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The roles of EP3 signaling in cervical cancer and recurrent miscarriages

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Contents

1. Abbreviations

AC	Adenylyl cyclase
ATP	Adenosine triphosphate
5-LOX	Arachidonate 5-lipoxygenase
cAMP	cyclic adenosine monophosphate
CIN	Cervical intraepithelial neoplasia
COX	Cyclooxygenase
COXibs	COX-2 inhibitors
CRP	C-reactive protein
DFS	Disease-free survival
EC	Endometrial cancer
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EGR1	Early growth response protein 1
ELISA	Enzyme-linked immunosorbent assay
EP3	Prostaglandin E_2 receptor 3
FGF2	Fibroblast growth factor 2
G_i	G protein alpha inhibitor
G_s	G protein alpha stimulator
HPV	Human papillomavirus
IFN-γ	interferon-γ
IL-1β	Interleukin-1β
IL-6	Interleukin 6
LRP	Low density lipoprotein receptor-related protein
MAPK	Mitogen-activated protein kinase cascade
MMP	Matrix metalloproteinase
mPGES-1	microsomal PGE synthase-1
mTORC1	mammalian target of rapamycin complex 1
NSAIDs	Nonsteroidal anti-inflammatory drugs
OS	Overall survival

PAI-1	Plasminogen activator inhibitor type 1
p-ERK1/2	phosphorylated extracellular signal-regulated kinases 1/2
PGE_2	Prostaglandin E_2
PGES	Prostaglandin G synthase
PGH_2	Prostaglandin H_2
PI3-k/Akt	phosphatidylinositol 3-kinase/protein kinase B
PLA2	Phospholipases A2
PPAR β/δ	Peroxisome proliferator-activated receptor β/δ
RM	Recurrent miscarriages
ROCK	Rho-associated coiled-coil kinase
RT-PCR	Rualitative reverse transcription-polymerase chain reaction
RXR	Retinoid X receptor
STAT	Signal transducers and activators of transcription
SUMO-1	Small ubiquitin-like modifier-1
TGF-β1	transforming growth factor β1
TNF	Tumor necrosis factor
TP	Thymidine phosphorylase
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator
uPAR	urokinase-type plasminogen activator receptor
uRPL	unexplained recurrent pregnancy losses
USF1/2	Upstream stimulatory factor proteins 1/2
VEGF	Vascular endothelial growth factor
VDR	Vitamin D receptor

2. Introduction

Abundant literature has demonstrated a strong correlation between chronic inflammation and cancer development since chronic inflammation contributes to the development of over 15% of malignancies worldwide[1]. Plenty of pro-inflammatory factors mediate a role in carcinogenesis, such as tumour necrosis factor (TNF), interleukin (IL)-1α, IL-1β, IL-6, IL-8, IL-18, matrix metallopeptidase-9 (MMP-9), vascular endothelial growth factor (VEGF), cyclooxygenase 2 (COX-2), and arachidonate 5-lipoxygenase (5-LOX)[2]. Serum levels of C-reactive protein (CRP), IL-6, and IL-1 receptor antagonist (IL-1Ra) are significantly associated with endometrial cancer risk when analyzing 246,000 women in ten European countries[3]. The signaling of cyclooxygenase 2-prostaglandin E_2-prostaglandin E_2 receptors (COX-2-PGE_2-EPs) is the central inflammatory pathway involved in the carcinogenesis. Based on the current information, this doctoral thesis aimed to supplement some additional knowledge in the carcinogenesis of cervical cancer and the pathology of recurrent miscarriage from the perspective of inflammation.

2.1 Biogenesis and signaling: COX-2-PGE_2-EPs

Arachidonic acid is released from the membrane phospholipids by phospholipase A2 (PLA2) and then metabolized by the enzyme of COX-1 and COX-2 into prostaglandin H_2 (PGH_2). PGH_2 is converted by specific isomerases (PGDS, PGES, PGFS and PGIS) and TXA synthase to various prostaglandins (PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2) and the thromboxane A_2 (TxA_2)[4] (**Figure 1**). All these prostaglandins (PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and TXA_2) act through relative specific G-protein coupled receptors (GPCR) to mediate their effects, referred to as the EP, DP, FP, IP and TP receptors[5] (**Figure 1**).

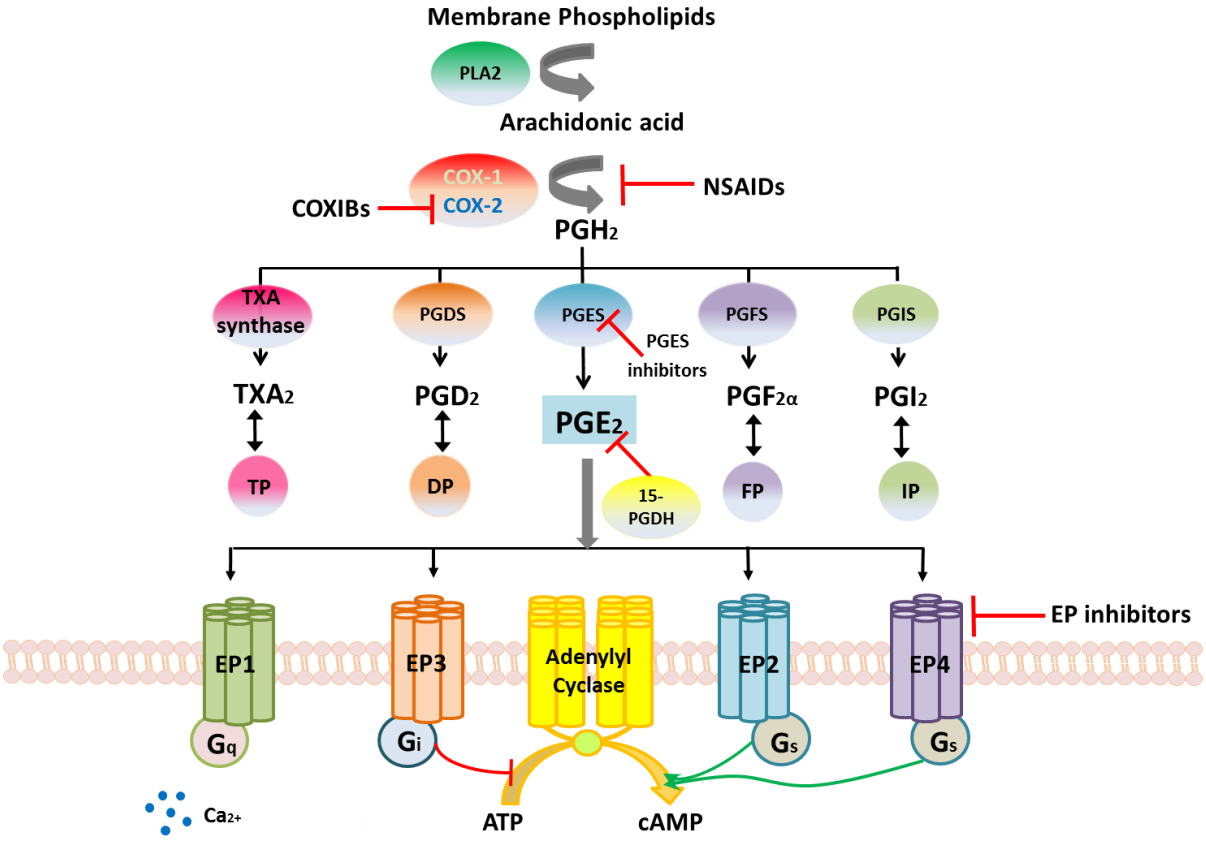


Figure1. COX-2- PGE_2 -EPs signaling pathway. Arachidonic acid is released from the membrane phospholipids by PLA2 and then is metabolized by COX-1 and COX-2 into PGH_2. PGH_2 is converted by specific isomerases (PGDS, PGES, PGFS and PGIS) and TXA synthase to multiple prostaglandins (PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2) and the thromboxane A_2[4]. Prostaglandins act through relative receptors (EP, DP, FP, IP and TP) to mediate their effects[5]. The inhibitors of COX-2- PGE_2 -EPs signaling pathway include nonsteroidal anti-inflammatory drugs (NSAIDs), COX-2 selective inhibitors (COXIBs), PGES inhibitor, 15-hydroxyprostaglandin dehydrogenase (15-PGDH) and EP inhibitors. NSAIDs inhibit the function of both COX-1 and COX-2 while COXIBs only inhibit the function of COX-2. PGE_2 is degraded by 15-PGDH into an inactive 15-keto PGE_2 after binding to EP receptors[6]. Both PGES inhibitors and EP inhibitors are novel inhibitors that have been investigating in these years. **Abbreviation**: PLA2, phospholipases A2; COX-1, cyclooxygenase-1 COX-2, cyclooxygenase-2; PGDS, prostaglandin D synthase; PGES, prostaglandin G synthase; PGFS, prostaglandin F synthase; PGIS, prostaglandin I synthase; PG, prostaglandin; EP prostaglandin E receptor; DP1.2, prostaglandin D receptor 1.2; FP, prostaglandin F receptor; IP, prostaglandin I receptor; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate.

COX enzymes are the primary enzymes in the synthesis of eicosanoids and exist in two isoforms: COX-1 is considered to be ubiquitously expressed[7], whereas COX-2 is expressed predominantly in inflammatory cells and upregulated in chronic and acute inflammations[8]. COX-1 and COX-2 are located on human chromosomes 9 and 1 respectively[9]. PGs produced by COX-1 are crucial for maintaining the integrity of gastric mucosa, normal platelet aggregation and renal function, while PGs derived by COX-2 contributes to cancer progression and metastasis[10]. The COX-2 expression is stimulated by different growth factors, cytokines and prostaglandins, which is associated with inflammatory response and is seen as a prognostic factor for malignancy[11,12]. Furthermore, upregulation of COX-2 and PGE_2 has been identified in many human cancers and precancerous lesions, and COX inhibitory drugs show a protective effects in colorectal cancer and breast cancer[13].

The three distinct synthases contributing to PGE_2 synthesis are consist of microsomal PGE synthase-1 (mPGES-1), mPGES-2 and cytosolic PGE synthase (cPGES)[14,15]. There are two separate PGE_2-biosynthetic routes: the cPLA2-COX-1-cPGES and cPLA2-COX-2-mPGES pathways[15]. COX-2 linked to mPGES is essential for delayed PGE_2 biosynthesis, which may be linked to inflammation, fever, osteogenesis, and cancer[15]. mPGES-1 is primarily responsible for increasing PGE_2 levels during inflammation and carcinogenesis, and elevated levels of mPGES-1 present in a number of human cancers, such as colon, lung, stomach, pancreas, cervix, prostate and head and neck squamous carcinoma[16].

PGE_2 is the most abundant prostaglandin in humans and is known as a key mediator in inflammation. The functions of PGE_2 are mainly facilitated by specific membrane-bound G-protein-coupled EP receptors (EP1-EP4) with various signaling pathways. EP1 is coupled to the G protein alpha q (G_q) to mobilize intracellular Ca^{2+}, EP2 and EP4 are coupled to the G protein alpha stimulator (G_s) to activate adenylyl cyclase (AC), and EP3 is mainly coupled to the G protein alpha inhibitor (G_i) to suppress AC[17]. The EP3 receptor can also be coupled to $G_{12/13}$ proteins, resulting in the activation of the small G protein Rho[18]. After binding its receptor, PGE_2 can be catalyzed by 15-hydroxyprostaglandin dehydrogenase (15-PGDH) into an inactive 15-keto PGE_2[6].

In cancer development, EP1 mediates tumor cell migration, invasion and adjustment to hypoxia enviroment; EP2 induces angiogenesis and suppresses the anti-tumor immune

response; EP4 can mediate tumor cell migration, metastasis, as well as promote aberrant DNA methylation in colon cancer[18]. The role of EP3 in carcinogenesis is still unclear with conflicting effects in distinct cancer cells. 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[19]. EP3 isoforms differ in the amino acid sequences in their specific C-terminal tails and signal transduction pathways by activating different second messengers[20,21]. This might increase the complexity of investigating the effects of EP3 on the pathological mechanism of cancer development.

2.2 Crosstalks with other signaling pathways in cancer

Wang et al. elucidated crosstalks interacting with COX-2-PGE_2-EPs signaling pathways in carcinogenesis, mainly consisting of the epidermal growth factor receptor (EGFR) pathway, nuclear receptor pathway, and Ras-mitogen-activated protein kinase cascade (Ras-MAPK) pathway[7]. The classic and most studied signaling pathway is EGFR pathway, since both COX-2 and PGE_2 are involved in the proliferation, migration and invasion of human colon carcinoma cells through EGFR[22,23]. Combining EGFR tyrosine kinase inhibitor (erlotinib) with COX-2 inhibitor (celecoxib) can inhibit the tumor cells proliferation of head and neck cancer cell lines and the tumor growth of nude mouse xenograft models compared with either single agent[24]. Moverover, the biomarker expression (antigen Ki67, phosphorylated S6 and CD34) of head and neck cancer is decreased in 11 cancer patients received the combined treatment with erlotinib and celecoxib[24]. PGE_2 can also trans-activate peroxisome proliferator activated receptor β/δ (PPAR β/δ) via phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling to promote cell survival of intestinal adenoma[25]. In the mice model, PGE_2 can stimulate tumor growth of intestinal adenoma in *Apc^{min}* mice, but not in *Apc^{min}* mice lacking PPAR β/δ[25]. As a nuclear transcription factor, PPAR β/δ binds as heterodimers with a retinoid X receptor (RXR) for transcription initiation[26], and the natural ligands for PPAR β/δ include fatty acid and PGE_2[27]. Additionally, PGE_2 activates Ras-MAPK cascade and high expression of PGE_2 can induce COX-2 expression in intestinal adenomas[28]. Studies concerning the COX-2-PGE_2-EPs signaling pathway is limited in gynecological cancers compared with that in gastrointestinal cancer and breast cancer[29].

2.3 Endometrial cancer

Endometrial cancer (EC) is the most common gynecological malignancy in developed countries, including the United States, Canada and Western Europe[30]. There estimated to be more than 61,000 new cases of EC and over 10,000 deaths in the United States according to the 2017 cancer statistics[31]. The main risk factor for EC is exposure to endogenous and exogenous estrogens, which is linked to obesity, diabetes, early age at menarche, null parity, late menopause and use of tamoxifen[32]. EC is classified into two subtypes: type I and type II. Type I is the most common subtype, and it is low-grade, endometrioid, diploid, hormone-receptor-positive endometrial cancer with a good prognosis[32]. By contrast, type II EC is high-grade, non-endometrioid, aneuploidy, hormone-receptor-negative, *TP53*-mutated with a poor prognosis and a higher risk of metastasis[32].

2.3.1 COX-2 and endometrial cancer

COX-2 is expressed in the cytoplasm of normal proliferative glandular epithelium and endometrial cancer cells[33]. The mRNA level of COX-2 is elevated in 51 cancerous endometria compared with 16 normal endometria[34]. COX-2 is proved to be a negative predictor of disease relapse for EC patients in the univariate analysis. COX-2 plays a key role not only in the maintenance of the endometrium during the menstrual cycle but also in EC carcinogenesis[35]. COX-2 overexpression increases angiogenesis, migration, invasiveness and tumor-induced immmunosuppression, as well as prevents apoptosis[35]. A combined treatment with celecoxib (a COX-2 inhibitor) and rapamycin (a mammalian target of rapamycin complex 1 inhibitor, a mTORC1 inhibitor) reduces EC progression in mouse models of EC and human EC cell lines[36]. Brasky et al. demonstrated that treatment of aspirin could reduce the risk of EC, especially in estrogen-mediated cases by analyzing 22,268 female Americans after up to ten years[37]. In clinical studies, the correlation of COX-2 expression and EC patients' prognosis still remains conflicting[38,39]. PGE_2 is associated with both endometrial functions and disorders. Ke et al. found that prostaglandin E synthase 2 (PTGES2) is upregulated in the 119 endometrial cancer tissues compared with 50 normal endometria, and PTGES2 is associated with the endometrial carcinoma stage, grade and the depth of myometrial invasion[40].

2.3.2 EPs expression in healthy normal endometrium

The expressions of EP receptors vary during different phases of normal endometrium. EP1 peaks during the early secretory phase and EP2-4 peak during the mid-secretory phase across the regular human menstrual cycle by reverse transcription polymerase chain reaction (RT-PCR)[41]. Besides, EP1 is located in different regions of glands depending on different phases[41]. However, Zhu et al. (2017) found that EP1 is expressed highly during the proliferative phase and decreases sharply during the early secretory phase in both epithelium and stroma cells of the normal endometrium with the method of immunohistochemistry[42]. Both studies indicate that EP1 may be regulated by sex hormones and may also play a role in mediating implantation.

2.3.3 EPs expression in endometrial cancer

Zhu et al. suggested that six patients with higher EP1 staining survived after seven years follow-up, although EP1 expression was not correlated to progression free survival or overall survival of endometrial cancer patients[42]. The biosynthesis of EP2, EP4 and cAMP are significantly elevated in response to PGE_2 in endometrial adenocarcinoma tissues compared with normal endometria by quantitative PCR[43]. PGE_2 stimulates vascular endothelial growth factor (VEGF) expression in Ishikawa cells (a human endometrial adenocarcinoma cell line) via EP2-cAMP-mediated transactivation of the epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinases 1/2 (ERK1/2) pathways[44]. Battersby and his colleagues proved that PGE_2 upregulates the expression of fibroblast growth factor 2 (FGF2) via the EP2 receptor in a cAMP-, c-Src-, epidermal growth factor receptor (EGFR)- and extracellular signal-regulated kinase (ERK)-dependent manner in Ishikawa cells[45]. FGF2 is a potent mitogenic and angiogenic factor, causing adenocarcinoma cell proliferation in nude mice transplanted subcutaneously with endometrial adenocarcinoma[46]. PGE_2 can enhance proliferation and invasion of two human endometrial cancer cells (Ishikawa and HEC-1B) by stimulating EP4 receptor and small ubiquitin-like modifier-1 (SUMO-1) via the Wnt/β-catenin signaling pathway[40].

The proliferation and angiogenesis of implanted tumor can be directly inhibited in *EP3*$^{-/-}$ mice, as well as suppressed by an EP3 antagonist (ONO-AE3–240) in wild-type mice[47]. EP3

mRNA is expressed abundantly in the uterus[48]. The latest study by our research group demonstrated that high expression of EP3 correlates with poor progression-free survival and overall survival in endometrial carcinoma[49]. Our group also proved that L-798,106 (a specific EP3 antagonist) induces the expression of estrogen receptor β and inhibites the activity of Ras, leading to decreased proliferation and migration of RL95-2 cells[49]. Overexpression of estrogen receptor β (ERβ) inhibits proliferation and invasion of tumor cells in breast cancer and endometrium[50,51].

2.4 Ovarian cancer

Ovarian cancer ranks the fifth as a cause of neoplastic death among women worldwide[30]. There would be around 22,440 new cases and 14,080 deaths in the United States in 2017[31]. Incidence rates are highest in more developed regions, with rates of more than 7.5 per 100,000[30]. The overall 5-year survival rate of ovarian cancer is just approximately 30-40%[52]. The fundamental problem in treating ovarian cancer is that it is not easy to discover it at an early stage and accomplish complete curative resection. Ovarian cancer histological groups include type I epithelial, type II epithelial, germ cell, sex cord-stromal, other specific non-epithelial and non-specific tumors[53]. Among all the subtypes, type II epithelial tumors are the most common in Oceania, North America and Europe, while type I epithelial tumors are more common in Asia during 2005-2009[53]. Type II epithelial tumors are associated with poorer survival than type I epithelial, germ cell and sex cord-stromal tumors[53].

2.4.1 COX and ovarian cancer

COX-2$^{-/-}$ female mice show defective ovulation, fertilization and implantation[54]. The mRNA expressions of COX-1, COX-2, EP2 and EP4 are detected in both granulosa and cumulus cells in mice periovulatory follicles during superovulation[55]. COX-1, COX-2, mPGES-1, EP1 and EP2 are expressed predominantly in epithelial cells of human epithelial ovarian cancer[56]. Kino et al. (2005) believed that COX-1 is the primary enzyme for producing PGE_2 instead of COX-2 in ovarian cancer cells. Because the elevated expression of COX-1 instead of COX-2 was detected in 22 ovarian cancer tissues compared with that in normal cases[57], and SC-560 (a COX-1 inhibitor) can suppress the production of PGE_2 in three ovarian cancer cell lines while NS-398 and rofecoxib (COX-2 inhibitors) can not[57]. However, the latest meta-analysis (2017)

suggested that COX-2 expression is correlated with FIGO stage, histological type and patients' age and the high expression of COX-2 is associated with reduced OS and DFS of ovarian cancer patients[58]. In addition, high expression of COX-2 is associated with a shorter progression time and overall survival time in the patients who firstly underwent explorative laparotomy and then received chemotherapy[59]. It implies that COX-2 is also correlated with chemotherapy resistance.

For *in vitro* studies, COX-2 can stimulate the proliferation, migration, apoptosis and angiogenesis of ovarian cancer cells. COX-2 enhances proliferation and migration of human ovarian cancer CAOV-3 cells mainly through activation of phosphatidylinositol 3-kinase/protein kinase B (PI3-k/Akt) pathway[60]. By analyzing the epithelial ovarian cancer (EOC) tissues and EOC cell lines (MDAH2274 and SKOV3), Uddin and his colleagues demonstrated that COX-2 modulates cell growth and apoptosis also through PI3K/AKT signaling pathway in EOC[61]. Furthermore, Uddin et al. proved that COX-2 could be a potential therapeutic target in EOC because treatment of xenografts together with aspirin can inhibit tumor growth in nude mice through decreasing the expression of COX-2 and Akt[61]. COX-2 protein levels correlate with VEGF protein levels and microvessel counts in ovarian carcinoma[62].

The expression of COX-2 is regulated by various cytokines in ovarian cancer cells, such EGF, vitamin D, IL-1β. EGF induces the production of both COX-2 and PGE_2 via the activation of the PI3K/Akt signaling pathway, resulting in an invasion of SKOV3 and OVCAR5 cells (two human ovarian cancer cell lines)[63]. A reduced level of vitamin D receptor (VDR) and an induced level of COX-2, 15-PGDH and PGE_2 are found in the serum of ovarian cancer patients older than 45 years[64], suggesting an interaction between PG and vitamin D-metabolism in ovarian cancer. The mRNA and protein expression of COX-2 can be stimulated by IL-1β and phorbol ester (TPA) in OVCAR-3 cells and by TPA in CAOV-3 cells[65].

2.4.2 EPs and ovarian cancer

PGE_2 can induce cell invasiveness via the increasing the expression of MMP-2 and MMP-9 in two human ovarian cancer cell lines (CaOV-3 and SKOV-3)[66]. PGE_2 stimulates the VEGF production in HEY ovarian cancer cells mainly via activating EP2 and EP4, which can be

reversed by AH23848 (an antagonist of both EP2 and EP4)[67]. In addition, PGE_2-induced EP4 receptor signaling induces MMP production and ovarian cancer cell invasion through Src-mediated EGFR transactivation[67]. EP2 plays a vital role in the process of ovulation and fertilization because cumulus expansion becomes decreased in *EP2*$^{-/-}$ mice[68]. By feeding hens 10% flaxseed-enriched or standard diet for four years, Eilati *et al.* proved a decreased expression of COX-2 and PGE_2, as well as a reduction in ovarian cancer severity and incidence[69].

2.5 Cervical cancer

Cervical cancer is the fourth most common cancer and the fourth most common cause of cancer-related death among women worldwide[70]. The incidence and prevalence of cervical cancer are higher in the developing countries than in the developed countries owing to a lack of screening, availability of vaccine, and awareness of HPV infections[71]. In the United States in 2017, there were an estimated 12,820 cases and 4,210 deaths from cervical cancer[31]. In many developing countries, cervical cancer causes more than one-quarter of a million deaths per year[72]. The 5-year survival rate of cervical cancer is between 63-79% in China, Singapore, South Korea and Turkey, less than 25% in Gambia and Uganda[73]. The two main malignant epithelial cervical cancer types are the squamous cell carcinoma and the adenocarcinoma[74].

The human papillomavirus (HPV) infection is the crucial risk factor for cervical cancer[75], and inflammation after the HPV infection is a driving force that increases cancer development[76]. HPV is present in more than 90 % of squamous cell cervical cancer cases worldwide and in nearly 90% of adenocarcinomas of women younger than 40 years old [77]. HPV serotype 16, 18, 45, 31, 33, 52, 58 and 35 are the most common serotype of HPV in women with cervical cancer[72]. High-risk HPV 16 encodes three oncoproteins: E5, E6 and E7[78]. E5 proteins induce COX-2 expression, and COX-2 increases PGE_2 secretion and EP4 expression[79]. E6 proteins can inactivate p53, block cell apoptosis, disrupt cell adhesion, alter transcription and reduce immune recognition[80]. E7 proteins inactivate pRb to force the host cell to keep cell division and induce genomic instability[81].

2.5.1 COX-2 and Cervical cancer

High expressions of COX-2 and PGE_2 have been found in the cervical carcinoma[82,83]. COX-2 is overexpressed in various types of cervical neoplasm such as cervical intraepithelial neoplasia (CIN), adenocarcinoma and squamous cell carcinoma, implying that COX-2 expression is highly associated with cervical carcinoma development and progression[10]. Many studies show that COX-2 contributes to carcinogenesis and progression of cervical cancer. High expression of COX-2 is related to poor overall survival (OS) and poor disease-free survival (DFS) in cervical cancer patients[84]. COX-2 is also associated with poor DFS in chemo-radiation subgroup, implying COX-2 is a chemo-radiation resistance predictive factor for cervical cancer[84]. The COX-2 expression is more frequently expressed in adenocarcinoma than in squamous cell carcinoma by immunohistochemistry[85]. The co-expression of COX-2 and thymidine phosphorylase (TP) is related to poor 5-year disease-free and overall survival rates, suggesting that the combination of COX-2 and TP is a prognosticator for squamous cell carcinoma of the cervical cancer[86].

The expression of mPGES-1 is higher in squamous intraepithelial lesions and carcinoma of the uterine cervix compared with the normal cervical epithelium[87]. Radilova et al. (2009) found that COX-1 is also coupled with mPGES-1 for co-regulating PGE_2 synthesis in human cervix cancer cells[88]. Dimethylcelecoxib (a non-COX-2 inhibitor) inhibits the early growth response protein 1 (EGR1) and transcription of mGPES-1 via an enhanced complex of NF-κB and histone deacetylase 1 (HDAC1) that binds to the EGR1 promoter in Hela cells[89].

2.5.2 EPs and Cervical cancer

Sales et al. (2001) reported that the syntheses of COX-2, PGE_2, EP2, EP4 and cyclic adenosine monophosphate (cAMP) are up-regulated in cervical cancer tissue compared to that in the healthy cervix, suggesting that PGE_2 may regulate neoplastic cell function via the EP2/EP4 receptors[82]. Sales and his colleagues further in 2002 proved that PGE_2 could induce the expression of COX-2, EP4 and cAMP in Hela cells which were transiently transfected with EP2 or EP4 cDNA[83]. However, this research did not detect whether the expression of cAMP would be decreased after knocking out or knocking down the expression of EP2 or EP4. Kurt J. et al. (2002) observed that rapid accumulation of cAMP is produced in Hela cells after

being stimulated with PGE_2, which is mediated via the cAMP-linked EP2/EP4 receptors[83]. Both studies imply that PGE_2 regulates the function of cervical cancer cells mainly via cAMP-linked EP2/EP4 signaling pathway.

Jung-Min et al. (2009) showed an increased expression of EP4 in 52 cervical cancer tissues compared with four healthy controls by immunohistochemistry[79]. This study also demonstrated that HPV16 E5 upregulated the activity of PGE_2-EP4-cAMP signaling pathways by inducing the binding of cyclic adenosine monophosphate response element binding protein (CREB) to a variant CRE site in the promoter of the human EP4 gene[79]. EP4 plays a role in the proliferation and angiogenesis of cervical cancer cells since GW627368X (a highly selective EP4 antagonist) inhibits the proliferation and angiogenesis of cervical carcinoma by blocking EP4/EGFR signaling pathway in cervical cancer cell lines (HeLa, SiHa and ME 180) and suppresses the tumor size in xenograft mice model[90].

It has recently demonstrated that overexpression of EP3 in cervical cancer patients is associated with impaired prognosis in overall survival rates when evaluating 250 cervical cancer patients with immunohistochemistry[91]. As an independent prognosticator for cervical carcinoma, the EP3 receptor is also significantly correlated with lymph node stage and FIGO stage[91]. However, the pathological mechanism of how EP3 signaling regulates in cervical cancer is still unclear.

2.6 Recurrent miscarriages

Trophoblasts and malignant tumors use the same biochemical mediators to degrade extracellular matrix degradation and suppress immune reaction of environmental conditions[92]. Recurrent miscarriages (RM) are defined as two or more consecutive failed pregnancies before the 20th week of pregnancy according to the Practice Committee of the American Society for Reproductive Medicine[93]. RM is also called recurrent pregnancy losses (RPL) affecting 1% of all women worldwide[94], and only a small part of reasons can be attributed by particular causative factors, comprising genetic abnormalities, structural abnormalities, infection, endocrine abnormalities, immune dysfunction and thrombophilic disorders[93]. Up to 50% of RM cases are classified as unexplained RM (uRM).

Inflammation is essential for successful blastocyst implantation, pregnancy maintenance and delivery, however, ultimately inflammatory over-reaction has a devastating effect on pregnancy outcome, including RM[95]. Laird et al. (2003) postulated that three mechanisms that result in RM: increased activity of uterine natural killer (uNK) cells or macrophages, various effects of cytokines on trophoblast and thrombotic effects of cytokines on vasculature[96]. Decreased expression of IL-1β and IL-6 mRNA has been detected in the endometrium of RM patients than healthy controls during the mid-sectory phase[97]. Elevated production of Th1 cytokines (interferon-γ, IL-2, IL-12, TNF-β) and downregulated levels of Th2 cytokines (IL-6) are found in RM women compared with controls by qualitative reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA)[98]. Abnormal inflammation in the fetal-maternal interface and unbalanced extracellular matrix (ECM) remodeling are also considered as key factors resulting in RM[99].

2.6.1 COX-2-PGE_2 and Recurrent miscarriages

COX-2 is expressed in human endometrial stroma cells[100] and it has influence in the blastocysts implantation in the early pregnancy phase [101]. So far, a limited amount of literature exists concerning COX-2 expression levels in RM with conflicting results. Wang et al. (2010) reported that lower mRNA and protein expressions of COX-2 have been found in the chorionic villi of women with unexplained RM[101], and Hua et al. (2013) showed the similar result in the mice embryo with autoimmune-type recurrent miscarriages[102]. Singh et al. (2017) reported that increased mRNA levels of MMP-2/MMP-9 were found in 135 C. trachomatis-positive RM patients than 120 age-matched controls, and MMP-2 positivley correlated with COX-2[103]. It implies that COX-2 might participate in both inflammatory reactions and endometrial matrix degradation in C. trachomatis-positive RM patients.

Banerjee et al. (2012) reported that expressions of PGE_2 and pro-inflammatory cytokines are also induced in the endometrium of women with unexplained RM, such as interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), and transforming growth factor β1 (TGF-β1) [104]. IL-1, TNF-α and IFN-γ belong to Th1 cytokines, and this Th1 predominance has been known to be one of the immunological reasons for RM[105]. Additionally, TNF-α can induce COX-2 gene expression in first-trimester trophoblasts[106]. PGE_2 is the major prostaglandin produced by the placenta[107]; it can modulate the Th1 and Th2 cell balance

responses[108], regulate chemokine productions[108] and inhibit lymphocyte alloreactivity during early pregnancy[109]. PGE_2 is found to be increased in cervical ovulatory mucus[110] and the endometrium[104,111] of RM women compared to healthy controls during implantation. Until now there is rare report on the EP receptors expression in the pathological mechanism of RM.

2.7 Drug targeting of COX-2-PGE_2-EPs signaling

Chemoprevention has long been recognized as an important prophylactic strategy to reduce the burden of cancer on health care system. Also, nonsteroidal anti-inflammatory drugs (NSAIDs) as chemoprevention chemicals have been proved to reduce the risk of several cancers in human, such as gastrointestinal cancer, breast cancer, prostate cancer, lung cancer and skin cancer[112]. Nan et al. (2015) found that regular use of aspirin or NSAIDs is linked to lower risk of colorectal cancer compared with no regular use after analyzing 8634 colorectal cancer cases and 8553 matched controls between 1976 and 2011[113]. Long-term use of COX inhibitors in humans leads to a 50% decrease in risk for colorectal cancer[25]. A meta-analysis by Banndrup et al. (2013) suggested the risk of invasive ovarian cancer is significantly reduced with the use of aspirin[114].

NSAIDs include aspirin, ibuprofen and naproxen, and act by inhibiting both COX-1 and COX-2. These unspecific inhibitors cause many adverse effects, such as gastrointestinal ulcers and bleeds, heart attack and kidney disease[115]. Selective COX-2 inhibitors (COXibs) has been successfully documented and showed less toxicity to gastrointestinal tract as compared to traditional NSAIDs[116]. However, the long-term use of COX-2 selective inhibitors still has other side effects. The adverse effects of COX-2 selective and non-selective inhibitors are summarized in the latest review by Rayar et al. (2017) including myocardial infarction, hypertension, stroke, reduced glomerular filtration rate and renal plasma flow, acute renal failure, acute interstitial nephritis, inhibition of ulcer healing, hepatic complications, allergy, fatal skin reaction, depression, delayed follicular rupture and so on[117]. Therefore, further exploration of novel anti-inflammation targets is needed.

Clinical studies show elevated levels of mPGES-1 are identified in colon, lung, stomach, pancreas, cervix, prostate, papillary thyroid carcinoma, head and neck squmaous carcinoma

and brain tumors, suggesting mPGES-1 inhibitors might be a potential chemopreventive agent[16]. However, a limited number of compounds that inhibiting mPGES-1 has not been successfully developed as anti-cancer agents, such as celecoxib, MF-63, NS-398, MK-866 and triclosan[16].

In recent years, extensive efforts have been made into elucidating the function of PGE_2 and the EP receptors in health and carcinogenesis with the aim of exploring promising targets and selective inhibitors as a novel therapy. Many researchers have found the strong correlation of EP2/EP4 receptors with colon cancer, skin cancer, mucosa cancer of the pharynx and the esophagus, prostate cancer, urothelial cancer and non-small cell lung cancer[18]. The EP4 promotes migration, invasion, angiogenesis and lymphangiogenesis of mammary tumor cells[118]. EP4 receptor is responsible for the PGE_2-induced colorectal tumor cell proliferation and morphogenic changes via PI3k/Akt signaling pathway[119]. Mice are not able to have inflammatory responses to PGE_2, IL-1β or lipopolysaccharide when lacking the EP3 receptor, but not EP1, EP2 or EP4 receptor[120]. Deletion or inhibition of EP3 receptors could ameliorate the neuronal apoptosis in the ischemic cortex in EP3 knock-out mice or EP3 antagonist-treated mice compared with wild-type mice or vehicle-treated mice, respectively. It suggests that EP3 is involved in the inflammatory and apoptotic reactions during stroke injury[121]. The investigations concerning EP receptors in gynecological cancers highlight the potential advantage of combining COX enzyme inhibitors with EP receptor antagonists as therapeutic agents in gynecological cancers.

Wang et al. proposed the possible PGE_2 downstream targets that might also serve as promising specific chemopreventive agents for cancer prevention and treatment, which include angiogenic factors (VEGF, bFGF), anti-apoptotic factors (Bcal-2), chemokines (MIP-1α, MIP-1β, RANTES, CXCR4) and their receptors, and immunosuppressive mediators[7].

2.8 EP3 and PAI-1 transcription

It is interesting to notice that EP3 can induce PAI-1 expression, which is also a prognosticator in carcinogenesis of numerous cancers. In the development of aortic stenosis, PGE_2 utilizes EP1/EP3 receptor to increase levels of plasminogen activator inhibitor type 1 (PAI-1) in cardiac fibroblasts resulting in elevated fibrin deposition[122]. PAI-1 is the prime inhibitor of

the fibrinolytic system, and its expression affects cell survival, migration and matrix remodeling in tumor cells as well as trophoblast cells. PAI-1 can inhibit trophoblasts invasion[123] while promoting tumor cell immigration. PAI-1 is a biomarker for malignancies with poor prognosis because it facilitates many tumor cell migration and invasion[123]. PAI-1 is a poor prognostic factor for early-stage endometrial cancer and advanced stage epithelial ovarian cancer patients[124,125]. Hazelbag et al. (2004) suggested that PAI-1 is also a negative prognosticator for the overall and disease-free survival of cervical cancer patients by analyzing 108 paraffin-embedded cervical carcinoma tissues[126]. PAI-1/uPA/uPA receptor (uPAR)/low density lipoprotein receptor-related protein (LRP)/integrin complexes are initiating an "adhesion–detachment–re-adhesion" cycle to promote tumor cell migration[123,127].

Most pieces of literature illustrate the gene transcription of PAI-1 is regulated by TNF-β, and the main signaling pathway includes MEK/ERK and Smad (**Figure 2**). Hua et al. (1998) proved that TGF-β signals through type I and type II receptor serine/ threonine kinases and induces PAI-1 gene transcription via Smad3, Smad 4 and transcription factor μE3 (TFE3)[128]. Winkins-Port and his colleagues (2007) suggested that in human cutaneous squamous cell carcinoma, TGF-β1 can trans-activate both MEK and EGFR, and then phosphorylate the downstream-activated MAP kinases consisting of ERK1/2 and p38[129]. Thereby TGF-β1 regulates the PAI-1 gene transcription with members of the Smad family and USF family in the nucleus[129]. Samarakoon et al. (2008) showed two distinct signaling pathways that TGF- β initiates PAI-1 transcription in vascular smooth muscle cells, which consist of the EGFR/pp60c-src/MEK-ERK pathway and Rho/ROCK-dependent SMAD2 activation[130]. Freytag et al. (2009) demonstrated that Smad 2/3 cooperates with p53 and upstream stimulatory factor proteins 1/2 (USF1/2) for maximal TGF-β directed PAI-1 transcription in human cutaneous squamous cell carcinoma[131]. Samarokoon et al. elucidated the extensive cross-talk among Smad2/3, EGFR, reactive oxygen species (ROS) and tumor suppressor p53 pathways are necessary for TGF-β1 inducing PAI-1 in renal fibrosis[132]. ROS production by TGF-β1 stimulation is crucial for activation of EGFR and p53, which cooperates with Smad3 to regulate PAI-1 transcription[132].

Numerous studies show that significant crosstalk exists between the EP receptors (EP1[133], EP2[44] and EP4[134]) and the EGFR signaling pathway. The EGFR signaling pathway can also activate several other signal transduction cascades, principally the MAPK, PI3K/Akt, signal transducers and activators of transcription (STAT) and Phospholipase C (PLC) signaling pathway, contributing to the pathogenesis and progression of numerous cancers[18]. However, it remains unknown if PGE_2-EPs signaling can work through EGFR signaling pathway to regulate PAI-1 gene transcription.

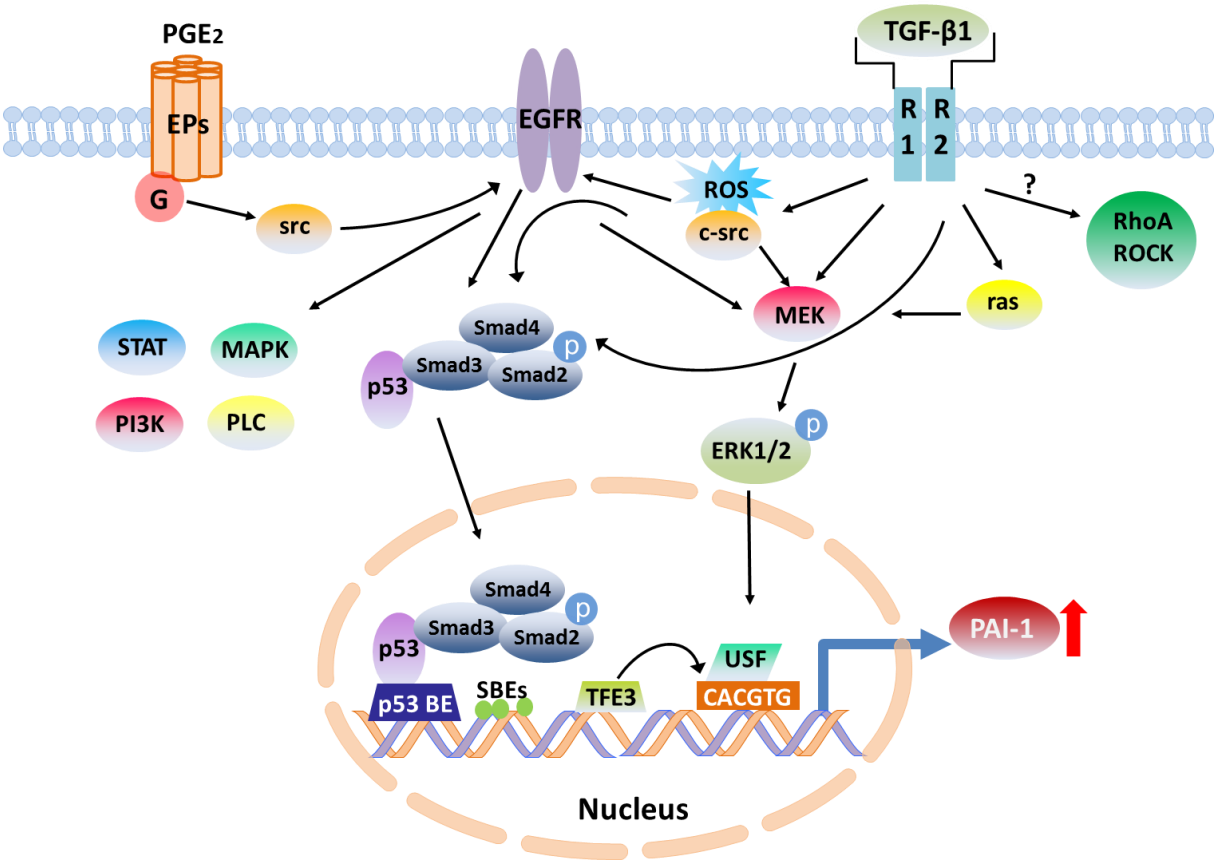


Figure 2. TGF-β1 induces PAI-1 gene transcription via complex signaling pathways. TGF-β1 can directly activate MEK through type I and type II receptor, and phosphorylate the downstream-activated MAP kinases consisting of ERK1/2, which is the main signaling pathway[129]. TGF-β1 can also transactivate EGFR via ROS or c-src, and both EGFR and c-src motivate MEK/ERK1/2[130]. TGF-β1 can also directly phosphorylate the Smad family including Smad 2, 3 and 4 as well as p53, which can bind with transcription factors. The transcription factors for PAI-1 include the Smad family[128,131], USF

family[131], p53 SE[131], SBE transcription factor μE3 (TFE3)[128] in the nucleus. TGF-β1 can also activate Ras and Rho/ROCK. The EP receptor signaling pathways also have crosstalk with the EGFR signaling pathway[18], which can activate several other signal transduction cascades, principally the MAPK, PI3K/Akt, STAT and PLC signaling pathways. It remains to be explored if PGE_2-EPs signaling can work through EGFR signaling pathway to regulate PAI-1 gene transcription. **Abbreviation:** TGF-β1, transforming growth factor β 1; PAI-1, plasminogen activator inhibitor type1; ERK, extracellular signal-regulated kinase; ROS, reactive oxygen species; ROCK, Rho-associated coiled-coil kinase; USF, upstream stimulatory factor proteins; TFE3, transcription factor μE3; MAPK, Mitogen-activated protein kinase cascade; PI3K/Akt, phosphatidylinositol 3-kinase/protein kinase B; STAT, signal transducers and activators of transcription; PLC, Phospholipase C.

2.9 Aim of the studies

The pathology underlying gynecological cancer development and recurrent miscarriages is not fully understood yet and an inflammatory over-action is one factor, which might play a role in the development of gynecological malignancies. COX-2-PGE_2-EPs signaling is the main pathway involved in inflammation, playing a role in carcinogenesis and miscarriages. A large number of studies have demonstrated the effects of COX-2 in gynecological cancer and proved high expression of COX-2 and PGE_2 benefits tumor development. Furthermore, NSAIDs or specific COX-2 inhibitors can successfully suppress tumor growth and inflammatory reaction. However, the side effects of NSAIDs or specific COX-2 inhibitor (COXibs) have also raised people's attention, including the gastrointestinal ulcers and bleeds, cardiology effects and stroke. Therefore, it is urgent to explore more effective and specific targets for cancer and RM therapy.

Limited information is available about the potential role for EPs in gynecological cancer as well as in recurrent miscarriages. Most of the studies have explored EP2/EP4 expression in the mechanism of cervical cancer and endometrial cancer. The effects of EP3 are still unclear on the cervical cancer, as well as on RM. We aimed to explore the EP3 signaling in the mechanism of cervical cancer and recurrent miscarriages from the perspective of inflammation. Therefore, this thesis is divided into two parts: 1) EP3 signaling in the carcinogenesis of cervical cancer; 2) EP3 signaling in the pathological mechanism of recurrent miscarriages. In addition, the possible correlation between EP3 signaling and plasminogen activating system during cancer development and miscarriages has been investigated. Based on the *in vivo* and *in vitro* studies, the intention was to explore the potential effects of EP3 antagonist (L-798,106) as a therapeutic approach to treat cervical cancer and recurrent miscarriages.

3. Material and methods

3.1 Cell culture and stimulation

CaSki cells (CRL-1550) and Hela cells (CCL-2) were used as cervical cancer cell models, while JEG-3 cells (ATCC HTB-36, Manassas, USA) and HTR-8/SVneo cells (ATCC CRL-3271, Manassas, USA) were used as trophoblast models. All of them were obtained from the American Type Culture Collection (ATCC). All cell lines were cultured in RPMI 1640 medium + Gluta MAXTM (Gibco, USA) supplemented with 10% fetal bovine serum (FCS, Gibco, USA) without antibiotics or antimycotics. 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) test, and 6-well plates for western blotting. After 6-8 hours, cell culture medium was replaced with fresh RPMI1640 with stimulation chemicals for the remaining 48 hours, which included dimethyl sulfoxide (DMSO, 0.5%) as vehicle control, PGE_2 (TOCRIS, USA), sulprostone (TOCRIS, USA) and L-798,106 (TOCRIS, USA).

3.2 Cell proliferation and viability assay

Both CaSki and Hela cells were cultured at a density of 1.5×10^4 cells/well in 96-well plates and were incubated with different concentrations (1, 10 and 100 nM) of sulprostone (TOCRIS, USA) for 24 h, 48 h and 72 h. JEG-3 cells were seeded at the density of 1.5×10^4 cells/well in 96-well plates and were incubated with different concentration of PGE_2 and L-798,106 for 48 h. The dimethyl sulfoxide (DMSO, 0.5%) always served as a vehicle control.

Cell proliferation was analyzed with a 5-bromo-2'-deoxy-uridine (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.

Cell viability assay was performed in JEG-3 cells, 20 µg MTT [3-(4,5-dimethhylthiaoly)-2,5-diphenyltetrazolium bromide] (Sigma) was added to each well for 1.5 h at 37 °C. After

removing MTT, 200 µl DMSO was added to each well and mixed thoroughly on the shaker for 5 min at room temperature. The optical density (OD) was examined at 595 nm using Elx800 universal Microplate Reader.

3.3 Wound Healing (Scratch) Assay

2.5×10^5/well of CaSki cells and 1.2×10^5/well of Hela cells were cultured in 24-well plates for two days. On the third morning 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) to remove the detached cells. Fresh RPMI1640 with DMSO (0.5%) and 100 nM sulprostone was added respectively. 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/). The cell migration area = area at 0 h – area at 24 h.

3.4 Western Blotting

Cell lysates were extracted from CaSki, Hela, JEG-3 and HTR-8/SVneo cells with radioimmunoprecipitation assay buffer (RIPA, Sigma-Aldrich, R0278-50ML). 20µg 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 hours at room temperature. Different primary antibodies were used as follows: rabbit polyclonal anti-EP3 antibody (Abcam, ab94496, 1:500), goat polyclonal anti-EP1 antibody (MyBioSource, MBS420141, 1:150), rabbit polyclonal anti-G_{i1} antibody (Novus Biologicals, NBP2-16558, 1:500), rabbit polyclonal anti-p-ERK1/2 antibody (Abcam, ab47339, 1:500), rabbit polyclonal anti-uPAR antibody (Abcam, ab218106, 1:300), mouse monoclonal anti-p53 antibody (Abcam, ab26, 1:200). B-actin was used as a housekeeping gene and mouse monoclonal anti-β-actin antibody was diluted as 1:1000 in 4% milk powder (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). Band intensities of EP3, EP1, G_{i1}, p-ERK1/2 and uPAR were normalized with band intensities of β-actin. The blots were repeated at least three times.

3.5 ELISA

3.5.1 PAI-1 and uPA

Both CaSki and Hela cells were cultured at a density of 5×10^4 cells/well in 24-well plates and were incubated with 10 nM of PGE_2, sulprostone and L-798,106, respectively. After 48 hours, the supernatants of both cell lines were harvested. The levels of PAI-1 in the supernatants of Hela and CaSki cells were measured utilizing 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. The intra- and inter-assay variability of PAI-1 was 4.6% and 8.7%, respectively. The levels of uPA in the supernatants of both cell lines were also evaluated using a commercially available ELISA kit (R&D system, DPUA00, Minneapolis, MN, USA). A standard curve of uPA was obtained for each assay and results were converted into pg/ml. The intra- and inter-assay variability of uPA was 2.1% and 7.1%, respectively.

3.5.2 β-hCG and progesterone

Both JEG-3 (50'000 cells/ well) and HTR-8/SVneo cells (60'000 cells/ well) were incubated with PGE_2, sulprostone and L-798,106 at different concentrations of 0.1, 1, 10, 100, 1000 nM and 10 µM in 24- well plates for 48 hours. Supernatants of JEG-3 and HTR-8/SVneo cells in 24-well plates were collected and centrifuged (13200g, 10min) to remove debris. The concentration of β-hCG and progesterone in the supernatant was detected according to the manufacturer's instructions on an ADVIA Centaur XP auto 23nalyser (Siemens Medical Solution Diagnostics) as described in our previous publications (32, 33). The concentration of total β-hCG was measured in a two-site sandwich immunoassay (ADVIA Centaur Total hCG Test; Siemens Medical Solutions Diagnostics). The detectable concentration of total β-hCG ranges from 2.0 mIU/ml to 1000 mIU/ml. The concentration of progesterone in supernatants was measured by an automated quantitative immunoassay (ADVIA Centaur Progesterone

Test; Siemens Medical Solutions Diagnostics) with a sensitivity of 0.2ng/ml. Intraobserver and interobserver coefficients of variation were 9.8% and 5.8%, respectively.

3.6 Clinical samples

3.6.1 cervical cancer tissues

We analyzed paraffin-embedded cervical cancer samples from 250 patients having undergone surgery 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 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 Tumor 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 250 cervical cancer patients are listed in Table 1, which includes age, follow-up months, stage, 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.

3.6.2 Placental tissue samples

We analyzed placenta tissues of 19 patients with a history of more than two consecutive pregnancy losses of unknown causes (uRM group) and of 19 healthy controls with legal pregnancy termination (control group) from a private practice in Munich, Germany. Exclusion criteria for the uRM group, described thoroughly in our previous studies[135], include infectious diseases, uterine anomalies, endocrinological dysfunctions, hyperprolactinemia, hyperandrogenemia, thyroidal dysfunctions, autoimmunologic disorders, deficiencies in

coagulation factors, as well as fetal and parental chromosomal disorders. All placentas were obtained within the first 24 hours after diagnosis without any prior hormonal pretreatment. Samples were obtained by dilatation and evacuation without any prior pharmaceutical induction. These samples were fixed immediately in 4% buffered formalin for 20-24 hours and embedded in paraffin for further immunohistochemical and double immunofluorescence analysis. The study was approved by the ethical committee of the Medical Faculty, Ludwig-Maximilian-University of Munich (Number of approval: 337-06) and informed consent was obtained from each patient in written form. Samples and clinical information were anonymized and encoded for statistical workup.

3.7 Immunohistochemistry

Paraffin-embedded slides (3µm-thick) were dewaxed in xylol and washed in 100% ethanol. For inhibition of the endogenous peroxidases, tissue samples were incubated in methanol with 3% H_2O_2 and rehydrated in a descending alcohol series. Slides were heated in a pressure cooker using sodium citrate buffer (pH=6.0), containing 0.1 M citric acid and 0.1 M sodium citrate in distilled water. After cooling and washing in phosphate-buffered saline (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 from cervical cancer patients were incubated with rabbit polyclonal anti-uPAR antibody (Abcam, ab218106, 1:300 dilution) for 16 hours at 4°C. The primary antibodies used for recurrent miscarriages patients were anti-COX rabbit IgG polyclonal antibody (Sigma, SAB4502491, 1:400 dilution), anti-EP3 rabbit IgG polyclonal antibody (Abcam, ab189131, 1:300 dilution) and anti-G_{i1} rabbit IgG polyclonal antibody (Novus Biologicals, NBP2-16558, 1:1000 dilution). After washing, the secondary antibodies/complexes of HRP-polymer (Zytochem-Plus HRP Polymer-kit, Zytomed, Berlin, Germany) were applied following the manufacturer's protocols to detect reactivity. uPAR immunostaining was visualized with the substrate and the chromogen-3, 3'-diaminobenzidine (DAB; Dako, Hamburg, Germany) after 3 min, 1 min for COX-2, 2.5 min for EP3 and 45 sec for G_{i1}.

Metastatic colon carcinoma was used as a positive and negative control for the immunohistochemical staining of uPAR to test antibody function and to determine an

appropriate dilution of the antibody for staining. Colon was used as a positive control for EP3 while Third-trimester placenta was used as a positive control for the immunohistochemical staining of COX-2 and G_{i1}. Positive cells showed a brownish color and the negative control, as well as unstained cells, appeared blue43. Negative controls were conducted with the same control tissues and were performed by replacement of a pre-immune serum (Negative Control for Super Sensitive Rabbit Antibodies, BioGenex, California, USA) at the same concentration as the anti-uPAR antibody.

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 used44, which was calculated via the multiplication of optical staining intensity (grades: 0=no, 1=weak, 2=moderate and 3=strong staining) and the percentage range of positive stained cells (0=no staining, 1= ≤10% of the cells; 2=11-50% of the cells; 3=51-80% of the cells and 4= ≥81% of the cells were stained with the anti-uPAR antibody, respectively).

3.8 Double Immunofluorescence staining

For the characterization of COX-2, EP3 and G_{i1}-expressing cells in the decidua, we applied the same paraffin-embedded slides with double immunofluorescence staining. The same experimental steps were carried out as for immunohistochemistry until the step of blocking. Slides were blocked with a blocking solution (Ultra V Block, Lab Vision, Fremont, CA, USA) to avoid non-specific staining and then incubated with various primary antibodies overnight at 4°C. Prolactin was used as a specific marker for stromal cells and HLA-G was used as a specific marker for trophoblast cells. Different primary antibodies were diluted with a diluting medium (Dako, Hamburg, Germany) according to the following ratios: 1:400 for rabbit anti-COX-2 polyclonal IgG (Sigma, SAB4502491) and 1:500 for mouse anti-Prolactin polyclonal IgG (Serotec, MCA712), 1:300 for rabbit anti-EP3 polyclonal IgG (Abcam, ab189131), 1: 300 for rabbit anti-G_{i1} polyclonal IgG (Novus Biologicals, NBP2-16558), 1:50 for mouse anti-HLA-G polyclonal IgG (Serotec, MCA2044). After washing, slides were incubated with Cy2-/Cy3-labeled antibodies (Jackson Dianova, Hamburg, Germany) as fluorescent secondary antibodies for 30 min at room temperature in darkness to avoid fluorescence quenching. Cy2-labeled secondary antibodies were at a dilution of 1:100 and Cy3-labelled antibodies were at a dilution of 1:500. Finally, the slides were embedded in mounting buffer

containing 4',6-diamino-2-phenylindole (DAPI, Vectastain, Vector Laboratories, Burlingame, CA, USA) for blue staining of the nucleus after washing and drying. Digital images were obtained with a digital camera system (Axiocam; Zeiss CF20DXC; KAPPA Messtechnik, Gleichen, Germany) and digitally saved.

3.9 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 non-parametric variables, such as the proliferation rate and cell migration area between the stimulation group and control group. Wilcoxon test was performed for the evaluation of PAI-1, uPA, β-hCG and progesterone expression levels and the band intensities of EP3, EP1, G_{i1}, p-ERK1/2 and uPAR between stimulation and control groups. Independent t-test was used to compare the clinical data. Mann-Whitney-U test was also applied for evaluating IRS scores of EP3, COX-2, G_{i1} and uPAR expression. 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 used a Cox-regression model for multivariate analyses. P-values <0.05 were regarded as statistically significant.

4 Results

4.1 EP3 signaling in cervical cancer

4.1.1 EP3 agonist stimulates the proliferation of CaSki and Hela cells

In our latest publication, we observed that high expression of EP3 (IRS≥2) is correlated with poor prognosis in overall survival rates of cervical cancer patients (P=0.016, Figure 1A)[91]. In the present study, we aimed to investigate the pathological mechanism of how EP3 signaling pathway has an effect on the proliferation and migration of cervical cancer cells. Therefore, for *in vitro* investigations, we used CaSki and Hela cells as our models of cervical cancer cells.

In vitro, BrdU assay was used to assess the relative proliferation rate of CaSki and Hela cells stimulated with different concentrations (1, 10 and 100 nM) of sulprostone. Sulprostone (an EP1/EP3 agonist) increased the proliferation rate of CaSki cells in a dose-dependent manner compared to the control group after 24 hours (each P<0.05, Figure 1B). 1, 10 and 100 nM sulprostone enhanced the proliferation rate of Hela cells compared to the control group after 72 hours (each P<0.05, Figure 1C).

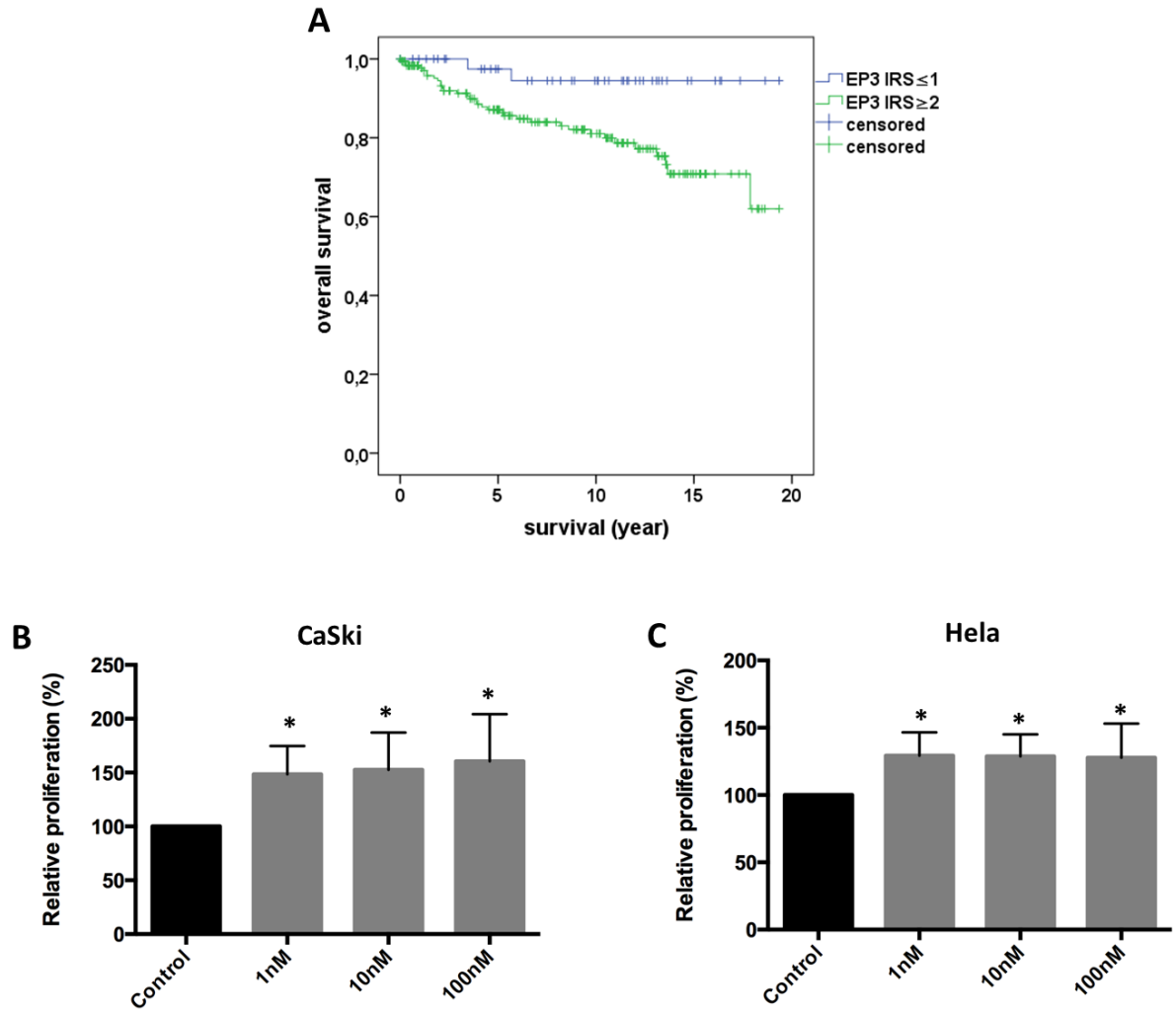


Figure 1. EP3 agonist stimulates proliferation of cervical cancer cells. (A) High EP3 expression (IRS≥2) is associated with a shorter overall survival (OS) in cervical cancer patients (*P*=0.016). (B) BrdU assay showed the relative proliferation rate of CaSki cells is increased by 1, 10 and 100 nM of sulprostone (an EP1/EP3 agonist) in a dose-dependent manner (**P*<0.05). (C) BrdU assay showed the relative proliferation rate of Hela cells is elevated by 1, 10 and 100 nM of sulprostone (**P*<0.05). Results are normalized to cell viability of the control group (0.5% (v/v) DMSO). Bar graphs represent mean ± SD (n=6).**P*<0.05 is considered as significantly different after comparison between the stimulation group and the control group.

4.1.2 EP3 agonist stimulates the migration of CaSki and Hela cells

To identify whether EP3 could facilitate the migration of cervical cancer cells, we performed wound healing assay. The results showed the relative migration rate was significantly enhanced in both CaSki (*P*=0.046, Figure 2A.2B) and Hela cells (*P*=0.015, Figure 2C.2D) after the treatment of 100 nM sulprostone for 24 hours compared to that in the control group.

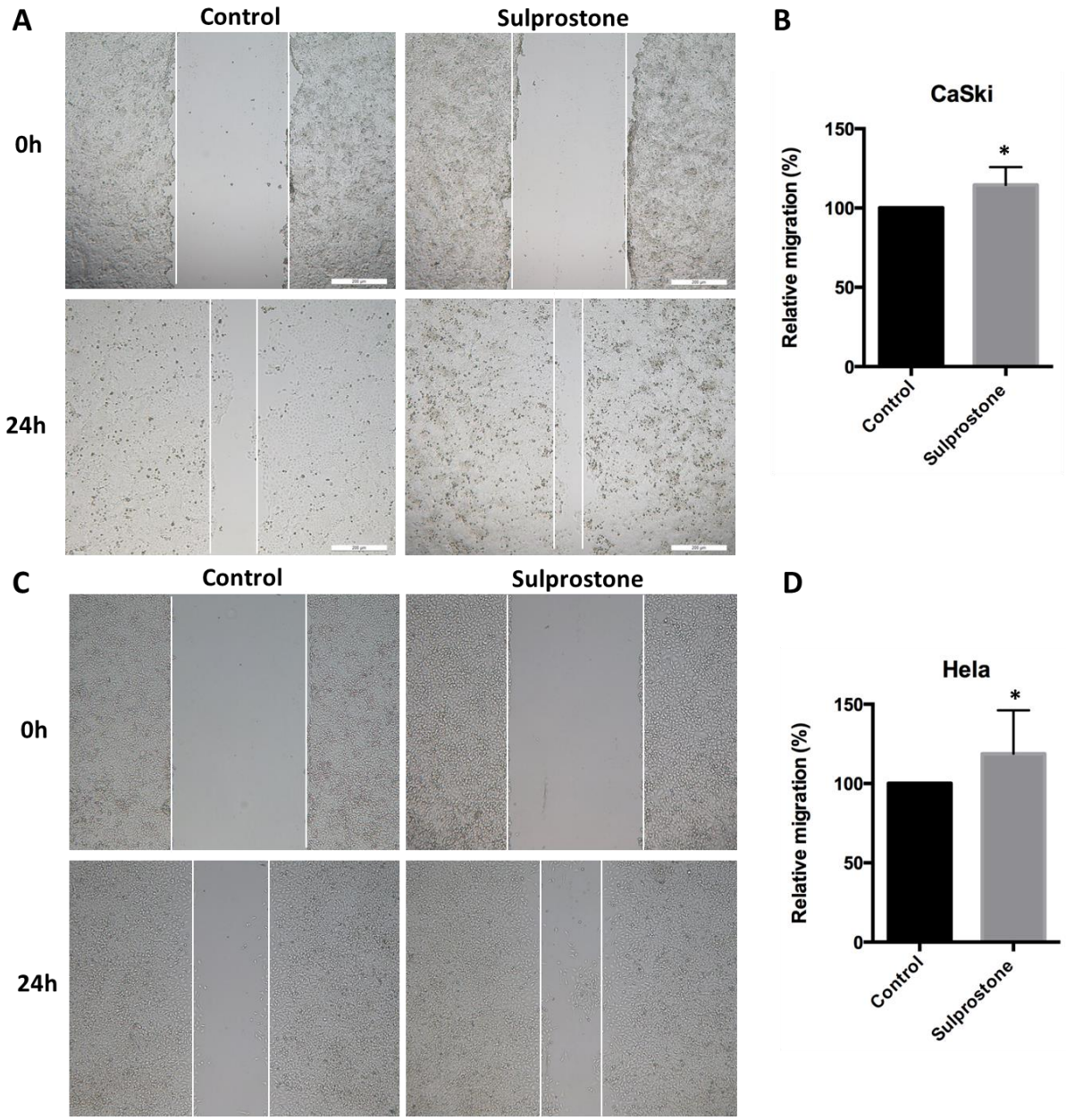


Figure 2. EP3 agonist stimulates migration of cervical cancer cells. (A) Representative photographs show the migration of CaSki cells into the wounded area in the control group and in the group treated with 100 nM sulprostone after 24 hours. (B) Wound healing assay observed the relative

migration rate of CaSki cells is enhanced in the group treated with 100 nM sulprostone compared to the control group (*P<0.05). (C) Representative images show the migration of Hela cells into the wounded area in the control group and in the group treated with 100 nM sulprostone after 24 hours. (D) Wound healing assay observed the relative migration rate of Hela cells is increased in the group treated with 100 nM sulprostone compared to the control group (*P<0.05). Bar graphs represent mean ± SD (n=6). *P<0.05 is considered as significantly different after comparison between the stimulation group and the control group.

4.1.3 Influence of the EP3 agonist and antagonist on the expression of EP3 signaling in CaSki and Hela cells

Western blotting was utilized to assess the expression of EP3 signaling (EP3, G_{i1}, p-ERK1/2 and p53) in CaSki and Hela cells stimulated with 100 nM of each PGE_2, sulprostone and L-798,106 for 48 hours (Figure 3A). The molecular weight of EP3 is around 35 kDa. EP3 expression was decreased by 17.0% in CaSki cells after treatment with 100 nM L-798,106 compared to the control group (P=0.012, Figure 3B). By contrast, the expression of EP3 was not altered by any stimulation substances in Hela cells. EP3 signals are primarily involved in inhibition of AC via activating G_{i1}, whose mass is 40 kDa. The expression of G_{i1} was downregulated by 14% via 100 nM PGE_2 in Hela cells compared to the control (P=0.024, Supplementary Figure 1A.1D). The rest of stimulators did not alter the expression of G_{i1} in either of two cell lines (Supplementary Figure 1A.1D.1E). The molecular weights of p-ERK1/2 are 44 and 42 kDa. The expression of p-ERK1/2 was decreased by 7.8% through 100 nM L-798,106 compared to the control group in CaSki cells (P=0.028, Figure 3C) and was increased by 47.8% through 100 nM sulprostone compared to the control group in Hela cells (P=0.028, Figure 3D). The band signals of p53 in CaSki and Hela cells could not be detected by western blotting.

Since sulprostone is an agonist for both EP3 and EP1, we also examined the expression of EP1 in CaSki and Hela cells by western blotting. The molecular weight of EP1 is around 70 kDa. There was no significant change in EP1 expression in both CaSki and Hela cells stimulated with 100 nM of each PGE_2, sulprostone or L-798,106 for 48 hours (each P>0.05, Supplementary Figure 1A.1B.1C).

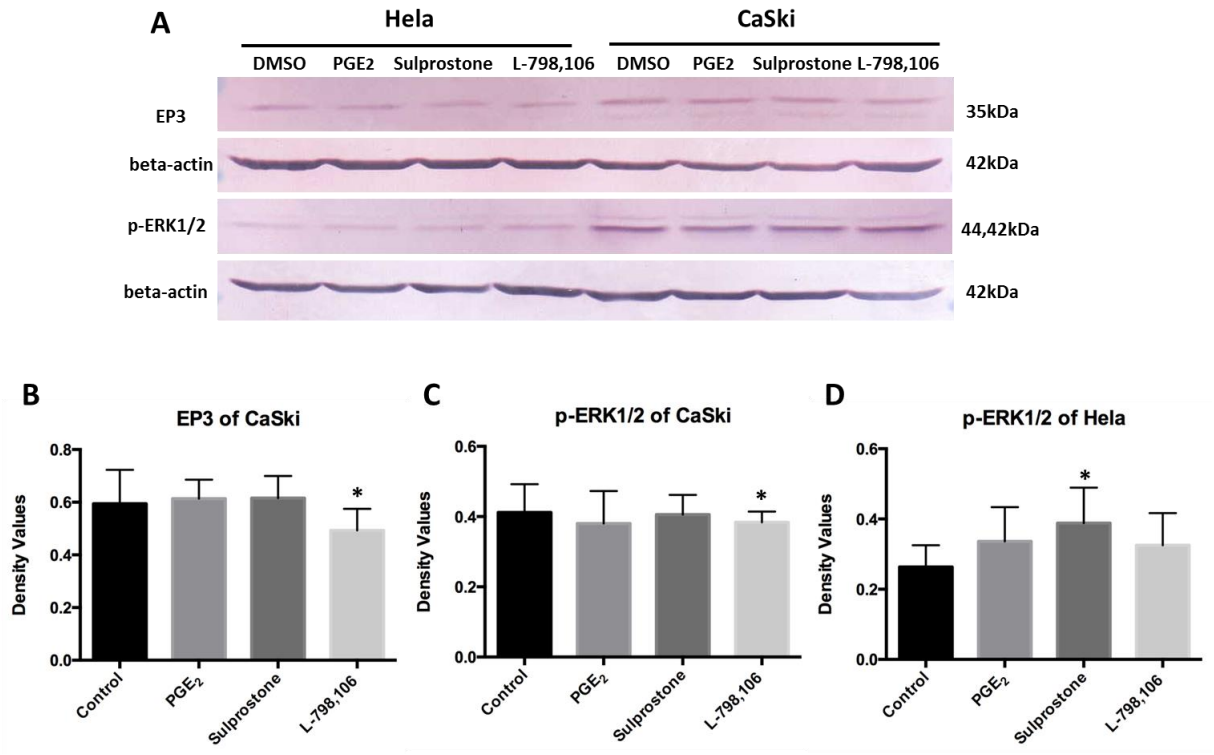
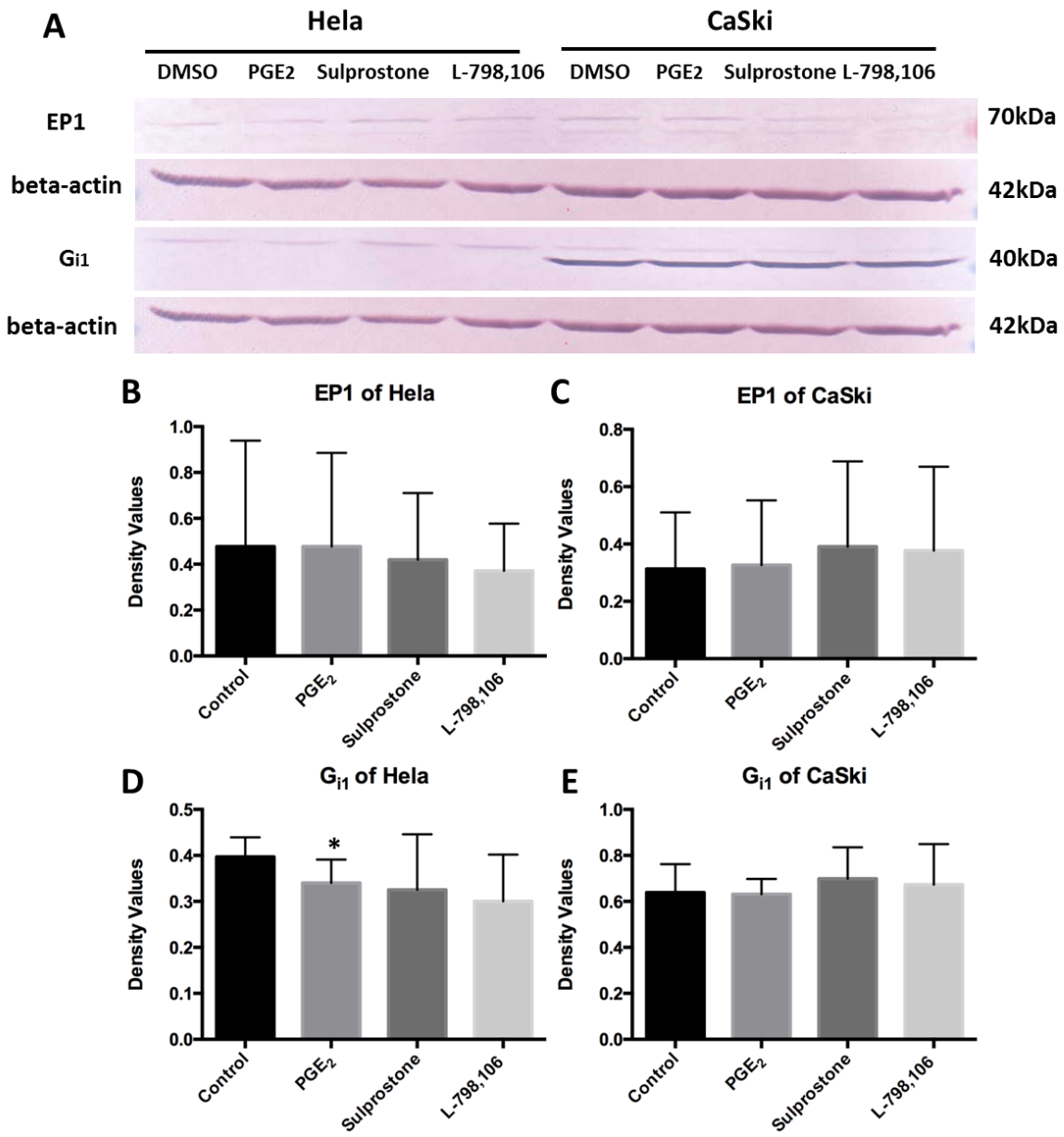
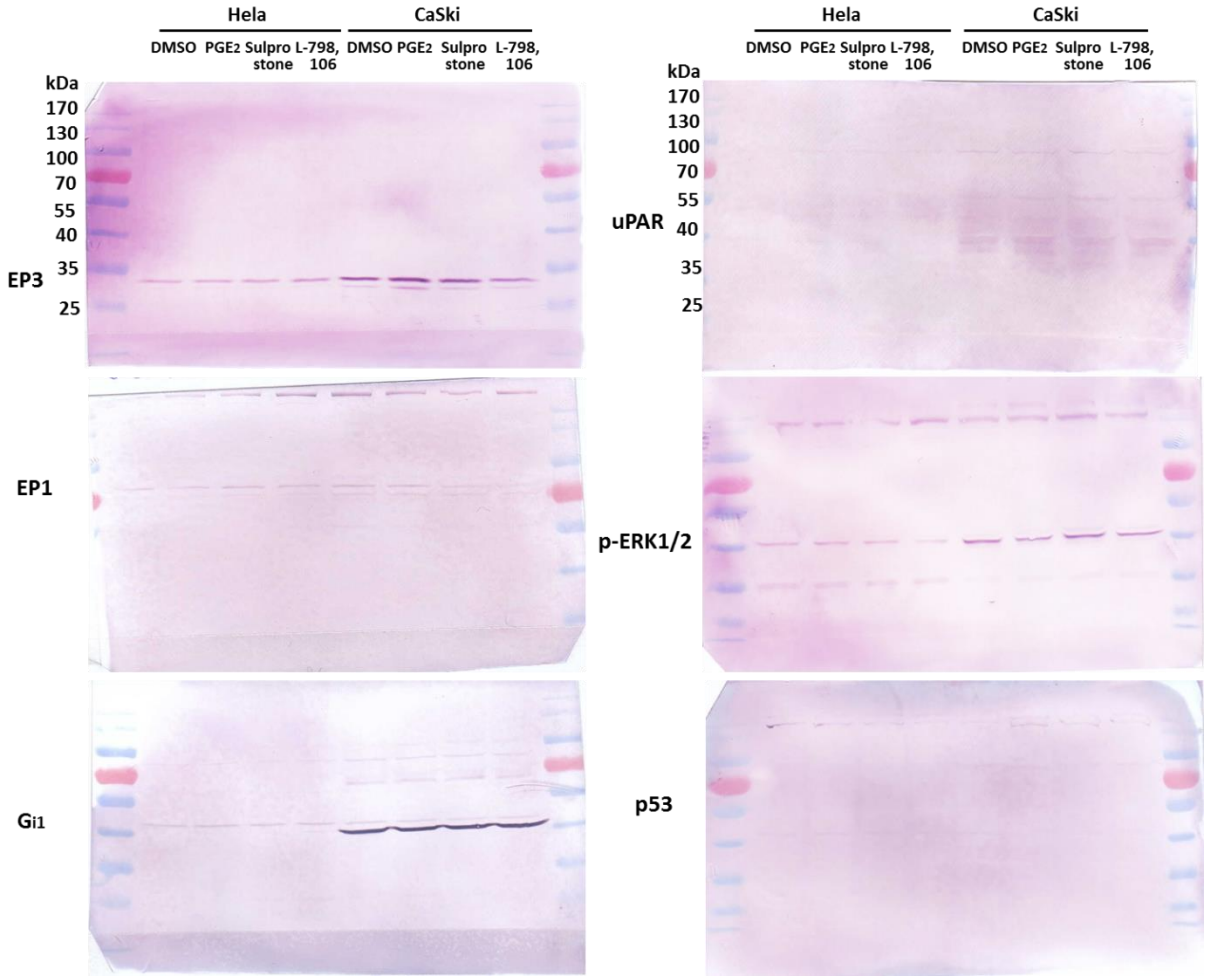


Figure 3. Expression of EP3 and p-ERK1/2 in Hela and CaSki cells influenced by PGE_2, sulprostone and L-798,106. (A) Western blotting analysis shows the expression of EP3 and phosphorylated extracellular signal-regulated kinases (p-ERK1/2) in CaSki and Hela cells following treatment with 100 nM of each PGE_2, sulprostone and L-798,106 for 48h. Beta-actin was used as a loading control and all the data was normalized to the β-actin band signals. (B) The histogram illustrates the expression of EP3 is inhibited by 100 nM L-798,106 in CaSki cells (*P<0.05). (C) The histogram illustrates the expression of p-ERK1/2 is inhibited by 100 nM L-798,106 in CaSki cells (*P<0.05). (D) The histogram illustrates the expression of p-ERK1/2 is stimulated by 100 nM sulprostone in Hela cells (*P<0.05). All data are shown as mean ± SD (n=3). Full-length blots are shown in Supplementary Figure 3. Statistically significant differences (P<0.05) between individual treatment groups and the control group are marked with an *.



Supplementary Figure 1. The expression of EP1 and G_{i1} in Hela and CaSki cells influenced by PGE_2, EP3 agonist and antagonist. (**A**) The western blotting analysis of EP1 and G_{i1} in CaSki and Hela cells following treatment with 100 nM of each PGE_2, sulprostone and L-798,106 for 48 h (n=3). (**B**) Histogram illustrates the expression of EP1 in CaSki cells is not altered by PGE_2, sulprostone or L-798,106 (Wilcoxon test). (**C**) Histogram shows the expression of EP1 in Hela cells is not changed by PGE_2, sulprostone or L-798,106 (Wilcoxon test). (**D**) Histogram illustrates the expression of G_{i1} is inhibited by 10 nM of PGE_2 in CaSki cells (*P<0.05, Wilcoxon test). (**E**) Histogram illustrates the expression of G_{i1} in Hela cells is not altered by PGE_2, sulprostone or L-798,106 (Wilcoxon test). Beta-actin was used as a loading control and all the data was normalized to the β-actin band signals. All data is shown as mean ± SD. Full-length blots are shown in **Supplementary Figure 3**. *P<0.05 is considered as significantly different after comparison between the stimulation group and the control

group.



Supplementary Figure 3. Full-length blots of EP3, EP1, Gi1, uPAR, p-ERK1/2 and p53.

4.1.4 Influence of the EP3 agonist and antagonist on the expression of PAI-1, uPA and uPAR in CaSki and Hela cells

The plasminogen activating system plays an important role in tumor progression and can be affected by EP3 signaling pathway. Therefore, we analyzed the expression of PAI-1 and uPA in the supernatants of CaSki and Hela cells stimulated with 10 nM of each PGE_2, sulprostone and L-798,106 using ELISA. PAI-1 levels in the supernatants of CaSki cells were elevated by 22.3% through sulprostone and by 25.4% through L-798,106 compared to the control group after 48 hours, respectively (both *P* values<0.001, Figure 4A). The same trend was observed

in Hela cells: PAI-1 levels in the supernatants were enhanced by 11.2% via sulprostone (P=0.033, Figure 4B) and by 5.7% via L-798,106 (P<0.001, Figure 4B) compared to the control group after 48 hours. The levels of uPA in the supernatants of CaSki cells were inhibited by 5.9% via 10 nM sulprostone (P<0.001, Figure 4C) and stimulated by 5.6% via 10 nM L-798,106 in CaSki cells (P=0.04, Figure 4C) compared to the control group.

The expression of uPAR was detected by western blotting and the molecular weight of uPAR is between 35-65kDa. In the western blotting, the expression of uPAR in CaSki cells was inhibited by 18.4% through L-798,106 compared to the control group (P=0.011, Figure 4D, Supplementary Figure 3). Although the expression of uPAR in CaSki cells was stimulated by 17.5% through sulprostone, there was no significant difference when compared to the control group (P=0.26, Figure 4D, Supplementary Figure 3). The expression of uPA and uPAR could not be detected in Hela cells.

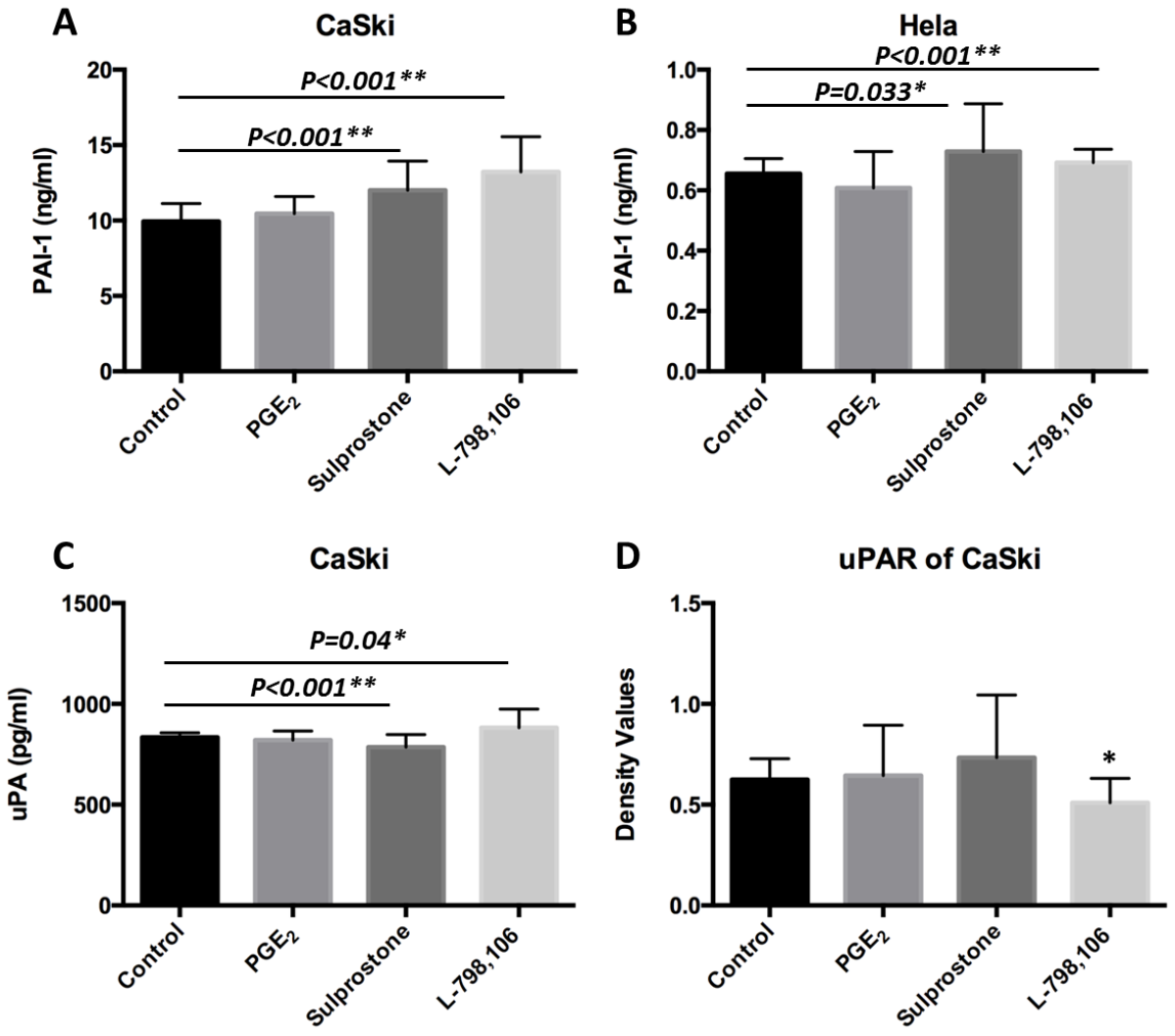


Figure 4. Expression of PAI-1, uPA and uPAR in CaSki and Hela cells influenced by PGE_2, sulprostone and L-798,106. (A) PAI-1 levels in the supernatants of CaSki cells are elevated after treatment with 10 nM of each sulprostone and L-798,106 for 48 h by ELISA (**P*<0.05, ***P*<0.001, n=12). (B) PAI-1 levels in the supernatants of Hela cells are increased after treatment with 10 nM of each sulprostone and L-798,106 for 48 h by ELISA (**P*<0.05, ***P*<0.001, n=12). (C) uPA expression levels in the supernatants of CaSki cells are inhibited by 10 nM sulprostone and are improved by 10 nM L-798,106 for 48 h via ELISA (**P*<0.05, ***P*<0.001, n=12). (D) Western blotting analysis of uPAR in CaSki cells following treatment with 100 nM of each PGE_2, sulprostone and L-798,106 for 48h (**P*<0.05, n=3). Full-length blots are shown in Supplementary Figure 3. All data are shown as mean ± SD. **P*<0.05 and ***P*<0.001 are considered as significantly different after comparison between the stimulation group and the control group.

4.1.5 Expression of uPAR in cervical cancer tissues

The *in vitro* studies imply the relevance of EP3 and uPAR, therefore, we further analyzed the expression of uPAR in 250 cervical cancer patients. 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 (Figure 5). The cut off of IRS 2 was obtained from receiver operator curve (ROC) analysis. To evaluate uPAR staining, we used metastatic colon carcinoma tissue with a very strong cytoplasmic expression of uPAR as negative and positive controls (Supplementary Figure 2A. B).

In the following analyses, we examined the correlation between uPAR expression and several clinical-pathological parameters such as T-status, N-status, M-status, grading, FIGO-classification and the expression of oncoproteins by noticing the distribution of these parameters in our study group (Table 2). For positive uPAR expression in cervical cancer tissues, 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) could be shown (Table 2). 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, Table 2) or between uPAR and mutant p53 in the nucleus (*P*=0.082, Table 2). A significant positive correlation between uPAR and histone H3 tri methyl K4 (H3K4me3) was also detected (*P*=0.041, Rho=0.134, Table 2). Although the EP3 antagonist could inhibit the expression of uPAR in CaSki cells, there was no significant correlation between uPAR expression and EP3 (*P*=0.822, Table 2) in cervical cancer specimens.

Also, we could show a significant negative correlation between the International Federation of Gynecology and Obstetrics (FIGO) status and uPAR expression (spearman's-rank correlation Rho=-0.165; *P*=0.012), suggesting that weak uPAR staining was correlated with a high FIGO stage (Table 2). Cervical cancer specimens demonstrated significantly decreased uPAR staining with a higher FIGO stage (*P*=0.046, Figure 6A). 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 (Figure 6A).

Table 1. Clinical-pathological variables of the patients included in the study

Clinical characteristics	**No./ Total No.**	**%**
Age (Median) [years]	40.5	
Follow-up (Median) [months]	126.5	
No. of Positive Nodes		
0	151/250	60.4
≥1	97/250	38.8
Not available	2/250	0.8
FIGO		
I and II	112/250	44.8
III and IV	44/250	17.6
Not available	94/250	37.6
Tumor Grade		
I	21/250	8.4
II	143/250	57.2
III	78/250	31.2
Not available	8/250	3.2
Tumor Subtype		
Squamous	202/250	80.8
Adenocarcinoma	48/250	19.2
Survival (over 235 months)		
Right censured	190/250	76.0
Died	49/250	19.6
Not available	11/250	4.4

Table 2. Staining results and correlation analysis

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

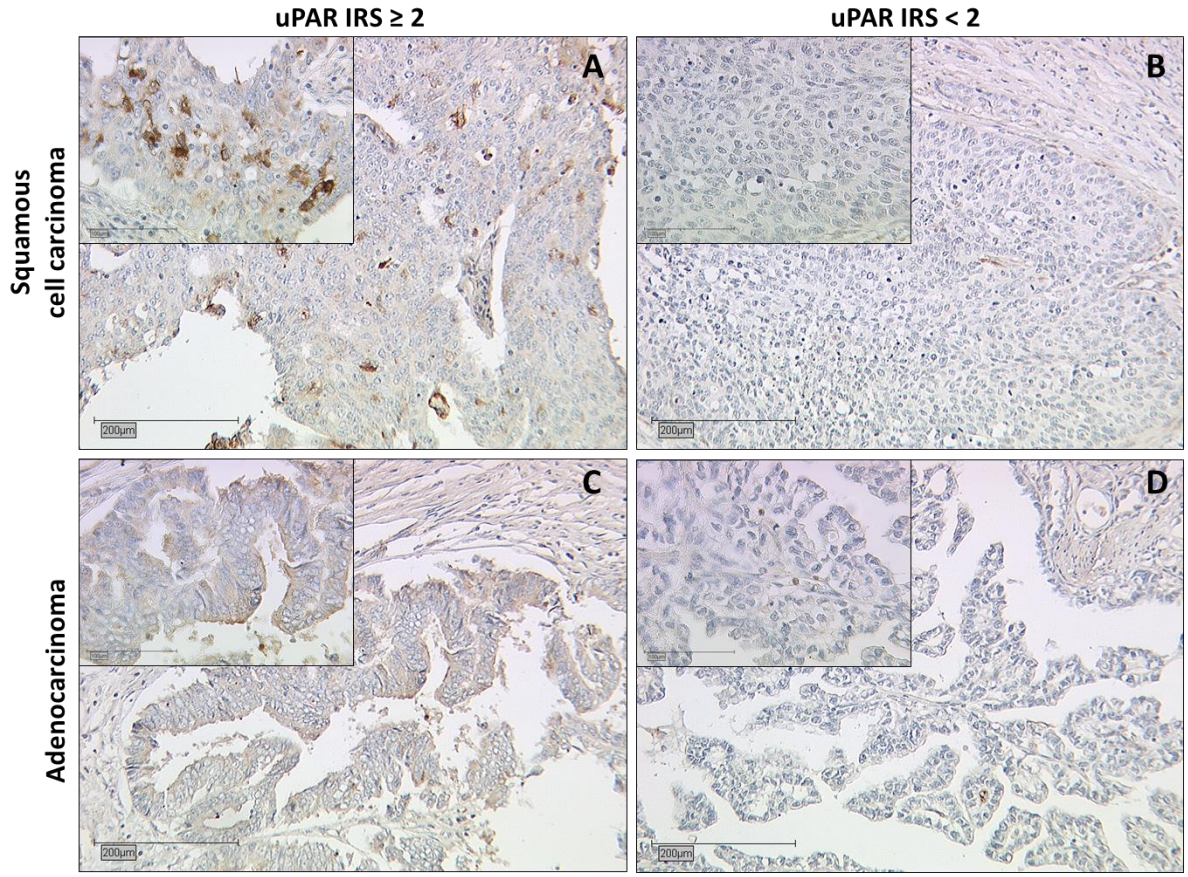
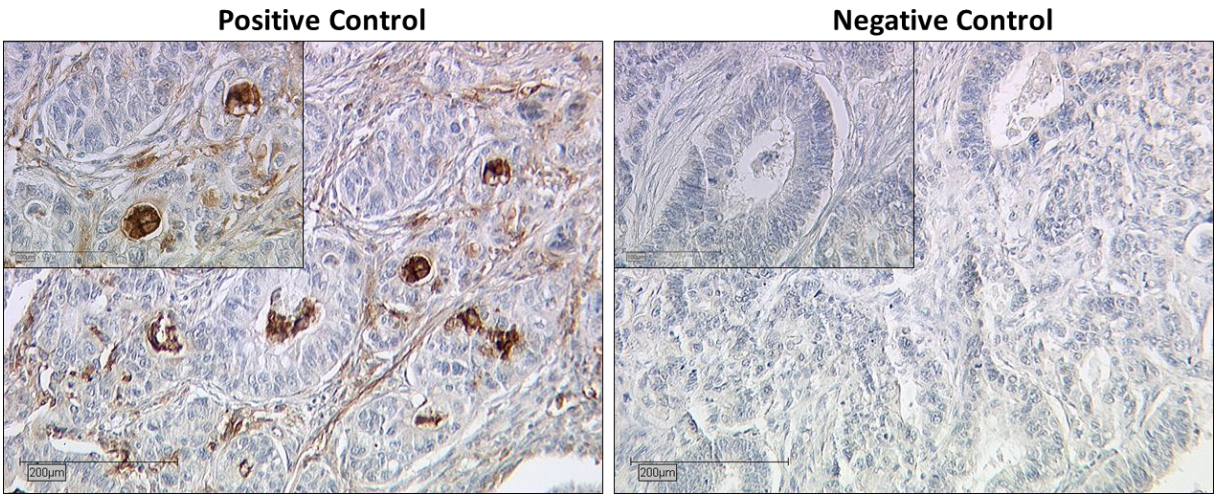


Figure 5. uPAR immunohistochemical staining in cervical carcinoma. (A) Representative photomicrographs of uPAR staining in cervical squamous cell carcinoma (FIGO IIIB) with uPAR IRS score of 3. (B) Representative photomicrographs of uPAR staining in cervical squamous cell carcinoma (FIGO IIIB) with IRS score of 0. (C) Representative photos of uPAR immunohistochemical staining in adenocarcinoma (FIGO IIIA) with IRS score of 4. (D) Representative photos of uPAR immunohistochemical staining in adenocarcinoma (FIGO IVB) with IRS score of 0. The scale bars in the outer pictures equal 200 µm (10x magnification) and the scale bars in the inserts equal 100 µm (50x magnification). FIGO = the International Federation of Gynecology and Obstetrics.



Supplementary Figure 2. Positive and negative controls of uPAR staining (**A.B**). We used the metastatic colon carcinoma for positive and negative controls.

4.1.6 Correlation of uPAR expression with OS of cervical cancer patients with FIGO Stages III and IV

uPAR positivity in general was not related to overall survival (OS) in the non-stratified patient sample. However, when patients had been stratified according to FIGO stage, the high expression of uPAR was correlated with poor prognosis in OS rates in cervical patients with FIGO stages III/IV as shown in the Kaplan-Meier curve (*P*=0.047, Figure 6B).

Among all the 44 advanced cervical cancer patients (FIGO III/IV), 33 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 shows no significant difference between these two histological subtypes (*P*=0.09). The subsequent survival analysis of the two main histological subtypes suggested that a significant negative correlation of uPAR with OS was observed in squamous cell carcinoma (*P*=0.006, Figure 6C), but not in cervix adenocarcinoma (*P*=0.536, Figure 6D). It implied that immunopositivity of uPAR is predictive for OS in cervical cancer patients of advanced stage (FIGO III/IV), especially among cases with squamous cell carcinoma.

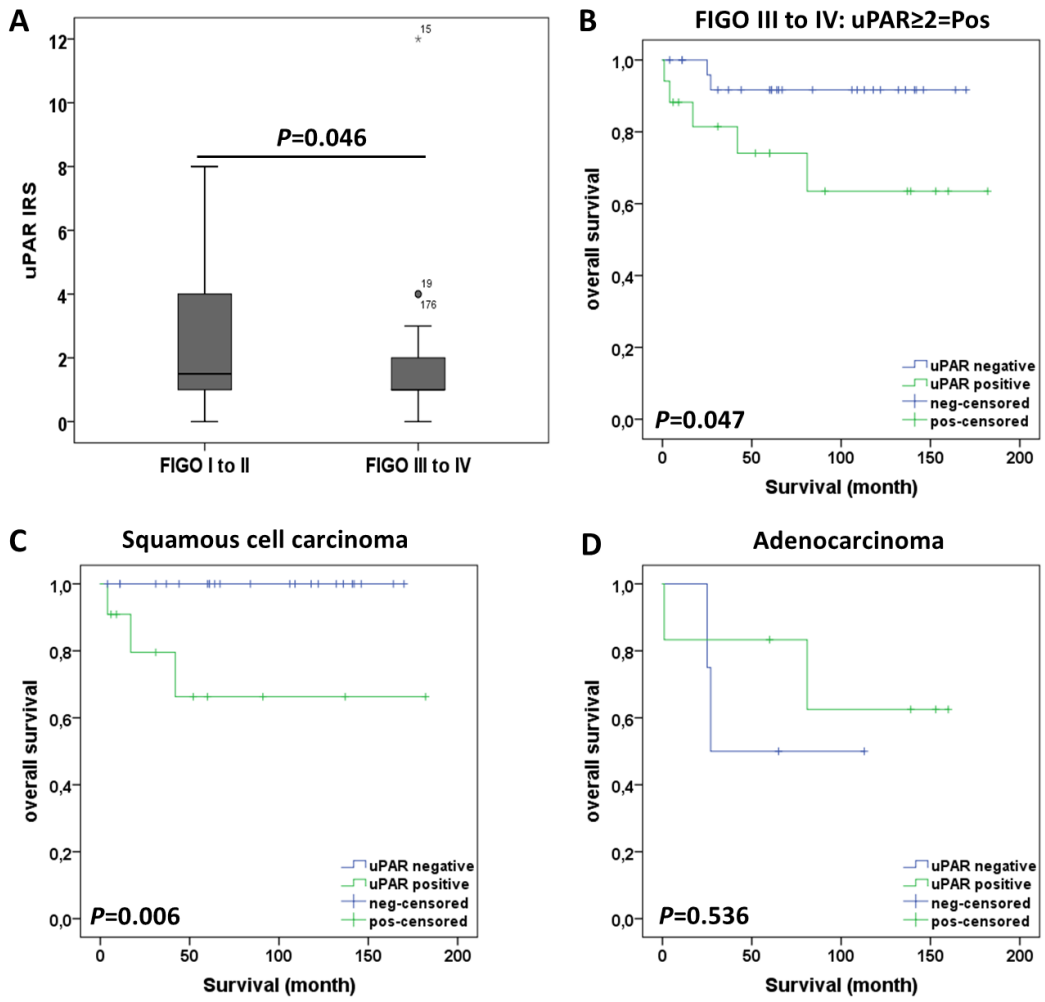


Figure 6. The role of uPAR in the overall survival of cervical cancer patients. (A) Boxplot shows uPAR staining in cervical cancer patients with FIGO I and II is higher than in patients with FIGO III and IV (*P*=0.046); (B) High uPAR expression (IRS≥2) is associated with a shorter OS in advanced cervical 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). FIGO= the International Federation of Gynecology and Obstetrics.

4.1.7 Multivariate Cox regression analysis of uPAR

Multivariate Cox regression analysis was performed to test which histopathological variables including uPAR expression, histology, tumor size (pT), nodal status (pN), tumor differentiation grade, FIGO-classification, and age were independent prognosticators for OS

in advanced cervical cancer patients (FIGO III/IV). Although no significant effect was detected for the histopathological variables, uPAR was nearly a promising prognosticator for advanced cervical cancer patient OS (*P*=0.067, Table 3).

Table 3. Cox regression of clinical-pathological variables regarding overall survival

Variable	Significance	Hazard Ratio of Exp (B)	Lower 95% CI of Exp (B)	Upper 95% CI of 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	4553661.9	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.

4.2 EP3 signaling in unexplained recurrent miscarriages (uRM)

4.2.1 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 and of the control group 35.78±5.88 years (*P*=0.41). 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). The gravidity times were decreased in patients with uRM (3.11±1.08) compared to healthy controls (3.42±1.90, *P*=0.002), while the parity times of two groups showed no significant differences (0.94±0.94 vs 1.63±1.12, *P*=0.35). Demographic and clinical data are illustrated in table 1.

Table 1: Demographic and clinical characteristics of the study population. Values are represented as mean± SD; the range is shown in parentheses.

Characteristic	**Normal pregnancy** n=19	**uRM** n=19	***P* Value** (**t-Test**)
maternal age (years)	35.78±5.88 (25-46)	37.76±4.88 (30-44)	0.41
gestational age (weeks)	9.71±1.88 (6-13)	9.09±2.17 (4-12)	0.66
gravidity	3.42±1.90 (1-7)	3.11±1.08 (2-5)	0.002
parity	1.63±1.12 (0-4)	0.94±0.94 (0-3)	0.35

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

Expression of COX-2, EP3 and G_{i1} was identified in the cytoplasm of cells in the syncytium and the decidua of first-trimester pregnancies in both the uRM group and the control group (Figure 1A-R). A significant increase in the expression of COX-2 could be observed in the syncytium of the uRM group in comparison to the control group (IRS 8.73 vs 5.59; *P*=0.007, Figure 1A.B.E). COX-2 expression in the decidua of the uRM group was also significantly increased in comparison to the control group (IRS 4.29 vs 2.00; *P*=0.004, Figure 1C.D.F).

Expression of both EP3 and G_{i1} in the decidua was significantly upregulated in patients with uRM compared to normal controls (Figure 1L.R), but no significant changes of EP3 and G_{i1} could be observed in the syncytium (Figure 1K.Q). In the uRM group, EP3-staining in the

decidua was more intense with a mean IRS of 4.15, which is higher than the mean IRS of the control group (IRS=2.22, *P*=0.001, Figure 1I.J.L). EP3-staining in the syncytium of the uRM group was similar to the control group (IRS 5.75 vs 6.32; *P*=0.350, Figure 1G.H.K). G_{i1}-staining was stronger in the decidua of the uRM group compared to the control group (IRS 4.48 vs 2.89; *P*=0.008, Figure 1O.P.R). There was no significant staining difference for G_{i1}-staining in the syncytium between the uRM group and the control group (IRS 5.27 vs 4.47; *P*=0.292, Figure 1M.N.Q). Both positive and negative controls are shown in the supplemental Figure 1.

Expressions of COX-2, EP3 and G_{i1} were all increased in the decidua of patients with uRM compared to healthy controls, implying that a correlation could exist between them. We further analyzed the correlation of each two of these three components in the decidua. Unfortunately, there was no statistically significant correlation between COX-2 and EP3 expression (r=-0.059; *P*=0.788), neither for EP3 and G_{i1} (r= 0,251; *P*= 0.261) nor for COX-2 and G_{i1} (r=0.158, *P*=0.450) in the decidua.

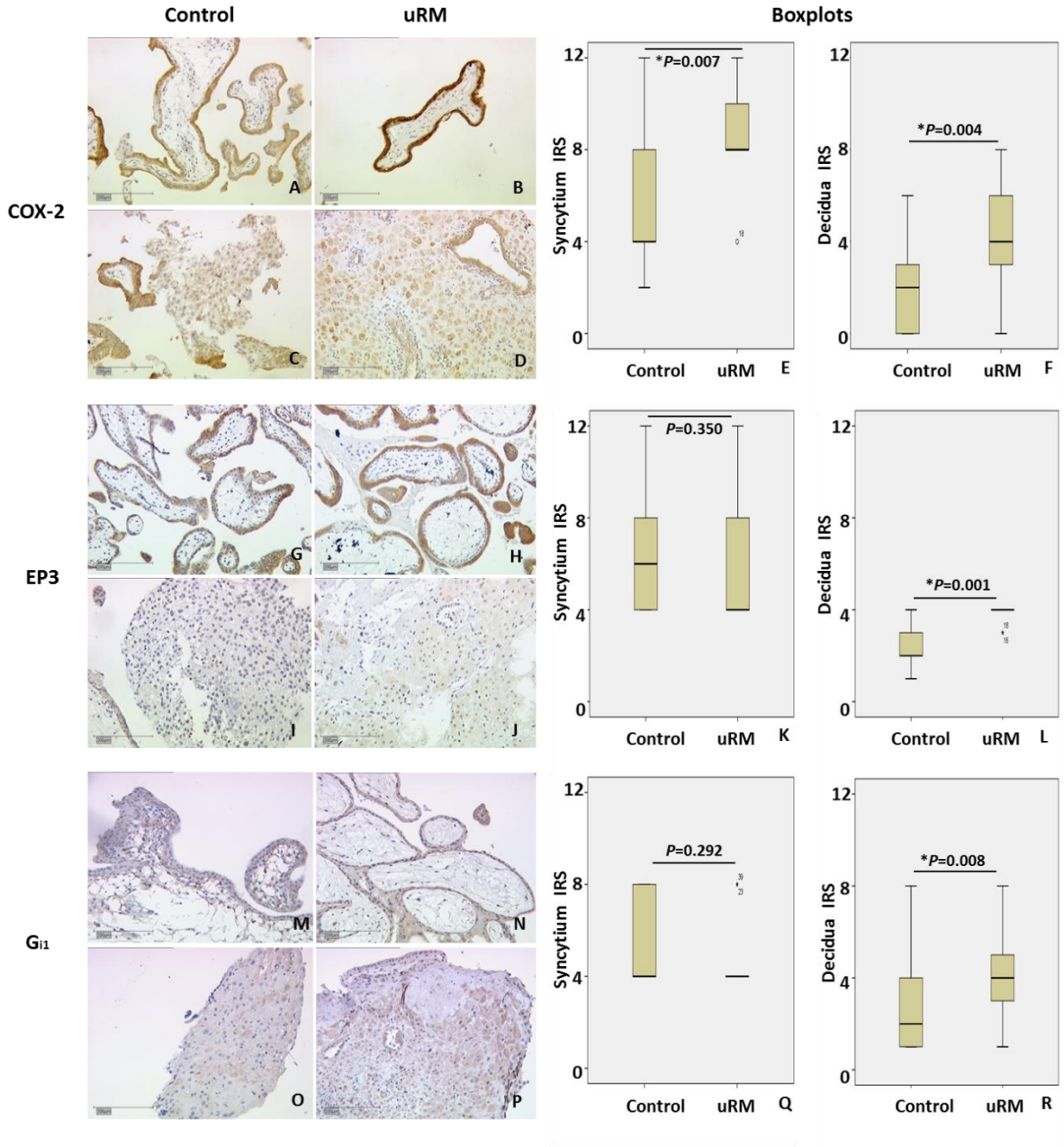
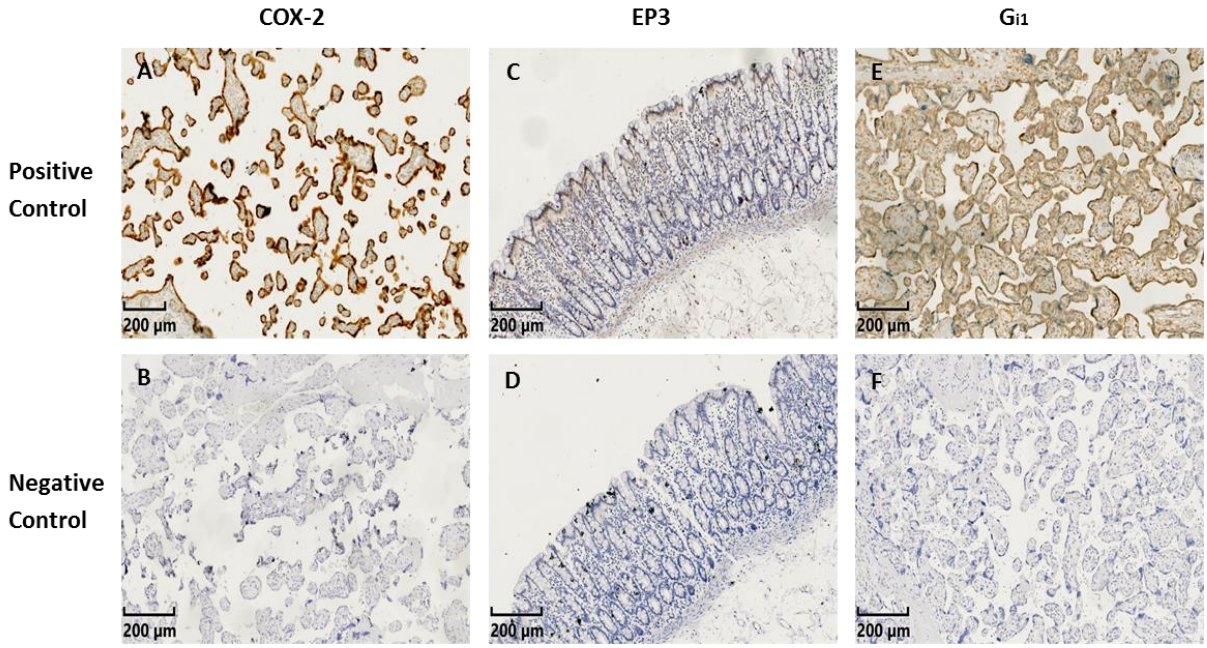


Figure 1. Immunohistological analyses of COX-2, EP3 and G_{i1} expression in placentas of uRM patients (**B.D.H.J.N.P**) and healthy controls (**A.C.G.I.M.O**) from the first trimester were measured via IRS score. In the syncytium, the stainings of COX-2 (**A.B**), EP3 (**G.H**) and G_{i1} (**M.N**) are compared between the uRM patients and controls, which are represented as box-plots (**E.K.Q**). In the decidua, the expressions of COX-2 (**C.D**), EP3 (**I.J**) and G_{i1} (**O.P**) are increased in uRM patients compared to controls, which are represented as box-plots (**F.L.R**). The range between the 25th and 75th percentiles is represented by the boxes with a horizontal line at the median. The bars show the 5th and 95th percentiles. Dots indicate values more than 1.5 box lengths from the 75th percentile. Magnification x10 lens, scale bar= 200 µm. uRM=unexplained recurrent pregnancy losses.



Supplementary Figure 1. Positive and negative controls of COX-2 (**A.B**), EP3 (**C.D**), and G_{i1} (**E.F**). For positive control of COX-2 and G_{i1}, third trimester placentas are used and colon is used as positive control for EP3.

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

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

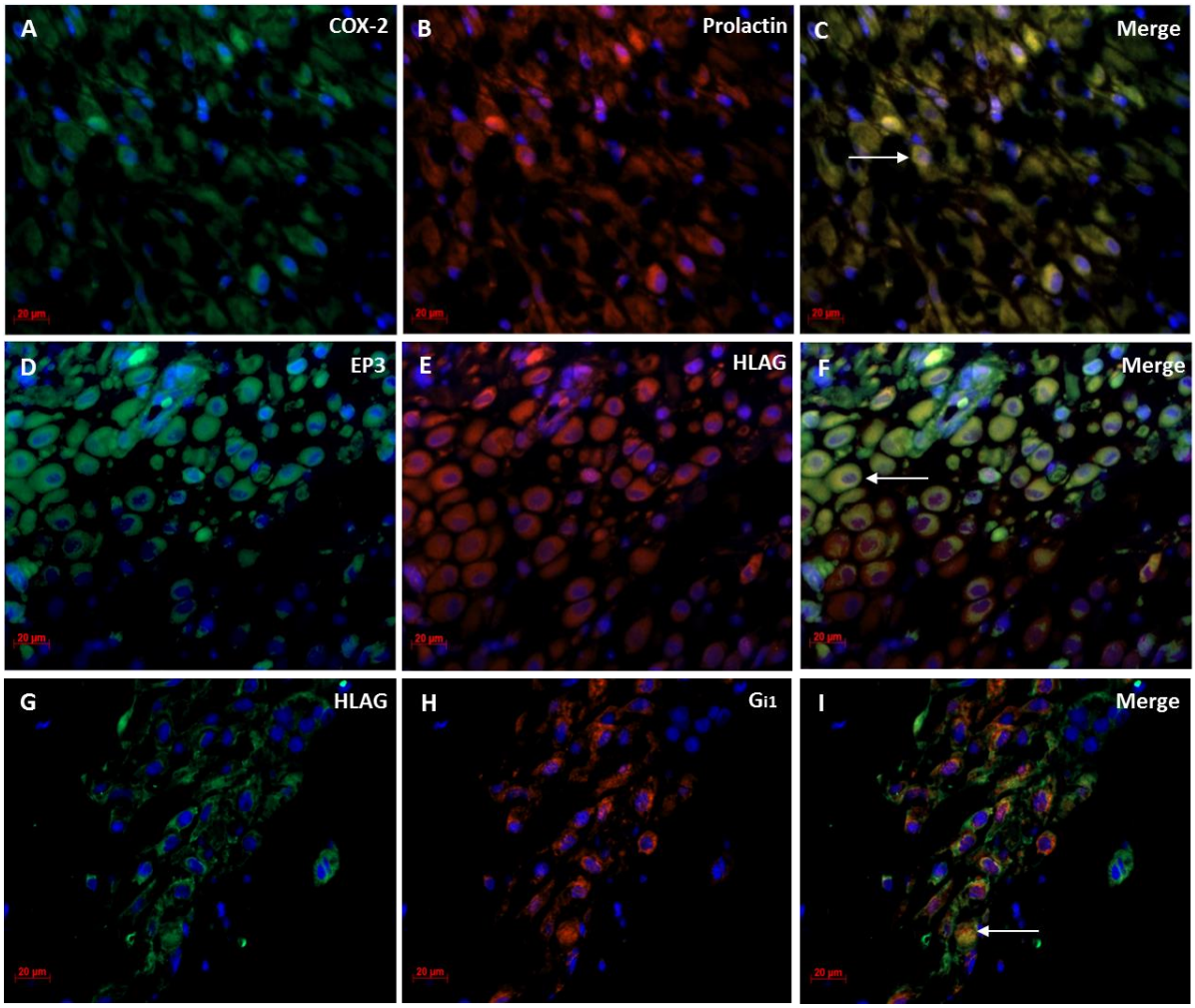


Figure 2. Localization of COX-2, EP3 and G_{i1} is shown in the decidua of patients with uRM. COX-2 is co-expressed with prolactin, which is a specific marker for stromal cells (**A.B.C**). EP3 is co-expressed with HLA-G, which is a specific marker for extravillous trophoblasts (**D.E.F**). G_{i1} is co-expressed with HLA-G in extravillous trophoblasts (**G.H.I**). Co-expression of COX-2, EP3 and G_{i1} is shown with →. Magnification x63, scale bar= 20 µm.

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

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

Firstly, the MTT assay was used to assess the viability of JEG-3 cells after 48 hours of treatment with 0.1, 1, 10, 100, 1000 nM and 10 µM PGE_2, L-798,106 or the vehicle control (DMSO, 0.1%). 10 nM PGE_2 and 10 nM L-798,106 significantly increased viability of JEG-3 cells compared to control group (P<0.05, data not shown).

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

Progesterone expression of JEG-3 cells was downregulated by PGE_2, sulprostone and L-798,106, independent of different concentrations (0.1 nM, 1 nM and 10 nM) in comparison to the vehicle after 48 hours (P= 0.028, each) (Figure 3B).

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

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

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

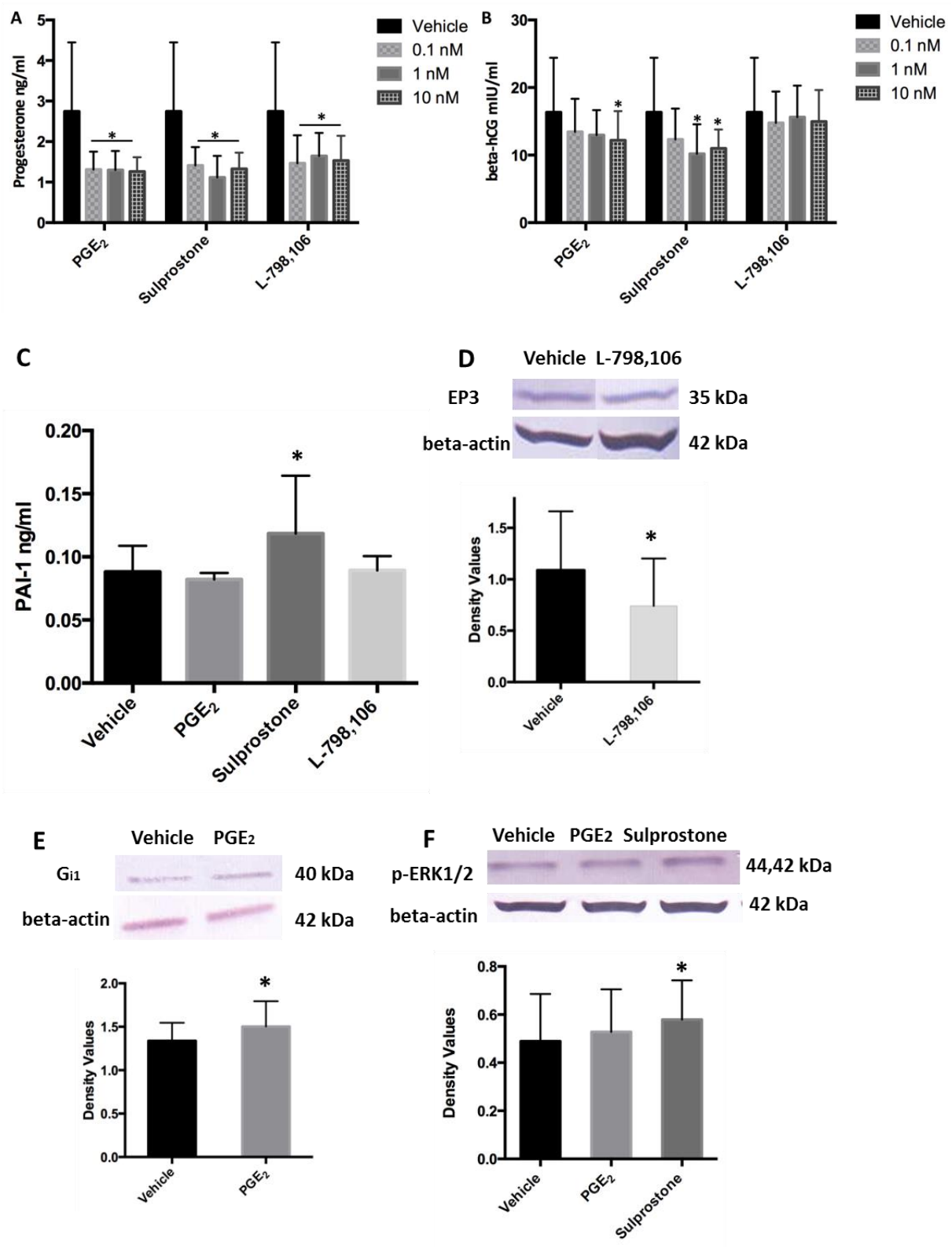
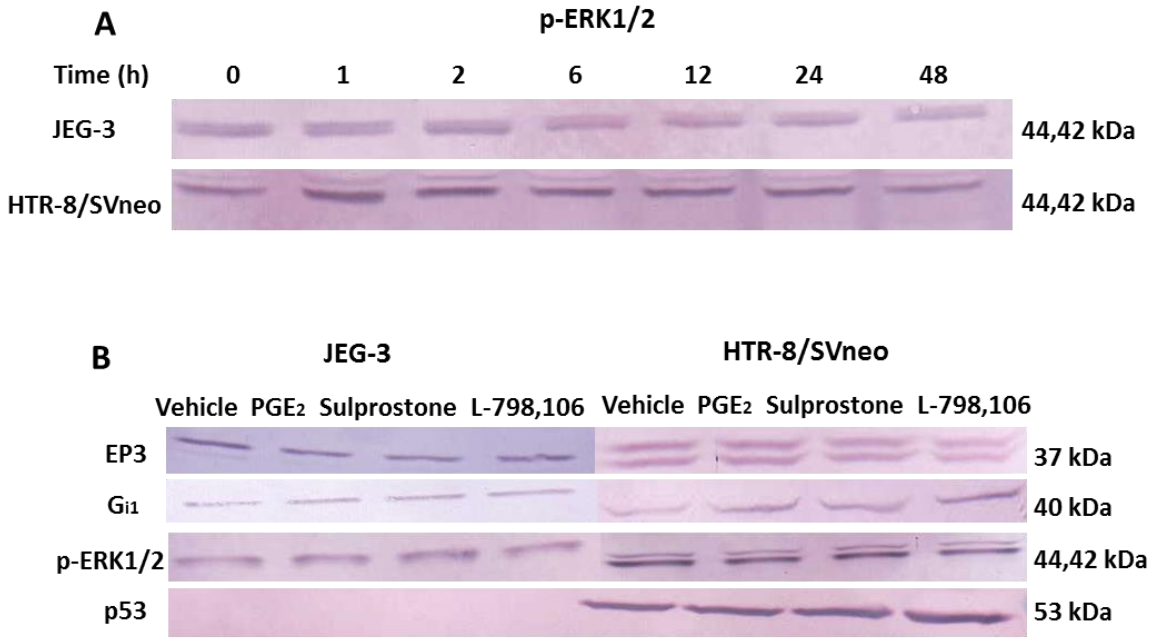


Figure 3. Expression of β-hCG, progesterone, plasminogen activator inhibitor (PAI-1), EP3, G_{i1}, p-ERK1/2 and p53 in JEG-3 cells after incubation with PGE_2, the EP1/EP3 agonist (sulprostone) and EP3 antagonist (L-798,106). β-hCG, progesterone and PAI-1 expression levels were measured via ELISA (each group n=6). EP3, G_{i1}, p-ERK1/2 and p53 expression extracted from JEG-3 cells were detected by

western blotting, following treatment with PGE_2 (10nM) and L-798,106 (10nM) for 48h (n=3). All data is shown as mean +/-SD and statistically significant differences ($P < 0.05$) between individual treatment groups and the vehicle are marked with an asterisk.



Supplementary Figure 2. Representative western blots. (A) Representative western blots of p-ERK1/2 expression when JEG-3 and HTR-8/SVneo cells were incubated with 0.5% DMSO for different time periods. (B) Representative western blots for the expression of EP3, G_{i1}, p-ERK1/2 and p53 in JEG-3 and HTR-8/SVneo cells stimulated by 10 nM of each PGE_2, sulprostone and L-798,106.

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

HTR-8/SVneo cells were incubated with PGE_2, sulprostone and L-798,106 at a concentration of 10 nM and 100 nM respectively. β-hCG levels of HTR-8/SVneo cells were down-regulated through PGE_2, sulprostone and L-798,106 for all concentrations after 48 hours (P<0.05, each) (Figure 4A). Progesterone levels could not be detected in the supernatants of HTR-8/SVneo cells. PAI-1 levels were reduced 3.7% through 10 nM L-798,106 than the vehicle, while no significant changes of PAI-1 expression were detected through 10 nM sulprostone (Figure 4B).

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

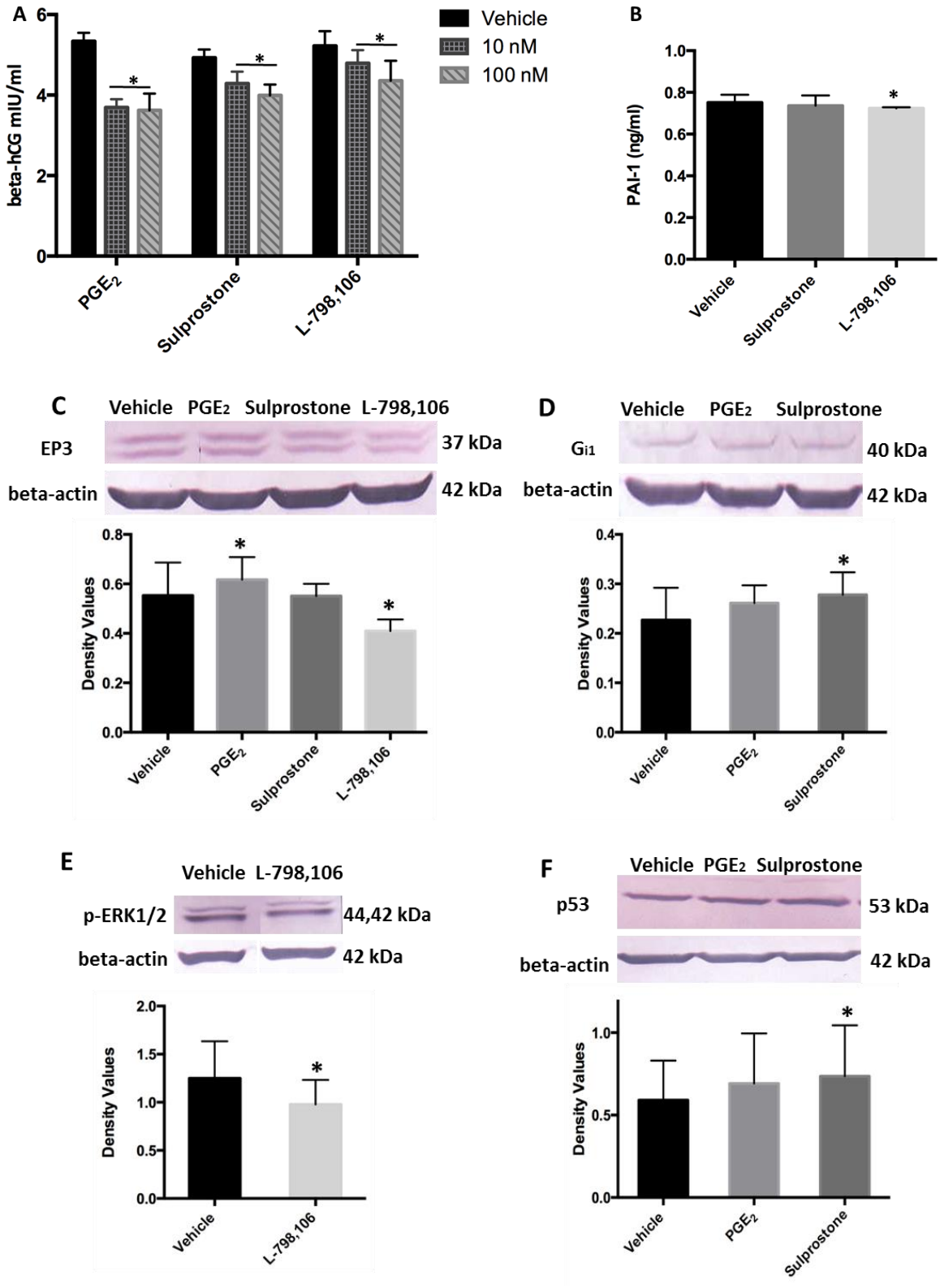


Figure 4. Expression of β-hCG, EP3, G_{i1}, p-ERK1/2 and p53 in HTR-8/SVneo cells after incubation with PGE_2, the EP1/EP3 agonist (sulprostone) and EP3 antagonist (L-798,106). The levels of β-hCG and PAI-1 were measured via ELISA (each group n=6). EP3, G_{i1}, p-ERK1/2 and p53 expression extracted from

HTR-8/SVneo cells were detected by western blotting, following treatment with PGE_2 (10nM), sulprostone (10nM) and L-798,106 (10nM) for 48h (n=3). All data are shown as mean ± SD and statistically significant differences ($P < 0.05$) between individual treatment groups and the vehicle are marked with an asterisk.

5 Discussion

5.1 EP3 signaling pathway in cervical cancer

5.1.1 The EP3 signaling pathway is induced in cervical cancer

High expressions of COX-2 and PGE_2 have been identified in cervical carcinoma. The research group of Sales et al. suggested that PGE_2 regulates the function of cervical cancer cells via cyclic adenosine monophosphate (cAMP)-linked EP2/EP4 signaling pathway[82,83]. In our latest publication, we found that high expression of EP3 (IRS≥2) in both squamous cell carcinoma and adenocarcinoma is associated with poor prognosis in overall survival rates of 250 cervical cancer patients[91]. It suggests that EP3 signaling pathway also participates in the carcinogenesis of cervical cancer. Therefore, in the present study, we aimed to explore the possible mechanism of how EP3 signaling pathway plays its role in cervical cancer cells.

Literatures have shown that EP3 regulates the carcinogenesis in numerous cancers through different signaling pathways. EP3 promotes the cell growth of CCLP1 human liver cancer cells by coupling to Gs protein and activating cAMP-protein kinase A (PKA)[136]. EP3 increases the migration of HCA-7 human colon cancer cells through the activation of phosphatidylinositol 3-kinase (PI3K) and the phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) signaling pathway[137]. Sulprostone improves the invasiveness of human doxorubicin-resistant MCF-7 breast cancer cells via enhancing the mRNA of matrix metalloproteinases (MMP)-2, MMP-9 and uPA[138]. On the contrary, sulprostone was suggested to decrease the proliferation of human prostate cancer cells[139] and the selective EP3 agonist ONO-AE-248 inhibits the viability of HCA-7 human colon cells[140]. *In vitro* studies, we proved that the EP1/EP3 agonist (sulprostone) stimulated the proliferation and migration of both CaSki and Hela cells, which is in line with the previous finding that EP3 is a negative prognosticator in cervical cancer patients[91]. We observed that the expression of EP1 was not altered by PGE_2, sulprostone or L-798,106, while the expression of EP3 was inhibited by the specific EP3 antagonist L-798,106. EP3 is mainly coupled to G_i to inhibit AC, leading to the decreased expression of cAMP. Regulation of G_i by the natural EP3 ligand PGE_2 seems to be in favour for an active EP3 signaling in cervical cancer cells, although there is no influence of sulprostone or L-798,106 on G_i expression in these cells. These findings suggest that EP3-G_i-

cAMP might not be the primary signaling pathway functioning in cervical cancer cells, although EP3 was proved to be a key hint in the carcinogenesis of cervical cancer.

In HCA-7 human colon cells, the phosphorylation of ERK1/2 was only mediated by EP3 receptor enhancing migration[137]. EP3 induces the mRNA expression of vascular endothelial growth factor (VEGF) through phosphorylation of ERK1/2 and activation of PI3K signaling, which contributes to tumor-related angiogenesis[141]. ERK1/2 are members of the mitogen-activated protein kinase superfamily that can mediate cell proliferation and apoptosis[142]. In our investigations, we showed that the expression of phosphorylated-ERK1/2 (p-ERK1/2) was upregulated by sulprostone in Hela cells and downregulated by L-798,106 in CaSki cells. Additionally, ERK1/2 is an upstream regulator of PAI-1 gene transcription[132]. The activation of EP3 signaling induces increased expression of p-ERK1/2 in cervical cancer cells, which could result in increased PAI-1 gene expression. It implies that EP3 might indirectly regulate the function of cervical cancer cells via induction of PAI-1 instead of cAMP signaling.

5.1.2 Plasminogen activating system is stimulated by EP3 in cervical cancer

Our data demonstrated that sulprostone increased the production of PAI-1 in the supernatants of both CaSki and Hela cells, which is in good agreement with the study that PGE_2 utilizes EP1/EP3 receptor to increase PAI-1 levels in cardiac fibroblasts[122]. It has been well-known that high expression of PAI-1 is correlated with worse overall and disease-free survival and is an independent prognosticator in cervical cancers[126,143]. High expression of PAI-1 can protect cervical tumor cells from degradation[126]. Furthermore, PAI-1 enhances tumor cell proliferation by encouraging S-phase entry[144] and increases migration by binding uPA/uPAR complex[145]. 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[145,146]. Interestingly, we also observed that L-798,106 could also increase the secretion of PAI-1 in the supernatants of both cell lines, which could imply that EP3 activates at least one signaling pathway except for plasminogen activating pathway and needs further investigations.

Apart from PAI-1, uPA and uPAR are also useful for predicting the prognosis of cervical cancer. Jing et al. suggested that the soluble uPAR in serum is a prognosis marker in cervical

cancer as well as a tumor biomarker for clinical diagnosis and treatment[147]. Sasaki et al. found that overexpression of uPAR mRNA is related to a shorter disease-free survival rate of cervical cancer patients, however, the immunohistochemical staining of uPAR was not very intense[148]. Using immunohistochemistry, we could demonstrate for the first time that uPAR expression (IRS≥2) was associated with a poor OS of cervical cancer patients with advanced stage (FIGO III/IV). uPA is useful for predicting the metastatic potential of lymph nodes in cervical cancer patients[149-151], implying that both uPA and uPAR are valuable biomarkers for advanced cervical carcinomas. The negative correlation of uPAR with OS of patients is significant in squamous cell carcinoma but not in cervix adenocarcinoma, which could be due to the smaller number of patients with adenocarcinoma (n=10). This result also agrees 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.

5.1.3 Correlation of uPAR with new biomarkers of cervical carcinomas

Several biomarkers has been analyzed for OS in cervical cancer patients in the last year, such as p16[152], MDM2[152], galectin-3[152], H3K9ac[153] and H3K4me3[153]. Therefore, we also performed the correlation analysis of uPAR with these oncoproteins in the same cervical cancer patients. The positive correlation of histone H3 tri methyl K4 (H3K4me3) and uPAR expression in our study is 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[153]. Additionally, 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[152]. In hepatocellular carcinoma cells, the silencing of galectin-3 attenuated the expression of uPAR and inhibited the proliferation, migration and invasion[154]. This study also agrees with our detections that low expression of uPAR was correlated with longer survival time in cervical cancer patients with advanced stage. E6 proteins induce rapid degradation of tumor suppressor protein p53 to prevent the host cell from inducing apoptosis[155]. Besides, overexpression of wild-type p53 causes the activation of ERK1/2[142] and both ERK1/2 and p53 are upstream regulators for PAI-1 transcription[132]. We found that wild-type p53 in the cytoplasm was downregulated in the cervical cancer cells that showed high expression of

uPAR, suggesting p53 might move into the nucleus from the cytoplasm to induce PAI-1 transcription.

One limitation of this investigation is that it is a retrospective study, which analyzes the data of the patients who had undergone surgery in one single hospital from 1993 to 2002. A multi-center prospective study should be carried out for further research.

In the part of cervical cancer, the findings of this study indicate that in human cervical cancer cells, the EP3 receptor is able to regulate cell proliferation and migration through phosphorylation of ERK1/2 and transcription of PAI-1. Further, it was observed that overexpression of uPAR was associated with poor prognosis in cervical cancer patients with advanced stage (FIGO III/IV) and therefore suggested that uPAR may represent a novel therapeutic target for the treatment of this malignancy. Several uPAR-targeted therapies already exist, such as specific uPA inhibitors, anti-uPAR antibodies blocking uPAR downstream signaling, and blockers breaking the interactions between uPAR and its binding partners[156]. In this investigation, L-798,106 could decrease the expression of both EP3 and uPAR in CaSki cell line, therefore a functional antagonist of EP3 could also be a promising agent for cervical cancer treatment.

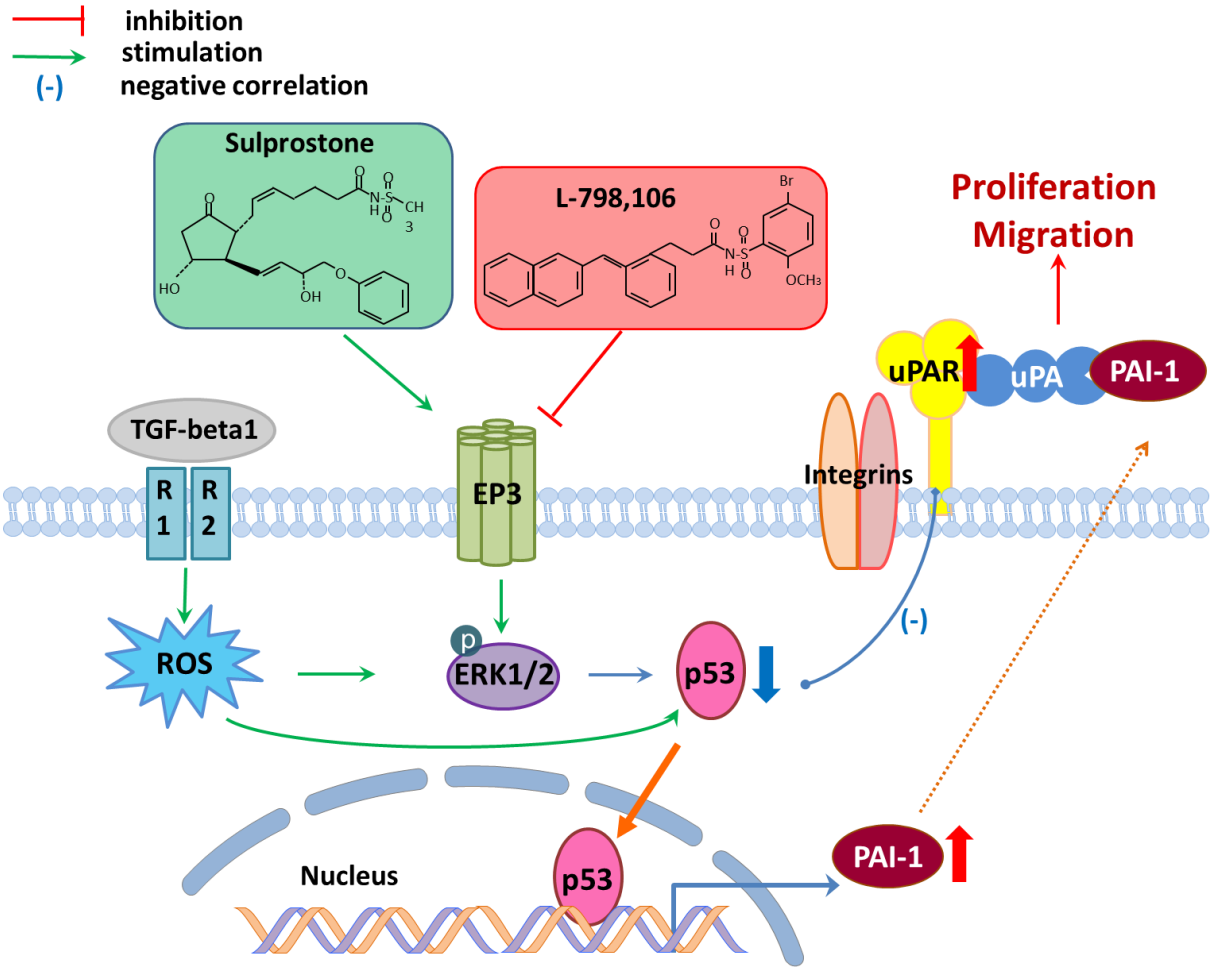


Figure 7. Schematic diagram of EP3 signaling in human cervical cancer cells. EP3 signaling regulates the proliferation and migration of cervical cancer cells through the plasminogen activating (PAI-1/uPA/uPAR) system. The EP3 agonist (sulprostone) coupling EP3 receptor stimulates phosphorylation of extracellular signal-regulated kinases (p-ERK1/2). Then the p53 in the cytoplasm translocates to the nucleus, inducing an increased transcription of plasminogen activator inhibitor type 1 (PAI-1). In addition, transforming growth factor-β 1 (TGF-β 1) can also induce PAI-1 gene expression via the rapid generation of reactive oxygen species (ROS), phosphorylation of ERK1/2 and the mobilization of p53 signaling[132]. The elevated expression of PAI-1 leads to a binding with urokinase-type plasminogen activator (uPA) and uPA receptor (uPAR), and then the trimeric PAI-1/uPA/uPAR complex is recognized by the low density lipoprotein receptor-related protein (LRP) and endocytosed together with integrins to promote tumor cells migration[145,146]. 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). The EP3 antagonist (L-798,106) can inhibit the expression of p-ERK1/2 and uPAR.

5.2 EP3 signaling in unexplained recurrent miscarriages (uRM)

5.2.1 COX-2-PGE_2-EP3 signalling pathway in uRM patients

An increased inflammatory reaction can contribute to uRM[157]. PGE_2 plays an important role in regulating immune balance and angiogenesis during implantation and early pregnancy[157] and it is able to stimulate inflammatory reactions via EP3[158]. The role of PGE_2 receptor signalling in the mechanism of uRM still remains unknown. After investigating the effects of EP3 in cervical cancer carcinogenesis, our research group also explored EP3 signalling pathway in pathological mechanisms of uRM. During normal placentation, extra villous trophoblast cells invade the uterus in a tumor-like manner and engraft to the maternal blood vessels.

5.2.2 COX-2 is highly expressed in the stromal cells of uRM patients

As the rate-limiting enzyme for PGE_2 synthesis, COX-2 is expressed by human endometrial stroma cells[100], which is in line with our finding. COX-2 is involved in blastocysts implantation in the early pregnancy phase[101]. So far, a small number of literatures exist concerning COX-2 expression levels in uRM with conflicting conclusions. Wang et al. and his research group reported that decreased expression levels of COX-2 have been found in the chorionic villi of women with uRM than healthy controls[101], and Hua et al. showed the similar result in mice embryos with the animal model of autoimmune-type recurrent miscarriages[102]. Our result is in accordance with another study group of Banerjee et al. who detected that the expression levels of COX-2 are upregulated in the endometrium of uRM women in comparison to healthy controls[104]. In this study, expression of both PGE_2 and pro-inflammatory cytokines is also increased in the endometrium of uRM women, such as interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), and transforming growth factor β1 (TGF-β1)[104]. IL-1, TNF-α and IFN-γ belong to Th1 cytokines, and this Th1 predominance has been known to be one of the immunological reasons for uRM[105]. TNF-α can induce COX-2 gene expression in first-trimester trophoblasts[106], indicating the pro-inflammatory cytokine can be a positive feedback for COX-2 expression.

5.2.3 PGE_2 is induced in uRM patients

The effects of PGE_2 on trophoblast cells are also contradictory for *in vivo and in vitro* studies. Ryantova et al. observed that PGE_2 levels in the cervical ovulatory mucus are increased in patients with uRM[110], which is mainly caused by overexpressed levels of COX-2. However, Biondi et al. proved that PGE_2 inhibits cell proliferation and migration of HTR-8/SVneo cells via upregulating cAMP[159], while Horita et al. demonstrated that PGE_2 stimulates migration of HTR-8/SVneo cells via leukemia inhibitory factor (LIF)[160]. We found that PGE_2 inhibited the production of β-hCG and progesterone in JEG-3 cells and the production of β-hCG in HTR-8/SVneo cells. Low expression levels of β-hCG and progesterone in the serum have been used to serologically diagnose early pregnancy losses[161,162]. Intravaginal PGE_2 decreases the excretion of β-hCG in the urine and progesterone in the plasma, which is in good agreement with our study that high expression levels of PGE_2 in the placenta are detrimental to pregnancy maintenance.

5.2.4 The expression of EP3 is induced in uRM patients

Prostaglandin E_2 receptors (EP1-4) are G protein-coupled receptors that are all activated by PGE_2 with various signalling pathways. Yamazaki et al. (2006) indicated that mRNA and protein expression of COX-2 and EP3 are increased in the placenta of rats after ischemia-reperfusion, which is associated with intrauterine growth restriction[163]. In our study, we observed that EP3 expression was mainly expressed in the extravillous trophoblasts and increased in extravillous trophoblast cells of uRM patients compared to normal pregnancies in the first trimester placenta. Trophoblast invasion takes place in a low oxygen environment in normal pregnancies, while shallow trophoblast implantation and ischemia in the placenta could lead to miscarriages. It is difficult to conclude that induced EP3 signalling pathway is the consequence or the cause of uRM. The EP3 gene formed nine distinct mRNAs encoding at least eight EP3 isoforms[19], and the anti-EP3 antibody applied in our study could detect the protein region within the internal sequence amino acids 360-409. It might partly explain different band signals of EP3 in JEG-3 and HTR-8/SVneo cells for *in vitro* studies.

Furthermore we investigated that sulprostone (an EP1/EP3 agonist) inhibited the secretion of β-hCG and progesterone in JEG-3 cells and β-hCG expression in HTR-8/SVneo cells.

Sulprostone, as a PGE_2 analog, can be used for medical termination of early pregnancy[164]. A reduced production of both β-hCG and progesterone is detrimental to blastocyst growth and pregnancy maintenance [165]. However, Biondi et al. (2006) reported that PGE_2 inhibited cell proliferation and migration of HTR-8/SVneo cells via EP2 and EP4 receptors instead of EP3[159]. In order to explore the exhaustive mechanisms of membrane receptors of PGE_2 in the placenta of uRM, the remaining membrane receptors of PGE_2 (EP1, EP2 and EP4) still need to be investigated, and the EP1 receptor represents an important receptor we have been focused on in a recent publication[166].

5.2.5 The effects of EP3 signaling in uRM patients

The activation of G protein α stimulator (G_s) stimulates the activity of adenylate cyclase (AC), enhances intracellular levels of cyclic adenosine monophosphate (cAMP) and activates protein kinase A (PKA), and this cAMP/PKA signaling cascade is a vital second messenger pathway for steroid biosynthesis[167]. G protein α inhibitor (G_i) inhibits AC, contributing to decreased levels of cAMP and inhibited activity of PKA[168]. We detected that the expression of G_{i1} was mainly expressed in extravillous trophoblasts and upregulated in the decidua of uRM patients. It indicates that the cAMP/PKA signaling pathway may be inhibited in the extravillous trophoblasts of uRM. cAMP increases α-hCG gene transcription in villous trophoblasts by interacting with the cAMP response element-binding protein (CREB)[169]. PKA stimulates progesterone synthesis in the human placenta by phosphorylation of enzymes[170]. We found that both PGE_2 and sulprostone stimulated the expression of G_{i1} and suppressed the production of β-hCG and progesterone, which could be caused by the stimulated EP3/G_{i1} and inhibited cAMP/PKA signaling.

Interestingly, we found the expression of PAI-1 was upregulated by sulprostone in JEG-3 cells, which is known as the main inhibitor of fibrinolysis[171]. High plasma levels of PAI-1 and high PAI activities are prevalent in women with uRM in comparison to healthy controls[172]. PGE_2 utilizes EP1/EP3 receptor to increase PAI-1 levels in cardiac fibroblasts, resulting in elevated fibrin deposition in aortic stenosis[122]. TGF-β1 can induce PAI-1 gene expression by phosphorylation of extracellular signal regulated kinases (ERK1/2) and p53[132]. The expression of p53 is upregulated in chorionic villi of RPL patients compared to healthy controls[173]. We found that sulprostone stimulated the expression of phosphorylated-ERK1/2 (p-ERK1/2) in

JEG-3 cells and the expression of p53 in HTR-8/SV neo cells. Additionally, the expression of p-ERK1/2 was inhibited by EP3 specific inhibitor L-798,106 in HTR-8/SV neo cells. The activation of EP3 signalling induces increased expression of p-ERK1/2 and p53 in extravillous trophoblasts, which results in increased PAI-1 gene expression and an imbalance of extracellular matrix degradation during first-trimester pregnancies. Both the *in vivo* study of Yamazaki et al[163] and also our study suggest that under pathological circumstances large differences in EP3 expression in the placenta can be observed. In contrast, only small magnitude changes of EP3 and PAI-1 were detected in our cell culture studies. It implies that trophoblast cells *in vitro* can partly mimic the *in vivo* delicate environment, however it still cannot replace the complex physical milieu of the placenta. Therefore, *in vivo* investigations of EP3 regulation in animal models or observational studies in humans should be the focus in future.

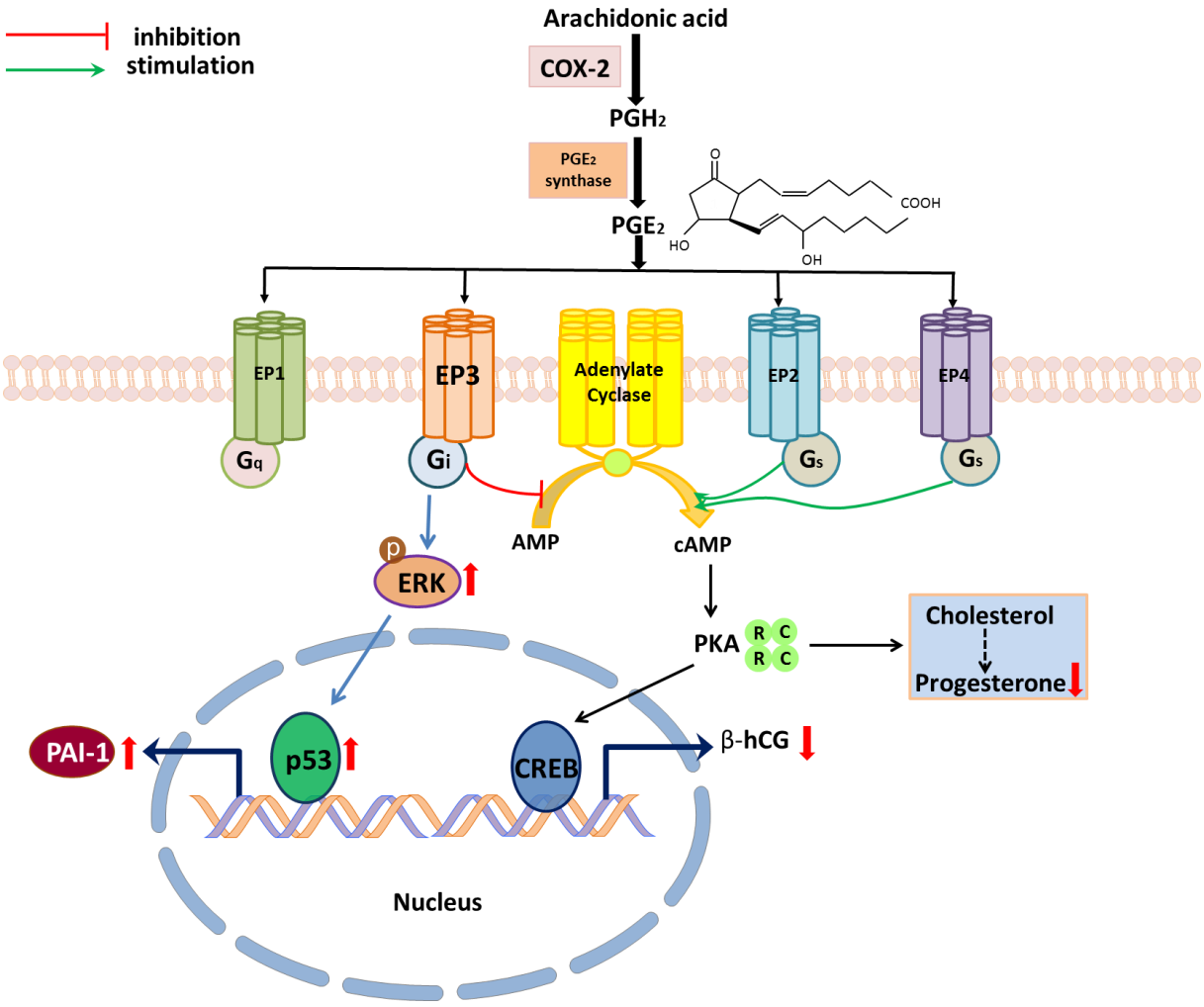


Figure 5. EP3 signaling is induced in the extravillous trophoblasts of uRM patients. Arachidonic acid is metabolized by COX-2 into PGH_2, which is converted into multiple prostaglandins, such as PGE_2[4]. PGE_2 acts through different membrane receptors (EP1-4)[174]. In the placenta of uRM patients, enhanced levels of COX-2 might be the reason for increased PGE_2 expression. PGE_2 coupling with EP3 induces G_{i1} and reduces cAMP, which can eventually lead to a downregulation of β-hCG and progesterone and an upregulation of PAI-1. The decreased cAMP/PKA signaling inhibits the secretion of progesterone via PKA[170] and the transcription of β-hCG via CREB[169]. The activated EP3/G_{i1} can stimulate p-ERK1/2 and p53, which eventually enhance PAI-1 gene transcription[132]. These changes could prevent trophoblast implantation and placentation, finally causing recurrent pregnancy losses. Abbreviation: COX-2, cyclooxygenase-2; PGH_2, prostaglandin H_2; PGE_2, prostaglandin E2; EP, PGE_2 receptor; Gq, Gprotein alpha q; Gs, G protein alpha stimulator; G_{i1}, G protein alpha inhibitor 1; cAMP, cyclic adenosine monophosphate; p-ERK1/2, phosphorylated extracellular signal–regulated kinases 1/2; PKA, protein kinase A; CREB, cAMP response element-binding protein; PAI-1, plasminogen activator inhibitor type 1; uRM, unexplained recurrent miscarriages.

6 Summary

6.1 Role of EP3 signaling in the carcinogenesis of cervical cancer

Elevated expression of COX-2 and PGE_2 are found in numerous cancers and are associated with tumor development and progression. Although epidemiological, clinical and preclinical studies have shown inhibiting of PGE_2 synthesis through NSAIDs or COX-2 inhibitors has the potential for cancer prevention and treatment, the side effects of inhibiting COX-2 functions have limited their use. Therefore, it is urgent for exploring novel targets and effective inhibitors which COX-2 activity may be reduced without inducing any reverse effects. In recent years, extensive efforts have gone into investigating the mechanism of PGE_2 and EP receptors in health and disease.

In our investigation concerning EP3 signaling in cervical cancer, the EP3 receptor can regulate cell proliferation and migration through modulation of the plasminogen activating pathway in human cervical cancer cells. *In vitro* studies, EP1/EP3 agonist sulprostone stimulated tumor cells growth and migration in both CaSki and HeLa cells, while the EP3 specific antagonist (L-798,106) inhibited the expression of EP3 in CaSki cells without affecting EP1 expression. At the same time, the expression of phosphorylated extracellular signal-regulated kinases (ERK1/2) was upregulated by sulprostone and was downregulated by L-798,106. Sulprostone increased the levels of PAI-1 in the supernatants of both CaSki and Hela cells via p-ERK1/2 and p53, implying a correlation might exist between EP3 signaling and plasminogen activating system in tumor development. *In vivo* studies showed that the overexpression of uPAR was associated with poor prognosis in cervical cancer patients with FIGO stages III and IV, which suggests that uPAR may represent another novel therapeutic target for the treatment apart from the EP3 receptor. L-798,106 could decrease the expression of both EP3 and uPAR in CaSki cells, which could be a promising agent for cervical cancers, especially for squamous cervical carcinoma.

6.2 Role of EP3 signaling in the pathological mechanism of uRM

In the study about recurrent miscarriages, we confirmed that enhanced levels of COX-2 were found in stromal cells of the placenta in unexplained RM. PGE_2 in combination with the EP3 receptor in extravillous trophoblasts contributed to a decreased production of β-hCG and

progesterone via G_{i1} and AC, thus contributing to reduced levels of cAMP and deactivated activity of PKA. We also proved a crosstalk between EP3 signaling and plasminogen activating system in the pathological mechanism of RM. The activated EP3/G_{i1} can enhance PAI-1 gene expression through stimulating p-ERK1/2 and p53. Upregulated production of PAI-1 can inhibit extracellular matrix degradation, contributing to intervillous fibrin deposition in the maternal-fetal interface. Downregulated secretion of β-hCG and progesterone and upregulated expression of PAI-1 could prevent trophoblast implantation and placentation, causing failed pregnancy maintenance. We explored that the EP3 antagonist (L-798,106) caused downregulated EP3 expression without influencing the β-hCG expression in JEG-3 cells. It indicates that L-798,106 might be a 'potential therapeutic candidate' for the treatment of unexplained RM.

In conclusion, EP3 signaling pathway plays a vital role in the cancer development of cervical cancer and the pathological mechanism of recurrent miscarriages via p-ERK1/2 and PAI-1. The strong connection between EP3 signaling and plasminogen activating system exists in both diseases and L-798,106 can effectively inhibit the key factors in the crosstalk. Future studies should apply animal models to examine the effects of L-798,106 or more specific EP3 antagonist on inhibiting the inflammatory reactions in cervical cancer and RM. Attention should also be put on the additional membrane receptors of PGE_2 (EP1, 2 and 4) and other members of G_i family (G_{i2} and G_{i3}) in cancer development and RM.

7. Zusammenfassung

7.1 Die Rolle des EP3 Signalwegs in der Karzinogenese des Cervixkarzinoms

Eine erhöhte Expression von COX-2 und PGE_2 wurde in zahlreichen Karzinomentitäten gefunden und ist mit der Entstehung und Progression von Tumoren assoziiert. Obwohl epidemiologische, klinische und präklinische Studien gezeigt haben, dass eine Inhibition der PGE_2 Synthese durch NSARs oder COX-2-Inhibitoren Potential für die Prävention und Therapie von Karzinomen besitzt, limitierten bisher die Nebenwirkungen von COX-2-Inhibitoren deren Nutzung. Aus diesem Grund ist es notwendig, neue Targets und effektive Inhibitoren zu untersuchen, mit welchen die COX-2 Aktivität reduziert werden kann, ohne entgegengesetzte Wirkungen zu induzieren. In den letzten Jahren wurden umfassende Untersuchungen vorgenommen um den Mechanismus von PGE_2 und EP Rezeptoren zu eruieren.

Unsere Untersuchungen bezüglich des EP3 Signalweges im Cervixkarzinom haben gezeigt, dass der EP3 Rezeptor in der Lage ist, die Zellproliferation und –migration durch Modulation des Plasminogen-aktivierenden Signalwegs in humanen Cervixkarzinomzellen zu regulieren. In *in vitro* Studien wurde nachgewiesen, dass Sulprostone, ein EP1/EP3 Agonist, das Tumorzellwachstum und die Tumorzellmigration in CaSki- und HeLa-Zellen stimuliert, während der EP3 spezifische Antagonist (L-798,106) die Expression in CaSki-Zellen inhibiert, ohne die EP1 Expression zu beeinflussen. Gleichzeitig wurde die Expression von phosphorylierten extrazellulären signal-regulierten Kinasen (ERK1/2) durch Sulprostone hoch-, durch L-798,106 hingegen herabreguliert. Sulprostone erhöhte das PAI-1 Level im Überstand sowohl bei CaSki- als auch bei HeLa-Zellen durch p-ERK1/2 und p53, was eine mögliche Korrelation zwischen dem EP3 Signalweg und dem Plasminogen-aktivierenden System in der Tumorentwicklung impliziert. *In vivo* Studien zeigten, dass die Überexpression von uPAR mit einer schlechteren Prognose bei Patientinnen mit Cervixkarzinom (FIGO III und IV) assoziiert ist, was suggeriert, dass uPAR, unabhängig vom EP3 Rezeptor, womöglich ein neues therapeutisches Ziel darstellt. L-798,106 konnte, als womöglich vielversprechende Substanz in der Therapie des Cervixkarzinoms, insbesondere des squamösen Subtyps, die Expression sowohl von EP3 als auch von uPAR in CaSki-Zellen verringern.

7.2 Die Rolle des EP3 Signalwegs im Pathomechanismus von rezidivierenden Spontanaborten

In eine Studie über rezidivierende Spontanaborte (RSA) haben wir bestätigt, dass in stromalen Zellen der Dezidua bei Patienten mit ungeklärten Fällen von RSA ein erhöhter COX-2 Spiegel gefunden wurde. PGE_2 hat in Kombination mit dem EP3-Rezeptor im extravillösen Trophoblast zu einer verringerten Produktion von β-hCG und Progesteron via G_{i1} und AC geführt, was letztendlich zu einem reduzierten cAMP Spiegel und einer verringerten Aktivität der PKA beiträgt. Wir haben ebenfalls Interaktionen zwischen dem EP3-Signalweg und dem Plasminogen-aktivierenden System im Pathomechanismus rezidivierender Spontanaborte nachweisen können. Aktiviertes EP3/G_{i1} kann die PAI-1 Genexpression durch eine Stimulation von p-ERK1/2 und p53 verstärken. Eine hochregulierte Produktion von PAI-1 kann den Abbau der extrazellulären Matrix verhindern, was zu einer intervillösen Fibrinablagerung in der maternal-fetalen Schnittstelle beiträgt. Die herab regulierte Sekretion von β-hCG und Progesteron sowie die erhöhte Expression von PAI-1 konnte die Einnistung des Trophoblasten und die Plazentation verhindern, was einen Abbruch der Schwangerschaft verursacht. Wir haben herausgefunden, dass der EP3-Antagonist L-798,106 die EP3-Expression herabsetzt ohne den β-hCG Spiegel in JEG3 Zellen zu beeinflussen, was L-798,106 zu einem potentiellen Kandidaten in der Therapie von RSA macht.

Zusammenfassend spielt der EP3 Signalweg über p-ERK1/2 und PAI-1 eine vitale Rolle sowohl in der Entstehung des Cervixkarzinoms als auch im Pathomechanismus von Spontanaborten. Ein starker Zusammenhang zwischen dem EP3-Signalweg und dem Plasminogen-aktivierenden System existiert in beiden Krankheitsbildern, wobei L-798,106 effektiv die Schlüsselfaktoren in ihrer Interaktion inhibiert. Zukünftige Studien sollten diesen Ansatz auf ein Tiermodell übertragen, um den Effekt von L-798,106 oder anderen spezifischen EP3 Antagonisten auf die Inhibition entzündlicher Reaktionen im Cervixkarzinom und in rezidivierenden Aborten zu untersuchen. Ein besonderes Augenmerk sollte zusätzlich auf die Membranrezeptoren PGE_2 (EP1, 2 und 4) und auf andere Mitglieder der G_i-Familie (G_{i2} und G_{i3}) im Hinblick auf rezidivierende Aborte und die Entstehung von Malignomen gelegt werden.

7. References

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