F-prostanoid receptor alters adhesion, morphology and migration of endometrial adenocarcinoma cells

KJ Sales, **SC Boddy**, and **HN Jabbour**
MRC Human Reproductive Sciences Unit, Medical Research Council, The Queen's Medical Research Institute, Edinburgh, UK

Abstract

Cellular adhesion to extracellular matrix is a central phenomenon for the maintenance of tissue integrity and cellular movement. Collectively, these processes are regulated by a fine-tuned balance between the formation and loosening of adhesive contacts, a process involving integrins, and the elevation and diminution of cytoplasmic signalling molecules. We demonstrate that prostaglandin (PG) $F_{2\alpha}$ stimulation rapidly increases the capacity of Ishikawa cells stably expressing the F-prostanoid receptor (FPS) to adhere to vitronectin. Coincident with this elevation in matrix adhesion, we demonstrate a profound $PGF_{2\alpha}$-induced alteration in cytoskeletal remodelling, characterized by polymerization of the actin cytoskeleton and recruitment of focal adhesion kinase at focal adhesions and enhanced cell migration. Moreover, we show that these $PGF_{2\alpha}$-induced alterations in adhesion and morphology on vitronectin and migration could be abolished by cultivating FPS cells in the presence of integrin $\alpha v \beta 3$ antibody or $\alpha v \beta 3$-directed tetrapeptide arg–gly–asp–ser or inhibition of FP receptor signalling with the FP receptor antagonist, chemical disruptors of the phospholipase C-β, protein kinase A, c-Src and epidermal growth factor receptor kinase pathways or inhibition of the monomeric G proteins Rho, Rac and CDC42. These results reveal a mechanism by which prostanoids regulate cell movement, which may be relevant to pathologies of the endometrium.

Keywords

$PGF_{2\alpha}$; FP receptor; focal adhesions; morphology; migration; chemotaxis

Introduction

Activation of G-protein–coupled receptors (GPCRs) like the F-prostanoid receptor (FPS) regulates heterotrimeric G-protein pathways to control cell growth and behaviour, via the phosphorylation and dephosphorylation of distal-signalling complexes (Daub *et al.*, 1997; Luttrell *et al.*, 1997). These signalling complexes either serve as scaffolds for the plasma membrane recruitment of guanine nucleotide exchange factors (GEFs) for small monomeric G proteins (Rho, Ras, Rac and CDC42) or are recruited to the cytoskeletal contact point with the plasma membrane, where they serve as docking proteins (Lefkowitz, 1993; Luttrell *et al.*, 1997, 1999). One such docking protein at the site where integrin- and proteoglycan-mediated adhesion links to the actin cytoskeleton is focal adhesion tyrosine kinase (FAK) (Wozniak *et al.*, 2004). FAK is a non-receptor tyrosine kinase that forms part of the plasma membrane focal adhesion complex, which assembles on integrin heterodimers following

Correspondence: Dr HN Jabbour, MRC Human Reproductive Sciences Unit, Medical Research Council, The Queen's Medical Research Institute, 47 Little France Crescent, Old Dalkeith Road, Edinburgh, EH16 4TJ, UK. E-mail: h.jabbour@hrsu.mrc.ac.uk.

integrin engagement of extracellular matrix (ECM) proteins (Morino *et al.*, 1995; Giancotti and Ruoslahti, 1999). ECM proteins interact with integrins via the arg–gly–asp (RGD) motif at focal adhesion sites to regulate cell signalling and motility (Giancotti and Ruoslahti, 1999; Brakebusch *et al.*, 2002; Wozniak *et al.*, 2004).

Integrin signalling via reorganization of the actin cytoskeleton at focal adhesions has been shown to activate the monomeric G proteins Rho, Rac and CDC42, resulting in membrane extension (Del Pozo *et al.*, 2002; Wozniak *et al.*, 2004) to facilitate cell spreading or migration as well as modulate the signalling of other effector molecules, complexed within the integrin-linked actin-bound scaffold (Clark and Brugge, 1995; Morino *et al.*, 1995). Agents that disrupt cytoskeletal assembly or that block activation of the small monomeric G-protein Rho have been shown to inhibit FAK phosphorylation and focal adhesions (Slack, 1998; Davidson *et al.*, 2004b).

Prostaglandins (PGs) are considered to play a major role in benign and neoplastic endometrial pathologies, where biosynthesis of PGE_2 and $PGF_{2\alpha}$ is elevated (Singh *et al.*, 1975; Lundstrom and Green, 1978; Lumsden *et al.*, 1983; Sales and Jabbour, 2003b). We have demonstrated elevated expression of FP receptor in human endometrial adenocarcinomas and ascertained a role for FP receptor in promoting angiogenic and tumourigenic gene expression both in Ishikawa FPS cells and endometrial adenocarcinoma biopsies (Sales *et al.*, 2004b, 2005; Jabbour *et al.*, 2005). Moreover in these studies we have shown that $PGF_{2\alpha}$–FP receptor signalling establishes a positive feedback loop to sustain the biosynthesis of prostanoids and transcription of cyclooxygenase-2 (COX-2) and fibroblast growth factor via the activation of $G_{q/11}$ and hydrolysis of phosphatidylinositol (Jabbour *et al.*, 2005; Sales *et al.*, 2007). In many cell types $G_{q/11}$ coupling leads to the activation of FAK in an adhesion-dependent manner via the activation of small monomeric G proteins such as Rho (Rodriguez-Fernandez and Rozengurt, 1996; Pierce *et al.*, 1999; Fujino *et al.*, 2000; Davidson *et al.*, 2004b). Disruption of focal adhesions can abolish activation of FAK (Slack, 1998), leading to an ablation of agonist-mediated cell signalling (Davidson *et al.*, 2004b). In colonic cancers, breast cancers and sarcoma, FAK is overexpressed and plays a role in cancer cell–ECM interactions, adhesion, cell spreading, invasion and metastasis (Weiner *et al.*, 1993).

In the present study we investigated the role of $PGF_{2\alpha}$ in mediating adhesion, morphology and migration of endometrial adenocarcinoma cells via the FP receptor using Ishikawa cells stably expressing FP receptor to the levels observed in endometrial adenocarcinomas.

Results

$PGF_{2\alpha}$–FP receptor stimulation of FPS cells induces alterations in cell morphology

In the present study we observed that upon stimulation of FPS cells, but not wild-type (WT) cells, with $PGF_{2\alpha}$ there are rapid changes in cellular morphology and lamellipodia formation coincident with reorganization of the actin cytoskeleton (Figure 1a, panel vi) and redistribution of microtubules within the cell (Figure 1b, panel vi). These morphological changes in response to $PGF_{2\alpha}$ were inhibited by co-incubation of FPS cells with the specific FP receptor antagonist AL8810 (Figures 1a and b, panel vii). These alterations in cellular morphology were not observed when WT or FPS cells were treated with 100 n$_M$ Iloprost, used as a control eicosanoid (Figures 1a and b, panel viii).

$PGF_{2\alpha}$–FP receptor stimulation enhances the adhesive capacity of FPS cells to ECM

Coincident with the agonist-induced cytoskeletal remodelling in FPS cells, we observed an enhanced capacity of FPS cells, but not WT cells, to adhere to the ECM vitronectin (Figure 2a). No alteration in adherence capacity of WT or FPS cells was observed on fibronectin,

laminin, collagen I or IV in response to $PGF_{2\alpha}$ treatment (Figure 2a). The cellular adherence effects of FPS cells to vitronectin in response to $PGF_{2\alpha}$ were abolished by co-incubation of FPS cells with the FP receptor antagonist AL8810 (Figure 2b). In addition to the morphological and adhesion alterations, $PGF_{2\alpha}$ also enhanced the migratory capacity of FPS cells via the FP receptor, since incubation of FPS cells with the specific FP receptor antagonist AL8810 abolished the $PGF_{2\alpha}$-induced increase in cell migration (Figure 2c).

Displacement of the extracellular region of the integrin from the matrix protein with the $\alpha v \beta 3$-directed tetrapeptide arg–gly–asp–ser (RGDS) abolished the agonist-induced cellular adherence effects of FPS cells to vitronectin as well as the ability of FPS cells to migrate towards the $PGF_{2\alpha}$ stimulus (Figures 2b and c). In contrast, co-incubation of FPS cells with arg–gly–glu–ser (RGES) tetrapeptide as a negative control did not alter the agonist-induced cell adhesion to vitronectin or increase in FPS cell migration (Figures 2b and c). Similar effects were observed using an integrin $\alpha v \beta 3$ antibody or immunoglobulin G (IgG) from the same host species (Figures 2b and c).

$PGF_{2\alpha}$-induced alterations in cell morphology is dependant on integrin αvβ3 engagement with vitronectin

We investigated whether the $PGF_{2\alpha}$-induced alterations in cell morphology was dependant on integrin $\alpha v \beta 3$ engagement with vitronectin. FPS cells incubated with RGDS tended to round up and become easily dislodged from the vitronectin-coated growth plate. Stimulation of these cells with vehicle (Figure 3Aiv) or 100 n_M $PGF_{2\alpha}$ (Figure 3Biv) for 4 h resulted in no apparent alteration in cell morphology as shown in the phase contrast fields. FPS cells treated with the control RGES tetrapeptide showed dramatic alterations in cell morphology after 4 h of $PGF_{2\alpha}$ treatment (Figure 3Div), compared with vehicle-treated cells (Figure 3Civ) as observed in the phase contrast fields.

The alteration in FPS cell morphology in response to agonist treatment in the presence of the control RGES tetrapeptide was associated with redistribution of the actin cytoskeleton at focal adhesions (Figure 3Di) compared to cells treated with vehicle (Figure 3Ci). This alteration in actin distribution at focal adhesions in the $PGF_{2\alpha}$-treated FPS cells in the presence of RGES was also associated with the coincident expression of FAK at focal adhesions. Here FAK (Figure 3Dii) was observed to co-localize (Figure 3Diii) with actin (Figure 3Di) in the $PGF_{2\alpha}$-treated FPS cells.

Disruption of the integrin–ECM interaction using the RGDS tetrapeptide caused a destabilization of the integrin α–β dimer and destruction of the focal adhesion complex. Under these conditions, actin and FAK were localized in separate subcellular compartments in presence (Figure 3Biii) or absence (Figure 3Aiii) of $PGF_{2\alpha}$. Similar effects in cellular morphology were observed using IgG or integrin $\alpha v \beta 3$ antibody in place of RGDS or RGES tetrapeptides (data not shown).

$PGF_{2\alpha}$–FP receptor interaction in FPS cells activates cellular structural-associated proteins

We investigated the signalling pathways activating various cellular structural-associated proteins involved in reorganization of the cytoskeleton. We found that $PGF_{2\alpha}$–FP receptor stimulation of FPS cells rapidly mobilized the soluble cytoplasmic second messenger system inositol 1,4,5 trisphosphate (IP3) to a greater extent in FPS cells compared with WT cells. This effect was abolished by co-incubation of cells with the FP receptor antagonist AL8810 or chemical inhibitor of phospholipase C-β (PLC-β) U73122 (Figure 4a). Stimulation of FPS cells with $PGF_{2\alpha}$ caused a dramatic time-dependent activation of the non-receptor tyrosine kinase c-Src (Figure 4b), the microtubule-associated protein kinase (also called

mitogen-activated protein kinase; MAPK) extracellular signal-regulated kinase (ERK; Figure 4c) and FAK (Figure 4d). These effects of $PGF_{2\alpha}$ stimulation on c-Src, ERK and FAK phosphorylation were significantly elevated in FPS cells compared with WT cells. Within our experimental paradigms, the activation of c-Src and ERK were very rapid, occurring maximally within 5 and 10 min, respectively. However the activation of FAK occurred at a much later time, with maximal phosphorylation occurring at 4 h. This time of activation of FAK by $PGF_{2\alpha}$ coincides with the agonist-induced alteration in cellular morphology and the co-expression of FAK at focal adhesions.

$PGF_{2\alpha}$-mediated activation of c-Src, ERK and FAK occurs in a sequential manner via the FP receptor

We investigated $PGF_{2\alpha}$–FP receptor signalling to c-Src at Tyr^{418}. We found that phosphorylation of c-Src^{y418}, after 5 min of treatment with $PGF_{2\alpha}$, was sensitive to inhibition of cell signalling with the specific FP receptor antagonist AL8810 (Figure 5a, lane 3) or small molecule chemical inhibitors of PLC-β (U73122; Figure 5a, lane 4) or protein kinase A (PKA) (4C3MQ; Figure 5a, lane 5). However phosphorylation of c-Src^{y418} by $PGF_{2\alpha}$ was insensitive to inhibition of cell signalling with the chemical inhibitor of the epidermal growth factor receptor (EGFR) tyrosine kinase, AG1478 (Figure 5a, lane 6).

We subsequently investigated the signalling pathways mediating the $PGF_{2\alpha}$–FP receptor activation of ERK. FPS cells were transiently transfected with an Myc-tagged ERK cDNA construct and either empty vector cDNA (pcDNA3) or cDNA encoding a dominant-negative (Dn) mutant isoform of FAK, EGFR, c-Src, ERK kinase (mitogen-activated protein kinase kinase (MEK)) or small monomeric G-protein Rho, Ras, Rac or CDC42. Cells were either treated with vehicle or 100 n_M $PGF_{2\alpha}$ following transfection or pre-treated with the same panel of chemical inhibitors used in Figure 5a, with the inclusion of the c-Src kinase inhibitor PP2, for 1h prior to agonist stimulation for 10 min. As shown in Figure 5b, $PGF_{2\alpha}$ stimulation of FPS cells and immunoprecipitation of Myc-ERK showed a profound elevation of ERK activity (Figure 5b, lane 2) compared with vehicle-treated cells (Figure 5b, lane 1). This $PGF_{2\alpha}$-mediated phosphorylation of Myc-ERK was abolished when cells were co-incubated with AL8810 (Figure 5b, lane 3), U73122 (Figure 5b, lane 4), 4C3MQ (Figure 5b, lane 5), PP2 (Figure 5b, lane 6) and AG1478 (Figure 5b, lane 7) or by co-expression of the Dn-EGFR (Figure 5b, lane 9), Rho (Figure 5b, lane 10), Ras (Figure 5b, lane 11), Rac (Figure 5b, lane 12), CDC42 (Figure 5b, lane 13), c-Src (Figure 5b, lane 14) and MEK (Figure 5b, lane 15) cDNA isoforms with Myc-ERK. However when the tyrosine mutant FAK isoform, which displays an inability to bind c-Src (Schlaepfer and Hunter, 1997), was co-expressed with the Myc-ERK, there was no attenuation in ERK phosphorylation (Figure 5b, lane 8), demonstrating that tyrosine phosphorylation of FAK is not an inherent requirement for ERK activation in FPS cells by $PGF_{2\alpha}$. Previously we showed that $PGF_{2\alpha}$ activation of ERK in FPS cells, occurred independently of protein kinase C (Sales *et al.*, 2005). Interestingly in the present study we show a requirement for PKA activity in transducing the signal from the FP receptor to ERK.

We next investigated the signalling pathways mediating the phosphorylation of FAK by $PGF_{2\alpha}$ in FPS cells by co-transfecting a hemagglutinin (HA)-tagged WT FAK cDNA construct with either empty vector cDNA or cDNA encoding the Dn isoforms of the small monomeric G-protein Rho, Rac, CDC42 or Dn isoforms of EGFR, MEK or c-Src. Following transfection, cells were stimulated with vehicle or 100 n_M $PGF_{2\alpha}$ for 4 h or pre-incubated with the FP receptor antagonist AL8810 or small molecule chemical inhibitors of PLC-β (U73122), PKA (4C3MQ), c-Src (PP2), EGFR (AG1478) or MEK (PD98059) for 1 h and then subjected to agonist stimulation for 4 h.

Agonist stimulation of FPS cells significantly phosphorylated FAK (Figure 5c, lane 2) compared with vehicle-treated cells (Figure 5c, lane 1). Co-expression of Dn-Rho (Figure 5c, lane 3), Rac (Figure 5c, lane 4), CDC42 (Figure 5c, lane 5), EGFR (Figure 5c, lane 6), MEK (Figure 5c, lane 7) and c-Src (Figure 5c, lane 8) attenuated the $PGF_{2\alpha}$-induced FAK phosphorylation. Similarly, FAK phosphorylation in FPS cells in response to $PGF_{2\alpha}$ (Figure 5d, lane 2) could be abolished by co-incubation of cells with AL8810 (Figure 5d, lane 3), U73122 (Figure 5d, lane 4), 4C3MQ (Figure 5d, lane 5), PP2 (Figure 5d, lane 6), AG1478 (Figure 5d, lane 7) and PD98059 (Figure 5d, lane 8). The temporal change in activation of FAK (after 4 h) compared with c-Src (5 min) and ERK (10 min) in FPS cells and the attenuation of FAK phosphorylation by Dn-MEK and PD98059 suggest that FAK activation occurs downstream of c-Src and ERK activation to coincide with the $PGF_{2\alpha}$-induced phosphorylation of FAK and actin at focal adhesions and alteration in cell morphology.

To determine whether $PGF_{2\alpha}$ signalling to ERK and FAK in FPS cells was dependant on engagement of integrin receptors to ECM, we incubated FPS cells with 1mM RGDS or RGES tetrapeptides for 16 h and then subjected cells to agonist stimulation. As shown in Figures 6a and b, respectively, active displacement of the extracellular region of the integrin from the ECM and disruption of the focal adhesion protein complex with RGDS abolished the $PGF_{2\alpha}$-induced phosphorylation of ERK (Figure 6a) and FAK (Figure 6b). Similar results were achievable using the integrin $\alpha v\beta 3$ antibody (data not shown). However co-treatment of FPS cells with the chemically similar RGES tetrapeptide, which fails to bind integrin receptors (or IgG, data not shown), had no effect on the $PGF_{2\alpha}$-induced phosphorylation of ERK and FAK. Thus the selective disruption of the focal adhesion complexes attenuated the ability of $PGF_{2\alpha}$ to coordinate the activation of the cytoskeleton-linked proteins microtubule-associated protein kinase (ERK) and FAK and direct the change in cell morphology.

$PGF_{2\alpha}$-FP receptor-induced change in cell morphology is dependant upon the EGFR-mediated activation of ERK

We found that the $PGF_{2\alpha}$-mediated changes in the actin cytoskeleton (Figure 6c, panel v) and distribution of FAK (Figure 6c, panel vi), which co-localized at focal adhesions in FPS cells (Figure 6c, panel vii), were abolished when cells are co-incubated with the FP receptor antagonist (Figure 6c, panel xi), EGFR kinase inhibitor AG1478 (Figure 6c, panel xv) or the MEK inhibitor PD98059 (Figure 6c, panel xix). In addition, the cellular distribution of actin and FAK and cell morphology in FPS cells treated with $PGF_{2\alpha}$ and AL8810 (Figure 6c, panel xi), AG1478 (Figure 6c, panel xv) or PD98059 (Figure 6c, panel xix) were similar to cells treated with vehicle alone (Figure 6c, panel iii). Similarly, cells incubated with chemical inhibitor on their own for the same length of time showed no alteration in cell morphology (data not shown).

Finally we have shown that treatment of FPS cells with the EGFR kinase inhibitor AG1478 or the MEK inhibitor PD98059, which abolishes the cytoskeletal reorganization, also abolishes the ability of cells to migrate towards the $PGF_{2\alpha}$ stimulus (Figure 7).

Discussion

PGs have been associated with endometrial pathologies such as endometriosis and cancer, however the cellular mechanisms whereby they can modulate cell adhesion and movement involved in such disorders is unclear (Singh *et al.*, 1975; Lumsden *et al.*, 1983; Sales and Jabbour, 2003a; Jabbour and Sales, 2004).

Recently, we have demonstrated a role for the FP receptor in endometrial adenocarcinomas (Sales *et al.*, 2004b, 2005, 2007). Here we show that activation of the FP receptor by $PGF_{2\alpha}$

leads to increased adherence of Ishikawa FPS cells to the ECM vitronectin coincident with the redistribution of microtubules within the cell and the formation of actin stress fibres and lamellipodia, resulting in a dramatic alteration in cell morphology and ligand-induced FPS cell migration.

Vitronectin, an adhesive glycoprotein found in the circulation and tissues, regulates cell adhesion, growth, differentiation and migration *in vitro* and *in vivo*. There is now much evidence to support a role for vitronectin and its integrin receptors in pathology (Preissner, 1991; Giancotti and Ruoslahti, 1999; Brakebusch *et al.*, 2002). In transformed cells, vitronectin interacts with integrin αvβ3 at focal adhesions via the minimum consensus motif RGD (Preissner, 1991; Giancotti and Ruoslahti, 1999; Brakebusch *et al.*, 2002). In ovarian cancer tissues vitronectin and integrin αvβ3 are abundantly expressed (Cruet *et al.*, 1999). Moreover, in primary ovarian carcinomas integrin αvβ3 is found at a significantly higher rate compared with ovarian tumours of low malignant potential (Liapis *et al.*, 1997), indicating a role for engagement of integrin αvβ3/vitronectin interaction in cancer progression. Similarly, in the endometrium, dysregulation of integrin αvβ3 expression has been associated with endometriosis, a disorder manifest by retrograde menstruation and adhesion of endometrial tissue and outgrowth in the peritoneal cavity and viscera (Lessey *et al.*, 1994; Sales and Jabbour, 2003a; Jabbour and Sales, 2004).

Clusters of integrins assembled at focal adhesion complexes are complexed with scaffold proteins and associate with the cytoskeleton at the plasma membrane contact point with the ECM, where they transduce the signal from GPCRs (Damsky and Werb, 1992; Giancotti and Ruoslahti, 1999). One such protein at the site of integrin–cytoskeleton contact is FAK (Wozniak *et al.*, 2004). In the present study we show that the $PGF_{2\alpha}$-induced adhesion to vitronectin and re-organization of the actin cytoskeleton also promotes the recruitment of FAK to focal adhesion sites in an EGFR- and ERK1/2-dependent manner.

We used specific small molecule chemical inhibitors of cell signalling and co-transfection studies with Dn mutant proteins targeted to specific signalling pathways to map out the signalling events activated following $PGF_{2\alpha}$– FP receptor engagement leading to the activation of c-Src, ERK1/2 and FAK. We have shown that $PGF_{2\alpha}$–FP receptor stimulation of FPS cells rapidly activates c-Src, ERK1/2 and FAK in a PLC-β-IP3, PKA, EGFR-dependent manner. In addition ERK and FAK phosphorylation in FPS cells in response to $PGF_{2\alpha}$ were also sensitive to inhibition of cell signalling with Dn mutant isoforms targeted against the small monomeric G-proteins Rho, Rac and CDC42. The small monomeric G proteins are typically activated through a growth factor receptor-induced association at the plasma membrane with GEFs (Egan *et al.*, 1993) to regulate the polymerization of actin to produce stress fibres and lamellipodia. In addition to stress fibres, Rho controls the assembly of focal adhesion complexes. Agents that block activation of Rho have been shown to inhibit FAK phosphorylation and focal adhesions in other model systems (Slack, 1998; Davidson *et al.*, 2004b). Indeed Pierce *et al.* (1999) have shown that agonist stimulation of FP receptor in HEK293 cells leads to cellular shape change, induction of actin stress fibre formation and activation of FAK in a Rho-dependent manner. Rac and CDC42 are also present at the periphery of migrating cells and direct the assembly of multimolecular focal complexes at the plasma membrane (Nobes and Hall, 1995; Wozniak *et al.*, 2004).

Disruption of focal adhesions and cytoskeletal assembly has been shown to inhibit FAK phosphorylation and signalling (Slack, 1998; Davidson *et al.*, 2004b). Similarly studies using FAK mutants, which fail to localize to focal adhesions, exhibit impaired autophosphorylation and an inability to bind FAK substrates and induce cell signalling (Wozniak *et al.*, 2004). In the present study, we have shown that disruption of integrin αvβ3–ECM interaction with an excess of an RGDS tetrapeptide abolishes the capacity of

FPS cells to adhere to ECM in response to agonist treatment, as well as the agonist-induced alteration in morphology, induction of actin stress fibres and the presence of FAK co-localized with actin at focal adhesions. Coincident with this abolition of integrin engagement, morphological change and cell migration we found that disruption of integrin–ECM interaction with the RGDS tetrapeptide also abolished cell signalling to ERK and FAK. These data suggest that integrin $\alpha v\beta 3$–ECM adherence and cytoskeletal reorganization is necessary for FPS cell signalling and migration in response to agonist treatment and suggest that chemical disruptors of integrin–ECM engagement may be potential therapy for inhibiting the adverse effects of $PGF_{2\alpha}$–FP receptor signalling *in vivo*. Indeed, blocking antibodies targeted against integrin $\alpha v\beta 3$ or cyclic RGD peptide antagonists have been demonstrated to induce tumour regression by influencing cell adhesion/migration and blocking proliferation and reducing tumour angiogenesis (Hynes, 2002). However the feasibility of such strategies remains a matter of debate.

Taken together, our findings herein provide a novel mechanism for the control of endometrial epithelial cell behaviour and movement by the FP receptor via integrin $\alpha v\beta 3$-ECM-mediated cell adhesion and migration. We believe that the findings we have presented herein may have relevance for understanding the molecular mechanisms regulating endometrial pathologies, such as endometriosis and cancer expressing aberrant levels of prostanoid receptors, and which are associated with cell movement metastasis and adhesion to distant sites of the body.

Materials and methods

Reagents

The anti-Myc-agarose, anti-phosphotyrosine (PY20) agarose, anti-phospho-c-Src[Y418], anti-phospho-FAK, anti-FAK, anti-HA-agarose pre-conjugate, IgG, anti-integrin $\alpha v\beta 3$, anti-β-tubulin and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Autogen-Bioclear, Wiltshire, UK). RGDS and RGES, alkaline phosphatase secondary antibodies, indomethacin, phosphate-buffered saline, bovine serum albumin, AL8810 (used at a final concentration of 50 μM) and $PGF_{2\alpha}$ (used at a final concentration of 100 nM) were purchased from Sigma Chemical Company (Dorset, UK). The Iloprost was purchased from Cayman chemical company (Axxora, Nottingham, UK). The Src kinase-specific inhibitor PP2 (used at a final concentration of 10 μM), EGFR kinase-specific inhibitor tryphostin AG1478 (used at a final concentration of 100 nM), PLC-β inhibitor U73122 (used at a final concentration of 10 μM), MEK1/2 inhibitor PD98059 (used at a final concentration of 50 μM), PKA inhibitor 4-cyano-3-methylisoquinoline (4C3MQ, used at a final concentration of 1 μM) were purchased from Calbiochem (Nottingham, UK). The Dn–MEK (Seger *et al.*, 1994; Jaaro *et al.*, 1997), Dn–EGFR (Benard *et al.*, 2001), Dn–Rho, Dn–Rac (Harris *et al.*, 2002), Dn–Ras (Benard *et al.*, 2001), Dn–CDC42 (Levi *et al.*, 1998) and Dn–FAK (Benard *et al.*, 2001) cDNA constructs were a kind gift from Prof Zvi Naor (Department of Biochemistry, University of Tel Aviv, Israel). The Myc-tagged ERK-2 construct, HA-tagged WT FAK cDNA and Dn-c-Src cDNA constructs (Davidson *et al.*, 2004a) were obtained from Prof Robert Millar (MRC Human Reproductive Sciences Unit, Edinburgh, UK). The FP receptor antagonist AL-8810 is a specific antagonist of the FP receptor (Griffin *et al.*, 1999). The mean potency of the FP receptor antagonist AL-8810 is EC50=261±44 nM and *E*max=19% compared with the FP receptor agonist cloprostenol: EC50=0.84 nM and *E*max=100. In this study the authors show that the FP receptor antagonist dose dependently inhibits 100 nM FP receptor agonist with 100% inhibition at 100 μM (Griffin *et al.*, 1999). We have shown that the FP receptor antagonist can inhibit 100 nM $PGF_{2\alpha}$ at a concentration of 10–50 μM (Sales *et al.*, 2005). Moreover at this concentration the FP receptor antagonist does not inhibit responses of TP, DP, EP2 or EP4 receptors (Griffin *et al.*, 1999).

Cell culture and transfection

Ishikawa WT (European Collection of Cell Culture, Wiltshire, UK) and Ishikawa cells stably expressing the FPS to the levels observed in endometrial adenocarcinomas (FPS cells) were maintained as described (Sales *et al.*, 2005). Transient transfections of FPS cells were performed using Superfect (Qiagen, Crawley, UK) as per the manufacturer's protocol. All experiments were conducted in the presence of 8.3 μ_M indomethacin (a dual COX enzyme inhibitor used to inhibit endogenous prostanoid biosynthesis). To actively displace integrins from their cell matrix attachments, FPS cells were incubated in serum-free media with $1m_M$ RGDS or RGES tetrapeptides, 50 μg ml^{-1} final concentration of anti-integrin $\alpha v \beta 3$ antibody or equivalent IgG from the same host species for 16 h (or 1 h for cell adhesion assays). Following agonist stimulation, any displaced and detached cells were collected with any adherent cells by centrifugation at $1000g$ for 5 min.

Protein extraction and western blot analysis

Protein extraction and immunoprecipitation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot analysis were performed as described previously (Sales *et al.*, 2004a, 2005). using specific primary antibodies as described in the figure legend and secondary antibodies conjugated to Alexafluor 680 (Molecular Probes Inc., Eugene, OR, USA) or IRdye 800 (Rockland Immunochemicals, Gilbertsville, PA, USA). Immunoreactive proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR Biosciences, Cambridge, UK). Relative density of immunoblots was calculated by dividing the value obtained from the phosphorylated immunoblots by the value obtained from total protein immunoblots or light-chain IgG and expressed as fold above vehicle controls.

Immunohistochemistry and confocal laser microscopy

Confocal laser microscopy was performed on a Zeiss (Jena, Germany) laser-scanning microscope LM510. Cells were plated upon vitronectin-coated slides, transfected with an HA-tagged WT FAK cDNA construct and serum starved for 16 h in the absence or presence of RGDS/RGES tetrapeptides, $\alpha v \beta 3$ antibody or IgG. Thereafter cells were either subjected to agonist stimulation for 4 h or pre-treated with chemical inhibitor or FP receptor antagonist for 1h prior to agonist stimulation as described in the figure legend. After stimulation, cells were fixed with 100% methanol, blocked using 5% normal rabbit serum before incubation with mouse anti-HA antibody at a dilution of 1:100 for 18 h at 4 °C. Thereafter sections were incubated sequentially with biotinylated rabbit anti-mouse (DAKO; Dako Corp., High Wycombe, UK) and fluorochrome streptavidin 546 Alexafluor (Molecular Probes Inc.) diluted 1 in 200. Sections were re-blocked with 5% normal rabbit serum and incubated sequentially with anti-actin/tubulin antibody at a dilution of 1:50 at 4 °C for 18 h and fluorochrome rabbit anti-goat fluorescein isothyocyanate (Molecular Probes Inc.) diluted 1 in 200 at 25 °C for 2 h. Nuclear staining was detected by incubating cells with a 1 in 2000 dilution of ToPro2 (Molecular probes Inc.) for 2 min. Control sections were incubated with IgG from the same host species.

Phosphatidylinositol hydrolysis

Accumulation of total inositol phosphates was measured in Ishikawa WT and FPS cells treated either with vehicle, 100 n$_M$ PGF$_{2\alpha}$, 100 n$_M$ PGF$_{2\alpha}$ and AL8810 or 100 n$_M$ PGF$_{2\alpha}$ and U73122 according to published protocols (Sales *et al.*, 2004b).

Cell adhesion assays

Agonist-induced cell-matrix adhesion was assessed using a CytoMatrix screening kit ECM 205 and CytoMatrix Human vitronectin cell adhesion strips ECM 102 (Chemicon, Temecula, CA, USA) according to the manufacturer's protocol. WT or FPS cells were

incubated in serum-free media in the presence/absence of 1m_M RGDS, 1m_M RGES, $50\,\mu_\text{M}$ AL8810, $50\,\mu\text{g}$ IgG or $50\,\mu\text{g}$ anti-integrin $\alpha\text{v}\beta3$ antibody for 1h prior to the addition of vehicle or $100\,\text{n}_\text{M}$ $PGF_{2\alpha}$. Thereafter cells were seeded onto the substrate-coated adhesion strips and incubated at 37 °C for 45 min. Adherent cells were fixed, stained and quantified according to the manufacturer's protocol. Fold increase was determined by dividing the absorbance of cells treated with $PGF_{2\alpha}$ by the absorbance of cells treated with vehicle.

Cell migration assays

Agonist-induced cell migration was assessed using the QCM Chemotaxis cell migration assay (Chemicon). FPS cells were incubated in 1ml of serum-free media in the presence/ absence of $200\,\text{n}_\text{M}$ AG1478, $50\,\mu_\text{M}$ PD98059, 1m_M RGDS, 1m_M RGES, $50\,\mu_\text{M}$ AL8810, $50\,\mu\text{g}$ IgG or $50\,\mu\text{g}$ anti-integrin $\alpha\text{v}\beta3$ antibody for 1h. Thereafter $100\,\mu\text{l}$ of each treatment containing 50 000 cells were placed into the migration (upper) chamber in triplicate. The lower chamber received either vehicle, $PGF_{2\alpha}$ or $PGF_{2\alpha}$ and $200\,\text{n}_\text{M}$ AG1478, $50\,\mu_\text{M}$ PD98059, 1m_M RGDS, 1m_M RGES, $50\,\mu_\text{M}$ AL8810, $50\,\mu\text{g}$ IgG or $50\,\mu\text{g}$ anti-integrin $\alpha\text{v}\beta3$ antibody for 24 h at 37 °C. Migratory cell number was determined according to the manufacturer's instruction. Fold increase was determined by dividing the fluorescence of cells treated with $PGF_{2\alpha}$ or $PGF_{2\alpha}$ and inhibitor by the fluorescence of cells treated with vehicle or vehicle and inhibitor.

Statistics

Where appropriate, data were subjected to statistical analysis with analysis of variance and Fisher's protected least significant difference tests (Statview 5.0; Abacus Concepts Inc., USA).

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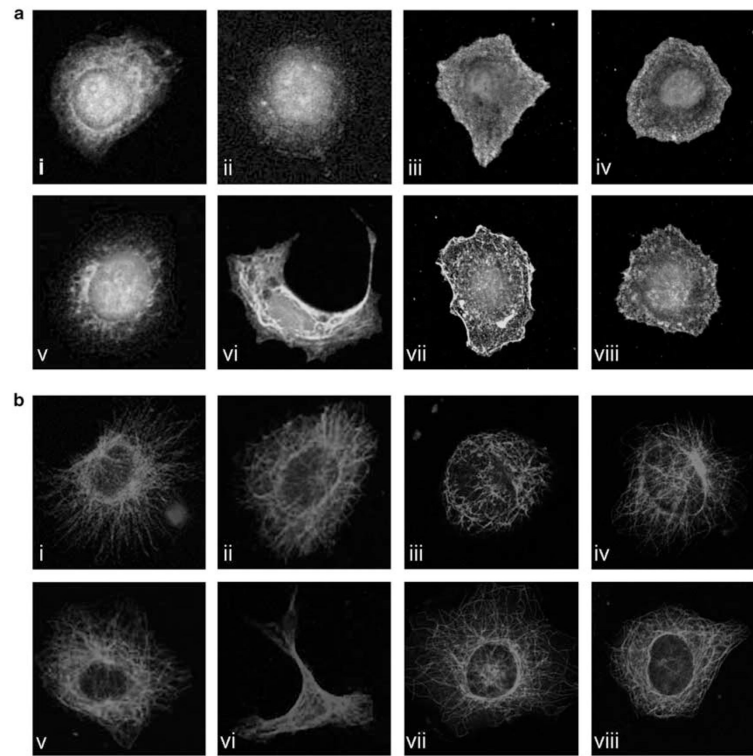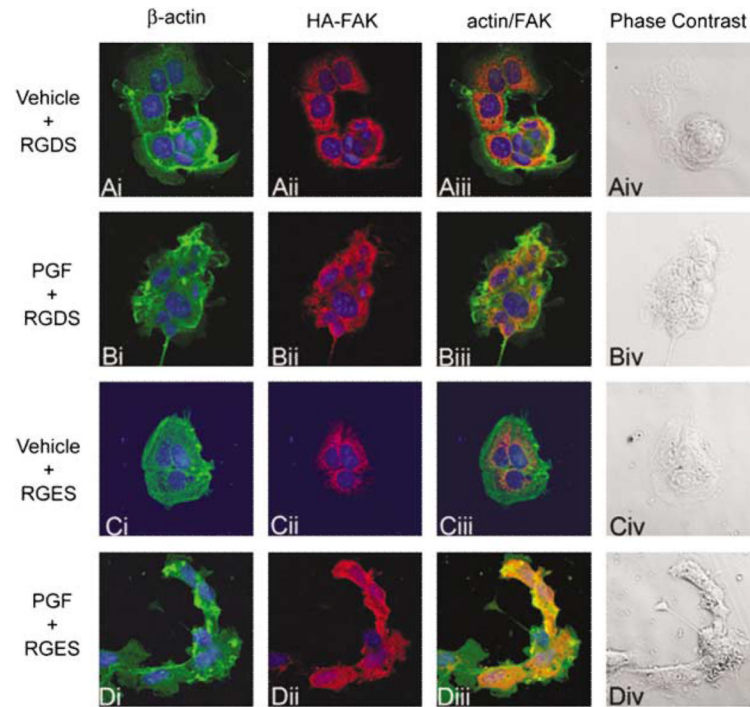
Figure 1.
$PGF_{2\alpha}$ induces ultrastructural changes in FPS cells. Confocal laser-scanning images showing the localization of β-actin and β-tubulin ((**a**) and (**b**), respectively) in methanol-fixed wild-type (WT) and FPS Ishikawa cells. Cells were serum starved before stimulation with vehicle (panels i and v for WT and FPS cells, respectively) or 100 nM $PGF_{2\alpha}$ (panels ii and vi for WT and FPS cells, respectively) or 100 nM $PGF_{2\alpha}$ and AL8810 (panels iii and vii for WT and FPS cells, respectively) or 100 nM Iloprost (panels iv and viii for WT and FPS cells, respectively) for 4 h. Data are shown as representative cells from at least four fields of view from three independent experiments.
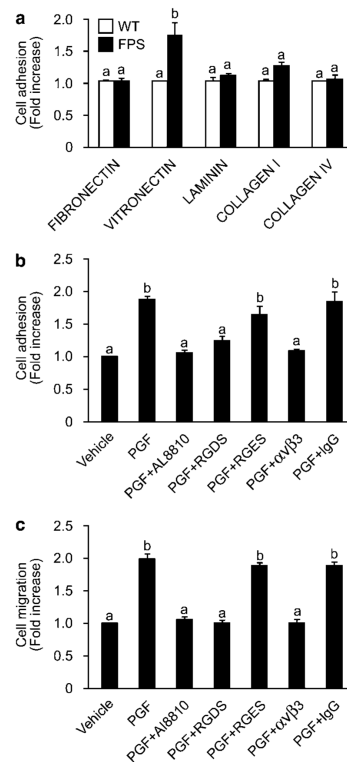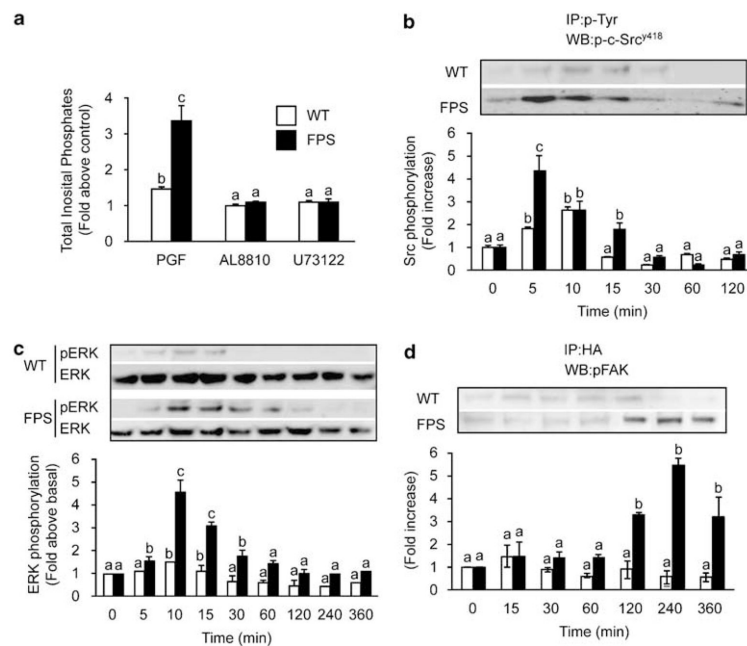
Figure 2.
Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) stimulation induces an increase in the adherent capacity of F-prostanoid receptor (FPS) cells to vitronectin and enhances cell migration. (**a**) Cell adhesion to extracellular matrix (ECM) as determined by CytoMatrix cell adhesion assay. Wild-type (WT) (open bars) and FPS cells (closed bars) were incubated with vehicle or 100 nM $PGF_{2\alpha}$ for 1h and cellular adherence to the ECM fibronectin, vitronectin, laminin, collagen I or IV was measured. (**b**) FPS cells were pre-incubated with either vehicle, the FP receptor antagonist AL8810, arg–gly–asp–ser (RGDS) or arg–gly–glu–ser (RGES) tetrapeptides, integrin $\alpha v \beta 3$ antibody or immunoglobulin G (IgG) for 1h and then seeded onto vitronectin-coated plates in the presence of vehicle or 100 nM $PGF_{2\alpha}$ for 1h. Cell adhesion induced by $PGF_{2\alpha}$ was calculated as fold increase above vehicle. (**c**) Cell migration was determined using a Chemotaxis cell migration assay. FPS cells were pre-incubated with either vehicle, the FP receptor antagonist AL8810, RGDS or RGES tetrapeptides, integrin $\alpha v \beta 3$ antibody or IgG for 1h and then seeded onto transwell chambers for 24 h. The lower chamber received either vehicle, $PGF_{2\alpha}$ or $PGF_{2\alpha}$ and AL8810, RGDS or RGES tetrapeptides, integrin $\alpha v \beta 3$ antibody or IgG as the chemoattractant. In all panels each bar represents the mean±s.e.m. of six independent experiments (b is significantly different from a; $P < 0.05$).
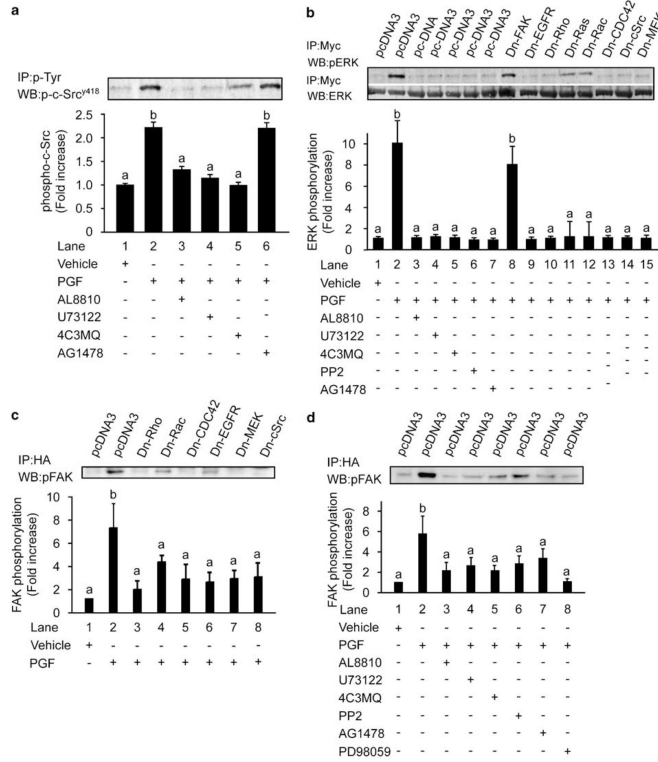
Figure 3.
Morphological changes are dependant upon integrin αvβ3 engagement with vitronectin. Phase contrast (column iv) and immunofluorescent laser confocal microscopy of FPS cells, showing the localization of β-actin (column i, green channel), focal adhesion tyrosine kinase (FAK) (column ii, red channel) and the co-localization of β-actin with FAK (column iii, yellow channel). FPS cells were seeded onto vitronectin-coated plates, transfected with hemagglutinin (HA)-tagged wild-type FAK cDNA and pre-incubated with 1m$_M$ arg–gly–asp–ser (RGDS) or RGES tetrapeptides for 16 h. Cells were stimulated with vehicle (rows A and C) for RGDS- and RGES-treated cells, respectively) or 100 n$_M$ prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) (rows B and D) for RGDS- and RGES-treated cells, respectively) for 4 h. In the PGF$_{2\alpha}$-treated FPS cells, the generation of β-actin and FAK-rich membrane extensions (lamellipodia) and change in cell morphology are evident in the RGES (D (i) and D (ii) for β-actin and FAK, respectively)-treated cells compared with vehicle-treated cells (C (i) for RGES-treated cells stained for β-actin and C (ii) for RGES-treated cells stained for FAK, respectively). These cytoskeletal alterations in β-actin and FAK staining were not observed in cells treated with PGF$_{2\alpha}$ and RGDS tetrapeptide (figure B (i) and B (ii) for β-actin and FAK, respectively). Data are shown as representative cells from at least four fields of view from three independent experiments.
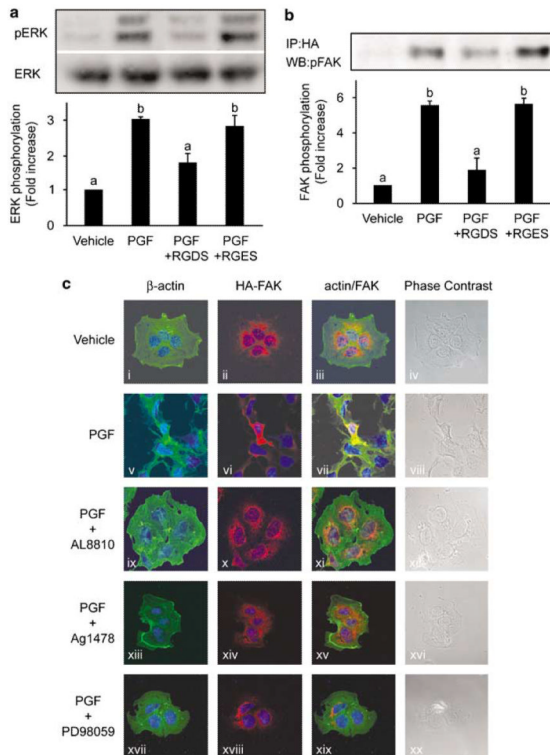
Figure 4.
Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) stimulation of F-prostanoid receptor (FPS) cells activates numerous structurally regulated proteins. (**a**) Total inositol phosphate hydrolysis was measured in wild-type (WT) (open bars) and FPS cells (closed bars) in response to administration of vehicle or 100 nM $PGF_{2\alpha}$ in the absence or presence of 50 μM AL8810 or 10 μM U73122. In figures (**b–d**), WT and FPS cells were stimulated with 100 nM $PGF_{2\alpha}$ for the time indicated or left unstimulated (0 min). (**b**) Cells were immunoprecipitated (IP) with anti-phosphotyrosine antibody (p-Tyr) and subjected to immunoblot analysis (WB) using specific antibodies recognizing the activated form of c-Src non-receptor tyrosine kinase phosphorylated at tyrosine 418. (**c**) Cell lysates were subjected to immunoblot analysis using antibody against phosphorylated extracellular signal-regulated kinase (ERK). Immunoblots were stripped and reprobed with antibody recognizing total ERK. (**d**) Cells were transiently transfected with hemagglutinin (HA)-tagged wild-type FAK cDNA. Cell lysates were immunoprecipitated with anti-HA-agarose antibody (HA) and subjected to immunoblot analysis using antibody recognizing phosphorylated FAK. For each, a representative immunoblot is shown, with semi-quantitative analysis determined as described in the 'Materials and methods' section. In all panels each bar represents the mean±s.e.m. of at least three independent experiments (**b** is significantly different from **a** and **c** is significantly different from **a** and **b**; $P < 0.05$).

Figure 5.
Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) activation of c-Src, extracellular signal-regulated kinase (ERK) and focal adhesion tyrosine kinase (FAK) are dependant on multiple signalling pathways and cell attachment to ECM. (**a**) FPS cells were stimulated with vehicle (lane 1), 100 nM $PGF_{2\alpha}$ (lane 2), 100 nM $PGF_{2\alpha}$ and AL8810 (lane 3), 100 nM $PGF_{2\alpha}$ and U73122 (lane 4), 100 nM $PGF_{2\alpha}$ and 4C3MQ (lane 5) or 100 nM $PGF_{2\alpha}$ and AG1478 (lane 6) for 5 min, immunoprecipitated with anti-phosphotyrosine antibody (p-Tyr) and immunoblotted for phosphorylated c-Src^{y418}, (**b**) FPS cells were transiently transfected with Myc-ERK cDNA and either empty vector cDNA (pcDNA3; lanes 1–7) or dominant-negative (Dn) mutant isoforms of FAK (lane 8), EGFR (lane 9), Rho (lane 10), Ras (lane 11), Rac (lane 12), CDC42 (lane 13), c-Src (lane 14) and MEK (lane 15) and subjected to stimulation with vehicle (lane 1) or 100 nM $PGF_{2\alpha}$ for 10 min. Empty vector transfected cells were stimulated with 100 nM $PGF_{2\alpha}$ (lane 2) 100 nM $PGF_{2\alpha}$ and AL8810 (lane 3), 100 nM $PGF_{2\alpha}$ and U73122 (lane 4), 100 nM $PGF_{2\alpha}$ and 4C3MQ (lane 5), 100 nM $PGF_{2\alpha}$ and PP2 (lane 6) or 100 nM $PGF_{2\alpha}$ and AG1478 (lane 7) for 10 min. Cells were immunoprecipitated with Myc-agarose-conjugated antibody and immunoblotted for phosphorylated ERK (upper panel). Immunoblots were stripped and re-probed with antibody recognizing total ERK (lower panel). (**c**) FPS cells were transiently transfected with HA-tagged wild-type FAK cDNA and empty vector cDNA (pcDNA3; lanes 1and 2) or dominant-negative (Dn) Rho (lane 3), Rac (lane 4), CDC42 (lane 5), EGFR (lane 6), MEK (lane 7) and c-Src (lane 8) cDNA and stimulated with vehicle or 100 nM $PGF_{2\alpha}$ for 4 h. Cells were immunoprecipitated (IP) with HA-agarose-conjugated antibody and subjected to immunoblot analysis (WB) for phosphorylated FAK. (**d**) FPS cells were transiently transfected with HA-tagged wild-type FAK cDNA and empty vector cDNA (pcDNA3; lanes 1–8). Cells were stimulated with vehicle (lane 1), 100 nM $PGF_{2\alpha}$ (lane 2), 100 nM $PGF_{2\alpha}$ and AL8810 (lane 3), 100 nM $PGF_{2\alpha}$ and U73122 (lane 4), 100 $PGF_{2\alpha}$ and 4C3MQ (lane 5), 100 nM $PGF_{2\alpha}$ and PP2 (lane 6), 100 nM $PGF_{2\alpha}$ and AG1478 (lane 7) or 100 nM $PGF_{2\alpha}$ and PD98059 (lane 8) for 4 h. Cells were

immunoprecipitated (IP) with HA-agarose-conjugated antibody and subjected to immunoblot analysis (WB) for phosphorylated FAK. In all panels each bar represents the mean±s.e.m. of at least four independent experiments (+ denotes addition of agent and − denotes absence of agent; **b** is significantly different from **a**; *P*<0.05).

Figure 6.

Morphological changes in FPS cells is dependant on FP receptor, EGFR and extracellular signal-regulated kinase (ERK) signalling. (**a**) FPS cells were pre-incubated with $1m_M$ arg–gly–asp–ser (RGDS) or arg–gly–glu–ser (RGES) tetrapeptides for 16 h and then stimulated with vehicle or 100 n_M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) for 10 min. ERK phosphorylation was detected by immunoblot analysis (upper panel) and normalized for protein loading against total ERK protein (lower panel) on the same blot. (**b**) FPS cells were transiently transfected with hemagglutinin (HA)-tagged wild-type FAK cDNA. FPS cells were preincubated for 16 h with 1 m_M RGDS or RGES tetrapeptides and stimulated for 4 h with vehicle or 100 n_M $PGF_{2\alpha}$. Cells were immunoprecipitated (IP) with HA-agarose-conjugated antibody and subjected to immunoblot analysis (WB) for phosphorylated FAK. In all panels each bar represents the mean±s.e.m. of at least four independent experiments (**b** is significantly different from **a**; $P<0.05$). (**c**) Phase contrast microscopy (panels iv, viii, xii, xvi and xx) and confocal laser-scanning images showing the localization of β-actin (panels i, v, ix, xiii and xvii), HA-tagged FAK (panels ii, vi, x, xiv and xvii) and co-localization of FAK with actin (panels iii, vii, xi, xv and xix) in FPS cells. Cells were transiently transfected with HA-tagged wild-type FAK cDNA construct, serum starved and treated with vehicle (panels i–iv), 100 n_M $PGF_{2\alpha}$ (panels v–viii), 100 n_M $PGF_{2\alpha}$ and AL8810 (panels ix–xii), 100 n_M $PGF_{2\alpha}$ and AG1478 (panels xiii–xvi) or 100 n_M $PGF_{2\alpha}$ and PD98059 (panels xvii–xx) for 4 h. In the $PGF_{2\alpha}$-treated FPS cells, the generation of β-actin (panel v) and FAK-rich membrane extensions (panel vi; lamellipodia) are consistent with change in cell morphology. Here FAK and actin co-localize at focal adhesions (panel vii) compared with vehicle-treated cells where FAK (panel ii) and actin (panel i) are localized in separate compartments (panel iii). The cytoskeletal alterations and co-localization of FAK with actin in $PGF_{2\alpha}$-treated cells were abolished by co-treatment of cells with AL8810 (panel xi), AG1478 (panel xv) and PD98059 (panel xix). Data are shown as representative cells from at least four fields of view from three independent experiments.
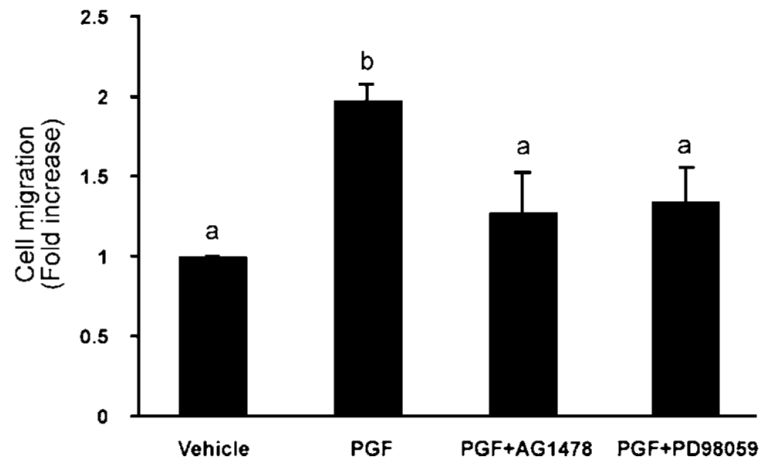
Figure 7.
FPS cell migration is dependant on EGFR and ERK signalling. Cell migration was determined using a Chemotaxis cell migration assay. FPS cells were pre-incubated with either vehicle, AG1478 or PD98059 for 1 h and then seeded onto transwell chambers for 24 h. The lower chamber received vehicle, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or $PGF_{2\alpha}$ and AG1478 or PD98059 as the chemoattractant. Each bar represents the mean±s.e.m. of at least four independent experiments (**b** is significantly different from **a**; $P<0.05$).