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Elevated expression of MITF counteracts B-RAF–stimulated melanocyte and melanoma cell proliferation

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The protein kinase B-RAF is a human oncogene that is mutated in ~70% of human melanomas and transforms mouse melanocytes. Microphthalmia-associated transcription factor (MITF) is an important melanocyte differentiation and survival factor, but its role in melanoma is unclear. In this study, we show that MITF expression is suppressed by oncogenic B-RAF in immortalized mouse and primary human melanocytes. However, low levels of MITF persist in human melanoma cells harboring oncogenic B-RAF, suggesting that additional mechanisms regulate its expression. MITF reexpression in B-RAF–transformed melanocytes inhibits their proliferation. Furthermore, differentiation-inducing factors that elevate MITF expression in melanoma cells inhibit their proliferation, but when MITF up-regulation is prevented by RNA interference, proliferation is not inhibited. These data suggest that MITF is an antiproliferation factor that is down-regulated by B-RAF signaling and that this is a crucial event for the progression of melanomas that harbor oncogenic B-RAF.

Introduction

Melanocytes are pigmented skin cells that protect us from ultraviolet radiation. The processes regulating melanocyte differentiation are intensely studied because melanocytes are thought to be the precursors of melanoma, a skin cancer whose incidence is increasing in Western societies. A master regulator of melanocyte differentiation is the microphthalmia-associated transcription factor (MITF; Widlund and Fisher, 2003). Strikingly, MITF levels are reduced in spontaneously transformed melanocytes (Selzer et al., 2002), and low MITF expression correlates with poor prognosis in melanoma (Salti et al., 2000). MITF regulation is complex. For example, the differentiation factor α-melanocyte–stimulating hormone strongly increases its expression in a cAMP and cAMP response element binding protein (CREB) transcription factor–dependent manner (Bertolotto et al., 1998). Another signaling module that regulates MITF is the RAS–RAF–MEK–ERK signaling cascade, which acts downstream of the receptor tyrosine kinase cKIT to stimulate MITF phosphorylation on serine 73 (S73) and enhances its transcriptional activity (Hemesath et al., 1998). However, extracellular regulated protein kinase (ERK)–mediated S73 phosphorylation also targets MITF for ubiquitin-dependent degradation through the proteasome pathway (Wu et al., 2000; Xu et al., 2000).

There are three RAS (H-RAS, K-RAS, and N-RAS) and three RAF (A-RAF, B-RAF, and C-RAF) genes in humans. N-RAS is mutated in 5–20% of melanomas, and B-RAF is mutated in 50–70% of melanomas (Davies et al., 2002). The most common mutation in B-RAF (~90%) is a glutamic acid for valine substitution at position 600 (formally identified as V599; Wellbrock et al., 2004a), which produces a highly active kinase that stimulates constitutive ERK signaling and stimulates melanoma cell proliferation and survival (Hingorani et al., 2003; Karasarides et al., 2004).

In this study, we show that V600E-B-RAF triggers MITF degradation in mouse and human melanocytes and that its reexpression inhibits proliferation. Furthermore, MITF up-regulation suppresses melanoma cell proliferation. These data suggest that high MITF levels are antiproliferative, and, therefore, its expression must be suppressed for transformation by oncogenic B-RAF.

Results and discussion

We previously described the generation of mouse melanocyte lines expressing myc-tagged versions of WT-B-RAF (melan-a–B-RAF) or V600EB-RAF (melan-a–V600E [VE]; Wellbrock et al., 2004b). We demonstrated that melanocytes expressing
V600E B-RAF show constitutive ERK signaling and proliferate in a factor-independent manner (Wellbrock et al., 2004b). Importantly, cells expressing high or low levels of WT B-RAF do not have elevated ERK activity or grow in a factor-independent manner, demonstrating that even high levels of WT B-RAF expression are not transforming. Melanocytes expressing V600E B-RAF (clone VE16; Fig. 1A) display dramatically reduced dendricity and pigmentation, which is similar to the morphology that is observed in melanocytes expressing oncogenic RAS (G12V RAS) or constitutively active MAPK and ERK kinase (MEK EE; Fig. 1B). In contrast, clones expressing low or high levels of WT B-RAF (clones B2 and B9), v600E B-RAF–expressing clones VE11, VE14, and VE16, and G12V RAS– or MEK EE–transformed melan-a cells under growing conditions. (C) Western blot analysis of MITF, phosphorylated ERK (ppERK), and ERK2 in melan-a cells, neo control clones, clonies B2 and B9, v600E B-RAF–expressing clones VE11, VE14, and VE16, and G12V RAS– or MEK EE–expressing cells.

MITF expression is lost in B-RAF–transformed melanocytes. (A) Western blot analysis of melan-a cells, a neo control line, v600E B-RAF–expressing clones B2 and B9, and v600E B-RAF–expressing clone VE16 probed for myc-tagged B-RAF, total B-RAF, and ERK2. (B) Bright field image of melan-a cells, neo control clones, B2, B9, and VE16, and G12V RAS– or MEK EE–transformed melan-a cells under growing conditions. (C) Western blot analysis of MITF, phosphorylated ERK (ppERK), and ERK2 in melan-a cells, neo control clones, v600E B-RAF–expressing clones [B2 and B9], v600E B-RAF–expressing clones (VE11, VE14, and VE16) and G12V RAS– or MEK EE–expressing cells.

V600E B-RAF show constitutive ERK signaling and proliferate in a factor-independent manner (Wellbrock et al., 2004b). Importantly, cells expressing high or low levels of WT B-RAF do not have elevated ERK activity or grow in a factor-independent manner, demonstrating that even high levels of WT B-RAF expression are not transforming. Melanocytes expressing V600E B-RAF (clone VE16; Fig. 1A) display dramatically reduced dendricity and pigmentation, which is similar to the morphology that is observed in melanocytes expressing oncogenic RAS (G12V RAS) or constitutively active MAPK and ERK kinase (MEK; MEK EE; Fig. 1B). In contrast, clones expressing low or high levels of WT B-RAF (clones B2 and B9) have a parental phenotype (Fig. 1B). The reduction in pigmentation and denticity that is induced by oncogenic B-RAF prompted us to examine known regulators of melanocyte differentiation. Importantly, we find that MITF is consistently down-regulated in cell lines expressing V600E B-RAF, G12V RAS, and MEK EE, and this loss correlates with constitutive ERK activation (Fig. 1C). Previous studies have shown that ERK phosphorylates S73 of MITF, targeting it for degradation (Wu et al., 2000; Xu et al., 2000), so we analyzed whether this mechanism underlies MITF loss in our cell lines. Transiently expressed HA-tagged MITF localizes to the nucleus of melan-a–VE cells (Fig. 2A). On SDS gels, it migrates as a single band whose mobility is increased when the cells are treated with the MEK inhibitor U0126 (Fig. 2B); these effects were previously attributed to ERK-dependent phosphorylation on S73 (Hemesath et al., 1998). Accordingly, MITF in which S73 is mutated to alanine (S73A MITF) comigrates with MITF in U0126-treated cells (Fig. 2B). In melan-a–VE lines, ectopic MITF is expressed at low levels, but these increase when the cells are treated with the proteasome inhibitor MG132 (Fig. 2C). This suggests that MITF is degraded by the ubiquitin pathway after S73 phosphorylation by ERK. However, when S73A MITF is expressed, it also fails to accumulate unless the cells are treated with MG132 (Fig. 2C, control), suggesting that additional mecha-
nisms regulate MITF expression. RT-PCR analysis revealed that MITF mRNA levels are significantly lower in melan-a–VE cells than in parental or melan-a–B-RAF cells (Fig. 2 D), but the cAMP-elevating agent forskolin still induces MITF expression (Fig. 2 E). Importantly, in our cells, MITF down-regulation did not occur by promoter silencing because it was still induced by cAMP.

To examine the biological consequences of MITF regulation by B-RAF, we reexpressed MITF in melan-a–VE cells. This caused a significant (73–84%) reduction in the number of colonies that were formed by these cells (Fig. 3 A). To clarify whether this effect was caused by inhibition of proliferation or induction of apoptosis, we developed melan-a–VE cell lines expressing an estrogen receptor (ER) version of MITF (ER-MITF; Carreira et al., 2005) that can be regulated by 4-hydroxytamoxifen (4-OHT) and developed a control cell line expressing only the ER fragment. Both proteins are expressed at similar levels (Fig. 3 B). ER-MITF activates the tyrosinase promoter in a 4-OHT–dependent manner, whereas the ER fragment does not (Fig. 3 C), demonstrating that ER-MITF is functional. Critically, ER-MITF activation does not induce apoptosis in melan-a–VE but significantly impairs its proliferation (38% reduction, P = 0.0116; Fig. 3 D).

Because V600E-B-RAF mutations occur in 50–70% of human melanomas (Davies et al., 2002), we examined whether our mouse cell studies were relevant to human melanocytes. First, we analyzed the RAF–MEK–ERK pathway in primary normal human melanocytes (NHMs). ERK inhibition by U0126 (Fig. 4 A) blocks DNA synthesis (Fig. 4 B), demonstrating that ERK signaling is essential for NHM proliferation, so we examined the contribution of individual RAF isoforms by RNA interference (RNAi). Depletion of A-RAF from these cells did not affect basal ERK activity (Fig. 4 C) or DNA synthesis (Fig. 4 D), whereas depletion of B-RAF or C-RAF suppresses ERK activity (Fig. 4 C) and significantly inhibits DNA synthesis (Fig. 4 D). Thus, A-RAF is not required for ERK-dependent melanocyte proliferation, whereas B- and C-RAF are both required. This contrasts with observations in melanoma cells harboring V600E-B-RAF in which only B-RAF is required for ERK activation but all three RAF kinases are required for proliferation (Karasarides et al., 2004). The observation that B- and C-RAF both contribute to ERK signaling and proliferation in NHM can be explained by the fact that in melanocytes, these isoforms stimulate nonredundant growth signals (Wellbrock et al., 2004a).

We have demonstrated that MEK–ERK signaling is essential for NHM proliferation and that in melanoma cells, this pathway is constitutively activated by V600E-B-RAF. Importantly, V600E-B-RAF also induces constitutive ERK activation in NHM (Fig. 4 E), and MITF protein levels are suppressed in ~92% of cells expressing V600E-B-RAF (Fig. 4, F and G). WT-B-RAF does not significantly affect ERK (Fig. 4 E) or MITF protein levels in NHM (Fig. 4, F and G).

Thus, MITF protein levels are significantly reduced in melanocytes in which B-RAF–ERK signaling is elevated. However, MITF is present in most melanoma cell lines expressing oncogenic B-RAF or RAS, albeit generally at reduced proportion.
levels compared with NHM (Fig. 5 A). Our data suggests that MITF is antiproliferative and that one function of oncogenic B-RAF is to suppress its expression to overcome its growth-inhibitory activity. This model is supported by our observation that MITF expression is reduced in NHM expressing V600E B-RAF (Fig. 4, F and G) and the finding that forskolin, which up-regulates MITF (Fig. 5 B), also inhibits DNA synthesis in these cells (Fig. 5 C). Forskolin also up-regulates MITF expression in Colo829 and WM266-4 cells (Fig. 5 D, lanes 1, 2, 5, and 6) and in two melanoma cell lines that express oncogenic B-RAF (Davies et al., 2002), and this is accompanied by reduced proliferation (Fig. 5 E). Importantly, when RNAi is used to prevent MITF up-regulation (Fig. 5 D, lanes 4 and 8), forskolin does not inhibit proliferation of Colo829 and WM266-4 cells (Fig. 5 E), clearly demonstrating that elevated MITF protein levels are growth inhibitory to melanoma cells.

Our data suggest that high levels of MITF hinder cell cycle progression, and it has been suggested that MITF-induced cell cycle exit is necessary for melanocyte differentiation (Loercher et al., 2005). Consistent with this, melanogenesis-inducing factors such as α-melanocyte-stimulating hormone and forskolin, which up-regulate MITF expression (Bertolotto et al., 1998), also block melanoma cell proliferation. Moreover, MITF regulates transcription of the cell cycle inhibitors p16^ink4a and p21^cip1 (Carreira et al., 2005; Loercher et al., 2005). However, Colo829 and WM266-4 cells are p16^ink4a negative (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200505059/DC1), and we did not observe increased p21 expression in response to forskolin (not depicted), sug-
gesting that MITF inhibits growth through alternative mechanisms in these cells.

The antiproliferative effects of high MITF levels in human melanoma is supported by the fact that MITF mRNA expression is frequently low or undetectable in human melanoma cells (Vachet et al., 2001). Importantly, MITF reexpression in transformed MITF-negative human melanocytes and melanoma cells reduces their tumorigenicity in vivo (Selzer et al., 2002), suggesting that elevated MITF is incompatible with melanoma progression. Notably, low MITF levels are linked to reduced survival rates and increased metastases in patients with intermediate thickness melanoma (Salti et al., 2000). Furthermore, MITF target genes such as melan-a/MART-1 or melanostatin/TRPM1 are generally down-regulated in more advanced melanomas (Duncan et al., 1998; Hofbauer et al., 1998), which is consistent with MITF not being expressed or being nonfunctional. Our data have clear clinical implications, suggesting that MITF has important prognostic value in melanoma, particularly if used in conjunction with B-RAF mutation status, which is an area that needs urgent investigation.

Previous studies have suggested that MITF protein levels are regulated by ERK-induced degradation (Wu et al., 2000; Xu et al., 2000) and, in agreement with this, we observe that MITF expression is significantly reduced in melanocytes in which ERK is constitutively active as a result of oncogenic B-RAF expression. However, we note that in melanoma cell lines and clinical samples of melanoma, MITF expression is not completely suppressed. We propose that MITF function cannot be completely abolished in melanoma and that low level expression must be maintained to stimulate survival and/or proliferation, possibly by regulating BCL2 (McGill et al., 2002) and CDK2 (Du et al., 2004) expression. Presumably, mechanisms exist to counteract the suppression of MITF expression by oncogenic B-RAF such as maintaining its expression at a level that is compatible with tumor progression but insufficient to suppress cell growth. We are currently developing genetic approaches to test this hypothesis. Our data demonstrate that MITF expression is carefully regulated in melanocytes and melanoma cells and that the regulation of its expression by oncogenic B-RAF warrants further study.

Materials and methods

Cell culture and transfection

Melan-a cells expressing B-RAF, V600E-B-RAF, MEKEE, and G12VRAS were described previously (Wellbrock et al., 2004b). Melan-a cells (gift of D. Bennett, St. George’s Hospital Medical School, London, UK) and melan-a-B-RAF cells were grown in RPMI/10% FCS supplemented with 200 nM TPA and 120 μM cholaerotoxin. Melan-a-VE and melan-a-VE-derived cells were cultured in RPMI/10% FCS. Melan-a-VE/ER-MITF cells expressed MITF that was fused to the ligand-binding domain of the ER (ER-MITF; gift from C. Goding, St. George’s Hospital Medical School, London, UK) and melan-a–B-RAF cells were grown in RPMI/10% FCS supplemented with 200 nM TPA and 120 μM cholaerotoxin. Melan-a-VE and melan-a-VE-derived cells were cultured in RPMI/10% FCS. Melan-a-VE/ER-MITF cells expressed MITF that was fused to the ligand-binding domain of the ER (ER-MITF; gift from C. Goding, St. George’s Hospital Medical School, London, UK) and melan-a–B-RAF cells were grown in RPMI/10% FCS supplemented with 200 nM TPA and 120 μM cholaerotoxin.

Cell lysis, Western blotting, and antibodies

Western blot analysis was performed with standard protocols with the following antibodies: A-RAF (C-20; Santa Cruz Biotechnology, Inc.), B-RAF (F-7; Santa Cruz Biotechnology, Inc.), CRAF (Transduction Laboratories), phosphorylated ERK (ppERK; MAPK7; Sigma-Aldrich), myc tag (9E10), HA tag (12CA5), ERK2 (C-14; Santa Cruz Biotechnology, Inc.), MITF (C5 and D5; Neomarkers and provided by D. Fisher, Dana-Farber Cancer Institute, Boston, MA), rabbit anti-myc (Abcam), and phosphorylated CREB (Cell Signaling).

RT-PCR

RNA was isolated using TRIzol (GIBCO BRL), and first-strand cDNA synthesis was performed with 1 μg of total RNA and random hexanucleotides. Specific genes were amplified under conditions in which amplification was still linear. Primers that were used are listed as follows: gapdh (5′-CCGGATCAACCGGATTTGGTCGTAT-3′ and 5′-AGCCTTCTCCATGGTGGA-3′) and mitf (5′-ATGCATTGAATGCTGAAAGAC-3′) and melanocyte-specific mitf (5′-ATGCATTGAATGCTGAAAGAC-3′ and 5′-GGTGCATTGAATGCTGAAAGAC-3′).

Immunofluorescence

Cells that were grown on glass coverslips were fixed in methanol/acetone, blocked with 1% BSA/PBS, and incubated with rabbit anti-myc (Abcam) and/or anti-MITF (clones C5 and D5; Neomarkers). Staining was revealed by secondary antibodies that were conjugated to Cy2 or Cy3 (Dianova), and nuclei were counterstained with DAPI. Cells were mounted in DABCO-glycerol.

Microscopy and image analysis

Fluorescence images were acquired at RT with a 40×/0.55 long working distance objective lens on a microscope (model TS-100; Nikon) with fluorescence optics. Bright field images were acquired at a 20×/0.4 long working distance objective lens on the same microscope. A camera (model DN-100; Nikon) was used for image processing using Nikon acquisition software.

Online supplemental material

Fig. S1 shows Western blot analysis of p16INK4a in melanoma cell lines SKMel2, A375, WM266-4, Colo205, and SKMel28 and in NHM, ERK2 serves as a loading control. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200505059/DC1.

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Thymidine incorporation, long-term growth, and colony formation assay

Cells were incubated with 0.4 μCi/ml [3H]-thymidine for 4 h before harvesting and were quantified by liquid scintillation counting. For long-term growth, melan-a-VE/ER and melan-a-VE/ER-MITF cells were seeded at 0.5 × 104 cells per 10-cm dish in 5% FCS (+/−4-OHT), counted every 3 d, and replated at 0.5 × 104 cells per dish. For clonogenic survival, cells were transfected with pCDNA3.1/V5-His-MITF (provided by H. Arnheiter, Porter Neuroscience Research Center, Bethesda, MD) or pCDNA3.1/V5-His plus 1:10 of pCDNA3.1/Hygro and were selected in Hygromycin for 5 d. After a further 10 d without Hygromycin, colonies were stained with crystal violet.

Cell lysis, Western blotting, and antibodies

Western blot analysis was performed with standard protocols with the following antibodies: ARAF (C-20; Santa Cruz Biotechnology, Inc.), B-RAF (F-7; Santa Cruz Biotechnology, Inc.), CRAF (Transduction Laboratories), phosphorylated ERK (ppERK; MAPK7; Sigma-Aldrich), myc tag (9E10), HA tag (12CA5), ERK2 (C-14; Santa Cruz Biotechnology, Inc.), MITF (C5 and D5; Neomarkers and provided by D. Fisher, Dana-Farber Cancer Institute, Boston, MA), rabbit anti-myc (Abcam), and phosphorylated CREB (Cell Signaling).

RNAi

Cells were transfected as described previously (Wellbrock et al., 2004b) using 20–120 nM A-RAF, C-RAF, scrambled (Karasarides et al., 2004), B-RAF (5′-AACAGUCUCAAGGGGAAGUG-3′), or melanocyte-specific MITF (5′-AGCAGTACGTTCTTACCC-3′) siRNA oligonucleotides.

Luciferase assays

2.5 × 105 cells were transfected with 0.6 μg pGL2 or pGL2hytr (Hemesath et al., 1998) with 0.4 μg pSV-β-galactosidase (Promega) using LipofectAMINE (GIBCO BRL) and were treated with 4-OHT after 24 h for a further 24 h before lysis in reporter lysis buffer (Promega) for luciferase activity analysis. Experiments were performed in triplicate, and luciferase activity was corrected for β-galactosidase expression.

Materials and methods

Cell culture and transfection

Melan-a cells expressing B-RAF, V600EB-RAF, MEKEE, and G12VRAS were described previously (Wellbrock et al., 2004b). Melan-a cells (gift of D. Bennett, St. George’s Hospital Medical School, London, UK) and melan-a–B-RAF cells were grown in RPMI/10% FCS supplemented with 200 nM TPA and 120 μM cholaerotoxin. Melan-a-VE and melan-a-VE-derived cells were cultured in RPMI/10% FCS. Melan-a-VE/ER-MITF cells expressed MITF that was fused to the ligand-binding domain of the ER (ER-MITF; gift from C. Goding, Marie Curie Research Institute, Oxted, UK; Carreira et al., 2005), and melan-a–B-RAF cells expressed only the ER fragment. They were created by transfecting melan-a–VE11 cells with pRKSHA.ER or pRKSHA.ER-MITF and 1:10 of pCDNA3.1/Hygro and were selected in 0.5 mg/ml Hygromycin for 1 wk. pRKS5ER and pRKS5ER-MITF were generated by cloning HA.ER and HA.ER-MITF as EcoRI fragments from pBABEpuroHA.ER and pBABEpuroHA.ER-MITF (Carreira et al., 2005) into pRK5. Human melanoma cell lines were grown in DMEM/10% FCS. NHM were cultured in medium 154 with HXG52 (Cascade Biologics, Inc.) and transfected with 5 μg DNA using a Nucleofector according to the manufacturer’s protocols (Amasya).
References


