

Oncogenic BRAF Is Required for Tumor Growth and Maintenance in Melanoma Models

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Abstract

The usual paradigm for developing kinase inhibitors in oncology is to use a high-affinity proof-of-concept inhibitor with acceptable metabolic properties for key target validation experiments. This approach requires substantial medicinal chemistry and can be confounded by drug toxicity and off-target activities of the test molecule. As a better alternative, we have developed inducible short-hairpin RNA xenograft models to examine the *in vivo* efficacy of inhibiting oncogenic BRAF. Our results show that tumor regression resulting from BRAF suppression is inducible, reversible, and tightly regulated in these models. Analysis of regressing tumors showed the primary mechanism of action for BRAF to be increased tumor cell proliferation and survival. In a metastatic melanoma model, conditional BRAF suppression slowed systemic tumor growth as determined by *in vivo* bioluminescence imaging. Taken together, gain-of-function BRAF signaling is strongly associated with *in vivo* tumorigenicity, confirming BRAF as an important target for small-molecule and RNA interference-based therapeutics. (Cancer Res 2006; 66(2): 999-1006)

Introduction

Some of the most frequently detected genetic alterations in epithelial cancers are in the *RAS* family, which plays a central role in both normal and malignant cell growth (1–3). The first identified downstream effectors of *RAS* were the RAF serine/threonine kinases (4, 5). The RAF family is composed of three members, ARAF, BRAF and RAF1, which exhibit a high degree of homology within three conserved regions. Each kinase has a *RAS*-binding domain and cysteine-rich domain that mediate interaction with GTP-bound *RAS* (6). On *RAS* association, RAF is recruited to the plasma membrane and the inhibitory intramolecular interaction of the RAF catalytic domain with the NH₂ terminus is alleviated. This leads to phosphorylation of sites within the catalytic loop of the activation domain (Thr⁵⁹⁹ and Ser⁶⁰² of BRAF; ref. 7). Although these initiating events for kinase activation are similar for all RAF isoforms, full activation of ARAF and RAF1 requires additional phosphorylation outside the catalytic domain (Tyr³⁴⁰, Tyr³⁴¹, and Ser³³⁸ in RAF1). Interestingly, in BRAF the corresponding residues are either substituted with aspartic acids (Asp⁴⁴⁸ and Asp⁴⁴⁹) or seem to be

constitutively phosphorylated (Ser⁴⁴⁶) in cells (8). This suggests a distinct and simplified activation mechanism that contributes to BRAF being the important physiologic mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) activator. This is further supported by gene disruption studies in mice wherein MEK/ERK activation was significantly disrupted in BRAF^{-/-} embryonic fibroblasts (9) but not in cells lacking either ARAF or RAF1 (10–12). Taken together, the higher propensity of BRAF towards MEK/ERK activation is thought to be a reason that only BRAF mutations, and not comparable mutations in the other RAF proteins, have been observed to be associated with malignancy.

Recently, activating somatic mutations in BRAF have been reported in 70% of melanomas (5, 13). These mutations are also present in premalignant atypical or dysplastic nevi and may thereby implicate BRAF activation as an initiating event in tumorigenesis (14). BRAF mutations also occur with high frequency in papillary thyroid carcinomas, serous ovarian cancers, and colorectal serrated adenocarcinomas whereas mutations in liver, pancreas, non-small-cell lung cancer, glioma, and acute myelogenous leukemia have been detected at lower frequency. Almost 90% of BRAF mutations are a T1799A transversion in exon 15 that results in a Val⁶⁰⁰Glu (V600E) amino acid substitution leading to constitutive kinase activation (15, 16). Interestingly, fewer than 1% of melanoma samples with this prevalent BRAF mutation also contain activating *RAS* mutations (which occur in more than 10% of melanomas), providing strong genetic evidence for a direct *RAS*/BRAF signaling pathway in this tumor type. Furthermore, in those cancers where neither *RAS* nor BRAF is mutated, sustained RAF/MEK/ERK signaling can arise from other mechanisms (17). These include both deregulated upstream activators, such as epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (ERBB2 or HER2), platelet-derived growth factor receptor (PDGFR), hepatocyte growth factor receptor (MET), and vascular endothelial growth factor receptor (VEGFR), and loss of downstream negative regulators such as MAPK phosphatase 3 (DUSP6) and RAF kinase inhibitor protein. It is thereby thought that the majority of human tumors, not just those with *RAS* or BRAF mutations, engage the RAF/MEK/ERK signaling cascade. Taken together, this makes BRAF an attractive therapeutic target.

In this context, several small-molecule BRAF inhibitors have been reported, with BAY 43-9006 being furthest along in clinical testing (18, 19). BAY 43-9006 was discovered through a combinatorial chemistry approach aimed at studying structural modifications to a bis-aryl urea chemical series (20). Although initially developed as a RAF kinase inhibitor, it has since been shown to have activity towards several additional kinases, including VEGFR2, VEGFR3, PDGFR β , FLT3, c-KIT, and p38 MAPK (21). Whereas the antitumor activity of this inhibitor is thought to result from the

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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combined effect of targeting angiogenesis (via receptor tyrosine kinases) and proliferation (via RAF/MEK/ERK-associated signaling), any correlation between BAY 43-9006 *in vivo* efficacy and BRAF mutation has yet to be established in ongoing clinical trials. For instance, in a phase II study of stage IV refractory melanoma patients receiving BAY 43-9006, Ahmad et al. (22) did not detect BRAF mutations in the majority of patients with stable disease. Flaherty et al. (23) also assessed BRAF mutational status in a phase I/II pharmacokinetic and pharmacodynamic trial of BAY 43-9006 in patients with metastatic melanoma and reported that BRAF^{V600E} mutation did not confer an advantage in terms of partial responses and stabilized disease. Hence, off-target receptor tyrosine kinase inhibition and antiangiogenic activity may be crucial to the therapeutic potential of BAY 43-9006.

Whereas efforts to design kinase inhibitors have exploited structural differences in the vicinity of ATP-binding pockets to achieve considerable molecular selectivity, there is still a great potential for cross-reactivity and rigorous experimental testing of compounds commonly used in the clinic have revealed numerous off-target interactions (24, 25). As shown for BAY 43-9006, mitigating the function of certain combinations of kinases with a single inhibitor may have a beneficial effect in particular disease states; however, interpreting biological results and elucidating the primary disease targets becomes challenging. Coupled to this, the most important consideration for successful therapy design remains the choice of a suitable target. This is highlighted by the clinical success of imatinib (Gleevec) which targets the key molecular drivers of chronic myeloid leukemia and gastrointestinal stromal tumors, Bcr-Abl and c-Kit/PDGFR α kinases, respectively (26). Taken together, the development of new tools to validate candidate kinases in a disease context is highly desired.

Here we describe tetracycline-inducible short-hairpin RNA (shRNA) interference method to directly test the antitumorigenic effect of *in vivo* BRAF inhibition in melanoma tumor progression. With this RNA interference-based approach, we show that oncogenic BRAF is causally involved in tumor growth and address key questions about the contribution of this oncogene to tumor growth, maintenance, and resistance to apoptosis.

Materials and Methods

Cell lines and antibodies. The A375M-luc cell line was a gift from Dr. Sanjiv Gambhir (Stanford University, Stanford, CA). A375 (American Type Culture Collection, Manassas, VA) and LOX-IMVI (NCI-60) cells were maintained at 37°C and 5% CO₂ in DMEM or RPMI 1640, respectively, with 10% tetracycline-free fetal bovine serum, 4 mmol/L L-glutamine, and penicillin/streptomycin. Antibodies used for Western blotting and immunohistochemistry were as follows: anti-ERK2, anti-p-ERK1/2 (Thr²⁰²/Tyr²⁰⁴), anti-MEK1, anti-p-MEK1 (Ser^{217/221}), anti-ARAF, and anti-cleaved caspase-3 (Cell Signaling Technology, Beverly, MA); anti-BRAF (F-7; Santa Cruz Biotechnology, Santa Cruz, CA); anti-RAF1 (BD Transduction Labs, San Jose, CA); anti-h-actin (Sigma Life Science, St. Louis, MO); anti-Ki-67 (MIB-1, DakoCytomation, Carpinteria, CA); anti-panendothelial cell marker (MECA-32, PharMingen, San Jose, CA); and horseradish peroxidase (HRP)-conjugated secondary antibodies (Pierce Biotechnology, Rockford, IL).

Inducible shRNA constructs. Hairpin oligonucleotides used in this study are as follows: BRAF shRNA-1 (sense) 5'-GATCCCCAGAAT-TGGATCTGGATCATTTCAGAGAATGATCCAGATCCAAATCTTTTTT-TGGAAA-3', BRAF shRNA-1 (antisense) 5'-AGCTTTTCCAAAAAAGAAT-TGGATCTGGATCATTCTTGAATGATCCAGATCCAAATCTGGG-3'; BRAF shRNA-2 (sense) 5'-GATCCCCGTACAGAGAAATCTCGATTTCAGAGAATCGAGATTTCTCTGTAGCTTTTTTGGAAA-3', BRAF shRNA-2 (antisense) 5'-AGCTTTTCCAAAAAAGCTACAGAGAAATCTCGATTCTCT-

TGAAATCGAGATTTCTCTGTAGCGGG-3'; luciferase shRNA (sense) 5'-GATCCCCCTTACGCTGAGTACTTCGATTCAAGAGATCGAAGTACT-CAGCGTAAGTTTTTTGGAAA-3', luciferase shRNA (antisense) 5'-AGC-TTTTCCAAAAAACTTACGCTGAGTACTTCGATCTCTTGAATCGAAGTACT-CAGCGTAAGGGG-3'; and enhanced green fluorescent protein (EGFP) shRNA (sense) 5'-GATCCCCAGATCCGCCACACATCGATTCAAGAGATC-GATGTTGTGGCGGATCTTGTTTTTTGGAAA-3', EGFP shRNA (antisense) 5'-AGCTTTTCCAAAAACAAGATCCGCCACACATCGATCTCTTGAATC-GATGTTGTGGCGGATCTGGG-3'. The complementary double-stranded shRNA oligos were inserted into our tetracycline-inducible retrovirus gene transfer vector using *Bgl*II and *Hind*III restriction enzyme sites as outlined for pTER (27). Our vector system is composed of a kanamycin-resistant, H1 promoter-driven shRNA expression shuttle plasmid and an ampicillin-resistant retroviral vector backbone that contains a codon-optimized Tet repressor-internal ribosomal entry site-puromycin cassette to enable Tet-regulated shRNA expression (Fig. 1A). Knockdown vectors are constructed by cloning shRNA oligos into the shuttle vector followed by a Gateway recombination reaction (Invitrogen) to transfer the shRNA cassette in the retroviral vector. All constructs were verified by sequencing. Gene knockdown using these shRNAs was first verified in transient assays.

Generation of inducible-shRNA cell clones. Retrovirus infection was done using Phoenix packaging cells according to the instructions of the manufacturer (Orbigen, San Diego, CA). As the puromycin resistance gene encoded in the vector is under the control of a constitutive β -actin promoter, 2 to 5 μ g/mL puromycin was used to select infected cells expressing shRNA. Stable clones were isolated, treated with 2 mg/mL doxycycline (BD Clontech, San Jose, CA) for 3 days, and endogenous BRAF knockdown was assessed by quantitative reverse transcription-PCR. Clones were further characterized for changes in Raf protein expression and p-ERK1/2 status by Western blotting.

Western blot analysis. Cells were lysed in modified radioimmunoprecipitation assay buffer containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Brij-35, 0.1% deoxycholate, protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN), and phosphatase inhibitor cocktail (Sigma, St. Louis, MO). SDS-PAGE (4-12% gel) was used to resolve the proteins in the lysate. After electrophoresis, the proteins were electrotransferred onto a polyvinylidene fluoride microporous membrane and immunodetected using standard procedures.

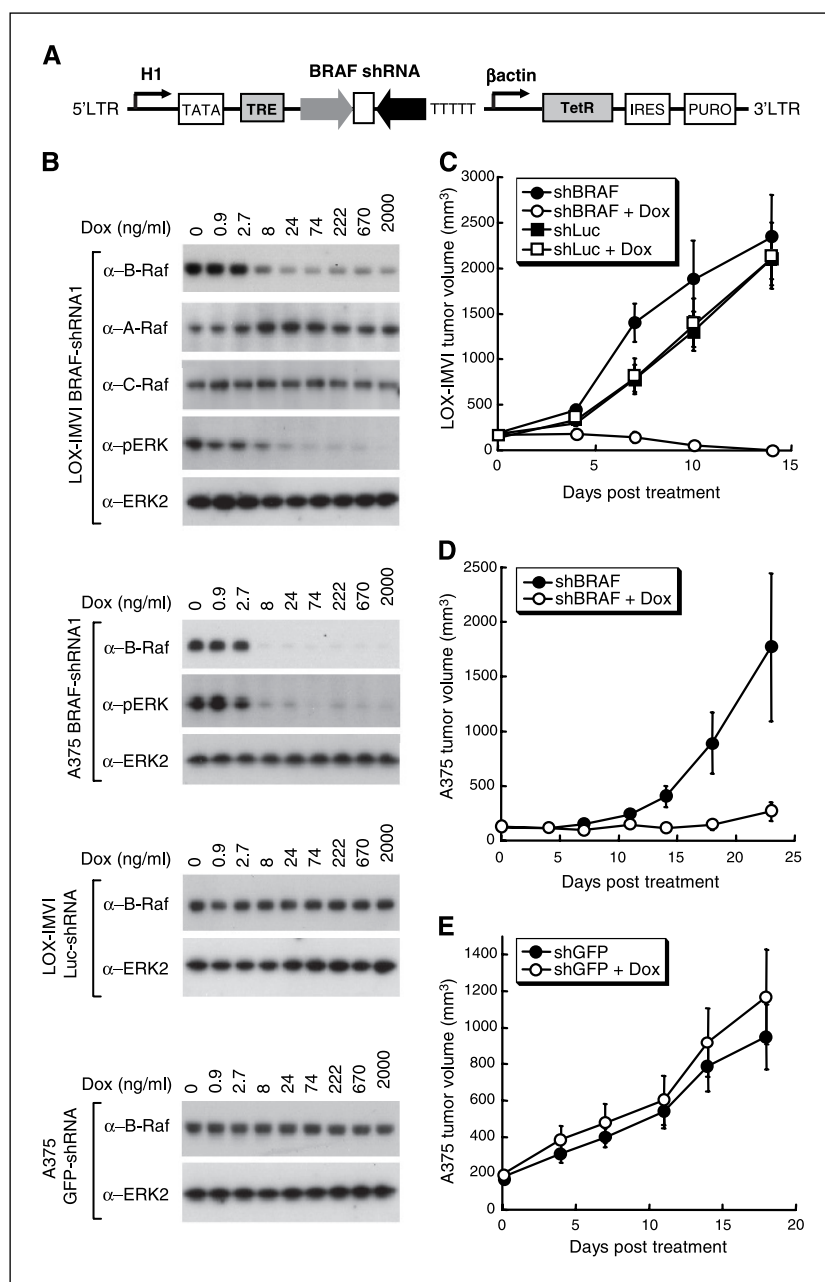
Xenograft models. Six- to eight-week-old female athymic *nu/nu* mice or *scid-beige* mice were purchased from Charles River Laboratories (Wilmington, MA) and maintained in the conventional animal facility of our institute. For s.c. tumor models, mice were injected in the right flank with either 3×10^6 human LOX-IMVI or 1×10^7 human A375 shRNA-containing cell clones resuspended in 200 μ L PBS. When tumors reached a mean volume of 100 to 150 mm³, the mice with similarly sized tumors were grouped into treatment cohorts. Mice received 5% sucrose only or 5% sucrose plus 1 to 2 mg/mL doxycycline for control and knockdown cohorts, respectively. All water bottles were changed thrice a week. Tumors were measured with calipers and mice weighed twice a week. Mice whose tumors reached 2,000 mm³ were euthanized. At the end of the dosing study, or as indicated, appropriate tumor samples were taken. Between 7 and 10 mice were used for each treatment group and results are presented as mean tumor volume \pm SE.

For metastatic tumor models, female *scid-beige* mice were injected i.v. with 50 μ L PBS containing 4×10^5 A375M-luc shRNA cell clones. Tumor progression was monitored by weekly bioluminescence imaging for luciferase and mice were monitored daily for survival. At least nine mice were used for each cohort.

Bioluminescence imaging. *Scid-beige* mice were injected i.p. with 250 μ L 200 mg/kg D-luciferin (Molecular Probes, Carlsbad, CA) and anesthetized using isoflurane. During image acquisition in a light-tight box, the animal was maintained on isoflurane via nose cone and body temperature was maintained using a warming pad. Bioluminescence images were acquired using a cooled intensified charge-coupled device camera. Image acquisition times were typically 1 minute. Images were processed by coregistering a reference image with the bioluminescence data image.

Immunohistochemistry. Formalin-fixed, paraffin-embedded specimens were collected and a routine H&E slide was first evaluated. Immunohistochemical

Figure 1. Inducible knockdown of BRAF expression prevents melanoma tumor growth. **A**, schematic representation of the retroviral vector for tetracycline/doxycycline (Dox)-inducible synthesis of shRNA. The Tet repressor is constitutively expressed from the β -actin promoter and H1 RNA polymerase III promoter-driven transcription is thereby repressed in the absence of doxycycline by binding of the Tet repressor protein to the Tet-responsive element sequence located immediately downstream of the TATA box. Addition of doxycycline results in dissociation of the Tet repressor protein and derepression of the shRNA transcriptional unit. TATA, TATA box; TRE, Tet-responsive element; TetR, Tet repressor protein; IRES, internal ribosomal entry site; PURO, puromycin resistance gene; LTR, long terminal repeat. **B**, experimental validation of BRAF knockdown in melanoma cell lines. LOX-IMVI and A375 cell clones stably expressing BRAF shRNA or control GFP and luciferase (Luc) shRNAs were treated with the indicated doxycycline concentrations for 72 hours. Lysates were then analyzed by immunoblotting. **C to E**, BRAF shRNA knockdown shows antitumor efficacy in xenograft models. LOX-IMVI and A375 inducible shRNA cells were implanted s.c. in the flank of athymic mice as described in Materials and Methods. Treatment in each experiment was initiated on the day when mice had tumors ranging in size from 100 to 150 mm³. Administration of 2 mg/mL doxycycline via drinking water produced regression in LOX-IMVI (C) or stasis in A375 tumors expressing an inducible BRAF-specific shRNA (D). GFP (E) or luciferase control shRNAs (E) did not affect tumor growth kinetics. No lethality or weight loss was observed.



staining was done on 5- μ m-thick paraffin-embedded sections using anti-Ki-67 (clone MIB-1, mouse anti-human with the DAKO ARC Kit), anti-panendothelial cell marker (clone MECA-32, monoclonal rat anti-mouse), and anti-cleaved caspase-3 (rabbit anti-human and anti-mouse) antibodies with a standard avidin-biotin HRP detection system according to the instructions of the manufacturer. Tissues were counterstained with haematoxylin, dehydrated, and mounted. In all cases, antigen retrieval was done with the DAKO Target Retrieval Kit as per instructions of the manufacturer.

Results

Induced RNA interference knockdown of BRAF interferes with s.c. tumor growth in melanoma xenograft models. Recently, van de Wetering et al. (27) described a stable system for tetracycline-inducible expression of shRNAs. We incorporated a

similar cassette into a single retroviral expression plasmid to direct conditional expression of shRNAs in proliferating cells of choice (Fig. 1A). In the "off" state, the Tet repressor protein binds a modified polymerase III promoter thereby preventing shRNA expression. However, in the presence of a tetracycline analogue, doxycycline, the Tet repressor protein is released from the promoter resulting in shRNA transcription and knockdown of endogenous BRAF expression. Using retroviral delivery followed by selection for puromycin resistance, cell clones with stable integration of this shRNA expression cassette can be rapidly generated.

To address the question of whether BRAF specifically could serve as a therapeutic target for melanoma, we employed this inducible-shRNA method to deplete the expression of BRAF in cultured melanoma tumor cells. LOX-IMVI cells, derived from a lymph node melanoma metastasis, and A375 cells from a malignant cutaneous

melanoma were genotyped for BRAF. PCR amplification and sequencing of exon 15 of BRAF revealed the presence of activated BRAF^{V600E} alleles in both LOX-IMVI and A375 cell lines. Consistent with this, strong BRAF/MEK/ERK signaling is observed in these cells. This suggests a role for BRAF in their tumorigenic potential. We generated stable cells that express either BRAF or control shRNA using previously published oligonucleotide sequences (28). Several independent LOX-IMVI and A375 clones were characterized to ensure against a clonal selection bias. In the uninduced state, cells expressing the inducible hairpins were not altered in their baseline growth properties and no discernable background expression could be detected (data not shown). As shown in Fig. 1B, dramatic BRAF suppression was observed in both cell lines by doxycycline-mediated hairpin targeting of BRAF but not GFP or luciferase. Densitometry quantitation of immunoblots revealed an effective BRAF protein knockdown of ~80% and ~98% for LOX-IMVI and A375, respectively (Supplementary Fig. S1). The suppression of BRAF protein levels was achieved in a dose-dependent manner with a doxycycline IC₅₀ of ~5 ng/mL. BRAF knockdown was reversible and time dependent, with the maximal mRNA depletion detected 2 days post induction and the corresponding protein depletion occurring at day 3 (data not shown). Induced BRAF-directed shRNA did not diminish the expression of ARAF and RAF1, as has been shown previously for these shRNAs (29). Consistent with the known BRAF phosphorylation-dependent activation of ERK1/2 via MEK1/2, increasing doxycycline concentration resulted in a reduction of p-ERK1/2 whereas total ERK1/2 levels remained unchanged (Fig. 1B). This indicates that BRAF-mediated ERK1/2 activation and signaling can be abrogated in these cells by doxycycline-mediated induction of BRAF shRNA.

In phenotypic analyses, we first investigated the effects of BRAF knockdown on the *in vitro* growth of these melanoma cells. On doxycycline addition, LOX-IMVI and A375 cells lacking BRAF show consistent changes in two-dimensional properties as compared with control shRNA-infected cells. These include a delay in cell cycle entry and reduced proliferation (Supplementary Fig. S2) and a flattened epithelial-like cell morphology change (data not shown). Of greater interest, we next tested whether ablation of BRAF function in LOX-IMVI and A375 cells might affect their ability to form tumors *in vivo*. Inoculation of 3×10^6 LOX-IMVI cells into immunodeficient *nu/nu* mice produced large tumors at 2 weeks (Fig. 1C), consistent with the reported strong tumorigenicity of this cell line. Mice bearing these inducible-shRNA xenografted tumors were given either 5% sucrose or 2 mg/mL doxycycline plus 5% sucrose and monitored for tumor progression. Strikingly, knockdown of BRAF completely inhibited LOX-IMVI tumorigenesis *in vivo* and led to tumor regression (Fig. 1C) even despite the incomplete depletion of BRAF as shown *in vitro* for the selected clone. Complete responses were observed in 6 of 10 animals in the doxycycline treatment cohort. In contrast, sucrose-treated LOX-IMVI/BRAF-shRNA tumors or LOX-IMVI tumors expressing a control shRNA directed against luciferase continued to increase in size. Similar results were obtained in a second LOX-IMVI study with the same dosing paradigm (data not shown). There was no difference in body weight between treatment groups and significant doxycycline-related toxicity was not observed.

We did a similar *in vivo* study with A375 melanoma cell lines to further substantiate the role of BRAF in tumorigenesis. In A375 xenografts, BRAF-shRNA induction also halted tumor progression (Fig. 1D); however, the tumors did not regress as observed in the

studies involving the LOX-IMVI cell line. There was no discernable effect on tumor growth observed with A375 cells expressing GFP control shRNA (Fig. 1E). Taken together, these results suggest that gain-of-function BRAF signaling is strongly associated with the *in vivo* tumorigenic ability of melanoma cells and confirm BRAF as an important therapeutic target.

Effect of conditional and dose-responsive BRAF gene suppression on tumorigenesis. Recent reports using inducible transgenic mouse models have begun to address the consequences of oncogene inactivation for the maintenance of an established neoplastic phenotype (30, 31). In some instances, even brief inactivation of a single oncogene can be sufficient to induce sustained tumor regression (30). To further explore the possibility of whether inactivation of oncogenic BRAF signaling may be effective in treating melanoma and to examine the mechanism by which oncogenic BRAF inactivation induces tumor regression, moribund mice with ~1,500-mm³ LOX-IMVI/BRAF-shRNA s.c. tumors (Fig. 1C) were switched to doxycycline. Within 5 days post BRAF shRNA induction, the tumor volume had visibly decreased, and after 2 weeks, the tumors had grossly regressed (Fig. 2A), showing that impairment of BRAF can result in rapid elimination of tumor cells. Despite the large starting tumor volume, growth inhibition was equally efficient as compared with early-onset BRAF knockdown in smaller volume tumors. Furthermore, we tested the effect of restoring BRAF expression in regressing tumors by discontinuing treatment in a doxycycline cohort (Fig. 1C). On doxycycline withdrawal, tumor recurrence was observed in two of eight mice (Fig. 2B). In cases where the tumor did not regrow, by the time shRNA induction was stopped by doxycycline removal the animals no longer had a palpable tumor, indicating that all the implanted tumor cells in these animals had already been eliminated. This indicates that whereas BRAF knockdown does not lead to an irreversible cascade of molecular events in these tumor cells, prolonged BRAF suppression is sufficient to promote elimination of well-established tumors.

The tetracycline-inducible shRNA induction system we describe here enables modulation of the shRNA expression level and, consequently, the abundance of target protein. This can result in distinct tumor phenotypes that are a function of the degree of target gene suppression. For drug development, partial knockdown of oncogenes could be used to assess the levels of physiologic target inhibition required for efficacy. We have characterized this modulation by bioluminescence imaging using shRNA targeting luciferase in SVT2 cells (data not shown). To explore this in the context of BRAF inhibition, we treated mice bearing LOX-IMVI/BRAF-shRNA tumors with 0, 0.02, 0.2, 0.5, 1.0, or 2.0 mg/mL doxycycline and monitored differences in tumor growth. Although all the examined doxycycline concentrations resulted in stasis or regression, there was a dose-dependent trend in tumor inhibition *in vivo* (Fig. 2C). Ongoing characterization of our Tet-inducible shRNA system suggests that 0.02 mg/mL doxycycline is near the lower threshold for *in vivo* gene knockdown.⁵ Hence, partial attenuation of BRAF may be sufficient for antitumor efficacy in a clinical setting.

LOX-IMVI tumor regression is specific to BRAF knockdown. Although RNA interference methods have great potential to elucidate gene function, it remains necessary to validate that the

⁵ D.P. Davis and D.C. Gray, unpublished observations.

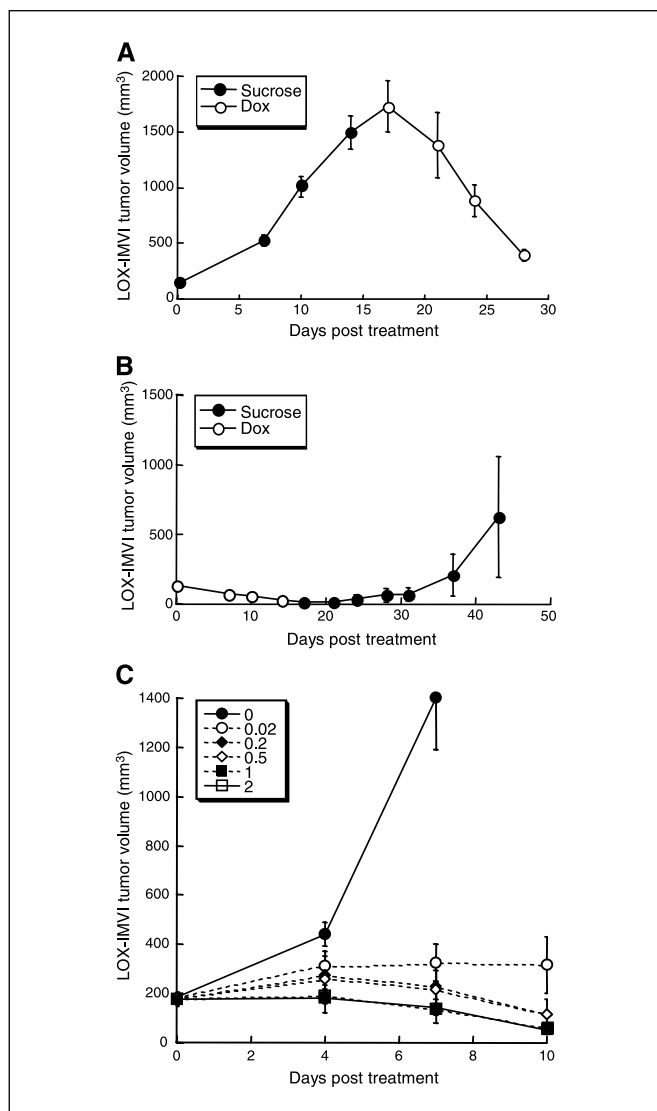


Figure 2. BRAF knockdown is reversible and tightly regulated *in vivo*. **A**, BRAF-dependent LOX-IMVI tumors were allowed to grow for 14 days before administration of doxycycline was initiated to knockdown BRAF-dependent signaling and tumorigenesis. **B**, doxycycline-treated mice with regressing s.c. tumors that are subsequently removed from doxycycline at day 14 undergo tumor recurrence. **C**, dose response of *in vivo* BRAF knockdown. Groups of mice with s.c. LOX-IMVI/BRAF-shRNA tumors of equivalent size were induced with 0, 0.02, 0.2, 0.5, 1.0, or 2.0 mg/mL doxycycline and 5% sucrose as indicated. *Points*, mean tumor volumes (mm³); *bars*, SD.

observed phenotypes result from gene silencing of the target gene and not of unintended, off-target transcripts (32). Thus, incorporation of multiple BRAF-specific shRNAs into these assays increases the confidence with which the observed changes in melanoma tumor growth can be directly linked to BRAF silencing. The first shRNA used for BRAF knockdown (Fig. 1) corresponds to the translated sequence just following the G-loop of the kinase domain (amino acids 461-467) in which no oncogenic mutations have been described to date. Accordingly, another hairpin specific to a distinct region of the BRAF transcript (encoding amino acids 597-603 and the V600E mutation) was also selected (28). Using this second retroviral construct (BRAF-shRNA2), we infected LOX-IMVI cells and clones were analyzed by quantitative PCR and Western blotting (Fig. 3A). Equally efficient doxycycline-mediated BRAF

knockdown and attenuated downstream signaling were observed as compared with that of BRAF-shRNA1 depicted in Fig. 1B. Moreover, in xenograft models, shRNA2-mediated knockdown of BRAF also showed robust tumor regression on doxycycline induction (Fig. 3B). Taken together, these results further support that the elicited phenotypes reflect silencing of the BRAF target gene and affirm the role of oncogenic BRAF in tumor growth.

BRAF mechanism of action. Understanding the mechanism of action of a drug target is a key step in the drug development cycle. To this end, in addition to tumor growth inhibition as the primary end point in these studies, we did histologic analysis to define the spectrum of cellular responses that can be caused by targeted BRAF inhibition. LOX-IMVI tumors from mice treated with doxycycline for 1 to 7 days were harvested and adjacent sections were probed for expression of Ki-67, activated caspase-3, or pan-endothelial cell marker (Fig. 4). Compared with xenografts from control animals, tumors from doxycycline-treated mice exhibited fewer neoplastic cells by H&E visualization. Immunohistochemical staining with an antibody to Ki-67 revealed a profound decrease in proliferating cells on BRAF knockdown. This result is consistent with the established role of BRAF/MEK/ERK signaling in driving cell cycle progression (17). LOX-IMVI tumors also showed increased inflammatory infiltrate and immunophenotyping with a macrophage marker (F480) revealed an increase in macrophages (Supplementary Fig. S3). The presence of inflammation in these xenografts raises the possibility of a response to apoptotic debris. In confirmation of this, whereas cleaved caspase-3-positive cells were infrequent in control tissues, following doxycycline treatment of LOX-IMVI xenograft mice the proportion of scattered apoptotic cells increased after 3 days and reached a stable maximum by day 4 (Fig. 4). Thereafter, the degree of apoptosis was consistent from 4 to 7 days post shRNA induction. This delay in observable apoptosis is consistent with the 2- to 3-day requirement for manifesting BRAF protein knockdown *in vitro*. Given that the activation of effector caspases rapidly leads to cell death, the extent of transient activated caspase-3 observed in knockdown tissues indicates a significant degree of apoptosis on BRAF ablation in the context of the LOX-IMVI tumor model. In contrast, although BRAF knockdown decreased VEGF secretion ~3-fold *in vitro* (data not shown),

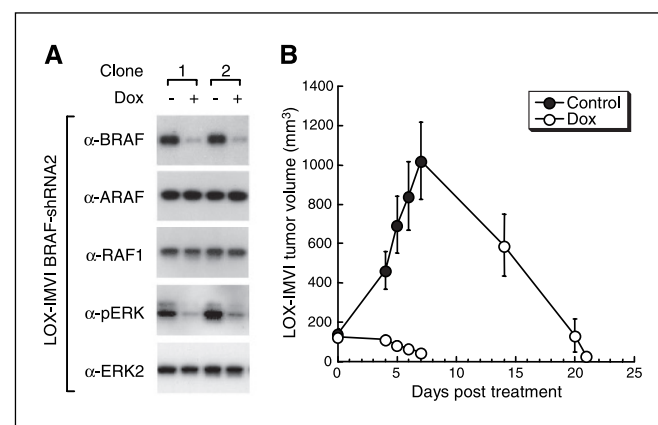


Figure 3. Conditional knockdown of BRAF using a second shRNA induces tumor regression. **A**, Western blot analysis of two LOX-IMVI/BRAF-shRNA2 cell clones for changes in BRAF protein expression and ERK1/2 phosphorylation on 2 μg/mL doxycycline treatment. **B**, LOX-IMVI/BRAF-shRNA2 xenograft mice exhibit tumor regression on 1 mg/mL doxycycline treatment. Each cohort consisted of 10 mice.

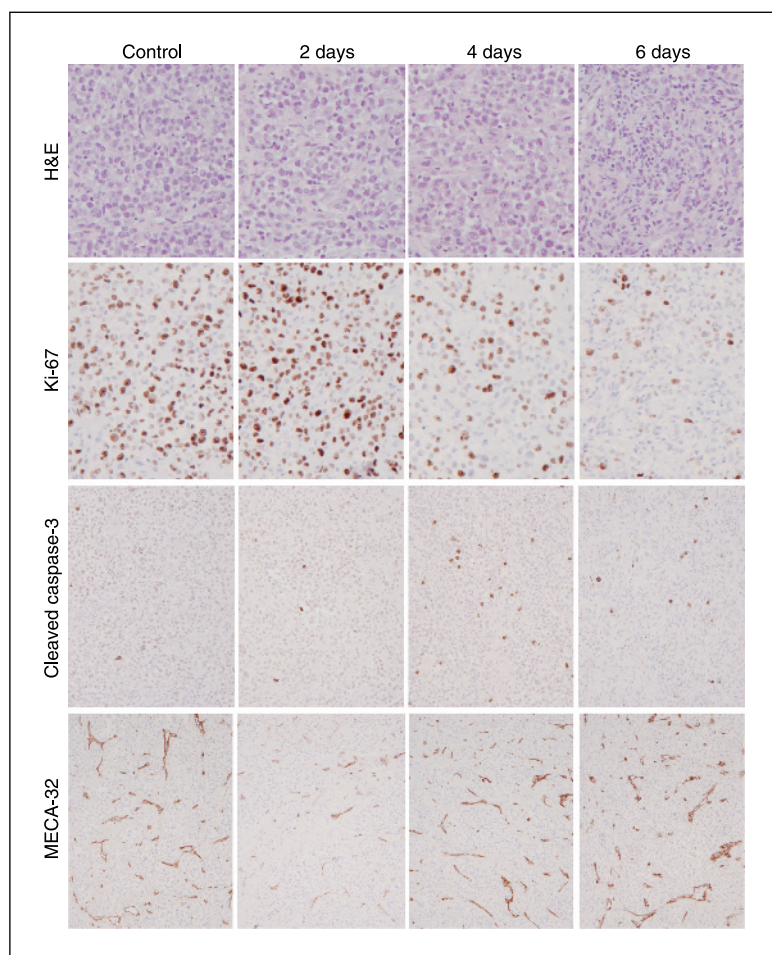


Figure 4. Histologic analysis of regressing tumors. LOX-IMVI/ BRAF-shRNA tumor-bearing mice were placed on 1 mg/mL doxycycline and sacrificed as indicated. Tumor tissue was analyzed by immunohistochemistry using antibodies specific for Ki-67, cleaved caspase-3, or MECA-32 (brown staining). No staining was observed in the naive immunoglobulin G control.

BRAF signaling does not play a pivotal role in regulating tumor vascularization as determined by staining of endothelial cells (Fig. 4). In summary, these results support the current view that BRAF signaling is important for mediating cellular proliferation and survival in tumorigenesis.

Attenuation of metastatic tumor development by BRAF knockdown. The spectrum of BRAF protein mutations has been shown to narrow significantly, with the BRAF^{V600E} variant being most prominent, in cutaneous/s.c. metastases compared with primary melanomas (33). However, the extent to which BRAF^{V600E} promotes the growth of tumor cells at metastatic sites has not yet been experimentally examined. Given that melanoma metastases are the predominant cause of melanoma-associated death (34), it would be of great interest to provide validation for targeting BRAF in the context of metastatic melanoma. To address this, we used A375M cells that were selected for high metastatic ability (35) and firefly luciferase expression (36) for whole-body noninvasive monitoring of tumor development via *in vivo* bioluminescence imaging. We engineered this cell line to stably express BRAF-shRNA for doxycycline-regulatable knockdown of BRAF protein and signaling (Fig. 5A). Pilot *in vivo* experiments showed that tail vein injection of 4×10^5 A375M cells into female *scid-beige* mice led to pulmonary, ovarian, and adrenal tumors that are detectable by bioluminescence imaging after a relatively short latency.

To evaluate the efficacy of BRAF knockdown in this systemic model, cohorts of mice with similarly increasing tumor burden

were divided into treatment groups. Mice were monitored longitudinally for tumor onset, progression, survival, and response to BRAF knockdown. In this melanoma metastasis model, BRAF ablation significantly slowed tumor growth and prolonged the survival of mice ($P < 0.0001$, log-rank test; Fig. 5B). When induction of knockdown was delayed until systemic tumors were well established, progression of disease was still partially inhibited as shown by representative images and bioluminescent quantification of pulmonary tumor burden (Fig. 5C and D). These experiments show anti-BRAF therapy to be a promising strategy to inhibit certain metastatic tumors and that the A375M bioluminescence imaging model can be used to measure the antitumoral efficacy of novel BRAF agents in development.

Discussion

Human tumor xenografts implanted into immunosuppressed mice have represented the mainstay of preclinical anticancer drug development for the past 25 years (37). Herein, we have shown that combination with regulatable RNA interference approaches represents a significant advance for these models and enables key proof-of-concept experiments for a particular target to assist in the optimization of pharmaceutically tractable molecules. Specifically, we have used inducible shRNAs of high specificity to study BRAF loss-of-function effects in preclinical melanoma xenografts. Our results show that BRAF is necessary for sustained neoplasia in

these models. The described data also lead to an expectation that specific targeting of BRAF will be an effective clinical therapy for melanomas carrying BRAF^{V600E} activating mutations. Of note, LOX-IMVI and A375 tumors show differential sensitivity to BRAF ablation (regression and stasis, respectively), the basis for which requires further study. Histopathologic analysis of LOX-IMVI regressing xenograft tumors over time showed marked differences correlating with the extent of BRAF knockdown (Fig. 4). Significant neoplastic contributions of BRAF^{V600E} include aberrant proliferation and cell survival. Consistent with our findings, a recent mechanism of action study using regenerated skin transplants showed hyperplasia arising from active BRAF (38). It is also striking that LOX-IMVI xenograft tumor vasculature was impervious to RNA interference-mediated targeting of BRAF in tumor cells. This is in contrast with the reduction of vascular development observed following BAY 43-9006 treatment of melanoma xenograft models (21, 39). Hence, we can conclude that BRAF inhibition alone is insufficient for the antiangiogenic activity of this compound and that changes in tumor vasculature induced by BAY 43-9006 result

from the additional targeting of receptor tyrosine kinases involved in angiogenesis (21).

Many of the challenges associated with conventional RNA interference methods are circumvented by use of a tetracycline-inducible shRNA system that can be introduced into tumor cell lineages (40). For instance, whereas numerous tissue culture applications are done using only transient siRNA knockdown, elucidating the role of an oncogene in transformation necessitates long-term shRNA induction for thorough *in vivo* analysis (Fig. 1C). Although some published studies have combined xenograft tumor experiments with transient knockdown using very optimal siRNA sequences (39), these very short studies are limited by variability in standard measurement of tumor growth (as with caliper measurement of s.c. tumors <100 mm³) and the dearth of tissues amenable to further biochemical or histologic analyses. Moreover, the standard plasmid- and viral-based methods for constitutive shRNA expression may unavoidably introduce significant selection pressure on the cells when targeting genes essential for cell viability or proliferation. Long-term suppression of gene expression can also result in compensatory or even nonphysiologic responses. Furthermore, as most tumor cell lines have undergone multiple genetic alterations and may have unstable genomes, stable cell lines generated by drug selection may have additional genetic damage. For this reason, an inducible gene silencing system allows for internal controls, thereby confirming the altered tumor growth phenotype as the result of the target gene loss-of-function rather than additional genetic hits. One caveat to consider for all RNA interference methods is that loss of a gene product leading to a molecular knockdown may not be equivalent to inhibiting only its catalytic function. To this end, cells depleted for endogenous proteins could be reconstituted with knockdown-resistant target genes rendered functionally inactive by point mutations to better mimic a chemical knockdown achieved through small-molecule inhibitors. Nevertheless, shRNA-mediated molecular knockdown provides a useful readout for consequences of target inhibition before a drug candidate becomes available. In addition, given the recent advances in *in vivo* delivery of siRNAs (41), it is quite conceivable that molecular knockdown studies will help address the feasibility of developing targeted siRNA-based therapies.

Having established the value of this inducible system for target validation in preclinical cancer research, it will be of interest to further examine inhibition of both oncogenic and wild-type forms of BRAF in cell lines that also include other indications. In addition, inducible BRAF knockdown may be combined with current standard of care agents to assess synergistic and adverse effects. This could potentially aid in developing an appropriate clinical trial design to test BRAF small-molecule inhibitors. Global analysis of the BRAF-regulated melanoma transcriptome or signaling pathways in inducible shRNA cells may also yield novel diagnostic or surrogate biomarkers for pathway activity. Taken together, this method allows for extensive interpretation of the biological outcome resulting from frequent BRAF activating mutations in melanoma.

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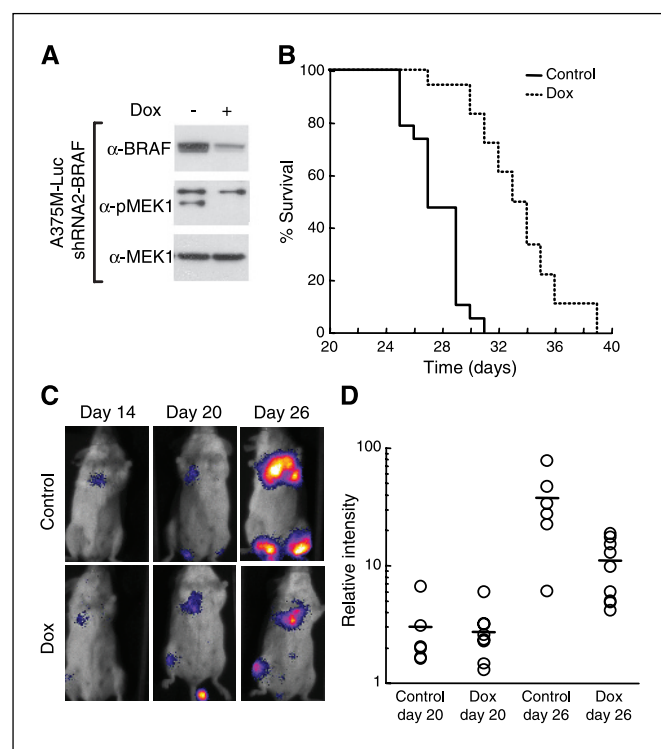


Figure 5. Reduction of A375M systemic tumor growth by BRAF shRNA knockdown. **A**, Western blot analysis showing expression of BRAF and phosphorylation of MEK1 in uninduced cells (lane 1) and cells treated with 2 mg/mL doxycycline for 72 hours (lane 2). Total MEK1 serves as an internal control to show equal loading. **B**, Kaplan-Meier survival data of *scid-beige* mice i.v. injected with 4×10^5 A375M-luc/shRNA-BRAF cells and receiving drinking water containing 5% sucrose only (control) or sucrose with 1 mg/mL doxycycline. Animals were monitored for tumor onset and illness until they reached a terminal stage and were euthanized. Each group consisted of at least 10 mice. The reduction in tumor growth conferred by doxycycline-mediated BRAF knockdown is significant according to the log-rank test, $P < 0.0001$. Median survival of all mice treated with 1 mg/mL doxycycline was 33.4 days, in comparison with 27.6 days for control mice. **C**, representative *in vivo* bioluminescence imaging of visible light emitted on injection of mice with luciferin; **D**, quantification of tumor burden of mice receiving doxycycline versus sucrose-treated control mice. Homogeneous cohorts of mice with established tumor lesions were divided into treatment groups 2 weeks after injection of A375M-luc/shRNA-BRAF cells. Bioluminescence is represented as relative to the intensity at day 14 for each animal.

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