A Central Role for RAF→MEK→ERK Signaling in the Genesis of Pancreatic Ductal Adenocarcinoma

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ABSTRACT
KRAS mutation is a hallmark of pancreatic ductal adenocarcinoma (PDA) but remains an intractable pharmacologic target. Consequently, defining RAS effector pathway(s) required for PDA initiation and maintenance is critical to improve treatment of this disease. Here, we show that expression of BRAFV600E, but not PIK3CAH1047R, in the mouse pancreas leads to pancreatic intraepithelial neoplasia (PanIN) lesions. Moreover, concomitant expression of BRAFV600E and TP53R270H result in lethal PDA. We tested pharmacologic inhibitors of RAS effectors against multiple human PDA cell lines. Mitogen-activated protein (MAP)/extracellular signal–regulated (ERK) kinase (MEK) inhibition was highly effective both in vivo and in vitro and was synergistic with AKT inhibition in most cell lines tested. We show that RAF→MEK→ERK signaling is central to the initiation and maintenance of PDA and to rational combination strategies in this disease. These results emphasize the value of leveraging multiple complementary experimental systems to prioritize pathways for effective intervention strategies in PDA.

SIGNIFICANCE: PDA is difficult to treat, in large part, due to recurrent mutations in the KRAS gene. Here, we define rational treatment approaches for the disease achievable today with existing drug combinations by thorough genetic and pharmacologic dissection of the major KRAS effector pathways, RAF→MEK→ERK and phosphoinositide 3′-kinase (PI3′K)→AKT. Cancer Discov; 2(8): 685–93. © 2012 AACR.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA) poses a major challenge in oncology due to our inability to diagnose the disease early in its progression, its aggressive clinical behavior, and the lack of effective systemic chemotherapy (1). The vast majority of PDAs harbor a mutationally activated form of KRAS (2). Moreover, KRAS mutation is an early event in PDA, as evidenced by its high prevalence in pancreatic intraepithelial neoplasia (PanIN) lesions, thought to be a benign precursor to malignant PDA (3). Furthermore, widespread expression of KRAS<sub>G12D</sub> throughout the developing mouse pancreas leads to multifocal PanIN formation, and when combined with lesions in p53, to PDA with high frequency in adult mice (4, 5).

Mutational activation of KRAS binds to a multiplicity of effector proteins including RAF kinases, phosphoinositide 3′-kinase (PI3K), and guanine nucleotide exchange factors for RAL and RHO GTPases, respectively (6). Because mutationally activated RAS remains an intractable pharmacologic target, defining relevant RAS effector pathway(s) in PDA is of considerable clinical importance. Because potent and specific inhibitors of key components of RAS effector pathways are being clinically deployed in a number of malignancies, it has become crucial to understand how best to implement these drugs in the clinical arena for maximal efficacy while minimizing toxicity. Unlike the scenario in melanoma or colorectal cancer, mutational activation of RAS effectors (e.g., BRAF or PIK3CA) is extremely rare in PDA and therefore uninformative as to the key downstream mediators of RAS signaling (7). This might suggest that numerous RAS effector pathways are essential for PDA and that effective targeting of cancers maintained by mutationally activated KRAS might require concomitant inhibition of 2 or more RAS effector pathways (8).

We examined the requirements of the RAF or PI3′K effector arms of KRAS signaling in the initiation, progression, and maintenance of PDA using genetically engineered mouse (GEM) cancer models and cancer cell lines derived from human or mouse PDA. Whereas pancreas-specific expression of BRAF<sup>V600E</sup> led to the rapid formation of multifocal PanIN lesions, similarly initiated expression of PIK3CA<sup>H1047R</sup> was without obvious effect. Furthermore, combined expression of BRAF<sup>V600E</sup> and gain-of-function TP53<sup>R270H</sup> uniformly led to lethal PDA in the mouse. Oral delivery of a mitogen-activated protein (MAP)/extracellular signal–regulated (ERK) kinase (MEK) inhibitor was effective in inhibiting ERK phosphorylation in vivo in an established, autochthonous model of PDA reported to exclude drugs and prolonged survival in a novel syngeneic model of PDA. Pharmacologic inhibition of MEK potency suppressed proliferation in a subset of PDA-derived cell lines in vitro but induced activation of AKT in both KRAS wild-type (w/t) or mutated human PDA cell lines. Finally, combined MEK and AKT inhibition showed synergistic interactions in most human PDA cells tested. Overall, our findings suggest the potential use of concerted clinical efforts to completely inhibit the RAS→RAF→MEK→ERK pathway at or below MEK in a subset of patients with PDA and to develop tolerable combination regimens of MEK and AKT inhibitors in this disease.

RESULTS

Expression of BRAF<sup>V600E</sup> But Not PIK3CA<sup>H1047R</sup> Is Sufficient for PanIN Formation

To test the consequences of activating the RAF→MEK→ERK pathway specifically in the pancreas, we crossed p48<sup>−/−</sup> mice with Braf<sup>C/A</sup> mice. As previously described, Braf<sup>C/A</sup> encodes normal BRAF but following Cre-mediated recombination is rearranged to encode activated BRAF<sup>V600E</sup> (9). p48<sup>−/−</sup> expresses Cre recombine in place of the Pitf gene. No compound p48<sup>−/−</sup>, Braf<sup>C/A</sup> progeny were detected at the time of weaning, leading us to conclude that widespread expression of BRAF<sup>V600E</sup> in the developing mouse pancreas is incompatible with development to adulthood. This lethality contrasts with the viability of p48<sup>−/−</sup>, Kras<sup>G12D</sup> mice (10). To circumvent this lethality, we generated compound Pdx1::CreERT2; Braf<sup>C/A</sup> mouse (BC mice hereafter) where expression of BRAF<sup>V600E</sup> is induced in the adult pancreas under the control of a conditionally active Cre recombinase driven by the Pax6 promoter (11). BC mice were born at normal Mendelian ratios and were healthy and fertile. In parallel, and as a comparator, we generated a cohort of Pdx1::CreERT2; Kras<sup>G12D</sup> mice (KC mice). Cohorts of BC and KC mice were treated with tamoxifen at P14 to initiate Cre activity and thereby BRAF<sup>V600E</sup> or Kras<sup>G12D</sup> expression in the pancreas. Mice were euthanized for analysis around P100, and all mice were healthy at the time of euthanasia.

Pancreatic expression of BRAF<sup>V600E</sup> led to near total replacement of the exocrine pancreas with PanIN lesions (Fig. 1A and B). These lesions were morphologically indistinguishable from those arising in KC mice and were of similar grade although greater in number (Fig. 1C and not shown). PanINs from BC mice expressed the ductal marker cytokeratin (CK) 19 (Fig. 1D), Ki67 (a marker of proliferation; Fig. 1E), and had abundant phosphorylated nuclear ERK1/2 (Fig. 1F), indicating activation of the RAF→MEK→ERK pathway. In addition, whereas primary cilia were observed in both pancreatic islets and normal ducts, PanIN cells from BC mice lacked primary cilia (Fig. 1G and H), consistent with previous findings in Kras<sup>G12D</sup>-induced induced PanIN lesions (12). Six BC mice aged 1 year showed no evidence of PDA upon euthanasia.

To test the ability of activated PI3′-kinase-α to initiate PanIN formation, we generated Pdx1::CreERT2; Pkhl<sup>−/−</sup> mice (PC mice). The Pkhl<sup>−/−</sup> allele encodes normal PI3′K-α before Cre-mediated recombination after which mutationally activated PI3K<sup>α/H1047R</sup> (PIK3CA<sup>H1047R</sup>) is expressed from the endogenous Pkhl<sup>−/−</sup> locus (13). We used a specific PCR to show that recombination (and thus activation) of the Pkhl<sup>−/−</sup> allele in the pancreas occurred (not shown) but found neither detectable PanIN lesions nor any other pancreatic abnormalities in PC mice up to 6 months after Cre induction with tamoxifen. These data indicate that mutationally activated BRAF<sup>V600E</sup>, but not PIK3CA<sup>H1047R</sup>, can initiate PanIN formation with an efficiency equal to that of Kras<sup>G12D</sup>.

BRAF<sup>V600E</sup> Cooperates with Gain-of-Function TP53<sup>R270H</sup> for PDA Formation

Mutationally activated Kras<sup>G12D</sup> cooperates with gain-of-function TP53<sup>R270H</sup> to promote development of PDA with...
high penetrance and striking histologic and clinical similarity to the human disease (5). We generated a cohort of Pdx1::CreER<sup>2</sup>; Braf<sup>CA/+, Trp53<sup>LSL-R270H/+</sup></sup> mice (BPC mice hereafter) to test whether oncogenic BRAF<sup>V600E</sup> might display similar cooperation. All BPC mice required euthanasia at 4.5 to 9 months because of abdominal distention, wasting and substantial loss of body weight. Mice typically presented with ascites and extra-pancreatic spread of metastatic disease, most often to the liver, peritoneal cavity, and lung (Fig. 2A–E). All BPC mice displayed clear evidence of PDA at necropsy. Analysis of tumor-derived genomic DNA confirmed recombination of the Braf<sup>CA</sup> allele and excluded spurious acquisition of activating mutations in either exon 1 of Kras or exon 20 of Pik3ca (data not shown). Histologic examination of these cancers showed them to be moderately differentiated PDA displaying robust proliferation (Ki67; Fig. 2F), heterogeneous CK19 expression (Fig. 2G), and abundant phosphorylated ERK (pERK; Fig. 2H). All mice succumbed to disease within a year (Fig. 2I). Interestingly, both BRAF<sup>V600E</sup>-induced PanINs and PDAs displayed abundant stroma and a desmoplasia similar to that seen within the human disease. We concluded that oncogenic BRAF<sup>V600E</sup> substitutes for most, if not all, of the oncogenic functions of KRAS<sup>G12D</sup> in the initiation and progression of PDA in the mouse.

Bioavailability of MEK1/2 Inhibitor PD325901 in PDA

The above results suggested to us that although mutant KRAS can serve many oncogenic functions in cancer, activation of the RAF pathway alone satisfied a genetic sufficiency argument for the PanIN and PDA initiation. To translate this finding into a more clinically relevant hypothesis, we next considered the clinical use of MEK inhibitors and potential barriers to successful trials with these agents in PDA. It has been recently reported that chemotherapeutic agents are excluded from PDA tissue due to poor drug perfusion into the primary tumor, which is in turn attributable to poor tumor vascularization (14, 15). To interrogate whether MEK1/2 inhibitor PD325901 was bioavailable to PDA tissue, we used an autochthonous Kras<sup>LSL-G12D</sup>, Trp53<sup>LSL-R270H/+ , p48<sup>CY</sup></sup> (KPC) mouse model similar to that previously described to exhibit high interstitial pressures and to exclude various
Figure 2. BrafV600E and Tps53R270H cooperate to form lethal PDA resembling the human disease. A, a 6-month-old Pdx1::CreERT2; BrafCA/+; Trp53LSL-R270H/+(BPC) mouse with ascites. B, gross images of primary pancreatic tumor (black arrow) and omental metastases (blue arrows). C, hematoxylin and eosin (H&E) staining of primary PDA arising in the pancreas of a BPC mouse. H&E staining of (D) liver metastases (black arrows) or (E) lung metastases (black arrow) from same. PDAs arising in BPC mice are proliferative (Ki67; F), heterogeneously express ductal markers (CK19; G), and display high levels of MAPK activation [pERK; H]. I, survival curves of mice of indicated genotypes.
RAF → MEK → ERK Pathway in Pancreatic Ductal Adenocarcinoma

Figure 3. MEK inhibition in vivo. Hematoxylin and eosin (H&E, A and B) or pERK (C and D) staining of KPC mice treated with either vehicle (A and C) or MEK1/2 inhibitor PD325901 (B and D). Day 7 (E and F) postimplantation, pretreatment bioluminescent images of FVB/n mice after orthotopic injection of syngeneic KrasLSL-G12D+/+, Cdkn2a−/− cells and subsequent treatment with vehicle (G) or MEK1/2 inhibitor PD325901 (H) for 2 weeks.

drugs from PDA tissues (14, 15). In this model, 2 daily treatments with MEK1/2 inhibitor PD325901 led to a profound reduction in pERK as detected by immunohistochemistry (Fig. 3A–D), suggesting that this agent is bioavailable to PDA cells in vivo at clinically achievable doses. We concluded that sufficient levels of MEK1/2 inhibition might be pharmacologically feasible in PDA, despite the drug delivery challenges posed by hypovascularity and desmoplasia in this disease.

For drug efficacy studies, we next developed 2 new in vivo, mouse syngeneic orthotopic models of PDA, denoted INK4.1LacZ/S and p53 2.1LacZ, using previously described mouse PDA-derived cell lines engineered to express luciferase (16). We found that implantation of either line in the pancreas of immune competent FVB/n mice reproducibly led to PDA with characteristics of the clinical disease including recruitment of activated stroma (Supplementary Fig. S1), ascites, cachexia, and bowel obstruction requiring euthanasia at 5 to 6 weeks postimplantation. The predictable kinetics and quantifiable tumor implantation allowed for relatively economical drug efficacy studies, as compared with the
autoclonal model (17). Following orthotopic engraftment, tumor-bearing mice were divided into equal tumor-bearing groups (as quantified by bioluminescence), treated with either vehicle or MEK1/2 inhibitor PD325901 by gavage for 14 days, and monitored clinically daily for disease progression. PD325901 led to pERK reduction 6 hours after a single oral gavage (Supplementary Fig. S2A and S2B), indicating that the drug gains access to tumor cells in this model as in the autotoclonal model. Treated mice were healthy while receiving drug, whereas control-treated mice began to decline. The experiment was terminated when the final vehicle-treated mouse required euthanization, as dictated by Institutional Animal Care and Use Committee (IACUC) protocols at our center. By this analysis, MEK inhibition resulted in a statistically significant survival advantage in mice bearing either INK4.1syn_Luc (log-rank, $P = 0.043$) or p53 2.1.Luc_Luc (log-rank, $P < 0.01$) syngeneic orthotypic xenografts. Despite this survival advantage, we noted that MEK inhibition was mostly cytostatic, as noted in vitro (18), and upon cessation of PD325901, all treated mice displayed rapid tumor growth (Fig. 3E–H).

**DISCUSSION**

The strikingly poor prognosis of patients with PDA is largely attributable to late diagnosis and general resistance to conventional cytotoxic or targeted therapeutics. Although mutational activation of KRAS is a signature genetic event of PDA, approaches to directly inhibit constitutively active, GTP-bound RAS proteins have so far failed. Consequently, considerable attention has shifted to pharmacologically tractable targets acting downstream of RAS-GTP on the arms of its various effector pathways. Chief among these are the RAF→MEK→ERK and the PI3′K→AKT pathways for 2 reasons. First, the RAF and PI3′K kinases are themselves frequently mutationally activated in human cancer whereas other putative RAS effectors are not. Second, components of these pathways are targeted with agents in clinical development. In this study, we sought to explore the relative importance of these RAS effector pathways in PDA initiation and maintenance to better prioritize treatment approaches with such pathway inhibitors and to prospectively define combinations of inhibitors likely to be of benefit specifically in this lethal disease. A key conclusion of this research is that induced expression of BRAFV600E, but not PIK3CAH1047R, signaling can recapitulate the PDA phenotype endowed by mutant KRASG12D in mice. Moreover, pharmacologic inhibition of MEK has antitumor effects against a subset of PDAs and broadly synergizes with AKT inhibition in this disease.

Whereas KRAS mutation is nearly universal in PDA, mutational activation of either BRAF or PIK3CA is very uncommon (7). It is perhaps surprising then that BRAFV600E is able to phenocopy the effects of KRASG12D in the mouse with such efficiency. These data suggest that, in the mouse, little more is required of KRASG12D than activation of the RAF→MEK→ERK axis for PDA initiation. In this capacity, activated BRAF (like activated KRAS), appears capable of activating additional pathways (e.g., Myc, NF-kB) and processes (inflammation, stromal recruitment) necessary for pancreatic tumorigenesis. Moreover, the absence of an overt pancreatic phenotype in Pdx1CtnvERK;Pkh3CagAllox mice further emphasizes the relative specificity of the RAF→MEK→ERK pathway in PDA initiation. These results are consistent with GEM models of KRASG12D-induced lung tumorigenesis wherein RAF→MEK→ERK signaling is both necessary (21) and sufficient (9, 22) for tumor initiation in lung and agrees with the requirement for RAF in RAS-induced skin cancer (23).

There are numerous inhibitors of RAF→MEK→ERK and PI3′K→PDK→AKT signaling currently in drug development (6). Our findings support the contraindication of RAF inhibitors in the treatment of cancers driven by mutationally
**Figure 4.** Combined inhibition of MEK and AKT leads to synergistic effects across a large panel of PDA cell lines. A, IC₅₀ measurements of human PDA cell lines treated with MEK inhibitor GSK1120212. Cell lines are on the x-axis and IC₅₀ (mol/L) is on the y-axis. Representative dose–response curves of (B) 3.27 or (C) Sw1990 treated with GSK690693 (triangles), GSK1120212 (diamonds), or a 5:1 mol/L fixed-dose combination ratio of GSK1120212:GSK690693 (squares) plotted as the dose of GSK1120212 in the combination. The x-axis is drug concentration in mol/L, and y-axis is percentage of growth inhibition at 72 hours. Error bars are ±SD. D, Immunoblotting of a subset of human PDA cell lines treated with dimethyl sulfoxide (DMSO), MEK inhibitor GSK1120212 (212) at 200 nmol/L, AKT inhibitor GSK690693 (69) at 1 μmol/L, or a 0.2:1 μmol/L (212:69) combination of the 2 agents for 24 hours.
activated RAS proteins due to their lack of efficacy and likely growth-stimulatory characteristics (24). We find that MEK1/2 inhibition has potent antitumor activity against human or mouse PDA cell lines and against orthotopically implanted tumors. We observed mostly cytostatic responses following MEK1 inhibition in vivo and observed induction of AKT signaling in response to MEK inhibition in PDA cells. This suggested a functional feedback loop as observed by others in breast or colorectal cancer lines harboring RAS mutations (25, 26). Indeed, we found that combined inhibition of MEK and AKT led to synergistic effects in the majority of human PDA cell lines tested, similar to findings in lung cancer (27). We interpret our findings with those of others to suggest that PDA cells, while relatively resistant to single-agent AKT inhibition, appear to consistently recruit this important survival pathway in response to acute MEK inhibition, possibly explaining the synergistic interactions seen with these 2 classes of agents.

Taken in total these findings emphasize the central role played by RAF→MEK→ERK signaling in both the genesis and maintenance of PDA. These results are important because, although KRAS remains a pharmacologically intractable target, there are numerous potent kinase inhibitors being developed against the downstream effectors of RAS. However, regimens to structure the combined use of such inhibitors remain to be established. We show that agents currently in clinical trials show potent synergy in PDA treatment. These findings strongly support the further development of combined MEK and AKT inhibition in PDA and suggest a clear direction for the implementation of pathway-targeted approaches in this disease with tremendous unmet medical need.

METHODS

Mouse Studies

All experiments were approved by the IACUC of the University of California, San Francisco. Braf(12) (9), KrasG12D.G19V (4), Trp53(−/−).Pten(−/−) (14), Pdx1-CreER2 (11), and PbkCal(−/−)/H19B2 (13) mice and their genotyping protocols have been described. Tamoxifen was dissolved in peanut oil and delivered in one intraperitoneal injection on day 14 of life. Derivation of the p53 2.1.1 and INK4.1 syn_Luc and p53 2.1.1 and INK4.1 lines from FVB/n mice in 20 weeks. Inhibitors were dissolved in dimethyl sulfoxide. PDA cell lines were plated on day 0, treated with nine, 2-fold dilutions of single drug or 1:1 mol/L (PD325901:GDC0879) or 5:1 mol/L (GSK690693:GSK1120212) ratio combinations of drugs, and read as described (16). I_{50} and synergy assessments were conducted using the SYNERGY program in R (see Supplementary Methods).

Immunoblotting

Cells were grown to 70% confluence and then either treated with 1 μmol/L GSK690693 or 200 nmol/L GSK1120212 for 24 hours. Cells were lysed using radioimmunoprecipitation assay buffer and loaded onto NuPAGE Bis-Tris SDS gels. Antisera included anti-phospho-ERK1/2, anti-ERK1, anti-phospho-AKT (pSer473), anti-pan-Akt, anti-phospho-PRAS40, anti-phospho-S6RP and phospho-4E-BP1 from Cell Signaling Technology, and anti-β-actin was obtained from Santa Cruz Biotechnology. The LiCOR Odyssey system was used to visualize immunoblots.

Immunohistochemistry and Immunofluorescence

Pancreata were fixed overnight in formalin, embedded in paraffin, cut into 5-μm sections, and placed on slides. Following citrate-mediated antigen retrieval, slides were incubated with monoclonal rabbit antibodies against Ki67 (Abcam), pERK1/2 (Cell Signaling Technology), rat anti-CXK1 (Hybridoma Bank at the University of Iowa, Iowa City, IA), pS356/44, pS364/47, and anti-smooth muscle actin A2547 (Sigma) overnight followed by incubation with biotinylated goat anti-rabbit or goat anti-rat IgG and horseradish peroxidase (Vector). Detection was conducted using the DAB Chromogen System (Dako). Primary cilia were stained and detected as described (12).

Disclosure of Potential Conflicts of Interest

J.W. Gray has commercial research grants from GlaxoSmithKline, Pfizer, and Susan G. Komen for the Cure and is a consultant/advisory board member for New Leaf Ventures, Agenda, and KromaTiD. M. McMahon has a commercial research grant from the National Comprehensive Cancer Network. No potential conflicts of interest were disclosed by the other authors.

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Activating KRAS mutations occur in the vast majority of pancreatic ductal adenocarcinomas (PDA), but attempts to pharmacologically inhibit RAS have been unsuccessful. Because several small-molecule inhibitors of RAS effectors are already in clinical development, defining the pathways required for KRAS-driven PDA initiation and progression could lead to readily testable therapeutic approaches. Collisson and colleagues evaluated the effects of constitutive activation of 2 downstream RAS effectors, BRAF and PI3K, in the pancreas. Mutationally activated BRAFV600E, but not PIK3CAH1047R, led to rapid formation of pancreatic intraepithelial neoplasia (PanIN) lesions that were morphologically indistinguishable from those caused by Kras mutation. Also, like oncogenic KRASG12D, BRAFV600E cooperated with dominant-negative p53 to rapidly induce PDA in 100% of mice. Taken together, these data suggest that RAF-mediated signaling accounts for most, if not all, of the effects of mutant KRAS in the initiation and progression of PDA, and that downstream blockade of RAF signaling by MEK1/2 inhibition may be an effective therapeutic approach. Indeed, a MEK1/2 inhibitor had a cytostatic effect on established PDA orthotopic xenografts that led to a significant survival advantage. MEK suppression also inhibited the growth of multiple human PDA cell lines, though some activated AKT in response to MEK inhibition. Combined MEK and AKT inhibition synergized to inhibit the growth of resistant PDA cell lines, further providing a rationale for the development of MEK and PI3K/AKT inhibitors in PDA.

See article, p. 685.

A STAT3 decoy oligonucleotide had pharmacodynamic activity in a phase 0 trial. Linkage or circulation of the 2 strands increased decoy stability without loss of activity. Systemic administration of a cyclic STAT3 decoy inhibited HNSCC xenograft growth.

Oligonucleotide decoys have been developed as a method to selectively inhibit transcription factor activity through competitive inhibition of binding to cis-regulatory elements. Sen and colleagues conducted an exploratory, first-in-human phase 0 trial to determine the pharmacodynamic activity of an oligonucleotide decoy for STAT3, which is constitutively active in head and neck squamous cell carcinomas (HNSCC) and many other cancers. A pretreatment biopsy was obtained prior to intratumoral injection of a STAT3 decoy or saline control solution during tumor resection surgery, and a post-treatment biopsy was collected from the site of injection after tumor resection. No adverse events were reported, and the trial met its primary endpoint of demonstrating that STAT3 decoy treatment led to decreased levels of STAT3 target gene products compared with the control. Because the linear double-stranded form of oligonucleotide decoys makes them sensitive to nuclease degradation if administered systemically, the authors generated modified STAT3 decoys with either a nucleotide or hexaethylene glycol linker connecting the 2 DNA strands at one or both ends. These modified STAT3 decoys had longer serum half-lives and higher melting temperatures than the parental decoy, and intravenous injection of the cyclic STAT3 decoy into tumor-bearing mice significantly suppressed tumor growth and STAT3 target gene expression and induced tumor regression in 2 of 10 mice. These findings will facilitate further clinical development of the STAT3 decoy and may provide a generalizable framework for inhibition of other transcription factors.

See article, p. 694.
RAF/MEK Dependence of KRAS-Mutant Pancreatic Ductal Adenocarcinomas

Aphrothiti J. Hanrahan and David B. Solit

Summary: Studies using genetically engineered mouse models indicate that RAF activation is sufficient to induce pancreatic intraepithelial neoplasms, suggesting that mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor–based combination approaches may have clinical use in patients with pancreatic ductal adenocarcinomas. Cancer Discov; 2(8):666–9. ©2012 AACR.

Commentary on Collisson et al., p. 685 (1).

Thirty years have passed since missense mutations in RAS were first identified as the transforming factors in the Harvey and Kirsten strains of the mouse sarcoma virus. Somatic mutations of the 3 RAS genes have since been shown to be among the most prevalent somatic alterations in human cancer. Studies using genetically engineered mouse models (GEMM) of pancreatic and lung cancer, among others, have confirmed that mutant RAS contributes to cancer initiation and maintenance of the transformed phenotype even in the setting of established, metastatic disease. These results have prompted intensive academic- and industry-led efforts to identify direct inhibitors of oncogenic RAS. These efforts have failed to date likely due to the high affinity of the RAS–GTP interaction, as have efforts to selectively inhibit the posttranslational modifications required for RAS activation. The latter approach was ineffective in KRAS- and NRAS-mutant tumors as geranylgeranylation modification can substitute for farnesylation in targeting KRAS and NRAS to the plasma membrane.

An alternate approach is to target the effector pathways responsible for RAS-mediated transformation. Biochemical studies have identified more than 20 distinct RAS effector molecules, the best characterized of which include the RAF proteins, the phosphatidylinositol 3-kinases (PI3K), and the RAL exchange factors. Our understanding of the contribution of individual RAS effectors to transformation remains incomplete, but is likely influenced by the spatial/temporal availability of effectors, the presence or absence of extracellular stimuli, and the pattern of coincident mutational events. In this issue of Cancer Discovery, Collisson and colleagues (1) set out to investigate which of the various RAS effectors are required for tumor initiation and progression in pancreatic ductal adenocarcinoma (PDA). An in-depth focus on PDA is justified by the high rate of KRAS mutation in this disease (>90%) and the urgent need to develop effective therapies for this common and almost universally lethal cancer.

Prior studies using GEMMs have shown that expression of mutant Kras leads to the formation of multifocal pancreatic intraepithelial neoplasms (PanIN; ref. 2). Furthermore, coincident loss of Ink4a/Arf or Trp53 function results in the development of invasive pancreatic adenocarcinomas that phenocopy the human disease (3, 4). To determine whether RAF activation is sufficient to initiate pancreatic tumor formation, the authors generated mice with constitutive or conditional expression of BrafV600E in pancreatic cells. The V600E mutation accounts for more than 90% of the Braf mutations found in human tumors and locks the kinase into a constitutively active conformation. In the Braf<sup>V600E</sup> mouse model generated by Dankort and colleagues (5), the Braf<sup>V600E</sup> allele contains an insert that includes a floxed cassette containing exons 15 to 18 of human wild-type Braf cDNA upstream of a modified exon 15, which harbors the V600E mutation. The wild-type Braf allele is expressed before Cre-mediated recombination, but upon expression of Cre, the wild-type exon 15 to 18 insert is excised, and the expression of Braf<sup>V600E</sup> is initiated under the control of the endogenous Braf promoter. Targeted expression of Braf<sup>V600E</sup> using this model in the mouse lung leads to the development of benign lung tumors that progress to adenocarcinoma in the setting of concomitant loss of Trp53 or Ink4A/Arf (5). Similarly, conditional melanocyte-specific expression of Braf<sup>V600E</sup> in mice using this model results in benign melanocytic hyperplasia, which in the setting of coincident Pten loss progresses to invasive melanoma (6).

In the current study, Collisson and colleagues (1) express Braf<sup>V600E</sup> in the mouse pancreas by crossing Braf<sup>CA</sup> mice
with mice that express Cre recombinase under the control of the p48/Ptf1 gene (p48\(^{-}\)/, Braf\(^{CA\text{A}}\)), a pancreas-specific transcription factor expressed at embryonic day 9.5. None of the progeny survived past weaning, which indicates that expression of Braf\(^{V600E}\) is toxic for normal pancreatic development, a result which contrasts with the viability of p48\(^{-}\)/;Kras\(^{LSL-G12D}\) mice. To bypass the embryonic lethality observed in the p48\(^{-}\)/;Braf\(^{CA\text{A}}\) model, the authors generated mice (Pdx1::CreER\(^{T2}\); Braf\(^{CA\text{A}}\)) in which conditional expression of Braf\(^{V600E}\) is activated postnatally via tamoxifen-induced Cre recombinase activity driven by the Pdx-1 promoter. In parallel, the authors used this system to generate mice expressing physiologic levels of activated Kras\(^{G12D}\) in the pancreas (Pdx1::CreER\(^{T2}\); Kras\(^{LSL-G12D}\)). Notably, constitutive expression of activated Braf\(^{V600E}\) in the adult pancreas phenocopied oncogenic activation of Kras\(^{G12D}\), including the development of PanIN lesions lacking primary cilia in the exocrine pancreas, upregulation of the ductal marker cytokeratin 19, increased proliferation, and increased expression of nuclear phosphorylated extracellular signal-regulated kinase (ERK)1/2. Moreover, concomitant expression of mutant p53 (Pdx1::CreER\(^{T2}\);Braf\(^{CA\text{A}I};\) Trp53\(^{LSL-R270H/+}\)) resulted in the development of PDA with abundant stroma and desmoplasia similar to that seen in Kras\(^{p48}\)/-/;Pdx1::CreER\(^{T2}\); Kras\(^{LSL-G12D}\) mice. Notably, this phenotype. These results suggest that RAF but not PI3K activation is sufficient to induce PanIN development. Limitations of this study include the possibility that oncogenic RAS does not exclusively activate p110\(\gamma\) PI3K, and that the spectrum of downstream effectors activated by the kinase domain mutant may differ from that regulated by an activated wild-type allele. Furthermore, in light of recent findings that the class IA PI3K p110\(\alpha\) is also overexpressed in PDA (7) and that deletion of Phn in mouse pancreatic centroacinar cells leads to ductal malignancy (8), the current data do not fully exclude the possibility that activation of PI3K signaling by other mechanisms may be sufficient to induce PanIN formation.

Overall, the results imply that inhibition of RAF signaling may be an effective therapeutic approach in patients with KRAS-mutant pancreatic tumors (Fig. 1). Highly selective RAF inhibitors were recently shown to prolong the survival of patients with BRAF\(^{V600E}\) melanoma. These agents, however, inhibit RAF activation in a mutant-selective manner and are thus ineffective in tumors that express activated RAS (9). Highly selective, allosteric inhibitors of mitogen-activated protein (MAP)/ERK kinase (MEK) have also shown promising activity in BRAF-mutant melanoma and provide an alternative approach to inhibiting ERK pathway activity in KRAS-mutant tumors (10). To determine whether MEK inhibitors could inhibit ERK signaling at a nontoxic dose, Collisson and colleagues (1) treated Kras\(^{LSL-G12D}\), Trp53\(^{LSL-R270H/+}\), p48\(^{Cre}\) mice with PD0325901, an allosteric inhibitor of MEK1 and MEK2. Treatment with PD0325901 potently downregulated ERK activity, as measured by a decrease in phosphorylated ERK expression, indicating that sufficient intratumoral levels of the MEK inhibitor could be achieved at nontoxic doses to potently inhibit ERK pathway activation. This result is notable, as resistance of pancreatic tumors to systemic cytotoxic therapies has been attributed to limited drug exposure resulting from poor intratumoral perfusion. To determine whether sufficient ERK pathway inhibition could be maintained to induce meaningful antitumor effects, the authors turned to an orthotopic, syngeneic model of PDA. In this model, treatment with the MEK inhibitor was associated with downregulation of phosphorylated ERK expression and an improvement in survival. In sum, the results provide strong rationale for clinical trials of MEK inhibitors in patients with advanced pancreatic cancer but also highlight the logistical challenges associated with the use of GEMMs for preclinical drug development.

Recently, the MEK inhibitor trametinib (GSK1120212) was shown to improve survival as compared with chemotherapy in a randomized trial of patients with metastatic melanoma whose tumors harbored BRAF\(^{V600E/K}\) mutations (10). On the basis of these results, U.S. Food and Drug Administration approval for the use of trametinib in patients with BRAF-mutant melanoma is anticipated. Notably, 22 patients with pancreatic cancer were treated with trametinib within the context of the phase I trial of this agent (11). One patient achieved a partial response and several additional patients were noted to have minor responses or stable disease. Although these results are disappointing in light of the GEMM studies reported by Collisson and colleagues (1), they are consistent with studies of human cancer cell lines conducted by this group and others showing that in contrast to BRAF-mutant cell lines, which are with rare exception sensitive to MEK inhibition, KRAS-mutant cell lines exhibit variable sensitivity to MEK inhibitors. The basis for this heterogeneity of MEK dependence in KRAS-mutant cell lines has been explored in colorectal cancer cell lines and in this context can be attributed, in part, to the presence of PIK3CA co-mutation in some models (12). While PIK3CA mutations are rarely observed in pancreatic cancers, Collisson and colleagues (1) show that MEK inhibition in KRAS-mutant PDA cell lines is associated with a reciprocal increase in the expression of phosphorylated AKT and that cotreatment with a selective inhibitor of AKT is associated with synergy in many, but not all, models.

In sum, the results reported by Collisson and colleagues (1) in concert with the clinical experience to date indicate that despite the sufficiency of RAF activation for PanIN development, MEK inhibitor–based combination approaches will be needed to induce durable tumor regressions in most patients with KRAS-mutant PDA. Future laboratory studies will be needed to define the molecular basis for the variable response of KRAS-mutant PDA tumors to MEK inhibition, as such studies would aid in the development of rational MEK inhibitor–based combination strategies.
Disclosure of Potential Conflicts of Interest

D.B. Solit is a consultant/advisory board member for Roche and GlaxoSmithKline. No potential conflicts of interest were disclosed by the other author.

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