

Akt/PKB-Mediated Phosphorylation of Twist1 Promotes Tumor Metastasis via Mediating Cross-Talk between PI3K/Akt and TGF- β Signaling Axes

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Metastatic breast tumor cells display an epithelial-mesenchymal transition (EMT) that increases cell motility, invasion, and dissemination. Although the transcription factor Twist1 has been shown to contribute to EMT and cancer metastasis, the signaling pathways regulating Twist1 activity are poorly understood. Here, we show that Twist1 is ubiquitously phosphorylated in 90% of 1,532 invasive human breast tumors. Akt/protein kinase B (PKB)-mediated Twist1 phosphorylation promotes EMT and breast cancer metastasis by modulating its transcriptional target TGF- β 2, leading to enhanced TGF- β receptor signaling, which in turn maintains hyperactive phosphoinositide 3-kinase (PI3K)/Akt signaling. Preventing phosphorylation of Twist1, as well as depletion of TGF- β 2, significantly impaired the metastatic potential of cancer cells *in vivo*, indicating a key role of phosphorylated Twist1 (phospho-Twist1) in mediating cross-talk between the PI3K/Akt and TGF- β /Smad signaling axes that supports metastatic tumor development. Our results describe a novel signaling event linking PI3K/Akt hyperactivation in tumor cells to direct regulation of Twist1 activation and tumor metastasis.

SIGNIFICANCE: We identified the first phospho-Twist1 transcriptional target TGF- β 2, which mediates cross-talk between PI3K/Akt and TGF- β signaling and promotes tumor metastasis. Our results thus illustrate a direct role of PI3K/Akt signaling in metastatic cancer development and suggest that Twist1 phosphorylation could be a potential therapeutic target in clinical cancer treatment.

INTRODUCTION

The metastasis of epithelial tumors, which comprise almost 90% of all cancers, occurs when tumor cells overcome fundamental cell controls maintaining cell-cell contacts and preventing migration. Accumulating experimental data indicate that these controls in epithelial tumor cells are overcome through the reactivation of a dormant developmental process, epithelial-mesenchymal transition (EMT), when epithelial cells transform into mesenchymal cells to allow migration into areas in which mesenchymal cells normally reside. The occurrence of EMT *in situ* has been illustrated in both mice and primary human breast cancer specimens (1).

The evolutionarily conserved basic helix-loop-helix transcription factor Twist1 plays an important role in morphogenesis during embryonic development by regulating cell migration (2, 3). In the pathologic setting of cancer, Twist1 overexpression is found in a variety of tumors (ref. 4; Supplementary Table S1) and correlates with tumor metastasis. Expression of Twist1 in untransformed epithelial cell

efficiently induces EMT. In a clinically relevant orthotopic breast cancer mouse model, Twist1 expression is essential for the formation of lung metastasis (5). In hepatocellular carcinomas, overexpression of Twist1 correlates with EMT-associated changes and metastasis (6). Twist1 expression can also negatively regulate programmed cell death (7) and overcome oncogene-induced senescence (4).

These studies indicate that Twist1 plays multiple roles in the regulation of antiapoptosis and metastasis during tumor progression. Active Akt directly phosphorylates Twist1 on serine 42 (S42) and protects cells from apoptosis induced by DNA damage (8). Twist1 promotes crucial prometastatic roles in tumor development and is frequently associated with aberrant hyperactivation of phosphoinositide 3-kinase (PI3K)/Akt. This prompted us to explore whether phosphorylation of Twist1 by Akt is part of a molecular mechanism contributing to Twist1 activity in cancer and particularly in metastasis.

We analyzed Twist1 phosphorylation in human cancer cell lines (with different genetic backgrounds) and invasive human breast tumors and addressed its functional importance in a mouse model of spontaneous breast tumor metastasis. We show that Twist1 is ubiquitously phosphorylated in invasive human breast tumors and is required for breast cancer lung metastasis *in vivo*. Twist1 phosphorylation supports metastasis by transcriptionally upregulating TGF- β 2, leading to enhanced activation of TGF- β R/Smad2 signaling, and these effects could be significantly suppressed by the knockdown of TGF- β 2 expression. These data suggest that Twist1 phosphorylation not only mediates a feedback loop allowing cross-talk with TGF- β to maintain hyperactivation of PI3K/Akt but can also potentiate cancer metastasis, at least in part, through enhanced TGF- β signaling in cancer cells. Collectively, our data uncover a novel mechanism

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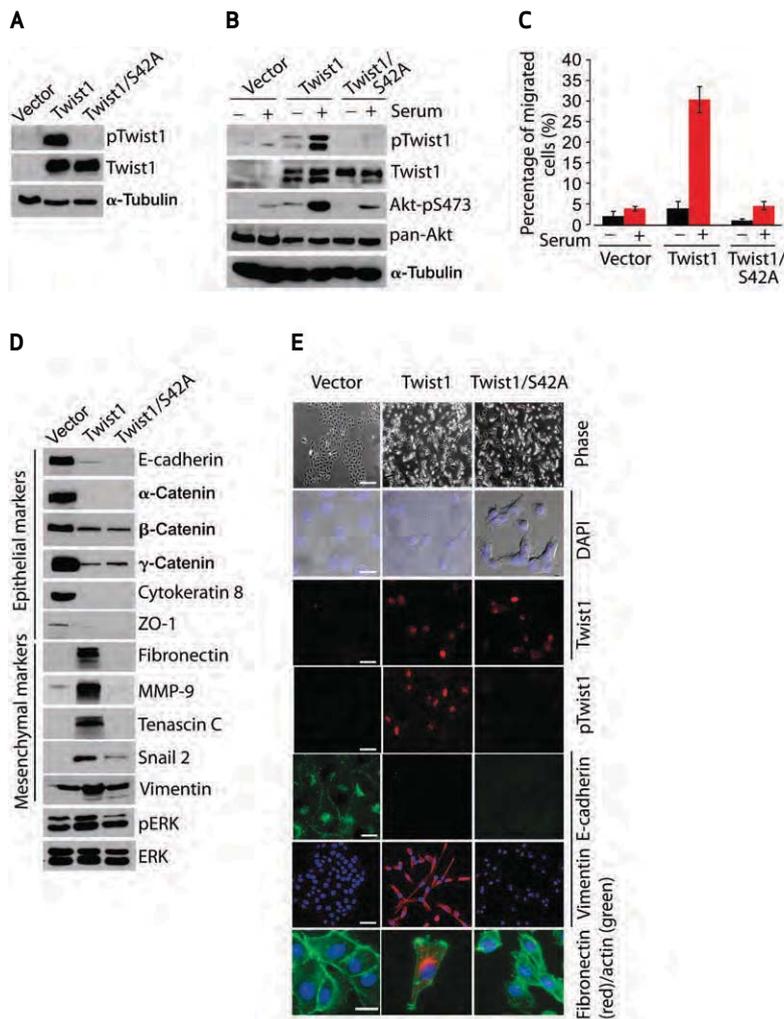


Figure 1. Expression of phosphorylated Twist1 in MDCK cells promotes EMT. **A**, expression and phosphorylation analysis of Twist1 in MDCK cells expressing empty vector, wild-type Twist1, and the mutant Twist1/S42A. **B**, MDCK cells expressing Twist1 and Twist1/S42A were cultured in serum-free medium for 16 hours and subsequently treated by adding 10% FBS for 1 hour. The lysates were subjected to Western blotting for Twist1 and Akt phosphorylation. **C**, MDCK cells expressing Twist1 and Twist1/S42A were serum-starved overnight and loaded into a Boyden chamber precoated with Matrigel. The invasiveness was quantified by counting the number of the cells that invaded into Matrigel and passed through the membrane. Error bars represent means \pm SEM of samples measured in triplicate. **D**, immunoblot analysis of indicated epithelial and mesenchymal proteins in MDCK cells expressing Twist1 and mutant Twist1/S42A. Correlation with Twist1 phosphorylation and increased ERK phosphorylation are also shown. α -Tubulin was used as a loading control. **E**, morphologic differences of MDCK cells expressing empty vector, wild-type Twist1, or Twist1/S42A mutant and cellular localization of examined markers. Scale bars, 40 μ m and 10 μ m (last row), respectively.

linking the PI3K/Akt pathway to tumor progression and malignancy. Furthermore, our results indicate that phosphorylated Twist1 (phospho-Twist1) is a potential new therapeutic target in the treatment of breast cancer.

RESULTS

Akt-Mediated Twist1 Phosphorylation Promotes Full EMT

Ectopically expressed wild-type Twist1 was phosphorylated on S42 in several epithelial cell lines (Fig. 1A). The specificity of S42 phosphorylation by Akt was demonstrated with the use of Akt1/2-deficient MEF cells (Supplementary Fig. S1A–S1C). Because elevated Twist1 expression is closely associated with increased cell motility, we performed Boyden chamber assays to examine whether regulation of cell invasion was controlled by Twist1 phosphorylation. Serum stimulation-mediated activation of Akt correlated with phosphorylation of Twist1 (Fig. 1B) and significantly enhanced invasion of MDCK cells expressing wild-type Twist1 by 8-fold compared with Twist1/S42A-expressing cells (Fig. 1C). Expression of wild-type Twist1 or Twist1/S42A strongly disrupted cell-cell contacts. Upregulation of the promigratory proteins

fibronectin, vimentin, metalloproteinase 9 (MMP-9), and tenascin C (TNC), commonly seen during EMT and metastasis, was only observed upon Twist1 S42 phosphorylation (Fig. 1D and E). These effects were confirmed by the use of phospho-mimicking mutant S42D (Supplementary Fig. S1D–S1F). Interestingly, unlike the phosphorylated Twist1, expression of Twist1/S42D did not upregulate MMP-9, TNC, and Snail2, indicating an additional complexity to the regulation of these targets.

Twist1 Is Phosphorylated in Metastatic Breast Cancer Cell Lines and Invasive Breast Tumors

The observation that phosphorylated Twist1 is crucial for EMT in untransformed cells prompted us to determine whether this is also the case in human tumor cells. Compared with 2 nonmetastatic cell lines, MCF-7 and BT-20, Twist1 is expressed in 7 metastatic breast cancer cell lines (Fig. 2A and B). Notably, constitutive Twist1 phosphorylation was detected in ErbB2-high cell lines, representing the luminal type of breast cancers (Fig. 2B), although no clear correlation with other genetic characteristics was observed. In ErbB2-low cell lines MDA-MB-231, MDA-MB-468, and MDA-MB-435, moderate

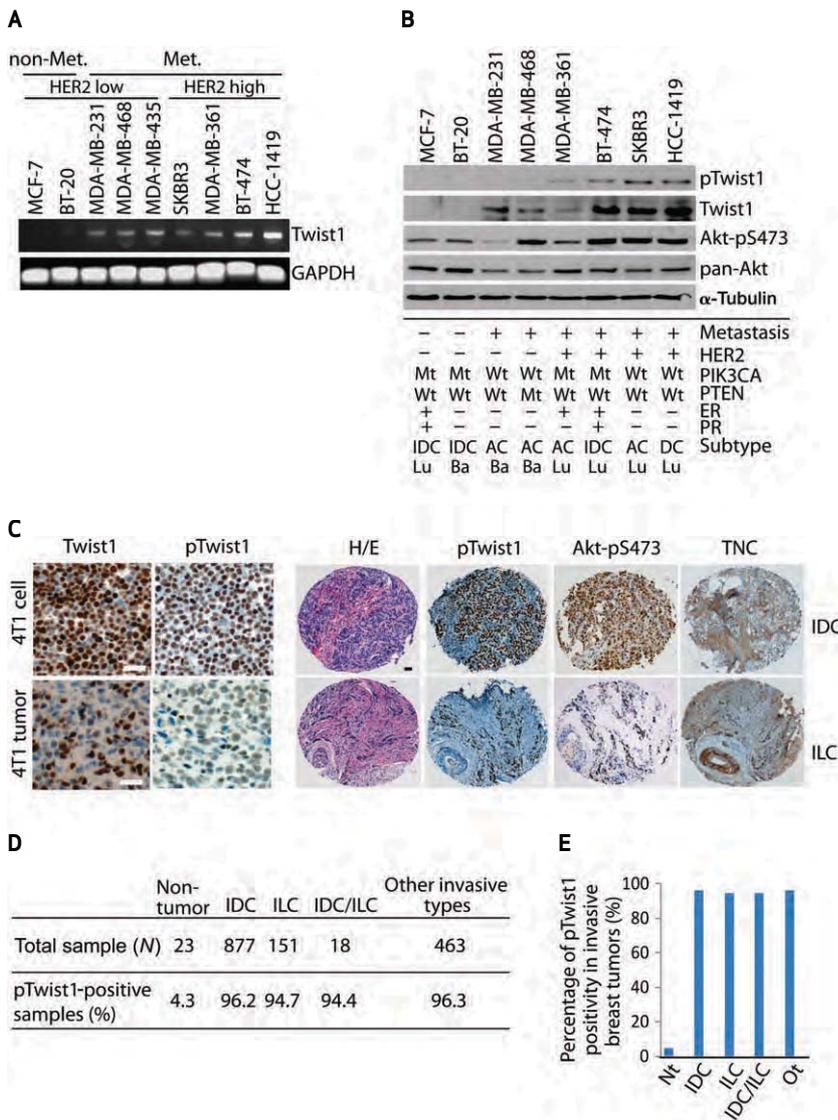


Figure 2. Phosphorylation of Twist1 in human breast cancer cell lines and tumors. **A**, Twist1 mRNA was analyzed in 9 human breast cancer cell lines categorized by different HER2 level (on the basis of the published data). Met, metastatic; non-Met, nonmetastatic. **B**, immunoblot analysis of Twist1 phosphorylation in breast cancer cell lines with distinct metastatic potential and known genetic background. **C**, 4T1 cells, 4T1 tumors, and invasive human breast tumors including IDC/ILC types were stained for phospho-Twist1, Akt-pS473, and prometastatic marker TNC. Scale bars, 20 μ m and 50 μ m, respectively. **D**, pathologic statistics of 1,532 invasive human breast tumors that have phospho-Twist1. Cells with > 30% stained were considered positive.

Twist1 expression could be observed, but phosphorylation was not detectable (Fig. 2B and Supplementary Fig. S2A and S2B). Because it was reported that growth factors such as epidermal growth factor (EGF) can activate Twist1 in MDA-MB-468 cells (9), we analyzed the status of Twist1 phosphorylation in selected breast cancer cell lines in the presence of EGF. Significantly, the addition of EGF rapidly induced Twist1 phosphorylation in ErbB2-low cell lines (Supplementary Fig. S2C and S2D) but did not further enhance Twist1 phosphorylation in ErbB2-high cell lines (Supplementary Fig. S2E).

When high-throughput tissue microarray analysis was used (10), Twist1 phosphorylation was detected in 90% of 1,532 invasive breast tumors, including clinically diagnosed invasive ductal carcinomas (IDC), invasive lobular carcinomas (ILC), and mixed IDC/ILC subtypes, and correlated with high levels of Akt phosphorylation and TNC, a newly proposed biomarker often upregulated in malignant tumors, including metastatic breast cancer (ref. 11; Fig. 2C and D).

Phosphorylation of Twist1 Is Required for Breast Tumor Metastasis in a Mouse Model

Twist1 expression correlates with invasive potential in 4 established mouse breast cancer cell lines that share the same genetic background. Constitutive Twist1 phosphorylation was observed in 4T1 cells (Supplementary Fig. S2A and S2B), a clinically relevant and highly metastatic model cell line for studying breast cancer metastasis in mice. Treating 4T1 cells with the PI3K/Akt pathway inhibitor LY294002 strongly decreased Twist1 phosphorylation on S42 (Fig. 3A) and led to significantly suppressed cell migration (Fig. 3B). To explore whether Twist1-mediated lung metastasis is dependent on S42 phosphorylation, we generated rescue clones expressing Twist1 and its variants in Twist1-depleted 4T1 cells (4T1_Tw1KD). Knockdown of Twist1 restored E-cadherin expression (Fig. 3C) at cell-cell contacts (Fig. 3D). Re-expressed Twist1 was phosphorylated on S42 (Fig. 3E), and intercellular junctions (Fig. 3F, black arrowhead) were again disrupted (Fig. 3F, yellow arrowhead), a phenotype also

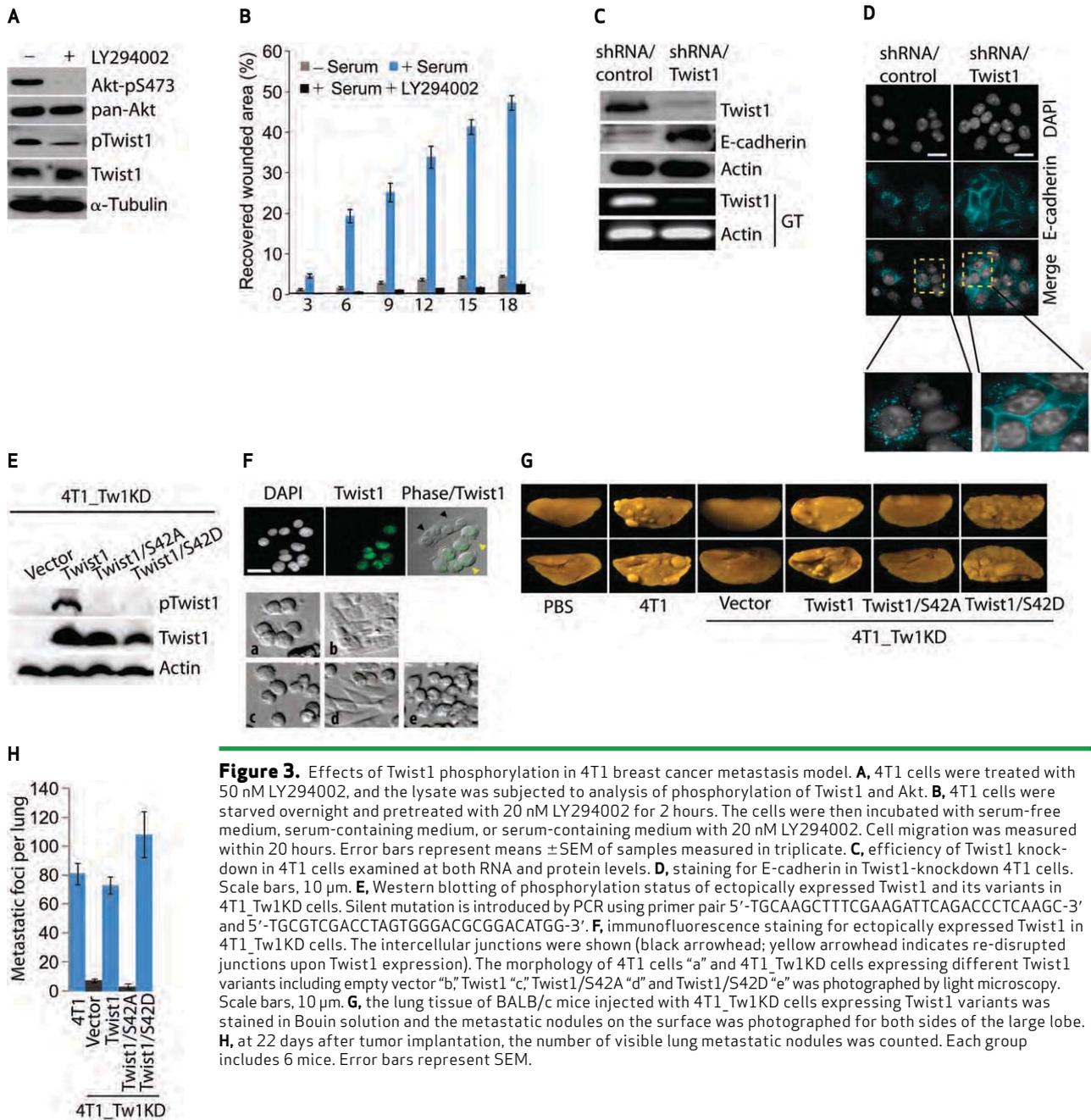


Figure 3. Effects of Twist1 phosphorylation in 4T1 breast cancer metastasis model. **A**, 4T1 cells were treated with 50 nM LY294002, and the lysate was subjected to analysis of phosphorylation of Twist1 and Akt. **B**, 4T1 cells were starved overnight and pretreated with 20 nM LY294002 for 2 hours. The cells were then incubated with serum-free medium, serum-containing medium, or serum-containing medium with 20 nM LY294002. Cell migration was measured within 20 hours. Error bars represent means \pm SEM of samples measured in triplicate. **C**, efficiency of Twist1 knock-down in 4T1 cells examined at both RNA and protein levels. **D**, staining for E-cadherin in Twist1-knockdown 4T1 cells. Scale bars, 10 μ m. **E**, Western blotting of phosphorylation status of ectopically expressed Twist1 and its variants in 4T1_Tw1KD cells. Silent mutation is introduced by PCR using primer pair 5'-TGCAAGCTTTCGAAAGATTTCAGACCCCTCAAGC-3' and 5'-TGCGTCGACCTAGTGGGACGCGGACATGG-3'. **F**, immunofluorescence staining for ectopically expressed Twist1 in 4T1_Tw1KD cells. The intercellular junctions were shown (black arrowhead; yellow arrowhead indicates re-disrupted junctions upon Twist1 expression). The morphology of 4T1 cells "a" and 4T1_Tw1KD cells expressing different Twist1 variants including empty vector "b," Twist1 "c," Twist1/S42A "d" and Twist1/S42D "e" was photographed by light microscopy. Scale bars, 10 μ m. **G**, the lung tissue of BALB/c mice injected with 4T1_Tw1KD cells expressing Twist1 variants was stained in Bouin solution and the metastatic nodules on the surface was photographed for both sides of the large lobe. **H**, at 22 days after tumor implantation, the number of visible lung metastatic nodules was counted. Each group includes 6 mice. Error bars represent SEM.

seen in Twist1/S42A-expressing clones (Fig. 3Fd), which was consistent with previous observations in MDCK cells.

However, loss of a flat cellular morphology and acquisition of a round cell morphology with downregulation of intercellular junctions, as displayed by parental 4T1 cells (Fig. 3Fa), was only observed upon re-expression of wild-type Twist1 (Fig. 3Fc) or S42D (Fig. 3Fe). *In vivo* experiments in BALB/c mice demonstrated that Twist1 was dispensable for 4T1 primary tumor growth (Supplementary Fig. S3A) but necessary for the formation of lung tumor nodules. Restoration of Twist1 or Twist1/S42D, but not Twist1/S42A, in Twist1-silenced cells (4T1_Tw1KD/Twist1; 4T1_Tw1KD/Twist1/S42D; 4T1_Tw1KD/Twist1/S42A, respectively) reconstituted tumor lung nodule formation (Fig. 3G-H). Consistent with

these macroscopic observations, the ratio of mamoglobin-positive cells in lung tissue sections was significantly greater in mice injected with 4T1_Tw1KD/Twist1 or 4T1_Tw1KD/Twist1/S42D (Supplementary Fig. S3B), illustrating that the lung tumor nodules originated from injected breast cancer tumor cells. This finding was further confirmed by neomycin gene expression in the lungs (Supplementary Fig. S3C).

Similar to our previous observations (Fig. 1D and Fig. 2C), the prometastatic matrix protein TNC that is expressed by both cancer cells and cancer stroma was observed in all primary tumors and strongly upregulated in the lung metastases of 4T1, 4T1_Tw1KD/Twist1, and 4T1_Tw1KD/S42D cell-injected mice (Supplementary Fig. S3D). This finding suggests that Twist1 phosphorylation promotes cancer cell

dissemination by differentially upregulating known pro-metastatic molecules. Nevertheless, Twist1 was not required for the formation of primary tumors, indicating that Akt-mediated Twist1 phosphorylation was specifically required for lung metastases formation.

Phosphorylation of Twist1 Regulates TGF- β R Signaling

To explore the mechanisms underlying the requirement of Twist1 phosphorylation for lung metastasis, we performed Affymetrix transcriptome analysis in 4T1-derivative cell lines used in the *in vivo* studies. We specifically focused on the genes upregulated by phospho-Twist1 by comparing those downregulated in 4T1_Tw1KD- and 4T1_Tw1KD/S42A-expressing cells versus parental 4T1 cells and those restored in 4T1_Tw1KD/Twist1- and 4T1_Tw1KD/S42D-expressing cells (Fig. 4A). A total of 68 genes were identified as potential targets of phospho-Twist1 (Supplementary Table S2), many of which are known to be deregulated in different cancer types. Two secreted cytokines, TGF- β 2 and Inhibin β -a (Inhba), that are closely linked to TGF- β signaling were selectively upregulated upon Twist1 phosphorylation. Interestingly, 4T1 cells are known to express high levels of growth factors, such as TGF- β , VEGF, platelet-derived growth factor (12), and fibroblast growth factor (13), which activate receptor kinase pathways to promote cancer cell invasion (14-16). Knockdown of Twist1 and re-expression of Twist1/S42A decreased TGF- β 2 by 2.9-fold and 1.2-fold, respectively, compared with parental 4T1 cells. Compensation of Twist1 and Twist1/S42D increased TGF- β 2 by 1.3-fold and 2.8-fold compared with Twist1-knockdown cells (Fig. 4B). Quantitative real-time (qRT)-PCR analysis revealed significant upregulation of TGF- β 2 that was differentially regulated by phospho-Twist1.

A further 5 targets among the 68 genes and 4 known tumor-promoting genes were selected for qRT-PCR validation. In contrast to the 4 known tumor-promoting genes, all 8 genes showed specific regulation by phosphorylated Twist1 (Supplementary Fig. S4A and S4B). ELISA analysis also showed TGF- β 2 secretion was increased in the medium of 4T1_Tw1KD cells expressing wild-type Twist1 (~180 pg/mL) and Twist1/S42D (~200 pg/mL) by more than 4-fold compared with Twist1/S42A-expressing cells (~50 pg/mL; Fig. 4C), strongly indicating that upregulation of TGF- β 2 was phospho-Twist1 dependent. Consistently, expression of phosphorylated Twist1 or Twist1/S42D, but not Twist1/S42A, led to greater Smad2 phosphorylation not only in the cancer cell lines (Fig. 4D) but also in the lung metastatic nodules (Fig. 4E and F), providing direct evidence for a correlation between phospho-Twist1-induced lung metastasis and activated TGF- β R/Smad2 signaling that is often observed in breast cancer development (17-19).

Phospho-Twist1-Upregulated TGF- β 2 Maintains Hyperactivation of PI3K/Akt and Promotes Lung Metastasis

Upon Twist1 phosphorylation, we noted enhanced phosphorylated Akt that colocalized with increased actin bundles on the membrane (Fig. 5A, arrowhead). In addition, Akt activation was more sensitive in response to serum stimulation

in cells harboring phospho-Twist1 (Fig. 1B). Furthermore, TGF- β activation induced by Twist1 phosphorylation was associated with increased Akt phosphorylation in 4T1 cells (Fig. 4D). In contrast to the nonmetastatic cell line 67NR, the highly metastatic 4T1 cells showed the greater phosphorylation level of Twist1 and Smad2 (Supplementary Fig. S5A), indicating that an active TGF- β pathway correlates with activated Akt/Twist1 axis and increased metastatic capacity. Inhibition of PI3K/Akt activity by BEZ235 and MK-2206, 2 mTOR/PI3K/Akt pathway inhibitors that are currently in preclinical trials, significantly reduced Twist1 phosphorylation associated with moderately decreased Smad2 phosphorylation (Fig. 5B). Similar to previous reports (12, 20-22), TGF- β signaling was enhanced upon TGF- β 2 treatment, indicating a functional TGF- β pathway in 4T1 cells. Conversely, inhibition of TGF- β signaling dramatically decreased phosphorylation of Akt and Twist1 (Fig. 5C), suggesting that PI3K/Akt signaling is strongly dependent on active TGF- β signaling in 4T1 cells. This signaling cross-talk has also been observed in several independent studies in both untransformed mammary epithelial cells and metastatic breast cancer cells, including 4T1 (23).

In the promoter region of TGF- β 2, there is an evolutionarily conserved functional E-box (E1; ref. 24) that represents a potential binding site for Twist1. Both phosphorylated Twist1 and Twist1/S42D could stimulate luciferase expression by ~2.5-fold and ~2-fold greater than Twist1/S42A in a TGF- β 2 promoter activity assay, respectively (Fig. 5D). These data demonstrate that Twist1 phosphorylated on S42 transcriptionally activates TGF- β 2 through an E1-responsive element, thus enhancing TGF- β signaling and consequently supporting hyperactivation of PI3K/Akt signaling to facilitate the survival and invasion of cancer cells. Indeed, chromatin immunoprecipitation (ChIP) assay clearly showed that phosphorylated Twist1, rather than Twist1/S42A, was observed to bind to the proximal promoter region of TGF- β 2 between -300 bp and -1 bp that harboring E1 box (Fig. 5E), indicating that TGF- β 2 is a direct transcriptional downstream target of phospho-Twist1.

To explore the importance of phospho-Twist1-stimulated upregulation of TGF- β 2 in tumor metastasis, TGF- β 2 was knocked down in 4T1 cells. Silencing of TGF- β 2 negatively regulated E-cadherin expression but had no effect on TGF- β R (Supplementary Fig. S5B) or TGF- β 1/3 (Supplementary Fig. S5C) expression. Furthermore, silencing TGF- β 2, although less efficiently than Twist1- and TGF- β R2-silencing (Fig. 3H and 5F), decreased 4T1 lung metastases by ~40% in BALB/c mice (Fig. 5F), confirming TGF- β 2 upregulation by phospho-Twist1 actively contributes, to a significant extent, to the metastatic capacity of 4T1 cells.

DISCUSSION

Several signaling pathways that induce EMT and metastasis (25, 26) often converge at or activate PI3K/Akt, which itself is frequently activated during tumor progression. Hyperactivation of Akt is closely associated with elevated invasiveness and metastasis, resulting in a poor prognosis and a greater probability of relapse in many different cancer types (27-29). Despite these observations, the molecular

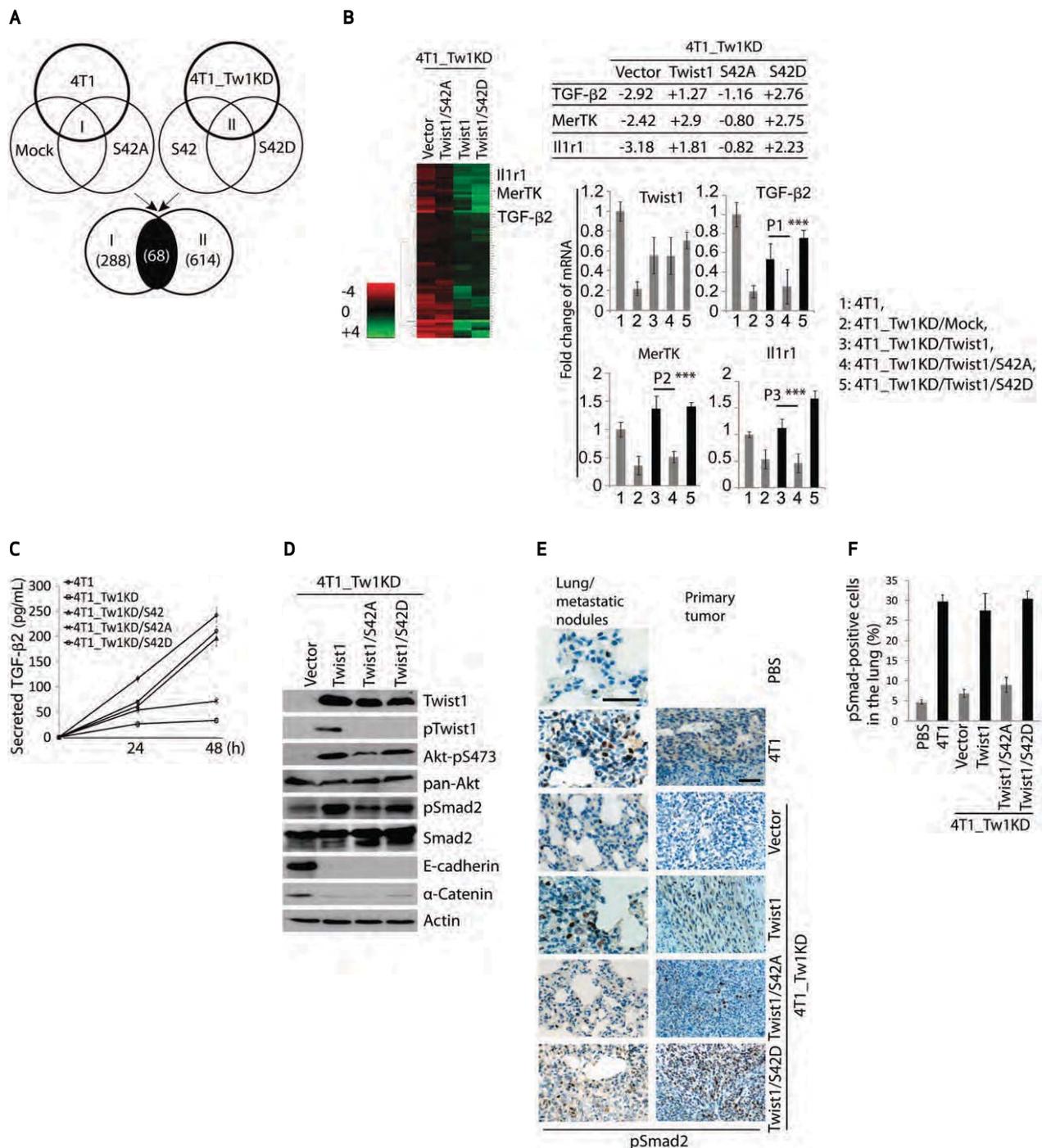


Figure 4. Microarray analysis of phospho-Twist1-responsive genes. **A**, in 4T1_Tw1KD cells, the downregulated genes when expressing Mock and S42A (compared with 4T1 cells, group I, 288 genes) were compared with the upregulated genes when expressing Twist1 and Twist1/S42D (compared with 4T1_Tw1KD cells, group II, 614 genes). A total of 68 overlapping genes represent potential targets of phospho-Twist1. **B**, differential regulation of 3 selected genes (TGF- β 2, MerTK, and Il1r1) was shown in heat map and relative expression fold-change of TGF- β 2, MerTK, and Il1r1. These 3 genes were validated by qRT-PCR and the fold-change of mRNA was indicated. Each sample was done in triplicate and the fold-change normalized against β -actin mRNA. Statistics were performed by *t* test: $P_1 = 0.00017$; $P_2 = 0.00053$; $P_3 = 8.89E-05$. **C**, parental 4T1 cell line and 4T1_Tw1KD expressing Twist1 and its variants were maintained in normal culture condition and the medium was collected after 24 or 48 hours for measuring the secreted TGF- β 2 ELISA. Average value for each sample was calculated from triplicates. **D**, immunoblot of phosphorylation of Smad2 and Akt in 4T1_Tw1KD cells expressing Twist1 variants. **E**, IHC staining of phospho-Smad2 in primary tumors and the lung tissues from BALB/c mice injected with PBS (as control), parental 4T1 cells, and 4T1_Tw1KD cells expressing Twist1 variants, 10 μ m and 50 μ m, respectively. **F**, the ratio of pSmad2-positive cells in the lung. Each group represents 4 different lung samples.

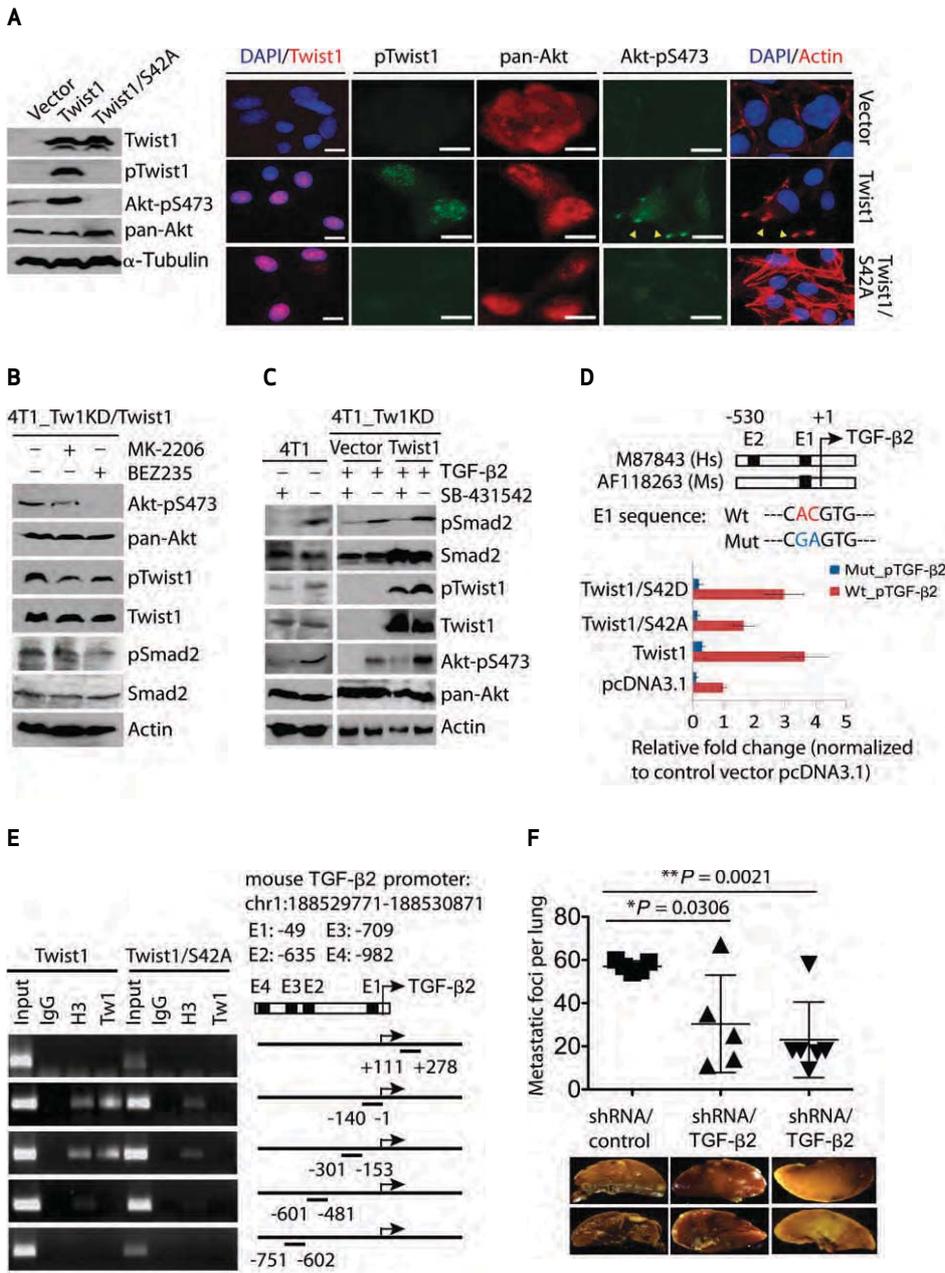


Figure 5. Phospho-Twist1-induced TGF-β2 acts as an essential mediator of signaling cross-talk between TGF-βR/Smad2 and PI3K/Akt axes. **A**, immunoblot analysis of Akt phosphorylation in MDCK cells expressing empty vector, wild-type Twist1, and the mutant Twist1/S42A. These cells were also subjected to immunofluorescence staining for phospho-Twist1, Akt, and reorganized cytoskeleton. Yellow arrowhead identifies the colocalization of phosphorylated Akt and strengthened actin bundles on the membrane at the migration front. Scale bars, 10 μm. **B**, 4T1_Tw1KD cells expressing phosphorylated Twist1 were treated with either BEZ235 (2 μM) or MK-2206 (0.5 μM) for 6 hours, and cell lysates were subjected to analysis for phosphorylation status of Smad2 and Akt. **C**, 4T1_Tw1KD cells expressing empty vector or Twist1 were incubated with TGF-β2 (10 ng/mL) for 24 hours in the presence/absence of SB-431542 (10 μM). The cells were lysed and subjected to analysis for indicated proteins. As control, parental 4T1 cells were treated with SB-431542 under the same condition. **D**, dual luciferase reporter assay of the effect of the Twist1 variants on activating wild-type mouse TGF-β2 promoter (wt_pTGF-β2) and its mutant (mut_pTGF-β2) in HEK-293 cells (pcDNA3.1 was used as control). Each sample was performed in triplicate. **E**, in 4T1_Tw1KD cells expressing either Twist1 or Twist1/S42A mutants, chromatin was immunoprecipitated using indicated antibodies [immunoglobulin G (IgG) as negative control and trimethyl-Histone H3 as positive control]. Corresponding amplified DNA fragments were indicated in the promoter region of TGF-β2 (the primers used are listed in Supplementary Fig. S8). **F**, TGF-β2- and TGF-βR2-depleted 4T1 cells were injected into BALB/c mice (5 mice per group) and the visible lung metastases were counted (statistics were done by t test).

mechanisms downstream of Akt that regulate EMT, invasion, and subsequent dissemination of cancer cells to distal sites remain mostly undefined.

Cells expressing phosphorylated Twist1 displayed increased actin polymerization at the migration front, which is known to mediate membrane protrusions that subsequently enhance cell migration (30). Activated Akt was often observed to colocalize with the actin bundles on the membrane (Fig. 5A) in motile cells. Consistently, cells expressing phosphorylated Twist1 have greater levels of promigratory proteins such as vimentin and MMP-9. Inhibiting Twist1 phosphorylation disrupted formation of filopodia-like structures and suppressed promigratory proteins associated with EMT. Snail2, another EMT-inducing factor recently shown to be a downstream mediator in Twist1-induced EMT and metastasis (31), was significantly increased upon Twist1 phosphorylation (Fig. 1D).

These findings indicate that the complete EMT phenotype and associated morphologic changes require activated PI3K/Akt-mediated Twist1 phosphorylation, placing the PI3K/Akt/Twist1 axis at a central position in mediating a complete EMT program involved in cell migration and invasion. Moreover, phosphorylation of Twist1 directly affects its transcriptional activities on TGF- β 2 (Fig. 5D, Supplementary Fig. S6), a polypeptide growth factor belonging to the cytokine TGF- β family that plays multiple important roles in embryonic development and tumor progression through the activation of the TGF- β signaling pathway (17, 32).

TGF- β signaling in cancer uses the canonical Smad pathway (17, 33) and initially exerts antiproliferative activities, whereas at later stages of tumor progression when it selectively loses signaling to a subset of antiproliferative genes, it promotes metastasis (34). This is often associated with hyperactivation of PI3K/Akt signaling in many cancers such as breast, melanoma, and prostate cancer, suggesting that the phosphorylation of Twist1 by Akt contributes to promoting the prometastatic capacity of TGF- β R/Smad2 signaling in late stages of cancer.

Indeed, very similar to our observations, in a recent paper researchers also reported increased Smad2 phosphorylation upon Twist1 expression (35). Moreover, a correlation of activated TGF- β signaling during Twist1-induced breast cancer metastasis has also been shown (36). Interestingly, Akt-mediated phosphorylation of Twist1 resulted in increased phosphorylation of Akt itself, illustrating a feedback loop between Twist1 phosphorylation and enhanced PI3K/Akt activity. Analysis of the affects of TGF- β 2 upregulation revealed that the observed increase of Akt phosphorylation was driven by TGF- β signaling (Fig. 5C). This illustrates a remarkable pro-oncogenic signaling feedback loop, whereby activated Akt phosphorylates Twist1, leading to increased secretion of TGF- β 2 and activation of TGF- β signaling, which in turn provides positive feedback to potentiate the activation of Akt, thereby perpetuating the invasive and metastatic progression of cancer cells. Importantly, this signaling occurs *in vivo* in a well-established 4T1 mouse model that recapitulates metastasis observed in the clinic (Fig. 3H).

Injection of phospho-Twist1-expressing 4T1 cells significantly enriched phospho-Smad2-positive cells in the lung (Fig. 4F), highlighting a functional cross-talk between PI3K/Akt/Twist1 and TGF- β in malignant breast cancer

progression. The authors of a previous report demonstrated the role of mTOR/PI3K/Akt pathway in TGF- β -induced cell invasion (37). In the current model, it appears that Twist1/S42A failed to couple to mTOR/PI3K/Akt pathway *in vivo* to mediate this signaling cross-talk. Twist1/S42D expression does not upregulate some of the classical mesenchymal markers *in vitro* but still promotes metastasis *in vivo*, which probably indicates a PI3K/Akt independent function of Twist1/S42D that needs further investigation.

Similar to the downregulation of Akt activity, blocking TGF- β activity dramatically inhibits lung metastasis in a number of different mouse models, including the 4T1 metastatic breast cancer model (12, 21, 23, 38). Similarly, when TGF- β 2 is depleted, the lung metastases are reduced by ~40% (Fig. 5F). Indeed, 4T1 xenograft tumors expressed a high level of TGF- β 2, which was suppressed upon Twist1 depletion (Fig. 6A). Re-expressing phospho-Twist1 notably rescued TGF- β 2 expression, demonstrating a link between Twist1 phosphorylation and TGF- β 2 expression *in vivo*. In addition, knockdown of TGF- β 2 in melanoma significantly inhibited brain metastasis (39), and a TGF- β 2 inhibitor has shown positive responses against malignant tumors in phase I/II clinical trials (40). Nevertheless, it should also be noted that the inhibition of the lung metastases upon knockdown of TGF- β 2 is not comparable with knockdown of Twist1, indicating that other yet-unknown downstream targets of Twist1 synergistically contribute to cancer cell invasion.

In several studies, investigators have also shown a synergy between TGF- β /Smad and EGFR/Ras/MAPK pathways that combine to activate PI3K/Akt signaling in breast cancer development (41–48). This signaling cross-talk often results in enhanced production of cytokines, including TGF- β , that potentiate cancer cell invasion. In human breast cell lines, Twist1 is constitutively phosphorylated in metastatic cancer cell lines with *ErbB2* amplification and is efficiently induced by the activation of EGFR signaling in metastatic cell lines without *ErbB2* amplification, such as MDA-MB-231 (Supplementary Fig. S2C). Indeed, a mouse xenograft tumor originated from injected MDA-MB-231 cells carries a large number of phospho-Twist1-expressing cancer cells (Supplementary Fig. S7A), thus further confirming that Twist1 phosphorylation is favored in the cancer environment *in vivo*. Because the PI3K/Akt axis is capable of being activated by receptor tyrosine kinases such as EGFR, it is possible that these pathways initiate PI3K/Akt activation at the early stage of tumor development to initiate and promote Twist1 phosphorylation.

Of a total of 1532 invasive human breast tumor samples, Twist1 was ubiquitously phosphorylated in invasive tumors and strongly correlated with Akt phosphorylation in both IDC and ILC types. This result indicates that Twist1 phosphorylation may act as one of the main driving forces regulating tumor dissemination. Not surprisingly, phosphorylated Twist1 also was detected in a small population of cancer cells that form lung metastatic nodules in both 4T1 and MDA-MB-231 mouse models (Fig. 6B and Supplementary Fig. S8). Because the mechanism of how Twist1 is inactivated/de-phosphorylated is unknown, it is not clear whether Twist1 is constitutively phosphorylated in these cancer cells or reactivated. However, it is obvious that these cancer cells harboring phospho-Twist1 are potentially predisposed to favor tertiary

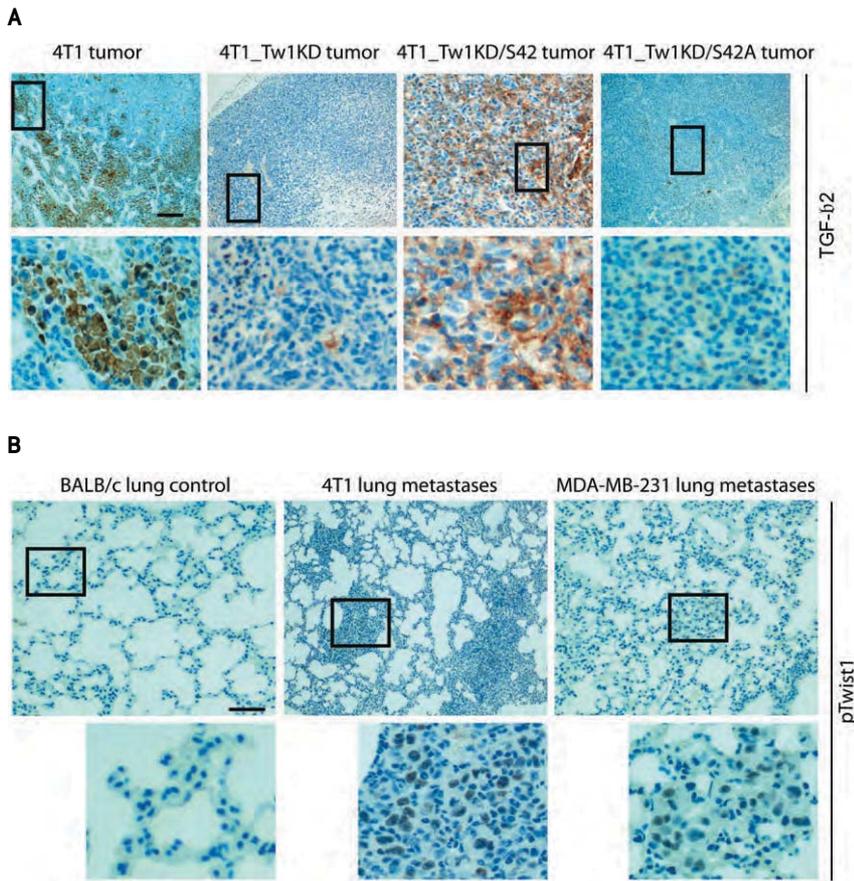


Figure 6. IHC staining of TGF-β2 and phospho-Twist1 in 4T1 and MDA-MB-231 xenograft tumors and matched lung metastases. **A**, TGF-β2 staining in primary breast tumors. Scale bars, 50 μm. **B**, phospho-Twist1 staining of the lung tissues from mice injected with 4T1 and MDA-MB-231. Scale bars, 50 μm. **C**, schematic model of phospho-Twist1 as a mediator of signaling cross-talk between PI3K/Akt and TGF-βR/Smad2 in cancer metastasis. Upon Akt activation by upstream signaling, Twist1 can be phosphorylated on S42. Phospho-Twist1 upregulates TGF-β2 to activate/enhance TGF-β receptor signaling. Consequently, enhanced TGF-β signaling maintains hyperactivation of PI3K/Akt, which further facilitates cancer cells' survival and invasiveness.

metastasis to other organs. Taken together, these data highlight the clinical importance of Twist1 phosphorylation associated with hyperactive PI3K/Akt in invasive breast cancers.

Our current model (Fig. 6C) places Twist1 as a new member of the PI3K/Akt pathway whose phosphorylation results in the metastatic dissemination of breast cancer cells to the lung. This represents a novel mechanism by which alterations in the PI3K/Akt pathway can communicate with the TGF-β signaling pathway to enhance malignant progression of cancers. Because the phosphorylation of Twist1 is observed in most malignant human breast tumors (this study), one might consider phospho-Twist1 as a potential biomarker to ascertain the efficacy of the PI3K/Akt inhibitors in the treatment of late-stage cancers with hyperactivated Akt. Finally, as Twist1 is important during embryogenesis but dispensable postnatally, it may also provide an effective therapeutic target for reducing metastatic spread.

METHODS

Constructs

Human Twist1 and its variants were subcloned into pcDNA3.1, pBA-BEpuro, and pLNCX2 (Clontech) vectors. Twist1-targeting shRNA

(Target sequence: 5'-AAGCTGA GCAAGATTCAGACC-3') was inserted into pSUPER.retro.puro vector. TGF-β2-targeting shRNAs were from Sigma-Aldrich (NM_009367, MISSION® shRNA). Plasmid-containing intact mouse TGF-β2 promoter was a gift from Angie Rizzino, and the mutations in the E-box region were introduced by PCR. Two promoter sequences were then inserted into the pGL3 for luciferase reporter assay.

Cell Lines and Retroviral Infection

67NR, 168FARN, 4TO7, 4T1, and MDCK cell lines were from Nancy Hynes (13). The Akt1 and Akt2 double-knockout MEF cell line (MEF_Akt1/2dKO) was a gift from Morris Birnbaum (49). To produce recombinant retrovirus, 20 μg of DNA was transfected into 5×10^6 of packaging cells growing on a 10-cm plate. After 24 hours, the medium was refreshed and incubated at 30°C for 48 to 72 hours. To infect targeting cells, the supernatant containing recombinant retroviruses was filtered (0.45 μm; Millipore), mixed with 4 μg/mL of polybrene (Sigma-Aldrich) and placed onto freshly split overnight cultures for 6 hours. After a 24-hour incubation in normal growth medium at 37°C, the infected cells were selected by appropriate antibiotics: MEF cells with 2 μg/mL puromycin, 4T1_Tw1KD cells with 12 μg/mL puromycin, MDCK cells expressing Twist1 variants with 4 μg/mL puromycin, and 4T1_Tw1KD cells expressing Twist1 variants with 5 μg/mL puromycin and 800 μg/mL G418.

Reagents

LY294002, SB-431542, and MK-2206 were from Alexis, Sigma-Aldrich, and ChemieTek, respectively. BEZ235 was from Novartis. EGF and TGF- β 2 were from PEPRO Tech. Polyclonal Twist1 and S42-phospho-specific Twist1 antibodies were described previously (8). The other antibodies against Akt1, Akt2 (Alexis); pan-Akt, Akt-pT308, Akt-pS473, Smad2, pSmad2, and MMP-9 (Cell Signaling); Actin (Santa Cruz Biotechnology); E-cadherin, N-cadherin, α -catenin, β -catenin, γ -catenin, Snail2, and fibronectin (BD Biosciences); vimentin V9 (Thermo Scientific); TGF- β 2 and TGF- β 2 (R&D Systems); trimethyl-Histone H3 (Lys4; Upstate); tenascin C (FMI); and cytokeratin 8 (Boehringer Mannheim) were applied according to the supplier's instructions. ELISA kit for TGF- β 2 was from Raybiotech, and the Dual-Luciferase Reporter Assay System was from Promega.

Immunocytochemistry and Immunohistochemistry

Cells were fixed with 4% paraformaldehyde for 20 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes at room temperature. All staining procedures followed the instructions of individual antibodies. The tissue samples were fixed in 4% paraformaldehyde and paraffin embedded. Staining was performed (Vantana Discovery XT) under standard conditions. The rabbit polyclonal Ki67 was from Neomarkers, and Mammaglobin was a gift from Hakan Hedman.

Transcriptome Analysis and qRT-PCR

Microarray analysis was performed by the use of the Affymetrix genechip, following the instructions provided by the manufacturer. qRT-PCR was performed with the ABI-7000 Real Time PCR System. Sequence design of primers was based on the "Primer databank" from Harvard University and validated. The experimental data of microarray analysis were submitted to the public databank Gene Expression Omnibus (GEO accession number: GSE29754).

Luciferase Reporter Assay

Analysis of luciferase activity was performed according to the instructions for the dual luciferase reporter system (Promega). pTK-RL plasmid (encoding Renilla luciferase) was used as the internal control. Conserved E-box cassette in the TGF- β 2 promoters of human and mouse (E1: at -45~-50, CACGTG) was mutated by PCR (mutated E1: CGAGTG) to result in a nonfunctional promoter.

Chromatin Immunoprecipitation

ChIP assay was performed as previously described (50). To summarize, cells were treated with 1% formaldehyde for 10 minutes and subjected to lysis and sonication. Pre-cleared with protein G Sepharose (GE Healthcare), the lysates were incubated with protein G conjugated with individual antibodies overnight. The associated DNA was extracted and purified for PCR analysis.

Clinical Breast Tumor Samples

The use of human breast tumor samples from the Biobank (Institute of Pathology, University Hospital, Basel, Switzerland) for retrospective studies was sanctioned by the ethics committee of Basel (EKBB, Switzerland). A total of 1,532 invasive breast tumor samples were evaluable and used in this study. The average age of the patients was 63 years. These tumors were clinically diagnosed as 59% of invasive ductal carcinoma, 11% of invasive lobular carcinoma, and 30% of other types.

Cell Migration and Invasion Assays

A wound healing assay was performed by use of the Ibidi chambers (Ibidi GmbH). After serum starvation overnight, the cell migration was visualized and recorded (Zeiss TILL5, LONG RUN, Axiovert 200M) for 20 to 40 hours at 37°C. Cell migratory potential was measured by the

size of uncovered area over the initial wounded area and quantified with Metamorph. A Matrigel-based invasion assay was conducted with the BioCoat Matrigel invasion chamber (BD Biosciences).

Mouse Model of Breast Cancer Metastasis

Animal maintenance and experimental procedures conformed to the Swiss Animal Protection Ordinance. Surgery was carried out on female 12- to 15-week-old BALB/c mice purchased from Charles River Laboratories. Tumor cells (5×10^5) were injected into the fat pad of mice anesthetized by isoflurane. At 21 days after injection, the size of primary tumors was measured weekly with calipers. Dissected primary tumors, the spleens, and the lungs were quickly rinsed with PBS and fixed in formaldehyde for histopathology analysis. Tumor nodules in lung were stained with Bouin solution and counted under a dissection microscope (Leica MacroFluo Z6 Apo).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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