Hypoxia-Inducible Factor-1α Is a Key Regulator of Metastasis in a Transgenic Model of Cancer Initiation and Progression

Debbie Liao,1,2 Courtney Corle,1 Tiffany N. Seagroves,1 and Randall S. Johnson1

1Division of Biological Sciences, Molecular Biology Section, and 2Department of Molecular Pathology, School of Medicine, University of California, San Diego, La Jolla, California

Abstract

Adaptation to hypoxia is a critical step in tumor progression and is, in part, regulated by the transcription factor hypoxia-inducible factor-1α (HIF-1α). Xenograft models have been extensively used to characterize the role of HIF-1α in experimental cancers. Although these models provide an understanding of tumor growth at terminal stages of malignancy, they do not address tumor initiation or metastatic progression. To elucidate these roles, HIF-1α was conditionally deleted in the mammary epithelium of a transgenic mouse model for metastatic breast cancer. Conditional deletion of HIF-1α in the mammary epithelium resulted in delayed tumor onset and retarded tumor growth; this was correlated with decreased tumor cell proliferation. Tumors with conditional deletion of HIF-1α were also less vascular during early tumor progression. Perhaps most surprisingly, deletion of HIF-1α in the mammary epithelium resulted in decreased pulmonary metastasis. These results show that whereas HIF-1α is not required for the initiation of breast tumor growth or tumor cell metastasis, the transcriptional activity of HIF-1α is a significant positive regulator of tumor progression and metastatic potential.

Introduction

Cellular adaptation to hypoxia is a critical step in tumor progression (1). In response to hypoxia, cells alter the expression of genes that encode protein products involved in increasing oxygen delivery and activate alternate metabolic pathways that do not require oxygen. This hypoxic response is chiefly regulated by the hypoxia-inducible factor-1 (HIF-1), a basic helix-loop-helix transcription factor composed of two subunits, HIF-1α and HIF-1β (2). The HIF-1α subunit is constitutively expressed, whereas expression of HIF-1α is regulated by oxygen tension. Under normal oxygen tension, HIF-1α is rapidly degraded by posttranslational ubiquitination–triggered proteolysis. However, under low oxygen tensions, HIF-1α is stabilized and heterodimerizes with HIF-1β mediating nuclear translocation and binding to hypoxic responsive elements within the promoter regions of target genes (3). HIF-1 regulates a significant number of genes involved in many biological processes, including angiogenesis, glycolytic metabolism, and cell survival and invasion (4).

Overexpression of HIF-1α has been shown in many cancers, including those of the breast (5). Multiple studies of HIF-1 and breast cancer have shown a significant association between HIF-1α overexpression and poor prognoses coupled to increased patient mortality (6–10). Additionally, Bos et al. have shown that levels of HIF-1α in human primary breast tumors increased with the progression of the pathologic stage and was correlated with increased proliferation and dedifferentiation of lesions; these lesions, in turn, are associated with more aggressive and invasive tumors (11). Interestingly, Vleugel et al. have recently shown that the prognostic outcome of breast cancer patients varies and depends on whether HIF-1α expression is perinecrotic or is diffuse and found throughout the tumor (7). These results suggest that HIF-1α is a negative prognostic factor in breast cancer, but that the contribution of HIF-1α in the progression of breast cancer in vivo remains unclear.

Significant work has been done to characterize the role of HIF-1α in experimental cancers. To date, all of this has been carried out in models using the injection of malignant cells (4). Although this aids in the understanding of tumor growth at terminal stages of malignancy, it does not address tumor initiation and progression and the role of HIF-1α during this process. It also does not address the key process of metastasis and the role of the HIF-1α response in that critical aspect of malignant progression.

We investigated the role of HIF-1α in tumorigenic initiation and metastatic progression of breast cancer by generating a mouse model in which HIF-1α can be conditionally deleted in the mammary epithelium. Our study shows that, in addition to being a positive regulator of mammary tumor progression, HIF-1α has an important role in the metastasis of malignant cells.

Materials and Methods

Animals. All animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International–approved facility, and animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Mammary epithelial tumor cell (MEC)–specific knock-out of HIF-1α was achieved by breeding mice (C57/Bl6) expressing the polyoma middle T (PyMT) oncoprotein under the promoter of the mouse mammary tumor virus (MMTV)-long terminal repeat (LTR) (12) to transgenic mice (FVB/C57/B6), with both alleles of exon 2 of HIF-1α flanked by LoxP sites (HIF-1αlox/lox) and expressing Cre recombinase under the promoter MMTV-LTR (line A; refs. 13, 14). Virgin female wild-type (HIF-1α+/+), genomic DNA isolated from either MECs or MEC wells and sexed (15). Mice were palpated once a week, and tumors were measured in two dimensions with digital calipers. Tumor onset was determined by the appearance of a palpable solid mass with dimensions of at least 2 × 2 mm. The end point was determined by the time at which any tumor had grown to a size of 1 × 1 cm.

Histology and immunohistochemistry. For whole-mount preparation of mammary glands, the inguinal gland was removed and fixed in 10%
phosphate-buffered formalin (Fisher Scientific, Fair Lawn, NJ) and dehydrated through a series of graded acetone and ethanol washes. Glands were stained for 3 h with Harris hematoxylin (VWR Int., West Chester, PA), and excess stain was removed by washing in water. Glands were destained in acidic 50% ethanol and dehydrated and stored in methyl salicylate (Fisher Scientific).

To visualize lung metastasis, lungs were inflated with 10% phosphate-buffered formalin by cannulation of the trachea, removed and fixed in 10% phosphate-buffered formalin. Lungs were embedded in paraffin wax, and 10-μm-thick serial sections were rehydrated through a graded series of xylenes and ethanol, stained with Gill's hematoxylin (Fisher Scientific) and eosin (Surgipath, Richmond, IL), dehydrated, and mounted with Cytoseal-XYL (Richard-Allan Scientific, Dalamazoo, MI).

Tumor hypoxia was detected using the Hypoxiprobe-1 Kit (2-nitroimidazole) for the Detection of Tissue Hypoxia (Chemicon Int. Inc., Temecula, CA) as previously described (16). Proliferating and apoptotic cells were detected by bromodeoxyuridine staining and the terminal nucleotidyl transferase–mediated nick end labeling assay, respectively, as previously described (17). Blood vessels were detected using anti-CD31 antibody, and microvessel density analysis was done as previously described (13). Glut1 was detected using a rabbit anti-mouse Glut1 antibody at 1:200 dilution (Alpha Diagnostics, San Antonio, TX). Vascular endothelial growth factor (VEGF) was detected using a goat anti-mouse VEGF antibody at 1:10 dilution (R&D Systems, Minneapolis, MN). Laminin was detected using a rabbit anti-mouse laminin antibody at 1:25 dilution (Sigma-Aldrich, St. Louis, MO).

**In vitro mammary epithelial cell culture.** MECs from end point tumors of virgin female wild-type (HIF-1α<sup>flox/flox</sup>) MMTV-PyMT positive, MMTV-Cre negative) mice were isolated as previously described (18). Briefly, tumors were minced manually and subject to collagenase digestion for 3 h at 37°C. Cells were then washed and maintained in growth medium: DMEM/F12 medium (Mediatech, Inc., Herndon, VA) supplemented with 2% FCS and 100 units of penicillin/10 units of streptomycin (Invitrogen, Grand Island, NY). Before normoxic (20% O<sub>2</sub>) or hypoxic (0.5% O<sub>2</sub>) culture, cells were infected with adeno-virus-<i>γ</i>-galactosidase or adeno-virus-Cre to generate HIF-1α<sup>flox/flox</sup> (wt) and HIF-α<sup>−/−</sup> (null) tumor MECs, respectively, as previously described (13).

**Western blot analysis.** Adenovirus-infected cells were cultured under normoxia or hypoxia for 24 h, and nuclear extracts were prepared as previously described (19). HIF-1α protein was detected using a rabbit anti-mouse HIF-1α antibody (Novus Biologicals, Littleton, CO) at 1:500 dilution.

**Quantitative real-time PCR analysis.** Adenovirus-infected cells were cultured at normoxia or hypoxia for 6 h, and gene expression of Glut1 and VEGF was quantified by real-time PCR and normalized to the expression of <i>rRNA</i> as previously described (13).

**Growth curve assay.** Adenovirus-infected cells were seeded in triplicate, and 1 day later, growth medium was replaced, and cells were placed under normoxic or hypoxic culture (0 h). Cells were trypsinized and counted every 24 h using a hemocytometer. Media were not changed for the duration of the experiment.

**Migration and invasion assays.** Adenovirus-infected cells were serum starved for 24 h before migration and invasion assays. Cells were seeded in triplicate into uncoated Costar transwells (8 μm pore size, Corning Costar Co., Corning, NY) for migration assays or Matrigel-coated transwells (8 μm pore size, BD Biosciences, Franklin Lakes, NJ) for invasion assays and cultured for 24 or 48 h, respectively, under normoxia (20% O<sub>2</sub>) or hypoxia (0.5% O<sub>2</sub>). For random migration and invasion assays, both upper and lower chambers contained DMEM/F12 supplemented with 5% fetal bovine serum. For directed migration and invasion assays, cells were seeded in serum-free DMEM/F12 medium in the upper chamber with DMEM/F12 medium supplemented with 5% fetal bovine serum in the lower chamber. Cells that had migrated across the transwell membrane were stained and quantified by Image J software.

**Statistical analysis.** Statistical analysis was done using Prism 4.0 software (GraphPad Software, San Diego, CA). Statistical significance was determined by unpaired <i>t</i> test (<i>P</i> < 0.05). The percentage of tumor-free mice and survival curves were analyzed using a Kaplan-Meier survival analysis, and statistical significance was determined using the log-rank test (<i>P</i> < 0.05).

**Results**

Delayed hyperplasia and increased tumor latency in mice with conditional deletion of HIF-1α in the mammary epithelium. To determine the effects of HIF-1α deletion on mammary gland tumor formation and metastasis, we targeted the conditional deletion of the HIF-1α gene in tumor MEC by crossing mice expressing the PyMT oncoprotein and Cre recombinase driven by the MMTV-LTR to mice with floxed alleles of exon 2 of HIF-1α, allowing specific deletion of HIF-1α in tumor MEC (HIF-1α<sup>MEC−/−</sup>) but not in the surrounding stroma. In vivo deletion efficiency of HIF-1α was determined by real-time PCR analysis of genomic DNA isolated from whole mammary glands and tumors (Fig. 1A). At 4 weeks of age, mean deletion of HIF-1α was 33.42 ± 0.54% and increased to 49.74 ± 2.62% by 10 weeks. At 16 weeks, mean deletion was 83.51 ± 9.96% and increased to 88.3 ± 6.71% by the end point. The increase in HIF-1α deletion observed over time is not surprising because the proportion of tumor MEC compared with stromal cells in the mammary gland increases over time as tumors expand to fill the mammary fat pad and begin to make up a larger fraction of cells overall in the gland. Although there was interanimal variability in the deletion efficiency of HIF-1α, this did not significantly alter the results of our study.

Virgin mouse mammary glands were palpated once per week beginning at 4 weeks of age. Tumor onset in HIF-1α<sup>MEC−/−</sup> mice was significantly delayed by 22.4 days compared with HIF-1α<sup>flox/flox</sup> (wild-type) mice (Fig. 1A). However, deletion of HIF-1α did not protect against tumor formation because both HIF-1α<sup>flox/flox</sup> and HIF-1α<sup>MEC−/−</sup> mice developed tumors (Fig. 1B). Whole-mount hematoxylin staining of inguinal glands at 4 and 8 weeks of age showed normal ductal development in both HIF-1α<sup>MEC−/−</sup> and HIF-1α<sup>flox/flox</sup> mice, consistent with previous findings from our lab that the deletion of HIF-1α in MEC does not affect ductal development (13). In whole-mounted glands, hyperplasia stained as dark clusters budding off the ductal tree. All mice with MMTV-PyMT gene expression developed mammary epithelial hyperplasias that eventually formed tumors, regardless of HIF-1α status of tumor MEC. Mammary glands from both HIF-1α<sup>flox/flox</sup> and HIF-1α<sup>MEC−/−</sup> mice contained small foci of hyperplasia restricted to the area under the nipple at 4 weeks of age (data not shown). However, hyperplasia was significantly reduced in HIF-1α<sup>MEC−/−</sup> glands compared with HIF-1α<sup>flox/flox</sup> glands at 8 weeks of age (Fig. 1C). H&E staining of mammary glands at 10 weeks of age revealed that most lesions in HIF-1α<sup>flox/flox</sup> glands had progressed to early carcinomas, whereas a majority of lesions in HIF-1α<sup>MEC−/−</sup> mice were still premalignant, consisting of well-defined lobular structures constrained by basement membrane, evident by laminin staining (Fig. 1D). Additionally, HIF-1α<sup>MEC−/−</sup> glands at this time point contained fewer and smaller lesions, and adipose cells and ductal structures were still apparent, whereas lesions from HIF-1α<sup>flox/flox</sup> glands contained mostly tumor MEC with few normal structures remaining.

Reduced tumor hypoxia and reduced induction of HIF-1α target genes in HIF-1α<sup>MEC−/−</sup> tumors. To determine the effect of HIF-1α deletion on tumor hypoxia, immunohistochemistry was done on formalin-fixed paraffin-embedded serial tumor sections obtained from HIF-1α<sup>flox/flox</sup> and HIF-1α<sup>MEC−/−</sup> mice. To
detect hypoxia, i.e., injections of 2-nitroimidazole were administered to mice 1.5 h before sacrifice, visualized with an antibody against the 2-nitroimidazole, and detected using either a peroxidase-based reaction (Fig. 2A and C) or immunofluorescence (Fig. 2E and G). Tumors from both HIF-1α<sup>flox/flox</sup> and HIF-1α<sup>MEC</sup>/C<sup>0</sup>/C<sup>0</sup>/C<sup>0</sup> mice contained areas of hypoxia; however, the intensity of hypoxia staining was increased in tumors from HIF-1α<sup>flox/flox</sup> mice.

The transcriptional regulation of hypoxia-inducible genes by HIF-1α plays a key role in tumor cell adaptation to hypoxic microenvironments created by rapid tumor growth (20). A key response to tumor hypoxia is the HIF-1α–dependent transcription of angiogenic factor VEGF and subsequent tumor angiogenesis (21). To determine if expression of VEGF correlated with tumor hypoxia and is dependent on HIF-1α, serial tumor sections from HIF-1α<sup>flox/flox</sup> and HIF-1α<sup>MEC</sup>/C<sup>0</sup>/C<sup>0</sup>/C<sup>0</sup> mice were stained with an antibody against VEGF and visualized using a peroxidase-based reaction (Fig. 2B and D). VEGF staining was prevalent throughout HIF-1α<sup>flox/flox</sup> tumors and correlated positively with hypoxia staining. In contrast, VEGF staining was greatly reduced in HIF-1α<sup>MEC</sup>/C<sup>0</sup>/C<sup>0</sup>/C<sup>0</sup> tumors despite the presence of hypoxia, indicating that the hypoxic induction of VEGF expression in these tumors is dependent on HIF-1α.
by increasing the transcription of genes in support of anaerobic fermentation as opposed to oxidative phosphorylation for the production of ATP (22, 23). One HIF-1α–dependent gene upregulated by this phenomenon is glucose transporter (Glut1) 1 (24). To determine if increases in tumor hypoxia correlated with increased expression of Glut1 and if expression is dependent on HIF-1α, serial tumor sections were stained with an antibody against Glut1 and visualized using immunofluorescence (Fig. 2F and H). Hypoxia staining correlated strongly with intense Glut1 staining in HIF-1α (C0) tumors. Although HIF-1α (C0) tumors stained positively for Glut1 in areas of necrosis, Glut1 staining was greatly reduced in non-necrotic areas despite the presence of tumor hypoxia. These results show that in mouse mammary tumors, HIF-1α promotes a hypoxic tumor microenvironment, resulting in the induction of hypoxia-inducible genes that is dependent on HIF-1α.

**Decreased tumor MEC proliferation in HIF-1α (C0) tumors.** Multiple studies have correlated HIF-1α staining with increased tumor cell proliferation in human breast tumors (10, 11). To determine if the delay in tumor progression of HIF-1α (C0) mice is due to changes in tumor cell proliferation, mice were injected with bromodeoxyuridine compound 2 h before sacrifice, and incorporation into proliferating cells was visualized using an antibody against bromodeoxyuridine and a peroxidase-based reaction. Positively stained cells were compared with total cell number in each tumor section as a percentage (Fig. 3A and B). At 10 weeks of age, the percentage of bromodeoxyuridine-positive cells was reduced by 2-fold in tumors from HIF-1α (C0) mice compared with HIF-1α (C0) mice (Fig. 3A). However, at end point, the percentage of bromodeoxyuridine-positive cells in HIF-1α (C0) tumors had increased to similar levels as in HIF-1α (C0) tumors, and there was no significant difference in tumor MEC proliferation between HIF-1α (C0)/C0 and HIF-1α (C0) mice (Fig. 3B).

To determine if the delay in tumor progression in HIF-1α (C0) mice could also be attributed to tumor MEC apoptosis, tumor sections were stained using the terminal nucleotidyl transferase–mediated nick end labeling method, and positively stained cells were compared with total cell number (Fig. 3C and D). There was no significant difference in the percentage of apoptotic MEC between HIF-1α (C0)/C0 and HIF-1α (C0) tumors at either 10-week time point (Fig. 3C) or at end point (Fig. 3D).

These results indicate that HIF-1α expression is a significant positive factor in tumor MEC proliferation during early stages of tumorigenesis. However, as tumor growth progresses, HIF-1α (C0) tumor MECs are able to increase proliferation by pathways not dependent on HIF-1α expression in these cells.

**Decreased tumor vascularization in HIF-1α (C0) tumors.** To determine if differences in HIF-1α–induced gene expression result in changes to tumor vascularization, blood vessels were visualized with a CD31 antibody using an alkaline phosphatase–based reaction (Fig. 4A and B). Vessel staining as well as microvessel density, quantified by microscopy using a Chaukley graticle eyepiece, was greatly reduced in HIF-1α (C0) tumors compared with HIF-1α (C0) tumors from 14-week-old virgin mice (Fig. 4A). However, at end point, there was no significant difference in either vessel staining or microvessel density between tumors from HIF-1α (C0)/C0 and HIF-1α (C0) mice (Fig. 4B). These results show that during early tumor progression, HIF-1α–dependent induction of hypoxic responsive genes by tumor MEC is required for induction of tumor angiogenesis. However, as tumor growth progresses, there are other factors that can facilitate tumor angiogenesis, allowing tumors in HIF-1α (C0) mice to achieve comparable vascularity compared with tumors from HIF-1α (C0) mice.

**Increased survival and reduced pulmonary metastasis in HIF-1α (C0) mice.** To determine if changes in tumor physiology caused by deletion of HIF-1α affect overall survival and pulmonary metastasis, mice were sacrificed when tumor dimensions reached 1 × 1 cm (end point). Before sacrifice, mice were weighed to obtain total body weight, and upon sacrifice, all mammary tumors were removed, and wet tumor weight was obtained. Wet tumor weight was then compared with total body weight as a percentage to determine tumor burden. HIF-1α (C0) mice had a significant increase in survival compared with HIF-1α (C0)/C0 mice (Fig. 5A). Mean survival of HIF-1α (C0) mice was increased by 4.6 weeks compared with HIF-1α (C0)/C0 mice (Fig. 5A). However, although HIF-1α (C0) mice had significantly prolonged survival, this did not correlate with a significant decrease in microvessel density, quantified by microscopy using a Chaukley graticle eyepiece, was greatly reduced in HIF-1α (C0) tumors compared with HIF-1α (C0) tumors from 14-week-old virgin mice (Fig. 4A). However, at end point, there was no significant difference in either vessel staining or microvessel density between tumors from HIF-1α (C0)/C0 and HIF-1α (C0) mice (Fig. 4B). These results show that during early tumor progression, HIF-1α–dependent induction of hypoxic responsive genes by tumor MEC is required for induction of tumor angiogenesis. However, as tumor growth progresses, there are other factors that can facilitate tumor angiogenesis, allowing tumors in HIF-1α (C0) mice to achieve comparable vascularity compared with tumors from HIF-1α (C0) mice.

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**Figure 2.** Effect of HIF-1α deletion on tumor hypoxia and expression of HIF-1α target genes in primary mammary tumors. Hypoxia probe and VEGF or Glut1 staining was done on representative serial cross-sections of tumors from end point virgin HIF-1α (C0)/C0 (A and B) and HIF-1α (C0) mice (C and D). G and H) mice. For hypoxia staining, tumor-bearing mice were injected with hypoxia probe 1.5 h before sacrifice, and an antibody against the probe was used to visualize incorporation (A and C, black; E and G, red). VEGF (B and D, black) and Glut1 (F and H, green) immunostaining was greatly reduced in HIF-1α (C0) compared with HIF-1α (C0) tumors. White arrowheads, areas of necrosis. White arrowheads, autofluorescent RBCs. Bar, 100 μm.

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in metastatic potential of tumor MEC in vitro were established from primary mammary tumors isolated from tumor MEC metastasis (Fig. 5).

Expression of HIF-1α is not required for mammary tumor formation or metastasis, genotype; Fig. 5). Mean metastatic foci count of 70.75 (10 wks of age, tumors from HIF-1α mice had a smaller percentage of bromodeoxyuridine-positive tumor MEC (brown nuclei stain) in proportion to total tumor MEC number (blue nuclei stain) compared with HIF-1α mice (P < 0.0001; flox/flox, n = 5; and MEC−/−, n = 4). B, at end point, the percentage of bromodeoxyuridine-positive cells in HIF-1α−/− tumors had increased to a similar level as HIF-1α−/− mice (flox/flox, n = 10; and MEC−/−, n = 11). C and D, tumor MEC apoptosis was detected via the terminal nucleotidyl transferase–mediated nick end labeling assay. Apoptotic tumor MEC is stained green. No significant difference exists in percentage of apoptotic tumor MEC at 10 wks (C; flox/flox, n = 5; and MEC−/−, n = 4) or at end point (D; flox/flox, n = 6; and MEC−/−, n = 6) between HIF-1α−/− and HIF-1α−/− mice.

To determine the effect of HIF-1α deletion on pulmonary metastasis, serial sections from end point lungs were stained with hematoxylin and eosin to visualize metastatic foci (Fig. 5C). The number of metastatic foci per lung was counted using a stereomicroscope. Although the end point tumor burden of HIF-1α−/− mice was comparable to HIF-1α−/− mice, deletion of HIF-1α resulted in a significant decrease in pulmonary metastasis. Lungs from HIF-1α−/− mice had a mean metastatic foci count of 146.5 ± 25.92 compared with HIF-1α−/− mice, which had a mean metastatic foci count of 70.75 ± 16.34 (P = 0.02, n = 12/genotype; Fig. 5C). These results indicate that although HIF-1α is not required for mammary tumor formation or metastasis, expression of HIF-1α accelerates tumor progression and promotes tumor MEC metastasis (Fig. 5D).

HIF-1α is required for hypoxia-inducible gene transcription in tumor MEC in vitro. To determine the contribution of HIF-1α in metastatic potential of tumor MEC in vitro, tumor MEC lines were established from primary mammary tumors isolated from wild-type (HIF-1α+/+/MMTV-PyMT positive, MMTV-Cre negative) mice. Before normoxic (20% oxygen) or hypoxic (0.5% oxygen) culture, HIF-1α−/− tumor MEC was generated by infection with a Cre-recombinase–expressing adenovirus to induce recombination and deletion of the conditional alleles of HIF-1α. HIF-1α−/− tumor MEC was infected with control adenovirus. Deletion of HIF-1α was verified by real-time PCR of DNA isolated from adenovirus-infected cells and ranged from 75% to 95% deletion efficiency (data not shown). Additionally, nuclear extracts from adenovirus-infected cells were assayed for HIF-1α protein expression (Fig. 6A). HIF-1α−/− tumor MEC had strong induction of nuclear HIF-1α protein expression during hypoxic culture, and this induction was greatly reduced in HIF-1α−/− tumor MEC.

To confirm that HIF-1α is required for hypoxia-inducible gene transcription in tumor MEC in vitro, cDNA was generated using RNA extracts from adenovirus-infected cells and subjected to real-time PCR analysis. HIF-1α−/− tumor MEC had reduced induction of hypoxia-inducible gene transcription during hypoxic culture. Glut1 and VEGF mRNA expression was reduced by 55% and 35%, respectively, in HIF-1α−/− tumor MEC compared with HIF-1α−/− tumor MEC during hypoxic culture (Fig. 6A). These results show that HIF-1α is expressed in murine tumor MEC lines and is required for hypoxic induction of gene expression in vitro.
HIF-1α regulates tumor MEC proliferation during hypoxia in vitro. To determine the effect of HIF-1α deletion on tumor MEC growth in vitro, adenovirus-infected cells were plated at equal density and cultured under normoxia and hypoxia for 96 h. Cell number was quantified every 24 h with a hemocytometer until cultures reached confluency. During normoxic culture, HIF-1α−/− tumor MEC grew at approximately the same rate as HIF-1αflox/flox tumor MEC (Fig. 6A). However, during hypoxic culture, the growth of HIF-1αflox/flox tumor MEC was maintained at approximately the same rate as during normoxic culture, whereas after 48 h of culture under hypoxia, the growth of HIF-1α−/− tumor MEC was significantly reduced compared with HIF-1αflox/flox tumor MEC (Fig. 6B). The growth rate of HIF-1α−/− tumor MEC continued to diminish at each time point beyond 48 h, whereas HIF-1αflox/flox tumor MEC was able to maintain exponential growth during hypoxic culture. These results support the in vitro observation that HIF-1α promotes tumor MEC proliferation.

Hypoxia regulates tumor MEC invasiveness in vitro. Tumor cell invasiveness depends on its ability to break down extracellular matrix components, migrate through the stroma, and enter into the vasculature. To determine if HIF-1α is involved in tumor MEC migration or invasion, we tested the random motility and directed chemotaxis of tumor MEC during culture under normoxic and hypoxic conditions. Uncoated Boyden chambers were used for migration assays, and Boyden chambers coated with Matrigel were used for invasion assays. To assay for random migration and invasion, tumor MECs were seeded into Boyden chambers with equal amounts of serum in both the upper and lower chambers. To assay for directed chemotaxis migration and invasion, tumor MECs were seeded into Boyden chambers with serum-free media in the upper chamber and media containing serum as a chemoattractant in the lower chamber. Tumor MECs were then cultured under normoxia or hypoxia for 24 and 48 h for migration and invasion assays, respectively. Tumor MECs that had migrated across the membranes were stained with dye and quantified using the Image J software. There was no significant difference in random motility between HIF-1αflox/flox and HIF-1α−/− tumor MEC cultured under normoxia. However, hypoxic culture significantly increased the random motility of both HIF-1αflox/flox and HIF-1α−/− tumor MEC (Fig. 6C). In contrast, directed chemotaxis migration, as well as random invasion and directed chemotaxis invasion of HIF-1α−/− tumor MEC, was significantly reduced during hypoxic culture compared with HIF-1αflox/flox tumor MEC (Fig. 6C and D). These results show that hypoxia contributes to tumor MEC invasiveness by increasing MEC motility and directed chemotaxis as well as HIF-1α-dependent invasion through the extracellular matrix.

Discussion

We have generated a mouse model in which the role of HIF-1α during de novo tumor growth, progression, and metastasis can be investigated. Using this model, we have shown that whereas HIF-1α is not required for mammary tumor formation in the PyMT tumor model, it is an accelerating factor during tumor progression. Loss of HIF-1α did not protect against mammary tumor formation because both HIF-1αflox/flox and HIF-1α−/− mice developed tumors. However, the presence of HIF-1α significantly accelerated tumor onset. Accelerated tumor onset in these mice can be attributed to an increase in tumor MEC proliferation. Areas of MEC hyperplasia in mammary glands examined at 10 weeks of age, before palpable tumors were detectable, revealed that HIF-1α−/− glands contained a significantly higher percentage of proliferating cells compared with hyperplasia in HIF-1αflox/flox glands and shows that HIF-1α is a significant contributing factor for tumor MEC proliferation during early tumor progression. This result is in contrast to previous studies by our laboratory showing that hypoxia causes HIF-1α-dependent cell cycle arrest in two different primary cell types (25). However, it is known that primary cells and tumor cells can differ in their responses to hypoxic stress (26). This is supported by findings showing that HIF-1α expression in human primary breast tumors are correlated with increased tumor cell proliferation (11) and by our finding here that HIF-1α is required to sustain exponential growth of tumor MEC exposed to hypoxic stress in vitro.

During tumor formation, the rapid rate of tumor MEC proliferation creates a hypoxic microenvironment, as the tumor acini increase in diameter and blood vessels supplying oxygen via diffusion become more and more distant. To sustain cell survival in...
a microenvironment low in oxygen, mammalian cells alter their gene expression to favor metabolism that uses anaerobic fermentation rather than oxidative phosphorylation (27). We have shown that this phenomenon is dependent on HIF-mediated transcription of genes encoding glycolytic enzymes (22). However, tumor cells also consume glucose at high rates, even under normal oxygen tensions, to meet the high metabolic requirement of rapidly dividing cells (27). These responses result in up-regulation of genes involved in glycolytic metabolism, as well as some of those that regulate glucose transport. Glut1, the primary glucose transporter used by the mammary gland, is expressed at high levels in HIF-1α/MEC during tumor progression. Glut1 expression in these lesions correlated with hypoxia staining and was significantly reduced in HIF-1αMEC−/− glands. Additionally, Glut1 mRNA levels was significantly reduced in HIF-1α−/− tumor MEC compared with HIF-1αMEC/−/− tumor MEC cultured under hypoxic conditions in vitro, evidence that Glut1 expression in these tumors is dependent on HIF-1α.

HIF-1α-dependent expression of Glut1 may play a vital role in supporting the high metabolic demands of rapid tumor MEC proliferation because it has been suggested that glucose transport is the first rate-limiting factor in sugar metabolism in cells (28). This idea is supported by Kang et al., who found that Glut1 expression in human breast carcinomas correlated significantly with nuclear grade, and the absence of Glut1 expression significantly increased disease-free survival (29).

Figure 5. Effects of conditional knock-out of HIF-1α on survival and pulmonary metastasis in MMTV-PyMT mice. Mammary tumor dimensions were measured weekly with digital calipers (HIF-1αMEC−/−, n = 15; and HIF-1αMEC−/−, n = 15). Mice were sacrificed when tumor size reached 1 × 1 cm (end point). A, HIF-1αMEC−/− mice had significantly prolonged survival compared with HIF-1αMEC−/− mice (left, P = 0.007). HIF-1αMEC−/− mice (black triangles) increased survival by an average of 4 wks compared with HIF-1αMEC−/− (black squares) mice (middle, P = 0.005). No difference in tumor burden exists between genotypes at end point (right). B, representative primary tumor sections from HIF-1αMEC−/−MICE (flox/flox) and HIF-1αMEC−/−MICE (MEC−/−) mice. White asterisks, areas of necrosis. Bar, 100 μm. C, representative serial sections of lungs from HIF-1αMEC−/−MICE (gray column) contained significantly fewer metastatic foci compared with lungs from HIF-1αMEC−/−MICE (black column) mice (P = 0.02). D, summary of tumor progression in HIF-1αMEC−/−MICE and HIF-1αMEC−/−MICE. a, normal mammary epithelial cells are composed of a single cell layer surrounding a lumen and constrained by a basement membrane. b, by 4 wks of age, both mammary glands from HIF-1αMEC−/−MICE and HIF-1αMEC−/−MICE have developed hyperplastic premalignant lesions. Cellular proliferation fills and expands the tumor acini. As distance increases from blood vessels, the microenvironment at the center of the tumor acini becomes hypoxic. c, by 10 wks of age, majority of lesions in HIF-1αMEC−/−MICE glands have progressed to early carcinomas. Tumor MECs are pleomorphic and have begun to invade the surrounding stroma. In contrast, majority of lesions in HIF-1αMEC−/−MICE glands are still premalignant and remain constrained within the basement membrane. At this stage, necrosis can be seen at the center of some tumor acini. d, by 14 wks of age, lesions in HIF-1αMEC−/−MICE glands have also progressed to carcinomas and now resemble HIF-1αMEC−/−MICE lesions observed at earlier time points.
In addition to altering cellular metabolism, hypoxia induces the expression of VEGF, a potent inducer of angiogenesis. The formation of new blood vessels is required to support tumor growth beyond 0.4 mm in diameter (30). Tumors from HIF-1α flox/flox mice showed strong VEGF staining that correlated with hypoxia staining. In these lesions, increased VEGF staining correlated with an increase in blood vessel staining as well as increased microvessel density during tumor progression. In comparison, VEGF staining, blood vessel staining, and microvessel density in HIF-1α MEC−/− lesions were greatly reduced, indicating that VEGF-induced angiogenesis during early tumor progression is dependent on HIF-1α. Reduced angiogenesis during tumor progression may also contribute to the decelerated tumor growth observed in HIF-1α MEC−/− mice.

Despite differences in cellular proliferation and vascularization observed between HIF-1α flox/flox and HIF-1α MEC−/− tumors during tumor progression, mammary tumors from HIF-1α MEC−/− mice eventually reach a size comparable to tumors from HIF-1α flox/flox mice. At end point, no differences were observed in cellular proliferation or vascularization between tumors from HIF-1α MEC−/− and HIF-1α MEC−/−/MEC−/− mice, and all tumors had developed into invasive carcinomas. However, tumors from HIF-1α MEC−/− mice required significantly more time to reach this stage when compared with HIF-1α flox/flox tumor MEC during hypoxic culture (P = 0.0005 and P = 0.03, respectively).

Figure 6. Effect of HIF-1α deletion on tumor MEC growth, migration, and invasion in vitro. HIF-1α+/+ (null) tumor MEC was generated by infection of HIF-1α flox/flox (wt) tumor MEC with adenovirus expressing Cre-recombinase. A, Western blot analysis (left) of nuclear protein extracts and real-time PCR (right) analysis of HIF target gene mRNA expression levels from adenovirus-infected cells cultured under normoxia (20% oxygen) or hypoxia (0.5% oxygen) for 6 h. Null tumor MECs were unable to express HIF-1α protein during hypoxic culture, resulting in decreased transcription of HIF target genes Glut1 and VEGF. B, growth of wt and null tumor MEC cultured under normoxia or hypoxia for 24 h. Hypoxia increased the migration of both HIF-1α MEC−/− and HIF-1α+/+ tumor MEC. However, chemotaxis-directed migration of HIF-1α−/− tumor MEC towards serum was significantly impaired compared with HIF-1α MEC−/−/MEC−/− tumor MEC during hypoxic culture (P = 0.004). D, random invasion (left) and chemotaxis-directed invasion (right) of tumor MEC cultured under normoxia or hypoxia for 48 h. Random and chemotaxis-directed invasion of HIF-1α−/− tumor MEC was significantly impaired compared with HIF-1α MEC−/−/MEC−/− tumor MEC during hypoxic culture (P < 0.0005 and P = 0.03, respectively).
variability in deletion efficiency, this did not significantly alter the early tumor progression, there are alternative pathways that do not require HIF-1α expression in tumor MEC that compensate for the absence of HIF-1α in these cells, facilitating cellular proliferation and angiogenesis albeit by a slower process. Despite having similar tumor burden as wild-type mice at end point, HIF-1α−/− mice have a significant reduction pulmonary metastasis even at terminal stages. This reduction in metastasis could be due to several possibilities.

First, our in vitro migration and invasion assays revealed that directed chemotaxis and the ability of tumor MEC to invade through Matrigel matrix during hypoxic stress was significantly impaired in HIF-1α−/− tumor MEC, suggesting that HIF-1α might play a central role in regulating tumor cell invasiveness. Indeed, multiple reports have linked hypoxia to elevated gene expression resulting in increased tumor cell invasiveness in vitro (31–34). Surprisingly, hypoxia induced the motility of both HIF-1α−/− mice and HIF-1α−/− tumor MEC. Because in vitro deletion of HIF-1α by adenovirus infection is not absolute, this phenomenon might be attributed to low expression levels of HIF-1α that are sufficient to induce increased tumor MEC motility under hypoxic conditions. Indeed, a similar finding has been reported by Semenza et al. showing that even a modest increase in HIF-1α protein levels was sufficient to significantly increase the invasion of human colon carcinoma cells (33). Second, although microvessel density is impaired in HIF-1α+ mice at the end point, the increase in hypoxia staining in HIF-1α+ mice suggests that not all newly formed vessels are functional (35). This could promote increases in VEGF expression, which could facilitate extravasation of tumor cells, promoting metastasis to distant organs. Third, HIF-1α might also play a role during tumor MEC homing to and intravasation into the secondary site by regulating genes, such as lysi oxidase (36), which can coordinate this process. Indeed, up-regulation of HIF-1α signaling pathways has been shown to be associated with the molecular signature of micrometastasis in human breast cancer (37). The mouse model we have generated can serve as a valuable tool for further investigation of HIF’s role in tumor cell metastasis.

The results we report here clearly show a role for tumor MEC HIF-1α expression as an accelerating factor in mammary tumor progression and metastasis. However, our study also emphasizes the importance of studying the tumor microenvironment as a whole during tumor progression and metastasis. Further investigation into the hypoxic interaction between tumor cells and stromal cells will allow for the development of more specifically targeted treatments that utilize HIF-1α target gene expression in tumors while minimizing interference with hypoxic responses associated with normal physiologic process vital to tissue repair and immune response.

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Debbie Liao, Courtney Corle, Tiffany N. Seagroves, et al.


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