–44)	0.41
Gestational age (weeks)	9.71±1.88 (6–13)	9.09±2.17 (4–12)	0.66
Gravidity	3.42±1.90 (1–7)	3.11±1.08 (2–5)	0.002
Parity	1.63±1.12 (0–4)	0.94±0.94 (0–3)	0.35

Values are represented as mean±S.D.; the range is shown in parentheses.
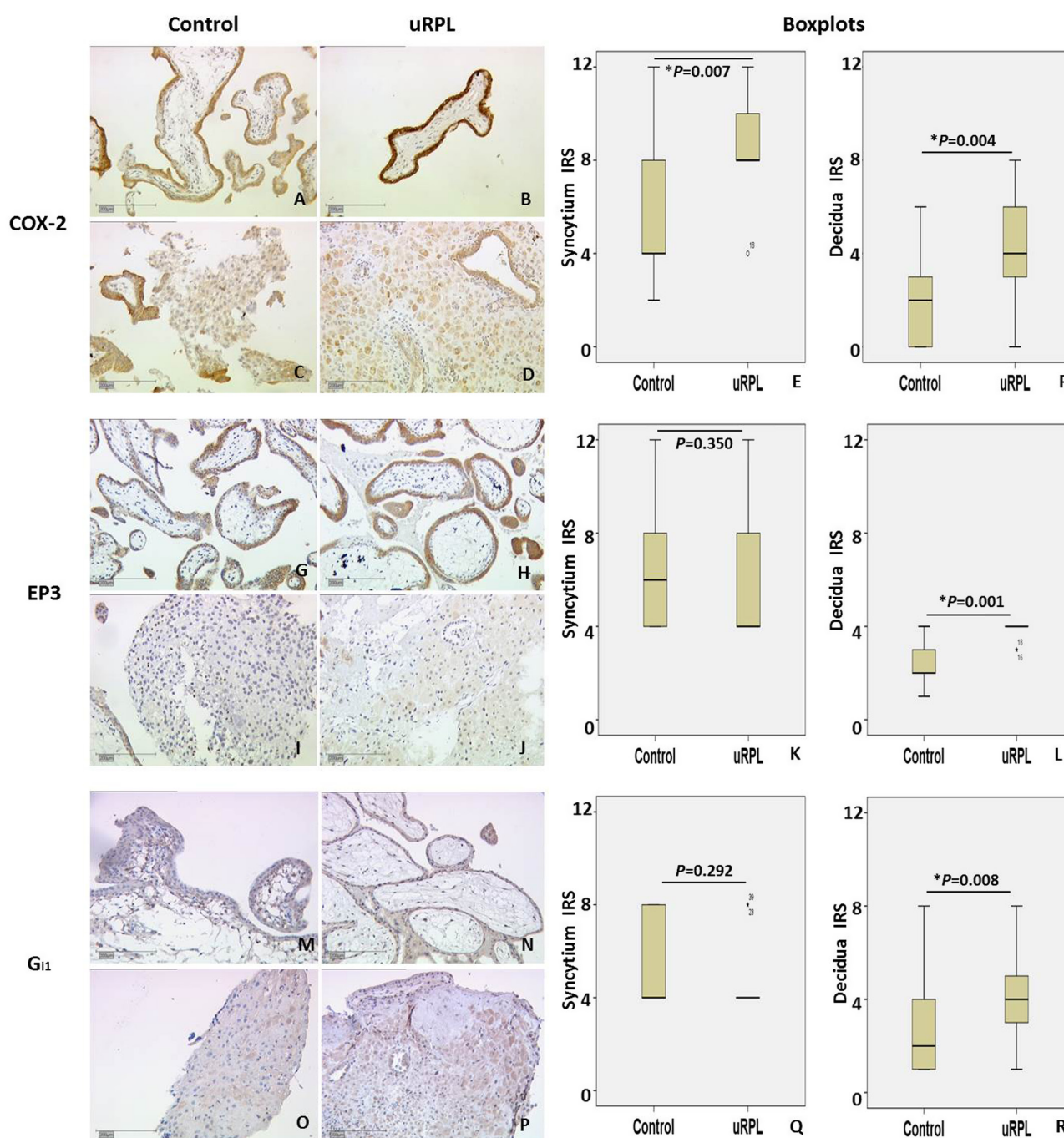
Figure 1

Immunohistological analyses of COX-2, EP3 and G_{i1} expression in placentas of uRPL patients (B, D, H, J, N and P) and healthy controls (A, C, G, I, M and O) from the first trimester were measured via IRS score. In the syncytium, the stainings of COX-2 (A and B), EP3 (G and H) and G_{i1} (M and N) are compared between the uRPL patients and controls, which are represented as box-plots (E, K and Q). In the decidua, the expressions of COX-2 (C and D), EP3 (I and J) and G_{i1} (O and P) are increased in uRPL patients compared to controls, which are represented as box-plots (F, L and R). The range between the 25th and 75th percentiles is represented by the boxes with a horizontal line at the median. The bars show the 5th and 95th percentiles. Dots indicate values more than 1.5 box lengths from the 75th percentile. Magnification ×10 lens, scale bar=200 μm. uRPL=unexplained recurrent pregnancy losses. COX-2, cyclooxygenase-2; EP3, prostaglandin E_2 receptor 3; G_{i1}, G protein alpha inhibitor 1; uRPL, unexplained recurrent pregnancy losses.
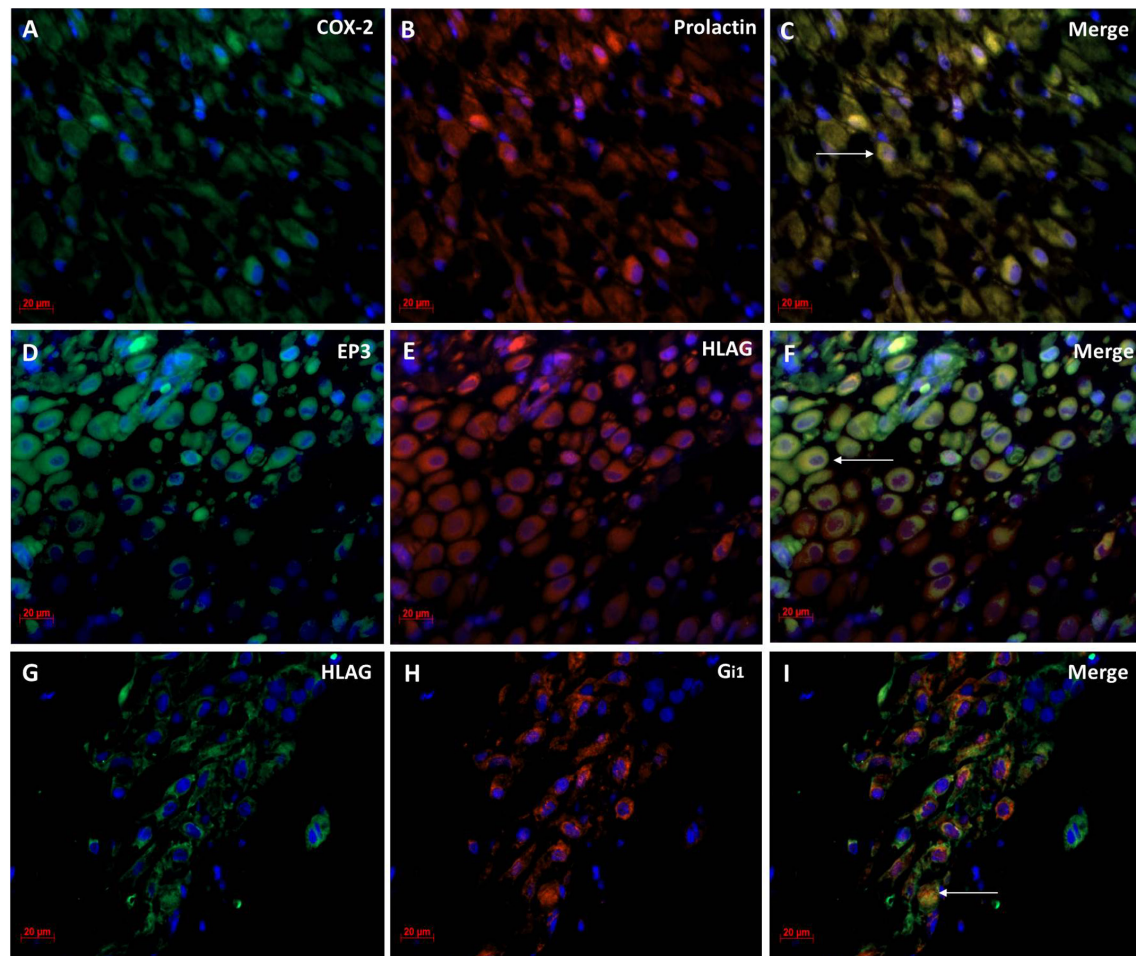
Figure 2

Localization of COX-2, EP3 and G_{i1} is shown in the decidua of patients with uRPL. COX-2 is co-expressed with prolactin, which is a specific marker for stromal cells (A, B and C). EP3 is co-expressed with HLA-G, which is a specific marker for extravillous trophoblasts (D, E and F). G_{i1} is co-expressed with HLA-G in extravillous trophoblasts (G, H and I). Co-expression of COX-2, EP3 and G_{i1} is shown with →. Magnification ×63, scale bar = 20 μm. COX-2, cyclooxygenase-2; EP3, prostaglandin E_2 receptor 3; G_{i1}, G protein alpha inhibitor 1; uRPL, unexplained recurrent pregnancy losses.

significantly increased viability of JEG-3 cells compared to control group ($P < 0.05$, data not shown).

For JEG-3 cells, the production of beta-hCG was suppressed from 16.33 ± 3.30 IU/mL in the vehicle to 12.20 ± 1.76 IU/mL by 10 nM PGE_2 ($P = 0.046$), while its concentration was not significantly influenced by 0.1 nM and 1 nM PGE_2 (Fig. 3A). Additionally, beta-hCG expression was inhibited from 16.33 ± 3.30 IU/mL in the vehicle to 10.19 ± 1.79 IU/mL and 10.99 ± 1.15 IU/mL when sulprostone concentration was 1 nM and 10 nM ($P = 0.028$, $P = 0.028$, respectively, Fig. 3A). However, beta-hCG levels were not altered by L-798,106 independent of the concentration of 0.1, 1 or 10 nM (Fig. 3A).
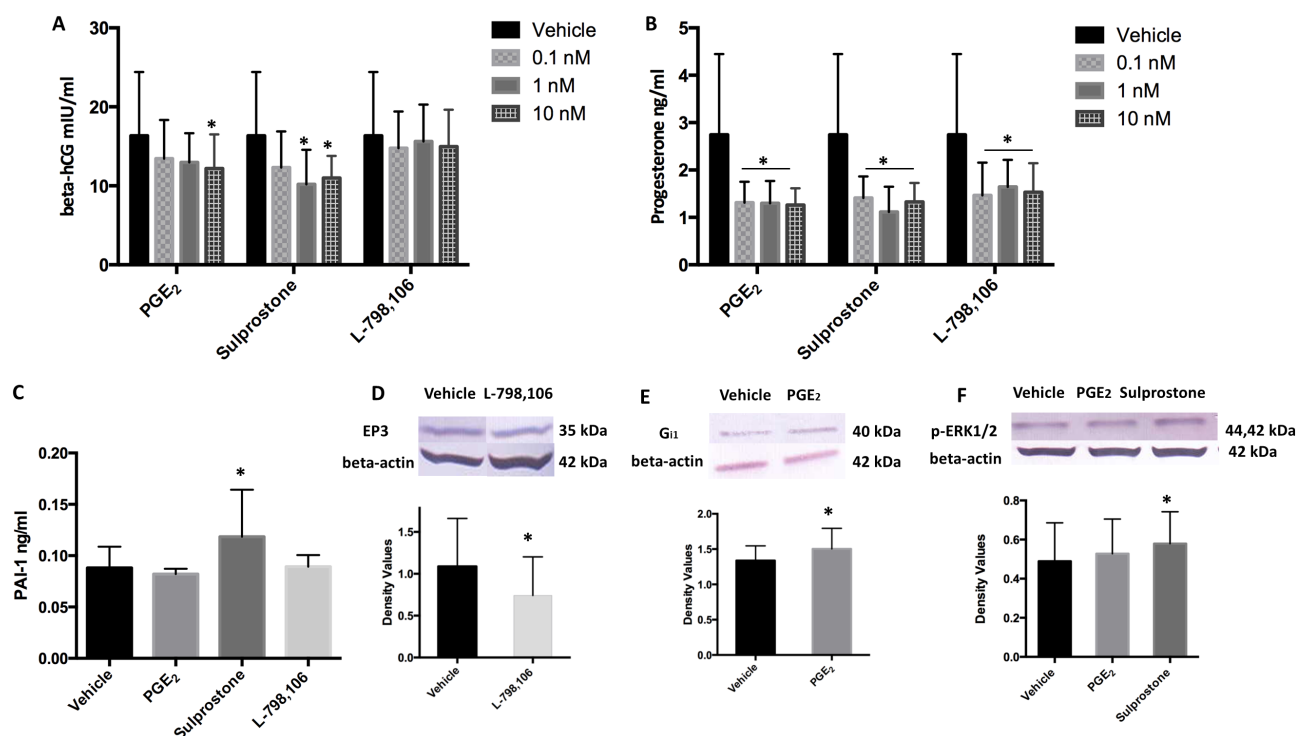
Progesterone expression of JEG-3 cells was downregulated by PGE_2, sulprostone and L-798,106, independent of

different concentrations (0.1, 1 and 10 nM) in comparison to the vehicle after 48 h ($P = 0.028$, each) (Fig. 3B).

PAI-1 levels were significantly altered 34% higher through 10 nM sulprostone than the vehicle, while no significant changes of PAI-1 expression were detected through 10 nM PGE_2 and 10 nM L-798,106 (Fig. 3C).

Western blotting was utilized to access the expression of EP3, G_{i1} and p-ERK1/2 in JEG-3 cells stimulated by PGE_2 and L-798,106, 10 nM each (Fig. 3D). The molecular weight of EP3 is 37 kDa and of G_{i1} it is 40 kDa. EP3 expression was decreased by 31.8% after treatment with 10 nM L-798,106 compared to the vehicle group ($P = 0.008$, Fig. 3D). G_{i1} expression was increased by 12.4% through 10 nM PGE_2 ($P = 0.012$, Fig. 3E). The molecular weights of p-ERK1/2 are 44 and 42 kDa.

Figure 3

Expression of beta-hCG, progesterone, PAI-1, EP3, G_{i1}, p-ERK1/2 in JEG-3 cells after incubation with PGE_2, the EP1/EP3 agonist (sulprostone) and EP3 antagonist (L-798,106). Beta-hCG, progesterone and PAI-1 expression levels were measured via ELISA (each group $n=6$). EP3, G_{i1}, p-ERK1/2 expression extracted from JEG-3 cells were detected by Western blotting, following treatment with PGE_2 (10 nM) and L-798,106 (10 nM) for 48 h ($n=3$). All data are shown as mean ± s.D. and statistically significant differences ($P<0.05$) between individual treatment groups and the vehicle are marked with an asterisk. EP3, prostaglandin E_2 receptor 3; G_{i1}, G protein alpha inhibitor 1; PGE_2, prostaglandin E_2; PAI-1, plasminogen activator inhibitor; p-ERK1/2, phosphorylated extracellular signal-regulated kinases 1/2.

JEG-3 cells were incubated with 0.5% DMSO for 1, 2, 6, 12, 24, 48 h and the results showed there was the strongest expression of p-ERK1/2 after 1 h incubation (Supplementary Fig. 2A). After 1 h incubation, the expression of p-ERK1/2 was stimulated by 18.3% through 10 nM sulprostone compared to the vehicle ($P=0.035$), while no significant change was detected in the group treated with 10 nM PGE_2 or L-798,106 (Fig. 3F).

Influence of PGE_2, sulprostone and L-798,106 on the expression of EP3 signaling in HTR-8/SVneo cells *in vitro*

HTR-8/SVneo cells were incubated with PGE_2, sulprostone and L-798,106 at a concentration of 10 nM and 100 nM, respectively. Beta-hCG levels of HTR-8/SVneo cells were downregulated through PGE_2, sulprostone and L-798,106 for all concentrations after 48 h ($P<0.05$, each) (Fig. 4A). Progesterone levels could not be detected in the supernatants of HTR-8/SVneo cells. PAI-1 levels were

reduced 3.7% through 10 nM L-798,106 than the vehicle, while no significant changes of PAI-1 expression were detected through 10 nM sulprostone (Fig. 4B)

In Western blotting, EP3 expression was increased by 11.6% through 10 nM PGE_2 ($P=0.011$, Fig. 4C) and decreased by 25.9% through 10 nM L-798,106 compared to the vehicle ($P=0.008$, Fig. 4C). G_{i1} expression was increased by 22.5% through 10 nM sulprostone ($P=0.046$, Fig. 4D), while no significant changes were observed through 10 nM PGE_2. The expression of p-ERK1/2 decreased when the incubation time with 0.5% DMSO is increased from 1 to 48 h and the strongest expression of p-ERK1/2 was after 1 h incubation (Supplementary Fig. 2A). The expression of p-ERK1/2 was inhibited by 21.8% after treatment with 10 nM L-798,106 for 1 h compared to the vehicle group ($P=0.011$, Fig. 4E). The molecular weight of p53 is 53 kDa, and its expression was stimulated by 17.0% through 10 nM PGE_2 ($P=0.028$, Fig. 4F) and by 24.5% through 10 nM sulprostone ($P=0.028$, Fig. 4F).
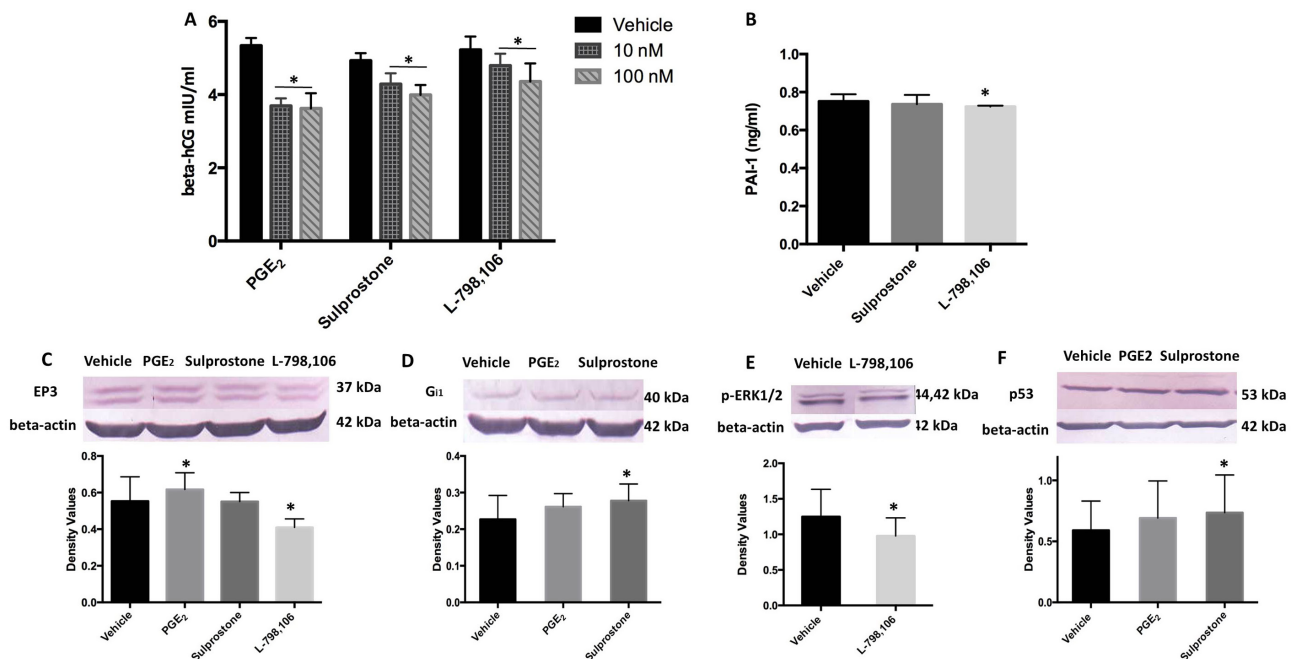
Figure 4

Expression of beta-hCG (A), PAI-1 (B), EP3 (C), G_{i1} (D), p-ERK1/2 (E) and p53 (F) in HTR-8/SVneo cells after incubation with PGE_2, the EP1/EP3 agonist (sulprostone) and EP3 antagonist (L-798,106). The levels beta-hCG and PAI-1 were measured via ELISA (each group $n=6$). EP3, G_{i1}, p-ERK1/2 and p53 expression extracted from HTR-8/SVneo cells were detected by Western blotting, following treatment with PGE_2 (10 nM), sulprostone (10 nM) and L-798,106 (10 nM) for 48 h ($n=3$). All data are shown as mean ± s.d. and statistically significant differences ($P<0.05$) between individual treatment groups and the vehicle are marked with an asterisk. EP3, prostaglandin E_2 receptor 3; PGE_2, prostaglandin E_2; PAI-1, plasminogen activator inhibitor; p-ERK1/2, phosphorylated extracellular signal-regulated kinases 1/2.

Discussion

An increased inflammatory reaction is one of the main causes of uRPL (8). PGE_2 plays a pivotal role in regulating immune balance and angiogenesis during early pregnancy (8), and it is able to stimulate inflammatory reactions via EP3 (4). The role of PGE_2 receptor signaling in the mechanism of uRPL still remains unknown. Our group is the first to investigate EP3 signaling pathway in placentas of patients with uRPL.

As the rate-limiting enzyme for PGE_2 synthesis, COX-2 is expressed by human endometrial stromal cells (34), and it is involved in the implantation of blastocysts in the early pregnancy phase (35). So far, a small number of publications exist about COX-2 expression levels in uRPL, but the results are conflicting. One research group reported that lower expression levels of COX-2 have been found in the chorionic villi of women with uRPL (35), and another group showed the similar result in the embryo of mice with autoimmune-type recurrent miscarriages (36). Our result is in accordance with a further study group who found that the expression levels of COX-2 are increased in the endometrium of uRPL women in comparison to healthy controls (13). In this study, expression of PGE_2

and pro-inflammatory cytokines is also induced in the endometrium of women with uRPL, such as interleukin 1beta, tumor necrosis factor alpha (TNF-alpha), interferon gamma (IFN-gamma) and transforming growth factor beta1 (TGF-beta1) (13). IL-1, TNF-alpha and IFN-gamma belong to Th1 cytokines, and this Th1 predominance has been known to be one of the immunological reasons for uRPL (37). In addition, TNF-alpha can induce COX-2 gene expression in first-trimester trophoblasts (38).

Ryantova *et al*. (2008) observed that PGE_2 levels in the cervical ovulatory mucus are increased in patients with uRPL (11), which is mainly caused by increased levels of COX-2. However, the effects of PGE_2 on trophoblast cells *in vitro* are contradictory. Biondi *et al*. (2006) proved that PGE_2 inhibits proliferation and migration of HTR-8/SVneo cells by enhancing cAMP (39), while Horita *et al*. (2007) suggested that PGE_2 promotes migration of HTR-8/SVneo cells by leukemia inhibitory factor (40). We investigated that PGE_2 inhibited the production of beta-hCG and progesterone in JEG-3 cells and the production of beta-hCG in HTR-8/SVneo cells. Low expression levels of beta-hCG and progesterone in the serum have been used to diagnose early pregnancy losses (41, 42). Intravaginal PGE_2 can decrease the excretion of hCG in the urine and

progesterone in the plasma (43), which is in line with our study that high expression levels of PGE_2 in the placenta are detrimental to pregnancy maintenance.

Prostaglandin E_2 receptors (EP1–4) are G protein-coupled receptors that both bind and are activated by PGE_2. Yamazaki *et al.* (2006) indicated that mRNA and protein expression of COX-2 and EP3 are increased in the placenta of rats after ischemia-reperfusion, which is associated with intrauterine growth restriction (24). In our study we could demonstrate that EP3 expression was mainly expressed in the extravillous trophoblasts and increased in extravillous trophoblast cells of uRPL patients compared to normal pregnancies in the first trimester of pregnancy. The EP3 gene formed nine distinct mRNAs encoding at least eight EP3 isoforms (44), and the anti-EP3 antibody we used could detect the protein region within the internal sequence amino acids 360–409. It might partly explain different band signals of EP3 in JEG-3 and HTR-8/SVneo cells for *in vitro* studies. We furthermore investigated that sulprostone (an EP1/EP3 agonist) inhibited the secretion of beta-hCG and progesterone in JEG-3 cells and beta-hCG expression in HTR-8/SVneo cells. Sulprostone, as a PGE_2 analog, can be used for medical termination of early pregnancy (10). Sulprostone coupling with EP1 or EP3 receptor may result in a reduced production of beta-hCG and progesterone in extravillous trophoblasts. Both are detrimental to blastocyst growth and pregnancy maintenance (45). However, Biondi *et al.* (2006) reported that PGE_2 inhibited proliferation and migration of HTR-8/SVneo cells via EP2 and EP4 receptors instead of EP3 (39). In order to investigate the exhaustive mechanisms of membrane receptors of PGE_2 in the placenta of uRPL, the remaining membrane receptors of PGE_2 (EP1, EP2 and EP4) still need to be explored, among which the EP1 receptor represents an important receptor we will focus on in future investigations (46).

The activation of G protein-coupled receptors stimulates the activity of adenylate cyclase (AC), increases intracellular levels of cAMP and activates protein kinase A (PKA), and this cAMP/PKA signaling cascade is a key second messenger pathway for steroid biosynthesis (47). G_i inhibits AC, resulting in decreased levels of cAMP and therefore inhibiting activity of PKA (48). We found that the expression of G_{i1} was mainly expressed in extravillous trophoblasts and upregulated in the decidua of women with uRPL. It suggests that the cAMP/PKA signaling pathway may be suppressed in the extravillous trophoblasts of uRPL. cAMP stimulates the transcription of the alpha-hCG gene expression in villous trophoblasts by interacting with the CREB (49). PKA can

stimulate progesterone synthesis in the human placenta by phosphorylation of enzymes (50). We investigated that both PGE_2 and sulprostone stimulated the expression of G_{i1} and suppressed the production of beta-hCG and progesterone, which can be explained by the activated EP3/G_{i1} and inhibited cAMP/PKA signaling.

Interestingly, we investigated that only sulprostone was able to upregulate the expression of PAI-1 in JEG-3 cells, which is known as the main inhibitor of fibrinolysis (51). High plasma levels of PAI-1 and high PAI activities are present in women suffering from uRPL in comparison to healthy controls (26). PGE_2 utilizes EP1/EP3 to increase PAI-1 levels in cardiac fibroblasts, contributing to elevated fibrin deposition in aortic stenosis (25). TGF-beta1 can induce PAI-1 gene expression by phosphorylation of extracellular signal-regulated kinases (ERK1/2) and p53 (52). The expression of p53 is increased in chorionic villi of RPL patients compared to healthy controls (53). We found that sulprostone stimulated the expression of phosphorylated-ERK1/2 (p-ERK1/2) in JEG-3 cells and the expression of p53 in HTR-8/SV neo cells. Additionally, the expression of p-ERK1/2 was inhibited by L-798,106 in HTR-8/SV neo cells. The activation of EP3 signaling induces increased expression of p-ERK1/2 and p53 in extravillous trophoblasts, which results in increased PAI-1 gene expression and an imbalance of ECM degradation during first-trimester pregnancies. Both the *in vivo* study of Yamazaki *et al.* (24) and also our study suggest that under pathological circumstances large differences in EP3 expression in the placenta can be observed. In contrast, only small magnitude changes of EP3 and PAI-1 were detected in our cell culture studies. It implies that trophoblast cells *in vitro* can mimic parts of the *in vivo* environment, however it still cannot replace the complex physical milieu of the placenta. Therefore, *in vivo* investigations of EP3 regulation in animal models or observational studies in humans should be the focus in future.

In conclusion, we suggest that the reason for increased PGE_2 expression in the placenta in women with uRPL might due to enhanced levels of COX-2 in stromal cells of the placenta. PGE_2 in combination with EP3 receptor of extravillous trophoblasts induces G_{i1} and inhibits AC activity, contributing to reduced levels of cAMP and inhibited activity of PKA (Fig. 5). The decreased cAMP/PKA signaling could suppress the production of beta-hCG and progesterone. At the same time, the activated EP3/G_{i1} can stimulate p-ERK1/2 and p53, enhancing PAI-1 gene expression (Fig. 5). Upregulation of PAI-1 can inhibit ECM degradation, contributing to intervillous fibrin
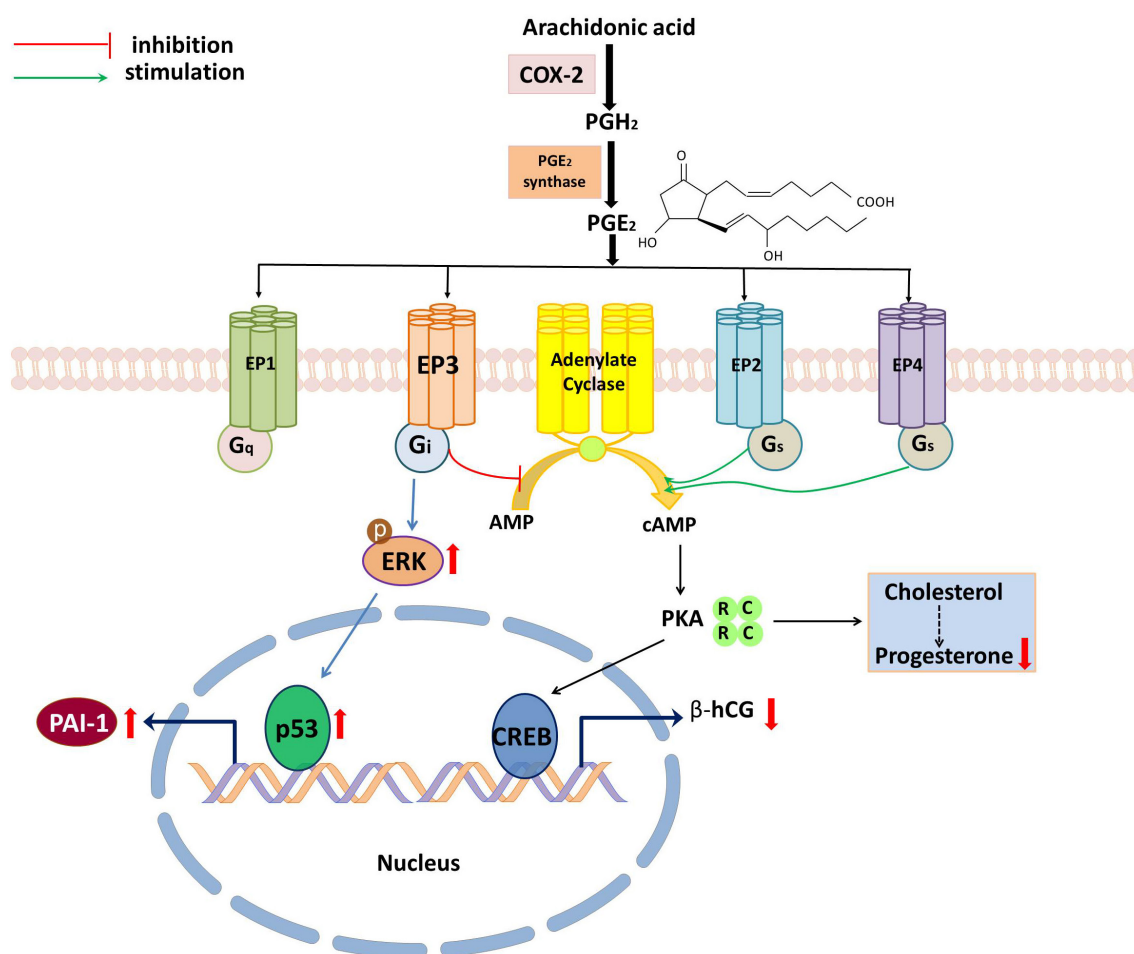
Figure 5

EP3 signaling is induced in the extravillous trophoblasts of uRPL patients. Arachidonic acid is metabolized by COX-2 into PGH$_2$, which is converted into multiple prostaglandins, such as PGE$_2$ (14). PGE$_2$ acts through different membrane receptors (EP1–4) (22). In the placenta of uRPL patients, enhanced levels of COX-2 might be the reason for increased PGE$_2$ expression. PGE$_2$ coupling with EP3 induces G$_{i1}$ and reduces cAMP, which can eventually lead to a downregulation of beta-hCG and progesterone and an upregulation of PAI-1. The decreased cAMP/PKA signaling inhibits the secretion of progesterone via PKA (50) and the transcription of beta-hCG via CREB (49). The activated EP3/G$_{i1}$ can stimulate p-ERK1/2 and p53, which eventually enhance PAI-1 gene transcription (52). These changes could prevent trophoblast implantation and placentation, finally causing recurrent pregnancy losses. COX-2, cyclooxygenase-2; G$_{i1}$, G protein alpha inhibitor 1; G$_q$, Gprotein alpha q; G$_s$, G protein alpha stimulator; PAI-1, plasminogen activator inhibitor type 1; p-ERK1/2, phosphorylated extracellular signal-regulated kinases 1/2; PGE$_2$, prostaglandin E$_2$; PGH$_2$, prostaglandin H$_2$; PKA, protein kinase A; uRPL, unexplained recurrent pregnancy losses.

deposition in the maternal-fetal interface. These changes could prevent trophoblast implantation and placentation, causing failed pregnancy maintenance. It is still unclear whether the observed findings are reflective of the uRPL or causative of it. We explored that the EP3 antagonist (L-798,106) led to a downregulation of EP3 expression without influencing the beta-hCG expression in JEG-3 cells. This suggests that L-798,106 might be a 'potential therapeutic candidate' for the treatment of uRPL. To further investigate the mechanism of uRPL, future studies are necessary to understand the role of the additional membrane receptors of PGE$_2$ (EP1, 2 and 4) and other members of G$_i$ family (G$_{i2}$ and G$_{i3}$) in the placenta.

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