UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of Hypoxia Inducible Factor–1alpha in Mammary Gland Tumorigenesis

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Molecular Pathology

by

Debbie Liao

Committee in charge:

Professor Randall S. Johnson, Chair
Professor Nigel A. Calcutt
Professor Richard Firtel
Professor Mark Kamps
Professor Mark Mercola

2007

The Dissertation of Debbie Liao is approved, and it is acceptable in quality and form for publication on microfilm:

_____

_____

_____

_____

_____

Chair

University of California, San Diego

2007

DEDICATION

With deepest gratitude, to my family,

my parents Patrick and Judy, Bob and Carol,

and my husband Dan.

Thank you for all your support during this adventure.

TABLE OF CONTENTS

Signature Page …… iii

Dedication …….. iv

Table of Contents ……... v

List of Figures …… vi

Acknowledgments ……..… viii

Vita and Publications ……..… x

Abstract ……... xii

Chapter I. General Introduction ……...…... 1

Chapter II. HIF-1α is a Positive Regulator of Metastasis in a Mouse Model of Tumor Initiation and Progression…….. 23

Chapter III. HIF-1α Regulates E-cadherin Localization Through a Vascular EndothelialGrowth Factor/Vascular Endothelial Growth Factor Receptor-1 Autocrine Signaling Pathway....……. 35

Chapter IV. General Discussion…….…….……... 65

LIST OF FIGURES

Chapter I

Figure 1.1: Normal mammary gland development in the mouse.....…… 14

Chapter II

Figure 2.1: Effects of conditional knock-out of HIF-1α on mammary tumor formation in MMTV-PyMT mice……... 26

Figure 2.2: Effect of HIF-1α deletion on tumor hypoxia and expression of HIF-1α target genes in primary mammary tumors……. 27

Figure 2.3: Histology of HIF-1$\alpha^{flox/flox}$ and HIF-1$\alpha^{MEC-/-}$ primary mammary tumors……...……. 28

Figure 2.4: Blood vessel staining of primary tumors from HIF-1$\alpha^{flox/flox}$ and HIF-1$\alpha^{MEC-/-}$ virgin mice…….…….. 29

Figure 2.5: Effects of conditional knock-out of HIF-1α on survival and pulmonary metastasis in MMTV-PyMT mice……. 30

Figure 2.6: Effect of HIF-1α deletion on tumor MEC growth, migration, and invasion in vitro…….……... 31

Chapter III

Figure 3.1: E-cadherin is not transcriptionally regulated by HIF-1α in mouse tumor MECs…….……... 55

Figure 3.2: Hypoxic reduction of cell surface E-cadherin expression is mediated by HIF-1α…… 56

Figure 3.3: HIF-1α mediated internalization of E-cadherin……..……...… 57

Figure 3.4: Hypoxic induction of VEGFR1 protein expression is dependent on HIF-1α transcriptional activity…….. 58

Figure 3.5: Inhibition of E-cadherin internalization by VEGFR1 blocking antibody…… 59

Chapter IV

Figure 4.1: Efficiency of Cre-mediated gene excision…….. 79

Figure 4.2. Orthotopic implantation of tumor cells isolated from end point HIF-1$\alpha^{flox/flox}$ and HIF-1$\alpha^{MEC-/-}$ mice……... 80

Figure 4.3: Tail-vein metastasis assay...……..…….…. 81

Figure 4.4: Decreased macrophage infiltration in HIF-1$\alpha^{MEC-/-}$ tumors at 14-weeks…….…… 82

Figure 4.5: Contribution of tumor microenvironment…..…… 83

ACKNOWLEDGMENTS

I would first like to thank Dr. Randall S. Johnson for giving me the opportunity to conduct my doctoral thesis research in his lab, and for being an invaluable mentor during the past five years.

I would like to thank Dr. Nigel Calcutt, Dr. Richard Firtel, Dr. Mark Kamps, and Dr. Mark Mercola, for dedicating time to serve on my thesis committee, and for their advice and guidance.

I would like to thank Dr. Tiffany N. Seagroves, for taking me under her wing and introducing me to the ins and outs of mammary gland biology. Her enthusiasm and encouragement will always be appreciated and never forgotten.

I would like to acknowledge and thank members of the Johnson laboratory, past and present, who have all contributed to the color of my graduate school experience. Special thanks to Courtney Corle, for her excellent technical assistance, to Nan Tang, for her friendship and insightful scientific discussions, and to Alexander Weidemann, for his positive attitude and for reviewing this manuscript.

I would like to give special thanks and acknowledgement to my family: the Liao, Defenbaugh, and Hickman clans. They are an incredible source of love and

support, and will forever be appreciated. Thank you to my parents Patrick and Judy Liao, and to my parents-in-law, Robert and Carol Defenbaugh, for always believing in me. Finally, a special thank you to my husband Dan, for keeping things in perspective for me by building a very happy and fulfilling home life.

Chapter 2, in full, is a reprint of the material as it appears in Cancer Research (Liao D, Corle C, Seagroves TN, Johnson RS. Hypoxia inducible factor-1alpha is a key regulator of metatasis in a transgenic model of cancer initiation and progression. Cancer Res 2007;67(2): 563-72). The dissertation author was the primary investigator and author of this paper, and the co-authors listed provided technical support and/or supervision. Permission to include this reprint was granted by the publisher and all co-authors.

VITAE

1999	B.S. in Animal Science University of California, Davis
1999-2002	Research Assistant The Salk Institute of Biological Studies, San Diego Laboratory of Dr. Ronald M. Evans
2002-2007	Graduate Student, Molecular Pathology Program School of Medicine, University of California, San Diego Advisor: Dr. Randall S. Johnson
2007	Ph.D. in Molecular Pathology School of Medicine, University of California, San Diego

PUBLICATIONS

Liao D, Corle C, Seagroves TN, Johnson RS. Hypoxia inducible factor-1alpha is a key regulator of metastasis in a transgenic model of cancer initiation and progression. Cancer Res 2007;*67(2):*563-72.

Liao D, Johnson RS. Hypoxia: A Key Regulator of Angiogenesis in Cancer. Cancer Metastasis Rev 2007;26(2) (in press).

Seagroves TN, Hadsell D, McManaman J, Palmer C, **Liao D**, McNulty W, Welm B, Wagner KU, Neville M, Johnson RS. HIF1alpha is a critical regulator of secretory differentiation and activation, but not vascular expansion, in the mouse mammary gland. Development 2003;*130:*1713-24.

Lee CH, Chawla A, Urbiztondo N, **Liao D**, Boisvert WA, Evans RM, Curtiss LK. Transcriptional repression of atherogenic inflammation: modulation by PPARdelta. Science 2003;*302:*453-7.

Chawla A, Lee CH, Barak Y, He W, Rosenfeld J, **Liao D**, Han J, Kang H, Evans RM. PPARdelta is a very low-density lipoprotein sensor in macrophages. Proc Natl Acad Sci U S A 2003;*100:*1268-73.

He W, Barak Y, Hevener A, Olson P, **Liao D**, Le J, Nelson M, Ong E, Olefsky JM, Evans RM. Adipose-specific peroxisome proliferator-activated receptor gamma

knockout causes insulin resistance in fat and liver but not in muscle. Proc Natl Acad Sci U S A 2003;*100:*15712-7.

Barak Y, **Liao D**, He W, Ong ES, Nelson MC, Olefsky JM, Boland R, Evans RM. Effects of peroxisome proliferator-activated receptor delta on placentation, adiposity, and colorectal cancer. Proc Natl Acad Sci U S A 2002;*99:*303-8.

Chawla A, Barak Y, Nagy L, **Liao D**, Tontonoz P, Evans RM. PPAR-gamma dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. Nat Med 2001;*7:*48-52.

Chawla A, Boisvert WA, Lee CH, Laffitte BA, Barak Y, Joseph SB, **Liao D**, Nagy L, Edwards PA, Curtiss LK, Evans RM, Tontonoz P. A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. Mol Cell 2001;*7:*161-71.

ABSTRACT OF THE DISSERTATION

The Role of Hypoxia Inducible Factor–1alpha in Mammary Gland Tumorigenesis

by

Debbie Liao

Doctor of Philosophy in Molecular Pathology

University of California, San Diego, 2007

Professor Randall S. Johnson, Chair

Maintenance of oxygen homeostasis is crucial at both the cellular and systemic level in mammals. In poorly oxygenated (hypoxic) microenvironments adaptation to hypoxia involves changes in gene expression crucial for cell and tissue viability, known as the hypoxic response. The master regulator of the hypoxic response is the transcription factor Hypoxia Inducible Factor (HIF)–1α. Deregulation of HIF-1α activity has been shown to contribute to various pathologies including cancer. The

aim of this dissertation is to elucidate the role of the hypoxic response, and the contribution of HIF-1α, during mammary gland tumor progression and metastasis.

The first chapter provides a brief overview of the hypoxic response and regulation of hypoxic gene expression by HIF-1α. A brief overview of normal mouse mammary gland development is discussed. Lastly, an introduction to the Polyoma Middle T (PyMT) transgenic mouse model of breast cancer used in these studies is provided.

The second chapter describes the results of conditional deletion of HIF-1α in the tumor mammary epithelial cells (MECs) in the PyMT mouse model. Loss of HIF-1α resulted in delayed tumor onset due to reduced tumor cell proliferation. Loss of HIF-1α also resulted in significantly increased survival and reduced pulmonary metastasis. These findings demonstrated that the transcriptional activity of HIF-1α is a significant regulator of tumor progression and metastatic potential.

The third chapter presents the regulation of E-cadherin expression by HIF-1α. Through Cre-mediated excision of the HIF-1α and VHL gene in tumor MECs, we demonstrate that E-cadherin is not transcriptionally regulated by HIF-1α. Instead, we show that HIF-1α activity promotes the internalization of E-cadherin protein through a VEGF/VEGFR1 autocrine signaling pathway during hypoxia stress.

The concluding chapter describes the initial results and future direction of continuing work to elucidate the contribution of HIF-1α mediated cell-autonomous changes versus changes in the tumor microenvironment during mammary gland tumor progression and metastasis.

CHAPTER I

General Introduction

1.1. Regulation of the hypoxic response.

Oxygen plays a central role in the function of mammalian tissues and conserved oxygen responsive pathways are expressed in almost every cell (1). In order to maintain oxygen homeostasis, exposure of mammalian cells to low oxygen environments triggers a hypoxic response. The aim of this response is to promote cellular adaptation to hypoxia by increasing the expression of genes involved in glucose uptake and glycolysis, as well as genes that promote angiogenesis, vascular permeability, and red blood cell maturation (1). The hypoxic response is tightly controlled and Hypoxia Inducible Factor 1 (HIF-1) has emerged as the master regulator of this response (2).

HIF-1 belongs to a family of basic helix-loop-helix (bHLH)/ Per-ARNT-Sim (PAS) transcription factors consisting of α/β-heterodimers that include HIF-1α, HIF-2α, HIF-3α and HIF-1β (3, 4). Each subunit contains two PAS domains that have roles in heterodimer formation and DNA binding (1). The transcriptional activities of the HIF-α subunits are mainly regulated by oxygen tension. Under normal oxygen tensions (normoxia) the α-subunits are rapidly degraded. Reoxygenation of cells exposed to hypoxia demonstrated the half-life of HIF-1α protein to be less than five minutes (3, 5). Unlike the oxygen regulated HIF-α subunits, HIF-1β, also known as the aryl hydrocarbon nuclear translocator or ARNT, is constitutively expressed and not responsive to oxygen. Together, the HIF-α/β-heterodimer activates transcription by binding DNA at hypoxia response elements (HREs) within the promoter region of

target genes. The expression of over 70 genes is known be directly activated at the transcriptional level by HIF (6).

Of the three HIF-α subunits, HIF-1α is the most ubiquitously expressed and functions as the main regulator of oxygen sensing in many cell types (6). HIF-1α was first identified as a nuclear protein capable of binding the hypoxia-inducible enhancer of the erythropoietin (EPO) gene in Hep3B human hepatoma cells, resulting in hypoxia induced increase in EPO expression (7). Since that time, *in vivo* expression of HIF-1α mRNA has been demonstrated in all human and mouse organs (8, 9). Additionally, HIF-1α mRNA was shown to be ubiquitously expressed by many cell types in the mouse embryo and plays an essential role during embryonic development (10, 11). HIF-1α is required for embryonic vascularization, and knock-out of HIF-1α in mice is embryonic lethal at stage E10.5 due to developmental arrest and cephalic and myocardial defects (11, 12). Additionally, the amino acid sequences of human and mouse HIF-1α share over 90% sequence identity, evidence of the gene's functional importance (2).

HIF-2α, also know as endothelial PAS-1 (EPAS1), was first cloned from the endothelium of mouse embryos (13). HIF-2α shares 48% amino acid sequence identity with HIF-1α and the two isoforms share overlap in the genes they regulate (14). However, the expression pattern of HIF-2α mRNA varies greatly compared to HIF-1α. In contrast to ubiquitous expression of the HIF-1α subunit, HIF-2α mRNA expression is restricted to a subset of cell types and varies in different organs (15, 16). Additionally, HIF-2α shows reduced induction by hypoxia and may have unique

target gene specificity compared to HIF-1α (17-19). In developing mouse embryos, expression of HIF-2α is limited to the endothelium (10, 13, 20, 21) and is implicated in cardiac function, lung development, and hematopoiesis, (22-25). These differences demonstrate that, despite overlap of their functional properties, HIF-1α and HIF-2α subunits have unique roles depending on the cell and tissue type in which they are expressed. In contrast, iPAS, a splice variant of HIF-3α, is involved in negative regulation of HIF-1α transcriptional activity (26, 27).

1.2. Regulation of HIF-1α activity.

Molecular regulation of the HIF-1α subunit is multifaceted, and involves control of mRNA expression, and protein stability and activity. Classical regulation of HIF-1α by oxygen tension is at the level of protein stability (3). Under normoxic conditions, oxygen dependent posttranslational hydroxylation of two proline residues occurs within the oxygen dependent degradation (ODD) domain of HIF-1α (28). Hydroxylation occurs via the activities of three prolyl hydroxylase domain proteins (PHD 1-3) in a reaction that is dependent on the presence of oxygen, 2-oxoglutarate, and iron (29, 30). Hydroxylation of the two proline residues in the ODD domain mediates binding of the tumor suppressor protein von Hippel-Lindau (pVHL) to the N-terminal transactivation domain (TAD-N) of HIF-1α (6, 31). Binding of pVHL, an E3 ubiquitin-protein ligase, results in polyubiquitin tagging of HIF-1α followed by rapid proteasomal degradation (32). However, under hypoxic conditions, hydroxylation does not occur and HIF-1α protein escapes degradation, resulting in

protein accumulation that enables heterodimerization with HIF-1β and subsequent binding to hypoxic responsive elements (HREs) within the regulatory regions of target genes (9, 33).

Oxygen dependent regulation of HIF-1α also occurs at the level of transcriptional activity. Inhibition of transcriptional activity is achieved by preventing co-activator binding to HIF-1α (34). Under normoxic conditions, an asparagine residue in the C-terminal trans-activation domain (TAD-C) of HIF-1α is hydroxylated by Factor Inhibiting HIF (FIH) (35). This hydroxylation event prevents binding of HIF-1α transcriptional co-activators p300/CBP, thus reducing HIF-1 transcriptional activity (36). In contrast, the transcriptional activity of HIF-1α is enhanced by the p42/p44 MAPK pathway (37). Activation of the p42/p44 MAPK pathway results in phosphorylation of serine residues in the N-terminal trans-activation domain (TAD-N), and results in nuclear accumulation of HIF-1α protein (37, 38). Unlike the activities of FIH, which is directly dependent on oxygen concentration, the activities of the p42/p44 MAPK pathway involves phosphorylation of HIF-1α in a process that does not require oxygen. However, phosphorylation of HIF-1α by this pathway is directly dependent on protein availability, and is therefore indirectly regulated by hypoxia (37).

Although regulation of HIF-1α activity occurs primarily at the posttranslational level, regulation of HIF-1α protein also occurs at the mRNA level via the PI(3)K/AKT signal transduction pathway. Deregulation of growth factors and their cognate receptor tyrosine kinases due to mutations involving loss of function in

tumor suppressor genes, or gain of function in oncogenes, can result in activation of the PI(3)K/AKT signaling pathway and has been shown to increase HIF-1α activity (39, 40). PI(3)K/AKT signaling mediates basic Fibroblast Growth Factor (bFGF) induced activation of HIF-1α in human breast tumor and glioblastoma cells (41, 42). Extracellular binding of platelet derived growth factor receptor (PDGFR) and epithermal growth factor receptor (EGFR) by ligand also activates the PI(3)K/AKT signaling cascade, which ultimately results in increased HIF-1α transcriptional activity (43). Additionally, inactivation of tumor suppressor PTEN, an inhibitor of AKT activation by PI(3)K, has also been shown to increase HIF-1α activity (44).

1.3. The hypoxic response in tumors.

In vivo studies using xenograft mouse models have demonstrated that HIF-1α is required for solid tumor growth. Initial gene deletion studies in mouse embryonic stem cells showed that loss of HIF-1α resulted in reduced tumor mass and increased apoptosis of teratocarcinomas (12). Additionally, subcutaneous injection of transformed mouse embryonic fibroblasts (mEFs) nullizygous for HIF-1α also resulted in decreased tumor growth (45). Subsequent studies using HIF-1α knockdown in cells isolated from human cancers and implanted into mice provided additional demonstration for the requirement of HIF-1α for solid tumor formation (46, 47).

Rapidly proliferating tumor cells have a higher metabolic demand compared to nonmalignant cells (48). However, *in vivo* studies have shown that in solid tumors,

oxygen availability can be limited due to inadequate tumor blood flow (48). In order to satisfy increased metabolic demands in hypoxic microenvironments, tumor cells switch from oxidative phosphorylation to glycolysis for the generation of ATP (49). HIF-1α directly regulates this switch by controlling the transcription of genes encoding glycolytic enzymes and glucose transporters (50). Demonstration of this was provided by conditional deletion of HIF-1α in transformed mEFs; when cultured under hypoxic conditions, they exhibited reduced growth rate that coincided with reduced expression of glycolytic enzymes (51). In human disease, increased expression of glucose transporter 1 (GLUT1), a major glucose transporter in mammalian cells, has been demonstrated in many tumor types (52). Expression of GLUT1 is increased significantly in breast cancer cells in comparison to healthy breast tissue (53). In a study of breast cancer patients, increased GLUT1 expression in tumors correlated significantly with decreased disease-free survival and was an indicator poor prognosis (54). Complimentary to this data is the finding that GLUT1 overexpression is associated with increased tumor cell invasiveness in multiple human breast cancer cell lines *in vitro* (55, 56). These studies provide evidence for the regulation of glycolytic metabolism as a significant contributor to tumor progression.

As a tumor cell mass expands, oxygen concentration in the center of the mass decreases due to increasing diffusion distance from local capillaries, and results in hypoxia (57). Sustained expansion of a tumor mass requires new blood vessel formation to provide rapidly proliferating tumor cells with an adequate supply of oxygen and metabolites (58).

It has been proposed that tumor growth consists of two phases: an avascular and vascular phase (59). During the avascular phase, tumor growth is constrained by an inadequate vascular supply (59). In order to overcome this constraint, the tumor must transition from an avascular phase of growth to a vascular phase (59). This phenomenon is known as the angiogenic switch and was first demonstrated during tumor progression in the RIP-Tag transgenic mouse model (60). In this model, cells isolated from late stage tumors, but not early stage tumors, were capable of eliciting an angiogenic response when co-cultured with endothelial cells *in vitro*. Hypoxia has emerged as the primary physiological regulator of the angiogenic switch (61). Vascular endothelial growth factor (VEGF), a potent endothelial cell mitogen, plays a central role in tumor angiogenesis and is directly regulated by HIF-1α (46, 62, 63). Histological studies of human cancers have correlated increased tumor expression of VEGF with more aggressive and malignant lesions (64, 65). Additionally, disruption of autocrine endothelial cell signaling between VEGF and its receptors reduced endothelial cell proliferation and tube formation *in vitro,* and severely impaired *in vivo* angiogenesis of tumor xenografts in mice (66). Together these studies implicate hypoxic regulation of VEGF expression as a key mediator of tumor growth through induction of angiogenesis.

Results from several clinical studies have suggested that HIF-1α may have a significant impact on tumor progression in human breast cancer. First, HIF-1α protein expression has been demonstrated by immunohistochemistry in solid tumors of the breast (67). Additionally, expression of HIF-1α protein, while absent in benign

tumors, has been demonstrated in preneoplastic and metastatic lesions, and has been shown to increase as the pathologic stage of breast cancer increases (67, 68). Lastly, studies have identified HIF-1α protein expression in primary tumors of breast cancer patients as a marker of poor prognosis, supporting the hypothesis that HIF-1α may play a significant role during breast cancer tumor progression and metastasis (68-71).

1.4. Perspective and Approach

Until recently, the most common *in vivo* experimental model used to study tumor progression involved injection of tumor cells into the subcutaneous space in immunodeficient mice (72). However the subcutaneous space is avascular, whereas the tissue-type of many organs in which cancers arise, such as the breast, are normally well vascularized. Additionally, the subcutaneous space is a very poor replicate of the stromal microenvironment in which carcinomas normally develop. This is relevant because tumor cells have been shown to modulate gene expression in non-transformed stromal cells resulting in significant affects on tumor growth (73).

Of particular significance is the indication that reliance of tumors on hypoxia-induced gene expression for growth seems to be dependent on the microenvironment in which it is located. A previous study conducted in our lab by Blouw et al. showed that when transformed astrocytes lacking HIF-1α activity were implanted into an avascular subcutaneous site in nude mice, tumor growth was significantly impaired, due to impaired angiogenesis (74). However, when the same astrocytes were implanted into the brain, a highly vascularized organ and the native microenvironment

for astrocytes, tumor growth was actually accelerated compared to the same cells grown subcutaneously (74). These results underscore the significance of the tumor microenvironment when using animal models to study tumor growth.

For these reasons, our study utilized a genetic approach to investigate the role of HIF-1α in tumor progression of breast cancer. Similar to humans, the mouse mammary gland is a complex tissue comprised of many cell types including epithelial cells, endothelial cells, adipocytes, fibroblasts, and inflammatory cells (75). Thus, the mouse is an ideal model for studying how hypoxic responses contribute to breast tumor growth in the context of disease progression within the mammary gland *in vivo*.

Mammary Gland Development in the Mouse

Mammary gland development in mice consists of several stages, with the majority of ductal growth occurring after birth. During embryonic development, at about day E.10, five pairs of mammary buds develop from migration of cells along the mammary streak (76). At about E.16, these cells undergo rapid proliferation to form a solid cord of mammary epithelial cells that penetrate the underlying dense mesenchyme, extending into the precursor tissue of the mammary fat pad (77). Within the fat pad precursor tissue, the epithelial cells undergo morphogenesis to form branching structures that establish the mammary anlage (76).

In newborn mice, the mammary gland consists of rudimentary ducts formed by a single layer of luminal epithelial cells that are separated from the surrounding stroma by a basement membrane (Fig. 1.1) (76). Proliferation of epithelial cells within the

terminal end buds (TEBs) results in ductal elongation. Cell proliferation during this stage is attributed to the residual effects of maternal and fetal hormones (77). At this time ductal elongation occurs in proportion with the overall growth rate of the animal (76, 77).

Pubertal growth of the mammary gland begins at 4-6 weeks of age and is triggered primarily by hormonal signals arising from the ovaries. During this stage, rapid elongation and branching of the ductal network occurs due to proliferating cells within the TEBs. Ductal outgrowth continues in this fashion until the limits of the mammary fat pad are reached and cell division is arrested (Fig. 1.1). Ductal morphogenesis occurs through a tightly regulated process of cell proliferation and cell death within the TEB. Differentiation of TEB cells gives rise to luminal epithelial and myoepithelial components. Once formed, primary ducts consist of a single layer of luminal epithelial cells surrounded by a layer of myoepithelial cells. The luminal epithelial cells form the channel through which milk will eventually flow. Myoepithelial cells are smooth muscle cells and make up the contractile unit that propels milk through the ducts. Once the ductal tree has fully infiltrated the mammary fat pad, the gland enters a state of dormancy with slight changes in proliferation and apoptosis of the cells within the TEBs in response to estrous cycles (77). In virgin mice, the epithelial cells do not undergo lobulo-alveolar growth until hormonal stimulation induced by pregnancy (78).

Conditional Deletion of HIF-1α in the PyMT Mouse Model

The Polyomavirus Middle T (PyMT) transgenic mouse has been shown to be a reliable model for recapitulation of human breast cancer disease, and was therefore chosen as the experimental model for these studies. Mammary tumors in PyMT mice arise spontaneously in virgin animals due to mammary epithelium directed expression of the PyMT antigen by the mouse mammary tumor virus (MMTV)-long terminal repeat (LTR) promoter (79). Though PyMT is not expressed in human breast tumor cells, the signal trasduction pathways through which PyMT exerts its tumorigenic effects are also altered in human breast cancers. Specifically, signal trasduction via the Src, Ras, and PI3K pathways have been shown to be required for cellular transformation by PyMT (80). Importantly, these signaling pathways are also activated by ErbB-2/Neu, a receptor tyrosine kinase that is commonly overexpressed in human breast cancers (80). Extensive histological characterization of tumor progression in the PyMT model has also demonstrated reliable recapitulation of the morphological changes that occur during human breast cancer progression (81). Additionally, changes in gene expression concurrent with tumor progression in the PyMT model correlates with changes in gene expression observed in human breast cancer disease (81). The PyMT model also provides an added advantage in the high rate of pulmonary metastasis that allows for the investigation of metastatic disease (79).

Transgenic mice with conditional deletion of HIF-1α specifically in the mammary epithelium were utilized to generate a mouse mammary tumor model in which the role of HIF-1α expression by tumorigenic mammary epithelial cells (MECs) during tumor progression could be investigated. Mice previously generated in our lab, with exon 2 of HIF-1α flanked by loxP sites (HIF-1$\alpha^{flox/flox}$) and expression of Cre-recombinase driven by the MMTV-LTR promoter (MMTV-Cre), results in a null allele of HIF-1α specifically in MECs. In these mice, mammary gland development occurs normally in virgin animals, thus making it an ideal model to combine with the PyMT tumor model (82). HIF-1$\alpha^{flox/flox}$/MMTV-Cre transgenic mice were crossed with PyMT transgenic mice to produce bigenic mice in which HIF-1α is specifically deleted in the tumor MECs, thus generating a mouse model in which the *in vivo* role of HIF-1α during tumor progression and metastasis can be investigated.

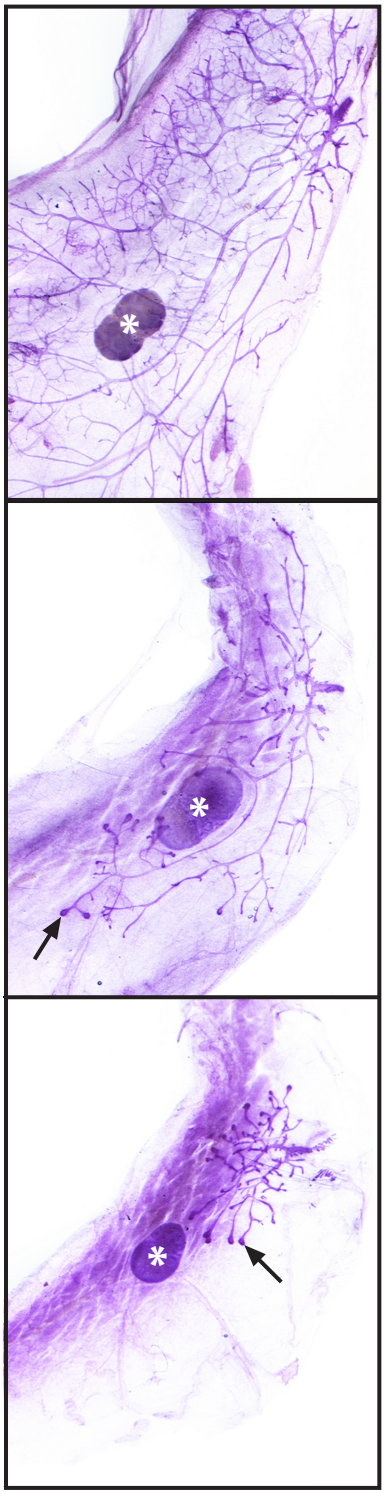


Figure 1.1. Normal mammary gland development in the mouse. Whole mount carmine staining of the #4 mammary fat pad from a 4-week old (left), 8-week old (middle), and mature adult (right) mouse. The lymp node is denoted by a white astrick. Black arrows denote terminal end buds. Magnification = 8x.

References

1. Bruick RK. Oxygen sensing in the hypoxic response pathway: regulation of the hypoxia-inducible transcription factor. Genes Dev 2003;*17:*2614-23.

2. Semenza GL. Hypoxia-inducible factor 1: master regulator of O2 homeostasis. Curr Opin Genet Dev 1998;*8:*588-94.

3. 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 U S A 1995;*92:*5510-4.

4. Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. J Biol Chem 1995;*270:*1230-7.

5. Yu AY, Frid MG, Shimoda LA, Wiener CM, Stenmark K, Semenza GL. Temporal, spatial, and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. Am J Physiol 1998;*275:*L818-26.

6. Semenza GL. Hydroxylation of HIF-1: oxygen sensing at the molecular level. Physiology (Bethesda) 2004;*19:*176-82.

7. Semenza GL, Nejfelt MK, Chi SM, Antonarakis SE. Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. Proc Natl Acad Sci U S A 1991;*88:*5680-4.

8. Wiener CM, Booth G, Semenza GL. In vivo expression of mRNAs encoding hypoxia-inducible factor 1. Biochem Biophys Res Commun 1996;*225:*485-8.

9. Semenza GL, Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 1992;*12:*5447-54.

10. Jain S, Maltepe E, Lu MM, Simon C, Bradfield CA. Expression of ARNT, ARNT2, HIF1 alpha, HIF2 alpha and Ah receptor mRNAs in the developing mouse. Mech Dev 1998;*73:*117-23.

11. Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, Semenza GL. Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev 1998;*12:*149-62.

12. Ryan HE, Lo J, Johnson RS. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. Embo J 1998;*17:*3005-15.

13. Tian H, McKnight SL, Russell DW. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev 1997;*11:*72-82.

14. Iyer NV, Leung SW, Semenza GL. The human hypoxia-inducible factor 1alpha gene: HIF1A structure and evolutionary conservation. Genomics 1998;*52:*159-65.

15. Wiesener MS, Jurgensen JS, Rosenberger C, Scholze CK, Horstrup JH, Warnecke C, Mandriota S, Bechmann I, Frei UA, Pugh CW, Ratcliffe PJ, Bachmann S, Maxwell PH, Eckardt KU. Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. Faseb J 2003;*17:*271-3.

16. Gordan JD, Simon MC. Hypoxia-inducible factors: central regulators of the tumor phenotype. Curr Opin Genet Dev 2007;*17:*71-7.

17. O'Rourke JF, Tian YM, Ratcliffe PJ, Pugh CW. Oxygen-regulated and transactivating domains in endothelial PAS protein 1: comparison with hypoxia-inducible factor-1alpha. J Biol Chem 1999;*274:*2060-71.

18. Park SK, Dadak AM, Haase VH, Fontana L, Giaccia AJ, Johnson RS. Hypoxia-induced gene expression occurs solely through the action of hypoxia-inducible factor 1alpha (HIF-1alpha): role of cytoplasmic trapping of HIF-2alpha. Mol Cell Biol 2003;*23:*4959-71.

19. Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC. Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. Mol Cell Biol 2003;*23:*9361-74.

20. Flamme I, Frohlich T, von Reutern M, Kappel A, Damert A, Risau W. HRF, a putative basic helix-loop-helix-PAS-domain transcription factor is closely related to hypoxia-inducible factor-1 alpha and developmentally expressed in blood vessels. Mech Dev 1997;*63:*51-60.

21. Ema M, Taya S, Yokotani N, Sogawa K, Matsuda Y, Fujii-Kuriyama Y. A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development. Proc Natl Acad Sci U S A 1997;*94:*4273-8.

22. Tian H, Hammer RE, Matsumoto AM, Russell DW, McKnight SL. The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine

homeostasis and protection against heart failure during embryonic development. Genes Dev 1998;*12:*3320-4.

23. Peng J, Zhang L, Drysdale L, Fong GH. The transcription factor EPAS-1/hypoxia-inducible factor 2alpha plays an important role in vascular remodeling. Proc Natl Acad Sci U S A 2000;*97:*8386-91.

24. Compernolle V, Brusselmans K, Acker T, Hoet P, Tjwa M, Beck H, Plaisance S, Dor Y, Keshet E, Lupu F, Nemery B, Dewerchin M, Van Veldhoven P, Plate K, Moons L, Collen D, Carmeliet P. Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. Nat Med 2002;*8:*702-10.

25. Scortegagna M, Morris MA, Oktay Y, Bennett M, Garcia JA. The HIF family member EPAS1/HIF-2alpha is required for normal hematopoiesis in mice. Blood 2003;*102:*1634-40.

26. Makino Y, Cao R, Svensson K, Bertilsson G, Asman M, Tanaka H, Cao Y, Berkenstam A, Poellinger L. Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature 2001;*414:*550-4.

27. Makino Y, Kanopka A, Wilson WJ, Tanaka H, Poellinger L. Inhibitory PAS domain protein (IPAS) is a hypoxia-inducible splicing variant of the hypoxia-inducible factor-3alpha locus. J Biol Chem 2002;*277:*32405-8.

28. Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A 1998;*95:*7987-92.

29. Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. Science 2001;*294:*1337-40.

30. Appelhoff RJ, Tian YM, Raval RR, Turley H, Harris AL, Pugh CW, Ratcliffe PJ, Gleadle JM. Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. J Biol Chem 2004;*279:*38458-65.

31. Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 2001;*107:*43-54.

32. Kondo K, Kaelin WG, Jr. The von Hippel-Lindau tumor suppressor gene. Exp Cell Res 2001;*264:*117-25.

33. Wenger RH. Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. Faseb J 2002;*16:*1151-62.

34. Mahon PC, Hirota K, Semenza GL. FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev 2001;*15:*2675-86.

35. Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev 2002;*16:*1466-71.

36. Arany Z, Huang LE, Eckner R, Bhattacharya S, Jiang C, Goldberg MA, Bunn HF, Livingston DM. An essential role for p300/CBP in the cellular response to hypoxia. Proc Natl Acad Sci U S A 1996;*93:*12969-73.

37. Richard DE, Berra E, Gothie E, Roux D, Pouyssegur J. p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1. J Biol Chem 1999;*274:*32631-7.

38. Mylonis I, Chachami G, Samiotaki M, Panayotou G, Paraskeva E, Kalousi A, Georgatsou E, Bonanou S, Simos G. Identification of MAPK phosphorylation sites and their role in the localization and activity of hypoxia-inducible factor-1alpha. J Biol Chem 2006;*281:*33095-106.

39. Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, Simons JW, Semenza GL. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res 2000;*60:*1541-5.

40. Blancher C, Moore JW, Robertson N, Harris AL. Effects of ras and von Hippel-Lindau (VHL) gene mutations on hypoxia-inducible factor (HIF)-1alpha, HIF-2alpha, and vascular endothelial growth factor expression and their regulation by the phosphatidylinositol 3'-kinase/Akt signaling pathway. Cancer Res 2001;*61:*7349-55.

41. Shi YH, Wang YX, Bingle L, Gong LH, Heng WJ, Li Y, Fang WG. In vitro study of HIF-1 activation and VEGF release by bFGF in the T47D breast

cancer cell line under normoxic conditions: involvement of PI-3K/Akt and MEK1/ERK pathways. J Pathol 2005;*205:*530-6.

42. Pore N, Liu S, Haas-Kogan DA, O'Rourke DM, Maity A. PTEN mutation and epidermal growth factor receptor activation regulate vascular endothelial growth factor (VEGF) mRNA expression in human glioblastoma cells by transactivating the proximal VEGF promoter. Cancer Res 2003;*63:*236-41.

43. Brat DJ, Mapstone TB. Malignant glioma physiology: cellular response to hypoxia and its role in tumor progression. Ann Intern Med 2003;*138:*659-68.

44. Zundel W, Schindler C, Haas-Kogan D, Koong A, Kaper F, Chen E, Gottschalk AR, Ryan HE, Johnson RS, Jefferson AB, Stokoe D, Giaccia AJ. Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev 2000;*14:*391-6.

45. Ryan HE, Poloni M, McNulty W, Elson D, Gassmann M, Arbeit JM, Johnson RS. Hypoxia-inducible factor-1alpha is a positive factor in solid tumor growth. Cancer Res 2000;*60:*4010-5.

46. Maxwell PH, Dachs GU, Gleadle JM, Nicholls LG, Harris AL, Stratford IJ, Hankinson O, Pugh CW, Ratcliffe PJ. Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci U S A 1997;*94:*8104-9.

47. Stoeltzing O, McCarty MF, Wey JS, Fan F, Liu W, Belcheva A, Bucana CD, Semenza GL, Ellis LM. Role of hypoxia-inducible factor 1alpha in gastric cancer cell growth, angiogenesis, and vessel maturation. J Natl Cancer Inst 2004;*96:*946-56.

48. Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res 1989;*49:*6449-65.

49. Shaw RJ. Glucose metabolism and cancer. Curr Opin Cell Biol 2006;*18:*598-608.

50. Semenza GL, Roth PH, Fang HM, Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem 1994;*269:*23757-63.

51. Seagroves TN, Ryan HE, Lu H, Wouters BG, Knapp M, Thibault P, Laderoute K, Johnson RS. Transcription factor HIF-1 is a necessary mediator of the pasteur effect in mammalian cells. Mol Cell Biol 2001;*21:*3436-44.

52. Macheda ML, Rogers S, Best JD. Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. J Cell Physiol 2005;*202:*654-62.

53. Brown RS, Wahl RL. Overexpression of Glut-1 glucose transporter in human breast cancer. An immunohistochemical study. Cancer 1993;*72:*2979-85.

54. Kang SS, Chun YK, Hur MH, Lee HK, Kim YJ, Hong SR, Lee JH, Lee SG, Park YK. Clinical significance of glucose transporter 1 (GLUT1) expression in human breast carcinoma. Jpn J Cancer Res 2002;*93:*1123-8.

55. Grover-McKay M, Walsh SA, Seftor EA, Thomas PA, Hendrix MJ. Role for glucose transporter 1 protein in human breast cancer. Pathol Oncol Res 1998;*4:*115-20.

56. Robey IF, Lien AD, Welsh SJ, Baggett BK, Gillies RJ. Hypoxia-inducible factor-1alpha and the glycolytic phenotype in tumors. Neoplasia 2005;*7:*324-30.

57. Brown JM, Giaccia AJ. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. Cancer Res 1998;*58:*1408-16.

58. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 1996;*86:*353-64.

59. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. Nat Rev Cancer 2003;*3:*401-10.

60. North S, Moenner M, Bikfalvi A. Recent developments in the regulation of the angiogenic switch by cellular stress factors in tumors. Cancer Lett 2005;*218:*1-14.

61. Giordano FJ, Johnson RS. Angiogenesis: the role of the microenvironment in flipping the switch. Curr Opin Genet Dev 2001;*11:*35-40.

62. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 1996;*16:*4604-13.

63. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. Nat Rev Mol Cell Biol 2006;*7:*359-71.

64. Jensen RL, Ragel BT, Whang K, Gillespie D. Inhibition of hypoxia inducible factor-1alpha (HIF-1alpha) decreases vascular endothelial growth factor (VEGF) secretion and tumor growth in malignant gliomas. J Neurooncol 2006;*78:*233-47.

65. Bos R, Zhong H, Hanrahan CF, Mommers EC, Semenza GL, Pinedo HM, Abeloff MD, Simons JW, van Diest PJ, van der Wall E. Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. J Natl Cancer Inst 2001;*93:*309-14.

66. Tang N, Wang L, Esko J, Giordano FJ, Huang Y, Gerber HP, Ferrara N, Johnson RS. Loss of HIF-1alpha in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis. Cancer Cell 2004;*6:*485-95.

67. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, Simons JW. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 1999;*59:*5830-5.

68. Gruber G, Greiner RH, Hlushchuk R, Aebersold DM, Altermatt HJ, Berclaz G, Djonov V. Hypoxia-inducible factor 1 alpha in high-risk breast cancer: an independent prognostic parameter? Breast Cancer Res 2004;*6:*R191-8.

69. Kronblad A, Jirstrom K, Ryden L, Nordenskjold B, Landberg G. Hypoxia inducible factor-1alpha 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:*2609-16.

70. Bos R, van der Groep P, Greijer AE, Shvarts A, Meijer S, Pinedo HM, Semenza GL, van Diest PJ, van der Wall E. Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. Cancer 2003;*97:*1573-81.

71. Dales JP, Garcia S, Meunier-Carpentier S, Andrac-Meyer L, Haddad O, Lavaut MN, Allasia C, Bonnier P, Charpin C. Overexpression of hypoxia-inducible factor HIF-1alpha predicts early relapse in breast cancer: Retrospective study in a series of 745 patients. Int J Cancer 2005;*116:*734-9.

72. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;*3:*721-32.

73. Fukumura D, Xavier R, Sugiura T, Chen Y, Park EC, Lu N, Selig M, Nielsen G, Taksir T, Jain RK, Seed B. Tumor induction of VEGF promoter activity in stromal cells. Cell 1998;*94:*715-25.

74. Blouw B, Song H, Tihan T, Bosze J, Ferrara N, Gerber HP, Johnson RS, Bergers G. The hypoxic response of tumors is dependent on their microenvironment. Cancer Cell 2003;*4:*133-46.

75. Wiseman BS, Werb Z. Stromal effects on mammary gland development and breast cancer. Science 2002;*296:*1046-9.

76. Imagawa W YJ, Guzman R, and Nandi S. Control of Mammary Gland Development. In: Knobil E aNJ. The Physiology of Reproduction. New York: Raven Press; 1994. p.1033-63.

77. Hennighausen L, Robinson GW. Think globally, act locally: the making of a mouse mammary gland. Genes Dev 1998;*12:*449-55.

78. Topper YJ, Freeman CS. Multiple hormone interactions in the developmental biology of the mammary gland. Physiol Rev 1980;*60:*1049-106.

79. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol 1992;*12:*954-61.

80. Dankort DL, Muller WJ. Signal transduction in mammary tumorigenesis: a transgenic perspective. Oncogene 2000;*19:*1038-44.

81. Lin EY, Jones JG, Li P, Zhu L, Whitney KD, Muller WJ, Pollard JW. 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:*2113-26.

82. Seagroves TN, Hadsell D, McManaman J, Palmer C, Liao D, McNulty W, Welm B, Wagner KU, Neville M, Johnson RS. HIF1alpha is a critical regulator of secretory differentiation and activation, but not vascular expansion, in the mouse mammary gland. Development 2003;*130:*1713-24.

CHAPTER II

HIF-1α is a Positive Regulator of Metastasis in a Mouse Model of Tumor Initiation and Progression

Research Article

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. Johnson[1]

[1]Division of Biological Sciences, Molecular Biology Section, and [2]Department 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. [Cancer Res 2007;67(2):563–72]

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-1β 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 promotor 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-1 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-1 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/B6) 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-1α$^{+f/+f}$) and expressing Cre recombinase under the promoter MMTV-LTR (line A; refs. 13, 14). Virgin female wild-type (HIF-1α$^{flox/flox}$: HIF-1α$^{+f/+f}$, MMTV-PyMT positive, MMTV-Cre negative) and HIF-1α MEC "null" (HIF-1α$^{MEC-/-}$: HIF-1α$^{+f/+f}$, MMTV-PyMT positive, MMTV-Cre positive) mice were used in these studies. To determine the *in vivo* deletion frequency of HIF-1α, genomic DNA isolated from mouse mammary glands was subjected to real-time PCR analysis as previously described (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%

Note: Current address for Tiffany N. Seagroves: Department of Pathology, University of Tennessee Cancer Institute, Memphis, TN 38163.

Requests for reprints: Randall S. Johnson, Department of Biology, University of California, San Diego, La Jolla, CA 92093-0377. Phone: 858-822-0509; Fax: 858-534-5831; E-mail: rjohnson@biomail.ucsd.edu.

doi:10.1158/0008-5472.CAN-06-2701

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 Hypoxiaprobe-1 Kit (2-nitro-imidazole) 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-1$\alpha^{flox/flox}$, 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 penincillin/10 units of streptomycin (Invitrogen, Grand Island, NY). Before normoxic (20% O_2) or hypoxic (0.5% O_2) culture, cells were infected with adenovirus-β-galactosidase or adenovirus-Cre to generate HIF-1$\alpha^{flox/flox}$ (wt) and HIF-$\alpha^{-/-}$ (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.

Quantative 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 rRNA 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_2) or hypoxia (0.5% O_2). 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 t test ($P < 0.05$). 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 ($P < 0.05$).

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-1$\alpha^{MEC-/-}$) 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. 1*A*). 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-1$\alpha^{MEC-/-}$ mice was significantly delayed by 22.4 days compared with HIF-1$\alpha^{flox/flox}$ (wild-type) mice (Fig. 1*A*). However, deletion of HIF-1α did not protect against tumor formation because both HIF-1$\alpha^{flox/flox}$ and HIF-1$\alpha^{MEC-/-}$ mice developed tumors (Fig. 1*B*). Whole-mount hematoxylin staining of inguinal glands at 4 and 8 weeks of age showed normal ductal development in both HIF-1$\alpha^{MEC-/-}$ and HIF-1$\alpha^{flox/flox}$ 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-1$\alpha^{flox/flox}$ and HIF-1$\alpha^{MEC-/-}$ 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-1$\alpha^{MEC-/-}$ glands compared with HIF-1$\alpha^{flox/lflox}$ glands at 8 weeks of age (Fig. 1*C*). H&E staining of mammary glands at 10 weeks of age revealed that most lesions in HIF-1$\alpha^{flox/flox}$ glands had progressed to early carcinomas, whereas a majority of lesions in HIF-1$\alpha^{MEC-/-}$ mice were still premalignant, consisting of well-defined lobular structures constrained by basement membrane, evident by laminin staining (Fig. 1*D*). Additionally, HIF-1$\alpha^{MEC-/-}$ glands at this time point contained fewer and smaller lesions, and adipose cells and ductal structures were still apparent, whereas lesions from HIF-1$\alpha^{flox/flox}$ glands contained mostly tumor MEC with few normal structures remaining.

Reduced tumor hypoxia and reduced induction of HIF-1 target genes in HIF-1$\alpha^{MEC-/-}$ 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-1$\alpha^{flox/flox}$ and HIF-1$\alpha^{MEC-/-}$ mice. To

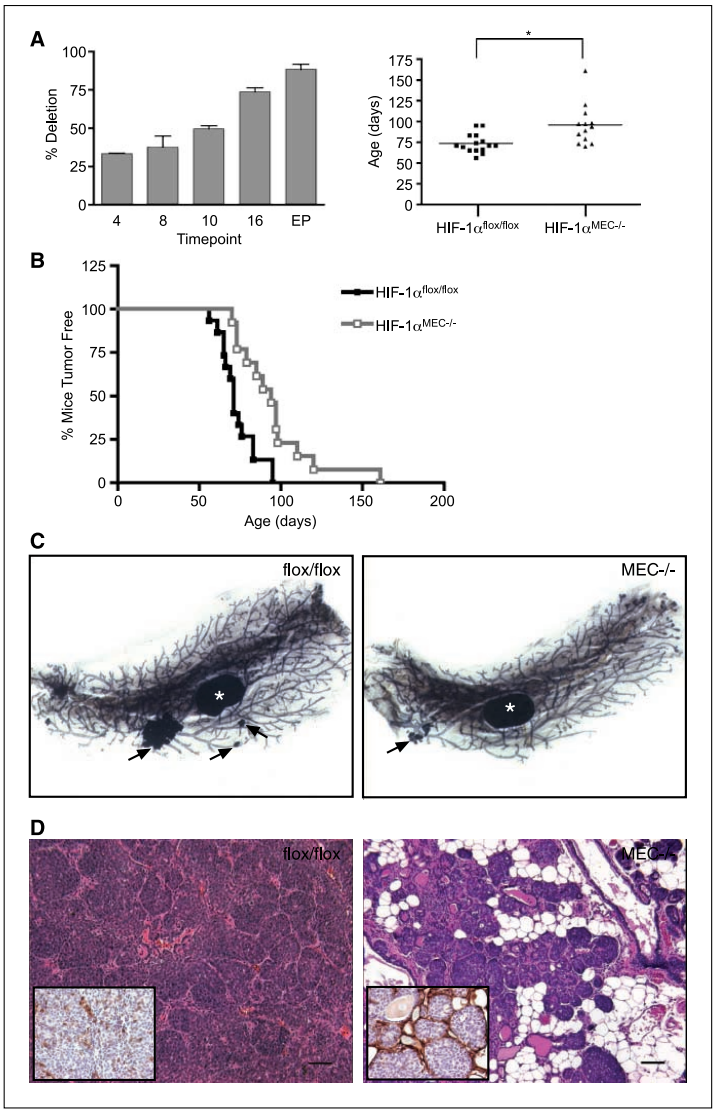


Figure 1. Effects of conditional knock-out of HIF-1α on mammary tumor formation in MMTV-PyMT mice. *A, left, in vivo* deletion of HIF-1α was determined by real-time PCR analysis of genomic DNA isolated from whole mammary glands and tumors at 4-, 8-, 10-, and 16-wk time points (n = 2/time point) and at end point (EP, n = 5). *Right*, mean age at tumor onset for HIF-1α$^{MEC-/-}$ mice was delayed by 22.4 d compared with HIF-1α$^{flox/flox}$ mice (P = 0.007). *B,* percentage of tumor-free mice in each group was calculated using a Kaplan-Meier survival analysis (HIF-1α$^{flox/flox}$, n = 15; and HIF-1α$^{MEC-/-}$, n = 13). Both HIF-1α$^{flox/flox}$ and HIF-1α$^{MEC-/-}$ mice developed tumors, although tumor onset was significantly delayed in HIF-1α$^{MEC-/-}$ mice (P = 0.001). *C,* representative whole-mount hematoxylin staining of the 8-wk-old virgin mammary gland from HIF-1α$^{flox/flox}$ (flox/flox) and HIF-1α$^{MEC-/-}$ (MEC−/−) mice (×6.3). The ductal tree is stained *blue*, as well as lymph nodes (*white asterisk*) and hyperplasias (*black arrow*). *D,* representative H&E staining of the 10-wk-old virgin mammary gland from HIF-1α$^{flox/flox}$ (flox/flox) and HIF-1α$^{MEC-/-}$ (MEC−/−) mice. *Inset, bottom left,* laminin staining (*red*) to detect basement membrane (×400). *Bar*, 100 μm.

detect hypoxia, i.p. 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. 2*A* and *C*) or immunofluorescence (Fig. 2*E* and *G*). Tumors from both HIF-1α$^{flox/flox}$ and HIF-1α$^{MEC-/-}$ mice contained areas of hypoxia; however, the intensity of hypoxia staining was increased in tumors from HIF-1α$^{flox/flox}$ 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$^{flox/flox}$ and HIF-1α$^{MEC-/-}$ mice were stained with an antibody against VEGF and visualized using a peroxidase-based reaction (Fig. 2*B* and *D*). VEGF staining was prevalent throughout HIF-1α$^{flox/flox}$ tumors and correlated positively with hypoxia staining. In contrast, VEGF staining was greatly reduced in HIF-1α$^{MEC-/-}$ tumors despite the presence of hypoxia, indicating that the hypoxic induction of VEGF expression in these tumors is dependent on HIF-1α.

It has previously been shown that HIF-1α also plays an important role in the metabolic adaptation of transformed cells

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 up-regulated by this phenomenon is glucose transporter (Glut) 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. 2*F* and *H*). Hypoxia staining correlated strongly with intense Glut1 staining in HIF-1α$^{flox/flox}$ tumors. Although HIF-1α$^{MEC-/-}$ 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$^{MEC-/-}$ 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$^{MEC-/-}$ 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. 3*A* and *B*). At 10 weeks of age, the percentage of bromodeoxyuridine-positive cells was reduced by 2-fold in tumors from HIF-1α$^{MEC-/-}$ mice compared with HIF-1α$^{flox/flox}$ mice (Fig. 3*A*). However, at end point, the percentage of bromodeoxyuridine-positive cells in HIF-1α$^{MEC-/-}$ tumors had increased to similar levels as in HIF-1α$^{flox/flox}$ tumors, and there was no significant difference in tumor MEC proliferation between HIF-1α$^{flox/flox}$ and HIF-1α$^{MEC-/-}$ mice (Fig. 3*B*).

To determine if the delay in tumor progression in HIF-1α$^{MEC-/-}$ 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. 3*C* and *D*). There was no significant difference in the percentage of apoptotic MEC between HIF-1α$^{flox/flox}$ and HIF-1α$^{MEC-/-}$ tumors at either 10-week time point (Fig. 3*C*) or at end point (Fig. 3*D*).

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$^{MEC-/-}$ tumor MECs are able to increase proliferation by pathways not dependent on HIF-1α expression in these cells.

Decreased tumor vascularization in HIF-1α$^{MEC-/-}$ 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. 4*A* and *B*). Vessel staining as well as microvessel density, quantified by microscopy using a Chaukley graticle eyepiece, was greatly reduced in HIF-1α$^{MEC-/-}$ tumors compared with HIF-1α$^{flox/flox}$ tumors from 14-week-old virgin mice (Fig. 4*A*). However, at end point, there was no significant difference in either vessel staining or microvessel density between tumors from HIF-1α$^{flox/flox}$ and HIF-1α$^{MEC-/-}$ mice (Fig. 4*B*). 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$^{MEC-/-}$ mice to achieve comparable vascularity compared with tumors from HIF-1α$^{flox/flox}$ mice.

Increased survival and reduced pulmonary metastasis in HIF-1α$^{MEC-/-}$ 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$^{MEC-/-}$ mice had a significant increase in survival compared with HIF-1α$^{flox/flox}$ mice (Fig. 5*A*). Mean survival of HIF-1α$^{MEC-/-}$ mice was increased by 4.6 weeks compared with HIF-1α$^{flox/flox}$ mice (Fig. 5*A*). However, although HIF-1α$^{MEC-/-}$ mice had significantly prolonged survival, this did not correlate with a significant decrease

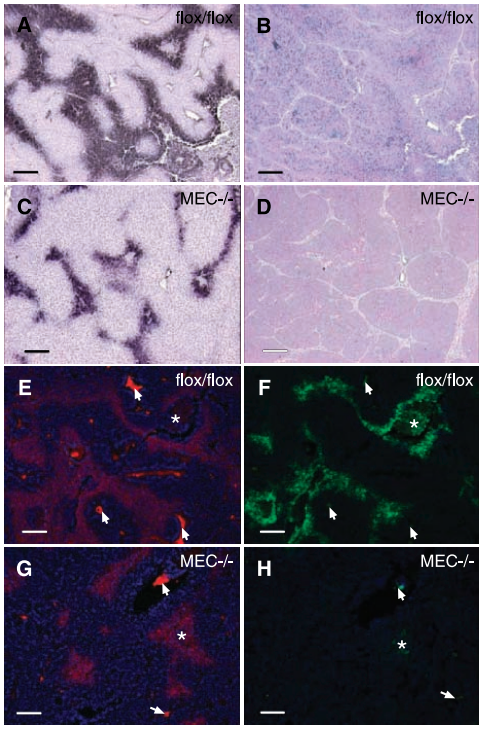


Figure 2. Effect of HIF-1α deletion on tumor hypoxia and expression of HIF-1α target genes in primary mammary tumors. Hypoxia probe and VEGF or Glut1 staining was done on representative serial cross-sections of tumors from end point virgin HIF-1α$^{flox/flox}$ (*A* and *B*, *E* and *F*) and HIF-1α$^{MEC-/-}$ (*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$^{MEC-/-}$ compared with HIF-1α$^{flox/flox}$ tumors. *White asterisks,* areas of necrosis. *White arrowheads,* autofluorescent RBCs. *Bar*, 100 μm.

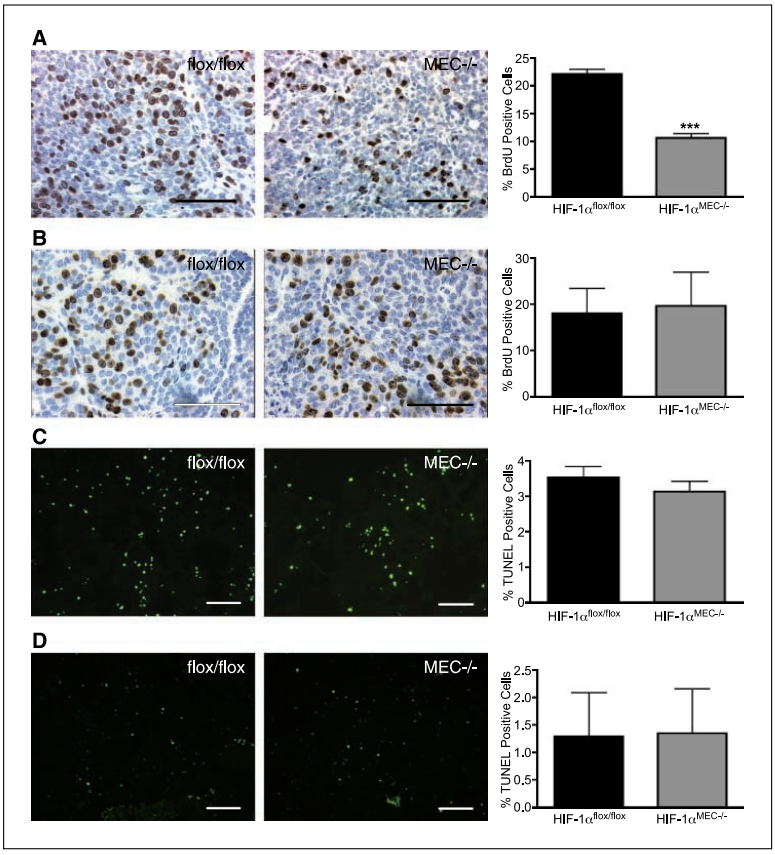


Figure 3. Histology of HIF-1α$^{flox/flox}$ (flox/flox) and HIF-1α$^{MEC-/-}$ (MEC−/−) primary mammary tumors. All staining was done on representative cross-sections of primary tumors from 10-wk-old virgin mice (*A* and *C*) and virgin mice at end point (*B* and *D*). *Bar*, 100 μm. *A* and *B,* proliferating tumor MECs with bromodeoxyuridine incorporation was visualized by immunostaining. *A,* at 10 wks of age, tumors from HIF-1α$^{MEC-/-}$ 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$^{flox/flox}$ 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$^{MEC-/-}$ tumors had increased to a similar level as HIF-1α$^{flox/flox}$ tumors (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$^{MEC-/-}$ and HIF-1α$^{flox/flox}$ mice.

in tumor burden compared with HIF-1α$^{flox/flox}$ mice at end point (Fig. 5*A*). Histology of tumor sections revealed that tumors from both HIF-1α$^{flox/flox}$ and HIF-1α$^{MEC-/-}$ mice had developed into invasive carcinomas (Fig. 5*B*).

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. 5*C*). The number of metastatic foci per lung was counted using a stereomicroscope. Although the end point tumor burden of HIF-1α$^{MEC-/-}$ mice was comparable to HIF-1α$^{flox/flox}$ mice, deletion of HIF-1α resulted in a significant decrease in pulmonary metastasis. Lungs from HIF-1α$^{flox/flox}$ mice had a mean metastatic foci count of 146.5 ± 25.92 compared with HIF-1α$^{MEC-/-}$ mice, which had a mean metastatic foci count of 70.75 ± 16.34 ($P = 0.02$, $n = 12$/genotype; Fig. 5*C*). 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. 5*D*).

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$^{+f/+f}$, 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$^{flox/flox}$ (wild-type) 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. 6*A*). HIF-1α$^{flox/flox}$ 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$^{flox/flox}$ tumor MEC during hypoxic culture (Fig. 6*A*). These results show that HIF-1α is expressed in murine tumor MEC lines and is required for hypoxic induction of gene expression *in vitro*.

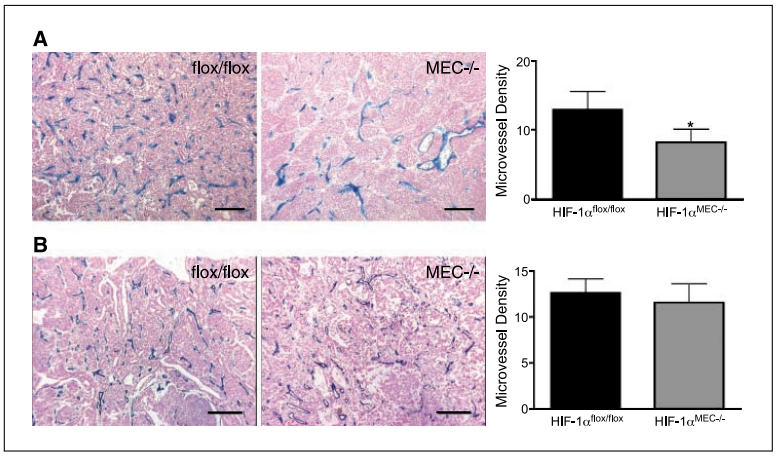


Figure 4. Blood vessel staining of primary tumors from HIF-1$\alpha^{flox/flox}$ (flox/flox) and HIF-1$\alpha^{MEC-/-}$ (MEC−/−) virgin mice. Endothelial cells were detected using CD31 antibody (*blue*). All of the staining was done on representative cross-sections of primary tumors from 14-wk-old virgin mice (*A*) and virgin mice at end point (*B*). *Bar*, 100 μm. Microvessel density was quantified using a Chaukley graticle eyepiece. *A,* CD31 staining and microvessel density in HIF-1$\alpha^{MEC-/-}$ tumors was significantly reduced compared with HIF-1$\alpha^{flox/flox}$ tumors at 14 wks (P = 0.02; flox/flox, n = 5; and MEC−/−, n = 4). *B,* no significant difference exists in CD31 staining or microvessel density between HIF-1$\alpha^{flox/flox}$ and HIF-1$\alpha^{MEC-/-}$ mice at end point (flox/flox, n = 10; and MEC−/−, n = 11).

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-1$\alpha^{-/-}$ tumor MEC grew at approximately the same rate as HIF-1$\alpha^{flox/flox}$ tumor MEC (Fig. 6*B*). However, during hypoxic culture, the growth of HIF-1$\alpha^{flox/flox}$ 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-1$\alpha^{-/-}$ tumor MEC was significantly reduced compared with HIF-1$\alpha^{flox/flox}$ tumor MEC (Fig. 6*B*). The growth rate of HIF-1$\alpha^{-/-}$ tumor MEC continued to diminish at each time point beyond 48 h, whereas HIF-1$\alpha^{flox/flox}$ tumor MEC was able to maintain exponential growth during hypoxic culture. These results support the *in vivo* 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-1$\alpha^{flox/flox}$ and HIF-1$\alpha^{-/-}$ tumor MEC cultured under normoxia. However, hypoxic culture significantly increased the random motility of both HIF-1$\alpha^{flox/flox}$ and HIF-1$\alpha^{-/-}$ tumor MEC (Fig. 6*C*). In contrast, directed chemotaxis migration, as well as random invasion and directed chemotaxis invasion of HIF-1$\alpha^{-/-}$ tumor MEC, was significantly reduced during hypoxic culture compared with HIF-1$\alpha^{flox/flox}$ tumor MEC (Fig. 6*C* 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-1$\alpha^{flox/flox}$ and HIF-1$\alpha^{MEC-/-}$ 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-1$\alpha^{flox/flox}$ glands contained a significantly higher percentage of proliferating cells compared with hyperplasia in HIF-1$\alpha^{MEC-/-}$ 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-1$\alpha^{flox/flox}$ tumor MEC during tumor progression. Glut1 expression in these lesions correlated with hypoxia staining and was significantly reduced in HIF-1$\alpha^{MEC-/-}$ glands. Additionally, Glut1 mRNA levels was significantly reduced in HIF-1$\alpha^{-/-}$ tumor MEC compared with HIF-1$\alpha^{flox/flox}$ 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).

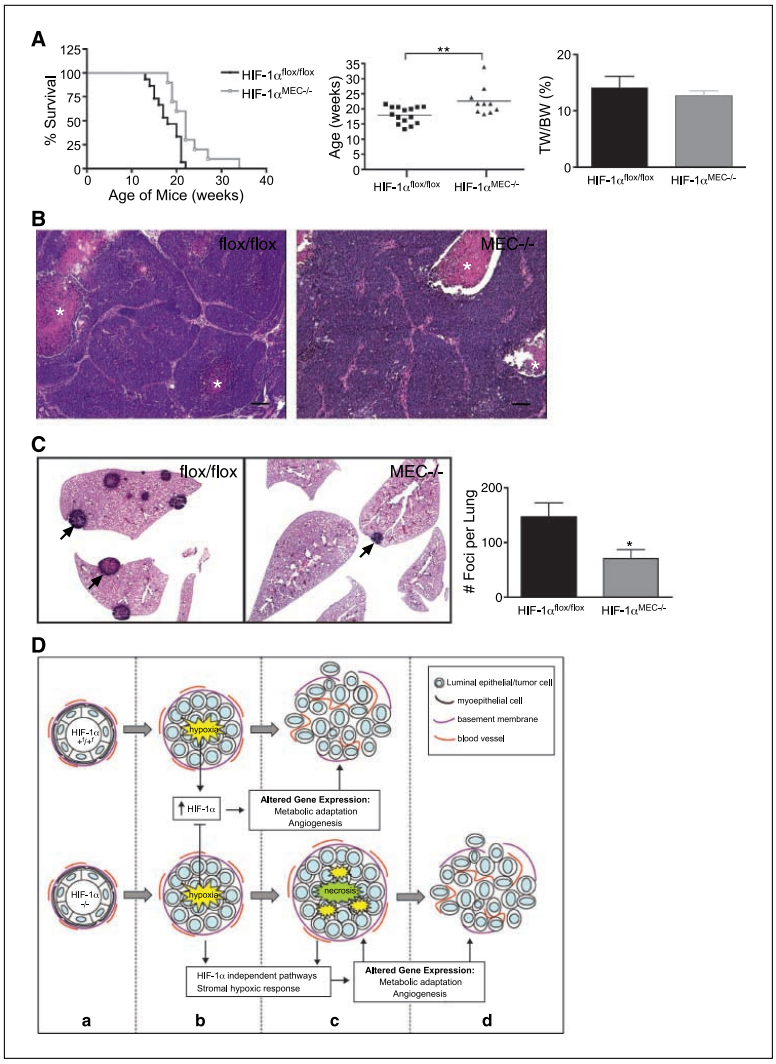


Figure 5. Effects of conditional knock-out of HIF-1α on survival and pulmonary metastasis in MMTV-PyMT mice. Mammary tumor dimensions were measured weekly with digital calipers (HIF-1$\alpha^{flox/flox}$, n = 15; and HIF-1$\alpha^{MEC-/-}$, n = 15). Mice were sacrificed when tumor size reached 1 × 1 cm (end point). *A,* HIF-1$\alpha^{MEC-/-}$ mice had significantly prolonged survival compared with HIF-1$\alpha^{flox/flox}$ mice (*left,* P = 0.007). HIF-1$\alpha^{MEC-/-}$ mice (*black triangles*) increased survival by an average of 4 wks compared with HIF-1$\alpha^{flox/flox}$ (*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-1$\alpha^{flox/flox}$ (flox/flox) and HIF-1$\alpha^{MEC-/-}$ (MEC−/−) mice. *White asterisks,* areas of necrosis. *Bar*, 100 μm. *C,* representative serial sections of lungs from HIF-1$\alpha^{flox/flox}$ and HIF-1$\alpha^{MEC-/-}$ mice stained with H&E to visualize metastatic foci (*black arrows*). Lungs from HIF-1$\alpha^{MEC-/-}$ mice (*gray column*) contained significantly fewer metastatic foci compared with lungs from HIF-1$\alpha^{flox/flox}$ (*black column*) mice (P = 0.02). *D,* summary of tumor progression in HIF-1$\alpha^{flox/flox}$ and HIF-1$\alpha^{MEC-/-}$ 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-1$\alpha^{flox/flox}$ and HIF-1$\alpha^{MEC-/-}$ 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-1$\alpha^{flox/flox}$ 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-1$\alpha^{MEC-/-}$ 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-1$\alpha^{MEC-/-}$ glands have also progressed to carcinomas and now resemble HIF-1$\alpha^{flox/flox}$ lesions observed at earlier time points.

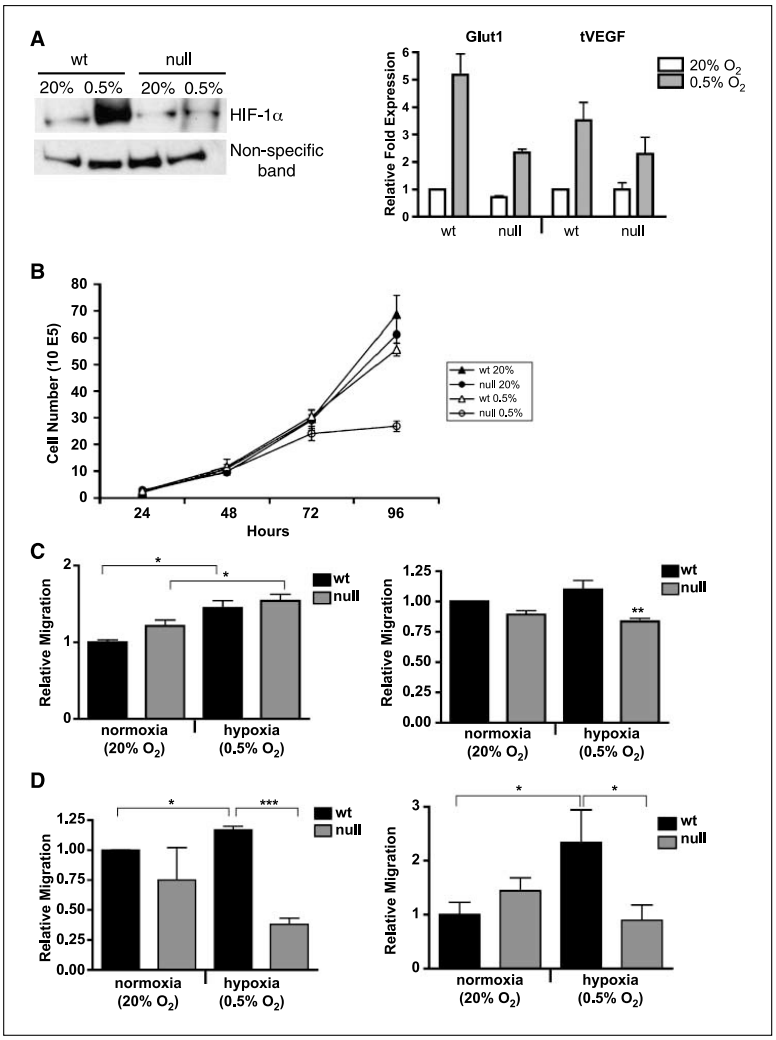


Figure 6. Effect of HIF-1α deletion on tumor MEC growth, migration, and invasion *in vitro*. HIF-1α$^{-/-}$ (*null*) tumor MEC was generated by infection of HIF-1α$^{flox/flox}$ (*wt*) tumor MEC with adenovirus-expressing Cre-recombinase. *A,* Western blot analysis (*left*) of nuclear protein extracts 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 96 h. In contrast to wt tumor MEC, null tumor MECs were not able to sustain exponential growth during hypoxic culture. *C,* random migration (*left*) and directed chemotaxis migration (*right*) of tumor MEC cultured under normoxia or hypoxia for 24 h. Hypoxia increased the migration of both HIF-1α$^{flox/flox}$ and HIF-1α$^{-/-}$ tumor MEC. However, chemotaxis-directed migration of HIF-1α$^{-/-}$ tumor MEC towards serum was significantly impaired compared with HIF-1α$^{flox/flox}$ 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$^{flox/flox}$ tumor MEC during hypoxic culture ($P < 0.0005$ and $P = 0.03$, respectively).

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$^{flox/flox}$ and HIF-1α$^{MEC-/-}$ 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$^{flox/flox}$ mice. The growth potential of HIF-1α$^{MEC-/-}$ tumors was not due to inefficient Cre-mediated deletion of HIF-1α *in vivo*; tumors examined from HIF-1α$^{MEC-/-}$ mice had on average 88.3% deletion of HIF-1α at end point by real-time PCR analysis of genomic DNA. Although there was interanimal

variability in deletion efficiency, this did not significantly alter the results seen. This suggests that whereas HIF-1α plays a key role in 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$^{MEC-/-}$ 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$^{flox/flox}$ 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 only 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 comparable in mammary tumors from HIF-1α$^{flox/flox}$ and HIF-1α$^{MEC-/-}$ mice at the end point, the increase in hypoxia staining in HIF-1α$^{flox/flox}$ tumors 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 lysyl 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.

Acknowledgments

Received 7/21/2006; revised 10/6/2006; accepted 11/14/2006.

Grant support: Department of Defense Breast Cancer Research Program grant W81XWH-04-1-0417 (D. Liao), NIH/National Cancer Institute grant CA082515.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Lesley G. Ellies and members of the Johnson Laboratory for helpful suggestions and assistance.

References

1. Dang CV, Semenza GL. Oncogenic alterations of metabolism. Trends Biochem Sci 1999;24:68–72.
2. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci U S A 1995;92:5510–4.
3. Semenza GL. Hydroxylation of HIF-1: oxygen sensing at the molecular level. Physiology (Bethesda) 2004;19:176–82.
4. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3:721–32.
5. Zhong H, De Marzo AM, Laughner E, et al. Overexpression of hypoxia-inducible factor 1α in common human cancers and their metastases. Cancer Res 1999;59:5830–5.
6. Bos R, van der Groep P, Greijer AE, et al. Levels of hypoxia-inducible factor-1α independently predict prognosis in patients with lymph node negative breast carcinoma. Cancer 2003;97:1573–81.
7. Vleugel MM, Greijer AE, Shvarts A, et al. Differential prognostic impact of hypoxia induced and diffuse HIF-1α expression in invasive breast cancer. J Clin Pathol 2005;58:172–7.
8. Gruber G, Greiner RH, Hlushchuk R, et al. Hypoxia-inducible factor 1α in high-risk breast cancer: an independent prognostic parameter? Breast Cancer Res 2004;6:R191–8.
9. Dales JP, Garcia S, Meunier-Carpentier S, et al. Overexpression of hypoxia-inducible factor HIF-1α predicts early relapse in breast cancer: retrospective study in a series of 745 patients. Int J Cancer 2005;116:734–9.
10. Kronblad A, Jirstrom K, Ryden L, Nordenskjold B, Landberg G. Hypoxia inducible factor-1α 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;118:2609–16.
11. Bos R, Zhong H, Hanrahan CF, et al. Levels of hypoxia-inducible factor-1α during breast carcinogenesis. J Natl Cancer Inst 2001;93:309–14.
12. 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;163:2113–26.
13. Seagroves TN, Hadsell D, McManaman J, et al. HIF1α is a critical regulator of secretory differentiation and activation, but not vascular expansion, in the mouse mammary gland. Development 2003;130:1713–24.
14. Wagner KU, McAllister K, Ward T, et al. Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. Transgenic Res 2001;10:545–53.
15. Cramer T, Yamanishi Y, Clausen BE, et al. HIF-1α is essential for myeloid cell-mediated inflammation. Cell 2003;112:645–57.
16. Blouw B, Song H, Tihan T, et al. The hypoxic response of tumors is dependent on their microenvironment. Cancer Cell 2003;4:133–46.
17. Grunstein J, Roberts WG, Mathieu-Costello O, Hanahan D, Johnson RS. Tumor-derived expression of vascular endothelial growth factor is a critical factor in tumor expansion and vascular function. Cancer Res 1999;59:1592–8.
18. Medina D, Kittrell F. Establishment of mouse mammary cell lines. In: Ip MM, Asch BB, editors. Methods in mammary gland biology and breast cancer research. New York: Plenum Publishers; 2000. p. 137–45.
19. Ryan HE, Lo J, Johnson RS. HIF-1α is required for solid tumor formation and embryonic vascularization. EMBO J 1998;17:3005–15.
20. Semenza GL. HIF-1 and tumor progression: pathophysiology and therapeutics. Trends Mol Med 2002;8:S62–7.
21. Carmeliet P. VEGF as a key mediator of angiogenesis in cancer. Oncology 2005;69 Suppl 3:4–10.
22. Seagroves TN, Ryan HE, Lu H, et al. Transcription factor HIF-1 is a necessary mediator of the Pasteur effect in mammalian cells. Mol Cell Biol 2001;21:3436–44.
23. Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab 2006;3:187–97.
24. Chen C, Pore N, Behrooz A, Ismail-Beigi F, Maity A. Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. J Biol Chem 2001;276:9519–25.
25. Goda N, Ryan HE, Khadivi B, et al. Hypoxia-inducible factor 1α is essential for cell cycle arrest during hypoxia. Mol Cell Biol 2003;23:359–69.
26. Brown JM, Giaccia AJ. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. Cancer Res 1998;58:1408–16.
27. North S, Moenner M, Bikfalvi A. Recent developments in the regulation of the angiogenic switch by cellular stress factors in tumors. Cancer Lett 2005;218:1–14.
28. Macheda ML, Rogers S, Best JD. Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. J Cell Physiol 2005;202:654–62.

29. Sung Soo Kang YKC, Min Hee Hur, et al. Clinical significance of glucose transporter 1 (GLUT1) expression in human breast carcinoma. Jpn J Cancer Res 2002; 93:1123–8.
30. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 1996;86:353–64.
31. 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;80:617–23.
32. Munoz-Najar UM, Neurath KM, Vumbaca F, Claffey KP. Hypoxia stimulates breast carcinoma cell invasion through MT1-MMP and MMP-2 activation. Oncogene 2005;25:2379–92.
33. Krishnamachary B, Berg-Dixon S, Kelly B, et al. Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. Cancer Res 2003;63:1138–43.
34. Lester RD, Jo M, Campana WM, Gonias SL. Erythropoietin promotes MCF-7 breast cancer cell migration by an ERK/mitogen-activated protein kinase-dependent pathway and is primarily responsible for the increase in migration observed in hypoxia. J Biol Chem 2005;280:39273–7.
35. Hashizume H, Baluk P, Morikawa S, et al. Openings between defective endothelial cells explain tumor vessel leakiness. Am J Pathol 2000;156:1363–80.
36. Erler JT, Bennewith KL, Nicolau M, et al. Lysyl oxidase is essential for hypoxia-induced metastasis. Nature 2006;440:1222–6.
37. Woelfle U, Cloos J, Sauter G, et al. Molecular signature associated with bone marrow micrometastasis in human breast cancer. Cancer Res 2003;63:5679–84.

Chapter 2, in full, is a reprint of the material as it appears in Cancer Research (Liao D, Corle C, Seagroves TN, Johnson RS. Hypoxia inducible factor-1alpha is a key regulator of metatasis in a transgenic model of cancer initiation and progression. Cancer Res 2007;67(2): 563-72). The dissertation author was the primary investigator and author of this paper, and the co-authors listed provided technical support and/or supervision. Permission to include this reprint was granted by the publisher and all co-authors.

CHAPTER III

HIF-1α Regulates E-cadherin Localization Through a VEGF/VEGFR1 Autocrine Signaling Pathway

Summary

E-cadherin function is important for maintaining epithelial cell polarity and cell-cell adhesions. Reduction of E-cadherin expression is associated with epithelial to mesenchymal transitions in human cancers and is correlated with increased invasiveness of tumor cells. The transcription factor HIF-1α is expressed by many types of human cancer cells, including those of the breast. We have previously shown that HIF-1α mediates tumor progression and metastasis in a mouse model of breast cancer *in vivo,* and that HIF-1α expression in mouse tumor MECs mediates hypoxia induced tumor cell invasion *in vitro*. HIF-1α has been shown to regulate the transcriptional repression of E-cadherin in CC-RCC cell lines, resulting in increased tumor cell invasiveness. Therefore, we sought to investigate the control of E-cadherin expression in mouse tumor MECs. We found that E-cadherin is not transcriptionally regulated by HIF-1α in mouse tumor MECs. Instead, we show that hypoxia induces the internalization of E-cadherin protein in mouse tumor MECs. Additionally, we show that the mechanism of E-cadherin internalization during hypoxia involves a HIF-1α mediated VEGF/VEGFR1 autocrine signaling pathway in mouse tumor MECs.

Introduction

Hypoxia, or low oxygen levels, is a hallmark of solid tumor formation. As cancer cells proliferate, diffusion distances from local capillaries increase, creating an oxygen gradient within the expanding cell mass. When the proliferation of tumor cells outpaces the supply of oxygen from local capillaries, tissue hypoxia results.

Mammalian cells respond to hypoxia by increasing the expression of several hypoxia-induced genes. This phenomenon, termed the hypoxic response, mediates cellular adaptation to hypoxic stress, and is primarily regulated by the transcription factor Hypoxia Inducible Factor (HIF)-1 (1). HIF-1 functions as a heterodimer comprised of α and β subunits (2). HIF-1β is constitutively expressed, while HIF-1α protein stability is induced by hypoxia. Under normoxic conditions, oxygen mediates binding of the VHL tumor suppressor protein, an E3 ubiquitin ligase, to HIF-1α, and leads to rapid proteasomal degradation of HIF-1α (3). Under hypoxic conditions, HIF-1α escapes degradation, and together the α/β-heterodimeric HIF complex activates transcription by binding to hypoxic responsive elements (HREs) within the promoter regions of target genes (1). HIF-1α activity is important for many normal physiological processes, however overexpression of HIF-1α protein is also associated with human pathologies including cancer (4). HIF-1α expression in solid tumors is associated with tumor angiogenesis and proliferation of tumor cells (4). Additionally, the expression of genes involved in extracellular matrix remodeling and cellular adhesion are also altered by hypoxia (5, 6).

E-cadherin belongs to the family of classical cadherins that mediate calcium dependent cell-cell adhesion (7). These single-span transmembrane-domain glycoproteins play an important role in maintaining cellular polarity, and loss of E-cadherin expression has been associated with malignant progression of many human cancers of epithelial origin (7, 8). Mutations in the E-cadherin gene, resulting in a non-functional protein, have been demonstrated in infiltrative lobular breast and gynecologic cancer (9, 10). The intracellular domain of E-cadherin interacts with catenin proteins to form cytoplasmic cell-adhesion complexes (CCCs) (7). CCCs have an essential role in maintaining strong cell-cell adhesions (7). Additionally, the sequestering of β-catenin by E-cadherin is also important for inhibiting β-catenin mediated activation of the WNT signaling pathway, which modulates the expression of many genes implicated in cell proliferation and tumor progression (7, 11, 12). In mouse mammary epithelial cells, inhibition of cell proliferation by E-cadherin expression was shown to be dependent on ability to bind β-catenin (13). Additionally, treatment of a highly invasive human breast cancer cell line with small interfering RNA directed against β-catenin suppressed tumor cell invasion *in vitro* (14).

Regulation of E-cadherin expression is multifaceted and includes transcriptional and post-transcriptional mechanisms. Post-transcriptional regulation of E-cadherin expression by receptor tyrosine kinases, which are commonly activated in cancer cells, occurs through targeted degradation of E-cadherin protein (7). Additionally, E-cadherin mediated cell-cell adhesion can also be disrupted by proteolytic degradation of E-cadherin protein by matrix metalloproteinases (7). At the

transcriptional level, members of the Snail family of zinc finger proteins have been shown repress E-cadherin transcription by binding to E2 boxes within the promoter region of E-cadherin (15). Hypoxia has been shown to attenuate expression of E-cadherin through up-regulation of Snail in ovarian cancer cell lines (16). Recent studies using human renal clear cell carcinoma cells, with inactivation of the von Hippel Lindau (VHL) tumor suppressor, have shown that constitutive activation of HIF-1α leads to increased transcription of Snail, which results in transcriptional repression of E-cadherin (17, 18). These results suggest that HIF-1α may mediate tumor cell invasiveness through control of E-cadherin expression.

Therefore, we investigated the role of HIF-1α in regulation of E-cadherin expression in tumor mammary epithelial cell (MEC) lines generated from a transgenic mouse model of breast cancer in which HIF-1α can be conditionally deleted. Characterization of this mouse model is detailed in Chapter 2. Importantly, mice with HIF-1α conditionally deleted in the tumor MECs had significantly reduced pulmonary metastasis (19). Additionally, we showed that HIF-1α null tumor MECs cultured under hypoxic conditions exhibited reduced invasiveness *in vitro* when compared to wild type tumor MECs (19). In this chapter we show hypoxia alters the cellular localization of E-cadherin in mouse tumor MECs. Additionally, we show that changes in E-cadherin localization are a result of HIF-1α mediated transcriptional activation of vascular endothelial growth factor receptor 1 (VEGFR1).

Results

E-cadherin is not transcriptionally regulated by HIF-1α in primary mouse tumor MEC.

Indirect transcriptional repression of E-cadherin expression by HIF-1α has been demonstrated in clear cell renal cancer cells (CC-RCC) and ovarian carcinoma cells, and shown to be mediated by HIF-1α transcriptional activation of the Snail transcriptional repressor (16-18, 20). To determine if E-cadherin is transcriptionally regulated by a similar fashion in mouse tumor MECs, tumor MEC cell lines were established from primary tumors isolated from virgin female mice with exon 2 of HIF-1α flanked by loxP sites (HIF-1$\alpha^{flox/flox}$), and expression of the polyoma middle T oncoprotein driven by the mouse mammary tumor virus (MMTV)-LTR promoter. HIF-1α wild type and null tumor MEC lines were generated by infection with adenovirus expressing β-galactosidase or Cre-recombinase, respectively. Deletion of HIF-1α was confirmed by real-time PCR on genomic DNA isolated from adenovirus-infected cells. For the following experiments, wild type and null tumor MECs were cultured at normoxia (20% O_2) or hypoxia (1.0% O_2).

To determine if E-cadherin transcription is regulated in a HIF-1α-dependent manner *in vitro*, cDNA was generated from wild type and null tumor MECs cultured for 6 hours under normoxia or hypoxia and analyzed by real-time PCR. E-cadherin and Snail mRNA expression levels were not significantly altered by culture in hypoxia in wild type and null tumor MECs (Fig. 3.1A). E-cadherin mRNA levels were also not significantly altered after 12 or 24 hours of hypoxic culture (data not shown).

Additionally, E-cadherin protein levels were assayed by Western blot of cell extracts obtained from wild type and null tumor MECs cultured at normoxia or hypoxia for 24 hours. E-cadherin protein expression levels were not significantly altered by hypoxic culture in wild type and null cells (Fig. 3.1A). In contrast, mRNA expression levels of Glut-1, a known HIF-1α specific target gene, was increased under hypoxic culture in wild type, but not null tumor MEC, demonstrating that HIF-1α is active in wild type but not in null tumor MECs (Fig. 3.1A). These results suggest that, contrary to CCRCC lines, E-cadherin is not transcriptionally regulated by HIF-1α in mouse tumor MECs.

However, previous demonstration of HIF-1α mediated transcriptional regulation of E-cadherin was shown in CCRCC lines with constitutive expression of HIF-1α due to loss of function mutations in the VHL gene. It is possible that the level of HIF-1α activation achieved during hypoxia is not sufficient to induce changes in E-cadherin gene expression when compared to the level of HIF-1α activation achieved due to loss of pVHL function. This is relevant because it has been shown that the degree of HIF-1α expression can result in the transcription of unique sets of genes (21). Therefore, we assayed for the expression of E-cadherin in tumor MECs in which the VHL gene is conditionally deleted. Tumor MECs were isolated from the same mouse tumor model, except with only exon 3 of the VHL gene flanked by loxP sites ($VHL^{flox/flox}$). VHL wild type and null tumor MECs were generated by infection with adenovirus as described above. Western blot analysis of nuclear extracts from VHL wild type and null tumor MECs showed that deletion of VHL results in increased HIF-

1α protein expression in VHL null tumor MECs compared to wild type MECs under normoxic culture conditions (Fig. 3.1B). Additionally, the increase in HIF-1α protein in VHL null tumor MECs also correlated with an increase in Glut1 mRNA expression by real-time PCR analysis (Fig. 3.1B). However, the expression of E-cadherin was not significantly different at either the mRNA or protein level in VHL null tumor MECs compared to wild type (Fig. 3.1B). These results confirm that regulation of E-cadherin transcription and protein levels is not mediated by HIF-1α in tumor MECs.

Hypoxia reduces cell surface expression of E-cadherin and is dependent on HIF-1α.

In addition to regulation by transcription, regulation of E-cadherin activity has also been shown to occur by endocytosis of E-cadherin protein in epithelial MDCK cells and in human keratinocytes and breast cancer cells (22-24). To determine if HIF-1α expression affects E-cadherin localization in mouse tumor MECs, HIF-1α wild type and null tumor MECs were plated onto gelatin coated glass chamber slides and allowed to reach confluency. Chamber slides were then cultured under normoxia or hypoxia for 24 hours. To detect E-cadherin, cells were fixed with paraformaldehyde, permeabilized, treated with an antibody against E-cadherin, and visualized using a fluorescent-conjugated secondary antibody. Cells were then imaged by deconvolution microscopy.

Both HIF-1α wild type and null tumor MECs cultured under normoxia showed membrane specific staining of E-cadherin in areas of cell-cell contacts (Fig. 3.2). In

contrast, membrane specific staining of E-cadherin in wild type cells cultured under hypoxia was significantly reduced. Instead, E-cadherin staining in hypoxic wild type cells was punctate and occurred in the cytoplasmic regions of the cells (Fig. 3.2), suggesting an increased internalization triggered by hypoxia. However, unlike wild type tumor MECs, HIF-1α null tumor MECs cultured under hypoxia showed membrane specific staining of E-cadherin similar to that observed during normoxic culture (Fig. 3.2). Taken together, these results implicate that HIF-1α signaling is involved in the hypoxic internalization of surface E-cadherin expression.

HIF-1α mediates hypoxia induced E-cadherin internalization.

To determine if the punctate cytoplasmic staining of E-cadherin in wild type tumor MECs cultured under hypoxia represents E-cadherin internalized from the cell surface, cells plated on gelatin coated glass chamber slides were treated with an antibody against the extracellular portion of E-cadherin prior to normoxic or hypoxic culture. Unbound antibody was removed by rinsing with media and cells were cultured at normoxia or hypoxia for 24 hours. Prior to fixing, antibody bound to E-cadherin at the cell surface was removed by an acid wash so that only internalized antibody would remain. The E-cadherin antibody was then visualized using a fluorescence-conjugated secondary antibody and imaged by deconvolution microscopy.

Both HIF-1α wild type and null tumor MEC cultured at normoxia had reduced expression of E-cadherin after acid washing, when compared to PBS control (Fig.

3.3). HIF-1α null tumor MECs cultured at hypoxia also had reduced expression of E-cadherin after acid washing, when compared to PBS control (Fig. 3.3). However, HIF-1α wild type tumor MECs retained E-cadherin expression after acid washing, and E-cadherin expression in these cells was present in the cytoplasmic regions (Fig. 3.3). These results show that the presence of E-cadherin in the cytoplasm of wild type tumor MECs after hypoxic culture represents protein that has been internalized from the cell surface in a HIF-1α dependent fashion.

HIF-1α regulates VEGFR1 expression in mouse tumor MECs.

We have shown previously that hypoxic induction of VEGF expression in mouse tumor MECs is dependent on HIF-1α (19). VEGF ligands bind to receptor tyrosine kinases known as VEGF receptors (VEGFRs), and have critical roles in endothelial cell proliferation and migration (25). Though VEGFR expression was though to be limited to endothelial cells, VEGFR expression has now been demonstrated in multiple human tumor cell lines (26-30). VEGFR expression by tumor cells has been shown to contribute to growth and survival in human breast cancer cells, and to migration and invasion in human pancreatic carcinoma cells *in vitro* (27, 28).

To determine if VEGFR1 is expressed in mouse tumor MECs, and transcriptionally regulated by HIF-1α, VEGFR1 mRNA levels in HIF-1α wild type and null tumor MECs cultured under normoxia or hypoxia was assessed by real-time PCR. Hypoxic culture of HIF-1α wild type tumor MECs resulted in a 2-fold increase

in VEGF mRNA expression level relative to normoxic culture (Fig. 3.4A). In contrast, hypoxic induction of VEGFR1 mRNA expression was absent in HIF-1α null tumor MECs (Fig. 3.4A). Additionally, western blotting of cell extracts from tumor MECs cultured at normoxia or hypoxia for 24 hours showed a hypoxia-induced increase in VEGFR1 protein levels in HIF-1α wild type tumor MECs (Fig. 3.4A). Hypoxic induction of VEGFR1 protein levels was absent in HIF-1α null tumor MECs (Fig. 3.4A). Additionally, *in vitro* VEGFR1 expression was assayed in HIF-1α wild type and null tumor MECs plated on gelatin coated glass slides cultured at normoxia or hypoxia for 24 hours. Immunoflourescence staining with an antibody against VEGFR1 revealed an increase in VEGFR1 expression in HIF-1α wild type cells cultured under hypoxia (Fig. 3.4B). In contrast, HIF-1α null tumor MECs cultured under hypoxia did not show an increase in VEGFR1 expression (Fig. 3.4B). These results demonstrate that VEGFR1 is expressed by mouse tumor MECs, and that hypoxic induction of VEGFR1 expression is transcriptionally regulated by HIF-1α.

HIF-1α dependent induction of VEGF/VEGFR1 expression drives an autocrine signaling loop that mediates E-cadherin internalization during hypoxia.

Activation of VEGFR by VEGF signaling has been shown to regulate VE-cadherin endocytosis in human vascular endothelial cells (HUVECs) (31). We have shown that deletion of HIF-1α in mouse tumor MECs reduces endocytosis of E-cadherin during hypoxic stress. Additionally, we have shown that hypoxic induction of both VEGF and VEGFR1 expression is regulated by HIF-1α, raising the possibility

that, in mouse tumor MECs, E-cadherin could also be regulated in a similar fashion as VE-cadherin in HUVECs.

To determine if VEGF/VEGFR1 autocrine signaling mediates hypoxia induced E-cadherin internalization, HIF-1α wild type and null tumor MECs were plated on gelatin-coated glass slides and a VEGFR1 blocking antibody was added to the media 1 hour prior to hypoxic culture. After 1 hour, cells, with media still containing VEGFR1 blocking antibody, were subject to 24 hours of hypoxic (1.0% O_2) culture. After hypoxic culture, the cells were fixed, treated with an anti-E-cadherin antibody, and imaged by immunoflourescence deconvolution microscopy. HIF-1α wild type tumor MECs without VEGFR1 blocking antibody treatment had punctate cytoplamic staining of E-cadherin after hypoxic culture, while HIF-1α null cells displayed unaltered membrane specific staining of E-cadherin (Fig. 3.5, left panel). However, when HIF-1α wild type tumor MECs was treated with VEGFR1 blocking antibody, E-cadherin staining was restored to the cell surface in a dose dependent manner (Fig. 3.5, middle and right panels). These results demonstrate that a HIF-1α dependent VEGF/VEGFR1 autocrine signaling pathway in mouse tumor MECs mediates the internalization of E-cadherin during hypoxic stress.

Discussion

E-cadherin function is important for maintaining cellular adhesion as well as inhibiting activation of WNT pathway signaling by β-catenin (7, 8). Alteration in E-cadherin expression has been shown to increase the invasiveness of human cancer cell lines (32-34). Additionally, loss of E-cadherin expression is associated with epithelial to mesenchymal transition during progression to malignancy in tumors of epithelial origin (35). In the present study, we propose a novel mechanism of E-cadherin regulation, whereby hypoxic activation of HIF-1α mediates E-cadherin internalization through activation of VEGFR1 autocrine signaling in mouse tumor MECs.

HIF-1α has been shown to mediate regulation of E-cadherin at the transcriptional level (17, 18, 20). Human RCC cells with mutations in the VHL gene have constitutive expression of HIF-1α and a concomitant decrease in E-cadherin protein expression, corresponding with a decrease in E-cadherin mRNA levels *in vitro* (17, 36). The decreases in E-cadherin mRNA levels was shown to occur indirectly through HIF-1α mediated induction of the E-cadherin specific transcriptional repressor Snail (18). Knockdown of HIF-1α by RNAi was shown to restore E-cadherin expression (20).

In contrast to human RCC cells, we found that E-cadherin expression is not regulated by HIF-1α mediated transcriptional repression in mouse tumor MECs. Induction of HIF-1α expression in wild type tumor MECs by hypoxia did not result in decreased E-cadherin protein levels when compared to normoxia. Additionally, deletion of HIF-1α by Cre-mediated recombination did not alter the expression E-

cadherin protein during hypoxia. Further confirmation was provided by real-time PCR analysis of cDNA generated from wild type and HIF-1α null mouse tumor MECs. Tumor MECs cultured at normoxia or hypoxia did not result in significant alterations of E-cadherin mRNA expression levels in either wild type or HIF-1α null tumor MECs. These results demonstrate that HIF-1α does not mediate transcriptional repression of E-cadherin in mouse tumor MECs.

However, regulation of E-cadherin activity has been shown to occur by mechanisms other than transcriptional repression. Multiple studies have demonstrated that E-cadherin is also regulated by cellular internalization (24, 37, 38). Cell surface expression of E-cadherin is important for formation of tight junctions and for maintaining epithelial cell polarity (39, 40). Cellular internalization of E-cadherin abolishes its functioning capacity and is associated with epithelial to mesenchymal transitions that typify malignant progression in cancers of epithelial origin (40). E-cadherin was shown to be actively internalized in confluent monolayers of epithelial MDCK cells upon disruption of the adhesive binding activity of the E-cadherin ectodomain by chelation of extracellular Ca^{2+} (38). Additionally, internalized E-cadherin was shown to be recycled back to the cell surface once extracellular Ca^{2+} was restored (38). Similar regulation of E-cadherin localization has also been demonstrated in human breast cancer cells (24).

Our data suggests that in mouse tumor MECs, HIF-1α activity triggers the internalization of E-cadherin during hypoxic stress. Whereas E-cadherin was expressed on the cell surface of wild type and HIF-1α null tumor MECs in areas of

cell-cell contact during normoxic culture, hypoxic culture resulted in a change from membrane expression to punctate cytoplasmic expression of E-cadherin in wild type tumor MECs. Additionally, E-cadherin internalization assays demonstrated that the cytoplasmic expression of E-cadherin represented protein that had been internalized from the cell surface. In contrast, tumor MECs lacking active HIF-1α retained cell surface expression of E-cadherin during hypoxic culture, demonstrating that HIF-1α plays a critical role in hypoxia-induced E-cadherin internalization.

Recent studies have revealed that activation of tyrosine kinases also mediate E-cadherin internalization (41). In particular, VEGF activation of VEGFR was shown to regulate endocytosis of VE-cadherin, an endothelial cell specific cadherin, in HUVECs (31). We have shown previously that HIF-1α activity is required for the hypoxic induction of VEGF expression in mouse tumor MEC both *in vivo* and *in vitro* (19). We now show that VEGFR1 is expressed by mouse tumor MECs *in vitro*. Additionally, hypoxia induced VEGFR1 expression is dependent on HIF-1α. Inhibition of VEGFR1 signaling during hypoxia by VEGFR1 blocking antibodies attenuated the internalization of E-cadherin and restored cell surface expression of E-cadherin.

Overexpression of HIF-1α and VEGFR, as well as reduced expression of E-cadherin in primary tumors, have all been associated with poor prognosis in human breast cancer patients (42). Our studies suggest that hypoxia induced HIF-1α signaling may play a role in promoting cancer cell invasiveness by inhibiting the normal functions of E-cadherin activity through autocrine VEGF/VEGFR signaling in

mammary epithelial tumor cells. This result is in contrast to previous studies showing HIF-1α mediated transcriptional regulation of E-cadherin expression in CC-RCCs, demonstrating that the mechanism of E-cadherin regulation may be cell-type specific. Never the less, these results present the possibility of targeting HIF-1α mediated regulation of E-cadherin expression for therapeutics aimed at inhibiting tumor cell invasiveness.

Material and Methods

Animals.

All animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International-approved facility. All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. HIF-1$\alpha^{flox/flox}$/MMTV-PyMT mice with exon 2 of HIF-1α flanked by lox P site and expressing the polyoma middle T (PyMT) oncoprotein under the promoter of the mouse mammary tumor virus (MMTV)-long terminal repeat (LTR) were generated as previously described (19). The VHL$^{flox/flox}$/MMTV-PyMT transgenic mouse was produced by crossing mice (C57BL/6) with exon 3 of the VHL gene flanked by loxP sites (43) with mice expressing MMTV-PyMT (C57BL/6).

Cell Culture.

Primary mammary epithelial tumor cell cultures were established from HIF-1$\alpha^{flox/flox}$/MMTV-PyMT and VHL$^{flox/flox}$/MMTV-PyMT tumor bearing mice as previously described (19). Briefly, tumors were minced manually and digested with collagenase for 2 hours at 30°C. Cells were then washed and plated in growth medium: DMEM/F12 medium (Mediatech, Inc., Herndon, VA) supplemented with 2% FCS and 100 units of penincillin/10 units of streptomycin (Invitrogen, Grand Island, NY). To generate HIF-1α null and VHL null tumor MEC lines, HIF-1$\alpha^{flox/flox}$ and VHL$^{flox/flox}$ cells were infected with adenovirus-β-galactosidase or adenovirus-Cre to generate HIF-1$\alpha^{flox/flox}$ (HIF-1α wt) or VHL$^{flox/flox}$ (VHL wt), and HIF-1$\alpha^{-/-}$ (HIF-1α

null) or $VHL^{-/-}$ (VHL null) tumor MECs, respectively, as previously described (44). Normoxic and hypoxic culture of cells was performed at 20.0% O_2 and 1.0% O_2, respectively.

Real-time PCR.

Total RNA was isolated from tumor MECs and used to generate cDNA as previously described (19). E-cadherin, Snail, Glut1, and VEGFR1 gene expression was quantified by real-time PCR and normalized to expression level of β-actin, as previously described (44).

Western Blotting.

Cells were cultured at normoxia or hypoxia for 24 hours and cytoplasmic and nuclear extracts were isolated as previously described (45). Antibody against VEGFR1 (Santa Cruz Biotechnology, Santa Cruz, CA) protein was used at 1:500 dilution. Antibody against E-cadherin (BD Biosciences, San Jose, CA) was used at 1:2500 dilution. Antibody again HIF-1α (Novus Biologicals, Littleton, CO) was used at 1:1000 dilution. Antibody against β-actin (Sigma, St. Louis, MO) was used at 1:5000 dilution.

Immunofluorescence.

Tumor MEC cultured on gelatin-coated glass chamber slides were fixed in 4% paraformaldehyde for 15 minutes at room temperature and permeabilized with 0.5% Triton X-100 for 5 minutes at room temperature. Antibody against VEGR1 (Santa

Cruz Biotechnology, Santa Cruz, CA) was used at 1:50 dilution. Antibody against E-cadherin (BD Biosciences, San Jose, CA) was used at 1:500 dilution. Antibody against the extracellular domain uvomorulin/E-cadherin (Sigma, Saint Louis, MO) was used at 1:250 dilution. Primary antibodies were detected using Alexa-Flour-488 conjugated secondary antibodies (BD Biosciences, San Diego, CA) used at 1:300 dilution. Cell nuclei were stained with DAPI (Sigma, St. Louis, MO) at 1:100 dilution. Slides were mounted with Vectashield Mounting Medium for Fluorescence (Vector Labs, Burlingame, CA) and sealed with nail polish. Deconvolution microscopy was performed using an inverted fluorescence microscope (model TE-300, Nikon) and DeltaVision Spectera imaging software.

E-cadherin internalization assay.

The protocol used for E-cadherin internalization assays was adapted from Xiao et al. (46). MECs were plated onto gelatin coated glass chamber slides and allowed to reach sub-confluency. Media was aspirated and replaced with fresh growth media containing a 1:250 dilution of monoclonal anti-Uvomorulin/E-cadherin antibody (Sigma, Saint Louis, MO), directed against the extracellular domain of E-cadherin. Cells were incubated at 4°C for 1 hour. Unbound antibody was removed by washing twice with ice-cold growth media. Cells were then given fresh growth media and plated at normoxia or hypoxia for 24 hours. To remove antibody bound to the cell surface while retaining internalized antibody, cells were washed with an acid wash at pH 2.7 (PBS, 25mM glycine, 3% BSA) for 20 minutes on ice at 4°C with gentle

agitation. Acid wash was aspirated and cells were rinsed once with ice cold PBS, fixed with 4% paraformaldehyde, and processed for immunofluorescence as described above.

VEGFR1 blocking assay.

HIF-1α wild type and null tumor MECs were plated onto gelatin-coated glass slides and allowed to reach sub-confluency. Prior to normoxic or hypoxic culture, fresh media containing 0, 5, or 8 μg/mL of VEGFR1 blocking antibody (R&D Systems, Minneapolis, MN) was added to the cells and incubated for 1 hour at 37°C under normoxic conditions. After VEGFR1 incubation, cells were then kept at normoxia or transferred to hypoxic culture. 24-hours after normoxic or hypoxic culture, cells were fixed on ice with ice-cold 4% paraformaldehyde for 15 minutes and immnostaining for E-cadherin was performed as described above.

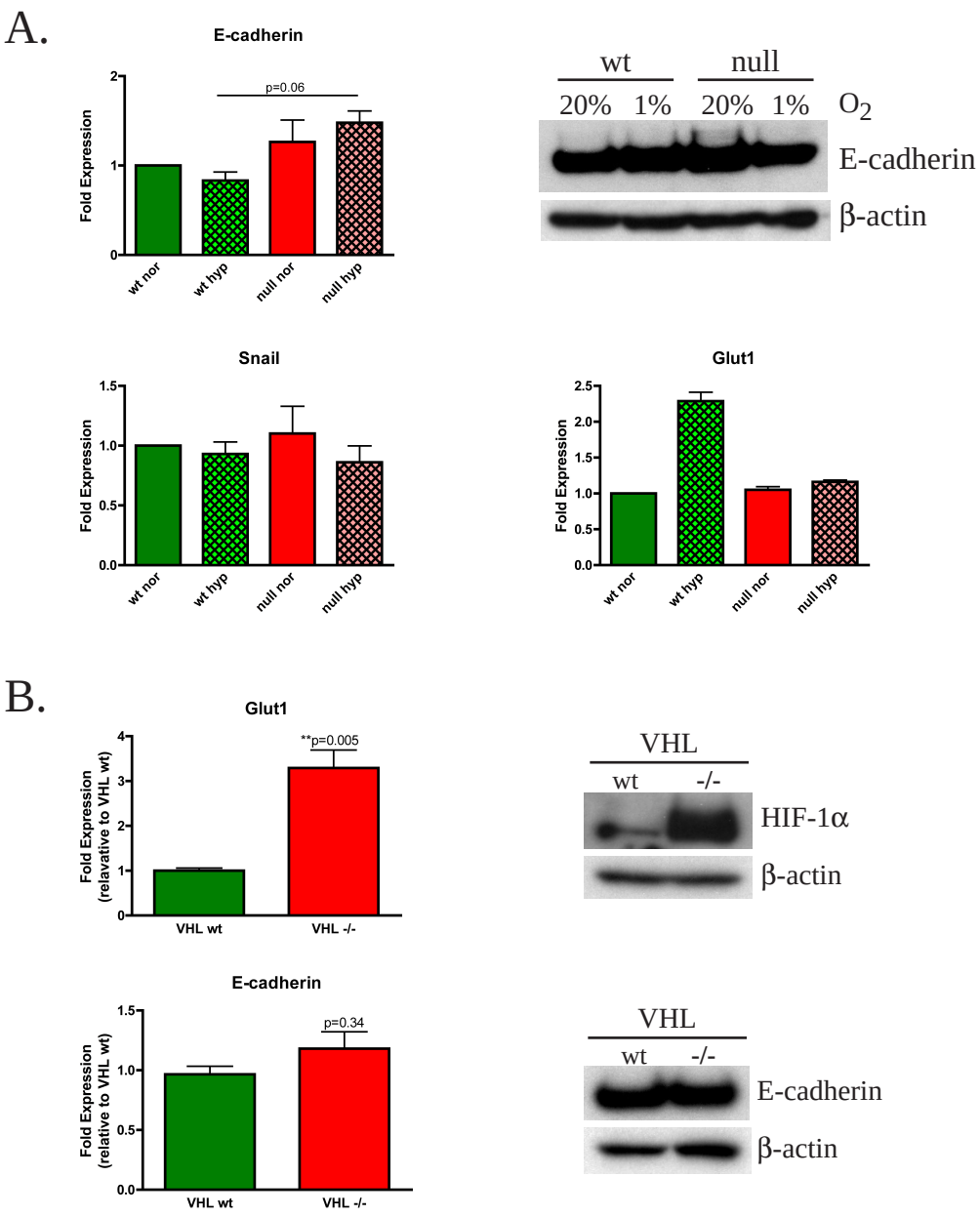


Figure 3.1. E-cadherin is not transcriptionally regulated by HIF-1α in tumor MECs. A, Real-time PCR (top left and bottom) analysis of E-cadherin, Snail, and Glut1 mRNA expression levels from HIF-1α$^{flox/flox}$ adenovirus-infected tumor MECs cultured under normoxia (20% O_2, solid bar) or hypoxia (1.0% O_2, thatched bar) for 6 hours. Western blot (top right) of cytoplasmic protein extracts from HIF-1α$^{flox/flox}$ adenovirus-infected tumor MECs cultured under normoxia (20% O_2) or hypoxia (1.0% O_2) for 24 hours. B, Real-time PCR (top and bottom left) analysis if Glut1 and E-cadherin mRNA levels from VHL$^{flox/flox}$ adenovirus-infected tumor MECs cultured under normoxia (20% O_2). Western blot analysis of nuclear (top right) and cytoplamsic (bottom right) protein extracts from VHL$^{flox/flox}$ adenovirus-infected tumor MECs cultured under normoxia (20% O_2).

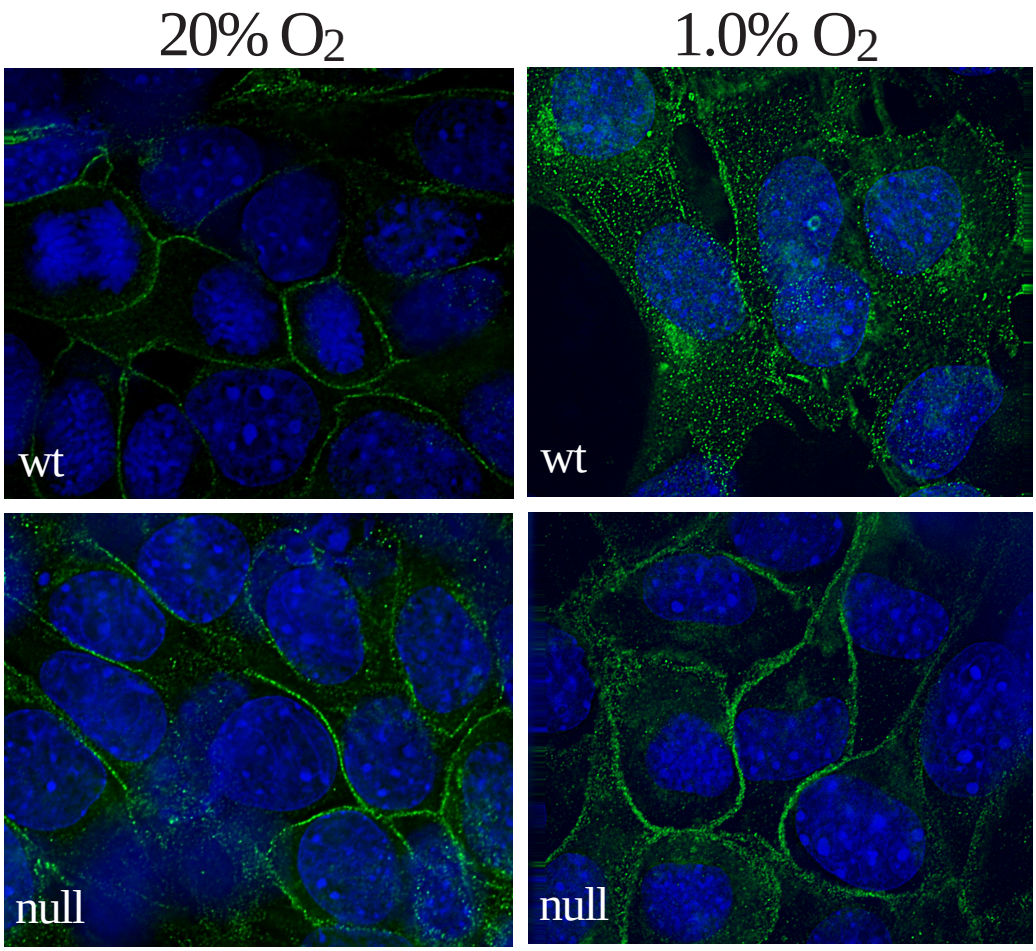


Figure 3.2. Hypoxic reduction of cell surface E-cadherin expression is mediated by HIF-1α. HIF-1α$^{flox/flox}$ (wt) and HIF-1α$^{-/-}$ (null) tumor MECs were plated on gelatin coated glass slides. Cells were cultured at normoxia (20% O_2) or hypoxia (1.0% O_2) for 24 hours and stained for E-cadherin (green). Cell nuclei are stained with Dapi (blue). Cells were imaged by deconvolution microscopy. Magnification = 600x.

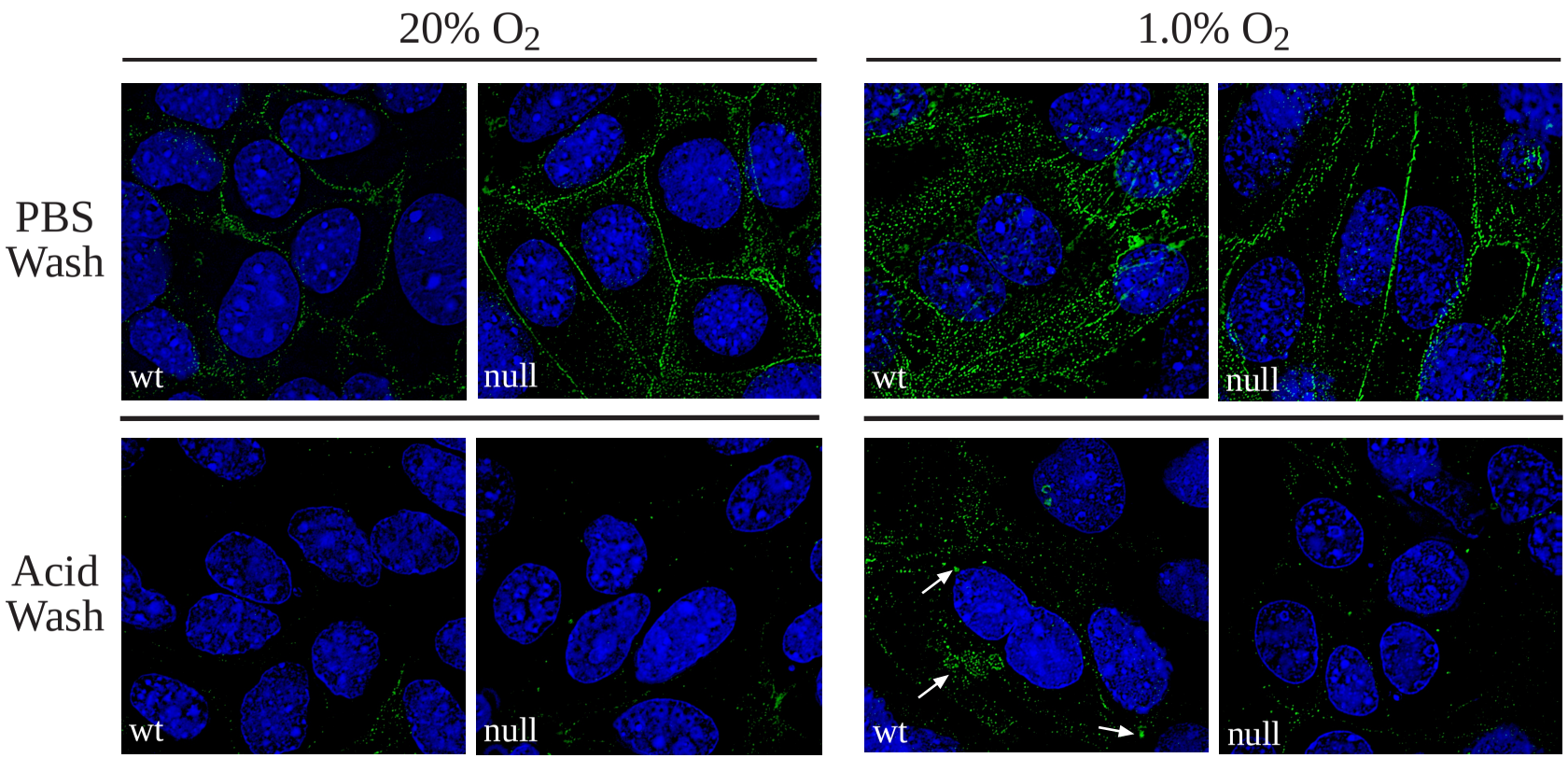


Figure 3.3. HIF-1α mediated internalization of E-cadherin. Prior to normoxic (20% O_2) or hypoxic (1.0% O_2) culture, HIF-1α wild type (wt) and null tumor MECs were incubated with an antibody against the extracellular portion of the E-cadherin. Unbound antibody was rinsed away with fresh media and cells were cultured at normoxia or hypoxia for 24 hours. After normoxic or hypoxic culture and prior to fixing, cells were washed with either an acid wash, to remove E-cadherin antibody retained at the cell surface, or PBS, as a negative control. E-cadherin staining was visualized by deconvolution microscopy. White arrows denote internalized E-cadherin. Magnification = 600x.

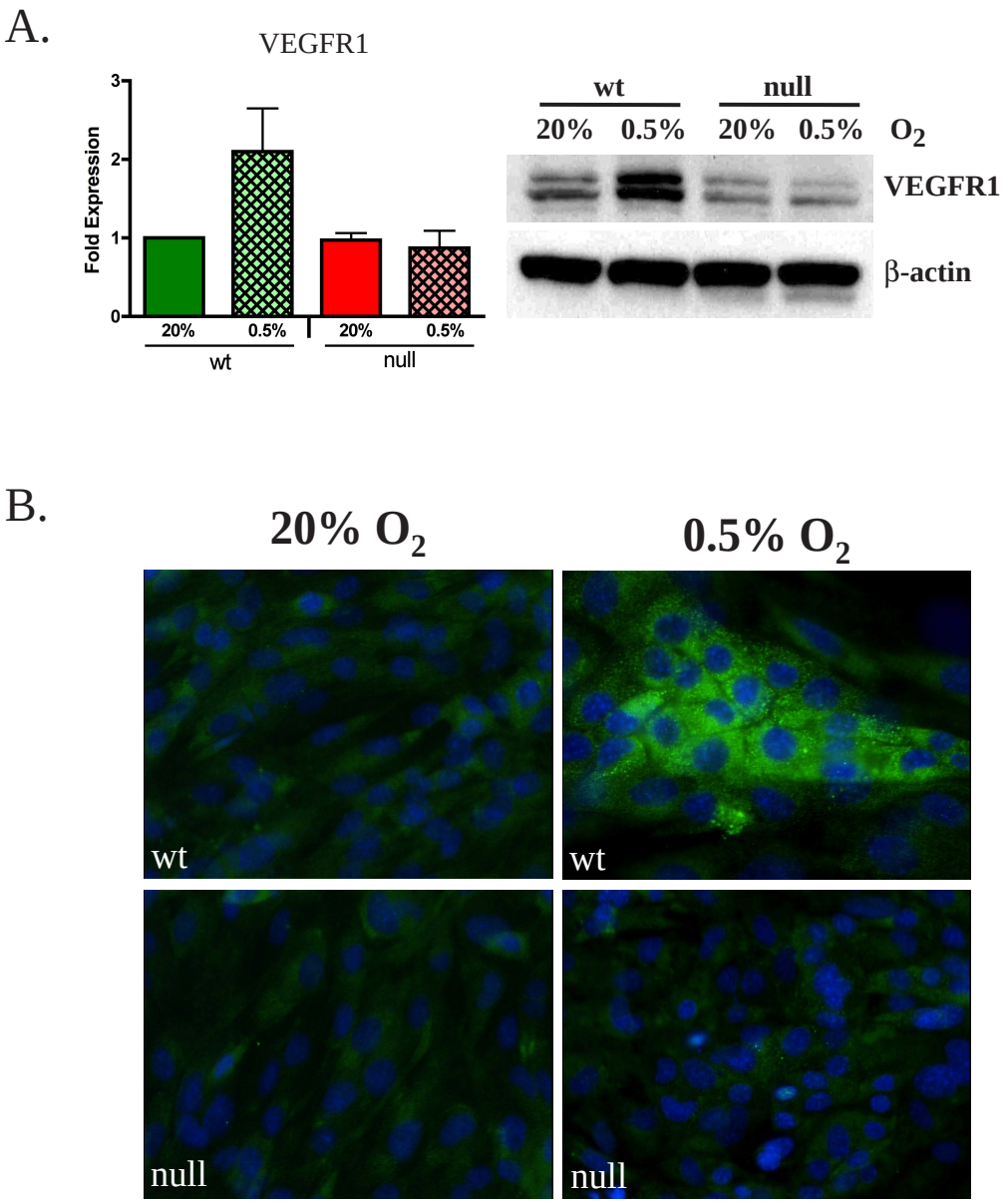


Figure 3.4. Hypoxic induction of VEGFR1 protein expression is dependent on HIF-1α transcriptional activity. A, Western blot analysis (right) of protein extracts and real-time PCR analysis (left) of VEGFR1 mRNA expression levels from HIF-$1\alpha^{flox/flox}$ (wt) and HIF-$1\alpha^{-/-}$ (null) tumor MEC cultured under normoxia (20% O_2) or hypoxia (0.5% O_2) for 24 and 6 hours, respectively. B, VEGFR1 (green) immuostaining of HIF-$1\alpha^{flox/flox}$ (wt) and HIF-$1\alpha^{-/-}$ (null) tumor MEC cultured under normoxia (20% O_2) or hypoxia (0.5% O_2) for 24 hours. Magnification = 400x.

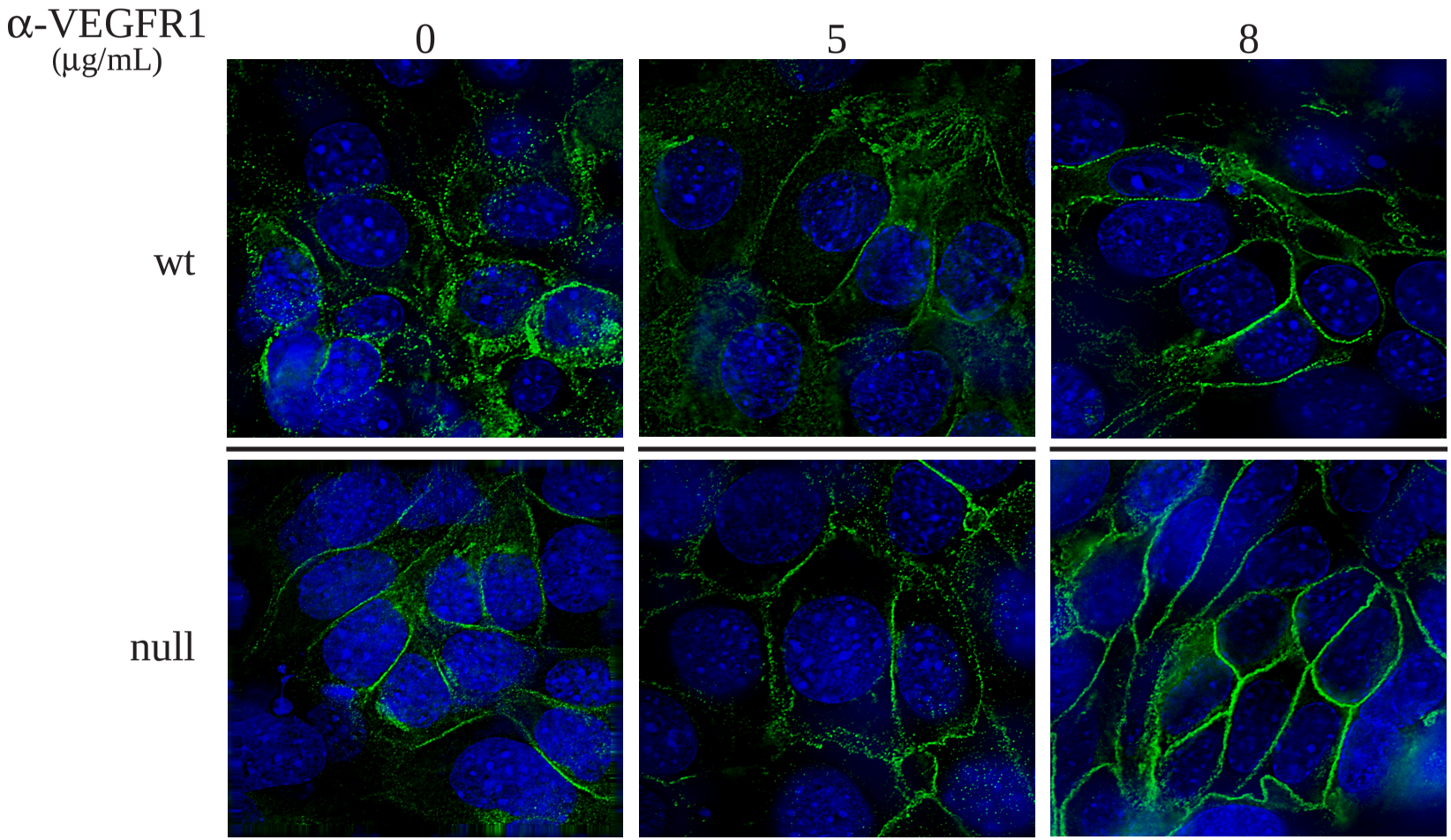


Figure 3.5. Inhibition of E-cadherin internalization by VEGFR1 blocking antibody. HIF-1$\alpha^{flox/flox}$ (wt) and HIF-1$\alpha^{-/-}$ (null) tumor MECs were cultured at hypoxia (1.0% O_2) for 24-hours in the presence of increasing concentrations of VEFGR1 blocking antibody (0, 5, and 8 μg/mL). E-cadherin (green) was detected by immunoflourescence. Cell nuclei were stained with Dapi (blue). Cells were imaged by deconvolution microscopy. Magnification = 600x.

References

1. Semenza GL. Hypoxia-inducible factor 1: master regulator of O2 homeostasis. Curr Opin Genet Dev 1998;*8:*588-94.

2. Semenza GL. Hydroxylation of HIF-1: oxygen sensing at the molecular level. Physiology (Bethesda) 2004;*19:*176-82.

3. Kondo K, Kaelin WG, Jr. The von Hippel-Lindau tumor suppressor gene. Exp Cell Res 2001;*264:*117-25.

4. Semenza GL. HIF-1 and tumor progression: pathophysiology and therapeutics. Trends Mol Med 2002;*8:*S62-7.

5. Le QT, Denko NC, Giaccia AJ. Hypoxic gene expression and metastasis. Cancer Metastasis Rev 2004;*23:*293-310.

6. Koike T, Kimura N, Miyazaki K, Yabuta T, Kumamoto K, Takenoshita S, Chen J, Kobayashi M, Hosokawa M, Taniguchi A, Kojima T, Ishida N, Kawakita M, Yamamoto H, Takematsu H, Suzuki A, Kozutsumi Y, Kannagi R. Hypoxia induces adhesion molecules on cancer cells: A missing link between Warburg effect and induction of selectin-ligand carbohydrates. Proc Natl Acad Sci U S A 2004;*101:*8132-7.

7. Cavallaro U, Christofori G. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. Nat Rev Cancer 2004;*4:*118-32.

8. Derksen PW, Liu X, Saridin F, van der Gulden H, Zevenhoven J, Evers B, van Beijnum JR, Griffioen AW, Vink J, Krimpenfort P, Peterse JL, Cardiff RD, Berns A, Jonkers J. Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. Cancer Cell 2006;*10:*437-49.

9. Risinger JI, Berchuck A, Kohler MF, Boyd J. Mutations of the E-cadherin gene in human gynecologic cancers. Nat Genet 1994;*7:*98-102.

10. Berx G, Cleton-Jansen AM, Nollet F, de Leeuw WJ, van de Vijver M, Cornelisse C, van Roy F. E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. Embo J 1995;*14:*6107-15.

11. Polakis P. Wnt signaling and cancer. Genes Dev 2000;*14:*1837-51.

12. Orsulic S, Huber O, Aberle H, Arnold S, Kemler R. E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation. J Cell Sci 1999;*112 (Pt 8):*1237-45.

13. Stockinger A, Eger A, Wolf J, Beug H, Foisner R. E-cadherin regulates cell growth by modulating proliferation-dependent beta-catenin transcriptional activity. J Cell Biol 2001;*154:*1185-96.

14. Wong AS, Gumbiner BM. Adhesion-independent mechanism for suppression of tumor cell invasion by E-cadherin. J Cell Biol 2003;*161:*1191-203.

15. Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, Garcia De Herreros A. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol 2000;*2:*84-9.

16. Imai T, Horiuchi A, Wang C, Oka K, Ohira S, Nikaido T, Konishi I. Hypoxia attenuates the expression of E-cadherin via up-regulation of SNAIL in ovarian carcinoma cells. Am J Pathol 2003;*163:*1437-47.

17. Esteban MA, Tran MG, Harten SK, Hill P, Castellanos MC, Chandra A, Raval R, O'Brien T S, Maxwell PH. Regulation of E-cadherin expression by VHL and hypoxia-inducible factor. Cancer Res 2006;*66:*3567-75.

18. Evans AJ, Russell RC, Roche O, Burry TN, Fish JE, Chow VW, Kim WY, Saravanan A, Maynard MA, Gervais ML, Sufan RI, Roberts AM, Wilson LA, Betten M, Vandewalle C, Berx G, Marsden PA, Irwin MS, Teh BT, Jewett MA, Ohh M. VHL promotes E2 box-dependent E-cadherin transcription by HIF-mediated regulation of SIP1 and snail. Mol Cell Biol 2007;*27:*157-69.

19. Liao D, Corle C, Seagroves TN, Johnson RS. Hypoxia-inducible factor-1alpha is a key regulator of metastasis in a transgenic model of cancer initiation and progression. Cancer Res 2007;*67:*563-72.

20. Krishnamachary B, Zagzag D, Nagasawa H, Rainey K, Okuyama H, Baek JH, Semenza GL. Hypoxia-inducible factor-1-dependent repression of E-cadherin in von Hippel-Lindau tumor suppressor-null renal cell carcinoma mediated by TCF3, ZFHX1A, and ZFHX1B. Cancer Res 2006;*66:*2725-31.

21. Dayan F, Roux D, Brahimi-Horn MC, Pouyssegur J, Mazure NM. The oxygen sensor factor-inhibiting hypoxia-inducible factor-1 controls expression of distinct genes through the bifunctional transcriptional character of hypoxia-inducible factor-1alpha. Cancer Res 2006;*66:*3688-98.

22. Akhtar N, Hotchin NA. RAC1 regulates adherens junctions through endocytosis of E-cadherin. Mol Biol Cell 2001;*12:*847-62.

23. Izumi G, Sakisaka T, Baba T, Tanaka S, Morimoto K, Takai Y. Endocytosis of E-cadherin regulated by Rac and Cdc42 small G proteins through IQGAP1 and actin filaments. J Cell Biol 2004;*166:*237-48.

24. Paterson AD, Parton RG, Ferguson C, Stow JL, Yap AS. Characterization of E-cadherin endocytosis in isolated MCF-7 and chinese hamster ovary cells: the initial fate of unbound E-cadherin. J Biol Chem 2003;*278:*21050-7.

25. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. Nat Rev Mol Cell Biol 2006;*7:*359-71.

26. Weigand M, Hantel P, Kreienberg R, Waltenberger J. Autocrine vascular endothelial growth factor signalling in breast cancer. Evidence from cell lines and primary breast cancer cultures in vitro. Angiogenesis 2005;*8:*197-204.

27. Wey JS, Fan F, Gray MJ, Bauer TW, McCarty MF, Somcio R, Liu W, Evans DB, Wu Y, Hicklin DJ, Ellis LM. Vascular endothelial growth factor receptor-1 promotes migration and invasion in pancreatic carcinoma cell lines. Cancer 2005;*104:*427-38.

28. Wu Y, Hooper AT, Zhong Z, Witte L, Bohlen P, Rafii S, Hicklin DJ. The vascular endothelial growth factor receptor (VEGFR-1) supports growth and survival of human breast carcinoma. Int J Cancer 2006;*119:*1519-29.

29. Ryden L, Linderholm B, Nielsen NH, Emdin S, Jonsson PE, Landberg G. Tumor specific VEGF-A and VEGFR2/KDR protein are co-expressed in breast cancer. Breast Cancer Res Treat 2003;*82:*147-54.

30. Yang AD, Camp ER, Fan F, Shen L, Gray MJ, Liu W, Somcio R, Bauer TW, Wu Y, Hicklin DJ, Ellis LM. Vascular endothelial growth factor receptor-1 activation mediates epithelial to mesenchymal transition in human pancreatic carcinoma cells. Cancer Res 2006;*66:*46-51.

31. Gavard J, Gutkind JS. VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. Nat Cell Biol 2006;*8:*1223-34.

32. Handschuh G, Candidus S, Luber B, Reich U, Schott C, Oswald S, Becke H, Hutzler P, Birchmeier W, Hofler H, Becker KF. Tumour-associated E-cadherin

mutations alter cellular morphology, decrease cellular adhesion and increase cellular motility. Oncogene 1999;*18:*4301-12.

33. Ino Y, Gotoh M, Sakamoto M, Tsukagoshi K, Hirohashi S. Dysadherin, a cancer-associated cell membrane glycoprotein, down-regulates E-cadherin and promotes metastasis. Proc Natl Acad Sci U S A 2002;*99:*365-70.

34. Strumane K, Bonnomet A, Stove C, Vandenbroucke R, Nawrocki-Raby B, Bruyneel E, Mareel M, Birembaut P, Berx G, van Roy F. E-cadherin regulates human Nanos1, which interacts with p120ctn and induces tumor cell migration and invasion. Cancer Res 2006;*66:*10007-15.

35. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002;*2:*442-54.

36. Clifford SC, Cockman ME, Smallwood AC, Mole DR, Woodward ER, Maxwell PH, Ratcliffe PJ, Maher ER. Contrasting effects on HIF-1alpha regulation by disease-causing pVHL mutations correlate with patterns of tumourigenesis in von Hippel-Lindau disease. Hum Mol Genet 2001;*10:*1029-38.

37. Suzuki K, Takahashi K. Induction of E-cadherin endocytosis by loss of protein phosphatase 2A expression in human breast cancers. Biochem Biophys Res Commun 2006;*349:*255-60.

38. Le TL, Yap AS, Stow JL. Recycling of E-cadherin: a potential mechanism for regulating cadherin dynamics. J Cell Biol 1999;*146:*219-32.

39. Takeichi M. Cadherins: a molecular family important in selective cell-cell adhesion. Annu Rev Biochem 1990;*59:*237-52.

40. Savagner P. Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. Bioessays 2001;*23:*912-23.

41. Fujita Y, Krause G, Scheffner M, Zechner D, Leddy HE, Behrens J, Sommer T, Birchmeier W. Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. Nat Cell Biol 2002;*4:*222-31.

42. Asgeirsson KS, JG Jiqm, Tryggvad inverted question markottir L, Olafsd inverted question markottir K, Sigurgeirsd inverted question markottir JR, Ingvarsson S, Ogmundsd inverted question markottir HM. Altered expression of E-cadherin in breast cancer. patterns, mechanisms and clinical significance. Eur J Cancer 2000;*36:*1098-106.

43. Haase VH, Glickman JN, Socolovsky M, Jaenisch R. Vascular tumors in livers with targeted inactivation of the von Hippel-Lindau tumor suppressor. Proc Natl Acad Sci U S A 2001;*98:*1583-8.

44. Seagroves TN, Hadsell D, McManaman J, Palmer C, Liao D, McNulty W, Welm B, Wagner KU, Neville M, Johnson RS. HIF1alpha is a critical regulator of secretory differentiation and activation, but not vascular expansion, in the mouse mammary gland. Development 2003;*130:*1713-24.

45. Ryan HE, Lo J, Johnson RS. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. Embo J 1998;*17:*3005-15.

46. Xiao K, Allison DF, Buckley KM, Kottke MD, Vincent PA, Faundez V, Kowalczyk AP. Cellular levels of p120 catenin function as a set point for cadherin expression levels in microvascular endothelial cells. J Cell Biol 2003;*163:*535-45.

CHAPTER IV

General Discussion

Cell autonomous or tumor microenvironment?

Tumor cell-intrinsic changes, such as overexpression of tyrosine kinase receptors and alterations in E-cadherin expression, affect proliferation, migration, and invasion of tumor cells *in vitro* (1). However, in the *in vivo* context, it is well recognized that cell-autonomous changes alone are not sufficient for tumor progression and metastasis to occur (1). The mammary gland stroma is composed of many cell types including endothelial and adipose cells, fibroblasts, and infiltrating inflammatory cells (2). Though transformation may occur in only the mammary epithelial cells, stromal cells are also capable of mediating tumor growth and dissemination of tumor cells through paracrine growth factor and cytokine signaling (1). Therefore, tumor cells work in concert with stromal components to shape the tumor microenvironment into one that is most conducive for growth. This paradigm increases the complexity of studying tumor progression using *in vivo* models; it's not just about the tumor cell.

Selection of tumor MECs during HIF-1α$^{MEC-/-}$ tumor progression.

The results presented in Chapter 2 strongly suggest that the tumor microenvironment mediates selection of tumor cells that can tolerate the loss of HIF-1α activity during tumor progression. Though HIF-1α$^{MEC-/-}$ tumors grew at a slower rate than HIF-1α$^{flox/flox}$ tumors, HIF-1α$^{MEC-/-}$ tumors were capable of achieving a similar mass as HIF-1α$^{flox/flox}$ tumors. Additionally, while the percentage of proliferating cells was decreased in HIF-1α$^{MEC-/-}$ tumors during early tumor progression, at the end point,

HIF-1α$^{MEC-/-}$ tumors contained a similar percentage of proliferating cells as HIF-1α$^{flox/flox}$ tumors. These results suggest that, during tumor progression, a selection process is occurring for tumor cells that have overcome the dependence on intrinsic HIF-1α activity for growth.

However, inefficient MMTV-Cre mediated gene excision in our model could also result in rescue of tumor growth in HIF-1α$^{MEC-/-}$ mice. In Chapter 2 we showed that HIF-1α wild type tumor MECs have a growth advantage over HIF-1α$^{-/-}$ tumor MECs during hypoxic stress. Therefore, inefficient deletion of HIF-1α in MMTV-PyMT/MMTV-Cre expressing animals could potentially result in outgrowth of tumor MECs that have not undergone Cre-mediated excision, if there is a heterogenous population of HIF-1α$^{flox/flox}$ and HIF-1α$^{-/-}$ tumor MECs within the same tumor.

To verify Cre-mediated gene excision efficiency in our model, HIF-1α$^{flox/flox}$/MMTV-PyMT/MMTV-Cre mice were bred to the ROSA26 reporter (R26R) transgenic mouse. In R26R mice, expression of Cre results in the removal of a loxP-flanked DNA stop codon that prevents expression of a lacZ gene. Therefore, in these mice lacZ is only expressed in cells that have undergone Cre-mediated excision of loxP-flanked DNA sequences. This system is a reliable reporter strain for the detection of Cre-mediated excision events *in vivo* (3).

Mammary glands were isolated from HIF-1α$^{wt/flox}$/MMTV-PyMT/R26R (HIF-1α^{floxflox}) and HIF-1α$^{wt/flox}$/MMTV-PyMT/MMTV-Cre/R26R (HIF-1α$^{MEC-/-}$) mice at 4- and 8-weeks of age. Mammary glands were fixed and subject to X-Gal whole mount staining, resulting in lacZ expressing tissues turning blue. No mammary epithelial

structures from the glands of HIF-1α^{floxflox} mice stained blue with X-Gal (Fig. 4.1A). However, glands isolated from HIF-1α$^{MEC-/-}$ mice exhibited extensive blue staining of mammary epithelial structures from both 4- and 8-week old mice (Fig. 4.1B). Areas of hyperplasia in HIF-1α$^{MEC-/-}$ glands also stained blue with X-Gal (Fig. 4.1B and 4.1C). Additionally, HIF-1α gene excision in tumor MECs from end point HIF-1α$^{flox/flox}$ and HIF-1α$^{MEC-/-}$ tumors was confirmed by real-time PCR (Fig. 4.1C). Genomic DNA derived from tumor MECs isolated by collagenase digestion was subject to real-time PCR to detect expression of exon 2 of HIF-1α. The expression of HIF-1α in tumor MECs derived from HIF-1α$^{MEC-/-}$ tumors was significantly reduced, and had a value similar to water negative control, when compared to tumor MECs derived from Cre-negative tumors (Fig. 4.1C). These results demonstrate efficient MMTV-Cre-mediated gene excision of HIF-1α our mouse model. Therefore, the rescue of tumor growth in end point HIF-1α$^{MEC-/-}$ mice cannot be attributed to outgrowth of tumor MECs with inefficient Cre-mediated HIF-1α gene excision.

If selection of HIF-1α$^{MEC-/-}$ tumor cells is occurring, then tumor cells isolated from HIF-1α$^{MEC-/-}$ tumors at end point should have gone through the selection process and have overcome their growth deficit. To test this hypothesis *in vivo*, two separate tumor MEC lines were established: one from end point HIF-1α$^{flox/flox}$ tumors and another from end point HIF-1α$^{MEC-/-}$ tumors (Fig. 4.2A). These two separate tumor MEC lines were then individually implanted orthotopically into the cleared fat pad of immunocompromised mice (Fig. 4.2A). Weekly palpation of these mice after implantation of tumor MECs revealed no significant difference in tumor growth rate

between mice which received HIF-1α wild type tumor MECS versus mice which received HIF-1$\alpha^{-/-}$ tumor MECs (Fig. 4.1B). Additionally, there was no significant difference in tumor weight between the two groups (Fig. 4.1B).

This result strongly suggests that there is indeed tumor MEC selection occurring during tumor progression. In support of this hypothesis, hypoxia induced selection for transformed mouse embryonic fibroblasts deficient in p53 within a heterogeneous cell population has been demonstrated *in vitro* (4). Based on these results, it would be of interest to perform genomic profiling of tumor MECs derived from HIF-1$\alpha^{flox/flox}$ tumors, and early and late stage HIF-1$\alpha^{MEC-/-}$ tumors, to identify genes that are differentially regulated between these groups that may compensate for the loss of HIF-1α activity during tumor progression. These genes would be relevant for the development of adjuvant therapies to counteract tumor cell selection when utilizing treatments based on using HIF-1α as a therapeutic target.

***In vivo* tail vein metastasis assay: tumor MEC intravasation.**

Once metastasis occurs and secondary tumors are established, the probability of long-term patient survival is reduced dramatically (5). Therefore, much research has been devoted to uncover the mechanisms that contribute to metastatic spread of breast cancer cells. In order for metastasis to occur, the tumor cell must dissociate from the primary tumor, invade through the stroma, intravasate into the blood vessel or lymphatic system, home to a secondary site, and extravasate into and colonize in the secondary site (6).

In Chapter 2, we have shown that pulmonary metastasis is reduced in HIF-1$\alpha^{MEC-/-}$ mice compared to HIF-1$\alpha^{flox/flox}$ mice. However, HIF-1α activity may be mediating the metastatic dissemination of tumor MECs at any one of steps listed above. A caveat of using in an *in vivo* model of *de novo* tumor growth to study metastasis is that it is not possible to separate events occurring within the primary tumor from events occurring after tumor cell intravasation in regards to their contribution to the dissemination of tumor cells. Therefore, in order to investigate the contribution of HIF-1α activity specifically during tumor cell extravasation, we utilized an *in vivo* assay for metastatis in which tumor cells are directly introduced into the circulatory system by injection into the tail vein of mice. This method has been used to investigate the ability of tumor cells to extravasate and colonize in the lung (7, 8).

First, a tumor MEC line was established by collagenase digestion of end point tumors from a HIF-1$\alpha^{flox/flox}$/MMTV-PyMT^{+} virgin female mouse. The established tumor MEC line was maintained at 20% O_2. Prior to injection into the tail vein, HIF-1α wild type and null tumor MEC lines were generated by infection with adenovirus expressing either β-galactosidase or Cre-recombinase, respectively (Fig. 4.3A). Tumor MECs were then injected into two separate groups of mice: one receiving only wild type cells, and the other receiving only null cells. 4-weeks after injection of tumor cells, half the mice from each group were sacrificed. The lungs from each mouse were isolated, fixed in formalin, and processed for whole mount hematoxylin staining to visualize metastatic foci. No gross metastasis was visible in the lungs of mice from

either group 4-weeks after injection of tumor MECs. 20.6-weeks after injection of tumor MECs, the remaining mice were sacrificed. At this time, none of the mice exhibited any overt sign of disease; therefore this time point was chosen arbitrarily. The lungs of each mouse was isolated and processed for whole mounts as described above. At this time point, gross metastatic foci were present in lungs from both mice that received wild type tumor MECs and mice that received null tumor MECs (Fig. 4.3B). For the quantification of metastatic foci, lungs were embedded in paraffin wax and serial sections through the entire lung were generated. Lung sections were then stained for hematoxylin and eosin and the number of metastatic foci was quantified by microscopy (Fig. 4.3B). Though there was a trend towards fewer metastatic foci in mice having received null tumor MECs compared to mice having received wild type tumor MECs, this number did not reach statistical significance due to small sample size. These results suggest that HIF-1α activity in tumor MECs may promote tumor cell extravasation, though it is not required for tumor cell extravasation. However, this hypothesis must be tested with a larger sample size.

Reduced macrophage infiltration in HIF-1α$^{MEC-/-}$ tumors.

HIF-1α is expressed in a wide range of cell types, including those found in the mammary gland. Though HIF-1α may be inactivated in the tumor MECs of HIF-1α$^{MEC-/-}$ mice, HIF-1α signaling pathways can still be activated in other cell types in response to hypoxia, and may have profound effects on tumor growth. In particular, tumor-associated macrophages (TAMs) are commonly found in solid tumors of the

breast (9). TAMs are known to express abundant HIF-1α (10). Additionally, multiple groups have demonstrated that TAMs promote tumor angiogenesis *in vivo*, and release cytokines, such as VEGF, when exposed to hypoxia *in vitro* (11-13). Based on these observations, it is possible that TAMs may contribute to angiogenesis and tumor progression in our mouse model.

To investigate this possibility, we analyzed TAM infiltration in tumors samples derived from 14-week old HIF-1$\alpha^{flox/flox}$ and HIF-1$\alpha^{MEC-/-}$ mice. As described in Chapter 2, tumors at the 14-week time point from HIF-1$\alpha^{MEC-/-}$ mice have significantly decreased microvessel density when compared to tumors from HIF-1$\alpha^{flox/flox}$ mice at the same time point. To determine whether this phenotype is associated with differential macrophage infiltration, tumor sections from 14-week old HIF-1$\alpha^{flox/flox}$ (n=6) and HIF-1$\alpha^{MEC-/-}$ (n=6) mice were subject to immunohistochemistry with an antibody against the macrophage specific antigen, F4/80 (Fig. 4.4A) (14). To quantify the relative amount of macrophage infiltration in each tumor, a minimum of 8 different fields per tumor were captured and analyzed using ImageJ software. The presence of tumor-associated macrophages was significantly decreased in HIF-1$\alpha^{MEC-/-}$ tumors compared to wild type (Fig. 4.4B).

Based on this result, it is possible that TAMs may promote tumor angiogenesis. However, using our mouse model, we cannot distinguish between tumor cell autonomous versus stromal cell contributions to angiogenesis, because both tumor MECs and TAMs produce and secrete angiogenic factors, such as VEGF. Therefore, this hypothesis must be substantiated with studies that address whether tumor

angiogenesis is a direct cause of angiogenic factors produced by tumor MECs, or an effect of angiogenic factors produced by TAMs attracted to the tumor in a HIF-1α dependent fashion.

To this end, we have already initiated studies to elucidate the role of HIF-1α mediated cross-talk between tumor MECs and TAMs, and the potential contribution of this interaction on tumor progression and tumor cell invasion. Breast cancer cells have been shown to produce CSF-1, a growth factor that promotes the proliferation and migration of macrophages (15-17). Therefore, we are investigating whether the transcription of genes known to promote proliferation and migration of macrophages, such as CSF-1, are transcriptionally regulated by HIF-1α in tumor MECs. Additionally, we are utilizing *in vitro* migration assays to test whether factors secreted by tumor MECs during hypoxic culture promote macrophage migration and invasion. Also, we are utilizing *in vitro* co-culture techniques to test whether HIF-1α signaling in tumor MECs affects macrophage proliferation or the production of factors that contribute to tissue remodeling, such as matrixmetalloprotenases. Additionally, TAMs also have an intrinsic hypoxic response. Therefore, our lab is also currently investigating the contribution of macrophage HIF-1α activity to tumor progression in a MMTV-PyMT mouse model with conditional deletion of HIF-1α in TAMs. These studies will help to elucidate the potential contribution of HIF-1α activity during tumor MEC and macrophage cross-talk and its affects on tumor progression and tumor cell invasion. These studies will help to elucidate the contribution of HIF to cell

autonomous and tumor microenvironment changes that are necessary for tumor progression and metastasis.

Conclusion

The work presented here demonstrates that, though HIF-1α expression in tumor MECs is a positive factor for tumor growth and metastasis *in vivo*, eliminating HIF-1α activity in tumor cells alone is not sufficient to stop tumor growth or eliminate metastatic disease. Hypoxia induced gene expression in cells within the tumor microenvironment may also contribute significantly to tumor progression and metastatic spread of tumor cells, and therefore cannot be overlooked. It is clear from these studies that tumors and tumor microenvironments have evolved many compensatory mechanisms to meet the demands of rapid growth and of processes required for tumor cell dissemination (Fig. 4.5). The ultimate goal of these studies is to enable the development of more effective and targeted therapies for the treatment/prevention of breast cancer and metastatic disease through a better understanding of the biology of breast cancer disease *in vivo*.

Materials and Methods

Animals.

All animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International approved facility. Animal experiments were conducted in accordance with NIH Guide for the Care and Use of Laboratory Animals. Mice with exon 2 of HIF-1α flanked by loxP sites and expressing MMTV-PyMT and MMTV-Cre were generated as described in Chapter 2. R26R ROSA reporter transgenic mice were acquired from the Jackson Laboratory (Stock # 003309). Athymic nude mice were acquired from Simonsen Laboratories .

X-Gal staining of mammary glands.

Mammary glands were isolated from HIF-1$\alpha^{wt/flox}$/MMTV-PyMT/MMTV-Cre virgin mice at 4- and 8- weeks of age. The #1 and #4 mammary glands were isolated an fixed in PBS containing 2% paraformaldehyde, 0.25% glutaraldehyde, and 0.01% NP-40 for 2 hours on ice. After fixation, X-gal staining of glands was performed as previously described (18). Briefly, tissues were permeabilized and treated with X-gal (1mg/mL) overnight at 37°C. Glands were then dehydrated through a graded series of alcohol and cleared in xylene overnight. For long-term storage, whole mounted glands were transferred to methyl salicylate. Whole mount images were captured using a Hamamatsu camera mounted on a stereomicroscope. To generate sections for microscopic viewing, glands were embedded in paraffin wax and 5μm sections were

generated. Sections were counterstained with Nuclear Fast Red (Vector Labs, Burlingame, CA) prior to dehydration and mounting.

Real-time PCR.

Genomic DNA was isolated from end point tumors of HIF-1$\alpha^{flox/flox}$/MMTV-PyMT and HIF-1$\alpha^{flox/flox}$/MMTV-PyMT/MMTV-Cre virgin female mice. Real-time PCR was performed using primers and probe specific for exon 2 of HIF-1α, which is flanked by loxP-sites: Forward-GGTGCTGGTGTCCAAAATGTA G, reverse-ATGGGTCTAGAGAGATAGCTCC ACA, probe-CCTGTTGGTTGCG CAGCAAGCATT.

Orthotopic *in vivo* implantation assay.

Two separate tumor MEC lines were isolated by collagenase digestion; one from end point tumors of a HIF-1$\alpha^{flox/flox}$/MMTV-PyMT virgin female mouse (wild type) and another from end point tumors of a HIF-1$\alpha^{flox/flox}$/MMTV-PyMT/MMTV-Cre virgin female mouse (null). Cells were cultured under normoxic (20% O_2) conditions in DMEM/F12 media supplemented with 2% fetal calf serum and 100U/penincillin 10U/streptomycin. The #4 fat pads of 3-week old athymic nude mice were surgically cleared of epithelial structures and mice were allowed to recover for 4-weeks. After recovery, $1.5x10^6$ of either wild type or null tumor MECs were injected into the cleared fat pad. Mice were palpated weekly and tumor dimensions were

measured with digital calipers. Mice were sacrificed when tumor had reached dimensions of 1cm x 1cm.

***In vivo* tail-vein metastasis assay.**

A tumor MEC line was established from end point tumors of a HIF-$1\alpha^{flox/flox}$/MMTV-PyMT+ virgin female mouse, as described in Chapter 2. Cells were cultured under normoxic (20% O_2) conditions as described above. Prior to injection, HIF-1α wild type and null lines were generated by infection of tumor MEC with adenovirus expressing β-galactosidase or Cre-recombinase, respectively. $1x10^6$ tumor cells in PBS were injected into the lateral tail vein of athymic nude mice (n=6/group). 4- and 20.6-weeks after injection, mice were sacrificed and lungs subjected to whole mount hematoxylin staining as described in Chapter 2. After images of whole mounted lungs were captured, lungs were embedded in paraffin wax and 10μm serial sections through the entire lung was generated. Sections were stained for hematoxylin and eosin as described in Chapter 2 and metastatic foci was counted using a stereomicroscope.

F4/80 immunostaining.

5μm tumor sections from HIF-$1\alpha^{flox/flox}$ and HIF-$1\alpha^{MEC-/-}$ mice (n=6/group) at 14-weeks of age were rehydrated in xylene and through a graded series of ethanol washes. Tumor sections were blocked with 10% goat serum (Sigma-Aldrich, St. Louis, MO) diluted in PBS containing 0.1% Tween-20 (10% NGS) for 1 hour at room

temperature. Sections were incubated with a 1:200 dilution of biotin-conjugated antibody against F4/80 (Serotec, Raleigh, NC) in 10% NGS overnight at room temperature. After washing to remove unbound antibody, F4/80 was detected using an avidin-biotin-HRP detection kit (Vector Labs, Burlingame, CA) and visualized with DAB substrate (Vector Labs, Burlingame, CA). Sections were then countered stained with hemayoxylin (Vector Labs, Burlingame, CA) and dehydrated through a series of graded ethanol washes and xylene. Sections were permanently mounted using Cytoseal (Richard-Allan Scientific, Kalamazoo, MI). For quantification of macrophage infiltration, 8-12 images (depending on tumor size) were captured at 200x magnification and analyzed by Image J software.

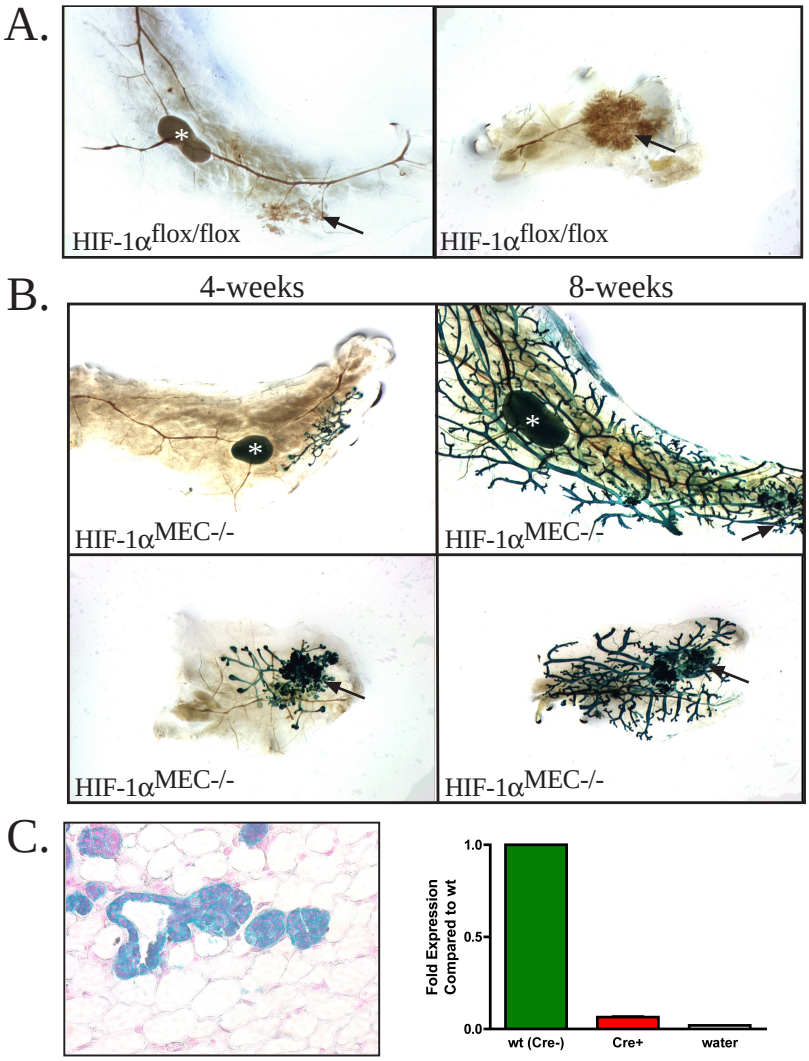


Figure 4.1. Efficiency of Cre-mediated gene excision. To verify the efficiency of Cre-mediated gene excision in the HIF-1α$^{flox/flox}$/MMTV-PyMT/MMTV-Cre (HIF-1α$^{MEC-/-}$) mouse tumor model, these mice were bred with the ROSA26-reporter (R26R) mouse. In R26R mice, expression of lacZ is dependent on Cre-recombinase mediated excision of loxP-flanked DNA segments. LacZ expression can be visualized by staining with X-Gal. All cells in which Cre-mediated excision has taken place will stain blue with X-Gal. A, Whole mount X-Gal staining of the #4 (left) and #1 (right) mammary gland from a Cre-negative (HIF-1α$^{flox/flox}$) mouse. B, whole mount X-Gal staining of the #4 (top row) and #1 (bottom row) mammary glands from 4- and 8-week old Cre-positive (HIF-1α$^{MEC-/-}$) mice. Lymph node is denoted by a white astrick. Black arrows denote hyperplasia. Magnification = 8x. C, Microscopic view of X-Gal staining in a Cre-positive mammary gland (left). Magnification = 400x. Real-time PCR (right) performed on genomic DNA purified from tumor MECs isolated from tumors of HIF-1α$^{flox/flox}$/MMTV-PyMT (wt, Cre-) and HIF-1α$^{flox/flox}$/MMTV-PyMT/MMTV-Cre (Cre+) mice at end point with a primer set specific for exon 2 of HIF-1α, which is excised upon Cre-expression. Water, containing no genomic DNA serves as a negative control.

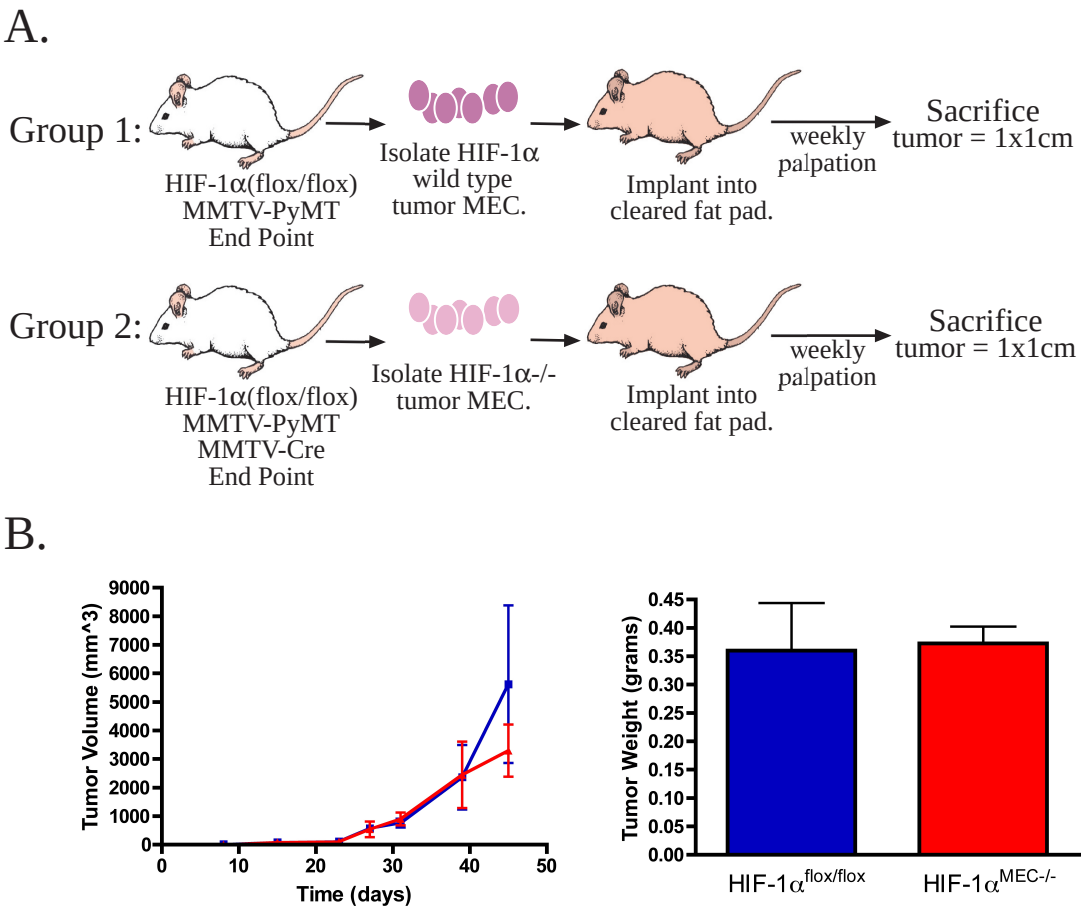


Figure 4.2. Orthotopic implantation of tumor cells isolated from end point HIF-1$\alpha^{flox/flox}$ and HIF-1$\alpha^{MEC-/-}$ mice. A, Schematic representation of the experiment. Two different lines of tumor MECs were established: one from a HIF-1$\alpha^{flox/flox}$ mouse at end point and another from a HIF-1$\alpha^{MEC-/-}$ mouse at end point. The inguinal fat pad of 3-week old athymic nude mice (pink) were cleared of mammary epithelial cells and mice were allowed to recover. After recovery, 1.5x10^6 cells or either HIF-1$\alpha^{flox/flox}$ (n=3) or HIF-1$\alpha^{MEC-/-}$ (n=4) tumor MECs were injected into the cleared fat pad. Mice were palpated once per week and tumor dimensions were measured using digital calipers. Mice were sacrificed when tumors reached a dimension of 1cm x 1cm. B, There is no significant difference between the tumor growth rate of implanted HIF-1$\alpha^{flox/flox}$ (blue) and HIF-1$\alpha^{MEC-/-}$ (red) tumor MECs (left). There was also no significant difference in tumor weight between tumors derived from HIF-1$\alpha^{flox/flox}$ and HIF-1$\alpha^{MEC-/-}$ tumor MECs (right).

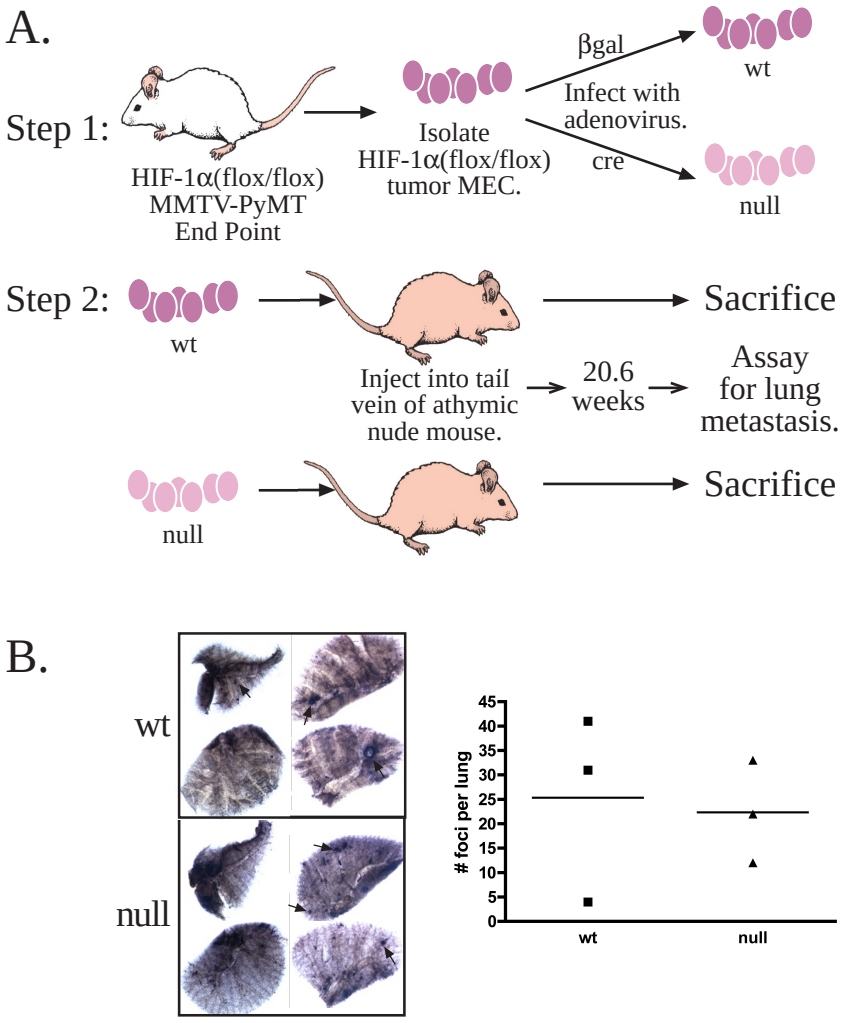
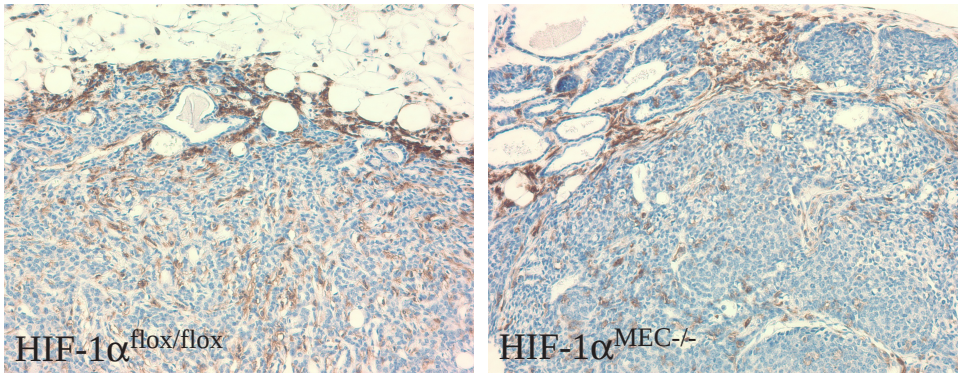


Figure 4.3. Tail vein metastasis assay. A, Schematic representation of the experiment. Tumor MECS were isolated from an end point HIF-1α$^{flox/flox}$ mouse by collagenase digestion and cultured at normoxia. Prior to injection into the tail vein, HIF-1α$^{flox/flox}$ tumor MECs were infected with adenovirus expressing either β-galatosidase (βgal) or cre-recombinase (cre) to generate HIF-1α wild type (wt) or HIF-1α$^{-/-}$ (null) tumor MECs, respectively. 1x10^{6} cells of either wt (n=6) or null (n=6) tumor MECs were injected into the tail vein of athymic nude mice (pink). At 8 and 20.6 weeks, 3 mice from each group were sacrificed. Lungs were perfused with formalin by intertracheal lavage and fixed in formalin. Wholemount hematoxylin staining was performed to visualize metastatic foci. At 8-weeks, there was no detectable lung metastasis in either mice inoculated with wt or null tumor MECs (not shown). B, Representative lung wholemounts from mice 20.6 weeks after inoculation with wt or null tumor MECs. Lung metastasis was present in mice inoculated with either wt or null tumor MECs. Metastatic foci are denoted by black arrows. Magnification = 8x. C, Lungs were serial-sectioned and stained with hematoxylin and eosin. Lung metastasis was quantified using a dissecting microscope. There was no significant difference in the number of lung metastasis between mice inoculated with wt

A.



B.

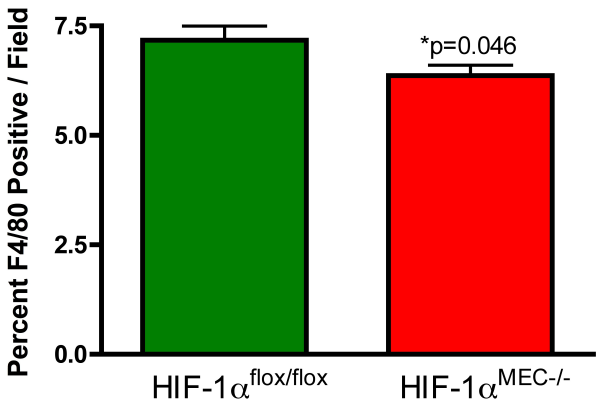


Figure 4.4. Decreased macrophage infiltration in HIF-1α$^{MEC-/-}$ tumors at 14-weeks. A, Representative F4/80 immunostaining (brown) of formalin fixed paraffin embeded tumor sections from 14-week old HIF-1α$^{flox/flox}$ (left) and HIF-1α$^{MEC-/-}$ (right) mice. Nuclei are counterstained with hematoxylin (blue). Magnification = 200x. Note the increase in macrophage infiltration into the inner region of the tumor in the HIF-1α$^{flox/flox}$ tumor compared to HIF-1α$^{MEC-/-}$ tumor. B, F4/80 immunostaining of tumor sections from HIF-1α$^{flox/flox}$ (n=6) and HIF-1α$^{MEC-/-}$ (n=6) mice were quantified using ImageJ software. For each tumor 5-12 images, depending on tumor size, were taken at 200x and used for quantification.

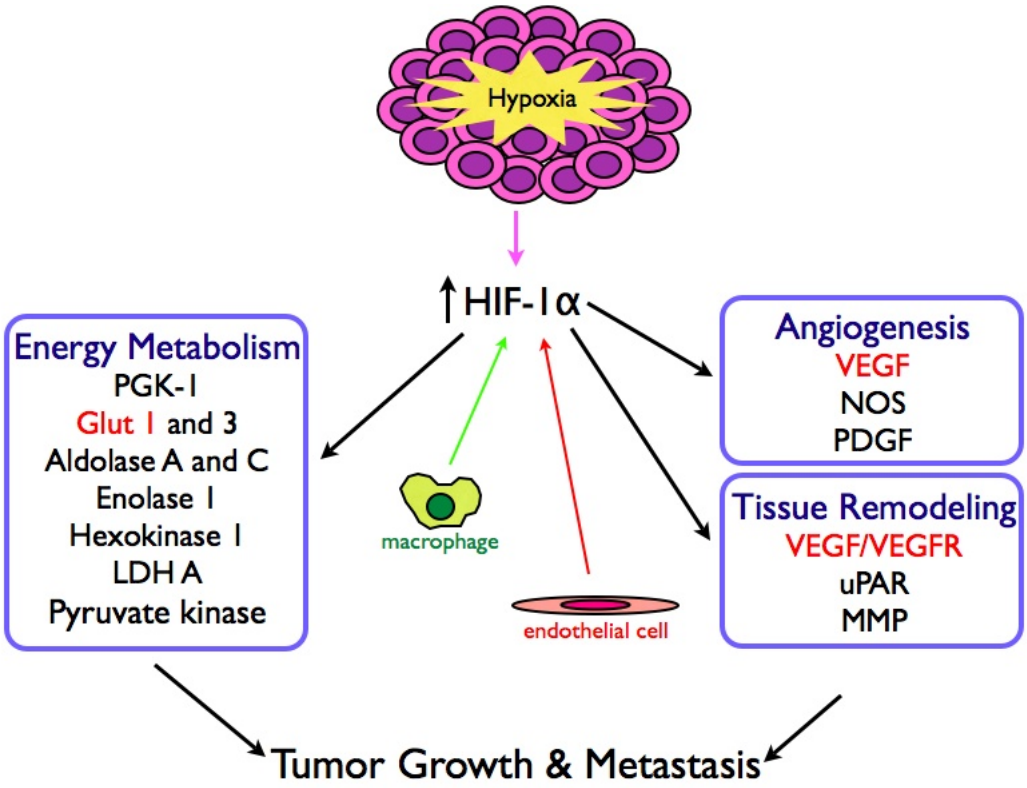


Figure 4.5. Contribution of tumor microenvironment. We have shown that HIF-1α signaling in tumor MECs (red) is important for tumor progression as well as tumor cell metastasis. However, in addition to tumor cells, the tumor microenvironment also contains many stromal cells which include, but are not limited to, endothelial cells and inflammatory cells, such as macrophages. HIF-1α signaling in endothelial cells has been shown to contribute to tumor angiogenesis and thus tumor growth. Tumor associated macrophages have also been shown to play a role in promoting tumor angiogenesis. Additionally, stromal cells have been shown to secrete various proteases which can contribute to tissue remodeling and facilitate dissemination of tumor cells. Therefore, although HIF-1α function in tumor MECs is important for tumor growth, stromal cells within the tumor microenvironment must also be taken to account when investigating tumor progression in the mammary gland *in vivo*.

References

1. Kopfstein L, Christofori G. Metastasis: cell-autonomous mechanisms versus contributions by the tumor microenvironment. Cell Mol Life Sci 2006;*63:*449-68.

2. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 2004;*4:*71-8.

3. Mao X, Fujiwara Y, Orkin SH. Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. Proc Natl Acad Sci U S A 1999;*96:*5037-42.

4. Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 1996;*379:*88-91.

5. Sierra A. Metastases and their microenvironments: linking pathogenesis and therapy. Drug Resist Updat 2005;*8:*247-57.

6. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer 2002;*2:*563-72.

7. Erler JT, Bennewith KL, Nicolau M, Dornhofer N, Kong C, Le QT, Chi JT, Jeffrey SS, Giaccia AJ. Lysyl oxidase is essential for hypoxia-induced metastasis. Nature 2006;*440:*1222-6.

8. Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL, Massague J. Genes that mediate breast cancer metastasis to lung. Nature 2005;*436:*518-24.

9. O'Sullivan C, Lewis CE. Tumour-associated leucocytes: friends or foes in breast carcinoma. J Pathol 1994;*172:*229-35.

10. Burke B, Tang N, Corke KP, Tazzyman D, Ameri K, Wells M, Lewis CE. Expression of HIF-1alpha by human macrophages: implications for the use of macrophages in hypoxia-regulated cancer gene therapy. J Pathol 2002;*196:*204-12.

11. Kerbel RS. Tumor angiogenesis: past, present and the near future. Carcinogenesis 2000;*21:*505-15.

12. Lin EY, Li JF, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, Qian H, Xue XN, Pollard JW. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer Res 2006;*66:*11238-46.

13. Harmey JH, Dimitriadis E, Kay E, Redmond HP, Bouchier-Hayes D. Regulation of macrophage production of vascular endothelial growth factor (VEGF) by hypoxia and transforming growth factor beta-1. Ann Surg Oncol 1998;*5:*271-8.

14. McKnight AJ, Macfarlane AJ, Dri P, Turley L, Willis AC, Gordon S. Molecular cloning of F4/80, a murine macrophage-restricted cell surface glycoprotein with homology to the G-protein-linked transmembrane 7 hormone receptor family. J Biol Chem 1996;*271:*486-9.

15. Kacinski BM. CSF-1 and its receptor in ovarian, endometrial and breast cancer. Ann Med 1995;*27:*79-85.

16. Stanley ER, Guilbert LJ, Tushinski RJ, Bartelmez SH. CSF-1--a mononuclear phagocyte lineage-specific hemopoietic growth factor. J Cell Biochem 1983;*21:*151-9.

17. Webb SE, Pollard JW, Jones GE. Direct observation and quantification of macrophage chemoattraction to the growth factor CSF-1. J Cell Sci 1996;*109 (Pt 4):*793-803.

18. Wagner KU, McAllister K, Ward T, Davis B, Wiseman R, Hennighausen L. Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. Transgenic Res 2001;*10:*545-53.