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The role of G-protein coupled prostaglandin E2 receptors (EP2 and EP3) in unexplained recurrent miscarriage and cervical cancer

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1 Abbreviations

PGE2	prostaglandins E_2
AA	arachidonic acid
PLA_2	phospholipase A_2
COX	cyclooxygenase
PGH2	prostaglandin H2
PGD2	prostaglandin D2
PGI2	prostaglandin I2
TXA_2	thromboxane A_2
PGF2α	prostaglandin F2α
GPCR	G-protein coupled receptors
PPAR	peroxisome proliferator activated receptors
PKC	protein kinase C
PLC	phospholipase C
IP_3	inositol trisphosphate
DAG	diacylglycerol
MAPK	mitogen-activated protein kinases
PI3K	phosphoinositide 3-kinases
NFAT	nuclear factor of activated T cells
c-Src	tyrosine-protein kinase Src
NF κB	nuclear factor-kappa B
AC	adenylate cyclase
cAMP	cyclic adenosine monophosphate
PKA	protein kinase A
CREB	cAMP response element binding protein
ERKs	regulated kinases
EGR-1	growth response factor-1
EVTs	extravillous trophoblasts
FGF	fibroblast growth factor
VEGF	vascular endothelial growth factor
NK	natural kill
ITG	integrins
FAK	focal adhesion kinase
EGFR	epidermal growth factor receptor
HCC	hepatocellular carcinoma
EMT	epithelial mesenchymal transformation
MMP	matrix metalloproteinases
GSK-3	glycogen synthase kinase 3
RAC1	ras related C3 botulinum toxin substrate 1
CDC42	control protein 42 homolog

ECM	extracellular matrix
TIMPs	tissue inhibitors of metalloproteinase
PAI-1	plasminogen activator inhibitor-1
DC	dendritic cell
VEGF	vascular endothelial growth factor
bFGF	basic fibroblast growth factor
TGFβ	transforming growth factor-β
PDGF	platelet derived growth factor
TNFα	tumor necrosis factor α
IL	interleukin
LIF	leukemia inhibitory factor
MDSC	myeloid derived suppressor cells
IFN	interferons
CCL	chemokines chemokine ligand
XCL	chemokine c motif ligand

2 Publication list

2.1 Expression of trophoblast derived prostaglandin E2 receptor 2 (EP2) is reduced in patients with recurrent miscarriage and EP2 regulates cell proliferation and expression of inflammatory cytokines

Lin Peng, and, Mullikin Heather, LiLi Lin, Christina Kuhn, Martina Rahmeh, Sven Mahner, Udo Jeschke , Viktoria von Schönfeldt

J Reprod Immunol. 2020 Oct 1;142:103210. doi: 10.1016/j.jri.2020.103210.

2.2 Prostaglandin E2 receptor 3 (EP3) signaling promotes migration of cervical cancer via urokinase-type plasminogen activator receptor (uPAR)

Yao Ye, **Lin Peng**, Aurelia Vattai, Eileen Deuster, Christina Kuhn, Christian Dannecker, Sven Mahner, Udo Jeschke, Viktoria von Schönfeldt, Helene H. Heidegger

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3 Introduction

Prostaglandin E_2 (PGE2) is the most biologically active prostaglandin and exists at different concentrations in nearly all cell types, regulating various physiological and pathological processes in the reproductive, cardiovascular, musculoskeletal, endocrine, nervous and immune systems [1-4]. Therefore, it also influences the disease severity in several conditions such as infertility, gynecologic cancers, endometriosis, polycystic ovary syndrome and pre-eclampsia in female reproductive system [5-8]. PGE2 contributes to the regulation of cellular functions, immune response and cell-cell interaction [9]. This thesis summarizes our current understanding of PGE2 biosynthesis and the role played by the cyclooxygenase 2 (COX-2)-prostaglandin E_2(PGE2)-prostaglandin E_2 receptors (EPs) signaling pathway in mediating both the similar and different functions it exerts within the placenta and cancer cells through cell proliferation, adhesion, migration, invasion, secretion of matrix metalloproteinases, angiogenesis and immunosuppression. We assume that these similarities and differences can yield new insights or improve the treatment of placental diseases and cancers.

3.1 PGE2 synthesis and signaling cascade

Prostaglandin (PG) synthesis can be divided into three steps: Firstly, membrane phospholipid from via the enzyme phospholipase A2 (PLA2) is the source of arachidonic acid (AA). Secondly, cyclooxygenase (COX) enzymes oxidize the free AA to form prostaglandin endoperoxides. As the rate-limiting enzymes, COX-1 or COX-2 convert the prostaglandin endoperoxides specifically prostaglandin G2 into bioactive prostaglandin H2 (PGH2), an unsteady transitional compound which is speedily converted into other prostanoids by specific terminal PG synthases. Thirdly, depending

on particular enzymes (PGDS, PGES, PGFS and PGIS). When modified, PGH2 produces five different metabolites known as prostaglandin F2α (PGF2α), prostaglandin D2 (PGD2), prostaglandin I2 (PGI2), thromboxane A_2 (TXA_2), and PGE2. Aspirin and indomethacin, which are non-steroidal anti-inflammatory drugs, perform their anti-inflammatory functions by suppressing COX enzymes, thus inhibiting the production of PGs. All of these prostaglandins (PGD2, PGE2, PGI2 PGF2α, and TXA_2) modulate a series of cellular functions by binding to cell surface G-protein coupled receptors (GPCR) to intercede their impacts, also known as the EP, DP, FP, IP and TP receptors [10]. PGE2 is the most abundant PG observed in tissues. It exerts versatile physiological and pathological actions in autocrine and paracrine glands. PGE2 receptors consist of 4 GPCR, called EP1, EP2, EP3 and EP4, each of which differs in terms of intracellular signaling transduction properties, and applies assorted functions throughout the body [11,12]. PGE2 can also function as a ligand for three nuclear receptors, peroxisome proliferator activated receptors (PPARα, PPARβ/δ, PPAR γ) and therefore activate nuclear transcription factors [13].

EP1 is coupled to the G-protein subunit Gq (Gq) and induces the Ca^{2+} protein channel leading to the increasing of intracellular Ca^{2+} and activation of protein kinase C (PKC) by phospholipase C (PLC) that elevates the second messenger inositol trisphosphate (IP_3) and diacylglycerol (DAG) [14]. PKC stimulates the mitogen-activated protein kinases (MAPK) and phosphoinositide 3-kinases (PI3K) flagging pathways, and furthermore actuates the record factors called atomic factor of initiated T-cells (NFAT), proto-oncogene tyrosine-protein kinase Src (c-Src), and atomic factor-kappa B (NF-κB) [15-17]. [15-17]. EP2 and EP4 are coupled to the G-protein subunit (Gs) and subsequently invigorate adenylate cyclase (AC), prompting raised cyclic adenosine monophosphate (cAMP) creation that initiates protein kinase A (PKA) just

as the cAMP response element (CRE)-binding protein (CREB) pathway [18]. Moreover, EP2 and EP4 have been shown to activate PI3K through the β-arrestin signaling pathway [19]. Subsequently, PI3K activation promotes extracellular signal regulated kinases (ERKs) signaling and induces early growth response factor-1 (EGR-1) [20]. ERG-1 has been shown to increase COX-2 expression levels and activate nuclear β-catenin [21]. EP3 is coupled with Gi and inhibits AC, thereby decreases cAMP via Gi, elicits Ca^{2+} mobilization and suppresses PKA and PI3K activation. Surprisingly, EP3 could also be coupled to Gas, then induce NF-κB and CREB [12]. Figure 1.

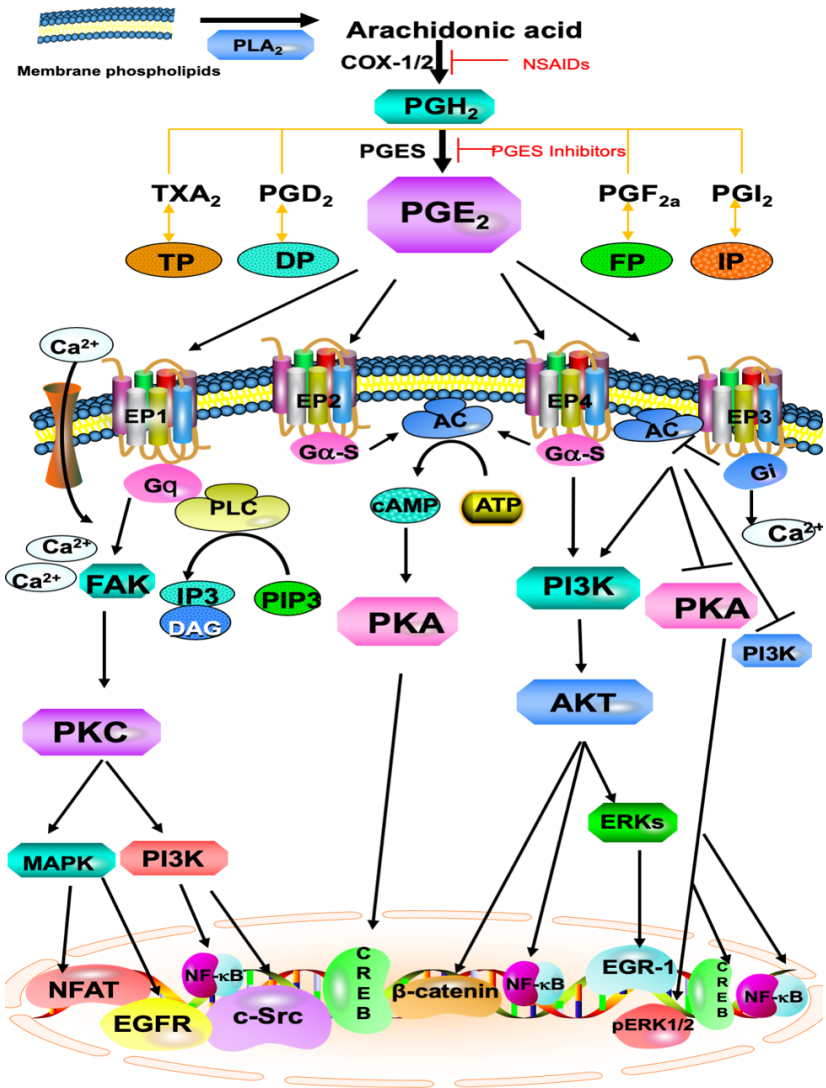

Figure 1: An overview of PGE2 biosynthesis and signaling cascade.

The membrane of phospholipids releases arachidonic acid (AA) by phospholipase A_2 (PLA_2). PGE2 is synthesized from arachidonic acid by cyclooxygenase (COX) enzymes and PGE_S via the unstable intermediate forms PGH2, which can be modified to TXA_2, PGD2, PGF2a and PGI2. NSAIDs exerts their anti-inflammatory actions by the inhibition of COX activity. PGE2 performs signaling in both paracrine and autocrine manners through four different types of EP-GPCRs, namely EP1, EP2, EP3, and EP4. EP1 couples with Gq and activate PLC which then increases IP_3 and DAG with the opening of Ca^{2+} channels leading to the increase of intracellular Ca^{2+} and activation of PKC that leads to the activation of nuclear factor of activated T-cells (NFAT), Proto-oncogene tyrosine-protein kinase Src (c-Src), nuclear factor-kappa (NFκB) and the MAPK pathways. EP2/EP4 couple with Gs and activates adenylate cyclase (AC) leading to higher levels of cyclic adenosine monophosphate (cAMP) that activates protein kinase A (PKA) as well as the cAMP response element-binding protein (CREB). EP3 couples with Gi to decrease cAMP levels. Multiple signaling pathways are involved, including EP3-PKA-pERK, EP3-PI3K, EP3-CREB and EP3-NFκB.

3.2 Similar characteristics in placenta and tumor cells

The placenta is a temporary organ that develops sustained apposition and communications between maternal and fetal tissues for gas and nutrient exchange, which shares significant similarities with cancer. Firstly, both the placenta and tumors have a lot of effectively partitioning cells, to be specific trophoblasts particularly cytotrophoblast cells in the placenta and cancer cells in tumors.

Secondly, placentation is a complex multistage process and can be divided into three distinct phases. The first phase, blastocyst apposition and attachment, is mediated by hormones secreted from the luminal and glandular epithelial cells. The second phase is regulated by endogenous steroidal hormones inducing changes in the epithelial, stromal cells and maternal vasculature by emitting embryonic signals. The final step is associated with trophoblast invasion and endometrial remodeling [22]. Cancer is also a multistage disease [23]. Tumorigenesis begins with a cluster of genetic mutations leading to abnormal cell proliferation, contributing to a monoclonal population outgrowth. Next, these neoplastic cells acquire the ability to resist apoptotic

signals and display increased angiogenesis. Finally, the adhesion of cancer cells breaks down the basement membrane, leading to cancer cells invasion and metastasis to other tissues and organs.

Thirdly, cytotrophoblast cells migrate through the anchoring villi with the capacity of proliferation, invasion, the ability to escape any attack from the maternal immune system, and survive under extreme hypoxic conditions and lack of blood supply, making them a model for comparison with tumors [24]. The invasion of these extravillous trophoblasts (EVTs) into the maternal decidua marks an essential step in embryo implantation and establishing a successful pregnancy. Broad vascularization and angiogenesis are standard highlights shared by malignancy cells and trophoblasts found at the maternal-fetal interface [25]. Both require blood provided by means of new vessels to develop. Cytokines, for example, fibroblast development factor (FGF) and vascular endothelial growth factor (VEGF) are essential parts that assume a pivotal job in tumor expansion, spiral artery remodeling, and angiogenesis [26].

Lastly, the maternal-fetal interface and the tumor microenvironment show amazingly comparable features. These comparable features consist of the similarities in immune cells involved including T cells, B cells, macrophages, natural killer (NK) cells, dendritic cells, and neutrophils. These infiltrating cells are more likely polarized to the immunosuppressive microenvironment. The placenta and tumor cells consist of similar cell types and express numerous common cytokines and chemokines.

3.3 Function of the PGE2 pathway in adhesion and proliferation

The focal adhesion pathway is a critical molecular pathway affected by suboptimal culture conditions during embryonic development and tumor progression [27,28].

PGE2 promotes embryo adhesion during the implantation window both in the natural cycle and in vitro fertilization [29]. PGE2 phosphorylates MAPK1/MAPK3 through EP2 receptors and boosts the adhesive capacity of trophoblasts by upregulating adhesion proteins such as focal adhesion kinase and intercellular adhesion molecule-1 [30]. Huang et al. reported that PGE2 lead to an increase in the expression levels of integrins (ITG) avβ3 which was associated with enhanced adhesion effect of trophoblast to the endometrium [31]. Similarly, PGE2 was also stated to be implicated in the adhesion of human hepatoma cell lines with increased secretion of ITG avβ3 [32]. Furthermore, PGE2 was shown to promote the adhesion of endothelial cells via cAMP and PKA dependent activation of Rac with increasing activity of avβ3 during tumor angiogenesis and inflammation [33]. Bai et al. demonstrated that PGE2 increased cell adhesion via EP1 and upregulated the expression of focal adhesion kinase (FAK) by activating the PKC/ c-Src and epidermal growth factor receptor (EGFR) signal pathway in hepatocellular carcinoma (HCC) [34].

Like cytotrophoblast cells, cancer cells have a high proliferation rate, but uncontrolled growth is elicited by malignant cells. Research projects on the role of PGE2 in trophoblast cell proliferation are still controversial. Nicola et al. came to conclusion that PGE2 did not influence HTR-8/SVneo cells at physiological concentrations, a human trophoblast derived cell line representing extravillous trophoblasts proliferation [14]. However, Biondi et al. proved that the proliferation of of HTR-8/SVneo cells is inhibited by PGE2 inhibits the proliferation [35]. Our work was based on of Biondi results, which demonstrated that the selective EP2 receptor antagonist (PF-04418948) reduced the proliferation of HTR-8/SVneo cells in vitro[36]. PPARα and PPARγ agonists promoted the proliferation of trophoblasts associated with increased synthesis of PGE2 [37]. We speculate that the effects elicited by PGE2 in

increasing or inhibiting trophoblasts proliferation seem highly dependent on its concentration levels and which G protein receptors are activated in cells. Besides, we deem that more in-depth research is necessary to obtain more accurate and comprehensive understanding of the effects of PGE2 on trophoblasts.

COX-2 could increase the proliferation of the ovarian cancer cell lines SKOV3 and OVCAR3 through PGE2 and NF-κB pathways [38-40]. Thanan et al. provided evidence suggesting that COX-2, which is involved in inflammation also regulates cell proliferation, demonstrating an increase of Oct3/4 and CD44v6 cells growth in bladder cancer [41]. PGE2 has been shown to promote the proliferation of cancer cells via multiple mechanisms. Ke et al. reported that PGE2 stimulates cellular proliferation via the EP4 receptor by activating the Wnt/β-catenin signaling pathway in endometrial cancer [42]. The Ras-MAPK kinase cascade was the main signaling pathway responsible for cell proliferation, and the downstream signaling Raf/MEK/ERKs and PI3K/AKT pathways are also involved [43]. PPARα has been associated with breast cancer proliferation, stimulating cyclin E expression, and mediating faster G1/S transition [44]. Previously published data indicated that PGE2 transactivates PPAR delta via PI3K-Akt signaling and promotes the development of colorectal adenomas [45]. These results point out that both PPARs and EPs are curial downstream mediators in PGE2 stimulated tumor growth.

3.4 Influence of the PGE2 pathway in invasion and migration

Following blastocyst adhesion, trophoblasts differentiate and acquire an invasive phenotype. Several pieces of evidence confirm that PGE2 facilitates trophoblast invasion. It has been reported that PGE2 produced from EVTs via leukemia inhibitory factor (LIF) and interleukin (IL)-1β stimulation promotes their invasion and migration

through EP1, EP2, and EP4 receptors [46]. Epithelial-mesenchymal transformation (EMT) is classically defined as an epithelial shift toward a more invasive and active mesenchymal state, modifying the adhesion molecules expressed within the cells [47]. EMT is involved in the process of migration and invasion, eliciting a decrease in the expression of the adherent junction protein E-cadherin, with concurrent elevation in the expression levels of vimentin, fibronectin, α-SMA, and Snail, ZEB [48]. EMT is an essential mechanism through which the highly plastic and dynamic germ layers and tissues proliferate during embryonic development and secrete exosomes to prepare for tissue invasion [49]; meanwhile, EMT is also involved in the process of invasion and metastasis of many solid tumors [50,51]. More research is needed to evaluate the mechanisms underlying the regulation of EMT by the COX-2-PGE2-EPs pathway. The COX-2-PGE2 axis was closely related to enhancing EMT in several cancer cells [52,53]. COX-2 elevated the level of PGE2, triggers EMT increased migration and invasion through matrix metalloproteinases (MMP) -2 and MMP-9 production and activates the NF-κB Pathway in colon cancer cells [54,55]. Additionally, the COX-2-PGE2-EP4 axis activated the PI3K-Akt-ERK and NF-κB pathways leading to miR-655 upregulation, and also enhances EMT in breast cancer [56]. The mechanism underlying the regulation of the nuclear transcription factor β-catenin by COX-2/PGE2 is responsible for the inhibition of EMT and invasion via the Gs -axin-GSK3β pathway in colon cancer cells [57]. Numerous papers indicated that PGE2 promotes cancer invasion by utilising multiple pathways, for instance the COX-2/PGE2-PI3K/AKT/GSK3β/β-catenin pathway in gastric cancer [58]. PGE2 has been proven to mediate prostate cancer cell invasion and migration through activation of the PI3K/AKT/mTOR pathway [59]. EP4 activated cAMP-PKA-pCREB and PKA are also linked to the WNT/β-catenin and NOTCH pathways, inhibiting glycogen synthase kinase 3(GSK-3) in breast cancer [60,61].

PGE2 has also been shown to promote the migration of EVTs by increasing the intracellular concentration of calcium and activating calpain via EP1 and EP4 receptors via cell division control protein 42 homolog (CDC42) and the activation of ras-related C3 botulinum toxin substrate 1(RAC1) and [15,46]. Nicola et al. found that the MAPK and ERK1/2 pathways related to PGE2 mediated stimulation of trophoblast migration [62]. PGE2 has been reported to induce migration by activating the phosphorylation of FAK and PKC/c-Src and EGFR signal pathways in hepatocellular carcinoma cells [34]. Woo SM et al. demonstrated that PGE2 regulates cell migration through EP2-PKA-CREB-Scr-STAT3 signaling pathways in renal cell carcinoma [63]. Kim Jae et al. emitted the idea that increased expression of the COX-2 lead to stimulation of PGE2 in the lung tumor microenvironment, which may initiate a mitogenic signaling cascade composed of EP4 -betaArrestin1-c-Src, hence promoting cancer cell migration [64]. PGE2 regulated β-catenin expression and promotes the growth and invasion of cholangiocarcinoma cells through the EP3-4R/Src/EGFR/PI3K/AKT/GSK-3β pathway [65]. Our findings were consistent with these results, supporting that PGE2 meditates EP3 to promote proliferation and migration with the activation of PAI-1, uPAR, and p-ERK1/2 in cervical cancer cells [66]. Other reports revealed that in HCC cells, different signaling pathways including p38 MAPK, MEK/ERK, PKC and PKA are involved in the tumor migration induced by PGE2 [67,68].

3.5 Effects of the PGE2 pathway on the extracellular matrix and angiogenic factors

The extracellular matrix (ECM) comprises extracellular macromolecules that maintain structural and biochemical support to surrounding cells, which regulates cell growth, migration, metastasis and differentiation [69]. Deregulation of the ECM is associated with the placenta's physiological development and tumorigenesis [70,71]. MMPs and

their inhibitors, tissue inhibitors of metalloproteinase (TIMPs), play a vital role in the balance of ECM remodeling, angiogenesis, embryogenesis, and cancer metastasis [72]. PGE2 induced trophoblast interferon secretion of MMP-1 and MMP-3 in ovine endometrial cells [73]. Li et al. provided evidence that PGE2 could mediate an increment in the secretion of MMP-9 after culturing trophoblasts with LPS in vitro [74]. Our previous study showed that the activation of COX-2-PGE2-Gi1-EP3-pERK1/2 could increase the expression levels of plasminogen activator inhibitor-1 (PAI-1) and inhibit ECM degradation in recurrent miscarriage patients [75]. PGE2 induced EP4 activation of the EGFR signaling pathway, which further results in degradation of the invadopodia-driven ECM and promotes invasion in breast cancer [76]. Furthermore, PGE2 can reduce dendritic cell (DC) migration by increasing TIMP-1 production in lung cancer.

Both the placenta and tumors need new vascular networks to support their highly proliferating and invading cells. Angiogenic factors are fundamental for the development and endurance of endothelial cells, and to invigorate the arrangement of new blood vessels, vascular endothelial cell migration and capillary development. Vascular endothelial growth factor (VEGF), transforming growth factor-β (TGFβ), basic fibroblast growth factor (bFGF), tumor necrosis factor α (TNFα), and platelet-derived growth factor (PDGF) are well-known major regulators of angiogenesis and play a key role in spiral artery remodeling, as well as tumor growth. Unlike to the firmly directed and organized vasculature of the placenta, the veins of tumors are profoundly atypical, dysfunctional, and uncontrolled. Research by Matsumoto et al. demonstrated the association between PGE2 and VEGF expression in COX-2 deficient mice during implantation [77]. Through its interaction with the EP2 and EP4 receptors, PGE2 was shown to irritate PKA pathway activation of AP2 and Sp1 as well as acetylation of

histone H3 to regulate the VEGF gene's transcriptional activity during placental and embryonic development [78]. The expression of COX-2 is highly correlated with TGF-β, VEGF and the microvascular density in neoplasms [79,80]. EP2/EP4 receptors mediate PGE2 induction of VEGF in ovarian cancer cells [81]

Interestingly, PGE2 is capable of inducing the pro-angiogenic chemokine called CXCL1, to increase tumor microvessel formation, cell invasion and cell growth in colorectal cancer [82]. Further evidence showed that VEGF and bFGF increase the production of COX-2 and PGE2 in endothelial cells [83]. We hypothesise that PGE2 regulates VEGF and bFGF via a positive feedback loop, which further increases the production of PGE2 and angiogenic factors.

3.6 Influence of the PGE2 pathway on immunosuppressive mediators

PGE2 is generally known as a major immunosuppressive middle person hampering supportive of incendiary reactions in different immune cells and building up an immunosuppressive microenvironment. PGE2 is generally known to suppress a wide range of immune cells, including Th1 cells, macrophages, neutrophils, cytotoxic T cells, and NK cells, while promoting Th2 cells, Th17 cells, and Treg cells in the microenvironment [84,85].

The maternal-fetal interface immune-microenvironment establishes maternal tolerance towards the semi-allogeneic fetus. Dysfunction of these immune cells leads to imbalances of the maternal-fetal interface, which may contribute to pathological conditions of pregnancy, such as recurrent miscarriages and preeclampsia. The effects of PGE2 systemic alterations on immune cells were also detectable, particularly concerning helper T cells type 2 polarization, which was evident in the second trimester of pregnancy [86,87]. Mir-494 in decidual mesenchymal stem cells reduced the

production of PGE2, which affects macrophage polarization of the M2 type and leads to preeclampsia [88]. Our research team reported that the lower expression levels of PPARγ are associated with M2 polarization in decidual macrophages leading to recurrent miscarriage [89]. Nonetheless, additional efforts are needed to further assess the role of the PGE2 Pathway in the maternal-fetal interface and the mechanism of its impact on immune cells during pregnancy.

There is a critical role played by the tumor-specific immunosuppressive microenvironment that leads to tumor progression and tolerance from immune surveillance. PGE2 can switch the Th1/ Th2 balance to promote Th2 responses, which tend to be dominant in neoplastic microenvironments. There were evidences that PGE2 has an effect on inhibiting the production of Th1 cells cytokines IL-2 and IFN-γ and increases the production of Th2 cytokines IL-4, IL-5 and IL-10 in cancer [90-92]. PGE2 induced M2 macrophage polarization via EP4 receptors promotes tumor growth and metastasis in lung cancer [93]. The COX-2-PGE2 axis played a central role in enhancing the function of myeloid-derived suppressor cells (MDSC) associated with immunosuppression and inhibits CD8+ T cells' ability to destroy malignant tumor cells [94,95]. Furthermore, Obermajer et al. elucidated that PGE2 inhibits the production of CXCL12, CXCR4 and blocks the recruitment of MDSC in the microenvironment of ovarian cancer [96]. PGE2 can promote the activation, maturation and migration of DC, but inhibits its ability to attract T cells to tumor cells [97,98]. The COX-2 - PGE2 axis decreased the expression of NK cell receptors on tumor cells [99,100]. PGE2 also suppressed the activity of NK cells by limiting their migration, secretion of cytotoxic substances, and enhanced interferon (IFN)-γ and TNF-α production by acting on EP2 and EP4 receptors during tumor progression in breast cancer [101]. More importantly, PGE2 produced by tumor cells annihilates the function of NK cells by decreasing the

secretion of chemokine ligand (CCL) 5 and Xcl1 chemokine (C motif) ligand 1, resulting in cancer immune evasion [102]. PGE2 also modulated the formation and function of Treg cells. Barateli et al. reported that the COX-2-PGE2 axis enhances FOXP3 gene expression and activates Treg functions of $CD4^{+}$T cells in lung cancer [103,104]. Additionally, Yuan et al. demonstrated that the COX-2-PGE2 axis attracted more Treg cells and inhibited cytotoxic T cell functions, leading to gastric cancer [105] and prostate cancer progression [106]. EP2 dependent signals from PGE2 have been proven to promote CD4+ T cells' polarization to the Th17 phenotype via the upregulation of IL23R in prostate cancer [107]. Finally, PGE2 induced IL-23 leads to Th17 cells expansion through the cAMP/PKA signaling transduction pathway in the tumor microenvironment [108].

3.7 Aims of the studies

Despite numerous studies concerning the COX-2-PGE2-EPs axis in regulation the process of pregnancy and the development of cancer, a precise understanding of EPs receptor in several physiological and various reproductive disease should be conducted in more studies. So that certain EPs ligands or antagonists can be used clinically to improve the reproductive rates and the disease-free survival rate of cancers.

3.7.1 Expression of EP2 in trophoblast with recurrent miscarriage

PGE2 involved in female reproduction, mainly in implantation, decidualization, blastocyst growth and development. It is necessary to understand the molecular regulation of EPs receptor in maternal-fetal interface, which help us more precise cure recurrent miscarriage. Previous research of our group showed that EP3 signaling contribute to remodel of extracellular matrix and produce of hormone in the maternal-

fetal interface of recurrent miscarriage. However, the expression and function other EPs in the maternal-fetal interface is still unclear and need to be explore.

Therefore, we aimed to identify the expression level of EP1, EP2 and EP4 in the maternal-fetal interface with recurrent miscarriage compared to normal pregnancy. We then further explore the potential function of EP2 in EVTs relevance with recurrent miscarriage progression *in vitro*.

3.72 The pathological mechanism of EP3 signaling in cervical cancer

PGE2 through EP2 and EP4 signaling pathway affected the proliferation and angiogenesis of cervical cancer cell lines. Our latest publication showed poor prognosis in overall survival rates of cervical cancer patients due to high expression of EP3 in both adenocarcinoma and squamous cell. To elucidate the biological molecular mechanism of EP3 involved in cervical cancer and the downstream targets of EP3 affects the cervical cancer development.

Thus, we searched the relevant evidences from publicly available databases by bioinformaitcs analysis and desiged the experiment to evaluate the effects of EP3 in cervival cancer cell proliferation and migration and to figured out the associated mechanisms.

3.8 Author contributions of two publications

The whole study was designed by Udo Jeschke and Viktoria von Schönfeldt. Statistical analysis and compiling the manuscript was done by Lin Peng and Yao Ye after performing the whole experiment. Christina Kuhn did the immunohistochemistry staining. Heather Mullikin, Aurelia Vattai, Christian Dannecker,Sven Mahner and Eileen Deuster revised the manuscript. Martina Rahmeh and Lili Lin guided the

expriments. To get into agreement on the whole document, all authors scrutinized the whole record and agreed with the publications of the hard copy.

4 Publication I

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Expression of trophoblast derived prostaglandin E2 receptor 2 (EP2) is reduced in patients with recurrent miscarriage and EP2 regulates cell proliferation and expression of inflammatory cytokines

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Expression of trophoblast derived prostaglandin E2 receptor 2 (EP2) is reduced in patients with recurrent miscarriage and EP2 regulates cell proliferation and expression of inflammatory cytokines

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ABSTRACT

Backgroud: Prostaglandin E2 (PGE2), an inflammatory mediator, modulates cytokines, regulates immune responses in reproductive processes and stimulates inflammatory reactions via the prostaglandin E2 receptor 2 (EP2). However, the regulatory effects of EP2 signaling on trophoblasts and its role in unexplained recurrent miscarriage (uRM) remains unclear.

Patients and methods: A total of 19 placentas from patients with a history of more than two consecutive pregnancy losses of unknown cause (uRM group) and placentas of 19 healthy patients following a legal termination of their pregnancy were used for PGE2 receptor (EP1, EP2 and EP4) expression analyses via immunohistochemistry. Double immunofluorescence was also used to identify EP2 expressing cells in the decidua. Finally, HTR-8/SVneo cells were used to clarify the role of EP2 in *in vitro* experiments.

Results: The expression of EP2 and EP4 was found to be reduced in the syncytiotrophoblast and decidua of uRM patients. A selective EP2 receptor antagonist (PF-04,418,948) reduced the proliferation and secretion of ß-hCG, inhibited interleukin -6 (IL-6) and interleukin-8 (IL-8) and up-regulated the production of the tumor necrosis factor-α (TNF-α) and plasminogen activator inhibitor type 1 (PAI-1) in HTR-8/SVneo cells *in vitro*.

Conclusion: PGE2-EP2 signaling pathway may represent a novel therapy option for uRM. The involvement of EP2 in uRM acts perhaps via inflammatory cytokines and indicates that the PGE2-EP2 signaling pathway might represent an unexplored etiology for uRM.

1. Introduction

Recurrent miscarriage (RM) is defined as the consecutive occurrence of two or more clinically (as assessed by an ultrasonographic or histopathological examination) failed pregnancies, according to the Practice Committee of the American Society for Reproductive Medicine (Anon, 2020). Related risk factors contributing to RM include anatomical factors, antiphospholipid syndrome (APS), embryonic factors, endocrine disorders, genetic factors, inherited thrombophilia and lifestyle factors (Anon, 2020). However, nearly 50 % of RM cases are still without identifiable factors and are therefore labelled "unexplained recurrent miscarriage" (uRM). Increasing evidence shows that uRM is linked to an abnormal inflammatory microenvironment at the maternal-fetal interface, which may be associated with cytokine dysregulation (Zenclussen et al., 2006; Kwak-Kim et al., 2009; Saifi et al., 2014; Galgani et al., 2015).

The inflammatory microenvironment of the maternal-fetal interface consists of inflammatory factors and placental cells. Most common are extravillous trophoblast cells, decidual stromal cells and decidual immune cells that consist, among others, of NK cells, T-cells, macrophages

Abbreviations: uRM, unexplained recurrent miscarriage; EP2, prostaglandin E2 receptor 2; IRS, immunoreactive score; IL-6, interleukin -6; IL-8, interleukin -8; TNF-α, tumor necrosis factor-α; IFN, interferon; PGE2, prostaglandin E2; ECM, extracellular matrix; Gq G, protein alpha q; AC, adenylyl cyclase; APS, antiphospholipid syndrome; IHC, immunohistochemistry; cAMP, cyclic adenosine monophosphate; CREB cAMP, response element binding protein.

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and dendritic cells. Experimental studies have demonstrated immunological defects in uRM patients, including an elevated Th1/Th2 or M1/M2 ratio, tregs deficiency and an excessive Th17 cell number (Wu et al., 2014; Dong et al., 2017; Zhu et al., 2017; Jiang et al., 2018). The local production of Th2 type cytokines, such as interleukin-4 (IL-4), IL-5, interferon-α(IFN-α) and IL-10, at the maternal-fetal interface assist in maintaining pregnancy. Meanwhile Th1 cells secrete pro-inflammatory cytokines, such as IL-1β, IL-6, IL-12 and TNF-α, that mediate fetal rejection (Saito et al., 2010; Yuan et al., 2015).

Prostaglandin E2 (PGE2) is a key mediator of inflammation and can modulate immune responses as well as regulate cytokine production in order to balance the Th1/Th2 ratio (Bao et al., 2011; Kalinski, 2012; Morimoto et al., 2014; Kawahara et al., 2015). Furthermore, PGE2 has been reported to play a pivotal role in female reproduction, particularly in blastocyst spacing, implantation, decidualization, blastocyst growth and development (Salleh, 2014; Niringiyumukiza et al., 2018). It exerts its effect by binding one of four G protein-coupled receptor subtypes (EP1, EP2, EP3, EP4), each of which has a distinct signal transduction properties, and exerts diverse physiological functions (Sugimoto and Narumiya, 2007; Kawahara et al., 2015). EP1 is coupled to the G protein alpha q (Gq) to mobilize intracellular Ca^{2+}, EP3 is mainly coupled to the G protein alpha inhibitor (Gi) to inhibit adenylyl cyclase (AC) and finally EP2 and EP4 are coupled to the G protein alpha stimulator (Gs) to activate AC (Kawahara et al., 2015). EP receptors are heptahelical transmembrane receptors, the localization of these receptors can be expected in the plasma membrane. But G protein coupled receptors (GPCR) can also be localized in intracellular membranes, including nucleus, endoplasmic reticulum, Golgi apparatus and endosomal bodies. Former work showed that EP1 and EP2 receptors were observed perinuclear in human skin (Konger et al. 2005). Moreover, EP1, EP3, and EP4 receptors can be located on the nuclear membrane in other cell types and tissues (Schlötzer-Schrehardt et al. 2002). PGE2 receptors are responsive to PGE2 production at the nucleus or the endoplasmic reticulum and are involved in nuclear second messenger signaling. The EP1 receptor is known to be coupled to nuclear calcium signaling in cells (Breyer et al. 2001). Our previous study demonstrated that EP3 signaling plays a vital role in the regulation of the inflammatory microenvironment, hormone production and extracellular matrix remodeling in the maternal-fetal interface of uRM patients (Ye et al., 2018). However, a deeper understanding of the contribution of PGE2 at the maternal-fetal interface in uRM patients and the role of the remaining membrane receptors of PGE2 (EP1, EP2 and EP4) still needs to be explored. Evidence from experiments with PGE2 receptor type2 (EP2)-deficient mice has shown disturbances in ovulation, fertilization, embryo development and implantation (Tilley et al., 1999). Moreover, Vilella et al. identified that the inhibition of EP2 prevents embryo adhesion, which can then be reversed by adding an EP2 agonist to an in vitro model of embryonic adhesion (Vilella et al., 2013). EP2 and EP4 have also been associated with an increase in cAMP, which is elevated by PGE2. Meanwhile, cAMP exerts similar Th1-selective suppression in arthritis (Chen et al., 2010). It has also been found that PGE2 stimulates the production of IL-1β and TNFα via EP2 and EP4 receptors in order to regulate the inflammatory microenvironment in the myometrium during pregnancy (Zhang et al., 2019). Another example, in chronic rhinosinusitis with nasal polyps, PGE2 activates the Akt and NF-κB signal pathways via EP2 and EP4 receptors to increase the production of IL-6 and IL-8 expression (Cho et al., 2014). However, despite our understanding on the role of PGE2, it remains unknown how it regulates inflammatory factors at the maternal-fetal interface through EP2.

We analyzed the expression of the remaining PGE2 receptors (EP1. EP2 and EP4) in first trimester placentas and found EP2 was downregulated in both the syncytium and decidua. Therefore, we hypothesize that the expression of EP2 in trophoblasts and decidual cells might play an important role at the maternal-fetal interface during placentation and early pregnancy maintenance. We believe that EP2 signaling may regulate the inflammatory microenvironment balance and hormone secretions, which may possibly be another pathological mechanism that leads to uRM.

2. Materials and methods

2.1. Ethics statement

Tissues samples for immunohistochemistry (IHC) and Immunofluorescence staining were obtained from 19 patients with a history of more than two consecutive pregnancy losses of unknown cause (uRM group) and of 19 healthy patients following a legal termination of their pregnancy (control group) in Munich, Germany. Exclusion criteria for the uRM group, described thoroughly in our previous studies (Rogenhofer et al., 2012), include infectious diseases, uterine anomalies, endocrinological dysfunctions, hyperprolactinemia, hyperandrogenemia, thyroidal dysfunctions, autoimmune disorders, deficiencies in coagulation factors as well as fetal and parental chromosomal disorders. All placentas were obtained within the first 24 h after diagnosis and without any prior hormonal treatment. The samples were obtained surgically via dilation and evacuation and without any pharmaceutical induction. These samples were fixed immediately in 4% buffered formalin for 20–24 h and then embedded in paraffin for conservation. The study was approved by the ethical committee of the Medical Faculty, Ludwig-Maximilian-University of Munich (Number of approvals: 337−06) and informed and written consent was obtained from each patient before the study. Samples and clinical information were anonymized and encoded for statistical work-up.

2.2. Immunohistochemistry

Immunohistochemical (IHC) staining has been previously described by our lab. The placentas of the two groups were conserved in paraffin slices. The tissue slides were then deparaffinised in xylol for 20 min, washed in 100 % ethanol, and then finally incubated in methanol/H2O2 for a further 20 min. This process was followed by the rehydration of the slides via distilled water. After washing the slides in PBS, they were then incubated with a blocking solution (Reagent 1, Zytochem-Plus HRP-Polymer-Kit (mouse/rabbit)) for 20 min in order to avoid non-specific binding of the primary antibodies at room temperature. Each slide was separately incubated with a primary antibody at 4 °C for 16 h. All antibodies used are listed in Table 1. After washing with PBS, the secondary antibodies/complexes of the ABC detection kit (Vector Laboratories) were applied following the manufacturer's protocols to detect reactivity. Visualization was reached after 2 min with a DAB substrate

Table 1
Antibodies used for immunohistochemical characterization and double immunofluorescence of placental tissue samples.

Antibody	Isotype	Clone	Dilution	Source
EP1	rabbit IgG anti-human	polyclonal	1:200 in PBS[a]	Abcam, Cambridge, UK
EP2	rabbit IgG anti-human	polyclonal	1:300 in PBS[a] 1:300 in Dako [b]	Abcam, Cambridge, UK
EP4	rabbit IgG anti-human	polyclonal	1:50 in PBS[a]	Abcam, Cambridge, UK
Prolactin	Mouse IgG anti-human	Monoclonal	1:500 in Dako [b]	Bio-Rad, California, USA
HLA-G	Mouse IgG anti-human	Monoclonal	1:200 in Dako [b]	Novus, Colorado, USA
Cy-3 [b]	goat IgG anti-rabbit	polyclonal	1:500 in Dako [b]	Dianova, Hamburg, Germany
Cy-2 [b]	goat IgG anti-mouse	polyclonal	1:100 in Dako [b]	Dianova, Hamburg, Germany

[a] antibodies used for immunohistochemistry, b antibodies used for immunofluorescence.

and chromogen (3, 3′-diaminobenzidine DAB, Dako, Glostrup, Denmark). Two independent observers read the expression intensity and distribution under a Leitz (Wetzlar, Germany) microscope by using a semi-quantitative immunoreactive score (IRS) [28]. The IRS is calculated by multiplying the intensity of cell staining (0: none; 1: weak; 2: moderate; 3: strong) with the percentage of positively stained cells (0: no staining; 1: <10 % of the cells; 2: 11–50 %; 3: 51–80 %; 4: >80 %). Antigen retrieval was performed in EDTA (pH 8) at 124 °C for 5 min. The placenta is divided into two main areas: the villous tissue and the decidua. In the villous tissue, the syncytiotrophoblast is the main tissue expressing EPs and was therefore evaluated by IRS in our paper. Within the decidua, we evaluated the extravillous trophoblast as well as decidual stroma cells via IRS. The samples were incubated in goat serum for 1 h to block nonspecific proteins, then incubated with primary antibody (Table 1) at 4 °C overnight. After a 1-h incubation with the secondary antibody at 37 °C, the nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The tissues were observed and images captured by fluorescence microscopy (Lecia DMi8 microscope, Leica Microsystems).

2.3. Double immunofluorescence staining

To examine the characteristics of EP2 within the placenta, we used the same paraffin-embedded slides but with a double immunofluorescence staining. The same experimental steps were performed as in the immunohistochemistry analysis. However, the slides were blocked with an ultraviolet blocking solution (Ultra V Block, Lab Vision, Fremont, CA, USA) and then incubated with the primary antibodies at 4 °C for 16 h. 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. All antibodies used are listed in Table 1. After incubation, the secondary antibodies, Cy3-labeled goat anti-mouse IgG (1:500 in background reducing antibody diluent (Dako)) and Cy2-labeled goat anti-rabbit IgG (1:100 in background reducing antibody diluent (Dako)), were applied (both antibodies form Dianova, Hamburg, Germany). Finally, after washing and drying, the slides were embedded in a DAPI containing solution (Vectastain, Vector Laboratories) in order to stain the nucleus blue. Confocal laser scanning microscope images were acquired with Zeiss LSM 880 with Airyscan model for high-resolution visualization and analyzed with ZEN blue software. Tissues incubated with the same concentration of secondary antibody served as background controls.

2.4. Cell culture

The HTR-8/SVneo (ATCC, CRL-3271) cell line is derived from human invasive extravillous trophoblast cells. It was created by transfecting the cells that grew out of chorionic villi explants from human first-trimester placentas with the gene encoding for simian virus 40 large T antigen. The cells were cultured in RPMI 1640 medium + Gluta MAXTM (Gibco, USA), supplemented with 10 % fetal bovine serum (Gibco, USA) and maintained at 37 °C in a humidified atmosphere with 5% CO2. The medium was changed every three to four days.

2.5. Cell viability assay

HTR-8/SVneo cells were seeded with the density of 8000 cells/well in 96-well plates in groups of six. After 24 h, the cells were incubated with different concentration of a selective EP2 receptor antagonist (PF-04,418,948) for a further 24 h. A solution of 5 mg/mL MTT [3-(4,5-dimethhylthiaoly)-2,5-diphenyltetrazolium bromide] (Sigma) in phosphate-buffered saline (PBS) was prepared. Following incubation, 20 µg MTT solution was added to each well for 1.5 h at 37 °C. The culture medium along with MTT was then removed. Finally, 200 µL of DMSO was added to each well to dissolve the visible formazan crystals and mixed thoroughly on the shaker for 5 min at room temperature. The MTT assay is a colorimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,2-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. The absorbance can be used to determine the vitality ability of cells to reduce MTT, which represents the activity of mitochondria. MTT assay assess can be used as an indicator of cell survival numbers.

The optical density (OD) was read at 595 nm using an Elx800 universal Microplate Reader.

2.6. BrdU proliferation assay

Cell proliferation was analyzed using a 5-bromo-2′-deoxyuridine (BrdU) labeling and detection kit (Roche). 8000 HTR-8/SVneo cells were seeded in each well of a 96-well culture plate for 24 h with varying substrate concentrations. According to the manufacturer's protocols, the cells were fixed and the incorporation of BrdU into the DNA was measured at 450 nm using an Elx800 universal Microplate Reader.

2.7. Hormone measurement

Supernatants of HTR-8 cells in a 24-well plate were centrifuged at 13,200 x g for 10 min to remove debris after incubation with different concentration of PF-04,418,948 **for 36 h.** ß-hCG and progesterone quantifications were carried out according to the manufacturer's instructions on an ADVIA Centaur XP auto analyzer (Siemens Medical Solution Diagnostics) as described in our previous publications (Ye et al., 2018).

2.8. Enzyme linked immunosorbent assay

After incubation of the different concentrations of PF-04,418,948 into RPMI1640 with 10 % FBS for 6 h, the levels of IL-6, IL-8, TNF-α, IL-1β and PAI-1 in the supernatants of HTR-8/SVneo cells were measured by Quantikine ELISA Human Serpin Human IL-6, Human IL-8/CXCL8, Human TNF-α, Human IL-1βand Human Serpin E1/PAI-1 Immunoassay (R&D Systems, Minneapolis, USA). We added different concentrations of PF-04,418,948 into RPMI1640 with 10 % FBS and incubated for 6 h in a 37 °C 5% CO2 incubator for testing the cytokine level.

Firstly, all standards and supernatants were incubated in a microplate coated with a specific monoclonal antibody for 2 h. After three washes, the human Serpin conjugate was incubated for another 2 h. Substrates were incubated in the dark for 30 min, stopped by the stop solution and then analyzed in a microplate reader (DYNEX Technologies, MRX II) using wavelengths between 450 and 570 nm. The concentration of IL-6, IL-8, TNF-α, IL-1β and PAI-1 were determined via comparison with a standard curve (according to the manufacturer's instructions).

2.9. Data analysis

All data is presented as the Mean ± Standard deviation (SD). Differences between two groups were analyzed using an independent sample *t*-test and among multiple groups were carried out by one-way ANOVA and the post-hoc Tukey's multiple comparison or Dunnett's test. P values less than 0.05 were considered to be statistically significant. All statistical values were calculated using GraphPad Prism, Version 7 (GraphPad Software, San Diego CA, USA).

3. Results

3.1. Clinical data of the uRM group and control group

The mean age of the women in the uRM group was 37.76 ± 4.88 years old and the control group was 35.78 ± 5.88 years old (P = 0.41,

student's *t*-test). Mean gestational age of the uRM group was 9.09 ± 2.17 weeks and of the control group 9.71 ± 1.88 weeks (P = 0.66, student's *t*-test). Both gravidity times (3.42 ± 1.90 vs 3.11 ± 1.08, P = 0.78, Mann-Whitney test) and the parity times of two groups showed no significant differences (0.94 ± 0.94 vs 1.63 ± 1.12, P = 0.06, Mann-Whitney test). Demographic and clinical data are illustrated in Table 2.

3.2. Expression of EP1, EP2, EP4 receptors in the first-trimester human placentas

First, to evaluate the expression of the other PGE2 receptor subtypes (EP1, EP2, EP4) in first trimester tissues, we performed an immunohistochemical analysis on 38 human placentas (19 uRM samples and 19 healthy controls). The placenta tissues were divided into the syncytium and decidual areas and scored via IRS separately.

Staining of EP1, EP2 and EP4 was observed in the cytoplasm of syncytium cells and decidual cells of first-trimester placentas in both the healthy control group and the uRM group (Fig. 1. A, B, D, E, H, I, K, L, N, O, Q, R). The expression of EP1 was upregulated in the syncytium and decidua in uRM tissues compared with control tissues; however, the differences were not statistically significant in the syncytium (P = 0.160, Fig. 1 A, B and C) or decidua (P = 0.309, Fig. 1 D, E and F) between the uRM group and the control group according to the IRS. A significant decrease in the expression of EP2 was observed in the syncytium (p = 0.003, Fig. 1 H, I and G) and decidua (p = 0.036, Fig. 1 K, L and M) samples in the uRM group compared to that of the control group. With regard to EP4, the expression of EP4 was significantly decreased in the syncytium (p = 0.008, Fig. 1 N, O and P) and decidua (p = 0.034, Fig. 1 Q, R and S) samples in the uRM group compared to that of the control group.

Our study aimed to analyze the separate roles of EP2 and EP4 signaling mechanisms of uRM. We started by exploring the role of the EP2 pathway within the pathogenesis of uRM.

3.3. Cellular localization of EP2 in first-trimester placentas

To further confirm the localization and expression of EP2 in paraffin-embedded sections from first-trimester human trophoblasts and maternal decidua, we conducted a double immunofluorescence staining. DAPI stained the nucleus, HLA-G was used as a marker for trophoblasts and prolactin was used as a marker for stromal cells.

EP2 co-expressed with HLA-G mainly in the cytoplasm of trophoblasts (Fig. 2 A–D) and co-expressed with prolactin predominantly in the cytoplasm of stromal cells (Fig. 2 E–H) in patients with uRM.

3.4. Influence of an EP2 antagonist on the proliferation of HTR-8/SVneo cells in vitro

Next, we investigated whether EP2 signaling was involved in the proliferation capacity of trophoblasts. To achieve this, we used HTR-8/SVneo cells as our model for human trophoblast cells.

After 24 h of treatment with PF-04,418,948 at concentrations of 1 nM, 10 nM, and 100 nM or the vehicle control (DMSO, 0.1 %), an MTT assay was used to assess the viability of HTR-8/SVneo cells. PF-04,418,948 (10 nM) significantly decreased the viability of cells by 14 % (P = 0.047, Fig. 3 A), and the 100 nM concentration decreased the viability by 15 % (P = 0.025, Fig. 3 A). However, the 1 nM concentration did not result in a significantly changed cell viability (P = 0.1184, Fig. 3 A). Given that the MTT assay is designed to measure the number of metabolically active cells, we next conducted a BrdU assay, which assesses the proliferation of cells by quantifying the BrdU incorporated into DNA during the S phase. A significant reduction in the proliferation of HTR-8/SVneo cells was seen with the 1 nM concentration (17 % reduction; P = 0.036, Fig. 2 B), the 10 nM concentration (23 % reduction; P = 0.0001, Fig. 2 B) and the 100 nM concentration (20 % reduction; P = 0.005, Fig. 2 B) compared with the proliferation in the control group. In agreement with the MTT assay, the BrdU assay also indicated that PF-04,418,948 inhibited cell proliferation. These results suggest that EP2 might play a key role in trophoblast proliferation.

Table 2
Demographic and clinical characteristics of the study population.

Characteristic	Normal pregnancy n = 19	uRM n = 19	*P* Value
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)	0.78
parity	1.63 ± 1.12 (0–4)	0.94 ± 0.94 (0–3)	0.06

3.5. An EP2 antagonist affects the secretory function of trophoblasts in vitro

Our next step was to explore the EP2 signaling pathway underlying the regulatory effects of hormones in HTR-8/SVneo cells. After 36 h of incubation with 1 nM, 10 nM, or 100 nM PF-04,418,948 or the vehicle control (DMSO, 0.1 %), ß-hCG production was shown to be inhibited by 24 % with the 100 nM concentration of PF-04,418,948 (P = 0.017, Fig. 4 A), while ß-hCG production was not significantly influenced by either the 1 nM concentration (P = 0.758, Fig. 4 A) or the 10 nM concentration (P = 0.998, Fig. 4 A). Meanwhile, progesterone levels in HTR-8/SVneo cells remained unaffected at the 1 nM concentration (P = 0.230, Fig. 4 B), the 10 nM concentration (P = 0.136, Fig. 4 B) and the 100 nM concretion (P = 0.121, Fig. 4 B) in comparison with the levels in the vehicle group.

To further confirm that PF-04,418,948 had a prominent effect on the expression of pro-inflammatory cytokines in the different concentration groups, we measured the levels of IL-6, IL-8, TNF-α, IL-1β and PAI-1 secreted into the culture medium by HTR-8/SVneo cells. PF-04,418,948 reduced the secretion of IL-6 by 14 % at 1 nM (P = 0.0004, Fig. 5 A), 8% at 10 nM (P = 0.103, Fig. 5 A) and 25 % at 100 nM (P < 0.0001, Fig. 5 A) compared to that in the control group. PF-04,418,948 significantly decreased IL-8 in a dose-dependent manner compared to the control group, with reductions of 21 %, 19.4 %, and 20 % at 1 nM, 10 nM, and 100 nM concentrations respectively (P = 0.004, 0.007, and 0.005 Fig. 5 B). Interestingly, PF-04,418,948 significantly promoted the secretion of TNF-α at concentrations as low as 1 nM (55.8 % increase; P = 0.003, Fig. 5 C) and as high as 100 nM (41 % increase; P = 0.023, Fig. 5 C). IL-1β levels were not significantly altered at concentrations of 1 nM (P = 0.633, Fig. 5 D), 10 nM (P = 0.639, Fig. 5 D) or 100 nM (P = 0.949, Fig. 5 D) when compared with the levels in the vehicle group. The production of PAI-1 was significantly increased by 63 % at the 100 nM concentration (P = 0.005, Fig. 5 E), while its level was not significantly influenced by the 1 nM (P = 0.923, Fig. 5 E) or 10 nM (P = 0.237, Fig. 5 E) concentrations.

4. Discussion

In the present study, we demonstrated that the expression level of EP2 was significantly decreased in trophoblasts and decidua at the maternal–fetal interface in samples from the uRM group compared to samples from the normal group. In addition, the selective EP2 antagonist (PF-04,418,948) inhibited the production of ß-hCG, IL-6 and IL-8 and elevated the secretion of PAI-1 and TNF-α by HTR-8/SVneo cells.

This data suggests a potential role of PGE2-EP2 signaling as a pathologic mechanism for uRM.

Four EP isotypes are expressed in human and non-human trophoblasts of placentas (Nicola et al., 2005; Waclawik et al., 2013). HTR-8/SVneo cells have been shown to express all four of these receptors (Nicola et al., 2005). There is evidence that PGE2 stimulates extravillous trophoblast (EVT) migration by signaling through EP1

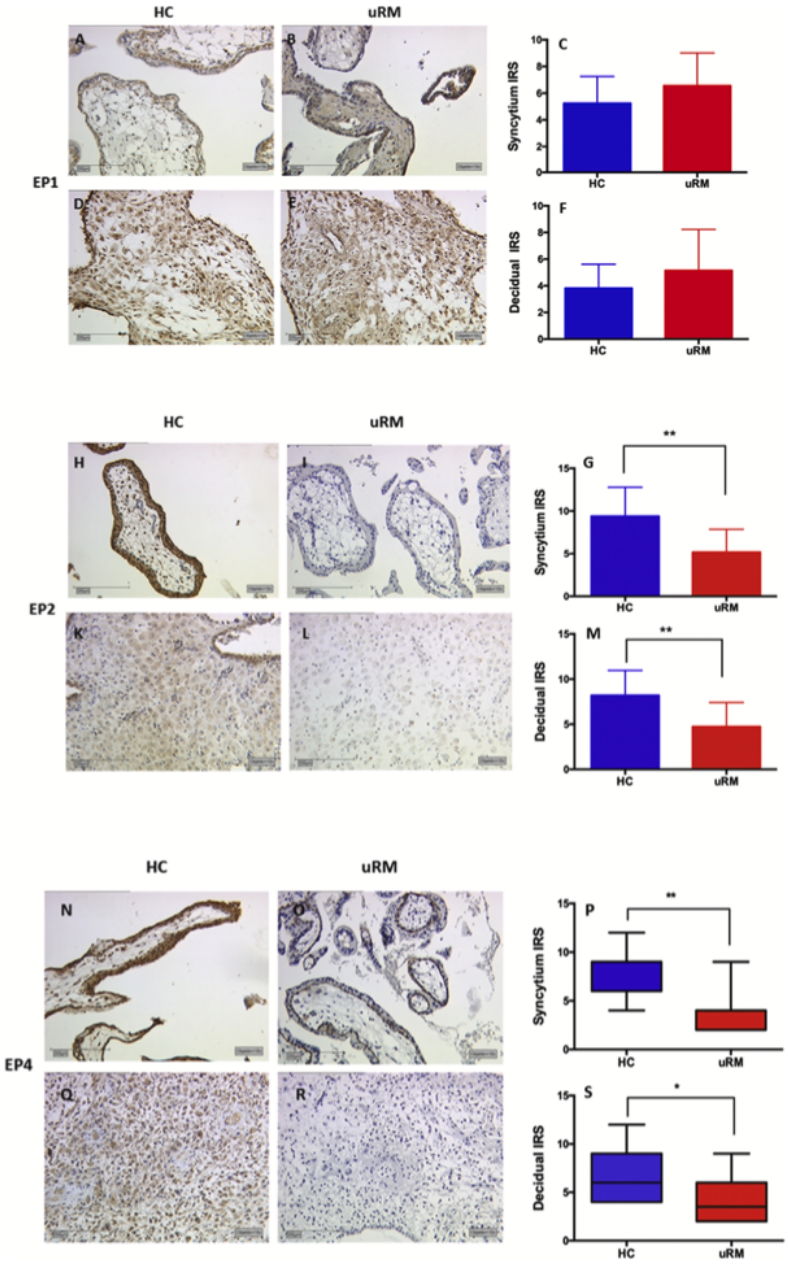

Fig. 1. Immunohistochemical staining of EP1, EP2 and EP4 in the first-trimester placentas of healthy controls (A, D, H, K, N and Q) and patients with uRM (B, E, I, L, Q and R) as measured by the IRS. In the syncytium, the expression of EP1 (A and B), EP2 (H and I) and EP4 (N and O) was compared between uRM tissues and healthy control tissues. The results are represented as box plots (C, G and P). In the decidua, the expression of EP1 (D and E), EP2 (K and L) and EP4 (Q and R) between uRM and healthy control tissues was compared and the results are represented as box plots (F, M and S). Magnification: x10 lens, scale bar = 200 μm, magnifications of inserted pictures: x25 lens, scale bar = 100 μm. EP1, prostaglandin E2 receptor 1; EP2, prostaglandin E2 receptor 2; EP4, prostaglandin E2 receptor 4; HC, healthy control; uRM, unexplained recurrent miscarriage. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

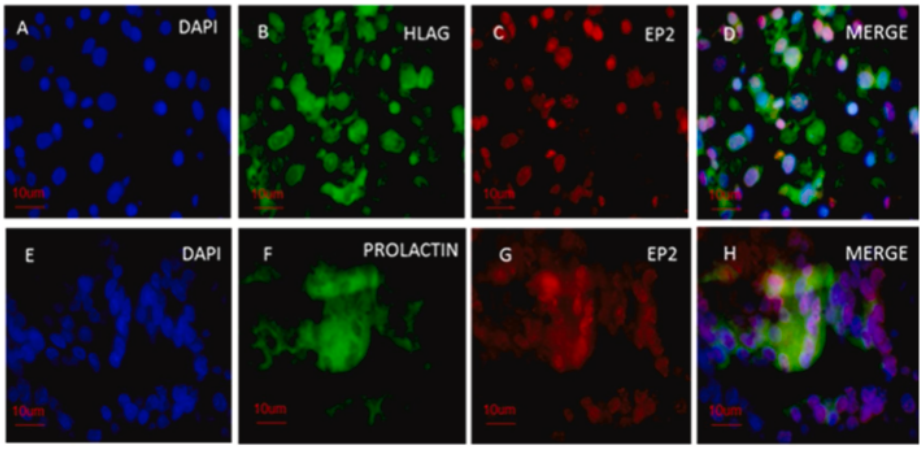

Fig. 2. Double immunofluorescence staining of EP2 in first-trimester placentas. HLA-G/prolactin (green), EP2 (red), and nuclei (blue) were stained. EP2 co-expressed with HLA-G in extravillous trophoblasts; EP2 co-expressed with prolactin in the decidua (A). Magnification: x100, scale bar = 10 μm. EP2, prostaglandin E2 receptor 2; HC, healthy control; uRM, unexplained recurrent miscarriage (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

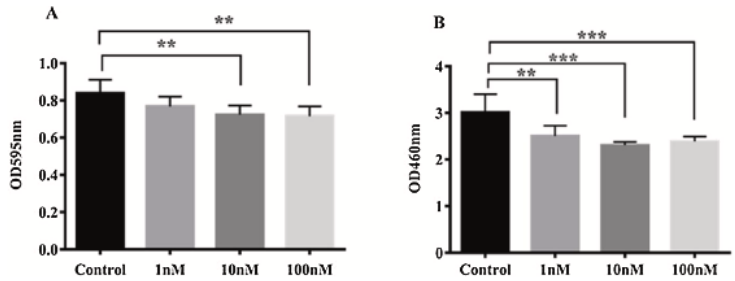

Fig. 3. MTT cell viability assay after PF-04,418,948 treatment at different concentrations in HTR-8/SVneo cells (A). BrdU assay after PF-04,418,948 treatment at different concentrations in HTR-8/SVneo cells (B). (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

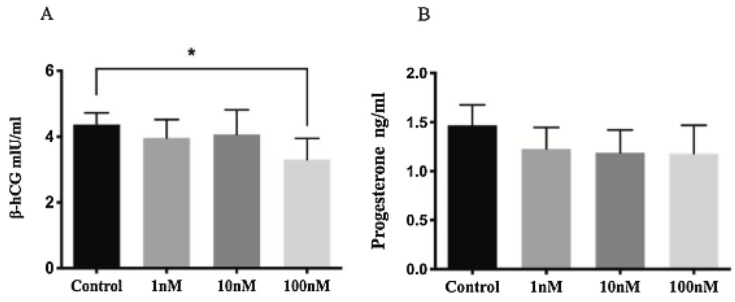

Fig. 4. Hormone levels were measured after PF-04,418,948 treatment in HTR-8/SVneo cells to test the production of ß-hCG and progesterone at drug concentrations of 1 nM, 10 nM and 100 nM. (8 $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

receptors, and elevation of cytosolic free [$Ca2^{+}$], and activating calpain (Nicola et al., 2005). Previously published data indicates that EP2 mRNA expression markedly increased in trophoblasts during the implantation and early placentation period when compared with trophoblasts preimplantation (Waclawik et al., 2013). Sakhila et al. observed that PGE2 elevated the proliferation of human endometriotic

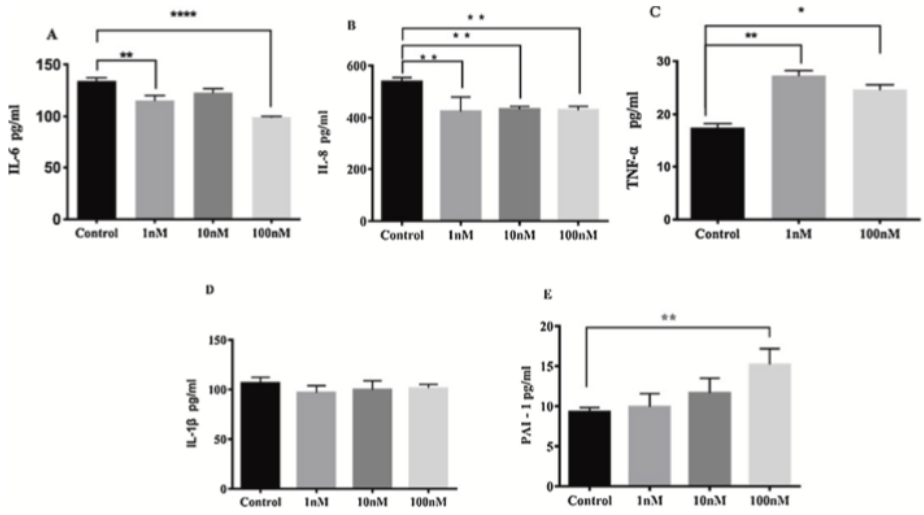

Fig. 5. ELISAs were performed after PF-04,418,948 treatment at different concentrations in HTR-8/SVneo cells to measure the secretion of IL-6, IL-8, TNF-α, IL-1β, and PAI-1 at different drug concentrations. IL, interleukin; TNF-α, tumor necrosis factor alpha. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

cells through EP2 and EP4 receptors by activating the ERK1/2, AKT, NF-κB and β-catenin signaling pathways. Meanwhile, selective inhibition of EP2 and EP4 promoted apoptosis activation via the caspase-3/PARP pathway (Banu et al., 2009). Masahiko et al. further observed that EP2 combined with EP4 promoted the proliferation of human endometrial stem cells via multiple trans-activating complex signaling pathways such as c-Src/β-arrestin 1/EGFR/ERK1/2, IL-1βR1/IκB/NFκB and Gsα/axin/β-catenin (Tsujii and DuBois, 1995). Our results are in accordance with these reports; demonstrating that a selective EP2 antagonist decreases HTR-8/SVneo cell proliferation. Meanwhile, ß-hCG controls trophoblast invasion, angiogenesis, endometrial vascularization and placentation and acts as the most classical biomarker for the clinical diagnosis of recurrent abortion (Licht et al., 2001; Fournier et al., 2015).

It has been found that Choriocarcinoma cells secrete increasing amounts of ß-hCG into culture medium in response to cAMP agonists (Hohn et al., 1998; Chen et al., 2013). cAMP stimulates the transcription of the ß-hCG gene expression in villous trophoblasts by interacting with CRE-binding protein (CREB) (Knofler et al., 1999; Forstner et al., 2019). Therefore, a decrease in EP2 inhibits cAMP, resulting in decreased levels of ß-hCG. Previous work by our group investigated whether PGE2 suppressed the production of ß-hCG and progesterone. And discovered that activating EP3/Gi1 lead to inhibition of cAMP/PKA signal pathway (Ye et al., 2018).

IL-6 plays an important role as a multifunctional cytokine in inflammatory responses and T cell differentiation (Choy and Rose-John, 2017). IL-6 deficiency is associated with elevated fetal resorption in genetic mouse models, emphasizing the pivotal role of IL-6 in pregnancy (Prins et al., 2012). Several studies have indicated that IL-6 increases the proliferation, invasion and migration of primary trophoblasts, HTR-8/SVneo cells and JEG-3 cells (Sengupta et al., 2003; Jovanovic and Vicovac, 2009; Sokolov et al., 2015). The transcriptional regulation of the IL-6 gene is complex and involves a cAMP response element (CRE)-binding protein (CREB) (Grassl et al., 1999). However, excessive production of IL-6 is associated with pregnancy complications, including infertility, miscarriage and preeclampsia (Prins et al., 2012). Zenclussen et al. reported increased IL-6 at the maternal-fetal interface in the case of fetal loss, as shown by an increased immunohistochemical expression in trophoblast cells (Zenclussen et al., 2003). Moreover, IL-6 alleviates the absorption ratio in the abortion-prone CBA x DBA/2 mouse combination model (Dubinsky et al., 2008). These contradictory results may be due to diverse roles of IL-6 in inflammation, as IL-6 can act as both a pro-inflammatory cytokine and an anti-inflammatory cytokine (Xing et al., 1998; Schuerwegh et al., 2003). In our study, we found that a selective EP2 antagonist inhibited the production of IL-6 by HTR-8/SVneo cells in vivo, so it can be assumed that an appropriate amount of IL-6 is beneficial in maintaining pregnancy. Consistent with our results, a recent study showed that the reduced levels of IL-6 and IL-8 observed in RM contribute to reduced trophoblast invasion and reduced spiral artery remodeling (Pitman et al., 2013). IL-8, derived from decidual natural killer cells and endometrial epithelial cells with increased levels of MMP-2 and MMP-9, promoted the invasion of extravillous trophoblast cells (De Oliveira et al., 2010). When we measured the levels of IL-6, IL-8, we incubated cells for 6 h in RPMI1640 with 10 % FBS with different concentrations of PF-04418948. This incubation time is suspected to be too short to affect cell proliferation and intracellular mRNA levels. As for progesterone and ß-hCG, we incubated cells for 36 h in RPMI1640 with 10 % FBS with different concentrations of PF-04418948. We suspect that 36 h' incubation could activate the EP2-Gas-cAMP-PKA-CREB pathway in cells, which regulates cell proliferation and hormone production. So, we thought the decreased levels of ß-hCG, progesterone, IL-6, and IL-8 is not caused by the reduced cell proliferation. These findings demonstrate that abnormal autocrine and/or paracrine IL-6 and IL-8 signaling controls trophoblast cell functions, thus disturbing placentation.

The levels of TNF-α in the plasma of women with preeclampsia, pregnant women with diabetes and women with RM were significantly higher than those in the plasma of healthy pregnant individuals (Vitoratos et al., 2010; Piosik et al., 2013; Mohammed and Aliyu, 2018). In a study by Alaa et al., a selective EP2 agonist (butaprost) and EP4 agonist (L-902,688) suppressed TNF-α production in human monocytic cells, blood and THP-1 cells (Kashmiry et al., 2018). TNF-α can induce an increase in COX-2 gene expression during the synthesis of PGE2 in first-trimester trophoblasts (Imseis et al., 1997). Tanaka et al. reported

that TNF-α promoted proliferation and independently inhibited apoptosis of BeWo cells (Tanaka et al., 2018). Studies using a human trophoblast-endothelium cell interaction model demonstrated that TNF-α can inhibit the invasion of JEG-3 cells into the maternal endothelium, decrease MMP-2 and block the switching of integrins from α6β4 to α1β1(Tanaka et al., 2018). Maternal immune cells attempt to attack allogeneic fetal placental trophoblasts by producing the Th1-type cytokines: TNF-α and IL-12 (Saito et al., 2010). TNF-α acts as a pro-inflammatory Th1 cytokine, and an elevated Th1/Th2 cytokine ratio is known to be harmful to maternal immune cells tolerating the fetus (Dong et al., 2017). Increased pro-inflammatory cytokines were shown to promote M1 macrophage polarization, which has been considered one of the immunological reasons for uRM (Jiang et al., 2018). Research from our group has demonstrated via gene array that the level of TNF-alpha is 2.5 times higher in placenta tissue with recurrent spontaneous abortion compared to regular pregnancies.

IL-1β is also known as a pro-inflammatory cytokine that affects the balance of the Th1/Th2 and M1/M2 ratios in the maternal-fetal environment (Yao et al., 2019). However, IL-1β levels were not significantly altered in the EP2 antagonist supernatant assay. Huber et al. reported that TNF-α stimulates PAI-1 levels in HTR-8/SVneo cells by activating the NF-κB pathway (Huber et al., 2006). Renaud et al. further suggested that macrophages participate in decreasing the depth of trophoblast invasion by secreting TNF-α, which promotes EVT to release PAI-1 during placentation (Renaud et al., 2005). Elevated PAI-1 can inhibit extracellular matrix (ECM)degradation, contributing to impeding the trophoblast invasion during implantation and placentation (Estella et al., 2012). Our previous work proved that in a similar cohort of patients with recurrent miscarriage the sera contained anti-trophoblast antibodies (ATAB) and in parallel increased concentration of PAI-1(Ye et al., 2019). Therefore, increased PAI-1 is also a potential marker for placental insufficiency and is associated with uRM.

Further research should focus on selective EP2 agonists at the maternal-fetal interface to determine whether EP2 regulates the inflammatory microenvironment and if it is conducive to the maintenance of pregnancy. EP4 functions in a similar signaling pathway as EP2, therefore, we are interested in exploring the potential mechanism by which EP4 regulates cytokines in uRM.

5. Conclusions

In the present study, we demonstrated that the expression level of EP2 was significantly decreased in trophoblasts and decidua at the maternal–fetal interface in the uRM group when compared with the level in normal healthy placentas. The reduced levels of EP2 observed in uRM may contribute to reduced trophoblast proliferation. In addition, selective EP2 antagonists inhibited trophoblast production of ß-hCG, IL-6 and IL-8 but elevated secretion of PAI-1 and TNF-α. The aberrant release of cytokines (IL-6, IL-8, and TNF-α) by trophoblasts can potentially affect trophoblast cell functions at the maternal–fetal interface.

We therefore conclude that EP2 plays a vital role in the maintenance of pregnancy and therefore altered levels of EP2 may contribute to uRM. EP2 may be a novel subject for research and a potential therapeutic target to treat uRM.

Author Contributions: V.S., and U.J. conceived and supervised the project. L.P. and LL.L. performed the IHC staining and ELISA with the help of C.K. and., M. under supervision of U.J. L.P. performed most analysis and wrote the first draft of the paper. Y.Y., V.S., U.J., S.M. and contributed to manuscript writing and editing. All authors have read and agreed to the published version of the manuscript. The study was funded by the Medical Faculty of the LMU Munich.

Declaration of Competing Interest

The authors declare no conflict of interest.

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5 Publication II

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Prostaglandin E2 receptor 3 (EP3) signaling promotes migration of cervical cancer via urokinase-type plasminogen activator receptor (uPAR)

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ORIGINAL ARTICLE – CANCER RESEARCH

Prostaglandin E2 receptor 3 (EP3) signaling promotes migration of cervical cancer via urokinase-type plasminogen activator receptor (uPAR)

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Abstract

Purpose Cervical cancer metastasis results in poor prognosis and increased mortality, which is not separated from inflammatory reactions accumulated by prostaglandin E2 (PGE2). As a specific G-protein coupled PGE2 receptor, EP3 is demonstrated as a negative prognosticator of cervical malignancy. Now, we aimed to investigate the pathological mechanism of EP3 in modulating cervical cancer carcinogenesis.

Methods Bioinformatics analysis was used to identify PAI-1 and uPAR correlations with EP3 expression, as well as the prognosis of cervical cancer patients. In vitro analyses were carried out to investigate the role of EP3 on cervical cancer proliferation and migration.

Results In vitro studies showed that sulprostone (an EP3 agonist) enhanced the proliferation and migration of cervical cancer cells, whereas silencing of EP3 inhibited their proliferation and migration. Furthermore, EP3 knockdown increased the expression of plasminogen activator inhibitor type 1 (PAI-1), urokinase-type plasminogen activator receptor (uPAR), and phosphorylated extracellular signal-regulated kinases 1/2 (p-ERK1/2), but decreased p53 expression. Bioinformatics analysis showed that both PAI-1 and uPAR were correlated with EP3 expression, as well as the prognosis of cervical cancer patients. The survival analysis further showed that uPAR overexpression (IRS≥2) was correlated with a lower overall survival rate of cervical cancer patients with advanced stages (FIGO III-IV).

Conclusion These results indicated that EP3 signaling pathway might facilitate the migration of cervical cancer cells through modulating uPAR expression. Therefore, EP3 and uPAR could represent novel therapeutic targets in the treatment of cervical cancer in advantaged stages.

Keywords Cervical cancer · Prostaglandin E_2 receptor 3 (EP3) · Urokinase-type plasminogen activator receptor (uPAR) · Plasminogen activator inhibitor type 1 (PAI-1)

Viktoria von Schönfeldt and Helene H. Heidegger contributed equally as senior authors to this study.

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Abbreviations

CESC	Cervical squamous cell carcinoma and endocervical adenocarcinoma
HPV	Human Papillomavirus
OS	Overall survival

DFS	Disease-free survival
PGE_2	Prostaglandin E_2
EP3	Prostaglandin E_2 receptor 3
uPAR	Urokinase-type plasminogen activator receptor
PAI-1	Plasminogen activator inhibitor type 1
ECM	Extracellular matrix
ERK1/2	Extracellular signal-regulated kinases 1/2
p-ERK1/2	Phosphorylated Extracellular signal-regulated Kinases 1/2
TGF-β1	Transforming Growth Factor-β1

Introduction

Cervical cancer is the fourth most common cancer in women worldwide and approximately 510,000 new cases of women will be diagnosed in 2030 as today (Ginsburg et al. 2017). According to the cancer statistics of the United States in 2019, there were an estimated 13,170 cases and 4250 deaths from cervical cancer (Siegel et al. 2019). Approximately half of the cervical cancer patients die from metastasizing tumors globally (Wright and Kuhn 2012). The two main malignant epithelial cervical cancer types are the squamous cell carcinoma and the adenocarcinoma (Young and Clement 2002). The human papillomavirus (HPV) infection is the crucial risk factor for cervical cancer and is the primary cause of cervical cancer (Schiffman et al. 2011). Inflammation after HPV infection is a driving force that increases cervical cancer development (Deivendran et al. 2014). Cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) are well-known inflammatory factors and up-regulated synthesis of both has been identified in the cervical carcinoma (). As the rate-limiting enzyme of PGE_2 synthesis, COX-2 is not only related to poor overall survival (OS) and poor disease-free survival (DFS) in cervical cancer patients, but also is associated with poor DFS in a chemo-radiation subgroup of cervical cancer patients (Huang et al. 2013).

The effects of PGE_2 are mainly facilitated by four specific membrane-bound G-protein-coupled EP receptors (EP1-EP4) with various signaling pathways (Sokolowska et al. 2015). It is suggested that PGE_2 regulates the function of cervical cancer cells mainly via cyclic adenosine monophosphate (cAMP) linked EP2/EP4 signaling pathway (). GW627368X (a highly selective EP4 antagonist) inhibits the proliferation and angiogenesis of cervical carcinoma by blocking EP4/epidermal growth factor receptor (EGFR) signaling pathway in cervical cancer cell lines (HeLa, SiHa and ME180) and suppresses the tumor size in xenograft mice model (Parida et al. 2016). Our latest publication demonstrated that high expression of EP3 is associated with poor prognosis in overall survival rates of cervical cancer patients in both squamous cell carcinoma and adenocarcinoma (Heidegger et al. 2017). EP3 is a unique PGE_2 receptor, since the human EP3 gene consists of ten exons and nine introns, encoding at least eight distinct EP3 splice variants (Kotani et al. 1997). EP3 has been reported to mediate the carcinogenesis in numerous tumors with conflicting effects (Fujino et al. 2011; Hoshikawa et al. 2009; Kang et al. 2011; Kashiwagi et al. 2013; Ma et al. 2013; Shoji et al. 2004; Yamaki et al. 2004; Zhu et al. 2018). However, the molecular pathological mechanism of EP3 in cervical cancer development is still unknown.

Plasminogen activator contributes to proteolytic degradation and intercellular interaction damage during tumor metastasis. Plasminogen activator inhibitor type 1 (PAI-1) is the main inhibitor of the plasminogen activating system, which consists of urokinase-type plasminogen activator (uPA) and uPA receptor (uPAR) (Zorio et al. 2008). PGE_2 combining with the EP1/EP3 receptor regulates the levels of PAI-1 in cardiac fibroblasts (Kassem et al. 2014). PAI-1 inhibits the activation of uPA and plays a crucial role in cancer invasion and metastasis by remodelling the extracellular matrix (ECM). PAI-1 enhances tumor cell proliferation by encouraging S-phase entry (Giacoia et al. 2014) and increases migration by binding uPA/uPAR complex (Andreasen et al. 2000). PAI-1/uPA/uPAR/low-density lipoprotein receptor-related protein (LRP)/integrin complexes are initiating an "adhesion–detachment–re-adhesion" cycle to promote tumor cell migration (Andreasen et al. 2000; Carter and Church 2009). Overexpressions of both PAI-1 (Hazelbag et al. 2004; Horn et al. 2002) and uPA (Fujishiro et al. 1994; Sugimura et al. 1992) are associated with poor prognosis in cervical cancer patients. However, Sato et al. proposed that lower levels of PAI-1 are produced in cervical cancer cells that distant from the basal membrane, especially in cervical cancer stem cells (Sato et al. 2016). These conflicting reports indicate the complex roles of PAI-1 in cervical carcinoma development, which requires further investigations. The uPAR protein in the serum (Jing et al. 2012) and uPAR mRNA in the specimen (Sasaki et al. 2014) are identified as new prognosticators of cervical cancers. uPAR can be cleaved into soluble uPAR, both full-length and cleaved uPAR are involved in cell signaling, proliferation, migration and invasion of tumor cells (Magnussen et al. 2017). However, the correlation between uPAR and overall survival of cervical cancer has not been clarified.

In the present study, we aimed to explore the functional roles of EP3 in the tumor genesis of cervical cancer, especially in the migration. In in vitro studies, we observed that EP3 silencing attenuated the proliferation and migration of cervical cancer cells and upregulated the expression of PAI-1 and uPAR. This was in accordance with the finding that EP3 was significantly correlated with PAI-1 and uPAR from publicly available databases. By immunohistochemistry, we demonstrated that high uPAR expression

was associated with the poor prognosis of cervical cancer patients with advanced stages (FIGO III–IV). Our present study shed light on the critical role of EP3 and uPAR in regulating migration in cervical cancer in advantaged stages.

Materials and methods

Bioinformatics

The gene set enrichment analysis (GSEA) software was performed to calculate the corresponding signaling pathways associated with EP3 (https://www.software.broadinstitute.org/gsea/index.jsp). The cut-off criteria for GSEA were nominal P value < 0.05 and false discovery rate (FDR) < 0.25. TIMER database was applied to identify the correlation between EP3 and PAI-1 or uPAR (https://cistrome.shinyapps.io/timer/). Both of GSEA and TIMER databased are based on the cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC) in the Cancer Genome Atlas (TCGA) dataset (https://www.cancer.gov). We analyzed the survival rate in groups with differently expressed PAI-1 and uPAR by screening out the relevant documents and clinical information related to CESC in GEPIA database (https://gepia.cancer-pku.cn/) and UALCAN database (https://ualcan.path.uab.edu/index.html), respectively.

Cell lines and culture

HeLa (RRID:CVCL_0030), SiHa (RRID: CVCL_0032), C-33A (RRID: CVCL_1094) and CaSki (RRID: CVCL_1100) cells were obtained from the American Type Culture Collection (ATCC) and were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) without antibiotics or antimycotics. According to the American Type Culture Collection (ATCC), HeLa cells are categorized as cervical adenocarcinoma, SiHa cells are squamous cell carcinoma, CaSki cells are categorized as epidermoid carcinoma and C-33A cells are categorized as cervical carcinoma. All experiments were performed with mycoplasma-free cells. To investigate the effect of EP3 knockdown, cells were cultured in 96-well plates for the cell proliferation assay, 24-well plates for the wound healing assay and the enzyme-linked immunosorbent assay (ELISA), and 6-well plates for real-time polymerase chain reaction (RT-PCR) and western blotting.

Real time-PCR (Taq Man)

Total RNA was obtained from cultured cells using a Rneasy Mini Kit (Qiagen, Hilden, Germany) and converted to cDNA with an MMLV Reverse Transcriptase First-Strand cDNA synthesis kit (epicenter, Madison, USA) as instructed by the protocol. The total EP3 mRNA levels were subjected to RT-PCR using two different primers (Applied Biosystems, EP3 Primer I, Nr. Hs00168755_m1, exon boundary 1–2; EP3 Primer II, Nr. Hs00988369_m1, exon boundary 4–5). 20 µl reaction mixture containing 1 µl TaqMan® Gene Expression Assay 20×, 10 µl TaqMan® Fast Universal PCR Master Mix 2×, 1 µl cDNA template and 8 µl RNase-free water were prepared per probe on an Optical Fast 96-well plate and covered by an optical adhesive film. PCR assays were run by utilizing Applied Biosystems 7500 Fast Real-time PCR system. The amplification conditions were 20 s at 95 °C; 40 cycles of 95 °C for 3 s and of 60 °C for 30 s. β-actin (Nr. Hs99999903_m1) was used as an endogenous control and the comparative CT method was applied for calculation.

EP3 silencing

Cervical cancer cells (HeLa, SiHa and C-33A) were seeded in six-well plates in 2 ml of RPMI-1640 medium to achieve 40–60% confluence after 24 h. 1.2 µl of EP3 siRNA or the negative control siRNA and 4 µl of Lipofectamine RNAiMAX (Invitrogen, California, USA) were first diluted in 200 µl Opti-MEM (Gibco, California, USA) medium separately. Then we combined and added the corresponding complex into each well, mixed gently, and incubated at 37 °C in 5% CO_2 for 48 h. The knockdown efficiency was assessed by RT-PCR.

Cell proliferation assay

HeLa, SiHa and C-33A cells were seeded into 96-well plates and siRNA-mediated EP3 knockdown was conducted with the siRNA-Lipofectamine RNAiMAX mixture on day two. Cell proliferation was analyzed with a 5-bromo-2′-deoxyuridine (BrdU) labeling and detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Cells were incubated with BrdU (20 µl/well) for 24 h, and then fixed with fixing solution for 30 min. After adding anti-BrdU-POD working solution (100 µl/well), BrdU incorporation into the cellular DNA was measured by an ELISA technique. The optical density (OD) was examined at 450 nm using Elx800 universal Microplate Reader. At least six replicates were performed with each cell line. 100 nM of PGE_2 and L-798,106 were incubated with HeLa, SiHa and C-33A cells and the dimethyl sulfoxide (DMSO, 0.5%) served as a vehicle control. The BrdU assay was performed as describe above.

Wound healing assay

HeLa and SiHa cells were cultured in 24-well plates, starved overnight and on day two siRNA-mediated EP3 knockdown was treated for 48 h. On day three, the central fields of

confluent monolayers were scratched with 200 µl pipette tips to make artificial wound gaps. Then each well was rinsed with phosphate-buffered saline (PBS) and was then added fresh RPMI1640 containing 1% FBS. Cell migration was monitored by photographing with an inverse phase contrast microscope (Leica Dmi1, Leica, Wetzlar, Germany) for 0 h and 24 h. Photos of cells migration area were analyzed with software Image J (https://imagej.nih.gov/ij/). Scratch area was measured at 0 h and 24 h by image J, and the cell migration area = scratch area at 0 h—scratch area at 24 h.

Western blotting

Cell lysates were extracted from cervical cancer cells with radioimmunoprecipitation assay buffer (RIPA, Sigma-Aldrich, R0278-50ML). 20 µg of cell lysates for western blotting were first separated in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane (Bio-Rad, USA). 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 follows: rabbit polyclonal anti-EP3 antibody (Abcam, ab94496, 1:500), mouse polyclonal anti-ERK1/2 antibody (Abcam, ab224313, 1:200), rabbit polyclonal anti-p-ERK1/2 antibody (Abcam, ab47339, 1:500), mouse monoclonal anti-p53 antibody (Santa Cruz, OD-1, 1:500) and rabbit polyclonal anti-uPAR antibody (Abcam, ab218106, 1:300). β-actin was used as a housekeeping gene and mouse monoclonal anti-β-actin antibody was diluted as 1:1000 (Sigma, A5441). Afterwards, the membrane was incubated with the goat-anti-rabbit/-mouse secondary antibody conjugated with alkaline phosphatase (1:1000 dilution, Jackson Immuno Research, UK), 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). The blots were repeated at least three times.

PAI-1 ELISA

Both HeLa and SiHa cells were cultured in 24-well plates and EP3 knockdown was conducted utilizing the siRNA-Lipofectamine RNAiMAX mixture on day two. After 48 h, the supernatants of both cell lines were harvested. The levels of PAI-1 in the supernatants were measured with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D system, DSE100, Minneapolis, MN, USA). A standard curve of PAI-1 was obtained for each assay and results were converted into ng/ml.

Patient samples

We analyzed paraffin-embedded cervical cancer samples from 250 patients having undergone surgeries for cervical cancer in the Department of Obstetrics and Gynecology in the Ludwig Maximilians University of Munich, Germany between 1993 and 2002. This study was approved by the ethical committee of the Medical Faculty, Ludwig Maximilian University of Munich (approval number: 259-16). The written informed consent was obtained from each patient and all methods were performed in accordance with the relevant guidelines and regulations. Staging and grading were assessed by two gynecological pathologists according to the criteria of FIGO and WHO. Follow-up data were received from the Munich Cancer Registry (Munich Tumour Center, Munich, Germany). Samples and clinical information were anonymized and encoded for statistical workup. All clinical information was blinded from the authors during experimental analysis.

Detailed clinic characteristics of these cervical cancer patients are summarized in supplementary Table 1, which includes age, follow-up months, stages, grading, histology and survival months. The outcome was assessed by patients' overall survival (OS). OS is defined as the time from diagnosis to the death or to the date of the last follow-up. 76% (190/250) of the cohort survived over 235 months and 19.6% (49/250) of the cohort died. The information of the rest 4.4% (11/250) of the cervical cancer patients is missing.

Table 1 Effects of EP3 knockdown in HeLa, Siha and C-33A cervical cancer cell lines

Cell lines	Effects of EP3 knockdown on			
	Proliferation	Migration	PAI-1	uPAR
HeLa	↓	↓	↑	No detection
Siha	↓	↓	↑	↑
C-33A	No change	-	-	-

↓ = inhibit; ↑ = enhance; - = unknown.

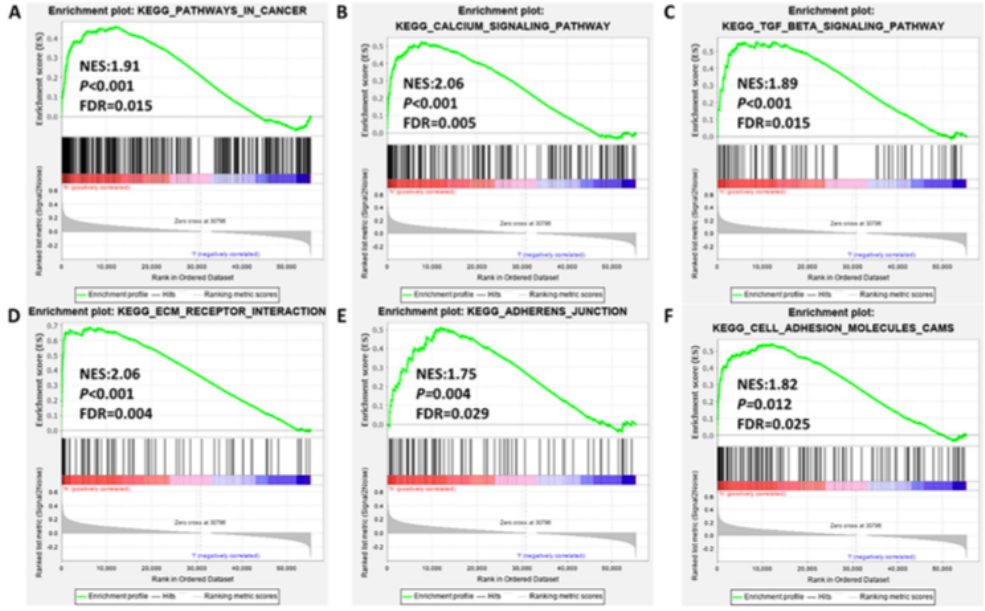


Fig. 1 EP3 is associated with KEGG signaling pathways of cancer (**a**), calcium signaling (**b**), transforming growth factor-β (TGF-β) (**c**), ECM receptor interaction (**d**), adheren junction (**e**) and cell adhesion molecules (CAMs) (**f**) in carcinogenesis. KEGG pathway gene sets in EP3 high versus low samples were obtained from The Cancer Genome Atlas (TCGA) dataset with the gene set enrichment analysis (GSEA) software (https://www.software.broadinstitute.org/gsea/index.jsp). Normalized enrichment score (NES), nominal *P* value and false discovery rate (FDR) are shown in each plot. The cut-off criteria for GSEA were nominal *P* value < 0.05 and false discovery rate (FDR) < 0.25

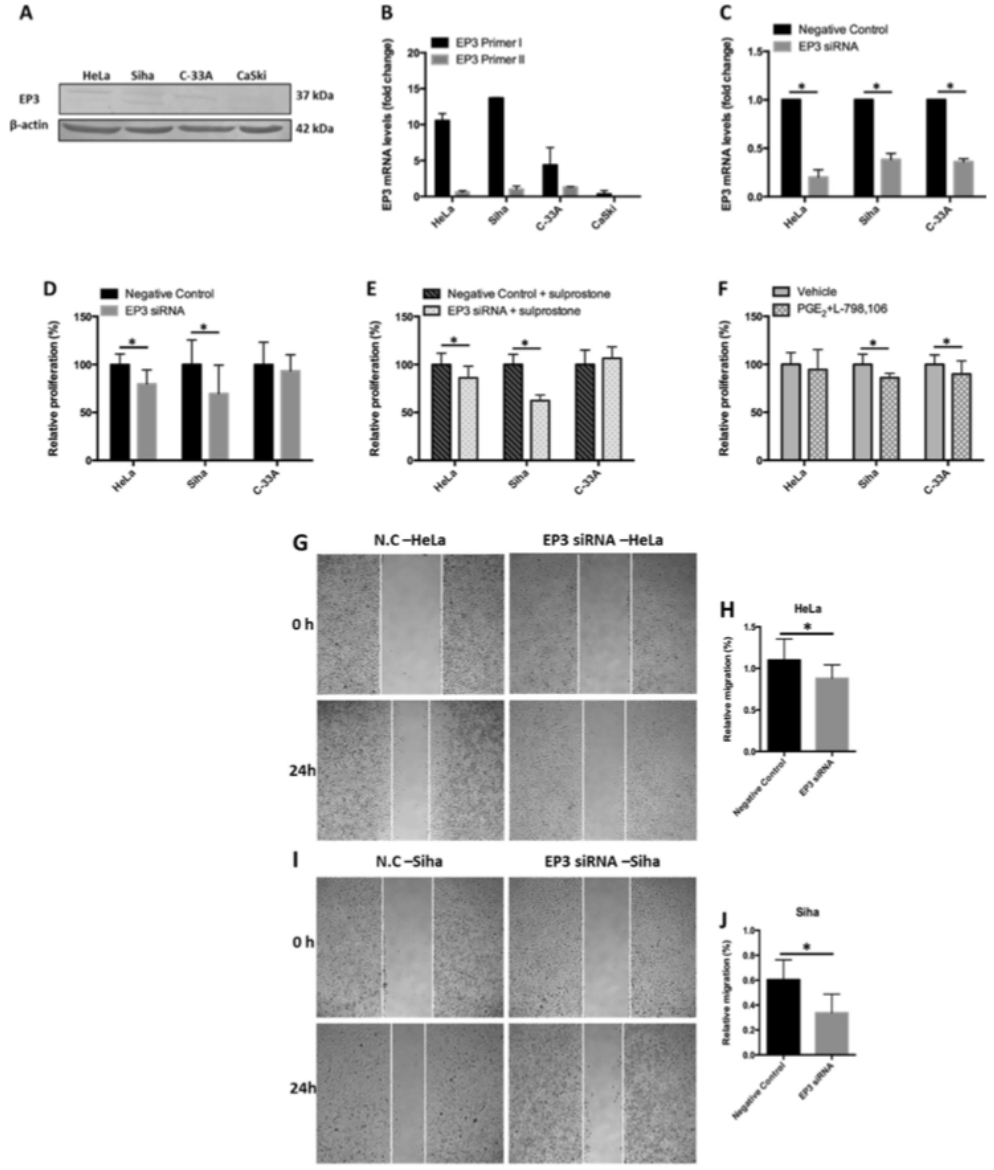
Immunohistochemistry

Paraffin-embedded slides (3 µm-thick) were dewaxed in xylol and washed in 100% ethanol, and then 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 a blocking solution [Reagent 1, Zytochem-Plus HRP-Polymer-Kit (mouse/rabbit)] for 30 min to avoid non-specific binding of the primary antibodies. The slides were incubated with rabbit polyclonal anti-uPAR antibody (Abcam, ab218106, 1:300 dilution) for 16 h at 4 °C. After washing, the secondary antibodies/complexes of HRP-polymer (Zytochem-Plus HRP Polymer-kit, Zytomed, Berlin, Germany) were applied. uPAR immunostaining was visualized with the substrate and the chromogen-3, 3′-diaminobenzidine (DAB; Dako, Hamburg, Germany) after 3 min. All slides were analyzed under the microscope by two independent observers using a Leitz (Wetzlar, Germany) photomicroscope. For the light microscopy analysis, a semi-quantitative IRS score was calculated via the multiplication of optical staining intensity and the percentage range of positive stained cells (Remmele and Stegner 1987).

Metastatic colon carcinoma was used as a positive and negative control for the immunohistochemical staining of uPAR. Positive cells showed a brownish color and the negative control, as well as unstained cells, appeared blue (Worbs et al. 2007).

Statistical analysis

All data were analyzed with SPSS Statistics 24 software (IBM Corporation, Armonk, NY, USA) and are expressed as the mean ± standard deviation (SD). Mann–Whitney *U* test was applied for evaluating the proliferation rate and cell migration area. Wilcoxon test was performed for the evaluation of PAI-1 expression levels and the band intensities of p-ERK1/2, ERK1/2, p53 and uPAR. Spearman's rank correlation analysis was adopted to evaluate the correlation between two monotonic, nonlinear variables. The ROC curve



was drawn to identify an appropriate cut-off value which can maximize the sum of sensitivity and specificity. Survival time was compared using Kaplan–Meier (long-rank) test method. We also applied a Cox-regression model for multivariate analyses. P-values < 0.05 were regarded as statistically significant.

◂**Fig. 2** EP3 knockdown inhibits the proliferation and migration of cervical cancer cells. **a** The expression of EP3 is higher in HeLa, SiHa and C-33A than CaSki cells in the protein level by western blots. **b** The expression of EP3 is higher in HeLa, SiHa and C-33A than CaSki cells in the mRNA level detected by primer I with RT-PCR. **c** The downregulated expression of EP3 mRNA is shown in HeLa, SiHa and C-33A detected by RT-PCR (*$P<0.05$). **d** BrdU assay suggests the proliferation rate of HeLa and SiHa is decreased by EP3 knockdown compared to the negative control after 48 h. **e** The proliferation rate of HeLa and SiHa is inhibited followed by stimulation of 100 nM sulprostone and EP3 siRNA compared to the negative control after 48 h (*$P<0.05$). **f** The proliferation rate of SiHa and C-33A is decreased by 100 nM of PGE_2 and L-798,106 compared to the vehicle control after 48 h (0.5% (v/v) DMSO, *$P<0.05$). **g** Representative photographs show the migration of HeLa cells into the wounded area treated with the EP3 siRNA and the negative control after 24 h. **h** We observed that the relative migration rate of HeLa cells is suppressed in the EP3 siRNA group compared to the negative control (*$P<0.05$). **i** Representative pictures represent the migration of SiHa cells into the wounded area followed by incubating EP3 siRNA and the non-targeting control for 24 h. **j** The relative migration rate of SiHa cells is inhibited in the EP3 siRNA group compared to the non-targeting control (*$P<0.05$). Bar graphs represent mean ± SD ($n=6$). *$P<0.05$ is considered as significantly different after comparison between the EP3 siRNA and the negative control (N.C)

Results

Associated EP3 signaling pathways were upregulated in cancer

Our latest publication observed that enhanced expression of EP3 (IRS ≥ 2) is correlated with a poor prognosis in the OS of 250 cervical cancer patients after a 20-year follow-up analysis (Heidegger et al. 2017). Additionally, increased EP3 expression is associated with higher tumor status, higher the International Federation of Gynecology and Obstetrics (FIGO)-classification, as well as with poorer survival (Heidegger et al. 2017). Based on this publication, we aimed to investigate the pathological mechanism of EP3 in the carcinogenesis of cervical cancer. First, we analyzed the relationship between EP3 expression and KEGG pathway gene sets with GSEA software (https://www.software.broadinstitute.org/gsea/index.jsp). Pathways in cancer, calcium signaling and transforming growth factor-β (TGF-β) signaling were significantly enriched (Fig. 1a–c), so were ECM receptor interaction, adheren junction and cell adhesion molecules (CAMs) signalings (Fig. 1d–f). This indicated that EP3 might be involved in the carcinogenesis, especially in tumor adhesion, migration and metastasis.

Knockdown of EP3 inhibits the proliferation and migration of HeLa and SiHa cells

Next, we investigated the effect of EP3 knockdown on the proliferation and migration of cervical cancer cells with in vitro cell culture. The EP3 expression levels in HeLa, SiHa, C-33A and CaSki cervical cancer cell lines were determined by western blotting and real-time polymerase chain reaction (RT-PCR) analyses. The protein expression of EP3 was higher in HeLa, SiHa and C-33A cells than CaSki cells detected by western blots (Fig. 2a). With the EP3 primer I, the expression of EP3 in the mRNA level (Fig. 2b) showed the similar result as western blots. With the EP3 primer II, the mRNA expression of EP3 was detected only in HeLa, SiHa and C-33A cells, and was not as high as with the EP3 primer I (Fig. 2b). Therefore, we used HeLa, SiHa and C-33A as cervical cancer models and the EP3 primer I for RT-PCR detection after the depletion of EP3 mRNA with siRNA. The EP3 mRNA level was downregulated by 80% in HeLa cells, 62% in SiHa cells and 64% in C-33A cells compared to the negative control, respectively (each $P<0.05$, Fig. 2c).

EP3 knockdown decreased the proliferation rate by 20.3% in HeLa cells ($P=0.028$, Fig. 2D) and by 30.5% in SiHa cells ($P=0.036$, Fig. 2d) compared to the relative negative control group after 48 h' incubation. Since sulprostone (an EP1/EP3 agonist) can enhance the proliferation of HeLa cells ($P=0.028$ at the concentration of 1, 10 and 100 nM, supplementary Fig. 1a), we tested the effect of EP3 siRNA on the proliferation of sulprostone-induced cervical cancer cells. As shown in Fig. 2e, co-incubation of EP3 siRNA and 100 nM sulprostone reduced the proliferation rate by 13.9% in HeLa cells ($P=0.043$) and by 37.7% in SiHa cells ($P=0.028$) compared with the non-targeting siRNA with 100 nM sulprostone after 48 h' incubation. By contrast, the proliferation rate of C-33A cells was neither significantly altered by EP3 siRNA ($P=0.33$, Fig. 2dD), nor by the combination of 100 nM sulprostone and EP3 siRNA ($P=0.075$, Fig. 2e).

The inhibitory effect was exhibited when incubating 100 nM of PGE_2 and L-798,106 (a specific EP3 antagonist) with SiHa and C-33A cells, although the effect was not as obvious as the effect of EP3 siRNA. The combination of PGE_2 and L-798,106 suppressed the proliferation rate by 14.0% in SiHa cells ($P=0.028$, Fig. 2f) and by 10.0% in C-33A cells ($P=0.046$, Fig. 2f) compared to the vehicle (0.05% DMSO) after incubating for 48 h, respectively.

To identify whether EP3 participates the migration of cervical cancer cells, we performed wound healing assay. Our pre-test showed that 100 nM sulprostone promoted the migration rate of HeLa cells by 13.6% for 24 h ($P=0.015$, supplementary Fig. 1b, c). In comparison, EP3 knockdown inhibited the migration rate by 20.0% in HeLa cells ($P=0.016$, Fig. 2g, h) and by 44.2% in SiHa cells ($P=0.006$, Fig. 2i, j) compared with the negative control. To wrap it up, downregulation of EP3 inhibited the proliferation and migration of HeLa and SiHa cells, while had no effect on C-33A cells. The impacts of EP3 knockdown on the proliferation,

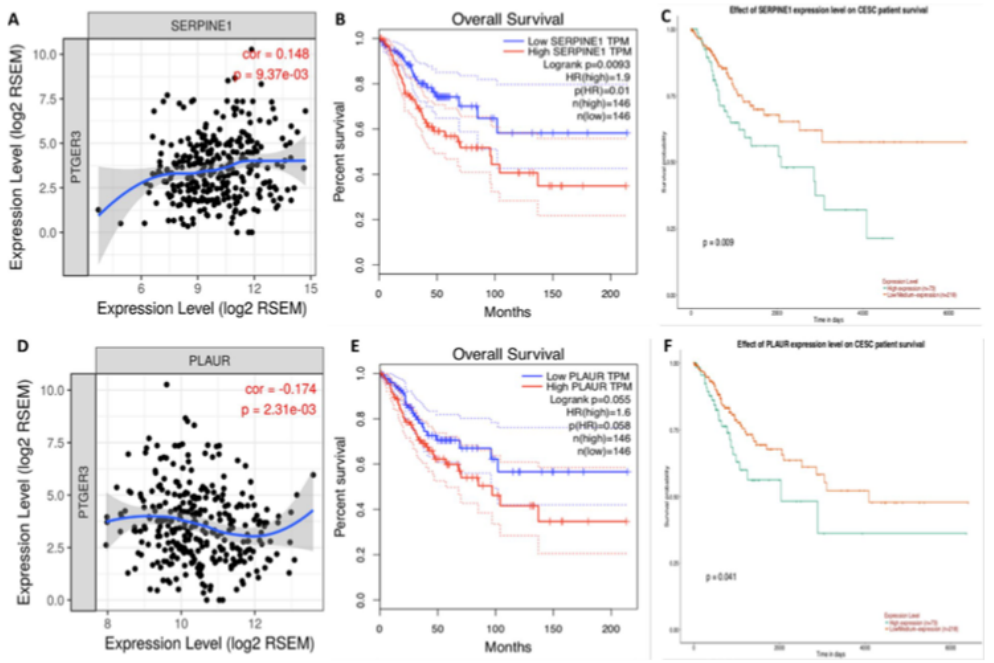


Fig. 3 EP3 is correlated with PAI-1 and uPAR in cervical cancer. **a, d** TIMER database was applied to identify the correlation between EP3 and PAI-1 or uPAR, which is based on the CESC (cervical squamous cell carcinoma and endocervical adenocarcinoma) in the Cancer Genome Atlas (TCGA) dataset (https://www.cancer.gov). **b, c** PAI-1 is associated with poor overall survival (OS) of cervical cancer patients both in GEPIA database (https://gepia.cancer-pku.cn/) and UALCAN database (https://ualcan.path.uab.edu/index.html). **e, f** The association of uPAR with poor prognosis of cervical cancer patients is significant in UALCAN database but not in GEPIA database

migration and expression of PAI-1/uPAR in HeLa, Siha and C-33A cells were summarized in Table 1.

EP3 is correlated with PAI-1 and uPAR in cervical cancer tissues

Migration and invasion are responsible for the majority of patients death from solid tumors in advanced stages (Paul et al. 2017), and both of PAI-1 and uPA are involved in the migration of cervical carcinoma (Fujishiro et al. 1994; Hazelbag et al. 2004; Horn et al. 2002; Sugimura et al. 1992). TIMER database was applied to identify the correlation between EP3 and PAI-1 or uPAR. The result showed that EP3 was positively correlated with PAI-1 ($r=0.148$, $P=9.37\times10^{-3}$, Fig. 3a) and negatively correlated with uPAR ($r=-0.174$, $P=2.31\times10^{-3}$, Fig. 3d). With the GEPIA and UALCAN databases we observed that the OS of the low PAI-1 expression group was higher than that of the high PAI-1 expression group in the long run ($P=0.0093$ in GEPIA, Fig. 3b; $P=0.009$ in UALCAN, Fig. 3c). The OS was not significantly different in the low and high uPAR expression groups in GEPIA database ($P=0.055$, Fig. 3e), whereas the OS of the low uPAR expression group was increased than that of the high uPAR expression group in UALCAN ($P=0.041$, Fig. 3f). Therefore, we examined the expression of PAI-1 and uPAR in HeLa and SiHa cells after knocking down EP3 and then testified uPAR expression in our 250 cervical cancer specimens because of the diverse results of two databases.

Knockdown of EP3 increases the expression of PAI-1 and uPAR

Knockdown of EP3 promoted the production of PAI-1 in the supernatants of both HeLa and SiHa cells. Downregulation of EP3 enhanced the production of PAI-1 by 38.7% in the supernatants of HeLa cells compared to the negative control (0.55 ± 0.09 vs 0.40 ± 0.12 ng/ml, $P=0.003$, Fig. 4b). The same trend was observed in SiHa cells, downregulation of EP3 increased the production of PAI-1 in the supernatants by 66.1% compared to the negative control (0.67 ± 0.07 vs

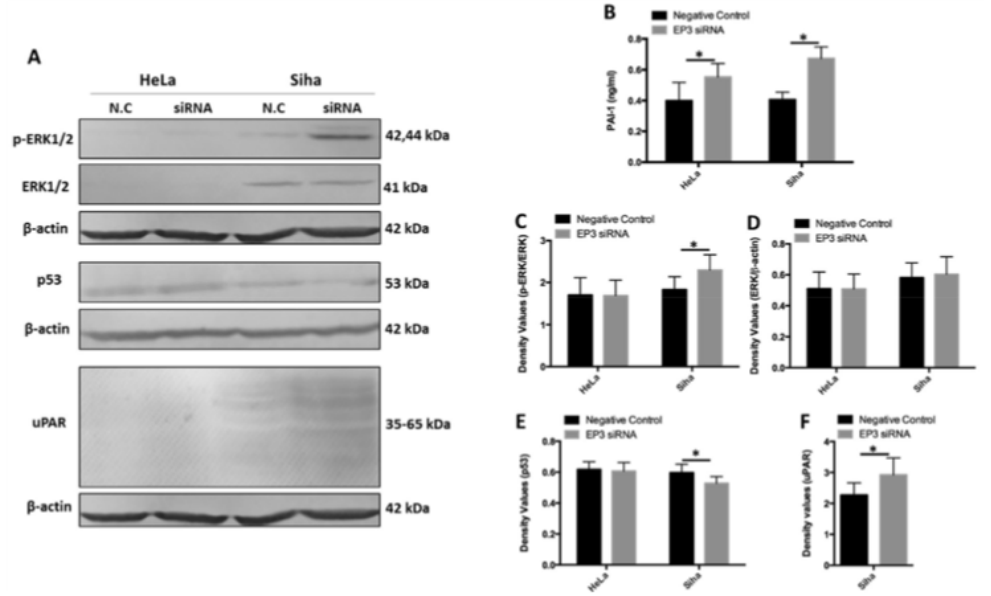


Fig. 4 Expression of plasminogen activator inhibitor type 1 (PAI-1) and urokinase-type plasminogen activator receptor (uPAR) is influenced by silencing EP3 gene. **a** Western blotting analysis shows the expression of phosphorylated extracellular signal-regulated kinases (p-ERK1/2), extracellular signal-regulated kinases (ERK1/2), p53 and uPAR in HeLa and SiHa cells following treatment with EP3 siRNA and the negative control (N.C) for 48 h. β-actin was used as a loading control and all the data was normalized to the β-actin band signals. **b** PAI-1 levels in the supernatants of HeLa and SiHa cells are enhanced after silencing EP3 compared with the negative control for 48 h by ELISA (*$P<0.05$, $n=6$). **c** The histogram illustrates the expression of p-ERK1/2 is increased after silencing EP3 gene for 48 h in SiHa cells (*$P<0.05$). **d** The histogram presents the expression of ERK1/2 is not altered by EP3 siRNA in HeLa and SiHa cells ($P>0.05$). **e** The histogram illustrates the expression of p53 is inhibited after downregulation of EP3 compared with the negative control for 48 h in SiHa cells (*$P<0.05$). **f** The histogram shows the expression of uPAR is stimulated after EP3 knockdown compared with the negative control for 48 h in SiHa cells (*$P<0.05$). Statistically significant differences ($P<0.05$) between EP3 siRNA group and the negative control group are marked with an *. All western blots data are shown as mean ± SD ($n=3$). Full-length blots are shown in Supplementary Fig. 2

0.41 ± 0.05 ng/ml, $P=0.003$, Fig. 4b). Both phosphorylated extracellular signal-regulated kinases 1/2 (p-ERK1/2) and p53 are the upstream regulators of PAI-1 gene transcription (Samarakoon et al. 2013; Wilkins-Port et al. 2007), hence we also analyzed the expression of p-ERK1/2 and p53 by western blots. The molecular weights of p-ERK1/2 are 44 and 42 kDa. In SiHa cells, EP3 blockade increased the expression of p-ERK1/2 by 25.5% ($P=0.028$, Fig. 4a, c) while did not change the expression of total ERK 1/2 ($P=0.753$, Fig. 4a, d) compared to the negative control group after incubating EP3 siRNA for 48 h. Silencing EP3 decreased the expression of p53 by 7.4% in SiHa cells ($P=0.011$, Fig. 4a, d). Additionally, the expression of uPAR was also analyzed by western blotting and the molecular weight of uPAR is between 35–65 kDa. EP3 knockdown improved the expression of uPAR by 28.6% in SiHa cells ($P=0.027$, Fig. 4a, e). No alteration of p-ERK1/2, ERK1/2 and p53 was observed in HeLa cells while the expression of uPAR was not able to be detected in HeLa cells (Fig. 4a).

Expression of uPAR in cervical cancer tissues

Finally, we analyzed uPAR expression in the same group of 250 cervical cancer patients as we previously conducted (Heidegger et al. 2017) and examined the correlation of uPAR expression with clinical-pathological parameters and several cervical cancer biomarkers. uPAR staining was observed in the cytoplasm of 93.6% (234/250) of cervical cancer tissue samples, and the median IRS for cytoplasmic uPAR expression was 2.05. Although EP3 was negatively correlated with uPAR in the TIMER database, there was no significant correlation between uPAR and EP3 expression

Table 2 Correlation analysis of uPAR and variables

Variables	*P* value	Correlation coefficient
Histology	0.247	0.076
pT	0.117	−0.103
pN	**0.017***	−0.156
pM	0.308	−0.067
Grading	0.397	0.056
FIGO	**0.012***	−0.165
EP3	0.822	−0.015
E6	0.836	0.014
p16	**0.05***	−0.130
Wild-type p53	0.118	−0.103
Mutant p53	0.082	−0.114
MDM2	0.938	−0.005
galectin-3	**0.002***	−0.202
GPER	0.52	−0.042
H3K9ac	0.121	−0.102
H3K4me3	**0.041***	0.134

Bold numbers represent significant correlations

pT tumor stage, *pN* lymph node stage, *pM* distant metastasis stage, *FIGO* the International Federation of Gynecology and Obstetrics, *EP3* prostaglandin E_2 receptor 3, *MDM2* MDM2 proto-oncogene, *GPER* G-protein-coupled estrogen receptor, *H3K9ac* histone H3 acetyl K9, *H3K4me3* histone H3 tri methyl K4

in our cervical cancer specimens ($P=0.822$, Table 2). However, a significant negative correlation was shown between uPAR expression and FIGO status (spearman's rank correlation Rho = −0.165; $P=0.012$), suggesting the weaker uPAR staining was correlated with a higher FIGO stage (Table 1). Additionally, decreased uPAR staining was observed in cervical cancer cases with higher FIGO stages ($P=0.046$, Fig. 5a). A total of 44.8% (112/250) of cervical cancer patients with FIGO stages I/II had a median IRS of 2.17 compared to 17.6% of patients (44/250) with FIGO stages III/IV and with a median IRS of 1.52 (Fig. 5a).

The cut off value of IRS 2 was obtained from receiver operator curve (ROC) analysis. We observed that uPAR positivity (IRS ≥ 2) in general was not related to OS in our non-stratified patient samples ($P=0.48$). However, when patients had been stratified according to FIGO stage, the high expression of uPAR was correlated with poor prognosis in OS of cervical cancer patients with FIGO stages III/IV as shown in the Kaplan–Meier curve ($P=0.047$, Fig. 5b). Among all the 44 advanced cervical cancer patients (FIGO III/IV), 34 cases with squamous cell carcinoma had a median IRS of 1.12 and 10 cases with adenocarcinoma had a median IRS of 2.9, which showed no significant difference between these two histological subtypes ($P=0.09$). The subsequent survival analysis of the two main histological subtypes suggested a significant negative correlation of uPAR with OS

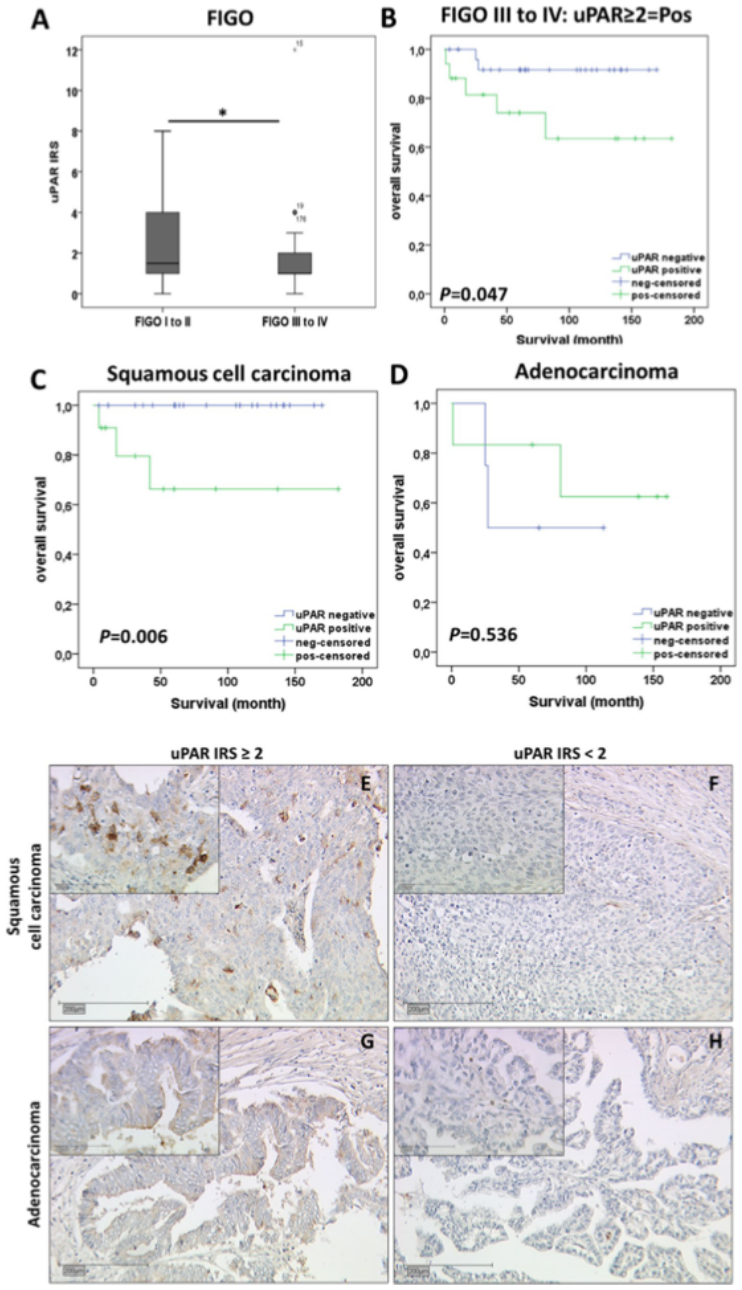
Fig. 5 The expression of urokinase-type plasminogen activator receptor (uPAR) in cervical cancer patients. **a** Boxplot shows uPAR staining in cervical cancer patients with FIGO I and II is higher than in cases with FIGO III and IV ($P=0.046$). **b** High uPAR expression (IRS ≥ 2) is associated with a shorter overall survival (OS) in advanced cervical cancer patients (FIGO III/IV) ($P=0.047$). **c** High uPAR expression (IRS ≥ 2) is associated with a shorter OS of advanced patients in cervical squamous cell carcinoma ($P=0.006$). **d** uPAR survival function of cervical adenocarcinoma in patients with FIGO stages III/IV ($P=0.536$). **e** Representative photomicrographs of uPAR staining in cervical squamous cell carcinoma (FIGO IIIB) with the IRS score of 3. **f** Representative photomicrographs of uPAR staining in cervical squamous cell carcinoma (FIGO IIIB) with the IRS score of 0. **g** Representative photos of uPAR immunohistochemical staining in adenocarcinoma (FIGO IIIA) with the IRS score of 4. **h** Representative photos of uPAR immunohistochemical staining in adenocarcinoma (FIGO IVB) with the IRS score of 0. The scale bars in the outer pictures equal 200 µm (×10 magnification) and the scale bars in the inserts equal 100 µm (×50 magnification). *FIGO* the International Federation of Gynecology and Obstetrics

in squamous cell carcinoma ($P=0.006$, Fig. 5c), but not in cervix adenocarcinoma ($P=0.536$, Fig. 5d). The representative cytoplasmic expression of uPAR in the squamous cell carcinoma and adenocarcinoma were shown in Fig. 5, and metastatic colon carcinoma tissues were applied as negative and positive controls (Supplementary Fig. 3A, B). It indicated that immunopositivity of uPAR was predictive for OS in cervical cancer patients of advanced stage (FIGO III/IV), especially among cases with squamous cell carcinoma. In advanced cervical cancer patients (FIGO III/IV), uPAR was nearly a promising prognosticator for advanced cervical cancer patient OS ($P=0.067$, Table 3) tested by multivariate Cox regression analysis.

Moreover, we detected a significant positive correlation of uPAR with histone H3 tri methyl K4 (H3K4me3, $P=0.041$, Rho = 0.134) and a significant negative correlation with pN ($P=0.017$, Rho = −0.156), p16 ($P=0.05$, Rho = −0.13) and galectin-3 ($P=0.002$, Rho = −0.202) in 250 cervical cancer tissues (Table 1). The percentage of uPAR positive staining cells was negatively associated with the intensity of wild-type p53 staining in the cytoplasm ($P=0.011$, Rho = −0.184, data not shown), although no correlation between uPAR and wild-type nuclear p53 ($P=0.118$) or between uPAR and mutant p53 in the nucleus ($P=0.082$).

Discussion

Our latest study demonstrated that high expression of EP3 (IRS ≥ 2) is associated with poor prognosis in the OS rate of 250 cervical cancer patients in both squamous cell carcinoma and adenocarcinoma (Heidegger et al. 2017). EP3 can increase the migration of HCA-7 human colon cancer cells through the activation of phosphatidylinositol 3-kinase (PI3K) and the phosphorylation of ERK1/2


A
FIGO
uPAR IRS
FIGO I to II
FIGO III to IV
B
FIGO III to IV: uPAR≥2=Pos
overall survival
P=0.047
Survival (month)
uPAR negative
uPAR positive
neg-censored
pos-censored
C
Squamous cell carcinoma
P=0.006
D
Adenocarcinoma
P=0.536
uPAR IRS ≥ 2
uPAR IRS < 2
Squamous cell carcinoma
Adenocarcinoma
E
F
G
H

signaling pathway (Fujino et al. 2011). In accordance to those findings, we found that sulprostone (an EP1/EP3 agonist) induced the proliferation and migration of HeLa cells, while silencing EP3 reduced the proliferation and migration of HeLa and SiHa cells. In contrast to colon cancer cells, EP3 silenced SiHa cells showed elevated expression of phosphorylated-ERK1/2. The latter was in accordance with a study demonstrating that activation of EP3 signaling reduced ERK phosphorylation in rat cerebellar astrocytes (Paniagua-Herranz et al. 2017).

With bioinformatics, signaling pathways of ECM receptor interaction, adheren junction and cell adhesion molecules were enriched when EP3 was upregulated in cancer microenvironment. Additionally, we observed that EP3 was positively associated with PAI-1 in cervical malignancy, and PAI-1 was correlated with the OS of cervical cancer patients in both UALCAN and GEPIA databases. Studies also proved that PAI-1 is an independent prognosticator in cervical cancer (Hazelbag et al. 2004; Horn et al. 2002). Therefore, we deduced that EP3 and PAI-1 are involved in the tumor migration of cervical tumor. PGE_2 can increase mRNA and protein levels of PAI-1 by binding with EP1/EP3 receptor in rat ventricular fibroblasts, contributing to elevated fibrin deposition in aortic stenosis (Kassem et al. 2014). However, Sato et al. suggested that TM5275 (a small molecular inhibitor of PAI-1) can increase the collagenase activity of SiHa and CaSki cells (Sato et al. 2016), implying that lower expression of PAI-1 benefits the ECM degration and cervical cancer migration. The latter study was in accordance with our study that silencing EP3 increased the production of PAI-1 and decreased the migration in HeLa and SiHa cells. Conflicting effects of PAI-1 on migration might due to the different distances of cervical cancer cells from basal membrane (Sato et al. 2016).

In comparison, EP3 expression was negatively associated with uPAR expression in CESC. The correlation of uPAR with OS of cervical cancer patients was significant in UALCAN but not GEPIA. Sasaki et al. testified that overexpression of uPAR mRNA is related to a shorter DFS of cervical cancer patients, however, the immunohistochemical staining of uPAR was not very intense (Sasaki et al. 2014). In the present study, we detected only those patients with an uPAR expression (IRS ≥ 2) showed a poor OS in the subgroup of advanced stage (FIGO III/IV) cases. The negative correlation of uPAR with OS of patients was significant in squamous cell carcinoma but not in cervix adenocarcinoma, which could be due to the smaller number of patients with adenocarcinoma ($n = 10$) or different pathological molecular mechanisms in squamous cell carcinoma and adenocarcinoma. This result agreed with multivariate Cox regression analysis, indicating that with a large scale of specimens, uPAR could be a promising prognosticator for OS of advanced cervical cancer patients.

Magnussen et al. (2017) proved that high production of PAI-1 can reduce uPAR cleavage to inhibit the migration of oral squamous cell carcinoma (OSCC) and the cleaved soluble uPAR is responsible for promoting the migration of OSCC cells (Magnussen et al. 2017). Jing et al. also suggested that the soluble uPAR in serum is a prognosis marker as well as a tumor biomarker for clinical diagnosis and treatment of cervical cancer (Jing et al. 2012). The in vitro studies showed knockdown of EP3 increased expression of uPAR and PAI-1 in SiHa cells. Therefore, we deduced that the upregulated secretion of PAI-1 decreased uPAR cleavage in EP3 knockdown SiHa cells causing less soluble uPAR in the ECM and leaving more uPAR on the membrane, and these might contribute to decreased migration of SiHa cells (Fig. 6).

Interestingly, TGF-β signaling pathway was found to be significantly enriched when EP3 gene was upregulated in the CESC. Many studies illustrate the gene transcription of PAI-1 is regulated by TGF-β1 through various signalling pathways, one of which is through phosphorylation of ERK1/2 (Wilkins-Port et al. 2007). The cross-talks among reactive oxygen species (ROS), tumor suppressor p53, and upstream stimulatory factor proteins 1/2 (USF1/2) are necessary for TGF-β1 inducing PAI-1 transcription (Freytag et al. 2009; Samarakoon et al. 2013). This gave a hint that EP3 signaling possibly modulates PAI-1 gene transcription through similar signalling pathways as TGF-β1. We proved that EP3 blockade increased the expression of PAI-1 and p-ERK1/2 and decreased the expression of p53 in SiHa cells. Furthermore, decreased expression of wild-type p53 in the cytoplasm of cervical cancer tissues was correlated to increased expression of uPAR. This in vivo finding was in good agreement with the previous report that HPV E6 oncoproteins induce rapid degradation of tumor suppressor protein p53 to prevent the host cell from inducing apoptosis (DeFilippis et al. 2003). It implied that p53 might translocate into the nucleus from the cytoplasm to induce PAI-1 transcription. However, this deduction concerning p53 translocation should be explored in further studies.

Our group previously found other biomarkers of cervical cancer, such as p16 (Stiasny et al. 2017), MDM2 (Stiasny et al. 2017), galectin-3 (Stiasny et al. 2017), H3K9ac (Beyer et al. 2017) and H3K4me3 (Beyer et al. 2017). Therefore, we also analyzed the correlation of uPAR with these biomarkers in the same cervical cancer patients. First, we found that a negative correlation of uPAR with both galectin-3 and p16 in cervical cancer patients. Stiasny et al. showed that galectin-3 expression was correlated with a shorter survival time in cervical cancer patients expressing no or very low p16 (Stiasny et al. 2017). In hepatocellular carcinoma cells, galectin-3 silencing attenuated uPAR expression and inhibited the proliferation, migration and invasion (Zheng et al. 2014). This study was also in line with our detections that low expression of uPAR

Table 3 Cox regression of clinical-pathological variables regarding overall survival in cervical cancer patients with FIGO III/IV ($n=44$)

Variable	Significance	Hazard ratio of exp (B)	Lower 95% CI of exp (B)	Upper 95% CI exp (B)
uPAR IRS	0.067	8.332	0.863	80.425
Histology	0.222	5.182	0.370	72.505
pT	0.231	2.056	0.632	6.687
pN	0.987	4,553,661.9	0.000	–
pM	0.314	0.193	0.008	4.734
Grading	0.255	3.664	0.391	34.350
FIGO	0.962	0.974	0.331	2.865
Age	0.588	1.031	0.922	1.153

IRS Immunoreactive score, *pN* lymph node stage, *pT* tumor stage, *pM* distant metastasis stage, *FIGO* the International Federation of Gynecology and Obstetrics

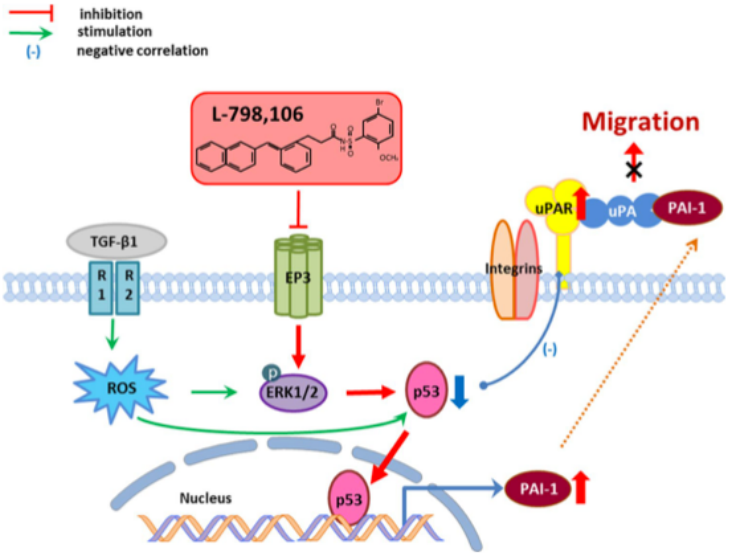


Fig. 6 Hypothetic schema of EP3 signaling in the migration of human cervical cancer cells. Inhibiting EP3 signaling contributes to phosphorylation of extracellular signal-regulated kinases (p-ERK1/2) and translocation of p53 from the cytoplasm to the nucleus, resulting in an increased transcription of PAI-1. High expression of PAI-1 reduces uPAR cleavage (Magnussen et al. 2017), thus leading to decreased migration of cervical cancer cells. The EP3 signaling pathway is similar to the one that transforming growth factor-β1 (TGF-β1) induces PAI-1 gene expression via the rapid generation of reactive oxygen species (ROS), phosphorylation of ERK1/2 and the mobilization of p53 signaling (Samarakoon et al. 2013; Wilkins-Port et al. 2007). In addition, cytoplasmic p53 is decreased in the cervical cancer cells with high expression of uPAR, which is correlated with poor prognosis in overall survival rates of cervical cancer patients with advanced FIGO stages (III/IV). Therefore, we believed that EP3 signaling regulates the migration of cervical cancer cells through plasminogen activator inhibitor type 1 (PAI-1) and urokinase-type plasminogen activator receptor (uPAR)

was correlated with longer survival time in cervical cancer patients with advanced stage. Additionally, the positive correlation of H3K4me3 and uPAR expression in our study was in accordance with the finding that H3K4me3 is related to poor prognosis in cervical cancer patients and is an independent marker of relapse-free survival (Beyer et al. 2017). Although EP3 seems not to correlate with the expression of the direct HPV marker protein E6 ($P=0.192$, data not shown) or with the indirect protein p16 ($P=0.267$, data not shown), there is a link between uPAR and p16 ($P=0.05$, Table 2). This link was already found in migrating keratinocytes. Migration seems to

be stimulated by a combined upregulation of both p16INK4a and an activated uPAR signaling (Darbro et al. 2005).

The upregulated expression of p-ERK1/2 was observed in SiHa cells while the expression of ERK1/2 was too low to draw any conclusion in HeLa cells. HeLa cells are categorized as adenocarcinoma and SiHa cells are squamous cell carcinoma according to the ATCC. The different pathological molecular mechanisms in cancer development should be investigated between squamous cell carcinoma and adenocarcinoma in the future. Another limitation of this investigation is that it is a retrospective study, which analyzed the data of patients who had undergone surgeries in one single hospital from 1993 to 2002. A multi-centre prospective study should be carried out for further research, as well as the xenograft mice experiments.

Conclusions

Taken all results together, EP3 might facilitate the migration of cervical cancer cells through modulating the production of PAI-1 and uPAR in advantaged stages of cervical malignancy. The high production of PAI-1 might due to the phosphorylation of ERK1/2 and translocation of p53 from the cytoplasm into the nucleus after sliencing EP3. The high expression of PAI-1 inhibits the cleavage of uPAR (Magnussen et al. 2017), contributing to inhibited migration of cervical cancer cells (Fig. 6). EP3 and uPAR might represent novel therapeutic targets for cervical cancer and specific antagonists or inhibitors of EP3 and uPAR could be promising therapeutic treatments for cervical cancer.

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Author contributions UJ: conceived and designed the whole study, and interpreted the data; YY: participated in the whole experimental work, performed the statistical analysis and wrote the manuscript; LP: performed the bioinformatic analysis; CK: participated in the immunohistochemistry assay; AV and ED: carefully reviewed the manuscript for important intellectual content; VS and HH: conceived of the study and participated in its design and coordination; CD and SM: conceived of the study and approved the final version of the manuscript. All authors read the manuscript and agreed with the publication of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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6 Summary

PGE2 biology is a complex regulatory process in different cell types through stimulating various receptors. Our latest research found that EP3 is involved in the pathology of recurrent miscarriage and cervical cancer. In order to investigate other EP receptors in recurrent miscarriage and attempt to elucidate the molecular mechanisms by which the EP3 signaling pathway is implicated in the development of cervical cancer, we focused on the potential function of EP2 in trophoblast with recurrent miscarriage and the molecular regulation of EP3 signaling in cervical cancer.

6.1 Expression and function of EP2 in trophoblast with recurrent miscarriage

Our group did some primary studies to identify the expression level of PGE2 receptors (EP1, EP2, EP3 and EP4) in the maternal-fetal interface with normal pregnancy and recurrent miscarriage patients via immunohistochemistry. We evaluated the results through IRS score found that the expression of EP2 and EP4 were reduced both in syncytium and decidual in the recurrent miscarriage group compared with normal pregnancy tissue. EP2 co-expression with HLA-G predominantly exist in the cytoplasm especially in the cell membrane of trophoblast cells. The results clearly showed that EP2 antagonist (PF-04418948) inhibited the proliferation and production of substances (β-hCG, progesterone, IL-6, IL-8, and TNF-α) and promoted the secretion of PAI-1 in HTR-8/SVneo in vitro. The PGE2-EP2 axis might be an important target to gain knowledge for future therapy options for a better treatment of recurrent miscarriage via regulating the underlying pathway and the inflammatory cytokines.

6.2 The molecular mechanism of EP3 signaling in cervical cancer

The cervical cancer patient's database was used to analyze the expression of EP3 was positively correlated with PAI-1 and negatively related with uPAR expression. With

bioinformatics analysis we observed that EP3 is involved in ECM interaction, adherent junction and cell adhesion in cervical cancer. We performed a serious in vitro experiment which suggested that silencing of EP3 declined the proliferation and migration of cervical cancer cells while EP3 agonist can reverse these effects. The underlying mechanism thought to decrease the migration in part via inhibition of EP3 increased the levels of PAI-1 and uPAR with the activation of pERK1/2 and P53 transport from cytoplasmic to nuclear. The uPAR high group had a lower overall survival rate and advanced stages compared to uPAR low group (IRS＜2) with the Kaplan-analysis. Hence, the targets of EP3 and uPAR might be prognostic predictor and provide beneficial effects in cervical cancer patients.

Taken together, EP2 and EP3 receptors play crucial roles in trophoblast cell function in the placenta and for the biology of cervical cancer. Future studies would focus on the role of COX-2-PGE2-EPs axis in immune cells, like T cells, macrophage, dendritic cells, NK cells, Treg and their cross-talk in the maternal-fetal interface with recurrent miscarriage. These findings might help us to obtain deeper understanding of the regulatory role of PGE2 in the pathological mechanism of reproductive diseases and improve the treatment of recurrent miscarriage and hopefully also cervical cancer.

7 Zusammenfassung

Die Biologie von PGE2 ist aufgrund der Stimulation verschiedener Rezeptoren kompliziert. Unsere neuesten Forschungsergebnisse zeigen, dass EP3 an der Pathologie wiederkehrender Fehlgeburten und Gebärmutterhalskrebs beteiligt ist. Um andere EP-Rezeptoren und den molekularpathologischen Mechanismus von EP3 bei der Entwicklung von Gebärmutterhalskrebs zu untersuchen, bestimmten wir die Expression von EP2 in Trophoblasten mit wiederkehrenden Fehlgeburten und den pathologischen Mechanismus der EP3-Signalübertragung bei Gebärmutterhalskrebs.

7.1 Expression und Funktion von EP2 in Trophoblasten bei rezidivierenden Fehlgeburten

Unsere Gruppe untersuchte zunächst die Expression von PGE2-Rezeptoren (EP1, EP2, EP3 und EP4) im Übergangsbereich zwischen Mutter und Fötus bei Patienten mit normaler Schwangerschaft und habituellen Aborten mittels Immunhistochemie. Die Expression von EP2 und EP4 im Synzytium und der Dezidua war signifikant verringert in der Gruppe mit wiederkehrenden Fehlgeburten im Vergleich zu der Gruppe mit normalen Schwangerschaften. Eine EP2-Coexpression mit HLA-G zeigte sich hauptsächlich im Zytoplasma insbesondere in der Zellmembran von Trophoblastenzellen. Die Ergebnisse zeigten deutlich, dass der EP2-Antagonist (PF-04418948) die Proliferation und Produktion von Substanzen (β-hCG, Progesteron, IL-6, IL-8 und TNF-α) inhibierte und förderte die Produktion von PAI-1 in HTR-8 / SVneo Zellen in vitro. Die PGE2-EP2-Achse könnte ein wichtiges Ziel sein, um Einblicke in zukünftige Therapieoptionen für ein besseres Management wiederkehrender Fehlgeburten durch Regulierung des zugrunde liegenden Signalwegs und entzündlicher Zytokine zu erhalten.

7.2 Der molekulare Mechanismus der EP3-Signalübertragung bei Gebärmutterhalskrebs

Die bioinformatische Analyse von Patientinnen mit Gebärmutterhalskrebs ergab eine Assoziation von EP3 mit PAI-1, uPAR sowie mit der Prognose Außerdem wurde ein Zusammenhang mit ECM-Interaktionen und Zelladhäsionen gefunden. Im Folgenden konnten wir zeigen,dass das Silencing von EP3 die Proliferation und Migration von Zervixcarzinomzellen in vitro verringert, während der EP3-Agonist diese Effekte in vitro umkehren kann. Als zugrundeliegender Mechanismus wäre denkbar, dass das Silencing von EP3 die Expression von PAI-1 und uPAR erhöht. In der Folge findet Phosphorylierung von ERK1 / 2 und die Translokation von p53 aus dem Zytoplasma in den Kern statt. Daraus lässt sich die Migration von Gebärmutterhalskrebszellen erklären. Die hohe uPAR-Gruppe hatte eine niedrigere Gesamtüberlebensrate und fortgeschrittene Stadien im Vergleich zur niedrigen uPAR-Gruppe (IRS $<$ 2) mit der Kaplan-Analyse. Daher könnten die Ziele von EP3 und uPAR prognostische Prädiktoren sein und bei Patienten mit Gebärmutterhalskrebs positive Auswirkungen haben.

Zusammenfassend kann festgestellt werden, dass EP2- und EP3-Rezeptoren eine entscheidende Rolle bei der Funktion von Trophoblastenzellen in der Plazenta und bei der Entwicklung von Gebärmutterhalskrebs spielen. Weitere Studien sind nötig, um auf die Funktion der COX-2-PGE2-EPs-Achse in Immunzellen wie Makrophagen, dendritischen Zellen, NK-Zellen und Tregs bei habituellen Aborten zu schließen. Dennoch können unsere Ergebnisse helfen, den Einfluss von PGE2 auf den Pathomechanismus von Fortpflanzungserkrankungen besser zu verstehen und die Behandlungsmöglichkeiten sowohl dafür, als auch für Gebärmutterhalskrebs, zu erweitern.

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