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ORIGINAL RESEARCH

Use of selective PGE_2 receptor antagonists on human endometriotic stromal cells and peritoneal macrophages

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ABSTRACT: Non-hormonal therapeutic strategies for endometriosis are needed. The aim of this study was to characterize the effects of prostaglandin (PG)E_2 receptor inhibitors to explore their potential as novel therapeutic strategies for endometriosis. The expression of PGE_2 receptors (EP2 and EP4) in donated tissues from human ovarian endometriosis, adenomyosis and peritoneal endometriosis was examined using immunohistochemistry. Human endometriotic stromal cells (ESC) isolated from ovarian endometriotic tissue and peritoneal macrophages were treated with EP2 and EP4 antagonists. cAMP accumulation and the effect of EP antagonists were measured using cAMP assays. DNA synthesis in ESC was detected using bromodeoxyuridine incorporation analysis. Interleukin (IL)-6 and IL-8 protein levels in ESC supernatants were measured using ELISAs. mRNA expression level for aromatase by ESC, and selected cytokines by peritoneal macrophages was measured using RT–PCR. EP2 and EP4 receptors were expressed in cells derived from control and diseased tissue, ovarian endometriotic, adenomyotic and peritoneal lesions. A selective EP2 antagonist reduced DNA synthesis, cAMP accumulation and IL-1β-induced proinflammatory cytokine secretion and aromatase expression. A selective EP4 antagonist negated IL-1β-induced IL-6 secretion and aromatase expression. In peritoneal macrophages, EP expression was elevated in endometriosis samples but the EP4 antagonist reduced cAMP levels and expression of vascular endothelial growth factor, chemokine ligand 2 and chemokine ligand 3 mRNA. EP2 and EP4 are functioning in endometriosis lesions and peritoneal macrophages, and their selective antagonists can reduce EP-mediated actions, therefore, the EP antagonists are potential therapeutic agents for controlling endometriosis.

Key words: adenomyosis / chemokine / endometriosis / interleukin / macrophages / ovary / peritoneum / prostaglandin E_2 / stromal cells / vascular endothelial growth factor

Introduction

Endometriosis is a disease characterized by endometrial growth beyond the uterus, commonly into the ovary and/or peritoneal cavity (Zondervan *et al.*, 2018). This condition may cause infertility, dysmenorrhea and chronic pelvic pain, and thereby remarkably deteriorates the state of the patient's health. Currently, therapeutic strategies for these disorders are limited to hormonal interventions; consequently, patients who desire to conceive cannot undertake these treatments (Zondervan *et al.*, 2018). Therefore, there is a strong necessity to identify potential non-hormonal cell signaling pathways as novel targets for controlling endometriosis.

Prostaglandin E_2 (PGE_2) is a well-established mediator of inflammatory disorders, including endometriosis. In endometriosis, synthesis of prostaglandins within lesions has been reported (Rakhila *et al.*, 2013) and the concentration of PGE_2 is higher in the peritoneal fluid from affected women (Dawood *et al.*, 1984; Wu *et al.*, 2002): these findings suggest that PGE_2 plays an important role in the growth of

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endometriosis lesions (Wu et al., 2010). Furthermore, cyclooxygenase2 (COX2) inhibitor blocks biosynthesis of PGE_2, and controls the symptoms (Cobellis et al., 2004) and progression (Laschke et al., 2007) of endometriosis; however, due to its adverse effects on cardiovascular (Cha and DuBois, 2007) and reproductive systems (Lim et al., 1997), the therapeutic uses of COX2 inhibitor are very limited.

The biological actions of PGE_2 are mediated via G protein receptors EP1, EP2, EP3 and EP4 by integrating multiple cell signaling pathways (Narumiya et al., 1999). Indeed, blocking EP receptor activity has been attempted for treating various diseases such as pancreatic ductal adenocarcinoma (Schmidt et al., 2017), breast cancer (Majumder et al., 2014), colon cancer (Ma et al., 2015) and acute inflammation (Morimoto et al., 2014). In endometriosis, Arosh et al. (2015) demonstrated that inhibiting EP2/EP4 decreased the growth and survival of endometriotic tissue in a mouse model, and Greaves et al. (2017) showed that an EP2 antagonist reduced hyperalgesia in a preclinical mouse model of endometriosis. In addition, an EP2/EP4 inhibitor induced apoptosis (Banu et al., 2009) and inhibited invasion (Lee et al., 2011); however, most of these studies were conducted using immortalized endometriotic cells. Further evidence regarding the effects of EP2/EP4 inhibitors on primary endometriotic and immune cells has, to date, been sparse.

The aim of this study was to obtain further evidence of the effects of EP2 and EP4 inhibitors on endometriotic tissue. For this purpose, we examined the expression of EP2 and EP4 receptors in human endometriotic tissue, adenomyosis lesions and peritoneal macrophages from patients with endometriosis. We then characterized the effects of an EP2 and EP4 antagonist on primary human endometriotic stromal cells (ESC) and peritoneal macrophages, using measures known to be significant in the context of the pathogenesis of endometriosis.

Materials and methods

Regents and materials

Type I collagenase, deoxyribonuclease I (DNase I) and recombinant interleukin (IL)-1β were purchased from Wako (Tokyo, Japan). Antibiotics (a mixture of penicillin, streptomycin and amphotericin B) and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM)/F-12 medium, Roswell Park Memorial Institute (RPMI) 1640 medium, 2.5% Trypsin, HEPES and 0.25% Trypsin-EDTA were from Gibco (Grand Island, NY, USA). Charcoal/dextran-stripped fetal bovine serum (FBS) was from HyClone (Logan, UT, USA). Ficoll-Paque PLUS was from GE Healthcare (Buckinghamshire, UK). The CD14 microbeads were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Polyclonal rabbit anti-EP2 antibody, polyclonal rabbit anti-EP4 antibody, PGE_2, PF-04418948 (EP2 antagonist)and CJ-042794 (EP4 antagonist) were from Cayman Chemical (Ann Arbor, MI, USA).

Patients and samples

The Institutional Review Board of the University of Tokyo approved this study and written informed consent for use of tissue was obtained from each donor. For immunohistochemistry studies, tissues derived from normal uterus, ovarian endometriosis, adenomyosis and peritoneal endometriosis were obtained from patients during surgical procedures for endometriosis or adenomyosis at the University of Tokyo Hospital. Five samples of each group were collected and immunohistochemistry was conducted. Endometriotic tissues were obtained from patients during laparoscopic surgery for ovarian endometriosis (n = 18, aged 38.3 ± 3.7 years, mean ± SD). Peritoneal fluid samples were collected during laparoscopic surgery from women (n = 6, aged 34.5 ± 3.0 years, mean ± SD) with benign gynecological diseases (controls), and from patients with endometriosis (n = 17, aged 36.2 ± 1.5 years, mean ± SD). In order to avoid the influence of menstrual cycle variation, peritoneal fluid was only collected from women who were within the first 10 days of their menstrual cycles. Diagnosis of endometriosis was confirmed by pathological examination. All patients had experienced regular menstrual cycles and had not received hormone therapy for at least 3 months before surgery. Endometriosis tissue samples were obtained from the cyst walls of the endometriotic ovaries under sterile conditions and transported to the laboratory on ice in DMEM/F-12. Peritoneal fluid samples were transported to the laboratory on ice.

Immunohistochemistry

Immunohistochemistry was conducted for EP2 and EP4. The following primary antibodies were used: polyclonal rabbit anti-EP2 antibody at 1:50 dilution, polyclonal rabbit anti-EP4 antibody at 1:50 dilution. Rehydrated sections were treated with 0.3% hydrogen peroxidase (H_2O_2) for 5 min to neutralize endogenous peroxidases and rinsed for 5 min with distilled water. Antigen retrieval was performed using Target Retrieval (DakoCytomation). After washing with PBS, slides were incubated in a moist chamber with primary antibody at 4°C for 1 h. The negative control slides were incubated with non-immune rabbit IgG. Slides were then incubated with anti-rabbit IgG-labeled polymer (DakoCytomation). All sections were visualized using ImmPACT™ (Vector Laboratories, Burlingame, CA, USA) for substrate. Qualitative assessment of staining intensity was by eye.

Isolation and culture of ESC

Isolation of ESC was conducted as described previously (Urata et al., 2014; Miyashita et al., 2015). Endometriotic tissue was minced and incubated in DMEM/F12 containing 0.25% type I collagenase, 15 U/ml DNase I, 0.006% trypsin and 0.02 mol/l HEPES for 60 min at 37°C. To isolate ESC, they were filtered through 100 µm and then 70 µm nylon cell strainers. ESC were cultured in DMEM/F-12 with 5% FBS and antibiotics and plated in a 100-mm culture plate and maintained at 37°C in a humidified 5% CO_2/95% air atmosphere. The purity of ESC was confirmed by >95% positive staining of vimentin and negative staining of cytokeratin and CD45. At the first passage, cells were plated at a density of 1×10^5 cells/ml into 96-well culture plates for bromodeoxyuridine (BrdU) incorporation assays and 2×10^5 cells/ml into 24-well culture plates for protein measurement assays.

Isolation and culture of peritoneal macrophages

Polymorphonuclear cells in the peritoneal fluid were obtained by centrifugation over a Ficoll density gradient. Peritoneal macrophages were

purified using magnetic-activated cell separation with CD14 microbeads. For mRNA expression assays, peritoneal macrophages were cultured in RPMI 1640 with 5% FBS and antibiotics and plated into 12-well culture plates at a density of 5 × 105 cells/ml and maintained at 37°C in a humidified 5% CO2/95% air atmosphere.

Treatment of ESC with EP antagonists

For cAMP assays, ESC were stimulated with PGE_2 (100 nM) in the absence or presence of 100 nM PF-04418948 (selective EP2 antagonist), 100 nM CJ-042794 (selective EP4 antagonist) or in combination for 20 min. For BrdU incorporation assays, ESC were treated with 100 nM PF-04418948, 100 nM CJ-042794 or in combination for 24 h. For ELISAs and aromatase mRNA expression assays, ESC were stimulated with IL-1β (5 ng/ml) in the absence or presence of 100 nM PF-04418948, 100 nM CJ-042794 or in combination for 24 h.

Treatment of peritoneal macrophages with EP antagonists

For cAMP assays, macrophages were stimulated with PGE_2 (100 nM) in the absence or presence of 100 nM PF-04418948 or 100 nM CJ-042794 or their combination for 20 min. For cytokines and growth factor mRNA expression assays, macrophages were stimulated with PGE_2 (100 nM) in the absence or presence of 100 nM PF-04418948 or 100 nM CJ-042794 or in combination for 24 h.

cAMP measurement using ELISA

The cAMP assay was performed using the Cyclic AMP XP® Assay Kit (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions. Briefly, ESC and peritoneal macrophages were treated with IBMX (0.5 nM) for 30 min, and then PGE_2 in the presence or absence of EP antagonists for 20 min. Cells were lysed and cell lysates were collected. cAMP found in the cell lysate competes with a fixed amount of horse-radish peroxidase (HRP)-linked cAMP for binding to an anti-cAMP antibody in a 96-well plate. The immune complexes (TMB substrate bound to HRP-linked cAMP) were detected by the subsequent substrate reaction, and the resultant color was read at 450 nm in an Epoch Microplate Spectrophotometer (BioTek).

The BrdU incorporation assay

The BrdU incorporation assay was performed using the Biotrak cell proliferation ELISA system (GE Healthcare) according to the manufacturer's instructions. Briefly, ESC were treated with 5% FBS with EP antagonists for 24 h, and then 10 µl BrdU solution was added and incubated at 37°C for an additional 2 h. After removing the culture medium, the cells were fixed and the DNA denatured by the addition of fixative solution (0.2 ml/well). The immune complexes (peroxidase-labeled anti-BrdU bound to BrdU incorporated into newly synthesized, cellular DNA) were detected by the subsequent substrate reaction, and the resultant color was read at 450 nm in an Epoch Microplate Spectrophotometer (BioTek).

Protein measurement using ELISA

Supernatants were obtained after cell culture for 24 h and immediately stored at −80°C until use. Concentrations of IL-6 and IL-8 in supernatants were measured using specific ELISAs (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

RNA extraction and quantitative real-time PCR

Total RNA from macrophages was extracted using an RNeasy minikit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. RNA (200 ng) was reverse transcribed to cDNA (20 µl in total) using an RT–PCR kit (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. Quantitative RT–PCR (qRT–PCR) was performed using a Light-Cycler 480 (Roche Diagnostics GmbH, Mannheim, Germany). Primers used for qRT–PCR analysis are listed in Table I. Primers were prevalidated by standard PCR and by generating standard curves using qRT–PCR. Each reaction buffer contained 5.0 µl Power SYBR® Green PCR Master Mix (2×), 0.5 µl primer pair (0.5 µM final), 3.5 µl nuclease-free H_2O and 1.0 µl cDNA, and each reaction was conducted in duplicate. The qRT–PCR cycling program consisted of a denaturing step (95°C for 10 min), annealing and extension step (95°C for 15 s and 60°C for 1 min) and dissociation step (95°C, 60°C and 95°C for 15 s each). In EP2 and EP4 mRNA expression assays, expression of each mRNA was normalized using PPIA (cyclophilin A) as an internal control. For aromatase (CYP19A1), cytokines and growth factor mRNA expression assays, expression of each mRNA was normalized using GAPDH as an internal control.

Statistical analysis

The differences between groups were calculated using non-parametric analysis (Mann–Whitney U test). Each group consisted of 6–10 samples from different patients. A P-value of <0.05 was considered as significant. The data were expressed as the mean (±SEM). Data analysis was conducted using Jmp software (version 10.0, SAS Institute Inc. Cary, NC, USA).

Results

Protein expression of EP2 and EP4 in endometriotic tissues

To begin to understand the role of EP proteins in the pathogenesis of endometriosis, we determined the expression of EP receptors in normal and diseased endometrium. As shown in Fig. 1A, EP2 proteins were expressed in epithelial cells and stromal cells of the normal endometrium and diseased tissue: ovarian endometriotic, adenomyotic and peritoneal lesions. The level of EP expression was lower in stromal cells than in epithelial cells. As depicted in Fig. 1B, EP4 proteins were expressed in epithelial and stromal cells of normal endometrium, and each of the diseased tissues, namely ovarian endometriotic, adenomyotic and peritoneal lesions. In each case, the staining was weaker in stromal cells than in epithelial cells.

The effect of EP antagonists on cAMP accumulation in ESC

To evaluate the functionality of EP2 and EP4 expressed by ESC and the effects of antagonists, we conducted cAMP assays (Fig. 2A).

Table I Primers used for quantitative real-time PCR analysis.

Gene name	Protein name	Accession no.	Forward primer (5′–3′)	Reverse primer (5′–3′)
GAPDH	GAPDH	NM002046	TGGACCTGACCTGCCGTCTA	CTGCTTCACCACCTTCTTGA
PPIA	PPIA	NM021130	CCCACCGTGTTCTTCGACATT	GGACCCGTATGCTTTAGGATGA
EP2	EP2	NM000956	TACAAGGCCGTGAAGACAAAAA	AGAGCACTATATCCGTGAGCG
EP4	EP4	NM000958	CGGCGGTGATGTTCATCTT	CCCACATACCAGCGTGTAGAA
CYP19A1	aromatase	NM000103	TGGAAATGCTGAACCCGATAC	AATTCCCATGCAGTAGCCAGG
IL-6	IL-6	NM000600	ACAAGCCAGAGCTGTGCAGATG	GTGCCCATGCTACATTTGCCGA
VEGF	VEGF	NM005429	TGGATGTCTATCAGCGCAGC	CGGCTTGTCACATTTTTGTTGTCT
CXCL2	CXCL2	NM009140	CGCCCAAACCGAAGTCAT	GATTTGCCATTTTTCAGCATCTTT
CXCL3	CXCL3	NM138522	CGCCCAAACCGAAGTCATAG	GCTCCCCTTGTTCAGTATCTTTT

PPIA, cyclophilin A; EP, prostaglandin receptor; CYP19A1, aromatase; IL, interleukin; VEGF, vascular endothelial growth factor; CXCL, chemokine ligand.

PGE_2 significantly increased intracellular cAMP accumulation in ESC. The EP2 antagonist reduced PGE_2-induced cAMP accumulation in ESC to 21.3% ± 4.9% (mean ± SEM, $P < 0.01$). The combination of EP2 and EP4 antagonists inhibited PGE_2-induced cAMP accumulation in ESC to 21.6% ± 4.4% ($P < 0.01$). EP4 antagonist alone did not change cAMP accumulation levels.

The effect of EP antagonists on ESC DNA synthesis

To determine the effect of EP proteins on DNA synthesis in ESC, the effect of EP antagonists on DNA synthesis was evaluated (Fig. 2B). The EP2 antagonist reduced BrdU incorporation in ESC DNA to 85.8% ± 5.1% of control levels (mean ± SEM, $P < 0.05$). The combination of EP2 and EP4 antagonists reduced BrdU incorporation into ESC DNA to 79.6% ± 7.3% ($P < 0.01$). The EP4 antagonist alone did not change BrdU incorporation, indicating no change in DNA synthesis levels.

The effect of EP antagonists on secretion of IL-6 and IL-8 by ESC

Proinflammatory cytokines, such as IL-6 and IL-8, have a role in the pathogenesis of endometriosis. To determine whether EP proteins have a role in stimulating the secretion of these cytokines, the effect of EP antagonists on the secretion of IL-6 and IL-8 was analyzed (Fig. 2C and D). Both EP2 and EP4 antagonists independently decreased IL-1β-induced IL-6 secretion by ESC to 64.1%± 8.3% (mean ± SEM, $P < 0.01$) and to 89.1% ± 11.1% ($P < 0.05$), respectively. The combination of EP2 and EP4 antagonist further decreased IL-1β-induced IL-6 secretion by ESC to 52.1% ± 10.0% ($P < 0.01$). As for IL-8 secretion, the EP2 antagonist alone decreased IL-1β-induced secretion by ESC to 77.9% ± 9.7% ($P < 0.05$), and the combination of EP2 and EP4 antagonist decreased IL-1β-induced IL-8 secretion in ESC to 66.1% ± 14.0% ($P < 0.01$). The EP4 antagonist alone did not affect the IL-8 secretion.

The effect of EP antagonists on aromatase expression by ESC

To elucidate a mechanism for EP action in the pathogenesis of endometriosis, the effect of EP antagonists on aromatase expression was investigated (Fig. 2E). Both EP2 and EP4 antagonists independently decreased IL-1β-induced aromatase expression by ESC, to 25.9% ± 3.5% (mean ± SEM, $P < 0.01$) and to 67.4% ± 10.2% ($P < 0.05$), respectively. The combination of EP2 and EP4 antagonist also decreased IL-1β-induced aromatase expression by ESC to 27.2% ± 11.5% ($P < 0.01$).

mRNA expression of EP2 and EP4 in peritoneal macrophages

Peritoneal macrophages are known to play an important role in the progression of endometriosis; therefore, we assessed the mRNA expression of EP2 and EP4 in peritoneal macrophage samples from endometriosis patients and controls (Fig. 3). EP2 mRNA expression level in peritoneal macrophages from endometriosis patients was significantly higher than control macrophages (Fig. 3A, $P < 0.05$). EP4 mRNA expression level in peritoneal macrophages was marginally higher in endometriosis patients, but the difference was not statistically significant (Fig. 3B, $P = 0.3657$).

The effect of EP antagonists on cAMP accumulation in peritoneal macrophages

We then evaluated the expression of functional EP2 and EP4 in peritoneal macrophages using a cAMP measurement assay (Fig. 4A). PGE_2 significantly increased intracellular cAMP accumulation in peritoneal macrophages. EP2 antagonist inhibited PGE_2-induced cAMP accumulation in peritoneal macrophages to 60.9% ± 3.8% (mean ± SEM, $P < 0.01$) and the EP4 antagonist produced a similar effect (50.0% ± 5.3%, $P < 0.01$). The combination of EP2 and EP4 antagonists inhibited PGE_2-induced cAMP accumulation in peritoneal macrophages to 46.1% ± 18.2% ($P < 0.01$).

The effect of EP antagonists on mRNA expression of IL-6, vascular endothelial growth factor, chemokine ligand 2 and chemokine ligand 3 in peritoneal macrophages

Finally, we studied the effect of EP antagonists on the mRNA expression of IL-6 (Fig. 4B), vascular endothelial growth factor (VEGF)

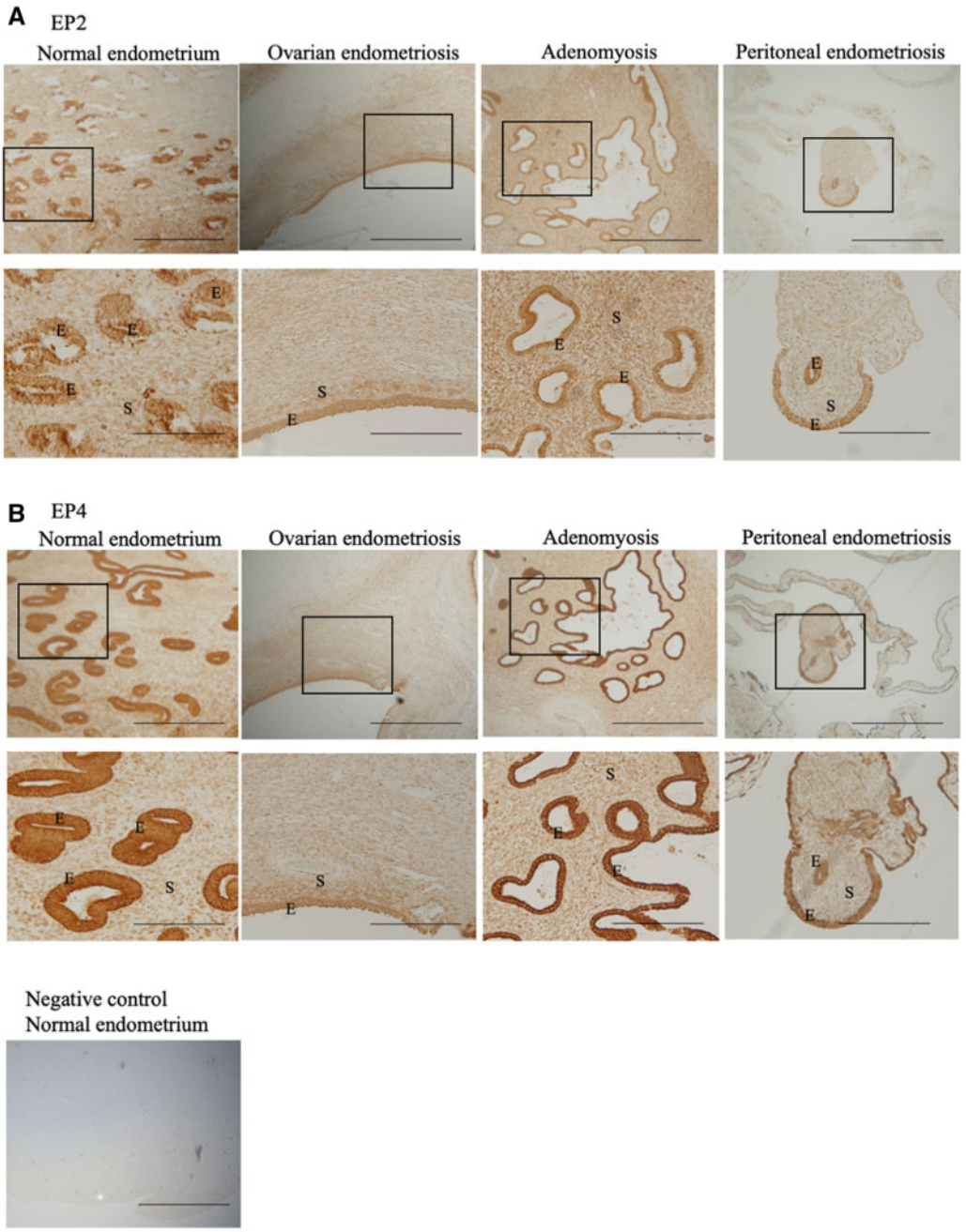

Figure 1. The expression of EP2 and EP4 proteins in normal human endometrium, and tissue samples from ovarian endometriosis, adenomyosis and peritoneal endometriosis. Immunohistochemical staining for the prostaglandin E_2 receptors EP2 (**A**) and EP4 (**B**) in tissue sections. The upper photomicrographs are at ×40 magnification and the lower photomicrographs are at ×100 magnification. EP2 and EP4 proteins were strongly expressed in epithelial cells but weakly expressed in stromal cells of the normal endometrium, and tissue samples from ovarian endometriosis, adenomyosis and peritoneal endometriosis. The figures are representative results of six samples from six patients. As a negative control, the normal endometrium was incubated with non-immune rabbit IgG. Black bars in ×40 photomicrographs represent 500 μM and bars in ×100 photomicrographs indicate 100 μM. 'E' indicates epithelium and 'S' indicates stroma.

(Fig. 4C), chemokine ligand 2 (CXCL2: Fig. 4D) and CXCL3 (Fig. 4E) in peritoneal macrophages. EP4 antagonist decreased PGE_2-induced VEGF, CXCL2 and CXCL3 mRNA expression to 72.2% ± 9.3% ($P < 0.05$), 81.5% ± 5.3% ($P < 0.05$) and 91.1% ± 5.8% ($P < 0.05$), respectively (mean ± SEM). The combination of EP2 and EP4 antagonist decreased PGE_2-induced IL-6, VEGF, CXCL2 and CXCL3 mRNA expression to 79.4% ± 5.3% ($P < 0.01$), 72.2% ± 9.3% ($P < 0.05$), 82.3% ± 7.9% ($P < 0.05$) and 86.0% ± 11.6% ($P < 0.05$), respectively.

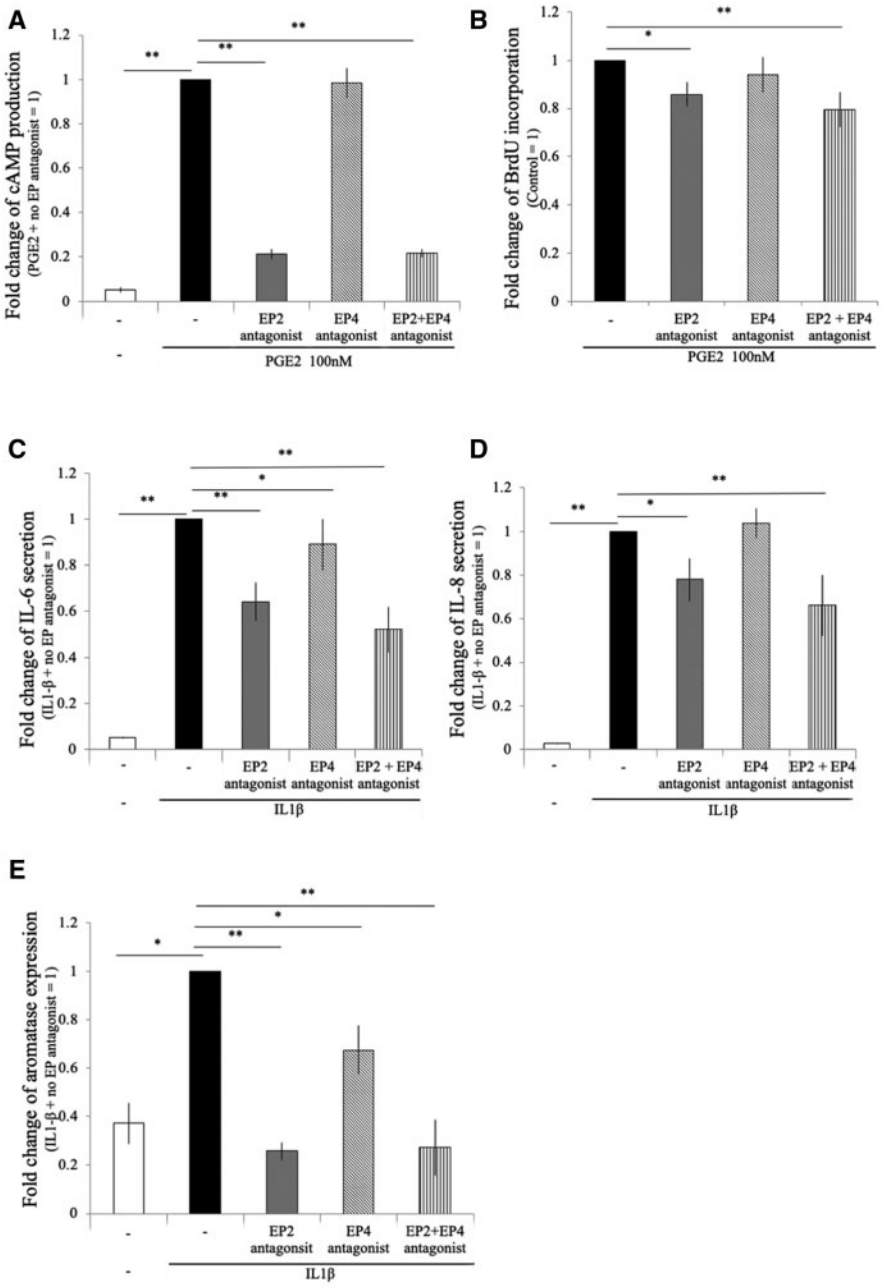

Figure 2. The effect of EP antagonists in human endometriotic stromal cells. (**A**) cAMP accumulation: Endometriotic stromal cells (ESC) were exposed to prostaglandin E_2 (PGE_2) (100 nM) in the absence or presence of EP2 antagonist (100 nM), EP4 antagonist (100 nM) or their combination for 20 min. Cells were lysed and cell lysates were collected. Concentrations of cAMP were measured using ELISA. The EP2 antagonist and the combination of EP2 and EP4 antagonists significantly decreased cAMP production in ESC, while the EP4 antagonist alone did not change cAMP production. (**B**) DNA synthesis: ESC were treated with EP2 antagonist (100 nM), EP4 antagonist (100 nM) or their combination for 24 h. DNA synthesis in ESC was detected using bromodeoxyuridine (BrdU) incorporation analysis. EP2 antagonist significantly reduced DNA synthesis in ESC. EP4 antagonist marginally reduced the DNA synthesis, although the difference was not statistically significant. Additive, but not synergistic, effects were observed for samples treated with the combination of each antagonist. (**C, D**) Cytokine secretion: ESC were exposed to interleukin (IL)1β (5 ng/ml) in the absence or presence of EP2 antagonist (100 nM), EP4 antagonist (100 nM) or their combination for 24 h. Supernatants were collected and concentrations of IL-6 (C) and IL-8 (D) were measured using ELISAs. EP2 and EP4 antagonist and their combination significantly decreased IL-6 secretion. As for IL-8, EP2 antagonist alone, and the combination of EP2 and EP4 antagonist significantly decreased the level of secreted protein, while the EP4 antagonist alone did not affect secretion levels. (**E**) Aromatase mRNA expression: ESC were exposed to IL-1β (5 ng/ml) in the absence or presence of EP2 antagonist (100 nM), EP4 antagonist (100 nM) or their combination for 24 h. Cells were harvested, mRNA was extracted and the expression of aromatase (CYP19A1) was measured using quantitative RT–PCR. GAPDH was used for normalization. EP2 and EP4 antagonists and their combination significantly decreased aromatase expression. No synergistic effect was observed when the antagonists were combined. Values are the mean (±SEM, n = 6, triplicates). *$P < 0.05$ versus control, **$P < 0.01$ versus control. The differences between groups were calculated using non-parametric analysis (Mann–Whitney U test).

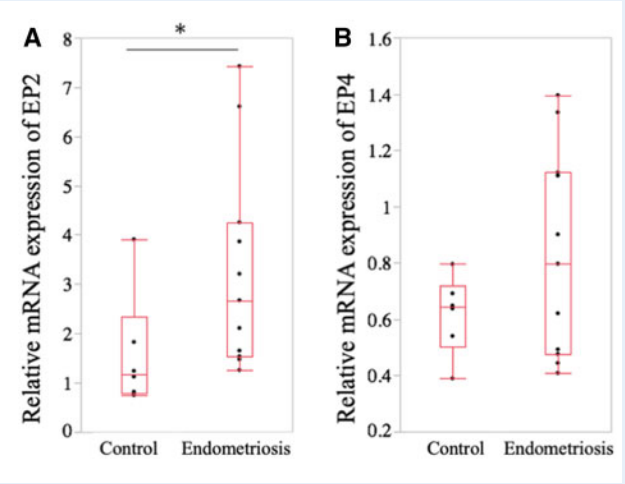

Figure 3. Expression of EP2 and EP4 in peritoneal macrophages from control women and patients with endometriosis. Peritoneal fluid samples were collected from control women and patients with endometriosis. Macrophages were purified using CD14 magnetic-activated cell separation selection and mRNA was extracted. The expression levels of EP2 and EP4 mRNA were quantified using quantitative RT–PCR. Cyclophilin A (Ppia) was used for normalization. The expression level of EP2 mRNA was significantly higher in macrophages from patients with endometriosis than in control samples (**A**). EP4 expression was marginally higher in macrophages from patients with endometriosis but the difference was not significant (**B**, $P = 0.3657$). The boxes represent the interquartile ranges and the whiskers indicate the maximum and minimum values. The bars within the boxes show the median values. *$P < 0.05$ versus control. The differences between groups were calculated using non-parametric analysis (Mann–Whitney U test).

EP2 antagonist alone had no effect on IL-6, VEGF, CXCL2 and CXCL3 expression in peritoneal macrophages and no synergistic effect was observed when the antagonists were combined.

Discussion

In this study, we demonstrated the expression of EP2 and EP4 receptors not only in the lesion of ovarian endometriosis but also in the peritoneal endometriotic and adenomyotic tissues. Using primary ESC, we made the novel discovery that the selective EP2 antagonist reduced the production of cAMP, DNA, proinflammatory cytokines and aromatase mRNA. The EP4 antagonist also controlled the expression of IL-6 and aromatase in ESC. Regarding peritoneal macrophages, the expression of EP2 and EP4 was enhanced in samples from patients with endometriosis in comparison with controls. Further novel findings in peritoneal macrophages were EP2 and EP4 antagonists reduced the production of cAMP, and the EP4 antagonist reduced the synthesis of VEGF, CXCL2 and CXCL3 mRNA. These findings imply that EP2 and EP4 are functioning in endometriosis lesions in the peritoneal cavity, and inhibiting these receptors may be a therapeutic strategy for controlling endometriosis.

EP2 and EP4 have been shown to be expressed in ovarian endometriosis (Banu *et al.*, 2009). Selective inhibition of the PGE_2 receptors EP2 and EP4 induces apoptosis of human endometriotic cells by suppressing extracellular signal-regulated kinase (ERK)1/2, AKT, nuclear factor-kappa b (NFκb) and beta-catenin pathways, and activating intrinsic apoptotic mechanisms (Banu *et al.*, 2009). The current study confirmed this using an immunohistochemical approach and further demonstrates that EP receptors are expressed in peritoneal endometriotic and adenomyotic tissues. These findings indicate that PGE_2 actions are not only confined to ovarian endometriotic tissue, but also may have a role in the pathogenesis of peritoneal endometriosis and adenomyosis. Indeed, EP2 and EP4 antagonists could potentially ameliorate these disease conditions by specifically blocking PGE_2/EP action.

In addition to the protein expression of EP2 and EP4 receptors, this study provides novel examinations of the function of these receptors in primary ESC by measuring intracellular cAMP levels, a molecule specifically synthesized through the activation of EP2 and EP4. We found that selective inhibition of EP2 suppressed the PGE_2-induced cAMP production, whereas inhibition of EP4 did not change cAMP levels. These findings suggest that only EP2 is functioning in ESC, even though both receptors are expressed. A discrepancy between EP expression and activity has been demonstrated in various types of cells. For instance, collecting duct cells express EP1, EP3 and EP4 but PGE_2 acts on EP1 only and thereby upregulates renin production by activating the protein kinase C (PKC)/cAMP/CRE-binding protein pathway (Gonzalez *et al.*, 2017). According to the current results, EP2 should be considered as a therapeutic target for controlling the progression of endometriosis. We have persisted, however, with testing the EP4 antagonist alone and in combination with the EP2 antagonist in further studies to determine whether it affects the behavior of ESC.

Firstly, we confirmed that the selective EP2 antagonist reduced the proliferation of ESC but the EP4 antagonist had no effect. Similar EP2 antagonist effects have been reported for other cell types such as colorectal cancer cells (Ma *et al.*, 2015). In endometriosis, Banu et al. (2009) tested antagonists of EP2 and EP4 on the cell survival of immortalized endometriotic epithelial and stromal cells and they found that EP2 and EP4 antagonists reduced cell survival via inducing apoptosis, but the current study demonstrated for the first time that the EP2 antagonist reduced the proliferation of ESC via reducing DNA synthesis.

We then focused on the effects of the antagonist on the production of proinflammatory cytokines and aromatase, given their roles in the pathology of endometriosis. Previously, using a murine endometriosis model, Arosh *et al.* (2015) demonstrated that inhibition of EP2/EP4 ameliorates disease with the reduction of proinflammatory cytokines and aromatase expression within the lesion; however, the direct effect of EP antagonists on human primary endometriotic cells has not been examined. A previous *in-vitro* study demonstrated that in immortalized endometriotic cells, PGE_2 mediates the IL-1β-induced production of proinflammatory cytokines such as IL-6 and IL-8, and aromatase (Wu *et al.*, 2007). Our novel findings reveal that these IL-1β-induced events can be controlled by antagonism of EP2 in primary ESC. Regarding IL-8 and aromatase expression, the EP4 antagonist had a slight inhibitory effect, although it is not clear whether this effect was EP4 specific.

The expression of EPs on macrophages and their role in the pathogenesis of diseases, such as cancer and aneurysms, has been

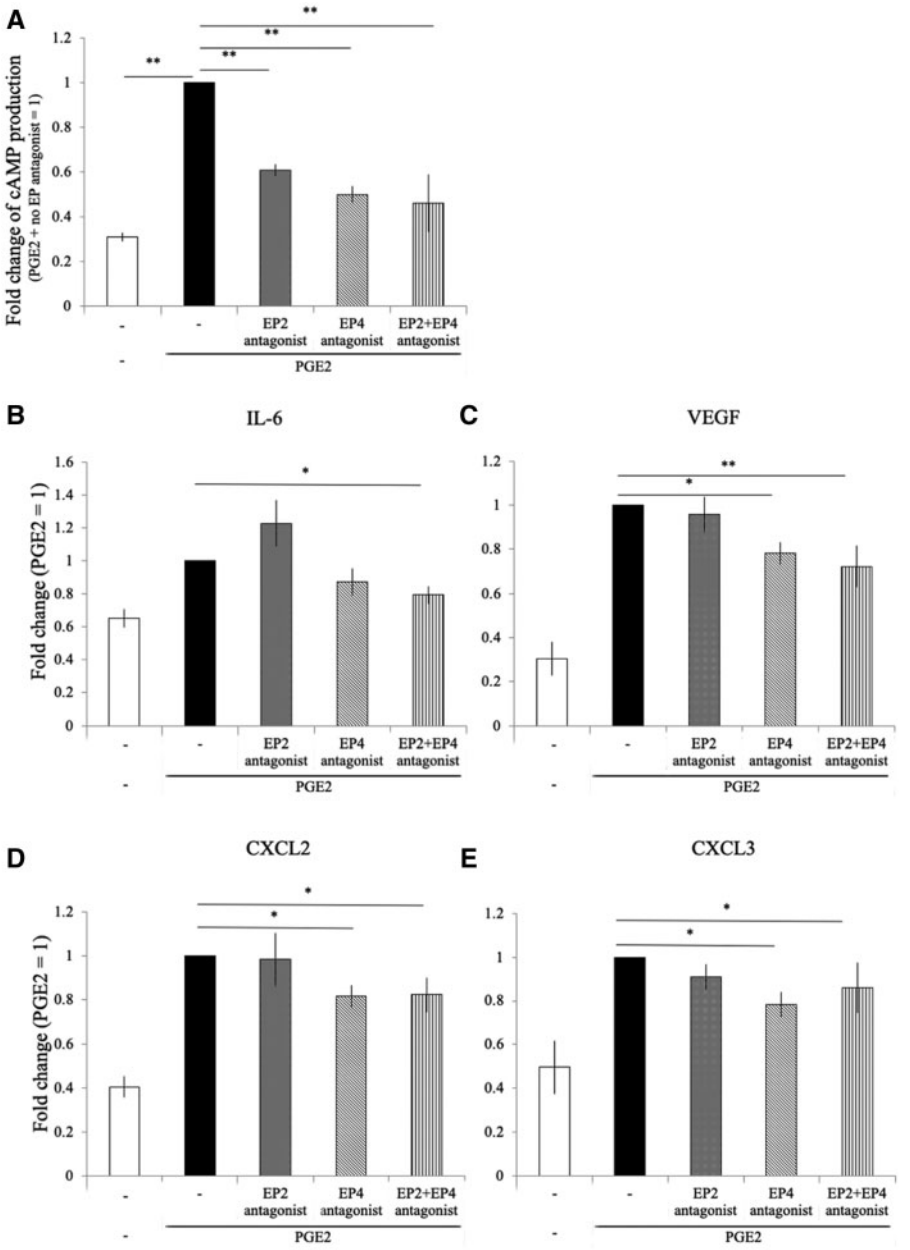

Figure 4. The effect of EP antagonists in human peritoneal macrophages. (**A**) cAMP accumulation: Peritoneal macrophages were exposed to PGE_2 (100 nM) in the absence or presence of EP2 antagonist (100 nM), EP4 antagonist (100 nM) or their combination for 20 min. Cells were lysed and cell lysates were collected. Concentrations of cAMP were measured using ELISA. Each EP antagonist and their combination significantly decreased cAMP production in peritoneal macrophages. (**B–E**) Cytokine and growth factor expression: Peritoneal macrophages were exposed to PGE_2 (100 nM) in the absence or presence of EP2 antagonist (100 nM), EP4 antagonist (100 nM) or their combination for 24 h. Cells were harvested, mRNA was extracted and the expression of IL-6, vascular endothelial growth factor (VEGF), chemokine ligand (CXCL)2 and CXCL3 mRNA were measured using quantitative RT–PCR. GAPDH was used for normalization. The EP4 antagonist significantly decreased the expressions of VEGF (C), CXCL2 (D) and CXCL3 (E) mRNA but the EP2 antagonist did not alter expression levels. No synergistic effect was observed when the two antagonists were combined. Values are the mean (±SEM, n = 6, triplicates). *$P < 0.05$ versus control, **$P < 0.01$ versus control. The differences between groups were calculated using non-parametric analysis (Mann–Whitney U test).

documented (Kalinski, 2012; Majumder *et al.*, 2014; Aoki *et al.*, 2017). The present study was a novel attempt to examine EP expression by peritoneal macrophages. We found that peritoneal macrophages express EP2 and EP4, which may suggest that these cells are also involved in the PGE_2-mediated pathogenesis of endometriosis. Additionally, inhibitors of these receptors, if given systemically, may exert their effects not only on the lesion *per se*, but on macrophages in the peritoneal cavity. More interestingly, EP2 expression levels were

significantly higher in macrophages from patients with endometriosis in comparison with macrophages from controls. Given that PGE_2 levels in the peritoneal fluid (Dawood *et al.*, 1984), and COX2 expression in the peritoneal macrophages (Wu *et al.*, 2002), are higher in endometriotic samples, the current results further support that the PGE_2 system is enhanced in the pelvic cavity of endometriosis patients. The mechanism by which the expression of EPs is induced in endometriosis is uncertain, but it is possible that the proinflammatory milieu in the diseased peritoneal cavity may induce EP2 and EP4 expression in macrophages, and thereby amplify inflammation within the environment.

In order to test whether the EP receptors on peritoneal macrophages were functioning, we treated macrophages with both PGE_2 and the EP2 or EP4 antagonist and measured intracellular cAMP. EP2 and EP4 on macrophages in other organs, such as the lung (Gill *et al.*, 2016), have been shown to be functional but this was the first study to examine their functionality in peritoneal macrophages. In contrast to their non-functional status in primary ESC, we found that EP4 receptor is functioning in peritoneal macrophages, and can thereby mediate PGE_2 actions in the peritoneal cavity.

We further investigated the involvement of EP2 and EP4 on peritoneal macrophages in the pathogenesis of endometriosis. It has been demonstrated that PGE_2 enhances the production of cytokine or growth factors from tumor-associated macrophages in malignant tumors such as breast cancer (Gan *et al.*, 2016) and gastric cancer (Zhang *et al.*, 2015), and thereby contributes to tumor growth. The receptor on which PGE_2 acts has not been elucidated in these contexts. In this study, we showed that PGE_2 enhances the production of IL-6, VEGF, CXCL2 and CXCL3 in peritoneal macrophages. All of these factors are enhanced in the peritoneal cavity of endometriosis cases (Mclaren *et al.*, 1996; Chen *et al.*, 2019; Jiang *et al.*, 2019). VEGF induces angiogenesis within the lesion, which is essential for the development of endometriosis (Taylor *et al.*, 2009). CXCL2 and CXCL3 are both ligands for CXCR2, which was recently identified as a key chemokine receptor for developing endometriosis (Tan *et al.*, 2018) and may induce an inflammatory reaction and angiogenesis via acting on ESC (Tan *et al.*, 2018) or monocyte-derived suppressor cells (Zhang *et al.*, 2018). Here, we show that production of these factors was partially but significantly reduced by the EP4 antagonist only. Indeed, PGE_2 is also known to reduce phagocytic activity in peritoneal macrophages in endometriosis (Wu *et al.*, 2005) and this reduction may be restored by EP2 or EP4 antagonist, although further studies are warranted to clarify these effects. Taken together, our results indicate that the EP antagonists should be further investigated as potential non-hormonal therapeutic agents for controlling endometriosis.

This study has several limitations. First, the study was conducted using primary stromal cells of endometriosis. Although it has been demonstrated that the EP2/EP4 antagonist induces cell apoptosis (Banu *et al.*, 2009) and reduces cell migration (Lee *et al.*, 2011) in immortalized endometriotic epithelial cells, the effect of EP antagonists on primary epithelial cells remains uncertain. Second, the peritoneal macrophages we isolated using CD14 microbeads were a mixed population of large and small peritoneal macrophages, and could also contain monocytes and dendritic cells; however, the current study does not reveal differences in the response of these subclasses to EP antagonists. Further studies, using primary epithelial cells, or peritoneal cells sorted using FACS with other cell-surface makers, are needed to clarify them.

In summary, our study demonstrates that EP2 is functional in the primary stromal cells of endometriosis, and EP4 is functional in peritoneal macrophages. Together with previous findings using mice models (Arosh *et al.*, 2015), the data suggest that inhibition of EP receptors using specific antagonists is a therapeutic strategy that needs to be considered for controlling endometriosis.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Authors' roles

T.M., K.K., H.N., M.A. and Y.O. contributed to the conception and design of the study. T.M. was responsible for acquisition of data. T.M. and K.K. analyzed the data. All authors were involved in data interpretations. T.M. drafted the manuscript, which was edited by K.K. and H.N. All authors have approved the final manuscript.

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Conflict of interest

The authors have nothing to disclose.

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