Tumor-Derived Fibronectin Is Involved in Melanoma Cell Invasion and Regulated by V600E B-Raf Signaling Pathway

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Melanomas are malignant tumors of melanocytes that, if not detected early, are highly aggressive and poorly treatable. Activation of extracellular signal-regulated (ERK)/mitogen-activated protein (MAP) kinase signaling is commonly found in melanomas mainly through oncogenic mutations of B-Raf. We previously reported that activation of ERK/MAP kinase stimulates synthesis of fibronectin by upregulating the transcription factor early growth response-1 (Egr-1). To further analyze the link between ERK/MAP kinase pathway and fibronectin in melanoma, we have studied the regulation and role of fibronectin produced by melanoma cells bearing oncogenic B-Raf mutation. We show that fibronectin is expressed *in situ* during tumor progression and that high fibronectin and Egr-1 levels are found in cells expressing this mutation. Expression of active mutants of B-Raf induces fibronectin, whereas endogenous fibronectin is inhibited by small interfering RNA (siRNA)-mediated depletion of B-Raf or Egr-1. In contrast, stimulation of ERK pathway is insufficient to promote fibronectin upregulation in normal melanocytes. Finally, we show that suppression of fibronectin by siRNA leads to decreased melanoma cell invasiveness *in vitro*. These results reveal a tumor-specific regulation of fibronectin by constitutive ERK/MAP kinase signaling and indicate that self-production of fibronectin may play a role in melanoma tumorigenesis, by promoting tumor cell invasion.

Journal of Investigative Dermatology (2007) **127,** 400–410. doi:10.1038/sj.jid.5700524; published online 7 September 2006

INTRODUCTION

Melanoma is a highly aggressive form of skin cancers that may progress to a deadly metastatic disease. Its incidence has been increasing worldwide over the past two decades. Although primary tumors can be successfully surgically removed, there is no satisfactory treatment for metastatic melanoma (Houghton and Polsky, 2002). Melanomas arise from malignant transformation of normal epidermal melanocytes (Goding, 2000; Herlyn *et al.*, 2000). Typically, primary lesions progress to malignant tumors through a multistep process including dysplasia, radial growth phase (RGP), invasive vertical growth phase (VGP), and metastasis. This transition is accomplished through the accumulation of genetic alterations in growth control pathways as well as molecular changes in cell–cell and cell–matrix interactions (Bogenrieder and Herlyn, 2002; Li *et al.*, 2002). Despite recent advances in the identification of genetic alterations involved in oncogenic transformation of melanocytes, the molecular mechanisms underlying the development and progression of melanoma are still poorly understood (Polsky and Cordon-Cardo, 2003).

Accumulating evidence point to the critical role of the Ras/Raf/MEK (mitogen-activated protein (MAP) kinase kinase)/ERK (extracellular signal-regulated kinase) MAP kinase pathway in melanoma development. Abnormal activation of this pathway occurs in melanoma because of oncogenic mutations in *BRAF* and *NRAS* genes or through autocrine growth factor stimulation (van Elsas *et al.*, 1996; Brose *et al.*, 2002; Cohen *et al.*, 2002; Davies *et al.*, 2002; Pollock *et al.*, 2003; Satyamoorthy *et al.*, 2003). Approximately, 60% of human melanomas have mutational activation of the *BRAF* gene (Davies *et al.*, 2002). Mutations of the *NRAS* gene has also been reported but with a lower frequency (van Elsas *et al.*, 1996). B-Raf is an immediate downstream mediator of Ras signaling that belongs to the Raf family of serine/threonine kinases. Once activated, Raf proteins stimulate a signaling cascade involving MEK and MAP kinases, ERK1 and ERK2, that ultimately regulates the activity of key transcription factors leading to the control of cell growth, differentiation, and survival (Hagemann and Rapp, 1999; Peyssonnaux and Eychene, 2001). The V600E single substitution within the

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Abbreviations: Egr-1, early growth response-1; ERK, extracellular signal-regulated kinase; HGF, hepatocyte growth factor; MAP, mitogen-activated protein; RGP, radial growth phase; siRNA, small interfering RNA; TRP, tyrosinase related protein; VGP, vertical growth phase

Received 5 January 2006; revised 5 June 2006; accepted 20 June 2006; published online 7 September 2006

activation loop in exon 15 of *BRAF* accounts for 90% of reported mutations and results in a constitutively active kinase (Davies *et al.*, 2002). V600E B-Raf leads to constitutive ERK activity, increased *in vitro* colony formation, and transformation of immortalized mouse melanocytes, whereas suppression of V600E B-Raf expression has been reported to cause inhibition of melanoma cells proliferation and survival *in vitro* and *in vivo* (Davies *et al.*, 2002; Calipel *et al.*, 2003; Hingorani *et al.*, 2003; Karasarides *et al.*, 2004; Sumimoto *et al.*, 2004; Wellbrock *et al.*, 2004). Thus, oncogenic V600E B-Raf-associated signaling pathway may support melanoma growth and survival. Identification of downstream events resulting from mutation of B-Raf is therefore a key issue to our understanding of melanoma biology.

Adhesive interactions between tumor cells and the extracellular matrix (ECM) regulate signaling events that promote cell adhesion, migration, growth, and survival. These interactions occur at multiple stages of metastasis (Fidler, 1996). Cellular adhesion is primarily mediated by integrins, a family of cell adhesion receptors, which provide both the connection to the adhesive substrate and signaling to the actin cytoskeleton (Giancotti and Ruoslahti, 1999). Alterations in expression of adhesion molecules such as integrins, MelCAM, cadherins, and cell surface proteoglycans have been well documented in the development of malignant melanoma (Bogenrieder and Herlyn, 2002; Li *et al.*, 2002). Abnormal expression of the fibronectin matrix protein has also been linked to metastatic melanoma progression. Fibronectin is one of the most important integrin ligand of ECM, which controls many fundamental biological processes (Hynes and Yamada, 1982). There is strong evidence that expression of fibronectin is tightly correlated with the acquisition of invasive and metastatic behavior of melanoma cells (Bittner *et al.*, 2000; Clark *et al.*, 2000; Hoek *et al.*, 2004; Gaggioli *et al.*, 2005). Besides melanoma, fibronectin is also expressed in several other tumor cell types including colorectal carcinomas, esophageal squamous cell carcinomas, papillary thyroid carcinomas, and breast carcinomas (Zhang *et al.*, 1997; Huang *et al.*, 2001; Jiang *et al.*, 2002; Zhang *et al.*, 2005). Importantly, blockade of fibronectin binding to melanoma cells by synthetic peptides prevented melanoma metastasis in mice (Humphries *et al.*, 1986). These observations support the hypothesis that during melanoma development, overexpression of fibronectin can influence the control and progression of metastasis by mediating integrin-associated signaling pathways.

The biochemical mechanisms that control upregulation of fibronectin in melanoma cells are beginning to unfold. We recently observed that, stimulation of ERK/MAP kinase signaling pathway by the hepatocyte growth factor (HGF) induced fibronectin expression in melanoma cells through upregulation and activation of the zinc finger transcription factor early growth response-1 (Egr-1) (Gaggioli *et al.*, 2005). Because the deregulation of the ERK/MAP kinase pathway arising from mutations in the *BRAF* gene occurs in most melanomas, we sought to analyze the contribution of this signaling pathway to fibronectin overexpression in melanoma cells. We now show that fibronectin is a target gene of B-Raf signaling in melanoma cell lines but not in normal melanocytes, and that tumor-derived fibronectin may contribute to melanoma tumorigenesis by promoting invasion of cells that contain the B-Raf V600E mutation.

RESULTS

Fibronectin is expressed *in situ* during melanoma progression

We have shown previously that fibronectin protein is expressed at much higher levels in human melanoma cell lines compared to normal melanocytes (Gaggioli *et al.*, 2005). Also, increase of fibronectin mRNA was reported to be associated with melanoma metastasis (Bittner *et al.*, 2000; Clark *et al.*, 2000). In this study, we extended the analysis of fibronectin expression to normal skin and melanoma samples to determine whether the increase of fibronectin also occurs in the tumor lesion itself. Immunofluorescent staining analysis was performed on normal human foreskin and primary melanomas biopsies (two RGPs and one VGP). In normal skin, melanocytes were stained for tyrosinase related protein (TRP)-1 to detect their location in the basal layer of the epidermis (Figure 1). Fibronectin was strongly positive in the dermis but virtually absent in epidermis. In melanoma sections, the intensity level of fibronectin staining associated with TRP-1-positive cells significantly increased compared to normal epidermis. In primary VGP melanoma no. 3, fibronectin staining tend to be uniform throughout the tumor, whereas in RGP melanomas nos. 1 and 2 the intensity varied in different areas of the tumor. This indicates that within a lesion, melanoma cells were heterogeneous for fibronectin expression and also that fibronectin immunostaining did not correlate with TRP-1 expression in these melanomas. Interestingly, in melanoma no. 1 lesion, cells appeared positive for fibronectin in the deep portion of the tumor nest and negative in the superficial portion. Finally, it may be worth noting that fibronectin localized in punctuate pattern that contrasted with the well-organized network of matrix observed in the dermis. These results indicate that fibronectin was expressed in primary melanomas and corroborate the aberrant fibronectin protein expression pattern observed in melanoma cell lines.

Overexpression of fibronectin and Egr-1 is associated with oncogenic mutation of B-Raf in human melanoma cell lines

The zinc-finger transcription factor Egr-1 regulates fibronectin gene expression via activation of the ERK/MAP kinase pathway in human MeWo melanoma cells (Gaggioli *et al.*, 2005). This observation led us to examine the expression profile of Egr-1 during melanoma progression and compare it with that of fibronectin. Real-time PCR was first used to evaluate fibronectin and Egr-1 mRNA levels in human primary melanocytes and metastatic melanoma cell lines. In agreement with our previous observation (Gaggioli *et al.*, 2005), cells expressing oncogenic B-Raf (A375, 1205Lu, and SKmel28) showed high levels of fibronectin mRNA (Figure 2a, left panel). In contrast, in MeWo cells that express wild-type B-Raf, fibronectin was expressed at a level comparable with that of normal melanocytes. A similar expression pattern was observed for Egr-1. Egr-1 mRNA was more abundant in

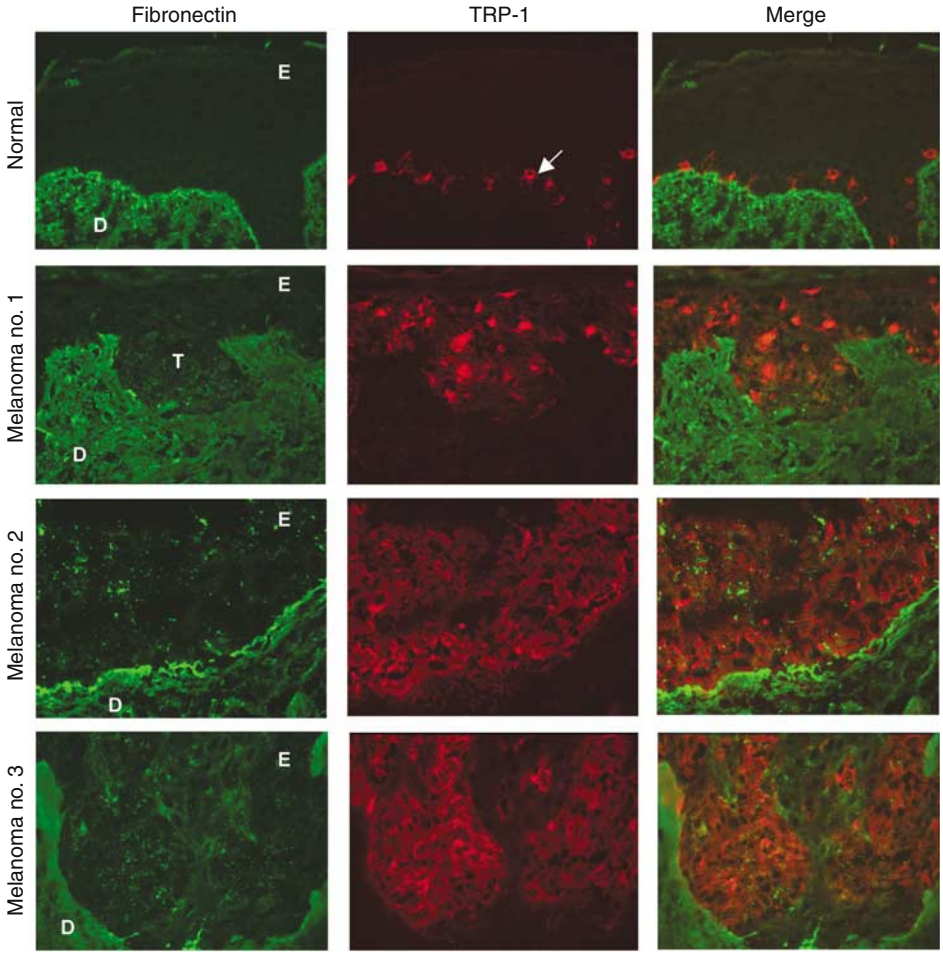

Figure 1. Detection of fibronectin in normal skin and melanoma specimens by immunofluorescence analysis. Frozen sections were fixed and stained, as indicated, for fibronectin (green), TRP-1 (red), and a control serum (data not shown). FITC-conjugated anti-rabbit and Texas Red-conjugated anti-rat secondary antibodies were used. TRP-1 staining was used to identify melanocytic skin cells. Slides were examined with a Zeiss Axiophot fluorescence microscope and pictures were taken at ×20 magnification. Arrow shows a melanocyte that resides on the dermal–epidermal junction. Panels for merge images are shown. Analysis of normal skin revealed strong fibronectin signals in dermis but no signal in epidermis. In melanoma sections, staining revealed a significant and punctuate expression pattern associated with TRP-1 signals. E, epidermis; D, dermis; and T, nest of tumor cells. Melanoma specimen no. 1, RGP, tumor thickness 0.40 mm, Clark level II. Melanoma specimen no. 2, RGP, tumor thickness 0.55 mm, Clark level II. Melanoma specimen no. 3, VGP, tumor thickness 0.80 mm, Clark level III.

the tumor cell lines having V600E mutation compared with MeWo or normal melanocytes (Figure 2a, right panel). Fibronectin and Egr-1 expression analysis was next extended to melanoma cell lines derived from primary RGP and VGP melanoma lesions. Total cell lysates from two RGP melanomas (WM35 and SBcl2), one VGP melanoma (WM793), and four metastatic melanomas (MeWo, 501mel, 1205Lu, and A375), together with melanocytes were analyzed by Western blotting with antibodies against fibronectin and Egr-1. ERK activities were also evaluated with an antibody specific for the dually phosphorylated and activated MAP kinases, ERK1 and ERK2. Figure 2b shows that Egr-1 and fibronectin protein levels were higher in WM35, WM793, 1205Lu, and A375 cells than in MeWo, 501mel, SBcl2, or normal melanocytes. Relatively low levels of fibronectin and Egr-1 were seen in 501mel cells, which contain the V600E B-Raf mutation but exhibited low level of activated ERK1/2. Note that in comparison with melanocytes, we observed that under the experimental conditions used, most of the wild-type B-Raf melanoma cells exhibited significant phospho-ERK levels. Immunoprecipitation using an Egr-1-specific antibody further confirmed increased levels of Egr-1 in 1205Lu and A375 cells compared to MeWo or 501mel cells (Figure 2c). These results indicate that Egr-1 is expressed in melanoma cell lines that harbor V600E B-Raf and increased ERK/MAP kinase activity, suggesting that Egr-1 is a target of this pathway in these cells. Moreover, the parallel expression pattern observed between Egr-1 and fibronectin is consistent with the hypothesis that Egr-1 modulates fibronectin expression in melanoma cells.

We next asked whether the increase in fibronectin expression might allow melanoma cells to accumulate fibronectin matrix on the cell surface. As expected, the fibronectin matrix was barely detectable by immunofluorescence on MeWo cells whereas WM35 and 1205Lu cells

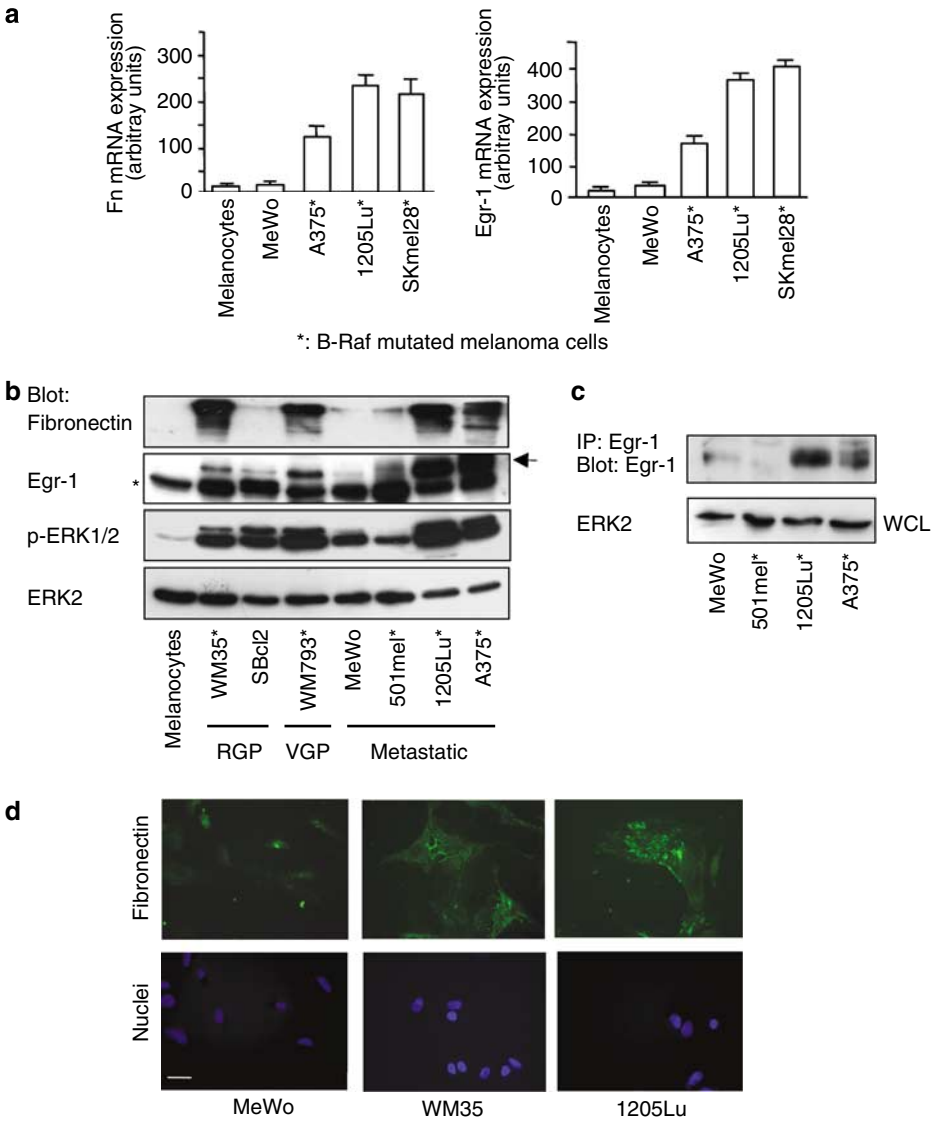

Figure 2. Fibronectin and Egr-1 are expressed at high levels in melanoma cells expressing V600E B-Raf. (**a**) Total RNA was isolated from primary melanocytes and wild type B-Raf (MeWo) or V600E B-Raf (A375, 1205Lu, and SKmel28) melanoma cell lines. Real-time reverse transcription–PCR was performed and data were normalized against four housekeeping genes as described in Materials and Methods. Fibronectin (Fn) and Egr-1 mRNA values were expressed as arbitrary units and represent means ± SD of triplicates and are representative of two independent experiments. (**b**) Whole-cell lysates were collected from primary melanocytes or from the indicated primary or metastatic melanoma cell lines, separated on SDS–PAGE and analyzed by Western blotting using antibodies against fibronectin, Egr-1, and phospho-ERK1/2. Antibody to ERK2 was used as loading control. *Non-specific band. Arrow points to Egr-1 protein. (**c**) Whole-cell lysates (WCL) were immunoprecipitated with anti-Egr-1 followed by anti-Egr-1 blot (upper panel) or directly analyzed with anti-ERK2 blot as loading control (lower panel). (**d**) Immunofluorescence staining of pericellular fibronectin on the surface of MeWo, WM35, and 1205Lu cells. Cells were fixed and stained with anti-fibronectin antibody (green) or 4,6-diamidino-2-phenylindole (blue) to reveal nuclei. Cells were viewed under a fluorescence microscope (Zeiss). Bar = 5 μm.

exhibited higher levels of the protein and displayed a moderately, developed pericellular fibronectin network (Figure 2d).

Fibronectin overexpression depends on ERK activity and requires V600E B-Raf

The correlation between oncogenic B-Raf signaling and fibronectin expression prompted us to examine the role of MAP kinase and B-Raf in particular in fibronectin regulation. We first examined the effect of B-Raf V600E expression on *fibronectin* gene transcription. The ability of V600E mutant to induce the *fibronectin* promoter was tested in MeWo cells, which have low levels of ERK activity. When *fibronectin*–luciferase construct was transfected along with increasing amount of B-Raf V600E expression vector, a dose-dependent induction in luciferase activity was observed, indicating that activation of ERK/MAP kinase pathway by expression of V600E B-Raf stimulates transcriptional activation of fibronectin (Figure 3a). We next tested if activated B-Raf could stimulate expression of endogenous fibronectin protein.

Adenoviruses expressing active B-Raf (B-RafCAAX) or vector control were administered to MeWo cells and infection was controlled by detection of hemagglutinin-tagged B-Raf protein in Western blot (data not shown). Figure 3b clearly showed that expression of B-RafCAAX stimulated ERK1/2 activity and increased levels of fibronectin protein. In addition, expression of B-RafCAAX resulted in an increase of Egr-1 protein amount. The latter observation is consistent with the possibility that B-Raf stimulates fibronectin expression via upregulation of Egr-1.

To further explore the role of constitutive ERK signaling on the basal activity of the *fibronectin* promoter in melanoma cells, we used the MEK inhibitor U0126 to inhibit ERK activity in these cells. After transfection with the *fibronectin*–luciferase construct, MeWo, 1205Lu, and A375 cells were treated with 10 μM of U0126 for 48 hours, and then

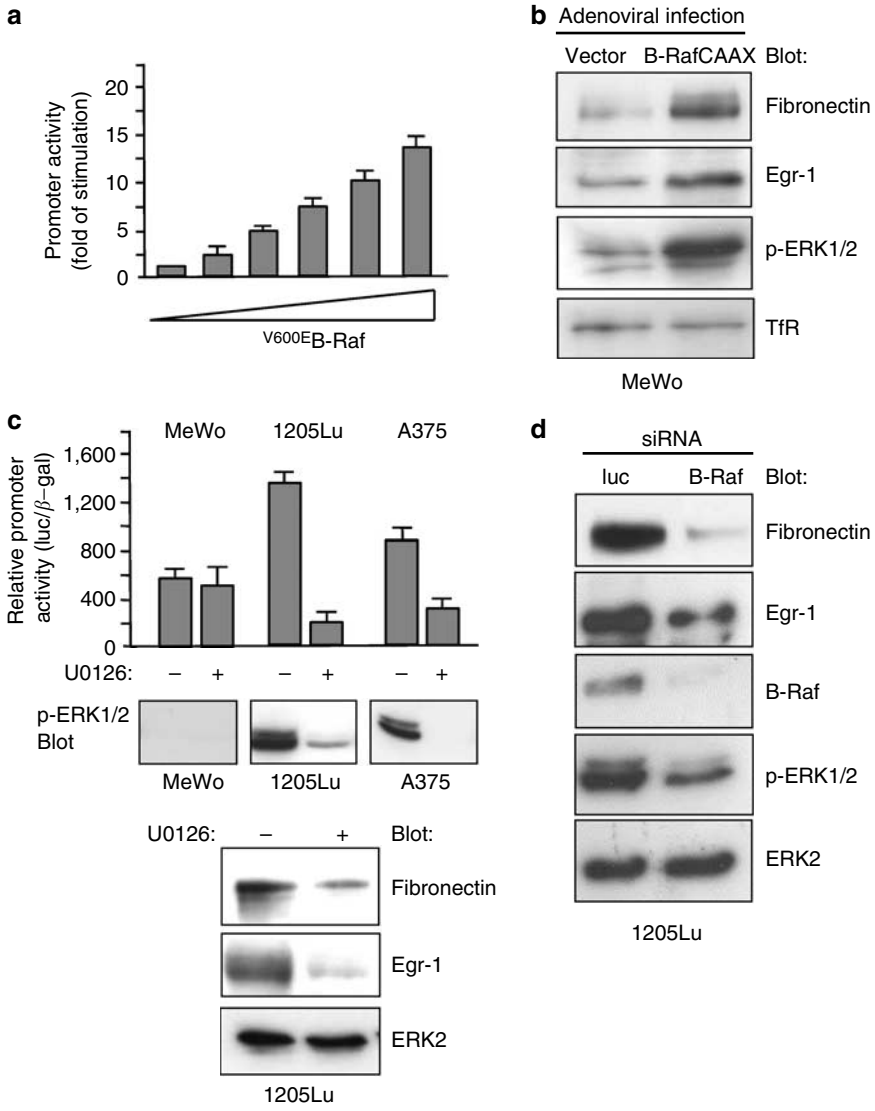

Figure 3. Regulation of endogenous fibronectin and Egr-1 by oncogenic B-Raf in melanoma cells. (**a**) Transcriptional activation of the *fibronectin* promoter by B-RafV600E. MeWo cells were transfected with the *fibronectin* promoter luciferase reporter (200 ng) and increasing amounts of a vector expressing V600E B-Raf (from 10 to 200 ng). Luciferase activities were determined 48 hours post-transfection. Activities were normalized to β-galactosidase activities and expressed as fold induction. (**b**) Expression of B-RafCAAX increases fibronectin and Egr-1 protein levels. MeWo cells were infected with either control or B-RafCAAX adenovirus. Fibronectin and Egr-1 protein levels as well as ERK1/2 phosphorylation were assayed by Western blot 48 hours after infection. TfR is shown as a loading control. (**c**) ERK/MAP kinase-dependent expression of fibronectin and Egr-1. Upper panel: MeWo, A375, and 1205Lu cells were transfected with the *fibronectin* promoter luciferase reporter, cultured with 10 μM of the MEK inhibitor U0126 for 48 hours, and harvested for luciferase activity. Values were normalized to β-galactosidase activities to eliminate differences in transfection efficiency. Insets show Western blot using anti-phospho ERK1/2 antibody of the indicated cell lines after 48 hours treatment with U0126. Lower panel: Western blot with anti-fibronectin and anti-Egr-1 antibodies showing levels of fibronectin and Egr-1 in control and U0126-treated 1205Lu cells. Total ERK2 is shown as a loading control. (**d**) siRNA against B-Raf downregulates fibronectin and Egr-1 expression in 1205Lu cells. Cells were transfected with luc siRNA or B-Raf siRNA as indicated. After 72 hours, cell lysates were blotted for total B-Raf, phospho-ERK1/2, total ERK2, Egr-1, and fibronectin as indicated. Results are representative of two independent experiments.

luciferase assays were performed. As expected, treatment of 1205Lu and A375 cells with U0126 eliminated constitutive phosphorylation of ERK1/2 (Figure 3c, upper panel). Compared with MeWo, 1205Lu cells showed high levels of *fibronectin* reporter activity that was abrogated by U0126 treatment. A similar ERK activity dependence was seen in A375 cells. Significantly, inhibition of the ERK pathway also reduced fibronectin and Egr-1 protein levels (Figure 3c, lower panel). These results indicate that upregulation of fibronectin and Egr-1 is a consequence of constitutive ERK/MAP kinase signaling.

We also examined regulation of fibronectin expression via small interfering RNA (siRNA)-mediated knockdown of V600E B-Raf in 1205Lu melanoma cells. This siRNA specifically depleted B-Raf but not c-Raf or ERK2, and led to suppression of the constitutive ERK signaling (Figure 3d and data not shown). Consistent with the results from U0126 treatment, we observed a significant reduction of fibronectin and Egr-1 protein levels upon depletion of B-Raf. Similar results were obtained in A375 melanoma cells (data not shown). These findings suggest that V600E B-Raf contributes to fibronectin and Egr-1 expression in 1205Lu melanoma cells.

Egr-1 is required for fibronectin production in melanoma cells

Our findings obtained so far raise the possibility that Egr-1 might be responsible for increasing fibronectin in cells that harbor V600E B-Raf. To test this, we infected 1205Lu cells with an adenoviral vector expressing nerve growth factor-IA binding protein (NAB2), a specific repressor of Egr-1 activity (Svaren *et al.*, 1996) (Figure 4a). Control cells were infected with an adenoviral vector expressing green fluorescent protein alone. After 48 hours, cells were analyzed for fibronectin expression by Western blotting. Figure 4a shows that expression of NAB2 inhibited the expression of fibronectin, and as expected, Egr-1 protein levels were not affected in cells infected with AdNAB2. This indicates that inhibition of Egr-1 transcriptional activity results in reduced fibronectin production. Next, we used siRNA to selectively knockdown Egr-1 protein levels in 1205Lu cells. Cells transfected with an Egr-1-specific siRNA showed significant downregulation of Egr-1 but also a decrease in fibronectin amount (Figure 4b). These results further suggest that Egr-1 contributes to fibronectin expression in these melanoma cells.

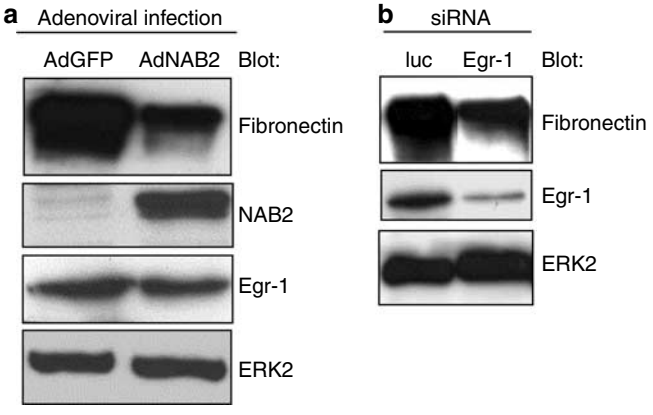

Figure 4. A role for Egr-1 in fibronectin expression in 1205Lu melanoma cells. (**a**) Cells were infected with adenoviruses encoding green fluorescent protein alone or NAB2 green fluorescent protein. NAB2, fibronectin, and Egr-1 protein levels as well as ERK2 were assayed by Western blot 72 hours after infection. (**b**) Cells were transfected with luc siRNA or Egr-1 siRNA as indicated. After 72 hours, cell lysates were blotted for Egr-1 and fibronectin as indicated. Results are representative of two independent experiments.

Lack of fibronectin regulation by activated ERK in normal melanocytes

To investigate whether the regulation of fibronectin by ERK signaling is specific to malignant melanocytes, we analyzed the expression of fibronectin in normal primary melanocytes expressing activated B-Raf or after 2 days of treatment with HGF, a potent inducer of the Ras/Raf/MEK/ERK pathway (Figure 5). Adenoviral infections revealed that expression of B-RafCAAX stimulated both ERK signaling and Egr-1 expression, but failed to increase fibronectin protein levels (Figure 5a). Similarly, no appreciable induction of fibronectin was seen in melanocytes exposed to HGF, despite the stimulation of ERK1/2 phosphorylation and upregulation of Egr-1 (Figure 5b). Similar results were obtained using melanocytes isolated from several donors (data not shown). These observations indicate that regulation of fibronectin expression by activated ERK/MAP kinase signaling pathway is limited to transformed melanocytes.

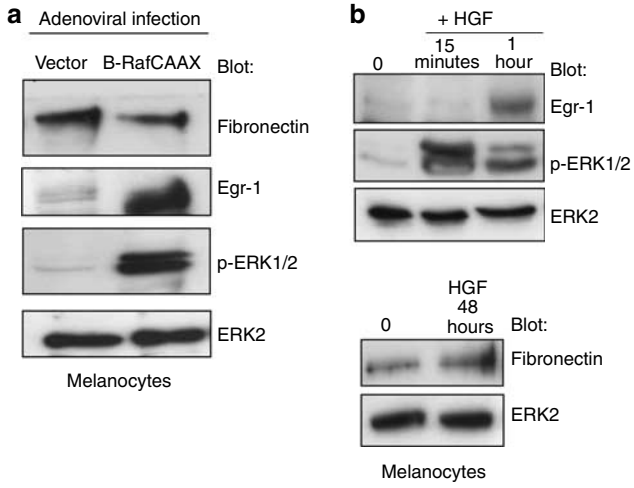

Figure 5. Activation of ERK/MAP kinase signaling pathway does not regulate fibronectin expression in normal melanocytes. (**a**) Expression of B-RafCAAX induces sustained activation of ERK1/2 and promotes Egr-1 expression. Human primary melanocytes were infected with either control or B-RafCAAX adenovirus. Fibronectin and Egr-1 protein levels as well as ERK1/2 phosphorylation were assayed by Western blot 48 hours after infection. Total ERK2 is shown as a loading control. (**b**) HGF stimulates ERK1/2 activation and Egr-1 upregulation. Human primary melanocytes were serum-starved overnight and then incubated with HGF (50 ng/ml) for the indicated times. Cell lysates were blotted for Egr-1, phospho-ERK1/2, total ERK2, and fibronectin as indicated. Notice that no change in fibronectin protein levels was observed. Similar results were obtained with three independent melanocyte cultures.

Tumor-derived fibronectin is involved in the invasive potential of melanoma cells

To examine the biological consequences of fibronectin production by melanoma cells, we designed an siRNA sequence to target fibronectin in 1205Lu cells. As shown in Figure 6a, Western blot analysis of whole-cell lysates 4 days after transfection with fibronectin siRNA revealed more than 80% reduction in fibronectin protein level without any significant modulation of the amount of ERK2 used as control. Consequently, staining of pericellular fibronectin in 1205Lu transfected with the fibronectin siRNA was completely negative (Figure 6b). We first compared the adhesive capacities of control and fibronectin siRNA-transfected cells and found that fibronectin silencing decreases melanoma cells anchorage to substratum and that fibronectin-depleted cells were more sensitive to detachment after trypsinization than control cells (data not shown). In subsequent experiments, the invasion ability of fibronectin-silenced melanoma cells was evaluated in Boyden chambers by using inserts coated with ECMatrix. As shown in Figure 6c, the number of cells that migrated through a reconstituted basement membrane was significantly lower in fibronectin-depleted cells than in control cells. These findings are consistent with the possibility that production of fibronectin by melanoma cells contributes to their invasive phenotype.

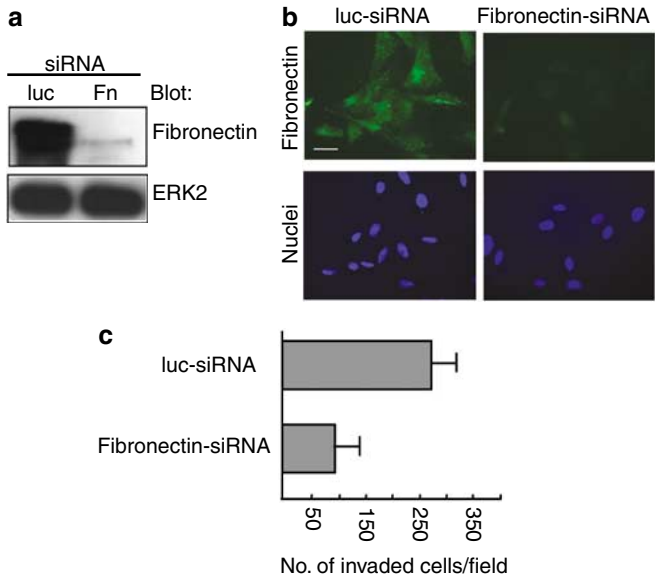

Figure 6. Fibronectin silencing leads to decreased invasion capacity of 1205Lu melanoma cells. (**a**) Western blot analysis of cell lysates 96 hours after transfection with siRNA against luciferase (luc) or against fibronectin (Fn). Cell lysates were blotted with specific antibodies to fibronectin. Total ERK2 is shown as a loading control. (**b**) Immunofluorescence of control and fibronectin siRNA-transfected cells with antibodies directed to fibronectin (green). Nuclei were stained with 4,6-diamidino-2-phenylindole (blue). Bar = 25 μm. (**c**) Cells were transfected with luc siRNA or fibronectin siRNA, starved overnight, detached, and then assayed for their ability to invade ECMatrix using a modified Boyden chamber assay. Invasion was quantitated by visual counting. Mean values of cell counts were obtained from five individual fields. Similar results were obtained in two independent experiments.

DISCUSSION

We have shown previously that fibronectin is overexpressed in several melanoma cell lines that display constitutive ERK/MAP kinase signaling, suggesting a role of this signaling pathway in fibronectin regulation. We also reported that HGF, a growth factor produced by melanoma cells, induces fibronectin expression through ERK/MAP kinase-dependent expression of Egr-1 (Gaggioli *et al.*, 2005). Moreover, global gene expression analyses have shown that an increase of fibronectin mRNA accompanies oncogenic transformation of melanocytes and correlates with acquisition of tumorigenic and metastatic potentials (Bittner *et al.*, 2000; Clark *et al.*, 2000; Hoek *et al.*, 2004). Collectively, these studies support the idea that self-production of fibronectin might contribute to the invasive behavior of melanomas. This study was designed to expand on these observations and to determine the role of oncogenic B-Raf and ERK/MAP kinase in general, on fibronectin regulation in melanoma cells. We first confirmed that increase in fibronectin expression observed in melanoma cell lines also occurs in the primary cancer lesion itself. Here, we have analyzed a limited number of melanomas and found for two lesions intratumoral differences in fibronectin staining that might reflect heterogeneity in fibronectin expression pattern in tumor cells within the same lesion. We are presently studying a large number of human primary and metastatic melanomas as well as nevi by immunohistochemistry in order to define with certainty the pattern of fibronectin expression in melanomas and during disease progression. Interestingly, the pattern of pericellular fibronectin observed in melanomas is distinguishable from the fibrillar network seen in dermis. This might be consistent with a cleveage of fibronectin by tumor-derived proteolytic enzymes such as matrix metalloproteases or tissue serine proteinases. Melanoma cells are known to secrete high levels of these ECM-degrading enzymes (Bogenrieder and Herlyn, 2002). Proteolysis of tumor-produced fibronectin will generate matrix fragments that might have beneficial effects on migratory properties. In this regard, upregulation of fibronectin may provide transformed melanocytes with a selective advantage to escape the epidermis and invade the underlying dermis. Alternatively, proteolysis of tumor-produced fibronectin may serve as a reservoir for growth factors or cytokines that will provide clues necessary for tumor growth and survival. Finally, another attractive possibility is that production of fibronectin may influence tumor implantation during the metastatic process. This might be of importance for the adaptation of tumor cells to local requirements. Studies are under way to examine these different hypotheses. As one step toward elucidation of the role of tumor-derived fibronectin in melanoma cells behavior, we tested whether its invalidation may affect adhesion and invasion of 1205Lu cells. We show that blocking fibronectin expression with specific siRNA partially inhibits invasive capabilities of these cells. This finding adds weight to the idea that fibronectin may support melanoma progression by promoting adhesion and invasion of tumor cells.

Oncogenic mutations of B-Raf resulting in constitutive activation of ERK/MAP kinase signaling pathway are a major

characteristic of melanoma (Davies *et al.*, 2002). Here, we provide several lines of evidence that this signaling pathway is required to maintain high fibronectin levels in melanoma cells. Thus, the presence of these activating mutations in the majority of melanoma may account for the overexpression of fibronectin found in these cells compared to normal melanocytes. Besides B-Raf mutations, more than 20% melanoma harbor oncogenic mutations in the *NRAS* gene occurring mainly in lesions from sun-exposed sites. *BRAF* and *NRAS* mutations appear to be equivalent in the constitutive activation of the MAP kinase signaling pathway and are generally found with mutual exclusion in melanoma (Brose *et al.*, 2002; Davies *et al.*, 2002; Pollock *et al.*, 2003; Garnett and Marais, 2004). Thus, it seems likely that fibronectin through Egr-1 expression would be a target of MAP kinase pathway in melanoma cells carrying activating mutations in N-Ras. This view is supported by our previously reported findings showing high fibronectin levels in N-Ras-mutated melanoma cells (Gaggioli *et al.*, 2005 and unpublished observations). Further, our previous observation that fibronectin can also be induced by activation of ERK/MAP kinase pathway in response to HGF suggests that in some melanomas not bearing activating B-Raf or N-Ras mutations, fibronectin may also be induced through autocrine growth factor stimulation (Gaggioli *et al.*, 2005). Altogether, these findings support the idea that expression of fibronectin is a general feature of melanoma cells.

Importantly, we have observed that oncogenic B-Raf upregulates the expression of fibronectin, most likely through Egr-1 transcriptional activity. These results are in agreement with previous studies showing that fibronectin is a direct Egr-1-responsive gene (Liu *et al.*, 2000; Gaggioli *et al.*, 2005). Interestingly, we observed that Egr-1 levels are correlated with fibronectin levels and with B-Raf-mediated ERK/MAP kinase signaling. This observation further emphasizes the involvement of Egr-1 in B-Raf-dependent fibronectin synthesis. To our knowledge, this is the first report showing an increase of Egr-1 expression in melanoma cells compared to normal melanocytes. The Egr-1 promoter contains several serum response elements and Ets binding sites that mediate its induction (Cohen *et al.*, 1996; Thiel and Cibelli, 2002). These elements are regulated by ternary complex factors, which include Elk-1. Elk-1 is a direct target of the MAP kinase pathway, and studies have shown that Elk-1 is continuously phosphorylated and activated by upstream MAP kinases in melanoma cells harboring B-Raf V600E mutation (Conner *et al.*, 2003; Satyamoorthy *et al.*, 2003). Based on this, it seems likely that Elk-1 may contribute to the transcriptional regulation of Egr-1 and subsequent fibronectin gene expression in these cells. In support to this, we have observed that expression of a dominant-negative mutant of Elk-1 inhibits Egr-1 expression in V600E B-Raf-positive cells (data not shown). As for fibronectin, we can predict that Egr-1 is upregulated by constitutive activated ERK/MAP kinase pathway in melanoma cells having oncogenic mutation of N-Ras. Besides fibronectin, Egr-1 has been shown to regulate the expression of the adhesion receptor CD44v6 in melanoma cells (Recio and Merlino, 2003). Together with this report, our study supports a potential role for Egr-1 in transcriptional induction of molecules implicated in the interaction between tumor cell and ECM. It will be interesting now to determine whether selective inhibition of Egr-1 expression (by siRNA knockdown) or activity (by NAB2 expression) modulates melanoma cells adhesion, migration, and/or invasion.

Interestingly, we have found that activation of ERK/MAP kinase signaling and expression of Egr-1 are not sufficient to promote fibronectin expression in normal melanocytes. This indicates that regulation of fibronectin by this signaling pathway is limited to transformed cell lines and likely reflects a tumor-specific regulation. Our findings suggest that in contrast to transformed cells, melanocytes are not programmed to produce fibronectin following ERK activation. Consistent with this, stimulation of Ras/B-Raf/ERK pathway by the melanocyte-differentiating agent cAMP did not induce fibronectin expression in normal melanocytes (unpublished results). The mechanism of tumor-specific regulation of fibronectin remains unclear, but could involve the regulation of additional targets in melanoma cells. One attractive candidate is the transcription factor Snail. Snail has been shown to promote fibronectin expression in carcinoma cells (Cano *et al.*, 2000), and is expressed in several melanoma cell lines but absent in melanocytes (Poser *et al.*, 2001; Robert *et al.*, 2006). These suggest that Snail may contribute to fibronectin regulation in melanoma cells. Alternatively, the β-catenin-dependent signaling pathway could be involved in this process as β-catenin signaling is activated in most melanoma cells and known to control fibronectin expression (Rubinfeld *et al.*, 1997; Gradl *et al.*, 1999). Another possibility is that a putative repressor prevents fibronectin transcription in melanocytes and that its expression will be lost during oncogenic transformation. In this regard, NAB proteins (NAB1 and NAB2) might be potential candidates for such repression by blocking Egr-1-dependent transcription (Russo *et al.*, 1995; Svaren *et al.*, 1996). However, it seems unlikely because we did not observe, in preliminary experiments, the modulation of NAB1 and NAB2 levels between normal melanocytes and melanoma cells (unpublished observations). Because changes in DNA methylation often occur during tumor development, we can also hypothesize that activity of the *fibronectin* promoter is subject of epigenetic inactivation in normal melanocytes. Interestingly, methylation is observed in the CpG islands of promoter regions and these sequences often match with Egr-1-binding sites (Cohen *et al.*, 1996; Thiel and Cibelli, 2002). This methylation will prevent melanocytes to increase fibronectin levels in response to ERK/MAP kinase signaling and Egr-1 activation. Oncogenic transformation of melanocytes would then be associated with demethylation of the *fibronectin* promoter. Future studies in our lab will address the mechanism(s) through which ERK/MAP signaling upregulates fibronectin in melanoma cells specifically. Whatever the mechanism(s) involved, production of this matrix protein by melanoma cells predicts a potential role in their transformed phenotype.

In conclusion, our work reveals a tumor-specific regulation of fibronectin by constitutive ERK/MAP kinase signaling

and provides novel insights into the oncogenic role of B-Raf in melanoma. Further it indicates that self-production of fibronectin may have important implications for the biology of melanoma progression.

MATERIALS AND METHODS

Cells, melanoma biopsies, and reagents

Human melanoma cell lines from RGP (WM35 and SBcl2), VGP (WM793), and metastatic melanoma (1205Lu) were generously provided by Dr M. Herlyn (Wistar Institute, Philadelphia, PA). Primary melanomas were maintained in modified chemically defined medium 153 with 20% Leibovitz L-15 medium, 2% fetal bovine serum, and 5 μg/ml insulin as described previously (Satyamoorthy *et al.*, 2003). Human 501mel, MeWo, SK-mel28, A375, and 1205Lu melanoma cells were cultured in DMEM supplemented with 7% fetal bovine serum. The *BRAF* mutational status has been determined previously (Davies *et al.*, 2002; Satyamoorthy *et al.*, 2003; Tanami *et al.*, 2004; Tsavachidou *et al.*, 2004; unpublished data). Human primary epidermal melanocytes were isolated from foreskin and maintained as described previously (Bertolotto *et al.*, 1998). All tissue culture reagents were purchased from Invitrogen (San Diego, CA) and Sigma Chemical Co (St Louis, MO). MEK inhibitor U0126 was from Cell Signaling Technology (Beverly, MA). Human recombinant HGF was obtained from PeproTech (Rocky Hill, NJ). Melanoma biopsies were obtained from the files of the Department of Dermatology of University Nice Hospital. The experiments using human tissue samples were conducted according to the Declaration of Helsinki Principles and had institutional approval.

Immunofluorescence detection of fibronectin

Five micrometers of frozen sections of human foreskin or cutaneous melanoma samples embedded in optimal cutting Temperature Tissue-Tek compound were fixed in 3% paraformaldehyde in phosphate-buffered saline for 10 minutes, and stained for fibronectin using a rabbit polyclonal antibody (dilution, 1:400; Sigma). Sections were also stained for the melanogenic enzyme TRP-1 using a rat polyclonal antibody (dilution 1:20), generously provided by Dr V. Hearing. After washing with phosphate-buffered saline, sections were incubated with FITC-conjugated anti-rabbit and Texas Red-conjugated anti-rat secondary antibodies (Molecular Probes, Eugene, OR). Slides were examined with a Zeiss Axiophot fluorescence microscope. Immunofluorescence staining of cell monolayers was carried out as described previously (Gaggioli *et al.*, 2005). Briefly, cells grown on glass cover slips were fixed with 3% paraformaldehyde, and incubated for 1 hour with the antibody to fibronectin diluted in phosphate-buffered saline containing 1% BSA. FITC-coupled anti-rabbit antibody was used for detection.

DNA constructs, cell transfection, and infection

The pGL2F1900 containing the rat *fibronectin* gene between positions -1908 and $+136$ fused to the luciferase reporter gene was kindly provided by Dr I.-S. Kim and described previously (Lee *et al.*, 2000). A pcDNA3 vector-encoding human B-Raf was a gift from Dr P.J. Stork. The point mutation (V600E) was generated by site-directed mutagenesis using the Transformer kit (BD-Clontech, San Jose, CA) and confirmed by DNA sequencing before expression. Transfections of melanoma cells were performed according to the lipofectamine protocol (Invitrogen). As an internal control, a LacZ-expressing plasmid was included in each transfection. Cells were harvested after 48 hours and luciferase activity was assessed using the luciferase assay kit (Promega, Madison, WI). Final values were corrected for transfection efficiency as determined by β-galactosidase activity and represent means $\pm$ SD from at least three independent transfections performed in triplicates.

Adenoviral vectors for NAB2 (Ad green fluorescent protein NAB2) and B-RafCAAX have been described previously (Ehrengruber *et al.*, 2000; Larribere *et al.*, 2004; Gaggioli *et al.*, 2005). Propagation and purification of adenoviruses were performed as described (Gaggioli *et al.*, 2005). Adenoviruses without cDNA insert and viruses expressing green fluorescent protein alone were used as controls. Cells were infected at multiplicity of infection of 20 and assayed 48 hours post-infection.

Real-time PCR

Total RNA was prepared and reverse-transcribed as described previously (Gaggioli *et al.*, 2005). Primer pairs for each cDNA were designed using Primer Express Software (Applied Biosystems, Courtaboeuf, France). Real-time PCR was performed using the standard SYBR Green detection protocol of ABI PRISM 5700 (Applied Biosystems). Relative expression level of target genes was normalized for RNA concentrations with four different housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase, β-actin, hypoxanthine-guanine phosphoribosyltransferase, and ubiquitin).

Cell lysis, immunoprecipitation, and Western blotting

Cells were solubilized in radioimmunoprecipitation assay buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 5 mM NaF, 2.5 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 0.1% SDS, 0.5% sodium deoxycholate, and a Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN) supplemented with 1 Nonidet P-40. Lysates were then cleared and adjusted to equal protein amounts. Proteins were separated by SDS–PAGE, and Western blot analysis was performed following standard protocols using the following antibodies: fibronectin (F3648; Sigma); B-Raf (F-7; Santa Cruz Biotechnology, Santa Cruz, CA); phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technologies); Egr-1 (C-19; Santa-Cruz Biotechnology); ERK2 (D-2; Santa Cruz Biotechnology); and transferrin receptor (TfR) (H68.4; Zymed Laboratories Inc., San Francisco, CA). Signal was detected using peroxydase-conjugated secondary antibody followed by development using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

siRNA treatments

siRNAs were purchased from Eurogentec (Seraing, Belgium) and annealing was performed as described (Elbashir *et al.*, 2001). Egr-1 Stealth RNAi was designed by Invitrogen. Transfection of duplex siRNAs (200 nM) was carried out using Oligofectamine (Invitrogen) according to the manufacturer's protocol and resulted in efficiencies of $>90\%$. Cells were transfected three times in 24 hours intervals and were assayed between 92 and 96 hours after the first transfection. The sense strand of the siRNA duplex used to target B-Raf was 5′-AgAAUUggAUCUggAUCAUTT-3′ and has been described previously (Hingorani *et al.*, 2003). The sense strand sequence used to target human fibronectin was 5′-GUggUCCU gUCgAAgUAUU-3′. As control, an siRNA

duplex designed to target firefly luciferase transcript was used (Harborth *et al*., 2001).

Cell invasion assay

The assay was carried out as described previously using the Chemicon cell Invasion Assay kit (Chemicon International, Temecula, CA) (Robert *et al*., 2006). Briefly, 1205Lu cells were transfected with 200 nM of siRNA for 72 hours and serum starved overnight. Cells were added to each well and allowed to migrate for 6 hours at 37°C in 5% CO_2. The lower compartment of the chamber was filled with culture media containing 0.2% BSA. Invading cells on the lower membrane surface were fixed in 1% paraformaldehyde, stained with 0.1% crystal violet, and counted (five random $\times 20$ fields/well).

CONFLICT OF INTEREST

The authors state no conflicts of interest.

ACKNOWLEDGMENTS

We thank Dr M. Deckert for useful discussions and for his help in real-time PCR analyses. We also thank Dr M. Ehrengruber for the NAB2 adenoviral vector, Dr A. Eychene for the B-Raf-CAAX construct, Dr P.J. Stork for the human B-Raf construct, and Dr I.-S. Kim for the fibronectin promoter reporter construct. In addition, we thank Dr M. Herlyn for melanoma cell lines and Dr V. Hearing for the antibody to TRP-1. K. Bille is also thanked for her technical help. This research was funded by Institut National de la Santé et de la Recherche Médicale (INSERM) and the Association pour la Recherche sur le Cancer (ARC), Grant No. 4709 to S.T.D. C.G. is a recipient of a doctoral fellowship from ARC. V.B. was supported by NIH/NCI R01 CA 102688-01.

REFERENCES

Bertolotto C, Abbe P, Hemesath TJ, Bille K, Fisher DE, Ortonne JP *et al.* (1998) Microphthalmia gene product as a signal transducer in cAMP-induced differentiation of melanocytes. *J Cell Biol* 142:827–35

Bittner M, Meltzer P, Chen Y, Jiang Y, Seftor E, Hendrix M *et al.* (2000) Molecular classification of cutaneous malignant melanoma by gene expression profiling. *Nature* 406:536–40

Bogenrieder T, Herlyn M (2002) Cell-surface proteolysis, growth factor activation and intercellular communication in the progression of melanoma. *Crit Rev Oncol Hematol* 44:1–15

Brose MS, Volpe P, Feldman M, Kumar M, Rishi I, Gerrero R *et al.* (2002) BRAF and RAS mutations in human lung cancer and melanoma. *Cancer Res* 62:6997–7000

Calipel A, Lefevre G, Pouponnot C, Mouriaux F, Eychene A, Mascarelli F (2003) Mutation of B-Raf in human choroidal melanoma cells mediates cell proliferation and transformation through the MEK/ERK pathway. *J Biol Chem* 278:42409–18

Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG *et al.* (2000) The transcription factor snail controls epithelial–mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* 2:76–83

Clark EA, Golub TR, Lander ES, Hynes RO (2000) Genomic analysis of metastasis reveals an essential role for *Rho*C. *Nature* 406:532–5

Cohen C, Zavala-Pompa A, Sequeira JH, Shoji M, Sexton DG, Cotsonis G *et al.* (2002) Mitogen-actived protein kinase activation is an early event in melanoma progression. *Clin Cancer Res* 8:3728–33

Cohen DM, Gullans SR, Chin WW (1996) Urea inducibility of egr-1 in murine inner medullary collecting duct cells is mediated by the serum response element and adjacent Ets motifs. *J Biol Chem* 271:12903–8

Conner SR, Scott G, Aplin AE (2003) Adhesion-dependent activation of the ERK1/2 cascade is by-passed in melanoma cells. *J Biol Chem* 278:34548–54

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S *et al.* (2002) Mutations of the BRAF gene in human cancer. *Nature* 417:949–54

Ehrengruber MU, Muhlebach SG, Sohrman S, Leutenegger CM, Lester HA, Davidson N (2000) Modulation of early growth response (EGR) transcription factor-dependent gene expression by using recombinant adenovirus. *Gene* 258:63–9

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411:494–8

Fidler IJ (1996) Critical determinants of melanoma metastasis. *J Investig Dermatol Symp Proc* 1:203–8

Gaggioli C, Deckert M, Robert G, Abbe P, Batoz M, Ehrengruber MU *et al.* (2005) HGF induces fibronectin matrix synthesis in melanoma cells through MAP kinase-dependent signaling pathway and induction of Egr-1. *Oncogene* 24:1423–33

Garnett MJ, Marais R (2004) Guilty as charged: B-RAF is a human oncogene. *Cancer Cell* 6:313–9

Giancotti FG, Ruoslahti E (1999) Integrin signaling. *Science* 285:1028–32

Goding CR (2000) Mitf from neural crest to melanoma: signal transduction and transcription in the melanocyte lineage. *Genes Dev* 14:1712–28

Gradl D, Kuhl M, Wedlich D (1999) The Wnt/Wg signal transducer beta-catenin controls fibronectin expression. *Mol Cell Biol* 19: 5576–5587

Hagemann C, Rapp UR (1999) Isotype-specific functions of Raf kinases. *Exp Cell Res* 253:34–46

Harborth J, Elbashir SM, Bechert K, Tuschl T, Weber K (2001) Identification of essential genes in cultured mammalian cells using small interfering RNAs. *J Cell Sci* 114:4557–65

Herlyn M, Berking C, Li G, Satyamoorthy K (2000) Lessons from melanocyte development for understanding the biological events in naevus and melanoma formation. *Melanoma Res* 10:303–12

Hingorani SR, Jacobetz MA, Robertson GP, Herlyn M, Tuveson DA (2003) Suppression of BRAF (V599E) in human melanoma abrogates transformation. *Cancer Res* 63:5198–202

Hoek K, Rimm DL, Williams KR, Zhao H, Ariyan S, Lin A *et al.* (2004) Expression profiling reveals novel pathways in the transformation of melanocytes to melanomas. *Cancer Res* 64:5270–82

Houghton AN, Polsky D (2002) Focus on melanoma. *Cancer Cell* 2:275–8

Huang Y, Prasad M, Lemon WJ, Hampel H, Wright FA, Kornacker K *et al.* (2001) Gene expression in papillary thyroid carcinoma reveals highly consistent profiles. *Proc Natl Acad Sci USA* 98:15044–9

Humphries MJ, Olden K, Yamada KM (1986) A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. *Science* 233:467–70

Hynes RO, Yamada KM (1982) Fibronectins: multifunctional modular glycoproteins. *J Cell Biol* 95:369–77

Jiang Y, Harlocker SL, Molesh DA, Dillon DC, Stolk JA, Houghton RL *et al.* (2002) Discovery of differentially expressed genes in human breast cancer using subtracted cDNA libraries and cDNA microarrays. *Oncogene* 21:2270–82

Karasarides M, Chiloeches A, Hayward R, Niculescu-Duvaz D, Scanlon I, Friedlos F *et al.* (2004) B-RAF is a therapeutic target in melanoma. *Oncogene* 23:6292–8

Larribere L, Khaled M, Tartare-Deckert S, Busca R, Luciano F, Bille K *et al.* (2004) PI3K mediates protection against TRAIL-induced apoptosis in primary human melanocytes. *Cell Death Differ* 11:1084–91

Lee BH, Kim MS, Rhew JH, Park RW, de Crombrugghe B, Kim IS (2000) Transcriptional regulation of fibronectin gene by phorbol myristate acetate in hepatoma cells: a negative role for NF-kappaB. *J Cell Biochem* 76:437–51

Li G, Satyamoorthy K, Herlyn M (2002) Dynamics of cell interactions and communications during melanoma development. *Crit Rev Oral Biol Med* 13:62–70

Liu C, Yao J, Mercola D, Adamson E (2000) The transcription factor EGR-1 directly transactivates the fibronectin gene and enhances attachment of human glioblastoma cell line U251. *J Biol Chem* 275:20315–23

Peyssonnaux C, Eychene A (2001) The Raf/MEK/ERK pathway: new concepts of activation. *Biol Cell* 93:53–62

Pollock PM, Harper UL, Hansen KS, Yudt LM, Stark M, Robbins CM *et al.* (2003) High frequency of BRAF mutations in nevi. *Nat Genet* 33:19–20

Polsky D, Cordon-Cardo C (2003) Oncogenes in melanoma. *Oncogene* 22:3087–91

Poser I, Dominguez D, de Herreros AG, Varnai A, Buettner R, Bosserhoff AK (2001) Loss of E-cadherin expression in melanoma cells involves up-regulation of the transcriptional repressor Snail. *J Biol Chem* 276:24661–6

Recio JA, Merlino G (2003) Hepatocyte growth factor/scatter factor induces feedback up-regulation of CD44v6 in melanoma cells through Egr-1. *Cancer Res* 63:1576–82

Robert G, Gaggioli C, Bailet O, Chavey C, Abbe P, Aberdam E *et al.* (2006) SPARC represses E-cadherin and induces mesenchymal transition during melanoma development. *Cancer Res* 66:7516–23

Rubinfeld B, Robbins P, El-Gamil M, Albert I, Porfiri E, Polakis P (1997) Stabilization of beta-catenin by genetic defects in melanoma cell lines. *Science* 275:1790–2

Russo MW, Sevetson BR, Milbrandt J (1995) Identification of NAB1, a repressor of NGFI-A- and Krox20-mediated transcription. *Proc Natl Acad Sci USA* 92:6873–7

Satyamoorthy K, Li G, Gerrero MR, Brose MS, Volpe P, Weber BL *et al.* (2003) Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. *Cancer Res* 63:756–9

Sumimoto H, Miyagishi M, Miyoshi H, Yamagata S, Shimizu A, Taira K *et al.* (2004) Inhibition of growth and invasive ability of melanoma by inactivation of mutated BRAF with lentivirus-mediated RNA interference. *Oncogene* 23:6031–9

Svaren J, Sevetson BR, Apel ED, Zimonjic DB, Popescu NC, Milbrandt J (1996) NAB2, a corepressor of NGFI-A (Egr-1) and Krox20, is induced by proliferative and differentiative stimuli. *Mol Cell Biol* 16:3545–53

Tanami H, Imoto I, Hirasawa A, Yuki Y, Sonoda I, Inoue J *et al.* (2004) Involvement of overexpressed wild-type BRAF in the growth of malignant melanoma cell lines. *Oncogene* 23:8796–804

Thiel G, Cibelli G (2002) Regulation of life and death by the zinc finger transcription factor Egr-1. *J Cell Physiol* 193:287–92

Tsavachidou D, Coleman ML, Athanasiadis G, Li S, Licht JD, Olson MF *et al.* (2004) SPRY2 is an inhibitor of the ras/extracellular signal-regulated kinase pathway in melanocytes and melanoma cells with wild-type BRAF but not with the V599E mutant. *Cancer Res* 64: 5556–5559

van Elsas A, Zerp SF, van der Flier S, Kruse KM, Aarnoudse C, Hayward NK *et al.* (1996) Relevance of ultraviolet-induced N-ras oncogene point mutations in development of primary human cutaneous melanoma. *Am J Pathol* 149:883–93

Wellbrock C, Ogilvie L, Hedley D, Karasarides M, Martin J, Niculescu-Duvaz D *et al.* (2004) V599EB-RAF is an oncogene in melanocytes. *Cancer Res* 64:2338–42

Zhang J, Zhi H, Zhou C, Ding F, Luo A, Zhang X *et al.* (2005) Up-regulation of fibronectin in oesophageal squamous cell carcinoma is associated with activation of the Erk pathway. *J Pathol* 207:402–9

Zhang L, Zhou W, Velculescu VE, Kern SE, Hruban RH, Hamilton SR *et al.* (1997) Gene expression profiles in normal and cancer cells. *Science* 276:1268–72