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

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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 MALIGNAL-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. Cancer Discov; 5(5); 520–33. © 2015 AACR.

See related commentary by Brooks and Wicha, p. 469.
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 yet long-lived 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)hiCD24lo 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 tumortivity in the primary tumors (Supplementary Fig. S2Bc); however, a high level of SMA also was observed in the stromal compartment in the CD29hiCD24lo-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 CD29hiCD24lo-derived 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 Asmx2 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 CD29hiCD24lo cells. The expression of Fzd7, encoding one of the WNT ligand receptors, and Ccr2, encoding the receptor for CXCL12, was also upregulated in the TIC population as compared with the non-TIC population (Fig. 1C and D).

**The CD29hiCD24lo Subpopulation Is Less Proliferative as Compared with the TIC Subpopulation**

Cell-cycle analysis performed on TICs and CD29hiCD24lo cells using 7-Aminoactinomycin D (7-AAD) and pyronin Y and

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**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: CD29hiCD24lo, CD29hiCD24hi, CD29loCD24hi, and CD29loCD24lo. The Lin−CD29hiCD24lo 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−CD29hiCD24lo subpopulation identified in most of the heterogeneous Trp53-null tumors studied [including estrogen receptor–positive (ER+)] and ER− (ER−) tumors, tumors expressing 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−CD29hiCD24lo) was able to generate tumors with as few as 10 cells. The Lin−CD29hiCD24lo 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 tumorigenicity when compared with the Lin−CD29hiCD24hi and Lin−CD29loCD24lo subpopulations, which represented the bulk (≥90%) of the tumor cells. However, FACS analysis of tumors arising from the Lin−CD29hiCD24lo subpopulation showed that they did not mimic the phenotype of the parental tumor; instead, an expansion of the TICs was observed from the Lin−CD29hiCD24lo-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 CD29hiCD24lo-derived tumors (Supplementary Fig. S2Bc).
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 < 0.05.

showed that 8.6% ± 1.3% (mean ± SD) of TICs were in G0-G1 phase (Fig. 2Aa), whereas 21.5% ± 3.3% of CD29hiCD24lo 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 CD29hiCD24lo cells were in the G0-phase and 3.2% ± 0.3% in the G1-phase (Fig. 2Aa and ab), indicating that a group of CD29hiCD24lo cells were quiescent. However, this cannot explain the low tumorigenic potential of this subpopulation, as the CD29hiCD24lo cells contain more cells in the G0-phase, and have a higher tumorigenic potential than the CD29loCD24hi (Supplementary Fig. S3, G0-G1: 9.6% ± 2.1%; Supplementary Fig. S3B, G0: 7.8% ± 1.9%), and CD29loCD24lo 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 cytopsin centrifugation followed by Ki67 staining. Although 60% of TICs (Fig. 2Ca) were proliferative, only 30% of CD29hiCD24lo cells were Ki67-positive (Fig. 2Cb).

The CD29hiCD24lo Niche Population Has Features of Mesenchymal and Claudin-Low Signatures

Because several genes expressed in the CD29hiCD24lo 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 CD29hiCD24lo population expresses mesenchymal markers (Fig. 2Ca). 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 CD29hiCD24lo subpopulation that also displays an increased expression of EMT features (Fig. 2Cb and c).

CD29hiCD24lo Cells Promote In Vitro Self-Renewal Capacity of TICs

Coculture of CD29hiCD24lo 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 CD29hiCD24lo cells alone (Fig. 3A and B). However, the coculture of TIC and CD29hi cells did not result in increased mammosphere frequency, indicating the unique interaction between the TICs and CD29hiCD24lo 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 CD29hiCD24lo 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 (Fig. 3C), but not size, of mammospheres as compared with that of the TICs by themselves. A marginally significantly (**, P = 0.051) higher mammosphere-forming efficiency of TICs was observed in the presence of TICs and niche cells as compared with that in the presence of niche cells 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 CD29 hi CD24 hi cells, were transfected 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 they were Transwell-cultured with the TICs alone (Fig. 3D), suggesting the secreted molecules from the niche cells were able to affect the differentiation potential of the TICs.

**Downregulation of Preferentially Expressed Genes in the Putative Niche Cells (CD29 hi CD24 hi) 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 CD29 hi CD24 hi 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 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. CD29 hi CD24 hi TICs were used to coculture with the niche cells with Cxcl12 shRNA knockdown.

FACS-sorted CD29 hi CD24 hi niche cells were transduced with a lentivirus expressing two different shRNAs to knockdown expression of either Wnt2 or Cxcl12 differentially expressed in the niche population. Two clones targeting Wnt2, one clone targeting Cxcl12, and their corresponding non silenced controls were included. Real-time qPCR confirmed the downregulation of Wnt2 and Cxcl12 in the Trp53-null...
T1 tumor at levels ranging from 50% to 60% (Fig. 4A and B). Genetically modified knockdown niche cells were then co-cultured with TICs in serum-free mammosphere medium under nonadherent conditions for 7 days. A reduced mammosphere-forming ability was observed for both knockdowns, indicating that the functional interaction between two cell types was disrupted, and the self-renewal potential of the TICs was inhibited (Fig. 4C and D).

**Downregulation of Fzd7 and Cxcr4 in the TICs (CD29<sup>hi</sup>CD24<sup>lo</sup>) 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 WNT-responsive 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 alone (Fig. 4G and H). In addition, when Il6 was knocked down in the niche cells (Supplementary Fig. S5A), and such modified niche cells were cocultured with TICs, a decrease in mammosphere-forming efficiency was observed (Supplementary Fig. S5B).

**CD29<sup>hi</sup>CD24<sup>lo</sup> 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<sup>hi</sup>CD24<sup>lo</sup> 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...
mesenchymal-like niche cells (CD29 hi CD24 lo) were able to enhance TIC tumor initiation by secreting factors that perhaps provide an improved microenvironment.

The time to tumor formation curves were also estimated and compared (Fig. 5B). Across all eight groups, there was a significant difference between groups ($P < 0.001$). 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 investigate 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 (pEI-T-TICs) and CD29⁺CD24⁻ Niche Cells (pEIZ-Niche Cells) Suggests that the TICs Contributed to the Majority of Tumor Growth**

TICs and putative niche cells (CD29⁺CD24⁻) 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⁺CD24⁻) 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

![Image](image_url)

**Figure 4.** Inhibition of WNT2 and CXCL12 signaling in the niche cells, and FZD7 and CXCR4 in the TICs with shRNAs, respectively, reduced the self-renewal of the TICs. A and B, Wnt2 or Cxcl12 shRNA lentiviruses (shRNA) or control lentivirus (Ctrl) were introduced into the dissociated CD29⁺CD24⁻ cells after being FACS-sorted from Trp53-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 TICs after primary culture in the mammosphere medium. □ TICs were cocultured with control (CD29⁺CD24⁻ infected with the empty vector). □ TICs were cocultured with CD29⁺CD24⁻ cells infected with Wnt2 shRNA1 (C). □ TICs were cocultured with CD29⁺CD24⁻ cells infected with Wnt2 shRNA2 (D). (continued on following page)
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 were observed with different combinations of TOP-GFP-transduced GFP+ TICs and Wnt2 shRNA knockdown niche cells as compared with those of cotransplantation of TICs and control niche cells, suggesting the tumorigenic potential of the TICs was affected by decreasing paracrine factors, such as WNT2 in the niche cells (Table 1 and Supplementary Table S2). With 10 WNT-responsive TICs, different types of niche cells were associated with different tumor-forming frequencies (see Supplementary Table S5 with Fig. S2). With 10 WNT-responsive TICs, different types of niche cells were associated with different tumor-forming frequencies (see Supplementary Table S5 with Fig. S2).

Kaplan–Meier curves were also generated for TICs = 10 groups in Table 1 (Fig. S1D). Across all eight groups, there is a significant difference between groups (P < 0.001). To test whether shRNA niche cells reduced the time to tumor formation relative to the control shRNA, four comparisons were undertaken. There was no difference observed between shRNA groups for 0 or 2 niche cells, a marginal difference for 10 niche cells, and a significant difference detected for 20 niche cells (Supplementary Table S6A–S6D).

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 differently to both conventional and targeted therapies. In a clinical study, residual breast cancer cells following treatment differently to both conventional and targeted therapies. In a clinical study, residual breast cancer cells following treatment differently to both conventional and targeted therapies. In a clinical study, residual breast cancer cells following treatment differently to both conventional and targeted therapies.
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 Tp53-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<sup>+</sup>CD24<sup>+</sup><sup>+</sup> 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 Wnt<sup>2</sup> 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 Wnt<sup>2</sup>/Fzd7 were knocked down

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**Figure 5.** CD29<sup>+</sup>CD24<sup>+</sup> niche cells enhanced TICs tumor initiation potential shown by limiting dilution cotransplantation assays. **A,** cotransplantation of increased numbers of niche cells caused increased TIC tumor formation frequencies. **B,** niche cells reduce time to tumor formation. **C,** shRNA knockdown niche cells are associated with different tumor-forming cell frequencies shown by limiting dilution cotransplantation assays. **D,** a significant difference between groups (P < 0.001) as shown by the Kaplan–Meier curves among cotransplantation of the 10 TICs and the shRNA knockdown niche groups (cell number in parenthesis) versus that of 10 TICs and control vector groups.
Cellular Heterogeneity in Trp53-Null Mammary Tumors

Table 1. Cotransplantation of CD29hiCD24lo putative niche cells with TICs

<table>
<thead>
<tr>
<th>(A) Cells cotransplanted</th>
<th>Efficiency of tumor formation (number of tumors formed/transplants)</th>
<th>Latency of tumor formation, wk (palpable 2–3 mm)</th>
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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 Il6 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.

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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, causing the expansion of the TIC population, especially during early tumor development (Fig. 6; ref. 2). CD29\textsuperscript{hi}CD24\textsuperscript{lo} 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

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**Figure 6.** Tumor cell plasticity and ITH within the tumors. **A,** the TIC population of cells is responsible for the generation of the vast majority of cells within a tumor. **B,** the other tumor cells derived from the TICs presumably as a function of both symmetric and asymmetric division and resulting epigenetic changes due to the microenvironment and factors such as hypoxia and TGFβ may promote tumor formation, especially when there are only a limited number of TICs.
Trp53-null BALB/c tumors 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 CD29\(^{+}\)CD24\(^{-}\), CD29\(^{-}\)CD24\(^{+}\), CD29\(^{-}\)CD24\(^{-}\), and CD29\(^{-}\)CD24\(^{-}\) 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-depth single cell sequencing. It is also likely that both genetic and epigenetic factors will play a role in generating ITH, and this may only be detected by single cell sequencing. It is also likely that both genetic and epigenetic factors will play a role in generating ITH, and this may only be detected by single cell sequencing. It is also likely that both genetic and epigenetic factors will play a role in generating ITH, and this may only be detected by single cell sequencing. It is also likely that both genetic and epigenetic factors will play a role in generating ITH, and this may only be detected by single cell sequencing.
mice. For all in vivo transplantation assays, 50% growth factor-reduced Matrigel (BD Biosciences) was added to make 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 Supplementary Data.

**Immunostaining and Microscopic Analysis**

Paraffin-embedded and paraformaldehyde (PFA)-fixed tumor tissues, and FACS-sorted and cytospun cells were stained with the antibodies against K5 (1:5,000), SMA (1:250), and Ki67 (1:200) as described in the Supplementary Data. Microscopic analysis was done on an Olympus BMAX 50 fluorescence microscope with details described in the Supplementary 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 Supplementary 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 the median expression of the gene lists published in corresponding articles as referenced. Statistical analyses were 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.F. Cam, C.M. Perou, S.G. Hilsenbeck, J.M. Rosen

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

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IN THE SPOTLIGHT

Tumor Twitter: Cellular Communication in the Breast Cancer Stem Cell Niche

Michael D. Brooks and Max S. Wicha

Summary: Communication between the diverse assortment of cells that constitute the tumor microenvironment plays an important role in tumor development. Using a p53-null mouse model, Zhang and colleagues describe a novel feedback loop involving breast cancer stem cells and their progeny mediated by WNT2, CXCL12, and IL6.

IN THE SPOTLIGHT continues...
A much higher tumor initiation rate compared with similar numbers of TICs alone. Furthermore, the ability of the mesenchymal niche cells to increase the tumor-initiating capacity of CSCs was abrogated by knockdown of WNT2 in the mesenchymal niche cells, demonstrating an important role for this signaling pathway in tumor initiation.

A key element of the findings of Zhang and colleagues is the positive feedback loop between cancer stem cells and mesenchymal tumor populations derived from them. As a result, these tumors produce high levels of WNT2, CXCL12, and IL6, which in turn drives the self-renewal of tumor-initiating cells, which then produce more mesenchymal cells, and so forth (Fig. 1A). This might provide an explanation for the observation that basal and claudin-low breast tumors have the highest proportion of cancer stem cells (8) and are among the most aggressive and difficult to treat. This also emphasizes the importance of targeting both bulk and CSC populations to achieve maximum therapeutic effect. Moreover, signaling molecules involved in these feedback loops represent rational therapeutic targets. In fact, inhibitors of WNT, CXCR4, and IL6 have entered early-phase cancer clinical trials.

The work of Zhang and colleagues provides important new data on cell-cell communication in the cancer stem cell niche. However, a number of questions remain. These include further characterization of the CD29<sup>hi</sup>CD24<sup>lo</sup> mesenchymal niche cell population. Although described as mesenchymal-like cells, they in fact have tumor-initiating capacity, albeit less than the CD29<sup>hi</sup>CD24<sup>hi</sup> CSC population. Furthermore, tumors generated from this mesenchymal-like population are primarily epithelial rather than mesenchymal in nature. Together, this suggests that at least a fraction of the CD29<sup>hi</sup>CD24<sup>lo</sup> population is better characterized as an EMT-like CSC, rather than as a fixed EMT-like cell. If this is the case, then it suggests that there may be interactions between different CSC populations that regulate their behavior, as illustrated in Fig. 1B. Further work will be required to determine the exact nature of these interacting cells.

The work of Zhang and colleagues adds to the growing body of literature demonstrating that CSCs are able to generate key components of their niche and that differentiated progeny may play important roles in CSC regulation. It has previously been demonstrated that the steroid hormones...
estrogen and progesterone regulate normal and malignant mammary stem cells through feedback loops involving FGF9 (9), WNT4, and RANKL generated by differentiated mammary luminal cells (Fig. 1B; ref. 10). The work of Zhang and colleagues adds to these findings by demonstrating that mesenchymal-like progeny can also regulate breast stem cells through feedback loops. In total, these studies add to our understanding of the high degree of complexity that characterizes the tumor microenvironment and contributes to intratumor cellular heterogeneity. Although this heterogeneity represents a formidable therapeutic challenge, novel technologies, including single-cell DNA sequencing and RNA-seq, provide important tools to deconvolute this complexity. These studies thus may lead to the development of more effective therapeutic strategies.

Disclosure of Potential Conflicts of Interest

M.S. Wicha reports receiving a commercial research grant from Dompe, has ownership interest (including patents) in Oncomed Pharmaceuticals, is a consultant/advisory board member for Verastem, and has provided expert testimony for MedImmune. No potential conflicts of interest were disclosed by the other author.

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