KRAS\(^{G12D}\) and BRAF\(^{V600E}\)-Induced Transformation of Murine Pancreatic Epithelial Cells Requires MEK/ERK-Stimulated IGF1R Signaling

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Abstract

Mutation of KRAS is a common initiating event in pancreatic ductal adenocarcinoma (PDAC). Yet, the specific roles of KRAS-stimulated signaling pathways in the transformation of pancreatic ductal epithelial cells (PDEC), putative cells of origin for PDAC, remain unclear. Here, we show that KRAS\(^{G12D}\) and BRAF\(^{V600E}\) enhance PDEC proliferation and increase survival after exposure to apoptotic stimuli in a manner dependent on MEK/ERK and PI3K/akt signaling. Interestingly, we find that activation of PI3K/AKT signaling occurs downstream of MAP–ERK kinase (MEK), and is dependent on the autocrine activation of the insulin-like growth factor (IGF) receptor (IGF1R) by IGF2. Importantly, IGF1R inhibition impairs KRAS\(^{G12D}\)– and BRAF\(^{V600E}\)-induced survival, whereas ectopic IGF2 expression rescues KRAS\(^{G12D}\)– and BRAF\(^{V600E}\)-mediated survival downstream of MEK inhibition. Moreover, we show that KRAS\(^{G12D}\)– and BRAF\(^{V600E}\)-induced tumor formation in an orthotopic model requires IGF1R. Interestingly, we show that while individual inhibition of MEK or IGF1R does not sensitize PDAC cells to apoptosis, their concomitant inhibition reduces survival. Our findings identify a novel mechanism of PI3K/AKT activation downstream of activated KRAS, illustrate the importance of MEK/ERK, PI3K/akt, and IGF1R signaling in pancreatic tumor initiation, and suggest potential therapeutic strategies for this malignancy. Mol Cancer Res; 10(9); 1228–39. ©2012 AACR.

Introduction

Pancreatic cancer is the 4th leading cause of cancer-related deaths in the United States, with a 5-year survival rate of less than 5% (1). Pancreatic ductal adenocarcinoma (PDAC) comprises the majority of pancreatic cancers and develops through a series of precursor lesions, known as pancreatic intraepithelial neoplasias, or PanINs (2). This progression is marked by a series of genetic alterations, including activating mutations in the KRAS oncogene, and the loss of the INK4A, TP53, and MADH4 tumor suppressor genes (2–4). Of these alterations, mutational activation of KRAS occurs in approximately 95% of PDAC cases and is present in early precursor lesions (4–6). The early occurrence and high incidence of KRAS mutation indicate that this is a critical step in the initiation of pancreatic tumor development. Mouse models for PDAC, generated through the pancreas-specific expression of an activated Kras allele, further support this hypothesis (7–9).

KRAS is a member of the Ras family of GTPases that cycle between inactive GDP- and active GTP-bound states (10). Mutations that disrupt the GTPase activity of KRAS, thereby rendering it constitutively active, are commonly observed in pancreatic cancer, resulting in the persistent activation of downstream signaling pathways (5). Perhaps the best-characterized KRAS-stimulated signaling pathway is the RAF/MEK/ERK signaling cascade (10). Members of the Raf family of serine/threonine kinases are key signal transducers in this pathway, and the gene BRAF, encoding the BRAF kinase, is commonly mutated in human malignancies including malignant melanoma and colorectal carcinoma (11). BRAF gene mutations are generally mutually exclusive with KRAS mutations; therefore, given the high rate of KRAS mutations in PDAC, BRAF mutations are infrequently seen in this disease (11). However, previous work by Kern and colleagues has shown that in the small subset of tumors that do not have activating KRAS mutations, 33% have activating mutations in BRAF (12). These findings raise the possibility that activating BRAF mutations may functionally substitute for KRAS gene mutations during pancreatic tumor initiation, yet the specific roles played by individual downstream effector pathways during pancreatic cancer initiation and progression remain unclear.
Pancreatic ductal epithelial cells (PDEC) are putative cells of origin for PDAC (2), and genetic manipulation of PDECs through the expression of oncogenes, or loss of tumor suppressor genes, provides a unique experimental system for modeling the initial transforming events in PDAC development (13–15). In addition, in comparison with commonly used cell culture models, such as NIH 3T3 cells, PDECs provide an excellent experimental model system for analyzing the signaling pathway perturbations that occur during the initiation of pancreatic tumorigenesis. Indeed, we have previously exploited this feature to show the effects of sonic hedgehog (SHH) on the stimulation of the RAF/MEK/ERK and PI3K/AKT signaling cascades (14).

We have also shown that activated KRAS promotes PDEC proliferation, as well as their survival after exposure to apoptotic stimuli (14). In addition, orthotopic implantation of KRASG12D-expressing PDECs that also lack the Ink4a/Arf tumor suppressor locus (alone or with concomitant Trp53 deletion) results in tumor formation (14). Using a similar experimental approach, Lee and Bar-Sagi recently showed a role for Twist in bypassing oncogenic cellular senescence (16). Thus, primary PDEC culture represents a unique system for the dissection of KRAS-induced signaling during pancreatic tumor initiation.

Therefore, in the present study we sought to elucidate the roles of the MEK/ERK and PI3K/AKT signaling pathways in KRAS-mediated transformation of pancreatic epithelial cells, and to determine whether an activated BRAF molecule functionally substitutes for activated KRAS in this cell type. We find that both KRAS and BRAF stimulate the proliferation and survival of PDECs in culture, and that the induced survival is dependent on signaling through both the MEK/ERK and PI3K/AKT signaling pathways. Strikingly, we show that activation of AKT occurs downstream of the MEK/ERK pathway and the type 1 insulin-like growth factor receptor (IGF1R) and that PDECs expressing activated KRAS and BRAF depend upon IGF2-stimulated IGF1R signaling for survival after exposure to apoptotic stimuli. Moreover, PDEC cell lines remain dependent on these signaling pathways for survival after exposure to apoptotic stimuli. Finally, we show that KRASG12D- and BRAFV600E-induced tumor formation in an orthotopic pancreatic tumor model is dependent on IGF1R expression. Collectively, these data provide new insights into the mechanisms underlying KRAS-mediated initiation of pancreatic tumorigenesis and pancreatic cancer cell survival.

Materials and Methods

Transgenic mice and animal care

The keratin-19-tva, Ink4a/Arflox/lox, Trp53lox/lox, and Ptf1a-cre strains have been previously described (14, 17–19). Nude mice were purchased from Charles River Laboratories. All mice were housed in a specific pathogen-free facility with abundant food and water under guidelines approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee.

Isolation, culture, and infection of mouse PDECs

Isolation, culture, and infection of mouse PDECs was done as previously described (15). The RCAS-GFP, RCAS-KRASG12D-IRES-GFP, RCAS-BRAFV600E (V5 tag), RCAS-BRAF (V5 tag), and RCAS-IGF2 vectors have been described previously (14, 20, 21). Construction of the RCAS-BRAFV600E (myc-tagged) vector is described in the Supplementary Methods. All proliferation and survival assays were conducted as previously described (14). The MAP–ERK kinase (MEK) inhibitor PD980059, the phosphoinositide 3-kinase (PI3K) inhibitor LY294002, and the IGF1R inhibitor AG1024 were used at final concentrations of 25 μmol/L, 20 μmol/L, and 20 μmol/L, respectively. For survival assays, inhibitors were added to the culture media 1 hour before treatment with the apoptotic stimulus. Gene knockdown was achieved by infecting PDECs with pGIPZ- or pLKO-based lentiviruses encoding targeting short hairpin RNA (shRNA; Open Biosoysms). Infected PDECs were grown in media containing 2 μg/mL puromycin for at least 4 days. For serum-starved shRNA-treated cells, puromycin was included in the media during serum starvation.

Culture and treatment of tumor cell lines

Cell lines were maintained as previously described (14). Murine cell lines were isolated and characterized upon isolation by immunoblotting and immunostaining in the Lewis laboratory. Low passage cells were frozen and used for subsequent experiments. Panc1 cells were obtained from American Type Culture Collection. They were not authenticated in the Lewis laboratory. Small molecule inhibitors were used as described earlier for PDECs. Gemcitabine (Gemzar, Eli Lilly) was used at a concentration of 50 nmol/L. Gene knockdown was conducted as described for PDECs. Infected cells were in media containing 2 μg/mL puromycin for at least 4 days. Proliferation and survival assays were conducted as previously described (14).

Immunoblotting

Cells for lysates were isolated in a 1 mg/mL collagenase V solution (Sigma), centrifuged at 1,000 rpm for 5 minutes at 4°C, and incubated in lysis buffer on ice for 30 minutes. Lysis buffer composition is as previously described (22). Where noted, cells were serum-starved for 48 hours before the generation of protein lysates. Protein lysates were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham). Immunoblotting was conducted as described (23). Antibody dilutions can be found in the Supplementary Methods.

Quantitative real-time–PCR

RNA was extracted from serum-starved PDECs using Trizol (Invitrogen), purified with an RNeasy Mini Kit (Qiagen), and treated with Turbo DNase (Ambion). cDNA was then generated using the Superscript III First Strand Synthesis System (Invitrogen). For Kras expression analysis, 10 ng of cDNA was mixed with TaqMan primers (Primier Set One: PN# Mm00517492_m1 and Primer Set Two PN# Mm01255197_m1) and TaqMan PCR Master Mix.
(Applied Biosystems) using the TaqMan standard protocol. For IGF- and EGF-family ligands, 10 ng of cDNA was combined with SYBR Green Reaction Mix (Quanta Biosciences), and 500 nmol/L of the appropriate primer pairs (IDT). Primer sequences can be found in Supplementary Methods. PCR amplification was conducted using an ABI 7300 Real Time PCR system using Applied Biosystems standard conditions.

Orthotopic implantation of PDECs

A total of 10⁶ PDECs were resuspended in 10 µL of Matrigel and injected into the pancreata of nude mice as previously described (14). For studies involving the knockdown of the IGF1R, PDECs were infected with pGIPZ shRNA targeting either IGF1R or GFP (as described earlier) at least 1 week before orthotopic implantation.

Immunostaining

Immunostaining was conducted as previously described (23). Antibody dilutions were as follows: rabbit anti-Ki-67 (1:1,000, Cat #NCL-Ki67p, Novocastra), rabbit antiamouse Pdx-1 (1:5,000, gift of Chris Wright), rabbit anti-Keratin-8 (1:50, Developmental Studies Hybridoma Bank, University of Iowa, Iowa city, IA).

Statistical analysis

Data are presented as the mean ± SD. The 2-tailed t test was used to compare the differences between groups. For all comparisons, P < 0.05 was considered statistically significant.

Results

KRASG12D and BRAFV600E enhance the proliferation and survival of PDECs

To investigate the effects of activated KRAS and BRAF on PDECs, we isolated PDECs from transgenic mice expressing the avian leukemia virus subgroup A (ALV-A) receptor, Tumor Virus A (TVA), under the control of the Keratin-19 (K19) gene promoter and enhancer elements (K19-tv-a; ref. 14). PDECs were also isolated from K19-tv-a mice with pancreas-specific deletion of the Ink4a/Arf and/or Trp53 tumor suppressor genes. TVA-positive PDECs were infected with RCAS viruses encoding Flag epitope-tagged KRASG12D, BRAFV600E, or GFP as a control (14). Infection of PDECs by RCAS-KrasG12D was confirmed by immunoblotting for the Flag epitope tag (Fig. 1A). Importantly, quantitative real-time-PCR (qRT-PCR) showed that expression of the ectopic KrasG12D resulted in only a 3-fold increase in mRNA levels (Supplementary Fig. S1A). Elevated levels of BRAF in RCAS-BrafV600E infected cells relative to RCAS-GFP infected controls were observed by immunoblotting (Fig. 1B). This increased expression was specific to BRAF as, increased levels of ARAF and CRAF were not observed (Supplementary Fig. S1B).

We next investigated the effect of activated KRAS and BRAF on the proliferation of PDECs. As previously shown, KRASG12D increased PDEC proliferation relative to GFP-expressing controls, and this effect was similar in both tumor suppressor wild-type and Ink4a/Arf, Trp53 null PDECs (Fig. 1C and F; ref. 14). BRAFV600E also increased PDEC proliferation relative to GFP controls, but notably this increase was less than that induced by KRASG12D (Fig. 1C and F). In addition, we observed that PDECs infected with RCAS viruses encoding wild-type BRAF did not display increased proliferation relative to GFP-expressing PDECs, indicating that mutational activation of BRAF is important for its ability to stimulate proliferation in PDECs (Supplementary Fig. S1C and S1D).

We next determined the effect of KRASG12D and BRAFV600E expression on PDEC survival when challenged with an apoptotic stimulus. For these assays, PDECs were treated with UV irradiation or cycloheximide, a cytotoxic agent that has been previously shown to cause apoptosis in PDECs and pancreatic cancer cells (14, 24). We found that both KRASG12D and BRAFV600E promoted PDEC survival after exposure to UV irradiation and cycloheximide (Fig. 1D, E, G, and H). Moreover, these effects were irrespective of tumor suppressor status and similar results were obtained in wild-type (Fig. 1D and E) as well as Ink4a/Arf (1H) and Ink4a/Arf, Trp53 null PDECs (Fig. 1G). Of note, UV-induced apoptosis in PDECs is p53 dependent, thus KRASG12D and BRAFV600E block both p53-dependent and p53-independent apoptosis. In addition, we observed increased survival, relative to GFP controls, in PDECs expressing wild-type BRAF following cycloheximide treatment (Supplementary Fig. S1E and S1F). These findings show that elevated BRAF expression, both mutant and wild type, is able to functionally substitute for activated KRAS to promote PDEC survival and suggest an important role for the RAF/MEK/ERK cascade in the survival of pancreatic epithelial cells.

Given that tumor suppressor gene status did not impact the proliferation and survival phenotypes, we used tumor suppressor deficient PDECs for subsequent experiments as they were easier to establish and grow in culture.

Expression of KRASG12D or BRAFV600E induces pancreatic tumor formation

We have previously shown that the expression of KRASG12D in PDECs lacking Ink4a/Arf, Trp53 is sufficient to induce tumor formation in an orthotopic mouse model (14). Therefore, having shown that BRAFV600E expression in PDECs is sufficient to at least partially recapitulate the increased proliferation and survival seen in KRASG12D-expressing PDECs, we next sought to determine if the expression of BRAFV600E in PDECs was sufficient to induce tumor formation. We found that implantation of KRASG12D- and BRAFV600E-expressing PDECs resulted in efficient pancreatic tumor formation, whereas the implantation of GFP expressing cells did not (Table 1). In the experiment shown in Table 1, mice injected with KRASG12D-expressing cells (4 weeks vs. 8 weeks). However, this accelerated tumor formation by KRASG12D, relative to BRAFV600E, was not consistently
Figure 1. KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} enhance proliferation and survival in PDECs. A, immunoblot confirming expression of ectopic Flag epitope-tagged KRAS\textsuperscript{G12D} in Ink4a/Arf, Trp53 null PDECs infected with RCAS-KRAS\textsuperscript{G12D}. β-Actin is used as a loading control. B, immunoblot confirming elevated expression of BRAF in Ink4a/Arf, Trp53 null PDECs infected with RCAS-BRAF\textsuperscript{V600E}. β-Actin is used as a loading control. Numbers represent relative BRAF/β-actin levels in RCAS-BRAF\textsuperscript{V600E}-infected cells relative to RCAS-GFP infected controls. C, cell numbers of tumor suppressor wild-type PDECs expressing KRAS\textsuperscript{G12D}, BRAF\textsuperscript{V600E}, or GFP at indicated time points after plating. Results are representative of at least 2 experiments. D and E, viability of tumor suppressor wild-type PDECs expressing KRAS\textsuperscript{G12D}, BRAF\textsuperscript{V600E}, or GFP treated with 100 μmol/L cycloheximide (D) or UV irradiation (E). White bars vehicle-treated (or untreated) cells; black bars cycloheximide- or UV-treated cells. Values are normalized such that viability of untreated cells is 1. Results are representative of at least 2 experiments. F, cell numbers of Ink4a/Arf, Trp53 null PDECs expressing KRAS\textsuperscript{G12D}, BRAF\textsuperscript{V600E}, or GFP at indicated time points after plating. Results are representative of at least 2 experiments. G, viability of Ink4a/Arf, Trp53 double null PDECs expressing KRAS\textsuperscript{G12D}, BRAF\textsuperscript{V600E}, or GFP, treated with 100 μmol/L cycloheximide, as measured by trypan blue exclusion. *, P < 0.01 compared with GFP expressing controls. H, viability of Ink4a/Arf null PDECs expressing KRAS\textsuperscript{G12D}, BRAF\textsuperscript{V600E}, or GFP following UV irradiation. White bars vehicle-treated (or untreated) cells; black bars cycloheximide- or UV-treated cells. Values are normalized such that viability of untreated cells is 1. Results are representative of at least 2 experiments. *, P < 0.01 compared with GFP expressing controls. All error bars, SD.
observed across multiple replicates of the experiment. Thus, we infer that KRAS<sup>G12D</sup> and BRAF<sup>V600E</sup> induce tumor formation with similar kinetics in the orthotopic model.

Hematoxylin and eosin staining of tumor tissue showed that transplantation of both KRAS<sup>G12D</sup> - and BRAF<sup>V600E</sup> -expressing cells primarily resulted in the formation of undifferentiated carcinomas (Supplementary Fig. S2A), occasionally with entrapped normal pancreatic tissue (Supplementary Fig. S2B) and regions with glandular differentiation (Supplementary Fig. S2C), consistent with our previously published findings (14). Surprisingly, tumors formed after the injection of BRAF<sup>V600E</sup>-expressing PDECs additionally contained regions with features of skeletal, cartilaginous, or bone differentiation (Supplementary Fig. S2D). Consistent with this, KRAS-induced tumors displayed cells with detectable cytokeratin 8 staining throughout the tumor, whereas BRAF-induced tumors displayed cytokeratin 8 staining only in glandular structures (Supplementary Fig. S2E and S2F). The mechanisms underlying this mesenchymal-like differentiation in BRAF<sup>V600E</sup> -induced tumors are undetermined.

Collectively, these data show that although there are subtle differences between KRAS<sup>G12D</sup> - and BRAF<sup>V600E</sup> -induced pancreatic tumors, the expression of BRAF<sup>V600E</sup> in PDECs is largely able to substitute for the expression of KRAS<sup>G12D</sup> during pancreatic tumor initiation.

### Signaling downstream of MEK and PI3K is necessary for survival in KRAS<sup>G12D</sup> - and BRAF<sup>V600E</sup> expressing PDECs

We have previously shown that enhanced survival in PDECs induced by ectopic SHH expression is dependent on the PI3K/AKT signaling axis but not the MEK/ERK pathway (14). To interrogate the roles of these signaling cascades in KRAS<sup>G12D</sup> - and BRAF<sup>V600E</sup> -mediated survival, we exposed PDECs to apoptotic stimuli in the presence of the MEK inhibitor PD98059 or the PI3K inhibitor LY294002. We found that treatment with either of these inhibitors abrogated KRAS<sup>G12D</sup> - and BRAF<sup>V600E</sup> -enhanced survival after apoptotic challenge, irrespective of the apoptotic stimulus used (Fig. 2A and B). In contrast, and consistent with our published data, SHH-expressing PDECs displayed reduced survival when the PI3K pathway, but not the MEK/ERK pathway, was blocked (Fig. 2A and B). Similar results were obtained when PDECs were treated with rapamycin, an inhibitor of mTOR (Supplementary Fig. S3A). Of note, combined inhibition of MEK and PI3K did not result in a further diminution of survival, indicating that inhibition of either pathway resulted in the maximum impairment in survival measurable by this assay (Supplementary Fig. S3B and S3C).

### Expression of KRAS<sup>G12D</sup> and BRAF<sup>V600E</sup> results in activation of the MEK/ERK and PI3K/AKT signaling cascades

The dependence of KRAS<sup>G12D</sup> - and BRAF<sup>V600E</sup> -expressing PDECs on both the MEK/ERK and PI3K/AKT pathways for survival was unexpected, as BRAF<sup>V600E</sup> directly stimulates the MEK/ERK signaling cascade, but not the PI3K/AKT pathway. Therefore, we ascertained the activation status of these signaling pathways in PDECs expressing KRAS<sup>G12D</sup> or BRAF<sup>V600E</sup>. PDECs were serum starved for 48 hours to eliminate pathway activation induced by exogenous growth factors, and protein lysates generated from the serum-starved cells. Immunoblotting of these lysates showed increased ratios of phosphorylated AKT (pAKT; at ser473) to total AKT, and phosphorylated ERK1 and ERK2 (pERK; at Thr202/Tyr204) to total extracellular signal-regulated kinase (ERK), in KRAS<sup>G12D</sup> - and BRAF<sup>V600E</sup> -expressing PDECs relative to GFP-expressing controls, confirming activation of the MEK/ERK and PI3K/AKT signaling pathways (Fig. 2C). These data suggest that BRAF<sup>V600E</sup> - and potentially KRAS<sup>G12D</sup> - stimulate PI3K/AKT signaling in an indirect manner. Interestingly, we also observed increased levels of total AKT in KRAS- and BRAF-expressing PDECs relative to GFP controls (Fig. 2C). Analysis of mRNA and protein levels of AKT family members indicated that this regulation occurred by translational or posttranslational mechanisms (V.A. Appleman and B.C. Lewis, unpublished observations).

### Activation of PI3K/AKT signaling in KRAS<sup>G12D</sup> and BRAF<sup>V600E</sup> expressing PDECs depends on signaling through IGF1R

Because BRAF does not directly activate PI3K, we next sought to determine the mechanism by which BRAF<sup>V600E</sup> stimulates the PI3K/AKT pathway in PDECs. We hypothesized that PI3K/AKT activation occurs downstream of the RAF/MEK/ERK signaling cascade, potentially through the activation of autocrine growth factor

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<th>Tumor incidence</th>
<th>Time to tumor development (wks)</th>
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<td>GFP</td>
<td>1/6</td>
<td>8</td>
<td>400 mm&lt;sup&gt;3&lt;/sup&gt; (±0)</td>
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<tr>
<td>KRAS&lt;sup&gt;G12D&lt;/sup&gt;</td>
<td>6/6</td>
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<td>1679 mm&lt;sup&gt;3&lt;/sup&gt; (±607)</td>
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<td>BRAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
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NOTE: Tumor volume calculated using the formula \(L \times W \times H\).
We first assessed the levels of IGF1R ligands in serum-starved PDECs expressing KRASG12D, BRAFV600E, or GFP by qRT-PCR. We found robustly increased levels of IGF2 mRNA in both KRASG12D- and BRAFV600E-expressing cells relative to GFP-expressing controls, and a modest increase in IGF1 mRNA levels (Fig. 3A). Insulin mRNA levels were unaffected by the expression of KRASG12D and BRAFV600E. Immunoblotting confirmed increased IGF2 levels in KRASG12D- and BRAFV600E-expressing PDECs relative to GFP-expressing controls (Fig. 3B). Moreover, immunoblotting showed increased phosphorylation of IGF1R, indicative of receptor activation, in protein lysates from serum-starved KRASG12D- and BRAFV600E-expressing cells (Fig. 3C). Consistent with our hypothesis that IGF2 gene expression is stimulated downstream of the MEK/ERK cascade, blockade of MEK, but not PI3K, reduced IGF2 mRNA levels in KRASG12D-expressing PDECs (Fig. 3D, and Supplementary Fig. S4A). In contrast to the stimulation of IGF gene expression, we did not see a similar increase in the expression level of any EGF family ligands by qRT-PCR (Supplementary Fig. S4B), indicating that this increase is specific to IGF ligands.

To determine if AKT activation in KRASG12D- and BRAFV600E-expressing PDECs depends upon signaling through MEK, and subsequently through IGF1R, we inhibited MEK, PI3K, and IGF1R, and ascertained the impact on ERK and AKT phosphorylation via immunoblot. As expected, we found that ERK phosphorylation (pERK) is dramatically reduced in the presence of PD98059. Surprisingly, we found that pERK levels are also reduced in the presence of LY294002 and the IGF1R inhibitor AG1024 in KRASG12D- and BRAFV600E-expressing PDECs but not GFP controls (Fig. 3E). These results suggest that a potential feedback loop between the RAF/MEK/ERK and PI3K/AKT pathways specifically occurs downstream of KRASG12D and BRAFV600E in PDECs.

Treatment with LY2904002 reduced AKT phosphorylation at Ser 473 (pAKT; Fig. 3E). Interestingly, pAKT levels were also strongly reduced in BRAFV600E-expressing cells treated with PD98059 or AG1024, demonstrating that AKT activation lies downstream of MEK and IGF1R (Fig. 3E). Treatment of KRASG12D-expressing PDECs with LY294002 robustly reduced pAKT levels, whereas PD98059 and AG1024 had a measurable, but more modest impact (Fig. 3E).

To further assess the impact of IGF1R on the MEK/ERK and PI3K/AKT signaling pathways, we used shRNA-mediated knockdown to reduce IGF1R levels and downstream signaling. Immunoblotting confirmed efficient knockdown of IGF1R in PDECs infected with a lentivirus encoding an IGF1R-targeting shRNA relative to PDECs expressing a nonsilencing control (Fig. 3F). Immunoblotting showed that pERK and pAKT levels were strongly inhibited in KRASG12D- and BRAFV600E-expressing PDECs following IGF1R knockdown (Fig. 3F). These findings suggest that AKT activation occurs downstream of IGF1R. Coupled with our finding that IGF2 induction occurs downstream of MEK (Fig. 3D), these data further suggest that a MEK–IGF2–IGF1R signaling axis regulates

Figure 2. KRASG12D- and BRAFV600E-induced survival in PDECs requires MEK/ERK and PI3K/AKT signaling. A, viability of Ink4a/Arf, Tp73 double null of PDECs expressing KRASG12D, BRAFV600E, SHH, or GFP treated with DMSO, PD98059, or LY2900042 plus 100 μmol/L cycloheximide. *P < 0.05 compared with DMSO-treated cells of identical genotype. B, viability of Ink4a/Arf null PDECs expressing KRASG12D, BRAFV600E, SHH, or GFP treated with DMSO, PD98059, or LY2900042 plus UV irradiation. White bars vehicle-treated (or untreated) cells; black bars cycloheximide. Values are normalized such that viability of untreated cells is 1. Results are representative of at least 2 experiments. *P < 0.05 compared with DMSO-treated cells of identical genotype. DMSO, dimethyl sulfoxide. C, immunoblot analysis of ERK (Thr202/Tyr204) and AKT (ser473) phosphorylation in serum-starved KRASG12D, BRAFV600E, and GFP expressing Ink4a/Arf, Tp73 null PDECs. Values indicate the ratio of phosphorylated AKT relative to total AKT as measured by densitometry and normalized such that GFP expressing PDECs have a ratio of 1. All error bars, SD.
AKT activation downstream of activated KRAS and BRAF in primary PDECs.

IGF1R inhibition impairs KRAS<sup>G12D</sup>- and BRAF<sup>V600E</sup>-mediated survival

We next investigated the impact of IGF1R inhibition on PDEC survival. We found that AG1024 inhibited the ability of KRAS<sup>G12D</sup> and BRAF<sup>V600E</sup> to enhance survival in PDECs challenged with either cycloheximide (Fig. 4A) or UV irradiation (Fig. 4B). Importantly, survival in SHH-expressing PDECs was not impacted by AG1024 treatment, consistent with SHH-enhanced survival occurring in a MEK-independent manner, and in line with the hypothesis that IGF1R activation occurs downstream of MEK (Fig. 4A and B). Similarly, IGF1R knockdown impaired survival after apoptotic challenge (Fig. 4C). Interestingly, insulin receptor (IR) knockdown resulted in a reproducible, but statistically insignificant reduction in survival after apoptotic challenge, suggesting a potential role for IGF1R/IR heterodimers in mediating IGF2-stimulated signaling (Fig. 4C).

If the hypothesis that IGF2 stimulates IGF1R-mediated signaling downstream of MEK is correct, then ectopic expression of IGF2 in KRAS<sup>G12D</sup> - and BRAF<sup>V600E</sup>-expressing cells should rescue survival in the setting of MEK inhibition but not PI3K inhibition. To test this hypothesis, we infected KRAS<sup>G12D</sup>- and BRAF<sup>V600E</sup>-expressing PDECs with RCAS viruses encoding IGF2 or GFP as a control, and challenged these cells with apoptotic stimuli in the presence of specific signaling pathway inhibitors. Consistent with our hypothesis, we found that ectopic IGF2 expression rescued KRAS<sup>G12D</sup>- and BRAF<sup>V600E</sup>-induced survival in PDECs after challenge with cycloheximide in the presence of PI3K inhibition, but not MEK inhibition.

**Figure 3.** AKT phosphorylation in KRAS<sup>G12D</sup>- and BRAF<sup>V600E</sup>-expressing PDECs depends on signaling through IGF1R. A, qRT-PCR measurement of Igf2 mRNA in KRAS<sup>G12D</sup>-, BRAF<sup>V600E</sup>-, and GFP-expressing Ink4a/Arf<sup>−/−</sup>, Trp53<sup>−/−</sup> null PDECs (black, gray, and white bars, respectively). β-Actin is used as an endogenous control. Ligand expression in GFP-expressing cells is normalized to 1. B, immunoblot analysis of Igf2 levels in serum-starved KRAS<sup>G12D</sup>-, BRAF<sup>V600E</sup>-, and GFP-expressing Ink4a/Arf<sup>−/−</sup>, Trp53<sup>−/−</sup> null PDECs. β-Actin is used as a loading control. C, immunoblot analysis of IGF1R phosphorylation in serum-starved KRAS<sup>G12D</sup>-, BRAF<sup>V600E</sup>-, and GFP-expressing Ink4a/Arf<sup>−/−</sup>, Trp53<sup>−/−</sup> null PDECs. Total IGF1R levels are also shown. β-Actin is used as a loading control. D, qRT-PCR measurement of Igf2 mRNA in serum-starved KRAS<sup>G12D</sup>-expressing Ink4a/Arf<sup>−/−</sup>, Trp53<sup>−/−</sup> null PDECs treated with PD98059, LY294002, and AG1024. β-Actin is used as an endogenous control. E, immunoblot analysis of ERK (Thr202/Tyr204) and AKT (Ser473) phosphorylation in serum-starved KRAS<sup>G12D</sup>-, BRAF<sup>V600E</sup>-, and GFP-expressing Ink4a/Arf<sup>−/−</sup>, Trp53<sup>−/−</sup> null PDECs expressing an IGF1R-targeting shRNA. All error bars, SD; DMSO, dimethyl sulfoxide.
IGF1R is required for KRAS<sup>G12D</sup>- and BRAF<sup>V600E</sup>-induced pancreatic tumorigenesis

The data above showed a critical role for IGF1R-mediated signaling in the survival of PDECs. To determine whether this effect contributes to KRAS<sup>G12D</sup>- and BRAF<sup>V600E</sup>-induced transformation of pancreatic epithelial cells, we used targeting shRNAs to knockdown IGF1R expression in In<sub>ka</sub>4/Arf and Trp53 double null PDECs expressing either KRAS<sup>G12D</sup> or BRAF<sup>V600E</sup>. Effective shRNA-mediated knockdown was confirmed by immunoblot (Supplementary Fig. S5A). Orthotopic implantation of 10<sup>6</sup> PDECs resulted in efficient tumor formation in KRAS<sup>G12D</sup>- and BRAF<sup>V600E</sup>-expressing PDECs, whereas tumor formation was robustly inhibited in mice implanted with cells that simultaneously expressed IGF1R shRNA (Table 2). Interestingly, if mice implanted with cells expressing the IGF1R-targeting shRNA were allowed to remain on the study for a longer period of time, tumors eventually formed. Analysis of IGF1R expression in these tumors showed equivalent IGF1R levels to tumors formed by cells expressing a nonsilencing control (Supplementary Fig. S5B). Together, these data suggest that IGF1R is required for KRAS<sup>G12D</sup>- and BRAF<sup>V600E</sup>-induced pancreatic tumorigenesis.

Combined MEK and IGF1R inhibition impairs the survival of pancreatic cancer cells

Given that IGF1R is required for PDEC survival after apoptotic challenge and for KRAS<sup>G12D</sup>- and BRAF<sup>V600E</sup>-induced pancreatic tumorigenesis, we asked whether IGF1R is similarly required for the survival of pancreatic cancer cells. We first assessed the effect of IGF1R inhibition on the proliferation of pancreatic cancer cells derived from our orthotopic model. Consistent with our previously published data (14), we found that inhibition of MEK or PI3K strongly impaired the proliferation of the 170#3 cell line that harbors KRAS<sup>G12D</sup> and deletion of In<sub>ka</sub>4/Arf and Trp53 (Supplementary Fig. S6A). Similarly, inhibition of IGF1R with AG1024 also reduced proliferation in this cell line (Supplementary Fig. S6A).

We next determined the effect of MEK inhibition, PI3K inhibition, and IGF1R inhibition on the survival of pancreatic cancer cells. We found that inhibition of PI3K in 170#3 cells reduced survival after challenge with cycloheximide (Fig. 5A). Interestingly, in contrast to our findings in PDECs, we found that individual inhibition of MEK or IGF1R did not significantly impact survival after apoptotic challenge in the 170#3 cell line (Fig. 5A). However, combined inhibition of MEK and IGF1R reduced survival to the levels seen with PI3K inhibition (Fig. 5A). Similar results were observed in the human PDAC cell line Panc1 (Fig. 5B).

To confirm that this phenomenon occurs following exposure to a clinically relevant compound, we treated 170#3 cells with a 50 nmol/L concentration of the standard of care chemotherapeutic gemcitabine (26), a concentration that normally fails to elicit significant death in this cell line, in combination with inhibition of MEK, PI3K, and IGF1R. We found that PI3K inhibition, or combined inhibition of
MEK and IGF1R (but not inhibition of MEK or IGF1R alone), sensitized PDAC cells to gemcitabine (Fig. 5C). Similar results were obtained when IGF1R knockdown was combined with small molecule-mediated inhibition of MEK, demonstrating that these findings are not the consequence of toxicity induced by simultaneous exposure to AG1024 and PD98059 (Supplementary Fig. S6B and S6C). Thus, our data indicate that combined inhibition of MEK and IGF1R sensitizes pancreatic cancer cells to apoptotic stimuli, suggesting that combined inhibition of these signaling molecules may represent a novel therapeutic strategy for this malignancy.

Discussion

Activating mutations in KRAS are commonly identified in PDAC, and are present in early precursor PanIN lesions (5, 27, 28). Given the critical importance of initiating oncogenic lesions in oncogene addiction, targeted inhibition of mutant KRAS is an attractive therapeutic strategy (29–34). However, previous attempts to directly target KRAS have not been successful (10). Therefore, attention has shifted toward targeting downstream effector proteins. Yet, the roles of specific signaling cascades downstream of activated KRAS during pancreatic tumor initiation remain unclear. Therefore, in this study, we elucidated the roles of the MEK/ERK and PI3K/AKT signaling cascades. Interestingly, we identified that IGF1R is also required for KRAS<sup>G12D</sup>- and BRAF<sup>V600E</sup>-stimulated cell survival. Significantly, our dissection of the signaling pathways activated by KRAS<sup>G12D</sup> and BRAF<sup>V600E</sup> showed that the activation of the PI3K/AKT pathway lies downstream of both MEK and IGF1R. Indeed, we showed that IGF1R expression is induced downstream of MEK, resulting in the autocrine activation of IGF1R and subsequent stimulation of the PI3K/AKT pathway. Thus, in primary pancreatic epithelial cells, KRAS<sup>G12D</sup>-induced PI3K/AKT activation occurs predominantly through an indirect mechanism rather than via direct activation of PI3K by KRAS.

Interestingly, the effect of IGF1R knockdown on pAKT levels was more robust than that observed upon AG1024 treatment. These data suggest that knockdown of IGF1R more completely inhibits downstream signaling than treatment with AG1024 at the concentration used for these studies (20 μmol/L), a concentration that inhibits IGF1R but not IRs (36, 37). However, our data also showed that knockdown of the IR produced a modest but measurable effect on sensitivity to apoptotic stimuli, suggesting that IGF1R/IR heterodimers may, in part, mediate signals downstream of IGF ligands. In vivo genetic studies may shed light on the role of IR on KRAS<sup>G12D</sup>-driven pancreatic tumorigenesis.

Significantly, shRNA-mediated knockdown of IGF1R inhibited KRAS<sup>G12D</sup>- and BRAF<sup>V600E</sup>-induced pancreatic tumorigenesis in an orthotopic mouse model, underlining the importance of IGF1R signaling for pancreatic tumorigenesis. Moreover, tumors that eventually developed in mice injected with cells expressing IGF1R-targeting shRNAs displayed normal levels of IGF1R, strongly suggesting that this receptor tyrosine kinase (RTK) is required

<table>
<thead>
<tr>
<th>Cells implanted</th>
<th>Tumor incidence</th>
<th>Time to tumor development (wks)</th>
<th>Average tumor volume (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRAS&lt;sup&gt;G12D&lt;/sup&gt; control</td>
<td>4/5</td>
<td>7</td>
<td>576 mm&lt;sup&gt;3&lt;/sup&gt; (±436)</td>
</tr>
<tr>
<td>KRAS&lt;sup&gt;G12D&lt;/sup&gt; IGF1R shRNA</td>
<td>0/6</td>
<td>7</td>
<td>N/A</td>
</tr>
<tr>
<td>BRAF&lt;sup&gt;V600E&lt;/sup&gt; control</td>
<td>6/6</td>
<td>7</td>
<td>1211 mm&lt;sup&gt;3&lt;/sup&gt; (±239)</td>
</tr>
<tr>
<td>BRAF&lt;sup&gt;V600E&lt;/sup&gt; IGF1R shRNA</td>
<td>5/6</td>
<td>7</td>
<td>72 mm&lt;sup&gt;3&lt;/sup&gt; (±20)</td>
</tr>
</tbody>
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NOTE: Tumor volume calculated using the formula L × W × H.

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for KRAS-induced pancreatic tumorigenesis. Together, our data provide the novel observation that IGF1R is activated downstream of mutant KRAS in primary pancreatic epithelial cells via an autocrine signaling loop, and that IGF1R-mediated signaling is required to promote KRAS-induced tumor initiation. Moreover, our findings provide functional insight into the reported observation of elevated IGF and IGF1R expression in PDAC, and the association of elevated IGF1R levels with poor prognosis in PDAC (38–40).

Our studies also highlight the importance of cellular context in evaluating the roles of signaling molecules at specific stages of pancreatic tumor development. For example, despite the impact of MEK and IGF1R inhibition on survival in PDECs, we found that individual blockade of these signaling proteins did not sensitize pancreatic cancer cells to apoptotic stimuli. Indeed, in contrast to our findings in PDECs, inhibition of MEK or IGF1R did not impact AKT phosphorylation in pancreatic cancer cells, suggesting that genetic and/or epigenetic changes that occurred during transformation reduced the reliance on MEK and IGF1R for PI3K/AKT pathway activation. Thus, our identification of IGF1R as a key signaling molecule stimulated downstream of MEK/ERK signaling likely would not have occurred in immortalized fibroblasts or cancer cell lines. Therefore, additional studies analyzing primary pancreatic epithelial cells may be uniquely suited to the identification of other critical molecules required for pancreatic tumor initiation.

In contrast to the insensitivity of pancreatic cancer cells to inhibition of MEK or IGF1R, their combined inhibition sensitized human and mouse pancreatic cancer cell lines to apoptotic stimuli. These findings suggest that combined inhibition of MEK and IGF1R could potentially be part of new therapeutic regimens for the treatment of PDAC. Significantly, there are several ongoing clinical trials using IGF1R inhibition in many solid tumors, including in PDAC in which it is used in combination with gemcitabine (41–43). Our results suggest that simultaneous inhibition of MEK and IGF1R in combination with gemcitabine may potentially be more efficacious. Interestingly, our findings about the efficacy of combined inhibition of MEK and IGF1R are consistent with recently published observations by Engelman and colleagues who found that RTKs, particularly IGF1R, controlled PI3K activation in KRAS mutant colon cancer cell lines, and that combined inhibition of MEK and IGF1R led to tumor regression in a xenograft model (44). Indeed, RTKs have emerged as common mediators of resistance to BRAF-targeted
therapies in melanoma and colorectal cancer (45–48). In many instances, these RTKs reanimate MEK/ERK signaling independently of BRAF, and in that way confer resistance to BRAF inhibitors. In other instances, these RTKs drive enhanced survival through the activation of P13K/AKT signaling, and potentially other mechanisms. Indeed, consistent with our findings, combined MEK/IGF1R inhibition or combined MEK/P13K inhibition induced massive apoptosis in BRAF inhibitor resistant cells (44, 48). Our studies raise the possibility that these mediators of resistance may be commonly activated downstream of MEK/ERK signaling during the early phases of tumor genesis.

In total, our data show a critical role for IGF1R signaling in pancreatic tumor initiation and pancreatic cancer cell survival, and suggest that IGF1R is a viable target in combinatorial therapeutic strategies in PDAC. Further studies about the role of the insulin receptor substrate (IRS) adapter proteins and other downstream molecules may shed further light on the role of IGF1R-stimulated signaling cascades in pancreatic tumorogenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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