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

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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 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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CANCER DISCOVERY

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.

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.

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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. In 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 (B1-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 in several tissues as both cycling and long-lived tumor cells showed that they did not mimic the phenotype of the stromal compartment in the CD29 hi CD24 lo -derived tumors (Supplementary Fig. S2Bc). 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 was also observed in the stromal compartment in the CD29 hi CD24 lo -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 CD29 hi CD24 lo 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 Tcf7, 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 CD29 hi CD24 lo cells. The expression of Fzd7, encoding one of the WNT ligand receptors, and Cox4, encoding the receptor for CXCL12, was also upregulated in the TIC population as compared with the non-TIC population (Fig. 1C and D).

The CD29 hi CD24 lo Subpopulation Is Less Proliferative as Compared with the TIC Subpopulation

Cell-cycle analysis performed on TICs and CD29 hi CD24 lo 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–G₁ phase and 3.2% ± 0.3% in the G₁ phase (Fig. 2Aa 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⁻ (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).

**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 (Fig. 3C), but not of mammospheres as compared with culturing the TICs (Fig. 3A) and CD29⁺CD24⁻ cells alone (Fig. 3B). However, the coculture of TICs 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.
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 lo 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 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.

**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 CD29 hi CD24 lo (Ab and Bb) subpopulations. **C**, the cells (a, TIC; b, CD29 hi CD24 lo ) were also cytospun and analyzed using an antibody against Ki67. **D**, quantification of Ki67–positive cells within the TIC and niche populations. **E**, the CD29 hi CD24 lo niche population has features of mesenchymal cells and claudin-low tumors. The CD29 hi CD24 lo population expresses core mesenchymal signature (Ea), claudin-low high gene expression signature (Eb), and claudin-low low gene expression signature (Ec) 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, CD29 hi CD24 lo; red, CD29 hi CD24 lo; green, CD29 lo CD24 hi.

**Downregulation of Preferentially Expressed Genes in the Putative Niche Cells (CD29 hi CD24 lo ) 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–marked TICs, and niche cells with Wnt2 shRNA knockdown. CD29 hi CD24 lo TICs were used to coculture with the niche cells with Cxcl12 shRNA knockdown. FACS-sorted CD29 hi CD24 lo 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 nonsilencing controls were included. Real-time qPCR confirmed the downregulation of Wnt2 and Cxcl12 in the Trp53-null...
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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. S5A), and such modified niche cells were cocultured with genetically modified TICs and niche cells were cocultured, a decrease in mammosphere-forming efficiency 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).

**Figure 3.** Coculture and Transwell culture of TICs with CD29hiCD24lo cells or CD29lo 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 CD29hiCD24lo cells display larger and an increased number of mammospheres compared with their individual cultures alone. Aa, TICs (green); Ab, CD29hiCD24lo (red). Ac, TICs (green) and CD29hiCD24lo (red) were mixed; Ad, TICs (green) and CD29hiCD24lo (red) were mixed. B, mammosphere-forming efficiency of TICs cultured alone, CD29hiCD24lo alone, a mixture of TICs and CD29hiCD24lo, and a mixture of TICs and CD29lo. 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.

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 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).

**CD29hiCD24lo 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 CD29hiCD24lo 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 1B).
“mesenchymal-like” niche cells (CD29 hi CD24 lo) were able to initiate tumorigenesis 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 CD29hiCD24lo Niche Cells (pEIZ-Niche Cells) Suggests that the TICs Contributed to the Majority of Tumor Growth**

TICs and putative niche cells (CD29hiCD24lo) 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. S6A and S6B); 120/80 (Supplementary Fig. S6C and S6D); 68/132 (Supplementary Fig. S6E and S6F); and 32/168 (Supplementary Fig. S6G and S6H), consistent with their self-renewal and differentiation ability.

**Downregulation of Wnt2 in the Niche Cells (CD29hiCD24lo) 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

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 CD29hiCD24lo 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 TIC cells after primary culture in the mammosphere medium. ■ TICs were cocultured with control (CD29hiCD24lo infected with the empty vector). □ TICs were cocultured with CD29hiCD24lo cells infected with Wnt2 shRNA1 (C). ◆ TICs were cocultured with CD29hiCD24lo cells infected with Cxcl12 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–CXCL12 shRNA knockdown at 72 hours of selection with puromycin, levels of Fzd7 and Cxcr4 were determined by qPCR. In TICs and control shRNA, were plated under mammosphere conditions. The dissociated cells were then cocultured with niche cells either infected with the empty vector or with Wnt2 shRNA, respectively, after primary mammosphere culture, in the mammosphere medium. ■ TICs infected with Fzd7 shRNA were cocultured with CD29+CD24− cells infected with the control shRNA; □ TICs infected with Fzd7 shRNA were cocultured with CD29+CD24− cells infected with the control shRNA; △ TICs infected with Fzd7 shRNA were plated under mammosphere conditions. The dissociated cells were then cocultured with niche cells either infected with the empty vector or with Cxcl12 shRNA, respectively, after primary mammosphere culture, in the mammosphere medium. ■ TICs infected with Fzd7 shRNA were cocultured with CD29+CD24− cells infected with the empty control vector; □ TICs infected with Fzd7 shRNA were plated under mammosphere conditions. The dissociated cells were then cocultured with niche cells either infected with the empty vector or with Cxcl12 shRNA, respectively, after primary mammosphere culture, in the mammosphere medium. ■ TICs infected with Fzd7 shRNA were cocultured with CD29+CD24− cells infected with the control shRNA; □ TICs infected with Cxcr4 shRNA were cocultured with CD29+CD24− cells infected with the control shRNA; △ TICs infected with Cxcr4 shRNA were cocultured with CD29+CD24− cells infected with Cxcl12 shRNA. Three repeats for each group (N = 3). The number of mammospheres formed was determined and quantitated at day 7. *, P < 0.0002; **, P < 0.01; ***, P < 0.002.

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 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.

**Figure 4. (Continued)** E and F, Fzd7 and Wnt2 shRNA lentiviruses (shRNA) and control lentivirus (Ctrl) were introduced into the dissociated TICs 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, in the mammosphere medium. ■ TICs infected with Fzd7 shRNA were cocultured with CD29+CD24− cells infected with the control shRNA; □ TICs infected with Fzd7 shRNA were cocultured with CD29+CD24− cells infected with the control shRNA; △ TICs infected with Fzd7 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. ■ TICs infected with Fzd7 shRNA were cocultured with CD29+CD24− cells infected with the empty control vector; □ TICs infected with Fzd7 shRNA were cocultured with CD29+CD24− cells infected with the control shRNA; △ TICs infected with Fzd7 shRNA were cocultured with CD29+CD24− cells infected with the control shRNA; □ TICs infected with Cxcr4 shRNA were cocultured with CD29+CD24− cells infected with Cxcl12 shRNA. Three repeats for each group (N = 3). The number of mammospheres formed was determined and quantitated at day 7. *, P < 0.0002; **, P < 0.01; ***, P < 0.002.
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 CD29hiCD24lo 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.

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**Figure 5.** CD29hiCD24lo 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 forming 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.
Table 1. Cotransplantation of CD29hiCD24lo putative niche cells with TICs

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<th>Cells cotransplanted</th>
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<th>Latency of tumor formation, wk (palpable, 2–3 mm)</th>
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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.
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). CD29hiCD24lo 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...
Cellular Heterogeneity in Trp53-Null Mammary Tumors

For mammosphere coculture, 5,000 TICs, 5,000 CD29+/CD24−, and 5,000 CD29−/CD24− cells were cultured 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 CD29+/CD24−, and 5,000 CD29−/CD24− cells, or 5,000 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 pEITomato Red lentivirus reporters were driven by an EF1α promoter, and the lentiviruses were packaged in 293T cells by cotransfection of pEIZsGreen (or pEITomato Red), pRSV-rev, psiMDG-pRRE, and pCMV-VSVG using Fugene transfection reagent (Roche). For fluorescence tracking, enzymatically digested and FACS sorted Trp53-null tumor cells (niches or TICs; 20,000/per well) were suspended into 24-well ultra-low attachment plates, and transduced with lentiviruses expressing either pEIZsGreen or pEITomato Red, 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 FACSc-sorted and collected in Hank’s Balanced Salt Solution (HBSS) medium, before transplantation.

For knockdown analysis, dissociated and FACS-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 Ccr4, Fzd7, or two different Wnt2 shRNA lentiviruses, one Cxcl12 shRNA, and two different Il6 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, FACSc-sorted CD29+/CD24− cells and TICs were mixed at designated numbers in HBSS medium.

FACS-sorted TICs and niche cells were also collected and transfected 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 was 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

**METHODS**

**Materials**

TOP-eGFP and its control vector, TOP-eGFP, were kind gifts from Dr. Irving Weissman (Stanford University, Stanford, CA). pEIZsGreen and pEITomato Red 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. 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. 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.

**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.
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) 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-fixed tissue sections, and FACS-sorted and cryospun 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. Boxplots of the signatures were constructed and median expression was marked according to the company’s protocol. Boxplots of the signatures were constructed and median expression was marked as referenced. Bosplots 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 Tpt53-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 Bioclassify, 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. Fan, C.M. Perou, S.-G. Hilsenbeck, J.M. Rosen

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**REFERENCES**


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