The Steroid Receptor Coactivator-1 Regulates Twist Expression and Promotes Breast Cancer Metastasis

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Abstract

In breast cancer, steroid receptor coactivator-1 (SRC-1) expression positively correlates with HER2 expression and poor prognosis. In mouse mammary tumor virus–polyoma middle T (PyMT) breast cancer mouse model, SRC-1 strongly promotes mammary tumor metastasis. However, the molecular targets and mechanisms that mediate the role of SRC-1 in metastasis are unknown. In this study, SRC-1 wild-type (WT) and knockout (KO) cell lines were developed from the mammary tumors of WT/PyMT and KO/PyMT mice. WT cells exhibited strong migration and invasion capabilities, reduced E-cadherin and β-catenin epithelial markers, gained N-cadherin and vimentin mesenchymal markers, and formed undifferentiated invasive structures in three-dimensional culture. In contrast, KO cells showed slow migration and invasion, retained E-cadherin, had less N-cadherin and vimentin, and developed partially differentiated three-dimensional structures. Importantly, WT cells expressed Twist, a master regulator of metastasis, at significantly higher levels versus KO cells. SRC-1 knockdown in WT cells reduced Twist expression, whereas SRC-1 restoration in KO cells also rescued Twist expression. Furthermore, SRC-1 was found to coactivate Twist transcription through physical interaction with the transcription factor PEA3 at the proximal Twist promoter. Accordingly, Twist knockdown in WT cells increased E-cadherin and reduced cell invasion and metastasis, and Twist expression in KO cells decreased E-cadherin and increased cell invasion. SRC-1 knockdown in human breast cancer cells also decreased Twist, cell migration, and invasion. Therefore, SRC-1 promotes breast cancer invasiveness and metastasis by coactivating PEA3-mediated Twist expression. Intervention of SRC-1 function may provide new strategies to inhibit breast cancer metastasis. [Cancer Res 2009;69(9):3819–27]

Introduction

Breast cancer mortality is largely attributed to metastasis. Despite its clinical importance, the molecular mechanisms of metastasis remain poorly understood partially due to lack of appropriate experimental models and difficulties of identifying metastasis-specific regulators and mediators (1). Cancer metastasis consists of several steps: epithelial-mesenchymal transition (EMT), local invasion, intravasation, transport in the circulation, extravasation, survival and proliferation in a secondary organ site, and formation of overt metastatic lesions (1, 2). Conceivably, each of these steps may be promoted by a specific group of factors. During EMT, for example, breast tumor cells express Twist, Snail, Slug, and/or SIP1, which mediate the transition through repression of epithelial markers, such as E-cadherin and β-catenin, and induction of mesenchymal markers, such as N-cadherin, fibronectin, and vimentin (3, 4). In addition to the above transcription factors that control EMT, many other proteins are also associated with breast cancer metastasis. These proteins include certain oncoproteins, such as ras, HER2, epidermal growth factor receptor, and c-Myc, motility factors, such as hepatocyte growth factor, extracellular matrix (ECM) degradative enzymes, such as urokinase-type plasminogen activator and matrix metalloproteinases (MMP), and posttranslational modification enzymes for protein glycosylation, such as Mgat5 (5–7). Proteins that enhance metastasis also include S100A4, Mta-1, Muc1, and osteopontin (6). However, none of these proteins functions exclusively in metastasis, as inactivation of each also inhibits tumor growth.

Steroid receptor coactivator-1 (SRC-1) is a member of the SRC family that also contains SRC-2 (TIF2 or GRIP1) and SRC-3 (AIB1 or ACTR; refs. 8). These coactivators promote transcription by interacting with nuclear receptors, such as estrogen and progesterone receptors, as well as other transcription factors, such as activator protein-1, nuclear factor-κB (NF-κB), Ets-2, and PEA3 (9–15). Studies using mutant mice show SRC family members to have both unique and partially redundant functions in development, growth, steroid response, metabolism, reproduction, vasoprotection, and inflammation (8, 16–21). In human mammary epithelial cells, SRC-1 is very low (14). However, SRC-1 in HER2-positive breast cancers is increased and correlated with disease recurrence and resistance to endocrine therapy (14, 22). SRC-1 expression inversely correlates with estrogen receptor β (ERβ) expression, a marker for better prognosis in breast cancer (23). In addition, SRC-1 interacts with Ets-2 to enhance c-Myc expression in endocrine-resistant breast cancer cells (11, 12) and with ERα to promote stromal cell–derived factor-1 expression, facilitating cell proliferation and invasion (24).

Unlike other oncogenes, SRC-1 functions specifically to promote metastasis (25). During tumorigenesis in mouse mammary tumor virus–polyoma middle T (PyMT) mice, SRC-1 is up-regulated. Inactivation of SRC-1 in these mice does not alter mammary tumor initiation and growth but drastically suppresses mammary tumor cell intravasation and lung metastasis. Although SRC-1 enhances HER2 expression, Akt activity, colony-stimulating factor-1 expression, and macrophage recruitment to the tumor site, the molecular mechanisms for SRC-1 to promote breast cancer metastasis are largely unknown (25).

In this study, we have developed cell lines from mammary tumors of wild-type (WT)/PyMT and SRC-$1^{-/-}$/PyMT mice and used these cell lines to investigate the molecular mechanisms by

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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which SRC-1 promotes metastasis. We find that SRC-1 promotes EMT, mammary cancer cell migration, and invasion by enhancing PEA3-mediated transcriptional activation of Twist (3).

Materials and Methods

For detailed description of Materials and Methods, please refer to Supplementary Data. Briefly, mammary tumor cell lines were developed from primary tumors in WT/PyMT and SRC-1$^{-/-}$/PyMT mice as described (15). Cell motility and invasion were assayed as described (15). Small interfering RNA (siRNA)–mediated knockdown and adenovirus-mediated expression of SRC-1 were done as described (15, 26). Twist was stably knocked down and expressed in mammary tumor cells using short hairpin RNA (shRNA) lentiviruses and the pQCXIH retroviral system, respectively. Three-dimensional culture, Western blot, real-time quantitative reverse transcription-PCR (qRT-PCR), cell transfection and luciferase assays, coimmunoprecipitation, and chromatin immunoprecipitation (ChIP) assays were also done as previously described (15, 27, 28).

Results

SRC-1 enhances mammary tumor cell migration and invasion. To investigate the role of SRC-1 in regulation of cell behaviors and molecular pathways relevant to breast cancer metastasis, we developed WT and SRC-1 knockout (KO) mammary tumor cell lines. Two independent WT cell lines, WT1 and WT2, were derived from mammary tumors of WT/PyMT mice, and two independent KO cell lines, KO1 and KO2, were derived from mammary tumors of SRC-1$^{-/-}$/PyMT mice. After culturing under selective conditions for epithelial cell growth, these established cell lines were positive to K8 immunostaining (Supplementary Fig. S1) and thus to be of mammary epithelial origin. Because metastatic potential correlates with the ability of cancer cells to migrate out of tumor mass and invade tissues, we subjected cell lines to migration and invasion assays. We traced the migration of individual cells on culture plates by precoated fluorescence beads. Within 17 hours, most WT1 and WT2 cells had migrated extensively and left much broader and longer trails than SRC-1–deficient KO1 and KO2 cells did (Fig. 1*A, a*). The average migration area swept by individual WT1 or WT2 cells was significantly larger compared with that swept by individual KO1 or KO2 cells (Fig. 1*A, b*). We also cultured WT and KO cells in chambers containing Matrigel barrier that simulates ECM and measured their invasion capabilities. We found that 17% of WT1 and 38% of WT2 cells invaded through the Matrigel barrier versus only 10% of KO1 and 5% of KO2 cells (Fig. 1*A, c*). The different invasion capabilities between WT1 and WT2 cells and between KO1 and KO2 cells might reflect variable genetic and epigenetic changes in different tumors. Overall, these results suggest that SRC-1 deficiency reduces migration and invasion capabilities of the mammary tumor cells.

To confirm the role of SRC-1 in promoting cell migration and invasion in WT1 and WT2 cells, we knocked down SRC-1 by transfecting these cells with SRC-1 siRNA. Scrambled siRNA served as a control (Fig. 1*B, a*). On SRC-1 knockdown, both WT1 and WT2 cell lines showed >50% reduction in their migration velocity and invasion capability (Fig. 1*B, b* and *c*). We also expressed SRC-1 in KO1 and KO2 cells by adenoviral infection to evaluate the role of SRC-1 in cell migration and invasion (Fig. 1*C, a*). SRC-1 restoration

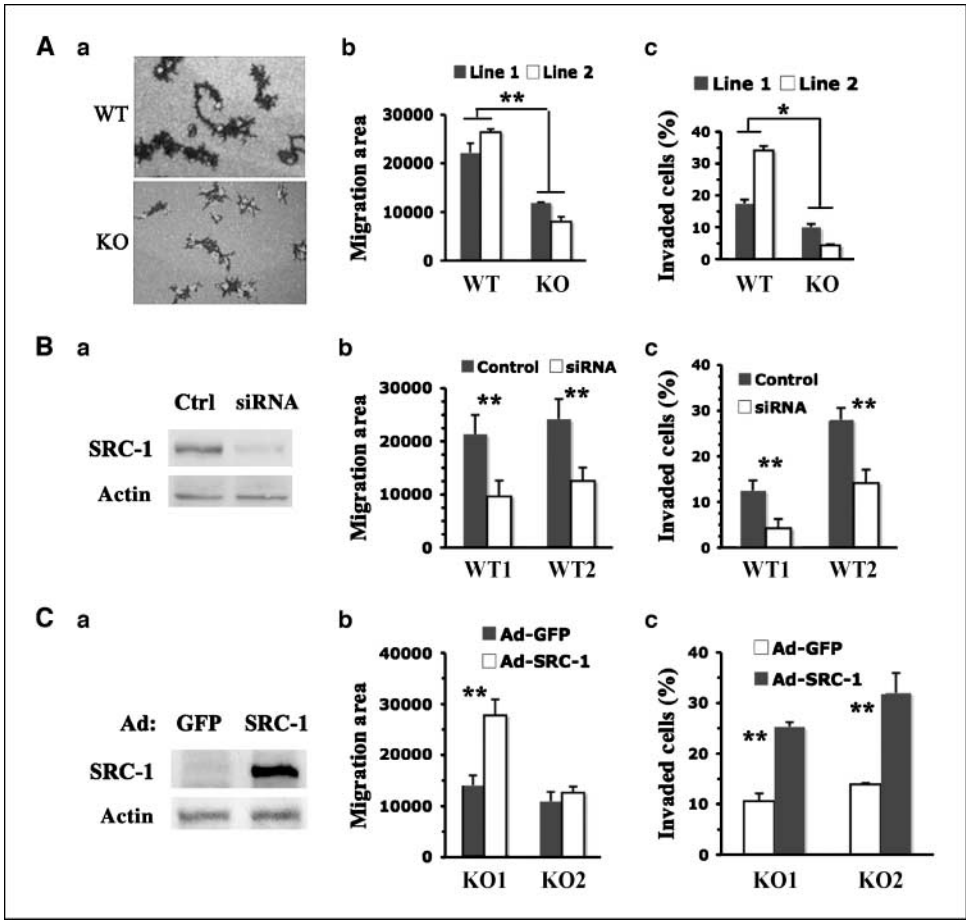

Figure 1. SRC-1 promotes mammary tumor cell migration and invasion. *A, a,* the trails of WT and SRC-1 KO cell migration were traced using fluorescence beads. The migration areas of at least 40 cells were individually measured by pixels. *b,* average migration areas per cell for WT1, WT2, KO1, and KO2 cell lines. *c,* data as percentages of invaded cells to total cells. *B, a,* immunoblotting for SRC-1 and β-actin (loading control) in WT1 cells transfected with either scrambled double-strand RNA (*Ctrl*) or SRC-1 siRNA. *b* and *c,* cell migration and invasion data for WT1 and WT2 cells treated with control or SRC-1 siRNA. *C,* immunoblotting confirmed SRC-1 expression in KO1 cells infected with SRC-1 adenoviruses. *a,* KO1 cells infected with GFP adenoviruses served as a control. *b* and *c,* cell migration and invasion data. *, $P < 0.05$; **, $P < 0.01$, unpaired *t* test.

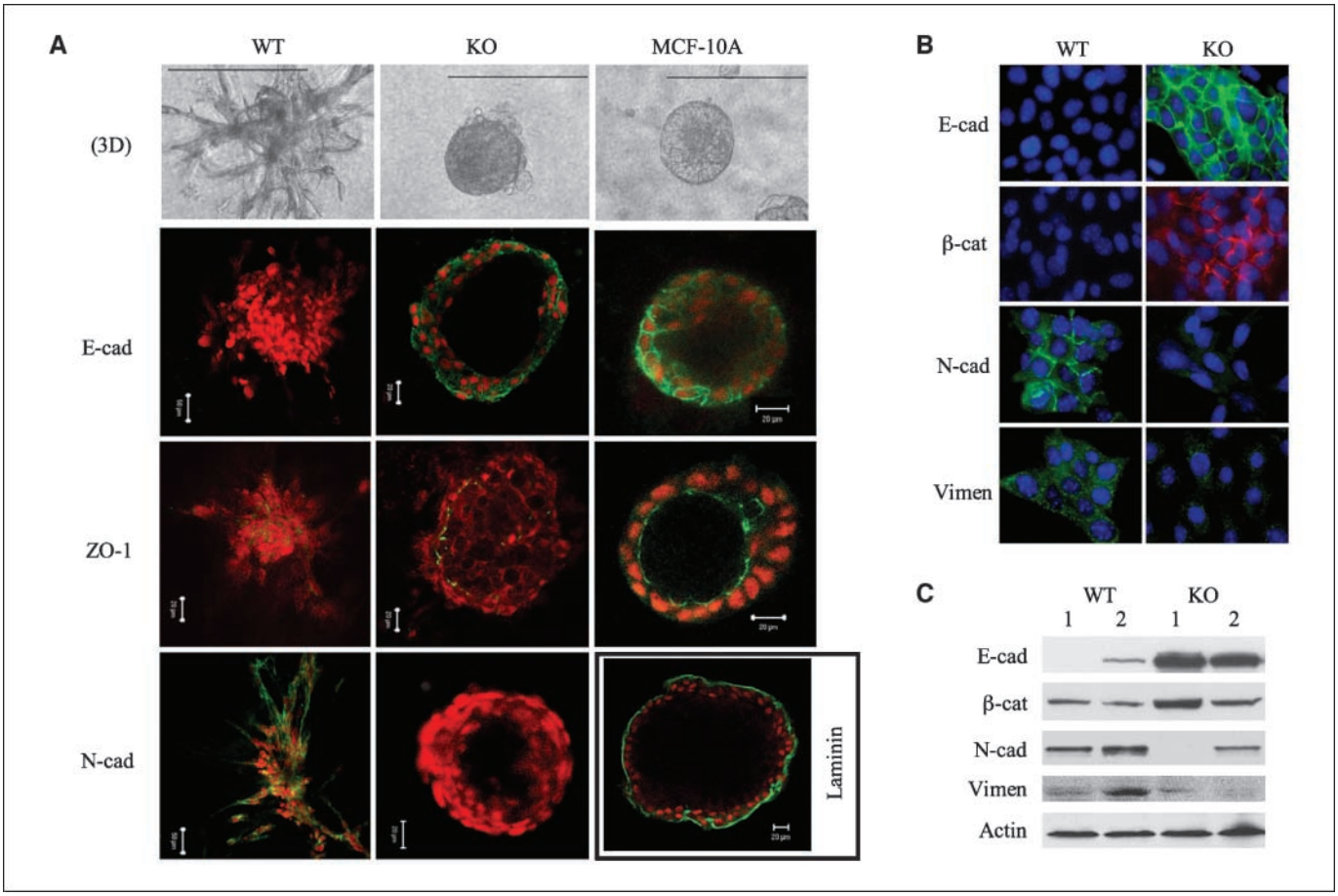

Figure 2. SRC-1 deficiency helps to retain epithelial differentiation of mammary tumor cells. *A,* three-dimensional (*3D*) structures formed from WT1, KO1, and MCF-10A cells. Immunofluorescence staining was done for E-cadherin (*E-cad*), ZO-1, N-cadherin (*N-cad*), and laminin. N-cadherin was not detected in the three-dimensional structures of MCF-10A cells (data not shown). Laminin was not detected in the three-dimensional structure of WT and KO mammary tumor cells (data not shown). *B,* immunofluorescence staining for E-cadherin, β-catenin (*β-cat*), N-cadherin, and vimentin (*Vimen*) in WT and KO mammary tumor cells cultured as monolayer. *C,* immunoblotting analyses of E-cadherin, β-catenin, N-cadherin, and vimentin in WT1, WT2, KO1, and KO2 mammary tumor cells. β-Actin served as a loading control.

in KO1 cells resulted in an increase in both cell migration and invasion versus green fluorescent protein (GFP) control. SRC-1 restoration in KO2 cells caused a similar increase in cell invasion but did not increase cell migration (Fig. 1*C, b* and *c*). Taken together, these results show that SRC-1 plays an important role in promotion of mammary tumor cell migration and invasion.

SRC-1 reduces mammary tumor cell differentiation and promotes EMT. To evaluate the degree of epithelial differentiation of mammary tumor cells with and without SRC-1, we did three-dimensional culture and examined the expression and distribution patterns of E-cadherin, ZO-1, laminin, and N-cadherin. In the three-dimensional culture system, the MCF-10A human mammary epithelial cells formed highly differentiated hollow spheres consisting of a single layer of polarized epithelial cells (Fig. 2*A*). E-cadherin was highly expressed and localized primarily at the lateral cell membrane for cell-cell adhesion. The tight junction protein ZO-1 was located at the apilateral membrane of polarized epithelial cells, and the basement membrane protein laminin was located around the sphere (Fig. 2*A*). N-cadherin, a marker of mesenchymal cells, was undetectable in MCF-10A cells (data not shown). In contrast, WT1 and WT2 cells were unable to develop hallow spheres and they only formed an undifferentiated dendritic architecture. In these invasive cellular architectures, E-cadherin, ZO-1, and laminin were undetectable but N-cadherin was detected, suggesting that WT1 and WT2 cells lost epithelial differentiation and polarity and gained certain mesenchymal features (Fig. 2*A*; data not shown). Interestingly, cultured KO1 and KO2 cells formed three-dimensional structures with a partially differentiated phenotype. These cells formed mammary epithelial spheres, although the shells of some spheres consisted of multiple layers of cells. E-cadherin was detected between most cells and ZO-1 was detected in ~50% of cells in the outer cell layer of the spheres (Fig. 2*A*; data not shown). Laminin and N-cadherin were undetectable. Overall, these results show that the SRC-1–deficient KO1 and KO2 mammary tumor cells were more differentiated than SRC-1–positive WT1 and WT2 cells but less differentiated than normal MCF-10A cells.

In WT1 and WT2 cells cultured as monolayer, immunofluorescent labeling did not detect epithelial markers E-cadherin and β-catenin but instead detected mesenchymal markers N-cadherin and vimentin. In contrast, parallel assays in KO1 and KO2 cells detected E-cadherin and β-catenin but not N-cadherin and vimentin (Fig. 2*B*; data not shown). Western blot analyses confirmed that E-cadherin and β-catenin levels were significantly higher in KO1 and KO2 cells versus in WT1 and WT2 cells, whereas N-cadherin and vimentin levels were higher in WT1 and WT2 cells

versus in KO1 and KO2 cells (Fig. 2*C*). These results suggest that SRC-1 plays an important role in promoting EMT of mammary tumor cells.

SRC-1 partially inhibits E-cadherin expression. Because loss of E-cadherin is an essential event in EMT during breast cancer progression (4), we focused our attention on addressing how SRC-1 regulates E-cadherin expression. E-cadherin mRNA was detected at high levels in KO1 and KO2 cells but at very low levels in WT1 and WT2 cells (Fig. 3*A*), which was consistent with the results from immunochemical analyses in Fig. 2. Southern blot analysis revealed that the genomic DNA of *E-cadherin* gene was present in both WT and KO cell lines, indicating that the loss of E-cadherin mRNA expression in WT cells was not due to a loss of the E-cadherin gene (Supplementary Fig. S2). To confirm the effect of SRC-1 on E-cadherin expression, we knocked down SRC-1 in WT1 and WT2 cells using siRNA, restored SRC-1 in KO1 and KO2 cells by adenovirus-mediated expression, and examined E-cadherin expression. E-cadherin protein and mRNA expression showed no change in WT cells treated with a short scrambled double-strand RNA as a control. However, when WT cells were treated with SRC-1 siRNA, E-cadherin protein and mRNA expression were induced in these cells (Fig. 3*B*). On the other hand, when KO cells were infected with SRC-1–expressing adenoviruses, E-cadherin protein became undetectable by immunostaining only in some cells with high SRC-1 expression; E-cadherin mRNA levels assayed by qRT-PCR were significantly reduced; and E-cadherin protein levels assayed by immunoblotting were partially reduced (Fig. 3*C*). Although it was unclear why short-term SRC-1 expression had a more prominent effect on E-cadherin mRNA than protein, these results show that SRC-1 levels inversely correlate with E-cadherin levels in these mammary tumor cells.

SRC-1 potentiates PEA3-mediated Twist expression. Several transcription factors, including Snail, SIP1, and Twist, can suppress E-cadherin promoter and promote EMT and cancer metastasis (3, 29). Because SRC-1, as a transcriptional coactivator, might enhance EMT and promote metastasis through influencing the expression levels of these master regulators, we measured the mRNA levels of Snail, SIP1, and Twist in WT and KO mammary tumor cells. Snail mRNA levels in KO1 and KO2 cells were similar, whereas it was lower in WT1 cells but slightly higher in WT2 cells. There was no statistical difference in average expression levels of Snail mRNA in WT and KO cells (Supplementary Fig. S3*A*). SIP1 mRNA levels were also similar in all four cell lines (Supplementary Fig. S3*B*). Twist mRNA levels, however, were markedly higher in WT1 and WT2 cells than in KO1 and KO2 cells (Fig. 4*A, a*). To confirm the role of SRC-1 in Twist expression, we also measured Twist mRNA levels in WT cells treated with SRC-1 siRNA and KO cells infected with SRC-1 expression adenovirus. Our measurements revealed that SRC-1 knockdown in WT cells decreased Twist expression >50%, whereas SRC-1 restoration increased Twist expression >5-fold in KO1 cells and 2-fold in KO2 cells (Fig. 4*A, b* and *c*). These results indicate that SRC-1 plays an important role in up-regulating Twist expression in mammary tumor cells.

To understand how SRC-1 regulates Twist promoter activity, we did transient transfection assays using a Twist promoter-driven luciferase reporter (Twist-Luc). The reporter showed much higher

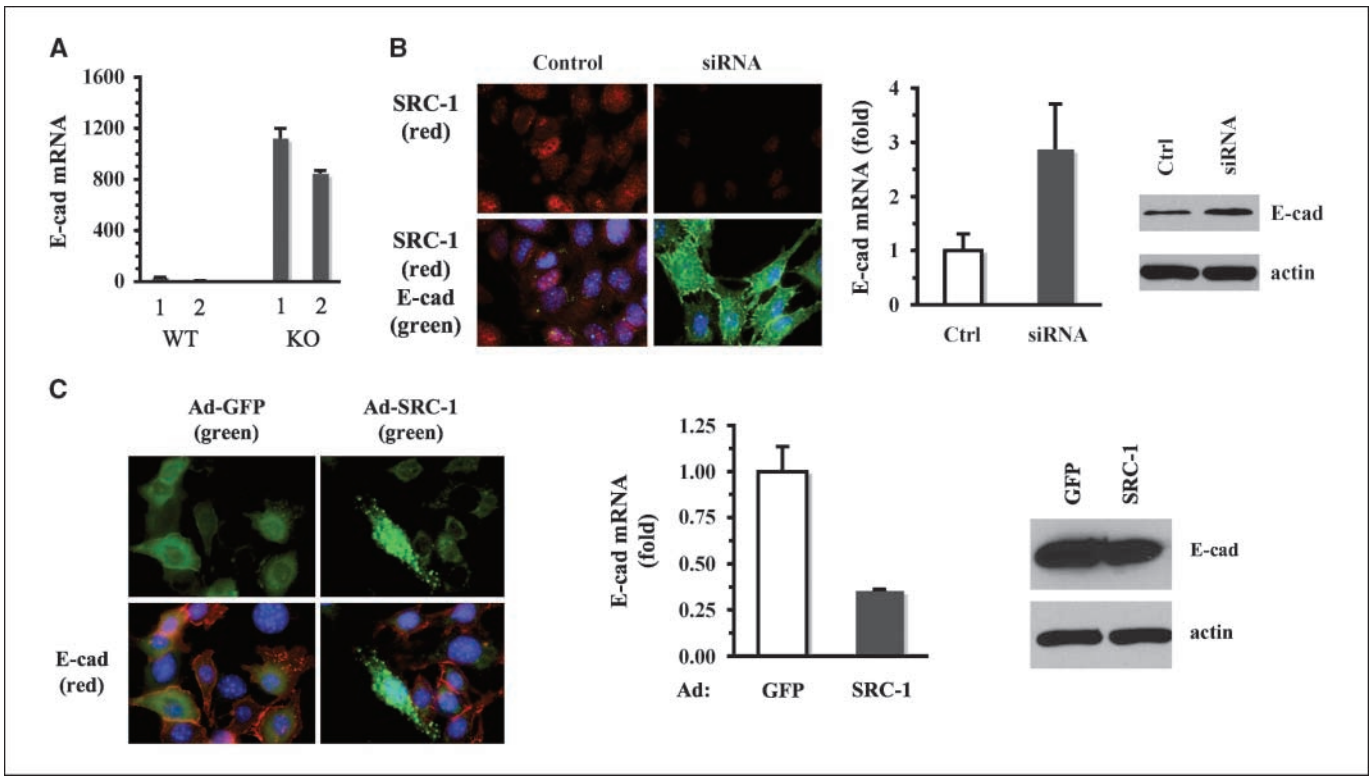

Figure 3. SRC-1 inhibits E-cadherin expression. *A,* qRT-PCR analysis of E-cadherin mRNA in WT1, WT2, KO1, and KO2 cells. The relative E-cadherin mRNA levels were normalized to the 18S RNA. *B,* SRC-1 knockdown and immunofluorescence staining of SRC-1 and E-cadherin in WT1 cells transfected with control or SRC-1 siRNA. The E-cadherin mRNA and protein levels were measured by qRT-PCR and immunoblotting, respectively. *C,* SRC-1 expression and immunostaining of SRC-1 and E-cadherin in KO1 cells with adenovirus-mediated GFP or SRC-1 expression. GFP signal and E-cadherin immunoreactive signal were imaged by fluorescence microscopy. The E-cadherin mRNA and protein levels in these cells were measured by qRT-PCR and immunoblotting, respectively.

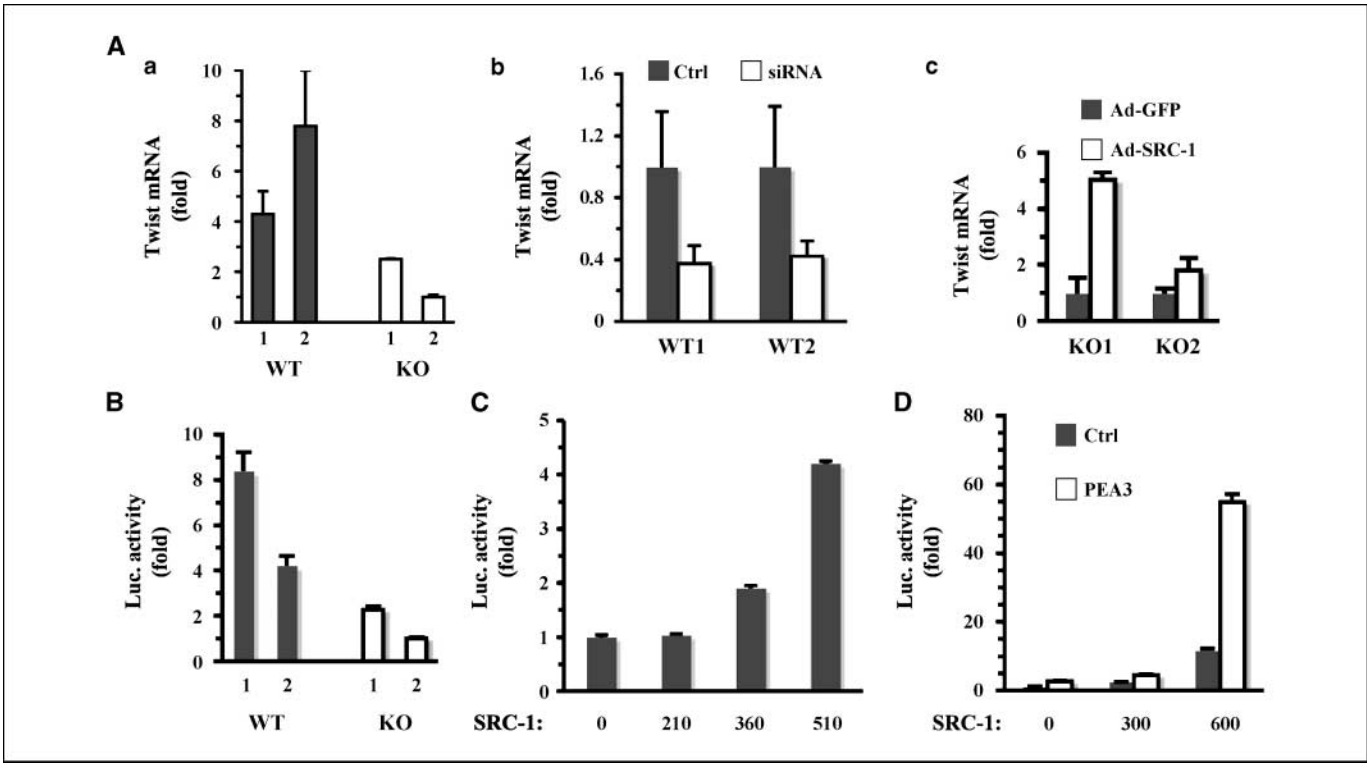

Figure 4. SRC-1 enhances Twist expression. *A,* qRT-PCR analyses of relative Twist mRNA levels in the following cells: WT1, WT2, KO1, and KO2 cells (*a*); WT1 and WT2 cells transfected with control or SRC-1 siRNA (*b*); and KO1 and KO2 cells with adenovirus-mediated GFP or SRC-1 expression (*c*). *B,* transfection assays of Twist-Luc promoter/reporter construct in WT1, WT2, KO1, and KO2 cells. *C,* HeLa cells were cotransfected with Twist-Luc and different amounts (ng) of SRC-1 expression plasmids as indicated. *D,* HeLa cells were transfected with Twist-Luc plasmids, with variable amounts of SRC-1 expression plasmids as indicated, and with PEA3 expression plasmids or its parent vector. In all of the above transfection assays, the reporter luciferase activities were normalized to β-galactosidase activity from a cotransfected expression vector.

activities in WT1 and WT2 cells compared with KO1 and KO2 cells (Fig. 4*B*). Furthermore, cotransfection of SRC-1 and Twist-Luc into HeLa cells activated Twist promoter in a SRC-1 dose-dependent manner (Fig. 4*C*). These results indicate that SRC-1 is required for Twist promoter activation.

The 2.8-kb Twist promoter region used in the Twist-Luc reporter contains binding elements for NF-κB, c-Jun, T-cell factor (TCF), hypoxia-inducible factor-1α (HIF-1α), E2F1, and PEA3, and some of these transcription factors have been shown to regulate Twist promoter (27, 30). To search for transcription factor candidates working with SRC-1, we cotransfected HeLa cells with Twist-Luc reporter, one of the above transcription factors, and SRC-1 expression plasmids. Our assays showed that expression of NF-κB, c-Jun, TCF, or PEA3 could slightly enhance Twist promoter activity, whereas expression of E2F1 or HIF-1α had no effect or an inhibitory effect on the Twist promoter (Fig. 4*D*; Supplementary Fig. S4). Coexpression of SRC-1 with NF-κB, c-Jun, TCF, HIF-1α, or E2F1 did not further enhance Twist promoter activity (Supplementary Fig. S4), suggesting that SRC-1 is not required for these transcription factors to activate Twist promoter. On the contrary, coexpression of SRC-1 with PEA3 synergistically activated Twist promoter activity, suggesting that SRC-1 may serve as a coactivator for PEA3 to potentiate Twist promoter activity (Fig. 4*D*). In addition, coexpression of SRC-1 with other Ets family members ERM and ER81 showed no increase in Twist promoter activity. Thus, the role of SRC-1 in promoting PEA3-mediated Twist promoter activation is quite specific.

SRC-1 is associated with PEA3 and Twist promoter for transcriptional activation. We also investigated the molecular mechanisms responsible for SRC-1 and PEA3 to regulate Twist promoter. From the total cell extract prepared from the metastatic MDA-MB-231 breast cancer cells, SRC-1 was specifically coimmunoprecipitated with PEA3 (Fig. 5*A*), which was consistent with a previous study showing physical interactions of SRC family members with Ets family transcription factors (13). Next, we used ChIP assays to address whether PEA3 and SRC-1 were recruited to Twist promoter. The 2.8-kb Twist promoter region contains 11 potential PEA3-binding sites. Five primer pairs at locations depicted in Fig. 5*B* were designed to amplify DNA fragments termed *a* to *e* that cover Twist promoter. Regions *a, c, d*, and *e* contained one or multiple PEA3-binding sites, and region *b* without PEA3-binding site served as a control (Fig. 5*B*). ChIP assays, done in MDA-MB-231 cells, revealed that both PEA3 and SRC-1 specifically associated with regions *a* and *e*, but not with regions *b, c*, and *d* (Fig. 5*C*). These results suggest that both PEA3 and SRC-1 are recruited to regions *a* and *e* of Twist promoter for transcriptional activation.

To determine whether region *a*, region *e*, or both regions were responsible for PEA3-mediated and SRC-1–mediated Twist promoter activation, we generated three luciferase reporter constructs with deletions of region *a* (termed Mut-a-Luc), region *e* (termed Mut-e-Luc), or both regions *a* and *e* (termed Mut-a&e-Luc; Fig. 5*B*) and did transfection assays with SRC-1 expression vector, PEA3 expression vector, or both SRC-1 and PEA3 expression vectors. Deletion of region *a* slightly reduced the Twist promoter activity only when PEA3 and SRC-1 were coexpressed. However, deletion of region *e* or of both regions *a* and *e* abolished PEA3/SRC-1–mediated Twist promoter activity (Fig. 5*D*). These results

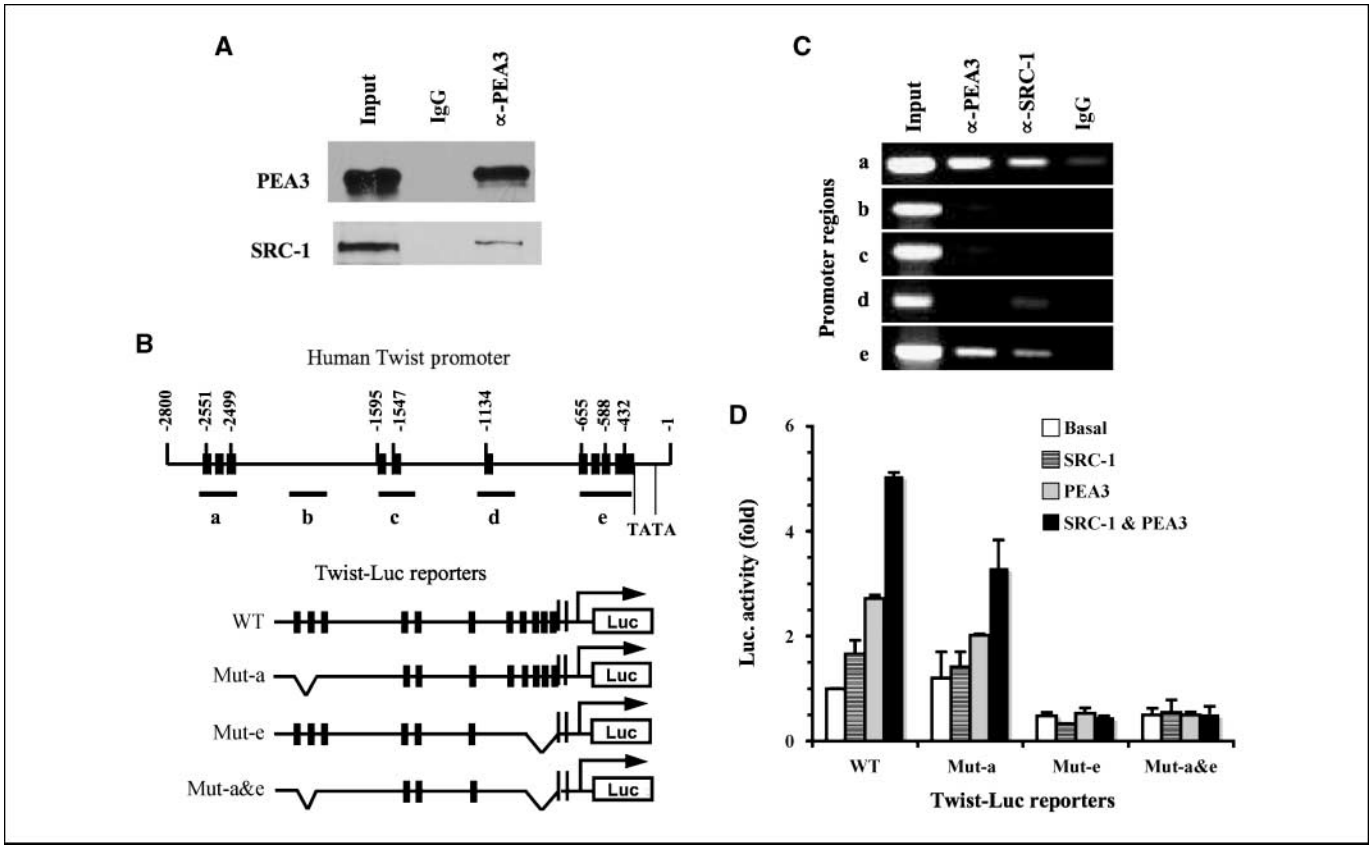

Figure 5. SRC-1 directly regulates PEA3-mediated Twist expression. *A,* MDA-MB-231 cell lysate was subjected to coimmunoprecipitation with PEA3 antibody and control IgG. Input represents 5% of coimmunoprecipitation lysate. Immunoblotting analyses were done with PEA3 and SRC-1 antibodies. *B, black bars,* the Twist promoter region (−1 to −2,800 bp) contains 11 PEA3-binding elements. Fragments *a* to *e* were chosen for PCR amplification in ChIP assays. Four Twist-Luc promoter/reporter constructs in the pGL3 vector are depicted, which are WT, Mut-a (with deletions of the three PEA3-binding sites in region *a*), Mut-e (with deletions of the five PEA3-binding sites in region *e*), and Mut-a&e (with deletions of the PEA3-binding sites in both regions *a* and *e*). *C,* ChIP assays using MDA-MB-231 cells. Input represents 3% of material used for ChIP analysis. ChIP analyses were done using PEA3 and SRC-1 antibodies and control IgG. PCR was done with specific primer pairs to amplify fragments *a* to *e* depicted in *B. D,* HeLa cells were transfected with WT or mutant Twist-Luc promoter/reporter constructs depicted in *B*. For each construct, cells also were cotransfected with parent vector (*Basal*), SRC-1, and/or PEA3 expression plasmids as indicated. *Columns,* mean of three repeat assays; *bars,* SD.

indicate that SRC-1 promotes PEA3-medicated Twist transcription mainly via the region *e* of Twist promoter. Region *e* (−655 to −432 bp) is adjacent to the first TATA box and contains five PEA3-binding sites.

Twist mediates the role of SRC-1 in inhibition of E-cadherin expression and promotion of breast cancer cell migration and invasion. The preceding results suggest that SRC-1 may be through up-regulation of Twist in breast cancer cells to suppress E-cadherin expression and promote EMT, cell motility, invasion, and metastasis. If Twist mediates SRC-1 function during breast cancer progression, Twist knockdown in WT mammary tumor cells with SRC-1 would block SRC-1–dependent inhibition of E-cadherin expression and tumor cell invasion. Conversely, expression of Twist in KO mammary tumor cells lacking SRC-1 would decrease E-cadherin expression and epithelial differentiation but increase EMT marker expression and cell invasiveness. Indeed, when Twist was stably knocked down in WT1 cells by shRNA (Fig. 6*A, a*), the morphology of some cells changed from spindle shape with loose cell-cell contact to cubic shape with tight cell-cell contact (Fig. 6*A, b*). E-cadherin expression in clones with Twist knockdown was markedly increased compared with clones with nontargeting control shRNA as assayed by qRT-PCR (Fig. 6*A, c*). Accordingly, real-time cell invasion assays revealed that Twist knockdown in WT1 cells significantly reduced their invasion through a Matrigel layer. Invasive behavior in one of the two examined Twist knockdown clones (shTwist#2) was almost completely blocked (Fig. 6*A, d*). Furthermore, NU/NU recipient mice with xenograft mammary tumors formed from WT/PyMT cells with stable Twist knockdown possessed a significantly lower frequency of tumor cells in their blood and metastatic tumors in their lungs compared with mice with tumors from control WT/PyMT cells (Supplementary Fig. S5). On the other hand, when Flag-tagged Twist was expressed in KO1 cells by adenoviral infection (Fig. 6*B, a*), E-cadherin expression was reduced in ~50% of cells, whereas vimentin was induced in a similar promotion of cells (Fig. 6*B, b*). Expression of Twist in KO1 cells also changed their epithelial morphology into fibroblast-like morphology and disrupted their capability to form partially differentiated three-dimensional structure in the three-dimensional culture system. These KO1 cells with exogenous Twist expression formed only undifferentiated and invasive structures (Fig. 6*B, b*), resembling structures formed by WT cells (Fig. 2*A*). These results show that Twist is a downstream mediator for SRC-1 in mammary tumor cells to regulate E-cadherin expression and promote EMT and malignancy.

Finally, we validated the findings obtained in mouse mammary tumor cells by recapitulating SRC-1 knockdown experiments in MDA-MB-231 metastatic human breast cancer cells. MDA-MB-231

cells express SRC-1 protein. SRC-1 knockdown in these cells by siRNA decreased Twist expression to 25% and cell migration and invasion to ~45% (Fig. 6*C*). These results indicate that SRC-1 also plays an important role to potentiate Twist expression, cell motility, and invasiveness in human breast cancer cells.

Discussion

In the SRC family, AIB1 is overexpressed in a subgroup of breast cancers (31). In mice, AIB1 overexpression causes mammary tumorigenesis, whereas its deficiency suppresses both mammary tumor formation and lung metastasis (15, 32, 33). These studies not only proved a crucial role of AIB1 in breast cancer but also raised the questions about possible roles for other SRC family members in breast cancer. To investigate the *in vivo* role of SRC-1 in breast cancer, we have recently generated WT/PyMT and SRC-$1^{-/-}$/PyMT mice and compared mammary tumor initiation, growth, and metastasis in these mice (25). Surprisingly, we found that inactivation of SRC-1 in mice does not affect primary mammary tumor initiation and growth but does drastically inhibit mammary tumor metastasis (25). Therefore, AIB1 promotes both primary mammary tumor formation and metastasis, whereas SRC-1 only promotes mammary tumor metastasis, although their differences in molecular mechanisms are unknown.

The present study is to address how SRC-1 promotes metastasis at cellular and molecular levels by developing and using SRC-1 WT and KO tumor cell lines. We found that SRC-1 plays important roles in promoting mammary tumor cell migration, invasion, epithelial depolarization, and EMT. These results were validated by analysis of SRC-1 knockdown in WT tumor cells, SRC-1 restoration in KO

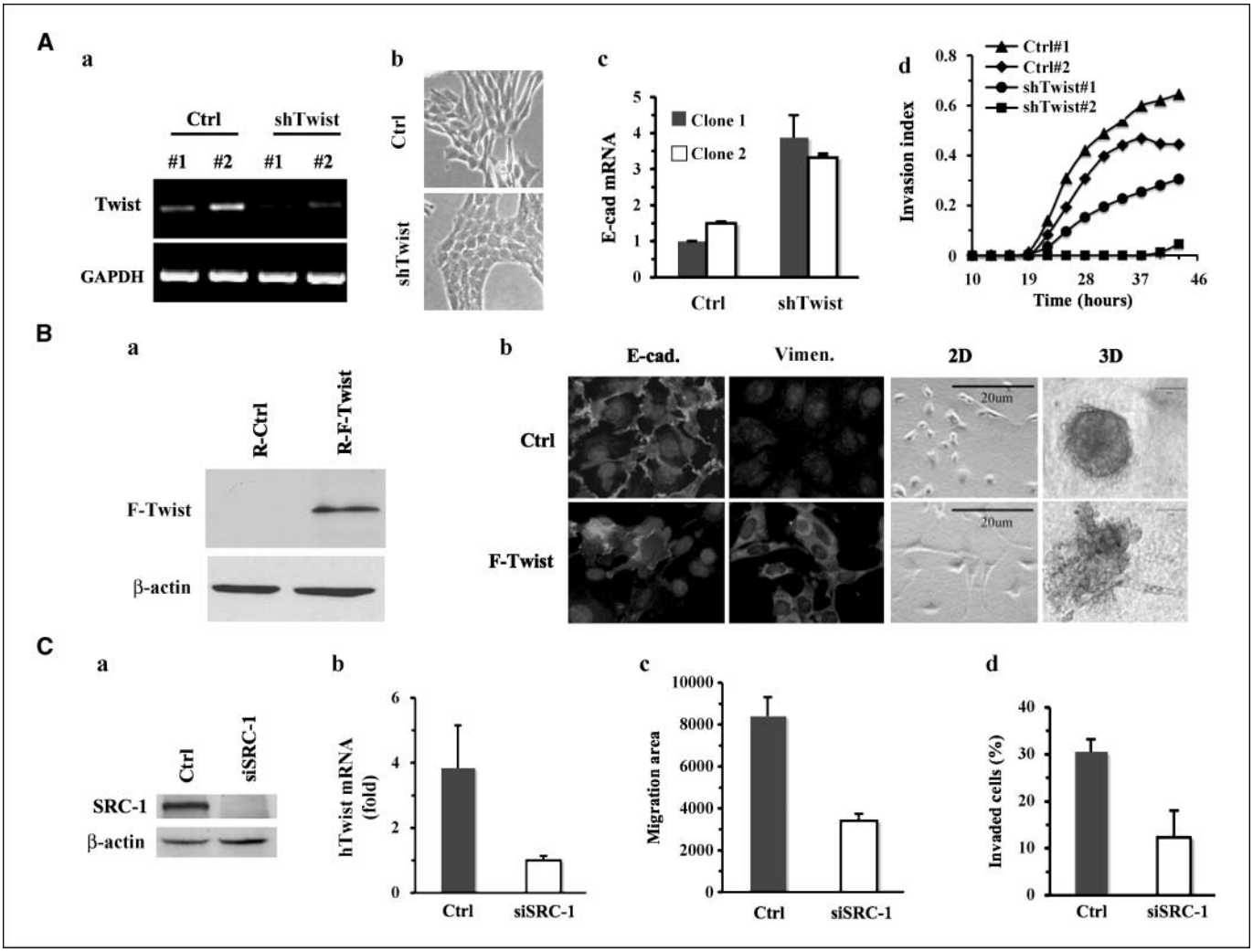

Figure 6. Twist mediates the role of SRC-1 in breast cancer cells. *A,* effects of Twist knockdown in WT1 cells. RT-PCR analysis revealed that Twist mRNA in WT1 cells was knocked down by two different shRNAs (*#1* and *#2*). *a,* lentivirus expressing nontargeting shRNAs served as a control (*Ctrl*). *b,* the images of WT1 cells expressing control or Twist shRNA were taken under a phase-contrast microscope. *c,* relative E-cadherin mRNA levels in WT1 cells expressing control or Twist shRNAs were measured by qRT-PCR. *d,* cell invasion indices were monitored in real time. *B,* effects of Twist restoration in KO1 cells. Flag-tagged human Twist (*F-Twist*) was expressed in KO1 cells using retroviruses. Empty retrovirus served as a control. *a,* the F-Twist protein was detected by Flag antibody. Immunofluorescent staining of control and F-Twist–expressing KO1 cells was done using E-cadherin and vimentin antibodies. *b,* cell images of two-dimensional (*2D*) cultures and morphologies of three-dimensional structures formed from control and F-Twist–expressing cells were taken under a phase-contrast microscope. *C,* effects of SRC-1 knockdown in MDA-MB-231 cells. Cells were transfected with control or SRC-1 siRNAs. *a,* SRC-1 was analyzed by immunoblotting. *b,* relative hTwist mRNA was measured by qRT-PCR. Cell migration was traced with fluorescence beads. *c,* average area (pixels) swept by each cell was obtained from measurements of at least 40 cells in each group. *d,* cell invasion assay chambers with a Matrigel layer were used to determine the percentages of invaded cells to total cells. The assays were done in triplicates.

tumor cells, cellular morphology of WT and KO cells in three-dimensional cultures, and epithelial/mesenchymal marker profile of each cell line. These findings, obtained from cultured cells, strongly correlate with and substantiate our former *in vivo* observations showing that SRC-1 is required for enhancing mammary tumor metastasis in mice (25).

EMT is an important event during cancer progression. In EMT, tumor cells lose polarity and epithelial differentiation and acquire certain mesenchymal features, which enable tumor cells to invade and survive in the stromal tissues, namely, the first step of metastasis (34). Tumor cell EMT is associated with increased cell migration, invasion, and metastasis. The well-established hallmark of EMT is the loss of E-cadherin, which disrupts the stable cell-cell adhesion between epithelial cells (34). Our data show that SRC-1 expression inversely correlates with E-cadherin expression, suggesting that SRC-1 may promote EMT through direct or indirect inhibition of E-cadherin expression.

Several transcription factors, including Twist, Snail, and SIP1, are known to suppress E-cadherin expression and induce tumor cell EMT (3, 4). Among these transcription factors examined in SRC-1 WT and KO tumor cell lines, Twist is significantly up-regulated in WT cells compared with KO cells. Furthermore, SRC-1 knockdown in WT tumor cells decreases Twist expression, whereas SRC-1 reexpression in KO tumor cells increases Twist expression. The Twist promoter activity is higher in WT tumor cells than in KO tumor cells, and it can be potentiated by SRC-1 in another type of cells in a dosage-dependent manner. Taken together, these results show, for the first time, that SRC-1 regulates Twist expression.

SRC-1 directly enhances Twist expression by serving as a coactivator to potentiate PEA3-mediated Twist mRNA transcription. First, SRC-1 synergistically potentiates PEA3-mediated *Twist* promoter activation in transfection assays; second, SRC-1 directly associates with PEA3 in human breast cancer cells; third, both PEA3 and SRC-1 are recruited to Twist promoter regions *a* and *e*; and fourth, deletion of region *e* that contains multiple PEA3-binding sites diminishes PEA3 and SRC-1 activities in activation of *Twist* promoter. Conceivably, SRC-1 may also enhance Twist expression through other transcription factors and future studies may lead to identification of these yet unknown transcription factors.

Finally, this study addressed if Twist, as a SRC-1 target, is responsible for the effect of SRC-1 on the mammary tumor cells. Our data show that Twist knockdown in WT tumor cells increases E-cadherin expression and decreases cell invasion capability, whereas expression of Twist in SRC-1 KO tumor cells reduces E-cadherin expression and increases cell invasiveness. Because Twist expression or knockdown recapitulates the phenotypes of SRC-1 expression or KO, SRC-1–regulated Twist expression should be responsible, at least in part, for SRC-1–controlled tumor cell morphology and invasiveness. In addition, SRC-1 depletion in MDA-MB-231 human breast cancer cells also decreases Twist expression and cell migration and invasion, which validates the regulatory relationship and functional significance between SRC-1 and Twist in breast cancer.

Short-term expression of either SRC-1 or Twist at medium levels was not sufficient to induce complete EMT of mammary tumor cells in culture. This could be due to the lack of *in vivo* tumor progression environment or insufficient time for completing EMT. Furthermore, in addition to SRC-1, several other coactivators have been implicated in breast cancer metastasis. For example, AIB1 coactivates PEA3-mediated MMP2 and MMP9 expression to promote mammary tumor cell invasion and metastasis (15). The metastasis tumor antigen 1 serves as a coactivator for HIF-1α to enhance breast cancer metastasis (35). The functional and molecular relationships among these coactivators in metastasis will continue being an interesting research topic.

The SRC-1–linked regulatory relationship between PEA3 and Twist has important implications in breast cancer. Although controversial reports exist, recent studies suggest that PEA3 expression in breast cancer associates with HER2 expression, metastasis, and poor prognosis (14, 15, 36, 37). Twist is an E-box–binding transcription factor that serves as one of the master regulators for EMT and breast cancer metastasis (3, 27). Because SRC-1 is overexpressed in many HER2-positive breast cancers (14) and it mediates PEA3-dependent Twist expression in these cancers, inhibition of SRC-1 function may provide a useful intervening point to control the deleterious roles of both PEA3 and Twist in HER2-positive breast cancer metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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