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Twist, a Master Regulator of Morphogenesis, Plays an Essential Role in Tumor Metastasis

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Summary

Metastasis is a multistep process during which cancer cells disseminate from the site of primary tumors and establish secondary tumors in distant organs. In a search for key regulators of metastasis in a murine breast tumor model, we have found that the transcription factor Twist, a master regulator of embryonic morphogenesis, plays an essential role in metastasis. Suppression of Twist expression in highly metastatic mammary carcinoma cells specifically inhibits their ability to metastasize from the mammary gland to the lung. Ectopic expression of Twist results in loss of E-cadherin-mediated cell-cell adhesion, activation of mesenchymal markers, and induction of cell motility, suggesting that Twist contributes to metastasis by promoting an epithelial-mesenchymal transition (EMT). In human breast cancers, high level of Twist expression is correlated with invasive lobular carcinoma, a highly infiltrating tumor type associated with loss of E-cadherin expression. These results establish a mechanistic link between Twist, EMT, and tumor metastasis.

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Introduction

Tumor metastasis is the most common cause of death in cancer patients. In carcinomas, the metastatic process is thought to consist of a number of distinct steps. The first step—invasion—requires neoplastic epithelial cells to lose cell-cell adhesion and to gain motility, which enables them to invade the adjacent tissue. During the second step, intravasation, tumor cells penetrate through the endothelium of blood or lymphatic vessels to enter the systemic circulation. Only some circulating tumor cells appear to be able to survive the passage through circulation. Some of these survivors manage to complete the next step—extravasation—as they extravasate through the capillary endothelium at distal sites. Finally, in the new host environment, an even smaller subset of such metastasizing cells succeeds in proliferating from minute growths (micrometastases) into malignant, secondary tumors (Fidler, 2003). A central aim in the study of tumor metastasis is to understand the nature of the distinct genetic and epigenetic changes that program these individual steps.

Recently, our understanding of metastasis has been expanded through microarray analyses of various human tumor samples. For example, several studies of human primary breast tumors have generated gene expression profiles that are predictive of metastasis or poor survival rate (Ramaswamy et al., 2003; van't Veer et al., 2002). Such analyses are very powerful for producing fingerprints of metastatic tumor cells, which could be used as prognostic markers of metastatic diseases. However, due to the lack of further experimental manipulation of human tumor samples, it has been very difficult to elucidate the specific contributions of such genes to tumor metastasis.

Experimental animal models have been successfully used to identify molecular elements during metastasis. For example, in an expression profile analysis comparing melanoma cells and their highly metastatic derivatives, *RhoC* was identified as essential for pulmonary metastasis by melanoma cells that were injected intravenously into mice (Clark et al., 2000). Recently, a set of genes, including *osteopontin* and *IL-11*, were shown to promote bone metastasis when human breast cancer cells were injected via the intracardiac route (Kang et al., 2003). Most of these models rely on the introduction of tumor cells directly into the systemic circulation. Such approaches obviate the steps of invasion and intravasation and therefore are unlikely to reveal genes involved in these early steps of metastasis.

Given the complexity of the process of metastasis, it is essential to exploit experimental models in which each of the steps of metastasis is represented and can be manipulated individually. In this report, we exploit a mouse mammary tumor model, in which a set of otherwise isogenic tumor cell populations is able to complete distinct steps of metastasis when implanted into the mammary glands of BALB/c mice (Aslakson and Miller,

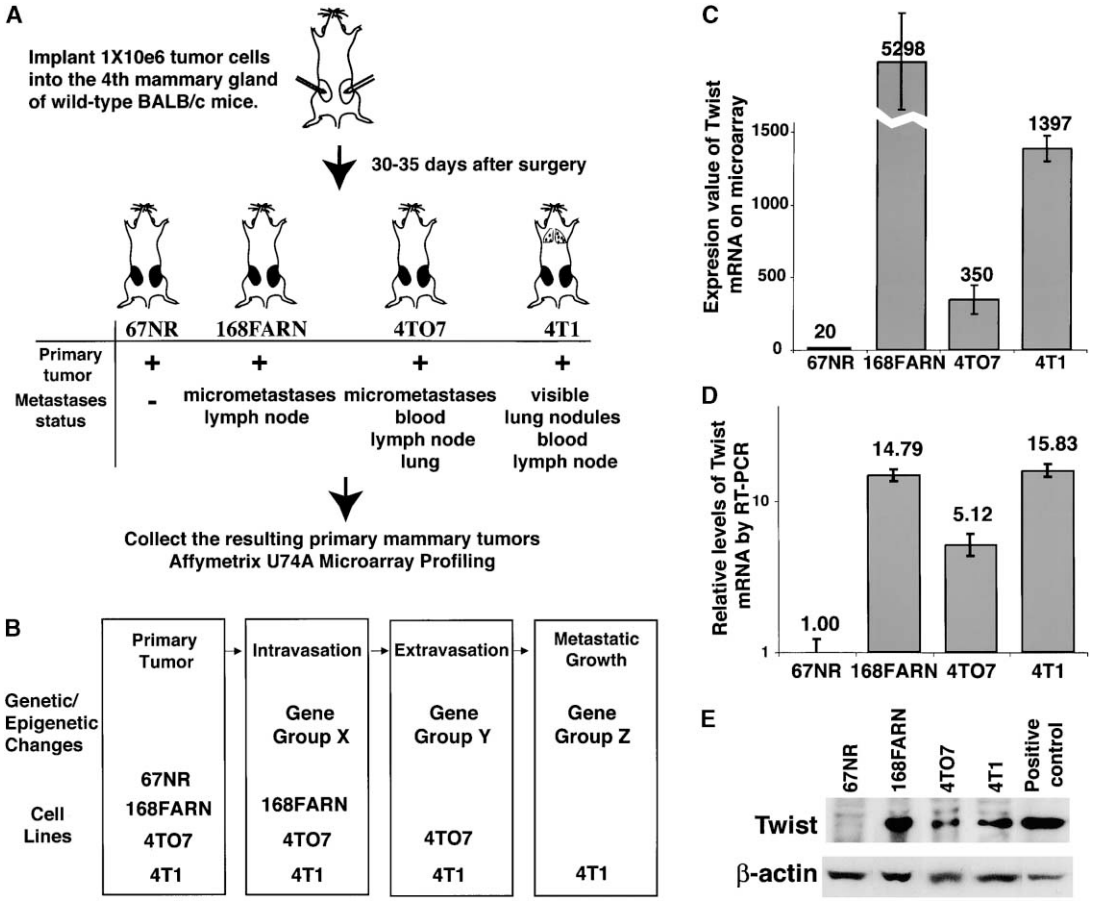

Figure 1. Microarray Analysis Identified Twist to Be Upregulated during the Metastatic Progression

(A) Schematic representation of the microarray experimental procedure.

(B) Gene expression profile scheme. Three groups of genes are classified based on their gene expression in primary tumors of four cell lines.

(C) The average values of Twist expression in four primary tumors formed by each cell line measured by microarray analysis. Each bar represents the mean ± SEM (standard error of the mean).

(D) The relative levels of Twist mRNA measured by real-time RT-PCR in the 168FANR, 4TO7, and 4T1 cells compared to 67NR cells. Each bar represents the mean ± SEM of the PCRs in triplicate.

(E) Expression of Twist protein was examined by immunoblotting in all four murine cell lines. The HMEC cells overexpressing mouse Twist is used as a positive control, and β-actin is used as a loading control.

1992). We have identified a transcription factor, Twist, as being essential for tumor metastasis and have characterized the molecular actions of Twist and its involvement in human breast cancer pathogenesis.

Results

Gene Expression Patterns Associated with Individual Steps of Mammary Tumor Metastasis

We utilized a mouse mammary tumor model that consists of four distinct tumor cell lines, 67NR, 168FARN, 4TO7, and 4T1. All four lines are derived from a single mammary tumor that arose spontaneously in a wild-type BALB/c mouse. When cells from these four cell lines are implanted into the mammary fat pads of syngeneic BALB/c mice, they form mammary carcinomas within a month (Aslakson and Miller, 1992). While these cell lines form primary tumors with equivalent kinetics, they differ dramatically in their metastatic potential. Thus, (1) cells of the line 67NR form primary tumors, but no tumor cells are detectable in any distant tissue, including blood, lymph nodes, and lung. (2) Cells of the 168FARN line disseminate from mammary fat pads and can be detected in lymph nodes; however, 168FARN cells are rarely detectable in lung, indicating that this cell line is unable to accomplish extravasation effectively. (3) Cells of the 4TO7 line are able to spread to lung, but cannot establish visible metastatic nodules. Moreover, removal of primary 4TO7 tumors results in the disappearance of clonogenic cells from the lung, suggesting that 4TO7 primary tumors can continuously seed distant sites, but fail to colonize these sites following their arrival. (4) Finally, cells of the 4T1 line are able to complete all steps of metastasis and form visible metastatic nodules in lung efficiently (Aslakson and Miller, 1992). The behavior of these tumor lines reflects the sequence of multistep metastasis progression. We therefore set out to compare the gene expression profiles of the four cell lines in order to dissect the specific genetic and

epigenetic changes associated with their respective metastatic abilities.

To compare the gene expression profiles, we injected cells from each of the four lines into the mammary glands of BALB/c mice (Figure 1A). At 30 days postinjection, all the cells formed primary tumors weighing 2–3 grams. We confirmed their respective metastatic phenotypes as previously reported (Aslakson and Miller, 1992). We utilized primary mammary tumors instead of cell lines growing in vitro for microarray analysis, because the host tissue environment can drastically influence their metastatic abilities and their gene expression profiles. In addition, histological analyses revealed that, in each case, major components of the tumor samples (>95%) were descendants of the introduced tumor cells with minimum host stromal contribution.

We hypothesized that each of the defined metastatic properties exhibited by these four tumor lines results from alterations in the expression of a specific group of genes. Accordingly, a group of genes involved in invasion and intravasation (Group X) should be altered in the 168FARN, 4TO7, and 4T1 cells, but not in the 67NR cells. Similarly, a set of genes altered in 4TO7 and 4T1 cells compared to 67NR and 168FARN cells (Group Y) are thought to enable these tumor cells to extravasate into distant sites. Finally, the Group Z genes are those that are specifically altered in the 4T1 cells compared to the other three lines, and are expected to be responsible for the ability of 4T1 cell to form visible metastatic nodules in the lung (Figure 1B). We compared the transcription profile of the four tumor lines and assigned differentially expressed genes (changes greater than 2.5-fold) to such three groups. The Group X genes consisted of 36 upregulated genes and 9 downregulated genes. The Group Y genes included 7 upregulated and 3 downregulated genes. The genes in the Group Z contain 38 upregulated genes and 59 downregulated genes (Supplemental Data available at http://www.cell.com/cgi/content/full/117/7/927/DC1).

Among the identified genes, several had previously been implicated in metastasis. For example, we identified the *CXCR3* chemokine receptor as a Group X gene. In fact, several members of the CXCR chemokine receptor family are highly expressed in malignant human breast cancers and melanomas and can elicit chemotactic and invasive responses (Muller et al., 2001). In addition, the matrix metalloproteinases *MMP9*, which plays key roles in tumor invasion and metastasis (Egeblad and Werb, 2002), were assigned to Group Z. These results indicated that in this mammary tumor metastasis model, tumor cells employ several known pathways of metastasis. Moreover, successful identification of these genes validated our approach to identify novel genes that may play equally important roles in metastasis.

Increased Expression of Twist Correlates with Tumor Invasion and Metastasis

Among the identified genes, the transcription factor Twist stood out as an attractive candidate for modulating metastasis. *Twist* was the second most strongly upregulated gene in the Group X list. The microarray analysis showed that *Twist* mRNA was expressed in 168FARN, 4TO7, and 4T1 tumors, but not 67NR tumors (Figure 1C). Real-time RT-PCR analysis confirmed that *Twist* mRNA was expressed 5–15-fold higher in 168FARN, 4TO7, and 4T1 tumor cells compared to 67NR cells (Figure 1D). We also observed that Twist protein was expressed in the 168FARN, 4TO7, and 4T1 tumor cells, but not in the 67NR cells (Figure 1E). In addition, we could not detect the expression of Twist in normal mammary epithelial cells, such as mouse EPH4 cells (data not shown) and immortalized human mammary epithelial cells (HMECs) (Figure 5C), indicating that Twist expression is specifically induced in 168FARN, 4TO7, and 4T1 tumor cells. These observations led us to pursue Twist as an attractive candidate for orchestrating some steps of metastasis in this mammary tumor model.

Our interest in Twist was further enhanced by its known functions as a master regulator of embryonic morphogenesis. Twist was originally identified as a key inducer of mesoderm formation in *Drosophila* (Thisse et al., 1987). In mice, Twist is essential for proper cell migration and localization during cranial neural tube morphogenesis (Chen and Behringer, 1995; Soo et al., 2002). Across phyla, Twist plays a major role in inducing cell movement and tissue reorganization during specific steps of embryonic development (Castanon and Baylies, 2002). Such cell migration and tissue remodeling also occurs during tumor invasion and metastasis. Therefore, we speculated that Twist expression might be derepressed during tumor progression. Given the complex nature of the multistep metastasis process, Twist, as a key transcription factor controlling morphogenesis, may be able to elicit multiple biochemical and cell biological alterations of metastasis.

Twist Is Essential for Tumor Metastasis from the Mammary Gland to the Lung

To determine whether Twist plays a causal role in tumor metastasis, we tested whether inhibition of Twist expression in the highly metastatic 4T1 cells would affect their metastatic ability. To do so, we chose several sequences in the coding region of the mouse *Twist* gene and designed short hairpin-interfering RNAs (siRNA) targeted against each sequence. To test whether these siRNAs could suppress Twist expression in 4T1 cells, we infected these cells with the lentiviral vector transducing a DNA segment specifying such siRNA sequences and then selected the cells stably expressing the siRNAs. As shown in Figure 2A, expression of all three siRNAs drastically reduced the expression of Twist protein in 4T1 cells. Specifically, the *Twist* siRNA3 suppressed the expression of Twist protein to a level that was undetectable by immunoblotting (Figure 2A). An siRNA, whose sequence did not match any known mouse gene, was also introduced into 4T1 cells. As anticipated, this control siRNA did not cause a reduction of Twist expression (Figure 2A).

We examined whether loss of Twist expression affects 4T1 cells to proliferate in vitro and form primary tumors in vivo. 4T1 cells carrying either *Twist*-siRNA3 or control-siRNA grew at similar rates in vitro (Figure 2B), indicating

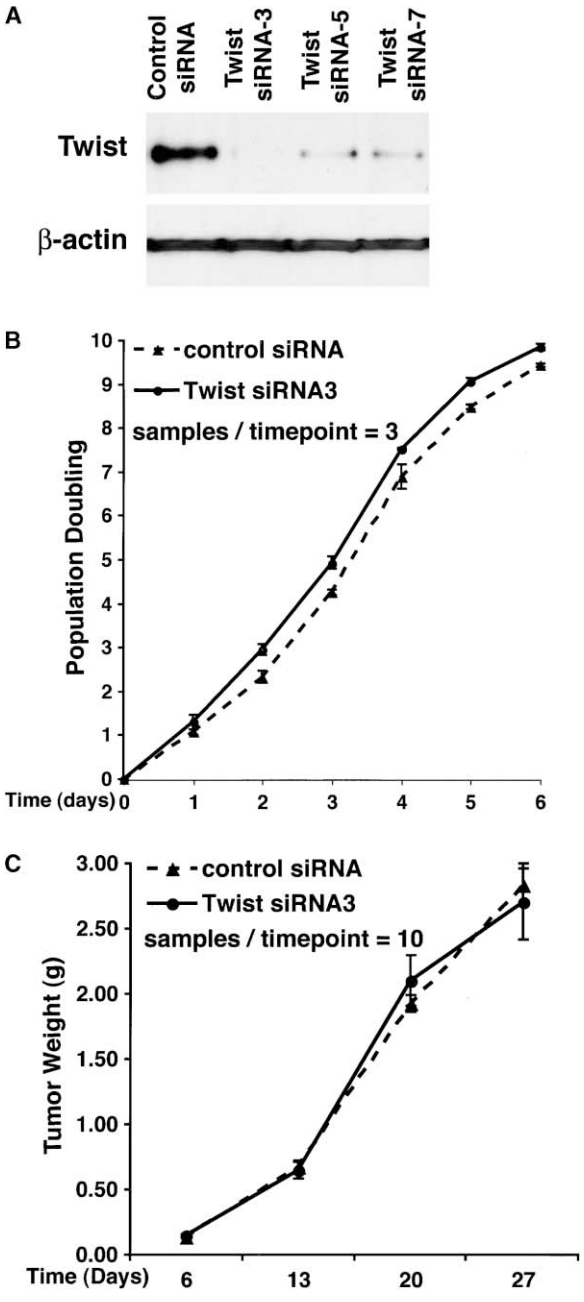

Figure 2. Suppression of Twist Expression by Stable siRNA Does Not Affect the Proliferation of 4T1 Cells In Vitro and In Vivo

(A) Expression of Twist protein was examined by immunoblotting in 4T1 cells stably expressing control-siRNA, Twist-siRNA3, *Twist*-siRNA5, or *Twist*-siRNA7.

(B) Population doublings of the 4T1 cells expressing either control-siRNA or *Twist*-siRNA3. Each data point represents the mean ± SEM number of cells counted in triplicate dishes.

(C) Growth patterns of primary mammary tumors formed by 4T1 cells expressing either control-siRNA or Twist-siRNA3. Each data point represents the mean ± SEM of ten primary tumors.

that Twist is not required for the proliferation of 4T1 cells in culture. We then injected such cells into the mammary fat pads of BALB/c mice and measured the weights of the resulting primary tumors weekly. 4T1 cells carrying either *Twist*-siRNA3 or the control-siRNA formed primary mammary tumors at identical rates (Figure 2C). These results demonstrated that Twist is not required for primary tumor formation by 4T1 cells.

To find out whether Twist expression continued to be suppressed by siRNA in tumors growing in mice, we collected primary tumors formed by either the 4T1 *Twist*-siRNA3 cells or the 4T1 control-siRNA cells and propagated the tumor cells in the presence of puromycin. We observed that expression of Twist in 4T1 cells continued to be suppressed by *Twist*-siRNA one month after tumor implantation (Figure 3E).

To determine whether loss of Twist expression affected the ability of 4T1 cells to metastasize, we first examined the metastatic behaviors of the 4T1 tumor cells expressing either *Twist*-siRNA3 or the control-siRNA. Four weeks after implantation of such cells in the mammary glands of BALB/c mice, we sacrificed the mice and examined lungs for metastatic lesions by inspection under a dissection microscope. While tumors expressing control-siRNA formed large numbers of macroscopically visible metastases in their lungs, those that expressed *Twist*-siRNA3 formed very few metastases (Figure 3A). The average number of visible metastatic nodules dropped from 105 ± 14 per lung in mice carrying 4T1 control-siRNA tumors to 14 ± 2 nodules per lung in mice carrying 4T1 *Twist*-siRNA3 tumors (Figure 3B). Histological analyses confirmed that the number of micrometastatic lesions was also drastically reduced in the lungs of mice carrying 4T1 *Twist*-siRNA3 tumors (Figure 3C). In addition, the sizes of individual metastatic nodules present in the lungs of both groups of mice were very similar. These results suggested that loss of Twist expression reduced the number of metastatic nodules present in the lung, rather than preventing micrometastases established in the lung from proliferating into visible nodules.

To exclude the possibility that the observed effect is due to nonspecific suppression of off-target genes by *Twist* siRNA-3, we further tested whether two additional *Twist* siRNA sequences (*Twist* siRNA5 and *Twist* siRNA7) can also suppress the ability of 4T1 cells to metastasize. Indeed, expression of either *Twist* siRNA5 or siRNA7 in 4T1 tumor cells also drastically reduced the formation of pulmonary metastatic nodules by 4T1 tumors in mice (Figure 3D). Together, these results demonstrate that continued Twist expression is essential for efficient execution of the metastatic program of the 4T1 cells.

We suspected that the presence of small numbers of nodules in the lungs of mice carrying 4T1 *Twist*-siRNAs tumors might be caused by incomplete suppression of Twist by siRNA in these polyclonal populations. This is due to variable numbers of integrated, siRNA-expressing proviral vectors generated by lentiviral infection in individual cells. Therefore, we analyzed the expression of Twist in the 4T1 *Twist*-siRNA3 and 4T1 control-siRNA tumor cells recovered from the lung samples. As shown in Figure 3E, the 4T1 *Twist*-siRNA3 tumor cells isolated from lungs continue to express a far lower level of Twist protein than did the 4T1 control-siRNA cells isolated

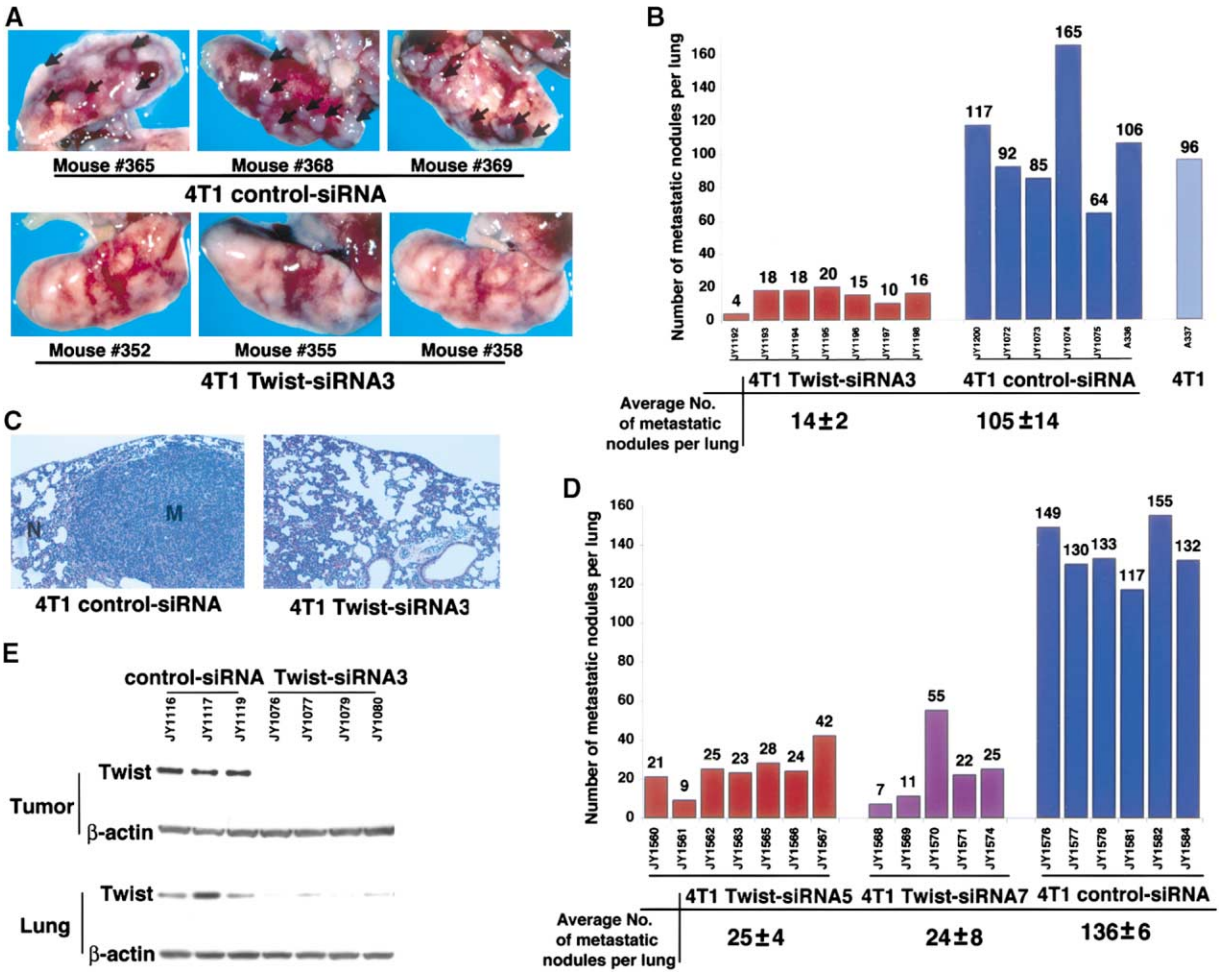

Figure 3. Suppression of Twist Expression Inhibited the Ability of 4T1 Cells to Metastasize from the Mammary Gland to Lung

(A) Representative photos of the lungs from mice carrying mammary tumors of 4T1 control-siRNA cells or 4T1 Twist-siRNA3 cells 30 days post tumor implantation. The arrows point to the metastatic nodules in the lung.

(B) Total numbers of lung metastatic nodules in individual mice were counted under the dissection scope. Two populations of 4T1 cells infected independently with *Twist*-siRNA3 lentivirus were used in four independent experiments (15–20 mice per experiment) and consistently yielded similar results.

(C) Representative H&E staining sections of the lungs from Figure 3A. N, lung tissue; M, metastatic nodule.

(D) Total numbers of metastatic nodules in the lung of individual mice carrying mammary tumors of 4T1 control-siRNA cells, 4T1 *Twist*-siRNA5 cells or 4T1 *Twist*-siRNA 7 cells at 28 days post tumor implantation.

(E) Expression of *Twist* protein was examined by immunoblotting of tumor cells recovered from both primary tumors and lungs of individual mice carrying either 4T1 control-siRNA tumors or 4T1 *Twist*-siRNA3 tumors. β-actin is used as a loading control.

from lungs. However, in many samples of 4T1 *Twist*-siRNA3 tumor cells recovered from lungs, low, but significant levels of Twist expression were detectable. In contrast, expression of Twist was undetectable in the 4T1 *Twist*-siRNA3 cells isolated from primary tumors (Figure 3E). These data argue that incomplete suppression of Twist by siRNA in a small subset of 4T1 *Twist*-siRNA cells account for the residual ability of such cells to form lung metastases.

Suppression of Twist Expression Reduces the Presence of Tumor Cells in the Blood Circulation

We attempted to determine the specific steps of the metastatic process to which Twist contributes. Histological examinations showed that the primary tumors formed by the 4T1 *Twist*-siRNA cells and the 4T1 control-siRNA cells were both undifferentiated mammary carcinomas (Figure 4A). To examine the ability of 4T1 *Twist*-siRNA cells to intravasate, we cultured blood samples from mice carrying primary tumors derived from 4T1 *Twist*-siRNA3 cells or 4T1 control-siRNA cells. The colonies formed by these cells were quantified to determine the number of 4T1 tumor cells present in such blood samples. We recovered colony-forming cells from the blood samples of 7 out of 9 mice bearing 4T1 control-siRNA tumors. In contrast, colonies were recovered from only 2 out of 9 mice bearing 4T1 *Twist*-siRNA3 tumors (Figure 4B). The average number of colonies recovered from the blood of the two mice bearing 4T1 *Twist*–

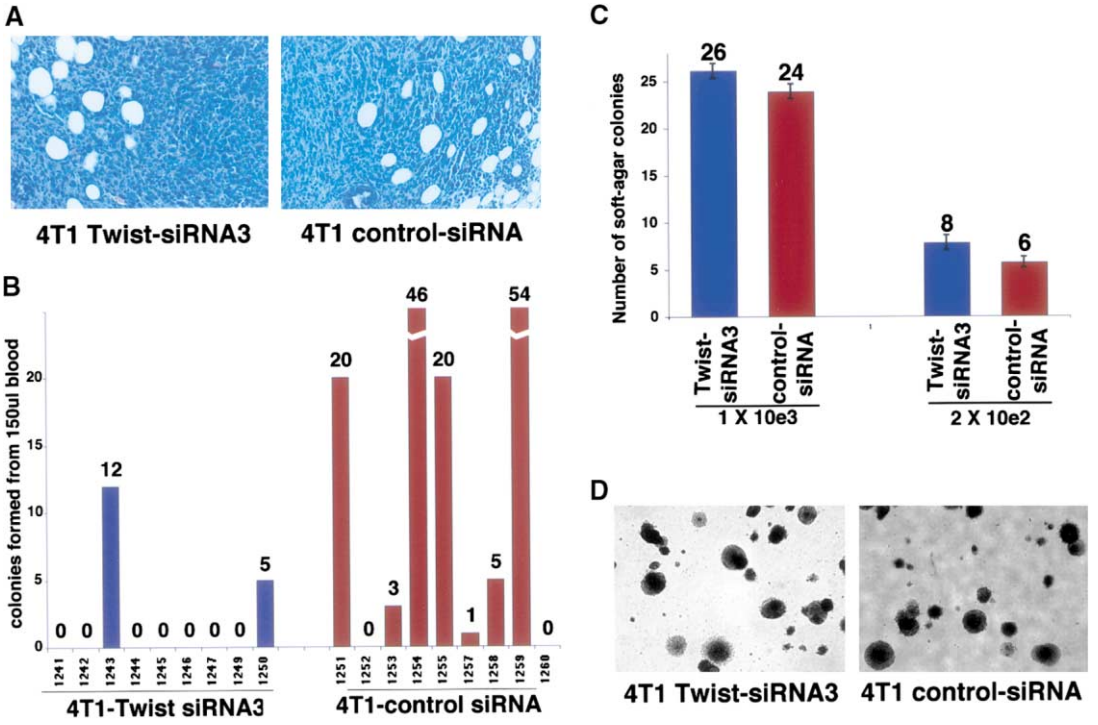

Figure 4. Suppression of Twist Expression Reduced the Presence of 4T1 Cells in the Blood Circulation

(A) Representative H&E staining sections of the primary mammary tumors formed by 4T1 control-siRNA cells or 4T1 *Twist*-siRNA3 cells. The large white cells are fat cells in mouse mammary glands.

(B) Total numbers of colonies formed by tumor cells presented in 150 μl blood from mice carrying mammary tumors of 4T1 control-siRNA cells or 4T1 *Twist*-siRNA3 cells.

(C) Anchorage-independent growth of 4T1 control-siRNA cells or 4T1 *Twist*-siRNA3 cells. 1×10^3 or 2×10^2 cells were seeded, and the number of soft agar colonies presented is the mean of colony counts in ten 40× microscopic fields from two dishes.

(D) Representative photos of soft agar colonies formed by 4T1 control-siRNA cells or 4T1 *Twist*-siRNA3 cells.

siRNA3 tumors (8 colonies/150 μl blood) were much smaller than those recovered from the seven mice bearing 4T1 control-siRNA tumors (21 colonies/150 μl blood) (Figure 4B). This observation suggests that expression of Twist in 4T1 cells enables continuous presence of these tumor cells in the blood.

The presence of 4T1 cells in the circulation indicates that 4T1 cells are able to complete intravasation and to survive anoikis due to loss of cell-matrix attachment. We tested whether Twist is required for 4T1 cells to survive and proliferate in an anchorage-independent manner. After growing in soft agar for 15 days, 4T1 cells carrying either *Twist* siRNA3 or the control siRNA formed very similar numbers of colonies (Figure 4C), and these colonies were of comparable size (Figure 4D). This result suggested that Twist does not necessarily affect the survival of 4T1 cells in the circulation; rather, Twist appears to allow the step of intravasation. The involvement of Twist in intravasation is further supported by our above observation that loss of Twist reduced the number of micrometastases in the lung, suggesting expression of Twist is necessary for 4T1 cells to leave their primary tumor and to reach the lung.

Twist Induces an Epithelial-Mesenchymal Transition (EMT) and Cell Migration

To understand the specific biological functions that Twist exerts during tumor metastasis, we took advantage of the known actions of this transcription factor during mesoderm formation in *Drosophila*. At gastrulation, Twist is induced to allow ventral furrow cells to migrate and form the mesodermal layer (Leptin and Grunewald, 1990). During this process, these cells lose cell-cell adhesion and undergo an epithelial-mesenchymal transition (EMT) (Hay, 1995). Such EMT is suggested to contribute to the dissemination of carcinoma cells from epithelial tumors (Thiery, 2002). We therefore speculated that the contribution of Twist to tumor metastasis might involve the induction of an EMT and cell motility.

We examined whether expression of Twist was able to promote an EMT in normal mammalian cells. To do so, we expressed Twist by retroviral infection in MDCK kidney epithelial cells. As shown in Figure 5A, MDCK cells maintain highly organized cell-cell adhesion and cell polarity; expression of Twist in these cells led to loss of cell-cell contacts and cell scattering. The cobblestone-like appearance of these epithelial cells was replaced by a spindle-like, fibroblastic morphology (Figure 5A). This morphological change represents one of the hallmarks of an EMT. To determine whether the molecular alterations of an EMT occurred in these cells, we examined the localization of several adherens junction proteins, such as E-cadherin, α-catenin, β-catenin, and γ-catenin. Immunostaining showed that all four proteins disappeared from cell membrane in the Twist-expressing MDCK cells compared to their strong membrane

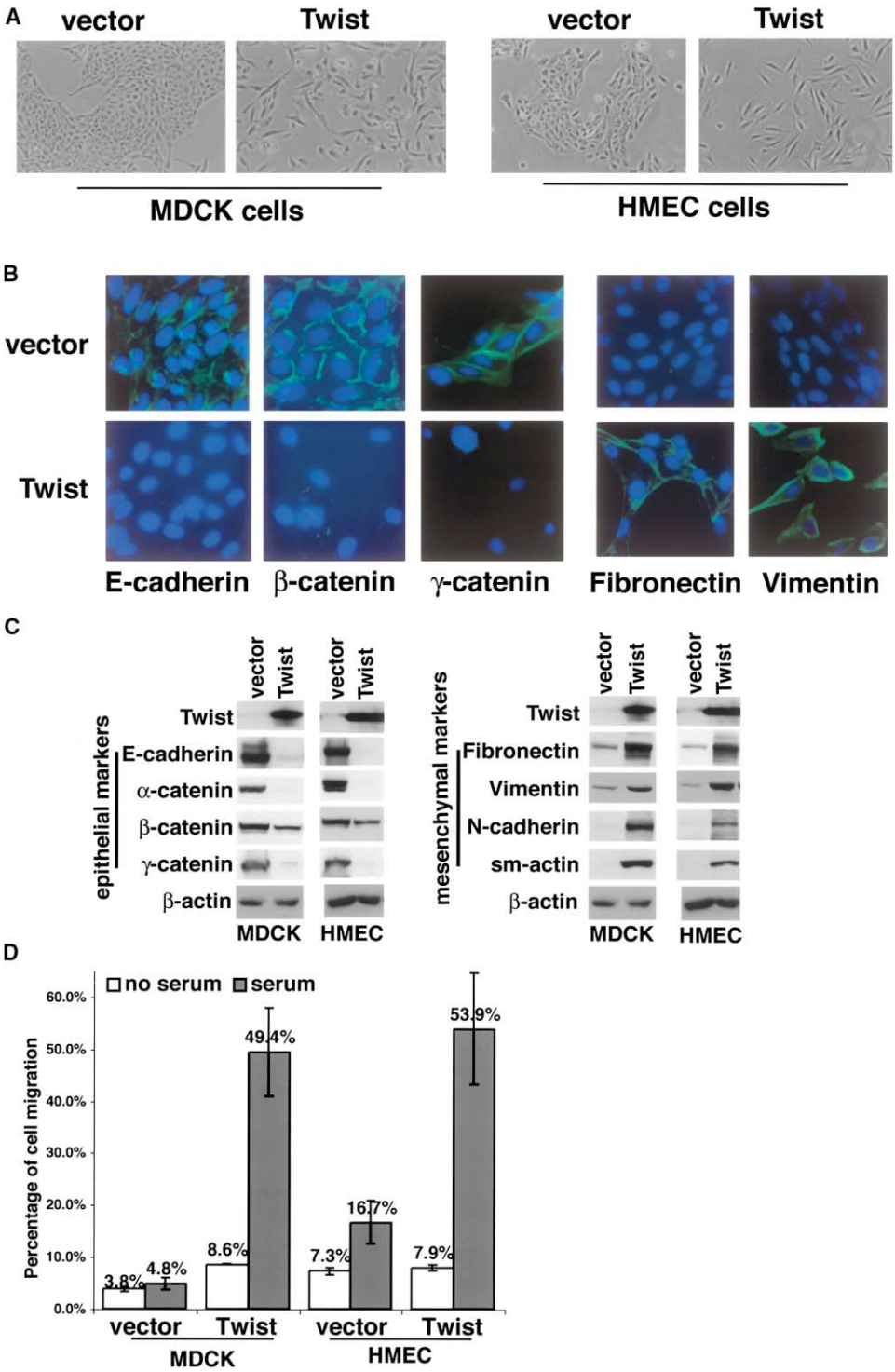

Figure 5. Twist Induces an EMT and Cell Migration

(A) The morphologies of the MDCK cells and HMEC cells expressing either the control vector pBabe-PURO or pBabe-PURO/Twist were revealed by phase contrast microscopy.

(B) Immunofluorescence staining of E-cadherin, β-catenin, γ-catenin, fibronectin,n and vimentin in the MDCK cells expressing either pBabe-PURO or pBabe-PURO/Twist. The green signal represents the staining of corresponding protein, and the blue signal represents the nuclear DNA staining by Hoechst.

(C) Expression of epithelial proteins, including E-cadherin, α-catenin, β-catenin, and γ-catenin, and mesenchymal proteins, including fibronectin, vimentin, N-cadherin, and smooth muscle actin was examined by immunoblotting in the MDCK cells and HMEC cells expressing either pBabe-PURO or pBabe-PURO/Twist. β-actin is used as a loading control.

(D) MDCK cells and HMEC cells expressing either pBabe-PURO or pBabe-PURO/Twist were induced to migrate toward serum (MDCK cells) or growth factors (HMEC cells). The migration toward growth factor-free media was examined as negative control for each cell line. The migration ability is presented as percentage of migrating cells over the total cells in the chamber. Each bar represents the mean ± SEM of samples measured in triplicate.

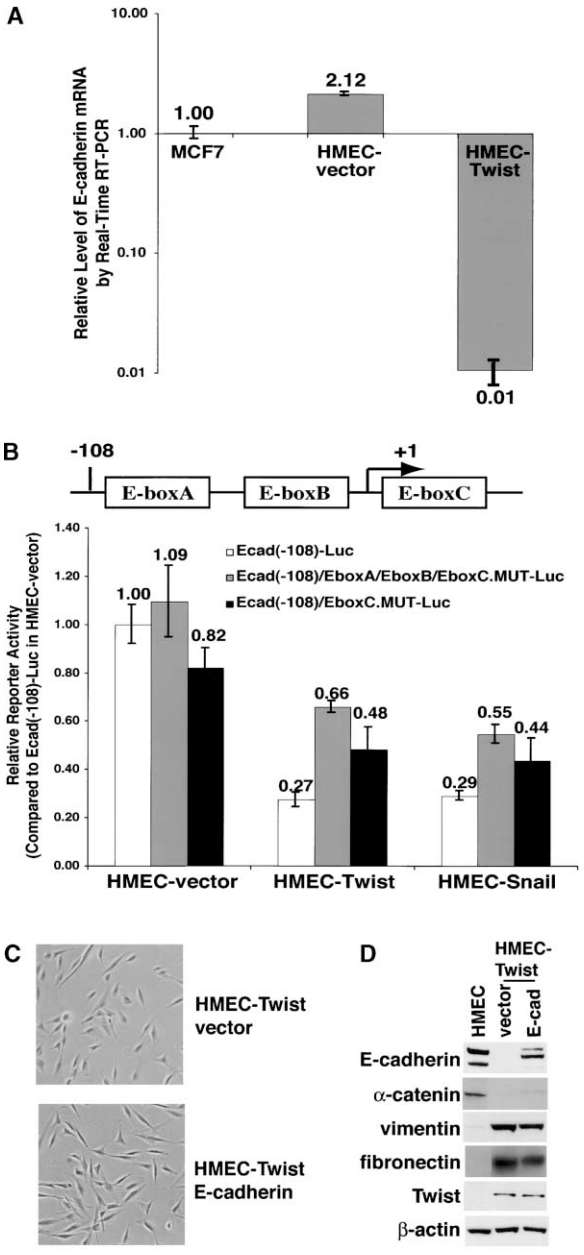

Figure 6. Expression of Twist Results in Repression of E-Cadherin Transcription

(A) The endogenous levels of *E-cadherin* mRNA was measured by real-time RT-PCR in the HMEC cells expressing either pBabe-PURO or pBabe-PURO/Twist. The level of *E-cadherin* mRNA in MCF7 cells is used as a positive control. Each bar represents the mean ± SEM of the PCR reactions in triplicate.

(B) The luciferase activity under the control of various human *E-cadherin* promoter constructs were measured in the HMEC cells expressing either pBabe-PURO, pBabe-PURO/Twist, or Snail. Ecad(-108)-Luc is the wild-type *E-cadherin* promoter construct; Ecad(−108)/EboxA/EboxB/EboxC.MUT-Luc is the *E-cadherin* promoter construct where consensus EboxA, EboxB, and EboxC were mutated; Ecad(−108)/EboxC.MUT-Luc is the *E-cadherin* promoter construct where consensus EboxC was mutated. Each bar represents the mean ± SEM of three experiments.

(C) Morphologies of the HMEC-Twist cells expressing either pBabe-Zeo or pBabe-Zeo/E-cadherin.

(D) Expression of exogenous E-cadherin was examined in the HMEC-Twist cells expressing either the control vector pBabe-Zeo or pBabe-Zeo/E-cadherin. The parental HMECs were used as a positive control for E-cadherin expression. Expression of epithelial protein α-catenin and mesenchymal proteins, fibronectin, and vimentin was also examined. β-actin is used as a loading control.

staining in the control cells (Figure 5B). We also observed complete loss of E-cadherin, α-catenin, and γ-catenin proteins by immunoblotting (Figure 5C). In contrast, the expression of fibroblast markers, including fibronectin, vimentin, smooth-muscle actin, and N-cadherin, whose expression has been shown to correlate positively with the EMT (Boyer and Thiery, 1993), was strongly induced (Figures 5B and 5C). Hence, both the morphological and molecular changes in the Twist-expressing MDCK cells demonstrated that these cells had undergone an EMT.

We wished to determine whether the ability of Twist to induce an EMT in MDCK cells could be extended to epithelial cells more relevant to breast cancers. Accordingly, we introduced Twist into immortalized human mammary epithelial cells (HMECs) (Elenbaas et al., 2001). We observed the identical set of changes associated with EMT in these cells (Figures 5A and 5C). Furthermore, both MDCK and HMEC cells expressing Twist, but not control cells, exhibited high levels of growth factor-induced directional migration (Figure 5D). Taken together, these data indicated that Twist is capable of inducing an EMT and associated migratory behavior in normal epithelial cells and suggested that Twist-induced EMT could contribute to the phenotypes of invasion and metastasis.

Loss of E-cadherin appears to be critical to an EMT. One major mechanism for inhibiting *E-cadherin* expression involves silencing of *E-cadherin* transcription through three E-boxes in its promoter (Thiery, 2002). We observed an over 100-fold reduction of *E-cadherin* mRNA level in HMEC cells expressing Twist (Figure 6A). To test whether this transcriptional repression is achieved through the three E-boxes in the *E-cadherin* promoter, we transiently transfected HMEC-Twist cells with a reporter construct containing the *luciferase* gene (*Luc*) under the control of the human *E-cadherin* promoter (Hajra et al., 2002). Indeed, Luc activity was efficiently suppressed in the HMEC-Twist cells compared to the HMEC-control cells. A similar degree of repression was also observed in the HMEC cells expressing Snail, a known repressor of E-cadherin expression (Figure 6B). When we introduced two *Luc* reporter constructs in which the E-box elements had been mutated (Hajra et al., 2002), the Luc activities were derepressed in the HMEC cells expressing Twist (Figure 6B). These data indicate that Twist directly or indirectly causes the transcriptional repression of E-cadherin through the E-box elements on the *E-cadherin* promoter.

To understand whether suppression of E-cadherin expression is the sole cause of the Twist-induced EMT, we examined whether ectopic expression of E-cadherin could revert the EMT phenotypes in HMEC-Twist cells. While the level of E-cadherin protein was restored to a similar level as seen in the parental HMEC cells, the HMEC-Twist cells expressing exogenous E-cadherin retained their spindle-like, fibroblastic morphology (Figure

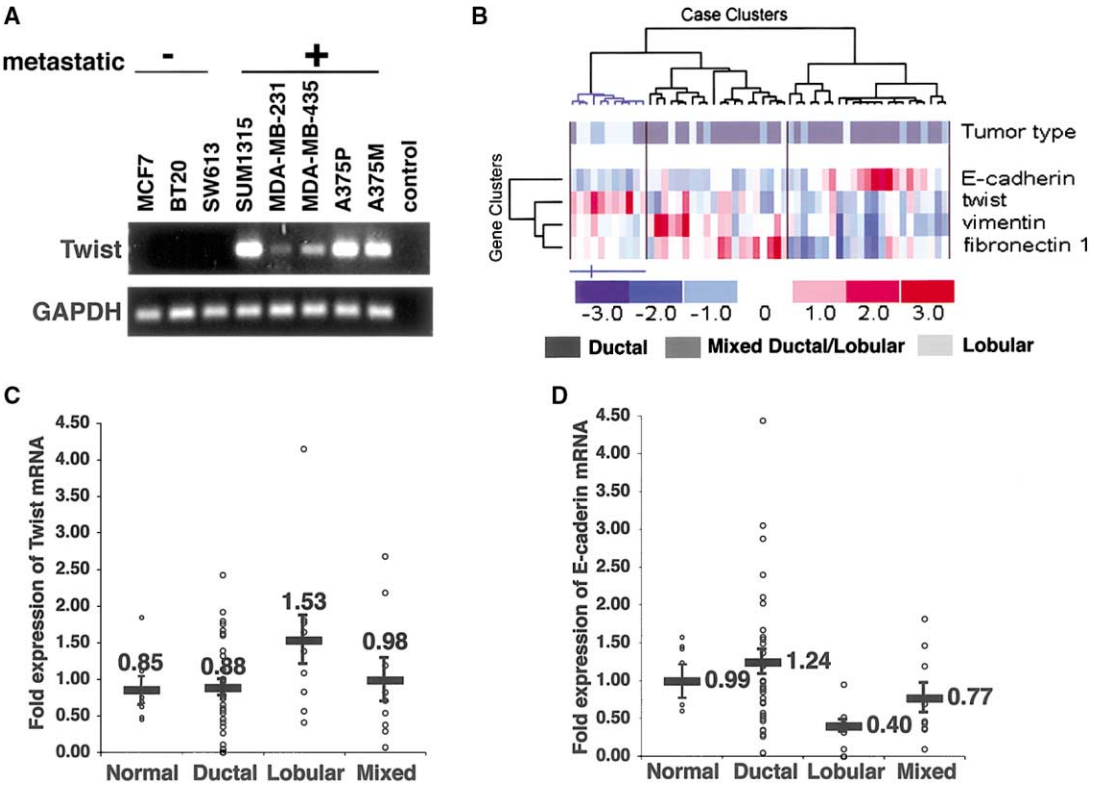

Figure 7. Expression of Twist in Human Tumor Cell Lines and in Human Breast Carcinomas

(A) Expression of *Twist* mRNA in a panel of human nonmetastatic or metastatic cell lines was examined by RT-PCR. The endogenous *GAPDH* mRNA level was measured as the internal control.

(B) Gene cluster analysis of 54 human invasive breast carcinoma samples using four genes: *Twist*, *E-cadherin*, *fibronectin*, and *vimentin*. For each gene, the average signal among all tumor samples is used as the baseline (1-fold).

(C) The relative levels of Twist expression in individual normal breast or tumor samples are plotted into three groups by their tumor types (open circle). Each bar represents the mean ± SEM of each group (solid strike). The difference of Twist expression between invasive ductal carcinoma and invasive lobular carcinoma has p-value = 0.021 based on two-tail Student's T-test.

(D) The relative levels of E-cadherin expression in individual normal breast or tumor samples were plotted into three groups by their tumor types (open circle). Each bar represents the mean ± SEM of each group (solid strike). The difference of E-cadherin expression between invasive ductal carcinoma and invasive lobular carcinoma has p-value = 0.008 based on two-tail Student's T-test.

6C). In addition, these cells continued to express fibroblastic markers, such as vimentin and fibronectin (Figure 6C). This result demonstrates that in addition to E-cadherin, Twist also modulates expression of additional important targets to promote an EMT.

Expression of Twist mRNA in Human Breast Tumor Samples

We wished to determine whether Twist expression was detectable in human breast tumors, in which it might play a role in the invasive or metastatic phenotypes. We observed that Twist was expressed in several invasive and metastatic human tumor cell lines, such as SUM1315, MDA-MB 231, and MDA-MB-435. In contrast, nonmetastatic tumor cell lines, such as MCF7 and BT20, did not express Twist (Figure 7A).

We then determined whether expression of Twist correlates with certain pathological phenotypes in clinical breast tumor samples. To do so, we analyzed a microarray gene expression data set from 57 invasive human breast tumors of three subtypes, specifically ductal carcinoma, mixed ductal/lobular type, and lobular carcinoma (Signoretti et al., 2002). The high Twist expression clustered around the group of invasive lobular carcinomas (ILC). About 70% invasive lobular carcinomas showed elevated Twist expression. In contrast, a far smaller proportion of invasive ductal carcinomas (32%) (IDC) and mixed ductal/lobular carcinomas (30%) had elevated Twist expression (Figure 7A). Moreover, most normal breast tissue samples did not show elevated Twist expression (Figure 7B), and cultured human mammary epithelial cells do not express any detectable level of Twist (Figure 5C).

In fact, invasive lobular carcinoma displays many features of cells that have undergone an EMT. It is defined histologically by its diffusely infiltrative growth, which constitutes dyshesive single tumor cells or single-file rows of cells. These characteristics are distinct from invasive ductal carcinomas, which normally maintain irregular mammary tubules and solid sheets of carcinoma cells. One unique molecular feature of invasive lobular carcinomas is their almost universal loss of E-cadherin expression, whereas only 50% of invasive ductal carci-

noma have reduced expression of E-cadherin (Acs et al., 2001; Gamallo et al., 1993; Moll et al., 1993). Indeed, 90% of invasive lobular carcinoma samples in our tumor set showed no expression of E-cadherin (Figure 7A). When the levels of Twist and E-cadherin expression in the ductal group and the lobular group were compared, we observed a statistically significant higher level of Twist transcript in the lobular group ($p = 0.021$) (Figure 7C). Moreover, we detected a significant loss of E-cadherin expression in the lobular group in comparison with the ductal group ($p = 0.008$) (Figure 7D). This inverse correlation between high Twist expression and low E-cadherin expression in human invasive lobular carcinoma is consistent with our finding that Twist can induce loss of E-cadherin-mediated cell-cell adhesion in epithelial cells. It also suggests that expression of Twist can contribute to the pathogenesis of human invasive lobular carcinomas.

Discussion

Activation of Twist in Tumor Metastasis

The present study utilized gene expression array analyses to identify genes involved in metastasis in a mouse mammary tumor model. Twist, a transcription factor important in embryonic development, was identified to be essential for the metastatic process. We have demonstrated that Twist induces loss of E-cadherin-mediated cell-cell adhesion and an EMT in epithelial cells. In human invasive lobular carcinomas, we also observed an inverse correlation between the expression levels of Twist and E-cadherin. Together, the present evidence leads us to propose that activation of Twist and Twist-induced EMT are important components of tumor metastasis.

The metastasis program is a complex series of biological steps, thus tumor cells must acquire a series of traits that enable them to overcome multiple barriers erected by the normal tissues that they encounter en route. Our results suggest that during the development of the metastatic phenotype, tumor cells activate latent morphogenetic programs that are normally active in early development in order to acquire the ability to execute multiple steps in the metastasis process. By exploiting these genes and their downstream pleiotropic regulatory program, cancer cells can concomitantly acquire many of the traits that are needed for them to invade and metastasize. A number of genes have been identified to participate in the Twist signaling pathway in *Drosophila* (Furlong et al., 2001). This suggests the possibility that not only Twist, but also other molecular components of the Twist-associated morphogenesis pathways, are good candidates to be examined for their possible involvements in tumor metastasis.

Biological Activities of Twist in EMT

The complex morphological changes observed during Twist-induced EMT raise the question of the identities of the transcriptional targets of Twist. Our observation that *E-cadherin* transcription is repressed during Twist-induced EMT is consistent with the hypothesis that loss of E-cadherin expression is a critical event of EMT. Interestingly, in *Drosophila* Twist can induce the expression of Snail, a known repressor of E-cadherin transcription (Ip et al., 1992). However, in spite of repeated attempts, we could not observe an induction of Snail in the human mammary epithelial cells that have undergone an EMT upon Twist expression (J.Y., A.S. Shieh, and R.A.W., unpublished data). This observation is consistent with the notion that Twist and Snail function independently in mice (Soo et al., 2002; Carver et al., 2001). It is also possible that Twist, acting as a bHLH transcription factor, binds directly to the E-boxes on the *E-cadherin* promoter to suppress its transcription.

In addition to silencing *E-cadherin* transcription, we have also found that Twist induces the expression of mesenchymal markers, such as fibronectin and N-cadherin, during an EMT. These events appear to be independent of E-cadherin expression, because we have found that ectopic expression of E-cadherin could not revert the EMT phenotype in Twist-expressing HMEC cells. Hence, Twist does not rely on E-cadherin repression to induce mesoderm-specific genes and may, as is the case during *Drosophila* gastrulation (Leptin, 1991), function as a potent transcriptional activator to induce such genes.

Involvement of Twist in Human Cancers

The experimental model that we used here, implantation of tumor cells in an orthotopic site in a syngeneic host, recapitulates many biological conditions in cancer patients. For example, the host tissue environment of primary tumors strongly affects their ability to metastasize and the target organ sites to which they spread (Gohji et al., 1997). Thus, we implanted mammary tumor cells in their orthotopic site—the mammary fat pad. The host immune system is also thought to play important roles in tumorigenesis and metastasis (Miller, 1993). In this study, the tumor cells proliferated in a wild-type BALB/c mouse, so we could examine their metastatic behaviors in an intact immune environment.

Interestingly, we have found a statistically significant correlation between expression of Twist and invasive lobular carcinoma. This observation is also supported by an independent study of promoter methylation of *Twist* gene in human breast cancers (Fackler et al., 2003). This study showed that the promoter of *Twist* gene was much less frequently methylated in invasive lobular carcinomas as compared to invasive ductal carcinomas, indicating that Twist expression is preferentially suppressed in ductal carcinomas.

In lobular carcinoma, an important molecular mechanism determining its histologic phenotype is thought to be loss of E-cadherin-mediated cell-cell adhesions. Several means of silencing *E-cadherin* gene expression in lobular carcinoma have been observed, including *E-cadherin* gene truncation mutations, loss of heterozygosity (LOH) of the *E-cadherin* locus, and methylation of the *E-cadherin* promoter (Berx et al., 1996; Huiping et al., 1999; Droufakou et al., 2001). However, these mechanisms seem to account for only a portion of the E-cadherin-negative invasive lobular carcinomas. Our

observation that Twist expression is associated with invasive lobular carcinoma provides the first indication that repression of the *E-cadherin* promoter by such transcription factors plays a role in pathogenesis of these tumors.

The involvement of Twist in infiltrative sub-types of carcinomas has also been observed in gastric cancers. Twist was found to be significantly upregulated in the diffuse-type gastric carcinomas when compared to the intestinal-type of gastric carcinomas (Rosivatz et al., 2002). Provocatively, the diffuse-type gastric carcinoma is also characterized pathologically by its infiltrative growth and is associated with loss of E-cadherin. Together with our results, these studies further suggest that Twist is likely to repress E-cadherin expression and promote infiltrative tumor growth in various human carcinomas. Moreover, they suggest that many such EMT-inducing transcription factors may be exploited opportunistically by different types of human carcinomas in order to acquire invasive and metastatic powers.

Experimental Procedures

Cell Cultures

67NR, 168FARN, 4TO7, and 4T1 cell lines were provided by Dr. Fred R. Miller at Wayne State University and cultured as described (Aslakson and Miller, 1992). The human mammary epithelial cell line was described previously (Elenbaas et al., 2001). To measure cell growth rates, cells were seeded at 5×10^4 per 100-mm dish. For each cell line, cells from three dishes were trypsinized and counted daily.

Plasmids

The mouse *Twist* cDNA was provided by Dr. Fabienne Perrin-Schmitt at Universite Louis Pasteur and subcloned into the pBabe-Puro vector. The siRNA-expressing U6 lentivirus system was provided by Dr. Richard Iggo at Swiss Institute for Experimental Cancer Research (Bridge et al., 2003). Three siRNA-coding oligos against mouse *Twist* were designed and verified to be specific to *Twist* by Blast search against the mouse genome. *Twist*-siRNA3-targetting sequence is AAGCTGAGCAAGATTCAGACC; *Twist*-siRNA5-targetting sequence is AGGTACATCGACTTCCTGTAC; and *Twist*-siRNA7-targetting sequence is AGCGGGTCATGGCTAACGTGC. The U6 promoter with *Twist*-siRNA3, *Twist*-siRNA5, or *Twist*-siRNA7 insert were subcloned into pSP108-PURO. A control siRNA oligo, which does not match any known mouse coding cDNA, was used as control.

Luciferase reporter gene constructs containing the *E-cadherin* promoter sequence and various E-box mutant forms (EboxC.MUT, EboxA+B+C.MUT) were provided by Dr. Eric Fearon at University of Michigan (Hajra et al., 2002).

The mouse *E-cadherin* cDNA was provided by Dr. Gerhard Christofori at University of Basel and subcloned into the pBabe-Zeo vector.

Viral Production and Infection of Target Cells

The production of lentiviruses and pBabe-amphotropic viruses, and infection of target cells were described previously (Stewart et al., 2003). The 4T1 cells were selected with 12 μg/ml puromycin. The HMEC cells were selected with 2 μg/ml puromycin, or 200 μg/ml Zeocin. The MDCK cells were selected with 4 μg/ml puormycin.

Mice and Mammary Gland Injection

Female BALB/cJ mice, 3–5 months, from Jackson Laboratory were used for surgery. Mice were anesthetized with 2,2,2-tribromoethanol. The skin was incised and tumor cells (5×10^5) in a 20 μl DME-10 were injected into the mammary gland.

Measurement of Primary Tumor Growth and Isolation of Tumors Cells from the Mammary Gland, Blood, and Lung

Post tumor cell injection, groups of 5–10 mice were sacrificed weekly. The mammary tumors were removed and weighed individually. Mice were bled from retroorbital sinus, and 150 μl blood samples are cultured in the presence of puromycin. The tumor colonies were counted three weeks later. Tumors or lungs were minced and digested in DME-10 with 1 mg/ml collagenase A for 2 hr at 37°C. The tissue mixtures were dispersed with an 18-gauge needle, washed, and plated in DME-10 media plus puromycin.

Microarray Hybridization, Data Collection, and Analysis

10 μg of total RNA from each tumor were used for microarray probe synthesis and hybridization as described in Affymetrix GeneChip manual. For each cell line, four tumors grown individually in four mice were hybridized to mouse U74A chips. The data collection was carried out using the Affymetrix GeneChip 3.1. Scaling was performed on the expression data set to minimize global differences in mean intensities among individual chips. Expression values below baseline (20) were set to 20. To find genes associated with individual metastatic phenotypes, we compared the gene expression profiles between each two sets of tumors. For each gene, three statistical analysis criteria were used to evaluate whether the expression difference is significant. (1) The difference between two mean expression values had to be >2-fold over their combined standard deviation; (2) The fold difference between two mean expression values had to be >2.5-fold; (3) The higher mean expression value between two values had to be >300 because most of the values below 300 were given ABSENT calls by the Affymetrix GeneChip program. Then we further grouped the resulting differentially expressed genes into the three Groups X, Y, and Z.

The gene cluster analysis of human invasive breast tumor samples was performed using dChip software as previously described (Signoretti et al., 2002).

SYBR-Green Real-Time RT-PCR

Mouse *Twist* RT-PCR forward primer is CGGGTCATGGCTAACGTG, and its RT-PCR reverse primer is CAGCTTGCCATCTTGGAGTC. Human *Twist* RT-PCR forward primer is GGAGTCCGCAGTCTTACGAG, and its RT-PCR reverse primer is TCTGGAGGACCTGGTAGAGG. Mouse and human *GAPDH* RT-PCR forward primer is GACCCCTT CATTGACCTCAAC, and its RT-PCR reverse primer is CTTCTCCAT GGTGGTGAAGA.

Total RNA was extracted using RNeasy Mini kit coupled with RNase-free DNase set (Qiagen) and reverse transcribed with Hexanucleotide Mix (Roche). The resulting cDNAs were used for PCR using SYBR-Green Master PCR mix (Applied Biosystem) in triplicates. PCR and data collection were performed on iCycler (BioRad). All quantitations were normalized to an endogenous control GAPDH. The relative quantitation value for each target gene compared to the calibrator for that target is expressed as $2^{-(Ct-Cc)}$ (Ct and Cc are the mean threshold cycle differences after normalizing to GAPDH). The relative expression levels of samples are presented by a semi-log plot.

Antibodies, Immunoblotting, Immunofluorescence, and Histology

Monoclonal Twist antibody was originally report by Gitelman (1997). The other antibodies utilized were β-actin (Abcam), E-cadherin, α-catenin, β-catenin, γ-catenin, Fibronectin and N-cadherin (BD Transduction), vimentin V9 (NeoMarkers), and smooth muscle actin (Sigma). All procedures were performed as described (Elenbaas et al., 2001).

Assay of Anchorage-Independent Growth

The soft agar assay was performed as described (Elenbaas et al., 2001).

Migration Assay

This assay was performed as described (Clark et al., 2000).

Luciferase Reporter Assay

Cells of 50% confluence in 12-well dishes were transfected using Fugene6 (Roche). 0.2 μg *E-cadherin* reporter gene construct and

0.2 ng of pRL-SV40 (Promega) were cotransfected per well. Cell extracts were prepared 40 hr after transfection, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). All experiments were performed in triplicates.

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