Breast cancer cells respond differentially to modulation of TGF-β2 signaling after exposure to chemotherapy or hypoxia


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Abstract

Intratumoral heterogeneity helps drive the selection for diverse therapy-resistant cell populations. In this study, we demonstrate the co-existence of two therapy-resistant populations with distinct properties that are reproducibly enriched under conditions that characterize tumor pathophysiology. Breast cancer cells that survived chemotherapy or hypoxia were enriched for cells expressing the major hyaluronic acid receptor CD44. However, only CD44<sup>hi</sup> cells that survived chemotherapy exhibited cancer stem cell (CSC) phenotypes based on growth potential and gene expression signatures that represent oncogenic signaling and metastatic prowess. Strikingly, we identified TGF-β2 as a key growth promoter of CD44<sup>hi</sup> cells that survived chemotherapy but a growth inhibitor of cells that survived hypoxia. Expression of the TGFβ receptor TGFβR1 and its effector molecule SMAD4 was required for enrichment of CD44<sup>hi</sup> cells exposed to the chemotherapeutic drug epirubicin, which suggests a feed-forward loop to enrich for and enhance the function of surviving CSCs. Our results reveal context-dependent effects of TGF-β2 signaling in the same tumor at the same time. The emergence of distinct resistant tumor cell populations as a consequence of prior therapeutic intervention or microenvironmental cues has significant implications for the responsiveness of recurring tumors to therapy.

Precis

Findings provide mechanistic insight into how chemotherapy-resistant cell populations emerge and illustrate the inherent flaws in any treatment strategy that does not address tumor heterogeneity as driven by genetic and epigenetic factors in tumor cells as well as the tumor microenvironment.
Introduction

Tumor cells that are resistant to therapy can fuel disease relapse and thus constitute a major barrier to achieving sustained clinical responses. Resistance is generally classified as intrinsic or developed and can be mediated by several mechanisms. Moreover, intratumoral heterogeneity may generate a diversity of resistance mechanisms that coexist within the same tumor. Sources of this heterogeneity include genetic and epigenetic changes, cellular plasticity, tumor microenvironment factors such as hypoxia, immune cell infiltration, exposure to therapeutic compounds, and stochastic changes in signal transduction and gene expression (1-4).

The cancer stem cell (CSC) model provides a mechanistic basis for intratumoral heterogeneity based on measurable phenotypes and thus constitutes a useful framework for studying therapeutic resistance. CSCs comprise the malignant subpopulation of cells in a tumor that drive tumor growth, metastasis and relapse (5,6). CSCs can be resistant to cytotoxic chemotherapies (1,7) and have also been observed adjacent to hypoxic regions of tumors, for example after antiangiogenic therapy (8,9). Recent studies have elucidated the coexistence of distinct CSC phenotypic states (10), though the factors that direct cells towards one state versus the other are not known.

Expression of the hyaluronic acid receptor CD44 is significantly enriched on CSCs in many tumor types (5,11,12). The tumor-forming capacity of cells isolated from breast tumors is defined in part by CD44 expression (11), and in breast cancer patients, the expression of CD44 in the tumor correlates with poor prognosis (13,14). In addition to its utility as a cell surface marker for CSCs, CD44 directly impacts tumor progression, cell proliferation and extracellular matrix organization (12,15). CD44 knockdown was previously shown to inhibit invasion,
spheroid formation, and tumor growth of MDA-MB-231 cells (16) and promote differentiation of primary breast cancer cells (17).

Transforming growth factor beta (TGFβ) has been implicated in CSC survival after chemotherapy and in the epithelial-mesenchymal transition (EMT), a process that has been associated with metastasis and CSCs (18,19). More generally, TGFβ signaling exerts pleiotropic and context-dependent effects on cancer cell growth. For example, TGFβ promotes cytostasis in normal mammary epithelial cells (20), and conversely the same signal promotes cell proliferation in late breast cancer (21). While the mechanisms that distinguish the pleiotropic effects of the signaling are not fully understood, the downstream effects of TGFβ can be modulated by many factors, including signals from the microenvironment, other oncogenic signaling pathways, and the expression of SMAD-binding coactivators and corepressors.

Here we identify conditions that reproducibly enrich for CSC-like populations that exhibit opposite responses to TGFβ signals. TGFβ promotes the regrowth of breast cancer cells after cytotoxic chemotherapy yet inhibits their regrowth after hypoxic stress. Moreover, pharmacological inhibition of TGFβ signaling elicits the opposite pattern of responses in the two cell populations. Since hypoxic regions of the tumor may not be accessible to chemotherapy, the distinct populations described here could coexist in tumors, with implications for the successful long-term treatment.
Materials and Methods

Cell Culture and Treatments

SKBR3 and MCF7 cells were authenticated by short tandem repeat analysis. SKBR3 cells were maintained in McCoy’s 5A Medium supplemented with 10% FBS and penicillin/streptomycin. MCF7 cells were maintained in Eagle’s Minimum Essential Medium supplemented with 10% FBS, 0.01 µg/mL insulin, 1% nonessential amino acids, and 1% sodium pyruvate. For FACS experiments, SKBR3 were treated with 60-90 nM epirubicin and MCF7 cells were treated with 150-200 nM, approximately IC₃₀-IC₅₀. For hypoxia experiments, cells were grown in 1% O₂ for 5-7 days. CD44 activity was stimulated with hyaluronic acid (HA) polymers of either 50K or 1000K size (Sigma) by adding 200 µg/mL to 3D cultures. CD44-HA interaction was blocked using the 5F12 antibody (Thermo Scientific) at 10-25 µg/mL. To examine TGFβ activity, cells were treated with 25 ng/mL TGFβ-2 or 1-2 µM SB431542 (R&D). Cells in 3D growth assays were treated by adding growth factor, HA, or drug to both Matrigel and media.

RNAi-mediated Knockdown

Cells were transduced with lentiviral particles containing specific shRNAs directed against CD44 or TGFβR1 (Sigma). Stably transduced cells were selected and grown in 2 µg/mL puromycin. SMAD4 siRNAs were transfected with Opti-MEM. Sequences are provided in Supplement.
Flow Cytometry

Cells were harvested using CellStripper nonenzymatic cell dissociation buffer (Mediatech) and resuspended in PBS with 3% BSA. Direct staining was performed for 30 min on ice, and CD44 was detected with a FITC-labeled G44-26 antibody (BD Biosciences). ALDH activity was determined by incubating cells with Aldefluor reagent (Stem Cell Technologies) according to manufacturer’s protocol for 1 hour at 37°C. Analysis was performed using a BD FACSCalibur, and sorting was performed using a BD FACSaria 3. Live, CD44^{hi}, and CD44^{-/lo} or Aldefluor^{hi} and Aldefluor^{-/lo} cells were isolated, spun at 1000 xg for 10 min and resuspended in media for subsequent analysis and 3D growth assays.

PCR

RNA (triplicate samples per condition) was isolated using an RNeasy Mini Kit (Qiagen) and cDNA was generated using Superscript VILO RT (Life Technologies). qPCR was performed using Taqman Fast Advanced Master Mix (Life Technologies) and specific primer-probe sets for total CD44, CD44v8-10, VEGF, TGFβR1, and TGFβ-2 (provided in Supplemental Information). Values reported are mean +/- SEM for at least 3 replicates.

Clonal Growth Assay

SKBR3 cells were embedded in Matrigel (Corning) at 1000 cells per well (unsorted) or 3500-5000 cells per well (sorted), while MCF7 cells were embedded in Reduced Growth Factor Matrigel (Corning) at 1000 cells per well and n ≥3 for each condition. Cells in matrigel were overlaid with Mammocult medium (Stem Cell Technologies). Colonies were counted at 7 days for untreated and 7-14 days after epirubicin or hypoxia. Representative pictures of complete
wells were taken using a GelCount instrument (Oxford Optronix) and individual colonies were taken at 20X on an Olympus IX51 microscope. Values reported are mean +/- SEM for at least 3 replicates within each experiment. Each result was reproduced in multiple independent biological experiments. Significance was tested using a two-tailed student’s t-test assuming unequal variance. Matrigel was used because without it, SKBR3 mammospheres exhibited a high degree of aggregation, especially after epirubicin, and thus were difficult to quantify.

**Western Blot**

Cells were lysed with MPER (Pierce) supplemented with protease and phosphatase inhibitors for 30min on ice and lysates were clarified by centrifugation for 5 min at 10,000g. 25-50 µg of lysates were loaded onto 4-12% SDS-PAGE gels and transferred onto PVDF membrane. Western blots were performed using GAPDH (Sigma), CD44 (Millipore), TGFβR1 (Life Technologies). Proteins were visualized using DyLight-conjugated secondary antibodies (ThermoFisher) and the LI-COR Odyssey scanner.

**Senescence-associated β-galactosidase staining**

Senescence was detected using the β-galactosidase staining kit from Clontech. SKBR3 cells (250,000) were seeded in 6-well plates, treated with epirubicin or placed in hypoxia, and stained using X-gal reagent overnight according to Clontech protocol. Representative pictures were taken at 20X on an Olympus IX51 microscope, and results were quantitated by counting stained and total cells in each picture. Values reported are mean +/- SEM for at least 3 replicates within each experiment. Each result was reproduced in multiple independent biological experiments.
**Microarray Analysis**

RNA from sorted cells was isolated using the RNEasy kit (Qiagen). cDNA synthesis was performed using the Ovation Pico WTA System (NuGEN) and Ribo-SPIA technique. Ovation Pico WTA products were fragmented and biotin labeled using the Encore Biotin Module (NuGEN). For each sample, 5 µg of biotin-labeled cDNA was hybridized to Human Genome U133 + 2.0 oligonucleotide arrays (Affymetrix) using buffers and conditions recommended by manufacturer. GeneChips were then washed and stained with Streptavidin R-phycoerythrin (Molecular Probes) using the GeneChip® Fluidics Station 450, and scanned with an Affymetrix GeneChip Scanner 3000. Gene expression data was processed by Micro Array Suite 5.0 (MAS5) algorithm. Probe sets were filtered to obtain robustly expressed qualifiers (average signal value of >=50, 100% present call (based on MAS5) in any of the sample group). Microarray data was deposited in the Gene Expression Omnibus (GEO; accession #GSE72362). Gene Set Enrichment analysis was conducted using the Gene Set Enrichment Analysis (GSEA) software (Broad Institute) (22).

**Master Regulator Analysis**

We interrogated context-specific regulatory or signaling networks and applied a network-based systems biology approach, the master regulator inference algorithm (23,24) to identify key transcriptional or signaling master regulators associated with CD44hi cell population induced by chemotherapy or hypoxia. We used a data-driven approach, ARACNe algorithm (25) to reconstruct breast cancer specific interactomes from The Cancer Genome Atlas (TCGA) (26) gene expression profiles in both microarray (N=359) and RNASeq (N=950) platforms, against ~800 transcription factors and ~2500 signaling proteins. The parameters of ARACNe were
configured as follows: p-value threshold \( p = 1 \times 10^{-7} \), DPI tolerance \( e = 0 \), number of bootstraps \( NB = 100 \), and adaptive partitioning algorithm for mutual information estimation. For GSEA method in master regulator inference algorithm, we applied ‘maxmean’ statistic to score the enrichment of the gene set and used sample permutation to build the null distribution for statistical significance. We used Fisher’s method to integrate MRs predicted from microarray- or RNASeq-based TCGA breast cancer networks.
Results

Chemotherapy and hypoxia enrich for a CD44\textsuperscript{hi} population

Cultured SKBR3 breast cancer cells and tumor xenografts established from them exhibit low expression of CD44, but when tumor-bearing animals were treated with epirubicin, a cytotoxic chemotherapy, the relapsed tumors exhibited high CD44 expression (27). To determine whether this observation could be reproduced \textit{in vitro}, which would enable mechanistic studies, SKBR3 cells were treated with epirubicin for 3 days at the concentration that inhibited growth by 50\% (IC\textsubscript{50}). Both the mRNA and protein levels of CD44 increased after the treatment, as revealed by immunoblot and qRT-PCR respectively (Figure 1A, 1B). The same effect was observed when the cells were cultured for 5-9 days in hypoxia (1\% O\textsubscript{2}), a stress that is common in untreated tumors and also can be induced by anti-angiogenic therapy (Figure 1A, 1B). Since drug perfusion through hypoxic regions can be minimal (28), it is conceivable that chemotherapy and hypoxic stress could simultaneously and differentially impact separate regions of the same tumor.

CD44 mRNA was induced to a much greater extent in hypoxia compared to chemotherapy (Figure 1B). The induction of CD44 in hypoxia required 3 days, compared to 1 day for induction of VEGF, and therefore CD44 does not appear to be a direct target of hypoxia inducible factor (HIF). Since alternatively spliced variants of CD44 yield protein isoforms with different binding specificities for extracellular matrix substrates, cell surface receptors and other signaling molecules (29), we analyzed the variants by RT-PCR after each treatment with exon-specific primers (30). CD44S and CD44v8-10 were similarly induced in epirubicin and hypoxia compared to untreated cells (Supplemental Figure S1A).
Flow cytometry was used to distinguish between two scenarios: induction of CD44 expression in all of the cells or enrichment of the CD44<sup>hi</sup> population. Treatment with epirubicin or incubation in hypoxia enriched for CD44<sup>hi</sup> cells as defined by CD44 cell surface expression (Figure 1C); moreover, treatment with paclitaxel had a similar effect (data not shown). The enrichment persisted long after each treatment: after one week in normal conditions, the fraction of CD44<sup>hi</sup> cells had increased further, and after two weeks, the fraction remained elevated in the epirubicin-treated culture and returned to its post-treatment level in the hypoxia-treated culture (Figure 1D, 1E). Thus the observed enrichments of CD44<sup>hi</sup> do not reflect transient or readily reversible processes. Despite these changes in CD44 expression, we did not observe any decrease in the expression of CD24 (Supplemental Figure S1B), another marker used in analysis of breast CSC populations and commonly used in combination with CD44. The expression of CD44 alone has been sufficient for isolation of CSC populations (11).

Several experiments were performed to study the mechanism that underlies the enrichment of CD44<sup>hi</sup> cells. Annexin V staining revealed that the apoptotic population in both treatments contained both CD44<sup>hi</sup> and CD44<sup>-/lo</sup> cells (Supplemental Figure S1C), which suggested that the enrichment was not merely the result of cell death that was restricted to CD44<sup>-/lo</sup> cells. Because CD44 expression can be induced in senescent fibroblasts (31), we examined whether chemotherapy and hypoxia induce senescence in this model. Senescence-associated β-galactosidase staining revealed an increased number of senescent cells after epirubicin treatment compared to hypoxia (Figure 1F, 1G). This result indicated that epirubicin could induce a quiescent state in cells and also suggested unique mechanisms of survival that gave rise to the CD44<sup>hi</sup> population in each condition.
CD44 is required for clonal growth of SKBR3 breast cancer cells

The above results suggested that CD44 could be an important determinant of therapeutic resistance in SKBR3 breast cancer cells. To determine whether CD44 was required for growth, expression of CD44 was reduced by short hairpin RNA (shRNA) technology (Figure 2A). The clonal growth of SKBR3 in Matrigel was dramatically inhibited in the absence of CD44: colony number was reduced by at least 80% with each of two independent CD44 shRNA constructs relative to non-targeting (NT) shRNA (Figure 2B). On tissue culture plastic, CD44 knockdown did not have an observable impact on cell growth at normal passaging density and had a modest effect on clonogenic growth, which might reflect the interaction of CD44 with extracellular matrix (32).

To further explore the function of the extracellular matrix in the observed phenotype, we investigated the effect of hyaluronic acid (HA), the primary ligand of CD44. HA increased the cell growth of SKBR3 but did not restore colony formation of CD44 knockdown cells (Figure 2C), confirming the CD44-based mechanism of the effect. Conversely, the addition of an antibody (clone 5F12) that blocks the interaction of CD44 with HA inhibited HA-stimulated cell growth in a dose-dependent manner (Figure 2D). Since the knockdown of CD44 had a greater effect on cell growth than 5F12 treatment, it is likely that functions of CD44 in addition to HA-based signaling are required for cell growth. Together these data demonstrate key functions of CD44 in SKBR3 cells, a finding consistent with results in other cell lines as well as the prognostic significance of CD44 in breast cancer (11,13,16).
The CSC phenotype of CD44$^{hi}$ cells depends on the treatment history

CSC populations have been enriched following chemotherapy treatment (1,7) as well as hypoxia that results from rapid tumor growth or antiangiogenic treatment (9,33). To determine whether the SKBR3 CD44$^{hi}$ cells that survived these treatments exhibited CSC phenotypes, viable CD44$^{hi}$ and CD44$^{-lo}$ populations were isolated by fluorescence-activated cell sorting (FACS) and then immediately counted and seeded in growth assays (Figure 3A and Supplemental Figure S2A). qRT-PCR confirmed higher CD44 mRNA levels in the sorted CD44$^{hi}$ cells, consistent with the cell surface expression (Supplemental Figure S2B-C).

Of the cells that survived epirubicin, CD44$^{hi}$ cells readily formed colonies, while CD44$^{-lo}$ cells rarely formed colonies (Figure 3B, 3C); comparable results were obtained with two concentrations of epirubicin (Figure 3C). In sharp contrast, of the cells that survived hypoxic stress, the CD44$^{hi}$ and CD44$^{-lo}$ populations exhibited comparable growth (Figure 3B, 3D); the same results were obtained at the extreme near-anoxic oxygen levels of 0.1% O$_2$ (Figure 3D). Moreover, increasing the stringency of growth conditions, for example with Cultrex or Growth factor-reduced Matrigel matrices, could not distinguish the clonogenic potentials of CD44$^{hi}$ and CD44$^{lo}$ (data not shown). Colonies from sorted cells from untreated, epirubicin-treated and hypoxia-treated cultures exhibited similar colony morphology (Supplemental Figure S2D), which enabled a direct comparison of colony number. Colonies that grew from cells sorted after chemotherapy and hypoxia were comprised predominantly of CD44$^{-lo}$ cells regardless of CD44 expression at the time of sorting (Supplemental Figure S2E). Overall, our results demonstrated a striking phenotypic difference in CD44$^{hi}$ populations following two treatments and suggested that epirubicin, but not hypoxia, was enriching for CD44$^{hi}$-marked CSCs.
We hypothesized that SKBR3 cells that survive epirubicin versus hypoxia would exhibit differential responses to salinomycin, a Wnt pathway modulator that selectively inhibited growth of CD44\textsuperscript{hi} breast CSCs (7). Indeed, salinomycin suppressed the enrichment of CD44\textsuperscript{hi} in epirubicin but not in hypoxia (Figure 3E). Thus three different phenotypes of CD44\textsuperscript{hi} cells that survive epirubicin versus hypoxia – mechanism of enrichment, growth potential and response to salinomycin – together indicated that surprisingly, CD44\textsuperscript{hi} enriches for CSC-like cells in epirubicin but not hypoxia.

**Unique transcriptional profiles of epirubicin-surviving CD44\textsuperscript{hi} cells**

To identify mechanisms that could explain the treatment-dependent phenotype of CD44\textsuperscript{hi} cells, transcriptional profiles were generated for sorted CD44\textsuperscript{hi} and CD44\textsuperscript{-lo} cells after each treatment. The comparison of two populations from the same culture provides a uniquely controlled basis for analysis. Replicate samples from four independent experiments clustered together (Figure 4A). Based on the CSC phenotypes specifically observed for CD44\textsuperscript{hi} cells after epirubicin, it was initially surprising that hypoxia induced the transcription of a substantially larger number of genes in CD44\textsuperscript{hi} vs. CD44\textsuperscript{-lo} compared to epirubicin (Figure 4B). However, function-based analyses of the transcriptional profiles revealed that epirubicin CD44\textsuperscript{hi} cells exhibited a response more reflective of tumor progression, consistent with their CSC phenotypes.

Gene set enrichment analysis (GSEA) revealed that more transcriptional signatures were enriched in CD44\textsuperscript{hi} vs. CD44\textsuperscript{-lo} after epirubicin vs. hypoxia (Figure 4C). CD44\textsuperscript{hi} cells that survived epirubicin exhibited broader enrichment for signatures related to stem cells, EMT, metastasis, Wnt signaling, cancer progression, and oncogenic signaling (Supplemental Table 1), consistent with the CD44\textsuperscript{hi} CSC phenotype after epirubicin but not hypoxia. Notably, multiple
gene sets related to β-catenin (CTNNB1), a key transducer of Wnt signaling, were observed exclusively after epirubicin treatment (Supplemental Table 1). There was also substantial overlap in the gene sets enriched by both treatments, and these shared sets are indicative of aggressive cancer growth and less differentiated tumors, as well as genes associated with cell adhesion and motility, which might be connected to the function of CD44.

CD44<sup>hi</sup> cells that survived both treatments were enriched for genes in the TGFβ pathway (Supplemental Table 1). The ligand TGFβ-2 was upregulated in CD44<sup>hi</sup> cells following both chemotherapy and hypoxia, as indicated by several microarray probes and confirmed by RT-PCR; the mRNA levels of TGFβ-2, like CD44, were induced to a greater extent by hypoxia than epirubicin, but the CD44<sup>hi</sup>/CD44<sup>-</sup>/lo ratios were comparable (Supplemental Figure S3A-B). Interestingly, endoglin, a coreceptor that regulates TGFβR1/R2 activity, was downregulated after hypoxia but maintained in epirubicin (Supplemental Figure S3C).

As a complement to GSEA, we performed an analysis of gene regulatory networks to identify master regulators (MRs) that may have implemented the observed transcriptional changes in CD44<sup>hi</sup> vs. CD44<sup>-</sup>/lo. MRs include transcription factors as well as upstream signaling factors and are defined by interactomes that were reconstructed from datasets in The Cancer Genome Atlas (TCGA) (25,26). There were more unique MRs in CD44<sup>hi</sup> vs. CD44<sup>-</sup>/lo cells after epirubicin compared to hypoxia (Figure 4D, Supplemental Table 2), similar to the finding in GSEA. Interestingly, two MRs related to Wnt signaling were only identified in epirubicin: Wnt5A (FDR = 0.01) and CITED1 (FDR = 0.009). CITED1 interacts with p300/CBP and SMAD4 to promote transcription in response to TGFβ and also plays a role in Wnt-mediated gene expression (34,35). These epirubicin-specific MRs are noteworthy in light of the salinomycin effect and β-catenin signatures that were also specific to epirubicin.
TGFβ exerts opposing effects on cells that survive epirubicin or hypoxia

The enrichment of TGFβ signaling factors in CD44hi cells was provocative in light of the documented interaction between CD44 and TGFβR1 to promote cell growth (36) and role of TGFβ signaling in CD44hi breast CSCs (37). We hypothesized that TGFβ signaling modulated the growth of the cells that survived epirubicin and hypoxia. To evaluate the effect of TGFβ on the growth of cells that survived the two treatments, sorted cells were supplemented with recombinant TGFβ-2 ligand. Strikingly, TGFβ-2 promoted the growth of CD44hi cells that survived epirubicin, yet inhibited the growth of CD44hi (and CD44+/lo) cells that survived hypoxia (Figure 5A-B, Supplemental Figure S4A-B). TGFβ-2 did not rescue the growth defects of CD44+/lo cells that survived epirubicin (Figure 5A, Supplemental Figure S4A). Similar effects were obtained with TGFβ-1 ligand (data not shown). Thus despite the upregulation of TGFβ-2 in both conditions, the ligand exerted opposite effects on the cells based on their treatment history.

In a complementary approach, TGFβ signaling was modulated pharmacologically with the TGFβR inhibitor SB-431542 and the effect on cell growth was determined. SB-431542 is a highly specific kinase inhibitor of type 1 TGFβ receptors (38). Consistent with the above results, SB-431542 inhibited the growth of CD44hi cells that survived epirubicin and promoted the growth of CD44hi and CD44+/lo cells that survived hypoxia (Fig. 5C-D).

Because CD44 did not mark a CSC-specific population after hypoxia, we examined whether another CSC marker defined a population that could regrow after exposure to low oxygen. Aldehyde dehydrogenase (ALDH) activity was previously identified as a marker of breast CSCs by staining cells with Aldefluor (39,40), and cells that exhibit this activity have also
been identified in hypoxic regions of breast tumors (33). Unlike CD44, there was no enrichment in Aldefluor$^\text{hi}$ cells in response to epirubicin or hypoxia; moreover, the CD44$^\text{hi}$ and Aldefluor$^\text{hi}$ populations had minimal overlap (Supplemental Figure S4C). However, of the cells that survived hypoxia, Aldefluor$^\text{hi}$ signal highly enriched for clonal growth (Figure 5E), which indicated that this activity enriched for a CSC-like population in this context. After hypoxia, Aldefluor$^\text{hi}$ cells responded to TGFβ-2 treatment in the same way as CD44$^\text{hi}$ and CD44$^\text{lo}$ cells: TGFβ-2 inhibited colony formation (Figure 5E). The effect of TGFβ on cells that survive hypoxia was thus independent of CD44. Conversely, the effect of TGFβ on cells that survive epirubicin was dependent on CD44: it was observed in CD44$^\text{hi}$ but not CD44$^\text{lo}$ or Aldefluor-sorted cells (Figure 5 and Supplemental Figure S4D).

To determine if the treatment-dependent effects of TGFβ in SKBR3 were also observed in other breast cancer cell lines, we performed similar experiments with MCF7 cells, which are ER-positive, HER2-negative and represent a different subtype of breast cancer than HER2-positive SKBR3. Consistent with the results in SKBR3, TGFβ-2 promoted the growth of MCF7 CD44$^\text{hi}$ cells that survived epirubicin and inhibited the growth of CD44$^\text{hi}$ cells that survived hypoxia (Figure 5F-G and Supplemental Figure S5). These data extended a key finding of this study to another breast cancer cell line and suggested that our conclusions may apply broadly across breast cancer. In addition, although the HER2 oncogene driver is amplified in SKBR3 and has been shown to cooperate with TGFβ (41,42), the MCF7 results suggest that the HER2-TGFβ interaction is unlikely to be relevant in this context.
CSC enrichment in epirubicin is dependent on TGFβ signaling

Based on the above results and cited literature (36,37), we hypothesized a direct mechanistic link between TGFβ signaling and the CD44^{hi} CSC phenotype in epirubicin. To determine whether TGFβ signaling was required for the enrichment, we reduced TGFβR1 expression in SKBR3 cells by shRNA and then exposed them to epirubicin or hypoxia. Strikingly, the enrichment of CD44^{hi} cells in epirubicin was prevented by TGFβR1 knockdown, while the enrichment in hypoxia was not (Figure 6A-B).

To establish whether the TGFβ–mediated enrichment of CD44^{hi} cells in epirubicin occurs at the transcriptional level, we knocked down the expression of SMAD4, a key transcriptional effector of TGFβ signaling, and exposed the cells to epirubicin. The reduction of SMAD4 levels prevented the enrichment of CD44^{hi} CSCs (Figure 6C). In addition, we measured CD44 mRNA levels in the TGFβR1 knockdown cells and observed that CD44 levels were reduced (Figure 6D). These results demonstrate SMAD-dependent transcriptional activation downstream of TGFβ engagement in order to enrich the CSC phenotype in epirubicin.

Based on these results we hypothesized that inhibition of CD44 or TGFβ signaling would sensitize SKBR3 cells to chemotherapy. To test this, cells with stable shRNA knockdown of CD44 or TGFβR1 were exposed to various concentrations of epirubicin for 24 hours and then allowed to form colonies. Indeed, SKBR3 cells with stable knockdown of CD44 or TGFβR1 exhibited reduced growth after epirubicin treatment relative to the control (Figure 6E).

Similarly, TGFβ inhibition increased the radiosensitivity of MCF7 and other breast cancer cells (43), which is likely due to the same mechanism since both epirubicin and radiation generate DNA damage. Notably, CD44 knockdown in SKBR3 did not modulate sensitivity to epirubicin in a cell proliferation assay, which suggests that the clonogenic effect is driven by cell survival.
The experiments could not be performed in Matrigel since CD44 knockdown inhibits growth in this condition (Figure 2B). Together these data demonstrate that TGFβ signaling is involved in the earliest response to chemotherapy and suggest a feedback loop in which, under certain conditions, TGFβ signaling induces CD44 expression and enriches the CSC phenotype, and then CD44 modulates the effect of TGFβ signals.
Discussion

Resistance to anti-cancer therapies is driven by a vast array of mechanisms and can vary across tumor types and patient populations. Our study has demonstrated the coexistence in the same tumor of resistant cells with opposite responses to TGFβ signals, and thus has revealed a novel layer of complexity of the TGFβ pathway in breast cancer. One interpretation of the data is that different cell populations in a tumor can exhibit early- or late-stage characteristics based on microenvironment, cellular history or other factors. For example, cells exposed to chemotherapy may interpret TGFβ signals as pro-growth, consistent with late-stage disease, while cells under hypoxic stress may interpret TGFβ signals as growth-suppressive until the region becomes vascularized.

This study has identified an unexpected duality of TGFβ pathway modulation that builds upon and refines the previously identified links between TGFβ and breast CSCs (18,37). We document opposite effects of TGFβ activity that are dependent on a cell’s previous treatment yet independent of CD44 expression (Figure 7); the data challenge the existing model of a general CD44-TGFβ functional interaction in breast CSCs as well as the assumption that all CD44hi breast cancer cells exhibit the same phenotypes. Importantly the data provide the first evidence that cells in the same tumor can have opposite signaling activities in response to TGFβ modulation; this dichotomous response is mechanistically distinct from heterogeneous signaling that reflects varied expression of a receptor. In addition, we document a role of TGFβ signaling, including SMAD4 dependence, in enriching for the CD44hi CSC phenotype in chemotherapy. TGFβ signaling may enrich for the CSC phenotype by inducing senescence transiently in epirubicin treatment (Figure 1F-G), consistent with previously characterized functions of TGFβ.
Thus our study provides one mechanistic basis for heterogeneity of CSC phenotypes and markers and suggests a feed-forward loop in which, in certain cellular contexts, TGFβ promotes the enrichment of CD44^hi CSC phenotype and then CD44 functionally interacts with the TGFβ pathway to promote cell growth in recovery.

The TGFβ pathway interacts with many pathways, and unique drivers may influence the proliferative versus anti-proliferative effects of TGFβ. Our results implicate a context-dependent interaction between CD44 and TGFβ receptors, which builds on previous observations (36). Phosphorylated SMADs could interact with distinct transcription factors after chemotherapy or hypoxia; for example, we identified the transcription factor CITED1 as a MR in CD44^hi cells that survived epirubicin but not hypoxia. Signatures of β-catenin, which can interact with SMAD proteins, were enriched in epirubicin but not hypoxia. Together TGFβ and β-catenin could promote an EMT phenotype and CSC-like growth. Both CITED1 and β-catenin illustrate the possible interaction between TGFβ and Wnt pathways in response to chemotherapy. Signal modulation also could occur at the receptor complex; for example, the reduced expression of TGFβR coreceptor endoglin in cells that survived hypoxia but not epirubicin (Supplemental Figure S3C) may favor the growth-inhibitory effects of TGFβ in hypoxia (45,46). Interestingly the cytokine IL8 was strongly induced in hypoxia but not epirubicin (Supplemental Figure S3D), which could be explained by the potent angiogenic properties of IL8, although IL8 was also implicated in paclitaxel-induced CSCs (18).

Our study builds on previous observations of multiple CSC states in breast cancer (10) by revealing specific conditions that enrich for CSC-like populations with distinct phenotypes. CD44 expression enriched these cells only after epirubicin, while Aldefluor activity enriched them after hypoxia; in addition, the CSCs exhibited opposite results to TGFβ modulation.
CD44<sup>hi</sup> cells that survived epirubicin exhibited a gene expression profile that reflects EMT, consistent with CD44<sup>+</sup> breast cancer patient samples (10). Conversely, ALDH1 expression in patient samples correlated with a mesenchymal to epithelial transition and increased proliferation (10). Our results suggest that CSC states and phenotypes may continually adapt to changes in the tumor microenvironment and therapeutic insults as well as cumulative genetic changes.

This study, along with several others (40,47), demonstrates that <i>in vitro</i> mechanistic studies are a necessary complement to <i>in vivo</i> tumor initiation studies in the characterization of intratumoral heterogeneity and CSC growth mechanisms. CD44<sup>hi</sup> cells isolated from dissociated tissues cannot be distinguished by their unique histories and phenotypes, and the characterization of those cells as one population compromises efforts to understand mechanisms of therapeutic resistance. In both breast cancer cell lines that we analyzed, our data suggest that CD44<sup>hi</sup> cells isolated from relapsed tumors would include subpopulations that exhibit opposite responses to TGFβ modulation, depending on their exposure to chemotherapy or hypoxic microenvironment. In the future, tumor cells that are genetically engineered to display their individual history of DNA damage and exposure to hypoxia, for example with fluorescent proteins, could be used to bridge <i>in vitro</i> and <i>in vivo</i> analyses. Other parameters that could also be monitored include intracellular levels of metabolites and administered compounds; the latter is relevant due to challenges of drug delivery in solid tumors that can result in different exposures in different regions of the tumor.

The characterization of the diversity of resistance mechanisms that may coexist in a tumor will ultimately inform therapeutic strategies to improve clinical outcome. Novel therapies or multidrug regimens that target resistant tumor cell populations may be enabled by recent technological advances, such as nanoparticles that can enable the timed delivery of two different
compounds (48). Our data has specific implications for the development of TGFβ pathway modulators such as Type 1 TGFβ receptor inhibitors (49,50). In addition our results suggest that microenvironment-based biomarkers might be valuable complements to tumor cell-based biomarkers in optimizing the design of therapeutic regimens.
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Figure Legends

Figure 1. CD44 is expressed in response to cytotoxic chemotherapy or hypoxia in SKBR3 cells.

A. CD44 total protein expression is increased in chemotherapy and hypoxia. Western blot was performed using total protein extracts of untreated, epirubicin-treated, or hypoxic cells for CD44 and GAPDH.

B. CD44 mRNA expression is induced in epirubicin treatment and hypoxia. cDNA samples were generated from SKBR3 cells treated with 60 nM epirubicin for 3 days or incubated in 1% O₂ for 1-8 days. VEGF mRNA was included as a control for HIF1-controlled transcription. Results shown are average of three or more replicates +/- SEM for a representative experiment. *, P ≤ 0.05.

C. Flow cytometry for CD44 expression. SKBR3 cells were incubated with 90 nM epirubicin for 3 days or placed in 1% O₂ for 7 days. Cells were harvested and stained with CD44-FITC antibody for analysis.

D. CD44 is expressed at the cell surface after treatment withdrawal. CD44 levels were measured by flow cytometry 0-14 days after epirubicin washout.

E. After incubation in hypoxia, cells were placed back in normoxia and CD44 expression was measured by flow cytometry.

F. Senescence-associated beta-galactosidase staining in untreated, epirubicin-treated, and hypoxic SKBR3.

G. Quantitation of beta-galactosidase staining. **, P ≤ 0.01. N/S, not significant.
Figure 2. CD44 expression and interaction with hyaluronic acid is necessary for clonal growth of SKBR3 cells.

A. Flow cytometry for CD44 cell surface expression in nontargeting and shCD44 SKBR3. Cells were incubated in 1% O₂ for 7 days and then stained with an isotype control or CD44-APC.

B. Matrigel colony formation assay with SKBR3 cells stably expressing nontargeting (shNT) shRNA or CD44-specific (shCD44) shRNA. Top, quantitation of colony formation; bottom, view of representative wells. Data shown represents the average of at least three samples +/- SEM. *, P ≤ 0.05.

C. Matrigel colony formation assay with shNT SKBR3 or shCD44 SKBR3 supplemented with hyaluronic acid (HA) of increasing size.

D. Matrigel colony formation of SKBR3 treated with a CD44-HA blocking antibody. 5F12 antibody or IgG and 200 µg of 1000K HA were added to both matrigel and media.

Figure 3. CD44^{hi} cells isolated after chemotherapy and hypoxia are phenotypically distinct.

A. Schematic for isolation of CD44^{hi} and CD44^{lo} cells by FACS after epirubicin treatment or hypoxia. Sorted cells were implanted in matrigel and colonies were counted.

B. Colony formation of SKBR3 CD44^{hi} and CD44^{lo} cells after epirubicin or hypoxia.

C. CD44^{hi} cells from epirubicin treatment form more colonies than CD44^{lo}. Data shown represents the average of at least three samples +/- SEM. *, P ≤ 0.05.

D. CD44^{hi} and CD44^{lo} cells from hypoxia form an equal number of colonies.

E. Chemotherapy-treated but not hypoxic CD44^{hi} cells are sensitive to salinomycin. SKBR3 cells were co-treated with 3 µM salinomycin and either 90 nM epirubicin or 1% O₂.
Figure 4. Gene expression patterns in SKBR3 CD44hi and CD44−/lo cells after treatment.

A. Heat map detailing expression patterns of genes from four independent experiments significantly upregulated (P ≤ 0.05) in CD44hi compared to CD44−/lo from epirubicin treatment or hypoxia.

B. Venn diagram comparing genes significantly upregulated in CD44hi vs. CD44−/lo in either epirubicin or hypoxia treatment and genes expressed in both conditions.

C. Venn diagram summarizing number of gene sets enriched (FDR ≤ 0.05) in CD44hi vs. CD44−/lo samples after either epirubicin or hypoxia treatment and gene sets enriched in both conditions.

D. Venn diagram summarizing master regulators of gene expression identified (z-score ≥ 2, FDR ≤ 0.05) in CD44hi vs. CD44−/lo samples after epirubicin or hypoxia treatment.

Figure 5. TGFβ signaling elicits different effects on chemotherapy-treated and hypoxic cells.

A. TGFβ-2 stimulates colony formation of CD44hi cells from epirubicin treatment. CD44hi and CD44−/lo cells were sorted and embedded in matrigel with 25 ng/mL TGFβ-2. Data shown represents the average of at least three samples +/- SEM. *, P-value ≤ 0.05.

B. TGFβ-2 inhibits matrigel colony formation of hypoxic SKBR3 cells.

C. Inhibition of TGFβR with 1-2 µM SB431542 decreases colony formation in CD44hi cells after epirubicin treatment. SB431542 was added to sorted cells in a matrigel colony formation assay.

D. SB431542 treatment enhances colony formation of CD44hi and CD44−/lo SKBR3 cells from hypoxia.
E. Colony formation of Aldefluor\textsuperscript{hi} and Aldefluor\textsuperscript{-lo} populations sorted from SKBR3 incubated in hypoxia. Sorted cells were embedded in matrigel with 0 or 25 ng/mL TGFβ-2. CD44\textsuperscript{hi} and CD44\textsuperscript{-lo} colony formation is shown for comparison.

F. TGFβ-2 stimulates colony formation of chemotherapy-treated CD44\textsuperscript{hi} MCF7 cells. Results represent the average of three replicates +/- SEM. *, P ≤ 0.05. Cells were sorted after treatment with 200 nM epirubicin and then embedded in matrigel.

G. TGFβ-2 inhibits colony matrigel formation of hypoxic MCF7 cells. Cells were sorted after exposure to hypoxia and then embedded in matrigel.

**Figure 6. TGFβ dependence of CSC enrichment in epirubicin**

A. Western blot for TGFβR1 in SKBR3 cells stably expressing NT or TGFβR1-specific shRNAs.

B. CD44 cell surface expression in shNT or shTGFβR1-SKBR3 cells was determined by FACS after epirubicin treatment or hypoxia. % CD44\textsuperscript{hi} is plotted for a representative experiment.

C. CD44 cell surface expression in SKBR3 cells with non-targeting (NT) or SMAD4 siRNA (SMAD4-1, -2 and -3) was determined by FACS after epirubicin treatment. Cells were transfected with siRNA 3 days prior to 60 nM epirubicin treatment for an additional 3 days.

D. mRNA was isolated from shNT or shTGFβR1-SKBR3, and qPCR was then performed using primer-probe sets for TGFβR1 and CD44. Data shown is the average of three replicates +/- SEM. *, P ≤ 0.05. **, P ≤ 0.01.

E. Clonogenic growth after epirubicin treatment of parental SKBR3 cells or cells stably transduced with non-targeting (NT), TGFβR1 or CD44 shRNA. Cells were treated with epirubicin for 24 hours starting the day after seeding, and colonies were counted 17 days after
seeding. Histogram shows the mean +/- standard deviation of triplicate samples, normalized to the untreated sample with appropriate error propagation.

**Figure 7. Dichotomy between TGFβ signaling in breast cancer cells that survive chemotherapy versus hypoxia.** Among cells that survive chemotherapy, CD44 expression marks the population with growth potential and various characteristics of CSCs, and TGFβ promotes the enrichment of CD44\textsuperscript{hi} cells in a SMAD-dependent manner and promotes their growth during recovery. In contrast, among cells that survive hypoxia, TGFβ elicits the opposite response during recovery by inhibiting the growth in a CD44-independent manner; CD44 does not enrich for CSC-like cells and is not required for growth during recovery.
Figure 1

A

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<th>Epirubicin</th>
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<th>Hypoxia</th>
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B

Relative mRNA Expression

- Untreated
- 60 nM Epirubicin
- Time in Hypoxia: 1d, 3d, 8d

C

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D

- 3d treatment
- Washout
- 7d
- 14d

Epirubicin

No Drug

E

7d treatment

Hypoxia

Normoxia

- 7d
- 14d

F

Untreated

Epirubicin

Hypoxia

G

% β-Gal Positive

- Untreated
- Epirubicin
- Hypoxia

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Figure 2

A

Count

CD44

B

Colonies/1000 Cells

NT  shCD44-1  shCD44-2

C

Colonies/1000 Cells

NT  shCD44-1

No HA  50K HA  1000K HA

D

Colonies/1000 Cells

No HA  HA only  10 µg  25 µg

αCD44-5F12 + HA

* N/S
Figure 3

A

SKBR3 or Hypoxia

CD44 FACS

Measure 3D growth

B

60nM Epirubicin

1% O₂

CD44<sup>hi</sup> vs CD44<sup>-/lo</sup>

C

Colonies/1000 cells

Live

CD44<sup>hi</sup>

CD44<sup>-/lo</sup>

60nM Epi

30nM Epi

D

Colonies/1000 cells

CD44<sup>hi</sup>

CD44<sup>-/lo</sup>

1% O₂

0.1% O₂

E

Untreated

Epirubicin

Hypoxia

CD44

FSC-H

CD44

FSC-H

CD44

FSC-H

Salinomycin

- Salinomycin

+ Salinomycin

4.31%

95.7%

22.4%

77.6%

21.3%

78.7%

4.50%

96.5%

10.3%

89.7%

20.1%

79.9%
Figure 4

A

B Gene Expression
C Gene Set Enrichment
D Master Regulators

118 38 260
281 283 126
164 70 39

Epirubicin CD44^hi vs CD44^lo
Hypoxia CD44^hi vs CD44^lo
Figure 6

A

TGFβR1
GAPDH

B

% CD44hi

Untreated
Epirubicin
Hypoxia

C

% CD44hi

Untreated
Epirubicin, 60 nM

Control
NT
SMAD4-1
SMAD4-2
SMAD4-3

D

Relative mRNA Expression

TGFβR1
CD44

E

Clonogenic growth (% of untreated)

Vehicle
1 nM
3 nM
10 nM

Epirubicin

Parental
NT shRNA
TGFβR1 shRNA
CD44 shRNA

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Figure 7

Chemotherapy
Treatment

Rearrangement

CD44, TGFβ-2, β-Catenin/Wnt, Metastasis

Growth

Recovery

Hypoxia
Treatment

CD44

TGFβ-2

TGFB

Growth

Recovery

IL-8; CD44; TGFβ-2

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Breast cancer cells respond differentially to modulation of TGF-β2 signaling after exposure to chemotherapy or hypoxia


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