HAL
open science

Progesterone receptor isoforms PRA and PRB differentially contribute to breast cancer cell migration through interaction with focal adhesion kinase complexes.

Catherine Bellance, Junaid A. Khan, Geri Meduri, Anne Guiochon-Mantel, Marc Lombès, Hugues Loosfelt

To cite this version:

Catherine Bellance, Junaid A. Khan, Geri Meduri, Anne Guiochon-Mantel, Marc Lombès, et al.. Progesterone receptor isoforms PRA and PRB differentially contribute to breast cancer cell migration through interaction with focal adhesion kinase complexes.. Molecular Biology of the Cell, 2013, 24 (9), pp.1363-74. 10.1091/mbc.E12-11-0807 . inserm-00812020

HAL Id: inserm-00812020

https://inserm.hal.science/inserm-00812020v1

Submitted on 13 Sep 2013

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

TITLE PAGE

Progesterone receptor isoforms PRA and PRB differentially contribute to breast cancer cell migration through interaction with focal adhesion kinase complexes

Authors: Catherine Bellance[a,b], Junaid A. Khan[a,b,e], Geri Meduri[a,b,c], Anne Guiochon-Mantel[a,b,c], Marc Lombès[a,b,d], and Hugues Loosfelt[a,b]

Author affiliations: [a]Inserm Unité 693, 63 rue Gabriel Péri, Le Kremlin-Bicêtre, F-94276, France; [b]Univ Paris-Sud, Faculté de Médecine Paris-Sud, UMR-S693, Le Kremlin-Bicêtre, F-94276, France; [c]Assistance Publique-Hôpitaux de Paris, Hôpital Bicêtre, Service de Génétique Moléculaire, Pharmacogénétique et hormonologie, Le Kremlin-Bicêtre, F-94275, France ; [d]Assistance Publique-Hôpitaux de Paris, Hôpital Bicêtre, Service d'Endocrinologie et Maladies de la Reproduction, Le Kremlin-Bicêtre, F-94275, France ; [e]Department of Physiology and Pharmacology, University of Agriculture, Faisalabad 38040, Pakistan.

Current email adresses : Catherine Bellance (bellance.catherine@laposte.net); Junaid A Khan (junaidali.khan@ymail.com); Geri Meduri (geri.m@free.fr); Anne Guiochon-Mantel (anne.mantel@bct.aphp.fr); Marc Lombès (marc.lombes@u-psud.fr); Hugues Loosfelt (hugues.loosfelt@u-psud.fr).

Corresponding Author: Dr Hugues Loosfelt, Inserm U693, Faculté de Médecine Paris Sud, 63 rue Gabriel Péri, Le Kremlin-Bicêtre, F-94276, France. Tel: 33 1 49 59 67 10; Fax: 33 1 49 59 67 32; E-mail: hugues.loosfelt@u-psud.fr

Running head: PR-dependent breast cancer cell motility

Author's contributions: CB performed and designed experiments as well as participated in the drafting of article, JAK designed the experiments and characterized the cell lines, HL and ML conceived the studies and wrote the article. AGM participated in discussions and data interpretation.

Abbreviations used: PR, progesterone receptor; PRA, progesterone receptor isoform A; PRB, progesterone receptor isoform B; ER, estrogen receptor ; P4, progesterone; R5020 (17,21-dimethyl-19-norpregna-4,9-dien-3,20-dione), RU486 (11β-(4-Dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one). FAK, focal adhesion kinase; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; PAI-1, plasminogen activator inhibitor type 1; FAK^{Y397p}, FAK phosphorylated at Tyr397 residue.

ABSTRACT

Progesterone receptor (PR) and progestins are known to impact mammary tumorigenesis, however the relative contribution of PRA and PRB isoforms in cancer cell migration remains elusive. By using a bi-inducible MDA-MB-231 breast cancer cell line expressing PRA and/or PRB, we analyzed the impact of conditional PR isoform expression. Surprisingly, unliganded PRB but not PRA strongly enhanced cell migration as compared to PR(-) cells. R5020 progestin limited this effect that was counteracted by antagonist RU486. Importantly, PRA co-expression potentiated PRB-mediated migration whereas PRA alone was ineffective. PR isoforms differentially regulated expressions of major players of cell migration such as urokinase plasminogen activator (uPA), its inhibitor PAI-1, uPA receptor (uPAR) and β1-integrin, known to impact Focal adhesion kinase (FAK) signaling. Moreover, unliganded PRB but not PRA enhanced FAK Tyr397 phosphorylation, and colocalized with activated FAK in cell protrusions. Since PRB as well as PRA co-immunoprecipitated with FAK, both isoforms may interact with FAK complexes depending on their respective nucleocytoplasmic trafficking. In addition, FAK degradation was coupled to R5020-dependent turnovers of PRA and PRB. Such impact of PRB/PRA expression on FAK signaling may thus affect adhesion/motility underscoring the implication of PR isoforms in breast cancer invasivity and metastatic evolution with underlain therapeutic outcomes.

INTRODUCTION

Human progesterone Receptor (PR) is a crucial transcription factor involved in development and differentiation of female reproductive tissue. It is expressed from a single gene as two isoforms PRA (94 kDa) and PRB (116 kDa) at similar level, PRA being truncated for the 164 N-terminal aminoacids of PRB. Upon hormone binding, PRA and PRB homodimers or heterodimers exhibit distinct transcriptional regulatory functions by targeting various subsets of genes (Graham *et al.*, 2005; Jacobsen *et al.*, 2005; Leo *et al.*, 2005; Khan *et al.*, 2012). However, unliganded PR has been shown to be transcriptionally activated by growth factor stimuli irrespectively of hormone status (Labriola *et al.*, 2003). PRA is mainly localized in the nucleus, whereas PRB continuously shuttles between nuclear and cytoplasmic compartments (Guiochon-Mantel *et al.*, 1994; Boonyaratanakornkit *et al.*, 2008). Thus, PRB can mediate either direct transcriptional events or rapid cytoplasmic changes by interacting with non-nuclear signaling pathways, such as MAPK, c-Src, PI3K/Akt and JAK2/Stat3 (Migliaccio *et al.*, 1998; Proietti *et al.*, 2005; Boonyaratanakornkit *et al.*, 2007; Hammes and Levin, 2007; Fu *et al.*, 2008b). Therefore, it is likely that both isoforms elicit distinct and coordinated functions in the two compartments through dynamic processes.

Association of estrogens and progestins has been shown to increase breast cancer risk factor in long term Hormone Replacement Therapy (HRT) patients (Rossouw *et al.*, 2002; Chlebowski *et al.*, 2003; Chlebowski *et al.*, 2010). Moreover, alteration of PRA/PRB expression ratio is frequently observed in breast cancer cells (Graham *et al.*, 1995; Mote *et al.*, 2002). Elevated PRA expression (McGowan and Clarke, 1999; Bagheri-Yarmand *et al.*, 2004) as well as loss of PR expression (Bogina *et al.*, 2011) is generally associated with poor prognosis. PR has been also implicated in breast cancer metastatic progression through unclear molecular mechanisms (Weigelt *et al.*, 2005). Although most of breast cancer metastases lack ER and PR expression, PR has been reported to facilitate metastasis evolution by increasing invasiveness of primary cancer cells through transcriptional regulation of key proteins involved in cellular migration and adhesion, such as matrix metalloproteases (MMPs), vascular endothelial growth factor (VEGF), the plasminogen activator (PA) system (Kato *et al.*, 2005; Carnevale *et al.*, 2007) and focal adhesion kinase (FAK) (Fu *et al.*, 2010).

The metastatic process mainly results from alterations in cell migration and invasivity through multiple signaling cross-talks such as extracellular matrix (ECM) degradation and cell adhesion/de-adhesion dynamics (Lauffenburger and Horwitz, 1996; Chiang and Massague, 2008; May *et al.*, 2011; Rosenthal *et al.*, 2011). In this regards, the PA system is strongly activated in aggressive tumor cells and is frequently involved in the development of metastatic phenotype (Chapman, 1997; Bernard-Trifilo *et al.*, 2006; Mitra *et al.*, 2006; Wei *et al.*, 2007; Michael *et al.*, 2009; Smith and Marshall, 2010). Urokinase plasminogen activator (uPA), the major actor of the PA system, is a serine protease targeting plasminogen and several MMPs, leading to the remodeling of ECM and activating cell migration. In addition, uPA binds to the membrane-anchored uPA receptor (uPAR) that exhibits multiple functions on proliferation, migration and adhesion-dependent signal transduction. This receptor is coupled to signaling factors such as integrins, c-Src kinases, focal adhesion kinase (FAK), PDGFR, GPCR, JAK/STAT (Busso *et al.*, 1994; Stahl and Mueller, 1994; Wei *et al.*, 1996; Chapman, 1997; Bernard-Trifilo *et al.*, 2006; Mitra *et al.*, 2006; Mitra and Schlaepfer, 2006; Wei *et al.*, 2007; Michael *et al.*, 2009; Smith and Marshall, 2010). Moreover, following binding to uPA, the plasminogen activator inhibitor type 1 (PAI-1) inhibits uPA serine protease activity and thus ECM proteolysis. Despite this function, PAI-1 is also able to promote cell migration (Chazaud *et al.*, 2002; Czekay and Loskutoff, 2004; Providence and Higgins, 2004; Wilkins-Port *et al.*, 2007; Fabre-Guillevin *et al.*, 2008) as well as proliferation processes (Olson *et al.*, 1992; Schneider *et al.*, 2000; McMahon *et al.*, 2001; Jo *et al.*, 2005) via multiple interactions with ECM components such as low-density lipoprotein receptor-

related protein (LRP) and integrins. Both PAI-1 and uPA have now been validated as major prognostic factors for breast cancer evolution (Janicke *et al.*, 2001; Harbeck *et al.*, 2004; Sakakibara *et al.*, 2004; Biermann *et al.*, 2008).

Interaction of uPA-bound uPAR with β1-integrin triggers clustering of the complex leading to autophosphorylation of the focal adhesion kinase (FAK) (Tang *et al.*, 1998; Bernard-Trifilo *et al.*, 2006; Mitra and Schlaepfer, 2006; Lim *et al.*, 2008). This tyrosine kinase functions as an integrator of multiple signaling pathways promoting formation and turnover of focal adhesion points, membrane ruffling, cell shape and cytoskeletal reorganization involved in cell motility processes (Luo and Guan, 2010; Schaller, 2010). Under the control of extracellular signalings mediated by integrins and other cell surface receptors allow FAK autophosphorylation on Tyr397 residue (Y397) creating a binding site for SH-2 containing proteins such as Src kinase. This association then leads to subsequent phosphorylations of FAK maximizing its activation (Luo and Guan, 2010). It has been shown that FAK Y397-dependent activation is strongly enhanced in metastatic cancer cells as compared to primary breast tumors (Sood *et al.*, 2004). Moreover, knockdown of FAK (Ilic *et al.*, 1995) or mutation on several phosphorylation sites results in a strong decrease in cancer cell motility (Luo and Guan, 2010).

Various studies have highlighted the possible impact of progestins and PR on such signaling pathways regulating cell migration (Marbaix *et al.*, 1992; Lin *et al.*, 2000; Lin *et al.*, 2001; Vincent *et al.*, 2002; Fu *et al.*, 2008a; Fu *et al.*, 2010; Hiscox *et al.*, 2010). PR was found to inhibit cell growth and induce cell spreading and focal adhesion in association with modifications of FAK activity and β1-integrin signaling in metastatic MDA-MB-231 cells stably expressing both PRA and PRB recombinants (Lin *et al.*, 2000). In contrast, P4 was found to enhance *in vitro* cell migration through matrix-coated membranes although it strongly inhibited uPA mRNA synthesis (Lin *et al.*, 2001). In non-metastatic T47D cells that endogenously express PRA and PRB, PR-dependent cellular proliferation involves the activation of c-Src/p21^{ras}/MAPK signaling pathway (Migliaccio *et al.*, 1998; Carnevale *et al.*, 2007). In such cells, P4 was found to enhance motility via activation of FAK signaling relying on c-Src tyrosine kinase activity (Fu *et al.*, 2010). This function was also recently reported to be effective in vascular endothelial cells (Zheng *et al.*, 2012). However, due to the lack of relevant models, the relative contribution of PR isoforms PRA and PRB has not been clearly investigated.

Herein, we re-addressed these controversial questions using our newly established cellular model conditionally expressing PRA and/or PRB in MDA-MB-231 metastatic breast cancer cells (MDA-iPRAB) (Khan et al, 2012). Such model allowed us to determine the differential impacts of PR isoform inductions on cell migration as a function of ligand status independently from estrogen signaling. We correlate the global pro-migratory effect of PRA and PRB co-expression with PR-responsive transcriptional modulation of factors involved in cell migration mechanism as well as with non-genomic regulations directly targeting FAK complexes.

RESULTS

PRA and PRB differentially enhance migration of MDA-MB-231 breast cancer cells

We previously established a bi-inducible cell line derived from metastatic breast cancer MDA-MB-231 (PR-, ERα-) cells (Khan *et al.*, 2012), conditionally expressing PRA and/or PRB. Addition of diacylhydrazine (RSL1) and/or doxycycline (DOX) as non-steroidal inducers to MDA-iPRAB cells triggers expression of PRA and/or PRB, respectively (Figure 1, upper panel). Such model led us to investigate in a single cell line the differential impact of PR isoform expression to cell motility in the absence as well as in the presence of ligand. As shown in Figure 1, the experimental conditions of PR isoform expressions was herein fixed to

a PRA/PRB ratio of 2 after 24 h treatment depending on the relative concentrations of inducers. We then performed wound healing repair assays on cycle-arrested cells by incubating them with 10^{-8} M R5020 or vehicle at various time in the absence of inducers up to 24 h. We have previously shown that inducer withdrawals did not markedly change expression of PRA and PRB for 12 h and reduced it to 50 % after 24 h (Khan *et al.*, 2012).. Surprisingly, as presented in Figure 1 for time 10 h, in the absence of ligand, induction of PRB expression led to strongly increase of MDA-iPRAB cells migration whereas induction of PRA alone had no significant effect. .When cells were treated with R5020, cell migration was still significantly enhanced in PRB-induced cells for a prolonged time until 24 h, although to a limited extent (2.5 fold) as compared to untreated PRB cells (5 fold). Cells co-expressing both isoforms (PRAB) migrated independently of the ligand at a rate similar to that observed for untreated PRB cells. We further controlled that neither Dox nor RSL1 inducers provoked any change in adherent cell number, thus excluding experimental bias in wound healing assays (not shown). Next, we determined whether RU486 antagonist might impact the R5020-dependent cell migration. As shown in Figure S1 in Supplemental Material, RU486 completely abolished the anti-migratory action of R5020, showing that this antagonist specifically restored the effect raised by unliganded PRB expression. Therefore, as compared to the high basal migration rate of MDA-MB-231 cells lacking PR, co-induction of PRA and PRB expressions provokes a global pro-migratory change in MDA-MB-231 cell behavior, that can be partially but specifically counteracted by R5020 only when PRA co-expression is low.

PRB regulates key genes of the PA system enhancing cell migration

Movements of cancer cells result in part from extracellular matrix (ECM) degradation at the leading edge of cell progression by different proteases and alteration of cell adhesion/de-adhesion processes, both regulated by the PA system. To determine whether the effect of PRB on cell migration could be associated to relevant transcriptional regulations, we quantified uPA and uPAR transcripts in iPRAB cells by real-time qRT-PCR (Figure 2). Unliganded PRB but not PRA increased urokinase (uPA) transcripts 2 fold as compared to the basal expression level observed in PR- cells. Moreover, induction of both PRA and PRB enhanced 3 fold uPA mRNA in the absence of hormone. In contrast, R5020 down-regulated uPA and uPAR mRNAs in PRB and PRAB cells as compared to ligand-free conditions. Of note, the hormone counteracted the constitutive effect of PRB on uPA transcripts in agreement with cell migration observed in wound-healing assays. We also analyzed expression of β1-integrin that is required for matrix-dependent signaling particularly by interacting with uPAR and promoting FAK autophosphorylation. As shown in Figure 2, unliganded PRB but not PRA significantly enhanced β1-integrin expression, while R5020 did not provoke any variation in this effect. β1-integrin synthesis might be thus constitutively enhanced in PRB expressing cells, potentially favoring cell progression.

We next determined whether PRA and PRB could regulate transcription of PAI-1, the main inhibitor of uPA proteolytic functions. PAI-1 mRNA was induced by R5020 but not by the unlinganded PRs (Figure 3A) suggesting the possible impact of this factor in the relative anti-migratory action of hormone observed in PRB-expressing cells. As shown in Figure 3B, RU486 inhibited the R5020-induced expression of PAI-1 gene supporting the PRB-specificity of the mechanism. We also controlled that neither R5020 nor RU486 had any effect on such transcription in PR- cells (not shown). Furthermore, as measured by ELISA (Figure 3C), transcriptional induction of PAI-1 transcript by R5020 was translated into secretion of PAI-1 protein in the culture medium,that was inhibited by RU486. To test the impact of PAI-1 on cell migration, we performed wound healing repair assays on PR- cells treated by increasing amounts of recombinant PAI-1 (Figure 3D left panel). Surprisingly, up to 100 ng/mL PAI-1

strongly enhanced migration whereas higher doses led to decreasing effects likely through cell surface desensitization process. Such pro-migratory effect of PAI-1 on malignant cells is supported by previously reported data (Waltz *et al.*, 1997; Croucher *et al.*, 2007; Fabre-Guillevin *et al.*, 2008). Moreover, as shown in Figure 3E, high amounts of PAI-1 failed to decrease migration of PRB cells. Therefore, this ruled out that R5020-mediated down-regulation of PRB-dependent cell migration could act via PAI-1 stimulation.

Together these results show that PRA and PRB regulate the PA system to different extents depending of ligand status. Mainly, PRB up-regulates uPA and β1-integrin in the absence of ligand, thus potentially inducing pro-migratory effects by facilitating proteolysis of ECM and activating uPAR signaling. In contrast, although ligand-bound PRB switched off uPA signal in agreement with its effect on migration, it induced at the same time PAI-1 gene transcription and enhanced secretion of PAI-1 protein having a pro-migratory incidence on MDA-MB-231 cells. Such effects on pro-migratory gene expression are consistent with a global pro-migratory mechanism triggered by PRB expression in cancer cells, irrespective of ligand conditions.

PRA and PRB- differentially impact regulation of FAK activity

Recent studies have shown that P4 enhances T47D breast cancer cell migration via extranuclear activation of FAK (Fu *et al.*, 2010) resulting from initial phosphorylation of Y397 residue. Since FAK signaling is involved in the regulation of FAs assembling/disassembling, we asked whether conditional induction of PRA and PRB in MDA-iPRAB cells could interfere onto it. We analyzed FAK phosphorylation at Tyr397 key residue (FAK^{Y397p}) and total FAK (FAK^{total}) expression. Following 24 h induction of expression (Figure 4A), unliganded PRB but not PRA was able to enhance both FAK^{Y397p} and total FAK^{total} to similar extent, i.e. without inducing any change in their ratio. This suggested that PRB but not PRA may selectively increase FAK expression in the absence of ligand. Furthermore, the 1 h time-course of hormone-dependent FAK phosphorylation (Figure 4B) revealed that the liganded PRA was unable to activate FAK in contrast to PRB inducing FAK phosphorylation as early as 5 min. Although PRB but not PRA expression slightly enhanced FAK mRNA level as compared to PR- cells (Figure S2 in Supplemental Material), addition of hormone did not significantly decrease this level, excluding that hormone-dependent down-regulation of cell migration could result from any drastic transcriptional repression of FAK gene.

These results showed that PRA and PRB inductions differentially impacted FAK activity depending on ligand status. In the absence of ligand, PRB but not PRA stabilized FAK^{Y397p} in agreement with unliganded PRB-dependent cell migration observed in wound healing assays. In contrast, R5020 led to rapid increase of PRB-dependent FAK phosphorylation, excluding that its down-regulating effect on migration could result from direct PRB-dependent inhibition of FAK activity.

PRB and FAK^{Y397p} are colocalized in focal adhesion points

Since PRB-dependent alteration of cell migration was detected as early as 3 h after exposure to R5020, the early activation of FAK by liganded PRB should result in enhanced cell migration, contrasting with the data obtained in wound healing assays., We hypothesized that PRB-specific, FAK-dependent migration could be affected by variation in cytoplasmic PRB expression. To clarify this point, using immunofluorescence experiments at various time intervals following hormone treatment, we compared PR isoform and FAK^{Y397p} distributions in iPRAB cells. High motility of MDA-MB-231 cells has already been correlated to their ability to produce cellular protrusions like filipodia and lamellipodia. These structures lead in sustained cell orientation with growing FAs at the leading edge contributing to cell expansion,

and cell movement. Such elements were clearly visible in PR- cells (Figure 5, left panel). While ligand-free PRB was found to be equally distributed within cytoplasmic and nuclear compartments, the hormone-bound PRB was, as expected, fully translocated in the nucleus in 30 min. In the absence of PR, FAK was expressed in the cytoplasm and the nucleus, and was especially condensed in sub-membrane speckles corresponding to FAs into the pseudopodia involved in migration. Interestingly, following 24 h induction of PRB expression, the ligand-free PRB was repeatedly found in such structures containing FAK, especially at the leading edge of migration and also in filamentous radiant elements such as lamellipodia (Figure 5). In addition, the number of cell protrusions with FAK-containing FAs was increased supporting that the unliganded PRB could somehow potentialize FAK-dependent migration. However, although FAK^{Y397p} as well as the apo PRB were also present in the nuclei, no colocalized speckles were detected in merged images of this compartment. Interestingly, upon hormone exposure, PRB-FAK^{Y397p} colocalization was impaired concomitantly to nuclear translocation of PRB. Time-course experiments showed that the subcellular localization of PRB was modified as early as 5 min after R5020 exposure (Supplemental Figure S3). PRB-FAK^{Y397p} co-localization in FAs was completely abolished after 30 min for 90 % of the cells owing to the complete nuclear translocation of PRB. Of note, some nuclear speckles were visible in the perinuclear region of few cells suggesting that PRB-FAK^{Y397p} complexes might be transiently present in the nuclear compartment. As opposed to R5020, RU486 antagonist resulted in a slower PRB nuclear translocation, RU486-bound PRB remaining in the cytoplasmic compartment at 30 min (Figure 5 right panel). Interestingly, PRB-RU486 complexes were also clearly present in FAK^{Y397p} -containing FAs similarly to unliganded PRB. Such characteristics were thus well correlated with the effects on cell migration observed in both ligand conditions. In contrast, the hormone-dependent decrease in PRB-mediated migration was likely related to PRB nuclear translocation limiting its impact on FAK phosphorylation.

Similar experiments were also performed in cells treated by RSL1 to induce PRA expression (Supplemental Figure S4). In contrast to PRB, unbound PRA was essentially localized in the nuclei with a perinuclear distribution. Although low expression of PRA was also slightly visible in the cytoplasm, we failed to identify any condensation points containing unliganded-PRA with FAK^{Y397p} in pseudopodia. However, several co-localized speckles were found into the nucleus supporting that PRA-FAK complexes could be there assembled. R5020 treatment did not alter cellular distribution of PRA. Confocal analysis profiles corresponding to overlayed images of Figures 5 and Figure S4 are shown in Supplemental Figure S5, clearly indicating that PRB but not PRA could be detected in the sub-membrane FAs present in the leading edges of migrating cells grown in the absence of ligand or in the presence of RU486.

Collectively, these experiments showed that unliganded PRB but not PRA is colocalized with autophosphorylated FAK^{Y397p} in FAs at the leading edge of migration, strongly suggesting that PRB can constitutively enhance cell motility via FAK-dependent mechanism independently of transcriptional regulations. In contrast, nuclear translocation of liganded PRB well correlated with the reduction of PRB-dependent cell motility by R5020.

PRA and PRB interactions with FAK^{Y397p} differentially regulate FAK turnover

To determine whether ligand-dependent cellular colocalization of PRB and FAK^{Y397p} might underlie a protein-protein interaction within the FAs, we immunoprecipitated FAK^{total} complexes from lysates of either PRB or PRA expressing cells using anti- FAK^{total} or -PR antibodies. Interestingly, as shown in Figure 6A, PRA and PRB were refolded in FAK-specific immunoprecipitates in the presence as well as the absence of hormone. Reverse co-immunoprecipitation experiments (co-IP) were performed (Supplemental Figure S6) confirming that FAK^{Y397p} was present only when PR isoforms were induced. Therefore, these co-IP provided evidence for the abilities of both isoforms to interact with complexes

containing FAK, independently of ligand status. This raised the question on whether PRA and PRB might alternately interact with a shared component of the FAK complexes. As shown in Figure 6 (right inset), induction of PRA expression in PRB-expressing cells resulted in a drastic decrease in PRB-FAK complexes suggesting that PRA could compete with PRB for the same binding sites.

Given that proteasome-mediated degradation of agonist-bound PRB is required for its transcriptional activity (Dennis *et al.*, 2005), we next asked whether PRB-dependent FAK activity could be also controlled by such dynamic processes. FAK expression was analyzed in iPRAB cells after 10 h hormonal treatment (Figure 6B). In PRB-expressing cells and in agreement with previous reports (Lange *et al.*, 2000; Khan *et al.*, 2011), R5020 induced proteasome-dependent degradation of PRB, with a 50 % decrease after 10 h in MDA-iPRAB cell line. In contrast, liganded PRA remained stable after 10 h hormone treatment in these cells. Interestingly, PRB but not PRA expression clearly led to hormone-dependent decrease of total FAK as well as FAK^{Y397p} expression, whereas co-expression of PRA strikingly prevented this effect. Therefore, FAK turnover was at least partially coupled to hormone-induced PRB degradation long after PRB nuclear translocation has been achieved. Since turnover dynamics might be cell-specific, experiments were repeated in two different cell lines expressing PRA or PRB (Figure 6C). As previously reported (Khan *et al.*, 2011), in endometrial cancer Ishikawa cells (ERα-) stably transfected by PRB or PRA, both isoforms are rapidly down-regulated following hormone exposure. In mammary cancer T47D cells, PRA and PRB are endogenously expressed independently of estrogen regulation although ERα is present. In both cell lines, FAK down-regulation was induced upon hormone treatment.

These results together provide evidence for ligand-independent interactions of both PRA and PRB with FAK, and establish a biological link between FAK and PR isoform turnovers depending on PRA and PRB degradation kinetics.

DISCUSSION

P4 was reported to induce cell spreading and adhesion in MDA-MB-231 stably expressing both PRA and PRB (Lin *et al.*, 2000; Lin *et al.*, 2001). In T47D cells endogenously expressing PRA and PRB, P4 increased cell migration (Fu *et al.*, 2008b; Fu *et al.*, 2010), whereas PRA but not PRB enhanced migration in PR-inducible T47D YiA and YiB cells (Jacobsen *et al.*, 2005). In sharp contrast, our unique bi-inducible cell line allowed us to unambiguously evaluate the relative contribution of both isoforms expression in the absence as well as presence of hormone. In our model, PRB and PRA clearly cooperate through multiple mechanisms accelerating migration. PRB but not PRA enhance uPA transcript level consistent with a positive effect on cell migration in the absence of progestins. Moreover, since addition of hormone decreased PRB-dependent up-regulation of uPA, this gene might be interestingly only sensitive to unliganded PRB but switched off by hormone. Therefore, unliganded PRB-dependent cell migration would result from synergetic activations of uPA signaling and FAK cascade via both transcriptional and non-genomic processes. Moreover, upon hormone addition, PAI-1 synthesis and secretion were enhanced in PRA and PRB expressing cells. Although PAI-1 was initially referred as an inhibitor of both uPA-mediated ECM proteolysis and uPA-inducible uPAR-signaling, several reports argued in favor of other PAI-1 functions linked to the endocytic receptor LRP and integrins signaling leading to stimulate adhesion/de-adhesion dynamics (Waltz *et al.*, 1997; Croucher *et al.*, 2007; Fabre-Guillevin *et al.*, 2008). Moreover, PAI-1 is considered as a metastasis prognostic marker (Leissner *et al.*, 2006). In this context, since PAI-1 strongly increased migration of MDA-MB-231 cells at low concentration, this factor may potentially mediate PRB-dependent migration through paracrine mechanism impacting FAK activity.

Independently of regulation of the PA system, PRB directly targets the FAs through interaction with FAK complexes. PRB clearly colocalized with FAK^{Y397p} which is required for focal adhesion disassembling (Hamadi *et al.*, 2005; Deramaudt *et al.*, 2011). P4 has been already found to rapidly enhance phosphorylation of Tyr397-FAK in T47D cells (Fu *et al.*, 2010) and we confirmed herein these results using R5020 progestin in our bi-inducible cell model. However, in contrast to this report, our studies argue for a direct interaction of PRB with FAK complexes that likely enhances FAK-mediated motility. Other converging data indicate that steroid receptors are able to cross-talk with FAK signaling. In this regards, the ligand-free estrogen receptor (ER) has been identified in the same complex as FAK via interaction with Src tyrosine kinase, that was disrupted by estrogens (Le Romancer *et al.*, 2008). Moreover, evidence for the interaction of FAK complexes with steroid receptor co-activator 3 (SRC-3) in MDA-MB-231 cells was reported (Long *et al.*, 2010). Tumor suppressor BRCA1 has been found to colocalize in FAs leading to decrease cell motility (Coene *et al.*, 2011). Interestingly, PRB has been shown to interact with ER (Ballare *et al.*, 2003), c-Src (Boonyaratanakornkit *et al.*, 2001), SRC3 (Long *et al.*, 2010), and BRCA1 (Ma *et al.*, 1999; Poole *et al.*, 2006). It is possible that, even in the absence of ER, cytoplasmic PRB might be directly recruited by FAK concomitantly to Src and other partners leading to modulate cell migration in a coordinated manner.

Under physiological conditions, PRB trafficking and shuttling are finely tuned through hormone-sensitive regulations (Guiochon-Mantel *et al.*, 1991; Tyagi *et al.*, 1998) that might strongly influence cell migration dynamics. In our cell model, hormone-induced PRB nuclear translocation led to rapid depletion of PRB in sub-plasma membrane structures as well as in cytoplasm that was correlated to decreased migration rate after few hours of hormone treatment. It was also surprising that hormone induced FAK degradation in PRB cells. PRB transcriptional hyperactivity is tightly coupled to its proteasome-dependent turnover (Dennis *et al.*, 2005). In a similar manner, the increase in hormone-dependent degradation of PRB-FAK complexes might be the signature of PRB-dependent FA disassembling. FAK^{Y397p} has been shown to translocate to the nucleus where it can interact with p53 (Golubovskaya *et al.*, 2005) and down-regulate its turnover by enhancing p53 ubiquitination (Lim *et al.*, 2008). Because PRA strongly interacted with FAK in cell lysates but neither colocalized with FAK at FAs nor activated FAK at Tyr397, PRA interaction with soluble FAK could mainly take place in the nuclear compartment. Anyhow the fact that PRA interfered with PRB-dependent FAK turnover and could compete with PRB for binding FAK suggests that such cross-talk might transiently occur into the nucleus with important consequences on FAK stability in this compartment.

We have previously reported that RU486 stabilizes PRB through a MAPK-dependent mechanism (Khan *et al.*, 2011), and concomitantly stabilizes SRC-1, a major co-activator of PRB (Amazit *et al.*, 2011). RU486 also inhibited the hormone-dependent spreading of MDA-MB-231 cells expressing PRB (Lin *et al.*, 2001). Accordingly, we found herein that this antagonist induced a delayed nuclear translocation as compared to R5020-treated cells, and abolished the relative down-regulating effect of hormone on cell migration. The pro-migratory action of RU486 likely resulted from both increased uPA expression and PRB stabilization that might favor its interaction with FAK in the cytoplasmic compartment. This highlights the need of selective PR antagonists that neither induce MAPK-dependent stabilization of PRB nor inhibit proteasome-dependent turnover, in contrast to RU486 and most of its derivatives.

Our results are schematically summarized in Figure 7. Induction of PRB expression enhances cell migration through transcriptional as well as non-genomic processes involving FAK dependent signaling. Based on the mechanistic model previously proposed for FAK assembling/disassembling kinetics (Hamadi *et al.*, 2005; Deramaudt *et al.*, 2011), cell

migration might be correlated to FAK time residency within the FAs. In PR expressing cells, association of stable unliganded PRB with FAK would enhance FAK-dependent cell migration in a sustained manner by favoring FAK recruitment within the FAs and subsequent activation of migration. Hormone-bound PRB transiently leads to rapid stimulation of FAK^{Y397p}, and enhancement in cell motility as compared to PR- cells. However, hormone-bound PRB cells migrate at a lower speed than unliganded PRB cells due to enhanced nuclear localization. PRB-mediated cell migration is thus dependent on cytoplasmic PRB abundance that is an intricate function of PRB neosynthesis, nucleocytoplasmic shuttling and proteasome-dependent degradation, as well as hormone status. Importantly, antagonist ligands such as RU486 that stabilize PR lead to sustained stimulation of migration as compared to hormone-treated cells. In addition, PRA would extend hormone-dependent migration of PRB-expressing cells by stabilizing FAK, leading to constitutively activate migration at high level. Our model predicts that the more PRB is stable and shuttles in cytoplasm the more it interacts with FAK and enhances migration. PRB over expression would thus favor mammary cancer cell expansion especially in the context of either low P4 status or disturbed PRB trafficking or any associated treatment with RU486-like antagonists. Interestingly, the more PRA is expressed the more it stabilizes FAK and amplifies PRB-dependent cell migration. Therefore, high expression of PRA might play a critical role in setting output signals for PRB-dependent cell migration independently of progestins, in agreement with the deleterious effects of high PRA expression level found in a majority of PR+ER+ breast cancers.

In sum, PRB and PRA differentially impact cell migration through multiple mechanisms activating FAK as a function of their relative expression level in cell compartments. We believe that our findings argue for the important roles that PRA/PRB ratio may play in regulation of cellular movements of PR expressing cells, and suggest a mechanistic scheme for cell motility and metastatic dissemination of ER+ PR+ breast cancers.

MATERIALS AND METHODS

Cell cultures and treatments

MDA-iPRAB cell line derivating from MDA-MB-231 human breast cancer cell line (American Type Culture Collection) has been previously described (Khan *et al.*, 2012). Briefly, these cells stably express all components of Rheoswitch (NE Biolabs) and T-Rex (Invitrogen) systems allowing a controlled expression using diacylhydrazine (RSL1) and doxycycline (Dox) non-steroidal inducers. They were also stably transfected by two vectors conditionally expressing either PRA in the presence of RSL1, or PRB in the presence of doxycycline in a dose-dependent manner. Cells were grown in DMEM supplemented with 5 % FBS as previously described (Khan *et al.*, 2012) in the presence of geneticin (500 µg/mL), blasticidine (2 µg/mL), zeocin (100 µg/mL) to maintain selective pressure on plasmid expression (Khan *et al.*, 2012). 24 h before each experiment, cells were starved in DMEM without phenol red with 5 % FBS stripped using the dextran-coated-charcoal method (DCC-FBS), with 100 U/mL penicillin, 100 µg/mL streptomycine. Then RSL1 (0.25 µM) and/or doxycycline (2 µM) were added in the medium to induce PRA and/or PRB expression respectively. Following induction for 24 h, steroids or vehicle (0.01 % ethanol) were added in the medium as indicated. Ishikawa cells expressing PRA or PRB and T47D cells were grown as previously described (Khan *et al.*, 2011). The experimental facility for material of human origin was granted approval (N° B94-043-12), with an authorization (75-978, ML) given by the French Administration (Prefecture du Val de Marne, Direction départementale des services vétérinaires du Val de Marne).

Real time quantitative RT-PCR

Total RNA was extracted from iPRAB cells treated or not by the indicated ligands for a given time using TRIZOL reagent (Invitrogen), and equal amounts (1 µg) were reverse transcribed for real time qPCR analysis as previously described (Khan *et al.*, 2011). Primers (300 nM) used are listed in Supplemental Table 1 in Supplemental Materials. Quantification of gene expression was normalized to 18S rRNA and expressed as means ± SEM from six experiments.

In vitro wound-healing repair assays

MDA-iPRAB cell migration was assessed according to previously reported method (Chen *et al.*, 2011). Briefly, 1.10^5 cells were grown to confluence into inserts onto graduated plastic micro-dishes (Ibidi). Following induction of PRA and/or PRB expression by RSL1 and/or Dox for 24 h, cell proliferation was arrested by adding 10 µg/mL mitomycin C (Sigma Aldrich) for 1 h. The insert was pulled out and cells debris were removed by washing with PBS. Width of each wounded area was measured using grids at three marked positions. The cells were then treated by either 10^{-8} M R5020 or 10^{-6} M RU486 or vehicle. The cultures were kept at 37°C in humidified incubator and photographed (40X magnification) at the indicated times to monitor migration of cells into the wounded area. Cell migration was quantified as the distance covered by cells in wound healed surface from the marked positions. Results are expressed as mean ± SEM of three independent experiments.

Immunoblots and immunoprecipitations

MDA-iPRAB cells were harvested in cold PBS and the pellet resuspended in lysis buffer (0.1 % Triton X-100, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM, NaCl, 0.2 % NaF, 1.3 % sodium pyrophosphate) containing a mixture of phosphatases and proteases inhibitors (Sigma). PAGE electrophoresis and immunoblotting were performed as previously described (Khan *et al.*, 2011). The membranes were incubated with the indicated primary antibodies at 4°C overnight, then incubated with secondary antibody conjugated to the horseradish peroxydase-conjugated or fluorescence before being developing using ECL plus detection reagents (Amersham, Biosciences Corp. Piscataway, NJ) or scanning using the Odyssey system (LI-COR). For co-immunoprecipitation experiments, the cells were lysed in lysis buffer containing 0.5 % Nonidet-P40. Supernatants (1mg total proteins) were incubated with 2 µg antibodies for 5 h at 4°C. The samples were then mixed with protein G magnetic beads (Millipore) according to manufacturer's instructions. Bound immunocomplexes were boiled in Laemmli loading buffer for 10 min and analyzed by western blot. The antibodies used were monoclonal anti-PR (Novocastra, NCL-LPGR-312/2), rabbit polyclonal anti-FAK C-terminal domain (C20, Santa Cruz, CA), rabbit polyclonal anti-phospho Tyr397-FAK (ab4803, Abcam), anti-α tubulin (Sigma).

Plasminogen activator inhibitor-1 antigen assays

Cells were grown at 80 % confluence in medium containing either RSL1 or Dox for 24 h. The cells were then treated by 10^{-8} M R5020, or/and 10^{-6} M RU486, or vehicle for 16 h. Conditioned media were immediately transferred to -20 °C until the following step. Total PAI-1 was measured using ELISA-kit (Gentaur, Paris) as described by the manufacturer. Results are expressed as mean ± SEM from 3 independent experiments.

Immunocytochemistry and confocal imaging.

MDA-iPRAB cells were plated on chambered slides (Lab Tek), fixed in 4 % paraformaldehyde, permeabilized with 0.1 % Triton and saturated with 5 % BSA and 0.1 % casein in PBS. The slides were incubated with primary anti-FAK^{Y397P} rabbit polyclonal antibody (ab4803, Abcam), anti-PRB monoclonal antibody let126 (Lorenzo *et al.*, 1988), anti-

PRB and PRA monoclonal antibody (Novocastra, NCL-LPGR-312/2), Cy5-conjugated secondary anti-rabbit antibody and Alexa fluor green conjugated secondary anti-mouse (Invitrogen). Nuclei were stained using To-PRO3 (Invitrogen) or DAPI (Invitrogen) and the slides were mounted using Fluorescence Mounting Medium (Dako, Trappes, France). The cells were analyzed by confocal fluorescence microscopy as previously described (Amazit *et al.*, 2011).

Statistical analysis.

All data are representative of at least three independent experiments and are presented as mean ± SEM. Non parametric Mann-Whitney or the student 2-tailed tests were used to determine statistical significance of difference between groups using the computer software inVivoStat (http://www.invivostat.co.uk). Statistical significance is indicated as $p<0.001$(***), $p<0.01$ (**) and $p<0.05$ (*).

Acknowledgements
The authors are grateful to Dr. Claude Labrie (Laval University Medical Research Center, Quebec) for his support on inducible expression system. We thank Philippe Leclerc for confocal photographs and technical assistance. This work was supported by grants from INSERM, the Université Paris-Sud 11 and Association pour la Recherche sur le Cancer (ARC). CB was recipient of a fellowship from the Conseil Régional de la Martinique, France. JAK was on study leave from the department of Physiology and Pharmacology, University of Agriculture, Faisalabad and was a recipient of doctoral scholarship from Higher Education Commission, Islamabad, Pakistan and a fellowship from La Ligue Contre le Cancer, France.

REFERENCES

Amazit, L., Roseau, A., Khan, J.A., Chauchereau, A., Tyagi, R.K., Loosfelt, H., Leclerc, P., Lombes, M., and Guiochon-Mantel, A. (2011). Ligand-dependent degradation of SRC-1 is pivotal for progesterone receptor transcriptional activity. Mol Endocrinol *25*, 394-408.

Bagheri-Yarmand, R., Talukder, A.H., Wang, R.A., Vadlamudi, R.K., and Kumar, R. (2004). Metastasis-associated protein 1 deregulation causes inappropriate mammary gland development and tumorigenesis. Development *131*, 3469-3479.

Ballare, C., Uhrig, M., Bechtold, T., Sancho, E., Di Domenico, M., Migliaccio, A., Auricchio, F., and Beato, M. (2003). Two domains of the progesterone receptor interact with the estrogen receptor and are required for progesterone activation of the c-Src/Erk pathway in mammalian cells. Mol Cell Biol *23*, 1994-2008.

Bernard-Trifilo, J.A., Lim, S.T., Hou, S., Schlaepfer, D.D., and Ilic, D. (2006). Analyzing FAK and Pyk2 in early integrin signaling events. Curr Protoc Cell Biol *Chapter 14*, Unit 14 17.

Biermann, J.C., Holzscheiter, L., Kotzsch, M., Luther, T., Kiechle-Bahat, M., Sweep, F.C., Span, P.N., Schmitt, M., and Magdolen, V. (2008). Quantitative RT-PCR assays for the determination of urokinase-type plasminogen activator and plasminogen activator inhibitor type 1 mRNA in primary tumor tissue of breast cancer patients: comparison to antigen quantification by ELISA. Int J Mol Med *21*, 251-259.

Bogina, G., Bortesi, L., Marconi, M., Venturini, M., Lunardi, G., Coati, F., Massocco, A., Manfrin, E., Pegoraro, C., and Zamboni, G. (2011). Comparison of hormonal receptor and HER-2 status between breast primary tumours and relapsing tumours: clinical implications of progesterone receptor loss. Virchows Arch *459*, 1-10.

Boonyaratanakornkit, V., Bi, Y., Rudd, M., and Edwards, D.P. (2008). The role and mechanism of progesterone receptor activation of extra-nuclear signaling pathways in regulating gene transcription and cell cycle progression. Steroids *73*, 922-928.

Boonyaratanakornkit, V., McGowan, E., Sherman, L., Mancini, M.A., Cheskis, B.J., and Edwards, D.P. (2007). The role of extranuclear signaling actions of progesterone receptor in mediating progesterone regulation of gene expression and the cell cycle. Mol Endocrinol *21*, 359-375.

Boonyaratanakornkit, V., Scott, M.P., Ribon, V., Sherman, L., Anderson, S.M., Maller, J.L., Miller, W.T., and Edwards, D.P. (2001). Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. Mol Cell *8*, 269-280.

Busso, N., Masur, S.K., Lazega, D., Waxman, S., and Ossowski, L. (1994). Induction of cell migration by pro-urokinase binding to its receptor: possible mechanism for signal transduction in human epithelial cells. J Cell Biol *126*, 259-270.

Carnevale, R.P., Proietti, C.J., Salatino, M., Urtreger, A., Peluffo, G., Edwards, D.P., Boonyaratanakornkit, V., Charreau, E.H., Bal de Kier Joffe, E., Schillaci, R., and Elizalde, P.V. (2007). Progestin effects on breast cancer cell proliferation, proteases activation, and in vivo development of metastatic phenotype all depend on progesterone receptor capacity to activate cytoplasmic signaling pathways. Mol Endocrinol *21*, 1335-1358.

Chapman, H.A. (1997). Plasminogen activators, integrins, and the coordinated regulation of cell adhesion and migration. Curr Opin Cell Biol *9*, 714-724.

Chazaud, B., Ricoux, R., Christov, C., Plonquet, A., Gherardi, R.K., and Barlovatz-Meimon, G. (2002). Promigratory effect of plasminogen activator inhibitor-1 on invasive breast cancer cell populations. Am J Pathol *160*, 237-246.

Chen, J.Y., Tang, Y.A., Huang, S.M., Juan, H.F., Wu, L.W., Sun, Y.C., Wang, S.C., Wu, K.W., Balraj, G., Chang, T.T., Li, W.S., Cheng, H.C., and Wang, Y.C. (2011). A novel sialyltransferase inhibitor suppresses FAK/paxillin signaling and cancer angiogenesis and metastasis pathways. Cancer Res *71*, 473-483.

Chiang, A.C., and Massague, J. (2008). Molecular basis of metastasis. N Engl J Med *359*, 2814-2823.

Chlebowski, R.T., Anderson, G.L., Gass, M., Lane, D.S., Aragaki, A.K., Kuller, L.H., Manson, J.E., Stefanick, M.L., Ockene, J., Sarto, G.E., Johnson, K.C., Wactawski-Wende, J., Ravdin, P.M., Schenken, R., Hendrix, S.L., Rajkovic, A., Rohan, T.E., Yasmeen, S., and Prentice, R.L. (2010). Estrogen plus progestin and breast cancer incidence and mortality in postmenopausal women. JAMA *304*, 1684-1692.

Chlebowski, R.T., Hendrix, S.L., Langer, R.D., Stefanick, M.L., Gass, M., Lane, D., Rodabough, R.J., Gilligan, M.A., Cyr, M.G., Thomson, C.A., Khandekar, J., Petrovitch, H., and McTiernan, A. (2003). Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial. JAMA *289*, 3243-3253.

Coene, E.D., Gadelha, C., White, N., Malhas, A., Thomas, B., Shaw, M., and Vaux, D.J. (2011). A novel role for BRCA1 in regulating breast cancer cell spreading and motility. J Cell Biol *192*, 497-512.

Croucher, D.R., Saunders, D.N., Stillfried, G.E., and Ranson, M. (2007). A structural basis for differential cell signalling by PAI-1 and PAI-2 in breast cancer cells. Biochem J *408*, 203-210.

Czekay, R.P., and Loskutoff, D.J. (2004). Unexpected role of plasminogen activator inhibitor 1 in cell adhesion and detachment. Exp Biol Med (Maywood) *229*, 1090-1096.

Dennis, A.P., Lonard, D.M., Nawaz, Z., and O'Malley, B.W. (2005). Inhibition of the 26S proteasome blocks progesterone receptor-dependent transcription through failed recruitment of RNA polymerase II. J Steroid Biochem Mol Biol *94*, 337-346.

Deramaudt, T.B., Dujardin, D., Hamadi, A., Noulet, F., Kolli, K., De Mey, J., Takeda, K., and Ronde, P. (2011). FAK phosphorylation at Tyr-925 regulates cross-talk between focal adhesion turnover and cell protrusion. Mol Biol Cell *22*, 964-975.

Fabre-Guillevin, E., Malo, M., Cartier-Michaud, A., Peinado, H., Moreno-Bueno, G., Vallee, B., Lawrence, D.A., Palacios, J., Cano, A., Barlovatz-Meimon, G., and Charriere-Bertrand, C. (2008). PAI-1 and functional blockade of SNAI1 in breast cancer cell migration. Breast Cancer Res *10*, R100.

Fu, X.D., Flamini, M., Sanchez, A.M., Goglia, L., Giretti, M.S., Genazzani, A.R., and Simoncini, T. (2008a). Progestogens regulate endothelial actin cytoskeleton and cell movement via the actin-binding protein moesin. Mol Hum Reprod *14*, 225-234.

Fu, X.D., Giretti, M.S., Baldacci, C., Garibaldi, S., Flamini, M., Sanchez, A.M., Gadducci, A., Genazzani, A.R., and Simoncini, T. (2008b). Extra-nuclear signaling of progesterone receptor to breast cancer cell movement and invasion through the actin cytoskeleton. PLoS One *3*, e2790.

Fu, X.D., Goglia, L., Sanchez, A.M., Flamini, M., Giretti, M.S., Tosi, V., Genazzani, A.R., and Simoncini, T. (2010). Progesterone receptor enhances breast cancer cell motility and invasion via extranuclear activation of focal adhesion kinase. Endocr Relat Cancer *17*, 431-443.

Golubovskaya, V.M., Finch, R., and Cance, W.G. (2005). Direct interaction of the N-terminal domain of focal adhesion kinase with the N-terminal transactivation domain of p53. J Biol Chem *280*, 25008-25021.

Graham, J.D., Yager, M.L., Hill, H.D., Byth, K., O'Neill, G.M., and Clarke, C.L. (2005). Altered progesterone receptor isoform expression remodels progestin responsiveness of breast cancer cells. Mol Endocrinol *19*, 2713-2735.

Graham, J.D., Yeates, C., Balleine, R.L., Harvey, S.S., Milliken, J.S., Bilous, A.M., and Clarke, C.L. (1995). Characterization of progesterone receptor A and B expression in human breast cancer. Cancer Res *55*, 5063-5068.

Guiochon-Mantel, A., Delabre, K., Lescop, P., and Milgrom, E. (1994). Nuclear localization signals also mediate the outward movement of proteins from the nucleus. Proc Natl Acad Sci U S A *91*, 7179-7183.

Guiochon-Mantel, A., Lescop, P., Christin-Maitre, S., Loosfelt, H., Perrot-Applanat, M., and Milgrom, E. (1991). Nucleocytoplasmic shuttling of the progesterone receptor. Embo J *10*, 3851-3859.

Hamadi, A., Bouali, M., Dontenwill, M., Stoeckel, H., Takeda, K., and Ronde, P. (2005). Regulation of focal adhesion dynamics and disassembly by phosphorylation of FAK at tyrosine 397. J Cell Sci *118*, 4415-4425.

Hammes, S.R., and Levin, E.R. (2007). Extranuclear steroid receptors: nature and actions. Endocr Rev *28*, 726-741.

Harbeck, N., Kates, R.E., Schmitt, M., Gauger, K., Kiechle, M., Janicke, F., Thomassen, C., Look, M.P., and Foekens, J.A. (2004). Urokinase-type plasminogen activator and its inhibitor type 1 predict disease outcome and therapy response in primary breast cancer. Clin Breast Cancer *5*, 348-352.

Hiscox, S., Barnfather, P., Hayes, E., Bramble, P., Christensen, J., Nicholson, R.I., and Barrett-Lee, P. (2010). Inhibition of focal adhesion kinase suppresses the adverse phenotype of endocrine-resistant breast cancer cells and improves endocrine response in endocrine-sensitive cells. Breast Cancer Res Treat *125*, 659-669.

Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature *377*, 539-544.

Jacobsen, B.M., Schittone, S.A., Richer, J.K., and Horwitz, K.B. (2005). Progesterone-independent effects of human progesterone receptors (PRs) in estrogen receptor-positive breast cancer: PR isoform-specific gene regulation and tumor biology. Mol Endocrinol *19*, 574-587.

Janicke, F., Prechtl, A., Thomssen, C., Harbeck, N., Meisner, C., Untch, M., Sweep, C.G., Selbmann, H.K., Graeff, H., and Schmitt, M. (2001). Randomized adjuvant chemotherapy trial in high-risk, lymph node-negative breast cancer patients identified by urokinase-type plasminogen activator and plasminogen activator inhibitor type 1. J Natl Cancer Inst *93*, 913-920.

Jo, M., Thomas, K.S., Marozkina, N., Amin, T.J., Silva, C.M., Parsons, S.J., and Gonias, S.L. (2005). Dynamic assembly of the urokinase-type plasminogen activator signaling receptor complex determines the mitogenic activity of urokinase-type plasminogen activator. J Biol Chem *280*, 17449-17457.

Kato, S., Pinto, M., Carvajal, A., Espinoza, N., Monso, C., Sadarangani, A., Villalon, M., Brosens, J.J., White, J.O., Richer, J.K., Horwitz, K.B., and Owen, G.I. (2005). Progesterone increases tissue factor gene expression, procoagulant activity, and invasion in the breast cancer cell line ZR-75-1. J Clin Endocrinol Metab *90*, 1181-1188.

Khan, J.A., Amazit, L., Bellance, C., Guiochon-Mantel, A., Lombes, M., and Loosfelt, H. (2011). p38 and p42/44 MAPKs Differentially Regulate Progesterone Receptor A and B Isoform Stabilization. Mol Endocrinol *25*, 1710-1724.

Khan, J.A., Bellance, C., Guiochon-Mantel, A., Lombes, M., and Loosfelt, H. (2012). Differential Regulation of Breast Cancer-Associated Genes by Progesterone Receptor Isoforms PRA and PRB in a New Bi-Inducible Breast Cancer Cell Line. PLoS One *7*, e45993.

Labriola, L., Salatino, M., Proietti, C.J., Pecci, A., Coso, O.A., Kornblihtt, A.R., Charreau, E.H., and Elizalde, P.V. (2003). Heregulin induces transcriptional activation of the progesterone receptor by a mechanism that requires functional ErbB-2 and mitogen-activated protein kinase activation in breast cancer cells. Mol Cell Biol *23*, 1095-1111.

Lange, C.A., Shen, T., and Horwitz, K.B. (2000). Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. Proc Natl Acad Sci U S A *97*, 1032-1037.

Lauffenburger, D.A., and Horwitz, A.F. (1996). Cell migration: a physically integrated molecular process. Cell *84*, 359-369.

Le Romancer, M., Treilleux, I., Leconte, N., Robin-Lespinasse, Y., Sentis, S., Bouchekioua-Bouzaghou, K., Goddard, S., Gobert-Gosse, S., and Corbo, L. (2008). Regulation of estrogen rapid signaling through arginine methylation by PRMT1. Mol Cell *31*, 212-221.

Leissner, P., Verjat, T., Bachelot, T., Paye, M., Krause, A., Puisieux, A., and Mougin, B. (2006). Prognostic significance of urokinase plasminogen activator and plasminogen activator inhibitor-1 mRNA expression in lymph node- and hormone receptor-positive breast cancer. BMC Cancer *6*, 216.

Leo, J.C., Wang, S.M., Guo, C.H., Aw, S.E., Zhao, Y., Li, J.M., Hui, K.M., and Lin, V.C. (2005). Gene regulation profile reveals consistent anticancer properties of progesterone in hormone-independent breast cancer cells transfected with progesterone receptor. Int J Cancer *117*, 561-568.

Lim, S.T., Chen, X.L., Lim, Y., Hanson, D.A., Vo, T.T., Howerton, K., Larocque, N., Fisher, S.J., Schlaepfer, D.D., and Ilic, D. (2008). Nuclear FAK promotes cell proliferation and survival through FERM-enhanced p53 degradation. Mol Cell *29*, 9-22.

Lin, V.C., Eng, A.S., Hen, N.E., Ng, E.H., and Chowdhury, S.H. (2001). Effect of progesterone on the invasive properties and tumor growth of progesterone receptor-transfected breast cancer cells MDA-MB-231. Clin Cancer Res *7*, 2880-2886.

Lin, V.C., Ng, E.H., Aw, S.E., Tan, M.G., and Bay, B.H. (2000). Progesterone induces focal adhesion in breast cancer cells MDA-MB-231 transfected with progesterone receptor complementary DNA. Mol Endocrinol *14*, 348-358.

Long, W., Yi, P., Amazit, L., LaMarca, H.L., Ashcroft, F., Kumar, R., Mancini, M.A., Tsai, S.Y., Tsai, M.J., and O'Malley, B.W. (2010). SRC-3Delta4 mediates the interaction of EGFR with FAK to promote cell migration. Mol Cell *37*, 321-332.

Lorenzo, F., Jolivet, A., Loosfelt, H., Thu vu Hai, M., Brailly, S., Perrot-Applanat, M., and Milgrom, E. (1988). A rapid method of epitope mapping. Application to the study of immunogenic domains and to the characterization of various forms of rabbit progesterone receptor. Eur J Biochem *176*, 53-60.

Luo, M., and Guan, J.L. (2010). Focal adhesion kinase: a prominent determinant in breast cancer initiation, progression and metastasis. Cancer Lett *289*, 127-139.

Ma, H., Hong, H., Huang, S.M., Irvine, R.A., Webb, P., Kushner, P.J., Coetzee, G.A., and Stallcup, M.R. (1999). Multiple signal input and output domains of the 160-kilodalton nuclear receptor coactivator proteins. Mol Cell Biol *19*, 6164-6173.

Marbaix, E., Donnez, J., Courtoy, P.J., and Eeckhout, Y. (1992). Progesterone regulates the activity of collagenase and related gelatinases A and B in human endometrial explants. Proc Natl Acad Sci U S A *89*, 11789-11793.

May, C.D., Sphyris, N., Evans, K.W., Werden, S.J., Guo, W., and Mani, S.A. (2011). Epithelial-mesenchymal transition and cancer stem cells: a dangerously dynamic duo in breast cancer progression. Breast Cancer Res *13*, 202.

McGowan, E.M., and Clarke, C.L. (1999). Effect of overexpression of progesterone receptor A on endogenous progestin-sensitive endpoints in breast cancer cells. Mol Endocrinol *13*, 1657-1671.

McMahon, G.A., Petitclerc, E., Stefansson, S., Smith, E., Wong, M.K., Westrick, R.J., Ginsburg, D., Brooks, P.C., and Lawrence, D.A. (2001). Plasminogen activator inhibitor-1 regulates tumor growth and angiogenesis. J Biol Chem *276*, 33964-33968.

Michael, K.E., Dumbauld, D.W., Burns, K.L., Hanks, S.K., and Garcia, A.J. (2009). Focal adhesion kinase modulates cell adhesion strengthening via integrin activation. Mol Biol Cell *20*, 2508-2519.

Migliaccio, A., Piccolo, D., Castoria, G., Di Domenico, M., Bilancio, A., Lombardi, M., Gong, W., Beato, M., and Auricchio, F. (1998). Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. Embo J *17*, 2008-2018.

Mitra, S.K., Lim, S.T., Chi, A., and Schlaepfer, D.D. (2006). Intrinsic focal adhesion kinase activity controls orthotopic breast carcinoma metastasis via the regulation of urokinase plasminogen activator expression in a syngeneic tumor model. Oncogene *25*, 4429-4440.

Mitra, S.K., and Schlaepfer, D.D. (2006). Integrin-regulated FAK-Src signaling in normal and cancer cells. Curr Opin Cell Biol *18*, 516-523.

Mote, P.A., Bartow, S., Tran, N., and Clarke, C.L. (2002). Loss of co-ordinate expression of progesterone receptors A and B is an early event in breast carcinogenesis. Breast Cancer Res Treat *72*, 163-172.

Olson, D., Pollanen, J., Hoyer-Hansen, G., Ronne, E., Sakaguchi, K., Wun, T.C., Appella, E., Dano, K., and Blasi, F. (1992). Internalization of the urokinase-plasminogen activator inhibitor type-1 complex is mediated by the urokinase receptor. J Biol Chem *267*, 9129-9133.

Poole, A.J., Li, Y., Kim, Y., Lin, S.C., Lee, W.H., and Lee, E.Y. (2006). Prevention of Brca1-mediated mammary tumorigenesis in mice by a progesterone antagonist. Science *314*, 1467-1470.

Proietti, C., Salatino, M., Rosemblit, C., Carnevale, R., Pecci, A., Kornblihtt, A.R., Molinolo, A.A., Frahm, I., Charreau, E.H., Schillaci, R., and Elizalde, P.V. (2005). Progestins induce transcriptional activation of signal transducer and activator of transcription 3 (Stat3) via a Jak- and Src-dependent mechanism in breast cancer cells. Mol Cell Biol *25*, 4826-4840.

Providence, K.M., and Higgins, P.J. (2004). PAI-1 expression is required for epithelial cell migration in two distinct phases of in vitro wound repair. J Cell Physiol *200*, 297-308.

Rosenthal, D.T., Iyer, H., Escudero, S., Bao, L., Wu, Z., Ventura, A.C., Kleer, C.G., Arruda, E.M., Garikipati, K., and Merajver, S.D. (2011). p38gamma promotes breast cancer cell motility and metastasis through regulation of RhoC GTPase, cytoskeletal architecture, and a novel leading edge behavior. Cancer Res *71*, 6338-6349.

Rossouw, J.E., Anderson, G.L., Prentice, R.L., LaCroix, A.Z., Kooperberg, C., Stefanick, M.L., Jackson, R.D., Beresford, S.A., Howard, B.V., Johnson, K.C., Kotchen, J.M., and Ockene, J. (2002). Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. Jama *288*, 321-333.

Sakakibara, T., Hibi, K., Kodera, Y., Ito, K., Akiyama, S., and Nakao, A. (2004). Plasminogen activator inhibitor-1 as a potential marker for the malignancy of esophageal squamous cell carcinoma. Clin Cancer Res *10*, 1375-1378.

Schaller, M.D. (2010). Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions. J Cell Sci *123*, 1007-1013.

Schneider, J., Lucas, R., Sanchez, J., Tejerina, A., and Ruibal, A. (2000). Cellular accumulation of uPA-PAI-1 [correction of UPA-PAI-1] and uPA-PAI-2 [correction of UPA-PAI-2] complexes in early (pT1) breast cancer: a new link in the uPA-UPAr-PAI chain. In Vivo *14*, 507-511.

Smith, H.W., and Marshall, C.J. (2010). Regulation of cell signalling by uPAR. Nat Rev Mol Cell Biol *11*, 23-36.

Sood, A.K., Coffin, J.E., Schneider, G.B., Fletcher, M.S., DeYoung, B.R., Gruman, L.M., Gershenson, D.M., Schaller, M.D., and Hendrix, M.J. (2004). Biological significance of focal adhesion kinase in ovarian cancer: role in migration and invasion. Am J Pathol *165*, 1087-1095.

Stahl, A., and Mueller, B.M. (1994). Binding of urokinase to its receptor promotes migration and invasion of human melanoma cells in vitro. Cancer Res *54*, 3066-3071.

Tang, H., Kerins, D.M., Hao, Q., Inagami, T., and Vaughan, D.E. (1998). The urokinase-type plasminogen activator receptor mediates tyrosine phosphorylation of focal adhesion proteins and activation of mitogen-activated protein kinase in cultured endothelial cells. J Biol Chem *273*, 18268-18272.

Tyagi, R.K., Amazit, L., Lescop, P., Milgrom, E., and Guiochon-Mantel, A. (1998). Mechanisms of progesterone receptor export from nuclei: role of nuclear localization signal, nuclear export signal, and ran guanosine triphosphate. Mol Endocrinol *12*, 1684-1695.

Vincent, A.J., Zhang, J., Ostor, A., Rogers, P.A., Affandi, B., Kovacs, G., and Salamonsen, L.A. (2002). Decreased tissue inhibitor of metalloproteinase in the endometrium of women using depot medroxyprogesterone acetate: a role for altered endometrial matrix metalloproteinase/tissue inhibitor of metalloproteinase balance in the pathogenesis of abnormal uterine bleeding? Hum Reprod *17*, 1189-1198.

Waltz, D.A., Natkin, L.R., Fujita, R.M., Wei, Y., and Chapman, H.A. (1997). Plasmin and plasminogen activator inhibitor type 1 promote cellular motility by regulating the interaction between the urokinase receptor and vitronectin. J Clin Invest *100*, 58-67.

Wei, Y., Lukashev, M., Simon, D.I., Bodary, S.C., Rosenberg, S., Doyle, M.V., and Chapman, H.A. (1996). Regulation of integrin function by the urokinase receptor. Science *273*, 1551-1555.

Wei, Y., Tang, C.H., Kim, Y., Robillard, L., Zhang, F., Kugler, M.C., and Chapman, H.A. (2007). Urokinase receptors are required for alpha 5 beta 1 integrin-mediated signaling in tumor cells. J Biol Chem *282*, 3929-3939.

Weigelt, B., Peterse, J.L., and van 't Veer, L.J. (2005). Breast cancer metastasis: markers and models. Nat Rev Cancer *5*, 591-602.

Wilkins-Port, C.E., Higgins, C.E., Freytag, J., Higgins, S.P., Carlson, J.A., and Higgins, P.J. (2007). PAI-1 is a Critical Upstream Regulator of the TGF-beta1/EGF-Induced Invasive Phenotype in Mutant p53 Human Cutaneous Squamous Cell Carcinoma. J Biomed Biotechnol *2007*, 85208.

Zheng, S., Huang, J., Zhou, K., Xiang, Q., Zhang, Y., Tan, Z., Simoncini, T., Fu, X., and Wang, T. (2012). Progesterone enhances vascular endothelial cell migration via activation of focal adhesion kinase. Journal of cellular and molecular medicine *16*, 296-305.

FIGURES LEGENDS

Figure 1. PRA and PRB differentially enhance migration of MDA-MB-231 breast cancer cells. (A) MDA-iPRAB cells were incubated with either vehicle (PR-) or RSL1 (inducing PRA) or Dox (inducing PRB) or both (inducing PRA and PRB) for 24 h. Immunoblot analysis was performed from whole cell extracts using anti-PR antibody recognizing both PR isoforms and anti-tubulin antibody for loading control. (B) MDA-iPRAB cells were induced as in A for 24 h and treated by mitomycin C for 1 h. At zero time point wound-healing repair assays were performed either in the presence of 10^{-8} M R5020 or vehicle for various time until 24 h. Photographs of each wounded area were taken at regular time and distance intervals (left panels). Results from 3 independent experiments are presented (right panels) as the random distance covered by cells after 10 h treatment (mean ± SEM, n=3). Statistical analyses were done using Mann-Whitney tests (stars).

Figure 2. PRB regulates key-genes of the PA system.
Following 24 h induction by Dox and/or RSL1 to induce or PRA and/or PRB, MDA-iPRAB cells were treated (black bars) or not (white bars) by R5020 (10^{-8} M) for 6 h. Urokinase Plasminogen Activator (uPA), Urokinase Plasminogen Activator Receptor (uPAR) and β1-integrin were quantified by real time RT-qPCR as described in material and methods. The data were normalized to 18S rRNA and expressed as mean ± SEM (n=6). Statistical analyses using the student 2-tailed test are shown by either crosses referring to PR- cells with vehicle or stars referring to PR+ (PRA and/or PRB) cells with R5020.

Figure 3. PR isoforms regulate expression of PAI-1.
(A) Following 24 h induction by Dox and/or RSL1 or vehicle, MDA-iPRAB cells were treated by 10^{-8} M R5020 (black bars) or vehicle (white bars) for 6 h. Plasminogen Activator Inhibitor type-1 (PAI-1) mRNA was measured by real time RT-qPCR as described in Material and Methods. Results and statistical analyses are calculated as in figure 2. (B) MDA-iPRAB cells were treated with Dox for 24 h to induce PRB expression, and as indicated by either 10^{-8} M R5020 and/or 10^{-6} M RU486 or vehicle for 6 h. PAI-1 transcript was measured as in A. (C) MDA-iPRAB cells were induced as in B and treated with either 10^{-8} M R5020 and/or 10^{-6} M RU486 or vehicle for 14 h. PAI-1 concentration was measured in conditioned medium by ELISA (see Material and Methods). (D) MDA-iPRAB cells were grown without inductors for 24 h and increasing amounts of soluble recombinant PAI-1 were added to the conditioned medium as described in Material and Methods. Cell migration was quantified after 10 h treatment as in figure 1, and results are expressed as per cent of basal migration obtained in the absence of PAI-1 (mean ± SEM, n=3). (E) MDA-iPRAB cells were grown with Dox to express PRB or with vehicle (PR-) for 24 h. Following addition of PAI-1 (200 ng/mL) or vehicle in the conditioned medium, cell migration was measured after 10 h as in D.

Figure 4. PRA and PRB- differentially impact regulation of FAK activity.
(A) MDA-iPRAB cells were grown in the presence Dox (PRB) or RSL1 (PRA) or vehicle (PR-) for 24 h and analyzed by western blot using alternately anti-FAK^{Y397p}, anti-FAK^{total} and anti-tubulin antibodies as described in Material and Methods. PR isoforms were analyzed on a separated gel using anti-PR antibody. A representative immunoblot is shown while FAK^{Y397p}, FAK^{total} and FAK^{Y397p}/FAK^{total} ratio were quantified and normalized to tubulin from 3 independent experiments. The corresponding graphs are presented as fold change of values obtained in PR- cells (means ± SEM, Mann-Whitney statistical test). (B) Cells were induced as in A and then treated or not by 10^{-8} M R5020 for the indicated times (min). Cell lysates

were analyzed as in A, and data are presented on the graphs as fold change of values obtained for the corresponding PR isoform at zero time point.

Figure 5. PRB and FAK^{Y397p} are colocalized in focal adhesion sites.
MDA-iPRAB cells were induced (PRB) or not (PR-) by Dox for 24 h then treated with either 10^{-8} M R5020 or 10^{-6} M RU486 or vehicle for 30 min. Immunofluorescence microscopy was performed as described in material and methods by analyzing FAK^{Y397P} (red) and PRB (green). The nuclei were counterstained with DAPI (blue). Photographs were taken by using confocal microscope at 400x magnification. The images a-d were further magnified in the lower panels to focus on representative structures.

Figure 6. PRA and PRB interact with FAK complexes and regulate their turnover.
(A) MDA-iPRAB cells were induced by RSL1 and/or Dox for 24 h and were then exposed to R5020 or to vehicle for 1 h. Cell lysates were incubated with either total FAK antibody or non-related antibody (IgG). Lysates (input) and immunoprecipitates (IP) were analyzed by western-blot for FAK^{Y397P}, FAK^{total}, PRA, PRB and tubulin. Framed inset: the co-IP experiments were repeated using iPRAB cells induced by either Dox or RSL1+Dox to induce PRB or PRA+PRB for 24 h. (B) iPRAB cells were induced by either RSL1 or Dox or both of them and then treated or not by 10^{-8} M R5020 for 10 h. Western blot were performed and quantified as described in Figure 4A for FAK^{Y397P}, FAK^{total}, PRA, PRB and tubulin (mean ± SEM, Mann-Whitney statistical test) (C) Ishikawa cells stably transfected by either PRA or PRB and T47D cells endogenously expressing PRA and PRB were treated either by 10^{-8} M R5020 or vehicle for 24 h. Western blot analyses were performed as in B.

Figure 7. Model for PRB-dependent regulation of cell migration.
Impaired FAK phosphorylation at Y397 site decreases FAK time residency at focal adhesion points (FAs) leading to reduce cell retractile activity and migration (migration OFF). Increased stabilization of FAK at FAs leads to sustained stimulation of phosphorylation cascades initiated by FAK^{Y397} phosphorylation, interactions with protein partners and disassembly of FAs (migration ON). Interaction of FAK with cytoplasmic (cyto) PRB at FAs leads to enhance FAK time residency within FAs (PRB-dependent migration HIGH). The hormone transiently enhances Y397 phosphorylation enhancing migration, and PRB-FAK is released from the FAs thus translocated in the nucleus and/or degraded (PRB-dependent migration LOW). The exchange of PRB with PRA within soluble FAK complexes inhibits FAK/PRB co-degradation potentializing the effect of hormone on PRB-dependent migration (PRB-dependent migration HIGH). The indicated PR-dependent transcriptional regulations impacting migration may further strengthen these effects at delayed time. RU486 antagonist counteracts all the previous hormone-dependent effects and potentiates PRB-dependent cell migration. The triangles symbols rely on R5020 (black), RU486 (grey) or PRA expression (white) variations leading to extend migration rate at the indicated high or low level.

SUPPLEMENTAL MATERIALS (PDF file).

Supplemental Table 1. List of primers used in RT-qPCR experiments.

Supplemental Figure S1. RU486 antagonist releases pro-migratory functions of PRB.
Following 24 h treatment by vehicle (PR-) or Dox to induce PRB expression, iPRAB cells were treated or not with RU486 (10^{-6} M) and/or R5020 (10^{-8} M) in the presence of mitomycin C. Cell migration was measured after 10 h as in article Figure 1. Results are expressed as % of migration obtained in PRB-induced cells treated by vehicle (mean ± SEM, n=3). Stars rely on significance of Mann-Whitney test for the indicated comparisons.

Supplemental Figure S2. R5020 does not inhibit FAK transcription in PRB cells.
FAK expression in PRB-induced cells treated or not by 10^{-8} M R5020 for 10 h was assessed by RT-qPCR as described in Materials and Methods. Data are represented as mean ± SEM (n=6), and stars rely on significance of Mann-Whitney test for the indicated comparisons (ns: non significant variation).

Supplemental Figure S3: Time course of PRB-FAK localization.
MDA-iPRAB cells were treated by vehicle (PR-) or Dox to induce PRB expression for 24 h and then by either vehicle or 10^{-8} M R5020 for the indicated time (min). Immunofluorescence labeling experiments were performed using anti-FAK^{Y397P} (red) and anti-PRA (green) antibodies or Dapi (blue) as in article Figure 5. The images were merged at the lower right corner of each panel.

Supplemental Figure S4. PRA does not colocalize with FAK^{Y397p} in plasma membrane region.
MDA-iPRAB cells were treated by RSL1 inducer of PRA expression for 24 h and then by either vehicule or 10^{-8} M R5020 for 30 min. Immunofluorescence labeling experiments were performed using anti-FAK^{Y397P} (red) and anti-PRA (green) antibodies as in article Figure 5.

Supplemental Figure S5. Confocal microscopy profiles of differential PR isoforms and FAK colocalizations.
Immunostaining experiments were performed as in article Figure 5 for time 30 min. The intensity scanning profiles of PR-FAK^{Y397P} colocalization obtained along the indicated line on the images of a representative cell are expressed as arbitrary units (AU). The intensity profiles are drawn either in green for PR isoform or in red for FAK^{Y397P} signal or in blue for nucleus marker. The indicated arrows in the graphs point to the identified colocalization speckles.

Supplemental Figure S6. Reverse co-IP of PR-FAK complexes using anti-PR antibodies.
Cells lysates were first incubated with PR antibody (Novocastra), then immunoprecipitations and immunoblot were performed as describe in article Figure 6A.

Figure 1

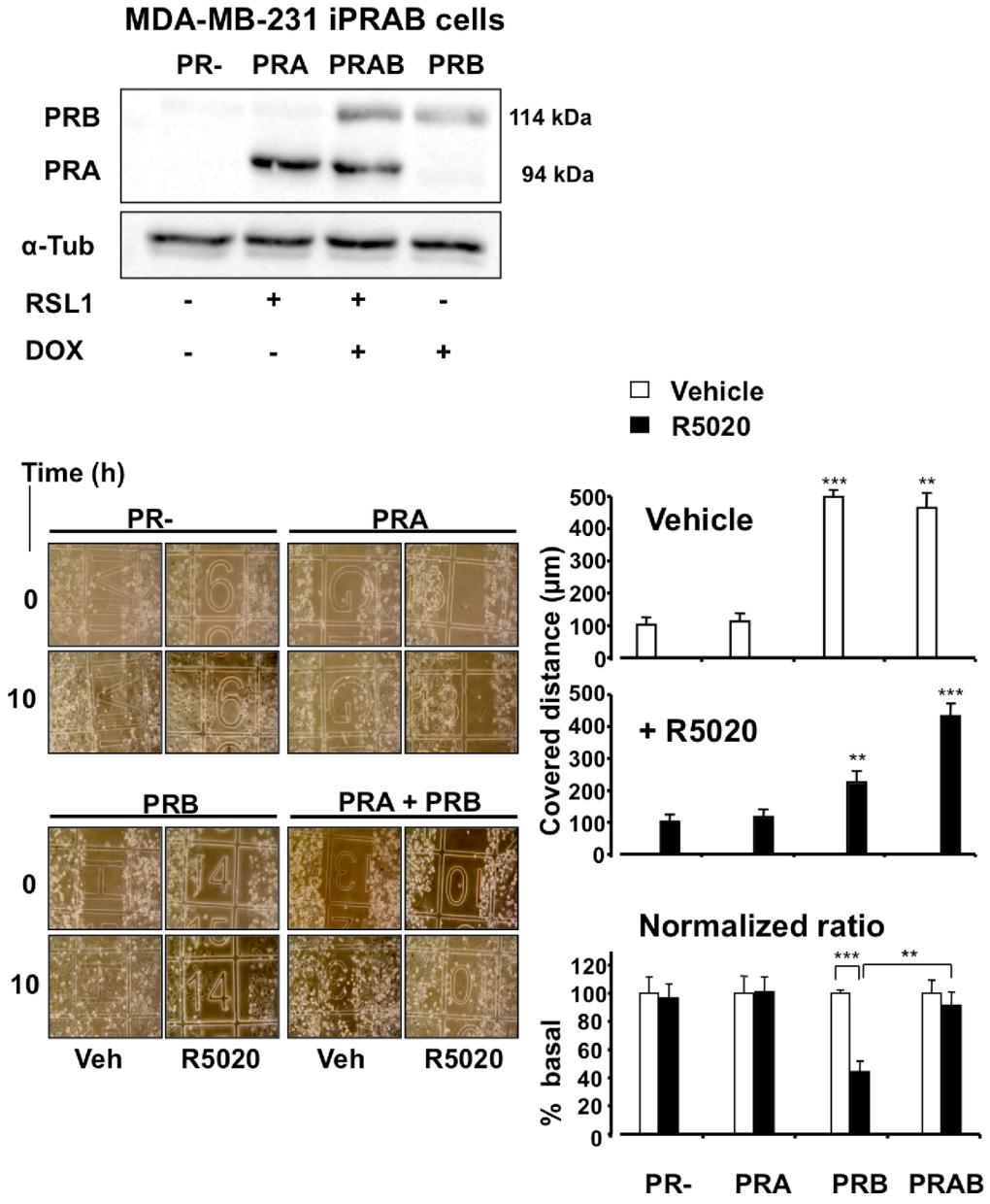

MDA-MB-231 iPRAB cells
PR- PRA PRAB PRB
PRB
PRA
114 kDa
94 kDa
α-Tub
RSL1 - + + -
DOX - - + +
Vehicle
R5020
Time (h)
PR-
PRA
PRB
PRA + PRB
0
10
Veh
R5020
Veh
R5020
Vehicle
+ R5020
Covered distance (µm)
Normalized ratio
% basal
PR-
PRA
PRB
PRAB

Figure 2

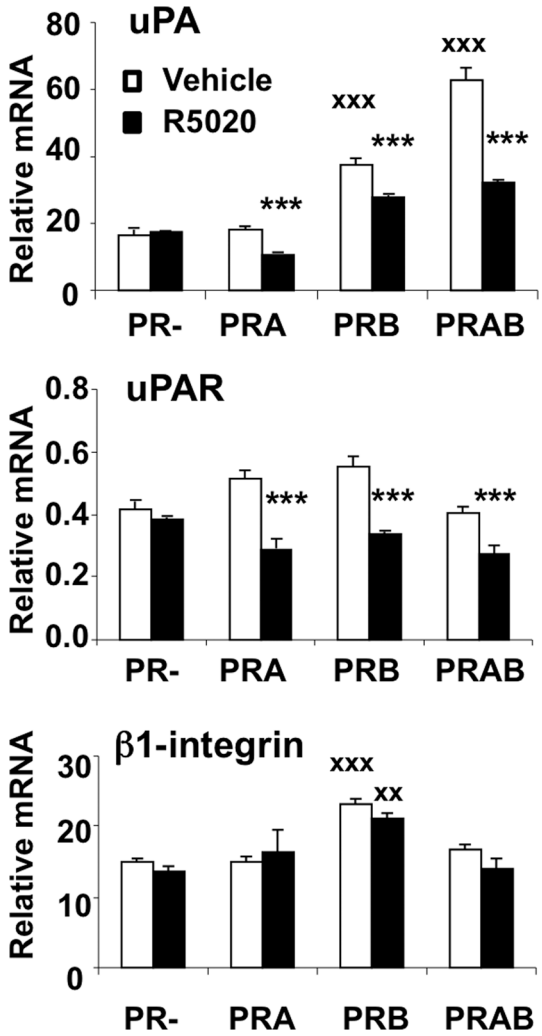

uPA
Vehicle
R5020
Relative mRNA
80
60
40
20
0
xxx
xxx
***
***
***
PR-
PRA
PRB
PRAB
uPAR
Relative mRNA
0.8
0.6
0.4
0.2
0.0
***
***
***
PR-
PRA
PRB
PRAB
β1-integrin
Relative mRNA
30
20
10
0
xxx
xx
PR-
PRA
PRB
PRAB

Figure 3

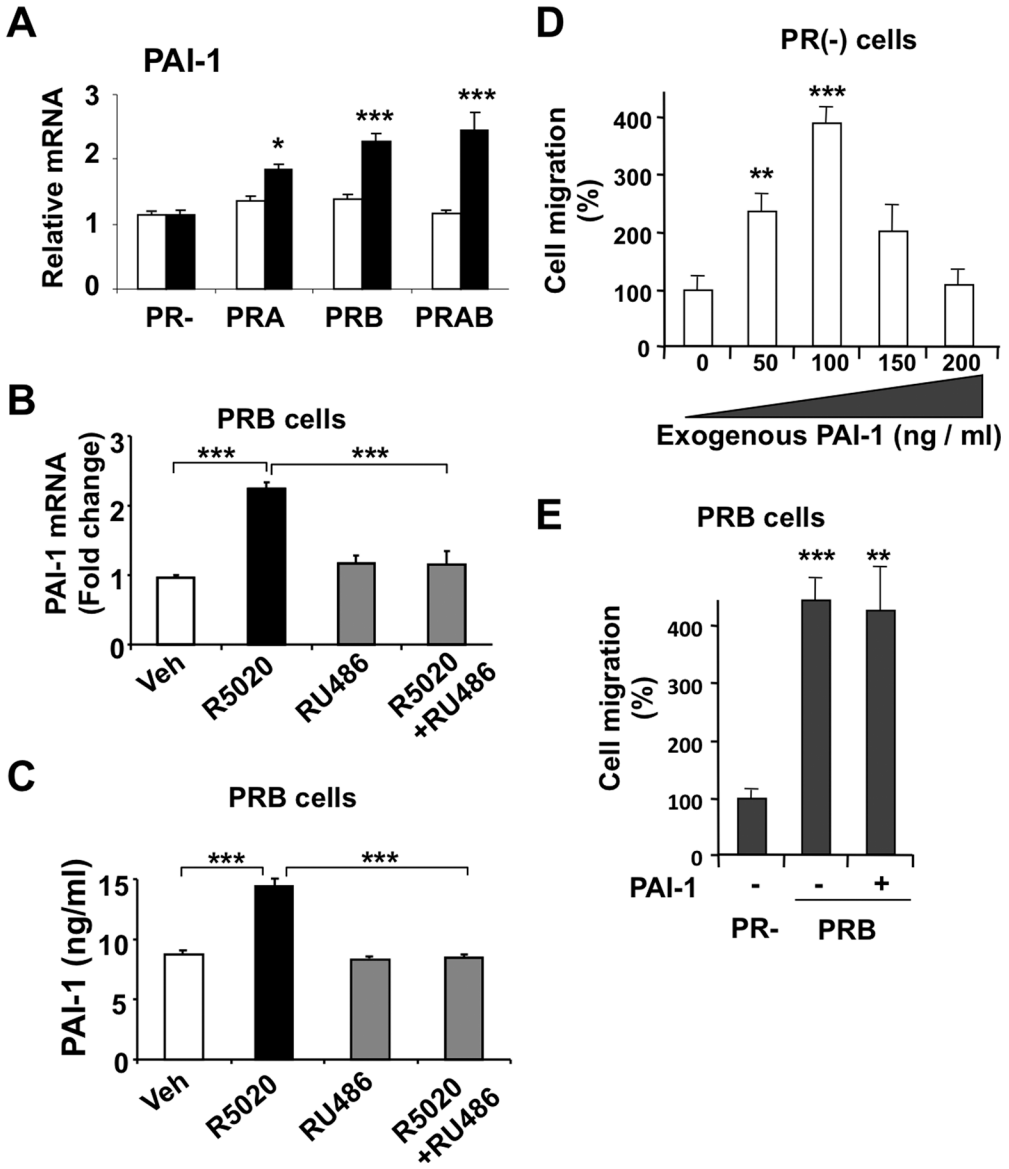

A
PAI-1
Relative mRNA
3
2
1
0
*
***
***
PR-
PRA
PRB
PRAB
B
PRB cells
PAI-1 mRNA
(Fold change)
3
2
1
0
***
***
Veh
R5020
RU486
R5020
+RU486
C
PRB cells
PAI-1 (ng/ml)
15
10
5
0
***
***
Veh
R5020
RU486
R5020
+RU486
D
PR(-) cells
Cell migration
(%)
400
300
200
100
0
**
***
0
50
100
150
200
Exogenous PAI-1 (ng / ml)
E
PRB cells
Cell migration
(%)
400
300
200
100
0
***
**
PAI-1
-
-
+
PR-
PRB

Figure 4

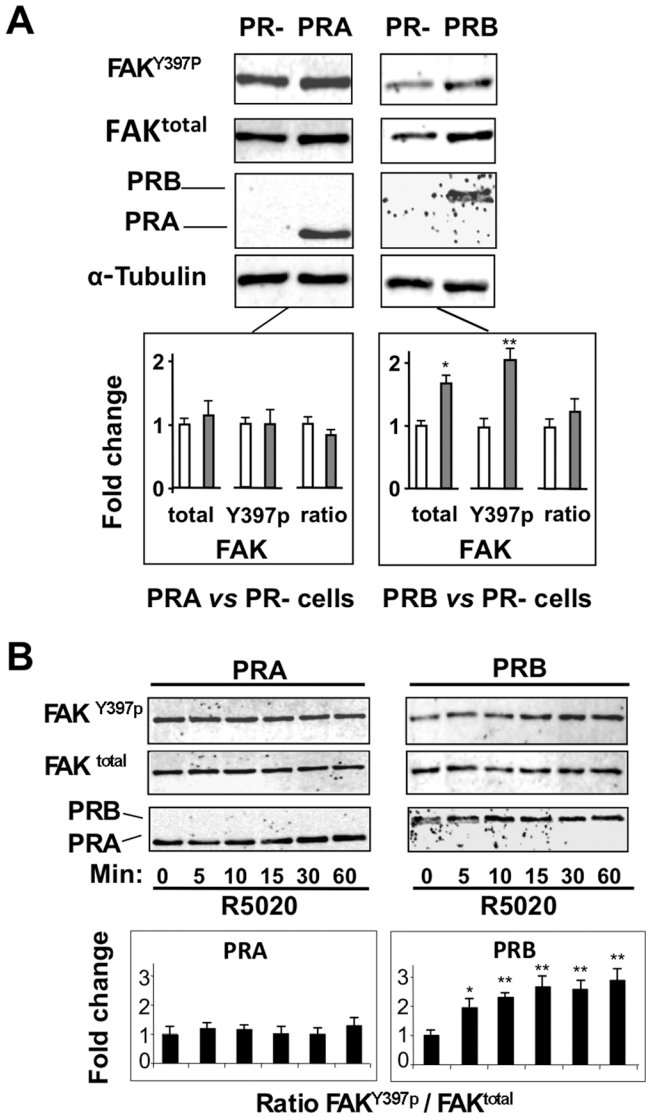

A
PR- PRA
PR- PRB
FAKY397P
FAKtotal
PRB
PRA
α-Tubulin
Fold change
total Y397p ratio
FAK
PRA vs PR- cells
PRB vs PR- cells
B
PRA
PRB
FAK Y397p
FAK total
PRB
PRA
Min: 0 5 10 15 30 60
R5020
Ratio FAKY397p / FAKtotal

Figure 5

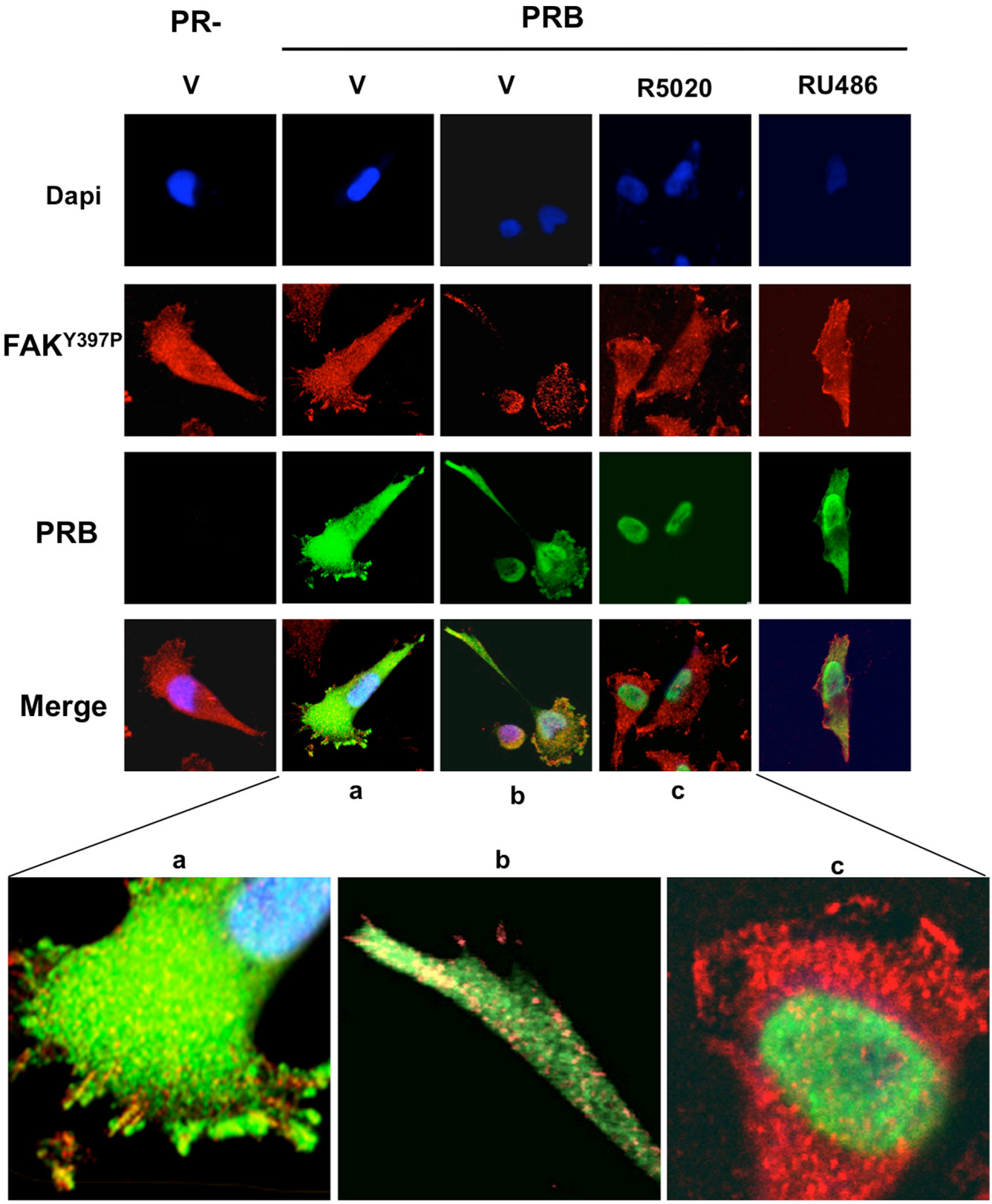


Figure 6

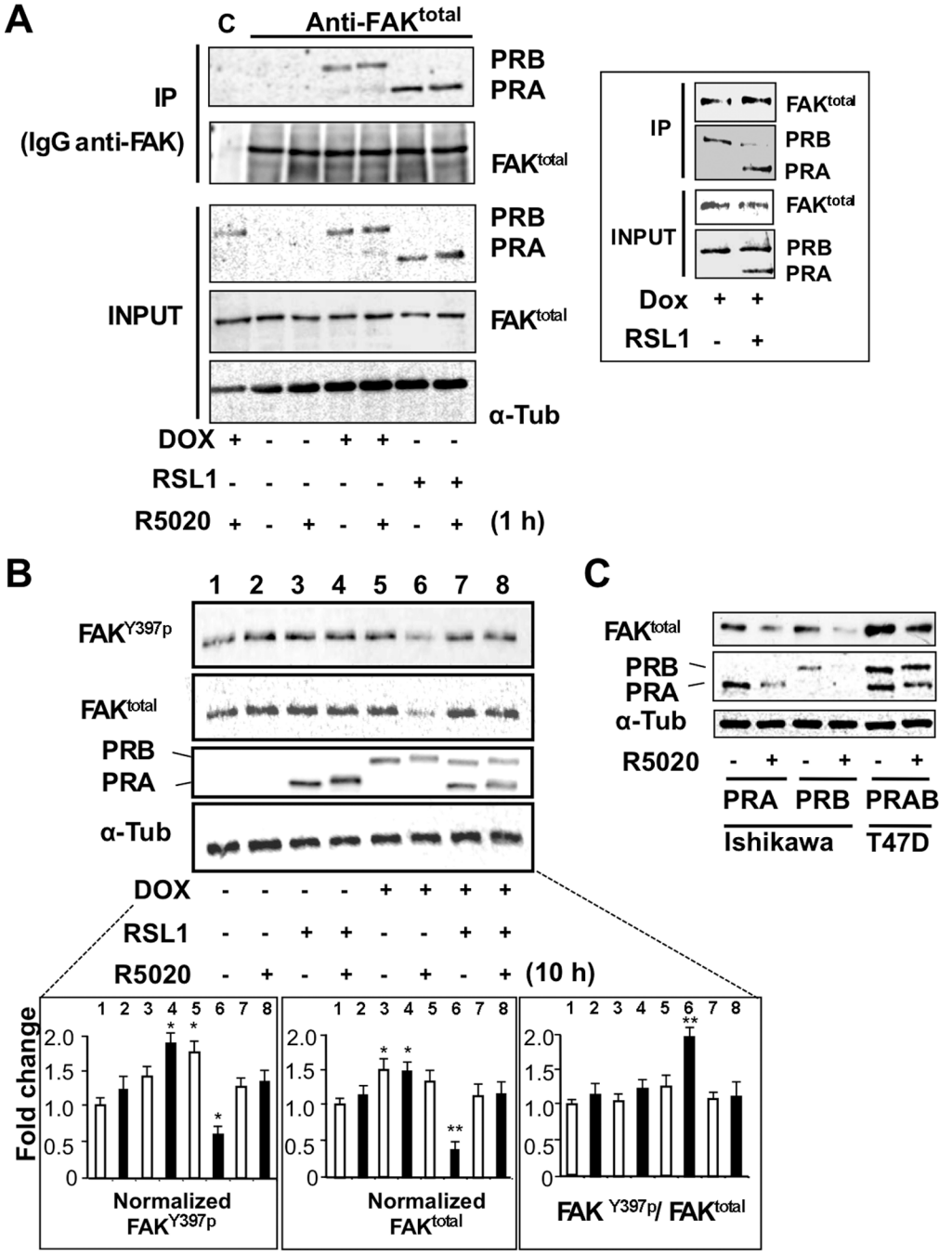
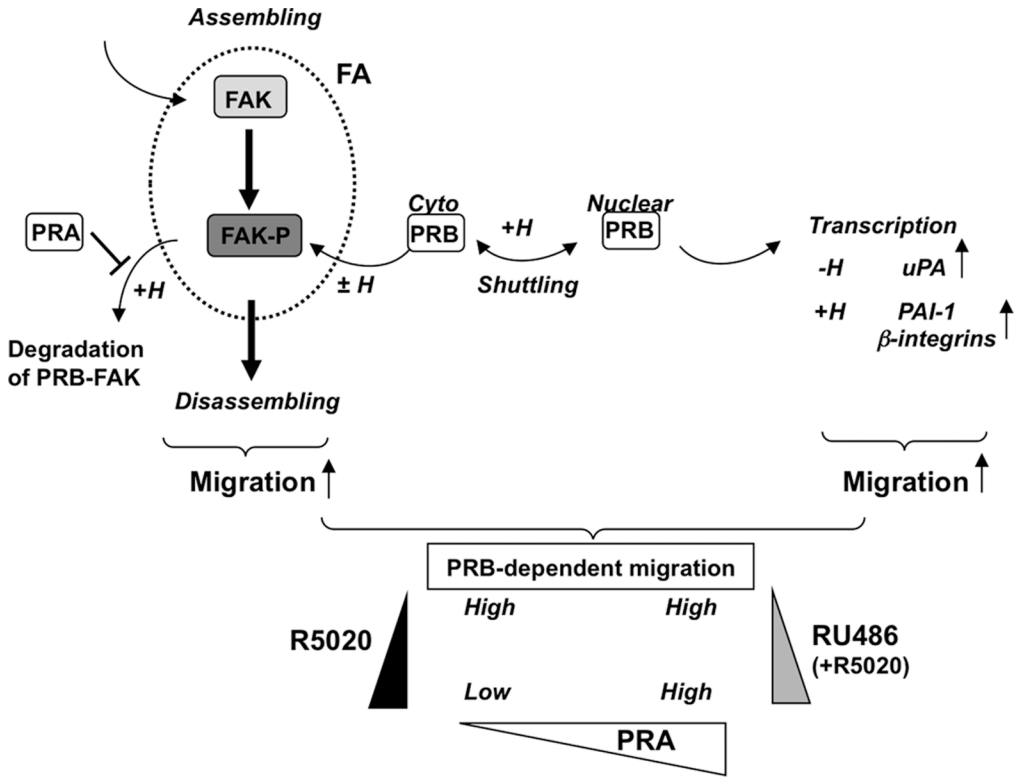

A
C
Anti-FAK^total
IP
(IgG anti-FAK)
PRB
PRA
FAK^total
INPUT
α-Tub
DOX
RSL1
R5020
(1 h)
IP
INPUT
Dox
RSL1
B
1 2 3 4 5 6 7 8
FAK^Y397p
FAK^total
PRB
PRA
α-Tub
DOX
RSL1
R5020
(10 h)
Fold change
Normalized FAK^Y397p
Normalized FAK^total
FAK Y397p/ FAK^total
C
FAK^total
PRB
PRA
α-Tub
R5020
PRA
PRB
PRAB
Ishikawa
T47D

Figure 7


Assembling
FA
FAK
FAK-P
PRA
+H
Degradation
of PRB-FAK
Disassembling
Cyto
PRB
± H
+H
Shuttling
Nuclear
PRB
Transcription
-H
uPA
+H
PAI-1
β-integrins
Migration
Migration
PRB-dependent migration
High
High
R5020
RU486
(+R5020)
Low
High
PRA

Supplemental Table 1

Primer sequences used for RT-qPCR experiments

Primer	Sequence (5' to 3')	Gene
uPA, *Fwd*	ACCACCAAAATGCTGTGTGC	*PLAU* : Urokinase Plasminogen activator (uPA)
Rev	AGTCAAAGTCATGCGGCCTT	
uPAR, *Fwd*	GCATTTCCTGTGGCTCATCA	*PLAUR*: uPA receptor (uPAR)
Rev	CTTTGGACGCCCTTCTTCAC	
PAI-1, *Fwd*	CATCCTGGAACTGCCCTACC	*SERPIN1*: uPA inhibitor-1 (PAI-1)
Rev	ATGTTGCCTTTCCAGTGGCT-	
ITGb1, *Fwd*	CGCGCGGAAAAGATGAAT	*ITGB1*: β1-integrin
Rev	CACAATTTGGCCCTGCTTG	
FAK1, *Fwd*	CCCTGCTGACAGCTACAACG	*PTK2* : Focal adhesion kinase (FAK1)
Rev	GCCCGTCACATTCTCGTACA	
18SR, *Fwd*	GTGCATGGCCGTTCTTAGTTG	*RNA18S5*: 18S ribosomal RNA (18S-RNA)
Rev	CATGCCAGAGTCTCGTTCGTT	

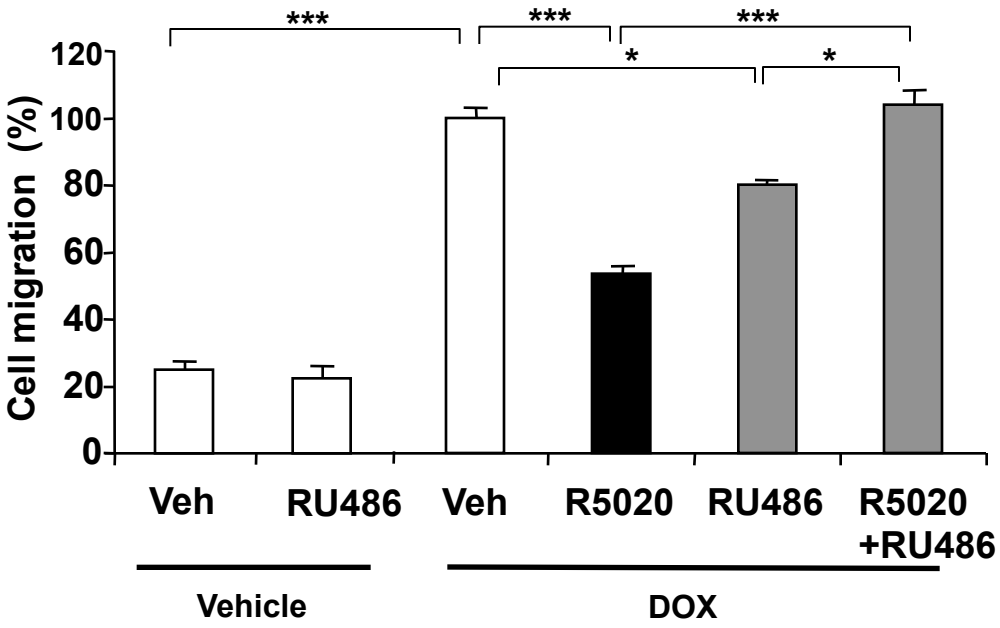


Figure S1. RU486 antagonist releases pro-migratory functions of PRB.
Following 24 h treatment by vehicle or Dox to induce PRB expression, iPRAB cells were treated with RU486 (10^{-6} M) and/or R5020 (10^{-8} M) or vehicle (veh) in the presence of mitomycin C. Cell migration was measured after 10 h as in article Figure 1. Results are expressed as % of migration obtained in PRB-induced cells treated by vehicle. Stars rely on significance of Mann-Whitney test for the indicated comparisons.

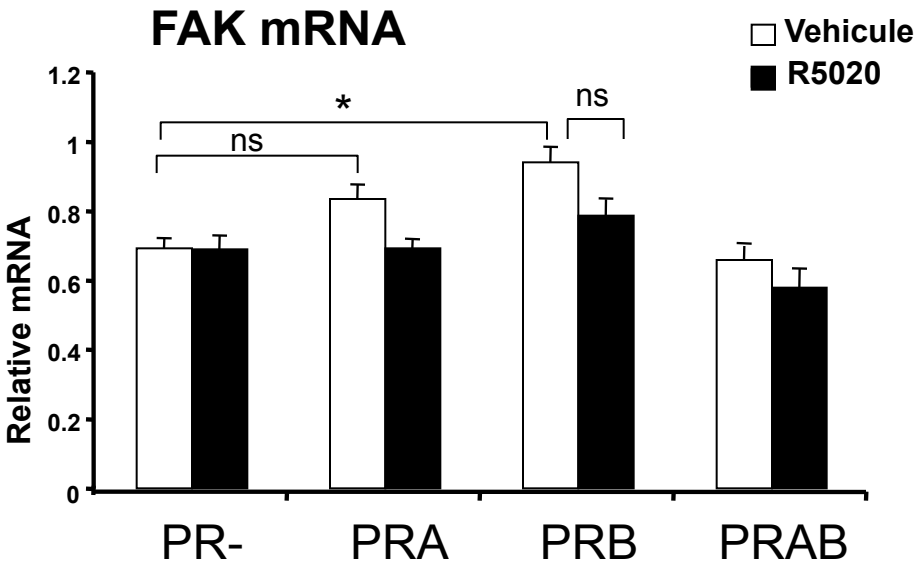


Figure S2. R5020 does not inhibit FAK transcription in PRB cells.
FAK expression in PRB-induced cells treated or not by 10^{-8} M R5020 for 10 h was assessed by RT-qPCR as described in Materials and Methods. Data are represented as mean ± SEM (n=6), and stars relies on significance of Mann-Whitney test for the indicated comparisons (ns: non significant variation).

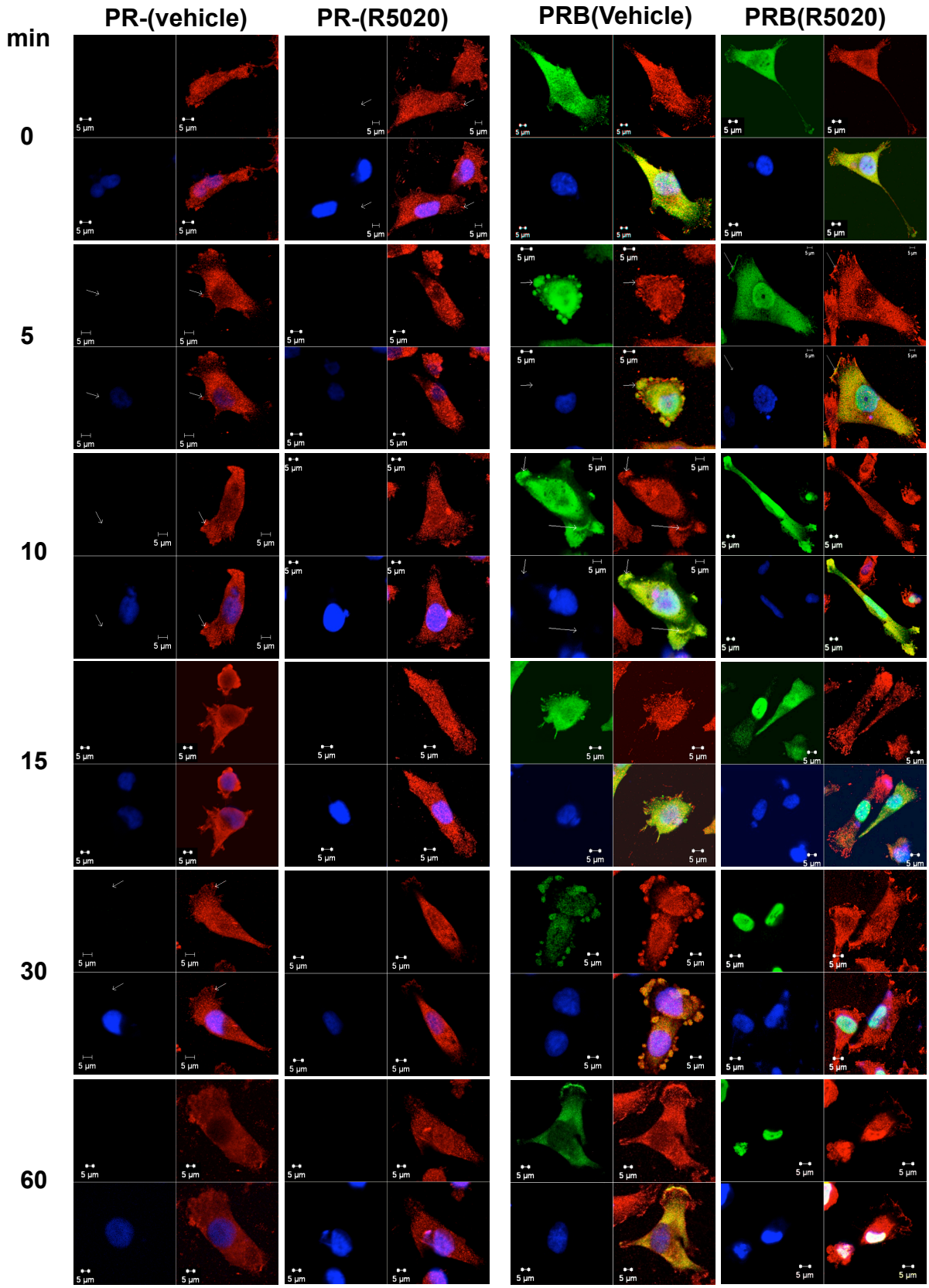


Figure S3. Time course of PRB-FAK localization.
MDA-iPRAB cells were treated by vehicle (PR-) or Dox to induce PRB expression for 24 h and then by either vehicle or 10^{-8} M R5020 for the indicated time (min). Immunofluorescence labeling experiments were performed using anti-FAK^{Y397P} (red) and anti-PRA (green) antibodies or Dapi (blue) as in article Figure 5. The image were merged at the lower right corner of each panel.

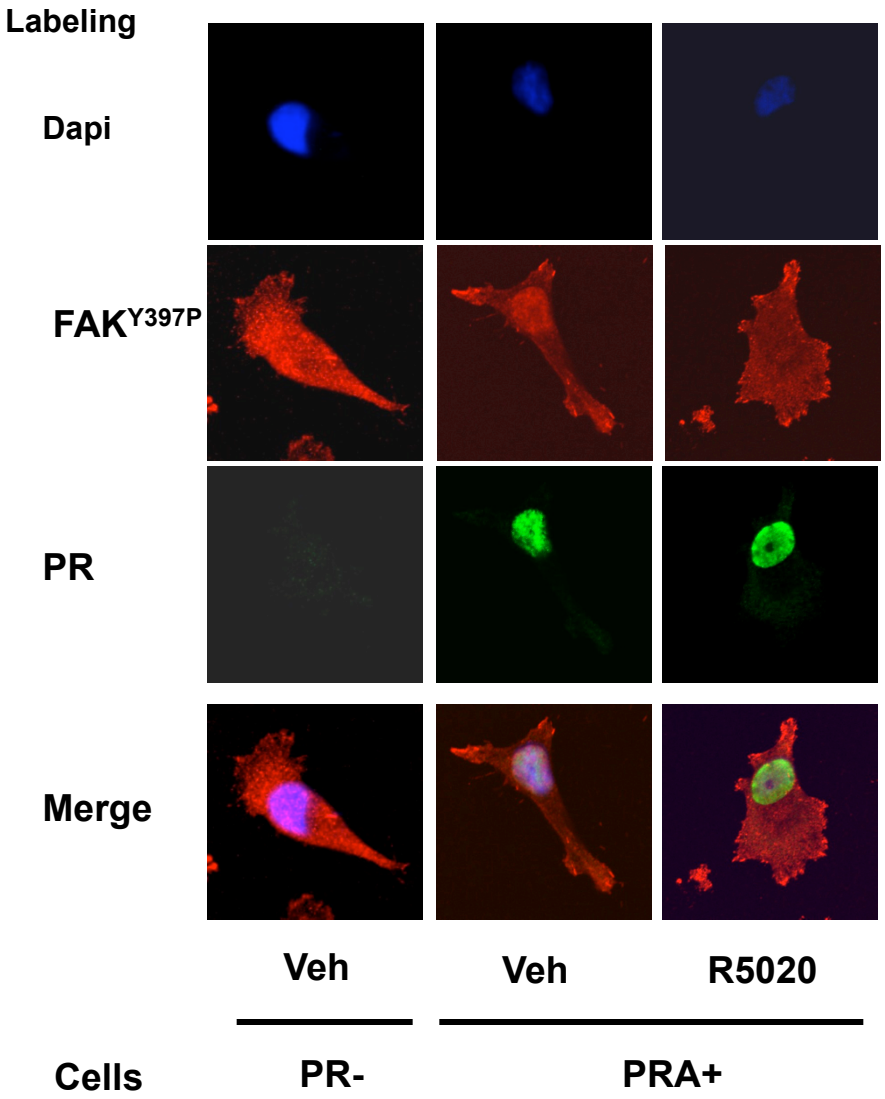


Figure S4. PRA does not colocalize with FAK^{Y397p} in plasma membrane region. MDA-iPRAB cells were treated by RSL1 inducer of PRA expression for 24 h and then by either vehicle or 10^{-8} M R5020 for 30 min. Immunofluorescence labeling experiments were performed using anti-FAK^{Y397P} (red) and anti-PRA (green) antibodies or Dapi (blue) as in article Figure 5.

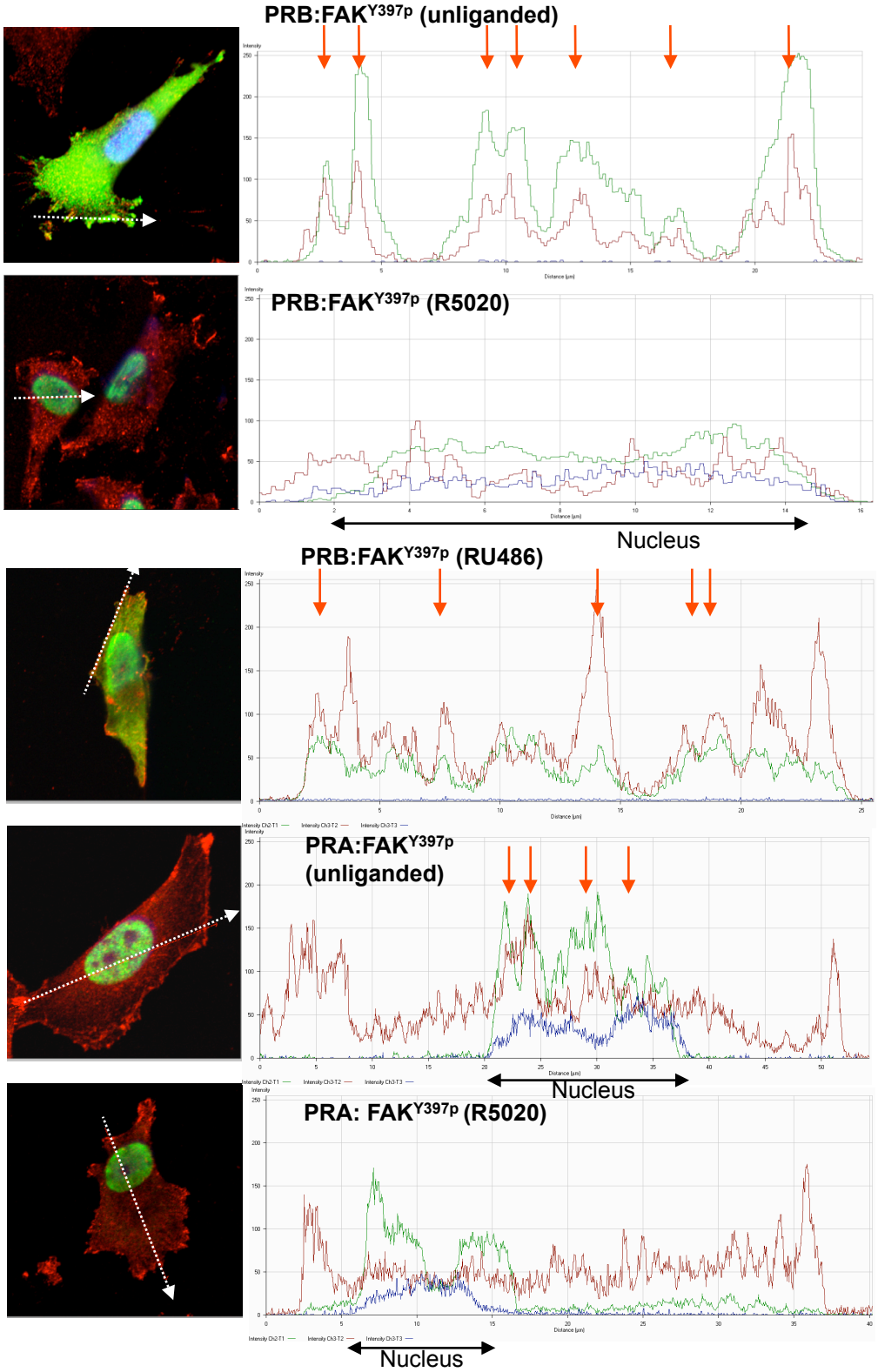


Figure S5. Confocal microscopy profiles of differential PR isoforms and FAK colocalizations. Immunostaining experiments were performed as in article Figure 5 for time 30 min. The intensity scanning profiles of PR-FAK^{Y397P} colocalization obtained along the indicated line on the images of a representative cell are expressed as arbitrary units (AU). The intensity profiles are drawn either in green for PR isoform or in red for FAK^{Y397P} signal or in blue for nucleus marker. The indicated arrows in the graphs point to the identified colocalization speckles.

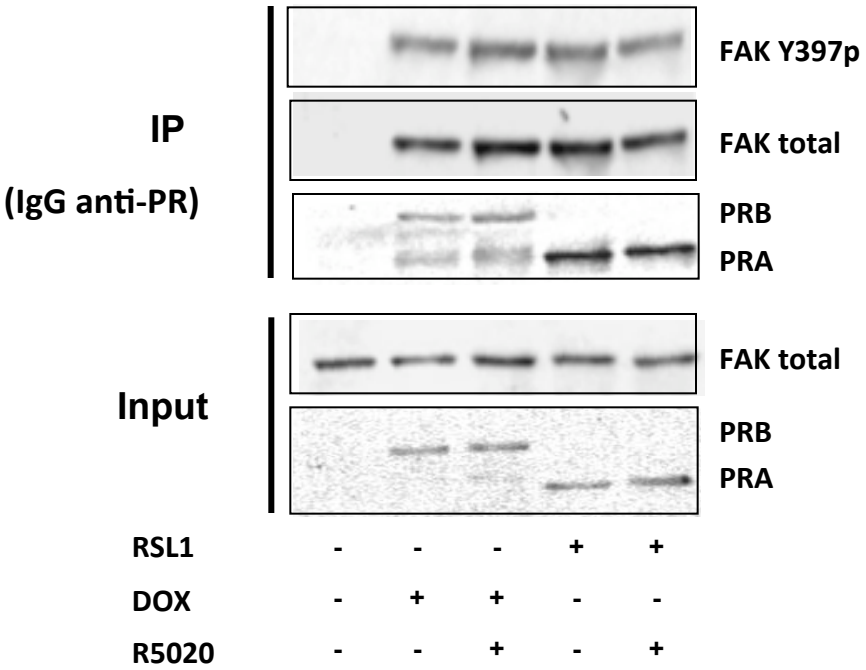


Figure S6. Reverse co-IP of PR-FAK complexes using anti-PR antibodies. Cells lysates were first incubated with PR antibody (Novocastra), then immunoprecipitations and immunoblot were performed as describe in article Figure 6A.