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Expression of Endogenous Oncogenic *V600E B-raf* Induces Proliferation and Developmental Defects in Mice and Transformation of Primary Fibroblasts

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Abstract

Mutations of the human *B-RAF* gene are detected in ~8% of cancer samples, primarily in cutaneous melanomas (70%). The most common mutation (90%) is a valine-to-glutamic acid mutation at residue 600 (V600E; formerly V599E according to previous nomenclature). Using a Cre/Lox approach, we have generated a conditional knock-in allele of *V600E B-raf* in mice. We show that widespread expression of *V600E B-Raf* cannot be tolerated in embryonic development, with embryos dying ~7.5 dpc. Directed expression of mutant *V600E B-Raf* to somatic tissues using the IFN-inducible *Mx1-Cre* mouse strain induces a proliferative disorder and bone marrow failure with evidence of nonlymphoid neoplasia of the histiocytic type leading to death within 4 weeks of age. However, expression of mutant B-Raf does not alter the proliferation profile of all somatic tissues. In primary mouse embryonic fibroblasts, expression of endogenous *V600E B-Raf* induces morphologic transformation, increased cell proliferation, and loss of contact inhibition. Thus, *V600E B-Raf* is able to induce several hallmarks of transformation in some primary mouse cells without evidence for the involvement of a cooperating oncogene or tumor suppressor gene.

Introduction

Raf oncogenes were first identified as transforming genes in retroviruses that caused tumors in mice and chickens (1, 2). Oncogenic homologues of *C-Raf* and *B-RAF* were associated with the induction of carcinomas, sarcomas, and cancers of the hemopoietic lineage in these species (3, 4). It has since been discovered that oncogenic mutations of the *B-RAF* gene are present in human cancer samples (5). This study analyzed >900 cancer samples and human cancer cell lines and detected the presence of *B-RAF* missense mutations in ~70% of human malignant melanomas. *B-RAF* mutations are also present at a high frequency in papillary thyroid cancer (36-53%; refs. 6-8), colorectal cancer (5-22%; ref. 9), and serous ovarian

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cancer (30%; ref. 10) and at a low frequency in a wide variety of other cancers (1-3%; refs. 5, 11, 12). Thus, in a similar way to *RAS* oncogenes that are detected in 15% to 30% of human cancers (13), *B-RAF* mutations are a critical event in the development of many human neoplasias.

Over 30 missense *B-RAF* mutations have now been identified in cancer samples (11, 14). The most common mutation, accounting for ~90% of all those detected, is T1799A resulting in a glutamic acid-to-valine substitution at position 600 within the activation segment of the B-Raf kinase domain. ^{V600E}B-Raf induces 500-fold more active basal B-Raf kinase activity than basal ^{WT}B-RAF and exceeds ^{G12V}RAS-induced ^{WT}B-RAF activity (14). Recent structural studies have shown that this is due to the ability of the V600E mutation to disrupt the normal hydrophobic interaction between the activation segment and P-loop that maintains basal B-Raf in an inactive conformation (14). In ^{WT}B-RAF, phosphorylation of residues Thr⁵⁹⁹ and Ser⁶⁰² normally disrupt this interaction, allowing activation of B-RAF. The increased activity of ^{V600E}B-RAF has the effect of stimulating endogenous, basal mitogen-activated protein/extracellular signal-regulated kinase (MAP/ERK) kinase (MEK) and ERK1/2 activation, leading to increased cell proliferation and survival (14-17). The effect of ^{V600E}B-RAF on proliferation in mouse melanocytes and melanomas can be reversed by treatment of these cells with MEK inhibitors or with small interfering RNA (siRNA) to B-RAF. Thus, this mutant is thought to transform and mediate its tumorigenic effects in a similar way to oncogenic *RAS*. This is reinforced by the fact that *B-RAF* and *RAS* mutations are rarely present in the same cancer samples, indicating that they may have overlapping functions in tumorigenesis (5, 11, 12).

Although ^{V600E}B-RAF has the ability to induce G₀-G₁-S progression in tissue culture systems, it is not clear whether this is the case in tumor development *in vivo*. Oncogenic *B-RAF* mutations have been detected in benign nevi and premalignant colon polyps, suggesting that additional mutations in other key oncogenes/tumor suppressor genes are required to unleash the tumorigenic effects of *B-RAF* (9, 18). Candidate genes for such additional mutation are *INK4A* and *PTEN* that are frequently found mutated in melanomas (19, 20). Work in tissue culture systems has also shown that the ability of the RAF/MEK/ERK cascade to induce G₀-G₁-S cell cycle progression is dependent on the cooperative activation of the phosphatidylinositol 3-kinase (PI3K)/PDK1/AKT pathway (21) or inactivation of cyclin-dependent kinase inhibitors (22, 23), including p16^{INK4A} (24, 25).

To investigate how *B-RAF* is involved in tumorigenesis, and whether oncogene/tumor suppressor gene cooperation has a key function in development of tumors with *B-RAF* mutations, we have produced a tractable mouse model that mimics the somatic expression of ^{V600E}B-Raf in human cancers. These transgenic mice contain a heterozygous knock-in mutation of ^{V600E}*B-raf* whose expression is determined by the presence or absence of a Lox-STOP-Lox (LSL) cassette. We show that following deletion of LSL by the Cre recombinase, expression of ^{V600E}B-Raf in somatic tissues leads to development of some hallmarks of cancer in mice.

Materials and Methods

Generation of *LSL-B-raf*^{V600E} mice

The LSL targeting vector was assembled by cloning left, middle, and right arms in the ploxP-neo-loxP vector. The right arm fragment contains exon 15 with the T1799A mutation. The middle arm contains a LSL cassette with a cDNA encoding exons 15 to 18 of mouse *B-raf*. This was PCR amplified from a mouse *B-raf* cDNA clone. The forward primer for this PCR reaction had the sequence 5'-

TACTAACCATGCCTTCTTCTTTTCTACAGATATATTTCTTCATGAAGACCTCAC

GGT-3' and contained 31 nucleotides corresponding to a splice acceptor sequence from the *α-globin* gene followed by 28 nucleotides corresponding to exon 15 of the mouse *B-raf* gene. The reverse primer had the sequence 5'-CGAGTGAGAGACACAAAAAATTCCAACACACTATTGTTGCTACCTTACTTTATTGTCTCACTC-3' and contained 35 nucleotides corresponding to the *α-globin* pause site and 28 nucleotides corresponding to the 3'-untranslated region of the mouse *B-raf* gene. The vector was electroporated into E14.1 embryonic stem cells, and homologous recombination events were identified. Positive embryonic stem clones were injected into mouse C57BL6 blastocysts and chimeric mice derived using standard procedures. Inheritance of the targeted allele was assessed by PCR genotyping (26, 27) of mouse tail DNA using primers A (5'-GCCAGGCTCTTTATGAGAA-3') and B (5'-GCTTGGCTGGACGTAAACTC-3') for the *LSL-B-raf^{V600E}*-targeted allele combined with primers A and C (5'-AGTCAATCATCCACAGAGACCT-3') for the wild-type allele (Fig. 1). A breeding colony of *LSL-B-raf^{V600E}* mice were established by backcrossing on the C57BL6 background. Genotyping of offspring from *LSL-B-raf^{V600E}* × *Cre* crosses was done by using a multiplex PCR reaction using primers A to C as well as primers D (5'-GTTTCGCAAGAACCTGATGGACA-3') and E (5'-CTAGAGCCTGTTTTGCACGTTC-3') for identifying the *Cre* gene. To detect the *Lox-B-RAF^{V600E}* allele, primers A and C were used. All PCRs were done using Reddymix PCR reagent (ABgene, Surrey, United Kingdom) with 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds.

Histology, immunohistochemistry, and proliferation assays

Mouse tissues were processed for histology by previously described methods (26). Sections were either stained with H&E or used for immunohistochemistry with antibodies for Ki67 (Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom), Mac-2 (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada), and phospho-ERK (Cell Signaling Technology, Beverly, MA) or used for terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) analysis as described previously (26, 27). To assess proliferation, mice were injected with 10 µg/mL bromodeoxyuridine (BrdUrd), and tissues were either analyzed by immunohistochemistry with an *α*-BrdUrd antibody or, in the case of the spleen, cells were dissociated by passing through a mesh, counted, and then stained with *α*-BrdUrd-FITC and analyzed by fluorescence-activated cell sorting (FACS) using the kit and method provided by BD Biosciences (San Jose, CA). Bone marrow was collected from the femurs of freshly culled mice, and cells were quantitated using a Coulter counter (Becton Dickinson, Mountain View, CA).

Northern blot analysis

Mouse spleens were snap-frozen in liquid nitrogen and ground to a powder. Total cellular RNA was isolated using the RNeasy-B technique (Qiagen, Crawley, United Kingdom). RNA was resolved by denaturing 1.2% agarose/18% (v/v) formaldehyde gel electrophoresis and transferred to GeneScreen (Dupont, Boston, MA), before UV cross-linking. cDNA inserts were labeled (Random primers DNA labeling system, Amersham Biosciences, Buckinghamshire, United Kingdom) using [*α*-³²P]dATP (Amersham; 3,000 Ci/mmol). Filters were hybridized in a solution containing 7% (w/v) SDS, 1% (w/v) bovine serum albumin, 0.25 mol/L NaCl, and 0.5 mol/L Na₂HPO₄ buffer (pH 7.2) at 65°C for 16 hours. They were washed sequentially at 65°C in 4× SSC, 0.5× SSC, and 0.2× SSC, each containing 0.1% SDS, and resolved by autoradiography.

Western blots and kinase assays

Mouse tissues were snap-frozen in liquid nitrogen and homogenized in NP40 lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% NP40, 5 mmol/L NaF] with 1

mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L aprotinin, 1 mmol/L leupeptin, and 1 mmol/L sodium orthovanadate. Western blots were carried out as described previously (28). B-Raf kinase assays were done using the immunoprecipitation kinase cascade assay as previously reported (29) using the B-Raf antibody provided by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Primary antibodies were a 1:1,000 dilution of a mouse monoclonal antibody against Thr²⁰²/Tyr²⁰⁴ phospho-p44/42 ERK1/2 (Cell Signaling Technology), a 1:1,000 dilution of a rabbit polyclonal antibody for ERK2 (Zymed Laboratories, Inc., South San Francisco, CA), a 1:1,000 dilution of a rabbit polyclonal antibody against Ser²¹⁷/Ser²²¹ phospho-MEK1/2 (Cell Signaling Technology), a 1:1,000 dilution of a cyclin D1 antibody (Cell Signaling Technology), a 1:1,000 dilution of an antibody for actin (Sigma, St. Louis, MO), and a 1:1,000 dilution of an antibody for p21^{CIP1} (Cell Signaling Technology).

Mouse embryonic fibroblast analysis

Mouse embryonic fibroblasts (MEF) were isolated and cultured from homogenized E14.5 embryos arising from timed matings between *LSL-B-raf*^{V600E} animals and wild-type C57BL/6 animals using standard methods (26). MEFs were transfected with the pCrePac plasmid using a Nucleofector under the MEF conditions recommended by the manufacturer (Amaxa Biosystems, Koln, Germany). Forty-eight hours following transfection, cells were photographed and processed for immunofluorescence analysis of the actin cytoskeleton by staining with FITC-phalloidin as described previously (30). For proliferation, mock-transfected and Cre-transfected cells, 24 hours after transfection, were placed in serum-free media for 20 hours and then either left unstimulated or stimulated with 10% (v/v) FCS for 16 hours. BrdUrd incorporation assays were done by using the BrdUrd labeling and detection kit (Roche, Indianapolis, IN). The percentage of BrdUrd-positive cells was visualized by fluorescence microscopy using a Zeiss Axiophot microscope. For protein analysis, 24 hours following transfection, cells were serum starved for 20 hours and then stimulated with FCS for 10 minutes or for 16 hours. Protein lysates were generated, and Western blots were carried out as described above. Focus-forming assays were done as described elsewhere (31), and agar assays were done as described previously (32).

Results

Numbering of the amino acids in B-Raf

The valine residue that is most commonly mutated in human cancer was originally reported as being at position 599 of the human B-Raf protein. However, as highlighted by Wellbrock et al. (33), more recent sequencing data have shown that there was a previous error in the sequencing of the *B-RAF* 5' coding region, such that this valine is now reclassified as being at residue 600. In the mouse, the 5' coding region has not yet been fully sequenced and thus the residue number of the equivalent valine is not known. For the present purposes, this residue will also be referred to as Val⁶⁰⁰.

Generation of *LSL-B-raf*^{V600E} mice

We used the Cre-LoxP system to generate transgenic animals containing a conditional knock-in allele that expresses ^{V600E}B-Raf only after Cre-mediated recombination (Fig. 1A). The targeting vector contained a left arm spanning exon 14 and a right arm containing exon 15 with the *B-raf*^{V600E} mutation, representing a T1799A nucleotide change. These two arms were separated from each other by the *neo*^R gene and a minigene cDNA encoding wild-type exons 15 to 18. Three LoxP sequences were present in the vector and two polyadenylation STOP sequences. In this arrangement, wild-type B-Raf is expressed from the targeted gene with exons 15 to 18 being encoded by the minigene. Introduction of the Cre recombinase allows deletion of the LSL cassette and expression of ^{V600E}B-Raf. The vector was introduced into embryonic stem cells, and homologous recombination events were

identified. Mice were generated from the positive clones, and a breeding colony was established on the C57BL6 background. PCR genotyping confirmed the inheritance of the *LSL-B-raf*^{V600E} allele (Fig. 1B).

Expression of ^{V600E}B-Raf in embryogenesis

We generated mice that permanently express V599EB-Raf in all tissues. This was achieved by crossing the *LSL-B-raf*^{V600E} mice to heterozygous *CMV-Cre* mice that express the Cre transgene in germ cells as well as in a mosaic manner during development (34). Multiplex PCR genotyping was used to identify *LSL-B-raf*^{V600E} + *CMV-Cre* animals (Fig. 1B). These were then analyzed by PCR for the presence of the Cre-deleted *Lox-B-raf*^{V600E} allele expressing ^{V600E}B-Raf (Fig. 1C). Genotyping of the surviving offspring revealed no viable *LSL-B-raf*^{V600E} + *CMV-Cre* animals (Table 1). The *LSL-B-raf*^{V600E} and *CMV-Cre* mice were thus mated at timed intervals, and embryos were harvested at various ages of gestation. *LSL-B-raf*^{V600E} + *CMV-Cre* embryos were obtained at the expected frequency at E7.5, but lethality was observed from this point onwards. At E7.5, all embryos with the *LSL-B-raf*^{V600E} allele were abnormal and in the process of being reabsorbed (Fig. 1D). Only those embryos that were mosaic for the *Lox-B-raf*^{V600E} and *LSL-B-raf*^{V600E} alleles survived to later ages of gestation (Table 1), but even these all died before birth. This indicates that the expression of ^{V600E}B-Raf in gestation is lethal to the animal, although the embryos are able to implant.

Somatic expression of ^{V600E}B-Raf

To examine the effect of the oncogenic ^{V600E}B-*raf* mutation on somatic tissues, we crossed the *LSL-B-raf*^{V600E} mice to *Mx1-Cre* mice and examined the offspring. The *Mx1-Cre* mouse strain expresses the Cre recombinase from an IFN-inducible promoter and can be induced to express the Cre recombinase either by injection of IFN or polyribonucleic/polyribocytidylic acid copolymer (pI-pC; ref. 35). Consistent with previous observations with the *Mx1-Cre* mice, we found that the *LSL-B-raf*^{V600E} + *Mx1-Cre* animals showed a phenotype even without injection of pI-pC (Fig. 2A; refs. 36, 37). This is likely because of endogenous IFN production in response to a subclinical infection or other stimulus. All of the *LSL-B-raf*^{V600E} + *Mx1-Cre* animals died before 4 weeks of age, whereas the *LSL-B-raf*^{V600E} - *Mx1-Cre* animals survived the normal life span of a mouse with no symptoms (Fig. 2A). Wild-type mice with the *Mx1-Cre* allele also showed no phenotype (data not shown). PCR analysis of DNA from various tissues of *LSL-B-raf*^{V600E} + *Mx1-Cre* animals indicated the presence of the *LSL-B-raf*^{V600E} allele in many tissues, including the spleen, liver, lung, and heart (Fig. 2B). However, this allele was present at considerably lower levels in some tissues, particularly, the kidney, testis, gut, and thymus.

Tissues with the *LSL-B-raf*^{V600E} allele were subjected to further investigation. No alterations were detected in the morphology or pathology of the lung, heart, kidney, gut, or testis (data not shown). However, the spleen and liver were abnormal. Splenomegaly was observed in all *LSL-B-raf*^{V600E} + *Mx1-Cre* animals (Fig. 2C). Histologic analysis of spleen sections showed the infiltration of a proliferating, pleomorphic population of cells with abundant eosinophilic cytoplasm and irregular nuclei as well as extramedullary sites of hemopoiesis (Fig. 2D). The spleens of the *LSL-B-raf*^{V600E} + *Mx1-Cre* mice also stained far more strongly with antibodies for the S-phase marker Ki67 as well as phospho-ERK than *LSL-B-raf*^{V600E} - *Mx1-Cre* spleens, but there were no noticeable changes in apoptosis, as measured by TUNEL analysis (Fig. 2D). To quantitate proliferation *in vivo*, mice were injected with BrdUrd, and incorporation into splenocytes was measured by FACS analysis (Fig. 2E). The splenocytes of *LSL-B-raf*^{V600E} + *Mx1-Cre* mice (mean = 51.1%, n = 6) proliferated 2-3× more than splenocytes from *LSL-B-raf*^{V600E} - *Mx1-Cre* mice (mean = 22.6%, n = 6, P = 0.0015). To identify which cell types were amplifying in the spleen,

Northern blot analysis was done on spleen RNA with probes for genes expressed in the myeloid (*myeloperoxidase* and *lysozyme M*), lymphoid (*CD19*, *rag-1*, and *VpreB1*), stem cell (*Sca1*), and erythroid (*erythropoietin receptor*) lineages. No consistent increase in expression of any of these genes was observed in *LSL-B-raf^{V600E} + Mx1-Cre* spleens compared with *LSL-B-raf^{V600E} - Mx1-Cre* spleens (Fig. 2F). However, the spleens from *LSL-B-raf^{V600E} + Mx1-Cre* mice stained strongly for Mac2 (Fig. 2G), indicating the presence of an amplified population of histiocytes.

Histologic analysis of the liver also indicated the presence of extramedullary sites of hemopoiesis. Ki67 staining indicated increased proliferation in the liver of *LSL-B-raf^{V600E} + Mx1-Cre* mice, associated with increased phospho-ERK staining (Fig. 3A). However, in addition to this, the liver of these mice was also in the process of undergoing apoptosis as indicated by increased TUNEL staining (Fig. 3A). The *Lox-Braf^{V600E}* allele was strongly detected in the bone marrow of *LSL-B-raf^{V600E} + Mx1-Cre* animals. This was associated with the virtual absence of RBC in the bone marrow and a reduction in the production of WBC by at least 50% (Fig. 3B). Circulating RBC and WBC were also significantly reduced (data not shown). Additionally, *LSL-B-raf^{V600E} + Mx1-Cre* animals developed *LSL-B-raf^{V600E}*-containing histiocytes (Fig. 2B) at extranodal sites that were predominantly observed on the skin (Fig. 3C), although occasionally on the gut wall. These were detectable at birth and contained proliferating, phospho-ERK positive cells with a typical histiocyte morphology that stained strongly for Mac2 (Fig. 3D). The appearance of splenomegaly and Mac2-positive staining cells in the spleen and at extranodal sites is consistent with a diagnosis of nonlymphoid leukemia of the histiocytic type (according to Bethesda classification of hemopoietic neoplasms).⁴

Expression of ^{V600E}B-Raf induces activation of the B-RAF/mitogen-activated protein/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase cascade

We did B-Raf kinase assays on protein lysates generated from spleen, liver, and lung of *LSL-B-raf^{V600E} ± Mx1-Cre* animals. B-Raf kinase activity was elevated by ~11-fold in the spleen and by ~3-fold in the liver and lung of *LSL-B-raf^{V600E} + Mx1-Cre* animals compared with controls (Fig. 4A). Western blot analysis also showed that levels of phospho-MEK and phospho-ERK were significantly increased in tissues affected by the expression of ^{V600E} B-Raf (i.e., the spleen and liver). With regard to cyclin D1, levels of expression were increased in the spleen of *LSL-B-raf^{V600E} + Mx1-Cre* mice but not the liver, probably because this tissue is also undergoing apoptosis (Fig. 3A). However, tissues that were not morphologically affected by the mutation (lung or thymus) showed an increase in levels of phospho-MEK but not an increase in phospho-ERK or cyclin D1 levels (Fig. 4B).

Expression of ^{V600E}B-Raf induces transformation of primary mouse embryonic fibroblasts

To further investigate the transforming effects of endogenous ^{V600E}B-Raf, we derived primary MEFs from *LSL-B-raf^{V600E}* mice and transfected these cells with a plasmid expressing the Cre recombinase. *LSL-B-raf^{V600E}* MEFs without Cre showed a typical nontransformed morphology (Fig. 5A). However, transfection with the Cre plasmid induced morphologic transformation and collapse of the actin cytoskeleton (Fig. 5A). In addition, *LSL-B-raf^{V600E}* MEFs with the Cre plasmid showed a significant increase in proliferation (Fig. 5B). When serum starved, 6.75% of mock-transfected cells entered S phase, whereas in the presence of Cre 9.57% of cells entered S phase ($P < 0.002$). In the presence of FCS, 11.5% of cells without Cre entered S phase, whereas in the presence of Cre, 26.2% of cells entered S phase ($P < 10^{-6}$). Thus, serum-starved, Cre-containing *LSL-B-raf^{V600E}* MEFs undergo proliferation at a similar rate to serum-stimulated, mock-transfected *LSL-B-*

⁴<http://emice.nci.nih.gov>.

raf^{V600E} MEFs, although proliferation of the Cre-containing *LSL-B-raf*^{V600E} MEFs was further stimulated by the presence of growth factors. Concomitant with the increase in proliferation in serum-starved, Cre-containing cells, levels of phospho-ERK (Fig. 5C) and cyclin D1 (Fig. 5D) were increased in these cells compared with nontransfected controls, but p21^{CIP1} levels were drastically reduced (Fig. 5D). *LSL-B-raf*^{V600E} primary MEFs transfected with the Cre plasmid also acquired the ability to grow to a very high density, with loss of contact inhibition and focus formation after 3 weeks in culture (Fig. 5E), and were able to form very small colonies when grown in soft agar (Fig. 5F).

Discussion

We report the generation of mice with a LSL conditional knock-in mutation of ^{V600E}*Braf*, whereby the expression of endogenous ^{V600E}B-Raf is dependent on delivery of the Cre recombinase and deletion of the LSL cassette. We show that somatic tissues expressing endogenous ^{V600E}B-Raf have elevated B-Raf kinase activity, elevated levels of phospho-MEK, phospho-ERK, and cyclin D1 expression, and increased proliferation. In addition, expression of ^{V600E}B-Raf in primary MEFs induces morphologic transformation as well as hyperproliferation, the loss of contact inhibition, and a weak ability to grow in an anchorage-independent manner. These results show that at least in some primary mouse cells, ^{V600E}B-Raf is able to induce several hallmarks of transformation without evidence for the involvement of a second cooperating oncogene or loss of a key tumor suppressor gene.

Classic studies have previously established a requirement for oncogene cooperation in transformation of primary cells (38). *RAS* and *RAF* oncogenes overexpressed in primary cells have been shown to induce cell senescence unless accompanied by mutations in the p19^{ARF}/p53 pathway (23-25). However, it has recently been shown that the expression of lower, physiologic levels of *G12DK-ras* in mice promotes proliferation and partial transformation in primary cells, without a cooperating event (39). These authors were able to indicate clear differences in the biological effects of overexpressed oncogenic *K-ras*, which induces senescence, versus oncogenic expression of endogenous *K-ras* at physiologic levels that induces proliferation. We have found that like *G12DK-Ras*, ^{V600E}B-Raf expressed at physiologic levels in mice can promote proliferation and partial transformation of some primary cell types. This is perhaps a more surprising result than that with oncogenic *RAS* as *RAS* has multiple effector pathways, notably, the RAF/MEK/ERK and PI3K/PDK1/PKB pathways, both of which can impinge on the cell cycle, whereas oncogenic RAF is only known to activate the MEK/ERK pathway. However, our observations are consistent with previous data showing that the effects of ^{V600E}B-Raf on proliferation of mouse melanocytes and human melanoma cells containing the ^{V600E}B-Raf mutation can be reversed by siRNA to *B-RAF* (15-17, 40). In the development of human malignant cancers *in vivo*, the situation may well be more complex as ^{V600E}B-Raf mutations are detected in a high proportion of benign melanocytic lesions (nevi) and premalignant colon polyps in which the cells are thought to be senescent (9, 18, 41, 42). This has been taken as evidence that *B-RAF* activation itself is not sufficient to induce all hallmarks of cancer *in vivo*, at least in melanocytes and colonic epithelial cells.

We expressed ^{V600E}B-Raf ubiquitously, by crossing the *LSL-B-raf*^{V600E} mice to *CMV-Cre* mice, and show that this cannot be tolerated in embryonic development with embryos harboring the *Lox-B-raf*^{V600E} allele embryos dying before E7.5. This result is similar to that obtained with the ubiquitous expression of endogenous *G12DK-ras*, which also induces early embryonic lethality (39). Using the *Mx1-Cre* strain, we found that the expression of ^{V600E}B-Raf in adult somatic tissues induces hyperproliferation as well as bone marrow failure. The effects on proliferation are highly tissue dependent. Pathologic effects on proliferation were observed in the *Lox-B-raf*^{V600E}-containing spleen and liver, and the histiocytes were

observed on the skin but not in the *Lox-B-raf^{V600E}*-containing lung, kidney, or thymus, which showed no evidence for the presence of histiocytes. Despite this, similar levels of B-Raf kinase activity were observed in the *Lox-B-raf^{V600E}*-containing lungs of *LSL-B-raf^{V600E} + Mx1-Cre* mice compared with the *Lox-B-raf^{V600E}*-containing livers. This observation may be related to the fact that there is no noticeable increase in levels of phospho-ERK in the lung of *LSL-B-raf^{V600E} + Mx1-Cre* animals, although phospho-MEK levels are increased, which would suggest an uncoupling between B-RAF/MEK and ERK in the lung, possibly due to the action of tissue-specific MAP kinase phosphatases. The liver is affected in a complex way by the expression of ^{V600E}B-Raf. Higher levels of apoptosis are observed as well as increased proliferation. The increase in apoptosis may be an indirect consequence of the bone marrow failure and reduction in circulating RBC rather than a direct consequence of ^{V600E}B-Raf expression in this tissue.

Expression of ^{V600E}B-Raf using the *Mx1-Cre* strain leads to the development of a form of hemopoietic dysplasia with some of the characteristics of nonlymphoid leukemia of the histiocyte type, in which there is an amplification of circulating cells of the histiocyte/macrophage lineage. At present, it is not clear why these cells in particular are susceptible to the proliferative effects of ^{V600E}B-Raf. Histiocytes have been observed in other mouse tumor models for cancer, particularly those on the C57BL6 strain background, including *Bax/ARF* double null mice (43) as well as mice expressing endogenous *G12V K-ras* (44). However, histiocytes are rare in humans and have not thus far been associated with the presence of *B-RAF* mutations. Indeed, despite the high level of expression of B-Raf in hemopoietic cells, *B-RAF* mutations are extremely rare in hemopoietic neoplasms, and none of those reported thus far represent *V600E B-RAF* mutations (45-47). By contrast, *NRAS* and *KRAS* mutations occur in ~30% of acute myeloid leukemias and myeloproliferative disorders (13), and somatic activation of *G12D K-ras* in mice using the *Mx1-Cre* strain induces a similar myeloproliferative disorder (37, 48), indicating that oncogenic *B-RAF* and *RAS* target different populations of *Mx1-Cre*-expressing cells.

Studies with *Ras* oncomice have clearly illustrated the importance of expression level in mediating the effects of oncogenic *Ras* on tumor development (39). We have developed a tractable strain of transgenic mouse that allows the somatic expression of ^{V600E}B-Raf at physiological levels. The next important step is to deliver the Cre recombinase to these mice in such a way that it allows the somatic expression of ^{V600E}B-Raf in cell types most affected by *B-RAF* mutations in the development of human tumors, most notably melanocytes, papillary thyrocytes, and colonic and ovarian epithelial cells, as well as cancer stem cells.

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Figure 1.

A, generation of *LSL-B-raf^{V600E}* and *Lox-B-raf^{V600E}* alleles. The targeting vector contains left and right arms with the right arm containing exon 15 with the T1799A mutation (*). These two arms are separated by the LSL cassette. This cassette contains three LoxP sequences (black arrows), a minigene (MG) encoding exons 15 to 18 of wild-type B-Raf with a splice acceptor (SA) sequence at the 5' end. Two STOP sequences, represented by polyadenylation (PA) sequences, are located at the 3' end of the minigene and at the 3' end of the *neo^R* cassette. Homologous recombination between the targeting vector and the wild-type *B-raf* gene in embryonic stem cells generated the *LSL-B-raf^{V600E}* allele. Expression of the Cre recombinase allows deletion of the LSL cassette and generation of the *Lox-B-*

raf^{V600E} allele. Location of primers used for PCR genotyping (arrows and A-C). B, PCR genotyping to detect *LSL-B-raf*^{V600E}, *WT B-raf*, and *Cre* alleles. Primers A-E were used in combination on tail DNA samples from intercrosses between heterozygous *LSL-B-raf*^{V600E} mice and *Cre* mice. In this example, the *WT B-raf* allele (466 bp), *Cre* allele (350 bp), and *LSL-B-raf*^{V600E} allele (140 bp) are analyzed in tail DNAs from heterozygous *LSL-B-raf*^{V600E} mice without *Cre* (lane 1), wild-type mice with *Cre* (lane 2), and heterozygous *LSL-B-raf*^{V600E} mice with *Cre* (lane 3). C, PCR genotyping to detect *Lox-B-raf*^{V600E} and *WT B-raf* alleles. Primers A and C were used on the same DNA samples described above in (B). A 518-bp product indicates the presence of the *Lox-B-raf*^{V600E}, and a 466-bp product indicates the presence of the *WT B-raf* allele. D, photographs of E7.5 embryos resulting from *LSL-B-raf*^{V600E} × *CMV-Cre* intercrosses showing *LSL-B-raf*^{V600E} – *CMV-Cre* embryo (left) and *LSL-B-raf*^{V600E} + *CMV-Cre* embryo (right). Bar, 250 μm.

Figure 2.

Analysis of mice resulting from *Lox-B-raf^{V600E}* × *Mx1-Cre* intercrosses. *A*, % survival of *LSL-B-raf^{V600E}* + *Mx1-Cre* animals versus *LSL-B-raf^{V600E}* − *Mx1-Cre* animals at various ages after birth. *B*, PCR genotyping to detect *Lox-B-raf^{V600E}* allele in DNA from various tissues derived from *LSL-B-raf^{V600E}* + *Mx1-Cre* mice. *C*, splenomegaly in *LSL-B-raf^{V600E}* + *Mx1-Cre* mice. *Left*, increase in size of the spleen of P21 animals. *Right*, pooled weight data from four spleens of *LSL-B-raf^{V600E}* + *Mx1-Cre* mice at P21. *LSL-B-raf^{V600E}* + *Mx1-Cre* mice have a mean spleen weight of 0.2 g compared with 0.04 g for the control *LSL-B-raf^{V600E}* − *Mx1-Cre* mice. *Columns*, means; *bars*, SD. *D*, histologic analysis of spleen sections from *LSL-B-raf^{V600E}* ± *Mx1-Cre* mice. Spleen sections were either stained with H&E, with antibodies for Ki67 or phospho-ERK, or processed for TUNEL analysis. Bar, 50 μm (the same for all sections). *E*, proliferation analysis of spleens from *LSL-B-raf^{V600E}* ± *Mx1-Cre* mice. Mice were injected with BrdUrd, splenocytes were isolated, and the % incorporation of BrdUrd was assessed by FACS analysis. *Columns*, means; *bars*, SD. *F*, Northern blot analysis of spleen RNA isolated from *LSL-B-raf^{V600E}* ± *Mx1-Cre* mice. Northern blots were probed with the markers indicated. *G*, histologic analysis of spleen sections from *LSL-B-raf^{V600E}* ± *Mx1-Cre* mice with a histiocyte marker. Spleen sections were stained with a Mac-2 antibody to detect histiocyte amplification. Bar, 50 μm.

Figure 3.

Analysis of tissues from mice resulting from *LSL-B-raf^{V600E}* × *Mx1-Cre* intercrosses. *A*, histologic analysis of liver sections from *LSL-B-raf^{V600E}* ± *Mx1-Cre* mice. Liver sections were either stained with H&E, with a Ki67 antibody, with a phospho-ERK antibody or processed for TUNEL analysis. *Right*, H&E section was photographed at higher magnification (×400) to indicate site of hemopoiesis (*black arrow*) in the mutant liver. Bar, 50 μm (the same for all sections) except for 25 μm (enlarged H&E section, *right*). *B*, analysis of bone marrow from *LSL-B-raf^{V600E}* ± *Mx1-Cre* mice. Bone marrow cells were isolated from the femurs of *LSL-B-raf^{V600E}* ± *Mx1-Cre* mice and analyzed by the Coulter counter. There are far fewer RBC and WBC in the bone marrow of *LSL-B-raf^{V600E}* + *Mx1-*

Cre mice (*shaded area*) than *LSL-B-raf^{V600E} – Mx1-Cre* mice (*clear area*). PCR analysis of DNA from bone marrow of *LSL-B-raf^{V600E} + Mx1-Cre* animal, indicating the presence of the *Lox-B-raf^{V600E}* allele. *C*, photograph of histiocyte arising on skin of *LSL-B-raf^{V600E} + Mx1-Cre* animal. *D*, histologic analysis of histiocyte sections from *LSL-B-raf^{V600E} ± Mx1-Cre* mice. Sections were either stained with H&E, with antibodies for Ki67, phospho-ERK, or Mac-2, or processed for TUNEL analysis. Bar, 50 μ m.

Figure 4. Analysis of B-Raf/MEK/ERK activation in tissues derived from *LSL-B-raf^{V600E} ± Mx1-Cre* mice. Protein lysates were harvested from various tissues and subjected to (A) B-Raf kinase assays and (B) Western blot analysis with antibodies for phospho-ERK, phospho-MEK, and cyclin D1. Immunoblotting with a total ERK antibody was used to confirm protein loading.

Figure 5.

Analysis of primary MEFs derived from *LSL-B-raf^{V600E}* mice. **A**, morphologic transformation. Primary *LSL-B-raf^{V600E}* MEFs were either mock-transfected (*-Cre*) or transfected with the pCrePac plasmid (*+Cre*). After 48 hours, the cells were either photographed (*top*) or subjected to immunofluorescence analysis with FITC-phalloidin (*bottom*). **B**, proliferation analysis. Mock- and Cre-transfected cells were serum starved for 20 hours (*open columns*) and then stimulated with serum for 16 hours (*gray columns*). % BrdUrd incorporation was assessed under each condition. *Columns*, means; *bars*, SD. The cells transfected with Cre undergo a high level of proliferation even in the absence of serum compared with mock-transfected controls. **C**, Western blot analysis of lysates prepared from Cre- and mock-transfected MEFs with an antibody for phospho-ERK. Immunoblotting with a total ERK antibody was used to confirm protein loading. **D**, Western blot analysis of lysates prepared from Cre- and mock-transfected MEFs with antibodies for cyclin D1 and p21^{CIP1}. Immunoblotting with an antibody for actin was used to confirm protein loading. **E**, focus formation assay of Cre- and mock-transfected *LSL-B-raf^{V600E}* primary MEFs. MEFs were grown to confluency and the media was changed every 2 to 3 days until foci formed for 18 days. **F**, soft agar assays of Cre- and mock-transfected *LSL-B-raf^{V600E}* primary MEFs.

Table 1
Genotypes of embryos arising from intercrosses between *LSL-B-raf^{V600E}* and *CMV-Cre* heterozygous animals

Age	<i>B-raf^{+/+}</i>	<i>B-raf^{+/+}</i> + <i>CMV-Cre</i>	<i>LSL-B-raf^{V600E}</i>	<i>LSL-B-raf^{V600E}</i> - <i>CMV-Cre</i>	<i>LSL-B-raf^{V600E}</i> + <i>CMV-Cre</i>	Untyped
E7.5	7	6	4	4	10	0
E8.5	5	4	5	5	5	1
E9.5	0	4	2	2	1*	0
E10.5	3	6	3	3	2*	5
E12.5	2	5	5	5	1*	9
Birth (P ₁)	13	12	13	13	0	0

NOTE: Analysis of embryos at E7.5 and E8.5 showed that *LSL-B-raf^{V600E}* + *CMV-Cre* embryos were obtained at the expected frequency at these developmental ages but were obtained at reduced frequencies at later developmental ages, and none survived to birth. At E7.5 and E8.5, all *LSL-B-raf^{V600E}* + *CMV-Cre* embryos containing the *Lox-B-raf^{V600E}* allele were abnormal and in the process of being reabsorbed (see Fig. 1D).

* These later surviving embryos were all mosaic for the *LSL-B-raf^{V600E}* and *Lox-B-raf^{V600E}* alleles.