Suppression of Breast Cancer Stem Cells and Tumor Growth by the RUNX1 Transcription Factor

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Abstract

Breast cancer remains the most common malignant disease in women worldwide. Despite advances in detection and therapies, studies are still needed to understand the mechanisms underlying this cancer. Cancer stem cells (CSC) play an important role in tumor formation, growth, drug resistance, and recurrence. Here, it is demonstrated that the transcription factor RUNX1, well known as essential for hematopoietic differentiation, represses the breast cancer stem cell (BCSC) phenotype and suppresses tumor growth in vivo. The current studies show that BCSCs sorted from premalignant breast cancer cells exhibit decreased RUNX1 levels, whereas ectopic expression of RUNX1 suppresses tumorsphere formation and reduces the BCSC population. RUNX1 ectopic expression in breast cancer cells reduces migration, invasion, and in vivo tumor growth (57%) in mouse mammary fat pad. Mechanistically, RUNX1 functions to suppress breast cancer tumor growth through repression of CSC activity and direct inhibition of ZEB1 expression. Consistent with these cellular and biochemical results, clinical findings using patient specimens reveal that the highest RUNX1 levels occur in normal mammary epithelial cells and that low RUNX1 expression in tumors is associated with poor patient survival.

Implications: The key finding that RUNX1 represses stemness in several breast cancer cell lines points to the importance of RUNX1 in other solid tumors where RUNX1 may regulate CSC properties. Mol Cancer Res, 16(12); 1952–64. ©2018 AACR.

Introduction

Breast tumors are heterogeneous, as they are comprised of several types of cells, including transformed cancer cells, supportive cells, tumor-infiltrating cells, and cancer stem cells (CSC). The CSC is acknowledged to be a significant component of growing tumors (1, 2). As the name implies, CSC can self-renew and reconstitute the cellular hierarchy within tumors (3, 4). Moreover, these stem-like cells are highly chemoresistant and metastatic (5, 6). Significantly, signaling pathways (TGFβ, WNT, Hedgehog, and Notch) and transcription factors (Snail, Twist, and Zeb) regulate stemness properties in CSCs; they are also involved in controlling an essential cellular process designated as epithelial–mesenchymal transition (EMT; refs. 7, 8), which is linked to chemoresistance and cancer metastasis (9–11). One such transcription factor is ZEB1, a well-known EMT activator, which is essential for cell plasticity and promotes stemness properties in breast and pancreatic cancers (12, 13). There remains a compelling requirement to understand the regulatory mechanisms that contribute to and sustain stemness of the CSC population. Identifying regulator(s) that maintain or repress the CSC phenotype can provide insights for novel therapeutic approaches. Recently, a list of 40 mutation driver genes for which deregulation contributes directly to breast tumor progression has been identified (14); among these is the transcription factor RUNX1 that has been shown to repress EMT. Here we address for the first time, the function of RUNX1 in regulating breast cancer stem cells (BCSC).

The Runx family, including RUNX1, RUNX2, and RUNX3, are evolutionarily conserved transcription factors and function as critical lineage determinants of various tissues (15). RUNX1 is well established as essential for definitive hematopoiesis and is a frequent target of translocations and other mutations in hematopoietic malignancies. For example, RUNX1-related chromosomal translocations, including RUNX1-ETO (16), TEL-RUNX1 (17), and RUNX1-EVI (18), are associated with distinct leukemia subtypes. Besides its function in the hematopoiesis lineage, RUNX1 is well documented to play a fundamental role in controlling the stem cell populations in the gastrointestinal tract (19), hair follicles (20, 21), and oral epithelium (22). As a master transcriptional regulator, RUNX1 is a central player in fine-tuning the balance among cell differentiation, proliferation, and cell-cycle control in stem cells during normal development (23). In the mammary gland, it has recently been shown that RUNX1 is involved in luminal development (24), and that loss of RUNX1 in mammary epithelial cells blocks differentiation into ductal and lobular
tissues. These findings suggest that RUNX1 is an essential regulator of normal mammary stem cells (24). In addition to its essential function during normal development, disrupting RUNX1 function(s) can cause cancer (15, 25).

Knowledge regarding the function(s) of RUNX1 in breast cancer is limited, and RUNX1 has been shown to be related to WNT pathway and key transcription factors including ERα and ELF5 (15, 26–28). Recent studies from our group have demonstrated that RUNX1 has tumor suppressor activity and maintains the epithelial phenotype and represses EMT (29). RUNX1 expression is decreased during breast cell EMT, and loss of RUNX1 expression in normal-like epithelial cells (MCF10A) and epithelial-like breast cancer cells (MCF7) initiates the EMT process (29). Complementary studies demonstrated that ectopic expression of RUNX1 reverts cells to the epithelial state. However, mechanisms underlying RUNX1 regulation of CSC properties and the consequences for tumor growth in vivo remain to be resolved.

On the basis of evidence that RUNX1 regulates stem cell properties during normal development (24, 30) and that loss of RUNX1 activates partial EMT in breast cancer (29), we hypothesized that RUNX1 represses the CSC population and/or stemness properties in breast cancer. We investigated whether altering RUNX1 levels by overexpression or knockdown in breast cancer cells changes the stemness phenotype, aggressive properties in vitro, and tumor progression in vivo. Our findings have identified a previously unknown function for RUNX1 in repressing the CSC population as well as tumour sphere formation, and demonstrated that RUNX1 has a significant impact in inhibiting breast cancer tumor growth in vivo.

Materials and Methods

Cell culture

MCF10AT1 and MCF10A cells were grown in DMEM/F12 (Hyclone: SH30271, Thermo Fisher Scientific) with 5% (v/v) horse serum (Gibco: 16050, Thermo Fisher Scientific) + 10 μg/mL human insulin (Sigma-Aldrich, H-1882) + 20 ng/mL recombinant hEGF (PeproTech, AF-100-15) + 100 ng/mL cholera toxin (Sigma-Aldrich, C-8052) + 0.5 μg/mL hydrocortisone (Sigma-Aldrich, H-0888), 50 μM penicillin/50 μg/mL streptomycin, and 2 mmol/L glutamine (Life Technologies), 15140-122 and 25030-081, respectively). MCF10CA1a cells were grown in DMEM/F12, 5% (v/v) horse serum with 50 μM penicillin and 2 mmol/L glutamine. MCF7 cells were maintained in DMEM high glucose (Thermo Fisher Scientific, MT-10-017-CM) supplemented with 10% (v/v) FBS (Atlanta Biologicals, S11550) and 50 μg/mL penicillin/100 μg/mL streptomycin. MCF10A, MCF10AT1, and MCF10CA1a cells were a kind gift from Jeffrey Nickerson’s lab. MCF10AT1 and MCF10CA1a were validated by short tandem repeat analysis in 2015 using the Promega GenePrint 10 system and karyotype at the UVM Cancer Center DNA Analysis Facility. All experiments were performed within 10 passages after thawing cells.

Lentiviral plasmid preparation and viral vector production

RUNX1 cDNA was cloned into lentivirus-based expression plasmids pLenti-CMV-Blast-DEST (Addgene). To generate lentivirus vectors, 293T cells in 10-cm culture dishes were cotransfected with 10 μg of pGPZIP shRunx1 or pGPZIP nonsilencing, 5 μg of psPAX2, and 5 μg of pMD2.G using Lipofectamine 2000 reagent (Life Technologies). Viruses were harvested 48 hours posttransfection. After filtration through a 0.45-μm pore-size filter, viruses were concentrated using LentivX concentrator (Clontech).

Gene delivery by transfection and infection

For exogenous expression of RUNX1, MCF10AT1 or MCF10CA1a cells were plated in 6-well plates (1 × 105 cells/well) and infected 24 hours later with lentivirus expressing RUNX1 or empty vector. Briefly, cells were treated with 0.5 mL of lentivirus and 1.5 mL complete fresh DMEM-F12 per well with a final concentration of 4 μg/mL polybrene. Plates were centrifuged upon addition of the virus at 1,460 × g at 37°C for 30 minutes. Infection efficiency was monitored by GFP coexpression at 2 days postinfection. Cells were selected with 2 μg/mL puromycin (Sigma-Aldrich, P7235-100MG) for at least two additional days. After removal of the floating cells, the remaining attached cells were subcultured for cell-based assays. shRunx1 virus was generated and delivered as has been described previously (29).

Western blotting

Cells were lysed in RIPA buffer and 2 × SDS sample buffer supplemented with cOmplete, EDTA-free protease inhibitors (Roche Diagnostics) and MG132 (EMD Millipore). Lysates were fractionated in an 8.5% acrylamide gel and subjected to immunoblotting. The gels are transferred to polyvinylidene difluoride membranes (EMD Millipore) using a wet transfer apparatus (Bio-Rad Laboratories). Membranes were blocked using 5% blocking Grade Blocker Non-Fat Dry Milk (Bio-Rad Laboratories). Membranes were washed using 5% Blotting Blocker Non-Fat Dry Milk (Bio-Rad Laboratories) on a Chemidoc XRS imaging system (Bio-Rad Laboratories).

Tumorsphere formation assay

Monolayer cells were enzymatically dissociated into single cells with 0.05% trypsin-EDTA and plated at 10,000 cells per well in 24-well low-attachment plates (Corning). Cells were grown for 7 days in DMEM/F12 supplemented with B27 (Invitrogen) in the presence of 10 ng/mL EGF and 10 ng/mL bFGF. Where indicated, the CDK4 inhibitor palbociclib (Sigma) was added at a final concentration of 100 nmol/L. Tumorsphere-forming efficiency was calculated as the number of spheres divided by the number of single cells seeded, expressed as a percentage.

CD24/CD44 flow cytometry

Flow cytometry for CD24 (PE-cy7, BioLegend 311120) and CD44 (APC, BD Pharmingen 559942) was performed using the optimized conditions for marker detection as described previously (31, 32). Cells were grown to subconfluence and dissociated with Accutase. The Accutase was quickly neutralized with serum and 1 × 106 cells were washed with 1 × PBS.
These cells were then resuspended in 475 μL of 1% FBS/1 × PBS, to which 25 μL of CD44-APC and 4 μL of CD24-PE-cy7 were added and incubated at room temperature for 30 minutes. Cells were then washed with PBS and strained (Falcon 352235) to obtain single-cell suspensions. Isotype controls were used to gate for negative signal in each replicate of the experiment.

Migration assays
For the scratch assays, cells were seeded in triplicate and when they reached 95%–100% confluence, they were serum starved with 0.1% FBS-containing media for 12 hours. Subsequently, a scratch was made across the cell layer using a P-200 pipette tip, and cell migration was monitored by recording images at indicated timepoints postscratch. The area of the scratch was quantified using the MetaBox plug-in for ImageJ software and plotted as a percentage of total area.

For the transwell migration assay, cells were trypsinized and resuspended in triplicate in migration chambers (BD Biosciences) in serum-free medium. Twenty-four hours (MCF10AT1 cells) or 48 hours (MCF10CA1a cells) after cell seeding, the experiment was performed and results quantified as described previously (33).

Invasion assay
For the invasion assay, cells were trypsinized and resuspended in triplicate in growth factor-reduced Matrigel invasion chambers (BD Biosciences) in serum-free medium. Twenty-four hours (MCF10AT1 cells) or 48 hours (MCF10CA1a cells) after cell seeding, the experiment was performed and results quantified as described previously (33).

Immunofluorescence microscopy
Cells grown on coverslips were fixed using 3.7% formaldehyde in PBS for 10 minutes. Cells were then permeabilized in 0.1% Triton X-100 in PBS, and washed in 0.5% BSA in PBS. Detection was performed using rabbit polyclonal to RUNX1 antibody (Cell Signaling Technology #4336), or mouse monoclonal to CD24 (Santa Cruz Biotechnology sc-11406). Staining was performed using fluorescent secondary antibodies; for rabbit polyclonal goat anti-rabbit IgG (H+L) secondary antibody, Alexa Fluor 568 conjugate (Life Technologies A-11011) was used, and for mouse monoclonal F(ab)’2-goat anti-mouse IgG (H+L) secondary antibody, Alexa Fluor 488 conjugate (Life Technologies A-11001) was used.

siRNA transfection
MCF10AT1 cells (~60%–70% confluent) were transfected with control nonsilencing siRNA (siNS, 500 nmol/L) or the OnTarget Plus human ZEB1 siRNA smartpool (Thermo Fisher Scientific; siZEB1 500 nmol/L) using Oligofectamine (Life Technologies) according to the manufacturer’s instructions. Protein knockdown of ZEB1 was assessed 48 hours after transfection, and tumorsphere formation assay was initiated 24 hours after transfection; CD24/CD44 flow cytometry was initiated 48 hours after transfection.

Animal studies
Female NCI SCID/NCr Mouse mice (Charles River Laboratories) 7 weeks of age were used for mammary fat pad injection. The mice were randomly divided into two groups (7 for each group). For each mouse, 1 × 10⁶ MCF10CA1a cells suspended in 0.1 mL of saline were mixed with 0.1 mL of Matrigel (BD Biosciences) and were injected under mammary fat pads. Bioluminescence images were acquired 5 minutes after injection of 150 mg/kg of D-Luciferin (Gold Biotechnology) in PBS using the IVIS Imaging System (Xenogen). All animals were housed in a pathogen-free environment and handled according to protocol number 12-051 approved by the Institutional Animal Care and Use Committee at the University of Vermont (Burlington, VT). In conducting studies using animals, the investigators adhered to the laws of the United States and regulations of the Department of Agriculture.

Analysis of RUNX1 expression and patient survival using public datasets
The PROGene database (www.compbio.iupui.edu/progene; refs. 34, 35) was used to analyze the patient survival using the public GEO datasets [www.ncbi.nlm.nih.gov/gds; GSE37751 (36), GSE7390 (37), TCGA (38)]. RUNX1 expression in different breast cancer stages was analyzed using the TCGA database (www.cbioportal.org; ref. 38).

Quantitative PCR
RNA was isolated with TRIzol (Life Technologies) and cleaned by DNase digestion (Zymo Research). RNA was reverse transcribed using SuperScript II and random hexamers (Life Technologies). cDNA was then subjected to quantitative PCR using SYBR Green technology (Applied Biosystems). The primers are human specific as they tested negative with RNA from mouse embryonic fibroblast (MEF) cells.

ChIp-qPCR
Chromatin immunoprecipitation (ChIP)-qPCR was performed essentially as described (39). Briefly, 2 × 10⁶ MCF10AT1 or MCF10CA1a cells were cross-linked with 1% formaldehyde, lysed, and sonicated to obtain DNA fragments mostly in the 200- to 1,000-bp range. Immunoprecipitation was performed at 4°C overnight with anti-RUNX1 antibody (4334, Cell Signaling Technology) at a 1:15 antibody to chromatin ratio. Primers used in ChIP-qPCR are listed below:

ZEB1 Forward: GTGTTAAGACCGGGGAGTTGCTC;
ZEB1 Reverse: GCCATCCCGCATGATCCTC;
ZEB1 Forward: GCCCATCAGCTTGTGATTGTTG;
ZEB1 Reverse: GCCATCCCGCATGATCCTC;
ZEB1 Forward: GCCCATCAGCTTGTGATTGTTG;
ZEB1 Reverse: GCCATCCCGCATGATCCTC;
ZEB1 Forward: GCCATCCCGCATGATCCTC;
ZEB1 Reverse: GCCATCCCGCATGATCCTC;
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ZNF333 (negative control 1) Forward: TGAAGACATCT-GCAACC,
ZNF333 Reverse: TCGCCGACTCATAAGTTTC;
ZNF180 (negative control 2) Forward: TGCAGCAAATAA-GTTCAGCA,
ZNF180 Reverse: TGCAGTCAATGTGGGAAGTC.

Tissue microarray
Tissue microarray data of RUNX1 in patients with breast cancer were obtained from Human Protein Atlas (www.proteinatlas.org; ref. 40).

Statistical analysis
Each experiment was repeated at least three times. The differences in mean values among groups were evaluated and expressed as mean ± SEM. A P value less than 0.05 was considered statistically significant (**, P < 0.05; ***, P < 0.01; ****, P < 0.001). Student t test was used to analyze the expression of cell surface markers, side population analysis, cell viability, relative mRNA levels, migrated cells, and invaded cells.

Results
Reduced RUNX1 expression is associated with decreased survival probability in patients with breast cancer
To investigate possible association between RUNX1 expression and breast cancer progression, we examined RUNX1 expression in normal tissues and breast cancer patient specimens using the Human Protein Atlas. Within normal breast tissues, RUNX1 is highly expressed in ducts of the mammary gland (Fig. 1A). However, in ductal carcinoma tissues, the level of RUNX1 is decreased in malignant regions compared with normal glandular tissues in the same tumor specimen (Fig. 1B). In the majority of ductal carcinoma specimens (9/12 samples) from the Human Protein Atlas, 75% of breast cancer tumors show low RUNX1 staining (Fig. 1C). We also analyzed TCGA data and found that RUNX1 levels are progressively decreased across early-stage breast cancer (stage 1 vs. stage 2; stage 2 vs. stage 3; Supplementary Fig. S1). These findings suggest that during breast cancer progression, RUNX1 levels are decreased along with the loss of the mammary gland tissue organization. The data are consistent with our previous report that RUNX1 is highly expressed in mammary gland tissue and in normal-like mammary epithelial MCF10A cells and reduced in a panel of breast cancer cell lines (29, 33). With reduced RUNX1 expression, mammary epithelial cells do not maintain their epithelial phenotype (29). On the basis of these observations of low RUNX1 in breast tumors and the concomitant loss of RUNX1 in normal epithelial cells with loss of epithelial properties, we hypothesized that loss of RUNX1 is promoting a breast cancer phenotype.

We therefore addressed whether there is a clinical relationship of RUNX1 expression in breast cancer patient tumors with survival. Using publicly available mRNA expression datasets, we analyzed the relationship of mean expression levels of RUNX1 in breast cancer patient tissue samples with patient survival. Our Kaplan–Meier analysis of RUNX1 expression in three separate datasets [GSE37751—‘Human Breast Cancer and Their Association with Tumor Subtypes and Disease Prognosis’ (36 high RUNX1 and 24 low RUNX1 patients), GSE7390—‘Strong Time Dependence of the 76-Gene Prognostic Signature’ (82 high RUNX1 and 116 low RUNX1 patients), and TCGA data of breast cancer patient mRNAs (304 high RUNX1 and 290 low RUNX1 patients)] indicated a statistically significant correlation (P < 0.01, P < 0.05, and P < 0.01, respectively) between high RUNX1 expression levels and longer patient survival time (Fig. 1D). These results suggested that reduction in RUNX1 expression is associated with low survival probability of patients with breast cancer. Thus, several in vitro studies combined with these clinical observations support a role for RUNX1 in repressing tumor growth.

RUNX1 is decreased in tumors formed in mouse mammary fat pad
To further investigate whether RUNX1 decreases during breast tumor growth in vivo, we utilized a mouse xenograft model to examine RUNX1 levels during tumor progression. MCF10CA1a cells, which are aggressive breast cancer cells, were injected into the mammary fat pad of SCID mice and tumor growth was monitored weekly. Tumors formed within 2 weeks (Fig. 2A), and one month post injection, mice were euthanized and tumors were removed to analyze for RUNX1 and other factors at both protein and mRNA levels. RUNX1 expression in tumor cells was quantitated by qRT-PCR using human-specific primer sets, which showed that RUNX1 mRNA is decreased specifically in the MCF10CA1a cancer cells within the tumor environment (Fig. 2B). The parental MCF10CA1a cells had a 3.3-fold higher RUNX1 protein level than the removed tumor, as quantified from Western blots (Fig. 2C). The epithelial marker E-cadherin was decreased in tumor samples, whereas the mesenchymal marker Vimentin was increased (Fig. 2C). To exclude the possibility that the altered gene expression is because of the mouse stromal cells in the tumor samples, we performed qPCR using human gene–specific primers. The human-specific mRNA levels of several cancer-related genes, such as VEGF, FN1, MMP13, MMP9, CXCR4, and CXCL12, are also upregulated in the tumor samples, while RUNX1 level is decreased (Fig. 2C and D). These findings indicate that the human breast cancer cells that formed a tumor in mouse mammary fat pads acquired a more aggressive phenotype and that RUNX1 expression is decreased during the period of tumor growth. Therefore, we have demonstrated that in the aggressive MCF10CA1a cells in vivo, RUNX1 expression is strikingly decreased when other markers related to EMT and metastasis increase during tumor progression.

RUNX1 reduces the aggressive phenotype of breast cancer cells in vitro
It has been suggested that RUNX1 represses cancer-related genes and pathways in breast cancer cells (26, 27, 29). On the basis of these data and the results that RUNX1 level is decreased in our in vivo studies (Fig. 2B), we next addressed whether RUNX1 in malignant breast cancer cells reduces the aggressive phenotype. RUNX1 was ectopically expressed using a lentivirus delivery system (pLenti-CMV) in premalignant MCF10AT1 and highly aggressive malignant MCF10CA1a cells (Fig. 3A). Upon RUNX1 expression, Vimentin expression is decreased in both cell lines (Fig. 3A). However, E-cadherin expression was not affected by RUNX1 overexpression, suggesting that the cells have not fully transitioned back to epithelial-like stage. Expressing RUNX1 in either MCF10AT1 or MCF10CA1a cells did not alter proliferation (Supplementary Fig. S2). To
evaluate the effect of RUNX1 in regulation of migration and invasion capacities of the breast cancer cells in vitro, we used both scratch and Transwell assays to measure migration. Figure 3B shows representative images of the scratch assay, both at the time of the scratch and 48 hours (MCF10AT1) or 16 hours (MCF10CA1a) later. RUNX1 expression decreased the ability of breast cancer cells to migrate. These results were confirmed using the transwell migration assay (Fig. 3C). Invasion of both MCF10AT1 and MCF10CA1a cells were also significantly inhibited when RUNX1 was overexpressed (Fig. 3D). We conclude from these studies that loss of RUNX1 in MCF10A and cancer cells is detrimental in promoting EMT in vitro (29) and in vivo (Fig. 2B), whereas exogenous expression of RUNX1 suppresses the migration and invasion of breast cancer cells in vitro.

RUNX1 represses tumor growth in vivo
Together, our data above and the earlier studies demonstrate that RUNX1 has tumor suppressor activity in vitro. However, to date there are no studies showing that RUNX1
RUNX1 inhibits tumor growth \textit{in vivo}. We tested the ability of RUNX1 to alter tumor growth \textit{in vivo} by using the metastatic MCF10CA1a breast cancer cells. MCF10CA1a/EV (control) and MCF10CA1a/RUNX1-overexpressing cells carrying a luciferase reporter (experiment) were injected into the mammary fat pad of SCID mice. Eighteen days post injection, tumors appeared in the control mice, with an average volume of 63 mm$^3$ (caliper measurement), while the experimental group had barely palpable tumors (Fig. 4A). At the endpoint of this experiment (4 weeks), we sacrificed the mice, excised the tumors, and measured tumor volume and weight (Fig. 4B and C). Mice injected with MCF10CA1a/EV RUNX1 cells had a significantly reduced tumor size (57%) and weight (47%) compared with tumors from control mice. Supplementary Figure S3A and S3B show the excised tumors and luminescence of tumors in all 7 mice from each group. MCF10CA1a cells with EV or overexpressing RUNX1 were validated before injection into the SCID mice (Supplementary Fig. S3C). Luminescence images of representative mice (Fig. 4D) confirm reduced tumor growth. Collectively, these data indicate that RUNX1 inhibits tumorigenesis and suppresses breast tumor growth \textit{in vivo}.

RUNX1 level is decreased in BCSCs

As BCSCs have been shown to be critical for tumor initiation and growth (11) and all of our data demonstrate a role for...
RUNX1 in decreasing tumorigenesis, we next investigated the potential role of RUNX1 in breast cancer stemness. We used FACs to isolate BCSCs from premalignant MCF10AT1 cells based on expression of the cell-surface antigen markers CD44 and CD24. These two markers have been successfully used to identify putative CSCs in primary breast tumors or mammary cell lines (CD44$^{\text{high}}$/CD24$^{\text{low}}$). We compared the BCSCs with bulk cells (CD44$^{\text{high}}$/CD24$^{\text{high}}$) as gated in Supplementary Fig. S4. The CD44$^{\text{high}}$/CD24$^{\text{low}}$ subpopulation from MCF10AT1 cells displayed lower levels of RUNX1 protein (33%) compared with the bulk cell population and the parental MCF10AT1 cells (Fig. 5A). To corroborate that CD24$^{\text{low}}$ cells have low RUNX1 expression, we performed immunofluorescence costaining of RUNX1 and CD24 in MCF10AT1 cells. Cells with high CD24 expression also have high RUNX1 expression (Supplementary Fig. S5). Moreover, the CD44$^{\text{high}}$/CD24$^{\text{low}}$ population displays many CSC-like properties; they are endowed with higher expression of CSC markers ZEB1 and Twist1 (Fig. 5A) and greater long-term self-renewal capacity as measured by tumorsphere formation assays (Fig. 5B). Collectively, these data provide evidence that cell populations with BCSC properties express lower levels of RUNX1 compared with the bulk and parental populations, consistent with RUNX1 influence on BCSC properties.

RUNX1 inhibits stemness properties in breast cancer cells

To further investigate the role of RUNX1 in regulating BCSC properties, we addressed the capability of RUNX1 to regulate tumorsphere formation from breast cancer cells. Tumorsphere formation assays were performed using nonadherent plates with nonserum medium. The ectopic expression of RUNX1 in both MCF10CA1a and MCF10AT1 cells significantly decreased the number of tumorspheres ($P < 0.05$; Fig. 5C and D). To better understand whether RUNX1 represses stemness properties in breast cancer, we used two lentiviruses to establish RUNX1 knockdown cell lines in MCF10AT1 cells (Fig. 5E). Depletion of RUNX1 in these cell lines activated an epithelial-to-mesenchymal transition with lower E-cadherin and higher Vimentin expression.
Significantly, the knockdown of RUNX1 resulted in increased tumorsphere formation efficiency in MCF10AT1 cells (51% and 41%, respectively; Fig. 5F). This ability of RUNX1 to repress stemness properties was also observed in additional cell lines, including normal-like MCF10A cells and ER-positive luminal-like MCF7 cells (Supplementary Fig. S6A and S6B), which reinforces that RUNX1 suppression of stemness is universal across breast cancer subtypes.

Further evidence for the influence of RUNX1 on the CSC population in MCF10AT1 cells was provided by flow cytometry analysis. As shown in Fig. 6A, ectopic expression of RUNX1 reduced the CD44hi/CD24lo subpopulation of premalignant MCF10AT1 cells from 22.3% to 15.1% (Fig. 6A). Moreover, knockdown of RUNX1 significantly increased the CD44hi/CD24lo subpopulation of MCF10AT1 cells by more than 2-fold (21.9% ns; 45.3% shR1-1; 45.6% shR1-2; Fig. 6B). Therefore, in MCF10AT1 cells, RUNX1 represses both tumorsphere formation efficiency and the CSC population. Conversely, ectopic expression of RUNX1 in metastatic MCF10CA1a cells did not change the percent of the CD44hi/CD24lo CSC population (Supplementary Fig. S7). Upon Figure 4.

RUNX1 represses the expression of ZEB1 in breast cancer cells

We further pursued the mechanism regulating RUNX1 function in reducing the CSC population. In Fig. 5A, we observed that decreased RUNX1 expression is coincident with activation of ZEB1 in BCSC in MCF10AT1 cells. ZEB1 is well known for its function in promoting EMT, cancer stemness, and metastasis in breast cancer (41). Therefore, we tested whether RUNX1 negatively regulates ZEB1 expression in breast cancer cells. ZEB1 protein is downregulated when RUNX1 is ectopically expressed in MCF10AT1 cells (Fig. 7A). This RUNX1-mediated ZEB1 repression was confirmed in MCF10AT1 RUNX1 knockdown cells, where ZEB1 expression is enhanced (Fig. 7B). We did not observe RUNX1 repression of ZEB1 expression in MCF10CA1a cells, which is a consequence of very low ZEB1 mRNA levels in MCF10CA1a cells compared with MCF10AT1 cells (Supplementary Fig. S8). To test whether RUNX1 can directly regulate ZEB1 in MCF10CA1a cells, we performed ChIP-qPCR for RUNX1 in the ZEB1 promoter region in both MCF10AT1 and MCF10CA1a cells (Supplementary Fig. S9). As shown in Fig. 7C, RUNX1 directly binds to the ZEB1 promoter in the two breast cancer cell lines relative to two negative control genes ZNF333 and ZNF180.
RUNX1 overexpression, the binding of RUNX1 is enhanced on the ZEB1 promoter, suggesting that RUNX1 has potential to directly regulate ZEB1 expression in both premalignant and metastatic breast cancer cell lines.

To better illustrate the role of ZEB1 in RUNX1-related phenotypes, we knocked down ZEB1 using siRNA in Runx1-depleted MCF10AT1 cells (Fig. 7D). Both flow cell cytometric analysis of CD44 high/CD24 low population and tumorsphere assay demonstrated that knockdown of ZEB1 partially rescued activated CSC phenotypes induced by RUNX1 knockdown (Fig. 7E and F). Knockdown of ZEB1 alone in MCF10AT1-NS control cells does not change the CSC phenotypes, which may be due to low expression level of ZEB1 in these cells (Supplementary Fig. S10). Interestingly, ZEB1 knockdown has a more dramatic effect on tumorsphere formation than on the population of CSCs, indicating that other factors may contribute to RUNX1 and ZEB1 phenotypes. Overall, these data suggest that ZEB1 is a significant downstream target of RUNX1 (Fig. 7G).

In summary, our findings provide several lines of evidence that RUNX1 has antitumor activity in breast cancer cells both in vivo and in vitro. From in vivo tumor growth of aggressive breast cancer cells (MCF10CA1a), the endogenous level of RUNX1 is decreased in a three-dimensional tumor environment. High RUNX1 expression correlates with better patient survival, which indicates that RUNX1 has the potential to be a prognostic marker. Both EMT and the BCSC population are repressed by RUNX1 in vitro, and in vivo, ectopic RUNX1 expression inhibits tumor growth by 50% in the mammary fat pad. Mechanistically, RUNX1-mediated repression of aggressive breast cancer phenotypes is through negative regulation of ZEB1 expression (Fig. 7G), which is a well-known

Figure 5.
RUNX1 level is decreased in BCSCs. A, Western blot analyses show RUNX1 is decreased and ZEB1, Twist1, and Vimentin level are increased in BCSC samples compared with Parental and Bulk MCF10AT1 cells. Right, protein quantification shows that RUNX1 is significantly decreased in BCSCs. B, Tumorsphere-forming efficiency for BCSC populations is significantly higher than bulk population. **, P < 0.01. C, RUNX1 overexpression in MCF10CA1a cells reduces tumorsphere formation efficiency. †, P < 0.05. Right, picture of tumorsphere. D, RUNX1 overexpression in MCF10AT1 cells reduces tumorsphere-forming efficiency. ††, P < 0.05. Right, picture of tumorsphere. E, Western blot analyses of lysates from MCF10AT1 cells treated with shRUNX1 show decreased protein expression of RUNX1 and E-cadherin and increased protein expression of Vimentin. F, RUNX1 knockdown in MCF10AT1 cells activates tumorsphere formation efficiency. ††, P < 0.05. Right, picture of tumorsphere.
activator for both EMT and CSCs in breast cancer (41). This study provides new insight into functional mechanisms of RUNX1 transcriptional regulation in contributing to the stemness and the plasticity of nonmetastatic MCF10AT1 breast cancer cells.

**Discussion**

Our findings provide compelling evidence that the loss of RUNX1 induces BCSCs and that high levels of RUNX1 expression can suppress the CSC population, which is responsible for metastasis, treatment resistance, and tumor recurrence in breast cancer. RUNX1 also reduces cell migration and invasion of breast cancer cells in vitro and tumor growth in vivo. Moreover, RUNX1 reduces the BCSC population and tumorsphere formation efficiency, thus indicating that RUNX1 represses stemness properties in breast cancer. RUNX1 overexpression and knockdown studies revealed that RUNX1 mediates the mechanisms of inhibition of breast cancer stemness and tumorigenesis through repression of ZEB1 expression.

An unresolved question is whether RUNX1 functions to promote or suppress tumor growth in breast cancer. Our studies of mammary fat pad injection using a metastatic breast cancer cell line ectopically expressing RUNX1 demonstrate suppression of tumor growth by RUNX1 in vivo. Increasing evidence indicates that loss of RUNX1 function contributes to EMT and thus mediates the progression of breast cancer (26, 27, 29). Mechanistically, loss of RUNX1 in ER+ breast cancer activates the WNT signaling pathway and ELF5 expression (26, 27) suggesting that RUNX1 represses breast cancer progression. Our previous study showed loss of RUNX1 promotes EMT in both normal and breast cancer cells indicating that RUNX1 has the potential to inhibit tumor growth (29). In this study, we clearly demonstrated that the level of RUNX1 is decreased during tumor growth, and that ectopic RUNX1 expression suppresses tumor growth in the mouse mammary fat pad. Together, this literature and our experiments establish that RUNX1 has tumor-suppressive activity by reducing CSC population. Clinically, RUNX1 expression is decreased in high histologic grade tumors compared with low/mid-grade tumors (42). In the past few years, RUNX1 loss-of-function somatic mutations have been identified in several subtypes of breast cancer (38, 43, 44). It is unclear whether RUNX1 could repress breast cancer metastasis in vivo. We did not observe metastases in mice injected with MCF10CA1a cells in our in vivo model, which may be due to the additional time required for metastasis. It is also possible that the genetic background of the mouse strain used in this study (NCI SCID/NCr Mouse) still have normal NK cells and macrophages which may prevent metastasis. Therefore, future investigations with other mice strains, such as NOD-SCID mice, may address whether RUNX1 represses metastasis in vivo.

We cannot rule out the possibility that RUNX1 may have other functions in breast cancer, especially in late-stage disease. For example, in the MMTV-PyMT mouse model, the level of RUNX1 is positively correlated with tumor progression (33) and regulates genes promoting tumor growth in late-stage MDA-MB-231 breast cancer cells (45). However, in this study, we found that metastatic MCF10CA1a cells with exogenous RUNX1 expression resulted in smaller tumors in vivo indicating that RUNX1 functions to reduce tumor growth. These contradictory results in late-stage breast cancer suggest that RUNX1 has dual functions (oncogene vs. tumor suppressor) that are highly dependent on cellular context.

**Figure 6.** RUNX1 reduces BCSC subpopulation. **A,** Flow cytometric analysis of CD44 and CD24 expression in MCF10AT1 cells with EV or RUNX1 overexpression. **B,** Flow cytometric analysis of CD44 and CD24 expression in MCF10AT1 cells stably expressing RUNX1 or nonsilencing shRNAs.
The antitumor growth activity of RUNX1 in breast cancer is likely through its properties in maintaining the normal mammary epithelial phenotype. For example, depletion of RUNX1 in normal-like MCF10A cells causes loss of epithelial morphology and activates mesenchymal genes (29). Furthermore, knockdown of RUNX1 in ER-positive luminal MCF7 breast...
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cancer cells transforms the cells into a partial EMT state (29). It has been suggested that partial activation of the EMT promotes plasticity that allows reprogramming of the epithelial cell to acquire both migratory and stem-like features (46). RUNX1 may also be involved in response to cellular stress, such as chemotherapy. For example, in lymphoid cells, RUNX1 protects cells from apoptosis and in acute megakaryocytic leukemia cells (47), and depletion of RUNX1 increases the sensitivity of the cells to chemotherapy agent, cytosome arabinoside (48). Therefore, it will be interesting in the future studies to examine the sensitivity of the breast cancer cells to chemotherapy agents upon alteration of RUNX1 expression.

We investigated whether RUNX1 might function by suppressing ZEB1, due to its well-known activity in increasing breast cancer stemness and as a marker of EMT. Our results show that RUNX1 directly binds to the ZEB1 promoter in both MCF10AT1 and MCF10CA1a cells and that binding is enhanced upon RUNX1 overexpression. In MCF10AT1 cells, RUNX1 negatively regulates ZEB1 expression. Together these findings indicate that the binding of RUNX1 on the ZEB1 promoter and the suppression of ZEB1 by RUNX1 reduce breast cancer stemness in the cells that retain plasticity. Consistent with this conclusion, overexpressing RUNX1 in MCF10CA1a cells does not change the expression of EMT markers to the same extent that it does in premalignant MCF10AT1 cells (Fig. 3A). These data and the fact that RUNX1 represses EMT in normal-like MCF10A cells (29), highlight its critical function in repressing tumor initiation and growth in early-stage breast cancer. Also of significance is that overexpression of RUNX1 in MCF10CA1a cells decreased tumor growth in vivo and tumor sphere formation efficiency in vitro, suggesting that RUNX1 can inhibit the aggressive phenotype in late-stage breast cancer cells.

Breast cancer is ranked as the second leading cause of cancer-related deaths in women after lung cancer (49). In 2017, approximately 63,400 cases of female breast carcinoma in situ were expected to be diagnosed (50). Despite the significant advances that have been achieved in early detection and treatment of breast cancer, understanding the mechanisms of breast cancer progression and metastasis still requires intensive study. Recently, using sophisticated next-generation sequencing technology, a 40 mutation-driver gene list was generated in human breast cancer (14). RUNX1, which is often mutated in breast tumors, is one of those genes. Mining the TCGA clinical datasets, we found that reduced RUNX1 levels in tumors correlate with breast cancer progression. Both tight control of RUNX1 expression and RUNX1 functional integrity are required to prevent breast cancer malignancy. Consequently, clinical strategies should consider RUNX1 as an informative biomarker for diagnosis and potentially as a therapeutic candidate.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Hong, A.J. Fritz, M.P. Fitzgerald, J.B. Lian, G. S. Stein
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