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A new mouse model to explore the initiation, progression, and therapy of BRAFV600E-induced lung tumors

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Mutational activation of BRAFV600E (BRAFVE) is detected in ∼6% of human malignancies and promotes sustained MEK1/2–ERK1/2 pathway activation. We have designed BRafCA mice to express normal BRaf prior to Cre-mediated recombination after which BRafVE is expressed at physiological levels. BRafCA mice infected with an Adenovirus expressing Cre recombinase developed benign lung tumors that only rarely progressed to adenocarcinoma. Moreover, BRafVE-induced lung tumors were prevented by pharmacological inhibition of MEK1/2. BRafVE expression initially induced proliferation that was followed by growth arrest bearing certain hallmarks of senescence. Consistent with Ink4a/Arf and TP53 tumor suppressor function, BRafVE expression combined with mutation of either locus led to cancer progression.}

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The RAS-activated RAF–MEK–ERK mitogen-activated protein (MAP) kinase signaling pathway is directly implicated in the development of a wide variety of human malignancies. Although mutational activation of RAS in human cancer was first demonstrated in 1982, somatic activating mutations in BRAF were only detected in 2002 (Davies et al. 2002). Mutationally activated BRAF is detected in melanoma (70%), colorectal (15%), papillary thyroid (40%), ovarian (30%), and non-small-cell lung cancers (NSCLCs) (40%) (Davies et al. 2002, Zebrasch and Troppmair 2006). Of the ∼40 BRAF mutations identified to date, a T1799A transversion encoding constitutively active BRAF-V600E [BRAFVE hereafter] accounts for ∼90% of all BRAF mutations (Wan et al. 2004). Mutationally activated BRAFVE promotes the sustained activation of the ERK1/2 MAP kinases, pleiotropic regulators of the aberrant physiology of the cancer cell.

Lung cancer is the most prevalent cancer in the industrialized world and was responsible for ∼165,000 deaths in the United States in 2005. Adenocarcinomas represent approximately one-half of all NSCLCs subtypes. Despite its prevalence and characteristically high mortality rates, the cellular and genetic origins of the disease remain obscure. Mutational activation of KRAS or BRAF has been detected in ∼25% of NSCLCs, and, while the majority of these harbor KRAS mutations, activating mutations in BRAF account for ∼3% of all NSCLCs (Brose et al. 2002, Davies et al. 2002, Naoki et al. 2002). In addition, somatic mutations in the genes encoding the EGF receptor or ERBB2, both of which activate RAS signaling, are detected in ∼13% of lung adenocarcinomas (Shigematsu and Gazdar 2006). Finally, ERK1/2 activation is associated with disease aggression, suggesting a more general role for this pathway in NSCLC pathogenesis (Vicent et al. 2004).

Here we describe mice carrying a genetically modified allele of BRaf, BRafCA, which expresses normal BRaf prior to Cre-mediated recombination after which BRafVE is expressed. BRafVE expression in the lung elicited the growth of benign neoplastic adenomas, reminiscent of the effects of KRasG12D expression (Jackson et al. 2001). Tumor formation was potently inhibited by pharmacological MEK1/2 inhibition. Evidence suggested that BRafVE-induced lung tumors arose as a consequence of an initial burst of cell proliferation followed by an indolent phase, characterized by decreased proliferation accompanied by expression of senescence markers. Indeed, rarely did BRafVE-induced adenomas display spontaneous progression to adenocarcinoma unless mice were deliberately engineered to lack the TP53 or Ink4a/Arf tumor suppressor genes (TSGs).

Results and Discussion

Generation of BRafCA 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 BRAFVE in cancer initiation, progression, and therapy with an emphasis on temporal and spatial control in 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 [BRafCA] (Fig. 1B). BRafCA is designed to express normal BRaf prior to Cre-mediated recombination at which time BRafVE 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 BRAFVE. To discriminate the expression of the various BRaf mRNAs,
V600E is indicated (*). (enzyme fragments are indicated. The modified exon 15 encoding germline. Breeding of BRafCA/+ and BRafVE alleles was 98% that of endogenous BRaf/H11032 are depicted. Red arrows depict fully processed BRaf the targeting vector used, and the proteins expressed from the locus (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). Furthermore, analysis of the earliest BRafVE-induced 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 BRafV600E-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 IC50 in cells and, unlike most protein kinase inhibitors, acts noncompetitively with its substrates (Sebolt-Leopold and Herrera 2004). Tumors were initiated in BRafV600E/− mice by administration of 5 × 106 PFU of Ad-Cre. Four weeks later, at a time when hyperplastic lung epithelium exists (Supplementary Fig. 2D), with the diagnostic digestion products indicated by blue arrows and red arrowheads, respectively. (C–L) BRafV600E/− mice were infected with 5 × 105 PFU (C–H) or 105 PFU (I–L) of Ad-Cre intranasally and were analyzed 7 wk following infection. (C) Macroscopic lesions are present on the surface of BRafV600E/− (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 BRafV600E/− (G,H). Note papillary adenomas in higher power magnification (H) and wide-scale involvement of lungs (I). (J,L) Adenomas stain negative for CCA (J,L) and positive for SP-C (K,L). Bars: F,H,J,L, 100 µm; E,G,I,K, 500 µm.

Figure 2. Expression of BRafV600E in lung induces adenomas. (A) PCR detection of BRafV600E rearrangement from lungs of BRafV600E/− (wt) or BRafV600E/− mice infected with Ad-Gal [B] or Ad-Cre [C]. The black arrowhead, blue arrow, and red arrowhead highlight the wild-type, the unarranged BRafV600E, and the BRafV600E alleles, respectively. (B) Detection of BRafV600E mRNA in BRafV600E mice following Ad-Cre infection. Total lung RNA from BRafV600E/− (wt) or BRafV600E/− mice isolated 8 wk after Ad-Cre infection (5 × 105 PFU) was subjected to RT-PCR analysis as in Figure 1D. BRafV600E and BRafV600E 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) Macroscopic lesions are present on the surface of BRafV600E/− (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 BRafV600E/− (G,H). Note papillary adenomas in higher power magnification (H) and wide-scale involvement of lungs (I). (J,L) Adenomas stain negative for CCA (J,L) and positive for SP-C (K,L). Bars: F,H,J,L, 100 µm; E,G,I,K, 500 µm.

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 BRafV600E 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 excrescences; however, at early time points (2–4 wk), there was no nodule formation (Fig. 4A). Papillary adenomas (1.2.1.2.2) were detected 6–8 wk after BRafV600E expression and appeared to increase in number and size. These lesions were bronchiole-centric, but did not appear to involve the terminal bronchioles. Rather, the lesions appeared to arise in alveolar ducts and expand outward and around bronchioles (Fig. 4B). At later times (14 wk) after BRafV600E induction, we detected changes in the papillary adenomas such that they appeared to undergo alterations at their periphery, changing to a more solid architectural appearance (Fig. 4C). The cells also displayed additional morphologic alterations where the cytoplasm became more abundant and eosinophilic. This is consistent with the appearance of mixed papillary and solid adenomas (1.2.1.2.3). It is striking that the BRafV600E-induced lung adenomas formed rapidly (6–8 wk), but did not appear to grow beyond ~900 µm in diameter. Moreover, we rarely detected spontaneous progression to adenocarcinoma in these mice. To date, only two out of 57 BRafV600E/− mice possessing adenomas displayed focal adenocarcinomas at the time of necropsy (Fig. 4D). The adenocarcinoma cells in these two mice displayed nuclear enlargement, hyperchromasia, and contour irregularities. The cells were arranged in solid sheets, histologically consistent with adenocarcinoma (1.2.3.2). Thus, expression of BRafV600E appears sufficient to rapidly induce benign lung tumors that only rarely progress to adenocarcinoma, suggesting that additional events are required for cancer progression.

By a similar strategy, others assessed the effects of BRafV600E expression in the hematopoietic system. In this case, BRafV600E elicits hematopoietic dysplasia with characteristics of histiocytic sarcoma (Mercer et al. 2005). In this model, BRafV600E expression is uniformly lethal due to bone marrow failure, but mice do not die due to acute myeloid leukemia, suggesting that BRafV600E induces a pre-malignant myelodysplastic state. It is interesting to note that the consequences of induced BRafV600E lung expression bear both interesting similarities and differences in comparison to those reported for KRasNSL2 (Jackson et al. 2001). Ad-Cre-induced expression of KRasNSL2 in the lungs of KRasNSL2 mice leads initially to development of...
Data suggest that either additional KRas effector pathways (Collado et al. 2005; Michaloglou et al. 2005). Recently, it was reported that response to inappropriate activation of RAS or its signaling mechanism restraining the proliferation of normal cells in regression.

Induced lung adenomas are more highly constrained from progression (van der Eerden et al. 2001). Eight weeks post-infection, all mice receiving vehicle development of mice and was sustained at the indicated concentrations for 28 d. Eight weeks post-infection, all mice receiving vehicle development of 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.

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 pathways are required for cancer progression or that BRafVE-induced adenomas are more highly constrained from progression. Oncogene-induced cell cycle arrest with features of senescence (OIS) is reported to be a cellular defense mechanism restraining the proliferation of normal cells in response to inappropriate activation of RAS or its signaling effectors (Collado et al. 2005; Michaloglou et al. 2005). Recently, 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 sections from lungs harvested at different times after BRafVE expression were stained for markers of cell proliferation (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 dramatically decreased even though the BRafVE-MEK-ERK signaling pathway remains active in these cells [Figs. 3A,B, 4G]. Further analysis of adenomatous lesions revealed 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 nucleoli, 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-βGal 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 carcinogenesis, then BRafVE expression combined with loss of TSG expression should lead to cancer progression. To this end, we crossed BRafCA mice to obtain mice homozygous 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 increased proliferation at ~8 wk post-infection with Ad-Cre when compared with BRafCA/+, TP53+/− 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 papillary 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 proliferative index [Fig. 4P; Supplementary Fig. 3], and cells displayed 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 proliferation. 2/4 BRafCAX/+, Ink4a/Arfllox/lox mice contained multiple 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 adenomas [Fig. 4Q].

The data presented here provide support for the hypothesis that oncogenic BRAF can provoke cell cycle arrest accompanied by induction of some, but not all, markers of OIS [Zhu et al. 1998; Collado et al. 2005].
That \( K_{\text{ras}}^{G12D} \) [Jackson et al. 2005] or \( B_{\text{raf}}^{V600E} \) 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 \( B_{\text{raf}}^{V600E} \) are Diagnostic PCR products of 185 bp, 308 bp, and 335 bp for \( BR_{\text{af}} \), \( BR_{\text{af}}^{CA} \), and the \( BR_{\text{af}}^{V600E} \) alleles, respectively.

**Adenoviral Cre delivery**

Ad-Cre and Ad-\( B_{\text{raf}} \) were purified and titred by standard means (Viraquest), and \( 10^3 \) to \( 5 \times 10^7 \) PFU were administered to the nasal septum of 6 to 8-wk-old mice by intranasal instillation as a calcium phosphate precipitate [Fasbender 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. Anti-\( P_{\text{16}} \) antibody (NeoMarkers; 1:200) with secondary antibodies and detection kits (Jackson et al. 2005). Immunohistochemical staining of serial lung sections from \( B_{\text{raf}}^{CA} \)-induced adenomas expresses p19\Mcell{ARF} (L) and Dec1 (I) and are negative for SA-\( \beta \)-Gal (K). (L–S) \( B_{\text{raf}}^{CA} \)-mice (L, O) and those homozygous for conditional alleles of \( TP_53 \) [M, P, R, S] or \( Inkla/Arf \) [N, Q] were analyzed at 7 wk (R, S), 9 wk (L, M, O, P) or 14 wk (N, Q) post-infection with Ad-Cre. (O–Q) Ki67 immunochemical staining of serial lung sections from L–N. (R, S) Low-powered (R) and high-powered (S) views of \( B_{\text{raf}}^{CA}, TP_53^{\text{lox/lox}} \) lungs demonstrate the extent of lesions and disordered morphology, enlarged size (yellow arrow), and heterochromatic staining of nuclei. Bars, A–E, E–G, L–Q, 100 \mu m; D, H, 500 \mu m; insets, 1 mm; R, 2 mm.

**Materials and methods**

**Generation of a conditionally active \( B_{\text{raf}} \) allele**

DNA encompassing mouse \( B_{\text{raf}} \) exons 14–16 was modified such that exon 16 was replaced with a HSV-thymidine kinase cassette. A \( \text{Loxp} \)-flanked cassette containing the 3' 382 base pairs (bp) of intron 14, the human \( B_{\text{raf}} \)-cDNA containing exons 15–18, the mouse \( B_{\text{raf}} \) polyadenylation sequences, and a pGK-neo cassette was inserted into intron 14 upstream of a modified exon 15 that encodes \( B_{\text{raf}}^{V600E} \) and a silent XbaI restriction site polyorphism. The details of construction, Southern blotting, and RNA analysis are available as Supplemental Material.

**Mice used in this study**

Mice carrying conditional alleles of \( TP_53 \) or \( Ink4a/Arf \) were genotyped as described in the Supplemental Material. \( B_{\text{raf}}^{CA} \) mouse genotypes were determined by standard PCR of tail DNA with primer pair AD [AD FwdA1, 5'–TGAGTAATTGTGCAACTGC–3'; and AD RevB1, 5'–CTCCTGCTGGGAAACCGGC–3'] to produce diagnostic PCR products of 185 bp, 308 bp, and 335 bp for \( B_{\text{raf}}^{CA} \), \( B_{\text{raf}}^{V600E} \), and the \( B_{\text{raf}}^{V600E} \) alleles, respectively.

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erate with \( K_{\text{ras}}^{G12D} \) or \( B_{\text{raf}}^{V600E} \) to initiate and/or maintain the OIS program. These observations contrast with those in cultured cells, where activation of RAF robustly induces p16\Mcell{INK4A} 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.

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