Hypoxia-inducible factors are required for chemotherapy resistance of breast cancer stem cells

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Triple negative breast cancers (TNBCs) are defined by the lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 expression, and are treated with cytotoxic chemotherapy such as paclitaxel or gemcitabine, with a durable response rate of less than 20%. TNBCs are enriched for the basal subtype gene expression profile and the presence of breast cancer stem cells, which are endowed with self-renewing and tumor-initiating properties and resistance to chemotherapy. Hypoxia-inducible factors (HIFs) and their target gene products are highly active in TNBCs. Here, we demonstrate that HIF expression and transcriptional activity are induced by treatment of MDA-MB-231, SUM-149, and SUM-159, which are human TNBC cell lines, as well as MCF-7, which is an ER\textsuperscript{+}/PR\textsuperscript{+} breast cancer line, with paclitaxel or gemcitabine. Chemotherapy-induced HIF activity enriched the breast cancer stem cell population through interleukin-6 and interleukin-8 signaling and increased expression of multidrug resistance 1. Coadministration of HIF inhibitors overcame the resistance of breast cancer stem cells to paclitaxel or gemcitabine, both in vitro and in vivo, leading to tumor eradication. Increased expression of HIF-1\textalpha or HIF target genes in breast cancer biospies was associated with decreased overall survival, particularly in patients with basal subtype tumors and those treated with chemotherapy alone. Based on these results, clinical trials are warranted to test whether treatment of patients with TNBC with a combination of cytotoxic chemotherapy and HIF inhibitors will improve patient survival.

Results

Paclitaxel Induces HIF Expression and Transcriptional Activity in TNBC Cells. MDA-MB-231 TNBC cells were exposed to 10 nM paclitaxel for 0–4 d and whole cell lysates were subjected to immuno blot assays, which revealed induction of HIF-1\textalpha protein expression (25). In this study, we demonstrate that when TNBCs are treated with paclitaxel or gemcitabine, induction of HIF activity is required for enrichment of BCSCs, both in vitro and in vivo.

Significance

Breast cancer stem cells play essential roles in tumor growth, maintenance, and recurrence after chemotherapy. We report that treatment of human breast cancer cells with chemotherapy results in an enrichment of breast cancer stem cells among the surviving cells, which is dependent upon the activity of hypoxia-inducible factors (HIFs). Studies in mouse tumor models suggest that combining chemotherapy with drugs that block HIF activity may improve the survival of breast cancer patients.
expression on day 4 (Fig. 1). Reverse transcription-quantitative real-time PCR (RT-qPCR) analysis of total RNA isolated from MDA-MB-231 cells revealed that paclitaxel significantly increased the expression of HIF-1α mRNA (Fig. 1B, Left). Exposure of MDA-MB-231, SUM-149, or SUM-159 TNBC cells to paclitaxel for 4 d at the concentration that inhibited growth by 50% (IC_{50}) induced HIF-1α protein expression (Fig. 1C). We have previously shown that digoxin and acriflavine are drugs that inhibit HIF activity, primary tumor growth, and breast cancer metastasis (26–29). Induction of HIF-1α in MDA-MB-231 cells treated with paclitaxel was blocked by coadministration of digoxin (100 nM) or acriflavine (1 μM) (Fig. 1D). Although previous studies indicated that exposure of cancer cells to acriflavine for 24 h blocked dimerization of HIF-1α and HIF-1β without affecting protein levels (27), exposure of TNBC cells to acriflavine for 4 d resulted in decreased HIF-1α protein levels, which may reflect decreased stability of the free (monomeric) HIF-1α subunit. Exposure of MDA-MB-231 or SUM-159 cells to paclitaxel for 4 d also induced expression of HIF-2α mRNA (Fig. 1B, Right) and protein (Fig. 1E), which was inhibited by coadministration of digoxin or acriflavine (Fig. 1E). Paclitaxel significantly increased the expression of CA9 and ENO1 mRNA, which are products of HIF target genes, whereas expression of RPL13A mRNA, which is not HIF regulated, was unaffected (Fig. 1F). To determine the effect of paclitaxel treatment on HIF transcriptional activity, MDA-MB-231 cells were cotransfected with p2.1, which is a HIF-dependent reporter plasmid that contains a 68-bp hypoxia response element from the human ENO1 gene upstream of a basal SV40 promoter and firefly luciferase coding sequences, and pSV-RL, a control reporter plasmid that contains Renilla luciferase coding sequences downstream of the SV40 promoter only. The ratio of firefly:Renilla luciferase activity serves as a measure of HIF transcriptional activity. In cotransfected MDA-MB-231 cells, paclitaxel treatment significantly increased HIF transcriptional activity (Fig. 1G).

Taken together, the data presented in Fig. 1 indicate that paclitaxel induces HIF-α expression and HIF transcriptional activity in multiple TNBC cell lines.

**Paclitaxel Treatment Increases the Percentage of BCSCs.** Several different assays identify subpopulations of breast cancer cells that are enriched for BCSCs. The Aldefluor assay is based on the activity of aldehyde dehydrogenases (ALDH), which generate a fluorescent product in BCSCs that can be identified by flow cytometry (30, 31). Many breast cancer cell lines, including MDA-MB-231, SUM-149, SUM-159, and MCF-7 cells, have been shown to contain an ALDH+ subpopulation that displays stem cell properties in vitro and in vivo (6, 23, 31). Approximately 1% of vehicle-treated MDA-MB-231 (Fig. 2A) or SUM-159 (Fig. 2B) TNBC cells were ALDH+. Treatment with digoxin had no significant effect, whereas treatment with 10 nM paclitaxel for 4 d increased the percentage of ALDH+ cells by 12-fold. Coadministration of digoxin significantly reduced the paclitaxel-dependent increase in the percentage of ALDH+ cells (Fig. 2A and B).

No single marker, such as ALDH activity, has complete sensitivity and specificity in the identification of BCSCs and a phenotypic assay is therefore desirable. To confirm that changes in the percentage of ALDH+ cells reflected changes in the percentage of BCSCs, we performed multiple assays to characterize the ability of BCSCs to generate multicellular spheroids in suspension culture (32, 33). Exposure of SUM-159 (Fig. 2C) or SUM-149 (Fig. 2D) TNBC cells to paclitaxel for 4 d significantly increased the number of mammosphere-forming cells and this effect was completely abrogated by coadministration of digoxin. Primary mammosphere formation is a reflection of tumorigenicity, whereas secondary mammosphere formation provides increased evidence of self-renewal. Exposure of SUM-159 cells to paclitaxel again increased formation of primary mammospheres, which when dissociated and replated gave rise to increased numbers of secondary mammospheres (Fig. 2E). Treatment with digoxin alone significantly decreased primary and secondary mammosphere formation and abrogated paclitaxel-induced mammosphere formation. Taken together, the data presented in Fig. 2 indicate that paclitaxel increases the percentage of BCSCs in multiple TNBC cell lines in a HIF-dependent manner.

**Paclitaxel-Induced Interleukin Expression Is Blocked by HIF Inhibitors.** Interleukin 6 (IL-6) and IL-8 have been shown to regulate BCSC survival and self-renewal (6, 31, 34–38). Treatment of MDA-MB-231, SUM-159, or SUM-149 TNBC cells with paclitaxel induced IL-8 mRNA expression on day 4 (Fig. 3A). The induction of IL-8 or IL-6 mRNA by exposure to paclitaxel for 4 d was inhibited by coadministration of digoxin or acriflavine (Fig. 3B). Expression of RPL13A mRNA was unaffected by exposure to paclitaxel, alone or in combination with digoxin or acriflavine.
increased the percentage of ALDH+ cells (Fig. 4C) in the NTC subclone, whereas neither HIF-1α, HIF-2α, nor IL-6 expression was induced and ALDH+ cells were markedly reduced in the DKD subclone, both before and after treatment with paclitaxel (Fig. 4 A–C). Analysis of subclones stably transfected with a lentiviral vector encoding shRNA targeting either HIF-1α or HIF-2α (shH1α and shH2α subclones, respectively) revealed that expression of both subunits was required for maximal enrichment of BCSCs in response to paclitaxel (Fig. 4D). Similarly, paclitaxel induced increased mammosphere formation in the NTC subclone but this response was markedly impaired in the shH1α, shH2α, and DKD subclones (Fig. 4E). Knockdown of HIF-1α or HIF-2α impaired the induction of IL8 and IL6 gene expression in the same cell lysates (Fig. 4S). Immunoblot assays confirmed that IL-6 protein levels increased in a dose-dependent manner in paclitaxel-treated SUM-159 and SUM-149 cells (Fig. 3C), which mirrored the induction of HIF-1α in the same cell lysates (Fig. 1C). Paclitaxel-induced expression of IL-6 protein was blocked by co-administration of digoxin or acriflavine (Fig. 3D). To determine whether paclitaxel-induced BCSC enrichment was dependent on IL-6 or IL-8 signaling, we coadministered IgG or neutralizing antibodies against IL-6 or IL-8 during the exposure of MDA-MB-231 or SUM-159 cells to paclitaxel. Whereas IgG had no effect, IL-6 or IL-8 neutralizing antibodies abrogated the increased percentage of mammosphere-forming cells (Fig. 3E) and ALDH+ cells (Fig. 3F).

HIF-1α and HIF-2α Are Required for Paclitaxel-Induced BCSC Enrichment. To further analyze the role of HIFs in the response to paclitaxel, we used MDA-MB-231 subclones that were stably transfected with a lentiviral expression vector encoding a non-targeting control (NTC) short hairpin RNA (shRNA) or shRNAs targeting HIF-1α and HIF-2α (double knockdown, DKD), which have been extensively validated and used to investigate the role of HIFs in breast cancer progression (29). Paclitaxel treatment markedly increased HIF-1α and HIF-2α (Fig. 4A) as well as IL-6 (Fig. 4B) protein levels and significantly
transfected with vector encoding an shRNA against HIF-1α. Paclitaxel increased SMAD2 phosphorylation in both NTC and DKD subclones (Fig. S2A), despite the marked loss of ALDH+ cells in the DKD subclone (Fig. 4C), indicating that SMAD activation is independent of HIF activity and is not sufficient for BCSC enrichment in response to paclitaxel. JAK2 → STAT3 signaling was also implicated as playing a critical role in the BCSC phenotype in TNBC (37). Paclitaxel induced STAT3 phosphorylation in a HIF-independent manner (Fig. S2B). Thus, activation of SMAD2 and STAT3 in response to paclitaxel is not sufficient to induce BCSC enrichment in the absence of HIF activity.

Paclitaxel-Induced SMAD2 and STAT3 Activity Is Insufficient to Induce BCSC Enrichment. A recent publication reported that TGF-β → SMAD2/4 → IL-8 signaling was necessary for paclitaxel-induced BCSC enrichment (6). Paclitaxel induced SMAD2 phosphorylation in both NTC and DKD subclones (Fig. S2A), despite the marked loss of ALDH+ cells in the DKD subclone (Fig. 4C), indicating that SMAD activation is independent of HIF activity and is not sufficient for BCSC enrichment in response to paclitaxel. JAK2 → STAT3 signaling was also implicated as playing a critical role in the BCSC phenotype in TNBC (37). Paclitaxel induced STAT3 phosphorylation in a HIF-independent manner (Fig. S2B). Thus, activation of SMAD2 and STAT3 in response to paclitaxel is not sufficient to induce BCSC enrichment in the absence of HIF activity.

TLR-4 and NF-κB Do Not Mediate Chemotherapy-Induced BCSC Enrichment. Previous studies suggested that paclitaxel induces expression of TLR-4, which in turn increases NF-κB activity (41). However, exposure of MDA-MB-231 or SUM-159 cells to concentrations of paclitaxel that enriched for BCSCs did not increase TLR-4 mRNA expression (Fig. S3A and B). Levels of phospho-NF-κBα were not affected by treatment with paclitaxel in the three TNBC cell lines analyzed (Fig. S3C). Our results are consistent with a recent report that the NF-κB pathway is constitutively activated in TNBC (42). Thus, induction of TLR-4 → NF-κB signaling does not play a role in paclitaxel-induced BCSC enrichment in TNBC, although these results do not rule out constitutive involvement of NF-κB in promoting the BCSC phenotype.

Increased ROS Levels Mediate Induction of HIF-1α and Enrichment of BCSCs. Short-term exposure of cancer cells to very high concentrations of paclitaxel (100 nM for 8 h) has been shown to increase ROS levels (43, 44). We exposed TNBC cells to paclitaxel at IC50 for 4 d and stained the cells with MitoSOX Red, which is rapidly and selectively targeted to mitochondria and produces fluorescence when oxidized by superoxide radicals, thereby serving as an indicator of ROS in live cells. Flow cytometry revealed a marked increase in the percentage of MitoSOX+ cells after paclitaxel treatment of SUM-149 (Fig. 5A) or SUM-159 (Fig. 5B) cells. The percentage of MitoSOX+ cells was significantly decreased when paclitaxel was coadministered with increasing concentrations of manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP), which is a cell-permeable superoxide scavenger (Fig. 5A and B). MnTMPyP decreased the paclitaxel-induced expression of HIF-1α in a dose-dependent manner in both SUM-149 (Fig. 5C) and SUM-159 (Fig. 5D) cells. MnTMPyP also abrogated the paclitaxel-induced increase in the number of mammosphere-forming cells in both SUM-149 (Fig. 5E) and SUM-159 (Fig. 5F) cells. Taken together, the results presented in Fig. 5 suggest that increased ROS levels trigger HIF-1 signaling leading to BCSC enrichment.

Paclitaxel-Induced MDR1 Expression Is Blocked by HIF Inhibitors. Treatment of MDA-MB-231 or SUM-159 TNBC cells with paclitaxel increased expression of MDR1 mRNA (Fig. 6A and B) and protein (Fig. 6C), which was abrogated by coadministration of digoxin or acriflavine. Analysis of flow-sorted MDA-MB-231
MDR1, significantly reduced the enrichment of BCSCs (Fig. 6H). Paclitaxel and 5-fluorouracil (5-FU) contribute to paclitaxel-induced BCSC enrichment. HIF-1α signaling, induction of MDR1 expression and activity represents another HIF-mediated molecular mechanism that contributed to paclitaxel-induced BCSC enrichment.

Taken together, these data indicate that in addition to IL-6 and IL-8 signaling, induction of MDR1 expression and activity represents another HIF-mediated molecular mechanism that contributes to paclitaxel-induced BCSC enrichment.

Digoxin Blocks Paclitaxel-Induced BCSC Enrichment in Vivo. To investigate whether paclitaxel elicited similar effects in vivo, we implanted MDA-MB-231 cells in the mammary fat pad (MFP) of female severe combined immunodeficiency (SCID) mice. When the orthotopic tumors reached a volume of 200 mm³ (designated day 1), the mice were randomized to receive i.p. injections of saline, digoxin (2 mg/kg/d), paclitaxel (10 mg/kg on days 5 and 12), or both digoxin and paclitaxel. Tumors were harvested on day 12. There was no significant change in body weight of the mice (Fig. S4A). Concurrent exposure of MDA-MB-231 cells to 10 nM paclitaxel for 4 d and mammosphere assays were performed. *P < 0.001 compared with vehicle-treated cells, and IP < 0.001 compared with paclitaxel-treated cells, by Student's t test.

(Fig. 6D) or SUM-159 (Fig. 6E) cells revealed a marked induction of MDR1 mRNA expression in ALDH<sup>+</sup> cells and an even greater increase in ALDH<sup>+</sup> cells. Deficiency of either HIF-1α or HIF-2α or both resulted in a dramatic reduction in MDR1 mRNA and protein expression in the absence or presence of paclitaxel (Fig. 6 F and G). Concurrent exposure of MDA-MB-231 cells to 10 nM paclitaxel and 5 μM verapamil, which is a competitive inhibitor of MDR1, significantly reduced the enrichment of BCSCs (Fig. 6H).

Taken together, these data indicate that in addition to IL-6 and IL-8 signaling, induction of MDR1 expression and activity represents another HIF-mediated molecular mechanism that contributes to paclitaxel-induced BCSC enrichment.

Digoxin Blocks Paclitaxel-Induced BCSC Enrichment in Vivo. To investigate whether paclitaxel elicited similar effects in vivo, we implanted MDA-MB-231 cells in the mammary fat pad (MFP) of female severe combined immunodeficiency (SCID) mice. When the orthotopic tumors reached a volume of 200 mm³ (designated day 1), the mice were randomized to receive i.p. injections of saline, digoxin (2 mg/kg/d), paclitaxel (10 mg/kg on days 5 and 10), or both digoxin and paclitaxel. Tumors were harvested on day 12. There was no significant change in body weight of the mice (Fig. S4A). The combination of paclitaxel and digoxin had a significantly greater inhibitory effect on tumor growth compared with either digoxin or paclitaxel alone (Fig. 7A). The freshly harvested tumors were dissociated into single cell suspensions and subjected to the Aldeflour assay, which revealed that compared with saline treatment, paclitaxel significantly increased the percentage of ALDH<sup>+</sup> cells (Fig. 7B). In contrast, digoxin significantly decreased the percentage of ALDH<sup>+</sup> cells and abrogated the paclitaxel-induced enrichment of ALDH<sup>+</sup> cells (Fig. 7B). In the same tumors, paclitaxel increased the expression of IL-6, IL-8, and MDR1 mRNA and this effect was inhibited by digoxin (Fig. 7C).

To validate our results in another model, we injected SUM-159 TNBC cells s.c. into female athymic (Nude) mice and started treatment when the tumor xenografts reached a volume of 500 mm³ (designated day 1). The final tumor volume on day 12 was significantly decreased in mice treated with both paclitaxel and digoxin compared with mice treated with saline (control) or paclitaxel alone (Fig. 7D). Compared with saline, paclitaxel treatment increased the fraction of ALDH<sup>+</sup> cells in the residual tumor and digoxin treatment inhibited the effect of paclitaxel (Fig. 7E). Paclitaxel treatment also increased the number of
mammosphere-forming cells and digoxin significantly blunt the effect of paclitaxel (Fig. 7F). None of the treatments had any significant effect on body weight (Fig. S4B). Taken together, these results indicate that combination therapy of TNBC with digoxin blocks paclitaxel-induced IL-6/IL-8/MDR1 expression and BCSC enrichment in vivo.

**Digoxin Blocks Gemcitabine-Induced IL-6 Expression and BCSC Enrichment.** To analyze the effect of another chemotherapeutic agent, we treated TNBC cells with gemcitabine, which is a nucleoside analog that blocks DNA replication and acts by a different mechanism of action than paclitaxel, which binds to microtubules and blocks their breakdown during cell division. HIF-1α protein expression was induced in a dose-dependent manner when MDA-MB-231 (Fig. 8A) or SUM-159 (Fig. 8B) cells were exposed to gemcitabine at 1C50 for 4 d. Treatment of MDA-MB-231 or SUM-159 cells with gemcitabine for 4 d increased the percentage of ALDH+ cells (Fig. 8C) and mammosphere-forming cells (Fig. 8D and Fig. S5A), respectively, and these effects were blocked by coadministration of digoxin. Gemcitabine induced expression of IL-6 and MDR1 mRNA expression,

![Fig. 7. Digoxin blocks paclitaxel-induced BCSC enrichment in TNBC orthografts and xenografts. (A–C) MDA-MB-231 cells were implanted into the mammary fat pad of female Scid mice. When tumor volume reached 200 mm³, mice were randomized to four groups, which were treated with: saline (control, C); digoxin (D; 2 mg/kg on days 1–12); paclitaxel (P; 10 mg/kg on days 5 and 10); or paclitaxel and digoxin (P/D). Tumor volumes were determined every 2–3 d (A). Tumors were harvested on day 12 for Aldefluor (E) or mammosphere (F) assays. Data are mean ± SEM (n = 3). *P < 0.001 compared with C, and #P < 0.001 compared with P, by Student’s t test. (D–F) SUM-159 cells were injected s.c. into female athymic Nude mice. When tumor volume reached 500 mm³, mice were randomized to four groups, which were treated as described above. Tumor volume was determined every 2–3 d (D). Tumors were harvested on day 12 for Aldefluor (E) or mammosphere (F) assays. Data are mean ± SEM (n = 3). *P < 0.001 compared with C, and #P < 0.01 compared with P, by Student’s t test. Representative photomicrographs of mammospheres are shown. (Scale bar, 2 mm.)

![Fig. 8. Gemcitabine induces BCSC enrichment in vitro and in vivo. (A and B) MDA-MB-231 and SUM-159 cells were treated with 0–20 nM gemcitabine (Gem) either alone or in combination with 100 nM digoxin (D) or 1 μM acriflavin (A) for 4 d and immunoblot assays were performed. (C and D) Cells were treated with vehicle (V), digoxin (D), gemcitabine (G; 20 nM for MDA-MB-231 and 10 nM for SUM-159), or gemcitabine and digoxin (G/D) for 4 d. Cells were trypanosed and subjected to Aldefluor (C) and mammosphere (D) assays (mean ± SEM; n = 3). *P < 0.001 compared with V, and #P < 0.001 compared with G, by Student’s t test. (E–G) MDA-MB-231 cells were treated with vehicle (V) or 20 nM gemcitabine, either alone (G) or in combination with 100 nM digoxin (G/D) or 1 μM acriflavin (G/A) for 4 d. RT-qPCR assays were performed for IL-6 (E), MDR1 (F), and IL-8 (G) mRNA relative to 18S rRNA, with expression normalized to vehicle-treated cells (mean ± SEM; n = 3). *P < 0.001 compared with V, and #P < 0.001 compared with G, by Student’s t test. (H) MDA-MB-231 cells were exposed to vehicle or gemcitabine (Gem), either alone or in combination with IgG or neutralizing antibody against IL-6 or IL-8 (500 ng/mL). After 4 d, the percentage of ALDH+ cells was determined (mean ± SEM; n = 3). *P < 0.001 compared with vehicle, and #P < 0.001 compared with Gem, by Student’s t test. (I and J) MDA-MB-231 cells were implanted into the mammary fat pad of female Scid mice. When tumor volume reached 200 mm³, the mice were randomized to four groups, which were treated with: saline control, digoxin (2 mg/kg on days 1–12), gemcitabine (20 mg/kg on days 5 and 10), or gemcitabine and digoxin. Tumor volumes were determined every 2–3 d (I). Tumors were harvested on day 12 and the percentage of ALDH+ cells was determined (J; mean ± SEM; n = 3). *P < 0.001 compared with control and #P < 0.001 compared with gemcitabine, by Student’s t test.}

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which was inhibited by combined treatment with digoxin or acriflavine, in MDA-MB-231 cells (Fig. 8E and F) and SUM-159 cells (Fig. S5 B and C). In contrast to paclitaxel, gemcitabine did not induce IL-8 mRNA expression (Fig. 8G). However, treatment with neutralizing antibodies revealed that IL-8 signaling was required for BCSC maintenance in the absence of chemotherapy and that both IL-6 and IL-8 signaling were required for gemcitabine-induced BCSC enrichment (Fig. 8H).

Next, we implanted MDA-MB-231 cells into the MFP of Scid mice. When the tumor volume reached 200 mm³, mice were randomized to receive i.p. injections of saline, digoxin (2 mg/kg/d), gemcitabine (20 mg/kg on days 5 and 10), or the combination of digoxin and gemcitabine. Combination therapy had a significantly greater inhibitory effect on tumor growth than either drug alone (Fig. 8I) without any significant effect on body weight (Fig. S4C). Gemcitabine treatment increased the percentage of ALDH+ cells in the residual tumor but digoxin abrogated the gemcitabine-induced increase in ALDH+ cells (Fig. 8J). Taken together, the data presented in Fig. 8 (and Fig. S5) indicate that digoxin blocks HIF induction, IL-6 expression, and BCSC enrichment induced by treatment of TNBC cells with gemcitabine.

**Coadministration of Digoxin with Gemcitabine Prevents Tumor Relapse.** Patients with TNBC have the highest rate of relapse within 1–3 y despite adjuvant chemotherapy (10,11). To examine the effect of HIF inhibition on tumor relapse, MDA-MB-231 cells were implanted into the MFP of Scid mice. When the tumor became palpable, the mice were randomized to receive i.p. injections of saline, gemcitabine (20 mg/kg on days 5, 10, 15, 20, and 25), or the combination of digoxin (2 mg/kg on days 1–25) and gemcitabine. Treatment was stopped when tumors were no longer palpable in the mice receiving digoxin/gemcitabine combination therapy. Although treatment with gemcitabine markedly reduced tumor growth, after treatment was discontinued, rapid tumor growth resumed. In contrast, gemcitabine/digoxin combination therapy not only eliminated the primary tumor but also prevented any immediate tumor relapse (Fig. 9). Taken together, the data in Figs. 8 and 9 indicate that inhibiting the HIF pathway blocks chemotherapy-induced BCSC enrichment in the primary tumor and thereby prevents rapid relapse of TNBC.

**Effect of Cytotoxic Chemotherapy on HIF-1α Expression and BCSC Enrichment in Other Breast Cancer Subtypes.** To investigate whether chemotherapy-induced BCSC enrichment is limited to basal subtype/TNBCs, we treated MCF-7 (luminal A subtype/ER+PR+) and HCC-1954 (HER2+) cells with paclitaxel at IC50. Exposure of MCF-7 cells to 10 nM paclitaxel for 4 d induced HIF-1α and HIF-2α protein expression, whereas the induction was abrogated by coadministration of digoxin or acriflavine (Fig. S6A). Paclitaxel increased the percentage of ALDH+ cells, whereas digoxin decreased the percentage of ALDH+ cells, and coadministration of digoxin significantly reduced the paclitaxel-dependent increase in the percentage of ALDH+ cells (Fig. S6B). Similarly, when HCC-1954 cells were treated with increasing doses of paclitaxel (Fig. S6C) or gemcitabine (Fig. S6E) for 4 d, there was a dose-dependent increase in HIF-1α protein expression, which was abrogated by coadministration of digoxin or acriflavine. However, there was no enrichment of ALDH+ cells after treatment with paclitaxel (Fig. S6D) or gemcitabine (Fig. S6F). The MCF-7 results demonstrate that chemotherapy-induced BCSC enrichment is not restricted to TNBC cells, whereas the HCC-1954 data indicate that induction of HIF-1α is not sufficient for chemotherapy-induced BCSC enrichment.

**HIF Signature Predicts Mortality of Patients Treated with Chemotherapy.** To investigate the clinical relevance of HIF transcriptional activity with regard to the survival of patients with breast cancer, we used a HIF-1 signature composed of 16 genes (PLOD1, VEGFA, LOX, P4HA2, NDRG1, SLC2A1, ERO1L, ADM, LDHA, PGK1, ANGPTL4, SLC243, C49, HIF1A, IL6, and IL8), which are expressed in human breast cancer cell lines in a HIF-1-dependent manner. This HIF-1 signature was compared with the PAM50 signature, which consists of 50 genes, the expression of which divides human breast cancer specimens into five groups, designated basal, HER2 enriched, luminal B, luminal A, and normal (45,46). Most TNBCs cluster in the basal subtype (12). Comparison of the HIF-1 and PAM50 signatures in 1,160 breast cancer specimens (47) revealed increased expression of HIF-1 target genes in the basal and, to a lesser extent, the HER2-enriched subtypes (Fig. S7). Analysis of HIF-1α mRNA expression in 3,458 human breast cancer specimens (48) revealed that levels above the median were associated with significantly decreased overall survival [P < 10−10; hazard ratio (HR) = 1.48; Fig. 10A], with an even larger survival difference when only breast cancers of the basal subtype were analyzed (HR = 1.56; Fig. 10B).

Use of the HIF-1 signature resulted in an even more significant difference in patient survival in the overall population (P < 10−15; HR = 1.63; Fig. 10C) and increased the hazard ratio in the subpopulation with breast cancers of the basal subtype (HR = 1.90; Fig. 10D). Finally, analysis of data from patients treated only with chemotherapy revealed that in this subpopulation, differences in expression of the HIF-1 signature were associated with significant differences in survival (HR = 1.58; Fig. 10E), especially among patients with basal subtype tumors (HR = 3.45; Fig. 10F). These results are consistent with our in vitro and in vivo studies demonstrating HIF-dependent BCSC enrichment after chemotherapy.

**Discussion.**

Effective treatment for TNBC and other advanced breast cancers requiring cytotoxic chemotherapy represents a major unmet clinical need. Recent studies have suggested that eradication of BCSCs is required to achieve a complete remission and to prevent disease recurrence, but BCSCs are resistant to chemotherapy (4–6). Previous studies have demonstrated induction of HIF-1α expression by doxorubicin (25) and increased sensitivity of HIF-1α-deficient mouse embryo fibroblasts to killing by carboplatin and etoposide (16). In the present study, we have demonstrated that treatment of three different TNBC cell lines with clinically relevant concentrations of paclitaxel or gemcitabine increases ROS levels, which induce HIF-1α and HIF-2α mRNA and protein expression, leading to the HIF-dependent expression.
of MDR1, IL-8, and/or IL-6, and to BCSC enrichment, both in vitro and in vivo (Fig. 11). Coordinate expression of IL-6 and IL-8 has been shown to play an essential role in TNBC tumorigenesis and is associated with decreased survival in patients with breast cancer (37). Both IL-6 (34, 37) and IL-8 (6, 35) have been implicated in the maintenance of BCSCs and their resistance to chemotherapy, particularly in basal subtype/TNBC.

It should be noted that cytotoxic chemotherapy induced HIF-1\(\alpha\) and HIF-2\(\alpha\) expression under nonhypoxic conditions and that both HIF-1\(\alpha\) and HIF-2\(\alpha\) were required for maximal chemotherapy-induced BCSC enrichment, whereas only HIF-1\(\alpha\) was required for BCSC enrichment in response to hypoxia (23), indicating differences in the mechanisms leading to BCSC enrichment in response to chemotherapy vs. hypoxia that remain to be delineated. Although both paclitaxel and gemcitabine induced HIF-dependent IL-6 expression and BCSC enrichment, only paclitaxel induced IL-8 expression. However, basal IL-8 signaling was still required for gemcitabine-induced BCSC enrichment, which is consistent with previous studies demonstrating that inhibitors of CXCR1, which is the cognate receptor for IL-8, have profound effects on BCSCs (35).

A previous study reported that paclitaxel-induced IL-8 expression and BCSC enrichment was dependent upon TGF-\(\beta\) pathway signaling to SMAD2/4 (6). Similarly, IL-6 expression was linked to STAT3 activation and BCSCs (37). However, we found that HIF deficiency in the MDA-MB-231-DKD subclone blocked paclitaxel-induced IL-8 and IL-6 expression and BCSC enrichment, yet phosphorylation of SMAD2 and STAT3 was still induced in the DKD cells, indicating that induction of SMAD2 and STAT3 activity was HIF independent and was not sufficient for BCSC enrichment. We also found that expression of HIF-1\(\alpha\), but not IL-8, was induced in gemcitabine-treated MDA-MB-231 cells, and that paclitaxel or gemcitabine treatment of HCC-1954 cells induced HIF-1\(\alpha\) expression but not BCSC enrichment, indicating that HIF-1\(\alpha\) expression is not sufficient for IL-8 expression or BCSC enrichment. Taken together, our data and prior studies suggest that HIFs, SMADs, and STAT3 all contribute to BCSC enrichment in response to cytotoxic chemotherapy. However, from a translational point of view, the critical result is that inhibition of HIF activity is sufficient to block the enrichment of BCSCs, regardless of the activity of other transcription factors.

IL-6 and IL-8 signaling promotes BCSC enrichment by inhibiting chemotherapy-induced apoptosis and by promoting the BCSC phenotype. In contrast, MDR1 expression acts to maintain cell viability by pumping cytotoxic chemotherapy agents out of cancer cells. Our data indicate that treatment of TNBC

![Fig. 10. Expression of HIF-1\(\alpha\) and HIF-1 target gene mRNAs in primary breast cancers predicts clinical outcome. Kaplan–Meier analyses of overall survival (120 mo) were performed based on clinical and molecular data for 2,785 breast cancer patients, who were stratified by expression levels in the primary tumor of HIF-1\(\alpha\) mRNA (A and B) or a 16-gene HIF-1 signature (C–F), which were greater (red) or less (black) than the median. The \(P\) value (log-rank test) and hazard ratio (HR) for each comparison are shown. Patients of all breast cancer types (A and C) or only basal-type breast cancer (B and D) were analyzed. Within these two groups, the subgroup of patients who received only chemotherapy (E and F) was also analyzed.](#)

![Fig. 11. Cytotoxic chemotherapy induces ROS-dependent expression of HIF-1\(\alpha\) and HIF-2\(\alpha\), leading to HIF-mediated expression of IL-6, IL-8, and MDR1, which promote the survival of BCSCs, a response that is a critical determinant of treatment failure and patient mortality.](#)
cells with either paclitaxel or gemcitabine increased MDR1 expression in a HIF-dependent manner in ALDH1+ cells and to an even greater extent in ALDH1− cells, providing a mechanism for increased resistance of BCSCs to chemotherapy relative to bulk cancer cells. IL-8 signaling also contributes to this differential resistance because CXCXR1 expression is restricted to BCSCs (35).

Combination therapy with digoxin reversed the effects of paclitaxel or gemcitabine at the molecular level (IL-6, IL-8, and MDR1 expression), resulting in improved tumor control and abrogation of chemotherapy-induced BCSC enrichment in orthotopic and xenograft models of TNBC. We recently reported that, in contrast to cytotoxic chemotherapy, treatment of mice bearing orthotopic TNBC tumors with ganetespib, which is a second-generation HSP90 inhibitor, induced the degradation of HIF-1α (but not HIF-2α), inhibited primary tumor growth, and reduced the number of ALDH1+ cells in the residual tumor (49), providing further evidence that HIF inhibition provides a mechanism to prevent BCSC enrichment. HIF inhibition by methylelenecysteine sensitized head and neck squamous cell carcinoma xenografts to the cytotoxic chemotherapy agent irinotecan (50), suggesting that HIF-mediated cancer stem cell enrichment in response to chemotherapy may not be restricted to breast cancer treated with gemcitabine or paclitaxel and may be blocked by other HIF inhibitors.

The clinical relevance of these results is underscored by studies demonstrating associations between: (i) ALDH expression and ER+/basal subtype breast cancer (51); (ii) HIF-1α and ALDH1+ cells in breast cancers, both before and after chemotherapy (51); (iii) ALDH1+ breast cancer cells and resistance to sequential paclitaxel and epirubicin chemotherapy (52); and (iv) increased ALDH1+ cells after chemotherapy and decreased disease-free survival (51, 53). Taken together with these clinical studies, our results provide compelling evidence in support of clinical trials combining HIF inhibitors and cytotoxic chemotherapy in women with TNBC. Several drugs have been shown to inhibit HIF activity (49, 50, 54) and are candidates for such trials.

Methods

Cell Culture. MDA-MB-231 and MCF-7 cells were maintained in high-glucose (4.5 mg/mL) Dulbecco’s modified Eagle medium (DMEM) with 10% (vol/vol) FBS and penicillin/streptomycin. SUM-159 and SUM-149 cells were maintained in DMEM/F12 (50:50) with 10% (vol/vol) FBS, hydrocortisone, insulin, and 1% penicillin/streptomycin. HCC-1545 cells were maintained in RPMI-1640 media with 10% (vol/vol) FBS and penicillin/streptomycin. MDA-MB-231 knockdown subclones were cultured in the presence of 0.5 μg/mL of puromycin. Cells were maintained at 37 °C in a 5% CO2 95% air incubator (20% O2). Paclitaxel, digoxin, acriflavine, and gemcitabine were obtained from Sigma-Aldrich and dissolved in DMSO at 1,000X relative to final concentration in tissue culture medium. MTT assay was performed from Calbiochem and dissolved in deionized water.

RT-qPCR. Total RNA was extracted from cells and tissue using TRIzol (Invitrogen) and treated with DNase I (Ambion). First-strand cDNA synthesis was performed using 1 μg of total RNA and the iScript cDNA Synthesis System (Bio-Rad), and qPCR was performed using human-specific primers and iQ SYBR Green Supermix (Bio-Rad). For each primer pair, annealing temperature was optimized by gradient PCR. The expression (D) of each target mRNA relative to 18S RNA was calculated based on the cycle threshold (Ct): D = 2–ΔΔCt, in which ΔΔCt = Ct(target) − Ct(18S) and ΔCt = Ct(target) − Ct(Internal Control). See Table S1 for primer sequences.

Immunoblot Assays. Whole-cell lysates were prepared in RIPA lysis buffer. Blots were probed with antibodies against HIF-1α, HIF-2α, IL-6, IL-8, MDR1, phospho-SMAD2, phospho-STAT3, SMAD2, and STAT3 (Novus Biologicals). HRP-conjugated anti-rabbit (Roche) and anti-mouse (Santa Cruz) secondary antibodies were used. Chemiluminescent signal was detected using ECL Plus (GE Healthcare). Blots were stripped and reprobed with anti-actin antibody (Santa Cruz).

Luciferase Assay. 2 × 10^4 MDA-MB-231 cells were seeded onto 24-well plates overnight and the cells were transfected with plasmid DNA using PolyJet (SignaGen). Reporter plasmids pSvRL (5 ng) and p2.1 (295 ng) were cotransfected. The media was changed after 6 h. Starting the next day, the cells were treated with either vehicle or 10 nM paclitaxel. The cells were lysed after 4 d and luciferase activities were determined with a multimwell luminescence reader (Perkin-Elmer Life Science) using a dual luciferase reporter assay system (Promega).

Aldefluor Assay. After treatment of cultured cells for 4 d, the Aldefluor assay (StemCell Technologies) was performed to identify cells with ALDH activity. Cultured cells were trypsinized, whereas tumor tissue was minced, digested with 1 mg/mL of type I collagenase (Sigma) at 37 °C for 30 min, and filtered through a 70-μm cell strainer. The number of live cells was determined by Trypan blue assay and 1 × 10^6 live cells were suspended in assay buffer containing the fluorogenic substrate BODIPY aminoacetaldehyde (1 μM) and incubated for 45 min at 37 °C. As a negative control, an aliquot of cells was treated with the ALDH inhibitor diethylaminobenzaldehyde (50 mM). Samples were then passed through a 35-μm strainer and analyzed by flow cytometry (FACSCalibur; BD Biosciences).

Mammosphere Assay. Single-cell suspensions were seeded in six-well ultra-low attachment plates (Corning) at a density of 5,000 cells per milliliter in Complete MammaCult Medium (StemCell Technologies). After 7 d, the cells were photographed under an Olympus TH4-100 microscope with a 4x apochromat objective lens. Mammosphere number and volume were determined using ImageJ software. Mammospheres with area >500 pixels were counted in images of three fields per well in triplicate wells and the mean number of mammospheres per field was determined. For secondary mammosphere formation, primary mammospheres were trypsinized, plated at a density of 5,000 cells per milliliter, incubated for 7 d, and analyzed as described above.

MitoxSOX Staining. Intracellular ROS levels were determined by incubating the cells in 5 μM MitoxSOX Red ( Molecular Probes) at 37 °C for 45 min in PBS with 5% FBS, followed by rinsing with PBS. Stained cells were filtered and subjected to flow cytometry (FACScan; BD Bioscience). All gain and amplifier settings were held constant for all samples.

Animal Studies. Animal protocols were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (55) and were approved by the Johns Hopkins University Animal Care and Use Committee. Female 5–7-wk-old Scid and Nude mice (National Cancer Institute) were studied. Paclitaxel, gemcitabine, digoxin, and saline for injection were obtained from the research pharmacy of The Johns Hopkins Hospital. Cells were harvested by trypsinization, rinsed with PBS, and resuspended at 2 × 10^5 cells per milliliter in a 1:1 solution of PBS/Matrigel. MDA-MB-231 cells were injected in the MFP of Scid mice and SUM-159 cells were injected s.c. in Nude mice. Primary tumors were measured in three dimensions (a, b, and c), and volume (V) was calculated as V = abc/2.52.

Statistical Analysis. Data are expressed as mean ± SEM. Differences between two groups and multiple groups were analyzed by Student’s t test and ANOVA, respectively. P values <0.05 were considered significant. For the HIF-1 signature, the Breast Invasive Carcinoma Gene Expression Dataset of 1,162 patients was analyzed (47). Kaplan–Meier curves were generated from a breast cancer dataset containing gene expression and overall survival data on 2,785 patients (48). The log rank test was performed to determine whether observed differences between groups were statistically significant. The analysis of HIF-1 signature expression according to breast cancer subtype was performed using the GOBO database (56).

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Fig. S1. RPL13A mRNA was analyzed as a representative non–HIF-regulated gene product. MDA-MB-231 (A), SUM-159 (B), and SUM-149 (C) cells were treated with vehicle or either 10 nM (MDA-231 and SUM-159) or 5 nM (SUM-149) paclitaxel, either alone (P) or in combination with 100 nM digoxin (P/D) or 1 μM acriflavine (P/A) for 4 d. RT-qPCR analyses were performed to assess the expression of RPL13A mRNA relative to 18S rRNA in the same sample and normalized to vehicle-treated cells (mean ± SEM; n = 3).
SMAD2 and STAT3 are phosphorylated in response to paclitaxel in the presence or absence of HIF activity. NTC and DKD subclones of MDA-MB-231 cells were treated with either vehicle or 10 nM paclitaxel for 4 d and immunoblot assays were performed using antibodies specific for phosphorylated (p-) and total (t-) SMAD2 (A) or STAT3 (B).

TLR-4 expression and IκB phosphorylation are not affected by paclitaxel treatment of TNBC cell lines. (A and B) MDA-MB-231 (A) and SUM-159 (B) cells were treated with vehicle (V) or 10 nM paclitaxel, either alone (P) or in combination with 100 nM digoxin (P/D) or 1 μM acriflavine (P/A), for 4 d. RT-qPCR analyses were performed to quantify TLR-4 mRNA relative to 18S rRNA in the same sample and normalized to V (mean ± SEM; n = 3). N.S., not significant. (C) MDA-MB-231, SUM-159, and SUM-149 cells were treated with vehicle or either 10 nM (MDA-MB-231 and SUM-159) or 5 nM (SUM-149) paclitaxel, either alone (P) or in combination with 100 nM digoxin (P/D) or 1 μM acriflavine (P/A) for 4 d, and immunoblot assays were performed using antibodies specific for phosphorylated IκBα.
Fig. S4. Body weights of tumor-bearing mice were not affected by treatment with chemotherapy or digoxin. Scid mice received mammary fat pad injections of MDA-MB-231 cells (A and C) and athymic Nude mice received s.c. injections of SUM-159 cells (B). Body weights were measured every 2–3 d during the time period of treatment with saline (control), digoxin (days 1–12), chemotherapy (paclitaxel or gemcitabine, days 5 and 10), or the combination of chemotherapy and digoxin.
Fig. S5. Gemcitabine treatment of SUM-159 cells induced BCSC enrichment and increased expression of IL-6 and MDR1 mRNA, which were inhibited by digoxin or acriflavine. (A) Cells were treated with vehicle, 100 nM digoxin, 20 nM gemcitabine, or gemcitabine and digoxin for 4 d. Cells were trypsinized and subjected to mammosphere assays. Representative photomicrographs of mammospheres are shown. (Scale bar, 2 mm.) (B and C) Cells were treated with vehicle (V) or 20 nM gemcitabine, either alone (G) or in combination with 100 nM digoxin (G/D) or 1 μM acriflavine (G/A) for 4 d. RT-qPCR assays were performed for IL-6 (B) and MDR1 (C) mRNA relative to 18S rRNA, with expression normalized to vehicle-treated cells (mean ± SEM; n = 3). *P < 0.001 compared with V, and #P < 0.001 compared with G, by Student’s t test.
Fig. S6. Effect of paclitaxel or gemcitabine treatment of other breast cancer cell types. (A and B) MCF-7 cells were treated with vehicle (V) or 10 nM paclitaxel, either alone (P) or in combination with 100 nM digoxin (P/D) or 1 μM acriflavine (P/A) for 4 d and immunoblot (A) or Aldefluor (B) assays were performed (mean ± SEM; n = 3). *P < 0.001 compared with V, and #P < 0.001 compared with P, by Student’s t test. (C) HCC-1954 cells were treated with vehicle (V), 2.5 or 5 nM paclitaxel alone, or 2.5 nM paclitaxel in combination with 100 nM digoxin (P/D) or 1 μM acriflavine (P/A) for 4 d, and immunoblot assays were performed. (D) HCC-1954 cells were treated with vehicle (V), digoxin (D; 100 nM), paclitaxel (P; 2.5 nM), or both paclitaxel and digoxin (P/D) for 4 d, and Aldefluor assays were performed (mean ± SEM; n = 3). (E) HCC-1954 cells were treated with vehicle (V), 1 or 2.5 nM gemcitabine alone, or 2.5 nM gemcitabine in combination with 100 nM digoxin (G/D) or 1 μM acriflavine (G/A) for 4 d, and immunoblot assays were performed. (F) HCC-1954 cells were treated with vehicle (V), digoxin (D; 100 nM), gemcitabine (G; 2.5 nM), or both gemcitabine and digoxin (G/D) for 4 d, and Aldefluor assays were performed (mean ± SEM; n = 3).
Fig. S7. Expression of HIF-1 target genes (HIF-1 signature) in primary breast cancers. (A) Expression of PAM50 (1) and HIF-1 signatures in 1,160 primary breast cancers was used to classify tumors into those with expression greater (red) or less (green) than the median expression level. (B) HIF-1 signature expression was significantly associated with basal subtype in an analysis of 1,160 breast cancers using the GOBO database (2). Lum, luminal.

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