Intratumoral Heterogeneity in a Trp53-Null Mouse Model of Human Breast Cancer

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**ABSTRACT**

Intratumoral heterogeneity correlates with clinical outcome and reflects the cellular complexity and dynamics within a tumor. Such heterogeneity is thought to contribute to radio- and chemoresistance because many treatments may target only certain tumor cell subpopulations. A better understanding of the functional interactions between various subpopulations of cells, therefore, may help in the development of effective cancer treatments. We identified a unique subpopulation of tumor cells expressing mesenchymal-like markers in a Trp53-null mouse model of basal-like breast cancer using fluorescence-activated cell sorting and microarray analysis. Both in vitro and in vivo experiments revealed the existence of cross-talk between these “mesenchymal-like” cells and tumor-initiating cells. Knockdown of genes encoding ligands upregulated in the mesenchymal cells and their corresponding receptors in the tumor-initiating cells resulted in reduced tumorigenicity and increased tumor latency. These studies illustrate the non–cell-autonomous properties and importance of cooperativity between tumor subpopulations.

**SIGNIFICANCE:** Intratumoral heterogeneity has been considered one important factor in assessing a patient's initial response to treatment and selecting drug regimens to effectively increase tumor response rate. Elucidating the functional interactions between various subpopulations of tumor cells will help provide important new insights in understanding treatment response and tumor progression.

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See related commentary by Brooks and Wicha, p. 469.

**INTRODUCTION**

Cell-cell interactions, through paracrine signaling, play critical roles in the regulation of mammary morphogenesis. Although most of these interactions involve luminal to basal signaling (1), a recent study has shown that basal cells may regulate luminal progenitor activity during pregnancy and lactation processes through p63–neuregulin signaling (2). Similarly, tumorigenesis is a multistep process involving acquired genetic and epigenetic alterations that generate individual cell populations with aberrant differentiation and proliferation potential. Intratumoral heterogeneity (ITH) has been considered one important factor in assessing a patient’s initial response to treatment and selecting drug regimens to effectively increase tumor response rates (3, 4). Understanding the molecular interactions between various subpopulations of tumor cells, as well as their interaction with the microenvironment, may provide new targets for treatment to help in eradicating both primary and recurrent tumors. Most studies have focused to date on the latter; that is, the interactions of tumor cells with the microenvironment, for example, tumor-associated fibroblasts, macrophages, endothelial cells, osteoblasts, etc. However, little is known about the importance of possible tumor cell–tumor cell and paracrine interactions that may be important within the intrinsic tumor cell population.

ITH has long been recognized from a pathologic point of view (5) and has been identified at the molecular level in the past few years through large-scale transcriptome and genome analyses (6, 7). ITH is thought to be generated through subclonal evolution during tumor progression (8), with different clones displaying various capabilities of tumor propagation and responses to therapy (8–12). Such variability within a tumor may be partially explained by the cancer stem cell (CSC) theory. Contrary to the monoclonal theory of cancer (5), the CSC theory has suggested that a subpopulation of CSCs [a.k.a. tumor-initiating cells (TIC)] can self-renew and differentiate along a particular lineage to generate the bulk of tumor “non-stem” cells (7). Using a strategy of transplanting fluorescence-activated cell sorting (FACS)–sorted single cells from solid tumors into immunodeficient mice, a small subpopulation of TICs has been identified from a variety of solid tumors, including breast tumors (13). The ability to form secondary mammospheres after plating the cells dissociated from primary spheres cultured on a nonadherent substratum also has been used as a surrogate for an *in vivo* stem cell self-renewal assay (14). The mammosphere and stem cell subpopulations have been shown to be more resistant to chemotherapy and radiation treatment than the total cells and non-stem cells, respectively (15–17). This has been hypothesized to result in cancer recurrence and metastasis after the initial treatment.

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Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

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Although the CSC theory may apply in many subtypes of cancer, including breast cancer, increasing evidence has suggested non-TICs, although less tumorigenic than the TICs, may generate aggressive TICs within a tumor (18). Li and Clevers (19) have proposed a theory of coexistence of both active and quiescent stem cells in several tissues as both cycling and noncycling populations of stem cells have been identified. However, “gold standard” limiting dilution transplantation assays most commonly used in the characterization of stem cells from various tissues might only identify active (cancer) stem cells. Therefore, investigation of ITH will provide important insights into the roles of stem cells as well as their interactions with other tumor cells in tumor initiation, progression, and treatment resistance.

Our previous studies defined a lineage-negative (Lin−)CD29 (β1-integrin)CD24hi subpopulation of TICs by both limiting dilution transplantation and in vitro mammosphere assays using a syngeneic Trp53-null mouse mammary tumor model (20). Using FACS and microarray analysis, these studies also identified a unique group of cells in these tumors expressing “mesenchymal-like” cell markers. Factors such as cytokines, chemokines, growth factors, and secretory WNT proteins that have been reported to function as niche components in various tissues were significantly increased within the mesenchymal-like tumor cell subpopulation. The stem cell niches characterized to date in the mouse use WNT signaling, NOTCH signaling, IL6, or CXCL12 to regulate stem cell function (21). All of these factors are important autocrine or paracrine cues that affect diverse processes in normal tissue development and tumorigenesis. The functional interaction between niche cells and TICs, therefore, was investigated by comparing the properties of the combined “mesenchymal-like” and TIC subpopulations to the individual isolated subpopulations alone. Cocultures and Transwell cultures of putative niche cells with TICs in serum-free suspension mammosphere assays revealed that both the in vitro self-renewal ability and the proliferation potential of the TICs were enhanced in the presence of the niche cells or factors secreted from the niche cells. In vivo cotransplantation assays indicated that the niche cells enhanced the TIC tumorigenic potential when a limited number of TICs was present. Transduction of niche cells with lentiviral expressed short hairpin RNAs (shRNA) directed against differentially expressed Wingless-type MMTV integration site family, member 2 (Wnt2) and Cxcl12 within the niche population resulted in reduced mammosphere frequency and decreased in vivo tumorigenic potential with increased latency. Knockdown of the receptors for these ligands in the TIC subpopulation also provided additional evidence of the importance of functional interactions between these tumor subpopulations.

RESULTS

A Lin− CD29hiCD24lo Subpopulation from Trp53-Null Mammary Tumors Displays a Mesenchymal-like Gene Expression Profile

Cell-surface markers CD29 and CD24 separated the dissociated Trp53-null tumor cells into four subpopulations: CD29hi CD24lo, CD29hi CD24hi, CD29lo CD24hi, and CD29lo CD24lo. The Lin− CD29lo CD24hi subpopulation displayed a significantly increased tumorigenic potential as compared with the other subpopulations (20). PCR genotyping performed using p53 primers (X7/X6.5 defining Trp53 wild-type, and X7/NEO19 defining Trp53-null) confirmed the Trp53-null status of all the individual subpopulations, suggesting their non-host cell of origin when 30 cycles of PCR were performed (Supplementary Fig. S1A, left). A small trace of Trp53 wild-type product was detected when 35 cycles of PCR were performed, most likely due to infiltrating immune cells within the tumors (Supplementary Fig. S1A, right).

To determine whether there exist genomic copy-number differences among the four subpopulations, we performed high-resolution mouse whole-genome bacterial artificial chromosome (BAC)-based comparative genomic hybridization (CGH) array that covers the entire mouse genome (22, 23). The syngeneic BALB/c mouse tail DNA was used as a control. The chromosomal copy-number profiles performed on the four subpopulations of the Trp53-null tumor did not show significant variations (Supplementary Fig. S1B).

We have previously shown that the Lin− CD29lo CD24hi subpopulation identified in most of the heterogeneous Trp53-null tumors studied [including estrogen receptor–positive (ER+) and ER–negative (ER–) tumors] expressed basal/myoepithelial markers K5/K14, as well as those only expressing luminal marker K8] was usually <5% of the total cell population. The TIC subpopulation (i.e., Lin− CD29lo CD24hi) was able to generate tumors with as few as 10 cells. The Lin− CD29hi CD24hi subpopulation was also able to generate tumors, but only when more cells were transplanted, indicating a reduced tumorigenic potential as compared with the TIC population (20). Nevertheless, such cells displayed increased tumorigenecity when compared with the Lin− CD29lo CD24hi and Lin− CD29hi CD24hi subpopulations, which represented the bulk (>90%) of the tumor cells. However, FACS analysis of tumors arising from the Lin− CD29lo CD24hi subpopulation showed that they did not mimic the phenotype of the parental tumor; instead, an expansion of the TICs was observed from the Lin− CD29lo CD24hi−derived tumors (Supplementary Fig. S2A and ref. 20). TIC-derived tumors, such as the primary tumors (Supplementary Fig. S2Ba), express smooth muscle actin (SMA) mainly in the ductal structures (Supplementary Fig. S2Bb); however, a high level of SMA also was observed in the stromal compartment in the CD29lo CD24hi−derived tumors (Supplementary Fig. S2Bc).

Microarray analysis (described in details in the Supplementary Data and reported previously; ref. 20) to compare the expression of the CD29lo CD24hi−cells with those of the other three subpopulations identified an increased expression of genes encoding WNT proteins, including WNT ligands WNT2 and WNT9a, CXCL12, and IL6 in this subpopulation (Supplementary Table S1). Interestingly, the TICs in the Trp53-null tumor T1, a squamous adenocarcinoma, expressed a higher level of Axin2 and Tgf7, both of which encode known targets of WNT signaling as demonstrated by qPCR (Fig. 1A and B), suggesting the possible interaction between the TICs and the CD29lo CD24hi cells. The expression of Fod7, encoding one of the WNT ligand receptors, and Cxcr4, encoding the receptor for CXCL12, was also upregulated in the TIC population as compared with the non-TIC population (Fig. 1C and D).

The CD29lo CD24hi Subpopulation Is Less Proliferative as Compared with the TIC Subpopulation

Cell-cycle analysis performed on TICs and CD29lo CD24hi cells using 7-Aminoactinomycin D (7-AAD) and pyronin Y and...
showed that 8.6% ± 1.3% (mean ± SD) of TICs were in G₀-G₁ phase (Fig. 2Aa), whereas 21.5% ± 3.3% of CD29⁺CD24⁻ cells were in the G₀-G₁ phase (Fig. 2Ab). A more detailed characterization of the 20% cells in the G₀-G₁ phase showed that 17.4% ± 3.7% of the CD29⁺CD24⁻ cells were in the G₀-phase and 3.2% ± 0.3% in the G₁-phase (Fig. 2Ba and b), indicating that a group of CD29⁺CD24⁻ cells were quiescent. However, this cannot explain the low tumorigenic potential of this subpopulation, as the CD29⁺CD24⁻ cells contain more cells in the G₀-phase, and have a higher tumorigenic potential than the CD29⁻CD24⁺ cells (Supplementary Fig. S3A, G₀-G₁: 9.6% ± 2.1%; Supplementary Fig. S3B, G₀: 7.8% ± 1.9%), and CD29⁺CD24⁻ cells (Supplementary Fig. S3C, G₀-G₁: 12.6% ± 1.9%; Supplementary Fig. S3D; G₀: 8.9% ± 1.6%), respectively. We further measured the proliferative potential of the individual populations after FACS and cytospin centrifugation followed by Ki67 staining. Although 60% of TICs (Fig. 2Ca) were proliferative, only 30% of CD29⁺CD24⁻ (Fig. 2Cb) cells were Ki67-positive (Fig. 2D).

The CD29⁺CD24⁻ Niche Population Has Features of Mesenchymal and Claudin-Low Signatures

Because several genes expressed in the CD29⁺CD24⁻ niche population have been associated with cells undergoing an epithelial-to-mesenchymal transition (EMT), we performed a comprehensive analysis of the RNA expression profiles of the individual populations using previously identified mesenchymal (24) and claudin-low gene signatures (25, 26). This analysis strongly supports the observation that the CD29⁺CD24⁻ population expresses mesenchymal markers (Fig. 2Ea). The mesenchymal gene expression signature was highly correlated with the claudin-low tumor subtype, with the claudin-low subtype-defining signature showing high expression in the CD29⁺CD24⁻ subpopulation that also displays an increased expression of EMT features (Fig. 2Eb and c).

Figure 1. qPCR analysis using p53-null tumors suggested that Axin2 (A), Tcf7 (B), Fzd7 (C), and Cxcr4 (D) were upregulated in TICs. Total RNA isolated from FACS-sorted subpopulations based on the expression of CD29 and CD24 were extracted using the RNA purification kit as mentioned in the Supplementary Data Microarray Analysis. *, P < 0.01.

CD29⁺CD24⁻ Cells Promote In Vitro Self-Renewal Capacity of TICs

Coculture of CD29⁺CD24⁻ cells (labeled with red fluorescent cell linker dye, PKH 26) with TICs (labeled with green fluorescent cell linker dye, PKH 67) in serum-free mammosphere assays generated both large and an increased number of mammospheres as compared with culturing the TICs or CD29⁺CD24⁻ cells alone (Fig. 3A and B). However, the coculture of TIC and CD29⁺ cells did not result in increased mammosphere frequency, indicating the unique interaction between the TICs and CD29⁺CD24⁻ cells (Fig. 3B). These results suggest that both the in vitro self-renewal ability and proliferation potential of the TICs are enhanced in the presence of the niche cells. To directly test this hypothesis, the levels of CXCL12 expression under different culture conditions were measured (Supplementary Fig. S4). The levels of CXCL12 secreted by CD29⁺CD24⁻ cells, cultured either alone or together with TICs, were significantly higher than observed with TICs alone, indicating that CXCL12 is both regulated and functioning via a paracrine mechanism to promote the in vitro self-renewal ability of TICs.

Next, we used a Transwell assay to determine whether direct cell–cell contact or secreted factors are required to enhance the self-renewal potential of TICs when cultured under the serum-free condition. A 0.4-μm filter was used to prevent the passage of both cell types through the membrane. Under these conditions, TICs cultured with the putative niche cells resulted in an increased number of mammospheres as compared with TICs cultured alone. This result suggests...
that soluble factors secreted from the putative niche cells support the self-renewal of TICs, but possibly not their proliferation. When TICs, after Transwell culture with or without CD29hiCD24lo cells, were transferred to growth factor–reduced Matrigel, branching structures were observed if the TICs were previously Transwell-cultured with the putative niche cells, whereas no branching structures were observed if the TICs were previously Transwell-cultured with the previous studies showing the TIC subpopulation identified using cell-surface markers (CD29 and CD24) overlapped with the active canonical WNT signaling cells identified using a WNT reporter system (17). We thus performed coculture experiments using different combinations of WNT reporter–marked TOP-GFP+ TICs, and niche cells with Wnt2 shRNA knockdown. CD29hiCD24lo TICs were used to coculture with the niche cells with Cxcl12 shRNA knockdown.

Downregulation of Preferentially Expressed Genes in the Putative Niche Cells (CD29hiCD24lo) Inhibits the Self-Renewal of TICs

We next determined the functional role of secreted factors previously identified by our microarray studies. Because WNT signaling and CXCL12 secretion are known to increase the self-renewal potential of TICs (27–29), we hypothesized that repression of WNT2 and CXCL12 expression in the niche cells alone might be inhibitory. FACS-sorted CD29hiCD24lo TICs have increased expression of Fed7, Tcf7, and Axin2 encoding components of the WNT signaling pathway as compared with the non-TICs (Fig. 1). This finding is consistent with the previous studies identifying the niche population as compared with the three other populations. Individual subpopulations of RNAs were isolated from pooled tumors of the same subtypes after FACS isolation. Three independent Trp53-null tumors (T1 ■, T2 ●, T7 ▲) were included. Blue, CD29hiCD24lo; red, CD29hiCD24lo; black, CD29loCD24hi; green, CD29loCD24lo.

**Figure 2.** Molecular characterization of the individual tumor cell subpopulations. A and B, cell-cycle analyses of the FACS-sorted TIC (Aa and Ba) and CD29hiCD24lo (Ab and Bb) subpopulations. C, the cells (a, TIC; b, CD29hiCD24lo) were also cytospun and analyzed using an antibody against Ki67. D, quantification of Ki67-positive cells within the TIC and niche populations. E, the CD29hiCD24lo niche population has features of mesenchymal cells and claudin-low tumors. The CD29hiCD24lo population expresses core mesenchymal signature (Ea), claudin-low high gene expression signature (E5), and claudin-low gene expression signature (Ec) as compared with the other signatures.
null mammary tumors cellular heterogeneity in Trp53 partial was analyzed using mammosphere assays. Both alone, a mixture of TICs and CD29 hi CD24 lo, and a mixture of TICs and CD29 lo. Six repeats for each group. T1 tumors were used in the study. Mammosphere formation was determined and quantitated at day 7. *, from the primary mammospheres in serum-free mammosphere medium under nonadherent conditions for 7 days. Mammosphere formation was determined and quantitated at day 7.

![Figure 3](image-url)

Figure 3. Coculture and Transwell culture of TICs with CD29 hi CD24 lo cells or CD29 lo cells. All cells used were dissociated from the primary mammosphere culture and labeled with green: PKH67 fluorescence or red: PKH26 fluorescence as designated. A and B, coculture of TICs and CD29 hi CD24 lo cells display larger and an increased number of mammospheres compared with their individual cultures alone. Aa, TICs (green); Ab, CD29 hi CD24 lo (red); Ac, TICs (green) and CD29 hi CD24 lo (red) were mixed; Ad, TICs (green) and CD29 lo (red) were mixed. B, mammosphere-forming efficiency of TICs cultured alone, CD29 hi CD24 lo, a mixture of TICs and CD29 hi CD24 lo, and a mixture of TICs and CD29 lo. Six repeats for each group. T1 tumors were used in the study. Mammosphere formation was determined and quantitated at day 7. C, Transwell cultures of the CD29 hi CD24 lo cells and TICs display increased self-renewal and differentiation potential of the TICs. C, Transwell cultures of the CD29 hi CD24 lo cells and TICs display increased self-renewal and differentiation potential of the TICs. D, TICs after Transwell cultured with the TICs or niche cells were collected and transferred to the B-well chamber slides precoated with growth factor-reduced Matrigel. Pictures were taken after 14 days. Scale bars, 50 μm.

T1 tumor at levels ranging from 50% to 60% (Fig. 4A and B). Genetically modified knockdown niche cells were then cocultured with TICs in serum-free mammosphere medium under nonadherent conditions for 7 days. A reduced mammosphere-forming ability was observed (Supplementary Fig. S5B).

Downregulation of Fzd7 and Cxcr4 in the TICs (CD29 hi CD24 lo) in Combination with Downregulation of Wnt2 and Cxcl12 in the Niche Cells Significantly Inhibits the In Vitro Self-Renewal of TICs

A higher expression of Fzd7 in the WNT-responsive TIC population than in the non-TIC population has suggested that Fzd7 may play a role in the interaction of TICs with the surrounding cells through WNT signaling. To determine the functional interaction between TICs and niche cells, Fzd7 and Cxcr4, Wnt2 and Cxcl12, were knocked down, respectively, in the TICs and niche cell populations, and their in vitro self-renewal potential was analyzed using mammosphere assays. Both Fzd7 and Cxcr4 expression were decreased by 75% and 55%, respectively, in the TICs as confirmed by qRT-PCR (Fig. 4E and F). When genetically modified TICs and niche cells were cocultured, a significant decrease of the mammosphere-forming ability was detected in both knockdowns, with the coculture of the knockdown in both TIC and niche populations exhibiting a greater reduction of mammosphere-forming ability as compared with the ligand knockdown alone (Fig. 4G and H).

CD29 hi CD24 lo Niche Cells Enhance the TICs Tumor-Initiation Potential as Shown by Limiting Dilution Cotransplantation Assays

These in vitro assays were suggestive of a functional interaction between the TICs and niche cells. This was confirmed using an in vivo limiting dilution analysis. Transplantation of 10 CD29 hi CD24 lo niche cells alone did not initiate tumor formation, whereas in contrast, at least 10 TICs were capable of initiating tumor formation (Table 1A and Supplementary Table S1).
“mesenchymal-like” niche cells (CD29 hi CD24 lo) were able to renew the TICs. A and B, Wnt2 or Cxcl12 shRNA lentiviruses (shRNA) or control lentivirus (Ctrl) were introduced into the dissociated CD29 hi CD24 lo cells after being FACS-sorted from Tp53-null T1 tumors. After 72 hours of selection with puromycin, levels of Wnt2 and Cxcl12 were determined by qPCR. C and D, dissociated tumor niche cells from T1 tumor, infected with control or Wnt2 shRNA, or Cxcl12 shRNA were plated under mammosphere conditions. The designated dissociated single cells from the primary sphere culture were cocultured with the dissociated TIC cells after primary culture in the mammosphere medium. **TICs were cocultured with control (CD29 hi CD24 lo infected with the empty vector). □TICs were cocultured with CD29 hi CD24 lo cells infected with Wnt2 shRNA1 (C). □TICs were cocultured with CD29 hi CD24 lo cells infected with Cxcl12 shRNA2 (D). (continued on following page)

Table 2). However, transplantation of 2 TICs, with a tumor formation frequency of 2 of 8, was able to initiate tumorigenesis when cotransplanted with 10 niche cells. Cotransplantation of increasing numbers of niche cells resulted in increasing numbers of tumors (Supplementary Table S3 and Fig. 5A). This limiting dilution analysis involving several doses of TICs revealed significant differences between groups defined by the numbers of niche cells (χ² 10.1753 on 2 degrees of freedom (DF), P = 0.006. Coinjection of 10 niche cells versus 0 niche cells showed a 4.7-fold increase in tumor formation (P = 0.0017), whereas the effect of coinjection of 2 niche cells was intermediate (about 3-fold) and not clearly different from either 0 or 10. Finally, with 20 TICs, tumor formation was faster with 10 niche cells as compared with 0 (P = 0.02; Supplementary Table S4B), whereas 2 niche cells were not different from either 0 or 10. Two groups (TICs = 0/Niche = 10 and TICs = 5/Niche = 0) did not have any tumors, but differences remained significant even after eliminating these two groups (P = 0.002). For fixed numbers of niche cells (i.e., niche cells = 10), tumor latency was decreased with increasing numbers of TICs (Supplementary Table S4A).

To determine whether niche cells reduced the time to tumor formation, two sets of comparisons were undertaken. With 10 TICs, tumor formation was more rapid with 10 niche cells as compared with 0 (P = 0.02; Supplementary Table S4B), whereas 2 niche cells were not different from either 0 or 10. Finally, with 20 TICs, tumor formation was faster with 10 niche cells as compared with 0 (P = 0.02; Supplementary Table S4C).

Cotransplantation of the Fluorescence-Labeled TICs (pEIT-TICs) and CD29 hi CD24 lo Niche Cells (pEIz-Niche Cells) Suggests that the TICs Contributed to the Majority of Tumor Growth

TICs and putative niche cells (CD29 hi CD24 lo) were individually infected with the lentiviral expression system ZsGreen and Tomato Red, and were individually or separately cotransplanted into the cleared fat pad of 3-week-old recipient mice. FACS analysis demonstrated that the majority of the resulting tumor cells were derived from the TICs when TICs and niche cells were mixed at different combinations of 200/0 (Supplementary Fig. S6Ba and S6Bb); 120/80 (Supplementary Fig. S6Bc and S6Bd); 68/132 (Supplementary Fig. S6Be and S6Bf); and 32/168 (Supplementary Fig. S6Bg and S6Bh), consistent with their self-renewal and differentiation ability.

Downregulation of Wnt2 in the Niche Cells (CD29 hi CD24 lo) Inhibits the In Vivo Self-Renewal of TICs Shown by Limiting Dilution Transplantation Assays

To determine whether the in vivo tumor-initiating ability of TICs was affected when secreting factors were repressed, we also cotransplanted the genetically modified (shRNA
knockdown of Wnt2) niche cells, together with the TICs (WNT-responsive cells), into the cleared fat pads of recipient mice. A decreased tumorigenic potential and a longer latency after being FACS-sorted from Trp53-null T1 tumors. After 72 hours of selection with puromycin, levels of Fzd7 and Cxcr4 were determined by qPCR.

G, dissociated TICs from T1 tumor, infected with control and Fzd7 shRNA, were plated under mammosphere conditions. The dissociated cells were then cocultured with niche cells either infected with the empty vectors or with Wnt2 shRNA, respectively, after primary mammosphere culture. The incidence and decreased the latency of tumor formation in TICs (%). H, dissociated TICs from T1 tumor, infected with control and Cxcr4 shRNA were plated under mammosphere conditions. The dissociated cells were then cocultured with niche cells either infected with the empty vectors or with Cxcl12 shRNA, respectively, after primary mammosphere culture, in the mammosphere medium. I, TICs infected with the control vector were cocultured with CD29+CD24− cells infected with the empty control vector; J, TICs infected with Fzd7 shRNA were cocultured with CD29+CD24− cells infected with the control shRNA; K, TICs infected with Fzd7 shRNA were cocultured with CD29+CD24+ cells infected with Wnt2 shRNA; L, TICs infected with Fzd7 shRNA were cocultured with CD29+CD24+ cells infected with the empty control vector; M, TICs infected with Fzd7 shRNA were cocultured with CD29+CD24+ cells infected with the control shRNA; N, TICs infected with Cxcr4 shRNA were cocultured with CD29+CD24+ cells infected with the empty control vector.

In summary, limiting dilution analyses and, alternatively, tumor latency analyses show that niche cells increased both the incidence and decreased the latency of tumor formation in a dose-dependent manner, and furthermore, that shRNA knockdown of Wnt2 reduced tumor formation, most noticeably in the presence of increased numbers of niche cells.

**DISCUSSION**

ITH correlates with clinical outcome (30), which also poses considerable challenges for tumor prognosis and therapy (31). Increasing evidence has emerged to show that various subpopulations of cells within solid tumors may respond differently to both conventional and targeted therapies. In a clinical study, residual breast cancer cells following treatment with either an aromatase inhibitor or chemotherapy showed an enrichment of the TIC subpopulation (32). However, whether cells are in a dynamic state under treatment conditions has not been determined. In addition, studies from glioblastoma, in which CD133+ has been identified as a stem cell marker, have shown a population of CD133+ cells can generate both CD133+ and CD133− progeny (33), suggesting a dynamic exchange between the CD133+ and CD133− cells. Thus, plasticity has to be considered as a factor that may...
influence tumor development. However, the mechanisms by which the different types of tumor cells interact with each other during tumor progression remain to be elucidated. Similarly, using the combined analyses of cellular differentiation markers (CD24, CD44, HER2, etc.) and genotypic alterations such as copy-number variation, Park and colleagues (34) uncovered a high level of genetic heterogeneity between stem-like cells and more differentiated cancer cell populations. These results questioned the validity of a unidirectional simple differentiation stem cell hierarchy. Therefore, the elucidation of the dynamic and functional relationship between various breast tumor cells may provide new therapeutic targets for drug development with the goal of both preventing breast cancer and reducing relapse and metastasis.

Using in vitro coculture, Transwell culture, and in vivo cotransplantation together with shRNA knockdown, we identified a group of mesenchymal-like tumor cells from the Trp53-null mammary tumors. Factors that have been reported to function as niche components in various tissues, such as cytokines, chemokines, and secretory WNT proteins, were significantly increased within our mesenchymal-like cell subpopulation. WNT2 expression has been detected at high levels in both epithelium and stroma in infiltrating carcinomas and fibroadenomas, indicating that an autocrine WNT signaling loop might exist within the tumor cells (35). Stem cells may generate their own niche or interact with the surrounding microenvironment via WNT signaling (36). We have demonstrated a marked overlap of the WNT-positive cells with the TIC population characterized as CD29+CD24− using a WNT reporter system (17). Consistently, a decreased self-renewal potential of the TICs when cotransplanted with the niche cells that were transduced with shRNAs mediating knockdown of Wnt2 indicated that the functional interaction between the TICs and the niche cells was disrupted. Thus, these various cell types functionally interact with each other using a mechanism similar to that used in the normal mammary gland. CXCL12 together with its receptor CXCR4 constitutes the chemokine–receptor axis that plays an important role in mammary tumorigenicity and metastasis (37). The interaction between CXCL12 and CXCR4 also plays an important role in maintaining the hematopoietic stem cell pool in the bone marrow (38). CXCL12 is also expressed in the cytoplasm of malignant ovarian epithelial cells (39). In our study, the ligand CXCL12 and its receptor, CXCR4, are highly expressed, respectively, in the tumor-derived niche cells and the TICs, suggesting the possible interaction between TICs and the mesenchymal-like niche. Therefore, the knockdown of both Cxcr4 in the TICs and the Cxcl12 in the niche subpopulation were performed to investigate the role of CXCL12 and CXCR4 in the interaction of our various tumor cells. The reduction in the mammosphere-forming efficiency when Wnt2/Fzd7 were knocked down...
### Table 1. Cotransplantation of CD29hiCD24lo putative niche cells with TICs

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<th>Cells cotransplanted</th>
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<td><strong>(C) Wnt2 shRNA-transduced niche cells</strong></td>
<td><strong>WNT-responsive cells (GFP+)</strong></td>
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was not as dramatic as that in the Cxcl12/Cxcr4 knockdown (Fig. 4G and H), although the expression level of Fzd7 was decreased by 70% as compared with 50% for Cxcr4 (Fig. 4E and F). This may be partially due to the presence of multiple redundant ligand/receptor components for WNT signaling as compared with the specific interaction between CXCL12 and CXCR4.

Our data also support a role for IL6 in TIC self-renewal as demonstrated by the reduced mammosphere-forming ability observed following Ile6 knockdown. These results are consistent with previous findings that IL6 regulates the breast TIC population through both autocrine and paracrine mechanisms (40, 41). Tumor cells have been shown to secrete IL6 to promote tumor growth (42) via an autocrine mechanism. It is also likely that the TICs produce factors that regulate the mesenchymal population. However, the level of CXCL12 secretion was extremely low when TICs were cultured alone, suggesting that a potential paracrine feedback pathway regulating CXCL12 expression may be important in this tumor model.

Both tumor formation frequency and tumor latency time after limiting dilution transplantation experiments reflect the process of tumor initiation, with the formation frequency representing the relative number of stem cells and/or the ability of the cell population to establish a niche to allow replication, whereas tumor latency represents the proliferative potential of these cells. In vivo cotransplantation demonstrated that such niche cells enhanced TIC tumor initiation, likely by providing an improved microenvironment, especially for those tumors initiated from extremely low numbers of TICs. The shortened latency observed in the presence of niche cells is consistent with the findings that WNT signaling promotes cell proliferation (43).

Although studies have suggested that there is a dynamic equilibrium among various cell subpopulations, the relevance of this “plasticity” in influencing treatment response, metastasis, and recurrence is unknown (44). It remains to be determined what factors contribute to ITH in solid cancers, and if plasticity in these subpopulations contributes to treatment resistance, metastasis, minimal residual disease, and recurrence.
One method to define self-renewal and differentiation properties of CSCs is through limiting dilution transplantation to identify cells capable of forming tumors that recapitulate the characteristics of the original tumor. Our results suggest that Trp53-null tumors contain cells with different degrees of self-renewal capacity. Although cell plasticity may exist between TICs and non-TICs, a majority of the resulting tumor cells were derived from the TICs (Supplementary Fig. S6 and Fig. 6; ref. 1). Mesenchymal cells resulting from an EMT are usually more migratory and less proliferative than their epithelial counterparts (45). Thus, it is likely that the widely used limiting dilution transplantation assay may preferentially identify the rapidly proliferative TICs, but not the less proliferative, more quiescent population that may also initiate tumor growth. Previous limiting dilution transplantation assays have shown that the niche cell population is 30-fold less tumorigenic than the TICs (17), and the niche cells, indeed, are more quiescent and less proliferative than the TICs. Notwithstanding these studies, in the absence of appropriate lineage tracing experiments, it is not feasible to definitively know the origin of these primary tumor cells. When TICs were cotransplanted with the mesenchymal-like tumor cells, the latter cells increased the self-renewal and tumorigenic potential of the TICs, especially during early tumor development (Fig. 6; ref. 2). CD29<sup>hi</sup>CD24<sup>lo</sup> cells fail to generate tumors with a low number of cells, and the resulting tumors exhibit a different phenotype and FACS profile as compared with the TIC-derived tumors, suggesting that these cancer cell subpopulations may interact and collaborate differently with the host microenvironment. Interactions of self-renewing tumor cells with both the microenvironment and the surrounding tumor cells determine the progression and phenotypic features of the tumors. The generation of cells with less self-renewal, but with mesenchymal features, was able to fuel tumor growth. Recently, using approaches including whole-genome sequencing and reverse phase protein arrays (RPPA), Li and colleagues (46) thoroughly characterized 13 patient-derived xenograft (PDX) lines along with their advanced primary breast tumors. The studies showed that although PDXs have relatively stable genomes without a significant accumulation of DNA structural rearrangements, minor mutant clones are retained in PDXs during multiple transplants, indicating the possibility of cooperation of clones during tumor evolution. Further characterization of the individual subpopulations using PDX lines will help us better understand the complexity of human breast cancer. Eliminating multiple subpopulations and blocking the transition between these populations will be an important consideration when designing effective cancer therapies.

Only a limited number of studies to date have been able to demonstrate the importance of functional ITH. For example, Cleary and colleagues (47) reported recently that both the luminal and basal populations were required for efficient tumor formation in the MMTV-driven WNT1 genetically engineered mouse model, which was dependent on luminal WNT1 expression. In this transgenic mouse model, Hras mutations were used as clonal markers identifying both distinct basal Hras-mutant and luminal wild-type tumor subclones. A similar requirement for WNT signaling was observed in our stochastic
null BALB/c tumors that are also a model for basal-like breast cancer (25). However, in the p53 model, a distinct TIC population has been identified, and, as discussed previously, the interaction with the mesenchymal-like subpopulation enhanced, but was not essential for, tumor formation.

To better understand clonal heterogeneity, Marusyk and colleagues (48) recently developed an experimental model in which factors previously implicated in tumor progression were overexpressed in the indolent MDA-MB-468 cell line. These investigators then generated sublines expressing different cytokines and used these to model how subclonal cooperation was required for metastasis. These studies support the conclusion that there are non-cell-autonomous drivers of tumor growth, and importantly that interclonal interactions can lead to new phenotypic properties.

In our studies, no large-scale genomic deletions or insertions among individual subpopulations of CD29hiCD24lo, CD29loCD24hi, and CD29loCD24lo cells were detected using CGH analysis. Therefore, in the Trp53-null BALB/c tumors, it appears that epigenetic factors may influence clonal heterogeneity, and that ITH may not be exclusively due to genetic differences among various subpopulations. Thus, even when introduced into a similar microenvironment, epigenetic modifications may allow different clones to develop into cells with markedly different tumorigenic potential and phenotypes. DNA sequencing has demonstrated an increased mutation frequency in human triple-negative breast cancers as compared with ER-positive luminal breast cancers (49), but many of these mutations occurred at low frequency. Therefore, it is likely that both genetic and epigenetic factors will play a role in generating ITH, and this may only be detected by in vitro studies, FACS-sorted CD29hiCD24lo cells and TICs were mixed under different selection pressures from those observed in somatic Trp53-null and mutant human breast cancers.

**METHODS**

**Materials**

TOP-eGFP and its control vector, FOP-eGFP, were kind gifts from Dr. Irving Weissman (Stanford University, Stanford, CA). pEiZsGreen and pEiTomoRed vectors were kindly provided by Dr. Bryan Welm (Oklahoma Medical Research Foundation, Oklahoma City, OK). The highly transfectable 293T cell line, which was used routinely for virus propagation, was purchased from the ATCC. No test and authentication was done by the authors. All other antibodies, lentiviral particles, and primers were purchased from commercial sources as listed in the Supplementary Data Materials and Methods.

**Preparation of Single Mammary Tumor Cells**

Mice were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Experimental Animals. All animal protocols were reviewed and approved by the Animal Protocol Review Committees of the University of Pittsburgh (Pittsburgh, PA) and Baylor College of Medicine (Houston, TX). Trp53-null mammary tumors were generated as previously described (20).

**Mammosphere and Transwell Coculturing Assays**

The protocol for mammosphere assays was as described by Dontu and colleagues (14) and in the Supplementary Data.

For mammosphere coculture, 5,000 TICs, 5,000 CD29hiCD24lo niche cells, or 2,500 TICs plus 2,500 niche cells, 2,500 TICs plus 2,500 CD29lo cells, or 2,500 TICs plus 2,500 niche cells with Cxcl12 or Wnt2 knockdown, and 2,500 TICs with Cxcr4 or Fzd7 knockdown plus 2,500 niche cells were cultured after dissociation of the primary mammospheres in 2 mL serum-free mammosphere medium under a nonadherent condition for 7 days. PKH26 (red fluorescent) and PKH67 (green fluorescent) cell linker kits (Sigma) were used to label individual cell subpopulations according to the manufacturer’s protocol.

For Transwell culture of mammospheres, 5,000 TICs, 5,000 CD29hiCD24lo niche cells, or 2,500 TICs plus 2,500 niche cells were Transwell-cultured with 5,000 dissociated TICs after dissociation of the designated cell types from the primary mammospheres in 3.6 mL serum-free mammosphere medium under a nonadherent condition in Transwell plates for 7 days.

**Lentiviral Transduction**

Lentiviral transduction was performed as described previously (17). The pEiZsGreen and pEiTomoRed lentiviruses were driven by an EF1α promoter, and the lentiviruses were packaged in 293T cells by cotransfection of pEiZsGreen (or pEiTomoRed), pRSV-rev, pMDG-eRRE, and pCMV-VSVG using Fugene transfection reagent (Roche). For fluorescence tracking, enzymatically digested and FACsorting Trp53-null tumor cells (niche cells or TICs; 20,000/per well) were suspended into 24-well ultra-low attachment plates, and transduced with lentiviruses expressing either pEiZsGreen or pEiTomoRed, allowing stable integration of ZsGreen and Tomato Red fluorescence reporter at a multiplicity of infection (MOI) of 10, respectively, for 24 hours in a final volume of 1 mL serum-free mammosphere medium. After transduction, ZGreen or Tomato Red-positive cells were FAC-sorted and collected in Hank’s Balanced Salt Solution (HBSS) medium, before transplantation.

For knockdown analysis, dissociated and FAC-sorted TICs and niche cells from the Trp53-null tumors (20,000/per well) were suspended into 24-well ultra-low attachment plates, and were transfected with empty shRNA vector control, panels of shRNA lentiviruses against Cxcr4, Fzd7, or two different Wnt2 shRNA lentiviruses, one Cxcl12 shRNA, and two different Ile6 shRNA lentiviruses, respectively, as designated in the main text, for 48 to 72 hours in a final volume of 1 mL serum-free mammosphere medium. The shRNA sequences (antisense) used in this study are listed in the Supplementary Data. Cells infected with viruses were selected in the presence of 2 μg/mL of puromycin. Downregulation of the target genes was verified by RT-PCR using TaqMan primer and probe sets (Life Technologies). Lentiviral pLKO.1 empty vector control (Dharmacon) was used as the control vector.

**In Vivo Transplantation into the Cleared Mammary Fat Pad**

Clearance of the mammary fat pad and transplantation procedures were performed as originally described (20). For cotransplantation studies, FACS-sorted CD29hiCD24lo cells and TICs weremixed at designated numbers in HBSS medium.

FACS-sorted TICs and niche cells were also collected and transduced with lentiviruses as described in the Lentiviral Transduction section above. Then, these two populations of cells were mixed and cotransplanted into the cleared fat pads of 3-week-old female recipient mice (BALB/c mice from Harlan) at the designated ratios of 200/0, 120/80, 68/132, and 32/168 (with a total number of cells of 200). The resulting tumors were FACs analyzed on the basis of expression of ZsGreen and TomatoRed.

For gene knockdown studies, after transduction with the Wnt2 shRNA, the designated number of cells were washed once with 1× PBS and cotransplanted with the freshly sorted WNT-responsive GFP+ cells into the cleared fat pads of 3-week-old female BALB/c
For all in vivo transplantation assays, 50% growth factor-reduced Matrigel (BD Biosciences) was added to the final volume of 2 μl before injection. Two weeks after transplantation, tumor formation was monitored daily. Mammary tumor tissues were removed when tumor size reached 1 cm in diameter.

Quantitative Reverse Transcriptase–Polymerase Chain Reaction

RNA (300 ng each) was used to generate cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the company’s protocol. qRT-PCR reactions were performed as described in the Supplemental Data.

Immunostaining and Microscopic Analysis

Paraffin-embedded and paraformaldehyde (PFA)-fixed tumor tissues, and FACS-sorted and cytopun cells were stained with the antibodies against Ki5 (1:5,000), SMA (1:250), and Ki67 (1:200) as described in the Supplemental Data. Microscopic analysis was done on an Olympus BMAX 50 fluorescence microscope with details described in the Supplemental Data.

Microarray Analysis

Statistical analyses for microarray were performed in the biostatistics core facility of the Dan L. Duncan Cancer Center at Baylor College of Medicine (A. Tsimelzon), and University of North Carolina at Chapel Hill (Chapel Hill, NC; C. Fan). Detailed analysis was described in ref. 20 and in the Supplemental Data. The complete array data can be accessed at Gene Expression Omnibus (GEO; GSE8863).

Mesenchymal and Claudin-Low Signature Analysis on Individual Populations

RNA microarray data obtained from the four individual subpopulations (CD29hiCD24lo, CD29hiCD24hi, CD29loCD24hi, and CD29loCD24lo) were analyzed. Each signature/module was built using the median expression of the gene lists published in corresponding articles as referenced. Boxplots of the signatures were constructed and ANOVA analysis for the signatures in the four different groups was performed using the R software package. Three independent tumors from the Tp53-null model were included in this analysis for each boxplot category.

Disclosure of Potential Conflicts of Interest

C.M. Perou has ownership interest (including patents) in Bioclassifier, LLC, and is a consultant/advisory board member for the same. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: M. Zhang, J.M. Rosen
Development of methodology: M. Zhang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Zhang, C.-H. Chang, A. Wolff, C.M. Perou
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Zhang, A. Tsimelzon, C. Fan, C.M. Perou, S.G. Hilsenbeck, J.M. Rosen
Writing, review, and/or revision of the manuscript: M. Zhang, C.-H. Fan, C.M. Perou, S.G. Hilsenbeck, J.M. Rosen

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REFERENCES

Cellular Heterogeneity in Trp53-Null Mammary Tumors


Intratumoral Heterogeneity in a Trp53-Null Mouse Model of Human Breast Cancer

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