

Progesterone promotes focal adhesion formation and migration in breast cancer cells through induction of protease-activated receptor-1

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Abstract

Progesterone and progestins have been demonstrated to enhance breast cancer cell migration, although the mechanisms are still not fully understood. The protease-activated receptors (PARs) are a family of membrane receptors that are activated by serine proteases in the blood coagulation cascade. PAR1 (F2R) has been reported to be involved in cancer cell migration and overexpressed in breast cancer. We herein demonstrate that PAR1 mRNA and protein are upregulated by progesterone treatment of the breast cancer cell lines ZR-75 and T47D. This regulation is dependent on the progesterone receptor (PR) but does not require PR phosphorylation at serine 294 or the PR proline-rich region mPRO. The increase in *PAR1* mRNA was transient, being present at 3 h and returning to basal levels at 18 h. The addition of

a PAR1-activating peptide (aPAR1) to cells treated with progesterone resulted in an increase in focal adhesion (FA) formation as measured by the cellular levels of phosphorylated FA kinase. The combined but not individual treatment of progesterone and aPAR1 also markedly increased stress fiber formation and the migratory capacity of breast cancer cells. In agreement with *in vitro* findings, data mining from the Oncomine platform revealed that *PAR1* expression was significantly upregulated in PR-positive breast tumors. Our observation that *PAR1* expression and signal transduction are modulated by progesterone provides new insight into how the progestin component in hormone therapies increases the risk of breast cancer in postmenopausal women.

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Introduction

Progesterone is an essential hormone in the process of breast development and differentiation (Horwitz *et al.* 1990). Given the requirement of breast cells to proliferate, migrate, and invade during the luteal phase and pregnancy, it has been speculated that progestins hijack these signaling pathways in breast cancer cells. Progesterone and progestins have been previously reported to stimulate breast cancer cell migration and invasion (Carvajal *et al.* 2005, Kato *et al.* 2005b, Fu *et al.* 2008). Indeed, in postmenopausal women receiving hormone replacement therapy (HRT), the presence of progestagenic compounds increases breast cancer incidence (Beral 2003). To deliver their proliferative and invasive responses, progestagenic compounds use the progesterone receptor (PR).

Post-translational modifications such as sumoylation and phosphorylation have been shown to alter PR transcriptional activity. Phosphorylation of PR Ser-294 augments PR degradation by the 26S proteasome and also blocks PR Lys-388 sumoylation, a reversible ligand-induced modification that blunts PR transcriptional response on certain progesterone-regulated genes (Lange *et al.* 2000, Daniel & Lange 2009). PRs engineered to be defective in either Ser-294 (S294A) or in the ability to bind to and activate c-Src-dependent intracellular signaling to MAPKs (PR-mPro) have shown the requirement for PR-induced rapid activation of c-Src and MAPKs in proliferative responses stimulated by progestagenic compounds (Favre *et al.* 2005).

For anchorage-dependent migration to occur, a coordinated interplay of filopodia and lamellipodia protrusions at the

leading cellular edge, the formation of focal contacts with the extracellular matrix and their maturation into focal adhesions (FAs), contraction of the cell body, and finally detachment at the rear of the cell is required (Abreu *et al.* 2011). One of the most studied components of the FA complexes in adherent cells is FA kinase (FAK), which was initially described as a downstream target of the viral oncogene v-Src, but has now been described as one of the major mediators of integrin signaling (Schaller *et al.* 1992, Zhao & Guan 2009). FAK belongs to a subfamily of cytoplasmic tyrosine kinases, which lack Src homology domains SH2 and SH3. In a nonactive state, the N-terminal FERM domain of the FAK protein prevents access to its catalytic loop and prevents autophosphorylation of the kinase domain. Upon activation, FAK associates with FA proteins allowing autophosphorylation at residue tyrosine 397, which serves as a docking site for SH2 domain containing Src and PI3K (through the p85 subunit). This interaction and initial phosphorylation event displaces the FERM domain and is followed by further phosphorylation at tyrosine 567/577 (Fu *et al.* 2008, Schaller 2008, Guan 2010). Mature FA complexes, together with phosphorylated FAK, require the further recruitment of proteins, such as vinculin, talin, and paxillin, among others (Burrige & Chrzanowska-Wodnicka 1996, Critchley 2000). A role of FAK in cell migration is now widely accepted through key experiments demonstrating that FAK overexpression promotes wound repair in *in vitro* assays, FAK knockout fibroblasts show decreased migration capabilities, and FAK inhibition prevents endothelial cell movement (Zhao & Guan 2009).

A recent study on the progesterone-responsive T47D breast cancer cell line demonstrated that progesterone could enhance FAK phosphorylation and increase the number of FA complexes within specialized cellular membrane protrusions (Fu *et al.* 2008). This phosphorylation at both tyrosine 397 and 567/577 was rapid and transient, occurring within 15 min and returning to basal levels at 1 h. Maximum stimulation occurred at the physiological concentration of 10 nM, a progesterone concentration previously reported to be optimal in both the PR-positive ZR-75 and T47D cell lines (Kato *et al.* 2005b). In the T47D cell line, a progesterone-dependent increase in FAK phosphorylation was shown to be dependent on both Src signaling and the PR, although whether this involved direct interaction between both signaling molecules through the Src interaction domain of PR was investigated (Boonyaratanakornkit *et al.* 2001, Pierson-Mullany & Lange 2004, Faivre & Lange 2007, Faivre *et al.* 2008, Fu *et al.* 2008). The same study further provided evidence that RHOA and ROCK2 signaling proteins are also essential. The ROCK protein kinase family has also been given a pivotal role within the signaling network controlling actomyosin contractility and stress fiber assembly (Leung *et al.* 1996, Pellegrin & Mellor 2007). Interestingly, migration of T47D breast cancer cells is blocked not only by PR and PI3K pathway inhibitors but also by inhibitors of G proteins and the MAPK pathway (MEK1/2), demonstrating that multiple signals are required to permit or maintain

FAK-mediated cell migration in response to progesterone signaling (Fu *et al.* 2008).

The protease-activated receptors (PARs) are a family of four vascular receptors that respond to local changes in the proteolytic environment. PAR1 (F2R) to PAR4 (F2RL3) are G protein-coupled receptors activated by serine proteases of the blood coagulation cascade pathways, notably thrombin (Hirano 2007, Landis 2007). Many of the PAR-activating proteases are produced during tissue damage, and thus PARs make important contributions to tissue responses to injury, which include hemostasis, cell survival responses, inflammation, pain, and repair (Ossovskaya & Bunnett 2004). However, only PAR1, PAR3 (F2RL3), and PAR4 are referred to as thrombin receptors due to thrombin's ability to specifically cleave the extracellular N-termini of the receptor (Yuan & Lin 2004). Signaling through PARs has been demonstrated to influence a wide range of physiological responses including inflammation, platelet activation, vascular tone maintenance, and barrier function (Leger *et al.* 2006). In tumor cells, the factor VIIa-tissue factor (TF) complex activates PAR2 and thereby regulates proangiogenic growth factor expression (Ruf & Mueller 2006). Interestingly, TF is strongly induced by progesterone in both the T47D and ZR-75 breast cancer cell lines (Kato *et al.* 2005b), which in turn increase the procoagulant activity of breast cancer cells (Kato *et al.* 2005b, Henriquez *et al.* 2011).

The ability of progesterone and progestins to regulate PAR expression appears to be cell specific. For example, levonorgestrel, a widely used progestin, was shown to downregulate *PAR1* expression in human endometrium (Hague *et al.* 2002). However, in vascular smooth muscle cells and the rat aorta, medroxyprogesterone acetate and several other progestins upregulate *PAR1* expression, yet levonorgestrel and the synthetic estrogen 17 α -ethinylestradiol do not confer the same effect (Herkert *et al.* 2001). In the ovine cervix, *PAR1* and *PAR2* are expressed in the luminal epithelium of the cervix throughout the estrus cycle, with expression peaking at estrus. The synchronization of estrus by a progesterone sponge increased *PAR2* but not *PAR1* expression (Mitchell *et al.* 2005). Furthermore, it has been suggested that *PAR1* plays a key role in signaling events preceding menstrual bleeding (Critchley *et al.* 2006). However, currently no reports exist of progesterone regulation of PARs in normal or malignant breast cells.

Overexpressed *PAR1* is a feature of many metastatic cancers (Even-Ram *et al.* 1998, Tellez & Bar-Eli 2003, Yin *et al.* 2003). Cancer cells overexpressing *PAR1* are more invasive *in vitro*, and this is further enhanced by a *PAR1*-activating ligand (Even-Ram *et al.* 2001). We have previously reported on a correlation between metastasis and the expression of TF, which is known to cleave and activate *PAR1* and *PAR2* (Kato *et al.* 2005a,b). Other clotting intermediates have also been associated with the cancer progression, angiogenesis, and metastasis, such as thrombin, factor Xa and plasminogen activator inhibitor, type 1 (Rickles 2006). These observations suggest that signaling

events downstream of these clotting cascade factors deliver a survival advantage to cancer cells. In this study, we examined the possibility that progesterone impacts on the behavior of breast cancer cells by engaging PAR-dependent signaling (Rickles 2006).

Materials and Methods

Cell culture and hormonal treatment

The breast cancer cell lines ZR-75 (Engel *et al.* 1978), T47Dco (T47D), T47D-YB (Sartorius *et al.* 1994), T47D PR S294A, and T47D PR mPRO (Pierson-Mullany & Lange 2004, Faivre & Lange 2007, Faivre *et al.* 2008) were maintained in DMEM/F12 media supplemented with 10% fetal bovine serum (FBS; Invitrogen). To obtain protein and RNA, cells were plated at 50% confluence in 10 cm² Petri dishes (Falcon, Becton-Dickinson, Franklin Lakes, NJ, USA). For immunocytochemistry, coverslips were added to the dishes. Culture medium was changed to charcoal-treated medium containing 5% serum for 24 h before hormone or epidermal growth factor (EGF) treatment. The steroid hormones progesterone or estrogen (17 β -estradiol (E₂), (both Sigma-Aldrich)) were dissolved in ethanol and added to the cells, at a final concentration of 10 nM. R5020 was purchased from NEN and used at 20 nM. EGF (Upstate Biotechnology, NY, USA) was added at a final concentration of 10 nM in water. An equal volume of ethanol was added as a control in the EGF treatments. For immunocytochemistry and migration experiments, a PAR1-activating peptide (aPAR1), H-Thr-Phe-Leu-Leu-Arg-NH₂ (TFLLRN purchased from Gen Script Corporation, NJ, USA), was added as stated in the figure legends.

Western blotting

Protein was isolated as previously published (Kato *et al.* 2005b), and 100 μ g protein extract was loaded in each lane. Breast cancer cells fixed onto coverslips were incubated for 18 h with PAR1 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a final dilution of 1:100. The secondary antibody was a goat anti-mouse/rabbit IgG secondary antibody coupled with HRP (1:5000, Bio-Rad Laboratories), used for 1 h at room temperature. Bound antibody was detected by chemiluminescence using the ECL western blot analysis system (NEN, Western lightning; Perkin-Elmer (Waltham, MA, USA)).

RT-PCR

Total RNA isolation and cDNA generation were as previously published (Kato *et al.* 2005b). Using PAR1 primers, sense: 5'-TGT ACG CCT CTA TCT TGC TCA TGA C-3', antisense: 5'-GCA GGT ATG CAA GTC GTA CAT CTG-3' (BiosChile, Santiago, Chile) semiquantitative PCRs were performed from cDNA generated from

progesterone, estrogen EGF, and RU486-treated samples, using Taq polymerase (Invitrogen). Cycle curves were performed for all sets of PCR primers, with the number of cycles used for each primer set being in the linear range of the curve. Primers amplifying a region of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to act as an internal loading control: sense: 5'-ccc ctg gcc aag gtc atc cat gac-3', antisense: 5'-ccc ctc ccc tct tca agg ggt cta c-3'; (BiosChile). NET1 primers have been previously reported (Bray *et al.* 2005) The MEK1/2 (U0126, 10 μ M; Calbiochem, La Jolla, CA, USA), Src (PP2, 10 μ M; Calbiochem), and EGFR (AG1478, 1 μ M; Calbiochem) pathway inhibitors were added 30 min before progesterone or vehicle addition. Semiquantitative densitometry of the bands was performed using NIH Image 1.62c Software package for Macintosh (NIH, Bethesda, MD, USA). Mann-Whitney analysis was used to determine significant variations, with a level of significance set at $P < 0.05$.

Migration assays

ZR-75 cells were seeded onto porous inserts (8 μ m, Nunc) previously coated with matrigel (Sigma-Aldrich). Progesterone (10 nM) or vehicle was added for 24 h. An activating PAR1 peptide was added 12 h before the end of the experiment. The Nunc inserts were fixed in cold methanol and immunocytochemistry against cytokeratin (Sigma-Aldrich) was performed. Observation under light microscopy determined the number of cells in each field that had passed through the porous membrane. In each dataset, 15 fields (400 \times) were counted and the mean number of cells was plotted with standard errors. Four separate experiments were performed for each reported point. Mann-Whitney analysis was used to determine significant variations, with a level of significance set at $P < 0.05$.

Immunocytochemistry

PAR1 antibody (Calbiochem) was added for 18 h at a dilution of 1:100. The coverslips received two 30 min washes before labeled Streptavidin Biotin, LSABTM (DAKO, CA, USA) addition for a further for 30 min. The image was developed using diaminobenzidine for 15 min in darkness and counterstained with Harris hematoxylin before mounting.

Immunofluorescence

ZR-75 cells (2×10^4 cells) were seeded on 12 mm coverslips inserted in 24-well plates and incubated for 24 h in DMEM/F12 containing 5% FBS. Progesterone (10 nM) was then added to the cells and incubated for an additional 24 h. The medium was then replaced by serum-free medium and after 30 min incubation, aPAR1 (A1, 100 μ M) was added for 15 min. Next, cells were fixed with 4% *p*-formaldehyde in 100 mM PIPES buffer, pH 6.8, containing 0.04 M KOH, 2 mM EGTA, and 2 mM MgCl₂, permeabilized with 0.1% Triton X-100 in Universal buffer (50 mM Tris-HCl,

pH 7.6, 0.15 M NaCl, and 0.1% sodium azide) for 10 min, and blocked with 2% BSA in Universal buffer for 30 min. Cells were then incubated with anti-phospho-FAK IgG, followed by FITC-conjugated anti-rabbit IgG, rhodamine-labeled phalloidin, and 4',6-diamidino-2-phenylindole (DAPI) for 1 h. Samples were then mounted onto slides with 10% Mowiol (Calbiochem) and 2.5% 1,4-diazabicyclo[2,2,2]octane (DABCO, Sigma) and visualized using an IX-81 spinning disc microscope (Olympus, Center Valley, PA, USA). Visualization of polymerized actin by rhodamine-labeled phalloidin allowed the quantification of the number of individual cells that contained stress fibers under each experimental condition. The number of FAs was counted as previously reported (Avalos *et al.* 2004).

Data mining using the OncoPrint platform

OncoPrint (Compendia Bioscience, Ann Arbor, MI, USA) was used for analysis and visualization. OncoPrint is an online tool that aggregates mRNA and copy number expression data from various studies related to cancer. OncoPrint contains data from over 18 000 cancer gene expression microarrays and allows a researcher to easily investigate the relative expression of a particular gene across various cancer datasets (including human tumors and cell lines; Rhodes *et al.* 2004, 2007). Herein, we employed the recently updated TCGA breast cancer dataset, where each tumor sample was genome sequenced and analyzed for mRNA expression, DNA copy number, DNA methylation, etc. The OncoPrint database collected and standardized this information for our analysis. *PAR1* mRNA expression was investigated in breast tumors and sorted based on PR status (as provided in the metadata for each tumor sample, i.e. PR clinical immunohistochemistry (IHC) report, either positive or negative).

Results

Progesterone regulates *PAR1* mRNA and protein in the ZR-75 breast cancer cell line

To investigate the regulation of *PAR1*, we determined the *PAR1* transcript levels in ZR-75 breast cancer cells stimulated for 9 h with progesterone (10 nM), E₂ (10 nM), or EGF (10 nM). Figure 1A demonstrates that only progesterone increases *PAR1* mRNA levels in these cells. Adding estrogens did not alter the ability of progesterone to increase *PAR1* transcript levels, and this effect was also replicated using the synthetic progestin medroxyprogesterone acetate (results not shown). To further dissect the progesterone regulation of *PAR1*, we performed a 24 h time course to examine mRNA. As seen in Fig. 1B, the progesterone upregulation is rapid and transient, being elevated at 3 h, reaching a maximum at 6 h, and plateauing out at 12 h, before the levels return to basal values at 18 and 24 h (Fig. 1B). The dependence on transcription and the PR was demonstrated by the inability

of progesterone to increase *PAR1* mRNA after 9 h in the presence of the competitive PR antagonist, RU486 (100 nM, Fig. 1C), and the transcription inhibitor actinomycin-D (10 µg/ml, Fig. 1D). To demonstrate that this increase in

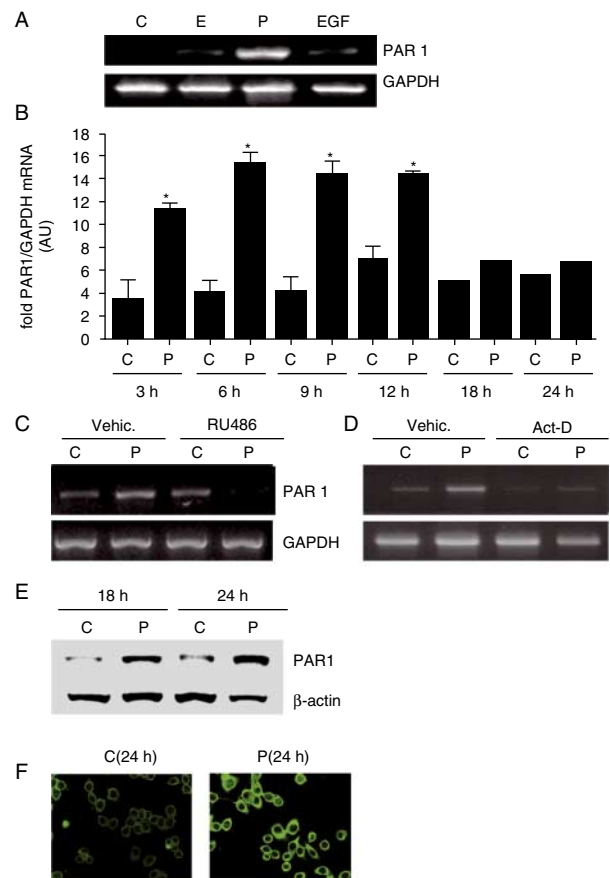


Figure 1 Progesterone upregulates *PAR1* mRNA and protein in the ZR-75 cell line. (A) The effects of ovarian hormones and EGF upon *PAR1* mRNA expression levels. ZR-75 cells were incubated for 9 h in DMEM/F12 with 5% charcoal-treated serum containing vehicle (C, ethanol), 10 nM progesterone (P), 10 nM 17 β -estradiol (E), or 10 nM EGF treatments. RT-PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. Equal starting RNA concentrations were confirmed by GAPDH amplification. (B) Time course of *PAR1* mRNA levels in response to progesterone in ZR-75 cells. Cells were treated with a single pulse of progesterone (10 nM) and harvested at indicated times between 0 and 24 h. Densitometric analysis was obtained from a minimum of three independent experiments. Mann-Whitney analysis was used to determine statistical significance, * $P < 0.05$. (C and D) RT-PCR in which progesterone (10 nM) treatment was for 9 h and RU486 (100 nM) or actinomycin-D (Act-D, 10 µg/ml) addition 30 min before progesterone addition. GAPDH was used as an internal control. (E) Cells were treated as earlier with 10 nM progesterone for 18 or 24 h before protein extraction and *PAR1* analysis by western blot. Equal protein loading was verified by an anti- β -actin antibody. (F) ZR-75 cells were grown on coverslips for 24 h in the presence of vehicle or progesterone under the conditions stated earlier. *PAR1* immunoreactivity was more pronounced in the presence of progesterone. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-11-0310>.

mRNA expression translated into increased protein levels, we examined *PAR1* levels by western blotting and immunocytochemistry in ZR-75 cells treated with progesterone for 18 or 24 h. As shown in Fig. 1E and F, progesterone strongly enhanced the cellular expression of this PAR family member at both time points.

Progesterone regulates *PAR1* mRNA and protein in the T47D breast cancer cell line

To demonstrate that the progesterone-dependent regulation of *PAR1* was not restricted to ZR-75 cells, we turned to T47D cells, another well-characterized progesterone-responsive breast cancer cell line (Horwitz *et al.* 1982). As shown in Fig. 2A, progesterone also increased *PAR1* mRNA levels in this cell line and the kinetics of this response appeared similar to that observed in ZR-75 cells. To determine whether PR-dependent transcription mediates the effect of progesterone on *PAR1* expression, we made use of a PR-negative T47D clone stably transfected with PR-B

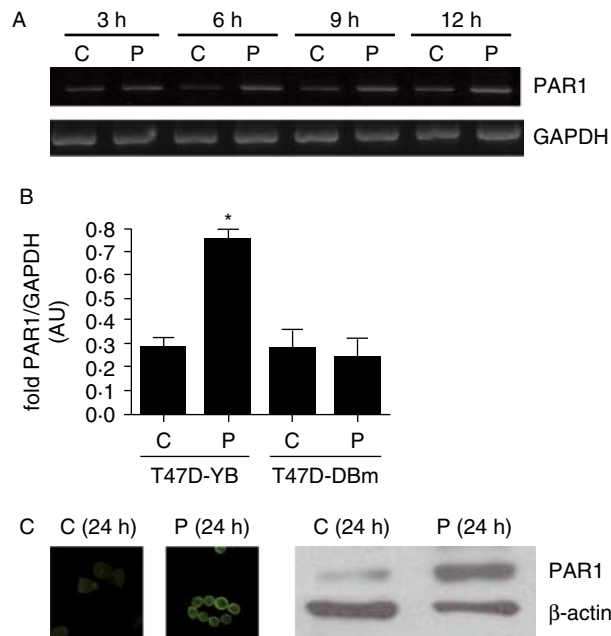


Figure 2 Progesterone upregulates *PAR1* mRNA and protein in the T47D cell line. (A) Representative time course of *PAR1* mRNA levels in response to progesterone in T47D cells. Cells were treated with a single pulse of progesterone (10 nM) and harvested at indicated times between 0 and 12 h. (B) *PAR1* mRNA levels in response to progesterone in the T47D-YB and T47D-DBM cell lines. Densitometric analysis from a minimum of three independent experiments is shown. Mann-Whitney analysis was used to determine statistical significance, * $P < 0.05$. In both panels A and B, GAPDH was used as an internal loading control. (C) T47D cells were treated with 10 nM progesterone (P) or vehicle (C) for 24 h before analysis of *PAR1* by immunoreactivity (left-hand panel) or by western blotting (right-hand panel). Equal protein loading was verified by an anti- β -actin antibody. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-11-0310>.

(T47D-YB) and a clone containing a PR mutant unable to bind DNA (T47D-DBM) (Sartorius *et al.* 1994). As shown in Fig. 2B, the progesterone-dependent regulation of *PAR1* expression was dependent on the presence of transcriptionally competent PR-B. Again, the increase in *PAR1* mRNA upon progesterone stimulation increases correspondingly protein levels in T47D cells (Fig. 2C).

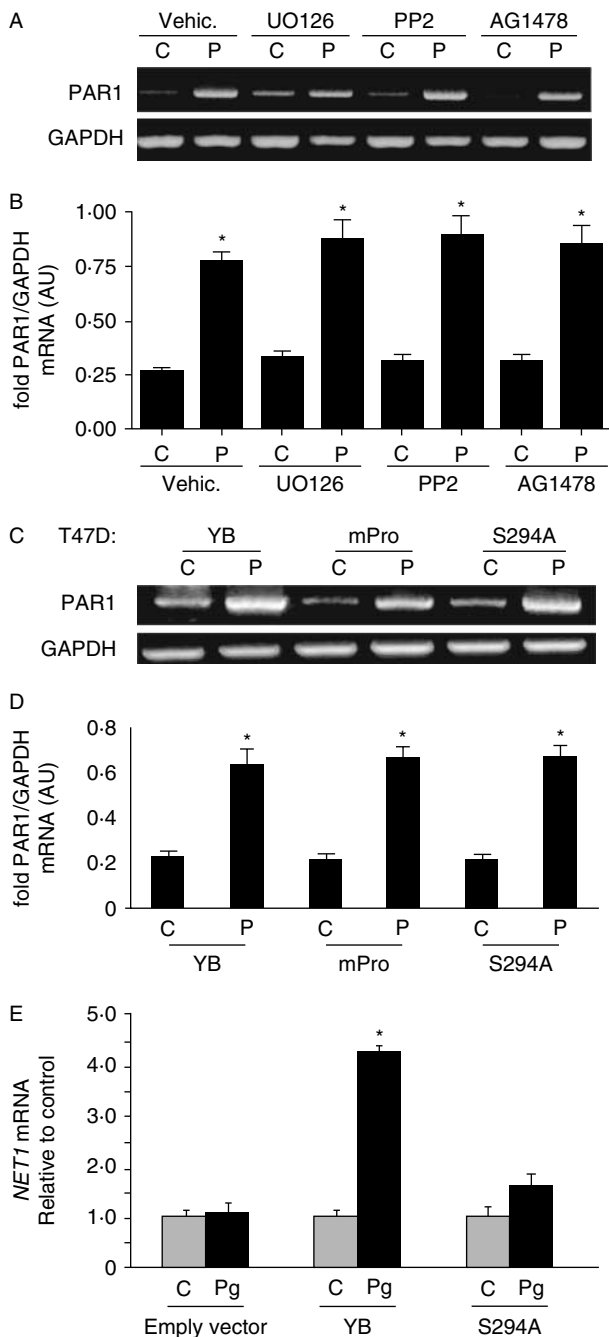
Progesterone-dependent regulation of *PAR1* expression does not require EGFR, MEK1, or c-Src signaling

As progesterone actions are modified through targeted phosphorylation of PR by the MAPK, c-Src, and the EGFR signal transduction pathways (Lange 2008), we investigated whether inhibitors of these pathways also impinge on the induction of *PAR1* transcripts. As shown in Fig. 3A and B, none of the pharmacological inhibitors, at concentrations previously validated in ZR-75 cells, attenuated the progesterone induction of *PAR1* transcripts. To confirm this result, we again made use of T47D cells stably expressing specific PR mutants that either lack the MAPK phosphorylation site (S294A) or the proline-rich Src interaction motif (mPRO) (Quezada *et al.* 2010, Pierson-Mullany & Lange 2004, Faivre & Lange 2007, Faivre *et al.* 2008). As anticipated from the inhibitor studies, these PR mutants were as effective as wild-type PR-B in inducing *PAR1* expression upon progesterone activation (Fig. 3C and D). As the mutant S294A PR-B had previously been reported to possess limited transcriptional activity (on selected endogenous genes), we tested whether our cell lines maintained this response on additional progesterone-regulated genes. As demonstrated in Fig. 3E, prostin is capable of regulating *NET1* mRNA levels via wild-type PR but not in cells stably expressing S294A PR. These data confirm that S294A mutant receptors function as previously reported and that this phosphorylation site positively impacts PR transcription in a promoter selective manner. By contrast, herein, our data clearly demonstrate that PR Ser294 is not required for progesterone-dependent regulation of *PAR1*.

Progesterone promotes focal contacts, stress fiber formation, and cell migration upon *PAR1* activation

To evaluate whether progesterone-dependent increase in *PAR1* expression is of biological relevance, we focused on cell migration. Cell migration requires formation of focal contacts, mediated by phosphorylated FAK, and the creation of stress fibers. The presence of focal contacts was confirmed by co-localization of phosphorylated FAK with vinculin (not shown). Although *PAR1* is expressed in ZR-75 cells in the absence of progesterone stimulation, treatment with a specific aPAR1 did not increase the number of focal contacts per cell. Similarly, progesterone treatment alone had no discernible effect. However, pretreatment with progesterone, which upregulates *PAR1* expression, followed by the addition of the activating peptide resulted in a significant increase in focal

contacts (Fig. 4A and B). The same pattern of regulation was observed upon the examination of stress fibers using phalloidin staining (Fig. 5A and B). Although progesterone treatment alone had no effect, pretreatment with progesterone followed by a *a*PAR1 doubled the number of cells that contained stress fibers. Finally, we evaluated whether the increase in focal contacts and stress fiber formation correlated with cellular migration. As shown in Fig. 6A, treatment with progesterone alone was sufficient to increase migration of



ZR75 cells. However, pretreatment with progesterone followed by exposure to *a*PAR1 significantly enhanced the migratory capacity of ZR-75 cells. Repeating this experiment in the T47D cell line showed that combined progesterone and *a*PAR1 treatment also statistically increased breast cancer cell migration. Despite showing tendencies to increase, progesterone or *a*PAR1 alone did not demonstrate statistical differences from control. Combining the data suggest that progesterone not only enhances the expression of *PAR1* in breast cancer cells but also sensitizes the cells to *PAR1*-dependent signaling.

Clinical data mining (Oncomine): *PAR1* is overexpressed in PR+ tumors

In the breast cancer cell lines, ZR-75 and T47D, we have demonstrated that progesterone regulates *PAR1* mRNA and protein levels. To investigate whether this *in vitro* observation was also observed in the clinical setting, we performed data mining in public breast cancer datasets using the Oncomine platform (Rhodes *et al.* 2004, 2007). We used the TCGA breast cancer mRNA expression dataset within the Oncomine database for our analysis, investigated *PAR1* (F2R) mRNA expression in breast tumors, and sorted based on their PR status. The same analysis is represented in two different panels (either a bar chart or box- and -whiskers plot) in Fig. 7. The data show that *PAR1* mRNA is overexpressed in PR+ve breast tumor samples ($n=191$) compared with PR-ve tumors ($n=128$). *PAR1* is 1.281-fold higher in PR+ve tumors, with a P value=0.000259 and t -test=3.516. Thus, Fig. 7 demonstrates that within the highly robust TCGA dataset, *PAR1* mRNA expression levels are statistically associated with PR-positive breast cancers.

Discussion

Herein, we demonstrate that progesterone regulates *PAR1* at the mRNA and protein levels. This regulation is dependent on the presence of the PR, yet is independent of the *c*-Src interaction domain (mPRO) and the serine 294

Figure 3 Progesterone does not require the MEK1, Src, or EGFR signaling pathways to upregulate *PAR1* mRNA. (A) Representative RT-PCR analysis of ZR-75 cells treated with 10 nM progesterone for 6 h. The MEK1 (U0126), Src (PP2), and EGFR (AG1478) pathway inhibitors were added 30 min before progesterone addition. (B) Densitometric analysis from a minimum of three independent experiments shown in panel A. (C) Representative RT-PCR analysis of T47D-YB, T47DmPRO, and T47D-S294A cells treated with 10 nM progesterone for 6 h. (D) Densitometric analysis from a minimum of three independent experiments shown in panel E. T47D cells stably expressing empty vector, wild-type PRB, or the PR mutant S294A were treated in the presence of progestin (R5020, 10 nM) for 6 h before the evaluation of *NET1* mRNA levels. Progestin is capable of regulating *NET1* mRNA via wild-type PR but not in cells expressing PR S294A. In panels B, D, and E, Mann-Whitney analysis was used to determine statistical significance, $*P<0.05$.

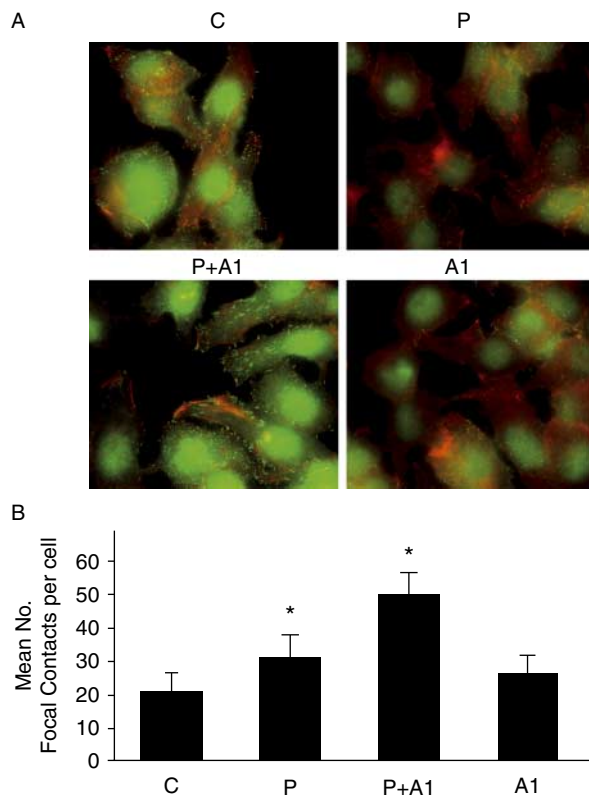


Figure 4 Progesterone pretreatment and PAR1-activating peptide increase the number of focal contacts. (A) ZR-75 cells treated with 10 nM progesterone for 24 h and/or the peptide that activates PAR1 for 15 min were stained with rhodamine-labeled phalloidin (red), DAPI (blue), and pFAK (green) to visualize F-actin, DNA, and focal contacts respectively. Representative images of merged colors or optical zoom of the focal contacts (black and white internal pictures) of the indicated regions are shown for each condition. (B) Analysis of focal contacts per cell is shown. Averages \pm S.E.M. of three independent experiments are shown. * $P < 0.05$. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-11-0310>.

phosphorylation domain. The mechanism of regulation is transcriptional, as demonstrated by the loss of regulation in the presence of the transcriptional inhibitor actinomycin-D, and is most probably through DNA binding as we demonstrated the requirement of an intact PR DNA binding domain and observed a lack of inhibition in the presence of Src and MEK pathway inhibitors. Interestingly, our results demonstrate that phosphorylation of PR at Ser294 does not enhance or alter the ability of progesterone to regulate *PAR1*. Notably, PR Ser294 phosphorylation prevents Lys388 sumoylation (a repressive modification). Mutant S294A PR is thus heavily sumoylated (at Lys388) relative to the wild-type (i.e. reversibly phosphorylated) receptor (Daniel *et al.* 2007, Daniel & Lange 2009). While this regulatory mechanism mediates phosphorylation-dependent PR promoter selection (and depression of transcription), not all endogenous genes are sensitive to these modifications of PR (Daniel & Lange 2009). As an internal control, we examined the regulation of

NET1, a progestin-responsive gene (Bray *et al.* 2005) that has been shown previously to be a sumo-sensitive PR target gene (Knutson *et al.* 2012). Herein, we show that cells stably expressing S294A PR fail to upregulate *NET1* relative to cells expressing wild-type PR. Notably, PR-dependent regulation of *NET1* and *PAR1* occurs at the same time point, demonstrating the selective nature of phospho-Ser294 PR on PR target gene regulation.

Although this is not the first observation that progesterone can regulate *PAR1*, this is, to our knowledge, the first to demonstrate regulation in breast cancer or mammary gland-derived cells. It has not escaped our notice that the timing and mechanism of *PAR1* regulation by progesterone is remarkably similar to the progesterone regulation of TF in both T47D and ZR-75 cells (Kato *et al.* 2005b, Quezada *et al.* 2010, Henriquez *et al.* 2011). Advanced breast cancer is associated

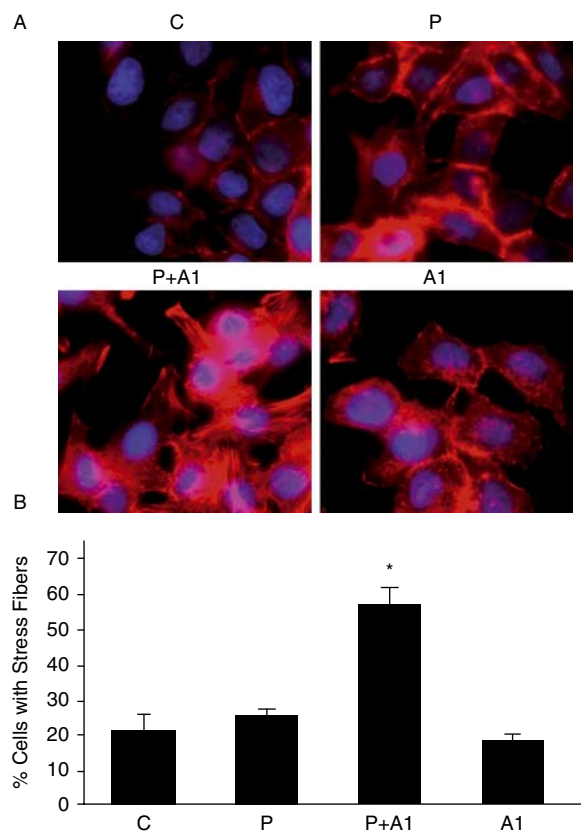


Figure 5 Progesterone pretreatment and PAR1-activating peptide treatment increase the number of cells with stress fibers. (A) ZR-75 cells treated with 10 nM progesterone for 24 h and/or the peptide that activates PAR1 for 15 min were stained with rhodamine-labeled phalloidin (red) to visualize polymerized actin. Images are representative of results obtained in at least three independent experiments. (B) The graph shows the quantification of the number of cells that contain stress fibers (examples shown in white arrows) in each condition (shown as a percentage). Values were averaged from three independent experiments. * $P < 0.05$ compared with control condition. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-11-0310>.

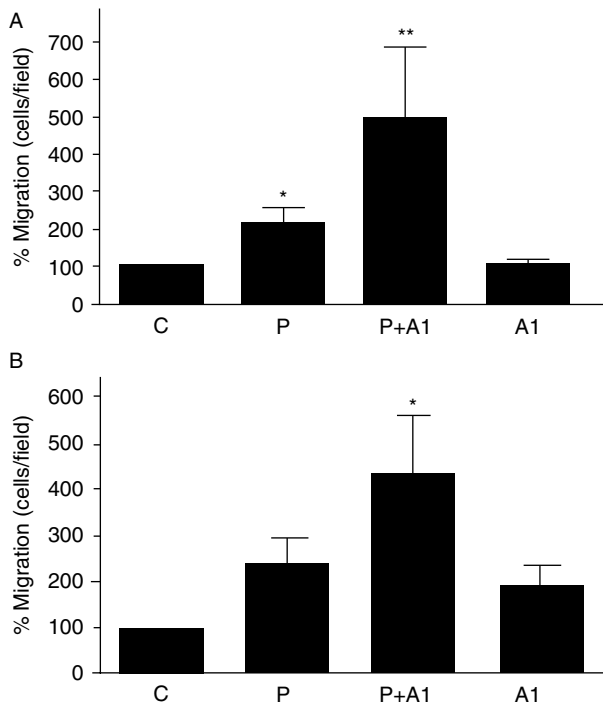


Figure 6 Progesterone pretreatment and PAR1-activating peptide treatment increase breast cancer cell migration. ZR-75 and T47D breast cancer cells were seeded over porous inserts and treated with progesterone (10 nM) or vehicle (control) for 12 h before the addition of PAR1-activating peptide (aPAR1) or vehicle for a further 12 h. Cells that had migrated through the pores were counted after immunostaining for a marker of epithelial cells, cytokeratin. The data show the mean cell migration (\pm S.E.M.) of four separate experiments. Statistical analysis was performed by the Mann–Whitney method (* $P < 0.05$ compared with control; ** $P < 0.05$ compared with progesterone alone).

with a hypercoagulable state that may be triggered or enhanced by the presence of TF and PAR1 on the surface of cancer cells (Ruf & Mueller 2006). TF, when bound to factor VIIa, activates the coagulation cascade resulting in FX and thrombin activation and PAR cleavage (Duarte *et al.* 2006, Albrektsen *et al.* 2007, Uusitalo-Jarvinen *et al.* 2007). Given the well-reported links of the coagulation cascade proteins TF and PAR1 with cancer cell angiogenesis, coagulation, migration, and invasion, their enhancement by progesterone may prime breast cancer cells for these functions, thus contributing to the increase in breast cancer incidence seen in postmenopausal women taking HRT. In a cohort of 319 patients from the TCGA dataset, data mining using the OncoPrint platform showed that *PAR1* mRNA is overexpressed in PR+ compared with PR- breast tumor samples. The statistically significant 1.281-fold increase in expression in the presence of PR supports our *in vitro* cell line data and may suggest that in PR+ breast cancer patients taking HRT, the presence of progestins may increase the levels of *PAR1* and deliver a more aggressive phenotype to the cancer cell. The link between *PAR1*, cancer cell invasion, and

poor prognosis is already well established (Even-Ram *et al.* 1998, Tellez & Bar-Eli 2003, Yin *et al.* 2003).

We also demonstrate that progesterone increases the formation of FA complexes as determined by an increase in pFAK in these structures. However, unlike the previous report at 15 min by Fu *et al.*, we concentrated on longer time points where we had previously shown that progesterone increased invasion of these cells. At 24 h after progesterone addition, we observed pFAK phosphorylation and a significant increase in FAs. However, progesterone pretreatment of breast cancer cells for 12 h to increase PAR1 protein levels followed by a further 12 h with aPAR1 significantly increased the number of FAs still further. It is noteworthy that in ZR-75 cells, aPAR1 had no effect after 12 h on the formation of FAs, despite being able to phosphorylate Erk-1/2. This demonstrates that PAR1 cannot directly phosphorylate FAK at this time point but requires either PR presence or a process enhanced by the PR, fueling previous speculations in the literature that the assembly at the intercellular domain of PAR1 can selectively mediate cellular processes.

Breast cancer cells are not the first steroid hormone-responsive cells to show a relation between PAR1 and pFAK. By immunohistochemistry, Grisar-Granovsky *et al.* (2005) observed pFAK in invasive ovarian carcinoma but not in the corresponding normal epithelium. The authors speculated that PAR1 levels in ovarian carcinoma may transmit signals leading to the phosphorylation of FAK, causing alterations in the integrin functional state and thus promoting ovarian cancer malignancy (Grisar-Granovsky *et al.* 2005).

In recent publications by Fu *et al.* (2008), it was demonstrated in breast cancer cells that PR signals to moesin, leading to rapid actin remodeling related to cell movement and invasion. The authors speculated that the liganded PR-A, but not PR-B, interacts with G coupled proteins, in particular $G\alpha_{13}$, allowing recruitment of the RhoA/ROCK-2 cascade and subsequent moesin phosphorylation, cell migration, and invasion. Notably, the expression of activated $G\alpha_{13}$ in cancer cells has been shown to increase invasion (Kelly *et al.* 2006). Interestingly, in the presence of pertussis toxin, PAR1 has been shown to induce cellular invasion through a $G\alpha_{12}/G\alpha_{13}$ and RhoA/Rho kinase (ROCK)-dependent signaling (Nguyen *et al.* 2002). PAR1 has recently been shown to signal through a $G\alpha_{13}$ -disheveled pathway to stabilize β -catenin. Furthermore, a dominant negative $G\alpha_{13}$ or a siRNA against disheveled inhibited or greatly reduced PAR1-induced matrigel invasion (Turm *et al.* 2010). Whether the increase in PAR1 by progesterone recruits the $G\alpha_{13}$ -disheveled pathway leading to increased migration is certainly a promising theory that needs to be investigated.

While invasion was shown to be further enhanced by a PAR1-activating ligand in cancer cell lines, the treatment of melanoma cells with the PAR activator thrombin dramatically increased the number of lung metastases in rats (Maragoudakis *et al.* 2000, Even-Ram *et al.* 2001). However, in our migration model, we did not observe notable effects with

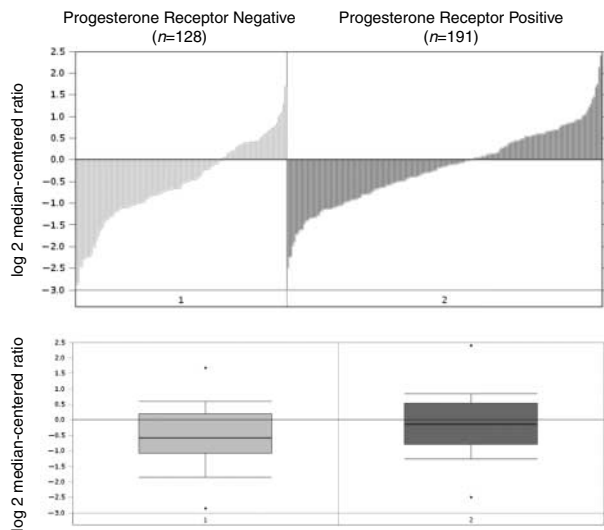


Figure 7 *PAR1* is overexpressed in PR+ tumors: results from the data mining platform, OncoPrint. Specifically using the TCGA breast cancer dataset, *PAR1* (*F2R*) mRNA expression in breast tumors sorted by PR status. The same dataset is represented by bar chart in the upper panel and by a box-and-whiskers plot in the lower panel. *PAR1* mRNA expression is overexpressed in the PR+ breast tumor samples ($n=191$) compared with the PR- tumors ($n=128$) 1.281-fold higher in PR+ tumors (P value=0.000259, t -test=3.516). In the upper panel, each bar represents one individual breast tumor sample; with more bars above the 0.0 mRNA expression line in the PR+ tumor subgroup. The Y-axis is normalized mRNA gene expression values (normalized to the median value of the dataset, and expression levels have been \log_2 transformed, where positive numbers indicate overexpression and negative numbers indicate underexpression). In the lower panel (box-and-whiskers plot), the dots represent the high/low values, the error bars represent the 90%/10% values, the top and bottom of the box represent the 75%/25% values, and the bar in the middle of the box is the median.

aPAR1 alone. No effect was observed in ZR-75 cells but only tended to increase (not statistically significant) in the T47D cell line. This may reflect the basal levels of PAR1 in the breast cancer cell lines analyzed herein and/or the tight control that progesterone possesses in progesterone-responsive tissue, allowing activation of certain signaling cascades only in concert with other progesterone-primed pathways.

In an ER- and PR-negative MDA-MB-231-derived cell line, where progesterone markedly inhibits cell growth, progesterone treatment increases the size and number of stress fibers, FA proteins, and focal contacts (Lin *et al.* 2000). Interestingly, it has also been reported that it is the loss of stress fibers in actin remodeling that is associated with cancer metastasis (Carragher & Frame 2004, Fu *et al.* 2008). Although these questions cannot be addressed in this paper, it is important to note that we are examining only one facet of progesterone regulation in the breast cancer cells and in the global picture of replication, proliferation, migration, and metastasis, the notable appearance of stress fibers and FAs on addition of progesterone and PAR1-activating ligand may

represent the initial traction that the cells require to stimulate their migratory capacity.

In conclusion, progesterone promotes FAK phosphorylation and breast cancer cell migration. Progesterone transiently regulates PAR1 mRNA and protein and this increase permits an enhancement in stress fiber and FA formation, providing the necessary adhesion to stimulate migration upon PAR1 activation. Because migration is stimulated under these conditions, a possibility exists that increased FAK phosphorylation is related to an elevated FA turnover, which would account for faster cell migration. Our results, which fit well with clinical data showing an association between increased PAR1 levels and PR+ tumors, bring to light new signaling complexities and deliver further understanding of progesterone-mediated breast cancer cell migration. PARs can be effectively targeted by pharmacological agents, such as small-molecule inhibitors, peptides, and antibodies (Leger *et al.* 2006), and thus, this study may promote the use of anti-PAR1 and progesterone strategies for the treatment of PR-positive breast cancer.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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