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ORIGINAL ARTICLE

Upregulation of PIP3-dependent Rac exchanger 1 (P-Rex1) promotes prostate cancer metastasis

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Excessive activation of G-protein-coupled receptor (GPCR) and receptor tyrosine kinase (RTK) pathways has been linked to prostate cancer metastasis. Rac activation by guanine nucleotide exchange factors (GEFs) plays an important role in directional cell migration, a critical step of tumor metastasis cascades. We found that the upregulation of P-Rex1, a Rac-selective GEF synergistically activated by Gβγ freed during GPCR signaling, and PIP3, generated during either RTK or GPCR signaling, strongly correlates with metastatic phenotypes in both prostate cancer cell lines and human prostate cancer specimens. Silencing endogenous P-Rex1 in metastatic prostate cancer PC-3 cells selectively inhibited Rac activity and reduced cell migration and invasion in response to ligands of both epidermal growth factor receptor and G-protein-coupled CXC chemokine receptor 4. Conversely, expression of recombinant P-Rex1, but not its 'GEF-dead' mutant, in non-metastatic prostate cancer cells, such as CWR22Rv1, increased cell migration and invasion through Rac-dependent lamellipodia formation. More importantly, using a mouse xenograft model, we showed that the expression of P-Rex1, but not its mutant, induced lymph node metastasis of CWR22Rv1 cells without an effect on primary tumor growth. Thus, by functioning as a coincidence detector of chemotactic signals from both GPCRs and RTKs, P-Rex1-dependent activation of Rac promotes prostate cancer metastasis.
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Introduction

Prostate cancer is the most commonly diagnosed non-cutaneous cancer in American men, and the second leading cause of cancer mortality (Jemal et al., 2007). Metastasis, the major cause of prostate cancer death, begins with the migration and invasion of the cancer cells into the surrounding tissues and lymphatics in response to chemotactic and structural signals (Steeg and Theodorescu, 2008). Chemoattractants, including chemokines, growth factors and matrix metallopro-teases, bind to specific membrane receptors on cancer cells to direct their migration and invasion (Kedrin et al., 2007). Earlier research focused on chemoattrac-tant stimulation of receptor tyrosine kinases (RTKs), but recent evidence from both preclinical and clinical studies has shown that G-protein-coupled receptor (GPCR) systems are excessively activated in prostate cancer cells (Daaka, 2004; Dorsam and Gutkind, 2007), and contribute to the migratory potential of cancer cells during metastasis (Dorsam and Gutkind, 2007).

Cell migration toward chemoattractants is dependent on actin cytoskeleton reorganization regulated by the Rho family of small GTPases, including Rac, Cdc42 and Rho (Yamazaki et al., 2005). Aberrant Rac signaling is found in some human cancers and mediates cancer metastasis (Sun et al., 2006; Yamaguchi and Condeelis, 2007) in part by generating the F-actin-rich thinned-out lamellipodia protrusions at the leading edge of migrat-ing cancer cells. Hyperactivation of Rac has been found in metastatic prostate cancer cells (Knight-Krajewski et al., 2004) and inhibition of Rac activation blocked prostate cancer cell migration and invasion (Gao et al., 2004), suggesting an important role of Rac activation in prostate cancer metastasis.

Rac acts as a molecular switch and cycles between an inactive GDP- and an active GTP-bound forms, catalysed by guanine nucleotide exchange factors (GEFs) (Servitja et al., 2003). Both GPCRs and RTKs can activate Rac through Rac-selective GEFs (Rac-GEFs; Ma et al., 1998; Schiller, 2006). Tiam1, a RacGEF, that contributes to the metastasis of colorectal and breast cancers (Minard et al., 2004), is also increased in prostate cancer (Engers et al., 2006).

However, Tiam1 is already elevated in precancerous high-grade prostatic intraepithelial neoplasia (Engers et al., 2006), suggesting that Tiam1 upregulation may not directly trigger prostate cancer metastasis.

During a recent screen of cancer cell lines, we found a selective elevation of P-Rex1 expression in metastatic prostate cancer cells. P-Rex1 is a GEF that selectively activates Rac through its catalytic Dbl-homology (DH) domain (Welch et al., 2002; Rosenfeldt et al., 2004; Hill et al., 2005). P-Rex1 helps regulate the function of neutrophils (Welch et al., 2005), macrophages (Wang et al., 2008) and neuronal migration in the developing nervous system (Yoshizawa et al., 2005). P-Rex1 is among the few RacGEFs that is known to be directly activated by free Gβγ subunits released from G-proteins during GPCR signaling (Welch et al., 2002). In the developing nervous system, P-Rex1 was also stimulated by agonists, such as nerve growth factor, which act through RTKs to activate phosphoinositide-3 (PI3) kinases and their downstream effectors (Yoshizawa et al., 2005). Interestingly, mammalian target of rapamycin (mTOR), which integrates nutrient and growth factor signals (Fingar and Blenis, 2004), can also activate P-Rex1 through mTOR complex 2 formation to activate Rac and promote cell migration (Hernandez-Negrete et al., 2007; Dada et al., 2008). Thus, P-Rex1 could function as a molecule that integrates signals from several input pathways simultaneously activated by the cell microenvironment and could be a novel therapeutic target in many pathological conditions, including human cancers (Hernandez-Negrete et al., 2007).

The purpose of this study was to determine the potential roles of upregulated P-Rex1 in human prostate cancer metastasis. Our studies revealed that immuno-histochemical staining for P-Rex1 was more intense and uniform in human lymph node metastases of prostate cancer than in the corresponding primary prostate tumors or adjacent benign tissues. Moreover, silencing endogenous P-Rex1 protein in metastatic prostate cancer PC-3 cells suppresses Rac-dependent cell migration and invasion, whereas exogenous expression of P-Rex1 in non-metastatic CWR22Rv1 increases cell migration and invasion through Rac activation in vitro and leads to the development of lymph node metastases following orthotopic transplantation in SCID mice. These data collectively identify P-Rex1 as a novel promoter of prostate cancer metastasis.

Results

P-Rex1 is upregulated in metastatic human prostate cancer cell lines
Expression levels of P-Rex1 mRNA were very low in human normal primary prostate epithelial cells (PrECs) and in non-metastatic prostate cancer cells, such as LNCaP and CWR22Rv1, but were remarkably higher in metastatic prostate cancer cells, such as PC3-LN4 and PC-3 (Figure 1a). Transwell chamber assays were used to measure the directional migration and invasion

abilities of those prostate cancer cells in response to NIH3T3 fibroblast conditioned medium (CM), which contains many cytokines and growth factors, and is commonly used for in vitro metastasis assays (Mensing et al., 1983; Albini and Benelli, 2007). As shown in Figure 1b, there is a direct correlation between the P-Rex1 mRNA expression levels in these cells, and their ability to migrate and invade through the Matrigel. Western blot analysis and immunofluorescent staining using P-Rex1-specific antibodies 6F12 (Welch et al., 2005) and 4A3 (Yoshizawa et al., 2005), respectively, confirmed that P-Rex1 protein was significantly higher in PC-3 cells than in LNCaP and CWR22Rv1 cells (Figure 1c). Most P-Rex1 in PC-3 cells is cytosolic, with a small fraction found near the plasma membrane (Figure 1c, right).

P-Rex1 protein is upregulated in human prostate adenocarcinoma and lymph node metastases
The P-Rex1 protein level in cells was measured immunohistochemically in primary tumors and a paired lymph node metastasis in archived samples from eight prostate cancer patients. Figure 1d shows representative results from primary tumors (d1 and d2) alongside the paired lymph node metastases (d3 and d4, respectively) for two cases. Compared with the non-cancerous adjacent prostate tissue in primary tumors (green arrows), P-Rex1 staining intensity is only slightly and heterogeneously increased in the localized prostate carcinoma (black arrows), but higher and more homo-geneous in lymph node metastases (red arrows). An Automated Cellular Imaging System (San Juan Capis-trano, CA, USA; Gao et al., 2005) was used to get the histoscore (H-score) indices of P-Rex1 protein levels shown in Figure 1d (right panel). The H-score was increased an average of 3.7-fold in lymph node metastases and 1.6-fold in localized prostate cancer relative to nearby non-cancerous prostate tissues, differences that were statistically significant. Further-more, the H-score was significantly higher in lymph node metastases than in the localized prostate cancer from the same patient, raising the possibility that the heterogeneous upregulation of P-Rex1 in the primary prostate tumor is associated with progression to the metastatic phenotype.

Silencing endogenous P-Rex1 suppresses metastatic prostate cancer cell migration and invasion by reducing Rac activity
To directly show a role for P-Rex1 in metastatic prostate cancer cells, endogenous P-Rex1 expression in PC-3 cells was silenced with a P-Rex1-specific siRNA (Yoshizawa et al., 2005). Both western blot assay (Figure 2a, upper panel) and immunostaining analysis (Figure 2a, lower panel) indicated that this P-Rex1 siRNA reduced the P-Rex1 protein level in PC-3 cells approximately 80% compared with P-Rex1 levels with mock or scramble siRNA transfection. Silencing P-Rex1 reduced PC-3-cell migration and invasion in response to NIH3T3 CM by approximately 50% when compared with the scramble
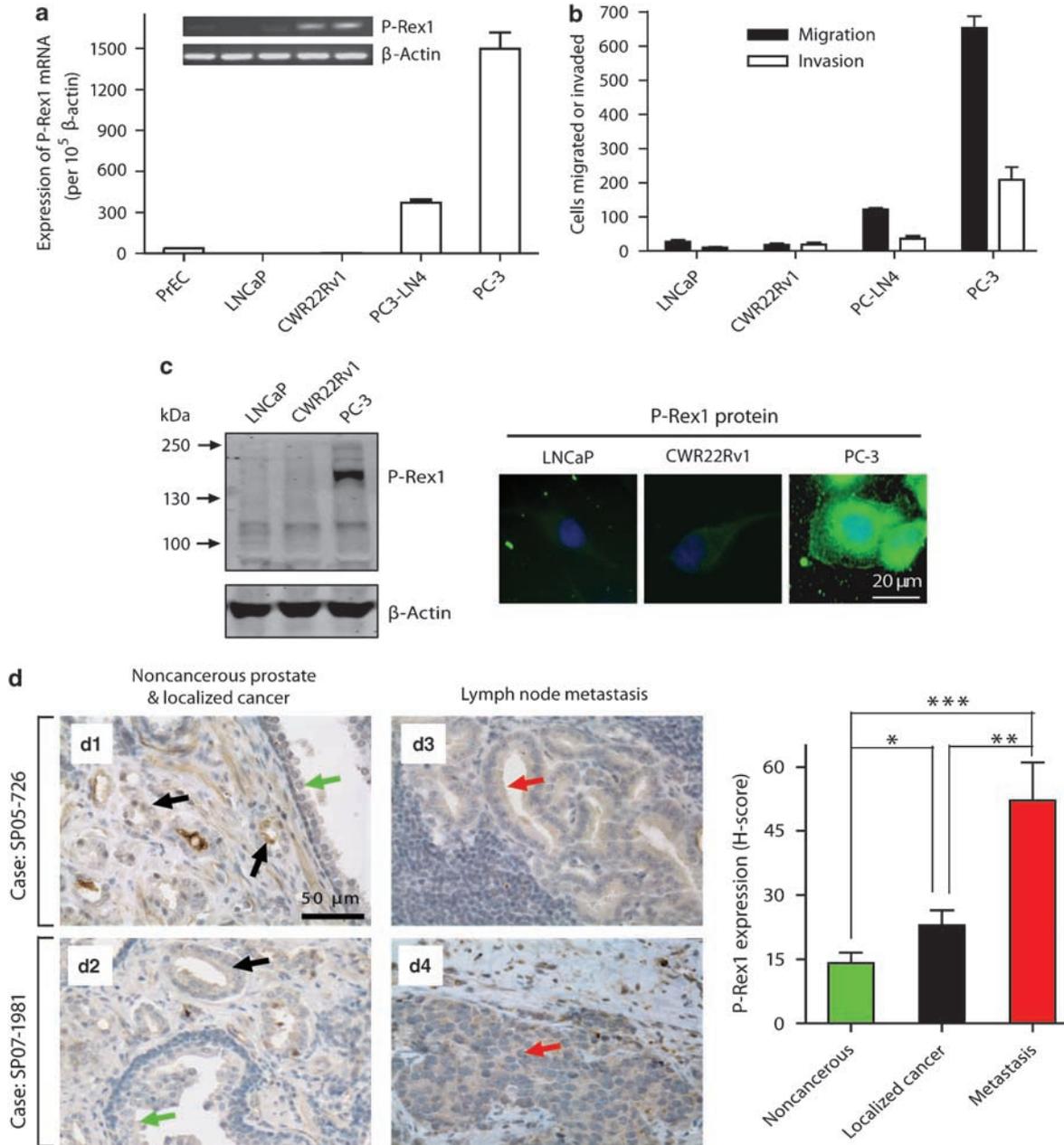
Figure 1 Upregulation of P-Rex1 in human metastatic prostate cancer cell lines and in human prostate cancer specimens. (**a**) Analysis of P-Rex1 mRNA expression in human prostate cell lines by quantitative real-time reverse transcriptase PCR (RT–PCR). Bars represent the mean ± s.e. of P-Rex1 mRNA levels ($n = 5$); inset: conventional RT–PCR for P-Rex1 and β-actin. (**b**) Transwell migration and invasion assays of prostate cancer cells toward NIH3T3 conditioned medium (CM). Results are represented as mean ± s.e. per 10 000 loaded cells ($n = 3$). (**c**) Left: western blot analysis of P-Rex1 protein using the anti-P-Rex1 antibody 6F12; right: representative immunofluorescence staining of prostate cancer cells for P-Rex1 protein with the anti-P-Rex1 antibody, 4A3, followed by AlexaF488 (green)-linked secondary antibody. 4′,6-Diamidino-2-phenylindole (DAPI) staining (blue) indicates the nuclei. (**d**) Immunohisto-chemical staining of human specimens for P-Rex1 protein using the antibody 4A3 (indicated in brown color). The left panel shows representative results from primary tumors in two cases (d1 and d2) as well as their paired prostate tumor metastases in lymph nodes (d3 and d4, respectively). Green and black arrows point to non-cancerous prostate epithelial cells and localized prostate cancer, respectively, whereas red arrows indicate prostate cancer metastasis to lymph nodes. The average *H*-score was used to grade P-Rex1 expression levels. The right panel shows the mean ± s.e of eight cases. *$P = 0.016$, **$P = 0.0055$, ***$P = 0.0004$.

siRNA (Figure 2b). In addition, silencing P-Rex1 caused a large decrease in the level of activated GTP-bound Rac1 with minimal effect on activated GTP-bound RhoA (Figure 2c, upper panel), confirming that endogenous P-Rex1 functions as an activator of Rac1, but not RhoA in PC-3 cells. These results were further

supported by the data shown in Figure 2c, lower panel, where silencing P-Rex1 significantly attenuated active Rac-dependent lamellipodia formation in PC-3 cells. To understand the relationship between the decreased Rac activity and reduced cell migration ability, we verified that NSC23766 (NSC), which selectively blocks Rac
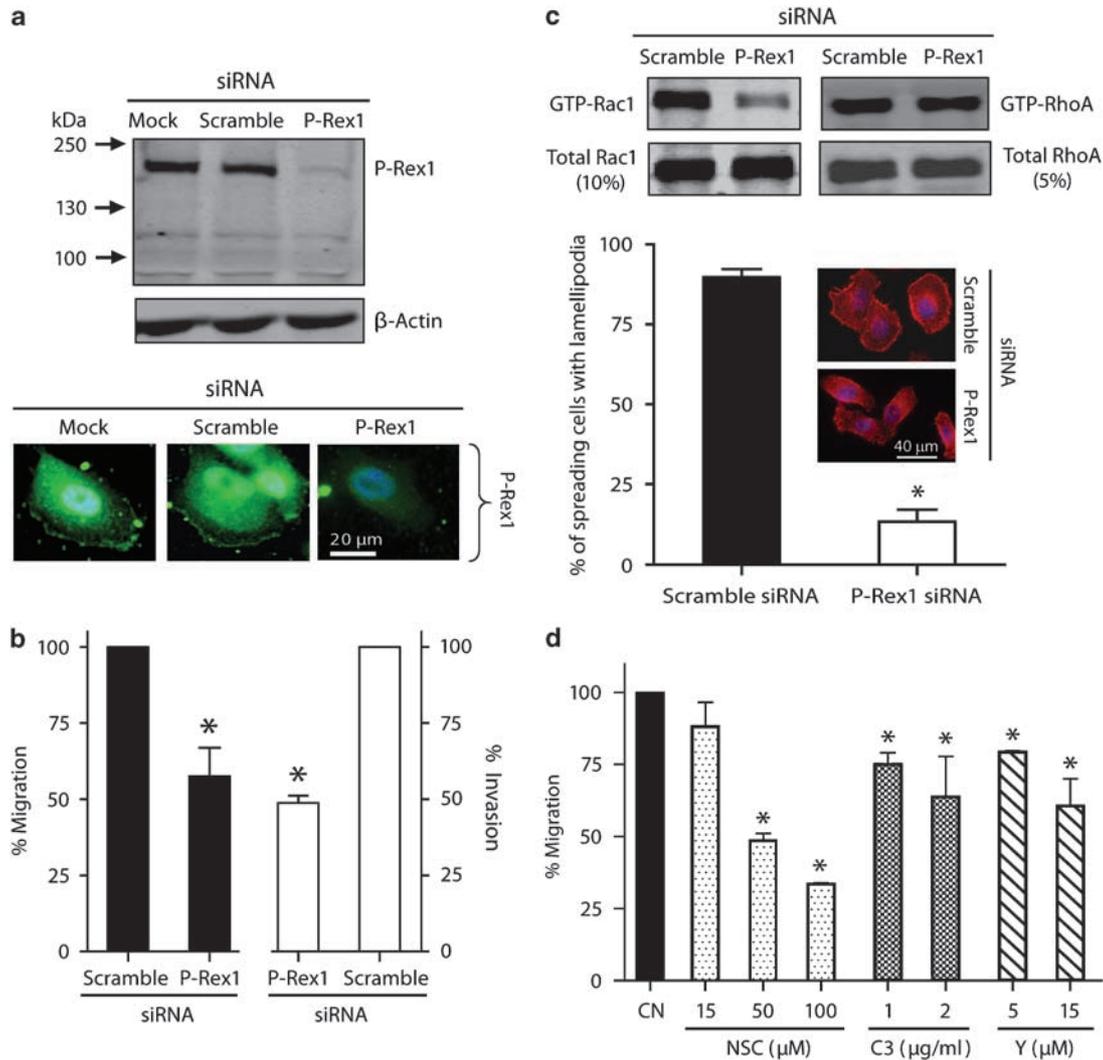
Figure 2 Silencing endogenous P-Rex1 suppressed prostate cancer PC-3-cell migration and invasion by reducing Rac activation. (**a**) Silencing P-Rex1 by siRNA as shown by western blot analysis (upper panel) and immunofluorescence staining (lower panel) of P-Rex1 protein in PC-3 cells. A mock transfection and scramble siRNA were used as controls. The western blot image is representative of three separate experiments. (**b**) Silencing P-Rex1 reduced PC-3-cell migration and invasion in response to NIH3T3 conditioned medium (CM). Bars show mean ± s.e. with *$P<0.05$ compared with control ($n=3$). (**c**) Silencing P-Rex1 in PC-3 cells reduced Rac1 activity (upper left panel) without affecting RhoA activity (upper right panel). Immunofluorescence staining of F-actin (lower panel) shows a corresponding loss of lamellipodia formation in P-Rex1-silenced PC-3 cells. Graphed data are mean ± s.e. ($n=3$). At least 50 cells were counted in each experiment with *$P<0.05$ when compared with scramble siRNA; inset images are representative cells. (**d**) Migration of PC-3 cells without (CN) or with indicated concentrations of Rac activation inhibitor NSC, Rho inhibitor C3 transferase (C3) or Rho-kinase inhibitor Y27632 (Y). Bars show the mean ± s.e. ($n=3$) with *$P<0.05$.

activation by Tiam1 (Gao et al., 2004), also blocked the P-Rex1/Rac1 interaction (see Supplementary information). NSC reduced PC-3-cell migration in a dose-dependent manner, causing about a 70% inhibition at 100 μm (Figure 2d). In contrast, Rho inhibition with 2 μg/ml of cell permeable C3 transferase (C3), a Rho inhibitor (recommended concentration is 0.5–1 μg/ml) or selective inhibition of downstream effector Rho kinase with 15 μM Y27632 (Y, $K_i = 140$ nM) only caused a 35% reduction. Altogether, our data show that silencing endogenous P-Rex1 impairs Rac activation, resulting in decreased lamellipodia formation and an inhibition of metastatic prostate cancer cell migration and invasion.

Endogenous P-Rex1 contributes to directional prostate cancer cell migration in response to both GPCR and RTK ligands

As NIH3T3 CM contains a range of known and unknown factors capable of acting on both GPCRs and RTKs, we next examined the effects of silencing endogenous P-Rex1 on GPCR- or RTK-dependent prostate cancer cell migration. CXCL12 is a selective agonist for the G-protein-coupled CXC chemokine receptor 4 (CXCR4). Epidermal growth factor (EGF) activates RTK pathways. Both CXCR4 and EGF receptor have been linked to prostate cancer metastasis (Ching et al., 1993; Arya et al., 2004). In the absence of CXCL12 or EGF, PC-3 cells displayed less spreading
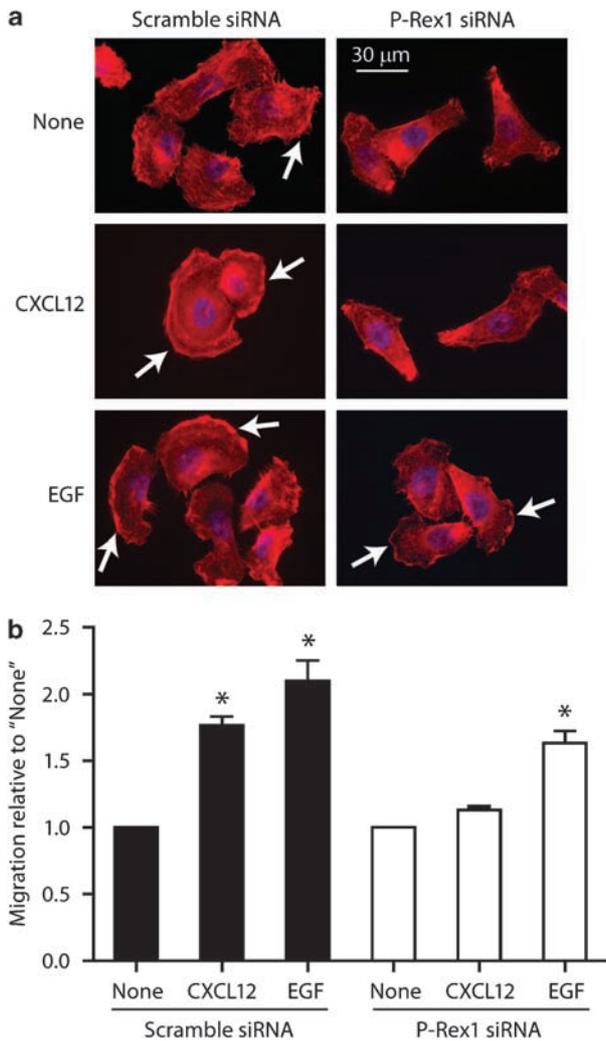
and less lamellipodia formation when transfected with P-Rex1 siRNA (Figure 3a). Silencing endogenous P-Rex1 caused a noticeable decrease in lamellipodia formation in response to CXCL12 (200 ng/ml). When PC-3 cells were treated with EGF (50 ng/ml), they were spread out with lamellipodia formation irrespective of whether they were transfected with scramble siRNA or P-Rex1 siRNA, with a tendency for lamellipodia formation to be decreased after P-Rex1 siRNA transfection. Figure 3b shows complementary data from

Figure 3 Endogenous P-Rex1 contributes to directional prostate cancer cell migration in response to both G-protein-coupled receptor (GPCR) and receptor tyrosine kinase (RTK) ligands. (**a**) PC3 cells, transiently transfected with scramble or P-Rex1 siRNA, were plated on coverslips. The cells were starved in RPMI 1640 medium for 14 h, stimulated without (none) or with CXCL12 (200 ng/ml) or epidermal growth factor (EGF) (50 ng/ml) for 30 min. Cells were stained for F-actin (red) and with 4',6-diamidino-2-phenylindole (DAPI) to visualize the nuclei (blue). (**b**) Silencing P-Rex1 reduced PC-3-cell migration in response to CXCL12 (200 ng/ml) or EGF (50 ng/ml). Bars show mean ± s.e. with *$P < 0.05$ compared with respective control ($n = 4$). The basal migratory abilities (none) of cells transfected with scramble and P-Rex1 siRNA were 272 ± 28 and 217 ± 25 per 10 000 cells loaded in the Transwell chamber, respectively; arrows point to lamellipodia.

Transwell migration assays (Lowell, MA, USA). When cells were transfected with scramble siRNA (Figure 3b), CXCL12 and EGF increased cell migration by about 1.7-fold and 2.1-fold, respectively, from a basal level of 272 ± 28 migrated cells per 10 000 cells loaded in the Transwell chamber. However, after transfecting cells with P-Rex1 siRNA, CXCL12 had little stimulatory effect 1.1-fold, whereas EGF still increased cell migration about 1.7-fold from a basal level of 217 ± 25 migrated cells per 10 000 cells loaded. Thus, transient transfection with P-Rex1 siRNA, which was capable of decreasing P-Rex1 protein levels about 80% (see Figure 2a) also caused about an 80% reduction in the relative ability of the PC-3 cells to migrate in response to CXCL12, but only reduced EGF-induced cell migration by 40%.
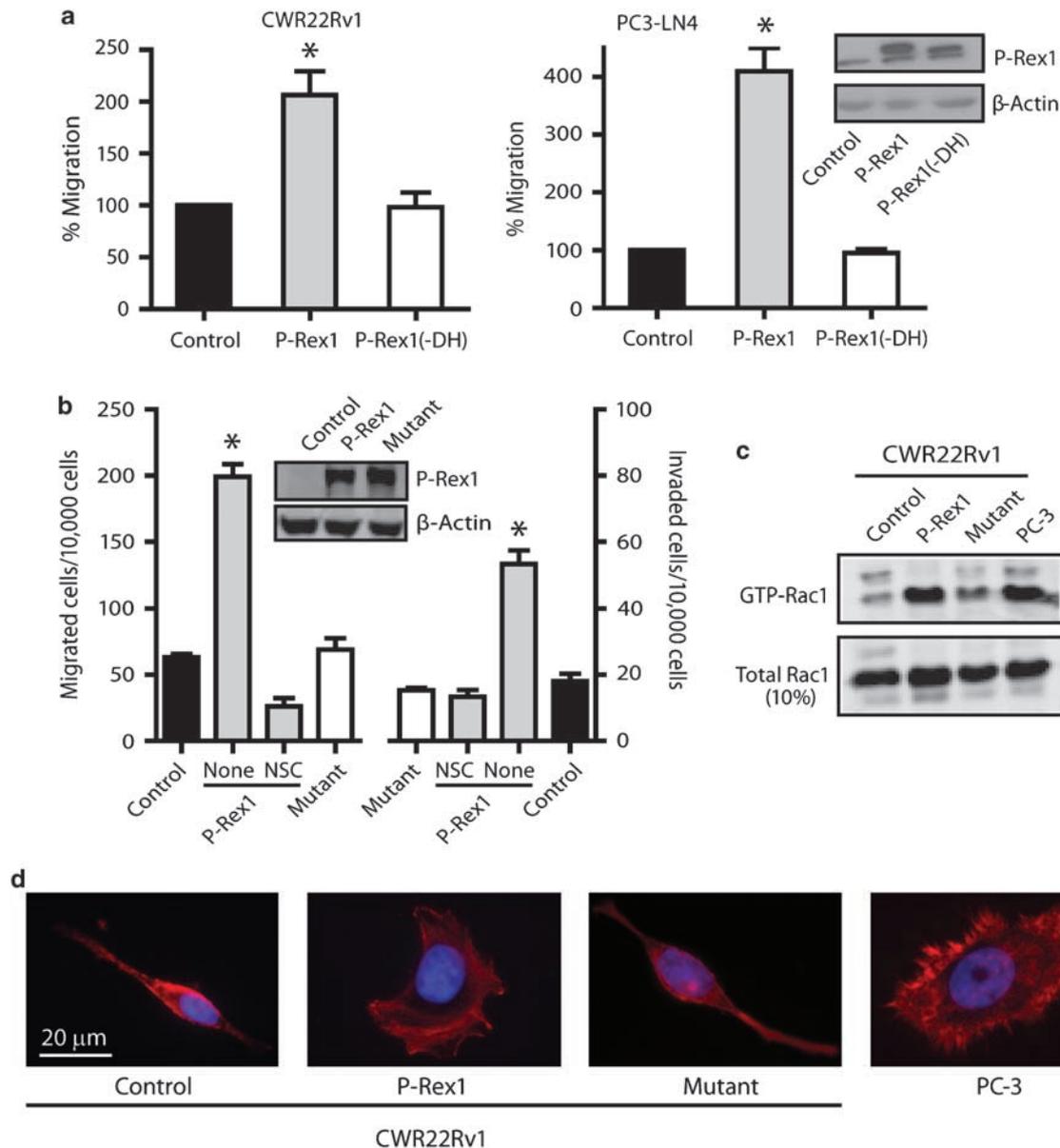
Exogenous P-Rex1 promotes prostate cancer cell migration and invasion through Rac activation
We next used non-metastatic prostate cancer cells, such as CWR22Rv1, as an *in vitro* model to investigate the effect of exogenously expressed P-Rex1 on prostate cancer metastasis. As shown in Figure 4a (left panel), with about 50% transfection efficiency, expression of P-Rex1 increased CWR22Rv1-cell migration by about twofold. Deletion of the DH domain of P-Rex1 completely abolished its ability to enhance the CWR22Rv1 cell migration. A similar dependence on the DH domain was seen in PC3-LN4 cells when green fluorescent protein (GFP)-tagged P-Rex1 or its P-Rex1 (−DH) mutant was transiently expressed at similar levels (Figure 4a, right panel). Thus, increasing P-Rex1 expression in prostate cancer cells increases their RacGEF-dependent migration ability.

Furthermore, we generated stable CWR22Rv1 cells expressing either wild-type P-Rex1 or a P-Rex1(E56A/N238A) 'GEF-dead' mutant (Hill *et al.*, 2005). Although P-Rex1 mutant expression was similar to wild-type P-Rex1 (Figure 4b, inset), cells expressing wild-type P-Rex1, but not its mutant, showed a threefold increase in cell migration and invasion (Figure 4b) when compared with control cells transfected with vector. Rac activation inhibitor, NSC, (50 μM) abolished the increases in migration and invasion observed in cells stably expressing wild-type P-Rex1 (Figure 4b), suggesting that stably expressed P-Rex1 in CWR22Rv1 cells functioned in a Rac-dependent manner similar to that seen in PC-3 cells. Indeed, CWR22Rv1 cells expressing wild-type P-Rex1 had significantly higher Rac activity, similar to PC-3 cells, when compared with CWR22Rv1 control cells or cells expressing the P-Rex1 mutant (Figure 4c). In addition, cell spreading was seen in PC-3 and CWR22Rv1 cells expressing wild-type P-Rex1 in association with active Rac-dependent lamellipodia (Figure 4d). In contrast, CWR22Rv1 control cells or cells expressing the P-Rex1 mutant had fusiform shapes and lacked lamellipodia. These findings are consistent with the notion that P-Rex1 stimulates Rac activity to induce the lamellipodia formation needed for effective migration and invasion of prostate cancer cells.

Figure 4 P-Rex1 promotes prostate cancer cell migration and invasion through Rac activation. (**a**) CWR22Rv1 cells (left panel) or PC3-LN4 cells (right panel) were transfected with control green fluorescent protein (GFP), GFP-tagged wild-type P-Rex1 or P-Rex1(−DH) mutant for 48 h, and then subjected to Transwell migration assay for 12 h (CWR22Rv1 cells) or 5 h (PC3-LN4) toward NIH3T3 conditioned medium (CM). Transfected PC3-LN4 cells were selected by flow cytometry cell sorting based on GFP fluorescence before migration assays due to low transfection efficiency (∼25%). Bars show mean ± s.e. with *$P < 0.05$ compared with control set at 100% ($n = 3$); inset: expression of GFP-tagged P-Rex1 and P-Rex1(−DH) protein detected by western blot analysis using anti-GFP antibody. (**b**) CWR22Rv1 cells stably expressing wild-type P-Rex1, P-Rex1 'GEF-dead' mutant or control vector were subjected to migration and invasion assays for 12 h. Cells stably expressing wild-type P-Rex1 were pretreated without (none) or with 50 μM NSC for 15 min. Bars show mean ± s.e. with *$P < 0.01$ compared with control ($n = 3$); inset: western blot analysis of expression levels of P-Rex1 and its mutant. (**c**) P-Rex1 activates Rac1 in prostate cancer cells. The amount of activated GTP-bound Rac1 was measured by an Rac activation assay. The image shown represents three separate experiments. (**d**) P-Rex1 increases lamellipodia formation. Serum-starved CWR22Rv1 cells or PC-3 cells (positive control) were incubated with NIH3T3 CM for 5 h and then stained with rhodamine-conjugated phalloidin (red) for detection of F-actin.

P-Rex1 does not affect prostate tumor growth but induces spontaneous lymph node metastasis in mouse models

Metastatic PC3-LN4 cells, CWR22Rv1 control cells and cells stably expressing wild-type P-Rex1 or its 'GEF-dead' mutant were subcutaneously injected into nude mice (six mice per group), and tumor volumes were measured once per week. There was no statistical difference in the subcutaneous tumor growth rate among these groups (Figure 5a), and tumor incidence was 100% in all four groups, suggesting that P-Rex1 does not have a direct impact on prostate tumor growth.

We next used an orthotopic model (Kovar *et al.*, 2006) to determine the role of P-Rex1 in the spontaneous metastasis of prostate cancer to lymph nodes. The
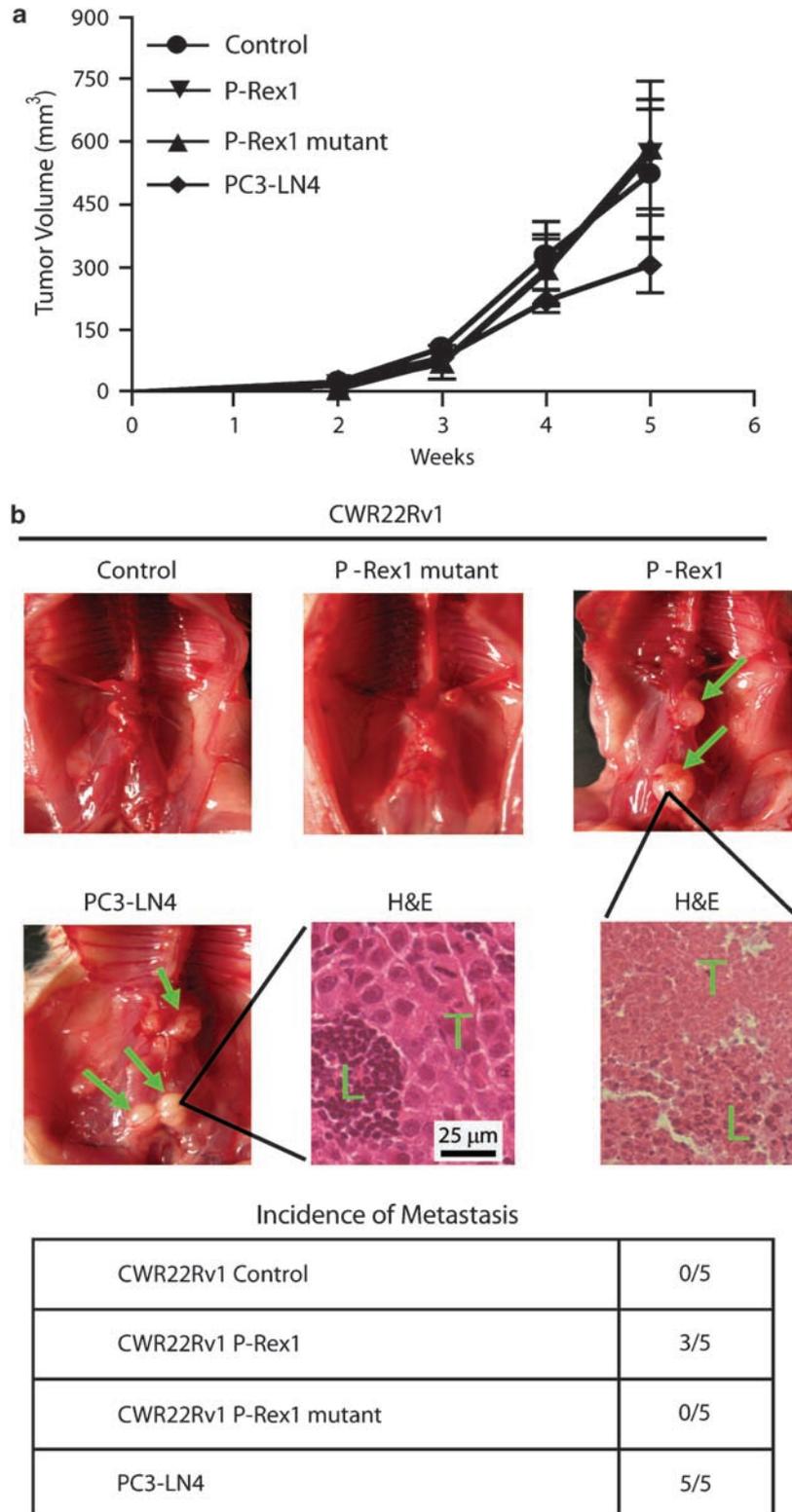
Figure 5 *In vivo* prostate tumor growth and spontaneous metastasis to mouse lymph nodes. (**a**) 1×10^6 PC3-LN4 cells or CWR22Rv1 cells stably expressing either wild-type P-Rex1, P-Rex1 'GEF-dead' mutant or vector were injected subcutaneously into Nu/Nu mice (six mice per group). Tumor growth was monitored once per week for 4 weeks. One-way ANOVA indicates no statistical differences in tumor growth among the four groups. (**b**) Intraprostatic injection of CWR22Rv1 or PC3-LN4 cells into male NOD/SCID mice (50 000 cells in 15 μl culture medium per mouse). At 9-week postinjection, mice were killed to examine spontaneous metastases (green arrow) of prostate tumor into lymph nodes. Hematoxylin and eosin (H&E) staining of mouse lymph nodes from the PC3-LN4 and the CWR22Rv1-expressing P-Rex1 groups show infiltration of tumor cells (T) into the lymph node tissue (L). The incidence of spontaneous metastasis in each group is shown in the table.

CWR22Rv1 stable transfectants were implanted into an anterior prostate lobe of immunodeficient male NOD/SCID mice. The metastatic PC3-LN4 cell line was used as a positive control. All mice bore large primary prostate tumors of similar size (data not shown). All five mice in the PC3-LN4 group had macroscopic lymph node metastases (green arrows, Figure 5b). There was no visible metastasis detected in any mice bearing tumors derived from CWR22Rv1 control cells or cells expressing the P-Rex1 mutant. In contrast, expression of wild-type P-Rex1 in CWR22Rv1 cells gave rise to lymph node metastases (three out of five mice). Hematoxylin and eosin staining of the mouse lymph nodes showed widespread infiltration of tumor cells (T) into the lymph node tissue (L) for both the PC3-LN4 group with the larger tumor cells (bottom middle panel) and the CWR22Rv1/P-Rex1 group with smaller tumor cells (bottom, right panel). These results show that P-Rex1 promotes spontaneous prostate cancer metastasis *in vivo*.

Discussion

Current evidence suggests that Rac activity is crucial for migration and invasion of cancer cells, and aberrant Rac signaling is often seen in cancer metastasis (Sun et al., 2006). As Rac mutations are rarely detected in cancer, research is focused on the factors that govern Rac activation. RTKs and GPCRs are among the plasma membrane proteins capable of initiating responses that lead to Rac activation through RacGEFs. Here, we report that P-Rex1, a RacGEF that functions downstream of both GPCR and RTK signaling, is markedly upregulated in metastatic prostate cancer and may function as one of the important molecules in metastatic signaling pathways in prostate cancer cells.

P-Rex1 is a relatively abundant protein normally expressed in cells of a hematopoietic or neuronal lineage (Welch et al., 2002). We found that endogenous P-Rex1 expression levels are very low in normal prostate epithelial cells and non-metastatic prostate cancer cells, but remarkably elevated in the metastatic prostate cancer cells. In addition, our studies, using human prostate cancer specimens, showed a progressive increase of P-Rex1 protein in cells from non-cancerous prostate tissues, localized prostate carcinoma and prostate cancer that has metastasized to a lymph node. Using a mouse xenograft model, we showed that P-Rex1 expression induced lymph node metastasis of non-metastatic prostate cancer cells, such as CWR22Rv1, without an effect on primary tumor development and growth. These results suggest that upregulated P-Rex1 may contribute to prostate cancer metastasis.

P-Rex1 is characterized by a DH domain, possessing its catalytic activity in tandem with a pleckstrin homology domain, two adjacent DEP (disheveled, EGL-10 and pleckstrin homology) domains, two adjacent PDZ (postsynaptic density disc-large zo-1) domains and a tail with polyphosphate 4-phosphatase

homology (Welch et al., 2002). The pleckstrin homology domain of RacGEFs binds to PIP3 in the cell membrane, which can either directly activate the RacGEF or anchor the molecule to facilitate its activation by other proteins. P-Rex1 is among the few RacGEFs also known to be selectively activated by Gβγ subunits (Welch et al., 2002; Rosenfeldt et al., 2004; Hill et al., 2005). Moreover, the binding of cytosolic P-Rex1 to both Gβγ subunits and PIP3 leads to a synergistic activation of this RacGEF (Welch et al., 2002; Barber et al., 2007). Interestingly, P-Rex1 has recently been shown to interact with the cellular nutrient sensor mTOR through a DEP domain, and functions as an effector-mediating Rac activation through the rapamycin-insensitive mTORC2 pathway (Hernandez-Negrete et al., 2007). Thus, P-Rex1 appears to be positioned as a coincidence detector, something that could be crucial for a cancer cell navigating through a range of chemotactic signal gradients reaching it from different directions.

Indeed, our data indicate that endogenous P-Rex1 can regulate metastatic prostate cancer cell migration and invasion. For instance, silencing endogenous P-Rex1 in metastatic PC-3 cells reduced their migration and invasion abilities toward chemoattractants in NIH3T3 CM by approximately 50%. As expected, silencing endogenous P-Rex1 also largely decreased Rac1 activity without a significant effect on RhoA activity. The loss of Rac1 activity is important for the reduction of the PC-3-cell migration as NSC, which blocks the activation of Rac1 by P-Rex1, also significantly reduced PC-3-cell migration. Those results indicate a significant contribution of endogenous P-Rex1 to the maintenance of Rac activity that is important for PC-3 cells to migrate in response to chemoattractants in NIH3T3 CM. Furthermore, silencing P-Rex1 attenuated the cell migratory response to CXCL12, an agonist of G-protein-coupled CXCR4, by over 80%, whereas RTK agonist EGF-stimulated cell migration was only reduced about 40%, suggesting that P-Rex1 is especially important for chemotactic factors that stimulate GPCRs. The relative insensitivity of EGF-stimulated responses to P-Rex1 silencing may be due to the activation of a greater range of RacGEFs following the stimulation of RTK pathways.

A wide range of evidence has appeared over the past several years supporting a role for activation of G-proteins by GPCRs during cancer growth and metastasis (Daaka, 2004; Dorsam and Gutkind, 2007). The Gα subunit of the G12 subfamily of G-proteins is directly linked to intracellular pathways that promote prostate cancer invasion (Kelly et al., 2006). Pertussis toxin, which selectively inactivates G_i-proteins, also suppresses prostate cancer metastasis in a mouse model (Bex et al., 1999), showing the importance of G_i-proteins. Several prostate cancer metastasis-associated GPCRs, including CXCR4 (Arya et al., 2004) and CC chemokine receptor 2 (Lu et al., 2007), are coupled to G_i-proteins (Moepps et al., 1997; Jimenez-Sainz et al., 2003). $G\alpha_i$ is not required for neutrophil chemotaxis mediated by G_i-coupled receptors (Neptune et al., 1999). Instead, disruption of Gβγ signaling inhibits this

neutrophil chemotaxis (Lehmann et al., 2008). Inhibition of Gβγ-mediated signaling has been shown to inhibit prostate cancer cell growth in vitro and prostate tumor formation in vivo (Bookout et al., 2003). In addition, we found (JQ, unpublished observations) that the migration and invasion of P-Rex1-expressing metastatic prostate cancer cells are significantly attenuated by pertussis toxin and M119, a Gβγ inhibitor (Bonacci et al., 2006). Therefore, it appears that chemotactic factors that stimulate GPCRs promote prostate cancer metastasis through a Gβγ-dependent P-Rex1/Rac activation pathway. Further investigation is in progress.

Characteristically, the DH domain accounts for the GEF activity leading to Rac activation (Hoffman and Cerione, 2002). We found that transient expression of recombinant P-Rex1, but not a DH-deleted P-Rex1 mutant, increases cell migration in both CWR22Rv1 cells and PC3-LN4 cells normally having undetectable and mid-range levels of endogenous P-Rex1, respectively. These results prompted us to establish stable CWR22Rv1 cells expressing P-Rex1 or its 'GEF-dead' mutant. Compared with control cells, cells stably expressing P-Rex1 have threefold higher migration and invasion abilities that could be largely abolished by NSC, the inhibitor of Rac activation. The failure of the P-Rex1 'GEF-dead' mutant to activate Rac caused a corresponding loss of its ability to induce lamellipodia formation in prostate cancer cells. Consequently, the P-Rex1 'GEF-dead' mutant failed to stimulate prostate cancer cell migration and invasion in vitro and spontaneous metastasis in an orthotopic xenograft model. Altogether, our studies showed the importance of the classical action of P-Rex1 as an RacGEF in prostate cancer metastasis.

Other chemotactic factors acting through RTKs can increase PIP3 production through the PI3K signaling pathway (Cantley, 2002), and contribute to prostate cancer metastasis (Mimeault and Batra, 2006). In fact, the silencing of P-Rex1 reduced EGF-stimulated migration by 40%, suggesting the importance of PIP3-dependent P-Rex1 activation in RTK-promoted prostate cancer metastasis. Furthermore, mTOR, the nutrient sensor in cells (Fingar and Blenis, 2004), also uses P-Rex1 to stimulate cell migration (Hernandez-Negrete et al., 2007). Thus, by functioning as a molecule that integrates the simultaneous, but separate inputs from GPCRs, RTKs and mTOR-nutrient sensing in its local microenvironment, the synergistic activation of P-Rex1 could function as a coincidence detector that helps regulate the direction of prostate cancer cell movement. Unlike Rac1, P-Rex1 has limited tissue expression and its knockout is not embryonically lethal (Welch et al., 2005). P-Rex1 contributes to the regulation of neutrophil function, but is not essential for either chemotaxis or degranulation (Welch et al., 2005). Thus, P-Rex1 could be an attractive drug target, as blockade of its expression or actions may impair metastatic prostate cancer cell navigation with less destructive side effects than traditional chemotherapy, thereby making the cancer a better target for surgery, radiation and immunotherapy.

Materials and methods

Cells and reagents
PC-3, LNCaP and CWR22Rv1 cell lines (ATCC) were grown in RPMI-1640 medium with 10% fetal bovine serum. The PC3-LN4 cell line (Melanie Simpson, University of Nebraska at Lincoln) was cultured in Dulbecco's modified eagle medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum, non-essential amino acids and 1.0 mM sodium pyruvate. Human prostate epithelial cell line PrEC (Zafar Nawaz, University of Miami, Miami, FL, USA) was cultured in PrEGM (Cambrex, Charles City, IA, USA).

Additional reagents were NSC23766, Y27632 and anti-GFP antibody (EMD Biosciences, Gibbstown, NJ, USA); cell permeable C3 transferase and F-actin visualization kit (Cytoskeleton, Denver, CO, USA); Rac activation assay kit, Rho assay reagent, anti-Rac1 antibody (clone 23A8), and Immobilon-FL (Millipore, Billerica, MA, USA); mouse anti-RhoA (26C4) and rabbit anti-HA and anti-β-actin antibodies (Santa Cruz, Santa Cruz, CA, USA); fetal bovine serum (Hyclone, Logan, UT, USA); Lipofectamine 2000, AlexaF488-labeled secondary antibody, RPMI-1640 and Dulbecco's modified eagle medium (Invitrogen); EGF, Matrigel and fibronectin (BD Biosciences, Bedford, MA, USA); CXCL12 (R&D Systems, Minneapolis, MN, USA); and IRDye-labeled secondary antibodies (LI-COR Biosciences). Descriptions of rat monoclonal anti-P-Rex1 antibody 4A3, untagged and GFP-tagged wild-type P-Rex1 and DH-domain deleted mutant have been published (Yoshizawa et al., 2005). P-Rex1 monoclonal antibody 6F12 was a gift from Marcus Thelen, Institute for Research in Biomedicine, Bellinzona, Switzerland. P-Rex1 'GEF-dead' mutant was generated as described previously (Hill et al., 2005).

Cell invasion and migration assays
Matrigel invasion assays were performed at 37 °C using 24-well Transwell inserts (Corning) coated with 30 μg of Matrigel. Cells (50 000) suspended in 200 μl of serum-free medium were seeded into the upper chamber with 600 μl of medium containing chemoattractants in the lower chamber. Cells that invaded through the membrane were stained, counted, and normalized relative to 10 000 seeded cells. Transwell cell migration assays were performed similarly, but without Matrigel. NIH3T3 CM (Albini and Benelli, 2007) was the chemoattractant in routine experiments. To study the effect of silencing endogenous P-Rex1 on the EGF receptor- or CXCR4-dependent cell migration, the insert membrane was pre-coated with fibronectin (2 μg/ml) and EGF or CXCL12 was used as the chemoattractant.

Conventional RT–PCR and quantitative real-time RT–PCR
Conventional and real-time RT–PCR analyses were done as described previously (Cao et al., 2006). P-Rex1 primers resulting in a 163-bp product are, forward 5′-CCTTCTTCCT CTTCGACAAC-3′ and reverse 5′-CCATCTTCCACATTCT CCAC-3′. Conventional PCR products were separated by 4% agarose gel electrophoresis and confirmed by DNA sequencing.

Fluorescence microscopy
P-Rex1 was visualized in methanol-fixed prostate cancer cells with antibody 4A3 and AlexaF488-conjugated secondary antibody. F-actin was stained with rhodamine-labeled phalloidin. Nikon Ti-80 microscope images were captured by a CoolSNAP-CF camera and processed with Image-Pro Plus

software (v6.1). Lamellipodia were identified as smooth convex stretches of perpendicular actin stain along cell edges. Images are representative of at least 50 cells.

Immunohistochemistry analysis of human prostate tissues
Formalin-fixed, paraffin-embedded blocks of prostate cancer were archived with informed consent for research purposes by the Creighton University Department of Pathology, and this use was approved by our Institutional Review Board. Immunohistochemistry was performed using standard techniques. Tissue sections were stained with the 4A3 anti-P-Rex1 antibody, counterstained with hematoxylin, and analysed by the Automated Cellular Imaging System (ChromaVision Medical Systems) as described previously (Gao *et al.*, 2005). Negative controls were performed without the primary antibody. The *H*-score was calculated from 10 randomly chosen $200 \times$ fields by multiplying the percentage (P) of cells staining positive for P-Rex1 with the average intensity (I), and averaged for each sample. The maximum *H*-score of 255 would be obtained if all the cells ($P = 1.0$) in the lesion stained with maximal intensity ($I = 255$).

RNA interference and stable cell lines
PC-3 cells in suspension were transfected with 50 nM scramble siRNA (negative control #1 siRNA, Ambion) or synthesized P-Rex1 siRNA (target sequence 5′-GCAACGACTTCAA GCTGGTGGAGAA-3′) using Lipofectamine 2000. After 5 h of incubation, 90% of the transfection medium was replaced with fresh culture medium. The following day, adherent PC-3 cells were re-transfected with the same siRNA. Two days later, cells were harvested for western blot analysis of P-Rex1 protein expression or used in Transwell assays. Stable CWR22Rv1 cell lines expressing wild-type P-Rex1, P-Rex1 mutant or vector were established with standard methods. Cells were selected with G-418 (400 μg/ml) for three weeks. Positive clones were amplified and verified by western blotting for P-Rex1 expression.

Protein extraction, electrophoresis and western blot analysis
Protein was extracted from cells using $1 \times$ RIPA complete lysis buffer (Santa Cruz). Prestained protein standards and protein samples (40 μg) were subjected to SDS–PAGE before transfer to Immobilon-FL. Primary antibodies were used to identify the relevant protein and loading control (β-actin). IRDye-labeled secondary antibodies were used for band detection with an Odyssey infrared imaging system (LI-COR Biosciences).

Rac and Rho activation assays
Cell lysates were assayed according to the Rac Activation Assay Kit or Rhotekin RBD Rho Assay Reagent instructions. Lysates and activated-Rac1 or -RhoA were subjected to western blot analysis using anti-Rac1 or anti-RhoA antibody, respectively.

Subcutaneous and orthotopic injections of prostate cancer cells
These experiments were approved by the Creighton University Institutional Animal Care and Use Committee. A prostate cancer cell suspension (10^6 cells in 200 μl of 1:1 culture medium and Matrigel) was injected subcutaneously in the flank region of male athymic Nu/Nu mice (Charles River, Lee's Summit, MO, USA; six mice per group). Tumor growth was monitored once per week and tumor volume was estimated using the ellipsoid formula. Orthotopic injections of tumor cells were performed as described elsewhere (Kovar *et al.*, 2006). Briefly, the left anterior prostate of male NOD/SCID mice (Charles River) was exposed through a small suprapubic incision for injection of 50 000 CWR22Rv1 or PC3-LN4 cells in 15 μl of growth media. Mice were monitored for 9 weeks. At the end point, primary prostate tumors were removed and the carcasses were dissected to examine para-aortic lymph nodes for visible tumor metastases.

Statistical analysis
Results are means ± s.e. of at least three determinations and statistical comparisons used Student's *t*-test. A probability (*P*) value of < 0.05 was considered significant.

Conflict of interest

The authors declare no conflict of interest.

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