Erythropoietin (EPO) is a hormone that induces red blood cell production. In its recombinant form, EPO is the one of most prescribed drugs to treat anemia, including that arising in cancer patients. In randomized trials, EPO administration to cancer patients has been associated with decreased survival. Here, we investigated the impact of EPO modulation on tumorigenesis. Using genetically engineered mouse models of breast cancer, we found that EPO promoted tumorigenesis by activating JAK/STAT signaling in breast tumor-initiating cells (TICs) and promoted TIC self renewal. We determined that EPO was induced by hypoxia in breast cancer cell lines, but not in human mammary epithelial cells. Additionally, we demonstrated that high levels of endogenous EPO gene expression correlated with shortened relapse-free survival and that pharmacologic JAK2 inhibition was synergistic with chemotherapy for tumor growth inhibition in vivo. These data define an active role for endogenous EPO in breast cancer progression and breast TIC self-renewal and reveal a potential application of EPO pathway inhibition in breast cancer therapy.
Erythropoietin promotes breast tumorigenesis through tumor-initiating cell self-renewal

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Erythropoietin (EPO) is a hormone that induces red blood cell production. In its recombinant form, EPO is the one of most prescribed drugs to treat anemia, including that arising in cancer patients. In randomized trials, EPO administration to cancer patients has been associated with decreased survival. Here, we investigated the impact of EPO modulation on tumorigenesis. Using genetically engineered mouse models of breast cancer, we found that EPO promoted tumorigenesis by activating JAK/STAT signaling in breast tumor-initiating cells (TICs) and promoted TIC self renewal. We determined that EPO was induced by hypoxia in breast cancer cell lines, but not in human mammary epithelial cells. Additionally, we demonstrated that high levels of endogenous EPO gene expression correlated with shortened relapse-free survival and that pharmacologic JAK2 inhibition was synergistic with chemotherapy for tumor growth inhibition in vivo. These data define an active role for endogenous EPO in breast cancer progression and breast TIC self-renewal and reveal a potential application of EPO pathway inhibition in breast cancer therapy.

Introduction

The glycoprotein hormone erythropoietin (EPO) is principally produced in the tubulointerstitial cells of the kidney in adults and is the chief regulator of erythropoiesis. EPO couples red blood cell production to tissue oxygenation through an oxygen-sensing system mediated by the HIF family of transcription factors, of which there are 3 family members (HIF1α, HIF2α, and HIF3α) (1). Genetic evidence suggests that EPO is regulated at the transcriptional level by HIF2α (2–5).

EPO activates the transmembrane EPO receptor (EPO-R), located primarily on erythroid progenitor cells (6–8). The EPO-R lacks intrinsic kinase activity and thus relies on its association with the intracellular kinase JAK2 to initiate context-dependent signal transduction cascades such as the PI3K, MEK/ERK, and STAT pathways (6–9).

Hypoproliferative anemia affects over 75% of all cancer patients and recombinant human EPO (rHuEPO) is FDA approved for the treatment of chemotherapy-induced anemia (10). Its positive effects on quality of life led to its widespread use in clinical oncology. However, recently, several prospective, randomized trials have shown that administration of erythropoiesis-stimulating agents (ESAs) during treatment results in both a shorter time to tumor progression and overall survival of cancer patients (11–17). Nonetheless, the negative impact of EPO in cancer patients remains controversial, as some other studies have also shown no detrimental effect of EPO if the target hemoglobin (Hgb) is approximately 10 g/dl (18–21).

A number of preclinical studies have examined the role of EPO with a wide range of in vitro findings (6–8, 22–27). In contrast, in various in vivo xenograft tumor models, exogenous EPO does not significantly promote tumor growth or result in resistance to therapy (6–8, 22, 23) with the exception of Lewis lung carcinoma xenografts and carcinogen-induced fibrosarcomas (28). Therefore, there is a disconnect between the in vitro results that suggest a protumorigenic role of EPO and the in vivo results, which do not.

We found that exogenous EPO did not have appreciable effects on cellular proliferation or protect from chemotherapy-induced apoptosis in vitro. In contrast, EPO promoted in vivo tumor progression of 2 autochthonous genetically engineered murine models (GEMMs) of breast cancer as well as cell lines derived from GEMM tumors when injected orthotopically. While the bulk populations of these tumor cell lines were not responsive to EPO in vitro, treatment of breast tumor-initiating cells (TICs) with EPO activated JAK/STAT signaling as well as promoted their self renewal. Finally, we show several lines of evidence that endogenous EPO plays a protumorigenic role in breast tumorigenesis. We show that breast cancer cells as well as tumor-associated endothelial cells can produce EPO and that EPO expression within human breast tumors correlates negatively with progression-free survival. Additionally, JAK2 inhibition cooperates with chemotherapy to decrease tumor growth. These findings suggest an active role for endogenous EPO in tumor progression and argue for a potential role of targeting the EPO pathway in breast cancer therapy.

Results

EPO does not affect human breast cancer cell lines in vitro. Based on previously reported data suggesting a protumorigenic role of EPO in
breast cancer, we wished to explore the impact of EPO in vitro. In our hands, neither proliferation nor cell-cycle progression were altered by increasing amounts of EPO (Figure 1, A and B). Similarly, EPO did not appear to protect against chemotherapy-induced apoptosis (Figure 1, C and D). Despite several of these cell lines expressing the EPO-R, exogenous EPO did not appear to appreciably increase JAK/STAT signaling (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI69804DS1). Thus, based on these assays, in our hands, EPO does not appear to be relevant for the in vitro growth properties or survival of breast cancer cells grown as monolayer cultures.

EPO decreases the survival of breast GEMMs but has no effect on GEMM-derived cell lines in vitro

The majority of in vivo studies have not demonstrated a protumorigenic effect of EPO (6, 7, 22, 23, 29–32). We noted that these studies utilized xenografts or carcinogen-induced tumors and hypothesized that GEMMs would serve as a more relevant context to address this issue, providing tumors arising in a native setting and allowing us to administer EPO for longer intervals. To this end, we administered clinically relevant doses of exogenous EPO that minimally elevated Hgb and Hct levels (Supplemental Figure 2A) to 2 independent breast cancer GEMMs (MMTV-Neu and C3-Tag) (33, 34). The percentage of mice living over time was significantly shorter in EPO-treated MMTV-Neu mice (P = 0.05, Figure 2A), with a median survival of 20.6 and 15.7 weeks in the saline- and EPO-treated groups respectively. Similar results were seen in the C3-Tag model (P = 0.04, Figure 2A). The decreased percentage of mice living appeared to be a reflection of a shortened tumor-free survival (Supplemental Figure 2, B and C). There were no apparent differences in H&E histology between PBS- and EPO-treated tumors (Figure 2A) or their proliferation and vascularity as assessed by Ki67 and CD31 staining (Supplemental Figure 3, A and B).

We next generated cell lines from C3-Tag tumors and obtained the NT2 line derived from MMTV-Neu tumors (E. Jaffee, Johns Hopkins University, Baltimore, Maryland, USA). We confirmed the origin of the C3-Tag cells by the detection of SV40 large T antigen (Supplemental Figure 4A). Similar to the human breast cancer cell lines, EPO did not affect proliferation (Figure 2, B and C) or chemotherapy-induced apoptosis (Figure 2, D and E) of these primary murine cell lines despite expressing detectable EPO-R
EPO increases the growth of orthotopically implanted C3-Tag and MMTV-Neu tumors. We next asked whether orthotopic reimplantation of luciferase-expressing C3-Tag cells [C3-Tag-luc] into the mammary fat pad would affect the cell’s sensitivity to exogenous EPO. To this end, C3-Tag-luc cells were orthotopically implanted into syngeneic female FVB/N mice. EPO significantly increased tumor burden at 21 days as measured by Xenogen IVIS (Figure 3, A and B) and caliper measurements (Supplemental Figure 5A) and was associated with the more rapid growth of established tumors (Supplemental Figure 5B) and a significantly decreased percentage of mice living over time (Figure 3D and E). Exogenous EPO also promoted the growth of NT2 cells orthotopically implanted into FVB mice (Figure 3C). Finally, exogenous EPO decreased the percentage of mice alive that had been tail-vein injected with C3-Tag cells (an experimental model of metastases) (Figure 3D) as well as C3-Tag tail-vein–injected mice concurrently treated with carboplatin (Figure 3E).
Thus, orthotopic implantation of C3-Tag or MMTV-Neu cells and metastatic C3-Tag models recapitulate the in vivo EPO responsiveness of autochthonous GEMM tumors.

**EPO increases mammosphere formation and promotes self-renewal.** We hypothesized that EPO’s tumor promoting effects are seen in vivo but not with in vitro assays because it affects a limited fraction of cells, such as breast TICs. Thus, its effects might only be seen with a longer period of EPO administration, such as those achieved in vivo. We hypothesized that if we enriched for the EPO-responsive fraction of cells, we might be able to see its protumorigenic effects more rapidly. Therefore, we compared the impact of exogenous EPO on the ability of FACS-sorted (Supplemental Figure 6) subpopulations of SUM149 cells: (a) CD44+CD24–EpCAM+ [TICs], (b) CD44+CD24 EpCAM- (non-TICs), and (c) CD44+CD24+EpCAM+ (non-TICs) to form mammospheres in vitro (Figure 4, A and B, and ref. 35). EPO significantly increased the number of spheres in the TIC fraction (Figure 4, A and B), but not in unsorted SUM149 cells or SUM149 cells from non-TIC fractions.

Furthermore, to determine the effects of EPO on breast TIC self renewal, PBS- and EPO-treated spheres were dissociated and passed for several generations. EPO-treated spheres had a higher sphere-forming efficiency and maintained a steady level of sphere-forming activity over serial passage (Figure 4C). In contrast, PBS-treated spheres had a progressive decrease in the capacity to form daughter spheres, with a significantly lower sphere-forming efficiency by passage 4. Thus, stringent isolation of breast cancer cell populations revealed that the effects of EPO are remarkably specific in breast cancer in vitro, promoting the self renewal of a highly tumorigenic TIC fraction.

**EPO promotes expansion of breast TICs in MMTV-Wnt1 breast tumors.** Previous studies have defined TICs from the autochthonous breast tumors of MMTV-Wnt1 mice (36). In keeping with the results in SUM149 cells, in vitro exposure to exogenous EPO appeared to increase sphere formation by MMTV-Wnt1 TICs (Lin-Thy1+CD24+) (Figure 4, D and E). We next wanted to see whether EPO can promote the expansion of breast TICs in vivo as well. To this end, we randomized female FVB/N mice with orthotopically implanted MMTV-Wnt1 tumors to PBS or EPO treatment (Figure 4F). After 4 weeks, the percentages of MMTV-Wnt1 TICs quantified by flow cytometry (Supplemental Figure 7A) were significantly higher in the tumors of mice that received EPO (Figure 4G and Supplemental Figure 7B). Moreover, cells from EPO-treated tumors showed...
increased tumor-initiating capacity by in vivo limiting dilution assays (Table 1) (tumor initiating frequency: PBS, 1 in 2,166 [95% CI of 1,205–3,896]; EPO, 1 in 673 [95% CI of 435–1,041]). These results in aggregate demonstrate that exogenous EPO promotes the expansion and self-renewal of breast TICs in vitro and in vivo.

**EPO stimulates JAK/STAT signaling in breast TIC.** EPO activates the JAK/STAT pathway in hematopoietic erythroid precursors, and recent work has shown that CD44^+CD24^- stem cell–like breast cancer cells require JAK2/STAT3 signaling for growth (37). We therefore asked whether exogenous EPO activates JAK/STAT signaling in breast TICs. We noted that SUM149 TICs had higher levels of EPO-R (Supplemental Figure 8) and higher basal levels of JAK/STAT activation, as evidenced by increased pSTAT3 in PBS-treated TICs relative to PBS-treated non-TICs (Figure 5A). In addition, EPO-treated TICs had induction of pJAK2 and pSTAT3, while EPO-treated non-TICs did not (Figure 5A).

Additionally, JAK/STAT activation in breast TICs resulted in changes in STAT-responsive genes, as microarrays performed on SUM149 TICs cultured overnight in the presence of EPO showed an enrichment of JAK/STAT gene signatures as well as gene signatures that characterize cancer stem cells or promote stem cell self-renewal, such as the notch and hedgehog pathways (Figure 5B). Therefore, treatment of breast TICs with exogenous EPO not only upregulates JAK/STAT signaling but also induces functional gene expression changes indicative of STAT, hedgehog, and notch transcriptional activation.
Neutralizing antibodies to EPO or EPO-R abrogate hypoxia-induced mammosphere formation. Hypoxia is known to increase TIC self-renewal, and EPO is a well-established hypoxia-inducible gene (38). To determine the contribution of endogenous EPO to hypoxia-induced mammosphere formation, we assessed sphere formation of SUM149 TICs in the presence of increasing concentrations of neutralizing antibodies to EPO, 2 independent EPO-R antibodies, or control antibody (anti-HA) (Figure 5, C and D). Both EPO and EPO-R neutralizing antibodies decreased EPO-induced ERK activation (Supplemental Figure 9) as well as hypoxia-induced mammosphere formation (Figure 5, C and D), suggesting that endogenous EPO and EPO-R signaling are necessary for the hypoxia-induced clonogenicity of breast TICs.

JAK2 inhibition abrogates EPO-induced mammosphere formation and is synergistic with chemotherapy in vivo. Exogenous EPO treatment of breast TICs promotes mammosphere formation (Figure 4, A–E) and JAK/STAT activation (Figure 5, A and B). We asked whether pharmacologic JAK2 inhibition using the orally available JAK2 kinase inhibitor TG101348 (TargetGen) (39) inhibits sphere formation. In keeping with the notion that JAK2 signaling is critical for mammosphere formation by breast TICs, we saw a dose-dependent reduction in sphere formation by TG101348 (Figure 5E).

While JAK2 inhibition may be effective at limiting breast TIC self-renewal (Figure 5E), the lack of JAK2 activation in the non-TICs suggested that it may not be effective on this population of cells. We therefore hypothesized that combining JAK2 inhibition with chemotherapy might be necessary to target TICs and non-TICs, respectively. In keeping with this notion, orthotopic Wnt1 tumors treated with the combination of carboplatin and TG101348 had a statistically significant decrease in tumor volume relative to vehicle treatment, treatment with single-agent carboplatin, or treatment with single-agent TG101348 (Figure 5F). Moreover, the addition of TG101348 to carboplatin did not appear to induce significantly more anemia (the primary dose-limiting toxicity of JAK2 inhibition) (Figure 5G). Therefore, the combination of chemotherapy with JAK2 inhibition appears to have potential for the treatment of breast cancer.

Endogenous EPO correlates negatively with relapse-free survival and is expressed by breast cancer cells and tumor-associated endothelial cells. We hypothesized that endogenous EPO exists in the tumor microenvironment and that EPO levels might therefore correlate with the clinical outcome of women with breast cancer. We interrogated 2 independent microarray data sets of early stage breast cancers (GEO GSE18229 and GSE26338) (40, 41) and found that high EPO expression was significantly associated with a decreased probability of relapse (Figure 6A) as well as enrichment in STAT gene signatures (Supplemental Figure 10). Thus, endogenous EPO and found that RNA from laser-captured, tumor-associated endothelial cells had higher levels of EPO than endothelial cells from adjacent normal tissue (Figure 6D and ref. 43). However, primary cultures of cancer associated fibroblasts (CAF) from human breast tumors of luminal (CAP9) and basal-like (CAF7J) breast cancers did not have appreciable hypoxia-inducible EPO mRNA or protein when cultured under hypoxia (Supplemental Figure 11, A and B), nor did HUVECs (Figure 6E).

In aggregate, these data enforce the notion of the biologic complexity of tumors and suggest that both breast cancer cells and tumor-associated endothelial cells may produce EPO and that increased EPO expression correlates with shortened relapse-free survival in breast cancer.

Discussion
rHuEPO was developed with the best of intentions. While it decreases the transfusion requirements and improves the quality of life of cancer patients, randomized trials have demonstrated that rHuEPO administration is associated with shorter progression-free and overall survival (11–17). A more definitive understanding of how EPO is protumorigenic is important. First, it would allow the design of novel ESAs that promote erythropoiesis but not tumorigenesis. Second, it might define whether all patients or only specific subgroups should be excluded from ESA use. Finally, it may give us insight into the role that endogenous EPO plays in tumor progression. Efforts to assess the protumorigenic effects of EPO have been particularly hampered by the lack of in vivo models to test hypotheses. Our experiments identify relevant GEMMs of breast cancer in which autochthonous breast tumors are sensitive to the growth-promoting effects of EPO and are sensitive to JAK/STAT inhibition.

Recent data from a loss-of-function screen identified the JAK2/STAT3 pathway as being necessary for the growth of basal-like, stem-like breast cancer cells and suggested that IL-6 was the primary mediator of normoxic JAK/STAT signaling (37). Notably however, breast TICs were never enriched for in this study, and therefore their data were derived primarily from comparisons of basal-like with luminal breast cancer cell lines. Our experiments therefore refine and extended these observations via a functional analysis of the effects of JAK/STAT signaling in a highly enriched population of TICs (the CD44+CD24 EpCAM− fraction) (35) and identify EPO as a hypoxia-induced activator of JAK/STAT signaling and cancer cell stemness. Along these lines, a previous report also demonstrated that EPO increased the numbers and self-renewal of breast cancer–initiating cells (CICs), yet in contrast to our studies, identified notch, rather than the JAK/STAT pathway, as a key regulator of self renewal (46).

Table 1
EPO promotes expansion of tumor-initiating capacity of breast TICs from MMTV-Wnt1 tumors

<table>
<thead>
<tr>
<th>Cells injected</th>
<th>2,000</th>
<th>1000</th>
<th>500</th>
<th>250</th>
<th>100</th>
<th>TIC capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS treated</td>
<td>5/6</td>
<td>3/10</td>
<td>2/12</td>
<td>1/13</td>
<td>0/6</td>
<td>1 in 2,166 (1,205–3,896)</td>
</tr>
<tr>
<td>EPO treated</td>
<td>6/8</td>
<td>10/6</td>
<td>6/12</td>
<td>3/13</td>
<td>1/6</td>
<td>1 in 673 (435–1,041)</td>
</tr>
</tbody>
</table>

*95% CI. Cells dissociated from PBS- or EPO-treated MMTV-Wnt1 orthotopic tumors were injected into the mammary fat pads of female FVB mice at the indicated cell numbers. Tumor-initiating capacity and CIs (95%) were calculated using L-Calc limiting dilution software 7 weeks after inoculation.

EPO expression in primary tumors correlates with clinical outcome.

While EPO is primarily produced in the kidney, a number of other tissues, a broad spectrum of cancer cell lines, and primary tumors have also been shown to produce EPO (6, 7, 42–44). We found that endogenous EPO was hypoxia inducible in breast cancer cell lines (Figure 6, B and C), but not human mammary epithelial cells (HMECs). Additionally, we examined several of the cellular compartments within breast tumors for their ability to produce endogenous EPO.
Figure 5
EPO activates JAK/STAT signaling in breast TICs, and JAK inhibition is a potential therapeutic target in breast cancer. (A) SUM149 cells were FACS sorted into TIC (CD44+CD24−EpCAM+) and non-TIC (not CD44+CD24−EpCAM+) populations, treated with PBS or EPO (1 IU/ml) for 10 minutes, and immunoblotted with the indicated antibodies. (B) Gene-expression profiles were generated from total RNA of sorted SUM149 TICs (CD44+CD24−EpCAM+), treated with PBS or EPO (1IU/ml) for 16 hours, and subjected to ssGSEA for the indicated JAK/STAT and cancer stem cell gene signatures. (C) Mammosphere formation of CD44+CD24−EpCAM+ SUM149 cells cultured at hypoxia (2% O2) in the presence of control antibody (anti-HA) or anti-EPO antibody at the indicated dilutions. (D) Mammosphere formation of CD44+CD24−EpCAM+ SUM149 cells cultured at hypoxia (2% O2) in the presence of control antibody (anti-HA) or 2 indicated anti-EPOR antibodies (1:200). (E) 20,000 (CD44+CD24−EpCAM+) SUM149 cells were plated in duplicate in the presence of EPO (10 IU/ml) and vehicle or EPO and the JAK inhibitor TG101348 at the indicated concentrations. Spheres were counted on day 5. (F) Orthotopic MMTV-Wnt1 tumors were grown to 5 mm in length and width (average tumor volume of all groups = 60 mm³) and treated with vehicle (NT), carboplatin (Carbo), TG101348 (TG), or the combination of carboplatin and TG101348. Caliper measurements were taken weekly. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. (G) Hematocrit (HCT) measurements were done at baseline and at end point in the indicated treatment groups.
A recent report suggests that EPO antagonizes treatment with the anti-HER2 antibody trastuzumab by activating EPO-R/JAK2 downstream effectors, effectively bypassing HER2 signaling (47). While mechanistically satisfying, this result only explains the therapeutic antagonism between EPO and trastuzumab in women with HER2+ breast cancer, which represents approximately 15%–20% of patients. Moreover, it should result in therapeutic antagonism only in women being actively treated with anti-HER2 agents. The fact that EPO’s negative effects have been seen in a wide variety of tumor types and in anemic cancer patients not undergoing treatment argue strongly that there are other, non-HER2–related ways in which EPO promotes tumor progression (17, 48). These clinical studies along with our data in both the C3-Tag and MMTV-Neu GEMMs are strong evidence that the negative effects of EPO are not merely a result of therapeutic antagonism to trastuzumab.

Finally, our studies support a role for endogenous EPO in breast tumorigenesis. Our studies suggest that both breast cancer cells and cancer-associated endothelial cells are potential sources of intratumoral EPO (Figure 7). While we did not see appreciable hypoxia-inducible EPO expression from CAFs, others have reported that tumor-associated stromal cells are a source of PDGF-dependent EPO production (49). Nonetheless, hypoxia-dependent breast TIC self renewal is dependent upon EPO and is correlated with relapse-free survival in breast cancer patients. Moreover, we identify the therapeutic potential of combining the JAK2 inhibitor TG101348 with carboplatin chemotherapy in vivo and postulate that this synergistic effect was seen because of the ability of TG101348 and carboplatin to target the TIC and bulk populations of breast cancer cells, respectively. This is particularly interesting in light of the fact that the JAK2 inhibitor ruxolitinib (Jakafi), is now FDA approved for the treatment of patients with JAK2 mutant myelofibrosis, and clinical trials using ruxolitinib are ongoing in women with breast cancer.

In summary, we have identified tractable GEMMs of breast cancer in which autochthonous tumors are sensitive to the protumorigenic effects of EPO. EPO promotes self-renewal and enhances TIC expansion of breast TICs in vitro and in vivo. Finally, our studies define an active role for endogenous EPO produced within tumors and show that JAK2 inhibition in combination with chemotherapy is a promising therapeutic strategy in the treatment of breast cancer.

Methods

Cell culture. MDA-MB231, SKBR3, and MCF7 cell lines were obtained from ATCC and cultured according to their recommendations. SUM149 cells (Asterand) were maintained in HuMEC basal medium and supplements (Gibco; Life Technologies) and 5% FBS and 100 U/ml penicillin/streptomycin. HMEC cells (50) were cultured in HuMEC ready medium (Gibco; Life Technologies). The C3-Tag cell line was established by dissociation of C3-Tag mammary tumors with 0.25% Trypsin, followed by differential trypsinization to remove fibroblasts. C3-Tag-luc cells were generated by stably trans-
protein assay, and 20 μg of whole-cell lysates was used for SDS-PAGE. Membranes were blocked with 5% milk or BSA diluted in 1 × TBST for 1 hour and incubated at 4°C overnight with one of the following primary antibodies: phospho-Jak2 (Tyr1007/1008) (cat. #3771; 1:1000; Cell Signaling Technology), Jak2 (D2E12) (cat. #3230, 1:1000; Cell Signaling Technology), phospho-Stat3 (Tyr705) (cat. #9145, 1:1000; Cell Signaling Technology), and Stat3 (cat. #8768; Cell Signaling Technology). Immunoblots against actin (sc-1615, 1:5000; Santa Cruz Biotechnology Inc.) were used as loading controls. The membranes were then probed with secondary antibodies (HRP-conjugated goat anti-rabbit and goat anti-mouse, cat. #31462 and #31432, respectively, 1:5000; Thermo Scientific), and immunoreactions were detected with Amersham Biosciences ECL (GE Healthcare).

EPO ELISA. Five million cells were plated in 10 cm dishes in 6 ml of serum-free medium and cultured under either normoxia (21% O2) or hypoxia (1% O2) for 16 hours. Media were then collected and concentrated to 200 μl using Ultra-15 centrifugal filter units (cut-off 10 kDa; Millipore). EPO levels were assayed in duplicate using a human ELISA kit (Stem Cell Technologies) following the manufacturer’s instructions.

Total RNA Isolation and real-time quantitative PCR. Total RNA was isolated using the RNAeasy Mini Kit (QIAGEN Sciences) following the manufacturer’s instructions. One microgram of purified total RNA was used for reverse transcription with ImProm-11 Reverse Transcription System (Promega) according to the manufacturer’s instructions. Expression of all transcripts was determined by using commercially available assays from Applied Biosystems. Transcript expression levels of human EPO (HS01071097), 18S ribosomal RNA (Hs03003631) and GAPDH protein assay, and 20 μg of whole-cell lysates was used for SDS-PAGE. Membranes were blocked with 5% milk or BSA diluted in 1× TBST for 1 hour and incubated at 4°C overnight with one of the following primary antibodies: phospho-Jak2 (Tyr1007/1008) (cat. #3771; 1:1000; Cell Signaling Technology), Jak2 (D2E12) (cat. #3230, 1:1000; Cell Signaling Technology), phospho-Stat3 (Tyr705) (cat. #9145, 1:1000; Cell Signaling Technology), and Stat3 (cat. #8768; Cell Signaling Technology). Immunoblots against actin (sc-1615, 1:5000; Santa Cruz Biotechnology Inc.) were used as loading controls. The membranes were then probed with secondary antibodies (HRP-conjugated goat anti-rabbit and goat anti-mouse, cat. #31462 and #31432, respectively, 1:5000; Thermo Scientific), and immunoreactions were detected with Amersham Biosciences ECL (GE Healthcare).

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Mammosphere assay. Mammosphere assays were performed according to the protocol from StemCell Technologies. Briefly, 20,000 sorted or unsorted SUM149 cells per well were grown in 6-well Ultra Low Attachment Plates (Corning) in 2 ml of Complete MammoCult Medium (MammoCult Basal Medium supplied with 10% Mammoctult proliferation supplement, 4 μg/ml heparin, and 0.48 μg/ml hydrocortisone; all purchased from StemCell Technologies). EPO was added (10 IU/ml) every other day, and spheres were counted on day 5.

neutralization experiments were performed using anti-EPO antibody (H-162; sc-7956; Santa Cruz Biotechnology Inc.). EPO-R neutralization was performed using anti-EPO antibodies (C-20 and M-20; Santa Cruz Biotechnology Inc.). TG101348 was synthesized by the UNC-CH Center for Integrative Chemical Biology and Drug Discovery based on its publicly available structure.

Serial sphere assay. Mammospheres were passed using 0.05% trypsin/0.53 mmol/l EDTA-4Na and filtered through a 40-μm cell strainer. 20,000 dissociated cells per well were replated in 2 ml mammosphere medium as above. Sphere-forming efficiency was calculated by dividing the number of mammospheres formed by the original number of single cells seeded and is expressed as a percentage. Each experiment was performed in triplicate.

MammTV-Wnt1 sphere assays. Mouse MMTV-Wnt1 tumors were minced with a razor blade and suspended in 10 ml of L-15 Leibovitz medium (Thermo Scientific) supplemented with 500 μl of collagenase/hyaluronidase (cat. #07912; Stem Cell Technologies). Tumors were digested for 2 hours at 37°C with pipetting every 30 minutes. Then, HBSS with 2% HICS was added to inactivate the collagenases. After pelleting, cells were treated serially with trypsin, dispase, DNase, and ACK lysis buffer. Cells were then stained with lineage cocktail (antibodies against CD31, CD45, and CD49f) and antibodies against CD49f and CD24 in HBSS with 2% HICS. Viable, Lin CD24hiCD49fhi cells were sorted and cultured in Matrigel with antibodies against CD140a and antibodies against CD49f and CD24 in HBSS with 2% HICS. Viable, Lin CD24hiCD49fhi cells were sorted and cultured in Matrigel with antibodies against CD140a and antibodies against CD49f and CD24 in HBSS with 2% HICS. Viable, Lin CD24hiCD49fhi cells were sorted and cultured in Matrigel with antibodies against CD140a and antibodies against CD49f and CD24 in HBSS with 2% HICS. Viable, Lin CD24hiCD49fhi cells were sorted and cultured in Matrigel with antibodies against CD140a and antibodies against CD49f and CD24 in HBSS with 2% HICS.

Mouse colony and orthotopic injections. FVB/NJ, C3-Tag, and MMTV-Neu mice were purchased from the Jackson Laboratory.

For the GEMMs of breast cancer studies, 6-week-old virgin C3-Tag mice were treated with i.p. injections of either saline (PBS) or Procrit (EPO; Amgen). MMTV-Neu mice began EPO treatment 4 weeks after parturition; mice were dosed i.p. 500 IU/kg biweekly (BIW). Mice were sacrificed when orthotopic tumors reached 5 mm in length and width, mice were randomized to treatment with vehicle, carboplatin (37.5 mg/kg/d, crow), or a combination of carboplatin and TG101348 (37.5 mg/kg/d and 240 mg/kg/d i.p. respectively).

LDA assay. MMTV-Wnt1 orthotopic tumors were dissociated and the indicated number of DAPI Lin+ tumor cells were then implanted into the mammary fat pads of FVB mice. Mice were observed weekly for 2 months for tumor formation. Tumorigenic cell frequencies were calculated using L-Calc (StemCell Technologies).

Complete blood counts. Hematocrit measurements were run on a Hemavet 950 hematology system (Drew Scientific).

Microarray and GSEA. TICs (CD44+CD24 EpCAM+) from SUM149 cells were sorted as described above. After sorting, 10,000 cells were reseeded in MammoCult Basal Medium and starved for 2 hours, then treated with either PBS or EPO (1 IU/ml) for 16 hours. Total RNA was extracted from 2 independent sortings. All samples were profiled as described previously using oligo microarrays (Agilent Technologies) and uploaded to NCBI Gene Expression Omnibus (GSE51433) (53). All microarray and patient clinical data are available in the UNC-CH Microarray Database (https://genome.unc.edu/pubsup/breastGEO/cli7calData.shtml). single-sample gene set enrichment analysis (ssGSEA) (Broad Institute) was performed on log-transformed median centered data. All gene sets are part of the Molecular Signature Database v3.1 curated gene sets (http://www.broad.mit.edu/gsea/msigdb/).

For additional information, see Supplemental Methods.

Statistics. Mouse Kaplan-Weier tumor-free or overall survival was analyzed using GraphPad Prism 4.0 software (GraphPad Software Inc.), and comparisons were made using the log-rank test. Tumor sizes were compared using an unpaired, 2-tailed t test. Human Kaplan-Weier relapse-free survival was analyzed in R (http://www.r-project.org/) using the library survival in the R project. All data are graphed as mean ± SEM, unless otherwise noted. All box and whisker plots represent 25th to 75th percentiles, with whiskers representing the range and the line denoting the median value. P < 0.05 was considered significant.

Study approval. All studies involving mice were performed with approval from the IACUCs at UNC-CH.

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