Supplementary Data for Li Qin et al.

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Supplementary Materials and Methods

Generation of mouse mammary tumor cell lines. Individual mammary tumors were collected from WT/PyMT and SRC-1$^{-/-}$/PyMT mice. Tumor cell lines were established from WT/PyMT and SRC-1$^{-/-}$/PyMT mammary tumors as described (15). The epithelial character of these cells was confirmed by immunocytochemistry for cytokeratin 8 (K8) as described (15).

Cell motility and invasion assays. Individual cell movement was traced for 18 hours in 96-well culture plate coated with blue fluorescent beads. Track areas were quantitatively analyzed using NIH image software as described (15). Cell invasion was assayed by using BioCoat Matrigel Invasion Chambers (BD Biosciences) as described (15). 25,000 cells in serum-free medium containing 0.2% BSA were loaded to the upper chambers. The lower chambers were filled with fibroblast-conditioned medium containing 5% fetal bovine serum (FBS) as chemo-attractant. After cultured for 22 hours, the invading cells were fixed, stained and counted. Real-time cell invasion assay was carried out using an RT-CES system with 16-well E-Plates equipped with a Matrigel-coated membrane with 8 μm pores (ACEA Biosciences Inc.). 50,000 cells in serum-free medium were loaded to the top chamber and medium with 5% serum was added to the bottom chamber. Cell invasion was monitored every 30min till the experimental endpoint.

SRC-1 Knockdown and adenovirus-mediated expression. To knock down SRC-1, WT/PyMT mouse cells and MDA-MB-231 human cells (5×10^5) in 6-well plates were transfected with mouse and human SRC-1 siRNA Smart Pools as described (Dharmacon) (15). Cells transfected with scrambled siRNA served as controls. The knockdown efficiency was examined by qPCR and Western blot analyses as described (15). For SRC-1 expression and its control, SRC-1$^{-/-}$/PyMT cells were infected with adenoviruses carrying either an SRC-1 or a control GFP expression cassette as described (15, 26). Exogenous expression of SRC-1 in SRC-1 knockout cells was examined by western blot as described (15).

Stable shRNA knockdown and retroviral expression of Twist. Five different shRNA lentiviruses targeting non-overlapping regions of the mouse Twist mRNA (SHVRS-NM-011658) as well as a non-targeting shRNA lentivirus (SHC002V) were purchased from Sigma. WT1 mammary tumor cells were infected with either the targeting lentiviral mixture or control non-targeting lentiviral particles. Infected cells were selected in puromycin-containing medium. Surviving clones were isolated and examined by real-time RT-PCR to determine Twist knockdown efficiency. To express Twist in KO1 cells, a DNA sequence for translational initiation and Flag tag were added to the 5' human Twist coding sequence and the resulting fusion DNA was cloned into the pQCXIH retroviral vector (BD Biosciences). The parent vector was used as a control. Retroviruses were produced in pT67 packaging cells. Stable KO1 cells with human Twist expression or control vector were generated by retroviral infection followed by hygromycin selection.

Three-dimensional (3D) culture. The 3D culture system for WT/PyMT and SRC-1$^{-/-}$/PyMT cells was set up as described (15). 5,000 cells in 400μl of growth medium containing 2% Matrigel were seeded on 8-well chamber slides (BD Biosciences) pre-coated with 40ul Matrigel and cultured for 8-18 days. Cells were fixed in methanol and acetone (1:1). Immunofluorescence staining was performed with primary antibodies against E-cadherin, N-cadherin (BD Biosciences) and ZO-1 (Zymed Lab.), and fluorescence-conjugated secondary antibodies. DNA was stained using propidium iodide (PI). Stained slides were mounted in 50% glycerol and examined by confocal microscopy.

Western blot. Cells in 80% confluent monolayer culture were extracted with a lysis buffer containing 0.05 M Tris-HCl, pH 7.6, 0.15 M NaCl, 1% NP-40, 0.5% Deoxycholate, 2 mM EDTA and protease inhibitor cocktail. Cell lysates with 50 μg protein were subjected to Western blot analyses using primary antibodies against SRC-1 (Abcam), E-cadherin, N-cadherin (BD Biosciences), β-catenin, vimentin, PEA3 (Santa Cruz), PEA3, Flag-Twist and β-actin (Sigma).

Horseradish peroxidase-conjugated secondary antibodies and a chemiluminiscence kit from Pierce Biotechnology were used to visualize the immunochemical signals.

Real-time quantitative RT-PCR (qPCR). Total RNA was isolated from cells using TRIZOL reagents (Invitrogen). cDNA template was produced by reverse transcription from 1 μg of RNA using Reverse Transcriptase Core kit (Eurogentec). The relative concentrations of mouse SRC-1, Twist, Snail, SIP1, E-cadherin, N-cadherin and vimentin mRNAs and human SRC-1 and Twist mRNAs were measured by qPCR using matched Universal Taqman probes and gene-specific primers (Roche). The measurement of 18s mRNA was used as an endogenous control.

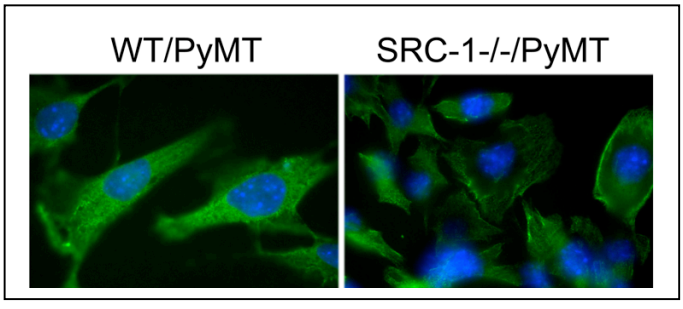
Cell transfection and luciferase assays. The pGL3-mTwist-Luciferase (Luc) promoter/reporter construct was provided by Dr. Howe (27). The pGL3-hTwist-Luc promoter/reporter plasmid was constructed with a 2300-bp DNA fragment amplified by PCR from the proximal enhancer/promoter region of the human Twist gene. The primers for PCR were 5'-GTTGGTACCGCTGTGGACTTGGTTTCTCC and 5'-GATAAGCTTCTGCAGACTTGGAGGCTCTT. WT/PyMT and SRC-1$^{-/-}$/PyMT cells were grown to 80% confluence in 12-well plate and transiently transfected with 200ng of pGL3-mTwist-Luc and 50ng of pSV40-gal plasmids as described (15). pSV40-gal supported constitutive expression of β-galactosidase (β-gal), which was used to normalize transfection efficiency. Hela cells were co-transfected with pSV40-gal, pCR3.1-SRC-1, pGL3-hTwist-Luc and expression plasmids for PEA3, ERM, ER81, NF-κB, c-Jun, TCF, HIF-1α or E2F1. Total DNA for each well was added to the same amount using respective parent vectors when different combinations of plasmids were applied. Cells were cultured for 48 hours and then lysed with Reporter Assay Lysis Buffer (Promega). Luciferase activity was measured using the Luciferase Assay System (Promega). β-gal activity was assayed as described (28). Relative luciferase activity was normalized to β-gal activity.

Co-immunoprecipitation (Co-IP). Cell lysates with 500μg protein prepared from MDA-MB-231 cells were pre-cleaned with protein G/A beads and subjected to Co-IP using 2μg of PEA3 antibody. Equal amount of IgG was used as negative control. Immuno-complexes were denatured by boiling in SDS-PAGE sample buffer and separated in 6% SDS-PAGE gels for Western blot using SRC-1 and PEA3 antibodies. All Co-IP assays were repeated for 3 times.

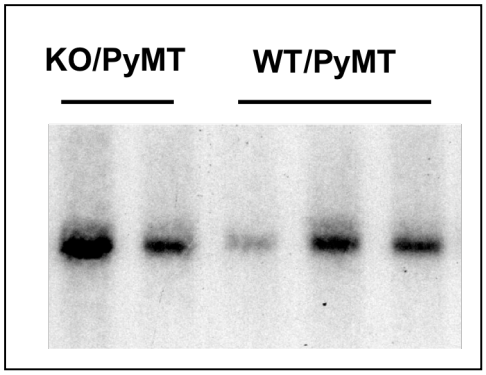
Chromatin immunoprecipitation (ChIP) assays. ChIP assays were performed with MDA-MB-231 cells, 2μg of antibodies against SRC-1 or PEA3 and the protein-G/A beads as described (15). PCR was performed with specific primer pairs to amplify proximal regions of the human Twist promoter that contain PEA3-binding sites (27).

Supplementary Figures: Fig. S1, Fig. S2, Fig. S3, Fig. S4 and Fig. S5

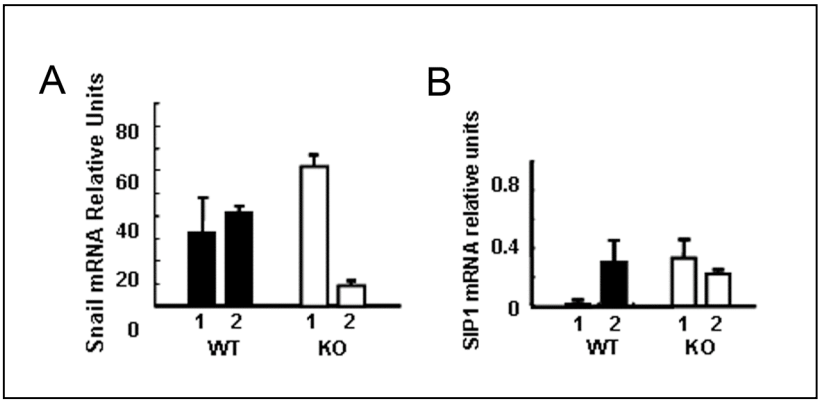
Supplementary Fig. S1. Both WT/PyMT and SRC-1$^{-/-}$ (KO)/PyMT mammary tumors cells express cytokeratin 8 (K8). Stable mammary tumor cells were developed from mouse primary tumors as described in Materials and Methods. Immunocytofluorescence labeling was performed with anti-K8 polyclonal antibody and FITC-conjugated secondary antibody. These results demonstrate that these stably established cell lines are derived from mammary epithelial tumor cells.



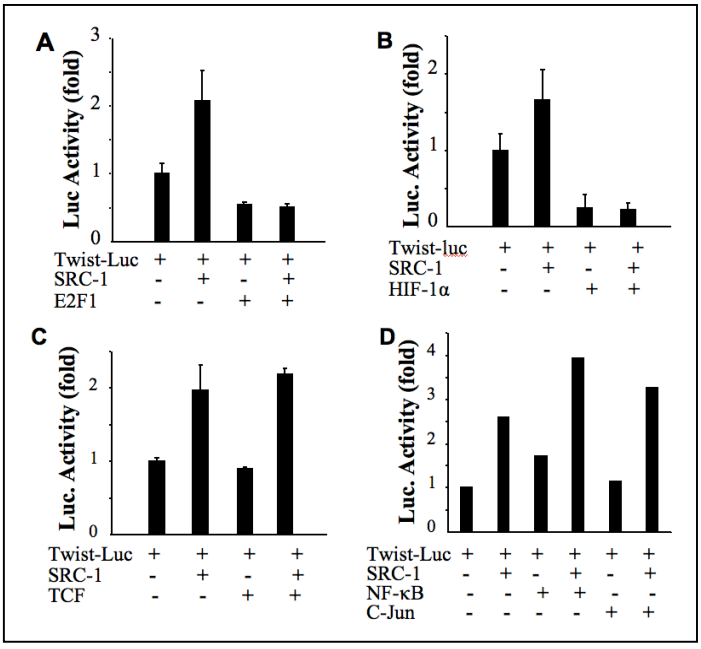
Supplementary Fig. S2. Southern blot analysis of E-cadherin genomic DNA of WT/PyMT and KO/PyMT mammary tumor cells. Genomic DNA samples were extracted from mammary tumor cell lines, and 5 μg of DNA of each sample was digested with restriction endonucleases Hind III and Ecol R1. Digested DNA was separated by running 2% agarose gel and blotted onto a nylon membrane. The blot was hybridized with a mouse E-cadherin genomic DNA probe labeled with ^{32}P-dCTP. The probe hybridization specifically detected one band with predicted molecular weight. These results suggest that all of these mammary tumor cells contain E-cadherin gene, so the loss or reduction of E-cadherin expression in WT/PyMT cells is not due to a loss of this gene.



Supplementary Fig. S3. SRC-1 deficiency does not exhibit consistent effect on Snail and SIP1 expression. Total RNA samples were prepared form WT1, WT2, KO1 and KO2 mouse mammary tumor cell lines and subjected to real-time RT-PCR analyses of relative Snail (panel A) and SIP1 (panel B) mRNA concentrations. The relative Snail and SIP1 mRNA concentrations were normalized to the concentration of 18 S RNA. The forward and reverse primer pairs were 5'-gtctgcacgacctgtggaa and 5'-caggagaatggcttctcacc for Snail and 5'-ccagaggaaacaaggatttcag and 5'-aggcctgacatgtagtcttgtg for SIP1. The fluorescence-labeled real-time PCR universal probes were #71 for Snail and #42 for SIP1 (Roche, Indianapolis, IN).



Supplementary Fig. S4. SRC-1 is not required for E2F1, HIF1a, TCF, NF-kB and C-jun-mediated Twist promoter activation. Hela cells in 24-well plates were co-transfected with the following plasmids: hTwist-Luc reporter (200 ng), pCR3.1-SRC-1 or pCR3.1 vector control (300 ng), and 50 ng of E2F1 (panel A), HIF1α (panel B), TCF (panel C), NF-κB (panel D) or C-Jun (panel D) expression vector. Cells were also co-transfected with 30 ng of RSV-β-galacrosidase expression vector for normalizing transfectional efficiency. Luciferase (Luc) activity of the reporter was assayed 36 hr after transfection.



Supplementary Fig. S5. Knockdown of Twist in WT/PyMT mammary tumor cells reduces their metastatic capability in NU/NU mice.

A. One pool of WT/PyMT cells expressing non-targeting shRNA and two pools of WT/PyMT cells expressing two different shRNAs (#95074 and #95077, SHVRS-NM-11658, from Sigma, St. louis, MO) that target two different regions of the mouse Twist mRNA were stably established by lentiviral infection and puromycin-resistant selection. Cells (10^7) were injected into the 4th pairs of mammary fat pads of 4-week-old female NU/NU mice (n = 5). Xenograft tumor growth was observed in 3 weeks after cell injection. Lung metastasis was examined at 7 weeks after cell injection. Metastatic tumors in the lung were observed in 2 out of 5 recipient mice with tumors of WT/PyMT cells expressing non-targeting shRNA, while no metastatic tumors could be seen in the lungs of all recipient mice (n = 4 and 5) with tumors of WT/PyMT cells expressing Twist shRNAs. Panel A shows the H&E-stained lung sections prepared from NU/NU recipient mice with xenograft mammary tumors of WT/PyMT cells expressing control non-targeting shRNA (Control) and Twist-targeting shRNAs #95074 and #95077.

B. To determine the number of mammary tumor cells in the blood circulation, 0.2 ml of blood was collected from the heart of each recipient mouse at the experimental endpoint. The cells in the blood were cultured in DMEM with 10% FCS for 10 days, and the formed tumor cell colonies were counted. Tumor cell colonies were found in 5 out of 5, 1 out of 4, and 1 out of 5 blood samples collected from recipient mice with xenograft tumors expressing control, #95074 (indicated as 74shTwist) and #95077 (indicated as 77shTwist) shRNAs, respectively.