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# Prostaglandin E and F activate the FP receptor and up-regulate cyclooxygenase-2 expression via the cyclic AMP response element

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1 **Prostaglandin E<sub>2</sub> and F<sub>2α</sub> activate the FP receptor and up-regulate cyclooxygenase-**  
2 **2 expression via the cyclic AMP response element.**

3

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19

20

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22

1 **Abstract**

2 In endometrial adenocarcinomas COX-2 and F-series prostanoid (FP) receptor  
3 expression and prostanoid biosynthesis (PGE<sub>2</sub> and PGF<sub>2α</sub>) are elevated. In the present  
4 study we investigated the effect of PGE<sub>2</sub> and PGF<sub>2α</sub> on expression of COX-2 via the FP  
5 receptor in endometrial adenocarcinoma cells stably expressing the FP receptor (FPS  
6 cells). Using chemical inhibitors of intracellular signaling pathways, reporter gene  
7 assays and quantitative RT-PCR analysis, we show that PGE<sub>2</sub> and PGF<sub>2α</sub> can mobilize  
8 inositol 1,4,5 trisphosphate, induce ERK1/2 phosphorylation via the phospholipase Cβ-  
9 protein kinase A-epidermal growth factor receptor pathway and induce cyclooxygenase-  
10 2 (COX-2) expression via the FP receptor. In addition we show that the PGE<sub>2</sub> or PGF<sub>2α</sub>-  
11 regulation of COX-2 via the FP receptor is mediated via the cAMP response element  
12 (CRE) binding site on the COX-2 promoter. These data indicate that PGE<sub>2</sub> and PGF<sub>2α</sub>  
13 biosynthesized locally within endometrial adenocarcinomas can regulate tumor cell  
14 function in an autocrine/paracrine manner via the FP receptor.

15

16

## 1 **Introduction**

2 Prostaglandin endoperoxide (PGH) synthase or cyclooxygenase (COX) catalyses  
3 the committed step in the conversion of arachidonic acid to prostaglandins (PG)  
4 (Marnett et al., 1999). Two COX enzymes, COX-1 and COX-2, which are the targets for  
5 non-steroidal anti-inflammatory drug treatment have been characterised (Vane and  
6 Botting, 1998; Smith et al., 2000). A third COX enzyme (COX-3), a variant of COX-1  
7 formed by retention of intron-1 and which is sensitive to acetaminophen, has been  
8 cloned more recently from canine cerebral cortex (Chandrasekharan et al., 2002).

9 COX-1 is constitutively expressed in many cell types and is overexpressed in  
10 certain cancers (Hwang et al., 1998; Bauer et al., 2000; Kirschenbaum et al., 2000; Sales  
11 et al., 2002). COX-2 is the more inducible form of the enzyme and is commonly  
12 associated with pathological conditions including tumorigenesis (DuBois et al., 1996;  
13 Vane et al., 1998). The existence of COX-3 protein in humans remains controversial and  
14 its role in pathologies undefined.

15 Following biosynthesis, prostaglandins exert their function through G protein  
16 receptor (GPCR)-mediated interaction. In the human endometrium, PGE<sub>2</sub> and PGF<sub>2α</sub> are  
17 the most abundantly biosynthesized prostanoids (Lumsden et al., 1983; Hofer et al.,  
18 1993). PGE<sub>2</sub> exerts its autocrine/paracrine action by binding to either of four main  
19 subtypes of GPCR (EP1, EP2, EP3 and EP4) to mobilize intracellular calcium and  
20 inositol 1,4,5 trisphosphate (InsP) via G<sub>q/11</sub> (EP1/EP3) or increase cAMP accumulation  
21 via G<sub>αs</sub> (EP2/EP4) (Sugimoto et al., 1993; Narumiya et al., 1999). Activation of FP  
22 receptors by PGF<sub>2α</sub> results in phospholipase C (PLC) activation, inositol 1,4,5  
23 trisphosphate (InsP) hydrolysis and intracellular calcium flux (Watanabe et al., 1994).

1 Activation of the cAMP/InsP second messenger systems by prostanoid-receptor  
2 interaction can in turn regulate target gene transcription via the phosphorylation and  
3 dephosphorylation of distal signaling pathways such as the extracellular signal-regulated  
4 kinase (ERK1/2) and phosphatidylinositol-3-kinase/protein kinase B pathways via  
5 transactivation of the epidermal growth factor receptor (EGFR) (Sheng et al., 2001;  
6 Regan, 2003; Jabbour and Sales, 2004; Sales et al., 2004a,b; Jabbour et al., 2005; Smith  
7 et al., 2006)

8 We have previously demonstrated elevated expression and signaling of COX-2  
9 and FP receptor in human endometrial adenocarcinomas and have ascertained a role for  
10  $\text{PGF}_{2\alpha}$ -FP receptor interaction in enhancing the proliferation of endometrial epithelial  
11 cells (Jabbour and Sales, 2004; Jabbour et al., 2005; Sales et al., 2004b) and promoting  
12 the expression of pro-angiogenic and inflammatory genes in endometrial  
13 adenocarcinoma cells and adenocarcinoma biopsy explants via activation of ERK1/2  
14 (Jabbour et al., 2005; Sales et al., 2005; Sales et al., 2007).

15 Although  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$  are considered to be the endogenous ligands for EP  
16 and FP receptors respectively,  $\text{PGE}_2$  can bind to the FP receptor with an affinity that is  
17 only 10-30 fold less than  $\text{PGF}_{2\alpha}$  (Abramovitz et al., 2000). The affinity of  $\text{PGF}_{2\alpha}$  for EP  
18 receptors is 100-300 fold less than it is for the FP receptor (Abramovitz et al., 2000).

19 Recently we have shown that COX-2 and  $\text{PGF}_{2\alpha}$  biosynthesis can be  
20 autoregulated in endometrial adenocarcinoma cells via the FP receptor (Jabbour et al.,  
21 2005). Given that both  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$  biosynthesis are elevated in endometrial  
22 pathologies (Lundstrom and Green, 1978; Lumsden et al., 1983; Sales and Jabbour,  
23 2003) and since  $\text{PGE}_2$  can act as an agonist of the FP receptor, we investigated the effect

- 1 of PGE<sub>2</sub> and PGF<sub>2α</sub> on regulation of COX-2 via the FP receptor in Ishikawa endometrial
- 2 epithelial cells stably transfected with the human isoform of the FP receptor.

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## 1 **Materials and Methods**

### 2 *Reagents*

3 Culture medium was purchased from Life Technologies (Paisley, UK).  
4 Penicillin-streptomycin and fetal calf serum (FCS) were purchased from PAA  
5 Laboratories (Middlesex, UK). Indomethacin, phosphate buffered saline (PBS), bovine  
6 serum albumin (BSA), AL8810 (10mM stock in ethanol), PGE<sub>2</sub> and PGF<sub>2α</sub> were  
7 purchased from Sigma Chemical Company (Dorset, UK). Rabbit anti-Phospho-p42/44  
8 and mouse anti-p42/44 monoclonal antibodies were purchased from Cell Signaling  
9 Technologies (New England Biolabs, Herts, UK). The Alexafluor 680 secondary  
10 antibody was purchased from Molecular Probes Inc. (Eugene, OR, USA). The IRDye<sup>TM</sup>  
11 800 secondary antibody was purchased from Rockland Immunochemicals (Gilbertsville,  
12 PA, USA). AH6809 (10mM Stock in dimethylsulfoxide, DMSO), U73122 (10mM Stock  
13 in DMSO), PD98059 (18.7mM Stock in DMSO), 4C3MQ (4-cyano-3-  
14 methylisoquinoline; 10mM stock in DMSO), GF109203x (10mM stock in DMSO) and  
15 AG1478 (10mM stock in DMSO) were purchased from Calbiochem (Nottingham, UK).  
16 EP4 antagonist (ONOAE2227, 10mM stock in ethanol, used at a final concentration of  
17 1μM) was chemically synthesised by Charnwood Molecular Ltd. (Leics, UK).

18 Doses of chemical inhibitors described in table 1 and antibodies were determined  
19 empirically by titration using the manufacturer's guidelines.

20

1 *Cell Culture*

2 Ishikawa endometrial adenocarcinoma cells were obtained from the European  
3 Collection of Cell Culture (Wiltshire, UK). Stable FP transfectant cells were  
4 constructed, characterised and maintained as described (Sales et al., 2005), with the  
5 addition of a maintenance dose of 200µg/ml G418.

6 *Total Inositol Phosphate (IP) Assays*

7 Total inositol phosphate (InsP) production was measured in Ishikawa WT and FPS  
8 cells and assayed as described previously (Berg et al., 1994; Sales et al., 2005). Cells  
9 were treated either with vehicle, PGE<sub>2</sub> or PGF<sub>2α</sub> in the absence or presence of receptor  
10 antagonist or chemical inhibitor as shown in the figure legends. Data are presented as  
11 mean ± SEM from at least 3 independent experiments.

12  
13 *cAMP assay*

14 cAMP accumulation was determined in response to administration of vehicle,  
15 PGE<sub>2</sub> or PGF<sub>2α</sub> in the absence or presence of receptor antagonist or chemical inhibitor  
16 as shown in the figure legend and was performed as described previously (Sales et al.,  
17 2002). Briefly, cells (2 x 10<sup>5</sup>) were seeded and allowed to attach overnight. The  
18 following day, the cells were serum starved by incubating with fresh serum free medium  
19 containing 8.4 µM indomethacin for at least 16 hours. Thereafter the culture medium  
20 was removed and replaced with serum-free medium containing 3-isobutyl-1-methyl  
21 xanthine (IBMX; Sigma) to a final concentration of 1mM and receptor  
22 antagonist/chemical inhibitor for 30 minutes at 37°C. Cells were then stimulated with  
23 ligand for the time indicated in the figure legend. Following stimulation, cells were

1 lysed in 0.1M HCl. cAMP concentration was quantified by ELISA using a cAMP kit  
2 (Biomol, Affiniti, Exeter, UK) according to the manufacturer's protocol and normalised  
3 to protein concentration of the lysate. Protein concentrations were determined using  
4 protein assay kits (Bio-Rad Laboratories, Hemel Hamstead, UK). Data are presented as  
5 mean  $\pm$  SEM from at least 4 independent experiments.

6

### 7 *In Cell Western Detection*

8 Cell signaling to ERK1/2 was investigated using an In-Cell Western Assay.  
9 Approximately 20000 cells were seeded per well in a 96 well microtitre plate and  
10 allowed to adhere overnight at 37°C. The following day cells were starved by serum  
11 withdrawal in serum free culture medium containing 8.4  $\mu$ M indomethacin for at least  
12 16 hours at 37°C. Cells were incubated for 30 minutes with vehicle, receptor antagonist  
13 or chemical inhibitor as described in the figure legend. Thereafter cells were stimulated  
14 with either vehicle PGE<sub>2</sub> or PGF<sub>2 $\alpha$</sub>  in the absence or presence of receptor antagonist or  
15 chemical inhibitor for the time indicated in the figure legend. Following stimulation,  
16 cells were washed with ice-cold PBS, fixed in 3.7% (v/v) Formaldehyde for 20 minutes  
17 at room temperature, and permeabilised with 0.1% Triton X-100 in PBS. Cells were  
18 then blocked for 45 minutes at room temperature with Odyssey Blocking buffer<sup>TM</sup> (LI-  
19 COR Biosciences, Cambridge, UK) before overnight incubation with primary rabbit  
20 phospho-p42/44 and goat p42/44 antibodies (diluted 1:100 in Odyssey blocking buffer)  
21 at 4°C. The following day, cells were washed and incubated with the goat anti-rabbit  
22 Alexafluor 680 (1:200) and goat anti-mouse IRDye<sup>TM</sup> 800 (1:800) for 60 minutes at  
23 room temperature.

1 Immunoreactive proteins were detected and quantified using the Odyssey infrared  
2 imaging system (LI-COR Biosciences). ERK1/2 phosphorylation was calculated by  
3 dividing the value obtained from the phosphorylated ERK1/2 channel (700nm) by the  
4 value obtained from total ERK1/2 channel (800nm) and expressed as fold above vehicle  
5 controls. Results are expressed as mean  $\pm$  SEM from at least 3 independent experiments  
6 performed in triplicate.

7

#### 8 *Taqman quantitative RT-PCR*

9 COX-2 mRNA expression in FPS cells was measured by quantitative RT-PCR  
10 analysis. FPS cells were starved by serum withdrawal for at least 12 hours in serum-free  
11 medium containing 8.4  $\mu$ M indomethacin. Thereafter medium was removed and  
12 replaced with fresh medium containing indomethacin with either vehicle, PGE<sub>2</sub> or  
13 PGF<sub>2 $\alpha$</sub>  in the absence or presence of receptor antagonist or chemical inhibitor as  
14 described in the figure legend. RNA was extracted using Tri-reagent (Sigma) following  
15 the manufacturers guidelines. Once extracted and quantified, RNA samples were reverse  
16 transcribed and subjected to RT-PCR analysis using an ABI Prism 7900 (Jabbour et al.,  
17 2005). COX-2 primers and probe for quantitative PCR were designed using the  
18 PRIMER express program (PE Applied Biosystems, Warrington, UK) as described  
19 previously (Jabbour et al., 2005). Data were analysed and processed using Sequence  
20 Detector v1.6.3 (PE Applied Biosystems). Expression of COX-2 was normalised to  
21 RNA loading for each sample using the 18S ribosomal RNA as an internal standard.  
22 Results are expressed as fold increase above vehicle treated from at least 4 independent  
23 experiments and represented as mean  $\pm$  SEM.

1 *Transfection of CRE and COX-2 Promoter with Deletions and Mutations*

2 Ishikawa FPS cells were transiently transfected for 6 hours using a liposomal  
3 transfection system (Superfect, Qiagen, Crawley, UK). Transfections were performed  
4 with CRE-Luciferase a specific cis-acting DNA binding sequence of the cAMP response  
5 element ligated with a Luciferase reporter plasmid (Clontech Laboratories, BD  
6 Bioscience, Cowley, UK) or the COX-2 promoter - performed using C2.1 (-917 to +49)  
7 966 base pair (bp) fragment of the COX-2 promoter and C2.1 with a series of deletions  
8 or site specific mutations ligated with a Luciferase reporter plasmid pGL3 basic  
9 (Promega, Southampton, UK) as described in Bradbury et al (Bradbury et al., 2003);  
10 kindly supplied by Dr Robert Newton, BioMedical Research Institute, Department of  
11 Biological Sciences, The University of Warwick, UK). Deletions consisted of Dra (-  
12 625/+49) 674bp, Sty (-358/+49) 407bp, Alu (190/+49) 239bp and RSA (-86/+49) 135bp  
13 fragments. The description of each clone is based on the restriction site used to generate  
14 the construct (Dra, Alu, Sty, RSA, HIN). Mutations consisted of CRE (-59/-53), a  
15 mutation in the cAMP response element. The HIN (-79/+53) 200bp and HIN CRE-  
16 mutation (HINcrem; -79/+53, 200bp) fragments were generated by polymerase chain  
17 reaction (PCR) using the Sty and Stycrem cDNA (Bradbury et al., 2003) as a template.

18 Amplification of HIN and HINcrem was carried out using standard PCR mix  
19 containing forward 5'-AAGGCGGAAAGAAACAGTCA-3' and reverse 5'-  
20 AACAGTACCGGAATGCC-AAG-3' primers containing the HindIII restriction site at  
21 the start for ease of cloning. To amplify by PCR, sample mix was denatured at 94°C for  
22 5 minutes and subjected to 40 cycles of 94°C for 1 minute, 58°C for 1 minute and 68°C  
23 for 1 minute, with a final extension step of 68°C for 7 minutes. After amplification,

1 samples were cooled to 4°C and visualised on 1% agarose gels. The PCR product was  
2 ligated into the pCR®II-TOPO vector (Invitrogen, De Schelp, Netherlands) followed by  
3 sequencing in both directions using a PE Applied Biosystems 373A automated  
4 sequencer. The HIN and HINcrem cDNA was ligated into the pGL3 basic (Promega)  
5 expression vector followed by sequencing.

6

### 7 *Luciferase Reporter Assays*

8 CRE or COX-2 promoter firefly Luciferase reporter vectors were co-transfected  
9 into Ishikawa FPS cells in triplicate with an internal control pRL-TK (containing the  
10 renilla Luciferase coding sequence; Promega) as described (Jabbour et al., 2005). The  
11 following day the cells were serum-starved for at least 16 hours 37°C with 8.4 µM  
12 indomethacin prior to stimulation for 4 hours with vehicle, PGE<sub>2</sub> or PGF<sub>2α</sub> in the  
13 absence or presence of receptor antagonist or chemical inhibitor as described in the  
14 figure legend. The activity of both firefly and renilla Luciferase was determined using  
15 the dual Luciferase assay kit (Promega) and total Luciferase activity was determined by  
16 dividing the relative light units generated by the firefly Luciferase by the relative light  
17 units generated by the renilla Luciferase in the same reaction. Fold increase in  
18 Luciferase activity was calculated by dividing the total Luciferase activity in cells  
19 treated with PGE<sub>2</sub> or PGF<sub>2α</sub> in the absence or presence of receptor antagonist or  
20 chemical inhibitor by the total Luciferase activity in cells treated with vehicle. Data are  
21 presented as mean ± SEM from at least 4 independent experiments.

22

1 *Statistics*

- 2           Data were subjected to statistical analysis with ANOVA and Fishers protected  
3 least significant difference tests (Statview 5.0; Abacus Concepts Inc., USA).

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## 1 **Results**

### 2 *PGE<sub>2</sub> and PGF<sub>2α</sub> mobilize inositol 1,4,5, trisphosphate in FPS cells*

3 We have previously reported elevated FP receptor expression in endometrial  
4 adenocarcinomas (Sales et al., 2004b) and constructed and characterized an endometrial  
5 adenocarcinoma (Ishikawa) cell line expressing FP receptor (FPS cells) to the levels  
6 observed in endometrial adenocarcinomas (Sales et al., 2005). In this latter study we  
7 showed that PGF<sub>2α</sub>-FP receptor interaction in FPS cells increases the hydrolysis of InsP  
8 in FPS cells via G<sub>q/11</sub> to a greater extent than in wild-type (WT) Ishikawa cells (Sales et  
9 al., 2005).

10 In the present study we found that PGE<sub>2</sub> could also dose-dependently mobilize  
11 InsP in FPS cells (Fig. 1A; P<0.05). No such increase in InsP production was observed  
12 in WT cells in response to PGE<sub>2</sub>, but a modest increase was observed in response to  
13 PGF<sub>2α</sub> (Fig. 1A). Co-incubation of FPS cells with the specific FP receptor antagonist  
14 AL8810 abolished the PGE<sub>2</sub> or PGF<sub>2α</sub>-mediated increase in InsP production at all  
15 concentrations of ligand administered (Fig.1B, P<0.05). These results suggest that the  
16 PGE<sub>2</sub>-mediated InsP hydrolysis in FPS cells was via the FP receptor.

17 We further confirmed that the InsP hydrolysis in response to 100nM PGE<sub>2</sub> or  
18 100nM PGF<sub>2α</sub> was not mediated via activation of either of the endogenous EP2 or EP4  
19 receptors or intracellular signaling cascades downstream of phospholipase Cβ (PLC) as  
20 neither the specific EP2 receptor antagonist (AH6809), the EP4 receptor antagonist  
21 (ONOAE2227; Fig.1C, P<0.05) nor the chemical inhibitors of the protein kinase A  
22 (PKA; 4-cyano-3-methylisoquinoline; 4C3MQ), protein kinase C (PKC;GF109203x)  
23 and ERK1/2 (PD98059) signaling pathways (Fig.1C, P<0.05, P<0.05) inhibited the

1 PGE<sub>2</sub> or PGF<sub>2α</sub>-mediated InsP production. As shown for the specific FP receptor  
2 antagonist (AL8810), the PLC inhibitor (U73122) also abolished the InsP produced in  
3 response to treatment of FPS cells with 100nM PGE<sub>2</sub> or 100nM PGF<sub>2α</sub> (Fig. 1C, P<0.05)  
4 further demonstrating the InsP production in response to ligand activation was mediated  
5 via the FP receptor-PLC pathway.

6

### 7 *PGE<sub>2</sub> and PGF<sub>2α</sub> promote cyclic adenosine 3, 5 monophosphate (cAMP) in FPS cells*

8 In addition to elevated FP receptor, Ishikawa FPS cells also express basal levels  
9 of EP2 and EP4 receptor, but not detectable EP1 receptor (data not shown), which  
10 couple to G<sub>s</sub> and mobilize intracellular cAMP. We investigated cAMP accumulation in  
11 FPS cells in response to treatment with 100nM PGE<sub>2</sub> or 100nM PGF<sub>2α</sub> for 0, 5 or 10  
12 minutes (Fig. 2A). We found that 100nM PGE<sub>2</sub> rapidly mobilized intracellular cAMP at  
13 5 and 10 minutes (Fig. 2A; P<0.05). By contrast 100nM PGF<sub>2α</sub> modestly increased  
14 cAMP following 10 minutes of treatment only (Fig. 2A; P<0.05). We confirmed that the  
15 cAMP produced in response to 100nM PGE<sub>2</sub> or 100nM PGF<sub>2α</sub> was not mediated by an  
16 intracellular mechanism via activation of the FP receptor or PLC, PKA, PKC or ERK1/2  
17 signaling cascades as neither the specific FP receptor antagonist (AL8810; Fig. 2B;  
18 P<0.05) nor the chemical inhibitors of the PKA (4C3MQ), PKC (GF109203x) or  
19 ERK1/2 (PD98059) signaling pathways (Fig. 2B; P<0.05) inhibited the PGE<sub>2</sub> or PGF<sub>2α</sub>-  
20 mediated cAMP production following ligand stimulation for 10 minutes. However co-  
21 treatment of FPS cells with the EP2 receptor antagonist (AH6809) or EP4 receptor  
22 antagonist (ONOAE2227) significantly reduced the PGE<sub>2</sub>- or PGF<sub>2α</sub>-mediated cAMP  
23 response. Moreover, the EP2 (AH6809) and EP4 (ONOAE2227) receptor antagonist in

1 combination totally abolished the PGE<sub>2</sub>, or PGF<sub>2α</sub> -mediated cAMP response following  
2 10 minutes of ligand stimulation (Fig. 2B; P<0.05).

3

#### 4 *PGE<sub>2</sub> and PGF<sub>2α</sub> signaling cascades converge on ERK1/2*

5 The effect of PGE<sub>2</sub> or PGF<sub>2α</sub> on the activation of the downstream extracellular  
6 signal regulated kinase (ERK1/2) signaling pathway was determined after treatment of  
7 FPS cells with vehicle, 100nM PGE<sub>2</sub> or 100nM PGF<sub>2α</sub> for 0, 5, 10 and 20 minutes (Fig.  
8 3A; P<0.05). Stimulation of FPS cells with PGE<sub>2</sub> or PGF<sub>2α</sub> caused a rapid time-  
9 dependent activation of ERK1/2 (Fig. 3A; P<0.05). The peak of ERK1/2 activation was  
10 observed after 5 minutes in FPS cells treated with 100nM PGE<sub>2</sub> and 10 minutes in cells  
11 treated with 100nM PGF<sub>2α</sub> (Fig. 3A, P<0.05).

12 We next evaluated the effect of the FP receptor antagonist (AL8810), EP2  
13 receptor antagonist (AH6809), EP4 receptor antagonist (ONOAE2227) and chemical  
14 inhibitors of PLC (U73122), PKC (GF109203x), PKA (4C3MQ), EGFR (AG1478) and  
15 ERK1/2 kinase (MEK; PD98059) on the PGE<sub>2</sub> or PGF<sub>2α</sub>-induced activation of ERK1/2  
16 signaling. As observed in Figure 3A, ERK1/2 phosphorylation was significantly  
17 elevated in FPS cells treated for 10 minutes with PGE<sub>2</sub> (Fig. 3B, P<0.05). The PGE<sub>2</sub>-  
18 induced elevation in ERK1/2 activation was significantly inhibited by co-treatment of  
19 FPS cells with FP receptor antagonist (AL8810), EP2 receptor antagonist (AH6809),  
20 EP4 receptor antagonist (ONOAE2227) and PLC inhibitor (U73122) and abolished by  
21 treatment of cells with the PKA (4C3MQ), EGFR kinase (AG1478) and ERK1/2 kinase  
22 (PD98059) inhibitors, but not the PKC inhibitor (GF109203x; Fig. 3B, P<0.05). The  
23 PGF<sub>2α</sub>-induced elevation in ERK1/2 activation was significantly inhibited by co-

1 treatment of cells with the EP2 antagonist (AH6809), but not the EP4 receptor  
2 antagonist (ONOAE2227) or PKC inhibitor (GF109203x), and was abolished by  
3 treatment with the FP receptor antagonist (AL8810), and inhibitors of PLC (U73122),  
4 PKA (4C3MQ), EGFR kinase (AG1478) and ERK1/2 kinase (PD98059; Fig. 3B,  
5  $P < 0.05$ ).

6

#### 7 *Activation of COX-2 Luciferase reporter and mRNA by PGE<sub>2</sub> and PGF<sub>2α</sub>*

8 The role of PGE<sub>2</sub> or PGF<sub>2α</sub> on the activation of COX-2 in FPS cells was  
9 investigated by Luciferase reporter gene assay (Fig. 4A) and quantitative RT-PCR  
10 analysis (Fig. 4B). Treatment of FPS cells with PGE<sub>2</sub> or PGF<sub>2α</sub> caused a significant  
11 time-dependent increase in COX-2 Luciferase reporter activity (Fig. 4A;  $P < 0.05$ ) and  
12 mRNA expression (Fig. 4B;  $P < 0.05$ ) which peaked at 4-6 hours. The PGF<sub>2α</sub>-induced  
13 increase in COX-2 reporter gene activation (Fig. 4A) and mRNA expression (Fig. 4B)  
14 was greater than that induced by PGE<sub>2</sub> ( $P < 0.05$ ).

15

#### 16 *COX-2 expression is mediated via activation of the ERK pathway*

17 We set out to determine the signaling pathways mediating COX-2 expression in  
18 FPS cells. Cells were treated with vehicle, 100nM PGE<sub>2</sub> or 100nM PGF<sub>2α</sub> in the  
19 presence/absence of the FP receptor antagonist (AL8810), EP2 receptor antagonist  
20 (AH6809), EP4 receptor antagonist (ONOAE2227), or chemical inhibitors of PLC  
21 (U73122), PKA (4C3MQ), PKC (GF109203x), EGFR kinase (AG1478) or ERK1/2  
22 kinase (PD98059). COX-2 promoter activation and mRNA expression was determined  
23 using a reporter cDNA construct containing the full length COX-2 promoter cDNA

1 fused upstream of the firefly Luciferase reporter (C2.1; Fig. 5A and 5C) and real-time  
2 quantitative RT-PCR analysis (Fig. 5B and 5D) respectively. The PGE<sub>2</sub>-induced  
3 elevation in COX-2 Luciferase (Fig. 5A) and mRNA expression (Fig. 5B) was  
4 significantly reduced by treatment of FPS cells with the FP receptor antagonist  
5 (AL8810), EP2 receptor antagonist (AH6809), EP4 receptor antagonist (ONOAE2227),  
6 and chemical inhibitors of PLC (U73122), PKA (4C3MQ), EGFR kinase (AG1478) and  
7 ERK1/2 kinase (PD98059), but not the PKC inhibitor (GF109203x; P<0.05). The  
8 PGF<sub>2α</sub>-induced elevation in COX-2 Luciferase (Fig. 5C) and mRNA expression (Fig.  
9 5D) was significantly inhibited by co-treatment of cells with the FP receptor antagonist  
10 (AL8810), and chemical inhibitors of PLC (U73122), PKA (4C3MQ), EGFR kinase  
11 (AG1478) and ERK1/2 kinase (PD98059), but not the EP2 receptor antagonist  
12 (AH6809), EP4 receptor antagonist (ONOAE2227) or PKC inhibitor (GF109203x; Fig.  
13 5C and 5D, P<0.05).

14

15 *Mutation of the CRE-binding site in the COX-2 promoter inhibits PGE<sub>2</sub> and PGF<sub>2α</sub>*  
16 *mediated Luciferase activity.*

17 To determine which transcription factors were involved in mediating COX-2  
18 expression in response to PGE<sub>2</sub> or PGF<sub>2α</sub>, FPS cells were transiently transfected with the  
19 966bp full length COX-2 promoter (C2.1) or COX-2 promoter containing a series of  
20 deletions as described in Bradbury et al (Bradbury et al., 2003). PGE<sub>2</sub> or PGF<sub>2α</sub>  
21 increased COX-2 Luciferase activity by 2.2 ± 0.1 and 2.6 ± 0.1 fold respectively (Fig.  
22 6A; P<0.05). There was no significant reduction in Luciferase activity with any of the  
23 sequential 5' deletions compared with the C2.1, suggesting that even the smallest HIN

1 fragment of the COX-2 promoter, which contains only the CRE, was able to induce  
2 COX-2 Luciferase activity (Fig. 6A). Transfection of FPS cells with the HIN COX-2  
3 promoter fragment with a mutated CRE (HINcre<sup>m</sup>; -59/-53) abolished the COX-2  
4 activity induced by PGE<sub>2</sub> or PGF<sub>2α</sub> (Fig. 6A; P<0.05), suggesting that this factor is  
5 necessary for COX-2 induction by prostanoids.

6

7 *CRE is activated by PGE<sub>2</sub> and PGF<sub>2α</sub> signaling to ERK*

8 We further confirmed the signaling pathways activating the CRE in FPS cells  
9 transfected with cDNA construct containing a specific cis-acting DNA binding sequence  
10 of the cAMP response element ligated with a Luciferase reporter plasmid. Cells were  
11 treated with vehicle, 100nM PGE<sub>2</sub> or 100nM PGF<sub>2α</sub> in the presence/absence of the FP  
12 receptor antagonist (AL8810), EP2 receptor antagonist (AH6809), EP4 receptor  
13 antagonist (ONOAE2227), PLC inhibitor (U73122), PKA inhibitor (4C3MQ), PKC  
14 inhibitor (GF109203x), EGFR kinase inhibitor (AG1478) or ERK1/2 inhibitor  
15 (PD98059) for 4 hours. CRE Luciferase activity in FPS cells was significantly elevated  
16 in response to PGE<sub>2</sub> (Fig. 6B) or PGF<sub>2α</sub> (Fig. 6C) treatment for 4 hours (P<0.05).

17 The PGE<sub>2</sub> (Fig. 6B) induced activation of CRE Luciferase was significantly  
18 reduced by treatment of FPS cells with the FP receptor (AL8810), EP2 receptor  
19 (AH6809), or EP4 receptor (ONOAE2227) antagonists or chemical inhibitors of PLC  
20 (U73122), PKA (4C3MQ), EGFR kinase (AG1478) or ERK1/2 kinase (PD98059), but  
21 not the PKC inhibitor (GF109203x; Fig. 6B P<0.05). The PGF<sub>2α</sub> (Fig. 6C) induced  
22 activation of CRE Luciferase was significantly inhibited by co-treatment of cells with  
23 the FP receptor antagonist (AL8810) or chemical inhibitors of PLC (U73122), PKA

1 (4C3MQ), EGFR kinase (AG1478) and ERK1/2 kinase (PD98059), but not the EP2  
2 receptor antagonist (AH6809), EP4 receptor antagonist (ONOAE2227) or PKC inhibitor  
3 (GF109203x; Fig. 6C;  $P < 0.05$ ).

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## 1 Discussion

2 COX-2 expression is up-regulated in numerous pathologies including those of  
3 the reproductive tract such as ovarian carcinoma, cervical carcinoma and endometrial  
4 adenocarcinoma (Dore et al., 1998; Tong et al., 2000; Jabbour et al., 2001; Sales et al.,  
5 2001; Sales et al., 2002). The augmented biosynthesis of prostaglandins produced as a  
6 consequence of elevated COX-2 expression has been shown to promote tumorigenesis  
7 (Watanabe et al., 2000; Sonoshita et al., 2001; Seno et al., 2002) by interacting with  
8 specific prostaglandin receptors, which are also overexpressed in the same tumours.

9 We and others' have shown that PGE<sub>2</sub>-EP receptor interaction can down regulate  
10 the expression of tumour suppressor genes (Sales et al., 2004c), increase cellular growth,  
11 migration and invasiveness (Sheng et al., 2001) and promote angiogenesis in in vitro  
12 and in vivo model systems (Watanabe et al., 2000; Sonoshita et al., 2001; Seno et al.,  
13 2002; Buchanan et al., 2003; Fujino et al., 2003; Sales et al., 2004a). Similarly PGF<sub>2α</sub>  
14 can also enhance cell growth rate and induce the expression of inflammatory and  
15 angiogenic genes, in Ishikawa cells stably expressing the FP receptor and endometrial  
16 adenocarcinoma explants, via the FP receptor (Sales et al., 2004b; Jabbour et al., 2005;  
17 Sales et al., 2005).

18 We have recently reported elevated expression of FP receptor in endometrial  
19 adenocarcinomas (Sales et al., 2004b; Sales et al., 2005). Since endometrial pathologies  
20 biosynthesize PGE<sub>2</sub> and PGF<sub>2α</sub> in the micromolar range (Lundstrom and Green, 1978;  
21 Smith et al., 1981; Rees et al., 1984; Adelantado et al., 1988) which can act locally at  
22 the site of production on prostaglandin receptors and since PGE<sub>2</sub> can bind to the FP  
23 receptor with an affinity that is only 10-30 fold less than PGF<sub>2α</sub> (Abramovitz et al.,

1 2000), we investigated the effect of PGE<sub>2</sub> and PGF<sub>2α</sub> on regulation of COX-2 via the FP  
2 receptor.

3 Using endometrial adenocarcinoma cells (Ishikawa cells) stably expressing the  
4 FP receptor to the levels observed in endometrial adenocarcinomas (FPS cells), we  
5 found that PGE<sub>2</sub> dose-dependently mobilized InsP hydrolysis in FPS cells in a similar  
6 manner to that observed for PGF<sub>2α</sub> via the FP receptor, since the specific FP receptor  
7 antagonist AL8810 abolished the PGE<sub>2</sub> and PGF<sub>2α</sub>-mediated increase in InsP  
8 production. Although the PGE<sub>2</sub>-induced mobilization of InsP via the FP receptor was  
9 less than that observed for PGF<sub>2α</sub>, which we postulated was due to the reduced affinity  
10 of PGE<sub>2</sub> for the FP receptor compared with the native ligand PGF<sub>2α</sub>, these data  
11 nevertheless demonstrate that elevated levels of PGF<sub>2α</sub> as well PGE<sub>2</sub> as can activate FP  
12 receptor signaling in tumors expressing elevated levels of FP receptor.

13 As FPS cells also express basal levels of EP2 and EP4 receptor (but not  
14 detectable EP1 or EP3 receptor), we found that PGE<sub>2</sub> and to a much lesser extent PGF<sub>2α</sub>  
15 could also mobilize intracellular cAMP. This effect of PGE<sub>2</sub> on intracellular cAMP  
16 accumulation could be inhibited with the selective EP2 or EP4 receptor antagonists  
17 AH6809 and ONOAE2227 and abolished with the combination of EP2 and EP4 receptor  
18 antagonists. Interestingly only the EP2 receptor antagonist, but not the EP4 receptor  
19 antagonist abolished the PGF<sub>2α</sub>- mediated increase in cAMP. We found that neither the  
20 PGE<sub>2</sub> nor PGF<sub>2α</sub> effects on inositol phosphate hydrolysis and intracellular cAMP release  
21 was mediated by an intracellular mechanism involving activation of the downstream

1 PKA, PKC or ERK1/2 pathways, as the chemical inhibitors of PKA, PKC and ERK1/2  
2 failed to reduced the prostanoid mediated effects on InsP and cAMP accumulation.

3 The integrated response to GPCR coupling and second messenger activation  
4 results in phosphorylation of numerous effector signaling pathways, including the  
5 MAPK pathway (Naor et al., 2000), to regulate gene transcription. The MAPK pathway  
6 is a key signaling mechanism that regulates many cellular functions such as growth,  
7 differentiation and transformation (Lewis et al., 1998; Naor et al., 2000). We  
8 investigated the signaling pathways mediating the effect of PGE<sub>2</sub> or PGF<sub>2α</sub> on ERK1/2  
9 in FPS cells. We found that PGE<sub>2</sub> and PGF<sub>2α</sub> stimulation of FPS cells induces ERK1/2  
10 phosphorylation via identical intracellular signaling pathways via the PLC-PKA-  
11 mediated activation of the EGFR since co-treatment of cells with the PLC, PKA or  
12 EGFR kinase inhibitors significantly inhibited the PGE<sub>2</sub> or PGF<sub>2α</sub> induced ERK1/2  
13 phosphorylation. We previously reported that PGF<sub>2α</sub> signaling to ERK1/2 in FPS cells is  
14 mediated via the PKA and not PKC pathway (Sales et al., 2005). In the present study we  
15 demonstrate that the PGE<sub>2</sub>-mediated signaling to ERK1/2 in FPS cells is also PKA  
16 dependent and PKC-independent, since the PKC inhibitor GF109203x failed to inhibit  
17 either the PGE<sub>2</sub> or PGF<sub>2α</sub>-mediated signaling to ERK1/2. Moreover, we found that the  
18 PGE<sub>2</sub>-mediated effects on ERK1/2 are mediated largely in FPS cells via the FP receptor  
19 as the FP receptor antagonist inhibited the PGE<sub>2</sub>-mediated ERK1/2 phosphorylation to a  
20 greater extent than the EP2 or EP4 receptor antagonists. These data suggest that the  
21 cAMP pathway activated by the endogenous EP2/EP4 receptors acts synergistically with  
22 the InsP pathway to augment the signaling of PGE<sub>2</sub> to ERK1/2 via the PKA-EGFR  
23 pathway.

1 We previously reported that  $\text{PGF}_{2\alpha}$  could regulate COX-2 expression in an  
2 autocrine/paracrine manner to establish a positive feedback system for regulating  
3 endometrial tumorigenesis (Jabbour et al., 2005). In the present study we have shown  
4 that  $\text{PGE}_2$  can regulate COX-2 promoter activity and mRNA expression in a similar  
5 manner via the FP receptor as observed for  $\text{PGF}_{2\alpha}$  via the PLC-PKA-EGFR-ERK1/2  
6 signaling cascade. However as observed for the  $\text{PGE}_2$ -mediated effects on second  
7 messenger production and intracellular signaling reported herein, the  $\text{PGE}_2$ -mediated  
8 increase in COX-2 expression via the FP receptor is also less than that produced by the  
9 native ligand  $\text{PGF}_{2\alpha}$ . We believe this difference is due to the lower binding affinity of  
10  $\text{PGE}_2$  for the FP receptor compared with  $\text{PGF}_{2\alpha}$  (Abramovitz et al., 2000). In addition,  
11 we found that the  $\text{PGE}_2$  activation of COX-2 promoter and mRNA expression was  
12 significantly reduced by the EP2 and EP4 receptor antagonists. This effect was not  
13 observed for  $\text{PGF}_{2\alpha}$ . Thus it would appear that unlike the  $\text{PGF}_{2\alpha}$ -mediated activation of  
14 COX-2 that occurs solely via the Gq activation of InsP, the  $\text{PGE}_2$  regulation of COX-2  
15 in FPS cells is mediated by the synergistic effects of the cAMP and InsP second  
16 messenger systems via the PKA-EGFR-ERK1/2 pathway.

17 We next investigated the transcription regulatory regions within the COX-2  
18 promoter activated by  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$  in FPS cells. The COX-2 promoter has binding  
19 sites for a number of transcription factors including nuclear factor (NF)- $\kappa\beta$ ,  
20 CCAAT/enhancer binding protein (C/EBP), AP-2 and cAMP response element (CRE)  
21 (Lukiw et al., 1998). Transfection studies with the cis-acting DNA binding sequence of  
22 the CRE or the full length C2.1 COX-2 promoter and a series of deletions containing  
23 key transcription factor-binding sites showed that promoter activity was maintained with

1 a construct that had a CRE region only (RSA/HIN). Mutation of the CRE region of this  
2 construct resulted in complete loss of COX-2 promoter activity in response to  
3 administration of either PGE<sub>2</sub> or PGF<sub>2α</sub> indicating that the CRE is essential for  
4 transcriptional activation of the COX-2 gene by prostaglandins. The regulation of the  
5 CRE by PGE<sub>2</sub> and PGF<sub>2α</sub> was further investigated by transient transfection studies using  
6 a cDNA construct containing the cis-acting DNA binding sequence of the CRE fused to  
7 a luciferase reporter system. These studies showed that CRE activation in FPS cells is  
8 mediated by PGE<sub>2</sub> and PGF<sub>2α</sub> via the same mechanisms regulating COX-2, namely by  
9 activation of the PKA-EGFR-ERK1/2 pathways further confirming the importance of  
10 the CRE in the regulation of COX-2 activity in FPS cells.

11 Because PGE<sub>2</sub> and PGF<sub>2α</sub> mobilize intracellular cAMP and can activate protein  
12 kinase A (PKA) and since the PKA inhibitor 4C3MQ inhibited both the PGE<sub>2</sub> and  
13 PGF<sub>2α</sub> -mediated signaling to ERK1/2 and COX-2 it is plausible that the mode of action  
14 of prostanoid signaling on activation of the CRE was by phosphorylation of CRE  
15 binding protein (CREB) at SER133, which can in turn bind the CRE and activate gene  
16 transcription. In the present study, we did not observe any significant phosphorylation of  
17 CREB at SER133 in response to agonist treatment by either PGE<sub>2</sub> or PGF<sub>2α</sub> by Western  
18 blot analysis (data not shown). Although activation of COX-2 by CREB binding to the  
19 CRE has been shown by Bradbury et al (Bradbury et al., 2003), Subbaramaiah et al  
20 (Subbaramaiah et al., 2002a; Subbaramaiah et al., 2002b) have shown that COX-2  
21 transcriptional activation via the CRE binding site can be mediated by activator protein  
22 (AP)-1. It is feasible that COX-2 transcriptional activation via the CRE in our study may

1 be regulated via the binding of an alternative transcription factor or transcription factor  
2 complex to CREB, such as AP1.

3 In conclusion, our data provide strong evidence that the elevated biosynthesis of  
4 PGE<sub>2</sub> and PGF<sub>2α</sub> produced locally within endometrial adenocarcinomas can act in an  
5 autocrine/paracrine manner to enhance the expression of COX-2 via the CRE by means  
6 of their integrative actions on intracellular signaling pathways such as the ERK1/2  
7 pathway. Moreover we believe that these data have implications for the use of  
8 prostaglandin synthase inhibitors targeted against PGF synthase as therapeutic  
9 intervention strategies as suggested for PGE synthase (Murakami and Kudo, 2006;  
10 Jachak 2007; Wang et al., 2006; Cheng et al., 2006). Our data highlight that in tumours  
11 expressing elevated levels of FP receptor, elevated biosynthesis of other prostanoids  
12 such as PGE<sub>2</sub> can in the absence of the native ligand PGF<sub>2α</sub> activate tumorigenic genes  
13 via the FP receptor. It is thus feasible that signaling pathways such as the EGFR or ERK  
14 pathways, which integrate the signaling from second messenger systems to target genes,  
15 may offer a better therapeutic target to reverse the adverse effects of prostanoid  
16 signaling, or indeed signaling in response to multiple prostanoids, in cancer.

17

18

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3 of the HIN and HINrem constructs.

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1 **Figure Legends**

2

3 **Fig 1. (A)** Total inositol phosphate (InsP) production was assessed in Ishikawa WT or  
4 FPS cells treated with increasing doses of PGE<sub>2</sub> or PGF<sub>2α</sub> for 1 hour at 37°C. **(B)** Total  
5 InsP production in FPS cells treated with increasing doses of PGE<sub>2</sub> or PGF<sub>2α</sub> in the  
6 absence or presence of 50μM of the specific FP receptor antagonist AL8810 for 1 hour  
7 at 37°C. **(C)** Total InsP production in FPS cells treated with 100nM PGE<sub>2</sub> or 100nM  
8 PGF<sub>2α</sub> in the absence/presence of the FP receptor antagonist AL8810 (50μM), EP2  
9 receptor antagonist (AH6809; 10μM), EP4 receptor antagonist (ONOAE2227; 1μM) or  
10 chemical inhibitors of phospholipase Cβ (U73122, 10μM), protein kinase A (4C3MQ,  
11 1μM), protein kinase C (GF109203x, 10μM) or ERK1/2 kinase (PD98059, 50μM) for 1  
12 hour at 37°C. Data are presented as mean ± SEM. b is significantly different from a, c is  
13 significantly different from a and b; P<0.05.

14

15 **Fig. 2. (A)** cAMP accumulation in FPS cells in response to treatment with 100nM PGE<sub>2</sub>  
16 or 100nM PGF<sub>2α</sub> for 0, 5 and 10 minutes. **(B)** cAMP accumulation in FPS cells in  
17 response to treatment with 100nM PGE<sub>2</sub> or 100nM PGF<sub>2α</sub> for 10 minutes in the  
18 presence/absence of the FP receptor antagonist AL8810 (50μM), EP2 receptor  
19 antagonist (AH6809; 10μM), EP4 receptor antagonist (ONOAE2227; 1μM) or chemical  
20 inhibitors of phospholipase Cβ (U73122, 10μM), protein kinase A (4C3MQ, 1μM),  
21 protein kinase C (GF109203x, 10μM) or ERK1/2 kinase (PD98059, 50μM). Data are

1 presented as mean  $\pm$  SEM. b is significantly different from a and c is significantly  
2 different from a and b;  $P < 0.05$ .

3

4 **Fig 3. (A)** ERK1/2 phosphorylation in FPS cells in response to treatment with 100nM  
5 PGE<sub>2</sub> or 100nM PGF<sub>2 $\alpha$</sub>  for 0, 2, 5, 10 and 20 minutes. ERK1/2 phosphorylation in FPS  
6 cells in response to treatment with 100nM PGE<sub>2</sub> **(B)** or 100nM PGF<sub>2 $\alpha$</sub>  **(C)** for 10  
7 minutes in the presence/absence of the FP receptor antagonist AL8810 (50 $\mu$ M), EP2  
8 receptor antagonist (AH6809; 10 $\mu$ M), EP4 receptor antagonist (ONOAE2227; 1 $\mu$ M) or  
9 chemical inhibitors of phospholipase C $\beta$  (U73122, 10 $\mu$ M), protein kinase A (4C3MQ,  
10 1 $\mu$ M), protein kinase C (GF109203x, 10 $\mu$ M), EGFR kinase (AG1478, 200nM) or  
11 ERK1/2 kinase (PD98059, 50 $\mu$ M). Data are presented as mean  $\pm$  SEM. b is significantly  
12 different from a and c is significantly different from a and b;  $P < 0.05$ .

13

14 **Fig 4. COX-2 luciferase activity (A) and mRNA expression (B)** in FPS cells in response  
15 to treatment with 100nM PGE<sub>2</sub> or 100nM PGF<sub>2 $\alpha$</sub>  for 0, 2, 4, 6, 8 and 24 hours. Data are  
16 presented as mean  $\pm$  SEM. b is significantly different from a, c is significantly different  
17 from a and b;  $P < 0.05$ .

18

19 **Fig 5. COX-2 luciferase activity (A, C) and mRNA expression (B, D)** in FPS cells in  
20 response to treatment with 100nM PGE<sub>2</sub> **(A and B)** or 100nM PGF<sub>2 $\alpha$</sub>  **(C and D)** for 4  
21 hours in the presence/absence of the FP receptor antagonist AL8810 (50 $\mu$ M), EP2  
22 receptor antagonist (AH6809; 10 $\mu$ M), EP4 receptor antagonist (ONOAE2227; 1 $\mu$ M) or  
23 chemical inhibitors of phospholipase C $\beta$  (U73122, 10 $\mu$ M), protein kinase A (4C3MQ,

1 1 $\mu$ M), protein kinase C (GF109203x, 10 $\mu$ M), EGFR kinase (AG1478, 200nM) or  
2 ERK1/2 kinase (PD98059, 50 $\mu$ M). Data are presented as mean  $\pm$  SEM. b is significantly  
3 different from a and c is significantly different from a and b, d is significantly different  
4 from a, b and c; P<0.05.

5

6 **Fig. 6. (A)** COX-2 luciferase activity in FPS cells transiently transfected with the full  
7 length COX-2 promoter C2.1 or series of 5' deletions (DRA, STY, ALU, RSA, HIN)  
8 and HIN with a mutation in the cAMP response element (HINcrem; -59/-53). FPS cells  
9 were treated with vehicle, 100nM PGE<sub>2</sub> or 100nM PGF<sub>2 $\alpha$</sub>  for 4 hours. CRE Luciferase  
10 activity in FPS cells transiently transfected with the cis-acting DNA binding sequence of  
11 the cAMP response element (CRE). FPS cells were treated with 100nM PGE<sub>2</sub> **(B)** or  
12 100nM PGF<sub>2 $\alpha$</sub>  **(C)** for 4 hours in the presence/absence of the FP receptor antagonist  
13 AL8810 (50 $\mu$ M), EP2 receptor antagonist (AH6809; 10 $\mu$ M), EP4 receptor antagonist  
14 (ONOAE2227; 1 $\mu$ M) or chemical inhibitors of phospholipase C $\beta$  (U73122, 10 $\mu$ M),  
15 protein kinase A (4C3MQ, 1 $\mu$ M), protein kinase C (GF109203x, 10 $\mu$ M), EGFR kinase  
16 (AG1478, 200nM) or ERK1/2 kinase (PD98059, 50 $\mu$ M). Data are presented as mean  $\pm$   
17 SEM. b is significantly different from a and c is significantly different from a and b;  
18 P<0.05.

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Table 1: List of reagents summarising the targets of each compound.

| <b>Compound</b> | <b>Target</b>  | <b>Reference</b>       |
|-----------------|--|------------------------|
| AL8810          | FP receptor antagonist                                       | Griffen et al., 1999   |
| AH6809          | EP2 receptor antagonist                                      | Woodward et al., 1995  |
| ONOAE2227       | EP4 receptor antagonist                                      | Mutoh et al., 2002     |
| U73122          | PLC beta inhibitor   | Bleasdale et al., 1990 |
| 4C3MQ           | Protein kinase A inhibitor                                   | Lu et al., 1996        |
| GF109203X       | Protein kinase C inhibitor                                   | Toullec et al., 1991   |
| AG1478          | Epidermal growth factor receptor tyrosine kinase inhibitor   | Eguchi et al., 1998    |
| PD98059         | Extracellular signal-regulated kinase kinase (MEK) inhibitor | Alessi et al., 1995    |

Figure 1

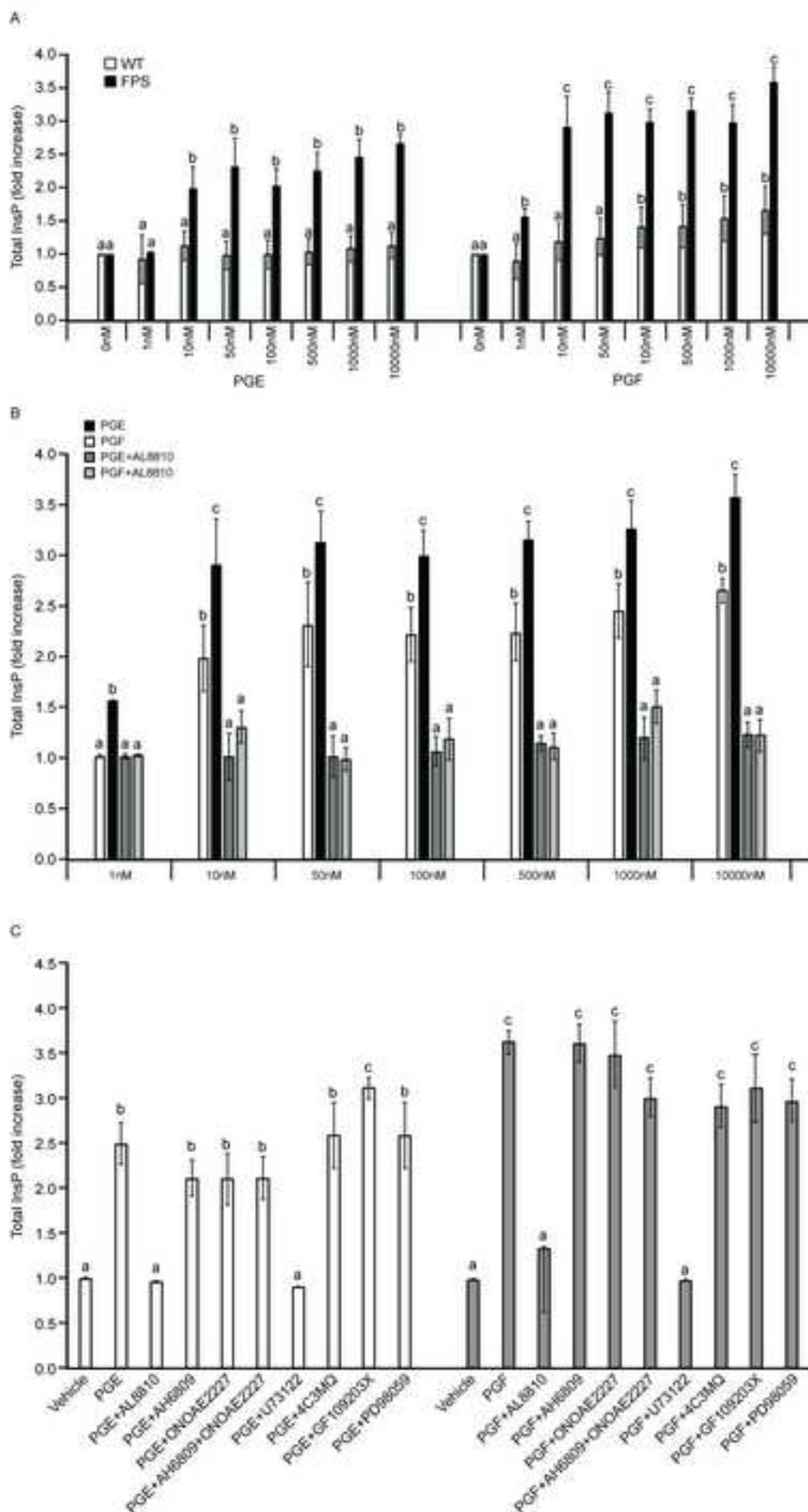
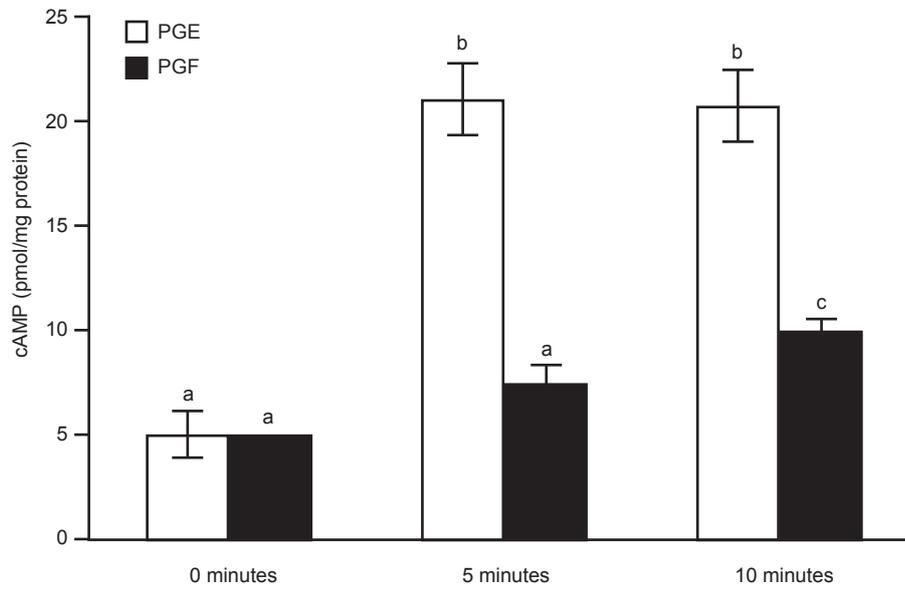


Figure 2

A



B

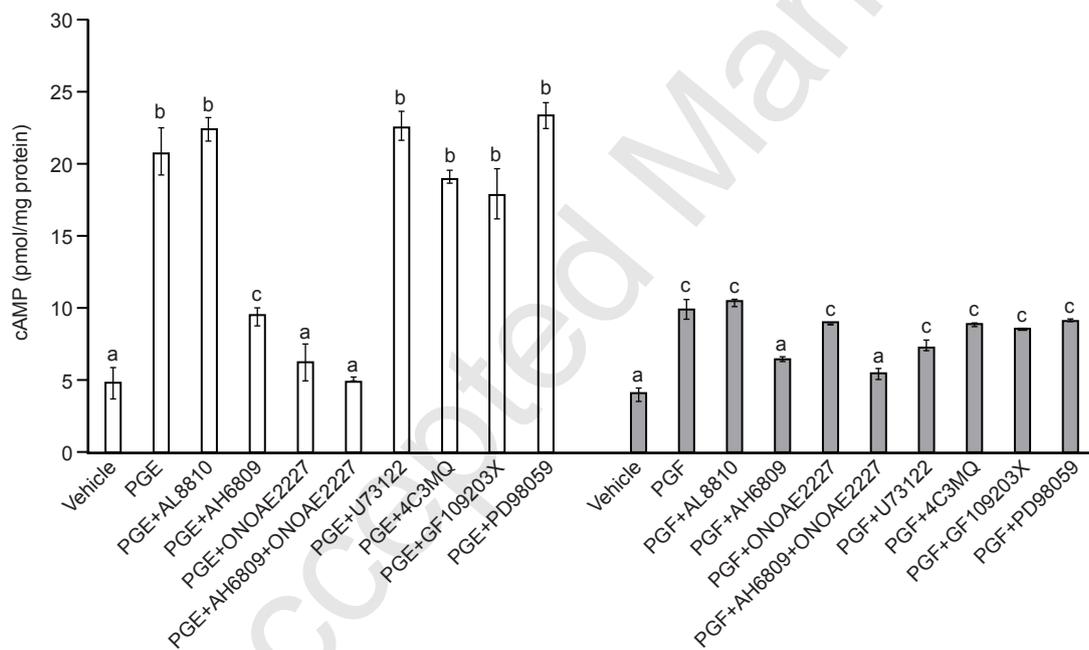


Figure 3

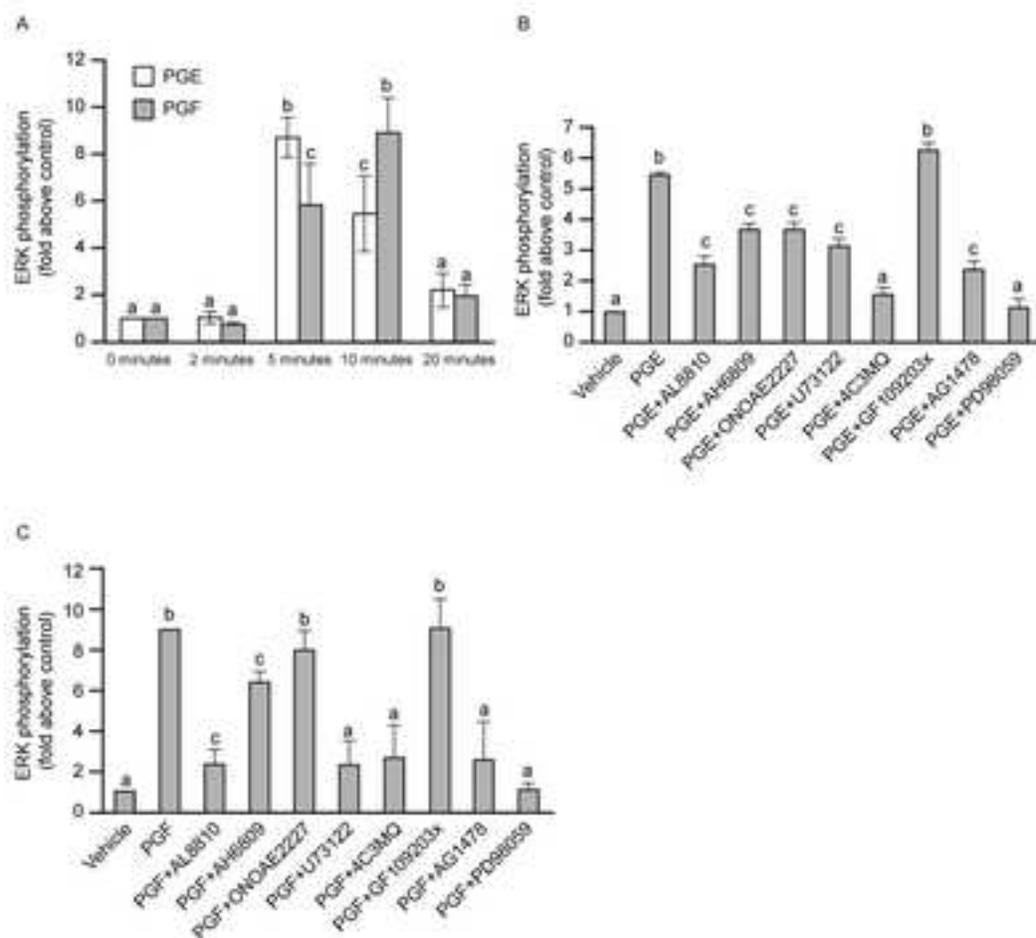
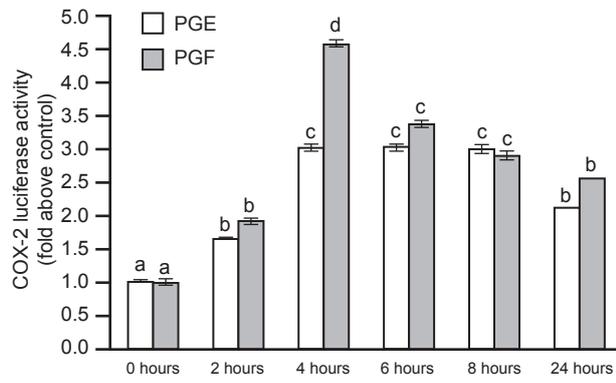


Figure 4

A



B

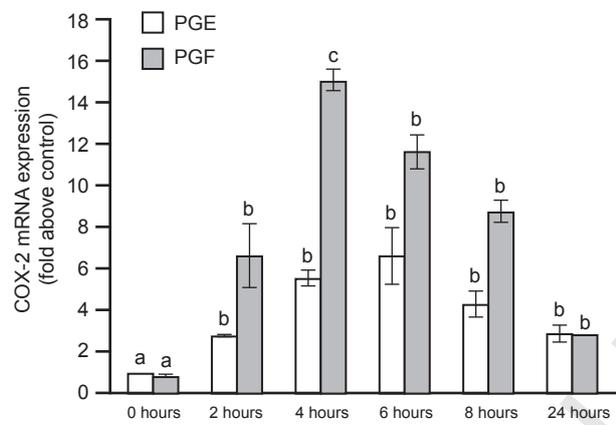
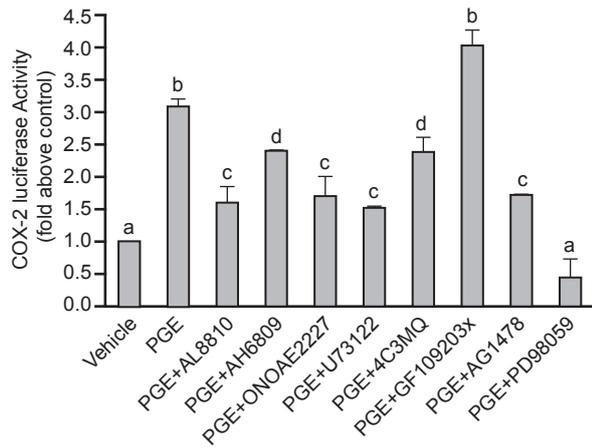
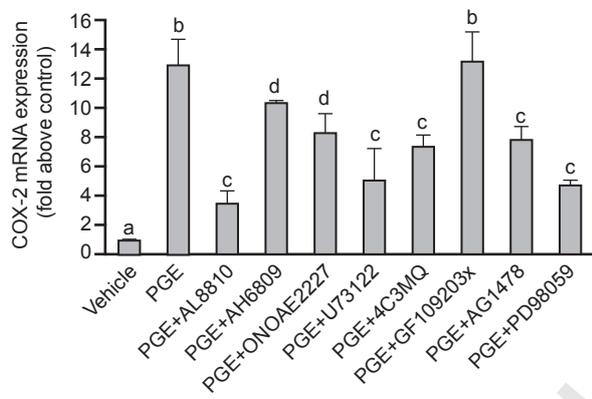


Figure 5

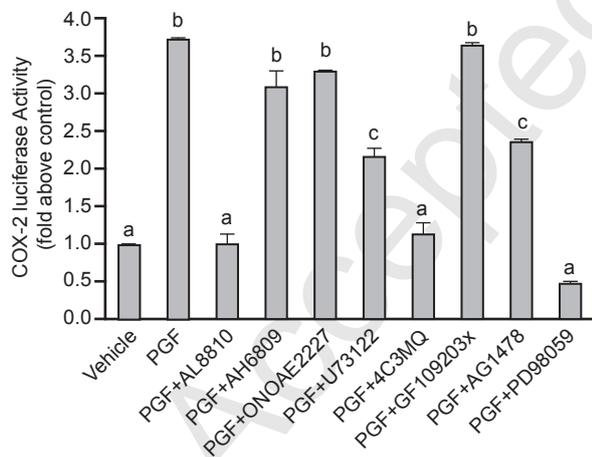
A



B



C



D

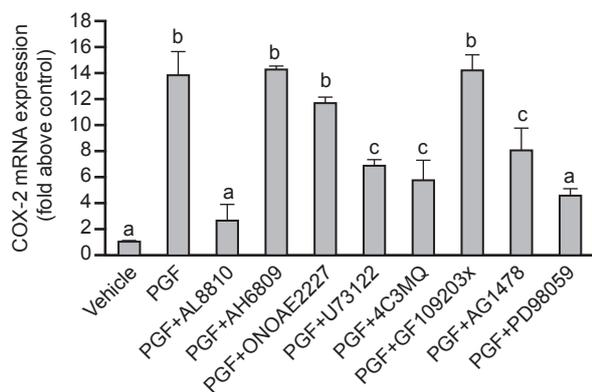


Figure 6

