Cancer stem cells from human breast tumors are involved in spontaneous metastases in orthotopic mouse models

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To examine the role of breast cancer stem cells (BCSCs) in metastasis, we generated human-in-mouse breast cancer orthotopic models using patient tumor specimens, labeled with optical reporter fusion genes. These models recapitulate human cancer features not captured by previous models, including spontaneous metastasis in particular, and provide a useful platform for studies of breast tumor initiation and progression. With noninvasive imaging approaches, as few as 10 cells of stably labeled BCSCs could be tracked in vivo, enabling studies of early tumor growth and spontaneous metastasis. These advances in BCSC imaging revealed that CD44+ cells from both primary tumors and lung metastases are highly enriched for tumor-initiating cells. Our metastatic cancer models, combined with noninvasive imaging techniques, constitute an integrated approach that could be applied to dissect the molecular mechanisms underlying the dissemination of metastatic CSCs (MCSCs) and to explore therapeutic strategies targeting MCSCs in general or to evaluate individual patient tumor cells and predict response to therapy.

breast cancer | human-in-mouse cancer models | fused optical reporters | bioluminescence imaging

Cancer stem cells (CSCs) were first identified in human leukemia (1, 2) and exhibited capacity to form tumors in immunodeficient mice. Because CSCs are characterized from various types of cancers, CD44 has been a useful marker for enriching CSCs not only for breast tumors but also a variety of other epithelial tumor models (3–17). We and others have previously reported that CSCs are more resistant to traditional cancer therapies (4, 18, 19). There is circumstantial evidence that CSCs may be involved in metastasis of solid tumors, including breast cancer. Breast CSCs (BCSCs) possess an “invasiveness” gene signature that correlates with poor overall survival and shortened metastasis-free survival in cancer patients (20). Importantly, BCSCs are enriched for cells that can undergo epithelial–mesenchymal cell transition (EMT), which likely plays a critical role in metastasis in at least some tumors (21). The observation that microRNAs in normal breast stem cells and BCSCs can regulate both EMT and self-renewal further suggests that CSCs might somehow play a role in metastasis (22). Nonetheless, there remains uncertainty surrounding the contributions of CSCs to metastasis.

Understanding the role of CSCs in metastasis requires a reliable, noninvasive measure of BCSC outgrowth and dissemination in representative and predictive models of human metastatic disease. Because of genetic differences in mouse tumors or genetic changes that occur with establishment of cell lines, the commonly used models to study metastases, including those involving human cancer cell lines, mouse tumor models, and/or metastatic tumor models via bloodstream injections, do not fully recapitulate human disease (9, 23–25). Here, by implanting patient tumors or BCSCs into mouse mammary fat pads and using noninvasive imaging strategies, we established representative human-in-mouse orthotopic xenograft tumor models that developed spontaneous metastases. The BCSCs were tagged through expression of optical fusion reporter genes to facilitate their visualization in the living mouse, enable their retrieval for subsequent flow cytometry and functional analyses, and guide the selection of cells for ex vivo or in vivo analysis and/or killing assays. Several early-passage xenograft tumors were identified in which the growth and dissemination of BCSCs could be monitored using noninvasive imaging techniques. The collection of live invasive cells from the primary tumors by use of an in vivo invasion assay (26) enabled phenotypic analysis of the invasive cells in these tumor models. By this approach, we observed an enrichment of the CD44+ population among the invasive breast cancer cells.

Optical imaging approaches that use multifunctional reporter genes permit thorough analyses of disease models by linking in vivo and ex vivo assays and guiding the experimental design (27–32). Such reporters include firefly luciferase (Luc) for whole-body tracking of cells via bioluminescence imaging (BLI) (33) and fluorescent proteins that facilitate intravitral imaging and ex vivo analyses (e.g., fluorescence microscopy and flow cytometry). Dual-function BLI-fluorescent reporter constructs constituting the Luc coding sequence fused to that of fluorescent proteins were used to label BCSCs. To maximize the BLI detection sensitivity of BCSCs, we used a modified, codon-optimized version of Luc, Luc2 (34). Cells expressing this reporter were 10–100 times brighter than those with previously reported versions of Luc (Luc+; SI Materials and Methods). In addition to the widely used enhanced GFP (eGFP), we also fused Luc2 to the red fluorescent protein td-Tomato (Tom) (35), which has improved detection


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in vivo relative to GFP. We established optimized parameters to transduce primary or passaged BCSCs with lentiviral vectors encoding Luc2-eGFP (L2G) or Luc2-Tom (L2T) fusion genes; thereby as few as 10 cells of labeled BCSCs could be noninvasively visualized at 16 h after implantation, thus greatly enhancing the accuracy and efficiency of tumorigenic assays compared with traditional monitoring of the formation of palpable tumors that takes 3–6 mo. Driven by the ubiquitin promoter, these optical reporters could be stably expressed over a series of tumor passages. More importantly, they enabled the retrieval and detection of invasive tumor cells (ITCs) collected in vivo (26, 36), as well as micrometastases from mouse lungs, which could not be readily detected by the more labor-intensive traditional invasive methods (e.g., H&E staining), therefore facilitating the identification and phenotypic analysis of invasive and metastatic cells. We demonstrated that CD44+ metastatic cancer cells isolated from the lungs were capable of regenerating tumors upon orthotopic transplantation, suggesting a metastatic role for BCSCs.

Our metastatic models, in combination with noninvasive imaging techniques, might be broadly applicable to rapidly stage disease, assess therapeutic responses, guide in vivo cell selection, and facilitate ex vivo analyses, and as such are essential for functional characterization of CSCs and for preclinical studies of therapies that target these cells.

Results

Generation of Human-in-Mouse Breast Cancer Xenograft Models in NOD/SCID Mice. According to gene expression profiling and hormone receptor status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2 or ErbB2), breast cancer is traditionally classified into four or five molecular subtypes: ER+ (luminal A or luminal B, usually PR− and Her2−), overexpressed Her2 (Her2+, usually ER ‘PR−), negative for ER, PR, and Her2 (ER ‘PR ‘Her2−, mostly basal-like), and normal-like (37–41). To generate human-in-mouse orthotopic xenograft tumor models representative of these subtypes, we collected fresh specimens from various types of breast cancer patients and then xenotransplanted sliced tumor specimen or sorted CD44+ lineage− breast cancer cells into mammary fat pads of nonobese diabetic (NOD)/SCID mice as previously described (3). From breast tumor specimens representing each of these subclasses, we generated eight xenograft tumor models, including two ER+ (E1, E2), two Her2+ (H1, H2), and four triple-negative (TN) tumors (TN1–TN4) (Fig. S1 and Table S1). Because TN breast cancers exhibit a more aggressive and metastatic phenotype in the clinic (42), we expected that these tumor-derived orthotopic xenografts would have a higher likelihood of metastasizing, and thus we focused primarily on these models for our studies. Using previously described methods (3), we identified tumorigenic populations from two TN early-passage xenograft tumor models, which we called TN1 and TN2. In both tumors, CD44+ cells were demonstrated to be tumorigenic, whereas CD44− cells were not (Fig. S1 and Table S2).

Visualizing 10 BCSCs in Vivo by BLI. To closely monitor BCSC fates and functions in mice, we labeled CD44+ cells from TN1 or TN2 tumors with dual-function reporters comprising the coding sequences of bioluminescent and fluorescent proteins (28–31, 43), which allow for both BLI in vivo and fluorescence imaging in vitro, along with flow cytometry analyses. We generated several lentiviral constructs, comprising different reporter genes and promoter sequences, and assayed their utility in labeling BCSCs under a variety of conditions (multiplicity of infection, or MOI, of 5–100) (Fig. S2). We found that the ubiquitin promoter-driven L2G or L2T (pFU-L2G/L2T) led to the most efficient or stable labeling and the brightest bioluminescent signals of BCSCs (Fig. S2), with a sensitivity of BLI detection of one cell in vitro (Fig. S3A and C) and minimum of 10 cells in vivo (Fig. L4). Tumor growth was observed from two of four injections of 10 BCSCs (Fig. S3B and C), and the label was stable for at least two or three serial transplantsations, with 80–90% of the cells maintaining labeling, as determined by flow cytometry (Fig. 1B). Importantly, we did not observe a significant change in tumor phenotypes associated with the lentiviral transduction, as revealed by tumor size, histological appearance, and flow cytometry profiles, and there was no infection of residual mouse stromal cells (Fig. S4A–D). We also observed comparable gene expression profiles between sorted eGFP+ tumor cells and the unlabeled tumor cells by real-time PCR of 30 genes (Fig. S4E) that have been shown to regulate invasion in vivo in mouse breast tumor models (36).

![Fig. 1. Visualizing BCSCs with optical reporters. (A) BLI of L2T-TN1 tumor cells (10–50,000) implanted into four separate fat pads of NOD/SCID mice. Transduced td-Tomato+ cells were sorted from the labeled parental tumor. (B) Stable integration of the optical reporters L2T or L2G into TN1 tumor cells after lentiviral transduction. Reporter expression in harvested cells was measured by flow cytometry in both parental tumors (parental) and passaged tumors (p1–p3). Sample flow cytometry dot plots are shown.](https://www.pnas.org/content/107/50/20676.full.pdf)
Monitoring the Early Growth of Breast Cancer Cell Subsets in Vivo. On the basis of the 10-cell detection sensitivity enabled by L2G or L2T, we were interested in improving the detection of tumor cell growth in vivo at early time points after implantation, information that is lacking in traditional CSC assays. TN1-BCSCs transduced by L2G (labeled passage 1) were visualized by BLI as early as 16 h after infection/implantation, and their signals intensified over time (Fig. 2A and B). Whereas the early cell growth curve was predominantly linear (Fig. 2B, Inset), the late stage of tumor growth showed an exponential burst of BLI signal that was consistent with the formation of palpable tumors. This finding underscores the utility of BLI in vivo detection methods for monitoring early events in tumorigenesis.

Whereas traditional tumorigenic assays are not amenable to monitoring the cell fate of nontumorigenic cells, we used BLI to more closely evaluate the contributions of different primary cell populations to tumor initiation or progression. We transduced different subsets of primary breast tumor cells from human specimens with L2T or L2G for in vivo implantation and imaging. Both CD44+ and CD44− human breast cancer cells were transduced by L2T and detected on day 2 after implantation (Fig. 2C and D). As shown by the growth curves, CD44+ cells grew at a steady rate over 5 mo. Surprisingly, the signal of CD44− cells increased slightly after injection but stabilized by day 50−90 and did not increase further over the next 60 d (Fig. 2D), indicating that these cells had a limited proliferative capacity. Although these CD44− tumor cell subsets did not form palpable tumors, BLI provided an efficient and improved method of assessing their proliferation at early time points after implantation in vivo.

Detection of Breast Cancer Metastases in Vivo. Optical imaging enabled the sensitive detection of BCSCs in vivo, suggesting that it could be used to guide the early detection of breast cancer spontaneous metastasis. Using L2G- or L2T-transduced BCSCs, we detected spontaneous lung metastasis of TN1 and TN2 tumors noninvasively in vivo, as well as ex vivo after dissection (Fig. 3A and Fig. S5A), even though macrometastases were not visually observed within the dissected lung lobules (Fig. S5B). We were able to detect lung metastases ex vivo with BLI from all TN1 (n = 53) and TN2 tumor-bearing mice (n = 20) after tumors became 2 to 3 cm in diameter. Furthermore, we detected local lymph node metastases in mice bearing TN1 tumors (Fig. 3B) and distant lymph node metastases in mice bearing TN2 tumors (Fig. S5A). This indicated that these human-in-mouse orthotopic models represented clinical features of patient tumors with both local and distant metastases. In addition, we labeled E1 (ER−PR−Her2+) and H2 (ER−PR−Her2+) tumor cells with L2T for mouse mammary fat pad implantation and detected spontaneous lung metastases of the E1 tumor-bearing mice with BLI (Fig. S5C).

By H&E staining of lung sections, we validated cancer metastases of these breast cancer xenograft models with or without reporter gene transduction (Table S1). The lung and lymph node metastases were confirmed in the labeled TN1 tumor-bearing mouse (Fig. 3C and Fig. S6). In addition, by H&E staining lung micrometastases were identified from all four TN xenografts.

Fig. 2. Analyzing the early growth of breast cancer cell subsets in vivo via BLI. (A and B) Monitoring tumor growth mediated by TN1 BCSCs (passage 2−5). CD44+ cells (1 × 10⁶) were transduced with L2G (MOI 50) or the mock and then transplanted into mouse mammary fat pads. Mice were imaged from day 1 to day 52 after implantation when palpable tumors formed. Quantification (total flux, photons per second, p/s) of the bioluminescent signal from the tumor regions in A is depicted in B. Error bars represent the SD of the mean for three experiments; *P < 0.01 for early-phase growth curve day 7 and P < 0.05 for day 52. (C and D) Analysis of primary breast tumor cell subsets into NOD/SCID or NSG (NOD/SCID with IL2R−γc−−) mice. Primary breast tumor cells were dissociated from clinical patient specimens, and 3,000 CD44+ or CD44− cells were sorted for transduction with L2T fusion reporter gene (MOI 10), implanted into mice, and analyzed by BLI over time. Representative images are shown in C and the light emission is quantified in D. Error bars represent SD of the mean for six replicate experiments (three from NSG mice).
result underscores the efficiency and sensitivity of BLI in metastasis detection as the imaging technique is used to guide tissue selection for these more labor-intensive assays. With our spontaneous metastasis models, we wanted to investigate whether BCSCs were directly involved in metastasis, particularly in the initial invasion step. Using an established in vivo invasion assay, whereby migration toward a cytokine gradient can be tested in the primary tumor microenvironment (26, 36), we first demonstrated that tumor cells from both TN1 and TN2 primary breast tumors were invasive and chemotactic to EGF in vivo (Fig. 4 A and B). Because of the limited numbers of cells we could collect from each tumor, we used a Guava microcapillary flow cytometer to analyze the expression of CD44 on sorted eGFP+ ITCs and average primary tumor cells (APTCs) from the TN1 breast tumors. Compared with APTCs, the ITCs were significantly enriched with CD44high cells (41% of ITCs vs. 17% of total APTCs from this tumor) (Fig. 4 C and D). These results clearly demonstrated the preferential invasive behavior of the BCSCs in vivo.

**Tumorigenic Assays of TN1 and TN2 Metastatic Cancer Cells.** To test whether metastatic cancer cells share properties with primary BCSCs, we analyzed the marker expression profiles and tumorigenic capacity of lung metastatic cells isolated from TN1 and TN2 breast cancers. Histological analysis revealed that in both TN1 and TN2 tumor models, lung metastatic cells and primary tumor cells had similar expression patterns of high-molecular-weight cytokeratins (CK3+BE12 or CK5/6) and proliferation indexes as assayed by Ki67 levels (Fig. S7A). Although metastatic cells were a rare population (0.5–2%) in the lungs (Fig. S7B), we took advantage of L2G- or L2T-transduced tumor models for clear identification and guided dissection of human cancer cells from lungs that shared similar expression profile of CD44 with parental breast cancer cells in the primary site (17–30% CD44+ cells) (Fig. S4).

To investigate whether metastatic cells contained BCSCs, we isolated lung cells from unlabeled TN1- or TN2-bearing mice and implanted bulk lung cells into mouse mammary fat pads for tumorigenic assays. The lung metastatic cells regenerated tumors upon implantation into the mammary fat pads (Fig. 5B), indicating the existence of BCSCs in the lung micrometastatic lesions. We further analyzed the expression profiles of CD44 in unlabeled lung metastatic cells excluding mouse stromal cells (Fig. 5B) and performed tumorigenic assays in vivo with sorted CD44+ and CD44− populations of TN1 or TN2 lung metastases. CD44+ metastatic cells from either TN1 or TN2 models were more tumorigenic than CD44− cells upon implantation into orthotopic mammary fat pads of NOD/SCID mice (Fig. 5B), and these CD44+ populations isolated from grown tumors were able to passage and differentiate again in mice (Fig. S7B). These data implicated the self-renewal and differentiation capacity of CD44+ cells.

Whereas the TN1 and TN2 patients only developed detectable metastases in the lungs and/or lymph nodes (Table S1), no metastasis was detected in the brain, liver, bones, or spleen in our mouse models, as determined by BLI (Fig. S7C). Surprisingly, after tail-vein injection of dissociated TN1 lung metastasis-derived breast tumor cells, we observed different patterns of homing, with most of the cells homed to the spleen, liver, or bones (Fig. S7D), which were not spontaneous metastatic sites. In agreement with other orthotopic injection models, these observations suggest that metastatic cells experience conditions that lead to different fates compared with spontaneous metastases from orthotopic tumors. This result also underscores
the relevance of our model for recapitulating the metastatic cas-

ocus in human breast cancer patients.

Discussion

We have developed a robust approach for imaging of BCSC
growth and dissemination, which permits both macroscopic
and microscopic analysis of cancer progression. In our studies,
the imaging assays facilitated development of visible human-in-mouse
 xenograft tumor models with spontaneous metastases to lungs or
local/distant lymph nodes. Our patient-tumor derived xenograft
models will be able to overcome some limitations of previous
metastatic models with human cancer cell lines or mouse tumor
models. Spontaneous metastases present representative features
of patient tumors that can be used in predictive models of me-
tasis and therapeutic response, but cannot be recapitulated by
lung/bone colonization models via tail-vein or intracardiac injec-
tions. This point is driven home by the observation that these
 xenografts metastasize to the lungs but not the bones when grown
in the mammary fat pads. This likely reflects that “triple-negative”
ER− breast cancers tend to metastasize to the lungs rather than
the bones (44). Surprisingly, when injected into the tail vein, the
cells lodged at different organs, such as the bones. The molecular
and cellular mechanisms underlying this phenomenon need to be
further investigated. The simplest explanation is that unlike cell
lines often used to study metastases, molecular pathways activated
in these early-passage xenograft cells that regulate the earlier
steps of metastasis (i.e., invasion and intravasation) are also
obligatory for the later steps of metastasis. Our ability to collect
the invasive/intravasating population of tumor cells from these
TN1 and TN2 tumors will allow direct analysis of this possibility.

Traditionally, the tumorigenic assays used to identify and ex-
amine CSCs from primary tumors are labor-intensive, time-con-
suming (3–6 mo), and provide little or no information on the
dynamics of early tumor growth or nontumorigenic cell function.
With our models and noninvasive imaging, we could monitor early
tumor growth and the activities of defined subsets of human breast
cancer cells. The relevance of CD44+ CSCs function and clinical
outcome has been questioned (17, 45, 46). This work further
supports reports from many other groups that CD44+ cells cor-
relate with EMT, unfavorable prognosis, and metastasis of clinical
breast cancer (17, 20, 21, 47). In some instances, mice were
injected with tumor cells at two different sites. Although it is
possible that tumors grown at one flank of the recipient mice may
regulate tumor cells implanted into the other flank (48), in our
studies we have not observed clear regulatory (suppressive or
promoting) effects, if any, of grown tumors on distant tumor cells
at other injection sites of mice.

By labeling BCSCs with optical reporters, we have been able to
isolate cells for further analysis or profiling. Most interestingly,
our study shows that BCSCs are significantly enriched in invasive
cells, suggesting that BCSC subsets are more capable of mediating
invasion than non-CSCs in vivo. This implies that CD44 serve
not only as markers of primary or parental BCSCs but also as
markers of metastatic CSCs (MCSCs). Increased CD44 expres-
sion in invasive cells can result from both transcriptional and
posttranscriptional regulations. It is not possible to determine the
 fate of CD44− cells in lung metastasis by tail-vein injection be-
cause they bypass the invasion or intravasation steps of meta-
stasis. Additional promoter-specific labeling of these cells may assist
in addressing this issue, although the CD44 expression levels and
functions are highly regulated posttranscriptionally. We also show
that a portion of metastatic cancer cells in the lungs share surface
markers (CD44+) and functional tumorigenic properties with
parental BCSCs. Thereby our work supports a colonization role
for disseminating CD44− CSCs during metastasis. However, it
may be possible that under pathophysiologic stimuli, the CD44−
breast cancer cells could undergo further genetic mutations or
epigenetic events that enable them to self-renew. This requires
further investigations with assistance of cell labeling approaches.

The optimized strategy for labeling BCSCs with optical reporter
genes can be applied to studies with CSCs associated with other
malignancies (colon cancer, brain tumor, head-and-neck cancer,
leukemia, etc.) and to evaluate other putative CSC markers (e.g.,
CD133, ALDH1, etc.) and various behaviors of subset pop-
ulations. The sensitivity of detection down to 10 cells in vivo led to
improved efficiency of the tumorigenic assays that were initiated
with a limited number of dissociated cancer cells. Theoretically, as
few as hundreds of cells in sorted subpopulations from tumor bi-
opsies could be labeled with L2T/L2G lentiviral vectors and sub-
jected to analyses of growth kinetics in vivo. Future studies will
aim to define CSC-specific and metastasis-regulating gene sets
and their promoters, label cancer cells with optical reporters
 driven by such unique promoters, and thereby evaluate the gene
expression dynamics in CSCs and MCSCs, as well as monitor cell—
cell interaction and cell fate and function during metastasis. Stabile expression of optical reporter genes in BCSCs will also enable future intravital imaging studies of BCSC-mediated early tumor initiation, invasion, circulation, and colonization via BLI and multiphoton microscopy. For these purposes, imaging will be an indispensable tool for revealing patterns of dissemination as well as determining the kinetics of tumor growth and spread to distant tissues after initiation with BCSCs. Such image analyses will also help to unravel the molecular mechanism underlying the dissemination of MCSCs by overexpression/knockdown of candidate players or regulators at genetic or epigenetic levels.

Therapeutic targeting of metastatic cells is the most challenging goal in clinical oncology. However, most of the molecular studies of metastatic mechanisms have been limited to human cancer cell lines and mouse models. We anticipate that our models and imaging strategies will be quite useful for preclinical therapeutic screenings and used as a guide to investigate therapies targeting CSCs in general or an individual patient’s own tumor cells. We will investigate the effects of biological therapies on CSCs, along with the promise of preclinical imaging in development of effective cancer therapies—a promising translational application of this imaging approach.

Materials and Methods

Experimental details are provided in SI Materials and Methods, including materials and protocols, tumor cell isolation and flow cytometry analysis, tumor transplantation in mice, cloning, lentivirus production and transduction, CSC transduction, bioluminescence imaging, in vivo invasion assay, Guava flow cytometry analysis of APTCs and invasive cells, immunohistochemistry, RNA extraction and PCR amplification, and statistical analysis with two-tailed Student’s t tests.

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