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Suppression of BRAF^{V599E} in Human Melanoma Abrogates Transformation[1]

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Abstract

Activating mutations in the *BRAF* serine/threonine kinase are found in >70% of human melanomas, of which >90% are *BRAF^{V599E}*. We sought to investigate the role of the *BRAF^{V599E}* allele in malignant melanoma. We here report that suppression of *BRAF^{V599E}* expression by RNA interference in cultured human melanoma cells inhibits the mitogen-activated protein kinase cascade, causes growth arrest, and promotes apoptosis. Furthermore, knockdown of *BRAF^{V599E}* expression completely abrogates the transformed phenotype as assessed by colony formation in soft agar. Similar targeting of *BRAF^{V599E}* or wild-type *BRAF* in human fibrosarcoma cells that lack the *BRAF^{V599E}* mutation does not recapitulate these effects. Moreover, these results are specific for *BRAF*, as targeted interference of *CRAF* in melanoma cells does not significantly alter their biological properties. Thus, when present, *BRAF^{V599E}* appears to be essential for melanoma cell viability and transformation and, therefore, represents an attractive therapeutic target in the majority of melanomas that harbor the mutation.

Introduction

Malignant melanoma will afflict >50,000 people in the United States this year and result in >7,000 deaths (1). The incidence of melanoma is rising among the most rapidly of all malignancies (2). When diagnosed early, melanoma is highly curable by wide surgical excision. However, in patients with deep local invasion, or with spread to lymph nodes or distant sites, the disease is highly resistant to all forms of therapy. The median survival for patients with metastatic melanoma is 6–9 months (3).

Recently, activating mutations in the *BRAF* gene were described in a majority of melanomas and benign nevi, suggesting an important role for this oncogene in melanocyte biology and disease (4–6). More than 60% of malignant melanomas were found to contain a specific mutation, *BRAF^{V599E}*, the product of which possesses constitutive kinase activity. *BRAF* is a member of the Raf family of serine/threonine kinases, along with *CRAF* and *ARAF*, which serve as immediate effectors of the *ras* GTPases (7). Activation of the Raf/MEK[3]/ERK, or MAPK, signaling cascade promotes cellular proliferation and survival. The highly homologous Raf family members have overlapping but distinct biochemical activities and biological functions. We therefore sought to determine whether Raf family members, and specifically *BRAF^{V599E}*, are required in melanoma cells for maintenance of the transformed state. Accordingly, the biochemical signaling properties and cellular phenotypes of melanoma cells were assessed after depletion of B-Raf, B-Raf^{V599E}, and C-Raf proteins by RNAi.

Materials and Methods

RNAi Sequences and Preparation. Small inhibitory duplex RNAs (PROLIGO, Boulder, CO) were prepared and reconstituted in annealing buffer as described (8, 9). The sense strands of the siRNA duplexes were as follows: Lamin A/C: CUggACUUCCAgAAgAACATT; Com-4: AgAAUUggAUCUggAUCAUTT; Mu-A: gCUACAgAgAAAUCUCgAUTT; C1: UgUgCgAAAUggAAUgAgCTT. Duplex shRNA oligos were cloned into the *Hind*III and *Bgl*II sites in pSUPER.retro (Oligoengine, Seattle, WA), and insert fidelity was confirmed by sequencing both strands with the following primers: forward – ttatccagccctcactcc; reverse – gtgttctgggaaatcacc. The sense strands of the shRNA pSUPER.retro inserts were as follows:

Com-1: gatccccTGGATACCGTTACATCTTCttcaagagaGAAGATGTAACGGTATCCAtttttggaaa.

Com-2: gatccccTCCCAGAGTGCTGTGCTGTttcaagagaACAGCACAGCACTCTGGGAtttttggaaa.

Com-3: gatccccTTGGTTGGGACACTGATATttcaagagaATATCAGTGTCCCAACCAAtttttggaaa.

Com-4: gatccccAGAATTGGATCTGGATCATttcaagagaATGATCCAGATCCAATTCTtttttggaaa.

Mu-A: gatccccGCTACAGAGAAATCTCGATttcaagagaATCGAGATTTCTCTGTAGCtttttggaaa.

Mu-B: gatccccGAGAAATCTCGATGGAGTGttcaagagaCACTCCATCGAGATTTCTCtttttggaaa.

C1: gatccccTGTGCGAAATGGAATGAGCttcaagagaGCTCATTCCATTTCGCACAtttttggaaa.

BRAF cDNA. Human wild-type *BRAF* and *BRAF^{V599E}* were cloned from mRNA and sequenced to confirm fidelity. 5′ HA epitope tags were cloned into both cDNAs by PCR. Full-length *BRAF* cDNAs were subsequently cloned into pBABE.puro.

Cell Culture and Transfection. WM793 melanoma cells were derived from a vertical growth phase tumor as described previously (10), and HT1080 and HEK cells were obtained from American Type Culture Collection. Cells were cultured under standard conditions (37°C in humidified atmosphere containing 5%CO_2) and grown in DMEM supplemented with 25 mM HEPES (pH 7.4), 10% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml). To achieve transient suppression of gene expression, cells were plated in six-well dishes at 50–60% confluency and transfected with 5 μg of duplex RNA plus 6 μl of OLIGOFECTAMINE (Life Technologies, Inc., Carlsbad, CA) per the manufacturer's recommendations and as described (8, 9). The specificity of the targeting sequences was determined by transient cotransfection of HEK cells with pBABE.puro.HA-tagged *BRAF* or pBABE.puro.HA-tagged *BRAF^{V599E}* and shRNA vectors (11). For stable transfection experiments, cells were plated at 50–80% confluency in 100-mm dishes and transfected with 4 μg of plasmid DNA and 12 μl of Fugene 6 (Roche, Indianapolis, IN) per the manufacturer's instructions. Twenty-four h after transfection, cells were selected in media containing 2 μg/ml Puromycin for 60–72 h and then collected for biochemical and cellular assays.

Immunoblotting. Adherent cells were washed with ice-cold PBS and lysed and scraped in boiling SDS lysis buffer (10 mM Tris, 1% SDS, 50 mM NaF, and 1 mM VO4). Lysates were boiled for 5 min, the DNA was sheared, and insoluble debris was removed by microcentrifugation (14,000 rpm for 10 min).

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[3] The abbreviations used are: MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; siRNA, small interfering RNA; HEK, human embryonic kidney; HA, hemagglutinin; shRNA, short hairpin RNA; MAPK, mitogen-activated protein kinase; RNAi, RNA interference; TBS, Tris-buffered saline.

Protein concentrations were determined with bicinchoninic acid (Pierce, Rockford, IL). Samples (15 μg of total protein per lane) were resolved by reducing SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked and incubated with primary antibodies in TBS [150 mM Tris-HCL (pH 8.0) and 150 mM NaCl] + 3% BSA for antiphospho antibodies or 5% nonfat dry milk/TBS for other antibodies. The membranes were subsequently washed (TBS/0.1% Tween 20), incubated with horseradish peroxidase-conjugated secondary antibodies, and washed again before being processed with enhanced chemiluminescence plus (Amersham Biosciences, Little Chalfont, United Kingdom). Membranes were probed sequentially for the indicated proteins after washing in stripping buffer [50 mM Glycine (pH 2.5) and 0.05% Tween 20] for 15 min at 55°C. Primary antibodies were procured from the following sources: antiphosphorylated and total MEK (Cell Signaling, Beverly, MA), anti-HA (Sigma, St. Louis, MO), and anti-Lamin A/C (Vector Laboratories, Burlingame, CA) and antibodies against actin, B-Raf, and C-Raf were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

Proliferation, Apoptosis, and Transformation Assays. After selection, shRNA-transfected cells were plated onto glass coverslips in media. The next day, cells were incubated with 1 mM BrdUrd (Sigma) for 4 h, and positive nuclei detected with anti-BrdUrd FITC per manufacturer's instructions (Roche, Indianapolis, IN). Apoptosis was detected with the In Situ Cell Death Detection kit per the manufacturer's instructions (Roche). Three high-powered fields were counted manually to determine the percentage of cells in S phase and the degree of apoptosis, respectively. Nuclear staining was detected with 4′,6-diamidino-2-phenylindole (Sigma). Soft agar assays were performed by plating 50,000 cells/60-mm dish in 0.34% agar/media suspension over a solidified 0.5% agar layer. Dishes were replenished every 5–7 days.

Results

The recent development of facile methods for both transient and stable suppression of gene expression by RNAi has provided powerful tools for the study of mammalian cell genetics (8, 11, 12). These methods exploit a conserved biological response to short duplex RNA that results in post-transcriptional gene silencing (13). To evaluate the role of *BRAF* expression in human melanoma cells, we generated a series of stably expressing vectors against discrete regions of the human *BRAF* coding sequence (Fig. 1*A*). We then tested the specificity of these reagents in HEK cells that had been transiently cotransfected with HA epitope-tagged wild-type and mutant *BRAF* vectors (Fig. 1*B*). Targeting sequences common to both wild-type and mutant alleles (Com-1, 2, 3, and 4) caused various degrees of knockdown of *BRAF* expression in HEK cells as demonstrated by immunoblots against the expressed protein, with Com-4 being the most effective. A mutant-specific vector (Mu-A) was also created that essentially completely abolished *BRAF*^{V599E} expression while keeping wild-type *BRAF* expression intact.

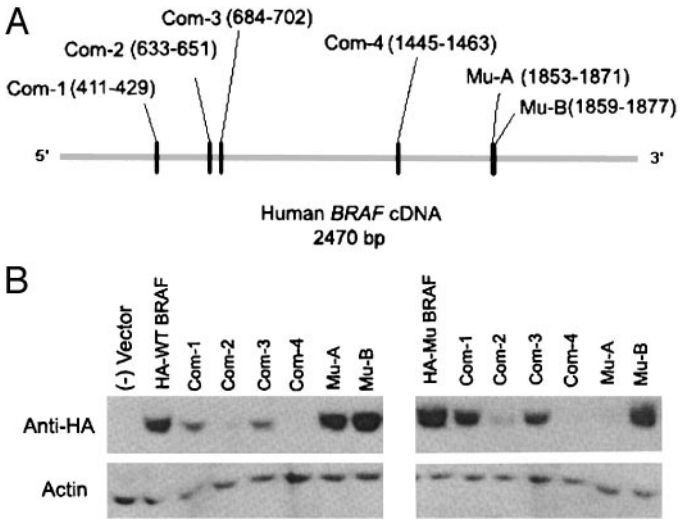

Fig. 1. Design and characterization of vector-based RNAi of human *BRAF*. *A*, schematic representation of human *BRAF* cDNA demonstrating sites selected for targeting. *B*, efficacy of shRNA vectors in suppressing *BRAF* and *BRAF*^{V599E} expression. Subconfluent adherent HEK cells were either not transfected (−) or cotransfected with pBABE.puro.HA-*BRAF* (*left panels*) or pBABE.puro.HA-*BRAF*^{V599E} (*right panels*), and the series of shRNA plasmid constructs were directed against *BRAF*. After transfection (48–72 h), whole cell extracts were prepared and analyzed for HA expression; actin was used as a loading control. This experiment was performed three times with similar results.

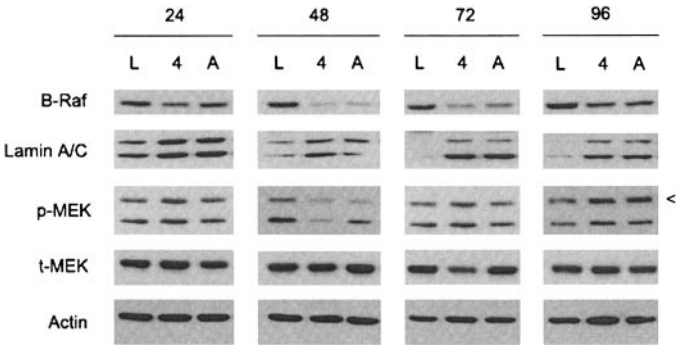

Fig. 2. Transient suppression of wild-type and mutant *BRAF* by siRNA in WM793 cells. Subconfluent cell cultures were transfected with siRNA directed against *Lamin A/C* (*L*), *BRAF* (*4*), and *BRAF*^{V599E} (*A*). Whole cell lysates were then prepared at 24-, 48-, 72-, and 96-h post-transfection and analyzed for specific protein expression by immunoblotting with the indicated antibodies. The position of the M_r 45,000 phospho-MEK 1/2 proteins is indicated; the identity of the faster migrating band is unknown. This experiment was performed three times with similar results.

After establishing the efficacy and specificity of various RNAi constructs described above, we designed corresponding duplex siRNA species to study the effects of endogenous *BRAF* knockdown in melanoma cells because of the higher transfection efficiencies achievable by this method and the ability to obviate the need for antibiotic selection. WM793 human melanoma cells were derived from a vertical growth phase tumor (10) and have been shown to harbor the *BRAF*^{V599E} allele (14). Com-4 and Mu-A siRNA significantly inhibited *BRAF* expression and its attendant downstream signaling as measured by phosphorylated MEK levels, although these effects were relatively short lived and reversed by 72–96 h after transfection (Fig. 2). Transfection of siRNA against Lamin A/C as a control revealed efficient and more durable suppression of target expression, reflecting either greater efficacy or stability of this transfected RNA duplex or perhaps lower endogenous levels of lamin mRNA synthesis.

Despite the marked biochemical sequelae to *BRAF* knockdown by siRNA, the effects were transient and rapidly reversible, and there were no overt phenotypic consequences. Thus, to study the potential biological consequences of more durable perturbations of these pathways, we used our panel of shRNA vectors to stably knock down *BRAF* in melanoma cells. Transfection of Com-4 and Mu-A shRNA vectors into WM793 cells effectively and stably suppressed *BRAF* expression and MEK phosphorylation (Fig. 3*A*). Cells transfected with control vector had no discernible effect on these parameters. On the other hand, although it was possible to stably suppress *BRAF* expression by Com-4 in HT1080 human fibrosarcoma cells, no corresponding inhibition of MEK phosphorylation was observed, indicating that these cells activate the MAPK pathway by another mechanism. As expected, transfection with Mu-A had no discernible effect on B-Raf levels because these cells do not contain the *BRAF*^{V599E} mutation.

Several cellular consequences to stable *BRAF* suppression were readily apparent. First, the morphology of WM793 cells changed dramatically, becoming much larger and flatter and also less refractile (Fig. 3*B*). In addition, far fewer WM793 cells were recovered after

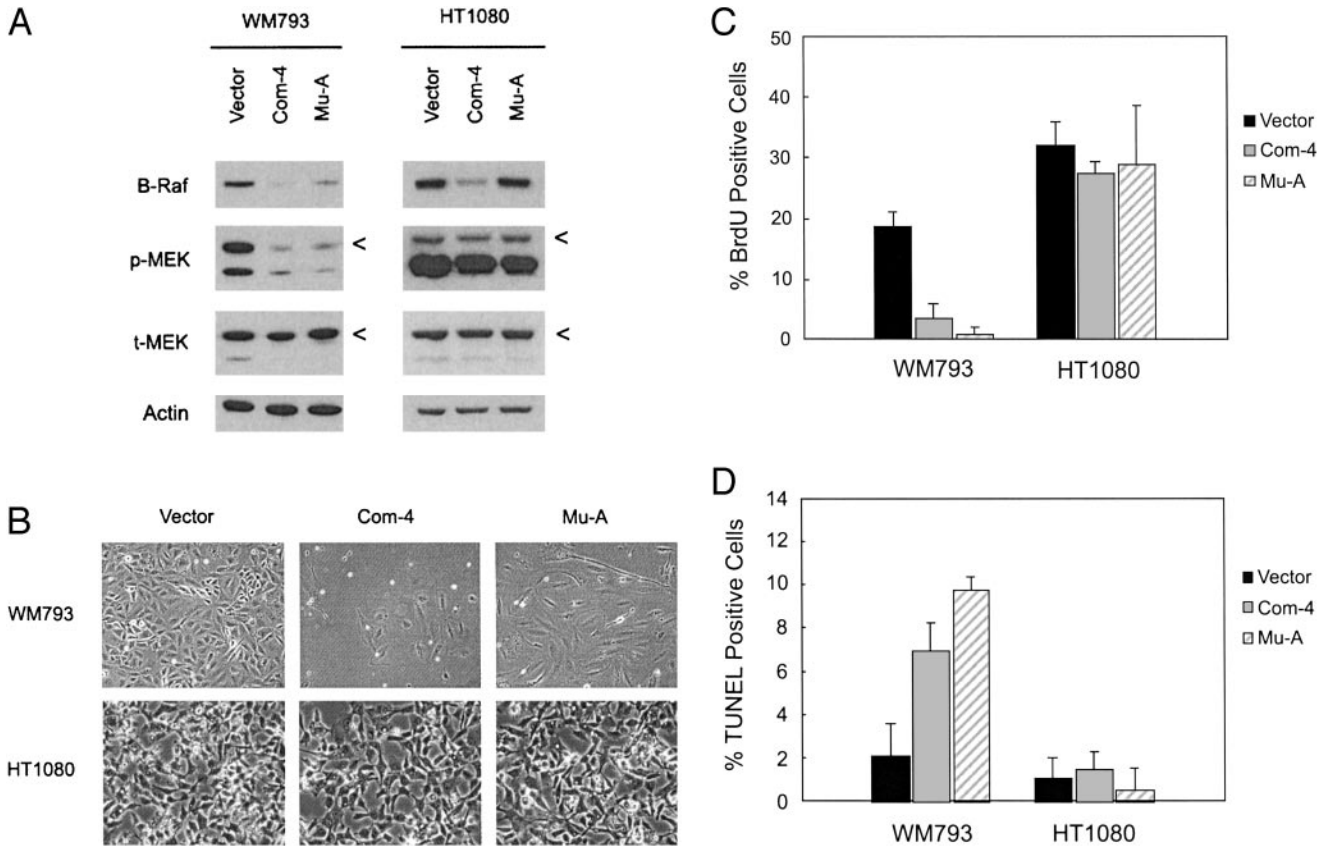

Fig. 3. Effects of stable suppression of endogenous *BRAF* and *BRAF*^{V599E} in WM793 and HT1080 cells. Subconfluent cultures of WM793 or HT1080 cells were transfected with pSUPER.retro (vector), shRNA directed against *BRAF* (Com-4), or shRNA directed against *BRAF*^{V599E} (Mu-A). After selection in puromycin, the biochemical and biological properties of these cells were assessed. *A*, immunoblot analysis of B-Raf, activated MEK (*p-MEK*), total MEK (*t-MEK*), and actin levels. The positions of the M_r 45,000 phospho- and total-MEK 1/2 proteins are indicated. *B*, phase contrast microscopy of cells in culture (magnification: ×100). *C*, proliferative index as measured by BrdUrd incorporation. *D*, degree of apoptosis as assessed by TUNEL reactivity. BrdUrd and TUNEL data are expressed as the means +/− SD of direct counting of three high-powered fields. These experiments were performed twice with similar results.

transfection with Com-4, as compared with control vector or Mu-A, suggesting that wild-type B-Raf function may also be important for the viability of these cells. No appreciable differences in morphology or cell number were noted in parallel transfections of HT1080 cells (Fig. 3*B*). In addition, Com-4 and Mu-A-transfected WM793 cells exhibited markedly lower proliferative rates; the percentage of cells in S phase was ~4 +/− 2% and 0.8 +/− 1%, respectively, as compared with 19 +/− 2% for cells transfected with empty vector (Fig. 3*C*). The proliferation of HT1080 cells was not affected by transfection with any of these vectors, again despite achieving significant knockdown of B-Raf levels (Fig. 3*C*). WM793 cells also exhibited increased levels of apoptosis after stable suppression of *BRAF*, whereas human fibrosarcoma cells again remained unaffected by similar manipulations (Fig. 3*D*).

These results demonstrated that *BRAF*-dependent signaling was necessary for the optimal proliferation and survival of human melanoma WM793 cells and dispensable for human fibrosarcoma cells. We wondered whether these effects were specific for the *BRAF* family member of the Raf kinases or extended to the heretofore more extensively studied homologue, *CRAF*. We therefore generated *CRAF*-specific duplex siRNA species and stably expressing shRNA vectors and tested their abilities to suppress *CRAF* expression and inhibit downstream phosphorylation of MEK. As shown in Fig. 4*A*, knockdown of C-Raf protein levels by siRNA followed a slower time course than that of B-Raf and remained more durably suppressed. Notably, however, there appeared to be no effect on MEK phosphorylation despite nearly complete suppression of *CRAF*. Thus, at least in WM793 melanoma cells, *CRAF* appears not to be required for MEK activation. A stably expressing shRNA vector directed against the same sequence similarly knocked down *CRAF* expression with no attendant affect on MEK phosphorylation (Fig. 4*B*).

Finally, the ability to manifest anchorage-independent growth, an established feature of cellular transformation, was assessed in melanoma cells after the knockdown of *BRAF*, *BRAF*^{V599E}, or *CRAF*. WM793 cells transfected with empty vector readily formed colonies in soft agar (Fig. 4*C*). Cells in which C-Raf levels had been stably knocked down formed colonies almost as readily. Transfection with either Com-4 or Mu-A, however, essentially completely abrogated the ability of these cells to manifest anchorage-independent growth. Therefore, *BRAF* is uniquely required for cellular transformation in WM793 cells.

Discussion

Oncogenesis is generally viewed as a multistep process characterized by the progressive acquisition of genetic mutations and functional capabilities (15). The hope for curative therapies lies in the proposition that key genetic events exist which represent unique points of vulnerability for cancer cells. Indeed, despite the complexity of genetic and epigenetic alterations in cancer cells and their microenvironment, recent evidence demonstrates that the specific inhibition of one, or perhaps a few, critical pathways in tumor cells may be sufficient to kill them and provide significant clinical benefit. As examples, treatment of patients with stable phase chronic myeloge-

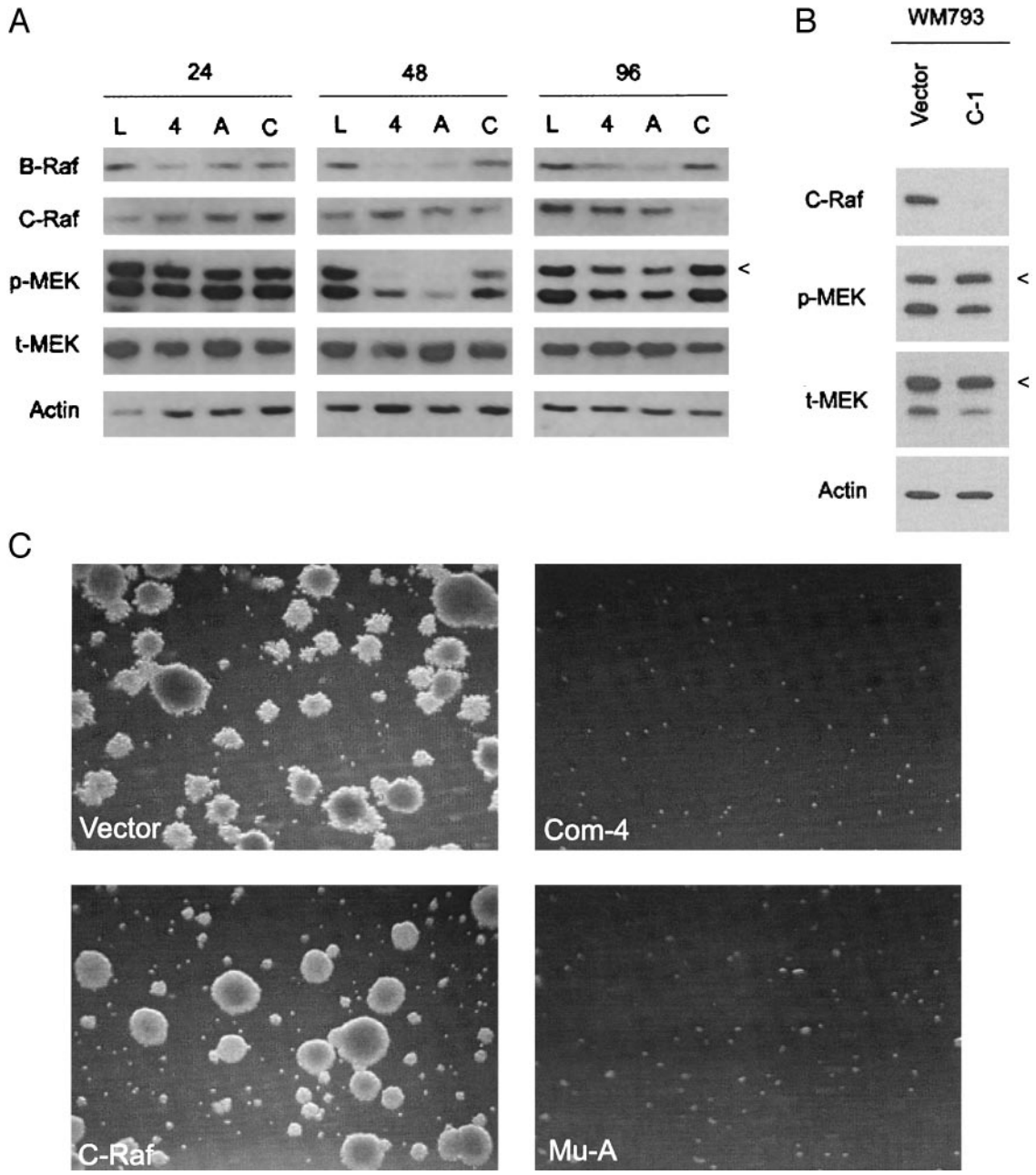

Fig. 4. Effects of transient and stable suppression of *BRAF* or *CRAF* expression in WM793 cells. In *A*, WM793 cells were transfected with siRNA directed against *Lamin A/C* (*L*), *BRAF* (*4*), *BRAF*^{V599E} (*A*), and *CRAF* (*C*) and analyzed for specific protein expression at 24, 48, and 96 h post-transfection. In *B*, WM793 cells were stably transfected with pSUPER.retro (vector) or shRNA directed against *CRAF* (C-1), subjected to antibiotic selection, and assessed for knockdown of C-Raf, activated MEK (*p-MEK*), total MEK (*t-MEK*), and actin levels. The positions of the M_r 45,000 phospho- and total-MEK 1/2 proteins are indicated. In *C*, WM793 cells were stably transfected with pSUPER.retro, Com-4 shRNA against BRAF, C-1 shRNA against C-Raf, or Mu-A shRNA against *BRAF*^{V599E} and plated onto semisolid media. Colonies were counted and photographed after 30 days of growth (magnification: ×40). These results are representative of two independent experiments.

nous leukemia or advanced gastrointestinal stromal tumors with imatinib mesylate, a small molecule inhibitor of the *ABL* and *KIT* tyrosine kinases, respectively, induces dramatic remissions with minimal toxicity (16, 17). Importantly, the responses of chronic myelogenous leukemia and gastrointestinal stromal tumor patients to imatinib correlates with the drug's ability to inhibit the kinase activities of Bcr-Abl and mutant c-kit (18, 19), respectively. These results suggest that essential pathways may exist in other malignancies and that biochemical confirmation of the effectiveness of molecularly targeted therapies may be predictive of clinical efficacy.

Our findings suggest that the *BRAF*^{V599E} mutation commonly found in malignant melanomas may represent a therapeutic target analogous to *BCR-ABL* and *KIT*. We have demonstrated here that knockdown of *BRAF* expression and inhibition of downstream signaling in WM793 human melanoma cells causes growth arrest and promotes apoptosis under adherent conditions, and prevents colony formation in suspension. These observations have been preliminarily extended to a second melanoma cell line known to contain the *BRAF*^{V599E} mutation (data not shown). These effects were specific to *BRAF*, as suppression of *CRAF* failed to inhibit downstream phosphorylation of MEK and did not appreciably alter the biological properties of these cells. Moreover, these effects were specific to melanoma cells, because human fibrosarcoma cells were impervious to suppression of *BRAF* expression.

Currently, a Raf kinase inhibitor, BAY 43–9006 (20), is undergoing worldwide clinical evaluation in Phase I and II trials in patients with a variety of malignancies, including melanoma. However, BAY 43–9006 inhibits both B-Raf and C-Raf kinase activities,[4] and any beneficial or adverse effects of treatment may therefore result from simultaneous inhibition of both kinases. Our results suggest that targeted inhibition of B-Raf specifically in such tumors may be equally efficacious and perhaps associated with less toxicity.

That *CRAF* expression was dispensable for the transformed phenotype in human melanoma cells was somewhat surprising. As the first of the three Raf family isoforms identified, a large body of evidence exists exploring the transforming properties of *CRAF* in mammalian cell systems. These properties, however, are exquisitely dependent on cellular context (7); for example, although a constitutively active form of *CRAF* can readily transform NIH 3T3 cells, it is unable to do so in RIE-1 cells (21). More recent experiments involving targeted disruption and mutation of Raf isoforms in mice implicate B-Raf as the more potent activator of MEK in many cell and tissue types (22). The minimal effects on transformation of *CRAF* suppression in the melanoma cells studied here suggest that this isoform may not always be the predominant effector of MAPK signaling in human cells either.

In summary, we find that suppression of *BRAF^{V599E}* in WM793 human melanoma cells abrogates their transformed phenotype and conclude, therefore, that agents that specifically inhibit activated *BRAF*, and not *CRAF*, might be particularly efficacious in melanomas, and perhaps other tumor types, that harbor activating mutations in this proto-oncogene.

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