

Mutant V600E *BRAF* Increases Hypoxia Inducible Factor-1 α Expression in Melanoma

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Abstract

Mutations in the *BRAF* serine/threonine kinase gene are frequently found in cutaneous melanomas. Activation of hypoxia inducible factor-1 α (HIF-1 α) in response to both hypoxic stress and oncogenic signals has important implications in cancer development and progression. Here, we report that mutant *BRAF*^{V600E} increases HIF-1 α expression in melanoma cells. Our microarray profiling data in 35 melanoma and melanocyte cell lines showed that *HIF-1 α* gene expression was significantly increased in melanomas harboring *BRAF*^{V600E} mutation. Stable suppression of mutant *BRAF*^{V600E} or both wild-type and mutant *BRAF*^{V600E} by RNA interference in melanoma cells resulted in significantly decreased HIF-1 α expression. Knockdown of mutant *BRAF*^{V600E} induced significant reduction of cell survival and proliferation under hypoxic conditions, whereas knockdown of both wild-type and mutant *BRAF*^{V600E} resulted in further reduction. The effects of *BRAF* knockdown can be rescued by reintroducing *BRAF*^{V600E} into tumor cells. Transfection of *BRAF*^{V600E} into melanoma cells with wild-type *BRAF* induced significantly more hypoxic tolerance. Knockdown of *HIF-1 α* in melanoma cells resulted in decreased cell survival under hypoxic conditions. Pharmacologic inhibition of *BRAF* by BAY 43-9006 also resulted in decreased HIF-1 α expression. Although HIF-1 α translational rate was not changed, the protein was less stable in *BRAF* knockdown cells. In addition, von Hippel-Lindau protein expression was significantly increased in *BRAF* knockdown cells. Our data show for the first time that *BRAF*^{V600E} mutation increases HIF-1 α expression and melanoma cell survival under hypoxic conditions and suggest that effects of the oncogenic V600E *BRAF* mutation may be partially mediated through the HIF-1 α pathway. [Cancer Res 2007;67(7):3177–84]

Introduction

Genetic alterations are common in both familial and sporadic melanomas; perturbations in the mitogen-activated protein kinase (MAPK) signaling pathway have been implicated in the development of 60% to 90% of melanomas, with activating *BRAF* mutations playing the most prominent role in this process (1). Activation of the *BRAF*/MAPK kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway has been shown to increase tumor cell

proliferation, survival, invasion, and tumor angiogenesis (2). Somatic mutations in *BRAF* have been reported in 70% of melanomas (3, 4). However, these mutations are also present in banal or dysplastic nevi and may thereby implicate *BRAF* activation as an initiating event in tumorigenesis (5). Nevertheless, how *BRAF* mutations contribute to melanocyte transformation and melanoma progression is largely unknown.

The RAF family is composed of three members [*ARAF*, *BRAF*, and *CRAF* (*RAF1*)] that exhibit a high degree of homology within three conserved regions. RAF family members are intermediate molecules in the MAPK pathway, which is a cellular signal transduction pathway that conveys extracellular signals from the cell membrane to nucleus through a series of phosphorylation events (6, 7) and leads to the expression of genes associated with cell growth and survival (8). Suppression of *BRAF* expression in cultured melanoma cells inhibits the MAPK cascade and leads to growth arrest and promotes apoptosis *in vitro* and inhibits tumor development in animals (9, 10).

The rapid proliferation of cancer cells often outgrow blood supply in solid tumors, resulting in a reduction in oxygen tension to drop below physiologic levels. Hypoxic areas are common features of rapidly growing malignant tumors and their metastasis. Tissue hypoxia due to inadequate blood supply typically occurs very early during tumor development beginning at a tumor diameter of a few millimeters (11). Hypoxia-inducible factor-1 (HIF-1) is a master mediator of cellular responses to hypoxia, which is composed of two subunits (HIF-1 α and HIF-1 β). HIF-1 α is an oxygen-regulated subunit, and its expression is stabilized under hypoxic conditions (12). Under normoxic conditions, hydroxylation of key proline residues within the regulatory oxygen-dependent domain of the HIF-1 α subunit facilitates von Hippel-Lindau protein (VHL) binding, which in turn allows ubiquitination and subsequent proteasome-targeted degradation (13). Under limiting O₂ conditions, proline hydroxylation is inhibited, thereby stabilizing HIF-1 α subunits, which can then translocate into the nucleus and bind to constitutively stabilized *HIF-1 β* subunits, forming the active HIF-1 complex (14). HIF-1 activates a multitude of O₂-responsive genes, such as vascular endothelial growth factor (*VEGF*) and *erythropoietin*, which are involved in various normal cell functions such as survival, apoptosis, glucose metabolism, and angiogenesis (15, 16).

HIF-1 α expression is regulated by the MAPK/ERK and Akt/phosphatidylinositol 3-kinase (PI3K) pathways (11, 17). It has been shown that Ras regulates HIF-1 α expression via the RAF/MEK/ERK pathway, and that both phorbol ester and epidermal growth factor induce HIF-1 α via the same pathway (18, 19). *BRAF* is essential for ERK activation and for vascular development in the placenta (20). *BRAF* knockout mice suffer from an impaired development of the vascular system (21), which is similar to mice lacking the *HIF-1 β* (22). In this study, we show for the first time that mutant V600E *BRAF* increases HIF-1 α expression and melanoma cell survival under hypoxic conditions.

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Materials and Methods

Cell culture. The melanoma cell lines used in this study were derived from human melanomas representing all stages of melanoma progression [3 radial growth phase (RGP), 10 vertical growth phase (VGP), and 17 metastatic melanomas]. Melanoma cells were cultured under 37°C humidified atmosphere containing 5% CO₂ and grown in MCDB153/L15 medium (4:1, v/v) supplemented with 2% fetal bovine serum, insulin (5 units/mL), CaCl₂ (2 mmol/L), 100 units/mL penicillin, and 100 mg/mL streptomycin. Five melanocyte cell lines were generated from neonatal foreskin specimens, and they were maintained in melanocyte medium.³ Hypoxia treatment was done in a well-characterized chamber system as we previously described (15).

DNA constructs and transfection. *BRAF* shRNAs in pSUPER.retro vectors (Oligoengine, Seattle, WA) were kindly provided to us by Dr. David Tuveson at University of Pennsylvania. The Com-4 construct (designated as BRAFsiRNA) targets sequences common to both wild-type and *BRAF*^{V600E} mutant alleles, and Mu-A construct (designated as BRAFmutsiRNA) targets only mutated alleles, which can essentially completely abolished *BRAF*^{V600E} expression while keeping wild-type *BRAF* expression intact. The specificity of these constructs has been extensively studied by a previous study (9).

The *myc*-tagged *BRAF*^{V600E} cDNA in the pEFP expression vector was kindly provided by Dr. Jeffrey Knauf (Memorial Sloan-Kettering Cancer Center, New York). For transient expression of BRAF^{V600E} protein, melanoma cells (1 × 10⁵ per well in six-well plate) were seeded and incubated overnight. The cells were transfected with 200 to 2,000 ng of plasmids using LipofectAMINE (Invitrogen, Carlsbad, CA) as described previously (15).

Expression profiling. Total RNA was extracted from cells grown to 70% to 80% confluence using the RNeasy Mini kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol. cDNA was synthesized from 10 µg total RNA using the Superscript Choice System for cDNA Synthesis (Invitrogen). *In vitro* transcription reactions to synthesize biotinylated cRNA were done with the Bioarray High Yield RNA Transcript Labeling Kit (T7; Enzo Life Sciences, Farmingdale, NY). Labeled cRNA was fragmented and hybridized to the Human Genome U133A Array (Affymetrix, Santa Clara, CA) that contains 14,500 genes. Hybridized gene chips were scanned, and data were normalized using Microarray Suite Software (v5.0). Data sets were analyzed using Genesifter software (VizX Labs, Seattle, WA).

RNA interference. A VGP melanoma cell line with *BRAF*^{V600E} mutation (WM793) was used for transfection. BRAFsiRNA, BRAFmutsiRNA, or vector control (pSUPER) plasmids were added to 1 × 10⁶ cells, which were previously washed in 1× PBS and resuspended in 100 µL of Nucleofector solution V (Amaxa, Inc., Gaithersburg, MD). For nucleofection, we used the built-in T-20 program in the nucleofector device (Amaxa) and immediately transfected into prewarmed 2% MCDB tumor medium. Transfected cells were seeded into six-well plates and incubated for 24 h under standard conditions. Twenty-four hours after transfection, cells were selected in media containing 5 µg/mL Puromycin for 4 to 5 days, and well-formed colonies were selected and expanded for biochemical assays.

Transient transfection with *HIF-1α* small interfering RNA (siRNA) was done using the HiperFect Reagent (Qiagen) according to the manufacturer's specifications. Briefly, all of these experiments were done in six-well plates using melanoma cell lines Sbcl2 (with wild-type *BRAF*) and WM793 (with *BRAF*^{V600E}). Tumor cells were incubated with *HIF-1α* siRNA (5 nmol/L, Hs_HIF-1A_5_HP validated siRNA, NM_001530; Qiagen), or scrambled control siRNA (5 nmol/L; Qiagen), mixed with HiPerFect transfection reagent (12 µL) in a 100 µL of serum-free MCDB medium. Twenty-four hours after transfection, the medium was changed, and cells were harvested 48 h after transfection.

Western blot. Adherent monolayer cells were washed with ice-cold PBS and lysed in-place for the analysis of total proteins. These melanoma cells were washed with ice-cold 1× PBS and lysed in Tissue Protein Extraction

Reagent (T-PER, Pierce, Rockford, IL) with (1×) Protease Inhibitor cocktail (Sigma, St. Louis, MO) and 1 mmol/L phenylmethylsulfonyl fluoride (Sigma), subsequently homogenized on ice, and centrifuged at 10,000 rpm for 5 min. Whole-cell lysates were normalized for protein concentration. Fifty micrograms of proteins were separated in Nu PAGE 4-12% Bis-Tris Gel (Invitrogen) and transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Biosciences, Little Chalfont, England). Membranes were blocked and incubated with primary antibodies (HIF-1α, BRAF, VHL, and VEGF; Santa Cruz Biotechnology, Santa Cruz, CA; 1:1,000 to 1:10,000) in 5% milk TBST buffer [150 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 5% nonfat dry milk, and 0.1% Tween 20]. The membranes were washed thrice with wash buffer for 5 min and incubated with horseradish peroxidase-conjugated secondary antibodies and washed again before being processed with chemiluminescence (ECL Western Blotting Detection System, Amersham Biosciences). Bands were scanned and quantified using a ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA).

MG132 and cycloheximide treatment. MG132 is a proteasomal inhibitor, and it is known to block the degradation of HIF-1α. To investigate the translational rate of HIF-1α, we seeded WM793 cells (5 × 10⁵) that were stably transfected with pSUPER or BRAFsiRNA plasmids and incubated at 37°C overnight, aspirated the medium, and fed serum-free medium with MG132 (10 µmol/L) in each plate. The plates were kept in 37°C for up to 6 h, then the cells were lysed in Laemmli protein lysis buffer (200 µL) and boiled immediately. CoCl₂ is a hypoxia mimic and is known to stabilize HIF-1α in the cells. For CoCl₂ treatment, melanoma cells were subjected to 100 µmol/L CoCl₂ for 24 h in a CO₂ incubator. For cycloheximide treatment, the above mentioned melanoma cells were subjected to 10 µmol/L cycloheximide. The cells were harvested at 0, 5, and 60 min after cycloheximide treatment. Ten microliters of protein samples from each plates were separated in Nu PAGE 4% to 12% Bis-Tris Gel and transferred to a PVDF membrane. Membranes were blocked and incubated with primary antibodies (HIF-1α; 1:200; Santa Cruz Biotechnology) in 5% milk TBST buffer [150 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 5% nonfat dry milk, and 0.1% Tween 20]. The membranes were washed thrice with wash buffer and incubated with horseradish peroxidase-conjugated secondary antibodies and washed again before being processed with chemiluminescence. Bands were scanned and quantified using a ChemiDoc XRS system (Bio-Rad Laboratories).

Real-time PCR. Total RNA was prepared using RNeasy kit (Qiagen), and cDNAs were prepared by using SuperScript First-Strand Synthesis system (Invitrogen) for reverse transcription-PCR according to the manufacturer's instructions. Real-time PCR was done same as described previously (23) with specific primers for *HIF-1α* (forward, 5'-CATAAAGTCTGCAACATG-GAAGGT-3'; reverse, 5'-ATTGATGGGTGAGGAATGGGT-3') and *β-actin* (forward, 5'-CTACCTCATGAAGATCCTCACCGA-3'; reverse, 5'-ACGTAGCA-CAGCTTCTCCTTAATG-3'). cDNA corresponding to 1 µg RNA was added to the iQ SYBER green supermix (Bio-Rad Laboratories). PCRs were carried out in a real-time PCR cycler (iCycler; Bio-Rad Laboratories) according to the manufacturer's instructions. The thermal profile was 95°C for 30 s and 56°C for 30 s. Melting curve analysis was done for each PCR reaction to confirm the specificity of amplification. At the end of each phase, fluorescence was measured and used for quantitative purposes. The *HIF-1α* expression data were normalized to *β-actin*, and relative transcript level was calculated.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The cell viability assay was done using CellTiter 96 Non-Radioactive Cell Proliferation Assay kit (Promega Corp., Madison, WI) according to the manufacturer's instructions. Briefly, melanoma cells were washed with PBS and suspended in a final concentration of 1 × 10⁵/mL in assay medium, and 50 µL of cell suspension was subsequently dispensed into 96-well plates. The plates were incubated at 37°C for 24 h in a humidified CO₂ incubator. The medium was aspirated from the wells; 100 µL of serum-free medium was added into each well, and cells were incubated for 24 h at 37°C in a hypoxic chamber (1% O₂). For color development, 15 µL of dye solution was added to each well, and the plates were incubated at 37°C for 4 h followed by addition of 100 µL of solubilization/stop solution to each well. Absorbance was recorded at 570 nm using a 96-well plate reader. Experiments were carried out in triplicate.

³ http://www.wistar.org/herlyn/resource_culture.htm for details.

WST-1 cell proliferation assay. Cell proliferation was measured using a WST-1 Cell Proliferation Assay (Roche Diagnostics, Indianapolis, IN). Briefly, melanoma cells were washed with PBS and suspended in a final concentration of 1×10^5 /mL in assay medium, and 50 μ L of cell suspension was dispensed into 96-well plates. The plates were incubated at 37°C for 24 h in a humidified CO₂ incubator. The medium was aspirated from the wells; 100 μ L of serum-free medium was added into each well, and cells were incubated for 24 h at 37°C in a hypoxic chamber (1% O₂). After the treatment period, WST-1 reagent was added to cell culture medium (10 μ L in 100 μ L media), mixed gently, and incubated at 37°C for 20 min. Plates were shaken vigorously on an orbital shaker for 1 min, and absorbance was measured at 450 nm using a 96-well plate reader. Experiments were carried out in triplicate. Trypan blue dye exclusion assay was done as we described previously (23).

Statistical analysis. One-way ANOVA, followed by Tukey's multiple comparison test, was used to analyze the expression data for *VEGF* and *HIF-1 α* in 5 melanocyte cell lines and 30 melanoma cell lines. Student's *t* test or one-way ANOVA was used to analyze other gene expression, cell viability, and proliferation data. Statistical significance was determined if two-sided $P_{\text{test}} < 0.05$.

Results

***HIF-1 α* gene expression is increased in melanomas with mutant V600E BRAF.** We did a microarray profiling study on 30 melanoma cell lines derived from various stages of human melanoma progression and 5 foreskin-derived melanocyte cell lines. One of three RGP, 7 of 10 VGP, and 12 of 15 metastatic melanomas carry the *BRAF*^{V600E} mutation; *BRAF* mutation status is unknown in two metastatic melanoma cell lines. We found that *HIF-1 α* expression was significantly higher in melanomas harboring the *BRAF*^{V600E} mutation in comparison with melanomas without the mutation, or melanocytes ($P < 0.01$; Fig. 1A). The up-regulation of *HIF-1 α* in the melanoma cells was not associated with a global up-regulation of genes. Many genes remained stable, such as *protein phosphatase 2* and *heat shock protein 90 kDa beta* etc. When we compared *HIF-1 α* gene expression during melanoma progression regardless of V600E mutation status, we found that there was a significant increase of *HIF-1 α* gene expression in melanoma cells compared with melanocytes ($P < 0.05$; Fig. 1B), suggesting that *HIF-1 α* expression may be regulated by other factors as well. Because a majority of melanoma cell lines carry the *BRAF*^{V600E} mutation in this study, it is not reliable to perform a statistical analysis on the effect of *BRAF*^{V600E} mutation on *HIF-1 α* expression after adjusting for tumor stage, despite cell lines with V600E *BRAF* mutation seemed to have higher *HIF-1 α* expression than cell lines with wild-type *BRAF* within the same stage (data not shown). Additional studies with more *BRAF* wild-type cell lines are necessary to show the effects of *BRAF* mutations on *HIF-1 α* expression after adjusting for tumor stage in the future. Because *VEGF* expression is directly regulated by HIF-1 α , we analyzed *VEGF* expression during melanoma progression and showed that *VEGF* expression pattern was similar to HIF-1 α (Fig. 1C).

***BRAF* knockdown resulted in decreased HIF-1 α expression.** To further investigate the effect of V600E *BRAF* mutation on HIF-1 α expression, we used two well-characterized RNA interference constructs to stably knockdown mutant V600E *BRAF* (*BRAF*mut-siRNA) or both wild-type and mutant V600E *BRAF* (*BRAF*siRNA) in WM793 melanoma cells. Similar to the previous results in 1205Lu melanoma cells (9), *BRAF*siRNA and *BRAF*mut-siRNA stable transfection significantly reduced *BRAF* expression in transfected melanoma cells (Fig. 2A). Although *BRAF*siRNA targets both wild-type and V600E mutant *BRAF*, the level of *BRAF* expression in

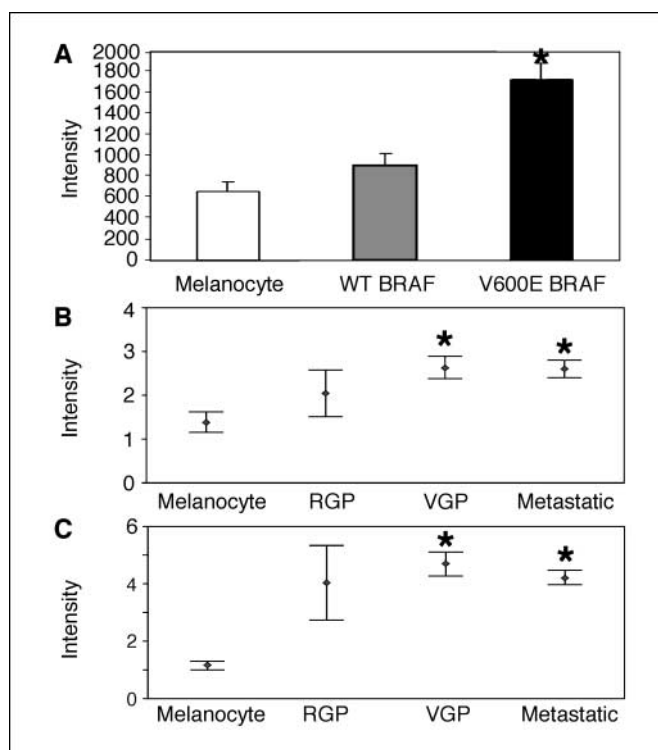


Figure 1. HIF-1 α mRNA expression in melanoma and melanocyte cell lines. **A,** *HIF-1 α* gene expression is increased in melanomas with *BRAF*^{V600E} mutation. *HIF-1 α* gene expression level was obtained by microarray analysis in 30 melanoma and 5 melanocyte cell lines, and it was significantly higher in melanoma cell lines with *BRAF*^{V600E} mutation comparing to the melanoma cell lines with wild-type *BRAF* (WT *BRAF*) or melanocytes. **B,** *HIF-1 α* gene expression during melanoma progression (regardless of *BRAF*^{V600E} mutation status). *HIF-1 α* gene expression level was significantly higher in VGP and metastatic melanomas than that in melanocytes. **C,** *VEGF* gene expression during melanoma progression. *VEGF* expression pattern was similar to *HIF-1 α* gene during melanoma progression. *, $P < 0.05$.

*BRAF*siRNA stably transfected cells was similar to that in *BRAF*mut-siRNA-transfected cells, suggesting different efficacy of the siRNA systems, or that it is still possible that siRNA for mutant *BRAF* may also down-regulate to some extent the expression of wild-type *BRAF*. *BRAF* knockdown cells seemed to have more dendritic processes than wild-type cells (Fig. 2D).

We examined HIF-1 α expression in multiple clones of *BRAF* knockdown cells, and all the clones showed decreased HIF-1 α expression (Fig. 2A). The controls were cells transfected with the pSUPER vector. Knockdown of both wild-type and mutated V600E *BRAF* decreased *HIF-1 α* gene expression by 15%, whereas knockdown of mutated *BRAF* resulted in 33% decrease of *HIF-1 α* gene expression ($P < 0.05$), compared with vector-transfected control cells (Fig. 2B). Similarly, *VEGF* expression was also significantly decreased in *BRAF* knockdown cells (Fig. 2C). However, *BRAF* knockdown did not result in a general down-regulation of protein expression. There was no change of erythropoietin receptor expression in these cells (data not shown). These data further suggest that HIF-1 α expression is regulated by *BRAF* in melanoma cells.

***BRAF* is essential for melanoma cell survival under hypoxic conditions.** To show biological function of decreased HIF-1 α expression in *BRAF* knockdown cells, we cultured these cells in moderate hypoxic conditions for 24 h. We assessed cell survival and proliferation using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue dye exclusion, and WST-1

cell proliferation assay. There was a 43% decrease of survival in the cells with mutant V600E *BRAF* knockdown in comparison with control cells under hypoxic conditions ($P < 0.01$; Fig. 3A). There was an additional 50% decrease of cell survival when both wild-type and mutant V600E *BRAF* were knocked down in comparison with cells with only mutant V600E *BRAF* knockdown using MTT assay ($P < 0.05$; Fig. 3A). Trypan blue dye exclusion assay showed same results as MTT assay (data not shown). Cell proliferation showed similar changes as cell survival after *BRAF* knockdown; however, the additional decrease of cell proliferation in cells with both wild-type and mutant *BRAF* knockdown was not as prominent comparing to cells with only mutant *BRAF* knockdown, despite the decrease was still statistically significant (Fig. 3A). Although the efficiency of the different siRNAs may account for the observations, it is likely that both wild-type and mutant V600E *BRAF* may contribute to melanoma cell adaptation to hypoxic conditions.

To eliminate the off-target effects of siRNA experiment, we did a rescue experiment using *myc*-tagged *BRAF*^{V600E} plasmids. We transfected the plasmids into both *BRAF* stable knockdown cell lines. Forty-eight hours after transfection, *BRAF* protein expression showed a dose-dependent increase in these cells (Fig. 3B). These *BRAF*-rescued cells were subjected to hypoxic condition for 24 h, and we measured the cell proliferation using WST-1 assay. Our data clearly showed that the addition of *BRAF*^{V600E} in *BRAF* knockdown cells rescued their hypoxic intolerance (Fig. 3C). The rescuing effects of *BRAF*^{V600E} was less prominent in cells with both wild-type and mutant V600E *BRAF* knockdown, further

suggesting that both wild-type and mutant V600E *BRAF* may contribute to melanoma cell adaptation to hypoxic conditions.

Overexpression of *BRAF*^{V600E} increases melanoma cell survival under hypoxic condition. To confirm that *BRAF*^{V600E} mutation plays a role in hypoxic adaptation for melanoma cells, we transiently transfected *BRAF* wild-type melanoma cells (Sbcl2 and WM3211) with *myc*-tagged *BRAF*^{V600E} plasmid. After transfection, tumor cells showed significantly increased *BRAF* and HIF-1 α mRNA and protein expression than the control cells (Fig. 4A and B). We then exposed these cells under hypoxic condition for 24 h and did cell survival and proliferation assays using MTT and WST-1 assays. There was a significant increase in cell survival in both Sbcl2 and WM3211 cells transfected with *BRAF*^{V600E} compared with the control cells ($P < 0.01$, Fig. 4C), whereas cell proliferation was significantly increased only in Sbcl2 cells but not in WM3211 cells. These data support that V600E *BRAF* mutation increases cell survival under hypoxic condition.

Mutant V600E *BRAF* increases cell survival in hypoxic condition through HIF-1 α . We next studied whether the effect of mutant V600E *BRAF* is mediated through HIF-1 α . We transiently knocked down *HIF-1 α* using commercially available *HIF-1 α* siRNA in Sbcl2 and WM793 melanoma cells (Fig. 4D). *HIF-1 α* knockdown decreased HIF-1 α expression in these cell lines and significantly decreased cell survival and proliferation of these cells in hypoxic condition (Fig. 4E). These data suggest that HIF-1 α mediates the effect of wild-type and mutant V600E *BRAF* in melanoma under hypoxic conditions.

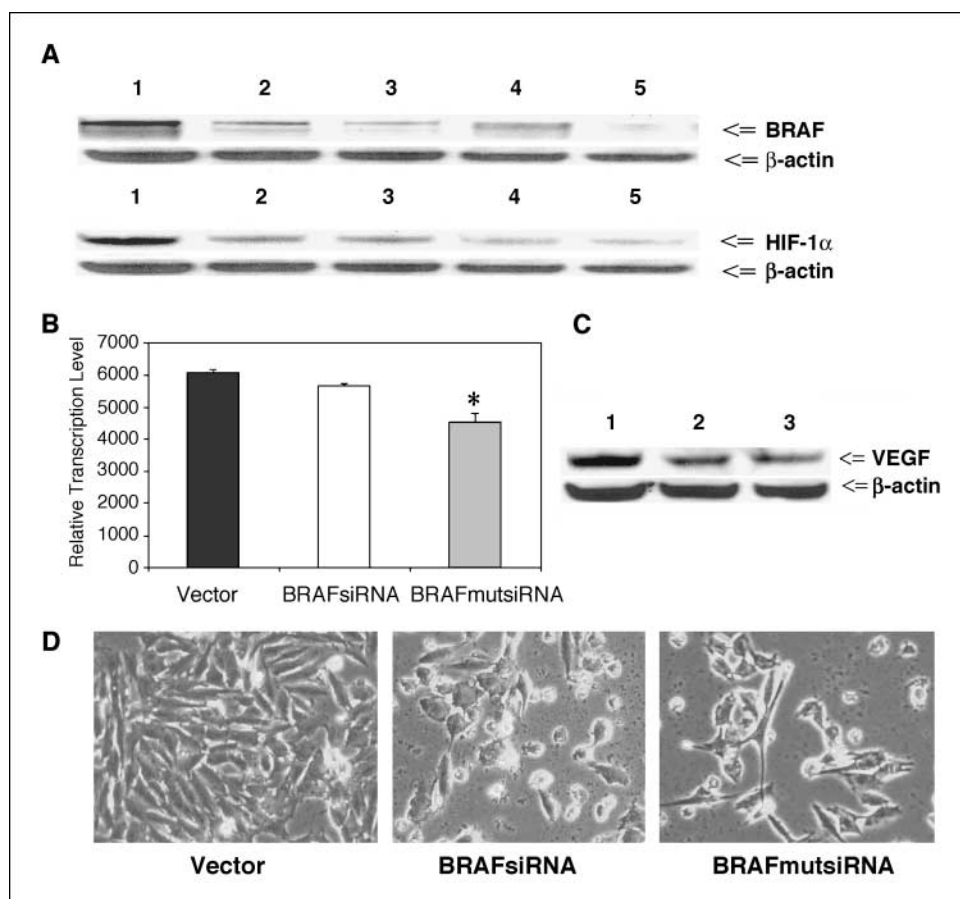


Figure 2. A, *BRAF* knockdown in melanoma cells. Western blot analysis of *BRAF* and HIF-1 α expression in WM793 melanoma cells stably transfected with pSuper-vector (lane 1), BRAFsiRNA (lanes 2 and 3), or BRAFmutsiRNA plasmids (lanes 4 and 5). The amount of protein loaded in each lane was normalized with β -actin. Cells from lanes 3 and 5 were used for further experiments. B, relative transcriptional level of *HIF-1 α* in *BRAF* knockdown melanoma cells compared with control cells. C, Western blot analysis of VEGF expression in WM793 melanoma cells stably transfected with pSuper-vector (lane 1), BRAFsiRNA (lane 2), or BRAFmutsiRNA plasmids (lane 3). The amount of protein loaded in each lane was normalized with β -actin. D, phase-contrast microscopy of transfected cells in the culture. WM793 cells were transfected with pSuper-vector, BRAFsiRNA, or BRAFmutsiRNA plasmids. The blots represent typical results from three independent experiments. *, $P < 0.05$.

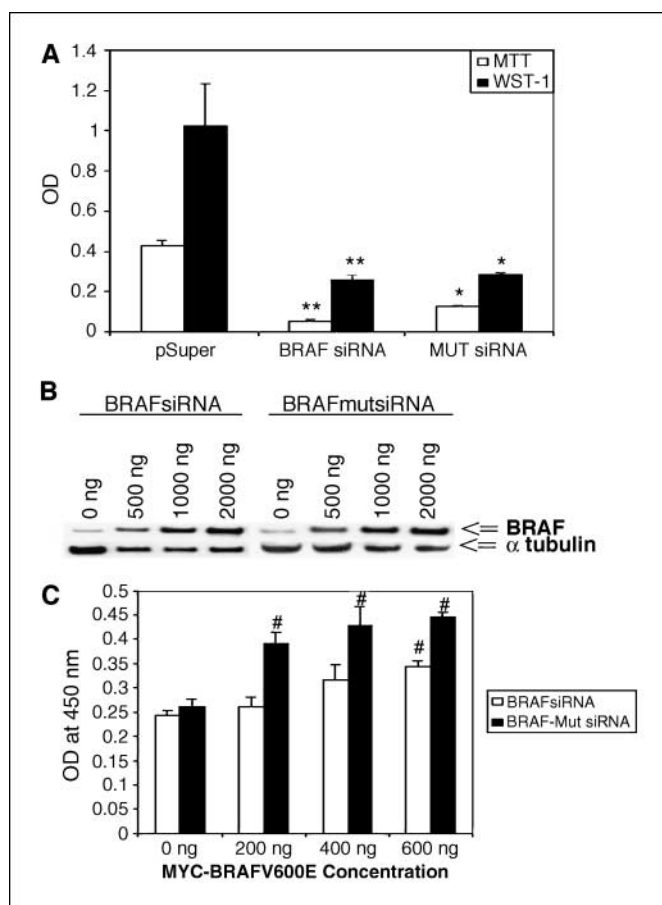


Figure 3. A, effects of stable *BRAF* knockdown on melanoma cells. Effects of *BRAF* knockdown on melanoma cell survival and proliferation under hypoxic conditions. Transfected cells were incubated under hypoxic conditions for 24 h, and cell survival or proliferation was measured by MTT or WST-1 assay. *BRAF* knockdown significantly decreased cell survival and proliferation under hypoxic conditions. B, *BRAF* knockdown rescuing experiment. *BRAF* stable knockdown cells were transfected with 0, 500, 1,000, or 2,000 ng of *myc*-tagged *BRAF*^{V600E} plasmid using LipofectAMINE transfection reagent. Cells were harvested 48 h after transfection and blotted against *BRAF* protein. *BRAF*^{V600E} plasmid increased *BRAF* level in both *BRAF* knockdown cell lines. α -Tubulin was used as a loading control. C, *BRAF*^{V600E} rescues the hypoxic intolerance of *BRAF* knockdown cells. Forty-eight hours after transfection with various concentrations of *BRAF*^{V600E} plasmids, the tumor cells were incubated in hypoxic condition for 24 h and then harvested. WST-1 cells proliferation assay was used to examine cell proliferation. The *BRAF*^{V600E} plasmids had a more profound effect in melanoma cells with only mutant *BRAF*^{V600E} knockdown. The MTT and WST-1 assays were done in triplicates. *, $P < 0.01$ comparing controls with *BRAF*mutsiRNA cells; **, $P < 0.05$ comparing *BRAF*siRNA with *BRAF*mutsiRNA cells. #, $P < 0.01$ comparing *BRAF* rescued cells with control cells. The blots represent typical results from three independent experiments.

BRAF inhibitor (BAY 43-9006) inhibits HIF-1 α expression.

We studied whether pharmacologic inhibition of *BRAF* would affect HIF-1 α expression. BAY 43-9006 was engineered to inhibit the *BRAF* kinase; however, it is not a specific *BRAF* kinase inhibitor as it is known to inhibit other proteins. The ability of BAY 43-9006 to block activation of the MAPK pathway was confirmed by measuring ERK1/2 phosphorylation in Sbc12 and WM793 cells. After cells were preincubated with BAY 43-9006 at different concentration (1, 2.5, 5, and 10 μ mol/L) for 2 h, there was a dose-dependent inhibition of phosphorylated MEK1/2 and phosphorylated ERK, whereas there were no significant changes in the expression of total ERK and MEK in melanoma cells (Fig. 5A). The hypoxia mimic CoCl_2 is known to stabilize HIF-1 α in cells. BAY 43-

9006 (10 μ mol/L) significantly inhibited HIF-1 α expression in melanoma cells in the presence of CoCl_2 (Fig. 5B). BAY 43-9006 also inhibited HIF-1 α mRNA level in melanoma cells (Fig. 5C), suggesting that BAY 43-9006 can inhibit HIF-1 α at transcriptional level. To further address whether the observed effects of V600E *BRAF* mutation on HIF-1 α expression is a result of ERK pathway activation, we treated WM793 cells with a MEK inhibitor UO126. Indeed, UO126 significantly decreased *HIF-1 α* gene and protein expression (Fig. 5B and C). Our findings suggest that pharmacologic inhibition of *BRAF* may result in decreased HIF-1 α expression, and the therapeutic effects of BAY 43-9006 may be mediated in part through the HIF-1 α pathway.

BRAF knockdown increases HIF-1 α degradation. To investigate the mechanism underlying the effects of *BRAF* on HIF-1 α expression, we studied whether *BRAF* knockdown in melanoma cells decreases the translational rate of HIF-1 α . MG 132 has been shown to inhibit the degradation of HIF-1 α *in vitro* (24). *BRAF*siRNA or control vector-transfected WM793 cells were treated with a proteasome inhibitor (10 μ mol/L MG 132) for 1, 2, and 6 h. Lower dose of MG132 did not completely block HIF-1 α degradation in these cells (data not shown). We collected cell lysates immediately after treatment and subjected the cell lysates to Western blot against HIF-1 α . The result showed that HIF-1 α protein accumulated at a similar rate in *BRAF* knockdown and control cells (Fig. 6A), suggesting that HIF-1 α translational rate is not changed. To test whether *BRAF* stabilizes the HIF-1 α transcripts, we measured HIF-1 α protein stability in the presence of cycloheximide, which blocks *de novo* protein synthesis. As shown in Fig. 6B, in the absence of HIF-1 α stabilizer CoCl_2 , HIF-1 α was stably expressed at a low level in pSuper-transfected cells, whereas it was degraded in the *BRAF* knockdown cells. In the presence of CoCl_2 and cycloheximide, HIF-1 α level persisted in these cells at a similar level. These data suggest that *BRAF* knockdown may destabilize HIF-1 α protein in melanoma cells.

Decreased expression of HIF-1 α in *BRAF* knockdown cells is associated with up-regulation of VHL. Stability of HIF-1 α protein depends upon its interaction with VHL. Under normoxic condition, HIF-1 α protein is constantly turned over due to hydroxylation, recognition by VHL, and subsequent destruction by the proteasome. To investigate whether the decreased HIF-1 α expression in *BRAF* knockdown cells is related to VHL expression, we did Western blot against VHL in *BRAF*siRNA- or *BRAF*mutsiRNA-transfected melanoma cells and found that there was indeed an increase in VHL protein expression in these cells compared with controls (Fig. 6C), suggesting that the increased degradation of HIF-1 α protein in *BRAF* knockdown cells may be due to increased expression of VHL protein.

Discussion

BRAF mutation is the most common genetic alteration in melanoma and thyroid cancer, occurring in about 70% of melanoma and 45% of sporadic papillary thyroid cancers (25, 26). This mutation is mutually exclusive with other common genetic alterations in both cancers such as *NRAS*, suggesting its independent oncogenic role (27). *BRAF* mutation is therefore likely involved in the pathogenesis and progression of these cancers, and it may represent a novel therapeutic target (28). Mutant V600E is the most common activating *BRAF* mutation in melanoma (29). Despite recently shown clinical efficacy of *BRAF* inhibitors, such as BAY 43-9006 in phase I and II clinical trials,

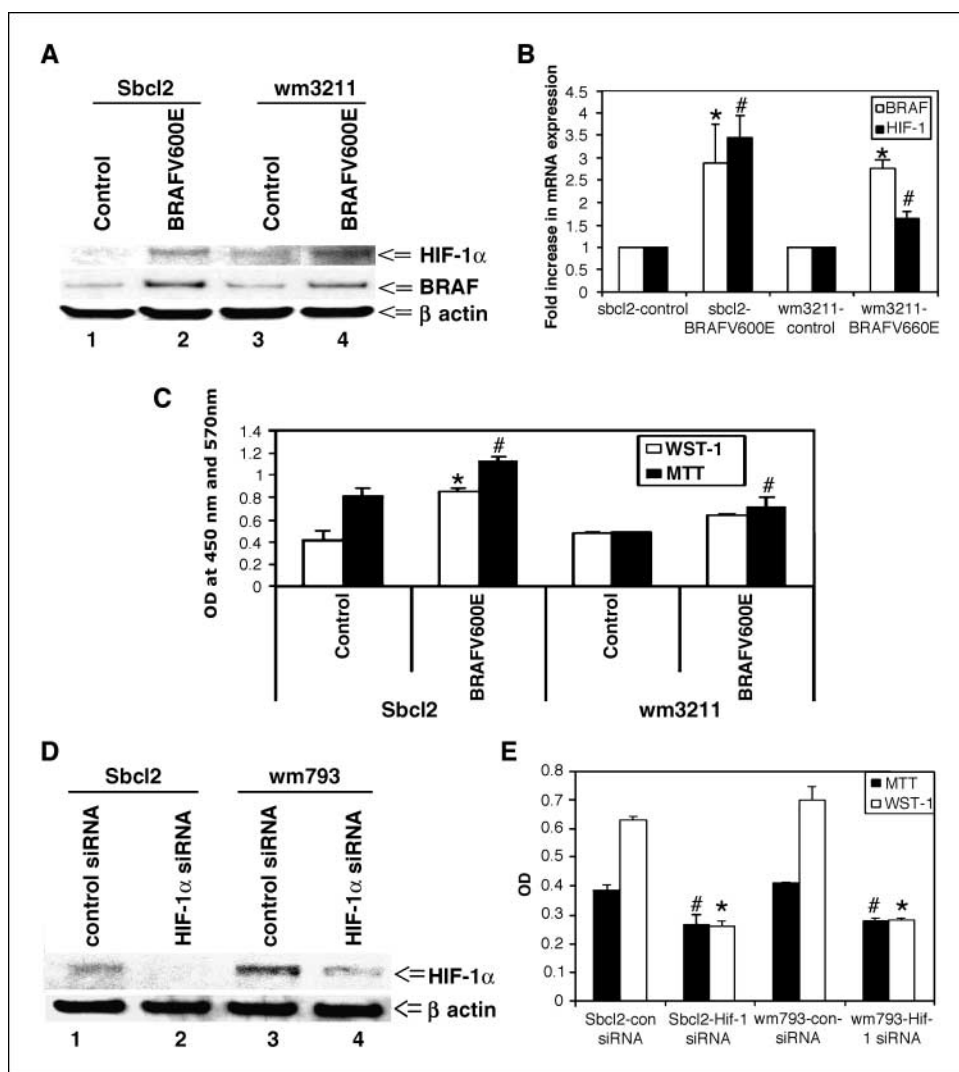


Figure 4. HIF-1 α mediates the effects of *BRAF*^{V600E} mutation under hypoxic conditions. **A**, overexpression of *BRAF*^{V600E} in *BRAF* wild-type melanoma cells resulted in increased HIF-1 α expression. Sbc12 and WM3211 cells were transfected with *BRAF*^{V600E} construct. Western blot against HIF-1 α showed increased HIF-1 α expression in transfected cells. β -Actin was used as a loading control. **B**, relative transcriptional level of *BRAF* and *HIF-1 α* in *BRAF*^{V600E}-transfected melanoma cells. The mRNA of *BRAF* and *HIF-1 α* was significantly increased in *BRAF*^{V600E}-transfected cells. **C**, effect of *BRAF*^{V600E} overexpression on melanoma cell survival in hypoxic condition. *BRAF*^{V600E}-transfected *BRAF* wild-type cells were incubated in hypoxic condition for 24 h, and cell survival and proliferation were measured by MTT and WST-1 cell proliferation assays. *BRAF*^{V600E} overexpression in *BRAF* wild-type cells resulted in significantly increased survival of these cells in hypoxic condition. **D**, *HIF-1 α* knockdown in melanoma cells. Lane 1, Sbc12 cells transfected with a scrambled control siRNA; lane 2, Sbc12 cells transfected with HIF-1 α siRNA; lane 3, WM793 cells transfected with a scrambled control siRNA; lane 4, WM793 cells transfected with HIF-1 α siRNA. The amount of protein loaded in each lane was normalized with β -actin. HIF-1 α siRNA decreased HIF-1 α level in these cells. **E**, MTT and WST-1 assays were done after these tumor cells were incubated in hypoxic condition for 48 h. *HIF-1 α* knockdown decreased cell survival under hypoxic conditions in both cell lines. The blots represent typical results from three independent experiments. MTT and WST-1 assays were done in triplicates. * and #, $P < 0.05$ compared with correspondent control cells.

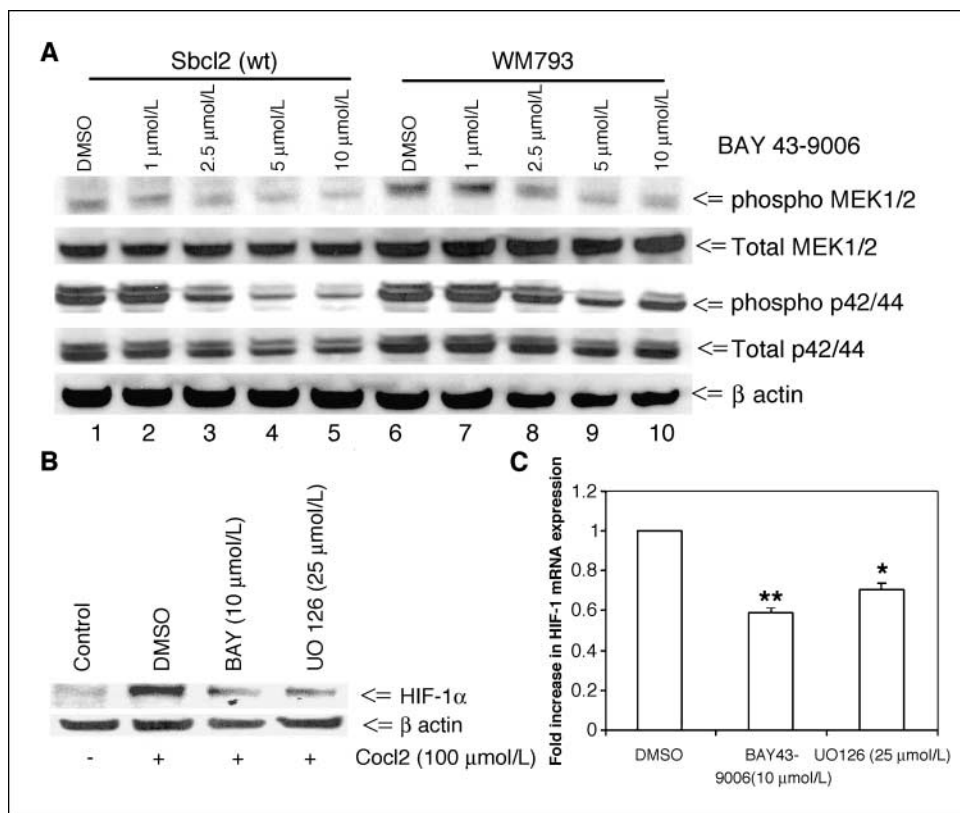
the role of *BRAF* mutations during melanoma initiation or progression is only started to be understood. In this study, we showed that *BRAF*^{V600E} mutation in melanoma is associated with increased HIF-1 α expression and cell survival under hypoxic conditions. Knockdown or pharmacologic inhibition of *BRAF* results in significant reduction of HIF-1 α expression in tumor cells. Decreased HIF-1 α expression in *BRAF* knockdown melanoma cells is likely resulted from both decreased transcription and increased degradation of HIF-1 α protein.

HIF-1 α is an essential mediator of O₂ homeostasis, it is instrumental in the oxygen-dependent regulation of a variety of genes, such as *erythropoietin*, *VEGF*, *nitric oxide synthase*, *heme oxygenase 1*, and *glucose transporters* (30, 31). HIF-1 α is also involved in tumor progression and metastasis, and its expression is correlated with early relapse and metastatic disease. Recently, it has been shown that HIF-1 α induces genetic instability by transcriptionally down-regulating mismatch repair system, which is crucial for maintaining cellular genetic integrity (32), and HIF-1 α -induced mutations may occur in the very early stages during tumor development. Because *BRAF* mutation occurs in precursor melanocytic lesions such as dysplastic nevi, mutant V600E *BRAF* induced HIF-1 α expression may potentially involved in the early transformation of the precursor lesions.

HIF-1 α activation has been shown to be regulated by the PI3K and MAPK pathways (33, 34). Our microarray results showed that melanomas with mutant V600E *BRAF* have significantly increased *HIF-1 α* gene expression. Papillary thyroid cancers (PTC) are also known to harbor V600E *BRAF* mutation. It has been shown recently that VEGF, one of the direct targets of HIF-1 α , is significantly up-regulated in V600E *BRAF*-positive PTCs, compared with V600E *BRAF*-negative PTCs, by immunohistochemistry (35). Therefore, it seems that the effect of V600E *BRAF* mutation on HIF-1 α is not confined to melanoma cells. Overexpression of mutant V600E *BRAF* in melanoma cells with wild-type *BRAF* increased hypoxic tolerance of these cells. Suppression of wild-type and/or mutant V600E *BRAF* resulted in marked reduction of HIF-1 α at both gene expression and protein level. Decreased HIF-1 α expression rendered melanoma cells more susceptible to hypoxia-induced cell death. The *BRAF* knockdown is specific, and it can be rescued by reintroducing a *BRAF*^{V600E} plasmid into *BRAF* stable knockdown cells. Our data suggest that mutant V600E *BRAF* promotes cell survival under hypoxic conditions through up-regulation of HIF-1 α expression in melanoma cells.

HIF-1 α expression is primarily regulated at the posttranslational level, and its degradation is regulated by O₂-dependent prolyl

Figure 5. Pharmacologic inhibition of BRAF decreases HIF-1 α expression. **A**, dose response of BAY 43-9006 on MEK-ERK phosphorylation. Sbc12 and WM793 cells were treated with different dose of BAY 43-9006 (0, 1, 2.5, 5, and 10 $\mu\text{mol/L}$) for 24 h. Phosphorylation specific MEK and ERK antibodies were used to determine the activation of MEK and ERK. Total MEK and ERK proteins as well as β -actin were used as loading controls. **B**, Western blot analysis showed CoCl₂ increased HIF-1 α protein in the tumor cells, whereas BAY 43-9006 (10 $\mu\text{mol/L}$) and MEK inhibitor UO126 (25 $\mu\text{mol/L}$) inhibited HIF-1 α protein expression. **C**, relative transcription level of HIF-1 α in WM793 cells after incubating with BAY 43-9006 compounds and UO126. **, $P < 0.01$ comparing HIF-1 α mRNA level before and after BAY 43-9006 treatment; *, $P < 0.05$ comparing HIF-1 α mRNA level before and after UO126 treatment. The blots represent typical results from three independent experiments.

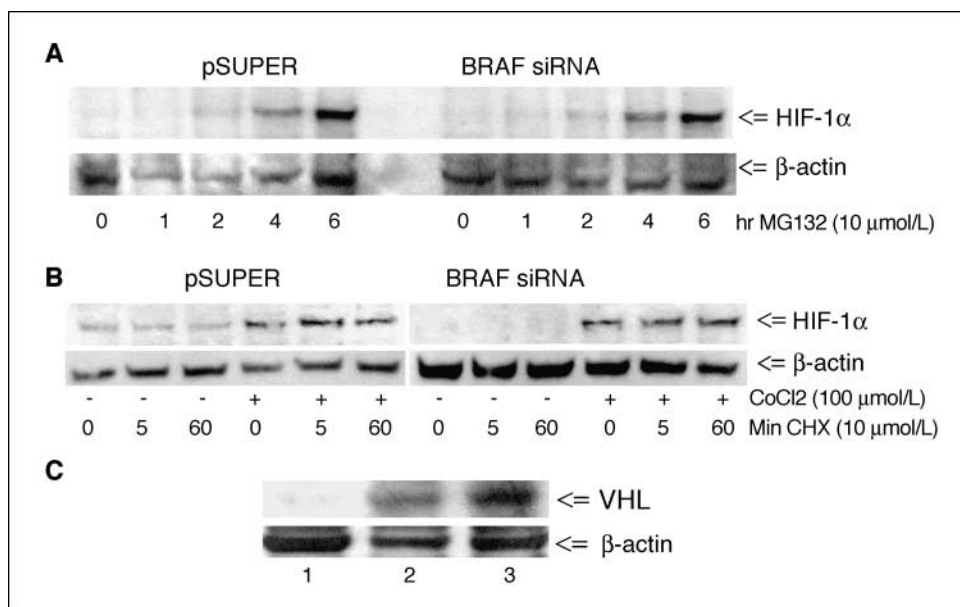


hydroxylation, which targets the protein for ubiquitylation and degradation by the proteasome (36). The degradation depends on VHL tumor suppressor protein, which binds specifically to hydroxylated HIF-1 α (37). We showed that *BRAF* knockdown does not change the translational rate of HIF-1 α but increases degradation of this protein with an associated up-regulation of VHL, suggesting that mutant V600E *BRAF* may regulate HIF-1 α posttranslational modification.

Recently, BAY 43-9006 has been used in several phase I and II clinical trials in patients with a variety of cancers, including

melanoma, and showed promising clinical efficacy when combined with other chemotherapeutic agents (38). This compound was initially thought to specifically inhibit BRAF; it was recently shown that it also inhibits other molecules such as VEGF and platelet-derived growth factor receptors (39, 40). Similar to a prior report using 1205Lu melanoma cells (10), we observed that BAY 43-9006 inhibited ERK and MEK phosphorylation in WM793 melanoma cells. BAY 43-9006 also significantly reduced HIF-1 α expression in melanoma cells, suggesting that some of the effects of this compound may be mediated through HIF-1 α inhibition.

Figure 6. HIF-1 α stability and VHL expression in *BRAF* knockdown cells. **A**, time course of HIF-1 α protein translation. A proteasome inhibitor (MG132) was used to inhibit degradation of HIF-1 α protein in these cells. Western blots of HIF-1 α in cells treated with MG132 for various time (0, 1, 2, 4, and 6 h) showed that HIF-1 α protein translational rate was similar before and after *BRAF* knockdown. The amount of protein loaded in each lane was normalized with β -actin. **B**, *BRAF* knockdown increases HIF-1 α degradation. In the absence of HIF-1 α stabilizer CoCl₂, HIF-1 α protein was rapidly degraded in *BRAF* knockdown cells, whereas the wild-type cells retained HIF-1 α protein. In the presence of CoCl₂ and cycloheximide (CHX), the HIF-1 α protein level was similar in *BRAF* knockdown and control cells. **C**, Western blot analysis of VHL protein expression in WM793 before and after *BRAF* knockdown shows that *BRAF* knockdown cells have higher VHL protein expression. Lane 1, control; lane 2, *BRAF*siRNA; lane 3, *BRAF*mutsiRNA. The blots represent typical results from three independent experiments.



In conclusion, our results show for the first time that mutant V600E *BRAF* increases HIF-1 α expression and melanoma cell survival under hypoxic conditions. These data suggest that the oncogenic effects of activating *BRAF* mutations are mediated in part through the HIF-1 α pathway. Involvement of activating mutations of *BRAF* in hypoxic signaling might be critical for tumor initiation and growth because *BRAF* gene mutations occur early during tumor progression.

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