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Constitutive HIF-1 activity in malignant melanoma

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ABSTRACT

The hypoxia-inducible factor-1 (HIF-1), which consists of the constitutive HIF-1β and the oxygen-responsive HIF-1α subunit, is the master activator of the cellular transcriptional response to hypoxia coordinating gene expression during reduced oxygen tension.

Overexpression of HIF-1 and increased transcriptional activity induced by hypoxia are linked to progression of many tumour types such as head and neck cancer, cervical carcinoma, leukaemia and renal cell carcinoma.

In this study, we demonstrate that HIF activity is increased in malignant melanoma cells already under normoxic conditions in contrast to other tumour types. HIF-1α and -2α knockdown by siRNA transfection revealed that this effect is due to constitutive HIF-1α expression. Furthermore, the inhibition or activation of reactive oxygen species (ROS) decreased or activated, respectively, HIF-1 activity and HIF-1α protein expression. Interestingly, the inhibition of the NFkappaB pathway also reduced the accumulation of HIF-1α assuming a context between ROS and NFkappaB, and suggesting that ROS and NFkappaB activity contribute to HIF-1α accumulation. In summary, we identified an increased HIF-1α protein expression and activity in melanoma under normoxia mediated by ROS and the NFkappaB pathway.

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1. Introduction

HIF-1 is the key transcriptional regulator of the cellular response to a hypoxic environment. It is involved in many cellular processes that help cells and organisms to cope with a reduced oxygen and energy supply.[1] HIF-1 is composed of two subunits: HIF-1α and HIF-β. HIF-β, which was previously identified as the aryl hydrocarbon receptor nuclear translocator (ARNT) and is also part of other transcription factors, is required to form a functional DNA-binding complex with the HIF-α subunit. HIF-1α is the oxygen-regulated subunit which is stabilised under hypoxic conditions.[2] In normoxia (21% O_2), HIF-1α is hydroxylated by specific prolyl hydroxylases (PHDs) which allows binding to the von Hippel–Lindau protein (VHL). By these means HIF-1α is targeted for ubiquitination and rapid proteasomal degradation.[3] In the absence of O_2, the prolyl-hydroxylation is inhibited and the HIF-1α subunit is stabilised. It translocates into the nucleus and forms a complex with the constitutively expressed HIF-β subunit.[4] The functional HIF-1 dimer binds to the HIF-binding site (HBS) with the consensus sequence RCGTG in hypoxia-responsive elements in the promoter or enhancer regions of its target genes.[5,6] The active HIF-1 complex induces O_2-responsive genes that are involved in increased anaerobic glucose metabolism and cell survival under hypoxic conditions and also in crucial aspects of cancer development and progression including angiogenesis and tumour invasion.[7–11] HIF-1α accomplishes these tasks by controlling the expression of

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GLUT-1 and many glycolytic enzymes, VEGF and several other angiogenic factors, growth factors, transferrin receptor 1 (TfR1), CXCR4 chemokine receptor[12] and lysyl oxidase,[13–18] whereas the induction of erythropoietin[19,20] was recently showed to depend primarily on the alternative HIF-α subunit HIF-2α.

In a hypoxic microenvironment melanoma cells show increased HIF-1α expression and induction of vasculogenic mimicry to acquire an adequate blood supply.[21,22]

However, regulation of HIF-1 activity seems to be complex and next to hypoxia further inducers of HIF activity are known: High amounts of ROS such as superoxide radicals (O_2^{-}), hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen ($^1O_2$) can induce cell damage. There is evidence that ROS can act as signalling molecules and control the activity of HIF-1.[23,24] Tumour cells possess increased ROS levels due to decreased levels of antioxidants that scavenge the produced ROS, which promote tumour progression.[25] In melanoma cells increased levels of ROS contribute to melanoma development.[26]

HIF-1α expression is also regulated at the translational and transcriptional level by the AKT/phosphatidylinositol 3-kinase (PI3K) and the MAPK/ERK pathways, respectively.[27–29] It has been shown that RAS regulates the RAF/MEK/ERK pathway and that BRAF is essential for ERK activation in malignant melanoma cells.[30] The mutant V600E BRAF increases HIF-1α expression in melanoma leading to a higher cell survival under hypoxic conditions.[31] It was shown that a hypoxic environment, which is found in normal skin, contributes to AKT-mediated melanocyte transformation. Downregulation of HIF-1α by the mTOR inhibitor rapamycin prevents the transformation under hypoxia. Therefore, Rapamycin was proposed as a therapeutic approach in melanoma treatment.[32,33]

Furthermore, Bonello and colleagues showed binding and activation of the *HIF*-1α promoter by NFkappaB.[34,35] Rius and colleagues[35] demonstrated for the first time *in vivo* by the use of IKK-β knockout mice that basal NFkappaB activity is crucial for HIF-1α gene transcription and HIF activation under hypoxia. Interestingly, the transcription factor NFkappaB is constitutively upregulated in malignant melanoma.[36]

In this study, we present evidence that HIF-1α is expressed and transcriptionally active under normoxic conditions in malignant melanoma cells, regulated by ROS and mediated by NFkappaB.

2. Materials and methods

2.1. Cell lines and cell culture conditions

The melanoma cell lines Mel Im, Mel Ju and HMB2 were derived from metastases of malignant melanoma.[37] Furthermore, the melanoma cell line 501 Mel, the colon carcinoma cell line SW480 (ATCC CCL-228) and the hepatocellular carcinoma cell line HepG2 (ATCC HB-8065) were used.

For tissue culture, the cells were maintained in Dulbecco's modified Eagle's medium supplemented with penicillin (400 U/ml), streptomycin (50 μg/ml), L-glutamine (300 μg/ml), and 10% v/v foetal calf serum (Invitrogene, Carlsbad, USA) and diluted 1:5 every 3 d. The cells were cultivated under a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Reagents

Echinomycin was purchased from Alexis Biochemicals (Lörrach, Germany), YC-1 from Merck (Darmstadt, Germany). All other chemicals were purchased from Sigma (St. Louis, USA). Incubation time for the chemicals was 16 h for histidine (30 μM), alpha-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone (POBN) (30 μM), Echinomycin (5 nM), YC-1 (30 μM), arsenic trioxide (As_2O_3) (2 μM, respectively, 5 μM), Pyrogallol (30 μM), Rapamycin (1 μM) and 2,2′dipyridyl (DP, 50 μM).

2.3. RNA isolation and reverse transcription

Total cellular RNA was isolated from cultured cells using the RNeasy kit (Qiagen, Hilden, Germany). cDNAs were generated by reverse transcriptase reaction performed in 20 μl reaction volume containing 2 μg of total cellular RNA, 4 μl of 5 × first strand buffer (Invitrogen), 2 μl of 0.1 M DTT, 1 μl of dN_6-primer (10 mM), 1 μl of dNTPs (10 mM), and DEPC-water. The reaction mixture was incubated for 10 min at 70 °C, 200 U of Superscript II reverse transcriptase (Invitrogen) were added and RNAs were transcribed for 1 h at 37 °C. Reverse transcriptase was inactivated at 70 °C for 10 min and the RNA was degraded by digestion with 1 μl RNase A (10 mg/ml) at 37 °C for 30 min.

2.4. Analysis of expression by quantitative PCR

Quantitative real-time PCR for HIF-1α was performed on a LightCycler (Roche). cDNA template (2 μl), 0.5 μl (20 mM) of forward and reverse primers (HIF-1α for: 5′-CAC AGG CCA CAT TCA CGT A-3′; HIF-1α rev: 5′-ATC CAG GCT GTG TCG ACT G-3′; MAGE-11 for: 5′-GCC ATC TTT GGG AGC CTA TCT-3′; MAGE-11 rev: 5′-GGG TCC ACT TCC TTC ACA TCA-3′; VEGF for: 5′-CAG CGC AGC TAC TGC CAT CCA ATC GAG A-3′; VEGF rev: 5′-GCT TGT CAC ATC TGC AAG TAC GTT CGT TTA-3′; AngPTL4 for: 5′-CAG GGT ACC TAA GAG GAT GAG CGG TGC-3′; AngPTL4 rev: 5′-CTG CTC GAG CTG CAG GAG TCC GTG C-3′) and 10 μl of SYBR Premix *Ex Taq* (TaKaRa Bio,USA) in a total of 20 μl were applied to the following PCR programme: 30 s at 95 °C (initial denaturation); 20 °C/s temperature transition rate up to 95 °C for 15 s, 3 s at 64 °C, 5 s at 72 °C, 82 °C acquisition mode single, repeated for 40 times (amplification). The PCR reaction was evaluated by melting curve analysis and checking the PCR products on 2% w/v agarose gels. β-Actin was amplified to ensure cDNA integrity and to normalise expression.

2.5. Transfection experiments and luciferase reporter gene assay

For transient transfections 2×10^5 cells were seeded into each well of a six-well plate and transfected with 0.5 μg plasmid DNA using the lipofectamine plus method (Gibco) according to the manufacturer's instructions. Transfection efficiency was normalised according to Renilla luciferase activity by cotransfecting 0.1 μg of the plasmid pRL-TK (Promega, Madison, USA). The 6 × HRE reporter plasmid previously described by Warnecke and colleagues[38] and the NFkappaB-luc plasmid (Promega, Mannheim, Germany) were used for the reporter gene assay. Cotransfections were performed using expression

plasmids of the NFkappaB subunits p50, p52 and p65 in pET4T.[39] The expression vector for dominant negative HIF-1α was generated by introducing a stop codon (TGA) after AA 380 in the wild type mouse HIF-1α coding sequence. The resulting truncated protein thus consists of the bHLH and PAS domains, but lacks the oxygen-dependent degradation domain and all transactivation domains. Therefore it competes with wild type HIF-1α for the dimerisation partner ARNT, and may bind to HREs but does not transactivate the respective target genes. Adenoviral transfection of Mel Im cells with the recombinant replication-deficient adenovirus Ad5IκB was previously described.[40,41] The cells were lysed 16 h after transfection and luciferase activities were determined. All transfections were repeated at least three times.

2.6. SiRNAs and transfection procedures

HIF-α siRNAs (HIF-1α sense 5′ CUGAUGACCAGCAACUUGAdTdT; HIF-2α sense 5′ CAGCAUCUUUGAUAGCAGUdTdT) were described before[42,43] and synthesised by Qiagen (Cologne, Germany). SiRNA against firefly luciferase comprised the pGL3 vector (3luc sense 5′ CUUACGCUGAGUACUUCGAdTdT) was used as control. Floating cells were transfected with the siRNAs (final concentration 50 nmol/l) by the use of HighPerfect Transfection Reagent (Qiagen, Karlsruhe Hilden, Germany) in serum-free medium according to the manufacturer's protocol. After 4 h, 10% v/v foetal calf serum was added and cells were incubated for 20 h. Then the cells were lysed for Western blot analysis or transfected with 6 × HRE plasmid using the lipofectamine plus method and luciferase reporter gene assays were performed after 20 h.

2.7. HIF-1α Immunoblot assays

Cells (2×10^6) were washed and lysed in 6.65 M urea, 10% v/v glycerol, 1% w/v SDS, 10 mM Tris HCl (pH 6.8), protease inhibitors, and 5 mM DTT. Equal amounts of protein were separated on 8.75% SDS–PAGE gels and subsequently blotted onto a PVDF membrane. After blocking for 1 h with 5% w/v milk powder/TBS, the membrane was incubated for 16 h with a 1:500 dilution of HIF-1α antibody (Novus Biological, NB100–449, Novus Biologicals, Littleton, CO, USA). A 1:4000 dilution of anti-mouse-AP (Chemicon, Temecula, CA, USA) was used as secondary antibody. Staining was performed using NBT/BCIP (Zytomed, Berlin, Germany).

2.8. Immunofluorescence

5×10^5 Mel Im and Mel Ju cells were grown on eight-well chamber slide for 1 d, washed with PBS and fixed with methanol for 15 min. For permeabilisation of the cell membrane, the cells were incubated for 5 min with 0.1% v/v Triton-X-100 (Sigma), washed again and covered with blocking solution (1% w/v BSA/PBS) for 1 h. Thereafter, cells were incubated with a 1:20 dilution of MAGE-11 antibody (Abcam, ab55439) for 1 h at 37 °C. After washing the cells, they were incubated with a 1:40 dilution of secondary antibody (FITC anti-mouse, DakoCytomation, Denmark) in PBS for 1 h followed by washing again. After mounting with Hard Set Mounting Medium with DAPI (Vectashield, H-1500) images were collected by fluorescence microscopy.

2.9. Immunohistochemistry

Human skin biopsies were collected in the Molecular Pathology of the University Hospital Regensburg. The formalin-fixed and paraffin-embedded biopsies were dewaxed and rehydrated. The biopsies were then boiled for 30 min in citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked in 3% v/v H_2O_2 in PBS for 10 min. The sections were placed in a humidified chamber and covered with blocking solution (Zytomed, Berlin, Germany) for 5 min. Sections were incubated with primary anti-HIF-1α antibody (Sigma–Aldrich) in a 1:50 dilution in TBS–Tween (0.1% v/v) overnight at 4 °C. After washing with TBS–Tween, sections were incubated with biotinylated secondary antibody for 20 min (Zytomed, Berlin, Germany), before incubation with Streptavidine-HRP conjugate for 20 min and AEC (3-amino-9-ethylcarbazole) for 30 min (Zytomed) according to the manufacturers' instructions. All slides were then counterstained with routine haematoxylin.

2.10. Determination of ROS

For measurement of intracellular ROS levels, the fluorescent dye H_2DCFDA (2,7-dichlorodihydrofluoresceindiacetate) was used. Cells were stained with H_2DCFDA (Molecular Probes, Invitrogen, USA) in a concentration of 10 μM for 10 min, harvested by trypsinisation and analysed in PBS buffer by a FACS Calibur.

2.11. Statistical analysis

The results are expressed as the means ± S.D. (range) or percentages. Comparison between groups was made using the Student's paired *t* test. A p-value < 0.05 was considered statistically significant. All calculations were performed using the GraphPad Prism software (GraphPad Software Inc, San Diego, CA).

3. Results

As several target genes of HIF are known to be strongly expressed in melanoma cells already under normoxic conditions like VEGF, integrin alpha v beta 3, TfR1 and Glut-1 we became interested in the role of HIF in melanoma.

3.1. Constitutive HIF activity in melanoma

We first determined the basal HIF activity in melanoma cells lines under normoxic conditions using a 6 × HRE-luciferase construct. Interestingly, we detected normoxic HIF activity in all four melanoma cell lines analysed whereas in the colon carcinoma cell line SW480 and hepatoma cell line HepG2 only minor activity was observed (Fig. 1A). Additionally, compared to SW480 and HepG2 cells the melanoma cell lines showed increased expression of HIF-1α mRNA as measured by qRT-PCR (Fig. 1B). In tissue samples from melanoma metastases nucle-

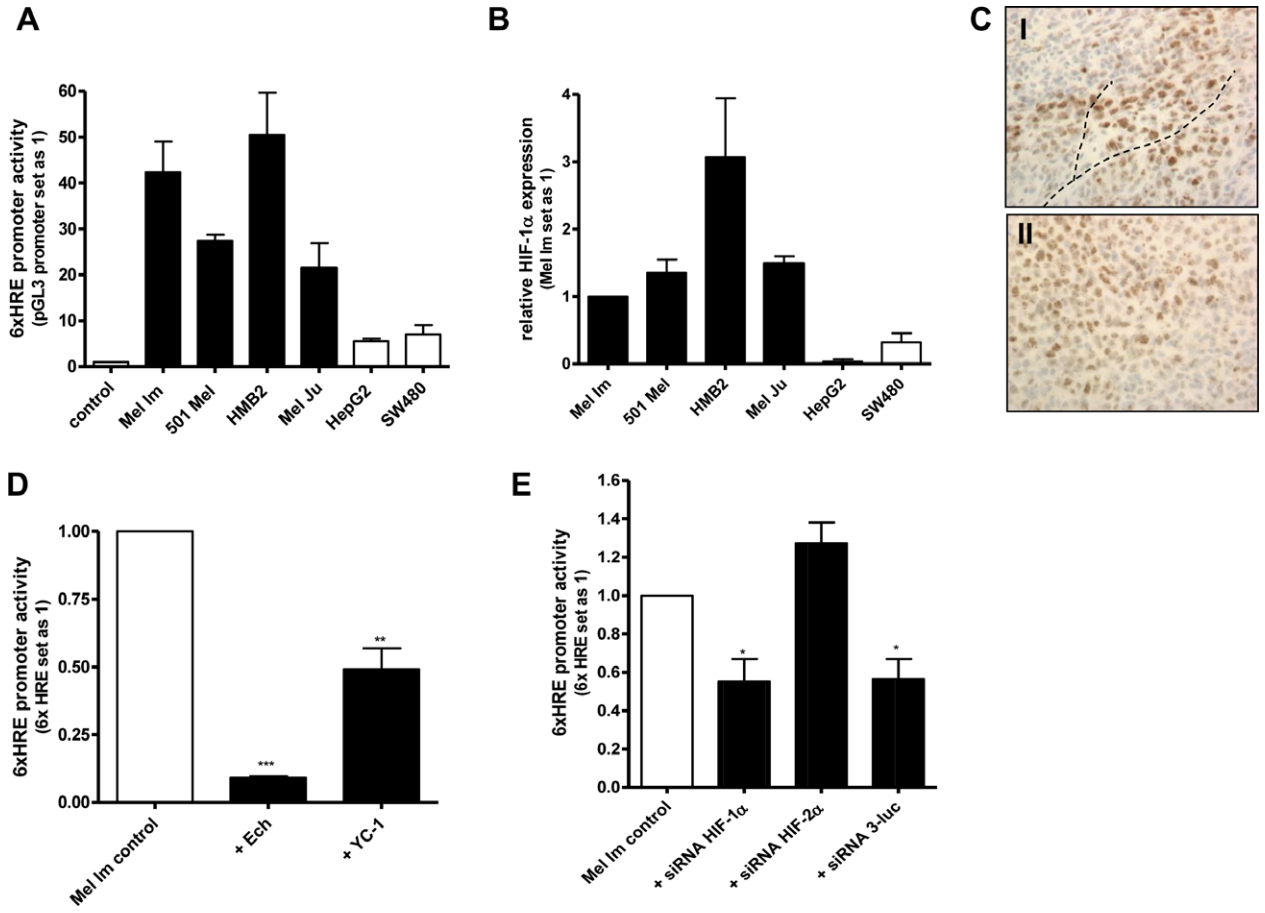

Fig. 1 – *HIF activity in melanoma under normoxia*. (A) HIF activity was determined in luciferase reporter assays using the 6 × HRE (hypoxia-responsive element) luciferase reporter which contains six copies of the phosphoglycerate kinase HRE upstream of the thymidine kinase (TK) promoter. The control vector contains the TK promoter but lacks the 6 HREs. Melanoma cell lines were compared with the hepatocarcinoma cell line HepG2 and the colon carcinoma cell line SW480. (B) The mRNA expression status of HIF-1α was determined by quantitative real-time PCR. The expression of HIF-1α in the melanoma cell line Mel Im was set as 1. The melanoma cell lines were again compared with HepG2 and SW480. (C) Immunohistochemistry was performed with an anti-HIF-1α antibody. Strong nuclear staining was detected in the tissue samples of two melanoma metastases (I, II; 400×). In (I), a blood vessel is indicated by the dotted line. (D) HIF activity was measured after incubation of the melanoma cell line Mel Im with the HIF inhibitors Echinomycin (5 nM) and YC-1 (30 μM) for 16 h. ($p < 0.01$, ***$p < 0.001$). (E) HIF activity was measured after transfection of the melanoma cell line Mel Im with the siRNA against HIF-1α and HIF-2α. Mel Im control shows the normoxic HIF activity determined by the use of the 6 × HRE promoter construct. Transfection of siRNA against the luciferase gene (siRNA3-luc) served as positive control. (*$p < 0.05$).**

ar HIF-1α expression was detectable in nests of melanoma cells as determined by immunohistochemistry (Fig. 1C). Interestingly, no differences in staining intensity were observed comparing cells next to vessels with cells more distant, suggesting that HIF-1α protein accumulation did not correlate with areas of regional hypoxia.

To manipulate the normoxic HIF-1 activity in melanoma, cells were treated with chemical inhibitors of HIF activity, Echinomycin (Ech), an inhibitor of DNA binding, and YC-1, which enhances HIF degradation. Exposure to either compound significantly reduced HIF activity (Fig. 1D).

Finally, treatment of the melanoma cell line Mel Im with siRNA against HIF-1α[42,43] resulted in downregulation of HIF-driven luciferase expression in comparison to negative control siRNA-transfected cells. SiRNA3-luc, which downregulates luciferase expression from the pGL3 luciferase vectors, was used as positive control (Fig. 1E). HIF-1α knockdown had equivalent effects on luciferase activities to the direct luciferase knock-down by the 3luc siRNA, which indicates that luciferase expression in Mel Im was completely dependent on HIF-1α. SiRNA transfection targeting HIF-2α, leading to 55% reduction of HIF-2α mRNA expression (data not shown), had no significant effect on HIF activity. We, therefore, concluded that HIF activity in melanoma was mainly due to activation of the subunit HIF-1α.

To confirm that the constitutive HIF-1α activity under normoxic conditions has effects on the expression of HIF target genes in melanoma we performed further assays. First of all, down-regulation of HIF-1α protein after siRNA transfection was confirmed by western blotting using an anti-HIF-1α antibody (Fig. 2A). In addition, a dominant negative variant of HIF-1α (HIF-1α dn) was transfected leading to reduction of

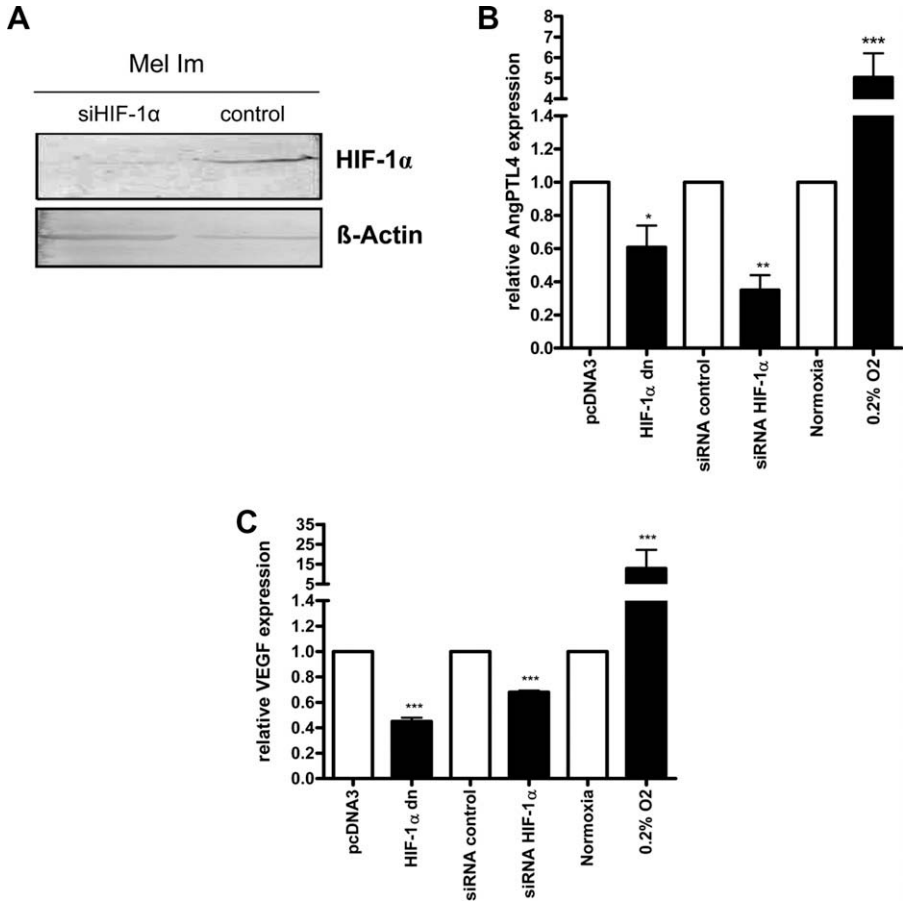

Fig. 2 – *HIF-1α induces target genes under normoxia*. (A) Protein extracts of Mel Im cells transfected with control siRNA and siRNA against HIF-1α were analysed in western blot. The elimination of the band by the siRNA transfection demonstrated the efficiency of the HIF-1α knockdown and confirmed that the band in the control siRNA-transfected samples represented indeed normoxic HIF-1α. (B and C) mRNA expression of the HIF-1α target genes AngPTL4 and VEGF under normoxic conditions after HIF-1α knockdown or overexpression of a dominant negative HIF-1α (HIF-1α dn; the empty pcDNA3 vector served as control). For comparison cells were also exposed to hypoxia (0.2% O_2). (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

HIF activity. Downregulation of HIF-1α by both means resulted in significantly reduced expression of the HIF-1α target genes AngPTL4 (Fig. 2B) and VEGF (Fig. 2C). As all experiments were performed under normoxic conditions, so far, we aimed to analyse the influence of hypoxic conditions on the expression of the target genes AngPTL4 and VEGF. The melanoma cell line Mel Im was kept under reduced oxygen tension (0.2%) and induction of target gene expression was determined (Fig. 2B and C). Interestingly, induction of expression of the target genes under hypoxia was weaker than expected and previously published for other cell types than melanoma.[42,44] We speculated that the high constitutive HIF-1α expression under normoxia in melanoma cells results in reduced inducibility.

3.2. *Regulation of HIF activity*

Next, we aimed to understand the regulation of HIF activity under normoxic conditions in melanoma. It is known that melanosomes contribute to increased oxidative stress and production of ROS in malignant melanoma cells.[45–47] Regarding this aspect, we analysed the intracellular ROS production in four melanoma cell lines in comparison to HepG2 (hepatocarcinoma cell line) and SW480 (colon carcinoma cell line) using H_2DCFDA as fluorescence marker. All melanoma cell lines showed significantly higher intracellular ROS production in comparison to HepG2. The cell lines 501 Mel and HMB2 produced higher levels of ROS than SW480 (Fig. 3A). Therefore, we analysed the involvement of ROS in the regulation of HIF activity under normoxic conditions. Treatment with ROS inhibitors (POBN, histidine) resulted in strong reduction of HIF-1 activity (Fig. 3B) and protein expression (Fig. 3C). Vice versa, induction of ROS by As_2O_3 or Pyrogallol-enhanced HIF activity and HIF-1α protein level (Fig. 3D and E).

3.3. *Involvement of NFkappaB for transcriptional HIF regulation*

ROS are known to induce NFkappaB activity.[34,21,48] Additionally, melanoma cell lines show constitutively activated NFkappaB.[36] We determined the NFkappaB activity in the four melanoma cell lines in comparison with HepG2 and SW480. The high NFkappaB activity in the melanoma cell lines is in accordance to the literature. HepG2 and SW480 showed significant lower NFkappaB activity than the melanoma cell

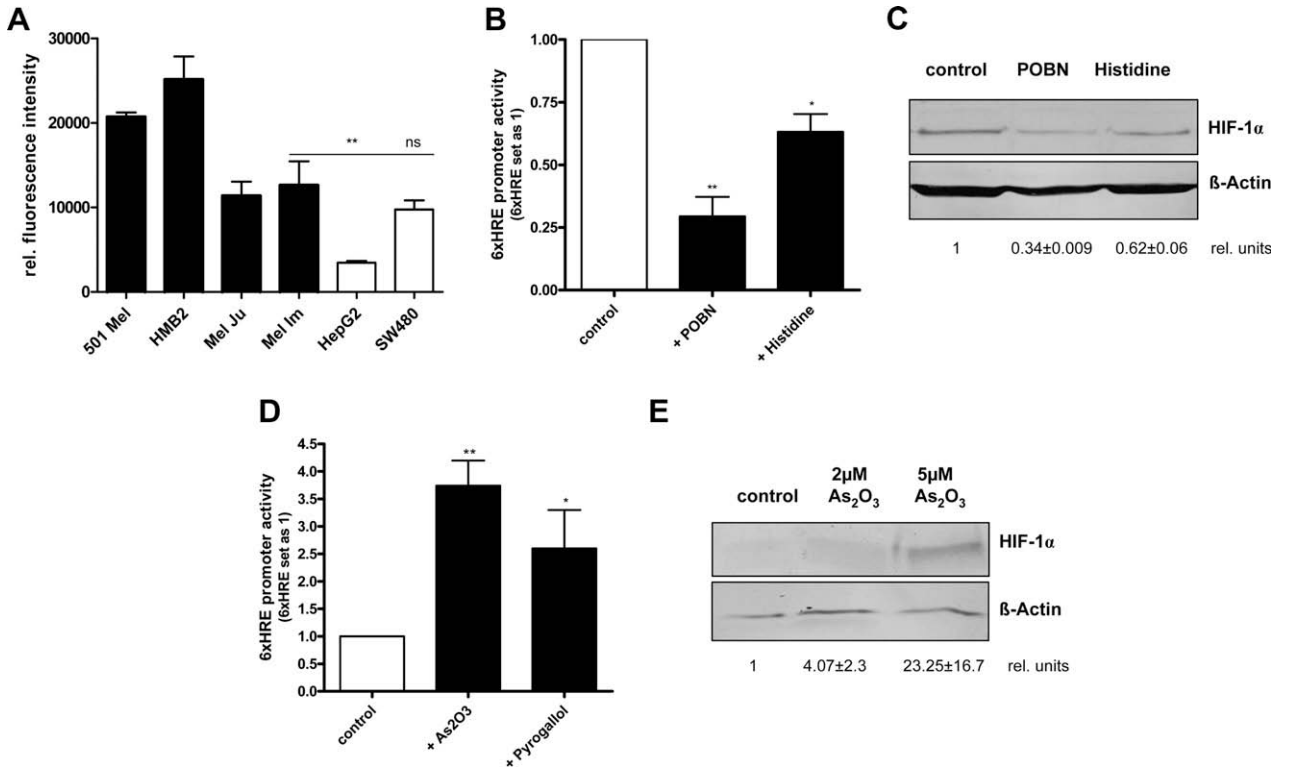

Fig. 3 – *HIF regulation through ROS under normoxia*. (A) Intracellular ROS levels were determined by H_2DCFDA staining and FACS analysis for the cell lines Mel Im, HMB2, Mel Ju, 501Mel, SW480 and HepG2. Analysis of the *p*-value shows significance (*$p < 0.05$) or no significance (ns) measured between Mel Im and HepG2 and Mel Im/SW480, respectively. (B) HIF activity was measured in luciferase assays using the 6 × HRE reporter. The construct lacking the HREs was used as control. The melanoma cell line Mel Im was treated with ROS quencher POBN (30 μM) and histidine (30 μM). (*$p < 0.05$; **$p < 0.01$). (C) Western blot with anti-HIF-1α antibody shows the modulation of HIF-1α after incubation of the cell line Mel Im with POBN (30 μM) and histidine (30 μM). Protein lysates (40 μg) were loaded. Data are mean ± SE from triplicate experiments normalised to β-actin. (D) HIF activity was measured in luciferase assays using the 6 × HRE reporter. The melanoma cell line Mel Im was treated with ROS inducer As_2O_3 (5 μM) and Pyrogallol (30 μM). (*$p < 0.05$; **$p < 0.01$). (E) Western blot with anti-HIF-1α antibody shows the modulation of HIF-1α after incubation of the cell line Mel Im with ROS inducer As_2O_3 (2 μM, 5 μM). Protein lysates (5 μg) were loaded. Data are mean ± SE from triplicate experiments normalised to β-actin.

lines (Fig. 4A). We then analysed the correlation between ROS and NFkappaB regarding normoxic HIF activity. Using inhibitors of ROS (histidine and POBN), we observed strong inhibition of NFkappaB activity in melanoma cells (Fig. 4B). We concluded that ROS are involved in regulating NFkappaB in melanoma cells. Overexpression of an inhibitor of NFkappaB activity, dominant active IkappaB, in the melanoma cell line Mel Im using adenoviral transduction resulted in significant reduction of HIF activity (Fig. 4C). Reduction of HIF activity by incubation of Mel Im cells with the dominant active IkappaB was similar to the effect of POBN and no significant synergistic effect was observable by simultaneous inhibition of ROS and NFkappaB. To confirm the regulation of HIF activity by NFkappaB, transfection of Mel Im cells with expression constructs for the NFkappaB subunits p50, p52 and p65 were performed. All led to significant induction of HIF activity (Fig. 4D). We, therefore, conclude that HIF expression is controlled by ROS via NFkappaB.

Since mTOR regulates HIF-1α translation[49,50] we also analysed the involvement of mTOR in HIF-1α activity in melanoma. Interestingly, we also observed an effect of Rapamycin on HIF activity and protein expression (Fig. 5A and B). However, no effect of Rapamycin on NFkappaB activity (Fig. 5C) or HIF1α mRNA expression was detectable (data not shown), indicating that two alternative HIF-inducing pathways are active in melanoma.

3.4. *Involvement of MAGE-11 in the HIF protein depletion in melanoma*

As it is known that HIF is regulated on the transcriptional level as well as on the posttranscriptional level (via modulation of protein translation on the one hand and oxygen-dependent protein degradation on the other), we hypothesised that we see a dual way of regulation. HIF-1α expression is induced on the transcriptional level via ROS and NFkappaB, and on the translational level through active mTOR. Only recently, a study revealed that MAGE-11 is a regulator of HIF protein stability by virtue of its ability to bind to and inhibit PHD activity.[51] We, therefore, analysed MAGE-11 mRNA expression in the melanoma cell lines and compared it with HepG2 and SW480 cells. We detected a high MAGE-11 expression in

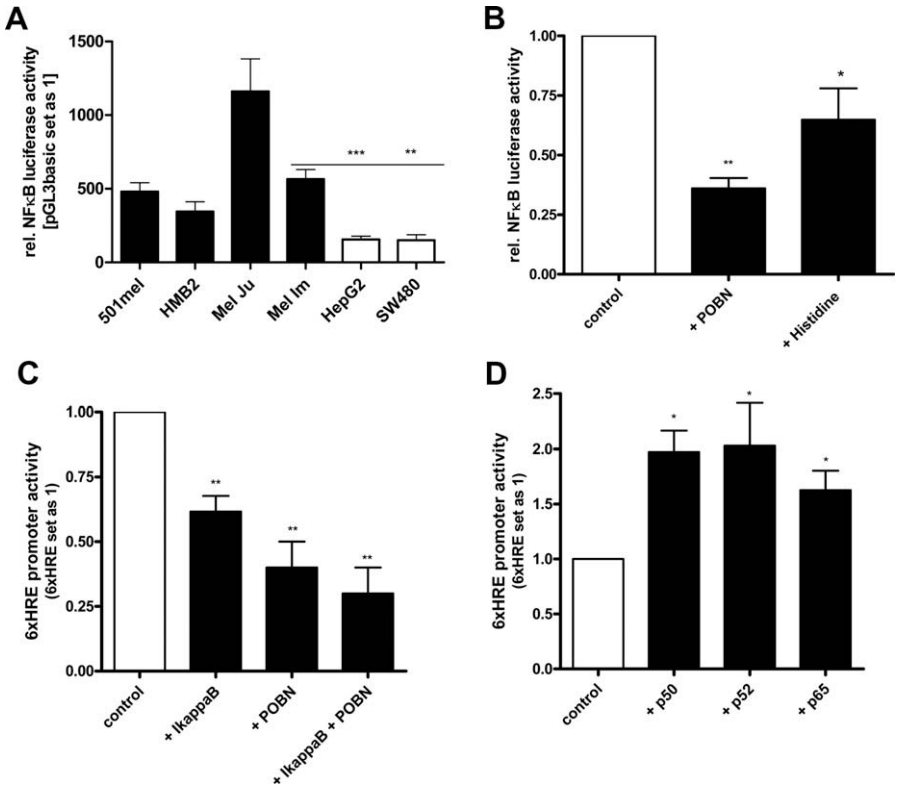

Fig. 4 – *Transcriptional regulation of HIF-1α through ROS is mediated by NFkappaB*. (A) NFkappaB activity was measured in luciferase assays. The pGL3basic vector was used as control. Analysis of the *p*-value shows significance (*$p < 0.001$; **$p < 0.01$) measured between Mel Im/HepG2 and Mel Im/SW480, respectively. (B) NFkappaB luciferase assay showing the regulation of NFkappaB transcriptional activity after incubation of the cell line Mel Im with the ROS quenching agents POBN (30 μM) and histidine (30 μM). (*$p < 0.05$; **$p < 0.01$). (C) HIF activity as measured by the use of the 6 × HRE reporter. The melanoma cell line Mel Im was transduced with an adenovirus expressing the NFkappaB inhibitor IkappaB and treated with ROS quencher POBN (30 μM). (**$p < 0.01$). (D) The melanoma cell line Mel Im was transfected with expression vectors for NFkappaB subunits p50, p52 and p65 to analyse the induction of HIF activity through NFkappaB (*$p < 0.05$).**

qRT-PCR (Fig. 6A). Additionally, immunofluorescence staining showed the MAGE-11 protein distributed in the cytoplasm of the melanoma cell lines Mel Im and Mel Ju (Fig. 6B).

4. Discussion

HIF-1 is the master regulator of the cellular and systemic adaptation to hypoxia and is recognised as a key factor in cancer development. Usually, HIF-1α is rapidly degraded by the ubiquitin–proteasome system under normoxic conditions, and it is up-regulated in a hypoxic microenvironment. Recently, it has also been shown that HIF-1α protein can be upregulated under normoxia in response to growth factors, hormones, cytokines,[52] UV irradiation and metal ions.[53,54,47] We became interested in analysing HIF activity in melanoma cells under normoxia as several of the HIF-1α-inducing factors such as IL-1beta and endothelins[55–57] are known to be overexpressed in melanoma. In addition, several HIF target genes are strongly expressed in melanoma already under normoxic conditions. Our results revealed a marked HIF-1α activity in the melanoma cell lines under normoxic conditions in contrast to other types of tumours. Immunohistochemistry of malignant melanoma showed the focal expression of HIF-1α in cancer tissue independent of regional hypoxia, in agreement with previous results of other groups.[58,59] Furthermore, we demonstrated that part of the normoxic expression of VEGF and AngPTL4 was dependent on HIF-1α. Interestingly, the incubation of cells under reduced oxygen tension did not lead to the strong, expected upregulation found in other cell,[60,61] supporting the already high expression under normoxia.

Melanoma cells and melanocytes show an unusual antioxidative system.[26] The melanocytes primary function is delivering melanin in melanosomes to keratinocytes resulting in protection against harmful effects of UV radiation. Within the melanocytes the synthesis of melanin results in generation of hydrogen peroxide and, if inappropriately processed, of hydroxyl radicals and other reactive oxygen species (ROS).[46,26] In particular, melanosomes within melanoma cells are characteristically abnormal, with fragmented melanin and disrupted membranes. The disruption of melanosomal melanin might be an early event in the aetiology and progression of melanoma, leading to increased oxidative stress, production of ROS and mutation of DNA.[45–47] In our study we revealed that ROS is responsible for the increased HIF activity under normoxia in melanoma. Quenching or inducing reagents strictly control activity and protein level of HIF. The major redox-sensitive transcription factors in mammalian

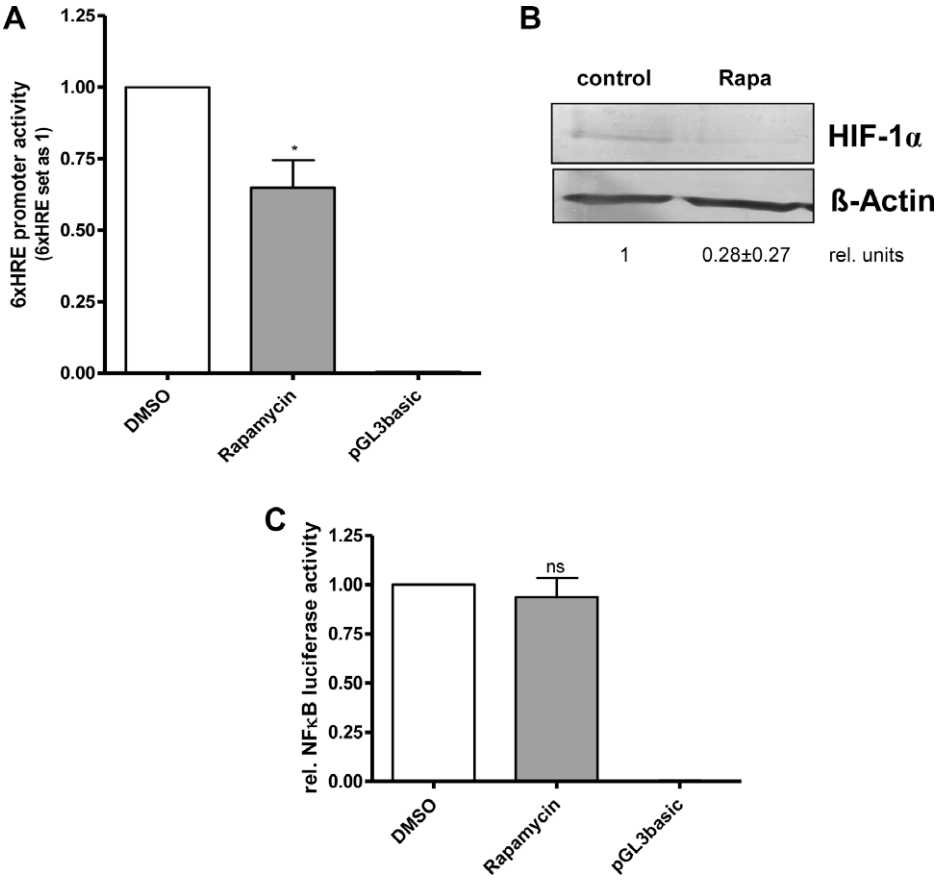

Fig. 5 – *mTOR is involved in the expression of HIF under normoxia*. (A) 6 × HRE luciferase assay. The melanoma cell line Mel Im was treated with the mTOR inhibitor Rapamycin (1 μM). (*$p < 0.05$). (B) Western blot analysis showing the modulation of HIF-1α after incubation of the cell line Mel Im with Rapamycin (1 μM). Data are mean ± SE from triplicate experiments. (C) NFkappaB luciferase assay shows no regulation of NFkappaB transcriptional activity after incubation of the cell line Mel Im with Rapamycin (ns: not significant).

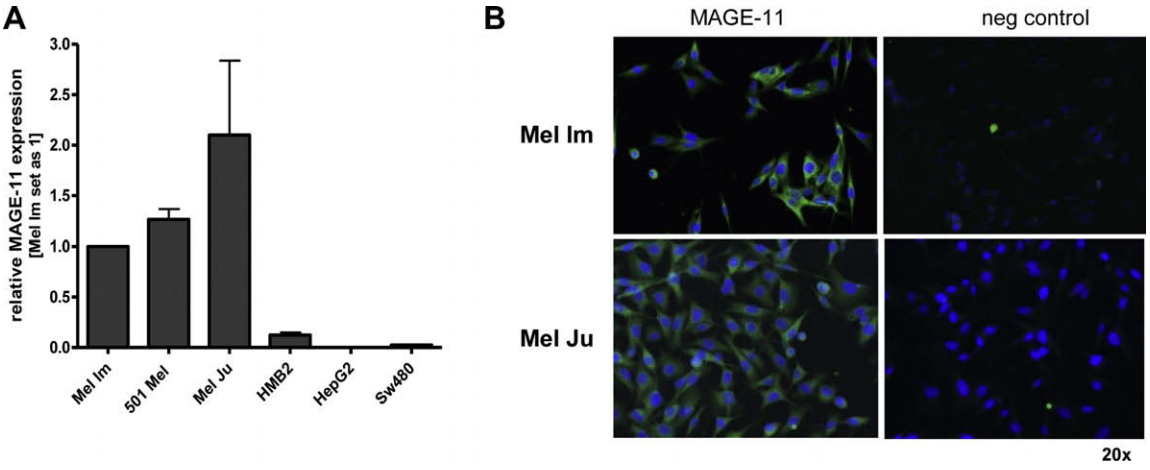

Fig. 6 – *MAGE-11 expression in melanoma*. (A) The expression level of MAGE-11 in four melanoma cell lines, the hepatocarcinoma cell line HepG2 and the colon carcinoma cell line SW480 was determined by quantitative real-time PCR. (B) Immunofluorescence images of MAGE-11 in the melanoma cell lines Mel Im and Mel Ju show the expression of MAGE-11 and localisation in the cytoplasm (20×).

cells are NFkappaB, AP-1 and thioredoxin.[62] It is also known that ROS can activate the transcription factor NFkappaB[21,48] which is constitutively activated in melanoma cells.[36] NFkappaB in turn can induce HIF-1α gene expression[34] and NF-kap-

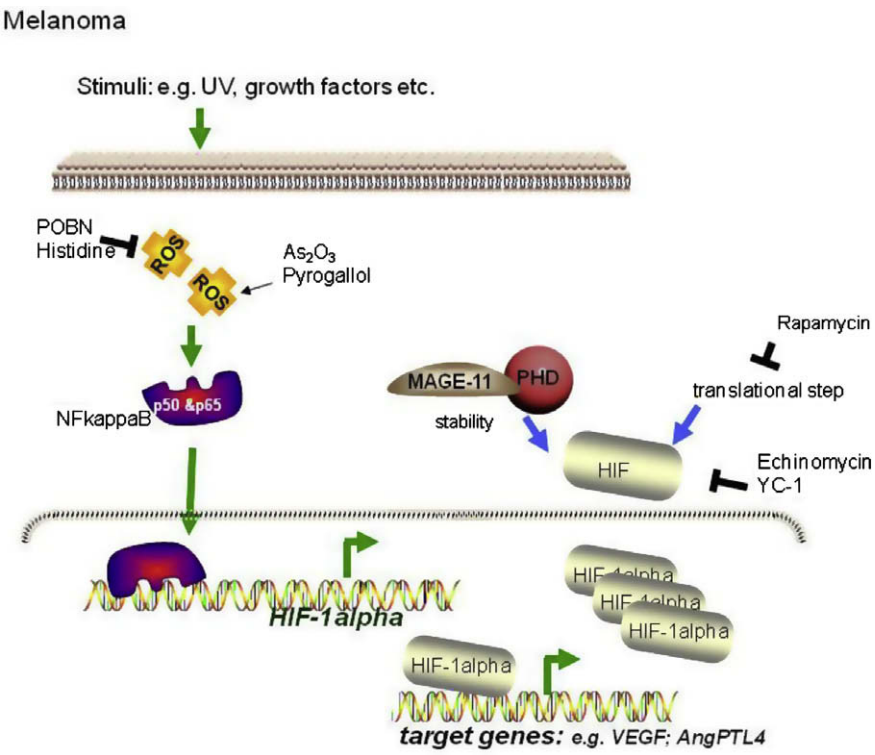

Fig. 7 – *Schematic overview of HIF regulation in malignant melanoma*. Stimuli like UV irradiation or growth factors could lead to production of ROS (reactive oxygen species). This was analysed using the ROS quencher POBN and histidine respectively the ROS inducer As_2O_3 and Pyrogallol. Our data demonstrated the regulation of NFkappaB activity through ROS. Enhanced NFkappaB activity in melanoma is involved in the transcriptional regulation of HIF-1α which in turn activates its target genes AngPTL4 and VEGF. Furthermore, HIF-1α protein stability is regulated under normoxic conditions in malignant melanoma. We believe that mTOR and MAGE-11 are responsible for enhanced normoxic HIF-1α protein stability.

paB-HIF-1 interaction contributes to breast cancer metastatic capacity.[63] We confirmed regulation of NFkappaB through ROS in malignant melanoma and demonstrated that inhibition of NFkappaB by adenoviral overexpression of the NFkappaB inhibitor IkappaB led to attenuation of HIF activity. These data support the concept of a transcriptional regulation of HIF1α by NFkappaB under normoxic conditions.

Apart from the described ROS-dependent regulation, HIF-1α is usually translationally regulated by the mammalian target of rapamycin (mTOR). The regulatory mechanisms and the involvement of molecular oxygen itself in this regulation of HIF by mTOR are poorly understood. It seems that under hypoxia mTOR is inactivated which led to the conclusion that the mTOR signalling to HIF is oxygen independently regulated.[50] Additionally, under severe hypoxia, no influence of mTOR inhibitors were observed; thus, stimulation of HIF-1α by mTOR may only be relevant under mild hypoxia or even normoxia.[50] Our results confirmed this hypothesis, as Rapamycin reduced HIF activity and protein expression under normoxia. In addition, our study showed that Rapamycin, in contrast to ROS and NFkappaB, has no influence on HIF-1α mRNA expression, suggesting posttranscriptional regulation. Recently, Aprelikova and colleagues described a novel role for the cancer-testis antigen melanoma antigen-11 (MAGE-11) as an inhibitor of PHD2.[51] We, therefore, speculated that the high expression of MAGE-11 in melanoma cells compared to other tumour cell types led to stabilisation of HIF under normoxia. Strong expression of MAGE-11 in different melanoma cell lines confirmed our hypothesis.

In summary, we could show that HIF-1α activity is induced in melanoma cells under normoxic conditions through at least two different complementary pathways (summarised in Fig. 7): a transcriptional upregulation by ROS and NFkappaB, and enhanced stabilisation through inhibition of PHD2 by MAGE-11. Furthermore, HIF-1α activity in melanoma is controlled by mTOR. As HIF plays a critical role in tumour development and progression, the induction of HIF-1α and, consequently, of target genes in melanoma cells under normoxia may be an important factor supporting tumour growth. The finding that not only the regulation of protein abundance, but also the transcriptional regulatory network is crucial in controlling HIF-1 levels in melanoma and other tumour types opens new therapeutic options to modulate HIF-1 activity under non-hypoxic conditions.[64]

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Conflict of interest statement

None declared.

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REFERENCES

1. Adams AE, Chudnovsky Y, Khavari PA. Oxygen deprivation provokes melanoma. *Nat Med* 2006;**12**:168–9.
2. Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. *J Biol Chem* 1995;**270**:1230–7.
3. Tanimoto K, Makino Y, Pereira T, Poellinger L. Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel–Lindau tumor suppressor protein. *EMBO J* 2000;**19**:4298–309.
4. Haddad JJ. Oxygen-sensitive pro-inflammatory cytokines, apoptosis signaling and redox-responsive transcription factors in development and pathophysiology. *Cytokines Cell Mol Ther* 2002;**7**:1–14.
5. Wenger RH, Gassmann M. Oxygen(es) and the hypoxia-inducible factor-1. *Biol Chem* 1997;**378**:609–16.
6. Kvietikova I, Wenger RH, Marti HH, Gassmann M. The hypoxia-inducible factor-1 DNA recognition site is cAMP-responsive. *Kidney Int* 1997;**51**:6.
7. Harris AL. Hypoxia–a key regulatory factor in tumour growth. *Nat Rev Cancer* 2002;**2**:38–47.
8. Krishnamachary B, Berg-Dixon S, Kelly B, et al. Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. *Cancer Res* 2003;**63**:1138–43.
9. Kimbro KS, Simons JW. Hypoxia-inducible factor-1 in human breast and prostate cancer. *Endocr Relat Cancer* 2006;**13**:739–49.
10. Brahimi-Horn C, Pouyssegur J. The role of the hypoxia-inducible factor in tumor metabolism growth and invasion. *Bull Cancer* 2006;**93**:E73–80.
11. Maxwell PH, Dachs GU, Gleadle JM, et al. Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. *Proc Natl Acad Sci USA* 1997;**94**:8104–9.
12. Staller P, Sulitkova J, Lisztwan J, et al. Chemokine receptor CXCR4 downregulated by von Hippel–Lindau tumour suppressor pVHL. *Nature* 2003;**425**:307–11.
13. Forsythe JA, Jiang BH, Iyer NV, et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol* 1996;**16**:4604–13.
14. Wenger RH. Cellular adaptation to hypoxia: O_2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O_2-regulated gene expression. *FASEB J* 2002;**16**:1151–62.
15. Haase VH. Hypoxia-inducible factors in the kidney. *Am J Physiol Renal Physiol* 2006;**291**:F271–81.
16. Wiesener MS, Maxwell PH. HIF and oxygen sensing; as important to life as the air we breathe? *Ann Med* 2003;**35**:183–90.
17. Rolfs A, Kvietikova I, Gassmann M, Wenger RH. Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1. *J Biol Chem* 1997;**272**:20055–62.
18. Erler JT, Bennewith KL, Nicolau M, et al. Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature* 2006;**440**:1222–6.
19. Rankin EB, Biju MP, Liu Q, et al. Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo. *J Clin Invest* 2007;**117**:1068–77.
20. Gruber M, Hu CJ, Johnson RS, et al. Acute postnatal ablation of Hif-2alpha results in anemia. *Proc Natl Acad Sci USA* 2007;**104**:2301–6.
21. Tobar N, Caceres M, Santibanez JF, Smith PC, Martinez J. RAC1 activity and intracellular ROS modulate the migratory potential of MCF-7 cells through a NADPH oxidase and NFkappaB-dependent mechanism. *Cancer Lett* 2008;**267**:125–32.
22. Sun B, Zhang D, Zhang S, et al. Hypoxia influences vasculogenic mimicry channel formation and tumor invasion-related protein expression in melanoma. *Cancer Lett* 2007;**249**:188–97.
23. Kietzmann T, Gorlach A. Reactive oxygen species in the control of hypoxia-inducible factor-mediated gene expression. *Semin Cell Dev Biol* 2005;**16**:474–86.
24. Klimova T, Chandel NS. Mitochondrial complex III regulates hypoxic activation of HIF. *Cell Death Differ* 2008;**15**:660–6.
25. Kamata H, Hirata H. Redox regulation of cellular signalling. *Cell Signal* 1999;**11**:1–14.
26. Wittgen HG, van Kempen LC. Reactive oxygen species in melanoma and its therapeutic implications. *Melanoma Res* 2007;**17**:400–9.
27. Kunz M, Ibrahim SM. Molecular responses to hypoxia in tumor cells. *Mol Cancer* 2003;**2**:23.
28. Jiang BH, Jiang G, Zheng JZ, et al. Phosphatidylinositol 3-kinase signaling controls levels of hypoxia-inducible factor 1. *Cell Growth Differ* 2001;**12**:363–9.
29. Minet E, Arnould T, Michel G, et al. ERK activation upon hypoxia: involvement in HIF-1 activation. *FEBS Lett* 2000;**468**:53–8.
30. Galabova-Kovacs G, Matzen D, Piazzolla D, et al. Essential role of B-Raf in ERK activation during extraembryonic development. *Proc Natl Acad Sci USA* 2006;**103**:1325–30.
31. Kumar SM, Yu H, Edwards R, et al. Mutant V600E BRAF increases hypoxia inducible factor-1alpha expression in melanoma. *Cancer Res* 2007;**67**:3177–84.
32. Bedogni B, Welford SM, Cassarino DS, et al. The hypoxic microenvironment of the skin contributes to Akt-mediated melanocyte transformation. *Cancer Cell* 2005;**8**:443–54.
33. Michaylira CZ, Nakagawa H. Hypoxic microenvironment as a cradle for melanoma development and progression. *Cancer Biol Ther* 2006;**5**:476–9.
34. Bonello S, Zahringer C, BelAiba RS, et al. Reactive oxygen species activate the HIF-1alpha promoter via a functional NFkappaB site. *Arterioscler Thromb Vasc Biol* 2007;**27**:755–61.
35. Rius J, Guma M, Schachtrup C, et al. NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. *Nature* 2008;**453**:807–11.
36. Kuphal S, Poser I, Jobin C, Hellerbrand C, Bosserhoff AK. Loss of E-cadherin leads to upregulation of NFkappaB activity in malignant melanoma. *Oncogene* 2004;**23**:8509–19.
37. Jacob K, Wach F, Holzapfel U, et al. In vitro modulation of human melanoma cell invasion and proliferation by all-trans-retinoic acid. *Melanoma Res* 1998;**8**:211–9.
38. Warnecke C, Zaborowska Z, Kurreck J, et al. Differentiating the functional role of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha (EPAS-1) by the use of RNA interference. erythropoietin is a HIF-2alpha target gene in Hep3B and Kelly cells. *FASEB J* 2004;**18**:1462–4.
39. Massoumi R, Chmielarska K, Hennecke K, Pfeifer A, Fassler R. Cyld inhibits tumor cell proliferation by blocking Bcl-3-dependent NF-kappaB signaling. *Cell* 2006;**125**:665–77.
40. Hellerbrand C, Jobin C, Iimuro Y, et al. Inhibition of NFkappaB in activated rat hepatic stellate cells by proteasome inhibitors and an IkappaB super-repressor. *Hepatology* 1998;**27**:1285–95.
41. Iimuro Y, Nishiura T, Hellerbrand C, et al. NFkappaB prevents apoptosis and liver dysfunction during liver regeneration. *J Clin Invest* 1998;**101**:802–11.
42. Warnecke C, Weidemann A, Volke M, et al. The specific contribution of hypoxia-inducible factor-2alpha to hypoxic gene expression in vitro is limited and modulated by cell

type-specific and exogenous factors. *Exp Cell Res* 2008;**314**:2016–27.
43. Sowter HM, Raval RR, Moore JW, Ratcliffe PJ, Harris AL. Predominant role of hypoxia-inducible transcription factor (Hif)-1alpha versus Hif-2alpha in regulation of the transcriptional response to hypoxia. *Cancer Res* 2003;**63**:6130–4.
44. Blancher C, Moore JW, Talks KL, Houlbrook S, Harris AL. Relationship of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression to vascular endothelial growth factor induction and hypoxia survival in human breast cancer cell lines. *Cancer Res* 2000;**60**:7106–13.
45. Fruehauf JP, Trapp V. Reactive oxygen species: an Achilles' heel of melanoma? *Expert Rev Anticancer Ther* 2008;**8**:1751–7.
46. Meyskens Jr FL, Farmer P, Fruehauf JP. Redox regulation in human melanocytes and melanoma. *Pigment Cell Res* 2001;**14**:148–54.
47. Gidanian S, Mentelle M, Meyskens Jr FL, Farmer PJ. Melanosomal damage in normal human melanocytes induced by UVB and metal uptake–a basis for the pro-oxidant state of melanoma. *Photochem Photobiol* 2008;**84**:556–64.
48. Li N, Karin M. Is NF-kappaB the sensor of oxidative stress? *FASEB J* 1999;**13**:1137–43.
49. Dutcher JP. Mammalian target of rapamycin inhibition. *Clin Cancer Res* 2004;**10**:6382S–7S.
50. Knaup KX, Jozefowski K, Schmidt R, et al. Mutual regulation of hypoxia-inducible factor and mammalian target of rapamycin as a function of oxygen availability. *Mol Cancer Res* 2009;**7**:88–98.
51. Aprelikova O, Pandolfi S, Tackett S, et al. Melanoma antigen-11 inhibits the hypoxia-inducible factor prolyl hydroxylase 2 and activates hypoxic response. *Cancer Res* 2009;**69**:616–24.
52. Zelzer E, Levy Y, Kahana C, et al. Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT. *EMBO J* 1998;**17**:5085–94.
53. Richard DE, Berra E, Pouyssegur J. Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1alpha in vascular smooth muscle cells. *J Biol Chem* 2000;**275**:26765–71.
54. Berra E, Milanini J, Richard DE, et al. Signaling angiogenesis via p42/p44 MAP kinase and hypoxia. *Biochem Pharmacol* 2000;**60**:1171–8.
55. Grimshaw MJ. Endothelins and hypoxia-inducible factor in cancer. *Endocr Relat Cancer* 2007;**14**:233–44.
56. Patel PS, Yang S, Li A, Varney ML, Singh RK. Capsaicin regulates vascular endothelial cell growth factor expression by modulation of hypoxia inducing factor-1alpha in human malignant melanoma cells. *J Cancer Res Clin Oncol* 2002;**128**:461–8.
57. Spinella F, Rosano L, Di C. V et al. Endothelin-1 and endothelin-3 promote invasive behavior via hypoxia-inducible factor-1alpha in human melanoma cells. *Cancer Res* 2007;**67**:1725–34.
58. Cheng Y, Luo Y, Zhou YS, Wu HY, Xie ZB. Expression of hypoxia-inducible factor-1 alpha and vascular endothelial growth factor in malignant melanoma. *Zhonghua Zheng Xing Wai Ke Za Zhi* 2009;**25**:134–6.
59. Giatromanolaki A, Sivridis E, Simopoulos C, et al. Hypoxia inducible factors 1alpha and 2alpha are associated with VEGF expression and angiogenesis in gallbladder carcinomas. *J Surg Oncol* 2006;**94**:242–7.
60. Zhong H, De Marzo AM, Laughner E, et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res* 1999;**59**:5830–5.
61. Marin-Hernandez A, Gallardo-Perez JC, Ralph SJ, Rodriguez-Enriquez S, Moreno-Sanchez R. HIF-1alpha Modulates Energy Metabolism in Cancer Cells by Inducing Over-Expression of Specific Glycolytic Isoforms. *Mini Rev Med Chem* 2009;**9**:1084–101.
62. Yamanishi DT, Buckmeier JA, Meyskens Jr FL. Expression of c-jun, jun-B, and c-fos proto-oncogenes in human primary melanocytes and metastatic melanomas. *J Invest Dermatol* 1991;**97**:349–53.
63. Bendinelli P, Matteucci E, Maroni P, Desiderio MA. NF-{kappa}B activation, dependent on acetylation/deacetylation, contributes to hif-1 activity and migration of bone metastatic breast carcinoma cells. *Mol Cancer Res* 2009;**7**:1328–41.
[64]. Gorlach A. Regulation of HIF-1 at the transcriptional level. *Curr Pharm Des* 2009;**15**:3844–52.