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The expression of prostaglandin E receptors and their roles in healthy and diseased endometrium

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1 Abbreviations

AA	arachidonic acid
AC	adenylyl cyclase
Bad	Bcl associated death protein
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-XL	Bcl-Xlong protein
bFGF	basic fibroblast growth factor
BrdU	5-bromo-2'-deoxyuridine
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
CDK	cyclin-dependent kinase
COX	cyclooxygenase
cPGES	cytosolic prostaglandin E synthase
cPLA2	cytosolic phospholipase A2
CREB	cAMP-response element binding protein
E2	estradiol
EC	endometrial cancer
ECL2	the second extracellular loop
EEC	endometrial epithelial cell
EGFR	epidermal growth factor receptor
EGR-1	early growth response protein 1
EP	prostaglandin E receptor
EPAC	exchange protein activated by cAMP
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
ESC	endometrial stromal cells
FAK	focal adhesion kinase
GPCR	G-protein coupled receptor
HGF	hepatocyte growth factor
HIF-1α	hypoxia-inducible factor-1α
ICL2	the second intercellular loop
IL-8	interleukin-8
iPLA2	Ca2+-independent phospholipase A2
IRS	immunoreactive score
LPS	lipopolysaccharide
Mφ	macrophages
MMP	matrix metalloproteinase
mPGES	membrane-bound PGES
MRP4	resistance protein 4
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVD	microvessel density
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NSAID	non-steroidal anti-inflammatory drug
P4	progesterone

PG	prostaglandin
PGES	prostaglandin E synthase
PGG2	prostaglandin G2
PGH2	prostaglandin H2
PGT	prostaglandin transporter
PI3K	phosphoinositide-3 kinase
PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
PRB	progesterone receptor B
Ras	rat sarcoma
RERG	Ras-like, estrogen-regulated, growth inhibitor
sPLA2	secretory phospholipase A2
SUMO-1	enhancing small ubiquitin-like modifier 1
TMVII	the seventh transmembrane domain
TXA2	thromboxane A2
VEGF	vascular endothelial growth factor

2 Publication list

2.1 Expression of EPs in healthy and diseased endometrium

Histochem Cell Biol. 2018 Feb;149(2):153-160. doi: 10.1007/s00418-017-1616-y. Epub 2017 Nov 13.

Prostaglandin E2 receptor EP1 in healthy and diseased human endometrium

Junyan Zhu, Doris Mayr, Christina Kuhn, Sven Mahner, Udo Jeschke, Viktoria von Schönfeldt

2.2 Role of EP3 in endometrial cancer

Oncotarget. 2017 Dec 9;9(1):982-994. doi: 10.18632/oncotarget.23140. eCollection 2018 Jan 2.

Prostaglandin receptor EP3 regulates cell proliferation and migration with impact on survival of endometrial cancer patients

Junyan Zhu, Fabian Trillsch, Doris Mayr, Christina Kuhn, Martina Rahmeh, Simone Hofmann, Marianne Vogel, Sven Mahner, Udo Jeschke, Viktoria von Schönfeldt

3 Confirmation of co-authors

3.1 Prostaglandin E2 receptor EP1 in healthy and diseased human endometrium

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Junyan Zhu
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Prostaglandin E2 receptor EP1 in healthy and diseased human endometrium
Title of publication

Histochemistry and Cell Biology. 2018 Feb;149(2):153-160.
Journal (Name, issue, year, etc.)

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- their agreement to the submission of the publications, and
- the article in question is not the subject of another (current or completed) dissertation.

Name of co-author	Extent of contribution (content-related and volume)	Signature of co-author
1. Doris Mayr	data collection and analysis	
2. Christina Kuhn	immunohistochemistry and reviewing the manuscript	
3. Sven Mahner	reviewing the manuscript	
4. Udo Jeschke	conception and design of the work, reviewing the manuscript	
5. Viktoria von Schoenfeldt	funding and reviewing the manuscript	

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3.2 Prostaglandin receptor EP3 regulates cell proliferation and migration with impact on survival of endometrial cancer patients

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- the article in question is not the subject of another (current or completed) dissertation.

Name of co-author	Extent of contribution (content-related and volume)	Signature of co-author
1. Fabian Trillsch	critical revision of the article	
2. Doris Mayr	data collection and analysis	
3. Christina Kuhn	immunohistochemistry and reviewing the manuscript	
4. Martina Rahmeh	western blot and reviewing the manuscript	
5. Simone Hofmann	real time RT-PCR and reviewing the manuscript	

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Cumulative dissertation September 2017

Name of co-author	Extent of contribution (content-related and volume)	Signature of co-author
6. Marianne Vogel	western blot and reviewing the manuscript	
7. Sven Mahner	reviewing the manuscript	
8. Udo Jeschke	conception and design of the work	
9. Viktoria von Schoenfeldt	funding and reviewing the manuscript	

4 Introduction

4.1 Human endometrium and its common diseases

The primary function of human endometrium is to orchestrate the success of implantation and subsequent fetal growth and maturation [1,2]. Human endometrium is composed of the superficial "functionalis" layer and the deeper "basalis" layer according to distinct responses to estrogen and progesterone (P4) and different functions [2,3]. The former one consists of luminal epithelium, majority of glands lined by epithelium, and stroma [2]. The latter one, containing fewer glands and compact stroma, acts as the seat of the regenerative functionalis layer and the main component of the postmenopausal endometrium [2,3]. Endometrial stromal cells within proper extracellular matrices regulate epithelial cells growth and differentiation [4]. In addition, cycle-dependent immune cells exist in both layers [5]. Human endometrium undergoes a cyclic morphological change in response to fluctuating sex hormones and inflammatory mediators before menopause [1,2]. It is classified into proliferative, secretory and menstrual phases (Fig. 1). During the proliferative phase, all the tissue components including glandular, stromal and vascular endothelial cells increase and peak between cycle days 8 and 10, mainly under the control of estradiol (E2). Following ovulation, under the influence of P4, the endometrium undergoes secretory differentiation, which is characterized by active apocrine secretion of glycoproteins, edema, coiling of spiral arterioles, and predecidualization of the stroma. In the absence of conception, withdrawal of estrogen and P4 leads to the shedding of the endometrium and this process is limited to the functionalis layer [3].

Endometriosis and endometrial cancer (EC) are common endometrial diseases [2]. Endometriosis is characterized by endometrial-like tissue appearing outside the uterine cavity [2]. Major phenotypes include ovarian endometrioma, superficial peritoneal endometriosis, and deep infiltrating endometriosis [6,7]. Pelvic pain, such as dysmenorrhea, dyspareunia, non-period chronic pelvic pain and dyschezia, and infertility are among the most common symptoms. In less frequent but more severe cases, endometriosis implants may infiltrate the pelvic organs and cause intestinal perforation and obstruction [8]. Although being a benign disorder, endometriosis greatly affects the patients' life quality. Although several theories have been proposed, the underlying pathogenesis remains unclear. Either drug treatments or surgeries are only effective for temporarily alleviating symptoms [7]. EC is a tumor originated from the endometrium and endometrioid adenocarcinoma is the most common histological subtype. Although it is often diagnosed at an early stage with good prognosis, its survival has actually not been improved since 1990 in European countries [9]. Furthermore, it receives more attention because of its growing morbidity. EC has become the most frequent gynecological carcinoma in more developed regions in 2012, where the incidence was estimated to be 14.7 per 100,000 [10,11]. For a long period, EC has been classified into two pathogenic types (type I and II) based on endocrine features to guide the treatment or predict the outcome [12]. However, this dualistic classification fails to explain the heterogeneity from biological and molecular features to prognosis [13]. Studies on molecular features of EC are emerging to provide more precise methods of defining high-risk patients [14].

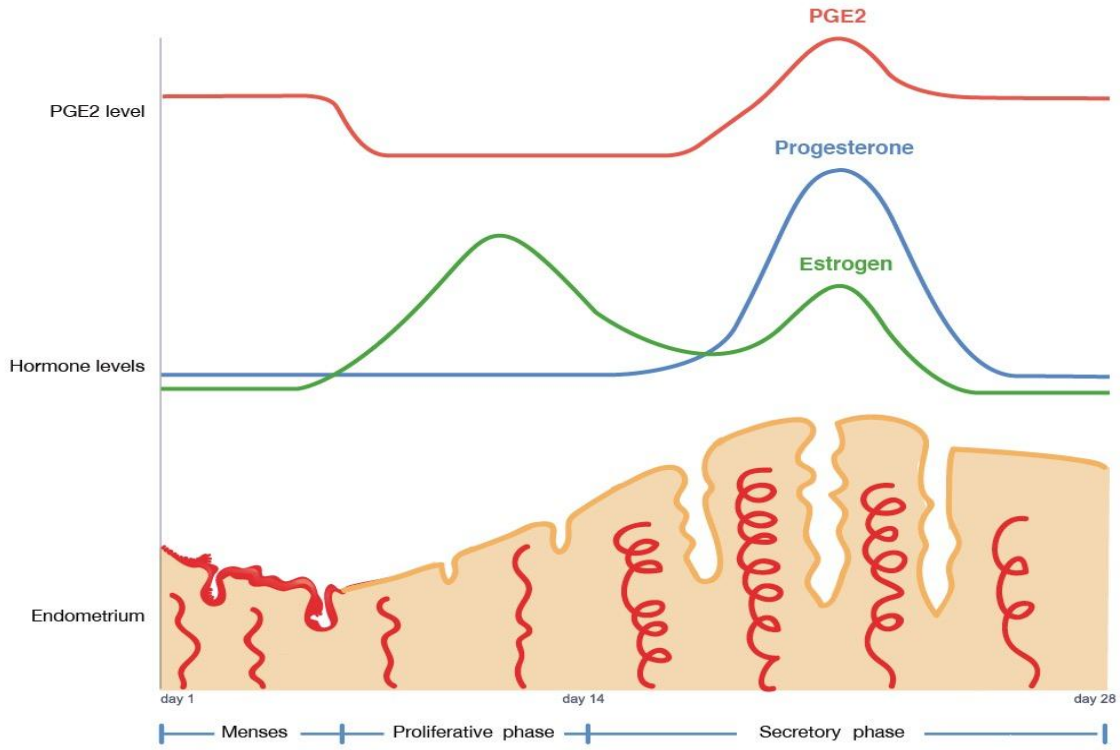

Figure 1. **The levels of ovarian hormones and prostaglandin E2 (PGE2) in the menstrual cycle.** This illustration depicts the change of endometrium under the control of estrogen and progesterone and the simultaneous fluctuation of PGE2.

4.2 Biosynthesis of prostaglandin E2

Prostaglandin (PG) E2, a member of the prostanoid family, is a lipid mediator formed ubiquitously in human bodies and plays an extensive role in immune response [15], inflammation [16], pain perception [17], fever [18], oncogenesis [19], female fertilization and parturition [20]. Non-steroidal anti-inflammatory drugs (NSAIDs) for the treatment of pain and inflammation are believed to function to a large extent via targeting the PGE2 signal [21-23]. PGE2 exerts its biological actions via binding to its seven-transmembrane, G-protein coupled receptors (GPCRs), termed prostaglandin E receptor (EP) 1, EP2, EP3 and EP4 [11,24]. It is well described to be associated with both endometrial functions [25] and disorders [26].

The biosynthesis of PGE2 is a three-step process and three types of enzymes are included (Fig. 2). Firstly, arachidonic acid (AA) is released from plasma membrane phospholipids, after hydrolysis of sn-2 bond by phospholipase A2 (PLA2). Although secretory PLA2 (sPLA2), cytosolic PLA2 (cPLA2) and Ca(2+)-independent PLA2 (iPLA2) are all involved in this reaction, only cPLA2alpha shows a preference for AA in the sn-2 position, which makes it a potential target for inhibiting the production of AA [27].

The released AA is then converted successively to prostaglandin G2 (PGG2) and prostaglandin H2 (PGH2), the two unstable intermediates by the action of cyclooxygenase (COX) [28]. Three isoforms of COX enzymes have been described: COX-1, COX-2, and COX-3. Because COX-1 is constitutively expressed at low levels in most tissues and at high levels in stomach and platelets, COX-1 was initially considered to be only involved in physiological processes, such as the protection of the gastric mucosa and platelet aggregation. As

research continues, the findings that COX-1 is also upregulated in inflammation [29,30], pain perception [31] and tumors [32,33] and highly selective COX-1 inhibitors can inhibit PGE2 production, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κB) activation and inflammatory cell influx, suggest a role of COX-1 in pathological phenomena [34,35]. Since COX-2 was discovered in 1991, a lot of research has been performed to compare these two isoenzymes in structure and function. Although the genes for COX enzymes are mapped to different chromosomes: the gene for COX-1 is on chromosome 9q32-q33.3, the gene for COX-2 is on chromosome 1q25.2-q25.3, the amino acid sequences of both COX proteins are highly conserved [36]. The differences in regulatory regions, as well as coding sequences of the genes, contribute to distinct expressions and functions [37,38]. COX-2 has been demonstrated to be an immediate-early gene and quickly induced by stimuli. In the inflammatory context, inducible PGE2 production, anorexia, hyperalgesia, and fever are mainly dependent on COX-2, while COX-1 plays a role in avoiding hypothermia [38-41]. Both COX-1 and COX-2 deficient mice show reduced tumorigenesis [42]. In a solid tumor, COX-2 has been found to prompt angiogenesis [43] and proliferation [44]. Consistently, exposure to COX-2 inhibitor induces apoptosis [45]. A recent study has revealed that aspirin's chemoprotective activity is due to inhibition of COX-1-mediated platelet activation and consequent blockade of the interaction between tumor cells and their microenvironment [46].

COX-3 is generated by alternative splicing from COX-1 and highly expressed in cerebral cortex and heart. The COX activity of COX-3 determined by PGE2 production shows only about 20% of that of COX-1 [47]. The precise role, which COX-3 plays in the physiological and pathological process, remains to be elucidated.

Finally, the unstable precursor — PGH2, is catalyzed to different prostanoids, including PGE2, PGF2α, PGD2, PGI2, and thromboxane A2 (TXA2) by their respective terminal PG synthases, PGE synthase (PGES), PGI synthase, PGD synthase, PGF synthase, and thromboxane synthase [24]. Membrane-bound PGES-1 (mPGES-1), mPGES-2, and cytosolic PGES (cPGES) are the three distinct forms of PGES [48].

mPGES-1 is mainly expressed in the urogenital system at an intermediate level and colocalized with COX enzymes on nuclear membranes. mPGES-1 is preferentially coupled with COX-2 to produce delayed and immediate induction of PGE2 [49]. In addition, a selective COX-2 inhibitor is showed to attenuate the induction of mPGES-1, suggesting COX-2 is essential to mPGES-1 and inhibition of either mPGES-1 or COX-2 affects the PGE2 biosynthesis [50]. mPGES-1 also shows the capability to couple with COX-1 by supplied with high concentration exogenous or endogenous AA [49]. It can be markedly induced by proinflammatory stimuli, tissue damage and tumor, which makes it a potential therapeutic target for several diseases [51].

mPGES-2 is located on Golgi membrane and constitutively expressed in heart, brain, lung, liver, and colon. Only the expression in liver and colon can be upregulated by lipopolysaccharides (LPS) stimulation. Consistent with that, its expression is elevated in colorectal cancer while keeping unchanged in inflammatory diseases and tissue damage. It can prompt immediate and delayed PEG2 biosynthesis via both COX enzymes with a slight COX-2-preference, suggesting it may be involved in organism homeostasis as well as diseases [51]. However, a recent report demonstrating PGE2 expression in mPGES-2 deficient mice was equal to that in healthy mice implies the little importance of mPGES-2 as a terminal synthase [52]. The possibility remains that the other two prostaglandin E synthases might compensate for the loss of mPGES-2.

RNA blot analysis identifies cPGES expression to be ubiquitous and constitutive with the exception that it could be induced remarkably following LPS treatment in brain tissue. Confocal microscopic analysis reveals it is located in the cytoplasm of the cells. cPGES pairs functionally with constitutive COX-1 to produce PGE2 from exogenous and endogenous AA [53]. Moreover, in cells lack of mPGES-1, cPGES is able to pair with COX-2 to produce a modest amount of PGE2 [54]. Thus, cPGES is essential to maintenance of tissue homeostasis.

Following synthesis, PGE2 is transported across the plasma membrane by multidrug resistance protein 4 (MRP4) [55] and activates its receptors (Fig. 2). Alternatively, newly synthesized extracellular PGE2 is imported back into cells via the prostaglandin transporter (PGT) to suppress the signal pathway by preventing PGE2 from attaching to EPs (Fig. 2) [56,57].

4.3 Structures and pathways of EPs

Although recognizing PGE2 as a ligand, the homology among these receptors is limited. Both EP1 and EP2 share more identities with other prostanoid receptors than with other EPs [58]. Among several conserved regions of EPs, the most highly conserved regions are located in the second extracellular loop (ECL2) and the seventh transmembrane domain (TMVII) [58]. All the EPs had been cloned by 1994. Afterward, the amino acid sequences have been compared to elucidate the prostaglandin receptor-specific events and understand the structure-function relationships of GPCRs. The aromatic amino acids in the second intercellular loop (ICL2) of EP2 is identified to mediate the Gs protein coupling, which offers an insight into the role of ICL2 of other GPCRs in coupling with G protein [59,60]. Other studies focusing on highly conserved residues among EPs of many species have identified that arginine residue in TMVII is essential to selective G protein coupling [61], while serine residue in TMVI and threonine residue in ECL2 are essential to ligand binding [62,63].

EP1 is mainly coupled to Gq/G11 and induces the Ca(2+) mobilization and activates protein kinase C (PKC) by phospholipase C (PLC) -dependent pathway (Fig. 2) [64,65]. Its downstream effectors include c-Src, focal adhesion kinase (FAK), NF-κB [66-68]. Recent studies show the EP1-mediated PKC is also responsible for cyclic adenosine monophosphate (cAMP) formation and the following cAMP/PKA/cAMP-response element binding protein (CREB) pathway, which might reveal the interaction among EPs in this pathway [69,70]. Additionally, that EP1 is able to upregulate the expression of hypoxia-inducible factor-1α (HIF-1α) via activation of phosphoinositide-3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling by coupling to Gi/Go has also been reported [65].

EP2 and EP4 have long been known to activate cAMP/PKA/CREB signaling pathway via Gs (Fig. 2). Besides, both receptors are able to induce the translocation of β-catenin, which is involved in promoting gene transcription [71]. Following activation of cAMP, either of them also induces a PKA-independent exchange protein activated by cAMP (EPAC) pathway [72-74]. However, consistent with different molecular structures, ligand infinities, and distributions, there are several fundamental differences in signaling transduction between EP2 and EP4. The most important one is that EP4, rather than EP2, couples to Gi/Go and is responsible for the subsequent PI3K pathway, including phosphorylation of the extracellular signal-regulated kinase (ERK)1/2 and induction of early growth response protein 1 (EGR-1) [75,76]. Furthermore, the translocation of β-catenin and the activation of CREB achieved by EP2 and EP4 occur through distinct upstream pathways. EP4-mediated

activation is PI3K-dependent to a large extent, while the one mediated by EP2 is PKA-dependent [71,77]. In addition, the findings that PI3K inhibits the activity of PKA underlie the connection between these two major pathways [77]. These similarities and differences in signaling and the close interaction among them indicate a fine and complex regulation of EP2 and EP4 signaling transduction.

EP3 stimulation mainly induces inhibition of adenylyl cyclase (AC) activity and enhances the Ca(2+) entry and mobilization via Gi-coupling (Fig. 2) [24,78]. However, among four EPs, EP3 is the most unique due to its diverse C-terminal tails by alternative splicing [11]. For example, human EP3 gene, containing ten exons and nine introns, generates nine distinct mRNAs and encodes at least eight isoforms [11,79]. Consistent with its multiple splicing variants, EP3 has been reported to activate AC and Rho protein via Gs and G12/G13 [80]. Therefore, its downstream targets include the majority of pathways of the other EPs, such as PKA/β-catenin [81,82], PI3K/AKT signaling [83,84]. In addition, one bovine isoform of EP3 is indicated to induce phosphatidylinositol turnover and intercellular Ca(2+) increase by coupling to Gq/G11 [85].

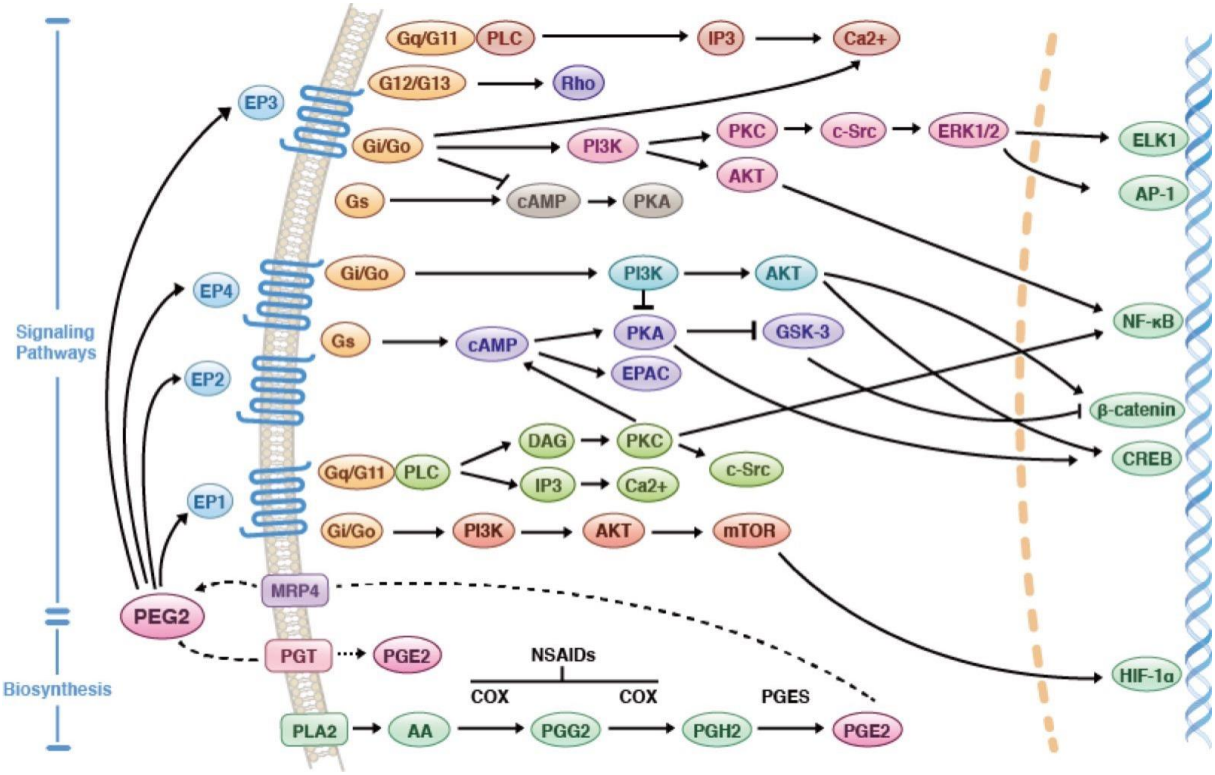

Figure 2. **PGE2 biosynthesis and signaling pathways.** AA is released and ultimately catalyzed into PGE2. Following transported across the plasma membrane, PGE2 binds to its receptors, EP1-4 and in turn, activates different associated G proteins and downstream signaling pathways.

4.4 PGE2 pathway in healthy and diseased endometrium

PGE2 is the predominant product by endometrium instead of by myometrium, implying its major role in endometrium [86]. So far, PGE2 has been confirmed to play an essential role in implantation, consistent with the fact that its secretion increases during the window of implantation and last through the menstrual period (Fig. 1) [87-89]. Inhibition of COX greatly decreases the mouse embryo adhesion to human endometrial epithelial cells (EECs), which can be completely reversed by adding PGE2. Similarly, activation of endometrial EP2 increases

the embryo adhesion by 20% [88]. Besides, PGE2 is also indispensable to induce the vascular permeability and angiogenesis and transform stromal cells into decidual cells during implantation [90]. Therefore, the concentration of PGE2 in endometrial fluid is considered as a biomarker of endometrial receptivity [88]. In addition, PGE2 is proposed to participate in the endometrial repair process in menstruation by upregulating an angiogenic factor, interleukin-8 (IL-8) [91].

COX-2 and mPGES-1 expression are higher in endometriotic lesions than in normal eutopic endometrium [92,93]. Concordantly, PGE2, secreted predominantly by endometrial stromal cells (ESCs) and macrophages (Mφ), also displays an increase in peritoneal fluid and menstrual fluid of women with endometriosis [94,95]. EP4 gene expresses the highest in ESCs among four EPs and together with EP3 mRNA, is reported to be significantly up-regulated in endometriotic tissue [96,97]. The over-expression of COX-2 and the resulting high production of PGE2 are closely linked to pain, infertility and a high risk of recurrence [98-100]. NSAIDs, which inhibit the COXs activity, are the most commonly used medication for relieving pain. A selective COX-2 inhibitor is effective to prevent the ectopic implants in rats [101,102].

The correlation between COX, PGE2, and EC has also been well identified. The expression of COX-2 and PTGES2, which is essential to PGE2 synthesis, is markedly elevated in EC as compared to the normal endometrium and this increased expression is associated with advanced stage, poor grade and deep myometrial invasion [103,104]. A study reported that COX-2 also predicted poor prognosis in EC [105]. COX-1 expression is more abundant in well-differentiated EC and therefore thought to be involved in the early stage of the disease [106]. Consistently, PGE2 secretion and EP2/EP4 mRNA are higher in tumor tissue [89,107]. And more remarkable, the synthesis of PGE2 is localized in neoplastic epithelial cells rather than in stromal cells, the usual localization in normal tissue. The transition from stroma to epithelium ensures the production of PGE2 is not affected by the loss of endometrial stroma during the evolution of EC, implying that PGE2 regulates the function of neoplastic cells in an autocrine/paracrine manner via EP2/EP4 [107,108]. Moreover, the high-frequency use of aspirin and the regular use of selective COX-2 inhibitors have been suggested to reduce the risk of EC in certain groups of women [109,110].

Feedback regulation of COX-2 by PGE2 has been reported in both cultured cells and animal models [111]. In several malignant endometrial epithelial cell lines, PGE2 is observed to be the positive regulator of COX-2 by activating COX-2 transcription and stabilizing COX-2 mRNA, which indicates PGE2 is able to accumulate continuously in the local tumor in an autocrine/paracrine manner [112-114]. Regarding endometriosis, one study hypothesizes a negative COX-2 feedback by PGE2, although it presents no convincing evidence [115].

4.5 The interaction between PGE2 and hormones in the endometrium

The endometrium is one of the tissues most affected by the sex steroid. Many reports suggest that sex steroid can also adjust and control PGE2 pathway in the endometrium, although the exact mechanisms by which estrogen and P4 function independently and/or together to regulate PGE2 pathway are not clear. E2 can increase the PGE2 secretion and P4 acts the opposite *in vitro*, although exposure to either of them upregulates COX-2 expression [116,117]. Further, EPs are demonstrated to be under the control of sex hormones. In ovariectomized rats, EP2 and EP4 are increased by E2, while EP1 and EP3 are increased only by the combination of E2 and P4.

Treatment with P4 alone has no effect on any of the receptors. The differential responses to estrogen might be due to the existence of two different estrogen receptors [118]. The steroidal regulation of PGE2 signaling is also supported by the temporal pattern in human endometrium across the menstrual cycle. COX-1 expression levels are generally higher in secretory phase and probably the highest in mid-secretory phase, while COX-2 expression increases significantly in late secretory following P4 withdrawal and maintains a high level throughout proliferative phase [119-121]. PGT sharply increases during the secretory phase, while MRP4 expression has no significant difference across the whole menstrual cycle [97]. mPGES-1, mPGES-2, and cPGES express quite different and peak at late secretory, mid-secretory, and menstrual phase, respectively [122]. Concerning with EPs, although some expressions also show a temporal-specific manner based on RNA levels, the patterns vary wildly between different studies. Compared to the present results, the only non-contradictory conclusion is that EP3 mRNA increases during the secretory phase, most probably during mid-secretory phase [25,97,122,123].

Endometrial diseases are all sex hormones related to some extent. Considering PGE2 and its related enzymes and receptors fluctuate with the menstrual cycle and play a role in the pathogenesis of endometrial diseases, it is proposed that PGE2 and sex hormones are also closely related in the endometrium under pathologic conditions (Fig. 3). In ESCs, PGE2 is demonstrated to be a potent stimulator of aromatase, a key enzyme catalyzing the final step of estrogen biosynthesis from testosterone, and estrogen receptor (ER) β expression and thus amplifies E2 synthesis through multi-mechanisms [124,125]. Meanwhile, suppression of aromatase diminishes the PGE level in peritoneal fluid in surgery-induced endometriosis mouse model [126]. Taken together, a positive feedback loop of COX-2/PGE2 and estrogen in endometriosis is established [127]. Besides interactions between them, E2/ERβ and PGE2/PKA work in conjunction to promote endometriotic cell proliferation by transcriptional and post-translational modulations of Ras-like, estrogen-regulated, growth inhibitor (RERG) [128]. A positive correlation between COX-2, ERα and aromatase is observed in type 1 EC [129], while another study reports COX-2 has a positive correlation with progesterone receptor (PR) A and PRB instead of with ER in EC [130]. On the other hand, the autocrine of estrogen is enhanced in EC cells in response to trigging COX-2/PGE2/aromatase signaling [131]. Progesterone could increase the oxytocin-induced PGE2 formation of Ishikawa cells via inactivation of oxytocinase on the cell surface [132]. All the evidence supports the notion that COX-2/PGE2 and sex hormones in EC go hand in hand, although the exact expression patterns and regulatory mechanisms between them are not yet fully understood.

4.6 Role of PGE2 in the pathology of the endometrium

Despite a discrepancy in correlation between endometriosis and the risk of EC according to recent large cohort studies [94,95], these two diseases share at least two pathogenic mechanisms: dysregulation of hormone and chronic inflammation [96-99] and four biological features: inducing angiogenesis, sustaining proliferation and evading apoptosis, activating migration and invasion, and avoiding immune destruction [133-136]. Growing evidence shows that PGE2 is implicated in the regulation of all these processes (Fig. 3).

4.6.1 Inducing angiogenesis

Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are potent angiogenic factors in health and disease [137,138] and both elevated in pathologic endometrium including endometriosis and endometrial cancer [139-142].

COX-2 silencing greatly interrupts the production of VEGF in either eutopic or ectopic ESC with endometriosis [143]. When mPGES-1 knockout mouse endometrial fragments were implanted to mPGES-1 knockout mice, the expression of VEGF and microvessel density (MVD) of implanted tissue was significant suppressed compared with wild-type to wild-type implants [144]. Several studies identify that VEGF protein and MVD increases with COX-2 expression in endometrial carcinoma tissue [130,145]. PGE2 could induce the bFGF expression via EP2 by activating cAMP-dependent PKA pathway and its downstream c-Src pathway and thus transactivating the epidermal growth factor receptor (EGFR) and ERK pathway in EC cells [146]. This induction could be attenuated by etodolac, a COX-2 selective inhibitor, in a dose-dependent manner [147]. Moreover, following the same signaling pathway and transactivation of EGFR, PGE2-EP2 could also increase the secretion of VEGF in endometrial adenocarcinoma cells [148].

4.6.2 Sustaining proliferation and evading apoptosis

Highly expressed COX-2 and increased PGE2 level have been shown to promote the proliferation and inhibit the apoptosis in pathological endometrium [104,105]. These effects are mainly achieved by modifying series cell-cycle regulator proteins, apoptosis-related proteins, and factors involved in oncogenesis. In EC tissue, elevated COX-2 protein levels are associated with increased Bcl-2 protein and survivin, two key components in inhibiting apoptosis, and decreased miR101, which could inhibit the growth of endometrial serous carcinoma cell line and is thus expressed rather low in endometrial serous adenocarcinomas [130,149,150]. Moreover, PGE2 could promote proliferation of endometrial cancer cells by enhancing small ubiquitin-like modifier 1 (SUMO-1) expression and inactivating tuberin, a tumor suppressor [104,151]. SUMO-1 is recently known to play a role in the growth of endometrial cancer cells by increasing SUMOylation of histone H4 and the effect of PGE2 on SUMO-1 is via EP4-mediated Wnt/β-catenin signaling pathway [104,152]. The blockade of PGE2/EP2 and PGE2/EP4 pathways are associated with anti-proliferation of human endometriotic epithelial cells and stromal cells by inducing cell cycle arrest through changing the expression of certain cyclins and cyclin-dependent kinases (CDKs) in a cell-specific manner [153]. Moreover, blocking the same pathways increases the interaction between antiapoptotic (Bcl-2 and Bcl-XL) and proapoptotic proteins (Bax and Bad), accelerates the release of cytochrome c, and ultimately activates intrinsic apoptosis in human endometriotic cells [154].

4.6.3 Activating migration and invasion

PGE2 mediated LPS-induced invasion and its level in culture media is positively correlated with migration and invasion in endometriotic cells [96,155]. Similarly, oxytocin and hepatocyte growth factor (HGF)-induced invasion and/or migration in EC cells are PGE2 and/or COX-2 dependent [156,157]. These actions might be mediated by enhancing matrix metalloproteinases (MMPs) secretion and activities, which is essential to extracellular matrix degradation and in turn, leads to tumor cell invasion and metastasis, via diverse EPs (EP1, EP2 or EP4) [104,156,158,159].

4.6.4 Avoiding immune destruction

Abnormal amount and function of immune cells, including T lymphocytes, B lymphocytes, natural killer cells and Mφ, participate in endometrial disorders initiation and progression. Experiments *in vitro* with immune cells derived from women with endometriosis show that PGE2's effect on immune suppression is achieved by inhibiting the growth of peripheral blood lymphocytes, the phagocytic ability of Mφ, the chemotaxis of peripheral blood polymorphonuclear leukocytes and natural killer (NK) cell activity [95,160,161]. Treatment with PGE2 ultimately leads to increased Escherichia coli colonies *in vitro* and endometriotic lesions *in vivo* [95,160]. In EC, it is reported that COX-2 restricts the filtration of cluster of differentiation (CD) 8+ T cells within cancer cell nest, and acts synergistically with tumor-derived mucins to escape from immune surveillance [162,163].

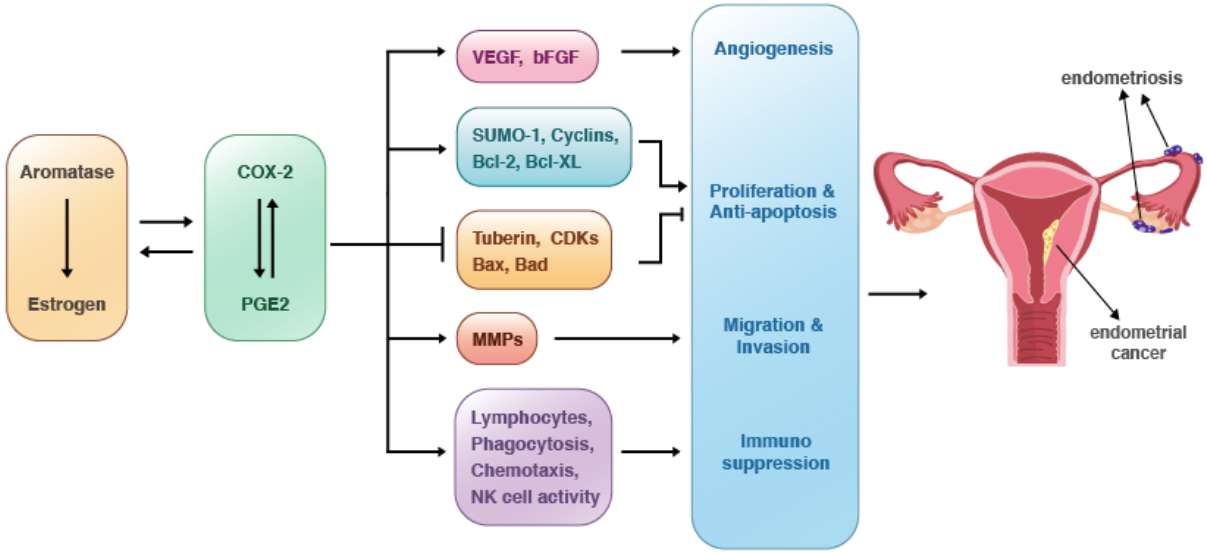

Figure 3. **The interaction between PGE2 and estrogen and the role of PGE2 in diseased endometrium.**

4.7 Aims of the studies

4.7.1 Expression of EPs in healthy and diseased endometrium

PGE2 is well described to be involved both in physiological functions, such as menstrual cycle and implantation, and in human endometrial pathologies [2]. Since PGE2 exerts its actions via its four receptors, it is necessary to understand the expression pattern of them in health and disease [2]. Although some expressions of them show a temporal-specific manner based on RNA levels across the menstrual cycle, the patterns vary wildly between different studies. Moreover, little research has been done into the expressions in endometriosis and EC. We hypothesized spatial-temporal expression of these receptors in health underlies the mechanisms that control the growth and function of the endometrium [2].

Therefore, we investigated the spatial-temporal expression of these receptors in healthy endometrium throughout the menstrual cycle at a protein level. Then in the pathological endometrium, we further investigated the receptors which changed their expression across the menstrual cycle.

4.7.2 Role of EP3 in endometrial cancer

EP3 is reported to regulate the oncogenesis and progression of various tumors, such as human prostate, breast, liver, colon, oral cancer [11]. Although the uterus is one of the organs with most abundant EP3, only little is known about the contribution of EP3 in EC so far [11,104,164]. Since our preliminary experiment demonstrated EP3 peeks during the early secretory phase, we hypothesized that EP3 is correlated with sex hormones and works synergistically with sex hormones to promote the EC progression.

Therefore, we investigated the EP3 expression in tissue samples of EC patients and its association with clinicopathologic characteristics and survival. We studied the mechanism of EP3's effect on EC using human EC cells and established the rationale of PGE2's tumor-promoting action in EC [11].

5 Publication I

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ORIGINAL PAPER

Prostaglandin E2 receptor EP1 in healthy and diseased human endometrium

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Abstract Prostaglandin E2 (PGE2) is well described to be associated with both endometrial functions and disorders. The primary aim of this study was to explore the underlying mechanisms that affect the growth and function of endometrial epithelium and stroma by assessing the staining intensity of PGE2 receptors (EP) in healthy endometrium across the menstrual cycle and in pathological endometrium, such as ovarian endometriosis and endometrial cancer. We retrospectively analyzed the EPs staining intensity in human non-pregnant endometrium throughout the menstrual cycle by immunohistochemistry and further focused on EP1 ($n = 42$). The variation of EP1 was compared among healthy endometrium, ovarian endometriosis ($n = 14$), and endometrial cancer ($n = 140$) crosswise. EP1 presented cyclical changes with increased intensity in both epithelium and stroma during the proliferative phase. EP1 staining in the epithelium was increased in endometriotic tissue compared to healthy endometrium and tumor tissue, while in the stroma, the staining in the tumor was lower than that in both normal tissue and endometriosis. No significant differences in EP1 intensity were detected for histological, stage, grading, metastatic and recurrent subtypes in endometrial cancer. EP1 was also correlated with neither progression-free survival nor overall survival of patients with cancer. EP1 staining in progesterone receptor B (PRB)-positive tumor was stronger compared to PRB-negative tumor. EP1 may play a role in human endometrial physiology and pathology. Further studies on the effect of EP1 on human endometrium are needed.

Keywords EP1 · Endometrium · Endometriosis · Endometrial cancer · Progesterone receptor B

Introduction

The primary function of human endometrium is to orchestrate the success of implantation and consequent fetal growth and maturation. In the absence of conception, human premenopausal endometrium undergoes a cyclic morphological

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change in response to fluctuating sex hormones and inflammatory mediators (Maybin and Critchley 2015). Human endometrium is classified into the superficial functional layer and the deeper basal layer according to distinct responses to estrogen and progesterone and different functions (Ferenczy and Bergeron 1991). The former one consists of luminal and glandular epithelium and stroma. Shedding, proliferation, and secretion are limited to this layer. The latter one, containing gland and compact stroma, acts as the seat of the regenerative functional layer and the main component of the postmenopausal endometrium (Ferenczy and Bergeron 1991). Also, there is the presence of cycle-dependent immune cells in both layers (Bulmer et al. 1991).

Endometriosis and endometrial cancer (EC) are common endometrial diseases. Endometriosis is characterized by endometrial-like tissue appearing outside the uterine cavity, such as on the peritoneum, ovary, other abdominal organs, and pelvic tissue. Although it is a benign disorder, endometriosis is found in an estimated 70% of patients with pelvic pain (Treloar et al. 2010) and 40% of infertile women (Eisenberg et al. 2017), which imposes a heavy burden on the quality of women's lives. EC has become the gynecological carcinoma with the highest incidence in developed regions (Ferlay et al. 2015).

Providing an insight into changes in the endometrium under physiological and pathological conditions is vital to understand the mechanism of implantation and endometrial disorders. Prostaglandin E2 (PGE2), a bioactive lipid metabolite of arachidonic acid, is well described to be involved in both normal physiological functions such as menstrual cycle (Milne et al. 2001) and implantation (Huang et al. 2017) and human endometrial pathologies (Jabbour et al. 2006).

As PGE2 exerts its actions via receptors, namely EP1, EP2, EP3, and EP4 (Narumiya et al. 1999), the present study aimed to explore the underlying mechanisms that control the growth and function of endometrium by identifying the spatial–temporal expression of these receptors in healthy endometrium throughout the menstrual cycle and in pathological endometrium.

Materials and methods

Patients and tissue collection

Formalin-fixed paraffin-embedded samples including 42 healthy endometria (14 from proliferative phase, 14 from early secretory phase, and 14 from late secretory phase), 14 endometriotic ovarian tissues, and 140 endometrial cancer tissues were collected at the Department of Obstetrics and Gynecology of the Ludwig-Maximilians-University. Informed consent from all the participants was obtained before surgery. Healthy endometrium was obtained from the women undergoing dilatation and curettage or hysterectomy for benign diseases and receiving no hormone therapy for 3 months before surgery. Menstrual cycle was determined using hematoxylin and eosin staining. Endometriosis patients underwent surgery between 2000 and 2002 and were diagnosed and staged according to the Revised American Fertility Society classification. Endometrial cancer patients received surgery between 1990 and 2002. Stage and grading diagnoses were assessed according to the criteria of the International Federation of Gynecology and Obstetrics and the World Health Organization. Follow-up data were acquired from the Munich Cancer Registry.

Ethics approval

The study was performed conforming to the Declaration of Helsinki 1975 and approved by the Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany (approval number 063-13). All samples used were remaining materials and no longer needed for clinical diagnosis.

Immunohistochemistry

Immunohistochemical staining has been previously described by our lab (Hutter et al. 2016; Scholz et al. 2012). Briefly, tissue specimens were deparaffinized in xylol for 20 min, followed by 100% ethanol. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide diluted in methanol for 20 min before rehydrating the slides in a graded alcohol series (100, 70, 50%). Slides were heated with citric acid-buffer in a pressure cooker to retrieve epitope. Blocking and antibody staining procedures were then performed using either VECTASTAIN Elite ABC Kit (Vector, Burlingame, USA) or Zytochem-Plus HRP Polymer-kit (Zytomed, Berlin, Germany) according to the manufacturer's protocol. Detailed information on blocking and staining procedure and antibodies is listed in Table 1. 3,3′-Diaminobenzidine (Dako, Hamburg, Germany) was used for the peroxidase substrate staining reaction for the appropriate time as described in Table 1. Finally, slides were counterstained with hemalum, dehydrated, and covered.

Immunohistochemical staining always included positive controls and a negative control using placenta tissue. Negative control in this study was the substitution of preimmune serum (Goat/Rabbit Super Sensitive™ Negative Control, Hague, the Netherland) as the primary antibody. The immunohistochemical staining was analyzed using a semiquantitative immunoreactive score (IRS), which evaluates both staining intensity (1 = low, 2 = moderate, and 3 = strong) and proportion of stained cells (0 = no staining, 1 ≤ 10%, 2 = 11–50%, 3 = 51–80%, and 4 ≥ 81%) (Remmele and Stegner 1987).

Table 1 Antibodies used and blocking, staining procedures

Ab	Host/Clonality	Dilution	Blocking	Incubation	Reaction system	DAB staining (min)
EP1 (MyBioSource)	Goat/polyclonal	1:300	Blocking serum (20 min)	Overnight (4 °C)	Vectastain Elite Kit	3
EP2 (Abcam)	Rabbit/polyclonal	1:300	Reagant 1 (5 min)	Overnight (4 °C)	ZytoChem Plus HRP Polymer Kit	2.5
EP3 (Abcam)	Rabbit/polyclonal	1:300	Reagant 1 (5 min)	Overnight (4 °C)	ZytoChem Plus HRP Polymer Kit	2.5
EP4 (Novus)	Rabbit/polyclonal	1:50	Reagant 1 (5 min)	Overnight (4 °C)	ZytoChem Plus HRP Polymer Kit	2

Ab antibody, *DAB* 3,3′-Diaminobenzidine

Statistical analysis

The Mann–Whitney *U* or Kruskal–Wallis test was used to compare between or among groups. *P* value < 0.05 was considered statistically significant for all analyses. The data were analyzed using the Statistical Product and Service Solutions (SPSS, IBM, Armonk, NY, USA) version 23.0.

Results

Patients' characteristics

The median age of healthy women and endometriotic patients at surgery was 40.8 years (range 26.9–59.0 years) and 35.1 years (range 23.7–42.7), respectively. Among 14 patients with endometriosis, eight patients had stage I disease and three patients had stage II disease. The numbers of patients with stage III and IV were one and two, respectively. The median EC patient age at diagnosis was 65.7 years (range 37.0–87.1 years) and the median follow-up time was 82.7 months (range 1–175.9 months). Further characteristics of 140 EC patients including histology, stage of disease, and grading are listed in Table 2.

Table 2 Clinical characteristics of the patients with endometrial cancer

Characteristics	*N*	%
Death	36	25.7
Histology		
Endometroid	102	72.9
Serous	11	7.9
Mucinous	6	4.3
Mixed cell	19	13.6
Undifferentiated	2	1.4
Stage		
I	104	74.3
II	9	6.4
III	23	16.4
IV	4	2.9
Grading		
1	67	47.9
2	46	32.9
3	27	19.3
Lymph node involvement ($n = 135$)	16	11.9
Metastasis ($n = 128$)	11	8.6
Recurrence ($n = 128$)	18	14.1
Co-morbidities		
Type 1 diabetes ($n = 128$)	14	10.9
Type 2 diabetes ($n = 128$)	4	3.1
Hypertension ($n = 128$)	52	40.1
Obesity ($n = 128$)	42	32.8
ERα ($n = 128$)	57	44.5
ERβ ($n = 128$)	15	11.7
PRA ($n = 128$)	57	44.5
PRB ($n = 128$)	61	47.7

ER estrogen receptor, *PR* progesterone receptor

Receptors' staining intensity in healthy endometrium

EP1 staining in epithelium was significantly different within three phases of the menstrual cycle ($p = 0.01$) and remarkably higher during the proliferative phase (median 4) compared to the early secretory phase (median 1, $p = 0.006$). However, the difference in EP1 staining between the late secretory phase (median 4) and the other two phases did not reach statistical significance ($p > 0.05$) (Fig. 1).

Statistical significance in EP1 intensity was also observed in the stroma among different phase subtypes, entirely consistent with the trend demonstrated in the epithelium ($p = 0.025$). The intensity during the proliferative phase (median 2.5) was much higher than that during the early secretory phase (median 0, $p = 0.012$) (Fig. 1). There was no significant difference in EP1 intensity between the late secretory phase and the other two phases ($p > 0.05$).

Besides EP1, EP3 also showed a change in its staining intensity during the menstrual cycle (unpublished data), indicating these two receptors might be regulated by sex hormones or play a role in endometrial proliferation. In this study, we focused on EP1.

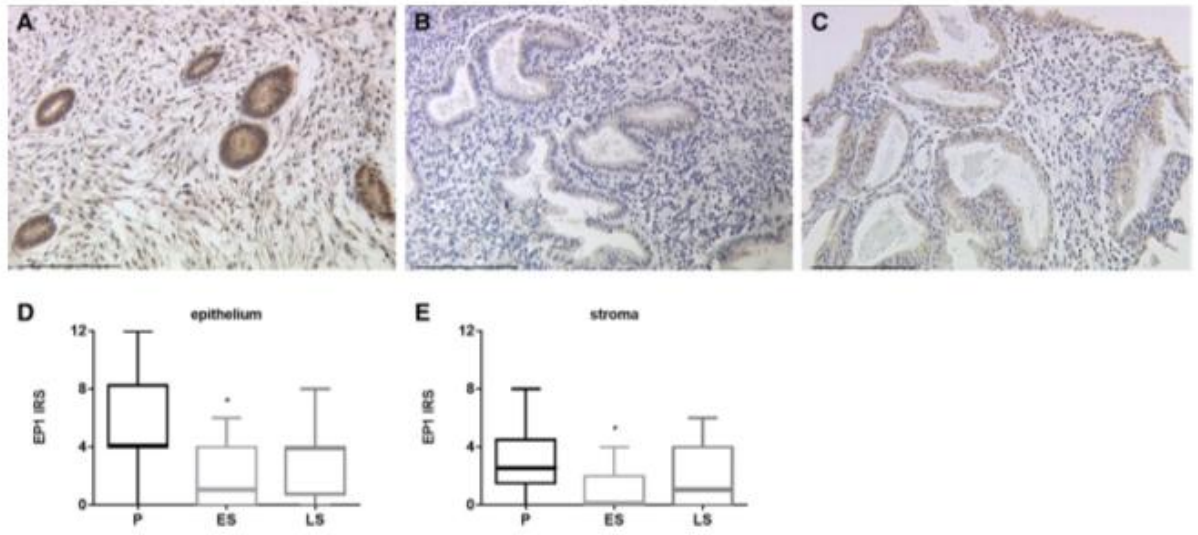

Fig. 1 EP1 was detected in the endometrium across the menstrual cycle by immunohistochemistry. Representative microphotographs of EP1 staining in the endometrium during proliferative (P), early secretory (ES), and late secretory (LS) phase are shown (**a**–**c**). EP1 expression was higher during proliferative phase compared to early secretory phase in both epithelium (**d**) and stroma (**e**). *Represents statistically significant differences compared to proliferative phase. Scale bars equal 200 μm

Comparison of EP1 staining intensity in healthy and diseased endometrium

EP1 immunoreactivity could be observed in the cytoplasm of both glandular epithelial cells and stromal cells of three kinds of endometria but not in the nucleus.

EP1 was differentially expressed among normal, endometriotic, and tumorous endometrium in both epithelium ($p = 0.001$) and stroma ($p < 0.001$). The intensity in the epithelium was increased in endometriotic tissue compared to healthy endometrium (median 4 vs. 4, $p = 0.042$) and tumor tissue (median 4 vs. 3, $p < 0.001$), while the latter two groups expressed similar EP1 (Fig. 2). In the stroma, the staining in the tumor was less abundant compared to both healthy tissue (median 0 vs. 1.5, $p < 0.001$) and endometriosis (median 0 vs. 2, $p < 0.001$), whereas there was no statistical difference between normal and endometriosis tissue (Fig. 2).

EP1 staining intensity in endometriosis

The highest intensity in epithelium was noted in stage IV (median 8). However, the number of other stages was too small to conduct statistical analyses.

EP1 staining intensity in endometrial cancer

There were no significant differences in EP1 staining either in epithelium or in stroma within histological, stage, grading, metastatic and recurrent subtypes ($p > 0.05$). Also, the immunoreactivity of EP1 was not significantly different comparing cases being co-morbidity-positive and co-morbidity-negative, although the intensity of patients with diabetes was higher than that without diabetes (median 4 vs. 3.5 in the epithelium, $p > 0.05$, median 0.5 vs. 0 in the stroma, $p > 0.05$).

Further, cases were analyzed according to groups based on hormone receptors expression, such as estrogen receptor (ER) α, ERβ, progesterone receptor (PR) A, and PRB. Interestingly, the EP1 staining intensity in epithelium varied by PRB status. The staining intensity in PRB-positive tumors was stronger compared to PRB-negative tumors (median 4 vs. 3, $p = 0.045$). However, there were no significant differences in the other hormone receptors status in either epithelium or stroma.

Survival analysis indicated that EP1 staining intensity in the tumor was associated with neither progression-free survival nor overall survival. However, it was worth noting that all patients with high EP1 staining intensity (IRS ≥ 6, $n = 6$) survived during the follow-up period.

Discussion

The data presented in this manuscript exhibit the intensity of EPs in the epithelium and the stroma of the human endometrium across the menstrual cycle. EP1, which has been confirmed to be strongly expressed in epithelial cells in

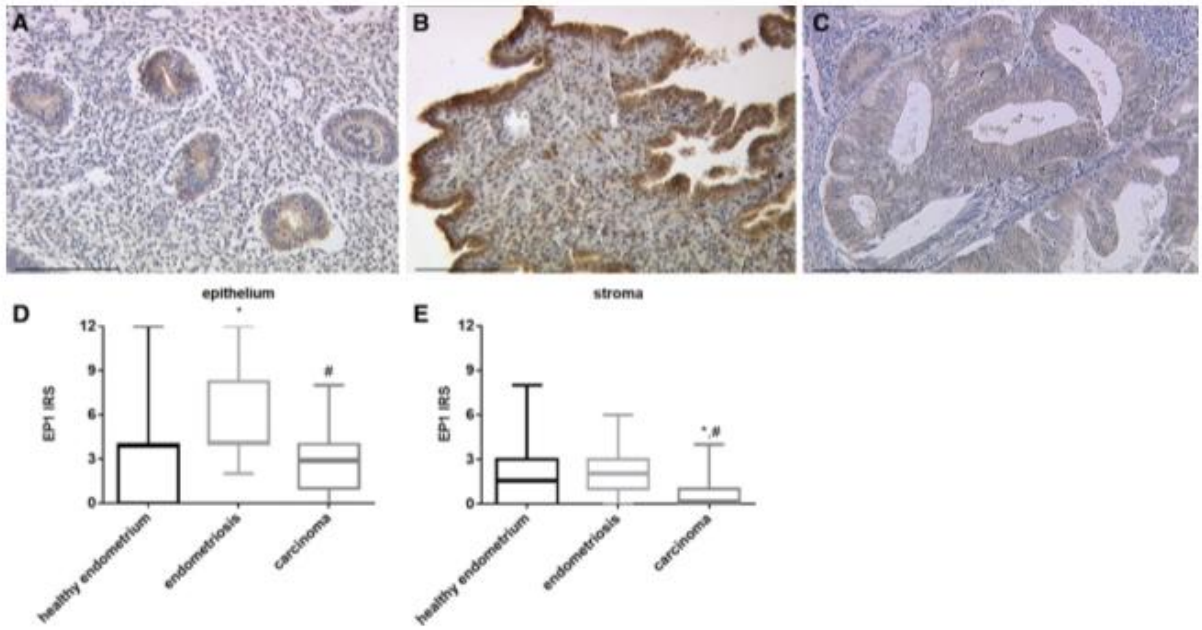

Fig. 2 EP1 was detected among healthy and diseased endometrium by immunohistochemistry. Representative microphotographs of EP1 staining in healthy endometrium (**a**), endometriosis (**b**), and endometrial cancer (**c**) are shown. EP1 expression in epithelium was upregulated in the endometriotic tissue compared to the healthy endometrium and tumor tissue (**d**). In the stroma, the staining in the tumor was downregulated compared to the normal tissue and endometriosis (**e**). * and # represent statistically significant differences compared to the healthy endometrium and endometriosis, respectively. Scale bars equal 200 µm

human endometrium (Carson et al. 2002), displayed cyclical changes with increased intensity during the proliferative phase. Also, EP1 staining intensity was increased in the epithelium of endometriosis compared to normal endometrium. There are only a few studies comparing EP1 expression in the healthy and diseased endometrium. All the published studies were based on real-time PCR (Catalano et al. 2011; Rakhila et al. 2015; Santulli et al. 2014). For the first time, we showed the staining intensity of EP1 in healthy and endometriotic endometrium by immunohistochemistry. One previous study showed the same trend of EP1 expression, which is almost 1.5-fold in proliferative endometrium compared to secretory tissue and twofold in endometriotic tissue compared to normal tissue during the secretory period, though the differences did not reach statistical significance (Rakhila et al. 2015). The other two showed unchanged EP1 mRNA between proliferative and early secretory phase or between normal and endometriotic endometrium (Catalano et al. 2011; Santulli et al. 2014). The inconsistent results might be explained by various methodologies, which detected different products and used different classification of the menstrual cycle.

During the proliferative phase, with the re-growth of the functional layer of the endometrium, angiogenesis is essential to maintain perfusion of new-born tissue (Girling and Rogers 2005). Activation of EP1 has been shown to increase endometrial PGE (PGE1 + PGE2) secretion (LaPorte et al. 2017), both of which are potent mediators of angiogenesis (Suzuki et al. 2013; Zhang and Daaka 2011). A previous study revealed that PGE1 signaling via EP1 is crucial to induce VEGF gene expression under non-hypoxic conditions (Suzuki et al. 2013). Moreover, T helper cells differentiate into two major subtypes of T cells, Th1 and Th2, which secrete different cytokines. The ratio of Th1 and Th2 dynamically changes across the menstrual cycle. The proliferative phase is characterized by higher Th1/Th2 ratio and the alteration of Th1/Th2 balance is involved in a variety of reproduction-related events, such as pregnancy and infertility (Chaouat et al. 2004; Faas et al. 2000). Researchers have demonstrated PGE2 acts through EP1 for the promotion of Th1 differentiation and Th1-mediated immune response in vivo (Nagamachi et al. 2007).

It has been well documented that the concentration of PGE2 was higher in peritoneal fluid of endometriotic patients and PGE2 is associated with the pathophysiology of this disease, e.g. abnormal immune response and upregulation of estrogen (De Leon et al. 1986; Sacco et al. 2012). Endometriosis is associated with chronic inflammation and an impaired immune response. A variety of inflammatory cytokines might contribute to creating a favorable milieu

for the development of endometriosis, especially interleukin (IL)-8, IL-6, and tumor necrosis factor (TNF)α (Lin et al. 2006; Sikora et al. 2017). EP1 signaling has been identified to induce IL-8 protein synthesis in human T lymphocytes via protein kinase C, Src family kinases, and Phosphatidylinositol 3-Kinase pathways and TNFα, IL-6 secretion in human microglia by using pharmacological and genetic approaches (Caristi et al. 2005; Li et al. 2011). Persistent estrogen stimulation is another major risk factor for endometriosis besides immune disorders (Kitawaki et al. 2002). PGE2 can induce estrogen production in ectopic endometrial cells via different regulatory pathways, one of which is to increase transcription of CYP19, the gene for aromatase cytochrome P450 (Sacco et al. 2012). It is reported that EP2 and EP4 mediate the action of peroxisome proliferator-activated receptor γ on estrogen biosynthesis (Lebovic et al. 2013). However, considering that PGE2 increases the activity of aromatase via EP1 in human stromal cells in breast cancer, we speculate that it might also work in stromal cells in endometriosis (Richards and Brueggemeier 2003). Several studies have shown that ovarian endometriosis accompanies with dysmenorrhea, non-menstrual pelvic pain, and dyspareunia (Gruppo Italiano per lo Studio 2001; Porpora et al. 1999). One report indicated that ovarian endometriosis is the only lesion significantly involved in severe dysmenorrhea and non-menstrual pelvic pain (Fedele et al. 1992). Intrathecal administration of EP1 antagonist and EP1 gene ablation suppress the sensitivity induced by inflammatory pain in vivo, suggesting that the signaling through EP1 mediates the algesia caused by inflammation to a great extent (Nakayama et al. 2002; Stock et al. 2001). Taken together, higher EP1 may contribute to the pathogenesis and symptoms associated with endometriosis.

Because of shared etiology and pathogenesis of both excessive estrogen and chronic inflammation, we did not expect the intensity of EP1 to be higher in endometriotic lesion and in stromal cells of normal tissue than that in endometrial cancer. It might be explained by diverse estradiol production pathways. Estrogen is formed in the majority of premenopausal endometriotic patients in ovaries and peripheral tissues. Aromatase is crucial for its synthesis, while most women with EC are postmenopausal and estradiol is formed by circulating estrone-sulfate under the action of steroid sulfatase instead of aromatase (Hevir-Kene and Rizner 2015; Secky et al. 2013). This is in line with a recent large prospective cohort identifying that endometriosis is not related to endometrial cancer risk (Poole et al. 2017). On the other hand, suppression of EP1 increases breast cancer metastasis in vivo and overall survival for patients with negative nuclear EP1 is significantly poorer (Ma et al. 2010). Nuclear EP1 staining also positively correlates with PR and inversely with lymph node involvement (Thorat et al. 2008). Our data suggest patients with the higher level of EP1 all survived during follow-up. Furthermore, EP1 intensity correlated with PRB, which is decreased sharply in EC and inhibits invasion and recurrence of EC. These findings are consistent with previous studies and suggest a protective role of EP1 in EC (Hanekamp et al. 2002; Pijnenborg et al. 2005; Sakaguchi et al. 2004). The result that EP1 intensity in the tumor was unable to predict prognosis might be explained by the small number of cases in our group with strong EP1 intensity.

In conclusion, we detected the distribution and intensity of EP1 in human endometrium by immunohistochemistry and compared the variation of EP1 in healthy and diseased endometrium crosswise. We propose EP1 might play a novel role in human endometrial physiology and pathology based on published data. Given that all the studies which evaluated the efficacy of EP1 in vivo were conducted in animal models and some results were obtained in other tissues such as breast, the effect of EP1 in endometrium should not be over-interpreted. Thus, further studies on the role of EP1 in human endometrium are needed.

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Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

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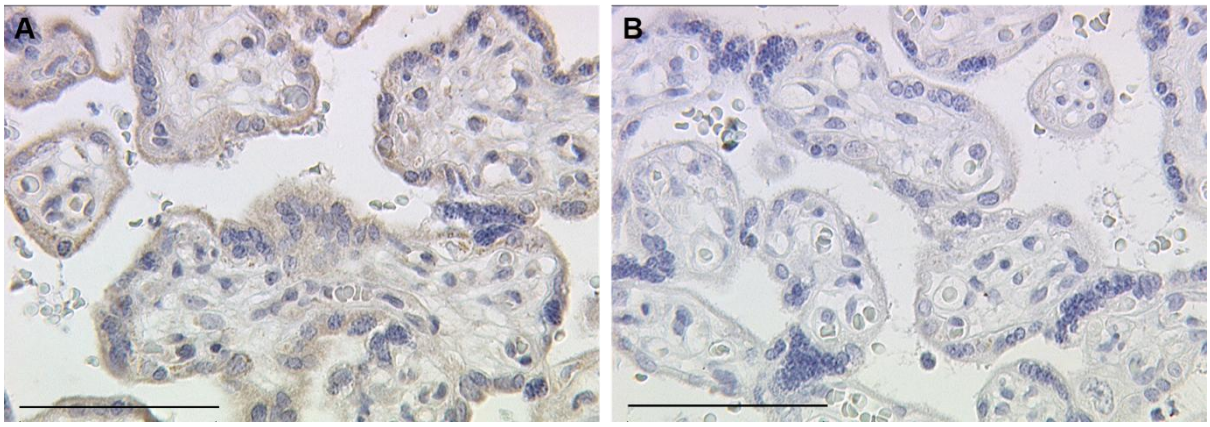

Supplementary material 1 Representative microphotographs of positive (A) and negative controls (B) for EP1 in placenta tissue are shown. Scale bars equal 100 μm

6 Publication II

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Research Paper

Prostaglandin receptor EP3 regulates cell proliferation and migration with impact on survival of endometrial cancer patients

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ABSTRACT

Background: Prostaglandin E2 (PGE2) receptor 3 (EP3) regulates tumor cell proliferation, migration, and invasion in numerous cancers. The role of EP3 as a prognostic biomarker in endometrial cancer remains unclear. The primary aim of this study was to analyze the prognostic significance of EP3 expression in endometrial cancer.

Methods: We analyzed the EP3 expression of 140 endometrial carcinoma patients by immunohistochemistry. RL95-2 endometrial cancer cell line was chosen from four endometrial cancer cell lines (RL95-2, Ishikawa, HEC-1-A, and HEC-1-B) according to EP3 expression level. Treated with PGE2 and EP3 antagonist, RL95-2 cells were investigated by MTT, BrdU, and wound healing assay for functional assessment of EP3.

Results: EP3 staining differed significantly according to WHO tumor grading in both whole cohort (p = 0.01) and the subgroup of endometrioid carcinoma (p = 0.01). Patients with high EP3 expression in their respective tumors had impaired progression-free survival as well as overall survival in both cohorts above. EP3 expression in the overall cohort was identified as an independent prognostic marker for progression-free survival (HR 1.014, 95%CI 1.003-1.024, p = 0.01) when adjusted for age, stage, grading, and recurrence. Treatment with EP3 antagonists induced upregulation of estrogen receptor β and decreased activity of Ras and led to attenuated proliferation and migration of RL95-2 cells.

Conclusions: EP3 seems to play a crucial role in endometrial cancer progression. In the context of limited systemic treatment options for endometrial cancer, this explorative analysis identifies EP3 as a potential target for diagnostic workup and therapy.

INTRODUCTION

With about 320,000 new cancer cases in 2012, endometrial cancer (EC) becomes the fifth most common tumor, following breast, colorectum, cervix uteri, and lung cancer. It represents 4.8% of cancer in women worldwide and is the most frequent gynecological carcinoma in developed regions [1]. Moreover, the incidence rate in

USA is expected to increase from 19.1 per 10,000 in 2012 to 42.13 per 10,000 in 2030 [1, 2].

Obesity, nulliparity, late menopause, diabetes, and use of tamoxifen are the best-known risk factors of EC, which can be summarized into unopposed endogenous and exogenous estrogen [3]. Several prospective studies focusing on postmenopausal EC patients and healthy control women have demonstrated a notable positive correlation between circulation estradiol level and EC [4, 5]. Estrogen receptors (ER), mediating the effect of estrogen, play a key role in differentiation and invasion of EC [6].

In numerous cancers, chronic inflammation has been linked to tumor progression and was recently demonstrated for EC as well [7]. Risk reductions of EC have been associated with a high-frequency use of aspirin, a non-steroidal anti-inflammatory drug (NSAID), decreasing prostaglandin (PG) synthesis via inhibiting the activity of cyclooxygenases (COXs) [8], especially in obese women according to the latest meta-analysis [9]. COX2 mRNA, protein expression and prostaglandin E2 (PGE2) synthesis are notably elevated in EC compared to healthy endometrium [10, 11]. Moreover, PGE2 has been shown to promote proliferation and invasion in EC [12]. PGE2 exerts its biological actions via binding to its seven-transmembrane, G-protein coupled receptors (GPCRs), termed EP1, EP2, EP3, and EP4 [13]. EP3 is reported to regulate the cancerogenesis and progression in various cancer cells, such as human prostate [14], breast [15], liver [16], colon [17], oral cancer cells [18]. Although the uterus is one of the organs with most abundant EP3 [19], only little is known about the contribution of EP3 in EC so far [12].

The present study aimed to examine the EP3 expression in tissue samples of EC patients and its association with clinicopathologic characteristics and survival. Also, we tried to find the mechanism of EP3's effect on EC using human EC cells and establish the rationale of PGE2's tumor-promoting action in EC.

RESULTS

Patients characteristics

Detailed medical records of 140 EC patients including age, stage of disease, histology, and grading are listed in Table 1. The median follow-up was 82.71 months and during the follow-up period, 18 (12.9%) patients recurred and 36 (25.7%) died.

EP3 expression in EC and correlation with clinicopathological characteristics

EP3 staining showed significant difference within the World Health Organization (WHO) grading in the overall cohort ($p = 0.011$) (Figure 1A-1D) as well as in the endometrioid adenocarcinoma subgroup ($p = 0.013$) (Figure 1E). In the overall cohort, the highest expression was in G3 (median = 30%), while the lowest expression was in G1 (median = 5%, $p = 0.013$). G2 staining showed no statistical differences compared to either G1 or G3 staining. The expression in endometrioid adenocarcinoma group followed the same trend. G1 staining (median = 5%) was much weaker than G2 (median = 15%, $p = 0.041$) and G3 staining (median = 65%, $p = 0.013$) and no differences were found between G2 and G3 group. EP3 expression among the different histological subtypes exhibited decreasing density for from undifferentiated (median = 45.5%), over mucinous cancer (median = 45%), serous carcinoma (median = 30%), mixed cell (median = 10%), and endometrioid histology (median = 7.5%), although the differences were not significant (Figure 1F-1I).

Besides that, no significant differences in EP3 expression were noted between different International Federation of Gynecology and Obstetrics (FIGO) stages. The expression of EP3 comparing cases being negative vs. positive for lymph node involvement or relapse was also not significantly different.

Prognostic significance of EP3 in EC

A cut-off value of 72.5 was identified by using Receiver Operating Characteristic (ROC) curve. The staining percentage below 72.5 was defined as the low EP3 expression in 111 EC tissues (79.3%), while the one above 72.5 as the high EP3 expression was identified in 29 EC tissues (20.7%). Survival analysis was performed in the whole cohort as well as in specific subgroups such as FIGO I, endometrioid cancers, and FIGO I endometrioid cancers groups. Kaplan-Meier analysis indicated that patients with high expression in tumor had impaired progression-free survival (PFS) and overall survival (OS) in the overall cohort (10-year-PFS rate, 62.1% vs. 74.7%, p=0.046, Figure 2A, 10-year-OS rate, 64.7% vs. 78.1%, p=0.022, Figure 2B) as well as in FIGO I endometrioid cancer group (10-year-PFS rate, 70.2% vs. 81.8%, p=0.047, Figure 2C; 10-year-OS rate, 74.0% vs. 83.9%, p=0.041, Figure 2D). Neither OS nor PFS in other subgroups showed significant differences, which is most likely related to the limited number of cases and a low number of events. In order to evaluate whether EP3 immunostaining is an independent prognostic factor, multivariate analyses were conducted. The biomarker and clinicopathological variables which have a great impact on the regression coefficient of EP3 were involved in the analysis, including age, stage, grading, and recurrence. After adjusting for these factors, the expression of EP3 in the overall cohort was showed to be an independent prognostic marker for PFS (HR 1.014, 95%CI 1.003-1.024, $p = 0.01$) (Table 2) but not for OS (HR 1.008, 95%CI 0.998-1.019, $p = 0.122$). C-index for PFS was 0.855, indicating that the Cox model was capable of predicting the prognosis accurately [20, 21].

Table 1: Clinical characteristics of included patients (n=140)

Clinical characteristics	All patients (n=140) No. (%)
Age (Median) [years]	65.7
Follow up (Median) [months]	82.7
Histology	
Endometrioid	102 (72.9)
Serous	11 (7.9)
Mucinous	6 (4.3)
Mixed cell	19 (13.6)
Undifferentiated	2 (1.4)
FIGO stage	
I	104 (74.3)
II	9 (6.4)
III	23 (16.4)
IV	4 (2.9)
WHO grading	
1	67 (47.9)
2	46 (32.9)
3	27 (19.3)
Lymph node involvement	
No	119 (85.0)
Yes	16 (11.4)
Unknown	5 (3.6)
Metastasis at first dignosis	
No	117 (83.6)
Yes	11 (7.9)
Unknown	12 (8.6)
Recurrence	
No	110 (78.6)
Yes	18 (12.9)
Unknown	12 (8.6)

FIGO: International Federation of Gynecology and Obstetrics; WHO: World Health Organization.

Correlations with other EC-related proteins

We performed a correlation analysis to evaluate the association of EP3 with proteins that are related to EC. The staining of EP3 showed no statistically significant correlation with ERα (correlation coefficient r = -0.15; p = 0.091), progesterone receptor A (PRA) (correlation coefficient r = -0.095; p = 0.286), PRB (correlation coefficient r = -0.023; p = 0.793), and glycodelin A (GdA) (correlation coefficient r = 0.153; p = 0.88). However, we found a significant negative correlation between EP3 and ERβ (correlation coefficient r = -0.225; p = 0.011) (Figure 3).

PGE2 enhances EP3 expression in EC cells

Available EC cell lines were analyzed for EP3 expression, identifying RL95-2 and HEC-1-A with

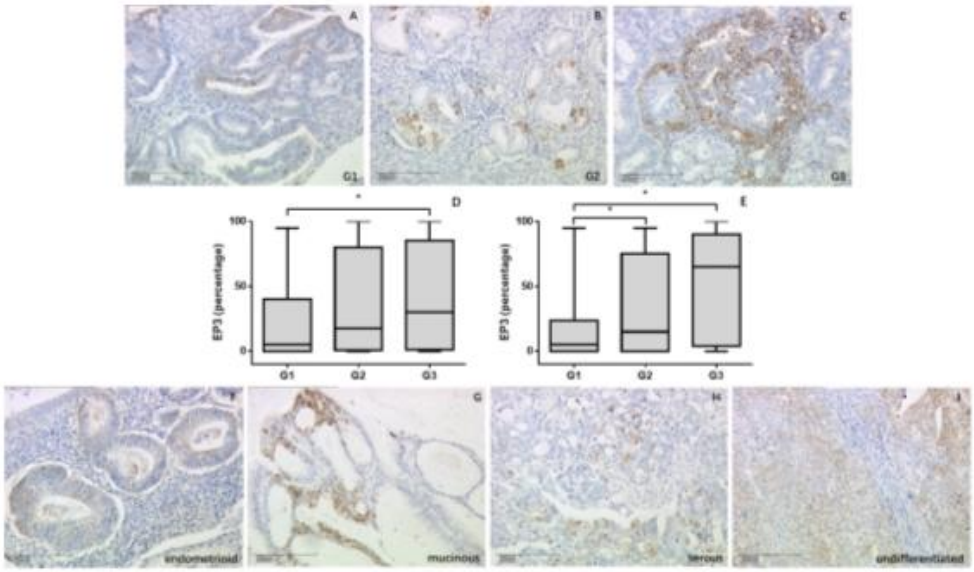

Figure 1: EP3 expression in EC tumor samples detected by immunohistochemistry. **(A-C)** Representative microphotographs of EP3 staining in different grading of EC tissue. **(D, E)** EP3 expression is associated with grading with weakest staining in G1 tumors in overall cohort and endometrioid tumor subgroup. $^{*}p < 0.05$. **(F-I)** Representative microphotographs of EP3 staining in different histological subtypes of EC tissue. Scale bars equal 200 μm.

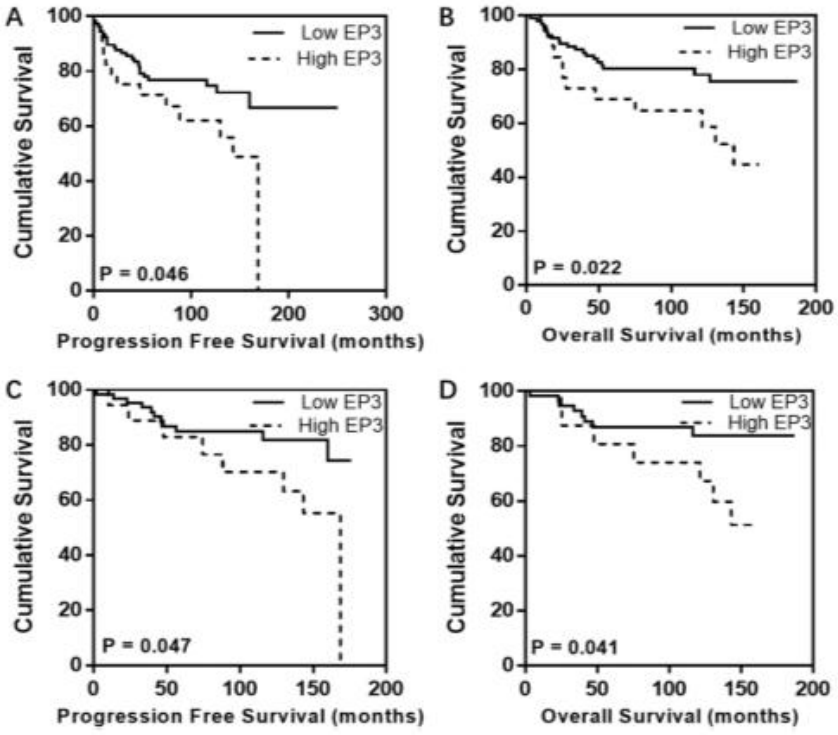

Figure 2: Kaplan-Meier analysis for EP3 in EC patients. Individuals with high EP3 expression exhibit impaired PFS and OS in both overall cohort **(A, B)** and FIGO I endometrioid cancer group **(C, D)** compared to those with low EP3 expression.

Table 2: Multivariate Cox regression analysis of all included patients regarding PFS (n=140)

Variable	Coefficient	HR (95%CI)	P Value
Age, y	0.038	1.039 (1.003-1.076)	**0.036**
FIGO stage (I vs. ≥ II)	1.038	2.824 (1.261-6.324)	**0.012**
WHO grading			0.426
1 vs. 2	-0.284	0.753 (0.309-1.835)	0.533
1 vs. 3	-0.604	0.546 (0.221-1.353)	0.192
EP3	0.013	1.014 (1.003-1.024)	**0.010**
Recurrence	2.957	19.240 (7.710-48.013)	**<0.001**

Significant results are shown in bold; HR: Hazard Ratio; CI: confidence interval.

high and moderate levels of EP3 protein, respectively, compared to Ishikawa and HEC-1-B with low and undetectable EP3 expression levels (Supplementary Figure 2). According to these results, we chose RL95-2 cell line as the model for our functional EP3 study. To confirm the effect of PGE2 on EP3, we examined both mRNA and protein levels of EP3 in RL95-2 cells after exposure to PGE2. Both EP3 mRNA and protein were noted to be increased following PGE2 treatment (Figure 4).

Proliferation of EC cells is inhibited by EP3 antagonist

After 48 hours of treatment with 10, 100, 1000 nM PGE2, the EP3 antagonist L-798,106 or the vehicle control (DMSO, 0.1%), MTT assay was used to assess viability. L-798,106 significantly decreased viability in a dose-dependent manner compared to control group, consistent with a pro-proliferative effect of EP3 (Figure 5A). Given that MTT assay is designed to measure the number of metabolically active cells, we conducted a BrdU assay, which assesses the proliferative cells by quantifying the BrdU incorporated into DNA during the S-phase [22]. Aligned with MTT, BrdU also indicated that EP3 antagonist inhibited the cells proliferation (Figure 5B). Contrarily, neither MTT nor BrdU showed a changed proliferation of RL95-2 following PGE2 exposure (Figure 5).

Migration of EC cells is inhibited by EP3 antagonist

To identify whether EP3 could facilitate metastasis of endometrial cells, we performed wound healing (scratch) assay. The results showed that after treatment of EP3 antagonist, the migration ability of RL95-2 cells was significantly suppressed compared to that in the vehicle group (Figure 6).

Inhibited EP3 increases ERβ expression and decreases Ras activity in EC cells

As immunohistochemistry showed a negative association of EP3 with ERβ, we further investigated whether EP3 acts upstream of ERβ. Both ERβ mRNA and protein were upregulated by L-798,106 treatment (Figure 7A, 7B). A previous study demonstrating that ERβ acts as a inhibitory signaling molecule upstream of Ras, prompted us to examine the activity of Ras in response to L-798,106 treatment [23]. As shown in Figure 7C, EP3 antagonist significantly decreased the activity of Ras in a time-dependent manner.

EP3 does not change the estradiol biosynthesis

Since EP3 can change the expression of ERβ, we speculated it might also have some effect on estradiol biosynthesis. According to a previous report, EC cell lines can form estradiol from estrone [24]. To verify our hypothesis, we estimated the estradiol concentration of EC cells exposed to L-798,106 together with 10% FBS, which contains estrone. Given that the conversion can be observed after 24 h incubation [24], we set the incubation time point as 24 h. However, EP3 antagonist did not affect the formation of estradiol (Supplementary Figure 3).

DISCUSSION

In this study of 140 EC tumor samples, we confirmed that EP3 is expressed in EC tissues and showed for the first time that EP3 expression in glandular epithelial cells correlates with tumor grade and is associated with impaired prognosis regarding PFS and OS. For EC, the results of our explorative analysis could show that EP3 might serve as a novel diagnostic and therapeutic target strongly deserving further investigation.

Furthermore, we demonstrated that EP3 might also play a role in regulating endometrial tumor growth.

In vitro, we could show that EP3 antagonist may attenuate proliferation and migration of EC cells, which supports the possible significance of EP3 in oncogenesis as previously reported. EP3 knockout mice have shown to markedly reduce tumor growth and tumor-associated angiogenesis [25]. By activating downstream signaling pathways, such as protein kinase A (PKA) [16], extracellular signal-regulated kinases 1 and 2 (ERK1/2) [26] and phosphoinositide 3-kinase (PI3K)/Protein Kinase B (AKT)/Glycogen synthase kinase 3 beta (GSK-3β) pathway [27], EP3 enhances the viability [16], proliferation [28], and invasiveness [15] of various cancer

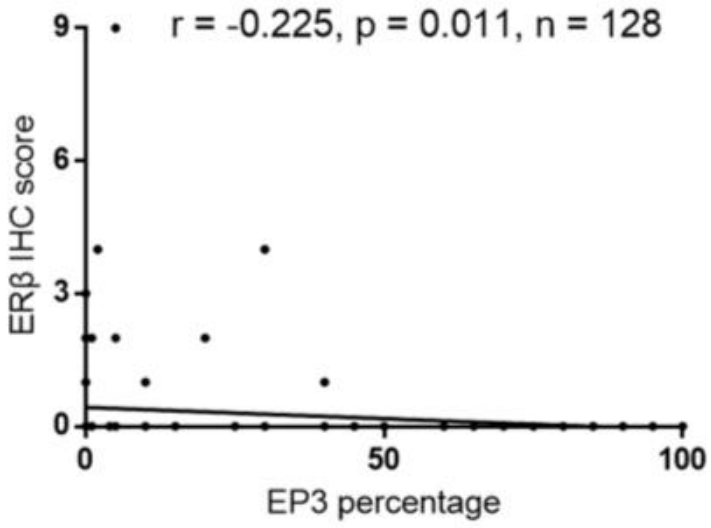

Figure 3: Correlation analysis of EP3 and ERβ in EC tissue (n=128).

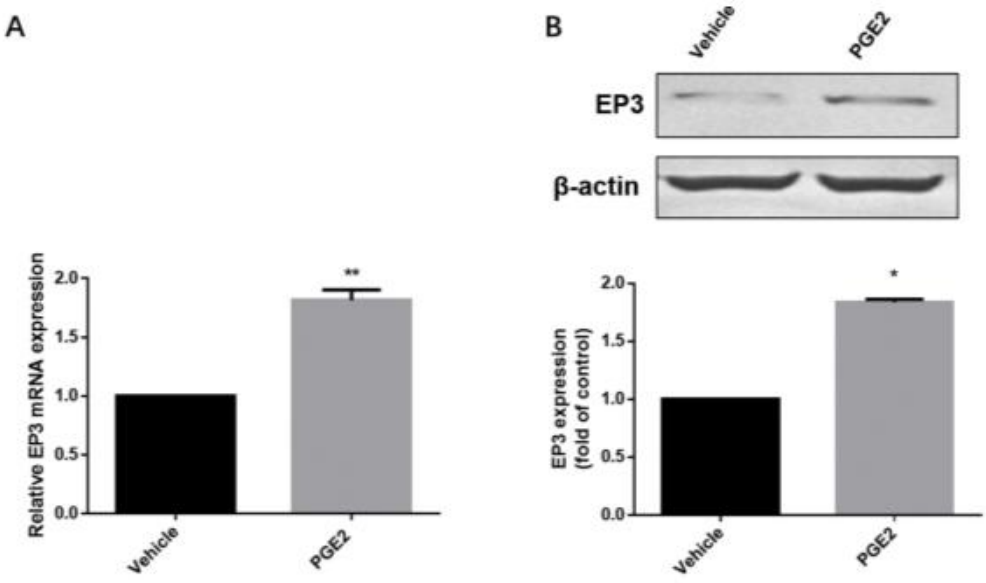

Figure 4: The effect of PGE2 on EP3 in RL-952 cells. (A) Cells treated either with vehicle (0.1% (v/v) DMSO) or 1μM PGE2 for 4h and subjected to RT-PCR. Bar graph represents mean ± SEM (n = 6). $^{**}p < 0.001$ (compared to vehicle treated control group). **(B)** Cells treated either by vehicle (0.1% (v/v) DMSO) or 1μM PGE2 for 12h and subjected to western blotting. Histogram represents the ratio of EP3 to β-actin as assessed with pooled densitometric data. Data was normalized to the expression of vehicle treated group. β-actin was used as loading control. Bar graph represents mean ± SEM (n = 3). $^{*}p < 0.01$ (compared to vehicle treated control group). For gel source data, see Supplementary Figure 4.

cells. Besides the direct influence on cancer cells, EP3 also promotes the tumor metastasis and angiogenesis by upregulating the matrix metalloproteinases (MMP)-9 of endothelial cells [29], which is an essential component of stroma and constitutes the tumor microenvironment. All these studies support our finding regarding the anti-cancer function of EP3 antagonist in EC cells.

In contrast to a previous report [12], our study did not confirm an effect of PGE2 on EC cells, even though PGE2 was proved to upregulate the expression of EP3 in our study. This might underline the fact that aspirin instead of non-aspirin NSAIDs reduces the risk of EC [9]. Aspirin has been reported to regulate the growth of prostate cancer cells in a PGE2-independent way [14]. There might also be a PGE2-independent pathway in EC cells. Alternatively, the nonspecific binding of PGE2 to other receptors, such as EP2 and EP4, which have been known to increase cyclic adenosine monophosphate (cAMP) via Gs protein [13], might counteract the effect of EP3. This is the main reason we chose L-798,106, a highly selective antagonist of EP3 for our study to avoid interference caused by nonspecific binding. The same study showed EP3 did not influence proliferation of Ishikawa cell line [12]. These discrepancies about EP3 and PGE2 might be due to the usage of different cell lines as an experimental model. The Ishikawa cell line, used by the other group,

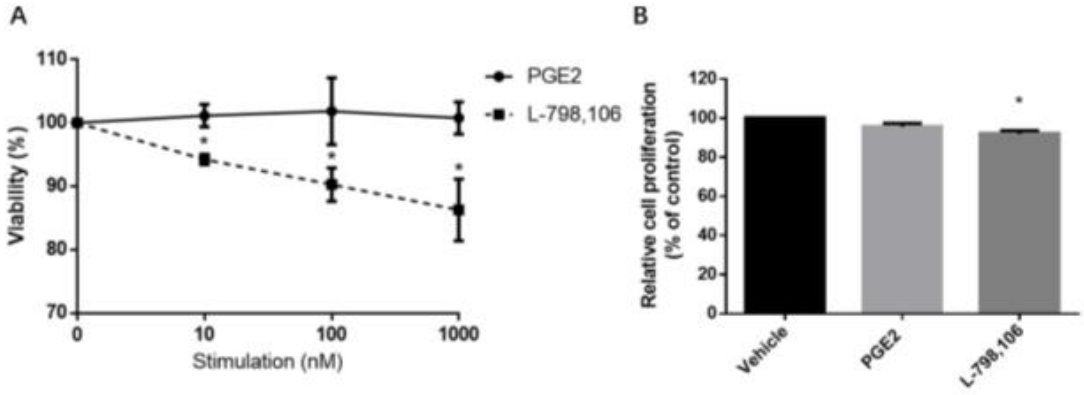

Figure 5: EP3 antagonist but not PGE2 suppresses proliferation of RL95-2 cells. (A) Cells cultured with indicated concentrations of PGE2 or L-798,106 for 48 h. The viability was determined by MTT assay. Results are normalized to cell viability of control group (0.1% (v/v) DMSO). Bar graphs represent mean ± SEM (n = 3). $^{*}p < 0.05$ (compared to vehicle treated control group). **(B)** Cells treated with vehicle (0.1% (v/v) DMSO), 1μM PGE2 or 1μM L-798,106 for 48 h. Proliferation was determined by BrdU assay. Results were normalized to cell proliferation of control group. Bar graphs represent mean ± SEM (n = 6). $^{*}p < 0.01$ (compared to vehicle treated control group).

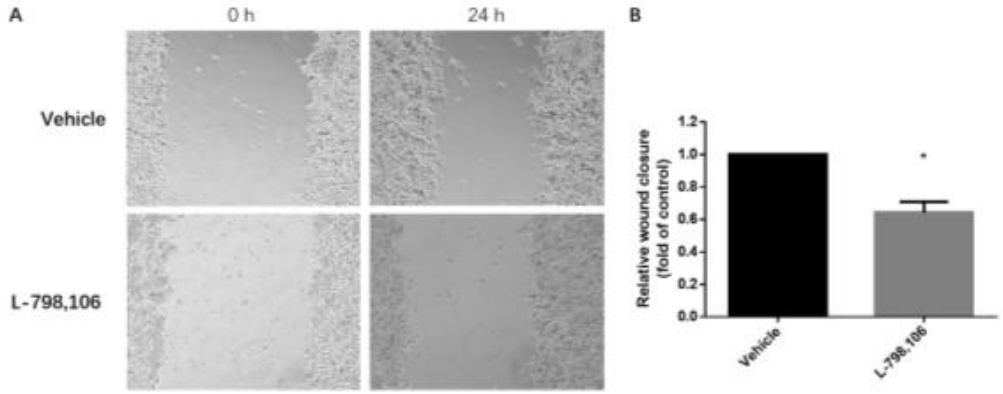

Figure 6: EP3 antagonist inhibits migration ability of RL95-2 cells. (A) Representative images show cell migration into the wounded area in vehicle treated group and 1 μM L-798,106 treated group. **(B)** Histogram compares migration in vehicle treated group and 1 μM L-798,106 treated group. Results were normalized to cell proliferation of control group. Bar graph represents mean ± SEM (n = 4). $^{*}p < 0.05$ (compared to vehicle treated control group).

barely expresses EP3 according to our data and thus could be less sensitive to EP3 pharmacological stimulation.

For the first time, we could show that EP3 was negatively associated with ERβ in EC tissue. Pharmacological research provides more information to verify that EP3 regulates ERβ, although the mechanism is far from clear. L-798,106 increased ERβ expression in RL95-2 cells. ERβ belongs to nuclear receptor superfamily and is clarified to be a modulator of ERα by functioning oppositely [30] and by repressing ERα transcriptional activity [31]. Although the effect of ERβ in EC has not been fully elucidated, accumulating evidence reveal that ERβ has a protective effect on the endometrium [32–34] and promotes differentiation and inhibits proliferation as well as invasion of endometrium [30, 35, 36]. In addition, ERβ is also shown to inhibit the migration of cancer cells [37].

Ras, a GTPase, transduces different downstream signalings by interacting with various Ras-effectors, such as Raf kinase, PI3K, and Ral guanine nucleotide exchange factors (Ral-GEFs) [38] and plays an important role in multistep carcinogenesis in EC [39, 40]. The activation of Ras can be induced by either mutant Ras or alteration of Ras protein expression. Here, we confirmed that EP3 antagonist decreased active Ras. Since ERβ can modulate Ras signaling [23], EP3 might regulate phenotypes of RL95-2 cells via ERβ/Ras.

As EP3 does not affect estradiol biosynthesis, the regulation of ERβ apparently was not induced by estradiol. The main effect of EP3 is to bind to the Gi protein and inhibit cAMP production [41], which is documented to increase the ERβ protein expression rather than ERα protein expression and promote ERβ transcriptional activation [42]. Therefore, EP3 might negatively regulate ERβ by adjusting the cAMP concentration.

Also, an inhibitory effect of EP3 on tumor development has been observed. In prostate and colon cancer, EP3 mRNA was remarkably reduced compared to corresponding normal tissue and EP3 impaired growth ability of these two cancer cell lines [14, 17]. Furthermore, EP3 was reported to inhibit the hormone-dependent growth of breast cancer by reducing aromatase activity of adipose stromal cells [43]. Among all the prostaglandin receptors, EP3 is the most complicated receptor because of its various isoforms. The human EP3 gene, consisting of ten exons and generating nine mRNAs, encodes at least eight distinct EP3 splice variants, which only differ at C-terminal tails [44]. The specific C-terminal tails produced by alternative splicing bind to different G proteins and activate different second messengers, which consequently determines the diverse physiological activity of EP3 receptor [19, 28, 41, 44–48]. This might partly explain these cell and tissue type specific phenomena. Further research on the specific isoforms expressed in EC and their effects should be performed.

Over the past years, molecular cancer biology has been integrated into the clinical routine of different tumor entities (e.g., breast cancer [49]). In this context, the Cancer Genome Atlas Research Network proposed a new classification of EC and categorized EC as POLE

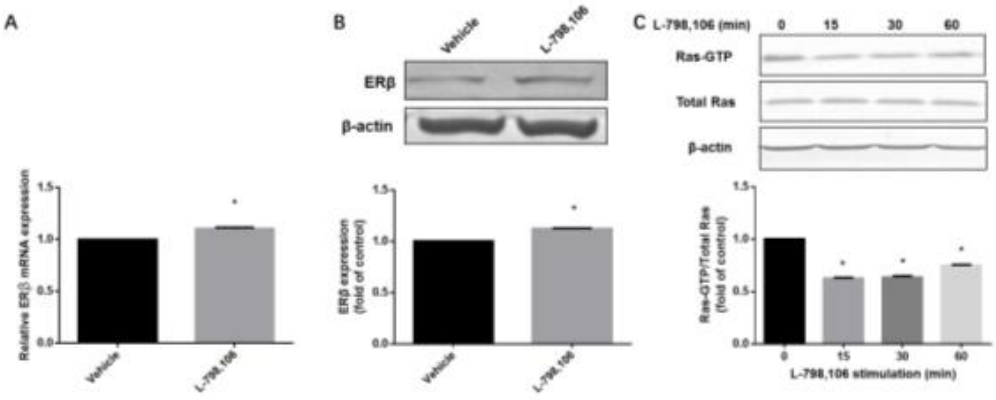

Figure 7: EP3 antagonist increases expression of ERβ and decreases activity of Ras in RL95-2 cells. **(A)** Cells treated either by vehicle (0.1% (v/v) DMSO) or 1μM L-798,106 for 4 h and subjected to RT-PCR. Bar graph represents mean ± SEM (n = 4). *p < 0.001 (compared to vehicle treated group). **(B)** Cells treated either by vehicle (0.1% (v/v) DMSO) or 1μM L-798,106 for 12 h and subjected to western blotting. Histogram illustrates the ratio of ERβ to β-actin as assessed with pooled densitometric data. Data was normalized to the expression of vehicle treated group. β-actin was used as loading control. Bar graph represents mean ± SEM (n = 3). *p < 0.001 (compared to vehicle treated group). **(C)** Cells incubated with 1μM L-798,106 for indicated time and subjected to western blotting. Data was normalized to the expression at 0 min. β-actin was used as loading control. Bar graph represents mean ± SEM (n = 3). *p < 0.001 (compared to 0 min group). For gel source data, see Supplementary Figure 4.

ultramutated, microsatellite instability hypermutated, copy-number low, and copy-number high [50]. The four TGCA subtypes are related to different clinical outcomes, among which the copy-number high subgroup has impaired PFS, while the POLE ultramutated subgroup has improved PFS [50]. Mutations of RPL22 are almost exclusively identified in the microsatellite instability (MSI) group [50]. Direct sequencing of RPL22 exons 2 and 4 in 226 MSI endometrial tumors confirmed 51.6% tumors were heterozygous for the 43delA mutation, which was also presented in RL95-2 cell line [51]. Consequently, we speculate that this cell line could represent the MSI group, the survival of which is centered. Although not yet fully implemented in clinical routine, this classification gains more prognostic significance so that our results on EP3 will need to be confirmed in this context in future investigations.

In conclusion, we demonstrate for the first time that EP3 expression in glandular epithelial cells is associated with advanced WHO grading and poor patients' prognosis. Inhibited EP3 mediates an anti-cancer effect in EC cells, which can be utilized for therapeutic interventions. As the results are partially contradictory to previous studies in other cell lines and tumor entities, our study indicates that EP3 seems to act in a cell and tissue type-specific manner. For EC, we could show that EP3 might serve as a novel diagnostic and therapeutic target strongly deserving further investigation.

MATERIALS AND METHODS

Patients

Formalin-fixed paraffin-embedded tissue of 140 patients, who received surgery for EC at the Department of Obstetrics and Gynecology of the Ludwig-Maximilians-University Munich between 1990 and 2002 was available. All patients provided informed consent before surgery. Staging and grading were assessed by two gynecological pathologists according to the criteria of FIGO and WHO. Follow-up data were obtained from the Munich Cancer Registry. PFS was defined as the time from operation to relapse or death from any cause, whereas OS was the time from diagnosis to the death or date of the last follow-up.

The study was performed according to the Declaration of Helsinki 1975. We used the remaining material of the tumor tissue after the initial histopathological diagnosis had been completed. The current study was approved by the Ethics Committee of the Ludwig Maximilians University, Munich, Germany (approval number 063-13). Authors were blinded for clinical information during experimental analysis.

Immunohistochemistry

Immunohistochemistry was performed as previously described by our lab [52]. Paraffin-embedded and formalin-fixed EC samples were incubation with the polyclonal rabbit IgG anti-EP3 antibody (Abcam), which was diluted at the ratio of 1:300, overnight. The signal was amplified with HRP-polymer (Zytochem-Plus HRP Polymer-kit, Zytomed, Berlin, Germany) for 30 min followed by incubating with diaminobenzidine (Dako, Hamburg, Germany) for 2.5 min. In the end, counterstaining with hematoxylin was carried out.

To support the validity of the EP3 staining, we used slides made from one normal colon tissue as the positive and negative control (Supplementary Figure 1). The negative control was performed by substituting for the primary antibody with a pre-immune serum (Rabbit Super Sensitive™ Negative Control, Hague, the Netherland).

The immunostaining of EP3 showed dots and an uneven distribution. The intensity of staining varied considerably within one slide. Therefore, the scoring was made according to the percentage of immunostained glands. The estimation of the percentage of EP3-positive glands was conducted by viewing the tumor area at 5x and 10x objectives and the results were recorded as an exact percentage. All slides were evaluated by two independent investigators. The staining and scoring with primary antibody ERα, ERβ, PRA, PRB, and GdA were performed as previously described by our research team [53, 54].

Cell culture and drugs

One well-differentiated cell line, Ishikawa and three moderate-differentiated cell lines, RL95-2, HEC-1-A, and HEC-1-B cells were purchased from European Collection of Cell Culture (ECACC, Salisbury, UK) and maintained in RMPI 1640 medium containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific) without antibiotic at 37°C in a humidified 5% CO_2 incubator. In each experiment, cells were seeded in wells overnight before being incubated with test substances or dimethyl sulfoxide (DMSO) unless otherwise indicated. PGE2, L-798,106, a highly selective EP3 antagonist, and EP3 agonist, sulprostone was purchased from Tocris Bioscience. The results of MTT and BrdU showed that the agonist we chose did not affect proliferation of EC cells. Although Abrahao et al. [55] and Fujino et al. [26] claimed sulprostone to be a selective EP3 agonist, it has been shown that sulprostone is an EP1/EP3 dual agonist [13, 48]. As other EP3 highly selective agonists such as ONA-AE-248 are currently not commercially available, we decided to concentrate on the antagonist L-798,106 to avoid interfering effects caused by non-specific binding.

RNA isolation and reverse transcription

Total RNA was obtained from cultured cells using a RNeasy Mini Kit (Qiagen, Hilden, Germany) and converted to cDNA with an MMLV Reverse Transcriptase First-Strand cDNA synthesis kit (epicentre, madison, WI, USA) as instructed by the manufacturer.

Quantitative real-time RT-PCR

20μL reaction mixture containing 1μL TaqMan® Gene Expression Assay 20 x (Applied Biosystems, target PTGER3, Nr. Hs00168755_m1, target ESR2, Nr. Hs01100353_m1, target ACTB, Nr. Hs99999903_m1), 10μL TaqMan® Fast Universal PCR Master Mix 2 x (Applied Biosystems), 1μL cDNA template and 8μL RNase-free water was prepared per probe on an Optical Fast 96-well plate (Applied Biosystems) and covered by an optical adhesive film. PCR assays were run by using Applied Biosystems 7500 Fast Real-time PCR system. Enzymes activation was performed at 95°C for 20 s on hold. Afterward, 40 cycles of qPCR denaturing at 95 °C for 3 s and annealing at 60°C for 30 s were run. The comparative CT method, also referred to as the ΔΔCT method was applied for the results. For ΔCT values calculation, β-actin was used as an endogenous control. The results are representative of at least three independent experiments.

Western blotting and Ras-GTP assay

The procedure and protocol of western blotting were previously described by our group [56]. Briefly, cell lysates were electrophoresed by SDS-PAGE and transferred to PVDF membranes. The membranes were incubated overnight with 1:2000 dilution of EP3 antibody (ab117998, Abcam), 1:200 dilution of ERβ antibody (53472, Anaspec) or 1:1000 dilution of β-actin antibody (A5441, Sigma). Afterwards, membranes were washed and incubated for 1 h with 1:1000 dilution of the corresponding alkaline phosphatase-conjugated secondary antibodies. Blotting was detected and visualized by BCIP/NBT Color Development Substrate (Promega). GTP-bound RAS was determined using the active Ras detection kit (8821, Cell Signaling) according to the manufacturer's instruction.

Images were analyzed by an image analyzer (Molecular Imager® Gel DocTM XR+, Bio-rad) using software Quantity One 4.6.7 (Bio-Rad, Munich, Germany). β-actin was used as an endogenous control (Supplementary Figure 4).

Cell viability assay

RL95-2 cells were seeded at the density of 1,5 × 10^4 cells/well in 96-well plates in sextuplicate. The next morning cells were incubated with different concentration of PGE2 or L-798,106 for 48 h. 5 mg/mL MTT [3-(4,5-dimethhylthiaoly)-2,5-diphenyltetrazolium bromide] (Sigma) in phosphate-buffered saline (PBS) was prepared. 20 μg MTT solution was added to each well for 1.5 h at 37°C. The culture medium along with MTT was then removed. 200 μL DMSO was added to each well to dissolve the visible formazan crystals, followed by mixed thoroughly on the shaker for 5 min at room temperature. The optical density (OD) was read at 595 nm using Elx800 universal Microplate Reader.

Cell proliferation assay

RL95-2 Cells were seeded in 96-well plates at 5 × 10^3 cells/well in sextuplicate. The next morning cells were incubated with different concentration of PGE2 or sulprostone for 48 h. 5-Bromo-2'-Deoxyuridine (BrdU) incorporation assay (11647229001, Roche) was used to determine the cell proliferation according to manufacturer's protocols. OD was quantified at 450 nm using Elx800 universal Microplate Reader.

Wound healing (scratch) assay

1.4 × 10^6/well RL95-2 cells were cultured in 24-well-plates overnight. The next morning the central fields of confluent monolayers were scratched with a 200 μl pipette tip to make artificial wound gaps. The detached cells were aspirated and rinsed once with PBS. 1 μM L-798,106 was added to treat cells. After 0 h, 24 h, cell migration was monitored by photographing using an inverse phase contrast microscope (Leica Dmi1, Leica, Wetzlar, Germany) with a camera (LEICA MC120 HD, Leica, Wetzlar, Germany). Photomicrographs of wounded areas covered by cells were analyzed by software Image J (http://rsb.info.nil.goc/ij). The cell migration area = area at 0 h – area at 24 h.

Estradiol measurements

3× 10^5/well RL95-2 cells were seeded in 24-well-plates. After treated by 1 μM L-798,106 for 24 hours, the supernatant was collected and centrifuged (13,200 g, 10 min) to remove cell debris. The estradiol concentration was subsequently determined using chemiluminescent immunometric assay (IMMULITE 2000 immunoassay system) (Siemens, Germany) as described by the manufacturer.

Statistical analysis

A Student's t-test (two-tailed) was used to analyze means of two groups. Mann-Whitney U or Kruskal-Wallis tests were conducted to compare non-parametric variables between or among groups. A Spearman rank test was performed for correlations between continuous variables. Survival times were compared using Kaplan-Meier (log-rank) test method. The ROC curve was

drawn to identify an appropriate cut-off. The ROC curve analysis is one of the most widespread methods used in cut-off point selection. The ROC curve is a plot, y-axis of which represents sensitivity and x-axis of which represents (1-specificity) [57]. Youdan index, defined as the maximum (sensitivity+specificity-1) [58], is applied to ensure the optimal cut-off which can maximize the sum of sensitivity and specificity [59, 60]. A cox-regression model for multivariate analyses was used. Harrell's c-index was performed to evaluate the accuracy of the Cox model using R 3.3.1 with Hmisc and rms packages (http://www.r-project.org). A p-value below 0.05 was considered statistically significant for all analyses. The data were analyzed using the Statistical Product for Social Science (SPSS, IBM, Armonk, NY, USA) version 23.0.

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CONFLICTS OF INTEREST

All authors declare no conflicts of interest with this study.

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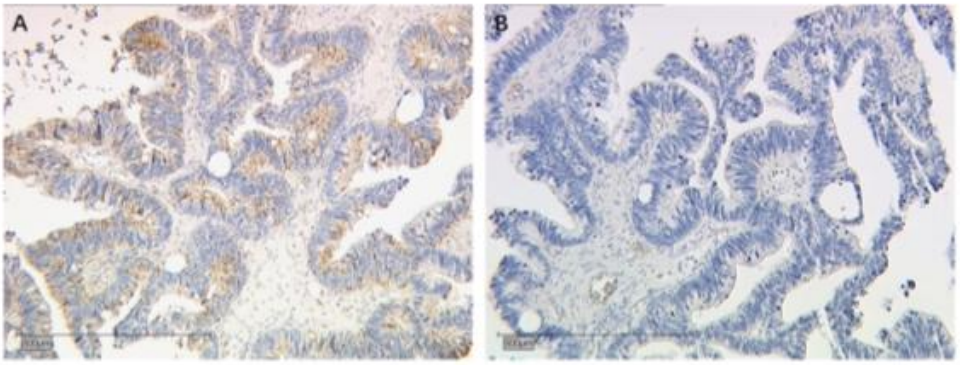
Prostaglandin receptor EP3 regulates cell proliferation and migration with impact on survival of endometrial cancer patients

SUPPLEMENTARY MATERIALS



Supplementary Figure 1: Representative microphotographs of positive and negative controls for EP3 in normal colon tissue. (A) Positive control of EP3 staining in normal colon tissue showed strong cytoplasm expression in more than 80% epithelial cells. **(B)** Negative control of EP3 staining in same normal colon tissue as positive control. Scale bars equal 500 μm.

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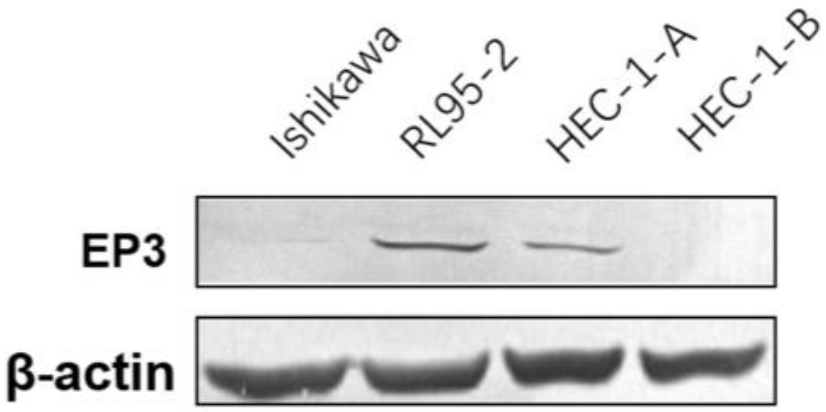

Supplementary Figure 2: EP3 protein expression in various EC cells. For gel source data, see Supplementary Figure 4.

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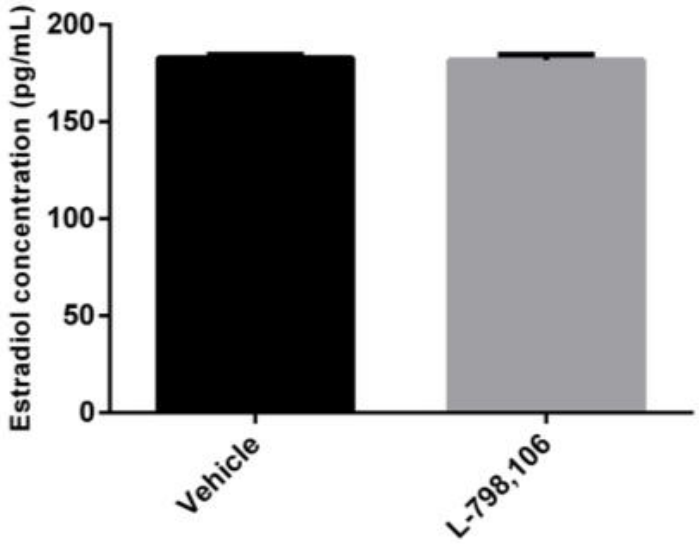

Supplementary Figure 3: EP3 does not change the formation of estradiol. RL95-2 cells incubated with vehicle (0.1% (v/v) DMSO) or 1 μM L-798,106 for 24 h. Levels of estradiol in the supernatant were estimated by chemi-luminescent immunometric assay. Bar graph represents mean ± SEM (n = 4).

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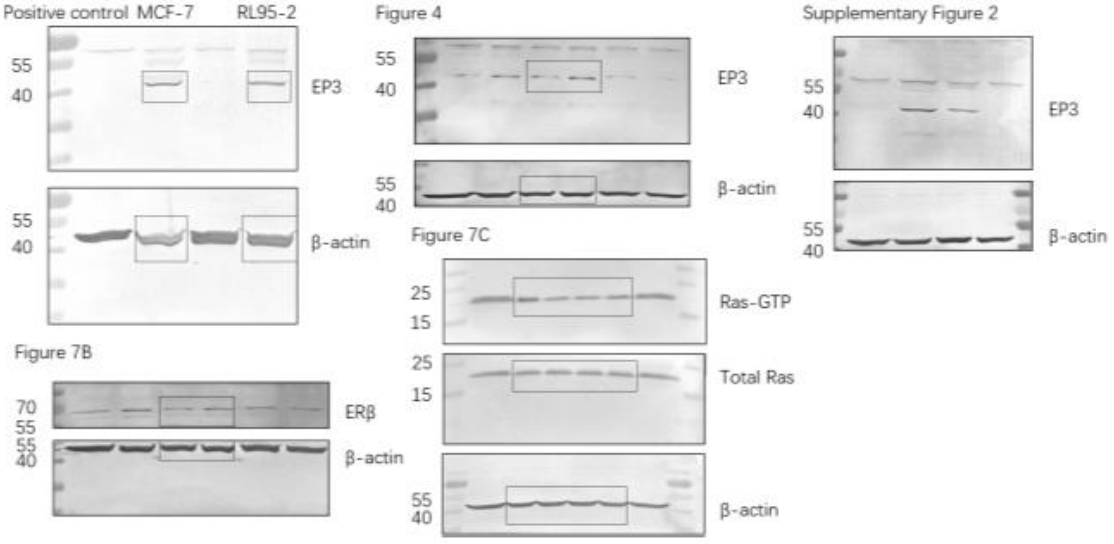

Supplementary Figure 4: Uncropped scans with marker indications.

7 Summary

In the absence of conception, human premenopausal endometrium undergoes a cyclic morphological change in response to fluctuating sex hormones and inflammatory mediators. PGE2 also changes according to the phases. Moreover, it is upregulated in both EC and endometriosis and well known to be involved in both physiological functions and human endometrial pathologies. Therefore, providing an insight into changes of its receptors in the endometrium under physiological and pathological conditions is vital to understand the mechanism of endometrial disorders. However, so far little is known about the expression patterns of them in health and disease.

We analyzed the expression patterns of EPs in human nonpregnant endometrium throughout the menstrual cycle by immunohistochemistry (n = 42) and found EPs were located in the cytoplasm of both epithelial cells and stromal cells but not present in the nucleus. Next, we found the expression of both EP1 and EP3 demonstrated cyclical changes by comparing the intensity of EPs in three phases of menstrual cycle using a semiquantitative immunoreactive score (IRS). EP1 expression decreases during the early secretory phase ($p = 0.006$), while EP3 expression behaves in the opposite way ($p < 0.05$). We thus focused our further analysis on these two receptor subtypes.

The expressions of EP1 were compared among healthy endometrium, ovarian endometriosis, and EC. There were significant differences among these tissues in both epithelium ($p = 0.001$) and stroma ($p < 0.001$). EP1 staining in the epithelium was increased in endometriotic tissue compared to the healthy endometrium ($p = 0.042$) and tumor tissue ($p < 0.001$), while in the stroma, the staining in the tumor was lower than that in both normal tissue ($p < 0.001$) and endometriosis ($p < 0.001$). However, no significant differences in EP1 intensity were detected among four endometriosis stages and among subtypes of histology, stage, grade, metastasis, and recurrence in EC. Consistently, EP1 was also correlated with neither progression-free survival nor overall survival of patients with cancer. Besides that, we made an interesting discovery that EP1 staining varied by PRB status. The staining in the PRB-positive tumor was stronger compared to the PRB-negative tumor.

EP3 has been revealed to regulate tumor cell proliferation, migration, and invasion in numerous cancers. However, the role of EP3 as a prognostic biomarker in EC remains unclear. Therefore, the primary aim of the further study was to analyze the prognostic significance of EP3 expression in EC. EP3 expression of 140 endometrial carcinoma patients was determined by immunohistochemistry. RL95-2 endometrial cancer cell line was chosen from four endometrial cancer cell lines (RL95-2, Ishikawa, HEC-1-A, and HEC-1-B) according to EP3 expression level. Treated with PGE2 and EP3 antagonist, RL95-2 cells were investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5-bromo-2′-deoxyuridine (BrdU) and wound healing assay for functional assessment of EP3. EP3 staining was statistically associated with tumor grade in both whole cohort ($p = 0.01$) and the subgroup of endometrioid carcinoma ($p = 0.01$). Patients with high EP3 expression in their respective tumors had impaired progression-free survival as well as overall survival in both cohorts above. EP3 expression in the overall cohort was identified as an independent prognostic marker for progression-free survival (HR 1.014, 95%CI 1.003-1.024, $p = 0.01$) when adjusted for age, stage, grade, and recurrence. Besides, a significant negative correlation between EP3 and ERβ (correlation coefficient $r = -0.225$; $p = 0.011$) was found. Treatment with EP3 antagonists induced the upregulation of ERβ and decreased the

activity of Ras and led to attenuated proliferation and migration of RL95-2 cells, while the treatment had no effect on E2 biosynthesis.

In conclusion, EP1 is upregulated during the proliferative phase, which is in accordance with its effect on inducing angiogenesis and promoting Th1 differentiation. Although the exact mechanism for EP1's action in endometriosis is unknown, we speculate it might be related to its role in proinflammation and production of estrogen according to previous studies. Furthermore, the expression of EP1 is positively correlated with PRB, which is decreased sharply in EC and inhibits invasion as well as recurrence of EC, suggesting a protective role of EP1 in EC. Regarding EP3, we confirm that its expression in glandular epithelial cells is associated with tumor grade and predicts poor prognosis of EC. EP3 plays a crucial role in EC progression by regulating proliferation and migration of tumor cells and modulating ERβ and the activity of Ras. In the context of limited systemic treatment options for endometrial cancer, this explorative analysis identifies EP3 as a potential target for diagnostic workup and therapy.

Taken together these studies, PGE2 not only participates in the physiology of the female reproductive system but also is involved in the benign and malignant endometrial diseases via its specific receptors. Thus, further studies are needed to connect the obtained knowledge of the molecular mechanisms of these receptors with a translation to the clinical practice under physiological and pathological conditions.

8 Zusammenfassung

Als Reaktion auf fluktuierende Sexualhormone und Entzündungsmediatoren unterliegt menschliches prämenopausales Endometrium ausserhalb einer Schwangerschaft zyklischen morphologischen Veränderungen. Auch die Expression von PGE2, das in physiologische sowie pathologische Prozesse des Endometriums involviert ist, unterliegt zyklischen Schwankungen. Zudem wird die Expression sowohl bei Endometriumskarzinomen, als auch in bei Endometriose hochreguliert. Daher scheint eine Untersuchung der Veränderungen der endometrialen Prostaglandinrezeptoren essentiell, um die Mechanismen endometrialer Störungen zu verstehen. Bislang jedoch ist nur wenig über das Expressionsverhalten dieser Rezeptoren unter physiologischen Bedingungen und bei Erkrankungen des Endometriums bekannt.

In der vorliegenden Arbeit haben wir retrospektiv die Expressionsmuster und -intensitäten von EPs in humanem endometrialem Gewebe (n=42) während des Menstruationszyklusses mittels Immunhistochemie untersucht. Die EP-Expression konnte im Zytoplasma sowohl von epithelialen Zellen, also auch von Stromazellen nachgewiesen warden, nicht aber in den Nuclei dieser Zellen. EP1 und EP3 zeigten zyklische Veränderungen in der Intensität ihrer Expression. Während sich die EP1-Expression in der frühen Sekretionsphase verminderte ($p=0.006$), verhielt sich die EP3-Expression gegenteilig ($p<0.05$).

In der Folge wurden die Unterschiede der EP1- und EP3- Expression bei gesundem endometrialem Gewebe, ovarieller Endometriose und Endometriumskarzinomen verglichen. Signifikante Unterschiede zeigten sich im endometrialen Epithel und Stroma: Bei Endometriose war die epitheliale EP1-Expression im Vergleich zu gesundem Gewebe ($p=0.042$) und zu Tumorgewebe erhöht ($p<0.001$). Die Expression im Stroma zeigte sich bei Tumorgewebe vermindert, verglichen mit gesundem Gewebe ($p<0.001$) und Gewebe von Endometriose-Patientinnen ($p<0.001$). Die EP1-Expression bei Endometriumskarzinomen unterschied sich nicht significant hinsichtlich Tumorhistologie, Stadium, Grad, Metastasierung und Wiederauftreten des Tumors. EP1 korreliert weder mit progressionsfreiem Überleben noch mit dem Gesamtüberleben der Patienten. Allerdings wiesen die PR-B-positiven Tumore eine stärkere EP1-Anfärbung auf als PR-B-negative.

Der Rezeptor EP3 reguliert die Proliferation, Migration und Invasion von Tumorzellen in einer Vielzahl von unterschiedlichen Karzinomen. Die mögliche Rolle von EP3 als prognostischer Biomarker im Endometriumskarzinom ist jedoch bislang unklar. Daher untersuchten wir auch die prognostische Signifikanz der EP3-Expression in Endometriumskarzinomen von 140 Patienten. Hierzu wurde die Endometriumskarzinomzellinie RL95-2 anhand ihres ähnlichen EP3-Exprssionslevels aus vier relevanten Ziellinien ausgewählt (RL95-2, Ishikawa, HEC-1-A, und HEC-1-B). Versetzt mit PGE2- und EP3-Antagonisten wurden die RL95-2-Zellen mit Hilfe von MTT-, BrdU- und „wound healing-“Assays analysiert, um die Funktionalität von EP3 zu beurteilen. Abhängig vom Tumorstadium konnten signifikante Unterschiede in der EP3-Anfärbung sowohl in der gesamten Kohorte ($p = 0,01$), als auch in der Subgruppe mit Endometriumskarzinom ($p = 0,01$) nachgewiesen werden: Patienten mit hoher EP3-Expression in ihren jeweiligen Tumoren zeigten ein schlechteres progressionsfreies Überleben sowie Gesamtüberleben. In der Gesamtkohorte wurde die EP3-Expression nach Adjustierung von Alter, Stadium, Grad und Rezidiv als unabhängiger Prognosefaktor für progressionsfreies Überleben identifiziert (HR 1.014, 95%CI 1.003-1.024, $p = 0,01$). Außerdem wurde eine signifikante negative Korrelation zwischen EP3 und ER-β (Korrelation Koeffizient

r = -0,225; p = 0,011) gefunden. Eine Behandlung mit EP3-Antagonisten induzierte eine Hochregulierung von ER-β, eine Verminderung der Ras-Aktivität und führte zur Abschwächung der Proliferation und Migration von RL95-2-Zellen. Kein Effekt konnte auf die Biosynthese von auf die nachgewiesen werden.

Somit scheint EP3 eine entscheidende Rolle in der Progression des Endometriumskarzinoms zu spielen. Im Kontext von limitierten systematischen Therapieoptionen für das Endometriumskarzinom, stellt diese descriptive Analyse von EP3 als potentiellem therapeutischem Target einen vielversprechenden translationalen Ansatz für diagnostische und therapeutische Entwicklungen dar.

Zusammenfassend für beide Teile der Arbeit ist festzustellen, dass Prostaglandine (PGE2) nicht nur die physiologischen Prozesse der Endometriumsdifferenzierung beeinflussen sondern auch benigne und maligne Transformationen des Endometriums über spezifische Rezeptoren steuern. Es sind noch weiterführende Studien notwendig, um gefundene Modulatoren dieser Rezeptoren im Sinne translationaler Prozesse in den klinischen Alltag übertragen zu können.

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