BRCA1 Suppresses Epithelial-to-Mesenchymal Transition and Stem Cell Dedifferentiation during Mammary and Tumor Development

Feng Bai1,2, Ho Lam Chan1,2, Alexandria Scott1,2, Matthew D. Smith3, Cheng Fan4, Jason I. Herschkowitz4, Charles M. Perou4,5, Alan S. Livingstone2, David J. Robbins1,2,3, Anthony J. Capobianco1,2,3, and Xin-Hai Pei1,2,3

Abstract

BRCA1 mutation carriers are predisposed to developing basal-like breast cancers with high metastasis and poor prognosis. Yet, how BRCA1 suppresses formation of basal-like breast cancers is still obscure. Deletion of p18Ink4c (p18), an inhibitor of CDK4 and CDK6, functionally inactivates the RB pathway, stimulates mammary luminal stem cell (LSC) proliferation, and leads to spontaneous luminal tumor development. Alternately, germline mutation of Brca1 shifts the fate of luminal cells to cause luminal-to-basal mammary tumor transformation. Here, we report that disrupting Brca1 by either germline or epithelium-specific mutation in p18-deficient mice activates epithelial-to-mesenchymal transition (EMT) and induces dedifferentiation of LSCs, which associate closely with expansion of basal and cancer stem cells and formation of basal-like tumors. Mechanistically, BRCA1 bound to the TWIST promoter, suppressing its activity and inhibiting EMT in mammary tumor cells. In human luminal cancer cells, BRCA1 silencing was sufficient to activate TWIST and EMT and increase tumor formation. In parallel, TWIST expression and EMT features correlated inversely with BRCA1 expression in human breast cancers. Together, our findings showed that BRCA1 suppressed TWIST and EMT, inhibited LSC dedifferentiation, and repressed expansion of basal stem cells and basal-like tumors. Thus, our work offers the first genetic evidence that Brca1 directly suppresses EMT and LSC dedifferentiation during breast tumorigenesis. Cancer Res; 74(21); 6161–72. ©2014 AACR.

Introduction

Mammary luminal and basal epithelial cells originate from multipotent progenitors in the embryo (1–2), and expansion and maintenance of these cells in adults are ensured by unipotent luminal stem cells (LSC) and basal stem cells (BSC), respectively (1, 3). Cancer stem cells (CSCs) are a subpopulation of cancer cells that shares characteristics with stem cells such as self-renewal ability and multipotency. CSCs could generate daughter cells, thus contributing to tumor growth, and are associated with radioresistance and chemoresistance, metastasis, and poor prognosis (4). Germline mutations in the tumor suppressor BRCA1 contribute to about half of familial breast cancer cases and increase the risk of developing basal-like breast tumors with high metastasis and poor prognosis. Basal-like tumors developed in BRCA1 mutation carriers were thought to originate from either mammary stem cells or basal progenitors (5, 6). Recently, we and others discovered that aberrant LSCs, not BSCs/progenitors, are likely the origin of basal-like tumors developed in patients harboring BRCA1 mutations as well as in germline Brca1−/−mutant mice (7–10). Furthermore, breast cancer stem cells are enriched in human BRCA1-mutant breast cancers (11, 12). However, whether BRCA1 functions in LSCs to maintain their unipotency and whether and how BRCA1 controls breast cancer stem cells and basal-like tumors in vivo, remain elusive.

Epithelial-to-mesenchymal transition (EMT) plays an important role in intratumoral heterogeneity, breast basal-like tumor development, and generation of breast epithelial and cancer cells with stem cell–like characteristics, directly linking EMT with the gain of stem cell properties (13, 14). A set of transcription factors, including TWIST1/2, SNAIL, SLUG, ZEB1/2, and FOXC1/2, was identified as EMT-inducing transcription factors (EMT-TF). Among these, TWIST1 (TWIST) is a master regulator of EMT and plays an essential role in tumor metastasis (15).
phosphorylates and functionally inactivates RB, p107, and p130. p18 expression is significantly lower in human breast cancers than normal breast (refs. 16, 17; F. Bai, unpublished data). RB has been identified as a major target for genomic disruption in basal-like breast cancers of BRCA1 mutation carriers (18), and loss of both RB and BRCA1 is, indeed, a feature of basal-like human breast cancers (17, 19). Deletion of Rb alone in mouse mammary epithelia does not induce tumors (20), and deletion of both Rb and p107 results in luminal type tumors (19), suggesting a role of the Rb pathway in controlling luminal tumorigenesis. Interestingly, deletion of p18, which functionally inactivates the Rb pathway, stimulates mammary LSC proliferation and results in luminal type tumors (16) as well as rescues the premature senescence caused by Brca1 deficiency. Thus, p18/Brcal double-mutant mice provide a unique mouse model with a genetically intact p53 pathway and functionally inactivated Rb pathway (10) to study the role of Brca1 in breast tumor suppression.

In this report, we used Brca1 germline and conditional mutant mice as well as human breast cancer cells and samples to determine the function and mechanism of Brca1 in suppressing EMT and basal-like tumors.

Materials and Methods

Mice, histopathology, and IHC

The generation of p18 and Brca1 germline mutant mice has been described previously (10, 16). Brcal/F in Tg(MMTV-Cre) 4Mam mice were obtained from the NCI Mouse Repository and JAX lab, respectively (21, 22). All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of North Carolina and University of Miami. Histopathology and IHC were performed as described previously (10, 16). Primary antibodies used are as follows: Ck5 (Covance), Ck8 (American Research Products), Ck14, SMA (Thermo Scientific), ERα, CD29, Brca1, Gata3, Foxc1, Foxc2 (Santa Cruz), E-cadherin (BD Biosciences), fibronectin, vimentin, Twist, Snail (Abcam), and Slug (Novus Biologicals).

Mammary cell preparation, FACS analysis, cell sorting, mammosphere assay, colony-formation assay, cell lines, transfection, and lentiviral infection

Mammary glands were dissected from female mice at the indicated ages, and the tissue was processed as previously described (10, 16, 23, 24). MCF-7, T47D (ATCC), SUM149 (Dr. Sendurai Mani, University of Texas, Houston, TX), and HCC1937 (Dr. Jennifer Hu, University of Miami, Miami, FL) cells were tested and authenticated (10, 25, 26). For ectopic expression of BRCA1, HCC1937 cells were transfected with pcDNA3-empty or pcDNA3-BRCA1 with FuGene. For BRCA1 knockdown (KD), pGIPZ-empty (Sh-Ctrl) and pGIPZ-shBRCA1 (Sh-BRCA1) lentiviral vectors were purchased from Open Biosystems.

Xenograft models of breast cancer

T47D and MCF-7 Sh-Ctrl and Sh-BRCA1 cells were suspended in a 50% solution of Matrigel (BD), and then inoculated into the left and right inguinal mammary fat pads of 6-week-old female NSG mice (Jackson Laboratory), respectively. Eighteen weeks after transplantation, animals were euthanized and mammary tumors were dissected for analyses. No estrogen was administered to animals during the course of the study.

Western blot, qRT-PCR, and chromatin immunoprecipitation assay

Western blot, QRT-PCR, and chromatin immunoprecipitation (ChIP) assay were carried out as previously described (10, 16, 27). Primary antibodies used for Western blot are as follows: Brca1, Gata3 (Santa Cruz), E-cadherin, fibronectin, tubulin-α (DM1A; NeoMarkers), and actin (ACTN05; NeoMarkers). Anti-BRCA1 antibody (D-9; Santa Cruz) or control mouse IgG was used to precipitate chromatin associated with BRCA1. qPCR was performed to determine the relative abundance of target DNA. Specific primers for the analysis of BRCA1 binding to TWIST are available upon request.

Human tumor samples

Formalin-fixed paraffin-embedded (FFPE) human breast cancer samples lacking patient-identifying information were obtained from the Tissue Bank Core Facility at the University of Miami. All samples obtained were nontreated invasive breast cancers with known estrogen receptor (ER) status. Regions from tumor samples were microdissected, and only samples with a consistent tumor cell content >75% of tissues were used for RNA extraction. The expression of BRCA1 was determined by qRT-PCR.

Patients and gene-expression datasets

The UNC337 human breast cancer dataset (28) with 337 breast cancer samples and the MetaBrC dataset (29) with 2,000 samples were analyzed. We compared gene expression versus six breast cancer subtypes using two-way ANOVA.

Results

Germline mutation of Brca1 transforms p18–/– luminal tumors into basal-like tumors with induction of EMT

In our previous studies, we reported that deletion of p18 in mice stimulates mammary LSC proliferation and leads to spontaneous luminal tumor development (16), and that germline mutation of Brca1 in p18-deficient mice blocks the expansion of LSCs and transforms luminal tumors into basal-like tumors (10). Prompted by the highly invasive heterogeneous mammary tumors developed in p18–/–; Brca1–/– mice with various degrees of whorls and clusters of spindle-shaped cells within these tumors—typical morphologic characteristics of mesenchymal cells (10)—we looked at molecular markers associated with EMT. We found that the majority of the luminal tumors from p18–/– mice highly expressed E-cadherin (Cdh1), an epithelial marker, whereas basal-like tumors from p18–/–; Brca1–/– mice expressed very weak and heterogeneous Cdh1. In contrast, most (77%, n = 13) of p18–/–; Brca1–/– tumors that developed after one year of age were stained positive for mesenchymal markers, including fibronectin (Fn), vimentin (Vim), and CD29, whereas only 11% (n = 19) of p18–/–;
tumors that developed at a similar age were positive for these markers (Fig. 1A–C; Supplementary Fig. S1A–S1D; Table 1). This observation suggests that heterozygous germline mutation of Brca1 activates EMT in mammary tumor progression.

Consistently, p18<sup>−/−</sup> Brca1<sup>+/−</sup> tumor cells that were positive for Ck5 expressed very low levels of Cdhl (Supplementary Fig. S1A) and the majority of Fn-positive cells coexpressed Ck5 (Fig. 1B; Supplementary Fig. S1C). These data suggest, at the least, that some Ck5<sup>+</sup> basal-like tumor cells lost their epithelial characteristics and gained mesenchymal features. In further analysis of these tumors for the expression of CD29, a basal and mesenchymal marker (14) demonstrated to be enriched in breast CSCs (23, 30), we found that 69% (n = 13) of p18<sup>−/−</sup>; Brca1<sup>+/−</sup> tumors expressed various degrees of CD29-positive tumor cells from 2% to 60%, whereas only 11% (n = 19) of p18<sup>−/−</sup> tumors were positive for CD29 in 2% to 3% of tumor cells (Fig. 1C and Table 1). These observations support the notion that EMT activation, as previously demonstrated (13, 14), results in cancer cells gaining stem cell properties. Primary p18<sup>−/−</sup> Brca1<sup>+/−</sup> tumor cells formed more and larger colonies in Matrigel than p18<sup>−/−</sup> tumor cells (Fig. 1E), and Ck5/Ck8 double-positive tumor cells were frequently detected in p18<sup>−/−</sup> Brca1<sup>+/−</sup> tumors, but rarely in p18<sup>−/−</sup> tumors (1.1% [67/6100] versus 0.04% [2/5120]; Fig. 1F; Supplementary Fig. S1E and refs. 10, 16), which further suggests increased CSCs in p18<sup>−/−</sup> Brca1<sup>+/−</sup> tumors. Together, these results indicate that heterozygous germline mutation of Brca1 induces EMT, increases CSCs, and transforms p18 null luminal tumors into basal-like tumors.

**Germline mutation of Brca1 activates EMT-TFs in mammary and tumor development**

We then determined the expression of EMT-TFs and observed that 77% (n = 13, >1 year of age) of p18<sup>−/−</sup>; Brca1<sup>+/−</sup> tumors were stained positive for Twist, Foxc1, Foxc2, Slug, and Snail in greater than 2% of cells per tumor, whereas 16% (n = 19, >1 year of age) of p18<sup>−/−</sup> tumors were positive at similar ages (Fig. 1D; Supplementary Fig. S2A–S2C; and Table 1). Tumors with high expression of EMT-TFs showed high histologic grade and strong invasive and metastatic potential as evidenced by EMT-TF–positive staining in the invasive front of tumors and metastasized cancers (Fig. 1D). The expression pattern and percentage of positive cells in tumors stained for EMT-TFs and EMT markers were highly correlated with its genotype—p18<sup>−/−</sup> or p18<sup>−/−</sup> Brca1<sup>+/−</sup>—which not only confirms that germline mutation of Brca1 promotes EMT in mammary tumors but that this induction of EMT is very likely a result of the aberrant activation of EMT-TFs in Brca1-mutant tumors. We next isolated mammary epithelial cells (MEC) from tumor-free virgin mice and found that Brca1<sup>+/−</sup> and p18<sup>−/−</sup>; Brca1<sup>+/−</sup> cells expressed significantly less Cdh1 and more EMT-TFs than wild-type (WT) or p18<sup>−/−</sup> cells (Supplementary cultured for 2 weeks, and colonies larger than 30 μm were counted. Bar graph, mean ± SD of two tumors per genotype. F, representative immunostaining of tumors from p18<sup>−/−</sup> Brca1<sup>−/−</sup> mice with Ck5 and Ck8. Ck5<sup>+</sup> Ck8<sup>+</sup> cells are indicated.
Table 1. Characterization of spontaneous mammary tumors derived from mutant mice

<table>
<thead>
<tr>
<th>Tumor</th>
<th>WT&lt;12 mo</th>
<th>12–27 mo</th>
<th>p18&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Brca1&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>p18&lt;sup&gt;−/−&lt;/sup&gt;;Brca1&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td>Mammary tumor</td>
<td>0/5</td>
<td>1/10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4/16</td>
<td>19/23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10%)</td>
<td>(25%)</td>
<td>(83%)</td>
<td>(9%)</td>
</tr>
<tr>
<td>Metastasis&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/1</td>
<td>0</td>
<td>1/19</td>
<td>0/1</td>
<td>0</td>
</tr>
<tr>
<td>EMT&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3/4</td>
<td>15/19</td>
<td>0/1</td>
<td>1/6</td>
<td>2/13</td>
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<td>% EMT&lt;sup&gt;+&lt;/sup&gt; cells/tumor</td>
<td>5%</td>
<td>3/19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0/1</td>
<td>1/1</td>
<td>4/6</td>
</tr>
<tr>
<td>% EMT&lt;sup&gt;+&lt;/sup&gt; cells/tumor</td>
<td>1%–5%</td>
<td>2%–40%</td>
<td>2%–40%</td>
<td>2%–20%</td>
<td>2%–95%</td>
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<tr>
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<td>2/19</td>
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<td>2/6</td>
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<td>(11%)</td>
<td>2%</td>
<td>1/1</td>
<td>3/6</td>
<td>10/13</td>
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*24-month-old tumor-bearing mouse.

*Most tumor-bearing mice were 12 to 16 months old, and the oldest was 22 months old. One male developed mammary tumor.

**Most tumor-bearing mice were 12 to 16 months old, and the oldest was 20 months old. One male developed mammary tumor.

***Mammary tumors metastasized mostly to the lung except one to a blood vessel in a p18<sup>−/−</sup>;Brca1<sup>−/−</sup> mouse.

†One tumor stained positive for Ck5 in approximately 5% tumor cells and the other two were positive in approximately 1% tumor cells.

‡Two tumors stained positive for Ck5 in approximately 95% tumor cells.

§At least two EMT markers (decreased Cdh1, increased Vim, Fn1, Sma, or Cd29) were detected in >2% tumor cells.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>0/1</th>
<th>0/4</th>
<th>3/19&lt;sup&gt;c&lt;/sup&gt;</th>
<th>1/1</th>
<th>3/6</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMT-TF&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(16%)</td>
<td></td>
<td>(100%)</td>
<td>(50%)</td>
<td>(77%)</td>
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Fig. S2D). These results indicate that EMT-TF activation in Brca1-mutant MECs occurs before tumor initiation.

Specific deletion of Brca1 in mammary epithelia activates EMT and induces aberrant differentiation of LSCs

To directly test the function of Brca1 in controlling and transforming MECs as well as to determine the implications of loss of Brca1 on mammary tumorigenesis, we generated Brca1<sup>−/−</sup>;MMTV-cre<sup>−/−</sup> and Brca1<sup>−/−</sup>;MMTV-cre<sup>−/−</sup> mice with and without p18 mutation, in which MMTV-cre (MC) is active in virgin epithelia but not in stroma (22, 31). Using these mice also enabled us to rule out the impact of Brca1-mutant stroma on mammary stem cell self-renewal and tumorigenesis.

Brca1<sup>−/−</sup>;MC and p18<sup>−/−</sup>;Brca1<sup>−/−</sup> ;MC breasts expressed <5% of Brca1 protein and mRNA relative to the levels in Brca1<sup>−/−</sup>;MC and p18<sup>−/−</sup>;Brca1<sup>−/−</sup> ;MC, indicating an efficient and near complete depletion of Brca1 in the mammary epithelia (Fig. 2A and B; Supplementary Fig. 3). Similarly, Brca1<sup>−/−</sup>;MC and p18<sup>−/−</sup>;Brca1<sup>−/−</sup> ;MC breasts expressed <20% of Brca1 protein and mRNA relative to the levels in MC and p18<sup>−/−</sup>;MC (data not shown). Consistent with the data from Brca1<sup>−/−</sup> mice (10), the expression of Gata3, Cdh1, and Epcam—genes associated with luminal differentiation—in Brca1<sup>−/−</sup>;MC and p18<sup>−/−</sup>;Brca1<sup>−/−</sup> ;MC breasts was significantly reduced relative to Brca1<sup>−/−</sup>;MC and p18<sup>−/−</sup>;Brca1<sup>−/−</sup> ;MC breasts (Fig. 2A and B; Supplementary Fig. 3), suggesting that loss of Brca1 impairs luminal differentiation. MECs from p18<sup>−/−</sup>;Brca1<sup>−/−</sup> ;MC mice showed increased mammosphere-forming ability than those from p18<sup>−/−</sup>;Brca1<sup>−/−</sup> ;MC mice. Most p18<sup>−/−</sup>;Brca1<sup>−/−</sup> ;MC mammospheres were 35 to 45 μm and none larger than 100 μm, whereas 10% to 15% of p18<sup>−/−</sup>;Brca1<sup>−/−</sup> ;MC mammospheres were larger than 100 μm. The average p18<sup>−/−</sup>;Brca1<sup>−/−</sup> ;MC mammosphere was significantly larger than that of p18<sup>−/−</sup>;Brca1<sup>−/−</sup> ;MC mammospheres (Fig. 2C). These results suggest that Brca1 deficiency increased the self-renewal capacity of p18<sup>−/−</sup> mammary stem cells. Accordingly, MECs from p18<sup>−/−</sup>;Brca1<sup>−/−</sup> ;MC mice formed more colonies than those from p18<sup>−/−</sup>;Brca1<sup>−/−</sup> ;MC mice and p18<sup>−/−</sup>;Brca1<sup>−/−</sup> ;MC mammospheres expressed significantly higher levels of EMT-TFs than those of p18<sup>−/−</sup>;Brca1<sup>−/−</sup> ;MC mice (Fig. 2D and E). These results confirm that loss of Brca1 activates EMT-TFs, which is likely responsible for the induction of EMT and increased mammosphere- and colony-forming potential in p18<sup>−/−</sup>;Brca1<sup>−/−</sup> ;MC mice.

We then performed FACS and found that p18<sup>−/−</sup>;Brca1<sup>−/−</sup> ;MC MECs had a reduced CD24<sup>+</sup> and blockage of LSCs, the latter of which is consistent with our findings derived from heterozygous germline Brca1-mutant mice (10).

FACS-sorted cells of the BSC-enriched population expressed higher basal genes (Twist2, Id4, and Tbx2) and lower luminal
genes (c-kit, Epcam, and Gata3) than those of the LSC-enriched population, confirming that these cell populations are, as reported (32), the basal and luminal cell-enriched populations, respectively (Supplementary Fig. 5H). LSCs derived from p18\(^{-/-}\);Brca1\(^{-/-}\);MC mice formed more colonies in Matrigel and expressed lower luminal and epithelial genes and significantly higher basal genes and EMT-TFs when compared with p18\(^{-/-}\);Brca1\(^{+/+}\);MC LSCs (Fig. 2G and H). Consistently, LSCs from p18\(^{-/-}\);Brca1\(^{-/-}\);MC mice also expressed lower luminal genes and higher basal genes and EMT-TFs than those from p18\(^{-/-}\); Brca1\(^{+/+}\);MC mice (Supplementary Fig. 5C). These results indicate that haploid or near complete loss of Brca1 in mammary epithelium not only inhibits the expression of luminal genes but also stimulates the expression of basal genes and EMT-TFs in p18\(^{-/-}\) LSCs. Interestingly, expression of basal genes and EMT-TFs was also significantly increased in the BSCs from p18\(^{-/-}\); Brca1\(^{-/-}\);MC mice relative to those from p18\(^{-/-}\); Brca1\(^{+/+}\);MC mice (Fig. 2I). Together, these results suggest that Brca1 deficiency leads to the expansion of BSCs, which is likely, at least partially, a result of the dedifferentiation of LSCs. We have previously analyzed five histologically distinct epithelial cell populations and defined the small light cell (SLC) and undifferentiated large light cell (ULLC) populations as enriched for stem and luminal stem/progenitor cells, respectively (16). To determine the impact of EMT on stem/progenitor cell populations in situ, we examined tumor-free mammary glands and found that Twist or Fn-positive MECs were frequently detected in p18\(^{-/-}\); Brca1\(^{-/-}\);MC or p18\(^{-/-}\); Brca1\(^{+/+}\);MC mammary glands (Fig. 2J–L).
mice but not in p18\(^{-/-}\)/MC mice and that most, if not all, Twist or Fn-positive cells were either SLC or ULLC. ULLC in particular (Fig. 2J and K). Furthermore, Ck5 and Ck8 double-positive epithelial cells were also frequently detected in p18\(^{-/-}\); Brca1\(^{+/+}\)/MC but not in p18\(^{-/-}\);Brca1\(^{+/+}\)/MC mammary (Fig. 2L; Supplementary Fig. 5; data not shown). These results further suggest that loss of Brca1 in MECs activates Twist, induces EMT, and leads to dedifferentiation of LSCs.

**Specific deletion of Brca1 in mammary epithelia recapitulates basal-like tumorigenesis and EMT activation**

To determine the tumorigenic impact of specific loss of Brca1 in mammary epithelia, we first examined Brca1\(^{+/+}\)/MC and p18\(^{-/-}\); Brca1\(^{+/+}\)/MC mice and found that no hyperplasia nor tumors developed in 5 female Brca1\(^{+/+}\)/MC mice at 10 to 12 months of age. Of the 8 p18\(^{-/-}\); Brca1\(^{+/+}\)/MC mice examined at similar ages, all developed mammary hyperplasia, though no mammary tumors, were detected. A majority (7/8) of p18\(^{-/-}\); Brca1\(^{+/+}\)/MC mice died at early ages from carcinomas in the pancreas, skin, pituitary, or lung (data not shown), very likely due to active MMTV-Cre expression and near complete deletion of Brca1 in these tissues (33), which prevented us from observing the relatively late-onset mammary tumorigenesis in these mice. These results, however, confirm the previous findings that loss of Brca1 alone is insufficient to promote tumorigenesis and that Brca1 cooperates with p18 to control tumorigenesis.

We then examined p18\(^{-/-}\); Brca1\(^{+/+}\)/MC and p18\(^{-/-}\); Brca1\(^{+/+}\)/MC mice and found that 1 of 4 p18\(^{-/-}\); Brca1\(^{+/+}\)/MC mice and 4 of 5 p18\(^{-/-}\); Brca1\(^{+/+}\)/MC mice developed mammary tumors in 12 to 16 months (Fig. 3). In accordance with the tumors developed in p18\(^{-/-}\); Brca1\(^{+/+}\)/MC, mammary tumors in p18\(^{-/-}\); Brca1\(^{+/+}\)/MC and p18\(^{-/-}\); Brca1\(^{+/+}\)/MC mice were also highly heterogeneous, poorly differentiated, and more aggressive than those developed in p18\(^{-/-}\) mice (Figs. 1, 3; Supplementary Fig. S1 and S2). About 25% to 30% p18\(^{-/-}\); Brca1\(^{+/+}\)/MC tumor cells were spindle-shaped and were positive for Twist and Fn (Fig. 3A and B), and more than 40% of the tumor cells were positive for Ck5 and negative for Cdh1 or Ck8 (Fig. 3C and D), indications of a basal-like tumor undergoing EMT. The p18\(^{-/-}\); Brca1\(^{+/+}\)/MC mammary tumors also expressed 1/3 of Brca1 and 1/5 of Gat3 relative to the tumor-free mammary tissues of the same mouse (Fig. 3E), confirming deficient Brca1 and downregulation of Gat3 in the tumor.

More than 25% of tumor cells were spindle-shaped in all four p18\(^{-/-}\); Brca1\(^{+/+}\)/MC mammary tumors, and two displayed more than 90% spindle-shaped cells (Fig. 3F). These tumors were also positive for Twist and Fn (Fig. 3G), indications of typical metaplastic breast carcinomas undergoing EMT. A p18\(^{-/-}\); Brca1\(^{+/+}\)/MC tumor expressed less than 10% Brca1 and Gat3 when compared with tumor-free mammary of the same mouse (Fig. 3I). FACS showed that the LSC-enriched population in p18\(^{-/-}\); Brca1\(^{+/+}\)/MC mammary tumors was significantly reduced in comparison with the tumor-free mammary tissues of the same mouse (6% vs. 56%) and when compared with p18\(^{-/-}\)/MC mammary tumor cells (6% vs. 57%). Contrastingly, the BSC-enriched population, also enriched with breast CSCs, was significantly expanded in p18\(^{-/-}\); Brca1\(^{+/+}\)/MC mammary tumors relative to the tumor-free mammary tissues of the same mouse (19% vs. 11%) and when compared with p18\(^{-/-}\) mammary tumor cells (19% vs. 4%; Fig. 3I). These results further support that p18\(^{-/-}\); Brca1\(^{+/+}\)/MC mammary tumors are basal-like tumors undergoing EMT that are enriched with CSCs, which is in line with the data derived from human patients showing that metaplastic breast carcinomas are basal-like breast cancers with EMT-like molecular makeup and are closely correlated with BRCA1 dysfunction (34).

Taken together, these results suggest that insufficient Brca1 in mammary epithelial cells represses Gat3, activates Twist and EMT, and results in basal-like tumorigenesis with an increase in the CSC population. Because p18\(^{-/-}\); Brca1\(^{+/+}\)/MC and p18\(^{-/-}\); Brca1\(^{+/+}\)/MC mice are in B6 and Balb/c mixed backgrounds, unlike p18\(^{-/-}\); Brca1\(^{+/+}\)/MC mice in pure Balb/c background, these data also suggest that the role of Brca1 controlling basal-like tumorigenesis and EMT is independent of genetic background.

**BRCA1 suppresses TWIST transcription and EMT**

We screened a panel of human breast cancer cell lines and found that MCF7 and T47D cells expressed higher CDH1 and GATA3 and lower VIM and EMT-TFs than SUM149 and HCC1937 cells (Supplementary Fig. S6), confirming that MCF7 and T47D cells are luminal/epithelial-like and SUM149 and HCC1937 cells are basal/mesenchymal-like cancer cells in our culture system (35). Transfection of WT BRCA1 into HCC1937 (Brca1 mutant, transcriptionally null) cells resulted in increase of CDH1 and decrease of VIM and FN, indicating that BRCA1 suppresses EMT. Importantly, ectopic expression of BRCA1 significantly repressed TWIST by more than 50% compared with control, moderately repressed FOXC2, but hardly repressed other EMT-TFs (Fig. 4A). A similar inhibitory effect on TWIST and FOXC2 expression was also detected in 293T cells transfected with BRCA1 (Supplementary Fig. S7). Because the ability of BRCA1 in regulating transcription controls normal differentiation and suppresses tumor development (36, 37), we determined whether BRCA1 is recruited to the TWIST promoter. A previous study demonstrated that GATA3 recruits BRCA1 to its binding sites in the FOXC1/2 promoters to repress their transcription (27). We performed bioinformatic analysis of the TWIST gene promoter and found that there exists, at the least, six putative GATA3 binding sites on the TWIST promoter (Fig. 4B), which are conserved in both human and mouse (data not shown). We then performed a ChIP assay and found that one of five amplicons that contained two GATA3 sites was specifically enriched in the immunoprecipitation of BRCA1 in HCC1937 cells transfected with WT BRCA1 compared with control (P5 in Fig. 4C). In sum, these results suggest that BRCA1 specifically binds to the TWIST promoter and negatively regulates its transcription.

To confirm the role of Brca1 in the suppression of Twist and tumor development in situ, primary mammary tumors derived from p18\(^{-/-}\); Brca1\(^{+/+}\)/MC mice were immunostained with antibodies against Brca1 and Twist. We found that tumor cells positive for Brca1 expressed very low or no Twist, whereas Brca1-mutant tumor cells expressed high levels of Twist, most...
of which were spindle-shaped basal-like cells (Fig. 4D), demonstrating that Brca1 inhibits Twist and EMT in mammary tumor development and progression.

**Knockdown of BRCA1 inactivates TWIST and EMT with enhanced tumor formation potential**

We knocked down BRCA1 in two human luminal cancer cell lines, MCF7 and T47D, using BRCA1 shRNA targeting 3 different sequences (Fig. 5A, data not shown), and transplanted these cells into the mammary fat pads of NSG mice. We found that mammary tumors from T47D-Sh-BRCA1 cells were palpable in 8 weeks, whereas tumors formed from T47D-Sh-Ctrl. cells were undetectable at this stage (data not shown). Eighteen weeks after transplantation, T47D-Sh-BRCA1 tumors were significantly bigger than T47D-Sh-Ctrl. tumors (Fig. 5B and C). Consistently, mammary tumors from MCF7-Sh-BRCA1 cells were palpable significantly sooner and were larger compared with tumors from MCF7-Sh-Ctrl. cells (data not shown). These results indicate that KD of BRCA1 in luminal cancer cells enhances their tumor formation potential. Histo- and pathologic analysis revealed that, unlike homogeneous and well-differentiated T47D-Sh-Ctrl. mammary tumors, T47D-Sh-BRCA1 tumors were highly heterogeneous with an abundance of large and poorly differentiated cells (Fig. 5D), suggesting that KD of BRCA1 induced the dedifferentiation of luminal tumor cells. IHC analysis indicated that most cells in T47D-Sh-BRCA1...


**BRCA1 and TWIST expression levels are inversely related in human claudin-low type breast cancers**

Gene-expression profiling analyses have categorized human breast tumors into six intrinsic subtypes: basal-like (BL), claudin-low (CL), Her2⁺ (H2), luminal A (LA), luminal B (LB), and normal breast-like (NBL), each of which has unique biologic and prognostic features (28). Of these subtypes of breast cancer, the CL subtype is characterized by the low to absent expression of luminal differentiation markers and high enrichment for EMT markers and cancer stem cell–like features. Clinically, the majority of CL tumors are poor prognosis triple-negative (ER⁻, PR⁻, and HER2⁻) invasive carcinomas with high frequencies of metastatic and medullary differentiation (28, 38, 39). To determine whether our mouse genetic analysis models human breast cancers, we queried the expression of *BRCA1* and EMT-TFs in the UNC337 breast cancer patient sample sets (28). We found that expressions of *BRCA1* and EMT-TFs were highly correlated with breast tumor–intrinsic subtypes (Fig. 6A). Specifically, the mRNA level of *BRCA1* was low, whereas that of EMT-TFs—TWIST, SNAIL, and SLUG—in particular—was high in the CL subtype. Pearson correlation analysis revealed an inverse correlation between *BRCA1* with TWIST mRNA levels, but not with SNAIL, SLUG, and FOXC1 (Fig. 6B). We performed similar analyses on the MetaBric dataset with 2,000 breast tumors (29) and detected similar results—*BRCA1* and TWIST mRNA levels were inversely correlated in all breast cancers and in the CL subtype in particular (Supplementary Fig. S9).

We screened 43 invasive breast cancers then selected five ER-positive and five ER-negative samples. RNA was prepared from microdissected FFPE sections of tumors. The expression levels of *BRCA1* in ER-negative tumors were significantly lower than in ER-positive tumors (1.16 ± 0.49 vs. 2.04 ± 0.56; *P* = 0.015), reflecting the downregulation of *BRCA1* mRNA in ER-negative tumors (Fig. 6D). IHC indicated that TWIST-positive tumor cells were only detected in ER-negative, CK5-positive tumors, not in ER-positive tumors. The expression of TWIST was closely associated with that of CK5 and was inversely correlated with the mRNA level of *BRCA1* (Fig. 6C and D). Furthermore, all TWIST-positive tumors were highly heterogeneous, poorly differentiated, and showed typical morphologic EMT features, evidenced by the various degrees of whorls and clusters of spindle-shaped cells within these tumors. Together, these clinical findings, consistent with our results in mice, further confirm that *BRCA1* suppresses TWIST and EMT in breast basal-like cancer development and progression.

**Discussion**

In this article, we confirm our previous finding that heterozygous mutation of *Brc1* in p18-deficient mice transforms luminal tumors into basal-like tumors (10) and report here that it activates EMT-TFs and induces EMT. Consistently, specific deletion of Brc1 in mammary epithelia led to enhanced self-renewal potential of stem cells, blockage of LSC expansion, impaired luminal gene expression, increased basal gene

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*tumors expressed very faint or no CDH1 and ERα, but high levels of TWIST, VIM, and FN in comparison with T47D-Sh-Ctrl tumors (Fig. 5E; G; Supplementary Fig. S8), indicating that T47D-Sh-BRCA1 tumor cells had undergone EMT. These results collectively suggest that KD of BRCA1 in breast luminal tumor cells activates TWIST and EMT, which is associated with increased tumor formation potential, further supporting the data derived from p18;Brc1 double-mutant mice.*

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**Figure 4.** BRCA1 suppresses TWIST and EMT in mammary tumor cells. A, HCC1937 cells were transfected with pcDNA3-empty (Ctrl) or pcDNA3-BRCA1 (BRCA1), and RNA was analyzed. Data are expressed as the mean ± SD from triplicates of two independent experiments. B, diagram showing the locations of putative GATA3 sites in the human TWIST gene. C, CHIP analysis of BRCA1 binding to putative GATA3 sites on the TWIST promoter in HCC1937 cells transfected with BRCA1. Data are expressed as the mean ± SD from triplicates of two independent experiments. D, mammary tumors from p18⁻−;Brc1⁻⁻ mice were stained with antibodies against Brc1 (green) and Twist (red).
expression, expansion of BSCs and CSCs, and induction of EMT and basal-like tumors. These results suggest that either germ-line mutation of Brca1 or mammary epithelia-specific deletion of Brca1 is responsible for the activation of EMT-TFs, induction of EMT, dedifferentiation of LSCs, expansion of BSCs and CSCs as well as the development of basal-like tumors. This study provides the first genetic evidence suggesting that Brca1 suppresses EMT and dedifferentiation of LSCs in mammary and tumor development. We also show that KD of BRCA1 in human luminal breast cancer cells activates EMT and increases tumor formation potential, further supporting the data derived from p18/Brca1 double-mutant mice.
Because of growth defects induced by Brca1 deficiency (40–42), mice carrying Brca1 mutation in mammary epithelia rarely develop tumors, making it difficult to identify the cells of origin of Brca1-mutant basal-like tumors. Most, if not all, genetic studies have used co-transformation of one of the genes in the p53 pathway to overcome the growth defects induced by mutation of Brca1 in mice (8–9, 40–43). Specific deletion of Brca1 in mammary epithelia by MMTV-Cre or Wap-Cre resulted in basal-like tumor development in p53-deficient mice (40, 43), supporting the notion that BRCA1-mutant breast cancer may also arise from basal stem/progenitor cells. Direct comparison of p53+/−;Brca1+/−;Blg-Cre tumors phenocopy human BRCA1-mutant basal-like breast cancers, whereas p53+/+;Brca1+/−;K14-Cre tumors do not resemble human BRCA1 breast cancers, further supporting the notion that Brca1-mutant basal-like tumors originate from luminal stem/progenitor cells (9). However, mutation of p53 in these studies may induce EMT and mammary tumors falling into multiple molecular subtypes, including the basal-like and claudin-low subtypes (45–48), masking the contribution of Brca1 mutation alone in basal-like tumorigenesis. Hence, it is imperative that the role of Brca1 in controlling mammary stem cells and tumorigenesis be determined under a genetically intact p53 background.

EMT-TFs orchestrate EMT, which plays an important role in intratumoral heterogeneity and breast basal-like tumor progression. The levels of BRCA1 and TWIST were inversely related to the CL subtype of human breast cancer. A, analysis of gene expression in UNC337 breast cancer patients according to tumor subtype. B, correlation analysis of the expression of BRCA1 and EMT-TFs for UNC337 breast cancer patients. C, serial sections from ER-positive and -negative invasive human breast cancers were stained with hematoxylin and eosin (H&E), CK5, and TWIST. The boxed areas were enlarged in the insets. Representative cytoplasmic staining of CK5+ cells and nuclear staining of TWIST+ cells are indicated by arrows. D, summary of expression of CK5 and TWIST by IHC and BRCA1 by qRT-PCR. The expression levels of CK5 and TWIST were scored by the percentage of positive tumor cells in total tumor cells. The expression of BRCA1 was determined by qRT-PCR. The levels of BRCA1 mRNA were expressed as the mean values from triplicates of the two sets of the primers.
Brca1 Suppresses EMT and Stem Cell Dedifferentiation

leads to claudin-low tumors with EMT features and stem cell-like characteristics (54), which molecularly and histologically resembles breast cancers developed in p18:Brca1 double-mutant mice.

Disclosure of Potential Conflicts of Interest
C.M. Perou is a board member for and has ownership interest (including patents) in Bioclassifier LLC and GeneCentric Diagnostics. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Bai, H.L. Chan, A.J. Capobianco, X.-H. Pei.
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.L. Chan, A. Scott, M.D. Smith, C. Fan, J.I. Herschkowitz, C.M. Perou, D.J. Robbins, X.-H. Pei.
Writing, review, and/or revision of the manuscript: H.L. Chan, A. Scott, M.D. Smith, J.I. Herschkowitz, C.M. Perou, A.S. Livingstone, D.J. Robbins, X.-H. Pei.
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Bai, H.L. Chan, M.D. Smith.
Others (advice on design and interpretation of data, manuscript review and revision): A.J. Capobianco.

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Feng Bai, Ho Lam Chan, Alexandria Scott, et al.


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