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A new mouse model to explore the initiation, progression, and therapy of BRAF<sup>V600E</sup>-induced lung tumors

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Mutationally activated BRAF<sup>V600E</sup> (BRAF<sup>VE</sup>) is detected in ~6% of human malignancies and promotes sustained MEK1/2–ERK mitogen-activated kinase signaling pathway activation. We have designed BRaf<sup>CA</sup> mice to express normal BRaf prior to Cre-mediated recombination after which BRaf<sup>VE</sup> is expressed at physiological levels. BRaf<sup>CA</sup> mice infected with an Adenovirus expressing Cre recombinase developed benign lung tumors that only rarely progressed to adenocarcinoma. Moreover, BRaf<sup>VE</sup>-induced lung tumors were prevented by pharmacological inhibition of MEK1/2. BRaf<sup>VE</sup> expression initially induced proliferation that was followed by growth arrest bearing certain hallmarks of senescence. Consistent with Ink4a/Arf and TP53 tumor suppressor function, BRaf<sup>VE</sup> expression combined with mutation of either locus led to cancer progression.

Results and Discussion

Generation of BRaf<sup>CA</sup> mice by homologous recombination in embryonic stem (ES) cells

The development of new mouse models of human cancer places great emphasis on temporal and spatial control of oncogene expression, with particular attention paid to the levels of oncogene expression. This is especially important for BRaf since relatively small differences in RAF activity can promote quiescence, proliferation, or cell cycle arrest/senescence [Woods et al. 1997; Zhu et al. 1998]. We sought to develop a mouse to accurately model the role of BRaf<sup>VE</sup> in cancer initiation, progression, and therapy with an emphasis on temporal and spatial control of tissues of interest. Using a suitably designed targeting vector [Fig. 1A], we used homologous recombination in ES cells to generate mice carrying a Cre-activated allele of BRaf<sup>CA</sup> [Fig. 1B]. BRaf<sup>CA</sup> is designed to express normal BRaf prior to Cre-mediated recombination at which time BRaf<sup>VE</sup> expression is initiated at physiological expression levels and subject to normal patterns of alternate splicing and differential exon usage [Fig. 1C]. By design, this model recapitulates the situation in human cells, where one copy of normal BRaf is converted by somatic mutation to BRaf<sup>VE</sup>. To discriminate the expression of the various BRaf<sup>CA</sup> mRNAs,

[Keywords: BRaf; mouse model; lung tumors; senescence]

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V600E is indicated (*). (enzyme fragments are indicated. The modified exon 15 encoding BRafVE germline. Breeding of and BRafVE alleles was 98% that of endogenous internal 5 BRaf are depicted. Red arrows depict fully processed the targeting vector used, and the proteins expressed from the locusations analogously to normal BRaf gene products. (A) The genomic BRaf locus (BRaf(wt)), the targeting vector used, and the proteins expressed from the locus are depicted. Red arrows depict fully processed BRaf mRNAs. Ex-ternal 5′ and 3′ probe locations and the size of XbaI restriction enzyme fragments are indicated. The modified exon 15 encoding V600E is indicated [*]. (B) The targeted BRafCA allele and following Cre-mediated recombination, BRafVE (C). (D) Semiquantitative RT–PCR analysis of targeted and wild-type ES cell-derived mRNAs using a radiolabeled reverse primer. PCR products amplified for the indicated numbers of cycles were treated with BamHI where indicated to distinguish BRaf transcripts. Expression of the BRafCA allele was 98% that of endogenous BRaf mRNA.

Multiple correctly targeted ES cell clones were identified by Southern blotting (Supplementary Fig. 1A) and by RT–PCR analysis of BRaf and BRafCA mRNA expression. The latter analysis confirmed that BRaf and BRafCA mRNAs were expressed at the same level [Fig. 1D], whereas BRafVE mRNA was not detected [data not shown]. Two independent clones gave rise to chimeric mice that transmitted the BRafCA allele through the germline. Breeding of BRafCA/+ mice to mice heterozygous for the CA allele, or a null allele, generated mice with all possible genotypes at normal Mendelian frequencies, demonstrating that the BRafCA allele functions analogously to normal BRaf prior to Cre-mediated recombination [Supplemental Material].

BRafVE expression in the lung elicits tumor formation

To test the utility of BRafCA mice, we initiated expression of BRafVE in the lungs by intranasal instillation of an adenovirus expressing Cre recombinase [Ad-Cre] [Fasbender et al. 1998]. We chose this strategy for four reasons: (1) Mutationally activated BRAF has been described in human NSCLC [Zebisch and Troppmair 2006]; (2) The use of Ad-Cre provides temporal control over BRafVE expression and therefore permits prospective evaluation of BRafVE effects. [3] Ad-Cre titration allows control of the number of cells in which BRafVE expression is initiated. [4] Others have previously described the effects of Raf-1- or Ad-Cre-induced KRasG12D expression in the lungs [Kerkhoff et al. 2000; Jackson et al. 2001; Tuveson et al. 2004].

Six- to eight-week-old wild-type or BRafCA/+ mice were administered with $5 \times 10^{7}$ PFU [plaque-forming units] of either Ad-Cre or Ad-βGal. Mice were monitored for 7–8 wk, at which time all of the BRafCA/+ mice administered with Ad-Cre were euthanized due to evidence of labored breathing and general wasting. BRafCA genomic rearrangement [Fig. 2A] and BRafVE mRNA expression [Fig. 2B] were Cre-dependent and entirely restricted to the lung, as we detected no abnormalities or induction of BRafCA somatic recombination in any other tissue analyzed. Lungs of BRafVE-expressing mice displayed evidence of the formation of multiple lung tumors with an adenomatous morphology [Fig. 2C–F]. Such lesions were not detected in BRafCA/+ mice even up to 6 mo after administration of a high dose of Ad-βGal or in wild-type mice injected with Ad-Cre. The effects of BRafVE expression in the lung were highly penetrant in that all BRafCA mice [n = 31] treated with Ad-Cre [107 PFU] developed lung tumors over the course of nine experiments. Moreover, induction of BRafVE expression in the lung using a ubiquitously expressed, conditionally active CreER transgene [Guerra et al. 2003] elicited the formation of multiple lung tumors of the same type.

The cellular origins of human lung adenocarcinoma and adenomas are unclear. NSCLCs frequently express markers of Clara cells or alveolar type II pneumocytes [ATII]. Indeed, it has been suggested that they arise from a common bronchio-alveolar stem cell [BASC] [Kim et al. 2005]. To assess the properties of BRafVE-induced tumors, immunohistochemical analyses to detect Clara Cell antigen [CCA/CC10] and Surfactant Protein-C (SP-C), a surface marker of ATII pneumocytes, were performed. Staining for CCA detected cells lining the bronchioles, but the BRafVE-induced tumors were largely CCA negative [Fig. 2J]. In contrast, the majority of cells within BRafVE-induced tumors expressed SP-C, suggesting that they have properties of ATII pneumocytes [Fig. 2K, L]. Furthermore, analysis of the earliest BRafVE-in-duced lesions [2 wk after Ad-Cre] revealed them to express SP-C.

Requirement for BRAFVE–MEK–ERK activity for lung tumor formation

Clear genetic and biochemical evidence indicates that the MEK–ERK pathway plays an essential role in cancer cell proliferation, yet recent studies have suggested that alternate RAF-regulated pathways may exist [Chen et al. 2001]. To address the effects of BRafVE on canonical ERK signaling, we assessed phosphorylation of downstream effector proteins MEK1/2, ERK1/2, and the ERK-dependent phosphorylation of the Ets1/2 transcription factors. Indeed, using serial tissue sections, positive staining for all three of these surrogate markers of BRafVE activity overlapped in all tested cases, demonstrating ERK MAP kinase activation [Fig. 3A, B, Supplementary Fig. 2A–C]. It is reported that KRasG12D-induced lung tumors do not routinely display elevated pERK1/2 but display stress-
activated MAP kinase (SAPK/JNK) activation (Lee et al. 2002). Hence, to address the importance of MEK1/2 in BRafVE-induced tumor formation, we used PD0325901, a highly specific and selective MEK1/2 pharmacological inhibitor (a generous gift of Pfizer, Inc.). PD0325901 displays a nanomolar IC\textsubscript{50} in cells and, unlike most protein kinase inhibitors, acts noncompetitively with its sub- strates (Sebolt-Leopold and Herrera 2004). Tumors were treated with PD0325901 dis- 

Figure 2. Expression of BRaf\textsuperscript{VE} in lung induces adenomas. [A] PCR detection of BRaf\textsuperscript{CA} rearrangement from lungs of BRaf\textsuperscript{CA/+} (wt) or BRaf\textsuperscript{CA/+} mice infected with Ad-Gal [B] or Ad-Cre [C]. The black arrowhead, blue arrow, and red arrowhead highlight the wild-type, the unarranged BRaf\textsuperscript{CA}, and the BRaf\textsuperscript{VE} alleles, respectively. [B] Detection of BRaf\textsuperscript{VE} mRNA in BRaf\textsuperscript{CA} mice following Ad-Cre infection. Total lung RNA from BRaf\textsuperscript{CA/+} (wt) or BRaf\textsuperscript{CA/+} mice isolated 8 wk after Ad-Cre infection (5 × 10\textsuperscript{7} PFU) was subjected to RT-PCR analysis as in Figure 1D. BRaf\textsuperscript{VE} and BRaf\textsuperscript{CA} transcripts are distinguished through the use of restriction polymorphisms (BamHI, B, XbaI, X) with the diagnostic digestion products indicated by blue arrows and red arrowheads, respectively. (C-L) BRaf\textsuperscript{CA/+} mice were infected with 5 × 10\textsuperscript{7} PFU [G–H] or 10\textsuperscript{7} PFU [I–L] of Ad-Cre intranasally and were analyzed 7 wk following infection. (C) Macroscopic lesions are present on the surface of BRaf\textsuperscript{CA/+} lungs (right and magnified in D) but not wild-type (left) lungs. (E-H) Hematoxylin and eosin staining of histological sections of wild-type [E,F] or BRaf\textsuperscript{CA/+} [G,H]. Note papillary adenomas in higher power magnification (H) and wide-scale involvement of lungs [G]. (I,J) Adenomas stain negative for CCA [I,J] and positive for SP-C [K,L]. Bars: F,H,I,L, 100 µm; E,G,J,K, 500 µm.

BRaf\textsuperscript{VE}-induced cell cycle arrest as a barrier to lung adenocarcinoma progression

Induction of BRaf\textsuperscript{VE} expression by high dose Ad-Cre induced the formation of sufficiently large numbers of tumors that such mice rapidly succumbed to breathing difficulties and general wasting. In subsequent experiments, we used lower Ad-Cre doses to elicit smaller numbers of tumors to determine if such lesions would display progression given sufficient time for additional events to occur. In addition, mice were euthanized at different times following Ad-Cre administration, and the histological appearance of the tumors was assessed. Lesions were characterized according to consensus criteria established by a panel of lung cancer biologists (Nikitin et al. 2004).

At early times after induced BRaf\textsuperscript{VE} expression, we detected evidence of epithelial hyperplasia (classified as 1.1.1.1) arising within the terminal bronchioles and within the central lung parenchyma. This hyperplastic epithelium displayed papillary ex- cresences; however, at early time points (2–4 wk), there was no nodule formation (Fig. 4A). Papillary adeno- 

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gression.

induced adenomas are more highly constrained from pro-

Figure 3. MEK1/2 dependency of BRafVE-induced adenoma formation. [A,B] Adenomas stain positive for active MEK (A) and active ERK (B). [C] Time line of PD0325901 treatment. BRafCA mice in-

fected with Ad-Cre [5 × 10⁶ PFU] were grouped randomly into one of

two arms: One arm received PD0325901, and the other received the vehicle control. Drug treatment was initiated 4 wk after initial in-

fection of mice and was sustained at the indicated concentrations

for 28 d. Eight weeks post-infection, all mice receiving vehicle de-

veloped multiple adenomas (D), whereas no mouse treated with

PD0325901 developed adenomas (E). [Insets] Lower magnification. Bars: A,B,D,E, 100 μm; A,B, insets, 1 mm; E,F, insets, 2 mm.

atypical adenomatous hyperplasia (AAH, ∼2 wk) that

progresses to adenoma (∼6 wk). Interestingly, the major difference appears to be that expression of KrasG12D in the lung leads to more rapid and consistent progression to adenocarcinoma than that elicited by BRafVE. These data suggest that either additional KRas effector path-

ways are required for cancer progression or that BRafVE-

induced adenomas are more highly constrained from pro-

gression.

Oncogene-induced cell cycle arrest with features of se-

nescence (OIS) is reported to be a cellular defense mecha-
nism restraining the proliferation of normal cells in re-
sponse to inappropriate activation of RAS or its signaling

effectors (Collado et al. 2005; Michaloglou et al. 2005). Recen
tly, it was reported that KrasG12D-induced lung tumors are arrested in the cell cycle and express OIS

markers (Collado et al. 2005). To determine if BRafVE-

induced lung tumors display evidence of OIS, tissue sec-

tions from lungs harvested at different times after

BRafVE expression were stained for markers of cell prolif-
eration (phospho-histone H3, Ki67, or BrdU incorporation) and for OIS markers (SA-βGal, p19ARF, Dec1). In hyperplastic lung tissues and in tumors induced at early
times after BRafVE expression, a high percentage of cells

stain positive for Ki67 (Fig. 4E,F), phospho-histone H3, and

BrdU incorporation. However, in tumors observed at later
times, the percentage of Ki67-positive cells is dra-

matically decreased even though the BRafVE-MEK-ERK

signaling pathway remains active in these cells (Figs.

3A,B, 4G). Further analysis of adenomatous lesions re-

vealed that they were positive for expression of p19ARF and

Dec1 (Fig. 4J). p19ARF expression was detected in

∼15% of tumor cells with staining localized to nucleioli,

a pattern consistent with previous data (Weber et al.

1999). While these markers of OIS were readily detected,

these adenomas were largely negative for SA-β-Gal activity [Fig. 4K]. In the BRafVE-induced adenocarcinomas found in the two mice displaying tumor progression, however, a very high rate of Ki67 staining was observed [Fig. 4H]. These data are consistent with the hypothesis that sustained activation of BRafVE promotes an initial period of cell proliferation followed by cell cycle arrest constraining further tumor progression.

BRafVE cooperates with TSG loss to promote adenocarcinoma formation

If BRafVE-induced senescence is a barrier to lung carci-
nogenesis, then BRafVE expression combined with loss

t of TSG expression should lead to cancer progression.

To this end, we crossed BRafCA/+ mice to obtain mice homo-

zygous for floxed alleles of either TP53 or Ink4a/Arf. These mice express normal levels of the various TSGs until the modified alleles undergo Cre-mediated deletion of critical exons. Concomitant loss of TP53 led to in-

creased proliferation at ∼8 wk post-infection with Ad-

Cre when compared with BRafCA/−/+ mice [Fig. 4L,M,O,P]. Histological analyses of lungs from BRafCA/−/−; TP53lox/lox mice revealed rapid cancer progression [Fig. 4L,M,R] and showed nodules composed of central papil-

lar structures with solid peripheral areas (similar to older BRafCA/+ mice) consistent with formation of mixed adenomas [1.2.1.2.3]. Several small airways also displayed papillary hyperplasia, which was never observed in BRafCA/+ mice. In each case analyzed (n = 5), mice lacking TP53 function displayed an increased prolifera-
tive index [Fig. 4P; Supplementary Fig. 3], and cells dis-

played altered nuclear morphology [Fig. 4S]. Collections of cells with enlarged nuclei stained positive for Ki67, indicating ongoing proliferation [Supplementary Fig. 3A–D]. Significantly, at ∼8 wk post-infection, all BRafCA/+ mice homozygous for the TP53lox allele had a normal lung phenotype [data not shown]. Upon euthanasia, 2/5

BRafCA/+; TP53lox/lox mice displayed prominent adenocarcinomas [Fig. 4R] composed of glandular structures lined by highly atypical cells with nuclear enlargement, hyperchromasia, and contour irregularities and displayed prominent nucleoli [Fig. 4S]. The adenocarcinomas possessed large areas of necrosis and showed evidence of vascular and lymphatic invasion. This is significant, as we have not detected adenocarcinoma in any BRafCA mice prior to 40 wk of age (n = 55).

Similarly, BRafVE expression in an Ink4a/Arflox/lox background led to scattered papillary adenomas with evidence of subpleural nodules harboring cords of atypical cells trapped within regions of mesenchymal prolifera-

tion. 2/4 BRafCA/+; Ink4a/Arflox/lox mice contained mul-
tiple lung tumors with a classical bronchioloalveolar component and, when tumor cells were present, growing along alveolar septa without architectural destruction. This pattern is peculiar and is not categorized in the recent classification scheme but displays phenotypic similarities to human bronchioloalveolar carcinoma. Ki67 staining demonstrates that these lesions display increased proliferation relative to BRafVE-induced adenoma-

mas [Fig. 4Q].

The data presented here provide support for the hy-

pothesis that oncogenic BRAF can provoke cell cycle ar-

rest accompanied by induction of some, but not all,

markers of OIS (Zhu et al. 1998; Collado et al. 2005).
That KRasG12D [Jackson et al. 2005] or BRAfVE can cooperate with loss of TSGs believed to mediate OIS is further, albeit circumstantial, evidence that OIS may serve as a bona fide tumor-suppressive mechanism in vivo. However, there remains at least one conundrum in this hypothesis. Adenomas that arise from expression of KRasG12D or BRAfVE in mice initiated by oncogene activation in a single cell that subsequently underwent ~15–20 population doublings to give rise to an adenoma. Clearly, the initial response to oncogene activation is not cell cycle arrest but a dramatic induction of cell proliferation. However, at some time temporally distinct from oncogene activation, mechanisms that promote cell cycle arrest/senescence are engaged. This interpretation is consistent with the observation that BRAfVE-induced melanocytic nevi are clonal, having undergone multiple rounds of proliferation (Pollock et al. 2003), but eventually undergo senescence [Michaloglou et al. 2005]. One possibility is that these biologically distinct outcomes reflect differences in the level of oncogene expression/activation at different stages of tumor progression. Alternatively, secondary signals may cooperate with KRasG12D or BRAfVE to initiate and/or maintain the OIS program. These observations contrast with those in cultured cells, where activation of RAF robustly induces p16INK4A and irreversible cell cycle arrest/senescence within 12–24 h and in the absence of any initial cell proliferation [Zhu et al. 1998]. While in vitro culture conditions may cooperate with RAF in the induction of senescence, the nature of the secondary trigger for OIS in vivo remains a key unanswered question.

Materials and methods

Generation of a conditionally active BRAf allele
DNA encompassing mouse BRAf exons 14–16 was modified such that exon 16 was replaced with a HSV-thymidine kinase cassette. A LoxP-flanked cassette containing the 3’ 382 base pairs (bp) of intron 14, the human BRAf cDNA containing exons 15–18, the mouse BRAf polyadenylation sequences, and a PGK-neo cassette was inserted into intron 14 upstream of a modified exon 15 that encodes BRAfV600E and a silent XbaI restriction site. Polymorphism. The details of construction, Southern blotting, and RNA analysis are available as Supplemental Material.

Mice used in this study
Mice carrying conditional alleles of TP53 or Ink4A/Arf were generated as described in the Supplemental Material. BRAfCA mouse genotypes were determined by standard PCR of tail DNA with primer pair AD [AD FwdA1, 5’-TGAGTATTTCGTTTGCAACTGTC-3’; and AD RevB1, 5’-CTCTGCTGGGAAAGCGGC-3’] to produce diagnostic PCR products of 185 bp, 308 bp, and 535 bp for BRAf, BRAf19, and the BRAfVE alleles, respectively.

Adenoviral Cre delivery
Ad-Cre and Ad-βGal were purified and titred by standard means (Virquest), and 10^7 to 5 x 10^7 PFU were administered to the nasal septum of 6 to 8-wk-old mice by intranasal instillation as a calcium phosphate precipitate [Fastenberg et al. 1998].

Immunohistochemistry
Animals were euthanized at the specified times or upon display of visible signs of disease. Lung tissues were prepared through standard techniques. Immunostaining was performed as described in the Supplemental Material. The details of construction, Southern blotting, and RNA analysis are available as Supplemental Material.
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References


on February 23, 2007