Contrasting Hypoxic Effects on Breast Cancer Stem Cell Hierarchy Is Dependent on ER-α Status

Hannah Harrison1, Lynsey Rogerson1, Hannah J. Gregson1, Keith R. Brennan2, Robert B. Clarke3, and Göran Landberg3,4

Introduction

Hypoxia plays a major role in cancer progression, and areas of hypoxia are common in both preinvasive and invasive breast cancer (1, 2). Tumor hypoxia is often linked to decreased survival in patients with breast cancer and has been shown to induce specific molecular changes in cells including changes that confer a more malignant phenotype such as increased proliferation (3), survival (4), invasion (5), and metastasis (6).

Cellular responses to hypoxia are mediated by the hypoxia-inducible factors (HIF). HIF-α is rapidly degraded in normoxia, meaning signaling does not occur. In hypoxia, however, HIF-α dimerises with HIF-β and activates downstream pathways including, but not limited to, VEGF (7), estrogen (8), and Notch (9).

Novel therapeutic strategies include inhibitors that target HIF-1α directly (10) or downstream targets such as VEGF. VEGF is required for angiogenesis and inhibitors, such as bevacizumab, show some therapeutic potential with increased disease-free survival. However, initially promising results from these drugs are short lived and generally followed by regrowth and progression with no increase in overall survival (11). It has also been noted that the hypoxia generated by angiogenesis inhibitors can result in more aggressive (12) and invasive (13) tumors.

Tumor hypoxia is often linked to decreased survival in patients with breast cancer and current therapeutic strategies aim to target the hypoxic response. One way in which this is done is by blocking hypoxia-induced angiogenesis. Antiangiogenic therapies show some therapeutic potential with increased disease-free survival, but these initial promising results are short lived and followed by tumor progression. We hypothesized that this may be due to altered cancer stem cell (CSC) activity resulting from increased tumor hypoxia. We studied the effects of hypoxia on CSC activity, using in vitro mammosphere and holoclone assays as well as in vivo limiting dilution experiments, in 13 patient-derived samples and four cell lines. There was a HIF-1α–dependent increase in ER-α–positive cancers following hypoxic exposure, which was blocked by inhibition of estrogen and Notch signaling. A contrasting decrease in CSC was seen in ER-α–negative cancers. We next developed a xenograft model of cell lines and patient-derived samples to assess the hypoxic CSC response. Varying sizes of xenografts were collected and analyzed for HIF1-α expression and CSC. The same ER-α–dependent contrasting hypoxic-CSC response was seen validating the initial observation. These data suggest that ER-α–positive and negative breast cancer subtypes respond differently to hypoxia and, as a consequence, antiangiogenic therapies will not be suitable for both subgroups. Cancer Res; 73(4); 1420–33. ©2012 AACR.
to assess the effects of hypoxia on breast CSC in cell lines and primary samples and to elucidate the mechanism by which these changes take place.

Materials and Methods

Patient samples

Tumors (MCRB Biobank, project ID:09_GOLA_02) were dissected into 1 mm pieces and incubated at 37°C for 16 hours in 1× collagenase/hyaluronidase mixture (Stem Cell Technologies) in Dulbecco’s Modified Eagle’s Medium (DMEM):F12/15 mmol/L HEPES (Sigma). Pleural effusion and ascites samples were collected during standard drain procedure. Cells were centrifuged at 200 g to collect epithelial cells and remove fibroblasts (22). Blood cells were removed using Lymphoprep solution (Axis Shield) and magnetic removal (Dynal Biotech). Cells were cultured in DMEM:F12/20% fetal calf serum (FCS)/0.1% nonessential amino acid solution/2.5 mmol/L L-glutamine/PenStrep. Where possible, cell suspensions were assessed for epithelial origin using Pan-Cytokeratin (DAKO) staining (Supplementary Fig. S1A and S1B).

Cell lines

MCF7, T47D, MDA-MB-231 (231), and MDA-MB–468 (468) were purchased from American Type Culture Collection. Lines were authenticated by multiplex-PCR assay using the AmpF/STR system (Applied Biosystems) and confirmed as mycoplasma free. Monolayers were grown in DMEM (DMEM/10% FCS/2 mmol/L L-glutamine/PenStrep, MCF7, and T47D), or RPMI medium (RPMI/10% FCS/1% sodium pyruvate/2 mmol/L L-glutamine/PenStrep). Cells were maintained in a humidified incubator at 37°C at an atmospheric pressure of 5% (v/v) CO2/air.

Hypoxic cell culture

Cells were incubated for 48 hours in the SCI-tiveN hypoxic workstation (Ruskinn) in 1% O2, 5% CO2, and 94% N2 in a humidified environment at 37°C. Cells were plated, cultured, and harvested within the workstation to maintain hypoxia at all times. Confirmation of hypoxic conditions was carried out using immunohistochemistry to measure expression of HIF-1α and qRT-PCR to assess upregulation of target genes at the RNA level (Supplementary Fig. S2A–S2C).

Mammosphere culture

Mammosphere culture was carried out as described in ref. (23), and spheres were counted on day 5 to avoid counting of any mammospheres that may have arisen from normal epithelial cells.

Clonogenic culture

Cells were plated at 50 cells per sq. cm in adherent conditions for 10 days. Colonies were fixed and stained with 1% crystal violet/70% EtOH and were identified microscopically. Colonies that had undergone 5 or more divisions, that is, containing 32 or more cells, were counted (24).

Western blotting

Protein was separated on an SDS–PAGE and transferred to Hybond-C Extra nitrocellulose membrane. Primary antibodies included: SP1-ER-α (RM-9101-S0, Thermo Fisher Scientific), Cleaved N1-ICD (100–401–407, Rockland), Jagged1 (Santa Cruz), Actin (Santa Cruz, sc-1616), and HIF-1α (610959, BD Biosciences). Densitometry was conducted using ImageJ software, which is freely available at http://rsb.info.nih.gov/ij/. Mean band intensity was measured and fold change from actin control was calculated.

Flow cytometry

A total of 1×10⁶ cells were resuspended in 1 mL and mixed with 5 μL of Aldefluor. A total of 0.5 mL was immediately transferred to a control tube containing diethylaminobenzaldehyde (DEAB). Samples were incubated at 37°C for 30 minutes before centrifugation to collect the cell pellet. Cells were then resuspended in assay buffer and analyzed using the FACS Calibur.

Quantitative Reverse Transcription PCR

RNA was extracted using the Qiagen RNAeasy kit according to the manufacturer’s instructions and quantified on the Nanodrop spectrophotometer (Thermo). cDNA was produced using the First Strand Kit (SABiosciences) and quantitative reverse transcription PCR (qRT-PCR) was conducted using RT kit (Sybergreen, SABiosciences) before analysis on the 7900 PCR machine (Applied Biosystems).

Inhibition of signaling

A total of 10 μmol/L YC1 (Cayman Chemicals), 10 μmol/L dibenzazepine (DBZ, a kind gift from Adrian Harris, The Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, in 0.5% Methocel/0.1% Tween 80), 1 μmol/L 4-hydroxytamoxifen, or 1 μmol/L gefitinib were added to monolayer culture at time of plating. HIF1 ON-TARGETplus-SMARTpool (L-004018-00-0010, Dharmacon) was used according to manufacturer’s instructions.

Inducible cell line production

For detailed description of cell line production please see ref. (25).

Transient ER-α expression

Cells were transfected with V16-ERtl (Addgene plasmid 11351; ref. 26) using Lipofectamine according to the manufacturer’s instructions. VP16 empty vector was used as a control. Cells were cultured in 21% or 1% oxygen for 48 hours following transfection and then plated in mammosphere culture.

In vivo tumor formation

Cells were injected subcutaneously into nude mice (MCF7, 231, and 468) and NOD scid gamma mice (primary metastatic, COB9). Ninety-day slow release estrogen pellets (0.72 mg) were implanted subcutaneously 2 days before injection (MCF7 only, Innovative Research of America). Upon collection, xenografts were cut and half of it was immediately placed in formalin for embedding. The remaining xenograft was dissociated in the same way as primary solid tumors.
A total of 20 mg/mL YC1 was administered via a single intraperitoneal injection when tumors reached approximately 100 mm³.

**Xenograft embedding and immunohistochemistry**

Tumors were formalin fixed and paraffin embedded. Antigen retrieval, blocking, and staining were carried out using the Biogenex i6000. Slides were blocked with hydrogen peroxide and casein before incubation with the primary antibody (HIF-1α—ab1, Abcam; Glut1—ab15309, Abcam). Slides were then incubated with Envision secondary followed by 3,3′-diaminobenzidine.

**Statistical analysis**

Data is represented as mean ± SEM taken over 3 independent experiments. Statistical significance was measured using parametric testing, assuming equal variance, in the majority of experiments with standard t tests for 2 paired samples used to assess difference between test and control samples. In the case of tumor growth rate, ANOVA with replicates was used for correlations. Pearson’s R² correlation was calculated for correlation between xenograft size, HIF-1α/Glut1, and mammosphere-forming cells (MFC).

**Results**

**The breast cancer cell hierarchy is affected by hypoxia**

To assess hypoxic effects on breast cancer cells, I3 patient-derived primary samples and 4 established cell lines were cultured in 21% or 1% oxygen. As physiologic oxygen levels vary greatly throughout the tumor, normoxia is extremely difficult to represent in vitro. With this in mind, atmospheric oxygen (21%) was compared with a more physiologic 8% to assess whether this would be suitable as a representation of “normoxia.” No alteration in CSC activity was seen at this oxygen level and, therefore, 21% oxygen was used to represent normoxia (Supplementary Fig. S3A). One percent oxygen was used to represent hypoxia, although levels of oxygen will also vary within hypoxic regions of tumors. Culture for 48 hours in hypoxia had no significant effect on proliferation or apoptosis (Supplementary Fig. S3B–S3D). CSC activity was assessed using well-established in vitro assays including mammosphere (25, 27) and clonogenic culture (28, 29) and fluorescence-activated cell sorting (FACS analysis of ALDH1 expression; ref. 30). CSC Activity was also measured in vivo using limiting dilution experiments.

MFC number increased significantly after hypoxic culture in all ER-α–positive primary samples and cell lines (Fig. 1A). In contrast to this, a significant decrease in MFC occurred in all ER-α–negative primary samples and cell lines. The same pattern was seen in an extended panel of 6 additional cell lines representing the same subtypes of breast cancer (Supplementary Fig. S3E). Inhibition of HIF-1α, with YC1 (ref. 31; Supplementary Fig. S4A and S4B), reduced the effect of hypoxic culture in all cell types (Fig. 1A) but had no effect in 21% oxygen (Supplementary Fig. S4C and S4D). The same effect was seen in cell lines using a targeted HIF1 siRNA (Fig. 1B).

As hypoxia is known to increase anoikis resistance (14), adherent clonogenic culture was used to verify that the changes in MFC were due to altered CSC activity rather than increased survival of non-CSC in nonadherent culture (ref. 29; Supplementary Fig. S5A–S5E). The same hypoxic response pattern was seen with the CSC-enriched fraction of holoclone-forming cells (HFC) increasing in ER-α–positive lines and decreasing in ER-α–negative lines after hypoxic culture (Fig. 1C). Treatment with YC1 had no effect in normoxic culture but blocked the hypoxia induced clonogenic changes in MCF7, 231, and 468 returning the HFC number to controls levels (Fig. 1C). The HFC remained significantly increased in T47D cells treated with YC1, but the effect was markedly reduced.

Numerous cell surface markers have been shown to identify a population that is enriched for CSC (25, 30, 32). We measured the expression of ALDH1 in cell lines following normoxic and hypoxic culture and, in support of the MFC and HFC data shown above, show increased ALDH1–positive cell number in ER-α–positive lines and a contrasting decrease in ER-α–negative lines (Fig. 1D).

Tumor initiation is considered to be the gold standard for CSC measurement, and so an in vitro limiting dilution experiment was carried out to assess changes in tumor-initiating cell number following hypoxic culture. Fifty percent tumor formation required 3.1 × 10⁵ hypoxia pretreated MCF7 cells, whereas 6.5 × 10⁵ control MCF7 cells were required for the same level of tumor formation suggesting a 2-fold increase in tumor-initiating cells (Fig. 1E). Conversely, 1.5 × 10⁶ hypoxia pretreated 468 cells are required for 50% tumor formation compared with 9.2 × 10⁵ control cells suggesting a 0.6-fold decrease in tumor-initiating cells following hypoxic exposure (Fig. 1E). Because of small sample size, the changes in tumor-initiating cell number were not significant (P > 0.05), but these data are supportive of the in vitro findings and suggest that hypoxia may have a positive effect on the tumor-initiating cell population in ER-α–positive breast cancers and a negative effect in ER-α–negative tumors.

**Hypoxia has long-term effects on the cell hierarchy**

We previously showed that the breast cancer cell hierarchy is strictly maintained and, when altered, the proportion of CSC returns to normal within 5 to 7 days (33). We were interested, therefore, to see whether the hierarchy changes seen in hypoxia would revert to normal once cells were returned to 21% oxygen. Following in vitro culture in hypoxia, cells were reexposed to...
atmospheric oxygen for up to 10 days before mammosphere culture. At all time points, MFCs remained significantly increased in ER-α-positive cells, which had previously been treated in hypoxia and significantly decreased in ER-α-negative cells (Fig. 1F). This finding shows that the hypoxia-induced changes are not immediately reversed when cells are reexposed to oxygen suggesting long-lasting effects on the cellular hierarchy.

**Estrogen signaling plays a role in the hypoxic CSC response**

As distinct hypoxic responses were observed between ER-α-positive and negative breast cancers, we hypothesized that the response seen in ER-α-positive cancers was downstream of ER-α. To confirm ER-α activation, known estrogen-responsive genes were assessed by qRT-PCR following hypoxic culture. Significant upregulation of AREG and TFF1 were seen in both MCF7 and T47D cells as well as upregulation of PIP in MCF7 cells only (Fig. 2A). To assess whether the ER-α activation is necessary for HIF-1α-induced CSC changes, signaling was inhibited with 4-hydroxytamoxifen (Tam). Tam significantly reduced the hypoxic-MFC increase in all ER-α-positive cells tested, although in 2 of 6 primary cases, the MFC remained significantly raised compared with control (Fig. 2B).

No effect was seen in ER-α-negative cells. These data support the hypothesis that ER-α plays an important role in the hypoxic CSC response in ER-α-positive cells and that signaling through this pathway is responsible, at least in part, for the increase in CSC.

It has previously been reported that the CSC within ER-α-positive breast cancer have low or no expression of ER-α (34) similar to normal breast stem cells (SC). In the normal breast, estrogen-activated ER-positive cells release amphiregulin, which binds to EGFR in the SC facilitating their response to estrogen despite their ER-negative status (35). To assess whether this is occurring within breast cancer cells in response to hypoxia-induced activation of ER-α, EGFR signaling was blocked with the inhibitor gefitinib. Similar to Tam, gefitinib blocked the hypoxic effect in ER-α-positive primary samples and cell lines but had no effect on ER-α-negative primary samples or cell lines (Fig. 2C). These findings are supportive of the hypothesis that HIF-1α activates ER-α signaling resulting in a downstream paracrine response through EGFR.

**Differential effects on gene expression were identified in ER-α-positive and negative cell lines**

Custom PCR array plates (SABiosciences, Supplementary Table S1) were used to assess changes within the total cell and CSC populations following hypoxic culture. The plates contained genes associated with proliferation and apoptosis, to confirm our in vitro findings that no changes occurred, as well as genes known to identify/enrich for CSC and those involved in CSC signaling. CSC enrichment was achieved by collection of aneikis-resistant cells as we have previously shown this population to be highly enriched for mammosphere and tumor-initiating cells (25). Cell lines were cultured for 48 hours in 21% and 1% oxygen and RNA was collected (termed total population). Alternatively, following normoxic and hypoxic culture, cells were plated in nonadherent culture for 16 hours and RNA was harvested from the CSC-enriched cells (termed aneikis-resistant (AB) population; Supplementary Fig. S6A shows experimental design). Three independent experiments were carried out for each cell line.

Comparison of the total population following hypoxic and normoxic culture showed significant gene changes in both ER-α-positive and negative lines (Table 1). It is interesting to note that when comparing these gene expression changes between ER-α-positive and negative lines, very little correlation was seen between the cell types, which is supportive of previously published data (36). Furthermore, no changes in expression of genes involved in proliferation or apoptosis were seen in the total population in any lines supporting our in vitro findings. These data also add further support to the activation or ER and Notch signaling in hypoxia with increased expression of ER and Notch target genes (highlighted in Table 1) in the ER-positive lines.

Significant changes in gene expression between aneikis-resistant and total population cells are shown in Table 1. Increased expression of genes such as ABCG2 (37), CD44 (32), DNER (38) and ALDH1 (30), and decreased expression of ESRI (34) support the use of this CSC enrichment method as all of these changes are expected in a CSC-enriched population. No significant differences were seen between the normoxic and hypoxic aneikis-resistant populations collected from MCF7 cells and a single significant gene change (PIB) was seen in T47D (Table 1) suggesting that the CSC-enriched population remains virtually unchanged following hypoxic culture and the increase in MFC, HFC, and tumor initiating cells is, therefore, due to expansion of the population, perhaps by increased symmetric self-renewal of the CSC or de-differentiation of early progenitor cells, rather than simply the acquisition of aneikis resistance in non-CSC.

In the case of ER-α-negative cells, however, there is a small but significant decrease in three genes; PITG1, JAG1 and KIF6 (Table 1) suggesting that the population is altered in hypoxia. Although small, the decrease in KIF6, for example, could suggest altered proliferation within the CSC enriched population and may explain the reduced CSC number in these cells as the total population continues to proliferate at a normal rate.

**Notch signaling is required for the hypoxic CSC response in ER-α-positive cells**

We, and others, have previously reported that Notch activation plays a role in maintenance and proliferation of breast CSC (25) and that Notch1 is a downstream paracrine mediator of ER-α (39). The gene expression data presented here also show upregulation of Notch signaling within ER-α-positive lines with increased expression of HES1 and JAK1 in response to hypoxia (Table 1). Activation of Notch signaling by hypoxia was further assessed by measuring the levels of activated Notch1-intracellular domain (N1-ICD) and downstream targets at the protein and RNA level. N1-ICD was increased in all ER-α-positive primary cells and lines following hypoxic culture (Fig. 3A). There was a small but significant decrease in ER-α-negative cells. Increased expression of JAG1, a ligand and
downstream target of the Notch pathway (40), in ER-α-positive lines, as well as upregulation of HES1, HEY2, and HEYL, gave further evidence for activation of Notch signaling (Fig. 3B and C).

To confirm that Notch plays a role in the hypoxic response of CSC, signaling was inhibited using a gamma secretase inhibitor (GSI), dibenzazepine, during monolayer culture. Addition of GSI significantly decreased MFC in all ER-α-positive samples.
**Table 1.** Significant gene expression changes in MCF7, T47D, and 231 cell populations

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and cell lines cultured in 1% oxygen reducing the effect of hypoxic culture. No significant effect on the hypoxic response was seen in ER-α–negative samples or lines and MFC remained at the same, low levels (Fig. 3D). To further verify Notch’s role, the pathway was inhibited using inducible shRNA cell lines (Supplementary Fig. S6B). Interestingly, when signaling through Notch1 and Notch4 were specifically blocked only inhibition of Notch1 reduced the hypoxic CSC response (Fig. 3E). This may suggest that the effect seen is occurring within an early progenitor cell population as Notch4 is thought to regulate the most primitive CSC population (25).

Expression of ER-α or N1-ICD causes a positive hypoxic CSC response in ER-α–negative cells

Our data suggest that estrogen and Notch1 regulate the hypoxic CSC response in ER-α–positive cells. We therefore asked whether activation of these pathways in ER-α–negative lines would modify their hypoxic CSC response. ER-α was transiently overexpressed in 231 and 468 cells using the V16-ERα plasmid (Addgene #11351) and expression was confirmed by Western blot analysis (Fig. 4A). Following hypoxic culture, the percentage MFC was significantly increased in both 231-V16 and 468-V16 (Fig. 4B).

Next, to assess the effect of Notch1 activation, 231 cells were produced that stably expressed doxycycline-inducible N1-ICD (231-YNICD; ref. 25). A significant increase in MFC was observed in 231-YNICD cells cultured in hypoxia similar to that seen in ER-α–positive cells (Fig. 4C).

These data support the hypothesis that HIF1α-ERα-Notch1 activation is responsible for the increased CSC seen in ER-α–positive cells and suggest that ER-α–negative cells can be converted to show a positive hypoxic CSC response by expression of ERα or Notch1.

The hypoxic effect on CSC can be modeled in vivo

In vitro analysis of CSC activity is a valuable and accurate technique, but an in vitro model with varying degrees of hypoxia would allow more realistic analyses of the link between tumor growth, CSC and the potential divergent influence of hypoxia on CSC activity. To mimic the in vitro hypoxic response in vivo, we developed a xenograft model based on the hypothesis that with increasing xenograft sizes, there will be a parallel increase in hypoxia due to rapid cell proliferation and restricted blood supply to the xenografts. MCF7, 231, and 468 lines and an ER-α–positive metastatic sample (COB) were implanted subcutaneously into mice, and xenografts were harvested at different time points; half was embedded in paraffin for immunohistochemical analysis, whereas half was dissociated for mammosphere culture. HIF-1α and GLUT1 staining was quantified by image analyses (Supplementary Fig. S7A and S7B) and a significant correlation between tumor size and both HIF-1α and Glut1 expression was observed in xenografts produced from all cell types validating the hypothesized link between xenograft size and the presence of hypoxia (Fig. 5A).

In ER-α–positive xenografts (MCF7 and COB, primary metastatic cancer cells), size was strongly correlated with MFC, and as the xenograft increased in volume, the proportion of MFCs within the tumor increased (Fig. 5Bi). Importantly, an inverse correlation was observed for ER-α–negative lines with a decrease in the proportion of MFC within the xenograft as tumor size increases (Fig. 5Bii). No correlations were seen

Table 1. Significant gene expression changes in MCF7, T47D, and 231 cell populations (Cont’d)

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aLog2, known estrogen-responsive genes.

bLog2, known Notch targets.
Figure 3. Hypoxic effect requires Notch signaling in ER-α-positive cancers. Densitometric analysis of 3 independent Western blots for activated Notch1 (N1-ICD; A) and Jagged1 (B) protein levels. C, expression changes of known Notch-responsive genes. D, following 48 hours in 21% or 1% oxygen, C6 GSI cells were plated in mammosphere culture. E, following 48 hours in 21% or 1% oxygen C6 Doxycycline (Dox)-inducible shRNA lines to Notch1 (MCF7Notch1) and Notch4 (MCF7Notch4) were plated in mammosphere culture. Mean ± SEM, P < 0.05.
between MFC number and growth rate or total viable cell number extracted (Supplementary Fig. S7C and S7D).

Intraperitoneal injection of the HIF-1α inhibitor, YC1, following tumor initiation reduces the hypoxic CSC effect in MCF7 xenografts and results in no correlation between xenograft size and MFC (Fig. 5C).

This xenograft model fully supported our in vitro findings of a contrasting link between hypoxia and CSC in ER-α–positive and negative breast cancer subtypes.

Discussion

We describe differing CSC responses to hypoxia in ER-α–positive and negative breast cancer with ER-α–positive cancers gaining increased CSC activity and ER-α–negative cancers showing reduced CSC. Earlier reports support the observed hypoxia induced increase in CSC in breast cancer cell lines (18–20), but the ER-α–dependent contrasting reaction has not been reported elsewhere. Although the differing effects of hypoxia reported here are in conflict with previously published data, where no contrasting effect was seen (18–20), our data was produced using 13 primary breast cancer samples, as well as established cell lines, and the opposing effects in hormone receptor–positive and negative tumors, measured using multiple in vitro and in vivo CSC assays, were consistent in all cases. Similar contrasting effects of hypoxia have been reported elsewhere relating to other key biological features such as cell-cycle regulation, growth, migration, and gene expression (6, 20, 41). The reason for these differential effects of hypoxia may be due to the complex regulation of HIF-1αt activities by various subunits, postranslational modifications, and the presence and effects of coregulators, which are known to vary between different cell types (6).

Interestingly, the observed effects of hypoxia were relatively long lasting (Fig. 1F) and were not reversed immediately upon re-exposure to oxygen. This suggests that occasional exposure to hypoxia either induced by treatments or endogenously present, as often observed in ductal carcinoma in situ and in high-grade tumors, could have profound long-term effects on the CSC content and consequently tumor aggressiveness. The findings may also elucidate the divergent behaviours of breast cancer metastases with the existence of late recurrences in ER-α–positive breast cancer but fewer late events in ER-α–negative cases. Dormant tumor cells in a hypoxic bone marrow niche (19) will be enriched for CSC in ER-α–positive breast cancer but depleted in ER-α–negative cancers profoundly influencing the capacity for late disease recurrence. How these long term effects occur is currently unknown and this requires further investigation. One possibility is that hypoxia induces epigenetic changes within the cells that can persist for some time. Similar lasting effects have previously been reported as risk factors for other diseases such diabetes with temporal spikes in glucose stress leading to long-term activating epigenetic changes in p65 and NF-κB (42).

To mechanistically explain the effect of hypoxia on CSC, we assessed the roles of ER-α and Notch signaling. Inhibition of ER-α blocked the hypoxic CSC response in all ER-α–positive cells, supporting the role of estrogen signaling in the hypoxic response. We also show that, like in the normal breast (35), estrogen signaling causes downstream activation of EGFR-ERK signaling and that this is required for the hypoxic response. Our findings suggest that this is also true in breast cancer as EGFR inhibition blocked the hypoxic CSC effect in ER-α–positive tumors.

Notch inhibition also blocked the hypoxic CSC effect in all ER-α–positive patient-derived samples and cell lines and...
specifically, Notch1 seemed to be central for the hypoxic CSC response. In the case of ER-α–negative breast cancer, no activation of Notch was observed suggesting, in this setting, that Notch is activated downstream of ER-α. There are contradictions in the literature regarding crosstalk between the estrogen and Notch pathways and some reports suggest that estrogen activation inhibits Notch signaling (43) whereas others, in line with the findings in this study, report Notch activation by estrogen (39). It is possible that Notch regulation by estrogen is context dependent, but our data clearly show that hypoxia activates estrogen signaling, resulting in increased Notch1 cleavage and activation of downstream targets.
As the mechanistic studies suggest that ER-α and Notch are central in mediating the unfavourable hypoxic CSC response in ER-α-positive breast cancer, they may offer an attractive combination treatment approach, which would consist of ER-α or Notch inhibitors combined with antiangiogenic drugs. This would bypass the potential adverse effects of hypoxia on the CSC fraction while limiting tumor progression due to the general vasculature effects.

Understanding the inherent negative hypoxic CSC response in ER-α-negative tumors will be of great value for the development of additional novel treatments as activating this response alone in cancer cells or alongside antiangiogenic treatments may offer a more successful treatment with loss of CSC activity and reduced breast cancer recurrences. There may be a certain level of complexity in elucidating the mechanisms involved in this negative response as we have shown that the response can be easily altered with expression of either ER-α or N1-ICD, and this suggests that the intrinsic hypoxic response of these cells can be overcome quite simply.

An important question is how the CSC fraction is altered by hypoxia. Changes in the CSC fraction in relation to non-CSC could be due to altered proliferation, self-renewal, or cell death rates or dedifferentiation of non-CSC into a less mature cell state any of which would result in a change to the balance between the 2 compartments (44, 45). Figure 6A and B show putative models of the hypoxic effects in breast cancer. As we did not observe any proliferation or apoptosis differences in the non-CSC or the CSC-enriched fraction in ER-α-positive cell lines, we hypothesize that either self-renewal symmetry is altered or cells are caused to dedifferentiate resulting in the increased CSC numbers seen. For ER-α-negative breast cancer, there was a slight difference in proliferation for the CSC-
enriched cells suggesting that for this subtype of breast cancer, it may be a combined differentiation and proliferation changes within different subpopulations causing the decrease in CSC under hypoxia, but this requires further investigation.

Our xenograft experiments clearly show that as xenograft size increases, and therefore, extent of hypoxia within the tumor increases, there is a profound effect on CSC number. However, the direction of this effect is dependent upon the ER-α status of the tumor mimicking our *in vitro* data. These results clearly indicate that analysis of future xenograft experiments should take careful note of the size of the xenografts as well as how hypoxia influences CSC in the specific cell line used. On the other hand, the model system presented within this study will be important in examining the effectiveness of new agents that affect the hypoxia-driven changes in CSC number in ER-α–positive and negative breast cancer.

Inhibiting angiogenesis, and thus limiting nutrient supply to a tumor, is an attractive method to treat cancer and so treatments targeting HIF-1α or VEGF have been developed and are currently in clinical trials (10, 11). Both of these novel therapies have, however, shown varying clinical results with disappointing cancer recurrences after initially promising responses (13, 46). The data presented in this article suggests that tumor subtype needs to be taken into account during treatment design as blocking angiogenesis and therefore increasing tumor hypoxia may be beneficial in the treatment of ER-α–negative breast cancer but could have catastrophic effects on ER-α–positive breast cancer. This is supported by recently published data in the *New England Journal of Medicine* (21), where patients with triple-negative breast cancer were seen to show increased complete pathologic response to bevacizumab treatment, with no detectable tumor at primary or metastatic sites, whereas no improvement was observed in hormone receptor–positive cases.

As ER-α is considered a reliable biomarker in immunohistochemical analyses and is already used to guide endocrine treatment it would be a suitable treatment predictive marker for antiangiogenic therapies. Future studies need to clarify whether smaller subgroups of breast cancer, defined according to expression array criteria, will behave in ways similar to ER-α–positive or negative breast cancer but it is clear that the 2 major disease subgroups, defined by ER-α status, show contrasting hypoxic CSC responses.

In summary, our novel data suggest that ER-α–positive and negative breast cancer subtypes respond differently to hypoxic exposure and as a consequence, anti-HIF-1α or antiangiogenic therapies will not be suitable for both subtypes. Finally, the findings will form a foundation for novel cancer treatment principles targeting or enhancing the hypoxic CSC response within various combination treatment approaches.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

Conception and design: H. Harrison, K.R. Brennan, G. Landberg

Development of methodology: H. Harrison, R.B. Clarke, G. Landberg

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Harrison, L. Rogerson, H.J. Gregson, R.B. Clarke

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Harrison, L. Rogerson, K.R. Brennan, G. Landberg

Writing, review, and/or revision of the manuscript: H. Harrison, K.R. Brennan, G. Landberg

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Harrison, L. Rogerson, H.J. Gregson

Study supervision: H. Harrison, K.R. Brennan, G. Landberg

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Contrasting Hypoxic Effects on Breast Cancer Stem Cell Hierarchy Is Dependent on ER-α Status

Hannah Harrison, Lynsey Rogerson, Hannah J. Gregson, et al.


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