Expression of Endogenous Oncogenic \( V^{600E}B-raf \)Induces Proliferation and Developmental Defects in Mice and Transformation of Primary Fibroblasts

Kathryn Mercer,^{1} Susan Giblett,^{1} Stuart Green,^{1} David Lloyd,^{1} Silvy DaRocha Dias,^{3} Mark Plumb,^{2} Richard Marais,^{3} and Catrin Pritchard^{1}

Departments of Biochemistry and Genetics, University of Leicester, University Road, Leicester, United Kingdom and Cancer Research UK Centre for Cell and Molecular Biology, Institute of Cancer Research, London, United Kingdom

Abstract

Mutations of the human \( B-RAF \) gene are detected in \( \sim \) 8% of cancer samples, primarily in cutaneous melanomas (70%). The most common mutation (90%) is a valine-to-glutamic acid mutation at residue 600 \( (V^{600E}; \text{ formerly } V^{599E}) \) according to previous nomenclature. Using a Cre/Lox approach, we have generated a conditional knock-in allele of \( V^{600E}B-raf \) in mice. We show that widespread expression of \( V^{600E}B-Raf \) cannot be tolerated in embryonic development, with embryos dying \( \sim 7.5 \) dpc. Directed expression of mutant \( V^{600E}B-Raf \) 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 \( V^{600E}B-Raf \) induces morphologic transformation, increased cell proliferation, and loss of contact inhibition. Thus, \( V^{600E}B-raf \) 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. (Cancer Res 2005; 65(24); 11493-500)

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 \( \sim 900 \) cancer samples and human cancer cell lines and detected the presence of \( B-Raf \) missense mutations in \( \sim 70\% \) 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 (9, 18). Candidate genes for such additional mutations have now been identified in \( 15\% \) 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. \( V^{600E}B-raf \) induces 500-fold more active basal \( B-Raf \) kinase activity than basal \( W^{500}B-raf \) and exceeds \( G^{12V}Ras \)-induced \( W^{500}B-raf \) activity (14). Recent structural studies have shown that this is due to the ability of the \( V^{600}E \) 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 \( W^{500}B-Raf \), phosphorylation of residues Thr\(^{599} \) and Ser\(^{602} \) normally disrupt this interaction, allowing activation of \( B-Raf \). The increased activity of \( V^{600E}B-Raf \) 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 \( V^{600E}B-Raf \) 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).

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 \( \sim 90\% \) 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. \( V^{600E}B-Raf \) induces 500-fold more active basal \( B-Raf \) kinase activity than basal \( W^{500}B-Raf \) and exceeds \( G^{12V}Ras \)-induced \( W^{500}B-Raf \) activity (14). Recent structural studies have shown that this is due to the ability of the \( V^{600}E \) 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 \( W^{500}B-Raf \), phosphorylation of residues Thr\(^{599} \) and Ser\(^{602} \) normally disrupt this interaction, allowing activation of \( B-Raf \). The increased activity of \( V^{600E}B-Raf \) 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 \( V^{600E}B-Raf \) 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 \( V^{600E}B-Raf \) has the ability to induce \( G_{0}/G_{1} \)-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 \( INK44 \) and \( PTEN \) that are frequently found mutated in melanomas (19, 20). Work in tissue culture systems has also shown that the ability of the \( RAS/MEK/ERK \) cascade to induce \( G_{0}/G_{1} \)-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 \( V^{600E}B-Raf \) in human cancers. These transgenic mice contain a heterozygous knock-in mutation of \( V^{600E}B-raf \) 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-RafV600E 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'-TACT-ACATTGCCTTTCTTCTTCTCAAGATATTATTTCCGTGAAGCCTCAGGGT-3' and contained 31 nucleotides corresponding to a splice acceptor sequence from the a-globin gene followed by 28 nucleotides corresponding to exon 15 of the mouse B-raf gene. The reverse primer had the sequence 5'-GGAGTGGAGACACAAATTTCCACACATTTGTGTCTACTCT-TACTTTATGCTCTCCT-3' and contained 35 nucleotides corresponding to the a-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 were generated using standard procedures. Inheritance of the targeted allele was assessed by PCR genotyping (26, 27) of mouse tail DNA using primers A (5'-GGCCGGCCTTCTATGAGAA-3') and B (5'-GTTGGTGGTACGATTCAC-3') for the LSL-B-RafV600E-targeted allele combined with primers A and C (5'-AGTCATACCTCCACAGAGACC-3') for the wild-type allele (Fig. 1). A breeding colony of LSL-B-RafV600E mice were established by backcrossing on the C57BL6 background. Genotyping of offspring from LSL-B-RafV600E × Cre crosses was done by using a multiplex PCR reaction using primers A to C as well as primers D (5'-GGTCCGAAAACAGCCGTGAAAGACCC-3') and E (5'-CTAGACCGTGTGTTTGGCACTGTC-3') for identifying the Cre gene. To detect the Lox-B-RafV600E 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) antibody or, in the case of the spleen, cells were dissociated by enzymatic digestion with 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 LSL-B-rafV600E cassette. Homologous recombination between the targeting vector and the wild-type B-raf gene in embryonic stem cells generated the LSL-B-rafV600E allele. Expression of the Cre recombinase allows deletion of the LSL cassette and generation of the Lox-B-rafV600E allele. Location of primers used for PCR genotyping (arrows and A-C), B, PCR genotyping to detect LSL-B-rafV600E, WT B-raf, and Cre alleles. Primers A-E were used in combination on tail DNA samples from intercrosses between heterozygous LSL-B-rafV600E mice and Cre mice. In this example, the WT B-raf allele (466 bp), Cre allele (350 bp), and LSL-B-rafV600E allele (140 bp) are analyzed in tail DNAs from heterozygous LSL-B-rafV600E mice without Cre (lane 1), wild-type mice with Cre (lane 2), and heterozygous LSL-B-rafV600E mice with Cre (lane 3). Primers A-C were used for the same DNA samples described above in (B). A 518-bp product indicates the presence of the Lox-B-rafV600E allele, and a 466-bp product indicates the presence of the WT B-raf allele. D, photographs of E7.5 embryos resulting from LSL-B-rafV600E × CMV-Cre intercrosses showing LSL-B-rafV600E – CMV-Cre embryo (left) and LSL-B-rafV600E + CMV-Cre embryo (right). Bar, 250 μm.
(pH 7.5), 150 mM NaCl, 0.5% NP40, 5 mM NaF] with 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 1 mM leupeptin, and 1 mM 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 (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 p21CIP1 (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-rafV600E animals and wild-type C57BL6 animals using standard methods (26). MEFs were transfected with the pCMV-Cre plasmid using a Nucleofector under the MEF conditions recommended by the manufacturer (Amx Swissys, Köln, 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, 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-rafV600E 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-rafV600E mutation, representing a T1799A nucleotide change. These two arms were separated from each other by the neo 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-rafV600E allele (Fig. 1B).

Expression of V600E-B-Raf in embryogenesis. We generated mice that permanently express V599EBRaf in all tissues. This was achieved by crossing the LSL-B-rafV600E 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-rafV600E + CMV-Cre mice (Fig. 1B). These were then analyzed by PCR for the presence of the Cre-deleted Lox-B-rafV600E allele expressing V600E-B-Raf (Fig. 1C). Genotyping of the surviving offspring revealed no viable LSL-B-rafV600E + CMV-Cre animals (Table 1). The LSL-B-rafV600E and CMV-Cre mice were thus mated at timed intervals, and embryos were harvested at various ages of gestation. LSL-B-rafV600E + 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 Lox-B-rafV600E allele were abnormal and in the process of being reabsorbed (Fig. 1D). Only those embryos that were mosaic for the Lox-B-rafV600E and LSL-B-rafV600E 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

Table 1. Genotypes of embryos arising from intercrosses between LSL-B-rafV600E and CMV-Cre heterozygous animals

<table>
<thead>
<tr>
<th>Age</th>
<th>B-raf+/+</th>
<th>B-raf+/+ + CMV-Cre</th>
<th>LSL-B-rafV600E – CMV-Cre</th>
<th>LSL-B-rafV600E + CMV-Cre</th>
<th>Untyped</th>
</tr>
</thead>
<tbody>
<tr>
<td>E7.5</td>
<td>7</td>
<td>6</td>
<td>4</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>E8.5</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>E9.5</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>1*</td>
<td>0</td>
</tr>
<tr>
<td>E10.5</td>
<td>3</td>
<td>6</td>
<td>3</td>
<td>2*</td>
<td>5</td>
</tr>
<tr>
<td>E12.5</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>1*</td>
<td>9</td>
</tr>
<tr>
<td>Birth</td>
<td>13</td>
<td>12</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE: Analysis of embryos at E7.5 and E8.5 showed that LSL-B-rafV600E + 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-rafV600E + CMV-Cre embryos containing the Lox-B-rafV600E allele were abnormal and in the process of being reabsorbed (see Fig. 1D).

*These later surviving embryos were all mosaic for the LSL-B-rafV600E and Lox-B-rafV600E alleles.
LSL-B-raf<sup>V600E</sup> 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 polyriboinosinic/polyribocytidylic acid colpolymer (pI-pC; ref. 35). Consistent with previous observations with the Mx1-Cre mice, we found that the LSL-B-raf<sup>V600E</sup> + 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<sup>V600E</sup> + Mx1-Cre animals died before 4 weeks of age, whereas the LSL-B-raf<sup>V600E</sup> – Mx1-Cre animals survived the normal life span of a mouse with no symptoms (Fig. 2B). 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<sup>V600E</sup> + Mx1-Cre animals indicated the presence of the Lox-B-raf<sup>V600E</sup> 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 Lox-B-raf<sup>V600E</sup> 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<sup>V600E</sup> + 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<sup>V600E</sup> – 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<sup>V600E</sup> – Mx1-Cre spleens, but there were no noticeable changes in apoptosis, as measured by TUNEL analysis (Fig. 2D). To quantify 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<sup>V600E</sup> + Mx1-Cre Cre mice (mean = 51.1%, n = 6) proliferated 2-3× more than splenocytes from LSL-B-raf<sup>V600E</sup> – 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<sup>V600E</sup> – Mx1-Cre spleens compared with LSL-B-raf<sup>V600E</sup> + Mx1-Cre spleens (Fig. 2F). However, the spleens from LSL-B-raf<sup>V600E</sup> + 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<sup>V600E</sup> + Mx1-Cre mice. The spleens of control mice and mice with the Lox-B-raf<sup>V600E</sup> allele did not stain for Ki67. The spleens of LSL-B-raf<sup>V600E</sup> + Mx1-Cre mice stained strongly for Mac2, indicating an increased population of histiocytes. Histologic analysis of liver sections from LSL-B-raf<sup>V600E</sup> – Mx1-Cre mice showed little or no Ki67 staining in the liver, consistent with the lack of histiocyte amplification in these animals.
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-rafG^{V600E} allele was strongly detected in
the bone marrow of LSL-rafG^{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-rafG^{V600E} × Mx1-Cre animals developed
Lox-rafG^{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 histiocyte type (according to
Bethesda classification of hemopoietic neoplasms).

Expression of V^{600E}B-Raf induces activation of the B-RAF/
mitogen-activated protein/extracellular signal-regulated
kinase/extracellular signal-regulated kinase cascade.
We did B-Raf kinase assays on protein lysates generated from
spleen, liver, and lung of LSL-rafG^{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-rafG^{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
V^{600E} B-Raf (i.e., the spleen and liver). With regard to cyclin D1,
levels of expression were increased in the liver and spleen of
LSL-rafG^{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 V^{600E}B-Raf induces transformation of
primary mouse embryonic fibroblasts. To further investigate the
transforming effects of endogenous V^{600E}B-Raf, we derived
primary MEFs from LSL-rafG^{V600E} mice and transfected these
cells with a plasmid expressing the Cre recombinase. LSL-
rafG^{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-rafG^{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-rafG^{V600E} MEFs undergo proliferation at a
similar rate to serum-stimulated, mock-transfected LSL-rafG^{V600E}
MEFs, although proliferation of the Cre-containing LSL-rafG^{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. 5D) and cyclin
D1 (Fig. 5D) were increased in these cells compared with
nontransfected controls, but p21^{CIP1} levels were drastically reduced.

in the p19ARF/p53 pathway (23–25). However, it has recently been shown to induce cell senescence unless accompanied by mutations and oncogene cooperation in transformation of primary cells (38).

Discussion

We report the generation of mice with a LSL conditional knock-in mutation of V600E B-Raf, 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 p19ARF/p53 pathway (23–25). However, it has recently been shown that the expression of lower, physiologic levels of G12D K-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 G12D K-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-rasV600E mice to CMV-Cre mice, and show that this cannot be tolerated in embryonic development with embryos harboring the Lox-B-rasV600E allele embryos dying before E7.5. This result is similar to that obtained with the ubiquitous expression of endogenous G12D K-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 hyper-proliferation as well as bone marrow failure. The effects on proliferation are highly tissue dependent. Pathologic effects on proliferation were observed in the Lox-B-rasV600E-containing spleen and liver, and the histiocytes were observed on the skin but not in the Lox-B-rasV600E-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-rasV600E-containing lungs of LSL-B-rasV600E + Mx1-Cre mice compared with the Lox-B-rasV600E-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-rasV600E + 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 histioyte type, in which there is an amplification of circulating cells of the histioyte/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 G12D 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 G12DK-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 V600EB-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 V600EB-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.

Acknowledgments


Grant support: Cancer Research UK project grant #C1362/A2402 (C. Pritchard and R. Marais) and core funding grant #C107/A3096 (R. Marais).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Peter Greaves (University of Leicester) for providing histopathological advice; Misha Roodbari (University of Leicester) for helping with the MEF proliferation assays; staff in Biomedical Services at Leicester for helping with mouse breeding colonies; and Graham Freeth (Animal Resource Centre, Western Australia) for providing CMV-Cre and Mx1-Cre mice.

References


Expression of Endogenous Oncogenic $B-raf^{V600E}$ Induces Proliferation and Developmental Defects in Mice and Transformation of Primary Fibroblasts

Kathryn Mercer, Susan Giblett, Stuart Green, et al.