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**Abstract**

Cancer stem-like cells contribute to tumor heterogeneity and have been implicated in disease relapse and drug resistance. Here we show the coexistence of distinct breast cancer stem-like cells (BCSC) as identified by ALDH$^+$ and CD29$^/$CD61$^+$ markers, respectively, in murine models of breast cancer. While both BCSC exhibit enhanced tumor-initiating potential, CD29$^/$CD61$^+$ BCSC displayed increased invasive abilities and higher expression of epithelial-to-mesenchymal transition and mammary stem cell--associated genes, whereas ALDH$^+$ BCSC were more closely associated with luminal progenitors. Attenuating the autophagy regulator FIP200 diminished the tumor-initiating properties of both ALDH$^+$ and CD29$^/$CD61$^+$ BCSC, as achieved by impairing either the Stat3 or TGFβ/Smad pathways, respectively. Furthermore, combining the Stat3 inhibitor Stattic and the Tgfβ-R1 inhibitor LY-2157299 inhibited the formation of both epithelial and mesenchymal BCSC colonies. In vivo, this combination treatment was sufficient to limit tumor growth and reduce BCSC number. Overall, our findings reveal a differential dependence of heterogeneous BCSC populations on divergent signaling pathways, with implications on how to tailor drug combinations to improve therapeutic efficacy.

**Introduction**

Breast cancer is the most common form of cancer among women worldwide and some of the key challenges faced when treating this widespread disease include therapeutic resistance, relapse, and metastasis (1). There is increasing evidence to indicate that these challenges persist due to the existence of a sub-population of cells within tumors termed cancer stem-like cells (CSC; refs. 2–4). Initial isolation of breast CSCs (BCSC) demonstrated that not all cells within a tumor are equal and it is the BCSCs that exhibit increased tumorigenicity and drive tumor growth (5). In fact, BCSCs have been shown to exhibit resistance to conventional therapies (3, 4) and drive metastasis (6). The presence of BCSCs presents an additional layer of heterogeneity within tumors alongside intratumor genetic diversity. Conventional chemotherapies that tend to eliminate just the bulk tumor population enrich for BCSCs and this can lead to more aggressive residual disease. Accordingly, therapeutic efforts now account for BCSCs rather than just targeting the bulk tumor population (7).

Meanwhile, plasticity exists where non-BCSC populations can acquire BCSC properties through pathways such as epithelial-to-mesenchymal transition (EMT; refs. 8, 9). In addition, distinct BCSC populations with epithelial and mesenchymal properties, respectively, have been described and the interconversion between the two states has been demonstrated (10). These distinct BCSCs were also shown to occupy different niches, where epithelial BCSCs were located in central regions of the tumor, whereas mesenchymal BCSCs were found at the invasive front. These observations raise the possibility that diverse BCSC populations may exist within a tumor and possibly have differential susceptibilities to BCSC-targeted therapeutics. With that notion, it would be imperative to account for heterogeneous BCSC populations and limiting the plasticity of these populations when considering therapeutic strategies.

Autophagy is a self-cannibalization process, which involves the sequestration of organelles, proteins, or lipids in vesicles termed autophagosomes for degradation and recycling. Degradation occurs upon fusion of autophagosomes with lysosomes and the degraded products are exported into the cytoplasm for recycling (11). Basal levels of autophagy are important to eliminate damaged organelles such as mitochondria and unfolded proteins. Autophagy can also be induced to maintain homeostasis under stressful conditions such as nutrient starvation. The physiologic importance of autophagy is illustrated by the involvement of autophagy-related (Atg) proteins in diseases such as cancer, neurodegeneration, and autoimmunity (11).

In terms of the role of autophagy in cancer, a duality exists where the autophagic process performs a tumor-suppressive function during tumorigenesis but promotes malignancy in advanced tumor progression (12). The homeostatic function of autophagy limits genotoxic stress, inflammation, and production of reactive oxygen species and this reduces the rate of mutagenesis (13). On the other hand, autophagy confers survival advantages in established tumors under stressful conditions such as hypoxia and allows tumor cells to sustain demanding metabolic needs (12, 14). It is also becoming apparent that autophagy impinges on...
features that are crucial for metastatic dissemination. Autophagy promotes resistance to anoikis, a trait that is important for the survival of tumor cells that have detached and are circulating (15, 16). In addition, autophagy has also been implicated in promoting tumor cell invasion (17, 18). For these reasons, it is no surprise that autophagy inhibitors such as hydroxychloroquine are actively trialed in the clinic for treatment of cancers and development of novel autophagy inhibitors are earnestly pursued (19). Recent studies also implicated a role of autophagy specifically in BCSC populations. Impairing autophagy can affect the maintenance of BCSCs through limiting EMT and the CD44+/CD24− phenotype (20). BecIn1 silencing could also abrogate the propagation of BCSCs in mammosphere cultures and their tumorigenicity in vivo (21). However, the underlying molecular mechanism is still not well understood, and it is not known whether autophagy may regulate different BCSC subsets through different mechanisms.

Materials and Methods

Reagents and antibodies

EGFR-WT plasmid was a gift from Matthew Meyerson (Addgene plasmid # 11011; ref. 22). Plasmids used for silencing Stat3 (TRCN71453, TRCN71454), Egfr (TRCN23482, TRCN23480), and Smad4 (TRCN25585, TRCN25581) were obtained from the Cincinnati Children’s Hospital Lenti-shRNA library core. Antibodies used for immunoblotting include β-actin (Sigma A5441), vinculin (Sigma V4505), EGFR (CST 4267), phospho-EGFR Y1068 (CST 3777), Jak2 (CST 3230), phospho-Jak2 Y1007/1008 (CST 3776), Stat3 (CST 9139), phospho-Stat3 (p-Stat3) Y705 (CST 9145), Smad2/3 (CST 3102), phospho-Smad2/3 (CST 8828), phospho-Smad2 (CST3101), Smad4 (CST 9515), Sox3 (CST 2932), and Pias3 (CST 9042). For flow cytometry, antibodies used were CD29-biotin (eBioscience 116212), Streptavidin-APCcy7 (Biolegend 405208), and CD45-APC (Biolegend 103112), CD24-PE (BD553262), CD31-APC (Biolegend 103112), Ter119-APC-V450 (BD 562155), and CD61-biotin for 20 minutes at 4°C. Dissociated single-cell suspensions from primary tumors [as described previously (24)] or cultured cells were incubated with Aldefluor buffer before sorting or analysis by FACS

Tumor mice and transplants

MMTV-PyMT, Ctrl-MT (Fip200WT, MMTV-PyMT), and cKO-MT (Fip200WT, MMTV-PyMT, MMTV-Cre) mice have been described previously (14). MMTV-Wnt1 mice were obtained from Dr. Yi Li of Baylor College of Medicine. Mice were housed and handled according to local, state, and federal regulations. All experimental procedures were carried out according to protocols approved by the Institutional Animal Care and Use Committee at University of Cincinnati (Cincinnati, OH). For transplantation experiments, cells were prepared in DMEM:Matrigel at a 1:1 ratio and the required number of cells were injected in a 50 μL volume orthotopically into the fourth inguinal mammary fat pads of athymic nude mice (Harlan). For limiting dilution transplants, mice were monitored for at least 3 months for the formation of tumors. Tumor growth measurements were obtained using calipers and volume was calculated as [(length)²] × width/2. For administering Stattic and LY-2157299, Stattic was purchased from ApexBio and LY-2157299 was kind gift from Eli Lilly. Drugs were prepared in vehicle [1% carboxymethylcellulose, 0.5% sodium laurel sulfate, 0.085% povidone, and 0.05% anti-foam (kind gift from Xiameter)] for administration via orogastric gavage. Mice with orthotopically transplanted PyMT cells were randomized into respective treatment groups when the volume of tumors reached ≥50 mm³. The treatment cohorts consisted of vehicle control, LY-2157299 only, Stattic only or LY-2157299 and Stattic. LY-2157299 was administered twice daily at 100 mg/kg doses, whereas Stattic was fed once daily at 20 mg/kg doses. Mice cohorts were treated for a total of 21 days in this treatment regimen with no observable side effects.

SDS–PAGE and immunoblotting

Lysates were prepared from cells using modified RIPA buffer as described previously (24) with the addition of protease and phosphatase inhibitors according to manufacturer's instructions (Thermo Fisher Scientific). Protein concentrations were then quantified by bicinchoninic acid method, subjected to SDS–PAGE and analyzed by immunoblotting as described previously (24).

RNA extraction and quantitative RT-PCR

Total RNA was isolated from cells using RNAeasy Kit (Qiagen) according to manufacturer's instructions. Equal amounts of RNA were then reverse transcribed using SuperScript III First-Strand Synthesis Kit (Invitrogen) with random hexamers as primers. cDNA samples were then subjected to qRT-PCR analysis with
SYBR Green in a BioRad CFXConnect thermocycler. List of primers used are detailed in Supplementary Figures.

RNA sequencing of sorted cell populations
RNA sequencing experiments were performed by the Genomics, Epigenomics, and Sequencing Core in University of Cincinnati. Briefly, RNA from sorted cells was isolated using mirVana miRNA Isolation Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions for total RNA isolation. Targeted RNA enrichment was achieved using NEBNext Poly(A) mRNA Magnetic Isolation Module (New England BioLabs) and PrepX mRNA Library Kit (WaferGen) combined with Apollo 324 NGS automated library prep system was used for library preparation. Cluster generation and HiSeq sequencing were carried out using the cBot and HiSeq systems (Illumina), respectively. To analyze differential gene expression, sequence reads were aligned to the genome using standard illumina sequence analysis pipeline, which was analyzed by The Laboratory for Statistical Genomics and Systems Biology in the University of Cincinnati.

IHC
Formalin-fixed paraffin-embedded sections were sectioned (5 μm) and stained for respective antigens as described previously (14). For antigen retrieval, slides were heated in citrate buffer in a pressure cooker.

Statistical analysis
Data were plotted as means ± SEM and statistical significance was determined using a two-tailed t test. Statistical differences between groups for limiting dilution transplants were performed using ELDA as described previously (25). For tumor growth curves, analysis of covariance (ANCOVA) test was used to determine significance. The threshold for significance of P values was 0.05.

Results
ALDH⁺ and CD29⁺CD61⁺ markers identify distinct BCSC populations in mammary tumors
The putative BCSC markers ALDH⁺ and CD29⁺CD61⁺ have been reported to enrich for CSC populations in mouse mammary tumors (24, 26, 27). Nonetheless, there is evidence to suggest that BCSCs with different characteristics can coexist within breast tumors (10). For that reason, we set out to address whether ALDH⁺ and CD29⁺CD61⁺ populations identify identical overlapping subsets of CSCs or they are separate entities within mammary tumors. First, we employed Lin⁻/CD24⁻/CD29⁺/CD61⁺ as a marker to analyze BCSC content in the MMTV-PyMT and MMTV-Wnt1 models, which represent luminal and basal-like subtypes of breast cancer, respectively (28). Under similar gating criteria, we found a higher fraction of BCSCs (i.e., % Lin⁻/CD24⁻/CD29⁺/CD61⁺) only, in sorted cells from both tumor populations, identifying distinct overlapping subsets of MECs that coincide within the MSC and ML enriched gate. On the other hand, MMTV-Wnt1 tumors are comprised mostly of cells that coincide within the MSC and ML gates (Fig. 1B). We next used Lin⁺/CD24⁺/ALDH⁺ as a BCSC marker to analyze both tumor types and found that consistent with results using Lin⁻/CD24⁻/CD29⁺/CD61⁺, MMTV-Wnt1 tumors contained higher amounts of ALDH⁺ cells than MMTV-PyMT tumors (Fig. 1C). However, surprisingly, we found that ALDH⁺ and CD29⁺CD61⁺ populations only overlap to a small degree in either of these two tumors (Fig. 1D).

Although numerous markers and criteria have been described to enrich for BCSCs in mouse mammary tumors, these markers have not been studied concurrently. Our observation that ALDH⁺ and CD29⁺CD61⁺ populations overlap only minimally led us to address whether both these populations are indeed enriched for BCSCs or perhaps just the overlapping fraction (i.e., ALDH⁺/CD29⁺CD61⁺) have BCSC activity. For that, we sorted cells from freshly harvested tumors into four fractions termed P1 (CD29⁺CD61⁺ only), P2 (ALDH⁺ only), P3 (ALDH⁺ and CD29⁺CD61⁺), and P0 (bulk of ALDH⁺, non CD29⁺CD61⁺) based on the sorting strategy described in Supplementary Fig. S1. Through limiting dilution transplantation of these populations, we found that in MMTV-PyMT tumors, P1, P2, and P3 populations have increased tumor-initiating ability relative to P0, which are the bulk of tumor cells (Fig. 1E). This shows that both ALDH⁺ and CD29⁺CD61⁺ populations overlap only minimally among tumors that form from respective sorted populations also display continuous activation of BCSC-associated pathways, namely, p-Stat3 (Supplementary Fig. S3A) and p-Smad2 (Supplementary Fig. S3B). Contrastingly, in Wnt1-driven tumors, we found that the CD29⁺CD61⁺ population was most tumorigenic, whereas the ALDH⁺ population was least tumorigenic (Fig. 1F). However, the tumors that developed in the recipient mice also showed similar features as examined by histology, tumor growth, and flow cytometry profiles of parental tumors (Supplementary Fig. S2A–S2D). The tumors that form from respective sorted populations also display continuous activation of BCSC-associated pathways, namely, p-Stat3 (Supplementary Fig. S3A) and p-Smad2 (Supplementary Fig. S3B). Contrastingly, in Wnt1-driven tumors, we found that the CD29⁺CD61⁺ population was most tumorigenic whereas the ALDH⁺ population was least tumorigenic (Fig. 1F). However, the tumors that developed in the recipient mice also showed similar features as examined by histology, tumor growth, and flow cytometry profiles of parental tumors (Supplementary Fig. S2A–S2D). These results demonstrate that ALDH⁺ and CD29⁺CD61⁺ markers identify distinct populations of BCSCs in MMTV-PyMT tumors, whereas mammary tumors driven by the Wnt1 oncogene primarily contain BCSCs enriched by CD29⁺CD61⁺ but ALDH⁺ is not a suitable marker for this subtype of breast cancer.

CD29⁺CD61⁺ tumor-initiating cells have enhanced invasive and mesenchymal properties
As ALDH⁺ and CD29⁺CD61⁺ both enriched for BCSCs in MMTV-PyMT tumors, we further investigated whether these two distinct BCSC populations have different characteristics. ALDH⁺ and CD29⁺CD61⁺ enriched BCSCs as well as those cells positive for both sets of markers showed no statistical differences for Ki67 staining, but all these three populations had less actively cycling cells compared with the bulk tumor cells (Fig. 2A). In contrast to the comparable lower proliferation rates for both ALDH⁺ and CD29⁺CD61⁺ BCSCs, CD29⁺CD61⁺ (as well as
Figure 1.
CD29⁺CD61⁺ and ALDH⁺BCSC populations overlap minimally in mouse models of breast cancer. A, dot plots showing the CD29 CD61 profile of Lin⁻CD24⁺ cells from PyMT and Wnt tumors and the overlay of both profiles. B, contour plots showing the CD29 CD61 profile of normal MECs, with gates identifying distinct MSC-enriched, luminal progenitor (LP) and mature luminal (ML) populations, alongside contour plots of PyMT and Wnt tumors indicating the CD29 CD61 distribution of these tumor cells with respect to the gates from normal MECs. C, dot plots of gated Lin⁻CD24⁺ALDH⁺ populations in PyMT and Wnt tumors relative to respective negative control (+DEAB). D, CD29 CD61 profiles of P1, CD29⁺CD61⁺ cells (red), P2, ALDH⁺ cells (green), and P3, ALDH⁺CD29⁻CD61⁺ cells (yellow) in PyMT and Wnt tumors. Bar charts show the percentage of respective populations from at least n = 6 tumors for each cohort. Limiting dilution transplants of sorted P0: ALDH⁻non-CD29⁻CD61⁺, P1: ALDH⁻CD29⁺CD61⁺, P2: ALDH⁺non-CD29⁻CD61⁺, and P3: ALDH⁺CD29⁺CD61⁺ cells from PyMT (E) or Wnt tumors (F). Freshly isolated PyMT or Wnt tumor cells were sorted and orthotopically transplanted into the fourth mammary glands of athymic nude mice. The frequency of tumor formation after 3 months was recorded and CSC frequency was calculated using ELDA software. Statistical significance was determined by pairwise χ² test of respective groups against P0; *, P ≤ 0.05; **, denotes P ≤ 0.01.
ALDH⁺CD29hiCD61⁺ BCSCs exhibited increased invasive abilities (Fig. 2B). Analysis of these populations for factors important in determining epithelial and mesenchymal features showed that the reduced proliferation of BCSCs (compared with the bulk tumor cells) correlated with the decreased E-cadherin levels, whereas the increased invasiveness of CD29hiCD61⁺ BCSCs correlated with higher vimentin levels and increased Twist1 expression (Fig. 2C). In Wnt1-driven tumors, CD29hiCD61⁺ BCSCs (P1) showed a significant reduction in proliferation, and also exhibited increased invasion, compared with the bulk tumor cells (P0; Fig. 2D and E). No difference in E-cadherin expression was found, but CD29hiCD61⁺ BCSCs showed increased vimentin and another EMT-inducing factor Slug (Fig. 2F). The ALDH⁺CD29hiCD61⁺ population (P3) in

Figure 2.
CD29hi CD61⁺ CSCs exhibit increased invasiveness and expression of EMT-associated genes. Freshly isolated tumor cells were sorted into P0: ALDH⁺ non-CD29⁺CD61⁺, P1:ALDH⁺ CD29hiCD61⁺, P2: ALDH⁺ non-CD29⁺CD61⁺, and P3: ALDH⁺ CD29hiCD61⁺ populations. PyMT (A) or Wnt (D) sorted populations were plated overnight under normal culture conditions and stained for Ki67 via IHC and the percentage of positive cells were quantified. PyMT (B) or Wnt (E) sorted populations were seeded in Matrigel-coated Boyden chambers for 24 hours and the number of cells that invaded were quantified. Sorted populations from PyMT (C) or Wnt (F) tumors were analyzed via qRT-PCR for gene expression levels of E-cadherin, vimentin, Twist1, Twist2, Snail, and Slug. Statistical significance was determined by two-tailed t test; *, P ≤ 0.05; **, P ≤ 0.01. G, RNA-sequencing data showing correlation of differentially expressed genes between sorted populations with genes differentially expressed in cells within the mammary epithelial hierarchy published in ref. 28.
Figure 3.
Ablation of Fip200 reduces the proportion of CD29hiCD61+ cells and Aldh1a3 transcript levels in PyMT tumors. Dot plots showing gated ALDH+ (A) or CD29hiCD61+ (B) populations from freshly isolated Ctrl-MT (Fip200F/F, PyMT) or cKO-MT (Fip200F/F, MMTV-Cre, PyMT) tumors. Bar charts show percentage marker positive cells (n = 5 tumors for each group). Dot plots showing gated ALDH+ (C) or CD29hiCD61+ (D) populations from Fip200F/F, PyMT, Cre-ER treated with vehicle control (VC) or 4-hydroxytamoxifen (4-OHT). Bar charts show percentage marker positive cells (n = 6 for each group). E, table showing limiting dilution transplants of sorted P0: ALDH+/C0 non-CD29hiCD61+, P1: ALDH+/C0 CD29hiCD61+, and P2: ALDH+ non-CD29hiCD61+ populations from Ctrl-MT and cKO-MT cells. Ctrl-MT and cKO-MT cells were sorted and orthotopically transplanted into the fourth mammary glands of athymic nude mice. The frequency of tumor formation after 3 months was recorded and CSC frequency was calculated using ELDA software. Statistical significance was determined by pairwise \( \chi^2 \) test between Ctrl-MT and cKO-MT of respective populations; *, \( P < 0.05 \); **, \( P < 0.001 \). F, qRT-PCR analysis of Aldh1a1, Aldh1a3, and Aldh2 transcript levels from Ctrl-MT (vehicle-treated FIP200+/+, PyMT, Cre-ER) or cKO-MT (4-OHT–treated FIP200+/+, PyMT, Cre-ER) cells. Data points represent n = 6 for each group. G, histograms showing ALDH positivity in Ctrl-MT (vehicle-treated FIP200+/+, PyMT, Cre-ER) or cKO-MT (4-OHT–treated FIP200+/+, PyMT, Cre-ER) cells with or without 100 \( \mu \)mol/L Daidzin (Aldh2 inhibitor) treatment along with respective +DEAB negative controls. The proportion of ALDH+ cells relative to vehicle controls were quantified for Ctrl-MT and cKO-MT cells (n = 6 per group). Statistical significance was determined by two-tailed t-test; *, \( P < 0.05 \); **, \( P < 0.001 \).
Wnt tumors also exhibited invasive and mesenchymal features (Fig. 2E and F) but were not enriched in BCSCs (Fig. 1F), possibly representing an intermediate between CD29\(^{hi}\)CD61\(^{+}\)BCSCs (P1) and non CSCs.

To characterize the different BCSC populations in PyMT tumors in more detail, we performed RNA-sequencing experiments to identify differentially expressed genes between CD29\(^{hi}\)CD61\(^{+}\)BCSCs (P1), ALDH\(^{+}\)BCSCs (P2), and the bulk tumor cells (P0). Interestingly, the differentially expressed genes illustrate a hierarchical difference between these populations, similar to differences observed in the hierarchy of normal MECs (28).

Comparison of CD29\(^{hi}\)CD61\(^{+}\) BCSCs (P1) with bulk tumor cells (P0) identifies the P1 population as being more MSC like (Fig. 2G). On the other hand, ALDH\(^{+}\) BCSCs (P2) had a gene expression that is more associated with both MSC and LPs when compared with P0 (Fig. 2G), suggesting that these cells may have features associated with primitive LPs. Direct comparison of the two BCSC populations, P2 with P1, indicates that P2 BCSCs are more LP like and P1 BCSCs are more MSC-like (Fig. 2G). Altogether, these findings illustrate the existence of distinct BCSC populations with differing hierarchical/EMT states in PyMT tumors.

Figure 4.
Fip200 depletion diminishes TGFβ/Smad signaling that is necessary for the CD29\(^{hi}\)CD61\(^{+}\) CSC phenotype. A, qRT-PCR analysis of Tgfb1, Tgfb2, and Tgfb3 transcript levels from Ctrl-MT or cKO-MT cells. Data points represent n = 6 for each group. B, immunoblots showing p-Smad2, Smad2/3, and actin levels in Ctrl-MT and cKO-MT cells. C, immunoblots showing levels of Smad4 and actin in Ctrl-MT cells stably transduced with nontarget shRNA, sh1 Smad4, or sh2 Smad4. D, dot plots showing CD29 CD61 profiles of Ctrl-MT cells stably transduced with nontarget shRNA, sh1 Smad4 or sh2 Smad4 along with quantification. E, dot plots showing ALDH activity of Ctrl-MT cells stably transduced with nontarget shRNA, sh1 Smad4, or sh2 Smad4 along with quantification. F, bar charts showing the levels of Aldh1a3 isoforms in Ctrl-MT cells stably transduced with nontarget shRNA, sh1 Smad4, or sh2 Smad4. G, dot plots showing CD29 CD61 profiles of Ctrl-MT or cKO-MT cells treated with vehicle control (red) or 10 ng/mL TGFβ (cyan) for 72 hours. Quantification of % CD29\(^{hi}\)CD61\(^{+}\) cells (H) and % ALDH\(^{+}\) cells (I) in Ctrl-MT or cKO-MT cells treated with vehicle control or 10 ng/mL TGFβ for 72 hours. Statistical significance was determined by two-tailed t test; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
mesenchymal and invasive, whereas ALDH+ BCSCs were more closely associated with LPs and relatively less invasive.

**Autophagy inhibition by FIP200 deletion impairs the tumor-initiating potential of ALDH+ and CD29hiCD61+ CSCs in MMTV-PyMT tumors**

Autophagy has been shown to play both tumor-suppressive and tumor-promoting functions under different contexts (12). We recently found that inhibition of autophagy by deletion of FIP200 (Rb1cc1), an essential gene for autophagy induction, decreased tumor development, and progression in MMTV-PyMT–driven mammary tumors (14). To examine whether autophagy contributes to the tumorigenicity of these tumors through regulation of the two BCSC populations with differing characteristics, we analyzed ALDH+ and CD29hiCD61+ BCSCs in MMTV-PyMT tumors with conditional knockout of FIP200 driven by MMTV-Cre (herein referred to as cKO-MT tumors; ref. 14). We found comparable levels of ALDH+ cells in Ctrl-MT and cKO-MT tumors (Fig. 3A), suggesting that autophagy is not important in the maintenance of ALDH+ BCSCs. However, a significant decrease in CD29hiCD61+ BCSCs was observed in cKO-MT tumors (Fig. 3B). To ensure that the reduced BCSC content was caused by intrinsic defects of FIP200-null tumor cells, we also examined our newly developed FIP200f/f;PyMT;CreER mammary tumor cells (23) following 4-OHT treatment to induce FIP200 deletion in vitro. The degree of autophagy inhibition in 4-OHT–treated cells is illustrated by p62 accumulation and inhibition of LC3 I conversion to LC3 II under both normal and sphere culture conditions (Supplementary Fig. S4). We found that deletion of FIP200 in these cells did not affect the percentage of ALDH+ BCSCs, but significantly reduced CD29hiCD61+ BCSCs compared with the isogenic (vehicle-treated) control cells (Fig. 3C and D).

To further investigate whether FIP200 deletion affects self-renewal and tumorigenicity of BCSCs in vivo, ALDH+, CD29hiCD61+ or bulk cKO-MT (i.e., FIP200f/f;PyMT;CreER mammary tumor cells treated with 4-OHT) and Ctrl-MT (i.e., vehicle-treated FIP200f/f;PyMT;CreER mammary tumor cells) tumor cells were transplanted at limiting dilutions into mammary fat pads of recipient nude mice and monitored for tumorigenesis. Whereas ALDH+ and CD29hiCD61+ BCSCs from Ctrl-MT mice generated tumors in the majority of recipient mice, ALDH+ and CD29hiCD61+ BCSCs from cKO-MT mice induced tumor formation in only a small fraction of recipients (Fig. 3E). Bulk tumor cells from cKO-MT and Ctrl-MT mice also only generated tumors in a smaller fraction of recipients, although a statistically significant reduction was also observed in cKO-MT tumor cells. These results indicate that autophagy plays a role in regulating the tumorigenicity of both ALDH+ and CD29hiCD61+ BCSC populations.

It was interesting to note that FIP200 deletion did not alter the percentage of ALDH+ cells but it decreased the tumor-initiating potential of this population (Fig. 3A, C, and E). One possible explanation for this observation is that ALDH+ BCSC characteristics but not ALDH enzymatic activity is dependent on FIP200. However, in breast cancers and normal mammary LP cells, the predominant ALDH isoform expressed is Aldh1a3 and the expression of this specific isoform has been implicated with BCSC traits (29–31). In the Aldefluor assay that we used to sort ALDH+ populations, cells were distinguished on the basis of the enzymatic activity of ALDH isoforms that are sensitive to the inhibitor DEAB. There is evidence to indicate that the Aldefluor assay can also detect ALDH2 activity and this lack of specificity may explain the discrepancy we observed (32). Indeed, we found that FIP200 deletion significantly decreased Aldh1a3 mRNA levels (Fig. 3F) and this correlated with the decreased tumor-initiating potential of ALDH+ cells from cKO-MT tumors (Fig. 3E). Conversely, Aldh2 levels were increased (Fig. 3F) and contributed significantly to Aldefluor assay activity, specifically in cKO-MT tumors (Fig. 3G). This is illustrated by the reduction of Aldefluor activity in the presence of an Aldh2 inhibitor, Daidzin, in cKO-MT but not Ctrl-MT cells. Together, these results demonstrate that inhibition of autophagy by FIP200 deletion reduced the CSC-associated isoform of ALDH (Aldh1a3) and tumor-initiating potential of ALDH+ BCSCs.

**FIP200 deletion diminishes TGFβ/Smad signaling that is necessary for the CD29hiCD61+ CSC phenotype**

Given the recent studies suggesting a role of EMT in promoting characteristics of BCSCs (8), we wondered whether autophagy may regulate CD29hiCD61+ BCSCs through EMT-inducing pathways. To examine such a possibility, we explored potential changes in TGFβ signaling, which is a well-described EMT-inducing pathway. We found that while TGFβ1 exhibited comparable levels in cKO-MT and Ctrl-MT cells, TGFβ2 and TGFβ3 mRNA levels were significantly decreased in cKO-MT cells (Fig. 4A).

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**Table 1. Limiting dilution transplants of sorted P0: ALDH+ non-CD29hiCD61+, P1: ALDH+ CD29hiCD61+, and P2: ALDH+ non-CD29hiCD61+ populations from Ctrl-MT cells transduced with either nontarget shRNA, shStat3 or shSmad4**

<table>
<thead>
<tr>
<th>PI: CD29hiCD61+</th>
<th>500 Cells</th>
<th>1,000 Cells</th>
<th>2,000 Cells</th>
<th>Estimated stem cell frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontarget</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>1/169 (1/696–1)</td>
</tr>
<tr>
<td>shStat3</td>
<td>1/3</td>
<td>3/3</td>
<td>3/3</td>
<td>1/153 (1/1,322–1/213)</td>
</tr>
<tr>
<td>shSmad4</td>
<td>1/3</td>
<td>3/3</td>
<td>3/3</td>
<td>1/169 (0.291–1/469)</td>
</tr>
</tbody>
</table>

| P2: ALDH+       | 2/3       | 3/3         | 3/3         | 1/154 (1/955–1/125)         |
| shStat3         | 0/3       | 1/3         | 1/3         | 1/4.453 (1/7,891–1/190)     |
| shSmad4         | 2/3       | 3/3         | 3/3         | 1/609 (1/1,492–1/249)       |

| P0: Bulk        | 1/3       | 2/3         | 2/3         | 1/325 (1/5,378–1/520)       |
| shStat3         | 1/3       | 2/3         | 1/3         | 1/998 (1/5,645–1/708)       |
| shSmad4         | 0/3       | 1/3         | 1/3         | 1/4.453 (1/7,891–1/1309)    |

**NOTE:** Ctrl-MT cells transduced with either nontarget shRNA, shStat3 or shSmad4 cells were sorted and orthotopically transplanted into the fourth mammary glands of athymic nude mice. The frequency of tumor formation after 3 months was recorded and CSC frequency was calculated using ELDA software. Statistical significance was determined by pairwise χ² test between nontarget shRNA and respective knockdowns.

*p < 0.01.
*<sup>pp</sup> < 0.001.
Moreover, we observed a concomitant decrease in Smad2/3 activation in FIP200-null tumor cells compared with control tumor cells (Fig. 4B). To further validate the role of TGFβ/Smad signaling in the regulation of the distinct BCSC populations, we silenced Smad4 in control tumor cells and found that the percentage of CD29hiCD61+ BCSCs was diminished (Fig. 4C and D). The tumor-initiating potential of CD29hiCD61+ BCSCs was also impaired upon Smad4 knockdown, as indicated by limiting dilution transplant experiments (Table 1). Interestingly, knockdown of Smad4 did not alter the percentage of ALDH+ cells, Aldh1a3 transcript levels or their tumor-initiating potential significantly (Fig. 4E and F; Table 1). Addition of recombinant TGFβ restored the decreased content of CD29hiCD61+ BCSCs in cKO-MT cells to a comparable level as in Ctrl-MT cells (Fig. 4G–I), suggesting that deficient TGFβ signaling after FIP200 deletion is responsible for the reduced CD29hiCD61+ BCSCs in these cells. Consistent with a lack of effect of Smad4 knockdown on Aldh1a3 expression, treatment with recombinant TGFβ did not increase the ALDH+ population (but rather decreased it slightly; Fig. 4I). These results suggest that autophagy plays an important role in the maintenance of CD29hiCD61+ BCSCs but not ALDH+ BCSCs through TGFβ/Smad signaling.

FIP200 deletion impairs Stat3 activation, which is essential for the tumor-initiating potential of ALDH+ cells

We next investigated whether altered Stat3 signaling is responsible for the decreased tumorigenicity of ALDH+ BCSCs upon deletion of FIP200, as both the BCSC properties and Aldh1a3 levels of ALDH+ BCSCs can be regulated by Stat3 signaling (33, 34). In FIP200 cKO-MT cells, we observed decreased p-Stat3 levels (Fig. 5A), suggesting that FIP200 is required to sustain Stat3 activation (Fig. 4B) and ALDH+ BCSCs in these cells. We found that knockdown of Egfr led to decreased p-Stat3 and Aldh1a3 transcript levels, indicating that Egfr is a major stimulus for Stat3 activation in these cells (Fig. 5B and C). Moreover, ectopic expression of wild-type Egfr in cKO-MT cells was sufficient to elevate Stat3 phosphorylation and Aldh1a3 transcript levels (Fig. 5D and E). The requirement for Stat3 in the regulation of Aldh1a3 and tumorigenicity of ALDH+ BCSCs was also demonstrated by silencing of Stat3 in Ctrl-MT cells, where cells transduced with sh1 Stat3 led to significant reductions in Aldh1a3 transcript levels (Fig. 5F and G) and tumor-initiating potential in vivo (Table 1). Together, these results suggest that deficient Stat3 signaling is responsible for the reduced tumor-initiating capacity of ALDH+ BCSCs upon FIP200 deletion.

Combination targeting of distinct BCSCs with Stat3 and TGFβR inhibitors enhances therapeutic outcomes

Our above observations indicate that ALDH+ and CD29hi CD61+ BCSCs that coexist in MMTV-PyMT tumors depend on EGFR/Stat3 and TGFβ/Smad signaling, respectively. These findings have important implications because the differential dependence could lead to therapeutic resistance and tumor relapse if both populations are not effectively eliminated. As such, we went on to address whether combinatorial targeting of ALDH+ and CD29hiCD61+ BCSCs with Static (Stat3 inhibitor) and LY-2157299 (TGFβR1 inhibitor) can lead to better therapeutic responses. From colony-forming assays, we found that...
LY-2157299 in combination with Static led to a greater reduction in the number of colonies formed when compared with either inhibitor alone (Fig. 6A). Interestingly, when the types of colonies that formed were analyzed (Fig. 6B), we found that LY-2157299 treatment resulted in formation of mostly epithelial colonies, whereas Static treatment promoted the formation of mesenchymal colonies (Fig. 6C). This observation is in line with our finding that TGFβ/Smad signaling promotes characteristics of mesenchymal BCSCs (CD29<sup>hi</sup>CD61<sup>+</sup>) and Egfr/Stat3 regulates the epithelial BCSC population (ALDH<sup>b</sup>).

In a preclinical setting, the effects of combining these two inhibitors were examined in vivo by treating transplanted PyMT tumors in nude mice when the size of tumors was about 50 mm<sup>3</sup> (Fig. 6D). Administration of either LY-2157299 or Static alone did not result in significant reductions in tumor volume (Fig. 6E). However, the combination of both inhibitors impaired tumor growth significantly (Fig. 6F). After 21 days of treatment, the percentage of CD29<sup>hi</sup>CD61<sup>+</sup> and ALDH<sup>b</sup> BCSCs were analyzed. Tumors treated with LY-2157299 or the combination of inhibitors significantly reduced the percentage of CD29<sup>hi</sup>CD61<sup>+</sup> BCSCs (Fig. 6F), an effect not seen in tumors treated with Static alone. On the other hand, only cohorts that received Static or the combination of drugs were effective in significantly reducing ALDH<sup>b</sup> BCSCs (Fig. 6G). Tumors that were treated with Static displayed diminished p-Stat3 staining, whereas tumors treated with LY-2157299 had diminished p-Smad2 staining, illustrating the efficacy of respective inhibitors at the doses administered (Fig. 6H). These results indicate that the combination of LY-2157299 and Static may be more effective due to its ability to target both CD29<sup>hi</sup>CD61<sup>+</sup> and ALDH<sup>b</sup> BCSCs, which depend on TGFβ/Smad and Stat3 signaling, respectively (Fig. 6I).

**Discussion**

The proposed CSC concept has played a significant role in the recent advance of cancer research, despite concerns on their existence or utility in some cancers (7, 36, 37). In this study, we describe for the first time that ALDH<sup>b</sup> and CD29<sup>hi</sup>CD61<sup>+</sup>, two widely used markers for BCSCs, enrich for two distinct BCSC populations with minimal overlap in one mouse model of breast cancer, but that only CD29<sup>hi</sup>CD61<sup>+</sup> markers enriched for BCSCs in another model. These results suggest that various markers (even those frequently employed) may not identify all CSCs, which may contribute to some potential conflicting data concerning the existence and/or role of CSCs.

Although sharing some common features like reduced proliferation compared with bulk tumor cells, CD29<sup>hi</sup>CD61<sup>+</sup> and ALDH<sup>b</sup> BCSCs displayed differential properties with CD29<sup>hi</sup>CD61<sup>+</sup> BCSCs exhibiting increased invasive activity and expression of mesenchymal markers. Interestingly, these distinct BCSC populations have expression profiles that are associated with MSCs and LPs, respectively. The fact that both BCSC populations have expression profiles associated with less differentiated cells in the mammary cell hierarchy suggests that a dedifferentiation process confers increased tumor-initiating potential, consistent with the CSC hypothesis. However, due to different degrees of dedifferentiation (i.e., MSC and LP), it is possible that BCSCs with differing properties may exist within a tumor. These findings may have potential clinical implications because it suggests that cell populations with luminal and basal-like characteristics, respectively, may coexist and that multiple disease subtypes that require different treatment modalities may be present within a single tumor. Accordingly, the two BCSC populations were found to be differentially reliant on separate signaling pathways. Stat3 inhibition either through genetic or pharmacologic means decreased the ALDH<sup>b</sup> BCSCs and their tumor-initiating activities, but not CD29<sup>hi</sup>CD61<sup>+</sup> BCSCs, in MMTV-PyMT tumors. This agrees with previous findings implicating a role for Stat3 in ALDH<sup>b</sup> BCSCs of breast and non–small cell lung tumors (33, 34, 38). Conversely, CD29<sup>hi</sup>CD61<sup>+</sup> BCSCs, but not ALDH<sup>b</sup> BCSCs, were preferentially inhibited by interfering with the TGFβ/Smad pathway in MMTV-PyMT tumors. It is notable that the markers used to isolate BCSCs with more mesenchymal features in this study were CD29 (β1-integrin) and CD61 (β3-integrin). The CD29 expression levels distinguish high-expressing basal cells from low-expressing luminal cells. While CD61 has been initially characterized as a LP marker in normal MECs of virgin female mice (27), more recently CD29<sup>hi</sup>CD61<sup>+</sup> cells have been shown to be pregnancy-associated MSCs (39). Apart from being a putative BCSC marker in mouse models (24, 26, 27), CD61 is a functional marker where its expression has been shown to be regulated by TGFβ2 and it contributes to BCSC properties through regulation of the EMT-associated transcription factor Slug (39). Therefore, the dependence of CD29<sup>hi</sup>CD61<sup>+</sup> BCSCs on the TGFβ/Smad pathway could possibly be due to the role of CD61 in regulating EMT-associated pathways and stemness.
In further support of the different characteristics of the ALDH− and CD29+/CD61− BCSCs, we found that while it affected both BCSCs, which is consistent with a number of previous reports (20, 21, 40), inhibition of autophagy by FIP200 depletion compromised ALDH+ and CD29+/CD61+ BCSCs through defects in the Stat3 and TGFβ/Smad pathways, respectively. Regulation of Stat3 by autophagy in breast cancer cells have been previously reported and this can be mediated by IL6 secretion that is controlled by autophagy-related proteins (18). However, secretion of IL6 was increased (data not shown), despite reduced p-Stat3 in FIP200-null MMTV-PyMT tumor cells. This indicates that the regulation of IL6 secretion is context dependent and indeed this was shown in a recent study comparing the effects of Atg7 or Beclin1 knockdown in two breast cancer cell lines (40). In MCF7 cells, silencing of autophagy genes led to increased IL6 secretion, whereas in MDA-MB-468 cells, the reverse was observed. It is also worth noting that AnG deletion in HRasG12V-transformed immortalized baby mouse kidney epithelial cells (iBMK) led to increased IL6 secretion in the presence of inflammatory stimuli (41). In the current study, however, the increased IL6 secretion upon FIP200 deletion does not contribute significantly to Stat3 phosphorylation and we can attribute this to low levels of IL6RA expression. On the other hand, we found that autophagy inhibition reduces Stat3 activation through decreased EGFR levels in MMTV-PyMT tumor cells. It is possible that autophagy inhibition leads to accumulation of the autophagy adapter protein Cbl, a well-characterized ubiquitin ligase that promotes lysosomal degradation of EGFR (42, 43), resulting in enhanced EGFR degradation and a consequent decrease in Stat3 activation after autophagy inhibition.

Several lines of evidence have implicated a role for autophagy in the regulation of EMT at separate levels. In hepatocellular carcinoma cell lines, starvation-induced autophagy could increase TGFβ levels which is required for induction of EMT and invasiveness (16). Apart from regulating TGFβ cytokine levels, silencing of autophagy-related genes has also been shown to impair TGFβ-induced expression of vimentin and human BCSCs enriched by CD24+/CD44+/CD0− markers (29). In MMTV-PyMT tumor cells, we observed that FIP200 deletion led to decreased TGFβ2 and TGFβ3 expression, suggesting that FIP200 regulates the TGFβ/Smad pathway at least in part through TGFβ2 and TGFβ3 transcript levels. The fact that TGFβ1 levels were not affected upon FIP200 deletion in these cells suggests that autophagy governs the activity of a transcription factor that can bind to TGFβ2 and TGFβ3 promoters but not the TGFβ1 promoter. In view of that, cyclic AMP-responsive elements (CRE) which can be bound by CREB/Atf1 transcription factors have been described in promoter regions of just TGFβ2 and TGFβ3 but not TGFβ1 (44). It will be interesting to investigate these intermediate mechanisms as cross-regulation between autophagy and CREB is possible (45).

We have shown that autophagy can regulate the separate pathways utilized by distinct BCSCs but it does not occur at critical nodes within the respective signaling cascades. From the mechanisms by which autophagy regulate BCSCs that we have shown in this study, it is possible that some redundancies may exist. For instance, paracrine TGFβ secretion by other cell types within the tumor microenvironment may be sufficient to bypass the need for autophagy in CD29+/CD61− BCSCs. As for the Stat3 pathway, activation by IL6 rather than EGFR may compensate for the maintenance of ALDH+ BCSCs. For that reason, we explored the potential of combining inhibitors which can target nodes in the TGFβ and Stat3 pathways, respectively. As we showed that ALDH+ BCSCs were more epithelial and dependent on Egrf/Stat3 signaling, it is coherent that Static treatment led to decreased epithelial colonies and an increased amount of mesenchymal colonies in colony-forming assays in vitro. On the other hand, LY-2157299 was effective in diminishing the number of mesenchymal colonies, concordant with our findings that CD29+/CD61+ BCSCs are dependent on the TGFβ/Smad pathway. The heterogeneity that is present due to the existence of distinct BCSCs is likely to exacerbate therapeutic resistance. This is illustrated by the limited effectiveness of either LY-2157299 or Static alone on both BCSC populations. The decreased tumor growth in vivo and reduction of both ALDH+ as well as CD29+/CD61+ BCSCs was only achieved when LY-2157299 was used in combination with Static.

Recent studies in human breast cancers showed that CD24+/CD44− BCSCs are associated with the basal-like phenotype, whereas ALDH+ cells correlate with luminal tumor subtypes (10, 48). Moreover, CD24+/CD44− BCSCs also display EMT-associated features in human breast tumors (10). Although CD24+/CD44− markers are not applicable in mouse models (49), the CD29+/CD61− markers we used in this study enriches for a BCSC population that is more invasive and express higher levels of EMT-associated genes. We also found that this population can expand after TGFβ treatment, suggesting that it is a mesenchymal BCSC population similar to CD24+/CD44− BCSCs described in human tumors and CD29+/CD61− (48) can be utilized in immune-competent mouse models of breast cancer to identify mesenchymal BCSCs. On the other hand, while several previous reports indicate that IL6/Stat3 signaling can regulate the more mesenchymal CD24+/CD44− BCSC population in human breast cancer cells (9, 35), we only observed a marginal effect on the tumorigenicity of the CD29+/CD61− population after Stat3 knockdown, albeit not significantly. On the basis of our observations that Stat3 activation is sustained by EGFR and not through IL6/Jak2 in MMTV-PyMT tumor cells, it is possible that these differences are due to additional IL6 downstream signals that are not induced by the EGFR pathway. We also cannot exclude the possibility that CD29+/CD61− cells are not a direct correlate for CD24+/CD44− cells, despite both of these populations being mesenchymal BCSCs.

BCSCs, defined as cells which are less differentiated and more tumorigenic than the bulk population, can possibly represent...
Regulation of Distinct Breast Cancer Stem Cells by Autophagy

distinct entities depending on the cell of origin and the degree as well as direction of dedifferentiation [7]. The characterization of these distinct BCSC populations in the MMTV-PyMT tumors provides support to the idea that multiple BCSC/progenitor-like populations with differing states on the EMT spectrum can be present and contribute to the heterogeneity within breast tumors [7]. To our knowledge, this is the first time in which distinct coexisting BCSC populations have been described in the highly metastatic MMTV-PyMT model and this allows the study of these distinct entities in an immune-competent host. Accordingly, this is advantageous when investigating immune system–modulated niches that regulate these heterogeneous BCSC populations and whether BCSCs exhibit unique immune tolerance or resistance to immune therapies (50).

On the whole, our findings support the notion that distinct BCSCs with differing characteristics and susceptibility to therapies can coexist within tumors. Accordingly, it would not be appropriate to consider CSCs as a single entity. While we have described ALDH1+ and CD29^hi CD61^+ BCSCs and their differences in PyMT tumors, we have not exhausted and addressed all reported BCSC markers in our models and such a systematic effort may unravel higher degrees of complexity. With a better understanding of the heterogeneity and the specific susceptibility of varying subpopulations, therapeutic combinations may then be utilized to prevent drug resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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Development of methodology: S.K. Yeo, S. Chen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.K. Yeo, J. Wen, S. Chen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.K. Yeo, J. Wen, S. Chen, J.-L. Guan
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Acknowledgments

The authors thank Dr. Yi Li of Baylor College of Medicine for his kind gift of MMTV-Wnt1 mice, Eli Lilly for providing LY-2157299, and the University of Cincinnati LAMS staff for their support. The authors appreciate the help from Glenn Doerman in preparation of figures. The authors are thankful to Drs. Cherran Wang, Shaogang Sun, and Hsin-Jung Wu for critical appraisal and suggestions in the preparation of this manuscript. The authors appreciate the assistance from the Laboratory for Statistical Genomics and Systems Biology as well as the Genomics, Epigenomics and Sequencing Core in University of Cincinnati funded by NIEHS grant P30-ES00696 to Shuk-Mei Ho.

Grant Support

The work in this study is funded by NIH grants R01CA150926 and R01CA163493 (J.-L. Guan).

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Received October 26, 2015; revised February 22, 2016; accepted March 7, 2016, published OnlineFirst April 13, 2016.

www.aacrjournals.org Cancer Res; 76(11) June 1, 2016 3409

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Cancer Res 2016;76:3397-3410. Published OnlineFirst April 13, 2016.

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