TGF-β inhibition enhances chemotherapy action against triple-negative breast cancer

Neil E. Bhola, … , Rebecca S. Cook, Carlos L. Arteaga


After an initial response to chemotherapy, many patients with triple-negative breast cancer (TNBC) have recurrence of drug-resistant metastatic disease. Studies with TNBC cells suggest that chemotherapy-resistant populations of cancer stem-like cells (CSCs) with self-renewing and tumor-initiating capacities are responsible for these relapses. TGF-β has been shown to increase stem-like properties in human breast cancer cells. We analyzed RNA expression in matched pairs of primary breast cancer biopsies before and after chemotherapy. Biopsies after chemotherapy displayed increased RNA transcripts of genes associated with CSCs and TGF-β signaling. In TNBC cell lines and mouse xenografts, the chemotherapeutic drug paclitaxel increased autocrine TGF-β signaling and IL-8 expression and enriched for CSCs, as indicated by mammosphere formation and CSC markers. The TGF-β type I receptor kinase inhibitor LY2157299, a neutralizing TGF-β type II receptor antibody, and SMAD4 siRNA all blocked paclitaxel-induced IL8 transcription and CSC expansion. Moreover, treatment of TNBC xenografts with LY2157299 prevented reestablishment of tumors after paclitaxel treatment. These data suggest that chemotherapy-induced TGF-β signaling enhances tumor recurrence through IL-8–dependent expansion of CSCs and that TGF-β pathway inhibitors prevent the development of drug-resistant CSCs. These findings support testing a combination of TGF-β inhibitors and anticancer chemotherapy in patients with TNBC.

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TGF-β inhibition enhances chemotherapy action against triple-negative breast cancer

Neil E. Bhola, Justin M. Balko, Teresa C. Dugger, María Gabriela Kuba, Violeta Sánchez, Melinda Sanders, Jamie Stanford, Rebecca S. Cook, and Carlos L. Arteaga

After an initial response to chemotherapy, many patients with triple-negative breast cancer (TNBC) have recurrence of drug-resistant metastatic disease. Studies with TNBC cells suggest that chemotherapy-resistant populations of cancer stem-like cells (CSCs) with self-renewing and tumor-initiating capacities are responsible for these relapses. TGF-β has been shown to increase stem-like properties in human breast cancer cells. We analyzed RNA expression in matched pairs of primary breast cancer biopsies before and after chemotherapy. Biopsies after chemotherapy displayed increased RNA transcripts of genes associated with CSCs and TGF-β signaling. In TNBC cell lines and mouse xenografts, the chemotherapeutic drug paclitaxel increased autocrine TGF-β signaling and IL-8 expression and enriched for CSCs, as indicated by mammosphere formation and CSC markers. The TGF-β type I receptor kinase inhibitor LY2157299, a neutralizing TGF-β type II receptor antibody, and SMAD4 siRNA all blocked paclitaxel-induced IL8 transcription and CSC expansion. Moreover, treatment of TNBC xenografts with LY2157299 prevented reestablishment of tumors after paclitaxel treatment. These data suggest that chemotherapy-induced TGF-β signaling enhances tumor recurrence through IL-8–dependent expansion of CSCs and that TGF-β pathway inhibitors prevent the development of drug-resistant CSCs. These findings support testing a combination of TGF-β inhibitors and anticancer chemotherapy in patients with TNBC.

Introduction

Triple-negative breast cancers (TNBCs) lack detectable hormone receptors and ERBB2 gene amplification and represent the most virulent subtype of this malignancy (1). Cytotoxic chemotherapies such as taxanes are initially effective in most patients with metastatic TNBC; however, the majority of these tumors recur after chemotherapy (2). Metastatic tumor relapses are characterized by rapidly proliferating, drug-resistant cancers, associated with a high mortality rate. An increasing body of evidence suggests that survival of a small population of cells with stem-like properties may be responsible for these tumor recurrences after an initial response to chemotherapy (3–6). This population, interchangeably called cancer stem-like cells (CSCs) or tumor-initiating cells (TICs), retains the capacity to self-renew and regenerate the total bulk of a heterogeneous tumor comprised mostly of non–stem cells. In this study, we sought to identify clinically targetable molecules or pathways driving the survival of chemotherapy-resistant CSCs in TNBC.

Recent data suggest that the TGF-β family of cytokines plays a role in breast cancer stem cells. Shipitsin and colleagues showed that subpopulations with CSC features (CD44+) within breast tumors overexpress TGF-β1 and the TGF-β type I receptor (TGF-βR1). TGF-β is a potent inducer of an epithelial-to-mesenchymal transition (EMT) in mammary cells, and this transformation has been associated with acquisition of tumor stem-like properties (7). Indeed, a TGF-βR1/2 kinase inhibitor was shown to reverse EMT and induce a mesenchymal-to-epithelial differentiation in CD44+ mammary epithelial cells (8). TGF-β ligands are often enriched in the TNBC tumor microenvironment and can be produced by tumor cells or by tumor-associated stromal and immune cells (9, 10). These data suggest the possibility that the TGF-β pathway is involved in maintenance of CSCs in breast carcinomas. TGF-β inhibitors have been proposed and are being developed as antimetastatic therapies in patients with cancer. However, the impact of these inhibitors on CSCs in breast cancer has not yet been explored. Using a small molecular weight TGF-βR1 kinase inhibitor and a neutralizing TGF-β type II receptor antibody currently in clinical development (11, 12), we determined the role of TGF-β signaling in chemotherapy-induced expansion of CSCs in TNBC cell lines and xenografts. We initially discovered enrichment of a TGF-β-responsive gene signature in chemotherapy-treated primary breast cancers. This signature correlated with TNBC cell lines with basal-like gene expression. In TNBC cell lines and xenografts, treatment with the chemotherapy agent paclitaxel expanded a population with CSC markers, high autocrine TGF-β signaling, and tumor-initiating capacity. These effects were abrogated by both TGF-β inhibitors as well as SMAD4 siRNA. Expression of IL-8 at the mRNA and protein level was also increased by chemotherapy. This induction required an intact TGF-β pathway, as it was blocked by the TGF-βR1 kinase inhibitor and SMAD4 siRNA. Finally, addition of the TGF-βR1 kinase inhibitor to paclitaxel abrogated expansion of the CSC fraction and IL-8 release in both cultured TNBC cell lines and xenografts established in athymic mice. These studies are the first to our knowledge to demonstrate the ability of TGF-β inhibitors to block the expansion of chemotherapy-resistant TICs in vivo. They provide a basis for future clinical studies testing their role in combination with chemotherapy in patients with TNBC.

Results

Chemotherapy-treated breast cancers display increased markers of TGF-β signaling and CSCs. We first examined gene expression signatures enriched by chemotherapy in primary breast cancers (Table 1). RNA extracted from 17 matched breast tumor biopsies before and after...
neoadjuvant chemotherapy was quantitated using NanoString nCounter analysis (13). A TGF-β-responsive gene signature score (described in the Methods) was significantly enriched by chemotherapy in the biopsies after treatment (Figure 1A). When we applied the TGF-β signature to a second cohort of 21 patients treated with neoadjuvant systemic therapy (4–6 months), similar results were obtained with publically available gene expression in the pretreatment biopsy and the residual cancer (ref. 14 and Supplemental Figure 3B; supplemental material available online with this article; doi:10.1172/JCI65416DS1). TGFBR2, TGFBR3, and SMAD4 genes were increased approximately 2 fold (Figure 1B), whereas TGFBR1, TGFBR1, TGFBR3, SMAD2, and SMAD7 were also increased but to a lesser degree (Supplemental Figure 3A).

Paclitaxel enriches a CSC population with increased TGF-β signaling. Paclitaxel is one of the most commonly used chemotherapies in patients with TNBC (16–19). IC50s for this drug in 4 TNBC cell lines were between 2 and 5 nM, respectively (Supplemental Table 1). These cells were treated with a concentration of paclitaxel (5 or 10 nM) that induced >50% cell death. CSCs, measured by flow cytometry of ALDEFLUOR+ SUM159 cells and CD44+/PROCR−/SMAD4− BT549 cells, were increased 7 to 9 fold (Figure 1B), whereas TGFBR1, TGFBR2, and TGFBR3, and CD44 were increased 7 to 9 fold, respectively in response to paclitaxel (Figure 1B). Paclitaxel also increased the CSC population in a dose-dependent fashion in the paclitaxel-treated tumors (Figure 1B). To confirm these findings with paclitaxel induced TGF-β signaling, we treated BT549 and SUM159 cells with recombinant TGF-β1 with or without the TGF-β1 receptor ALK5 serine/threonine kinase (11). Cotreatment with LY2157299 abrogated TGF-β-induced P-SMAD2 in both SUM159 and BT549 cells (Figure 3A). In SUM159 cells, exogenous TGF-β increased the proportion of ALDH+ cells by >3 fold and the mammosphere number by >2 fold. Both of these effects were blocked by LY2157299. In BT549 cells, TGF-β also increased the CD44+/PROCR− fraction, and this effect was abrogated by LY2157299 (Figure 3A, B, and C). Although TGF-β did not increase mammosphere formation in BT549 cells, treatment with LY2157299 alone markedly reduced baseline mammosphere number (Figure 3C), further suggesting that tumor-initiating properties are regulated by autocrine TGF-βR1 function.

Like TGF-β, the cytokine IL-8 has been shown to regulate CSCs (21). Therefore, we sought to determine whether there was a link among paclitaxel, TGF-β, and IL-8 in regulating CSCs. Paclitaxel significantly induced IL8 mRNA expression in SUM159 and BT549 cells (Figure 4A). Exogenous TGF-β also induced IL8 mRNA expression in SUM159 and MDA231 cells (Supplemental Figure 6A). Further, TGF-β–induced secretion of IL-8 was completely abrogated by LY2157299 (Figure 4B). Knockdown of SMAD4 by siRNA also reduced >50% of basal IL8 mRNA levels (Supplemental Figure 6B) and reduced both baseline and TGF-β–induced mammosphere formation (Figure 3E) and Supplemental Figure 8) in SUM159, BT549, and MDA231 cell lines. These data further suggest that tumor-initiating properties in TNBC are regulated by autocrine TGF-β/SMAD4 signaling.

The type I TGF-β receptor ALK5 phosphorylates SMAD2/3, which, in turn, forms a complex with SMAD4. This complex translocates to the nucleus, in which it binds DNA to induce the transcription of TGF-β–dependent genes (20). Thus, we examined whether the effects of LY2157299 were mirrored by downregulation of SMAD4. Transfection with SMAD4 siRNA oligonucleotides downregulated SMAD4 protein levels (Figure 3D) and reduced both baseline and TGF-β–induced SMAD4 protein levels (Figure 3E and Supplemental Figure 8) in SUM159, BT549, and MDA231 cell lines. These data further suggest that tumor-initiating properties in TNBC are regulated by autocrine TGF-β/SMAD4 signaling.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinical pathological characteristics of patients from whom biopsies were acquired before and after chemotherapy</th>
</tr>
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<tbody>
<tr>
<td>Characteristics</td>
<td>Patients (n = 17)</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>0.9–14</td>
</tr>
<tr>
<td>Grade 1</td>
<td>n = 1</td>
</tr>
<tr>
<td>Grade 2</td>
<td>n = 4</td>
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<td>Grade 3</td>
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<td>n = 3</td>
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<tr>
<td>Mitotic index 3</td>
<td>n = 10</td>
</tr>
<tr>
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<td>n = 5</td>
</tr>
<tr>
<td>Subtype ER+/PR HER2- TNBC</td>
<td>n = 6</td>
</tr>
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<td>n = 6</td>
</tr>
<tr>
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<td>Stage III</td>
<td>n = 4</td>
</tr>
<tr>
<td>Stage IV</td>
<td>n = 2</td>
</tr>
</tbody>
</table>

All patients received anthracyclines and taxanes, either paclitaxel or docetaxel, before mastectomy.
ods: (a) RNAi targeting the IL-8 receptors CXCR1 and CXCR2 and (b) an IL-8–neutralizing antibody. Knockdown of CXCR1 and CXCR2 inhibited basal and TGF-β–induced SUM159 mammosphere formation (Figure 4D and Supplemental Figure 7A). However, treatment with the neutralizing IL-8 antibody only reduced basal but not TGF-β–induced mammosphere formation (Supplemental Figure 7B). Of note, addition of recombinant human IL-8 only modestly rescued the inhibition of mammosphere formation by MDA231 cells stably expressing SMAD4 shRNA (Supplemental Figure 8). These data imply that (a) the effects of TGF-β on TICs are at least in part mediated by the IL-8/CXCR1/2 axis; (b) the ability of IL-8 to induce mammosphere formation still requires TGF-β signaling, which is disabled upon stable SMAD4 knockdown; and (c) IL-8 is not the only mediator of TGF-β–induced CSCs.

Finally, we determined the effect of TGF-β inhibition on paclitaxel-induced IL-8 expression. Both transfection of SMAD4 siRNA (Figure 4E) and treatment with LY2157299 (Figure 4, F and G) reduced paclitaxel-induced IL8 mRNA and protein levels. These findings suggest that paclitaxel-induced IL-8 levels are regulated by the TGF-β pathway.

**Paclitaxel requires TGF-β signaling to expand the CSC population.** In SUM159 and BT549 cells, treatment with LY2157299 abrogated paclitaxel-induced phosphorylation of SMAD2 and the concurrent expansion of CSCs, as measured by flow cytometry for ALDH or CSC surface markers (Figure 5, A and B, and Supplemental Figure 9A). In addition, LY2157299 reduced the paclitaxel-enriched MDA231 CSC population measured by FACS analysis (Supplemental Figure 9B). Similarly, primary mammosphere formation by paclitaxel-treated cells was inhibited by treatment with LY2157299 (Figure 5C) and SMAD4 siRNA oligonucleotides (Figure 5D).

SMAD4 also mediates the transcriptional effects of BMP and activin receptors (22). Further, type I TGF-β receptor (ALK5) inhibitors like LY2157299 have been shown to inhibit the kinase activity of ALK4 and ALK7, the type I receptors for activin (23–25). Therefore, to determine whether the effects observed with SMAD4 siRNA and LY2157299 were specific to inhibition of ALK5, we used a monoclonal antibody (TR1) that specifically neutralizes the type II TGF-β receptor (TGF-βR2) (12). TR1 functions by preventing the binding of TGF-β to TGF-βR2 and induces the internalization of TGF-βR2. Cotreatment with TR1 also decreased the paclitaxel-induced expansion of the ALDH−SUM159 cells (Supplemental Figure 9C), suggesting that this expansion is the result of drug-induced autocrine activation of TGF-βR2. Finally, we flow sorted ALDH− and ALDH+ SUM159 cells prior to plating them in a 3D-Morphogenesis (Matrigel) assay. Under these conditions, ALDH+ cells but not ALDH− cells
continued to grow in the presence of paclitaxel. However, addition of LY2157299 eliminated the 3D growth of ALDH+ cells treated with paclitaxel (Figure 5E). These results suggest that TGF-β inhibition can reduce the chemotherapy-resistant CSC populations in TNBC.

**Pharmacological inhibition of TGF-β in vivo reduces the paclitaxel-resistant CSC population.** We next established SUM159 xenografts in female athymic mice. Mice (n = 10 per group) with tumors measuring ≥75 mm³ were randomized to treatment with vehicle, paclitaxel (20 mg/kg/d i.p. 5 times), LY2157299 (100 mg/kg twice a day p.o. 14 times), and a combination both drugs (Figure 6A). Some xenografts were harvested 5 days immediately after completing treatment with paclitaxel. Immunoblot analysis of these tumor lysates showed higher levels of P-SMAD2 in paclitaxel-treated xenografts compared with those in untreated xenografts. P-SMAD2 was undetectable in tumors from mice treated with paclitaxel.
LY2157299 (Figure 6B). Interestingly, P-SMAD2 levels were maintained in paclitaxel-treated and control tumors harvested on day 14, even though treatment with the taxane had been discontinued on day 5. Tumors previously treated with LY2157299 exhibited very low or undetectable levels of P-SMAD2 (Figure 6C). On day 14, tumor volume was significantly lower in the group treated with LY2157299 and paclitaxel compared with that of tumors treated with paclitaxel alone (Figure 6D). In all 4 treatment arms, mice maintained their body weight, and there were no signs of heart or liver toxicity upon histopathological evaluation (data not shown).

Dissociated cell suspensions from freshly harvested tumors were assessed for CSCs by flow cytometry. The ALDH + fraction of cell suspensions from xenografts harvested on day 14 was significantly lower in tumors treated with LY2157299 compared with that of paclitaxel-treated and control xenografts (Figure 6E). Finally, mice treated with paclitaxel alone exhibited >5-fold higher serum levels of TGF-β1 compared with mice treated with either LY2157299 or paclitaxel alone (Figure 6F).
of tumor-derived IL-8 compared with untreated controls; these levels were reduced by >50% in mice treated with the combination of LY2157299 and paclitaxel (Figure 6F).

TGF-β inhibition in vivo abrogates tumor-initiating potential after chemotherapy. Upon cessation of LY2157299 treatment at day 14, mice (n = 5) previously receiving paclitaxel (for 5 days) with or without LY2157299 were monitored for an additional 9 days to assess tumor growth. During this follow up, tumors in mice previously treated with LY2157299 and paclitaxel exhibited a slower rate of growth compared with that of tumors in mice treated with paclitaxel alone (Figure 6G). Tumors from all 4 groups were harvested on day 14; single tumor cell suspensions were prepared and injected in limiting dilutions (10²–10⁴) into tumor-free mice, which were monitored for subsequent tumor formation for 25 days. This limiting dilution assay is considered a rigorous test of cells with tumor-initiating capacity within a cancer population. SUM159 cells from tumors previously treated with LY2157299 alone or in combination with paclitaxel exhibited reduced tumor-forming potential (Table 2; P < 0.01). The combination of LY2157299 and paclitaxel significantly reduced the CSC frequency (Table 2). Taken together, these data suggest that TGF-β signaling sustains the tumor-initiating capacity of TNBC stem cells, at least in part, through regulation of IL-8. Thus, we propose the use of TGF-β pathway inhibitors in combination with chemotherapy to target drug-resistant TICs in TNBC.

Discussion

It has been proposed that tumor recurrences following treatment with anticancer chemotherapy are driven by a subpopulation of CSCs (26, 27). Compared with the bulk tumor cell population, CSCs are thought to possess intrinsic resistance to chemotherapy. Indeed, gene expression data from tumors that remain in the breast after neoadjuvant chemotherapy for invasive breast cancer have revealed enrichment of a cancer stem cell-like signature (26). Consistent with this notion, persistence of tumor cells in the breast after chemotherapy...
is associated with a high rate of metastatic recurrences and shorter survival compared with that in patients that exhibit a pathological complete response (2, 26). In an effort to discover targetable signaling pathways present in drug-resistant cells, we examined gene expression signatures enriched by chemotherapy in primary breast cancers. RNA extracted from primary breast tumors before and after chemotherapy showed enrichment of CSC-related genes and a TGF-β-responsive gene signature that was also associated with the more aggres-
sive basal-like subtype of breast cancer. We also examined levels of phosphorylated SMAD2 in tumor cell nuclei by IHC. Staining intensity and percentage of P-SMAD2–positive nuclei were not different in archival tumor sections before and after treatment (data not shown). These results concur with recent findings that a TGF-β–responsive signature was a better predictor of recurrence than P-SMAD2 tumor levels in a large cohort of patients with breast cancer (28).

In this study, treatment of TNBC cells with the microtubule inhibitor paclitaxel induced TGF-β reporter activity and upregulated genes in the TGF-β pathway. In some cases this was associated with increased phosphorylation of SMAD2. These data suggest an effect of paclitaxel on TGF-β signaling, which could be direct and/or indirect through negative selection, that is by sparing those CSCs with high TGF-β signaling. However, data in Figure 5 argue for a direct effect, albeit by molecular mechanisms that require further investigation: treatment with the type I TGF-β receptor kinase inhibitor LY2157299 and RNAi-mediated knockdown of SMAD4 inhibited paclitaxel-induced SUM159 and BT549 mammosphere formation and ALDH+ or CD44hi/PROCR+ cells, respectively. Our data do not exclude a simultaneous effect through negative selection. Nonetheless, data in Figure 5E suggest that might be the case: paclitaxel eliminates ALDH– cells but spares ALDH+ cells. Addition of LY2157299 eliminates paclitaxel-treated ALDH+, suggesting that these cells harbor autocrine TGF-β signaling causally associated with resistance to paclitaxel.

We also showed that paclitaxel induces transcription and secretion of the CSC-associated cytokine IL-8 to drive CSC expansion. Fas ligand and IL-8 are released by tumors upon treatment with chemotherapy (29). Of note, an IL8 gene expression signature correlates with poor prognosis in patients with basal-like breast cancer (30), and IL-8 has been shown to expand the CSC fraction in breast cancers (21, 31). Blockade of IL-8 has also been reported to abrogate chemotherapy-induced enrichment of CSCs (21). Further, like TGF-β, IL-8 is an osteolytic factor that can facilitate bone metastasis (32, 33). Both cytokines can expand the CSC population, which may explain the high recurrence rates observed in patients with TNBC. Taken together, these data suggest that following anticancer therapy, upregulation of autocrine/paracrine TGF-β signaling at tumor sites and in situ levels of IL-8 contribute to a tumor microenvironment that sustains CSCs in the primary cancer and also facilitates progression at metastatic sites.

We also showed a causal association between TGF-β signaling and IL-8 expression, since the latter was blocked by treatment with the TGF-βR1 kinase inhibitor LY2157299, a TGF-β type II...
improve the outcome of patients with this subtype of breast cancer. Breast tumor recurrences following chemotherapy and, as a result, kinase inhibitors or therapeutic antibodies, should limit basal-like

β

These data also imply that anticancer chemotherapy in combination

ated with metastatic cancer recurrences and poor patient outcome.

β

Invasion and IL-11 secretion in SMAD4-null MDA468 cells (40). In summary, results presented herein link anticancer chemotherapy, autocrine TGF-

β

Further, the combination of LY2157299 with paclitaxel significantly reduced IL-8 expression, stem cell markers, and the tumor-initiating potential of SUM159 cancer cells compared with either drug alone.

The TGF-β type II receptor–neutralizing antibody, TR1 (12), also blocked chemotherapy-induced expansion of CSCs (Supplemental Figure 9C) by FACS analysis. Since this antibody blocks access of endogenous ligands to TGF-βRII (12), this result implies that chemotherapy-mediated induction of IL-8 follows autocrine activation of the type II receptor. This is also consistent with previous reports showing that TGF-βRII can activate MAPK (38) and PI3K (39) independently of TGF-βRI and that RNA interference of p38MAPK abrogates IL-8 expression in a mouse model of MDA231 bone metastasis (40). Further, TGF-β can still induce p38MAPK-dependent invasion and IL-11 secretion in SMAD4-null MDA468 cells (40).

In data not shown herein, treatment with LY2157299 abrogated paclitaxel-induced enrichment of CSCs in MDA468 cells. Because of the potential pharmacological advantages of antibodies and the high specificity of TR1 to TGF-βRII and the TGF-β pathway, this monoclonal antibody should also be pursued in the clinic in parallel to LY2157299 for the treatment of patients with TNBC.

In summary, results presented herein link anticancer chemotherapy, autocrine TGF-β signaling, IL-8 expression, and the expansion of cells with phenotypic markers of stem cells with tumor-initiating capacity. We surmise that, in the clinic, this phenomenon is associated with metastatic cancer recurrences and poor patient outcome. These data also imply that anticancer chemotherapy in combination with systemic antagonists of TGF-β signaling, either small molecule kinase inhibitors or therapeutic antibodies, should limit basal-like breast tumor recurrences following chemotherapy and, as a result, improve the outcome of patients with this subtype of breast cancer.

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of tumor cells used for inoculation</th>
<th>Tumors formed (n = 5)</th>
<th>CSC frequency (per 1 in.)</th>
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<tbody>
<tr>
<td></td>
<td>1 x 10^4</td>
<td>1 x 10^3</td>
<td>1 x 10^2</td>
</tr>
<tr>
<td>Vehicle</td>
<td>4/5</td>
<td>3/5</td>
<td>2/5</td>
</tr>
<tr>
<td>LY2157299</td>
<td>4/5</td>
<td>3/5</td>
<td>2/5</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>4/5</td>
<td>4/5</td>
<td>2/5</td>
</tr>
<tr>
<td>LY2157299 + paclitaxel</td>
<td>2/5</td>
<td>2/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

SUM159 xenografts from mice treated with vehicle, LY2157299, paclitaxel, or both drugs were dissociated into single cell suspensions and injected in the number 4 mammary fat pad of nude mice in limiting dilutions (10,000; 1,000; 100 cells). Tumor formation was observed for 4 weeks following inoculation. CSC frequency was calculated using extreme limiting dilution analysis (ELDA). The difference in CSC frequency among all 4 treatment groups was significant, with a P value of 0.031.

receptor–neutralizing antibody and SMAD4 siRNA. However, we acknowledge the inconsistency between the efficacy of LY2157299 observed in vitro and in vivo in this article. We would like to point out that the variable monothecurapy efficacy of TGF-β inhibitors in 2-dimensional and 3-dimensional culture systems has been observed and reported (34). Phase I safety and dose-finding clinical trials with LY2157299 have shown that it is well tolerated (35, 36). Efficacy studies using an intermittent schedule of this small molecule are currently ongoing in patients with hepatocellular carcinoma and multiple myeloma (37). In the xenograft studies shown herein, mouse body weight and histological examination of organs did not reveal any evidence of toxicity related to the TGF-β inhibitor. Further, the combination of LY2157299 with paclitaxel significantly reduced IL-8 expression, stem cell markers, and the tumor-initiating potential of SUM159 cancer cells compared with either drug alone.

The TGF-β type II receptor–neutralizing antibody, TR1 (12), also blocked chemotherapy-induced expansion of CSCs (Supplemental Figure 9C) by FACS analysis. Since this antibody blocks access of endogenous ligands to TGF-βRII (12), this result implies that chemotherapy-mediated induction of IL-8 follows autocrine activation of the type II receptor. This is also consistent with previous reports showing that TGF-βRII can activate MAPK (38) and PI3K (39) independently of TGF-βRI and that RNA interference of p38MAPK abrogates IL-8 expression in a mouse model of MDA231 bone metastasis (40). Further, TGF-β can still induce p38MAPK-dependent invasion and IL-11 secretion in SMAD4-null MDA468 cells (40).

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score by subtracting the mean score and dividing by the standard deviation.

NanoString gene expression analysis. Genes (n = 132) significantly regulated by TGF-β in 4 individual breast cell lines (41) were used to construct a TGF-β gene expression signature (TBRS). Genes upregulated (n = 101) and downregulated (n = 51) in this signature were quantified by NanoString in 17 paired breast tumors biopsied before and after neoadjuvant chemotherapy. Data from 96 upregulated genes and 49 downregulated genes met quality controls and were available for analysis. The TGF-β signature score "i" was calculated as:

\[
s = \frac{n \times \sum w_i x_i}{\sum w_i}
\]

(Equation 1)

where \( w \) is the weight +1 or -1, depending on whether the gene was upregulated or downregulated in the signature (41), and \( x \) is the normalized gene expression level.

Cell lines and inhibitors. SUM159, BT549, SUM149, and MDA231 cells were from ATCC. SUM159 and SUM149 cells were cultured in Ham’s F-12/5% FBS and 500 ng/ml hydrocortisone; BT549 cells were cultured in RPMI1640/10% FBS; and MDA231 cells were cultured in DMEM/10% FBS. All cells were maintained at 37°C in 5% CO₂. LY2157299 and TR1 were provided by Eli Lilly and ImClone, respectively. Paclitaxel for in vitro studies was obtained from Sigma-Aldrich. Paclitaxel for in vivo studies was obtained from the Vanderbilt Hospital Outpatient Pharmacy. The IL-8–neutralizing antibody was from R&D Systems.

Quantitative RT-PCR. RNA was harvested using the RNAeasy Kit (Qagen) according to the manufacturer’s instructions and used to synthesize cDNA (iSCRIPT cDNA Synthesis Kit, Bio-Rad) also according to the manufacturer’s instructions. PCR reactions were performed using SYBR Green Master Mix (Bio-Rad) and the Bio-Rad IQ5 cycler. Relative mRNA levels were standardized to GAPDH mRNA levels. The TGF-β/BMP PCR Array (SA Biosciences), containing primers for 84 genes, was performed according to the manufacturer’s instructions. Gene expression was verified in cells independently treated with paclitaxel and using primer sets different than the ones used in the TGF-β/BMP Array.

Flow cytometry. The ALDEFLUOR assay (Stemcell Technologies) was performed according to the manufacturer’s guidelines to identify cells with high ALDH activity. Cells were passed through a 35-μm filter, suspended in ALDEFLUOR assay buffer plus BODIPY aminoacetaldehyde (BAAA), and incubated for 45 minutes at 37°C in the presence or absence of the ALDH inhibitor diethylaminobenzaldehyde (DEAB). CD44 (BD Biosciences, 1:25) and PROCR (BD Biosciences, 1:5) were incubated with single cells in PBS/1% FBS for 30 minutes at 4°C. Cells were stained with propid-
an alcohol solution, 1× PBS, 1 mM sodium vanadate, and 1 μM sodium orthovanadate. After fixation with 4% paraformaldehyde and permeabilization with 0.1% Triton X-100, cells were incubated with primary antibodies (Cell Signaling, 1:1000), P-SMAD2 (Cell Signaling, 1:500), and β-actin (Cell Signaling, 1:1000) overnight at 4°C. Immunoreactive bands were visualized by enhanced chemiluminescence reagents (Santa Cruz Biotechnology) for 1 hour at room temperature. Incubation with HRP-conjugated anti-rabbit and anti-mouse secondary antibodies at 4°C overnight or at room temperature for 2 hours, followed by treatment with a chemiluminescent substrate. Bands were visualized using an imaging system (Bio-Rad). Immunoblot analysis was performed using pCAGA-actin (Cell Signaling, 1:1000), P-SMAD2/3 (Santa Cruz Biotechnology), and β-actin (Cell Signaling, 1:1000), and IL8RA and IL9RB (Santa Cruz Biotechnology, 1:500).

Transcriptional reporter assays. Following treatment with paclitaxel, cells were seeded in 24-well dishes. Twenty-four hours later, cells were transfected with pCAGA-Luc and CMV-Renilla (Promega) using Fugene 6 transfection reagent (Roche). pCAGA-luciferase consists of 12 Smad3/Smad4-binding sequences (CAGA boxes) and the luciferase-coding sequence. Twenty-four hours later, firefly and Renilla reniformis luciferase activity were measured using the Dual Luciferase Kit (Promega) according to the manufacturer's instructions in a MoLight 3010 luminometer (Analytical Luminescence Laboratory).

RNAi experiments. SAMD4 and CXCR1/2 siRNA (Dharmacon) were transiently transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. SAMD4 shRNA retroviral vectors were provided by Yibin Kang (Princeton University, Princeton, New Jersey, USA) and transfected into Phoenix cells (ATCC) to generate retroviral particles. MDA-231 cells were transduced with retrovirus-containing media and selected in puromycin. Single cell clones were obtained and expanded.

IL-8 ELISA assay. Cells in 6-well plates were treated with LY2157299, paclitaxel, or both for 3 days in growth medium; after this time, the cell medium was collected and centrifuged to remove cellular debris. For each treatment group cell number was determined. IL-8 ELISA (R&D Systems) was performed according to the manufacturer's instructions. IL-8 readings were normalized to cell number, and the fold change compared with control group was calculated and graphed using GraphPad Prism Software.

For in vivo serum IL-8 analysis, IL-8 levels were normalized to tumor volume and the fold change compared with the control group.

Statistics. Statistical differences were determined using the Student's t test. For the paired tumor NanoString analyses (before and after chemotherapy), paired t tests were used. For animal studies, the differences among treatment groups were determined by Mann-Whitney U test with Bonferroni post-hoc corrections. Extreme limiting dilution assay analysis was performed to determine the statistical differences in stem cell-like frequency among the treatment groups as described previously (46). A P value of less than 0.05 was considered to be statistically significant.

Study approval. Animal studies were approved and performed in accordance with the Vanderbilt Institutional Animal Care and Use Committee. Breast tumor blocks were identified retrospectively from a cohort of patients who had consented to use of any deidentified tissues for research purposes under the auspices of an IRB-approved protocol (Vanderbilt IRB no. 030747). Selection was based on the following criteria: (a) the patient received neoadjuvant chemotherapy and had residual cancer in the breast in the mastectomy specimen; (b) sufficient formalin-fixed paraffin-embedded (FFPE) tissue was available for nucleic acid purification; and (c) pretreatment biopsies were available for comparison. Samples were included only if they were deemed by an expert pathologist to contain ≥20% tumor cells in the entire section or if they contained a region with ≥20% tumor cells that could be macrodissected. In total, 17 patients were included. Nucleic acid purification was performed using the RNAeasy FFPE Kit (Qiagen) according to the manufacturer's instructions. RNA samples were provided to NanoString for analysis. The raw transcript counts were normalized by dividing by the geometric mean of 7 preselected normalization “housekeeper” genes (WAS, CD40, B2M, NAGA, TUBB, NPAS2, and POLR1B), which cover a range of levels of constitutive expression.

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