HIF-1-dependent expression of angiopoietin-like 4 and L1CAM mediates vascular metastasis of hypoxic breast cancer cells to the lungs

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Most cases of breast cancer (BrCa) mortality are due to vascular metastasis. BrCa cells must intravasate through endothelial cells (ECs) to enter a blood vessel in the primary tumor and then adhere to ECs and extravasate at the metastatic site. In this study we demonstrate that inhibition of hypoxia-inducible factor (HIF) activity in BrCa cells by RNA interference or digoxin treatment inhibits primary tumor growth and also inhibits the metastasis of BrCa cells to the lungs by blocking the expression of angiopoietin-like 4 (ANGPTL4) and L1 cell adhesion molecule (L1CAM). ANGPTL4 is a secreted factor that inhibits EC–EC interaction, whereas L1CAM increases the adherence of BrCa cells to ECs. Interference with HIF, ANGPTL4 or L1CAM expression inhibits vascular metastasis of BrCa cells to the lungs.

Oncogene (2012) 31, 1757–1770; doi:10.1038/onc.2011.365; published online 22 August 2011

Keywords: breast cancer; metastasis; hypoxia

Introduction

Metastasis is the process that transforms breast cancer (BrCa) from a disease that is local and curable to one that is systemic and lethal. Metastatic dissemination of cancer cells may occur via the vascular or lymphatic system. At the time of primary tumor excision, metastasis may have already occurred in patients who eventually die from BrCa (Talmadge and Fidler, 2010). Vascular metastasis involves intravasation of cancer cells between endothelial cells (ECs) and into blood vessels, through which they are transported to distant tissues and arrest in capillary beds at the metastatic site, followed by extravasation out of the blood vessel and proliferation at the metastatic site (Liotta and Kohn, 2000). Several genes have been implicated in BrCa metastasis to the lungs (Minn et al., 2005; Gupta et al., 2007; Paik et al., 2008).

BrCa is characterized by intratumoral hypoxia, with a mean $P_{O_2}$ of 10 mm Hg (~1.5% $O_2$) as compared with 65 mm Hg (~9.5% $O_2$) in normal breast tissue (Vaupel et al., 2004). Patients who have a primary BrCa with $P_{O_2} < 20$ mm Hg are at increased risk of metastasis and mortality, independent of tumor size, stage, histology, grade or nodal status (Vaupel et al., 2004). Intratumoral hypoxia and resulting necrosis are even observed in ductal carcinoma in situ, the pre-invasive stage of BrCa (Bos et al., 2001; Dewhirst et al., 2008). Exposure of cancer cells to hypoxic conditions ex vivo increases their invasive and metastatic potential in vivo, indicating a cause-and-effect relationship (Subarsky and Hill, 2003; Tafani et al., 2010).

Reduced $O_2$ availability leads to activation of hypoxia-inducible factor 1 (HIF-1), a transcription factor that regulates the expression of genes that have roles in many critical aspects of cancer biology including angiogenesis, metabolism, stem cell renewal, invasion, immune avoidance and therapeutic resistance (Semenza, 2010). HIF-1 is composed of $O_2$-regulated HIF-1$\alpha$ and constitutively expressed HIF-1$\beta$ subunits (Wang et al., 1995). Increased HIF-1$\alpha$ protein levels in tumor biopsies are associated with increased risk of metastasis and mortality in node-negative (Bos et al., 2003), node-positive (Schindl et al., 2002; Kronblad et al., 2006), human epidermal growth factor receptor 2-positive (Giatromanolaki et al., 2004), estrogen receptor-positive (Generali et al., 2006) and unselected (Dales et al., 2005; Vleugel et al., 2005; Trastour et al., 2007; Yamamoto et al., 2008) BrCa patients. HIF-2$\alpha$ is also $O_2$ regulated, dimerizes with HIF-1$\beta$, and its expression is associated with BrCa metastasis and mortality (Helecynska et al., 2008). Increased HIF target gene expression is also associated with BrCa mortality (Buffa et al., 2010).

The correlative data from clinical studies have been complemented by studies in mouse models, which have
demonstrated that HIF-1 has a key role in primary tumor growth and vascularization (Semenza, 2010). Metastasis of autochthonous BrCa to the lungs was decreased in mice with knockout of HIF-1α in mammary epithelial cells (Liao et al., 2007), although specific target genes involved in promoting metastasis were not identified. The hypoxia-induced and HIF-1α-dependent expression of lysyl oxidase promoted a pro-metastatic microenvironment in the lungs of mice bearing orthotopic BrCa xenografts (Erler et al., 2006, 2009). However, the molecular mechanisms by which intratumoral hypoxia promotes metastasis of BrCa through blood vessels to the lungs remain to be determined.

Further delineation of the role of HIFs in BrCa metastasis is clinically relevant because of the identification of drugs that inhibit HIF activity (Melillo, 2007; Verheul et al., 2008; Chintala et al., 2010), including digoxin, which has been used for decades to treat heart failure and which potently inhibits the growth of hepatic and prostate cancer xenografts (Zhang et al., 2008). In this study, we utilized a mouse orthotopic model to investigate the cellular and molecular mechanisms by which HIF activity promotes vascular metastasis of BrCa cells from primary tumors to the lungs and to determine whether this process can be inhibited by digoxin therapy.

Results

Inhibition of HIF expression impairs breast tumor growth and lung metastasis

The MDA-MB-231 cell line was established from metastatic cells in the pleural fluid of a BrCa patient (Cailleau et al., 1978). Transplantation of MDA-MB-231 cells into the mammary fat pad (MFP) of severe combined immunodeficiency (SCID) mice results in growth of a primary breast tumor that spontaneously metastasizes to the lungs. To analyze the role of HIFs in BrCa progression, we inhibited HIF expression by stably transfecting MDA-MB-231 cells with an expression vector encoding a short hairpin RNA (shRNA) against HIF-1α (sh1) or HIF-2α (sh2); vectors encoding shRNAs against both HIF-1α and HIF-2α (double knockdown (DKD)); or empty vector (EV). Immunoblot analyses confirmed decreased levels of HIF-1α and HIF-2α protein under non-hypoxic (20% O2) and hypoxic (1% O2) conditions (Figure 1a). There was no significant difference in the proliferation of the subclones (Supplementary Figure S1A).

The growth of primary tumors derived from sh1α, sh2α and DKD subclones was significantly decreased compared with tumors derived from EV cells (Figure 1b). The inhibition of primary tumor growth in the absence of direct effects on cell proliferation in tissue culture is consistent with the well-established role of HIFs in regulating tumor vascularization (Semenza, 2010). HIF-1α levels were reduced in DKD as compared with EV primary tumors, whereas β-actin levels were similar (Figure 1c); HIF-2α protein expression was below the limits of detection in both EV and DKD tumors. Lung sections were analyzed for spontaneous metastases (Figure 1d). The number of lung metastases was significantly decreased in mice bearing sh1α, sh2α or DKD as compared with EV primary breast tumors (Figure 1e). To obtain a more sensitive estimate of overall lung metastatic burden, genomic DNA was isolated from the contralateral lung and quantitative real-time PCR (qPCR) was performed using primer pairs that only amplify human DNA (Supplemental Figure S1B). The number of metastatic cells in the lungs of mice bearing sh1α, sh2α or DKD tumors was significantly reduced as compared with EV tumors (Figure 1f).

To complement this genetic approach, in which HIF activity was inhibited in cancer cells before implantation, we treated mice bearing established tumors with digoxin, which inhibits translation of HIF-1α and HIF-2α mRNA into protein by an mTOR-independent mechanism (Zhang et al., 2008). SCID mice were subjected to MFP injection of parental MDA-MB-231 cells and treated with daily intraperitoneal injection of digoxin or saline starting on day 14. Digoxin treatment did not cause weight loss (Supplementary Figure S1C) or any other sign of toxicity, but significantly inhibited GLUT1 and HK1 mRNA expression in primary breast tumors (Supplementary Figures S1D and E) and reduced their growth (Figure 1g). Histological analysis of lung sections (Figure 1h) revealed that both the number of lung metastases (Figure 1i) and their size (Figure 1h) were significantly decreased by digoxin, resulting in a marked reduction in total lung metastatic burden (Figure 1j). Thus, genetic or pharmacological inhibition of HIF activity impairs BrCa growth and metastasis.

HIF activity in BrCa cells modulates EC–EC and EC-cancer cell interactions

To metastasize via blood vessels, BrCa cells must invade through the EC monolayer that defines the vascular lumen. We hypothesized that HIFs mediate production of secreted proteins that promote cancer cell invasion by inhibiting EC–EC interaction. To test this hypothesis, EC monolayers on Boyden chamber inserts were exposed to MDA-MB-231 conditioned medium (CM). The medium was then removed, and the upper chamber was seeded with naïve 5-chloromethylfluorescein diacetate-labeled MDA-MB-231 cells. CM from hypoxic cells significantly increased MDA-MB-231 invasion of the EC monolayer (Figure 2a) and the effect of CM on EC invasion was inhibited by knockdown of HIF-1α and HIF-2α (Figure 2b) or digoxin treatment (Figure 2c). To demonstrate that the CM inhibited EC–EC interaction, we measured trans-endothelial electrical resistance (TER). When EC monolayers were incubated with CM from hypoxic EV cells, a significant reduction in TER was observed (Figure 2d). TER was increased when ECs were incubated with CM from DKD cells or digoxin-treated cells (Figure 2d). These results indicate that a hypoxia induced, HIF-dependent factor that inhibits EC–EC interaction is secreted by BrCa cells.
In order for BrCa cells to extravasate from blood vessels at a metastatic site, they must adhere to ECs. We hypothesized that hypoxia-induced HIF activity promotes cancer cell-EC interaction. To test this hypothesis, adherence of MDA-MB-231 cells to ECs was determined. Hypoxia significantly increased MDA-MB-231 adherence to ECs in a HIF-dependent manner (Figure 2e). Thus, the results shown in Figure 2 support...
The experiment was repeated but the lungs were not harvested until 3 weeks after injection to determine whether isolated cells present at 1 week proliferate to form metastatic foci. Analysis of lung sections (Figure 3d) revealed significant effects of hypoxia and HIF activity on lung focus formation (Figure 3e) and total lung BrCa burden (Figure 3f).

To complement the analysis of genetically manipulated cells, mice were intravenously injected with MDA-MB-231-Luc cells that express firefly luciferase and treated with digoxin or saline. Bioluminescent imaging 10 and 21 days after injection revealed decreased tumor cells in the lungs of digoxin-treated mice at both time points (Figure 3g). Lung BrCa burden, as determined by qPCR on day 21, was also decreased (Figure 3h). The data presented in Figure 3 provide a mechanistic link between the effects of hypoxia and HIF activity on spontaneous metastasis from primary breast tumors to the lung (Figure 1) and BrCa induced changes in EC–EC and EC-cancer cell interactions (Figure 2).

HIF activity mediates induction of ANGPTL4 expression in hypoxic BrCa cells

To search for effectors of the HIF-mediated metastasis of hypoxic MDA-MB-231 cells, the expression of a panel of 88 metastasis-related genes was quantified by reverse transcriptase qPCR and the data were analyzed to determine whether any of the hypoxia-induced mRNAs encoded a secreted protein that might promote vascular metastasis.

HIF activity promotes extravasation of tumor cells from the lung vasculature

We hypothesized that exposure of BrCa cells to hypoxia in the primary tumor before intravasation may induce the expression of proteins that subsequently facilitate extravasation in the lungs. To test whether HIF activity promotes the egress of blood-borne BrCa cells from the pulmonary circulation, we cultured EV and DKD cells under non-hypoxic (20% O2) or hypoxic (1% O2) conditions for 48 h, labeled with 5-chloromethylfluorescein diacetate, and their adhesion to HUVEC monolayers was determined (mean ± s.d.; n = 3; *P < 0.05 vs EV-20%; #P < 0.05 vs EV-1%; Student's t-test). EV and DKD cells were cultured at 20 or 1% O2 for 48 h in the presence (EV + digoxin) or absence (EV and DKD) of 100 nM digoxin, labeled with 5-chloromethylfluorescein diacetate, and their adhesion to HUVEC monolayers was determined (mean ± s.d.; n = 6; *P < 0.05 vs EV-20%; #P < 0.05 vs EV-1%; Student's t-test). EV and DKD cells were cultured at 20 or 1% O2 for 48 h.
inhibit EC–EC interaction. Expression of mRNA encoding angiopoietin-like 4 (ANGPTL4) was induced by hypoxia in MDA-MB-231 cells and subsequent analysis of subclones revealed that the hypoxia-induced expression of ANGPTL4 mRNA (Figure 4a) and protein (Figure 4b) was dependent upon HIF-1α and,
to a lesser extent, HIF-2α as described in other cell types (Belanger et al., 2002; Manalo et al., 2005). Digoxin inhibited the hypoxia-induced expression of HIF-1α and ANGPTL4 protein with similar dose dependency (Figure 4c). ANGPTL4 mRNA levels were reduced in tumors (analyzed in Figure 1g) from digoxin-treated as compared with saline-treated mice (Figure 4d).

To investigate whether ANGPTL4 is a direct HIF-1 target gene, we searched for potential HIF-1-binding sites and identified the sequence 5′-ACGTGCCACCA-3′ located 1.6 kb 5′ to the human ANGPTL4 translation initiation codon. Several known hypoxia response elements (HREs) contain the consensus sequence 5′-RC GTG[N1-8 ]CACA-3′ (Fukuda et al., 2007). Chromatin
immunoprecipitation assays demonstrated hypoxia-inducible binding of HIF-1α at this site in MDA-MB-231 cells comparable to its binding to an established HRE in the LDHA gene, whereas hypoxia had no effect on binding to the RLPI3A gene, which is not HIF-1 regulated (Figure 4e). To determine whether this HIF-1 site was embedded in a transcriptionally active HRE, a 56-bp sequence spanning the site (Supplementary Figure S2) was cloned into a reporter plasmid (pGL2), which contains firefly luciferase coding sequences downstream of an SV40 promoter. Cells were co-transfected with pGL2 or pGL2-HRE and pSV-Renilla (which contains Renilla luciferase sequences downstream of the SV40 promoter) and exposed to 20 or 1% O2 for 24 h. The 56-bp sequence mediated increased firefly luciferase activity under hypoxic conditions (Figure 4f), thereby fulfilling the criteria for an HRE. The results presented in Figures 4a–f demonstrate that ANGPTL4 is a direct HIF-1 target gene in BrCa cells.

**ANGPTL4 expression inhibits EC–EC interaction and promotes EC monolayer invasion**

To determine whether ANGPTL4 contributes to the effects of CM from hypoxic BrCa cells on ECs, we generated MDA-MB-231 subclones that were transfected with a vector encoding either of two different shRNAs against ANGPTL4 (shA4-2, shA4-4) or a non-targeting control shRNA (shNT). Expression of shA4-2 or shA4-4, but not shNT, reduced ANGPTL4 mRNA and protein (Figure 4g). TER was increased in EC monolayers incubated with CM from shA4-2 and shA4-4 cells as compared with medium from A4-4 cells, especially under hypoxic conditions (Figure 4i). The stimulatory effect of CM from hypoxic MDA-MB-231 cells on invasion through an EC monolayer was decreased when cells with ANGPTL4 knockdown were the source of CM (Figure 4i).

To complement loss-of-function studies, a gain-of-function approach was used by stably transfecting the MDA-MB-231 DKD subclone, which has reduced ANGPTL4 levels, with a vector encoding ANGPTL4 (pAngptl4) or EV (pBabe). Immunoblot assays demonstrated that DKD.pAngptl4 cells have increased ANGPTL4 levels compared with DKD.pBabe (Figure 4j). Compared with DKD.pBabe, CM from non-hypoxic DKD.pAngptl4 cells significantly reduced TER (Figure 4k) and promoted EC monolayer invasion by naïve MDA-MB-231 cells (Figure 4l). The data in Figures 4g–l demonstrate that ANGPTL4 expression in hypoxic BrCa cells mediates changes in EC–EC interaction that promote vascular metastasis.

**ANGPTL4 promotes extravasation of BrCa cells in the lungs**

To test whether ANGPTL4 promotes extravasation of BrCa cells from the pulmonary vasculature, DKD.pBabe and DKD.pAngptl4 subclones were injected via tail vein and 1 week later lung sections were isolecitin stained. Fluorescence microscopy (Figure 5a) revealed a greater number of extravasated GFP⁺ DKD.pAngptl4 cells as compared with DKD.pBabe cells (Figure 5b). Lung BrCa burden, as determined by GFP qPCR, was also increased in lungs from mice injected with DKD.pAngptl4 cells (Figure 5c). Thus, ANGPTL4 expression rescues the defective extravasation of DKD cells. To determine whether the difference in extravasated cells leads to stable differences in lung BrCa burden, the experiment was repeated and lungs were harvested after 3 weeks. The GFP qPCR signal was increased 3 weeks after injection of DKD.pAngptl4, as compared with DKD.pBabe, cells (Figure 5d). Conversely, the increase in lung BrCa burden that was observed when MDA-MB-231 cells expressing shNT were incubated under hypoxic conditions before injection was impaired in cells expressing shA4-2 (Figure 5e).

To determine whether ANGPTL4 is required for spontaneous metastasis of BrCa cells to the lung, the shNT and shA4.2 subclones were transplanted into the MFP. There was no significant difference in the growth of primary breast tumors (Figure 5f). In contrast, lung metastasis was markedly inhibited by loss of ANGPTL4 expression, as determined by histological analysis (Figures 5g and h) and qPCR assays (Figure 5i). Taken together, the finding that HIFs activate ANGPTL4 expression and that ANGPTL4 specifically promotes the metastasis of MDA-MB-231 cells to the lungs, provides a mechanism by which HIFs directly affect lung metastasis independent of their known effects on primary tumor growth.

**HIF-1-mediated expression of L1 cell adhesion molecule (L1CAM) promotes EC-cancer cell interaction**

We also analyzed the panel of metastasis-related genes to identify a hypoxia-induced mRNA encoding a cell surface receptor that might mediate interaction of BrCa cells with ECs. Expression of the cell adhesion molecule L1CAM was induced by hypoxia in MDA-MB-231 cells and analysis of subclones demonstrated that HIF-1α was required for L1CAM mRNA (Figure 6a) and protein (Figure 6b) expression, especially under hypoxic conditions. Digoxin treatment inhibited hypoxia-induced expression of HIF-1α and L1CAM protein with similar dose dependency (Figure 6c). L1CAM mRNA levels were decreased in primary breast tumors from digoxin-treated as compared with saline-treated mice (Figure 6d).

MDA-MB-231 cells were transfected with a vector encoding either of two different shRNAs against L1CAM (shL1-3, shL1-5) or a control non-targeting shRNA (shNT). Expression of shL1-3 or shL1-5 but not shNT reduced L1CAM mRNA and protein (Figure 6e). Knockdown of L1CAM expression reduced adherence of both hypoxic and non-hypoxic BrCa cells to ECs (Figure 6f). In contrast, CM from shL1-3, shL1-5 or shNT cells had similar effects on TER (Supplementary Figure S3) indicating that L1CAM affects EC-cancer cell, but not EC–EC, interaction.

To complement loss-of-function studies, MDA-MB-231 DKD cells were transfected with vector encoding the full-length, membrane-bound form of L1CAM
(pL1CAM) or EV (pcDNA3). DKD,pL1CAM cells expressed levels of L1CAM protein comparable to EV cells exposed to 1% O2 (Figure 6g). L1CAM expression in DKD cells increased their adherence to ECs (Figure 6h). Thus, HIF-1-mediated L1CAM expression contributes to increased EC-cancer cell interaction.

**Figure 5** ANGPTL4 enhances breast cancer (BrCa) cell metastasis to the lungs. (a–c) pBabe and pAngptl4 subclones of MDA-MB-231 DKD cells were injected into the tail vein of SCID mice. After 1 week, lung tissues were harvested, sections stained with isolectin B4 and GFP-expressing cancer cells (a) were counted under fluorescent microscopy (b). To determine the lung BrCa burden, lung DNA was analyzed by qPCR with GFP primers and the results (mean ± s.e.m., n = 5) were normalized to pBabe (c). *P < 0.05 (Student’s t-test). (d) pBabe and pAngptl4 subclones were injected into the tail vein of SCID mice. After 3 weeks, lung DNA was analyzed by qPCR with GFP primers and results (BrCa burden; mean ± s.e.m., n = 5) were normalized to pBabe. *P < 0.05 (Student’s t-test). (e) MDA-MB-231 cells stably expressing shNT or shA4-2 were cultured at 20 or 1% O2 for 48 h and injected into the tail vein of SCID mice. After 3 weeks, lung DNA was analyzed by qPCR with HK2 primers and results (mean ± s.e.m., n = 5) were normalized to shNT-20%. *P < 0.05 vs shNT-20%; #P < 0.05 vs shNT-1% (Student’s t-test). (f–i) MDA-MB-231 cells stably expressing shA4-2 or shNT were implanted in the MFP of SCID mice. Primary tumor volume (f) was determined every 3–5 days; NS, not significant (analysis of variance). Lung sections were stained with hematoxylin-eosin (g) and metastatic foci were counted (h). Lung DNA was subjected to qPCR with human-specific HK2 primers (i). Mean ± s.e.m. data are shown (n = 5); *P < 0.05 vs shNT (Student’s t-test).
L1CAM promotes extravasation

Compared with DKD, pcDNA3, cells, DKD, pL1CAM cells manifested increased extravasation from the pulmonary vasculature (Figures 7a and b) and increased lung BrCa burden (Figure 7c) at 1 week after intravenous injection. At 3 weeks post-injection, lung metastatic burden was increased by L1CAM gain of function (Figure 7d) and decreased by L1CAM loss of function (Figure 7e). L1CAM loss of function resulted in a modest effect on primary tumor growth (Figure 7f). However, L1CAM knockdown significantly impaired spontaneous metastasis of MDA-MB-231 BrCa cells to the lungs (Figures 7g–i).

HIF activity is required for metastasis of MDA-MB-435 cells to the lungs

Results presented in Figures 1–7 demonstrate a critical role for HIF-1-mediated ANGPTL4 and L1CAM expression in vascular metastasis of hypoxic MDA-MB-231 cells to the lungs. Inhibition of primary tumor growth may also have contributed to the reduction in lung metastasis. To demonstrate that these processes are a general feature of BrCa cells and to analyze a cell line that results in macro-metastases (as opposed to the micro-metastases observed in mice bearing MDA-MB-231 tumors), we utilized MDA-MB-435 cells. Although there has been controversy regarding their derivation,

**Figure 6** L1CAM is regulated by HIF-1 and stimulates EC-cancer cell interaction. (a) MDA-MB-231 subclones were cultured at 20 or 1% O2 for 24 h and L1CAM mRNA was analyzed by reverse transcription (RT)-qPCR (mean ± s.d., n = 3). *P < 0.05 vs EV-20%; #P < 0.05 vs EV-1%; Student’s t-test. (b) MDA-MB-231 subclones were cultured at 20 or 1% O2 for 48 h and L1CAM and β-actin protein levels were determined by immunoblot assay. The ratio of L1CAM:β-actin signal intensity, normalized to EV-20%, is shown. (c) Parental MDA-MB-231 cells were cultured at 20 or 1% O2 for 48 h in the presence of the indicated concentration of digoxin and cell lysates were subjected to immunoblot assays. (d) L1CAM mRNA expression in primary tumors (from Figure 1g) was analyzed by RT-qPCR (mean ± s.d.; n = 5; *P < 0.05 vs saline). (e–f) MDA-MB-231 cells stably expressing a non-targeting shRNA (shNT) or either of two independent shRNAs targeting L1CAM (shL1-3, shL1-5) were cultured at 20 or 1% O2 and analyzed for: (e) L1CAM mRNA and protein (inset) expression; and (f) adherence to human umbilical vein endothelial cell (HUVEC) monolayer (mean ± s.d.; n = 3; *P < 0.05 vs shNT-20%; #P < 0.05 vs NT-1%; Student’s t-test). (g, h) MDA-MB-231 EV cells and DKD cells stably transfected with EV (pcDNA3) or vector encoding L1CAM (pL1CAM) were cultured at 20 or 1% O2 for 48 h and analyzed for: (g) L1CAM, HIF-1α and β–actin protein expression; and (h) adherence to HUVEC monolayer (mean ± s.d.; n = 3; *P < 0.05 vs pcDNA3; Student’s t-test).
recent evidence has confirmed their identity as BrCa cells (Chambers, 2009) and they represent an excellent model of aggressive metastasis from breast to lungs. Subclones with knockdown of HIF-1α (sh1α), HIF-2α (sh2α), both (DKD) or neither (EV) were generated. Hypoxia-induced expression of both ANGPTL4
Figure 8  HIF-1 regulates ANGPTL4 and L1CAM expression and promotes metastasis of MDA-MB-435 cells to the lungs. (a–c) MDA-MB-435 cells stably transfected with a lentiviral vector encoding short hairpin RNA directed against HIF-1α (sh1α), HIF-2α (sh2α) or both (DKD) or with EV were cultured at 20 or 1% O2 for 24 (a, b) or 48 (c) h. ANGPTL4 (a) and L1CAM (b) mRNA expression was determined by reverse transcription (RT)–qPCR, relative to EV-20% (mean ± s.d., n = 3); *P < 0.05 vs EV-20%; #P < 0.05 vs EV-1% (Student’s t-test). Protein expression was determined by immunoblot assay (c). (d–g) MDA-MB-435 cells were implanted in the MFP of SCID mice (n = 5 each), which were treated with daily intraperitoneal (IP) injections of saline or digoxin (2 mg/kg) starting 7 days after implantation. Tumor volumes were determined every 3–5 days (d). *P < 0.05 vs saline (analysis of variance). Lung sections were stained with hematoxylin-eosin (e) and the percentage of total lung area occupied by metastases was determined (f). Lung DNA was analyzed by qPCR with HK2 primers and results (mean ± s.e.m., n = 5) were normalized to saline (g). *P < 0.05 vs saline (Student’s t-test). (h) Effect of combined therapy with digoxin and doxorubicin. Mice bearing MDA-MB-231 xenografts were treated, starting at time 0 with daily digoxin (1 mg/kg IP) or weekly doxorubicin (2 mg/kg intravenous) injections or both. Tumor volume was determined weekly (mean ± s.e.m., n = 8). *P < 0.05 (analysis of variance) for indicated comparison. (i) Role of HIF-1-dependent ANGPTL4 and L1CAM expression in vascular metastasis of hypoxic BrCa cells to the lungs. Primary BrCa and lung metastasis are indicated by white and yellow arrows, in top and bottom panel, respectively.
HIF-1, ANGPTL4 and L1CAM promote lung metastasis

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and L1CAM (Supplementary Figure S4B) mRNA was inhibited by digoxin treatment, resulting in decreased ANGPTL4 and L1CAM protein levels (Supplementary Figure S4C). Following MFP injection of MDA-MB-435 cells, mice were treated with digoxin, starting on day 7 because of the increased metastatic properties of MDA-MB-435 compared with MDA-MB-231 cells. Digoxin treatment resulted in reduced primary breast tumor growth (Figure 8d). Histological analysis (Figure 8e) revealed that 17.3% of total lung area was occupied by metastases in saline-treated mice compared with 0.9% in digoxin-treated mice (Figure 8f). Metastatic burden was reduced by >96% in the lungs of digoxin-treated mice (Figure 8g), demonstrating a profound effect of HIF inhibition on MDA-MB-435 lung metastasis. The almost complete elimination of MDA-MB-435 metastasis to the lungs in digoxin-treated mice despite only a modest reduction in primary tumor growth provides compelling evidence for a specific effect of HIF-1 on lung metastasis.

Anthracycline chemotherapeutic agents such as doxorubicin are used as adjuvant therapy and first-line treatment for metastatic disease in BrCa patients not previously exposed to them (Palmieri et al., 2010). We hypothesized that the anti-cancer effects of digoxin might improve the outcome of treatment with doxorubicin. To test this translational hypothesis, we treated mice with MDA-MB-231 xenografts with daily low-dose digoxin (1 mg/kg intraperitoneal), weekly low-dose doxorubicin (2 mg/kg intravenously) or both. Combination therapy resulted in significantly improved tumor control compared with doxorubicin alone (Figure 8h).

Discussion

In this study, we demonstrate that HIF activity promotes primary tumor growth and spontaneous metastasis of human MDA-MB-231 and MDA-MB-435 cells to the lungs of SCID mice and provide two distinct cellular and molecular mechanisms by which HIFs mediate this effect (Figure 8i). First, hypoxic BrCa cells produce secreted factors that inhibit EC-EC interactions thereby facilitating extravasation of metastatic cells and colonization of the lungs by blood-borne cancer cells. We identified ANGPTL4 as a secreted factor with these properties and show that ANGPTL4 transcription is directly regulated by binding of HIF-1 to an HRE in the 5′-flanking region of the gene. Although HIF-1 regulates the expression of many genes required for primary tumor growth, our results demonstrate that ANGPTL4 is a HIF-1 target gene that specifically contributes to vascular metastasis with no major role in primary tumor growth.

Increased expression of ANGPTL4 was previously demonstrated in MDA-MB-231 subclones that were selected for increased lung metastasis in mice as well as in primary BrCa of patients with lung metastases (Minn et al., 2005). Transforming growth factor (TGF)β1 treatment increased ANGPTL4 expression in BrCa cells (Padua et al., 2008), but the transcription factor directly responsible for this effect was not determined. Previous studies reported that HIF-1 activates transcription of the genes encoding TGFβ1 and TGFβ3 (Caniggia et al., 2000; Nishi et al., 2004; Manalo et al., 2005) and that TGFβ3 activates HIF-1 transcriptional activity (Ueno et al., 2011). Thus, crosstalk between these pathways may drive ANGPTL4 expression in BrCa cells in response to hypoxia, TGFβ or both. Whereas the work cited above supports our conclusion that ANGPTL4 promotes BrCa metastasis, it is important to note that in melanoma ANGPTL4 has been shown to inhibit metastasis by reducing vascular permeability (Galau et al., 2006), underscoring the need for further study to delineate the mechanisms by which ANGPTL4 exerts its effects on ECs.

Second, exposure of BrCa cells to hypoxia increases HIF-1-dependent expression of L1CAM, which promotes adherence of BrCa cells to ECs. L1CAM, which is associated with metastasis and patient mortality in several cancer types, engages in homophilic interactions as well as heterophilic interactions with integrins, CD24 and neuropilin-1 (Issa et al., 2009). Whereas HIF-1 promotes primary tumor growth (for example, through effects on angiogenesis), as well as metastasis, the downstream targets identified in this study, primarily (L1CAM) or exclusively (ANGPTL4) for metastasis.

Taken together, our results have delineated molecular mechanisms by which hypoxia increases vascular metastasis of BrCa cells to the lungs (Figure 8i). MDA-MB-231 is a model for triple-negative BrCa, which lacks expression of estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2; is highly aggressive with frequent metastasis to lungs and brain; and has a high recurrence rate after neoadjuvant anthracycline chemotherapy (Pal et al., 2011). HIF-dependent activation of ANGPTL4 and L1CAM expression demonstrated here provides potential targets for BrCa therapy. Inhibitors of ANGPTL4 or L1CAM are not currently available. In contrast, digoxin is a drug that has been administered orally to safely treat cardiac disease for decades. We previously reported that digoxin blocks the synthesis of HIF-1α and HIF-2α at nanomolar concentrations and inhibits hepatocarcinoma and prostate cancer xenograft growth in a HIF-1-dependent manner (Zhang et al., 2008). In this study, we have demonstrated that digoxin inhibits primary tumor growth and lung metastasis in two models of BrCa. The similar effects of genetic knockdown and digoxin treatment in every assay that was performed suggest that digoxin inhibits BrCa growth and metastasis by blocking HIF activity. The therapeutic range for digoxin is well established and, based on the data presented above, clinical trials are warranted to determine whether drug levels achievable in patients are sufficient to block HIF activity and, in combination with established chemotherapy, inhibit BrCa growth and metastasis, particularly in cases where high HIF-1α expression is demonstrated in the primary tumor.
Materials and methods

Vectors and cell lines

Construction of expression vectors, establishment of stably transfected subclones, maintenance of cell lines and exposure of cells to hypoxia are described in the Supplementary Information.

Immunoblot, quantitative reverse transcriptase–PCR, chromatin immunoprecipitation and reporter gene assays

Protein, mRNA, chromatin and transcriptional analyses are described in the Supplementary Information.

TER assay

The assay was performed as described previously (Krishnamachary et al., 2006). See Supplementary Information for details.

TransEC migration assay

Boyden chambers were inserted with human umbilical vein endothelial cell monolayers, which were exposed to CM from MDA-MB-231 cells cultured for 48 h in 20 or 1% O2 with/without digoxin. After overnight incubation, the CM was removed and parental MDA-MB-231 cells labeled with 5 μM CellTracker Green 5-chloromethylfluorescein diacetate (Invitrogen) were seeded onto the human umbilical vein endothelial cells in serum-free EGM-2 (Lonza, Walkersville, MA, USA). Dulbecco’s modified eagle medium with 10% Fetal bovine serum was used as chemoattractant in the bottom chamber. After 20 h, non-invaded cells on top were removed and invaded cells on the bottom were fixed with methanol and counted under fluorescence microscopy.

Cell adhesion assay

MDA-MB-231 cells were cultured for 48 h in 20 or 1% O2 with/without digoxin, stained with 5-chloromethylfluorescein diacetate (5 μM) and seeded onto human umbilical vein endothelial cell monolayers at 37°C in a 24-well plate. After 30-min incubation, non-adherent cells were washed off with phosphate buffered saline (PBS), adherent cells were lysed and fluorescence was measured using a plate reader.

Animal studies

Studies using 5–7 week-old female SCID mice (NCI) were performed according to protocols approved by the Johns Hopkins University Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Digoxin, doxorubicin and saline for intraperitoneal injection were obtained from the pharmacy of The Johns Hopkins Hospital. XenoLight Rediject D-luciferin was from PerkinElmer (Boston, MA, USA). Dulbecco’s modified eagle medium with 10% Fetal bovine serum was used as chemoattractant in the bottom chamber. After 20 h, non-invaded cells on top were removed and invaded cells on the bottom were fixed with methanol and counted under fluorescence microscopy.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We are grateful to Karen Padgett of Novus Biologicals for generous gifts of antibodies against HIF-1α, HIF-2α and L1CAM. This work was supported by the Emerald Foundation, the National Institutes of Health (U54-CA143865) and the Johns Hopkins Institute for Cell Engineering. DMG was supported by the Postdoctoral Training Program in Nanotechnology for Cancer Medicine (T32-CA130840). GLS is the C Michael Armstrong Professor at Johns Hopkins University School of Medicine.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)