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## Targeting Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase Kinase in the Mutant (V600E) B-Raf Signaling Cascade Effectively Inhibits Melanoma Lung Metastases

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### Abstract

Malignant melanoma has a high propensity for metastatic spread, making it the most deadly form of skin cancer. *B-RAF* has been identified as the most mutated gene in these invasive cells and therefore an attractive therapeutic target. However, for uncertain reasons, chemotherapy inhibiting B-Raf has not been clinically effective. This has raised questions whether this pathway is important in melanoma metastasis or whether targeting a protein other than B-Raf in the signaling cascade could more effectively inhibit this pathway to reduce lung metastases. Here, we investigated the role played by <sup>V600E</sup>B-Raf in melanoma metastasis and showed that targeting this signaling cascade significantly reduces lung metastases. Small interfering RNA (siRNA)-mediated inhibition was used in mice to reduce expression (activity) of each member of the signaling cascade and effects on metastasis development were measured. Targeting any member of the signaling cascade reduced metastasis but inhibition of mitogen-activated protein kinase/extracellular signal-regulated kinase (Mek) 1 and Mek 2 almost completely prevented lung tumor development. Mechanistically, metastatic inhibition was mediated through reduction of melanoma cell extravasation through the endothelium and decreased proliferative capacity. Targeting B-Raf with the pharmacologic inhibitor BAY 43-9006, which was found ineffective in clinical trials and seems to act primarily as an angiogenesis inhibitor, did not decrease metastasis, whereas inhibition of Mek using U0126 decreased cellular proliferative capacity, thereby effectively reducing number and size of lung metastases. In summary, this study provides a mechanistic basis for targeting Mek and not B-Raf in the mutant <sup>V600E</sup>B-Raf signaling cascade to inhibit melanoma metastases.

### Introduction

Malignant melanoma is the most deadly form of skin cancer due to its highly metastatic nature (1–3). The disease develops in a stepwise manner as evolving cancer cells undergo cellular

changes, resulting from genetic alteration, which promote development of more aggressive metastatic tumors (1,4). These changes include accelerated cell growth, enhanced survival under conditions that would kill a normal cell, development of blood vessels as tumors increase in size, and, finally, invasion of cancer cells into surrounding tissues culminating in metastasis to distant organs (1,4). Although clinical trials have tested a wide range of approaches ranging from immunotherapy, radiotherapy, and chemotherapy, no effective therapy exists for patients to inhibit the metastatic spread of melanoma (5–8).

Several studies have implicated the mitogen-activated protein (MAP) kinase [Ras/Raf/MAP kinase-extracellular signal-regulated kinase (Erk) kinase (Mek)/Erk] pathway in melanoma metastasis by promoting processes such as cell proliferation, survival, invasion, and tumor angiogenesis (9–12). The signaling cascade is activated in ~60% of melanomas by a single-base mutation in *B-RAF* converting T to A at nucleotide 1,799, substituting a valine for a glutamic acid at codon 600 (V600E) in exon 15 (13). The mutation was originally misidentified as occurring at codon 599 instead of codon 600; therefore, it is referred to as either V600E or V599E in the literature (13–15). *B-RAF* mutation is acquired during development of sporadic melanomas and therefore not inherited in familial melanomas (16–18).

The role of B-Raf in the signaling cascade is as an intermediate kinase relaying extracellular signals via an ordered series of consecutive phosphorylation events from cell surface through Ras to nucleus (9,11). The <sup>V600E</sup>B-Raf mutation leads to constitutive activation of B-Raf and increased activity or expression of downstream members of the signaling cascade (13–15). For example, increased phosphorylation of Mek and Erk, as well as increased expression of cyclin D1, has been reported in cells expressing <sup>V600E</sup>B-Raf protein (14,15). Metastasis requires that melanoma cells pass into the vascular system (intravasation), survive the stresses encountered during blood flow, pass through the endothelial lining of vessels into the tissue (extravasation), and finally proliferate in the new tissue environment (19). The role played by mutant <sup>V600E</sup>B-Raf protein to facilitate these processes remains uncertain.

Because *B-RAF* is the most mutated gene in melanomas, it is an attractive therapeutic target to inhibit metastatic spread (14). Unfortunately, targeting B-Raf in melanomas using a Raf kinase inhibitor, called BAY 43-9006 (Sorafenib), has not been clinically effective (14,20). This has raised concerns about the importance of this pathway in promoting melanoma metastasis (15,21,22). Furthermore, it remains uncertain whether lack of therapeutic success was due to the mechanism of action of the Raf kinase inhibitor BAY 43-9006 or whether targeting mutant <sup>V600E</sup>B-Raf is not the most efficient strategy to inhibit this signaling cascade (14,20,23–25). Targeting another member of this signaling cascade might more effectively inhibit this pathway, thereby more efficiently reducing melanoma metastasis (26–28). Finally, the mechanism by which <sup>V600E</sup>B-Raf promotes metastasis remains uncertain (14).

This study investigates these questions by identifying the role played by <sup>V600E</sup>B-Raf in melanoma metastasis and determining whether targeting this signaling cascade reduces metastatic tumor development. Results show that targeting any member of the signaling cascade (<sup>V600E</sup>B-Raf, Mek, Erk, or cyclin D1) reduced metastasis; however, simultaneous inhibition of Mek 1 and Mek 2 almost completely prevented tumor development in the lungs. Mechanistically, inhibition of metastasis occurred through a reduction of melanoma cell extravasation into lung tissue and by slowed cell proliferation in the lung microenvironment. Thus, these studies provide a rationale and a mechanistic basis for therapeutically targeting mutant <sup>V600E</sup>B-Raf signaling to inhibit melanoma metastasis.

## Materials and Methods

### Human melanoma cell lines, culture conditions, and *B-RAF* mutational status

The human melanoma cell lines 1205 Lu, A375M, C8161.C19, UACC 903, and UACC 903M were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Logan, UT). Green fluorescent protein (GFP)-tagged versions of 1205 Lu, UACC 903M, and C8161.C19 were used for metastasis assays. UACC 903M, a highly metastatic variant of the UACC 903 cell line, was derived by i.v. injecting GFP-tagged UACC 903 cells into nude mice and, 30 days later, establishing cell lines from tumors, which had formed in the lungs. The resultant cell line called UACC 903M was highly metastatic to the lungs following i.v. injection of the cells. The presence or absence of the T1799A *B-RAF* mutation in cell lines was undertaken as previously described (29).

### *In vitro* siRNA studies

SiRNA (100 pmol) was introduced into  $1 \times 10^6$  1205 Lu, A375M, C8161.C19, or UACC 903 cells via nucleofection with an Amaxa Nucleofector (Koeln, Germany), as described in refs. 12,30. Solution R/program K-17 was used for 1205 Lu, C8161.C19, and UACC 903 cell lines whereas Solution R/program A-23 was used for A375M cells. The resultant transfection efficiency following nucleofection was >90%. Duplexed Stealth siRNA (Invitrogen) was used for these studies. The sequence for each respective siRNA is as follows: scrambled siRNA, AAUUCUCCGAACGUGU-CACGUGAGA; *C-RAF*, GGUCA AUGUGCGAAAUGGAAUGAGC; WT *B-RAF* (Com4 or 4), GGACAAAGAAUUGGAUCUGGAUCAU; WT/MUT *B-RAF* (MuA or A), GGUCUAGCUACAGAGAAAUCUCGAU; *MEK 1*, CCCGCAAUCCGGAAC-CAGAUCAUAA; *MEK 2*, GAACUCAAAAGACGAUGACUUCGAAA; *ERK 1*, CCCUGGAAGCCAUGAGAGAUGUCUA; *ERK 2*, CCGAAGCACCAUUCAA-GUUCGACAU; and *CYCLIN D1*, CCACAGAUGUGAAGUUCAUUCCAA. Following nucleofection, cells were replated and, 72 hours later, protein lysates were harvested for Western blot analysis. Duration of siRNA knockdown was measured in replated cells harvested 0, 2, 4, 6, and 8 days following nucleofection with siRNA to *B-RAF*, *C-RAF*, *MEK 1*, *MEK 2*, *ERK 1*, *ERK 2*, and *CYCLIN D1* and cell lysates were subjected to Western blot analysis.

### Western blot analysis

For Western blot analysis, cell lysates were harvested in Petri dishes by the addition of lysis buffer containing 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10 mmol/L EDTA, 10% glycerol, 1% Triton X-100, 1 mmol/L sodium orthovanadate, 0.1 mmol/L sodium molybdate, 1 mmol/L phenylmethylsulfonyl fluoride, 20  $\mu$ g/mL aprotinin, and 5  $\mu$ g/mL leupeptin. Whole-cell lysates were centrifuged ( $\geq 10,000 \times g$ ) for 10 minutes at 4°C to remove cell debris. Protein concentration was quantified using the BCA Assay from Pierce (Rockford, IL) and 30  $\mu$ g of protein lysates per lane were loaded onto a NuPage Gel (Life Technologies, Inc., Carlsbad, CA). Following electrophoresis, samples were transferred to polyvinylidene difluoride membrane (Pall Corporation, Pensacola, FL). The blots were probed with antibodies according to the recommendations of suppliers: anti-pErk and anti-pMek from Cell Signaling Technologies (Beverly, MA); antibodies to B-Raf, C-Raf, Erk 2, cyclin D1, p27, and  $\alpha$ -enolase from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies were conjugated with horseradish peroxidase and obtained from Santa Cruz Biotechnology. The immunoblots were developed using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

### ***In vitro* extravasation assays**

Three days after nucleofection, migration of melanoma cells across an endothelial-like cell layer under flow conditions was examined using a modified 48-well Boyden flow migration chamber (31,32). The device contains 48 wells in the bottom plate with an inlet and outlet connected to the upper chamber to enable flow conditions. A monolayer of confluent EI cells was grown (typically 36–48 hours after cell seeding) on a sterile 8- $\mu$ m pore size polyvinylpyrrolidone-free polycarbonate filter (NeuroProbe, Gaithersburg, MD) precoated for 3 hours with 30  $\mu$ g/mL fibronectin (Sigma, St. Louis, MO). EI cells, fibroblast L-cells transfected to express human E-selectin and intercellular adhesion molecule 1, were generously provided for these studies by Dr. Scott Simon (University of California at Davis, Davis, CA) and maintained as previously described (33). The 12 center wells in the bottom plate were filled with soluble type IV collagen chemoattractant (100  $\mu$ g/mL in RPMI 1640/0.1% BSA; BD Biosciences, Lexington, KY). Surrounding control wells were filled with RPMI 1640 supplemented with 0.1% BSA. Apparatus was assembled by placing filter on plate followed by addition of a sealing gasket and top plate. Flow was initiated using 37°C medium to purge bubbles from the system. For migration assays,  $5 \times 10^5$  melanomas cells only or melanoma cells together with neutrophils ( $5 \times 10^5$  of each) were pipetted into the chamber subjected to static conditions or 4 dyn/cm<sup>2</sup> shear flow for 4 hours in a 37°C, 5% CO<sub>2</sub> incubator. Human neutrophils were isolated according to a protocol approved by The Pennsylvania State University Institutional Review Board that involved isolating cells from whole blood using a Ficoll-Hypaque (Histopaque, Sigma, Milwaukee, WI) density gradient according to the protocol of the manufacturer. Following isolation, neutrophils were stored in PBS supplemented with 0.1% human serum albumin at 4°C until use. Migration was quantified by staining of the filter using Protocol Brand Hema3 solution (Fisher Scientific, Pittsburgh, PA). Melanoma cells migrating to the underside of the filter were photographed using an inverted microscope (Diaphot 330, Nikon, Japan) with NIH Image software (v.β4.0.2) and counted.

### ***In vivo* siRNA and pharmacologic studies**

Animal experimentation was undertaken according to protocols approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University College of Medicine. SiRNA (100 pmol) was nucleofected into GFP-tagged 1205 Lu, UACC 903M, or C8161.C19 cells, which were then replated in culture dishes. Thirty-six hours later,  $1 \times 10^6$  1205 Lu or C8161.C19 cells or  $0.5 \times 10^6$  UACC 903M cells in 0.2 mL of HBSS were injected i.v. into the lateral tail vein of nude mice. Mice were sacrificed 17 days later, necropsied, and lungs were analyzed for the presence of fluorescent tumors using a Nikon SMZ 1500 dissecting microscope with a Plan Apo 1.6 $\times$  objective and fluorescence detection capabilities. Images of five random fields were photographed at a magnification of 4.8 $\times$  from the ventral surface of each lung and the number of fluorescent tumors as well as area scored in pixels occupied by each tumor was quantified using IP lab imaging software (Scanalytics, Fairfax, VA). Duplicate experiments consisting of eight animals were used per group. For tumor proliferation studies,  $5 \times 10^6$  1205 Lu cells nucleofected with siRNA were s.c. injected into mice and tumors harvested 4 days later to measure changes in cell proliferation or apoptosis, as previously described (12,30,34).

Effect of the Mek inhibitor U0126 (35) or Raf kinase inhibitor BAY 43-9006 (12,24,36) on metastasis development was measured by pretreatment of mice with compound (U0126, 10 mg/kg body weight; BAY 43-9006, 75 mg/kg body weight) by i.p. injection twice (–4 and –2 days) before i.v. injection of 1205 Lu, UACC 903M, or C8161.C19 cells and every 2 days thereafter. Tumor proliferation rates following U0126 or BAY 43-9006 treatment were determined by comparing tumors of the same size exposed to DMSO vehicle 4 days after s.c. injection of melanoma cells into nude mice. U0126 was obtained from Cell Signaling whereas

the BAY 43-9006 compound used for these studies was synthesized as described in refs. 12, 37.

Cell proliferation rates in formalin-fixed tumor sections were measured using the RPN 20 cell proliferation kit (Amersham Biosciences) that uses bromodeoxyuridine (BrdUrd) incorporation and immunocytochemistry. Two hours before sacrificing, 1 mg/100 gm body weight of BrdUrd was injected i.p. into mice and tumors were processed according to the proliferation kit instructions. The number of BrdUrd stained cells was scored as the percentage of total cells of tumors treated with siRNA, U0126, BAY 43-9006, or vehicle (DMSO). For all tumor analyses, a minimum of four different tumors with four to six fields per tumor were analyzed and results represented as the average  $\pm$  SE.

### Cell doubling time

The *in vitro* doubling time of 1205 Lu, A375M, and UACC 903 cells nucleofected with control or experimental siRNA was estimated by plating  $3 \times 10^3$  per well for UACC 903,  $2.5 \times 10^3$  per well for A375M, or  $4 \times 10^3$  per well for 1205 Lu in 200  $\mu$ L of DMEM supplemented with 10% FBS in multiple rows of wells in five 96-well plates. Growth was measured colorimetrically using the sulforhodamine B binding assay (Sigma Chemical Co., St. Louis, MO) every 24 hours over a period of 5 days on one plate each day. Absorbance was measured at 570 nm using a Perkin-Elmer HTS 700+ Bioassay Plate Reader (Foster City, CA). Doubling times were calculated using the exponential equation of a best-fit line ( $y = y_0e^{ax}$ ) for each condition (12). Values are averages  $\pm$  SE obtained from three independent experiments by calculating the amount of time ( $x$ ) it took the absorbance ( $y$ ) to double.

### Statistics

For statistical analysis, the Student's *t* test was used for pairwise comparisons and the ANOVA or the Kruskal-Wallis one-way ANOVA test was used for groupwise comparisons, followed by the appropriate post hoc tests (Dunnett's, Tukey's, or Dunn's test). Results were considered significant at  $P < 0.05$ .

## Results

### Targeting mutant <sup>V600E</sup>B-Raf signaling in melanoma cells inhibits development of lung metastases

Although mutant <sup>V600E</sup>B-Raf protein has been shown to play an important role in melanoma tumor development (12,38,39), its role in metastasis remains uncertain (14). To develop a more thorough understanding of its function in metastasis, expression (activity) of B-Raf was decreased in metastatic human melanoma cells containing <sup>V600E</sup>B-Raf (1205 Lu, A375M, and UACC 903) and compared with the C8161.C19 cell line lacking the T1799A mutation. siRNA specifically designed to inhibit expression of mutant <sup>V600E</sup>B-Raf protein (MuA or A) or to simultaneously reduce expression of both wild-type and mutant protein (Com4 or 4) were used for this study (12,38). Experimental metastasis development was studied by nucleofecting siRNA into melanoma cells, allowing recovery in culture for 1.5 days (36 hours) followed by i.v. injection of cells into the tail vein of nude mice. Seventeen days later, the lungs of mice were removed and number as well as area occupied by GFP-expressing tumors was quantified. Nucleofection resulted in siRNA transfection efficiencies of >90% (12,30) and knockdown of B-Raf protein in 1205 Lu melanoma cells persisted beyond 8 days in culture compared with control cells nucleofected with scrambled siRNA (Fig. 1A; ref. 12). Similar levels and durations of siRNA-mediated B-Raf protein knockdown were observed for UACC 903M and C8161.C19 cell lines (not shown). Reducing expression (activity) of <sup>V600E</sup>B-Raf significantly retarded the metastatic potential of 1205 Lu (Fig. 1B–E;  $P < 0.05$ , one-way ANOVA) and UACC 903M (Fig. 1F;  $P < 0.05$ , *t* test) cells containing mutant <sup>V600E</sup>B-Raf but not of C8161.C19 cells lacking



it (not shown). Fewer fluorescent tumors were observed in the lungs of animals injected with melanoma cells in which  $V^{600E}$ B-Raf had been inhibited, suggesting that less cells extravasated through the endothelial lining or become entrapped in lung vessels and proliferated in the lung tissue (Fig. 1C). In contrast, control animals injected with cells nucleofected with scrambled siRNA or buffer had significantly more metastases, occupying a larger overall area of lung than cells in which B-Raf had been inhibited (Fig. 1D;  $P < 0.05$ , one-way ANOVA). Metastases were placed into two pixel-based categories based on size (i.e., those  $< 1,500$  and  $> 1,500$  pixels). A significant 5- to 7-fold decrease in tumors  $> 1,500$  pixels was observed following  $V^{600E}$ B-Raf inhibition compared with control 1205 Lu cells nucleofected with buffer or scrambled siRNA (Fig. 1E). Similar results were observed in UACC 903M cells following  $V^{600E}$ B-Raf inhibition in which there was a ~2- to 3-fold decrease in metastases  $> 1,500$  pixels compared with a scrambled siRNA control (Fig. 1F;  $P < 0.05$ ,  $t$  test). In stark contrast, inhibition of *B-RAF* in the C8161.C19 cell line lacking the T1799A mutation had no effect on metastatic potential of these cells (not shown). Collectively, these data show that inhibition of B-Raf in melanoma cells containing, but not those lacking, the T1799A B-Raf mutation reduces development of metastatic lung tumors.

### Inhibition of proteins downstream of mutant $V^{600E}$ B-Raf retarded metastasis development and identified Mek 1/2 proteins as target to most effectively inhibit metastasis

Whereas inhibiting  $V^{600E}$ B-Raf can reduce melanoma cell lung metastasis (Fig. 1), it is unknown whether targeting another protein in the signaling cascade would more efficiently inhibit this process. To investigate this unknown possibility, signaling through the mutant  $V^{600E}$ B-Raf pathway was initially confirmed by measuring changes in phosphorylation or expression levels of downstream proteins in the MAP kinase pathway following siRNA-mediated knockdown of  $V^{600E}$ B-Raf protein (Fig. 2A; refs. 12,38,39). Western blot analysis showed reduced  $V^{600E}$ B-Raf expression in 1205 Lu cells 72 hours after siRNA introduction with a corresponding decrease in phosphorylation (activity) levels of downstream targets Mek and Erk as well as reduced amounts of cyclin D1. In contrast, no changes were observed in phosphorylation levels of Mek, Erk, or cyclin D1 in control untransfected or cells transfected with buffer, scrambled siRNA, or siRNA against *C-RAF* (Fig. 2A). Because changes in cyclin D1 levels were subtle following  $V^{600E}$ B-Raf knockdown 72 hours (3 days) after introduction of siRNA into cells, a time course was undertaken examining levels 2, 4, 6, and 8 days after siRNA-mediated knockdown of  $V^{600E}$ B-Raf protein expression (Fig. 2B). Cyclin D1 levels decreased significantly between days 4 and 6 in 1205 Lu cells, indicating that targeting  $V^{600E}$ B-Raf reduced downstream levels of this protein whereas inhibition of *C-Raf* did not. Signaling through the mutant  $V^{600E}$ B-Raf signaling cascade was also verified in UACC 903 (Fig. 2C) and A375M (Fig. 2D) melanoma cell lines yielding similar results. A corresponding increase in p27 levels accompanied declining cyclin D1 levels in all cell lines (Fig. 2B–D). Low levels of pErk in A375M cells made altered activity levels difficult to measure; however, downstream cyclin D1 levels were decreased. These results confirmed that the mutant  $V^{600E}$ B-Raf signaling cascade relayed signals from  $V^{600E}$ B-Raf to Mek 1/2 to Erk 1/2 to cyclin D1 in melanoma cells, thereby regulating cellular proliferation. Thus, all these proteins were potential targets in the signaling cascade that could inhibit metastasis development.

Having verified the signal relay through members of the  $V^{600E}$ B-Raf pathway in melanoma cells, we next examined whether any particular protein of the signaling cascade would more efficiently inhibit metastatic tumor development in the lungs of animals compared with targeting mutant  $V^{600E}$ B-Raf. Members of the signaling cascade, including  $V^{600E}$ B-Raf, Mek 1/2, Erk 1/2, and cyclin D1, were inhibited using siRNA specific against each gene and consequences on lung metastasis development were compared. Each siRNA knocked down expression of its target protein to approximately equivalent levels, which persisted beyond 8

days in culture (not shown). Reduced protein expression 72 hours (3 days) after nucleofection of each respective siRNA into 1205 Lu cells is shown in Fig. 3A. Following knockdown of protein expression, number and size of fluorescent tumors present in the lungs of mice were measured 17 days after i.v. injecting cells nucleofected with either control or siRNA to each respective member of the  $V^{600E}$ B-Raf signaling cascade (Fig. 3B). Targeted inhibition of each member of the  $V^{600E}$ B-Raf signaling cascade significantly retarded development of lung metastases compared with buffer or scrambled siRNA controls (Fig. 3B;  $P < 0.05$ , Kruskal-Wallis ANOVA). However, the most dramatic reduction in metastasis development occurred following simultaneous inhibition of both Mek 1 and Mek 2, which nearly eliminated metastasis formation. In contrast to combined Mek 1/2 inhibition, targeting each independently or Erk 1 and Erk 2 simultaneously were not as effective at reducing metastasis development. Collectively, these data showed that siRNA-mediated inhibition of downstream members of the mutant  $V^{600E}$ B-Raf signaling pathway reduced metastasis development. However, simultaneous inhibition of Mek 1 and Mek 2 most dramatically inhibited formation of metastases, suggesting that these proteins in the  $V^{600E}$ B-Raf pathway should be targeted to inhibit effectively melanoma lung metastases.

### Reduced melanoma cell extravasation and proliferation following inhibition of $V^{600E}$ B-Raf signaling retard melanoma lung metastases

Melanoma cell lung metastasis involves extravasation through the endothelial lining of lung vessels or entrapment in the lung microvasculature followed by active proliferation in lung tissue (19). Disruption of any of these processes can reduce development of melanoma metastases (19). To determine which of these processes  $V^{600E}$ B-Raf signaling regulated to promote metastasis, the pathway was inhibited and effect on extravasation and proliferation examined. Extravasation of melanoma cells through the endothelial lining of lung vessels was evaluated using an *in vitro* flow-migration assay (31,32). Using this model, migration of melanoma cells alone or in combination with neutrophils across an endothelial-like layer was measured by placing cells into a chamber in which fluid movement could be controlled and quantifying migration across an endothelial cell like layer in response to collagen IV chemoattractant placed beneath the cell layer (Fig. 4A). Migration under flow conditions (4 dyn/cm<sup>2</sup>) recapitulates the physical dynamics of a blood capillary where cell adhesion to the endothelial-like layer corresponded to binding to the vessel wall and migration of tumor cells from the top to the bottom compartment represented extravasation into the tissue space (31, 32,40). Under static conditions and in the absence of neutrophils, melanoma cell migration was significantly reduced following B-Raf inhibition (Fig. 4A, *Static*;  $P < 0.05$ , one-way ANOVA). In contrast, under flow conditions of 4 dyn/cm<sup>2</sup>, no migratory difference was observed between control cells or those in which B-Raf had been inhibited. However, combining neutrophils with 1205 Lu melanoma cells promoted significant migration under flow conditions of 4 dyn/cm<sup>2</sup> (Fig. 4A, +*Neutrophil*;  $P < 0.05$ , one-way ANOVA) compared with melanoma cells alone (Fig. 4A, -*Neutrophil*). In the presence of neutrophils, inhibiting  $V^{600E}$ B-Raf in melanoma cells significantly retarded migration ( $P < 0.05$ , one-way ANOVA). Thus, inhibition of mutant  $V^{600E}$ B-Raf signaling significantly reduced extravasation of melanoma-neutrophil cell complexes or melanoma cells alone across an endothelial-like cell layer, suggesting one potential mechanism for preventing metastasis development.

Extravasated or entrapped cells within lung tissue need to proliferate to form secondary tumors (19). To determine whether reduced growth of melanoma cells following  $V^{600E}$ B-Raf inhibition also contributed to decreased lung metastases, growth of tumor cells was measured following siRNA-mediated inhibition of  $V^{600E}$ B-Raf, Mek 1/2, Erk 1/2, and cyclin D1 and compared with controls (Table 1). 1205 Lu, A375M, and UACC 903 cells nucleofected with buffer or scrambled siRNA doubled in number *in vitro* every ~41, ~33, and ~27 hours, respectively (Table 1). In contrast, cells nucleofected with siRNA against  $V^{600E}$ B-Raf grew

29% to 41% slower than controls, similar to prior reports (12,38,39). To identify which proteins downstream of B-Raf contributed to decreased proliferation, each downstream protein (Mek 1, Mek 2, Erk 1, Erk 2, or cyclin D1) was initially inhibited individually. No consistent pattern was observed that was common to all three cell lines. This result suggested that knockdown of one isoform might be compensated for by another isoform. Therefore, we simultaneously targeted Mek 1 and Mek 2 or Erk 1 and Erk 2. Similar to results observed following knockdown of  $V^{600E}$ B-Raf, targeting Mek 1 and Mek 2 or Erk 1 and Erk 2 together consistently reduced cellular proliferation by 18% to 63% for all cell lines.

Consistent with the reduced proliferative capacity observed *in vitro*, decreased proliferation was observed in 1205 Lu tumors following inhibition of  $V^{600E}$ B-Raf (Fig. 4B). Rates of tumor cell proliferation and apoptosis were measured 4 days after s.c. injection in nude mice. Whereas no significant difference was detected in numbers of cells undergoing apoptosis (not shown), siRNA-mediated inhibition of B-Raf led to 2- to 3-fold fewer proliferating cells in tumors compared with control cells nucleofected with buffer, scrambled siRNA, or *C-RAF* siRNA (Fig. 4C). The ~75% reduction in proliferative capacity of tumor cells nucleofected with *B-RAF* siRNA showed that decreased proliferative capacity contributed to reduced metastasis development. Thus, inhibition of the mutant  $V^{600E}$ B-Raf signaling pathway decreased tumor cell extravasation and proliferation to inhibit metastasis.

### Pharmacologic inhibition of Mek, but not of B-Raf, retards development of melanoma lung metastases

Because siRNA-mediated inhibition of mutant  $V^{600E}$ B-Raf signaling reduced lung metastasis development, the effects of pharmacologic inhibition of B-Raf or Mek were examined next to determine whether a Mek inhibitor more effectively inhibited metastasis than a B-Raf inhibitor. BAY 43-9006, a nonspecific Raf kinase inhibitor, and U0126, a specific Mek inhibitor, were evaluated for effectiveness in preventing lung metastasis development. Mice were pretreated with each respective compound (75 mg/kg BAY 43-9006 or 10 mg/kg U0126) 4 and 2 days before i.v. injection of metastatic melanoma cells and every 2 days following i.v. injection of GFP-tagged 1205 Lu or UACC 903M tumor cells up to day 17 (12,24,35,36). Both compounds inhibited the  $V^{600E}$ B-Raf signaling pathway in culture (not shown), consistent with our prior reports and those of others (12,23). However, in animals, BAY 43-9006 treatments had no effect on metastasis development of 1205 Lu (Fig. 5A) or C8161.C19 cells (not shown). Thus, irrespective of whether the metastatic cells contained or lacked  $V^{600E}$ B-Raf, treatment with BAY 43-9006 had no effect on metastasis. In contrast, Mek inhibition using U0126 significantly reduced metastasis in both 1205 Lu and UACC 903M cell lines compared with mice exposed to DMSO vehicle (Fig. 5A;  $P < 0.05$ , *t* test). Both number and size of tumors developing in the lungs were reduced following Mek inhibition but not following B-Raf inhibition. Mechanistically, fewer proliferating cells were observed in mice exposed to the Mek inhibitor U0126 (Fig. 5B;  $P < 0.05$ , *t* test) compared with mice treated with the B-Raf inhibitor BAY 43-9006 (Fig. 5B;  $P = 0.34$ , *t* test). No significant difference was observed in apoptosis levels (not shown). Thus, pharmacologic inhibition of Mek in melanoma cells containing mutant  $V^{600E}$ B-Raf reduced metastasis by decreasing the proliferative potential of the cells.

### Discussion

Although mutant  $V^{600E}$ B-Raf protein has been shown to play an important role in development of advanced stage melanomas by regulating proliferation, vascular development, and metastasis (12,38,39,41), the mechanistic basis by which it regulates metastasis remained uncertain (14). Through use of a unique strategy involving transient siRNA-mediated knockdown of  $V^{600E}$ B-Raf protein expression (activity), experimental lung metastases could



be inhibited 4- to 5-fold in metastatic melanoma cells containing mutant B-Raf but not those lacking the mutant protein. The model focused on lung metastasis because lungs are a major organ to which melanoma cells metastasize; however, melanoma are highly aggressive and capable of invading any organ. It is important to note that inhibiting B-Raf in metastatic cells lacking the mutation did not reduce lung metastasis. Thus, targeting the MAP kinase signaling cascade may only be effective for ~60% of the melanoma patients whose tumors contain mutant <sup>V600E</sup>B-Raf protein (13,42–44). This observation is supported by a recent study showing that melanomas containing mutated B-Raf are more responsive to agents targeting the MAP kinase pathway than those wild type for the protein or harboring a Ras mutation (45). Therefore, ascertaining the mutational status of *B-RAF* before use of therapeutics targeting the MAP kinase signaling cascade would be a potentially important indicator of clinical efficacy. This is also the first study to identify that targeting Mek 1/2 rather than <sup>V600E</sup>B-Raf in the signaling cascade can more efficiently inhibit lung metastasis development. siRNA-mediated inhibition of <sup>V600E</sup>B-Raf and proteins downstream in the signaling cascade (Mek 1/2, Erk 1/2, and cyclin D1) all decreased metastasis development with simultaneous inhibition of Mek 1/2 having the most dramatic effect. This is not the first study to identify an important role for Mek in metastasis; pharmacologic inhibition of Mek has been shown previously to reduce melanoma metastasis (46). It is also intriguing that simultaneously targeting Erk 1/2 did not inhibit metastasis development to the same extent as Mek 1/2 inhibition, which further shows the importance of targeting Mek 1/2 to inhibit the <sup>V600E</sup>B-Raf pathway in melanoma cells.

A lack of clinical responsiveness in targeting B-Raf using the pharmacologic agent BAY 43-9006 has raised serious doubt about the usefulness of therapeutically targeting this signaling cascade to inhibit melanoma development (14,20). We have shown that siRNA-mediated inhibition of mutant <sup>V600E</sup>B-Raf signaling reduced lung metastases, indicating that this pathway is important for melanoma metastasis. Furthermore, because siRNA-mediated inhibition of <sup>V600E</sup>B-Raf reduced lung metastasis development, it is reasonable to assume that a pharmacologic inhibitor of B-Raf would do the same (24,47). However, our results show that Mek inhibition using U0126 could inhibit melanoma metastasis more efficiently than occurs when inhibiting B-Raf using BAY 43-9006. It is possible that the *in vivo* mechanism of action of BAY 43-9006 is not conducive for metastasis inhibition. In support of this possibility, BAY 43-9006 has been shown to inhibit melanoma tumor development in preclinical models by reducing vascular development (angiogenesis) mediated through decreased vascular endothelial growth factor (VEGF) secretion by the melanoma cells and through inhibition of VEGF receptors (12,48,49). Furthermore, others have reported that BAY 43-9006 did not block lung adenoma formation whereas Mek inhibitor CI-1040 did (23). It is confounding that both compounds inhibit <sup>V600E</sup>B-Raf signaling in melanoma cells in culture but are not equally effective at inhibiting metastasis development. Thus, *in vitro* efficacy does not necessarily translate into *in vivo* effectiveness. A possible explanation is that the mechanism of action of BAY 43-9006 involves regulating angiogenesis, which is not required for initial lung metastasis, making the compound ineffective (50,51). In contrast, Mek inhibition reduced cell proliferation, which effectively decreased the number and size of metastasis forming in the lung stroma (23,46). It remains to be determined whether other B-Raf inhibitors more specific than BAY 43-9006 would inhibit proliferation and thereby inhibit metastasis formation. However, even if a specific B-Raf inhibitor were developed, siRNA-mediated inhibition data presented in this manuscript show that targeting Mek and not mutant B-Raf in melanomas might be the most efficient approach to inhibit melanoma metastasis.

Metastasis can be inhibited by disrupting extravasation through the endothelial lining of lung vessels or by reducing proliferation in the lung microenvironment (19). We have shown that <sup>V600E</sup>B-Raf plays a critical role regulating both of these processes thereby regulating metastasis. Interaction of melanoma cells with neutrophils facilitates attachment to the

endothelium, thereby promoting extravasation into lung stroma (52,53). We have provided novel insight into these processes showing that, in melanoma, <sup>V600E</sup>B-Raf promotes metastasis by facilitating extravasation across the endothelial layer under flow conditions and by conferring increased proliferative potential in the lung microenvironment. <sup>V600E</sup>B-Raf enhanced extravasation by facilitating interaction with neutrophils, which aided in tethering the melanoma cell to the endothelial lining under flow conditions, thereby promoting movement of the melanoma cell through the endothelial lining (52,53). Once extravasation has occurred into lung stroma, cells need to proliferate, also facilitated by <sup>V600E</sup>B-Raf. SiRNA-mediated inhibition of <sup>V600E</sup>B-Raf reduced the proliferative capacity of melanoma cells by 29% to 75%, the most dramatic inhibition occurring following Mek 1/2 inhibition.

In conclusion, we identified the role played by <sup>V600E</sup>B-Raf in melanoma metastasis and showed that targeting this signaling cascade would reduce metastatic tumor development. Targeting any member of the signaling cascade reduced metastasis, but inhibition of Mek 1 and Mek 2 almost prevented it. Inhibition is mediated through reduction of melanoma cell extravasation into lung tissue and decreased proliferative potential within the lung microenvironment. This study provides a mechanistic basis for therapeutically targeting mutant <sup>V600E</sup>B-Raf signaling to inhibit melanoma metastasis.

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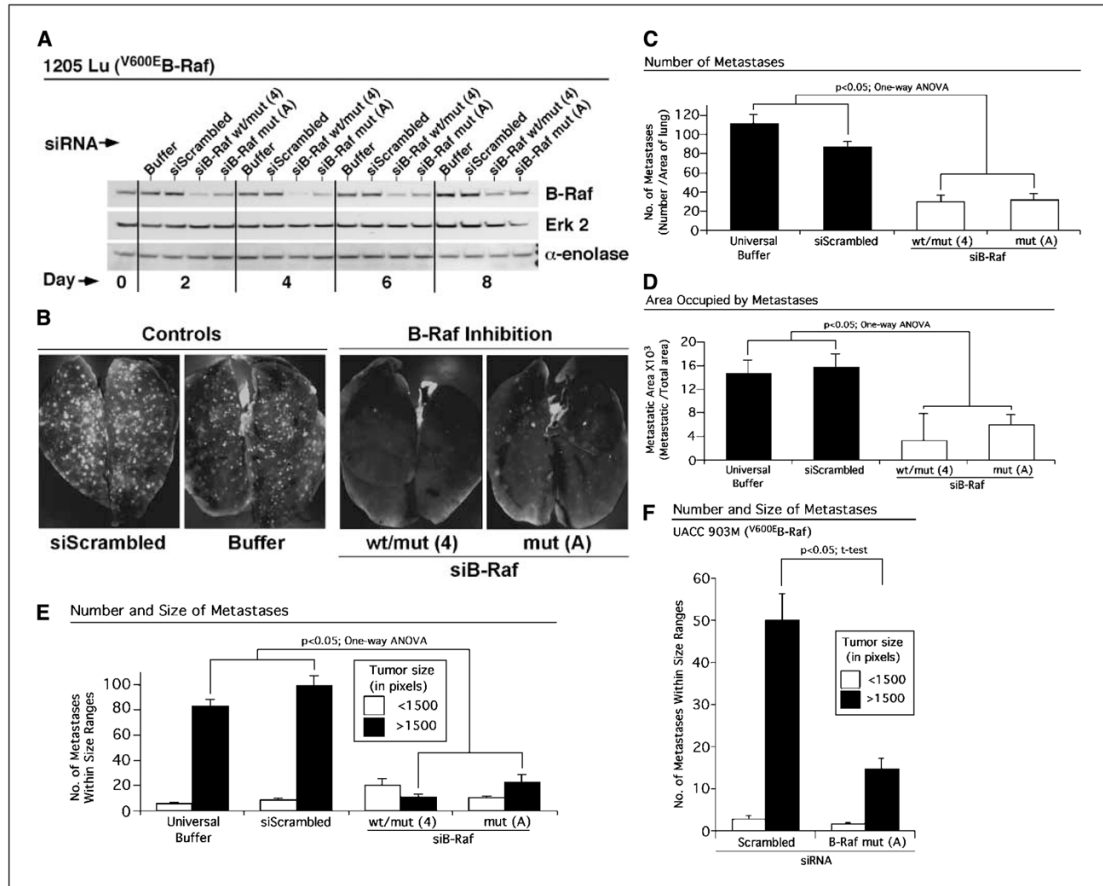
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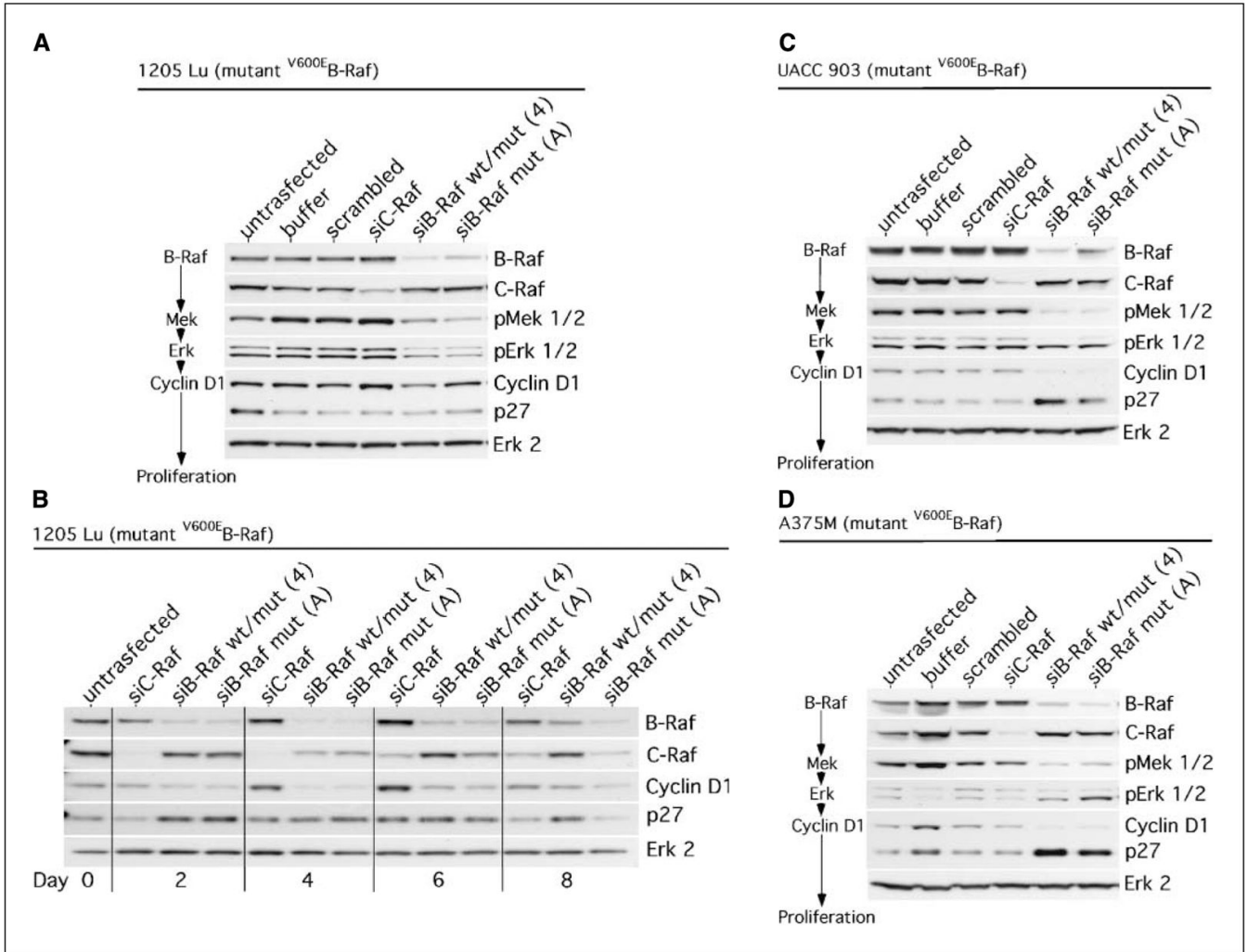
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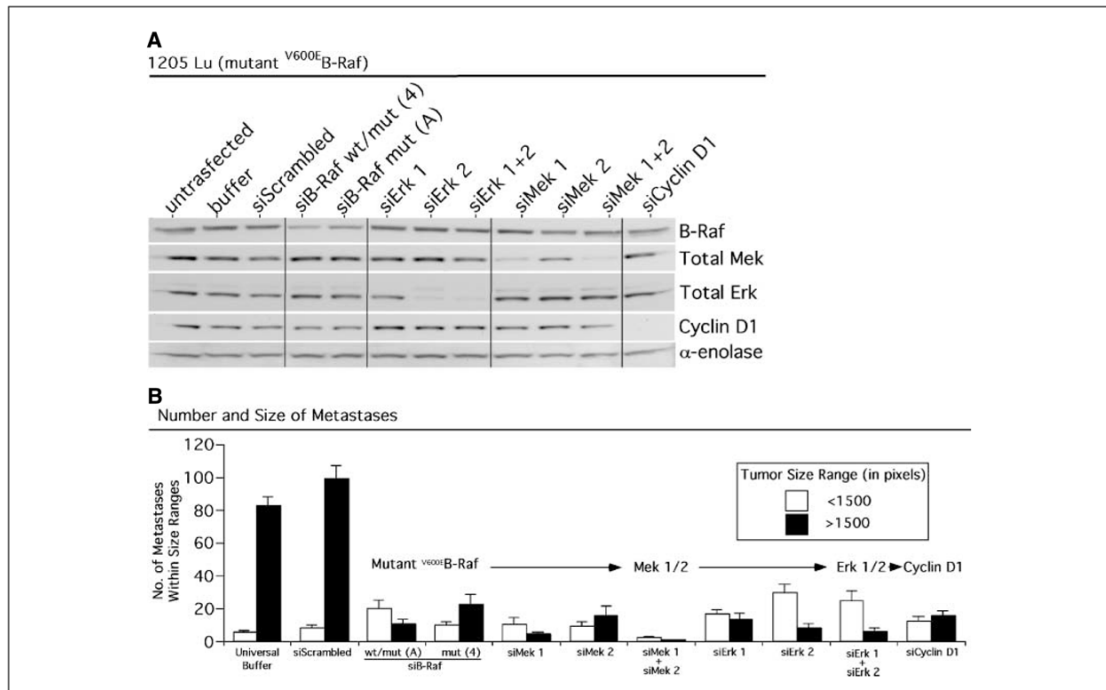
**Figure 1.**

Inhibition of mutant <sup>V600E</sup>B-Raf reduces the metastatic potential of melanoma cells. *A*, siRNA-mediated reduction of mutant <sup>V600E</sup>B-Raf protein expression persists for 8 to 10 days in melanoma cells. SiRNA-mediated knockdown of B-Raf reduces protein expression in cells for 8 to 10 days following nucleofection compared with controls nucleofected with buffer or scrambled siRNA. Erk 2 and α-enolase were used as controls for protein loading. *B*, siRNA-mediated reduction of mutant <sup>V600E</sup>B-Raf protein decreased formation of melanoma metastases in the lungs of nude mice. SiRNA against *B-RAF* or scrambled siRNA was introduced into GFP-tagged 1205 Lu cells; 36 hours later, cells were i.v. injected into nude mice. Photographs show presence of GFP-tagged tumors in the lungs of mice 17 days later. Control cells were nucleofected with buffer only or scrambled siRNA. SiRNA mediated down-regulation of B-Raf protein expression reduced tumor number (*C*), area (*D*), and large-sized metastases (*E*) in the lungs of nude mice. Number of tumors and area occupied by GFP-tumors were quantified in a minimum of six fields per lung from 5 to 10 animals. Tumors were grouped into two pixel-based size ranges (<1,500 or >1,500 pixels). *F*, siRNA-mediated reduction of mutant <sup>V600E</sup>B-Raf expression (activity) in UACC 903M cells decreased formation of lung metastases. SiRNA against <sup>V600E</sup>B-RAF or a scrambled siRNA control were introduced into GFP-tagged UACC 903M cells; 36 hours later, cells were i.v. injected into nude mice, and 17 days later, lung metastases were quantified. Numbers of tumors within particular size ranges (<1,500 or >1,500 pixels) were quantified in a minimum of six fields per lung from 5 to 10 animals.

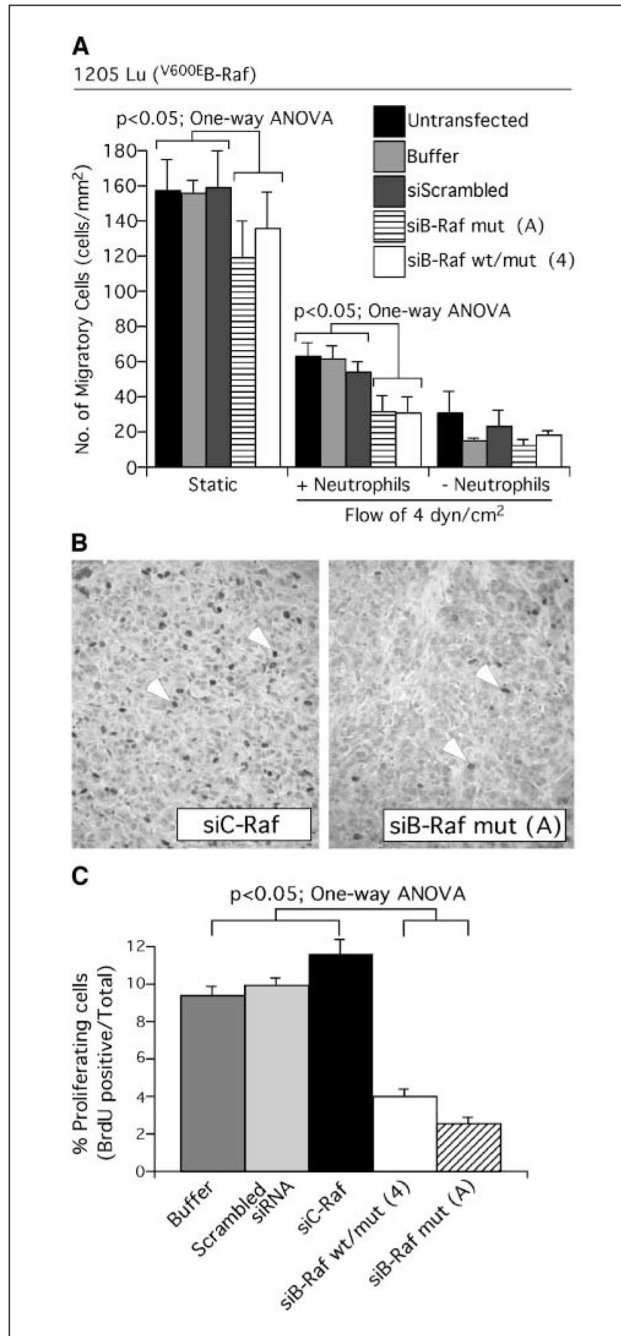




**Figure 2.** SiRNA-mediated reduction of mutant <sup>V600E</sup>B-Raf expression reduces the downstream activity of Mek and Erk as well as levels of cyclin D1 protein in melanoma cells. *A*, siRNA-mediated knockdown of <sup>V600E</sup>B-Raf, but not of C-Raf, reduces phosphorylation (activity) levels of downstream proteins Mek and Erk in 1205 Lu cells 72 hours after nucleofection. Western blots of cells probed for the various proteins in the <sup>V600E</sup>B-Raf signaling cascade or control proteins following introduction of siRNA into cells. Erk 2 served as a control for protein loading. *B*, decreased cyclin D1 levels were detected in 1205 Lu cells following knockdown of <sup>V600E</sup>B-Raf expression. A 2-, 4-, 6-, and 8-day time course is shown for 1205 Lu cells following mutant <sup>V600E</sup>B-Raf inhibition. A decrease in cyclin D1 levels was observed starting at day 2 and ending at day 6. A corresponding increase in p27 levels was detected at day 2. Erk 2 was used as a protein loading control. SiRNA-mediated reduction of <sup>V600E</sup>B-Raf expression (activity) in melanoma cell lines UACC 903 (*C*) and A375M (*D*) led to significant decreases in activity and expression of downstream proteins 72 hours after nucleofection into cells. Increased p27 levels accompanied decreased cyclin D1 levels. Scrambled siRNA or buffer alone were used as controls. Erk 2 served as a control for protein loading.



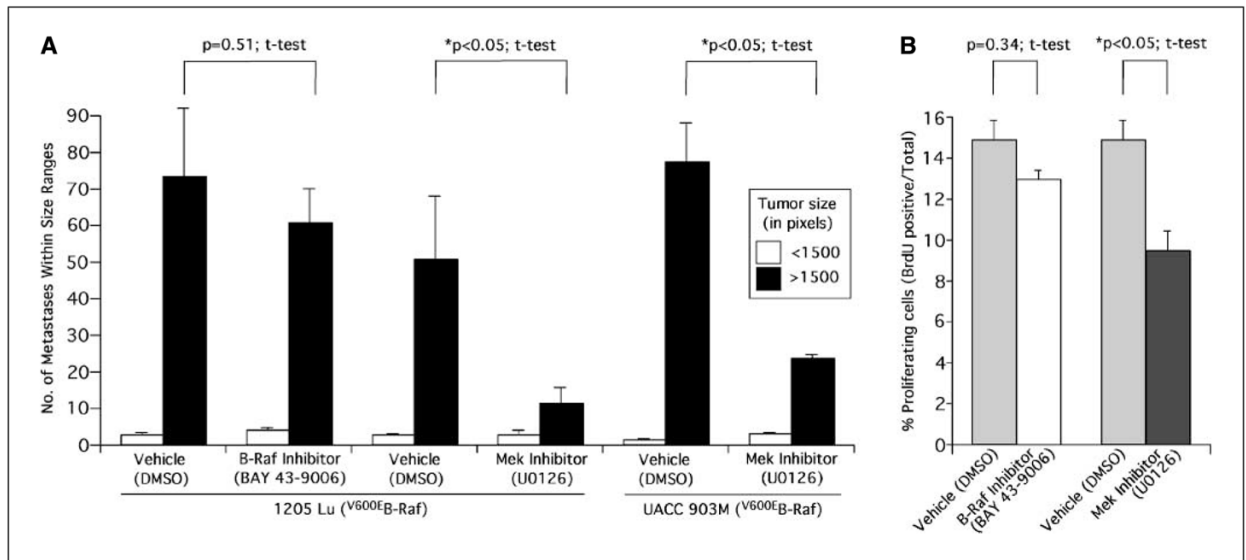
**Figure 3.** Inhibition of each protein downstream of mutant <sup>V600E</sup>B-Raf reduces formation of metastatic tumors in the lungs of mice. *A*, siRNA-mediated reduction of mutant <sup>V599E</sup>B-Raf, Mek, Erk, and cyclin D1 reduced expression of each respective protein 72 hours after nucleofection into 1205 Lu melanoma cells. Western blots probed with antibodies against each respective protein indicates specificity of knockdown.  $\alpha$ -Enolase served as a control for protein loading. *B*, siRNA-mediated reduction of mutant <sup>V600E</sup>B-Raf, Mek, Erk, and cyclin D1 protein decreased formation of melanoma metastases in the lungs of mice. SiRNA against each respective protein or a scrambled siRNA control was introduced into GFP-tagged 1205 Lu cells; 36 hours later, cells were i.v. injected into nude mice, and 17 days later, lung metastases were quantified. Numbers of tumors within particular size ranges (<1,500 or >1,500 pixels) were scored in a minimum of six fields per lung from 5 to 10 animals.



**Figure 4.** Inhibition of mutant <sup>V600E</sup>B-Raf in melanoma cells reduces neutrophil-mediated extravasation through the endothelium and lowers the proliferative capacity of melanoma cells. **A**, siRNA-mediated inhibition of <sup>V600E</sup>B-Raf decreased neutrophil-mediated extravasation across an endothelial-like cell layer. Following siRNA-mediated knockdown of <sup>V600E</sup>B-Raf, migration of 1205 Lu melanoma cell across an endothelial-like cell layer in response to collagen IV (100  $\mu\text{g}/\text{mL}$ ) over a 4-hour period was quantified by scoring number of migrated cells. Comparisons were made under static or flow conditions of 4  $\text{dyn}/\text{cm}^2$  in the presence or absence of neutrophils. Untransfected cells or cells nucleofected with scrambled siRNA or buffer alone were used as controls for comparison. **B**, siRNA-mediated knockdown of B-Raf protein

reduced the number of proliferating cells in tumors. Percentage proliferating cells was determined by scoring BrdUrd-positive cells in tumors established from 1205 Lu cells nucleofected with siRNA against *V600E*-RAF and compared with control tumors formed from cells transfected with buffer, scrambled siRNA, or siRNA against *C-RAF*. *C*, siRNA-mediated inhibition of *V600E*-Raf reduced melanoma tumor proliferative capacity by 2- to 3-fold.

*Columns*, mean percentage of proliferating cells determined from four to six fields analyzed from each of six tumors; *bars*, SE. Untransfected cells or cells nucleofected with scrambled siRNA or buffer alone were used as controls for comparison.

**Figure 5.**

Pharmacologic inhibition of Mek, but not of <sup>V600E</sup>B-Raf, retards the metastatic potential of melanoma cells by altering proliferative capacity. *A*, pharmacologic inhibition of Mek, but not of <sup>V600E</sup>B-Raf, reduced the metastatic potential of melanoma cells. Four and two days before i.v. injection of metastatic melanoma cell lines 1205 Lu or UACC 903M into mice, animals were pretreated i.p. with a Raf inhibitor (BAY 43-9006; 75 mg/kg) or a Mek inhibitor (U0126; 10 mg/kg) dissolved in DMSO vehicle. Treatment was continued every 2 days up to day 17 when number and size of GFP-tagged tumors (<1,500 or >1,500 pixels) were quantified.

*Columns*, mean of a minimum of six fields per lung from 5 to 10 animals; *bars*, SE. Controls were animals treated with DMSO vehicle only. *B*, pharmacologic inhibition of Mek, but not of <sup>V600E</sup>B-Raf, reduced the number of proliferating cells in tumors. Percentage proliferating cells was determined by scoring BrdUrd-positive cells in tumors 4 days after s.c. injection into mice. Animals treated with the DMSO vehicle served as controls. *Columns*, mean of four to six fields analyzed from six different tumors; *bars*, SE.



Table 1

Growth properties of 1205 Lu, A375M, and UACC 903 cells treated with scrambled siRNA or siRNA against *B-RAF*, *MEK*, *ERK*, or *CYCLIN D1*

siRNA treatment	Melanoma cell line doubling time <i>in vitro</i> (hours $\pm$ SE)			
	1205 Lu	A375M	UACC 903	
Controls				
Scrambled	38 $\pm$ 3	33 $\pm$ 2	26 $\pm$ 1	
Universal buffer	44 $\pm$ 7	32 $\pm$ 3	28 $\pm$ 0	
Average	41	33	27	
Experimental				
B-Raf mut (A)	53 $\pm$ 10	46 $\pm$ 6	38 $\pm$ 3	$\uparrow$ 41%
Mek 1	41 $\pm$ 3	36 $\pm$ 3	32 $\pm$ 0	$\uparrow$ 19%
Mek 2	39 $\pm$ 3	29 $\pm$ 2	26 $\pm$ 0	NC
Mek 1 + Mek 2	60 $\pm$ 9	39 $\pm$ 2	44 $\pm$ 5	$\uparrow$ 63%
Erk 1	53 $\pm$ 0	34 $\pm$ 4	26 $\pm$ 0	NC
Erk 2	43 $\pm$ 4	34 $\pm$ 2	31 $\pm$ 1	$\uparrow$ 15%
Erk 1 + Erk 2	64 $\pm$ 3	40 $\pm$ 2	37 $\pm$ 1	$\uparrow$ 37%
Cyclin D1	52 $\pm$ 0	36 $\pm$ 3	29 $\pm$ 0	NC
Average	51	36	33	$\uparrow$ 22%

NOTE: To the right of each experimental value is shown relative changes in proliferative capacity between the control average and experimental value; NC, no significant change between control average and experimental value (i.e., a difference of  $\leq 3$  hours).