Dermato-Endocrinology 5:1, 239–251; January/February/March 2013; © 2013 Landes Bioscience

Putative role of HIF transcriptional activity in melanocytes and melanoma biology

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Keywords: HIF-1, skin, keratinocytes, melanocytes, melanoma, oncogenesis

Hypoxia-inducible factor-1α (HIF-1α) is a highly oxygen sensitive bHLH protein that is part of the heterodimeric HIF-1 transcription factor. Under hypoxic stress, HIF-1 activity is induced to control expression of multiple downstream target genes, including vascular endothelial growth factor (*VEGF*). The normal epidermis exists in a constant mild hypoxic microenvironment and constitutively expresses HIF-1α and HIF-2α. Expression of HIF-1α and/or HIF-2α has been suggested to correlate with the increased malignant potential of melanocytes, therefore, failures of melanoma therapies may be partially linked to high HIF activity. Notably, melanomas that have the V600E *BRAF* mutation exhibit increased HIF-1α expression. We have utilized a bioinformatics approach to identify putative hypoxia response elements (HREs) in a set of genes known to participate in the process of melanogenesis (including*TRPM1, SLC45A2, HRAS, C-KIT, PMEL* and *CRH*). While some of the mechanistic links between these genes and the HIF pathway have been previously explored, others await further investigation. Although agents targeting HIF activity have been proposed as novel treatment modalities for melanoma, there are currently no clinical trials in progress to test their efficacy in melanoma.

Introduction: Overview of the Biology of the Hypoxia-Inducible Factors (HIFs)

The hypoxia-inducible factors HIF-1α and HIF-2α are oxygen-responsive basic helix-loop-helix (bHLH)-PAS domain proteins expressed in all metazoan organisms. They are key components of the HIF-1 or HIF-2 transcription factors when they heterodimerize with their common subunit, the aryl hydrocarbon receptor nuclear translocator (ARNT, also known as HIF-1β). Whereas ARNT is constitutively expressed, the HIF-α subunits are stabilized in response to decreased oxygen tension, increased reactive oxygen species (ROS) levels or growth factor activated intracellular signaling pathways.[1] Hydroxylation of the HIF-α subunits promotes their physical interaction with the von Hippel-Lindau protein (VHL), which targets HIF subunits for destruction by the 26S proteasome. Under hypoxic conditions, ROS are also generated that, in turn, cause the oxidation of iron from the ferrous to ferric state in the catalytic centers of prolyl hydroxylases (PHD), thereby leading to impaired PHD activity, loss of VHL interaction and stabilization of the HIF-α subunits. Both HIF-1 and HIF-2 bind to hypoxia response elements (HREs) in DNA and recruit the co-activators CREB binding protein (CBP) and p300, which are required for maximal induction of downstream target genes.

The HIF transcription factors regulate the transcription of hundreds of genes in a cell type-specific manner, as some tissues are more dependent on HIF-1 rather than HIF-2 activity and vice versa.[2] In addition, gene knockdown, chromatin immunoprecipitation and sequencing approaches (ChIP-seq) have each identified genes that can be regulated by both HIF-1 and HIF-2, or that are preferentially regulated by either HIF-1 or HIF-2. Therefore, control of HIF activity is highly cell type and microenvironment context dependent.[3-5]

Some of the classic HIF target genes include glucose transporters 1 and 4 (*GLUT1* and *GLUT4*), multiple enzymes in the glycolytic pathway, including lactate dehydrogenase A (*LDHA*) and phosphoglycerate kinase 1 (*PGK1*), *VEGF*, other angiogenic factors, growth factors, transferrin receptor 1, chemokine receptors (*CXCR4)* and lysyl oxidase (*LOX*).[6-10] HIF-1α is overexpressed in a variety of human cancers, including melanoma.[11] HIF-1α activation in human cancers also promotes several hallmarks of malignancy. For example, HIF activity stimulates immortalization through control of telomerase and phosphoglycerate mutase (*PGM*) expression, an enrichment of a progenitor/stem cell phenotype, de-differentiation via regulation of inhibitor of DNA binding 2 (ID2) and genetic instability (via regulation of MutS homologs 2 and 6). In addition, HIF activity controls neoangiogenesis (VEGF), cellular metabolism, autocrine growth factor expression [including insulin-like growth factor 2 (*IGF2*)], transforming growth factor α (*TGF*α), invasion/metastasis (e.g., via E-cadherin and *LOX*), as well as chemotherapy resistance via regulation of the ABC transporters *ABCB1* and *ABCG2*.[12]

HIF-1 expression and the role of HIF-1α in normal keratinocytes and non-melanoma skin cancers. The majority of our knowledge about the hypoxic response in the skin is derived from studies on HIF-1α, therefore, this review will primarily focus on the role of HIF-1 in the skin and in melanoma. Often quoted to be body's largest immune/endocrine organ (comprising ~15% of total body weight and covering an average surface area of ~2 sq. m), the skin is a source of multiple peptide and cytokine

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Submitted: 09/07/12; Revised: 10/23/12; Accepted: 10/26/12
http://dx.doi.org/10.4161/derm.22678

mediators that act not only locally but also systemically.[13,14] On the other hand, the cells in the epidermis and dermis also respond to systemic mediators delivered by the vasculature by modifying their phenotype to better preserve body homeostasis.[13,14] Of note, the epidermis does not produce its own vasculature, being nourished instead through the exchange of substances via the most superficial regions of the papillary capillaries.[15] The dermal vasculature forms superficial and deep dermal plexuses that are connected by the straight collaterals, whereas the superficial plexus sends papillary loops toward the skin surface. The presence and absence of local circulation are reflected in the oxygenation levels of the dermis and the epidermis. Although the human dermis is well-vascularized and therefore, well-oxygenated, the epidermis is mildly hypoxic (~5% O_2), with portions of some sebaceous glands and hair follicles shown to be moderately to severely hypoxic.[16] Hypoxic conditions in the epidermis lead to readily detectable levels of the HIFs, including HIF-1α and HIF-2α by immunostaining.[16-18]

The main cellular constituent of the epidermis, the keratinocytes, are either replicating in the basal layer (~50%) or are undergoing differentiating as they migrate toward the epidermal surface (~50%). The keratinocytes of subsequent layers gradually flatten to form a solid, cornified layer that is desquamated. The intermediate filaments, cytokeratins, are the most important structural elements of the keratinocyte, whereas the cornified layer of skin is comprised of various cross-linked proteins and lipids.

UV (UV) radiation, in particular UVB exposure, causes damage of keratinocytes,[19] leading to cell cycle arrest to allow DNA repair pathways to be activated, or to apoptosis when UV damage is beyond repair. In sun-damaged skin, the apoptotic keratinocytes are known as sunburn cells. A current dogma in the field is that the main pathway linking UVB exposure to DNA damage is tumor protein 53 (p53)-dependent.[20] However, it has been shown previously that keratinocytes with inactive p53 still undergo UVB-induced apoptosis.[21] One example of p53-independent induction of apoptosis in keratinocytes is stimulation of cyclin-dependent kinase inhibitor 1 (p21*)* by HIF-1α. p21 is a pivotal regulator of the G1/S cell cycle checkpoint.[22] The other example of p53-independent pro-apoptotic pathway induced by UV is mediated by HIF-1α and phorbol-12-myristate-13-acetate-induced protein 1 (NOXA). HIF-1α expression, induced through mitogen activated kinase p38 (p38 MAPK) and C-jun terminal kinase (JNK) activation, also stimulates NOXA, a protein that targets the anti-apoptotic protein myeloid cell leukemia sequence 1 (MCL-1) for degradation. In the absence of MCL-1, B-cell lymphoma 2 (BCL-2)–associated X (BAX) mediates mitochondrial outer membrane permeabilization, leading to apoptosis.[23]

Although there is evidence that HIF-1α positively correlates with increased microvessel density, poor survival or decreased response to therapeutic intervention in various epithelial neoplasms, such as non-small cell lung cancers, breast cancers, cervical cancers and cancers of head and neck area,[24-27] the clinical significance of the expression and/or cellular localization of HIF-1α in epidermal neoplasms, such as squamous cancers and basal cell carcinomas has not been studied extensively. There are a few reports that suggest indirect involvement of HIF-1α in skin cancers since VEGF, a major direct target gene of HIF-1α, is expressed in squamous cell carcinomas[28] and papillomas.[29] Non-melanoma skin cancers present in renal transplant recipients have also been shown to have high numbers of VEGF-positive lymphocytes.[30]

Expression and role of HIF-1α in normal melanocytes and melanoma. The melanocytes are derived from neural crest cells primarily located at the dermo-epidermal junction. Their density in the skin varies among different parts of the body from 1 in 4 to 1 in 10 basal keratinocytes. Melanin, a protective pigment produced by these cells, is transferred from melanocytes through dendrites to approximately 36 neighboring keratinocytes by the process of apocopation.[15] Melanin not only absorbs UV irradiation, but also serves as a scavenger of ROS and miscellaneous chemical compounds.[31,32] Melanocytes also play sensory and regulatory functions within the epidermis[33,34] and hair follicle[35,36] with intermediates of melanogenesis serving as bioregulators.[37,38]

Melanoma is a malignant neoplasm derived from melanocytes. Constitutive expression of HIF-1α has been shown in melanomas by immunohistochemical staining of tissue sections, by quantitative PCR, by western blotting and by immunonofluorescent staining of cultured cells.[39-41] In one study, the expression of HIF-1α in patient melanomas was detectable in the majority of samples (87.6%), but expression was not correlated with any clinicopathological variables, including patient prognosis or survival.[40] In another study, the expression of HIF-1α was increased at each step of progression compared with the previous phase. It was higher in vertical growth phase than in horizontal growth phase and the highest in the metastatic melanomas.[41] In addition, overexpression of HIF-1α promoted soft colony formation and invasion through Matrigel. Moreover, in a separate study, it was observed that increased HIF-1α expression was present not only in the melanoma cells but also in melanoma-associated stromal cells, including pericytes.[42] Finally, increased expression of HIF-1α and HIF-2α was found to be correlated with VEGF expression.[43] In this particular study, HIF-2α, rather than HIF-1α, had a stronger association with VEGF expression and with poor prognosis in univariate and multivariate analyses. Of note, in these studies, Breslow's thickness had prognostic value only in univariate analysis. Although the authors suggested that HIF-2α is a better prognosticator than HIF-1α, the overall cumulative data generated to date point to a contribution of both HIF-1α and HIF-2α, as well as VEGF, in melanoma progression and/or metastasis.

In accordance with data reported in literature and discussed in this review, we also detected by immunohistochemistry the expression of HIF-1α in malignant melanoma, with a trend of higher expression in melanoma cells vs. benign nevi (**Fig. 1**). HIF-1α staining was localized in both the nucleus and the cytoplasm of melanoma cells. Interestingly, an increased expression of nuclear HIF-1α was also observed in the keratinocytes in the epidermis that was colonized by melanoma cells in comparison to expression observed in the epidermis distant from melanoma cells.

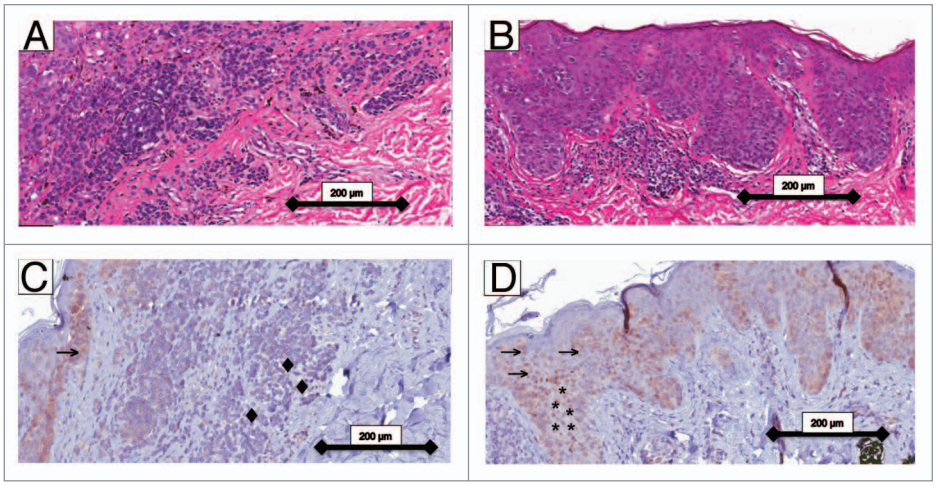

Figure 1. HIF-1α expression in an invasive malignant melanoma arising in melanocytic nevus. (**A**) H&E-stained section of the nevus side of the lesion. (**B**) H&E-stained section of the melanoma side of the same lesion. (**C**) HIF-1α localization in the section containing the nevus side. (**D**) HIF-1α localization in the sections containing the melanoma side. Arrows indicate melanoma cells with cytoplasmatic localization of HIF-1α signal. Asterisks indicate keratinocytes with nuclear HIF-1α positivity. Diamonds indicate the benign nevus cells with negligible to negative HIF-1α staining. H&E staining was performed with standard methodology using a Leica tissue stainer. HIF-1α immunostaining was performed using an anti-HIF1A rabbit polyclonal antibody (HPA001275, 1:50, Sigma) and detected using the using iVIEW DAB system with a VENTANA automatic stainer.

Significance of HIF-1α in current melanoma treatments. As of today there is no effective treatment of melanoma. Various modalities have been attempted but they typically do not increase survival of the patients to any significant degree.[44-48] There are attempts to explain why some of these treatments fail,[47,49,50] however, the recent Bayesian network meta-analysis suggest that overall survival time of patients receiving ipilimumab is expected to be better than with other therapies during management of pretreated patients with unresectable stage III or IV melanoma.[51] Interestingly, there is evidence that stimulation of the hypoxic response is partially responsible for attenuation of therapeutic responses. Isolated limb-perfusion treatment and agents inducing ROS are two examples of scenarios in which HIF-1α plays a role in limiting therapeutic efficacy in either approach. Agents targeting BRAF (v-raf murine sarcoma viral oncogene homolog B1) represent another approach for which the inhibition of HIF activity may be part of the mechanism responsible for killing melanoma cells.

Isolated limb-perfusion is a treatment modality used for isolated, unresectable regional in-transit disease. Tumor necrosis factor α (TNF-α) or other therapeutic agents are administered in such a way that delivery is restricted to local tissues, without being distributed in the whole body.[52] In an isolated limb-perfusion mouse model, in which melanoma cells were engrafted into either ischemic or non-ischemic limbs, tissue ischemia was shown to be a major stimulant of increased HIF-1α activity.[53] In this study, ischemia induced HIF-1α activity and increased the number of new vessels in tumors and the invasiveness of the engrafted melanomas. The expression of HIF-1α, metalloproteinases and VEGF was higher in the ischemic group than in the non-ischemic controls.[53] TNF-α has been previously shown to stimulate both HIF-1α and VEGF expression, which ultimately results in the promotion of blood vessel formation. Therefore, induction of HIF activity by TNFα may be involved in the potential mechanism of failure of this treatment modality.[54]

Dacarbasine is an Food and Drug Administration (FDA)-approved agent utilized to treat advanced melanoma.[46] It primarily acts through the generation of ROS.[55] ROS are important mediators of HIF-1α activity in melanoma. Increased production of ROS in melanoma cells stimulates expression of HIF-1α.[39] In addition, melanoma cells respond to hypoxic conditions by the mitochondrial release of ROS that is generated through the electron transfer chain complex III, leading to stabilization and activation of HIF-1α. Furthermore, induction of ROS with subsequent increased expression of HIF-1α leads to activation of the proto-oncogene *MET (*hepatocyte growth factor receptor). In turn, MET expression increases the spread of melanoma cells on extracelullar matrix, demonstrating increased motility, invasion, growth of metastatic colonies and the formation of vessel-like structures.[56]

Vemurafenib, an inhibitor of BRAF, is also being used with limited success for treatment of advanced melanoma.[45,47] The BRAF (V600E) mutation is the most common mutation found in cutaneous melanomas.[57] Data collected through microarray analyses of melanomas and melanocytic cell cultures has shown that *HIF1A* mRNA levels were significantly increased in lesions containing the V600E mutation.[58] Transfection of the BRAF(V600E) mutant into BRAF wild type (WT) melanoma cells also increased their hypoxic tolerance. Pharmacologic inhibition of BRAF by the compound BAY 43–9006 results in decreased HIF-1α expression, primarily due to reduced protein stability, without affecting the production of the HIF-1α protein.[58] From this study, the authors concluded that the oncogenic activity of the V600E BRAF mutation in melanoma may be partially mediated directly through the HIF pathway.

Exogenous compounds affecting HIF-1α in melanoma. The variety of novel anticancer agents that inhibit HIF-1α and HIF-2α activity has been recently reviewed by Semenza.[59] These drugs act through a variety of mechanisms. Aminoflavone, the active component of AFP-464, a drug currently studied in phase I trials for breast and kidney cancer treatment, inhibits both *HIF1A* mRNA and HIF-1α protein expression.[59] Translation of HIF-1α is inhibited by a variety of compounds, including the mammalian target of rapamycin (mTOR) inhibitors, such as rapamycin, cardiac glycosides, such as digoxin, topoisomerases inhibitors, such as topotecan, microtubules inhibitors, such astaxotere and, even specific oligonucleotides (ENZ-2968).[59] Several other compounds induce

HIF-1α protein degradation. This class of drugs encompasses the heat shock protein (HSP) inhibitors (17-AAG; 17-allylamino-17-demethoxygeldanamycin), antioxidants (ascorbate), a thioredoxin inhibitor (PX-2), a histone deacetylase (LAQ824), G-rich oligonucleotides, berberine, Se-methylselenocysteine, and a commonly utilized guanylate cyclase inhibitor (YC-1).[59] The antimicrobial drug acriflavine was shown by the Semenza laboratory to heterodimerize with HIF, thus, effectively blocking its transcriptional function. Anthracyclines and echinomycin block binding of the HIFs to DNA, leading to diminished expression of HIF-driven genes. Finally, the proteasome inhibitor nortezomib interferes with the domain of HIF-1α that binds to the co-activator p300, attenuating the hypoxic transcriptional response. This drug has been recently approved for treatment of hematopoietic malignancies.[59]

There are several reports in which various inhibitors described above have been tested in melanoma models. In addition, a variety of other inhibitors commonly used for melanoma treatment were also found to affect the HIFs indirectly. For example, MONCPT, a topoisomerase I inhibitor, decreases B16F10 melanoma metastases in C57BL/6 mice by decreasing ERK (extracellular regulated kinase) phosphorylation, HIF-1α expression and the secretion of VEGF and metalloproteinase 9.[60] The imidazoacridinone C-1311 acts as both a DNA-reactive topoisomerase II and receptor tyrosine kinase inhibitor, and represses HIF-1 activity and downstream VEGF expression. In a cell-free system, C-1311 prevented HIF-1α from binding to an oligonucleotide encompassing a canonical HRE, but did not directly interfere with HIF-1α protein production. In mice bearing murine melanoma B16-F10 cells, C-1311-induced significant downregulation of VEGF, resulting in a 70% reduction of angiogenesis.[61] Combined treatment with Nutlin-3, RITA (reactivation of p53 and induction of tumor cell apoptosis) and topotecan inhibited HIF-1α and the delayed growth of B16-F10 melanoma cells.[62] A complex consisting of transferrin, which binds to transferrin receptor, with a short hairpin RNA (shRNA) targeting *HIF1A* inhibited growth of melanoma tumors in the A375 MM xenograft model.[63] In MDA-MB-435 cells, which share features of breast cancer and melanoma cells, a small molecule inhibitor of HIF-1α known as DJ12 inhibited expression of HIF target genes by blocking transactivation of the HRE response element.[64] Telomere homolog oligonucleotides (T-oligos) act through Wermer and ataxia teleangiectasia mutated (ATM) kinase proteins and inhibit the activity of HIF-1, leading to decreased expression of VEGF, VEGF receptor 2, angiopoieitin-1, angiopoieitin-2. These oligos also decreased tumor vascularity in melanoma xenografts in immunocompromised mice.[65] Vernolide-A inhibited radiation-induced tumor angiogenesis in B16F-10 melanoma cells in C57BL/6 mice through regulation of HIF-1α, metalloproteinases 2 and 9 and VEGF.[66] It has been shown that berberine has anti-angiogenic effects in melanoma and it possibly acts through HIF-1α, VEGF and nitric oxide.[67] Selenium inhibits metastatic potential of murine melanoma cells and acts through inhibition of HIF-1α, VEGF and interleukin 18.[68] Vitamin K3 (menadione) attenuates self-ubiquitination of the seven in absentia homolog 2 (Siah2), increases expression of PHD3, thereby leading to decreased activity of HIF-1α and phosphorylated extracellular signal-regulated kinases. Vitamin K3 also decreased the growth of melanoma xenografts.[69] Lenalidomide, used for patients with myelodysplasia with a chromosome 5q abnormality, has anti-angiogenic effects by acting on cadherin 5, β-catenin and cluster of differentiation 31 (CD31), and was found to decrease significantly lung metastasis by B16-F10 cells in mice.[70]

Role of HIF-1α in the control of the behavior of melanoma cells. HIF-1α regulates the expression of several genes that control motility, invasion, homing to distant organs, interactions of tumor cells with endothelial cells and neo-angiogenesis in variety of tumor types. Some of the key genes relevant to melanoma will be discussed here. First, HIF-1α is a positive regulator of connective tissue growth factor (*CTGF*) activity, which modulates function of the transforming growth factor β (TGFß) family, including TGFß-1, -2, -3 and bone morphogenic protein 4 and 7 (BMP4 and 7).[71] These proteins are known to be important in melanoma cell invasion, spreading and motility. Another hypoxia-regulated target that contributes to melanoma is BCL-2, which acts not only as an inhibitor of apoptosis, but also stimulates formation of vessel-like structures. Specifically, under hypoxic conditions BCL-2 increases HIF-1 activity and the expression of *VEGF* in melanoma cells.[72] The effects of BCL-2 on *VEGF* expression were shown to be dependent on its Bcl-2 homology (BH)4, but not its BH1 or BH2 domains,[73] suggesting that the BCL-2 domains engaged in angiogenesis are distinct from those that promote apoptosis.

Hypoxia is also pro-angiogenic in melanoma since it negatively regulates expression of a class of axonal sprouting inhibitors known as class 3 semaphorins (SEMA3AF). This family of proteins functions as tumor suppressors in a variety of tumor types, including melanomas, and functionally interacts with the neuropilin-2 (NRP2) receptors. Hypoxia inhibits NRP2 expression by melanoma cells. Silencing of *HIF1A* abrogates hypoxia-induced NRP2 repression,[74] whereas overexpression of HIF-1α directly inhibits *NRP2* promoter activity. Loss of NRP2 expression in tumor cells inhibited SEMA3F-dependent activities, such as depolymerization of F-actin, and inhibition of tumor cell migration. On the other hand, loss of NRP2 expression in tumor cells increased VEGF protein levels in conditioned media. This increase in VEGF protein levels promoted paracrine signaling in endothelial cells, including VEGF receptor-2 phosphorylation, and the activation of downstream signaling proteins such as mitogen activated protein kinase p44/42 and p38. In addition, the elevated VEGF levels induced migration and sprouting of endothelial cells, two key steps of tumor angiogenesis in vivo. Thus, HIF-dependent inhibition of NRP2 increases melanoma invasiveness and angiogenesis.[74]

The factor-inhibiting HIF (FIH) protein is another key modulator of HIF activity. Its action is via interactions with the C-terminal activation domain (C-TAD), of the hypoxia-inducible factors. Silencing of FIH severely reduced cell proliferation and in vivo tumor growth in A375 melanoma cells. Also, silencing of FIH significantly increased both the total and phosphorylated forms of the tumor suppressor p53, which led to an increase in the cell cycle inhibitor p21. Thus, FIH activity is essential for

tumor growth through the suppression of the p53-p21 axis, the major barrier that prevents cancer progression.[75]

Identification of putative HIF-dependent target genes implicated in melanogenesis and melanocyte functions. Melanogenesis, a process of transformation of L-phenylalanine and L-tyrosine to melanin pigment, is a highly regulated within the melanocyte (for most comprehensive reviews see refs. 31, 76–78). Melanocyte development is controlled by v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (C-Kit) and endothelins [acting through endothelin receptors B (EDNRB)]. Tyrosinase (TYR) is a key enzyme responsible for transformation of l-tyrosine into subsequent melanogenic intermediates that finally form polymers known as eumelanin and pheomelanin. Tyrosinase related proteins (TYRPs) stabilize and regulate activity of tyrosinase or have their own enzymatic activity. It was also proposed that these melanogenesis related proteins have additional regulatory functions aside of their role as enzymes.[38,79,80] Interestingly, active melanogenesis can affect intracellular metabolism including glycolysis and pentose phosphate pathway.[81-84] These properties make melanogenesis an extremely interesting pathway to study its interaction with HIF within melanoma tumor environment.[37]

The function of the melanogenic pathway is prominently regulated by proopiomelanocortin (POMC)-derived melanocyte stimulating hormone (MSH) and ACTH acting through MSH receptor (MC1R).[31,85] The main transcription factors involved in the process are microphtalmia associated transcription factor (MITF) and Sry-related HMG box (SOX) 10. Corticotropin releasing hormone (CRH) and related peptide urocortin acting through CRH receptors stimulating production of POMC derived peptides by skin cells.[86-89] Other genes that affect pigmentation or melanocyte function are: SOX5, SOX9, SOX18, TRPM1, SLC45A2, GPCR143, BRAF, HRAS, INK4/p16, MLANA, PMEL, VDR, CYP27B1 and CYP27A1.[31,34,76] These genes will be introduced and described in detail below.

Identification of High Matrix Score HREs in Melanogenesis Genes

In order to identify potential direct links between HIF activity and melanocyte physiology, we have searched the promoter regions of genes involved in regulating melanogenesis for the presence of consensus HRE sites that may bind the HIF-1 and/or HIF-2 transcription factors. The results of this bioinformatics-based approach are presented in **Table 1**. A total of 33 genes that encode proteins regulating melanocyte behavior were analyzed for HREs. Of these genes, six were identified as having highly significant matrix scores for the presence of an HRE ($p < 0.001$) (**Fig. 2**). These included genes important in regulation of melanin synthesis (*SLC45A2*,[90,91] *PMEL*[92]), in melanocyte genesis during embryonal development and differentiation (*TRPM1)*[93] and (*c-KIT),*[94] a master regulator of systemic and local responses to stress (*CRH*)[86,89] and an important oncogene (*HRAS*).[95]

SLC45A2 (solute carrier family 45 member 2) is also known as a membrane-associated transporter protein (MATP) or melanoma antigen AIM1.[90,91] It is believed that its main role is to regulate the transport of melanogenic proteins into melanosomes, where melanogenesis in vivo takes place. It regulates skin, hair and eye pigmentation and mutations in this gene are known to be the cause of oculocutaneous albinism type 4.[91] Expression of the *SLC45A2* gene is regulated by MITF.[96] It is also considered a melanoma associated gene.[97] There are seven putative HREs located upstream of the transcription start site from -237 to -1326 nucleotides.

PMEL17 (also known as GP*100/SILV*) is the human homolog of mouse silver locus,[92] which may function in the structure of the melanosome,[98] its morphogenesis[99] and in the synthesis of 5,6-dihydroxyindole-2-carboxylic acid (DHICA).[100] It is transcriptionally regulated by MITF in both normal and malignant melanocytes.[101] There are 2 HREs located at -1011 and -1086 positions upstream of the transcription start site.

TRPM1 (melastatin) is a member of the transient receptor potential (TRP) cation channel superfamily playing important roles in a variety of cellular processes.[102] TRPM1 serves as the tumor suppressor gene in melanoma;[93] its expression correlates with terminal melanocytic differentiation and loss of expression has been identified as an important diagnostic and prognostic marker for primary cutaneous melanoma.[102-105] Its gene codes two transcripts, the TRPM1 channel protein is encoded by the exons[93] and miR-211 is encoded in one of the introns.[106,107] Because of the above observations, we have proposed a dual role for TRPM1 protein to regulate melanogenesis and $Ca2^{+}$ homeostasis, whereas the expression of miR-211 may be linked to its tumor suppressor function.[102] Of note, three HREs are located at the -1074, -1103 and -1265 nucleotides of the promoter region of the *TRPM1* gene, suggesting that the HIF pathway may play a regulatory role in both TRPM1 mRNA and miR-211 synthesis.

CRH is the central regulator of the hypothalamic-pituitary-adrenal (HPA) axis, the main organizer of the body's response to stress. Briefly, stress induces the hypothalamic production and release of CRH, which in the anterior pituitary activates CRH receptor 1 (CRHR1) to stimulate adrenocorticotropin (ACTH) release with POMC expression.[108,109] In addition, CRH regulates behavioral, autonomic, endocrine, reproductive, cardiovascular, gastro-intestinal, immune and metabolic functions both centrally and in the periphery.[108,110] CRH is also expressed in the skin,[111] where it regulates local homeostasis directly (reviewed in refs. 85 and 89) or indirectly through activation of the local POMC system (reviewed in ref. 112), and its expression is stimulated by UVB.[113-115] The direct effects include regulation of cell proliferation, differentiation and immune interactions (reviewed in refs. 86 and 89), defining it as a novel growth factor/pleiotropic cytokine,[116] while indirect effects include chain of reactions secondary to CRH induced expression of POMC in melanocytes[88,117] and other skin cells[87,118] or other signaling pathways as proposed.[119] Therefore, CRH appears to be one of the major players in the regulation of skin homeostasis[13] and melanocytic functions.[34,88,116,117,120]

Three HREs sites were identified at positions 260, 280 and 338 within the first exon (**Table 1**; **Fig. 2**). Of interest, we noted that the CRH peptide is coded by exon 2[85] but that that no HRE site was detected in the CRHR1 regulatory regions, indicating

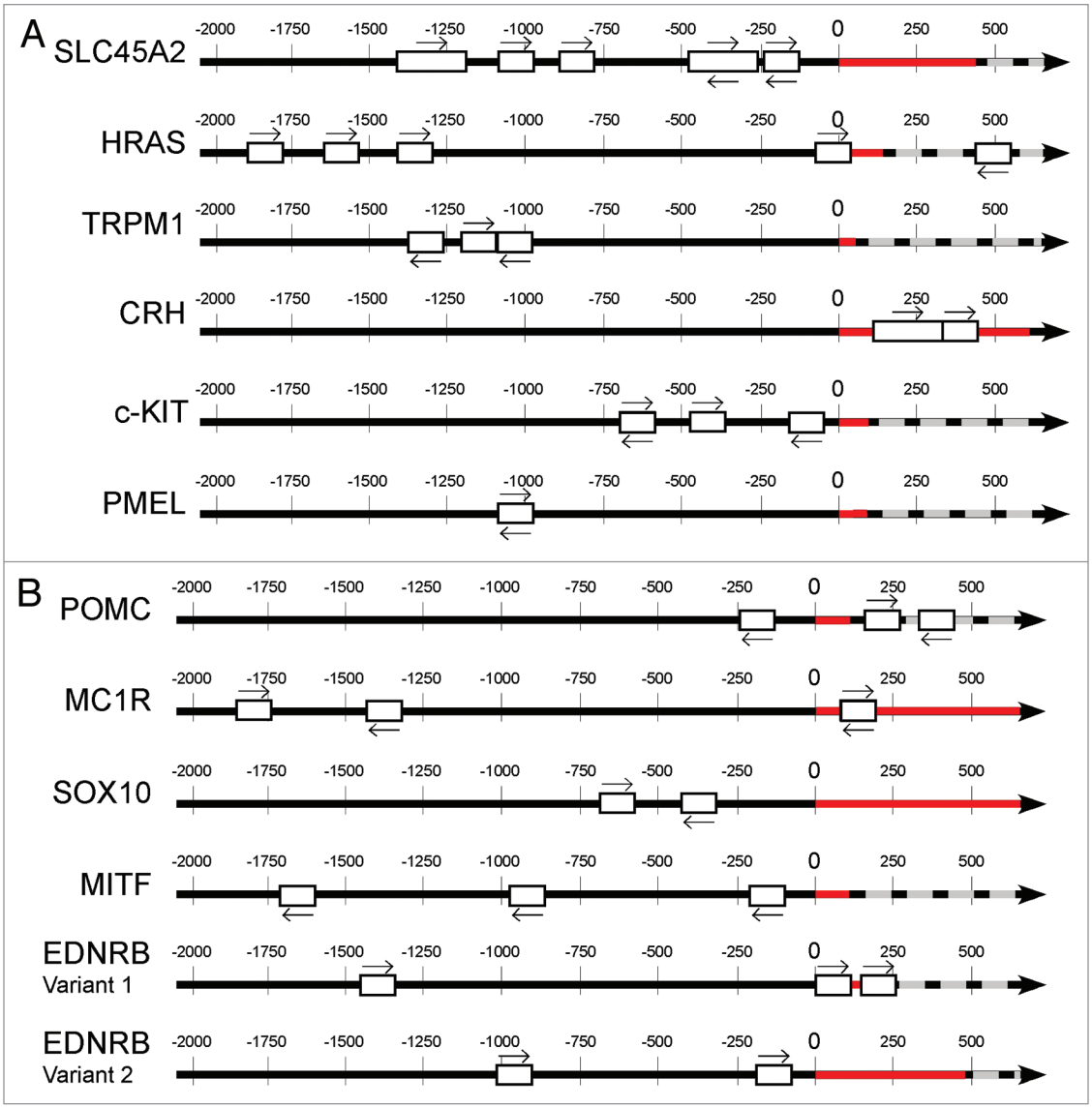

Figure 2. Overview of the position and localization of putative hypoxia response elements (HREs) in genes involved in the regulation of melanogenesis. The number '0' refers to the transcriptional start site of the gene. Positive numbers indicate specific base pairs located after the start site, and negative numbers indicate specific base pairs before the start site. The red line refers to the first exon of the gene and the black and gray dashed lines refer to the first intron. White boxes represent the location of the HRE identified in the gene. The forward and reverse arrows refer to the HRE direction, i.e., the forward or reverse strand, respectively. (**A**) Reviews the HREs identified in genes that produced strong matrix scores during database analysis ($p < 0.001$). (**B**) Lists the HREs identified in key genes involved in the regulation of the melanocyte differentiation program.

pleiotropic regulation at the level of the ligand but not the receptor (CRH also serves as the ligand for CRHR2). Interestingly, another ligand for CRHR1, urocortin 1 (UCN), also contains two HREs, one being upstream at the -311 with second located within exon 1 at +161 (**Table 1**). Furthermore, two other related peptides (UCN2 and UCN3) that act on CRHR2 but not CRHR1 had three (-524, -542, -1557 and -1875) and two (-20 and +134) HREs, with CRHR2 itself having one HRE (-476) (**Table 1**). This is consistent with a complexicity of CRH/UCN signaling in regulation of melanogenesis and hair growth as we have discussed previously.[89,120] In addition, the presence of putative HREs in the CRH gene may explain of the observation of the hypoxia-induced release of CRH[121,122] and the described phenomenon of ischemia[123] or anemia-induced activity of the HPA axis.[124]

C-kit is important for development of melanocytes and their migration from the neural crest to their final destinations.[125] It is also responsible for piebaldism[126] and also has a known role in mediating the development of mucosal, acral and melanomas that arise in sun-damaged skin.[127] Four HREs were identified at the -142, -404, -687 and -726 positions within the promoter region of the *C-kit* gene. Notably, HIF-1α may be an important part of the signaling transduction pathways that lead to the activation of c-kit in several malignancies. In small cell lung cancers and neuroblastoma, the stimulation of c-kit leads to increased expression of VEGF that mediates angiogenesis.[128,129] In pancreatic cancers, c-kit

Table 1. Summary of identification of putative HREs in genes regulating melanocyte and melanoma behavior

Gene	Accession Number	Number of HREs upstream of Start Site	Number of HREs after start site	Location after start site	Genes identified with a strong matrix score
POMC	NM_000939	1 (-248)	2 (209 and 383)	First Intron	
MC1R	NM_002386	2 (-1372 and -1799)	1 (143)	First Exon	
MITF	NM_198159	3 (-198, -950 and -1699)	0	-	
SOX10	NM_006941	2 (-387 and -640)	0	-	
SOX5 (transcript variant 1)	NM_006940	1 (-720)	0	-	
SOX5 (transcript variant 2)	NM_152989	2 (-1218 and -1577)	0	-	
SOX9	NM_000346	0	0	-	
SOX18	NM_018419	1 (-1240)	0	-	
EDNRB (transcript variant 1)	NM_000115	1 (-1422)	2 (28 and 220)	First Exon	
EDNRB (transcript variant 2)	NM_003991	2 (-128 and -1007)	0	-	
TRPM1	NM_002420	3 (-1074, -1103 and -1265)	0	-	Strong Matrix Scores, p = 4.73E-05
SLC45A2	NM_016180	7 (from -237 to -1326)	0	-	Strong Matrix Scores, p = 4.25E-10
GPCR143	NM_000273	2 (-806 and -1661)	2 (230 and 446)	First Intron	
BRAF	NM_004333	0	0	-	
HRAS	NM_005343	4 (-36, -1322, -1684 and -1811)	1 (472)	First Intron	Strong Matrix Scores, p = 4.32E-10
INK4/p16	NM_000077	1 (-1216)	1 (148)	First Intron	
c-Kit	NM_000222	4 (-142, -404, -687 and -726)	0	-	Strong Matrix Scores, p = 1.93E-04
TYR	NM_000372	0	0	-	
TYRP1	NM_000550	0	1 (126)	First Exon	
TYRP2	NM_001922	0	1 (231)	First Exon	
MLANA	NM_005511	1 (-721)	0	-	
PMEL	NM_006928	2 (-1011 and -1086)	0	-	Strong Matrix Scores, p = 4.32E-04
VDR	NM_000376	0	0	-	
CYP27B1	NM_000785	0	1 (248)	First Intron	
CYP27A1	NM_000784	1 (-993)	2 (346 and 400)	First Intron	
CRH	NM_000756	0	3 (260, 280 and 338)	First Exon	Strong Matrix Scores, p = 3.81E-05
CRHR1	NM_004382	0	0	-	
CRHR2	NM_001883	1 (-476)	0	-	
UCN	NM_003353	1 (-311)	1 (161)	First Exon	
UCN2	NM_033199	4 (-524, -542, -1557 and -1875	0	-	
UCN3	NM_053049	1 (-20)	1 (134)	First Exon	

The promoter regions of genes known to regulate melanocyte activity and melanoma were scanned for the presence of putative HIF response elements (HREs) using the publicly available web server, Transcription Factor Matrix (TFM) Explorer.[156-158] A 2,500 base pair sequence (2,000 base pairs upstream of the start site and 500 base pairs after the start site) of each gene was scanned using weight matrices derived from JASPER and TRANSFAC® for the HRE sequence 5'-RCGTG-3', where R is any purine (A or G). Using this approach, a few genes were identified as having a significant matrix scores for the presence of an HRE ($p < 0.001$). The remaining genes were either not considered by these parameters to be significant for the presence of a consensus HRE site, or no HRE was identified in the scanned sequence submitted for analysis. A summary of all of the analyzed genes and the locations of the HREs identified in the database search are presented.

expression results in increased invasiveness.[130] In studies of various melanoma cell lines having the most common c-kit mutations, HIF-1α was found to be essential for their proliferative potential, such that mutated melanoma cells proliferated only in hypoxic conditions or in the presence of overexpressed HIF-1α. Finally, the RAS (rat sarcoma viral oncogene homolog)/RAF (murine sarcoma viral oncogene homolog)/MEK (mitogen-activated protein kinase kinase)/ERK pathways were engaged in this process.[131]

Harvey ras (HRAS) belongs along with KRAS and NRAS to the RAS family. These proteins operate as GTPases and trigger

MAPK-dependent pathways.[132] HRAS is responsible for the development of Noonan, Costello and cardio-facio-cutaneous syndromes.[132] Homozygous single nucleotide polymorphism for rs 12628C portends a potential risk for bladder, colon, gastrointestinal, oral, and thyroid carcinoma and also for melanoma.[95] Of note, amplification of HRAS is characteristic of Spitz nevi.[133] There are five HREs from positions -2000 to +500 of the *HRAS* gene (**Fig. 2**; **Table 1**). H-ras also mediates the levels of HIF-1α in response to vitamin D3 in breast cancer cells. This effect on HIF-1α expression is mediated at the transcriptional level in the absence of HRAS, but in the presence of HRAS, HIF-1α expression is mediated via proteosomal degradation.[134]

Additional Melanogenesis Genes with Putative HRE Sites

POMC and MC1Rs are important in the regulation of melanogenesis (reviewed in refs. 31 and 85). Specifically, binding of -αMSH (one of the derivatives of POMC) to MC1R stimulates cAMP in the melanocyte that, in turn, leads to activation of downstream transcription factors. Of note, variants of MCR1 are important in the determination of skin types, hair color, response to tanning and propensity to develop melanoma.[31,135] One HRE site was found to be located at the -248 position and two HREs were found to be located at the +209 and +383 positions of *POMC* gene. There are three HREs identified at the -1799, -1372 and +143 positions of *MC1R* gene. HIF1-α has been shown to control POMC expression in the hypothalamus of mice in the setting of POMC-expressing neurons responsible for hypothalamic glucose sensing.[136] In addition, in renal carcinomas, in which *VHL* is frequently mutated, leading to overexpression of the HIFs, overexpression of POMC has also been observed, being mediated by Nurr77.[137]

MITF is considered to be a master regulator of melanogenesis on transcriptional level, as reviewed in reference 31. MITF regulates the expression of *TYR, TYRP1* and *TYRP2* genes. Mutation of *MITF* leads to the development of variant IIA of Waardenburg syndrome.[138] Three putative HRE sites are located at -198, -950 and -1699 positions of *MITF* gene (**Table 1**). A hypoxic environment leads to inhibition of expression of MITF in melanoma cells, which, in turn, increases their malignant potential. This is mediated through HIF-1α and the basic helix-loop-helix family, member e40 (BHLHB2) pathway.[139] Although MITF is a key factor in melanoma, the effects of hypoxia on MITF downregulation are not melanocyte-cell specific and can also be observed in human mast cell lines and human clear cell sarcoma.[140]

The Sry-related HMG box (SOX) group of transcription factors has also been implicated in the normal physiological development of a wide array of cellular functions from maintaining stem cell progeny, to lineage restriction, and in the determination of cell fate. SOX10 plays a role in melanocyte development, SOX9 affects pigment formation and SOX5 and SOX18 play a more regulatory role.[141] *SOX10* is known to be mutated in variant IV of Waardenburg syndrome.[142] SOX genes are integral in the transcriptional regulation of the MITF.[143] In metastatic melanomas, three *SOX10* gene mutations have been found, two of which generate frame shift mutations before the DNA binding domain, leading to truncated proteins and inactivated SOX10 transcriptional activity. The third mutation causes a frame shift mutation at the C-terminus and this mutant *SOX10* has reduced activity on the dopachrome tautomerase promoter, causing a loss of heterozygosity of *SOX10* within the tumor.[144] *SOX10* is expressed not just in melanoma cells but also in congenital nevi.[145] It is responsible for preservation of oncogenic properties of human melanoma cells.[145] Two putative HREs are located at the -387 and -640 of the promoter region of the *SOX10* gene. One HRE is located at the -720 promoter region of the *SOX5* gene (variant 1) and two located at -1218 and -1577 of the *SOX5* gene (variant 2). One HRE is located at the -1240 of the promoter region of the *SOX18* gene. No HREs were identified as associated with the *SOX9* gene. The relationship between SOX and HIF-1α activities is apparent in the process of new cartilage formation under hypoxic conditions since HIF-1α was found to be essential for the activation of SOX9, which led to the formation of chondrocytes from primitive mesenchymal cells.[146]

Several genes analyzed for HREs are related to structure of melanocytes and to the enzymatic pathway of the melanogenesis. TYR, TYRP1 and TYRP2 are of the most prominence here. Of note, no possible HRE sites were identified in the promoter of *TYR* and only a singular putative HRE region in the first exon was identified in either *TYRP1* (at +126) or*TYRP2* (at +231), which might suggest an indirect hypoxic regulation of *TYR* through MITF, which contains several HREs (**Fig. 2**; **Table 1**). In a *C. s elegans* model, HIF-1 was found to increase expression of TYR-2 that in turn downregulated the effect of CEP-1, which is equivalent to mammalian p53.[147] Since p53 is a major stimulant of apoptosis, the anti-apoptotic effect of HIF-1 mediated by in part by the key genes of the melanogenesis machinery has obvious connotations for melanoma. Indeed, if expression of TYRP-2 is turned off in melanoma, the cells will undergo apoptosis.[147]

Endothelin 3 and EDNRB are important in migration of melanocytes during embryonal development, their proper positioning in the body and survival.[148] Mutation of EDNRB is present at the of Waardenburg syndrome IV that is characterized by absent neural networks in the colon.[148] One putative HRE is located at the -1422 of the promoter region and two HREs were identified at +28 and +220 after start site of the *EDNRB* (variant 1) gene. In addition, two HREs were identified at the -128 and -1007 of the promoter region of the *EDNRB* (variant 2) gene. Tumor growth, vessel formation and invasiveness of melanoma cells defined by expression of VEGF, COX, prostaglandin E2 production is increased by endothelin 3 binding to its receptor. This behavior is mediated by HIF-1α.[149]

BRAF is a serine-threonine kinase that affects MAP pathway. It is considered to the most commonly mutated gene in melanoma. Vemurafenib targeting mutated BRAF was recently FDA approved for the treatment of advanced melanomas.[150] Of note, no possible HRE sites were identified in the promoter of *BRAF*. Resistance to vemurafenib is being increasingly recognized and is subject of ongoing studies. Vemirafenib-resistant melanomas respond to treatment with vemurafenib with an increase in HIF-1α levels, which thus serves as one of the mechanism of their

resistance.[151] Indeed it is thought that HIF-1α is responsible for pro-proliferative effects of BRAF V600E mutation.[58]

Finally, G protein coupled-receptor 143 (GPCR143) is involved in the process of pigmentation of eyes and skin. Mutation of GPCR143 leads to ocular albinism.[152] Two putative HREs were identified at the -806 and -1661 of the promoter region and two HRE sites were identified at +230 and 446 after start site of the *GPCR143* gene. Constitutive activation of one of GPCRs is known to lead to increased expression of VEGF through activation of HIF-1α.[153] Cyclin-dependent kinase inhibitor 2A (p16) is an important inhibitor of cell cycle. p16 is mutated in familial melanoma.[154] One HRE was identified at the -1216 of the promoter region and one at + 148 after start site of the *p16* gene. p16 forms a physical complex with HIF-1α and modifies expression of VEGF in breast cancer cells.[155]

Summary and Perspective

The hypoxic transcriptional response mediated by HIF-1 and HIF-2 is activated in response to decreased oxygen levels, increased ROS or growth factor stimulation. One of the classical hypoxic response target genes, VEGF, is a key mediator of tumor neo-angiogenesis. Unlike many other organs in the body, the human epidermis exists in a constant mild hypoxic state and constitutively expresses the HIFs, including HIF-1α and HIF-2α. In keratinocytes, HIF-1α blocks the cell cycle and stimulates apoptosis by stimulating p53. In addition, HIF-1α and HIF-2α have been shown to be overexpressed in melanoma, with expression positively correlating directly with the expression of VEGF in melanoma cells, with melanoma progression and with metastatic potential. Hypoxia also stimulates expression of the CTGF, BCL-2 and SEMA3AF protein. Moreover, increased HIF-1α expression and activity seems to be one of the common explanation described as why various melanoma treatments failed (including in the isolated-limb perfusion model, and in response to treatment with dacarbazine and vemurafenib). Since drugs targeting the HIF pathway are already being studied in humans for treatment of other malignancies, their use for melanoma treatment is highly probable in the future. More importantly, our novel analysis of several genes involved in melanogenesis and melanocyte behavior has revealed that the majority of the key players in these processes contain putative functional HREs and, therefore, may be direct transcriptional targets of the HIF transcription factors. These putative direct target genes include in particular *TRPM1, SLC45A2, HRAS, C-KIT, PMEL* and *CRH* but also POMC, MC1R, MITF, SOX5, 10 and 18, EDNRB, TYRP1 and 2, MLANA, CYP27B1 and A1, CRHR2, UCN, UCN2 and 3. Melanocyte development and melanin production is a multistep process that engages multiple players. Most connections between HIF-1α and those genes have been previously studied in non-skin and non-melanoma models. However, in the skin, HIF-1α has a role in the proliferation of melanomas with *C-KIT* mutations and HIF-1α participates in the downregulation of MITF expression in hypoxic environment. HIF-1α also mediates increased tumorogenic potential of melanoma cells upon binding of endothelin 3 to EDNRB.

We have shown in agreement with prior reports that melanoma cells express HIF-1α in both the nucleus and the cytoplasm. Surrounding keratinocytes adjacent to invading melanoma cells also have increased nuclear expression of HIF-1α. Although the significance of the cytoplasmic localization of HIF-1α is not clear, cytoplasmic localized HIF-1α might play other roles in skin biology than its well-established role as a transcription factor in the nucleus. These mechanisms remain to be elucidated. In summary, there is great potential for additional basic and clinical research to reveal the potential cooperative relationship of HIF activity and key melanogenesis genes in controlling melanocyte physiology, melanoma behavior and therapeutic resistance to warrant that the HIF pathway is an attractive pharmacological target for the treatment of melanoma.

Acknowledgments

We thank Michael Lee, MD for the excellent contribution to this work he has made during his one month elective rotation in the dermatopathology program at the University of Tennessee Health Science Center. The writing of this review was in part supported by NIH grants 1R01AR056666–01A2 to AS and by 1R01CA138488 to TS, and dermatopathology fellowship program at the UTHSC. We also thank Dr. Meiyun Fan at UTHSC for her assistance in training Danielle Peacock to perform database searches to identify HREs in various genes of interest.

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