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Hypoxia Contributes to Melanoma Heterogeneity by Triggering HIF1α-Dependent Phenotype Switching

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We have previously reported a model for melanoma progression in which oscillation between melanoma cell phenotypes characterized by invasion or proliferation is fundamental to tumor heterogeneity and disease progression. In this study we examine the possible role of hypoxia as one of the microenvironmental influences driving metastatic progression by promoting a switch from a proliferative to an invasive phenotype. Immunohistochemistry on primary human cutaneous melanoma biopsies showed intratumoral heterogeneity for cells expressing melanocytic markers, and a loss of these markers correlated with hypoxic regions. Furthermore, we show that the downregulation of melanocytic markers is dependent on hypoxia inducible factor 1α (HIF1α), a known regulator of the hypoxic response. *In vitro* invasion assays showed that a hypoxic environment increases the invasiveness of proliferative melanoma cell cultures in a HIF1α-dependent manner. In contrast, invasive phenotype melanoma cells showed no increase in invasive potential upon exposure to hypoxia. Thus, exposure of proliferative melanoma cells to hypoxic microenvironments is sufficient, in a HIF1α-dependent manner, to downregulate melanocytic marker expression and increase their invasive potential.

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INTRODUCTION

Melanoma is a growing public health burden because it is one of the few cancers still escalating in incidence (Jemal *et al.*, 2009). Despite advances in understanding the molecular nature of melanoma in recent decades, few treatment options are available for metastatic melanoma that only marginally increase overall patient survival (Lui *et al.*, 2007; Bollag *et al.*, 2010; Hodi *et al.*, 2010; Joseph *et al.*, 2010; Chapman *et al.*, 2011; Robert *et al.*, 2011; Flaherty *et al.*, 2012). One reason why melanoma is difficult to treat is its heterogeneity (Fidler, 1978). The source of this heterogeneity is unclear, but evidence suggests that it is driven by microenvironmental factors (Postovit *et al.*, 2006).

In the process of studying melanoma heterogeneity, we described different subtypes of melanoma cells *in vitro* (Hoek *et al.*, 2006) that can be distinguished by gene-expression analysis (Widmer *et al.*, 2012). These differences in gene expression correlate with alterations in *in vitro* cell morphology, proliferation rate, invasion, transforming growth factor-β susceptibility, and *in vivo* tumor growth kinetics (Hoek *et al.*, 2006; Hoek *et al.*, 2008a). These experiments have identified a proliferative phenotype and an invasive melanoma cell phenotype. Previous work showed that primary and metastatic lesions are commonly composed of a mixture of both phenotypes (Eichhoff *et al.*, 2010, 2011). We subsequently hypothesized that melanoma cells, responding to changes in microenvironmental conditions, change their transcription programs to switch back-and-forth between proliferative and invasive states and thereby drive metastatic progression (Hoek *et al.*, 2008a).

As other cancers, melanoma tumors include regions of hypoxia and anoxia caused by an imbalance in both oxygen supply and consumption. It has long been known that the perfusion of tumors is heterogeneous resulting in a wide range of pO_2 levels both within and between tumors (Chaplin *et al.*, 1986). It is estimated that the proportion of solid tumors with hypoxic (or anoxic) areas is up to 50–60% (Vaupel and Mayer, 2007). The negative impact of tumor hypoxia has been studied extensively. Early studies have shown that hypoxia-induced gene amplification can lead to resistance to treatment (Rice *et al.*, 1987; Luk *et al.*, 1990). In many cancer types, the induction of an epithelial-to-mesenchymal transition (EMT)-like process by exposure to an intralesional hypoxic microenvironment is currently under investigation. Tumor hypoxia is known to reduce the sensitivity of solid tumors to radiation therapy and can negatively influence treatment outcome and patient survival in multiple cancer types (Hockel *et al.*, 1996; Vergis *et al.*, 2008).

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Abbreviations: EMT, epithelial-to-mesenchymal transition; GLUT1, glucose transporter 1; HIF1α, hypoxia inducible factor 1α; MITF, microphthalmia-associated transcription factor; qRT-PCR, quantitative real-time reverse-transcriptase–PCR; siRNA, silencing RNA

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Two recent studies of hypoxia in melanoma demonstrate a role for hypoxia in downregulating the master regulator of melanocyte differentiation, microphthalmia-associated transcription factor (MITF). A study by Cheli *et al.* (2012) showed that hypoxic conditions lead to a decrease in MITF expression and that a deletion of MITF is sufficient to increase the metastatic potential of mouse and human melanoma cells *in vivo*. They identified BHLHE40 (BHLHB2, DEC1) as a mediator of the observed hypoxia inducible factor 1α (HIF1α)-dependent inhibitory effect on MITF. In another study also published in 2011, the authors (Feige *et al.*, 2011) found BHLHE40 to be responsible for hypoxia-induced downregulation of MITF.

To investigate the role of hypoxia in melanoma progression, we describe the effects of hypoxia on proliferative and invasive melanoma cell cultures. The results suggest that hypoxia, through HIF1α, alters the gene-expression pattern of proliferative melanoma cells, making them more invasive in *in vitro* assays.

RESULTS

To gain more insight into the nature of intratumor heterogeneity in patients, we stained Clark's level IV primary human cutaneous melanoma biopsies for key markers of melanocytic function, hypoxia response, proliferation, and vascularization. Immunohistochemical stainings show that the tumor is mostly composed of tumor cell nodules (Figure 1a″–c″), which are surrounded by stroma and endothelial cells (Figure 1c″, arrowheads). CD31 (*PECAM-1*), which is expressed constitutively on the surface of adult and embryonic endothelial cells (Pusztaszeri *et al.*, 2006), was used to stain blood vessels. Glucose transporter 1 (GLUT1) (*SLC2A1*), a glucose transporter that has been shown to be upregulated in hypoxic tissue (Bashan *et al.*, 1992; Loike *et al.*, 1992), was upregulated with increasing distance from blood vessels in multiple regions of the tumor (Figure 1c′, arrowheads). Panel a of Figure 1 shows that the majority, but not all, of the cells in the tumor express melanoma antigen recognized by T cells 1 (MLANA) that is involved in melanosome biogenesis and has been widely used to identify melanocytic lesions (Busam *et al.*, 1998). In the hypoxic areas, which are indicated by arrowheads in a′, we found downregulation of the melanocytic marker MLANA (Figure 1a′, arrowheads as compared with Figure 1b′) and MITF (data not shown).To further illustrate the presence of hypoxia in the tumor, we co-stained tumor slides for HIF1α and GLUT1 (Supplementary Figure S2 online). As proliferative phenotype cell cultures strongly express these melanocytic markers, whereas invasive phenotype cell cultures do not express them at all, their expression indicates a dedifferentiation of the melanoma cells and points toward a gain of invasive phenotype characteristics (Hoek *et al.*, 2006, 2008a; Eichhoff *et al.*, 2010, 2011; Zipser *et al.*, 2011). These data suggest that by exposure to a hypoxic microenvironment, melanoma cells downregulate melanocytic marker genes.

Hypoxia-regulated EMT genes are differentially expressed between the two phenotypes

Intratumoral hypoxia has been shown to induce regulators of EMT and metastasis in breast-, colon-, prostate-, and non-small cell lung cancer (Luo *et al.*, 2006; Peinado and Cano,

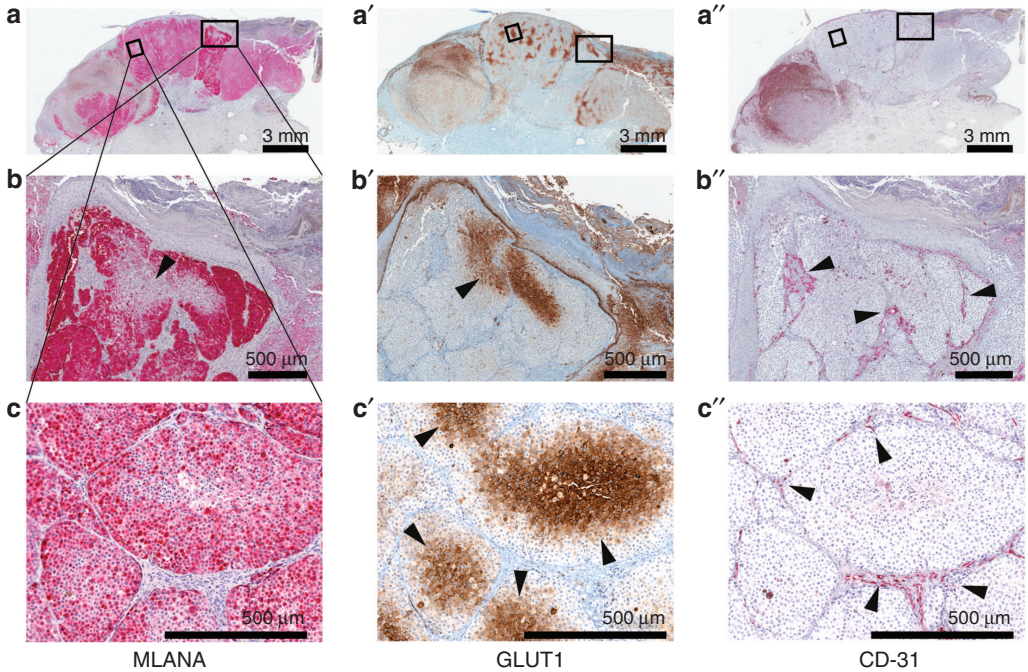

Figure 1. Immunohistochemistry of a primary cutaneous melanoma. Overview pictures of a representative melanoma tumor are shown in the top row (**a**, **b**, **c**); boxes on the right-hand side of **a**, **b**, and **c** indicate regions of higher magnification shown in **a′**, **b′**, and **c′**, and boxes on the left side highlight the magnified region in **a″**, **b″**, and **c″**. Staining for MLANA (**a**, **a′**, and **a″**) shows regions with high expression and regions that have lost expression of this melanocytic marker (arrowhead). GLUT1 (**b**, **b′**, and **b″**) stains hypoxic areas (arrowheads). CD31 shows blood vessels (**c**, **c′**, and **c″**, arrowheads). Scale bars = 3 mm (**a**), 500 µm (**b**), and 500 µm (**c**). MLANA, melanoma antigen recognized by T cells 1.

2008; Hung *et al.*, 2009; Jo *et al.*, 2009; Sipos and Galamb, 2012). An expression analysis of some of the genes known to be involved in this process shows a phenotype-specific expression pattern (Supplementary Figure S1 online). *LOX, ZEB1,* and *MMP2*, which are upregulated in the invasive phenotype, have been shown to be involved in EMT, but also in hypoxia-induced tumor invasiveness and metastasis (Ray and Stetler-Stevenson, 1994; Postigo and Dean, 1997; Hojilla *et al.*, 2003; Stamenkovic, 2003; Erler *et al.*, 2006; Peinado *et al.*, 2008; Spaderna *et al.*, 2008; Baranwal and Alahari, 2009; Elloul *et al.*, 2010; Jia *et al.*, 2012; Xiong *et al.*, 2012). In development, as well as in the process of EMT and cancer progression, E-cadherin (CDH1) is repressed and leads to a loss of the extracellular matrix as well as of adherens and tight junctions (Peinado *et al.*, 2007). However, mesenchymal cadherins, such as N-cadherin (CDH2), are upregulated during EMT (Oda *et al.*, 1998; Peinado *et al.*, 2004). This cadherin switch can also be observed in proliferative and invasive melanoma phenotypes, with an upregulation of CDH1 in proliferative cells and an upregulation of CDH2 in invasive cells (Supplementary Figure S1 online). These findings suggest that an EMT-like process is involved in the switching of melanoma cells from proliferative to invasive phenotypes.

Hypoxia changes *in vitro* invasive potential in proliferative melanoma cells in a dose-dependent manner

To further test if hypoxia drives proliferative cells to a more invasive state, we treated proliferative phenotype cells with 1% O_2 for 48 hours and tested their invasiveness *in vitro* by performing Boyden chamber experiments. The proliferative cell cultures treated with hypoxia showed a significant increase in invasion compared with the non-treated control cells (Figure 2a). The relative invasion increased by 2-fold for M080423, 2.3-fold for M000921, and even 4.2-fold for M010817. However, invasive phenotype cells (i.e., M080307) in identical treatment conditions did not show any change in invasiveness (*P*-value = 0.399) (Figure 2a). To test if the gain of invasive ability required the continued lack of oxygen, we returned the cells to normoxia for 48 hours after a hypoxia treatment period of 48 hours. In Figure 2b (dark gray bar), we show that the effect remained significant after cells were returned to normoxia 48 hours before testing invasion. When the cells were exposed to hypoxia for 96 hours, the effect was intensified (Figure 2b, white bar). However, proliferative melanoma cells in hypoxic conditions do not alter their proliferation rate (Supplementary Figure S3 online).

Downregulation of *MLANA* during hypoxia is mediated by HIF1α

The inverse correlation of hypoxic areas with the expression of melanocytic markers like MLANA (Figure 1) raises the question whether the decrease in the expression of this protein is mediated by HIF1α, a known regulator of the hypoxic response. To test this possibility, we treated proliferative phenotype melanoma cells (i.e., M000921) with chemical compounds to either suppress or activate signaling through

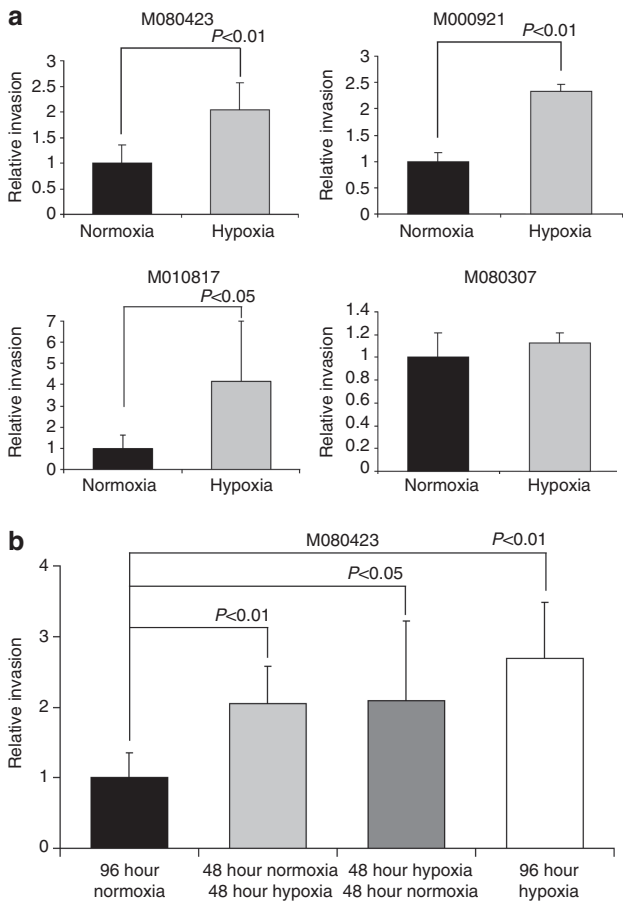

Figure 2. Hypoxia increases *in vitro* invasion in proliferative melanoma cells. *In vitro* invasiveness of proliferative (M080423, M010817, and M000921) and invasive (M080307; *P*-value = 0.399) phenotype melanoma cell cultures (**a**). Hypoxia treatment of a proliferative melanoma cell culture (M080423) induces increased invasiveness in a dose-dependent fashion (**b**). Light gray bar: incubated in normoxia for 48 hours and subsequently analyzed in a Boyden chamber assay in hypoxia for 48 hours; dark gray bar: vice versa. The *P*-values are calculated by a *t*-test; the error bars represent the s.d.

HIF1α. To inhibit HIF1α transcriptional activity, we treated the cells with Echinomycin during exposure to hypoxia (1% O_2 for 72 hours) (Kong *et al.*, 2005). The induction of HIF1α as well as the downregulation of MLANA could be inhibited under hypoxia (Figure 3a), and the hypoxic induction of *in vitro* invasion (Figure 2a) could be significantly abrogated by about 8-fold through HIF1α knockdown (Figure 3b). The same results on marker expression could be seen when we enhanced HIF1α degradation with the chemical compound YC-1 (Chun *et al.*, 2001) (Figure 3c). $CoCl_2$ stabilizes HIF1α by inhibiting proline hydroxylases, leading to HIF1α expression also under normoxic conditions (Epstein *et al.*, 2001), leading to downregulation of MLANA (Figure 3c). Because these compounds might have off-target effects and because their specificity is unclear, we also performed silencing RNA (siRNA)-mediated knockdown of HIF1α using two different siRNAs against the HIF1α transcript (Figure 3d). The results confirm a HIF1α-dependent downregulation of MLANA in

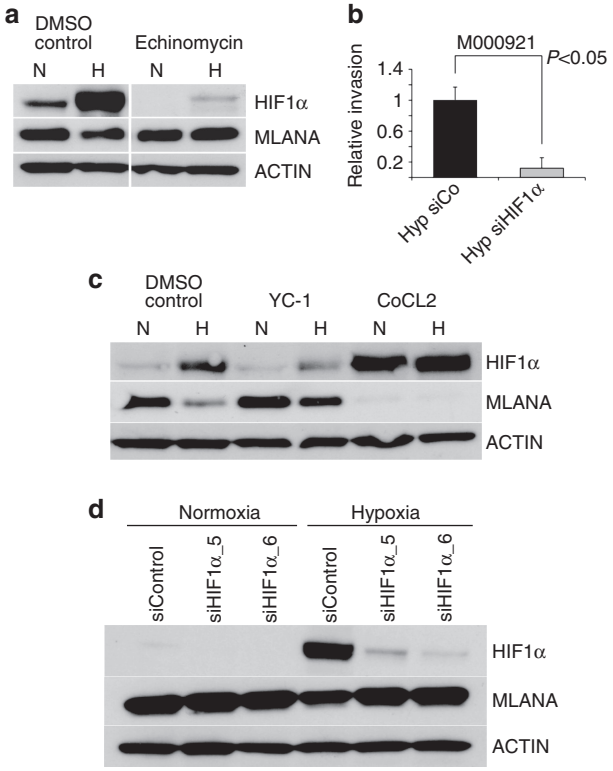

Figure 3. Downregulation of MLANA and upregulation of *in vitro* invasion upon hypoxia treatment is hypoxia inducible factor 1α (HIF1α) dependent. Echinomycin enhances HIF1α degradation under hypoxic conditions (**a**). Relative invasion under hypoxic culture conditions and treatment with silencing RNA (siRNA) against HIF1α (Hyp siHIF1α) and control siRNA (Hyp siCo), *P*-value <0.05, done in quadruplicate (**b**). Treatment of proliferative melanoma cell cultures with YC-1 decreases HIF1α levels. $CoCl_2$ stabilizes HIF1α under normoxic conditions (**c**). Under normoxia, no HIF1α could be detected (**d**). Treatment with siRNA against HIF1α (siHIF1 α _5/_6) and control siRNA (siControl). The *P*-value is calculated by a *t*-test; the error bars represent the s.d. MLANA, melanoma antigen recognized by T cells 1.

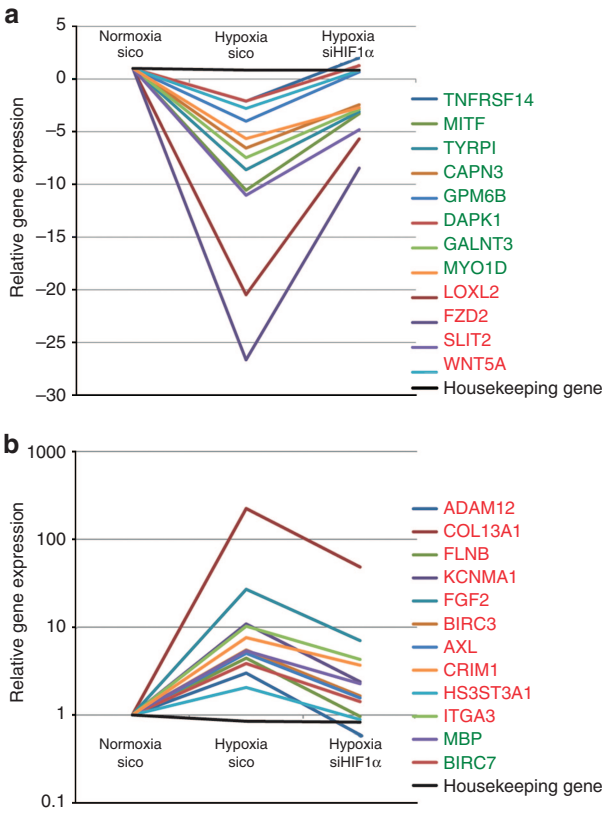

Figure 4. qRT-PCR using RNA from hypoxia- and/or siHIF1α-treated proliferative phenotype melanoma cell cultures. Genes that were at least 2-fold downregulated when grown in a hypoxic environment (hypoxia sico), but at least 2-fold upregulated when *HIF1α* was knocked down (hypoxia siHIF1α) (**a**). Genes that were at least 2-fold upregulated when grown in a hypoxic environment (hypoxia sico), but at least 2-fold downregulated when *HIF1α* was knocked down. This experiment was performed in duplicates with two different siRNAs against *HIF1α* (**b**). Gene names in green highlight genes that were upregulated in the proliferative phenotype melanoma cell cultures. Gene names in red indicate genes that were upregulated in the invasive phenotype cell cultures.

hypoxia, as well as a HIFα-dependent hypoxic induction of invasive properties, suggesting a role for HIF1α in the phenotype switch induced by tumor hypoxia.

Hypoxia regulates a phenotype-specific gene set through HIF-1α

It is likely that there are intermediate factors acting downstream of HIF1α that repress *MLANA* gene expression. To find HIF1α targets that might repress MLANA, we performed siRNA knockdown of HIF1α followed by quantitative real-time reverse-transcriptase–PCR (qRT-PCR) analysis. We tested 90 genes that have been previously identified to be differentially expressed between a large number of proliferative and invasive phenotype melanoma cell lines (Widmer *et al.*, 2012). These genes were assembled on a custom qRT-PCR array. This array was used to find genes that are downregulated when treated with hypoxia, but rescued when *HIF1α* is knocked down. The results show 12 genes that are at least 2-fold downregulated upon hypoxia treatment (hypoxia sico) (Figure 4a) and at least 2-fold upregulated when *HIF1α* was knocked down under hypoxic conditions (hypoxia siHIF1α) compared with normal hypoxia (hypoxia sico). Of the 12 genes, 8 were upregulated in the proliferative phenotype cell cultures (Figure 4a, gene names in green) and 4 were upregulated in invasive phenotype cell cultures (Figure 4a, gene names in red). We were also interested in genes that were upregulated at least 2-fold upon hypoxia treatment (hypoxia sico), but had an at least 2-fold decreased expression when HIF1α is knocked down under hypoxic conditions (hypoxia siHIF1α) compared with normal hypoxia (hypoxia sico). Of the 12 genes fulfilling these criteria, 10 were upregulated in the invasive phenotype cells (Figure 4b, gene names in red), whereas 2 genes were upregulated in the proliferative phenotype cell cultures (Figure 4b, gene names in green). The results show that 18 (75%) out of the 24 genes regulated by HIF1α were differentially regulated between the proliferative and invasive melanoma cell lines in such a way that proliferative genes were downregulated and invasive genes are upregulated by HIF1α (i.e., genes with green text in Figure 4a, and genes with red text in Figure 4b).

DISCUSSION

The characterization of different subsets of melanoma cells in a tumor lesion is of major clinical relevance, because all the subpopulations of melanoma cells need to be killed for successful therapeutic intervention. The exceptional transcriptional and phenotypic plasticity of melanoma cells has so far hampered the discovery of single prognostic factors or target molecules appropriate for general melanoma therapy. In our previous work, we demonstrated that invasive phenotype melanoma cells are less susceptible to MAPK inhibition and that proliferative phenotype melanoma cells temporarily switch their phenotype to one with more invasive features during drug treatment (Zipser *et al.*, 2011). Furthermore, immunotherapy targeting melanocytic markers will not eradicate the invasive phenotype cells, because these markers are downregulated upon phenotype switching.

Recently published studies have shown inter- and intratumoral heterogeneity for driver mutations like *BRAF* or *KIT*, suggesting a limited scope for current therapies in personalized medicine that target specifically mutated serine or tyrosine kinases (Sakaizawa *et al.*, 2012; Yancovitz *et al.*, 2012). Not only is intrinsic genetic heterogeneity of importance as a basis for tumor progression, but acquired heterogeneity that is induced by microenvironmental factors may also lead to therapeutic resistance. To overcome intrinsic as well as acquired resistance, it is imperative to understand tumor heterogeneity at the genetic and transcriptional levels.

We have previously shown that melanoma cells with high MITF and MLANA expression are only weakly invasive *in vitro*, whereas melanoma cells with no MITF and MLANA expression are highly invasive (Hoek *et al.*, 2006; Eichhoff *et al.*, 2011; Zipser *et al.*, 2011). Immunohistochemical stainings of melanoma tumors show regions with high GLUT1 expression, indicating hypoxic regions which anti-correlate with the melanocytic markers MITF and MLANA (Figure 1). These results suggest that in a hypoxic microenvironment, melanocytic markers are downregulated, which is a sign of cell dedifferentiation that is also evident in invasive phenotype cells. These data confirm the results of an earlier study in which proliferative markers (i.e., MLANA and MITF) were anti-correlated with invasive markers (WNT5A) and hypoxia (GLUT1) in melanoma (Eichhoff *et al.*, 2010). Conversely to what Eichhoff *et al.* reported, we also observed GLUT1 expression in many nested, nicely organized tumor regions (Figure 1). Although the staining for CD31 suggests the presence of blood vessels around the tumor cell nests, it seems that either the nests are too large or the blood vessels are not fully functional so that the melanoma cells become hypoxic.

To identify the role that hypoxia might have in melanoma progression, we measured the effect of hypoxic conditions on the *in vitro* invasive potential of proliferative melanoma cells by performing Boyden chamber invasion assays. The results showed a significant increase in invasion from 2- to more than 4-fold for all tested proliferative cell cultures (Figure 2a). An invasive cell line (i.e., M080307) did not display such an increase in invasive potential (Figure 2a). We also showed that the higher *in vitro* invasiveness observed in proliferative melanoma cultures is dose dependent, lasts at least 48 hours, and increases with longer exposure time up to 96 hours (Figure 2b).

Because one of the mediators of hypoxic responses in many cell types is the HIF1α protein, we modulated its expression level and could show that the hypoxic downregulation of MLANA is HIF1α dependent (Figure 3). We demonstrate that a knockdown of HIF1α under hypoxic conditions decreases *in vitro* invasion by about 8-fold, thereby suggesting a requirement for HIF1α in the hypoxic-induced invasive ability of proliferative melanoma cell cultures (Figure 3b). Possible mediators for this effect were identified by qRT-PCR analysis of 90 genes, which are differentially expressed between the proliferative and invasive melanoma phenotypes (Widmer *et al.*, 2012). Analysis of these data showed 12 genes to be downregulated by hypoxia treatment, but not when *HIF1α* is knocked down (Figure 4). Of these, eight genes are commonly downregulated in the invasive melanoma cell cultures and therefore potentially involved in the switch from a proliferative to an invasive phenotype. Interestingly, *MITF* and its target tyrosinase-related protein 1are among these genes (Figure 4). Also, *TNFRSF14*, *CAPN3*, *GPM6B*, and *DAPK1*, which are potential targets of *MITF* that we have previously identified (Hoek *et al.*, 2008b), are differentially expressed. This suggests an important role for HIF1α-regulated *MITF* in the process of hypoxia-induced phenotype switching. Furthermore, two of these genes are known to have a pro-apoptotic function: calpain 3 (*CAPN3*) (Moretti *et al.*, 2009) and death-associated protein kinase 1 (*DAPK1*) (Lin *et al.*, 2007). In addition, glycoprotein M6B (*GPM6B*) has been shown to be downregulated in highly invasive melanoma cells versus poorly invasive lines (Seftor *et al.*, 2002).

Furthermore, a set of 12 genes is induced by hypoxia but not when *HIF1α* is knocked down (Figure 4). Ten of these genes are upregulated in the invasive phenotype melanoma cell cultures and are therefore of particular interest (Figure 4). One of the most interesting genes encodes fibroblast growth factor 2, a mitogenic and angiogenic factor that can act as an autocrine growth factor for melanoma cells (Halaban *et al.*, 1988; Becker *et al.*, 1989). *AXL*, a receptor tyrosine kinase, has been shown to be expressed only in MITF-negative melanoma cells (Sensi *et al.*, 2011), confirming our own results (Hoek *et al.*, 2006; Widmer *et al.*, 2012). Sensi *et al.* also showed that *AXL* increases the motility and invasion of melanoma cells. *HIF1α* appears also to induce the expression of multiple genes that encode proteins involved in interactions with the extracellular matrix, such as ADAM12, a disintegrin and metalloproteinase (COL13A1), a transmembrane collagen chain (ITGA3), an integrin alpha subunit, and FLNB, a filamin known to bind to the actin cytoskeleton (Prockop and Kivirikko, 1995; Takafuta *et al.*, 1998; Danen and Sonnenberg, 2003; Lendeckel *et al.*, 2005; Kveiborg *et al.*, 2008). The upregulation of *BIRC3*, a gene encoding an apoptosis inhibitor, is consistent with the downregulation of two pro-apoptotic genes (*CAPN3* and *DAPK1*). The potassium channel KCNMA1, which is also upregulated in the presence of hypoxia in a HIF1α-dependent manner, could be regulated

by MITF over the microRNA miR-211 (Mazar *et al.*, 2010). Further studies have to determine if inhibition of one or more of these factors can abrogate the tumor-promoting effect of HIF1α.

The data presented here suggest that hypoxia is a possible microenvironmental stimulus triggering a switch from a proliferative to an invasive melanoma cell phenotype. By downregulating melanocytic genes, HIF1α induces an EMT-like dedifferentiation to a more invasive type of melanoma cell. Upregulation of pro-angiogenic and pro-tumorigenic factors, as well as of extracellular matrix-modifying genes along with a downregulation of pro-apoptotic genes, may allow the invasive melanoma cells to contribute to melanoma progression.

MATERIALS AND METHODS

Cell culture

M-series primary melanoma cell cultures were set up from surplus material from cutaneous melanoma and melanoma metastases in our laboratory. Many of these lines have been previously published and have been shared with multiple international laboratories for *in vitro*, genomic, and xenografting assays (Lin *et al.*, 2008; Hoek *et al.*, 2008a; Eichhoff *et al.*, 2011; Zipser *et al.*, 2011; Kiowski *et al.*, 2012; Widmer *et al.*, 2012). Written informed consent was approved by the local IRB (EK647 and EK800). Clinical diagnosis was confirmed by histology and immunohistochemistry. Melanoma cells were brought into culture as previously described (Geertsen *et al.*, 1998). Melanoma cell cultures were grown in RPMI (Invitrogen, Carlsbad, CA) including 5 mM glutamine, 1 mM sodium pyruvate, and 10% heat-inactivated fetal bovine serum (GIBCO 10500; Invitrogen), and they were grown at 37 °C and 5% CO_2. The study was conducted according to the Declaration of Helsinki Pinciples.

Quantitative real-time reverse-transcriptase–PCR

Total RNA was extracted from primary melanoma cell cultures using TRIzol according to the manufacturer's instructions (Invitrogen). Total RNA was used for cDNA synthesis using Promega's Reverse Transcription System (Promega, Madison, WI) or RT HT First Strand cDNA kit for the qRT-PCR arrays according to the supplied protocols. Gene expression was quantified using the FastStart Universal SYBR Green Master (ROX; 04913914001; Roche, Basel, Switzerland) and the Viia7 system from Applied Biosystems (Carlsbad, CA). The primers for MITF and WNT5A were purchased from Qiagen (Venlo, the Netherlands). The qRT-PCR arrays (Custom RT2 Profiler PCR Array (CAPH10586E-6)) were purchased from SA Biosciences (Qiagen, Venlo, the Netherlands).

Western blot analyses

Cells were washed twice with cold phosphate-buffered saline and lysed at 4 °C in RIPA protein lysis buffer as previously reported (Dissanayake *et al.*, 2007). Proteins were separated by SDS-PAGE using the NuPAGE SDS-PAGE Gel System (Invitrogen) on 4–12% Tris-glycine gels (Invitrogen) under reducing conditions according to the manufacturer's instructions and transferred onto nitrocellulose membranes (Invitrogen). Primary antibodies used were: HIF1α: mouse monoclonal anti-HIF1alpha 1:1000 (610959; BD Biosciences, San Jose, CA); MLANA: mouse monoclonal anti-MLANA 1:1000, (ab3168; Abcam, Cambridge, UK); ACTIN: goat polyclonal anti-Actin 1:1000 (sc-1616; Santa Cruz Biotechnology, Dallas, TX). Secondary antibodies used were: rabbit polyclonal anti mouse IgG-HRP (ab97046, Abcam) and Rabbit anti-goat IgG-HRP (sc-2768, Abcam). Bound antibodies were detected using ECL Western Blotting Detection Reagent (28906836; GE Healthcare).

In vitro invasion assay (Boyden chamber assays)

In vitro invasion assays were performed using Matrigel-coated cell inserts (BD Biosciences; 354480) and uncoated cell inserts (BD Biosciences; 353097) according to the manufacturer's instructions. A total of 5×10^4 cells in 500 μl starving medium were seeded onto cell culture inserts. RPMI containing 10% fetal calf serum was used as chemoattractant. After incubation at 37 °C for 48 hours, the cells on the membranes were fixed, stained, cut out, and mounted on a glass slide. The number of cells, which had moved through the pores to the other side of the membrane, was assessed by microscopy. Invasion values were calculated by dividing the number of cells migrating through Matrigel-coated inserts by the number of cells migrating through uncoated inserts.

Proliferation assay

Melanoma cells were counted after 24, 48, and 72 hours, and doubling time was calculated.

Immunohistochemical stainings

All tissue used for immunohistochemistry was fixed in 4% paraformaldehyde and embedded in paraffin. Sections were deparaffinized in xylene and rehydrated. Epitope retrieval was performed in antibody specific buffers. Staining was performed using kits supplied by Ventana (Tucson, AZ) or Dako (Glostrup, Denmark) REAL Detection System (kit 5005). Antigen-specific antibodies were applied and visualized with either the iVIEW DAB detection kit (Ventana) or the Chem-Mate detection kit (Dako). Slides were counterstained with haematoxylin. Antibodies used were: MLANA (Novocastra, Leica Biosystems, Nussloch, Germany; NCL-L-MLANA; EDTA; 1:50), GLUT1 (Cell Marque, Rocklin, CA; 355A-16; EDTA; 1:100), CD31 (Dako; M0823; Protein kinase K; 1:40), and Hif1alpha (Abcam; mgc3, ab16066).

Hypoxia treatment

Cells were cultured in a Modular Incubator Chamber (MIC-101; Billups-Rothenberg, Del Mar, CA), flushed with 20 l min^{-1} (flow meter; RMA-23-SSV; Dwyer, Michigan City, IN) with certified premixed gas composed of 1% O_2, 5% CO_2, and 94% N_2 (Carbagas, Guemligen, Switzerland). The O_2 concentration inside the chamber was measured with an oxygen sensor (VTI-122, Disposable Polarographic Oxygen Cell, 100122; Vascular Technology, Nashua, NH). The hypoxia chamber was placed in an incubator at 37 °C. Medium exchange and splitting of the cells was performed outside the hypoxic environment. To stabilize HIF1α expression in melanoma cells under normoxic conditions, cells were treated with 100 μM $CoCl_2$ (Sigma-Aldrich, St Louis, MO) for 24, 48 or 72 hours. To inhibit expression or stabilization of HIF1α in melanoma cells under hypoxic conditions, cells were treated with 60 μM YC-1 (Calbiochem, Merck, Darmstadt, Germany) or 5 nM of Echinomycin Streptomyces sp (Calbiochem).

siRNA transfections

siRNA transfection of melanoma cells was carried out using INTERFERin transfection solution according to the manufacturer's protocol (Polyplus Transfection, Illkirch, France). Cells were transfected with

10 nM of siRNA (Qiagen) for 24, 48 or 72 hours before RNA or protein was extracted. As control siRNA, the All-Star negative siRNA sequence (Qiagen) was used. siRNA used were: Hs_HIF1A_5 (NM_001530; S102664053, Qiagen FlexiTube) and Hs_HIF1A_6 (NM_001530; S102664431, Qiagen FlexiTube).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Baranwal S, Alahari SK (2009) Molecular mechanisms controlling E-cadherin expression in breast cancer. *Biochem Biophys Res Commun* 384:6–11

Bashan N, Burdett E, Hundal HS *et al.* (1992) Regulation of glucose transport and GLUT1 glucose transporter expression by O2 in muscle cells in culture. *Am J Physiol* 262:C682–90

Becker D, Meier CB, Herlyn M (1989) Proliferation of human malignant melanomas is inhibited by antisense oligodeoxynucleotides targeted against basic fibroblast growth factor. *EMBO J* 8:3685–91

Bollag G, Hirth P, Tsai J *et al.* (2010) Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature* 467:596–9

Busam KJ, Chen YT, Old LJ *et al.* (1998) Expression of melan-A (MART1) in benign melanocytic nevi and primary cutaneous malignant melanoma. *Am J Surg Pathol* 22:976–82

Chaplin DJ, Durand RE, Olive PL (1986) Acute hypoxia in tumors: implications for modifiers of radiation effects. *Int J Radiat Oncol Biol Phys* 12:1279–82

Chapman PB, Hauschild A, Robert C *et al.* (2011) Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* 364:2507–16

Cheli Y, Giuliano S, Fenouille N *et al.* (2012) Hypoxia and MITF control metastatic behaviour in mouse and human melanoma cells. *Oncogene* 31:2461–70

Chun YS, Yeo EJ, Choi E *et al.* (2001) Inhibitory effect of YC-1 on the hypoxic induction of erythropoietin and vascular endothelial growth factor in Hep3B cells. *Biochem Pharmacol* 61:947–54

Danen EH, Sonnenberg A (2003) Integrins in regulation of tissue development and function. *J Pathol* 201:632–41

Dissanayake SK, Wade M, Johnson CE *et al.* (2007) The Wnt5A/protein kinase C pathway mediates motility in melanoma cells via the inhibition of metastasis suppressors and initiation of an epithelial to mesenchymal transition. *J Biol Chem* 282:17259–71

Eichhoff OM, Weeraratna A, Zipser MC *et al.* (2011) Differential LEF1 and TCF4 expression is involved in melanoma cell phenotype switching. *Pigment Cell Melanoma Res* 24:631–42

Eichhoff OM, Zipser MC, Xu M *et al.* (2010) The immunohistochemistry of invasive and proliferative phenotype switching in melanoma: a case report. *Melanoma Res* 20:349–55

Elloul S, Vaksman O, Stavnes HT *et al.* (2010) Mesenchymal-to-epithelial transition determinants as characteristics of ovarian carcinoma effusions. *Clin Exp Metastasis* 27:161–72

Epstein AC, Gleadle JM, McNeill LA *et al.* (2001) C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107:43–54

Erler JT, Bennewith KL, Nicolau M *et al.* (2006) Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature* 440:1222–6

Feige E, Yokoyama S, Levy C *et al.* (2011) Hypoxia-induced transcriptional repression of the melanoma-associated oncogene MITF. *Proc Natl Acad Sci USA* 108:E924–33

Fidler IJ (1978) Tumor heterogeneity and the biology of cancer invasion and metastasis. *Cancer Res* 38:2651–60

Flaherty KT, Robert C, Hersey P *et al.* (2012) Improved survival with MEK inhibition in BRAF-mutated melanoma. *N Engl J Med* 367:107–14

Geertsen RC, Hofbauer GF, Yue FY *et al.* (1998) Higher frequency of selective losses of HLA-A and -B allospecificities in metastasis than in primary melanoma lesions. *J Invest Dermatol* 111:497–502

Halaban R, Kwon BS, Ghosh S *et al.* (1988) bFGF as an autocrine growth factor for human melanomas. *Oncogene Res* 3:177–86

Hockel M, Schlenger K, Aral B *et al.* (1996) Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res* 56:4509–15

Hodi FS, O'Day SJ, McDermott DF *et al.* (2010) Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 363:711–23

Hoek KS, Eichhoff OM, Schlegel NC *et al.* (2008a) In vivo switching of human melanoma cells between proliferative and invasive states. *Cancer Res* 68:650–6

Hoek KS, Schlegel NC, Brafford P *et al.* (2006) Metastatic potential of melanomas defined by specific gene expression profiles with no BRAF signature. *Pigment Cell Res* 19:290–302

Hoek KS, Schlegel NC, Eichhoff OM *et al.* (2008b) Novel MITF targets identified using a two-step DNA microarray strategy. *Pigment Cell Melanoma Res* 21:665–76

Hojilla CV, Mohammed FF, Khokha R (2003) Matrix metalloproteinases and their tissue inhibitors direct cell fate during cancer development. *Br J Cancer* 89:1817–21

Hung JJ, Yang MH, Hsu HS *et al.* (2009) Prognostic significance of hypoxia-inducible factor-1alpha, TWIST1 and Snail expression in resectable non-small cell lung cancer. *Thorax* 64:1082–9

Jemal A, Siegel R, Ward E *et al.* (2009) Cancer statistics, 2009. *CA Cancer J Clin* 59:225–49

Jia B, Liu H, Kong Q *et al.* (2012) Overexpression of ZEB1 associated with metastasis and invasion in patients with gastric carcinoma. *Mol Cell Biochem* 366:223–9

Jo M, Lester RD, Montel V *et al.* (2009) Reversibility of epithelial-mesenchymal transition (EMT) induced in breast cancer cells by activation of urokinase receptor-dependent cell signaling. *J Biol Chem* 284:22825–33

Joseph EW, Pratilas CA, Poulikakos PI *et al.* (2010) The RAF inhibitor PLX4032 inhibits ERK signaling and tumor cell proliferation in a V600E BRAF-selective manner. *Proc Natl Acad Sci USA* 107:14903–8

Kiowski G, Biedermann T, Widmer DS *et al.* (2012) Engineering melanoma progression in a humanized environment in vivo. *J Invest Dermatol* 132:144–53

Kong D, Park EJ, Stephen AG *et al.* (2005) Echinomycin, a small-molecule inhibitor of hypoxia-inducible factor-1 DNA-binding activity. *Cancer Res* 65:9047–55

Kveiborg M, Albrechtsen R, Couchman JR *et al.* (2008) Cellular roles of ADAM12 in health and disease. *Int J Biochem Cell Biol* 40:1685–702

Lendeckel U, Kohl J, Arndt M *et al.* (2005) Increased expression of ADAM family members in human breast cancer and breast cancer cell lines. *J Cancer Res Clin Oncol* 131:41–8

Lin WM, Baker AC, Beroukhim R *et al.* (2008) Modeling genomic diversity and tumor dependency in malignant melanoma. *Cancer Res* 68:664–73

Lin Y, Stevens C, Hupp T (2007) Identification of a dominant negative functional domain on DAPK-1 that degrades DAPK-1 protein and stimulates TNFR-1-mediated apoptosis. *J Biol Chem* 282:16792–802

Loike JD, Cao L, Brett J *et al.* (1992) Hypoxia induces glucose transporter expression in endothelial cells. *Am J Physiol* 263:C326–33

Lui P, Cashin R, Machado M *et al.* (2007) Treatments for metastatic melanoma: synthesis of evidence from randomized trials. *Cancer Treat Rev* 33:665–80

Luk CK, Veinot-Drebot L, Tjan E *et al.* (1990) Effect of transient hypoxia on sensitivity to doxorubicin in human and murine cell lines. *J Natl Cancer Inst* 82:684–92

Luo Y, He DL, Ning L *et al.* (2006) Hypoxia-inducible factor-1alpha induces the epithelial-mesenchymal transition of human prostatecancer cells. *Chin Med J (Engl)* 119:713–8

Mazar J, DeYoung K, Khaitan D *et al.* (2010) The regulation of miRNA-211 expression and its role in melanoma cell invasiveness. *PLoS One* 5:e13779

Moretti D, Del Bello B, Cosci E *et al.* (2009) Novel variants of muscle calpain 3 identified in human melanoma cells: cisplatin-induced changes in vitro and differential expression in melanocytic lesions. *Carcinogenesis* 30:960–7

Oda H, Tsukita S, Takeichi M (1998) Dynamic behavior of the cadherin-based cell-cell adhesion system during Drosophila gastrulation. *Dev Biol* 203:435–50

Peinado H, Cano A (2008) A hypoxic twist in metastasis. *Nat Cell Biol* 10: 253–4

Peinado H, Moreno-Bueno G, Hardisson D *et al.* (2008) Lysyl oxidase-like 2 as a new poor prognosis marker of squamous cell carcinomas. *Cancer Res* 68:4541–50

Peinado H, Olmeda D, Cano A (2007) Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer* 7:415–28

Peinado H, Portillo F, Cano A (2004) Transcriptional regulation of cadherins during development and carcinogenesis. *Int J Dev Biol* 48:365–75

Postigo AA, Dean DC (1997) ZEB, a vertebrate homolog of Drosophila Zfh-1, is a negative regulator of muscle differentiation. *EMBO J* 16:3935–43

Postovit LM, Seftor EA, Seftor RE *et al.* (2006) Influence of the microenvironment on melanoma cell fate determination and phenotype. *Cancer Res* 66:7833–6

Prockop DJ, Kivirikko KI (1995) Collagens: molecular biology, diseases, and potentials for therapy. *Annu Rev Biochem* 64:403–34

Pusztaszeri MP, Seelentag W, Bosman FT (2006) Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand factor, and Fli-1 in normal human tissues. *J Histochem Cytochem* 54: 385–95

Ray JM, Stetler-Stevenson WG (1994) The role of matrix metalloproteases and their inhibitors in tumour invasion, metastasis and angiogenesis. *Eur Respir J* 7:2062–72

Rice GC, Ling V, Schimke RT (1987) Frequencies of independent and simultaneous selection of Chinese hamster cells for methotrexate and doxorubicin (adriamycin) resistance. *Proc Natl Acad Sci USA* 84:9261–4

Robert C, Thomas L, Bondarenko I *et al.* (2011) Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* 364: 2517–26

Sakaizawa K, Goto Y, Kiniwa Y *et al.* (2012) Mutation analysis of BRAF and KIT in circulating melanoma cells at the single cell level. *Br J Cancer* 106:939–46

Seftor EA, Meltzer PS, Kirschmann DA *et al.* (2002) Molecular determinants of human uveal melanoma invasion and metastasis. *Clin Exp Metastasis* 19:233–46

Sensi M, Catani M, Castellano G *et al.* (2011) Human cutaneous melanomas lacking MITF and melanocyte differentiation antigens express a functional Axl receptor kinase. *J Invest Dermatol* 131:2448–57

Sipos F, Galamb O (2012) Epithelial-to-mesenchymal and mesenchymal-to-epithelial transitions in the colon. *World J Gastroenterol* 18:601–8

Spaderna S, Schmalhofer O, Wahlbuhl M *et al.* (2008) The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer. *Cancer Res* 68:537–44

Stamenkovic I (2003) Extracellular matrix remodelling: the role of matrix metalloproteinases. *J Pathol* 200:448–64

Takafuta T, Wu G, Murphy GF *et al.* (1998) Human beta-filamin is a new protein that interacts with the cytoplasmic tail of glycoprotein Ibalpha. *J Biol Chem* 273:17531–8

Vaupel P, Mayer A (2007) Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Metastasis Rev* 26:225–39

Vergis R, Corbishley CM, Norman AR *et al.* (2008) Intrinsic markers of tumour hypoxia and angiogenesis in localised prostate cancer and outcome of radical treatment: a retrospective analysis of two randomised radiotherapy trials and one surgical cohort study. *Lancet Oncol* 9:342–51

Widmer DS, Cheng PF, Eichhoff OM *et al.* (2012) Systematic classification of melanoma cells by phenotype-specific gene expression mapping. *Pigment Cell Melanoma Res* 25:343–53

Xiong H, Hong J, Du W *et al.* (2012) Roles of STAT3 and ZEB1 proteins in E-cadherin down-regulation and human colorectal cancer epithelial-mesenchymal transition. *J Biol Chem* 287:5819–32

Yancovitz M, Litterman A, Yoon J *et al.* (2012) Intra- and inter-tumor heterogeneity of BRAF(V600E))mutations in primary and metastatic melanoma. *PLoS One* 7:e29336

Zipser MC, Eichhoff OM, Widmer DS *et al.* (2011) A proliferative melanoma cell phenotype is responsive to RAF/MEK inhibition independent of BRAF mutation status. *Pigment Cell Melanoma Res* 24:326–33