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Differential Tks5 isoform expression contributes to metastatic invasion of lung adenocarcinoma

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Metastasis accounts for the vast majority of cancer-related deaths, yet the molecular mechanisms that drive metastatic spread remain poorly understood. Here we report that Tks5, which has been linked to the formation of proteolytic cellular protrusions known as invadopodia, undergoes an isoform switch during metastatic progression in a genetically engineered mouse model of lung adenocarcinoma. Nonmetastatic primary tumor-derived cells predominantly expressed a short isoform, Tks5short, while metastatic primary tumor- and metastasis-derived cells acquired increased expression of the full-length isoform Tks5long. This elevation of Tks5long to Tks5short ratio correlated with a commensurate increase in invadopodia activity in metastatic cells compared with nonmetastatic cells. Further characterization of these isoforms by knockdown and overexpression experiments demonstrated that Tks5long promoted invadopodia in vitro and increased metastasis in transplant models and an autochthonous model of lung adenocarcinoma. Conversely, Tks5short decreased invadopodia stability and proteolysis, acting as a natural dominant-negative inhibitor to Tks5long. Importantly, high Tks5long and low Tks5short expressions in human lung adenocarcinomas correlated with metastatic disease and predicted worse survival of early stage patients. These data indicate that tipping the Tks5 isoform balance to a high Tks5long to Tks5short ratio promotes invadopodia-mediated invasion and metastasis.

[Keywords: Tks5; invadopodia; metastasis; lung adenocarcinoma; non-small-cell lung cancer; mouse model]

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Despite the high rates of mortality associated with metastatic lung cancer (Keshamouni et al. 2009; Siegel et al. 2013), the molecular mechanisms underlying disease progression remain incompletely understood. Metastasis accounts for the vast majority of all lung cancer fatalities, as even 30%–50% of early stage patients who undergo surgical resection eventually succumb to metastatic relapse, and patients with metastatic disease are almost always incurable (Keshamouni et al. 2009). The development of more effective therapeutic interventions for this disease will rely on improving our understanding of metastasis at the molecular level.

Tks5 (also known as Sh3pxd2a) has been previously implicated in promoting metastasis because of its role in invasive cellular structures known as invadopodia (Courtneidge 2012). Invadopodia are actin-rich, proteolytic membrane protrusions that were initially identified in Src-transformed mouse embryonic fibroblasts (Chen et al. 1984; Tarone et al. 1985) and later observed in a variety of cultured human cancer cells, including breast cancer, melanoma, and head and neck squamous cell carcinoma (Seals et al. 2005; Bowden et al. 2006; Clark et al. 2007). While invadopodia formation is spontaneous in some cancer cells in culture, it can be further stimulated by activating integrin β1 and receptors for epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Nakahara et al. 1998; Yamaguchi et al. 2005; Philippar et al. 2008; Eckert et al. 2011). These cellular “invasive feet” have been demonstrated to produce a variety of proteases, including metalloproteases (MMP2, MMP9, MT1-MMP, and the ADAM family of sheddases) and serine proteases (seprase and uPAR) (Linder 2007), and are capable of digesting various components of the extracellular matrix in vitro (Kelly et al. 1994). Given their proteolytic
capabilities, invadopodia are thought to facilitate metastasis by enabling tumor cells to breach the basement membrane, degrade the extracellular matrix, and invade into the stroma during the intravasation and extravasation steps of the metastatic cascade [Murphy and Courtneidge 2011]. In fact, it has been proposed that invadopodia in cancer cells are a co-opted and dysregulated version of normal cellular structures known as podosomes that are found in untransformed cells such as osteoclasts, macrophages, dendritic cells, endothelial cells, and smooth muscle cells [Murphy and Courtneidge 2011]. Although it is still unclear whether invadopodia have any physiological function in metastatic invasion during normal tumor progression [Linder 2009; Sibony-Benyamini and Gil-Henn 2012], animal transplant studies and intravitral imaging have provided some in vivo evidence for a role for invadopodia in mediating metastasis [Philippar et al. 2008; Gligorijevic et al. 2012].

Tks5 is an important component of invadopodia and mediates invadopodia formation by acting as a Src-dependent scaffolding protein [Lock et al. 1998]. Upon phosphorylation by Src, the N-terminal phox [PX] homology domain of Tks5 is thought to be released from intramolecular interactions and becomes free to bind membrane phosphoinositides, including PI(3,4)P2, thereby localizing Tks5 to the site of invadopodia formation [Abram et al. 2003; Okawa et al. 2008]. Tks5 also contains five C-terminal Src homology 3 [SH3] domains, which recruit effector proteins [including AFAP-110, cortactin, and ADAM metalloproteases] to initiate actin polymerization and matrix degradation [Abram et al. 2003; Crimaldi et al. 2009]. Knockdown of Tks5 [targeting all isoforms simultaneously] abrogates invadopodia formation and proteolytic function in cultured human breast cancer and melanoma cells [Seals et al. 2005] and reduces lung metastasis formation by Src-transformed NIH-3T3 mouse embryonic fibroblasts and Ras-transformed human mammary epithelial cells after intravenous injection or subcutaneous transplantation [Blouw et al. 2008; Eckert et al. 2011].

Despite the evidence that Tks5 is important for invadopodia formation in cell lines, its contribution to the metastatic process in naturally evolving tumors has not been elucidated. Moreover, previous studies have not accounted for the presence of the two functionally distinct isoforms [which we termed Tks5long and Tks5short] that we characterize in this study. These two Tks5 variants were first detected by immunoblotting in Src-transformed NIH-3T3 cells when Tks5 was initially identified [Lock et al. 1998]; however, no subsequent functional studies have taken into account the existence of these isoforms. Therefore, the distinct roles of Tks5long and Tks5short in invadopodia function and cancer invasion are unknown.

Here we report that Tks5long and Tks5short play distinct and opposing roles in regulating invadopodia-mediated invasion in lung adenocarcinoma. We show that metastatic primary tumor- and metastasis-derived cells acquired an elevated ratio of Tks5long to Tks5short expression and a commensurate increase in invadopodia activity compared with nonmetastatic cells. We further demonstrate that the ratio of Tks5long to Tks5short expression regulates invadopodia function in vitro and influences metastatic potential in transplant models and a genetically engineered mouse model of lung cancer. Finally, we provide evidence that the relative expression of Tks5long and Tks5short represents an important prognostic factor in human lung cancer. These results highlight the isoform-dependent roles of Tks5 in invadopodia and metastasis.

**Results**

**Metastatic and nonmetastatic lung adenocarcinoma cells exhibit differential expression of Tks5long and Tks5short**

To better characterize the cell state changes and molecular alterations that accompany tumor progression and metastasis in lung adenocarcinoma, we recently developed a K-ras<sup>G12D</sup>/WT, p53<sup>lox/lox</sup> mouse model for studying metastatic and nonmetastatic primary tumors [Winslow et al. 2011]. This model harbors genetic mutations frequently found in human lung adenocarcinoma [Rodenhuis et al. 1988; Takahashi et al. 1989] and closely recapitulates the histopathological progression of the human disease [Jackson et al. 2005]. Although these mice develop multiple K-ras<sup>G12D</sup>, p53<sup>−/−</sup> lung tumors after inhalation of lentivirus expressing Cre recombinase, only a subset of tumors eventually acquire full metastatic potential, suggesting that progression to metastasis requires additional genetic and/or epigenetic events. Importantly, this model allows the identification of metastatic versus nonmetastatic primary tumors, as the metastases that form in these mice can be matched to their primary tumor of origin based on the common lentiviral integration site in their genome. Thus, primary tumors [T<sub>Met</sub>] that have given rise to metastatic lesions can be distinguished from primary tumors for which no metastasis was found [T<sub>nonMet</sub>]. Cell lines derived from these T<sub>Met</sub> and T<sub>nonMet</sub> tumors were examined for their gene expression profiles via exon microarrays, which identified expression alterations in various genes, including Nkx2-1 and Hmga2, that were associated with metastatic progression [Winslow et al. 2011].

To identify gene isoform expression changes that could be caused by alternative splicing or differential promoter utilization in our collection of autochthonous tumor-derived T<sub>nonMet</sub> and T<sub>Met</sub> cells, we developed an algorithm that allowed us to query our exon array data for changes in isoform usage. The most striking result from this analysis was a change in Tks5 isoform expression between T<sub>nonMet</sub> and T<sub>Met</sub> cells [Supplemental Fig. S2]. We identified two Tks5 isoforms by referencing sequences published on the University of California at Santa Cruz genome browser [http://genome.ucsc.edu, assembly NCBI37/mm9, gene Sh3pxd2a]: Tks5long, which contains exons 1−15, and Tks5short, which contains a distinct 5′ sequence from intron 7 followed by exons 8−15 [Fig. 1A]. Both transcripts encode five SH3 domains in the C terminus, but only Tks5long contains the N-terminal PX homology domain [Fig. 1A].
We confirmed the differential expression of Tks5\textsubscript{long} and Tks5\textsubscript{short} in T\textsubscript{nonMet} and T\textsubscript{Met} cells by performing isoform-specific quantitative RT–PCR (qRT–PCR) on a panel of three T\textsubscript{nonMet} cell lines as well as five T\textsubscript{Met} cell lines and their five matching metastasis cell lines (Met). Consistent with the microarray data, Tks5\textsubscript{long} transcript levels were, on average, fourfold higher in T\textsubscript{Met}/Met cells compared with T\textsubscript{nonMet} cells, while Tks5\textsubscript{short} was transcribed at a similar level between T\textsubscript{Met}/Met and T\textsubscript{nonMet} cells [Fig. 1B]. As a result, the ratio of Tks5\textsubscript{long} to Tks5\textsubscript{short} was, on average, sixfold higher in T\textsubscript{Met}/Met cells than T\textsubscript{nonMet} cells [Fig. 1B]. Consistent with the mRNA expression patterns, the ratio of Tks5\textsubscript{long} to Tks5\textsubscript{short} proteins (150 kDa and 140 kDa, respectively) is higher in T\textsubscript{Met} cells compared with T\textsubscript{nonMet} cells [Fig. 1C].

Interestingly, despite this increase in Tks5\textsubscript{long} transcripts, because Tks5\textsubscript{long} only accounted for a fraction of total Tks5 expression, the levels of total Tks5 transcript did not vary significantly between T\textsubscript{nonMet} and T\textsubscript{Met}/Met cell lines in our exon microarray or in our qRT–PCR analysis using primers targeting a common region shared by Tks5\textsubscript{long} and Tks5\textsubscript{short} transcripts [Fig. 1B]. Thus, the T\textsubscript{nonMet} and T\textsubscript{Met}/Met cell lines could be distinguished by their Tks5\textsubscript{long} expression or Tks5\textsubscript{long} to Tks5\textsubscript{short} ratio but not by total Tks5 level. 5’ RACE and H3K4me3 chromatin immunoprecipitation sequencing (ChIP-seq) analyses suggest that the two isoforms are transcribed from distinct promoters [data not shown].

Expression of Tks5\textsubscript{long} and Tks5\textsubscript{short} in T\textsubscript{nonMet} and T\textsubscript{Met} cells correlates with invadopodia formation and function

Given the differences in Tks5 isoform levels between T\textsubscript{Met} and T\textsubscript{nonMet} cells and the previously reported role of Tks5 in mediating invadopodia activity, we compared three T\textsubscript{nonMet} and four T\textsubscript{Met} cell lines for invadopodia formation and function. To measure invadopodia formation, we performed immunofluorescence staining to detect the colocalization of two essential invadopodia components: cortactin and F-actin [Fig. 2A]. T\textsubscript{Met} cells, which have a higher Tks5\textsubscript{long} to Tks5\textsubscript{short} ratio, displayed...
a higher frequency of colocalized cortactin and F-actin than TnonMet cells [Fig. 2B]. To measure invadopodia function, we examined invadopodia-mediated proteolysis in TnonMet and TMet cells by culturing them on a thin layer of FITC-labeled gelatin. The degraded areas can be observed by fluorescence microscopy as FITC-negative patches that frequently coincide with cortactin/F-actin-stained invadopodia foci [Fig. 2A]. The gelatin degradation assay is a more sensitive method to measure invadopodia activity compared with cortactin/F-actin immunofluorescence staining because the effect of degradation is cumulative over time, while the presence of invadopodia is transient. Quantification of the degradation area showed a strong correlation between the proteolytic capability of these TnonMet and TMet cells and their ratio of Tks5long to Tks5short expression: TMet cells with higher Tks5long to Tks5short ratios were more proteolytic on the gelatin matrix compared with TnonMet cells [Fig. 2C]. Importantly, we did not observe significant differences in other invadopodia components at either the total gene expression or isoform level by exon array analysis [analyzed for Tks4, Src, Crtactin, Araf110, p190 RhoGAP, Arg, N-WASP, Arp2/3 complex subunits, Wave1, Cdc42, Coflin, Gelsolin, MT1-MMP, MMP2, MMP9, ADAM12, ADAM15, and ADAM19] or the protein phosphorylation level by Western blot [analyzed for Src] (data not shown). Taken together, these observations suggest that an increased ratio of Tks5long to Tks5short is associated with the enhanced invadopodia formation and function that we observed in TMet cells compared with TnonMet cells.

Knockdown of Tks5long Impairs invadopodia activity and metastasis formation

Since the distinct functions of Tks5long and Tks5short in invadopodia have not been previously reported, we tested whether Tks5long was specifically required for invadopodia formation and function in two TMet cell lines. Stable RNAi-mediated depletion of Tks5long using two isoform-specific shRNAs reduced Tks5long expression in TMet cells by 55%–65% without a significant effect on Tks5short [Fig. 3A,B]. Tks5long knockdown impaired the ability of TMet cells to form invadopodia as measured by immunofluorescence staining for cortactin/F-actin foci [Fig. 3C,D, Supplemental Fig. S3A,B]. These TMet-shTks5long cells also exhibited significantly reduced extracellular matrix proteolysis capability when cultured on FITC gelatin (Fig. 3C,E, Supplemental Fig. S3A,C). Because these observations were consistent for both shTks5long shRNAs, it is unlikely that they were the results of off-target effects. Collectively, these data indicate that Tks5long is necessary for invadopodia activity in metastatic lung cancer cells.

To determine whether the effects of Tks5long knockdown on invadopodia activity in vitro translate to the inhibition of metastatic ability in vivo, we transplanted TMet-shTks5long cells subcutaneously into athymic nude mice to assess the metastatic potential of tumor cells. TMet cells with Tks5long knockdown exhibited a significantly diminished ability to disseminate from the subcutaneous site and form lung tumor nodules compared with parental cells 8 wk after subcutaneous injection [Fig. 3F]. Of note, the sizes of the subcutaneous tumors were comparable between the two mouse cohorts, suggesting that loss of Tks5long expression had no effect on primary tumor growth (Supplemental Fig. S3D). Furthermore, in a separate experiment, we transplanted cells intrasplenically to assess their ability to extravasate and colonize the liver after draining into the hepatic portal vein from the spleen. Consistent with data from the subcutaneous transplant experiment, intrasplenically
injected T<sub>Met-shTks5<sub>long</sub> cells showed a substantially reduced ability to form liver nodules 3 wk after transplantation compared with controls [Fig. 3G]. Importantly, insufficient liver colonization by T<sub>Met-shTks5<sub>long</sub> cells was not a result of reduced cell proliferation or increased apoptosis, as the infrequent liver nodules that were formed by T<sub>Met-shTks5<sub>long</sub> cells displayed similar mitotic and apoptotic indices compared with parental cells [Supplemental Fig. S3E,F]. These experiments suggest that Tks5<sub>long</sub>-induced invadopodia are not only important for promoting cell invasion during intravasation at the primary site, but also required for extravasation and/or colonization at the metastatic sites, potentially by facilitating metastatic cell exit from blood vessels and/or invasion at secondary sites.

**Increased Tks5<sub>long</sub> expression promotes invadopodia activity and metastasis formation**

To ask whether exogenous expression of Tks5<sub>long</sub> alone is sufficient to enhance invadopodia formation and function in T<sub>nonMet</sub> cells, we generated a lentivirus that allows doxycycline-inducible expression of Flag-tagged Tks5<sub>long</sub> (Fig. 4A). Increased expression of Tks5<sub>long</sub> in two independent T<sub>nonMet</sub> cell lines promoted invadopodia formation compared with parental cells as measured by immunofluorescence staining for foci of cortactin/F-actin colocalization [Fig. 4B,C; Supplemental Fig. S4A]. Moreover, these cells also displayed a dramatic sixfold to 14-fold increase in matrix proteolysis in the FITC gelatin degradation assay [Fig. 4B,D; Supplemental Fig. S4B]. Our data thus demonstrate that changing the Tks5<sub>long</sub> to Tks5<sub>short</sub> ratio by increasing Tks5<sub>long</sub> expression is sufficient to promote invadopodia formation and function in nonmetastatic lung adenocarcinoma cells.

We then sought to determine whether Tks5<sub>long</sub> also facilitates tumor metastasis in vivo. To test this in a physiologically relevant model of tumor progression, we infected K-ras<sup>LSL-G12D/WT</sup>, p53<sup>lox/lox/flox</sup>, Rosa26-LSL-TdTomato; CCSP-rtTA mice with a PGK-Cre/TRE-Tks5<sub>long</sub> lentivirus [Fig. 4E]. Inhalation of the virus initiates RFP-positive lung tumors through the concomitant activation of oncogenic K-ras and deletion of p53. Infected mice were then fed with a doxycycline diet starting at 4 wk post-infection to induce Flag-tagged Tks5<sub>long</sub> expression, allowing us to study the effects of Tks5<sub>long</sub> specifically on tumor progression and not initiation. We confirmed inducible expression of our construct through detection of doxycycline- and rtTA-dependent expression of Flag-tagged Tks5<sub>long</sub> in these lung tumors [Supplemental Fig. S4C]. At 6 mo post-infection, although widespread distant metastases had not yet developed, we observed a significant acceleration in primary tumor progression in Tks5<sub>long</sub> mice (n = 9 mice; 221 tumors) compared with the control mice (n = 14 mice; 397 tumors); Tks5<sub>long</sub> tumors had a smaller proportion of low-grade tumors and a commensurate increase in high-grade lesions [Supplemental Fig. S4D]. These high-grade tumors were invasive into stromal tissues surrounding the blood vessels and were thus categorized as grade 4 lesions [Supplemental Fig. S4E].

**Figure 4. Tks5<sub>long</sub> is sufficient to promote invadopodia activity in vitro and metastasis formation in vivo.** (A) Immunoblot detection of Tks5 isoforms in 394T4 T<sub>nonMet</sub> cells and T<sub>nonMet</sub>-Flag Tks5<sub>long</sub> cells with or without doxycycline induction of Flag-Tks5<sub>long</sub>. Tubulin was used as a loading control. (B) Immunofluorescence staining shows that colocalization of invadopodia components (cortactin is in green, and F-actin is in red) and FITC-negative areas of gelatin matrix degradation are more readily observed in T<sub>nonMet</sub>-Tks5<sub>long</sub> cells compared with parental T<sub>nonMet</sub> cells, both treated with 2 μg/mL doxycycline. Magnified views of the regions indicated by the boxed area are shown at the right. Representative images of T<sub>nonMet</sub> cells (394T4) are shown. (C) Effects of increased Tks5<sub>long</sub> expression on invadopodia formation in T<sub>nonMet</sub> cells (394T4). At least 100 cells were scored for colocalization of cortactin and F-actin in three independent experiments. All values are mean ± SEM. (**) P-value < 0.05, paired t-test. (D) Effects of increased Tks5<sub>long</sub> expression on FITC gelatin matrix degradation in T<sub>nonMet</sub> cells [394T4]. Areas of degradation were quantified using ImageJ and normalized to number of cells per field. At least 40 fields containing a total of 1800 cells were analyzed per condition. All values are mean ± SEM. (***). P-value < 0.001, Student’s t-test. (E) Induction of lung adenocarcinomas with doxycycline-dependent overexpression of Tks5<sub>long</sub> in an autochthonous mouse model. K-ras<sup>LSL-G12D/WT</sup>, p53<sup>lox/lox/flox</sup>; CCSP-rtTA mice were infected with a PGK-Cre/TRE-Tks5<sub>long</sub> lentivirus. The Cre recombinase initiates lung adenocarcinomas upon infection. Doxycycline diet later induced Tks5<sub>long</sub> expression in these tumors to study the effects of Tks5<sub>long</sub> on tumor progression without affecting tumor initiation. (F) Examples of distant metastases observed in mice with increased Tks5<sub>long</sub> expression. All tumors are RFP-positive because of a Rosa26-LSL-TdTomato allele in the mice. (BF) Bright field. (G) Mice with increased Tks5<sub>long</sub> expression (n = 15) developed more distant metastases compared with control mice (n = 8). P < 0.03, χ² test; P < 0.04, Fisher’s exact test, two tailed. (H) Mice with increased Tks5<sub>long</sub> expression (n = 15) developed more metastases overall (including distant metastases and pleural metastases) compared with control mice (n = 8). P < 0.003, χ² test; P < 0.008, Fisher’s exact test, two tailed.
Consistent with these observations, at 8 mo post-infection, when mice had developed both pleural metastases and distant metastases in the liver, kidneys, and distant lymph nodes [Fig. 4F], Tks5long-expressing mice developed significantly more distant metastases [11 of 15 mice] than the control group [two of eight mice; \( P < 0.05 \) by both \( \chi^2 \) test and Fisher’s exact test] [Fig. 4G]. In addition, 15 of 15 Tks5long mice developed pleural metastases and/or distant metastases, while four of eight mice in the control group remained metastasis-free [\( P < 0.003 \) by \( \chi^2 \) test and \( P < 0.008 \) by Fisher’s exact test] [Fig. 4H]. Importantly, we did not observe any significant difference in the primary lung tumor sizes and total lung tumor burden in the Tks5long mice versus control mice [Supplementary Fig. S4F,G], suggesting that the effect of Tks5long on tumor progression and metastasis was not a consequence of increasing the rate of tumor growth.

Collectively, these data from this mouse model indicate that Tks5long plays an important role in promoting metastasis in vivo and are consistent with our in vitro evidence that Tks5long promotes invadopodia-mediated invasion. Whether tumor progression can be further stimulated by other mechanisms—including, for example, growth factor shedding during invadopodia-mediated degradation of the extracellular matrix—remains an open possibility and will be addressed in future studies.

**Elevated expression of Tks5short reduces gelatin matrix degradation and invadopodia lifetime**

In addition to modulating Tks5long expression, the Tks5long to Tks5short ratio can also be altered by increasing the expression of the less well-studied isoform Tks5short. While Tks5short levels in TnonMet and TMet/Met cells were similar, we were interested whether Tks5short could also participate in regulating invadopodia activity. Although we attempted to knock down Tks5short by shRNA, this approach proved to be challenging, since the unique sequence in the Tks5short transcript is very short. Therefore, we chose to infect TMet cells with a lentivirus that allowed doxycycline-inducible expression of HA-Tks5short [Fig. 5A]. Interestingly, two independent TMet cell lines with exogenous Tks5short expression both had drastically reduced gelatin matrix proteolysis [Fig. 5B], suggesting that Tks5short may act in a dominant-negative manner over Tks5long in regulating invadopodia activity.

We additionally determined the localization of Tks5short by using an antibody specific for the HA tag and found that HA-Tks5short exhibited a diffuse localization in TMet Tks5short cells [Fig. 5C]. This distribution is in contrast to that of endogenous Tks5 in TMet cells stained with a pan-Tks5 antibody, where the protein [presumably the dominant Tks5long isoform in TMet cells] is concentrated at foci of cortactin/F-actin colocalization [Fig. 5C]. The diffuse distribution of Tks5short suggests that it cannot localize to invadopodia foci on the cell membrane, conceivably due to lack of a PX homology domain.

Moreover, even though cortactin/F-actin-positive foci of invadopodia could be observed in TMet-Tks5short cells [Fig. 5C], when we measured the lifetime of invadopodia in these cells using time-lapse fluorescence imaging, they exhibited shortened invadopodia lifetime compared with parental TMet cells [Fig. 5D]. Previous studies have shown that nascent invadopodia remain nonproteolytic for at least 1 h before maturing into fully functional invadopodia that are capable of mediating degradation [Yamaguchi et al. 2005; Oser et al. 2009]. Thus, our data suggest that Tks5short may destabilize invadopodia and interfere with their maturation into a proteolytic state. Hence, the ratio between Tks5short and Tks5long, rather than the absolute levels of either isoform or total Tks5, appears to be important for invadopodia-mediated cell invasion.
High expression of Tks5\textsubscript{long} and low expression of Tks5\textsubscript{short} correlate with metastatic progression and poor survival in lung adenocarcinoma patients

We next explored whether a high Tks5\textsubscript{long} to Tks5\textsubscript{short} ratio correlates with tumor progression and metastasis in human lung cancer. For this purpose, we analyzed RNA sequencing [RNA-seq] data from lung adenocarcinoma patients deposited in The Cancer Genome Atlas [TCGA] for the expression ratio of Tks5\textsubscript{long} to Tks5\textsubscript{short}. The human Tks5\textsubscript{short} transcript homologous to mouse Tks5\textsubscript{short} was the most abundant isoform alternative to Tks5\textsubscript{long} in these lung tissues [data not shown], although additional transcripts with slight variations exist in other tissue types (S. Courtneidge, pers. comm.; http://genome.ucsc.edu, assembly GRCh37/hg19, gene Sh3pxd2a). Importantly, while the expression patterns of Tks5\textsubscript{long} and Tks5\textsubscript{short} in these lung adenocarcinomas were diverse, there was a trend toward high Tks5\textsubscript{long} to Tks5\textsubscript{short} expression ratios in patients with stage III and IV disease [characterized by metastatic invasion in the thoracic cavity and distant organs, respectively; \(n = 59\)] compared with patients with stage IA disease [characterized by a single, small, localized lesion without detectible metastases; \(n = 57\), \(P < 0.009\) by \(\chi^2\) test and \(P < 0.013\) by Fisher’s exact test] [Fig. 6A], suggesting that high Tks5\textsubscript{long} and low Tks5\textsubscript{short} expression contributes to promoting metastatic progression.

In addition, we examined Tks5\textsubscript{long} and Tks5\textsubscript{short} expression in an independent cohort of 102 patients with stage I/II lung adenocarcinoma from the University of Michigan. Interestingly, we observed that higher Tks5\textsubscript{long} expression and lower Tks5\textsubscript{short} expression correlated with worse disease-free survival and overall survival by Kaplan-Meier analysis [Fig. 6B; Supplemental Fig. S5] and reflected poor prognosis in a multivariate analysis by the Cox proportional hazard model after adjustment for gender, age, stage, and tumor differentiation state [Table 1; Supplemental Table S1]. Importantly, total expression of Tks5 did not demonstrate any survival correlation or prognostic values in these analyses [Fig. 6B; Table 1; Supplemental Fig. S5; Supplemental Table S1], suggesting that the distinction between Tks5 isoforms is critical in analyzing these clinical data. As disease-free survival and, to a lesser extent, overall survival reflect the rate of post-resection tumor relapse and thus, most likely, the magnitude of early micrometastatic spread prior to surgery, our data are consistent with the conclusion that Tks5\textsubscript{long} promotes metastasis in human lung cancer, while Tks5\textsubscript{short} exerts the opposite effect, and a shift in the balance of the two isoforms may influence the clinical outcomes in lung adenocarcinoma patients. In addition, Tks5\textsubscript{long} and Tks5\textsubscript{short} may serve as prognostic factors for identifying high-risk patients with early stage disease who may benefit from adjuvant treatment following tumor resection.

Discussion

Metastasis accounts for the vast majority of cancer-related deaths, underscoring the need for a better understanding of the molecular mechanisms that enable tumor cells to escape from their primary site and spread to other parts of the body. In this study, we report a shift in the isoform expression of an invadopodia component, Tks5, that helps explain the increased invasiveness of metastatic cells during lung adenocarcinoma progression. Our data indicate that as primary tumors progress from a non-metastatic state [\(T_{\text{nomMet}}\)] to a metastatic state [\(T_{\text{Met}}\)] and eventually form secondary lesions [\(T_{\text{Met}}\)], tumor cells acquire an increase in Tks5\textsubscript{long} to Tks5\textsubscript{short} expression, despite a lack of significant increase in total Tks5 expression. Using functional experiments in cultured cells and mouse models, we demonstrate distinct and opposing roles for Tks5\textsubscript{long} and Tks5\textsubscript{short}. Tks5\textsubscript{long} promotes invadopodia activity and metastasis formation, as knockdown of Tks5\textsubscript{long} impairs invadopodia function in vitro and metastasis formation in vivo, while elevated expression of Tks5\textsubscript{long} has the opposite effects. Tks5\textsubscript{short}, on the other hand, acts as a negative regulator of invadopodia function, as increased expression of Tks5\textsubscript{short} interferes with invadopodia stability and inhibits gelatin proteolysis. Hence, it is the balance of Tks5\textsubscript{long} and Tks5\textsubscript{short} expression, rather than total Tks5 level, that appears to be important for metastatic invasion. Consistent with these functional analyses, our clinical data demonstrate that a high level of Tks5\textsubscript{long} expression and a low level of Tks5\textsubscript{short} expression [but not total Tks5 expression] correlate with
metastatic progression in lung adenocarcinoma patients and predict poor survival of patients with early stage disease.

These experiments provide insight into the roles of Tks5 isoforms in metastasis. Previous studies have demonstrated a role of Tks5 in invadopodia and invasion [Seals et al. 2005; Blouw et al. 2008], however, the specific roles of its isoforms have not been defined. Our data revise the current notion that Tks5 generally promotes invadopodia and metastasis and support a model in which Tks5long acts to promote invadopodia formation by binding to the cellular membrane and recruiting effector proteins for actin polymerization and protease secretion, while Tks5short acts to regulate invadopodia function by interfering with their stability and maturation. A shift in the balance of Tks5long and Tks5short expression in cancer cells may have a profound impact on tumor progression. While our data indicate that Tks5short interferes with invadopodia stability, the specific mechanism of this regulation remains to be elucidated. It is conceivable that Tks5short acts by sequestering invadopodia components away from the cell membrane via its multiple SH3 domains, proline-rich regions, and phosphorylation sites. Previous biochemical assays have shown that these functional domains of Tks5 bind to multiple invadopodia components, including N-WASP, Nck, and ADAM family metalloproteases [Abram et al. 2003; Oikawa et al. 2008; Stylli et al. 2009]. Whether these protein interactions mediate the inhibitory function of Tks5short in invadopodia deserves future investigation. In addition, given the differential isoform expression of Tks5 in metastatic and nonmetastatic tumors, it will be of great interest to identify the regulatory mechanism of this isoform switch. Preliminary data from our 5’ RACE and H3K4me3 ChIP-seq analyses suggest that the two isoforms are transcribed from distinct promoters [C Li, unpubl.]. Future work will dissect the regulatory mechanisms of promoter choice that lead to the increased expression of Tks5long during tumor progression.

This study also underscores the in vivo role of invadopodia as critical mediators of metastasis in natural tumor progression. While previous studies have provided important evidence for a role of invadopodia in mediating metastatic invasion by using cell culture-based invasion assays and transplant models [Seals et al. 2005; Blouw et al. 2008; Philippar et al. 2008; Eckert et al. 2011; Gligorijevic et al. 2012], these experimental systems often do not fully recapitulate natural tumor progression and metastatic spread. Here we further establish the role of invadopodia in promoting metastasis in vivo by using an autochthonous mouse model of metastatic lung cancer. Our data thus help address the question of whether invadopodia play a physiologically relevant role in metastasis in vivo [Linder 2009; Sibony-Beniamini and Gil-Henn 2012]. The data presented by our study and others provide a mechanism that explains one of the ways in which tumor cells might overcome the multiple physical barriers presented by stromal tissues, the extracellular matrix, and endothelial cells during the intravasation, extravasation, and colonization steps of metastasis.

Interestingly, while the gain of Tks5long expression in both TMet and Met cells in our lung adenocarcinoma model suggests that invadopodia function in both the invasion/intravasation step at the primary tumor and the extravasation/colonization step at the metastatic site, the specific contribution of invadopodia to each step of the metastasis cascade may be context-dependent and cell type-dependent. Whereas knockdown of Tks5long in lung adenocarcinoma cells [this study] and knockdown of total Tks5 in Ras-transformed mammary epithelial cells [Eckert et al. 2011] diminished the number of metastases formed, similar total Tks5 knockdown experiments in Src-transformed mouse embryonic fibroblasts did not lead to more metastases but an increase in the volume and vascularization of metastatic nodules [Blouw et al. 2008]. Thus, the specific contribution of invadopodia to each step of the invasion–metastasis cascade in different cancer types remains to be further dissected in the future.

In addition, this study carries clinical implications for lung cancer patients. Previous clinical studies of another invadopodia component, cortactin, indicate that its elevated expression and dysregulated cellular localization correlate with poor survival in laryngeal carcinoma and lung adenocarcinoma, respectively, underlining the relevance of invadopodia activity in predicting clinical outcomes [Gibcus et al. 2008; Hirooka et al. 2011]. Consistent with these studies, our data show that a high level of Tks5long expression and a low level of Tks5short expression correlate with metastatic progression of lung adenocarcinoma patients and predict poor survival of patients with early stage disease, suggesting that the Tks5long to Tks5short ratio may serve as a prognostic marker for assessing the metastatic potential of primary tumors and for identifying early stage patients who bear higher risks for metastasis and may benefit from adjuvant therapy after tumor resection. Furthermore, future development of molecular therapeutic strategies that inhibit Tks5long function or strengthen Tks5short activity could potentially help inhibit metastatic progression.

Finally, this study demonstrates the value of mouse models and their derivative cell lines in allowing molecular characterization of the cell state changes that accompany tumor progression and metastasis and is representative of recent developments of animal models for lung adenocarcinoma [Jackson et al. 2005; Politi et al. 2006; Dankort et al. 2007; Ji et al. 2007]. Importantly, our approach demonstrates that in order to fully understand the metastatic process, analysis of gene expression at the isoform level in addition to the total gene expression level is important. Given the lethal effects of the metastatic phase of cancer, a deeper insight into the molecular determinants of metastasis will have a significant impact on cancer mortality and morbidity.

**Materials and methods**

**Cell lines**

TnonMet, TMet, and Met cell lines were derived from autochthonous tumors in K-rasLSL-G12D/WT, p53lox/lox mice as described
in both TnonMet and TMet groups, and (3) significant deviation of isoform expression, (2) robustly detectible gene expression statistical significance in Partek’s ANOVA-based test for alternative isoform switches between groups TnonMet and TMet using the Partek Genomics suite software package (version 6.4) with a high-ranking genes were then further evaluated by manual expression analysis and/or functional experiments in this study.

Exon arrays and differential isoform expression detection
Affymetrix GeneChip Mouse Exon 1.0 ST arrays [Gene Expression Omnibus GSE26874] of four TnonMet [368T1, 393T1, 394T4, 802T4, and 2557T1], six TMet cell lines [373T1, 373T2, 389T2, 393T3, 393T5, and 482T1], and five Met cell lines [373N1, 393N1, 393M1, 482N1, and 482M1] were used for subsequent gene expression analysis and/or functional experiments in this study.

FITC gelatin degradation assay
Class-bottomed 35-mm plates [MatTek] were coated with FITC-gelatin as described in Bowden et al. (2001) with some modifications. Briefly, MatTek plates were treated with HCl, followed by 50 μg/mL poly-L-lysine, and then coated with a thin layer of FITC-labeled 0.2% gelatin (Sigma) for 1 h. The gelatin coating was then cross-linked with ice-cold 0.8% glutaraldehyde [Electron Microscopy Sciences]/PBS for 15 min at 4°C and then for 30 min at room temperature. Plates were successively washed in PBS [three times for 5 min each], 5 mg/mL sodium borate in PBS (once for 3 min), and PBS [three times for 5 min each], before being incubated for 30 min with complete tissue culture medium. Cells (8 x 10^4) were cultured on the gelatin-coated plates for 72 h and subsequently processed using standard fluorescence microscopy procedures.

Immunofluorescence staining for invadopodia components
Cells (8 x 10^4) were grown overnight at 37°C on 35-mm MatTek plates coated with either 0.2% FITC-labeled gelatin or 0.2% plain gelatin (Sigma). Cells were then fixed in 3.7% formalde-hyde [Electron Microscopy Sciences] in PBS for 20 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and blocked with 1% BSA and 1% PBS in PBS. Subsequently cells were stained for immunofluorescence microscopy. Primary antibodies included Tks5 [1:100, Santa Cruz Biotechnology, M300; detects both Tks5long and Tks5short], HA tag [1:103; Cell Signaling, 3724], and cortactin [1:100, Millipore, 4F11]. F-actin was stained with phalloidin [Invitrogen].

Live-cell fluorescence microscopy
Cells transfected with a pcDNA3 RFP-β-Actin construct (a generous gift from F. Gertler) were plated on gelatin-coated MatTek dishes in L-15 medium (Leibovitz); placed in an envi-ronmental chamber with constant 37°C temperature, CO2, and humidity; and imaged every 2.5 min for at least 12 h. The lifetimes of at least 150 invadopodia from three independent measurements per condition were analyzed using ImageJ.

Subcutaneous transplant
Nude mice were injected with 5 x 10^4 TnonMet cells (393T3 parental cells or 393T3 cells expressing sh1 shRNA against Tks5long) resuspended in 100 μL of PBS under the skin on their hind flank. Subcutaneously injected mice were analyzed 8 wk after injection. To quantify lung tumor nodules, all of the visible surface tumors were counted under a dissecting microscope.

Intrasplenic transplant
Nude mice were injected with 5 x 10^4 TMet cells (373T1 parental cells or 373T1 cells expressing sh1 shRNA against Tks5long) resuspended in 200 μL of PBS via the spleen, as described previously [Winslow et al. 2011]. Briefly, the animals were given 0.1 mg/kg buprenorphine prior to surgery and anaesthetized with a continuous flow of isoflurane throughout the procedure. Once the animals were under deep anesthesia, the abdominal area was disinfected with betadine and 70% ethanol. The spleen was exposed through a small incision. Cells were injected into the spleen with a single injection using an insulin syringe. Cells were given 10 min to travel through the vasculature to the liver, after which the entire spleen was removed to prevent the formation of a large splenic tumor mass. To remove the spleen, a dissolvable 4.0 suture was tied snugly around the base of the spleen, including the major splenic vasculature, and the spleen was removed. The muscle wall was closed with 4-0 dissolvable sutures, and the skin incision was closed with sterile 7-mm wound clips [Roboz]. Intrasplenically injected mice were analyzed 3 wk after injection. Quantification of liver tumor nodules was performed by counting all of the visible surface tumors under a dissecting microscope.

Lentiviral infection of autochthonous mouse model of lung adenocarcinoma
Tumors were initiated by intratracheal infection of mice as described previously [DuPage et al. 2009]. Lentivirus was produced from 293T cell transfection as described above. Virus was recovered from the supernatant by ultracentrifugation at 25,000 rpm for 90 min and resuspended in an appropriate volume (200–2000 μL) of PBS. A lentiviral dose of 1000–4000 viral particles induced 25–50 lung tumors per mouse and allowed 6 mo of survival after tumor initiation, while a lentiviral dose of 500 viral particles induced ~10 tumors per mouse and allowed 8 mo of survival post-initiation.

Clinical analysis
For TCGA data set, an index of Tks5 isoform expression was calculated by [total Tks5/Tks5long] based on RNA-seq align-
The University of Michigan data set, Tks5long expression and total Tks5 expression were measured by qRT-PCR in 102 primary tumor samples from patients with stage I/II lung adenocarcinoma. Levels of Tks5long expression were calculated by subtracting Tks5short expression from total Tks5 expression (see the Supplemental Material for details). Based on expression level, patients were divided into a high-expression group (top two-thirds) and a low-expression group (bottom one-third) for Tks5long, total Tks5, or Tks5short to total Tks5 ratio. Five-year disease-free survival and overall survival were analyzed by Kaplan-Meier curves and log-rank test. Multivariate analysis by the Cox proportional hazard model (adjusted by gender, age, stage, and tumor differentiation state) was performed using a continuous value of Tks5 mRNA level to assess survival results. P-values (two-tailed) of <0.05 were considered statistically significant.

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