Published in final edited form as:

Arch Biochem Biophys. 2014 December 1; 563: 79–93. doi:10.1016/j.abb.2014.06.030.

The role of melanogenesis in regulation of melanoma behavior: Melanogenesis leads to stimulation of HIF-1 α expression and HIF-dependent attendant pathways

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Abstract

To study the effect of melanogenesis on HIF-1 α expression and attendant pathways, we used stable human and hamster melanoma cell lines in which the amelanotic vs melanotic phenotypes are dependent upon the concentration of melanogenesis precursors in the culture media. The induction of melanin pigmentation led to significant up-regulation of HIF-1 α , but not HIF-2 α , protein in melanized cells for both lines. Similar upregulation of nuclear HIF-1 α was observed in excisions of advanced melanotic vs. amelanotic melanomas. In cultured cells, melanogenesis also significantly stimulated expression of classical HIF-1-dependent target genes involved in angiogenesis and cellular metabolism, including glucose metabolism and stimulation of activity of key enzymes in the glycolytic pathway. Several other stress related genes containing putative HRE consensus sites were also upregulated by melanogenesis, concurrently with modulation of expression of HIF-1-independent genes encoding for steroidogenic enzymes, cytokines and growth factors. Immunohistochemical studies using a large panel of pigmented lesions revealed that higher levels of HIF-1 α and GLUT-1 were detected in advanced melanomas in comparison to melanocytic nevi or thin melanomas localized to the skin. However, the effects on overall or disease free survival in melanoma patients were modest or absent for GLUT-1 or for HIF-1 α , respectively. In conclusion, induction of the melanogenic pathway leads to robust upregulation of HIF-1-dependent and independent pathways in cultured melanoma cells, suggesting a key role for melanogenesis in regulation of cellular metabolism.

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Keywords

melanoma; melanogenesis; HIF-1; metabolism; glycolysis; melanoma progression

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Introduction

Cutaneous melanoma is the most rapidly increasing malignancy in Caucasian populations, and causes a high mortality rate due to onset of therapeutic resistance in stages III and IV of the disease [1-3]. Recently, multiple strategies have been proposed or implemented that target the hallmark features of cancer progression [4], with an expectation of finding a cure for this devastating disease [1-3, 5-8]. Unfortunately, efficacies of these modalities last only a few months before the disease re-emerges, leading to death of the patients. Overall, most therapies lead only to modest improvements in overall survival (OS) and are accompanied by toxic side effects and very high costs of treatment [2, 5, 7, 9-11]. Therefore, there is an urgent need for additional research in order to better define the molecular mechanisms that underline the ability of melanomas to re-wire metabolic and homeostatic responses that are permissive to development of resistance to targeted therapies [11]. Melanoma behavior is driven by several growth factors and cytokines that interact with their corresponding receptors [1, 7, 11], as well as various hormones, neuropeptides and neurotransmitters [12, 13]. These include proopiomelanocortin (POMC)-derived peptides ([14, 15] corticotropin releasing hormone (CRH) [16], steroids [17], biogenic amines and melatonin [18, 19].

Normal and malignant melanocytes are armed with an unique metabolic pathway that begins with tyrosinase mediated oxidation of L-tyrosine to L-DOPA [20, 21], followed by a tightly regulated series of oxidation reactions that involve production of several intermediates, including toxic compounds such as quinones, semiquinones and reactive oxygen species (ROS) [21-26]. The melanin synthesis apparatus serves not only as a differentiation marker but also, and more importantly, protects normal and malignant melanocytes and epidermal keratinocytes containing melanin from a myriad of physical and chemical insults [12, 25]. These latter properties can lead to melanotic melanomas that are relatively resistant to chemo-, radio- and phototherapy [12, 25, 27, 28]. Melanogenesis also influences the behavior of normal and malignant melanocytes and their surrounding microenvironment [26, 27, 29] through the action of intermediates of melanogenesis [30-32], oxygen consumption and stimulation of aerobic glycolysis [26, 33] or interaction with other metabolic pathways [12, 34-36].

There is an abundance of evidence that malignant neoplasms rely on aerobic glycolysis, known as the “Warburg effect” [37] to provide energy to the tumor [4, 38-41]. Changes in cellular metabolism, including stimulation of glycolysis, play a central role in tumor development, progression and therapeutic resistance. In fact, targeting dysregulated tumor metabolism has emerged as a promising approach to prevent drug resistance [42, 43]. Key master regulators of cellular metabolism and therapeutic resistance are the Hypoxia-Inducible Factors (HIF)-1 α and HIF-2 α , oxygen-responsive, basic helix-loop-helix-PAS domain proteins expressed in all metazoan organisms [44]. In partnership with aryl hydrocarbon nuclear receptor translocator (ARNT), HIF-1 α or HIF-2 α , from the HIF-1 or

HIF-2 transcription factors, respectively, which regulate transcription of hundreds of direct gene targets by binding to hypoxic response elements (HREs) in gene regulatory regions [45]. Protein stability of the HIF α subunits is regulated by hydroxylation of two key proline residues in the C-terminus by a family of prolyl hydroxylase (PHD) enzymes. Hydroxylation of the oxygen-dependent degradation (ODD) domain of the HIF α subunits promotes direct interaction with the von Hippel-Lindau protein (VHL), which functions as an E3 ubiquitin ligase, leading to HIF α destruction by the 26S proteasome [38, 39, 44]. HIF-1 and HIF-2 regulate gene transcription in a cell type-specific and microenvironment context dependent manner [46-49]. Some of the classic HIF target genes include multiple enzymes in the glycolytic pathway, such as lactate dehydrogenase A (LDH-A), pyruvate dehydrogenase kinase, isozyme 1 (PDK1), aldolase A, fructose-bisphosphate (ALDOA), hexokinase 2 (HK2), BNIP3 (BCL2/adenovirus E1B 19kDa interacting protein 3), sodium hydrogen exchanger (NHE1), monocarboxylate transporter 4 (MCT4), glucose transporters 1 and 4 (GLUT-1 and GLUT-4) and angiogenic factors, including vascular endothelial growth factor A (VEGF), growth factors and transferrin receptor 1 [38, 50-54]. HIF-1 α is over-expressed in several types of human cancers, including melanoma [55, 56] and directly impacts several hallmarks of malignancy [39].

Since melanogenesis has the potential to regulate tumor behavior [26, 27], we have tested the effects of stimulation of melanogenesis on the expression of HIF-1 α and HIF-2 α proteins, the genes regulated downstream of the HIF transcription factors, as well as the activity of select enzymes in the glycolytic pathway. For these studies, we used human melanoma and hamster melanoma lines whose amelanotic versus melanotic phenotypes are regulated by the concentration of melanin precursors added to culture medium, as previously described [30, 32, 57, 58]. These studies were complemented by pathological analysis of clinical samples to examine the relationships between HIF-1 α or GLUT-1 expression and the progression of melanocytic lesions and the clinical outcome of metastatic disease.

Materials and Methods

Patients

We analyzed 106 samples obtained from 84 patients treated in the Oncology Centre - Prof. Franciszek Łukaszczyk Memorial Hospital, Bydgoszcz, Poland. Samples of normal skin (n=5) were obtained from patients who underwent surgery not related to skin disorders. The characteristics of the patients and pigmented lesions are presented in Table 1. This study was approved by the Committee of Ethics of Scientific Research of Collegium Medicum of Nicolaus Copernicus University, Poland.

Cell culture

Human SKMEL-188 [59] and AbC1 hamster [57] melanoma cells were cultured in Ham's F10 supplemented with 5% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin/amphotericin, Sigma-Aldrich, St. Louis, MO) at 37°C in 5% CO₂ and the media were changed every second day. Although Ham's F-10 contains a low concentration of melanin precursor (10 μ M of L-tyrosine), cells cultured in this medium remained amelanotic [30, 57]. To induce production of melanin pigment, the medium was changed to

Dulbecco's Modified Eagle's Medium (DMEM) containing 400 μ M of L-tyrosine or Ham's F-10 plus 400 μ M of L-tyrosine and cells were cultured for 2, 3 or 6 days and collected as described previously [30, 57]. Non-pigmented cells were grown in parallel in Ham's F10 medium [30, 57] and processed using the same procedures as the pigmented cells.

Western Blot analyses

The expression of HIF-1 α and HIF-2 α was compared in non-pigmented and pigmented melanoma cells, with Caki cells as a positive control for HIF-2 α [60], and *Hif1a* wild type (WT) and knockout (KO) mammary tumor epithelial cells (MTECs) derived from the polyoma virus middle T transgenic mouse (MMTV-PyMT) [61] as controls for HIF-1 α . Cells were grown to 80% confluence and harvested after culture for 6 hours at either normoxia (ambient air; 5% CO₂) or hypoxia (0.5% O₂, 5% CO₂). Cell extracts were prepared using a modified RIPA buffer to produce a whole cell extract (WCE) comprised of cytoplasmic and nuclear proteins. Insoluble material remaining after preparation of WCE was then re-extracted in high-salt (HS) (400 mM NaCl) buffer containing the deubiquitinase inhibitor N-ethylmaleimide (NEM, 0.5 μ M) to enrich for nuclear proteins. HS-WCE was resolved on 3% to 8% Tris-acetate gels (10 μ g/lane; Invitrogen) and transferred onto polyvinylidene fluoride (PVDF) membrane. Membranes were blocked in 5% milk prior to addition of anti-HIF1A (1:5,000, Novus Biologicals, NB100-479) or anti-HIF2A (1:2,000, Novus Biologicals, NB100-122) primary antibodies, which were incubated overnight at 4°C followed by incubation in anti-rabbit IgG-HRP (Jackson Laboratories, 1:40,000) for 45 minutes and detection using enhanced chemiluminescence (ECL).

Reverse transcription (RT) and quantitative polymerase chain reaction (qPCR)

RNA was harvested from SKMEL-188 melanoma cells cultured for two or three days in F10 media (non-pigmented) or in DMEM media (pigmented), supplemented with 5% FBS, using the Absolutely RNA Miniprep kit (Stratagen) as previously described [62]. Reverse transcription was performed using the Transcriptor First Strand cDNA Synthesis kit (Roche). Primers were designed using either the Universal Probe Library software (UPL Roche, Germany) or as previously reported in [63-74]. Primer sequences are listed in Table 1. Real-time PCR (qPCR) data were generated from input cDNA using a SYBR Green Master Mix (n=3 technical/replicates/gene) that was amplified using standard settings on a Roche LC480 LightCycler machine. Gene expression (expressed as crossing point values, Cp) was normalized using either cyclophilin B or β -actin by the Cp method. Changes in gene expression are presented as a relative quantities based on the mean difference between target gene and reference gene in the cycle of appearance in time $\pm$ SD. For each gene, data were compared between pigmented and non-pigmented cells using a Student's *t*-test. It must be noted that the lower is the number presented in tables or figures the higher is relative gene expression level. Since the Cp value represents the cycle at which signal is first detected above a threshold, increases in the relative abundance of the PCR product detected are reflected by a lower Cp numeric value for each particular gene (PCR product) of interest

Enzyme assays

Enzyme assays were performed using protocols described in *Methods in Enzymology* (Volume 9) [75-77]. Briefly, the cell pellets were suspended in cold 50 mM potassium phosphate buffer containing 10% glycerol, 10 mM DTT and a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO). Suspensions were sonicated followed by centrifugation at 12,000g and the supernatants were collected for enzyme assays. Extracts were resuspended in 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM Sodium pyruvate and 0.13 mM NADH for LDH (lactate dehydrogenase) assays, or 20 mM Tris-HCl buffer (pH 7.6) containing 15 mM glucose, 20 mM MgCl₂, 0.13 mM NADP, 0.01mM EDTA, 1 mM ATP and 0.2 units of glucose-6-phosphate dehydrogenase (Sigma-Aldrich, St. Louis, MO) for the hexokinase assay. An aldolase assay was performed by resuspending extracts in a buffer containing 50 mM glycylglycine (pH 7.5) containing 0.2 mM NADH, 10 mM FDP and 15 units of glycerophosphate dehydrogenase (Sigma-Aldrich, St Louis, MO). Changes in optical density were measured at room temperature at a wavelength of 340 nm and the units of activity were reported as the changes in optical density per minute per 1 mg protein, as in [75-77]. Total protein content per extract was measured using Coomassie brilliant blue (Sigma-Aldrich, St. Louis, MO).

NAD and NADH ratios

NAD and the NADH ratio were measured using a commercial kit (Sigma-Aldrich, St. Louis, MO) per manufacturer instructions. Briefly, SKMEL-188 cells were grown in Ham's F10 media + 5% FBS and 400 μ M L-tyrosine for 6 days or in DMEM/Ham's F10 (75/25) +10% FBS for 2 days to generate pigmented cells. For the non-pigmented cells, L-tyrosine was either omitted, or Ham's F10 + 5% FBS was used. At harvest, cells were washed with PBS and total NAD and NADH were extracted using the buffer provided in the kit, followed by filtration using a 10 kDa cut-off spin filter (Abcam, Cambridge, MA). NAD was decomposed by heating at 60°C for 30 min for detection of NADH only. After the reaction with an enzyme mix and buffer provided in the kit and addition of the NADH developer, the absorbance was measured at 450 nM.

Immunohistochemistry staining and analysis

Immunohistochemistry was performed on 4 μ m sections of formalin-fixed paraffin-embedded samples of human melanocytic lesions following protocols described previously [78]. The characteristics of this large panel of pigmented lesions isolated from different patients are listed in Table 2. After antigen retrieval in high pH buffer in a PT-Link device (Dako, Carpinteria, CA), endogenous peroxidase was blocked in H₂O₂-based blocking reagent (Dako, Carpinteria, CA). For GLUT-1 immunostaining, sections were incubated with a monoclonal anti-GLUT-1 antibody (Abcam, Cambridge, UK) at a dilution of 1:75 for 1 hour. After excessive washing and incubation (30 minutes) with anti-mouse EnVision +System-peroxidase labeled polymer (Dako, Carpinteria, CA), antibody-antigen interactions were visualized using a peroxidase substrate, ImmPACT NovaRED (Vector Laboratories Inc., Burlingame, CA). For HIF1 α immunostaining, a TSA (tyramide signal amplification) kit (Life Technologies, Carlsbad, CA) was used per the manufacturer's protocol. A mouse monoclonal anti-HIF1A antibody (Santa Cruz Biotechnology, sc-53546) was applied at a

dilution of 1:750 for 1 hour at RT, followed by overnight incubation at 4°C. After washing and incubation for 1 hour with HRP-conjugated goat anti-mouse secondary antibody (1:75, Abcam, Cambridge, UK), antibody complexes were visualized using Vector Red substrate (Vector Laboratories Inc., Burlingame, CA). All sections were counterstained with Harris hematoxylin and mounted in non-aqueous medium (Consul Mount; Thermo Fisher Scientific Inc. Waltham, MA, USA).

Both HIF-1 α and GLUT-1 immunostaining were scored semi-quantitatively as described previously [78, 79]. Briefly, the level of GLUT-1 was calculated based on the portion of positive cells and the mean staining intensity evaluated from 0 to 3 arbitrary units (A.U.) with 0 as negative (0), weak (1), moderate (2) and strong (3). Staining intensity was evaluated with reference to immunostaining of erythrocytes, which scored as strong. The level of HIF-1 α was also assessed according to the above formulation. The percentage of HIF-1 α -positive cell nuclei per field was also analyzed with reference to clinical pathology morphological data.

Bioinformatics analysis for putative Hypoxic Response Elements (HREs)

The promoter regions of a panel of genes involved in melanogenesis were scanned for the presence of putative HIF binding sites (HREs). The publicly available web server, Transcription Factor Matrix (TFM) Explorer [80] was used for analysis of a total of 2,500 base pairs (bp) of sequence [2000 bp upstream of the transcription start site (TSS) and 500 base pairs after the TSS]. Each gene was scanned using weight matrices obtained from JASPER [81] and TRANSFAC® [82] for the HRE consensus sequence 5'-RCGTG-3', where R is any purine A or G. A list of genes and their identified putative HRE locations is summarized in Table 3.

Statistical analysis

Statistical analysis was performed with Prism 5.0 (GraphPad Software, San Diego, CA). To test the significance of differences between cohorts, either one way ANOVA or Student's *t*-tests were performed as appropriate. To examine the relationship between two variables, Pearson's correlation was used. Survival was compared using Kaplan-Meier plots and the log-rank test. In all tests, a *p*-value of <0.05 was considered statistically significant.

Results and Discussion

Medium rich in melanin precursors stimulates melanogenesis in melanoma cells

Medium containing high concentrations of melanin precursors induces rapid melanization of Bomirski hamster amelanotic AbC1 melanoma [57] and SKMEL-188 human melanoma [28, 30]. When cells are switched from Ham's F10 (containing 10 μ M of L-tyrosine) to DMEM media (containing 400 μ M L-tyrosine), the production of melanin pigment is observed after two days of culture, with an increase in melanization levels observed on day 3 that is accompanied by rounding of heavily melanized cells and their detachment from the culture surface into the media as described previously [57, 83, 84]. In contrast, melanoma cells cultured in Ham's F10 medium (control medium) remain amelanotic and are tightly attached to the culture surface with multipolar morphology during the entire culture period.

Melanogenesis stimulates expression of HIF-1 α in melanoma cells

Expression of HIF-1 α , but not HIF-2 α , protein is dramatically up-regulated in response to induction of melanogenesis in melanoma cells (Fig. 1; data representative of two independent experiments). The specificity of the HIF-1 α and HIF-2 α bands detected by western blotting was confirmed using both positive (HIF-1 WT MTECs, Caki) and negative control cell lines (HIF-1 KO MTECs) (Fig. 1). To evaluate whether this relationship is also observed *in vivo* in human melanoma clinical specimens, we performed immunocytochemical analyses on 75 excisional biopsies or excisions of human melanomas, including 34 amelanotic and 41 melanotic tumors. Melanin production in these specimens was heterogeneous, with higher melanin levels detected in less advanced melanomas (pT1-2, 66%) relative to advanced tumors (pT3-4, 47%, Chi square test: $\chi^2=7.341$, $p=0.0067$). We next compared nuclear HIF-1 α immunostaining in pigmented vs. amelanotic melanomas and observed significantly higher levels of HIF-1 α expression in melanotic tumors (Fig. 2A-C). When samples were stratified according to the pT, this relationship was even more pronounced in advanced melanomas (pT3-4), with loss of correlation for melanoma at pT1-2. It should be also noted that high levels of nuclear HIF-1 α immunostaining were observed in cells located close to the areas of necrosis, as expected (data not shown).

Therefore, these combined *in vitro* and *in vivo* data demonstrate that stimulation of melanogenesis increases the overall expression of HIF-1 α and its nuclear localization. It is possible that HIF-1 α is induced by the production of intermediates of melanogenesis, including ROS and the consumption of intracellular oxygen by the melanogenic process [12, 26]. Both ROS and hypoxic environments are known to efficiently stimulate nuclear HIF-1 α localization in other tumors [38, 44]. Further support for our hypothesis is that melanogenesis induces a hypoxic intracellular oxidative environment is supported by the significant increase in the NAD/NADH ratio in melanized vs. non-melanized cells (Table 4).

Melanogenesis stimulates the expression of glycolysis related genes, glycolytic enzyme activity and VEGFA

Next we investigated the effect of melanogenesis on the expression of genes known to be regulated by HIF-1 as well as the enzymatic activity of key enzymes in the glycolytic pathway (Table 5 and Fig. 3). Table 5 shows that induction of melanogenesis significantly stimulates the expression of genes coding vascular endothelial growth factor A (VEGFA), glucose transporter 1 (GLUT-1), monocarboxylate transporter 2 (MCT2), sodium hydrogen exchanger (NHE1), which regulates intracellular acidity, and the glycolytic enzymes pyruvate dehydrogenase kinase, isozyme 1 (PDK1), aldolase A, fructose-bisphosphate (ALDOA), lactate dehydrogenase A (LDHA) and hexokinase 2 (HK2) ($p < 0.0001$). These changes were also associated with increased expression of tyrosinase (Tyr) mRNA, a HIF-1-independent marker that indicates induction of the melanogenic pathway. As results are based on relative crossing point (Cp) values, it should be noted that a lower Cp value corresponds to higher gene expression, because all data are presented as the mean Cp between the target and reference genes (refer to Materials and Methods).

The induction of melanogenesis is accompanied by cell rounding, followed by the detachment of heavily pigmented cells from the culture dish. Therefore, we also analyzed

the time dependent expression of selected genes in pigmented vs. non-pigmented cells (Table 6). Again, we observed that the stimulation of HIF-1-dependent genes (*VEGFA*, *GLUT-1*, *LDHA*, *PDK1*, *ALDOA*, *HK2* and *MCT4*) was highly reproducible for all-time points and conditions tested. It was previously shown that increases in HIF-1 dependent mRNAs for genes in the glycolytic pathway are accompanied by increases in total protein levels [85]. Therefore, changes in mRNA levels were confirmed at the protein level by measuring enzymatic activities of several glycolytic enzymes, including LDHA, ALDOA, HK2 and PDK1 enzymes activities, all of which increased in melanized vs. amelanotic melanomas (Fig. 3A-C). These data are also in agreement with previous observations showing that one intermediate of melanogenesis, L-DOPA, can stimulate lactate production in melanotic but not amelanotic melanomas [33]. In summary, stimulation of melanogenesis leads to the increased transcription of classical target genes regulated by HIF-1, and is accompanied by increased activities of several key enzymes that mediate glycolysis. Therefore, as is the case for other tumors, increased HIF-1 activity in melanized melanomas leads to up-regulation of the glycolytic pathway [38, 41, 44].

Yet, the inhibition of expression of *Tyr* (Table 6) following melanization requires a separate explanation. After 3 days of culture, when melanoma cells became heavily pigmented, the relative expression of *Tyr* mRNA decreased, approaching values of the control amelanotic cells. *Tyr* expression levels became undetectable in detached pigmented cells (Table 5). The decrease of *Tyr* may be explained by a feedback-dependent inhibition of enzyme activity and gene expression in heavily melanized cells, as described and discussed previously [86-89].

Differential effect of melanogenesis on expression of stress related, steroidogenic, immune and growth related genes

As melanin pigmentation leads to increased HIF-1 α protein levels and HIF-1 transcriptional activity, a panel of genes involved in stress response, regulation of melanogenesis, immunity, angiogenesis and cell growth were screened for the presence of putative Hypoxia Response Elements (HRE; 5'-RCGTC, where R is any purine, A or G) within each gene's regulatory regions using publicly available bioinformatic tools (Table 3). This screen identified several potential HIF target genes that are known to be involved in stress-responses, the HPA axis, steroidogenesis and the regulation of melanogenic activity, as well as genes involved in immunity, angiogenesis and cell proliferation. The two most striking genes identified as putative HIF target genes were *CRH* and *DHCR7*. There are three putative HRE sites identified within the regulatory region of *CRH*, each with a significant matrix score ($p < 0.001$, based on TRANSFAC and JASPAR transcription factor binding matrix models) [81, 82]. Multiple putative HREs were also identified for *DHCR7*: five HRE sites were identified within the first 1000 bp of the transcriptional start site (TSS; $p < 0.05$ to $p < 0.001$). Additional HPA axis genes, including *PCKS1*, *PCKS2*, *MC2*, and the previously identified *POMC* and *MC1* each contained at least two putative HREs within the regulatory regions [56]. In agreement with this analysis, we discovered that expression of these genes is also stimulated by melanogenesis, as shown in Table 7, which suggests involvement of HIF-1 α . It must be noted that the PCSK1 and PCSK2 convertase enzymes differentially process pro-CRH and POMC to CRH peptide and POMC-derived ACTH, α -MSH and β -

endorphin, respectively [14]. Each of these factors can stimulate melanogenesis through activation of their corresponding receptors [12, 14-16, 90, 91]. Collectively, these observations strongly suggest that HIF-1 is involved in regulation of the stress responses through the activation of pro-hormones, convertases, CRH and the MC1 and MC2 coding genes, which in a context-dependent manner could regulate either the HPA or melanogenic activity following schemes previously outlined [12, 13, 16]. Additional mechanisms of induction of pro-melanogenic molecules could include a direct action of intermediates of melanogenesis on POMC and MC1 [26], since stimulation of POMC by L-DOPA [92] and of MSH receptors by L-tyrosine and L-DOPA have already been reported [58, 59, 93]. It is possible that induction of these classes of genes (see Table 7) could be secondary to HIF-1 transcriptional activity and/or may alternatively represent the cellular response to intermediates of melanogenesis [26] or of oxidative stress.

Excluding *7DHCR* described above (which encodes for the 7 reductase that transforms 7-dehydrocholesterol, 7DHC, to cholesterol), many genes involved in steroidogenesis did not contain any putative HREs using our search parameters, including *CYP11A1*, *HSD3B1*, *CYP17A1*, *CYP21A2*, *HSD11B1*, and *HSD11B2*. Only one putative HRE was identified for *CYP11B1*, but it was detected below the matrix significance level ($p > 0.05$; Table 3). Interestingly, we observed melanogenesis-dependent stimulation of *CYP11A1*, *CYP11B1*, *3βHSD*, but inhibition of *CYP17A1*, *CYP21A2*, *HSD11B1*, *HSD11B2* and *7DHCR* (Table 7). Together, these results imply that there is HIF-1 independent regulation of steroidogenesis in pigment cells, similar to the complexity of the pathways operating in the skin [reviewed in [13, 17, 18]]. In the context of downregulation of *7DHCRF*, which contains several putative HREs, we speculate that HIF-1 might be involved in the inhibition of cholesterol production by attenuation of reduction of unsaturated B ring and the corresponding accumulation of 7DHC. In the skin, UVB exposure will transform 7DHC to vitamin D3 [94], which can be further hydroxylated by *CYP27A1* and *CYP27B1* [95] to its active form, 1,25-dihydroxyvitamin D3 (calcitriol). Of note, both of these CYP genes were previously shown to contain putative HRE elements [56].

Genes for which no HREs were identified included *CRHR1* and *NQO2* (Table 3). Therefore, their stimulation (see Table 7) could be secondary to the action of intermediates of melanogenesis or oxidative stress. Since *CRHR1* is a part of the HPA axis, this relationship could link its activity with oxidative stress. Stimulation of *NQO2* by melanogenesis could result from a protective role of the protein product quinone reductase 2 (originally considered as a melatonin receptor type 3 and ubiquitously expressed in the skin) against oxidative damage and quinone compounds [96, 97], since both ROS and quinones are produced during melanogenesis [12].

TGFB2 and *PDGFA*, genes involved in regulation of cell growth and immunity, each contained five putative HRE sites ($p < 0.05$) (Table 3). Whereas *TGFB2* expression was stimulated by melanogenesis, *PDGFA* was inhibited by melanogenesis (see Table 7). In addition, *TFGB1*, *TFRC*, *IL1B* and *CCL5* contained at least two HREs (Table 3), of which the expression of only *TFRC* was downregulated by melanogenesis, with other enzymes showing steady rate of expression of mRNA (see Table 7). Additional genes in this category, including *IL5*, *IL10*, *IL17A*, *IFNA1*, *IFNB1*, *TFGA*, *EGFR*, and *NPL* did not

contain any putative HREs (Table 3). Interestingly, *IL-5*, *IL-10* and *IL17A* expression levels were stimulated by melanogenesis, but there was no effect on the expression of other variety of growth factors or cytokines, including *ICAM1*, *INFA*, *INFB*, *INFG*, *TGFA*, *TGFB1*, *EGFR*, *IL1A*, *IL1B*, *IL2*, *IL2*, *IL4*, *IL6*, *IL8*, *IL10*, *RANTES* or *NPL* (see Table 7). The above pattern suggests limited effects of melanogenesis on expression of growth factors or cytokines in melanoma cells and that there are selective as well as both HIF-1 -dependent and independent effects on gene expression for these classes of genes.

Concluding remarks for cell culture studies

In conclusion, induction of melanogenesis affects the expression of multiple genes involved in the regulation of melanocyte/melanoma behavior, including the metabolic switch to glycolysis, coordinated by HIF-1, with accompanying changes in stress related genes. However, it is not yet clear if these genes are exclusively HIF-dependent since analysis is by complicated by potential secondary effects on gene expression, and since the HRE sites have not been shown to be functional using chromatin immunoprecipitation or other assays. The proposed interactions on the cellular level of these complex biochemical and biological networks are outlined in Fig. 4. Similar studies using normal human melanocytes remain to be performed to evaluate whether similar relationships are operating under physiological conditions of normal skin.

Changes in expression of HIF- α during progression of melanocytic lesions: clinico-pathological analyses

In the context of above studies and the literature on the constitutive expression of HIF-1 α in melanomas [56, 98-100], we have evaluated a relationship between HIF-1 α expression and the progression of melanocytic lesions in a large panel of pigmented lesions obtained from patients listed in Table 2. Representative immunostaining of HIF-1 α in melanocytic nevi and melanomas is shown in Fig 5. In normal skin, not involved in pathology, expression of HIF-1 α was below the detection limits of our protocols. In melanocytic nevi and melanomas, HIF-1 α was expressed both in the cytoplasm and nuclei (Fig. 6), with significant differences in expression between these groups found for nuclear immunoreactivity. There was no difference in immunostaining between different types of nevi including the dysplastic type. Cells with HIF-1 α -positive nuclei were found more frequently in malignant lesions (Chi square test: $\chi^2=6.697$, $p=0.0097$) in comparison to melanocytic nevi. The nuclear HIF-1 α -positivity was found in 60% of melanocytic nevi and in 77% of malignant melanomas. The percentage of HIF-1 α -positive nuclei was 18.7% in melanomas and 9.1% in melanocytic nevi (Fig. 5A-F, 6A). When melanomas were stratified according to different types, a higher portion of HIF1 α -positive nuclei was found in nodular (24.0%) in comparison to superficial spreading type (12.7%) melanomas (Fig. 5C-F, 6B). Stratification of melanomas according pTNM stage revealed a positive correlation between cytoplasmic HIF-1 α and the pT ($r=0.2705$, $p=0.0128$), pN ($r=0.2029$, $p=0.0407$) or pM ($r=0.2872$, $p=0.0131$) stages. The increases for both mean cytoplasmic and nuclear HIF-1 α levels with the pT or pM stages were observed, with the highest levels of HIF-1 α detected in melanomas at the advanced stages (pT3-4, pM1) (Fig. 5C-F, 7A-C). For melanomas stratified according pN stage, only cytoplasmic HIF-1 α expression increased with pN advancement (Fig. 7B). The percentage of HIF-1 α -positive cell nuclei correlated

also with pT ($r=0.2511$, $p=0.0195$, data not shown). In addition, thorough analysis of portion of HIF-1 α -positive cell nuclei in melanomas stratified according pT and overall stage revealed no differences between pT1 and stage 1 melanomas and nevi (Fig. 7D,E). There was also lack of correlation between OS and DFS of melanoma patients and tumor HIF-1 α levels (data not shown).

Thus, analysis of 26 cases of melanocytic nevi and 75 cases of melanoma demonstrates distinct and significant increases of HIF-1 α expression in melanomas at advanced stages as indicated by higher expression in nodular vs. superficial spreading melanomas, or in thicker (pT3 and pT4) vs. thinner (pT1) melanomas or localized (pN0 or pM0) vs. metastatic melanomas that involve multiple lymph nodes (pN3) or visceral sites (pM1). This was further supported by higher expression in highly advanced stages (stage 4) vs. localized (stage 1) disease. These results are in agreement with studies by Mills et al., 2009 [100] who showed the highest expression of HIF-1 α in metastatic melanomas and a higher expression in the vertical growth phase vs. radial growth phase. Our data are also in agreement with a study by Giatrmanolaki et al., who demonstrated a contribution of HIF-1 α to melanoma progression and/or metastasis [101]. Other investigators have not detected a clear correlation between the expression of HIF-1 α and any clinicopathological variables, including patient prognosis or survival [99]. Overall, our studies are in partial agreement with these analyses as we also found no significant correlation between HIF-1 α expression and either OS or DFS. However, a significant correlation was observed between progression and HIF-1 α expression in highly advanced melanomas, with higher HIF-1 α levels in thicker (pT2) but not thin (pT1) melanomas in comparison to melanocytic nevi. A similar trend was reported previously by other investigators [56]. Despite the limitations listed above, our pathologic data support the hypothesis that overexpression of HIF-1 α can contribute to the increased aggressiveness of melanoma, which is in agreement to prior reports in the literature on melanomas [100, 102, 103] and other tumor types [38, 44]. However, it is unlikely that HIF-1 α acts alone in driving melanoma progression.

Changes in expression of GLUT-1 during progression of melanocytic lesions: clinico-pathological analyses

Comparisons of GLUT-1 expression in the same specimens revealed both cytoplasmic and membranous patterns of localization (Fig. 8A, J). The membrane-localized GLUT-1 was observed only in melanomas (55%). Cytoplasmic GLUT-1 was more frequently observed in malignant (49%) vs. benign (20%) melanocytic lesions (Chi square test: $\chi^2=18.61$, $p<0.0001$). GLUT-1 immunostaining in melanomas was heterogeneous, being clustered in patches of melanomas cells in various areas of tumor. A high level of membrane-localized GLUT-1 immunostaining was observed around necrotic areas (data not shown). Comprehensive analysis of GLUT-1 levels in reference to pathomorphological features revealed significantly higher membranous GLUT-1 expression in pT3-4 stages and pN3 melanomas as compared to less advanced tumors (Fig. 8B-F). The OS and DFS in melanomas with membranous GLUT-1 expression was shortened (Fig. 8G,H). However, there was lack of such correlation for cytoplasmic GLUT-1 staining. In normal skin, GLUT-1 was localized predominantly to the keratinocytes' cytoplasm, while in keratinocytes immediately surrounding melanomas GLUT-1 expression increased having

predominant membranous localization (Fig. 8 I-K). There were no significant differences in GLUT-1 immunostaining levels and either melanin level, pM or overall melanoma stage. We also observed that OS and DFS in melanomas expressing membrane-localized GLUT-1 was shorter. Other laboratories have reported trends between GLUT-1 expression and some melanoma features of increased tumor aggressiveness [104, 105]. However, GLUT-1's role in melanoma progression requires further investigation using large number of specimens as other investigators have reported a decreased frequency of GLUT-1 in aggressive melanomas as compared to melanocytic nevi [106], or have reported variable effects on glucose uptake in melanomas [107-109].

Final concluding remarks

Induction of melanogenesis in melanoma cells is associated with dramatic increases of nuclear HIF1- α expression accompanied by upregulation of HIF-1 dependent multiple genes involved in the regulation of glucose metabolism, angiogenesis and stress responses. It is, however, unclear whether these regulatory pathways are exclusively or predominantly coordinated by HIF-1, since melanogenesis had several secondary effects on gene expression that are HIF-1 independent. Therefore, we propose a complex set of interactions between melanogenesis, glucose metabolism and HIF-1-dependent- or independent pathways that can regulate both cellular metabolism and cell behavior (Fig. 4). The immunocytochemical analyses revealing higher levels of HIF-1 α and of GLUT-1 in advanced melanomas suggest that HIF-1 might also be involved in melanoma progression at local levels.

Acknowledgments

This work was supported by grants from NIH/NIAMS (2R06AR052190 and 1R01AR056666 to AS), NIH/NCI (1R01CA138488 to TS) and the West Clinic Cancer Foundation awards to AS and TS. The paper is dedicated to Dr. Andrzej Bomirski, who established Bomirski melanomas that are transplantable to Syrian golden hamsters [110]. We thank Dr. Zbigniew Pawłowicz, Director of Oncology Centre - Prof. Franciszek Łukaszczyk Memorial Hospital for generously supporting collaborations advantageous for our research.

Nonstandard abbreviations

VEGFA	vascular endothelial growth factor A
GLUT-1	glucose transporter 1
Tyr	Tyrosinase
LDHA	lactate dehydrogenase A
MCT	monocarboxylate transporter
NHE1	sodium hydrogen exchanger
PDK1	pyruvate dehydrogenase kinase, isozyme 1
ALDOA	aldolase A, fructose-bisphosphate
HK2	hexokinase 2
Cyp11A1	(cytochrome P450, family 11, subfamily A, polypeptide 1) exon 4

Cyp11B1	cytochrome P450, family 11, subfamily B, polypeptide 1
Cyp17A1	cytochrome P450, family 17, subfamily A, polypeptide 1
Cyp21 A2v2	cytochrome P450, family 21, subfamily A, polypeptide 2
HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1
HSD11B2	hydroxysteroid (11-beta) dehydrogenase 2
7DHCR	7-dehydrocholesterol reductase
3βHSD	3-beta-hydroxysteroid dehydrogenase
CRH	corticotropin releasing hormone
CRHR1	corticotropin releasing hormone receptor 1
POMC	proopiomelanocortin
MC	melanocortin receptor
PCSK	proprotein convertase subtilisin/kexin type
NQO2	NAD(P)H dehydrogenase, quinone 2
EGFR	epidermal growth factor receptor
ICAM1	intercellular adhesion molecule 1
IFN	interferon
IL	interleukin
CXCL	chemokine ligand
TNF	tumor necrosis factor
PDGFA	platelet-derived growth factor alpha polypeptide
TGFB	transforming growth factor, beta
RANTES	CCL5 chemokine (C-C motif) ligand 5
TRANSFERRIN RC	transferrin receptor
NPL	N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)
HIF	Hypoxia-Inducible Factor
HRE	HIF responsive element
SM	stimulators of melanogenesis
ROS	reactive oxygen species
QS	quinones and semiquinones
IOM	intermediates of melanogenesis
OS	overall survival

DFS

disease free survival

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Highlights

Melanogenesis stimulates expression of HIF-1 α and downstream regulators of metabolism

Melanogenesis affects expression of stress related genes

Advanced melanomas show increased expression of HIF-1 α protein

Advanced melanomas show increased expression of membrane-bound GLUT-1

Figure 1.

The induction of melanin pigmentation is associated with up-regulation of HIF-1α, but not HIF-2α protein. For each independent experiment, experiment 1 (upper panels) and experiment 2 (lower panels), respectively, images of cell pellets obtained from two stable melanoma cell lines [Bomirski hamster AbC1 (1, 3) and human SKMEL-188 (2, 4)] that were cultured as described in the Materials and Methods and western blots for HIF-1α and HIF-2α are shown. For the western blots, lanes correspond to the following HS-WCE: A and C: amelanotic (non-pigmented) AbC1 and SKMEL-188 cells, respectively; B and D: melanotic (pigmented) AbC1 and SK-MEL 188 cells, respectively; E, Caki cells cultured 6h at hypoxia; F, Caki cells cultured at normoxia; G and H, HIF-1 WT (G) and HIF-1 KO (H) PyMT MTECs cultured at hypoxia (6h at 0.5% O₂). Arrows to the left indicate the molecular weight (MW, kDa), whereas arrows to the right point to either HIF-1α, HIF-2α or cross-reactive material (CRM).

Figure 2. Relationship between maximal HIF-1α expression and melanin production (A). Statistically significant differences are denoted using p values as determined by Student's t -test. Amel: amelanotic melanomas, Pigm: pigmented melanomas. Representative immunostaining in amelanotic (B) and pigmented (C) melanomas. Arrows show nuclear HIF-1α immunostaining, whereas arrowheads show cytoplasmic HIF-1α immunostaining, asterisks indicate melanin; scale bar: 50 μm.

Figure 3.

Melanogenesis induces gene expression associated with increased HIF-1 α expression. SKMEL-188 cells were grown in Ham's F10 containing 5% FBS, followed by incubation in fresh Ham's F10 containing 5% FBS for non-pigmentation or 75% DMEM and 25% Ham's F10 containing 5% FBS for pigmentation. The cells were harvested after 2 days (B) or 3 days (C) for enzyme assays and after 2 days for qPCR (D). A-1, non-pigmented cells at day 2; A-2, pigmented cells at day 2; A-3, non-pigmented cells at day 3; A-4, pigmented cells at day 3. Panels B and C summarize the enzyme activities as determined using whole cell protein extracts as described in the methods. Panel D shows the mean values of C_p for each gene (with lower values indicative of relatively higher gene expression levels). Data are presented as the mean $\pm$ SE ($n = 3$). ** $p < 0.01$; *** $p < 0.001$ by Student's t -test.

Figure 4.

The proposed interactions between melanogenesis, glucose metabolism and potential HIF-1 dependent genes/pathways that culminate to regulate cellular metabolism. SM: stimulators of melanogenesis, ROS: reactive oxygen species, QS: quinones and semiquinones, IOM: intermediates of melanogenesis, POMC: proopiomelanocortin, CRH: corticotropin releasing hormone, PCSK1/2: proprotein convertase subtilisin/kexin types 1 and 2.

Figure 5.

Representative immunostaining with antibodies against HIF-1 α in nevi (A, B) and melanomas (C-F). Dashed lines separates cases with these same pathomorphological features, arrows show nuclear HIF-1 α immunostaining, arrow-head shows cytoplasmic HIF-1 α immunostaining, asterisks indicates melanin; scale bar 50 μ m.

Figure 6. Differences in HIF-1α immunostaining between nevi (n=26) and melanomas (n=75) (A) and between superficial spreading (SSM) (n=36) or nodular (NMM) (n=37) melanomas (B). Statistically significant differences are denoted with *p* values as determined by Student's *t*-test.

Figure 7.

Expression of cytoplasmic (A,B,C) and nuclear (A,C) HIF-1α antigen in melanomas stratified according pT (A), pN (B) and pM (C) stage. The percentage of HIF-1α-positive tumor cell nuclei in nevi and melanomas stratified according pT (D) and overall stage (E). Statistically significant differences are denoted with *p* values determined by Student's t-test and with asterisks by ANOVA (*p < 0.05). Refer to Table 1 for information regarding the number of cases per melanoma stage.

Figure 8.

GLUT-1 expression in nevi and melanomas (A), membrane GLUT-1 expression and melanoma stratified according pT (B) and pN (C) classification. Representative GLUT-1 immunostaining in pT1 (D), pT2 (E) and pT4 (F) melanomas. Relationship between OS (G) or DFS (H) and membrane GLUT-1 levels. Representative immunostaining of GLUT-1 in normal skin (I), in the epidermis above the nevus (J) or in melanoma (K). Statistically significant differences are denoted with *p* values as determined by Student's *t*-test. Arrows show membrane GLUT-1 immunostaining, whereas arrowheads indicate cytoplasmic GLUT-1 immunostaining, and asterisks indicate melanin, MM: melanomas, ker: keratinocytes, nev: nevus cells, mel: melanoma cells; scale bar 50 μ m. Refer to Table 2 for information regarding the number of cases per melanoma stage.

Table 1

Sequences of primers used for qPCR

Genes	Description	Primers
CYCB	cyclophilin B	L 5'-tgtggtgtttggcaagttc-3' R 5'-gtttatcccgctgtctgtc-3'
β -actin	β -actin	L 5'-ccaaccgcgagaagatga-3' R 5'-ccagaggcgtacaggatag-3'
ALDOA	aldolase A, fructose-bisphosphate	L 5'-cggaagaaggagaacctg-3' R 5'-gaccgctcggagtgtacttt-3'
PDK1	pyruvate dehydrogenase kinase, isozyme 1	L 5'-ccgctctccatgaagcagtt-3' R 5'-ttccgcagaaacataatgag-3'
HK2	hexokinase 2	L 5'-caaagtgcagtggtgtgtg-3' R 5'-gccaggctctcactgtctc-3'
MCT2	monocarboxylate transporter 2	L 5'-atcctgggcttcattgacat-3' R 5'-atcctgggcttcattgacat-3'
MCT4	monocarboxylate transporter 4	L 5'-atcctgggcttcattgacat-3' R 5'-atggagaagctgaagagga-3'
GLUT1	glucose transporter 1	L 5'-ccattggctccggtatcgt-3' R 5'-tgctcgtccaccacaac-3'
NHE1	sodium hydrogen exchanger	L 5'-tctccgtctccactgtctcca-3' R 5'-ccctcagctcctcattacca-3'
LDHA	lactate dehydrogenase A	L 5'-accagtttccaccatgatt-3' L 5'-cccaaatgcagggaacact-3'
VEGFA	vascular endothelial growth factor A	L 5'-tgggaattggattcgccattt-3' R 5'-tgggtgggtgtgtctacagga-3'
TYR	tyrosinase	L 5'-ggctgtttgtactgctgct-3' R 5'-aggagacacaggctctaggga-3'
Cyp11A1, exon 4	cytochrome P450, family 11, subfamily A, polypeptide 1, exon 4	L 5'-ccagacctgtccgtgtgtt-3' R 5'-aaatcacgtccatgcag-3'
Cyp11B1	cytochrome P450, family 11, subfamily B, polypeptide 1	L 5'-aggtggacagcctgcac-3' R 5'-ccattcaggccattcag-3'
Cyp17A1	cytochrome P450, family 17, subfamily A, polypeptide 1	L 5'-gcatcatagacaacctgagca-3' R 5'-gggtttgtgggaaaac-3'
Cyp21A2v2	cytochrome P450, family 21, subfamily A, polypeptide 2, variant2	L 5'-gttgccctgactcagaaat-3' R 5'-gacaccagctgtgttcag-3'
HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1	L 5'-caatggaagcattgtgtcg-3' R 5'-ggcagcaaccattggataag-3'
HSD11B2	hydroxysteroid (11-beta) dehydrogenase 2	L 5'-gtcaaggtcagcatcatcca-3' R 5'-cactgaccacgtttctac-3'
7DHCR	7-dehydrocholesterol reductase	L 5'-ttgtaaaagaattgctgtgaat-3' R 5'-gccatggtcaagggtac-3'
3 β HSD	3 β -hydroxysteroid dehydrogenase	L 5'-cttgacaaggccttcagac-3' R 5'-tcaagtacagtcagctgtctc-3'
CRH	corticotropin releasing hormone	L 5'-ctccgggaagtcttggaaat-3' R 5'-gttctgtgagcttgcgtg-3'
CRH, exon2	corticotropin releasing hormone, exon 2	L 5'-actcagagaccaagtc ca-3' R ctcccaggcgcttcgcaggt-3'
CRHR1	corticotropin releasing hormone receptor 1	L 5'-tggatgttcattgcattgg-3' R 5'-tgccaaccagcacttctc-3'
POMC, exon3	proopiomelanocortin, exon 3	L 5'-_agcctcagcctgctggaa-3' R 5'-cagcaggttgctttccgtgtg-3'

Genes	Description	Primers
MC1	melanocortin receptor 1	L 5'-actcgtctgtccaatgac-3' R 5'-agagctggcagcaaatg-3'
MC2	melanocortin receptor 2	L 5'-tctcagcctgtctgtgattg-3' R 5'-ggcacaggatgaagaccag-5'
PCSK1	proprotein convertase subtilisin/kexin type 1	L 5'-tgatccacaacgagaaca-3' R 5'-tgtgattattgtctgcatgg-3'
PCSK2	proprotein convertase subtilisin/kexin type 2	L 5'-agcatacaactccaaggttc-3' R 5'-aggcctcgatgatgtctgc-3'
NQO2	NAD(P)H dehydrogenase, quinone2	L 5'-ttgacatcccaggattctac-3' R 5'-ccgtggttacggaagag-3'
EGFR	epidermal growth factor receptor	L 5'-acacagaatctatacccaccagagt-3' R 5'-atcaactcccaacgggtcac-3'
ICAM1	intercellular adhesion molecule 1	L 5'-ccttctcaccgtgtactgg-3' R 5'-agcgtagggttaaggttcttc-3'
IFN α 1	interferon α 1	L 5'-ccctctttatcaacaacttc-3' L 5'-ttgtttcatgttgaccaga-3'
IFN β 1	interferon β 1	L 5'-gatgagtacaaaagtcctgatcca-3' R 5'-ctgcagccactggttctgt-3'
IL1 α	interleukin 1 α	L 5'-ggttgagtttaagccaatcca-3' R 5'-tgctgacctaggctgatga-3'
IL1 β	interleukin 1 β	L 5'-crgtcctcggttgaaaga-3' R 5'-ttggtaattttgggatctaca-3'
IL2	interleukin 1	L 5'-aagttttacatgccccagaagg-3' R 5'-aagtgaagtttttgccttgagcta-3'
IL4	interleukin 3	L 5'-caccgagttgacctaacag-3' R 5'-gccctgcagaaggttcc-3'
IL5	interleukin 5	L 5'-ggtttgtgcagccaaagat-3' R 5'-tcttgccctcattctact-3'
IL6 (IFN β 2)	interleukin 6	L 5'-gaagctctatctgcctcca-3' R 5'-agcaggcaacaccaggag-3'
IL8 (CXCL-8)	interleukin 8, chemokine ligand 8	L 5'-agacagcagagcacacaagc-3' R 5'-atggttcctccgggtgt-3'
IL10	interleukin 10	L 5'-tgggggagaaacctgaagac-3' R 5'-ccttgcctgttttcacagg-3'
IL17A	interleukin 17A	L 5'-tggaagacctcattggtgt-3' R 5'-ggatttcgtgggatttgat-3'
TNF α	tumor necrosis factor alpha	L 5'-cagcctcttctcctctgat-3' R 5'-gccagaggctgattagaga-3'
IFN γ	interferon gamma	L 5'-ggcattttgaagaattggaaag-3' R 5'-tttgatgctctggtcatctt-3'
PDGFA	platelet-derived growth factor alpha polypeptide	L 5'-gcagtcagatccacagcatc-3' R 5'-tccaaagaatcctcactcccta-3'
TGFA	transforming growth factor, alpha	L 5'-ttgctgccactcagaagac-3' R 5'-atctgccacagtcacctg-3'
TGFB1	transforming growth factor, beta 1	L 5'-gcagcacgtggagctgta-3' R 5'-cagccggttgctgaggtta-3'
TGFB2	transforming growth factor, beta 2	L 5'-ccaaagggtacaatccaac-3' R 5'-cagatccttctggattatgtatt-3'
RANTES	CCL5 chemokine (C-C motif) ligand 5	L 5'-tgccacatcaaggagtattt-3' R 5'-tttcgggtgacaagacga-3'

Genes	Description	Primers
TRANSFERRIN RC	transferrin receptor	L 5'-ttgagaaaacaatgcaaatgtg-3' R 5'-cccagttgctgctgatataga-3'
NPL	N-acetylneuraminate pyruvate lyase	L 5'-ccagagatttatcaactttgtgtca-3' R 5'-gaggctttctgcagtggaag-3'

Table 2

Clinical, pathological and morphometry characteristic of patient samples.

Features	Number of cases
Nevi	26
Junctional	6
Intradermal	7
Compound	8
Dysplastic	5
Melanomas	75
Superficial spreading	36
Nodular	37
Lentiginous	2
Age	
Nevi	Median 38,2 years (range 20-85)
20 years	2
21–40 years	15
41–60 years	7
61–80 years	1
>80 years	1
Melanomas	Median 58,0 years (range 25-100)
20 years	0
21–40 years	5
41–60 years	36
61–80 years	28
>80 years	6
Female/male ratio	
Nevi	
Female/Male	18/8
Melanomas	
Female/Male	36/39
pT	
pT1	21
pT2	11
pT3	15
pT4	28
pN	
pN0	38
pN1	16
pN2	11
pN3	10

Features	Number of cases
pM	
pM0	66
pM1	9
overall stage	
1	22
2	15
3	29
4	9

Table 3

Identification of putative HREs in genes related to stress response, steroidogenesis, immunity and cell growth.

Gene name or symbol	Accession	HREs	Location relative to TSS	Strand	<i>p-value</i>
CRH	NM_000756	3	+ 260	forward	<.001
			+ 280	forward	<.001
			+ 338	forward	<.001
CRHR1	NM_004382	0	NA	NA	NA
POMC	NM_000939	2	- 248	reverse	NS
			+ 382	reverse	NS
PCSK1	NM_000439	2	- 1582	forward	<.005
			- 1294	reverse	NS
PCSK2	NM_002594	2	- 1292	forward	<.05
			- 1287	forward	<.005
MC2R	NM_000529	2	- 1530	forward	<.05
			- 829	reverse	<.05
NQO2	NM_000904	0	NA	NA	NA
TGFB1	000660	2	- 936	forward	NS
			- 133	reverse	NS
TGFB2	NM_003238	5	- 790	reverse	<.001
			- 767	reverse	<.001
			- 466	reverse	<.001
			- 762	forward	<.05
			+ 42	forward	<.05
CYP11A1	NM_000781	0	NA	NA	NA
CYP11B1	NM_000497	1	- 75	reverse	NS
HSD3B1	NM_000862	0	NA	NA	NA
CYP17A1	NM_000102	0	NA	NA	NA
CYP21A2	NM_000500	0	NA	NA	NA
HSD11B1	NM_005525	0	NA	NA	NA
HSD11B2	NM_000196	0	NA	NA	NA
DHCR7	NM_001360	5	- 901	forward	<.05
			- 760	forward	<.05
			- 637	forward	<.05
			- 622	forward	<.005
			- 251	forward	<.001
IL1A	NM_000575	0	NA	NA	NA
IL1B	NM_000576	2	- 1123	forward	NS
			- 296	forward	<.05
IL5	NM_000879	0	NA	NA	NA

Gene name or symbol	Accession	HREs	Location relative to TSS	Strand	<i>p-value</i>
IL10	NM_000572	0	NA	NA	NA
IL17A	NM_002190	0	NA	NA	NA
PDGFA	NM_002607	5	– 1972	reverse	<.05
			– 1549	reverse	<.05
			– 1432	forward	<.05
			– 1401	forward	<.005
			– 574	forward	<.005
TFRC	NM_003234	3	– 1112	forward	<.05
			– 327	reverse	NS
			– 19	forward	<.05
IFNA1	NM_024013	0	NA	NA	NA
IFNB1	NM_024013	0	NA	NA	NA
TGFA	NM_003236	0	NA	NA	NA
EGFR	NM_005228	0	NA	NA	NA
CCL5	NM_002985	2	+ 32	forward	NS
			+ 468	forward	NS
NPL	NM_030769	0	NA	NA	NA

The promoter regions of a panel of genes involved in melanogenesis were scanned for the presence of putative HIF binding sites (HREs) as described in the methods. A summary of each genes profile, its accession number at the National Center for Biotechnology Information (NCBI), and the identified putative HRE locations are listed; NA-not applicable (no binding sites identified), NS-not significant.

Table 4

NAD/NADH ratio in pigmented and non-pigmented human melanoma cells.

Media used for pigmentation	NAD / NADH ratio	
	Pigmented SKMEL-188	Non-pigmented SKMEL-188
Ham's F10 with L-tyrosine	3.39 ± 0.23	2.73 ± 0.06 **
DMEM/Ham's F10 (75/25)	4.11 ± 0.15	3.56 ± 0.19 **

**
 $p < 0.01$ by Student's *t*-test

Table 5

Melanogenesis stimulates expression of several genes regulated by HIF-1.

Gene	Mean Cp value $\pm$ S.D.	
	Non-pigmented cells	Pigmented cells
<i>VEGF</i>	6.04 $\pm$ 0.31	0.85 $\pm$ 0.18
<i>GLUT1</i>	6.55 $\pm$ 0.28	0.11 $\pm$ 0.31
<i>Tyr</i>	4.68 $\pm$ 0.22	3.05 $\pm$ 0.10
<i>LDHA</i>	9.34 $\pm$ 0.16	3.05 $\pm$ 0.10
<i>MCT2</i>	3.52 $\pm$ 0.17	0.16 $\pm$ 0.15
<i>NHE1</i>	9.34 $\pm$ 0.16	-3.09 $\pm$ 0.26
<i>PDK1</i>	-2.12 $\pm$ 0.33	-8.25 $\pm$ 0.16
<i>ALDOA</i>	0.04 $\pm$ 0.35	-5.11 $\pm$ 0.31
<i>HK2</i>	0.38 $\pm$ 0.16	-4.77 $\pm$ 0.32

Expression of mRNAs in non-pigmented versus pigmented SKMEL-188 melanoma cells grown as described as in the methods was compared to β -actin using a comparative Cp method. Relative gene expression data were calculated using the Cp method. Changes in gene expression are presented as a relative quantities using the mean Cp (normalized target) $\pm$ S.D. to calculate the difference between the target gene and the reference gene by comparing the first cycle of appearance above threshold (crossing point, Cp) using standardized algorithms in the Roche LC480 1.5 software package. All data (pigmented vs non-pigmented cells) are statistically significant, $p < 0.0001$. VEGFA (vascular endothelial growth factor A); GLUT-1 (glucose receptor 1); Tyr (Tyrosinase); LDHA (lactate dehydrogenase A); MCT2 (monocarboxylate transporter 2); NHE1 (sodium hydrogen exchanger); PDK1 (pyruvate dehydrogenase kinase, isozyme 1); ALDOA (aldolase A, fructose-bisphosphate); HK2 (hexokinase 2). A lower Cp numeric value reflects a more abundant signal for each particular gene (PCR product) of interest.

Table 6

Time- and pigmentation-dependent changes in gene expression in human melanoma cells.

Gene	2 days culture, mean Cp $\pm$ S.D.		3 days culture, mean Cp $\pm$ S.D.		
	Non-pigmented cells	Pigmented cells	Non-pigmented cells	Pigmented cells	Pigmented and detached cells
<i>ALDOA</i>	12.95 $\pm$ 0.27	11.57 $\pm$ 0.22 **	11.49 $\pm$ 0.51	4.71 $\pm$ 0.44 ****	2.91 $\pm$ 0.95 ***
<i>PKD1</i>	5.5 $\pm$ 0.18	3.24 $\pm$ 0.18 ****	4.79 $\pm$ 0.43	-1.19 $\pm$ 0.22 ****	-2.41 $\pm$ 0.34 ****
<i>HK2</i>	11.17 $\pm$ 0.21	10.25 $\pm$ 0.28 **	10.13 $\pm$ 0.53	5.54 $\pm$ 0.22 ***	3.27 $\pm$ 0.37 ****
<i>GLUT1</i>	17.02 $\pm$ 0.11	13.56 $\pm$ 0.22 ****	14.68 $\pm$ 0.46	9.61 $\pm$ 0.37 ***	8.29 $\pm$ 0.41 ****
<i>LDHA</i>	14.91 $\pm$ 0.22	10.22 $\pm$ 0.19 ****	13.4 $\pm$ 0.36	8.85 $\pm$ 0.17 ****	6.37 $\pm$ 0.16 ****
<i>VEGFA</i>	17.02 $\pm$ 0.11	6.39 $\pm$ 0.14 ****	17.42 $\pm$ 0.36	11.87 $\pm$ 0.16 ****	11.85 $\pm$ 0.43 ****
<i>Tyr</i>	9.61 $\pm$ 0.21	0.26 $\pm$ 0.31 ****	3.23 $\pm$ 0.39	2.83 $\pm$ 0.18	ND
<i>MCT4</i>	8.48 $\pm$ 0.102	7.1 $\pm$ 0.11 ****	7.89 $\pm$ 0.16	6.24 $\pm$ 0.11 ****	8.48 $\pm$ 0.102

Relative mRNA levels present in of Sk-Mel-188 non-pigmented and pigmented cells grown for 2 or 3 days after stimulation of melanogenesis were compared to reference the gene Cyclophilin B. Relative gene expression data were calculated using Cp method. Changes in gene expression are presented as a relative quantities using mean Cp (normalized target) $\pm$ S.D. as the difference between target gene and reference gene in cycle of appearance in time (Cp). Data were compared between pigmented and non-pigmented cells using Student's *t*-test

* $p < 0.05$

ND-not detected. A lower Cp numeric value reflects a more abundant signal for each particular gene (PCR product) of interest.

**
 $p < 0.01$

***
 $p < 0.001$

****
 $p < 0.0001$

Table 7

Pigmentation-dependent gene expression in human SKMEL-188 melanoma cells.

Gene	Mean Cp $\pm$ S.D.	
	Non Pigmented Cells	Pigmented Cells
CRH	4.51 $\pm$ 0.37	3.14 $\pm$ 0.63 *
CRH, exon 2	5.24 $\pm$ 0.28	3.14 $\pm$ 0.63 **
CRHR1	3.45 $\pm$ 0.36	1.53 $\pm$ 0.72 *
POMC, exon 3	5.24 $\pm$ 0.28	2.02 $\pm$ 0.66 **
MC1	5.24 $\pm$ 0.28	-3.01 $\pm$ 0.69 ****
MC2	3.07 $\pm$ 0.31	2.02 $\pm$ 0.67
PCSK1	5.24 $\pm$ 0.28	3.14 $\pm$ 0.63 **
PCSK2	5.24 $\pm$ 0.28	-31.86 $\pm$ 0.001 ****
NQO2	5.24 $\pm$ 0.28	-2.89 $\pm$ 0.65 ****
Cyp11A1, exon 4	5.24 $\pm$ 0.26	3.14 $\pm$ 0.63 **
Cyp11B1	4.16 $\pm$ 0.31	0.17 $\pm$ 0.64 ***
β HSD	5.24 $\pm$ 0.28	3.14 $\pm$ 0.63 **
Cyp17A1	-10.70 $\pm$ 0.3	0.15 $\pm$ 0.63 ****
Cyp21A2v2	-29.76 $\pm$ 0.001	3.14 $\pm$ 0.063 ****
HSD11B1	-12.86 $\pm$ 0.42	-4.38 $\pm$ 0.64 ****
HSD11B2	-13.48 $\pm$ 0.45	-4.04 $\pm$ 0.7 ****
7DHCR	-1.87 $\pm$ 0.34	3.14 $\pm$ 0.63 ***
ICAM1	11.41 $\pm$ 0.19	11.78 $\pm$ 0.25
IFN α 1	-0.07 $\pm$ 0.20	0.25 $\pm$ 0.26
IFN β 1	7.41 $\pm$ 0.31	7.83 $\pm$ 0.26
IFN γ	-23.59 $\pm$ 0.1	-23.22 $\pm$ 0.1
TNF α	7.02 $\pm$ 0.35	7.12 $\pm$ 0.32
IL1 α	11.41 $\pm$ 0.19	11.78 $\pm$ 0.25
IL1 β	11.38 $\pm$ 0.20	11.41 $\pm$ 0.41
IL2	11.41 $\pm$ 0.19	11.78 $\pm$ 0.25
IL4	9.47 $\pm$ 0.22	9.77 $\pm$ 0.81
IL5	-6.13 $\pm$ 0.36	11.14 $\pm$ 0.41 ****
IL6	7.28 $\pm$ 0.21	7.86 $\pm$ 0.32
IL8 (CXCL-8)	11.41 $\pm$ 0.19	11.78 $\pm$ 0.25
IL10	10.57 $\pm$ 0.41	11.56 $\pm$ 0.46 *

Gene	Mean Cp $\pm$ S.D.	
	Non Pigmented Cells	Pigmented Cells
IL17A	-4.89 $\pm$ 0.47	7.78 $\pm$ 0.25 ****
RANTES	11.41 $\pm$ 0.19	11.78 $\pm$ 0.25
PDGFA	-0.61 $\pm$ 0.21	6.84 $\pm$ 0.30 ****
TGFA	3.59 $\pm$ 0.34	4.29 $\pm$ 0.40
TGFB1	9.33 $\pm$ 0.24	9.82 $\pm$ 0.27
TGFB2	0.37 $\pm$ 0.20	-23.22 $\pm$ 0.001 ****
EGFR	7.10 $\pm$ 0.34	7.57 $\pm$ 0.28
TRANSFERRIN RC	-11.69 $\pm$ 0.37	-5.61 $\pm$ 0.42 ****
NPL	11.41 $\pm$ 0.19	11.78 $\pm$ 0.25

Changes in gene expression of human SKMEL-188 melanoma cells grown for three days in culture in F10 media (non-pigmented) or in DMEM media (pigmented) as described in the methods, after normalization to the reference gene cyclophilin B. Changes in gene expression are presented as a relative quantities using mean Cp (normalized target) $\pm$ S.D. as the difference between target gene and reference gene in cycle of appearance in time (Cp). Data were compared between pigmented and non-pigmented cells using Student's *t*-test. Genes profiled include: CYP11A1 (cytochrome P450, family 11, subfamily A, polypeptide 1) exon 4; CYP11B1 (cytochrome P450, family 11, subfamily B, polypeptide 1); CYP17A1 (cytochrome P450, family 17, subfamily A, polypeptide 1); CYP21 A2v2 (cytochrome P450, family 21, subfamily A, polypeptide 2); HSD11B1 (hydroxysteroid (11-beta) dehydrogenase 1); HSD11B1 (hydroxysteroid (11-beta) dehydrogenase 2); 7DHCR (7-dehydrocholesterol reductase); 3 β HSD (3-beta-hydroxysteroid dehydrogenase); CRH (corticotropin releasing hormone); CRHR1 (corticotropin releasing hormone receptor 1); POMC (proopiomelanocortin); MC (melanocortin receptor); PCSK (proprotein convertase subtilisin/kexin type); NQO2 (NAD(P)H dehydrogenase, quinone 2); EGFR (epidermal growth factor receptor); ICAM1 (intercellular adhesion molecule 1); IFN (interferon); IL (interleukin); CXCL (chemokine ligand); TNF (tumor necrosis factor); PDGFA (platelet-derived growth factor alpha polypeptide); TGFB (transforming growth factor, beta); RANTES (CCL5 chemokine (C-C motif) ligand 5); TRANSFERRIN RC (transferrin receptor); NPL (N-acetylneuraminase pyruvate lyase (dihydrodipicolinate synthase))

A lower Cp numeric value reflects a more abundant signal for each particular gene (PCR product) of interest.

*
 $p < 0.05$

**
 $p < 0.01$

***
 $p < 0.001$

****
 $p < 0.0001$.