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Role of stat3 in regulating hif-1alpha expression and tumor angiogenesis

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Role of Stat3 in Regulating HIF-1alpha Expression and Tumor Angiogenesis

by

Jon J. Briggs

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Medical Microbiology and Immunology
College of Medicine
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Keywords: STAT, HIF, VEGF, IL-6, angiogenesis, hypoxia

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Stat3 is required for HIF-1α induction by EGF

Stat3 is required for HIF-1α induction by hypoxia

Stat3 is required for the hypoxic induction of VEGF and MMP-2

Stat3 is activated by hypoxia

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<th>Description</th>
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<tr>
<td>Abl</td>
<td>Abelson kinase</td>
</tr>
<tr>
<td>AP</td>
<td>Activator protein</td>
</tr>
<tr>
<td>ASO</td>
<td>Antisense oligonucleotide</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic Fibroblast growth factor</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic-Helix-loop-helix</td>
</tr>
<tr>
<td>CHX</td>
<td>Cyclohexamide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FIH</td>
<td>Factor inhibiting HIF</td>
</tr>
<tr>
<td>FRAP</td>
<td>FKBP/rapamycin-associated protein</td>
</tr>
<tr>
<td>gp130</td>
<td>glycoprotein 130</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia response element</td>
</tr>
<tr>
<td>hSIE</td>
<td>high affinity Sis-Inducible Element</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Mek</td>
<td>MAPK/ERK kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapomycin</td>
</tr>
<tr>
<td>ODD</td>
<td>Oxygen dependent degradation domain</td>
</tr>
<tr>
<td>PAS</td>
<td>Per/Arnt/Sim</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl hydroxylase domain</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue deleted on chromosome 10</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TK</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>VHL</td>
<td>von Hippel-Lindau protein</td>
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</table>
Role of Stat3 in Regulating HIF-1alpha Expression and Tumor Angiogenesis

Jon Briggs

ABSTRACT

Increased vascularization (angiogenesis) is a required adaptation for sustained tumor growth, and the primary mediator of de novo blood vessel formation is vascular endothelial growth factor (VEGF). The central transcriptional activator of VEGF is hypoxia inducible factor-1 (HIF-1), a heterodimeric transcription factor composed of an inducible HIF-1α subunit and a constitutively expressed HIF-1β subunit. In addition to HIF-1, it has recently been reported that signal transducer and activator of transcription 3 (Stat3) is required for VEGF production and angiogenesis. Although it is known that Stat3 is an important mediator of many of the oncogenic signaling pathways that regulate HIF-1α, it was not known if Stat3 regulates HIF-1α. To answer this important question, the effect of blocking Stat3 signaling on both HIF-1α and VEGF expression was examined. Treatment of cells with IL-6, a potent activator of Stat3, resulted in HIF-1α and VEGF induction during normoxia. By blocking protein synthesis with cycloheximide, it was determined that IL-6 induction of HIF-1α resulted from increased translation. When Stat3 was silenced with siRNA, both basal level expression and IL-6 induction of HIF-1α and VEGF were significantly reduced. Furthermore, it is likely that Stat3 is required for HIF-1α induction by a variety of growth signals, as both HIF-1α and VEGF expression resulting from EGF and heregulin were abolished when Stat3 signaling
was blocked. Because we had observed that Stat3 was required for induction of HIF-1α by growth signals, we wanted to determine if Stat3 was also required for HIF-1α induction by hypoxia. When stat3 was silenced and cells exposed to hypoxia, HIF-1α expression was again abolished. Furthermore, the hypoxic induction of VEGF and MMP-2 was also prevented. These results, along with loss of basal expression of HIF-1α, suggest that Stat3 is fundamentally involved with the cellular response to hypoxia. This idea is reinforced by our observation that Stat3 is activated by hypoxia in several cell lines. These findings position Stat3 as an important target for antiangiogenic therapy, not only through its direct regulation of the VEGF gene, but also because it is required for expression of the other most important transcriptional activator of VEGF.
INTRODUCTION

Tumor hypoxia and angiogenesis

As solid tumors grow beyond a diameter of 1-2 millimeters, they outgrow the existing blood supply (52). As a result, areas of low oxygen (hypoxia) develop. The presence of hypoxic regions was originally hypothesized in response to the observation that necrosis increased as distance from the vasculature increased (199). Confirmation of this theory, and more accurate measurements of tumor oxygen levels were achieved with oxygen electrodes. It was observed that oxygen levels in tumors were highly variable, with areas as low as 0.7% O$_2$ (206). For their continued growth and survival in a hypoxic environment, tumor cells encourage growth of new blood vessels from the pre-existing vasculature in a process known as angiogenesis (60). It is clear that tumors have an absolute requirement for vascularization, and in the absence of angiogenesis, sustained tumor growth cannot occur (59, 60, 87, 118).

In response to hypoxia, cell signaling pathways and transcription factors are activated in the tumor cells that help them cope with the harsh tumor microenvironment (87). Activation of these pathways results in changes in gene expression encouraging survival under conditions of low oxygen, low nutrients, and high acidity. There is increased need for glucose uptake and metabolism due to low oxygen and nutrients, and as a result there is increased expression of glucose transporters and glycolytic enzymes.
Because it is a toxic environment characterized by hypoxia and acidosis, there is elevated expression of growth factors and survival pathways. Angiogenesis is facilitated by the release of soluble factors such as VEGF that bind to receptors on vascular endothelial cells and encourage their migration and growth. Furthermore, factors are released which result in the breakdown and restructuring of the extracellular matrix, allowing endothelial cell migration. In this manner, tumors restructure the local vasculature to fulfill their need for oxygen and nutrients.

A poor clinical outcome correlates with both microvessel density (118, 216) and tumor hypoxia (93). The reason for this is that although many tumors are highly vascularized, the tumor vasculature is often poorly formed and inefficient (24). The tumor vascular system is characterized by leaky blood vessels, haphazard patterns of connections, loose attachment, and an abnormal basement membrane (107). These abnormalities result in a tumor environment that although vascularized, is characterized by hypoxia, acidosis, and necrosis (107). Tumors with a high degree of hypoxia amount to a poor prognosis for patients for a variety of reasons. Cells adapted to hypoxia tend to upregulate genes associated with angiogenesis and metastasis (171). It is clear that hypoxia exerts selective pressure for formation of a tumor with greater resistance to apoptosis (77). Furthermore, the environment tends to increase mutation rates (232, 233). These adaptations result in a tumor with greater potential for survival and metastasis. In addition, hypoxic tumors tend to have greater resistance to treatment with drugs and radiation (16, 45, 81). The implication is that tumor cell survival is facilitated by both encouragement of angiogenesis and adaptation to hypoxia, and therefore tumor progression correlates with both hypoxia and vascularization.
Antiangiogenic therapy was proposed due to the observation that solid tumors require vascularization for sustained growth (60). By preventing growth of new blood vessels, the tumor could be deprived of oxygen and nutrients and continued growth would be inhibited. It is clear that tumors have an absolute requirement for vascularization, and inhibition of angiogenesis can prevent tumor growth (59, 60, 86, 87, 118). Furthermore, there have been reports of successful clinical trials (115, 226). Importantly, it has been reported that antiangiogenic therapy in conjunction with radiation and chemotherapy resulted in an improved response (75, 100, 122, 134, 146, 197). Despite these successful trials, many of the antiangiogenesis inhibitors when used alone have not been effective (12, 54). A likely explanation for this is that they were not effective enough, as the majority of them have only targeted an individual angiogenic factor such as VEGF, and it is known that vascularization is a complex process involving a variety of factors (54, 118). When drugs are developed with greater potency that are capable of blocking a wide variety angiogenesis inducers, it is likely that clinical trials will be more successful either alone or in conjunction with traditional radiation and chemotherapy (12, 97).

Vascular Endothelial Growth Factor

It had long been known that tumor cells secrete a factor capable of initiating angiogenesis, however the stimulus responsible for its production was not known (53, 61). Subsequently, it was demonstrated that macrophages exposed to a hypoxic environment were shown to secrete an angiogenesis-stimulating factor (124). Vascular
endothelial growth factor (VEGF) was then purified, sequenced and cloned, leading to its identification and characterization as an exceptionally potent angiogenic mitogen (117, 135). Following this, it was shown that high VEGF expression occurs close to necrotic and presumably hypoxic areas, demonstrating probable in vivo stimulation of angiogenesis by a hypoxic environment (187).

VEGF is a soluble factor capable of stimulating blood vessel growth, and is important for processes such as embryogenesis and wound repair (52, 54). Furthermore, it is well established that VEGF is an important mediator of tumor angiogenesis and progression (53, 84, 151, 162, 187). Tumors with high expression of VEGF have been shown to be of increased grade and result in a poor clinical outcome (163, 203, 216). VEGF is demonstrated to be an important therapeutic target in both animal models (9, 97, 101, 103, 121-123, 221, 231) as well as patients (100, 115, 226). It is clear from these reports that VEGF is essential for tumor angiogenesis, and inhibition of VEGF can prevent angiogenesis and tumor growth.

The biologically active form of vascular endothelial growth factor is VEGF-A (54), usually referred to as VEGF, and it forms a 45 kDa homodimer (55). VEGF undergoes splice variation to form four main variants of the protein denoted by subscript numbers representing the number of amino acids: VEGF_{121}, VEGF_{165}, VEGF_{189}, and VEGF_{206} (96, 202). These different isoforms bind with varying affinities to both the extracellular matrix and receptors expressed on most endothelial cells (54). The two major VEGF RTKs are VEGF receptor 1 (VEGFR-1, Flt-1) and VEGF receptor 2 (VEGFR-2, KDR, FLK) (40, 198). Although both of these receptors are upregulated by
hypoxia and important for angiogenesis, VEGFR-2 is the primary mediator of the growth and angiogenic properties of VEGF (54, 183).

**Regulation of VEGF**

Regulation of VEGF expression may result from transcriptional, post-transcriptional or translational mechanisms. Increased transcription results from hypoxia or growth signals and is facilitated by transcription factors such as HIF-1 and Stat3. Concerning post-transcriptional modification, proteins binding to the 3’-untranslated region of VEGF mRNA result in its stabilization in response to hypoxia (31, 36, 136, 190). Furthermore, VEGF may be regulated at the translational level by a mechanism dependent on the PI3K/AKT/mTOR pathway (30, 150).

In addition to hypoxia (187) and acidosis (185), VEGF transcription can be induced by a variety of soluble factors such as growth factors, cytokines, and hormones. Examples of these include transforming growth factor-β (TGF-β) (63, 160), tumor necrosis factor (TNF) (63, 174), platelet-derived growth factor (PDGF) (56, 209), keratinocyte growth factor (KGF) (63), Interleukin-6 (IL-6) (33, 38), hepatocyte growth factor (HGF) (235), Interleukin-1β (IL-1β) (139), fibroblast growth factor-2 (FGF-2) (43, 174), epidermal growth factor (EGF) (32, 63), and insulin-like growth factor 1 (IGF-1) (32, 72). Many of these signals are commonly overexpressed by tumor cells, and result in paracrine induction of VEGF which is essential for tumor vascularization (84).

Tumor cells often produce high levels of VEGF without any external stimulation. This results from genetic changes undergone by the tumor cells themselves, such as loss
of tumor suppressor function and oncogene overexpression. Concerning tumor suppressors, loss of von Hippel-Lindau (VHL) gene (102), p53 (120, 154), and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) (238) are associated with increased expression of VEGF. In addition to negative regulation by tumor suppressors, VEGF is upregulated by a variety of receptor and non-receptor tyrosine kinases. Examples of membrane bound TKs capable of increasing VEGF production include EGFR (161) and HER2 (133, 161). Various non-receptor TKs also stimulate VEGF production, examples include c-Src (47, 155, 218), v-Src (109, 154), and Ras (83, 166).

The best documented signaling pathways capable of stimulating VEGF production are PI3K/AKT and the Ras/Raf/MEK/ERK pathway. Many of the growth signals or genetic alterations responsible for upregulating VEGF expression are dependent on the kinases PI3K and Akt (32, 110, 133, 209). Furthermore, loss of function of the tumor suppressor PTEN results in activation of the PI3K/ AKT pathway (85, 189, 222) and increased VEGF expression (238). Concerning the Ras/Raf/MEK/ERK cascade, VEGF is upregulated by mutant Ras (166), v-Ras (83) and v-Raf (83). Furthermore, VEGF is required for Ras-mediated tumorigenesis (84).

Increased transcription of the VEGF gene results from activation of a limited set of transcription factors. The VEGF promoter contains several confirmed transcription factor binding sites, including HIF-1 (62, 137), Stat3 (159, 214), AP-1 (174), and Sp1 (56, 174, 184), and AP-2 (69, 202). Induction of VEGF by hypoxia involves HIF-1α, and this process is well characterized (137, 140). Furthermore, it has been demonstrated that AP-1 becomes activated under hypoxia (173, 227), and possibly contributes to the HIF-1
response to hypoxia (1, 35, 130). It has recently been reported that Stat3 and HIF-1α are both required for maximal VEGF induction by hypoxia (82).

Other angiogenic factors

Angiogenesis is a complex process requiring not only endothelial cell growth and migration, but also processes involving reorganization of the extracellular matrix and basement membrane (94). As a result, a variety of factors in addition to VEGF are required. Several other factors facilitate angiogenesis in a direct manner by stimulating growth of vascular endothelial cells, these include basic fibroblast growth factor (bFGF, or FGF-2) (50) and IL-8 (125). Other factors act in an indirect manner by increasing VEGF production, and these were described in the previous section. In addition to factors that stimulate endothelial cell growth, others have been described that regulate angiogenesis in negative manner. An important example of a negative regulator of angiogenesis is thrombospondin 1 (TSP-1), which functions by inhibiting endothelial cell proliferation and inducing apoptosis (112, 175). Furthermore, as with VEGF, it has been reported that many of these factors are regulated in response to hypoxia, including PDGF (126, 129), IL-8 (44, 224) and bFGF (129).

In addition to endothelial cell migration and growth, angiogenesis requires breakdown of the extracellular matrix and basement membranes, allowing endothelial cells to migrate toward the source of the angiogenic stimulus. Important factors involved in this process are the matrix metalloproteinase enzymes, in particular MMP-2 (gelatinase A) and MMP-9 (gelatinase B) (149). It has been demonstrated that MMP-2 is upregulated
by hypoxia (128), and is important for tumor angiogenesis (104), as well as invasion and metastasis (95, 104, 178).

Hypoxia inducible factor

Hypoxia inducible factor 1 (HIF-1) was originally described as a transcription factor that binds to the 3’-enhancer of the erythropoietin (EPO) gene, a protein important for synthesis of haemoglobin, upregulating its production in response to hypoxia (182). It rapidly became apparent that this transcription factor was instrumental in the hypoxic regulation of cellular processes beyond haemoglobin synthesis, as the enhancer sequence was still responsive in cells incapable of producing EPO (211). Furthermore, it was discovered that a common transcriptional complex inducible by hypoxia was involved with the regulation of a variety of angiogenic factors (71, 73, 140, 181). The demonstration that HIF-1 was required for VEGF induction by hypoxia confirmed the importance of HIF-1 in the cellular response to hypoxia (62). A number of subsequent studies confirmed that HIF-1 was induced by hypoxia and facilitated transcription of an array of genes essential for angiogenesis (165).

Hypoxia inducible factor is a heterodimeric transcription factor composed of an alpha and beta subunit (210, 212). Both subunits belong to a family of transcription factors which contain a basic-helix-loop-helix (bHLH) and Per/Arnt/Sim (PAS) domain (210), sequences important for dimerization and DNA binding. The HIF-1β subunit, also known as aryl hydrocarbon nuclear transporter (ARNT), dimerizes with multiple members of the bHLH-PAS family of TFs (145). Although both subunits are for the most
part continuously transcribed, HIF-1α is rapidly degraded under hypoxia and is subjected to extensive post-translational regulation. On the other hand, HIF-1β is constitutively expressed and is not subject to regulation by hypoxia (99). Following translocation to the nucleus, HIF-1 binds to promoters of genes containing hypoxia response elements and increases their transcription (180) (Figure 1).

The alpha subunit exists in three isoforms, HIF-1α, HIF-2α, and HIF-3α. The HIF-1α isoform is the best characterized and seems to be the most important in the cellular response to hypoxia (99). The HIF-2α subunit, also known as endothelial PAS domain protein 1 (EPAS 1) or HIF-related factor (HRF), shares 48% sequence identity with HIF-1α, is inducible by hypoxia and binds to the HREs of hypoxia inducible genes (49, 58, 201, 220). Although HIF-1α and HIF-2α share significant structural and functional similarities, they appear to be inducible by different stimuli (22) and have different patterns of tissue distribution (219). The third variant HIF-3α, also known as inhibitory PAS domain protein (IPAS), undergoes an alternative splicing event which removes the transactivation domain, and by binding to HIF-1α rather than HIF-1β functions in a dominant negative manner (143, 144).

It has been estimated that HIF-1 is capable of directly regulating approximately 60 genes (180). These genes can be categorized into several groups based on their physiological roles, including angiogenesis, growth and apoptosis, ECM metabolism, oxygen transport and iron metabolism, and energy metabolism (Table 1) (177). Although expression of some of these genes, such as VEGF, are under the control of HIF-1 in a wide variety of cell types, many are regulated in a cell-type specific manner (180). As
FIGURE 1. Overview of HIF-1α regulation. Under normal oxygen conditions (normoxia), HIF-1α levels are very low. This is due to the activity of tumor suppressors such as VHL and p53, which facilitate HIF-1α ubiquitination and degradation by the proteosome. When oxygen levels are low (hypoxia), interaction with the tumor suppressors is disrupted and HIF-1α rapidly accumulates. In addition to its stabilization by hypoxia, HIF-1α levels can increase due to activation of the PI3K-AKT-FRAP growth signaling pathway. HIF-1α accumulation by growth signaling results from an increased rate of synthesis.
TABLE 1. Examples of HIF-1 target genes.

<table>
<thead>
<tr>
<th>Category</th>
<th>Gene</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiogenesis</td>
<td>VEGF</td>
<td>Vascular endothelial cell growth</td>
<td>(62)</td>
</tr>
<tr>
<td></td>
<td>FLT-1 (VEGFR-1) FGFs</td>
<td>VEGF receptor</td>
<td>(68)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vascular endothelial cell growth</td>
<td>(129)</td>
</tr>
<tr>
<td></td>
<td>PDGF</td>
<td>Vascular endothelial cell growth</td>
<td>(71, 129)</td>
</tr>
<tr>
<td>Growth and apoptosis</td>
<td>Nip3</td>
<td>Apoptosis</td>
<td>(21)</td>
</tr>
<tr>
<td></td>
<td>Insulin-like growth factor</td>
<td>Binds and sequesters IGF</td>
<td>(196)</td>
</tr>
<tr>
<td></td>
<td>binding protein 1 (IGFBP-1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECM metabolism</td>
<td>(UPAR) urokinase plasminogen</td>
<td>Degradation of ECM</td>
<td>(78)</td>
</tr>
<tr>
<td></td>
<td>activator receptor</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Matrix metalloproteinase-2</td>
<td>Degradation of ECM</td>
<td>(128)</td>
</tr>
<tr>
<td></td>
<td>(MMP-2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Collagen prolyl-4-hydroxylase</td>
<td>Collagen synthesis</td>
<td>(194)</td>
</tr>
<tr>
<td>Energy metabolism</td>
<td>Glucose transporter-1</td>
<td>Glucose transport</td>
<td>(46)</td>
</tr>
<tr>
<td></td>
<td>6-phosphofructo-1-kinase</td>
<td>Glucose metabolism</td>
<td>(181)</td>
</tr>
<tr>
<td></td>
<td>Phosphoglycerate kinase 1</td>
<td>Glucose metabolism</td>
<td>(57)</td>
</tr>
<tr>
<td>Oxygen transport and iron</td>
<td>Transferrin</td>
<td>Iron transport protein</td>
<td>(172)</td>
</tr>
<tr>
<td>metabolism</td>
<td>Transferrin receptor</td>
<td>Mediates cellular iron uptake</td>
<td>(141, 192)</td>
</tr>
<tr>
<td></td>
<td>Erythropoietin</td>
<td>Haemoglobin synthesis</td>
<td>(182)</td>
</tr>
</tbody>
</table>

Genes in bold have confirmed HIF-1 binding sites, others are inducible by hypoxia and are suspected to have HIF-1 binding sites.
many of these genes are important for growth and survival, they are oncogenic and their
disregulation can lead to tumor development and progression. Because of this, HIF-1α is
often overexpressed in a wide variety of cancer types (180).

Because HIF-1 is important for the regulation of so many genes, many of which
are oncogenic proteins, expression and activity of the HIF-1α subunit is tightly
controlled. Although there are some reports of regulation of HIF-1α mRNA (109, 200,
210), the most frequently documented modes of HIF-1 regulation concerns abundance of
the alpha subunit and HIF-1 transcriptional activation. Regulation of HIF-1α levels, as
with all proteins, is determined by the rate of its synthesis versus the rate of its
degradation. It is well documented that the rate of synthesis is determined by the activity
of growth signaling pathways, and the rate of degradation by the availability of oxygen.
Transcriptional activation of HIF-1 is controlled by both growth signaling pathways and
oxygen availability. These modes of regulation will be discussed in greater detail in
subsequent sections.

Regulation of HIF-1α levels and activity occur primarily as a result of
postranslational modification, and these modifications include hydroxylation, acetylation,
and phosphorylation. Hydroxylation of multiple residues facilitates the response of HIF-
1α to hypoxia, and affects both the rate of degradation and the level of transcriptional
activation. This will be discussed in greater detail in the following section. It is known
that HIF-1α is subject to acetylation by the ARD1 acetyltransferase, and this reportedly
facilitates its degradation by increasing interaction with VHL (108). Several residues of
HIF-1α are phosphorylated (152, 169), as treatment with a phosphatase causes the
multiple bands associated with HIF-1α western blot return to the predicted size of 104
kDa (152). The function of HIF phosphorylation is not entirely clear (11), but appears to be facilitated by MAPK and probably results in transcriptional activation by encouraging interaction with CBP/p300.

**Regulation of HIF-1 by hypoxia**

The most notable aspect of HIF biology is induction of the alpha subunit during hypoxia. Under normal oxygen conditions and without stimulation by growth/oncogenic signals, HIF-1α levels in vitro are very low and usually undetectable. This is due to its continual and rapid destruction via proteosomal degradation in the presence of O₂. Degradation of HIF-1α during normoxia is extremely rapid, and the half-life has been estimated to be less than 5 minutes (98, 210). As the level of O₂ decreases, proteosomal degradation ceases and HIF-1α protein accumulates. In this manner, HIF-1 is capable of responding rapidly to changes in O₂ concentrations without having to wait for the lengthier processes of transcription and translation.

The destruction of HIF-1α in the presence of O₂ is facilitated by tumor suppressors such as VHL. Loss of VHL, as occurs in von Hippel-Lindau (VHL) disease, results in normoxic expression of HIF-1α and re-introduction of wildtype VHL eliminates HIF-1α expression (147). It was determined that VHL physically interacted with HIF-1α in a region known as the oxygen-dependent degradation domain (ODD), mediating its ubiquitination and proteosomal degradation (figure 2) (195). Although it was known that interaction of HIF-1α and VHL only occurred in the presence of O₂, and this interaction was abolished during hypoxia or treatment with iron chelators such as
FIGURE 2. Structure of HIF-1α and regulation by prolyl hydroxylation. The basic helix-loop-helix (bHLH) domain and Per-Arnt-Sim (PAS) domain are required for dimerization and DNA binding. There are two transactivation domains, an amino-terminal transactivation domain (TAD-N, or NAD) and a carboxyl-terminal transactivation domain (TAD-C, or CAD). An asparagine residue in the CAD is subjected to hydroxylation by the factor inhibiting HIF (FIH), resulting in increased transcriptional activity during hypoxia. Stability of the alpha subunit is mediated by the oxygen dependent degradation (ODD) domain. Two prolyl residues within the ODD are subjected to hydroxylation by the prolyl hydroxylase (PHD) enzymes during normoxia, which facilitate interaction with the E3 ubiquitin ligase VHL and result in HIF-1α destruction.
CoCl$_2$, it was not known how the actual level of oxygen was sensed. The mechanism of oxygen sensing became apparent with the discovery that VHL was only able to interact with HIF-1$\alpha$ when several critical conserved proline residues within the ODD were in a hydroxylated state (105, 106, 229). The enzymes responsible for hydroxylation of proline residues 402 and 564 were found to belong to a group of prolyl hydroxylase domain containing (PHD) enzymes. It was subsequently determined that there were three non-heme containing iron dependent PHD enzymes that share significant homology and facilitate HIF-1$\alpha$ hydroxylation (145). During normoxia, the proline residues in the ODD domain are fully hydroxylated, facilitating interaction with the E3 ubiquitin ligase VHL and destruction of HIF-1$\alpha$. As O$_2$ levels decrease, proline hydroxylation also decreases and the ability of VHL to interact with HIF-1$\alpha$ is diminished. In this manner, PHD enzymes control the rate of HIF-1$\alpha$ degradation in response to variations in oxygen availability.

In addition to stabilization during hypoxia, it was known that HIF-1 transcriptional activity was also elevated during hypoxia (111, 164). It was eventually determined that this was dependent on the hydroxylation state of asparagine residue 803 in the transactivation domain (TAD) of HIF-1$\alpha$. The enzyme responsible for its hydroxylation during hypoxia was determined to be an asparaginyl hydroxylase, also known as factor inhibiting HIF-1 (FIH-1) (132). During normoxia, the asparagine residue 803 is hydroxylated disrupting interactions between HIF-1$\alpha$ and the transcriptional coactivator CBP/p300 (10, 48, 90, 131, 132). During hypoxia, asparaginyl hydroxylation is diminished, and interaction with CBP/p300 is increased. In this manner, both the amount of HIF-1$\alpha$ protein and its transcriptional activity is elevated during hypoxia.
Regulation of HIF-1 by growth signaling

In addition to regulation by changes in oxygen levels, HIF-1α is regulated by receptor and non-receptor tyrosine kinases activity. Examples of growth signals, hormones, and cytokines capable of inducing HIF-1α are numerous, and include insulin (191, 204, 234), insulin-like growth factor-1 (IGF-1) (234), insulin-like growth factor-2 (IGF-2) (51), PDGF (170), EGF (236), HGF (193), TNFα (89), IL-1β (89, 191, 200), angiotensin-2 (170) and thrombin (74, 170) (Table 2). Not only is HIF-1α induced by ligand stimulation of membrane bound receptors, multiple studies have shown that oncogene (TKs and RTKs) overexpression (or overactivtity) can also elevate HIF-1α levels. Examples of these include HER2NEU (133), Ras (28, 148), v-Src (109).

In contrast to stabilization of HIF-1α as occurs during hypoxia, its induction by growth signals has been shown to function by increasing the rate of its synthesis. This was demonstrated through the use of the translation inhibitor cyclohexamide and pulse labeling experiments (64, 133, 204). The majority of these studies implicated members of the kinase cascade involving the tyrosine kinase PI3-kinase (PI3K) and the serine-threonine kinases AKT (protein kinase B) and FRAP (FKBP/rapamycin-associated protein, also known as mTOR, mammalian target of rapamycin), as critical for this enhanced rate of translation. The importance of these kinases was demonstrated repeatedly through the use chemical inhibitors (64, 191, 204, 236) and dominant negative versions (236). In addition, loss of the tumor suppressor phosphatase and tensin
TABLE 2. Regulators of HIF-1\(\alpha\) expression or function.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Effects on HIF-1(\alpha)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ligands of tyrosine kinase receptors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>Increased translation</td>
<td>(236)</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Increased translation</td>
<td>(234)</td>
</tr>
<tr>
<td>IGF-2</td>
<td>Increased translation</td>
<td>(51)</td>
</tr>
<tr>
<td>Insulin</td>
<td>Increased translation</td>
<td>(191, 204, 234)</td>
</tr>
<tr>
<td>Heregulin</td>
<td>Increased translation</td>
<td>(133)</td>
</tr>
<tr>
<td>PDGF</td>
<td>Increased translation</td>
<td>(170)</td>
</tr>
<tr>
<td><strong>Ligands of other receptors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin</td>
<td>Increased translation</td>
<td>(74, 170)</td>
</tr>
<tr>
<td>Angiotensin</td>
<td>Increased translation</td>
<td>(170)</td>
</tr>
<tr>
<td><strong>Oncogenes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPK</td>
<td>Increased transactivation</td>
<td>(169)</td>
</tr>
<tr>
<td>RAS</td>
<td>Increased translation</td>
<td>(28)</td>
</tr>
<tr>
<td>v-Src</td>
<td>Increased translation, increased transcription</td>
<td>(109)</td>
</tr>
<tr>
<td>c-Src</td>
<td>Increased translation</td>
<td>(116)</td>
</tr>
<tr>
<td>PI3K/AKT</td>
<td>Increased translation</td>
<td>(64, 191, 204, 236)</td>
</tr>
<tr>
<td><strong>Tumor suppressors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTEN</td>
<td>Decreased translation</td>
<td>(238)</td>
</tr>
<tr>
<td>VHL</td>
<td>Increased degradation</td>
<td>(147)</td>
</tr>
<tr>
<td>P53</td>
<td>Increased degradation</td>
<td>(167)</td>
</tr>
</tbody>
</table>
homologue deleted on chromosome 10 (PTEN), a negative regulator of AKT, or expression of constitutively active AKT also increased levels of HIF-1α (236, 238). Furthermore, induction of HIF-1α by non-receptor tyrosine kinases such as c-Src (pp60c-src) (116), v-Src (109), and Ras (28, 148) function via increased translation which is dependent on the PI3K/AKT/FRAP pathway.

The ability of the PI3K/AKT/FRAP kinase cascade to increase the rate of HIF-1α synthesis probably depends on the ability of FRAP to phosphorylate two important components of the translational machinery; eIF-4E binding protein 1 (4E-BP1) and p70 S6 kinase (S6K) (11) (Figure 3). Following phosphorylation, 4E-BP1 loses the ability to inhibit eukaryotic translation initiation factor 4E (eIF-4E), resulting in increased recruitment of 40S ribosomal subunit to the 5’ cap structure of the mRNA (11). In addition, phosphorylated FRAP activates S6K which enables activation of the 40S ribosomal protein S6, resulting in increased transcription of mRNAs with a 5’ polypyrimidine tract (70, 208). The ability of the PI3K/AKT/FRAP kinase cascade to increase the rate of HIF-1α synthesis is dependent on the presence of the 5’-UTR of the HIF-1α mRNA, as removal of this sequence abolished the effects of heregulin (133).

In addition to HIF-1α regulation by the PI3K/Akt pathway, it has been reported that MAPK signaling is instrumental in receptor mediated activation of HIF-1 (64). Although its role in regulation of HIF-1α is not as well studied as PI3K/AKT, activation of p42/p44 MAPK seems to result in transcriptional activation of HIF-1 (152, 169, 188). Direct phosphorylation of HIF-1α by MAPK has been reported (152, 169), and the probable result of HIF-1α phosphorylation is increased transcriptional activation by
FIGURE 3. Mechanism of HIF-1α regulation by growth signaling. Activation of PI3K results in a kinase cascade involving AKT and mTOR. Activated mTOR phosphorylates p70 S6 kinase (S6K) which in turn phosphorylates S6, increasing transcription of mRNAs with a 5’ polypyrimidine tract. Furthermore, activated mTOR phosphorylates eIF-4E binding protein 1 (4E-BP1), which consequently loses its ability to inhibit eukaryotic translation initiation factor 4E (eIF-4E). Unrepressed eIF-4E facilitates recruitment of 40S ribosomal subunit to the 5’ cap structure of the mRNA.
disrupting interactions with FIH, the transcriptional suppressor of HIF-1α (11).

Furthermore, several groups have reported MAPK activation by hypoxia in several cell lines (34, 148, 152), so it is possible that MAPK activity enhances both signal mediated and hypoxic transcriptional activation of HIF.

**Signal transducer and activator of transcription**

Signal transducers and activators of transcription (STATs) are a group of transcription factors that in the unphosphorylated form exist in a latent state in the cytoplasm (138). STATs undergo tyrosine phosphorylation by a variety of tyrosine kinases, including both receptor tyrosine kinases (RTKs) and non-receptor TKs (figure 4). Many membrane bound receptors do not have intrinsic tyrosine-kinase activity, and therefore require the assistance of the Janus Kinase (JAKs) (23). Furthermore, STAT phosphorylation can result from the activity of non-receptor tyrosine kinases such as Src and Abl (37, 228). Phosphorylation of a single tyrosine residue near the carboxy terminus results in homo- or hetero-dimerization via interaction with the Src-homology-2 (SH2) domain of the other STAT protein (186). Furthermore, several STAT proteins undergo serine phosphorylation in the carboxy-terminal transactivation domain resulting in increased transcriptional activity (217). Following tyrosine phosphorylation and translocation to the nucleus, Stats bind to promoters containing the consensus sequences TT(N₄)AA and TT(N₅)AA (179). Stats control a variety of genes, particularly those involved with cell growth, survival, and immune function (230).
FIGURE 4. Stat3 signaling pathway. Binding of growth factors or cytokines to their receptors results in activation of intrinsic receptor tyrosine kinase activity or recruitment and activation of receptor associated kinases such as janus kinase (JAK). The cytoplasmic tail of the receptor undergoes phosphorylation providing a docking site for non-phosphorylated latent Stat monomers. Recruited Stats undergo tyrosine phosphorylation, followed by dimerization and translocation to the nucleus. Phosphorylated Stat homo- or hetero-dimers bind to and activate promoters of genes involved with survival, growth, immunity and angiogenesis.
There are seven isoforms of Stat proteins, Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6. Of these different isoforms, Stat1, Stat3, and Stat5 are commonly activated in a variety of cancers (23). However, because Stat1 is primarily growth suppressive, it is not likely that it contributes to oncogenesis (19). There is a wealth of evidence to indicate that Stat3 and Stat5 are important for tumor progression. Stat5 is commonly activated in and appears to be important for various leukemias (23). On the other hand, Stat3 is activated in a wide variety of cancer types, including leukemia (76), multiple myeloma (25), breast cancer (66, 213), head and neck cancer (80), prostate cancer (157) and melanoma (158).

There is an abundance of evidence defining a critical role for Stat3 in oncogenesis (15, 17). Transfection of cells with Stat3C, in which substitution of two residues with cysteine results in spontaneous dimerization and constitutive activity, results in transformation and tumor formation in nude mice (20). Furthermore, Stat3 is necessary for cellular transformation mediated by v-Src (18, 205). It is well established that Stat3 signaling contributes to oncogenesis by preventing apoptosis (25, 79, 80) and blocking Stat3 signaling induces apoptosis in a variety of tumors (25, 80, 158). Inhibition of Stat3 signaling prevents expression of Bcl-xL, an important anti-apoptotic protein (25, 80, 158). Furthermore, it has also been demonstrated that Stat3 controls cell cycle progression by regulating D1 (20). These reports and others firmly establish Stat3 as an important facilitator of oncogenesis.

Although Stat3 itself is not mutated in tumors, it is activated by and required for the transforming capabilities of many oncogenic proteins. For example, Stat3 is activated in cells transformed with v-Src and v-Abl oncogenes (37, 228) and is required for the
transforming ability of v-Src (14, 18, 205). Furthermore, Stat3 is induced by a variety of cytokines and growth factors commonly over-expressed by cancer cells, including EGF, PDGF, and IL-6 (14, 25, 80, 157). These reports and others demonstrate that Stat3 is an important facilitator for many oncogenic proteins.

**Stat3 and angiogenesis**

There is accumulating evidence that Stat3 is an important facilitator of tumor angiogenesis. Stat3 activation correlates with VEGF production in a variety of human cancers, including breast, head and neck, melanoma, and pancreatic cancer cell lines (159, 214) as well as VEGF mRNA in pancreatic cancer specimens from patients (214). Furthermore, many of the growth/oncogenic signaling pathways capable of increasing VEGF production also result in activation of Stat3. For example it has been reported that activation of both EGFR and Src signaling pathways result in increased production of VEGF (47, 155, 161, 218), and it is well documented that stimulation of these pathways results in Stat3 activation (18, 66, 67, 79, 205, 228). These data indicate that there is a strong correlation between Stat3 activation and VEGF expression in a variety of cancers.

Several publications have shown that Stat3 is required for VEGF expression in various cancer cell lines. Blocking constitutive Stat3 activity in tumor cells with a dominant negative version downregulates basal levels of VEGF expression (159, 214). Furthermore, it has been shown that Stat3 is necessary for VEGF induction resulting from both growth signals and tyrosine kinases. In cells transformed with v-Src there is high expression of VEGF and Stat3 activity, and blocking Stat3 significantly reduces VEGF
levels (159). Induction of VEGF in by IL-6 or glycoprotein (gp) 130 (a subunit of the IL-6 receptor) is dependent on Stat3 signaling (65, 215). Furthermore, Stat3 is required for VEGF induction by oncostatin-M (OSM) in an astroglioma cell line (168).

Transfection with Stat3C results in constitutive Stat3 activation and induces VEGF expression in both normal fibroblasts and B16 tumor cells (159). Furthermore, B16 cells transfected with Stat3C demonstrated increased angiogenesis when placed in matrigel and inserted into C57BL/6 mice (159). A putative Stat3-responsive element (SRE) sequence spanning –842 to –849 successfully competed with hSIE probe (159) and itself was able to function as a probe in EMSA (214). Furthermore, VEGF promoter activation by v-Src and constitutively activated Stat3 was abrogated when this same sequence was mutated (159, 214). Stat3 binding to the VEGF promoter in vivo was confirmed by chromatin immunoprecipitation assays in v-Src transformed NIH3T3 cells (159). These reports establish that Stat3 increases VEGF production by directly binding to and activating its promoter.

In addition to its effects on VEGF, Stat3 has been implicated as a facilitator of angiogenesis by other mechanisms. It has recently been demonstrated that Stat3 regulates expression of both MMP-2 and MMP-9, important facilitators of both angiogenesis and metastasis (41, 149, 223). It has also been reported that Stat3 is necessary for signaling by receptors associated with angiogenic ligands, including bFGFR and VEGFR (7, 42, 176). Furthermore, it has been demonstrated that Stat3 is necessary for the mitogenic effects of the angiogenic factor PDGF (14). It has also been reported that Stat3 is required for endothelial cell migration and microvascular tube formation (225). These data implicate Stat3 as a key facilitator of angiogenesis beyond regulation of VEGF.
It has recently been reported that there is a combined requirement for both Stat3 and HIF-1 for maximum VEGF induction by hypoxia or Src (82). Through the use of CHIP assays, the authors show that HIF-1 and Stat3 bind simultaneously to the VEGF promoter in response to activated Src or the hypoxia mimic CoCl$_2$. They also demonstrate that both Stat3 and HIF-1 are required for maximum activity of a luciferase reporter construct under the control of the VEGF promoter. When either HIF-1 or Stat3 was blocked with a dominant negative version, there was an equivalent loss of reporter activity. Furthermore, they show that both HIF-1$\alpha$ and Stat3 coimmunoprecipitate with each other as well as the transcriptional activator p300. It is not entirely surprising that they could form a complex on the VEGF promoter, as the HIF-1 binding site spans –985 to –935, and the Stat3 binding site is 86 bp downstream at –849 to –842 (62, 159). Although the authors demonstrate that both HIF-1$\alpha$ and Stat3 are required for VEGF expression, they do not show the effects of blocking Stat3 activity on HIF-1$\alpha$ expression.
OBJECTIVES

The overall objective of the studies conducted in this dissertation was to investigate the role of Stat3 in regulating tumor angiogenesis, in particular expression of HIF-1α and VEGF. It had recently been determined that Stat3 is a transcriptional activator of VEGF, and is essential for angiogenesis (159, 214). However, the role of Stat3 in regulating HIF-1α, the other main transcriptional regulator of VEGF, had not been examined. Results from preliminary experiments suggested that blocking Stat3 prevented both basal level expression and the hypoxic induction of HIF-1α. These findings lead to the hypothesis that Stat3 is required for HIF-1α expression, and blocking Stat3 signaling could abrogate both tyrosine kinase and hypoxia induced HIF-1 activity and VEGF expression. In order to verify this hypothesis, the following aims were pursued.

Aim I. To determine the role of Stat3 in HIF-1α expression induced by growth signals.

(A) To determine the mechanism by which IL-6 induces HIF-1α expression. It had been reported that stimulation of a variety of receptor tyrosine kinases results in increased HIF-1α levels during normoxia (64, 133, 204, 236). However, it had not been determined if stimulation with IL-6 resulted in elevated levels of HIF-1α. Because Stat3 is an
important facilitator of IL-6 signaling, the role of Stat3 in IL-6 induced HIF-1α expression was examined.

(B) To determine if Stat3 is required for HIF-1α induction by oncogenic growth signals other than IL-6. Multiple growth signals that activate Stat3 also result in elevated expression of HIF-1α during normoxia. For example, stimulation with epidermal growth factor (EGF) increases both Stat3 activation and HIF-1α expression (236, 237). Because it had been observed that Stat3 was required for HIF-1α induction by IL-6, experiments were performed to determine if Stat3 was also required for increased HIF-1α expression resulting from heregulin and EGF.

Aim II. To determine the role of Stat3 in the cellular response to hypoxia.

(A) To determine if Stat3 is required for the hypoxic induction of HIF-1α and hypoxia inducible genes. It has been reported that inhibiting the AKT/PI3K pathway does not limit the induction of HIF-1α by hypoxia (2, 6). This suggests that expression of HIF-1α due to growth signaling and hypoxia result from separate and independent mechanisms. Because it had been observed that blocking Stat3 signaling prevented both basal HIF-1α expression and its induction by growth signals, experiments were performed to determine if the hypoxic induction of HIF-1α was also prevented. Furthermore, the role of Stat3 in the hypoxic induction of VEGF and MMP-2 was examined.
(B) To characterize Stat3 activation by hypoxia. HIF-1 is an important regulator of the VEGF gene in response to hypoxia. Because it has been reported that Stat3 is also a transcriptional regulator of VEGF, and preliminary experiments in the laboratory suggested that Stat3 was necessary for HIF-1α induction by hypoxia, Stat3 activation by hypoxia was examined. Furthermore, the time-course of Stat3 activation in relation to HIF-1α and VEGF induction was studied.
MATERIALS AND METHODS

Reagents and antibodies

The following reagents and antibodies were purchased from various companies as indicated: Interleukin 6 (BD Pharmingen); heregulin (Sigma); EGF (Sigma); Cyclohexamide (Calbiochem); G418 (Cellgro); CoCl$_2$ (sigma); Anti-VEGF monoclonal antibody (R&D); Anti-HIF-1α polyclonal antibody and Anti-β-actin monoclonal antibody (Santa Cruz Biotechnology); Anti-HIF-1β monoclonal antibody (NOVUS Biologicals); Anti-Phospho-Akt (Cell Signaling). Anti-Akt1 monoclonal antibody was a kind gift from Dr. J. Cheng (University of South Florida College of Medicine, Tampa).

Cell lines and cell culture

BALB/c-3T3 fibroblasts and v-Src-transformed BALB/c 3T3 fibroblasts were grown in Dulbecco’s modification of Eagle’s medium (DMEM) with 10% calf serum (CS) and 1% antibiotic-antimycotic (Gibco). MCF-7, DU-145, and A2058 tumor cells were grown in RPMI 1640 with 10% FBS and 1% antibiotic-antimyocotic. BALB/c fibroblasts were a gift from Dr. Jack Pledger (H. Lee Moffitt Cancer Center, Tampa, Florida). BALB/c fibroblasts transformed with v-Src were a gift from Dr. Richard Jove (H. Lee Moffitt Cancer Center, Tampa, Florida). A2058 human melanoma, DU145 human prostate
cancer and MCF-7 human breast cancer cells were obtained from ATCC (Rockville, MD). For culture under hypoxia, cells were placed in a modular incubator chamber (Billups-Rothenberg). The chamber was sealed and flushed with a gas mixture containing 1% O₂, 5% CO₂, and 94% N₂ for 10-15 minutes, and then incubated at 37°C.

**Treatment With Src Tyrosine Kinase Inhibitors**

PD166285 (127) and PD180970 (127) were dissolved in 100% dimethyl sulphoxide (DMSO) and stored at -80°C. The stocks were diluted into the media, immediately prior to use.

**Generation of cell lines stably transfected with Stat3 siRNA**

The Stat3 siRNA oligonucleotide, AATTAAGGGTTTTGGCTGTCTGTTTCAAATTCT CTTGAATTGACCAGCAATCCACTCC, was inserted into pSilencer 1.0-U6 siRNA expression vector (Ambion). To generate siRNA/Stat3 stable tumor cell clones, the pSilencer 1.0-U6 Stat3 siRNA expression vector (1µg) was co-transfected with pcDNA3 (0.1µg) into MCF-7 and A2058 cells using Lipofectamine (Invitrogen) as described below, followed by selection with hygromycin (300 µg/ml). MCF-7 and A2058 clones stably transfected with the empty pSilencer/pcDNA3 were used as a control.
Retrovirus infection

The stable retrovirus-producing cell lines HEK 293T expressing Stat3D and MSCV (the control retrovirus vector) were generous gifts from Dr. D. Link (Washington University, Seattle). For retrovirus infection, supernatant was collected from confluent retrovirus-producing cells following a 48 hour incubation. The supernatant was supplemented with 8 µg/ml polybrene. BALB/c-3T3 v-Src cells were seeded at 1.5 x 10⁶ in a 10cm plate the day prior to infection. The following day, cells were cultured in viral supernatant for 4-8 hours, then the viral supernatant was replaced with normal growth media. This was repeated up to three times until 100% infection efficiency had been achieved, as visualized by fluorescent microscopy.

Anti-sense oligonucleotide and Stat3 siRNA transient transfections

For transfection with antisense oligonucleotide, human tumor cells were seeded in 6-well plates at 2.5 x 10⁵ or 10cm dishes at 1.5 x 10⁶ for 18-24h prior to transfection. Cells were transfected with either 1 µg of plasmid DNA or 300 nM oligonucleotide using Lipofectamine and Lipofectamine plus reagents (Life Technologies), according to the manufacturer’s protocol. The sequence for Stat3 antisense oligonucleotide was 5’-GCTCCAGCATCTGCTGCTTC-3’. The sequence for control oligonucleotide was 5’–GCTCCAATACCCCGTTGCTTC-3’. For transient transfection with Stat3 or HIF-1α (Dharmacon), cells were seeded at 2.5 x 10⁵ per well of 6-well plates 18-24h prior to
transfection. Cells were transfected with 10-50nM siRNA using transit TKO transfection reagent (Mirus), according to the manufacturer’s protocol.

**Preparation of whole cell and nuclear extracts**

For preparation of whole cell extracts, cells were washed 3x with ice cold phosphate-buffered saline (PBS), scraped into PBS and pelleted by centrifugation at 2500 x g for 5 min at 4°C. Pellets were resuspended in RIPA triple detergent lysis buffer (50 mM Tris (pH 7.4), 5 mM EDTA, 150 mM NaCl, and 0.5% Triton-X-100, 1 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 2 µg/ml pepstatin), then incubated on ice for 30 min. Lysates were centrifuged at 15000 x g for 30 min. Supernatants were collected and protein concentration was determined using the Bradford assay. For preparation of nuclear extracts, cells were washed 3x with ice cold PBS (supplemented with 100 mM Na₃VO₄, 100 mM NaF), scraped into PBS and pelleted by centrifugation at 2500 x g for 5 min at 4°C. Pellets were resuspended in hypotonic buffer A (10mM Tris-HCl (pH 7.6), 1.5 mM MgCl₂, 10 mM KCl, 2mM DTT, 0.4 mM PMSF, 1mM Na₃VO₄, 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 2 µg/ml pepstatin) then centrifuged at 2500 x g. Cells were resuspended in buffer A + 1% NP-40 then incubated on ice for 10 min. Cells were centrifuged for 10 min at 10,000 x g. The supernatant (cytoplasmic extract) was discarded, and the pellet was resuspended in hypertonic buffer C (0.42 M KCl, 20 mM Tris-HCl (pH 7.6), 20% glycerol, 1.5 mM MgCl₂, 2mM DTT, 0.4 mM PMSF, 1 mM Na₃VO₄, 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 2 µg/ml pepstatin). Following
stirring for 30 min at 4°C, extracts were spun at 15,000 x g for 30 min. Supernatants were collected (nuclear extract) and concentration was determined using the Bradford assay.

**Western Blotting**

Equal amounts of whole cell or nuclear extract (30-50 µg of NE or 100 µg of WCE) were dissolved in Laemmli SDS-PAGE sample buffer (100mM Tris-HCl (pH 6.8), 4% SDS, 0.2% Bromophenol, 20% glycerol, 2% 2-mercaptoethanol, 1mM DTT) and boiled at 100°C for 5 min prior to separation by SDS-polyacrylamide gel electrophoresis. Samples were applied to 8% polyacrylamide gels for nuclear extracts and 12% polyacrylamide gels for whole cell extracts. Electrophoresis was generally performed at 50 V for approximately 16 hours. For transfer, the Gel, PVDF membrane (Millipore), and filter papers were soaked in transfer buffer (25 mM Tris base, 192 mM glycine, 20% Methanol) for approximately 5 min. The gel and membrane were sandwiched between the filter papers and placed in a semidry electroblotter (Owl Separation Systems). Transfer was generally performed at 200 mAmps for 4 hours (current varied depending on gel size, but never exceeded Area (cm) X 3 mAmps). Following transfer, even loading and transfer was confirmed by staining the membrane with Ponceau S solution (Sigma). The membrane was blocked by incubation for 1 hour at room temperature in blocking buffer (PBS containing 5% non-fat dry milk and 0.1% Tween). Membranes were probed with primary antibody dissolved in blocking buffer at the following concentrations: HIF-1α rabbit polyclonal (H-206) (1:500 dilution), HIF-1β mouse monoclonal (1:1,500 dilution), B-actin mouse monoclonal (1:5000), AKT1 mouse monoclonal (1:1,000 dilution),
dilution), phospho-AKT rabbit polyclonal (1:1,000 dilution), and VEGF mouse monoclonal antibody (1:500 dilution). Probing with primary antibodies was performed for 3 hours at room temperature or overnight at 4°C. Membranes were washed with washing buffer (PBS with 0.1% Tween) twice for 5-10 min each. Membranes were probed with secondary antibody dissolved in blocking buffer at the following concentrations for one hour: Horseradish peroxidase-conjugated sheep anti-mouse (1:2,500) and donkey anti-rabbit (1:5,000). Following incubation with secondary antibody, membranes were washed with washing buffer for 15 min three times. The signal was developed with SuperSignal West Pico Chemiluminescent Substrate (PIERCE) according to manufacturer’s instructions.

**Electrophoretic Mobility Shift Assay (EMSA)**

Binding reactions were carried out in a total volume of 20ul containing 4-8 µg of normalized nuclear extract in (10 mM HEPES, 10% glycerol, 1 mM DTT, 0.1µg/µl poly (dI:dC), 0.5 g/ul BSA, 80mM NaCl). The $^{32}$P-radiolabeled hSIE (Stat3 and Stat1 specific high affinity Sis-Inducible Element, 5’-AGCTTCATTTCCGTAAATCCCTA) probe was added and samples were incubated for 30 min at 30°C. Following the binding reaction, protein-DNA complexes were resolved by 5% non-denaturing polyacrylamide gel electrophoresis (PAGE). Electrophoresis was carried out at 200 V in 0.25 X Tris-borate-EDTA (TBE) (1x TBE is 89 mM Tris-HCl, 89 mM boric acid, and 5 mM EDTA) for 2-3 hours. Gels were fixed with fixing buffer (10% Methanol, 10% acetic acid),
vacuum dried at 80°C for 2 h, and autoradiographed with intensifying screens at –80°C for 1 to 5 days

**RNA isolation and Northern blotting**

Total RNA was isolated (from 1.5 x 10^6 cells seeded in 10-cm dishes) using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. 15-µg aliquots were added to 15ul loading buffer (Sigma) and samples were denatured at 65°C for 15 min. Samples were loaded into a 1% agarose-formaldehyde gel (containing ethidium bromide) and electrophoresed at 80 volts for approximately 5 hours in 1X MOPS (10X Mops is 0.4 M Morpholinopropanesulfonic acid, 0.1 M Sodium acetate–3 H2O, 10 mM EDTA-Na2-2 H2O, pH 7.2) solution. Prior to transfer, the gel was photographed under UV light to verify equal loading by visualization of the 18S and 28S rRNA bands. The gel, nylon membrane (PerkinElmer), and whatman filter paper were presoaked in 10X SSC prior to transfer. RNA was transferred to membrane in 10x standard saline citrate (SSC) by upward capillary transfer. The membrane was washed briefly in 2X SSC and ultra-violet cross-crosslinked using a UV-Stratalinker 2400 (Stratagene, La Jolla, CA, USA).

For preparation of the HIF-1α probe, the entire HIF-1α cDNA (3.2 Kbp) was 32P-radiolabeled using All-in-One™ random primer DNA labeling mix (-dATP) (Sigma) according to the manufacturer’s protocol. The radiolabeled cDNA was purified using a G-25 sephadex column (Roche) according to the manufacturer’s protocol. To detect the HIF-1α transcript, the membrane was first pre-hybridized at 68°C for at least 5 min in hybridization buffer (Sigma). The radiolabelled cDNA was denatured by heating to
100°C for 10 min followed by placement on ice for 2 min. The denatured probe was added to the warm pre-hybridization solution, and the membrane was hybridized overnight at 42°C. The following day, the membrane was washed at least twice at 60°C with 2x SSC for 15 min, and then exposed to a phosphoimaging plate for 3 hours (Molecular Dynamics).
RESULTS

Aim I: To determine the role of Stat3 in HIF-1α expression induced by growth signals.

IL-6 increases levels of HIF-1α protein

Multiple studies have shown that HIF-1α expression is increased by a variety of growth/oncogenic signals. Examples of these signals include heregulin (133), EGF (236), IGF-1 (234), TNFα (89), IL-1β (89, 191, 200) and others. In addition to HIF-1α, many of these signals also increase expression of VEGF (32, 63, 139, 174). Although it has been reported that stimulation with IL-6 results in increased production of VEGF (33, 38), it has not been demonstrated that it increases HIF-1α expression.

Here we demonstrate that stimulation of MCF-7 human breast cancer with IL-6 results in increased expression of HIF-1α protein in a dose dependent manner (Figure 5). MCF-7 cells were serum starved for 20 hours followed by stimulation with IL-6 in serum free media at the indicated concentrations for 6 hours. Nuclear extracts were collected and western blot analysis was performed. Nuclear extracts are used throughout this dissertation for the detection of HIF-1α by Western blot. Although HIF-1α can be detected using whole cell extracts, levels are low and the bands are often weak due to the extremely short half-life of HIF-1α under normoxia. Band intensity and broadness is significantly increased through the use of nuclear extracts.
FIGURE 5. IL-6 increases HIF-1α protein in MCF-7 breast cancer cells. MCF-7 cells (1.5 x 10^6) were cultured for 20h in serum free media followed by treatment with IL-6 in serum free media at the indicated concentrations for 6h. Nuclear extracts (30ug) were analyzed by western blotting using the indicated antibodies. HIF-1β and β-actin are used to verify equal loading.
The vast majority of reports concerning HIF-1α regulation by growth signals and cytokines indicate that increased expression of HIF-1α occurs without a concurrent increase in mRNA (11), however exceptions have been reported (200). To determine if this was true for IL-6, total RNA was collected from MCF-7 cells following treatment with IL-6 for 6h and analyzed by Northern blotting (Figure 6). Results demonstrate that the increase in HIF-1α levels resulting from IL-6 stimulation of MCF-7 cells cannot be accounted for by changes in levels of mRNA.

**IL-6 increases HIF-1α expression by increasing its rate of synthesis**

A majority of studies show that increased expression of HIF-1α resulting from stimulation with growth/oncogenic signals is due to an increase in the rate of its synthesis. Examples include heregulin (133), insulin (204), and IGF-1 (64), in which the authors directly show that elevated levels of HIF-1α were the result of an increased rate of synthesis. This was demonstrated through the use of the translation inhibitor cyclohexamide (CHX) and/or pulse labeling experiments. In contrast, increased HIF-1α expression resulting from hypoxia is due to protein stabilization, or cessation of the continuous degradation that occurs during normoxia.

To determine if IL-6 stimulation results in an increased rate of HIF-1α synthesis or in a decreased rate of degradation, cyclohexamide was utilized. If IL-6 stimulation stabilizes HIF-1α protein, the addition of cyclohexamide to halt protein synthesis
FIGURE 6. IL-6 does not increase HIF-1α mRNA in MCF-7 breast cancer cells. MCF-7 cells (1.5 x 10^6) were cultured for 20h in serum free media followed by treatment with IL-6 in serum free media at the indicated concentrations for 6h. RNA (15ug) was analyzed by Northern blotting using a HIF-1α cDNA probe. A photograph of the ethidium bromide-stained gel indicates equal loading as demonstrated by the 18S and 28S rRNA bands.
following pre-treatment with IL-6 would not affect levels of HIF-1α. However, if IL-6 increases the rate of HIF-1α synthesis, then the addition of CHX would counteract this and a rapid loss of HIF-1α protein would occur. To determine which of these was occurring, MCF-7 cells were treated with IL-6 for 6h followed by extraction of nuclear proteins and Western blot analysis (Figure 7). HIF-1α levels were significantly decreased as soon as 15 min following addition of CHX and returned to baseline within 1 hour, demonstrating that increased HIF-1α expression resulting from IL-6 stimulation is due to an increased rate of synthesis.

Stat3 is required for IL-6 induction of HIF-1α

Many of the growth/oncogenic signals that increase HIF-1α expression also result in activation of Stat3. It is well established that Stat3 is activated in response to stimulation with IL-6 (237). Furthermore, it has been reported that IL-6 results in increased production of VEGF (33, 38) and Stat3 is essential for this effect (215). We have shown here that IL-6 stimulation upregulates HIF-1α expression by increasing the rate of its synthesis. Because it was known that blocking Stat3 signaling prevented VEGF induction by IL-6 (215), as well as tumor angiogenesis (159, 214), we wanted to determine if HIF-1α expression resulting from IL-6 stimulation was also dependent on Stat3 signaling.

MCF-7 cells were stably transfected with pSilencer expressing Stat3 siRNA, or with empty pSilencer. These cells were treated with IL-6 for 6 hours in duplicate and both whole cell and nuclear extracts were collected. Western blot analysis was performed
FIGURE 7. IL-6 induced HIF-1α levels are due to synthesis and not cessation of degradation. MCF-7 cells (1.5 x 10^6) were cultured for 20h in serum free media followed by treatment with IL-6 (20 ng/ml) in serum free media for 6h. Cyclohexamide (100µM) was added for the indicated times. Nuclear extracts (30µg) were analyzed by western blotting using the indicated antibodies. HIF-1β and β-actin are used to verify equal loading.
to determine the levels of HIF-1α and VEGF. Results indicate that when Stat3 is silenced in MCF-7 cells, levels of HIF-1α are significantly lower compared to the control in both untreated and IL-6 treated cells (Figure 8). Furthermore, VEGF levels are dramatically reduced both at the basal level and following induction by IL-6 (Figure 8).

It is well documented that induction of HIF-1α by growth/oncogenic signals is due to increased synthesis. Furthermore, this increased rate of synthesis is the result of activation of growth signaling pathways, in particular the PI3K/AKT pathway (11). It is known that stimulation with IL-6 results in PI3K/AKT activation (88). Also, through correspondence with Dr. J. Cheng (University of South Florida College of Medicine, Tampa), we learned that Stat3 is a direct transcriptional activator of the AKT-1 gene. This suggested to us the potential mechanism that Stat3 may be regulating HIF-1α induction by controlling expression of AKT-1.

To determine if Stat3 regulates Akt expression in MCF-7 cells, both total levels of Akt1 and phosphorylated levels of Akt1 and Akt2 were examined in cells stably transfected with Stat3 siRNA. Treatment with IL-6 resulted in increased levels of total Akt1 and phosphorylated Akt1 and Akt2 (figure 9). Following silencing of Stat3, basal levels of Akt1 as well as its induction by IL-6 was abolished. Furthermore, phosphorylated levels of both Akt1 and Akt2 were dramatically reduced. These results suggest a potential mechanism by which Stat3 regulates HIF-1α expression.
FIGURE 8. Silencing Stat3 blocks IL-6 induction of HIF-1α. MCF-7 cells were stably-transfected with empty pSilencer/pcDNA3 or pSilencer/pcDNA3 with Stat3 siRNA. Cells were serum starved for 20h followed by treatment with or without IL-6 (20 ng/ml). Nuclear extracts (30µg) (Top panel) and whole cell extracts (100µg) (middle panel) were analyzed by Western blotting using the indicated antibodies. Stat3 DNA binding was determined by EMSA using nuclear extracts (8ug) (bottom panel).
FIGURE 9. Silencing Stat3 decreases AKT levels. MCF-7 cells (1.5 x 10^6), stably-transfected with empty pSilencer or pSilencer-Stat3 siRNA, were cultured in serum free media for 20h followed by treatment with IL-6 for 6h. Whole cell extracts (100µg) were obtained and analyzed by Western blotting using the indicated antibodies. The antibody used for detecting phospho-Akt (pAkt) recognizes both Akt1 and Akt2. For the total Akt protein detection, the antibody is specific for Akt1.
Stat3 is required for HIF-1α induction by Heregulin

Many growth signals capable of activating Stat3 also increase expression of HIF-1α. Examples of these include EGF (236, 237) and PDGF (64, 170, 207). Because we have observed that blocking Stat3 limits the ability of IL-6 to induce HIF-1α, we wanted to know if induction of HIF-1α by other growth signals was also prevented when Stat3 was silenced.

Comparable to published results (133), we observed a dose dependent induction of HIF-1α when MCF-7 cells were stimulated with heregulin for 6h (Figure 10). Furthermore, coordinate Stat3 activation was observed as determined by EMSA. To determine if Stat3 is required for HIF-1α induction by heregulin, MCF-7 cells expressing Stat3 siRNA were again utilized. Following stimulation with heregulin for 6h, HIF-1α was induced in control cells but not in Stat3 siRNA cells (Figure 11). Furthermore, VEGF expression was increased by stimulation with heregulin only in control cells. As previously seen in MCF-7 cells, basal level expression of both HIF-1α and VEGF was blocked. These data show that Stat3 is required for the induction of both HIF-1α and VEGF by heregulin in MCF-7 breast cancer cells.

Stat3 is required for HIF-1α induction by EGF

In addition to heregulin, it has been reported that stimulation with EGF results in both increased HIF-1α expression (236) and Stat3 activation (237). Because we had observed that silencing Stat3 prevented the induction of HIF-1α by Heregulin and IL-6,
**FIGURE 10. Heregulin increases both Stat3 binding and HIF-1α protein in MCF-7 breast cancer cells.** MCF-7 cells (1.5 x 10^6) were cultured for 20h in serum free media followed by treatment with heregulin at the indicated concentrations for 6h. Nuclear extracts (30µg) were analyzed by western blotting using the indicated antibodies. Stat3 DNA binding was determined by EMSA using nuclear extracts (8ug) (bottom panel).
FIGURE 11. Stat3 siRNA blocks heregulin induced HIF-1α expression. MCF-7 cells were stably-transfected with empty pSilencer/pcDNA3 or pSilencer/pcDNA3 containing Stat3 siRNA. Cells were serum starved for 20h followed by treatment with or without heregulin (100ng/ml). Nuclear extracts (30µg) (Top panel) and whole cell extracts (100µg) (middle panel) were analyzed by Western blotting using the indicated antibodies.
FIGURE 12. EGF increases both Stat3 binding and HIF-1α protein in prostate tumor cell line DU-145. DU-145 cells (1.5 x 10^6) were cultured for 20h in serum free media followed by treatment with EGF at the indicated concentrations for 6h. Nuclear extracts (30µg) were analyzed by western blotting using the indicated antibodies. Stat3 DNA binding was determined by EMSA using nuclear extracts (8µg) (bottom panel).
we wanted to know if this was also true for EGF. To verify that EGF induced both HIF-1α and Stat3 expression in DU145 prostate cancer, cells were treated with various concentrations of EGF for 6h. Consistent with previous reports (236), treatment with EGF results in increased expression of HIF-1α, as well as significant activation of Stat3 (Figure 12). To determine if knocking down Stat3 expression could prevent induction of HIF-1α by EGF, antisense oligonucleotide was utilized. Following transfection with ASO, cells were treated with EGF for 6 hours and nuclear extracts were obtained for Western blot analysis. Results demonstrate that transfection of cells with ASO results in significantly diminished levels of HIF-1α in both nontreated and EGF treated samples. (Figure 13). These results and those described in previous sections show that Stat3 signaling is necessary for both basal expression of HIF-1α and for its induction by growth signals and cytokines.

Aim II. To determine the role of Stat3 in the cellular response to hypoxia.

Stat3 is required for HIF-1α induction by hypoxia

It is well documented that increased levels of HIF-1α resulting from growth signals are due to increased synthesis, and increased levels by hypoxia result from stabilization and therefore accumulation. It is also reported that both hypoxic stabilization and growth signal induced translation can increase HIF-1α levels in a cumulative manner. However, several studies indicate that blocking the PI3K/AKT pathway does not limit the ability of hypoxia to increase HIF-1α levels (2, 6, 113). If silencing Stat3
FIGURE 13. Stat3 is required for EGF mediated HIF-1α expression. DU-145 cells were transiently transfected with control or Stat3 ASO, serum starved for 20h followed by treatment with EGF (30ng/ml) for 6h. Nuclear extracts (30ug) were analyzed by Western blotting using the indicated antibodies.
decreases HIF-1α levels only by affecting AKT, then blocking Stat3 signaling should not affect HIF-1α induction by hypoxia. However, if silencing Stat3 also limits HIF-1α induction by hypoxia, then there is likely some other mechanism also at work. Because we had proposed that Stat3 regulates HIF-1α via AKT, we wanted to know if blocking Stat3 signaling could prevent the induction of HIF-1α by hypoxia.

Our initial experiments involving HIF-1α induction by hypoxia utilized BALB/c-3T3 fibroblasts transformed by the oncoprotein v-Src. These cells have high levels of Stat3 activation as well as HIF-1α expression during normoxia (figure 14). The multiple bands associated with HIF-1α in this figure result from post-translational modifications. When they were transduced with retrovirus carrying the dominant negative Stat3D or empty vector MSCV, Stat3 DNA binding was almost completely ablated. Furthermore HIF-1α expression resulting from v-Src activity was eliminated. When these cells were treated with the hypoxia mimic CoCl$_2$, HIF-1α expression was strongly induced in the MSCV control cells but not in cells expressing Stat3D (Figure 14).

To be certain that the effects we were witnessing in mouse cells also occurred in human cells, we examined the role of Stat3 in the hypoxic induction of HIF-1α in A2058 human melanoma cells. Stat3 is constitutively activated in these cells due to activity of c-Src (158). When A2058 cells were treated with inhibitors of c-Src, Stat3 activation as well as HIF-1α induction by hypoxia was significantly reduced (Figure 15). To determine the effects of silencing Stat3 on HIF-1α induction by hypoxia, A2058 cells stably expressing Stat3 siRNA were created. When these cells were exposed to either 1% O$_2$ or CoCl$_2$, HIF-1α induction was significantly limited in Stat3 siRNA but not in control cells.
FIGURE 14. Stat3 is required for hypoxia induced HIF-1α expression in v-Src transformed Fibroblasts. BALB/c fibroblasts transformed with v-Src were stably transduced with empty vector MSCV or Stat3D. Cells were cultured for 20h in serum free media followed by treatment with the hypoxia mimic CoCl₂ (125μM) for 6h. (A) Nuclear extracts (30ug) were analyzed by Western blotting using the indicated antibodies. SP-1 and β-actin are used to verify equal loading. (B) Stat3 DNA binding was determined by EMSA using nuclear extracts (8ug).
FIGURE 15. Treatment with Src inhibitors prevents HIF-1α by hypoxia. A2058 human melanoma cells were seeded at $1.5 \times 10^6$ approximately 24 h prior to treatment with inhibitors. Cells were treated with Src inhibitors in serum free media for approximately 18 hours, and then incubated under hypoxia in the presence of inhibitors for 6 hours. Nuclear extracts (30µg) were analyzed by Western blot using the indicated antibodies.
FIGURE 16. Stat3 is required for HIF-1α induction by hypoxia in A2058 melanoma cells. A2058 cells were stably-transfected with empty pSilencer or pSilencer-Stat3 siRNA. Cells were serum starved for 20h followed by culture under conditions of 21% O₂, 1% O₂, or treatment with CoCL₂ (150uM). Nuclear extracts (30µg) (Top panel) and whole cell extracts (30µg) (middle panel) were analyzed by Western blotting using the indicated antibodies.
(Figure 16). These data clearly indicate that inhibition of Stat3 signaling prevents HIF-1α expression by Src as well as its induction by hypoxia. Because of the possibility that Stat3 was directly acting on the HIF-1α promoter, levels of HIF-1α mRNA were examined. In BALB/c-3T3-v-Src cells, HIF-1α mRNA expression was significantly decreased following transduction with Stat3D (data not shown). However, examination of HIF-1α mRNA in A2058 human melanoma showed no difference in RNA expression, or possibly even increased levels in cells in which Stat3 had been silenced (Figure 17). Because it has been reported that Src may regulate HIF-1α at the level of mRNA in non-human cells but not in human cells (109), and our findings seemed to corroborate these reports, we did not investigate these results further. We conclude that at least in A2058 human melanoma cells, regulation of HIF-1α protein by Stat3 cannot be accounted for by changes in mRNA.

Stat3 is required for the hypoxic induction of VEGF and MMP-2

The evidence that Stat3 is an important regulator of angiogenesis is continuously increasing. Several reports have indicated that Stat3 is required for VEGF production, both at the basal level in various cancer cells (159, 214) as well as for its induction by IL-6 (215). Furthermore, recent reports have indicated that Stat3 regulates MMP-2 (223) and MMP-9 (41), both of which are important for the reorganization of the extracellular matrix during angiogenesis (149). The metalloproteinase genes are potentially regulated
FIGURE 17. HIF-1α mRNA in A2058 melanoma treated with CoCl$_2$. A2058 cells were stably-transfected with empty pSilencer or pSilencer-Stat3 siRNA. Cells were serum starved for 20h followed by treatment with CoCL$_2$ (125uM) for the indicated times. Total RNA (15ug) was isolated and analyzed by Northern blotting using a HIF-1α cDNA probe. A photograph of the ethidium bromide-stained gel verifies equal loading as demonstrated by the 18S and 28S rRNA bands.
FIGURE 18. Stat3 is required for VEGF induction by CoCl$_2$ in A2058 melanoma. A2058 cells stably expressing Stat3 siRNA were exposed to CoCl$_2$ for 48 hours in serum free media. Whole cell extracts (100ug) were analyzed by Western blotting using the indicated antibodies.
by HIF-1α, as they have been shown to be inducible by hypoxia (128). However, it is not known if Stat3 is required for the hypoxic induction of these genes.

To examine Stat3 regulation of VEGF in response to hypoxia, A2058 cells stably transfected with Stat3 siRNA were utilized. These cells were treated with the hypoxia mimic CoCl₂ for 48h prior to harvest of whole cell extracts and western blot analysis (Figure 18). The long timepoint was utilized in order to obtain maximum VEGF induction. VEGF was induced significantly in control cells, but not in cells expressing Stat3 siRNA. These results not only reinforce the importance of Stat3 in VEGF production, but also for VEGF induction by hypoxia.

BALB/c-3T3 fibroblasts transformed with v-Src were used to study the role of Stat3 in the hypoxic regulation of MMP-2. These cells were treated with 0.1% O₂ or CoCl₂ for 24h followed western blot analysis of whole cell extracts (Figure 19). It can be seen that both hypoxia and the hypoxia mimic resulted in induction of MMP-2 in MSCV control cells. However, in cells stably transduced with the dominant negative Stat3D, MMP-2 induction was prevented. Although it has been reported that Stat3 is necessary for MMP-2 expression, the requirement for its induction by hypoxia had not been demonstrated.

**Stat3 is activated by hypoxia**

Hypoxia results in increased expression of genes important for angiogenesis, and this increase in gene transcription is dependent on the activation of a limited set of transcription factors. The best documented transcription factor to be activated by hypoxia
FIGURE 19. Stat3 is required for MMP-2 induction by hypoxia in Fibroblasts transformed with v-Src. BALB/c-3T3 fibroblasts transformed with v-Src were transduced with dominant negative Stat3D or control MSCV. Cells were exposed to 0.1% O₂ or CoCl₂ for 24h hours in serum free media. Whole cell extracts (100ug) were analyzed by Western blotting using the indicated antibodies.
FIGURE 20. Stat3 is activated in human cancer cells exposed to hypoxia. DU145 cells (1.5 x 10^6) were incubated under 21% or 1% O_2 in serum free media. Nuclear extracts (30ug) were analyzed by Western blotting using the indicated antibodies. Stat3 DNA binding was determined by EMSA using nuclear extracts (8ug) (bottom panel).
is HIF-1. Very recently, there was a report of increased Stat3 phosphorylation following treatment with CoCl$_2$ in a pancreatic and a prostate cancer cell line (82). Since it is known that Stat3 directly regulates the VEGF promoter as well as other hypoxia inducible genes, we wanted to determine if Stat3 was activated by hypoxia.

To examine Stat3 activation by hypoxia, DU145 prostate cancer cells were exposed to 1% O$_2$ for 24 hours, then nuclear extracts were collected and EMSA was performed (Figure 20). A significant increase in Stat3 DNA binding was observed. To understand this activation in more detail, we wanted to determine the time course of Stat3 activation. Because we could not be certain that O$_2$ concentrations were equal between multiple chambers, the hypoxia mimic CoCl$_2$ was utilized instead. When BALB/c-3T3 fibroblasts were treated with CoCl$_2$ for various time points, significant Stat3 activation was seen as soon as 1 hour (Figure 21). Next we examined the time course of Stat3 activation in DU145 human prostate cancer cells. Increased Stat3 activation was seen as early as 6h, and was highest at the 24h timepoint (Figure 22). At 48h there was loss of Stat3 activation, however many cells had died or appeared apoptotic. Because HIF-1 is the main transcription factor to be activated by hypoxia and to facilitate the cellular response, we wanted to know if Stat3 activation coincided with or followed HIF-1$\alpha$ induction. HIF-1$\alpha$ levels accumulate rapidly and are already visible at the earliest timepoint of 30 minutes, indicating that HIF-1$\alpha$ accumulation precedes Stat3 activation (Figure 22). Since both HIF-1 and Stat3 are transcriptional activators of VEGF, we next examined the time course of VEGF production. VEGF accumulation correlated closely with Stat3 activation, peaking at 24h and diminishing at 48h. Conversely, HIF-1$\alpha$ levels were still elevated at 48h despite massive cell death and diminishing VEGF production.
FIGURE 21. Time course of Stat3 activation in BALB/c fibroblasts treated with hypoxia mimic. BALB/c fibroblasts (1.5 x 10⁶) were treated with CoCl₂ (125uM) for the indicated times. Stat3 DNA binding was determined by EMSA using nuclear extracts (8ug). NIH3T3 fibroblasts expressing EGFR were stimulated with EGF and used as a marker for Stat3 and Stat1 dimers bound to labeled hSIE probe.
DU145 cells (1.5 x 10⁶) were treated with CoCL₂ (150μM) for the indicated times in serum-free media. Nuclear extracts (30μg) (Top panel) and whole cell extracts (100μg) (middle panel) were analyzed by Western blotting using the indicated antibodies. HIF-1β and β-actin are used to verify equal loading. Stat3 DNA binding was determined by EMSA using nuclear extracts (8ug) (bottom panel). NIH3T3 fibroblasts expressing EGFR were stimulated with EGF and used as a marker for Stat3 and Stat1 dimers bound to labeled hSIE probe.
From these data it is apparent that Stat3 is activated by hypoxia, and this activation follows and may result from HIF-1α accumulation.
DISCUSSION

The data in this dissertation confirms and expands the role of Stat3 in angiogenesis. It is shown for the first time that Stat3 is required for HIF-1α expression resulting from a variety of growth signals in multiple cell lines. In addition, it is demonstrated that Stat3 is required for the hypoxic induction of HIF-1α and several hypoxia inducible genes. Furthermore, activation of Stat3 by hypoxia suggests importance in the cellular response to hypoxia. Overall, these data firmly establish Stat3 as an extremely important regulator of angiogenesis, through the combined regulation of both VEGF and HIF-1α.

**Stat3 is required for HIF-1α induction by growth/oncogenic signals**

It is demonstrated here for the first time that stimulation with IL-6 results in increased expression of HIF-1α. MCF-7 breast cancer cells were treated with IL-6 for six hours in serum free media, followed by western blot analysis of nuclear extracts. Although HIF-1α induction by IL-6 is modest when compared to its induction by hypoxia, it is significant when compared to that seen by other well documented growth signals (133). Elevated HIF-1α levels following treatment with IL-6 did not result from increased transcription as verified by Northern blotting. These data show that IL-6 can be
added to the long list of cytokines and growth signals capable of increasing HIF-1α expression during normoxia.

Since the majority of reports indicate that increased production of HIF-1α by growth signals results from an elevated rate of protein synthesis, we wanted to determine if this was also true for IL-6. To achieve this, cyclohexamide was added to MCF-7 cells for various times following treatment with IL-6 for 6 hours. If increased HIF-1α expression was due to cessation of degradation, the addition of cyclohexamide would have no effect as protein levels would remain high over time. If the effect of IL-6 was to increase the rate of protein synthesis, it would be counteracted by the addition of cyclohexamide and HIF-1α levels would quickly return to that of baseline. Since HIF-1α levels returned to that of cells not treated with IL-6 within one hour, it was concluded that the effect of IL-6 on HIF-1α results from an increased rate of protein synthesis.

Since Stat3 is an important effector of IL-6 signaling, we wanted to determine if it was required for induction of HIF-1α. To achieve this, MCF-7 cells were stably transfected with Stat3 siRNA, and western blot was performed following stimulation with IL-6. There was limited induction of HIF-1α in response to IL-6 in cells expressing Stat3 siRNA when compared to control cells. This effect seemed to be applicable to HIF-1α induction by a variety of growth signals, as induction of HIF-1α by heregulin was also prevented in MCF-7 cells expressing Stat3 siRNA. Furthermore, HIF-1α induction by EGF was prevented in DU145 cells treated with Stat3 antisense oligonucleotide. These findings demonstrate that Stat3 is required for HIF-1α induction by a variety of growth signals.
In addition to a requirement for Stat3 in the induction of HIF-1α by growth signals, we observed loss of basal level expression of HIF-1α when Stat3 was silenced. Both of these cell lines, MCF-7 breast cancer and DU-145 prostate cancer cells, have low but detectable basal levels of HIF-1α expression during normoxia. Although Stat3 activation is weak when cultured in serum free media, silencing Stat3 clearly affected basal level HIF-1α expression. As constitutive HIF-1α expression often occurs in various cancers during normoxia, Stat3 is also required for this constitutive expression at least in these cell lines.

It is well established that increased expression of HIF-1α occurs via activation of the PI3K/AKT pathway. Since it is known that the PI3K/AKT pathway is activated in response to IL-6 (88), the effects of treatment with IL-6 on HIF-1α induction were not entirely surprising. Because we were aware of the work of Dr. Chen’s research, we knew that there was a possible role for Stat3 in the regulation of Akt1 at the transcriptional level. To verify that this was occurring in our system, protein levels of Akt1 were determined following silencing of Stat3 in MCF-7 cells. As was expected, total levels of Akt1 were reduced in Stat3 siRNA cells relative to the control. In addition, phosphorylated levels of Akt1 and Akt2 were also abolished. In accordance with published data demonstrating a requirement for Akt in HIF-1α expression, we conclude that the likely mechanism for Stat3 mediated IL-6 induction of HIF-1α is through regulation of Akt.
Stat3 is required for VEGF induction by growth/oncogenic signals

In addition to its effects on HIF-1α, it is shown here that blocking Stat3 signaling prevents induction of VEGF by multiple growth signals. It has previously been demonstrated that Stat3 is required for constitutive VEGF expression in several cancer cell lines, and for expression resulting from transformation with the v-Src oncogene (159, 214). Furthermore, it has been shown that Stat3 is required for VEGF induction by IL-6 (215). We elaborate on these findings by presenting data showing that Stat3 is essential for VEGF induction by heregulin and EGF. Furthermore, Stat3 is necessary for basal level VEGF production in MCF-7 breast cancer cells. These results firmly establish the requirement for Stat3 in expression of VEGF in multiple cell lines, both at the basal level and for its induction by various growth factors and soluble signals.

It has been reported that inhibition of Stat3 but not PI3K/Akt prevents the induction of VEGF by IL-6 (215). Since the only known mechanism of Akt regulation of VEGF is through HIF-1, this finding emphasizes the importance of Stat3 as a direct transcriptional regulator of VEGF. Although it is well documented that HIF-1 is an important regulator of the VEGF gene, Stat3 is probably more important at least for some inducers.

Because several reports have shown that Stat3 regulates VEGF production by directly binding to its promoter (159, 214), it is likely that the effects we are witnessing on VEGF production are both direct and indirect. First, by blocking Stat3 signaling, we are directly downregulating VEGF production by decreasing availability of one of its
transcriptional activators. Second, we are decreasing availability of another critical transcriptional activator, HIF-1α. This idea of Stat3 as a dual regulator is consistent with the dramatic decreases in VEGF levels that we are witnessing, as levels of VEGF are entirely eliminated when Stat3 is blocked. However, in many of these same experiments HIF-1α induction is only partially prevented. So it is likely that the dramatic decreases in VEGF observed following silencing Stat3 results from the combined effect of loss of Stat3 as well as HIF-1α.

Stat3 is required for HIF-1α induction by hypoxia

Induction of HIF-1α by hypoxia has been extensively studied and is well characterized. Under conditions of normal oxygen, HIF-1α is rapidly and continuously degraded. When O₂ becomes limiting, HIF-1α is stabilized and protein rapidly accumulates. This process occurs independently from the increased rate of synthesis that occurs due to PI3K/AKT activation. Both increased synthesis and decreased degradation can coordinately increase HIF-1α levels in an additive manner. However, there are conflicting reports concerning whether or not the PI3K/AKT pathway is required for the hypoxic induction of HIF-1α. Several groups have shown that inhibition of PI3K with chemical inhibitors or dominant negative forms could prevent HIF induction by hypoxia (27, 91, 236). Furthermore, it has been reported that overexpression of PTEN, a negative regulator of PI3K, prevented the hypoxic induction of HIF-1α (238). However, other reports indicate that HIF-1α induction by hypoxia is not prevented by genetic inhibition of PI3K/AKT pathway or by treatment with chemical inhibitors (2, 6, 8, 113).
Importantly, chemical inhibition of PI3K prevented AKT activation while simultaneously having no effect on the hypoxic induction of HIF-1α (2, 8). These reports make a convincing argument that AKT is not required for the induction of HIF-1α by hypoxia. Since we had observed loss of AKT expression following silencing of Stat3, we concluded that this accounts for decreased HIF-1α expression following stimulation with IL-6 or growth signals. However, decreased AKT expression should not prevent HIF-1α induction by hypoxia. Therefore, if silencing Stat3 also prevented HIF-1α induction by hypoxia, it is likely that some other mechanism is responsible.

To determine if Stat3 is necessary for HIF-1α induction by hypoxia, 3T3 fibroblasts expressing v-Src were transduced with Stat3D. Following treatment with CoCl₂, HIF-1α induction was prevented in cells expressing Stat3D. We next examined HIF-1α induction in A2058 human melanoma cells, which have constitutively activated c-Src (158). When these cells were treated with Src inhibitors, the hypoxic induction of HIF-1α was prevented. These findings verify published reports indicating that c-Src is necessary for HIF-1α induction by hypoxia (82). When these same cells were stably transfected with Stat3 siRNA, HIF-1α induction by hypoxia was prevented. These data demonstrate that Stat3 is required for HIF-1α induction by hypoxia in both 3T3 fibroblasts expressing v-Src and A2058 human melanoma.

These findings suggest that the effect of Stat3 on HIF-1α is not entirely due to the ability of Stat3 to regulate AKT expression. This reasoning is underscored by the fact that we continuously witness decreased basal level expression of HIF-1α when Stat3 is silenced or blocked with Stat3D. Although Stat3 clearly regulates AKT expression and
this will affect HIF-1α induction by growth/oncogenic signaling pathways, it is likely that Stat3 influences HIF-1α by some additional mechanism. Two other potential ways that Stat3 could regulate HIF-1α expression are via transcriptional control and/or control over its rate of degradation.

To determine if blocking Stat3 signaling affected levels of HIF-1α mRNA, Northern blotting was performed. In Balb/c-3T3 fibroblasts transformed with v-Src, there was decreased levels of HIF-1α RNA when these cell were transduced with Stat3D. This was not entirely surprising, as it had been reported that v-Src transformation resulted in increased HIF-1α mRNA levels in rat fibroblasts (109). However, in human cells transfected with activated c-Src there was elevated HIF-1α protein without a concurrent increase in mRNA (116). When we examined mRNA levels in A2058 human melanoma cells, silencing Stat3 did not reduce HIF-1α mRNA. Surprisingly, there appeared to be slightly elevated levels of RNA in Stat3 siRNA cells. Regardless, RNA could not account for the effects we were witnessing at the protein level in the human cells. Since these results seemed to comply with previously published findings, we concluded that mRNA regulation plays no role in Stat3 control of HIF-1α expression, at least in A2058 human melanoma.

Since we had eliminated transcriptional regulation as a potential mechanism in A2058 cells, we investigated the possibility that Stat3 regulates HIF-1α stability. Previous work in Dr. Yu’s lab demonstrated reciprocal regulation of p53 by Stat3. Because it has been shown that silencing p53 results in stabilization of HIF-1α during normoxia (167), we investigated this as another potential mechanism for Stat3 regulation.
of HIF-1α. When Stat3 was silenced in A2058 melanoma cells, we observed increased p53 expression and decreased HIF-1α expression during hypoxia. If the effects of Stat3 on HIF-1α were through p53, we theorized that loss of p53 would abolish Stat3 control of HIF-1α. However, when we silenced p53 in these cells we observed no change in HIF-1α levels, either during normoxia or hypoxia. Therefore, p53 was eliminated as a potential mechanism, at least in this cell line.

Another potential mechanism for Stat3 regulation of HIF-1α degradation concerns PHD enzymes and prolyl hydroxylation. It has been reported that in cells transformed with v-Src and RasV12 oncogenes, there was HIF-1α stabilization during normoxia and loss of hydroxylated proline residue 564 (26). Since loss of prolyl hydroxylation should theoretically only occur as oxygen levels decrease, the authors attributed this to oncogenic downregulation of prolyl hydroxylase enzyme or activity. They also reported that transfection with a constitutively activated version of AKT resulted in increased HIF-1α expression without loss of hydroxylation, suggesting that this second mode of oncogenic regulation of HIF-1α occurs independently of AKT. This idea of an additional mode of oncogenic regulation of HIF-1α is consistent with our findings, and it seems likely that Stat3 influences HIF-1α levels both through increased synthesis via PI3K/AKT and by influencing hypoxia regulated degradation. Because PHD-2 is the enzyme primarily responsible for hydroxylation of HIF-1α prolyl residues (4), and results in stabilization HIF-1α when silenced, we wanted to determine if it was regulated by Stat3. The idea is that silencing Stat3 will result in elevated levels of PHD-2, which in turn will increase HIF-1α degradation. However, we observed no change in
levels of PHD-2 when Stat3 was silenced in A2058 cells or when fibroblasts were transduced with Stat3D. The authors of the original study made use of an antibody specific for the hydroxylated form of HIF-1α, to which we did not have access. Through the use of this antibody it would be possible to further study the role of prolyl hydroxylation in Stat3 regulation of HIF-1α.

The requirement for Stat3 in the hypoxic induction of HIF-1α we observed occurred in cell lines expressing v-Src or those that had activated c-Src. However, we did not examine the hypoxic regulation of HIF-1α in other cell lines. It has recently been reported that both Stat3 and HIF-1 are required for VEGF expression in response to Src activation or hypoxia (82). They suggest that Src, Stat3, and HIF-1 cooperatively induce VEGF expression in response to hypoxia. These findings are not entirely surprising, because Src is required for the hypoxic expression of both VEGF (155) and HIF-1α (82). Furthermore, Src is activated by hypoxia (47, 156) and transfection with v-Src or activated c-Src increases HIF-1α levels (109, 116) as well as activating Stat3(228). It is possible that Src is a key player in the hypoxic response and cooperatively utilizes HIF-1α and Stat3 to facilitate that response.

**Stat3 is required for induction of several hypoxia inducible genes**

To further study the role of Stat3 as a facilitator of angiogenesis, we wanted to determine if Stat3 was required for the hypoxic induction of several hypoxia inducible genes. It has been reported that Stat3 is required for oncogenic expression of VEGF (159, 214, 215). Here we demonstrate that in addition to preventing its induction by oncogenes
and growth signals, silencing Stat3 prevents the hypoxic induction of VEGF. This finding verifies a recently published report showing that Stat3 is required for the hypoxic induction of VEGF (82). In addition, it has been shown that Stat3 is required for production of the matrix metalloproteinase enzymes MMP-2 and MMP-9 (41, 223). These enzymes are essential for the degradation of the extracellular matrix as occurs during angiogenesis, and are inducible by hypoxia (128). We report here that in BALB/c-3T3 fibroblasts transformed with the v-Src oncogene, expression of MMP-2 is significantly reduced in cells expressing the dominant negative Stat3D. Furthermore, there is induction of MMP-2 by hypoxia in control MSCV cells, but this induction is abolished in cells expressing Stat3D. These data demonstrate that Stat3 is required for the hypoxic induction VEGF and MMP-2. Since it has been demonstrated that Stat3 controls both of these genes at the transcriptional level, it is likely that they are directly under its control. However, they are also under transcriptional control by HIF-1α. Since we report here that Stat3 is necessary for HIF-1α expression, blocking Stat3 likely results in their inhibition both directly and indirectly through HIF-1α.

Stat3 is activated by hypoxia

The manner by which HIF-1α is activated by hypoxia is beginning to be well understood. Briefly, HIF-1α is hydroxylated by the PHD enzymes only in the presence of oxygen. VHL binds to hydroxylated HIF-1α and facilitates its degradation. As O2 levels decrease, HIF-1α is no longer hydroxylated, interaction with VHL is impeded and degradation ceases. Although HIF-1α activation by hypoxia is well characterized,
activation of growth signaling pathways by hypoxia is not clearly understood. It has been reported that activation of the PI3K/AKT pathway, as measured by AKT phosphorylation, occurs in some cell lines (2, 3, 8, 29, 238). However, hypoxic activation of AKT is not universal as with HIF-1α, and was not observed in other cell lines including breast cancers and prostate cancers (2, 13, 236). Furthermore, it has been reported that PI3K/AKT activation, when it did occur, followed HIF-1α activation (2).

Concerning Stat3 activation by hypoxia, it has recently been reported that there are increased levels of phosphorylated Stat3 following treatment with CoCl₂. Previously, it had been shown that cardiotrophin-1 activates Stat3 via a gp130-dependent signaling pathway in response to hypoxia (92). In addition it has been reported that Stat5 is activated by hypoxia (114). Furthermore, it has been shown that c-Src is activated by hypoxia (47, 156), and this could account for increased Stat3 activation by hypoxia. Because Stat3 is required for expression of HIF-1α and several hypoxia inducible genes, we decided to investigate its activation by hypoxia in more detail.

In DU145 prostate cancer cells, there was significant Stat3 activation following 24h incubation in hypoxia. Because we could not measure the exact oxygen concentration in the hypoxia chamber, and were not certain that levels were equal between chambers, we could not accurately determine the time course of Stat3 activation using hypoxia. As an alternative, the hypoxia mimic CoCl₂ was employed. In BALB/c fibroblasts, there was a significant activation of Stat3 within one hour which increased up to 4 h. Longer time points were not examined in these cells. In DU145 human prostate cancer cells, there was detectable activation of Stat3 as early as 2h, and this peaked at 24h. Stat3 activation was significantly diminished at 48h, coinciding with massive cell
death. In addition to detection of Stat3 activation by EMSA, both nuclear extracts and whole cell extracts were analyzed by western blot to determine levels of HIF-1α and VEGF. In contrast to Stat3 activation, HIF-1α accumulation was extremely rapid with detectable protein at the earliest time point of 30 minutes. HIF-1α levels increased steadily up to the 24h timepoint. At 48h when Stat3 signaling had diminished and cells were beginning to die, HIF-1α levels remained high. Expression of VEGF closely paralleled that of Stat3 activation, with detectable levels at 6h that peaked at 24h and diminished at 48h. These data emphasize the close relationship between Stat3 activation and VEGF expression. Although we observed hypoxic activation of Stat3 in DU145 cells, this was not observed in A2058 human melanoma cells. Therefore, similar to reports concerning AKT activation, it seems likely that Stat3 activation by hypoxia is cell type specific. Furthermore, Stat3 activation occurred following HIF-1α induction as reported for the hypoxic activation of AKT.

The only known transcription factor reported to increase VEGF production in response to hypoxia is HIF-1α. However, it has recently been reported that hypoxic induction of VEGF occurred in the absence of HIF-1α expression (153). Furthermore, a tumor cell line expressing constitutively activated AKT but lacking HIF-1 was able to form large and well vascularized tumors (5, 142). These reports suggest that other transcription factors are able to stimulate VEGF production in response to hypoxia independently of HIF-1α. Because Stat3 is also a transcriptional activator of VEGF, and we observed Stat3 activation following incubation in hypoxia in both BALB/c fibroblasts and human prostate cancer, it should be determined if Stat3 can facilitate the hypoxic induction of VEGF independently of HIF-1α. Although we had found that Stat3
activation occurred following induction of HIF-1α, it does not necessarily mean that Stat3 activation is dependent on HIF-1α. It is possible that Stat3 activation occurs in the absence of HIF-1α but just not as rapidly. Additional experiments should be performed to determine if Stat3 activation and increased VEGF production occur in response to hypoxia following silencing of HIF-1α. If both Stat3 activation and VEGF production occur independently of HIF-1α, further studies may be warranted. Reporter systems driven by the VEGF promoter containing mutated HIF-1 and Stat3 binding sites could be constructed to determine the relative importance of each transcription factor for the hypoxic response.

Stat3 as an antiangiogenic target

The data in this dissertation, along with recently published reports, position Stat3 as an important target for antiangiogenic therapy. It has been demonstrated repeatedly that Stat3 is a direct regulator of the VEGF gene, and is required for VEGF production in response to a variety of stimuli in different cell types. Furthermore, it has been shown that Stat3 is required for other important facilitators of angiogenesis such as MMP-2 and MMP-9. Importantly, it is demonstrated here that Stat3 is essential for expression of HIF-1α, the best documented transcriptional activator of VEGF and a wide variety of other angiogenic and invasive genes. Therefore, blocking Stat3 not only directly prevents production of multiple factors essential for angiogenesis, but also blocks production of the best documented angiogenesis transcription factor. These results strongly suggest that
targeting Stat3 could completely abrogate angiogenesis by simultaneously eliminating the activity of its two most important transcription factors.

For successful antiangiogenic therapy to succeed, it is necessary to have extremely potent angiogenesis inhibitors (12). The classes of angiogenesis inhibitors have been divided into ‘direct’ and ‘indirect’ based on which cells they target (118). Direct inhibitors are those that inhibit growth of vascular endothelial cells by targeting them directly. One advantage to the use of direct inhibitors is that endothelial cells are genetically stable, and therefore less likely to acquire drug resistance (119). Indirect angiogenesis inhibitors are those that target the cancer cells, generally by blocking expression of angiogenic proteins. Any inhibitor that could successfully act in a both direct and indirect manner would have greater potency and better likelihood for success. In addition to the requirement of Stat3 for angiogenic factor production by tumor cells, several reports have indicated that Stat3 is essential for vascular endothelial cell growth, migration, and microvascular tube formation. Stat3 is activated by and necessary for signaling by both the bFGF receptor and VEGFR2 (7, 42, 176). Furthermore, Stat3 is required for endothelial cell migration and microvascular tube formation (225). Therefore, any drug targeting Stat3 would be functioning simultaneously as both a primary and secondary angiogenesis inhibitor, and would likely have greater potency because of this.

Although antiangiogenic therapy has demonstrated efficacy in some clinical trials, many have had limited success (12, 54, 115, 226). The inhibitors used in these trials targeted only individual inducers of endothelial cell growth, such as VEGF, EGFR, VEGFR, and HER-2/neu (118). Since there are a variety of factors involved with
promoting angiogenesis, targeting only one of these may not be effective. However, the activity of many of these pathways is dependent on a relatively limited set of transcription factors. Because of this, selective inactivation of a few TFs could potentially inactivate a large array of angiogenic factors (39). There is current work investigating the effect of targeting HIF-1\(\alpha\) for anti-angiogenic therapy (180). Since we show here that Stat3 is necessary for both HIF-1\(\alpha\) expression and endothelial cell growth, Stat3 may prove to be the better transcription factor to target for antiangiogenic therapy.

In summary, there are multiple reasons why Stat3 inhibition will likely be an extremely effective antiangiogenic strategy. Stat3 is required for the production of a variety of angiogenic factors, in response to both growth/oncogenic signaling and hypoxia. Furthermore, Stat3 signaling is required for expression of HIF-1\(\alpha\), and therefore its inhibition could potentially prevent expression of a large array of angiogenic and invasive factors. Also, any angiogenesis inhibitor capable of preventing endothelial cell growth in both a direct and indirect manner simultaneously would have a distinct advantage. Furthermore, it is well documented that Stat3 is an important facilitator of cell survival and growth, so blocking Stat3 could make both tumor cells and endothelial cells more prone to apoptosis. Finally, targeting transcription factors will likely prove more effective because of their role as convergence points for a multitude of angiogenic factors. For these reasons, drugs that inhibit Stat3 will likely be important therapeutic tools for antiangiogenic treatments.
REFERENCES


64. **Fukuda, R., K. Hirota, F. Fan, Y. D. Jung, L. M. Ellis, and G. L. Semenza.**


Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element. Blood **90:**3322-31.


The 72 kDa type IV collagenase is modulated via differential expression of alpha v beta 3 and alpha 5 beta 1 integrins during human melanoma cell invasion.

Cancer Res **53**:3411-5.


Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem **269**:23757-63.


231. **Yuan, F., Y. Chen, M. Dellian, N. Safabakhsh, N. Ferrara, and R. K. Jain.**


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