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The Expression and Function of Hypoxia inducible factor-1α in Human Melanoma

Sandeep S. Joshi

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The Expression and Function of Hypoxia inducible factor-1α in Human Melanoma

Dissertation submitted to the
Graduate college of
Marshall University
in partial fulfillment of the requirement for
the degree of
Doctor of Philosophy

by
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April 2010
ABSTRACT

The Expression and Function of Hypoxia inducible factor-1α in Human Melanoma

By Sandeep S. Joshi

Hypoxia inducible factor-1α (HIF-1α) protein, a key regulator of oxygen homeostasis, is stabilized under hypoxia and degraded under normal oxygen tension. Here, the human melanoma cells were found to express an elevated amount of HIF-1α mRNA and protein relative to normal human melanocytes under normoxic conditions. The amount of HIF-1α expressed is roughly correlated with the stage of melanoma from which the cell line was established. In addition, a splice variant mRNA of HIF-1α785 is expressed at higher levels than full-length HIF-1α mRNA in the more aggressive melanoma cells. This splice variant lacks part of the oxygen regulation domain. Ectopic expression of HIF-1α in a radial growth phase melanoma cell line increased their ability to form colonies in soft agar and their ability to invade and migrate through Matrigel. Conversely, knockdown of HIF-1α expression in metastatic melanoma cell line decreased their ability to form colonies in soft agar as well as their ability to invade and migrate through Matrigel. Further, it has been shown that the ERK1/2 MAPK pathway is probably not involved in the expression of HIF-1α under normoxic conditions. In addition, the normoxic expression of HIF-1α directly regulates the microphthalmia-associated transcription factor (MITF) mRNA and protein expression in human metastatic melanoma cell line. There was clear shift in MITF expression from MITF-M, a specific isoform in melanocytes, to the predominantly MITF-A in metastatic melanoma. The MITF-A promoter contains two hypoxia response elements while none were found in the MITF-M promoter region. Reporter gene and chromatin immunoprecipitation assays confirmed
that HIF-1α directly binds and transactivates the MITF-A promoter in metastatic melanoma cells. Knockdown of MITF expression in metastatic melanoma cell line decreased their viability through induction of apoptotic pathways. Overall data suggest that increased normoxic expression of HIF-1α contributes to some of the malignant properties of melanoma and increases its importance as a therapeutic target. Further, HIF-1α activates the expression of MITF-A whose expression contributes to the survival of metastatic melanoma cells.
TO MY PARENTS AND WIFE
ACKNOWLEDGEMENTS

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<td>AKT</td>
<td>RAC serine/threonine-protein kinase</td>
</tr>
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<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ARD1</td>
<td>ADP-ribosylation factor domain protein 1</td>
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<tr>
<td>Arnt</td>
<td>Ah receptor nuclear translocator protein</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B-cell lymphoma gene 2</td>
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<tr>
<td>bHLH</td>
<td>basic helix loop helix</td>
</tr>
<tr>
<td>BRAF</td>
<td>v-raf murine sarcoma viral oncogene homolog B1</td>
</tr>
<tr>
<td>CBP</td>
<td>creb binding protein</td>
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<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>cyclin-dependent kinase inhibitor 2A</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
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<tr>
<td>DCT</td>
<td>dopachrome tautomerase</td>
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<tr>
<td>DFO</td>
<td>desferrioxamine</td>
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<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<tr>
<td>EMSA</td>
<td>electromobility shift assay</td>
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<td>EPAS1</td>
<td>endothelial PAS domain protein 1</td>
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<tr>
<td>EPO</td>
<td>erythropoietin</td>
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<tr>
<td>ERK</td>
<td>extracellular signal- regulated protein kinase</td>
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<tr>
<td>FIH1</td>
<td>factor inhibiting HIF-1</td>
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<tr>
<td>FL</td>
<td>full length</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
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<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
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<td>HIF-1</td>
<td>hypoxia-inducible factor 1</td>
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<td>HMGS</td>
<td>human melanocyte growth serum</td>
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HRE  hypoxia response element
ID   Inhibitory domain
IGF2 insulin-like growth factor 2 (somatomedin A)
INK4 cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
IPAS inhibitory PAS molecule
JNK  c-Jun N-terminal kinase
KIT  v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
MAPK mitogen activated protein kinase
MC1R melanocortin 1 receptor (alpha melanocyte stimulating hormone receptor)
MET  met proto-oncogene
MITF microphthalmia-associated transcription factor
MM  metastatic melanoma
MSC  melanocyte stem cells
mTOR mammalian target of rapamycin
NF-KB nuclear factor KB
NHM  normal human melanocytes
NO  Nitric oxide
ODDD oxygen dependent degradation domain
PAS  per-armt-sim
PAX3 paired box gene 3
PBS  phosphate buffered saline
PCR  polymerase chain reaction
Per  period circadian protein
PHD  prolyl hydroxylase
PI3K phosphoinositide-3-kinase
PKA  protein kinase A
PKC  protein kinase c
PTEN  phosphatase and tensin homolog
NRAS  neuroblastoma RAS viral (v-ras) oncogene homolog
RGP  radial growth phase
RT-PCR  reverse transcriptase-polymerase chain reaction
SDS-PAGE  sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM  standard error mean
Sim  single-minded protein
SOX10  SRY (sex-determining region Y)-box 10
SUMO  sumoylation
TAD  transactivation domain
TBS  tris-buffered saline
TBST  tris-buffered saline with Tween-20
TBX2  T-box2
TCF-4  T cell factor 4
TGFα  transforming growth factor α
TYR  tyrosinase
TYRP1  tyrosinase related protein 1
VEGF  vascular endothelial growth factor
VGP  vertical growth phase
VHL  von Hippel Lindau tumor suppressor
WNT  wingless
WS  waardenburg syndrome
CHAPTER ONE

INTRODUCTION

Melanoma

Melanoma originates from melanocytes and is the most deadly form of all skin cancers. Melanocytes are responsible for skin pigmentation and are situated at the base of the epidermis. The melanin produced by the melanocytes protects skin against the sun’s harmful rays. The incidence and death due to melanoma over the past decade has increased more rapidly than any other type of cancer (Melanoma Research Foundation). The increased incidence of melanoma is also associated with the type of human skin coloration and environmental sun exposure. The highest incidence of melanoma is found among the fair skin, red haired individuals that reside in the sun-belt area of Australia (Bowden, 2004). Melanoma, if diagnosed early can be surgically removed, however metastatic melanoma is notoriously resistant to chemotherapies and the median lifespan of these patients is less than 12 months (Melanoma Research Foundation).

The Breslow and Clark number are often used to predict prognosis of melanoma patients. Breslow’s measurements are based on thickness of a melanoma tumor while Clark’s measurements are based on how deep the tumor has penetrated into the layers of skin. The greater the thickness or deeper the penetration worsen the prognosis of melanoma patients. According to the widely respected Clark model of tumorigenesis, melanoma development and progression include several clinical stages. These include 1) common nevus, 2) melanocytic dysplasia, 3) radial growth phase (RGP), 4) vertical growth phase (VGP) and 5) metastatic melanoma (MM). (Figure 1.1). (Clark et al., 1984; Meier et al., 1998)
Figure 1.1: Melanoma development and progression. The model, developed by Drs. Clark, Elder, and Guerry, implies that melanoma commonly develops and progresses in a sequence of steps from nevic lesions which can be histologically identified in approximately 35% of cases. Thus, melanoma may also develop directly from normal (and precursor cells). The roles of melanocyte stem cells, melanoblasts (immature melanocytes) in melanogenesis remain poorly defined. Cells from lesions show persistence but non-tumorigenic lesions tend to disappear through apoptotic or differentiation pathways which are also poorly defined (Meier et al., 1998).

For the melanoma to develop the normal melanocyte cells needs to acquire characteristics such as clonal expansion, avoiding the senescence and escape of apoptosis (Bennett, 2008).

Melanoma commonly develops and progresses in a sequence of steps from nevic lesions which can be histologically found in approximately 35% of cases (Meier et al., 1998). However, melanoma may also develop from normal melanocyte cells through various genetic changes. According to Bennett’s model, probably no one particular genetic change is crucial in melanoma genesis, but rather any set of changes generating a specific set of biological outcomes is important. The biological outcomes that are involved in generation of melanoma from normal melanocytes include; 1) initiation of clonal expansion 2) avoiding the melanocyte senescence,
and 3) escape from apoptosis. The lesions with changes in step one and two are thought to grow only in epidermis, like RGP melanomas. RGP is an early stage melanoma that grows horizontally on the skin surface without undergoing differentiation. The changes leading to suppression of apoptosis might be an essential step for further progression of RGP to the VGP stage (Bennett, 2008). VGP cells have the ability to invade and grow vertically into the dermis. This stage shows maximum cytogenetic abnormalities suggesting considerable genetic instability. Additional genetic changes are necessary for the transition from VGP to metastatic melanomas. Micro-environmental factors such as cell-matrix and cell-cell signaling could be critical for inducing metastatic phenotype. Metastatic cells have the ability to migrate to distant organs and form tumors when the microenvironment is favorable. (Meier et al., 1998)

Recently, through various genetic and molecular studies several key molecules that play a role in melanoma development have been identified. The major genetic alteration found in approximately 60% of human melanomas is BRAF (Davies et al., 2002; Rodolfo et al., 2004). This mutation leads to constitutive activation of the extracellular signal-regulated kinase / mitogen activated protein kinase (ERK/MAPK) pathway. Some melanomas (20%) have an N-RAS mutation which also leads to activation of the ERK/MAPK pathway (Rodolfo et al., 2004; Eskandarour et al., 2004). These mutations are mutually exclusive i.e., melanomas have one or the other, but not both at the same time. A paradox is that these mutations are also found in nevi. Thus, additional changes are required for the development of melanoma. In addition to activation of an oncogene, the loss of several tumor suppressor genes has been observed in melanoma. The various familial melanomas that are caused due to mutations in the tumor suppressor genes include cell cycle regulators such as CDKN2A and CDK4. Mutation in these genes leads to an increase in cell cycle progression and thereby an increase in cell growth. The
CDKN2A mutations appear in 25-40% of familial melanomas (Levy et al., 2006). Another tumor suppressor that is found to be mutated in ~30% of melanomas is PTEN (Celebi et al., 2000). The loss of PTEN enzyme activity leads to activation of the PI3K pathway, which permits cells to divide uncontrollably. Although, significant progress has been made in understanding the mechanisms responsible for melanoma development, the key regulators involved in the advancement of disease are yet to be determined.
Hypoxia inducible factor-1 (HIF-1)

Hypoxia inducible factor-1 (HIF-1) is the most important factor involved in sensing and adapting cells to respond to a decrease in O$_2$ levels. Hypoxia, a state in which O$_2$ demand exceeds the supply, induces acute, as well as long term, physiological responses such as erythropoiesis, neovascularization and glycolysis. These processes increase the O$_2$ supply to the hypoxic region and active adaptive metabolic pathways such as glycolysis which is the only metabolic pathway that can produce energy in the absence of oxygen (Lisy and Peet 2008; Semenza 2003). The genes that are induced by hypoxia include erythropoietin (EPO), vascular endothelial growth factor (VEGF), and certain glycolytic enzyme genes containing unique promoter DNA sequences called hypoxia response elements (HRE) (Semenza and Wang 1992). These DNA elements were found to bind the heterodimeric transcription factor, HIF-1 (Wang and Semenza 1995).

HIF-1 a master regulator of oxygen homeostasis is a heterodimeric protein complex consisting of a constitutively expressed HIF-1$\beta$ subunit and a HIF-1$\alpha$ subunit whose expression is highly regulated. HIF-1$\alpha$ expression is regulated at both the transcriptional and translational level. Transcriptional regulation is O$_2$-independent, while post-translational regulation is O$_2$-dependent. Over 70 HIF-1 regulated genes have been identified and many of these genes are involved in crucial aspects of tumor biology including angiogenesis, cell survival, glucose metabolism and invasion (Semenza 2003; Mazure et al., 2004; Hampton-Smith and Peet, 2009).

HIF-1 belongs to the PER-ARNT-SIM (PAS) subfamily of the basic-helix-loop-helix (bHLH) family of transcription factors. The HIF-1 family consists of various isoforms such as HIF-1$\alpha$, HIF-2$\alpha$, and HIF-3$\alpha$ which heterodimerizes with HIF-1$\beta$ also known as aryl
hydrocarbon receptor nuclear translocator (ARNT). HIF-1α and HIF-2α are closely related both structurally and functionally. HIF-2α is also known as endothelial PAS domain protein 1 (EPAS1) due to its selective expression in endothelial cells. HIF-3α is also known as Inhibitory PAS Domain protein (IPAS) due to lack of a transactivation domain that is present in factors 1α and 2α. It therefore act as a negative regulator of hypoxia-inducible gene expression (Jang et al., 2005; Weidemann and Johnson, 2008).

The HIF-1α and β subunits are similar in structure. Both contain a bHLH and a PAS domain at the N-terminus that are required for DNA binding and dimerization respectively. The bHLH domains are found in specific DNA- binding proteins that act as transcription factors and are usually 60-100 amino acids long. bHLH regions form homo- and heterodimers. The PAS domain was named after three proteins that the domain occurs in: Per- period circadian protein, Arnt- aryl hydrocarbon receptor nuclear translocator protein and Sim- single-minded protein (Hogenesch and Bradfield, 2010). The C-terminus of HIF-1α and β contains a transactivation domain (TAD) where transcriptional co-regulators bind.

In addition to these domains, the C-terminal half of the HIF-1α subunit contains an oxygen-dependent degradation domain (ODDDD) that confers oxygen-dependent instability. The ODDD domain in HIF-1α is a highly regulated domain that is subject to various post-translational modifications, including hydroxylation, acetylation, sumoylation and phosphorylation. These modifications are necessary to inhibit HIF-1α responsive genes in normoxia either by inducing protein degradation or by inhibiting its activity. HIF-1α also contains two separate transactivation domains (N-TAD and C-TAD). N-TAD is present within the ODDD and between N-TAD and C-TAD lies an inhibitory domain (ID) that is a negative regulator of TAD (Figure 1.2) (Jiang et al., 1997; Weidemann and Johnson, 2008).
Figure 1.2: Domain structure of HIF-1α and targeted residues involved in its regulation. HIF-1α possesses bHLH and PAS domains which are involved in dimerization with HIF-1β and DNA binding. The HIF-1α subunit contains two TAD, the N- and the C- TADs. The N-TAD lies within the ODDD. The ODDD regulates the stability of HIF-1α via recognition, by the von Hippel-Lindau E3 ubiquitin ligase (pVHL), of the hydroxylation state of Proline402 and/or Proline564 residues. This hydroxylation is catalyzed by the enzyme PHD (prolyl hydroxylase domain protein). Acetylation of the Lys532 residue by the ARD1 acetyltransferase also favors interaction with pVHL. The hydroxylation state of the Asparate803 residue, by the enzyme FIH-1 (factor inhibiting HIF-1) inhibits binding of p300/CBP, a HIF-1α co-activator. S-nitrosation of Cys800, in the C-TAD, also promotes HIF-1 transcriptional activity. Three consensus sequences at Lys-391, -477, and -532 may be modified by SUMO. The domain from 531 to 826 has been shown to be phosphorylated (Mazure et al., 2004)

Factors regulating HIF-1α protein stability

PHDs

PHDs are prolyl hydroxylase enzymes with the isozymes PHD1, PHD2 and PHD3 being the most studied and well characterized (Masson et al., 2001). PHD1 is found specifically in the nucleus, PHD2 in the cytoplasm and PHD3 is found throughout the cell. In the presence of oxygen, 2-oxoglutarate and ascorbate, PHD, an iron-dependent enzyme, converts proline
residues to hydroxyproline. Proline residues (Pro\textsuperscript{402} and Pro\textsuperscript{564}) within the ODDD of HIF-1\textalpha protein and have been shown to be hydroxylated (Epstein \textit{et al}., 2001; Smith \textit{et al}., 2010). Upon hydroxylation of these proline residues in normoxic conditions, the HIF-1\textalpha protein is subjected to ubiquitin based proteasomal degradation. PHD enzymes are defined as oxygen sensors because their activity depends on the oxygen concentration in the cell. Oxygen concentrations also control the PHD mRNA levels. Since PHD enzymes are iron-dependent, iron chelators like desferrioxamine (DFO) and the iron antagonist cobalt chloride (CoCl\textsubscript{2}) are used as hypoxia mimetic drugs to stabilize HIF-1\textalpha protein. HIF-1\textalpha can also control its own activity by regulating the expression of PHD2 (Mazure \textit{et al}., 2004).

\textit{pVHL}

Von Hippel-Lindau (VHL) is a tumor suppressor gene; a germline mutation in this gene is the basis of a rare familial VHL syndrome (Kaelin 2002). This syndrome induces a predisposition to a variety of malignant and benign tumors, in particular clear-cell renal carcinomas, and cerebellar hemangioblastomas (Friedrich 2001; Smaldone and Maranchie, 2009). The protein encoded by this gene is a component of the E3 ubiquitin-protein ligase complex that targets proteins for ubiquitin based proteasomal degradation (Lisztwan \textit{et al}., 1999). This protein is involved in the ubiquitination and degradation of proline hydroxylated form of HIF-1\textalpha protein (Maxwell \textit{et al}., 1999; Ohh \textit{et al}., 2000; Weidemann and Johnson, 2008) (Figure 1.3). The multiple mutations or downregulation of VHL leads to accumulation of HIF-1\textalpha in cells grown under normoxic conditions (Hughston \textit{et al}., 2003).
Figure 1.3: Oxygen dependent regulation of HIF-1α. Oxygen dependent regulation of HIF-1α is post-translational. HIF-1α protein is constitutively expressed in all cells. In normal oxygen tension HIF-1α protein is hydroxylated and acetylated by PHD2 and ARD1 enzymes respectively. Upon hydroxylation and acetylation, VHL an ubiquitin ligase enzyme binds to HIF-1α protein that leads to polyubiquitination and proteasomal based degradation. However, in hypoxic condition these enzymes PHD2 and ARD1 cannot hydroxylate or acetylate HIF-1α protein respectively. Thereby, VHL cannot bind to HIF-1α protein and making it stable under hypoxic conditions. Upon stabilization HIF-1α protein translocates to the nucleus heterodimerizes with HIF-1β and transactivates genes that play role in adapting cells in hypoxic stress.

ARD1

Arrest-defective-1 protein (ARD1) is an acetyltransferase enzyme that is responsible for acetylation of HIF-1α protein. lysine within the ODDD of HIF-1α is the target for acetylation.
Upon acetylation of lysine\textsuperscript{532} which is in close proximity to hydroxyproline\textsuperscript{564}, the pVHL protein is recruited and results in the ubiquitination and proteasomal degradation of HIF-1\(\alpha\) (Jeong \textit{et al.}, 2002). HIF-1\(\alpha\)\textsubscript{785} splice variant of a full length HIF-1\(\alpha\) is missing the segment encoded by exon 11 and thus, ARD1 cannot acetylate Lysine\textsuperscript{532}. Lack of this acetylation leads to less stable interactions between the protein and VHL, and thereby less proteasomal based degradation (Chun \textit{et al.}, 2003). Thus, HIF-1\(\alpha\)\textsubscript{785} is thought to be more stable under normoxic conditions relative to full length HIF-1\(\alpha\). ARD1 activity is not known to be dependent on oxygen and, therefore, the HIF-1\(\alpha\) protein acetylation occurs under both normoxic and hypoxic condition. Other than ARD1, the acetylation HIF-1\(\alpha\) is also regulated by histone acetyltransferases (HATS) and deacetylation by histone deacetylases (HDACs) (Ellis \textit{et al.}, 2009).

\textit{Nitric oxide (NO)}

NO has also been shown to increase HIF-1\(\alpha\) protein instability. NO does so by increasing intracellular oxygen resulting in increased PHD-mediated proline hydroxylase-dependent degradation of the HIF1\(\alpha\) protein (Mazure \textit{et al.}, 2004; Dehne and Brune, 2009).

\textbf{Factors regulating HIF-1\(\alpha\) protein activity}

\textit{Factor inhibiting HIF-1(FIH-1)}

FIH-1, like the PHDs, is an iron-dependent hydroxylase enzyme but instead of proline residues it is responsible for hydroxylation of asparagine\textsuperscript{803} within the HIF-1\(\alpha\) protein. FIH-1 does not influence HIF-1\(\alpha\) protein stability, but instead inhibits the binding of co-activator p300/CBP at the C-TAD region and thereby represses HIF-1\(\alpha\) transcriptional activity (Mazure \textit{et al.}, 2004; Webb \textit{et al.}, 2009).
Mitogen activated protein kinase (MAPK)

Stabilization of HIF-1α under hypoxic conditions results in phosphorylation by the MAPK in vivo as well as in vitro (Richard et al., 1999). Moreover, phosphorylation of HIF-1α is associated with increased transcriptional activity by enhancing the interaction between p300 and the C-TAD (Sang et al., 2003).

Small ubiquitin-related modifier (SUMO)

SUMO is a member of the ubiquitin-like protein family. The process for SUMO modification is similar to ubiquitination. However, SUMO does not target the protein for degradation. Instead sumoylation regulates protein localization and regulates the activity of a transcription factor usually in the negative direction. HIF-1α is shown to undergo sumoylation by the sumo E3 ligase, RanBP2, in vitro at Lys\textsuperscript{391}, Lys\textsuperscript{477} and Lys\textsuperscript{532} (Mazure et al., 2004). The physiological effect of HIF-1α sumoylation is still under investigation.
Figure 1.4: Oxygen independent regulation of HIF-1α. Oxygen independent regulation of HIF-1α is transcriptional. It occurs mainly in growing cells and is regulated by growth signaling pathways. Growth factor binding to a cognate receptor tyrosine kinase activates the ERK-MAPK and PI3-AKT pathways. Activation of these pathways by growth factors leads to HIF-1α mRNA synthesis and thereby increasing the HIF-1α protein levels.

Oxygen-independent regulation of HIF1α

The oxygen-independent regulation of HIF-1α is at the transcriptional level mainly through growth-factor stimulation (Figure 1.4). The growth-factor mediated increase in HIF-1α mRNA is cell type specific, while the hypoxia mediated increase of HIF-1α levels occurs in all cell types (Semenza 2003). Moreover, the hypoxic regulation of HIF-1α expression is due to a decrease in HIF-1α protein degradation while growth-factors, cytokines and other signaling molecules stimulate HIF-1α mRNA expression through activation of phosphatidylinositol 3-
kinase (PI3K) or mitogen-activated protein kinase (MAPK) (Fukuda et al., 2002; Zhong et al., 2000). Among HIF-1α’s target genes are various growth factors such as insulin-like growth factor (IGF2) and transforming growth factor (TGF-α). These growth factors upon binding to their respective receptors on cell surface could activate signal transduction pathways involved in stimulating HIF-1α expression, thereby setting up a positive feedback loop (Feldser et al., 1999; Krishnamachary et al., 2003).

**Figure 1.5: Splice variants of HIF-1α.** HIF-1α consist of various isoforms; full length (FL), 785, 736, 557, 516 and 417 that originate due to splicing. The longest HIF-1α isoform consist of 826 amino acids and contains all the conserved domains and has 14 exons. HIF-1α785 is similar to FL but lacks exon 11. The missing exon 11 contains Lys532, a critical component of ODDD, which is responsible for acetylation by ARD1 leading to interaction between HIF-1α and VHL. This interaction directs for ubiquitination based proteasomal degradation under
normoxic condition. Thus HIF-1α785 is thought to be more stable than full length HIF-1α under normoxic conditions (Lee et al., 2004).

HIF-1α isoforms

HIF-1α consists of various isoforms; full length (FL), 785, 736, 557, 516 and 417 that originate due to alternate splicing (Figure 1.5). The longest HIF-1α isoform consists of 826 amino acids and contains all the conserved domains described above. FL is ~120kDa and has 14 exons. HIF-1α785 is similar to FL but lacks exon 11 and is ~91kDa. The missing exon 11 contains lys532, a critical component of ODDD, which is responsible for acetylation by ARD1 leading to interaction between HIF-1α and VHL. This interaction directs ubiquitination-dependent proteasomal degradation under normoxic conditions. Thus, HIF-1α785 is thought to be more stable than full length HIF-1α under normoxic conditions (Lee et al., 2004).

HIF-1α736 lacks exon 14 which carries C-TAD and it is thought to be a tumor suppressor. It is 3 times less active than FL HIF-1α. The 557 isoform lacks exon 12 and is also named as HIF-1αZ. It is regulated by zinc. It does not contain a nuclear localization signal (NLS) and is known to play a dominant negative role by sequestering HIF-1β in the cytosol. HIF-1α516 is similar to 557, but, in addition it is missing the TAD. HIF-1α417 is the shortest isoform and is missing the ODDD and TAD domains. It heterodimerize with HIF-1β and uses HIF-1β’s TAD to initiate transcription of target genes (Lee et al., 2004).

HIF-1α inhibitors

GL331 is a potent inhibitor of tumor-induced angiogenesis in lung cancer cells through inhibition of VEGF. The underlying mechanism responsible for angiogenic inhibition by GL331
is through downregulation of HIF-1α expression by transcriptional repression (Chang et al., 2003).

Echinomycin (NSC-13502) is a small-molecule known to bind DNA in a sequence-specific fashion. NSC-13502 inhibited the binding of HIF-1α and HIF-1β proteins to the HRE present in the promoter region of VEGF; echinomycin did not affect the AP-1 / NF-kB binding to the promoter regions of their respective target genes. Accordingly, NSC-13502 inhibited hypoxic induction of luciferase in U251-HRE cells and VEGF mRNA expression in U251 cells (Kong D et al., 2005). Other small molecules like synthetic pyrrole-imidazole polycamide (Olyenuk et al., 2004) and DJ12 (Jones et al., 2006) are also found to inhibit HIF-1α DNA binding to endogenous promoters.

TX-402, a potent hypoxia-selective cytotoxic agent, was shown to reduce the expression of VEGF and glucose transporter type 3 (GLUT-3) under hypoxic conditions. The mechanism appears to involve suppression of HIF-1α mRNA and protein levels (Nagasawa et al., 2003). The National Cancer Institute Diversity Set of 2000 compounds was screened for potential HIF-1α inhibitors. NSC-134754, a semisynthetic analogue of emetine (a natural alkaloid) and NSC-643735, a structural analog of actinomycin D aglycone, were both shown to have HIF-1 inhibitory effects. NSC-134754 inhibited hypoxia-induced HIF-1 activity, Glut-1 expression and HIF-1α protein expression. Both compounds were able to inhibit growth factor-induced HIF-1α protein expression (Chau et al., 2005).

The dietary component curcumin has been shown to inhibit hypoxia-stimulated in vitro angiogenesis and to downregulate HIF-1α and VEGF mRNA expression in endothelial cells (Bae et al., 2006). Additional dietary constituents such as quercetin, epigallocatechin gallate (EGCG),
resveratrol, inositol, etc. have been reported to affect HIF-1α expression or function in different types of cancer cells (Zang et al., 2006; Wu et al., 2008; Gu et al., 2009)

**Physiological role of HIF-1**

HIF-1 is functional in all mammalian cell types while the HIF-1–VHL–PHD system is fully conserved from *D. melanogaster* to *C. elegans* to *H. Sapiens* (Wiesener and Maxwell 2003). At the cellular level when oxygen levels drop, the oxygen sensor stimulates a signal which is responsible for increased expression of HIF-1α. The HIF-1α-β dimer then transactivates genes including those encoding glycolytic enzymes for anaerobic metabolism, VEGF for angiogenesis, inducible nitric oxide synthase and hemoxygenase-1 for the production of vasodilators, EPO for erythropoiesis and tyrosine hydroxylase for dopamine production to increase breathing (Figure 1.6). These gene products help cells to survive under a low oxygen supply and act to restore normal oxygen levels (Guillemin and Krasnow 1997; Semenza, 2008). Most of these genes are stimulated in all hypoxic cells, however, some genes like EPO are cell type specific and thus require tissue specific regulators. The orphan nuclear receptor hepatic nuclear factor-4 is thought to be a tissue specific regulator that works in association with HIF-1 to regulate EPO expression in the liver and kidney (Galson *et al.*, 1995).
Figure 1.6: HIF-1α targets involved in different physiological processes. HIF-1α protein on stabilization heterodimerizes with HIF-1β and target various genes that are involved different physiological processes. HIF-1α transactivated genes such as glycolytic enzymes involved in anaerobic metabolism, VEGF involved in angiogenesis, erythropoietin involved in erythropoiesis, iNOS and HO-1 involved in vasodilation and tyrosine hydroxylase involved in increased breathing (Guillemin and Krasnow 1997).

HIF-1α is required for embryonic development. In the homozygous HIF-1α-null mouse embryonic development is arrested at day 8, and the embryos are dead by day 10 due to cardiac malformations, vascular regression and mesenchymal cell death (Iyer et al., 1998; Kotch et al., 1999). The heterozygous-null (HET) mice develop normally and are indistinguishable from wild type; however they have impaired responses for hypoxia and ischemia (Yu et al., 1999). The HET mice revealed that HIF-1α plays a critical role in the pathophysiology of hypoxia-induced pulmonary hypertension. The ventilator response to chronic hypoxia, mediated through the carotid body, was lost in HET mice. These results suggest that HIF-1α is required for carotid body function. HIF-1α is also required for the cardiovascular and ventilatory response to intermittent hypoxia (Semenza 2006).
HIF-1 protein stabilization has been detected in certain ischemic conditions. In mice with oxygen-induced ischemic retinopathy, HIF-1α levels were shown to be increased in the retina (Ozaki et al., 1999). Brain ischemia also results in increased HIF-1α transcriptional activity. HIF-1α and HIF-1β protein levels were also significantly increased after intraperitoneal injection of CoCl$_2$ (Bergeron et al., 2000). The kidney demonstrates a marked potential for upregulation of HIF, but accumulation of HIF-1α and HIF-2α is selective with respect to cell type, kidney zone, and experimental conditions. HIF-1α was mainly induced in tubular cells, including proximal segments with exposure to anemia/carbon monoxide, in distal segments with CoCl$_2$ treatment, and in connecting tubules and collecting ducts with both stimuli plus renal ischemia. HIF-2α was not expressed in tubular cells but was expressed in endothelial cells of a small subset of glomeruli, in peritubular endothelial cells and in fibroblasts. In addition, upregulation of HIF-1α was also shown to be associated with the renal ischemia (Rosenberger et al., 2002). The functional role of this activation is still under investigation. However, since stabilization of HIF-1 leads to angiogenesis and hypoxia-induced metabolic adaptation, this adaptive response should prevent excessive death of kidney cells (Wiesener and Maxwell, 2003).

HIF-1 has generated a new hope to counteract neurodegenerative diseases. The cerebral ischemic stroke leads to various neurological dysfunctions due to reduction in blood supply that provides glucose and oxygen for the normal brain function (Correia and Moreira, 2010). The activation of HIF-1 through hypoxia-mimetic agents like CoCl$_2$ and DFO has shown to provide protection against cerebral ischemic damage (Baranova et al., 2007). The underlying mechanism responsible for HIF-1-mediated cytoprotection is thought to be due to HIF-1-induced EPO expression. A clinical trial conducted in 13 patients by intravenous administration of recombinant EPO showed marked reduction in the infarct size compared to control (Ehrenreich
et al., 2002). Moreover, VEGF another HIF-1α target induced by hypoxia leads to growth of new vessels after cerebral ischemia, thereby minimizing the detrimental effects of cerebral ischemia (Marti et al., 2000).

Alzheimer’s disease (AD) is a progressive and fatal disease that is characterized by neuronal loss, cognitive dysfunction and dementia. The pathological lesions found in AD patients consist of plaques composed of amyloid-β (Aβ) peptides and intracellular neurofibrillary tangles containing hyperphosphorylation of tau protein (Correia and Moreira, 2010). Glucose metabolism and uptake is required for normal brain function, and this process is highly impaired in AD patients. Accumulating evidence suggests that impaired glucose uptake and metabolism is the leading cause and not the consequence of the disease (Hoyer 2004). Recently, Liu et al (2008) reported that the glucose transporters GLUT-1 and GLUT-3 were strongly downregulated in the brain of AD patients. In addition, he found HIF-1α protein was also decreased in these patients. In a single clinical study conducted in AD patients, by intramuscular administration of DFO, an iron chelator and HIF-1 inducer, slowed cognitive decline by more than 50% in 2 years (Crapper McLachlan et al., 1991). In another in vivo study using APP2576 transgenic mice, an animal model for AD, oral treatment with CQ a Cu/Zn chelator that stabilizes HIF-1 protein induced marked inhibition of Aβ deposition (49% decrease) in the absence of any neurotoxicity (Cherny et al., 2001). In a phase II clinical trial, CQ treated patients showed less cognitive decline and a decrease in plasma Aβ levels relative to patients treated with placebo (Ritchie et al., 2003).

In other neurological disorders such as Parkinson and Huntington disease, increased HIF-1 levels induced neuroprotective events (Correia and Moreira 2010). DFO a hypoxia mimetic
agent and 3,4-dihydroxybenzoate, a pharmacological inhibitor of PHDs, prevent neurotoxicity in
the MPTP-mouse model of Parkinson’s disease through stabilization of HIF-1 protein levels (Lee
et al., 2009). The CQ treatment of a transgenic Huntington disease mouse model improved the
behavioral and pathological phenotype, decreased accumulation of huntingtin protein and striatal
atrophy. It also normalized blood glucose and insulin levels and extended lifespan (Nguyen et
al., 2005).

Role of HIF-1 in cancer

The identification of the HIF-1 pathway for cellular response to hypoxia led to numerous
studies that provide evidence for its role in cancer. Strong support for this conclusion is provided
by the VHL mutation that leads to cancer of the kidney. This disease is due to a germline
mutation in the vhl gene that leads to constitutive activation of HIF-1. In addition, solid tumors
are known to have hypoxic regions within their core, which is the consequence of inadequate
vasculature and high metabolic demand. To survive under hypoxic conditions, tumor cells must
overcome the tight regulation of HIF-1 activity. The stabilization of HIF-1 protein leads to
transactivation of genes involved in angiogenesis and anaerobic metabolism (Maxwell 2005).
These are some of the characteristics which tumor cells acquire during malignant progression,
suggesting that HIF-1α is a potential regulator of cancer progression.

Immunohistochemical analyses demonstrate that HIF-1α is overexpressed in many human
cancers including breast, cervix, brain, ovary, oropharynx, and uterus. In addition, its
overexpression is correlated with the poor clinical outcomes in these patients (Semenza 2003). In
contrast, HIF-1α overexpression was also found to be associated with increased survival in the
patients with head and neck cancer and also non-small cell lung cancer. Not all of the more than
70 genes regulated by HIF-1 favor cellular adaptation and survival. BCL2-interacting protein (BNIP3) is HIF-1 target protein which is an inducer of apoptosis. In some cell types, HIF-1 activation was shown to play a pro-apoptotic role (Sowter et al., 2001; Bruick 2000)) and is also a mediator of cell cycle arrest in hypoxia (Schipani et al., 2001; Goda et al., 2003)). Thus, the contribution of HIF-1α activation to oncogenesis could also result from the environmental, epigenetic and genetic parameters (Maxwell 2005). In addition, the function of HIF-1α may also be dependent on the type of cancer as well as the stage of the cancer progression (Semenza 2003).

Pancreatic cancer cells, PCI-10, which were overexpressing exogenous HIF-1α showed a significant increase in the frequency of xenograft growth post injection (Akakura et al., 2001). This study revealed that transfection of HIF-1α into a series of pancreatic cancer cells that were not expressing HIF1α at high levels, made these cells more resistant to apoptosis and also resulted in increased tumorigenicity. Hypoxia-induced or exogenous overexpression of HIF-1α directly increased in vitro invasion by the human colon adenocarcinoma cells, HCT116 (Krishnamachary et al., 2003). HIF-1α overexpression in tumor xenografts of HCT116 cells resulted in increased growth and angiogenesis (Ravi et al., 2000).

In addition to overexpression of HIF-1α, the inhibition of this subunit has also revealed the relevance of HIF-1 to cancer pathology. Overexpression of a dominant-negative form of HIF-1α in PCI-43, pancreatic cancer cells, resulted in an increase in apoptotic cells and a decrease in their ability to form tumors in SCID mice (Chen et al., 2003). Inhibition of HIF-2α by siRNA was recently shown to significantly decrease the growth of neuroblastoma tumor xenografts in athymic mice. HIF-2α was shown to mediate the chronic response of the cells to
hypoxia, while HIF-1α was implicated in the acute hypoxia response (Holmquist-Mengelbier L et al., 2006).

Recently, HIF-1α was shown to be overexpressed in preneoplastic hepatocytic lesions from a very early stage of hepatocarcinogenesis in mice and humans. Interestingly, oxygen tension within the lesions was not different compared to surrounding normal hepatic tissue, indicating HIF-1α expression was hypoxia independent. In addition, this hypoxia-independent expression of HIF-1α in hepatocarcinoma was shown to be regulated by the PI3K/AKT pathway (Tanaka et al., 2006). In a separate study, the AKT pathway was also shown to be involved in hypoxia-independent upregulation of HIF-1α. This factor was shown to contribute to angiogenesis in human gastric cancer (Lee et al., 2007).

**Role of HIF-1 in melanoma**

Although, HIF-1 has been extensively studied in various cancers, information about its role in melanoma biology is relatively limited. This area of research is critical, since the normal human melanocyte resides at the base of the epidermis where there is a partial hypoxic environment (Stewart et al., 1982). Evidence has been provided that this partial hypoxia contributes to melanomagenesis. The hypoxic stress is thought to contribute to the Ras and AKT-induced transformation of normal human melanocytes (Michaylira and Nakagawa, 2006). In another study an active AKT pathway in the presence of hypoxia was sufficient to transform melanocytes. Interestingly, this effect was mediated via HIF-1α activation, and inhibition of HIF-1α inhibited AKT-hypoxia induced transformation of melanocytes (Bedogni et al., 2005). In another study, Notch-1, an effector of AKT signaling, along with hypoxia was shown to promote melanoma development (Bedogni et al., 2008).
A majority of human melanomas have constitutively active MAPK/extracellular signal-regulated kinase (ERK) due to BRAF or N-Ras mutations (Omholt et al., 2003; Daniotti et al., 2004). Activation of this pathway is correlated with the upregulation of HIF-1α mRNA in human melanoma (Kumar et al., 2007; Chun et al., 2003). A recent study investigated the involvement of HIF-1 in uveal melanoma migration, invasion and adhesion (Victor et al., 2006). We recently reported HIF-1α is over expressed in melanoma cells under non-hypoxic conditions and its enhanced expression contributes to some of their malignant properties. In addition, the experiments described in this report have demonstrated that the ERK1/2 MAPK pathway is not likely involved in the upregulation of HIF-1α in melanoma cells (Mills et al., 2009).
Microphthalmia-associated transcription factor (MITF)

Microphthalmia-associated transcription factor (MITF) is a master regulator of melanocyte development, survival and function through activation of genes that play a role in differentiation and cell-cycle progression. Melanocytes are pigment producing cells derived from the neural crest. During development neural crest cells emerge from the neural tube and migrate to the ectoderm where melanoblasts fully differentiated to melanocytes. These melanoblasts also function as melanocyte stem cells and reside in a niche within the bulge region of hair follicles. Melanocytes are found to be present in hair follicles, the basal layer of the epidermis, the inner ear, the eye choroid, the Harderian glands and the heart. The major function of melanocyte in skin and hair is the production of the melanin pigment to protect skin against harmful solar UV radiation. Researchers have intensely studied the mechanism of melanocyte development, melanin production, transport of this pigment through the melanosome and finally transfer of the melanosome and release of melanin to the surrounding keratinocytes (Goding 2000; Cheli et al., 2009).

The molecular mechanism responsible for the melanocyte development was dissected in 1993-94. The analysis of the promoter region of tyrosinase (TYR), tyrosinase related protein 1 (Tyrp-1) and others that play a role in melanin synthesis share a common 11bp element AGT-CATGTG, termed the M box, which is required for their expression (Lowings et al., 1992). The core CATGTG (E-box) motif within M box was known to bind basic-helix-loop-helix (bHLH) and bHLH-Leucine Zipper (bHLH-LZ) families of transcription factors. It was found that Mitf, a gene that encodes a bHLH-LZ transcription factor, was responsible for regulating melanin synthetic genes as well, as many other genes involved in melanocyte development and
differentiation (Hodgkinson et al., 1993; Hughes et al., 1993). Also in melanocytes, MITF acts as a mediator for the α-melanin stimulating hormone (α-MSH) induced differentiation effects on melanin production.

MITF belongs to the MiT family that consists of other related factors, including transcription factor EB (TFEB), TFE3, and TFEC that bind E-box sequences. These transcription factors share a highly conserved transactivation domain and bHLH-LZ domain that mediates DNA binding and dimerization respectively. MITF can heterodimerize with TFEB, TFE3 and TFEC under in vitro conditions but cannot heterodimerize with other bHLH-LZ transcription factors such as myc, max or USF. Unlike cell type specific MITF, other members of this family are thought to be ubiquitously expressed and do not appear to play a role in melanocytic development. Although, TFE3 was found to be overexpressed in B16 melanoma, it fails to coimmunoprecipitate along with MITF protein. This and other studies indicate that MITF is the only MiT family member that functions as a homodimer and is essential for the normal development of melanocytes (Goding 2000).

Characterization of the Mitf gene has led to the identification of at least nine different MITF isoforms that differ in their transcriptional initiation sites: MITF-A, -B, -C, -D, -E, -H, -J, -M and -MC. (Figure 1.7). The MITF variants differ in exon one while they have identical exons from two to nine. Structurally, the functional domains like TAD and bHLH-LZ are encoded in these common exons two to nine (Levy 2006). Whether, the unique N-teminal of these MITF isoforms confers distinct properties on each protein, or whether they simply reflect the fact that correct temporal or spatial expression of MITF could only be achieved using different promoters, is not known. MITF-A is the largest and –M is the shortest isoform. Some of these isoforms are
cell type specific such as MITF-M, which is specifically expressed in melanocytes and –MC, which is expressed only in mast cells. In contrast -A is ubiquitously expressed. Among all of these isoforms, MITF-M, has been the target of most experimental studies (Goding 2000).

**Figure 1.7:** MITF isoform differ in their transcriptional initiation sites. In humans, nine different promoters have been described for MITF. These promoters give 50 specificity for each isoform. However, all the different isoforms have in common exons 2 to exon 9, which encode the functional domains of the transcription factors. These domains are the transactivation domain (TAD) and the b-HLH-Zip. Some of the isoforms are expressed in specific cell types, such as the isoform M, which is expressed specifically in melanocytes, and the isoform MC, which is expressed selectively in mast cells (Levy et al., 2006).

**Transcriptional and post-translational regulation of MITF**

MITF-M is transcribed from an alternate promoter region located between exon one and
two and is often referred to as M promoter. The transcription factors that are known to regulate the M promoter have great significance in neural crest development and signaling. The transcription factors that are included in this list are paired box gene 3 (PAX3), cAMP-responsive element binding protein (CREB), SRY (sex-determining region Y)-box 10 (SOX10), lymphoid enhancer-binding factor 1 (LEF1), one cut domain 2 (ONECUT-2) and MITF itself (Levy 2006). The well studied signaling mechanism that regulates MITF-M expression is the α-MSH stimulated adenyl cyclase pathway. α-MSH binds to the melanocortin 1 receptor (MC1R), which activates adenyl cyclase activity resulting in increased level of cAMP. This increase in cAMP activates PKA, which among other substrates, phosphorylates CREB. This transcription factor stimulates the MITF-M promoter by binding to a cAMP response element (CRE) (Price et al., 1998). Although the adenyl cyclase pathway is ubiquitous, the activation of MITF-M promoter is melanocyte specific. The reason for this specificity is that CREB mediated expression of MITF-M is dependent on SOX10 and, moreover, SOX10 mediated expression of MITF-M is also CRE based. Thus, there is a certain level of co-operativity between these two transcription factors restricted to only neural crest derived cells (Huber et al., 2003). The wingless (WNT) signaling pathway is also important for the differentiation of melanocytes from neural crest cells. Activation of this pathway leads to the stabilization of β-catenin, which in turn interacts with LEF1 and is involved in activation of the MITF-M promoter (Dorsky et al., 2000; Takeda et al., 2000; Widlund et al., 2002).

MITF protein can also be regulated at the post-translational level. MITF protein is phosphorylated by MAPK, ribosomal S6 kinase (RSK), glycogen synthase-3β (GSKβ) and p38; the change in phosphorylation can modulate its transcriptional activity during specific environmental responses. Activation of c-kit in melanocytes results in phosphorylation of MITF
at Ser\textsuperscript{73} by ERK2 and Ser\textsuperscript{409} by p90RSK. Although phosphorylation of MITF at Ser\textsuperscript{73} can increase MITF activity by recruiting the transcriptional co-activator p300 (CBP), phosphorylation at this site also subjects MITF to ubiquitination and degradation. The MITF protein also gets sumolyated by protein inhibitor of activated STAT3 (PIAS3), and this seems to affect its transcriptional activity in a target-gene-specific manner. MITF was also found to be a substrate for proteolytic degradation at its C-terminus by caspase 3 (Levy, 2006).

PIAS3 and protein kinase C interacting protein 1 (PKCI) have been shown to be involved in repressing MITF transcriptional activity by directly interacting with and inhibiting its DNA binding activity. The phosphorylation pattern of MITF is shown to affect PIAS3 interaction. Phosphorylation at Ser\textsuperscript{73} increases the interaction between PIAS3 and MITF while phosphorylation at Ser\textsuperscript{409} decreases this interaction (Levy 2006).

**MITF target genes**

Seventeen years after the role of MITF was first described in melanocyte biology, more than 40 genes have been identified as targets of this key transcription factor. MITF is involved in a complex cascade of gene regulation that plays a role in melanocyte growth, survival and differentiation (Figure 1.7) (Cheli et al., 2009).

In the melanocyte differentiation program, MITF regulates genes that are involved in melanin production, melanosome biogenesis, melanosome transport and signaling pathways. Melanin synthesis is an enzymatic mechanism that converts tryrosine to a melanin pigment. The first two steps of melanin production are the hydroxylation of tyrosine to dihydroxyphenylalaanine (DOPA) and oxidation of DOPA to DOPA quinine. Both of these steps
involve tyrosinase (TYR) enzyme as a catalyst. Two other enzymes, tyrosinase-related proteins 1 (TYRP1) and dopachrome tautomerase (DCT), are also involved in melanin synthesis (Cheli et al., 2009). MITF transactivates the TYR, TYRP1 and DCT promoters as demonstrated by reporter gene assays and further directs binding to the promoter region of these genes within whole cells (Bentley et al., 1994; Bertolotto et al., 1998; Yavuer et al., 1995; Cheli et al., 2009).

Melanin synthesis takes place in the melanosomes, which are lysosome-related organelles. The biogenesis and transport of the melanosome are also key determinants of melanin synthesis and skin pigmentation. In addition to TYR and DCT, other proteins in melanosomes can also play an important role in melanogenesis. The Silver homologue (SILV) and Melan-a (MLANA) are involved in the formation of the melanosomal matrix and melanosomal maturation. Loss of function by these proteins leads to pigmentary defects. Initially, MITF was predicted to regulate SILV as its homologue, Pmel17, expression was undetectable in MITF<sup>−/−</sup> embryos. Confirmation was obtained through reporter gene assays and ChIP assays. Therefore, MITF directly binds and transactivates the SILV and MALNA genes (Baxter and pavan 2003; Du et al., 2003; Cheli et al., 2009). Other proteins that are involved in melanosomal biogenesis such as ocular albinism type 1 (GPR143) have also been reported as a direct target gene of MITF. GPR143 is a G-protein coupled receptor protein that is located at the plasma membrane and is involved in melanosomal maturation and size (Cheli et al., 2009).

Rab27A a small GTP binding protein plays a role in melanosomal transport along with melanophillin and MyosinVa. Mutation in any one of these 3 genes leads to hypopigmentation as found in Griscelli syndrome. By knockdown / overexpression studies and reporter gene assays it was found that MITF is involved in transcription of Rab27A, indicating that MITF controls
melanosome transport (Cheli et al., 2009). The human MC1R promoter contains an E-box and is activated by MITF in gene reporter assays. MC1R is a G-protein coupled receptor that binds α-MSH and plays a major role in skin pigmentation by activating MITF expression through the adenylyl cyclase/cAMP/PKA/CREB pathway (Busca and Ballotti 2000).

**Figure 1.8: MITF regulation and role in melanocyte differentiation, cell cycle progression and survival.** MITF is activated by various signaling pathways such as growth signaling pathway involving ERK-MAPK activation, α-MSH mediated activation of adenyl cyclase pathway and Wnt signaling pathway. MITF forms a homodimer and binds to E-box. MITF transactivate various genes involved in cell cycle progression, differentiation and pigmentation, motility and anti-apoptosis.
In melanocyte development, MITF regulates genes that are involved in melanocyte precursor growth and survival. During the embryonic development of mutant MITF\textsuperscript{mi-ew} (eyeless white) mice, melanoblasts were detected in the early stage but not in the late stages. These mutant mice lacked melanocytes in the skin likely due to a defect in proliferation (Nakayama \textit{et al.}, 1998) leading to growth arrest or cell senescence. In this context, MITF was shown to be the target transcription factor TBX2, a member of the T-box family, which inhibits cell senescence through downregulation of the p21 and p19 genes. MITF regulates TBX2 expression through the E-box sequence located in its promoter region. TBX2 was the first MITF regulated gene identified that is not directly involved in melanin synthesis. Other studies found that cyclin-dependent kinase (CDK2), a gene involved in cell cycle progression, was also regulated by MITF. The promoter region of human CDK2 had a CATGTG motif (a same motif involved in the regulation of SILV by MITF), located -1315 bp from the transcription start site. MITF was found to regulate the expression of CDK2 by binding to its promoter region containing these motifs (Cheli \textit{et al.}, 2009).

MITF was further linked to cell cycle progression by stimulating the transcription of diaphanous-related formin (DIAPH1). Inhibiting the expression of MITF in melanoma was found to upregulate p27 expression, a gene that is involved in inhibiting cell cycle progression (Carreira \textit{et al.}, 2006). The upregulation of p27 was associated with the downregulation of diaphanous-related formin (DIAPH1), which controls the expression of p27 through ubiquitination and degradation (Mammoto \textit{et al.}, 2004; Schulman \textit{et al.}, 2000). Analysis of the DIAPH1 promoter showed three potential MITF binding sites. ChIP, electromobility shift assays (EMSA) and reporter gene assays demonstrated that MITF binds and transactivates the DIAPH1 promoter. In addition this study also showed that silencing of MITF blocks melanoma cell cycle
progression in G0/G1. However, MITF may also directly regulate p21 and p16 that results in G1-S arrest in melanocyte and melanoma. An E-box has been identified in the promoter of p21 but whether a similar sequence is in p16 has not been determined. Silencing of MITF in a uveal melanocyte cell line decreased p16 expression and increased cell proliferation while over expression of MITF increased the expression of both p16 and p21 expression and blocked the cell proliferation in a fibroblast cell line (Cheli et al., 2009).

The first evidence for the role of MITF in melanocyte survival comes from the microarray analysis of MITF dependent gene expression. Through this study BCL2, an anti-apoptotic gene was identified as a potential target gene of MITF. Further analysis of the BCL2 promoter revealed the presence of an E-box motif and ChIP, EMSA and reporter gene assays confirmed BCL2 as the direct target of MITF. Furthermore, overexpression of BCL2 rescued the apoptotic effect induced by dominant negative MITF (Mcgill et al., 2002). Thus, indicating that MITF-stimulated BCL2 controls melanoma cell survival. In addition, BCL2 knockout mice are born pigmented but later turn grey due to loss of melanocyte stem cells, thus indicating its role in survival of melanocyte precursors (Mak et al., 2006; Veis et al., 1993). Recently, other anti-apoptotic protein, BIRC7 (ML-IAP, LIVIN or KIAP) have been identified as direct targets of MITF (Dynek et al., 2008).

Two independent reports showed that MITF can regulate met proto-oncogene (hepatocyte growth factor receptor) (MET) expression. The MITF binding E-box motif was found in the promoter of MET but only one of these studies confirmed that the regulation of MET promoter activity by MITF was mediated by these motifs (Mcgill et al., 2006; Beuret et al., 2007). However in both studies, MET activation was associated with the migration of melanoma cells
and protected melanoma and melanocyte cells from apoptosis. MITF also regulates growth signaling receptors such as KIT and NGFR through direct transactivation of their genes. KIT and NGFR are tyrosinase kinase receptors involved in activating ERK/MAPK and PI3K signaling pathways that play a role in protecting cells against apoptosis and favor cell survival (Cheli et al., 2009).

Recently, MITF was found to play a role in protecting cells against the damage induced during oxidative stress. The apurinic/apyrimidinic endonuclease1/redox factor-1 (APEX1) was shown to be a direct target gene of MITF and its forced expression reversed ROS-induced apoptosis in MITF silenced cells (Liu et al., 2009). In addition HIF-1α was recently found as a direct target of MITF in melanoma cells. MITF stimulated HIF-1α expression was also shown to protect these cells against apoptotic stimuli (Busca et al., 2005).

**Physiological role of MITF**

In humans, mutation in the *Mitf* locus leads to Waardenburg syndrome (WS) type IIA. It is a dominantly inherited auditory-pigmentary syndrome mainly due to the absence of melanocytes in the eye, inner ear, skin and hair. The most serious consequences of *Mitf* mutation in affected individuals is hearing impairment that ranges from mild to severe. In the inner ear, melanocytes reside in the stria vascularis of the chochlea where it maintains extracellular K⁺ in the endolymphatic fluid. There are different types of WS; WS type I and type III are caused by mutated PAX3, which is found to be expressed in neural crest cells and is a modulator of MITF. PAX3 also affects lineages other than melanocytes. Thus, the WS type I and III patients, in addition to melanocytic abnormalities, also exhibit musculoskeletal abnormalities. WS type IV is caused by a mutation in SOX10, endothelin-1 and its receptor. It is characterized by melanocyte
related deficiencies as seen in type I and also it exhibits Hirschsprung syndrome that involves enlargement of the colon (Read and Newton 1997; Levy et al., 2006).

Studies on mutations of the Mitf gene in mice found alterations in phenotypes characterized by deafness, bone hyperdensity, small eyes and a lack of pigmentation in eyes and skin. Mutations in the Mitf gene that inhibit its function result in complete loss of pigmentation due to an absence of melanocytes. However, the heterozygous mutants showed either no pigmentation or a white spot on their belly. These white belly mice have a mutation within the basic DNA binding domain of MITF while the HLH-Zip region responsible for dimerization is unchanged. The ventral spotting in these dominant mutant mice is due to the defect in neural crest migration of melanocyte precursors (Levy et al., 2006). According to hypomorphic allele studies in mice, MITF is also necessary in adulthood and loss of function could lead to premature gray hairs due to low maintenance of melanocyte stem cells (MSC) in the hair follicle bulge region niche (Lerner et al., 1986; Nisthimura et al., 2005).

In addition to MITF’s role in melanocyte mediated pigmentation, it also seems to play a role in development of melanocyte precursors from neural crest cells. The study conducted by Hornyak et al. in Mitf<sup>mi/mi</sup> mutant mice revealed MITF is required for the survival and development of melanoblasts during or immediately after the migration from the dorsal neural tube. It also directly or indirectly affects the rate at which the number of melanoblasts migrate from the neural tube. MITF is also related to MSC which are a self renewing population of melanocytes that reside in the bulge region niche of the hair follicle. MSCs are involved in maintenance of hair coloration and loss of MSC could lead to premature graying of the hair (Hornyak et al., 2001). The Mitf<sup>vit</sup> mutant mice have premature loss of the MSC population
resulting in gray hair, a process normally associated with aging (Nisthimura et al., 2005). Moreover, the anti-apoptotic gene BCL2 which is a direct target gene of MITF was shown to be involved in maintenance of MSC in postnatal mice (Nisthimura et al., 2005; Steingrimsson et al., 2005).

In addition to MITF’s role in melanocyte function, it is also found to be expressed in mast cells and osteoclasts. Mast cells play a central role in generating innate immunity against bacterial and parasitic infections. They also serve as a mediator of allergic hypersensitivity reactions in various pathological conditions. The Mitfi/mi mutant mice which showed defects in mast cells were more susceptible to parasitic infection relative wild type mice. The various proteins such as proteases, protease inhibitors, adhesion molecules, metabolic enzymes and growth factor receptors that play roles in mast cell maturation and function have been identified as MITF target genes. Analogous to mast cells, osteoclasts arise from hematopoietic stem cells and are found to play a role in bone remodeling. Osteoclast dysfunctionality in humans and mice leads to osteoporosis making bones more brittle. The semi-dominant Mitf mutant mice have shown to develop osteoporosis and reintroduction of intact MITF have rescued some of these effects. MITF is shown to target genes such as TRAP, cathepsin K, OSCAR, E-cadherin, OSTM1 and Clcn7 that are involved in osteoclast function (Cheli et al., 2009).

**Role of MITF in melanoma**

In addition to MITF’s role in melanocyte function, survival and differentiation, it was found to be amplified as an oncogene in a fraction of melanomas. Analysis of primary melanoma tissue microarrays found MITF to be amplified in 10-20% of cases of advanced melanoma. Moreover, amplification of MITF in melanoma was associated with a decrease in the five year
survival rate relative to patients who did not have this gene amplification. In addition, the ectopic expression of MITF in conjunction with B-RAF (V600E), p53 and CDK4 mutations transformed immortalized primary human melanocytes, thus defining MITFs role as an oncogene in melanoma development (Garraway et al., 2005; Levy et al., 2006).

MITF expression is variable among melanoma specimens. In some cases, it was shown to be more highly expressed in early stage relative to late stage melanoma (Salti et al., 2000; Selzer et al., 2002). MITF levels seem to correlate with the levels of transient receptor potential cation channel M1 (TRPM1) which is a target gene of MITF. TRPM1 expression is found to be highly expressed in benign nevi, dysplastic nevi and melanomas in early stage and low in invasive melanoma and virtually absent in metastatic melanomas. MITF expression was found to follow the same trend as TRPM1 expression in melanomas. Recently, MITF has been identified as a marker in the proliferative signature of melanoma cells that were less motile than invasive cells. Melanoma cells with a proliferative signature initiated tumor growth in 14 days post injection relative to melanoma cells have an invasive signature, which took 59 days (Hoek et al., 2008). In another study MITF was identified as an oncogene in melanoma specimens representing the transition point for tumor progression and metastasis (Riker et al., 2008). Because of these opposing findings, there is controversy regarding the role of MITF in melanoma development and progression.

The growth and survival function of MITF in melanoma remains a matter of debate. It has been demonstrated that overexpression of MITF in melanoma cell lines reduced their xenograft tumor growth (Selzer et al., 2002) by activating cell cycle inhibitors INK4 (Loercher et al., 2005) and p21CIP1 (Carreira et al., 2005). However, MITF has been shown to up-regulate, the
anti-apoptotic gene BCL2 (McGill et al., 2002) and to promote melanoma cell growth by stimulating CDK2 expression (Du et al., 2004). Targeting MITF through direct injection of siRNA into tumors drastically reduced the growth of B16 melanomas in syngenic mice by inducing apoptosis (Nakai et al., 2007). This latter study, suggests that in some melanomas MITF is a pro-survival factor.

Over the years, MITF has been described as a diagnostic marker in clinical determination of melanoma. Its high sensitivity and specificity as an immunohistochemical marker is considered superior to other melanoma markers such as S-100 and HMB-45 (King et al., 1999). One of the immunohistochemical markers which is currently used in clinical diagnosis of melanoma is the MITF-specific antibody D5. However, MITF serves more as a melanocyte-lineage specific marker and cannot distinguish between benign vs malignant melanoma lesions (Levy et al., 2006).
Experimental objective

The major objective of this study was to determine if HIF-1α, a master regulator of oxygen homeostasis, also plays a role in melanoma cancer progression. In this study we have found that hypoxia-independent expression of HIF-1α protein in melanoma cells contributes to its progression. In addition, we have found that MITF, a melanocyte specific protein (Levy et al., 2006), is a novel direct target gene of HIF-1α in melanoma cells and high expression of MITF plays a role in melanoma cell survival.
CHAPTER TWO

Expression and function of hypoxia inducible factor-1 alpha in human melanoma under non-hypoxic conditions

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Abstract

Hypoxia inducible factor-1 alpha (HIF-1α) protein is rapidly degraded under normoxic conditions. When oxygen tensions fall HIF-1α protein stabilizes and transactivates genes involved in adaptation to hypoxic conditions. We have examined the normoxic expression of HIF-1α RNA and protein in normal human melanocytes and a series of human melanoma cell lines isolated from radial growth phase (RGP), vertical growth phase (VGP) and metastatic (MM) melanomas. HIF-1α mRNA and protein was increased in RGP vs melanocytes, VGP vs RGP and MM vs VGP melanoma cell lines. We also detected expression of a HIF-1α mRNA splice variant that lacks part of the oxygen-dependent regulation domain in WM1366 and WM9 melanoma cells. Over-expression of HIF-1α and its splice variant in the RGP cell line SbCl2 resulted in a small increase in soft agar colony formation and a large increase in matrigel invasion relative to control transfected cells. Knockdown of HIF-1α expression by siRNA in the MM WM9 melanoma cell line resulted in a large decrease in both soft agar colony formation and matrigel invasion relative to cells treated with non-specific siRNA. There is a high level of ERK1/2 phosphorylation in WM9 cells, indicating an activated Ras-Raf-MEK-ERK1/2 MAPK pathway. Treatment of WM9 cells with 30 μM U0126 MEK inhibitor, decreased ERK1/2 phosphorylation and resulted in a decrease in HIF-1α expression. However, a 24 h treatment with 10 μM U0126 totally eliminated Erk1/2 phosphorylation, but did not change HIF-1alpha levels. Furthermore, siRNA knockdown of MEK siRNA did not change HIF-1alpha levels. We speculate that metabolic products of U0126 decrease HIF-1α expression through "off target" effects. Overall our data suggest that increased HIF-1α expression under normoxic conditions contributes to some of the malignant phenotypes exhibited by human melanoma cells. The expanded role of HIF-1α in melanoma biology increases its importance as a therapeutic target.
Introduction

The incidence of melanoma is increasing more rapidly than any other tumor site. Melanoma accounts for 4% of all skin cancers, but for 79% of all skin cancer related deaths in the United States (Melanoma Research Foundation). Metastatic melanoma is highly resistant to both chemo- and radiotherapy (Soengas and Lowe, 2003). Cutaneous melanoma arises from melanocytes, presumably due to early childhood exposure of the skin to UV radiation. A predisposing factor for melanoma may be the melanocortin receptor. It has been found that individuals having a mutation that affects the function of the melanocortin receptor have an increased risk of developing cutaneous melanoma (Sturm 2002).

Hypoxia-inducible factor-1 (HIF-1) is a master regulator of O₂ homeostasis in cells. It consists of a heterodimeric transcriptional complex of two proteins, HIF-1β and HIF-1α. HIF-1β is constitutively expressed whereas HIF-1α protein is stabilized only under hypoxic conditions, despite its continuous synthesis under normoxic conditions (Semenza 2003). When O₂ tension is normal, HIF-1α is hydroxylated at specific proline residues by the enzyme prolyl hydroxylase-domain (PHD). This hydroxylation is required for the von Hippel Lindau (VHL) tumor suppressor protein to bind to HIF-1α leading to subsequent ubiquitination and proteasome-targeted degradation (Maxwell et al., 1999). VHL binding is also enhanced by acetylation of lys⁵³² catalyzed by the acetyltransferase, ADP-ribosylation factor domain protein 1 (ARD1) (Jeong et al., 2002). Under hypoxic conditions, proline hydroxylation decreases thereby stabilizing HIF-1α, which in turn moves to the nucleus and transactivates various genes containing hypoxia response elements (Semenza 2002).

HIF-1α controls the expression of over 60 genes involved in many aspects of
oncogenesis, including tumorigenesis (Kondo et al., 2005; Zhang et al., 2004) anti-apoptosis (Zhang et al., 2004; Greijer and Wall 2004), and genetic instability (Koshiji et al., 2005). HIFα has also been implicated in the malignant progression of several cancers including mammary gland, prostate, brain, and lung (Goda et al., 2003). HIF-1α is the regulatory subunit of HIF-1. It is regulated at the protein level by both oxygen-dependent and independent pathways (semenza 2002). HIF-1α is highly expressed in early stage of mouse hepatocarcinogenesis independent of hypoxia (Tanaka et al., 2006). The hypoxia independent increase in HIF-1α is thought to be activated by growth signaling pathways. A majority of human melanomas have constitutively active MAPK/extracellular signal-regulated kinase (ERK) due to BRAF or N-Ras mutations (Omholt et al., 2003; Daniotti et al., 2004). Activation of this pathway is correlated with the upregulation of HIF-1α mRNA in human melanoma (Kumar et al., 2007; Chun et al., 2003). However the biological significance of upregulated HIF-1α under normoxic conditions for initiation and progression of melanoma has not been elucidated.

In this study, we examined the normoxic expression and biological functions of HIF-1α in human melanoma. We found that both full length and a splice variant, HIF-1α785, are expressed in human melanoma cell lines while essentially undetectable in normal human melanocytes. Ectopic HIF-1α expression in a low expressing RGP cell line stimulated Matrigel invasion, while knockdown of HIF-1α in a high expressing MM cell line inhibited both soft agar colony formation and Matrigel invasion. Knockdown of MEK1/2 and loss of phosphorylated ERK1/2 did not decrease HIF-1α expression. U0126 MEK inhibitor at 10 μM eliminated ERK1/2 phosphorylation, but did not decrease HIF-1α expression.
Results

Expression of HIF-1α in human melanoma cells

In addition to the well known pathway of HIF-1alpha protein stabilization under hypoxic conditions, it has been established that many oncoproteins and growth factor signaling pathways up-regulate HIF-1α expression under normoxic conditions (Stiehl et al., 2002; Vogelstein and Kinzler 2004). However, there are few investigations into the normoxic expression of HIF-1α in human melanoma and its role in the malignant progression of this disease. Here, we show that in human melanoma cells, the oxygen-labile HIF-1α protein as well as its mRNA is expressed endogenously under normoxic conditions. Figure 2.1A shows that HIF-1α protein is highly expressed in WM9 cells relative to normal human melanocyte (HEMn-LP), but radial growth phase (SbCl2), and vertical growth phase (WM1366) also express a higher amount of HIF-1α protein relative to human melanocytes. Similar results are seen in second set of RGP, VGP and MM melanoma cell lines (Figure 2.1B). HIF-1α was detected as 120 kD protein in nuclear extracts while no protein was detected in cytoplasmic extracts (data not shown).
Hypoxic stabilization of HIF-1α occurs at the protein level (Semenza 2003). Since HIF-1α protein was increased in human melanoma cells under normoxic conditions, we determined whether this increase might be due to increased HIF-1α mRNA levels. Initially we used semi-quantitative RT-PCR to assess expression of HIF-1α full length (FL) and a splice variant HIF-1α785 that is missing the acetylation site lys^{532} due to lack of exon 11 (Figure 2.2A). This splice variant encodes HIF-1α protein that has been reported to be stable under normoxic conditions (Chun et al., 2003; Lin et al., 2004). Primers were designed so that full length HIF-1α would exclude HIF-1α785 by targeting exon 11, which is absent in HIF-1α785. Primers for HIF-1α785 excluded HIF-1α by targeting the exon 10:12 boundary only present in HIF-1α785. Figure 2.2B shows that human melanoma cell lines express both full-length and the 785 splice variant HIF-1α mRNA at a level that appeared to be higher than normal human melanocytes.
Figure 2.2 Expression of HIF-1αFL & HIF-α785 mRNA in human melanoma cells. (A) Schematic representation of the functional domains of both HIF-1α full length and HIF-1α785. Both HIF-1αFL and 785 have various domains in common such as basic helix loop helix (bHLH), Per/Arnt/Sim (PAS), Oxygen Dependent Degradation Domain (ODDD), N-terminal transactivation domain N-TAD, inhibitory domain (ID), nuclear localization signal (NLS) and C-terminal transactivation domain C-TAD. Upon loss of exon 11 in HIF-1α785, part of the ODDD is deleted. This missing region contains the important lysine 532 residue which is acetylated by ARD1 leading to increased stable interaction of HIF-1α with the von Hippel Lindau tumor suppressor. This interaction directs HIF-1α to the ubiquitin-proteasome pathway for degradation under normoxic conditions (Jeong et al., 2002). (B) Total RNA was extracted from HEMn-LP, SbCl2, WM1366, and WM9. RNA was reverse transcribed using the Advantage RT-for PCR kit®. Five μL of the resulting cDNA was used in the PCR reaction as described in the Advantage cDNA kit® manual. Primers (see Methods for sequence) for HIF-1α and HIF-1α785 were designed to specifically amplify each variant with no cross-amplification. Primers for the housekeeping control gene GAPDH were included in the Advantage cDNA kit®. Control primers amplifying a fragment of the control plasmid included in the Advantage cDNA kit® were used to ensure optimal PCR conditions.

These findings were verified by qRT-PCR measurement of full-length and HIF-1α785 mRNA levels (Figure 2.3). All melanoma cell lines had increased expression of HIF-1α mRNA relative to normal human melanocytes. In addition VGP and MM cell lines expressed more of the 785 HIF-1α mRNA than full length HIF-1α mRNA. Overall the WM9 metastatic
melanoma expressed the highest amount of 785 HIF-1α mRNA (~79× higher than normal human melanocytes).

**Figure 2.3 Quantitative analysis of HIF-1αFL & HIF-1α785 mRNA expression in human melanoma cells.** Total RNA was extracted from HEMn-LP, SbCl2, WM1366, and WM9 cells at 72 h (A) after seeding. RNA was then converted to cDNA using the High Capacity cDNA Archive Kit (ABI). Real-Time PCR analysis was performed using TaqMan probes directed at HIF-1αFL or HIF-1α-785 as well as β-actin. The reactions were performed under conditions specified in the ABI TaqMan Gene Quantification assay protocol. Data was corrected for efficiency and loading using the Pfaffl method. Data is expressed as fold change, corrected for β-actin, relative to HeMn-LP. (B) Real-time PCR analysis was performed using Taqman probes directed at HIF-1αFL or HIF-1α785 as well as 18S using RNA extracted from HEMn-LP, WM3211, WM3248, and WM239 cells. Data is representative of at least 2 separate experiments.

**HIFαFL and HIF-1α785 gain-of-function in radial growth phase SbCl2 cells**

Gain of function studies means overexpressing HIF-1α in RGP cells and measuring its
effect on their biological properties. The level of HIF-1α protein is low in the radial growth phase SbCl2 cells relative to VGP or MET cell lines. We determined the effect of HIF-1αFL or HIF-1α785 overexpression on SbCl2 anchorage-independent growth and Matrigel invasion. HIF-1αFL and HIF-1α785 were cloned into the pLenti-V5-D-TOPO vector and transiently overexpressed in SbCl2 cells (Figure 2.4A). HIF-1α785 overexpression resulted in a small, but statistically significant increase in anchorage-independent growth, relative to mock or lacz transfected cells (Figure 2.4C and 2.4D). In contrast overexpression of both HIF-1αFL and HIF-α785 in SbCl2 resulted in a large and significant 3-fold increase in Matrigel invasion relative to mock or Lacz transfected cells (Figure 2.4B).

Figure 2.4 Effect of HIF-1αFL and HIF-1α785 overexpression in radial growth phase SbCl2 cells on anchorage-independent growth and invasion. SbCl2 cells were transiently transfected at 80% confluence with either mock (no plasmid DNA), pLenti-V5-Lacz, pLenti-V5-D-TOPO-HIF-1αFL or pLenti-V5-D-TOPO-HIF-1α785 using FuGene 6 transfection
(A) After 48 h nuclear protein was extracted and over-expression was confirmed by western blot using HRP-conjugated anti-V5 antibody and mouse monoclonal anti-HIF-1α antibody (1 μg/ml). LaminB1 was used as loading control. (B) SbCl2 cells 24 h post transfection with either mock (no plasmid DNA), Lacz, HIF-1αFL or HIF-1α785 were subjected to Matrigel invasion assay as described in "Material and Methods". Results are expressed as percent invasive cells corrected for invasion level of cells seeded in Matrigel (-) chambers. (C&D). SbCl2 cells transfected either with reagents alone (mock), Lacz, HIF-1αFL or HIF-1α785 were subjected to CytoSelect 96 well Cell Transformation Assay® (Cell Biolabs, Inc.) as described in "Material and Methods" for 4 (C) and 5 days (D). Results are expressed as Relative Fluorescent Units. Data is expressed as the mean ± SEM of triplicate values. ANOVA with TUKEY for multiple pairwise comparison was used to analyze the data and all P values ≤ 0.05 are relative to Mock and Lacz overexpressing cells. The entire experiment was repeated two additional times with similar results.

**HIF-1α loss-of-function in human metastatic melanoma WM9 cells**

HIF-1α protein is highly expressed under normoxic conditions in the WM9 human metastatic melanoma cell line. To determine whether HIF-1α could be contributing to the malignant characteristics of these cells, we knocked down its expression and examine how this affected anchorage independent growth and Matrigel invasion. WM9 cells were treated with 100 nM siRNA targeting HIF-1α (Dharmacon) which consistently decreased its expression by ~75-85% (Figure 2.5A). Colony formation after 5 days in soft agarose was inhibited by 70% in HIF-1α-siRNA transfected WM9 cells in comparison to cells transfected with control siRNA (Figure 2.5B). A photo (Figure 2.5C) of the colonies formed at this time point in control vs. HIF-1α transfected cells verifies this decrease in soft agar colony formation. Matrigel invasion was also significantly decreased in HIF-1α-siRNA transfected WM9 cells compared to control siRNA transfected WM9 cells (Figure 2.5D). Measurement of cell viability in the Matrigel chambers shows no difference between control vs. HIF-1α siRNA transfected cells (Figure 2.5E). These knock down studies suggest that increased non-hypoxic expression of HIF-1α plays an important role in key malignant properties exhibited by these human melanoma cells.
Figure 2.5 The effect of loss of HIF-1α expression in human metastatic melanoma WM9 cells on anchorage-independent growth and invasion. (A) WM9 cells were treated with either 100 nM HIF-1α siRNA or 100 nM control non-targeting siRNA (Dharmacon, Inc.) using the RNAfect® transfection reagent (Qiagen, Inc.). Knock down of HIF-1α was confirmed by western blot at 72 h post transfection. (B) At 48 h post transfection Ctrl siRNA or HIF-1α siRNA treated WM9 cells, were assayed for anchorage independent growth using the CytoSelect 96 well Cell Transformation Assay® (Cell Biolabs, Inc.). Briefly, the cells were seeded at 8.0 × 10^3 cells/well into a 0.4% agar layer poured over a 0.6% agar layer of a 96 well plate. Wells lacking cells served as a blank control. On day 5 after seeding, agar layers were solubilized, cells were lysed, and nucleic acid stained with CyQuant dye. Fluorescence intensity in each well was determined by a plate reader set at 485/520 nm. Results are expressed as Relative Fluorescent Units. (C) Photomicrograph of a representative field of colonies form in the Cell Transformation Assay by control siRNA transfected cells (left) vs. HIF-1α siRNA transfected cells. (D) Matrigel invasion assay. At 48 h post transfection, the Ctrl siRNA or HIF-1α siRNA transfected WM9 cells were seeded into 6-well Matrigel (+) chambers, and as a control, 6-well Matrigel (-) chambers (BD Biosciences) at 7.0 × 10^4 cells per well. The method and counting of invading cells was done as described in "Experimental Procedures". Results are expressed as % invasion of HIF-1α siRNA treated-WM9 cells relative to invasion by Control siRNA treated-WM9 cells corrected for invasion by similarly treated cells seeded in Matrigel (-) chambers. (E) Cell viability assay was done in WM9 cells transfected with control siRNA or HIF-1α siRNA at 48, 72 and 96 h following transfection by the trypan blue exclusion method. Data is expressed as the mean ± SEM of triplicate samples. The entire experiment was repeated twice with similar results. Student paired t test was used to analyse the data and all P values ≤ 0.01 or 0.05 are relative to control siRNA treated cells.

Regulation of HIF-1α expression in human melanoma by the ERK1/2 MAPK pathway

Hypoxia independent expression of HIF-1α is thought to be regulated by growth signaling pathways (Stiehl et al., 2002; Vogelstein and Kinzler 2004) and the majority of melanomas have constitutively active ERK1/2 MAPK pathway due to BRAF or N-Ras mutations (Omholt et al., 2003; Daniotti et al., 2004). Therefore, we determined whether HIF-1α expression in human metastatic melanoma WM9 cells was dependent on activation of ERK1/2 MAPK signaling. These cells have an active ERK1/2 MAPK pathway as evidenced by the high phosphorylation of ERK (Figure 2.6A). Treatment of WM9 cells with 30 μM U0126, a selective U0126 MEK inhibitor, decreased ERK1/2 phosphorylation and led to a time-dependent decrease in HIF-1α protein expression (Figure 2.6A). Although 30 μM U0126 has
been used in published studies to selectively inhibit MEK (Kumar et al., 2007; Tisciuoglio et al., 2004), the original paper describing this inhibitor (Favata et al., 2004) used much lower concentrations to achieve high selectivity. Therefore we repeated this experiment using 10 μM U0126 (Figure 2.6B). At 24 h of treatment, 10 μM U0126 completely suppressed the phosphorylation of ERK1/2, yet there was minimal change in the level of HIF-1α relative to control cells. With further time of inhibitor treatment, phosphorylation of ERK was not totally suppressed, but HIF-1α levels decreased. We also used siRNA specifically targeting MEK1 and 2 in WM9 cells to inhibit ERK1/2 phosphorylation. Treatment of WM9 cells with siRNA targeting MEK1 and 2 consistently decreased its expression by greater than 90% and also decreased ERK1/2 phosphorylation. However, knockdown of MEK1 and 2 did not decrease the normoxic expression of HIF-1α protein in human metastatic melanoma WM9 cells (Figure 2.6C).
Figure 2.6 Effect of ERK1/2 MAPK inhibition on HIF-1α expression in human melanoma cells. WM9 cells were treated with either 30 (A) or 10 (B) μmol/L U0126 a MEK1/2-specific inhibitor or vehicle (DMSO). Inhibition of ERK phosphorylation and HIF-1α expression was determined by western blotting at 24, 48 and 72 h after treatment using both total ERK, phospho-specific ERK and HIF-1α antibodies respectively. C. WM9 cells were also treated with either 100 nM MEK1&2 siRNA or 100 nM control non-targeting siRNA (Dharmacon, Inc.) using the RNAiFect® transfection reagent (Qiagen, Inc.). Knock down of MEK1&2 was confirmed by western blot at 72 h post transfection. Inhibition of ERK phosphorylation and HIF-1α expression was examined by western blot at the same time point in nuclear extracts by using both total ERK, phospho-specific ERK, and HIF-1α antibodies. The entire experiment was repeated two additional times with similar results.
Discussion

Melanocytes - the cells responsible for producing the skin-coloring pigment, melanin, are the point of origin for melanoma. Melanoma, if diagnosed and treated early, has a high cure rate (Balch et al., 2001). If the melanoma progresses, it can metastasize regionally to lymph nodes, and then to distant organs such as the lungs, and the brain (Houghton and Polsky 2002). Metastatic melanoma is very difficult to treat and has a high mortality rate. Several studies have confirmed that HIF-1α is a survival factor, as well as a key regulator of metastasis in various cancers (Harris 2002; Liao et al., 2007).

HIF-1α regulates the adaptive responses to O₂ tensions at cellular levels. It controls the expression of many genes involved in different aspects of tumor biology, including angiogenesis, cell survival, invasion (Semenza 2003), tumor growth (Kondo et al., 2005; Zhang et al., 2004), anti-apoptosis (Zhang et al., 2004; Greijer and Wall 2004), and genetic instability (Koshiji et al., 2005). HIF-1α is rapidly degraded under normoxic condition and is stabilized under hypoxic condition (Semenza 2003). We found that HIF-1α protein and RNA is expressed under normoxic conditions in several human melanoma cell lines and that the levels of HIF-1α expression correlates with the stage of cancer from which the melanoma cell line was established. In contrast, HIF-1α protein was undetectable in normal human melanocytes. Normoxic expression of HIF-1α has been found in a number of cancer cell types (Tanaka et al., 2006; Kumar et al., 2007; Lee et al., 2007). Activation of the ERK1/2 MAPK (Kumar et al., 2007) and phosphotidylinositol 3-kinase (PI3K) (Stiehl et al., 2002) pathways has been implicated in stimulating normoxic expression of HIF-1α.

The epidermis of the skin is a partial hypoxic environment (Stewart et al., 1982).
Evidence has been provided that this partial hypoxia contributes to melanomagenesis (Bedogni et al., 2005; Bedogni et al., 2008). Therefore, one possibility is that the increase in HIF-1α that we observed in melanoma cells is due to the hypoxic adoptive response maintained by the cells in culture. However, hypoxia stabilizes the HIF-1α protein and does not increase the level of HIF-1α mRNA. We found that the increased HIF-1α protein in the melanoma cells was correlated with an increase in HIF-1α mRNA. Also, the normal human melanocytes used in our study came from the partial hypoxic environment of the skin and yet in culture, they do not express detectable levels of HIF-1α protein. Therefore, we think it more likely that the increased HIF-1α mRNA and protein is due to an inappropriately activated signaling pathway.

We also found that an mRNA splice variant, HIF-1α785, was expressed at higher levels than full length HIF-1α mRNA in the VGP and metastatic human melanoma cell lines. HIF-1α785 is missing the acetylation site lys532 due to lack of exon 11 and is thought to be more stable under normoxic conditions in comparison to full length HIF-1α (Chun et al., 2003; Lin et al., 2004). In several non-melanoma cell lines it was found that phorbol ester stimulated the expression of HIF-1α785 mRNA under normoxic conditions via a redox-dependent ERK1/2 MAPK pathway (Kumar et al., 2007). How this alternatively spliced isoform of HIF-1α mRNA is increased in melanoma is currently under investigation.

We examined the biological consequences of HIF-1α FL and 785 splice variant gain of function and HIF-α FL loss of function in selected human melanoma cells. The SbCl2 radial growth phase melanoma cells have low levels of HIF-1α protein expression and a limited capacity to form colonies in soft agar and to invade through Matrigel. This cell line was chosen to determine the biological effects of HIF-1α overexpression. Transient ectopic expression of FL HIF-1α did not result in a statistically significant increase in SbCl2 colony formation in soft
agar. However, transient overexpression of HIF-1α785 resulted in a small, but statistically significant increase in soft agar colony formation. The effect on anchorage-independent growth may be limited by the transient nature of the overexpression of HIF-1. Overexpression of either HIF-1αFL or 785 led to a large and statistically significant increase in the ability of SbCl2 cells to invade Matrigel. There have been a few reports on the effect of HIF-1α overexpression on the in vitro biologic properties of cancer cells. Hypoxia-induced or exogenous overexpression of HIF-1α increased in vitro invasion by human colon adenocarcinoma cells (Chun et al., 2003), while stable normoxic overexpression of HIF-1α promoted anchorage-independent growth in melanocytes having an activated AKT signaling pathway (Bedogni et al., 2008). SbCl2 cells overexpressing HIF-1α785 showed a somewhat greater increase in soft agar colony formation as well as invasion ability compared to full length HIF-1α. Whether this difference is due to a longer half-life for the splice variant protein relative to the full length protein is currently under investigation.

HIF-1α loss of function experiments were carried out in the WM9 metastatic melanoma cell line. This cell line was chosen due to its high level of normoxic expression of HIF-1α. The WM9 is an aggressive metastatic melanoma cell line that has a high level of anchorage-independent growth and Matrigel invasion ability. We found a significant decrease in both anchorage-independent growth and Matrigel invasion upon silencing of normoxic expression of HIF-1α by siRNA treatment. These decreases were not due to a loss of cell viability as has been reported for knockdown of HIF-1α under hypoxic conditions (Kumar et al., 2007). This decrease in invasion might be due to decreased expression of HIF-1α regulated genes involved in invasion such as matrix metalloproteinase 2 (MMP2), urokinase plasminogen activator receptor (uPAR), and cathepsin D (Krishnamachary et al., 2003; Luo et al., 2006). Loss of
anchorage-independent growth in HIF-1α silenced cells may be due to ERK/MAPK, PI3K/Akt and HIF-1 pathway interactions. The PI3K/Akt pathway is one of the most critical pathways involved in anchorage-independent growth (Wang 2004).

Hypoxia independent expression of HIF-1α is thought to be regulated by growth signaling pathways (Semenza 2002). The majority of melanomas have constitutively active ERK1/2 MAPK pathway due to BRAF or N-Ras mutations (Omholt et al., 2003; Daniotti et al., 2004). In particular, human metastatic melanoma WM9 cells have a constitutively active ERK1/2 MAPK pathway most likely due to the V600E BRAF mutation found in these cells. Treatment of these cells with 30 μM of the selective MEK inhibitor, U0126, decreased ERK1/2 phosphorylation and also resulted in a time dependent decrease in HIF-1α protein expression.

This 30 μM concentration chosen for our initial studies was based on two other published papers that used this amount of U0126 to demonstrate the involvement of the ERK1/2 MAPK pathway in the regulation of HIF-1α (Kumar et al., 2007; Tisciuoglio et al., 2004). In the original report describing U0126, it was stated that the Ki for intracellular inhibition of ERK phosphorylation in COS-7 cells was 0.1 μM (Favata et al., 1998). Thus the concentration used in our study and others (Kumar et al., 2007; Tisciuoglio et al., 2004) is 300 times higher than the Ki. Therefore we repeated the MEK inhibition studies using 10 μM U0126. In contrast to 30 μM U0126, the lower concentration completely eliminated ERK1/2 phosphorylation after a 24 h incubation with WM9 human metastatic melanoma cells. Despite this inhibition of ERK1/2 phosphorylation, there was no change in the expression of HIF-1α protein. At the later time point of 48 and 72 h of inhibitor treatment the phosphorylation of ERK1/2 was not completely suppressed and the level of HIF-1α protein was decreased. We also inactivated ERK1/2 signaling by knocking down the expression of MEK. We needed to
use siRNA against both MEK1 and MEK2 in order to obtain a > 90% decrease in expression of these enzymes. Although this knockdown inhibited ERK phosphorylation, there was no decrease in HIF-1α protein expression at any time point assayed (up to 6 days). In the course of analyzing these data, we were informed by Promega, the manufacturer of U0126, that the compound is unstable in tissue culture media and produces metabolites that have poor MEK inhibitory activity. Considering the sum of our data, we hypothesize that the metabolites of U0126 are responsible for the decrease in HIF-1α protein levels. This would explain the lack of change in HIF-1α protein in cells treated for 24 h with 10 μM U0126 despite complete inhibition of ERK1/2 phosphorylation and the fact that siRNA knockdown of MEK resulting in decrease ERK1/2 phosphorylation also did not result in a decrease in HIF-1α protein levels.

TABLE 1: Genotyping data of melanoma cell lines

<table>
<thead>
<tr>
<th>Mutation</th>
<th>NRAS</th>
<th>WT/WT</th>
<th>V600E BRAF</th>
<th>V600D BRAF</th>
</tr>
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<tbody>
<tr>
<td>Sbcl2</td>
<td>++</td>
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<td></td>
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<tr>
<td>WM3211</td>
<td></td>
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<tr>
<td>WM1366</td>
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<tr>
<td>WM3248</td>
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<tr>
<td>WM9</td>
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<td>++</td>
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</tr>
<tr>
<td>WM239</td>
<td></td>
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<td>++</td>
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</tbody>
</table>

The N-RAS or BRAF mutational status of melanoma cell lines RGP (Sbcl2, WM3211), VGP (WM1366, WM3248), MM (WM9, WM239).

Analysis of BRAF and NRAS mutations in our human melanoma cell lines (Table 2.1)
shows that WM3211 cells do not have the common activating mutations in these genes, yet these cells express increased amounts of HIF-1α protein and mRNA under normoxic conditions. Overall our data suggest normoxic expression of HIF-1α is not regulated by the ERK1/2 MAPK pathway, *at least in the WM9 human metastatic melanoma cell line*. The hypoxia independent expression in melanoma cells, like other cancers, might be regulated by phosphotidylinositol 3-kinase (PI3K) (Tanaka et al., 2006; Stiehl et al., 2002), NFκB (Jung et al., 2003) or JAK/STAT (Niu et al., 2008) pathways.

In conclusion, HIF-1α is overexpressed, in melanoma cell lines under normoxic conditions in a manner that correlates with the aggressiveness of the tumor from which the cell line was established. We also show that the novel splice variant HIF-1α785, which is missing part of the oxygen regulation domain is overexpressed in these melanoma cell lines. Manipulation of HIF-1α expression in several of our melanoma cell lines suggests that this transcription factors regulates, in part, anchorage-independent growth and Matrigel invasion. Our results suggest that development of new therapeutic agents that inhibit HIF-1α function may be of use in the treatment of human melanoma regardless of the hypoxic condition of the tumor.

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

All authors significantly contributed to the design of the study, data and manuscript drafts. SSJ and CNM contributed equally in carrying out the experiments reported in this study. All
Acknowledgements

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Materials and methods

Cell lines and cell culture conditions

SbCl2, WM3211 (RGP), WM1366, WM3248 (VGP), and WM9, WM239 (Metastatic melanoma) cells were a generous gift from Meenhard Herlyn's lab at the Wistar Institute (University of Pennsylvania). All cells were grown in a humidified incubator with 5% CO₂ and 95% air at 37°C. The SbCl2 cells were cultured in MCDB153 media (Invitrogen, Carlesbad CA), supplemented with 2% fetal bovine serum, 5 μg/ml insulin (Sigma Chemical, St. Louis, MO), 1.68 mM CaCl₂, 100 units/mL penicillin streptomycin solution (Invitrogen Corp., Carlsbad CA). WM3211 cells were cultured similar to Sbcl2 media except for 5% fetal bovine serum and no CaCl₂. WM1366, WM3248, WM9 and WM239 cells were cultured in RPMI medium (Invitrogen Corp., Carlsbad CA) supplemented with 10% fetal bovine serum and 100 units/mL penicillin streptomycin solution. Normal human melanocytes (HEMn-LP) were derived from human foreskin (Cascade Biologics, Portland, OR) and maintained in Medium
254 supplemented with 50 mL HMGS (Cascade Biologics, Portland, OR) and 1 mL PSA solution (Cascade). Maintaining all cell lines in RPMI media for 48 h did not change their level of HIF-1α expression levels

*Western Blot analysis*

Nuclear extracts from each cell line were isolated using the NePER kit® (Pierce, Rockford, IL) according to the manufacturer's protocol. Protein concentration was determined using the BCA protein assay reagents from Pierce as per the manufacturer's instructions. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes using the BioRAD MiniProtean3® system. Equal loading was also determined by Ponceau staining of the nitrocellulose membranes. Blots were blocked using ChemiBlocker reagent (Chemicon, Temecula, CA) for 1 hr at room temperature and probed overnight at 4°C with anti-HIF-1α mouse monoclonal antibody at 1 μg/mL (R&D Systems, Minneapolis, MN), 1:1000 phospho-p44/42 MAPK (Cell Signaling Tech Inc, Danvers, MA), p44/42 MAPK (Cell signaling Tech Inc, Danvers, MA), total MEK1/2 (Cell Signaling Tech Inc, Danvers, MA), 1:2000 V5-HRP (Invitrogen, Carlsbad, CA), 1 μg/mL Lamin B1 or Lamin A (Abcam, Cambridge, MA). Monoclonal mouse secondary IgG antibody (GE Healthcare, Piscataway, NJ) or rabbit secondary IgG antibody (Cell signaling) conjugated with HRP was applied after two 1× TBS + 0.05% Tween (TBS-T) washes. Blots were incubated with secondary antibody at 1:3000 for 1 h at room temperature and subsequently washed 2× with 1× TBS-T. A final 5 min wash with TBS (no Tween) was performed just prior to incubating the blot with ECL reagent (GE Healthcare, Piscataway, NJ). Blots were then autoradiographed and the density of immunoreactive bands determined by a BioRad imaging system after correction by an internal
protein standard such nuclear lamin.

**RNA isolation and RT-PCR**

Total RNA was extracted using Tri-Reagent (Sigma Chemical Co., St. Louis, MO). The purified RNA samples were dissolved in RNase-free water and quantified by Nanodrop spectrophotometer (NanoDrop Technology, Inc., Wimington, DE). Each RNA sample had an A260/A280 ratio of 1.8 or above. The quality of RNA was determined on the Agilent 2100 Bioanalyzer, using the RNA 6000 Nano Assay kit (Agilent Tehcnologies, Wilmington, DE) and reverse transcribed using the RT-for-PCR kit (Clontech, Palo Alto, CA) as per manufacturer's instructions. Primers were designed to either amplify only HIF-1α or HIF-1α785 exclusively. HIF-1α primers excluded HIF-1α785 by targeting exon 11, which is absent in HIF-1α785. HIF-1α785 primers were designed to exclude HIF-1α by targeting the exon 10:12 boundary only present in HIF-1α785. Sequence of the HIF-1α forward primers were: 5'-AAAGTTCCACCTGAGCCTAAT-3', and reverse 5'-TAAGAAAAAGCTCAGTTAAC-3'. The sequence of HIF-1α785 forward primers were 5'-AAAGTTCCACCTGAGGACAC-3', and reverse 5'-TAAGAAAAAGCTCAGGTAAC-3'. Primers for the housekeeping gene control GAPDH were included in the Advantage cDNA kit®. Control primers amplifying a fragment of the control plasmid included in the Advantage cDNA kit® were used to ensure optimal PCR conditions. PCR was performed using thermocycler (Biometra Tgradient, Goettingen, Germany) conditions of 94°C for 1 min; 25 cycles of 94°C for 30 sec, 68°C for 4 min; 68°C for 5 min and 15°C soak.

For quantitative PCR, total RNA was extracted from the different cell lines. RNA was then converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems Inc.
qPCR analysis was performed using TaqMan probes for HIF-1α (ABI Catalog number Hs00936366) or HIF-1α785 (ABI Custom Primer Order) as well as β-actin (ABI Catalog number 4326315E). The reactions were performed under conditions specified in the ABI TaqMan Gene Quantitation assay protocol. Data was corrected for efficiency and loading using the Pfaffl method (Tichopad et al., 2003). Data is representative of at least 3 separate experiments.

DNA constructs

The pLenti-V5-D-TOPO vector was used in gain of function experiments in the SbCl2 radial growth phase human melanoma cells. HIF-1α or HIF-1α785 was cloned into this vector by amplifying these genes using primers specific for both the 5' and 3' ends of HIF-1α. The linearized pLenti-V5-D-TOPO Vector contains 5' GTGG overhangs at one end while the insert is Taq Amplified to contain a 5' CACC overhang at one end. Following amplification, 20 μL of the 50 μL PCR amplification reactions were separated on a 1% agarose gel stained with ethidium bromide to ensure that the correct size amplicon was present. HIF-1α and HIF-1α785 amplicons were purified from the remainder of the PCR reaction using the Zymo DNA Clean & Concentrate Kit® (Zymo Research Inc., Orange, CA). After ligation, plasmids were transformed into Stbl3 competent cells (Invitrogen Corp., Carlsbad, CA) and plated onto agar plates containing 100 μg/mL ampicillin. Plates were incubated at 37°C overnight and then colonies were screened for intact insert in the correct orientation. Plasmids were isolated from positive colonies and analyzed by DNA sequencing to ensure the correct plasmid expression construct. Control plasmid, pLenti-V5-LacZ, was supplied in the ViraPower kit® (Invitrogen Corp., Carlsbad, CA).
**Transient expression studies**

SbCl2 cells were transfected at ~80% confluence with either transfection reagent alone (mock), pLenti-LacZ, pLenti-V5-D-TOPO-HIF-1α, or pLenti-V5-D-TOPO-HIF-1α785 using FuGene 6 transfection reagent as per manufacturer's protocol (Roche, Palo Alto, CA). After 48 h protein was extracted and overexpression was confirmed by western blot as described above.

**siRNA inhibition of HIF-1α**

WM9 cells seeded into 6 well plates at $2.0 \times 10^5$ were treated 24 h after seeding with either 100 nM HIF-1α siRNA or 100 nM control non-targeting siRNA (Dharmacon, Inc. Lafayette, CO) using the RNAifect® transfection reagent (Qiagen, Inc.) as per the manufacturer's instructions. HIF-1α inhibition was confirmed by western blotting at 48, 72, 96, and 120 h after transfection. There was ~70% - 80% decrease in HIF-1α protein relative to control siRNA treated WM9 cells at each time point. Cell viability assays were performed using WM9 cells transfected with control siRNA or HIF-1α siRNA at 48, 72 and 96 h by the trypan blue exclusion method.

**Matrigel invasion assay**

SbCl2 cells overexpressing either HIF-1α, HIF-1α785, or LacZ for 24 h, or WM9 cells transfected with Control siRNA or HIF-1α siRNA for 48 h were seeded into 6-well Matrigel (+) chambers, and, as a control, 6-well Matrigel (-) chambers (BD Biosciences) at $7.0 \times 10^4$ cells per well. At 24 hours post-seeding, the Matrigel was removed from the chambers using a
cotton-tipped applicator. After all the Matrigel on the inner part of the chambers was removed, invading cells were fixed with 80% methanol for 5 minutes and then stained with 0.5% crystal violet for 5 minutes. After staining, the cells/chambers were extensively washed with distilled water. Once excess stain was removed, cells were manually counted using a grid system covering the entire lower surface of the chamber.

*Anchorage-independent growth assay*

CytoSelect 96 well Cell Transformation Assay® (Cell Biolabs, Inc.) was used to determine anchorage-independent growth of SbCl2 cells overexpressing either HIF-1α, HIF-1α785, or LacZ and WM9 cells transfected with Control siRNA or HIF-1α siRNA. Cells were seeded at a density of $1.0 \times 10^4$ into a 0.4% agar layer poured over a 0.6% agar layer in wells of a 96 well plate and incubated for 4-5 days as per manufacturer's instructions. Wells lacking cells served as a fluorescent blank control. Agar layers were solubilized, cells were lysed, and nucleic acid content stained with CyQuant dye. The amount of Cyquant dye in each well was determined using a fluorescent plate detector (Molecular Devices' Spectra Max GEMINI EM Microplate spectrofluorometer; Biocompare Inc., CA) at 485/520 nm. Anchorage-independent growth of WM9 cells transfected with Control siRNA or HIF-1α siRNA for 5 days was also confirmed by microscopic examination (20×).

*ERK inhibition*

WM9 cells were seeded into 10 cm dishes at a density of $5.0 \times 10^5$ and the next day were treated with either 30 or 10 μmol/L U0126 (Promega, Madison, WI), a MEK1/2-specific inhibitor to block ERK1/2 activation or vehicle (DMSO). ERK inhibition was verified by
western blotting at 24, 48 and 72 h using both total and phospho-specific ERK antibodies.

WM9 cells seeded into 6 well plates at $2.0 \times 10^5$ were treated 24 h after seeding with either 100 nM MEK1 and MEK2 siRNA or 100 nM control non-targeting siRNA (Dharmacon, Inc. Lafayette, CO) using the RNAifect® transfection reagent (Qiagen, Inc.) as per the manufacturer's instructions. Both MEK1 and MEK2 inhibition was confirmed by western blotting at 72 h after transfection. There was ~80 - 90% decrease in MEK1 and MEK2 protein relative to control siRNA treated WM9 cells at each time point.

Statistics

Statistical analysis of the data was performed using the student paired $t$ test or ANOVA as appropriate. The statistical test used for each data set is stated in the figure legends; $p < 0.05$ was considered to be significant. Error bars in all figures represent SEM.
 CHAPTER THREE

Hypoxia inducible factor-1α directly upregulates Microphthalmia-associated transcription factor expression in human melanoma and enhances cell survival

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Running title: HIF-1α regulates MITF expression.

Submitted for publication
Abstract

We have previously shown that human melanoma cells express hypoxia factor-1α (HIF-1α) under normoxic conditions. Gene expression profiling identified Microphthalmia associated transcription factor (MITF) as a target gene of HIF-1α produced under normoxic conditions. Knockdown of HIF-1α significantly reduced the expression of MITF mRNA and protein in metastatic melanoma cells. We analyzed the expression of MITF-A and –M in human melanocytes and melanoma cell lines. MITF-M mRNA was highly expressed in normal human melanocytes relative to all melanoma cell lines. MITF-A and –M expression was low in cells established from radial and vertical growth phase melanomas, but MITF-A was highly expressed in two different cell lines established from metastatic melanomas. Analysis of these data showed a distinct shift from MITF-M mRNA in melanocytes to predominantly MITF-A in the melanoma cells. Computer analysis of the MITF-A promoter revealed two hypoxia response elements. Mutation of these sites decreased MITF reporter gene activity in melanoma cells expressing high levels of HIF-1α. Chromatin immunoprecipitation revealed direct in vivo binding of HIF-1α to the MITF-A promoter in WM9 cells. Decreasing MITF levels using siRNA, resulted in a decrease in the number of viable WM9 cells. These treated cells also had increased amounts of annexin V and cleaved caspase 3. Overall our data suggest that MITF is a direct target gene of HIF-1α and its enhanced expression contributes to the survival of metastatic melanoma.

Keywords: HIF-1α; Microphthalmia associated transcription factor; melanoma; MITF-A; apoptosis
**Introduction**

Melanoma, a malignant tumor arising from melanocytes, is the most aggressive type of skin cancer and is typically resistant to chemo and radio therapy (Soengas and Lowe, 2003). Over the past decade, the incidence and mortality of melanoma has increased more rapidly than any other tumor types. Although significant progress has been achieved in understanding the mechanism responsible for melanoma development, the key factors that regulate progression of disease are not yet well defined.

Microphthalmia-associated transcription factor (MITF) is a basic helix-loop-helix leucine zipper dimeric transcription factor (Hodgkinson et al. 1993). MITF is a master regulator of melanocyte development and function. It regulates melanocyte development by inducing genes involved in differentiation such as those genes involved in melanogenesis. It also contributes to melanocyte cell survival by inducing genes involved in cell cycle progression and anti-apoptosis (Levy et al., 2006). The MITF family consists of various isoforms that differ in transcription initiation sites. MITF-A is the largest and the most ubiquitous form while the MITF-M is missing exon 1 and is selectively expressed in melanocytes.

Although, MITF modulates melanocyte differentiation, it is found to be amplified in 10-20% of aggressive melanoma (Levy et al., 2006). It also has an oncogenic role in human clear cell sarcoma (Davis et al., 2006). Analysis of MITF expression with melanoma tissue microarrays, showed it was highly expressed in 10–20% cases of metastatic melanomas. MITF gene amplification in metastatic melanoma is also associated with a decrease of five year
survival rate relative to melanomas having normal mitf gene copy number (Garraway et al., 2005). These findings have generated a hypothesis that MITF when highly expressed leads to differentiation and pigmentation in melanocytes, at intermediate and low levels leads to proliferation and invasion in melanoma, while its silencing could promote apoptosis in normal melanocytes and melanoblasts (Carriera et al., 2006).

Hypoxia-inducible factor-1 (HIF-1α) is a master regulator of O₂ homeostasis in cells. It consists of a heterodimeric transcriptional complex (HIF-1β and HIF-1α). Oxygen-dependent regulation is at the post-translational level and results in HIF-1α protein stabilization (Semenza, 2003). HIF-1α controls over 60 genes involved in many aspects of oncogenesis, including tumorigenesis, anti-apoptosis, angiogenesis and genetic instability.

Recently we have shown that HIF-1α is expressed in human melanoma cell lines grown in normoxic conditions. Further, we have found that normoxic expression of HIF-1α increased as a function of malignant progression (Mills et al., 2009). During the course of determining whether normoxic expression of HIF-1α stimulated different gene targets compared to hypoxic expression of HIF-1α we found MITF as a potential target gene of HIF-1α.

The objective of this study was to determine whether MITF is a direct target gene of HIF-1α. Also, we investigated the MITF isoforms expressed in human melanoma cell lines isolated from different stages of melanoma progression. Lastly, we determined whether enhanced MITF expression contributed to human melanoma growth and survival.
Results

Regulation of MITF by HIF-1α in melanoma cells

HIF-1α protein, a key regulator of oxygen homeostasis, is shown to be highly expressed in WM9 metastatic melanoma cells under normoxic conditions and our data suggest it contributes to some of its malignant characteristics (Mills et al., 2009). Subsequently, we used gene expression profiling to determine if different sets of genes were regulated by HIF-1α under normoxic vs hypoxic conditions. MITF was identified as a potential target gene of HIF-1α under normoxic conditions (data not shown). To confirm these findings, we used siRNA to knock down normoxic expression of HIF-1α in melanoma cells and examined the expression of MITF. WM9 cells treated with 100 nM HIF-1α siRNA had only 10-15% of the amount of HIF-1α protein found in control cells (Figure 3.1A). The expression of MITF mRNA (Figure 3.1B) as well as protein (Figure 3.1C) was downregulated in HIF-1α siRNA treated WM9 cells relative to control. To determine which MITF isoform, -A or -M, was regulated by HIF-1α in WM9 melanoma cells, we performed quantitative RT-PCR using probes specific for each transcript. We found MITF-A mRNA levels were downregulated > 50% and MITF-M mRNA levels were downregulated > 40% in HIF-1α siRNA treated WM9 cells relative to control cells (Figure 3.1D & E). These experiments suggest that the normoxic expression of HIF-1α increases MITF mRNA and protein levels in WM9 melanoma cells.
Figure 3.1 MITF mRNA and protein is regulated by Hif-1α in human melanoma cells. (A) WM9 cells were treated with either 100nM HIF-1α or control non-targeting siRNA for 72h and nuclear extracts were analyzed by western blot analysis to detect HIF-1α protein levels. Total Erk was used as a loading control. MITF mRNA expression was measured in HIF-1α siRNA treated cells relative to control siRNA treated WM9 cells by semi-quantitative RT-PCR (B) and MITF protein level were measured by western blot (C). GAPDH was used as a loading control for both RT-PCR and western blot. HemnLP cell extracts were used as a positive control for MITF expression. The amount MITF isoforms –A (D) and –M (E) mRNA in HIF-1α siRNA treated WM9 cells was determined by quantitative RT-PCR as described in methods. Data was corrected for efficiency and loading using the Pfaffl method (Trichopad et al., 2003). Data is expressed as fold change, corrected for 18S, relative to control siRNA treated cells. * P value < 0.01

MITF expression in melanoma cells

Although, MITF is known to modulate melanocyte differentiation, it is found to be
amplified in a fraction of melanomas (Levy et al., 2006). The MITF family consists of various isoforms whose expression patterns are not well defined in human melanoma cells relative to human melanocytes. In these studies we have examined the expression of MITF isoforms -A and -M in human melanocytes and various melanoma cell lines representing different stages of progression. They include the radial growth phase (RGP) Sbcl2 and WM3211, Vertical growth phase (VGP) WM1366 and WM3248 and metastatic (MM) WM9 and WM239. MITF-A mRNA levels were decreased in RGP cell lines and tend to increase as melanoma progresses from RGP to VGP to MM. They are highly expressed in the MM melanoma cell lines WM9 and WM239 relative to normal human melanocytes (Figure 3.2A). In contrast, MITF-M mRNA levels were high in melanocytes and low in all of the other melanoma cell lines we tested, irrespective of the stage of melanoma from which the cell lines was established (Figure 3.2B). When the data are expressed as a ratio of MITF-A to –M it clearly shows a shift in the mRNA expression from MITF-M to –A in melanoma cell lines relative to melanocytes which had a ratio of only 0.2 (Figure 3.2C). We also examined the expression pattern of total MITF protein in all these cell lines and found that MITF protein was highly expressed in melanocytes and down regulated in RGP and VGP melanoma cell lines while expression was elevated in MM melanoma relative to RGP and VGP cell lines (Figure 3.2D).
Figure 3.2 MITF-A and -M expression in human melanoma cells. Total RNA was extracted from HEMn-LP, WM3211, SbCl2, WM3248, WM1366, WM239 and WM9 cells. RNA was reverse transcribed and quantitative PCR was performed using TaqMan probes directed at MITF-A (A), MITF-M (B) and 18S. Data was corrected for efficiency and loading using the Pfaffl method (Trichopad et al., 2003). Data is expressed as fold change, corrected for 18S, relative to HEMn-LP. (C) The ratio of MITF-A to –M mRNA levels for all cell lines stated above was calculated using the data obtained from (A) & (B). (D) Nuclear extracts from HEMn-LP, WM3211, SbCl2, WM3248, WM1366, WM239 and WM9 cells were subjected to western blotting to detect MITF protein expression. GAPDH was used as a loading control.

*HIF-1α binds and transactivates the MITF-A promoter in melanoma cells*

To elucidate the underlying mechanism by which HIF-1α regulates MITF expression in melanoma cells, we performed MITF-A promoter activity assays. The MITF-A promoter
region containing two hypoxia response elements (HRE) was cloned upstream of a luciferase reporter gene as described in the Methods section and then transfected into MM melanoma cell lines. The MITF-A promoter activity, monitored by luciferase levels was increased by more than 80 and 20 folds in WM9 and WM239 melanoma cells respectively compared to cells transfected with the luciferase reporter gene without the MITF-A promoter. When we decreased HIF-1α expression in WM9 and WM239 cells the MITF-A promoter deriven luciferase levels were significantly reduced by more than 50% (Figure 3.3B & C). To determine whether HIF-1α regulates MITF-A promoter activity through the HRE’s present in the promoter region of MITF-A, we generated two mutant plasmids Mut1 & 2, containing a mutated sequence of either one or both of the HRE’s. Transfection of these mutated constructs into the WM9 cells showed that MITF-A promoter activity decreased relative to WT promoter. The reduction in Mut2 promoter activity relative to the wild type MITF-A promoter activity was significantly different (P Value < 0.05) (Figure 3.3D).
Figure 3.3 MITF-A promoter activity in WM9 melanoma cells. (A) A schematic representation of the MITF locus illustrates that MITF-A & -M use two different promoters. MITF-M starts transcription from exon 2 and uses the promoter region between exon 1 & 2 (Davis et al., 2006; Levy et al., 2006). MITF-A uses the promoter region upstream of exon 1 and this region also contains two hypoxia response elements (HRE). The sequence of the MITF-A promoter region (highlighted grey) containing both HRE (bold) was cloned into a luciferase reporter plasmid from Panomics. WM9 (B) and WM239 (C) cells were treated with either 100nM HIF-1α or control non-targeting siRNA. After 24h of siRNA treatment, cells were subjected to transfection of the luciferase reporter plasmid containing either the MITF-A promoter or an empty vector. Cells were co-transfected with a β-gal expression plasmid to correct for transfection efficiency. At 48h post transfection, cell extracts were assayed for Luciferase and β-galactosidase activity. Data is expressed as relative fluorescence units (RFU) and luciferase activity is corrected for β-galactosidase activity. (D) WM9 cells were transfected with the luciferase reporter plasmid containing either no MITF promoter sequence (Control), wild type MITF-A promoter (MITF-A WT) or an MITF-A promoter containing a mutation in the downstream (MITF-A mut1) or mutations in both HRE elements (MITF-A mut2). Cells were co-transfected with β-gal to correct the transfection efficiency and 48h post transfection cell extracts were assayed for Luciferase and β-galactosidase activity. Data is expressed as relative fluorescence units (RFU) and luciferase activity is corrected for β-galactosidase activity. * P Value < 0.01 and # P Value < 0.05
To demonstrate *in vivo* binding of HIF-1α to the MITF-A promoter region in melanoma cells, we performed a chromatin immunoprecipitation assay. Chromatin complexes were immunoprecipitated from WM9 cells by using a CHIP grade HIF-1α antibody and PCR was performed using specific primers to the human MITF-A promoter region. As shown in Figure 3.4 (upper panel), we detected a specific amplification of the MITF-A promoter region in HIF-1α immunoprecipitated genomic DNA sample. There was no or very little amplification observed in IgG and no antibody samples. To validate our immunoprecipitation technique, we used an RNA polymerase antibody to immunoprecipitate genomic DNA from WM9 cells as a positive control and specific primers were used to amplify the human GAPDH promoter region. We detected a specific amplification of the GAPDH promoter region in the RNA polymerase immunoprecipitated genomic DNA sample, while there was no amplification observed in IgG and no antibody samples (Figure 3.4 down panel). Collectively these data indicate that HIF-1α protein regulates MITF-A promoter activity by directly binding to the proximal region of its promoter in WM9 melanoma cells.
**Figure 3.4 HIF-1α binds to the MITF-A promoter.** Chromatin immunoprecipitations were performed on extracts from WM9 melanoma cells using a specific anti-HIF-1α antibody and primers spanning the MITF-A promoter region. Magna ChiP™ G kit (Millipore Billerica, MA) was used and for more details refer to methods. A control PCR amplification was performed using genomic DNA from WM9 cells, which showed a 500 bp band corresponding to the amplification of the MITF-A promoter regions. The lower panel was a positive control for immunoprecipitation in which human GAPDH promoter region (150bp) was amplified only when RNA polymerase antibody or DNA input was used while no band was observed in no antibody or IgG antibody cells.

**MITF expression as a cell survival factor in melanoma**

MITF was highly expressed in the WM9 human metastatic melanoma cell lines. To determine whether MITF could be contributing to the biologic properties of these cells, we knocked down its expression and examined how this affected cell replication. Treatment of WM9 cells with 100 nM MITF siRNA consistently decreased its expression by ~65% relative to a control siRNA (Figure 5B & C). Knockdown of MITF expression resulted in a significant decrease in the number of trypan blue excluding cells relative to control siRNA treated WM9 cells (Figure 5A). These data suggest that MITF could be responsible for cell survival in WM9 melanoma cells. To further define MITF’s role in cell survival, we measured the expression of the pro-apoptotic markers cleaved caspase-3 and Annexin V. Knockdown of MITF expression in WM9 cells increased the amount of cleaved caspase-3 (17 & 19 kDa) relative to control siRNA (Figure 6A). The levels of early (Annexin V^+ve / 7-AAD^-ve) and late (Annexin V^+ve / 7-AAD^+ve) apoptotic markers were significantly higher in MITF siRNA treated WM9 cells relative to control siRNA (Figure 6B & C). Overall these data suggest MITF is involved in the cell survival of WM9 melanoma cells.
Figure 3.5 Decreased MITF expression in human metastatic melanoma WM9 cells inhibits cell proliferation and viability. (A) WM9 cells were transfected with control siRNA or MITF siRNA and cell viability assays were performed by counting number of tryphan blue excluding cells on 2\textsuperscript{nd}, 4\textsuperscript{th} and 6\textsuperscript{th} day. (B) Knock down of MITF expression in WM9 cells was confirmed by western blot on the 5\textsuperscript{th} day of post transfection. (C) Densitometric analysis of MITF protein expression for the western blot showed in (B). \# P Value < 0.01 and * P Value = 0.055
Figure 3.6 Decreased MITF expression in human metastatic melanoma WM9 cells increases apoptosis. (A) Cytoplasmic extracts from WM9 cells transfected with either control siRNA or MITF siRNA were analyzed by western blotting for cleaved caspase-3. GAPDH was used as loading control. WM9 cells transfected with either mock or control siRNA or MITF siRNA were labeled with annexin V pacific blue and 7-AAD and subjected to flowcytometric analysis. The percentage of WM9 cells positive for annexin-V and negative for 7-AAD is defined as early apoptotic, (B) while percentage of WM9 cells positive for both annexin-V and 7-AAD is defined as late apoptotic (C). * P Value < 0.01
Discussion

HIF-1α is the master regulator of cellular and systemic adaption to hypoxia and controls the expression of genes that are involved in crucial aspects of tumor biology, including angiogenesis, glycolytic metabolism and cell survival (Semenza, 2003). HIF-1α protein is rapidly degraded under normoxic conditions and is stabilized under hypoxia. Recently, we (Mills et al., 2009) and others (Kuphal et al., 2010) have shown that HIF-1α is expressed under normoxic conditions in human melanoma cells and its expression increases in cells isolated from more advanced stages of this disease. Further, we have shown that the normoxic expression of HIF-1α in human melanoma contributes to its malignant characteristics (Mills et al., 2009). In an attempt to distinguish genes regulated by HIF-1α under normoxic conditions compared to hypoxic conditions, we discovered MITF as a candidate gene stimulated by HIF-1α. Thus, the major focus of this study was to characterize MITF as a novel, directly transactivated gene of HIF-1α. Also, we have identified differential expression of MITF-A and -M isoforms in human melanoma relative to normal human melanocytes.

We demonstrate for the first time that the normoxic expression of HIF-1α regulates MITF at the transcriptional level and leads to increased expression of MITF mRNA and protein. Transcription factor binding site analysis of the promoters within the MITF gene identified two HRE’s upstream of exon 1. These HRE’s were present only in the promoter region of the MITF-A isoform while no HRE was found in the MITF-M promoter region located between the first and second exons. This information suggests that MITF-A is the isoform regulated by HIF-1α. However, quantitative RT-PCR showed mRNA expression of both isoforms MITF-A and -M decreased when HIF-1α expression was knocked down. It is not
clear how HIF-1α might also regulate MITF-M. Future experiments will need to test MITF-M promoter activity in melanoma cells where HIF-1α expression can be experimentally increased or decreased.

MITF-A promoter activity containing both HRE’s showed increased luciferase activity in both MM cell lines WM9 and WM239. This activity was significantly inhibited when HIF-1α expression was knocked down. When both MITF-A HRE sequences were mutated the luciferase activity was significantly inhibited. However the degree of inhibition was 33% compared to a 50% decrease in reporter gene activity when HIF-1α expression was inhibited. The reason for this could be that the four nucleotides we mutated were not sufficient to completely block the promoter binding of HIF-1α and the possibility of loose binding still exists. To obtain maximal inhibition of HIF-1α binding to the MITF-A promoter, a broader region of the promoter may need to be mutated. Chromatin immunoprecipitation results showed the direct endogenous binding of HIF-1α to the MITF-A promoter. Collectively these data provide convincing evidence that MITF-A is a direct target gene of HIF-1α.

MITF is a large family consisting of various isoforms that differ in transcription initiation sites. MITF-A is the largest and most ubiquitously found isoform while MITF-M is the smallest and is missing exon one. MITF-M is selectively expressed in melanocytes and its role in differentiation, pigmentation and melanocytic survival has been documented in many reports (Levy et al., 2006). MITF expression is variable among melanoma specimens. In some cases, it was shown to be more highly expressed in early stage relative to late stage melanoma (Salti et al., 2000; Selzer et al., 2002). However, MITF is reported to be highly expressed in 10-20% of advanced melanoma cases and was associated with decreased survival (Garraway et al., 2005). Recently, MITF has been identified as a marker in the proliferative signature of
melanoma cells that were less motile than invasive cells (Hoek et al., 2008). In another study MITF was identified as an oncogene in melanoma specimens representing a transition point for tumor progression and metastasis (Riker et al., 2008). Because of these opposing findings, there is controversy regarding the role of MITF in melanoma development and progression. In many of these studies the MITF isoforms being measured was not determined. Here, we have examined the expression of MITF isoforms A and M in melanoma cell lines representing different stages of melanoma progression relative to melanocytes. Both MITF-A and –M mRNA are expressed in melanocytes with MITF-M more highly expressed relative to –A. MITF-A and -M mRNAs are both downregulated in RGP and VGP melanoma cell lines, but significantly higher expression of MITF-A was found in the metastatic melanoma cell lines. This pattern of MITF-A expression correlates with the significantly higher normoxic expression of HIF-1α in these metastatic melanoma cell lines (Mills et al., 2009). MITF-A mRNA has been shown to be expressed in B16 melanoma cells (Murakami et al., 2007). In the study documenting mitf gene amplification in 10-20% of metastatic melanomas (Garraway et al., 2005), the PCR primers used to confirm elevated MITF mRNA expression in the clinical specimens were not specifically designed to detect the MITF-M isoform and could also detect MITF-A expression. We speculate that MITF-A mRNA might be expressed from those melanomas containing amplification of the mitf gene.

MITF regulates melanocyte development, function and survival by modulating the expression of genes involved in differentiation and cell cycle progression (Levy et al., 2006). In melanoma, MITF, in addition to targeting genes involved in cycle progression, also stimulates genes involved in anti-apoptosis and cell motility. In mouse melanoma cells, HIF-1α is identified as a direct target of MITF and was shown to be involved in pro-survival (Busca et
al., 2005). In this study, MITF targeting HIF-1α was activated by exogenous stimulation of the α-MSH/cAMP pathway. This pathway is known to transactivate MITF-M through a cAMP-response element (CRE) located at promoter region between exon one and two (Price et al., 1998; Levy et al., 2006). However, MITF-M is not highly expressed nor is the α-MSH/cAMP pathway exogenously activated in our metastatic melanoma cells. Thus, this information suggests that MITF-M is not involved in normoxic expression of HIF-1α in our human metastatic melanoma cell lines.

We found that knock down of MITF expression in WM9 metastatic melanoma cells led to a decrease in the number of viable cells. Our subsequent finding that knockdown of MITF expression resulted in increased annexin V binding and increased amounts of cleaved caspase 3 suggest that the decrease in viable cells was due to an increase in apoptosis. Although, MITF inhibits melanoma tumor xenograft (Selzer et al., 2002) and cell growth by activating cell cycle inhibitors INK4 (Loercher et al., 2005) as well as p21 CIP1 (Carreira et al., 2005), there is increasing evidence for its role in cell survival. Recently, MITF has been shown to up-regulate, the anti-apoptotic gene BCL2 (McGill et al., 2002) and to promote melanoma cell growth by stimulating CDK2 expression (Du et al., 2004). Targeting MITF through direct injection of siRNA into tumors drastically reduced outgrowth of B16 melanomas in syngenic mice by inducing apoptosis (Nakai et al., 2007). This latter study, together with our findings, suggests that in some melanomas MITF is a pro-survival factor. The fact that decreasing MITF in some melanoma cells leads to apoptosis is reminiscent of the phenomenon of “oncogene addiction” (Weinstein, 2002; Weinstein and Joe, 2006). We speculate that the switch from predominantly MITF-M in melanocytes to predominantly MITF-A in some metastatic melanomas may contribute to their anti-apoptotic phenotype. In summary we have shown that normoxic
expression of HIF-1α in more aggressive human melanoma cells activates the direct expression of the MITF-A gene and that expression of MITF-A contributes to melanoma cell survival. It will be interesting to determine whether MITF-A has a slightly different set of target genes, perhaps more in pro-survival pathways compared to MITF-M.

Conflict of interest

The authors declare that they have no conflict of interest.

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Materials and methods

Cell lines and cell culture conditions

SbCl2, WM3211 (RGP), WM1366, WM3248 (VGP), and WM9, WM239 (Metastatic melanoma) cells were a generous gift from Dr. Meenhard Herlyn’s lab at the Wistar Institute (University of Pennsylvania). All cells were grown in a humidified incubator with 5% CO2 and 95% air at 37°C. The SbCl2 and WM3211 cells were cultured in MCDB153 media (Invitrogen, Carlesbad CA) as described in (Mills et al., 2009). WM1366, WM3248, WM9 and WM239 cells were cultured in RPMI medium (Invitrogen Corp., Carlsbad CA) supplemented with 10% fetal
bovine serum and 100 units/mL penicillin plus streptomycin solution. Normal human melanocytes (HEMn-LP) were derived from human foreskins (Cascade Biologics, Portland, OR) and maintained in Medium 254 supplemented with 5 mL human melanocyte growth supplements (HMGS) (Cascade Biologics, Portland, OR) and 1 mL PSA solution (Cascade) (Mills et al., 2009).

Western Blot analysis

Nuclear extracts from each cell line were isolated using the NePER kit® (Pierce, Rockford, IL) according to the manufacturers protocol. Protein concentration was determined using the BCA protein assay reagents from Pierce as per the manufacturer’s instructions. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes using the BioRAD MiniProtean3® system. Equal loading was also determined by Ponceau staining of the nitrocellulose membranes. Blots were blocked using 5% non-fat dry milk for 1 hr at room temperature and probed overnight at 4°C with either anti-HIF-1α mouse monoclonal antibody at 1 ug/mL (R&D Systems, Minneapolis, MN), 1:1000 p44/42 MAPK (Cell signaling Tech Inc, Danvers, MA), 1 ug/mL MITF (C5) (Abcam, Cambridge, MA), GAPDH (Chemicon, Billerica, MA), or titer Cleaved caspase 3 (Cell signaling Tech Inc, Danvers, MA). Monoclonal mouse secondary IgG antibody (GE Healthcare, Piscataway, NJ) or rabbit secondary IgG antibody (Cell signaling) conjugated with HRP was applied after two 1 x TBS + 0.05% Tween (TBS-T) washes. Blots were incubated with secondary antibody at 1:3000 for 1 h at room temperature and subsequently washed 2 x with 1 x TBS-T. A final 5 minute wash with TBS (no Tween) was performed and immunoreactive bands were detected using the ECL system (GE Healthcare, Piscataway, NJ).
RNA isolation and RT-PCR

Total RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA). The purified RNA samples were dissolved in RNase-free water and quantitated using a Nanodrop spectrophotometer (NanoDrop Technology, Inc., Wilmington, DE). Each RNA sample had an A260/A280 ratio of 1.8 or above. The quality of RNA was determined on the Agilent 2100 Bioanalyzer, using the RNA 6000 Nano Assay kit (Agilent Tehenologies, Wilmington, DE) and reverse transcribed using the RT-for-PCR kit (Clontech, Palo Alto, CA) as per manufacturer’s instructions. Primers were designed to amplify only MITF-A. MITF-A primers were designed to target exon 1, which is absent in MITF-M. The sequences of MITF-A primers were: forward primers **5’- ATGCAGTCCGAATCGGGGAT -3’**, reverse **5’- TGCCCTGTTTTGCTCTTCAA -3’**. Primers for the housekeeping gene control GAPDH were included in the Advantage cDNA kit®. Control primers amplifying a fragment of the control plasmid included in the Advantage cDNA kit® were used to ensure optimal PCR conditions. PCR was performed using thermocycler (Biometra Tgradient, Goettingen, Germany) conditions of 94°C for 1min; 29 or 32cycles of 94 °C for 30sec, 55 °C for 30sec, 68 °C 1min; 68 °C for 7min and the PCR product stored at 4°C.

For quantitative PCR, total RNA was extracted from HEMn-LP, WM3211, SbCl2, WM3248, WM1366, WM239 and WM9 cells. RNA was then converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems Inc. (ABI), Foster City, CA). qPCR analysis was performed using TaqMan probes for MITF-A (ABI Catalog number Hs01115553_m1) or MITF-M (ABI Catalog number Hs00165156_m1) as well as 18S (ABI Catalog number 4310893E). The reactions were performed under conditions specified in the ABI TaqMan Gene
Quantitation assay protocol. Data was corrected for efficiency and loading using the Pfaffl method (Trichopad et al., 2003).

**siRNA inhibition of HIF-1α and MITF**

WM9 cells were seeded (2.0 x 10^5 cells/well) into 6 well plates and treated 24h after seeding with either 100nM HIF-1α siRNA, or 100nM MITF siRNA or 100nM control non-targeting siRNA (Dharmacon, Inc. Lafayette, CO) using the RNAiFect® transfection reagent (Qiagen, Inc.) as per the manufacturer’s instructions. Decreased levels of HIF-1α and MITF proteins was confirmed by western bloting.

**DNA constructs, Transfections and Luciferase reporter assay**

The pTL-Luc luciferase reporter vector (Panomics, Affymetrix, Inc. Fremont, CA) was used in luciferase reporter assay experiments following transfection into WM9 metastatic melanoma cells. The MITF-A promoter region (Figure 3.3A) was amplified and cloned into this plasmid by using the following primers: forward 5’-AAAAGCTAGCCTCCTCGTTGTTCCAATCC – 3’ and reverse 5’ –AAAAAGATCTGAGTTTACACTCGCACCCGG – 3’ containing Nhe1 and Bgl2 restriction enzyme sites that are also present in the pTL-Luc vector. To amplify the MITF-A promoter region, genomic DNA isolated from WM9 cells was used as a template. Following amplification, 20uL of the 50uL PCR reaction was separated on a 1% agarose gel and stained with ethidium bromide to ensure the correct size amplicon was present. The remainder of amplified MITF-A promoter and the pTL-Luc vector were digested with Nhe1 and Bgl2 restriction enzymes and purified using the Qiagen PCR/DNA kit (Qiagen, Valencia, CA). The linearized pTL-Luc Vector
and digested PCR product of MITF-A promoter were ligated by the One shot ligation kit (Roche Applied bioscience. Indianapolis, IN) and then plasmids were transformed into Top10 competent cells (Invitrogen Corp., Carlesbad, CA) and plated onto LB agar plates containing 100ug/mL ampicillin. Plates were incubated at 37°C overnight and then colonies were screened for intact insert in the correct orientation. Plasmids were isolated from positive colonies and analyzed by DNA sequencing to verify that we had obtained the correct MITF promoter sequence. For mutation of HRE elements in the MITF-A promoter, we used a site-directed mutational kit (Strategene, La Jolla, CA) and generated mutant clones as per manufacturer’s protocol. The mutation primers were: upstream 5', -TAAAATAAAGCAGTGGACACCCCTGCAGATGTCTGAGC – 3’ and downstream HRE is 5’ - TGGGAGCTGTAGTTTTACACGGAGCGGCTCCCCAGG – 3’. Mutant 1 (Mut 1) had only downstream HRE sequence mutated as only one primer i.e., downstream HRE primer was used while Mutant 2 (Mut 2) had both upstream as well as downstream HRE sequences were mutated as both upstream and downstream HRE sequences were used.

WM9 cells in 6 well plates were transfected at ~80% confluence with 5ug of pTL-Luc, pTL-Luc-MITF-A, pTL-Luc-MITF-A Mut1 pTL-Luc-MITF-A Mut2 or mock (no plasmid) using FuGene 6 transfection reagent as per manufacturer’s protocol (Roche, Palo Alto, CA). 1ug of pSV-βGal (Promega, Madison, WI) was cotransfected with the reporter plasmids to correct for transfection efficiency in the reporter assays. Cells were harvested 48h after transfection by passive lysis buffer (Promega, Madison, WI), incubated for 15min at RT, scraped from the wells and transferred to microcentrifuge tubes on ice. Cells were centrifuged at 12000xg for 2min at 4°C and the supernatant collected. Cell extracts were assayed for Luciferase and β-galactosidase activity by appropriate kits from Promega. Luciferase assays were evaluated in the linear range
and values were normalized to β-galatosidase activity. All transfections were performed in triplicate dishes and the experiments were repeated three times.

**Chromatin immunoprecipitation assay (ChIP)**

WM9 cells were grown to ~80-90% confluency in 150mm dishes. For ChIP assay 2x10^6 cells per ml were used. Chip assay was performed by using Magna ChIP™ G kit (Millipore Billerica, MA) and the procedure was followed as per manufacture’s instruction. DNA was crosslinked by treating cells with 1% formaldehyde for 10 min at RT. The reaction was then neutralized by addition of glycine. Cells were harvested in lysis buffer and chromatin sheared to 200-1000 base pairs in length by sonication 6 times in 15sec pulses with a rest of 50sec between pulses. The sheared chromatin was then immunoprecipitated with or without a specific monoclonal antibodies to HIF-1α or RNA polymerase. After immunoprecipitation, the cross-link was reversed by heat treatment (62°C for 2hr and proteinase K digestion). The captured genomic fragments were then recovered by DNA purification spin columns. Identification of the captured MITF-A or GAPDH promoter fragments was performed by PCR analysis using the promoter primers previously described for MITF-A or GAPDH promoter primers that were provided with the kit. PCR reactions were performed for 30 cycles and the amplified products were analyzed on a 1% agarose gel.

**Cell viability assay**

Cell viability assays were performed using WM9 cells transfected with control siRNA or MITF siRNA at the 2nd, 4th and 6th day post transfection using the trypan blue exclusion method.
**Apoptotic assay**

To evaluate for apoptosis, WM9 cells 72h post treatment with mock (no siRNA), control siRNA (non-targeting sirNA) or MITFsiRNA were trypsinized and pelleted after counting. The cells were resuspended in 1X binding buffer and 1 x 10^5 cells were used for the assay. The cells were stained with annexin-V-pacific blue (Invitrogen Corp., Carlesbad, CA) and 7-amino-actinomycin D (7-AAD; BD Pharmingen, Franklin lake, NJ) as per manufactures instruction mentioned in Annexin-V Apoptosis detection kit (BD Pharmingen, Franklin lake, NJ). A minimum of 10,000 events per sample was then acquired on a BD FACS flow cytometer and data analysis was done using Flow Jo software v.7.5.5 (Treestar, Ashland, OR). The percentage of WM9 cells positive for annexin-V and negative for 7-AAD was defined as early apoptotic while the percentage of WM9 cells positive for both annexin-V and 7-AAD was defined as late apoptotic. In addition, cleaved caspase-3 was also used as marker of apoptosis and was measured as described under the section on western blotting.

**Statistics**

Statistical analysis of the data was performed using the student paired t test or ANOVA as appropriate. The statistical test used for each data set is stated in the figure legends; \( p < 0.05 \) was considered to be significant. Error bars in all figures represent SEM.
CHAPTER FOUR

OVERALL DISCUSSION AND FUTURE DIRECTIONS

Melanoma is a malignant tumor that arises from melanocytes. Melanoma incidence is increasing more rapidly than any other type of tumor and accounts for most skin cancer related deaths (Melanoma Research Foundation). Melanoma if diagnosed early could be treated with high success rate but in later stages is notoriously resistant to both chemo- and radio-therapy (Soengas and Lowe 2003). Although, the mechanism responsible for the development of melanoma is beginning to be elucidated, the factors that play a role in the progression of melanoma are not yet established.

HIF-1α protein, a key regulator in adapting cells to hypoxia, is known to be degraded under normoxic conditions and stabilizes only under hypoxia. Upon stabilization HIF-1α transactivates genes that play a role in angiogenesis, anaerobic metabolism (Semenza 2003) and other processes which help the cell survive under adverse conditions. Thus, HIF-1α is thought to be a regulator of cancer progression. In this study we have found that hypoxia-independent expression of HIF-1α protein in melanoma cells contributes to in vitro malignant properties. In addition we have found that MITF, a melanocyte specific protein (Levy et al., 2006), is a novel direct target gene of HIF-1α in melanoma cells and its high expression appear to play role in melanoma cell survival.

HIF-1α mRNA as well as protein expression increased from RGP to VGP and VGP to metastatic melanoma cell lines, while it was undetected in normal human melanocytes.
Normoxic expression has been found in several other non melanoma types of cancer (Tanaka et al., 2006; Lee et al., 2007). Subsequent to publication of Chapter one (Mills et al., 2009), another laboratory confirmed the normoxic expression of HIF-1α in malignant melanoma cells and this expression was associated with an increase in ROS activity (Kuphal et al., 2010). The major distinguishing factor for normoxic versus hypoxic expression of HIF-1α is that normoxic regulation is at the transcriptional level, while hypoxic is at the translational level (Semenza 2003). The increase in expression of HIF-1α in melanoma cells from RGP to VGP and VGP to MM could be an important and essential factor for the progression of melanoma.

We have also found the expression of an mRNA splice variant, HIF-1α785, at higher levels, relative to full length HIF-1α, in more advanced melanoma cell lines. The HIF-1α785 variant is missing a part of ODD domain due to lack of exon 11 and the protein encoded by this mRNA is thought to be more stable under normoxic condition in comparison to full length HIF-1α (Chun et al., 2003; Lin et al., 2004). In non-melanoma cell lines HIF-1α785mRNA levels were found to be regulated by stimulation of cells with phorbol ester and involved a redox-dependent ERK1/2 MAPK pathway (Chun et al., 2003). The mechanism responsible for the increase of spliced 785 isoform of HIF-1α in melanoma is not known. The arginine-serine-rich splicing factor protein (ASF/SF2/SFRS1/SR proteins) controls splicing events in various proto-oncogenes and tumor suppressors frequently modify their cellular activity (Ward and Cooper 2010). Many SR proteins are up-regulated in number of cancer cell types (Fischer et al., 2004; Karni et al., 2007). Over expression of SF2/ASF has been shown to transform immortalized fibroblast cells and these transformants form sarcomas in nude mice (Karni et al., 2007). In another study SF2/ASF are shown to regulate the switch from the anti-angiogenic VEGFb isoform to angiogenic VEGF isoform by altering splicing sites (Qiu et al., 2009). Thus,
such splicing factors could be responsible for the increased expression of the splice variant HIF-1α785 in melanoma cells.

To further investigate the biological effects of HIF-1α expression on melanoma biology, we took the approach of gain of function and loss of function studies. In the former case, we choose a low HIF-1α expressing SbCl2 (RGP) cell line and transiently over expressed HIF-1αFL and 785. These cell lines are also known to have limited ability to form soft agar colonies and to invade through Matrigel. Gain of function studies in low HIF-1α expressing RGP cells revealed that overexpression of full length and 785 HIF-1α induced a small increase in anchorage-independent growth, but much larger and statistically significant increase in matrigel invasion. The smaller increase in anchorage independent growth could be limited by transient over expression of HIF-1α in RGP cells, which last for not more than 48 hours and the requirement of this assay is for duration of 5 days. This could be further investigated by stably expressing HIF-1α in RGP cells and testing their ability to increase anchorage-independent growth. Another explanation could be that HIF-1α alone might not be sufficient, and other factors or genetic changes are also necessary to acquire anchorage-independent growth characteristics in RGP cells. The overexpression of stable HIF-1α under normoxic conditions promotes anchorage-independent growth in melanocytes having an activated AKT signaling pathway (Bedogni et al., 2008).

RGP cells overexpressing HIF-1α785 splice variant had a slight increase in both soft agar colony formation and Matrigel invasion relative to full length HIF-1α. This difference might be due longer half life of the splice variant 785 compare to full length HIF-1α under normoxic condition (Chun et al., 2003; Lin et al., 2004). Although, the splice variant is thought to be more stable compared to full length under normoxic conditions, the protein stability of 785 has
never been investigated. In future I would like to measure the half life of the 785 protein relative to full length HIF-1α under non-hypoxic condition which we think might be a major contributor to the total expression of HIF-1α protein in melanoma cells.

In the loss of function study we choose the high HIF-1α expressing WM9 metastatic melanoma cell line. This cell line has a high ability to form colonies in soft agar and to invade through Matrigel. Loss of function studies in high HIF-1α expressing metastatic melanoma cells revealed that loss of HIF-1α significantly diminished their ability to form colonies in soft agar and to invade Matrigel compared to control cells. HIF-1α regulates genes involved in invasion and metastasis (Krishnamachary et al., 2003; Luo et al., 2006) which might account for HIF-1α-mediated Matrigel invasion of WM9 cells. Twist, a developmental protein, is a master regulator of epithelial-mesenchymal transition that is an essential step required by cells to invade into the surrounding tissue (Yang et al., 2004). Twist was shown to be a direct target gene of HIF-1α in head and neck cancer cells and correlates with the higher incidence of cancer metastasis (Yang et al., 2008). In my microarray analysis, Twist was found to be regulated by HIF-1α in WM9 cells (data not shown).

The normoxic expression of HIF-1α and its biological role in melanoma progression described here are based on cell culture studies and these findings need to be translated to in vivo models. The stable overexpression of HIF-1αFL or its 785 splice variant in RGP cells should increase the potential of these cells to invade and metastasize when injected in immunocompromised nude mice. The inducible knockdown of HIF-1α expression in metastatic melanoma cells should decrease the potential of these cells to invade and metastasize when tumor bearing mice bearing are treated with the inducing agent. Further, the normoxic expression of HIF-1αFL and 785 splice variant mRNA could be examined in different stages of
tumor tissues removed from patient with melanoma. This analysis is complicated, however, by the probability that certain regions of the tumor may have been hypoxic. These experiments will further suggest that inhibiting HIF-1α expression might be beneficial in treatment of melanoma patients.

The dietary component curcumin has been shown to inhibit hypoxia-stimulated *in vitro* angiogenesis and to downregulate HIF-1α and VEGF mRNA expression in endothelial cells (Bae *et al.*, 2006). It will be interesting to test whether curcumin could inhibit normoxic expression of HIF-1α in melanoma cells. Additional dietary constituents such as quercetin, epigallocatechin gallate (EGCG), resveratrol, inositol, etc. have been reported to affect HIF-1α expression or function in different types of cancer cells (Zang *et al.*, 2006; Wu *et al.*, 2008; Gu *et al.*, 2009) and could be tested to see if they also reduce full length and or 785 HIF-1α mRNA expressions in melanoma cells.

Hypoxia-independent expression of HIF-1α is thought to be regulated by the ERK-MAPK and PI3K pathways (Semenza 2003). Most melanomas have constitutively active ERK1/2 MAPK pathway due to mutations in either BRAF or N-RAS (Omholt *et al.*, 2003; Daniotti *et al.*, 2004). I investigated the involvement of this pathway in the normoxic expression of HIF-1α in WM9 cells. When a chemical inhibitor (U0126) at higher concentrations of 30 µM was used to inhibit the ERK1/2 MAPK pathway, it down regulated the hypoxia independent expression of HIF-1α in metastatic melanoma cells. However, a lower inhibitor concentration (10 µM) and more specific approaches, such as siRNA targeting of MEK1 and 2 leading to loss of ERK1/2 MAPK, did not affect the expression of HIF-1α protein. These results indicate that the ERK1/2 MAPK pathway is not involved in the normoxic expression of HIF-1α. The initial downregulation of HIF-1α expression in melanoma cells with higher concentrations (30 µM) of
chemical inhibitor could be due to off-target effects. This could be further investigated by determining the involvement of other MAPK’s, such as p38 or JNK, in normoxic expression of HIF-1α in melanoma cells.

Although a lower concentration (10 µM) of chemical inhibitor did not affect the expression of HIF-1α in WM9 cells at 24 hours, at the later time points (48 and 72 hours) treated cells had lower expression of HIF-1α protein. The U0126 compound is unstable in tissue culture media and produces metabolites that have poor MEK inhibitory activity (Favata et al., 1998). This information suggests that metabolites of U0126 were responsible for the decrease of HIF-1α protein at later time points. Future studies are needed to determine the pathway responsible for the normoxic expression of the HIF-1α gene in melanoma cells. Other than the ERK1/2 MAPK pathway, the PI3K/AKT pathway is also known to regulate the normoxic expression of HIF-1α (Semenza 2003). Recently, Kupal and colleagues have hinted at an involvement of ROS and the NFkB pathway in regulating normoxic expression of HIF-1α in melanoma cells (Kupal et al., 2010). The AKT pathway is known to regulate the NFkB pathway. The role of these pathways in the normoxic expression of HIF-1α in melanoma cells needs to be investigated.

In an attempt to distinguish genes regulated by HIF-1α under normoxic conditions compared to hypoxic conditions, we have found MITF as a potential target gene of HIF-1α. Thus, the major focus in the second part of my dissertation was to determine whether MITF was a novel direct target gene of HIF-1α. Also, I have identified the differential expression of MITF-A and -M in human melanoma cell lines relative to normal human melanocytes.

I have shown for the first time that normoxic expression of HIF-1α regulates MITF mRNA and protein expression in metastatic melanoma cells. In the process of determining the underlying mechanism responsible for the regulation of MITF by HIF-1α, I found two hypoxia
response elements in the MITF-A promoter region (proximal to exon one) while none were found in the MITF-M promoter region (between exon one and two). As predicted by this computer analysis, the MITF-A gene is transactivated by HIF-1α in metastatic melanoma cells. However, quantitative RT-PCR showed that MITF-M as well as MITF-A mRNA were downregulated when HIF-1α expression was knocked down in WM9 cells. How HIF-1α regulates MITF-M mRNA levels in WM9 cells is not clearly known. HIF-1α regulation of MITF-M might involve another transcription factors as an intermediate regulator of MITF-M promoter. In future experiments MITF-M promoter activity will need to be measured in cells having altered levels of HIF-1α.

Reporter gene assays showed that HIF-1α regulated the transactivation of an MITF-A promoter construct containing both HREs transfected into metastatic melanoma cells. Mutation in these sites reduced the activation of MITF-A reporter gene activity in WM9 cells which express high levels of HIF-1α. However, the inhibition due to mutations was less than the inhibition of reporter gene activity when HIF-1α expression was suppressed. The reason for this could be that the four nucleotides we mutated were not sufficient to completely block the promoter binding of HIF-1α and the possibility of loose binding to this DNA region still exists. In addition, HIF-1α is also known to heterodimerize with other bHLH transcription factors such as myc (Haung 2008). Thus, HIF-1α might be hetero-dimerizing with myc which could be responsible for the activation of MITF-A gene. Future studies should focus on the role of myc or other bHLH transcription factors DNA binding sites that might be present in MITF-A promoter region. Finally, chromatin immunoprecipitation results provided convincing evidence for the direct in vivo binding of HIF-1α protein to the MITF-A promoter in WM9 metastatic melanoma cells.
MITF is a large family consisting of various isoforms that differ in their transcription initiation sites. MITF-A is the largest and the most ubiquitously found isoform, while MITF-M is the shortest due to lack of exon one and is selectively expressed in melanocytes. The role of MITF-M is well documented in melanocytic differentiation, pigmentation and survival (Levy et al., 2006). MITF is also found to be amplified in 10-20% of advanced melanoma cases and is associated with decreased survival (Garraway et al., 2005). Although, the MITF-M isoform has a well-defined role in melanocytes, its roles in melanoma are not yet determined. I examined the expression of MITF isoforms A and M in melanoma cell lines representing different stages of melanoma progression relative to melanocytes. MITF-M, is highly expressed in human melanocytes and downregulated in melanoma cells. However, MITF-A, while at low levels in early stage melanoma, is highly expressed in advanced metastatic melanoma cells. This pattern of MITF-A expression correlates with significantly higher normoxic expression of HIF-1α in these metastatic melanoma cells (Mills et al., 2009). Expression of these data as a ratio of MITF-M to MITF-A indicates that there is a clear shift in the expression of MITF-M mRNA in melanocytes to the predominantly MITF-A mRNA expression in metastatic melanoma cells.

MITF regulates melanocyte development, function and survival by modulating the expression of genes involved in differentiation and cell cycle progression (Levy et al., 2006). In melanoma, MITF, in addition to targeting genes involved in cycle progression, also stimulates genes involved in anti-apoptosis and cell motility. In mouse melanoma cells, HIF-1α was identified as a direct target of MITF and was shown to be involved in pro-survival (Busca et al., 2005). In this study, MITF induction of HIF-1α was activated by exogenous stimulation of the α-MSH/cAMP pathway. This pathway is known to transactivate MITF-M through a
cAMP-response element (CRE) located at promoter region between exon one and two (Price et al., 1998; Levy et al., 2006). However, MITF-M is not highly expressed, nor is the α-MSH/cAMP pathway exogenously activated in our metastatic melanoma cells. Thus, this information suggests that MITF-M is not involved in normoxic expression of HIF-1α in our human metastatic melanoma cell lines. For future studies it will be interesting to determine the effect knockdown of MITF on the normoxic expression of HIF-1alpha in WM9 cells. This study will determine whether any positive feedback loop exists which could further stimulate the expression of HIF-1α via MITF expression in metastatic melanoma cells.

Knockdown of MITF expression in WM9 cells decreased their cell viability. The decrease in expression of MITF in these cells resulted in an increase in apoptotic markers such as Annexin V and cleaved caspase-3. MITF up-regulates the anti-apoptotic gene BCL2 (McGill et al., 2002) and promotes melanoma cell growth by stimulating CDK2 expression (Du et al., 2004). Direct injection of siRNA targeting MITF into tumors reduced Bl6 melanoma outgrowth in syngeneic mice by inducing apoptosis (Nakai et al., 2007). Thus, my data suggest that the switch from predominant MITF-M in melanocytes to a predominant MITF-A in some metastatic melanomas may contribute to their enhanced cell survival.

The regulation of MITF-A expression by HIF-1α in melanoma cells could be further confirmed by determining the levels of MITF-A mRNA when expression of HIF-1α is increased through ectopic transfection and expression in RGP melanoma cells. RGP cells have low expression of both HIF-1α and MITF-A. Expressing MITF-A in RGP cells to investigate its effects on cell proliferation and survival could further define its role in melanoma development and progression. It will be interesting to determine whether MITF-A has a somewhat different set of target genes compared to MITF-M. I hypothesize that pro-survival
genes will be major targets of MITF-A in melanoma cells. Finally, the MITF-A promoter which is exclusively regulated by normoxic expression of HIF-1α in melanoma cells could be used in targeted gene therapy. Genetically engineered viral constructs containing a tumor suppressor gene downstream of the MITF-A promoter could be transfected into the HIF-1α expressing melanoma cells. The high expression of HIF-1α in melanoma cells would be expected to transactivate the tumor suppressor gene through the MITF-A promoter leading to selective inhibition of melanoma tumor growth.

In summary, HIF-1α is overexpressed in melanoma cells under normoxic conditions and contributes to some of their malignant behavior (anchorage-independent growth and cell invasion). The expression of a splice variant HIF-1α785, which is missing a part of ODD domain, was higher in more advanced melanoma relative to full length HIF-1α. Although the ERK-MAPK pathway was not found to be involved in normoxic expression of HIF-1α, the PI3K-AKT pathway could be involved and will need to be investigated. In addition, the normoxic expression of HIF-1α directly transactivates MITF-A gene expression in more aggressive melanoma cells and the enhanced expression of MITF contributes to melanoma cell survival (a model illustrating these conclusions is shown in Fig 4.1). This suggests inhibiting either normoxic expression of HIF-1α or HIF-1α mediated expression of MITF might be beneficial in treatment of metastatic melanoma.
In normoxic melanoma cell, HIF-1α protein is found to be expressed at higher levels. These elevated levels of HIF-1α contributed to some of their malignant properties. A novel HIF-1α785 splice variant is also found to be overexpressed in normoxic melanoma cells. The growth signaling pathways such as ERK-MAPK and PI3K-AKT pathways are thought to be responsible for the non-hypoxic expression of HIF-1α in growing cells. However, in WM9 cells ERK-MAPK pathway was not found to be involved in the normoxic expression of HIF-1α and the involvement of PI3K-AKT pathway is need to be investigated. Additionally, the non-hypoxic expression of HIF-1α transactivated MITF-A gene by directly binding to the hypoxia response elements (HRE) located at its promoter region. The enhanced expression of MITF contributes to the malignant properties of melanoma cells by increasing their cell survival.
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