Published in final edited form as:

Prostate. 2014 February ; 74(2): 134–148. doi:10.1002/pros.22735.

Src-Dependent Tks5 Phosphorylation Regulates Invadopodia-Associated Invasion in Prostate Cancer Cells

Karen L. Burger¹, Brian S. Learman¹, Amy K. Boucherle¹, S. Joseph Sirintrapun², Scott Isom³, Begoña Díaz⁴, Sara A. Courtneidge⁴, and Darren F. Seals^{5,*}

¹Department of Cancer Biology, Wake Forest Comprehensive Cancer Center, Wake Forest School of Medicine, Winston-Salem, North Carolina

²Department of Pathology, Wake Forest Comprehensive Cancer Center, Wake Forest School of Medicine, Winston-Salem, North Carolina

³Department of Biostatistical Sciences, Wake Forest Comprehensive Cancer Center, Wake Forest School of Medicine, Winston-Salem, North Carolina

⁴Cancer Center, Sanford-Burnham Medical Research Institute, La Jolla, California

⁵Department of Biology, Appalachian State University, Boone, North Carolina

Abstract

BACKGROUND—The Src tyrosine kinase substrate and adaptor protein Tks5 had previously been implicated in the invasive phenotype of normal and transformed cell types via regulation of cytoskeletal structures called podosomes/invadopodia. The role of Src-Tks5 signaling in invasive prostate cancer, however, had not been previously evaluated.

METHODS—We measured the relative expression of Tks5 in normal (n = 20) and cancerous (n = 184, from 92 patients) prostate tissue specimens by immunohistochemistry using a commercially available tumor microarray. We also manipulated the expression and activity of wild-type and mutant Src and Tks5 constructs in the LNCaP and PC-3 prostate cancer cell lines in order to ascertain the role of Src-Tks5 signaling in invadopodia development, matrix-remodeling activity, motility, and invasion.

RESULTS—Our studies demonstrated that Src was activated and Tks5 upregulated in high Gleason score prostate tumor specimens and in invasive prostate cancer cell lines. Remarkably, overexpression of Tks5 in LNCaP cells was sufficient to induce invadopodia formation and associated matrix degradation. This Tks5-dependent increase in invasive behavior further depended on Src tyrosine kinase activity and the phosphorylation of Tks5 at tyrosine residues 557

© 2013 Wiley Periodicals, Inc.

*Correspondence to: Darren F. Seals, Department of Biology, Appalachian State University, Boone, NC 28608. sealsdf@appstate.edu. The present address of Karen L. Burger is Department of Cancer Biology, The Scripps Research Institute, Scripps Florida, Jupiter, FL 33458

The present address of S. Joseph Sirintrapun is Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

and 619. In PC-3 cells we demonstrated that Tks5 phosphorylation at these sites was necessary and sufficient for invadopodia-associated matrix degradation and invasion.

CONCLUSIONS—Our results suggest a general role for Src-Tks5 signaling in prostate tumor progression and the utility of Tks5 as a marker protein for the staging of this disease.

Keywords

podosome; cytoskeleton; motility; metastasis; biomarker

INTRODUCTION

For solid cancers, patient prognosis generally declines as the primary tumor spreads to distant anatomic sites. For prostate cancer patients with distant metastases, less than a third will survive after five years with 33,000 men succumbing to this disease in the United States annually [1]. A molecular understanding of invasive prostate cancer as a means of improving patient treatment and overall survival remains an unmet medical challenge.

Migratory cancer cells capable of remodeling the surrounding tumor stroma define an invasive phenotype. Cancer cell motility and extracellular matrix degradation are supported by cytoskeletal structures called invadopodia [2,3]. Invadopodia, and the related podosomes of normal cell types, are actin-based cell surface protrusions that enable adherence to and degradation of extracellular matrix proteins. Though they share some of the same cytoskeletal regulatory machinery (integrins, tyrosine kinases, Arp2/3, WASp) as other adhesion structures, they are distinguished by marker proteins (cortactin, dynamin2, Tks5) and metalloproteinases (MT1-MMP) that uniquely support focalized matrix remodeling activity [2–5]. Invadopodia may therefore confer invasive behavior onto cancer cells and support tumor metastasis [6].

Src is the namesake member of a family of non-receptor tyrosine kinases and the first described protooncogene [7]. Src is frequently upregulated in advanced stage cancers, and activation of Src tyrosine kinase activity transforms cells to a neoplastic phenotype with enhanced survival, growth, and migration [8–10]. Src activation also commonly promotes podosome/invadopod formation [4,11–13]. This is supported by the presence of tyrosine phosphorylated proteins at these structures, many of which are Src substrates [14–18].

Tks5 is a substrate of Src and an adaptor protein for lipids and proteins [19]. An amino terminal Phox homology domain mediates binding to phosphatidylinositol phosphates and supports the attachment of Tks5 to membranes [13,20]. Five SH3 domains along with several polyproline motifs enable the association of Tks5 with other proteins including WASp, cortactin, Nck, Grb2, and ADAMs family metalloproteinases [13,16,20,21]. In Src-transformed NIH3T3 cells (Src3T3) and human breast cancer and melanoma cell lines, Tks5 silencing diminishes podosome/invadopod development, matrix remodeling activity, and invasion [22]. Tks5-silenced Src3T3 cells also exhibit diminished primary tumor growth and a diminished size of lung lesions in an experimental metastasis assay [23]. Tks5 has Src phosphorylation sites located between the third and fourth SH3 domains [19]. We and others have demonstrated that Tks5 phosphorylation is important for podosome development and

associated matrix degradation in macrophages and osteoclasts [24,25]. In melanoma cells Src-dependent phosphorylation of Tks5 at tyrosine 557 is important for binding to Nck, for Nck recruitment to invadopodia, and for invadopodia-associated matrix degradation activity [16].

While Src is commonly upregulated in prostate cancer cell lines and inhibition of Src activity inhibits prostate cancer cell proliferation and prostate tumor growth [8,26], our knowledge of Src-Tks5 signaling and invadopodia development in the context of prostate cancer remains unexplored. In this study, we demonstrate a significant role for Src-Tks5 signaling in prostate cancer cell invasion by showing that Tks5 is upregulated in a high Gleason score prostate tumor specimens, that Tks5 expression is sufficient to drive invadopodia formation in prostate cancer cells, and that Src-dependent Tks5 phosphorylation at positions 557 and 619 modulates invadopodia-associated matrix remodeling activity and invasion.

MATERIALS AND METHODS

Immunohistochemistry

To conduct an immunohistochemical analysis of Tks5 protein, we purchased a high density prostate tumor microarray (Folio Bioscience, Powell, OH) containing 92, duplicate cases of formalin-fixed human prostatic adenocarcinoma and 20 cases of normal and cancer-adjacent normal prostate tissue each organized into 1-mm diameter, 5- μ m thick core sections. Tumor microarray slides were deparaffinized and incubated with a Tks5 antibody (Proteintech Group, Chicago, IL) at 1:50 dilution according to the manufacturer's protocol. Slides were developed using 3,3'-diaminobenzidine as substrate. Slides were digitalized using ScanScope XT (Aperio, Vista, CA). The staining intensity of Tks5 was evaluated on a scale from 1 (low) to 4 (high) by three, independent observers, including a board-certified pathologist with subspecialty expertise in urologic pathology. Tks5 staining intensity for each specimen was converted to a rounded mean score then plotted relative to defined Gleason score categories (normal, 6, 7, 8–10) according to the combined Gleason scores assigned by the vendor (Folio Bioscience; Supplemental Table SI). No core specimen exhibited negative Tks5 staining.

Cell Culture

LNCaP and PC-3 cells (ATCC, Manassas, VA) were cultured at 37°C and 5% CO₂ in RPMI-1640 media (Thermo Scientific Hyclone, Logan, UT) formulated with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10 mM HEPES, and 110 mg/L sodium pyruvate and supplemented with 10% fetal bovine serum (FBS; Sigma–Aldrich, St. Louis, MO) and 1% penicillin/streptomycin (Hyclone).

Cell Line Modification

Stable modifications to LNCaP and PC-3 cells were generated following infection with viral vector constructs and the selection of antibiotic-resistant cell lines as previously described [24]. LNCaP cells expressing firefly luciferase (LNCaP-luc) were generated with Ubc-GFP_{Luc}, a generous gift from Dr. Purnima Dubey (Department of Pathology, Wake Forest

School of Medicine). LNCaP-luc cells were further modified for Tks5 overexpression using pBABEpuro-Tks5 [24]. PC-3 cells were modified for the expression of activated Src using pBABEpuro-Src-Y527F. Tks5 silencing in the parental and Src-activated PC-3 cells was generated with a pLNeo lentiviral vector construct incorporating a human Tks5-selective shRNA (GCCCATCGA-GAAGTCTCAGTT). Control PC-3 cells expressed a scrambled shRNA construct. The pLNeo vector was generously provided by Dr. Guangchao Sui (Department of Cancer Biology, Wake Forest School of Medicine). All of the Src- and Tks5-modified LNCaP and PC-3 cell lines were maintained under standard cell culture conditions except that 300 µg/ml hygromycin (Invitrogen, Carlsbad, CA), 0.5 µg/ml puromycin (Sigma), and/or 500 µg/ml G418 (EMD, Gibbstown, NJ) were supplemented as appropriate.

Acute changes in Src and Tks5 expression were achieved by Amaxa-based electroporation. Briefly, prostate cancer cells (LNCaP: 2×10^6 ; PC-3: 1×10^6) were mixed with plasmid constructs (1–6 µg) or siRNA pools (6 µg) and the appropriate Nucleofector™ Kit (LNCaP: Kit R; PC-3: Kit V) in a final volume of 100 µl. Electroporations were carried out with an Amaxa Nucleofector™ II (Lonza, Basel, Switzerland) in accordance with manufacture guidelines. After electroporation, the cells recovered for 10 min in micro-centrifuge tubes containing 500 µl pre-warmed media before being plated in the indicated assays. All cDNAs for wild-type, constitutively active (Y527F), and kinase-inactivated (K298M) Src, and for wild-type and mutant (Y552F, Y557F, Y619F) Tks5 were subcloned into the pSGT mammalian expression vector [27]. Tks5-selective (siTks5; #M-006657-02) or nontargeting control (siNTC; #D-001206-13-20) siRNA pools were from Dharmacon (Waltham, MA).

Immunoblotting

Lysates were prepared from cells grown on cell culture dishes under described assay conditions. Cell lysis, protein concentration assays, SDS–PAGE, and immunoblotting were all as previously described [24]. Immunoblotting antibodies included Tks5 (1:1,000; #sc-30122; Santa Cruz Biotechnology, Santa Cruz, CA), Src (1:1,000; #2108; Cell Signaling, Danvers, MA), phospho-Src Y416 (1:1,000; #2101; Cell Signaling), phosphotyrosine (1:1,000; clone 4G10; #05-321; Millipore, Billerica, MA), GAPDH (1:1,000; #sc-25778; Santa Cruz), and the appropriate species-specific peroxidase-conjugated secondary antibodies (1:2,500; GE Health-care, Piscataway, NJ). In order to detect Tks5 phosphorylation, Tks5 was immunoprecipitated from whole cell lysates and immunoblotted with a phosphotyrosine antibody as previously described [24].

Invadopodia Formation

Invadopodia formation was monitored in Tks5-modified LNCaP cells cultured directly on glass cover-slips (Carolina Biological Supply, Burlington, NC) or on coverslips coated with gelatin (50 µg/ml; #G1890-100G; Sigma) or poly-L-lysine (50 µg/ml; #P6282; Sigma) under described assay conditions. At the end of the experiment, the coverslips were fixed and processed as previously described [24]. Immunolabeling antibodies included Tks5 (1:200, 1,736.9 [19]), cortactin (1:200; clone 4F11; #05-180; Millipore), and the appropriate species-specific fluorescent-conjugated secondary antibodies (1:1,000; Molecular Probes, Eugene, OR). Filamentous actin was labeled with fluorescent-conjugated phalloidin (1:200;

Molecular Probes). Images were captured with an Olympus IX70 inverted fluorescent microscope equipped with a Retiga 2000R digital color camera and using a UPlanFI 60X/1.25 objective (Olympus, Center Valley, PA). Image processing was conducted with Image Pro Plus 5.1 software.

In Situ Zymography

Invadopodia-associated matrix degradation (*i.e.*, gelatin degradation by in situ zymography) was monitored in Src- and Tks5-modified LNCaP and PC-3 cell lines under the indicated assay conditions as previously described [24]. The combined data from three, independent experiments (minimum of 75 cells and 8 random images per experiment) were plotted as the area of degradation per cell per image.

Migration and Invasion Assays

Motility and invasion was monitored in Src- and Tks5-modified LNCaP and PC-3 cells. Stable LNCaP cell lines and recently electroporated PC-3 cells were washed in PBS, counted, and transferred in triplicate (LNCaP: 1×10^4 ; PC-3: 5×10^4) to the upper well of rehydrated, transwell motility (8 μ ; #353097; BD Biosciences, San Jose, CA) or Matrigel-coated invasion (8 μ ; #354480; BD Biosciences) chambers in RPMI-1640 media containing 0.5% FBS in a final volume of either 100 μ l (motility) or 500 μ l (invasion). Gelatin-coated invasion chambers were generated by adding 50 μ l gelatin (200 μ g/ml in PBS; Sigma) to the upper well of motility chambers, incubating for 1–2 hr at 37°C, and washing in PBS. LNCaP motility and invasion assays also contained a fibronectin undercoating, generated by applying 100 μ l fibronectin (10 μ g/ml in PBS; BD Biosciences) to the underside of the upper well, incubating for 1–2 hr at room temperature, and washing in PBS. The lower wells of all assays contained 500 μ l of RPMI-1640 media supplemented with 10% FBS. Motility and invasion assays proceeded for 24 (LNCaP) or 48 (PC-3) hr at 37°C. Transwell chambers were fixed and stained as previously described [24]. Migration and invasion were based on the average number of cells on the underside of the membrane in 10 random images generated at 20 $\times$ magnification per chamber, and was normalized to the results from control cells.

Zymography

MMP2 and MMP9 gelatinase activity were measured in the serum-free conditioned media of confluent vector control and Tks5-modified LNCaP cell cultures after 24 hr as previously described [24].

Statistical Analysis

Graphical representation and descriptive statistics were used to describe the measurements discussed in this article. A Student's *t*-test was used to test the fold change between groups for several of the outcomes in this article. Associations of Tks5 with Gleason scores, gelatin degradation, and invadopodia development were analyzed using mixed models to account for the correlation between samples from a similar source. Contrasts were set up to test specified differences between the groups.

RESULTS

Tks5 is Expressed in Prostate Cancer Specimens and Cell Lines

Tks5 is a Src tyrosine kinase substrate and adaptor protein previously implicated in the invasive behavior of breast and melanoma cell lines [16,22]. Because invasive disease is also correlated with patient survival in prostate cancer, we examined the extent of Src-Tks5 signaling in prostate cancer patient specimens and cell lines. It has already been documented that activated Src family kinases correlate with the development of androgen-independent prostate cancer, metastasis to the bone, and overall patient survival [28–30]. Here, while carrying out an immunohistochemical analysis of Tks5 in a prostate tumor microarray, we observed an initial decline in Tks5 between normal prostate tissue and low Gleason score tumor specimens followed by increasing Tks5 protein levels such that high Gleason score tumors exhibited the greatest Tks5 staining intensity of all specimens analyzed (Fig. 1A).

Normal prostate tissue showed significant immunological staining for Tks5, with the mean intensity on a scale from 1 (low) to 4 (high) being 2.55 (Fig. 1A). Staining was most dramatic in basal epithelial cells making some of the glands to appear to have a striated epithelium (Fig. 1B). However, Tks5 staining was also seen in secretory epithelial cells as well as in the connective tissue surrounding the glands. In tumors with low combined Gleason scores (≤ 6), overall Tks5 expression routinely declined (mean intensity of 2.33), though this did not achieve statistical significance with the data set in this array (Fig. 1A). The loss of Tks5 was observed in both epithelial cells and connective tissue (Fig. 1C). Despite the initial decline in Tks5 in low Gleason score specimens, Tks5 staining intensity in many Gleason score 7 tumors (mean intensity of 2.57) and to a much greater extent in aggressive tumors with Gleason scores of 8–10 (mean intensity of 2.94) were higher (Fig. 1A, D, and E). Indeed Tks5 staining intensity in Gleason score 8–10 tumors were significantly higher than all other Gleason categorizations, including normal prostate tissue (Fig. 1A). We also noted that some individual tumor cells appeared to exhibit intense Tks5 staining along the periphery of the cytoplasm suggesting that Tks5 was localized to the membrane of these cells (Fig. 1E).

To gain further insights into the role of Src-Tks5 signaling in prostate cancer, we also examined the lysates from LNCaP and PC-3 cells, two well-characterized human prostate cancer cell lines. Both Src and Tks5 protein were detected in these cell lines (Fig. 2). However, while Src levels were similar between LNCaP and PC-3 cells, the protein levels of an activated form of Src phosphorylated at tyrosine position 416, of other tyrosine phosphorylated proteins, used here as a surrogate marker of tyrosine kinase activity, and of Tks5 were all much higher in PC-3 cells (Fig. 2). These data suggest that the two prostate cancer cell lines provide unique models of Src-Tks5 signaling. LNCaP cells represent a model in which Src activity and Tks5 expression are at low levels, while PC-3 cells model elevated Src activity and Tks5.

Tks5 Induces Invadopodia Development in LNCaP Cells

Since we observed relatively low levels of Src-Tks5 signaling in the LNCaP cell line, we first wanted to determine the effect of Tks5 overexpression in these cells. To this end, stable

cell lines were generated from parental LNCaP cells virally infected with an empty control vector (pBABEpuro) or with one containing a wild-type, murine Tks5 construct (Fig. 3A). We noted that there were no significant differences in the growth rate these of vector control and Tks5-modified LNCaP cell lines (Supplemental Fig. S1). Since Tks5 expression had previously been shown to regulate matrix remodeling activity in other cancer cell lines [16,22,24], we cultured each stable LNCaP cell line on a fluorescently labeled gelatin matrix to determine if these cells were similarly capable of gelatin degradation. As shown in Figure 3B, vector control LNCaP cells were unable to degrade gelatin. This was also true of parental LNCaP cells (data not shown). In contrast, fine, punctate, and spidery patterns of gelatin degradation were seen underneath or in the general vicinity of Tks5-over-expressing LNCaP cells after a 24 hr incubation on this substratum (Fig. 3C).

Given that Tks5 overexpression in LNCaP cells conferred gelatin degradation activity in situ, we naturally wondered whether Tks5 could sufficiently alter the cytoskeletal structure of these cells and generate invadopodia. To test this, vector control and Tks5-modified LNCaP cells were cultured on poly-L-lysine-coated glass coverslips for 12 hr, then fixed and stained for filamentous actin (F-actin). Vector control LNCaP cells generally exhibited an elongated morphology (Fig. 3D). F-actin was distributed throughout the cell, though enriched along the membrane and bundled at some of the termini of long cell extensions. Numerous actin-rich filopodia-like structures were also seen in these cells. Much of this cytoskeletal organization was also retained in the Tks5-overexpressing LNCaP cells (Fig. 3E). However, there were also numerous punctate F-actin structures scattered throughout the cell, and with a size and morphology reminiscent of invadopodia. We also noted that these invadopodia-like structures were more pronounced when the Tks5-overexpressing cells were cultured on poly-L-lysine as compared to gelatin or glass (Supplemental Fig. S2). In consideration that Tks5 overexpression had induced invadopodia formation in LNCaP cells, we tested the distribution of known invadopodia markers in this cell line. Indeed, we observed that Tks5, the invadopodia marker protein cortactin, and F-actin all co-localized at these punctate structures (Fig. 3F and G, Supplemental Fig. S3). Tks5 and cortactin also co-localized with punctate regions of gelatin degradation in these cells (Fig. 3H and I).

Given both the induction of gelatin degradation and the formation of punctate structures with co-localized invadopodia markers, Tks5 overexpression alone appears to be sufficient to induce invadopodia development in the LNCaP prostate cancer cell line.

Tks5 Regulates LNCaP Cell Invasion

Because invadopodia-associated matrix degradation was conferred by Tks5 overexpression, we next wanted to determine whether invasion was also altered in these cells. We typically use commercially available Matrigel-coated invasion chambers for this purpose, an assay that measures the ability of cells to permeate this matrix barrier while moving down a gradient comprised of growth factors and fibronectin. However, while Tks5 overexpression was sufficient to induce the degradation of a gelatin matrix, there was no significant difference in invasion through Matrigel between the vector control and Tks5-modified cells (Fig. 4). To further explore this matrix-related disparity in invasive behavior, we carried out the invasion assay using a gelatin matrix, and this time observed a significant twofold

increase in invasion in the Tks5-modified cells (Fig. 4). Such a difference between Matrigel and gelatin invasion was not explained by the functional gelatinase (MMP2, MMP9) levels in these cell lines, which remained unchanged (Fig. 3A). We also did not observe any differences in chemotaxis in the absence of a matrix barrier (Fig. 4). We therefore conclude that Tks5-associated invadopodia development specifically modulates the invasive phenotype of LNCaP cells, though this invasion appears to remain sensitive to the complement of matrix proteins being engaged by the cells.

Src Tyrosine Kinase Activity Regulates Tks5-Dependent Gelatin Degradation in LNCaP Cells

Because Tks5 is a Src tyrosine kinase substrate [19], we also wanted to examine the importance of Tks5 phosphorylation to invadopodia-associated gelatin degradation. This was first approached with pharmacological agents and mutant constructs that modify Src signaling. When Tks5-modified LNCaP cells were treated for 24 hr with the Src inhibitor PP2, there was a significant reduction in gelatin degradation relative to vehicle control-treated cells (Fig. 5A–C). Likewise, transient expression of kinase-inactivated Src-K298M, used here as a dominant-negative inhibitor of Src signaling [31,32], also significantly reduced gelatin degradation when compared to vector control cells (Fig. 5D–F and H). Conversely, Tks5-modified LNCaP cells transiently expressing constitutively active Src-Y527F exhibited a significant enhancement in gelatin degradation activity (Fig. 5D, E, G, and H). Thus, Src activity is also important for Tks5-regulated invadopodia development, underscoring the potential importance of Src-Tks5 signaling for invadopodia activity in this prostate cancer cell line.

Tks5 Phosphorylation is Necessary for Invadopodia Development in LNCaP Cells

Tks5 has several tyrosine residues between the third and fourth SH3 domains that resemble the consensus phosphorylation sites of known Src substrates [19]. Some of those sites also appear to be phosphorylated in the presence of activated Src [16,25]. Since Src activity was relatively low in parental LNCaP cells (Fig. 2), and there was an increase in gelatin degradation following the transient expression of activated Src in Tks5-modified LNCaP cells (Fig. 5), we wondered whether Src-dependent tyrosine phosphorylation of Tks5 might also regulate gelatin degradation activity in this cell line. To test that hypothesis, we monitored Tks5 phosphorylation and gelatin degradation in LNCaP cells following the transient expression of mutant Tks5 constructs harboring tyrosine to phenylalanine mutations at three potential Src phosphorylation sites located at positions 552, 557, and 619. First, as with the stable Tks5-modified LNCaP cells, we showed that the acute expression of a wild-type Tks5 construct into parental LNCaP cells supported an increase in gelatin degradation activity (Fig. 6A–C, and L). This increase in gelatin degradation was also observed in LNCaP cells transfected with a mutant form of Tks5 harboring a tyrosine to phenylalanine mutation at position 552 (Fig. 6A, B, D, and L). In contrast, similar tyrosine to phenylalanine mutations at positions 557 or 619 failed to stimulate gelatin degradation (Fig. 6A, B, E, F, and L). These data suggest that tyrosine phosphorylation at residues 557 and 619 (but not 552) may be necessary for Tks5-mediated gelatin degradation, however, these assay conditions were unable to detect any tyrosine phosphorylation of Tks5 (Fig. 6A).

We surmised that our inability to detect Tks5 phosphorylation in LNCaP cells was due, in part, to the relative insensitivity of the assay and because of the limited Src tyrosine kinase activity in this cell line (Figs. 2 and 6A). To remedy this, we repeated the previous experiment, but this time in the presence of constitutively activated Src-Y527F. Immunoblot analysis of cell lysates confirmed the expression of Src, shown here via detection of phosphorylated Src (Src-pY416) in LNCaP cell lysates (Fig. 6A). First, we observed that the co-expression of both activated Src and wild-type Tks5 stimulated gelatin degradation beyond that of Tks5 overexpression alone (Fig. 6A, C, H, and L). More importantly, we were also able to detect the tyrosine phosphorylation of Tks5 in these cells (Fig. 6A). When testing the Tks5 mutants in this assay, we noted that the relative increase in gelatin degradation was similarly dependent on the specific site of Tks5 mutation, and that gelatin degradation correlated with the extent of Tks5 phosphorylation. That is, the Tks5-Y552F mutation significantly enhanced gelatin degradation activity in a manner similar to that of wild-type Tks5, and retained much of the tyrosine phosphorylation seen with wild-type Tks5 as well (Fig. 6A, H, I, and L). In contrast, the Tks5-Y557F and Tks5-Y619F mutations largely failed to stimulate gelatin degradation activity (Fig. 6A, J, K, and L), and in correlative fashion demonstrated much reduced tyrosine phosphorylation relative to wild-type Tks5 (Fig. 6A).

Taken together, these data show that each tyrosine modification can affect Tks5 function, and that specifically residues 557 and 619 have the greatest impact on Tks5 phosphorylation, and the invadopodia-associated gelatin degradation stimulated by Tks5 overexpression in the LNCaP prostate cancer cell line.

Tks5 Phosphorylation is Necessary for Invadopodia-Associated Gelatin Degradation and Invasion in PC-3 Cells

Encouraged by our discovery that Tks5 is sufficient to initiate invadopodia formation and matrix-remodeling activity in LNCaP cells, we further hypothesized that reducing Tks5 expression in the PC-3 prostate cancer cell line would abrogate their invasive behavior. Consistent with a previous report, we recognized early on that parental PC-3 cells, like parental LNCaP cells, were only weakly competent for gelatin degradation activity in situ [33], and that these cells could be rendered a more efficient producer of matrix-remodeling invadopodia through the addition of an activated Src-Y527F construct (Fig. 6 and Supplemental Fig. S4). We therefore used stable, Src-activated PC-3 cells to determine whether their invadopodia activity was Tks5-dependent. In order to induce acute silencing of Tks5, we introduced a pool of Tks5-selective siRNAs into PC-3 cells and confirmed Tks5 protein knockdown by immunoblot analysis (Fig. 7A). In corresponding analysis of cells cultured on a fluorescent gelatin matrix, we observed a significant reduction in gelatin degradation in the cells silenced for Tks5 expression relative to cells treated with a nontargeting control siRNAs (Fig. 7B–D). We further observed that when the Src-activated, Tks5-silenced PC-3 cells were cultured on Matrigel-coated invasion chambers for 48 hr, there was a significant reduction in the number of invading cells relative to cells treated with non-targeting control siRNAs (Fig. 7E). This reduction in invasion was confirmed in stable Tks5 knockdown PC-3 cell lines, both in cells that were expressing activated Src (Fig. 7F) and in parental PC-3 cells as well (Fig. 7G). PC-3 cells are therefore sensitive to the loss of

Tks5 for their invasive behavior, an observation made previously in other invasive cancer cell lines [13,22,34].

To determine whether there was any role for Tks5 phosphorylation in the invasive behavior of PC-3 cells, stable Tks5 knockdown PC-3 cells were transiently reintroduced to either wild-type Tks5 or to the Tks5 tyrosine phosphorylation mutant constructs, all in the presence of activated Src-Y527F. Figure 8A confirms the expression of activated Src (Src-pY416) and the wild-type and mutant Tks5 constructs in these cells. In PC-3 cells, as with the LNCaP cells before, both wild-type and Y552F mutant Tks5 constructs were phosphorylated in the presence of Src, and the Y557F and Y619F mutant Tks5 constructs were not (Fig. 8A). Also like the LNCaP cells, the invasive behavior of PC-3 cells could be correlated with the extent of Tks5 phosphorylation. Wild-type and Y552F mutant Tks5, for example, supported a significant increase in gelatin degradation, while the Y557F and Y619F Tks5 mutants did not (Fig. 8B). Likewise, chemotactic invasion through Matrigel trended upward with wild-type Tks5 and the Y552F mutant, but was reduced in the presence of the Y557F and Y619F mutant Tks5 constructs (Fig. 8C). Indeed, expression of the Y619F Tks5 construct significantly inhibited the basal invasive behavior of Src-activated, Tks5-silenced PC-3 cells suggesting a dominant-negative effect on invasion by this Tks5 mutation.

DISCUSSION

Morbidity and mortality in prostate cancer patients are strictly associated with metastatic disease. Still the mechanisms driving invasion and metastasis remain poorly understood. This study considers the role of Src-Tks5 signaling and of specialized cytoskeletal structures called invadopodia in the invasive behavior of prostate cancer cells. Invadopodia are focalized sites for cell–matrix adhesion and protease-associated matrix remodeling [2,12,35–41]. In LNCaP cells we show for the first time that Tks5 overexpression alone leads to invadopodia formation, a remarkable feature for a scaffolding protein with no known enzymatic function. Additionally, in LNCaP and PC-3 cells the invadopodia-associated invasive phenotype is dependent on Src tyrosine kinase activity and Tks5 tyrosine phosphorylation. We also demonstrate in this study the upregulation of Tks5 in aggressive, high Gleason score specimens from prostate cancer patients. Our results therefore highlight a general role for Src-Tks5 signaling in processes associated with prostate tumor progression.

Already there is evidence for Src potentiation of prostate tumor progression [42]. Src is expressed in both prostate cancer cell lines and tumor specimens [26,28,43,44], and it was recently observed that increased levels of activated Src at the membrane correlated with a decrease in overall prostate cancer patient survival [28]. Prostate tumor progression is typically associated with the development of growth independence from the hormone androgen. New data indicate that this may also rely on enhanced Src signaling. For example, tumor tissue specimens and prostate cancer cells isolated from castrated mice harboring androgen-refractory tumors contained higher levels of activated Src than similar samples isolated from noncastrated littermates [30]. Apparently, Src phosphorylates the androgen receptor to promote the growth of androgen-independent tumors. It is therefore worth

speculating whether Src activation precedes the development of and is required for prostate cancer metastasis.

What is clear is that Src can regulate invasive cell behavior through cytoskeletal rearrangements leading to podosome/invadopod formation and function [5,16,24,25,45–48]. This appears to be true in prostate cancer where at least three previous studies have connected Src signaling with invadopodia development. In one study, the expression of osteopontin, an autocrine/paracrine factor important for prostate cancer growth and metastasis to the bone, directly correlated with the number of punctate invadopodia-like structures, as revealed by the co-staining of F-actin, vinculin, WASp, and MMP9 [49]. This correlated with MMP9-dependent degradation of gelatin and invasion through collagen. Importantly, osteopontin-dependent invasion was also mediated by a signaling pathway involving the integrin receptor $\alpha_v\beta_3$, PKC α , and the activation of Src [50]. In another study, ERK5 was identified as a regulator of PC-3 invasive behavior and its overexpression created a punctate pattern of gelatin degradation consistent with invadopodia development [33]. This agrees with a previous report showing that Src-transformed mouse embryonic fibroblasts isolated from ERK5 knockout mice are incapable of forming podosomes, and that re-expression of ERK5 restores their development [51]. Finally, invadopodia formation in prostate cancer cells can be regulated by the guanine nucleotide exchange factor Fgd1 [52]. In endothelial cells, TGF- β triggers Src-mediated Fgd1 phosphorylation and its recruitment to the cell membrane. Once there, Fgd1 activates Cdc42, modulates the actin cytoskeleton, and promotes podosome formation [53]. In PC-3 cells, Fgd1-silencing inhibited invadopodia-mediated matrix degradation activity. A similar observation has been made in a human melanoma cell line [52].

In this study, we implicate the Src substrate and adaptor protein Tks5 in invadopodia-associated prostate cancer cell invasion. Using site-directed mutagenesis, we demonstrated in both LNCaP and PC-3 cells that tyrosine phosphorylation of Tks5 at positions 557 and 619 was essential for Tks5-mediated invadopodia development and invasion. Two previous studies have demonstrated the importance of these tyrosine residues in Src-mediated Tks5 phosphorylation and podosome/invadopod function. First, the combined mutation of tyrosine 557 and 619 to phenylalanines in osteoclasts inhibited Tks5 phosphorylation and decreased podosome formation [25]. It was also noted that the combined mutation blocked an association between FLAG- and GFP-tagged Tks5 constructs. The potential dimerization of Tks5 en route to osteoclast podosome development implies that Tks5 phosphorylation may alter its conformation and open previously masked Tks5 scaffolding sites [25]. In keeping with this idea, one or more of these same residues seem to regulate the binding of Tks5 to WASp (557 and 619) and Nck (557) as their interactions cease in the presence of the indicated Tks5 tyrosine to phenylalanine mutations [16]. The binding of Tks5 to Nck occurs directly at the site of Tks5 phosphorylation, and mutations at either tyrosine 557 of Tks5 or the phosphotyrosine-binding SH2 domain of Nck abolish actin polymerization at invadopodia and therefore invadopodia-associated gelatin degradation activity. In the context of prostate cancer, it is not clear whether Tks5 binds to WASp or Nck (or to other proteins), but since Tks5 alone is sufficient to stimulate new actin polymerization and focalized matrix remodeling at sites of invadopodia in LNCaP cells, such a prospect is likely. Clearly, identifying Tks5-binding partners, their potential regulation by Tks5

phosphorylation, and their collective role in invadopodia development will be informative of the invasive processes employed by prostate cancer cells and the cells of other tumor origins.

During our analysis of Tks5 in prostate cancer, we noted a robust effect of ectopic Tks5 expression on invadopodia-associated gelatin degradation, but a more modest effect on invasion. We speculate that these differences may, in part, reflect on the matrix proteins being engaged by the cells. Matrigel, which is primarily laminin, collagen IV, and enactin, and gelatin, which is largely hydrolyzed collagen I, may differentially activate proteases needed for matrix degradation and invasion [54]. For example, aortic endothelial cells that form linear podosome-related structures fail to degrade gelatin, but have no difficulty degrading a mixture of gelatin and fibrillar collagen I [55]. Src-transformed fibroblasts also show enhanced degradation activity when gelatin and collagen I are mixed. Apparently collagen I can trigger the activation of a protease cascade in which MT1-MMP activates the gelatinase MMP2 [56–58]. While we do not see changes in MMP2 (or MMP9) activity in the Tks5-overexpressing LNCaP cells, it remains possible that the inclusion of gelatin or collagen I during cell culture may stimulate their activity (or that of other proteases) or may impact the clustering of proteases at invadopodia. We also cannot rule out the role that matrix rigidity or stiffness might play in the Tks5-modulated invasion of prostate cancer cells. When breast cancer cells are plated on increasing concentrations of gelatin, there is a direct relationship between gelatin thickness and invadopodia-associated matrix remodeling activity [59].

As with Src, Tks5 could be a potential biomarker for staging prostate tumor progression or for predicting patient outcome. When staining a prostate tumor microarray with a Tks5 antibody, we initially observed a decline in Tks5 from normal prostate tissue to low Gleason score tumors (6), but this was followed by a steady increase in Tks5 such that high Gleason score tumors [8–10] exhibited the highest Tks5 staining intensity of all tissue specimens analyzed, including that of normal prostate tissue. We also noted that some tumor cells exhibited especially high Tks5 staining intensities near the cell surface (Fig. 1E, lower panel). It is thus worth speculating whether, consistent with recent in vitro models, some tumor cells exhibit sufficient Src-Tks5 signaling to enable the accumulation of Tks5 at the cell surface where tyrosine phosphorylation, PX domain exposure, and binding to phosphatidylinositol phosphates and other scaffolding molecules facilitates invadopodia development [13,16,60]. Indeed, the presence of higher levels of Tks5 in potentially aggressive, high Gleason score prostate tumors reflects the results of our in vitro studies where Tks5 expression correlated with invadopodia-associated matrix degradation and invasion. High Tks5 protein levels have also been observed in advanced stage tumors of the brain, sometimes in association with patient survival [61]. The biological importance of the initial decrease in Tks5 staining between normal prostate tissue and low Gleason score tumors remains to be determined. Perhaps the initial process of tumor development that relies on changes in proliferative/anti-apoptotic activity is less dependent on Tks5 than on changes associated with tumor progression, such as the epithelial–mesenchymal transition in which Tks5 has been linked [62]. Another possibility is that there are different versions of Tks5 expressed in normal prostate cells relative to tumor cells. There are, for example, alternatively splice forms of Tks5 whose potentially differential functions might be critical

towards prostate tumor growth, invasion, and metastasis [19]. There is also a recent report suggesting that alternative Tks5 promoter sites generate long (with the PX domain) and short (without the PX domain) Tks5 isoforms, and that high long:short Tks5 isoform ratios are associated with invadopodia-mediated metastasis and may be prognostic for metastatic progression in lung adenocarcinoma patients [63].

CONCLUSIONS

In sum, we have demonstrated an important role for Tks5 in prostate cancer cell invasion. Expression of Tks5 alone is sufficient to drive invadopodia development and associated matrix-remodeling activity. Furthermore, Src-mediated Tks5 phosphorylation at tyrosines 557 and 619 is necessary for this invadopodia activity. Continued efforts will determine whether Tks5 is a valid biomarker for prostate cancer and whether the catalytic or structural features of invadopodia machinery represent a viable therapeutic target with the potential to benefit prostate cancer patients with an aggressive disease profile.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to express their gratitude to the following individuals who have assisted in this study: Drs. Guangchao Sui and Purnima Dubey for constructs used in prostate cancer cell line construction, Dr. Linda Metheny-Barlow for use of her laboratory's Amara Nucleofector II, Christopher Abdullah and Manishha Patel for preliminary characterization of the Tks5 antibody used for the immunohistochemistry experiments, and Robbin Newlin of the Sanford-Burnham Histopathology Core Facility for the Tks5 immunohistochemistry slide scanning. Special thanks are also extended to Dr. Steve Kridel for supplemental resources, helpful scientific discussions, and for the critical reading of this manuscript. D.F.S. further acknowledges the financial support of Mr. and Mrs. Ben Reynolds, the Wake Forest School of Medicine, and Appalachian State University. This work was supported by grants from the National Cancer Institute (T32-CA079448 to K.L.B. and CA153065, CA154002 to S.A.C.) and Department of Defense (W81XWH-08-1-0041 to D.F.S.).

References

1. Cancer facts & figures. Vol. 2012. Atlanta, GA: American Cancer Society; 2012.
2. Linder S. The matrix corroded: Podosomes and invadopodia in extracellular matrix degradation. *Trends Cell Biol.* 2007; 17(3):107–117. [PubMed: 17275303]
3. Gimona M, Buccione R. Adhesions that mediate invasion. *Int J Biochem Cell Biol.* 2006; 38(11): 1875–1892. [PubMed: 16790362]
4. Gimona M, Buccione R, Courtneidge SA, Linder S. Assembly and biological role of podosomes and invadopodia. *Curr Opin Cell Biol.* 2008; 20(2):235–241. [PubMed: 18337078]
5. Luxenburg C, Parsons JT, Addadi L, Geiger B. Involvement of the Src-cortactin pathway in podosome formation and turnover during polarization of cultured osteoclasts. *J Cell Sci.* 2006; 119(Pt 23):4878–4888. [PubMed: 17105771]
6. Courtneidge SA. Cell migration and invasion in human disease: The Tks adaptor proteins. *Biochem Soc Trans.* 2012; 40(1):129–132. [PubMed: 22260678]
7. Frame MC. Newest findings on the oldest oncogene; how activated src does it. *J Cell Sci.* 2004; 117(Pt 7):989–998. [PubMed: 14996930]
8. Chang YM, Bai L, Liu S, Yang JC, Kung HJ, Evans CP. Src family kinase oncogenic potential and pathways in prostate cancer as revealed by AZD0530. *Oncogene.* 2008; 27(49):6365–6375. [PubMed: 18679417]

9. Zhang XH, Wang Q, Gerald W, Hudis CA, Norton L, Smid M, Foekens JA, Massague J. Latent bone metastasis in breast cancer tied to Src-dependent survival signals. *Cancer Cell*. 2009; 16(1): 67–78. [PubMed: 19573813]
10. Summy JM, Gallick GE. Src family kinases in tumor progression and metastasis. *Cancer Metastasis Rev*. 2003; 22(4):337–358. [PubMed: 12884910]
11. Destaing O, Sanjay A, Itzstein C, Horne WC, Toomre D, De Camilli P, Baron R. The tyrosine kinase activity of c-Src regulates actin dynamics and organization of podosomes in osteoclasts. *Mol Biol Cell*. 2008; 19(1):394–404. [PubMed: 17978100]
12. Linder S, Aepfelbacher M. Podosomes: Adhesion hot-spots of invasive cells. *Trends Cell Biol*. 2003; 13(7):376–385. [PubMed: 12837608]
13. Oikawa T, Itoh T, Takenawa T. Sequential signals toward podosome formation in NIH-src cells. *J Cell Biol*. 2008; 182(1):157–169. [PubMed: 18606851]
14. Oser M, Mader CC, Gil-Henn H, Magalhaes M, Bravo-Cordero JJ, Koleske AJ, Condeelis J. Specific tyrosine phosphorylation sites on cortactin regulate Nck1-dependent actin polymerization in invadopodia. *J Cell Sci*. 2010; 123(Pt 21):3662–3673. [PubMed: 20971703]
15. Park SJ, Suetsugu S, Takenawa T. Interaction of HSP90 to N-WASP leads to activation and protection from proteasome-dependent degradation. *EMBO J*. 2005; 24(8):1557–1570. [PubMed: 15791211]
16. Styli SS, Stacey TT, Verhagen AM, Xu SS, Pass I, Courtneidge SA, Lock P. Nck adaptor proteins link Tks5 to invadopodia actin regulation and ECM degradation. *J Cell Sci*. 2009; 122(Pt 15): 2727–2740. [PubMed: 19596797]
17. Badowski C, Pawlak G, Grichine A, Chabadel A, Oddou C, Jurdic P, Pfaff M, Albiges-Rizo C, Block MR. Paxillin phosphorylation controls invadopodia/podosomes spatiotemporal organization. *Mol Biol Cell*. 2008; 19(2):633–645. [PubMed: 18045996]
18. Tehrani S, Tomasevic N, Weed S, Sakowicz R, Cooper JA. Src phosphorylation of cortactin enhances actin assembly. *Proc Natl Acad Sci USA*. 2007; 104(29):11933–11938. [PubMed: 17606906]
19. Lock P, Abram CL, Gibson T, Courtneidge SA. A new method for isolating tyrosine kinase substrates used to identify fish, an SH3 and PX domain-containing protein, and Src substrate. *EMBO J*. 1998; 17(15):4346–4357. [PubMed: 9687503]
20. Abram CL, Seals DF, Pass I, Salinsky D, Maurer L, Roth TM, Courtneidge SA. The adaptor protein fish associates with members of the ADAMs family and localizes to podosomes of Src-transformed cells. *J Biol Chem*. 2003; 278(19):16844–16851. [PubMed: 12615925]
21. Crimaldi L, Courtneidge SA, Gimona M. Tks5 recruits AFAP-110, p190RhoGAP, and cortactin for podosome formation. *Exp Cell Res*. 2009; 315(15):2581–2592. [PubMed: 19540230]
22. Seals DF, Azucena EF Jr, Pass I, Tesfay L, Gordon R, Woodrow M, Resau JH, Courtneidge SA. The adaptor protein Tks5/Fish is required for podosome formation and function, and for the protease-driven invasion of cancer cells. *Cancer Cell*. 2005; 7(2):155–165. [PubMed: 15710328]
23. Blouw B, Seals DF, Pass I, Diaz B, Courtneidge SA. A role for the podosome/invadopodia scaffold protein Tks5 in tumor growth in vivo. *Eur J Cell Biol*. 2008; 87(8–9):555–567. [PubMed: 18417249]
24. Burger KL, Davis AL, Isom S, Mishra N, Seals DF. The podosome marker protein Tks5 regulates macrophage invasive behavior. *Cytoskeleton (Hoboken)*. 2011; 68(12):694–711. [PubMed: 22021214]
25. Oikawa T, Oyama M, Kozuka-Hata H, Uehara S, Udagawa N, Saya H, Matsuo K. Tks5-dependent formation of circumferential podosomes/invadopodia mediates cell–cell fusion. *J Cell Biol*. 2012; 197(4):553–568. [PubMed: 22584907]
26. Nam S, Kim D, Cheng JQ, Zhang S, Lee JH, Buettner R, Mirosevich J, Lee FY, Jove R. Action of the Src family kinase inhibitor, dasatinib (BMS-354825), on human prostate cancer cells. *Cancer Res*. 2005; 65(20):9185–9189. [PubMed: 16230377]
27. Erpel T, Superti-Furga G, Courtneidge SA. Mutational analysis of the Src SH3 domain: The same residues of the ligand binding surface are important for intra- and intermolecular interactions. *EMBO J*. 1995; 14(5):963–975. [PubMed: 7534229]

28. Tatarov O, Mitchell TJ, Seywright M, Leung HY, Brunton VG, Edwards J. SRC family kinase activity is up-regulated in hormone-refractory prostate cancer. *Clin Cancer Res*. 2009; 15(10): 3540–3549. [PubMed: 19447874]
29. Goldenberg-Furmanov M, Stein I, Pikarsky E, Rubin H, Kasem S, Wygoda M, Weinstein I, Reuveni H, Ben-Sasson SA. Lyn is a target gene for prostate cancer: Sequence-based inhibition induces regression of human tumor xenografts. *Cancer Res*. 2004; 64(3):1058–1066. [PubMed: 14871838]
30. Guo Z, Dai B, Jiang T, Xu K, Xie Y, Kim O, Nesheiwat I, Kong X, Melamed J, Handratta VD, Njar VC, Brodie AM, Yu LR, Veenstra TD, Chen H, Qiu Y. Regulation of androgen receptor activity by tyrosine phosphorylation. *Cancer Cell*. 2006; 10(4):309–319. [PubMed: 17045208]
31. Kim M, Tezuka T, Tanaka K, Yamamoto T. Cbl-c suppresses v-Src-induced transformation through ubiquitin-dependent protein degradation. *Oncogene*. 2004; 23(9):1645–1655. [PubMed: 14661060]
32. Ahn S, Maudsley S, Luttrell LM, Lefkowitz RJ, Daaka Y. Src-mediated tyrosine phosphorylation of dynamin is required for beta2-adrenergic receptor internalization and mitogen-activated protein kinase signaling. *J Biol Chem*. 1999; 274(3):1185–1188. [PubMed: 9880482]
33. Ramsay AK, McCracken SR, Soofi M, Fleming J, Yu AX, Ahmad I, Morland R, Machesky L, Nixon C, Edwards DR, Nuttall RK, Seywright M, Marquez R, Keller E, Leung HY. ERK5 signalling in prostate cancer promotes an invasive phenotype. *Br J Cancer*. 2011; 104(4):664–672. [PubMed: 21266977]
34. Diaz B, Shani G, Pass I, Anderson D, Quintavalle M, Courtneidge SA. Tks5-dependent, nox-mediated generation of reactive oxygen species is necessary for invadopodia formation. *Sci Signal*. 2009; 2(88):ra53. [PubMed: 19755709]
35. Buccione R, Orth JD, McNiven MA. Foot and mouth: Podosomes, invadopodia and circular dorsal ruffles. *Nat Rev Mol Cell Biol*. 2004; 5(8):647–657. [PubMed: 15366708]
36. Clark ES, Whigham AS, Yarbrough WG, Weaver AM. Cortactin is an essential regulator of matrix metalloproteinase secretion and extracellular matrix degradation in invadopodia. *Cancer Res*. 2007; 67(9):4227–4235. [PubMed: 17483334]
37. Clark ES, Weaver AM. A new role for cortactin in invadopodia: Regulation of protease secretion. *Eur J Cell Biol*. 2008; 87(8–9):581–590. [PubMed: 18342393]
38. Artym VV, Zhang Y, Seillier-Moisewitsch F, Yamada KM, Mueller SC. Dynamic interactions of cortactin and membrane type 1 matrix metalloproteinase at invadopodia: Defining the stages of invadopodia formation and function. *Cancer Res*. 2006; 66(6):3034–3043. [PubMed: 16540652]
39. Chen WT, Wang JY. Specialized surface protrusions of invasive cells, invadopodia and lamellipodia, have differential MT1-MMP, MMP-2, and TIMP-2 localization. *Ann N Y Acad Sci*. 1999; 878:361–371. [PubMed: 10415741]
40. Nakahara H, Howard L, Thompson EW, Sato H, Seiki M, Yeh Y, Chen WT. Transmembrane/cytoplasmic domain-mediated membrane type 1-matrix metalloproteinase docking to invadopodia is required for cell invasion. *Proc Natl Acad Sci USA*. 1997; 94(15):7959–7964. [PubMed: 9223295]
41. Monsky WL, Lin CY, Aoyama A, Kelly T, Akiyama SK, Mueller SC, Chen WT. A potential marker protease of invasiveness, seprase, is localized on invadopodia of human malignant melanoma cells. *Cancer Res*. 1994; 54(21):5702–5710. [PubMed: 7923219]
42. Fizazi K. The role of Src in prostate cancer. *Ann Oncol*. 2007; 18(11):1765–1773. [PubMed: 17426060]
43. Sridhar SC, Miranti CK. Tetraspanin KAI1/CD82 suppresses invasion by inhibiting integrin-dependent crosstalk with c-Met receptor and Src kinases. *Oncogene*. 2006; 25(16):2367–2378. [PubMed: 16331263]
44. Recchia I, Rucci N, Festuccia C, Bologna M, MacKay AR, Migliaccio S, Longo M, Susa M, Fabbro D, Teti A. Pyrrolopyrimidine c-Src inhibitors reduce growth, adhesion, motility and invasion of prostate cancer cells in vitro. *Eur J Cancer*. 2003; 39(13):1927–1935. [PubMed: 12932673]
45. Murphy DA, Diaz B, Bromann PA, Tsai JH, Kawakami Y, Maurer J, Stewart RA, Izpisua-Belmonte JC, Courtneidge SA. A Src-Tks5 pathway is required for neural crest cell migration during embryonic development. *PLoS ONE*. 2011; 6(7):e22499. [PubMed: 21799874]

46. Thompson O, Kleino I, Crimaldi L, Gimona M, Saksela K, Winder SJ. Dystroglycan, Tks5 and Src mediated assembly of podosomes in myoblasts. PLoS ONE. 2008; 3(11):e3638. [PubMed: 18982058]
47. Tatin F, Varon C, Genot E, Moreau V. A signalling cascade involving PKC, Src and Cdc42 regulates podosome assembly in cultured endothelial cells in response to phorbol ester. J Cell Sci. 2006; 119(Pt 4):769–781. [PubMed: 16449321]
48. Boateng LR, Cortesio CL, Huttenlocher A. Src-mediated phosphorylation of mammalian Abp1 (DBNL) regulates podosome rosette formation in transformed fibroblasts. J Cell Sci. 2012; 125(Pt 5):1329–1341. [PubMed: 22303001]
49. Desai B, Ma T, Chellaiah MA. Invadopodia and matrix degradation, a new property of prostate cancer cells during migration and invasion. J Biol Chem. 2008; 283(20):13856–13866. [PubMed: 18337256]
50. Jain S, Chakraborty G, Kundu GC. The crucial role of cyclooxygenase-2 in osteopontin-induced protein kinase C α /c-Src/IkappaB kinase α /beta-dependent prostate tumor progression and angiogenesis. Cancer Res. 2006; 66(13):6638–6648. [PubMed: 16818637]
51. Schramm M, Ying O, Kim TY, Martin GS. ERK5 promotes Src-induced podosome formation by limiting Rho activation. J Cell Biol. 2008; 181(7):1195–1210. [PubMed: 18573916]
52. Ayala I, Giacchetti G, Caldieri G, Attanasio F, Mariggio S, Tete S, Polishchuk R, Castronovo V, Buccione R. Faciogenital dysplasia protein Fgd1 regulates invadopodia biogenesis and extracellular matrix degradation and is up-regulated in prostate and breast cancer. Cancer Res. 2009; 69(3):747–752. [PubMed: 19141649]
53. Daubon T, Buccione R, Genot E. The Aarskog-Scott syndrome protein Fgd1 regulates podosome formation and extracellular matrix remodeling in transforming growth factor beta-stimulated aortic endothelial cells. Mol Cell Biol. 2011; 31(22):4430–4441. [PubMed: 21911474]
54. Hughes CS, Postovit LM, Lajoie GA. Matrigel: A complex protein mixture required for optimal growth of cell culture. Proteomics. 2010; 10(9):1886–1890. [PubMed: 20162561]
55. Juin A, Billottet C, Moreau V, Destaing O, Albiges-Rizo C, Rosenbaum J, Genot E, Saltel F. Physiological type I collagen organization induces the formation of a novel class of linear invadosomes. Mol Biol Cell. 2012; 23(2):297–309. [PubMed: 22114353]
56. Azzam HS, Thompson EW. Collagen-induced activation of the M(r) 72,000 type IV collagenase in normal and malignant human fibroblastoid cells. Cancer Res. 1992; 52(16):4540–4544. [PubMed: 1322793]
57. Theret N, Lehti K, Musso O, Clement B. MMP2 activation by collagen I and concanavalin A in cultured human hepatic stellate cells. Hepatology. 1999; 30(2):462–468. [PubMed: 10421655]
58. Ruangpanit N, Price JT, Holmbeck K, Birkedal-Hansen H, Guenzler V, Huang X, Chan D, Bateman JF, Thompson EW. MT1-MMP-dependent and -independent regulation of gelatinase A activation in long-term, ascorbate-treated fibroblast cultures: Regulation by fibrillar collagen. Exp Cell Res. 2002; 272(2):109–118. [PubMed: 11777335]
59. Alexander NR, Branch KM, Parekh A, Clark ES, Iwueke IC, Guelcher SA, Weaver AM. Extracellular matrix rigidity promotes invadopodia activity. Curr Biol. 2008; 18(17):1295–1299. [PubMed: 18718759]
60. Murphy DA, Courtneidge SA. The ‘ins’ and ‘outs’ of podosomes and invadopodia: Characteristics, formation and function. Nat Rev Mol Cell Biol. 2011; 12(7):413–426. [PubMed: 21697900]
61. Stylli SS, IST, Kaye AH, Lock P. Prognostic significance of Tks5 expression in gliomas. J Clin Neurosci. 2012; 19(3):436–442. [PubMed: 22249020]
62. Eckert MA, Lwin TM, Chang AT, Kim J, Danis E, Ohno-Machado L, Yang J. Twist1-induced invadopodia formation promotes tumor metastasis. Cancer Cell. 2011; 19(3):372–386. [PubMed: 21397860]
63. Li CM, Chen G, Dayton TL, Kim-Kiselak C, Hoersch S, Whittaker CA, Bronson RT, Beer DG, Winslow MM, Jacks T. Differential Tks5 isoform expression contributes to metastatic invasion of lung adenocarcinoma. Genes Dev. 2013; 27(14):1557–1567. [PubMed: 23873940]

Fig. 1.

Tks5 expression in prostate cancer specimens. Histological samples of normal and cancerous prostate tissue were stained with a Tks5 antibody. **A:** Tks5 staining intensity was evaluated on a scale of 1 (low) to 4 (high) by three, independent observers. The bar plot shows the relative percentage of each of the four Tks5 staining intensities for all specimens in each Gleason score category. The mean Tks5 staining intensity for each of the indicated Gleason score categories (normal, 6, 7, 8–10) is also shown. Statistical analysis of Tks5 staining intensity across each categorical Gleason score was done using a mixed model ($*P < 0.05$; $***P < 0.0001$). **B–E:** Representative core specimens from the tumor microarray are shown. The demarcated areas in the upper panels (scale bars, 500 μm) are magnified in the lower panels (scale bars, 100 μm).

Fig. 2.

Tks5 expression in prostate cancer cell lines. Total cell lysates prepared from LNCaP and PC-3 prostate cancer cell lines were evaluated for Tks5, Src, activated Src (pY416), and general tyrosine phosphorylation (pY) by immunoblot analysis.

Fig. 3.

Effect of Tks5 on invadopodia development and invadopodia-associated gelatin degradation in LNCaP cells. **A:** Vector control (pBABE) and Tks5-overexpressing (Tks5) LNCaP cells were evaluated for Tks5 and GAPDH protein levels in total cell lysates by immunoblot analysis and for MMP2 and MMP9 gelatinase activity in conditioned media by zymography. **B,C:** Vector control and Tks5-overexpressing LNCaP cells were cultured on AlexaFluor488-gelatin (green) for 24 hr, fixed and stained with DAPI to visualize nuclei (blue), and gelatin degradation (arrows) was visualized by fluorescence microscopy. **D,E:** Vector control and Tks5-overexpressing LNCaP cells were cultured on poly-L-lysine-coated coverslips for 12 hr, then fixed and stained with AlexaFluor546-phalloidin to visualize F-actin (red) and with DAPI to visualize the nuclei (blue). The arrows indicate punctate invadopodia-like structures. **F,G:** Tks5-overexpressing LNCaP cells were cultured on poly-L-lysine-coated coverslips for 12 hr, then fixed and stained with AlexaFluor488-phalloidin to visualize F-actin (green) and with antibodies to Tks5 or cortactin (red). **H,I:** Tks5-overexpressing LNCaP cells were cultured on AlexaFluor488-gelatin (green) for 12 hr, then fixed and stained with antibodies to Tks5 or cortactin (red). Note that the larger composite images in panels (F)–(I) are demarcated to depict the results for each individual detection agent in the inset images. Scale bars, 20 μ m.

Fig. 4.

Effect of Tks5 on LNCaP cell invasion. Vector control and Tks5-overexpressing LNCaP cells were plated in triplicate into the upper well of uncoated (motility), Matrigel-coated, or gelatin-coated trans well chambers in the presence of 0.5% FBS, and exposed to a chemotactic gradient composed of 10% FBS and 10 μ g/ml fibronectin. All assays proceeded for 24 hr, after which the cells were fixed and stained with Crystal Violet, and counted as the average number of invading cells/10 random images/chamber. All data are plotted as the fold change relative to vector control cells, and are the averages and standard deviations

from three, independent experiments. Statistical comparisons between vector control and Tks5-overexpressing cells were done using a Student's *t*-test ($^*P < 0.05$).

Fig. 5. Effect of Src on Tks5-dependent gelatin degradation in LNCaP cells. **A–C:** Tks5-overexpressing LNCaP cells were cultured on AlexaFluor488-gelatin for 24 hr in the absence (A) or presence (B) of PP2, and gelatin degradation (dark patches) was visualized by fluorescence microscopy. **C:** Matrix-remodeling invadopodia activity was quantified as the area of gelatin degradation/cell/image. Data are the averages and standard deviations from three, independent experiments. **D–H:** Tks5-overexpressing LNCaP cells were electroporated with the indicated vector control (pSGT) and mutant Src constructs (Y527F, K298M). **D:** Total cell lysates generated from cells cultured on plastic dishes for 24 hr were evaluated for Src, activated Src (pY416), and GAPDH by immunoblot analysis. **E–G:** Cells cultured on AlexaFluor488-gelatin for 24 hr were evaluated for gelatin degradation by fluorescence microscopy, and (H) quantified as the area of gelatin degradation/cell/image. Data are the combined averages and standard deviations from three, independent experiments. Statistical comparisons between DMSO and PP2 as well as between pSGT and either K298M or Y527F were done using a mixed model (* $P < 0.05$; ** $P < 0.01$). Scale bars, 20 μm .

Fig. 6.

Effect of Tks5 phosphorylation on invadopodia development in LNCaP cells. **A**: Total cell lysates (TCL) from LNCaP cells electroporated with the indicated vector control (pSGT), wild-type and mutant (Y552F, Y557F, Y619F) Tks5, and activated Src-Y527F constructs were evaluated for Tks5, activated Src (pY416), and GAPDH by immunoblot analysis. Total cell lysates were also immunoprecipitated with a Tks5 antibody (Tks5 IP) and immunoblotted with either aTks5 antibody or a phosphotyrosine antibody in order to monitor Tks5 tyrosine phosphorylation. **B–L**: Cells cultured on AlexaFluor488-gelatin for 24 hr were evaluated for gelatin degradation by fluorescence microscopy and (L) quantified as the area of gelatin degradation/cell/image. Data are the combined average and standard deviations from six, independent experiments. Statistical comparisons were done using a mixed model (* $P < 0.05$; ** $P < 0.01$). Scale bar, 20 μ m.

Fig. 7.

Effect of Tks5 on invadopodia-associated gelatin degradation and invasion in PC-3 cells. **A–E:** Src-activated PC-3 cells were electroporated with a pool of nontargeting control (siNTC) or Tks5-selective (siTks5) siRNAs. **A:** Total cell lysates from cells cultured on plastic dishes for 24 hr were evaluated for Tks5 and GAPDH by immunoblot analysis (n/e, nonelectroporated control sample). **B,C:** Cells cultured on Alexa-Fluor488-gelatin for 24 hr were evaluated for gelatin degradation by fluorescence microscopy and **(D)** quantified as the area of gelatin degradation/cell/image. Data are the combined average and standard deviations from three, independent experiments. Statistical comparisons were done using a mixed model (** $P < 0.01$). **E:** Cells cultured in transwell invasion chambers were evaluated for movement through a Matrigel barrier and normalized to the invasion of siNTC-treated PC-3 cells. **F,G:** Matrigel invasion of stable Tks5 knockdown (shTks5) cells was normalized to control (shSCR) cells in the background of **(F)** Src-activated (SrcY527F) and **(G)** parental PC-3 cells. All the invasion data are the average and standard deviations from three, independent experiments. Statistical comparisons were done using a Student's t -test (* $P < 0.05$; ** $P < 0.01$). Scale bar, 20 μ m.

Fig. 8.

Effect of Tks5 phosphorylation on invadopodia-associated gelatin degradation and invasion in PC-3 cells. **A–C:** Stable Tks5 knockdown PC-3 cells were transiently nucleofected with the indicated vector control (pSGT), wild-type and mutant (Y5527F, Y557F, Y619F) Tks5, and activated Src-Y527F constructs. **A:** Total cell lysates (TCL) from cells cultured on plastic dishes for 24 hr were evaluated for activated Src (pY416), Tks5, and GAPDH by immunoblot analysis. Total cell lysates were also immunoprecipitated with aTks5 antibody (Tks5 IP) and immunoblotted with either aTks5 antibody or a phosphotyrosine antibody in order to monitor Tks5 tyrosine phosphorylation. **B:** Cells cultured on AlexaFluor488-gelatin for 24 hr were imaged by fluorescence microscopy and gelatin degradation quantified as the area of gelatin degradation/cell/image. Data are the combined average and standard deviations from three, independent experiments. Statistical comparisons for the gelatin degradation assay between pSGT and either Tks5 or Tks5-Y552F were done using a mixed model (** $P < 0.01$). **C:** Matrigel invasion of the Tks5-modified cells was normalized to vector control cells. Data are the average and standard deviations from three, independent experiments. Statistical comparisons for the invasion assay between pSGT and Tks5-Y619F were done using a Student's *t*-test (* $P < 0.05$).