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14. ABSTRACT

The transcription factor Twist1 is an important mediator of breast cancer metastasis by driving the epithelial-mesenchymal transition. We find that Twist1 promotes metastasis by inducing the formation of invadopodia, subcellular structures that localize protease activity and secretion to areas of the cell in contact with the basement membrane. Twist1 regulates invadopodia formation by increasing Src kinase activity through upregulation of platelet-derived growth factor (PDGF) receptors. In addition, Twist1 regulates focal adhesion formation and adhesion through upregulation of a disintegrin and metalloprotease 12 (ADAM12). ADAM12 disrupts focal adhesion formation or stability. As focal adhesions antagonize invadopodia formation, this further promotes invadopodia formation, invasion, and metastasis downstream of Twist1.

15. SUBJECT TERMS
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Introduction

During metastasis, carcinoma cells acquire the ability to invade surrounding tissues and intravasate through the endothelium to enter systemic circulation. Both the invasion and intravasation processes require degradation of basement membrane and extracellular matrix (ECM). Although proteolytic activity is associated with increased metastasis and poor clinical outcome, the molecular triggers for matrix degradation in tumor cells are largely unknown.

Invadopodia are specialized actin-based membrane protrusions found in cancer cells that degrade ECM via localization of proteases (Tarone et al., 1985; Chen, 1989). Their ability to mediate focal ECM degradation suggests a critical role for invadopodia in tumor invasion and metastasis. However, a definitive role for invadopodia in local invasion and metastasis in vivo has not yet been clearly demonstrated. As actin-based structures, invadopodia contain a primarily branched filamentous actin (F-actin) core and actin regulatory proteins, such as cortactin, Wiscott-Aldrich Syndrome protein (WASp), and the actin-related protein 2/3 complex (Arp2/3 complex) (Linder, 2007). The SH3-domain-rich proteins tyrosine kinase substrate 4 (Tks4) (Buschman et al., 2009) and Tks5 (Seals et al., 2005) function as essential adaptor proteins in clustering structural and enzymatic components of invadopodia. The matrix degradation activity of invadopodia has been associated with a large number of proteases, including membrane type 1 metalloproteases (MT1-MMP) (Linder 2007). Invadopodia formation requires tyrosine phosphorylation of several invadopodia components including cortactin (Ayala et al., 2008), Tks4 (Buschmann et al., 2009), and Tks5 (Seals et al., 2005) by Src family kinases.

Previous studies found that the Twist1 transcription factor, a key regulator of early embryonic morphogenesis, was essential for the ability of tumor cells to metastasize from the mammary gland to the lung in a mouse breast tumor model and was highly expressed in invasive human lobular breast cancer (Yang et al., 2004). Since then, studies have also associated Twist1 expression with many aggressive human cancers, such as melanomas, neuroblastomas, prostate cancers, and gastric cancers (Peinado et al., 2007). Twist1 can activate a latent developmental program termed the epithelial-mesenchymal transition (EMT), thus enabling carcinoma cells to dissociate from each other and migrate.

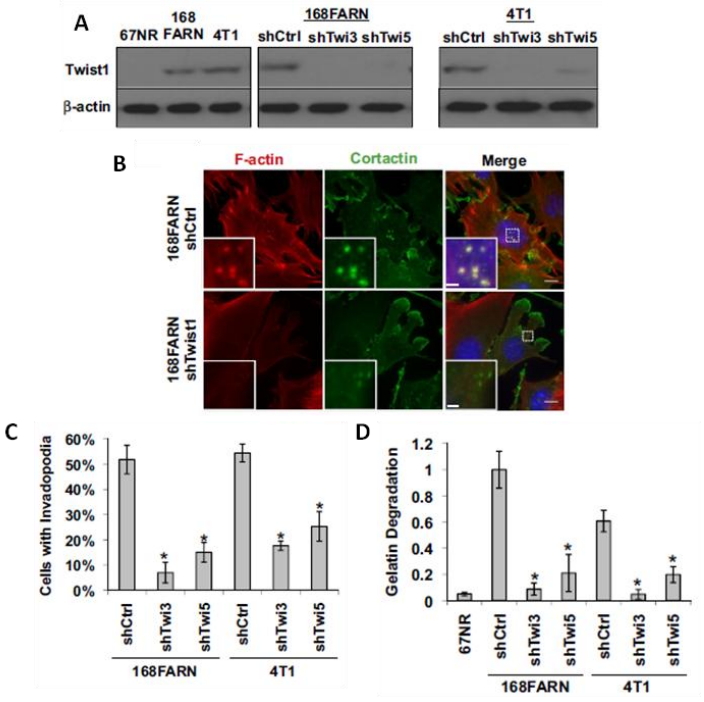

Figure 1: Twist is necessary for invadopodia formation. (A) Immunoblot demonstrating high expression of Twist1 in 168FARN and 4T1 cells; shRNA constructs against Twist1 are efficient at reducing Twist1 expression; (B) Representative image of invadopodia in 168FARN cells expressing control or shTwist1 constructs showing a decrease in invadopodia formation upon knockdown of Twist1; (C) Quantification of percentage of cells forming invadopodia (as measured by colocalized puncta of F-actin and cortactin) in the indicated cell lines demonstrating a requirement for Twist1 in invadopodia formation; (D) Quantification of relative gelatin degradation for the indicated cell lines, demonstrating that Twist1 is required for efficient invadopodia-mediated gelatin degradation. Scale bar = 5um; Error bars are SEM, *p<0.05.

The EMT program is a highly conserved developmental program that promotes epithelial cell dissociation and migration to different sites during embryogenesis. During EMT, cells lose their epithelial characteristics, including cell adhesion and polarity, and acquire a mesenchymal morphology and the ability to migrate (Hay, 1995). Biochemically, cells downregulate epithelial markers such as adherens junction proteins epithelial cadherin (E-cadherin) and catenins and express mesenchymal markers including vimentin and fibronectin (Boyer and Thiery, 1993). In addition to Twist1, the zinc-finger transcription factors, including Snail, Slug, zinc finger E-box binding 1 (ZEB1), and ZEB2 (Peinado et al., 2007), can also

activate the EMT program by directly binding the E-boxes of the E-cadherin promoter to suppress its transcription. However, it is unclear how Twist1, as a basic helix-loop-helix (bHLH) transcription factor, controls the EMT program. Over the course of this project, we investigated the role of two proteins induced by Twist1 expression, platelet derived growth factor alpha (PDGFRα) and a disintegrin and metalloprotease 12 (ADAM12,) in regulating invadopodia formation and metastasis.

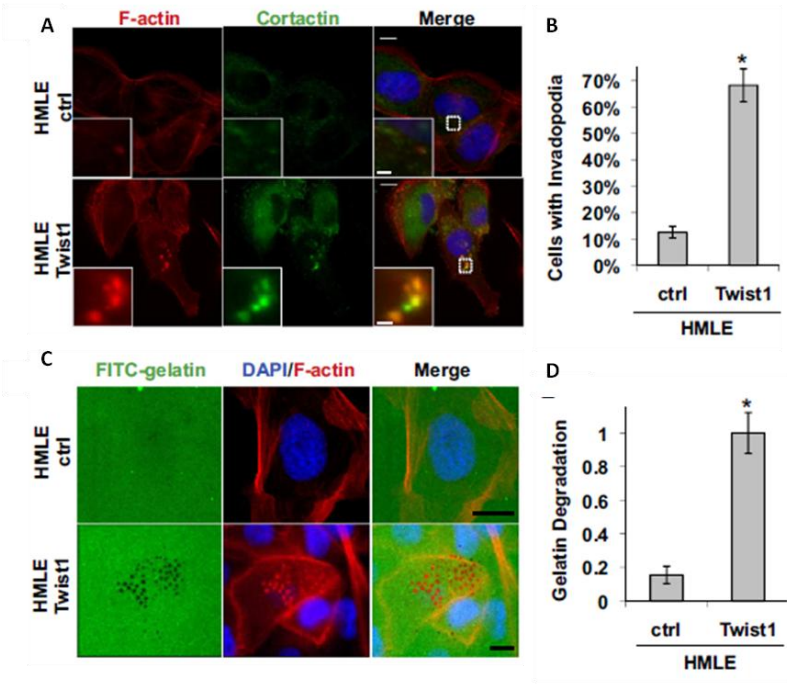

Figure 2: Twist is sufficient for invadopodia formation. (A) Representative immunofluorescent images showing increase in invadopodia formation in HMLE cells overexpressing Twist1; (B) Quantification of percentage of cells that form invadopodia upon overexpression of Twist1; (C) Representative images of gelatin degradation assay demonstrating an increase in degradation in HMLE cells overexpressing Twist1; (D) Quantification of gelatin degradation in the respective cell lines showing that Twist1 increases invadopodia-mediated gelatin degradation. Scale bars are 5 um; Error bars are SEM, *p<0.05.

In preliminary experiments, we investigated the requirement for Twist1 in invadopodia formation in 168FARN and 4T1 cells. Both of these cell lines express high levels of Twist1 that can be effectively silenced with shRNA constructs (Figure 1A). Upon knockdown of Twist 1, we observed a dramatic reduction in invadopodia formation, as visualized by colocalization of the invadopodia markers F-actin and cortactin, in both 168FARN and 4T1 cells expressing the shTwist constructs (Figure 1B and data not shown). When quantified, there was a significant reduction in invadopodia upon knockdown of Twist1 (Figure 1C). Gelatin degradation can be assayed in these cells be performing a gelatin degradation assay in which cells are plated on top of fluorescent labeled gelatin; areas where invadopodia degrade the gelatin become no longer fluorescent. Relative gelatin degradation can be easily assayed by quantifying the area of degraded fluorescent gelatin and normalizing it overall cell number. Importantly, the decrease in invadopodia formation was also associated with a significant reduction in the ability of 168FARN and 4T1 cells to degrade fluorescently-labeled gelatin upon Twist1 knockdown (Figure 1D). 67NR cells, which express very low levels of endogenous Twist1, failed to form invadopodia or degrade gelatin (Figure 1A, C-D). In addition, when Twist1 was over expressed in normal human mammary epithelial cells (HMLE cells), we observed a significant increase in invadopodia formation (Figure 2 A-B) as well as increase in gelatin degradation (Figure 2C-D)

Our preliminary data suggested that Twist1 was both necessary and sufficient for invadopodia formation in multiple cell lines. We therefore sought to determine the targets of Twist1 transcriptional regulation that were responsible for the regulation of invadopodia formation and understanding the potential role for this targets, and invadopodia, in regulating metastasis. For this project, we focus on PDGFRs and ADAM12.

Body

Aim 1: Identify the transcriptional targets of Twist1 responsible for invadopodia formation.

Twist1-induced PDGFRs are necessary for invadopodia formation.

In identifying downstream targets of Twist1 involved in regulation of invadopodia formation, we first focused on potential roles for the growth factor receptors PDGFRs. These were particularly interesting targets as activation of PDGFRs is associated with a direct activation of Src kinase by the intracellular domain of PDGFR (Kypta et al, 1990). Following overexpression of Twist1 in HMLE cells, there was a strong induction of both PDGFRα and β at both the mRNA (data not shown) and protein levels (Figure 3A). Interestingly, both PDGFRs were activated under normal culture conditions, as

evidence by the fact that they are phosphorylated at residues corresponding to receptor activation (Figure 3A). To probe the potential roles of PDGFRs in Twist1-induced invadopodia we inhibited PDGFR activation with a monoclonal blocking antibody directed against PDGFRα and generated two knockdown constructs targeting PDGFRα. Treatment of HMLE-Twist1 cells (Twist1 overexpressing HMLE cells) with either the monoclonal blocking antibody or expression of shRNAs targeting PDGFRα led to a dramatic reduction in activation of PDGFRs, as measured by receptor phosphorylation (Figure 3A). This gave us the tools to probe the potential roles of PDGFRs in regulating invadopodia formation downstream of Twist1.

Treatment of HMLE-Twist1 cells with the monoclonal PDGFRα blocking antibody led to a dramatic reduction in invadopodia formation, as assayed by immunofluorescence for the presence of cortactin/F-actin positive puncta in the cells (Figure 3B). The reduction in invadopodia was significant when quantified (Figure 3C). In addition, both treatment with the monoclonal antibody and knockdown of PDGFRα led to a dramatic reduction in gelatin degradation associated with invadopodia formation (Figure 3D). This data indicated that PDGFR-mediated signaling was required for invadopodia formation. The signaling pathways regulated by PDGFR that induce invadopodia formation are discussed further in the Aim 3 subsection, below.

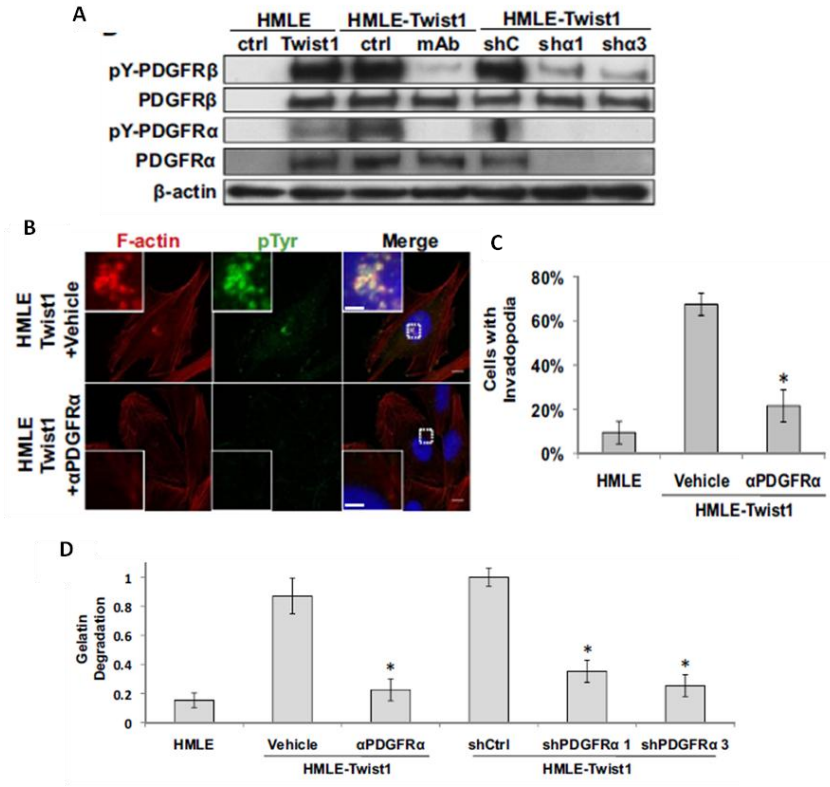

Figure 3: Induction of PDGFRs is necessary for Twist1-induced invadopodia formation. (A) Immunoblot demonstrating increase in PDGFR expression in cells overexpressing Twist1; two knockdown constructs agains PDGFRα (shα1 and shα3) were effective at reducing protein levels; treatment with a blocking antibody (mAb) was also effective at reducing PDGFR activation; (B) Representative immunofluorescent image of invadopodia formation in HMLE-Twist1 cells treated with control vehicle or with the PDGFRα blocking antibody revealing a requirement for PDGFR signaling in invadopodia formation; (C) Quantification of percentage of cells that form invadopodia in HMLE and HMLE-Twist1 cells under the indicated conditions revealing a significant decrease in invadopodia formation upon inhibition of PDGFR signaling; (D) Quantification of gelatin degration under the indicated conditions. Inhibition of PDGFR signaling or knockdown of PDGFR protein levels led to a significant decrease in invadopodia-mediated gelatin degradation. Scale bars are 5 um. Error bars are SEM; *p<0.06. Blocking antibody (mAb) was used at a concentration of 5 mg/ml.

Induction of ADAM12 is necessary for Twist1-induced invadopodia formation.

In addition to upregulation of PDGFRs, we also observed upregulation of ADAM12, an atypical metalloprotease with cysteine-rich and disintegrin domains involved in the regulation of cellular adhesion and integrin-mediated signaling (Kveiborg et al., 2008). Interestingly, ADAM12 has been described as an integral invadopodia protein that directly interacts with the invadopodia-specific scaffolding protein Tks5 (Abram et al, 2003). We were therefore very interested in characterizing the role of this protein in Twist1-induced invadopodia formation.

ADAM12 is strongly induced at the protein level following expression of Twist1 in HMLE cells (Figure 4A). In addition, we were able to effectively reduce ADAM12 protein levels with two different knockdown constructs (Figure 4A). Importantly, when we knocked down ADAM12 in HMLE-Twist1 cells, we observed a dramatic reduction invadopodia formation, as assayed by immunofluorescence for F-actin and cortactin colocalization in punctate invadopodia (Figure 4B). Quantification of both invadopodia formation and gelatin degradation revealed that ADAM12 was necessary for both the initial formation of invadopodia and the function of invadopodia in degrading extracellular matrix

components (Figure 4C-D). Experiments were carried out in parallel in Hs578t cells, a human breast cancer cell line that expresses high levels of Twist1. There, we found that ADAM12 was similarly required for invadopodia formation and function (data not shown). Potential mechanisms and signaling pathways regulated by ADAM12 expression are discussed more fully under Aim 3, below.

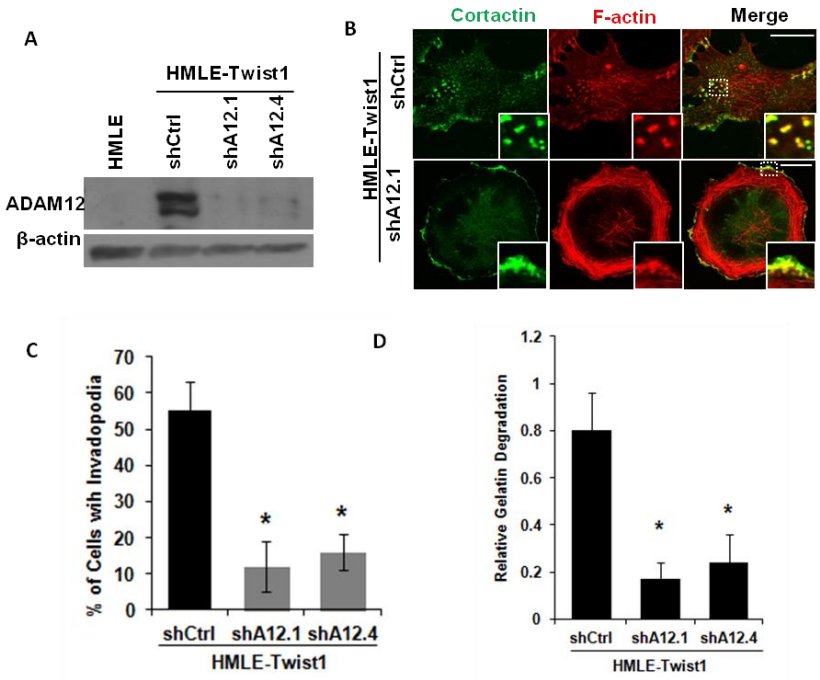

Figure 4: ADAM12 is necessary for Twist1-induced invadopodia formation. (A) Immunoblot demonstrating increase in ADAM12 expression upon overexpression of Twist1 in the indicated cell lines; multiple shRNA constructs are effective at reducing ADAM12 protein expression; (B) Representative immunofluorescent images showing a decrease in invadopodia formation upon knockdown of ADAM12 in the indicated cell lines stained with cortactin and F-actin; (C) Quantification of invadopodia formation in the indicated cell lines, indicating that ADAM12 is required for invadopodia formation; (D) Quantification of invadopodia-mediated gelatin degradation in the indicated cell lines, indicating an essential role for ADAM12 in regulation of ECM degradation. Scare bars are 5 um. Error bars are SEM, *p<0.05. shA12.1 = shADAM12.1; shA12.4 = shADAM12.4, two different shRNA constructs.

Aim 2: Determine if invadopodia are responsible for Twist1-induced metastasis.

PDGFRα and invadopodia are required for Twist1-induced metastasis.

Although we had evidence that PDGFRs and invadopodia were required for in vitro models of invasion through the gelatin degradation assays and Matrigel invasion assays (data not shown), we wanted to determine 1) if PDGFR signaling was required for Twist1-induced metastasis; and 2) if invadopodia-mediated degradation were required for Twist1-induced metastasis. To answer this questions, we used HMLE-Twist1 cells expressing the PDGFR shRNA constructs to determine if PDGFR signaling was required for Twist1-induced metastasis; to determine if invadopodia were actually necessary for this metastatic process, we used HMLE-Twist1 cells expressing shRNA constructs targeting Tks5. These cells were labeled with GFP and transformed with oncogenic Ras (V12G-Ras) and injected subcutaneously into the flanks of nude mice at a concentration of 1 million cells per injection, mixed with Matrigel. In this model, HMLE-Twist1 cells metastasize readily to the lung with the rate of metastasis easily quantifiable by investigation of the GFP signal in the lungs with fluorescence microscopy (Yang et al., 2004). Importantly, we observed no major differences in growth rate between cells expressing PDGFR or Tks5 specific shRNA constructs and those cells expressing control shRNA constructs.

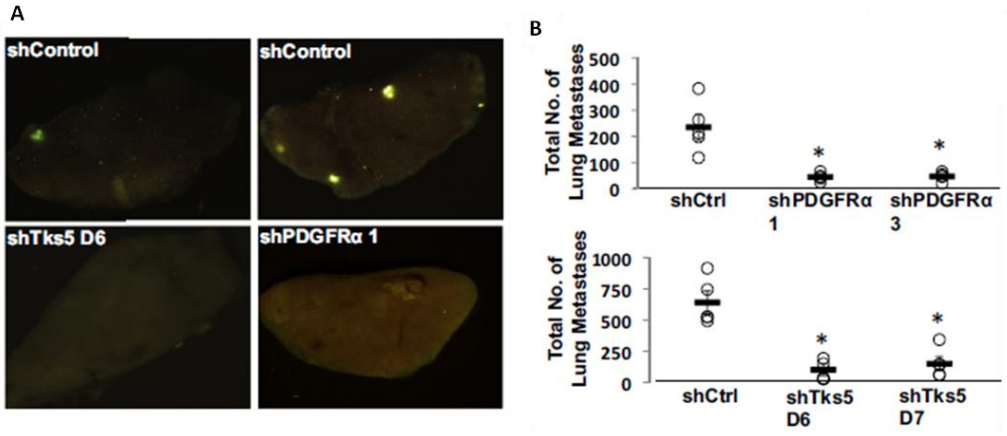

Figure 5: PDGFRα and invadopodia are necessary for Twist1-induced metastasis. (A) Representative images of lungs from mice injected with HMLER-Twist1 cells expressing the indicated constructs and GFP. Metastases appear as green dots on the lung surface. A clear reduction in the number of GFP-positive puncta corresponding to metastases can be observed upon knockdown of both Tks5 and PDGFRα; (B) Quantification of number of GFP-positive metastases to the lung in mice injected with HMLER-Twist1 cells expressing the indicated constructs. A significant decrease in lung metastasis was observed upon knockdown of both PDGFRα and Tks5, indicating role for both PDGFR signaling and Tks5-dependent invadopodia in mediating Twist1-induced metastasis. *p,0.05.

After allowing the tumors to reach 1.5 cm in diameter, the mice were sacrificed and the lungs analyzed for GFP-positive metastases. In HMLE-Twist1 cells expressing the control shRNA constructs, there was evidence of numerous micrometasases and some macrometastases throughout the lung through fluorescence imaging (Figure 5A). Importantly, when the number of GFP-positive puncta were quantified to determine the extent of metastases, there was a significant reduction in metastasis upon knockdown of both PDGFRα and Tks5 (Figure 5B-C). This data allowed us to conclude that induction of invadopodia formation by Twist1, through upregulation of PDGFRs, is essential for efficient lung metastasis in a mouse model of breast cancer.

ADAM12 is required for Twist1-induced metastasis to the lungs.

Due to the dramatic effect on invadopodia formation upon knockdown of ADAM12 in HMLE-Twist1 cells, we were curious if ADAM12 was also essential for Twist1-induced metastasis. To investigate this question, we essentially performed experiments identical to those completed in the above section to investigate the requirement for invadopodia and PDGFRs in Twist1-induced metastasis. Briefly, HMLE-Twist1 cells expressing control or shADAM12 constructs were transformed with oncogenic Ras and labeled with GFP and injected subcutaneously into the flanks of nude mice. After allowing the tumors to reach 1.5 cm in diameter, the mice were sacrificed and the lungs analyzed for the presence of GFP-positive metastases. Again, we observed a dramatic reduction in the number of GFP-positive nodules and micrometastases in the lungs of mice injected with cells expressing shADAM12 constructs compared to those mice injected with cells expressing shControl constructs (Figure 6A). Importantly, when we quantified the number of metastases by quantifying the number of GFP-positive puncta in the lungs of the mice, we observed a significant decrease in lung metastasis upon knockdown of ADAM12 (Figure 6B). This data suggested that ADAM12 also plays essential roles in regulating the metastatic process downstream of Twist1, likely through invadopodia formation.

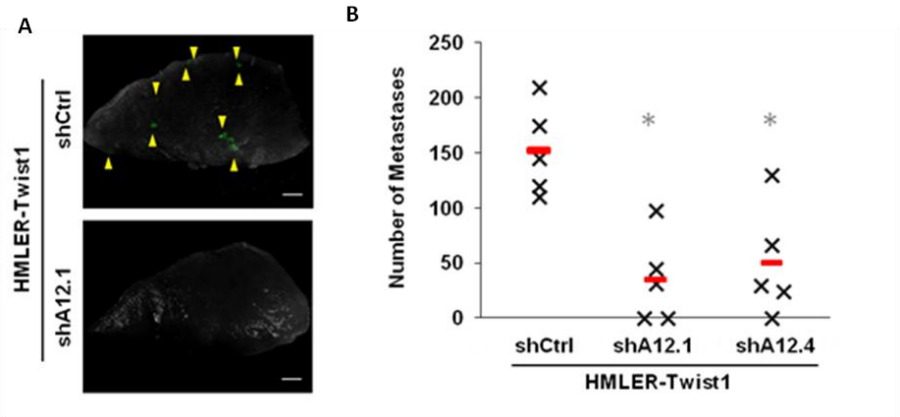

Figure 6: ADAM12 is necessary for Twist1-induced metastasis. (A) Representative images of lungs taken from mice injected subcutaneously with HMLER-Twist1 cells expressing the indicated constructs. Cells were labeled with GFP to allow visualization of metastases to the lung. Numerous GFP positive metastases (yellow arrows) are observed in shCtrl-expressing cells, while knockdown of ADAM12 reduces the number of GFP-positive puncta observed; (B) Quantification of the number of GFP positive metastases in the lungs of mice injected with HMLER-Twist1 cells expressing the indicated shRNA constructs. Scale bar = 1 mm; *p<0.05.

<u>Aim 3: Characterize the pathways responsible for inducing and mediating the formation and stability of Twist1-induced invadopodia.</u>

Induction of Src activity downstream of PDGFRs is necessary for invadopodia formation.

Src activity is required for invadopodia formation and regulates the initial steps of invadopodia formation (Chen 1989). Phosphorylation of Tks5 by Src leads to interaction with the adaptor protein Nck, which leads to eventual induction of focal Arp2/3 mediated actin polymerization via an N-WASP-dependent mechanism (Murphy et al., 2011). PDGFR signaling is directly upstream of activation of Src kinase, so we therefore investigated the status of Src signaling in cells overexpressing Twist1. Upon overexpressing Twist1 in HMLE cells, we observed a dramatic increase in Src activation (as measured by immunoblotting for the active form of Src, phosphorylated on tyrosine-416) (Figure 7A). In addition to the increase in Src activation, we also observed an increase in tyrosine phosphorylation of the invadopodia component protein cortactin upon expression of Twist1 (Figure 7A). This strongly implied that Twist1 could be regulating invadopodia formation by increasing Src activity, leading to

phosphorylation of invadopodia component proteins and eventual invadopodia formation. We therefore investigated if Src activity was required for invadopodia in our cells. Treatment of HMLE-Twist1 cells with either the Src kinase specific inhibitor SU6656, or expression of a dominant negative Src construct led to a dramatic decrease in both gelatin degradation and invadopodia formation (Figure 7B-C).

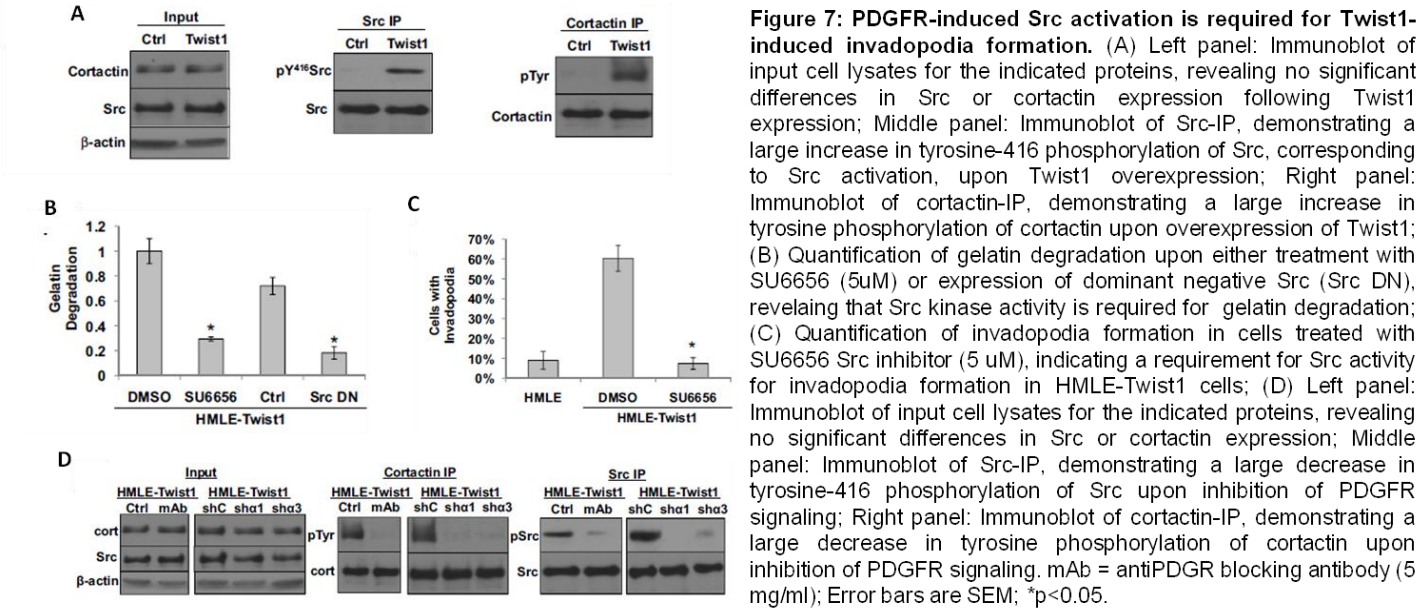

Figure 7: PDGFR-induced Src activation is required for Twist1-induced invadopodia formation. (A) Left panel: Immunoblot of input cell lysates for the indicated proteins, revealing no significant differences in Src or cortactin expression following Twist1 expression; Middle panel: Immunoblot of Src-IP, demonstrating a large increase in tyrosine-416 phosphorylation of Src, corresponding to Src activation, upon Twist1 overexpression; Right panel: Immunoblot of cortactin-IP, demonstrating a large increase in tyrosine phosphorylation of cortactin upon overexpression of Twist1; (B) Quantification of gelatin degradation upon either treatment with SU6656 (5uM) or expression of dominant negative Src (Src DN), revelaing that Src kinase activity is required for gelatin degradation; (C) Quantification of invadopodia formation in cells treated with SU6656 Src inhibitor (5 uM), indicating a requirement for Src activity for invadopodia formation in HMLE-Twist1 cells; (D) Left panel: Immunoblot of input cell lysates for the indicated proteins, revealing no significant differences in Src or cortactin expression; Middle panel: Immunoblot of Src-IP, demonstrating a large decrease in tyrosine-416 phosphorylation of Src upon inhibition of PDGFR signaling; Right panel: Immunoblot of cortactin-IP, demonstrating a large decrease in tyrosine phosphorylation of cortactin upon inhibition of PDGFR signaling. mAb = antiPDGR blocking antibody (5 mg/ml); Error bars are SEM; *p<0.05.

In Aim 1, we characterized an essential role for PDGFR in mediating Twist1-induced invadopodia formation. We were therefore curious if knockdown or inhibition of PDGFR led to changes in Src activation state in our system. When HMLE-Twist1 cells were treated with the monoclonal PDGFRα blocking antibody or when PDGFRα was knocked down in HMLE-Twist1 cells, we observed a significant decrease in both tyrosine phosphorylation of cortactin (Figure 7D). In addition, both inhibition with the blocking antibody and expression of PDGFRα knockdown constructs led to an attenuation of Src activation, as measured by immunoblotting of tyrosine-416 Src phosphorylation (Figure 7D). Combined with our earlier data from Aim 1, in which we observed that Twist1-induced invadopodia formation is dependent on PDGFR signaling, we concluded that Twist1 regulates invadopodia formation by upregulation of Src signaling through transcriptional regulation of PDGFRs.

ADAM12 may regulate the balance between focal adhesions and invadopodia.

In addition to roles as a metalloprotease, ADAM12 can also regulate integrin-mediated adhesions and focal adhesions through its disintegrin domain (Huang et al., 2005). We were therefore curious to determine if ADAM12 effected the formation of focal adhesions in cells expressing ADAM12 knockdown constructs. In HMLE-Twist1 cells expressing shADAM12 constructs, we observed a dramatic increase in focal adhesion formation, as measured by colocalization of the focal adhesion marker vinculin with F-actin on the cell periphery (Figure 8A). When quantified, a significant increase in focal adhesion formation was observed (Figure 8B). When we probed for a marker of focal adhesion activation, focal adhesion kinase (FAK) phosphorylation at tyrosine residue 396, we observed a significant increase in this biochemical marker of focal adhesion formation (Figure 8C). Similar experiments were also performed in Hs578t cells, with a similar increase in focal adhesion formation observed upon knockdown of ADAM12 (data not shown). This was very interesting, as previous reports have suggested that focal adhesions can inhibit invadopodia formation by sequestering signaling molecules, such as Src, at focal adhesions (Chan et al., 2009).

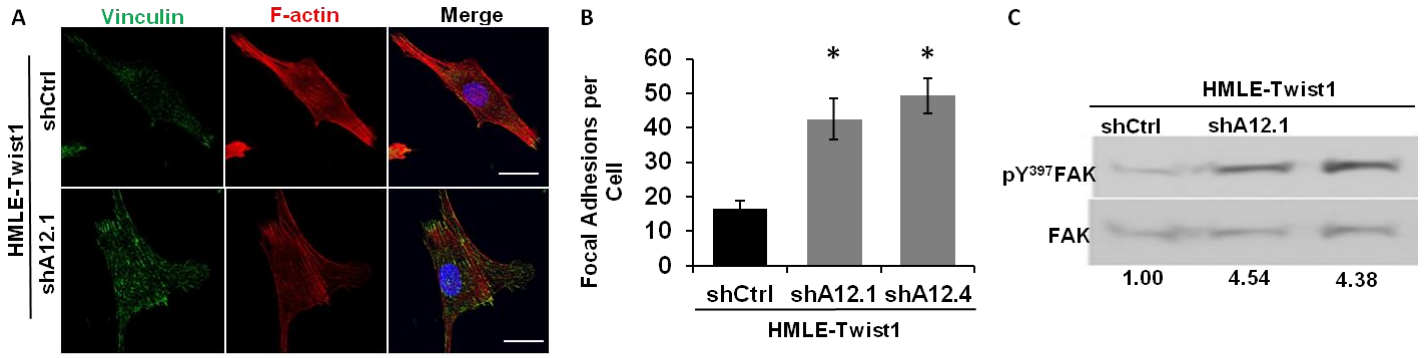

Figure 8: Knockdown of ADAM12 increases focal adhesion formation and FAK signaling. (A) Representative immunofluorescence image of focal adhesion formation (vinculin staining) in HMLE-Twist1 cells expressing the indicated constructs. A large increase in the number of vinculin-positive focal adhesions can be observed in cells expressing the shADAM12 construct; (B) Quantification of number of focal adhesions in HMLE-Twist1 cells expressing the indicated constructs demonstrating a large increase in focal adhesion formation in cells in which ADAM12 has been knocked down; (C) Immunoblot for total FAK and active FAK phosphorylated at residue tyrosine-397 in HMLE-Twist1 cells expressing the indicated shRNA constructs. Relative tyrosine phosphorylation is quantified below the blot, showing a more than 4-fold increase in FAK phosphorylation upon knockdown of ADMA12. Scale bar = 5 um; Error bars are SEM; *p<0.05.

To more carefully probe the potential effects of ADAM12 in regulating invadopodia and focal adhesions, we generated a panel of mutant ADAM12 constructs with which to rescue the shADAM12 defect. Briefly, mutants were created using PCR-mutagenesis which 1) lacked metalloprotease activity; 2) had mutations in the disintegrin domain that inhibited interactions with beta integrins; and 3) had a cytoplasmic truncation to remove the cytoplasmic tail (Jacobsen et al., 2008). These mutants allowed us to investigate the potential roles of the metalloprotease activity of ADAM12, the integrin-modulation abilities of ADAM12, and the possible effects of the cytoplasmic tail on invadopodia formation and focal adhesion formation. Initial experiments investigating the effect of re-expression of the mutant proteins in Hs578t cells expressing shADAM12 constructs determined that the disintegrin domain of ADAM12 play roles in regulating invadopodia formation in these cells (Figure 9A). These results suggest that ADAM12 may regulate invadopodia formation indirectly, rather than directly, through inhibition of focal adhesions that may inhibit invadopodia formation (Chan et al., 2009). Conversely, it may be the case that is an alteration in focal adhesion dynamics in shADAM12 knockdown cells that additionally negatively regulates invadopodia formation.

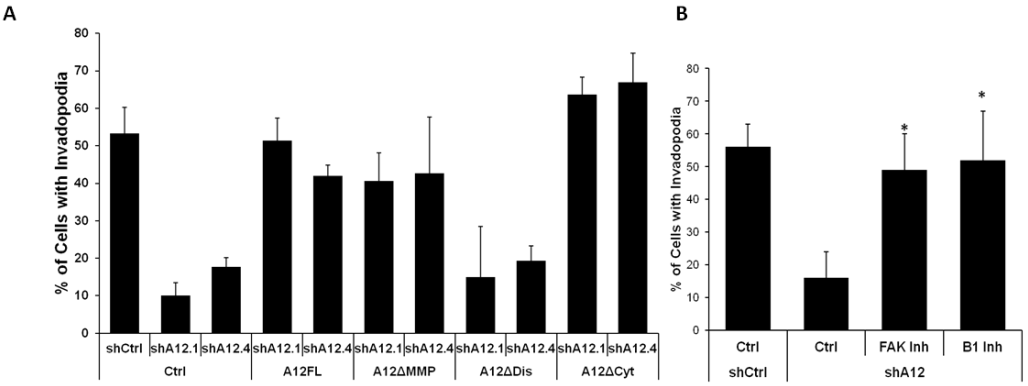

Figure 9: The disintegrin domain of ADAM12 is necessary for invadopodia formation. (A) Quantification of percentage of cells that form invadopodia (F-actin/cortactin colocalization) in Hs578t cells transfected with the following contructs: Ctrl=control vector; A12FL = full-length, wild-type ADAM12, A12ΔMMP=ADAM12 metalloprotease domain point mutation, A12ΔDis=ADAM12 disintegrin domain point mutation, A12ΔCyt = ADAM12 cytoplasmic domain truncation mutation. Only the disintegrin mutation failed to rescue the phenotype of reduced invadopodia formation in cells expressing shADAM12 constructs, implicating a role for regulation of focal adhesions or focal adhesion dynamics in invadopodia formation; (B) Quantification of percentage of cells forming invadopodia in Hs578t cells expressing the indicated constructs. Treatment with a FAK inhibitor PF228 (5 uM) or treatment with a β1 inhibitory antibody (AIIB2, 5 mg/ml) led to a rescue of invadopodia formation, further implicating focal adhesions in regulation of invadopodia formation. Error bars = SEM, *p<0.05.

To additionally probe the signaling pathways regulated by ADAM12, particularly integrins and focal adhesion, we performed addition experiments in the Hs578t cell lines expressing shADAM12 constructs. Treatment of these cells with either a FAK inhibitor or a β1 integrin inhibitory antibody (AIIB2 antibody) led to a dramatic increase in invadopodia formation (Figure 9B). In fact, treatment

with these inhibitors led to an almost complete rescue of the invadopodia formation defect. This strongly implies a potential role for ADAM12 in regulating invadopodia formation by indirectly regulating focal adhesions. We hypothesize that ADAM12 may either directly reduce the number of focal adhesions that form, or affect the overall lifetime of focal adhesions. Future experiments are focused on characterization of the status of important downstream signaling molecules such as Src, which is also regulated by focal adhesions. In addition, experiments are being performed to determine if the rescue mutants have complementary effects on the formation of focal adhesions. Finally, live-cell imaging of focal adhesion dynamics are in progress to determine if ADAM12 regulates focal adhesion formation or focal adhesion lifetime using an mCherry-paxillin fusion protein and fluorescence imaging.

Key Research Accomplishments

- Described an essential role of PDGFR signaling in regulating Src activity to promote invadopodia formation downstream of Twist1 expression
- Characterized the role of ADAM12 in promoting invadopodia formation by disrupting focal adhesion formation
- Determined both invadopodia formation and ADAM12/PDGFR upregulation by Twist1 are essential for metastasis in mouse models of breast cancer
- Participated in weekly journal and data discussions with laboratory to discuss recent publications and research within the lab
- Attended pharmacology research discussions to discuss ongoing research in the pharmacology department
- Meetings with PI occurred on a weekly basis to discuss ongoing work and future directions of research in the lab
- Experience preparing both primary and review literature
- Receiving mentoring experience through directing the work of undergraduate and rotating graduate students under my supervision

Reportable Outcomes

2009

- N/a

2010

- Published paper describing central role of Twist1 in mediating breast cancer metastasis through induction of invadopodia formation: 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): 372-86. See attached file in appendix.

2011

- Attended 2011 DOD BCRP Era of Hope Conference, Orlando, FL
- Published invited perspective article: Eckert MA, Yang J. Targeting invadopodia to block breast cancer metastasis. *Oncotarget*. 2011 Jul;2(7):562-8. See attached file in appendix.
- Manuscript in preparation: “ADAM12 promotes invadopodia formation by disrupting focal adhesion formation.”

Conclusion

Combined, our data suggest important roles for invadopodia in mediating Twist1-induced metastasis in breast cancer. We observed that Twist1 was both necessary and sufficient to promote invadopodia formation and associated degradation of ECM components. Multiple components of

invadopodia, including ADAM12 and PDGFRα and β were upregulated following Twist1 expression. Upregulation of PDGFRs, particularly PDGFRα, appear to be key in regulating pathways such as Src activation that are essential for efficient invadopodia formation. Inhibition of PDGFR or Src activity through a combination of knockdown constructs or chemical inhibition led to a dramatic reduction in invadopodia formation. Most importantly, however, we found that both PDGFRα-mediated signaling and invadopodia themselves were necessary for efficient metastasis to the lung in a mouse model of breast cancer. This provided firm data to conclude that invadopodia play important roles during the EMT process in promoting metastasis. Although characterization of the role of ADAM12 in invadopodia are ongoing, there is growing evidence that ADAM12 also modulates the formation of invadopodia downstream of Twist1. Surprisingly, our data thus far suggests that ADAM12 may regulate invadopodia indirectly through regulation of focal adhesions. We hypothesize that ADAM12 may either be necessary for the inhibition of focal adhesions, as focal adhesions may inherently inhibit invadopodia formation, or that ADAM12 induces dynamic turnover of focal adhesions that is required for efficient invadopodia formation (Chan et al., 2009). More experiments are clearly necessary to more fully understand the contribution of ADAM12 to Twist1-induced invadopodia formation and metastasis, although we do have evidence that ADAM12 is, in fact, required for Twist1-induced metastasis in a mouse model of breast cancer. Experiments are in progress to better understand the role of this interesting protease in invasion and metastasis. Better understanding the functional roles of downstream targets of Twist1 in metastasis will allow for the identification of new biomarkers as well as new therapeutic targets. Promisingly, both ADAM12 and PDGFRs are extremely druggable targets with catalytically active domains to which small-molecule inhibitors can be developed.

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Cancer Cell
Article

Twist1-Induced Invadopodia Formation Promotes Tumor Metastasis

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SUMMARY

The Twist1 transcription factor is known to promote tumor metastasis and induce Epithelial-Mesenchymal Transition (EMT). Here, we report that Twist1 is capable of promoting the formation of invadopodia, specialized membrane protrusions for extracellular matrix degradation. Twist1 induces PDGFRα expression, which in turn activates Src, to promote invadopodia formation. We show that Twist1 and PDGFRα are central mediators of invadopodia formation in response to various EMT-inducing signals. Induction of PDGFRα and invadopodia is essential for Twist1 to promote tumor metastasis. Consistent with PDGFRα being a direct transcriptional target of Twist1, coexpression of Twist1 and PDGFRα predicts poor survival in breast tumor patients. Therefore, invadopodia-mediated matrix degradation is a key function of Twist1 in promoting tumor metastasis.

INTRODUCTION

During metastasis, carcinoma cells acquire the ability to invade surrounding tissues and intravasate through the endothelium to enter systemic circulation. Both the invasion and intravasation processes require degradation of basement membrane and extracellular matrix (ECM). Although proteolytic activity is associated with increased metastasis and poor clinical outcome, the molecular triggers for matrix degradation in tumor cells are largely unknown.

Invadopodia are specialized actin-based membrane protrusions found in cancer cells that degrade ECM via localization of proteases (Tarone et al., 1985, Chen, 1989). Their ability to mediate focal ECM degradation suggests a critical role for invadopodia in tumor invasion and metastasis. However, a definitive role for invadopodia in local invasion and metastasis in vivo has not yet been clearly demonstrated. As actin-based structures, invadopodia contain a primarily branched F-actin core and actin regulatory proteins, such as cortactin, WASp, and the Arp2/3 complex (Linder, 2007). The SH3 domain-rich proteins Tks4 (Buschman et al., 2009) and Tks5 (Seals et al., 2005) function as essential adaptor proteins in clustering structural and enzymatic components of invadopodia. The matrix degradation activity of invadopodia has been associated with a large number of proteases, including membrane type MMPs (MT1-MMP) (Linder, 2007). Invadopodia formation requires tyrosine phosphorylation of several invadopodia components including cortactin (Ayala et al., 2008), Tks4 (Buschman et al., 2009), and Tks5 (Seals et al., 2005) by Src family kinases.

Our previous study found that the Twist1 transcription factor, a key regulator of early embryonic morphogenesis, was essential for the ability of tumor cells to metastasize from the mammary

Significance

Studies suggest that the EMT-inducing transcription factors play critical roles in tumor metastasis. A major question is what are the cellular functions and transcriptional targets of individual EMT-inducing transcription factors required for tumor metastasis. Our study identifies a unique function of Twist1 in promoting invadopodia-mediated matrix degradation, which is essential for its ability to promote metastasis. Formation of invadopodia and loss of cell adhesion are regulated by different transcription factors. This explains why multiple factors need to be activated coordinately to promote carcinoma cells to undergo EMT and invade. We also identify PDGFRα as a direct transcriptional target of Twist1 in promoting invadopodia formation and tumor metastasis, therefore suggesting that PDGFRs might be potential targets for anti-metastasis therapies.

gland to the lung in a mouse breast tumor model and was highly expressed in invasive human lobular breast cancer (Yang et al., 2004). Since then, studies have also associated Twist1 expression with many aggressive human cancers, such as melanomas, neuroblastomas, prostate cancers, and gastric cancers (Peinado et al., 2007). Twist1 can activate a latent developmental program termed the epithelial-mesenchymal transition (EMT), thus enabling carcinoma cells to dissociate from each other and migrate.

The EMT program is a highly conserved developmental program that promotes epithelial cell dissociation and migration to different sites during embryogenesis. During EMT, cells lose their epithelial characteristics, including cell adhesion and polarity, and acquire a mesenchymal morphology and the ability to migrate (Hay, 1995). Biochemically, cells downregulate epithelial markers such as adherens junction proteins E-cadherin and catenins and express mesenchymal markers including vimentin and fibronectin (Boyer and Thiery, 1993). In addition to Twist1, the zinc-finger transcription factors, including Snail, Slug, ZEB1, and ZEB2 (Peinado et al., 2007), can also activate the EMT program by directly binding the E-boxes of the E-cadherin promoter to suppress its transcription. However, it is unclear how Twist1, as a bHLH transcription factor, controls the EMT program. In this study, we test the hypothesis that Twist1 plays a major role in regulating ECM degradation to promote tumor metastasis.

RESULTS

Twist1 Is Necessary and Sufficient for Invadopodia Formation and Function

Our previous studies found that Twist1 expression was associated with increased metastatic potentials in a series of mouse mammary tumor cell lines, including 67NR, 168FARN, and 4T1 (Yang et al., 2004). Furthermore, Twist1 is required for the ability of 4T1 cells to metastasize from the mammary gland to the lung. To dissect the cellular functions of Twist1 in promoting tumor metastasis, we first tested whether expression of Twist1 was associated with increased ability to degrade ECM. 67NR, 168FARN, and 4T1 cells were plated onto FITC-conjugated gelatin matrix to assess their abilities to degrade matrix. We found that Twist1-expressing metastatic 168FARN and 4T1 cells potently degraded ECM in 8 hr, while nonmetastatic 67NR cells that do not express Twist1 failed to do so (Figures 1A–1C). To test whether Twist1 is required for the ability of 168FARN and 4T1 cells to degrade ECM, 168FARN and 4T1 cells expressing two independent shRNAs against Twist1 were processed for the matrix degradation assay (Figure 1A). Indeed, we found that suppressing Twist1 expression resulted in a potent reduction in matrix degradation in both cell types (Figures 1B and 1C). Together, these results demonstrate that Twist1 is required for ECM degradation ability in tumor cells.

Localized matrix degradation can be mediated through actin-based subcellular protrusions called invadopodia. Colocalization of F-actin with the actin-bundling protein cortactin (Bowden et al., 2006) or the unique adaptor protein Tks5 (Abram et al., 2003) can be used to identify invadopodia. To determine whether invadopodia are present in 168FARN and 4T1 cells and whether Twist1 is required for invadopodia formation, we examined the presence of invadopodia in 168FARN and 4T1 cells by immunofluorescence. Invadopodia are transient structures, so only a fraction of cells possess invadopodia at any given time. Indeed, over 50% 168FARN and 4T1 cells contain invadopodia, while suppression of Twist1 expression reduced the occurrence of invadopodia to 5%–20% in both cell lines (Figures 1D–1F; see Figures S1A and S1B available online). These data indicate that Twist1 is necessary for the formation of invadopodia for ECM degradation.

Since 168FARN and 4T1 mouse tumor cells contain additional genetic and epigenetic changes essential for their tumorigenic and metastatic abilities (Mani et al., 2007), we next tested whether Twist1 is sufficient to promote invadopodia formation and matrix degradation in HMLE cells, immortalized normal human mammary epithelial cells. As reported, expression of Twist1 induced EMT in HMLE cells (Yang et al., 2004). We examined the presence of invadopodia and found that over 60% of HMLE cells expressing Twist1 contained invadopodia, compared with 10% of HMLE control cells with invadopodia (Figures 2A and 2B; Figure S2A). Importantly, these invadopodia were all localized to the basal surface of the cell directly adjacent to the underlying matrix when examined with Z-sectioning (Figure 2C). To determine whether these Twist1-induced invadopodia are functional, we compared the ability of these two cell lines to degrade matrix using the FITC-gelatin degradation assay. Expression of Twist1 increased matrix degradation by approximately 10-fold (Figures 2D and 2E). Strikingly, focal matrix degradation precisely colocalized with F-actin positive puncta (Figure 2D), indicating that Twist1 is sufficient to promote the formation of functional invadopodia in HMLE cells. Furthermore, Twist1-induced matrix degradation is protease-driven since suppression of metalloproteases by GM6001 inhibited the ability of HMLE-Twist1 cells to degrade FITC-gelatin (Figure 2E). Together, these data demonstrate that Twist1 is both necessary and sufficient to promote invadopodia formation and focal matrix degradation.

Twist1-Mediated Matrix Degradation Is Invadopodia-Driven and Src Dependent

Since both invadopodia-associated proteases and secreted proteases can mediate matrix degradation, we next set out to determine whether invadopodia, not secreted proteases, are solely responsible for Twist1-induced matrix degradation. In HMLE-Twist1 cells, we expressed shRNAs against Tks5, an adaptor protein that is required for invadopodia formation, but not MMP secretion (Seals et al., 2005). Both shRNAs effectively suppressed Tks5 expression (Figure S3A), and gelatin zymography showed that knockdown of Tks5 did not affect the secretion of proteases, mainly MMP2, into conditioned media (Figure S3B). In contrast, suppression of Tks5 significantly reduced their abilities to form invadopodia (Figures 3A and 3B) and degrade FITC-gelatin matrix (Figure 3C). Complementary to these data, Boyden chamber migration and invasion assays showed that suppression of Tks5 inhibited the ability of HMLE-Twist1 cells to invade through Matrigel, but did not affect cell migration (Figures S3C and S3D). Together, these results demonstrate that the protease activity associated with invadopodia is the sole mediator of Twist1-induced matrix degradation.

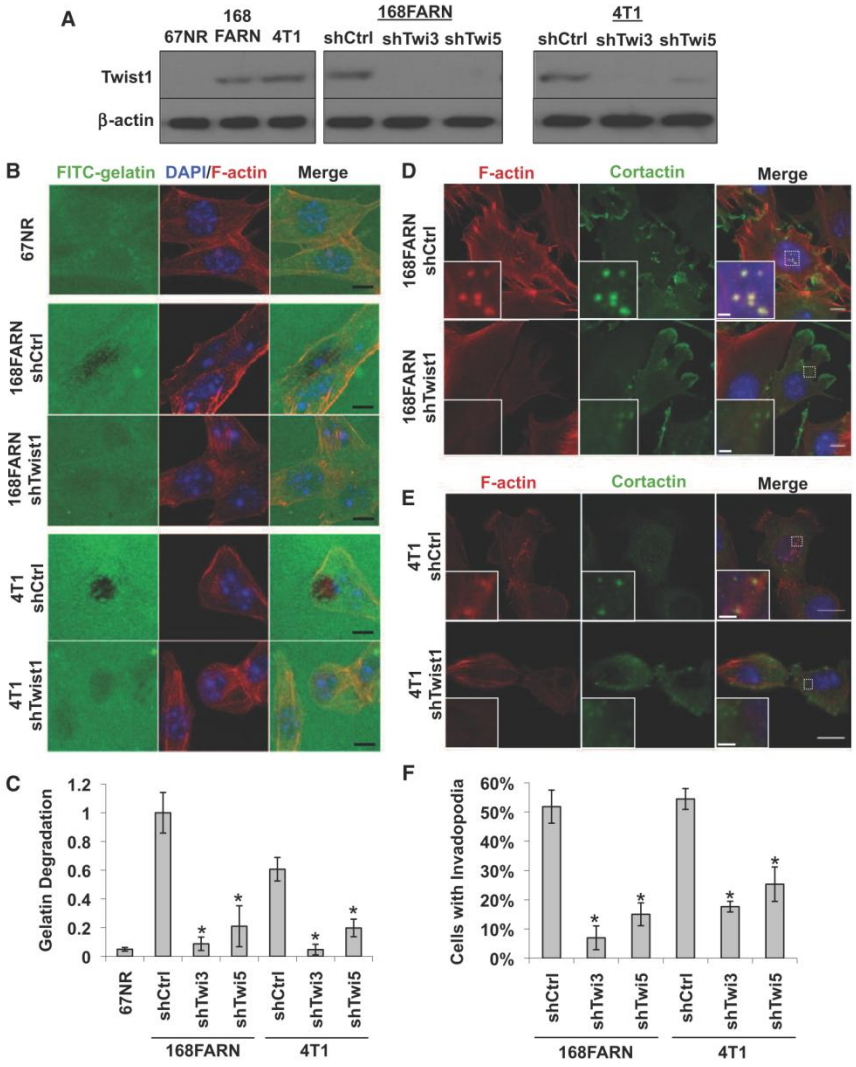

Figure 1. Twist1 Is Necessary for Invadopodia Formation

(A) Indicated cell lysates were analyzed by SDS-PAGE and probed for Twist1 and β-actin.

(B) 67NR, 168FARN (expressing control or Twist1 knockdown shRNA), and 4T1 (expressing control or Twist1 knockdown shRNA) cells were plated on FITC-conjugated gelatin (green) for 8 hr. F-actin was stained with phalloidin (red) and nuclei with DAPI (blue). Areas of gelatin degradation appear as punctuate black areas beneath the cells.

(C) Quantification of FITC-gelatin degradation. N = 150 cells/sample. *$p < 0.02$.

(D and E) 168FARN and 4T1 cells expressing control or Twist1 shRNAs were stained with phalloidin (red), DAPI (blue), and cortactin (green).

(F) Quantification of percentage of cells with invadopodia. N = 150 cells/sample. *$p < 0.02$.

Error bars are standard error of mean (SEM). Scale bars are 1 μm for insets, 5 μm for full images.

See also Figure S1.

We next set out to understand how Twist1 promotes invadopodia formation. While no transcription factor has been implicated in invadopodia regulation, tyrosine phosphorylation of invadopodia components, including cortactin and Tks5, is necessary for invadopodia formation (Ayala et al., 2008). We therefore assessed whether tyrosine phosphorylation at invadopodia was increased in HMLE-Twist1 cells. Immunofluorescence staining with a phosphotyrosine antibody revealed enrichment of phosphotyrosine at invadopodia (Figure 3D). Cortactin immunoprecipitated from HMLE-Twist1 cells also showed increased tyrosine phosphorylation compared to HMLE control cells (Figure 3E).

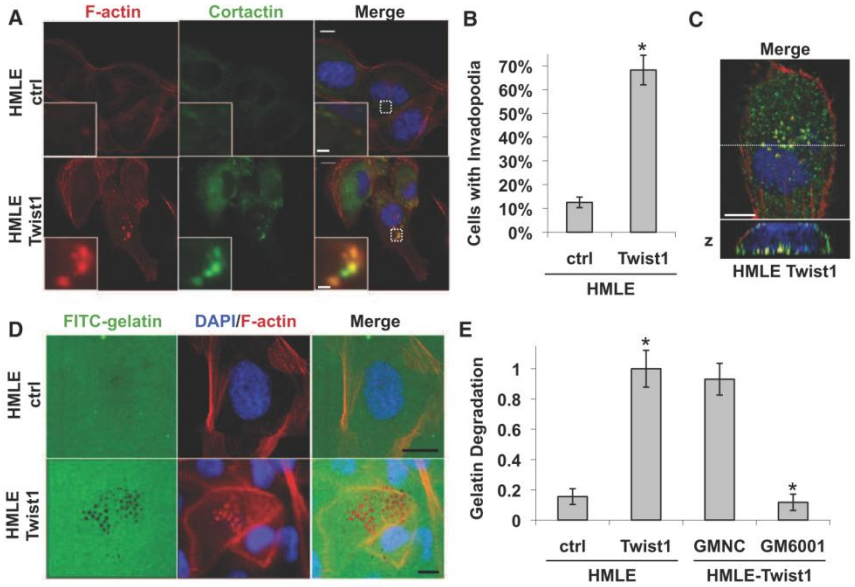

Figure 2. Twist1 Is Sufficient to Promote Invadopodia Formation

(A) HMLE cells expressing a control vector or Twist1 were plated on 0.2% gelatin matrix for 72 hr and invadopodia were visualized by colocalization of cortactin (green) and F-actin (red).

(B) Quantification of cells with invadopodia. N = 150 cells/sample. *$p < 0.02$.

(C) Colocalization of F-actin (red) and cortactin (green) is restricted to the basal side of cells in direct contact with the underlying matrix.

(D) HMLE control or HMLE-Twist1 cells were plated on FITC-gelatin for 8 hr and stained for F-actin (red) and nuclei (blue).

(E) Quantification of degradation by HMLE-ctrl and HMLE-Twist1 cells and HMLE-Twist1 cells treated with 25 μM GM6001 Negative Control (GMNC) or 25 μM GM6001 for 8 hr. N = 150 cells/sample. *$p < 0.02$.

Error bars are SEM. Scale bars are 1 μm for insets, 5 μm for full images.

See also Figure S2.

Src family kinases are the major kinases that promote tyrosine phosphorylation and formation of invadopodia. We therefore examined whether Twist1 induced expression of any of the three major Src family kinases, Src, Yes, and Fyn. Both real-time RT-PCR and immunoblotting analyses showed that none of the three Src kinases were greatly induced by Twist1 (Figures S3E and S3F; Figure 3E). Interestingly, when we probed for the activation status of Src, Yes, and Fyn in HMLE-Twist1 cells using an antibody recognizing the active form of Src family kinases (phosphotyrosine 416), Src was significantly activated upon Twist1 expression (Figure 3E), while Yes and Fyn phosphorylation remained constant (Figure S3F). These data suggest that activation of Src kinase activity, but not transcriptional induction of Src kinase expression, might be responsible for tyrosine phosphorylation at invadopodia in HMLE-Twist1 cells. To determine whether Src kinase activity is required for Twist1-induced invadopodia function, we treated HMLE-Twist1 cells with SU6656, a selective inhibitor of Src family kinases (Blake et al., 2000) (Figure S3G) or expressed a dominant-negative Src ($Src^{K295M/Y527F}$) (Figure S3H). Both treatments reduced the ability of HMLE-Twist1 cells to degrade matrix by 5-fold (Figure 3F), indicating that Src kinase activity is essential for Twist1-mediated invadopodia function. Treatment with SU6656 also inhibited colocalization of the phosphotyrosine signal with F-actin (Figure 3D) and caused a significant reduction in the number of cells that formed invadopodia (Figure 3G). Together, these results indicate that Twist1-induced invadopodia formation and function is dependent on activation of the Src kinase.

Twist1-Induced PDGFR Expression and Activation Is Required for Invadopodia Formation

As a transcription factor, Twist1 cannot directly activate Src kinase, so we probed how Twist1 promotes activation of Src in HMLE-Twist1 cells. Since activation of Src kinase is downstream of growth factor receptor (GFR) activation, we examined induction of known GFRs upstream of Src by Twist1. Using an inducible Twist1 (Twist1-ER) construct (Mani et al., 2008), we found that expression of PDGFRα mRNAs increased 3-fold within 3 hr of Twist1 activation and reached over 6000-fold induction at Day 15, while induction of PDGFRβ mRNAs occurred significantly later (Figure 4A). PDGFRs can directly activate Src family kinases by tyrosine phosphorylation (Kypta et al., 1990), and activation of a PDGF autocrine loop is associated with the EMT program (Jechlinger et al., 2003). We found that PDGFRα and β proteins were also induced in HMLE-Twist1 cells and both PDGFR α and β were phosphorylated at tyrosine residues corresponding to their active states (Figure 4B). This activation of PDGFR without exogenous PDGF ligands implies the existence of an autocrine activation loop in vitro most likely mediated by PDGF-C, the only PDGF ligand significantly expressed and

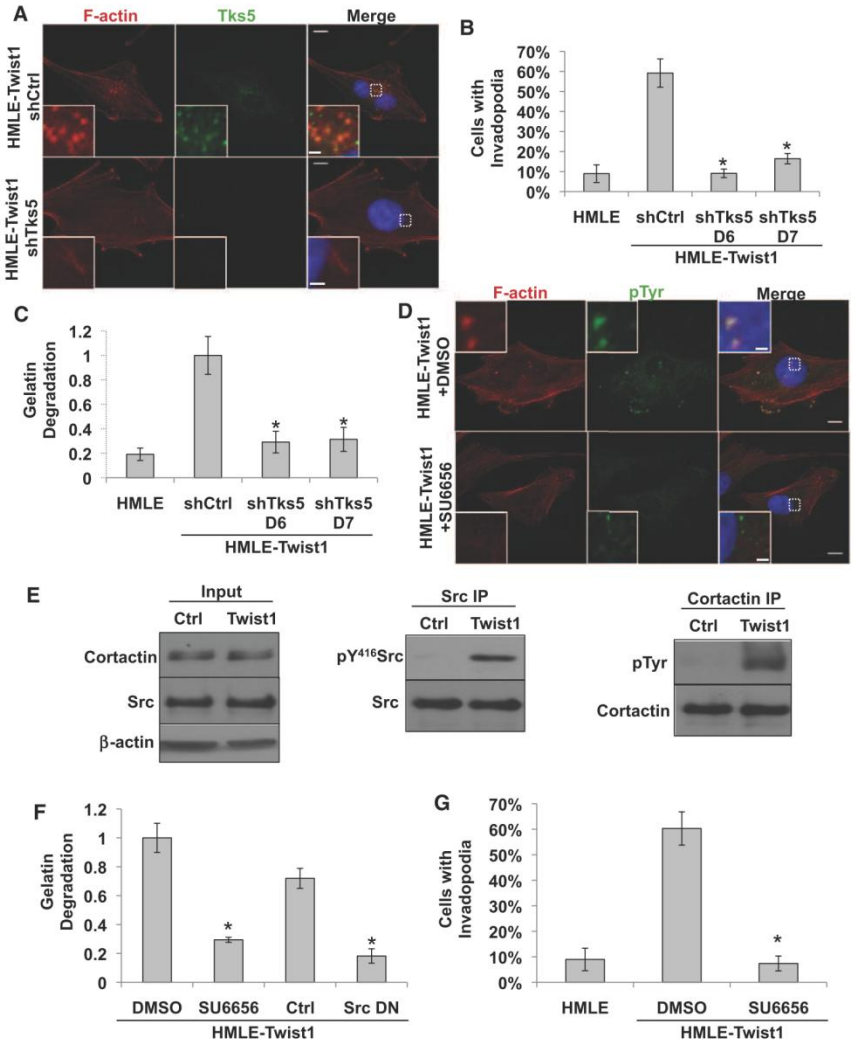

Figure 3. Twist1-Mediated Matrix Degradation Is Invadopodia Driven and Src Dependent

(A) HMLE-Twist1 cells expressing a control or Tks5 shRNA were plated on 0.2% gelatin and stained for Tks5 (green) or phosphotyrosine (green) and F-actin (red).

(B) Quantification of cells with invadopodia. N = 150 cells/sample. *$p < 0.02$.

(C) Quantification of FITC-gelatin degradation. N = 150 cells/sample. *$p < 0.02$.

(D) HMLE-Twist1 cells were plated on 0.2% gelatin and treated with treated with DMSO or 5 μM SU6656 for 12 hr and stained for phosphotyrosine (green) and F-actin (red).

(E) Cortactin and Src were immunoprecipitated from HMLE control and HMLE-Twist1 cell lysates, analyzed by SDS-PAGE, and probed for cortactin and phosphotyrosine and Src and $pTyr^{416}Src$, respectively. Input lysates were probed for β-actin, Src, and cortactin.

(F) Quantification of FITC-gelatin degradation. Indicated cells were treated with 5 μM SU6656 or DMSO for 12 hr or transfected with control or SrcK295M/Y527F vectors. N = 150 cells/sample. *$p < 0.02$.

(G) Quantification of cells with invadopodia. N = 150 cells/sample. *$p < 0.02$.

Error bars are SEM. Scale bars are 1 μm for insets, 5 μm for full images.

See also Figure S3.

upregulated upon activation of Twist1 in HMLE cells (Figure S4A). Upregulation of PDGFRs by Twist1 therefore presented a potential mechanism for activation of Src by Twist1.

We next set out to determine whether activation of PDGFRs is required for Twist1-induced invadopodia formation and matrix degradation. Given the immediate and robust induction of PDGFRα upon Twist1 activation, we focused on inhibiting PDGFRα to examine its role in mediating Twist1-induced Src activation and invadopodia formation. We first treated the HMLE-Twist1 cells with a monoclonal blocking antibody

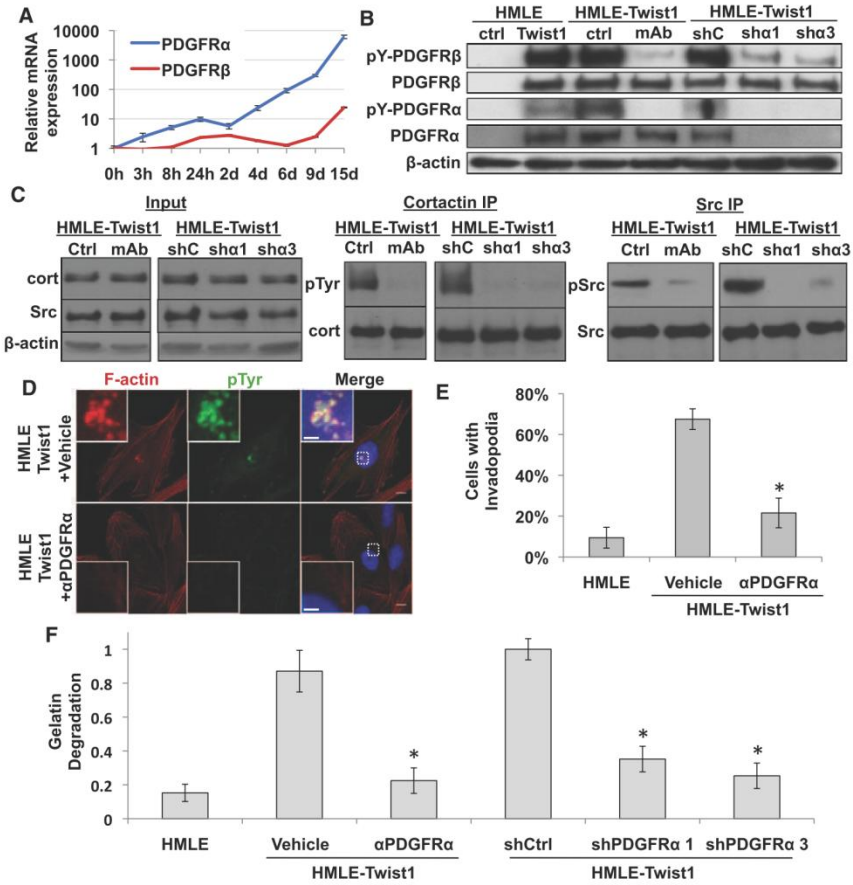

Figure 4. Twist1-Induced PDGFR Expression and Activation Is Required for Invadopodia Formation

(A) Real-time PCR analysis of PDGFRα and PDGFRβ expression in HMLE-Twist1-ER cells treated with 20 nM 4-hydroxy-tamoxifen.

(B) Cell lysates from HMLE control, HMLE-Twist1 cells, HMLE-Twist1 cells treated with vehicle or 8 μg/ml PDGFRα blocking antibody (ctrl and mAb), and HMLE-Twist1 cells expressing control (shC) or PDGFR (shα1 and 3) shRNA were analyzed by SDS-PAGE and probed for β-actin, PDGFRα, PDGFRβ, pTyr^{754}PDGFRα, and pTyr^{1009}PDGFRβ.

(C) Cortactin and Src were immunoprecipitated from cell lysates of HMLE-Twist1 cells treated with 8 μg/ml PDGFRα blocking antibody (mAb) or vehicle control (ctrl) or HMLE-Twist1 cells expressing indicated shRNAs (control, shC; shPDGFRα, shα1 and shα3) and probed for total cortactin and phosphotyrosine or total Src and pTyr^{419}Src, respectively. Input lysates were probed for β-actin, cortactin, and total Src.

(D) HMLE-Twist1 cells were seeded on 0.2% gelatin and treated for 24 hr with 8 μg/ml PDGFRα blocking antibody (αPDGFRα) or vehicle control and stained for phosphotyrosine (green), F-actin (red), and nuclei (blue). Scale bars are 1 μm for insets, 5 μm for full images.

(E) Quantification of cells with invadopodia. N = 150 cells/sample. *$p < 0.02$.

(F) Quantification of FITC-gelatin degradation. N = 150 cells/sample. *$p < 0.02$.

Error bars are SEM.

See also Figure S4.

against PDGFRα and examined invadopodia formation and matrix degradation. This antibody effectively inhibited PDGFRα activation (Figure 4B), Src activation, and tyrosine phosphorylation of cortactin in HMLE-Twist1 cells (Figure 4C). This PDGFRα blocking antibody significantly inhibited invadopodia formation and tyrosine phosphorylation at invadopodia and suppressed the ability of HMLE-Twist1 cells to degrade FITC-gelatin by over 5-fold (Figures 4D–4F). To verify the results observed with the PDGFRα blocking antibody, we also expressed two independent shRNAs against PDGFRα in HMLE-Twist1 cells to stably suppress and inhibit PDGFRα signaling. Both shRNAs potently suppressed PDGFRα expression (Figure 4B), Src activation, and cortactin phosphorylation (Figure 4C), and effectively suppressed the ability of HMLE-Twist1 cells to degrade matrix (Figure 4F). Importantly, expression or secretion of proteases was not affected by PDGFRα knockdown as measured with gelatin zymography (Figure S4B). Together, these data indicate that PDGFRα expression and activation is required for Twist1-induced invadopodia formation and invasion.

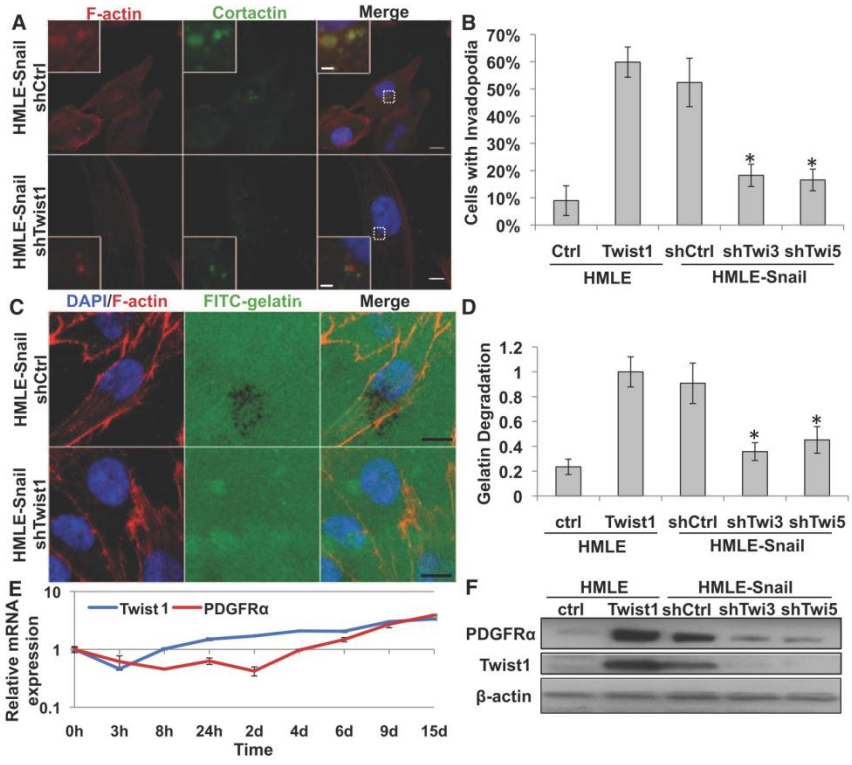

Figure 5. Twist1 Is Required for Snail-Induced Invadopodia Formation

(A) HMLE-Snail cells expressing indicated shRNA were seeded on 0.2% gelatin for 72 hr, and stained for cortactin (green), F-actin (red), and nuclei (blue).

(B) Quantification of cells with invadopodia. N = 150 cells/sample. *$p < 0.02$.

(C) HMLE-Snail cells expressing control or Twist1 shRNA were seeded on FITC-gelatin (green) for 8 hr and stained for F-actin (red) and nuclei (blue).

(D) Quantification of FITC-gelatin degradation. N = 150 cells/sample. *$p < 0.02$.

(E) Real-time PCR analysis of PDGFRα and Twist1 mRNA expression in HMLE-Snail-ER cells treated with 20 nM 4-hydroxy-tamoxifen.

(F) Cell lysates from indicated cells were analyzed by SDS-PAGE and probed for PDGFRα, Twist1, and β-actin.

Error bars are SEM. Scale bars are 1 μm for insets, 5 μm for full images.

See also Figure S5.

We also examined expression of PDGFRα in 168FARN cells expressing control and Twist1 knockdown constructs. PDGFRα was highly expressed in control cells and significantly reduced upon knockdown of Twist1 (Figure S4C). These results provide further evidence that expression of PDGFRα depends on the presence of Twist1 in breast tumor cells.

Twist1 Is a Central Mediator of Invadopodia Formation in Response to EMT-Inducing Signals

Since other inducers of EMT, such as TGFβ and Snail, have also been associated with tumor invasion and metastasis, we sought to understand whether invadopodia formation also occurs in response to other EMT-inducing signals and whether Twist1 mediates invadopodia formation in response to these signals.

To do so, we first tested the ability of Snail, another EMT-inducing transcription factor, to promote invadopodia formation and matrix degradation. As previously reported, Snail overexpression induces EMT similarly to Twist1 in HMLE cells (Mani et al., 2008). HMLE-Snail cells have similar numbers of invadopodia and ECM-degradation activities as HMLE-Twist1 cells (Figures 5A–5D). To determine whether Snail, like Twist1, could induce the expression of PDGFRα to promote invadopodia formation, we examined the expression of PDGFRα mRNA in HMLE cells that express an inducible Snail (Snail-ER) construct. In contrast to the immediate induction of PDGFRα upon Twist1 activation, PDGFRα mRNA only began to increase 6 days after Snail activation, indicating that induction of PDGFRα by Snail is indirect (Figure 5E). Interestingly, endogenous Twist1 mRNA levels increased significantly after 4 days of Snail activation, before PDGFRα mRNA began to increase (Figure 5E). These data suggest that induction of endogenous Twist1 could be responsible for PDGFRα expression and invadopodia formation upon Snail activation.

To assess whether Twist1 mediates the induction of invadopodia and PDGFRα in HMLE-Snail cells, we expressed shRNAs against endogenous Twist1 in HMLE-Snail cells. Indeed, suppression of endogenous Twist1 significantly inhibited expression of PDGFRα in HMLE-Snail cells (Figure 5F).

Significantly, suppression of Twist1 expression inhibited invadopodia formation in HMLE-Snail cells and reduced their ability to degrade matrix (Figures 5A–5D). Importantly, HMLE-Snail cells that express shRNAs against Twist1 presented an EMT phenotype with loss of E-cadherin expression and a mesenchymal morphology (Figures S5A and S5B), indicating that suppression of E-cadherin by Snail and induction of invadopodia by Twist1 are regulated independently. Treating HMLE-Snail cells with the PDGFRα blocking antibody also significantly suppressed the ability of HMLE-Snail cells to degrade FITC-gelatin (Figures S5C and S5D). Together, these results indicate that Twist1 and PDGFRα are responsible for invadopodia formation in response to Snail activation.

To further generalize our finding, we also investigated the role of Twist1 and PDGFRα in regulating invadopodia formation in response to TGFβ. In EpH4 mouse mammary epithelial cells, TGFβ has been shown to collaborate with Ras to promote EMT and activates an autocrine PDGF loop (Jechlinger et al., 2003). When we examined the invadopodia formation and matrix degradation in EpH4-Ras cells treated with TGFβ, we found that TGFβ treatment induced over 5-fold increase of invadopodia formation and matrix degradation in 2D culture (Figures 6A–6C). When these cells grew in 3D culture with TGFβ, invadopodia were visible at the leading edge of cells invading out of the organoids (Figure 6E). Interestingly, both Twist1 and PDGFRα were induced in response to TGFβ treatment (Figure 6D). When endogenous Twist1 induction was inhibited by shRNAs, invadopodia formation and matrix degradation were significantly reduced in 2D and 3D cultures (Figures 6A–6C and 6E). Importantly, knocking down Twist1 abolished induction of PDGFRα in EpH4-Ras cells treated with TGFβ (Figure 6D), but did not prevent induction of EMT morphogenesis and loss of E-cadherin (Figures S6A and S6B), similar to knockdown of Twist1 in HMLE-Snail cells. Furthermore, treating EpH4-Ras cells with the PDGFRα inhibitor ST1571 significantly suppressed their ability to degrade FITC-gelatin in response to TGFβ treatment (Figures S6C and S6D). Importantly, treatment with STI571 did not revert the EMT phenotype (Figure S6E). Together, these results support our conclusion that Twist1 is a central mediator of invadopodia formation and matrix degradation via induction of PDGFRα in response to EMT-inducing signals.

Twist1-Induced Metastasis Is Mediated by Invadopodia In Vivo and Requires PDGFRα

Twist1 is required for mammary tumor cells to metastasize from the mammary gland to the lung. We then tested whether PDGFRα and invadopodia are required for the ability of Twist1 to promote tumor metastasis in vivo. To do so, we generated HMLE-Twist1 cells that were transformed with oncogenic Ras (HMLER-Twist1) and expressed shRNAs against either PDGFRα or a control shRNA. These cells also expressed GFP to allow identification of tumor cells in mice. Individual cell lines were injected subcutaneously into nude mice. Suppression of PDGFRα did not affect cell proliferation in culture or tumor growth rate in vivo (Figures S7A and S7B). Six weeks after tumor implantation, we sacrificed the mice and examined primary tumors for histology and invadopodia. Since HMLER-Twist1 tumors expressing large T antigen, we used an antibody against large T antigen to stain implanted tumor cells. Interestingly, HMLER-Twist1 tumor cells invaded into surrounding stroma and adjacent adipose tissue, while PDGFRa knockdown inhibited local invasion and tumor cells remained encapsulated (Figure 7A). Staining for invadopodia using cortactin and Tks5 in sections of primary tumor tissue revealed that HMLER-Twist1 tumor cells contained abundant invadopodia, while knocking down PDGFRα significantly reduced their occurrence (Figures 7B and 7C). To test whether PDGFRα is required for distant metastasis, examination of lung lobes and sections revealed clusters of HMLER-Twist1 shControl cells throughout the lungs (Figure 7E; Figure S7E). Significantly, suppression of PDGFRα expression significantly reduced the number of disseminated tumor cells in the lung (Figure 7D). These results strongly indicate that induction of PDGFRα is required for the ability of Twist1 to form invadopodia and promote tumor metastasis without affecting primary tumor growth in vivo.

To demonstrate that invadopodia are required for the ability of Twist1 to metastasize in vivo, we expressed shRNAs against Tks5 to inhibit invadopodia formation in HMLER-Twist1 cells. Knockdown of Tks5 did not affect cell growth rate in vitro (Figure S7C), which is consistent with a previous study (Blouw et al., 2008).''. These cells were implanted subcutaneously into nude mice to follow primary tumor growth and lung metastasis. Consistent with the results from the PDGFRα knockdown experiments, Tks5 knockdown inhibited local tumor invasion and significantly reduced the numbers of tumor cells that disseminated into the lung, while primary tumor growth was not affected (Figures 7A, 7D, and 7E; Figure S7D). Together, these data demonstrate that induction of invadopodia formation via PDGFRα activation is essential for the ability of Twist1 to promote tumor metastasis in vivo.

PDGFRα Is a Direct Transcription Target of Twist1 and Expression of Twist1 and PDGFRα Are Tightly Linked in Human Breast Tumors

Given the immediate induction of PDGFRα by Twist1 and their tight association in various tumor cells, we set out to determine whether PDGFRα is a direct transcriptional target of Twist1. We examined the human PDGFRα promoter for potential Twist1-binding E-box sequences (CANNTG). We designed three sets of primers on the putative promoter: primer sets 1 and 2 target the identified E-box, and primer set 3 targets an adjacent region lacking the putative E-box (Figure 8A). By chromatin immunoprecipitation, we found that Twist1 directly bound to the E-box on the putative PDGFRα promoter (Figure 8B). Twist1 was able to activate the isolated human PDGFRa promoter in an E-box-dependent fashion in a luciferase reporter assay (Figures S8A and S8B). Furthermore, this consensus E-box sequence is highly conserved between all mammalian species examined and chickens (Figure 8C), indicating that induction of PDGFRα by Twist1 is direct and evolutionally conserved.

To more directly probe the in vivo association between Twist1 and PDGFRα in human breast tumor samples, we analyzed four published large human breast tumor gene expression data sets summarizing 860 primary breast cancers (Pawitan et al., 2005; Sotiriou et al., 2006; Wang et al., 2005; Miller et al., 2005). In each data set, we calculated the rank-based Spearman correlation coefficient between Twist1 and all 22282 genes on the array, including PDGFRa. PDGFRα was consistently among the top

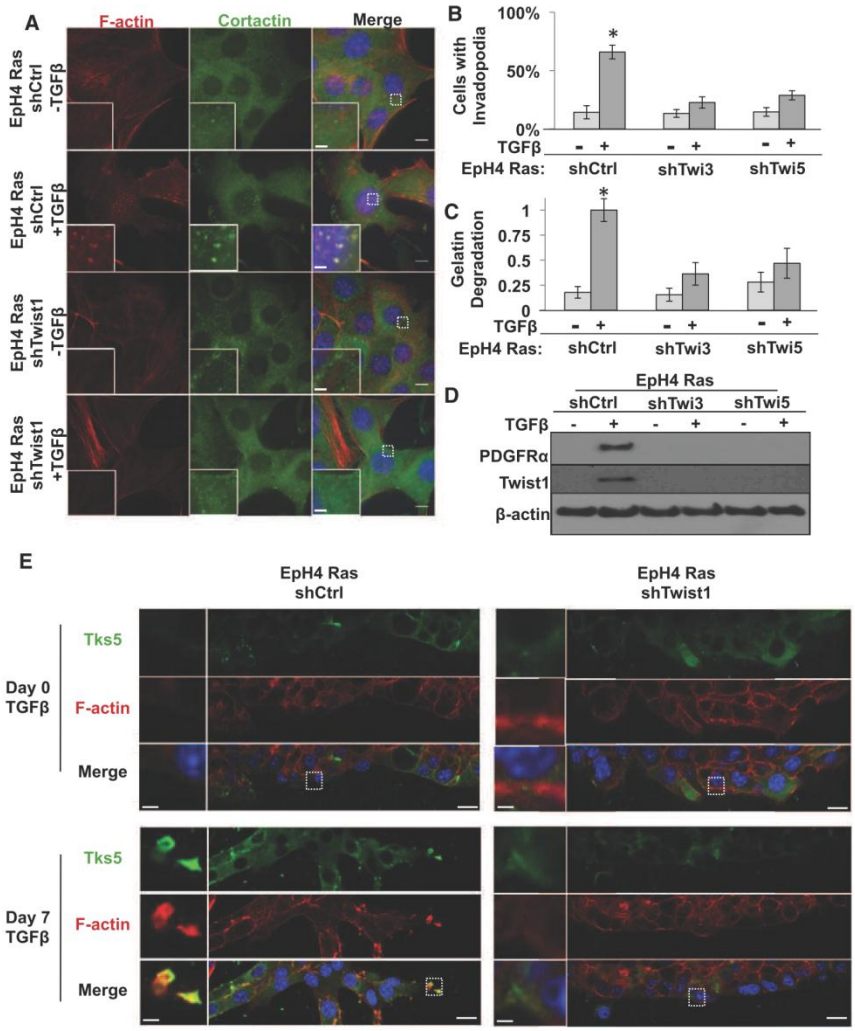

Figure 6. Twist1 Is Required for TGFβ-Induced Invadopodia Formation in Eph4Ras Cells

(A) Eph4Ras cells expressing control or Twist1 shRNAs were seeded on 0.2% gelatin for 72 hr before and after treatment with 5 ng/ml TGFβ1 for 7 days and stained for cortactin (green), F-actin (red), and nuclei (blue).

(B) Quantification of cells with invadopodia before and after 7 days of 5 ng/ml TGFβ1 treatment for EpH4Ras cells expressing indicated shRNAs. N = 150 cells/sample. *$p < 0.02$.

(C) Quantification of FITC-gelatin degradation for cells expressing indicated shRNA before and after 7 days of 5 ng/ml TGFβ1 treatment. N = 150 cells/sample. *$p < 0.02$.

(D) Cell lysates from indicated cells before and after treatment with 5 ng/ml TGFβ1 for 7 days were analyzed by SDS-PAGE and probed for PDGFRα, Twist1, and β-actin.

(E) Indicated cells were embedded in 1:1 mixture of Matrigel and collagen, allowed to form 3D structures, and processed for IF before and after 7 days of induction with 7 ng/ml TGFβ1. Cells were stained for Tks5 (green) and F-actin (red).

Error bars are SEM. Scale bars are 1 μm for insets, 5 μm for full images.

See also Figure S6.

ranked genes associated with Twist1 (4th, 17th, 47th, and 54th out of 22,282 genes) in all four breast cancer data sets (Figure 8D; Figure S8D). Expression of Twist1 and PDGFRα were positively correlated with correlation coefficients ranging from 0.56 to 0.70 (Figure 8D; Figure S8C). Furthermore, in all four data sets, PDGF ligand expression correlated with PDGFRα and Twist1 expression in over 95% of tumor samples (Table S1), indicating that PDGFRα could be active in these samples. To further access

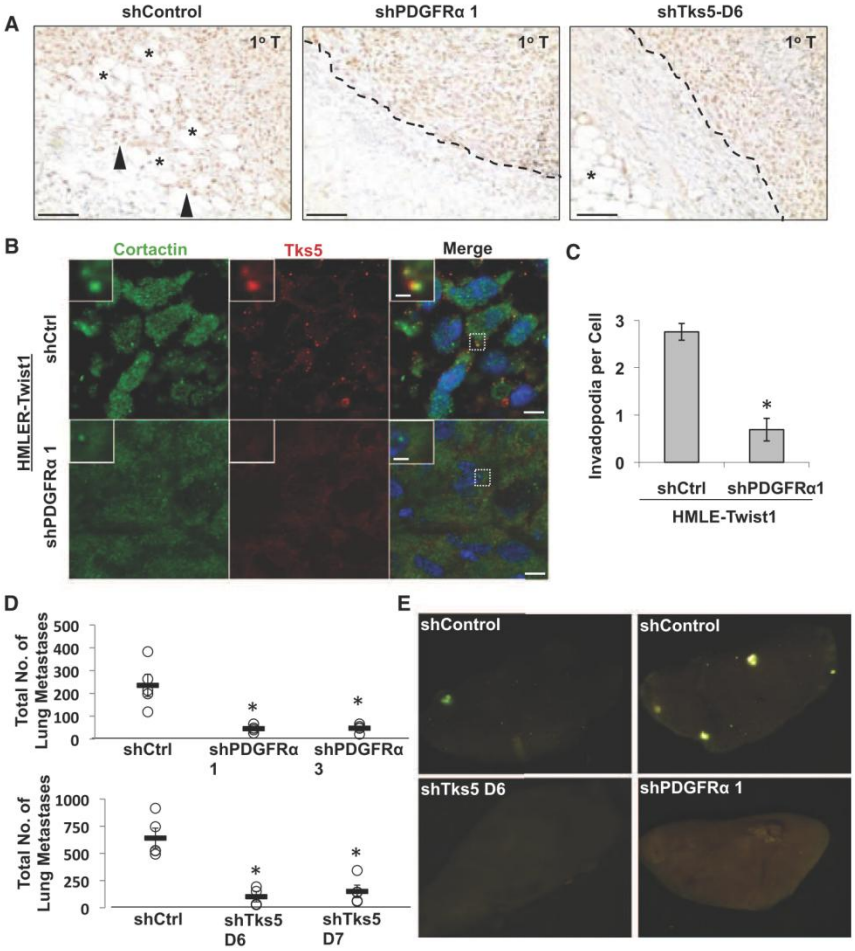

Figure 7. Twist1-Induced Metastasis Is Mediated by Invadopodia In Vivo and Requires PDGFRα

(A) Representative images of primary tumor paraffin tissue sections stained with SV40 Large-T antigen IHC and counterstained with hematoxylin. Tumor margin is indicated with dashed line when apparent. Closed triangles indicate invasive, Large-T positive tumor cells. Asterisks indicate adjacent adipose tissue. Scale bars are 100 μm.

(B) Images of sections of primary tumors stained with cortactin (green), Tks5 (red), and DAPI (blue). Scale bars are 1 μm for insets, 5 μm for full images.

(C) Quantification of number of invadopodia (cortactin/Tks5 colocalization) per cell. N = 150 cells/sample. *$p < 0.02$.

(D) Quantification of total number of GFP positive tumor cells (HMLER-Twist1 cells expressing indicated shRNAs) in individual lungs. N = 5 mice per group.

(E) Representative images of lungs from mice injected with HMLER-Twist1 cells expressing indicated shRNAs show a decrease in dissemination of GFP positive tumor cells (green) to the lungs upon knockdown of PDGFRα or Tks5.

Error bars are SEM.

See also Figure S7.

whether coexpression of Twist1 and PDGFRα could affect survival in breast tumor patients, we stained Twist1 and PDGFRα in a set of human invasive breast tumor tissue array samples and found that coexpression of Twist1 and PDGFRα was negatively associated with long-term survival (Figures 8E and 8F). Together, these data provide further support for a direct and functional association between Twist1 and PDGFRα in human breast cancers and suggest that regulation of invadopodia by Twist1 and PDGFRα contributes to human breast cancer progression.

DISCUSSION

Our study has identified a unique function of the Twist1 transcription factor in promoting invadopodia formation and matrix degradation during tumor metastasis. We demonstrate that transcriptional induction of PDGFRα and activation of Src by Twist1 are essential for invadopodia formation and matrix degradation. Induction of PDGFRα and invadopodia formation is also essential for the ability of Twist1 to promote metastasis in vivo. Twist1

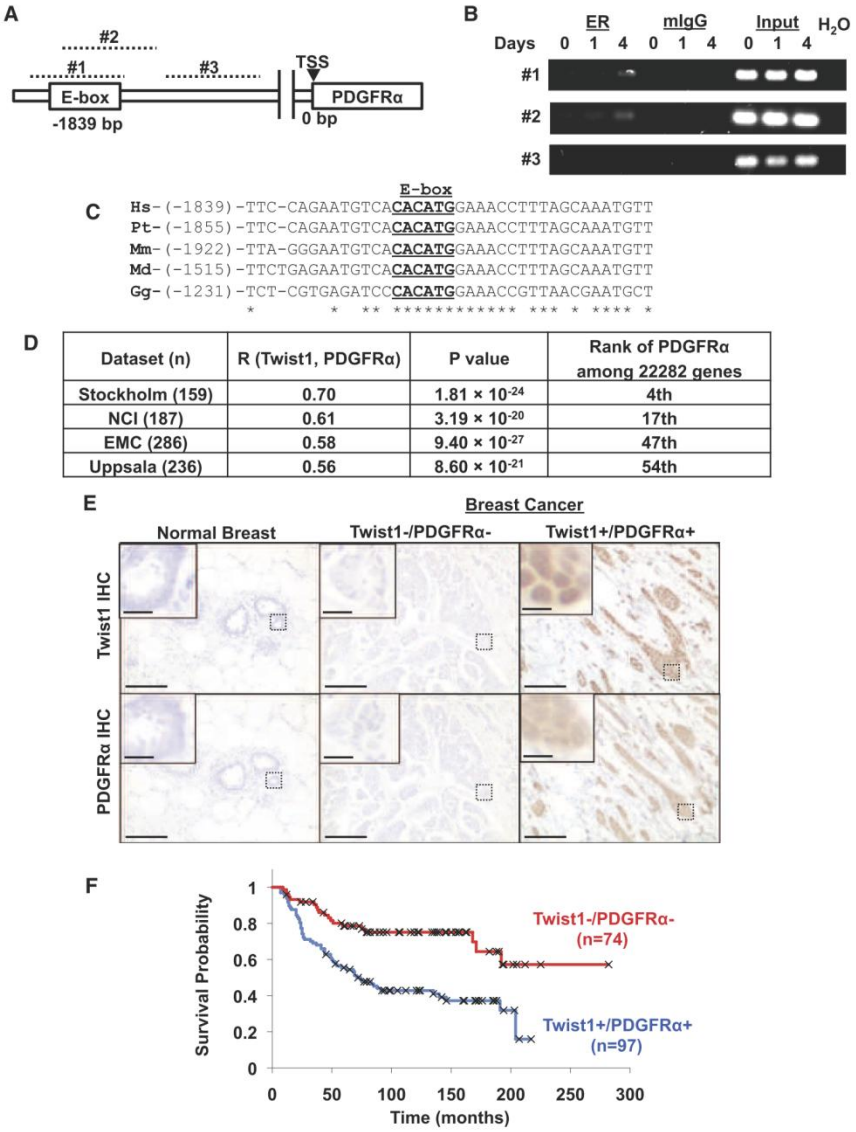

Figure 8. PDGFRα Is a Direct Target of Twist1 and Expression of Twist1 and PDGFRα Is Negatively Correlated with survival

(A) Schematic of the human PDGFRα gene promoter region with conserved E-box element 1839 bp upstream of transcriptional start site (TSS), and regions targeted by three primer pairs (#1-3, dashed lines). Primer pairs #1 and #2 target the putative E-box while primer pair #3 targets a downstream region lacking a conserved E-box.

(B) HMLE-Twist1-ER cells were treated with 20 nM 4-hydroxytamoxifen for 0, 1, or 4 hr. Chromatin was immunoprecipitated using estrogen receptor antibody and PCR was performed on the ChIP product using three primer pairs.

(C) Alignment of conserved E-box (underlined) in PDGFRα promoter. Number in parenthesis indicates distance upstream of transcription start site. Hs = *Homo sapiens*, Pt = *Pan troglodytes*, Mm = *Mus musculus*, Md = *Monodelphus domesticus*, Gg = *Gallus gallus*.

(D) Correlation of Twist1 and PDGFRα in four human breast cancer expression array data sets. R is the correlation coefficient.

(E) Representative images of normal human breast tissue or human breast cancer samples stained for Twist1 and PDGFRα. Scale bar is 5 μm for inset, 100 μm for full images.

(F) Kaplan-Meier survival curve for samples classified as high PDGFRα/high Twist1 expression and low PDGRα/low Twist1 by IHC analysis. Censored data are indicated with X.

See also Figure S8 and Table S1.

and PDGFRα are central mediators of invadopodia in response to several EMT-inducing signals. Finally, we provide evidence for a tight association between Twist1 and PDGFRα in human breast tumor samples.

Induction of Invadopodia by Twist1 Plays a Key Role in Extracellular Matrix Degradation and Metastasis

ECM degradation is considered a key step promoting tumor invasion and metastasis. Extensive studies have largely focused on secreted MMPs as key proteases in tumor invasion. More recent studies suggest a role for invadopodia and their associated proteases in localized matrix degradation during cell invasion. Conceptually, invadopodia provide an elegant solution to restrict protease activity to areas of the cell in direct contact with ECM, thus precisely controlling cell invasion in vivo. In this study, we show that Twist1, a key transcription factor in tumor metastasis, is both necessary and sufficient to promote invadopodia formation. Importantly, invadopodia formation is required for the ability of Twist1 to promote tumor metastasis in vivo. Together, these results demonstrate an essential role for invadopodia in tumor invasion and metastasis in vivo.

How invadopodia formation is regulated at the molecular level is still not well understood. Our current study indicates that Twist1 directly induces the expression and activation of PDGFRα, thus promoting Src kinase activation and invadopodia formation. Although we did not detect induction of several important invadopodia proteins, including cortactin, Tks4, Tks5, and MT1-MMP, by Twist1 (data not shown), we are actively exploring additional mechanisms by which Twist1 regulates invadopodia.

Another question arising from our study is whether invadopodia function is required for the EMT process. Epithelial cells sit on top of a layer of basement membrane. For the EMT program to occur in vivo, these cells must breach the underlying basement membrane to dissociate (Nakaya et al., 2008). Little is known about the functional relationship between basement membrane integrity and the EMT program. In HMLE-Snail cells and EpH4-Ras cells treated with TGFβ, knockdown of Twist1 inhibited invadopodia formation, while these cells underwent the morphological changes associated with EMT and lost E-cadherin expression. Additionally, knockdown of Tks5, a required component of invadopodia, did not revert the EMT phenotype in HMLE-Twist1 cells. These results indicate that invadopodia function is not essential for EMT to occur in 2D cultures. However, it is plausible that the EMT program requires activation of Twist1 and invadopodia formation to allow degradation of the basement membrane in vivo. Studies in vivo or in 3D cultures with intact basement membrane are required to fully answer this question.

Twist1 and Snail Have Distinct Cellular Functions and Transcriptional Targets

The EMT program is considered a key event promoting carcinoma cell dissociation, invasion, and metastasis. Several transcription factors, including Snail, Slug, ZEB1, ZEB2, and Twist1, promote EMT in epithelial cells (Peinado et al., 2007). During mesoderm formation and neural crest development, these transcription factors are activated to allow the dissociation and migration of epithelial cells. A major unsolved question is to determine the distinct cellular functions and molecular targets of individual EMT-inducing transcription factors. Extensive studies in recent years have demonstrated that Snail (Batlle et al., 2000; Cano et al., 2000), Slug (Hajra et al., 2002), and ZEB2 (Comijn et al., 2001), all zinc-finger-containing transcriptional repressors, directly bind to the E-boxes on the E-cadherin promoter and suppress its transcription. In this study, we identified a unique function of Twist1 in promoting matrix degradation via invadopodia. We show that Twist1 functions as a transcriptional activator to directly induce the expression of PDGFRα, in contrast to the EMT-inducing Zn-finger transcription factors.

Vertebrate Twist1 lacks a transcription activation domain and requires dimerization with other bHLH transcription factors to activate transcription. Previous studies have shown Twist1 heterodimers with MyoD function as transcriptional repressors (Hamamori et al., 1997). In contrast, heterodimerization with E12 enables Twist1 to activate FGF2 transcription (Laursen et al., 2007). Here, we demonstrate that Twist1 functions as a transcriptional activator to directly induce the transcription of PDGFRα. Twist1 might function as an activator or repressor of transcription based on dimerization partners under different physiological and cellular environments. The factors that heterodimerize with Twist1 to activate PDGFRα transcription remain unknown, although the E12/E47 proteins could perform this function.

The Pathway Linking Twist1, PDGFR, and Invadopodia Is Likely to Play a Conserved Role in Matrix Degradation during Both Tumor Metastasis and Embryonic Morphogenesis

Twist1 has been associated with increased metastasis in both experimental tumor metastasis models and in many types of human cancers. Interestingly, PDGFRα overexpression and activation have also been observed in aggressive human breast tumors (Seymour and Bezwoda, 1994; Jechlinger et al., 2006). Activation of PDGFRs was first observed in TGFβ-induced EMT and shown to be involved in cell survival during EMT and experimental metastasis in mice (Jechlinger et al., 2006). Here, we demonstrated a role of PDGFRα in invadopodia formation and matrix degradation during tumor metastasis. Interestingly, suppression of PDGFRα had no significant effects on cell proliferation or survival in vitro and in vivo. These results could be due to the greater specificity of shRNAs compared with chemical inhibition as well as differences in cellular and signaling contexts. Indeed, we found that STI571 (Gleevec), a c-ABL and c-Kit inhibitor that also inhibits PDGFR at a higher concentration, suppressed Twist1-induced invadopodia formation and matrix degradation. However, long-term (4 days) treatment with STI571 resulted in cell toxicity in HMLE-Twist1 cells (data not shown).

Our analyses identified Twist1 as a transcription inducer of PDGFRα and demonstrate a tight correlation between the expression level of Twist1 and PDGFRα in four large human breast tumor gene expression studies. Interestingly, PDGF ligand was also present in over 95% of tumor samples that expressed Twist1 and PDGFRα, indicating PDGFRα is activated in these tumors. Although these two genes alone are not sufficient to predict survival with statistical significance in these studies, these data, together with our metastasis data in mice and human breast cancer tissue array data, strongly suggest

Immunohistochemistry

Paraffin sections of human or mouse samples were rehydrated through xylene and graded alcohols. Antigen retrieval was accomplished using a pressure cooker in 10 mM sodium citrate 0.05% Tween. Samples were incubated with 3% H_2O_2 for 30 min followed by 5 hr blocking in 20% goat serum in PBS. Endogenous biotin and avidin were blocked using a Vector Avidin/Biotin blocking kit. Primary antibodies were incubated overnight at 4°C in 20% goat serum. Biotinylated secondary antibody and Vectorstain ABC kit were used as indicated by manufacturer. Samples were developed with diaminobenzidine (DAB) and samples counterstained with hematoxylin and mounted with Permount.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures, one table, and Supplemental Experimental Procedures and can be found online at doi:10.1016/j.ccr.2011.01.036.

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Targeting invadopodia to block breast cancer metastasis

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ABSTRACT:

Better understanding the mechanisms underlying the metastatic process is essential to developing novel targeted therapeutics. Recently, invadopodia have been increasingly recognized as important drivers of local invasion in metastasis. Invadopodia are basally-localized, actin-rich structures that concentrate protease activity to areas of the cell in contact with the extracellular matrix. We recently found that the transcription factor Twist1, a central regulator of the epithelial-mesenchymal transition (EMT), promotes invadopodia formation via upregulation of platelet-derived growth factor receptor (PDGFR) expression and activity. This finding, combined with other investigations into the mechanisms of invadopodia formation, reveal several novel targets for clinical inhibition of invadopodia. Here, we provide an overview of clinically-relevant targets for intervention in invadopodia, including Src signaling, PDGFR signaling, and metalloprotease activity.

BREAST CANCER METASTASIS, EMT, AND THE STATE OF THERAPEUTIC INTERVENTIONS

Breast cancer is the most common invasive cancer among women worldwide, with virtually all patients succumbing to not the primary disease, but distant metastases[1, 2]. Metastasis, the spread of cancer cells from the primary tumor to distant organs, is a multistep process in which cancer cells must invade through the extracellular matrix (ECM), intravasate into the bloodstream, survive transport through the circulatory system, and finally extravasate at distant organs[3]. As metastatic breast cancer is largely considered an incurable disease, better understanding the metastatic process and its regulation has the potential to not only identify new prognostic markers but also develop targeted therapeutic regimens.

Recently, aberrant activation of a developmental program termed the epithelial-mesenchymal transition (EMT) has been recognized as an important driver of the metastatic process[4].EMT is a conserved developmental process in which epithelial cells lose E-cadherin-mediated junctions and apical-basal polarity and become motile and invasive [5]. This program is accompanied by expression changes in a host of genes, among which genes associated with epithelial characteristics (E-cadherin and ZO-1) are downregulated while others associated with mesenchymal cells (smooth muscle actin, vimentin, and N-cadherin) are upregulated. A group of transcription factors, including Twist1, Snai1, Snai2, Zeb1, and Zeb2, play key roles in driving EMT during tumor metastasis[6, 7].

Current therapeutic standards for breast cancer involve surgical resection of the tumor supplemented with radiation therapy and chemotherapy[8]. Cytotoxic drugs and hormone-blocking therapeutics are the most often used chemotherapeutics, generally chosen for their effects on cell growth and apoptosis. Generation of new therapeutic agents targeting invasion and metastasis have the potential to improve survival in populations that do not respond well to conventional therapies. Despite the growing evidence linking EMT to metastasis in breast and other cancers, therapeutically targeting EMT may be difficult. Directly inhibiting the transcription factors that drive EMT is currently infeasible, as targeting large binding interfaces is not amenable to small-molecule inhibition[9, 10]. Instead, downstream targets of these transcription factors essential for their role in invasion and metastasis are more realistic targets of therapeutic

intervention.

TWIST1 AND INVADOPODIA

Although the role of EMT in metastasis is gradually becoming clearer, the exact molecular mechanisms underlying how EMT induces local invasion and metastasis are still not well understood[11]. Disruption of epithelial cell-cell contact is necessary for metastasis, but it is not sufficient[12]. We therefore sought to determine what pathways or mechanisms Twist1 induces to drive active local invasion and metastasis. We did not observe significant changes in secreted proteolytic activity in cells overexpressing Twist1, although they gained the ability to invade through Matrigel and metastasize to the lung in a subcutaneous tumor model[7]. We therefore hypothesized that Twist1 induces local invasion and eventual metastasis by inducing the formation of membrane protrusion structures called invadopodia.

Invadopodia are actin-rich protrusions that localize proteolytic activity to areas of the cell in contact with extracellular matrix(ECM)[13-15]. Invadopodia are observed in many invasive cancer cell lines [16]. A wide variety of actin-interacting proteins and scaffolding proteins are involved in invadopodia formation, including cortactin, Tks5, fascin, N-WASP, and Arp2/3[17]. In particular, the actin-bundling protein cortactin and the adaptor proteins Tks4/5appear to play integral roles in invadopodia formation[18, 19]. Both metalloproteases and serine proteases localize to invadopodia, including both secreted (MMP2 and MMP9) and transmembrane proteases (MT1-MMP, ADAM12, FAPα, and DPP-iv) [20]. Src kinase activity and phosphorylation of Tks4 [21], Tks5[18], and cortactin[19]are absolute requirements for invadopodia formation. Upregulation of invadopodia formation by Twist1 would therefore present a novel mechanism by which Twist1 could induce local invasion without changing secreted protease activity[22].

In order to investigate whether Twist1 was necessary for invadopodia, we generated knockdowns of Twist1 in 168FARN and 4T1 cell lines, two mouse mammary carcinoma cell lines that express high levels of Twist1. By staining for markers of invadopodia (colocalization of F-actin with either cortactin or Tks5) we found that knockdown of Twist1 significantly reduced invadopodia formation in both 168FARN and 4T1 cells[23]. Importantly, knockdown of Twist1 also dramatically reduced ECM degradation.Similar analyses in normal mammary epithelial cells overexpressing Twist1 demonstrated that Twist1 was also sufficient to promote invadopodia formation and function. Importantly, Twist1-inducedinvadopodia formation requires both metalloprotease and Src-kinase activities, consistent with their known roles in invadopodia.

TWIST1 INDUCES INVADOPODIA FORMATION BY UPREGULATING PDGFRA

We were therefore interested in the mechanism by which Twist1 was both necessary and sufficient for invadopodia formation. None of the structural or enzymatic proteins found in invadopodia that we investigated, including Tks5, cortactin,and MT1-MMP, were transcriptionally regulated by Twist1. We did, however, observe a significant upregulation of Src kinase activation upon Twist1 expression. Microarray analysis of genes upregulated by Twist1 revealed that only one family of growth factor receptors upstream of Srcactivation was induced by Twist1: platelet-derived growth factor receptors (PDGFR) α and β. PDGFRα, in particular, was immediately and dramatically upregulated. Importantly, PDGFRα was phosphorylated and activated under normal culture conditions, implying the existence of an autocrine loop upon Twist1 activation.

There are two PDGFRs, PDGFRα and β, which differ primarily in their responsiveness to PDGF ligands[24]. In mammalian systems, PDGFR expression is abundant in mesenchymal and vascular tissues and is particularly involved in angiogenesis[25, 26]. Importantly, PDGFRs are directly upstream of Src kinase activity[27]. Upon stimulation by their ligands, the receptors dimerize and can directly activate Src kinase[28]. PDGFR signaling has previously been implicated as required for metastasis in a TGF-β-induced EMT model, although the mechanism for this inhibition was not clearly understood[29]. Encouragingly, a previous study found that PDGFR activation increased invadopodia formation in vascular smooth muscle cells[30].

Knockdown or inhibition of PDGFRα with a mouse monoclonal blocking antibody significantly reduced Twist1-induced invadopodia formation and function. Chromatin immunoprecipitation and luciferase reporter assays also revealed that PDGFRα was a direct target of Twist1. Activation of PDGFR signaling by Twist1 therefore appeared to be a direct requirement for Twist1-mediated invadopodia formation.

INVADOPODIA AND PDGFRA ARE NECESSARY FOR METASTASIS AND IMPLICATED IN HUMAN BREAST CANCER

To better understand the role of invadopodia and PDGFRα in metastasis, we utilized a subcutaneous tumor implantation model in which Twist1-expressing human breast tumor cells carrying shRNAs against PDGFRα or Tks5 were injected subcutaneously in nude mice. Knockdown of Tks5, an essential invadopodia component protein with no other known functions, allowed us to test whether invadopodia were required for

Twist1-mediated metastasis. Knockdown of PDGFRα was used to determine whether PDGF signaling induced by Twist1 for invadopodia formation was required for metastasis. Although no significant differences in growth rate were observed, both Tks5 and PDGFRα knockdowns dramatically suppressed local invasion, with the primary tumors remaining relatively well-encapsulated in a layer of fibrotic tissue. In contrast, tumors expressing control knockdown constructs invaded through the local ECM, often as single cells. Furthermore, knockdown of both Tks5 and PDGFRα significantly reduced dissemination to the lungs, as measured by quantification of GFP-positive puncta in the lungs.

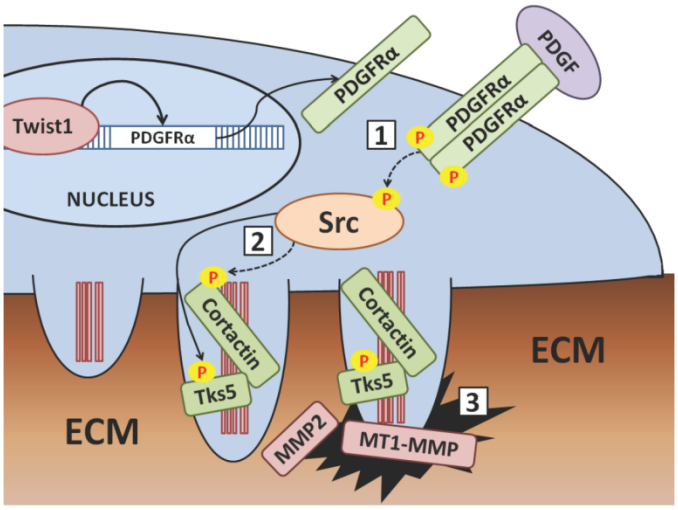
In microarray data sets of human breast cancer tumor samples, we found a strong correlation between Twist1 and PDGFRα expression, with PDGFRα consistently ranking within the top 1% of genes correlated with Twist1. Furthermore, in a Stage II breast cancer tissue array from the National Cancer Institute coexpression of Twist1 and PDGFRα was significantly associated with patient survival, indicating the importance of this pathway in human breast tumor progression.

Druggable targets regulating invadopodia formation and function

The connection between Twist1, PDGFR signaling, and invadopodia is exciting as it highlights several new therapeutic targets for targeting local invasion in the metastatic process. Namely, PDGFRα, Src, and metalloproteases localized in invadopodia are appealing, druggable targets for targeting invasion in breast cancer metastasis (see Figure). As metastasis may occur via invadopodia-independent mechanisms in patient populations, Twist1 and PDGFRα coexpression may be appealing markers for patient stratification for treatment regimens targeting invadopodia.

PDGFRα and EGFR

As a direct target of Twist1 tightly associated with survival in human breast cancer patient tissue samples, PDGFRα is an especially appealing target for therapeutic intervention in breast cancer metastasis. The most well-known and studied PDGFR inhibitor is imatinib mesylate (Gleevec, Novartis), which also inhibits Abl and c-Kit tyrosine kinases[31]. Data from clinical trials involving use of imatinib in advanced breast cancers has been discouraging with no clear objective responses[32]. If PDGFR signaling is important for invasion and metastasis, however, improved survival in these patients with late stage disease would be unlikely as the cancer had already widely metastasized. Often severe gastrointestinal side effects of imatinib treatment also severely limited its utility in at least one trial[33]. Another tyrosine kinase inhibitor, Sunitinib (Sutent, Pfizer), targets PDGFRs, VEGFs, Kit, RET, and CSF[33]. Encouragingly, Sunitinib is much better tolerated and has had some effectiveness in preliminary clinical trials of metastatic breast cancer[34]. The promiscuous inhibitory profile of Sunitinib makes it difficult to determine whether its effects on disease outcome are through inhibition of PDGFRs. In light of our discovery, it is important to examine patient tumor samples to determine whether Sunitinib suppresses invadopodia



Recent research in our lab revealed that Twist1 directly induces transcription of PDGFRα. Upregulation of PDGFRα leads to an increase in Src kinase activity that induces the formation or stabilization of invadopodia by phosphorylation of invadopodia component proteins by Src kinase. Invadopodia formation involves discrete steps in which formation of the F-actin core is an early event, followed by recruitment and phosphorylation of invadopodia component proteins like cortactin and Tks5 before proteases are recruited to the mature invadopodia. Promising targets for chemical inhibition include [1] PDGFR signaling, [2] Src kinase activity, and [3] metalloprotease activity at invadopodia (including MMP2, MMP9, and MT1-MMP).

and local invasion. To truly understand the utility of these novel inhibitors in breast cancer, it will be necessary to identify patient populations that will respond best to the therapy. Development of more specific inhibitors that target only PDGFRs, including humanized monoclonal antibodies, may address some of the side effects due to off targeting.

Epidermal growth factor receptor (EGFR) signaling is also known to play an important role in regulation of invadopodia formation. The most characterized role of EGFR signaling in invadopodia is its function upstream of Src activation[35]. HER-2 (human epidermal growth factor receptor 2) status is an important clinical marker for treatment, with about 30% of patients presenting increased levels of HER-2expression[33]. Patients that are HER-2 positive are considered candidates for treatment with HER-2 inhibitors, including trastuzumab (Herceptin, Genentech) and lapatinib (Tykerb/Tyverb GSK)[36]. Recently, new small molecule-based therapeutics targeting EGFR, including erlotinib (Tarceva, OSI Pharmaceuticals), have proven useful in other cancers with upregulation of EGFR signaling[37]. OSI Pharmaceuticals investigated the properties of cancer cells resistant to EGFR inhibition and found that this subpopulation of cells displayed properties of EMT, including an increased dependence on PDGFR signaling[38, 39]. During EMT, PDGFR signaling may largely supplant or supplement the role of EGFR signaling in promoting invadopodia formation in breast cancer cells. This also suggests that the EMT process may play roles in not only mediating local invasion and metastasis, but also providing an escape mechanism from growth factor inhibition.

Src and its effectors

As the first proto-oncogene discovered, there is a large body of research focusing on not only the role of Src in cancer but also potential therapeutic interventions. It is generally recognized that Src plays multiple roles in carcinomas, promoting both proliferation and survival and driving invasion[40]. The essential role of Src activation in invadopodia formation suggests that Src inhibitors should effectively prevent invadopodia formation and ECM degradation in tumors. Several Src inhibitors are already in the clinic and used to treat chronic myelogenous leukemia by virtue of their ability to also inhibit Abl kinase[41]. Src activity is also upregulated in a wide variety of solid cancers, including colon, breast, gastric, and ovarian cancers[42]. Several pharmaceutical companies have therefore developed Src kinase inhibitors with varying levels of success. Most Src inhibitors that have progressed to clinical trials in solid tumors (Dasatinib, Bristol-Meyers Squibb; Saracatinib, AstraZeneca; and Bosutinib, Wyeth) work by competitively binding the ATP-binding site of Src[40]. Initial results from clinical trials of Src inhibitors in breast cancer have been mixed, with most single-agent trials resulting in no significant differences in survival or progression[43]. Combination therapeutics have resulted in more positive, although modest, effects [42].It is important to note, however, that all clinical trials regarding Src inhibitors in breast cancer have been conducted in unselected patient populations and the main readout for effectiveness has been tumor size and growth, not invasion. There are some indications, however, that patient stratification can predict responsiveness to Src kinase inhibition[44]. Novel Src inhibitors targeting the peptide binding site of Src rather than the ATP-binding site (KX2-391, Keryx Biopharmaceuticals) may also prove to be more effective in solid tumors, although clinical trials involving these compounds are still preliminary and underway[45].

Interestingly, a recent publication elucidated a detailed mechanisms for Src-kinase induced invadopodia formation[35]. Rather than directly phosphorylating cortactin, Src instead activates the Abl-related non-receptor tyrosine kinase Arg that is responsible for cortactin phosphorylation. In this system, cortactin tyrosine phosphorylation is transiently required for cortactin-mediated actin polymerization in invadopodia. This is particularly interesting, as Gleevec, a drug often used to target PDGFR signaling, also inhibits Arg activity[31]. The promiscuity of Gleevec could therefore target multiple levels of the signaling pathways regulating invadopodia formation, making it a promising target in selected patient populations[46].

Metalloproteases

Several metalloproteases are enriched at invadopodia, including MMP2, MMP9, and MT1-MMP[47]. The transmembrane metalloprotease MT1-MMP is essential for invadopodia proteolytic activity: knockdown of MT1-MMP in multiple cell lines almost completely eliminates associated matrix degradation[19, 48]. In addition, recent work has also elucidated the vital role of MT-MMPs in mediating invasion through three-dimensional matrices[49].The central role of MT-MMPs in mediating extracellular proteolysis at invadopodia could be due to either its intrinsic collagenase/gelatinase activity or via activation of soluble MMPs by MT1-MMP[50]. There is also evidence that hydroxymate metalloprotease inhibitors prevent not only ECM proteolysis, but also invadopodia formation through an unknown mechanism[19].

As cancer cells must invade through both basement membranes and the ECM during metastasis, metalloproteases were quickly recognized as appealing targets to inhibit metastasis. Although results were promising in preclinical models, metalloprotease inhibitors have universally failed in clinical trials[51]. Once again, clinical trials with metalloprotease inhibitors to date have invariably used unselected patient populations, often with late-stage disease. Additionally, early metalloprotease

inhibitors were broad-spectrum inhibitors of multiple metalloproteases and often had acute toxicities that severely limited therapeutic doses[51]. MMPs may also play anti-tumor functions in many tumors, as well. For example, MMP8-/- mice developed more papillomas upon carcinogen treatment[52].In recent years, there has been a reemergence of interest in more targeted inhibition of metalloproteases. In particular, the fully human monoclonal antibody DX-2400 (Dyax Corp.) that targets MT1-MMP, has shown great promise in preclinical models in inhibiting invasiveness of cancer cell lines[53]. In addition, a novel class of metalloprotease inhibitors, triple-helical transition state analogues, specifically targets the gelatinase and collagenase activities of metalloproteases (specifically MMP2 and 9)[54]. Clinically addressing the role of metalloproteases in breast cancer metastasis will involve not only designing trials to maximize the impact of the therapeutics, but also finding novel inhibitors with greater specificity and fewer negative side-effects.

CONCLUSIONS

In order to directly target metastasis, essential regulators of the metastatic process must be identified. In addition, these targets should ideally be kinases or proteases with moieties amenable to chemical inhibition. Although EMT is beginning to be recognized as a key player in breast cancer, promising targets of inhibition regulating this process have been lacking. Our identification of PDGFRα and invadopodia as essential mediators of EMT-induced metastasis opens the door for clinical intervention of pathways regulating invadopodia function. These pathways include Src kinase, PDGFRα, and invadopodia-specific proteases. In order to test the hypothesis that inhibiting such pathways is effective, clinical trials most likely benefit from careful selection of patient populations based on our knowledge of invadopodia regulation. In addition, for both PDGFRα and metalloproteases, generation of more selective compounds may be necessary to realize positive clinical outcomes. Our study suggests that Twist1 and PDGFRα are effective predictors, not only of patient survival, but also for patient selection in clinical trials targeting invadopodia formation and function.

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