Published in final edited form as:

Future Oncol. 2013 November ; 9(11): 1623–1636. doi:10.2217/fon.13.92.

Role of hypoxia-inducible factors in breast cancer metastasis

Daniele M Gilkes^{1,2} and Gregg L Semenza^{*,1,2,3}

¹Vascular Program, Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

²McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

³Departments of Pediatrics, Oncology, Medicine, Radiation Oncology & Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

Abstract

Human breast tumors contain regions of hypoxia in which cells that are located far from a functional blood vessel have significantly reduced oxygen concentrations when compared with normal mammary tissue. Breast cancer cells adapt to hypoxic conditions by increasing levels of hypoxia-inducible factors (HIFs), which induce the expression of multiple genes involved in angiogenesis, glucose utilization, resistance to oxidative stress, cell proliferation, resistance to apoptosis, invasion and metastasis. Breast cancer patients with increased HIF expression levels in primary tumor biopsies are at increased risk of metastasis. This is an important finding since 90% of breast cancer deaths are the result of metastasis, primarily to the bone, lungs, liver, brain and regional lymph nodes. Although the prognostic significance of reduced oxygen levels in primary breast tumors of cancer patients is well recognized, the mechanisms underlying hypoxia-induced, HIF-dependent breast cancer metastasis are just beginning to be uncovered. Recent studies have implicated HIF target genes in every step of the metastatic process. Drugs, such as digoxin, show the potential therapeutic effects of blocking HIF activity by decreasing primary tumor growth, vascularization, invasion and metastasis in animal models of breast cancer.

Keywords

breast cancer; digoxin; HIF-1; HIF-2; hypoxia; metastasis

Hypoxia & breast cancer

Worldwide, breast cancer is the most common cancer in women. Upon diagnosis, the patients treatment options are determined by the presence or absence of three key receptors; ER, PR and HER2, as well as clinical staging based on size, lymph node involvement and

* Author for correspondence: Fax: +1 443 287 5618, gsemenza@jhmi.edu.

Financial & competing interests disclosure: The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

tumor histology. Despite all of these assessments, there are still no means of definitively identifying patients who will relapse or whose tumor will metastasize. Clinically, this is an important challenge since 90% of breast cancer patient deaths are due to metastasis, which reflects the fact that patients with metastatic disease respond poorly to currently available therapies. Evidence is mounting that the oxygen (O₂) content of tumor tissue is an important determinant of metastasis. Intratumoral hypoxia has been identified as an adverse indicator for patient prognosis independent of all of the histopathological parameters described above [1]. Hypoxia within a solid tumor arises from an increase in O₂ utilization due to an increase in rapidly dividing cancer cells, and a decrease in oxygen availability due to structurally and functionally abnormal vessels that form within solid tumors [2]. O₂ availability decreases as distance from the nearest blood vessel increases. Data from 125 studies describing the pretreatment oxygenation status of solid tumors were compiled and show that the mean partial pressure of oxygen (PO₂) in breast tumors ranges from 2.5 to 28 mm of mercury (Hg), with a median value of 10 mm Hg, as compared with 65 mm Hg in normal human breast tissue [3]. Intratumoral PO₂ values of <10 mm Hg have been associated with an increased risk of metastasis and mortality [4]. Given these dramatic findings, identification of key alterations that occur under hypoxic conditions will define important mechanisms that promote metastasis.

Hypoxia-inducible factors & breast cancer

Similar to their normal cell counterparts, cancer cells respond to decreased oxygen availability by increasing the activity of the hypoxia-inducible factors (HIFs), HIF-1 and HIF-2 [5]. HIF-1 functions as a heterodimeric protein composed of an O₂-regulated HIF-1 α subunit and a constitutively expressed HIF-1 β subunit [6]. Under normal (i.e., well oxygenated) conditions, the HIF-1 α subunit is hydroxylated on proline residue 564 and/or 402. Hydroxylation is required for binding of the von Hippel–Lindau E3-ubiquitin ligase complex, which targets HIF-1 α for subsequent proteasomal degradation. Three O₂-dependent prolyl hydroxylase enzymes, PHD1, PHD2 and PHD3, control the abundance of HIFs. Under hypoxic conditions, prolyl hydroxylation is decreased, resulting in HIF-1 α accumulation and dimerization with HIF-1 β [7]. The HIF-1 heterodimer binds to the consensus DNA sequence 5'-RCGTG-3' present within hypoxia response elements, recruits coactivator proteins, and activates the transcription of HIF-1 target genes. HIF-2 α is also O₂-regulated and binds with HIF-1 β to form the HIF-2 heterodimer. While HIF-1 α and HIF-2 α share a high degree of sequence similarity, HIF-2 stimulates some but not all of the genes activated by HIF-1 [8]. HIF target genes encode proteins involved in cell survival, angiogenesis, metabolic reprogramming, immortalization, epithelial–mesenchymal transition (EMT), stem cell maintenance, resistance to radiation and chemotherapy, invasion and metastasis [9]. Based on genome-wide chromatin immunoprecipitation combined with DNA sequencing or mRNA microarrays, the number of direct HIF target genes is currently greater than 800 [10,11]. In addition to intratumoral hypoxia, common genetic alterations that occur in cancer also regulate HIF-dependent expression of target genes. In breast cancer, this includes but is not limited to alterations in EGFR, AKT, PI3K, PTEN, mTOR, TP53 and HER2 signaling pathways [12]. The generation of reactive oxygen species is increased under hypoxic conditions [13]. Reactive oxygen species, in turn, inactivate PHD2

causing accumulation of HIFs [14]. Nitric oxide has also been shown to stabilize HIF-1 α [15]. Additional regulatory mechanisms of HIFs include interactions with histone demethylases such as JMJD2C, which selectively interacts with HIF-1 α , but not HIF-2 α , and mediates the recruitment of JMJD2C to the hypoxia response elements, resulting in increased transcription of HIF target genes [16].

The extensive list of HIF target genes provides the molecular basis for the effect of intratumoral hypoxia on cancer progression and the reported association between HIF-1 α overexpression and adverse outcomes in many studies of breast cancer patients [17–25]. Increased HIF-1 α levels have been demonstrated by immunohistochemistry in a subset of biopsies analyzed from both lymph node-negative [24] and lymph node-positive [25] breast cancer patients. Regardless of lymph node status, survival was significantly decreased in those patients with the highest HIF-1 α levels in their diagnostic breast cancer biopsies. A recent study, which aimed to standardize quantitative immunohistochemical assays to predict outcome among node-negative patients, identified a highly predictive signature consisting of five markers, including HIF-1 α , that could predict patient outcome in over 90% of breast cancer cases analyzed [26,27]. Furthermore, experiments of enriched circulating tumor cells from breast cancer patients revealed that HIF-1 α was detected in 76% of the samples [28]. These data are consistent with studies showing HIF-1 α overexpression within metastatic lesions arising from breast tumors [29]. HIF-2 α also correlates with distant recurrence and poor outcome in invasive breast cancer [30]. We have limited the scope of this review to genes regulated by HIF-1 and/or HIF-2 under hypoxic conditions (Table 1) and current literature indicates their importance in breast cancer patient prognosis.

Breast cancers are highly heterogeneous, and have been classified into at least five different subtypes based on their molecular profiles: the luminal A and luminal B groups (characterized by the expression of luminal/epithelial markers); the HER2 group (overexpressing the *ERBB2* oncogene); the normal-like group (closest to the molecular profile of a normal mammary gland); and the basal-like group (high expression of myoepithelial/mesenchymal markers) [31]. Basal-like tumors are the most aggressive and are associated with the highest rate of metastasis and recurrence. The basal-like subgroup has also been called the triple-negative breast cancer subgroup because most basal-like breast cancers are negative when tested for high-level expression of ER, PR and HER2. As a result, these patients do not respond to treatment with tamoxifen, aromatase inhibitors or trastuzumab. Instead, they are treated with conventional chemotherapy with rapid development of resistance and disease progression. A recent meta-analysis performed by the The Cancer Genome Atlas Network, which compared genes differentially activated between the basal and luminal breast cancer subtypes, highlighted increased expression of HIF-1 target genes in the basal breast cancer subgroup [32]. Preclinical studies highlighted in this review demonstrate that inhibition of HIF-1 activity in triple-negative breast cancer cells has a dramatic effect on primary tumor growth as well as both hematogenous and lymphatic metastasis.

HIFs regulate breast cancer metastasis

Tumor metastasis is the dissemination of cancer cells from the initial site of tumor growth to distant organs followed by the establishment of secondary tumors. Cancer cells can spread via two routes: blood vessels or lymphatic vessels. Cancer cells can access the bloodstream directly from a blood vessel in the primary tumor or indirectly via the lymphatic system. Either way, the metastatic process can be deconvoluted into a series of discrete steps beginning with the EMT, in which cells lose cell-to-cell contact, become motile and locally invade the surrounding stroma. Local tissue invasion, which requires extracellular matrix (ECM) degradation, leads to intravasation, which occurs when cancer cells penetrate the wall of a blood vessel or lymphatic vessel. Once breast cancer cells have intravasated, they must survive within the circulation during transit to distant organs, where they have the potential to extravasate by repenetrating through the vessel wall. The metastatic site must be primed so that it presents a suitable microenvironment for cancer cell survival (the premetastatic niche).

Although significant work has been performed to characterize the role of HIFs in experimental cancers, only recently has the direct requirement for HIFs in breast cancer metastasis been demonstrated. Breast cancers arising in conditional knockout mice lacking HIF-1 α expression in mammary epithelial cells demonstrated significantly reduced lung metastasis compared with breast cancers arising in wild-type mice, demonstrating that HIF-1 α promotes breast cancer metastasis [33]. In orthotopic transplants of human breast cancer cells injected into the mammary fat pad of immunodeficient mice, HIF-1 was also shown to be essential for the hematogenous metastasis of breast cancer to the lungs [34,35]. Recent studies implicate the transcriptional activation of HIF target gene products in every step of the metastatic process (Figure 1).

Regulation of EMT & cell motility

The EMT is a process by which epithelial cells lose their polarity and transition to a mesenchymal cell phenotype. Hypoxia-inducible genes that regulate EMT have been implicated in a wide range of cancers [36]. Many of these genes, including *SNAIL1*, *SLUG* (*SNAIL2*) and *TWIST*, function in EMT at least in part by downregulating expression of E-cadherin. E-cadherin is localized at adherens junctions and is critical for epithelial cell–cell adhesion and tissue architecture. Loss or reduction of E-cadherin expression is frequently observed at the invasive front of advanced-stage human carcinomas including ductal carcinoma of the breast [37]. In breast cancer cells, several studies have shown that hypoxia leads to an increase in the expression of two transcriptional repressors of E-cadherin, SNAIL1 and SLUG, by modulating the NOTCH1 signaling pathway [38–40]. However, only recently has HIF-1 transactivation of both the SNAIL1 and SLUG promoters been demonstrated [41,42].

HIF-1 α also directly regulates the expression of TWIST in breast cancer cells. During development, TWIST promotes gastrulation and mesoderm specification. In breast cancer, TWIST expression results in loss of E-cadherin-mediated cell–cell adhesion, activation of mesenchymal markers and induction of cell motility [43]. Hypoxia or overexpression of

HIF-1 α induces a metastatic phenotype in otherwise non metastatic breast cancer cells via a TWIST-dependent mechanism *in vitro* and *in vivo*; TWIST siRNA reversed these effects [44].

A second pathway of enhanced cell motility under hypoxic conditions occurs by HIF-1 activation of *MET* transcription [45]. In a study of node-negative breast cancer patients, co-overexpression of HIF-1 α and c-MET was a significant independent predictor of distant metastasis, and patients with co-overexpression had a significantly worse 10-year disease-free survival rate [46]. Autocrine motility factor (AMF), which is a secreted form of the glycolytic enzyme glucose phosphate isomerase, is also regulated by HIF-1 [47]. Increased AMF expression correlates with breast cancer progression and poor prognosis [48]. Ectopic expression of AMF induces EMT in untrans-formed breast epithelial cells. Inhibition of AMF expression triggers the mesenchymal–epithelial transition in metastatic breast cancer cells.

Regulation of invasion & intravasation

Cells at the invasive front of solid tumors are frequently found to have high expression levels of HIF-1 α [49]. In order for invasion to occur, breast cancer cells must degrade the surrounding basement membrane. Matrix metallo proteinases (MMPs) are a family of zinc-dependent endopeptidases that degrade ECM components. MMP-2 and MMP-9 degrade type IV collagen, which is a major component of the basement membrane. Increased levels of MMP-2 in breast cancer biopsies were associated with poor prognosis [50]. Hypoxia induces increased expression and activity of MMP-2 and MMP-9 through a HIF-1-dependent process [51,52]. In addition to secreted MMPs, which are activated by extra cellular proteolytic cleavage, hypoxia also induces the membrane-bound MT1-MMP in cancer cells by direct binding of HIF-2 to the *MMP14* gene [53].

In addition to MMPs, hypoxic breast cancer cells have increased proteolytic activity as a result of increased urokinase-type plasminogen activator receptor (PLAUR) expression [54]. Although many studies show that PLAUR is activated under hypoxic conditions in cancer cells, only recently has the HIF transcriptional binding site in the PLAUR promoter been described [55]. PLAUR promotes cell invasion by altering interactions between integrins and the ECM. When PLAUR expression levels were modulated by shRNA expression, cells with reduced levels of PLAUR were incapable of intravasation [56]. In addition to the direct regulation of genes involved in proteolysis, HIF-1 has also been shown to regulate expression of miRNA-372/373, which targets RECK mRNA [57]. Decreased expression of the tumor suppressor RECK results in increased MMP-9 activation. Recent clinical studies indicate that low expression of RECK is associated with decreased survival of breast cancer patients [58].

Although degradation of the ECM by proteases has been established as an important mechanism for tumor cell invasion, recent evidence shows that collagen, in particular type I collagen, provides a roadway for cell migration during invasion. Using mouse models that recapitulate the histological progression of human breast cancer, mammary tumors exhibit a localized increase in collagen deposition, which occurs early in tumor formation [59,60]. As

tumor size increases, collagen fibers straighten, bundle and align [61]. Several groups have observed that tumor cells preferentially invade along aligned collagen fibers [61–63]. Furthermore, the pattern and extent of collagen alignment has a prognostic significance in breast cancer [64]. Together this suggests that ECM matrix remodeling is highly dynamic. Cells must both degrade and reform a scaffold to support their migration within the tumor mass.

We recently discovered that HIF-1 α plays a critical role in collagen biogenesis in breast tumors by upregulating the expression of P4HA1, P4HA2, PLOD1 and PLOD2 hydroxylases [65–67] in addition to its previously established role in the regulation of lysyl oxidase family members (LOX, LOXL2 and LOXL4) under hypoxia [34,68], which is described further in the discussion of premetastatic niche formation. Proper hydroxylation is required for folding of newly synthesized procollagen polypeptide chains into stable triple-helical molecules and is a requirement for subsequent secretion into the extracellular space [69,70]. The knockdown of HIF-1 α , P4HA1 or P4HA2 decreases tumor fibrosis and P4HA1 or P4HA2 knockdown completely abrogates the spontaneous metastasis of mammary fat pad-implanted human breast cancer cells to the lungs and lymph nodes of immune-deficient mice [67]. In contrast with the effects of P4HA1 or P4HA2 knockdown, the knockdown of PLOD2 expression does not suppress collagen deposition but instead compromises collagen crosslinking. PLOD2 knockdown significantly impairs the spontaneous metastasis of mammary fat pad-implanted breast cancer cells to mouse lungs and lymph nodes [65].

The formation of new blood vessels (angiogenesis) is crucial for the growth and persistence of primary solid tumors and their metastases, and angiogenesis is also required for metastatic dissemination. HIF-dependent expression of VEGF under hypoxic conditions [71] increases micro-vascular density and permeability [72], which contribute to an increased probability of intravasation [73]. Elevated levels of circulating VEGF have been measured in breast cancer patients and are associated with increased tumor vascularity, metastasis, resistance to chemo therapy and poor patient prognosis [74]. The HIF-1 target gene *ANGPT2* was found to be closely correlated with VEGF expression and increased microvessel density in breast cancer [75,76]. High expression of both VEGF and *ANGPT2* has a strong prognostic significance in breast cancer. In an orthotopic model of breast cancer metastasis, attenuating *ANGPT2* decreased pulmonary vascular permeability and inhibited spontaneous lung metastasis [71,77].

In addition to VEGF, the HIF-2 target gene product ephrin-A1 is the prototypic ligand for EphA receptor tyrosine kinases and ephrin-A1 promotes tumor angiogenesis [78]. It is overexpressed in vascularized tumors relative to normal tissue. siRNA-mediated ephrin-A1 knockdown in metastatic breast cancer cells significantly diminishes lung metastasis [79]. Overexpression of ephrin-A1 promotes endothelial cell (EC) migration toward breast cancer cells, suggesting that activation of ephrin-A1 on ECs is one mechanism by which ephrin-A1 may regulate angiogenesis.

Regulation of extravasation

The contribution of HIFs to cancer cell extravasation has been directly assessed in mice by injecting human breast cancer cells, which were stably transfected with a control vector or vectors encoding shRNAs targeting both HIF-1 α and HIF-2 α , directly into the tail vein of immune-deficient mice (in order to bypass all steps that are required for cancer cells to access the vasculature) [35]. The cancer cells were exposed to 20% O₂ (PO₂ = 140 mm Hg) or 1% O₂ (PO₂ = 7 mm Hg) for 48 h prior to injection. Histological analysis of lung sections from these mice revealed that exposure to 1% O₂ (to simulate hypoxia within the primary tumor) increased the extravasation of control cells, an effect that was abrogated in HIF-knockdown cells. In this study, HIF activity also promoted the ability of hypoxic breast cancer cells to invade through an EC monolayer *in vitro*. The invasion of naive breast cancer cells through EC monolayers was increased when the cells were exposed to conditioned medium from hypoxic, when compared with nonhypoxic breast cancer cells. The hypoxia-induced effect was lost when HIF-knockdown breast cancer cells were used as the source of conditioned medium. Additionally, transendothelial electrical resistance, a biophysical measure of EC–EC interactions, was decreased when ECs were incubated in conditioned medium from hypoxic breast cancer cells. This effect was abrogated when conditioned media from hypoxic breast cancer cells expressing HIF-knockdown shRNAs were used [35].

Extravasation involves modulation of tumor cell adhesion to the endothelium of blood vessels, which is also known as margination. HIF target genes that promote extravasation by promoting cancer cell margination have recently been identified [35]. L1CAM, a protein involved in cell–cell adherence by homophilic interactions and by heterophilic interactions with integrins, neuropilin 1 and CD24, has been identified as a direct transcriptional target of HIF. Breast cancer cells exposed to hypoxia for 48 h had increased adherence to ECs, whereas cells with either reduced expression of HIF-1 or L1CAM had significantly reduced adherence to ECs. Furthermore, overexpression of L1CAM in cancer cells with reduced HIF expression had increased numbers of extravasated cancer cells in the lung after tail vein injection [35]. Interestingly, a recent study demonstrated that hypoxia-induced expression of the L1CAM-interacting protein CD24 by HIF-1 binding to a functional hypoxia responsive element in the CD24 promoter [80]. Although the CD24-knockdown studies were not performed in breast cancer cells, immunohistochemical staining expression of CD24 in early primary invasive breast cancers has been significantly associated with poor prognosis [81].

ANGPTL4 was also found to be a hypoxia-induced and HIF-dependent gene product that promoted extravasation of breast cancer cells [35,82]. TGF- β induction of ANGPTL4 expression in cancer cells disrupts vascular EC–cell junctions, increases the permeability of lung capillaries, and facilitates the transendothelial passage of tumor cells [82]. *In vitro* assays using conditioned medium from breast cancer cells suggest that this is a result of increased extravasation due to reduced EC–EC adherence. Similar results were obtained using conditioned medium from hypoxic breast cancer cells but not from HIF-knockdown cells [35]. Conditioned media from breast cancer cells stably transfected with an ANGPTL4 expression vector inhibited EC–EC interactions as measured by transendothelial electrical resistance and breast cancer invasion assays. When injected via the tail vein, HIF-knockdown breast cancer cells overexpressing ANGPTL4 extravasated more readily to the

lungs [35]. Lung metastasis from *ANGPTL4*-knockdown breast cancer cells following mammary fat pad implantation were also significantly reduced compared with mice bearing control tumors. Additional *in vivo* studies to assess vascular permeability and metastasis performed using *ANGPTL4*-knockout and wild-type mice injected with either control or *ANGPTL4*-knockdown tumors confirmed that *ANGPTL4* induced vascular leakiness and facilitated lung metastasis in mice [83]. Clinically, *ANGPTL4* is expressed at increased levels in the primary breast cancers of women with lung metastases [82].

Regulation of premetastatic niche formation

HIF expression in primary breast cancers has been found to influence metastatic seeding at distant organs prior to cancer cell arrival by regulating premetastatic niche formation [34,68,84,85]. The premetastatic niche comprises bone marrow-derived cells (BMDCs) that home to tumor-specific premetastatic sites, forming cell clusters that precede tumor cell arrival [86]. Recently, the induction of multiple members of the lysyl oxidase (LOX) family, including *LOX*, *LOXL2* and *LOXL4*, have been identified as key hypoxia-induced and HIF-1-regulated genes involved in BMDC recruitment to promote breast cancer premetastatic niche formation [34,68,84,85,87]. LOX family members are extracellular enzymes secreted from breast cancer cells that catalyze intra- and inter-molecular crosslinking of collagen at hydroxylated lysine residues. LOX enzymes promote metastasis by crosslinking collagen in metastatic tissues, which is required for subsequent BMDC recruitment. Two BMDC populations that promote metastasis in animal models are *CD11b⁺/Ly6C^{med}/Ly6G⁺* myeloid cells and *CD3⁻/NK1.1⁺* natural killer cells [88]. Furthermore, accumulation of *CD11b⁺/Ly6C^{med}/Ly6G⁺* cells was found to be enhanced by hypoxic breast cancer cell-derived MCP-1, which is a member of the chemokine- β subfamily that is known to regulate the recruitment of inflammatory cells.

Clinically, LOX expression was associated with both intratumoral hypoxia and distant metastases in breast cancer patients [68]. Interestingly, LOX, LOXL2 and LOXL4 are overexpressed (relative to surrounding normal tissue) in different combinations in human breast cancers and breast cancer cell lines [34]. Knockdown of either LOX [68] or LOXL4 [34] in MDA-MB-231 cells significantly impairs premetastatic niche formation and lung metastasis, which suggests that the functions of these enzymes are not redundant. Additional studies also demonstrated a role for LOX in invasion and migration by regulating FAK activity, suggesting multiple roles for HIF-induced LOX expression and activity in metastatic disease [89].

In addition to a premetastatic niche that is rich in BMDCs, homing of breast cancer cells to metastatic sites may also be governed by interactions between chemokine receptors on cancer cells and ligand secretion in target organs. For example, the SDF-1/CXCR4 signaling complex may play a role in breast cancer metastasis [90]. Expression of the genes encoding SDF-1 and CXCR4 is induced by hypoxia in a HIF-dependent manner in various cell types [91–94]. *In vivo* studies inhibiting CXCR4 expression by siRNA or blocking its function with either neutralizing antibodies or synthetic peptides inhibited the metastasis of breast cancer cells in orthotopic cell models [95,96]. Additionally, SDF-1/CXCR4 signaling in ECs under hypoxic conditions leads to tube formation and adhesion of breast cancer cells to ECs,

and stimulates transendothelial migration in a HIF-dependent manner [97]. Breast cancer cell adhesion and migration through a human umbilical vein EC monolayer was significantly reduced by inhibiting CXCR4 or treating ECs with an SDF-1-neutralizing antibody. This suggests an additional role for SDF-1 and CXCR4 in intravasation. Hypoxia-induced and HIF-dependent expression of additional chemokines and their ligands, such as CXCR6, CCR5 and CCL5, has also recently been implicated in enhancing directed migration of breast cancer cells [98,99].

Regulation of signaling interactions in the tumor microenvironment

The assembly and collective contributions of the assorted cell types that constitute the tumor microenvironment are beginning to be appreciated as a complex signaling network that contributes to cancer metastasis. Many stromal cell types including fibroblasts, myo fibroblasts, tumor-associated macrophages, bone marrow-derived angiogenic cells and mesenchymal stem cells (MSCs) are recruited to the tumor and enhance primary tumor growth and/or promote metastasis [100]. Recently, we found that hypoxia induces the recruitment of bone marrow-derived MSCs to primary breast tumors [101]. The interaction of MSCs with breast cancer cells induces CXCL10, CCL5 and VEGFR1 expression by MSCs and CXCR3, CCR5 and PGF expression by breast cancer cells. MSC–breast cancer cell signaling is mediated by CCL5–CCR5 and CXCL10–CXCR3 signaling. Breast cancer cell–MSC signaling is mediated by PGF–VEGFR1 signaling, which induces CXCL10 expression in MSCs. The result is a positive feedback loop between the two cell types. PGF–VEGFR1 signaling is important for MSC homing, whereas CXCL10–CXCR3 and CCL5–CCR5 signaling promote breast cancer metastasis in an orthotopic mouse model by stimulating the expression of genes that enhance invasion and the metastasis of breast cancer cells to the lungs and lymph nodes. *CXCR3* and *PGF* gene expression in breast cancer cells is directly regulated by HIFs [101].

Regulation of cell survival at metastatic sites

Although the microenvironment of the metastatic tumor site is different to the primary site, suggesting unique factors may be necessary to promote cancer cell proliferation, it is probable that many of the HIF-inducible genes that play a role in cell survival and resistance to apoptosis will also function in secondary tumor growth. Cancer cells must establish a new blood supply in the secondary organ to support their growth and the role of HIF-1 in tumor angiogenesis has long been established [102]. Future studies will likely identify additional metastatic site-specific cellular adaptations that may be attributable to HIF regulation.

Regulation of lymphatic metastasis

Rather than directly accessing blood vessels within the primary tumor, breast cancer cells may also reach the circulation by lymphatic drainage. Lymphatic capillaries lack the tight interendothelial junctions typically seen in blood vessels, as well as the surrounding layers of pericytes/smooth muscle cells and basement membranes, suggesting that these vessels may provide a more readily accessible route for intravasation [103]. Clinical studies have found that increased HIF-1 α levels in primary breast tumors are positively correlated with lymphatic vessel density within the primary tumor [104]. Likewise, the density of lymphatic

vessels within a breast tumor is correlated with lymph node metastasis [105]. Recently, the direct involvement of HIFs in the lymphatic metastasis of breast cancer was demonstrated [106]. The expression of genes in the *VEGF*, *FGF*, *IGF* and *PDGF* families were interrogated in breast cancer cells, since they were previously implicated in the growth of new lymphatic vessels (lymphangiogenesis) in other cancer types [107]. In addition to *VEGFA*, *PDGFB* expression was found to be upregulated by hypoxia in a HIF-dependent manner in breast cancer cells [106]. HIF or PDGF-B loss of function resulted in decreased lymphatic vessel density in the primary tumor and decreased lymph node metastasis. Inhibition of HIF activity or PDGF-B signaling by treatment of tumor-bearing mice with digoxin or imatinib, respectively, also decreased lymph node metastasis and lymphatic vessel density. Furthermore, HIF-1 α and PDGF-B expression was highly correlated in grade 2 and grade 3 human breast cancer biopsies [106]. Thus, HIF-1 regulates both hematogenous and lymphatic dissemination of breast cancer cells.

Regulation of metastatic organotropism

Recent work in the area of metastatic organ-otropism for breast cancer has utilized mouse models to identify organ-specific metastasis gene signatures that suggest distinct sets of genes are utilized for colonizing different organs [108–110]. Although the role of hypoxia in organ-specific breast cancer metastasis is currently unknown, several known hypoxia-inducible and HIF-1-regulated genes are included in each of the gene signatures. The gene expression signature of breast cancer metastasis to the brain identified the HIF-1 target gene *COX2* as a mediator of brain metastasis [110]. Breast cancer cell metastasis to the brain was decreased by *COX2* knockdown in animal models. *COX2*, *ANGPTL4* and *MMP2* are HIF-1-regulated genes with increased expression in breast cancers that metastasize to the lungs [35,52,111]. On the other hand, *CXCR4*, *OPN*, *IL-6* and *IL-8* are hypoxia-inducible genes that have been identified in the bone-specific metastatic gene signature and have been shown to be induced by HIF in some cell types [108,112–114]. Inhibiting HIF-1 activity significantly inhibits breast cancer metastases to lymph node, bone and lung in animal models, establishing HIF-1 as a promising therapeutic target for breast cancer metastasis [35,84,115].

HIF inhibitors

Multiple drugs have been shown to inhibit HIF-1 activity, block tumor growth and vascularization, and improve responses to chemoand radiation-therapy in a variety of mouse models. For example, anthracyclines (doxorubicin and daunorubicin) have been shown to inhibit binding of HIF-1 to DNA and reduce prostate cancer growth in a mouse xenograft model [116]. HIF-1 α protein levels and xenograft tumor growth have been shown to be reduced by disruption of microtubule polymerization by 2-methoxyestradiol [117]. HIF-1 α transactivation has been shown to be inhibited by bortezomib, a proteasome inhibitor that is used to treat relapsed multiple myeloma and mantle cell lymphoma [118]. Agents that inhibit HIF-1 α protein accumulation and demonstrate anti-tumor effects include the topoisomerase I inhibitor, topotecan, as well as the cardiac glycoside digoxin [35,84,106,119]. Digoxin was also shown to inhibit the growth of human prostate cancer xenografts and its effect was dependent on its inhibition of HIF-1 α expression [120].

Acriflavine, an inhibitor of HIF-1 dimerization, was shown to decrease HIF-1 transcriptional activity and decrease tumor growth and angiogenesis *in vivo* [121].

In orthotopic mouse models of breast cancer metastasis, treatment of tumor-bearing mice with acriflavine (4 mg/kg) or digoxin (2 mg/kg) by daily intraperitoneal injection significantly inhibited primary tumor growth and metastasis of cancer cells from breast to lungs concomitant with decreased expression of HIF target genes (*ANGPTL4*, *LICAM*, *LOX*, *LOXL2* and *LOXL4*) [35,84]. Since HIF-1 α can be induced by hypoxia-independent signaling pathways, such as mTOR, ERBB2 (HER2^{neu}) and MAP kinase, the therapeutic benefits of targeting these pathways may also be partially explained by a decrease in HIF-1 α levels [122].

Conclusion & future perspective

Intratumoral hypoxia is a strong selective pressure that promotes the outgrowth of malignant cells with the ability to overcome low oxygen conditions rather than undergo apoptosis. The cellular response to low oxygen tension involves the stabilization of HIFs and complex transcriptional regulation patterns that may be unique for different breast cancers; HIFs appear to play a role in every step of the metastatic cascade (Figure 1). To colonize a distant organ, disseminated tumor cells must have the capacity to interact with a new microenvironment efficiently enough to have a survival advantage, suggesting that this may be a rate-limiting step in metastasis. Intravital microscopy shows that while circulating tumor cells generally extravasate with high efficiency, only a small subset form micrometastases and only a subset of these micrometastases form vascularized macrometastases [123]. Cancer cells that have survived and have been reprogrammed by hypoxic conditions have a survival advantage at distant sites. Clinically, this is supported by the finding that patients with primary tumors that have high HIF-1 α levels have a greater likelihood of developing metastatic relapse and a shorter survival time [122].

Currently, it is impossible to predict which breast cancer patients are likely to develop metastatic disease. However, HIF-1 α expression has been implicated as an independent predictor of poor outcome for breast cancer patients [24]. A HIF-dependent gene signature has also been shown to differentiate luminal and basal subtypes of breast cancer [32]. Taken together, this suggests that HIF-1 α levels in the diagnostic tumor biopsy could be used to identify patients that are at increased risk to develop metastasis. It also suggests that HIF inhibitors may be particularly useful in the treatment of triple-negative breast cancers, which have high HIF transcriptional activity and respond poorly to currently available therapies.

The efficacy of many HIF inhibitors has been convincingly demonstrated in animal models of breast cancer metastasis. Clinical trials are warranted to determine if the addition of one or more HIF inhibitors to current therapeutic regimens will increase the survival of breast cancer patients. In the future, as personalized medicine for cancer progresses, clinical markers of HIF activity in patient biopsies could be utilized to predict which patients would benefit the most from HIF inhibitors. This would be particularly useful for the management of lymph node-negative breast cancer patients, who develop distant metastasis in 30–40% of cases. The subset of patients with high HIF activity in their primary tumors might benefit

from more aggressive therapy that would include a HIF inhibitor. This type of patient stratification may also help to avoid unnecessary therapies and their side effects in lymph node-negative patients with a more favorable prognosis.

Acknowledgments

Work in the authors' laboratory is partially funded by grants from the NCI (U54-CA143868) and funds from the Johns Hopkins Institute for Cell Engineering. GL Semenza is the C Michael Armstrong Professor at Johns Hopkins University School of Medicine and an American Cancer Society Research Professor. DM Gilkes is the recipient of a Susan G Komen Breast Cancer Foundation Post-Doctoral Fellowship Award.

References

Papers of special note have been highlighted as:

- of interest

1. Vaupel P. Prognostic potential of the pretherapeutic tumor oxygenation status. *Adv Exp Med Biol.* 2009; 645:241–246.
2. Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene.* 2010; 29(5):625–634. [PubMed: 19946328]
- 3. Vaupel P, Hockel M, Mayer A. Detection and characterization of tumor hypoxia using pO₂ histography. *Antioxid Redox Signal.* 2007; 9(8):1221–1235. Data from 125 studies describing the pretreatment oxygenation status of tumors compared with normal tissue. [PubMed: 17536958]
4. Vaupel P, Mayer A, Hockel M. Tumor hypoxia and malignant progression. *Methods Enzymol.* 2004; 381:335–354. [PubMed: 15063685]
5. Semenza GL. Hypoxia-inducible factors in physiology and medicine. *Cell.* 2012; 148(3):399–408. [PubMed: 22304911]
6. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci USA.* 1995; 92(12):5510–5514. [PubMed: 7539918]
7. Kaelin WG, Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell.* 2008; 30(4):393–402. [PubMed: 18498744]
8. Loboda A, Jozkowicz A, Dulak J. HIF-1 and HIF-2 transcription factors – similar but not identical. *Mol Cells.* 2010; 29(5):435–442. [PubMed: 20396958]
9. Semenza GL. Advances in cancer biology and therapy. *J Mol Med (Berl).* 2013; 91(4):409. [PubMed: 23515622]
10. Schodel J, Oikonomopoulos S, Ragoussis J, Pugh CW, Ratcliffe PJ, Mole DR. High-resolution genome-wide mapping of HIF-binding sites by ChIP-seq. *Blood.* 2011; 117(23):e207–e217. [PubMed: 21447827]
11. Xia X, Lemieux ME, Li W, et al. Integrative analysis of HIF binding and transactivation reveals its role in maintaining histone methylation homeostasis. *Proc Natl Acad Sci USA.* 2009; 106(11):4260–4265. [PubMed: 19255431]
12. Semenza GL. Molecular mechanisms mediating metastasis of hypoxic breast cancer cells. *Trends Mol Med.* 2012; 18(9):534–543. [PubMed: 22921864]
13. Kaelin WG. ROS: really involved in oxygen sensing. *Cell Metab.* 2005; 1(6):357–358. [PubMed: 16054083]
14. Gao P, Zhang H, Dinavahi R, et al. HIF-dependent antitumorigenic effect of antioxidants *in vivo*. *Cancer Cell.* 2007; 12(3):230–238. [PubMed: 17785204]
15. Quintero M, Brennan PA, Thomas GJ, Moncada S. Nitric oxide is a factor in the stabilization of hypoxia-inducible factor-1 α in cancer: role of free radical formation. *Cancer Res.* 2006; 66(2):770–774. [PubMed: 16424008]

16. Luo W, Chang R, Zhong J, Pandey A, Semenza GL. Histone demethylase JMJD2C is a coactivator for hypoxia-inducible factor 1 that is required for breast cancer progression. *Proc Natl Acad Sci USA*. 2012; 109(49):E3367–E3376. [PubMed: 23129632]
17. Giatromanolaki A, Koukourakis MI, Simopoulos C, et al. c-erbB-2 related aggressiveness in breast cancer is hypoxia inducible factor-1 α dependent. *Clin Cancer Res*. 2004; 10(23):7972–7977. [PubMed: 15585632]
18. Dales JP, Garcia S, Meunier-Carpentier S, et al. Overexpression of hypoxia-inducible factor HIF-1 α predicts early relapse in breast cancer: retrospective study in a series of 745 patients. *Int J Cancer*. 2005; 116(5):734–739. [PubMed: 15849727]
19. Vleugel MM, Greijer AE, Shvarts A, et al. Differential prognostic impact of hypoxia induced and diffuse HIF-1 α expression in invasive breast cancer. *J Clin Pathol*. 2005; 58(2):172–177. [PubMed: 15677538]
20. Generali D, Berruti A, Brizzi MP, et al. Hypoxia-inducible factor-1 α expression predicts a poor response to primary chemoendocrine therapy and disease-free survival in primary human breast cancer. *Clin Cancer Res*. 2006; 12(15):4562–4568. [PubMed: 16899602]
21. Kronblad A, Jirstrom K, Ryden L, Nordenskjold B, Landberg G. Hypoxia inducible factor-1 α is a prognostic marker in premenopausal patients with intermediate to highly differentiated breast cancer but not a predictive marker for tamoxifen response. *Int J Cancer*. 2006; 118(10):2609–2616. [PubMed: 16381002]
22. Trastour C, Benizri E, Ettore F, et al. HIF-1 α and CA IX staining in invasive breast carcinomas: prognosis and treatment outcome. *Int J Cancer*. 2007; 120(7):1451–1458. [PubMed: 17245699]
23. Yamamoto Y, Ibusuki M, Okumura Y, et al. Hypoxia-inducible factor 1 α is closely linked to an aggressive phenotype in breast cancer. *Breast Cancer Res Treat*. 2008; 110(3):465–475. [PubMed: 17805961]
24. Bos R, Van Der Groep P, Greijer AE, et al. Levels of hypoxia-inducible factor-1 α independently predict prognosis in patients with lymph node negative breast carcinoma. *Cancer*. 2003; 97(6):1573–1581. [PubMed: 12627523]
25. Schindl M, Schoppmann SF, Samonigg H, et al. Overexpression of hypoxia-inducible factor 1 α is associated with an unfavorable prognosis in lymph node-positive breast cancer. *Clin Cancer Res*. 2002; 8(6):1831–1837. [PubMed: 12060624]
26. Charpin C, Secq V, Giusiano S, et al. A signature predictive of disease outcome in breast carcinomas, identified by quantitative immunocytochemical assays. *Int J Cancer*. 2009; 124(9):2124–2134. [PubMed: 19142869]
27. Charpin C, Tavassoli F, Secq V, et al. Validation of an immunohistochemical signature predictive of 8-year outcome for patients with breast carcinoma. *Int J Cancer*. 2012; 131(3):e236–e243. [PubMed: 22120430]
28. Kallergi G, Markomanolaki H, Giannoukaraki V, et al. Hypoxia-inducible factor-1 α and vascular endothelial growth factor expression in circulating tumor cells of breast cancer patients. *Breast Cancer Res*. 2009; 11(6):R84. [PubMed: 19919679]
29. Zhong H, De Marzo AM, Laughner E, et al. Overexpression of hypoxia-inducible factor 1 α in common human cancers and their metastases. *Cancer Res*. 1999; 59(22):5830–5835. [PubMed: 10582706]
30. Helczynska K, Larsson AM, Holmquist Mengelbier L, et al. Hypoxia-inducible factor-2 α correlates to distant recurrence and poor outcome in invasive breast cancer. *Cancer Res*. 2008; 68(22):9212–9220. [PubMed: 19010893]
31. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature*. 2000; 406(6797):747–752. [PubMed: 10963602]
32. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature*. 2012; 490(7418):61–70. [PubMed: 23000897]
- 33. Liao D, Corle C, Seagroves TN, Johnson RS. Hypoxia-inducible factor-1 α is a key regulator of metastasis in a transgenic model of cancer initiation and progression. *Cancer Res*. 2007; 67(2):563–572. Shows that metastasis of autochthonous breast cancer cells to mouse lungs was decreased in mice with a knockout of hypoxia-inducible factor (HIF)-1 α in mammary epithelial cells. [PubMed: 17234764]

- 34. Wong CC, Gilkes DM, Zhang H, et al. Hypoxia-inducible factor 1 is a master regulator of breast cancer metastatic niche formation. *Proc Natl Acad Sci USA*. 2011; 108(39):16369–16374. Demonstrates the requirement for LOX, LOXL2 and LOXL4 in breast cancer metastasis. It is the first study to show that *LOXL4* is a direct HIF target. [PubMed: 21911388]
- 35. Zhang H, Wong CC, Wei H, et al. HIF-1-dependent expression of angiopoietin-like 4 and L1CAM mediates vascular metastasis of hypoxic breast cancer cells to the lungs. *Oncogene*. 2012; 31(14):1757–1770. [PubMed: 21860410]
- 36. Jiang J, Tang YL, Liang XH. EMT: a new vision of hypoxia promoting cancer progression. *Cancer Biol Ther*. 2011; 11(8):714–723. [PubMed: 21389772]
- 37. Graff JR, Gabrielson E, Fujii H, Baylin SB, Herman JG. Methylation patterns of the E-cadherin 5' CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. *J Biol Chem*. 2000; 275(4):2727–2732. [PubMed: 10644736]
- 38. Chen J, Imanaka N, Griffin JD. Hypoxia potentiates Notch signaling in breast cancer leading to decreased E-cadherin expression and increased cell migration and invasion. *Br J Cancer*. 2010; 102(2):351–360. [PubMed: 20010940]
- 39. Xing F, Okuda H, Watabe M, et al. Hypoxia-induced Jagged2 promotes breast cancer metastasis and self-renewal of cancer stem-like cells. *Oncogene*. 2011; 30(39):4075–4086. [PubMed: 21499308]
- 40. Lundgren K, Nordenskjold B, Landberg G. Hypoxia, Snail and incomplete epithelial–mesenchymal transition in breast cancer. *Br J Cancer*. 2009; 101(10):1769–1781. [PubMed: 19844232]
- 41. Luo D, Wang J, Li J, Post M. Mouse snail is a target gene for HIF. *Mol Cancer Res*. 2011; 9(2):234–245. [PubMed: 21257819]
- 42. Storci G, Sansone P, Mari S, et al. TNF α up-regulates SLUG via the NF- κ B/HIF1 α axis, which imparts breast cancer cells with a stem cell-like phenotype. *J Cell Physiol*. 2010; 225(3):682–691. [PubMed: 20509143]
- 43. Yang J, Mani SA, Donaher JL, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell*. 2004; 117(7):927–939. [PubMed: 15210113]
- 44. Yang MH, Wu MZ, Chiou SH, et al. Direct regulation of TWIST by HIF-1 α promotes metastasis. *Nat Cell Biol*. 2008; 10(3):295–305. Demonstrates that *TWIST* is a direct HIF target gene. [PubMed: 18297062]
- 45. Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. *Cancer Cell*. 2003; 3(4):347–361. Shows that *c-MET* is a direct HIF target gene. [PubMed: 12726861]
- 46. Chen HH, Su WC, Lin PW, Guo HR, Lee WY. Hypoxia-inducible factor-1 α correlates with MET and metastasis in node-negative breast cancer. *Breast Cancer Res Treat*. 2007; 103(2):167–175. [PubMed: 17028975]
- 47. Funasaka T, Yanagawa T, Hogan V, Raz A. Regulation of phosphoglucose isomerase/autocrine motility factor expression by hypoxia. *FASEB J*. 2005; 19(11):1422–1430. [PubMed: 16126909]
- 48. Funasaka T, Hogan V, Raz A. Phosphoglucose isomerase/autocrine motility factor mediates epithelial and mesenchymal phenotype conversions in breast cancer. *Cancer Res*. 2009; 69(13):5349–5356. [PubMed: 19531650]
- 49. Horree N, Van Diest PJ, Sie-Go DM, Heintz AP. The invasive front in endometrial carcinoma: higher proliferation and associated derailment of cell cycle regulators. *Hum Pathol*. 2007; 38(8):1232–1238. [PubMed: 17490724]
- 50. Duffy MJ, Maguire TM, Hill A, McDermott E, O'Higgins N. Metalloproteinases: role in breast carcinogenesis, invasion and metastasis. *Breast Cancer Res*. 2000; 2(4):252–257. [PubMed: 11250717]
- 51. Munoz-Najar UM, Neurath KM, Vumbaca F, Claffey KP. Hypoxia stimulates breast carcinoma cell invasion through MT1-MMP and MMP-2 activation. *Oncogene*. 2006; 25(16):2379–2392. [PubMed: 16369494]
- 52. Krishnamachary B, Berg-Dixon S, Kelly B, et al. Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. *Cancer Res*. 2003; 63(5):1138–1143. [PubMed: 12615733]

53. Petrella BL, Lohi J, Brinckerhoff CE. Identification of membrane type-1 matrix metalloproteinase as a target of hypoxia-inducible factor-2 α in von Hippel-Lindau renal cell carcinoma. *Oncogene*. 2005; 24(6):1043–1052. [PubMed: 15592504]
54. Graham CH, Forsdike J, Fitzgerald CJ, Macdonald-Goodfellow S. Hypoxia-mediated stimulation of carcinoma cell invasiveness via upregulation of urokinase receptor expression. *Int J Cancer*. 1999; 80(4):617–623. [PubMed: 9935166]
55. Buchler P, Reber HA, Tomlinson JS, et al. Transcriptional regulation of urokinase-type plasminogen activator receptor by hypoxia-inducible factor 1 is crucial for invasion of pancreatic and liver cancer. *Neoplasia*. 2009; 11(2):196–206. [PubMed: 19177204]
56. Kim J, Yu W, Kovalski K, Ossowski L. Requirement for specific proteases in cancer cell intravasation as revealed by a novel semiquantitative PCR-based assay. *Cell*. 1998; 94(3):353–362. [PubMed: 9708737]
57. Loayza-Puch F, Yoshida Y, Matsuzaki T, Takahashi C, Kitayama H, Noda M. Hypoxia and RAS-signaling pathways converge on, and cooperatively downregulate, the RECK tumor-suppressor protein through microRNAs. *Oncogene*. 2010; 29(18):2638–2648. [PubMed: 20154725]
58. Zhang Y, Cheng S, Zhang G, et al. Low expression of RECK indicates a shorter survival for patients with invasive breast cancer. *Cancer Sci*. 2012; 103(6):1084–1089. [PubMed: 22404079]
59. Maglione JE, McGoldrick ET, Young LJ, et al. Polyomavirus middle T-induced mammary intraepithelial neoplasia outgrowths: single origin, divergent evolution, and multiple outcomes. *Mol Cancer Ther*. 2004; 3(8):941–953. [PubMed: 15299077]
60. Lin EY, Jones JG, Li P, et al. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. *Am J Pathol*. 2003; 163(5):2113–2126. [PubMed: 14578209]
61. Provenzano PP, Eliceiri KW, Campbell JM, Inman DR, White JG, Keely PJ. Collagen reorganization at the tumor-stromal interface facilitates local invasion. *BMC Med*. 2006; 4(1):38. [PubMed: 17190588]
62. Wyckoff JB, Wang Y, Lin EY, et al. Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer Res*. 2007; 67(6):2649–2656. [PubMed: 17363585]
63. Wang W, Wyckoff JB, Frohlich VC, et al. Single cell behavior in metastatic primary mammary tumors correlated with gene expression patterns revealed by molecular profiling. *Cancer Res*. 2002; 62(21):6278–6288. [PubMed: 12414658]
64. Conklin MW, Eickhoff JC, Riching KM, et al. Aligned collagen is a prognostic signature for survival in human breast carcinoma. *Am J Pathol*. 2011; 178(3):1221–1232. [PubMed: 21356373]
65. Gilkes D, Bajpai S, Wong CC, et al. Procollagen lysyl hydroxylase 2 is essential for breast cancer metastasis. *Mol Cancer Res*. 2013; 11(5):456–466. [PubMed: 23378577]
66. Gilkes DM, Bajpai S, Chaturvedi P, Wirtz D, Semenza GL. HIF-1 promotes extracellular matrix remodeling under hypoxic conditions by inducing P4HA1, P4HA2, and PLOD2 expression in fibroblasts. *J Biol Chem*. 2013; 288(15):10819–10829. [PubMed: 23423382]
67. Gilkes DM, Chaturvedi P, Bajpai S, et al. Collagen prolyl hydroxylases are essential for breast cancer metastasis. *Cancer Res*. 2013; 73(11):3285–3296. [PubMed: 23539444]
68. Erler JT, Bennewith KL, Nicolau M, et al. Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature*. 2006; 440(7088):1222–1226. [PubMed: 16642001]
69. Myllyharju J. Prolyl 4-hydroxylases, the key enzymes of collagen biosynthesis. *Matrix Biol*. 2003; 22(1):15–24. [PubMed: 12714038]
70. Myllyharju J, Kivirikko KI. Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends Genet*. 2004; 20(1):33–43. [PubMed: 14698617]
71. Forsythe JA, Jiang BH, Iyer NV, et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol*. 1996; 16(9):4604–4613. [PubMed: 8756616]
72. Ravi R, Mookerjee B, Bhujwalla ZM, et al. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1 α . *Genes Dev*. 2000; 14(1):34–44. [PubMed: 10640274]
73. Sullivan R, Graham CH. Hypoxia-driven selection of the metastatic phenotype. *Cancer Metastasis Rev*. 2007; 26(2):319–331. [PubMed: 17458507]

74. Toi M, Kondo S, Suzuki H, et al. Quantitative analysis of vascular endothelial growth factor in primary breast cancer. *Cancer*. 1996; 77(6):1101–1106. [PubMed: 8635130]
75. Kelly BD, Hackett SF, Hirota K, et al. Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. *Circ Res*. 2003; 93(11):1074–1081. [PubMed: 14576200]
76. Simon MP, Tournaire R, Pouyssegur J. The angiopoietin-2 gene of endothelial cells is up-regulated in hypoxia by a HIF binding site located in its first intron and by the central factors GATA-2 and Ets-1. *J Cell Physiol*. 2008; 217(3):809–818. [PubMed: 18720385]
77. Huang Y, Song N, Ding Y, et al. Pulmonary vascular destabilization in the premetastatic phase facilitates lung metastasis. *Cancer Res*. 2009; 69(19):7529–7537. [PubMed: 19773447]
78. Yamashita T, Ohneda K, Nagano M, et al. Hypoxia-inducible transcription factor-2 α in endothelial cells regulates tumor neovascularization through activation of ephrin A1. *J Biol Chem*. 2008; 283(27):18926–18936. [PubMed: 18434321]
79. Brantley-Sieders DM, Fang WB, Hwang Y, Hicks D, Chen J. Ephrin-A1 facilitates mammary tumor metastasis through an angiogenesis-dependent mechanism mediated by EphA receptor and vascular endothelial growth factor in mice. *Cancer Res*. 2006; 66(21):10315–10324. [PubMed: 17079451]
80. Thomas S, Harding M, Smith SC, et al. CD24 is an effector of HIF-1 driven primary tumor growth and metastasis. *Cancer Res*. 2012; 72(21):5600–5612. [PubMed: 22926560]
81. Ahmed MA, Aleskandarany MA, Rakha EA, et al. A CD44(-)/CD24(+) phenotype is a poor prognostic marker in early invasive breast cancer. *Breast Cancer Res Treat*. 2012; 133(3):979–995. [PubMed: 22119938]
82. Padua D, Zhang XH, Wang Q, et al. TGF β primes breast tumors for lung metastasis seeding through angiopoietin-like 4. *Cell*. 2008; 133(1):66–77. [PubMed: 18394990]
83. Huang RL, Teo Z, Chong HC, et al. ANGPTL4 modulates vascular junction integrity by integrin signaling and disruption of intercellular VE-cadherin and claudin-5 clusters. *Blood*. 2011; 118(14):3990–4002. [PubMed: 21841165]
84. Wong CC, Zhang H, Gilkes DM, et al. Inhibitors of hypoxia-inducible factor 1 block breast cancer metastatic niche formation and lung metastasis. *J Mol Med (Berl)*. 2012; 90(7):803–815. [PubMed: 22231744]
85. Erler JT, Bennewith KL, Cox TR, et al. Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. *Cancer Cell*. 2009; 15(1):35–44. [PubMed: 19111879]
86. Kaplan RN, Riba RD, Zacharoulis S, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the premetastatic niche. *Nature*. 2005; 438(7069):820–827. [PubMed: 16341007]
87. Schietke R, Warnecke C, Wacker I, et al. The lysyl oxidases LOX and LOXL2 are necessary and sufficient to repress E-cadherin in hypoxia: insights into cellular transformation processes mediated by HIF-1. *J Biol Chem*. 2010; 285(9):6658–6669. [PubMed: 20026874]
88. Sceneay J, Chow MT, Chen A, et al. Primary tumor hypoxia recruits CD11b⁺/Ly6C^{med}/Ly6G⁺ immune suppressor cells and compromises NK cell cytotoxicity in the premetastatic niche. *Cancer Res*. 2012; 72(16):3906–3911. [PubMed: 22751463]
89. Levental KR, Yu H, Kass L, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell*. 2009; 139(5):891–906. [PubMed: 19931152]
90. Muller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature*. 2001; 410(6824):50–56. [PubMed: 11242036]
91. Tafani M, Russo A, Di Vito M, et al. Up-regulation of pro-inflammatory genes as adaptation to hypoxia in MCF-7 cells and in human mammary invasive carcinoma microenvironment. *Cancer Sci*. 2010; 101(4):1014–1023. [PubMed: 20151982]
92. Staller P, Sulitkova J, Lisztwan J, Moch H, Oakeley EJ, Krek W. Chemokine receptor CXCR4 downregulated by von Hippel–Lindau tumour suppressor pVHL. *Nature*. 2003; 425(6955):307–311. [PubMed: 13679920]

93. Lu X, Yan CH, Yuan M, Wei Y, Hu G, Kang Y. *In vivo* dynamics and distinct functions of hypoxia in primary tumor growth and organotropic metastasis of breast cancer. *Cancer Res.* 2010; 70(10):3905–3914. [PubMed: 20442288]
94. Ceradini DJ, Kulkarni AR, Callaghan MJ, et al. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat Med.* 2004; 10(8):858–864. [PubMed: 15235597]
95. Liang Z, Wu T, Lou H, et al. Inhibition of breast cancer metastasis by selective synthetic polypeptide against CXCR4. *Cancer Res.* 2004; 64(12):4302–4308. [PubMed: 15205345]
96. Liang Z, Yoon Y, Votaw J, Goodman MM, Williams L, Shim H. Silencing of CXCR4 blocks breast cancer metastasis. *Cancer Res.* 2005; 65(3):967–971. [PubMed: 15705897]
97. Jin F, Brockmeier U, Otterbach F, Metzen E. New insight into the SDF-1/CXCR4 axis in a breast carcinoma model: hypoxia-induced endothelial SDF-1 and tumor cell CXCR4 are required for tumor cell intravasation. *Mol Cancer Res.* 2012; 10(8):1021–1031. [PubMed: 22767589]
98. Lin S, Wan S, Sun L, et al. Chemokine C-C motif receptor 5 and C-C motif ligand 5 promote cancer cell migration under hypoxia. *Cancer Sci.* 2012; 103(5):904–912. [PubMed: 22380870]
99. Lin S, Sun L, Hu J, et al. Chemokine C-X-C motif receptor 6 contributes to cell migration during hypoxia. *Cancer Lett.* 2009; 279(1):108–117. [PubMed: 19231068]
100. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell.* 2012; 21(3):309–322. [PubMed: 22439926]
101. Chaturvedi P, Gilkes DM, Wong CC, et al. Hypoxia-inducible factor-dependent breast cancer-mesenchymal stem cell bidirectional signaling promotes metastasis. *J Clin Invest.* 2013; 123(1):189–205. [PubMed: 23318994]
102. Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med.* 2003; 9(6):677–684. [PubMed: 12778166]
103. Alitalo K, Carmeliet P. Molecular mechanisms of lymphangiogenesis in health and disease. *Cancer Cell.* 2002; 1(3):219–227. [PubMed: 12086857]
104. Schoppmann SF, Fenzl A, Schindl M, et al. Hypoxia inducible factor-1 α correlates with VEGF-C expression and lymphangiogenesis in breast cancer. *Breast Cancer Res Treat.* 2006; 99(2):135–141. [PubMed: 16555123]
105. Mohammed RA, Ellis IO, Elsheikh S, Paish EC, Martin SG. Lymphatic and angiogenic characteristics in breast cancer: morphometric analysis and prognostic implications. *Breast Cancer Res Treat.* 2009; 113(2):261–273. [PubMed: 18293084]
106. Schito L, Rey S, Tafani M, et al. Hypoxia-inducible factor 1-dependent expression of platelet-derived growth factor B promotes lymphatic metastasis of hypoxic breast cancer cells. *Proc Natl Acad Sci USA.* 2012; 109(40):E2707–E2716. [PubMed: 23012449]
107. Tammela T, Alitalo K. Lymphangiogenesis: molecular mechanisms and future promise. *Cell.* 2010; 140(4):460–476. [PubMed: 20178740]
108. Kang Y, Siegel PM, Shu W, et al. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell.* 2003; 3(6):537–549. [PubMed: 12842083]
109. Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. *Nature.* 2005; 436(7050):518–524. [PubMed: 16049480]
110. Bos PD, Zhang XH, Nadal C, et al. Genes that mediate breast cancer metastasis to the brain. *Nature.* 2009; 459(7249):1005–1009. [PubMed: 19421193]
111. Kaidi A, Qualtrough D, Williams AC, Paraskeva C. Direct transcriptional up-regulation of cyclooxygenase-2 by hypoxia-inducible factor (HIF)-1 promotes colorectal tumor cell survival and enhances HIF-1 transcriptional activity during hypoxia. *Cancer Res.* 2006; 66(13):6683–6691. [PubMed: 16818642]
112. Wan J, Ma J, Mei J, Shan G. The effects of HIF-1 α on gene expression profiles of NCI-H446 human small cell lung cancer cells. *J Exp Clin Cancer Res.* 2009; 28:150. [PubMed: 20003295]
113. Colla S, Tagliaferri S, Morandi F, et al. The new tumor-suppressor gene inhibitor of growth family member 4 (ING4) regulates the production of proangiogenic molecules by myeloma cells and suppresses hypoxia-inducible factor-1 α (HIF-1 α) activity: involvement in myeloma-induced angiogenesis. *Blood.* 2007; 110(13):4464–4475. [PubMed: 17848618]

114. Suva LJ, Griffin RJ, Makhoul I. Mechanisms of bone metastases of breast cancer. *Endocr Relat Cancer*. 2009; 16(3):703–713. [PubMed: 19443538]
115. Dunn LK, Mohammad KS, Fournier PG, et al. Hypoxia and TGF- β drive breast cancer bone metastases through parallel signaling pathways in tumor cells and the bone microenvironment. *PLoS One*. 2009; 4(9):e6896. [PubMed: 19727403]
116. Lee K, Qian DZ, Rey S, Wei H, Liu JO, Semenza GL. Anthracycline chemotherapy inhibits HIF-1 transcriptional activity and tumor-induced mobilization of circulating angiogenic cells. *Proc Natl Acad Sci USA*. 2009; 106(7):2353–2358. [PubMed: 19168635]
117. Carbonaro M, O'Brate A, Giannakakou P. Microtubule disruption targets HIF-1 α mRNA to cytoplasmic P-bodies for translational repression. *J Cell Biol*. 2011; 192(1):83–99. [PubMed: 21220510]
118. Kaluz S, Kaluzova M, Stanbridge EJ. Proteasomal inhibition attenuates transcriptional activity of hypoxia-inducible factor 1 (HIF-1) via specific effect on the HIF-1 α C-terminal activation domain. *Mol Cell Biol*. 2006; 26(15):5895–5907. [PubMed: 16847340]
119. Kummar S, Raffeld M, Juwara L, et al. Multihistology, target-driven pilot trial of oral topotecan as an inhibitor of hypoxia-inducible factor-1 α in advanced solid tumors. *Clin Cancer Res*. 2011; 17(15):5123–5131. [PubMed: 21673063]
120. Zhang H, Qian DZ, Tan YS, et al. Digoxin and other cardiac glycosides inhibit HIF-1 α synthesis and block tumor growth. *Proc Natl Acad Sci USA*. 2008; 105(50):19579–19586. [PubMed: 19020076]
121. Lee K, Zhang H, Qian DZ, Rey S, Liu JO, Semenza GL. Acriflavine inhibits HIF-1 dimerization, tumor growth, and vascularization. *Proc Natl Acad Sci USA*. 2009; 106(42):17910–17915. [PubMed: 19805192]
122. Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer*. 2003; 3(10):721–732. [PubMed: 13130303]
123. Macdonald IC, Groom AC, Chambers AF. Cancer spread and micrometastasis development: quantitative approaches for *in vivo* models. *Bioessays*. 2002; 24(10):885–893. [PubMed: 12325121]
124. Bentovim L, Amarilio R, Zelzer E. HIF-1 α is a central regulator of collagen hydroxylation and secretion under hypoxia during bone development. *Development*. 2012; 139(23):4473–4483. [PubMed: 23095889]

Executive summary

Hypoxia & breast cancer

- Intratumoral hypoxia is present in most breast tumors. The partial pressure of oxygen that has been measured within breast tumors (2.5–28 mm of mercury) is significantly less than in normal human breast tissue (~65 mm of mercury).
- Hypoxia within a solid tumor arises from an increase in oxygen utilization relative to supply, due to increased cell numbers and blood vessels that are structurally and functionally abnormal.

Hypoxia-inducible factors & breast cancer

- Breast cancer cells respond to decreased oxygen availability by increasing the activity of the hypoxia-inducible factors (HIFs) HIF-1 and -2.
- HIF-1 α overexpression is predictive of adverse outcomes for breast cancer patients.
- HIFs regulate genes implicated in every step of the metastatic cascade, although only a subset of these genes are activated by HIF-1 in any given cancer cell.

HIF regulation of breast cancer metastasis

- HIFs induce expression of *SNAIL1*, *SNAIL2* and *TWIST1*, which are repressors of E-cadherin gene transcription.
- HIFs promote cell motility by increasing *MET* and *AMF* gene expression.
- HIFs induce tissue invasion by increasing *MMP2*, *MMP9* and *PLAUR* gene expression.
- HIFs promote intravasation by increasing *VEGFA* and *ANGPT2* expression.
- HIFs promote cancer cell extravasation by increasing *LICAM* and *ANGPTL4* expression.
- HIFs promote lymphatic metastasis by inducing *VEGFA* and *PDGFB* expression.
- HIFs contribute to metastatic niche formation by activating *LOX*, *LOXL2*, *LOXL4* and *CXCR4* gene expression.
- HIFs regulate tumor fibrosis by activating *P4HA1*, *P4HA2* and *PLOD2* gene expression.
- HIFs directly regulate signaling with stromal cells by inducing *CXCR3* and *PGF* expression.

Therapeutically targeting HIFs

- The efficacy of many HIF inhibitors in blocking primary tumor growth, lymph node metastasis and lung metastasis has been convincingly demonstrated in orthotopic mouse models of breast cancer metastasis.

- Clinical trials in which HIF inhibitors are administered in combination with current therapeutic regimens will be necessary to assess the potential benefit to breast cancer patients.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1. Hypoxia-inducible factors promote breast cancer metastasis

Hypoxia in the primary tumor promotes HIF-1 α stabilization in breast cancer cells, leading to increased transcription of genes encoding proteins that regulate breast cancer metastasis. The genes included in this figure have demonstrated clinical relevance in breast cancer prognosis. Genes in bold promote spontaneous metastasis in experimental mouse models of breast cancer.

[†]Gene in which a functional hypoxia response element has been identified.

ECM: Extracellular matrix; EMT: Epithelial–mesenchymal transition

Table 1
Hypoxia-inducible factor-regulated genes implicated in breast cancer metastasis

Gene	HIF regulation	HIF binding	Ref.
<i>AMF</i>	HIF-1 (HIF-2 not tested)	Binding not tested	–
<i>ANGPT2</i>	HIF-1 only	HIF-1 only	[76]
<i>ANGPTL4</i>	HIF-1 and HIF-2	HIF-1 and HIF-2	[35]
<i>COX2</i>	HIF-1 (HIF-2 not tested)	HIF-1 (HIF-2 not tested)	[111]
<i>CXCR3</i>	HIF-1 (HIF-2 not tested)	HIF-1 (HIF-2 not tested)	[101]
<i>CXCR4</i>	HIF-1 and HIF-2	HIF-1 [†] and HIF-2 [†]	[92]
<i>Ephrin-A1</i>	HIF-2 (HIF-1 not tested)	HIF-2 (HIF-1 not tested)	[78]
<i>IL-8</i>	HIF-1 and HIF-2	Binding not tested	–
<i>LICAM</i>	HIF-1 only	HIF-1 only	[35]
<i>LOX</i>	HIF-1 and HIF-2	HIF-1 (HIF-2 not tested)	[68]
<i>LOXL2</i>	HIF-1 only	HIF-1 [†] (HIF-2 not tested)	[87]
<i>LOXL4</i>	HIF-1 and HIF-2	HIF-1 and HIF-2	[34]
<i>Met</i>	HIF-1 (HIF-2 not tested)	Binding not tested	–
<i>MMP2</i>	HIF-1 (HIF-2 not tested)	Binding not tested	–
<i>MT1-MMP</i>	HIF-2 (HIF-1 not tested)	Binding not tested	–
<i>OPN</i>	HIF-1 (HIF-2 not tested)	Binding not tested	–
<i>P4HA1</i>	HIF-1 only	HIF-1 (HIF-2 not tested)	[124]
<i>P4HA2</i>	HIF-1 only	HIF-1 (HIF-2 not tested)	[124]
<i>PLOD2</i>	HIF-1 only	Binding not tested	–
<i>PGF</i>	HIF-1 (HIF-2 not tested)	HIF-1 (HIF-2 not tested)	[101]
<i>PDGFB</i>	HIF-1 only	HIF-1 only	[106]
<i>PLAUR</i>	Specificity not determined [‡]	Specificity not determined [‡]	[55]
<i>SDF-1</i>	HIF-1 (HIF-2 not tested)	HIF-1 (HIF-2 not tested)	[94]
<i>Snail1</i>	HIF-1 and HIF-2	HIF-1 and HIF-2	[41]
<i>Twist</i>	HIF-1 (HIF-2 not tested)	HIF-1 (HIF-2 not tested)	[44]
<i>VEGF</i>	HIF-1 and HIF-2	HIF-1 [†] (HIF-2 not tested)	[71]

Genes included in the table above are hypoxia-inducible in a HIF-dependent manner. Chromatin immunoprecipitation or electrophoretic mobility assays have been utilized to demonstrate HIF-1 and/or HIF-2 binding to the target gene as indicated by the studies referenced.

[†]Electrophoretic mobility assays were used.

[‡]HIF-1 β binding was assayed by chromatin immunoprecipitation.

HIF: Hypoxia-inducible factor.