Hypoxia modulates the stem cell population and induces EMT in the MCF-10A breast epithelial cell line

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Abstract. A common feature among pre-malignant lesions is the induction of hypoxia through increased cell propagation and reduced access to blood flow. Hypoxia in breast cancer has been associated with poor patient prognosis, resistance to chemotherapy and increased metastasis. Although hypoxia HAS BEEN correlated with factors associated with the latter stages of cancer progression, it is not well documented how hypoxia influences cells in the earliest stages of transformation. Using the immortalized MCF-10A breast epithelial cell line, we used hypoxic culture conditions to mimic reduced O_2 levels found within early pre-malignant lesions and assessed various cellular parameters. In this non-transformed mammary cell line, O_2 deprivation led to some changes not immediately associated with cancer progression, such as decreased proliferation, cell cycle arrest and increased apoptosis. In contrast, hypoxia did induce other changes more consistent with an increased metastatic potential. A rise in the $CD44^+CD24^-$/low-labeled cell sub-population along with increased colony forming capability indicated an expanded stem cell population. Hypoxia also induced cellular and molecular changes consistent with an epithelial-to-mesenchymal transition (EMT). Furthermore, these cells now exhibited increased migratory and invasive abilities. These results underscore the contribution of the hypoxic tumour microenvironment in cancer progression and dissemination.

Introduction

Breast cancer is the most common malignancy in woman worldwide, and the second leading cause of cancer-related deaths in females (1). Considering that metastasis is responsible for 90% of these deaths (2), understanding the mechanisms which contribute to this endpoint is fundamental in the design of treatment strategies to alleviate breast cancer mortality. The pathological progression of breast cancer is well documented (3). However, the factors which govern this progression are less characterized (4). Considerable attention has been paid to the contribution of somatic mutation and epigenetic alterations in cancer initiation and progression (5-7). However, the role of tumour-associated microenvironmental changes may be equally contributive and are only recently gaining impetus (8).

Hypoxia exemplifies one microenvironmental change associated with tumourigenesis. In the earliest stages of tumour development, abnormal proliferation and accumulation of cells can lead to increased cellular mass, elevated intra-tissue pressure, insufficient perfusion and subsequent O_2 deficiency (9). In breast cancer patients, intra-tumour measurements conducted *in situ* have revealed substantial levels of O_2 deprivation compared to normal breast tissue (10). Hypoxia in breast cancer has been associated with poor patient prognosis (11-13), resistance to chemotherapy (14,15) and increased metastasis (13,16,17).

How hypoxia influences cancer progression is not fully defined. It is known that cells respond to reduced O_2 availability by increasing the activity of hypoxia-inducible factors (HIF-1α and HIF-2α) which, in turn, mediate global transcriptional changes (18). These transcriptional changes involve many genes and may alter various cellular processes which contributes to cancer progression (18). Numerous studies connecting hypoxia and cancer have been conducted on transformed cells isolated from animal models, patient tumours and established cancer cell lines (19). However, these cells harbour many cancer-associated genetic and epigenetic changes. How hypoxia affects breast epithelial cells in the earlier stages of transformation remains less well defined.

In the present study, we used the untransformed MCF-10A breast epithelial cell line and hypoxic culture conditions to replicate conditions found within early hyperplastic breast lesions. Using this model we were able to study the effects of O_2 deprivation independent from the contribution of cancer-associated genetic and epigenetic changes. We demonstrated that reduced O_2 availability induced a number of changes consistent with increased metastatic potential. Proliferation and

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cell cycle progression were perturbed along with an increase in apoptosis. A rise in the $CD44^{+}CD24^{-/low}$ cells coupled with an increased colony forming ability indicated a rise in the stem cell population. Cells underwent cellular and molecular changes consistent with epithelial-to-mesenchymal transition (EMT). Furthermore, hypoxia increased the migratory and invasive capabilities of these cells. Collectively, these results highlight the contribution of hypoxic microenvironmental changes in cancer progression and dissemination.

Materials and methods

Human tissue samples and ethics statement. Surplus breast tissue initially removed surgically for diagnostic purposes was used in the present study following informed patient consent. Archived paraffin-embedded tissue was obtained from Bristol Royal Infirmary under ethical approval from the NHS Health Research Authority and UWE Ethics Committee (Ref. 11/SW/0127). All methods were performed in accordance with the NHS Health Research Authority guidelines and regulations.

Cell culture and hypoxia. MCF-10A cells were purchased from the American Tissue Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Ham supplemented with 100 ng/ml cholera toxin (Sigma, St. Louis, MO, USA), 20 ng/ml epidermal growth factor (EGF) (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 10 μg/ml insulin, 500 ng/ml hydrocortisone and 5% heat-inactivated horse serum (all from Sigma). Experiments were conducted in the aforementioned media mixture excluding EGF (media was replaced at least 24 h before experiments). MCF-10A cells were subjected to no >8 passages in culture before experiments. Whilst control cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2 and ~21% O_2 (termed normoxia), hypoxic conditions (termed hypoxia) were induced using an airtight modular incubator chamber (Billups-Rothenberg, Inc., San Diego, CA, USA). Briefly, the cells were sealed in the modular incubator chambers with a sterile phosphate-buffered saline (PBS) reserve to maintain humidity, and then purged with a reduced O_2 gas mixture (1% O_2, 5% CO_2 and 94% N_2). The chamber was then sealed and placed in an incubator at 37˚C for 72 h.

Immunofluorescence microscopy. Paraffin blocks containing embedded human breast tissue were sectioned at 4 μm using a microtome (Leica RM2235) and mounted on Superfrost Plus slides (Thermo Fisher Scientific, Inc.). Sections were then deparaffinized with Histoclear (National Diagnostics, Atlanta, GA, USA) and rehydrated using a series of ethanol concentrations and dH_2O. Antigen unmasking was performed by heating in citrate buffer (pH 6.0) using a water bath for 30 min (95-100˚C) and then allowing the sections to cool to room temperature (RT) in the buffer. Cultured MCF-10A cells were fixed with ice cold 4% paraformaldehyde for 20 min and then stored at 4˚C in 70% ethanol. The slides and/or fixed cells were incubated in blocking serum [goat serum (Vector Laboratories, Burlingame, CA, USA) diluted in Tris-buffered saline (TBS)] for 30 min at RT, and then incubated in a primary antibody overnight at 4˚C. Antibodies used were anti-human and are as follows: CA IX (a kind gift from Professor M. Ladomery), Ki-67 (Thermo Fisher Scientific, Inc.), cleaved caspase-3 (Cell Signaling Technology, Inc., Beverly, MA, USA), E-cadherin, β-catenin and vimentin (all from BD Biosciences, Franklin Lakes, NJ, USA). The following day, the slides and/or cells were washed in TBS and then incubated with suitable fluorescent-labelled secondary antibodies [Alexa Fluor (Thermo Fisher Scientific, Inc.)] for 1 h at RT. Subsequently, the slides and/or cells were washed with TBS, then mounted using Vectashield Hardset Mounting Media with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). All images were obtained using a fluorescence microscope (Nikon Eclipse 80i).

Western blot analyses. Cells were harvested in lysis buffer (10 mM Tris-HCl, 50 mM sodium chloride, 5 mM EDTA, 15 mM sodium pyrophosphate, 50 mM sodium fluoride and 100 μM sodium orthovanadate) supplemented with phosphatase (Roche Applied Science, Indianapolis, IN, USA) and protease (Sigma) inhibitor cocktails at 4˚C for 30 min. Following collection, the cells were sonicated on ice using Soniprep 150 (MSE Ltd., London, UK), then centrifuged at 15,000 x g for 15 min at 4˚C and then the supernatant was collected. The protein concentration was determined using a Coomassie (Bradford) protein assay kit (Thermo Fisher Scientific, Inc.). An equal amount of protein from each sample was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto a nitrocellulose membrane (GE Healthcare, Little Chalfont, UK). After blocking with 5% milk powder for 1 h at room temperature, the membranes were incubated in a primary antibody overnight at 4˚C. The antibodies used were anti-human and are as follows: CA IX (a gift from Professor M. Ladomery), E-cadherin, β-catenin, vimentin (all from BD Biosciences) and β-actin (Thermo Fisher Scientific, Inc.) which was used as a loading control. The blot membrane was washed, then incubated with a horseradish peroxidase-conjugated secondary antibody, and signals were revealed using a chemiluminescence kit (Thermo Fisher Scientific, Inc.).

Proliferation and apoptosis scoring. Proliferation and apoptosis were assessed as a percentage of Ki-67-positive cells and a percentage of cleaved caspase-3-positive cells, respectively. Briefly, 10 evenly distributed x40 fields of view were imaged using a fluorescence microscope (Nikon Eclipse 80i) for each independent group. Positively-labeled cells were counted and scored as a percentage of total cells. Experiments were performed at least in triplicate for each group.

Flow cytometry. Cells were washed once with Hanks' balanced salt solution (HBSS) (Thermo Fisher Scientific, Inc.), and then harvested with 0.05% trypsin/0.025% EDTA (Thermo Fisher Scientific, Inc.). Detached cells were washed with HBSS containing 2% horse serum (Sigma) (wash buffer), and re-suspended in the wash buffer (10^6 cells/100 μl). Anti-human CD24-FITC-conjugated (BD Biosciences) and anti-human CD44-APC-conjugated (BioLegend, Inc., San Diego, CA, USA) antibodies or the respective isotype controls were added to the cell suspension, as recommended by the manufacturer, and incubated at 4˚C in the dark for 30 min. Subsequently, the

labelled cells were washed in wash buffer and then analysed on an Accuri C6 cytometer using CFlow Plus software (both from BD Biosciences).

Cell cycle analysis. Cells were harvested, washed with ice-cold PBS, and then fixed in 70% ethanol for at least 30 min at 4˚C. Before analysis, the cells were washed again in PBS, then incubated in staining buffer [100 μg/ml RNase and 50 μg/ml propidium iodide (PI) (Sigma)] in the dark at 4˚C for 30 min. The samples were analysed by flow cytometry using an Accuri C6 cytometer (BD Biosciences). CFlow plus software (BD Biosciences) was used to calculate the percentage of cells in the G0/G1, S and G2/M phases. All studies were performed in triplicate.

Mammosphere forming assay. Six-well culture plates were coated with poly(2-hydroxyethyl methacrylate) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to obtain an ultra-low adhesion surface. Following treatment, the cells were trypsinized and mechanically disrupted to obtain single-cell suspensions. The single-cell suspensions were then plated at $1x10^3$ in 1 ml MCF-10A medium in the ultra-low adhesion wells. The cells were left to form spheres for 10 days, and mammospheres were considered cell aggregates >50 μm in diameter. The mammospheres were imaged, counted and measured using a phase-contrast inverted microscope (Nikon Eclipse TE300). Each experiment was repeated in triplicate.

Wound healing assay. Cells were plated in 6-well culture plates, and wounds were inflicted upon the cell monolayers using a sterile plastic 200-μl micropipette tip. Phase-contrast microscopy images were immediately obtained after wounding and again 48 h later using an inverted microscope (Nikon Eclipse TE300). The experiments were independently performed in triplicate, and the migration distance under each condition was assessed by analyzing the images using ImageJ software (National Institutes of Health, Rockville, MD, USA).

Transwell invasion assay. Transwell inserts (Millipore, Billerica, MA, USA) containing polycarbonate filters with 8-μm pores were used in the assay. The inserts were coated with 50 μl of Matrigel matrix (1 mg/ml) according to the manufacturer's recommendations (Thermo Fisher Scientific, Inc.). The cells were seeded in the upper chambers of the inserts at a density of $2x10^5$ cells in 1 ml serum-free MCF-10A medium. MCF-10A medium (2 ml) containing serum was placed in the lower chambers. Following 72 h of treatment, the cells on the upper surface of the membrane were removed using a methanol coated cotton swab. The cells on the lower chamber were fixed in 4% paraformaldehyde and stained with hematoxylin (Sigma). For each membrane, the number of cells was counted in 10 evenly distributed x40 fields of view using a light microscope (Nikon Eclipse 80i). Each experiment was repeated in triplicate.

Statistical analysis. Data for each group are presented as the mean ± SD. Statistical analyses were performed using SPSS for Windows, version 20.0 (IBM SPSS, Inc., Chicago, IL, USA). Values of $P<0.05$ were deemed statistically significant.

Results

Hypoxic conditions induce upregulation of carbonic anhydrase (CA IX). CA IX is a downstream target of HIF-1α and a robust marker of hypoxia (20). To assess the consequence of abnormal breast cell propagation on intracellular O_2 levels, sections of hyperplastic breast tissue were labeled for CA IX and compared to control tissue (Fig. 1A). Whilst CA IX expression was undetectable in control tissue, upregulation was prominent within hyperplastic tissue with the highest expression observed within the center of lesions corresponding to areas with the most limited access to blood supply.

To model hypoxic conditions found within the breast tumour microenvironment and delineate-associated consequences, MCF-10A cells were cultured in hypoxic conditions (1% O_2) for 72 h and compared to cells cultured in normoxia (21% O_2). Previous studies have suggested a level of ~1% O_2 is found within the breast tumour microenvironment (21). Whilst control MCF-10A cells cultured in normoxia displayed undetectable levels of CA IX expression, MCF-10A cells cultured under hypoxic conditions displayed an increase in CA IX expression detected by both fluorescence microscopy (Fig. 1B) and western blot analysis (Fig. 1C).

Hypoxia reduces proliferation, induces apoptosis and perturbs cell cycle progression. Increased cell division and evasion of cell death are both prominent features in most tumours (22). To assess the effects of hypoxia on cell division, proliferation was analysed by monitoring changes in Ki-67 expression (Fig. 2A). A statistically significant reduction in the percentage of Ki-67 positive cells was observed in MCF-10A cells cultured under hypoxic conditions in comparison to those cultured in normoxia (2.50±1.28 compared to 29.53±3.89% respectively; $P<0.05$) (Fig. 2B). To assess the effects of hypoxia on cell death, apoptosis was analysed using cleaved caspase-3 expression (Fig. 2C). A statistically significant increase in the percentage of cleaved caspase-3 positive cells was observed in MCF-10A cells cultured under hypoxic conditions compared with those cultured in normoxia (3.91±1.12 compared to 0.35±0.18% respectively; $P<0.05$) (Fig. 2D).

Given the decrease in proliferation and increase in apoptosis in MCF-10A cells cultured in hypoxia and the link between these parameters and cell cycle progression, cell cycle distribution analysis was performed using PI staining and flow cytometry (Fig. 2E). 'Gating' was performed in analyses to include live cells in the G0/G1, S or G2/M phases whilst excluding debris and/or necrotic cells. MCF-10A cells cultured in normoxia had the following distribution: G0/G1 phase, 74.27±0.81%; S phase, 5.63±0.32%; and G2/M phase, 18.57±1.30%, whilst MCF-10A cells cultured in hypoxia had this distribution: G0/G1 phase, 63.83±1.63%; S phase, 3.33±0.25%; and G2/M phase, 32.40±3.15%. As shown in Fig. 2F, MCF-10A cells cultured in hypoxic conditions had a statistically significant decrease in the percentage of cells in the G0/G1 and S phases ($P<0.05$), compared to MCF-10A cells cultured in normoxia. Conversely, a statistically significant increase in the percentage of cells in the G2/M phase ($P<0.05$) was observed in MCF-10A cells cultured in hypoxia compared to MCF-10A cells cultured in normoxia. Collectively, these

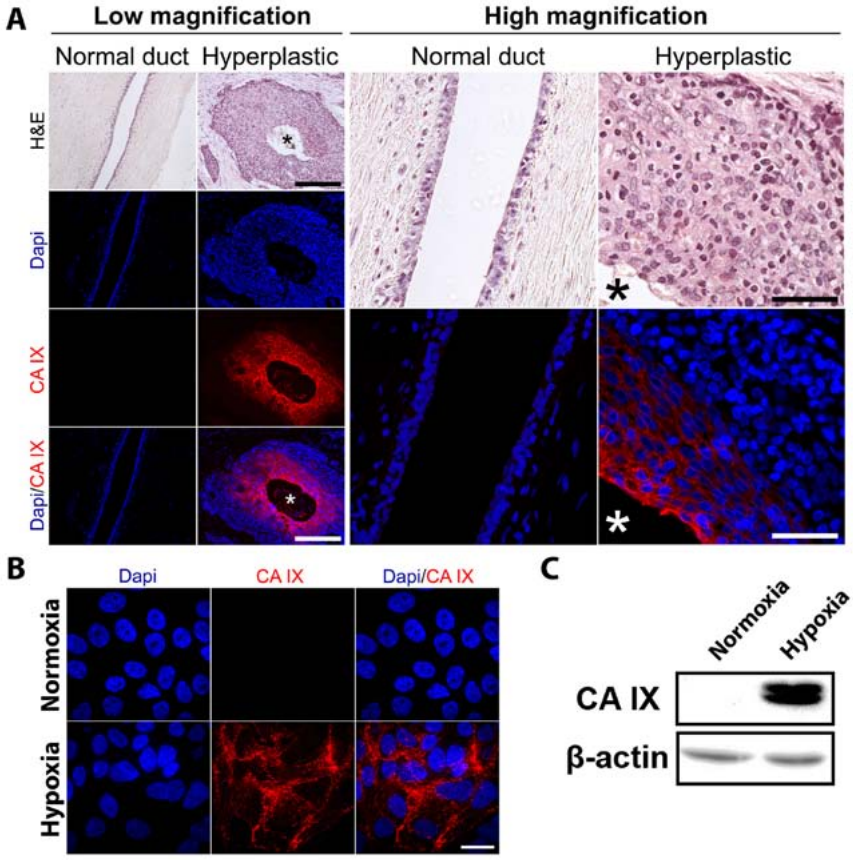

Figure 1. CA IX expression marks hypoxia in human breast tissue. (A) Serial sections of human breast tissue were stained with H&E or labeled for CA IX using fluorescent immunohistochemistry (scale bar represents 200 μm in low magnification; 50 μm in high magnification; * denotes the region furthest away from blood supply). (B) MCF-10A cells were placed in 20% O_2 or 1% O_2 culture conditions for 72 h then labeled for CA IX using fluorescent immunohistochemistry (scale bar represents 10 μm). (C) The expression levels of CA IX in MCF-10A cells were detected by western blotting, β-actin served as a loading control.

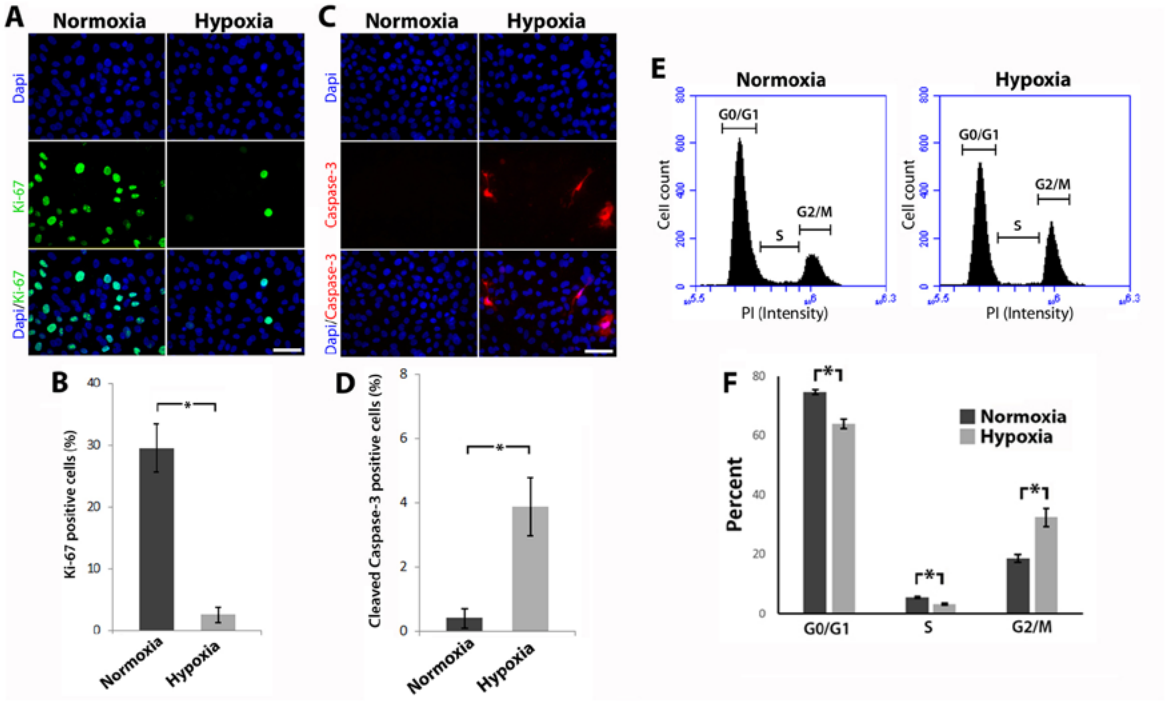

Figure 2. Hypoxia reduces proliferation, perturbs cell cycle progression and induces apoptosis. (A) Cells were labeled for Ki-67 using fluorescent immunohistochemistry (scale bar, 25 μm). (B) Percentage of Ki-67 positive cells (error bars ± standard deviation, *P≤0.05, Mann-Whitney U test, n≥3). (C) Cells were labeled for cleaved caspase-3 using fluorescent immunohistochemistry (scale bar, 25 μm). (D) Percentage of cleaved caspase-3 positive cells (error bars ± standard deviation, *P≤0.05, Mann-Whitney U test, n≥3). (E) Cell cycle distribution was evaluated using flow cytometry. (F) Graph displays the cell cycle phase expressed as a percentage of total cells (error bars ± standard deviation, *P≤0.05, Mann-Whitney U test, n≥3).

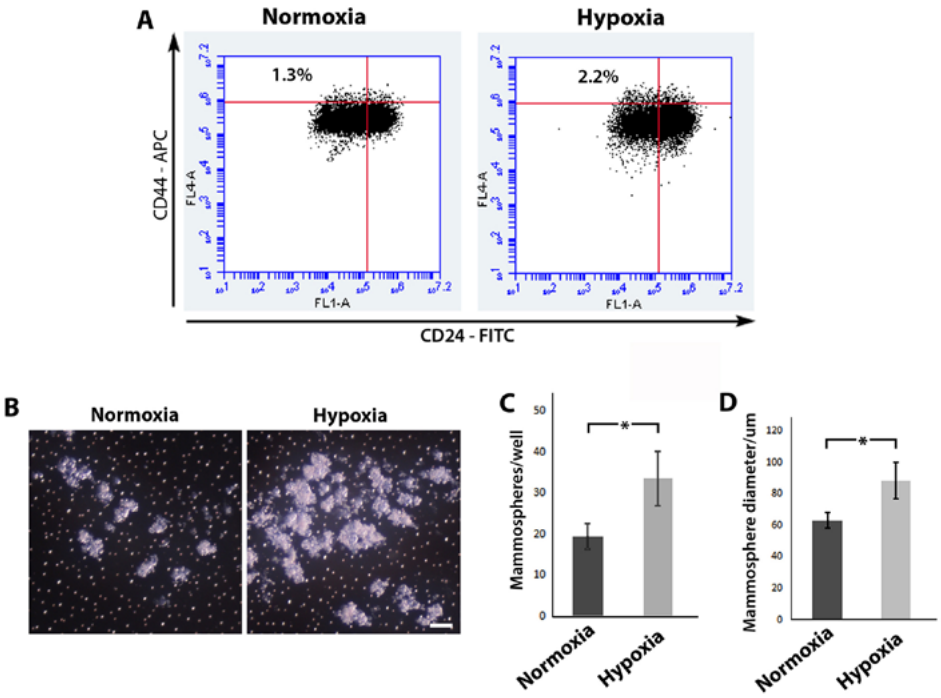

Figure 3. Hypoxia increases the stem/progenitor cell population. (A) Flow cytometric analysis of CD44 and CD24 cell surface markers. Percentage of $CD44^{+}CD24^{-/low}$ stained cells displayed. (B) Photomicrographs of mammospheres formed after 7 days (scale bar represents 100 μm). (C) Average number of mammospheres formed/well (error bars ± standard deviation, $^{*}P \leq 0.05$, Mann-Whitney U test, n≥3). (D) Average size of formed mammospheres (error bars ± standard deviation, $^{*}P \leq 0.05$, Mann-Whitney U test, n≥3).

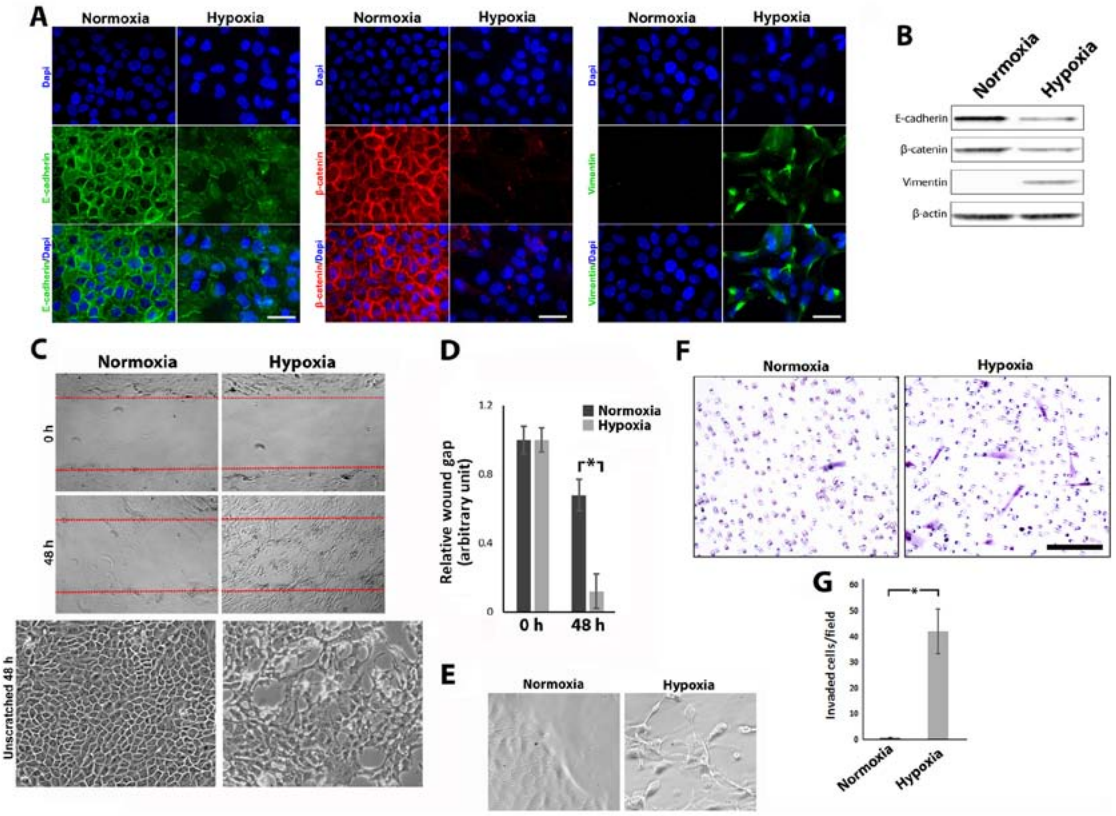

Figure 4. Hypoxia induces EMT, increased migration and invasion. (A) Cells were labeled for E-cadherin, β-catenin and vimentin using fluorescent immunohistochemistry (scale bar, 25 μm). (B) The levels of protein expression of E-cadherin, β-catenin and vimentin were detected by western blotting, β-actin served as a loading control. (C) Scratch wound migration assays were performed on confluent cells. Red dotted lines indicate the wound borders at the beginning of the assay. Lower panel displays comparative unscratched area. (D) Relative wound gap calculated as a ratio of the remaining wound gap at 48 h and the original wound gap at 0 h (error bars ± standard deviation, $^{*}P \leq 0.05$; Mann-Whitney U test, n≥3). (E) Phase contrast images of migratory leading edge. (F) Photomicrographs of invaded cells in Matrigel Transwell assay. (G) Average number of invaded cells/field of view (error bars ± standard deviation, $^{*}P \leq 0.05$; Mann-Whitney U test, n≥3).

data revealed that hypoxia can regulate cell growth and can block cell cycle progression in the G2/M phase.

Hypoxia increases the stem cell population. Previous studies have utilized the cell surface markers CD44 and CD24 to distinguish a $CD44^+CD24^-$/low sub-population which is enriched for stem cells/cancer stem cells (23,24). Using flow cytometric analysis (Fig. 3A), we revealed that MCF-10A cells cultured under hypoxic conditions displayed a higher percentage of cells in the $CD44^+CD24^-$/low sub-population in comparison to MCF-10A cells cultured in normoxia (2.2% in comparison to 1.3%, respectively). Mammosphere assays have been previously used as a surrogate reporter of stem cell activity (25,26), and an increase in the number and/or size of formed colonies are indicative of an expanded stem cell population. MCF-10A cells grown in normoxia or hypoxia were seeded in low adhesion culture vessels, left to form spheres in normoxia and then compared (Fig. 3B). Following re-oxygenation, MCF-10A cells cultured in hypoxia displayed a statistically significant increase in both mammosphere forming efficiency (33.50±6.56/well compared to 19.50±3.11/well, respectively; $P<0.05$) (Fig. 3C) and mammosphere size (88.78±11.57 compared to 63.49±4.77 μm respectively, $P<0.05$) (Fig. 3D) in comparison to MCF-10A cells initially cultured in normoxia. Collectively, these results suggest that hypoxic conditions lead to an expansion of the stem cell population.

Hypoxia induces EMT, increases migration and invasion. EMT is a process in which epithelial cells lose epithelial characteristics and acquire mesenchymal properties. It is recognized as an important event in the progression and dissemination of cancer (27). MCF-10A cells cultured in hypoxia or normoxia were labeled for E-cadherin, β-catenin and vimentin using fluorescent immunohistochemistry (Fig. 4A). MCF-10A cells cultured in hypoxic conditions displayed a loss of total and membrane bound E-cadherin, a loss of total and membrane bound β-catenin (although no nuclearization was apparent) concomitant with an upregulation of vimentin expression. Collectively, these expression changes along with characteristic changes noticed in cell shape are indicative of EMT (28,29). Total levels of protein expression, as detected by western blotting, confirmed global changes (Fig. 4B). Migratory and invasive capabilities are further traits acquired by cells to allow cancer metastasis (30). Scratch wound assays were used to assess the migratory ability of MCF-10A cells cultured in hypoxia vs. normoxia (Fig. 4C). MCF-10A cells cultured under hypoxic conditions displayed an increase in migratory ability. Comparative unscratched areas demonstrate highly polarized and tightly packed cells following normoxic culture conditions whilst following hypoxia, cells lose their tightly packed formation and appear more sporadic. Collectively, this suggests that wound closure is due to increased migratory abilities rather than an increase in cell numbers. Quantitative analysis of wound gap closure (Fig. 4D) revealed a statistically significant increase in gap closure and thus a higher rate of migration in MCF-10A cells cultured under hypoxic conditions (relative gap remaining after 48 h in normoxic culture conditions 0.68±0.091 compared to 0.12±0.1 in cells cultured under hypoxic conditions; $P<0.05$). Higher magnification of MCF-10A cells at the leading edge of migration revealed the extent of changes in cell shape and a more 'mesenchymal/fibroblastic' appearance of cells cultured in hypoxia as opposed to normoxia (Fig. 4E). A Matrigel Transwell invasion assay was used to compare and analyze the invasive capacity of MCF-10A cells cultured in hypoxia vs. normoxia. Whilst cells cultured in normoxia displayed a limited ability to move across the Matrigel barrier, cells cultured in hypoxic conditions were readily observed on the bottom of the insert (Fig. 4F). Quantification of these cells revealed a significant increase in the number of invaded cells in cells cultured under hypoxic conditions in comparison to those cultured in normoxia (42.2±8.57/field of view compared to 0.77±0.05/field of view respectively; $P<0.05$). Collectively, these results revealed that O_2 deprivation in MCF-10A cells can lead to changes consistent with EMT, increased migratory ability and increased invasive capabilities.

Discussion

As metastasis is responsible for ~90% of cancer-related deaths (2), underscoring the mechanisms that contribute to cancer dissemination are vital in our understanding of the disease and may thus help expose potential preventative strategies. Whilst considerable attention has been paid to the contribution of genetic and epigenetic alterations in this pathological process (5-7), the importance of tumour microenvironmental changes are beginning to be exposed (8). In the present study, we used hypoxic conditions to replicate an important microenvironmental change associated with tumourigenesis. MCF-10A cells were exposed to low O_2 levels to replicate conditions found within the earlier stages of breast tumourigenesis. O_2 deprivation led to some changes not immediately associated with tumourigenesis, such as decreased proliferation, cell cycle arrest and increased apoptosis. In contrast, hypoxia-induced changes were more consistent with a progression towards metastatic disease, such as an increase in 'stemness', induction of EMT and increased migratory and invasive capabilities.

Previous studies have linked hypoxia with reduced proliferation in breast cancer cell lines and ductal carcinoma *in situ* (31), a precursor of invasive ductal carcinoma. HIF-1α expression has been observed at this early stage of breast tumour development (31,32), and has been revealed to both inhibit transcription (33), and promote degradation (34) of c-MYC, an essential regulator of cellular growth and the cell cycle (35). Cell cycle progression was attenuated at the G2/M phase in our model and may represent a further mechanism for our observed reductions in proliferation. Previous studies have linked hypoxia with several G2/M checkpoint regulators and blockage of cell cycle progression at this phase (36,37) and this has been reported to contribute to increased chemo- and/or radio-resistance in some tumours (38,39). The induction of apoptosis in our model may also be explained through *HIF-1α* expression. HIF-1α has previously been reported to promote apoptosis (40,41). This, in part, could be explained by stabilization of p53 by HIF-1α (42) and/or by increased transcription of HIF-1α targets which are pro-apoptotic such as *NIP3* (43).

Given that sustaining proliferative signaling and resisting cell death are 'hallmarks' of cancer (22), reduced proliferation,

cell cycle arrest and increased apoptosis induced by hypoxia may seem disadvantageous for tumourigenesis. However, previous studies have demonstrated that hypoxia can exert a selective pressure whereby cells with accumulated genetic alterations, such as the loss of p53 (44), gain a selective advantage and constitute the tumour (44,45). Furthermore, it is known that hypoxia can increase mutation frequency and lead to genomic instability (46). This is most likely due to the effects of hypoxia on the DNA damage response exerted through both HIF-1α dependent (47) and HIF-1α independent means (48). Collectively, these changes can lead to the generation of cells which possess the genetic and epigenetic adaptations essential for tumour progression into metastatic disease.

Hypoxia in our model also induced an increase in the stem cell population. The link between stem cells and cancer is well documented (49,50), and cancer stem cells are reportedly responsible for initiating metastatic growth in various cancers including breast (23,51,52). Previous studies have reported a less differentiated phenotype and/or an increase in stemness induced by hypoxia in breast tumour tissue (31,53,54). Increases in stemness can, in part, be explained by the ability of HIF-1α and HIF-2α to induce various transcriptional programs, some of which include pluripotency factors (55,56). The consequence of hypoxia-induced increases in stem cell numbers in early neoplastic lesions and the contribution of this to metastatic disease may be two-fold. First, an increase in stem cell numbers due to hypoxia along with the increased rate of mutation may increase the chance of oncogenic mutations occurring within stem cell populations leading to cancer stem cells. Second, hypoxia leading to an increase in the number of cancer stem cells may lead to an increase in metastatic potential. These reasons may contribute to why hypoxia is linked to increased metastatic disease (13,16,17).

A more direct involvement of hypoxia in metastasis is elucidated from the induction of EMT observed in our model along with the increased migratory and invasive behavior of these cells. As mentioned previously, EMT is an important event in the progression and dissemination of cancer (27). Previous studies have linked hypoxia, EMT, increased migration and invasion to various cancer cell lines including breast (57-59). This most likely occurs through HIF-1α- and HIF-2α-related transcriptional changes (60). In the present study we demonstrated that hypoxia-induced EMT, increased migratory and invasive behavior in untransformed cells. Given that metastasis occurs in the later stages of cancer progression following an accumulation of genetic and epigenetic alterations, the significance of this finding remains unclear. However, a mechanistic dissection of the roles of HIF-1α and HIF-2α isoforms at this early stage of transformation and the relevance of their true input warrant further investigation and should be the basis of further experiments.

To conclude, the present study provided evidence that tumour-associated microenvironmental changes have a substantial role alongside genetic and epigenetic alterations in the progression of breast cancer. Hypoxia can occur in the earliest stages of tumourigenesis and influence various cellular processes associated with metastatic potential. Although the present study uses a simplistic approach to delineate the contributions of the hypoxic microenvironment from the myriad of genetic and epigenetic alterations found in human tumours; in reality, understanding the interactions between these co-contributors may elucidate the true factors driving metastasis in human disease.

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