Cancer Science
The official journal of the Japanese Cancer Association

Simultaneous suppression of MITF and BRAF^{V600E} enhanced inhibition of melanoma cell proliferation

Kenji Kido,[1,2] Hidetoshi Sumimoto,[1,4] Sakiyo Asada,[1] Starlyn M. Okada,[1] Tomonori Yaguchi,[1] Naoshi Kawamura,[1] Makoto Miyagishi,[3] Toshiaki Saida[2] and Yutaka Kawakami[1,4]

[1]Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, Shinjuku-ku, Tokyo; [2]Department of Dermatology, Shinshu University School of Medicine, Matsumoto City, Nagano; [3]Department of Gene Diagnostics and Therapeutics, Research Institute, International Medical Center of Japan, Shinjuku-ku, Tokyo, Japan

(Received April 28, 2009/Revised June 19, 2009/Accepted June 19, 2009/Online publication July 30, 2009)

Microphthalmia-associated transcription factor (*MITF*) is a master gene regulating differentiation of melanocytes, and a lineage survival oncogene mediating pro-proliferative function in malignant melanoma. However, high expression of MITF also has an anti-proliferative effect. To clarify the therapeutic implication of MITF as a molecular target for human melanoma, we evaluated the role of MITF in cell proliferation in a panel of human melanoma cell lines which express different levels of MITF. We found that both MITF depletion and forced expression of MITF significantly inhibited proliferation, suggesting that endogenous MITF is regulated at an appropriate level for melanoma cell proliferation, and could be a molecular target for melanoma. However, half of the melanoma cell lines in this study were relatively resistant to MITF depletion, indicating other treatment strategies are required for therapy. Our microarray analysis indicated that regulation of several cell growth–associated molecules may be independent of MITF and dependent on BRAF^{V600E}. Thus to enhance the anti-proliferative effect of MITF down-regulation, we combined shRNA-mediated *MITF* depletion with *BRAF^{V600E}* inactivation, another known molecular target for melanoma. Indeed, simultaneous depletion of both *MITF* and *BRAF^{V600E}* significantly inhibited melanoma growth even for the melanoma cell lines resistant to MITF depletion. These results suggest MITF may be an important molecular target for human melanoma and simultaneous inhibition of MITF and MAPK signaling may be an attractive strategy for melanoma treatment. (*Cancer Sci* 2009; 100: 1863–1869)

Microphthalmia-associated transcription factor (MITF) is a basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factor which is essential for the differentiation, proliferation, and survival of pigment cells including melanocytes.[1–3] MITF gene amplification was reported in 10–21% of melanoma patients and has been associated with poor prognosis.[4] Introduction of dominant-negative MITF into human melanoma cell lines inhibited cell proliferation and enhanced sensitivity to chemotherapeutic agents *in vitro*, indicating that MITF is a human melanoma lineage survival oncogene.[4] Although MITF has been shown to be an effective therapeutic target in the B16 murine melanoma model,[5] increased MITF expression inhibits cell growth in human melanocytes and melanoma cells.[6] In mouse fibroblasts and human melanocytes, MITF inhibits cell proliferation by inducing p16^{INK4A} and/or p21^{Cip1} mRNA expression.[7,8] These results suggest that MITF has dual functionality in melanocyte and melanoma proliferation. However, the functional role and therapeutic implication of endogenous MITF has remained to be defined in multiple human melanoma cell lines expressing different levels of MITF.

In this study, we have found that endogenous MITF acts as a pro-proliferative factor in a panel of eight human melanoma cell lines regardless of their expression levels. Although MITF over-expression was found in only 10–21% of melanoma cases,[4] melanoma cells with low MITF expression levels were also sensitive to MITF depletion, indicating that MITF could be a potential therapeutic target for most melanoma. Interestingly, half of the melanoma cell lines in this study were found to be relatively resistant to MITF depletion. However, we demonstrated that the combined inhibition of *MITF* and *BRAF^{V600E}* enhanced melanoma growth suppression even for melanoma cells relatively resistant to *MITF* shRNA alone. Therefore, we have clarified that MITF is an attractive target for melanoma treatment, and propose combined targeting against BRAF and MITF for effective melanoma treatment.

Materials and Methods

Cell lines. Melanoma cell lines 397mel, 501mel, 526mel, 624mel, 888mel, 1363mel, SKmel23, and WM266 were kindly provided by Dr S.A. Rosenberg (Surgery Branch, National Cancer Institute, Bethesda, MD, USA). A375 and MeWo were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). 501mel, SKmel23, and WM266 do not contain the mutated *BRAF^{V600E}*. 397mel, 526mel, 624mel, 888mel, and 1363mel are heterozygous for *BRAF^{V600E}*. A375 is homozygous. *BRAF^{V600E}* mutation status for MeWo was unknown. The copy number of the *MITF* increased by 1.5- to 4.5-fold in melanoma cell lines compared to melanocytes, but the copy number did not show clear correlation to the mRNA or protein expression of MITF (data not shown). These cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Human primary melanocytes were purchased from Cascade Biologics (Portland, OR, USA) and maintained in Medium254 with Human melanocell growth set (HMGS) (Cascade Biologics).

HIV vectors. The HIV vectors expressing various shRNAs were constructed from the HIV-U6i-GFP plasmid, which was described previously.[9–11] Briefly, HIV-U6i-GFP is a self-inactivating vector lacking its own promoter sequence in the 3′-LTR, and has two expression units; one is an shRNA expression cassette, from which an shRNA is transcribed under the control of a human U6 promoter, and the second is a GFP expression unit, from which GFP expression is driven by a CMV promoter. For shRNA expression, complementary oligonucleotides for target sequences were annealed *in vitro* and were inserted into the two BspMI sites downstream of the human U6 promoter. The shRNA target sequences were selected: for MITF, (MITF#2) GATCCAAACTGGAAGACAT and (MITF#4) GACCTAATAA CCTGTACAACAA; for mutated BRAF^{V600E}, (BRAF^{V600E}) GCTACAGAGAAATCTCGAT; for firefly luciferase (control

[4]To whom correspondence should be addressed.
E-mail: yutakawa@sc.itc.keio.ac.jp, sumimoto@sc.itc.keio.ac.jp

doi: 10.1111/j.1349-7006.2009.01266.x

shRNA, GL3B) GTGCGCTGCTGGTGCCAAC. An oligonucleotide containing a 5′-target sense sequence, loop segment (ACGTGTGCTGTCCGT), the target antisense sequence and the stop sequence (TTTTT)-3′, and its complementary oligonucleotide, were synthesized and annealed. The annealed double-stranded oligonucleotides with 5′-protruding ends complementary to the two BspMI sites in the HIV-U6i-GFP plasmid were then subcloned into the HIV-U6i-GFP.

For the construction of an HIV-MITF/BRAF^{V600E} vector, an XbaI-NheI fragment from the HIV-U6-BRAF#1′-GFP plasmid, containing the entire shRNA expression unit for the mutated BRAF^{V600E}, was subcloned into the NheI site of the HIV-U6-MITF#4 plasmid, in which the complementary ends between the 5′-NheI of HIV-U6-MITF#4 and the 5′-XbaI of the U6-BRAF#1′, and between the two 3′-NheI sites were ligated, creating dually expressed shRNA expression cassettes.

The third generation HIV vectors were produced as previously described.[9–11] Briefly, 293T cells were transfected with HIV plasmid vectors, pCAG-HIVgp (3rd generation packaging plasmid) and pCMV-VSV-G-RSV-Rev (VSV-G env and Rev expression plasmid) by a calcium phosphate transfection method. The culture supernatant was collected and used as the virus stock after concentration. The viral titer was calculated by evaluating the GFP expression in infected 293T cells.

***In vitro* growth inhibition assay.** A total of eight melanoma cell lines (397mel, 526mel, 624mel, 888mel, 1363mel, SKmel23, WM266, A375) (50 000 cells) and human primary melanocytes (5000 cells) were infected with shRNA HIV lentiviral vectors against MITF (MITF#2 or #4), BRAF^{V600E} (BRAF^{V600E}), both MITF and BRAF^{V600E} (MITF/BRAF^{V600E}), or firefly luciferase (GL3B) at 50 or 100 MOI. Cell numbers were determined on days 3 and 6 in triplicates by Trypan-blue dye exclusion method.

Forced expression of MITF-M. MITF-M cDNA was amplified by RT-PCR. PCR condition was as follows: MITF-M forward primer, 5′-GTACGCTAGCATGCTGGAAATGCTAGAATATAA-3′; MITF-M reverse primer, 5′-GTACGAATTCCTAACAAGTGTGCT CCGTCT-3′; initially denatured at 94°C for 2 min, followed by 28 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min 20 s, followed by final extension at 72°C for 7 min. The PCR product was subcloned into a pGEM-T easy vector (Promega, Madison, WI, USA), and after confirming the sequence, the PCR product was cloned into the NheI and EcoRI sites in the multicloning site of the HIV-IRES-puro plasmid, which expresses a puromycin resistance gene under the control of the internal ribosomal entry site (IRES). The HIV-MITF-M-puro vector was produced as described in 'HIV vectors'. Melanoma cells were infected with HIV-MITF-M-puro, and cell lines overexpressing MITF were established by puromycin selection.

Western blot analysis. Cell lysates were prepared with lysis buffer (20 mM Tris-HCl [pH 7.5], 12.5 mM β-glycerophosphate, 2 mM EGTA, 10 mM NaF, 1 mM benzamide, 1% NP-40, protease inhibition cocktail [complete, EDTA-free; Roche, Indianapolis, IN, USA], and 1 mM Na_3VO_4). Protein concentration was determined with the DC protein assay kit (Bio-Rad, Hercules, CA, USA). Primary antibodies used for analysis included the following: anti-MITF (Abcam, Cambridge, UK), a monoclonal Ab against a synthetic human MITF peptide able to detect both melanocytic and non-melanocytic MITF isoforms, anti-p21^{Cip1} (BD Pharmingen, San Jose, CA, USA), anti-p27^{Kip1} (BD Transduction, Lexington, KY, USA), anti-cylin-dependent kinase 2 (CDK2) (Upstate Biotechnology, currently Millipore, Billerica, MA, USA), anti-ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ppERK 1/2 (Cell Signaling Technology, Danvers, MA, USA), anti-BRAF (Santa Cruz Biotechnology), and anti-actin (Sigma, St. Louis, MO, USA). An HRP-conjugated anti-IgG antibody was used for the secondary antibody, and the reaction was detected with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Rockford, IL, USA).

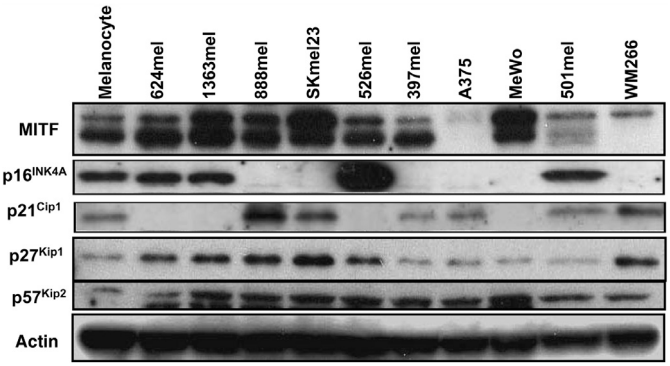

Fig. 1. Expression of microphthalmia-associated transcription factor (MITF) and cylin-dependent kinase (CDK) inhibitors in melanocyte and melanoma cell lines. The western blot shows varying degrees of MITF protein expression in melanocytes and melanoma cell lines. The upper and lower band indicates phosphorylated and unphosphorylated MITF, respectively. While MITF can be easily observed in melanocytes and most melanomas, it was only detected in low levels in A375 and WM266. p16^{INK4A} and p21^{Cip1} were expressed in melanocytes as well as in 4/10 and 6/10 of the melanoma cell lines, respectively. p27^{Kip1} and p57^{Kip2} were expressed at differing levels in all of the cell lines tested. In the bottom row, actin was used as a loading control.

DNA microarray analysis. Total RNA was extracted from 1363mel, 526mel, and 624mel cells after infection with one of the three shRNA HIV vectors (GL3B, MITF#4, or BRAF^{V600E}), respectively. Cy3-labeled cRNA was generated for each RNA sample using the Low RNA Input Linear Amplification Kit (Agilent Technologies, Palo Alto, CA, USA). The labeled cRNA was hybridized to the Whole Human Genome (4×44K) by using the Gene Expression Hybridization Kit and Hybridization Oven (Agilent). The hybridized spot was scanned using Agilent Microarray Scanner (Agilent) and extracted using Feature Extraction (Agilent). The data was analyzed using Gene Spring 7.3.1 (Agilent).

Statistical analysis. All statistical analysis was performed with the unpaired Student's *t*-test. *P*-values < 0.05 were considered significant.

Results

Different sensitivity of human melanoma cells for their growth inhibition by MITF depletion. The functional significance of endogenous MITF in the proliferation of human melanoma cells is not clear. It was reported that depletion of MITF with siRNA resulted in G1 cell cycle arrest in 501mel cells.[12] However, it is important to evaluate MITF functions as a therapeutic target by using various melanoma cell lines with different levels of endogenous MITF and other MITF-related cell cycle regulators, because MITF was reported to have dual functionality in cell proliferation and its expression varies among melanoma cells. To clarify the effect of MITF on melanoma proliferation, we down-regulated the endogenous MITF in eight human melanoma cell lines and primary cultured human melanocytes with different MITF levels, and different p16^{INK4A} or p21^{Cip1} expression (Fig. 1) using two MITF shRNA (#2 and #4). The effect of MITF depletion could not be evaluated in MeWo and 501mel (Fig. 1) due to the high toxicity of the shRNA viruses.

The two MITF shRNA viruses significantly inhibited *in vitro* cell growth in all of the evaluated melanoma cell lines and the melanocytes regardless of the status of CDK inhibitors or MITF levels ($P < 0.001$) (Figs 1 and 2a). MITF depletion induced G1 cell cycle arrest in all the cell lines as reported (data not

doi: 10.1111/j.1349-7006.2009.01266.x

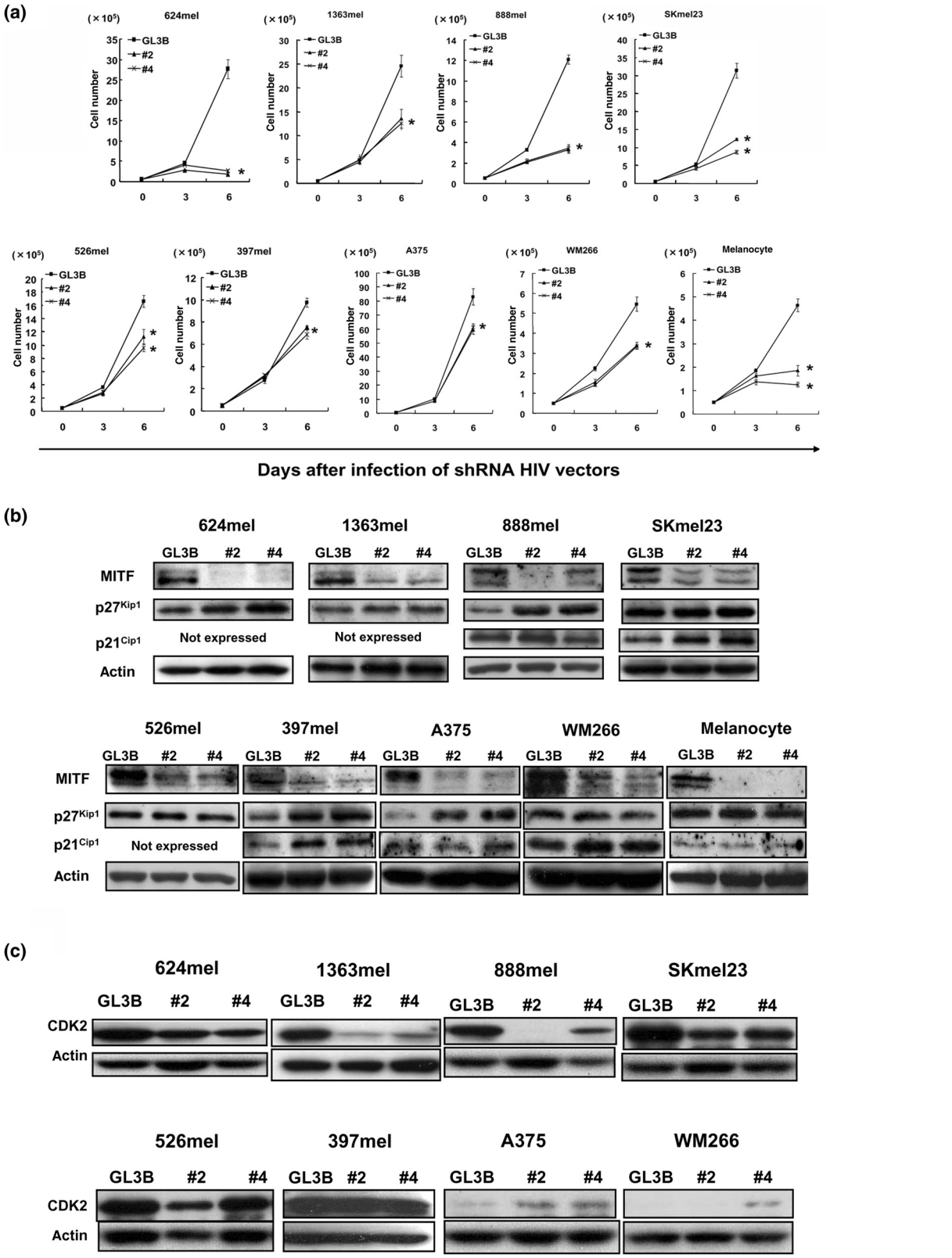

Fig. 2. Inhibition of *in vitro* cell growth induced by MITF shRNA. (a) *In vitro* cell growth of primary cultured melanocytes and the eight melanoma cell lines was evaluated as described in 'Materials and Methods'. MITF shRNA significantly suppressed *in vitro* cell growth in all of the cell lines (*$P < 0.001$). The vertical bars indicate the SD of the assays completed in triplicates. These line graphs are representative of the growth inhibition after MITF shRNA treatment of all of the cells evaluated from two to three independent experiments with similar results. (b) Western blot analysis showed that MITF depletion was associated with increased $p27^{Kip1}$ protein levels in 624mel, 1363mel, 888mel, 397mel, and A375, and also with increased $p21^{Cip1}$ protein levels in SKmel23, 397mel, and WM266. Actin was used as a loading control. The levels of GFP expression after MITF shRNA transfections were equivalent among the shRNA groups for each treated cell line (data not shown). (c) Western blot showed that MITF depletion was associated with decreased CDK2 protein levels in 624mel, 1363mel, 888mel, and SKmel23.

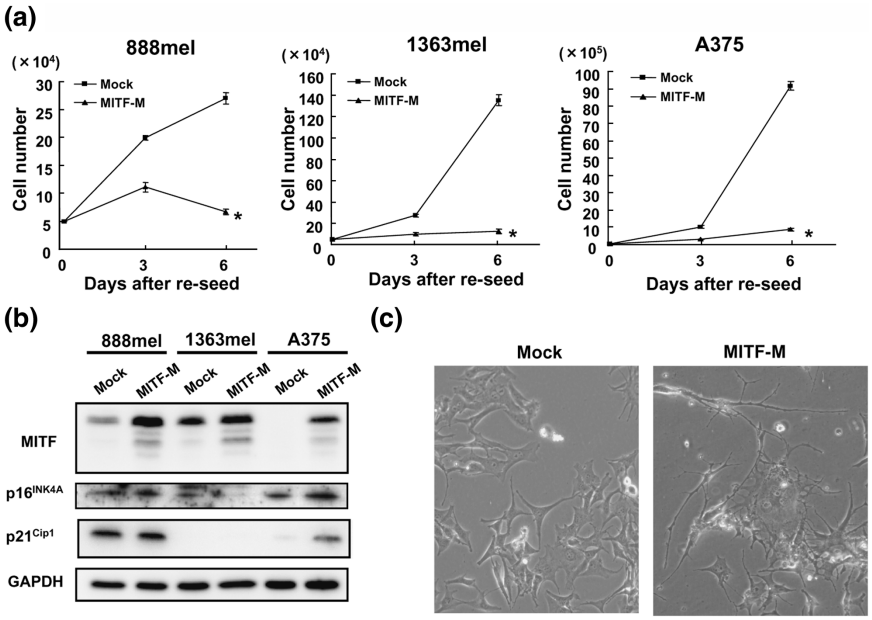

Fig. 3. Inhibition of *in vitro* cell growth and morphological changes induced by over-expression of MITF-M. (a) Cell proliferation was inhibited by MITF-M over-expression. Stably transfected MITF-M or mock 888mel, 1363mel, and A375 melanoma cells were harvested and re-seeded at 5×10^4 cells, and proliferation was compared at days 3 and 6. *In vitro* cell growth was significantly suppressed by the increased expression of MITF-M in all three cell lines (*$P < 0.00001$). The vertical bars indicate the SD of the assays completed in triplicates. (b) Western blot showing MITF, $p16^{INK4A}$, and $p21^{Cip1}$ expression in all the MITF-M transfectants. $p16^{INK4A}$ was slightly increased in 888mel and A375 cells, but decreased in 1363mel cells. $p21^{Cip1}$ expression levels did not change in 888mel cells, were absent in 1363mel, and increased in A375 cells. GAPDH was used as a loading control. (c) Representative image of typical morphological changes after increasing MITF-M in melanoma cells. Over-expression of MITF-M in melanoma cells promotes melanocyte-like dendritic morphology. Images are magnified ×400 and representative of two independent experiments with similar results.

shown).[12] 624mel showed a slightly increased sub-G1 population upon MITF depletion (#2, 4, 7.2–7.6% *vs* control, 1.4%) along with cleavage of caspase-3 and caspase-9 (data not shown), indicating apoptosis. However MITF depletion did not induce apoptosis in the other cell lines, suggesting that apoptosis is not the main mechanism for inhibiting cell proliferation upon MITF depletion in melanoma cells.

MITF depletion increased $p27^{Kip1}$ levels in five melanoma cell lines, 624mel, 1363mel, 888mel, 397mel, and A375; and also slightly increased $p21^{Cip1}$ in three melanoma cell lines, SKmel23, 397mel, and WM266 (Fig. 2b). Some of these observations are consistent with the prior report that MITF-mediated Dia1 expression subsequently increases Skp2 which leads to the ubiquitination and proteasomal degradation of $p27^{Kip1}$.[12] Since Skp2 also ubiquitinates $p21^{Cip1}$,[13] the increased $p21^{Cip1}$ after MITF down-regulation may also occur by the same mechanism. Although MITF directly regulates $p21^{Cip1}$ transcription,[7] we did not observe any significant decrease of $p21^{Cip1}$ protein in all the cell lines upon MITF depletion, contrary to the previous study with 501mel.[12] The balance between the protein stability and transcriptional activity may determine the $p21^{Cip1}$ protein levels.

MITF depletion also decreased CDK2 protein in four melanoma cell lines, 624mel, 1363mel, 888mel, and SKmel23, (Fig. 2c) which was consistent with a previous report showing that MITF enhances melanoma growth by up-regulation of CDK2 transcription.[14] However, CDK2 may not be regulated by MITF in all melanoma cells since MITF depletion did not decrease CDK2 in the other four melanoma cell lines, 526mel, 397mel, A375, and WM266 (Fig. 2c). In our preliminary experiments, RNAi targeting CDK2 in 526mel cells modestly decreased *in vitro* cell growth (data not shown). However, no additive or synergistic decrease of cell proliferation was observed with simultaneous depletion of both MITF and CDK2 (data not shown), suggesting that CDK2 may have a minor contribution in regulation of cell proliferation in the MITF-resistant melanoma cells.

These results suggest that endogenous MITF promotes cell proliferation via suppression of $p27^{Kip1}$ or $p21^{Cip1}$ and/or increased CDK2 in melanoma cells; however, the dominant regulatory mechanism by MITF differs according to the melanoma cell line. Interestingly, the inhibition of melanoma cell growth by down-regulating MITF appears to be positively correlated with the decrease of CDK2, suggesting that a possible determinant for the melanoma sensitivity to the MITF depletion is a MITF–CDK2 dependency. Although 624mel showed only a modest decrease of CDK2 (Fig. 2a), 624mel sensitivity to MITF depletion may be due to its apoptosis-prone nature. Overall, the effect of MITF depletion on melanoma cell growth varied depending on the cell line. Reduction of MITF did not dramatically suppress proliferation in some melanoma cell lines including 526mel, 397mel, A375, and WM266, even though MITF was depleted by shRNAs equally well in these cell lines (Fig. 2b). Therefore, additional therapeutic strategies are required for such resistant melanoma cells.

Endogenous MITF level required for cell proliferation differs among human melanoma cell lines. Regardless of different endogenous level, MITF still exhibits a pro-proliferative function in the melanoma cell lines. We noticed a possible correlation between the MITF protein level (Fig. 1) and its sensitivity to MITF depletion for cell proliferation (Fig. 2a). We then evaluated whether increased MITF levels in the melanoma cells with low endogenous MITF level enhance melanoma cell proliferation. However, over-expression of MITF in A375 cells with very low endogenous MITF (Fig. 3b) significantly inhibited proliferation ($P < 0.00001$, Fig. 3a), indicating that A375 cell proliferation is regulated by its low MITF level. Similarly, increasing MITF in 888mel and 1363mel, which express more endogenous MITF than A375 (Fig. 1) and were sensitive to MITF depletion (Fig. 2a), also significantly inhibited cell proliferation ($P < 0.00001$, Fig. 3a), confirming that melanoma proliferation is influenced by cell line–specific MITF levels. Over-expression of MITF was reported to suppress cell cycle via induction of $p21^{Cip1}$ or $p16^{INK4A}$ in mouse fibroblasts and human melanocytes.[7,8] In A375 cells with increased MITF, more $p21^{Cip1}$ was detected, and greater levels of $p16^{INK4A}$ were observed in 888mel and A375 after increasing MITF expression. (Fig. 3b) However these molecules did not increase in MITF-M-transfected 1363mel cells, suggesting that molecular mechanisms other than increased $p16^{INK4A}$ or $p21^{Cip1}$ may be involved in the MITF-induced growth inhibition in some melanoma cells.

Additionally, forced MITF over-expression in 1363mel was associated with melanocyte-like dendritic cellular morphology (Fig. 3c), indicating differentiation as previously reported.[12,15] The morphologic changes were observed in all three cell lines evaluated after the over-expression of MITF-M but increased pigmentation was not seen (data not shown).

doi: 10.1111/j.1349-7006.2009.01266.x

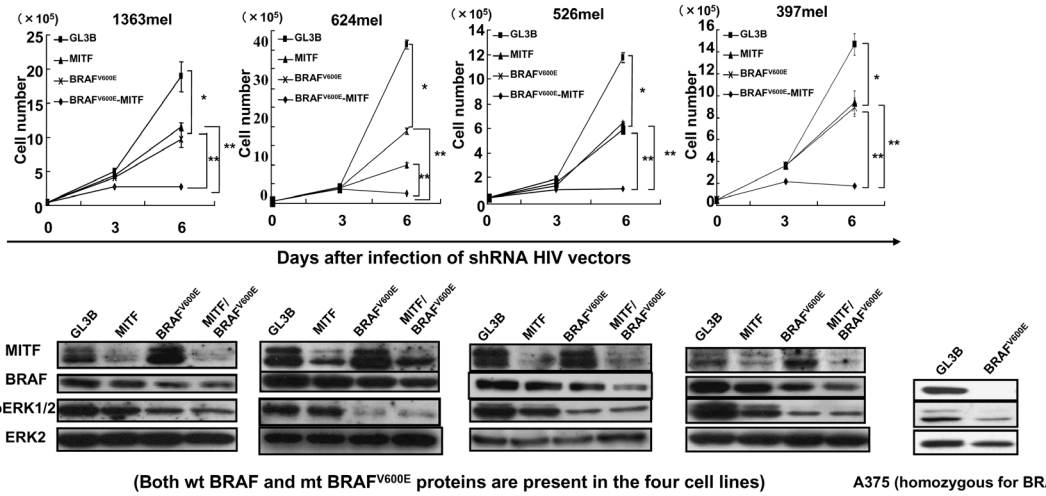

Fig. 4. Enhanced inhibition of cell growth *in vitro* of melanoma cells by simultaneous suppression of both MITF and BRAF^{V600E}. 1363mel, 624mel, 526mel, and 397mel cells were infected with shRNA HIV lentiviral vectors expressing GL3B (control), BRAF^{V600E}, MITF, or MITF/BRAF^{V600E} shRNA at 50 or 100 MOI, and the cell numbers were counted on days 3 and 6. The depletion of MITF or BRAF^{V600E} alone, in comparison with GL3B shRNA treatment, significantly suppressed cell proliferation *in vitro* in all cell lines (*$P < 0.005$). Simultaneous down-regulation of both MITF and BRAF^{V600E} (MITF/BRAF^{V600E}) inhibited growth in all of the cell lines even further than shRNA treatment against MITF or BRAF^{V600E} alone (**$P < 0.001$ compared to the single suppression). The vertical bars indicate the SD of the assays completed in triplicate. The western blots show MITF, BRAF, ppERK1/2, and ERK2 expression after the various shRNA treatments. Although MITF shRNA down-regulates MITF protein levels, BRAF^{V600E} shRNA does not seem to greatly decrease BRAF proteins levels in general. Specific targeting of BRAF^{V600E} in melanoma cells which are heterogeneous for the BRAF^{V600E} mutation may not deplete wild-type BRAF. Western blot analysis also revealed decreased phosphorylated ERK1/2 with either BRAF^{V600E} or MITF/BRAF^{V600E} shRNAs, suggesting successful inhibition of active BRAF^{V600E}. BRAF protein was completely depleted by BRAF^{V600E} shRNA in A375 which is homozygous for BRAF^{V600E}, confirming the specificity. BRAF^{V600E} shRNA increased the MITF protein levels in all of the cell lines except 526mel cells. The western blot is representative of two to three independent experiments with similar results. mt BRAF^{V600E}, mutant BRAF^{V600E}.

These results suggest that the endogenous MITF levels required for cell proliferation may differ for the various melanoma cell lines, and that MITF expression level may be regulated to the level appropriate for efficient cell proliferation in each melanoma cell line. It is important to note that the endogenous MITF could be a molecular target regardless of its expression level in human melanoma cells.

Differential regulation of genes associated with melanoma cell proliferation by MITF and BRAF. Half of the melanoma cell lines were found to be relatively resistant to cell growth inhibition via MITF depletion, indicating that other therapeutic strategies in addition to reducing MITF are needed for these populations. We then investigated the possibility of down-regulating both MITF and mutant BRAF^{V600E}, another known molecular target for melanoma. We compared changes of gene expression associated with melanoma cell proliferation between MITF depletion and BRAF depletion which we previously found to inhibit melanoma cell proliferation.[10] We compared the microarray gene expression profiles of the shMITF- or shBRAF^{V600E}-transduced melanoma cell lines for the eight melanoma cell lines evaluated in this study. The genes with decreased expression by more than two-fold in the melanoma cells treated either with MITF shRNA only, or with BRAF^{V600E} shRNA only, compared to control GL3B shRNA-transduced cells, were identified, and a gene list was made for each cell line, but no genes were shared by all of the eight melanoma cells. However, cluster analysis (Pearson's correlation) of the microarray data revealed one cluster consisting of 1363mel, 526mel, and 624mel, and commonly regulated genes were found in this cluster (Supporting Information Tables 1 and 2). In this group, several growth-related genes were independently regulated by either MITF (e.g. CDK2, PRDM7, MET, EMP1, NRG2) or BRAF^{V600E} (e.g. ADAM19, FOSL1, HIF1A, HMGA2), suggesting that inactivation of both MITF and BRAF^{V600E} could inhibit the growth of melanoma, particularly melanoma cells which were found to be relatively resistant to MITF depletion.

Enhanced inhibition of melanoma cell growth by combined depletion of both MITF and BRAF^{V600E}. We next investigated if combined depletion of both MITF and BRAF^{V600E} could actually enhance the anti-proliferative effects by MITF depletion alone, particularly for the melanoma cells relatively resistant to MITF depletion. We constructed a dual shRNA HIV lentiviral virus (MITF/BRAF^{V600E}) which contains two shRNA-expression cassettes, one for BRAF^{V600E} and another for MITF. Four melanoma cell lines (MITF-sensitive melanoma, 1363mel and 624mel; and MITF-resistant melanoma, 526mel and 397mel) were infected with one of the shRNA viruses (GL3B for firefly luciferase, MITF for *MITF*, BRAF^{V600E} for *BRAF^{V600E}*,[10] MITF/BRAF^{V600E} for both *MITF* and *BRAF^{V600E}*) and the resulting effects on melanoma cell proliferation were compared. Consistent with our previous results,[10] melanoma growth was significantly inhibited by *BRAF^{V600E}* shRNA as well as with *MITF* shRNA ($P < 0.005$) compared to the control GL3B in all the melanoma cell lines (Fig. 4). Simultaneous down-regualtion of both *BRAF^{V600E}* and *MITF* with the MITF/BRAF^{V600E} shRNA enhanced the suppression of melanoma cell growth significantly in comparison to either *MITF* or *BRAF^{V600E}* shRNAs in all of the melanoma cell lines tested ($P < 0.001$) (Fig. 4).

The growth inhibition was associated with G1 cell cycle arrest with the highest %G0/G1 caused by MITF/BRAF^{V600E} shRNA, but not with apoptosis (data not shown). Since all of the four melanoma cell lines expressed both wild-type BRAF and mutant BRAF^{V600E}, BRAF^{V600E} or MITF/BRAF^{V600E} shRNA did not seem to greatly decrease total BRAF protein; however, decreased phosphorylated ERK1/2 (ppERK1/2) (Fig. 4) suggested that active BRAF^{V600E} was successfully inhibited. BRAF protein was completely depleted in A375 cells which are homozygous for BRAF^{V600E} (Fig. 4), confirming the specificity of BRAF^{V600E} shRNA as previously reported.[9,10] Consistent with the prior study, BRAF^{V600E} shRNA increased MITF in all cell lines except 526mel (Fig. 4),[6] suggesting that activated MAPK signaling could induce proteasomal degradation of MITF through Ser73

phosphorylation.[16] Based on these results, the combined targeting against both MITF and $BRAF^{V600E}$ is an effective treatment even for the melanoma cells which were relatively resistant to suppression of cell growth by MITF-targeted treatment.

Discussion

MITF was identified as a lineage survival oncogene which was amplified in malignant melanoma using integrated genomic analysis.[4] MITF has also been demonstrated to have anti-proliferative activity when expressed at high levels,[6–8,15] indicating that it plays a dual role in regulating melanoma cell proliferation. To date, the functional significance of endogenous MITF in melanoma cell proliferation has not been fully investigated by using multiple human melanoma cell lines with heterogeneous MITF expression, which is an important issue to be addressed for consideration of MITF as a target for melanoma treatment. We demonstrated that human melanoma cells exhibit various degrees of sensitivity to MITF depletion, and associated cell proliferation inhibition mechanisms. Furthermore, promotion of melanoma cell proliferation requires different levels of endogenous MITF expression depending on the melanoma cell line. We found that endogenous MITF acts as a pro-proliferation factor in melanoma cell lines regardless of cell line–specific MITF level, confirming MITF as a potential target for treatment of melanoma.

MITF depletion inhibited cell proliferation in all of the melanoma cell lines evaluated, and increased $p21^{Cip1}$ and/or $p27^{Kip1}$. This up-regulation of $p27^{Kip1}$ following MITF depletion caused by decreased Dia1 and the resulting down-regulation of Skp2 may be the primary mechanism of G1 cell cycle arrest.[12] In our study, however, the decreased cell proliferation was accompanied by increased $p27^{Kip1}$ in five melanoma cell lines, but not in the other three melanoma cell lines. In two of these three melanoma cell lines, inhibition of cell proliferation was accompanied by increased $p21^{Cip1}$ which is also regulated by Skp2.[13] We have previously reported that Skp2 protein levels were low in most of the human melanoma cell lines evaluated, including 526mel cells, and that Skp2 depletion does not affect cell proliferation *in vitro* in low Skp2-expressing cells,[9] indicating that the MITF–Dai1–Skp2–$p27^{Kip1}$ pathway may not explain all of the inhibitory mechanisms caused by down-regulating MITF in melanoma cells. We also found that expression levels of the CDK2 protein in the half of the melanoma cells were decreased by MITF depletion. Our results suggest that primary regulatory mechanisms for human melanoma cell proliferation by MITF may differ among the various human melanoma cells.

In this study, half of the tested melanoma cell lines were relatively resistant to inhibition of cell growth by MITF depletion. MITF-sensitive melanoma cells appear to express a relatively high level of endogenous MITF, and reduced CDK2 expression was observed when MITF was down-regulated, indicating that MITF-sensitive melanoma cells may be more dependent on the MITF/CDK2 pathway for regulation of their proliferation. However, forced expression of MITF also caused significant inhibition of cell proliferation in A375 melanoma cells which express low levels of MITF, suggesting that endogenous MITF is self-regulated at appropriate levels for melanoma cell proliferation. To enhance the anti-proliferative activity of MITF-targeting therapy, we attempted to down-regulate both *MITF* and $BRAF^{V600E}$ which enhanced inhibition of melanoma cell growth in all of melanoma cell lines tested, including the relatively MITF-resistant melanoma cell lines 397mel and 526mel. This effect may be explained by differential regulation of genes related with melanoma proliferation by *MITF* and $BRAF^{V600E}$ as shown in our DNA microarray analysis.

Although MITF levels were increased in three of the four melanoma cell lines after $BRAF^{V600E}$ depletion, cell proliferation significantly decreased, suggesting that the increased MITF levels were still within a threshold range allowing observed pro-proliferative effects, or the increased MITF remained inactive due to the lack of MAPK-dependent phosphorylated Ser73.[16] Wellbrock *et al*. showed the transcriptional up-regulation of the *MITF* gene by active MAPK signaling mediated by $BRAF^{V600E}$ through the induction of the transcription factor BRN2 (N-Oct3).[17] However, our DNA microarray analysis does not support their observation in the melanoma cell lines evaluated. Depletion of $BRAF^{V600E}$ modestly decreased MITF mRNA level in A375 cells as previously shown,[17] but the effects of BRAF depletion on MITF mRNA levels vary among the melanoma cell lines. MITF mRNAs were not altered in three (average signal, 0.94–1.18; relative to the control cell line), slightly increased in three (1.31–2.96), and decreased in two (0.77–0.78) of the eight melanoma cell lines, respectively. The changes of MITF mRNA expression paralleled those of the BRN2 mRNA levels. Furthermore, MITF protein levels were increased after the depletion of $BRAF^{V600E}$ in three of the four melanoma cell lines, 1363mel, 624mel, and 397mel (Fig. 4), indicating reduction of MITF was not a main mechanism for the effects observed with the combined MITF/$BRAF^{V600E}$ depletion treatment.

In summary, we have demonstrated for the first time the heterogeneous nature of human melanoma cells in terms of the functional role of MITF in the melanoma cell proliferation. The requirement of endogenous MITF for cell proliferation, and sensitivity to MITF-targeted treatment and its inhibitory mechanisms differ among human melanoma cells. Nevertheless, we have confirmed in this study that MITF is an attractive molecular target for melanoma therapy, and simultaneous inhibition of both *MITF* and $BRAF^{V600E}$ is very effective for melanoma cells with heterogeneous molecular backgrounds, including relatively resistant melanoma cells.

Acknowledgments

This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (nos. 19590321, 17016070), and a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan (15-10 and 19-7) for the Second Term Comprehensive 10-year Strategy for Cancer Control, The Sagawa Foundation for Promotion of Cancer Research, and the Keio Gijuku Academic Development Funds.

We thank Dr H. Miyoshi (Subteam for Manipulation of Cell Fate, Bio-Resource Center, RIKEN Tsukuba Institute, Tsukuba, Japan) for providing us with an HIV vector packaging system.

Disclosure Statement

The authors state no conflict of interest.

References

1 Hodgkinson CA, Moore KJ, Nakayama A *et al*. Mutations at the mouse microphthalmia locus are associated with defects in a gene encoding a novel basic-helix-loop-helix-zipper protein. *Cell* 1993; **74**: 395–404.

2 Opdecamp K, Nakayama A, Nguyen MT, Hodgkinson CA, Pavan WJ, Arnheiter H. Melanocyte development *in vivo* and in neural crest cell cultures: crucial dependence on the Mitf basic-helix-loop-helix-zipper transcription factor. *Development* 1997; **124**: 2377–86.

3 Tachibana M, Takeda K, Nobukuni Y *et al*. Ectopic expression of MITF, a gene for Waardenburg syndrome type 2, converts fibroblasts to cells with melanocyte characteristics. *Nat Genet* 1996; **14**: 50–4.

4 Garraway LA, Widlund HR, Rubin MA *et al*. Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. *Nature* 2005; **436**: 117–22.

5 Nakai N, Kishida T, Shin-Ya M *et al*. Therapeutic RNA interference of malignant melanoma by electrotransfer of small interfering RNA targeting Mitf. *Gene Ther* 2006; **14**: 357–65.

doi: 10.1111/j.1349-7006.2009.01266.x

6 Wellbrock C, Marais R. Elevated expression of MITF counteracts B-RAF-stimulated melanocyte and melanoma cell proliferation. *J Cell Biol* 2005; **170**: 703–8.
7 Carreira S, Goodall J, Aksan I *et al*. Mitf cooperates with Rb1 and activates p21Cip1 expression to regulate cell cycle progression. *Nature* 2005; **433**: 764–9.
8 Loercher AE, Tank EM, Delston RB, Harbour JW. MITF links differentiation with cell cycle arrest in melanocytes by transcriptional activation of INK4A. *J Cell Biol* 2005; **168**: 35–40.
9 Sumimoto H, Hirata K, Yamagata S *et al*. Effective inhibition of cell growth and invasion of melanoma by combined suppression of BRAF (V599E) and Skp2 with lentiviral RNAi. *Int J Cancer* 2006; **118**: 472–6.
10 Sumimoto H, Miyagishi M, Miyoshi H *et al*. Inhibition of growth and invasive ability of melanoma by inactivation of mutated BRAF with lentivirus-mediated RNA interference. *Oncogene* 2004; **23**: 6031–9.
11 Sumimoto H, Yamagata S, Shimizu A *et al*. Gene therapy for human small-cell lung carcinoma by inactivation of Skp-2 with virally mediated RNA interference. *Gene Ther* 2005; **12**: 95–100.
12 Carreira S, Goodall J, Denat L *et al*. Mitf regulation of Dia1 controls melanoma proliferation and invasiveness. *Genes Dev* 2006; **20**: 3426–39.
13 Bornstein G, Bloom J, Sitry-Shevah D, Nakayama K, Pagano M, Hershko A. Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase. *J Biol Chem* 2003; **278**: 25752–7.
14 Du J, Widlund HR, Horstmann MA *et al*. Critical role of CDK2 for melanoma growth linked to its melanocyte-specific transcriptional regulation by MITF. *Cancer Cell* 2004; **6**: 565–76.
15 Selzer E, Wacheck V, Lucas T *et al*. The melanocyte-specific isoform of the microphthalmia transcription factor affects the phenotype of human melanoma. *Cancer Res* 2002; **62**: 2098–103.
16 Wu M, Hemesath TJ, Takemoto CM *et al*. c-Kit triggers dual phosphorylations, which couple activation and degradation of the essential melanocyte factor Mi. *Genes Dev* 2000; **14**: 301–12.
17 Wellbrock C, Rana S, Paterson H, Pickersgill H, Brummelkamp T, Marais R. Oncogenic BRAF regulates melanoma proliferation through the lineage specific factor MITF. *PLOS One* 2008: e2734.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Gene list of down-regulated genes by more than twofold after microphthalmia-associated transcription factor (MITF) RNAi, but not after $BRAF^{V600E}$ RNAi

Table S2. Gene list of down-regulated genes by more than twofold after $BRAF^{V600E}$ RNAi, but not after microphthalmia-associated transcription factor (MITF) RNAi

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.