Immune-Induced Epithelial to Mesenchymal Transition In vivo Generates Breast Cancer Stem Cells

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Abstract

The breast cancer stem cell (BCSC) hypotheses suggest that breast cancer is derived from a single tumor-initiating cell with stem-like properties, but the source of these cells is unclear. We previously observed that induction of an immune response against an epithelial breast cancer led in vivo to the T-cell–dependent outgrowth of a tumor, the cells of which had undergone epithelial to mesenchymal transition (EMT). The resulting mesenchymal tumor cells had a CD24−/CD44+ phenotype, consistent with BCSCs. In the present study, we found that EMT was induced by CD8 T cells and the resulting tumors had characteristics of BCSCs, including potent tumorigenicity, ability to reestablish an epithelial tumor, and enhanced resistance to drugs and radiation. In contrast to the hierarchical stem cell hypothesis, which suggests that breast cancer arises from the transformation of a resident tissue stem cell, our results show that EMT can produce the BCSC phenotype. These findings have several important implications related to disease progression and relapse.

Introduction

A current goal among breast cancer scientists worldwide is to reduce the risk of breast cancer recurrence following therapy (1). This risk, which remains high for at least one decade, suggests that treated patients still maintain a small population of tumorigenic, albeit dormant, cancer stem cells. Although previously described (2), it was not until 2003 that interest in breast cancer stem cells (BCSC) was renewed (3). In that study, identification of the CD24−/CD44+ tumorigenic breast cancer cells provided a valuable marker framework for testing hypotheses on BCSC involvement in the clinical course of breast cancer and its pathogenesis.

A prevalent BCSC hypothesis proposes that breast tumors are initiated and maintained by a small fraction of tumorigenic cells (4). What remains unclear about the BCSC hypothesis is the origin of the tumorigenic cells. One hypothesis is that transformed resident tissue stem cells undergo asymmetrical division, occasioning a renewal of a copy of themselves but most often generating proliferative epithelial daughter progeny with limited tumorigenicity. An alternative hypothesis is that BCSCs are derived from transformed, differentiated epithelial cells by acquisition of stem cell attributes. Determining the origin and biology of BCSCs is important for the development of therapies to reduce the risk of breast cancer relapse.

In prior work, we found that immunoediting of breast tumors in the neu-transgenic (neu-tg) mouse resulted in neu antigen-loss variant tumors (5–8). Our first observation was that the variants had a different proteomic profile and reduced inflammatory and danger signals (7). Subsequent analyses revealed that antigen-loss tumor cells had undergone T-cell–dependent epithelial to mesenchymal transition (EMT; refs. 5, 6). EMT, a dedifferentiation program that converts epithelial cells into a mesenchymal phenotype, is involved in embryogenesis and used pathologically during cancer progression (9). Because our studies showed that mesenchymal antigen-loss variants were CD24−/CD44+, we assessed whether these antigen-loss tumor cells had characteristics of CD24−/CD44+ BCSCs observed by Al-Hajj and colleagues (3, 6). Furthermore, we aimed to determine what T-cell subset was involved in the induction of EMT. Herein, we show that EMT induction in vivo involves CD8 T cells and generates BCSCs.

Materials and Methods

Animals. Female neu-tg mice on the FVB background and FVB/N mice were maintained as a colony in accordance with institutional policy.

Cell lines. The epithelial cell line (E) was derived as previously described (10). Four cell lines (M1, M2, M3, and M4) were obtained from relapsed tumors that underwent EMT following injection of E tumor cells into parental nontransgenic FVB/N mice (6). All cell lines were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% sodium pyruvate, 2.5% HEPEs, and 2 mMol/L L-glutamine.

In vivo tumorigenicity assays. Neu-tg mice were inoculated s.c. on the mid-dorsum unless otherwise specified, with doses of tumors cells ranging from 100 to 106 cells. Tumors were measured every other day with vernier calipers, and volumes were calculated as the product of length × width × height × 0.5236.

Antibodies for in vivo cell depletion. Anti-CD4 (GK1.5) and anti-CD8 (53.6.72) monoclonal antibodies were prepared by the Mayo Antibody Core Facility (Rochester, MN). Mice were injected daily for 5 d with 100 μg of antibody i.v. and E tumor cells were injected 48 h later. For secondary depletion of CD8a, mice previously injected with E tumor cells received five more daily doses of anti-CD8 and were rechallenged with tumors.

Proliferation of M3 cells in response to CD8 T-cell conditioned media. CD8 T cells were derived from lymph nodes and spleens of naïve FVB mice with a mouse CD8a T-cell isolation kit per manufacturer's recommendations using an autoMACS Separator (Miltenyi Biotec, Inc.).

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

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Two million untouched CD8+ T cells per well were plated in six-well plates. Unstimulated wells contained CD8s and media. CD8s were treated with 2 million irradiated splenocytes (3,300 rad) and 250 μg of concanavalin A. Anti-CD3/CD28 received 400,000 mouse Dynabeads (Invitrogen) per well (~1 bead/5 T cells). One hundred microliters of 18-hour conditioned media were added to mesenchymal (M) cells previously seeded into 96-well plates. [3H]Thymidine was added at 0.273 nCi per well; 24 h later, cells were harvested and read on a TopCount NXT scintillation counter (Perkin-Elmer).

**Flow cytometry.** Cells were incubated with primary antibodies at 4°C for 20 to 30 min, washed, followed by secondary antibody (if needed) for 20 min, washed, and fixed with 0.5% formaldehyde. Samples were run on a BD FACSscan or a FACScalibur flow cytometer (BD Bioscience). Sorting of M3 cells was done on a BD FACSVerse Cell Sorter and data were analyzed using BD CellQuest Pro software (version 4.0.2). Occasionally, results depicted the relative mean fluorescent intensity (ratio of specific marker intensity to the isotype or secondary antibody staining).

**Flow cytometry reagents.** Antibodies and reagents from BD Pharmingen included anti-CD24 FITC (M1/9), anti-CD44 FITC, phycoerythrin (PE)-Cy5 (IM7), anti-Scal FITC (D7), anti-Annexin V allophycocyanin, and 7-amoactinomycin D (7-AAD). Antibodies from eBioscience were anti-CD24 PE (30-F1), anti-CD34 FITC (RAM34), and anti-CD133/Prominin 1 FITC (13A4). Rabbit anti-claudin 3 (Z23.JM) was from Zymed/Invitrogen. The mouse monoclonal antibody against rat neu (7.16.4) was previously described (6). Secondary antibodies from Jackson ImmunoResearch Laboratories were FITC goat anti-rabbit IgG and FITC anti-mouse IgG. Anti mouse IgG2a/2b FITC (B2–10) was from BD Pharrmingen. Isotype antibodies were PE Rat IgG2b (eBioscience), FITC Rat IgG2b (BD Pharmingen), and PE-Cy5 Rat IgG2b (195-1, BD Pharmingen), BD Cytofix/ Cytoperm Fixation/Permeabilization solution kit (BD Pharmingen) was used for claudin-3 staining.

**In vitro coculture of E cells with primed CD8s.** FVB mice were injected with 5 million E tumor cells, and spleens removed 7 d later to isolate primed CD8 T cells as described above. In six-well plates, 2.5 × 10^5 E cells and 2 × 10^6 CD8 T cells were added per well. CD8 T cells were irradiated to 3,300 rad. Cells were cocultured for 24 h, T cells washed off, and tumor cells stained for neu and CD24 and analyzed by flow cytometry.

**PCR analysis.** RNA was isolated using RNeasy (Qiagen). PCR primers, selected with MacVector software (MacVector, Inc.), were from Invitrogen. Reverse transcription-PCR (RT-PCR) used SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen) using 100 or 200 ng of RNA in a Bio-Rad MyCycler. Samples were electrophoresed on 1% agarose gels, imaged on a Gel Doc XR (Bio-Rad), and band intensities quantified with Quantity One software (version 4.2.2). DNA repair gene expression analysis was done using DNA Damage Signaling RT2 Profiler PCR Array qPCR kit (SuperArray) according to the manufacturer’s protocol and run on a Mx3000P thermal cycler (Stratagene). Expression was normalized to housekeeping genes and expressed as fold expression relative to the E cell line.

**Cell photography.** Images of cell lines at ×40 magnification were taken using an Axiovert 200M inverted microscope and AxioCam HRm camera (Carl Zeiss). Images of spheroids at 40 magnification were obtained with a Leica DC 200 microscope (Leica Microsystems) and Fujifilm FinePix 6800 Zoom camera (Fujifilm).

**Histology.** M3 and E tumors from neu-tg mice were frozen in optimum cutting temperature compound (Tissue-Tek, Sakura Finetek USA), cryosectioned, and stained with H&E. Images (20×) were gathered using a NanoZoomer Digital Pathology (Bacus Laboratories, Inc.) slide scanner and captured using WebSlide Enterprise software. Staining and imaging of frozen tumor sections was done by the Tissue and Cell Molecular Analysis of the Mayo Clinic Cancer Center using standard techniques.

**Western blotting.** Protein was isolated from whole cell lysates using cell extraction buffer (Invitrogen), and concentration determined using the BCA assay (Pierce/Thermo Fisher Scientific, Inc.). Protein (80–150 μg) was subjected to SDS-PAGE electrophoresis and transferred onto polyvinylidene difluoride membranes, which were blocked for 1 h with skim milk (3% w/v of PBS with Tween 20) and incubated overnight (4°C) with monoclonal antibodies against BCRP (BXP-21) or PGP (C219) from Calbiochem diluted 1:50 and 1:500, respectively. Membranes were washed and incubated with alkaline phosphatase–conjugated antiserum IgG (Chemicon/Millipore) for 1 h (1:5,000 dilution) followed by detection with enhanced chemiluminescence (GE Healthcare). Anti–β-actin was used as loading control (AC-74, Sigma Aldrich).

**Chemotherapy assays.** Mitoxantrone dihydrochloride was from Sigma Aldrich; 1,3-bis(2-chlorethyl)-1-nitrosourea (BCNU) from the Chemical Synthesis Branch of National Cancer Institute; and etoposide from Biomol International. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cellular proliferation assays were used (11) for mitoxantrone chemosensitivity. E and M cells (5,000) were plated in 96-well plates in complete medium overnight and thereafter in serum-free medium. Cell lines were exposed to increasing concentrations (0, 2.5, 5, 10, and 20 μmol/L) of mitoxantrone (diluted in 95% ethanol) for 72 h. Medium was aspirated from wells and 15 μL/well of MTT reagent (Sigma Aldrich) was added and incubated for 4 h at 37°C followed by 100 μL of stop solution. The following day, plate absorbances were measured at 562 nm in an ELISA SpectraMax 190 reader (Molecular Devices). Percent inhibition of growth was measured as a percentage of control (no mitoxantrone). For apoptotic assays, cells were plated and treated with 2 μmol/L etoposide for 24 h followed by staining with Annexin V and 7-AAD. The percentage of apoptotic cells was determined by flow cytometry as described (12).

**Clonogenic assays.** Five hundred cells were plated in 96-well dishes and treated 24 h later with irradiation or BCNU. Irradiation was done with a 320 Cs laboratory irradiator to generate a dose curve of 0, 4, 8, 16, and 32 Gy, or cells were exposed to increasing doses (0, 2.5, 5, 10, and 20 μmol/L) of BCNU. After 8 d, cells were washed, stained with crystal violet, and colonies counted.

**Matrix metalloproteinase activity assay.** The EnzoLyt 520 Generic MMP Fluorometric Assay Kit (AnaSpec) was used according to the manufacturer’s directions. Cell culture supernatants were incubated with 1 mmol/L of 4-aminophenylmercuric acetate for 24 h at 37°C to activate pro-MMPs. Fluorescence was measured at 490/520 nm Ex/Em using a Victor plate reader (Perkin-Elmer).

**RNA microarray.** Affymetrix microarray analysis was done by Mayo Advanced Genomic Technology Center as previously described (6) using GeneChip Mouse Genome 430 2.0 arrays. The expression analysis was done with Affymetrix GeneChip Operating Software with a scaling target signal of 500 and normalization of L. Microarray data are posted on Gene Expression Omnibus as GSE13259.

**Spheroids.** Cells from monolayer cultures were harvested and resuspended at a density of 4 × 10^6 viable cells/mL in serum-free MEM (Cambrex Bioscience) supplemented with 2% B27 (Invitrogen), 100 μg/mL mercaptoethanol, 4 μg/mL insulin (Sigma), 1 ng/mL hydrocortisone, 1% penicillin/streptomycin, 0.25 μg/mL amphotericin B, and 20 μg/mL gentamicin sulfate and then seeded into 10-cm dishes coated with 1% agarose. Cultures were fed weekly.

**Statistical analyses.** Statistical analysis was done using Prism 4 for Windows (version 4.03, GraphPad Software). Data were analyzed using one-way ANOVA when comparing E with each of the M cell lines with the Bonferroni multiple comparison test (Kruskal-Wallis as nonparametric) or one-tailed Student’s t tests. Statistical significance was set at P < 0.05. The number of replicates is indicated with each figure.

**Results**

**Induction of EMT in vivo requires CD8 T cells.** Immune rejection of neu+ epithelial (E) breast tumors in the FVB/N mouse, where neu is a foreign antigen, results in T-cell– and IFN-γ–dependent induction of neu antigen-loss (neu−) variant tumors that have undergone EMT (5, 6). To determine which T-cell subset was responsible, we depleted CD4 or CD8 T cells from FVB/N mice before challenge with neu+ E tumor cells. Control mice developed tumors at ~40 days, whereas those depleted of CD4 T cells developed tumors at ~20 days (Fig. 1A; ref. 6). Consistent with...
prior work, control mice developed neu− variants, whereas those depleted of CD4 T cells had neu+ tumors (data not shown). Mice depleted of CD8 T cells, however, remained disease-free (Fig. 1A). Long-term observation (>100 days) suggested complete tumor rejection (data not shown). Four possible reasons could explain why tumors did not grow in CD8 T-cell–depleted mice. The first is that CD8 T-cell depletion permitted a more robust immune response against neu− EMT variants. To test this, CD8 T-cell–depleted mice that rejected tumor were rechallenged with neu− EMT variants. If robust immunity against neu− EMT variants existed, these mice should reject tumor, which we did not observe (Fig. 1B). The second possible reason is that CD8 T cells preferentially support enhanced growth of the neu− EMT variants contained as a small population within the E cell line. To test this, conditioned medium from CD8 cells was applied to neu− EMT variants in vitro. Neu− EMT variant tumor cells exposed to conditioned medium did not grow any better than those incubated with control medium (P > 0.05), ruling out the likelihood that growth of these cells was enhanced by growth factors derived from CD8 T cells (Fig. 1C). A third possibility is that CD8 T cells promote the malignant transformation of stromal cells that associate with the nascent tumor. This is ruled out because these mesenchymal tumors contain the neu oncogene, which is not in the FVB/N genome (5). The only remaining possibility is that CD8 T cells directly induce neu− EMT variants. These findings, along with prior work, show that the generation of neu− EMT variants in the FVB/N mouse is an active, rather than a selective, process (5). To further support this, we found that E cells cultured with tumor-primed CD8 T cells lost neu and CD24 expression (P = 0.0009; Fig. 1D). This was partially ablated by prior irradiation of the CD8 T cells, which is known to blunt T-cell proliferation and cytokine production. Naïve CD8 T cells failed to generate antigen-negative tumor cells (data not shown).

**Stable mesenchymal cell lines were established.** Four neu− EMT variant cell lines (M1, M2, M3, and M4) were established from four different tumors. All were neu− as assessed by RT-PCR (data not shown). Consistent with having undergone EMT, the M cell lines had significantly reduced levels of the epithelial markers E-cadherin and claudin 3 (P = 0.0001; Fig. 2A). Moreover, mesenchymal markers N-cadherin and Snail were up-regulated in M cell lines (Fig. 2B). Increased mesenchymal function, as measured by matrix metalloproteinase (MMP) activity, was increased significantly (P < 0.01) in all lines, except M1 relative to the parental E cell line (Fig. 2B). Visual inspection (Fig. 2C) showed that M cell lines had a scattered, spindle-shaped appearance, whereas the E cell line had strong cellular junctions with a cobblestone appearance. Fluorescence in situ hybridization karyotyping revealed that each M cell line contained, like the parental E cells, a single integration site for rat neu within chromosome 3 (Supplementary Fig. S1).
Mesenchymal cells are CD24−/loCD44+ and highly tumorigenic. Human BCSCs have been distinguished from nontumorigenic cells by the cell surface marker profile CD24−/loCD44+ (3). Because our prior work showed that CD24 was down-regulated in neu− EMT variant tumors, we hypothesized that M cells may have a BCSC profile. As shown in Fig. 3A, the M cell lines were largely CD24−/lo and CD44+ in contrast to the CD24+CD44+E cells. CD24 expression was significantly lower in all M cells relative to the E cells (P = 0.004). CD34, a sialomucin expressed on mesenchymal stem cells (13), was not significantly elevated (P = 0.63) in M cell lines relative to E cells (Supplementary Fig. S2). Consistent with prior work, the M cells had low and intermediate expression of stem cell antigen-1 (Sca1), and although elevated relative to E cells, it was not statistically significant (P = 0.23; Supplementary Fig. S2). CD133, a marker of hematopoietic stem cells, was also examined given its association with human cancer stem cells (14). Staining revealed similarly low expression in both tumor types (P = 0.74; Supplementary Fig. S2).

A key property of cancer stem cells is their ability to seed tumors at very low numbers. In vivo studies showed that M tumor cells were far more tumorigenic than E cells (Fig. 3B; Table 1). M cells formed tumors in 100% of mice at doses of ≥1,000 cells, and M3 formed tumors with as few as 100 cells. E cells only formed tumors when 10⁶ cells were injected (Fig. 3B; Table 1). Despite large differences in tumorigenicity, both cell types were able to form spheroids in vitro under conditions that prevented attachment (Supplementary Fig. S3). However, the M cells generated spheroids with greater efficiency than E cells (P < 0.0001; Fig. 3B).

Mesenchymal tumor cells generate neu−CD24hiCD44+ tumors in neu-tg mice. Another unique feature of BCSCs is the ability to give rise to epithelial progeny that constitute the bulk of a tumor. To examine whether the mesenchymal cells could reconstitute an epithelial tumor, M tumor cells were injected into the neu-tg mouse, which carries the neu transgene and will not reject neu+ tumors. Because some M cells showed a minor population with moderate CD24 expression, we sorted CD24−CD24hi tumor cells from CD24hi/int tumor cells. Both fractions formed tumors on injection of 10⁵, 10⁴, or 10³ cells with nearly equal efficacy (data not shown). M cells were able to reconstitute tumors that regained CD24 expression and maintained high CD44 expression levels (Fig. 3C). Histologic analysis of tumors from E and M3 cells shows no remarkable differences (Supplementary Fig. S4). However, neu expression was poorly up-regulated, a finding consistent with our prior study showing that the neu-transgene mouse mammary tumor virus (MMTV) promoter in the neu− EMT variant tumors is heavily methylated (5). Indeed, neu expression could be regained by injection of M tumor cells into the mammary fat pad, a site of preferential activation of the MMTV promoter. In fact, M1 cells regained complete neu expression in the mammary fat pad but not in the flank (P = 0.002; Fig. 3C). CD24 and neu expression were also induced, albeit moderately, in M1 cells grown under spheroid forming conditions (Supplementary Fig. S5).

Figure 2. Stable cell lines from tumor cells that underwent EMT were established. A, expression levels of E-cadherin and claudin 3 in E and M cell lines. The E-cadherin experiments were repeated independently thrice with similar results. Claudin 3 was measured by flow cytometry; columns, mean of two separate experiments; bars, SE. B, N-cadherin and Snail expression and MMP activity for all cell lines. Columns, signal intensity derived using RT-PCR, RT-PCR results are representative of two experiments that gave nearly identical results. For MMP activity, each column is the mean of three samples; bars, SE. C, light microscope pictures (×40) of the cell lines. Bar, 100 μm.
Mesenchymal cell lines have elevated expression of drug pumps and DNA repair enzymes and are resistant to cytotoxic agents. Another hallmark of BCSCs is their ability to resist environmental insults. Both mesenchymal cells and BCSCs have been linked to resistance to drugs and radiation in recent studies (15). Cytotoxic resistance is often due to elevated expression of drug pumps. Two pumps associated with resistance in breast cancer are BCRP and PGP (16). We observed higher expression of both pumps in mesenchymal cells relative to parental cells (Fig. 4A). Enhanced chemoresistance of M cells to mitoxantrone or etoposide (substrates for BCRP and PGP, respectively) suggests that these pumps are active (Fig. 4A).

Our mesenchymal tumor cells also have elevated expression of key DNA repair enzymes. One enzyme involved in drug resistance in human cancers is O\textsuperscript{6}-methylguanine DNA methyltransferase (MGMT), a ubiquitous DNA repair protein that removes O\textsuperscript{6}-alkylguanine lesions from damaged DNA and contributes to the resistance of brain cancers to \(\alpha\)-chloro-nitrosourea (BCNU; ref. 17). Figure 4B shows that M cells have high expression of MGMT (>200-fold higher relative to E cells) and are resistant to BCNU treatment (\(P = 0.0002\) at 20 \(\mu\)mol/L). Because human BCSCs are resistant to ionizing radiation (18), we evaluated this and the expression of double-stranded DNA repair pathway components (i.e., Brca2, H2afx, Mre11a, Prkdc, Rad52, and Xrcc6). Relative to E cells, M tumor cell lines showed elevated expression of H2afx and Xrcc6 (Fig. 4C). Activation of H2afx enhances DNA repair efficiency (19), and the KU-70 protein (encoded by Xrcc6) has a key role by recruiting the DNA-dependent protein kinase (20). In general, levels of double-stranded DNA repair genes were similar to E cells, except Prkdc, the gene encoding the catalytic subunit of the DNA-dependent protein kinase, whose expression was reduced in two M cell lines. Nonetheless, this altered expression of the double-stranded DNA repair pathway was associated with the strong resistance of M cells to \(\gamma\)-irradiation (\(P = 0.003\) at 16 Gy; Fig. 4C). Additional irradiation experiments showed that E cells were more sensitive to irradiation as compared with M cells when assessed by apoptosis assays (data not shown).

Gene expression of luminal or basal epithelial markers in mesenchymal cells suggests that EMT is incomplete. The normal mammary epithelium contains two layers, basal and luminal.
These layers are distinguished by unique cytokeratins (Ck; ref. 21). Ck5 and Ck14 are expressed by the basal layer and Ck8 and Ck18 by the luminal layer (21, 22). Because Ck expression is confined to epithelial cells, we investigated whether EMT was complete based on loss of Ck expression in our cell lines. E tumor cells have the characteristic signature of high Ck8 and low Ck14 expression consistent with their luminal differentiation (Fig. 5A). There was no consistent pattern of modulation of Cks observed in the mesenchymal cells. Whereas the M1 cell line showed loss of Ck8 and maintained low level of Ck14 expression, M2, M3, and M4 cell lines maintained Ck8 and increased Ck14 expression. Other frequently cited markers of luminal (EpCAM, MUC1, galectin 3, claudin 4, and Ck18) and basal (CD10, osteonectin, S100, myosin light chain, and integrin α6) epithelial phenotypes were also assessed (Fig. 5B and C). Of the luminal markers, EpCAM (P < 0.0001) and claudin 4 (P < 0.0001) were consistently down-regulated in all M lines relative to the E lines, and MUC1 was not expressed by any line. Like Ck8, Ck18 expression was inconsistent. Of the basal markers, only osteonectin was convincingly elevated (P < 0.0001), an expected finding given its expression in mesenchymal stem cells (ref. 23; Fig. 5C). The other basal markers did not change and failed to distinguish E from M tumor cells. Collectively, the results show that EMT is incomplete.

Table 1. Frequency of tumor development following injection of E and M cell lines

<table>
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<tr>
<th>Cell line</th>
<th>Tumors injected</th>
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*Numerator represents number of mice developing tumor; Denominator is number of mice injected; n.d.: not done.

These layers are distinguished by unique cytokeratins (Ck; ref. 21). Ck5 and Ck14 are expressed by the basal layer and Ck8 and Ck18 by the luminal layer (21, 22). Because Ck expression is confined to epithelial cells, we investigated whether EMT was complete based on loss of Ck expression in our cell lines. E tumor cells have the characteristic signature of high Ck8 and low Ck14 expression consistent with their luminal differentiation (Fig. 5A). There was no consistent pattern of modulation of Cks observed in the mesenchymal cells. Whereas the M1 cell line showed loss of Ck8 and maintained low level of Ck14 expression, M2, M3, and M4 cell lines maintained Ck8 and increased Ck14 expression. Other frequently cited markers of luminal (EpCAM, MUC1, galectin 3, claudin 4, and Ck18) and basal (CD10, osteonectin, S100, myosin light chain, and integrin α6) epithelial phenotypes were also assessed (Fig. 5B and C). Of the luminal markers, EpCAM (P < 0.0001) and claudin 4 (P < 0.0001) were consistently down-regulated in all M lines relative to the E lines, and MUC1 was not expressed by any line. Like Ck8, Ck18 expression was inconsistent. Of the basal markers, only osteonectin was convincingly elevated (P < 0.0001), an expected finding given its expression in mesenchymal stem cells (ref. 23; Fig. 5C). The other basal markers did not change and failed to distinguish E from M tumor cells. Collectively, the results show that EMT is incomplete.

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Discussion
Whereas great strides in breast cancer treatment have been made, women treated for the disease remain at high risk for recurrence. Recent hypotheses suggest that recurrence (and possibly tumor initiation) is caused by a subset of tumor cells with stem cell qualities including self renewal, ability to differentiate and reconstitute a tumor, and resistance to chemotherapeutic drugs and radiation. Whereas studies have confirmed the existence of this tumorigenic subset, their origin is unclear. Understanding the origin and biology of these cells may reveal strategies for targeting them therapeutically. In this study, we made three novel observations that provide an additional framework for BCSCs. These observations are that (a) EMT was induced in vivo by CD8 T cells; (b) EMT generates mesenchymal tumor cells with BCSC properties; and (c) EMT (and BCSC genesis) is not associated with complete loss of epithelial characteristics.

Although much evidence shows the importance of EMT in embryogenesis, investigation of its role in cancer has been confined mainly to in vitro model systems. As a result, the relevance of natural EMT to in vivo malignancy is controversial. In the current study, we found that EMT was induced in vivo without prior manipulation of tumor cells. Our conclusion that the tumor cells had undergone EMT was based on a number of molecular and cellular changes, including (a) loss of the epithelial markers CD24, E-cadherin, and claudin 3; (b) gain of validated mesenchymal markers N-cadherin and Snail; (c) gain of MMP activity; and (d) acquisition of a scattered phenotype. Prior studies showed that mesenchymal tumors develop in the neu-tg mouse, but it remained unclear whether the appearance of the mesenchymal tumors was
due to selection or induction (5, 6, 24). Our findings clarify this by showing CD8 T-cell–dependent EMT induction in vivo. Various immune effector cells are known to produce factors capable of inducing EMT, such as transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α; refs. 25, 26). Of those, the notable example is TGF-β. Whereas CD4 T regulatory cells are the predominant source of TGF-β among T cells, newer studies show that chronically stimulated effector CD8 T cells can also become regulatory and produce TGF-β (27). Another potential mediator is the recently discovered product of the EMAM3 gene, interleukin-like EMT inducer (ILEI). Although little is known of ILEI, it seems to be highly expressed in lymphocytes associated with chronic inflammatory lesions (e.g., arthritis and tumors; ref. 24). Bates and Mercurio (25) observed that TGF-β and TNF-α synergistically induced EMT in human colon cancer cells. The requirement of multiple stimuli for EMT is supported by our prior studies showing that engagement of the IFN-γ receptor is necessary for the generation of antigen-loss variants in vivo, but IFN-γ stimulation in vitro leads only to a modest loss of neu antigen (5). Whatever mechanism is operative, the induction of EMT and the generation of the BCSC phenotype were observed in vivo, suggesting that our results may be biologically relevant events. Our findings in mice are also consistent with prior studies showing that CD8 T-cell infiltration is associated with lymph node metastasis in breast cancer patients (28).

EMT was accompanied by the acquisition of the BCSC properties, including tumorigenicity, resistance to environmental insults, ability to re differentiate into an epithelial tumor, and ability to form spheroids. These findings are consistent with those of Mani and colleagues (29) who recently showed that forced expression of EMT-associated molecules such as Snail and Twist or treatment with TGF-β resulted in cells with a cancer stem cell phenotype. Similarly, Morel and colleagues (30) showed with the same cell lines that adding active Ras generated a population of CD24+/CD44− cells that underwent EMT and had BCSC attributes. One key characteristic of cancer stem cells called into question by our findings could be important for identifying evidence of EMT in breast cancer lesions with strict marker paradigms that include the BCSC phenotype (40). The implications of these data, we have found stemness associated with the CD24+/CD29hi and CD24+Sca1+ (21). In contrast to these data, we have found stemness associated with the CD24−/CD29lo cells. Although not directly addressed by our study, there are at least two possibilities to explain these discrepancies. First, there may be multiple types of cells generated during malignant transformation and progression that have BCSC-like properties. Second, markers used to identify BCSC traits may be variable and reflect the context or microenvironment from which the cells were derived, as has been described by Bissell and colleagues (38).

Although our results show that the tumor cells underwent EMT, as assessed using well-established markers, gene transcription analysis suggests that EMT was incomplete because luminal and basal epithelial associated genes remained expressed. These results are consistent with the observation that EMT is associated with some, but not all, genetic changes ordinarily associated with the stromal or mesenchymal phenotype (39). In other words, EMT may be incomplete or aberrant. Our results are also consistent with a recent study in human breast cancer that revealed that a small fraction (1-10%) of the disseminated CD4+ cells show the CD24−/CD29+ BCSC phenotype (40). The implications of our findings could be important for identifying evidence of EMT in breast cancer lesions with strict marker paradigms that include the absence of Cks and E-cadherin expression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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