Background: The significance of Pak1 in prostate cancer remains unclear.

Results: Pak1 knockdown impaired prostate tumor growth via increased expression of TGFβ and reduced secretion of MMP9.

Conclusions: We demonstrated that Pak1 is a more potent mediator of prostate cancer cell migration and tumor growth than Pak6, the predominant isoform in the prostate.

Significance: A novel role of Pak1 in prostate cancer is identified.

SUMMARY

P21 activated kinases (Paks) are major effectors downstream of small Rho family of GTPases. Among the six isoforms, Pak1 is the most ubiquitous and the best characterized member. Previous studies have shown that inhibition of Pak6, which is predominantly present in the prostate compared to other tissues, inhibit prostate tumor growth in vivo. Even though Pak1 has been identified in normal prostatic epithelial cells and cancer cells, its specific role in the development of prostate cancer remains unclear. We report here that highly invasive prostate cancer cells express significantly higher levels of Pak1 protein compared to non-invasive prostate cancer cells. Furthermore, prostate tumor tissues and prostate cancer metastasized to lungs showed higher expression of Pak1 compared to normal tissues. Interestingly, Pak6 protein expression levels did not change with the invasive/metastatic potential of the cancer cells or tumors. While inhibition of Pak1, and not Pak6, resulted in impaired PC3 cell migration, the effects of Pak1 knockdown on transendothelial migration (microinvasion), tumor growth and tumor angiogenesis was higher compared to Pak6 knockdown. Finally, gene array data revealed reduced expression of MMP9 with the ablation of either Pak1 or Pak6 gene expression in PC3 cells, whereas protein levels of TGFβ was significantly elevated with specific modulation of Pak1 activity or ablation of Pak1 gene. Our observations suggest that although some level of functional redundancy exists between Pak1 and Pak6 in prostate cancer cells, targeting Pak1 is a potential option for the management of prostate tumor growth, microinvasion and metastasis.

INTRODUCTION

P21 activated kinases (Paks) are a family of six serine-threonine kinases which are categorized into Group-I and Group-II Paks based on their mechanism of activation (1). Group-I Paks differ from their Group-II counterparts on their activation by small Rho GTPases such as Rac and cdc42 (2) as well as their specific involvement in inducing cytoskeletal changes, lamellipodia and filopodia formation in mammalian cells in the promotion of cell motility (1, 3). Group-II Paks lack the auto inhibitory domain, acidic and Pix-binding regions as well as Rac/cdc42 binding CRIB domain, which are present in all Group-I Pak isoforms (4-6).
Among the Pak isoforms, Group-I Paks (Pak1 and Pak2 in particular) is the best-characterized and most deregulated in cancers (1, 3). We have previously shown that Pak1 is necessary for inducing cytoskeletal changes in normal cells (7) and in reconciling the effects of Akt and ERK pathways, two major pathways deregulated in multiple cancers, thus mediating oncogenic transformation (8). Although present in the prostate, Group-I Paks are the least studied in prostate cancer, probably because the prostate is known for its higher expression of Group II Paks, Pak4 and Pak6 (9-10), which are less expressed and less known for cancer incidences in other tissues. In support of this assumption, inhibition of either Pak4 or Pak6 has been reported to inhibit prostate cancer cell function in vitro and tumor growth in vivo (11-12). Our recent study has demonstrated that activation of Rac1 driven by the dimerization of protein 14-3-3ζ induces cytoskeletal changes in prostate cancer cells thus augmenting their motility and transendothelial migration (13), strongly suggesting a role for Group-I Paks in prostate cancer.

The current study was designed to establish the biological significance of the expression of predominant Group-I Pak isoform Pak1 in prostate cancer cells in comparison with Pak6, the predominant Group II counterpart, on tumor growth and transendothelial migration. In addition to the analysis of Pak1 and Pak6 protein expression in human prostate cancer patient samples and cell lines of various tumor and metastatic potential, we generated stable PC3 cells with ShRNA-mediated knockdown of Pak1, Pak6 or a combination of both using a Lentiviral approach. Cells were then transiently transfected with either control vector (pBabe-Puro) or plasmids expressing constitutively active Pak1 (CA-Pak1; Pak1 E235) or dominant negative Pak1 (DN-Pak1; Pak1 R299). Transfected PC3 cells were used for the analysis of prostate cancer cell functions in vitro such as migration, proliferation, colony formation and transendothelial migration (microinvasion) as well as tumor growth in vivo along with qRT-PCR arrays in order to identify the novel genes involved in the process. Our study demonstrated that Pak1 is necessary for prostate tumor growth and micrometastasis mainly via regulation of prostate cancer cell migration and proliferation through enhanced expression of various tumor promoting factors such as MMP9 and reduced expression of inhibitors of tumor growth such as TGFβ.

EXPERIMENTAL PROCEDURES

Reagents, cell lines and antibodies — Human PC3 cells (ATCC, Manassas, VA) were used and maintained in DMEM (HyClone, Thermo Scientific, Logan, UT) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO2 atmosphere at 37°C. Primary antibodies against Pak1 were purchased from Cell Signaling (Boston, MA). Anti-Pak6 antibody as well as primary antibodies against β-actin and laminin were purchased from Sigma, St. Louis, MO. Anti-HRP secondary antibodies were obtained from BioRad (Hercules, CA). Alexa Fluor secondary antibodies and Alexa Fluor 555-labeled phalloidin were purchased from Invitrogen (Carlsbad, CA). ShPak1 and ShPak6 lentiviral particles were purchased from Origene (Rockville, MD).

Transfections — Human LNCaP, PC3 and LNCaP C4-2 cells were transiently transfected with CA-Pak1 plasmid using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. LNCaP C4-2 cells were stable transfected with ShPak1 and ShPak6 using lentiviral particles (5x10⁹ pfu) and selected with puromycin (or in a combination of ShPak1 with ShPak6). Scrambled shRNA was used as a control.

Processing of human prostate cancer patient samples — Tissue blocks of biopsy samples for the normal human prostate, benign prostatic hyperplasia (BPH), prostate tumor and prostate cancer metastasized to lymph node and lung tissues were obtained from the Pathology department of the Charlie Norwood VA Medical Center in Augusta as per the approval of the Georgia Health Sciences University Institutional Review Board and VAMC Research & development department. Tissue samples were subjected for immunohistochemical analysis of Pak1 and Pak6 using DAB staining kit (Life Technologies, Carlsbad, CA) to unveil their intra-tissue and intra-cellular localization compared to H&E stained sections. Percentage of Pak1 or Pak6 positive area in prostate cancer and lung metastasis samples as determined by the NIH Image J software.

Migration assay — Migration assays were performed as previously described (14). Briefly, prostate cancer cells, after stable and transient transfections were grown on 12-well plates either with or without specific ECM proteins (1 μg/ml) to reach confluence (approximately 12 h) and then serum starved for 3 h.
A scratch was made in the monolayer and pictures were taken at 0, 12 and 24 h. The rate of migration (as measured by scratch recovery) was calculated using the following equation \((1-T_f/T_0) \times 100\), where \(T_f\) is the area at the endpoint and \(T_0\) is the area at the time 0. Depending upon the conditions in individual experiments, treatments were used as follows: 5, 10 and 15 \(\mu\)M IPA3 (Pak1 inhibitor) for 12 and 24 h; 0.1, 1.0 and 5.0 ng/ml of TGFβ.

**Transendothelial migration assay** — Trans-endothelial migration of prostate cancer (PC3) cells was measured using Electric Cell-substrate Impedance Sensing (ECIS) equipment (14) with human dermal micro-vascular endothelial cells (ATCC, Manassas, VA) plated on 8W10E+ array chips (Applied Biophysics, Troy, NY). Following this, control PC3 as well as transfected cells were directly added onto endothelial cell monolayer at a density of 5x10^4 cells per well in 50 \(\mu\)l medium. Cells were detached from plate by using cell dissociation buffer (20mM EDTA in PBS, pH 7.4) to avoid integrin/receptor loss due to trypsin digestion. Real-time measurements on the trans-endothelial migration of PC3 cells were recorded by the ECIS up to 12 h.

**Immunocytochemistry** — The immuno-fluorescence staining of the cells was performed as described previously (7). Briefly, PC3 cells, after stable transfections or treatment with IPA3 inhibitor, were plated on cell culture chamber slides (Fisher scientific, Pittsburgh, PA) followed by fixation with 1% paraformaldehyde in PBS. Cells were permeabilized with 0.1% triton X-100 in PBS. The non-specific staining was blocked with 2% BSA for 1 h at room temperature. The fixed and permeabilized cells were incubated with standardized dilution Alexa Fluor 555-labeled phalloidin applied for 30 min in RT and washed. The slides were mounted with Vectashield (Vector Laboratories) and the images were taken by Zeiss fluorescent microscope (Zeiss Axiovert100M, Carl Zeiss, Germany).

**Cell proliferation assay** — Proliferation of PC3 cell was determined using the nonradioactive BrDU-based cell proliferation assay (Roche, Basel, Switzerland) according to the manufacturer's protocol. Briefly, PC3 cells, after stable and transient transfections were seeded in 96-well plates at a density of 5x10^3 cells per well. After 12 h, the cells were subject to a 5-bromo-2-deoxyuridine assay using the BrDU Labeling and Detection Kit III (RocheApplied Science), according to the manufacturer's protocol. BrDU incorporation into the DNA was determined by measuring the absorbance at both 450 and 690 nm on ELISA plate reader. The data are presented as mean ± SD (n=3).

**Colony formation assay** — Colony formation assay was performed using standardized protocol in the lab (15). In this approach, PC3 cells, after stable and transient transfections were cultured on 12-well. 5 days after trasfection, each of the wells was counted for the number of colonies and compared to control. Plates were fixed using crystal violet and counted visually or using Image J software. The data are presented as mean ± SD (n=3).

**In vivo nude mouse tumor xenograft model** — All animal procedures listed in this article were performed as per the protocol approved by the Institutional Animal Care and Use Committee at the Charlie Norwood Veterans Affairs Medical Center, Augusta, GA (protocol 09-07-011, dated July 10, 2009). PC3 cells after stable transfection with either ShPak1 or ShPak6 were grown to confluence in 250-ml flasks. Cells were re-suspended in saline to a concentration of 1.7x10^7/ml. Cell suspension (100 \(\mu\)l) was injected subcutaneously in 8-week-old nude mice (athymic nude mice; Harlan, Indianapolis, IN) (16). The respective controls were injected intraperitoneally with 0.9% saline. Mice were sacrificed on day 20, and tumors were dissected, weighed, and snap frozen using dry ice for further processing to use on western blot analysis or qRT-PCR.

**Immunohistochemistry** — Immuno-fluorescence staining of the tumor sections for Ki67 (proliferation) and laminin (blood vessels) was performed according to the manufacturer's protocol (Sigma, St. Louis, MO). Briefly, formalin fixed, frozen prostate (PC3) xenograft tumor sections from nude mouse were subjected to the standard xylene-ethanol dehydration process and permeabilized with 0.3% triton x-100 in 1 X PBS The non-specific staining was blocked with 5 % goat serum for 1 h at room temperature. The dehydrated, permeabilized and blocked tissue sections were incubated with primary antibody against Ki67 antigen (dilution 1:1000) or Laminin (dilution 1:1000) overnight at 4°C followed washing with 1 X PBS (4 X for 15 min each). Next, anti-mouse Alexa Fluor 488-labeled secondary antibodies (1:500) was applied for 1 h at room temperature and washed 4 times for 15 min with 1 X PBS. The slides were mounted with Vectashield (Vector Laboratories) and the images
were taken by Zeiss fluorescent microscope (Zeiss Axiovert100M, Carl Zeiss, Germany).

**qReal-Time PCR arrays** — PC3 cells stable transfected with either ShPak1 or ShPak6 were grown until reaching 75% of confluency in 6-well plates and subjected to RNA isolation, followed cDNA synthesis and qPCR quantification (15). Briefly, cells were lysed and RNA was isolated according to manufacturer’s protocol using RNAeasy Plus Mini Kit (Qiagen, Valencia, CA). Next, 25 μl of cDNA was produced by RT² First Strand Kit (SABioscience, Frederick, MD), mixed with qPCR SyberGreen master mix and loaded into Human Cancer Pathway RT² Profiler PCR Array (Catalog number: PAHS-033; SABiosciences, Frederick, MD) that detects 85 different cancer related gene expression changes per array and the data normalized to 8 different house-keeping genes. Reading was completed in Eppendorf Mastercycler realplex 2 instrument.

**Western analysis** — Whole cell lysates were prepared using lysis buffer [50 mM Tris-HCl (pH=7.4), 1% TritonX-100, 150mM NaCl, 1mM EDTA, 2mM Na₃VO₄, and 1X Complete protease inhibitors (Roche Applied Science, Indianapolis, IN)]. Proteins from sample paraffin tissue blocks were extracted using the previously published protocols (17-18). The protein concentration was measured by the DC protein assay (Bio-Rad, Hercules, CA). Approximately 30 μg of tissue lysates and 45 μg of cell lysates were used for loading onto Western gel apparatus. Western analyses were performed using standard Laemmle’s method as done previously (19). Densitometry was done using NIH Image J software.

**Statistical Analysis** — All the data are presented as means ± SD. To determine significant differences between treatment and control values, we used the Student’s two-tailed t test. The significance was set at 0.05 levels (marked with symbols wherever data are statistically significant).

**RESULTS**

Since the expression levels of Pak1 in various prostate cancer cells is not yet known, we first examined the protein levels of Pak1 in comparison with Pak6, the predominant Pak isoform expressed in prostate gland, in various androgen-dependent (LNCaP) and -independent (LNCaP C4-2, PC3 and VCaP) prostate cancer cell lines. In agreement with the previous reports, robust expression of Pak6 was observed in prostate cancer cells of all stages. Due to the possible differences in the specificities of the antibodies, it was not practically possible from our data to determine which among the Pak1 and Pak6 is the predominantly expressed isoform in prostate cancer cells. Interestingly, while protein expression levels of Pak6 was constant in all the cell lines that we tested, Pak1 expression was observed to be significantly higher in invasive/metastatic prostate cancer cells as compared to non-invasive LNCaP cells (Figure 1a and b).

In order to test the specific role of Pak1 and Pak6 in prostate cancer, we first utilized PC3 cells with ShRNA based knockdown of Pak1 or Pak6 and those transiently expressing control plasmid or plasmid encoding CA-Pak1, and subjected them for transendothelial migration (microinvasion) assays on monolayers of human dermal microvascular endothelial cells (HMVEC) plated in array chips suitable for measurements in the ECIS equipment. Our data indicated that PC3 cells expressing CA-Pak1 undergoes microinvasion more efficiently as compared to cells expressing control vector (Figure 1d and e). While ShRNA-mediated knockdown of both Pak1 and Pak6 significantly impaired microinvasion of PC3 cells, inhibition of Pak1 appeared to be more effective as compared to Pak6 inhibition (Figure 1d and e). A combined knockdown of Pak1 and Pak6 did not exhibit an effect beyond the effect of Pak1 knockdown. In contrast, impaired microinvasion of Pak6-deficient PC3 cells was significantly rescued by expression with CA-Pak1, thus indicating that Pak1 predominantly promotes microinvasion of prostate cancer cells.

To confirm our findings from in vitro studies, we utilized normal human prostate, BHP, tumor and metastatic (lymph node and lung) biopsy samples and subjected them to immunohistochemical and western blot analysis for Pak1 and Pak6 expression. In concert with our in vitro findings, Pak6 was expressed in all tissues, with modest, but significant increase in expression in tumor and metastatic tissues (Figure 2a-d; Supplemental Figure 1). Interestingly, Pak1 was relatively less expressed in normal and BPH tissues, but its expression was significantly elevated (~4-fold) in tumor and metastatic lymph-node and lung tissues (Figure 2a-d; Supplemental Figure 1).
Since cell motility in response to ECM proteins is the key for invasion and metastasis of prostate cancer cells, we subjected PC3 cells with Pak1 or Pak6 gene knockdown for a cell migration assay utilizing a monolayer scratch wound healing method (14). Our data indicated that expression of PC3 cells with CA-Pak1 resulted in significantly enhanced migration of PC3 cells on all ECM proteins we tested (Figure 3a-f). In contrast, Pak1 knockdown resulted in significantly impaired cell migration (~4 fold in the absence of ECM proteins and ~2-3 fold inhibition when plated on ECM proteins). Although modest inhibition in PC3 cell migration was observed with Pak6 knockdown, the data was not statistically significant. More importantly, Pak6 knockdown did not reverse the enhanced cell migration of PC3 cells expressing CA-Pak1 (Figure 3a-f). Next, we determined whether Pak1 has a differential role to play in androgen-responsive vs. androgen-insensitive prostate cancer cells and if ShRNA-mediated Pak1 knockdown leads to any off-target effects. We prepared PC3 cells over-expressing CA-Pak1 or DN-Pak1, LNCaP cells over-expressing CA-Pak1 and LNCaP C4-2 cells with stable ShRNA-mediated knockdown of Pak1. Our data indicated that while expression with CA-Pak1 in PC3 and LNCaP cells enhanced cell migration of fibronectin (Figure 4a and b), expression with DN-Pak1 in PC3 cells resulted in impaired cell migration (Figure 4b). In support of this, LNCaP C4-2 cells with stable knockdown of Pak1 exhibited impaired migration on fibronectin (Figure 4c). Together, our study demonstrated that directional migration of prostate cancer cells is reliant on the activation of Pak1 and not Pak6.

Even though Pak1 has been implicated in the tumor progression and metastasis of multiple cancers (1, 3), Group-I Paks are the least studied in prostate tumor growth. Hence we attempted to test the efficacy of Pak1 knockdown, in comparison with Pak6, on the growth of PC3 tumor xenografts in an athymic nude mouse model in vivo. Our data indicated that knocking down either Pak1 or Pak6 significantly inhibited prostate tumor growth in vivo by ~4 fold and ~3 fold, respectively (Figure 5a-d). While our data is in agreement with the previous reports that inhibition or gene ablation of group II Paks (Pak4 and Pak6) in mice inhibits prostate tumor growth (9, 11-12), we observed that Pak1 knockdown yielded higher rate of inhibition on PC3 tumor growth in nude mice as compared to Pak6 (Figure 5a-d). In order to determine the involvement of Pak1 and Pak6 in the regulation of prostate tumor angiogenesis, we subjected the frozen sections from control, Pak1 knockdown, and Pak6 knockdown PC3 tumor xenografts for fluorescence immunohistochemistry with antibodies specific for laminin, an ECM protein abundantly expressed in vascular basement membrane and a marker for vasculature (20). We observed that laminin-positive vascular area was significantly lesser in PC3 cells with impaired Pak1 and Pak6 activities (by 4 fold and 3 fold, respectively), compared to control cells (Figure 5e-f). Interestingly, the impact of Pak1 knockdown on prostate tumor angiogenesis was significantly higher as compared to Pak6 knockdown, thus demonstrating the predominant role of Pak1 in prostate tumor growth as compared to Pak6. Our data also suggests that Pak1 may be a potential mediator of tumor angiogenesis in vivo.

Although Pak1 predominantly regulated motility and microinvasion of prostate cancer cells, its effects on other cellular functions in vitro such as proliferation and colony formation appeared similar to that of Pak6. Our data showed that expression with CA-Pak1 significantly enhanced PC3 cell proliferation by 22% and colony formation by 20%. In contrast, knockdown of either Pak1 or Pak6 resulted in a ~3 fold decrease in the rate of PC3 cell proliferation and colony formation, compared to control (Figure 6a and b). A combined knockdown of Pak1 and Pak6 in PC3 cells did not exhibit any added effects on cellular functions. Interestingly, impaired proliferation and colony formation in PC3 cells with Pak6 knockdown was significantly reversed upon co-expression with CA-Pak1 (Figure 6a and b). These results corroborated with our findings from tumor xenografts in vivo that showed that the number of Ki67 positive proliferating tumor cells are less in ShPak1 and ShPak6 tumor sections as compared to control tumors (Figure 6c and d). Together these data indicated that the role of Pak1 and Pak6 in the regulation of proliferation and colony formation is highly redundant.

We next determined if pharmacological inhibition of Pak1 with a specific inhibitor (IPA3) will provide desired results on inhibition of cell migration. Our study indicated that treatment with IPA3 resulted in a dose-dependent inhibition of cytoskeletal organization and cell migration of PC3 cells plated on fibronectin (Figure 6e and f) thus indicating the therapeutic
potential of Pak1 inhibition for the prevention of prostate cancer invasion.

Since ShRNA-mediated knockdown of Pak1 and Pak6 in PC3 cells resulted in tumor regression, we sought to identify the genes involved in the process. In order to do that, we performed qRT-PCR-based gene arrays for control, Pak1 knockdown and Pak6 knockdown PC3 cells using arrays specific for genes involved in the cancer growth and progression. Our gene array analysis identified several candidate genes that are likely involved in the prostate tumor growth, invasion and metastasis (Figure 7). Candidate genes with increased expression associated with Pak1 knockdown include Angiopoitein1, TGFβ, and tumor necrosis factor-25, while expression of genes such as p21/cip1, interferon α1, insulin-like growth factor (IGF), metastasis associated proteins 1/2, integrin αβ1 and TGFβ1. Among these MMP9 mRNA as well as protein expression was reduced by both Pak1 and Pak6 (Figure 7 and 8a-d). In order to confirm the findings from gene arrays, we subjected tumor lysates for Western blot analysis using specific antibodies against MMP9, MMP2 and TGFβ. Our data indicated that MMP9 protein expression was significantly reduced in PC3 cells with the knockdown of either Pak1 or Pak6 by more than 10 fold. Notably, neither Pak1, nor Pak6 knockdown exhibited any changes in the protein expression levels of MMP2 (Figure 8b-c). Interestingly, we observed significant elevation in the protein expression levels of TGFβ (~3 fold), a widely known tumor suppressive growth factor (21) in PC3 tumors with Pak1 knockdown, but no changes was observed in tumors with Pak6 knockdown (Figure 8b-c). We further determined if these changes occurred independent of the tumor microenvironment. Our analysis of control and CA-Pak1 expressing PC3 cells indicated reduced protein expression of MMP9 (~3 fold) and enhanced protein expression of TGFβ (~4 fold) (Figure 6b and d). Finally, we tested the effect of TGFβ on prostate cancer cell motility in vitro. Our results indicated that TGFβ inhibited PC3 cell motility in a dose- and time-dependent manner (Figure 8e). Together, these data demonstrated that Pak1-mediated prostate tumor growth and invasion involved enhanced MMP9 and reduced TGFβ expression.

DISCUSSION

Our previous studies have demonstrated that 14-3-3β-Rac-Pak1 signaling is necessary for the migration of normal fibroblasts and endothelial cells (7). We also showed that Pak1 mediates oncogenic transformation in a Rat1 fibroblast model by reconciling the effects of Akt and cRaf pathways, two major signaling cascades that are often deregulated in multiple cancers (8). Our recent study demonstrated that 14-3-3ζ-Rac signaling, an upstream activator of Group I Paks, is also necessary for the extracellular matrix (ECM) specific directional migration and transendothelial migration of non-invasive LNCaP and highly invasive PC3 prostate cancer cells, respectively (13). In support of these findings, we hypothesized that, although not a predominant Pak isoform in prostate cancer cells, Pak1 is important for the prostate tumor progression and metastasis. In the current study, using multiple approaches, we studied the importance of Group I Paks, Pak1 in particular in the regulation of prostate tumor growth and metastasis, in comparison with Pak6, a Group II counterpart that is predominantly expressed in prostate.

As reported in the literature (9, 22), our data confirmed that prostate cancer cells express robust amount of Pak6 irrespective of their tumorigenic and metastatic potential. Although protein expression levels of Pak1 was relatively lesser in non-invasive LNCaP cells, expression of Pak1 in highly invasive prostate cancer cells such as PC3, LNCaP C4-2 and VCaP were significantly higher compared to less-aggressive LNCaP cells, thus suggesting the predominant role of Pak1 in prostate cancer cell invasion and metastasis. Similar to the results from cell based studies, normal human prostate and BPH tissues expressed negligible amount of Pak1. However, expression of Pak1 was significantly elevated in prostate tumor and prostate cancer metastatic lymph node and lung tissues. This is highly in agreement with a previous observation based on prostate cancer patient samples, where significantly elevated Pak1 expression was observed in invasive prostate cancer tissues as compared to tissues with benign prostatic hyperplasia (BPH) (23), which suggested a predominant role for Pak1 in prostate cancer invasion and metastasis. To our knowledge, this is the first report on a correlation between enhanced protein expression of Pak1 with tumorigenic and metastatic potential of prostate cancer.
Next, we sought to determine the significance of elevated Pak1 levels in prostate cancer with respect to its precise role in tumor progression and invasion. We have previously shown that Rac-Pak1 signaling is necessary for inducing cytoskeletal changes, lamellipodia formation and cell protrusion through actin remodeling in normal and prostate cancer cells (7, 14). This is also in agreement with another report that HGF utilizes Pak1 in prostate cancer cells in inducing cytoskeletal changes (24). We also previously demonstrated that Pak1 inhibition prevents oncogenic transformation and tumor growth in vivo through uncoupling of Akt from cRaf-ERK pathways (8). Although Pak1 has been implicated in the attainment of migratory and invasive potential by a variety of cancer cell types (25-26), this is the least studied Pak isoform in prostate cancer probably because prostate is well known for its higher expression of group II Paks such as Pak4 and Pak6, compared to Group I Paks such as Pak1 and Pak2. Since Group II Paks are not common effectors downstream of Rac1 activation (1), we hypothesized that our previous observation of Rac1-mediated activation of prostate cancer cells (13) is mediated via utilizing a mechanism independent of Pak6. Since Pak1 expression is significantly enhanced in invasive prostate cancer cells compared to non-invasive LNCaP cells, we assumed a role for Pak1 in the regulation of cell motility in prostate cancer cells. Our study revealed that while Pak6 knockdown had no significant effect on prostate cancer cell motility on ECM proteins, Pak1 deficiency resulted in significant impairment of motility of the PC3 cells plated on ECM proteins such as fibronectin, vitronectin, laminin, osteonectin and osteopontin. Similar effect was also observed in hormone-responsive LNCaP cells and metastatic LNCaP C4-2 cells. Furthermore, treatment with Pak1 specific inhibitor IPA3 significantly blunted EGF-mediated cell migration in PC3 cells.

Interestingly, the isoform specific difference in Paks in the regulation of cell motility was not apparent in the transendothelial migration (microinvasion) of PC3 cells. Transendothelial migration of prostate cancer cells is a measure of its ability to cross the endothelial-barrier to get into the systemic circulation (intravasation) and come out of circulation (extravasation) to the site of metastasis such as lung and bone (14). Since microinvasion, also termed as 'micrometastasis', is a pre-requisite for metastasis of cancers to distant tissues, this process is considered a key step to target for the prevention of cancer metastasis. Surprisingly, ShRNA-mediated knockdown of both Pak1 and Pak6 resulted in impaired microinvasion of PC3 cells. Notably, the effect of Pak1 knockdown on microinvasion of PC3 cells was much more robust compared to Pak6 knockdown. Furthermore, impaired microinvasion of PC3 cells with Pak6 knockdown was significantly reversed by co-expression with constitutively active Pak1, demonstrating that Pak1 is predominantly involved in the regulation of prostate cancer microinvasion.

Since Pak1 is a well-known effector downstream of Rac1 activation in the regulation of cytoskeletal remodeling and integrin activation (7, 14), its predominant role in cell migration is expected. However, why ablation of both Pak1 and Pak6 resulted impaired microinvasion was perplexing. In order to characterize the mechanisms involved in this process we performed gene arrays on PC3 cells with Pak1 and Pak6 gene knockdown compared to control vector expressing PC3 cells. Candidate genes identified to increase their expression with Pak1 knockdown included Angiopoitein1, TGFβ, and TNFR25, while expression of genes such as IFNβ1, IGF, metastasis associated proteins 1/2, integrin αv, and MMP9 were reduced with Pak1 knockdown. In contrast, Pak6 knockdown resulted in increased expression of IL-8, MMP2 and thrombospondin1, and reduced expression of MMP9. Since MMP9 is necessary for the transendothelial migration of cancer cells (27-29), this would answer why both Pak1 and Pak6 is important for microinvasion. Predominant effect of Pak1 on prostate cancer microinvasion over Pak6 may be due to its specific role in mediating cytoskeletal remodeling and in the expression of integrin αv, which when partnered with integrin β6 is a receptor for laminin in the vascular basement membrane (29). Our findings, in combination with these reports, unequivocally demonstrate that Pak1 is a potent mediator of prostate cancer cell migration and microinvasion through cytoskeletal remodeling, intercellular interactions and expression/repression of various genes.

Finally, to examine the role of Pak1 in prostate tumor growth, we utilized an athymic nude mouse tumor xenograft model for PC3 cells with Pak1 and Pak6 genes ablated using specific ShRNAs. While Pak1
knockdown resulted in >50% reduction in the rate of tumor growth, Pak6 knockdown was associated with >25% reduction in tumor growth. Impaired tumor growth was also associated with >70% and >40% reduction in the number of laminin positive blood vessels with Pak1 and Pak6 knockdown, respectively. We recently showed that TGFβ acts as a tumor suppressor for prostate and bladder cancer cells (30). Our data also indicated that TGFβ can significantly inhibit migration of PC3 cells on fibronectin. Hence enhanced expression of TGFβ and reduced expression of MMP9 as a result of Pak1 knockdown in PC3 cells may be the predominant factors that resulted in reduced tumor growth.

In conclusion, we show for the first time that expression of Pak1 is significantly elevated in invasive prostate cancer cells as well as prostate tumor and metastatic tissues as compared to non-invasive prostate cancer cells and normal prostate tissue, respectively. We demonstrated that inhibition of Pak1 expression or activity can inhibit prostate cancer cell migration, microinvasion and tumor growth via modulation of the expression of number genes such as TGFβ, MMP9, Integrin α4, Insulin-like growth factor (IGF) etc. Considering our data, it is possible that targeting Pak1, coupled with one or more of the existing chemotherapeutic agents such as docetaxel (Taxotere®) could prove to be an effective, multi-pronged treatment strategy for patients harboring advanced stage prostate cancer.
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FOOTNOTES
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Abbreviations used are: BPH, benign prostatic hyperplasia; CA-Pak1, constitutively active Pak1; ECIS, electric cell-substrate impedance sensing; ECM, extracellular matrix; BSP, bone sialoprotein; SPARC, secreted protein acidic and rich in cysteine; LNCaP, lymph node cancer of the prostate; MMP, matrix metalloprotease; TGFβ, Transforming growth factor-beta.
FIGURE LEGENDS

FIGURE 1 Pak1 controls micrometastasis of prostate cancer cells. (a, b) Western blot showing expression levels of Pak1 and Pak6 in human prostate cancer LNCaP, PC3, LNCaP C4-2 and VCaP cell lines normalized to β-actin. (c) Stable human prostate cancer (PC3) cells expressing ShRNA for scrambled (control), Pak1 or Pak6 were made using lent-viral particles following selection with puromycin. Control PC3 cells as well as those expressing ShPak1 and ShPak6 were also transiently transfected with control vector (pBape-Puro) or constitutively active Pak1 (CA-Pak1; Pak1 E423). Figure shows Western blot analysis of control and transfected PC3 cells with antibodies specific for Pak1, Pak6 and β-actin. (d, e) Trans-endothelial migration (microinvasion) of prostate cancer (PC3) cells was measured using Electric Cell-substrate Impedance Sensing (ECIS) equipment with human dermal microvascular endothelial cells plated on 8W10E+ array chips (14). Control and transfected PC3 cells, detached from the plate by using cell dissociation buffer (20mM EDTA in PBS, pH 7.4) to avoid receptor loss due to trypsin digestion, were directly added onto endothelial cell monolayer at a density of 5x10^4 cells per well in 50 μl serum-free DMEM. Real-time measurements on the trans-endothelial migration of PC3 cells were recorded by the ECIS up to 5 h. The data is presented as mean ± s.d. (n=3 of triplicate experiments; *p<0.001, Δp<0.01 and #p<0.05 vs. control experiments within the same group).

FIGURE 2 Expression of Pak1 and Pak2 are elevated during prostate tumor growth, but only Pak1 is elevated in prostate tumor metastasis in humans. (a, b) Western blot showing expression levels of Pak1 and Pak6 in human prostate tumor and metastasized lung tissues normalized to β-actin (n=3). (c) Immunohistochemical analysis of Pak1 and Pak6 in human prostate cancer and metastasized lung tissues revealing their intra-tissue and intra-cellular localization compared to H&E stained sections. Paraffin embedded prostate cancer tissues were obtained from the Pathology department of the Charlie Norwood VA Medical Center in Augusta and were subjected for immunohistochemistry analysis for Pak1 and Pak6 using DAB staining kit. (d) Percentage of Pak1 or Pak6 positive area in prostate cancer and lung metastasis samples as determined by the NIH Image J software (n=6).

FIGURE 3 Inhibition of Pak1, but not Pak6, inhibits extracellular matrix specific directional migration of prostate cancer cells. (a-f) Stable human prostate cancer (PC3) cells expressing ShRNA for scrambled (control), Pak1 or Pak6 and control PC3 cells (pBape-Puro) as well as transiently transfected with constitutively active Pak1 (CA-Pak1; Pak1 E493) were subjected for migration assay utilizing a monolayer scratch recovery assay. Cells were plated on 12-well pates pre-coated with ECM proteins (0.5 μg/well) such as fibronectin, vitronectin, laminin, SPARC/osteonectin and bone sialoprotein/osteopontin, respectively and treated with 50 μM EGF (R&D Systems, Minneapolis, MN). The data is presented as mean ± s.d. (n=3 of quadruplicate experiments; *p<0.001 and #p<0.05 vs. control experiments within the same group).

FIGURE 4 Modulation of Pak1 activity in androgen-responsive and androgen-insensitive prostate cancer cells inhibits directional migration of prostate cancer cells. (a-c) Human androgen-responsive LNCaP and androgen-insensitive PC3 and LNCaP C4-2 cells were either over-expressed with CA-Pak1 or DN-Pak1 (PC3 and LNCaP), or made stable cell lines of Pak1 knockdown (LNCaP C4-2) and then subjected for migration assay on fibronectin in the presence of 50 μM EGF. The data is presented as mean ± s.d. (n=3 of quadruplicate experiments; *p<0.001 and #p<0.05 vs. control experiments within the same group).

FIGURE 5 Pak1 is a more potent mediator of prostate tumor growth and angiogenesis in vivo as compared to Pak6. (a, b) Stable human prostate cancer (PC3) cells expressing ShRNA for scrambled (control), Pak1 or Pak6 were implanted subcutaneously into athymic nude mice (Harlan, Indianapolis, IN) at a concentration of 1.5X10^6 cells in 100 μl sterile saline) (16). Images show tumor xenographs collected on day 20. The levels of Pak1 and Pak6 protein expression in the tumor lysates was verified by western
Pak1 in prostate cancer

blot analysis. (e) Tumor measurements were made using a caliper on every 4th day after tumor cell administration until day 20. Figure shows quantification of the growth of Sh-control, ShPak1 and ShPak6 expressing tumor xenografts. The data is presented as mean ± s.d. (n=8; Δp<0.01 and #p<0.05 vs. control group). (d) Tumors were collected and weight was measured on day 20. Bar graph shows quantification of tumor weight (g) on day 20. The data is presented as mean ± s.d. (n=8, *p<0.001). (e) Tumor angiogenesis was measured by fluorescent immunohistochemistry analysis of frozen tumor sections using antibodies specific for laminin, and the slides were viewed under Zeiss fluorescent microscope. Pictures show laminin positive blood vessels in the frozen sections of Sh-control, ShPak1 and ShPak6 expressing PC3 tumor xenografts. (f) Bar graph showing quantification of the vascular area using Image J software. The data is presented as mean ± s.d. (n=8; Δp<0.01 and #p<0.05).

FIGURE 6 Both Pak1 and Pak6 is necessary for prostate cancer cell proliferation but only Pak1 is necessary for motility (a) Stable human prostate cancer (PC3) cells expressing ShRNA for scrambled (control), Pak1 or Pak6 or PC3 cells expressing CA-Pak1 were subjected for proliferation assays. Bar graph shows proliferation of PC3 cells after expression of control vector (pBabe-Puro and Sh-Scambled), CA-Pak1, ShPak1, and ShPak6, respectively or in combination of ShPak1 with ShPak6 and CA-Pak1 with rate of proliferation determined by the measurement of the amount of BrDU incorporated to the PC3 cells (Roche, Indianapolis, IN). (b) Stable human prostate cancer (PC3) cells expressing ShRNA for scrambled (control), Pak1 or Pak6 or PC3 cells expressing CA-Pak1 were subjected for colony (foci) formation assays. Bar graph shows quantification of colonies formed by PC3 cells after expression of control vector (pBabe- Puro), CA-Pak1, ShPak1, and ShPak6, respectively or in combination of ShPak1 with ShPak6 and CA-Pak1 with ShPak6. (c) Images show day 20 PC3 tumor (Scrambled, Sh-Pak1 and ShPak6) xenograft frozen sections stained with Ki67 antibodies. (d) Bar graph showing the number of Ki67 positive cells in day 20 PC3 tumor (Scrambled, Sh-Pak1 and ShPak6) xenograft frozen sections. (e) Images of PC3 cells treated with 0, 5, 10 and 15 µM of Pak1 inhibitor IPA3 stained with phalloidin. (f) PC3 cells were treated with 0, 5, 10 and 15 µM of Pak1 inhibitor IPA3 and were subjected for migration assay utilizing a monolayer scratch recovery assay. Cells were plated on 12-well pates pre-coated with fibronectin (0.5 µg/well) and treated with 50 µM EGF (R&D Systems, Minneapolis, MN). All data are presented as mean ± s.d. (n=4 of quadruplicate experiments; *p<0.001, Δp<0.01 and #p<0.05).

FIGURE 7 Genes modulated by Pak1 in prostate cancer cells. (a) Quantitative RT-PCR arrays (SA Biosciences, Valencia, CA) showing genes up- or down-regulated due to the ShRNA-mediated knockdown of Pak1 in PC3 cells. (b) Quantitative RT-PCR arrays (SA Biosciences, Valencia, CA) showing genes up- or down-regulated due to the ShRNA-mediated knockdown of Pak6 in PC3 cells. A 2-fold difference was considered as a change in gene expression.

FIGURE 8 Pak1 inhibition results in reduced MMP-9 expression and increased TGFβ expression in prostate cancer cells and tumor xenografts. (a) Stable human prostate cancer (PC3) cells expressing ShRNA for scrambled (control), Pak1 or Pak6 were subjected for qRT-PCR arrays for the identification of changes in cancer related genes according to the manufacturer’s protocol. Chart indicates three major genes whose RNA expression levels were modulated with ShRNA-mediated knockdown of either Pak1 or Pak6. (b-d) Western blot analysis of PC3 cells expressing Shscrambled, ShPak1 or Sh-Pak6 and those expressing control plasmid (PBabe-Puro) or CA-Pak1 for the expression of MMP2, MMP9 and TGFβ. Quantification of the changes in the protein expression levels of MMP2, MMP9 and TGFβ normalized to β-actin is also shown. (e) PC3 cells were treated with 0, 0.1, 1.0 and 5.0 ng/ml TGFβ (R&D Systems, Minneapolis, MN) and were subjected for migration assay utilizing a monolayer scratch recovery assay. Cells were plated on 12-well pates pre-coated with fibronectin (0.5 µg/well). The data is presented as mean ± s.d. (n=3; *p<0.001 and #p<0.05).
Figure 1

a) Western blot analysis of Pak1 and Pak6 in LNCaP, PC3, C4-2, and VCaP cells compared to β-actin as a loading control.

b) Bar graph showing normalized protein expression levels of Pak1 and Pak6 in LNCaP, PC3, C4-2, and VCaP cells. * indicates statistical significance.

c) Representative Western blots showing Pak1 and Pak6 protein levels in various cell lines.

d) Graph depicting normalized resistance over time (h) for different treatments: Control, CA-Pak1, shPAK1, shPAK6, shPAK1+shPAK6, CAP AK1+shPAK6. 

E) Graph showing normalized resistance over time (h) for different treatments: Control, CA-Pak1, ShPAK1, ShPAK6, Sh+Sh6, CA-Pak1+Sh6. # indicates statistical significance.
Figure 2

(a) Western blot analysis of Pak1, Pak6, and β-actin in normal, BPH, tumor, and lung metastasis tissues. OD values are shown in (b).

(c) H&E stain and Pak1/Pak6 positive area staining in normal, BPH, tumor, and lung metastasis tissues. OD values are shown in (d).

**Note:** Error bars indicate standard deviation.
Figure 3

(a) Osteonectin
(b) Fibronectin
(c) Vitronectin
(d) Laminin
(e) Osteonectin
(f) Osteopontin
Figure 4

(a) PC3

(b) LNCaP

(c) LNCaP C4-2

Migration (% of recovery)

- Control
- CA-PAK1
- DN-PAK1

Migration (% of recovery)

- Control
- CA-Pak1

Migration (% of recovery)

- Control
- IPA3 20 µM
- ShPak1
- ShPak6

*  *  *
Figure 5

(a) Control, ShPak1, ShPak6

(b) Control shPak1 shPak6

Pak1

Pak6

β-actin

(c) Tumor size (mm²)

Day 0 Day 4 Day 8 Day 12 Day 16 Day 20

0 20 40 60

Control ShPak1 ShPak6

(n=8)

(d) Tumor weight (g)

Day 20

Control ShPak1 ShPak6

(* * *)

(e) Control ShPak1 Laminin ShPak6 DAPI

Vascular Area (%)

Control ShPak1 ShPak6

(n=8)
Figure 6

(a) Proliferation (% of control) comparison among different treatments: Control, CA-pak1, Shpak1, Shpak6, Shpak1 + Shpak6, CA-pak1 + Shpak6, with significant differences indicated by symbols.

(b) Number of colonies (% of control) showing similar trends as in (a).

(c) Representative immunofluorescence images showing Ki67-positive cells under different conditions: Control, Shpak1, Shpak6.

(d) Ki67 positive cells (%) for the same treatments as in (c), with significant differences indicated.

(e) Migration (% of recovery) in response to IPA3 at concentrations 3 µM, 10 µM, and 15 µM, demonstrating a dose-dependent effect.

(f) Migration (% of recovery) with IPA3 treatments at 12 H and 24 H, showing significant differences at higher concentrations.

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Figure 7

Fold Change

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Fold Change

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Figure 8

(a) Table showing gene expression changes:

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<th>shPak6 (fold change)</th>
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<td>MMP9</td>
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<tr>
<td>TGF-β</td>
<td>2.6↑</td>
<td>1.5↑</td>
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(b) Western blots showing protein expression:
- **MMP-2**
- **MMP-9**
- **TGFβ**
- **Pak1**
- **Pak6**
- **β-actin**

(c) Bar graph showing X fold of change:

(d) Bar graph showing X fold of change:

(e) Bar graph showing迁移（% of recovery）
P21 activated kinase-1 (Pak1) promotes prostate tumor growth and microinvasion via inhibition of TGF β expression and enhanced MMP9 secretion

Anna Goc, Ahmad Al-Azayzih, Maha Abdalla, Belal Al-Husein, Sravankumar Kavuri, Jeffrey Lee, Kelvin Moses and Payanigal R. Somanath

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