Title

Contrasting hypoxic effects on breast cancer stem cell hierarchy is dependent on ERα status

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Running Title

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Abstract

Tumour hypoxia is often linked to decreased survival in breast cancer patients and current therapeutic strategies aim to target the hypoxic response by, for example, blocking hypoxia induced angiogenesis. Anti-angiogenic therapies show some therapeutic potential with increased disease free survival but initially promising results are short lived and followed by tumour progression with no increase in overall survival. We hypothesised that this may be due to altered cancer stem cell (CSC) activity resulting from increased tumour 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 4 cell lines. There was a HIF1-alpha-dependent CSC increase in ER-alpha-positive cancers following hypoxic exposure which was blocked by inhibition of oestrogen and Notch signalling. A contrasting decrease in CSC was seen in ER-alpha-negative cancers.

We next developed a xenograft model of cell lines and patient-derived samples to assess the hypoxic-CSC response *in vivo*. Varying sizes of xenografts were collected and analysed for HIF1-alpha expression and CSC. The same ER-alpha-dependent contrasting hypoxic-CSC response was seen validating our novel observation.

These data suggest that ER-alpha-positive and negative breast cancer sub-types respond differently to hypoxia and, as a consequence, anti-angiogenic therapies will not be suitable for both subgroups.

Introduction

Hypoxia plays a major role in cancer progression and areas of hypoxia are common in both pre-invasive and invasive breast cancer (1, 2). Tumour hypoxia is often linked to decreased survival in breast cancer patients as 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 hypoxic-inducible factors (HIF). HIFα is rapidly degraded in normoxia meaning signalling does not occur. In hypoxia, however, HIFα dimerises with HIFβ and activates downstream pathways including, but not limited to, VEGF (7), oestrogen (8) and Notch (9).

Novel therapeutic strategies include inhibitors that target HIF1α 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 re-growth 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) tumours.

How tumour cells gain resistance and/or avoid these therapies and which patients may benefit from these treatments is currently unknown. One possible mode of therapy avoidance arises from the increased hypoxia within the tumours following anti-angiogenic treatments. Breast cancer cells which survive in hypoxia share many characteristics with breast cancer stem cells (CSC) such as loss of oestrogen receptor-alpha (ERα) expression (1),

increased anoikis resistance (14) and increased resistance to radiotherapy (RT) and chemotherapy (CT) (15, 16). Hypoxia is also known to activate CSC pathways, such as Notch (11), and to initiate epithelial-mesenchymal transition (EMT) (17). Recent reports show increased CSC activity following hypoxic exposure in breast cancer cell lines (18-20) but no reports have studied this rare population in primary human breast cancer samples.

We hypothesised that breast CSC numbers would be altered in hypoxia via dedifferentiation of non-CSC or by expansion of the CSC sub-population. As it is known that patients respond differently to anti-HIF1α and anti-angiogenic treatments (21) we proposed that these changes would not be consistent in all sub-types of the disease. The aims of this study, therefore, were 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.

Experimental Procedures

Patient Samples

Tumours (MCRC Biobank, project ID:09_GOLA_02) were dissected into 1mm pieces and incubated at 37^0C for 16 hours in 1x Collagenase/Hyaluronidase mixture (Stem Cell Technologies, USA) in DMEM:F12/15mM HEPES (Sigma). Pleural effusion and ascites samples were collected during standard drain protocol with fully informed consent (Ethics#05/Q1403/159). Cells were centrifuged at 200g to collect epithelial cells and remove fibroblasts (22). Blood cells were removed using Lymphoprep solution (Axis Shield) and magnetic removal of CD45 positive cells according to the manufacturer's instructions (Miltenyi Biotech). Cells were cultured in DMEM:F12/20% FCS/0.1% non-essential amino acid solution/2.5mM L-glutamine/PenStrep. Where possible cell suspensions were assessed for epithelial origin using Pan-Cytokeratin (DAKO) staining [FigureS1A-B]

Cell Lines

MCF7, T47D, MDA-MB-231 (231) and MDA-MB-468 (468) (ATCC). Lines were authenticated by multiplex-PCR assay using the AmpF/STR system (Applied Biosystems) and confirmed as mycoplasma free. Monolayers were grown in DMEM medium (DMEM/10% FCS/2mM L-glutamine/PenStrep, MCF7 and T47D) or RPMI medium (RPMI/10% FCS/1% Sodium pyruvate/2mM L-glutamine/PenStrep, 231 and 468). Cells were maintained in a humidified incubator at 37^oC at an atmospheric pressure of 5% (v/v) carbon dioxide/air.

Hypoxic Cell Culture

Cells we incubated for 48hr in the SCI-tiveN hypoxic workstation (Ruskinn) in 1% O_2, 5% CO_2 and 94% N_2 in a humidified environment at 37^oC. Cells were plated, cultured and harvested within the workstation to maintain hypoxia at all times. Confirmation of hypoxic conditions

was carried out using IHC to measure expression of HIF1 and qRT-PCR to assess up-regulation of target genes at the RNA level (FigureS2A-C).

Mammosphere Culture

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

Clonogenic Culture

Cells were plated at 50 cells /cm^2 in adherent conditions for 10 days. Colonies were fixed and stained with 1% crystal violet/70% EtOH and were identified microscopically. Colonies which had undergone 5 or more divisions, i.e. containing 32 or more cells, were counted (24).

Western Blotting

Protein was separated on an SDS–polyacrylamide gel and transferred to Hybond-C Extra nitrocellulose membrane. Primary antibodies included: SP1-ERα (RM-9101-SO, Thermo Fisher Scientific), Cleaved N1-ICD (100-401-407, Rockland), Jagged1 (Santa Cruz), Actin (Santa Cruz, sc-1616) and HIF1α (610959, BD Biosciences). Densitometry was performed 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

$1x10^6$ cells were resuspended in 1ml and mixed with 5µl of Aldefluor. 0.5ml was immediately transferred to a control tube containing 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 analysed using the FACS Calibur.

Quantitative Real Time PCR

RNA was extracted using the Qiagen RNAeasy kit according to manufacturer's instructions and quantified on the Nanodrop spectrophotometer (Thermo). cDNA was produced using the First Strand kit (SABiosciences) and RT-PCR was performed using RT kit (Sybergreen, SABisciences) before analysis on the 7900 PCR machine (Applied Biosystems).

Inhibition of Signalling

10μM YC1 (Cayman Chemicals), 10μM Dibenzazepine (DBZ, a kind gift from Adrian Harris, Oxford) (in 0.5% Methocel/0.1% Tween 80), 1μM 4-hydroxytamoxifen or 1μM 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 (25).

Transient ERα Expression

Cells were transfected with V16-ERα (Addgene plasmid 11351 (26)) using lipofectamine according to 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 MS culture.

In Vivo Tumour Formation

Cells were injected sub-cutaneously into Nude mice (MCF7, 231 and 468) and NSG mice (primary metastatic, COB9). 90 day slow release oestrogen pellets (0.72mg) were implanted sub-cutaneously 2 days before injection (MCF7 only, Innovative Research of America). Upon collection xenografts were cut and half was immediately placed in formalin for embedding. The remaining xenograft was dissociated in the same way as primary solid tumours.

20mg/ml YC1 was administered via a single intraperitoneal injection when tumours reached approximately 100mm^3.

Xenograft Embedding and Immunohistochemistry

Tumours were formalin fixed and paraffin embedded. Antigen retrieval, blocking and staining were carried out using the Biogenix i6000. Slides were blocked with hydrogen peroxide and casein before incubation with the primary antibody (HIF1α - ab1, Abcam, Glut1 – ab15309, Abcam). Slides were then incubated with Envision secondary followed by DAB.

Statistical Methods

Data is represented as mean±SEM taken over three independent experiments. Statistical significance was measured using parametric testing, assuming equal variance, in the majority of experiments with standard t-Tests for two paired samples used to assess difference between test and control samples. In the case of tumour growth rate analysis of variance with replicates was used for correlations. Pearson's R^2 correlation was calculated for correlation between xenograft size, HIF1α/Glut1 and MFC.

Results

The breast cancer cell hierarchy is affected by hypoxia

To assess hypoxic effects on breast cancer cells, 13 patient-derived primary samples and 4 established cell lines were cultured in 21% or 1% oxygen. As physiological oxygen levels vary greatly throughout the tumour, normoxia is extremely difficult to represent in vitro. With this in mind atmospheric oxygen (21%) was compared to a more physiological 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 [FigureS3A]. 1% oxygen was used to represent hypoxia although levels of oxygen will also vary within hypoxic regions of tumours. Culture for 48 hours in hypoxia had no significant effect on proliferation or apoptosis [FigureS3B-D]. CSC activity was assessed using well established *in vitro* assays including mammosphere (MS) (25, 27) and clonogenic culture (28, 29) and FACS analysis of ALDH1 expression (30). CSC activity was also measured *in vivo* using limiting dilution experiments.

MS forming cell (MFC) number increased significantly after hypoxic culture in all ERα-positive primary samples and cell lines [Figure 1A]. In contrast to this, a significant decrease in MFC occurred in all ERα-negative primary samples and cell lines. The same pattern was seen in an extended panel of 6 additional cell lines representing the same sub-types of breast cancer [FigureS3E]. Inhibition of HIF1α, with YC1 (31) [Figure S4A-B], reduced the effect of hypoxic culture in all cell types [Figure1A] but had no effect in 21% oxygen [FigureS4C]. The same effect was seen in cell lines using a targeted HIF1 siRNA [Figure1B]

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 non-adherent culture (29) [Figure S5A-E]. The same hypoxic response pattern was seen with the CSC enriched fraction of holoclone forming cells (HFC) increasing in ERα-positive lines and decreasing in ERα-negative lines after hypoxic culture [Figure 1C]. Treatment with YC1 had no effect in normoxic culture [FigureS4D] but blocked the hypoxia induced clonogenic changes in MCF7, 231 and 468 returning the HFC number to controls levels (Figure 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 which 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, demonstrate increased ALDH1 positive cell number in ERα-positive lines and a contrasting decrease in ERα-negative lines (Figure 1D).

Tumour initiation is considered to be the gold standard for CSC measurement and so an *in vivo* limiting dilution experiment was performed to assess changes in tumour initiating cell number following hypoxic culture. 50% tumour formation required 3.1×10^5 hypoxia pre-treated MCF7 cells, whereas 6.5×10^5 control MCF7 cells were required for the same level of tumour formation suggesting a 2-fold increase in tumour initiating cells (Figure 1E). Conversely, 1.5×10^6 hypoxia pre-treated 468 cells are required for 50% tumour formation compared to 9.2×10^5 control cells suggesting a 0.6-fold decrease in tumour initiating cells following hypoxic exposure (Figure 1E). Due to small sample size, the changes in tumour initiating cell number were not significant ($P>0.05$) but these data are supportive of the *in vitro* findings and suggest that hypoxia may a positive effect on the TIC population in ERα-positive breast cancers and a negative effect in ERα-negative tumours.

Hypoxia has long term effects on the cell hierarchy

We previously demonstrated that the breast cancer cell hierarchy is strictly maintained and that when altered the proportion of CSC returns to normal within 5-7 days (33). We were interested, therefore, to see if 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 re-exposed to atmospheric oxygen for up to 10 days before MS culture. At all time points MFC remained significantly increased in ERα-positive cells which had previously been treated in hypoxia and significantly decreased in ERα-negative cells (Figure 1F). This finding shows that the hypoxia-induced changes are not immediately reversed when cells are re-exposed to oxygen suggesting long lasting effects on the cellular hierarchy.

Oestrogen signalling plays a role in the hypoxic CSC response

As distinct hypoxic responses were observed between ERα-positive and negative breast cancers, we hypothesised that the response seen in ERα-positive cancers was downstream of ERα. To confirm ERα activation, known oestrogen responsive genes were assessed by qRT-PCR following hypoxic culture. Significant up-regulation of AREG and TFF1 were seen in both MCF7 and T47D cells as well as u-regulation of PIP in MCF7 cells only (Figure2A). To assess whether the ERα activation is necessary for HIF1α induced CSC changes, signalling was inhibited with 4-Hydroxytamoxifen (Tam). Tam significantly reduced the hypoxic-MFC increase in all ERα-positive cells tested although two out of six primary cases the MFC remained significantly raised compared to control [Figure 2B].

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

It has previously been reported that the CSC within ERα-positive breast cancer have low or no expression of ERα (34) similar to normal breast stem cells. In the normal breast, oestrogen activated ER-positive cells release amphiregulin which binds to EGFR in the SC facilitating their response to oestrogen despite their ER negative status (35). To assess whether this is occurring within breast cancer cells in response to hypoxia induced activation of ERα, EGFR signalling was blocked with the inhibitor Gefitinib. Similar to Tam, Gefitinib blocked the hypoxic effect in ERα-positive primary samples and cell lines but had no effect on ERα-negative primary samples or cell lines [Figure 2C]. These findings are supportive of the hypothesis that HIF1α activates ERα signalling resulting in a downstream paracrine response through EGFR.

Differential Effects on Gene Expression Were Identified in ERα Positive and Negative Cell Lines

Custom PCR array plates (SABiosciences, TableS1) 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 signalling. CSC enrichment was achieved by collection of anoikis resistant (AR) cells as we have previously shown this population to be highly enriched for MS and tumour 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 non-adherent culture for 16 hours and RNA was harvested from the CSC enriched cells (termed AR population) [FigureS6A shows experimental design]. Three independent experiments were performed for each cell line.

Comparison of the total population following hypoxic and normoxic culture showed significant gene changes in both ERα-positive and negative lines [Table1]. It is interesting to note that when comparing these gene expression changes between ER 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 signalling in hypoxia with increased expression of ER and Notch target genes (highlighted in Table1) in the ER positive lines.

Significant changes in gene expression between AR 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 *ESR1* (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 AR populations collected from MCF7 cells and a single significant gene change (PIP) was seen in T47D [Table1] suggesting that the CSC enriched population remains virtually unchanged following hypoxic culture and the increase in MFC, HFC and tumour 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 anoikis resistance in non-CSC. In the case of ERα-negative cells, however, there is a small but significant decrease in three genes; *PTTG1*, *JAG1* and *KI67* [Table1] suggesting that the population is altered in hypoxia. Although small, the decrease in *KI67*, 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 signalling is required for the hypoxic CSC response in ERα-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 down-stream paracrine mediator of ERα (39). The gene expression data presented here also demonstrate up-regulation of Notch signalling within ERα-positive lines with increased expression of *HES1* and *JAK1* in response to hypoxia [Table 1]. Activation of Notch signalling by hypoxia was further assessed by measuring the levels of activated Notch1-intracellular domain (N1-ICD) and down-stream targets at the protein and RNA level. N1-ICD was increased in all ERα-positive primary cells and lines following hypoxic culture [Figure 3A]. There was a small but significant decrease in ER negative cells. Increased expression of JAG1, a ligand and downstream target of the Notch pathway (40), in ERα-positive lines, as well as up-regulation of *HES1*, *HEY2* and *HEYL*, gave further evidence for activation of Notch signalling [Figure 3B-C].

To confirm that Notch plays a role in the hypoxic response of CSC, signalling was inhibited using a gamma secretase inhibitor (GSI), Dibenzazepine, during monolayer culture. Addition of GSI significantly decreased MFC in all ERα-positive samples and cell lines cultured in 1% oxygen reducing the effect of hypoxic culture. No significant effect on the hypoxic response was seen in ERα-negative samples or lines and MFC remained at the same, low levels [Figure 3D]. To further verify Notch's role, the pathway was inhibited using inducible shRNA cell lines [FigureS6B]. Interestingly, when signalling through Notch1 and Notch4 were specifically blocked only inhibition of Notch1 reduced the hypoxic-CSC response [Figure 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 ERα or N1-ICD causes a positive hypoxic-CSC response in ERα-negative cells

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

Next, to assess the effect of Notch1 activation, 231 cells were produced which stably express doxycycline inducible N1-ICD [231-YNICD] (25). A significant increase in MFC was observed in 231-YNICD cells cultured in hypoxia similar to that seen in ERα-positive cells [Figure 4C].

These data support the hypothesis that HIF1α-ERα-Notch1 activation is responsible for the increased CSC seen in ERα-positive cells and suggest that ERα-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 modelled in vivo

In vitro analysis of CSC activity is a valuable and accurate technique but an *in vivo* model with varying degrees of hypoxia would allow more realistic analyses of the link between tumour growth, CSC and the potential divergent influence of hypoxia on CSC activity. In order 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 xenograft cells. MCF7, 231 and 468 lines and an ERα-positive metastatic sample (COB) were implanted sub-cutaneously into mice and xenografts were harvested at different time points; half was embedded in paraffin for immunohistochemical analysis, whilst half was dissociated for MS culture. HIF1α and GLUT1 staining was quantified by image analyses [FigureS7A-B] and a significant correlation between tumour size and both HIF1α and Glut1 expression was observed in

xenografts produced from all cell types validating the hypothesised link between xenograft size and the presence of hypoxia [Figure 5A].

In ERα-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 mammosphere forming cells within the tumour increased [Figure 5Bi]. Importantly, an inverse correlation was observed for ERα-negative lines with a decrease in the proportion of MFC within the xenograft as tumour size increases [Figure 5Bii]. No correlations were seen between MFC number and growth rate or total viable cell number extracted [FigureS7C-D].

Intraperitoneal injection of the HIF1α inhibitor, YC1, following tumour initiation reduces the hypoxic-CSC effect in MCF7 xenografts and results in no correlation between xenograft size and MFC [Figure 5C].

This xenograft model fully supported our *in vitro* findings of a contrasting link between hypoxia and CSC in ERα-positive and negative breast cancer sub-types.

Discussion

We describe differing cancer stem cell (CSC) responses to hypoxia in ERα-positive and negative breast cancer with ERα-positive cancers gaining increased CSC activity and ERα-negative cancers showing reduced CSC. Earlier reports support the observed hypoxia induced increase in CSC in breast cancer cell lines (18-20) but the ERα-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 tumours, 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 HIF1α activities by various subunits, post-translational modifications and the presence and effects of co-regulators which are known to vary between different cell types (6).

Interestingly, the observed effects of hypoxia were relatively long lasting (Figure 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 DCIS and in high grade tumours, could have profound long term effects on the CSC content and consequently tumour aggressiveness. The findings may also elucidate the divergent behaviours of breast cancer metastases with the existence of late recurrences in ERα positive breast cancer but fewer late events in ERα-negative cases. Dormant tumour cells in a hypoxic bone marrow niche (19) will be enriched for CSC in ERα-positive breast cancer but depleted in ERα 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 which 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 NFkB (42).

To mechanistically explain the effect of hypoxia on CSC, we assessed the roles of ERα and Notch signalling.

Inhibition of ERα blocked the hypoxic-CSC response in all ERα-positive cells supporting the role of oestrogen signalling in the hypoxic response. We also demonstrate that, like in the normal breast (35), oestrogen signalling causes downstream activation of EGFR-ERK signalling 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 ERα-positive tumours.

Notch inhibition also blocked the hypoxic-CSC effect in all ERα-positive patient derived samples and cell lines and specifically Notch1 seemed to be central for the hypoxic-CSC response. In the case of ERα-negative breast cancer no activation of Notch was observed suggesting, in this setting, that Notch is activated downstream of ERα. There are contradictions in the literature regarding cross-talk between the oestrogen and Notch pathways and some reports suggest that oestrogen activation inhibits Notch signalling (43) while others, in line with the findings in this study, report Notch activation by oestrogen (39). It is possible that Notch regulation by oestrogen is context dependent but our data clearly shows that hypoxia activates oestrogen signalling resulting in increased Notch1 cleavage and activation of downstream targets.

As the mechanistic studies suggest that ERα and Notch are central in mediating the unfavourable hypoxic-CSC response in ERα-positive breast cancer they may offer an attractive combination treatment approach which would consist of ERα or Notch inhibitors combined with anti-angiogenic drugs. This would bypass the potential adverse effects of hypoxia on the CSC fraction whilst limiting tumour progression due to the general vasculature effects.

Understanding the inherent negative hypoxic-CSC response in ERα-negative tumours will be of great value for the development of additional novel treatments as activating this response alone in cancer cells or alongside anti-angiogenic 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 ERα 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 de-differentiation of non-CSC into a less mature cell state any of which would result in a change to the balance between the two compartments (44, 45). Figure 6A-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 ERα-positive cell lines we hypothesise that either self-renewal symmetry is altered or cells are caused to de-differentiate resulting in the increased CSC numbers seen. For ERα-negative breast cancer there was a slight difference in proliferation for the CSC-enriched cells suggesting that for this sub-type of breast cancer it may be a combined differentiation and

proliferation changes within different sub-populations causing the decrease in CSC under hypoxia but this requires further investigation.

Our xenograft experiments clearly demonstrate that as xenograft size increases, and therefore extent of hypoxia within the tumour increases, there is a profound effect on CSC number. However, the direction of this effect is dependent upon the ERα status of the tumour 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 influence 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 ERα positive and negative breast cancer.

Inhibiting angiogenesis, and thus limiting nutrient supply to a tumour, is an attractive method to treat cancer and so treatments targeting HIF1α 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 paper suggests that tumour sub-type needs to be taken into account during treatment design as blocking angiogenesis and therefore increasing tumour hypoxia may be beneficial in the treatment of ERα-negative breast cancer but could have catastrophic effects on ERα-positive breast cancer. This is supported by recently published data in the *New England Journal of Medicine* (21) where triple negative breast cancer patients were seen to show increased complete pathological response to

Bevacizumab treatment, with no detectable tumour at primary or metastatic sites, whilst no improvement was observed in hormone receptor positive cases.

As ERα 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 anti-angiogenic therapies. Future studies need to clarify if smaller subgroups of breast cancer, defined according to expression array criteria, will behave in ways similar to ERα-positive or negative breast cancer but it is clear that the two major disease subgroups, defined by ERα status, show contrasting hypoxic-CSC responses.

In summary, our novel data suggest that ERα-positive and negative breast cancer subtypes respond differently to hypoxic exposure and as a consequence, anti-HIF1α or anti-angiogenic therapies will not be suitable for both sub-types. Lastly, the findings will form a foundation for novel cancer treatment principles targeting or enhancing the hypoxic-CSC response within various combination treatment approaches.

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HH was involved in study design, carried out the majority of the practical experimentation and drafted the manuscript. LR and HJG assisted with practical experimentation. KRB and RBC provided assistance with experimental design and manuscript preparation. GL conceived of the study and participated in manuscript production.

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MCF7 1% v 21%			MCF7 AR v Total			MCF7 AR 1% v 21%		
Gene	FC	P	Gene	FC	P	Gene	FC	P
WNT1	16.6085	0.032306	**FOSL1**[#]	7.245	0.005357	No Significant Changes		
TLR3	16.4556	0.04585	**COL1A2**	6.7279	0.018229			
HES1[#]	4.0483	0.015925	**ABCG2**	3.3276	0.027509			
AREG*	3.1825	0.043581	**DNER**	3.1636	0.050016			
BAMBI*	1.8923	0.046104	**CSF1**	1.8455	0.032669			
PIP*	1.5704	0.049938	**CSF1**	1.8455	0.032669			
			CD44	1.5503	0.024561			
			NFKB1	-1.731	0.046624			
			BCL2	-2.5799	0.039347			
			MSI1	-2.7023	0.049981			
			JAK1[#]	-2.8123	0.044749			
			VIM	-4.7073	0.002217			
			WNT4	-4.8657	0.00134			
			ESR1*	-7.338	0.01216			
			BAMBI*	-9.2361	0.022085			
			ID1	-15.9783	0.020758			
T47D 1% v 21%			T47D AR v Total			T47D AR 1% v 21%		
Gene	FC	P	Gene	FC	P	Gene	FC	P
EPO	16.022	0.010133	**DNER**	253.514	0.001475	**PIP***	-2.8342	0.035929
MYO1B*	1.8541	0.036707	**FOSL1**[#]	14.8296	0.000051			
JAK1[#]	1.8131	0.013465	**ABCG2**	10.7202	0.030977			
HES1[#]	1.6529	0.038997	**CD44**	7.8285	0.001109			
PROCR	-1.9183	0.029291	**JAG1**[#]	5.7859	0.000739			
GSC	-2.1919	0.014095	**AXIN2**	2.4642	0.008222			
TLR3	-2.404	0.054619	**LEF1**	2.2384	0.006673			
			MYO1B*	1.51	0.012522			
			MMP9	-1.5564	0.013801			
			BAG1	-1.6772	0.011334			
			ANK3	-2.0065	0.038898			
			BID	-2.3323	0.003406			
			JAK1[#]	-2.3774	0.001109			
			GSC	-2.3796	0.017204			
			AREG*	-2.4744	0.025721			
			ACTA2	-2.5196	0.00577			
			PROCR	-2.6686	0.00856			
			TFF1	-2.7346	0.027106			
			TGFB1	-2.8237	0.02609			
			VIM	-2.9029	0.047745			
			TWIST1	-3.392	0.001721			
			TLR3	-6.7592	0.002246			
			CXCL12	-7.4051	0.021454			
			BCL2	-17.263	0.02799			
			TCF4	-22.345	0.000223			
231 1% v 21%			231 AR v Total			231 AR 1% v 21%		
Gene	FC	P	Gene	FC	P	Gene	FC	P
IL6	3.269	0.001	**ALDH1A1**	3.777	0.001	**Ki67**	-0.697	0.019
WNT10A	2.956	0.038	**WNT10A**	3.587	0.048	**PTTG1**	-0.728	0.016
EPO	2.438	0.002	**TFF1**	3.479	0.024	**JAG1**[#]	-0.932	0.018
CSF1	1.545	0.044	**MMP9**	3.097	0.033			
BMP1	1.282	0.031	**IL6**	2.401	0.023			
TGFB1	1.267	0.042	**TGFB1**	2.241	0.03			
DNER	1.196	0.026	**SFRP1**	1.978	0.046			
SFRP1	1.19	0.002	**CSF1**	1.883	0.015			
SNAI2	1.116	0.017	**TLR3**	1.874	0.001			
JAK1[#]	-0.123	0.048	**WNT3A**	1.825	0.019			
MYO1B*	-0.16	0.035	**BMP1**	1.534	0.009			
WNT4	-0.228	0.004	**PCNA**	-1.568	0.002			
CDKN1A	-0.274	0.003	**FOSL1**[#]	-1.573	0.001			
SOX9	-0.484	0.046	**ID1**	-1.665	0.026			
ABCG2	-0.559	0.012	**LEF1**	-1.957	0.001			
RUNX1	-0.876	0.017	**BCL2**	-2.728	0.001			
NFKB1	-0.905	0.036	**DKK1**[#]	-5.52	0.002			
NOTCH4[#]	-0.994	0.002						

Table1: Significant gene expression changes in MCF7, T47D and 231 cell populations (Log_2, *known oestrogen responsive genes [#] known Notch targets

Figure 1: Hypoxic Effect on Breast Cancer Stem Cells in Different Cell Types

A) Following 48-hours in 21% or 1% oxygen +/-YC1 cells were plated in mammosphere culture. B) HIF1 siRNA was used to block the hypoxic response before mammosphere culture.

Hypoxic effect on C) adherent holoclone culture and D) Percentage ALDH1-positive cells.

(E) *In vivo* tumour formation for ER positive (MCF7) and negative (468) cells following 21% or 1% pre-treatment (numbers represent mice in group).

F) Cells were grown in 21% oxygen following hypoxic culture for 1-10 days before mammosphere culture.

Means ±SEM *P<0.05 [See also FigureS5/6]

Figure 2: Hypoxic Effect Requires Oestrogen and EGFR Signalling in ERα Positive Cells

(A) Expression changes of known oestrogen-responsive genes.

Following 48-hours in 21% or 1% oxygen +/- (B) Tamoxifen or (C) Gefitinib cells were plated in mammosphere culture.

Means ±SEM *P<0.05

Figure 3: Hypoxic Effect Requires Notch Signalling in ERα Positive Cancers

Densitometric analysis of 3 independent Western blots for (A) activated Notch1 (N1-ICD) and (B) Jagged1 protein levels. (C) Expression changes of known Notch-responsive genes.

D) Following 48-hours in 21% or 1% oxygen +/-gamma secretase inhibitor (GSI) cells were plated in mammosphere culture. E) Following 48-hours in 21% or 1% oxygen +/-Doxycycline (Dox) inducible shRNA lines to Notch1 ($MCF7^{Notch1}$) and Notch4 ($MCF7^{Notch4}$) were plated in mammosphere culture.

Means ±SEM *P<0.05

Figure 4: Hypoxic Effect is altered in ERα Negative Cells by Expression of ERα or Notch1-ICD

(A) Representative Western blot measuring expression of V16-ERα plasmid, upper band V16-ERα, lower band endogenous ERα. B) Following 48-hours in 21% or 1% oxygen cells were plated in mammosphere culture. C) N1-ICD was expressed in 231 cells in 21% or 1% oxygen and cells were plated in mammosphere culture. **UT** Untransfected, **CV** control vector.

Means ±SEM *P<0.05

Figure 5: In Vivo Model of Hypoxic CSC Effect

(A) HIF1α and Glut1 expression in i) ERα positive and ii) negative xenografts compared to tumour size (mm^3).

B) Tumour size (mm^3) and percentage mammosphere forming cell number following *in vivo* growth in i) ER-positive and ii) ER-negative cells.

C) MCF7 xenografts were treated with YC1 by IP-injection before collection for i) mammosphere culture and ii) Glut1 measurement.

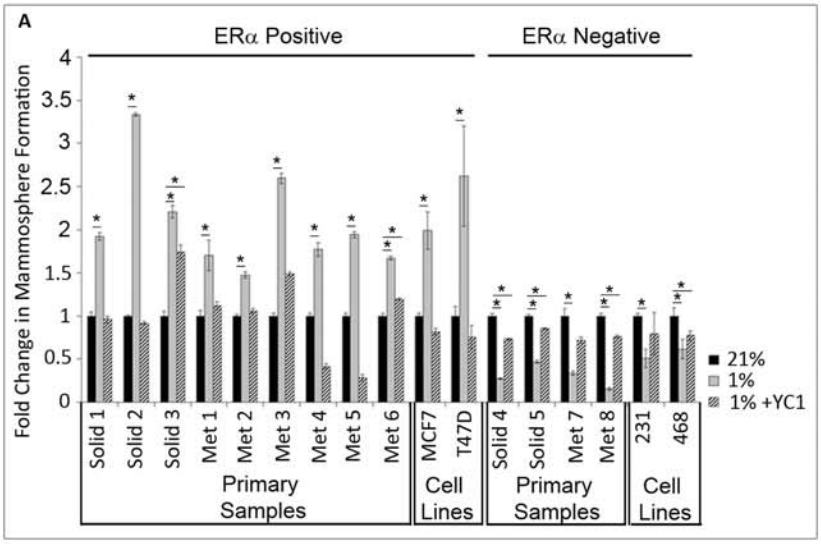
Figure 6: Putative Model of Breast Cancer Cell Hierarchy and the Hypoxic Effect

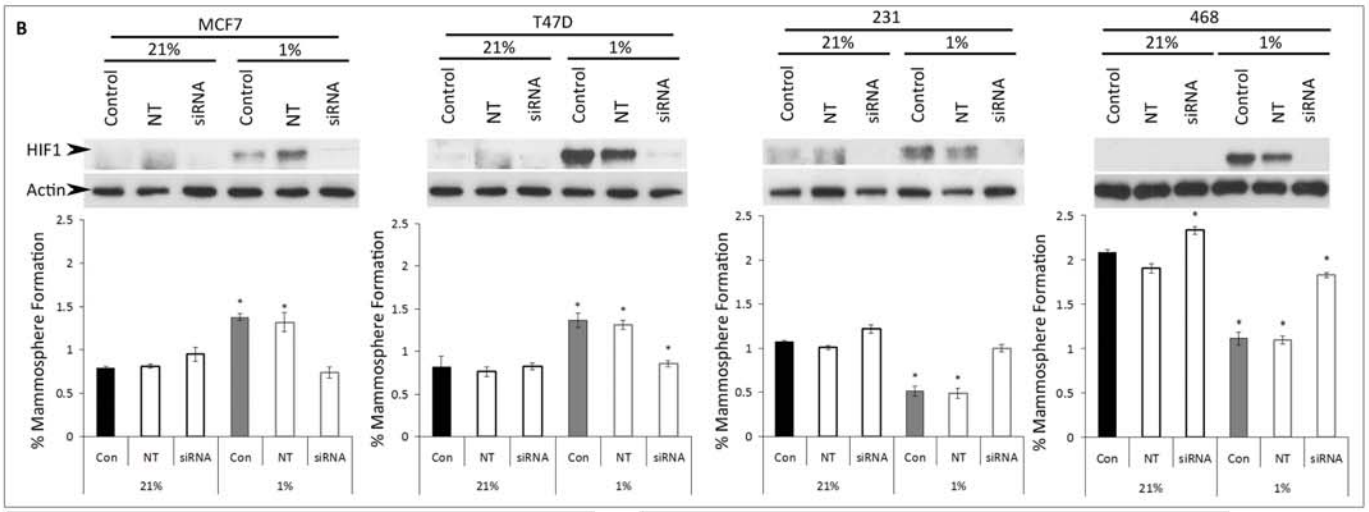
(A) Hypothesised model for interaction between CSC and non-CSC in response to hypoxic activation of ER

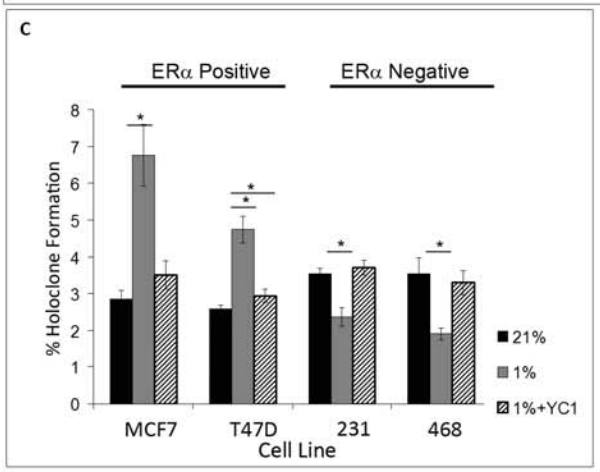
(B) Breast cancer cells are hierarchically organised and contain primitive CSC, early progenitor cells with some limited *in vivo* and *in vitro* stem cell activity, transit amplifying cells and more differentiated cells. Possible modes of hierarchy alteration are suggested as a) increased self-renewal, b) de-differentiation of early progenitor cells and c) blocked proliferation ERα-positive and negative tumours.

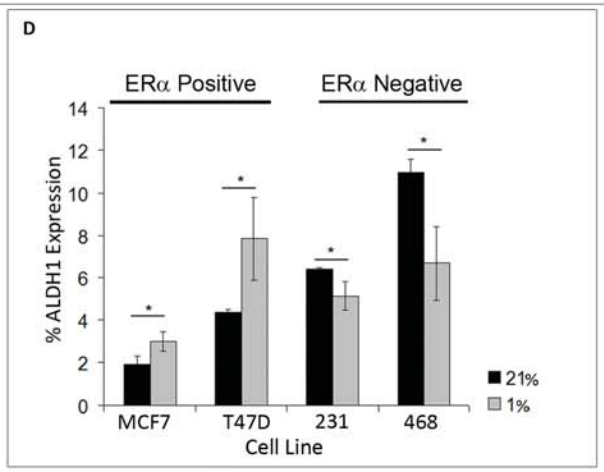
Table 1: Significant Gene Expression Changes

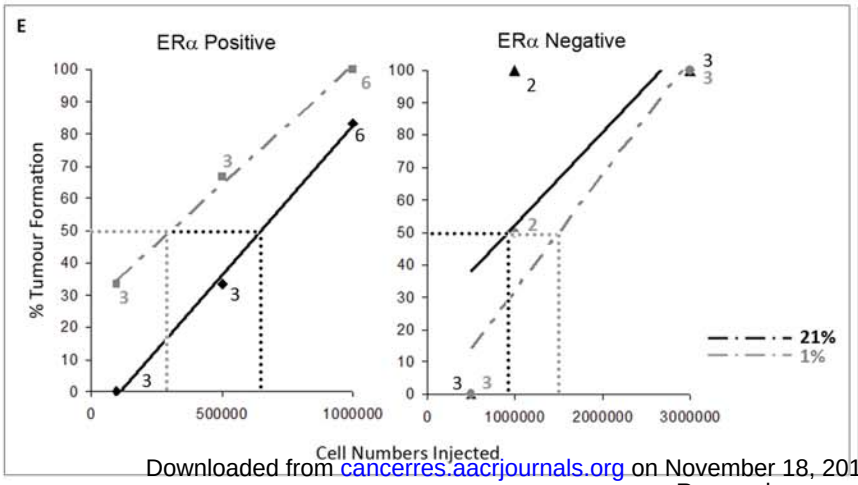
Gene expression changes (Log_2) in MCF7, T47D and 231 cells.

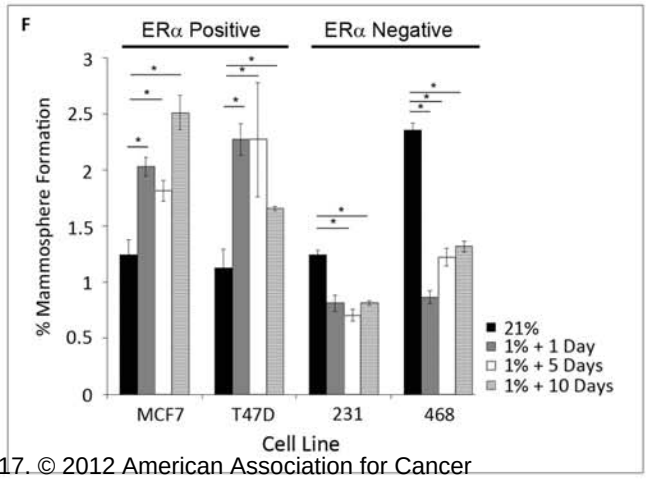




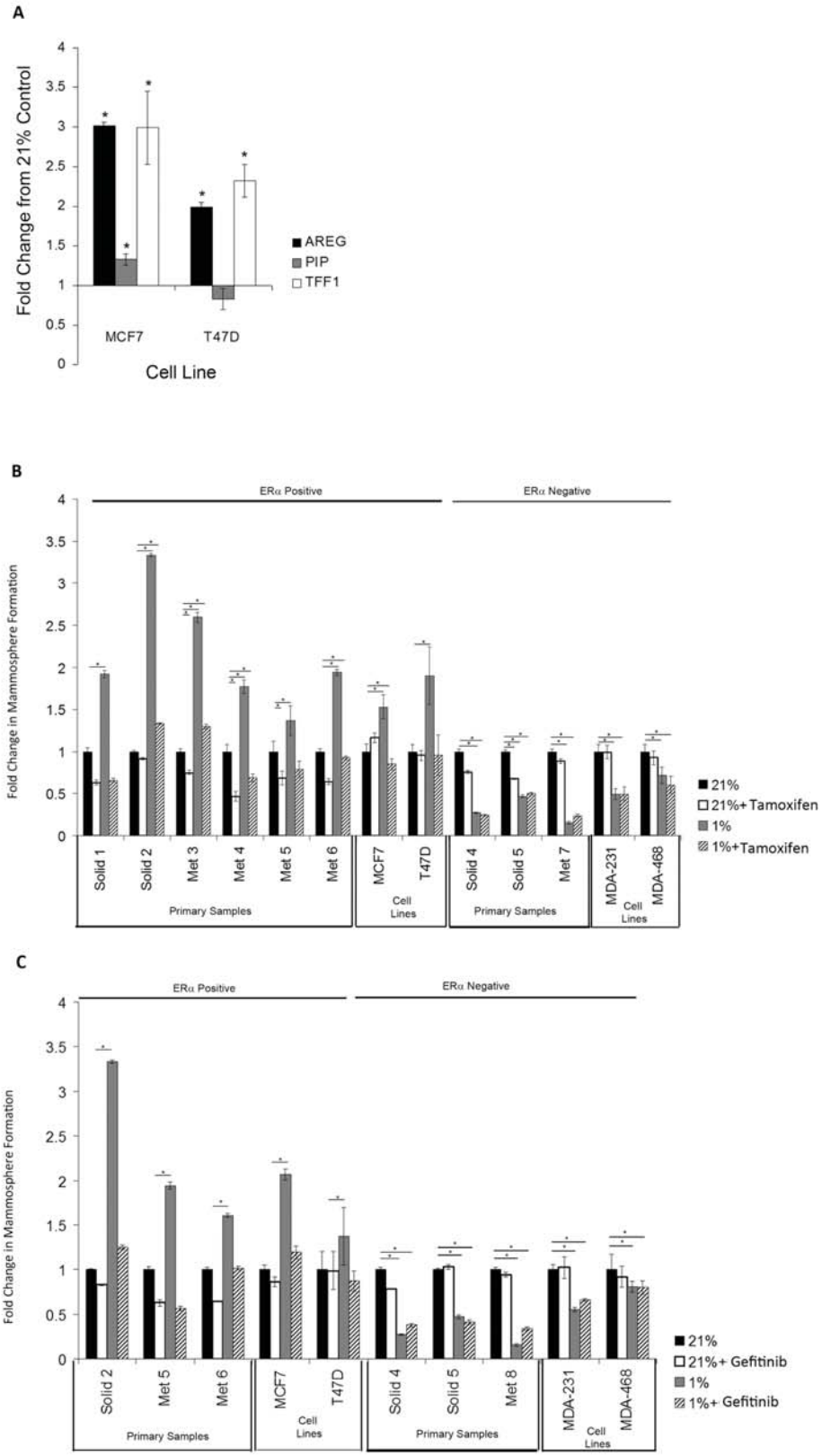




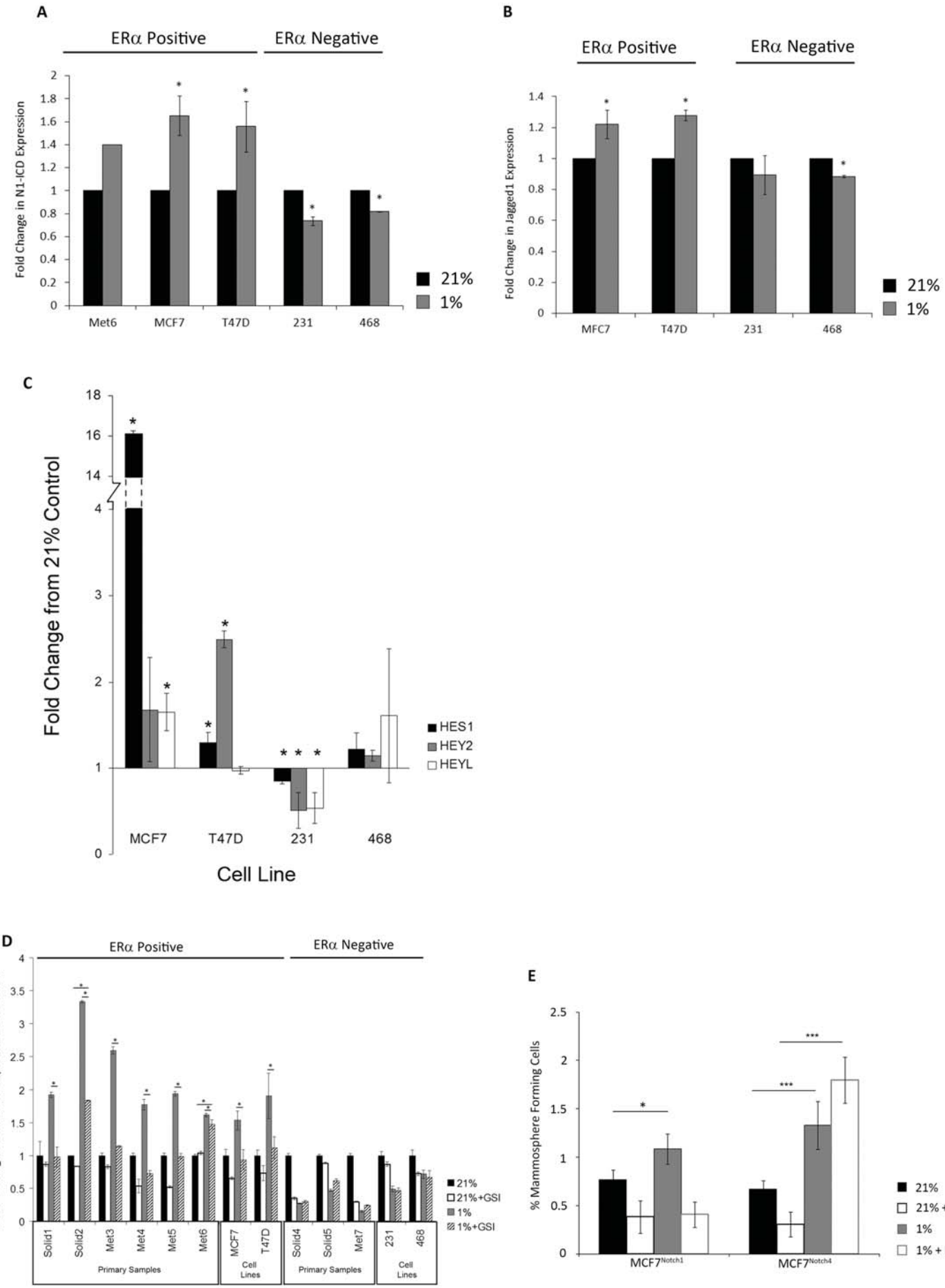




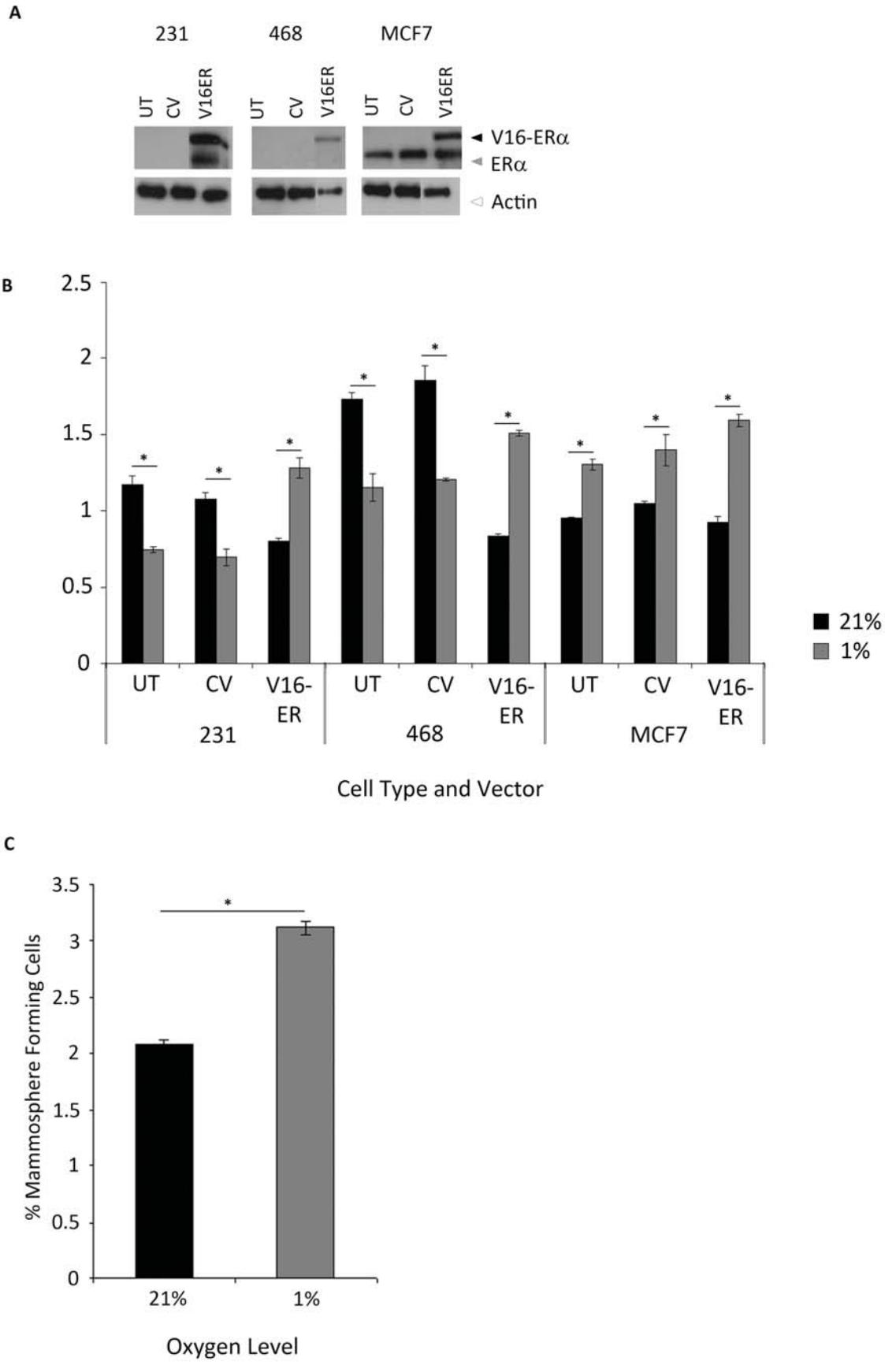
Harrison et al Figure 2



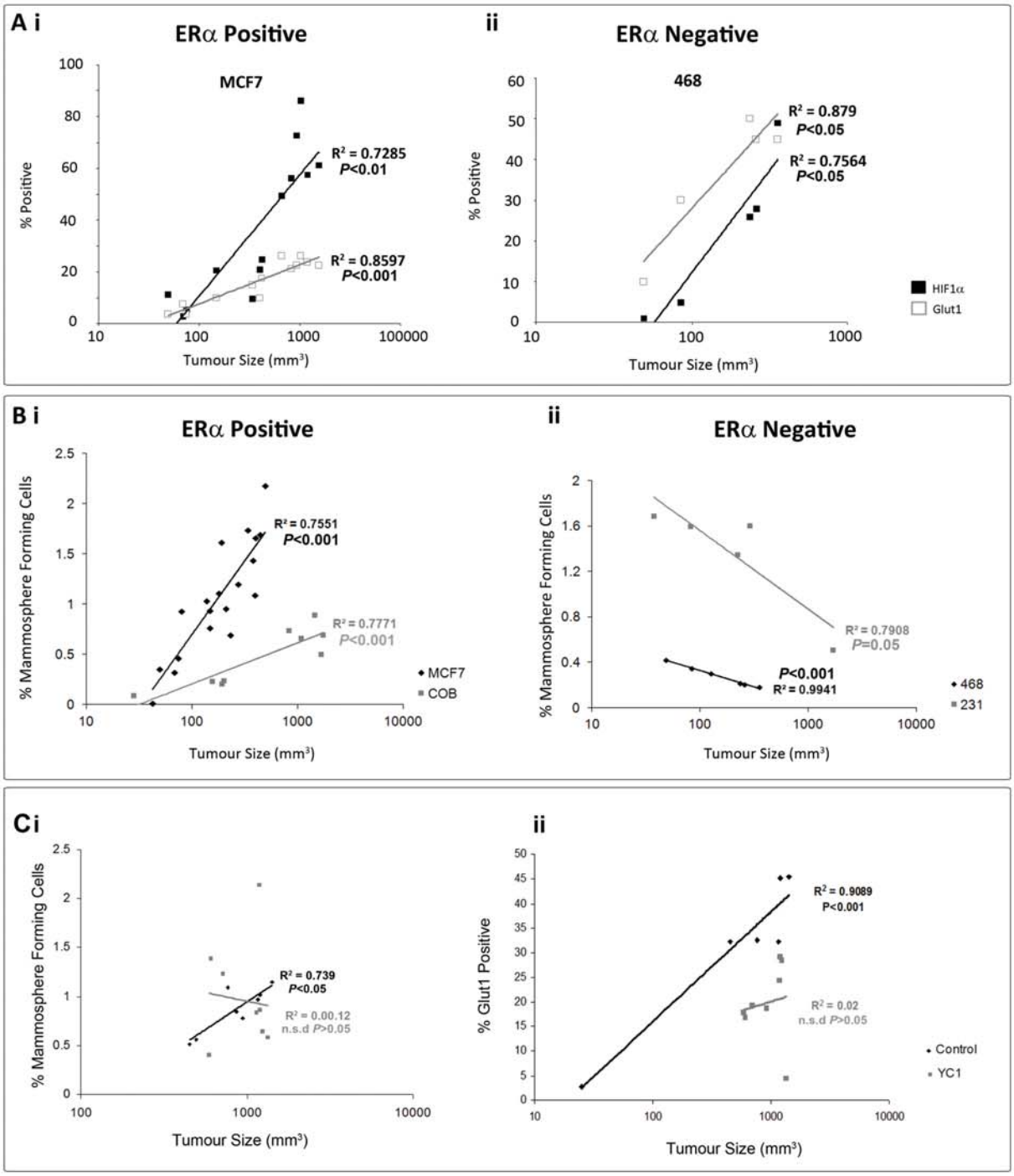
Harrison *et al* Figure 3



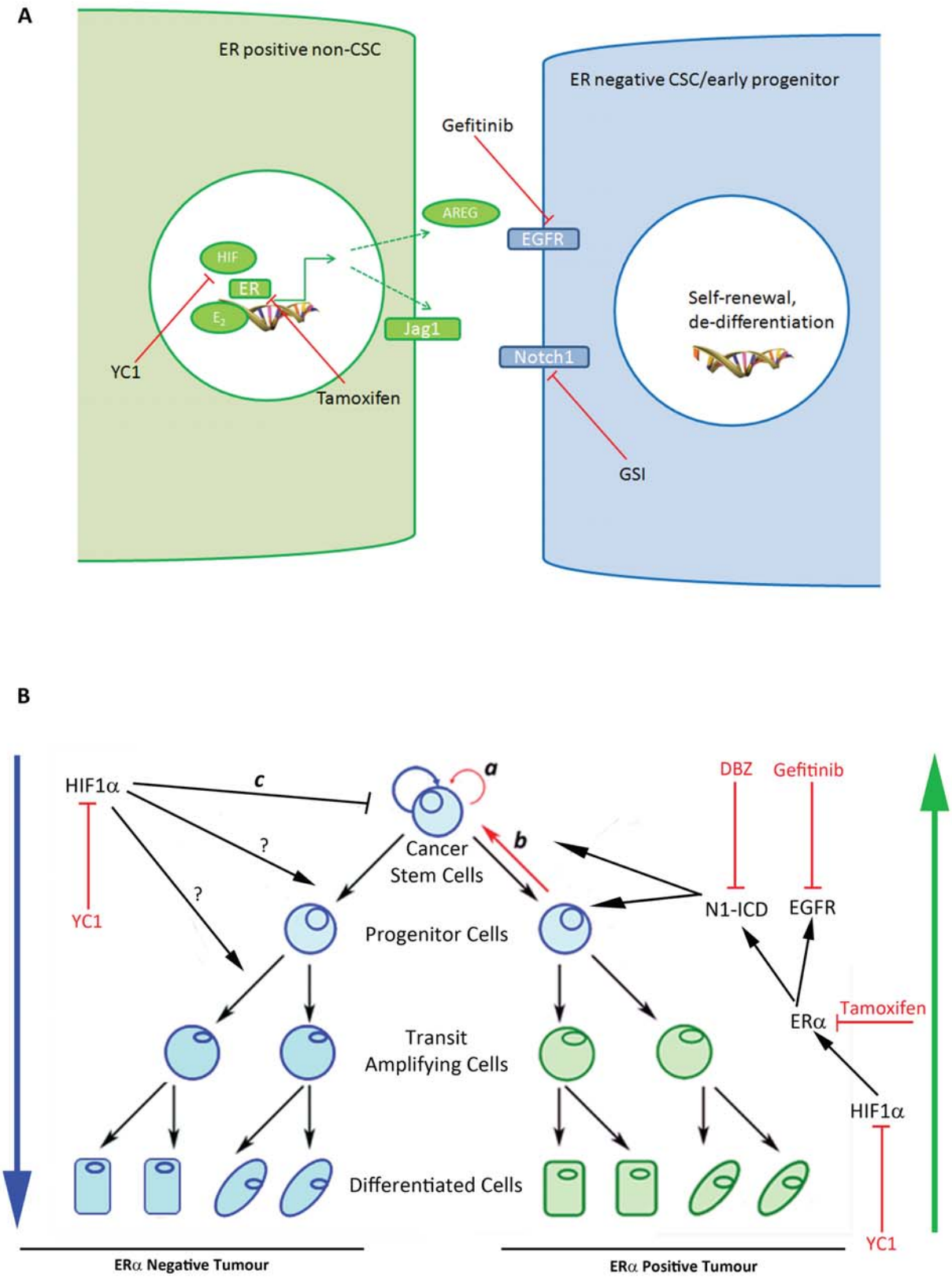
Harrison *etal* Figure 4



Harrison et al Figure 5



Harrison *et al* Figure 6



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Contrasting hypoxic effects on breast cancer stem cell hierarchy is dependent on ER α status

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