PTK6 Activation at the Membrane Regulates Epithelial–Mesenchymal Transition in Prostate Cancer

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Abstract

The intracellular tyrosine kinase protein tyrosine kinase 6 (PTK6) lacks a membrane-targeting SH4 domain and localizes to the nuclei of normal prostate epithelial cells. However, PTK6 translocates from the nucleus to the cytoplasm in human prostate tumor cells. Here, we show that while PTK6 is located primarily within the cytoplasm, the pool of active PTK6 in prostate cancer cells localizes to membranes. Ectopic expression of membrane-targeted active PTK6 promoted epithelial–mesenchymal transition in part by enhancing activation of AKT, thereby stimulating cancer cell migration and metastases in xenograft models of prostate cancer. Conversely, siRNA-mediated silencing of endogenous PTK6 promoted an epithelial phenotype and impaired tumor xenograft growth. In mice, PTEN deficiency caused endogenous active PTK6 to localize at membranes in association with decreased E-cadherin expression. Active PTK6 was detected at membranes in some high-grade human prostate tumors, and PTK6 and E-cadherin expression levels were inversely correlated in human prostate cancers. In addition, high levels of PTK6 expression predicted poor prognosis in patients with prostate cancer. Our findings reveal novel functions for PTK6 in the pathophysiology of prostate cancer, and they define this kinase as a candidate therapeutic target. Cancer Res; 73(17); 5426–37. ©2013 AACR.

Introduction

Prostate cancer is the second most common cancer and second leading cause of cancer-related death in American men (1). Most prostate cancer-related deaths are due to advanced metastatic disease, resulting from lymphatic, blood, or contiguous local spread. Tumors of the prostate originate from epithelial cells and there is a clinical correlation between the degree of differentiation and clinical outcomes.

Protein tyrosine kinase 6 (PTK6, also known as BRK or Sik) is a SRC-related intracellular tyrosine kinase that is expressed in epithelial cells. Unlike SRC-family members, PTK6 lacks an amino-terminal SH4 domain that promotes lipid modification and membrane association (2). Absence of palmitoylation and/or myristoylation facilitates flexibility in its intracellular localization. The intracellular localization of PTK6 may have a profound impact on signaling, due to its differential access to substrates and associated proteins in different cellular compartments (3–5). Currently, the prostate provides the only known physiologically relevant example of PTK6 relocalization in vivo. PTK6 is primarily nuclear in epithelial cells of the normal human prostate, but nuclear localization is lost in prostate cancer (6). Cytoplasmic retention of PTK6 promoted growth of the PC3 prostate cancer cell line, whereas expression of nuclear-targeted PTK6 significantly decreased cell proliferation (7).

Expression of PTK6 is elevated in several epithelial-derived cancers such as breast, colon, head and neck, melanoma, and ovarian cancer (reviewed in refs. 8, 9). Increased levels of PTK6 mRNA were detected in metastatic human prostate cancer samples, suggesting a role for PTK6 in prostate tumor metastasis (5). PTK6 promotes cancer cell proliferation, migration, and survival through activating oncogenic signaling pathways involving AKT, Paxillin, p190RhoGAP, p130CAS, STAT3, STAT5b, EGF receptor (EGFR), HER2, MET, and insulin-like growth factor-I receptor (IGF-IR; reviewed in refs. 8, 9). PTK6 directly phosphorylates and promotes AKT activation in response to EGF in BPH1 cells (10). It directly phosphorylates the CRK-associated substrate p130CAS, leading to formation of peripheral adhesion complexes and enhanced cell migration in PC3 cells (5). Recently, PTK6 was also shown to phosphorylate and activate focal adhesion kinase (FAK) to promote resistance to anoikis (11).

Elevated expression and/or activation of tyrosine kinases are often associated with the epithelial–mesenchymal transition (EMT), in which loss of the epithelial marker E-cadherin and elevation of the mesenchymal marker vimentin are observed (12). Activated SRC induces disorganization of E-cadherin-dependent cell–cell contacts and vimentin expression in the KM12C colon cancer cell line. Deregulation of E-cadherin and formation of peripheral adhesions induced by active SRC kinase...
Oncogenic Functions for PTK6 at the Membrane

relies on integrin, FAK, and extracellular signal–regulated kinase (ERK)1/2 signaling cascades (13, 14). Recent studies indicate that the EMT of tumor cells is also coupled with increased cell survival and drug resistance (reviewed in ref. 15).

We report endogenous PTK6 activation at the membrane in prostate epithelial cell lines, Pten-null mice, and human prostatic tumors. We found that membrane-targeted active PTK6 causes a cell-scattering phenotype in PC3 cells and promotes the EMT, cell migration, and invasion. This is achieved at least partially through increased activation of AKT. Knockdown of PTK6 in PC3 cells promotes an epithelial phenotype and dramatically reduces metastases in vivo. In contrast, activation of PTK6 at the plasma membrane is associated with deregulation of E-cadherin in mouse and human prostates. High levels of PTK6 also predict a poor prognosis for patients. Our studies show a novel role for PTK6 in the EMT and suggest that PTK6 can be a target for treating metastatic prostate cancer.

Materials and Methods

Antibodies

Anti-human PTK6 (C-18, G-6), mouse PTK6 (C-17), SP1 (PEP2), E-cadherin (H-108), ZEB1 (H-102), p63 (4A4), and anti-phospho-tyrosine (PY20) antibodies were purchased from Santa Cruz Biotechnology. Anti-phospho-tyrosine (clone 4G10) and anti-P-PTK6 (Try342) antibodies were purchased from Millipore. Antibodies directed against AKT, P-AKT (Thr308), P-AKT (Ser473), P-GSK3β (Ser9), SLUG, and Myc-tag (9B11) were obtained from Cell Signaling Technology. Antibodies directed against β-catenin and BrdUrd were obtained from BD Pharmingen. Anti-β-actin (AC-15) and vimentin antibodies were purchased from Sigma-Aldrich. Anti-C5K antibodies were a gift of Dr. G. Paolo Dotto (University of Lausanne, Switzerland). Anti-Ck8 and K67 antibodies were purchased from Abcam. Donkey anti-rabbit or sheep anti-mouse antibodies conjugated to horseradish peroxidase were used as secondary antibodies (Amersham Biosciences) and detected by chemiluminescence with SuperSignal West Dura extended duration substrate from Pierce.

Plasmids and siRNAs

The Myc-tagged Palm-PTK6-YF construct in the pBABE-puro vector has been described previously (10). The siRNAs (Dharmacon) targeting p130CAS: 5′-GGTCGACAGTGGTGTTGATT-3′ and AKT: 5′-CACCCGTGACAT- AAACCTGG-3′. Human E-cadherin: (forward) 5′-ATGCTAAGTGCCTTGGCAATCC-3′ and (reverse) 5′-TCCAAGCCCTTGCTGTTTTTTCC-3′. Human vimentin: (forward) 5′-CCCTGGCATCTTGGGCAC-3′ and (reverse) 5′-AGAGGTCTGCTTGGAG-3′. Human ZEB1: (forward) 5′-AACCGTCTTCCCCATTCTGCG-3′ and (reverse) 5′-GAGATGCTCTGTGAGCCTGTTCTGG-3′. Human SLUG: (forward) 5′-GCTCAGAAAAGC- CCCATATTGATG-3′ and (reverse) 5′-GCCAGGCCCCAGAAAAGTTGAAATAG-3′. Human Twist: (forward) 5′-GTCGCCCAGTCTTACGCCAGGG-3′ and (reverse) 5′-CCACGCTGGAGGAGTCTGAC-3′. and human PTK6: (forward) 5′-GCTATGGTCCCAACAATCC-3′ and (reverse) 5′-CTCCGACAGCCTGAATCC-3′.

Cell culture and fractionation

The human prostate cancer cell lines PC3 [American Type Culture Collection (ATCC); CRL-1435] and DU145 (ATCC; HTB-81) were certified by ATCC and cultured according to the ATCC guidelines. The benign prostatic hyperplasia epithelial cell line BPH-1 (kindly provided by Simon Hayward, Vanderbilt University, Nashville, TN; ref. 17) was cultured in RPMI-1640 containing 5% FBS. No additional authentication of cell lines was conducted. Cell fractions were carried out using the ProteoExtract Subcellular Proteome Extraction Kit (EMD Millipore) according to the manufacturer’s instructions. The method used for preparation of total cell lysates has been described previously (10).

Retrovirus production and transduction

pBABE-puro plasmids were transfected into Phoenix-Ampho cells using Lipofectamine 2000 (Invitrogen). Retrovirus was collected 48 and 72 hours later. PC3 and BPH1 cells were infected with retrovirus at a multiplicity of infection (MOI) of 100 for 24 hours. Stable cell pools were selected in growth medium containing 2 μg/mL puromycin for 1 week.

Primers and quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). After DNase I digestion (Promega), 500 ng of RNA was used to generate cDNA using a cDNA synthesis kit (Bio-Rad). Real-time (RT)-PCR was conducted using the following mixture: 1 x SYBR Green Supermix (Bio-Rad), 100 nmol/L of each primers, and 1 μL of cDNA in a 25 μL total volume. Reactions were amplified and analyzed in triplicate using a MyiQ single-color RT-PCR detection system (Bio-Rad). The following primers were used: human cyclophilin: (forward) 5′-GCAGACAAGGTCCAAAAGACAG-3′ and (reverse) 5′-CACCCGTGACAT- AAACCTGG-3′. Human E-cadherin: (forward) 5′-ATGCTAAGTGCCTTGGCAATCC-3′ and (reverse) 5′-TCCAAGCCCTTGCTGTTTTTTCC-3′. Human vimentin: (forward) 5′-CCCTGGCATCTTGGGCAC-3′ and (reverse) 5′-AGAGGTCTGCTTGGAG-3′. Human ZEB1: (forward) 5′-AACCGTCTTCCCCATTCTGCG-3′ and (reverse) 5′-GAGATGCTCTGTGAGCCTGTTCTGG-3′. Human SLUG: (forward) 5′-GCTCAGAAAAGC- CCCATATTGATG-3′ and (reverse) 5′-GCCAGGCCCCAGAAAAGTTGAAATAG-3′. Human Twist: (forward) 5′-GTCGCCCAGTCTTACGCCAGGG-3′ and (reverse) 5′-CCACGCTGGAGGAGTCTGAC-3′. and human PTK6: (forward) 5′-GCTATGGTCCCAACAATCC-3′ and (reverse) 5′-CTCCGACAGCCTGAATCC-3′.
a Transwell (24-well insert; pore size, 8 μm; Corning) and incubated with 1% FBS containing medium. Twenty percent FBS-containing medium was added to the lower chamber as a chemoattractant. After 18 hours, cells that did not migrate through the pores were removed by a cotton swab, and the cells on the lower surface of the membrane were stained by crystal violet. BD BioCoat Matrigel Invasion Chambers (BD Pharmin-gen) were used for invasion assays, which were conducted in a similar way to migration assays, except that 50 ng/mL HGF was used as a chemoattractant and the incubation time was 24 hours. Images were taken under the phase-contrast microscope using ×10 magnification.

**Immunostaining**

Cells were washed with PBS, fixed in Carnoy's solution (6:3:1 ethanol:chloroform:acetic acid), then blocked with 3% bovine serum albumin for 1 hour, and incubated with primary antibodies overnight. Fluorescein isothiocyanate (FITC)—conjugated anti-mouse secondary antibodies (Sigma-Aldrich) were used to detect primary antibodies made in mouse (green), and biotinylated anti-rabbit secondary antibodies (Vector Laboratories) were used and then incubated with rhodamine-conjugated avidin to detect primary antibodies made in rabbit (red). Slides were mounted with Vectashield fluorescent mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories).

For staining of prostate tissues and tumors, antigen retrieval was conducted in 10 mmol/L sodium citrate buffer on a hot plate at a temperature above 90°C for 20 minutes. Immunohistochemistry was conducted using the VECTASTAIN Elite ABC Kit [rabbit immunoglobulin G (IgG)] or the mouse on mouse (M.O.M.) kit as per the manufacturer's instructions (Vector Laboratories). Reactions were visualized with FITC or rhodamine-conjugated avidin, and slides were mounted in Vectashield fluorescent mount media containing DAPI, or with 3,3'-diaminobenzidine (DAB; Sigma-Aldrich) and counterstained with hematoxylin (Vector Laboratories). Staining controls were conducted with normal rabbit or mouse IgG.

**Xenograft and murine prostate cancer models**

To monitor metastases in vivo, pFU-L2G, which expresses optimized luciferase (L2) and GFP (G; ref. 18), was introduced into PC3 cells. Cells, selected for GFP expression, were transfected with PTK6 siRNA or control siRNA twice before intravenous injection into 6-week-old male SCID (IcrTac:ICR-Prkdc^+/−); Taconic) mice. Tumor growth and metastases were monitored weekly following injection of α-luciferin using the Xenogen IVIS Spectrum in vivo imaging system (Caliper Life Sciences, Inc.). Alternatively, cells were introduced by intracardiac injection and mice were sacrificed at 10 weeks, and internal organs were formalin-fixed and paraffin-embedded. Generation and characterization of the PB-Cre4 and Pten^fl/fl^ mice have been described previously (19). C57BL/6j PB-Cre4 Pten^fl/fl^ mice were sacrificed at 6 months of age and prostates were formalin-fixed and paraffin-embedded. All mouse experiments were reviewed and approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

**Statistical analysis**

Datasets containing 363 and 140 primary prostate cancer samples and the patient information were extracted from the Oncomine database (Compendia Bioscience). These include the Setlur Prostate Dataset, National Center for Biotechnology Information (NCBI) dataset GSE8402 (20), and the Taylor prostate dataset, NCBI dataset GSE21035 (21). Patients were categorized into "PTK6 high," "PTK6 medium," and "PTK6 low" groups according to their PTK6 RNA expression levels. The PTK6 high group represents the top 10%, whereas the PTK6 low group represents the bottom 25% of the patients according to PTK6 RNA levels. The PTK6 medium group represents the remaining patients with intermediate PTK6 expression levels. The survival curve or recurrence rate was estimated using the Kaplan–Meier method and the differences among three groups was tested using the log-rank test. The analysis was conducted using SAS 9.2. PTK6 and E-cadherin mRNA levels were analyzed in a NCBI human genome microarray dataset GDS2545, which contains 171 human prostate samples including normal prostate tissue, normal tissue adjacent to the primary tumor, primary tumor, and metastatic tumors. Results are shown as the mean ± SE. A linear regression model is set up using E-cadherin mRNA as a dependent variable and PTK6 as an independent variable. For all the other cell studies, data represent the mean of at least 3 independent experiments ± SD. P values were determined using the one-tailed Student t test (Microsoft Excel 2010) and two-sided Fisher exact test (GraphPad Prism 5). A difference was considered statistically significant if the P value was equal to or less than 0.05.

**Results**

**Membrane-targeted PTK6 causes a cell-scattering phenotype in prostate epithelial cells**

PTK6 relocates from the nucleus to the cytoplasm in prostate epithelial tumor cells, as human prostate cancer progresses (6). Phosphorylation of PTK6 at tyrosine residue 342 within its activation loop promotes activation (22). We examined the localization of total PTK6 and active PTK6, phosphorylated on tyrosine residue 342 (PY342), in three prostate epithelial cell lines PC3, DU145 (metastatic), and BPH1 (benign hyperplasia; ref. 17). Cells were fractionated into cytoplasmic, membrane/organelle, and nuclear compartments. In all three cell lines, total PTK6 is primarily localized in the cytoplasm (Fig. 1A). However, immunoblotting for PY342 revealed that active PTK6 is localized at the membrane (Fig. 1A).

To explore functions of membrane-associated active PTK6, PC3 and BPH1 cell lines stably expressing membrane-targeted active PTK6 (Palm-PTK6-YF) were generated. Palm-PTK6-YF contains dual fatty acylation sites for palmitoylation/myristoylation from the SRC-family kinase LYN at the amino terminus for membrane association (referred to here as Palm), and mutation of the negative regulatory tyrosine at position 447 to phenylalanine (YF; ref. 4). Ectopic expression of Palm-PTK6-YF was confirmed by immunoblotting (Fig. 1B). Compared with vector control cells, both PC3 and BPH1 cells expressing Palm-PTK6-YF undergo profound morphologic changes, which include a ruffled membrane and fewer cell-cell contacts (Fig. 1C). The ruffled membrane suggests the
formation of PTK6-induced peripheral adhesion complexes as reported previously (5). Formation of these peripheral adhesion complexes was dependent upon PTK6 kinase activity and did not form in cells expressing membrane-targeted kinase defective PTK6 (5).

A cell-scattering phenotype is often coupled with the EMT (12). Because both BPH1 and PC3 cells express E-cadherin, we examined whether E-cadherin expression and localization are altered by Palm-PTK6-YF expression. In both PC3 and BPH1 cells, expression of Palm-PTK6-YF led to a reduction in E-cadherin at the plasma membrane that was accompanied by activation of phospho-tyrosine signaling in peripheral adhesion complexes (Fig. 1D and E). BPH1 cells that do not form peripheral adhesion complexes with high levels of phospho-tyrosine still contain E-cadherin at cell–cell contacts (Fig. 1E), suggesting that phospho-tyrosine signaling is involved in deregulating E-cadherin.

**Active PTK6 at the plasma membrane promotes the EMT**

Loss of E-cadherin is one of the hallmarks of EMT (12). Reduced E-cadherin levels were detected in the presence of Palm-PTK6-YF in PC3 cells (Fig. 2A). We examined expression of other EMT markers and found that levels of vimentin and the E-cadherin transcriptional repressor ZEB1 are increased in cells expressing Palm-PTK6-YF (Fig. 2A). Following cell fractionation, we found that ectopic expression of Palm-PTK6-YF largely increases the pool of active PTK6 at the membrane (Fig. 2B; PY342). Endogenous membrane-associated phospho-PTK6 is the main band detected by immunoblotting of control PC3 cell lysates with anti-PY342 (Fig. 2C, vector lanes, arrowhead). Ectopic transfected Palm-PTK6 migrates slightly above the endogenous band (Fig. 2C; Palm-YF). Expression of Palm-PTK6-YF leads to decreased membrane association of E-cadherin, increased ZEB1 in the nucleus, and increased vimentin in the cytoplasm and membrane (Fig. 2B). Levels of mRNAs...
encoding EMT markers were also measured by either quantitative real-time PCR (qRT-PCR) or semiquantitative PCR. Consistent with protein levels, expression of E-cadherin mRNA is decreased, whereas levels of mRNAs encoding the mesenchymal intermediate filament protein vimentin, and transcriptional repressors of E-cadherin SLUG, Twist, and ZEB1 mRNAs are increased (Fig. 2D and E).

AKT participates in PTK6-mediated induction of the EMT

AKT is a crucial regulator of the EMT in squamous cell carcinoma lines (23). Previously, we observed increased AKT activation in response to FBS stimulation in Palm-PTK6-YF–expressing cells (5), and therefore examined whether AKT and downstream signaling are involved in the PTK6-mediated EMT. Phosphorylation of AKT at Thr308 and Ser473, which is required for its activation, is increased in PC3 cells expressing Palm-PTK6-YF relative to total AKT (Fig. 3A). This is accompanied by increased inhibitory phosphorylation of GSK3β, a direct target of AKT (Fig. 3A). AKT has been reported to regulate the SNAIL family member SLUG (SNAI2), a transcription factor that represses E-cadherin (24). We see increased expression (Fig. 3A) and nuclear localization (Fig. 3B) of SLUG in Palm-PTK6-YF–expressing PC3 cells.

To test whether AKT activation is required for the PTK6-induced EMT, we used siRNAs to knockdown endogenous AKT in PC3 cells. Following knockdown of AKT, E-cadherin levels are increased and vimentin levels are decreased in both Palm-PTK6-YF and vector control cells (Fig. 3C). However, expression of E-cadherin in Palm-YF–expressing cells treated with AKT siRNA remains lower than vector control cells treated with scrambled siRNA (Fig. 3C), indicating that AKT knockdown only partially rescues PTK6-induced EMT and that other mechanisms are involved. We also used siRNAs to knockdown the scaffold protein p130CAS, which is crucial for AKT activation (5). Following knockdown of p130CAS, AKT activity is reduced, whereas total AKT levels are not changed (Fig. 3D). Decreased AKT activity is accompanied by decreased GSK3β phosphorylation, increased E-cadherin expression, and decreased vimentin levels in both Palm-PTK6-YF and vector control cells (Fig. 3D). As in the AKT siRNA experiment, reduction of AKT activation through knockdown of p130CAS only partially rescues EMT induced by Palm-PTK6-YF (Fig. 3D).

Palm-PTK6-YF promotes tumorigenicity and invasiveness of PC3 cells

We examined the tumorigenic and invasive ability of PC3 cells stably expressing Palm-PTK6-YF in vitro and in vivo. Expression of Palm-PTK6-YF promotes anchorage-independent growth of PC3 cells in soft agar (Fig. 4A), while not affecting cell proliferation (data not shown), suggesting that
and no metastases were detected in internal organs in the 4 control cells, only 1 of 5 mice was found dead after 8 weeks, indicating better survival of these tumor cells in vivo, leading to increased metastases at day 50 (Fig. 4E). These data show that membrane-targeted active PTK6 promotes the EMT by conferring resistance to anoikis, as well as stimulating anchorage-independent growth and cell migration, resulting in increased metastasis in vivo.

**PC3 cells are less tumorigenic and invasive after knockdown of PTK6**

To determine if endogenous PTK6 participates in the EMT and regulates tumorigenicity of PC3 cells, PTK6 was knocked down using an siRNA-based approach. Knockdown of PTK6 persisted for at least 6 days posttransfection (Fig. 5A). Following PTK6 knockdown, E-cadherin levels increased, whereas vimentin and ZEB1 levels decreased (Fig. 5A). In addition, knockdown of PTK6 resulted in decreased proliferation (Fig. 5B), colony formation (Fig. 5C), and anchorage-independent growth in soft agar (Fig. 5D). After PTK6 knockdown, the ability of PC3 cells to invade through the extracellular matrix layer to the bottom side of the membranes in invasion chamber assays was diminished (Fig. 5E).

We conducted xenograft studies to monitor the impact of PTK6 knockdown on metastasis in SCID mice. Luciferase-expressing PC3 control cells and PTK6 knockdown cells were injected intravenously into SCID mice and monitored in vivo following injection with luciferin. Knockdown of PTK6 by siRNA effectively reduced survival and metastasis of PC3 cells, compared with control siRNA-treated cells, which metastasized by day 36 (Fig. 5F).

**Activation of endogenous PTK6 at the membrane in Pten-null mouse prostates correlates with the EMT**

To investigate the significance of PTK6 relocalization in vivo, we used a murine prostate cancer model (PB-Cre, Pten^flx/flx^; ref. 19). Compared with wild-type control mice, disruption of Pten led to an abnormally enlarged anterior prostate (AP) at the age of 8 months in male mice (Fig. 6A, white hatch marks). Consistent with previous reports, loss of both Pten alleles results in early murine prostatic intraepithelial neoplasia (PIN) formation that can progress to adenocarcinoma (25). Preexisting prostatic ductules and acini in PB-Cre, Pten^flx/flx^ mice were filled with cells derived from the hyperproliferative epithelium, whereas a single layer of epithelial cells was present in the control mice (Fig. 6B). Knockout of Pten and activation of AKT were observed in prostate epithelial cells in PB-Cre, Pten^flx/flx^ mice (Fig. 6B). As expected, PTK6 was detected within nuclei of normal prostate epithelial cells in wild-type
mice, but it was primarily cytoplasmic in the *Pten* null prostate (Fig. 6B; PTK6). Interestingly, in addition to relocalization of PTK6 from nucleus to cytoplasm, we detected significant association of activated endogenous PTK6 phosphorylated at the tyrosine residue 342 with the plasma membrane in the *Pten* null prostates (Fig. 6B; *Pten*fl/fl, PY342). In the wild-type prostate, active PTK6 is largely confined to the nucleus (Fig. 6B; *Pten*wt/wt, PY342).

To determine the lineage of the cells with active membrane associated PTK6, dual immunostaining was conducted using antibodies specific for markers that identify subpopulations of human and murine prostatic epithelial cells (25). Anti-phospho-tyrosine and anti-PTK6 PY342 antibodies recognized the same group of cells with high phospho-tyrosine signaling at the plasma membrane (Fig. 6C, a). An expanded pool of cytokeratin 5 (CK5)+, p63+ (basal cell marker) cells was observed within prostatic ductules upon homozygous *Pten* deletion, consistent with a previous report (25). However, cells with activated PTK6 at the membrane do not express CK5 and p63 (Fig. 6C, b–e), but are CK8 (luminal cell marker)-positive (Fig. 6C, f and g), suggesting they are derived from luminal secretory cells. Most of the phospho-tyrosine-positive cells are not proliferative as evidenced by Ki67 and bromodeoxyuridine (BrdUrd) staining, although there are more proliferating cells in the *Pten* null prostates compared with normal prostate in control mice (Fig. 6C, h–j). Phospho-tyrosine-positive cells are larger than surrounding phospho-tyrosine-negative cells, which led us to examine proteins involved in cell–cell contacts. The cells with activated PTK6 signaling at the plasma membrane show decreased E-cadherin and increased E-cadherin endocytosis (Fig. 6C, k–m). In addition, increased levels of vimentin, a mesenchymal marker, were detected in most of the prostate tumor cells in *Pten* null prostates (Fig. 6C, n and o). These data suggest that cells with high phospho-tyrosine signaling and active PTK6 at the plasma membrane are undergoing the EMT.

**High levels of PTK6 predict poor prognosis for prostate cancer patients**

To understand the clinical significance of PTK6 in human prostate cancer, a dataset containing 363 primary prostate cancer patients was analyzed. We found that higher expression of PTK6 was associated with worse clinical outcomes, including shorter disease-free survival and overall survival. These data suggest that PTK6 may be a valuable biomarker for prognosis and therapeutic targeting in human prostate cancer.
cancer samples and patient information was extracted from the Oncomine database (20). Patients were categorized into PTK6 high, PTK6 medium, and PTK6 low groups according to their relative PTK6 mRNA level. The Kaplan–Meier survival curve indicated that patients with higher PTK6 mRNA expression have significantly poorer survival outcomes, whereas lower PTK6 expression levels were associated with better overall survival ($P < 0.005$; Fig. 7A). Analysis of another dataset containing 140 prostate carcinoma samples with recurrence information was also extracted and analyzed using the Kaplan–Meier method (21). Higher PTK6 expression was associated with earlier recurrence ($P < 0.05$; Fig. 7B). We have reported that PTK6 expression is significantly increased in human metastatic prostate cancer (5). Analysis of the same dataset reveals decreased levels of E-cadherin in metastatic prostate cancer (Fig. 7C). Importantly, linear regression analyses show an inverse correlation of PTK6 and E-cadherin mRNA in normal tissue and metastatic cancer groups, indicating one unit change of PTK6 can be used to predict change in E-cadherin levels (Fig. 7D).

**Discussion**

A variety of studies indicate that PTK6 has context and condition-specific functions. PTK6 negatively regulates
proliferation, promotes differentiation, and mediates apoptosis in normal cells of the intestinal tract and skin, whereas it promotes proliferation, migration, and survival in breast, colon, ovarian, and prostate tumor cells (reviewed in refs. 8, 9, 26). Differences in PTK6 expression, activation, and intracellular localization, as well as expression of distinct sets of substrates and associated proteins in different cell types, would facilitate activation of distinct signaling pathways in normal and cancer cells. In normal cells, PTK6 is induced and activated in response to differentiation (27, 28) or stress such as DNA-damage (29, 30). On the other hand, the expression of PTK6 is significantly induced in various cancer cells, including breast cancer and prostate cancer (5, 31), where high levels of PTK6 predict poor prognosis in human patients (Fig. 7; ref. 32).

Our data are the first to show that activation of PTK6 at the membrane can positively contribute to the EMT. In vivo studies show that endogenous mouse PTK6 protein is active at the membrane in the Pten null prostate, and this correlates with reduced E-cadherin expression. In addition, we found that PTK6 is activated at the membrane in invasive human tumor samples. Expression of membrane-targeted PTK6 in PC3 cells led to repression of E-cadherin expression, a more mesenchymal phenotype, as well as increased tumorigenicity and metastases in xenograft models, further supporting a direct role for PTK6 in promoting the EMT. Recently, knockdown of PTK6 in a subline of human MCF-7 breast cancer cells engineered to overexpress HER2, led to increased E-cadherin and decreased mesenchymal marker expression, suggesting PTK6 also regulates the EMT in other cancers (33).

Membrane association of SRC kinases through amino-terminal lipid modification is critical for them to be able to transform cells (34). We have shown that even though PTK6 is not myristoylated/palmitoylated, the active endogenous protein can be found at the membrane (5, 11). Previously, we reported that membrane-targeted PTK6 has transforming potential, whereas nuclear PTK6 is growth inhibiting (4, 7). We showed that membrane-targeted PTK6 transforms mouse embryonic fibroblasts lacking the SRC-family members SRC, YES, and FYN (11). We detected nuclear localization of endogenous PTK6 in normal prostates, and relocalization of PTK6 to the cytoplasm and membrane in prostate tumors (Fig. 6; ref. 6). Activation and translocation of PTK6 in prostate cancer could lead to phosphorylation and activation of non-nuclear substrates to which it does not normally have access. Mechanisms regulating PTK6 intracellular shuttling are not well understood, but may be mediated through protein–protein interactions that could be modulated by expression of different PTK6 isoforms encoded by differentially spliced mRNAs (35).

PTK6 participates in several signaling pathways associated with cell migration, survival, and metastasis (reviewed in...
It regulates signaling by ERBB receptors (37, 38), the hepatocyte growth factor (HGF) receptor MET (39), and IGF-I (32, 40). Its substrates include Paxillin (41), AKT (10), EGFR (42), p130CAS (5), and FAK (11). PTK6 also regulates p190RhoGAP (43) and ERK5 (44) activity. PTK6-mediated deregulation of E-cadherin could involve several PTK6 downstream players, including AKT, p130CAS, FAK, and ERK5. AKT signaling promotes the EMT in different cancer cell lines (23, 45, 46). Here, we show that Palm-PTK6 promotes AKT activation and regulation of its downstream targets, including GSK3β and the E-cadherin repressor SLUG (Fig. 3), and this contributes in part to PTK6-mediated regulation of the EMT. Knockdown of the PTK6 substrate p130CAS impairs AKT activation (5), and partially rescues E-cadherin downregulation induced by Palm-PTK6-YF (Fig. 3D). Previously, we have shown that ERK5 plays an important signaling role downstream of p130CAS in cells expressing membrane-targeted active PTK6 (5). ERK5 has been implicated in breast cancer cell metastasis (48), and is required for HGF-induced cell migration in breast cancer cells (39).

In prostate cancer, decreased levels of E-cadherin are associated with high prostate tumor grade and poor prognosis. Patients with normal E-cadherin expression have a significantly higher overall survival rate than patients with low expression (49, 50). Here, we show that PTK6 is aberrantly expressed and activated in prostate tumor cells in some patients, and its levels are inversely correlated with E-cadherin expression in metastatic prostate cancer (Fig. 7). Targeting PTK6 using siRNAs...
dramatically reduces the metastatic potential of human prostate cancer cells in a mouse xenograft model (Fig. 5F). Our findings suggest that PTK6 is a novel gene marker in categorizing prostate cancer patient groups, and a potential gene target for personalized medicine.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Zheng, Z. Wang, A.L. Tyner
Development of methodology: Y. Zheng, P.M. Brauer, R.E. Perez White, J. Li
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Zheng, Z. Wang, V. Nogueira, N. Hay, D.A. Tonetti, A. Kajdacsy-Balla
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Zheng, Z. Wang, V. Macias, A.L. Tyner
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Correction: PTK6 Activation at the Membrane Regulates Epithelial–Mesenchymal Transition in Prostate Cancer

In this article (Cancer Res 2013;73:5426–37), which was published in the September 1, 2013, issue of Cancer Research (1), the citation and order of some of the References were incorrect due to a production error. These errors have been corrected in the online version of the article, which now no longer matches the print version.

Reference


Published OnlineFirst September 23, 2013.
doi: 10.1158/0008-5472.CAN-13-2555
©2013 American Association for Cancer Research.
PTK6 Activation at the Membrane Regulates Epithelial–Mesenchymal Transition in Prostate Cancer
