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SHORT REPORT

Effects on proliferation and melanogenesis by inhibition of mutant *BRAF* and expression of wild-type *INK4A* in melanoma cells

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Activating *BRAF* mutations and loss of wild-type *INK4A* expression both occur at high frequencies in melanomas. Here, we present evidence that *BRAF* and *INK4A* have different effects on melanogenesis, a marker of melanocytic differentiation. Human melanoma cell line 624Mel harbors mutations in both *BRAF* and *INK4A*. The *in vitro* and *in vivo* growth of these cells was inhibited by either reduced expression of mutant *BRAF* using stable retroviral RNA interference (RNAi) or retrovirus-mediated stable expression of wild-type *INK4A* cDNA. Consistent with the observed growth inhibition, phosphorylation of S780 and S795 in pRB, both CDK4/6 targets, was suppressed in cells expressing either mutant *BRAF* RNAi or wild-type *INK4A*. Interestingly, melanoma cells expressing mutant *BRAF* RNAi had increased pigmentation, produced more mature melanosomes and melanin and expressed higher levels of tyrosinase and tyrosinase-related protein-1, whereas melanogenesis was not induced by wild-type *INK4A*. We found that the melanocyte lineage-specific master control protein microphthalmia-associated transcription factor was upregulated by inhibition of mutant *BRAF,* which may be the cause for the melanogenic effect of *BRAF* RNAi. The results suggest that, although both *BRAF* and *INK4A* lesions promote cell growth and tumor formation, mutant *BRAF* may also induce dedifferentiation in melanoma cells.

Key words: ^{T1796A}*BRAF*; *INK4A*; melanogenesis; differentiation; melanoma

Melanoma is becoming one of the most prevalent malignancies with a dismal prognosis. Further understanding of melanoma biology is required to design better strategies in the treatment of this devastating disease. *BRAF* mutations have been identified in 70% of human malignant melanomas.[1] A T1796A transversion in axon 15, resulting in a V599E substitution in the BRAF kinase domain, accounts for >90% of *BRAF* mutations detected in melanoma samples.[1] BRAF is a component of the RAS–RAF–MEK–ERK signaling pathway that plays essential roles in cell proliferation, differentiation and survival.[2] BRAF is one of 3 members of the RAF family,[2] which are serine/threonine kinases that transduce regulatory signals from RAS through MEK to ERK. ^{V599E}BRAF has increased kinase activity; causes intrinsic ERK activation in cultured NIH3T3 cells, COS cells and human melanocytes; and leads to elevated transforming activity of cultured NIH3T3 cells and human melanocytes.[1,3–5] Suppression of ^{V599E}BRAF expression has been reported to cause inhibition of melanoma cell proliferation and survival *in vitro* and *in vivo*.[6–8]

Apart from *BRAF* mutation, most melanoma cells have lost expression of wild-type *INK4A,* through either DNA deletion/mutation or promoter hypermethylation.[9–12] *INK4A* encodes the cell cycle regulator $p16^{INK4A}$, which binds to and inhibits CDK4/6 and promotes cell cycle arrest *via* the RB tumor-suppressor pathway.[13,14] Consistent with a tumor-suppressor role of *INK4A* in melanomas, a germline R24C mutation in CDK4 has been identified in familial melanoma patients.[15,16] This mutation abolishes CDK4 inhibition by $p16^{INK4A}$ and, thus, is believed to be functionally equivalent to $p16^{INK4A}$ loss. Transient transfection of *INK4A* cDNA into melanoma cells has suppressed cell growth in culture.[17]

BRAF and *INK4A* lesions can have overlapping roles in melanoma cells. CDK4/6 may be activated either by mutant BRAF through upregulation of cyclin D *via* ERK signaling or by loss of $p16^{INK4A}$ activity. Activated CDK4/6 can phosphorylate and inactivate RB proteins, resulting in liberation of E2F transcription factors and cell cycle progression, which may contribute to the observed hyperphosphorylation of RB proteins and activation of E2F transcription in advanced melanoma cells.[18]

Malignant melanomas often consist of a fraction of cells that are depigmented, suggesting loss of the balance between proliferation and differentiation in these cells. During normal development, differentiation stimuli trigger the activation of MITF to initiate and maintain melanogenesis.[19] Melanogenesis is a multistep biochemical process resulting in the formation of melanin in pigment cells. Tyrosinase and TRP-1 participate in the melanogenic pathway and are important melanocyte differentiation markers.[19,20] Tyrosinase and TRP-1 are expressed in cultured normal human melanocytes, but their expression was repressed in melanoma cell lines.[21] Levels of tyrosinase correlate inversely with melanoma clinical stage.[22] These data suggest that the normal melanogenesis program is inhibited in melanoma cells.[23]

We examined the roles of *BRAF* and *INK4A* lesions in melanoma cells. Expression of either mutant *BRAF* RNAi or wild-type *INK4A* cDNA suppressed the *in vitro* and *in vivo* growth of 624Mel melanoma cells. Suppression of mutant *BRAF* also induced melanogenesis, which may represent a reversion of these melanoma cells to a more differentiated state. Melanogenesis was not induced by wild-type *INK4A,* revealing the nonoverlapping roles of the 2 most common genetic lesions in melanoma cells.

Material and methods

BRAF and INK4A mutation analysis

A PCR forward primer (5′-GTAAAAATAGGTGATTTTGGTCTAGCTGAAG-3′) was used to amplify *BRAF* exon 15, which creates, in cases of a mutant A1796 nucleotide in the DNA template, an MboII site GAAGA(N8)↓. The wild-type allele with T1796 does not contain the MboII recognition sequence at the corresponding position. The reverse primer (5′-AAAGTTAAAAAATCTATTTACATAAAAAATAAGAACACTGATTTTCTTCATAC-3′) also has an MboII site for enzyme digestion and

Abbreviations: CDK, cyclin-dependent kinase; ERK, extracellular signal–regulated kinase; LOH, loss of heterozygosity; luc, luciferase; MEK, mitogen-activated protein kinase/ERK kinase; MITF, microphthalmia-associated transcription factor; pRS, pRETRO-SUPER; RFLP, restriction fragment length polymorphism; RNAi, RNA interference; shRNA, short hairpin RNA; TRP-1, tyrosinase-related protein-1.

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size control. PCR amplification was as described.[4] PCR amplicons were digested with MboII (New England Biolabs, Beverly, MA). Restriction fragments were separated by electrophoresis on a 3% agarose or 10% polyacrylamide gel. The method was validated by direct sequencing of PCR products. *INK4* exons 1α, 2 and 3 and the alternatively spliced exon 1β of *ARF* were PCR-amplified from genomic DNA. Primer sequences and amplifications were as described.[24] PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Direct sequencing was performed by the Mount Sinai DNA Core Facility using the ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA).

Cell culture

624Mel, 1363Mel, A375, RPMI, WM35 and A101D melanoma cell lines were kindly provided by Dr. S. Aaronson (Mount Sinai School of Medicine, New York, NY). Cells were maintained in DMEM supplemented with 10% FBS and 50 units/ml penicillin–streptomycin (Invitrogen, Carlsbad, CA).

Construction and expression of ^{T1796A}BRAF RNAi and wild-type INK4A cDNA

pSUPER and pRS-puro[25] were used to express ^{T1796A}*BRAF* shRNA. The oligo 5′-gatccccTAGCTACAGAGAAATCTCGttcaagagaCGAGATTTCTCTGTAGCTAttttggaaa-3′ was ligated into pSUPER and then subcloned into pRS-puro (capital letters are the 19 nucleotide *BRAF* target sequence, the 1796G→T transversion is underlined). A 19 bp firefly luc sequence was used as RNAi control, as described.[26] Transient cotransfection experiments in 293T cells were done using Fugene (Roche, Nutley, NJ). Wild-type *INK4A* cDNA was subcloned into pBabe-neo retroviral vector. For stable expression of RNAi and *INK4A* constructs, retroviruses were produced and used to infect melanoma cells, as described.[26] Mass culture was selected and maintained in medium containing 1 μg/ml puromycin (RNAi) or 750 μg/ml G418 *(INK4A)*.

Analyses of melanin and melanosomes

Melanin contents were measured as described.[27] Cells were washed with PBS and dissolved in 1 N NaOH at 100°C for 1 hr. For electron microscopic analysis of melanosomes, cultured cell pellets and dissected xenograft tumors were fixed in 3% glutaraldehyde in a 0.2 M sodium cacodylate buffer (pH 7.4). Tissue was routinely processed through graded steps of ethanol, cleared with propylene oxide and embedded in embed 812 (Electron Microscopy Sciences, Hatfield, PA). Plastic sections (1 μm) were cut, stained with methylene blue and azure II and observed by light microscopy. Representative areas were chosen for ultrathin sectioning. Low- and high-magnification electron micrographs were taken with a Hitachi (Tokyo, Japan) H7000 TEM.

Immunoblotting assay and antibodies

Western blotting was performed as described.[26] The following antibodies were used: BRAF (Upstate Biotechnology, Lake Placid, NY); p16^{INK4A}, tyrosinase and cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA); phospho-MEK, MEK-1 and phospho-pRB (Cell Signaling, Beverly, MA); TRP-1 and cyclin D3 (BD Biosciences, San Diego, CA); MITF (Novus Biologicals, Littleton, CO); tubulin (Sigma-Aldrich, St. Louis, MO); and horseradish peroxidase–conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA).

In vivo *studies*

BALB/c nude mice, 5 weeks old (Taconic Laboratories, Germantown, NY), were used. Cells were injected s.c. into flanks of nude mice. Tumor growth was monitored weekly by caliper measurement in 3 dimensions. Tumor volume was calculated using the standard formula: $A \times B^2 \times 0.52$, where A is the longest diameter and B is the shortest diameter. Animals were treated according to NIH guidelines for animal care and use.

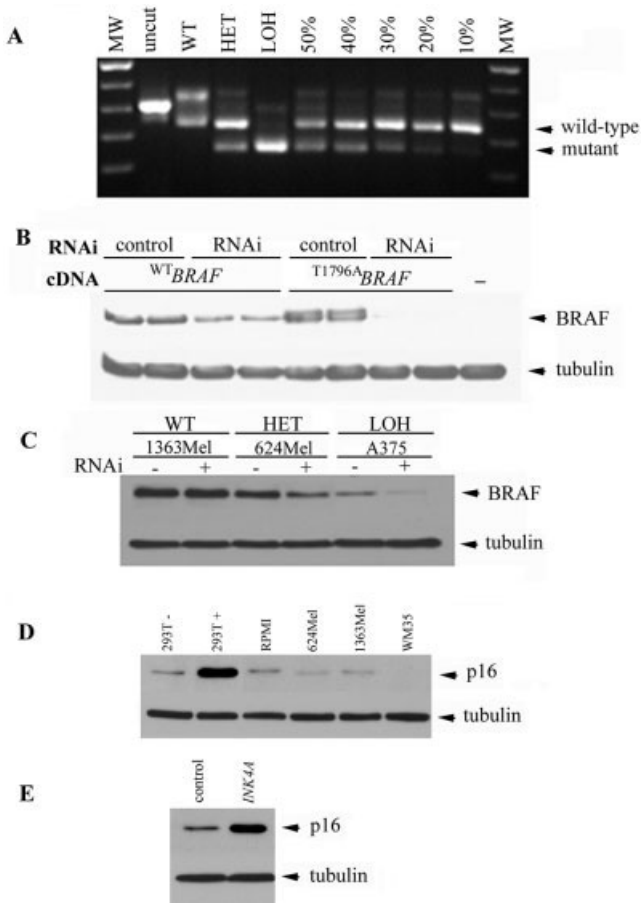

FIGURE 1 – Specific ^{T1796A}*BRAF* inhibition and reconstitution of wild-type *INK4A*. *(a)* PCR-RFLP analysis of *BRAF* T1796A mutation. The PCR amplicon, wild-type and mutant bands are 255, 218 and 178 bp, respectively. Serial dilutions containing 50%, 40%, 30%, 20% and 10% of mutant alleles in total DNA were used to determine assay sensitivity. It was possible to detect the mutant allele at 20% dilution in total DNA. Arrows, wild-type and mutant bands. Background bands were heteroduplexes. *(b)* Specificity and efficacy of ^{T1796A}*BRAF* RNAi. ^{WT}*BRAF* or ^{T1796A}*BRAF* cDNA was cotransfected with control vector or vector expressing ^{T1796A}*BRAF* RNAi in 293T cells. Forty-eight hours after transfection, cells were collected. Western blotting was performed as described.[26] The last lane (–) was control cell lysate from untransfected cells. *(c)* Endogenous *BRAF* inhibition by ^{T1796A}*BRAF* RNAi. 624Mel, 1363Mel and A375 melanoma cells were stably infected with luc-RNAi control (–) or ^{T1796A}*BRAF* RNAi retroviruses (+) as described.[26] Western blots were probed with BRAF and tubulin antibodies. *(d)* Endogenous *INK4A* expression. 293T control (–) and cells transfected with wild-type *INK4A* cDNA for 48 hr (+) were used as controls. Western blots were probed with p16^{INK4A} (p16) and tubulin antibodies. *(e)* Exogenous expression of wild-type p16^{INK4A} in 624Mel cells. Cell lysates of 624Mel cells expressing vector control or wild-type *INK4A* were separated by PAGE and probed with p16^{INK4A} and tubulin antibodies.

Results

Detection and specific inhibition of ^{T1796A} BRAF expression

We designed a sensitive PCR-RFLP assay to analyze the *BRAF* gene for the T1796A mutation (Fig. 1*a*). Using this method, we identified melanoma cell lines that are wild-type (1363Mel),

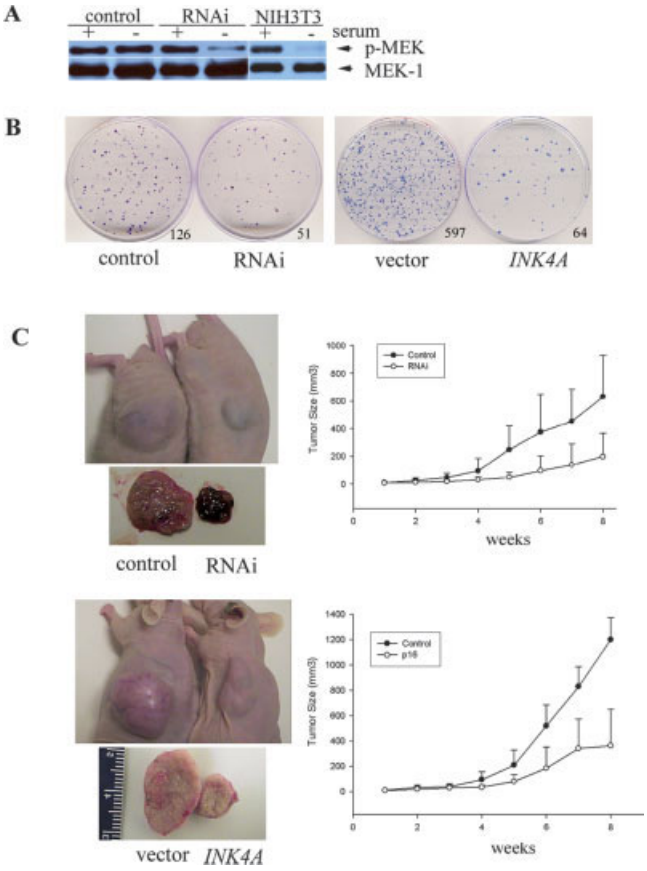

FIGURE 2 – ^{T1796A}*BRAF* RNAi and exogenous wild-type *INK4A* inhibited the growth of 624Mel melanoma cells. *(a)* ^{T1796A}*BRAF* RNAi suppressed intrinsic ERK signaling in melanoma cells. 624Mel cells stably expressing luc control or ^{T1796A}*BRAF* RNAi (RNAi) and nontransformed NIH3T3 cells were cultured in regular medium with serum (+) or serum-starved for 16 hr (–). Levels of phospho-MEK and total MEK-1 were detected by Western blotting. *(b)* Colony formation assay. 624Mel cells (1 x 10^3) stably expressing luc-RNAi (control) or ^{T1796A}*BRAF* RNAi (RNAi), vector control or wild-type *INK4A* were plated in triplicate in 35 mm diameter plates and grown in medium with 5% (RNAi) or 10% *(INK4A)* serum for 3 weeks. Colonies were fixed and stained. *(c)* Mouse xenograft assay. 624Mel luc control and ^{T1796A}*BRAF* RNAi cells (5 x 10^5) or 1 x 10^6 624Mel vector control and *INK4A*-expressing cells were injected s.c. into flanks of nude mice ($n = 6$) and monitored for tumor growth. Pictures were taken 8 weeks after cell inoculation. Average tumor volumes were calculated and plotted.

heterozygous (624Mel) or show LOH (A375) for the T1796A mutation. We used mutant specific RNAi to inhibit the expression of ^{T1796A}*BRAF* (Fig. 1*b,c*). The specificity and efficacy of RNAi in suppressing the expression of ^{T1796A}*BRAF* was examined by transient coexpression experiments in 293T cells. Wild-type or ^{T1796A}*BRAF* cDNA (pCEV29-^{WT}*BRAF* or pCEV29-^{T1796A}*BRAF*) was cotransfected with control or pSUPER-^{T1796A}*BRAF* RNAi. Mutant *BRAF* RNAi reduced ^{T1796A}*BRAF* to undetectable levels. Inhibition of ^{WT}*BRAF* also occurred but to a much lesser extent (Fig. 1*b*). Retroviruses of the luc control and ^{T1796A}*BRAF* RNAi were then produced using the pRS-puro vector and used to infect melanoma cells. Stable pools of infected cells were puromycin-selected and endogenous BRAF levels examined by immunoblotting. As shown in Figure 1*c,* mutant *BRAF* RNAi reduced the BRAF level in ^{T1796A}*BRAF*-positive cells but not in *BRAF* wild-type melanoma cells. BRAF expression was most significantly reduced in A375 melanoma cells, which show LOH at the *BRAF* locus and thus express only ^{T1796A}*BRAF*.

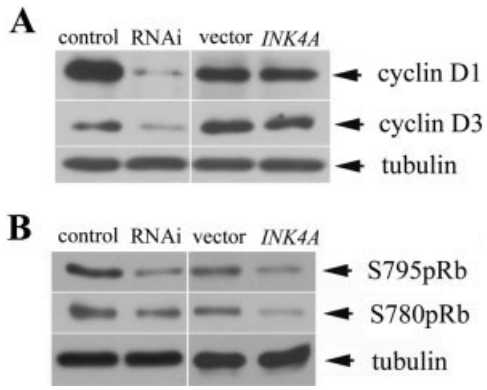

FIGURE 3 – Expression of cyclin D1/D3 and phospho-pRB. Western blotting was performed using cell lysates from 624Mel control cells and cells expressing ^{T1796A}*BRAF* RNAi or *INK4A* and probed with cyclin D1 and cyclin D3 *(a)* or Ser795 and Ser780 phosphorylated pRB *(b)*.

Mutation analysis and expression of wild-type INK4A

Mutation analysis of *INK4A* showed that 624Mel cells have an 18 bp in-frame deletion of codons 32–37 (CTGGAGGCG-GGGGCGCTG) in exon 1α. The deleted sequence is located in the first ankyrin repeat and encodes an evolutionarily conserved 6 amino acids (LEAGAL).[28] Deletions and mutations affecting these amino acid have been reported in melanomas and other cancers and shown to significantly affect the CDK- and cell cycle-inhibitory activities of $p16^{INK4A}$.[29–31] The sequence shows LOH (not shown), suggesting that either the wild-type copy of the gene is deleted or this is a homozygous deletion. Sequence analysis also showed that *INK4A* is wild-type in RPMI and 1363Mel and deleted in WM35 melanoma cells (not shown). Mutant $p16^{INK4A}$ is expressed at relatively low levels in 624Mel cells compared to wild-type $p16^{INK4A}$ in RPMI and 1363Mel cells (Fig. 1*d*). 624Mel cells were infected with either vector control or pBabe-neo-*INK4A* retroviruses. After G418 selection of mass culture, immunoblotting showed that $p16^{INK4A}$ was appropriately expressed at levels about 5-fold more than endogenous $p16^{INK4A}$ in 624Mel cells (Fig. 1*e*).

Growth inhibition by both suppression of mutant BRAF and expression of wild-type INK4A

We found that 624Mel cells that are positive for the T1796A mutation had intrinsic MEK activation, which was inhibited by ^{T1796A}*BRAF* RNAi (Fig. 2*a*), consistent with earlier reports.[3,6–8] Both mutant *BRAF* RNAi and wild-type *INK4A* significantly inhibited the growth of these cells in tissue culture, as measured by cell count (not shown) and colony formation assay. Population doubling times of ^{T1796A}*BRAF* RNAi and wild-type *INK4A* expressing 624Mel cells were on average 36 hr and 50 hr, respectively, compared to controls of about 24 hr (not shown). The colony numbers shown are the average colony counts from 3 plates (Fig. 2*b,* $p < 0.001$, two Poisson parameters test). The effect on tumorigenesis was examined in a nude mice xenograft assay. Tumor growth was significantly inhibited by both ^{T1796A}*BRAF* inhibition and wild-type *INK4A* expression (Fig. 2*c,* $p < 0.01$, *t*-test).

Both RAF/ERK signaling and $p16^{INK4A}$ regulate the activity of RB pocket proteins and cell cycle progression.[13,14] We found that cyclin D1 and D3 were downregulated in cells expressing ^{T1796A}*BRAF* RNAi (Fig. 3*a*). Phosphorylation of S780 and S795 of pRB, known cyclin D1/CDK4 targets,[32–34] was reduced by both *BRAF* RNAi and *INK4A* (Fig. 3*b*). These results are consistent with the observed growth inhibitory effects.

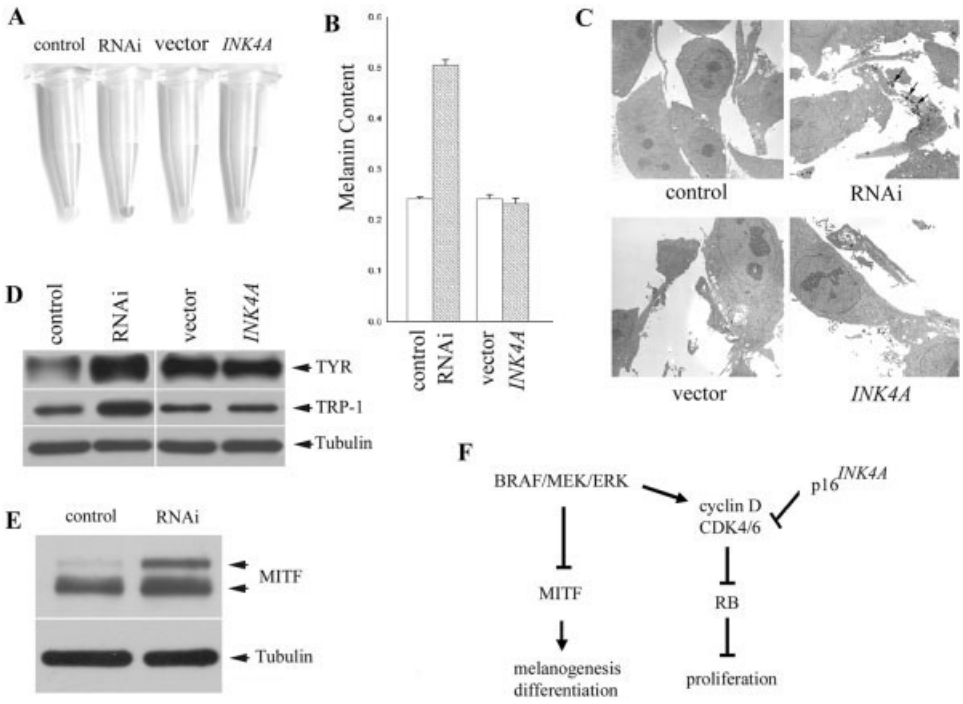

FIGURE 4 – Effect on melanogenesis. *(a)* Color of cell pellets. 624Mel controls (5 x 10^6) and cells expressing ^{T1796A}*BRAF* RNAi or *INK4A* were pelleted, and a visible color difference was noted in cells expressing ^{T1796A}*BRAF* RNAi. *(b)* Melanin contents; 5 x 10^6 cells were used. Data are means ± SE from 3 experiments performed in triplicate ($p < 0.001$, *t*-test). *(c)* Representative electron micrographs of cultured 624Mel controls and cells expressing ^{T1796A}*BRAF* RNAi or *INK4A*. Note the increased numbers of mature melanosomes in RNAi-expressing cells (arrows). Magnification x2,000. Similar changes were obtained using dissected xenograft tumors. *(d)* Expression of tyrosinase (TYR) and TRP-1 proteins. Western blot was probed with TYR, TRP-1 and α-tubulin antibodies. *(e)* Increased MITF in cells expressing ^{T1796A}*BRAF* RNAi, shown by Western blotting. *(f)* Model of separate regulation of proliferation and differentiation by mutant *BRAF* and p16^{INK4A} in melanoma cells.

Increased melanogenesis by mutant BRAF RNAi but not by wild-type p16^{INK4A}

624Mel cells expressing ^{T1796A}*BRAF* RNAi not only grew slower but were also darker in color (Figs. 2*c,* 4*a*) as a result of increased production of melanin (Fig. 4*b*) and mature melanosomes (Fig. 4*c*). This effect on pigmentation was not limited to 624Mel cells because pigmentation and melanin were increased by ^{T1796A}*BRAF* RNAi in other melanoma lines that harbor the hot-spot *BRAF* mutation (A101D, A375; not shown). Tyrosinase, the rate-limiting enzyme in melanin production that is associated with melanocytic differentiation,[35] was upregulated in cells expressing ^{T1796A}*BRAF* RNAi (Fig. 4*d*). Expression of TRP-1 was correlated with changes of tyrosinase in these cells (Fig. 4*d*). However, wild-type p16^{INK4A}-reconstituted 624Mel cells were not darker in color (Figs. 2*c,* 4*a*), did not produce more melanin and mature melanosomes (Fig. 4*b,c*) and did not have increased expression of tyrosinase and TRP-1 (Fig. 4*d*).

Pigment cell–specific expression of tyrosinase and TRP-1 is predominantly regulated by MITF.[19,36,37] MITF expression and activity are regulated by the ERK signaling pathway. Consistently, we found that MITF is increased in 624Mel cells expressing mutant *BRAF* RNAi (Fig. 4*e*).

Discussion

We found that melanogenesis is induced by inhibition of mutant *BRAF* but not by expression of wild-type p16^{INK4A}, suggesting the existence of different mechanisms in the malignant transformation of melanoma cells by *BRAF* and *INK4A* lesions. The observed growth inhibition by mutant *BRAF* RNAi and wild-type *INK4A* is consistent with their regulation of the cyclin D–CDK4/6–RB pathway (Fig. 4*f*). Since melanosome maturation and melanin production are signatures of melanocyte differentiation, by that criteria, melanoma cells expressing ^{T1796A}*BRAF* RNAi are more differentiated. The effect on melanogenesis suggests that inhibition of cellular differentiation could be a key transforming activity of *BRAF* lesions. Thus, mutant *BRAF* plays a role in the regulation of this process. The melanogenic effect has not been described in other published studies on mutant *BRAF* inhibition in melanoma cells,[6–8] probably due to differences in the levels of *BRAF* inhibition, the cell lines used or culture conditions.

Unlimited proliferation and defects in cellular differentiation are characteristic of cancer growth, and cancer is thought to be a disease of cellular differentiation.[38,39] However, how the abnormal proliferation and pigmentation/differentiation are interconnected in the development of melanoma and other cancers is largely unknown.[38,40] Since restoring p16^{INK4A} expression did not increase pigmentation, though cell proliferation was also suppressed, the differentiation effect of *BRAF* inhibition was not merely the result of general growth suppression. Rather, mutant *BRAF* actively participates in the differentiation program while simultaneously inducing proliferation. Dedifferentiation is characteristic of tumor cells.[39] It is well known that malignant cells, including those of melanomas, maintain their differentiation program and sensitivity to differentiation modulators.[41] Therefore, understanding mutant *BRAF* in the regulation of the differentiation process may provide strategies by targeting mutant *BRAF* to reverse melanoma cells to a more mature and less malignant state.

Inhibition of intrinsic ERK signaling in melanoma cells causes induction of melanogenesis and cellular differentiation.[42,43] Suppression of mutant *BRAF* inhibited ERK signaling (Fig. 2*a*), which may be attributed to the observed melanogenic effects. However, both activation and inhibition of ERK signaling cause increased pigmentation. For example, although melanogenesis in melanocytes is suppressed both *in vitro* and *in vivo* by exogenous expression of *Ras* oncogene,[44–46] trans-

genic mice expressing *Ras* under a mouse tyrosinase promoter demonstrated hyperpigmentation and melanocytic hyperplasia.[47] Activation of ERK signaling by the c-Kit receptor plays a crucial role in the differentiation of pigment cells during development,[48] whereas constitutive ERK signaling in melanoma cells could be inhibitory to melanogenesis.[42,43] Although our results demonstrate a melanogenic effect by inhibiting mutant *BRAF,* it is worth noting that activating *BRAF* mutations have been found in 70–80% of benign nevi that are dark in color, and some malignant melanomas that are highly pigmented may have *BRAF* mutation. Thus, although *BRAF* mutation is important in blocking the melanogenic process in melanoma cells, it is not sufficient by itself to cause depigmentation. It is clear that the roles of *BRAF* mutation and ERK signaling in melanogenesis and melanocytic differentiation depend on the cellular context. Precisely what role is played by *BRAF* mutation and how it interacts with other signaling pathways in the determination of pigmentation phenotype need to be further studied.

Our data suggest that MITF is a candidate factor in the mediation of the melanogenic effects of mutant *BRAF* in melanoma cells. It is interesting that MITF could be positively and negatively regulated by ERK signaling.[49–51] Phosphorylation of S73 and S409 in MITF by the ERK signaling cascade causes ubiquitination and degradation of MITF protein.[49–51] However, phosphorylation of these residues by the ERK signaling pathway has also been linked to an increase in MITF transcription activity through promoting its interaction with CBP and target DNA.[37] Clearly, much more needs to be done to understand how MITF is regulated by mutant *BRAF* in melanoma cells, including effects on MITF expression, phosphorylation, stability, protein–protein interaction, DNA binding and transcription activities.

(Note: *BRAF* nucleotide and codon numbers are based on NCBI entry NM_004333. However, this sequence misses a codon in exon 1.[52] Sequence numbering after exon 1 should be changed. Therefore, the hot-spot mutation should be V600 and not V599, T1799A and not T1796A. However, to avoid confusion with the published data, we have used here the original numbering.)

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