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EFFECTS OF ERYTHROPOIETIN AND GLYCOGEN SYNTHASE KINASE 3 ON HYPOXIA INDUCIBLE FACTOR-1α

A Dissertation Presented

by

Sarah Alison Hale

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy Specializing in Cell and Molecular Biology

May, 2008
Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, specializing in Cell and Molecular Biology.

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Cancerous tumors adjust their signaling pathways to facilitate survival in a hypoxic environment. The pro-angiogenic transcription factor hypoxia inducible factor -1α (HIF-1α) is stabilized in hypoxia and promotes tumor cell survival, proliferation, and metastasis by inducing transcription of many targets including vascular endothelial growth factor (VEGF). High expression levels of HIF-1α have been correlated with aggressive tumor types. As such, HIF-1α is an attractive candidate for targeted therapies in ovarian cancer. Though there have been attempts at developing chemotherapies that inhibit HIF-1α, to this date there remains no approved anti-angiogenic drug that directly targets HIF-1α.

Because the cytokine EPO (erythropoietin) is transcriptionally regulated by HIF-1α, we hypothesized that negative feedback inhibition from EPO to HIF-1α would decrease HIF-1α protein levels. In fact, exogenous EPO significantly inhibited hypoxia-induced HIF-1α expression in cultured human ovarian cancer cells in a dose-dependent fashion and significantly reduced hypoxia-induced VEGF transcription to near basal levels. Furthermore, although EPO is a growth factor, there was no difference in cell growth with EPO treatment in either the normoxic or hypoxic condition at any time point tested. The negative feedback of EPO on HIF-1α was not limited to ovarian cancer cells as EPO efficiently inhibited hypoxia-induced HIF-1α stabilization in MCF-7 breast cancer cells.

Initially, we hypothesized that EPO inhibition of HIF-1α was through activation of GSK3 (glycogen synthase kinase). Though EPO likely does not signal through activation of GSK3, initial experiments investigating this pathway generated promising results and prompted the continued exploration into the regulation of HIF-1α by GSK3. Therefore, the second goal of this dissertation was to investigate the role of GSK3 phosphorylation on HIF-1α protein levels and proteosomal degradation.

Recently, GSK3 has been implicated in the degradation of HIF-1α, the role of GSK3 in the regulation of HIF-1α in hypoxia has yet to be studied. We hypothesized that phosphorylation by GSK3 would target HIF-1α for proteosomal degradation in an oxygen-independent manner. In support of this hypothesis, we show that GSK3 inhibition in hypoxia lead to an increase in stabilization of HIF-1α that was echoed by an increase in VEGF transcription, suggesting an augmentation of HIF-1α transcriptional activity. Overexpression of GSK3 significantly decreased both HIF protein and VEGF transcription. Further, these studies identified five putative GSK3 sites within the HIF-1α protein that were necessary for phosphorylation by GSK3. Mutation of these sites also induced HIF-1α stabilization in normoxia and hypoxia beyond that of wild-type HIF-1α. From these results, we propose a model whereby GSK3 phosphorlates and subsequently destabilizes HIF-1α thus halting HIF-1α-dependent VEGF transcription. The work presented in this dissertation illustrates two ways in which the HIF-1α pathway can be modulated. Further exploration and exploitation of these pathways could prove to be therapeutically advantageous in the treatment of cancer.
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DEDICATION

This dissertation is dedicated to my grandparents Miriam and Rev. Murdock Hale, grandfather, Audie Martin, grandmother, Isabel Martin, who lost her battle with ovarian cancer and to my uncle, David Hale, who lost his battle with pancreatic cancer.
ACKNOWLEDGEMENTS

Ten years ago, I was ready to finish my undergraduate degree and move on to veterinary school. In the spring semester of my senior year at UVM, Dr. John Bramley and Dr. Karen Plaut both suggested that I would be “bored” as a veterinarian and I should consider research. To that, I thought, “Research? No way!” However, here I am after a M.S. and now, finishing my Ph.D. To Drs. Bramley and Plaut: thank you for your support and exceptional mentoring.

I have had the wonderful opportunity to be mentored by Dr. Karen Lounsbury during my dissertation research. She has been a great influence and role model, and I wish to thank her for all her encouragement, especially when things weren’t going so well. Also, I’d like to thank all the lab members I’ve had the opportunity to work with for their help and discussions. In particular, thank you to Terry Wellman. She’s an absolutely wonderful friend, fabulous technician and a beautiful person.

A heartfelt thank-you goes to my parents, Elizabeth and Titus Hale, siblings, Kendra, Rachel and Bradford, and friends for being incredibly supportive and putting up with my absence as a functional family member or friend as I completed this endeavor. Thank you to Anne Peyton for allowing me to form one of the most cherished relationships in my life, and to Rio, for helping me to maintain balance in my life.

Finally, thank you to my husband, Kyle, whose steadfast faith, support, love, patience and culinary aptitude I could not have done without.
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CHAPTER 1: LITERATURE REVIEW
INTRODUCTION TO CANCER

Brief history of cancer

Cancer has afflicted humans for thousands of years. Evidence of different types of carcinoma have been found in Egyptian mummies and fossilized hominid remains. The first known documentation of cancer dates to 1600 B.C. and was recorded on an Egyptian papyrus and described different types of breast tumors. Hippocrates (460 – 370 B.C.) is credited with being the “Father of Medicine” and the first to identify the difference between ulcer-forming and non-ulcer-forming tumors. He referred to the disease as carcinos and carcinoma, meaning ‘crab’ in Greek. Much later, during the Renaissance period, Rembrandt painted Bathsheba at Her Bath and may have documented a case of breast cancer indicated by the shadow on the model’s left breast.

In 1761, Giovanni Morgagni of Padua began performing autopsies and relating pathologies identified during the autopsy to the patient’s death. Soon after, John Hunter, a Scottish surgeon suggested that cancer might be curable simply by removing the tumor if it had not already invaded other tissues. These innovations and prior discoveries began the pioneering science of oncology.

Cancer defined

Cancer is a group of diseases that are characterized by genomic instability, unrestricted growth, lack of apoptosis, lack of antigrowth signals, a propensity towards invasion and metastasis, and pervasive angiogenesis (Hanahan and Weinberg, 2000). It is through the multiplicity of these aberrations that cancer is triggered (Hanahan and Weinberg, 2000).
CHARACTERISTICS OF MALIGNANCY

Genomic Instability

Genomic instability refers to defects in DNA such as impaired response to DNA damage and defective DNA repair mechanisms as well as inherent chromosomal abnormalities. Genomic instability can be induced by ionizing radiation or chemicals as well as genetically acquired mutations, hypoxia and reactive oxygen and nitrogen species and results in impaired DNA repair leading to changes in number and structure of chromosomes as well as other chromosomal abnormalities (Weinberg, 2007). These alterations in DNA that confer genomic instability can establish the premise for other hallmarks of cancer. In particular, the mutation of a gene to an oncogene facilitates cell survival and proliferation. An oncogene, by definition, possesses the ability to transform a normal cell into a tumor cell (Weinberg, 2007). Upon activation of an oncogene, downstream signaling pathways that stimulate cell survival, proliferation and inhibit apoptosis are induced leading to potentiation of malignant pathways.

Unrestricted growth

A normal cell maintains a healthy level of cell growth and division by controlling growth signals. However, a cancer cell has the unique ability to alter normal growth control signals thus facilitating tumor cell proliferation and survival. Often this is achieved by either continuous binding of the ligand to its cognate growth factor receptor by truncation of the extracellular domain of the growth factor receptor (e.g. epidermal growth factor receptor), by continuous production of growth factors, or simply
by receptor overexpression (e.g. Her2/neu) (Hanahan and Weinberg, 2000; Weinberg, 2007). Two of the most frequently perturbed growth factor signaling pathways are downstream of receptor tyrosine kinases: the Ras-Raf and the phosphatidylinositol 3 kinase (PI3K)/Akt pathways. Perpetuation of these pathways enables cells to have unlimited growth potential thereby contributing to an oncogenic phenotype (Mansour et al., 1994; Williams et al., 1993).

Ras proteins are GTPases that act as a molecular signaling switch controlling many cellular processes. Ras activation occurs upon binding of GTP. In its GTP-bound state, Ras binds an effector, such as Raf, and initiates the PI3K-Akt and MAPK signaling pathways. Mutations that prevent GTP hydrolysis inhibit Ras from becoming inactive inducing continuous stimulation of these downstream signaling cascades and leading to uncontrolled cell growth, differentiation and migration (Dhillon et al., 2007).

Activation of the PI3K pathway, potentially, though not exclusively, can occur through Ras activation and leads to activation of Akt (protein kinase B). Akt controls many normal cellular processes including cell growth and survival. Akt primarily promotes cell growth through regulation of mammalian target of rapamycin (mTOR). Downstream signaling through mTOR induces translation initiation, cell cycle progression and ribosome biogenesis (Jiang and Liu, 2007). Another way Akt mediates cell proliferation is through phosphorylation and inhibition of glycogen synthase kinase 3 (GSK3). GSK3 is involved in both proapoptotic and anti-proliferative mechanisms as it is responsible for initiating degradation of transcription factors involved in cell survival, cell cycle progression and activation of proapoptotic proteins (Manning and Cantley,
Therefore, inactivation of GSK3 through Akt-catalyzed phosphorylation of GSK3 facilitates cell cycle progression and cell survival. In malignancy, continuous activation of Akt serves to hijack the previously regulated, and therefore normal cellular processes, perpetuating the malignancy. In line, Akt is often overexpressed in carcinomas and more specifically, Akt2 expression in ovarian cancer has been linked to more aggressive cancer (Bellacosa et al., 1995; Larue and Bellacosa, 2005).

**Antiapoptotic potential**

In addition to potentiating pro-growth signals, cancer cells also often have decreased apoptotic ability and thereby increased survival. In normal cells, apoptosis and cell cycle arrest can be triggered by many different physiologic stimuli including DNA damage and hypoxia (Graeber et al., 1996). The tumor suppressor and transcription factor p53 is a key protein involved in the “sensing” of DNA damage. In unstressed or normal cells, both p53 activity and protein level is low. P53 activation upon detection of DNA damage initiates apoptotic cascades inhibiting the survival of cells harboring aberrations in DNA. Because of p53’s role in maintenance of genomic integrity, p53 is often referred to as the “guardian of the genome” (Kastan, 2007; Vogelstein et al., 2000). Disruption of p53 expression including loss of a p53 allele, mutation of the protein or altered expression of the protein can decrease normal apoptosis and cell cycle arrest preserving malignancy (Vousden and Prives, 2005). In fact, inactivation of p53 is seen in greater than 50% of cancers and has been shown to lead to many different cancers including but not exclusively, colon, prostate, breast, liver, cervix, brain and stomach (Hanahan and Weinberg, 2000; Vogelstein et al., 2000).
Akt, in addition to facilitating cell proliferation, also has a significant role in preserving cell survival primarily through the inhibition of proapoptotic proteins. In particular, Akt phosphorylation of Mdm2, the E3 ubiquitin ligase responsible for the degradation of p53, initiates p53 degradation thus causing loss of p53-induced apoptosis (Manning and Cantley, 2007). Akt also is responsible for the inhibitory phosphorylation on the proapoptotic family of transcription factors, FOXO, and the aforementioned p53. Phosphorylation of FOXO and p53 by Akt prevents the transcription of apoptotic and cell-cycle arrest proteins (Manning and Cantley, 2007)

**Invasion and metastasis**

During malignant progression cells change the nature of their attachment to the extracellular matrix (ECM) thus potentiating the movement of cells into the vasculature. Upon release from the ECM, cancer cells extravasate and invade a new location resulting in colonization of cancer cells at a site distant from the primary tumor. It is estimated that 90% of cancer deaths are attributed to this process of metastasis (Hanahan and Weinberg, 2000).

In order for invasion to occur, cancer cells must undergo a process called epithelial-mesenchymal transformation (EMT). EMT refers to the process by which epithelial cells acquire mesenchymal characteristics such as reduced intercellular adhesion and increased motility. Normally, this process occurs primarily during embryogenesis, however, post-embryogenesis, adaptations of cellular pathways can commit a cell to EMT thereby facilitating invasion. EMT can be induced by multiple signaling pathways including growth factors, the Wnt/β-catenin, Src and Ras pathways
and integrin activation (Larue and Bellacosa, 2005). One potent activator of EMT is activation of the PI3K/Akt pathway. Activated Akt signaling involves loss of cell adhesion, morphological changes, loss of cell polarization, induction of cell motility and decrease in cell-matrix adhesion; all characteristics of EMT (Larue and Bellacosa, 2005).

To facilitate the metastatic process, expression of matrix metalloproteinases (MMPs), by both epithelial and stromal cells, enhances digestion of the ECM and promotes ECM remodeling. In fact, MMPs are upregulated in many different types of carcinoma and their expression is correlated with poor patient survival (Egeblad and Werb, 2002). The substrates of MMPs are not limited to components of the ECM. Through disruption of cell-cell contacts, MMPs can access and cleave growth-factor-binding proteins, receptors and cell-adhesion molecules thereby regulating many facets of EMT (Egeblad and Werb, 2002).

Integrins are proteins involved regulating cell-ECM communication, cell shape, stress-fibre formation and migration (Hood and Cheresh, 2002). Considering the roles of integrins in various cell processes, it follows that changes in integrin expression patterns would be important for cell migration and invasion. In fact, expression of specific integrins may provide a way for cancer cells to promote their detachment from the ECM and it seems that specific integrin expression can be correlated with malignancy. For example, αVβ3 integrin is overexpressed in some malignancies, in particular, ovarian and breast cancers (Brooks et al., 1994; Davidson et al., 2003; Rolli et al., 2003).

Though above discussion has primarily focused on epithelial cell contributions to EMT, there is a clear contribution of the stroma to EMT. Stromal cells exert their
effects in a paracrine manner by secreting growth factors or matrix-degrading proteases that act on neighboring cells and the ECM, respectively. Fibroblasts within a cancer microenvironment produce growth factors that act in a paracrine fashion by binding to nearby epithelial cells. The tumor stroma also includes myofibroblasts that have been implicated in producing migratory cues that induce carcinoma cells to metastasize (Moustakas and Heldin, 2007).

The transition from an epithelial to a mesenchymal cell type aids the cell in movement from the primary tumor thus setting the stage for metastasis. Metastasis can occur through cells climbing through stromal tissue to colonize a new location or through cells using the circulatory system to facilitate relocation. The latter method of metastasis is potentiated by the process of angiogenesis primarily through increased accessibility of tumor cells to blood vessels.

**ANGIOGENESIS**

Tumor survival relies on the tumor cells resisting apoptosis, having unlimited replicative potential, unlimited growth potential and the ability to invade and successfully proliferate in other tissues. Many of those properties of cancer cells are dependent upon angiogenesis to promote tumor cell health by providing the tumor with oxygen and nutrients. To accomplish this, new vasculature must be constructed. This process is often termed “the angiogenic switch”. The angiogenic switch has been shown to occur at various stages during carcinogenesis and is dependent upon tumor type and tumor environment and often begins prior to malignancy, in a premalignant lesion (Bergers and Benjamin, 2003). By increasing blood vessel growth around the tumor, the tumor can not
only be fed nutrients and oxygen, but, the new blood vessels provide a roadway for tumor
cells to escape into the systemic circulation, leave the bloodstream and colonize a
location distinct from the primary tumor.

The vasculature in the adult is largely quiescent with just a few situations such
as inflammatory conditions and regenerating tissue, requiring angiogenesis during
adulthood. For angiogenesis to occur in the adult, the balance of proangiogenic factors to
antiangiogenic factors must be tipped to favor angiogenesis. Likewise, in angiogenic
situations, the expression of growth factors is favored while the expression of growth
inhibitors such as thrombospondin-1 and the statins (angiostatin, endostatin, canstatin and
turnstatin) is decreased (Bergers and Benjamin, 2003). In normal angiogenic processes
within the adult, the neovasculature matures rapidly and stabilizes whereas in tumor
angiogenesis, the new blood vessels do not become quiescent allowing constant
neovascularization. Hence, cancer is referred to as the “wound that never heals”
(Dvorak, 1986).

The vasculature’s failure to stabilize in tumorigenesis leads to an architecturally
different vessel as compared to normal blood vessels. The malignant tumor vasculature
is chaotic and circuitous with irregular shapes and dilation giving it a curly appearance.
The overproduction of capillary networks, lack of defined venules, arterioles and
capillaries, tortuousness and dead ends within the vessels differentiate tumor vasculature
from the regular grid-like pattern of normal vasculature. Further, tumor blood vessels are
highly permeable and thereby quite leaky (Ruoslahti, 2002).
New blood vessel growth begins with the migration of endothelial cells. Vascular endothelial growth factor-A (VEGF-A) has a dual function in that it also increases vasodilatation and permeability of existing blood vessels assisting extravasation of plasma proteins that lay down an interim matrix on which the activated endothelial cells migrate (Bergers and Benjamin, 2003). Concurrently, pericytes, cells that are related to vascular smooth muscle cells, are dislodged from the existing endothelium. Interestingly, pericytes are thought to lend stability to blood vessels. Further, there is a decrease in pericytes on tumor vessels (Lamalice et al., 2007; Ruoslahti, 2002). After the pericytes are dislodged, the basement membrane and ECM is degraded through tumor-cell-secreted MMPs, allowing migration of the endothelial cells towards the angiogenic stimuli. Once the endothelial cells have migrated, they proliferate filling the perivascular space creating a column on which other endothelial cells adhere. The basement membrane is then deposited, followed by limited attachment of pericytes to the endothelial cells (Bergers and Benjamin, 2003).

The expression and coordination of growth factors, growth factor receptors and integrins mediates the process of angiogenesis and creates a molecular profile that is very different from normal vasculature. The two largest differences between normal and tumor angiogenesis are the increased expression of growth factor receptors and the selective expression of tumor-specific integrins on both epithelial and endothelial cells (Ruoslahti, 2002). The differential expression profile of growth factor receptors and integrins promote the formation of new blood vessels on tumor-associated cells.
Endothelial cell migration and survival is mediated primarily through VEGF, basic fibroblast growth factor (bFGF) and angiopoietins. VEGF secretion is initiated by tumor cells that induce both a stabilization in VEGF mRNA and increased production of VEGF (Tonini et al., 2003). Paracrine and autocrine secretion of other growth factors such as PDGF, epidermal growth factor (EGF), tumor necrosis factor-α (TNF-α), TGF-β1, interleukin-1 by tumor cells also contribute to increased VEGF protein. VEGF and bFGF work to maintain survival of the newly mature endothelial cells by increasing expression of the antiapoptotic protein, Bcl-2 (Kim et al., 2000). BFGF induces VEGF production leading to an increase in MMP-1 and inhibiting tissue inhibitor of matrixmetalloproteinase-1 (TIMP-1) thus allowing pericyte invasion by ECM degradation (Lamalice et al., 2007).

**Vascular endothelial growth factor**

There are six VEGF family members including VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF). VEGF-A has five isoforms of which VEGF_{165} is the most abundant and active and often only referred to as VEGF. VEGF is not only produced by tumor cells themselves, but also by endothelial cells and vascular smooth muscle cells. VEGF signals through its tyrosine kinase receptors, VEGFR-1 (Flt-1), VEGFR-2 (Flk1Kdr), and VEGFR-3 (Flt4). The importance of the VEGFRs in angiogenesis and neovascularization is poignantly illustrated in the knockout mice for VEGFR1 and VEGFR-2 that show embryonic lethality as a result of vascular defects (Fong et al., 1995; Shalaby et al., 1995). Interestingly, the αVβ3 integrin can
associate with VEGFR-2, activating the receptor. The binding of the integrin to the ECM increases VEGF signaling in cell cultures (Borges et al., 2000; Soldi et al., 1999).

The role of angiopoietins (Ang) in neovascularization is largely promotion of endothelial survival via paracrine action; angiopoietins have low mitogenic or proliferative properties. There are four different angiopoietins with Ang1 and Ang2 having most importance in cancer. Both Ang1 and Ang2 induce endothelial cell migration and sprouting of new vessels. However, while Ang1 can induce angiogenesis alone, Ang2-mediated angiogenesis requires the presence of VEGF (Lamalice et al., 2007).

**Tumor hypoxia**

While hypoxia is not a traditional hallmark of cancer, in the context of angiogenesis, hypoxia is certainly essential to the process of new vascular growth. In this vein, hypoxia could be considered a hallmark of cancer.

Tissue hypoxia is a result of an inadequate supply of oxygen caused by poor oxygen diffusion, poor delivery of oxygen, low oxygen tension in the systemic blood, reduced tissue perfusion or inability of cells to use oxygen (Hockel and Vaupel, 2001). Tumor cells located more than 100 μm need to recruit new blood vessels as they quickly outgrow their blood supply. Without a blood supply and access to nutrients and oxygen nearby, the tumor cannot grow beyond one to two cubic millimeters. Solid tumors are frequently hypoxic in part because tumors quickly outgrow their blood supply thereby losing their oxygen reserve and also, because of defects in the delivery of oxygen.
Namely, this includes loss of the structural integrity of the vessel and the tortuous nature of tumor vasculature impeding efficient oxygen delivery.

Tissue hypoxia is estimated to be lower than 8 – 10 mmHg oxygen. Oxygen tension below this threshold induces many changes within the cell. Perhaps the largest adaptation that cells must make in a hypoxic environment is the switch from aerobic respiration to glycolysis. Normally, in the presence of oxygen, oxidative phosphorylation is carried out by the mitochondria and glycolysis is inhibited. However, in hypoxia, cells must adapt from making ATP in an oxygen-rich environment to manufacturing ATP from glycolytic mechanisms. This entails the conversion of glucose to pyruvate followed by generation of the waste product lactic acid. This process is termed anaerobic glycolysis. Therefore, in hypoxic cells, oxidative phosphorylation is impaired, ATP is depleted, levels of inorganic phosphate increase and intracellular acidosis occurs (Hockel and Vaupel, 2001).

While anaerobic glycolysis is a successful adaptation of hypoxic cancer cells, some of the byproducts of anaerobic glycolysis result in cellular damage. Increased acid production is one of the major byproducts generated by anaerobic glycolysis. This leads to significant decreases in extracellular pH resulting in p53 or caspase mediated apoptosis (Gatenby and Gillies, 2004). To combat the increased acid production, cells upregulate H⁺ transporters. Indeed, H⁺-ATPases have been found to be upregulated in cancer cells and may lead to resistance of apoptosis (Gatenby and Gillies, 2004). Interestingly, increased acid production can also work to benefit tumor invasion, metastasis and angiogenesis through acid-induced degradation of ECM (Gatenby and Gillies, 2004).
Another adaptation of hypoxic cancer cells in anaerobic glycolysis is the upregulation of select glucose transporters. As with H\textsuperscript{+}/ATPases, glucose transporters have also been observed to be higher in cancer cells (Gatenby and Gillies, 2004).

Alterations in cellular metabolism induce proteomic and genomic changes. These changes are estimated to occur between 1 – 15 mmHg and 0.2 – 1 mmHg oxygen, respectively (Hockel and Vaupel, 2001). The genomic instability induced by hypoxia promotes increased genomic heterogeneity and selection of clonal variants that exhibit resistance to apoptosis and an increase in angiogenic potential.

Hypoxia induces genomic instability in two ways. First, hypoxia can cause alterations in gene expression by creating DNA point mutations, double-strand breaks and chromosomal rearrangements as well as gene amplification potentially due to the generation of reactive oxygen species (Hockel and Vaupel, 2001). Often loss or mutation of p53 occurs in hypoxia due to hypoxic-induced genomic instability. Proliferation of the p53 null cells leads to population of clones that do not express p53 and therefore lack a key apoptotic mechanism (Huang et al., 2007). Thus, p53 null cells will potentially possess an increase in genetic defects. The second way hypoxia promotes genomic instability is through repression of DNA repair. Because ATP concentrations are low in hypoxic cells, cellular conservation of ATP occurs and is accomplished by curtailing DNA repair. This loss of DNA repair contributes to the generation of clonal variants and hypoxia-induced malignant progression (Hockel and Vaupel, 2001; Huang et al., 2007).

Hypoxic tumors also have increased resistance to chemotherapy and radiation. Radiation and some chemotherapeutic agents depend on oxygen radicals to induce DNA
damage. Radiosensitivity and chemotherapeutic efficacy is greatly reduced in hypoxia, where there is an inherent lack of oxygen and therefore less formation of oxygen radicals (Hockel and Vaupel, 2001; Huang et al., 2007). Further, the multitude of hypoxia-induced cell adaptations also contributes to chemoresistance. Characteristics such as inhibition of cell proliferation, high glycolytic rate coupled with increased tissue acidosis, loss of apoptotic potential and upregulation of genes involved in transmembrane glucose and drug transport, coordinate to increase chemoresistance (Hockel and Vaupel, 2001).

Patients harboring hypoxic tumors often have poor prognosis. Not only does the hypoxic environment encourage malignancy, survival adaptations employed by the tumor cell in hypoxia also exacerbates hypoxia-induced malignancy by facilitating growth of the tumor in hypoxia. It is the perpetuation of hypoxia and hypoxia-induced malignancy that leads to ensuing angiogenesis, metastasis and drug resistance.

**Hypoxia and angiogenesis in epithelial ovarian cancer**

The prognosis for epithelial ovarian cancer (EOC) is the most discouraging of all gynecological cancers. EOC accounts for 50% of deaths caused by female genital tract malignancies (Bamberger and Perrett, 2002). Because of the non-specificity of symptoms, EOC is usually diagnosed at later stages rather than at earlier stages. Patients often present at stage III or IV with abdominal swelling due to the accumulation of ascitic fluid from malignant ascites formation (Bamberger and Perrett, 2002).

As with many other solid tumors, angiogenesis is crucial to the growth and metastasis of solid tumors and EOC is not an exception. In general, a greater degree of neovascularization is associated with a poorer prognosis (Alvarez et al., 1999). As with
other tumors, VEGF plays a key role in driving angiogenesis in EOC and probably the neovascularization seen in EOC is at least partially due to overexpression of VEGF (Bamberger and Perrett, 2002). Indeed, expression of VEGF and VEGFR has been directly correlated with poor prognosis in EOC. In a comparison normal and malignant ovarian tissue, Wong et al. found that 75% of ovarian tumor sections express VEGF while normal ovarian sections did not express VEGF (Wong et al., 2002).

Not only do the epithelial cancer cells themselves show an increase in VEGF, but other cancer-associated tissues and fluids have also shown an increase in expression of VEGF (Bamberger and Perrett, 2002). Ovarian cancers frequently develop cysts that fill with fluid secreted from the tumor cells. Notably, VEGF concentrations in malignant cyst fluid are increased and further, serum of ovarian cancer patients also has increased levels of VEGF. The presence of VEGF in serum is also correlated with abbreviated survival (Hazelton and Hamilton, 1999; Tempfer et al., 1998).

Another prognostic indicator for EOC is microvessel density (MVD). MVD has been evaluated in colorectal, prostate, breast and ovarian cancer (Concato et al., 2007; Merritt and Sood, 2007; Rajaganeshan et al., 2007; Uzzan et al., 2004). In general, increased MVD is correlated with poor prognosis and increased metastasis. Further, MVD is also increased in EOC and this correlates with VEGF expression (Bamberger and Perrett, 2002). This finding is not surprising as VEGF is a strong propagator of endothelial migration and, as such, VEGF is an essential component in the progression of ovarian cancer through angiogenesis and metastasis.
HYPOXIA INDUCIBLE FACTOR-1α

Historical perspective

While cancer had been described for thousands of years and documented for hundreds, it wasn’t until the early 1970’s, that angiogenesis was first clearly defined in the context of cancer (Folkman et al., 1971). Approximately fifteen years later, the role of hypoxia in angiogenesis was discovered and work by Semenza’s group in the early 1990’s showed induction of erythropoietin (EPO) under hypoxic conditions (Folkman, 1985; Semenza and Wang, 1992). The same report isolated a nuclear protein called hypoxia inducible factor (HIF) from hepatoblastoma cells (Semenza and Wang, 1992). Almost concurrently, VEGF was found to be induced by hypoxia (Shweiki et al., 1992). A few years later, HIF was identified as the transcription factor responsible for VEGF induction in hypoxia (Forsythe et al., 1996).

HIF-1α in cancer

Hypoxia-inducible factor 1α is arguably one of the most influential transcription factors in tumorigenesis and certainly in angiogenesis. Its role in vascular pathologies is echoed in the HIF-1α knockout mouse that is lethal at embryonic day 10.5 due to cardiovascular malformations (Ryan et al., 1998). In cancer, overexpression of HIF-1α increases invasiveness, elicits poor prognosis and in some cases, decreases survivability (Table 1). The overall potentiating effect of HIF-1α in cancer can be distilled down to the individual regulation of genes by HIF-1α that are involved in tumorigenic process. HIF-1α regulates genes involved in cellular metabolism, invasion, metastasis, angiogenesis, drug resistance, ECM metabolism, cell adhesion, apoptosis, cell survival,
motility and vascular tone, among many other cellular processes. Through modulation of these pathways, HIF-1α can facilitate the malignant progression.

As stated earlier, in hypoxia, VEGF transcription is strongly driven by HIF-1α. Considering the increases in VEGF seen in EOC, it might be expected that HIF-1α levels are elevated as well. Indeed, HIF-1α and VEGF expression are correlated in EOC tumor sections isolated from patients (Wong et al., 2003). Further, though HIF-1α and VEGF are increased in stage III and IV, this study failed to detect a correlation between stage of EOC and expression of VEGF or HIF-1α (Wong et al., 2003). The correlation between HIF-1α and VEGF in EOC has been corroborated by a xenograft mouse model using human EOC cells (Jiang and Feng, 2006). Here, the authors found a correlation between HIF-1α and VEGF expression and further correlated HIF-1α expression and MVD.

Studies evaluating the use of HIF-1α as an independent prognostic indicator have not been as rewarding as the VEGF studies due to the contradictory nature of the results. In sum, HIF-1α overexpression has been found to be both a suitable and unsuitable prognostic indicator (Birner et al., 2001). Some reports suggest that HIF-1α is only a good prognostic indicator when overexpressed with p53 (Birner et al., 2001; Osada et al., 2007). Despite its controversial role as a prognostic indicator, expression of HIF-1α is clearly a very important element of EOC, probably through hypoxia-driven transcriptional regulation of many genes, including VEGF.

As shown, HIF-1α overexpression is found in various tumor types and in some cases is correlated with increased tumor progression. Loss of von Hippel Lindau protein
(VHL), an essential component of the HIF-1α degradation pathway, is another way increases in HIF-1α protein and subsequent transactivation can be accomplished. In fact, germ-line mutations in VHL lead to a familial tumor syndrome called von Hippel Lindau disease. Mutations in VHL have also been found in sporadic clear cell carcinomas of the kidney (Kapitsinou and Haase, 2008). VHL disease is characterized by highly vascularized tumors often found in the retina, central nervous system and kidney.

Table 1: Overexpression of HIF-1α levels in cancer

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Result of increased HIF-1α expression</th>
<th>Compounding genetic alterations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear cell renal</td>
<td>Increased tumor progression</td>
<td>Loss of VHL</td>
<td>(Di Cristofano et al., 2007)</td>
</tr>
<tr>
<td>Breast</td>
<td>Increased invasion</td>
<td>Loss of BRCA1</td>
<td>(van der Groep et al., 2007)</td>
</tr>
<tr>
<td>Ovarian</td>
<td>Correlation with progression and advanced stage</td>
<td>Loss of VHL</td>
<td>(Lee et al., 2007; Wong et al., 2003)</td>
</tr>
<tr>
<td>Endometrial</td>
<td>Correlation with progression and advanced stage</td>
<td></td>
<td>(Pansare et al., 2006)</td>
</tr>
<tr>
<td>Gastric</td>
<td>Increased invasion and no impact on survivability</td>
<td></td>
<td>(Cabuk et al., 2007)</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>Correlation with progression</td>
<td></td>
<td>(Ide et al., 2007)</td>
</tr>
<tr>
<td>Lung adenocarcinoma</td>
<td>Decreased survivability</td>
<td></td>
<td>(Enatsu et al., 2006)</td>
</tr>
</tbody>
</table>

The HIF-1 complex

HIF-1 is composed of an alpha and a beta subunit that together comprise an active transcription factor complex of the basic helix-loop-helix Per/ARNT/Sim (PAS) family of DNA-binding proteins (Wang et al., 1995). Expression patterns of human HIF-
messenger RNA show a ubiquitous distribution throughout tissues and organs with elevated expression levels in the kidney (Maynard and Ohh, 2007). In particular, HIF-1α expression has been found in epithelial cells, endothelial cells and vascular smooth muscle cells (Maynard and Ohh, 2007). Because of HIF-1α’s role in various vascular pathophysiologies, most research has focused on the expression of HIF-1α in these cells.

In contrast to many other proteins and transcription factors, regulation of HIF transcription is limited. Rather, regulation of the HIF-1 complex is primarily through posttranslational modification of the HIF-1α subunit. The HIF-1β subunit is an aryl hydrocarbon nuclear translocator protein (ARNT) that is constitutively transcribed and ubiquitously expressed (Carver et al., 1994). Interestingly, HIF-1α mRNA is constitutively synthesized, yet the protein is immediately degraded via ubiquitin-mediated proteosomal degradation unless the cell is in a low oxygen environment (Maxwell et al., 1999). In normoxia, HIF-1α has an extremely short half-life of approximately five minutes (Ke and Costa, 2006). Some caveats exist regarding the oxygen-dependant degradation of HIF-1α, as the protein can also be stabilized in normoxia by reactive oxygen species, reactive nitrogen species, growth factors and various cytokines (Fukuda et al., 2002; Sumbayev and Yasinska, 2007; Xia et al., 2007; Zhou et al., 2003).

**Structure of HIF-1α**

HIF-1α is a 125 kD protein composed of 826 amino acids (aas) with multiple domains important for its signaling (Figure 1). It contains a nuclear import signal at the N-terminus (17-33 aas), followed by the basic-helix-loop-helix (bHLH) motif between
amino acid residues 33 and 70. The cooperation of the bHLH and PAS (Per-ARNT-Sim) domains with HIF-1β induces maximal binding of the HIF-1 complex to the hypoxia response element (HRE) on DNA (Jiang et al., 1996). Likewise, the bHLH and PAS domains are required for both optimal DNA binding and heterodimerization. The oxygen-dependent degradation consists of amino acids 401-603, and, is not only the oxygen-regulated area, but also the most highly regulated region of the protein. Degradation of HIF-1α is triggered through modification of this domain in low oxygen tension (Huang et al., 1998). The HIF-1α protein also contains two transactivation domains, the N-terminal (N-TAD) located within the ODD from 531 – 575 aas and C-terminal (C-TAD) between 813 – 826 aas. The TADs, in particular, the C-TAD, are responsible for binding coactivators essential to the transcriptional activation of HIF-1α (Arany et al., 1996; Ebert and Bunn, 1998).

Transcriptional regulation of HIF-1α

The HIF-1α gene is constitutively transcribed, and, surprisingly, regulation of HIF-1α transcription is limited though there are some cell-specific differences (Wenger et al., 1997). Most regulation of HIF-1α occurs at the protein level either through modulation of protein translation pathways or by posttranslational modifications that
affect the stability of the protein. Hence, only a few reports discuss the regulation of HIF-1α transcription.

The HIF-1α promoter belongs to the TATA-less promoter family and is thus dependent upon Sp1, nuclear factor-1 (NF-1) and/or initiator sites located +1 either upstream or downstream of the transcription start site. Likewise, the HIF-1α promoter contains GC rich sequences that are putative Sp1 binding sites. The promoter also contains cis-acting hypoxia response elements (HIF-1 DNA binding sites), suggesting a possible positive autoregulatory mechanism for HIF-1α transcription by HIF-1α protein expression (Minet et al., 1999). Other elements within the HIF-1α promoter include NF-kB sites, AP-1, AP-2 and c-Ets-1 sites (Minet et al., 1999). While induction of NFκB has been found to increase HIF-1α transcription, the effect of AP-1 and 2, and Ets sites on HIF-1α gene transcription has not been evaluated (Bonello et al., 2007).

The regulation of HIF-1α mRNA by hypoxia is cell type specific. Many epithelial cell types have the same level of HIF-1α mRNA regardless of oxygenation status, other cells such as hepatoma cells, seem to have hypoxia-induced HIF-1α transcription (Minet et al., 1999; Wang et al., 1995; Wenger et al., 1997). Yet in endothelial cells hypoxia leads to a decrease in HIF-1α mRNA (Minet et al., 1999).

**Regulation of HIF–1α by prolyl hydroxylases**

Regulation of HIF-1α by oxygen is governed by the prolyl hydroxylase enzymes. The prolyl hydroxylases were originally discovered in *Caenorhabditis elegans* and called egg-laying abnormal (EGL) proteins (Epstein et al., 2001). Upon
identification of the mammalian homologs the proteins were renamed prolyl hydroxylase 1, 2 and 3 (PHD1, 2 and 3) (Epstein et al., 2001). The PHDs are a class of enzymes belonging to the iron and 2-oxoglutarate-dependant dioxygenase superfamily. Their oxygen-sensing capability is attributed to their need for oxygen as a cosubstrate in the hydroxylation reaction. Hence, in low oxygen conditions the PHDs can not function. The hydroxylation reaction also requires 2-oxoglutarate as a cosubstrate and Fe$^{2+}$ and ascorbate as cofactors (Berra et al., 2006) (Figure 2).

![Catalytic function of the prolyl-hydroxylase-domain proteins](image)

**Figure 2: Catalytic function of the prolyl-hydroxylase-domain proteins**

Figure 2: Catalytic function of the prolyl-hydroxylase-domain proteins. These enzymes need O$_2$ and 2-oxoglutarate as co-substrates, and Fe$^{2+}$ and ascorbate as co-factors. The hydroxylation reaction forms hydroxyproline and succinate. PHDs, prolyl-hydroxylase-domain proteins.

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The PHDs specifically target the sequence motif L-X-X-L-A-P. Two prolines within HIF-1α, 402 and 564, are hydroxylated by the PHDs. Although all three PHDs can hydroxylate Pro564, only PHD1 and PHD2 can hydroxylate Pro402 (Chan et al., 2005; Epstein et al., 2001). Additionally, the two sites are differentially regulated depending on the oxygen concentration, adding another layer to oxygen-dependant regulation of HIF-1α (Chan et al., 2005). Under conditions of low oxygen, Pro402 hydroxylation is lost first, followed by Pro564 hydroxylation. However, when oxygen is not limiting, Pro564 is hydroxylated first and enhances hydroxylation of Pro402 (Chan et al., 2005). Though not investigated, it may be that the seemingly differential roles of specific PHDs may be more reflective of varying PHD activity rather than the location of the sites and 3-dimensional folding of HIF-1α.

Although HIF-1α is almost exclusively detected in the nucleus, the location of its degradation has remained in question. This is in part due to the location of the PHDs. PHD1 is present only in the nucleus, PHD2 is located in the cytoplasm and PHD3 is found equally in both the cytoplasm and nucleus (Metzen et al., 2003). However, PHD2 can shuttle between the cytoplasm and nucleus. As follows, HIF-1α can be degraded in both locations and the specific location is dependent upon both cell type and proteasomal activity (Berra et al., 2001; Pereira et al., 2006).

In normoxia, destabilization of HIF-1α by the prolyl hydroxylation is accompanied by hydroxylation of an asparagine residue. Asp803 within the HIF-1α C-TAD is hydroxylated by factor-inhibiting HIF (FIH). The role of FIH and Asp803 in the
modulation of HIF-1α protein is two-fold. First, the binding of FIH to HIF-1α creates a binding pocket for interaction between FIH, HIF-1α and its E3 ubiquitin ligase, von-Hippel Lindau (VHL) (Lee et al., 2003). Second, the location of Asn803 within the C-TAD inhibits HIF-1α transcriptional activity by preventing binding of coactivators (Taylor, 2008). Therefore, posttranslational modification by the prolyl and asparaginyl hydroxylases is essential for oxygen-dependant degradation and the inhibition of HIF-1α transactivation in normoxia (Figure 3).

The hydroxylation of the HIF-1α prolines within the ODD provides a site for the β domain of VHL to bind (Tanimoto et al., 2000). VHL makes contact with HIF-1α in two distinct sites: between HIF-1α residues 560 – 567 and between residues 571 – 577 (Hon et al., 2002; Min et al., 2002) (Figure 3). Mutational analysis has shown another amino acid residue within the ODD to be important for the VHL-mediated degradation of HIF-1α. In vitro, Leu574 has been implicated in the recruitment of PHD2 for hydroxylation of Pro564 and appears to be essential for VHL recognition (Kageyama et al., 2004).

Upon binding of VHL to HIF-1α, elongin B and C are recruited to bridge the interaction between VHL and Cullin 2 (Krek, 2000). Cullin 2 assists E2 in the attachment of ubiquitin to sites Lys532, Lys538 and Lys547 thus targeting HIF-1α for degradation by the 26S proteasome (Clifford et al., 2001). Additionally, in normoxia, acetylation of Lys532 by arrest-deficient 1 protein (ARD1), an acetyl transferase, enhances the binding of HIF-1α to VHL (Jeong et al., 2002). Conversely, in hypoxia,
ARD1 expression is decreased and the affinity of ARD1 for HIF-1α hypoxia is reduced (Jeong et al., 2002) (Figure 3). Figure 3 shown below is a linearized HIF-1α, and not intended to be interpreted as a 3-dimensional representation.

Figure 3: Posttranslational modifications within HIF-1α that facilitate degradation

Figure 3: Hydroxylation on P402 is accomplished by PHD 1 or 2 while P564 is hydroxylated by PHD 1, 2 or 3. ARD1 acetylates K532 and FIH-1 hydroxylates N803. Upon hydroxylation of P402 and P564, VHL binds to HIF-1α. Acetylation of K532 and hydroxylation of N803 potentiates VHL-mediated degradation. Hydroxylation of FIH-1 also inhibits HIF-1α transactivation.

In vitro, HIF-1α stability can be induced chemically simply by the inhibition of prolyl hydroxylase activity. This effect can be accomplished by treatment of cells with transition metal ions or depletion of intracellular ascorbate. Cobalt and nickel have both been shown to stabilize HIF-1α in normoxia. Their inhibitory effect on the PHDs seems
to be three-fold. First, metal ions deplete intracellular ascorbate through inhibiting uptake by cells and second, by inhibiting PHDs through the oxidation of ascorbate (Salnikow et al., 2004). Thirdly, metal ions can substitute for the iron required of the PHDs thereby inhibiting the reaction (Davidson et al., 2006; Kaczmarek et al., 2007). Recently, induction of HIF-1α through angiotensin II treatment was shown to be via depletion of ascorbate by reactive oxygen species (Page et al., 2008).

The intricacies of the PHD reaction are subject to regulation by many factors including reactive oxygen and nitrogen species. In the context of cancer, reactive oxygen and nitrogen species may add another layer to the modulation of PHD activity. Through the generation of reactive oxygen and nitrogen species PHD activity may be modulated thereby affecting HIF-1α levels especially in normoxia.

**Regulation of HIF-1α by reactive oxygen and nitrogen species**

Reactive oxygen species (ROS) are found at high levels in cancer and may be involved in facilitating cell proliferation and migration through growth factor signaling (Kamata and Hirata, 1999; Liu et al., 2006; Xia et al., 2007). Further, high levels of ROS have been shown to induce activation of PI3K, MAPK and nuclear factor κB (NFκB) signaling, all signaling pathways known to be deregulated in cancer (Bowie and O'Neill, 2000; Rayet and Gelin, 1999; Stevenson et al., 1994). Considering 1) the role of HIF-1α in cancer, 2) the regulation of HIF-1α by oxygen and 3) the interconnection between HIF-1α and growth factor pathways, it follows that ROS may influence HIF-1α stability. Indeed, HIF-1α stabilization is induced by ROS in normoxia (Liu et al., 2006; Xia et al., 2007). Further, normoxic induction of VEGF by growth factors is attenuated with the
administration of a ROS scavenger suggesting that HIF-mediated angiogenesis requires
the generation of ROS (Liu et al., 2006; Xia et al., 2007). Liu et al. further delineated the
pathway by treating human ovarian cancer epithelial cells with epidermal growth factor
(EGF) and found EGF signaling generates ROS, activating Akt/p70S6K1 and leading to
HIF-1α stabilization and VEGF induction. Others have suggested an alternative
hypothesis that in normoxia, ROS stabilization of HIF-1α is due to the decreased
availability of reduced iron needed for the PHD reaction (Gerald et al., 2004).

Despite current research, the effect of ROS on HIF-1α remains controversial. In
particular, it is difficult to reconcile the effect of ROS on HIF-1α in hypoxia. ROS-
dependent HIF-1α expression, whether it be increased or decreased, may be reliant upon
the integrity of the mitochondria and especially, the electron transport chain. Some
researchers believe that hypoxic induction of ROS inhibits PHDs (Berra et al., 2006).
However, the inhibition of PHDs by ROS in hypoxia is accompanied by a loss of HIF-1α
protein levels (Berra et al., 2006). This effect is not predicted as upon loss of PHD
activity one might expect an increase in HIF-1α levels, not decrease. Others have
suggested a more likely explanation for ROS-induced HIF-1α destabilization in that any
insult in mitochondrial respiration or disruption of the electron transport chain, leads to
the destabilization of HIF-1α in hypoxia due to the release of oxygen from the
mitochondria (Gerald et al., 2004; Taylor, 2008).

As with the effect of ROS on HIF-1α, the regulation of reactive nitrogen species
(RNS) on HIF-1α is not clear. Both the cellular oxygen status and the species of RNS
can alter the effect of RNS on HIF-1α stabilization. In normoxia, RNS inhibit PHDs and thus HIF-1α is stabilized (Sumbayev and Yasinska, 2007). In hypoxia, it is suggested that RNS signaling can lead to mitochondrial damage that induces release of oxygen which is then available for use by the PHDs (Sumbayev and Yasinska, 2007). This theory is supported by a number of studies showing that inhibition of mitochondria leads to an induction of PHD activity and a loss of HIF-1α stability in hypoxia (Taylor, 2008).

Nitric oxide donors may have a direct effect on HIF-1α. This idea is supported by two studies that found HIF-1α to be a target for S-nitrosylation through generation of nitric oxide (Inna and Vadim, 2003; Li et al., 2007). Nitrosylation of HIF-1α on Cys800 facilitates the interaction between HIF-1α and its transactivating cofactors thus increasing HIF-1α transcriptional activity (Inna and Vadim, 2003; Yasinska and Sumbayev, 2003). Interestingly, nitrosylation of HIF-1α has also been shown to occur within the ODD on Cys533 to prevent the degradation of HIF-1α (Li et al., 2007). Further, disruption of the Cys533 nitrosylation of HIF-1α abrogates both radiation-induced and macrophage-induced stabilization of HIF-1α. In this way, RNS may directly modify HIF-1α affecting its stability. Whether there is coordination between the two nitrosylated HIF-1α sites and other posttranslational modifications on HIF-1α is not known.

**Cytokine regulation of HIF-1α**

Inflammation and cancer are two interconnected pathologies. As such, HIF-1α has become an interesting topic in the context of inflammatory versus angiogenic
signaling pathways and their cooperative effects on cancer. Unfortunately, studies evaluating the effect of cytokines on HIF-1α are few. Most reports focus on the effect of the NFκB pathway on HIF-1α and use tumor necrosis factor-α (TNF-α) or interleukin-1β (IL-1β) as activators of the NFκB inflammatory pathway.

In normoxia, Zhou et al. found that TNF-α treatment in normoxia resulted in HIF-1α accumulation, but also an increase in ubiquitinated HIF-1α. They also showed that inhibition of NFκB abrogates the TNF-α induced HIF-1α accumulation pointing to the importance of an intact NFκB pathway for the TNF-α dependent increase in HIF-1α protein (Zhou et al., 2003). Further, concurrent treatment with hypoxia and TNF-α augments HIF-1α DNA binding above TNF-α treatment alone (Hellwig-Burgel et al., 1999). Though the mechanism is not completely understood, the effect of TNF-α on HIF-1α stabilization may be through the generation of ROS (Sandau et al., 2001).

Besides TNF-α, IL-1β has been of particular interest in HIF-1α regulation. Like TNF-α, IL-1β induces DNA binding in both normoxia and hypoxia (Hellwig-Burgel et al., 1999). IL-1β has also been shown to increase HIF-1α protein synthesis increasing normoxic HIF-1α protein levels through NFκB activation (Jung et al., 2003).

**Regulation of HIF-1α by oncogenes, growth factors and phosphorylation signaling cascades**

Oxygen independent stabilization of HIF-1α can be achieved through a variety of pathways. Oncogene expression, increased growth factor and cytokine expression and potentiation of phosphorylation signaling cascades such as PI3K and MAPK pathways can both contribute to the stabilization of HIF-1α in normoxia. Normoxic stabilization of
HIF-1α is of particular importance to cancer cells as the cell is not reliant upon hypoxia to initiate angiogenic pathways. Further, oncogenes, growth factors, cytokines and their cognate signaling cascades are often perturbed in cancer. By stimulating HIF-1α expression and subsequently inducing HIF-1α transactivation, oncogenes, growth factors, and cytokines exponentially increase their individual roles in oncogenesis.

Oncogenic induction of HIF-1α was first alluded to in early work indicating a correlation between hypoxic-induced VEGF expression and both protooncogene c-Src and its oncogene counterpart, v-Src, expression (Mukhopadhyay et al., 1995). Semenza’s group later found that in normoxia, v-Src, but not c-Src, induced both HIF-1α expression and transcriptional activity (Jiang et al., 1997a). Though this was the first report to show that expression of an oncogene could induce HIF-1α expression, the authors did not suggest a mechanism. Concurrently, however, a report was published elucidating the mechanism of oncogenic stabilization of HIF-1α in normoxia (Mazure et al., 1997). Though this study did not evaluate v-Src, the authors showed activation of the Ras oncogene increasing HIF-1α and VEGF expression through activation of the PI3K pathway.

Activation of the Ras pathway affects both the MAP kinase pathway and the PI3K/Akt pathway thus influencing HIF-1α stabilization by two methods (Sodhi et al., 2001). Specifically, MAP kinase activation through oncogenic Ras stimulation, regulates the transactivation of HIF-1α through direct phosphorylation (Sodhi et al., 2001).
Moreover, inhibition of the Ras pathway activates glycogen synthase kinase 3 (GSK3), a kinase implicated in the phosphorylation of HIF-1α within the ODD (Sodhi et al., 2001).

In normoxia, both the PI3K and MAP kinase pathways are crucial in the regulation of HIF-1α translation and transactivation. These pathways are activated primarily through increased expression of growth factors such as EGF, insulin-like growth factor (IGF-1) and insulin (Treins et al., 2005). These growth factors signal through receptor tyrosine kinases to activate the PI3K/Akt and/or MAPK pathway.

Work by Semenza’s group investigated the role of the receptor tyrosine kinase Her2 in the stabilization of HIF-1α. They showed that both overexpression of Her2 and treatment of cancer cells with a Her2 agonist increases HIF-1α protein level through an increase in protein synthesis (Laughner et al., 2001). Induction of HIF-1α under these conditions requires PI3K, Akt and mTOR activity and implicates a role for the PI3K pathway in HIF-1α protein synthesis. This was the first report illustrating regulation of HIF-1α protein synthesis rather than modulation of the HIF-1α degradation pathway.

Treatment of cancer cells with IGF-1 also leads to stabilization of HIF-1α in normoxia (Fukuda et al., 2002). Here, inhibition of both the PI3K and the MAPK pathway abrogates the IGF-1 induced stabilization of HIF-1α. Inhibition of the PI3K pathway inhibits phosphorylation of key translational regulatory proteins such as 4E-BP1 and p70S6 kinase suggesting that IGF-1 regulates HIF-1α protein translation (Figure 4).

Only a minority of studies have investigated the effect of growth factors on HIF-1α in hypoxia. However, the few that have, show that in hypoxia, growth factor
signaling also leads to increases in HIF-1α protein translation. For example, Zhong et al. reported that in hypoxia, EGF signaling through PI3K/Akt and Akt’s effector, mTOR, can also lead to increased HIF-1α protein and target gene expression (Zhong et al., 2000). Here, treatment with rapamycin, an inhibitor of mTOR, and inhibitors of PI3K decreased HIF-1α expression. Further, expression of phosphatase and tensin homologue (PTEN), a tumor suppressor that is known to inhibit extracellular signal regulated kinase 1/2 (ERK1/2) and PI3K, reduced HIF-1α expression and gene transcription. This finding further implicated the PI3K pathway in HIF-1α regulation and more importantly, illustrates the vulnerability of HIF-1α to endogenous (PTEN) and exogenous (rapamycin) negative regulators of the PI3K pathway.

Though the specific mechanism whereby mTOR regulates HIF-1α is unknown, it has been suggested that mTOR directly phosphorylates HIF-1α, stabilizing the protein for initiation of target gene transcription (Land and Tee, 2007). In fact, HIF-1α does have an mTOR signaling motif and HIF-1α mutants lacking this motif are unable bind DNA and initiate transcription (Land and Tee, 2007). This mechanism may be an independent, yet parallel way for PI3K to mediate HIF-1α protein. Indeed, Akt has been found to increase HIF-1α protein translation independently of mTOR (Pore et al., 2006).

Activation of the PI3K pathway is known to generate reactive oxygen species (Kim et al., 2005; Liu et al., 2006; Park et al., 2004). As the PI3K pathway can clearly affect HIF-1α protein translation and possibly HIF-1α DNA transactivation, and ROS can increase HIF-1α stabilization in normoxia, it follows that the PI3K pathway could
affect HIF-1α purely through the generation of ROS. In line with this, EGF-generated ROS leads to increased stabilization of HIF-1α in normoxia, which is reversible upon treatment with a reactive oxygen scavenger (Liu et al., 2006).

In sum, activation of the PI3K/Akt pathway may have three separate effects on HIF-1α: 1) PI3K/Akt may increase HIF-1α protein translation, 2) PI3K/Akt signaling may increase ROS thus inhibiting PHD activity thus increasing HIF-1α stabilization, and 3) downstream PI3K/Akt signaling may inhibit a kinase involved in HIF-1α oxygen-independent degradation (Figure 4).

![Figure 4: Regulation of HIF-1α by PI3K](image)

**Figure 4: Regulation of HIF-1α by PI3K**

Figure 4: Activation of the PI3K/Akt pathway has three separate effects on HIF-1α: 1) PI3K/Akt increases HIF-1α protein translation through phosphorylation of eIF-4E and p70S6K, 2) PI3K/Akt signaling may increase ROS, and 3) downstream PI3K/Akt signaling inhibits GSK3 inducing HIF-1α oxygen-independent degradation.
The MAP kinase signaling cascade also contributes to the regulation of HIF-1α by directly phosphorylating HIF-1α and increasing its transcriptional activity. The MAP kinases are involved in a myriad of cell functions such as cell growth, differentiation, nucleotide metabolism and protein synthesis (Berra et al., 2000). They can be activated by growth factors and cellular stress, including hypoxia. Thus, it is not surprising that MAP kinases and growth factors have been shown to coordinate to positively affect HIF-1α dependant gene expression (Sutton et al., 2007). Rather than affecting the translational pathways, MAP kinases alter the transcriptional activity of HIF-1α through direct phosphorylation (Mylonis et al., 2006; Richard et al., 1999). Specifically, phosphorylation of HIF-1α by ERK1/2, members of the MAP kinase family, occurs at Ser641 and Ser643. This phosphorylation event has been proposed to enhance the transcriptional activity of HIF-1α by inhibiting nuclear export and thereby promoting nuclear accumulation of HIF-1α (Mylonis et al., 2006). Moreover, inhibition of MAP kinase phosphatase-1 (MKP-1), an inhibitor of ERK1/2, facilitates binding of p300/CBP, a cofactor essential to HIF-1α transcriptional activity, to HIF-1α and leads to an increase in HIF-1α activity (Liu et al., 2005). Taken together, this data suggests that ERK1/2 phosphorylation is essential for maximal HIF-1α transcriptional activity (Figure 5).
Figure 5: Regulation of HIF-1α by the MAP kinase pathway

Figure 5: HIF-α is phosphorylated by ERK1/2 thus enhancing CBP/p300 binding and increasing HIF-1α transactivation.

More recently, some of the nuances of ERK1/2 regulation of HIF-1α have been revealed. The effect of ERK1/2 on HIF-1α activation appears to be specific to the stimulus activating the MAP kinase pathway (Sutton et al., 2007). IGF-1 induction of HIF-1α in normoxia is dependent upon ERK1/2 activation for full transactivation of HIF-1α, but ERK1/2 does not seem to be important for hypoxia-induced HIF-1α transcriptional activity (Sutton et al., 2007). This effect is characteristic of HIF-1α regulation in that often the same stimulus generates different effects on HIF-1α depending on whether it is applied in normoxia versus hypoxia.
Though it is evident that MAP kinase activation facilitates HIF-1α transcriptional activity by directly phosphorylating HIF-1α within the C-TAD, the exact transcriptional mechanism remains unknown. One theory is that phosphorylation by ERK1/2 may enhance binding of transcriptional cofactors (Liu et al., 2005).

**Glycogen synthase kinase 3 regulation of HIF-1α**

GSK3 was originally identified as one of the proteins that phosphorylates and inactivates glycogen synthase (Frame and Cohen, 2001). Since its identification, GSK3 has since been recognized as a kinase involved in many key cellular processes and the diverse application of GSK3’s actions is exemplified by the number of pathologies in which its loss or gain is evident. Aberrant expression of GSK3 has been linked to muscle hypertrophy, cancer, bipolar mood disorder, Alzheimer’s disease, schizophrenia and diabetes (Frame and Cohen, 2001). In fact, disregulation of GSK3, including constitutive inhibition of GSK3, has been found in hepatocellular carcinoma, prostate cancer and squamous cell carcinoma of the tongue (Jope et al., 2007).

There are two isoforms of GSK3: GSK3α and GSK3β. While they share 97% sequence similarity, the difference lies in the N-terminal glycine-rich tail that is within GSK3α. Most research has focused on GSK3β and consequently, little is known about GSK3α. Recently, the GSK3α knockout mouse was developed and in stark contrast to the GSK3β knockout mouse, was viable but exhibited an increase in glucose tolerance and insulin sensitivity (MacAulay et al., 2007). The GSK3β knockout mouse is lethal at E14, about mid-gestation, from massive TNF-α induced hepatocyte apoptosis (Hoeflich
et al., 2000). These studies point to the different roles of GSK3α and β in embryogenesis and survival. Unless indicated otherwise, the following discussion refers to GSK3β.

GSK3 is inhibited by activation of PI3K pathway as activated Akt inactivates GSK3 by phosphorylating GSK3α on Ser21 and Ser9 on GSK3β. GSK3α/β can also be phosphorylated on Ser9/Ser21 by the terminal kinase in the MAP kinase pathway, ribosomal S-6 kinase (RSK or p70S6K), protein kinase A (PKA) and protein kinase C (PKC) (Jope and Johnson, 2004). Thus, growth factors signaling through the MAP kinase pathway can also affect GSK3 activation. Rapamycin has been shown to activate GSK3 activation by inhibiting mTOR and therefore, suppressing the activation of p70S6K1 (Armstrong et al., 2001). Interestingly, binding of p53 to GSK3 activates GSK3 and promotes the transcriptional and apoptotic actions of p53 (Watcharasit et al., 2002).

GSK3 is most active in the nucleus and mitochondria, and is present, though less active, in the cytoplasm (Jope and Johnson, 2004). Nuclear GSK3 levels fluctuate with the cell cycle peaking at S-phase to phosphorylate and inhibit cyclin D1. Nuclear levels of GSK3 also rise during apoptosis as GSK3 is involved in modulation the apoptotic process (Jope and Johnson, 2004).

GSK3 has many targets, including CREB, cyclin D1, jun, myc, nuclear factor activated T cells (NFAT), eukaryotic initiation factor 2B, tau and β-catenin among many others. The phosphorylation of a target protein by GSK3 results in one of three effects on the target protein: 1) the protein is targeted for ubiquitination and degradation, 2) the
protein is stabilized or 3) substrate phosphorylation facilitates the interaction between two proteins (Frame and Cohen, 2001).

GSK3 phosphorylates proteins through the consensus sequence Ser/Thr-X-X-X-Ser/Thr (Frame and Cohen, 2001). Often a priming phosphorylation event is needed at the C-terminal Ser/Thr in order to facilitate GSK3 phosphorylation. Casein kinase 2 (CK2), dual-specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1A) and PKA have all been shown to perform the priming phosphorylation event (Cohen and Goedert, 2004).

In the context of cancer, the effect of GSK3 on the transcription factor β-catenin is perhaps the most well-known. In the absence of a Wnt (wingless) signal, β-catenin is phosphorylated by GSK3 and targeted for ubiquitin-mediated proteasomal degradation (Cadigan and Liu, 2006). The phosphorylation of β-catenin by GSK3 creates a recognition sequence for the E3 ubiquitin ligase, β-transducin repeat containing protein (TrCP) to bind thus targeting β-catenin for proteasomal degradation and in effect, inhibiting β-catenin transcriptional activity (Cadigan and Liu, 2006).

**Degradation of HIF-1α through other pathways**

HIF-1α may be subject to degradation pathways other than ones initiated by PHDs and GSK3. Hypoxic degradation has been also shown to occur via a p53/Mdm2 mediated mechanism (Ravi et al., 2000). Loss of p53 enhances HIF-1α protein stability and transactivation in hypoxia (Ravi et al., 2000). However, the role of p53 and Mdm2 in HIF-1α stability is controversial as others have found that overexpression of Mdm2
results in an increase in HIF-1α protein stability and transactivation (Nieminen et al., 2005). Another oxygen-independent mechanism of HIF-1α degradation is through receptor of activated protein kinase C-1 (RACK1). In both normoxia and hypoxia, RACK1 induces HIF-1α proteosomal degradation (Liu et al., 2007). RACK1 binds within the amino-terminal half of HIF-1α and recruits Elongin B and C initiating the proteosomal degradation of HIF-1α (Liu et al., 2007).

**Regulation of HIF-1α by sumoylation**

Sumoylation is an important emerging cellular regulatory mechanism of HIF-1α. Sumoylation has been linked to regulation of many cellular functions including regulation of protein localization, stability and activity (Geiss-Friedlander and Melchior, 2007). In the case of HIF-1α, sumoylation has been shown to increase protein stability (Bae et al., 2004; Carbia-Nagashima et al., 2007). SUMO effects on HIF-1α transcriptional activity are less clear as studies have shown both an increase and decrease in HIF-1α transcriptional activity with SUMO modification (Bae et al., 2004; Berta et al., 2007). The location of the SUMO modification may determine the effect of sumoylation on HIF-1α transcriptional activity. One study showed HIF-1α sumoylation on Lys391 and Lys477 that confers an increase in transcriptional activity (Bae et al., 2004). A contrasting study identified the same sites, but indicated that sumoylation of HIF-1α decreases transcriptional activity (Berta et al., 2007). The majority of reports show that HIF-1α stability and transcriptional activity is increased by sumoylation however, there is insufficient research to definitively ascertain the effect of SUMO on HIF-1α.
HIF-1 transactivation

HIF-1 is responsible for initiating transcription of genes involved in cell processes such as proliferation, survival, apoptosis, motility, cytoskeletal structure, cell adhesion, angiogenesis and many more (Semenza, 2003) (Figure 6).
Figure 6: Genes that are transcriptionally activated by HIF-1

Genes that are involved in many processes are transcriptionally activated by HIF-1.

ADM, adrenomedullin; ALDA, aldolase A; ALDC, aldolase C; AMF, autocrine motility factor; CATHD, cathepsin D; EG-VEGF, endocrine-gland-derived VEGF; ENG, endoglin; ET1, endothelin-1; ENO1, enolase 1; EPO, erythropoietin; FN1, fibronectin 1; GLUT1, glucose transporter 1; GLUT3, glucose transporter 3; GAPDH, glyceraldehyde-3-P-dehydrogenase; HK1, hexokinase 1; HK2, hexokinase 2; IGF2, insulin-like growth-factor 2; IGF-BP1, IGF-factor-binding-protein 1; IGF-BP2, IGF-factor-binding-protein 2; IGF-BP3, IGF-factor-binding-protein 3; KRT14, keratin 14; KRT18, keratin 18; KRT19, keratin 19; LDHA, lactate dehydrogenase A; LEP, leptin; LRP1, LDL-receptor-related protein 1; MDR1, multidrug resistance 1; MMP2, matrix metalloproteinase 2; NOS2, nitric oxide synthase 2; PFKBF3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3; PFKL, phosphofructokinase L; PGK1, phosphoglycerate kinase 1; PAI1, plasminogen-activator inhibitor 1; PKM, pyruvate kinase M; TGF-α, transforming growth factor-α; TGF-β3, transforming growth factor-β3; TPI, triosephosphate isomerase; VEGF, vascular endothelial growth factor; UPAR, urokinase plasminogen activator receptor; VEGFR2, VEGF receptor-2; VIM, vimentin.
HIF-1 transactivation is regulated through the oxygen-sensitive N-TAD and posttranslational modifications within the C-TAD on HIF-1α. Though HIF-1β has a C-TAD, it appears to be dispensable for transactivation (Lisy and Peet, 2008). While the N-TAD lies within the ODD of HIF-1α and therefore may be controlled in part by oxygen, regulation of the C-TAD is largely oxygen-independent (Jiang et al., 1997b). Thus it is not surprising that the two transactivation domains are regulated independently of one another and are responsible for regulating transcription of their own set of genes (Dayan et al., 2006). Most HIF-1α target genes are under C-TAD regulation and thereby transcribed in the absence of prolyl and asparaginyl hydroxylation. However, the minority of HIF-1α transactivation that results from purely N-TAD activation occurs upon inhibitory hydroxylation of Asn803 within the C-TAD (Dayan et al., 2006).

Upon HIF-1α stabilization and nuclear translocation, HIF-1α dimerizes with HIF-1β and binds to hypoxia response elements (HREs). HIF-1 directly contacts the consensus sequence 5’-(A/G)CGTG-3’ initiating recruitment of transcriptional coactivators begins.

Despite studies that have shown HIF-1 transcriptional coactivators to include CREB binding protein (CBP) and p300, SRC-1 and transcription intermediary factor 2 (TIF-2), direct interactions have only been detected between HIF-1 and CBP/p300 (Lisy and Peet, 2008). CBP and p300 have two indispensable functions in HIF-1 transactivation. First, CBP/p300 provide a link between HIF-1 and the basal transcriptional apparatus. Second, CBP/p300 has histone acetyltransferase activity that is necessary for chromatin modification prior to transcription (Lisy and Peet, 2008). In
hypoxia, SRC-1 and TIF-2 work in concert with CBP/p300 to synergistically increase HIF-1 transcriptional activity (Carrero et al., 2000).

Research to date suggests that many posttranslational modifications control HIF-1α transcriptional activity, however, the data is conflicting. Some have reported that loss of the asparaginyl hydroxylation event in essential to HIF-1α transactivation (Cho et al., 2007; Lando et al., 2002). Others shown that Val802, Leu808, Leu814, Leu815 and Leu818 are crucial for HIF-1α transactivation, though the posttranslational modification occurring on these residues is not known (Ruas et al., 2002). The role of MAP kinase phosphorylation in HIF-driven transcription is controversial. Early studies suggest that MAP kinase phosphorylation of p300 enhanced the interaction between CBP/p300 and HIF-1α (Sang et al., 2003). Others show that MAP kinase phosphorylation within the HIF-1α C-TAD increases the transcriptional activity of HIF-1 (Sodhi et al., 2000). Still others show that MAP kinase phosphorylation does not have a role in transactivation, but rather has a role in inhibiting nuclear export of HIF-1α and that full HIF-1α transcriptional activity is attributable to the gain of nitrosylation and loss of asparaginyl hydroxylation (Cho et al., 2007; Mylonis et al., 2006; Yasinska and Sumbayev, 2003). Certainly posttranslational modifications within the C-TAD of HIF-1α are important for the regulation of HIF-1α-driven transcription, however, there is a lack of studies investigating the details of these posttranslational events.
SUMMARY AND UNANSWERED QUESTIONS

The concatenation of genomic instability, unrestricted growth, loss of antigrowth signals, antiapoptotic potential, invasion and metastatic pathways and angiogenesis initiates and propagates malignancy. Through tumor hypoxia, hypoxia inducible factor-1α exerts pleiotropic effects on many aspects of the hallmarks of cancer.

Though much is known about the crude regulation of HIF-1α by growth factors, cytokines and posttranslational modifications, many of the subtleties of HIF-1α regulation and transactivation remain unknown. As researchers gain insight to these subtle regulatory mechanisms, new ways to control HIF-1 may become evident thus generating new HIF-1-targeted therapies for cancer. With therapeutic targeting of HIF-1α in malignant cells, many of the aberrant cellular processes crucial to the survival of cancer cells would be inhibited. As such, HIF-1α is an attractive therapeutic target.

Although angiogenesis is clearly critical to the morbidity and mortality of ovarian cancer and with HIF-1α expression elevated in ovarian cancer and patients presenting with HIF-1α expressing tumors exhibiting a poorer prognosis, few studies have investigated the regulation of angiogenic signaling by hypoxia in ovarian tumor cells and more specifically, the effect of angiogenic signaling on HIF-1α. Both VEGF and EPO are transcriptionally regulated by HIF-1 and expression of both has been found to be elevated in ovarian and breast cancer as well as many other types of cancer (Acs et al., 2003; Acs et al., 2002; Geisler et al., 2007). Consequently, elucidating the regulation of these proteins is of great importance.
Clinically, exogenous EPO is given to cancer patients with chemotherapy related anemia. In addition to alleviating fatigue associated with chemotherapy, some studies have suggested that there is an improved response to chemotherapy with exogenous administration of EPO (Littlewood et al., 2001; Mittelman et al., 2004).

**Hypotheses**

The focus of this project has been on investigating the role of EPO and GSK3 in HIF-1α regulation and signaling. Because EPO is transcriptionally regulated by HIF-1α, possible feedback inhibition from EPO to HIF-1α was explored. Moreover, the effect of PI3K/Akt/GSK3 downstream signaling was investigated.

The hypotheses addressed in this work are as follows: 1) exogenous EPO exerts negative feedback on HIF-1α decreasing its stabilization and HIF-1-mediated VEGF transcription and 2) GSK3 phosphorylates HIF-1α targeting it for proteosomal degradation (Figure 7).
Figure 7: Hypotheses: Regulation of HIF-1α by EPO and GSK3

Figure 7: Hypothesis 1: EPO signaling inhibits HIF-1α protein stabilization and transactivation. Hypothesis 2: GSK3 phosphorylates HIF-1α thus targeting HIF-1α for ubiquitin-mediated proteasomal degradation.
CHAPTER 2: ERYTHROPOIEIN DISRUPTS HYPOXIA-INDUCIBLE FACTOR SIGNALING IN OVARIAN CANCER CELLS
Erythropoietin Disrupts Hypoxia-Inducible Factor Signaling in Ovarian Cancer Cells

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Running Title: EPO INHIBITS HIF-1\(\alpha\) SIGNALING

Keywords: HIF, VEGF, hypoxia, angiogenesis, cancer chemotherapy
Abstract

Objective: The objective of this study was to evaluate the effects of recombinant erythropoietin (EPO) on HIF-1α induced angiogenic pathways in ovarian cancer cells.

Methods: Using Western blots and both quantitative and non-quantitative RT-PCR, HIF-1α protein and VEGF transcription levels were assessed. Cell growth was measured using flow cytometry.

Results: EPO treatment decreased hypoxia-induced HIF-1α protein levels and VEGF transcription, with no effect on cell growth. Inhibition of HIF-1α signaling by EPO was also observed in MCF-7 breast cancer cells.

Conclusion: These novel findings suggest that EPO may exhibit anti-angiogenic properties thus encouraging further exploration of signaling pathways between EPO and HIF-1α.
Introduction

Angiogenesis is a key factor in the growth and metastasis of ovarian tumors [1-3]. The hypoxic core of the tumor initiates a signaling cascade that leads to expression of pro-angiogenic factors. Vascular endothelial growth factor (VEGF) is a potent angiogenic growth factor whose expression is regulated by hypoxia through the transcription factor hypoxia inducible factor -1 (HIF-1) [4]. HIF-1 is comprised of a constitutively expressed β subunit and an α subunit that is sensitive to cellular oxygen tension [5]. In normoxia, HIF-1α is targeted for ubiquitin-directed proteosomal degradation, through its interaction with the von Hippel Lindau tumor suppressor (VHL) [5]. Because oxygen-dependent prolyl hydroxylation is required for VHL binding to HIF-1α, hypoxia leads to HIF-1α stabilization, thus promoting transcription of target genes including VEGF and erythropoietin, as well as genes involved in glycolysis, adaptation to pH and apoptosis [5]. High levels of VEGF and HIF-1α have been correlated with aggressive tumor types, in particular, gynecologic malignancies [6, 7]. In addition, our laboratory has reported a strong correlation between high expression of VEGF and HIF-1α in advanced stages of ovarian cancer [7]. As such, HIF-1α is an attractive target for the development of therapies to combat VEGF-mediated angiogenesis in ovarian cancer.

Recombinant human erythropoietin (Epoetin alfa, EPO) is used to combat anemia in patients treated for chronic kidney disease and in cancer patients undergoing chemotherapy [8]. Given that erythropoietin expression is regulated by HIF-1α, we hypothesized that EPO may have an anti-angiogenic effect through negative feedback regulation of HIF-1α, resulting in reduced VEGF transcription. Here we present evidence
that EPO inhibits hypoxia-induced HIF-1α stabilization and VEGF transcription in ovarian cancer cells without affecting cell growth rate. This effect is not restricted to ovarian cancer cells, suggesting that EPO may exert anti-angiogenic activity in a variety of cancer cells.
Materials and Methods

Cell Culture Conditions. SK-OV-3 human ovarian cancer cells were maintained in McCoy’s Media (Mediatech, Herndon, VA) supplemented with 10 % FBS (Gibco, Carlsbad, CA). Penicillin/Streptomycin (Gibco) and L-glutamine (Gibco), MCF-7 cells, a gift from Dr. Mercedes Rincon (The University of Vermont, Burlington, VT) were maintained in supplemented RPMI 1640 (Gibco). For 2% O\textsubscript{2} hypoxia exposure, cells were incubated at 2% O\textsubscript{2} by N\textsubscript{2} injection into a humidified CO\textsubscript{2} incubator (Forma, Marietta, OH). For 0.1% O\textsubscript{2} hypoxia exposure cells were incubated in the above incubator with injection of pre-mixed gas containing 5% CO\textsubscript{2}, 95% N\textsubscript{2} and regulated by a ProOx oxygen controller (BioSpherix, Redfield, NY). EPO (Epoetin Alfa, PROCRIT®) was obtained from Ortho Biotech, Bridgewater, NJ. Cell culture reagents were obtained from Gibco (Carlsbad, Ca).

Semi-quantitative Reverse transcriptase-polymerase chain reaction (RT-PCR). RNA extraction was performed using the Trizol-chloroform method as described [9]. First strand cDNA was synthesized from 25 ng of total RNA by Sensiscript reverse transcriptase following the manufacturer’s protocol (Qiagen, Valencia, CA). PCR reactions were carried out following the manufacturer’s protocol (Invitrogen, Carlsbad, CA) with oligonucleotides recognizing human EPO-R (5’-GTGCTGGACAAATGGTTGCTG; 3’-AGGAGGATGCTTCTGAGCCTTC), 109 bp product), HIF-1α [10], VEGF [10], and β-actin [10] (Qiagen). The resulting products were analyzed by ethidium bromide/agarose gel separation and quantified using a Fluor-S Multi-imager (Bio-Rad, Hercules, CA) and Quantity One™ analysis software.
**Quantitative RT-PCR.** Extraction of total RNA was performed as described above. First strand cDNA was synthesized from 1.5 μg of total RNA by Omniscript reverse transcriptase following the manufacturer’s protocol (Qiagen, Valencia, CA). The PCR primers and Taqman probes for β2-microglobulin and VEGF were Assays-on-Demand products from Applied Biosystems (Foster City, CA). The PCR reactions were mixed and subsequent quantification of gene expression performed as described in [11]. Temperature cycling and real-time fluorescence measurement were performed using an ABI prism 7700 Sequence Detection System (Applied Biosystems). The relative quantitation of gene expression was performed using the comparative C_T (ΔΔC_T) method [11].

**Immunoblot.** Cultured SK-OV-3 and MCF-7 cells were grown to ~80% confluence. After treatments, cells were washed with ice-cold PBS, pH 7.4 and then harvested into hypotonic lysis buffer (HLB) containing: 25 mM Tris-HCl, pH 8, 2 mM MgCl_2, 5 mM KCl, 1 mM phenyl-methyl-sulfonamide, 20 μg/ml aprotinin, and 4 μg/ml leupeptin. Extracts were homogenized and centrifuged at 1,000 g to sediment the nuclear fraction. Nuclear pellets were washed, resuspended in 100 μl HLB and passed through a 26 ½ gauge syringe to shear DNA. Protein was determined by Bradford assay, and 25 μg from each sample separated by 8% SDS-PAGE. Proteins were transferred to nitrocellulose and analyzed by immunoblot using mouse monoclonal anti-HIF-1α (1:250; BD Biosciences, Bedford, MA) or rabbit polyclonal anti-EPO-R (1:200; Santa Cruz Biotechnology, Inc, Santa Cruz, CA) as described in Lounsbury et al. [12]. Reactive
bands were detected by chemiluminescence (LumiGLO, Kirkegaard & Perry, Gaithersburg, MD) and quantified using Quantity One™ analysis software.

**Immunofluorescence.** Cultured SK-OV-3 cells were grown to ~80% confluency on glass coverslips in 6-well culture dishes. After treatment, the cells were washed with ice-cold PBS, fixed with 4% paraformaldehyde in PBS for 15 min, and analyzed by immunofluorescence as described [10] using monoclonal mouse anti-HIF-1α (1:200; BD Biosciences, Bedford, MA) and CY3-anti-mouse IgG (1:500; Jackson IR Labs). Cells were counterstained with YOYO-1 ((1:10,000); Molecular Probes) containing 250 μg/ml RNase A for 30 min at 37°C. After being washed extensively with PBS, coverslips were mounted with Aqua Poly/Mount (Polysciences, Inc., Warrington, PA). Immunofluorescence was detected using a Bio-Rad 1000 laser scanning confocal microscope with a 40X objective.

**Flow cytometry.** Cells were seeded at low density, allowed to grow for 24 h then serum starved (0.1% FBS) 24 h. After serum starving, cells were placed in either normoxia or hypoxia and treated with or without 250 U/ml EPO for six days. Treatment media was replenished on days 2 and 5. On Days 0 (24 h after serum starving), Day 1, Day 3 and Day 6 cells were harvested for flow cytometry analysis. Cells were trypsinized, resuspended in PBS and centrifuged at 1200 g for 3 min. Cells were resuspended in 2% BSA in PBS. Each sample was counted twice for 60 s on an EPICS® XL / XL-MCL Flow Cytometry System with 488 nm Argon laser.
**Statistical Analysis.** One-way ANOVA was performed on all data. A Kruskal-Wallis adjustment was used where necessary. All pairwise multiple comparisons were assessed using the Newman-Keuls method of ANOVA.
Results

EPO prevents HIF-1α stabilization by hypoxia in ovarian cancer cells. To test our hypothesis that EPO exerts negative feedback on HIF-1α, SK-OV-3 cells were treated with EPO under either hypoxic or normoxic conditions, and then HIF-1α was detected in nuclear extracts by Western blot or in fixed cells by immunofluorescence. EPO significantly decreased HIF-1α levels induced by hypoxia at doses as low as 10 U/ml without altering normoxic levels of HIF-1α (Fig. 1A,B). Note that levels of HIF-1α and effect of EPO were not significantly different for hypoxia levels of 2% O₂ vs. 0.1% O₂. The inhibitory effect of EPO on HIF-1α protein was selective to the extent that exposure to VEGF did not significantly affect HIF-1α stabilization by hypoxia (HIF-1α protein levels relative to hypoxia alone (1.0): 0.754 ± 0.17 and 0.846 ± 0.09 for 5 and 50 ng/ml VEGF respectively; p > 0.1). Immunofluorescence confirmed that EPO did not reduce HIF-1α levels due to nuclear export (Fig. 1C). Furthermore, there was no effect of EPO on HIF-1α mRNA (Fig. 1D), suggesting that, as with other HIF-1α regulators [15], its reduction is likely caused by protein destabilization or decreased translation rather than by a decline in HIF-1α transcription.
Figure 1: EPO inhibits HIF-1α protein levels induced by hypoxia
Figure 1: A, SK-OV-3 cells were treated for 16 h with the indicated doses of EPO under normoxic or hypoxic (2% O₂) conditions. Shown is a representative Western blot of nuclear lysates (25 μg) using an antibody recognizing HIF-1α. “Loading” designates a non-specific reacting band that was used as a loading control. B, SK-OV-3 cells were treated for 16 h with the indicated doses of EPO under normoxia or hypoxic conditions (0.1% or 2% O₂, combined data) and analyzed by Western blot as in A. Shown are average HIF-1α band intensities, normalized to the loading control, and expressed as a percent of the hypoxia response; *, p<0.05, ** p < 0.01, ***p<0.001, n=4. C, Cells were analyzed by immunofluorescence using an antibody recognizing HIF-1α (red). Nuclei were stained with YoYo-1 (green). Shown are representative images, n=3. D, EPO does not affect HIF-1α mRNA levels. RNA was extracted from SK-OV-3 cells exposed to normoxia or hypoxia (2% O₂). Following reverse transcription, HIF-1α cDNA was specifically amplified using PCR. Shown is an agarose gel of the resulting RT-PCR amplified HIF-1α. β-actin was amplified as a reaction and loading control, n=3.
EPO treatment decreases VEGF transcription in SK-OV-3 ovarian cancer cells. Because HIF-1α has been shown to be the primary transcription factor leading to hypoxia-induced VEGF transcription [16, 17], the inhibition of hypoxia-induced HIF-1α protein levels by EPO was likely to result in a loss of hypoxia-induced VEGF mRNA. As expected, EPO significantly reduced hypoxia-induced VEGF transcription in a dose-dependent fashion determined using both semi-quantitative (Fig. 2A) and quantitative (Fig. 2B) RT-PCR analysis. These results confirm that EPO exerts a functional loss of HIF-1α function during hypoxia and support an important role for HIF-1α in hypoxia-driven VEGF transcription.
Figure 2: EPO decreases hypoxia-induced transcription of VEGF in SK-OV-3 cells

Figure 2: A, Cells were treated with EPO for 48 h under normoxic or hypoxic (2 % O₂) conditions. VEGF and β-actin mRNA levels were detected by RT-PCR. Shown is a representative agarose gel, n=3. B, Cells were treated as in A (0.1% or 2% O₂, combined data) followed by quantitative RT-PCR analysis of VEGF transcription; * p < 0.05, **p<0.01, ***p<0.001, n=4.
**EPO-R is expressed in SK-OV-3 ovarian cancer cells.** To verify that the observed effects of EPO on HIF-1α and VEGF could be mediated through the erythropoietin receptor (EPO-R), the expression of EPO-R was measured at the mRNA level using RT-PCR and at the protein level by Western blot. As shown in Fig. 3, EPO-R mRNA and protein were both readily detected, and hypoxia had no apparent effect on the level of EPO-R protein. Detection of EPO-R protein in MCF-7 cells was used as a positive control based on previous studies [18, 19]. Our results corroborate these studies and further indicate that the opportunity exists for a signaling pathway between EPO-R and HIF-1α in ovarian cancer cells.

**EPO does not affect the growth rate of SK-OV-3 ovarian cancer cells.** To determine if the EPO effects on angiogenic regulators were regrettably countered by increased cell growth, the rate of increase in cell number over time was determined by flow cytometry. Treatment of SK-OV-3 cells with EPO did not significantly alter the rate of normal cell growth under either normoxic or hypoxic conditions at any time point tested (Fig. 3C).
Figure 3: Erythropoietin receptor (EPO-R) is expressed in SK-OV-3 cells, and EPO does not affect cell growth rates

Figure 3: A, RNA was extracted from SK-OV-3 cells and EPO-R mRNA was amplified using RT-PCR. Shown is an agarose gel of DNA marker (M, lane 1) and amplified EPO-R (Lane 2). B, Cells were treated for 16 h in normoxia (N) or 2 % O$_2$ (hypoxia; H) followed by Western blot analysis of cell lysates using an antibody recognizing EPO-R. MCF-7 cell lysate was analyzed as a positive control. C, Cells were seeded at low density and treated where indicated with 250 U/ml EPO. Treated media was replaced on
day 2 and 5. Cell counts were measured by flow cytometry and values were normalized to day 0; n=3.

**Reduction of HIF-1α by EPO is not limited to ovarian cancer cells.** To explore the negative feedback of EPO on HIF-1α in other cancer cell types, we tested the effect of EPO on hypoxia-induced HIF-1α in the breast cancer cell line, MCF-7. Unlike SK-OV-3 cells, MCF-7 cells had undetectable levels of HIF-1α in normoxia. Similarly to SK-OV-3 cells, EPO efficiently inhibited the HIF-1α stabilization induced by hypoxia, suggesting that the observed negative feedback of EPO on HIF-1α is not limited to ovarian cancer cells.
Figure 4: EPO inhibits HIF-1α induction by hypoxia in other cancer cell lines

Figure 4: Cells were treated for 16 h with the indicated doses of EPO under normoxic and hypoxic (2 % O₂) conditions. Nuclear lysates (25 μg) were analyzed by Western blot using an antibody recognizing HIF-1α; n=2. Equal loading was verified using a non-specific band (Loading).
Discussion

Here we present a novel approach for reducing HIF-1α signaling in tumor cells by revealing an inhibitory effect of exogenous EPO on signaling pathways in hypoxic ovarian cancer cells. While previous studies have evaluated the expression levels of EPO and its receptor (EPO-R) and endogenous secretion of EPO in malignant tumors [20] our studies demonstrate direct inhibitory effects of EPO on hypoxic signaling through HIF-1α in ovarian and breast cancer cells.

Based on the known growth factor activity of erythropoietin [8], our observed decrease in hypoxia induction of HIF-1α levels in the presence of EPO was not inherently predicted. Several reports have shown that growth factors and cytokines upregulate HIF-1α protein and transcriptional activity [21-23]. However, unlike other growth factors and cytokines, erythropoietin is fundamentally regulated by HIF-1α, thus increasing the likelihood that it may exert a negative feedback role in its regulation. The current study corroborates other studies showing regulation of HIF-1α at the posttranslational level rather than at the transcriptional level as HIF-1α mRNA levels did not change with EPO treatment.

Our finding that the loss of HIF-1α response also corresponded with loss of VEGF transcription emphasizes the importance of HIF-1α in the hypoxia response. Although the dose-response effects paralleled those of HIF-1α protein, the inhibition of VEGF transcription was not as extensive, suggesting that the transcription response may have a HIF-1α-independent component. We and others have observed similar results in other
systems, leading to the proposal that VEGF transcription can occur through mechanisms independent of HIF-1α transcriptional regulation, or through different isoforms of HIF [11, 24-26].

One of the most detrimental effects of cancer progression is unrestricted cell growth caused in part by an increase in growth factors. EPO signals through the EPO-R triggering activation of the Janus kinase (JAK/STAT) pathway, which is associated with increased growth and survival of cancer cells [27]. In results presented here, hypoxia significantly abrogated the rate of cell growth as expected [28], and EPO did not alter the growth rate under either normoxic or hypoxic conditions. Though not significant, treatment with EPO slightly enhanced the inhibitory effect of hypoxia, suggesting that EPO does not rescue the growth suppression phenotype of cells exposed to hypoxia.

There has been considerable debate over whether it is detrimental to administer EPO to cancer patients receiving chemotherapy. Cancer patients undergoing chemotherapy who have received EPO exhibit a trend toward improved survival [29, 30], however a recent report showed that exogenous EPO may facilitate the growth of tumors and contribute to a decrease in survivability [19]. There are reports of EPO exerting both pro-angiogenic and anti-apoptotic effects on endothelial cells and MCF-7 cells respectively [9, 31] and molecular studies have implicated high levels of erythropoietin and erythropoietin receptor (EPO-R) in progression of tumor growth [18, 19].

Our results support an anti-angiogenic mechanism for EPO in dividing ovarian cancer cells under hypoxic conditions, and do not indicate an effect of EPO on growth rate. Importantly, these results are supported by an in vivo antitumor effect of EPO on ovarian
cancer when combined with cisplatin [32]. The discrepancies between results related to anti-apoptotic effects are likely due to our observations of signaling effects under hypoxic conditions, where signaling pathways are likely altered. Also, while correlations of EPO and tumor progression are indicative of a relationship, it is difficult to define whether the expressed EPO is itself acting to increase tumor progression. Nevertheless, despite our confidence that EPO has anti-HIF-1α activity, the potential for unfavorable effects of EPO encourage further studies defining the scope of the observed effect and the mechanisms underlying HIF-1α inhibition by EPO.

Together these studies reveal a novel approach to inhibiting angiogenesis through the discovery that hypoxia-induced VEGF transcription is disrupted by administration of EPO in ovarian cancer cells. Angiogenesis is critical for the growth and metastasis of multiple tumor types [6]. Thus, these results broaden the potential impact that EPO administration may have in reducing angiogenesis in a variety of cancers and encourage pursuit of both the molecular mechanism of EPO action as well as the effect of EPO on ovarian tumor angiogenesis in vivo.
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References


**Article Précis**

Erythropoietin decreased HIF-1α protein and VEGF transcription in ovarian cancer cells with no effect on cell growth, suggesting an inhibitory role for erythropoietin in angiogenesis.
CHAPTER THREE: GSK3 TARGETS HIF-1α FOR PROTEASOMAL DEGRADATION DURING HYPOXIA THROUGH MODIFICATION OF THE OXYGEN-DEPENDENT DEGRADATION DOMAIN
GSK3 targets HIF-1α for proteasomal degradation during hypoxia through modification of the oxygen-dependent degradation domain

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Running Title: GSK3 regulation of HIF-1α

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Abstract

The transcription factor hypoxia inducible factor-1α (HIF-1α) facilitates many oncogenic processes including cell survival, proliferation, angiogenesis and metastasis through transcriptional activation of vascular endothelial growth factor (VEGF) among many other targets. Despite current research describing HIF-1α signaling and regulation, the role of phosphorylation of HIF-1α during hypoxia remains uncertain. The oxygen-dependent degradation domain within HIF-1α contains consensus sequences for phosphorylation by GSK3. Considering the canonical role of GSK3 in proteasomal degradation of other oncoproteins such as c-Myc and β-catenin, we tested whether phosphorylation by GSK3 targets HIF-1α for proteasomal degradation. Treatment of ovarian cancer cells with a specific GSK3 inhibitor led to a time-dependent increase in stabilized HIF-1α and an increase in VEGF transcription. Conversely, overexpression of constitutively active GSK3β in HEK293 cells led to both a decrease in hypoxia-driven HIF-1α protein levels and VEGF transcription. Interaction between GSK3β and HIF-1α was also detected through co-immunoprecipitation. Mutation of the putative GSK3 phosphorylation sites within HIF-1α promoted an increase in protein level and a decrease in ubiquitination of the HIF-1α mutant protein. This study illustrates a mechanism in which posttranslational modification by GSK3 attenuates hypoxic HIF-1α through ubiquitination and subsequent proteasomal degradation thus decreasing VEGF transcription and modulating downstream oncogenic pathways.
Introduction

Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor subunit that when expressed in cancer is often a predictor of increased mortality (Semenza, 2003). This correlation has been demonstrated in a variety of cancers including, but not limited to, breast, cervical, gastrointestinal and ovarian cancer (Semenza, 2003). HIF-1α is constitutively transcribed, yet the translated protein undergoes rapid oxygen-dependent degradation thus is primarily stabilized only in hypoxia. Under normoxic conditions, two prolines, P402 and P564, within the HIF-1α oxygen-dependent degradation (ODD) domain are hydroxylated by the oxygen-dependent prolyl hydroxylases (Ivan et al., 2001; Jaakkola et al., 2001). This modification allows von Hippel Lindau protein (VHL), the recognition component of the E3 ubiquitin ligase complex, to bind and target HIF-1α for proteasomal degradation.

HIF-1α is subject to a number of other posttranslational modifications that can also radically alter its activity. For example, hydroxylation of an asparaginyl residue within the COOH-terminal transactivation domain prevents binding of CBP/p300, a transcriptional coactivator (Lando et al., 2002). This hydroxylation in concert with the two proline hydroxylations are required for full inactivation and destabilization of HIF-1α in normoxia (Lando et al., 2002). Other modifications on HIF-1α include nitrosylation, sumoylation and phosphorylation (Bae et al., 2004; Berta et al., 2007; Carbia-Nagashima et al., 2007; Li et al., 2007; Mylonis et al., 2006; Richard et al.,
1999). These modifications largely lead to stabilization of HIF-1α, although the effect of the particular modification is dependent upon the location of the modifications within the HIF-1α protein.

Stabilized HIF-1α translocates to the nucleus and dimerizes with its constitutively expressed β subunit, HIF-1β/ARNT, forming the active HIF transcription factor complex that targets hypoxia response elements (HREs). One of the key target genes activated by HIF is vascular endothelial growth factor (VEGF). VEGF promotes tumor progression through increasing vascular permeability and angiogenesis thus providing both tumor oxygen supply and a mechanism for nonadherent cells to circulate and metastasize (Connolly et al., 1989).

Aside from hypoxia-mediated stabilization, HIF-1α is also known to be stabilized by activators of the PI3 kinase pathway including reactive oxygen species and growth factors (Chandel et al., 2000; Daniel et al., 2002; Sandau et al., 2000). Activation of the PI3K pathway, leading to downstream activation of Akt (protein kinase B), has also been implicated in increasing the translation of HIF-1α (Mottet et al., 2003; Pore et al., 2006; Treins et al., 2002; Zhong et al., 2000). Among targets of Akt is glycogen synthase kinase 3 (GSK3). Activation of Akt results in inhibition of glycogen synthase kinase 3 (GSK3) via direct Akt phosphorylation (Cross et al., 1995). Although GSK3 was initially identified as a regulator of glycogen synthesis, it is now known that GSK3 is a negative regulator of numerous oncogenic transcription factors including β-catenin, c-Jun, c-Myc, and cyclin D (Gregory et al., 2003; Ikeda et al., 1998; Nikolakaki et al.,
Further, it is a putative negative regulator of HIF-1α (Mottet et al., 2003; Sodhi et al., 2001).

GSK3 confers substrate specificity through a distinct sequence present within the substrate. Substrates for GSK3 contain the consensus sequence: Ser/Thr-X-X-X-Ser/Thr (Fiol et al., 1987). For many substrates, phosphorylation by GSK3 requires a priming phosphorylation event on the C-terminal Ser/Thr before GSK3 can phosphorylate the remaining site(s). GSK3 phosphorylation of β-catenin and c-Myc exerts negative regulation through initiating the process of protein ubiquitination and proteasomal degradation while the effect of GSK3 phosphorylation on other transcription factors includes inhibition of DNA binding and transactivation (Gregory et al., 2003; Ikeda et al., 1998; Nikolakaki et al., 1993; Piwien-Pilipuk et al., 2001).

Although the HIF-1α degradation pathway through oxygen-dependent binding to VHL and proteasomal degradation has been relatively well characterized, few mechanisms of HIF-1α regulation during hypoxia have been identified. Due to the role of GSK3 in the targeting of oncogenic transcription factors such as c-Myc and β-catenin for degradation, we considered GSK3 as a candidate for hypoxia-specific modification of the oxygen-dependent degradation domain of HIF-1α. Here, we provide data in support of the hypothesis that HIF-1α is a target for negative regulation by GSK3 phosphorylation during hypoxia, leading to its ubiquitination and proteasomal degradation.
Materials and Methods

Cell Culture Conditions. SK-OV-3 human ovarian cancer cells were maintained in McCoy’s Media (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA), penicillin/streptomycin (Gibco) and L-glutamine (Gibco). HEK293 human embryonic kidney cells were maintained in DMEM (Mediatech, Herndon, VA) supplemented with 10% bovine growth serum (Hyclone, Logan, UT), penicillin/streptomycin and L-glutamine. Hypoxia (2% O$_2$) was achieved by N$_2$ injection into a humidified CO$_2$ incubator outfitted with an O$_2$ sensor (Forma, Marietta, OH). For ubiquitin experiments, cells were treated with 10 μM MG132 (Calbiochem, San Diego, CA). For GSK3 inhibitor experiments BIO (Calbiochem) was used.

Plasmids and Transfection. Mammalian expression plasmids (pcDNA3) expressing wild-type HIF-1α and the constitutively active form of GSK3β (GSK3S9A) were obtained from Erik Huang, NCI, and James Woodgett, University of Toronto, respectively. Mutations in wild-type HIF-1α were performed using Qiagen’s site-directed mutagenesis kit (Quik Change multi, Valencia, CA) following the manufacturer’s directions. Primer sequences used are as follows: for T498V: forward: 5’ CAGATTCAGGATCAGGTACCTAGTCCTTCCG 3’, reverse: 5’ CGGAAGGACTAGGTACCTGATCCTGAATCTG 3’; for S502A: forward: 5’ GACACCTAGTCCTGCCGATGGAAGCAC 3’, reverse: 5’ GTGCTTCCATCGGCAGGACTAGGTGTC 3’; for T506V: Forward: 5’ CCTGCCGATGGAGCCGTTAGACAAAGTGAC 3’, Reverse: 5’
GTGCACTTTGTCTAAGGGCTCATCGGCAGG 3'; and for S510A: forward: 5' GAAGCACTAGACAAAGTGCACCTGAGCCTAATAG 3', reverse: 5' CTATTAGGCTCAGGTGCACTTTGTCTAGTGCTTT 3'.

For transfections, HEK293 cells were seeded 24 h prior to transfection at 1.25 x 10^6 cells/100 mm plate. Cells were transfected using Superfect™ (Qiagen) following the manufacturer’s instructions, using 2.5 μg DNA for the HIF-1α constructs and 1 or 5 μg for the GSK3S9A construct. Cells were incubated for 24 h post-transfection before treatment was initiated.

Quantitative RT-PCR. Extraction of total RNA was performed using an RNeasy kit (Qiagen) following the manufacturer’s instructions. First strand cDNA was synthesized from 1.5 μg of total RNA by Omniscript reverse transcriptase following the manufacturer’s protocol (Qiagen). The PCR primers and Taqman probes for β2-microglobulin, VEGF, and HIF-1α were Assays-on-Demand products from Applied Biosystems (Foster City, CA). PCR temperature cycling and real-time fluorescence measurement were performed using an ABI prism 7700 Sequence Detection System (Applied Biosystems). The relative quantitation of gene expression was performed using the comparative C_T (ΔΔC_T) method (Dong et al., 2004).

Immunoblot. Cell plates were immediately placed on ice, washed with ice-cold PBS, pH 7.4 and then harvested into 300 μl hypotonic lysis buffer (HLB) containing: 25 mM Tris-HCl, pH 8, 2 mM MgCl_2, 5 mM KCl, or modified RIPA buffer (1% NP40, 0.5%
Sodium deoxycholate, 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol) containing 1 mM phenyl-methyl-sulfonamide, 20 μg/ml aprotinin, and 4 μg/ml leupeptin. Extracts were homogenized or sonicated with 10 pulses on 4 watts (HEK293 cells). Protein was determined by Bradford assay, and 100 μg from each sample separated by 8% SDS-PAGE. Proteins were transferred to nitrocellulose and analyzed by immunoblot as described (Lounsbury et al., 1994), using mouse monoclonal anti-HIF-1α (1:250; BD Biosciences, Bedford, MA) mouse monoclonal anti-ubiquitin clone P4D1 (1:500; Santa Cruz Biotechnology, Inc, Santa Cruz, CA), or mouse monoclonal anti-HA (1:6000; 12CA5 clone, a gift from Nicholas Heintz, University of Vermont) and β-actin (1:1000; Cell Signaling, Ipswich, MA).

**Immunoprecipitation.** For HIF-1α and GSK3 kinase assay immunoprecipitations:

Whole cell extracts were obtained as described for Immunoblot. Following preclear with 50% Sepharose beads (Sigma), extracts were incubated with 2.5 μg mouse anti-HA or 3 μg rabbit anti-HIF-1α (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at 4°C. Protein A/G Sepharose beads (Santa Cruz Biotechnology) were added for 2 h at 4°C, and precipitates were washed 2X with mRIPA buffer, 1X with high salt buffer (500 mM LiCl/100 mM Tris pH 8.6), 1X with low salt buffer (1:5 dilution of the high salt buffer) and then 2X with mRIPA buffer (as described above). For ubiquitin immunoprecipitations: whole cell extracts were obtained as described for Immunoblot. SDS was added to lysates to a final concentration of 1%, followed by incubation at 55°C for 10 minutes. Immunoprecipitation was performed as described above, except
immunoprecipitates were washed 1X with RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 1mM EDTA) following first two washes in mRIPA. The remaining washes were the same as above. Immunoprecipitates were then resolved using 8% SDS-PAGE (6% for ubiquitin immunoprecipitations) and transferred to nitrocellulose for immunoblotting.

**In-vitro Kinase Assay.** Cell lysates were obtained as described for Immunoblot. 400 U lambda phosphatase (Ppase) (New England Biolabs (NEB), Ipswich, MA) was added to 1.5 mg lysate supplemented with 1X lambda Ppase buffer (NEB) and 2 mM MnCl₂ (NEB). Lysates were incubated at 30°C for 30 m. Immunoprecipitation of HA-HIF-1α was performed as described above except immunoprecipitates were washed an additional 2X with GSK3 kinase buffer (NEB). Immunoprecipitates were incubed at 30°C for 30 min with 1X GSK3 buffer and 500 U active GSK3β supplemented with 200 μM ATP, 1 mM β-glycerophosphate and 1 mM NaF.

**Statistical Analysis.** One-way ANOVA was performed on all data. A Kruskal-Wallis adjustment was used where necessary. All pairwise comparisons were assessed using the Student’s t-test.
Results

Inhibition of the PI3K pathway decreases HIF-1α stabilization while inhibition of GSK3 enhances HIF-1α stabilization in hypoxia.

Others have found a correlation between PI3 kinase and an increase in HIF-1α protein, yet the mechanism has not been well characterized. In agreement with these findings, inhibition of the PI3 kinase with LY294002 reduced the hypoxic induction of HIF-1α protein in SK-OV-3 cells (Figure 1a). Because PI3 kinase activity leads to inactivation of GSK3, we tested whether a selective GSK3 inhibitor would mimic an endogenous PI3 kinase effect. Treatment with the GSK3 inhibitor led to increased stabilization of HIF-1α and VEGF transcription that was significant at 4 and 8 h (Figure 1b, d). The GSK3 inhibitor was further able to delay the loss of HIF-1α protein levels during reoxygenation (Figure 1c). These data indicate that GSK3 exerts negative regulation of HIF-1α protein level and function and suggest that inhibition of GSK3 may contribute to the mechanism whereby PI3 kinase induces HIF-1α.
Figure 1: PI3 kinase inhibition decreases HIF-1α stabilization and GSK3 inhibition enhances HIF-1α stabilization and transcriptional activity in hypoxia.

(a) SK-OV-3 cells were treated concurrently with hypoxia and DMSO (vehicle) or 50 μM LY294002 for times indicated. HIF-1α and β-actin proteins were detected by immunoblot. (b) SK-OV-3 cells were treated concurrently with hypoxia and DMSO or 1 μM glycogen synthase kinase 3 inhibitor (GSK3 Inh) for the times indicated. HIF-1α and β-actin proteins were evaluated as in (a). (c) SK-OV-3 cells were exposed to 2% O₂ for 4 h in the presence of DMSO or 1 μM GSK3 inhibitor and then returned to 21% O₂ (Reox) for the times indicated. HIF-1α and β-actin protein were evaluated as in (a). (d) SK-OV-3 cells were treated as in (b). Quantitative RT-PCR was performed to evaluate VEGF transcription. Statistical comparisons were made between the 1, 4, 8 and 16 h
time points. VEGF transcription was significantly different between the 4 h treatments and the 8 h treatments. Data shown are representative of three independent experiments; * p < 0.01.

**Overexpression of a constitutively active GSK3β decreases HIF-1α protein stabilization and HIF-1α transcriptional activity in hypoxia.**

To confirm that GSK3β acts as a negative regulator of HIF-1α during hypoxia, an HA-tagged constitutively active mutant of GSK3β, GSK3S9A, was expressed in cells prior to hypoxia exposure. Cells expressing GSK3S9A exhibited a decreased hypoxic HIF-1α protein level that was also reflected by a 30% decrease in VEGF transcription (Figure 2). There was no effect of GSK3S9A in normoxia, presumably because endogenous GSK3β is active during normoxia, and levels of HIF-1α are already low due to VHL-mediated ubiquitination.

![a](image1.png)  

**Figure 2: Overexpression of GSK3 decreases HIF-1α stabilization and transcriptional activity.** (a) HEK293 cells were transfected with either 1 μg or 5 μg of pcDNA3 (empty vector) or pGSK3S9A, a constitutively active GSK3 plasmid. 24 h after
transfection, cells were exposed to 2% O$_2$ for 16 h. Protein for HIF-1α, HA, and β-actin was analyzed using immunoblotting. (b) Cells were transfected with 5 μg of pcDNA3 or pGSK3S9A and treated as in (a). RNA was then isolated followed by measurement of relative VEGF mRNA levels using RT-qPCR. RNA content between samples was normalized using β2-microglobulin levels. The data shown are representative of at least three independent experiments; *p< 0.01.

**HIF-1α interacts with constitutively active GSK3β**

To further verify that HIF-1α can be regulated by GSK3β, co-immunoprecipitation experiments were used to establish whether HIF-1α interacts with expressed GSK3S9A. As shown in Figure 3, immunoprecipitation with anti-HA antibodies resulted in specific pull-down of HIF-1α that was increased when proteasomal activity was blocked using MG132 (Figure 3a). Conversely, immunoprecipitation using anti-HIF-1α antibodies resulted in specific pull-down of GSK3S9A (Figure 3b). Together these data indicate that HIF-1α is capable of physically interacting with GSK3β, further supporting the hypothesis that HIF-1α can be directly regulated by GSK3β.
Figure 3: HIF-1α interacts with GSK3β. (a) HEK293 cells were transfected with the indicated plasmids and then exposed to 2% O₂ for 8 h in the absence or presence of the proteasome inhibitor MG132 (10 μM). Lysates (1.5 mg) were immunoprecipitated with rabbit anti-HIF-1α, followed by immunoblot using mouse anti-HIF-1α. Normal rabbit IgG was used as a negative control (IgG). Bottom panel: Input lysates (100 μg) were analyzed by immunoblot using mouse anti-HIF-1α to show relative input HIF-1α levels. (b) Cells treated as in (a) were immunoprecipitated with rabbit anti-HIF-1α, followed by immunoblot using mouse anti-HA antibodies to detect HA-GSK3S9A. Arrow denotes HA-GSK3-specific band. Bottom panel: Input lysates (100 μg) were analyzed by immunoblot using mouse anti-HA antibodies to show relative input GSK3S9A levels. (c) Cells treated as in (a) were immunoprecipitated with mouse anti-HA, followed by immunoblot using mouse anti-HIF-1α. Normal mouse IgG was used as a negative control (IgG).
Mutational analysis of GSK3 phosphorylation sites within HIF-1α.

VHL is the canonical E3 ubiquitin ligase that is responsible for targeting HIF-1α for degradation via prolyl hydroxylation sites (Jaakkola et al., 2001; Maxwell et al., 1999) (Figure 4a). However, prolyl hydroxylase effects on HIF-1α are restricted to normoxia, whereas our results indicate the GSK3β effects are seen during hypoxia. To determine whether HIF-1α can act as a direct substrate for GSK3β, cells expressing HA-tagged HIF-1α were exposed hypoxia for 16 h followed by treatment of lysates with phosphatase to remove existing phosphates. HIF-1α immunoprecipitates were then subjected to a kinase assay using recombinant GSK3β. As shown in Figure 4b, phosphatase treatment resulted in a collapse of the characteristic HIF-1α electrophoresis pattern of bands at 120 and 130 kD to only the lower form. In vitro exposure to recombinant GSK3β returned the upper phosphorylated band, suggesting that HIF-1α can serve as a substrate for GSK3β.

Considering the number of GSK3β consensus sequences within the HIF-1α ODD domain along with our data and that of others showing regulation of HIF-1α by altering GSK3β activity, we designed two mutant HA-tagged HIF-1α constructs. The first construct, S551A T555A S589A (STS), was created based on previous findings suggesting these sites as GSK3β phosphorylation sites within a fragment of HIF-1α containing the ODD (Flugel et al., 2007). The second construct, T498V S502A S505A T506V S510A (TSSTS), was based on our analysis of likely GSK3β sites based on consensus sequence comparisons to other GSK3β targets such as β-catenin (Figure 4a).
When these mutants were compared with wild-type HIF-1α (WT) in the *in vitro* GSK3β phosphorylation assay, an interesting pattern emerged (Figure 4c). WT HIF-1α resolved as an approximately 130 kD protein after *in vitro* phosphorylation with GSK3β, whereas the STS mutant ran at 125 kD and the TSSTS band ran at 120 kD (the same size as phosphatase treated HIF-1α). This banding pattern suggests that full-length HIF-1α likely acts as a substrate for GSK3β at multiple sites within the ODD domain. Loss of phosphorylation sites in the STS mutant form of HIF-1α results in partial loss of GSK3 phosphorylation and loss of sites in the TSSTS mutant form prevents GSK3β-mediated phosphorylation.

![Diagram](image)

**Figure 4:** TSSTS mutant HIF-1α cannot be rephosphorylated by GSK3. (a) In normoxia, von-Hippel Lindau (VHL), a component of the E3 ubiquin ligase complex, recognizes two hydroxylated prolines within the oxygen-dependent degradation domain.
(ODD) and binds to HIF-1α targeting it for degradation. Blowup: HIF-1α protein sequence similarity to other substrates of GSK3 that are targeted for degradation. (b) HEK293 cells were transiently transfected with 2.5 μg WT HA-HIF-1α. After 24 h, cells were exposed to 2% O2 for 16 h. Lysates were obtained and treated with λ phosphatase and HA-HIF-1α was immunoprecipitated with an anti-HA antibody and HIF-1α detected by immunoblot. A GSK3 kinase reaction was performed on the immunoprecipitate, followed by immunoblot to detect phosphorylated forms of HIF-1α. (c) HEK293 cells were transiently transfected with the WT, TSSTS and STS HIF-1α constructs as in (a). Cells were treated and immunoprecipitation performed as in (a). GSK3 kinase reaction was conducted in the indicated samples and forms of HIF-1α were resolved and detected using HIF-1α immunoblot.

The TSSTS form of HIF-1α is present at higher levels in normoxia and hypoxia.

To determine if loss of GSK3β phosphorylation sites resulted in a change in HIF-1α protein stability, HIF-1α levels were measured in cells expressing WT or the putative GSK3β-resistant forms of HIF-1α. Using equal amounts of transfected DNA, the TSSTS mutant form was clearly more visible than WT by immunoblot in both normoxia and hypoxia (Figure 5a). There was no significant difference between the STS mutant form and WT. Similar to the in vitro GSK3β kinase experiments, the TSSTS mutant resolved with a pattern of smaller sized bands, suggestive of non-phosphorylated HIF-1α. There was, however, an increase in the slower resolving form of HIF-1α as well, possibly due to phosphorylation by other kinases at different sites (Mylonis et al.,
The TSSTS form of HIF-1α was also more resistant to reoxygenation as HIF-1α was detected at time points beyond that seen for WT HIF-1α (Figure 5b). Surprisingly, there was no significant effect of the TSSTS mutant on VEGF transcription when compared with WT HIF-1α (Figure 5c). The finding that expression of WT HIF-1α itself does not induce VEGF over endogenous levels may suggest that any increase in signal by expression of the TSSTS is masked by endogenous WT HIF-1α activity.

**Figure 5:** Mutation of TSSTS sites within HIF-1α stabilizes HIF-1α protein. (a) HEK293 cells were transiently transfected with pcDNA3 (empty vector), wild-type (WT), STS and TSSTS mutant HIF-1α plasmids. 24 h after transfection, cells were exposed to 2% O2 for 16 h. Cell lysates were obtained and immunoblot performed using antibodies to HIF-1α using β-actin as a loading control. (b) Cells were transfected with WT or TSSTS HIF-1α plasmids as in (a). 36 h after transfection, cells were exposed to 2% O2 for 4 h, followed by return to 21% O2 (Reox) for the times indicated. The
resulting cell lysates were analyzed by immunoblot as in (a). (c) Cells were treated as in (a) followed by isolation of mRNA and detection of VEGF mRNA levels using RT-qPCR. Data were calculated as fold-change (Rq value) normalized to β2-microglobulin, and values represent the average of 3 independent experiments.

**Evidence that GSK3 enhances ubiquitination of HIF-1α in hypoxia, and that the TSSTS mutant HIF-1α is not a good substrate for ubiquitination.**

GSK3 plays a role in initiating the degradation cascade of many proteins including β-catenin and mcl-1, so we tested the hypothesis that GSK3β phosphorylation of HIF-1α targets it for ubiquitination and degradation (Ikeda et al., 1998; Maurer et al., 2006). To rule out the possibility that higher levels of the TSSTS mutant were due to increased transcription, we tested the effect of overexpressing GSK3S9A on the relative mRNA levels of HIF-1α by RT-qPCR. As expected, GSK3β did not significantly affect HIF-1α transcript levels (Figure 6a). However, expression of GSK3S9A did increase the detection of ubiquitinated forms of HIF-1α when compared with untransfected or cells transfected with vector alone (Figure 6b). Furthermore, the TSSTS mutant was substantially less ubiquitinated as compared to the WT or STS forms of HIF-1α (Figure 6c). There was no apparent difference between the WT and STS HIF-1α. These results indicate that HIF-1α is subject to GSK3-mediated ubiquitination in hypoxia and that loss of GSK3β phosphorylation sites reduces its ability to serve as a substrate for ubiquitination in hypoxia.
Figure 6: GSK3 enhances ubiquitination of HIF-1α in hypoxia and ubiquitination of TSSTS mutant HIF-1α is decreased. (a) HEK293 cells were transiently transfected with 5 μg pcDNA3 (empty vector) or pGSK3S9A. 24 h after transfection, cells were exposed to 2% O₂ for 16 h, followed by analysis of HIF-1α mRNA levels using RT-qPCR. Data were calculated as fold-change (Rq value) normalized to β2-microglobulin, and values represent the average of two independent experiments. (b) Cells were transfected as in (a), followed by exposure to 2% O₂ in the presence of 10 μM MG132 for 8 h. Lysates were collected and HIF-1α was immunoprecipitated using rabbit anti-HIF-1α. Ubiquitination was assessed by immunoblot using mouse anti-ubiquitin (Ubq).
Arrow indicates ubiquitinated HIF-1α. (c) HEK293 cells were transiently transfected with 2.5 μg of wild-type (WT) or mutant HIF-1α plasmids. After transfection, cells were treated as in (b) and then HA-HIF-1α was immunoprecipitated from cell lysates using an anti-HA antibody. Ubiquitination was assessed by immunoblot using mouse anti-ubiquitin (Ubq).

**Discussion**

Expression and stabilization of HIF-1α, especially in hypoxia, is of paramount importance in the propagation of angiogenesis, tumor growth and metastasis. While many studies describe posttranslational regulation of HIF-1α via sumoylation, nitrosylation and phosphorylation, most of these modifications result in positive regulation of HIF-1α (Bae et al., 2004; Inna and Vadim, 2003; Mylonis et al., 2006). Activation of Akt by the PI3K pathway leads to increased HIF-1α protein levels, and some reports indicate that Akt activity leads to increased translation of HIF-1α through mechanisms requiring p70S6K1 or MDM/p53 (Fang et al., 2005; Mottet et al., 2003; Pore et al., 2006; Treins et al., 2002; Zhong et al., 2000; Zhong et al., 2004). Recently, GSK3β, which is phosphorylated and inhibited by Akt, has been identified as a possible negative regulator of HIF-1α. Here, we confirm and expand these findings by presenting evidence that GSK3 negatively regulates HIF-1α during hypoxia by facilitating proteasomal degradation of HIF-1α. Furthermore, we show that proteasomal degradation of HIF-1α by GSK3 is accomplished through phosphorylation of the HIF-1α protein at select sites within the ODD domain.
Although the activation state of GSK3 in hypoxia is debated, activity of its inhibitor, Akt, is low in early hypoxia, but is then stimulated at 6 h and persists for 24 h (Alvarez-Tejado *et al.*, 2001; Beitner-Johnson *et al.*, 2001; Chen *et al.*, 2001). Thus it is probable that lowered Akt activity in early hypoxia correlates with an increase in GSK3 activity. Our work agrees with this model in that our data show a transient effect of GSK3 on HIF-1α in early hypoxia. Further, when cells are introduced to normoxic conditions following a hypoxic treatment, loss of GSK3 activity slows the loss of HIF-1α protein level. Taken together, these data suggest that GSK3 is likely involved as a control mechanism to restrain HIF-1α protein and transcriptional activity in early hypoxia and during re-oxygenation.

The canonical GSK3 consensus sequence is S/T-X-X-X-S/T, and most substrates require a priming phosphorylation event by another kinase on the carboxy-terminal end of the consensus sequence (Cohen and Frame, 2001). Examination of the HIF-1α amino acid sequence within the ODD domain revealed a number of GSK3 consensus sequences, and our *in vitro* kinase experiments suggest that HIF-1α is a substrate for direct phosphorylation by GSK3β. Unlike other substrates for GSK3, in our system, HIF-1α did not require a priming phosphorylation event. However, it must be considered that our phosphorylation assay was conducted *in vitro* and a priming phosphorylation event may be required *in vivo*.

Recently, GSK3β has been shown to directly phosphorylate a fragment of HIF-1α containing the ODD domain (Flugel *et al.*, 2007; Sodhi *et al.*, 2001). This report identified STS as sites necessary for GSK3 phosphorylation and that mutation at these
residues increased HIF-1α stabilization, suggesting an effect of GSK3 on HIF-1α protein stability (Flugel et al., 2007). In contrast, our results did not find significant effects of the STS mutant, and identified T498-S502-S505-T506-S510 (TSSTS) as sites necessary for GSK3 phosphorylation. It is possible that the discrepancy in location of GSK3 phosphorylation sites is due to differences in cell conditions or because we evaluated full-length HIF-1α rather than an ODD domain fragment of HIF-1α. Folding of the full-length HIF-1α protein may thus affect the availability of sites to GSK3 and other cofactors.

Surprisingly, although the TSSTS mutant construct greatly stabilized HIF-1α protein levels, there was no apparent effect on HIF-1α transcriptional activity. It would be expected that expression of the TSSTS mutant HIF-1α would increase VEGF transcription, similar to that seen with GSK3 inhibition. Because endogenous HIF-1α was present in these expression experiments, the inability to see differences between the wild-type HIF-1α and TSSTS transcriptional activity could be attributed to an excess of endogenous HIF-1α saturating the transcriptional machinery. However, this possibility is not probable as there were no differences in VEGF transcription with the TSSTS mutant even at reoxygenation timepoints when endogenous HIF-1α protein and VEGF transcription were undetectable (Data not shown).

A more likely possibility is that one or more of the mutated residues are phosphorylation sites that potentiate the transcriptional activity of HIF-1α. For example, T498 is followed by a proline and is thus a potential MAP kinase phosphorylation site.
Phosphorylation by ERK1/2 facilitates HIF-1α transcriptional activity, but these sites have been identified as S641 and S643, distinct from sites mutated in the TSSTS mutant (Mylonis et al., 2006). For some transcription factors, such as c-Myc and c-Maf, transcriptional activity is inversely correlated with protein stability because transcriptional activators can also mediate ubiquitination and proteasomal degradation (Rocques et al., 2007; von der Lehr et al., 2003). Thus it is possible that the sites we mutated serve a dual function in regulating both the transcriptional activity and stability of HIF-1α. Either of these regulatory functions point to GSK3β as a potential target to modulate HIF-1α function.

GSK3 mediates proteasomal degradation of a number of proteins including the transcription factor β-catenin (Aberle et al., 1997). Thus, it is not surprising that the findings of the current study showed expression of GSK3 leading to an increase in ubiquitination of HIF-1α protein. The degradation of HIF-1α initiated by GSK3 could be reversed by proteasome inhibition supporting the hypothesis that GSK3 phosphorylation targets HIF-1α for proteasomal degradation. The current study is consistent with earlier work studying the effect of GSK3 on HIF-1α (Flugel et al., 2007). However, our work differs from Flugel et al. in that we observed a decrease in ubiquitination of our TSSTS mutant, but not their previously described STS mutant when compared to the wild-type. This disparity could be due to the differential oxygen concentrations; 8% was used in Flugel et al. while the current study used 2%. It is possible that different oxygen concentrations may induce variable kinase signaling pathways that have disparate effects on HIF-1α and its ability to act as a substrate for GSK3.
In contrast to most proteins including transcription factors, HIF-1α is primarily regulated by posttranslational modification rather than by regulation of transcription. Here we present evidence for yet another mechanism of HIF-1α regulation via a posttranslational modification by GSK3. Importantly, the regulation of HIF-1α by GSK3 is functional during hypoxia, when oxygen-dependent regulatory systems are disabled. We identified the sites that GSK3 phosphorylates HIF-1α thus targeting it for ubiquitination and eventual proteasomal degradation. This study thus presents another layer of HIF-1α regulation that may be manipulated or exploited for therapeutic purposes in the treatment of angiogenic tumor growth.

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CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS
DISCUSSION: Walking with the ghost of HIF-1α

HIF-1α expression can be manipulated in normoxia and hypoxia particularly by oxygenation status and growth factor signaling. This differential regulation presents an interesting paradox where in normoxia, HIF-1α expression can be induced through growth factor signaling and ROS and, conversely, in hypoxia, the same growth factor and ROS-induced signaling decreases HIF-1α protein levels. When considering the paradoxical nature of HIF-1α in normoxia versus hypoxia, the question arises, could researchers take advantage of the “alleviation of hypoxic stress pathway” to decrease hypoxic HIF-1α? Is it possible to trick the cell into thinking its oxygenated thereby decreasing HIF-1α stability and activity? Perhaps one might take advantage of ROS-induced destabilization of hypoxic HIF-1α? In line with this theory, the work presented in the first section of this dissertation suggests another mechanism for the regulation of HIF-1α via cytokine-induced destabilization of hypoxic HIF-1α. In investigating a putative negative feedback pathway between EPO and HIF-1α we exploited the paradoxical nature of HIF-1α. Often a treatment applied in normoxia will have the opposite effect on hypoxic HIF-1α. Here, though EPO did not induce an increase in HIF-1α in normoxia, both hypoxia-induced HIF-1α stabilization and transactivation were disrupted.

Initially, we explored the hypothesis that EPO-mediated destabilization of HIF-1α was due to the effects of activated GSK3 on HIF-1α. Though most literature points to EPO signaling through both PI3K pathway and the Jak/STAT pathway; two pathways
that should lead to increases in HIF-1α, we considered that exogenous EPO could signal through an alternative pathway activating GSK3 thus leading to GSK3-mediated proteosomal degradation of HIF-1α. We concluded that EPO likely does not signal through GSK3 to decrease HIF-1α stabilization and transactivation, however, because of the dramatic effects of GSK3 inhibition on HIF-1α, we pursued investigating the effect of GSK3 regulation of HIF-1α. The effect of GSK3 on HIF-1α is presented in the second section of this dissertation.

**Future Directions: EPO effect on HIF-1α**

EPO signaling through a heterotrimer consisting of the EPO receptor (EPOR) and the β common receptor (βCR) leads to both JAK2/STAT5 and PI3K signaling (Feng, 2006). Others have shown that signaling through this receptor confers the therapeutic, tissue-protective and anti-inflammatory actions of EPO (Feng, 2006). In turn, EPO signaling through this heterotrimeric receptor may have a permissive effect on HIF-1α destabilization.

There are a number of explanations for the effect of EPO on HIF-1α all linked by the idea that ROS mediates hypoxic HIF-1α levels. First, the inhibition of ROS by EPO signaling could inhibit HIF-1α in hypoxia. In support of this, treatment of cells with antioxidants has been shown to decrease hypoxia-induced HIF-1α stabilization (Taylor, 2008). However, the majority of studies show ROS activation in hypoxia, leading to a decrease of HIF-1α. The second way EPO may decrease HIF-1α levels is through disruption of the mitochondrial electron transport chain. Studies have shown that
a functional mitochondrion is essential for stabilization of HIF-1α in hypoxia (Klimova and Chandel, 2008). Further, nitric oxide (NO) is known to impair mitochondrial respiration thereby causing a release of molecular oxygen from the mitochondria into the cytoplasm (Erusalimsky and Moncada, 2007). Some researchers have suggested that the release of molecular oxygen from mitochondria enables PHD function and resulting in an inactive and hydroxylated HIF-1α (Taylor, 2008). Accordingly, through the generation of NOS, EPO may facilitate the generation of NO, in turn, facilitating oxygen release from the damaged mitochondrion. Following the release of oxygen from the mitochondria, PHDs are activated, HIF-1α is hydroxylated and subsequently degraded (Figure 1).
Figure 1: EPO decreases HIF-1α in hypoxia through RNS

Figure 1: EPO signaling through PI3K generates NO, inhibiting the electron transport chain and inducing the release of molecular oxygen from the mitochondria. As oxygen availability increases, PHD activity increases, HIF-1α is hydroxylated and degraded.

A third way treatment with EPO may decrease HIF-1α is through decreased levels of inflammatory cytokines. EPO has been shown to decrease inflammatory cytokines and oxidative stress caused by myocardial infarction (Li et al., 2006). Additionally, increased inflammatory cytokines have been linked to and increase in HIF-1α suggesting that inflammatory cytokines play a role in maintaining the stabilization of HIF-1α. Signaling by EPO through the EPO-R/βCR that induces a decrease in TNF-α and IL-1β, in particular, may lead to a decrease in HIF-1α (Figure 2).
Figure 2: EPO signaling inhibits TNF-α-stabilized assisted stabilization of HIF-1α in hypoxia

Concerns regarding the side-effects of EPO on patients, namely thrombosis, have cautioned clinician’s use of recombinant EPO. However, as the medical and research communities develop new drugs for targeting HIF-1α, EPO-mediated regulation of HIF-1α should be considered as an advantageous pathway in the control of HIF-1α. Of note is development of a carbamalished EPO (cEPO) derivative that is currently being evaluated for similar effects on HIF-1α (Fantacci et al., 2006). Further investigations into the HIF-EPO mechanism could provide insight to new therapies for targeting HIF-1α. Exploitation of the EPO signaling pathway especially through the EPO-R/βCR also could be an important therapeutic target for HIF-1α.
GSK3-mediated degradation of HIF-1α

As with β-catenin, HIF-1α has been identified as a putative GSK3 substrate (Flugel et al., 2007). The first indication that GSK3 might target HIF-1α was shown by Sodhi et al.. These authors showed, for the first time, that glycogen synthase kinase 3 (GSK3), a kinase downstream of PI3K and inhibited by active Akt, directly phosphorylates a piece of HIF-1α containing the ODD. Further studies have shown that in mild hypoxia, inhibition of GSK3 increases HIF-1α stabilization, suggesting that GSK3 may repress HIF-1α protein levels. Overexpression of GSK3 decreases HIF-1α levels and increases ubiquitination of HIF-1α. Overexpression of GSK3 also decreases HIF-1α transcriptional activity as assessed in vitro by DNA binding assays. The putative GSK3 phosphorylation sites within HIF-1α have been identified as S551 T555 and S589 (Flugel et al., 2007). Oddly, this is not a traditional GSK3 consensus sequence as the C-terminal serine is located 39 amino acids downstream of the two other sites. The E3 ubiquitin ligase involved in GSK3-mediated degradation of HIF-1α is currently unknown.

Our exploration into the GSK3-HIF-1α pathway was occurring simultaneously with the Flugel et al. study. Though there were some disparities between our work and the Flugel et al. study, the general result was the same. Like the Flugel et al. study, we found that GSK3 inhibition increased HIF-1α protein levels and conversely, GSK3 overexpression decreased HIF-1α protein. This effect translated to an increase in VEGF transcription upon GSK3 inhibition and likewise, a decrease in VEGF transcription with
GSK3 overexpression. We also found that GSK3 targeted HIF-1α for ubiquitination and proteasomal degradation.

Despite the many similarities in results between our study and the Flugel et al. work, there are three large discrepancies. First, the Flugel et al. report employed mild hypoxia- 8% oxygen- and our studies were performed in 2% oxygen, a level of oxygen that is accepted in the research community to be “true hypoxia” and is routinely used to study the relationship between hypoxia and malignancy (Klimova and Chandel, 2008). Second, we identified different GSK3 phosphorylation sites on HIF-1α than the Flugel et al. study. We suggest the oxygen concentration may determine the posttranslational modification within HIF-1α thereby dictating the specific folding of HIF-1α and predicting the subsequent posttranslational modifications. To this end, we propose the following: at 2% oxygen, GSK3 phosphorylates HIF-1α on T498, S500, S502, T506 and S510 while at 8% oxygen GSK3 phosphorylates HIF-1α on S551, T555 and S589.

The third discrepancy is also one involving oxygen concentration. We did not find an effect of GSK3 on normoxic HIF-1α while Flugel et al. reported that GSK3 targeted normoxic HIF-1α for proteasomal degradation. In fact, they reported that GSK3 inhibition preferentially acts under normoxic conditions to increase HIF-1α. Our study does not support this model as GSK3 inhibition did not increase normoxic HIF-1α levels. In line with this, GSK3 inhibition also did not increase VEGF transcription and conversely, GSK3 overexpression did not decrease VEGF transcription. This disparity in effect could be due to cell specific differences such as E3 ubiquitin ligase availability.
**Future Directions: GSK3 effect on HIF-1α**

There are two situations in particular to consider when investigating the mechanism through which GSK3 operates to target HIF-1α for proteasomal degradation. However, at this time, only one seems likely. GSK3 phosphorylation of HIF-1α and subsequent degradation may be through one of the following pathways: 1) oxygen-independent binding of VHL or 2) an E3 ubiquitin ligase other than VHL. These scenarios will be addressed in the following discussion.

Though it is possible that VHL may participate in GSK3-mediated degradation of HIF-1α, it is not probable. First, the binding of VHL is dependent on intact hydroxylation sites within the ODD of HIF-1α protein. The hydroxyprolines generated by the PHD reaction are critical for binding of VHL to HIF-1α therefore the absence of the hydroxyprolines abrogates the VHL-HIF-1α interaction in hypoxia (Min et al., 2002). Further, others have shown that VHL does not cooperate with GSK3 to target HIF-1α for degradation (Flugel et al., 2007). Though others have shown that VHL is not the E3 ubiquitin ligase involved in GSK3-mediated HIF-1α degradation, this still deserves confirmation in our system.

GSK3 phosphorylation of HIF-1α may facilitate binding of an E3 ubiquitin ligase other than VHL. There are two E3 ubiquitin ligases that are good candidates for targeting HIF-1α. The first is β-transducin repeat containing protein (β-TrCP). β-TrCP is a member of the SCF (complex of Skp1, Cul1 and F-box protein)-type ubiquitin ligases (Fuchs et al., 2004). GSK3 phosphorylates β-catenin within the β-TrCP recognition
sequence of D-S-G-X-X-S/T thus targeting it for proteosomal degradation (Fuchs et al., 2004). Though HIF-1α does not have the classical β-TrCP recognition motif, it does have a D-G-S sequence within the GSK3 sites we identified. The D-G-S sequence has been shown to provide an adequate recognition site for β-TrCP to bind to myeloid cell leukemia-1 (mcl-1) (Ding et al., 2007). With this in mind, we hypothesized that β-TrCP is the E3 ubiquitin ligase that targets HIF-1α after GSK3 phosphorylation. Preliminary experiments using siRNA against β-TrCP have indicated that HIF-1α is likely not targeted by β-TrCP for degradation. To confirm this finding, we will continue experiments using a constitutively active β-TrCP plasmid to evaluate the effect of overexpressed β-TrCP on HIF-1α protein.

Fbw7 (F-box and WD repeat domain-containing 7) is another likely E3 ubiquitin ligase candidate in the HIF-1α-GSK3 pathway. Fbw7 is also a component of the SCF-type ubiquitin ligases and like β-TrCP, targets proteins such as cyclin E, myc and jun, for degradation following GSK3 phosphorylation (Welcker and Clurman, 2008). Fbw7 targets proteins containing a consensus Cdc4-phospho-degron (CPD) motif (Welcker and Clurman, 2008). The HIF-1α putative CPD is closest to Fbw7 target protein Notch1 where the Notch1 sequence is L-T-P-S-P-E-S-P and the HIF-1α sequence is Q-T-P-S-P-S-D-G. The bold T indicates the putative GSK3 phosphorylation site and upstream bold E and S indicates the acidic or phospho-amino acid essential for Fbw7 binding (Welcker and Clurman, 2008). The similarity of CPDs between HIF-1α and Notch1 indicates that HIF-1α may be a target for GSK3-Fbw7-mediated ubiquitination and proteasomal
degradation (Figure 3). Thus the provocative question remains: is HIF-1α targeted by Fbw7? Certainly more investigation into the intricacies of HIF-1α regulation by GSK3 is warranted.

![Figure 3: GSK3-Fbw7 mediated degradation of HIF-1α](image.png)

**Figure 3: GSK3-Fbw7 mediated degradation of HIF-1α**

Figure 3: Glycogen synthase kinase 3 phosphorylates HIF-1α thus targeting HIF-1α for Fbw7 ubiquitin-mediated proteasomal degradation.

Our work suggests that activation of GSK3 ameliorates HIF-1α stabilization and transactivation seen in hypoxia thus underscoring the importance our findings in the context of HIF-1α regulated cancer progression and evoking further investigation into the use of GSK3 as a modulator of HIF-1α activity. As such, activation of GSK3 may
present a unique opportunity to mitigate not only HIF-1α activity, but also other transcription factors involved in cancerous signaling pathways. In fact, the idea of activating GSK3 in cancer to target transcription factors upregulated in cancer has been suggested by others (Jope et al., 2007). Controlled activation of GSK3 in cancer could provide a multifaceted way to abrogate cancer signaling pathways.

**CONCLUSIONS**

The development of anti-angiogenic agents has been promising, nevertheless, also has complications. One concern here is that anti-angiogenic agents might facilitate selection and survival of hypoxic cells. The targeting HIF-1α could provide a way to circumvent this problem. As HIF-1α expression is an extremely important element in the adaptation to hypoxia and the survival of clonal variants, elimination of HIF-1α would decrease the survival of hypoxic cells and abrogate the angiogenic pathway. Further, HIF-1α target genes collectively have a large role in facilitating malignant progression, especially, angiogenesis. As such, HIF-1α is an attractive drug target. In fact, some approved chemotherapeutic agents that are currently in use or in clinical trials, also inhibit HIF-1α activity (Semenza, 2003).

This dissertation outlines two ways in which HIF-1α could be targeted by therapeutics. One method with an already approved drug and another method still far from clinical trials. Both EPO and GSK3 are promising in the application of therapeutically targeting HIF-1α and both pathways demand more investigation. With luck, further investigations yielding more information on both of these pathways could lead to a future drug that effectively eliminates HIF-1α protein and transactivation.
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