# The hypoxic microenvironment of the skin contributes to Akt-mediated melanocyte transformation

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## Summary

**Constitutive activation of Akt characterizes a high percentage of human melanomas and represents a poor prognostic factor of the disease. We show that Akt transforms melanocytes only in a hypoxic environment, which is found in normal skin. The synergy between Akt and hypoxia is HIF1α mediated. Inhibition of HIF1α decreases Akt transformation capacity in hypoxia and tumor growth in vivo, while overexpression of HIF1α allows anchorage-independent growth in normoxia and development of more aggressive tumors. Finally, we show that mTOR activity is necessary to maintain the transformed phenotype by sustaining HIF1α activity. Taken together, these findings demonstrate that Akt hyperactivation and HIF1α induction by normally occurring hypoxia in the skin significantly contribute to melanoma development.**

## Introduction

Over the past 50 years, the incidence of melanoma has shown an alarming rate of increase worldwide. Although melanoma accounts for only 10% of all skin cancers, it is responsible for 80% of all skin cancer deaths (Houghton and Polsky, 2002). Melanoma is an aggressive tumor, with a propensity to metastasize, and is resistant to most current therapeutic regimens (Satyamoorthy et al., 2001). Environmental stresses together with genetic alterations are key players in melanoma development.

UV exposure, particularly in childhood, represents a key environmental risk factor in melanomagenesis (Williams and Ouhtit, 2005). Recent studies have identified the $p16^{INK4A}$/Rb and $p14^{ARF}$/p53 pathways as the main UV targets disrupted in the early stages of melanoma development (Kannan et al., 2003). *$p16^{INK4A}$* and *$p14^{ARF}$* are encoded by the *CDKN2a* locus at 9p21 via alternative reading frames. *CDKN2a* is considered a melanoma susceptibility locus because its loss of function, commonly seen in the human disease, is necessary although not sufficient for melanoma development (Sharpless and Chin, 2003). Additional cooperating genetic lesions are required.

Mutations or alterations of the Ras pathway are common events in melanoma development. Gain-of-function mutations of *NRas* and *BRaf* are found in a large number of primary melanomas and nevi (Davies et al., 2002; Demunter et al., 2001; Papp et al., 1999; Pollock et al., 2003), while *Akt3* has been found hyperactivated in 43%–60% of sporadic melanomas as a result of gene amplification and decreased PTEN protein activity (Stahl et al., 2004). Interestingly, although activating *BRaf* mutations are frequent in melanoma, *BRaf* appears to be insufficient for the development of the disease in model systems (Chudnovsky et al., 2005; Pollock et al., 2003), suggesting that additional molecular defects may be required. On the other hand, data obtained in transgenic mice (Chin et al., 1997, 1999; Powell et al., 1999), and more recently in a human melanoma model (Chudnovsky et al., 2005), have shown that both *Ras* and *phosphoinositide 3-kinase* [*PI(3)Kinase*] are oncogenes in melanoma, as active mutants of the two genes are sufficient to induce invasive melanocytic neoplasia. In both cases, concomitant disruption of the $p16^{INK4A}$-CDK4-Rb and ARF-HDM2-p53 pathways is necessary for melanoma development, confirming in experimental models what is commonly observed in the human disease.

In vivo models allow the study of melanoma development in a more physiological context where environmental factors can potentially play a role in the transformation process. In

### SIGNIFICANCE

**Melanoma is an aggressive tumor that can metastasize early in the course of the disease and, most importantly, is resistant to most current therapeutic regimens. Understanding the genetic and environmental factors driving melanomagenesis is essential for the development of new therapies. We show that the microenvironment in which melanocytes reside is hypoxic and that this oxygen level is necessary to allow melanocyte transformation initiated by Akt; that the synergy between hypoxia and Akt is HIF1α dependent; that the mTOR pathway is required to maintain the transformed phenotype partially by maintaining HIF1α activity; and that rapamycin efficiently decreases melanoma development. We therefore propose that rapamycin represents a promising hypoxia-related therapeutic approach in the treatment of melanoma.**

particular, the oxygen concentration in tissues is lower than that in the atmosphere where in vitro experiments are normally performed. Skin, in fact, has been shown to be mildly hypoxic (Evans and Naylor, 1967; O'Hara et al., 1997; Stewart et al., 1982), likely due to the distance from the superficial blood vessels. Thus, it seems reasonable to consider such factors when modeling diseases in vivo and in vitro.

Cells respond to hypoxia by activating the heterodimeric transcription factor hypoxia-inducible factor-1 (HIF-1), composed of HIF1α and the constitutively expressed HIF1β (ARNT). Under normoxic or atmospheric $O_2$ levels (21%), hydroxylation of key proline residues within the regulatory oxygen-dependent degradation domain (ODD) of the HIF1α subunit facilitates von Hippel-Lindau protein (pVHL) binding, which in turn allows ubiquitylation and subsequent proteasome-targeted degradation (Jaakkola et al., 2001; Yu et al., 2001). Under limiting $O_2$ conditions, proline hydroxylation is inhibited, thereby stabilizing HIF1α subunits, which can then translocate into the nucleus and bind to constitutively stabilized HIF1β subunits, forming the active HIF-1 complex.

HIF-1 activates a multitude of $O_2$-responsive genes involved in various normal cell functions such as survival, apoptosis, glucose metabolism, and angiogenesis. In physiologic hypoxic conditions, these functions are finely tuned and contribute to the maintenance of tissue homeostasis. In pathological states, such as neoplasms, severe hypoxia (<0.5% $O_2$) becomes toxic for normal and transformed cells unless the latter undergo genetic and adaptive changes that will allow them to survive. In this condition, hypoxia significantly contributes to tumor progression (Harris, 2002). On the other hand, mild hypoxia (1%–5% $O_2$) has been shown to promote cell proliferation and survival of different cell types (Alaluf et al., 2000; Balin and Pratt, 2002; Studer et al., 2000); to increase the life span of fibroblasts and vascular smooth muscle cells (Minamino et al., 2001; Parrinello et al., 2003); and to counteract replicative senescence (Chen et al., 1995; Lee et al., 1999), mainly by decreasing oxidative DNA damage and by activating telomerase via a HIF1α-dependent mechanism (Nishi et al., 2004; Yatabe et al., 2004).

Given the various growth-promoting effects of mild hypoxia through the action of HIF-1, mild hypoxia may be a previously unappreciated tumor-promoting environmental factor present in both in vivo models and human disease. In melanoma, this may be particularly relevant, as nuclear HIF1α staining has been shown in normal skin (Distler et al., 2004), suggesting that hypoxic levels of the skin are compatible with HIF1α activity. Melanocytes reside in such hypoxic milieu, which together with environmental stresses and genetic alterations, may represent a promoting factor for melanocyte transformation.

In this work, we show that constitutively active (c.a.) Akt is able to transform melanocytes in a hypoxic environment that recapitulates in vitro the hypoxic status of the skin. HIF1α is shown to be required for Akt-dependent transformation of melanocytes and to be able to enhance Akt oncogenic potential. Moreover, we show that treatment with rapamycin, a mammalian target of rapamycin (mTOR)-specific inhibitor, significantly reduces tumor growth and is associated with inhibition of HIF1α activity. Together, these findings highlight the role of Akt and hypoxia in melanocyte transformation and support further investigation of new prevention and therapeutic strategies for melanoma aimed at the inhibition of HIF1α and Akt pathways.

## Results

### Human and mouse melanocytes reside in a hypoxic environment

Both human and mouse skin have been shown to be mildly hypoxic, with oxygen levels ranging between 1.5% and 5% (Evans and Naylor, 1967; O'Hara et al., 1997; Stewart et al., 1982). As shown in Figure 1, normal human and mouse skin present hypoxic regions as indicated by positive staining of the hypoxia markers carbonic anhydrase IX (CAIX) (Figure 1B) (Potter and Harris, 2003) and 2-nitroimidazole EF5 (EF5) (Figure 1G) (Koch, 2002), respectively. These markers correlate with glucose transporter-1 (Glut-1) staining, a well-known HIF1α direct target gene (Figures 1C and 1H), which further supports the idea that the microenvironment where melanocytes reside is hypoxic and suitable for HIF1α activity. Indeed, HIF1α localizes in nuclei in human skin (Figure 1E) and correlates with Glut-1 staining (Figure 1F). Melanocytes are localized in the dermal epidermal junction in humans (upper left panels in Figure 1C) and in hair follicles in mice (Figures 1K–1N). These observations support the notion that the normal microenvironment of the skin is hypoxic, and this could, potentially, influence melanocyte viability and proliferation.

Stringent hypoxia is generally associated with cell death, whereas mild hypoxia has been shown to increase cell life span and to promote cell immortalization (Parrinello et al., 2003), a phenomenon that normally preludes transformation. Cells of the epidermal basal layer stain for the proliferation marker Ki67 (Figure 1I), indicating that the environment, although hypoxic, is suitable for cell viability. Normal human and mouse skin did not stain positive for phospho-Akt (Figures 1D and 1J, respectively).

These findings demonstrate that hypoxia and HIF1α are a feature of normal skin and may influence the fate of cells acquiring oncogenic alterations.

### Hypoxia cooperates with Akt in promoting melanocyte transformation

Although hypoxia is typically thought of as a common characteristic of solid tumors (Harris, 2002), it also appears to be a characteristic of normal skin. To investigate the contributory role of hypoxia to transformation of melanocytes, we infected *Ink4a/ARF* knockout cells with c.a. Akt or Ras ($^{V12}$Ras) and then plated them in soft agar in normoxic or hypoxic conditions. As previously shown (Laughner et al., 2001), Akt increased HIF1α levels under normoxia. Target genes such as Glut-1 and a hypoxia response element (HRE)-driven reporter construct were also increased. Under hypoxic conditions, HIF1α is further elevated in both control (pBabe) and Akt cells (Figures 2C and 2D). Similar results are seen in Ras cells as well (Figure S4D in the Supplemental Data available with this article online). Cells expressing c.a. Akt showed no or poor growth in normoxia despite a high level of GSK3 phosphorylation, while $^{V12}$Ras-expressing melanocytes formed numerous colonies in soft agar (Figure 2A and Figure S4A). On the contrary, the capacity of c.a. Akt-expressing melanocytes to grow in the absence of substrate was restored in a low-oxygen environment (2% $O_2$). These results suggest that hypoxia is required for efficient melanocyte transformation driven by Akt (Figure 2A, lower panel) and may aid in transformation by Ras. Indeed, a slight increase in colony number was also

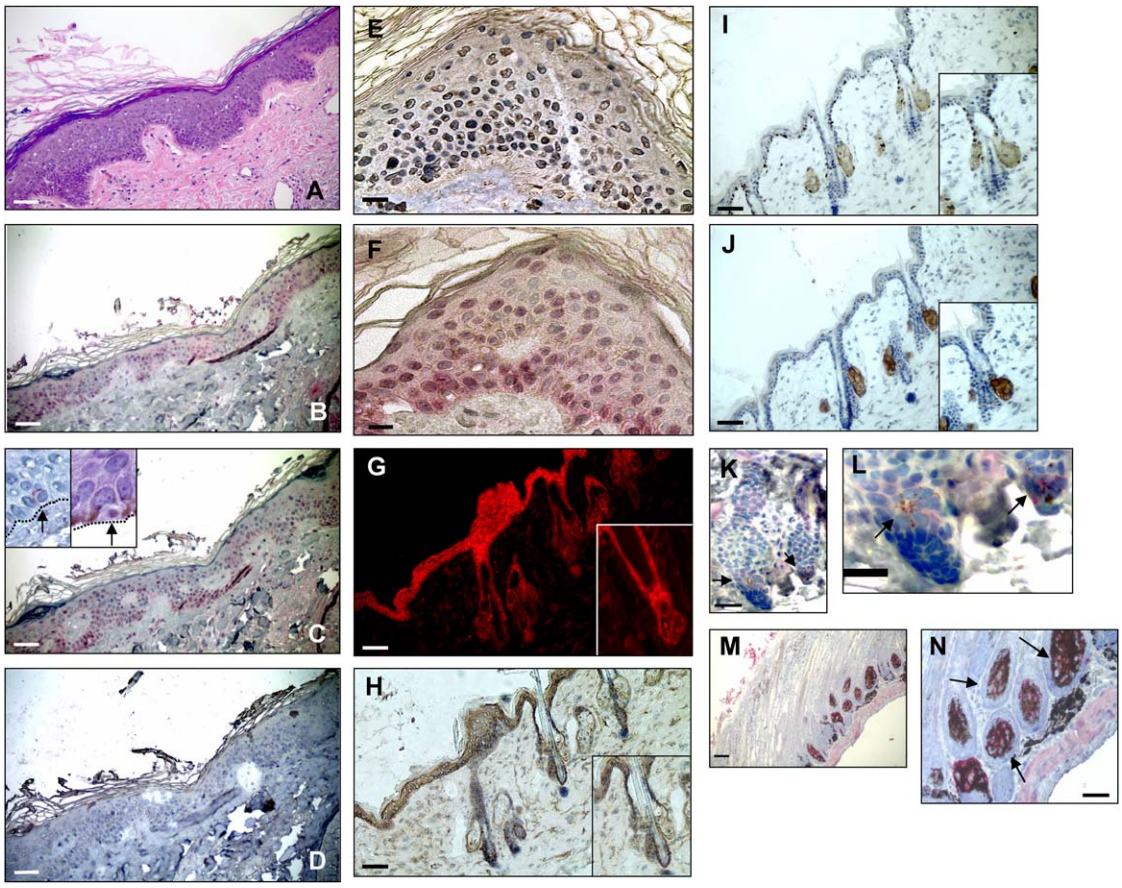


**Figure 1.** Normal human and mouse skin are hypoxic with low levels of Akt activity

**A:** Archival normal human skin specimens stained with H&E. **B:** Normal human skin shows positive staining for the hypoxia marker CAIX. **C:** The HIF1α-specific target gene Glut-1 in normal human skin is expressed along the epidermal/dermal junction where melanocytes reside. Upper left panels: melanocytes are localized in the epidermal/dermal junction and are positive for the melanocyte-specific marker Tyrp-2 (red stain of cytoplasm). **D:** Absence of activated Akt in normal human skin as shown by lack of staining with a specific anti-phospho-Akt antibody. **E:** Normal human skin positively stains for HIF1α. **F:** Glut-1 staining of the same human specimen used in **E**. **G:** Normal skin from C3H/HeJ mice injected with the hypoxic-specific marker EF5. Hypoxic areas in hair follicles and epidermis appear in bright red. **H:** Normal mouse skin shows intense staining for the HIF1α-dependent target Glut-1 in a pattern similar to the hypoxia marker. **I:** Ki67 staining of normal mouse skin along the basal layer. **J:** Akt activity is low or absent in normal mouse skin as shown by staining with a specific anti-phospho-Akt antibody. Insets from **G** to **J** show staining in hair follicles. **K** and **L:** Melanocytes in the hair matrix of the hair follicle of adult C3H/HeJ mice stain positive for the specific melanocyte marker Tyrp-1 (arrows show melanocytes). **M** and **N:** Melanocytes positive for Tyrp-1 in 5-day-old TP-Ras/C3H/HeJ mice (Powell et al., 1999). Photographs were taken of serial 5 μm sections of paraffin-embedded normal human skin or frozen mouse skin. Scale bars in **A**–**D**, **G**–**K**, and **M**, 50 μm; scale bars in **E**, **F**, and **N**, 20 μm. **L** shows a detail from **K**.

observed for $^{V12}$Ras-expressing melanocytes grown in hypoxia (Figure S4). Thus, mild hypoxia appears to be a more suitable environment for efficient transformation in general.

Active Akt has been shown to increase glucose consumption (Elstrom et al., 2004). We examined whether glucose depletion from the culture media might inhibit colony formation in soft agar. We found glucose consumption to be minimal overall with a slight increase in cells cultured in hypoxia (Figure S1). These results suggest that a glucose limitation does not affect colony growth of Akt-expressing melanocytes.

When injected in immunodeficient mice, Akt-expressing melanocytes formed tumors, although they grew at a slower rate with respect to Ras-expressing cells (Figure 2B). This was similar to what we observed in vitro. Since hypoxia is normally occurring in the skin, it is likely that this hypoxic environment is contributing to cell growth and tumor formation in vivo.

### HIF1α is necessary for Akt-dependent melanocyte transformation, and its overexpression allows anchorage-independent growth under normoxia

HIF1α is a major mediator of the cellular hypoxic response. The levels of HIF1α increase in cells in vitro after exposure to an oxygen concentration of <6%–8% (Jiang et al., 1996). HIF1α becomes rapidly stabilized in response to hypoxia and returns to basal levels after 12–24 hr (Uchida et al., 2004). In our system, melanocytes exposed to hypoxia exhibit sustained HIF1α stabilization and activity for up to 20 days (Figure S2), indicating that for the duration of the agar assay (about 3 weeks) cells continue to express HIF1α and its target genes. We wanted therefore to determine whether constant HIF1α activity was required for Akt to transform melanocytes. To test this, HIF1α levels were decreased by the use of a specific short interfering RNA (siRNA). Cells stably expressing this siRNA showed a

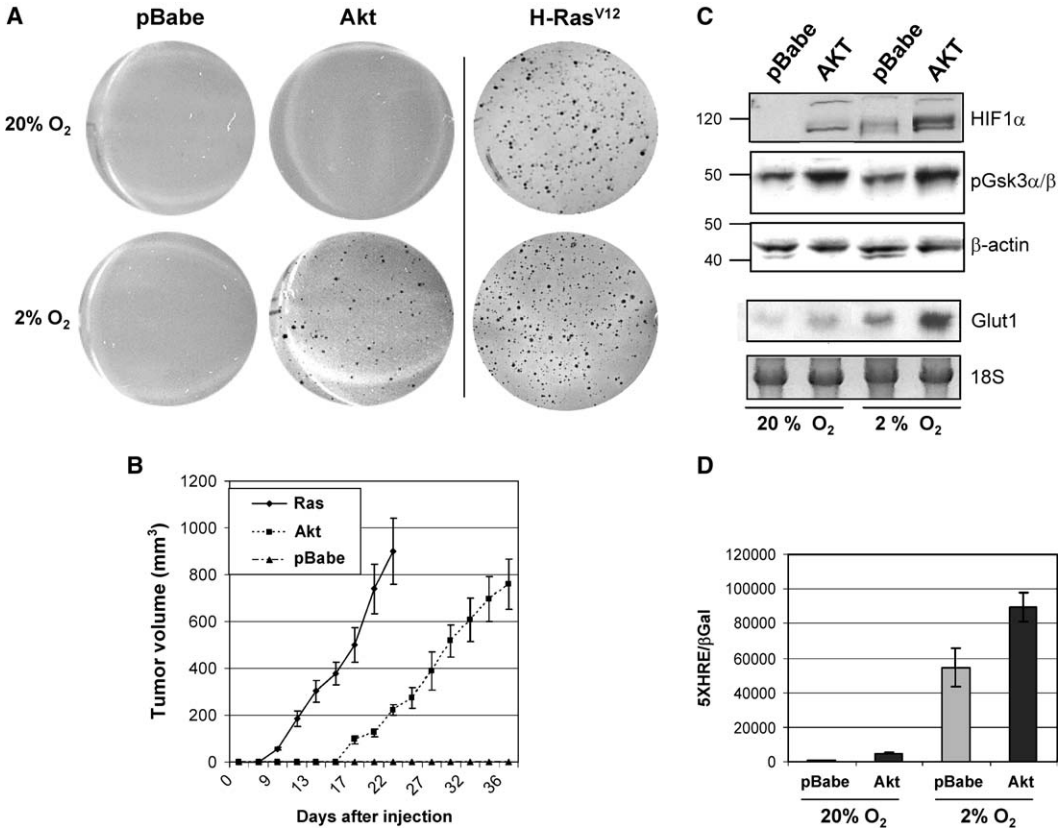


**Figure 2.** Akt and hypoxia synergize in transforming melanocytes

**A:** pBabe-, c.a. Akt-, and $^{V12}$Ras-expressing cells were seeded in soft agar and incubated in either normoxia (20% $O_2$) or hypoxia (2% $O_2$).
**B:** Cells described in **A** were injected s.c. in SCID mice at a density of $10^6$ cells. Tumor volumes were measured and are represented as average ± SE.
**C:** Activity of the c.a. Akt was assessed by phosphorylation of its downstream target, GSK3. HIF1α protein accumulates in hypoxia. HIF1α activity was measured by the induction of Glut-1 transcription.
**D:** HIF1α activity was assessed by the induction of 5XHRE-driven luciferase expression. Luciferase units were normalized for transfection efficiency with β-galactosidase expression. Results are the mean values ± SD of triplicates. Results are representative of at least three independent experiments.

decreased capacity to upregulate HIF1α under hypoxia (Figure 3C) and decreased HIF1α activity as demonstrated by reduced Glut-1 expression and decreased activity of an HRE-driven reporter construct (Figures 3C and 3D). Reduced HIF1α activity resulted in a reduced capacity to grow in soft agar (Figure 3A) and a significant reduction of tumor growth in vivo. In fact, tumors formed by cells expressing the siRNA were half the size of control tumors expressing a scrambled sequence (Figure 3B).

To further assess the role of HIF1α in melanocyte transformation, a constitutive form of the molecule was stably transduced into melanocytes expressing either a control vector (pBabe) or Akt. This HIF1α species lacks the ODD and therefore is stable in normoxic conditions. While the endogenous HIF1α is virtually undetectable (data not shown), HIF1α-$_{ODD}$ (60–70 kDa) is highly expressed and active in atmospheric oxygen (Figures 4C and 4D). Concomitant presence of active Akt and stable HIF1α promotes melanocyte transformation in vitro in normoxia and confers an increased tumor growth rate of the cells in SCID mice (Figures 4A and 4B).

These data suggest that HIF1α is a major mediator of the hypoxic effect on Akt-driven melanocyte transformation. Interestingly, expression of c.a. HIF1α alone was not able to transform melanocytes. This observation suggests that HIF1α may act mainly as a promoting factor rather than an oncogene per se.

### Inhibition of mTOR by rapamycin results in decreased anchorage-independent growth of Akt- and Akt/HIF1α-$_{ODD}$-expressing cells

Akt activates a plethora of signaling cascades involved in a number of functions, many of which can play a role in tumorigenesis (Testa and Bellacosa, 2001). mTOR is a downstream effector of the PI(3)Kinase/Akt pathway, and its functions have been found to be altered in a variety of tumors bearing alterations of PTEN, Akt, or other molecules along the pathway. Tumors with alterations in the PI(3)Kinase/Akt/mTOR pathway generally respond well to treatment with rapamycin, a specific mTOR inhibitor. This suggests that mTOR has an essential role in maintaining the transformed phenotype. Although the role of mTOR in melanomas has not been fully addressed, it is conceivable that Akt-driven melanomas may respond well to rapamycin treatment. In order to investigate the responsiveness of Akt-expressing melanocytes to rapamycin, cells were treated with increasing doses of the drug, and anchorage-independent growth capacity was analyzed (Figure 5A). The number of colonies in soft agar decreased significantly at increasing drug concentrations

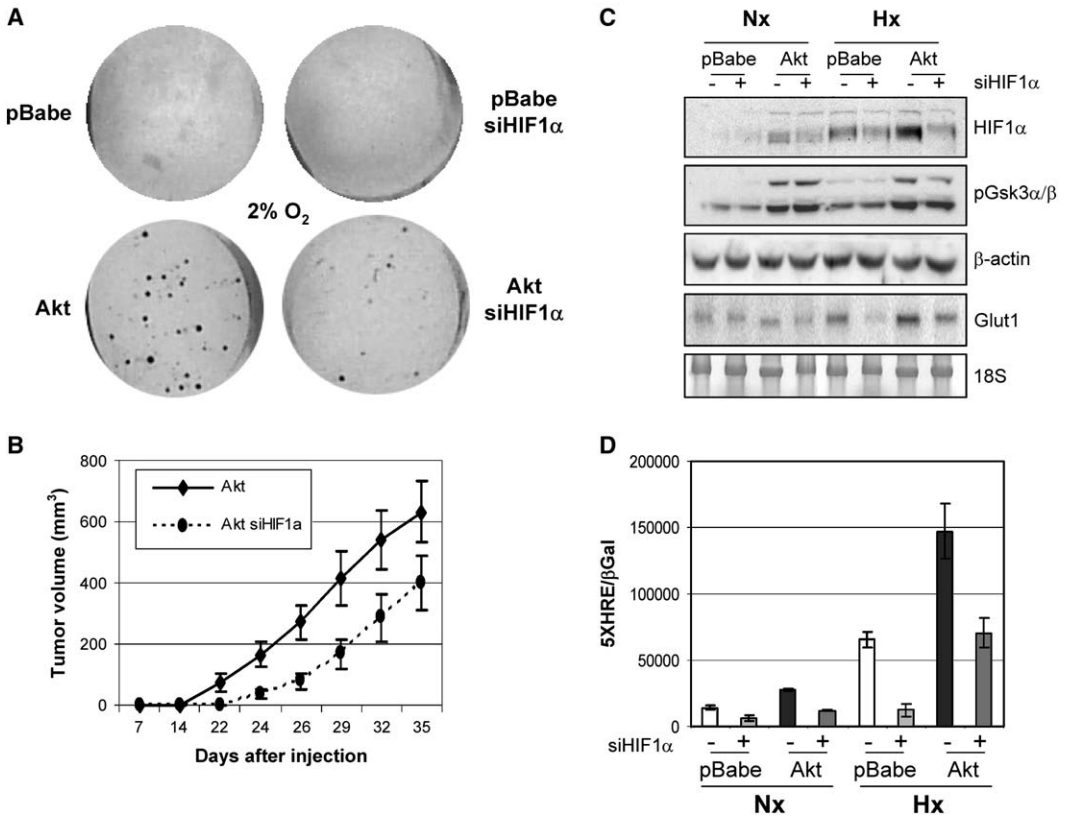


**Figure 3.** HIF1α is required for Akt-dependent melanocyte transformation

**A:** pBabe- and c.a. Akt-expressing cells stably transfected with either a siRNA specific for mouse HIF1α or a scrambled siRNA sequence were seeded in soft agar and incubated in 2% $O_2$ for 3 weeks. Colonies were stained with GIEMSA for detection.

**B:** Cells described in **A** were injected s.c in SCID mice at a density of $10^6$ cells. Tumor volumes are represented as average ± SE.

**C:** Cells using an anti-HIF1α-specific antibody on Akt cells expressing either a siRNA specific for HIF1α (+) or the scrambled sequence (−). HIF1α activity was determined by induction of Glut-1 transcription. 18S ribosomal RNA was used as a loading control.

**D:** HIF1α activity was assessed using a 5XHRE-luciferase reporter construct. Results are the mean values ± SD of triplicates. Results are representative of three independent experiments.

($p < 0.001$) for both Akt- and Akt/HIF1α-$_{ODD}$-expressing melanocytes, indicating that both cell types depend on mTOR function. On the other hand, rapamycin shows reduced efficacy in inhibiting colony growth in soft agar of Ras-expressing melanocytes (Figure S4A).

Phosphorylation of 4E-BP1, a downstream target of mTOR, is comparable in cells under normoxia or hypoxia, and rapamycin inhibits phosphorylation in a dose-dependent manner (Figure 5B). Similar results were seen for Akt/HIF1α-$_{ODD}$-expressing cells under normoxia (Figure 5C) and for Ras-expressing melanocytes (Figure S4C). Phosphorylation of GSK3, a downstream target of Akt that is independent of mTOR, and phosphorylation of extracellular signal-regulated kinases (Erk) do not appear to be inhibited, indicating that rapamycin is specific for its target molecule.

It has been reported that hypoxia inhibits protein synthesis by both inactivation (via phosphorylation) of the eukaryotic initiation factor eIF2α and inhibition of mTOR (Wouters et al., 2005). In our system, however, we did not observe this inhibitory effect at 2% oxygen. In fact, mTOR function is maintained, protein synthesis is slightly increased (Figure S5A), and eIF2α does not appear to be phosphorylated (Figure S5B). This may be due to the levels of oxygen used in our experimental settings or to melanocytes specifically. Therefore, inhibition of protein synthesis does not seem to affect our system.

### Inhibition of mTOR by rapamycin results in tumor growth inhibition associated with decreased HIF1α activity

In vitro, rapamycin significantly inhibits anchorage-independent growth, a characteristic of transformed cells. We next assessed whether rapamycin would inhibit the growth of Akt and Akt/HIF1α-$_{ODD}$ melanomas in vivo. Cells ($10^6$ of each type) were injected in SCID mice and allowed to grow until tumors reached about 150 $mm^3$ in volume. Tumors were then topically treated with different doses of rapamycin until the control tumor reached a volume of about 1–1.2 $cm^3$. As shown in Figure 6, tumor types originating from both Akt-expressing (Figure 6A) and Akt/HIF1α-$_{ODD}$-expressing (Figure 6B) cells respond well to rapamycin, showing a significant ($p < 0.05$) decrease in growth, especially at the highest dose used. At 100 μM, both tumor types are about 70% smaller than control tumors. The effect of rapamycin appears to be mainly cytostatic. In fact, whereas no significant difference was observed in TUNEL staining between ethanol-treated and rapamycin-treated tumors (data not shown), positivity to the proliferation marker Ki67 was significantly reduced in rapamycin-treated melanomas (Figure 7C).

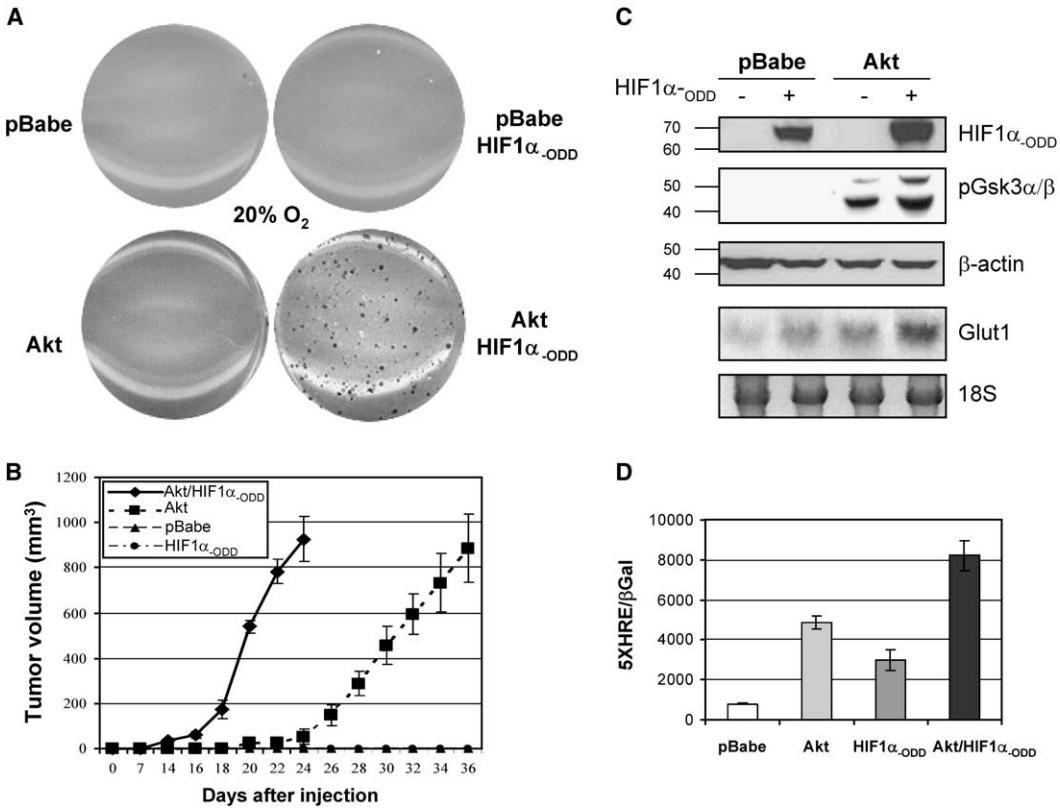


**Figure 4.** Overexpression of stable HIF1α promotes transformation of Akt-expressing melanocytes in normoxia

**A:** pBabe- and c.a. Akt-expressing melanocytes were stably transfected with HIF1α-$_{ODD}$, a normoxia-stable form of HIF1α. Cells were seeded in soft agar for 3 weeks in 20% $O_2$ (normoxia).

**B:** Cells indicated in **A** were injected s.c. in SCID mice ($10^6$ cells/flank), and mean tumor volume ± SE is represented.

**C:** Akt activity is indicated by the phosphorylation of GSK3. Expression of HIF1α-$_{ODD}$ was measured by Western blotting. The molecular weight of HIF1α-$_{ODD}$ is lower than that of endogenous HIF1α. Glut-1 expression was measured to assess HIF1α-$_{ODD}$ activity.

**D:** HIF1α-$_{ODD}$ activity was evaluated by using a 5XHRE-luciferase reporter construct. Results are the mean values ± SD of triplicates. All experiments were performed in normoxia to avoid interference with the endogenous HIF1α.

Less intense staining with a phospho-specific antibody against 4E-BP1 in rapamycin-treated tumors indicates that the treatment was effective against its specific target.

Inhibition of mTOR results in decreased HIF1α activity both in vitro and in vivo and a slight decrease in HIF1α protein accumulation in hypoxia (Figure 7A, upper panel). HIF1α activity was assessed in vitro as the capacity to transcribe Glut-1 and to activate an HRE-luciferase construct (Figures 7A and 7B). In vivo, inhibition of mTOR results in reduced Glut-1 expression as well (Figure 7C).

In parallel studies, we examined the effect of rapamycin in Ras-expressing melanomas. These tumors show a slight resistance to rapamycin treatment. Tumor size is reduced by only 30% with respect to the size of control tumors and only when the highest concentration of drug was used (Figure S4B). This effect is accompanied in vitro by a slight reduction of HIF1α protein and activity (Figure S4D). This is possibly due to the regulation of Akt by Ras, since Akt/mTOR have been shown to regulate HIF1α translation (Laughner et al., 2001).

In conclusion, the data presented here demonstrate that mTOR activity is essential for the formation of Akt-driven melanomas, potentially through regulation of protein translation and HIF1α activity.

## Discussion

Disregulation of the PI(3)Kinase/Akt pathway is found in a variety of cancers (Vivanco and Sawyers, 2002). Recently, active PI(3)kinase, together with inactivation of the $p16^{INK4A}$-CDK4-Rb and ARF-HDM2-p53 pathways, has been shown to be able to transform human melanocytes (Chudnovsky et al., 2005). These findings are consistent with observations in human melanomas in which inactivation of the *CDKN2A* locus is quite frequent and where high Akt activity has been associated with development and progression of melanoma and correlated with poor prognosis (Dai et al., 2005; Stahl et al., 2004). Using murine melanocytes lacking the *CDKN2A* locus, we found that an active form of Akt can transform such cells in vitro and induce tumor growth in vivo. Moreover, we demonstrate that in order to efficiently transform melanocytes, Akt requires hypoxia and the activity of HIF-1.

Most of the experiments evaluating the transformation potential of oncogenes in vitro have been carried out in atmospheric oxygen. This condition is not representative of the much lower oxygen tension found in tissues that constitute the native environment in which transformation processes are likely to happen. It has been reported that human skin is mildly hypoxic, with

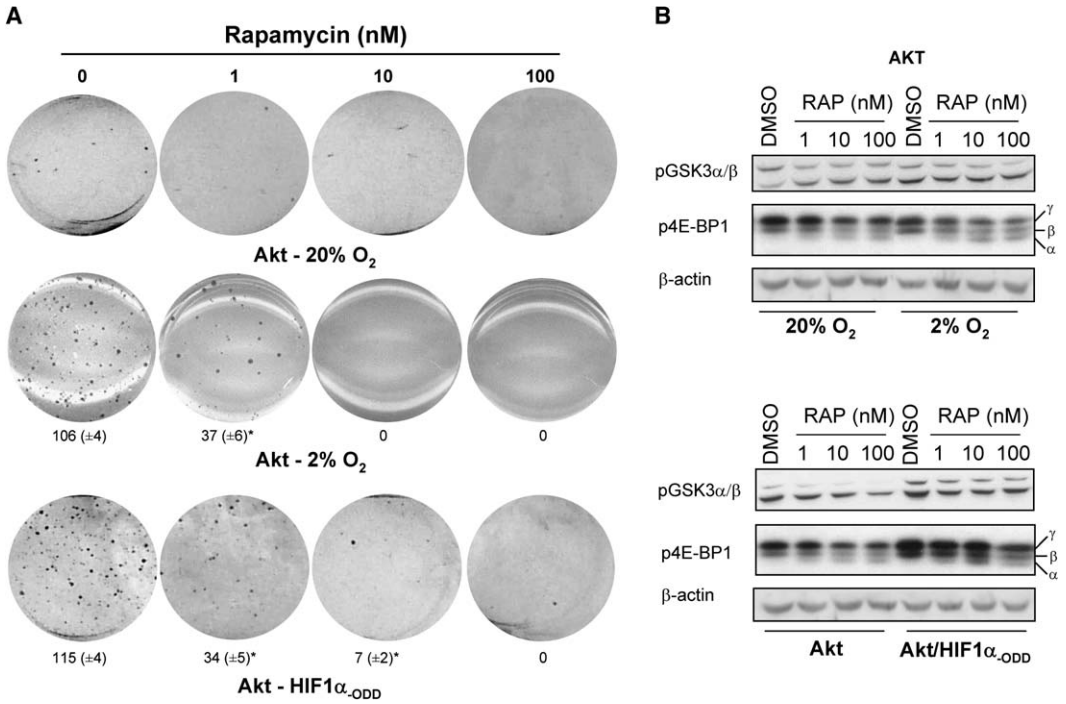


**Figure 5.** Inhibition of mTOR activity by rapamycin reduces melanocyte transformation by Akt and HIF1α in vitro

**A:** Akt- or Akt/HIF1α-$_{ODD}$-expressing melanocytes were seeded in soft agar containing increasing concentrations of rapamycin (1 nM, 10 nM, and 100 nM). Cells were incubated in normoxia (20% $O_2$) and hypoxia (2% $O_2$), as indicated, for 3 weeks. Average numbers of colonies ± SD from four independent experiments are indicated. Differences between rapamycin- and vehicle-treated cells (0 μM rapamycin) are statistically significant (*$p < 0.001$, Student's t test).
**B:** The efficacy of the rapamycin treatment on Akt- and Akt/HIF1α-$_{ODD}$-expressing melanocytes was evaluated as the inhibition of activation (phosphorylation) of the mTOR target 4E-BP1 in both normoxia and hypoxia. The Akt downstream effector GSK3 was used as a control for specificity of the treatment; β-actin was used as a loading control.

oxygen levels ranging from 1.5% to 5% $O_2$ (O'Hara et al., 1997). Here, we show that normal human skin presents high levels of CAIX and Glut-1 (Airley et al., 2003) and stains positively for HIF1α, strongly suggesting that hypoxia is a normal characteristic of the tissue microenvironment. This was further confirmed in mice injected with EF5. EF5 is a specific metabolic marker of hypoxia, as it binds to cellular macromolecules only in regions characterized by low oxygen concentrations (Koch, 2002). In mouse skin, we observed intense staining of the marker, which also correlated with high expression of Glut-1. Interestingly, cells in this hypoxic environment stained positive for the proliferation marker Ki67, indicating that the hypoxic levels found in skin are compatible with cell viability and replication.

Our finding is in contrast with other reports in which hypoxia was shown to inhibit cell growth by inducing p27 and by hypophosphorylating pRB (Gardner et al., 2001; Green et al., 2001). However, in these other studies very low oxygen tensions (0.01%) were used. On the contrary, it has been demonstrated in other experimental settings that mild hypoxia (2%–3% $O_2$) can increase cell life span by decreasing oxidative DNA damage and by increasing telomerase activity (Minamino et al., 2001; Nishi et al., 2004; Parrinello et al., 2003; Yatabe et al., 2004). Similar experiments have not been reported for melanocytes. However, melanocytes reside in the epidermal/dermal junction in humans and are mainly localized in the hair follicles in mice (Botchkareva et al., 2003), regions that we show to be normally mildly hypoxic, with constitutive HIF1α activity. Thus, melanocytes appear to benefit from the mild hypoxic environment in which they reside as opposed to the stressful conditions of severe hypoxia.

In the genesis of melanoma, melanocytes acquire a series of modifications that are the result of disruption of skin homeostasis and genetic alterations. Although Akt activity is absent in normal skin, it increases during melanomagenesis. Dysplastic nevi show a mild positivity for phospho-Akt, which increases with progression toward an invasive tumor (Dai et al., 2005; Stahl et al., 2004). PI(3)kinase has been shown to be able to transform human melanocytes in an in vivo model of regenerated skin (Chudnovsky et al., 2005). We show that overexpression of Akt, a critical downstream effector of PI(3)kinase, transforms melanocytes, which become able to form tumors. Interestingly, these cells show little or no capacity to grow in soft agar unless they are placed in mild hypoxia. Preliminary data with NIH3T3 fibroblasts expressing Akt suggest that the transforming capacity of the oncogene in hypoxia may be cell specific. In fact, hypoxia did not seem to increase transformation of these cells (data not shown). These observations suggest that the physiologic environment of the skin is playing a role in promoting melanocyte transformation and that hypoxia is promoting tumor growth in vivo.

HIF1α plays a major role in the cellular response to hypoxia (Giaccia et al., 2003). Downregulation of HIF1α in Akt-expressing cells inhibits their ability to grow in soft agar in hypoxia and significantly reduces tumor growth. On the other hand, by expressing a stable HIF1α, these cells display a fully transformed phenotype in normoxia, clearly demonstrating that HIF1α is the mediator of the hypoxia-Akt synergism. This is in

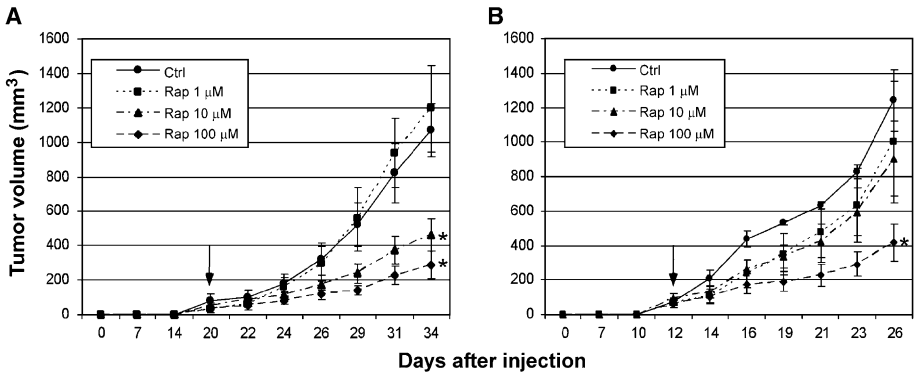


**Figure 6.** Rapamycin treatment inhibits growth of Akt- and Akt/HIF1α-$_{ODD}$-expressing melanocytes in vivo

**A:** Cells ($10^6$) expressing c.a. Akt were injected in each flank of three SCID mice for a total of six tumors per group of treatment. Tumors were treated topically with rapamycin (1 μM, 10 μM, or 100 μM) when tumor volumes reached approximately 150 mm$^3$. Treatments were done every other day for the duration of the experiment. Average tumor volumes ± SE are shown.

**B:** Akt/HIF1α-$_{ODD}$-expressing melanocytes ($10^6$) were injected s.c. in each flank of three SCID mice for a total of six tumors. Treatment with rapamycin started when tumors were approximately 150 mm$^3$. Mean tumor volumes ± SE are represented. Results are representative of three independent experiments. Statistically significant differences from the vehicle-treated tumors are indicated (*p < 0.05, Student's t test).

apparent disagreement with a previous report in which Akt and HIF1α were shown to independently enhance tumor growth and angiogenesis in a murine hepatoma cell line model (Arsham et al., 2004). It is possible that a synergism between the two molecules may be critical in the early events of melanocyte transformation, whereas already established tumor cells may contain additional alterations that allow them to grow and induce angiogenesis independently of HIF1α.

HIF1α gain-of-function mutations have not been found in tumors, likely because various factors can contribute to its

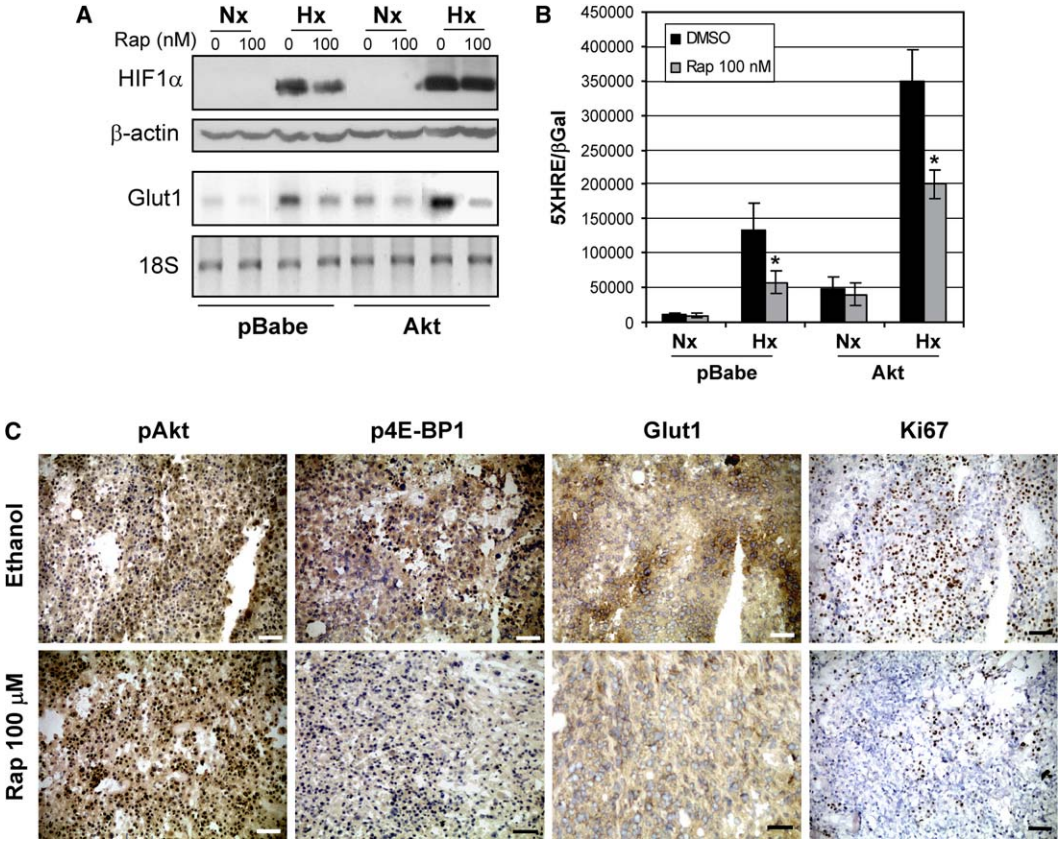


**Figure 7.** Suppression of mTOR inhibits HIF1α activity and reduces cell proliferation of Akt-dependent tumors

**A:** Melanocytes expressing c.a. Akt were treated overnight with 100 nM rapamycin either in normoxia or in hypoxia. HIF1α accumulation in hypoxia was assessed by hybridization with an anti-HIF1α-specific antibody on total protein lysates. Anti-β-actin-specific antibody was used as loading control. HIF1α activity in treated and untreated cells was determined by induction of Glut-1 transcript. Ribosomal RNA (18S) was included as a loading control.

**B:** HIF1α activity was assessed by using a 5XHRE reporter construct, and luciferase units were normalized for transfection efficiency against β-galactosidase expression. Values are mean ± SD of triplicates. Rapamycin-treated cells show a significant decrease in activity in hypoxia (*p < 0.05, Student's t test).

**C:** Phospho-Akt, phospho-4E-BP1, Glut-1, and Ki67 staining of sections from Akt-expressing tumors treated with ethanol (vehicle) or 100 μM rapamycin. Scale bars, 50 μm.

stabilization. Inactivation of the von Hippel-Lindau (*VHL*) tumor suppressor gene, which is associated with development of sporadic hemangioblastomas and clear cell renal carcinomas, stabilizes HIF1α due to lack of degradation (Kaelin, 2002). Ras, commonly mutated in a variety of human cancers, contributes to HIF1α stabilization by inhibiting prolyl hydroxylation (Chan et al., 2002). Akt/mTOR, a pathway activated in various human tumors, increases HIF1α synthesis, resulting in increased protein and activity in normoxia (Laughner et al., 2001). Recently, it has been shown that the melanocyte-specific transcription factor microphthalmia-associated transcription factor (MITF) increases HIF1α by inducing its transcription and contributing in this way to melanoma survival (Busca et al., 2005). Finally, hypoxia is certainly the most relevant environmental factor that allows HIF1α stabilization by inhibiting prolyl hydroxylases. Therefore hypoxia, through HIF1α, contributes to melanocyte transformation in the absence of genetic alterations of HIF1α itself.

Growing evidence supports a role for the mTOR pathway as a downstream effector of Akt-dependent tumorigenesis. The mTOR protein regulates cap-dependent translation and ribosome biogenesis, controlling general protein biosynthesis (Altomare et al., 2004; Bjornsti and Houghton, 2004; Neshat et al., 2001; Wendel et al., 2004). It has been shown that HIF1α activity is, at least in part, regulated by mTOR (Hudson et al., 2002; Laughner et al., 2001). Although protein synthesis is inhibited under severe hypoxia, we show that under a mild hypoxic environment, protein synthesis is maintained if not slightly increased. This observation correlates well with the fact that cells in the skin are viable and actively proliferating, and that Akt-expressing melanocytes actively grow and form colonies in agar in 2% $O_2$. In view of these observations, mTOR may represent a critical mediator of Akt-HIF1α-dependent melanocyte transformation. In fact, treatment with rapamycin abolishes the capability of the cells to grow in agar and significantly reduces tumor growth in vivo. This inhibitory effect correlates with a significant decrease in HIF1α activity that suggests, as it has been observed elsewhere (Majumder et al., 2004), that the antitumor activity of rapamycin is mediated, at least in part, through the inhibition of cellular responses to hypoxic stress. Rapamycin exerts a reduced effect on Ras-expressing melanocytes both in vitro and in vivo. In some models, Ras-dependent tumors have been shown to depend on mTOR (Shao et al., 2004; Wislez et al., 2005). However, Ras activates other signaling pathways that can contribute to its tumorigenicity. The Raf/MEK/Erk pathway, for example, is not inhibited by rapamycin, and it has been previously shown by us and others to be required by Ras for the maintenance of the malignant phenotype (Bedogni et al., 2004; Sebolt-Leopold et al., 1999). Ras-expressing melanocytes may therefore be less dependent on mTOR for survival and proliferation as observed in other experimental systems (Xing and Orsulic, 2005).

The data presented here show that Akt is an oncogene in melanoma and that the hypoxic environment of normal skin acts as an environmental promoting factor in melanomagenesis. While our data support a protumorigenic role of HIF1α, recent studies proposed a tumor suppressor role for the transcription factor (Acker et al., 2005). Moreover, Moeller et al. (2005) have shown that the overall impact of HIF1α on tumor radiosensitivity is dependent on the microenvironment. This apparent contrast between studies suggests that HIF1α may play different roles in different systems and environmental contexts and underlines the importance of understanding what functions HIF1α exerts in a specific model when targeting the molecule as a therapeutic approach. Here, we show that disruption of the PI(3)Kinase/Akt/mTOR pathway significantly reduces Akt-dependent melanoma development. Moreover, although the mechanism of HIF1α regulation by the mTOR pathway is not fully resolved, and both translational and posttranslational mechanisms may contribute to its induction, we show that, in the presence of an mTOR inhibitor, decreased tumor formation correlates with inhibition of HIF1α activity, supporting the notion that HIF1α is required for melanomagenesis. Inhibition of HIF1α activity by rapamycin may therefore represent a potential mechanism for its inhibitory effect on melanoma growth.

In view of these findings, we suggest that both Akt and HIF1α may serve as promising therapeutic targets for melanoma treatment. Rapamycin and its analogs have demonstrated impressive antitumor activity in vivo against different tumors (Rowinsky, 2004), and rapamycin has been shown to resensitize lymphomas to conventional chemotherapies (Wendel et al., 2004). Rapamycin may therefore represent a new promising treatment of melanoma.

## Experimental procedures

### Chemicals

Dimethyl sulfoxide (DMSO) and cholera toxin (CT) were purchased from Sigma. Phorbol 12-myristate 13-acetate (TPA) was from Alexis. The mTOR inhibitor rapamycin was obtained from LC Laboratories and dissolved in DMSO at a concentration of 2 mM. For in vitro studies, the final rapamycin concentrations were 100 nM, 10 nM, and 1 nM. In vivo, rapamycin was used at 100 μM, 10 μM, and 1 μM. EF5 was kindly provided by Dr. C. Koch (Koch et al., 1995). A 10 mM solution was prepared in sterile PBS and stored at 4ºC.

### Cell lines

Mouse *Ink4a/ARF* knockout melanocytes were maintained in RPMI 1640 supplemented with 10% FBS, 1% glutamine, 1% penicillin-streptomycin, 200 pM CT, and 200 nM TPA.

### Plasmids

Human myrAkt Δ4-129 (a gift from Dr. Richard Roth, Stanford University) was subcloned into the retroviral vector pWZL-hygro (hygromycin resistance) between BamHI and SalI restriction sites. Human HIF1α-$_{ODD}$ (deletion of aa 401 to 603) was kindly provided by Dr. H. Franklin Bunn (Harvard Medical School) and subcloned into pWZL-blast3 (blasticidin-3 resistance) between BamHI and ClaI. The HRE luciferase reporter construct was previously described (Shibata et al., 2000).

Oligos for mouse HIF1α-specific siRNAs were designed according to the manufacturer's instructions (BD Biosciences) and synthesized by the Stanford Protein and Nucleic Acid Biotechnology Facility. The annealed sequences were cloned between BamHI and EcoRI restriction sites into RNAi-Ready pSiren-RetroQ vector (BD Biosciences). Sequences are as follows: siHIF1α, GTCTAG AGATGCAGCAAGA; scrambled siHIF1α, TCAGAACGATGACTGAGAG.

### Luciferase assays

Cells ($5 \times 10^4$/well, in 24-well plates) were transfected using Lipofectamine plus reagent (Invitrogen) as per the manufacturer's instructions. After 24 hr, plates were placed at 2% $O_2$ for 16 hr or left in normoxia. Cells were lysed in 100 μl lysis buffer (Promega), and luciferase activity was determined by mixing 10 μl of cell extracts and 100 μl of luciferase assay reagent (Promega). Light production was measured for 10 s in a Monolight 2010 Luminometer (Analytical Luminescence Laboratory). A β-galactosidase reporter construct driven by a CMV promoter was cotransfected with the HRE reporter at a ratio of 1:5, respectively, to assess transfection efficiency. β-galactosidase expression was measured as per the manufacturer's instructions (Promega).

### Soft agar assay

Melanocytes overexpressing Akt, Akt/siHIF1α, and Akt/HIF1α-$_{ODD}$ ($2.5 \times 10^4$ cells/well, 12-well plates) were suspended in RPMI + 10% FBS containing 0.3% agar and spread onto a 0.6% agar layer. For each experiment, two sets of plates were prepared; one was left in normoxia, and one was placed in a three gas incubator set at 2% $O_2$ (Forma Scientific). After 3 weeks, colonies were stained with GEIMSA (Schwartz and Ashwell, 2001), and results were recorded by scanning the plates (Scan Jet 7400 C).

### Western blot analysis

All cell types ($10^6$ cells/dish, 100 mm dishes) were plated in RPMI-1640 plus 10% FBS, allowed to adhere, and then placed for 16 hr in low-serum media (0.5% FBS). Total protein was extracted with lysis buffer (9 M urea, 75 mM Tris-HCl [pH 7.5], and 100 mM 2-mercaptoethanol), and 30 μg per sample was separated on an 8% (for HIF1α, phospho-GSK3 and HIF1α-$_{ODD}$) or 12% (for phospho-4E-BP1) SDS-PAGE gel and transferred onto nitrocellulose membranes. Membranes were probed with anti-phospho-GSK3α/β (Ser21/9), anti-phospho-4E-BP1 (Thr37/46) (1:1000; Cell Signaling Technologies), and anti-HIF1α (1:1000; Bethyl Laboratories) antibodies. Bands were detected using ECL Western Blotting Detection Reagents (Amersham Biosciences). Loading was checked with anti-β-actin antibody (C-11, Santa Cruz Biotechnology). For samples collected in hypoxia, cells were incubated for 16 hr in a humidified hypoxic workstation (Invivo$_2$, Ruskinn Technologies) set at 2% $O_2$, and lysis was performed in the chamber to avoid reoxygenation.

### Northern blot analysis

Total RNA from all cell types was harvested using TRIzol Reagent (Invitrogen) as per the manufacturer's instructions. Hypoxic specimens were collected after 16 hr incubation in a 2% $O_2$ chamber. RNA samples were resolved on a 1% formaldehyde gel and transferred to Hybond-N+ membrane (Amersham Biosciences). Blots were hybridized with α-$^{32}$P-labeled mouse Glut-1 cDNA. The 18S band of ribosomal RNA was used as loading control.

### In vivo experiments

Three- to five-week-old male SCID (B6.CB17) mice supplied by Stanford University Animal Facility were housed in the same AALAC-approved facility with 12 hr light cycles. Food and water were provided ad libitum. All experimental protocols were APLAC approved. Cells ($1 \times 10^6$) were injected subcutaneously (s.c.) in the dorsal flank of mice (three for each treatment group) so that each mouse bore two tumors. Treatment with rapamycin started when Akt and Akt/HIF1α-$_{ODD}$-dependent tumors were about 150 mm$^3$ in size. Rapamycin was dissolved in ethanol at concentrations of 100 μM, 10 μM, 1 μM, and 200 μl of these solutions were topically applied on alternate days for the duration of the experiment. Mice were sacrificed at 34 days (Akt-dependent tumors) or 26 days (Akt/HIF1α-$_{ODD}$-dependent tumors) postinjection. Two hours prior to sacrifice, mice were injected with EF5 (10 mM, 10 ml/kg). Gross pathology of each tumor was recorded. Tumors were measured, and tumor volume was calculated by the following formula: $(W^2 \times L) \times 0.52$ (W, width; L, length) (Arbiser et al., 1997). Tumors were frozen in OCT, and 5 μm sections were cut. Untreated mice were also injected with EF5 and skin frozen in OCT. All experiments were performed at least three times.

### Immunohistochemistry

Formalin-fixed, paraffin-embedded archival human specimens of normal skin were treated as follows. Sections (5 μm) were deparaffinized and rehydrated in xylene/ethanol baths and antigen unmasked in 10 mM sodium citrate (pH 6) at 95ºC–100ºC for 20 min. For HIF1α staining, slides were also subjected to treatment in proteinase K solution (25 μl 20 mg/ml proteinase K, 50 mM Tris-Cl [pH 8], 10 mM EDTA [pH 8]) for 10 min, to increase unmasking of nuclear antigens. Slides were then incubated overnight in primary antibodies followed by biotinylated secondary antibody (1:200; Jackson Laboratory) and streptavidin/alkaline phosphatase or streptavidin/horseradish peroxidase (Vector Labs). Hematoxylin was used as a counterstain. Frozen mouse skin and tumor sections were incubated with primary antibodies followed with biotinylated secondary antibody and Streptavidin/horseradish peroxidase (Vector Labs). Hematoxylin was used as a counterstain. Sections were stained with the following antibodies: rat anti-mouse Ki67 (1:50; Dako Corporation), rabbit anti-phospho-Akt (Ser473) IHC specific (1:50; Cell Signaling Technologies), rabbit anti-phospho-4E-BP1 (Thr37/46) (1:150; Cell Signaling Technologies), rabbit anti-Glut-1 antibody (1:200; Chemicon International), mouse anti-CAIX (clone MN75; 1:50), rabbit anti-tyrosinase-related protein 1 and 2 (Tyrp-1 and Tyrp-2) (1:100; a gift from Dr. Vincent J. Hearing, National Cancer Institute), mouse anti-HIF1α (1:20; clone ESEE122; Novus Biologicals). Staining of hypoxic areas was as previously described (Koch et al., 1995). Briefly, frozen skin sections were fixed 1 hr in 4% paraformaldehyde in PBS at 0ºC and then blocked overnight in 10% skim milk/5% mouse serum at 4ºC. Finally, sections were incubated for ~6 hr at 4ºC with antibody ELK-51 (50 μg/ml; Cye-3 conjugated), mounted, and visualized under a fluorescent microscope.

### Supplemental data

The Supplemental Data include five supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/8/6/443/DC1/.

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