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Poised Chromatin at the ZEB1 Promoter Enables Breast Cancer Cell Plasticity and Enhances Tumorigenicity

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SUMMARY

The recent discovery that normal and neoplastic epithelial cells re-enter the stem cell state raised the intriguing possibility that the aggressiveness of carcinomas derives not from their existing content of cancer stem cells (CSCs) but from their proclivity to generate new CSCs from non-CSC populations. Here, we demonstrate that non-CSCs of human basal breast cancers are plastic cell populations that readily switch from a non-CSC to CSC state. The observed cell plasticity is dependent on ZEB1, a key regulator of the epithelial-mesenchymal transition. We find that plastic non-CSCs maintain the ZEB1 promoter in a bivalent chromatin configuration, enabling them to respond readily to microenvironmental signals, such as TGFβ. In response, the ZEB1 promoter converts from a bivalent to active chromatin configuration, ZEB1 transcription increases, and non-CSCs subsequently enter the CSC state. Our findings support a dynamic model in which interconversions between low and high tumorigenic states occur frequently, thereby increasing tumorigenic and malignant potential.

INTRODUCTION

Metastatic dissemination and disease relapse are critical determinants of cancer prognosis. The mechanisms underlying both processes remain poorly understood. Recent advances in understanding cellular hierarchies present within a variety of tumors have changed our perspective of neoplastic cell population organization. In particular, cell-surface antigen markers have revealed distinct subpopulations of neoplastic cells within tumors, showing pronounced differences in tumor-initiating and metastatic powers (Visvader and Lindeman, 2012). Such evidence indicates that, within individual tumors, genetically identical cancer cells may nonetheless reside in distinct phenotypic states.

Importantly, tumors derived from implanting highly tumorigenic subpopulations of cells exhibit the phenotypic heterogeneity of their predecessor tumors in that they contain both highly and weakly tumorigenic cells (Visvader and Lindeman, 2012). Implicit is the notion that highly tumorigenic cells can self-renew and also divide asymmetrically into daughter cells with low tumorigenic potential. Parallels identified with cell hierarchies operating in normal adult tissues have led to coining of the term "cancer stem cell" (CSC) to describe the subset of neoplastic cells that reside in a highly tumorigenic state.

The simplest depiction would portray CSCs as residing at the apex of a cellular hierarchy and spawning, in a unidirectional manner, more differentiated non-CSC progeny. Cells in a number of cancer types conform to that model (Bonnet and Dick, 1997; Visvader and Lindeman, 2012). These studies imply that, once a CSC has exited the CSC state, it cannot re-enter it. This principle of unidirectionality holds great importance, given the significance of CSCs for cancer development and, quite possibly, progression to metastatic disease.

A small number of studies now suggest that not all cancers strictly conform to the unidirectional hierarchical CSC model. We and others have recently demonstrated that non-CSCs can acquire CSC-like activity under certain conditions (Chaffer et al., 2011; Gupta et al., 2011; Roesch et al., 2010). These studies open the door to the possibility that there is likely to be greater plasticity in cancer cell populations—yielding bidirectional interconversions between CSC and non-CSC states—than is depicted in the simplest version of the CSC model.

It has remained unclear whether these interconversions are confined to specific types of cancer, how frequently they occur in vivo, and how they are achieved mechanistically. These interconversions are potentially important for cancer diagnosis, prognosis, and therapy, given the now-extensive evidence that CSCs are intrinsically more prone to disseminate and, at the same time, exhibit resistance to many existing antitumor therapies (Dean et al., 2005; Malanchi et al., 2012). In the present study, we aimed to address the role of non-CSC-to-CSC conversions by

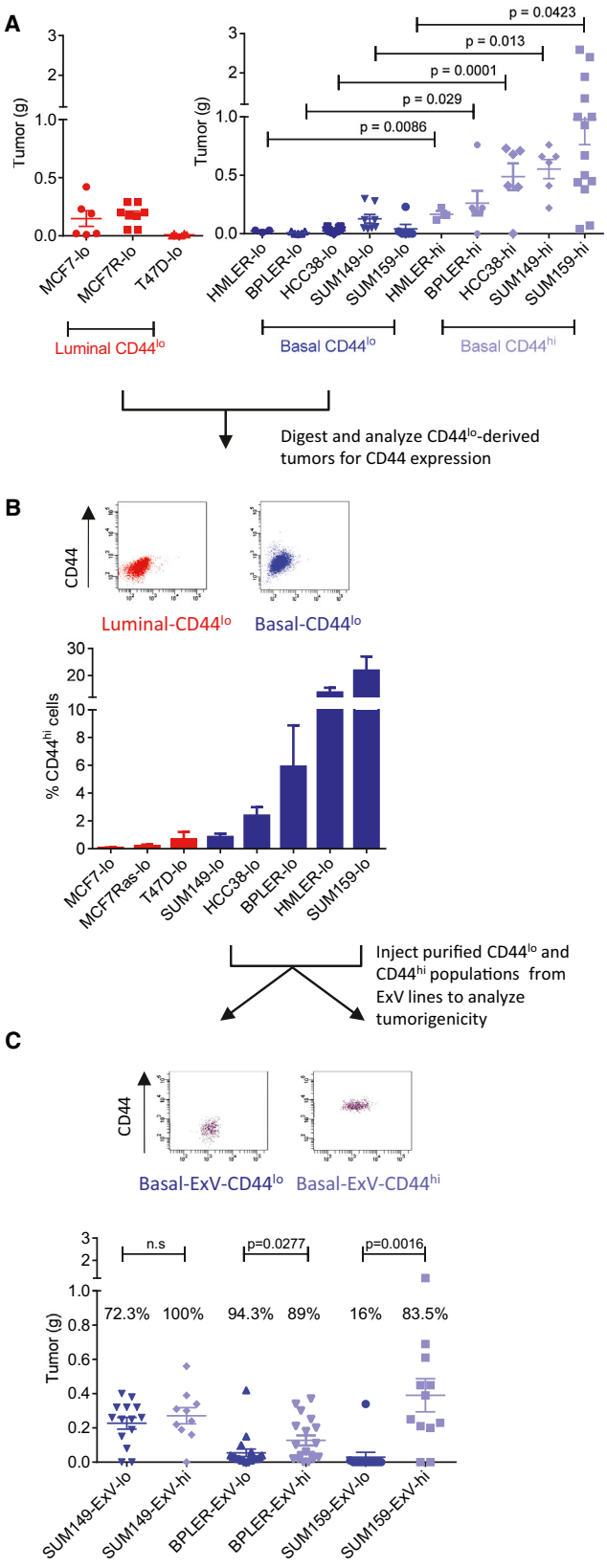


Figure 1. Basal Breast Cancer CD44^{lo} Non-CSC Cell Populations Spontaneously Switch to a CD44^{hi} CSC State In Vivo

(A) Tumorigenicity of FACS-purified luminal BrCa CD44^{lo} cells or basal BrCa CD44^{lo} and CD44^{hi} cell populations following orthotopic injection into NOD/SCID mice (n ≥ 6/group).

(B) Representative FACS plots for CD44 expression and quantification of CD44^{hi} cells generated from luminal or basal CD44^{lo}-derived tumors generated in (A).

(C) Basal CD44^{lo}-digested tumors from (B) were cultured in vitro to generate ex vivo cell lines (ExV). ExV lines were purified by FACS into CD44^{lo} and CD44^{hi} components and were injected orthotopically into NOD/SCID mice (n ≥ 8/group). Tumor incidence displayed as percentages on the graph.

Data are represented as mean ± SEM. See also Figure S1 and Tables S1–S4.

determining their frequency in a cohort of breast cancer (BrCa) cell lines.

In fact, we find that non-CSC-to-CSC conversions occur frequently in certain subtypes of BrCa, but not in others, and have uncovered a mechanism governing this transition. From a therapeutic standpoint, the plasticity that we describe suggests efforts to improve therapeutic outcome for cancer patients by specifically targeting CSCs must be further enhanced by coupling them with strategies designed to eliminate non-CSC-to-CSC interconversions or, at the very least, to eliminate the subpopulations of non-CSCs that are poised to become CSCs.

RESULTS

CD44 Status and Tumorigenic Potential

Cell-surface antigens, such as CD44, CD24, and ESA, have been successfully used to isolate CSC-like populations from BrCa cell lines and primary tissues. Among these antigens, it is widely accepted that breast CSCs are contained exclusively in the CD44^{hi} cell compartment (Al-Hajj et al., 2003; Fillmore and Kuperwasser, 2008; Mani et al., 2008; Visvader and Lindeman, 2012).

To test the notion that CD44^{hi} status on its own would allow enrichment of CSCs from BrCa cell lines, we analyzed five basal BrCa lines (SUM149, SUM159, HCC38, HMLER, and BPLER) and three luminal BrCa lines (MCF7, MCF7R, and T47D). (In the clinic, luminal BrCa generally are less aggressive and hold a better prognosis, whereas basal BrCa behave in the opposite fashion.) CD44^{hi} and CD44^{lo} populations were evident to various extents in all basal BrCa lines examined, whereas all luminal BrCa lines consisted only of CD44^{lo} populations (Figure S1A available online and data not shown). To compare the relative tumorigenic potentials of these subpopulations, we purified by fluorescence-activated cell sorting (FACS) CD44^{lo} fractions from the luminal lines and CD44^{lo} and CD44^{hi} populations from the basal lines. Purified populations were injected immediately into the mammary fat pads of NOD/SCID mice. In all of these experiments, cell populations were only implanted in hosts if they were greater than 99.7% pure as judged by FACS (Figures S1A–S1C).

We found that CD44^{lo} luminal lines required more cells and longer incubation times in vivo to generate tumors of equivalent size to those seeded by purified CD44^{lo} basal lines (luminal, 1 × 10^6 cells and 12–16 weeks in vivo; basal, 5 × 10^5 cells and 6–10 weeks in vivo; Figure 1A and Table S1). In

addition, basal CD44^{hi}-derived tumors were 3- to 40-fold larger than their CD44^{lo} counterparts when equal cell numbers were injected (Figure 1A and Table S2). Limiting dilution analysis showed that basal CD44^{hi} cell fractions were significantly enriched for CSC frequency compared to their CD44^{lo} counterparts (~10-fold, Figure S1D and Table S3). These data demonstrate that CD44^{hi} expression enriches for cells that naturally reside in basal BrCa cell lines and possess higher intrinsic tumor-initiating and growth potential. Moreover, they raised the question of how certain BrCa populations that apparently lacked all traces of tumor-initiating CSCs were able to generate tumors when injected into host mice.

CD44^{hi} CSCs Arising from Basal CD44^{lo} Cell Populations In Vivo

In previous work, we demonstrated that non-CSCs derived from experimentally transformed human mammary basal epithelial cells (HMECs) could spontaneously generate de novo CSCs both in vitro and in vivo (Chaffer et al., 2011). In the present work, we first undertook to test the idea that non-CSC-to-CSC conversions occur frequently in a broad array of BrCa cell lines.

Accordingly, we used FACS to analyze the tumors described above that arose from basal or luminal CD44^{lo} cells. We found that luminal CD44^{lo}-derived tumors comprised almost entirely CD44^{lo} cells with a small but detectable subpopulation (average, <0.32%) of CD44^{hi} cells. This suggested that luminal BrCa cells apparently lacking CD44^{hi} tumor-initiating cells were nonetheless able to seed tumors by generating new CD44^{hi} cells, albeit at a low frequency. In marked contrast to the behavior of luminal cells, basal CD44^{lo}-derived tumors contained CD44^{hi} subpopulations ranging in size from 2%–22% of tumor cells (Figure 1B). These findings indicate that basal CD44^{lo} populations efficiently generate CD44^{hi} populations in vivo, whereas luminal CD44^{lo} populations do so with dramatically lower efficiency.

Functional Analysis of CD44^{hi} Cells Created In Vivo

We next sought to demonstrate that CD44^{hi} cells arising in vivo from basal CD44^{lo} cells were functionally equivalent to CSCs that are naturally present in basal BrCa cell lines. To begin, we derived several cell lines from tumors arising from implanted CD44^{lo} basal cells (SUM149-, SUM159- and BPLER-CD44^{lo} tumors) depicted in Figure 1A, terming them ExV (reflecting their ex vivo derivation). Each of these tumor-derived ExV-cell lines contained both CD44^{lo} and CD44^{hi} cells (Figure 1B), which we termed ExV-CD44^{lo} and ExV-CD44^{hi} cells. We then used FACS to isolate pure (>99%) populations of ExV-CD44^{lo} and ExV-CD44^{hi} cells and immediately injected them orthotopically into NOD/SCID mice (Figures 1C and S1E–S1G). In most cases, ExV-CD44^{hi} cells generated 13- to 23-fold larger tumors compared to their ExV-CD44^{lo} counterparts (SUM159 and BPLER ExV lines) and displayed increased tumor-initiating ability (SUM159 and SUM149 ExV lines) (Figure 1C and Table S4). These results illustrate that CD44^{hi} cells arising in vivo from basal CD44^{lo} cells behave much like the CSCs that are naturally present in basal BrCa cell populations in that they exhibit higher tumor-initiating and tumor growth potential than their CD44^{lo} counterparts.

ZEB1 Drives CD44^{lo}-to-CD44^{hi} Cellular Plasticity

We then undertook to shed light on the mechanism(s) enabling the observed in vivo CD44^{lo}-to-CD44^{hi} conversions. Given the inherent difficulties of uncovering these mechanisms in an in vivo setting, we first sought mechanistic insights from an in vitro model system that we had previously developed in which nontransformed basal mammary epithelial cells (HME-flopc cells) spontaneously undergo CD44^{lo}-to-CD44^{hi} conversions with high frequency (Chaffer et al., 2011). As demonstrated at the time, conversions of nontransformed immortalized human mammary epithelial cells closely paralleled the behavior of their corresponding transformed derivatives.

In this instance, we also drew from earlier work demonstrating that CD44^{hi} stem-like cells (SC) are more mesenchymal than their CD44^{lo} counterparts (Chaffer et al., 2011; Mani et al., 2008). This and subsequent work (Guo et al., 2012) demonstrated that passage through the cell-biological program termed the epithelial-mesenchymal transition (EMT) placed cells close to the epithelial SC state. (This EMT program is largely studied for its ability to confer mesenchymal traits on epithelial cells.) Accordingly, we purified CD44^{lo} and CD44^{hi} subpopulations from HME-flopc cells and confirmed that CD44^{hi} cells, which contained the SC-like cells, indeed resided in a more mesenchymal state than their more epithelial CD44^{lo} counterparts (Figure 2A). Subsequently, to identify a key mediator of non-CSC-to-CSC plasticity, we analyzed the expression of various transcription factors (EMT-TFs) known to govern the EMT program. Here we found that *ZEB1* expression was significantly higher (~10-fold) in CD44^{hi} compared to CD44^{lo} cells (Figure 2B).

To determine whether ZEB1 contributed in a critical way to mediating the transition from the CD44^{lo} to CD44^{hi} state, we analyzed CD44^{lo}-to-CD44^{hi} conversions in HME-flopc-CD44^{lo} cells expressing either doxycycline-inducible control or *ZEB1*-targeted shRNAs. We first confirmed that, following doxycycline-mediated induction, each shRNA vector was capable on its own of achieving 80%–90% ZEB1 knockdown (ZEB1-kd) in CD44^{hi} cells. We also noted no differences in cell proliferation between cell populations expressing the control shRNA and those expressing shRNAs targeting *ZEB1* mRNA (Figures S2A–S2B).

We proceeded to purify CD44^{lo} cells expressing the various shRNA vectors by FACS, introduced them into two-dimensional (2D) cultures, and used FACS to monitor resulting cultures propagated in the presence or absence of doxycycline over the next 16 days. In the continued presence of doxycycline, the ability of CD44^{lo} ZEB1-kd cells to convert into the CD44^{hi} state was reduced by 75% (sh1) and 67% (sh2) relative to cultures expressing the control shRNA (Figure 2C). However, when doxycycline was withdrawn at day 8, permitting cells to continue growing in the absence of doxycycline for an additional 8 days, FACS analysis showed that CD44^{lo} ZEB1-kd cells soon regained their ability to convert to the CD44^{hi} state (Figure 2C). As such, the ability of CD44^{lo} cells to activate ZEB1 expression appeared to be an important determinant of their ability to enter into the CD44^{hi} state and thus an important determinant of cell plasticity in this model system.

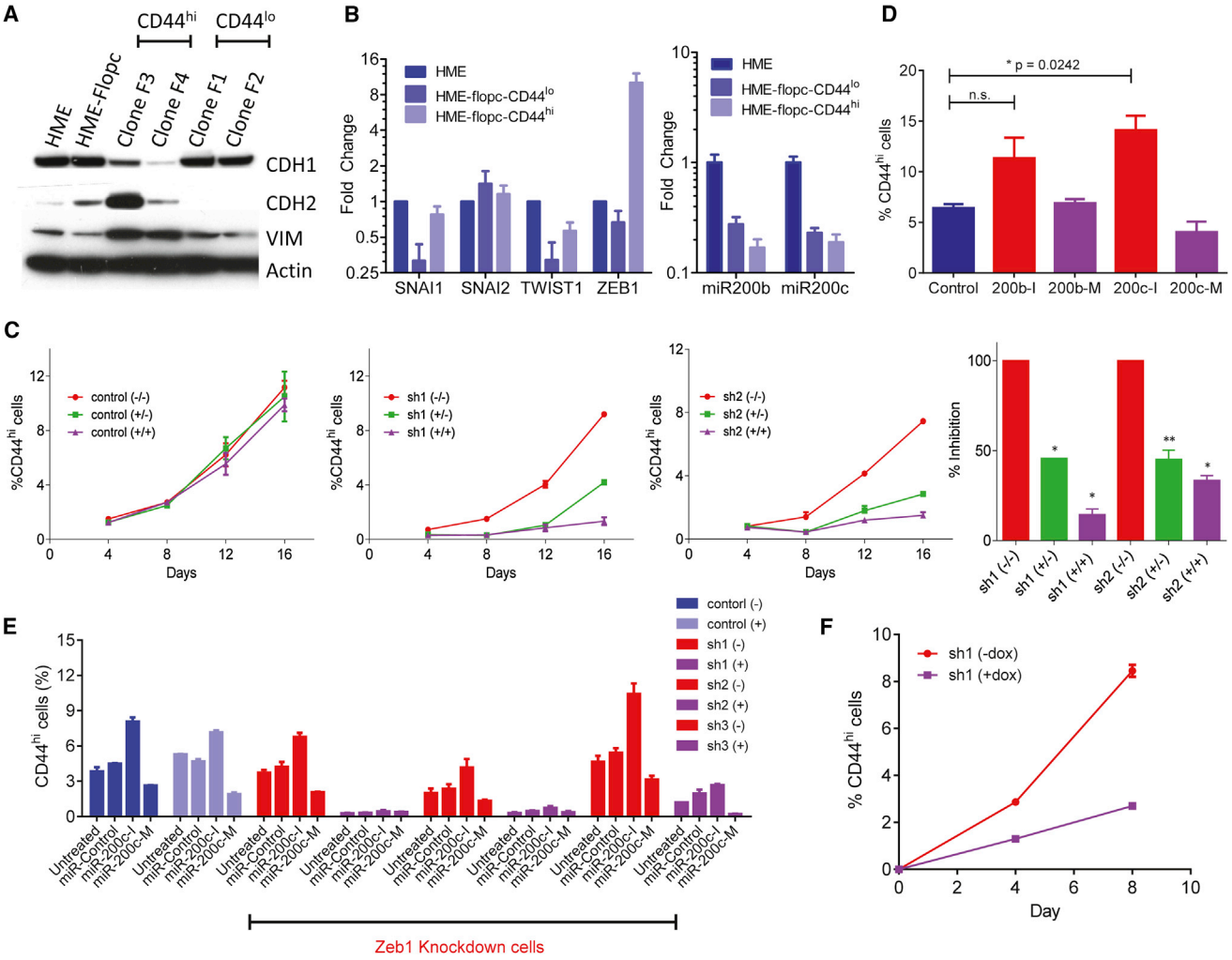


Figure 2. ZEB1 Is an Essential Mediator of CD44^{lo}-to-CD44^{hi} Cell Transitions

(A) Western blot for markers of the epithelial (CDH1) or mesenchymal (CDH2, VIM) phenotype in immortalized human mammary epithelial cells (HME), HME-flopc, and single cell clones derived from HME-flopc population enriched for the CD44^{lo} phenotype (clones F1 and F2) or CD44^{hi} phenotype (clones F3 and F4).

(B) qPCR for EMT transcription factors and *MIR200B/C* in nontransformed CD44^{lo} (HME and HME-flopc-CD44^{lo}) or HME-flopc-CD44^{hi} cells.

(C) FACS analysis for the ability of HME-flopc-CD44^{lo} cells to switch to the CD44^{hi} cell state. Cells express a doxycycline (dox)-inducible control shRNA (control) or shRNA targeting *ZEB1* (sh1 and sh2). −/−, no dox; +/−, dox on for 8 days then removed for the remaining 8 days; +/+, dox on for the duration of the experiment. Inhibition (%) at day 16 is also shown (*p < 0.0001, **p < 0.0008, compared to −/−).

(D) Purified HME-flopc-CD44^{lo} cells treated with *MIR200B/C* inhibitors (I) or mimetics (M) to determine effects on switching from CD44^{lo} to CD44^{hi} cell state. Data are mean ± SEM.

(E) Purified HME-flopc-CD44^{lo} cells expressing a dox-inducible control shRNA (control) or shRNA targeting *ZEB1* (sh1, sh2, or sh3) were analyzed for their ability to switch to the CD44^{hi} state in the presence (+) or absence (–) of dox and in response to a *MIR200C* inhibitor (I) or mimetic (M).

(F) Transformed HME-flopc-CD44^{lo} cells (with *SV40-Early Region* and *RAS* oncoprotein) expressing a dox-inducible shRNA targeting ZEB1 (sh1) were analyzed for conversion to the CD44^{hi} state in the presence (+) or absence (–) of ZEB1 knockdown. Cells were monitored by FACS for 8 days.

Data are represented as mean ± SEM. See also Figure S2.

Modulation of CD44^{lo}-to-CD44^{hi} Conversions by the MIR200 Family

The expression of the *ZEB1* gene is tightly regulated by an interactive network involving ZEB1 itself, its relative ZEB2, and members of the MIR200 family of microRNAs (Gregory et al., 2008; Wellner et al., 2009). Thus, ZEB1 can serve to repress expression of the MIR200 miRNAs, whereas the latter can both inhibit the function and/or reduce the stability of the mRNAs specifying ZEB1 and ZEB2; hence, these mutually antagonistic elements constitute a circuit that operates as a bistable switch, governing the residence of cells in either the mesenchymal or epithelial state. Accordingly, we confirmed that ZEB1-kd in our system resulted in a decrease in *ZEB2* mRNA and concomitant increases in *MIR200B* and *MIR200C* levels (Figures S2A and S4).

In light of the mutually antagonistic actions of ZEB1 and the MIR200 miRNAs, we determined whether addition of synthetic

inhibitors or mimetics (chemically synthesized, single-stranded, modified RNAs) influenced spontaneous $CD44^{lo}$-to-$CD44^{hi}$ conversions in HME-flopc-$CD44^{lo}$ cells. Indeed, as we found, miR200b or miR200c inhibitors significantly increased the rate of $CD44^{lo}$-to-$CD44^{hi}$ conversions (Figure 2D).

We next undertook to determine whether the ability of the MIR200 family to affect $CD44^{lo}$-to-$CD44^{hi}$ conversions derived largely from modulation of *ZEB1* transcript levels or, alternatively, from the involvement of other MIR200 targets. Here we found that, in the presence of ZEB1-kd, the miR200c inhibitor was unable to provoke a $CD44^{lo}$-to-$CD44^{hi}$ conversion (Figure 2E).

Together, these various results highlight two important points: (1) ZEB1 is a key mediator of spontaneous $CD44^{lo}$-to-$CD44^{hi}$ conversions in nontransformed HMECs, acting through repression of the *MIR200* family and, quite possibly, other still-unidentified targets and (2) *MIR200* family modulation of $CD44^{lo}$-to-$CD44^{hi}$ conversion derives from effects on *ZEB1* mRNA levels.

We extended these observations by examining the consequences of *ZEB1* knockdown in HME-flopc-$CD44^{lo}$ cells that had previously been transformed with *SV40-Early Region* and oncogenic *RAS* in vitro (Figure 2F). We found that similar dynamics observed previously in the untransformed HME-flopc cells operated in their transformed derivatives.

Together, these results demonstrat that ZEB1 is a key mediator of $CD44^{lo}$-to-$CD44^{hi}$ conversions in both nontransformed and transformed HMECs in vitro and support the previously reported notion that the dynamics of epithelial versus mesenchymal plasticity are quite similar in hTERT-immortalized cells and their transformed derivatives (Chaffer et al., 2011).

ZEB1/MIR200c Are Differentially Expressed in BrCa Cell Populations

To determine whether ZEB1 functions as a key player in driving BrCa cell plasticity, we assessed whether ZEB1 and MIR200 expression was indicative of the $CD44^{lo}$ versus $CD44^{hi}$ state in a broader array of BrCa lines. To do so, we first analyzed ZEB1 protein expression in luminal, basal $CD44^{lo}$, and basal $CD44^{hi}$ BrCa cell lines. ZEB1 was not detected in all luminal lines; however, in basal BrCa cell lines, ZEB1 was detectable in both populations, being 4-fold higher in matched pairs of $CD44^{hi}$ compared to $CD44^{lo}$ cells (Figure 3A).

Given the tight regulatory loop between ZEB1 and the *MIR200* family, we next examined how ZEB1 protein levels are regulated in these BrCa cell lines. Accordingly, we analyzed *ZEB1*, *MIR200B*, and *MIR200C* mRNA expression levels. We found that luminal lines expressed very low levels of *ZEB1* mRNA and very high levels of *MIR200B/C*. Conversely, basal $CD44^{lo}$ cells generally expressed modest but nonetheless detectable levels of both *ZEB1* mRNA and *MIR200B/C*, whereas basal $CD44^{hi}$ cells expressed high levels of *ZEB1* mRNA and low-to-absent *MIR200B/C* levels (Figures 3B and 3C). We further confirmed that these patterns of *ZEB1* and *MIR200B/C* differential expression were maintained in the basal ExV-$CD44^{lo}$ and ExV-$CD44^{hi}$ cell populations (Figure S3A).

These observations demonstrate that, as predicted from their known interactions, the expression of ZEB1 and *MIR200B/C* varies inversely in these various BrCa cell lines and that the ZEB1 EMT-TF is expressed at far higher levels in basal BrCa cells, which represent a class of tumors that generally carry worse clinical prognosis. Moreover, they indicated that the expression pattern initially observed in the HME-flopc cells lines was closely echoed by the human basal BrCa lines examined.

Role of ZEB1-Mediated $CD44^{lo}$-to-$CD44^{hi}$ Conversions in Tumor Initiation and Growth

The data implicating ZEB1 in $CD44^{lo}$-to-$CD44^{hi}$ cell plasticity in vitro did not shed light on whether it plays a similar role in vivo, specifically in basal BrCa cells. Consequently, we introduced the same doxycycline-inducible control or ZEB1-targeted shRNAs used earlier into cell populations of the HCC38 and SUM159 human breast cancer cell lines and the experimentally transformed HMLER cells (Figure S3B). We confirmed that ZEB1-kd achieved in the presence of doxycycline did not affect cell proliferation rates in monolayer culture (Figure S3C). FACS-purified $CD44^{lo}$ populations of control or doxycycline-induced ZEB1-kd cells were then injected orthotopically into NOD/SCID mice immediately following FACS purification in order to analyze the effects of ZEB1-kd on $CD44^{lo}$-to-$CD44^{hi}$ conversions in vivo and on tumorigenicity. Animals were administered doxycycline (2 g/1 L) for the duration of the experiment.

Strikingly, HMLER-$CD44^{lo}$ cells gave rise to tumors of 0.1 g on average after 8–10 weeks of growth in vivo, whereas their ZEB1-kd counterparts failed to form tumors (Figure 3D), indicating that ZEB1-mediated $CD44^{lo}$-to-$CD44^{hi}$ conversions were essential for tumor-initiating potential. That is, the ability of HMLER-$CD44^{lo}$ carcinoma cells to initiate tumors appeared to depend critically on the ability of these cells to spontaneously generate CSCs in vivo, which depended in turn on their ability to activate expression of their own endogenous *ZEB1* gene. Similar results were obtained with HCC38-$CD44^{lo}$ cells. In SUM159-$CD44^{lo}$ ZEB1-kd cells, tumor size was significantly decreased (0.39 g average for controls; 0.03 g and 0.18 g on average for sh1 and sh2, respectively), and tumor-initiating potential was decreased from 100% in control cells to 67% in ZEB1-kd cells. Together, these data demonstrate that the ability of $CD44^{lo}$ basal BrCa cell populations to upregulate ZEB1 expression is generally a critical determinant of their tumor-initiating potential and overall tumor growth.

Role of ZEB1 in the $CD44^{hi}$ Stem Cell State

Given the importance of ZEB1 in enabling spontaneous $CD44^{lo}$-to-$CD44^{hi}$ conversions, we wondered whether the continued expression of ZEB1 was required thereafter for maintenance of the resulting $CD44^{hi}$ cell state; alternatively, other regulatory loops might become activated that then obviate the need for high ZEB1 expression for initial entrance into the $CD44^{hi}$ state. Accordingly, we used FACS to monitor the ability of purified nontransformed HME-flopc-$CD44^{hi}$ cells to maintain their $CD44^{hi}$ marker profile in culture over a 16 day period in the presence or absence of ZEB1-kd. Interestingly, we found that $CD44^{hi}$ ZEB1-kd cells maintained their $CD44^{hi}$ marker profile (Figures 4A and S4).

Next, we functionally tested the SC activity of $CD44^{hi}$ ZEB1-kd cells by mammosphere-forming ability in 3D culture (Dontu et al.,

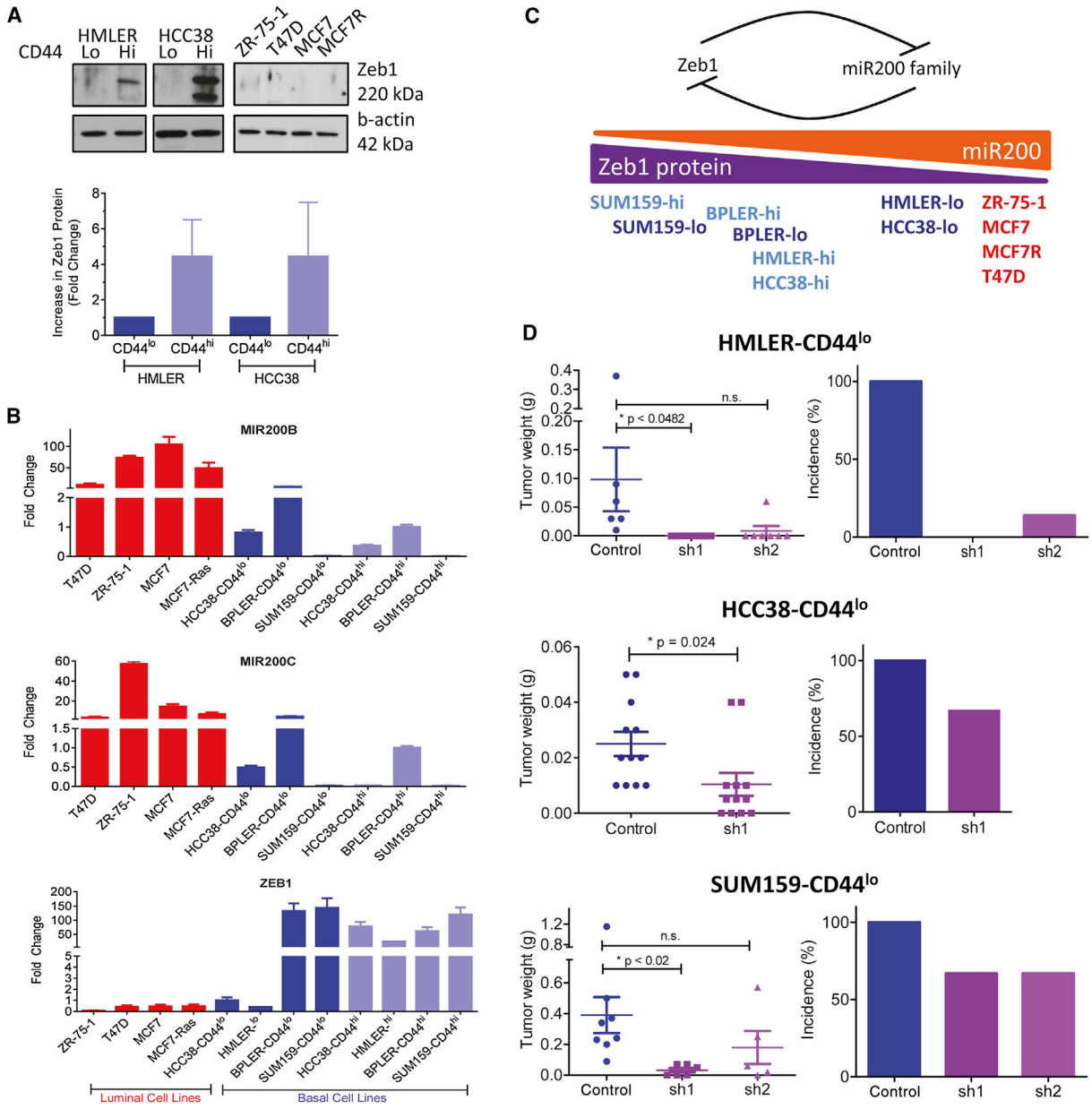


Figure 3. Inhibition of CD44^{lo}-to-CD44^{hi} Conversions by Blocking ZEB1 Decreases Tumorigenicity

(A) Western blot comparing the expression of ZEB1 in basal BrCa cell lines (HMLER and HCC38) purified for CD44^{lo} or CD44^{hi} subpopulations, and luminal BrCa cell lines (ZR-75-1, T47D, MCF7 and MCF7R). Quantification of differential ZEB1 expression in basal cell lines (n = 4).

(B) qPCR assessing *ZEB1*, *MIR200B*, and *MIR200C* mRNA expression in BrCa cell lines.

(C) Schematic illustrating expression of *MIR200* family members and ZEB1 protein expression in basal (CD44^{lo} and CD44^{hi} subpopulations) and luminal BrCa cell lines.

(D) Purified CD44^{lo} cells from HMLER or HCC38 and SUM159 basal BrCa cell lines expressing dox-inducible control shRNA (control) or shRNA targeting *ZEB1* (sh1 and sh2) were analyzed for tumorigenic potential. Final tumor mass and incidence are represented (n $\geq$ 5/group).

Data are represented as mean ± SEM. See also Figure S3.

2003). We found that mammosphere formation was reduced by 80%–99% compared to control. Furthermore, addition of miR200 inhibitors to CD44^{hi} ZEB1-kd cells could not restore mammosphere-forming ability (Figure 4B). Together, these data demonstrate that ZEB1 is required for initial acquisition of both CD44^{hi} expression and stem-like activity of CD44^{hi} cells and subsequent maintenance of SC activity but is not required for long-term maintenance of high cell-surface CD44 expression.

We further examined the effect of ZEB1-kd in HME-flopc-CD44^{hi} cells that had been transformed by the introduction of

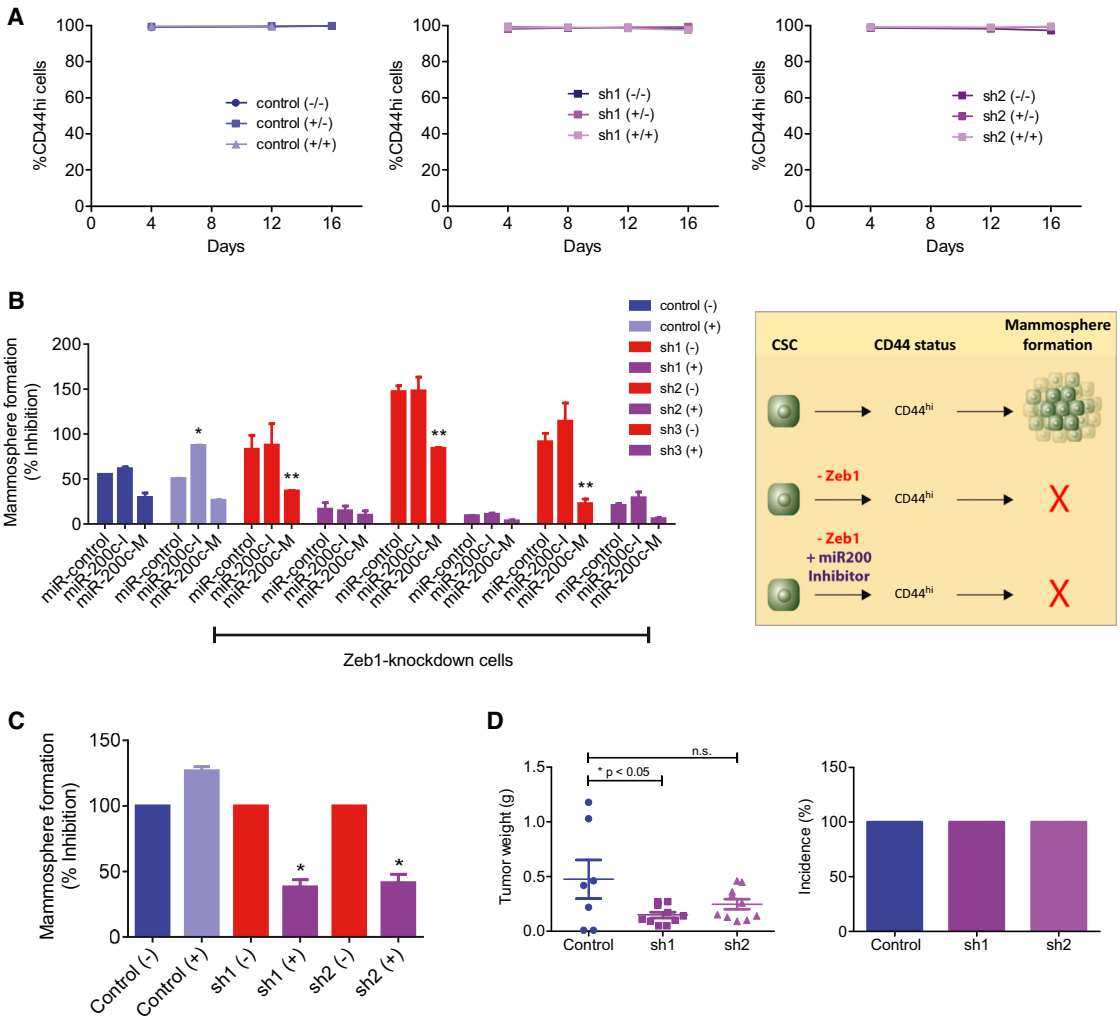


Figure 4. ZEB1 Is Essential for the Stem Cell/CSC Activity of CD44^{hi} Cells

(A) FACS analysis for CD44 expression in purified HME-flopc-CD44^{hi} cells. Cells express a doxycycline (dox)-inducible control shRNA (control) or shRNA targeting *ZEB1* (sh1 and sh2). −/−, no dox; +/−, dox on for 8 days then removed for the remaining 8 days; +/+, dox on for the duration of the experiment. The percentage of spontaneously arising CD44^{lo} cells was determined by FACS over a 16 day time period.

(B) Purified HME-flopc-CD44^{hi} cells expressing control or shRNA-targeting *ZEB1* (sh1, sh2, and sh3) were assessed for mammosphere-forming ability with or without dox induction. Cells were treated with a *MIR200C* inhibitor (I) or mimetic (M). $p < 0.001$, two-way ANOVA followed by Tukey's multiple comparisons test. *, different to miR-control and miR-200c-M; **, different to miR-control and miR-200c-I.

(C) Transformed HME-flopc-CD44^{hi} cells expressing control, sh1, or sh2 were assessed for mammosphere formation with or without dox induction ($p < 0.0001$, one-way ANOVA followed by Tukey's multiple comparisons test; *, different to sh(–)).

(D) Transformed HME-flopc-CD44^{hi} cells (control, sh1, and sh2) were purified by FACS and implanted into the fat pad of NOD/SCID mice (n = 8/group). Tumor weight and incidence are shown.

Data are represented as mean ± SEM. See also Figure S4.

SV40-Early Region and oncogenic *RAS* genes, i.e., the HMLER-flopc cells. Similarly, FACS analysis confirmed that transformed CD44^{hi} ZEB1-kd cells maintained their CD44^{hi} phenotype (data not shown). We also observed that ZEB1-kd decreased tumorsphere formation, an in vitro surrogate measure of CSC-like activity (60% inhibition, $p < 0.003$), and significantly reduced tumor burden in vivo ($p < 0.05$) (Figures 4C and 4D).

To summarize, together with our earlier results, these findings showed that ZEB1 is required for conversion from the CD44^{lo} to CD44^{hi} state and also for maintenance of CD44^{hi} stem-like/CSC-like activity. Once cells are residing in the CD44^{hi} state, however, ZEB1 and CD44 expression can be uncoupled, in that cells with ZEB1-kd functionally lose their stem-like/CSC features while still maintaining high CD44 expression. Stated differently, these data suggest that CD44^{hi} cells can constitute heterogeneous cell populations in which CD44^{hi}ZEB1^{+} signifies great enrichment of cells residing in a CSC-like state, whereas CD44^{hi} ZEB1^{-} signifies cells that are non-CSCs.

Phenotypic Plasticity and Chromatin Configuration of the *ZEB1* Promoter

The above-described experiments provided clear indication that ZEB1 function is necessary for the generation of CD44^{hi} stem-like cells from CD44^{lo} cells. Still, these observations did not provide insight into why ZEB1 was readily induced in basal CD44^{lo} cells but not in luminal CD44^{lo} cells. We reasoned that current models of epigenetic regulation might illuminate these differences in behavior, as global epigenetic differences have been observed between luminal and basal-type BrCa (Maruyama et al., 2011). More specifically, we chose to use chromatin immunoprecipitation followed by quantitative real-time PCR (ChIP-qPCR) to examine the chromatin state at the *ZEB1* promoter.

The functional state of chromatin has been defined largely by patterns of covalent modifications to the N-terminal domains of histones and is indicative of transcriptional activity. Thus, trimethylation of lysine 4 of the histone H3 subunit (H3K4me3) is associated with transcriptional initiation (Guenther et al., 2007), dimethylation of lysine 79 of the same subunit (H3K79me2) is associated with transcriptional elongation (Mueller et al., 2007), and the combination of H3K4me3 and H3K79me2 indicates an actively transcribed gene. In contrast, trimethylation of lysine 27 (H3K27me3) is often associated with transcriptional repression mediated by the Polycomb group of proteins (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002).

In embryonic stem (ES) cells, the promoters of many genes encoding key developmental regulators are associated with both the permissive H3K4me3 and the restrictive H3K27me3 modifications (yielding so-called ''bivalent'' domains) (Bernstein et al., 2006). This bivalent chromatin is thought to keep these genes repressed but nonetheless poised for rapid transcriptional activation in response to subsequent signaling decisions favoring differentiation. These findings indicate that this combination of histone modifications may be a signature of regulators that are required to rapidly switch cell state. Indeed, through publically available databases, we determined that ZEB1 maintains a bivalent chromatin configuration in ES cells (Figure S5A).

We speculated that ZEB1 might exhibit a bivalent chromatin state in those cells in which it was possible to switch between low and high ZEB1 expression. To pursue this notion, we first analyzed purified CD44^{lo} or CD44^{hi} HME-flopc populations. CD44^{hi} cells displayed chromatin methylation patterns indicating active transcription at the *ZEB1* promoter, as determined by the presence of both H3K4me3 and H3K79me2 marks (Figures 5A and 5B). In contrast, we found that CD44^{lo} cells exhibited bivalent chromatin methylation patterns at the *ZEB1* promoter, as determined by the presence of both H3K4me3 and H3K27me3. These data indicate that immortalized untransformed basal mammary epithelial cells with the ability to spontaneously switch to a CD44^{hi} stem-like state do indeed maintain their *ZEB1* promoter in a poised, bivalent configuration.

We wished to extend these observations to human BrCa cell lines, including luminal, basal CD44^{lo}, and basal CD44^{hi} cell populations. Our previous data had shown that luminal cell lines express very low levels of *ZEB1* mRNA (Figures 3A and 3B). Perhaps unsurprisingly, we found that all luminal cell lines exhibited only repressive chromatin methylation patterns at *ZEB*1, as determined by the presence of H3K27me3 and the relative absence of both H3K4me3 and H3K79me2 (Figure 5C). Further, we found that the chromatin at the *ZEB1* promoter in basal CD44^{hi} cells is characterized by the presence of H3K4me3 and H3K79me2 and the relative absence of H3K27me3, indicating active transcription. These chromatin modifications conform with our earlier expression data, indicating that all basal CD44^{hi} cells express high levels of ZEB1 (Figures 3A–3C).

Provocatively, we found that the *ZEB1* promoter in basal CD44^{lo} BrCa cells resided in a bivalent chromatin state in which both H3K4me3 and H3K27me3 modifications were detected, much as we had found in the immortalized CD44^{lo} HME cells. To demonstrate that both histone modifications reside simultaneously in specific regions in the basal CD44^{lo} BrCa cells, we performed sequential-ChIP analysis for H3K4me3 followed by H3K27me3 ChIP, as well as the reverse ChIP experiment (Figure S5B). Together, these results demonstrate that both immortalized and neoplastic basal CD44^{lo} populations maintain ZEB1 in a unique bivalent chromatin state poised for activation and provide a mechanistic insight as to why *ZEB1* is readily induced in basal CD44^{lo} cells, but not in luminal CD44^{lo} cells, which maintain the *ZEB1* promoter in a repressed state.

Microenvironmental Stimuli and CD44^{lo}-to-CD44^{hi} Conversions

Extensive evidence indicates that activation of the EMT program and entrance into a stem cell state is generally triggered by contextual signals received by normal and neoplastic epithelial cells (Mani et al., 2008; Thiery et al., 2009). Among these signals, TGFβ has been shown to potently upregulate ZEB1 expression (Gregory et al., 2011). Accordingly, we examined whether TGFβ could enhance the spontaneous CD44^{lo}-to-CD44^{hi} transitions of immortalized HMECs. Indeed, TGFβ induced a dose-dependent increase, whereas the SB431542 TGFβ receptor inhibitor inhibited such conversions. In the context of ZEB1-kd, however, the ability of TGFβ to induce transitions was abolished (Figures 6A and S6A and S6B). Similarly, we also found that transformed HME-flopc-CD44^{lo} cells could not transition from the CD44^{lo} to CD44^{hi} state in response to TGFβ if ZEB1 expression was inhibited (Figure 6B). Hence, TGFβ can enhance CD44^{lo}-to-CD44^{hi} transitions in both normal and transformed cells in a fashion that is dependent upon induction of ZEB1.

To further confirm that TGFβ-driven CD44^{lo}-to-CD44^{hi} conversions were achieved through ZEB1, we showed that *ZEB1* mRNA was induced and inhibited by TGFβ and SB431542 treatment, respectively (Figure S6C). As controls, we found that TGFβ target genes (PAI-1 and GADD45B) were induced and repressed in these cells in response to modulation of TGFβ signaling (Figure S6C), confirming that, overall, TFGβ signaling operated in these cells as anticipated.

We extended these findings to a series of human BrCa cell lines and speculated that basal CD44^{lo} cells, but not luminal CD44^{lo} cells, would readily transit to a CD44^{hi} state in response to TGFβ. To test this notion, luminal (MCF7Ras and ZR-75-1) and basal (HMLER and HCC38) CD44^{lo} cells were treated for 4 days with TGFβ (2 or 20 ng/ml). FACS analysis at day 4 showed that the luminal cells did not generate any CD44^{hi} cells in

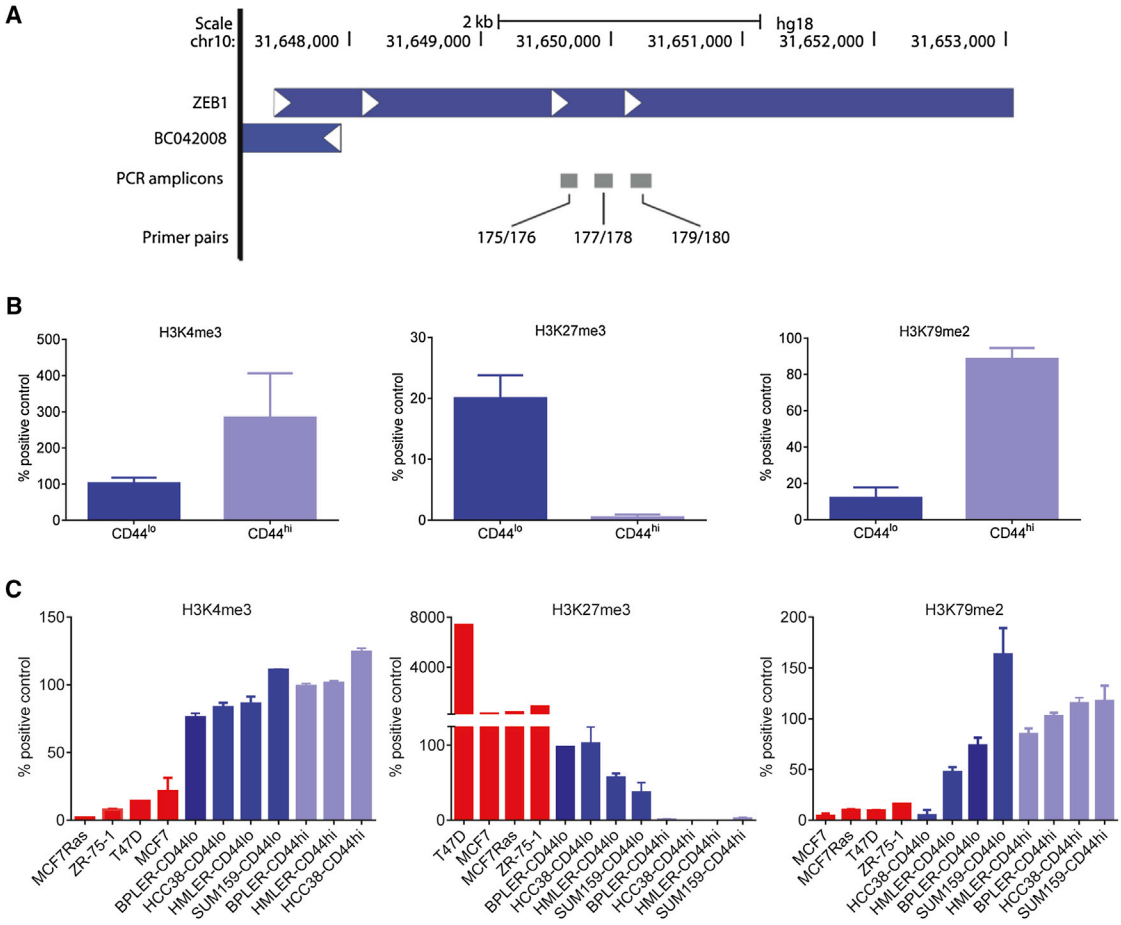


Figure 5. The ZEB1 Promoter Is Maintained in a Bivalent Chromatin State in Basal CD44^{lo} Non-CSCs

(A) Schematic showing the location of primer sets used for ChIP-qPCR.

(B and C) ChIP-qPCR for the H3K4me3, H3K27me3, and H3K79me2 histone modifications at the *ZEB1* promoter in (B) nontransformed CD44^{lo} or CD44^{hi} cells and (C) luminal CD44^{lo} cells and basal CD44^{lo} and CD44^{hi} sorted populations.

Data are mean ± SEM of biological duplicates performed as technical replicates. See also Figure S5.

response to TGFβ treatment, whereas the basal CD44^{lo} cells responded in a dose-dependent manner to TGFβ by generating CD44^{hi} cells (Figure 6C). We further demonstrate that TGFβ stimulation led to an increase in ZEB1 protein levels (Figure S6E). Together, these data indicate that one important contextual signal, TGFβ, enhances the rate of CD44^{lo}-to-CD44^{hi} transitions in basal breast cancer CD44^{lo} cell populations and that this response is dependent upon induction of ZEB1 expression.

Modulation of the Chromatin Status at the ZEB1 Promoter by TGFβ

Having shown that TGFβ induces CD44^{lo}-to-CD44^{hi} conversions in basal CD44^{lo} cells, we examined whether this effect coincided directly with changes in the histone modification patterns at the *ZEB1* promoter. To do so, we performed ChIP-qPCR on basal HME-flopc-CD44^{lo} cells expressing doxycycline-inducible *ZEB1*-shRNA treated with control (HCl), TGFβ (2 ng/ml), or the SB431542 TGFβ receptor inhibitor (10 μM).

To maintain a homogeneous CD44^{lo} population, performing this experiment in a ZEB1-kd intracellular environment was essential; otherwise, TGFβ treatment would cause the CD44^{lo} cells to transition to a CD44^{hi} state, in which case, the CD44^{hi} cells harboring active histone modifications at the *ZEB1* promoter would mask any changes occurring specifically in CD44^{lo} cells.

ZEB1 knockdown in basal CD44^{lo} cells was induced by exposure to doxycycline for 5 days, and cells were subsequently treated with TGFβ, SB431542, or control (PBS or DMSO). After an additional 5 days in the continued presence of doxycycline, FACS analysis confirmed that the CD44^{lo}-ZEB1-kd cells remained as pure CD44^{lo} populations (Figure 6D).

We then performed ChIP-qPCR at the *ZEB1* promoter to compare changes in the levels of histone modifications in control versus TGFβ or SB531542 treatment. Though no significant differences in the H3K4me3 or H3K79me2 methylation patterns were observed across all treatment groups, we found that

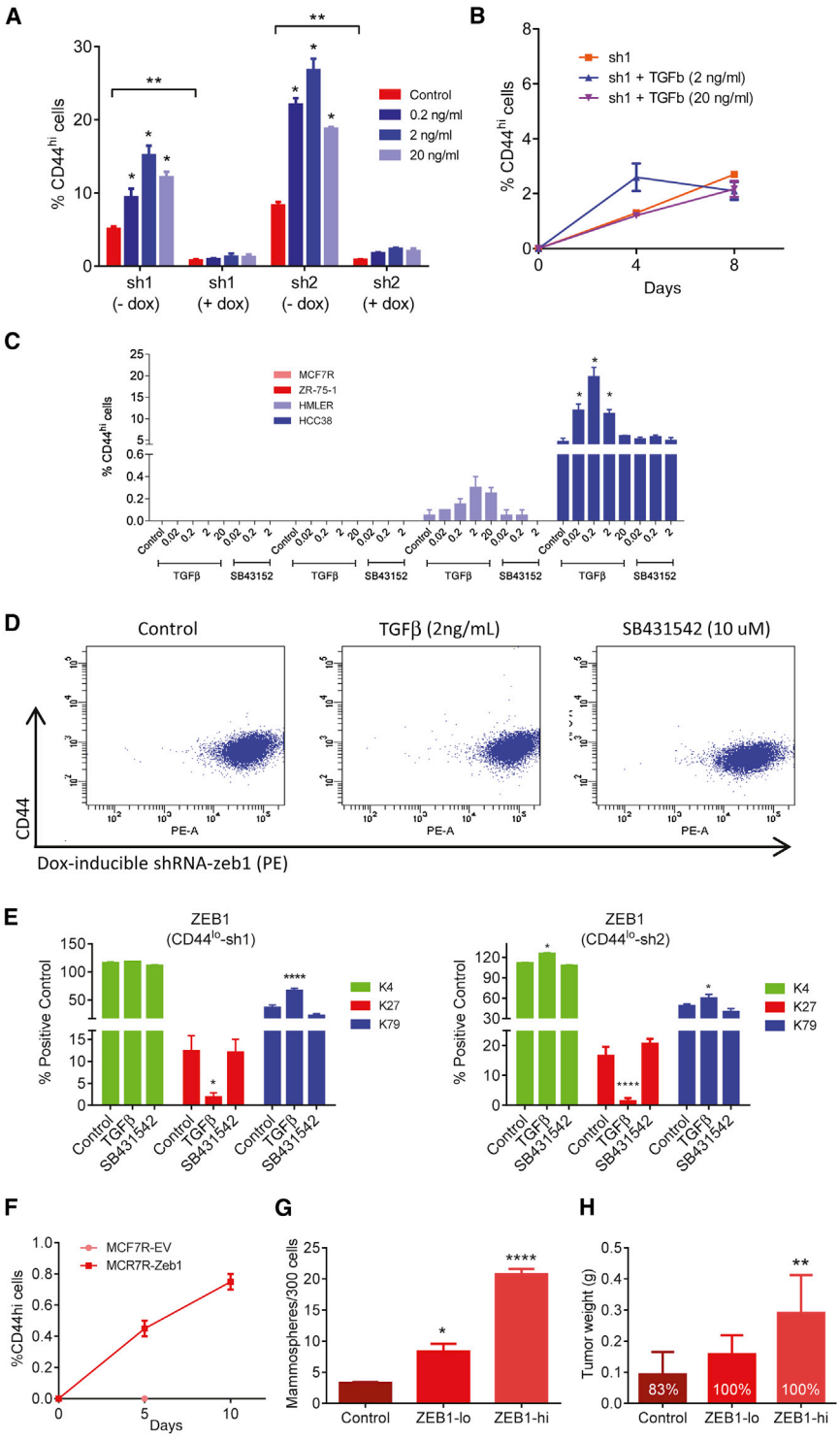


Figure 6. TGFβ Can Induce CD44^{lo}-to-CD44^{hi} Switching and Modulates the Chromatin at the ZEB1 Promoter

(A) Purified HME-flopc-CD44^{lo} cells expressing dox-inducible shRNA targeting *ZEB1* (sh1 and sh2) were monitored by FACS for their ability to switch to the CD44^{hi} state following TGFβ treatment in vitro. *p < 0.0001, different to control; **p < 0.001, different to control (–dox).

(B) Transformed HME-flopc-CD44^{lo} cells expressing sh1-targeting *ZEB1* were treated with TGFβ and monitored by FACS for switching to the CD44^{hi} state.

(C) Purified CD44^{lo} cells from luminal (MCF7R and ZR-75-1) and basal (HMLER and HCC38) BrCa cell lines monitored by FACS for switching to the CD44^{hi} state following TGFβ or SB431542 treatment in vitro. *p < 0.0001; two-way ANOVA followed by Tukey's multiple comparisons test.

(D) Representative FACS plots of HME-flopc-CD44^{lo} cells expressing sh1- or sh2-targeting *ZEB1* treated with control (PBS), TGFβ (2 ng/ml), or SB431542 (10 μM).

(E) ChIP-qPCR for the H3K4me3, H3K27me3, and H3K79me2 histone modifications at the *ZEB1* promoter in cells from (D) (*p < 0.0001, n = 4, two-way ANOVA followed by Tukey's multiple comparison test, different to control and SB431542).

(F–H) MCF7R cells expressing a dox-inducible empty vector (control) or *ZEB1* overexpression construct were treated with dox and monitored by FACS for their ability to switch to the CD44^{hi} state (F), for the ability to form tumorspheres in vitro (*p < 0.0001; one-way ANOVA and Tukey's multiple comparisons test), different to control (G), and for tumorigenicity in vivo (tumor-initiating ability marked as percentages on each bar; *p = 0.03; one-way ANOVA and Tukey's multiple comparisons test), different to control and ZEB1-lo (H).

Data are represented as mean ± SEM. See also Figure S6.

TGFβ treatment did indeed lead to a marked decrease in the repressive H3K27me3 mark associated with the *ZEB1* promoter in basal CD44^{lo} cells (Figure 6E). Together, these data demonstrate that TGFβ enables cells to transition from the bivalent chromatin status to the active chromatin state at the *ZEB1* promoter—doing so, at least in part, through the removal of the H3K27me3 repressive mark.

Wishing to put into context these changes in the histone modifications at the *ZEB1* promoter, we also followed changes in histone marks following control, TGFβ, or SB531542 treatment at the promoters of two TGFβ-responsive genes (GADD45B, PAI-1) and a TGFβ-nonresponsive gene (HPRT1). We found that the magnitude of changes in histone modifications causing active transcription at known TGFβ target genes closely correlates with the changes that we had previously observed at the *ZEB1* promoter in response to TGFβ treatment (Figure S6D). These data provide further evidence that *ZEB1* is a bona fide TGFβ target gene in these cells.

Intrinsic Responsiveness of Luminal BrCa Cells to Exogenous ZEB1

Knowing that luminal cells are indeed responsive to TGFβ treatment (as determined by TGFβ-mediated upregulation of pSMAD2; Figure S6E), we reasoned that the inability of luminal cells to undergo a $CD44^{lo}$-to-$CD44^{hi}$ switch in response to TGFβ treatment might be due to their inability to activate *ZEB1* transcription or, quite possibly, to an intrinsic lack of responsiveness of these cells to ZEB1 signaling. To explore these alternatives, we forced expression of ZEB1 in MCF7Ras cells (Figure S6F). We observed a progressively increasing population of $CD44^{hi}$ cells in MCF7R-ZEB1 cells over a 2 week time course (Figure 6F). Furthermore, we found that ZEB1 overexpression increased tumorsphere formation in vitro and tumorigenicity in vivo in a dose-dependent manner (Figures 6G and 6H). These data indicate that, although the endogenous *ZEB1* promoter in these luminal cells is repressed, it is nevertheless intrinsically responsive to this EMT-TF if its expression is forced.

Assessment of ZEB1 and MIR200B/C in Clinical Cases of Breast Cancer

As described above, we found that ZEB1 pays an important role in promoting $CD44^{lo}$-to-$CD44^{hi}$ conversions and in maintaining the CSC-like state in cells that already reside in the $CD44^{hi}$ state. Indeed, both of these processes contribute to enhanced tumor initiation and growth. We were interested in relating these observations to the properties of clinical cases of breast cancer. To pursue this question, we accessed data from the Cancer Genome Atlas Network (CGAN, 2012).

We first assessed the relative abundance of MIR200 family members across all breast tumors represented in the CGAN database and found that *MIR200C* accounts for 93% (SD = 5%) of mature miRNA in the MIR200BC family, with minority representation of the related *MIR200B* (6%) and *MIR429* (1%) family members (Figure 7A); given the dominant presence of *MIR200C* over its other family members, this allowed us to focus subsequent measurements on levels of *MIR200C.* We also found that the levels of *ZEB1* and *MIR200C* expression are inversely correlated in basal ($p = 9.4 \times 10^{-4}$; $r^2 = 0.13$), luminal A ($p = 2.8 \times 10^{-4}$; $r^2 = 0.07$), and luminal B ($p = 6.8 \times 10^{-4}$; $r^2 = 0.12$) subtypes, but not in *HER2*-overexpressing BrCa cells (Figure 7B). We then compared the absolute abundance of ZEB1 and *MIR200C* in BrCa subtypes and found that, surprisingly, in contrast to the observations described above, *ZEB1* mRNA appeared to be significantly more abundant in normal and luminal A BrCa subtypes compared to the basal subtype (Figure 7C).

Given our earlier observations in cultured cells that luminal BrCa lines do not express *ZEB1* and that the chromatin at the ZEB1 promoter resides in a repressed state in those same lines, we reasoned that this apparent conflict with the relatively high levels of *ZEB1* and *MIR200C* mRNA in clinical cases of luminal A-type BrCa might be explained in either of two ways: our studies of cultured cancer cell lines failed to properly reflect the behavior of corresponding cells in living tissues, or the data in the CGAN database was confounded by strong contamination of carcinoma cells with adjacent stromal cells expressing high levels of *ZEB1*.

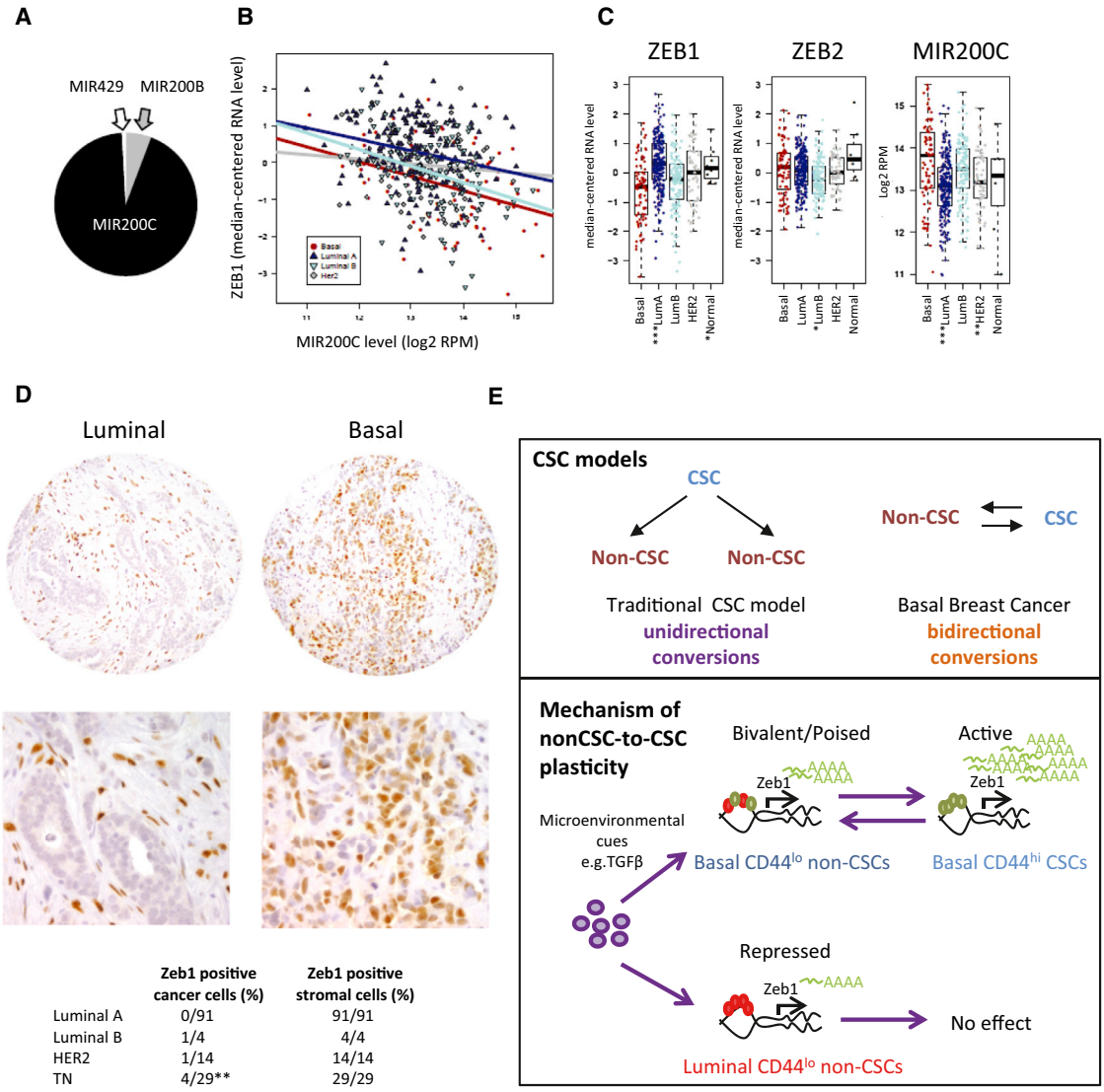
To resolve these alternatives, we analyzed ZEB1 protein expression in a tissue microarray of breast cancer biopsies (Figures 7D and S7). We found that ZEB1 protein is present at high levels in the stromata of all breast cancer subtypes. Interestingly, however, comparison of ZEB1 protein specifically in cancer cells showed that triple-negative (TN) BrCa cells have significantly higher levels of ZEB1 protein compared to luminal A cancer cells (Figure 7D, $p = 0.017$). In fact, ZEB1 was not present in the carcinoma cells of any luminal A BrCa sample examined (0/91 samples). From these data, we conclude that the strong *ZEB1*-positive signature produced by the TCGA analysis data in luminal A BrCa compared to TN BrCa (Figure 7C) is entirely attributable to stromal cells present as significant contaminants in the luminal A samples. Our data further highlight the difficulty of interpreting such global genomic analyses performed on whole-tumor digests, in which the relative representations of both carcinoma cells and stromal cells cannot be accounted for.

Together with our demonstration in BrCa cell lines that ZEB1 is the driver of the de novo generation of CSCs from non-CSC cell populations, the high expression of ZEB1 in TN BrCa cells raises the possibility that the more aggressive nature of clinical TN-type BrCa compared to luminal-type BrCa may be in part attributable to the ability of TN BrCas to readily activate *ZEB1* expression in response to microenvironmental stimuli and, subsequently, to create a continuous source of highly tumorigenic CSCs.

DISCUSSION

The present work reveals that the dynamics of interconversion between epithelial non-CSC and mesenchymal/CSC states are important determinants of normal and neoplastic epithelial tissue behavior. In contrast to the widely accepted CSC model, in which CSCs give rise to non-CSC progeny in a unidirectional manner, we have demonstrated that, in certain carcinoma subtypes—notably, basal carcinomas of the breast—neoplastic cells can readily convert from a $CD44^{lo}$ to a $CD44^{hi}$ state (Figure 7E). Given the strong enrichment of CSCs in the $CD44^{hi}$ state and their virtual absence in $CD44^{lo}$ cells (Al-Hajj et al., 2003), this suggested an interconversion between non-CSCs and CSCs, as indeed we demonstrated directly.

Our analyses indicate that this plasticity is not a universal property of all breast carcinomas. Plastic behavior was associated with four out of five basal-type BrCa cell lines, whereas luminal $CD44^{lo}$ populations (four out of four) were extremely inefficient at switching from the $CD44^{lo}$-to-$CD44^{hi}$ phenotype. These findings demonstrate fundamental differences in the biology driving basal- versus luminal-type tumors and suggest that the well-documented aggressive behavior of basal-type BrCas may be traced, in no small part, to this plasticity and the associated ability to generate carcinoma cells with enhanced tumor-initiating powers. The discovery of ZEB1, a well-characterized EMT-TF, as a key mediator of $CD44^{lo}$-to-$CD44^{hi}$ plasticity is consistent with the idea that the epithelial-mesenchymal transition generates cells with CSC-like activity (Mani et al., 2008; Morel et al., 2008). Although we do not rule out the possibility that other EMT transcription factors functioning upstream of ZEB1 may also drive non-CSC-to-CSC conversions, we have



	Zeb1 positive cancer cells (%)	Zeb1 positive stromal cells (%)
Luminal A	0/91	91/91
Luminal B	1/4	4/4
HER2	1/14	14/14
TN	4/29**	29/29

Figure 7. ZEB1 in Clinical Cases of Breast Cancer

(A) *MIR200C* accounts for 93% (SD = 5%) of mature miRNA in the *MIR200BC* family, which also includes *MIR200B* (6%) and *MIR429* (1%), so subsequent analysis uses *MIR200C* to represent the *MIR200BC* family.

(B) Levels of *ZEB1* and *MIR200C* are inversely correlated in basal ($p = 9.4 \times 10^{-4}$; r2 = 0.13), luminal A ($p = 2.8 \times 10^{-4}$; r2 = 0.07), and luminal B ($p = 6.8 \times 10^{-4}$; r2 = 0.12) subtypes (but not Her2). *ZEB1* is shown as median-centered values and *MIR200C* by log2-transformed reads per million mapped reads (RPM).

(C) mRNA abundance of *ZEB1*, *ZEB2*, and *MIR200C* by subtype. Asterisks indicate a difference compared to the basal subtype ($p < 0.05$; ANOVA with Dunnett post hoc). *$p < 0.05$; **$p < 0.01$.

(D) Human BrCa tissue array stained with an antibody targeting ZEB1 (100× and 400× images provided). **$p = 0.017$; TN compared to luminal A, Fisher's exact test followed by Bonferroni correction for multiple hypothesis testing. See also Figure S7.

(E) Schematic depicting: (1) an alternative CSC model for basal-type BrCa that includes bidirectional conversions between CSCs and non-CSCs and (2) a model of non-CSC-to-CSC conversion that includes a microenvironmental stimulus acting on non-CSCs harboring bivalent chromatin marks at the *ZEB1* promoter, enabling a rapid activation of *ZEB1* and switch to a CSC state.

See also Figure S7.

demonstrated that ZEB1 plays a critical, rate-limiting role in governing basal BrCa cell plasticity.

We found that the chromatin configuration associated with the *ZEB1* promoter in luminal CD44^{lo} cells was repressed, whereas in basal CD44^{lo} cells it was maintained in a bivalent/poised configuration, corresponding with the respective inability and ability of these two BrCa cell types to generate de novo CSC-like cells. As we argue here, differences in chromatin configuration appear to be responsible for the profound differences in cell plasticity. This yields, in turn, the interesting notion that the aggressiveness of certain breast carcinomas may not be determined by their steady-state concentrations of CD44^{hi} stem-like

cells; instead, their content of non-CSC cells with a proclivity to readily spawn CD44^{hi} stem-like derivatives may strongly influence the overall malignant behavior of these tumors. Stated differently, the bivalency of the *ZEB1* promoter in carcinoma cells may represent a useful prognostic parameter of tumor aggressiveness, a notion that will require extensive clinical testing and validation. Because such bivalent chromatin is already present in certain immortalized, nontransformed human mammary epithelial cells, this might suggest that the establishment of such bivalency occurs during the normal ontogeny of this lineage differentiation.

The nature of *ZEB1*-associated poised chromatin in basal CD44^{lo} cells suggests that those cells may readily and efficiently re-enter a stem-like state, given the appropriate stimulus. Indeed, we demonstrated that TGFβ, a well-known EMT-inducing stimulus (Gregory et al., 2011), can efficiently promote non-CSC-to-CSC conversion. The same TGFβ stimulus failed to induce luminal CD44^{lo} cells to convert to the CSC state (Figure 7E). Here, bivalency associated with the *ZEB1* promoter permits basal-type non-CSCs to respond to the same stimulus in a qualitatively different manner than luminal type non-CSCs. In that regard, it is plausible that basal non-CSCs located in an inflammatory microenvironment that is rich in EMT-inducing heterotypic signals may respond to local stimuli by switching to a CD44^{hi} CSC state; the resulting cells may then significantly enhance the aggressiveness of the tumors in which they reside.

At present, it seems plausible that disseminating CSCs are the principal agents of metastasis, as they are endowed with multiple traits that are essential for completion of most of the steps of the invasion-metastasis cascade (Thiery et al., 2009). However, in light of the plasticity that we can now ascribe to basal CD44^{lo} non-CSCs, it is conceivable that they too may leave a primary tumor and, following arrival at secondary tissue sites, create pools of newly formed CSCs that are critical to subsequent spawning of metastatic deposits. If validated, this would suggest that certain tumors are clinically aggressive because they can dispatch non-CSCs (which are usually far more numerous than CSCs) to distant sites as founders of new metastatic colonies following non-CSC-to-CSC conversions. In the same manner, plastic basal-CD44^{lo} cells may also contribute to disease recurrence. These and other considerations suggest that therapies directed at preventing non-CSC-to-CSC conversions should be considered as essential components of adjuvant therapies for breast cancer patients and, quite possibly, patients suffering other types of neoplastic disease.

EXPERIMENTAL PROCEDURES

Animals

All mouse studies were performed under the supervision of MIT's Division of Comparative Medicine in accordance with protocols approved by the Institutional Animal Care and Use Committee. NOD/SCID mice were bred in house. Mice were 2–4 months of age at time of injections. Tumor cells were resuspended in 20% Matrigel/MEGM (20 μl) for mammary fat pad injections. Tumors were dissected at the end of the experiment and weighed. GFP-positive lung metastases were counted from individual lobes by fluorescent microscopy.

Cell Culture

Cells were cultured as described in Table S5.

Vectors and Viral Infections

pBabe SV40-ER (Zeocin), pBabe H-Ras (Puromycin), PRRL-GFP, pLV-Tomato vectors, production of virus, and infection of target cells have been previously described (Elenbaas et al., 2001; Shaner et al., 2004). Infected cells were selected with Zeocin (100 μg/ml) and Puromycin (2 μg/ml). shRNA were purchased from Open Biosystems.

RNA Preparation and qRT-PCR Analysis

Total RNA was isolated using the RNeasy Micro kit (QIAGEN). Reverse transcription was performed with miScript II RT Kit; miScript and Qantitect Primer Assays were used to detect miRNAs and mRNA (QIAGEN).

ChIP-qPCR

Chromatin immunoprecipitation was performed as previously described (Lee et al., 2006).

Mammosphere Culture

Mammosphere culture was performed as previously described (Dontu et al., 2003).

Statistical Analysis

Data are presented as mean ± SEM. Student's t test was used to compare two groups ($p < 0.05$ was considered significant) unless otherwise indicated.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.06.005.

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