Taxane-Induced Hedgehog Signaling is Linked to Expansion of Breast Cancer Stem-Like Populations After Chemotherapy

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Recurrence of breast cancer after chemotherapy is thought to arise from resistant breast cancer stem cells which are eventually able to repopulate the tumor. The Hedgehog (HH) signaling pathway has been shown to regulate the proliferation and survival of breast cancer stem cells, and has been shown to promote resistance to chemotherapy through the activation of multi-drug resistance and pro survival pathways. Here we report that exposure of heterogenous breast cancer cell lines to docetaxel (DOC) resulted in release of Sonic Hedgehog ligand (SHH) and activation of the HH pathway as evidenced by increased expression and nuclear translocation of the downstream effector Gli-1 at 4–24 h after DOC treatment. This activation had little effect on the bulk of the tumor cell population as inhibition of HH signaling failed to increase apoptosis in response to DOC. However, HH pathway activation was required for clonogenic growth of cell lines after DOC. Increases in stemness markers as well as mammosphere formation were observed after treatment with DOC suggesting an increase in the breast cancer stem cell populations. These increases were similar to that of cell lines cultured in the presence of recombinant SHH and could be eliminated by co-treatment with HH inhibitors. These results suggest that HH pathway activation induced by DOC treatment does not have a chemosensitizing effect on the heterogeneous tumor population, but may be required for survival and expansion of breast cancer stem cells after chemotherapy. © 2014 Wiley Periodicals, Inc.

Key words: hedgehog; chemotherapy; breast cancer; recurrence; stem cells

INTRODUCTION

Locally advanced breast cancer is commonly treated with neoadjuvant chemotherapy (NCT) prior to surgical resection. The majority of women treated with NCT will experience a reduction in tumor size greater than 50% [1] allowing for breast conserving surgery. However, a less than pathological complete response to NCT is associated with higher local and systemic recurrence rates and lower survival, even in patients who have undergone mastectomy and nodal dissection [2].

Although the mechanisms leading to breast cancer recurrence are not well understood, several studies have suggested that an expansion of stem-like breast cancer cells may be responsible for disease recurrence and metastasis in breast cancer patients [3,4]. While conventional chemotherapy is able to eliminate the bulk of the tumor cells, highly aggressive resistant cells survive, and eventually repopulate the tumor. Recent studies have identified a subpopulation of highly tumorigenic cells in recurrent breast cancer that resemble mammary stem cells. These cells, referred to as breast cancer stem-like cells (BCSC) are phenotypically similar to mammary stem cells, and are characterized by high expression of CD44 (CD44high), and low or no expression of CD24 (CD24low/−). BCSC have the ability to self-renew, generating progeny that are identical to themselves, and to differentiate into a heterogeneous population of tumor cells. BCSCs show differential expression of genes involved in cell cycle arrest, DNA repair, drug transport, and survival pathways compared to other tumor cells [5–8] and are increased in residual breast tumors after neoadjuvant chemotherapy [9]. Although expansion of BCSC has been observed after treatment with chemotherapy, the mechanisms responsible for this expansion are unclear.

The Hedgehog (HH) signaling pathway is necessary for self-renewal and maintenance of mammary stem cells [10]. Activation of HH signaling occurs when
secreted HH ligands (Sonic Hedgehog [SHH], Desert and Indian) bind to and inhibit the cell surface receptor PATCHED (PTCH). This binding relieves the PTCH-mediated suppression of the transmembrane protein SMOOTHENED (SMO) leading to multiple intracellular events that result in the stabilization, nuclear translocation and activation of the Gli family of transcription factors (Gli-1, 2 and 3) [10,11]. Transcriptional targets of Gli include genes controlling cell cycle, cell adhesion, signal transduction, vascularization and drug transport. PTCH and Gli-1 are also targets for Gli mediated transcription, providing feedback for HH signaling. Thus, PTCH and Gli-1 expression is increased on cells with active HH signaling. Increased levels of SHH, Gli-1 and Gli-2 have been reported in CD44high/CD24−/low breast cancer cells isolated from human tumors, compared to the bulk of tumor cells [12,13]. HH signaling has also been shown to promote proliferation of both mammary and BCSC [12].

Abnormal activation of HH signaling has been associated with chemoresistance in gastrointestinal tumors. Upregulation of SHH and Gli-1 are associated with chemoresistance in esophageal and pancreatic tumors, in part by upregulation of ABC drug transporters and increased expression of survival proteins [14,15]. Although increased expression of HH signaling has been associated with poor survival in breast cancer [16], its role in chemoresistant breast cancer is unclear. In this study, we show that HH signaling is induced in breast cancer cells upon treatment with docetaxel (DOC), and supports expansion of BCSC.

MATERIALS AND METHODS

Cell Lines and Reagents

Breast cancer cell lines T-47D, SKBR3 and MCF-7 were obtained from ATCC (Manassas, VA). SUM159 cells were obtained from Asterand (Detroit, MI). T-47D and SKBR3 lines were cultured in DMEM plus 10% Fetal Bovine Serum (FBS). MCF-7 cells were cultured in DMEM, 10% FBS and 10 μg/ml insulin (Invitrogen, Grand Island, NY). SUM159 cells were cultured in Hams F12 media supplemented with 5% FBS, 2 μg/ml insulin and 1 μg/ml hydrocortisone (Invitrogen). Cells were maintained at 37°C in 5.0% carbon dioxide. Docetaxel (DOC) was purchased from LC Labs (Woburn, MA) and resuspended in ethanol at a stock concentration of 100 mM. Visnagide (GDC-0449) was purchased from LC Labs and resuspended at a stock concentration of 10 mM in DMSO. Hybridoma supernatants containing the HH neutralizing antibody clone 5E1 were obtained from the Developmental Studies Hybridoma Bank (UIOWA, developed under the auspices of the NICHD and maintained by The University of Iowa). Recombinant SHH ligand (clone C24II) was purchased from R&D systems (Minneapolis, MN) and reconstituted at a concentration of 100 μg/ml in PBS.

Western Blot

For treatment experiments, cells were plated in normal culture media and cultured overnight. Cells were then treated with DOC at a concentration of 50 nM and cultured for 2–6 h. Control cells were treated with ethanol. Media was removed and replaced with normal culture media. Cells were then cultured for the indicated time-point and lysed. Protein was harvested using RIPA buffer (Sigma-Aldrich, St. Louis, MO) and reconstituted at a concentration of 100 μg/ml in PBS. Supernatant from T-47D and SKBR3 cells either untreated or treated with DOC for 4 or 24 h were assayed with a SHH ELISA kit (Abcam) as described in the manufacturer’s protocol. Briefly, supernatants were collected and centrifuged to remove cells and debris. Cells were subsequently trypsinized and counted by trypan blue exclusion using a Countess automated cell counter (Invitrogen). Each sample or standard was added to each well and incubated overnight at 4°C with continued agitation. The following day blots were washed three times with PBS and incubated for 1 h at RT with an HRP conjugated anti-rabbit or antimouse antibody (Jackson Immunoresearch Laboratories, West Grove, PA). The blots were incubated with Super Signal West Dura Extended Duration Substrate (Pierce) and imaged on a G:Box ChemiXP4 Imaging System (Syngene, Frederick, MD).

ELISA

Supernatant from T-47D and SKBR3 cells either untreated or treated with DOC for 4 or 24 h were assayed with a SHH ELISA kit (Abcam) as described in the manufacturer’s protocol. Briefly, supernatants were collected and centrifuged to remove cells and debris. Cells were subsequently trypsinized and counted by trypan blue exclusion using a Countess automated cell counter (Invitrogen). Each sample or standard was added to each well and incubated overnight at 4°C with shaking. Wells were washed and incubated with the Biotinylated Sonic HH Detection antibody for 1 h at room temperature with gentle shaking. After washing, samples were incubated with a HRP–streptavidin solution for 45 min at room temperature with gentle shaking. After washing, samples were incubated with the TMB One-Step Substrate Reagent for 30 min at room temperature with gentle shaking in the dark. Finally, the absorbance was read at 450 nm after addition of the Stop Solution. The concentration of SHH in the media was calculated using the standard curve, and normalized to cell number.
Molecular Carcinogenesis

Breast cancer cell lines were seeded onto chamber slides (Nunc, Roskilde, Denmark) and grown overnight. Cells were treated with 50 nM DOC, and incubated for 4 h. Media was replaced with normal culture media and cells were incubated for an additional 20 h. Media was removed and cells or spheres were washed two times with 1XPBS. Following washes, cells were fixed with 100% methanol at −20°C for 5 min. Slides were rinsed with 1 ml of 1XPBS, and blocked with 1% BSA in PBS for 1 h at room temperature. The primary antibody, Gli-1 (clone H-300, Santa Cruz Biotechnology, Dallas, TX) was added at a 1:100 dilution in 1% BSA/PBS and was incubated overnight at 4°C. Slides were washed three times with PBS and incubated with Alexa-555 conjugated secondary antibody (Invitrogen). Slides were washed three times with PBS. DAPI Prolong Gold Antifade (Invitrogen) was added and coverslips were placed on slides. Slides were analyzed by immunofluorescence microscopy using a Zeiss motorized AxioObserver Z1 microscope.

Fluorescence Microscopy

Breast cancer cell lines were seeded onto chamber slides (Nunc, Roskilde, Denmark) and grown overnight. Cells were treated with 50 nM DOC, and incubated for 4 h. Media was replaced with normal culture media and cells were incubated for an additional 20 h. Media was removed and cells or spheres were washed two times with 1XPBS. Following washes, cells were fixed with 100% methanol at −20°C for 5 min. Slides were rinsed with 1 ml of 1XPBS, and blocked with 1% BSA in PBS for 1 h at room temperature. The primary antibody, Gli-1 (clone H-300, Santa Cruz Biotechnology, Dallas, TX) was added at a 1:100 dilution in 1% BSA/PBS and was incubated overnight at 4°C. Slides were washed three times with PBS and incubated with Alexa-555 conjugated secondary antibody (Invitrogen). Slides were washed three times with PBS. DAPI Prolong Gold Antifade (Invitrogen) was added and coverslips were placed on slides. Slides were analyzed by immunofluorescence microscopy using a Zeiss motorized AxioObserver Z1 microscope.

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Clonogenic Assays

Cells were seeded into 6 well plates at a density of 100–1000 cells per well. Cells were treated with 50 nM DOC, 10 μM GDC-0449, or a combination of 50 nM DOC and 10 μM GDC-0449. Plates were cultured for 10–14 days. Colonies (>50 cells) were fixed with formalin and stained with 0.5% crystal violet. Colonies were counted and colony-forming efficiency (colonies counted/cells plated) was determined.

Sphere Forming Assays

Breast cancer cell lines were seeded into 96 well low attachment plates at densities of 100–1000 cells/well in MEM supplemented with 1X B27 (Invitrogen), 20 ng/mL epidermal growth factor (EGF; Invitrogen), 20 ng/mL basic fibroblast growth factor (bFGF; Invitrogen) with and without the addition of 50 nM DOC, 10 μM GDC-0449, or 3 μg/ml 5E1, or 100 ng/ml recombinant SHH. Spheres were allowed to form for 7–10 days and counted. Spheres were dissociated by incubation in Acutase dissociation reagent (Invitrogen) for 10 min, washed with PBS and replated in low attachment plates for secondary sphere forming assays and retreated. Spheres were recounted 7–10 days later. Sphere forming efficiency was determined as number of spheres counted/original cell density plated.

Flow Cytometry Analysis

Cells were seeded with 50 nM DOC with and without addition of 5E1 and allowed to grow for 72 h. Control cells were mock treated with ethanol in PBS. Cells were trypsinized, washed with PBS containing 5% FBS and resuspended at a density of 1 × 10^6 cells/ml in cold PBS plus 5%FBS. Cells were stained with PE-conjugated CD44 (clone F10-44-2, Abcam) and FITC-conjugated CD24 (clone SN3, Abcam) for 30 min at RT. Samples were centrifuged, washed and passed through BD Falcon cell strainer cap tube for FACS analysis. Samples were analyzed on a BD FACS Aria II flow cytometer and samples were gated on an unstained control.

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism 6 software (Graph Pad, La Jolla, CA). The mean
values of data were evaluated using ANOVA followed by an unpaired test. For all tests, \( P \) values less than 0.05 were considered to be significant.

RESULTS

Hedgehog Expression Is Induced in Breast Cancer Cells Treated With Docetaxel

HH signaling is activated after chemoradiotherapy in esophageal cancer, and is associated with increased treatment resistance [18]. Thus, we sought to determine if HH signaling is activated in breast cancer after treatment with chemotherapy. We examined expression of SHH in breast cancer lines before and after treatment with DOC. These cell lines were chosen due to their low level of endogenous HH activity and their ability to undergo canonical HH activation as evidenced by Gli-1 nuclear translocation after treatment with SHH ligand (data not shown), and their differing levels of stem cell populations, low (T-47D, and MCF-7), moderate (SKBR3) and high (SUM159). Increased SHH protein expression was observed as early as 4 h after treatment with DOC and was maintained at 24 h post-treatment in SKBR3 and T-47D cells (Figure 1a and b). Increases in SHH protein expression were also observed 24 h after DOC in two other breast cancer cell lines, MCF-7 and SUM159 (Figure 1c and d). Release of SHH ligand was confirmed by ELISA (Figure 1e) in SKBR3 and T-47D cells. Increased concentrations of SHH levels were observed in supernatants of both cell lines after treatment with DOC, where levels peaked between 4–24 h for T-47D, but remained increased at 24 h in SKBR3 cells. Secretion of SHH correlated with increased Gli-1 expression and nuclear localization in all cell lines treated with DOC treated cells, as detected by immunofluorescence (Figure 2a and b; Supplementary Figure S1a and b). Classical HH activation occurs by ligand dependent activation of the PTCH receptor, leading to Gli-1 nuclear localization. However, in cancer cells there have been reports of noncanonical activation of Gli-1 [19]. In order to determine if DOC activation of Gli was due to ligand dependent HH signaling, we performed blocking studies with the SHH binding antibody 5E1. This antibody binds to the SHH at its pseudoactive site and interferes with binding to PTCH, blocking canonical activation of the pathway [20]. Nuclear expression of Gli-1 could be blocked by co-treatment of the SHH blocking antibody 5E1, indicating canonical pathway activation. Previous studies have shown that the HH receptor PTCH is a direct transcriptional target of Gli-1 and upregulation of PTCH expression is often observed in cells with activated HH signaling. As shown in Figure 2c and d, an increase in PTCH expression was observed in cells treated with DOC, suggesting an increase in nuclear Gli-1 activity. To confirm nuclear activation of Gli-1, we performed a Gli-1 luciferase reporter assay. Breast cancer lines were transfected with a firefly luciferase gene driven by a minimal CMV promoter and tandem repeats of Gli-1 response element to examine Gli-1 transcriptional activity with or without treatment with DOC. As shown, a statistically significant increase in relative luciferase activity was observed in DOC treated cells as early as 4 h after treatment (Figure 2e and f). At 24 h after treatment, a greater than threefold increase in Gli-1 reporter activity was observed in both cell lines. Gli-1 activity was significantly decreased by the addition of 5E1, indicating that the increase Gli-1 activity was associated with ligand-dependent HH signaling.

Inhibition of Hedgehog Signaling Does Not Sensitize Breast Cancer Cells to Chemotherapy

HH signaling has been reported to regulate expression of ABC drug transporters [14], increase expression of anti-apoptotic pathways [21] and has been shown to sensitize multiple tumor types to chemotherapy [14,15]. Therefore, we sought to determine if inhibitors to HH signaling could sensitize breast cancer cells to DOC treatment. Treatment of the breast cancer cell lines (Figure 3a–d) with 50 nM DOC or 10 μM of the SMO inhibitor vismodegib (GDC-0449) resulted in a decrease in cell survival at 72 h post-treatment compared to untreated cells. However, no synergistic effect was observed with the combination of DOC and GDC-0449, indicating that HH inhibition does not sensitize these cells to chemotherapy. Similar results were obtained with a SHH blocking antibody 5E1, indicating that inhibition of canonical HH signaling does not alter survival in response to chemotherapy. These results were further confirmed by an apoptosis assay in which inhibition of HH signaling failed to increase caspase 3 activation in response to DOC treatment (Figure 3e and f). These results indicate that although HH expression is activated in the majority of tumor cells after treatment with chemotherapy, this activation does not promote survival of the bulk of the tumor cells.

Hedgehog Expression Is Necessary for Clonogenic Expansion of Breast Cancer Cells After Chemotherapy

In order to further explore the role of HH signaling in recurrent breast cancer, we sought to determine the effect of HH inhibitors on colony formation of breast cancer cells with and without chemotherapy. Clonogenic assays were performed in SKBR3, T-47D and SUM159 cells treated with DOC, GDC-0449 or a combination of the two. As shown in Figure 4, treatment with either compound resulted in significantly reduced colony formation. In contrast to our previous study, which assessed short-term survival after treatment, the combination of DOC and HH inhibition had a synergistic effect on the growth of colonies after treatment, indicating that HH signaling is associated with survival and/or expansion of colony forming cells after chemotherapy.

Molecular Carcinogenesis
Figure 1. Release of SHH ligand by breast cancer cells after docetaxel treatment. Western blots of (a) SKBR3, (b) T-47D, (c) MCF-7 and (d) SUM159 cells showing upregulation of SHH protein expression after treatment with DOC. The intensities of each band were quantified and normalized to the housekeeping gene β-actin. (e) Results of SHH ELISA showing the fold increase in SHH release over mock-treated control cells in T-47D and SKBR3 cells. Error bars represent standard deviation.
Figure 2. Docetaxel induces canonical Hedgehog signaling. (a,b) Immunofluorescence analysis showing increased expression and nuclear translocation of Gli-1 (red) in (a) SKBR3 and (b) T-47D cells treated with DOC compared to control cells. Nuclei are stained with DAPI (blue). Nuclear staining of Gli-1 is observed in cells treated with DOC (pink). Expression was blocked by co-treatment with 5E1. (c,d) Western analysis showing upregulation of PTCH-1 expression in (c) SKBR3 and (d) T-47D cells. The intensities of each band were quantified and normalized to the housekeeping gene β-actin. (e,f) Gli-1 reporter assay showing increase in luciferase activity in (e) SKBR3 (f) and T-47D cells treated with DOC compared to control cells. Data is reported as ratio of firefly luciferase (Gli-activity) to renilla luciferase (transfection control). Error bars represent standard deviation. Significance is represented by asterisk: *P ≤ 0.05, **P ≤ 0.001.
Figure 3. Inhibition of Hedgehog signaling does not increase cell death in cells co-treated with docetaxel. (a–d) MTT assay of (a) SKBR3, (b) T-47D, (c) MCF-7 and (d) SUM159 cell lines treated with 50nM DOC, 10μM GDC-0449 or a combination of the two or 50nM DOC and 3μg/ml 5E1. Cell viability was determined 72 h following treatment. Data is represented as percentage of untreated cells. Error bars represent standard deviation. (e-f) Apoptosis assay showing caspase 3 activation in SKBR3 (e) and T-47D (f) cells treated with 50nM DOC, 10μM GDC-0449 or a combination of the two. Significant differences relative to untreated cells are represented by asterisks. *P < 0.05, **P < 0.001, ***P < 0.0001, n.s. indicates no significant difference between DOC treated cells and combination treatment.
Hedgehog Expression Is Necessary for Expansion of Breast Cancer Stem Cell Populations After Docetaxel Treatment

Treatment of breast cancer with taxane-based chemotherapies has been reported to increase cancer stem-cell populations among breast cancer cells [22–24]. CD44high/CD24low stem cell populations in breast cancer cell lines have been reported to have doubling times of 50–72 h [25]. Therefore, we sought to determine the effect of DOC on this population at 72 h post-treatment. Consistent with previous studies, we observed an increase in BCSC populations 72 h post-treatment with 50 nM DOC. Flow cytometry analysis revealed a 4.5 ± 0.7 and a 10.7 ± 2.6 fold increase in CD44+/CD24low in SKBR3 and T-47D cells, respectively, as compared to untreated cells (Figure 5a,b). Cotreatment with 5E1 abolished this increase, suggesting that HH signaling is driving proliferation of BCSC after chemotherapy. We also examined the expression of stem cell markers OCT-4 and SOX2 after treatment with DOC. Western analysis revealed a slight upregulation in global protein expression of both markers (Supplemental Figure S2a and b). To determine if differences in OCT-4 expression were evident in a subpopulation of cells, we employed an OCT-4 GFP reporter. Cells were transfected with a GFP reporter driven by the human OCT-4 promoter. We observed significant increases in OCT-4 positive cell populations using this method (Figure 5c and d; Supplemental Figure S2c–e) in all cell lines. Increases in OCT-4 transcription after DOC could be blocked by cotreatment with a HH inhibitor. These results are consistent with the previous experiment and indicate expansion of a minor resistant population of cells.

To further support the hypothesis that HH signaling is required for expansion of stem cell populations after DOC treatment, we examined mammosphere-forming efficiency in treated versus untreated cells. The ability to form non-adherent spheres is associated with self-renewal capacity; the number of spheres formed upon serial passage is representative of the number of mammary stem cells. Treatment of breast cancer cell lines with DOC or SHH ligand increased sphere formation (Supplemental Figure S3). A significant increase in sphere formation was observed in all cell lines in primary mammosphere forming assays (Figure 6a–d). Serial passage of mammospheres has been shown to increase stem cell markers and drug resistance [26]. Therefore, we sought to determine the effects of DOC in secondary sphere cultures. Primary mammospheres were dissociated and re-plated in mammosphere media containing DOC or DOC and HH inhibitors (Figure 6e and f). Consistent with our previous experiment, an increase in sphere formation was observed in DOC treated cells and this increase in stemness was blocked by the addition of 5E1 or GDC-0449.
Figure 5. Blocking of Hedgehog signaling prevents increases in stem-like populations observed after docetaxel treatment. (a,b) Flow cytometry analysis in (a) SKBR3 and (b) T-47D cells showing an increase in CD44^high^CD24^low^ cells 72 h after treatment with DOC. This increase could be blocked by co-incubation with 5E1. (c) Representative flow cytometry analysis of T-47D cells transfected with an OCT-4 GFP reporter. An increase in GFP positive cells is observed after treatment with DOC. This increase is blocked by addition of GDC. (d) Quantification of GFP positive cells in cell lines transfected with an OCT-4 GFP reporter. Significant changes in expression are marked with an asterisk. *P < 0.05, **P < 0.001, ***P < 0.0001. Unless otherwise indicated, significance is relative to control cells. Error bars represent standard deviation between three separate experiments.
Figure 5. (Continued)
Figure 6. Blocking of Hedgehog signaling prevents increases in mammosphere formation after docetaxel treatment. Reduction of sphere forming efficiency in primary and secondary sphere assays by co-treatment with DOC and GDC-0449 or 5E1 in primary mammosphere forming assays conducted in (a) SKBR3, (b) T-47D, (c) MCF-7 and (d) SUM159. Similar findings were observed in secondary mammosphere cultures in (e) SKBR3 and (f) T-47D cells. Data are reported as Mammosphere forming efficiency (MFE) which is the number of spheres counted/ number of cells plated. Error bars represent standard deviation. Significant differences relative to untreated cells are represented by asterisks. *P ≤ 0.05, **P ≤ 0.001, ***P ≤ 0.0001.
Recurrence of breast cancer after chemotherapy has been hypothesized to be driven by a subpopulation of breast cancer cells that resemble mammary stem cells [27]. Support for this theory comes from gene expression studies, which reveal an increase in stem-like signatures in residual breast tumors following NCT [28]. Furthermore, an increase in the population of tumor initiating cells bearing the CD44<sup>high</sup>/CD24<sup>low</sup> phenotype has been observed in recurrent breast cancers [27] and is associated with higher rates of metastasis and poor survival [27,29]. In this study, we show that treatment of breast cancer cells with DOC results in an increase of cells bearing a CD44<sup>high</sup>/CD24<sup>low</sup> phenotype. Furthermore, we show that SHH is released from breast cancer cells upon treatment and that activation of the HH signaling pathway is required for expansion of BCSC populations after chemotherapy.

HH signaling is important for self-renewal of stem cells from many tissues [12,30–32]. In breast cancer, treatment of cells with exogenous SHH ligand has been shown to increase mammosphere forming ability and self-renewal of breast cancer cells [12]. Furthermore, Liu et al. reported high expression of downstream HH family members Gli-1, Gli-2 and PTCH in CD44<sup>high</sup>/CD24<sup>low</sup> cells from breast cancer cell lines. Upon differentiation of these cells, HH activation significantly decreased [12]. HH signaling has been reported to induce expression of “stemness” related genes such as Oct-4, SOX2 and BMI-1, further supporting its role in stem cell self-renewal [33]. Although we found only a slight upregulation of stemness markers in the total cell population after treatment with chemotherapy, there was a HH dependent increase in a minor population of cells positive for OCT-4 expression.

Activation of HH signaling has been associated with poor outcome in breast cancer. High expression of SHH, Gli-1 and Gli-2 mRNA in primary breast tumors is positively correlated with recurrence [34]. In a study of 292 patients with ductal carcinoma of the breast, O’Toole et al. reported that high intensity staining of SHH in carcinoma cells was associated with higher rates of metastasis and breast cancer specific death [35]. In addition to its role in self-renewal of stem cells, HH signaling has also been reported to induce chemoresistance through the upregulation of ABC drug transporters, and increased expression of anti-apoptotic proteins in esophageal and pancreatic cancers [14,15,36]. In contrast to these studies, our studies show that HH signaling does not protect against chemotherapy induced cell death in breast cancer cell lines. However, HH signaling is necessary for expansion of resistant stem-like cells after treatment with taxane-based chemotherapies.

Taxane resistance in BCSC has been well documented. Similar to our findings, previous studies have reported enrichment in the proportion of stem like cells in breast cancer cell lines after treatment with paclitaxel [37]. Over expression of multidrug resistance proteins (MDR) MDR1 and ABCG2 in BCSC has been linked to resistance to many types of chemotherapy [38]. Additionally, increased expression of anti-apoptosis proteins including BCL-2 [39] and NFκB [40] as well as overexpression of cell cycle proteins associated with taxane resistance including Aurora A Kinase [41], CDK2 and Cyclin E1 [42] have been shown to contribute to taxane resistance in CD44<sup>high</sup>/CD24<sup>low</sup>/ populations. HH signaling has been shown to regulate many of these pathways in numerous cell types [14,15,21,43,44]. In particular, HH signaling has been shown to directly promote expression of MDR transporters in multiple tumor types [14,44]. In contrast to other tumor types, our data indicate that taxane-induced HH signaling does not promote upregulation of MDR in the bulk of breast tumor cells, which are destined to die despite activation of HH signaling. HH signaling is required for maintenance of ABCG2 expressing breast cancer side population cells [45], suggesting a role for it in the regulation of MDR expression in BCSC. The role of HH signaling in direct regulation of MDR expression by CD44<sup>high</sup>/CD24<sup>low</sup>/ populations has not been investigated. Whether taxane mediated activation of HH signaling mediates further increases in the expression of multi-drug resistance transporters in BCSC are currently under investigation by our group.

Other developmental and inflammatory pathways have been implicated in the expansion of BCSC cells after DOC treatment. Increases in TGF-β signaling have been reported in breast cancer cells after DOC treatment, which promote IL-8 dependent expansion of CD44<sup>high</sup>/CD24<sup>low</sup> populations and increased mammosphere forming ability [22]. Additionally, Zhang et al. reported an increase in ALDH1<sup>+</sup> and CD133<sup>+</sup>/CD44<sup>+</sup> stem-like subpopulations after DOC treatment in mouse models of breast cancer. This increase was associated to an increase in NOTCH signaling, and epithelial-mesenchymal transition (EMT) [23]. The interactions of these developmental signaling pathways with the HH pathway remains to be investigated. Further, it is unknown if DOC-induced HH activation treatment contributes to the increase in EMT observed upon treatment, as HH signaling has been implicated in the regulation of many EMT proteins [46].

Our results indicate that release of SHH by breast cancer cells after treatment with DOC promotes survival and expansion of BCSC, but has a limited effect on the bulk of the tumor cells. These results may indicate that HH activation may drive survival and proliferation of BCSC after chemotherapy treatment. These results may be important for determining treatment regimens combining chemotherapy and HH inhibitors. It is likely that HH inhibition may not have a significant effect on reducing tumor burden in...
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a neoadjuvant setting, but may rather inhibit the expansion of microscopic disease that may result in tumor recurrence. Future work will examine the role of HH activation in relationship to other developmental pathways and stem cell markers in animal models of breast cancer recurrence.

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SUPPORTING INFORMATION

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