Identification of Tumorsphere- and Tumor-Initiating Cells in HER2/Neu-Induced Mammary Tumors

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Abstract

A variety of human malignancies, including breast cancer, are thought to be organized in a hierarchy, whereby a relatively minor population of tumor initiating cells (TIC) is responsible for tumor growth and the vast majority of remaining cells is nontumorigenic. Analysis of TICs in model systems of breast cancer would offer uniform and accessible source of tumor cells and the power of mouse genetics to dissect these rare cells. The HER2/Neu proto-oncogene is overexpressed in an aggressive form of human breast cancer. Mouse mammary tumor virus (MMTV)-Neu transgenic mice develop mammary tumors that mimic human HER2 subtype breast cancer. Here, we report on the functional identification of mouse HER2/Neu TICs that can induce tumors after transplantation into the mammary gland of recipient mice. Secondary tumors formed after injecting MMTV-Neu TICs resemble primary tumors in the original transgenic mice and are organized in a hierarchy containing TICs as well as their nontumorigenic descendants. To study MMTV-Neu TICs in vitro, we grew tumorspheres under nonadherent culture conditions. Tumorsphere forming units (TFU) capable of producing tumorspheres retained tumorigenic potential and were indistinguishable by several criteria from TICs. Interestingly, MMTV-Neu TICs and TFUs were committed to the luminal cell fate when induced to differentiate in vitro. Our data define reproducible characteristics of the MMTV-Neu TIC and TFU, which help to explain marker expression profiles of HER2-positive breast cancer. In addition, the similarity between TICs and TFUs in this system provides a rationale for TFU-based screens to target tumor-initiating cells in HER2+ breast cancer. [Cancer Res 2007; 67(18):8671–81]

Introduction

HER2/ErbB2/Neu, a transmembrane tyrosine kinase receptor of the epidermal growth factor (EGF) receptor family, is overexpressed in 25% to 30% of human breast cancer (1). HER2-positive breast tumors are particularly aggressive (2). The causative role of HER2 and its overexpression in both tumors and metastatic sites renders this oncoprotein an ideal therapeutic target. Indeed, trastuzumab (Herceptin) a humanized monoclonal antibody against the extracellular portion of HER2, has been reported to improve overall response (3). In particular, the addition of trastuzumab to the adjuvant therapy of HER2+ early breast cancer has resulted in a 50% reduction in the risk of recurrence (4). Yet, for patients with HER2+ metastatic cancer treated with optimal combination therapy of chemotherapeutic drugs plus Herceptin, the median progression-free survival is less than a year (5). Novel approaches to target this aggressive form of breast cancer are urgently needed.

There is growing evidence that the majority of cancer cells is nontumorigenic. Tumor initiating cells (TIC), capable of forming new tumors when transplanted into immunocompromised recipient mice and presumably primary tumors/metastatic disease in cancer patients, are relatively rare (6–8). TICs were initially identified in acute myeloid leukemia (AML) in which cells capable of inducing human AML in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were shown to possess differentiative and proliferative capacities, and the potential for self-renewal characteristic of a leukemic stem cell (9, 10). Although the AML TIC resembles and probably originates from the transformation of a stem cell, it is possible that other TICs originate from transformation of early or late progenitor cells. Indeed, in mixed lineage leukemia (MLL), oncogenic transformation can occur within progenitor rather than stem cells, in part by inducing self-renewal genes (11). Thus, the definition of a TIC is not related to the cell of origin for a tumor but rather to its ability to self-renew, initiate cancer, and give rise to more differentiated cells that have lost self-renewal and tumorigenic potential.

The notion that cancer is driven by TICs has obvious therapeutic implications (12). The efficacy of tumor response to systemic therapy has traditionally been assessed on the bulk of tumor cells by monitoring for changes in tumor size (13). However, if only a small fraction of TICs is capable of initiating cancer, then curative therapy should be designed to target these rare TICs rather than the bulk of nontumorigenic cells. Thus, analysis of TICs may unravel novel therapeutic approaches/targets.

Analysis of human breast cancer revealed that TICs in this disease also represent a small fraction within an heterogeneous population of mostly nontumorigenic cells (ref. 14; reviewed in refs. 7, 15–19). Human breast TICs express early epithelial markers such as epithelial specific antigen (ESA) and the cell surface marker CD44, whereas nontumorigenic cells express CD24 (14). ESA+/CD44low or CD44+/CD24low but not CD24high cells sorted from pleural effusions induced tumors when injected into the fat pad of NOD/SCID mice (14). The CD44+/CD24− tumor cells gave rise to a heterogeneous tumor population that was again composed of CD44+/CD24− TICs as well as their nontumorigenic CD24+ descendants. TICs have subsequently been identified in brain, colon, ovarian, prostate and pancreatic tumors (20–24).

In mice, mammary stem cells (MSC) capable of repopulating an empty fat pad and generating luminal-epithelial, myoepithelial, and alveolar-epithelial cells have been functionally identified. They were initially observed as stem cell antigen (Sca1)−positive cells (25). Interestingly, the Sca1+ population is expanded in...
MMTV-Wnt1 tumors but reduced in MMTV-Neu mammary tumors (26–28). MSCs were subsequently identified as positive for the heat-labile CD29 antigen and either β1-integrin (CD29) or α6-integrin (CD49f; ref. 29). CD29hi/CD24+ cells express the myoepithelial marker K14, but not the luminal-epithelial marker K18, highlighting the basal nature of these stem cells. The CD29hi/CD24+ stem cell population is expanded in preneoplastic mammary glands of MMTV-Wnt1 but not MMTV-Neu mice (29, 30). However, the relationship between MSCs and TICs in mouse models using cell surface markers and functional analysis has not been addressed.

Here, we report on identification of TICs from MMTV-Neu mammary tumors. We show that tumorsphere-forming units (TFU) and TICs both segregate with the same cell surface marker profile and that tumorspheres contain TICs, suggesting that they originate from the same cell. Our identification of MMTV-Neu TFUs and TICs should facilitate analysis of these rare cells, the pathways controlling their biology, as well as the screening and identification of TIC-specific therapeutic targets.

Materials and Methods

Mice and PCR-based genotyping. MMTV-Wnt1 and MMTV-Neu mice both on pure FvB background were maintained in accordance with the Canadian Animal Care Council. Primers for PCR-based genotyping of tail DNA were as follows: Wnt1-forward 5′-GGACTTGTCTCTTCTCATAGCC; reversed 5′-CCACACAGGCATAGTGTCGTC. Neo-forward 5′-CTAGGC-CACAGAATGGAAGATCT; reversed 5′-GTAGGGGAATCTCATGATC.

Tissue harvesting, single-cell preparation, and enrichment of Lin− epithelial cells. Mammary tumors (0.5–1.0 cm in diameter) or glands were dissected from MMTV-Neu, MMTV-Wnt1 mice, and their wild-type littermates. A portion of the tumor/tissue was fixed in 4% paraformaldehyde in PBS at 4°C overnight and sequently dehydrated (30 min each in PBS, saline, 50% ethanol/saline, 70% ethanol/saline) and paraffin embedded. The remainder of the tumor (or gland) was minced into paste with sterile razor blades, washed in PBS, and digested in 100 units/mL collagenase (Sigma) for 2 h at 37°C with occasional mixing. The samples were washed with 5 volumes of HBSS (Sigma, phenol red free) + 2% fetal bovine serum (FBS) and 2 mmol/L EDTA (HFE) and centrifuged at 450 × g for 5 min. The pellet was dissociated in 2 mL dispase (Life Technologies) for 1 min at room temperature, followed by passing through 25G needles.

Mammary/tumorsphere culturing in vitro. Single-cell suspension of Lin− mammary epithelial cells or sorted cells from tumors or mammary glands were plated on ultra–low attachment plates (Corning, Costar) in DMEM/F-12/12% FBS, 5 μg/mL insulin, 1 μg/mL hydrocortisone, 5 ng/mL EGF, and penicillin/streptomycin for 3 to 5 days. Differentiation was induced for 24 h in medium containing DMEM/F-12, 5 μg/mL insulin, 1 μg/mL hydrocortisone, 3 μg/mL prolactin, and penicillin/streptomycin. Cells on coverslips were fixed in 3.5% paraformaldehyde for 5 min at room temperature, washed with PBS (3×, 3 min each), and permeabilized in OPAS buffer (100 mmol/L PIPEs, 1 mmol/L EGTA, 100 mmol/L KOH, 4% PEG 8000, 0.1% Triton X-100) for 5 min. After washing with PBS (3×, 3 min each), samples were incubated with 1% bovine serum albumin for 5 min followed by primary antibodies for 45 min. Antibodies were rabbit anti-smooth muscle actin (SMA, 1:200 dilution, Novus Biologicals), mouse anti–keratin 18 (K18, 1:200 dilution, Fitzgerald), and rabbit anti–keratin 14 (K14, 1:200 dilution, Panomics). Cells were washed in PBS (3×, 3 min each) and incubated for 45 min with goat anti-rabbit Alexa 488 (green, 1:200 dilution, Molecular Probes) or goat anti-mouse Alexa 568 (red, 1:200 dilution, Molecular Probes). Nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI, Sigma). Slides were washed thrice with PBS, mounted (DakoCytomation), and analyzed under Zeiss Axioskop 2 fluorescent microscope.

Histology and immunofluorescence staining. Paraffin-embedded tissue sections were sectioned (4 μm) using a Reichert Ultracut E microtome. Sections were deparaffinized by treating twice with xylene for 10 min each and sequentially hydrated in 100%, 90%, and 70% ethanol, respectively. For histologic studies, samples were stained with hematoxylin for 15 min; treated with 70% ethanol/1% HCl; and then stained with eosin for 5 min, dehydrated, and mounted in Permount (Sigma). For immunofluorescence staining, slides containing tissue sections were deparaffinized and hydrated as above. Antigen retrieval was done by boiling samples in 10 mmol/L sodium citrate (pH 6.0), for 10 min in microwave followed by 30 min gradual cooling at room temperature. The slides were incubated with M.O.M. mouse immunoglobulin blocking reagent for 1 h (Vector M.O.M Immunodetection kit, Vector Laboratories) and then with primary antibodies (diluted in M.O.M. diluent) in a humidified chamber at 4°C overnight. Secondary antibodies (goat anti-rabbit Alexa 488 and goat anti-mouse Alexa 568, both 1:200 dilution) and DAPI were added for 1 h at ambient temperature. Primary antibodies were as above plus mouse monoclonal SMA antibody (1:800 dilution, Sigma).

Transplantation. Sorted cells, dissociated tumorspheres, or whole tumorspheres were resuspended in 10 μL medium and mixed at 1:1 ratio with 10 μL Matrigel (BD Bioscience) on ice. The samples (total 20 μL) were injected into no. 4 mammary glands of immunocompromised Rag1−/− mice and in subsequent experiments into syngeneic FVB mice anesthetized with isoflurane. Liquid bandage (NewSkin) was applied to prevent sample leakage followed by 9-mm autoclip (Clay Adams Brand).

Calculation and statistical analysis. The limit-dilution calculation was based on a Poisson probability distribution: ln(y) = rx + ln(a), where y is the ratio of non–tumor-generating injections, x is the number of cells injected, and r is the frequency of TICs; a is the y intercept and it was
assumed that with no cells injected, no tumors are formed (therefore at 100% non–tumor generation, $a = 1$). The frequency of TICs ($r$) was calculated from the slope of line best fit to the data points. Statistical analysis was done using ANOVA and the Bonferroni tests for post hoc analysis. Differences between values were considered statistically significant at $P < 0.05$.

**Results**

Flow cytometry analysis with CD24 plus Sca1 but not CD49f discerns between MMTV-Neu and MMTV-Wnt1 tumors. MMTV-Neu transgenic mice carrying unactivated Neu proto-oncogene develop undifferentiated SMA-negative adenocarcinomas after long latency (28, 31). To identify tumor-initiating cells in MMTV-Neu mammary tumors, we used a series of enrichment steps. Nonepithelial cells were immunodepleted using hematopoietic (CD45/Ter119), endothelial (CD31), and stromal (CD140a) antibodies (see Materials and Methods). 7AAD, a fluorescent dye that accumulates in dead cells, was included to select for live cells. Lineage negative (Lin$^-$), live, single-cell populations were gated and subsequently analyzed or sorted on the basis of cell surface marker expression. Previous work showed an expansion of CD29$^{hi}$:CD24$^+$ progenitor/stem cell population in preeclastic mammary glands from MMTV-Wnt1 but not MMTV-Neu mice (29, 30). We have reproduced these data using CD49f in place of CD29, which

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Figure 1. Sca1 and CD24 cell surface markers discern between MMTV-neu and MMTV-Wnt1 tumors. A, representative flow cytometry analysis of CD140a$^-$, -CD31$^-$, CD45$^-$, TER119$^-$ (Lin$^-$), and 7AAD$^-$ mammary epithelial cells purified from wild-type, MMTV-Neu, and MMTV-Wnt1 mammary glands with CD24-FITC and CD49f-PE antibodies. B, flow cytometry analysis of Lin$^-$ mammary epithelial cells purified from MMTV-Neu and MMTV-Wnt1 mammary tumors with CD24-FITC and CD49f-PE antibodies. C, flow cytometry analysis of Lin$^-$ mammary epithelial cells purified from MMTV-Neu and MMTV-Wnt1 mammary tumors with CD24-FITC and Sca1-PE antibodies. D, percentage of epithelial cells from wild-type mammary gland (WT), MMTV-Neu tumor (NeuT), and MMTV-Wnt1 tumor (WntT) expressing both Sca1 and CD24 (Sca1$^+$:CD24$^+$), CD24 only (CD24$^+$), or Sca1 only (Sca1$^+$). Data were collected from three to five independent experiments. Columns, percentage of total; bars, SE. *, $P < 0.05$, statistically significant (ANOVA).
Figure 2. Differentiation potential of Neu and Wnt1 mammary tumor cells. Lin− mammary epithelial cells from MMTV-Neu and MMTV-Wnt1 tumors were sorted on the basis of Sca1 and CD24 expression. The sorted cells were plated on collagen-coated slides, induced to differentiate, and subjected to immunofluorescence with myoepithelial (SMA, cytokeratin 14) and luminal-epithelial (keratin 18) cell markers. A, SMA (green) plus K18 (red). B, K14 (green) and K18 (red). Nuclei were visualized by DAPI (blue). Original magnification in A and B, ×400. C, percentage of cells (columns) expressing SMA (n = 4), K18 (n = 6), or K14 (n = 2) was calculated after counting >100 cells from each quadrant; bars, SD.
was also used in combination with CD24 to identify MSCs. Mammary glands from control wild-type littermate, MMTV-Neu, and MMTV-Wnt1 mice exhibited ~13%, 6%, and 20% CD49f⁻; CD24⁺ double-positive cells, respectively (Fig. 1A). The percentage of CD49f⁻;CD24⁺ cells varied depending on the mouse age, but the pattern was similar in multiple mice (n > 5), showing a reproducible expansion of the CD49f⁻;CD24⁺ population in Wnt1 but not Neu mammary glands. In contrast to these values obtained from preneoplastic mammary glands, we have found a dramatic increase in the CD49f⁻;CD24⁺ cell populations (~50%) in both MMTV-Neu and MMTV-Wnt1 mammary tumors (Fig. 1B). Thus, flow cytometry with CD24 and CD49f cannot be used to distinguish these two mammary tumor types.

The Sca1⁺ fraction is enriched for MSCs (25) and is abundant in Wnt1 but not Neu mammary tumors (28). We therefore asked whether double labeling with CD24 and Sca1 would distinguish Wnt1 and Neu mammary tumors. Relative to wild-type FvB nulliparous mammary glands, MMTV-Wnt1 tumors exhibited a 2-fold increase in the Sca1⁻;CD24⁺ cell population (Fig. 1C and D). In contrast, MMTV-Neu tumors contained less Sca1⁺;CD24⁺ cells relative to control mammary glands and instead displayed an expansion (70–80%) of the Sca1⁺;CD24⁺ cell population. As the combination of CD24 and Sca1 antigens allowed us to discern between Neu and Wnt1 tumors, we used these markers in all subsequent experiments.

**Differentiation potential of sorted MMTV-Neu tumor cells.**

To define the lineage potential of tumor cell populations sorted on the basis of Sca1 and CD24, purified Lin⁻, 7AAD⁻ MMTV-Neu, and MMTV-Wnt1 tumor cells were sorted for Sca1 and CD24, plated on collagen 1–coated coverslips, and cultured in growth medium containing 10% FBS, insulin, hydrocortisone, and EGF. After 3 to 5 days, differentiation was induced for 24 h by switching to medium containing insulin and prolactin. The cells were fixed and stained with SMA, a myoepithelial cell marker, and cytokeratin 18 (K18), a luminal-epithelial marker. As shown in Fig. 2A, Sca1⁺ cells sorted from MMTV-Neu tumors were mostly SMA positive (~85%), CD24⁺ cells were almost exclusively K18 positive, and the Sca1⁺;CD24⁺ double-positive population contained both cell types. Sorted MMTV-Wnt1 tumor cells gave similar results except that the ratio of K18:SMa in the Sca1⁺;CD24⁺ double-positive quadrant was nearly 1:1 in Wnt1 tumors but 4.5:1 in cells from Neu tumors (Fig. 2C). Together with the flow cytometry profiles of these tumors (Fig. 1C and D), these data suggest that Neu tumors contain an increased number of progenitor cells capable of differentiating into luminal cell types, whereas Wnt1 tumors contain a large population of bipotent stem/early progenitor cells.

We next analyzed the expression of cytokeratin 14 (K14) after sorting and differentiation on collagen 1 plates. K14, a bona fide myoepithelial marker, is highly enriched in the MSC compartment (30). Remarkably, in MMTV-Neu tumors, K14 expression did not segregate with Sca1⁺ cells, as did SMA (Fig. 2A versus B). Instead, K14 was expressed in differentiating CD24⁺ and Sca1⁺;CD24⁺ cells. As shown below, these populations include TICs and TFUs. Interestingly, all K14⁺ cells also expressed K18 (Fig. 2B, yellow arrows; inset, K14 staining alone without keratin 18). In addition, some cells exclusively expressed K18 (Fig. 2B, red arrow). In contrast to the tight segregation of K14⁺ precursor cells in MMTV-Neu tumors, K14 expression in sorted MMTV-Wnt1 tumor cells was found in all three quadrants, including the Sca1⁺ cells (Fig. 2B and C). In addition, as opposed to the Neu-derived cells, most differentiated Wnt1 tumor cells did not coexpress K14 and K18 (green arrows). Some Sca1⁻;CD24⁻ double-negative cells also expressed SMA, K14, or K18, and may represent cells that had differentiated before the FACS sorting.

**Identification of MMTV-Neu TFUs.** TICs resemble stem cells in their ability to grow as spheres when cultured under conditions in which they cannot attach to a solid substratum (32, 33). To test for the ability of MMTV-Neu and MMTV-Wnt1 tumor cells to grow as spheres in culture, purified (Lin⁻) tumor cells were plated onto ultra–low-attachment plates in defined medium containing EGF, fibroblast growth factor (FGF), heparin, and B27 (34, 35). After 3 to 4 weeks in culture, ~80% of MMTV-Neu tumorspheres become symmetrically encapsulated to form “golf ball”–like structures (Fig. 3A). Figure 3B shows the kinetic of primary MMTV-Neu tumorsphere formation over a 1-month period. The golf ball spheres became hollow and did not grow or expand if left in culture for 1 month or longer. To circumvent this problem, we dissociated tumorspheres every 2 to 3 weeks before they differentiated. Interestingly, only ~20% of secondary Neu tumorspheres developed into golf ball structures; the majority appeared like normal mammospheres or Wnt1 tumorspheres.

In contrast, tumorspheres from MMTV-Wnt1 tumors did not form golf ball–like structures even when cultured for long periods.
Figure 4. Differentiation, tumorigenicity, and drug response of Neu and Wnt1 tumorspheres. A, flow cytometry analysis of Neu tumorsphere cells from MMTV-Neu and MMTV-Wnt1 tumors using the CD49f-CD24 (top row) and Sca1-CD24 (bottom row) cell surface markers. Tumorspheres were cultured as indicated for 3 or 5 wks before analysis. Ab, antibody. B, enzymatically dissociated primary or secondary tumorsphere cells were differentiated on collagen-coated slides and cell types were determined by immunocytostaining with myoepithelial (SMA, green) and luminal-epithelial (K18, red) specific antibodies. Original magnification, ×400. Data were collected from three or more experiments with >100 cells counted. C, tumorspheres contain TICs. Secondary tumors developed after injecting 1,000 cells from dissociated Neu tumorspheres into recipient mammary glands. D, effect of 0.2 and 1.0 μg/mL doxorubicin (Dox) on MMTV-Neu and MMTV-Wnt1 tumorsphere formation compared with vehicle alone (DMF, dimethylformamide). Tumorspheres were dissociated, treated with dimethylformamide or doxorubicin for 4 h, plated on ultra–low attachment plates, and then counted and photographed 2 wks later. A representative experiment done in duplicate. Columns, TFU; bars, SD. Insets, MMTV-Neu tumorspheres in the presence and absence of 0.2 μg/mL doxorubicin. Original magnification, ×100.
without dissociation (Fig. 3A). Upon dissociation and replating, the secondary and subsequent cultures formed spheres that resembled mammospheres obtained from normal mammary glands (Fig. 3A). When Sca1-CD24- cells from both MMTV-Wnt1 and MMTV-Neu tumors were sorted and then seeded in ultra–low–attachment plates, they both retained their characteristic tumorsphere shape (see below and data not shown).

To determine the incidence of TFUs in tumorspheres, 3-week-old primary tumorspheres were dissociated into single cells through light trypsinization and replated. The frequency of TFUs was ~1/700 and 1/800 for the Neu and Wnt1 tumorspheres, respectively. As shown below, the frequency of CD49f-CD24+ double-positive cells is higher in fresh versus old tumorspheres; hence, the frequency of TFU may reflect specific culture conditions and frequency of trypsinization/replating of cultures.

Differentiation and tumorigenic potential of MMTV-Neu TFUs. To test whether tumorspheres recapitulate the initial tumors from which they were derived, we did flow cytometry analysis on tumorspheres propagated for 3 weeks in ultra–low attachment plates. As shown in Fig. 4.4, Neu and Wnt1 tumorspheres maintained their characteristic flow cytometry profiles. Thus, Neu tumorspheres were enriched for CD24+ cells, whereas Wnt1 tumorspheres contained a substantial population of CD24+Sca1- double-positive cells. In addition, like the original tumors, Neu and Wnt1 tumorspheres both contained a large population of CD49f-CD24+ double-positive cells (Figs. 1B and 4A). Similar results were obtained from independent samples maintained for several months in culture, as long as they were dissociated on a regular basis every 2 to 3 weeks. If kept in culture without dissociation, yet with constant supply of fresh growth factors (EGF and FGF), the flow cytometry profiles of tumorspheres were reduced to Sca1-CD24+ or CD49f-CD24+ double negative. This trend is shown in Fig. 4A for CD49f-CD24 markers: 80% to 90% of 3-week-old secondary tumorspheres from MMTV-Wnt1 and MMTV-Neu tumors were CD49f-CD24+ double-positive cells, whereas only ~30% to 36% of ~5-week-old tumorsphere cells were double positive.

To investigate the differentiation potential of TFUs, the tumorspheres were dissociated, plated on collagen 1–coated coverslips, and stained with SMA and K18. Interestingly, primary tumorspheres from both tumor types were bipotent, giving rise to both luminal (K18+) and myoepithelial (SMA+) cells (Fig. 4B). However, primary MMTV-Neu tumorspheres were enriched for luminal cells (87%), whereas MMTV-Wnt1 tumorspheres were enriched for myoepithelial cell types (62%). When secondary tumorspheres were analyzed as above, those derived originally from MMTV-Wnt1 continued to differentiate into both luminal and myoepithelial cell types. In contrast, secondary MMTV-Neu tumorspheres differentiated exclusively into K18+ cells (Fig. 4B). Thus, Wnt1 tumorspheres are bipotent, whereas Neu tumorsphere cells are committed to the luminal cell fate.

We next tested whether tumorspheres contained tumor-initiating cells. To this end, tumorspheres were dissociated and 1,000 cells were mixed with Matrigel and transplanted into fat pads of recipient Rag-1−/− mice. Full-blown tumors were detected after 1 and 2 months from Wnt1 and Neu tumorspheres, respectively (Fig. 4C). Tumorspheres cultured for as long as 3 months under ultra–low attachment conditions gave rise to tumors after transplantation.

Inhibition of Neu and Wnt1 tumorsphere growth by cytotoxic drugs. As we show below, the TFU is similar if not identical to the TIC. We therefore asked whether we could screen for TFU inhibitory drugs. Primary tumorspheres were dissociated into single cells and treated with Adriamycin (doxorubicin), an anthracycline used in single-agent treatment of breast cancer (36, 37). The ability of the cells to form secondary tumorspheres in the absence of the drug was determined by counting spheres 2 weeks later. A 4-h exposure to high a dose of Adriamycin (1 μg/mL) reduced the number of MMTV-Neu and MMTV-Wnt1 tumorspheres by 6- and 3-fold, respectively (Fig. 4D). Thus, this assay can be used to screen for drugs/inhibitors that can suppress the proliferation or survival of TFUs.

MMTV-Neu TFUs and TICs both arise from the Sca1-CD24+ and Sca1-CD24- cell populations. We next sought to identify MMTV-Neu tumor cells capable of giving rise to tumorspheres. Lin+ tumor cells were sorted on the basis of 7AAD exclusion (i.e., live cells) CD24 and Sca1 expression and then cells from each quadrant were plated under tumorsphere growth conditions. When plated at ~5,000 live cells per well, MMTV-Neu tumorspheres only formed from CD24- or CD24+Sca1+ cells (Fig. 5A and B). The number of TFUs per plated cell was ~1/400 and ~1/2,000 for CD24+ and CD24+Sca1+ cell populations, respectively (Fig. 5B). Importantly, tumorspheres obtained from sorted cells and subsequently transplanted into recipient mice also induced secondary tumors (data not shown). Thus, cells capable of forming tumorspheres in culture (TFUs) cosegregate with and may be...
highly related or identical to cells that can initiate tumors in vivo (TICs).

We finally asked whether TICs are derived from the same fraction of sorted cells that contain TFUs. To this end, Lin− 7AAD− single tumor cells were labeled with CD24 and Sca1 fluorescent antibodies, sorted into four quadrants, mixed with Matrigel, and transplanted into FvB mouse female fat pads (Fig. 6A, left). Incidence of secondary tumors was evaluated 6 months after injection (Fig. 6B). When 1,000 to 5,000 Lin− sorted cells were transplanted into recipient mammary glands, all quadrants gave rise to secondary tumors. However, when 500, 250, 100, or 50 MMTV-Neu tumor cells from double-negative or Sca1+/CD24− cells were transplanted, no tumors were detected. In contrast, secondary tumors developed from the Sca1+/CD24− or Sca1+/CD24+ quadrants after transplanting as few as 50 cells. The frequency of TICs was calculated to be 1/303 cells and 1/286 cells from the Sca1+/CD24+ and Sca1− populations, respectively. The sorted Sca1−/CD24− cells remained single cells, were devoid of doublets or aggregates, and contained very few 7AAD− dead cells, suggesting that the sorting process did not compromise the properties or viability of the TICs (Fig. 6A, right).

We note that as with TFUs, MMTV-Neu TICs were found exclusively in CD24− populations (Sca1+/CD24− or Sca1−/CD24−). However, although the frequency of TFU (1/400) and TIC (1/286) in the Sca1+/CD24− quadrant was relatively similar, the Sca1+/CD24− double-positive cell fraction contained significantly higher levels of TICs (1/303) than TFUs (∼1/2,000; Fig. 5 and 6). These disparity may reflect differences in the dependency of Sca1+/CD24− and Sca1−/CD24− TICs on the mammary gland microenvironment.

Flow cytometry analysis revealed that the newly developed secondary tumors exhibited similar CD24−/Sca1 profiles as the original MMTV-Neu tumors. Remarkably, sorted Sca1+/CD24− and Sca1−/CD24− populations both induced tumors with similar Sca1+/CD24− profiles (Fig. 6A). The histology of secondary tumors, undifferentiated adenocarcinoma, was also indistinguishable from that of primary tumors (Fig. 6C). Moreover, immunofluorescent analysis with antibodies against K18, SMA, and K14 revealed very similar patterns of marker expression in primary and secondary tumors (Fig. 6D). Both primary and secondary MMTV-Neu tumors expressed patches of K18-positive areas and no K14− or SMA− positive cells. In contrast, primary MMTV-Wnt1 tumors contained multiple, yet not continuous, areas with K18−, SMA−, and K14− cells. We conclude that the Sca1+/−:CD24− MMTV-Neu cell populations contain bona fide TICs capable of regenerating the initial tumors.

Discussion

We here report on the identification and similarities between TICs and TFUs in MMTV-Neu tumors. One parsimonious model that encompasses all our results is that Neu tumors originate from the transformation of luminal precursors. First, MMTV-Neu tumors have increased number of Sca1+/CD24− cells that represent luminal precursors (Figs. 1C and 2). Second, MMTV-Neu TICs were found within bipotent or luminal (Sca1+/CD24−) progenitor cell compartments (Figs. 1 and 6). Third, TFUs also were found within bipotent/luminal (Sca1+/−:CD24−) progenitor compartments in Neu tumors (Fig. 5). Fourth, Neu tumors do not express myoepithelial markers (Fig. 6D). Fifth, MMTV-Neu secondary TFUs differentiate exclusively into luminal (K18+) cells (Fig. 4B). Together, these observations support the notion that Neu TICs represent transformed luminal precursor cells.

However, other observations are not easily reconciled with this model. First, like MMTV-Wnt1 tumors, MMTV-Neu tumor cells exhibit expansions of the CD49f−/CD24+ cell population, which marks MSCs (Fig. 1B). Second, although Neu tumors do not contain myoepithelial cells expressing SMA or K14, sorted Sca1+/CD24− cells from Neu tumors as well as primary tumorspheres do differentiate in vitro into cells that express these myoepithelial markers (Figs. 2 and 4B). Third, although sorted Wnt1 tumor cells differentiated into cells expressing either luminal (K18) or myoepithelial (K14) markers, all differentiated Neu tumor cells that expressed K14 also expressed K18 (Fig. 2B).

The model described above can nonetheless be extended to incorporate these observations as well. The Neu oncogene may target luminal progenitors but induce them to revert into less differentiated stem/early progenitor–like cells capable of self-renewal, reexpress K14, and differentiate into luminal as well as myoepithelial cell types under favorable conditions. In this view, despite the fact that Neu tumor cells do not express K14 or SMA, they may posses some potential to differentiate into both major epithelia lineages; however, this does not occur in vivo. Similarly, MLL can be induced by oncogenic transformation of progenitor cells and activation of self-renewal genes (11).

In a second model, the Neu oncogene may transform stem/early progenitor cells but provide instructive cues to differentiate specifically into luminal cell fate or to specifically restrict differentiation into the myoepithelial lineage. Such a function would be reminiscent of the Notch-RBP-J pathway, which maintains luminal cell fate during pregnancy by preventing basal cell proliferation (38). Both models are consistent with the gene profiles associated with HER2-positive tumors that include luminal as well as Basal "A" (K5 and K14 positive; ref. 39 and references therein). Indeed, as we have shown herein, although MMTV-Neu mammary tumors are invariably negative for SMA and K14 (Fig 6), Neu tumor cells do differentiate into SMA+ and K14+ cells in vitro (Fig. 2). These opposing models may be tested by sorting MSCs and luminal progenitor cells, inducing transformation by activated Neu oncogene and then analyzing which transformed cell type resembles MMTV-Neu/HER2 tumors. An additional approach may involve the analysis of myoepithelial cell differentiation after inhibiting Neu signaling.

**Figure 6.** Neu TICs are found in the CD49f−, CD31−, CD45−, TER119−, 7AAD−, Sca1+/−:CD24−, or Sca1+/−:CD24− cell populations. A (left), sorted Sca1+/−:CD24− and Sca1−:CD24− cells contain TICs capable of inducing tumors that harbor TICs as well as their descendants. Lin− MMTV-Neu tumor cells were sorted on the basis of Sca1 and CD24 (left column). The sorted Sca1+/−:CD24− cells (bottom) or Sca1+/−:CD24− cells were transplanted into mammary glands of recipient mice. The Sca1+/−:CD24− profiles of the secondary tumors (right column) were indistinguishable from the original tumor. Right, additional quality tests of sorted Sca1+/−:CD24− cells. B, frequency of TICs after transplantation of the indicated number of sorted cells into the mammary glands of FvB mice. TIC/cell was calculated from limit-dilution analysis based on Poisson distribution. C, histologic comparison of primary and secondary MMTV-Neu tumors by H&E staining. Both tumors are undifferentiated adenocarcinoma. D, marker analysis of primary and secondary MMTV-Neu tumors by immunofluorescent staining for myoepithelial (SMA, green, top; and K14, green, bottom) and luminal (K18, red) cells. MMTV-Wnt1 primary tumors were used as positive control for SMA and K14. Nuclei were visualized by DAPI staining (blue). Original magnification in C and D, ×400.

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The ability of Neu TICs and TFUs to induce secondary tumors and tumorspheres shows their capacity to self-renew. In this context, we note that by reverse transcription-PCR analysis, we have detected Sox2, Oct4, and Nanog, which are involved in maintenance of stemness in embryonic stem cells, in RNA extracted from embryonic stem cells but not from Wnt1 or Neu tumorspheres. Whether these genes are expressed exclusively in TFUs but turned off in more differentiated progenitor cells remains to be seen and will require purification of TFUs and TIC to near homogeneity. Interestingly, Notch and Notch ligands, which are implicated in self-renewal and are elevated in aggressive forms of breast cancer (40), were readily identified in Wnt1 tumorspheres; their expression in Neu tumorspheres is yet to be determined. A nonbiased microarray analysis of highly purified TICs may unravel whether MSCs, Wnt1, and Neu TICs share a common set of self-renewal genes.

Mammary colony-forming cells (CFC), defined as progenitors that produce colonies in low cell density adherent cultures, can be physically and functionally separated from mammary repopulating units (MRU), which mark MSCs (30). Notably, however, mammary CFCs are cultured under different conditions than mammospheres. It would therefore be important to establish the relationship between cells capable of forming mammospheres in nonadherent cultures and TICs. Our observation that produce colonies in low cell density adherent cultures can be seen and will require purification of TFUs and TIC to near homogeneity. Interestingly, Notch and Notch ligands, which are implicated in self-renewal and are elevated in aggressive forms of breast cancer (40), were readily identified in Wnt1 tumorspheres; their expression in Neu tumorspheres is yet to be determined. A nonbiased microarray analysis of highly purified TICs may unravel whether MSCs, Wnt1, and Neu TICs share a common set of self-renewal genes.

Mammary colony-forming cells (CFC), defined as progenitors that produce colonies in low cell density adherent cultures, can be physically and functionally separated from mammary repopulating units (MRU), which mark MSCs (30). Notably, however, mammary CFCs are cultured under different conditions than mammospheres. It would therefore be important to establish the relationship between cells capable of forming mammospheres in nonadherent cultures (as opposed to mammary CFC) and MRUs. Indeed, our results suggest that TICs are similar, if not identical, to TFUs. These include our observations that (a) TICs and TFUs cofractionate; (b) the flow cytometry profiles of primary tumors and tumorspheres is highly similar; and most importantly (c) tumorspheres arising from sorted cells can give rise to secondary tumors after transplantation into recipient mice. Thus, screening for inhibitory drugs or small interfering RNAs that can suppress TFU growth in vitro may lead to the identification and development of TIC-specific therapy. Large-scale screening for tumorsphere growth inhibitors could be readily adopted on the basis of Fig. 4D. Of particular interest would be the identification of inhibitors that alone or together with conventional chemotherapeutic agents may lead to the EGF/FGF pathways, required for maintaining both tumorspheres and mammospheres in culture, or various essential, noncancerous processes. Such screens can be easily extended to tumorspheres derived from other important mouse models of sporadic and heritable forms of breast cancer.

The use of mouse models of mammary tumors described here offers several advantages over the use of human breast tumors or pleural effusions. The mouse mammary tumor models provide ample and accessible source of primary tumors with relatively uniform genetic background. In addition, the power of mouse genetics may allow us to modulate gene expression and test for effect of various signaling/developmental pathways and specific inhibitors on the growth of mammary tumorspheres and TICs both in vitro and in vivo.

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Identification of Tumorsphere- and Tumor-Initiating Cells in HER2/Neu-Induced Mammary Tumors

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