PTEN Loss Accelerates Kras$^{G12D}$-Induced Pancreatic Cancer Development

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Abstract

KRAS mutations are found in ~90% of human pancreatic ductal adenocarcinomas (PDAC). However, mice genetically engineered to express Kras$^{G12D}$ from its endogenous locus develop PDACs only after a prolonged latency, indicating that other genetic events or pathway alterations are necessary for PDAC progression. The PTEN-controlled phosphatidylinositol 3-kinase (PI3K)/AKT signaling axis is dysregulated in later stages of PDAC. To better elucidate the role of PTEN/PI3K/AKT signaling in Kras$^{G12D}$-induced PDAC development, we crossed Pten conditional knockout mice (Pten$^{lox/lox}$) to mice with conditional activation of Kras$^{G12D}$. The resulting compound heterozygous mutant mice showed significantly accelerated development of acinar-to-ductal metaplasia (ADM), malignant pancreatic intraepithelial neoplasia (mPanIN), and PDAC within a year. Moreover, all mice with Kras$^{G12D}$ activation and Pten homozygous deletion succumbed to cancer by 3 weeks of age. Our data support a dosage-dependent role for PTEN, and the resulting dysregulation of the PI3K/AKT signaling axis, in both PDAC initiation and progression, and shed additional light on the signaling mechanisms that lead to the development of ADM and subsequent mPanIN and pancreatic cancer. Cancer Res; 70(18); 7114–24. ©2010 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest of all cancers with a 5-year survival rate of <5%. Genetically engineered mouse models that recapitulate the human disease (1) have become essential tools in the dissection of the cellular and molecular pathogenesis of PDAC and the preclinical assessment of novel biomarkers and therapeutics. The most studied model to date has been the Pdx1-Cre-mediated expression of a knock-in mutant allele of Kras with a glycine to aspartic acid substitution (Pdx1-Cre$^{Kras^{G12D/G12D}}$) from its endogenous locus. These mice slowly develop pre-malignant pancreatic intraepithelial neoplasias (mPanIN) with prolonged latencies and incomplete penetrance for the development of PDAC (2). However, PDAC developed far more rapidly when Pdx1-Cre$^{Kras^{G12D/G12D}}$ mice were crossed with mice deficient in one of the genes commonly associated with PDAC, such as Ink4a/Arf (3), transforming growth factor-β (TGF-β) receptor type 2 (Tgfbir2; ref. 4), and Smad4 (5), or carrying a mutation in p53 (6), although these molecular alterations alone do not lead to the development of pancreatic cancer in the absence of oncogenic Kras. These data indicate that additional genetic events beyond Kras mutation are responsible for pancreatic cancer progression. To date, the function of phosphatase and tensin homologue (PTEN) tumor suppressor, which is mutated in PDAC far less frequently (7, 8), has yet to be examined in the context of mutant Kras$^{G12D}$ activation. In this study, we examined the effects of concomitant conditional Pten deletion and Kras$^{G12D}$ activation and observed an accelerated and accentuated phenotype of acinar-to-ductal metaplasia (ADM), leading to mPanIN and malignant progression. Our findings indicate that concurrent dysregulation of the PTEN/phosphatidylinositol 3-kinase (PI3K)/AKT and RAS/RAF/mitogen-activated protein kinase (MAPK) pathways acts synergistically to promote pancreatic cancer initiation and progression. This model provides us with a means to explore the role of PTEN and the PI3K-AKT signaling axis in pancreatic tumor development and treatment.

Materials and Methods

Mouse strains

To generate Pdx1-Cre$^{+/-}$;Kras$^{G12D/+}$ and Pdx1-Cre$^{+/-}$;Pten$^{lox/lox}$;Kras$^{G12D/+}$ mice, we backcrossed the Kras$^{G12D/+}$ line (on a C57 background; ref. 2) to the Pten$^{lox/lox}$ line (on a 129/BALB/c background) twice to generate Kras$^{G12D/+}$;Pten$^{lox/lox}$.
mice. We then crossed \(\text{Kras}^{\text{G12D}/-}\cdot\text{Pten}^{\text{floxed/lox}}\) mice to \(\text{Pdx1-Cre}^{+}\); \(\text{Pten}^{\text{lox/lox}}\) mice we had previously generated (9) to produce experimental animals on a mixed C57/BL/6,129/BALB/c background. All studies were performed under the regulation of the Division of Laboratory Animal Medicine at the University of California at Los Angeles (UCLA). \(\text{Kras}^{\text{G12D}}\) primers, designed to recognize the \(\text{Kras}^{\text{G12D}}\) mutant allele (10), and \(\text{Pten}\) primers, which detect the \(\text{Pten}\) floxed allele (11), were used to confirm the genotypes of mice from the breeding crosses.

**Histology and immunohistochemistry**

Immunohistochemical analysis was performed on formalin-fixed, paraffin-embedded tissue. Antigen retrieval was performed by heating the slides at 95°C in citrate buffer (pH 6.0) for 15 minutes before staining. The following primary antibodies were used: rabbit anti–phospho- Akt (Ser\(473^{\beta}\); 1:50; Cell Signaling), guinea pig anti-Cytokeratin \(\alpha\) (1:5,000; a gift from Chris Wright, Vanderbilt University, Nashville, TN), rabbit anti–phospho-extracellular signal-regulated kinase (ERK) 1/2 (p\(44^{\beta}/p42^{\beta}\); 1:100; Sigma), rabbit anti–Ki67 (1:1,000; Vector Labs), rabbit anti–amylose (1:500; Sigma), mouse anti–cytokeratin 19 (CK19; 1:100; Abcam), mouse anti–pan-cytokeratin (1:500; Abcam), mouse anti–phospho-S6 (Ser\(240/244^{\beta}\); 1:100; Cell Signaling), rat anti-mouse/human CD44 (1:50; eBiosciences), mouse anti–smooth muscle actin (SMA) \(\alpha\) (1:1,000; Sigma), rabbit anti–platelet-derived growth factor receptor \(\beta\) (PDGF\(\beta^{\beta}\); 1:100; Cell Signaling), and rabbit anti–cyclin D1 (1:25; Cell Signaling). Biotinylated \(\text{Dolichos biflorus}\) lectin (Vector Labs) was used at 1:250 in HEPES/NaCl.

The collection of all human tumor samples used for this study was approved by the UCLA Institutional Review Board. The UCLA Pathology databases were used to identify patients with PDAC. Slides from the selected cases were reviewed. Anonymously labeled sections for immunohistochemistry studies, as detailed above, were prepared by the UCLA Department of Pathology Translational Pathology Laboratory Core facility. Additionally, an H&E-stained slide was made from each block to confirm the diagnosis.

**Proliferation index and statistical evaluation**

The proliferation index for ADMs and mPanINs was determined by averaging the percentage of Ki67-positive cells per field for 10 fields at \(\times 400\) magnification \(n = 4\). Each field selected contained only ADMs or only mPanINs. The percentage of Ki67-positive cells was determined as the number of Ki67-positive cells per field (excluding positive cells in the stroma or untransformed acinar cells)/total cells per field (excluding cells in the stroma). Differences between mice in each cohort were evaluated by Student’s \(t\) test. \(P < 0.05\) was considered of statistical significance.

**Histopathologic scoring of murine pancreatic lesions**

H&E-stained sections were reviewed by R.H. and H.W. and two pathologists with extensive experience in murine and human pancreas pathology (D.W.D. and S.D). Histopathologic lesions in mouse pancreata were scored in blinded fashion using serial step sections (20 μm apart, four sections per mouse) and consensus criteria established at the 2004 Penn Workshop (12).

**Laser capture microdissection and loss of heterozygosity analysis**

Laser capture microdissection (LCM) of H&E-stained pancreatic sections was performed using an Arcturus PixCell IIe LCM system. Approximately 50 cells from mPanINs or PDACs in \(\text{Pdx1-Cre}^{+}\cdot\text{Kras}^{\text{G12D}/-}\cdot\text{Pten}^{\text{lox/lox}}\) mice were collected on CapSure HS LCM caps (Molecular Devices). DNA was extracted by 16-hour incubation (55°C) in the ExtracSure sample extraction devices (Molecular Devices) with lysis buffer [10 mmol/L Tris-HCl (pH 8.0), 1% Tween 20, 100 μg proteinase K]. Following incubation, proteinase K was inactivated by incubation at 99°C for 10 minutes, and 1-μL aliquots of DNA were used for PCR analysis. PCR was performed for detection of wild-type (WT), floxed, and recombined alleles of \(\text{Pten}\) using previously described primers (11).

**Results**

**PTEN dosage controls pancreatic cancer progression in the \(\text{Pdx1-Cre}^{+}\cdot\text{Kras}^{\text{G12D}/+}\) mouse model**

The prolonged latencies and incomplete penetrance for the development of PDAC observed in the \(\text{Pdx1-Cre}^{+}\cdot\text{Kras}^{\text{G12D}/+}\) mouse model prompted us to determine whether alteration of the PTEN/PDK pathway could promote accelerated PDAC initiation and progression. For this, we crossed the \(\text{Pdx1-Cre}^{+}\cdot\text{Kras}^{\text{G12D}/+}\) model (2) with the \(\text{Pten}\) conditional knockout line we had previously generated (9). We found that \(\text{Pten}\) haploinsufficiency significantly shortened the life span of \(\text{Pdx1-Cre}^{+}\cdot\text{Kras}^{\text{G12D}/+}\cdot\text{Pten}^{\text{lox/lox}}\) mice to a median survival of ~3.5 months (green line in Fig. 1A; \(\text{Pdx1-Cre}^{+}\cdot\text{Kras}^{\text{G12D}/+}\cdot\text{Pten}^{\text{lox/lox}}\); \(n = 45\)). Most \(\text{Pdx1-Cre}^{+}\cdot\text{Kras}^{\text{G12D}/+}\cdot\text{Pten}^{\text{lox/lox}}\) mice exhibited abdominal distension due to a combination of ascites fluid and pancreatic enlargement, at which time the animals were sacrificed. Nearly 100% of the \(\text{Pdx1-Cre}^{+}\cdot\text{Kras}^{\text{G12D}/+}\cdot\text{Pten}^{\text{lox/lox}}\) mice succumbed to cancer burden by 12 months compared with 90% survival of the \(\text{Pdx1-Cre}^{+}\cdot\text{Kras}^{\text{G12D}/+}\) model (blue line in Fig. 1A; \(n = 25\)). We examined sections from the pancreata of each transgenic model to score the presence of different grades of mPanIN or PDAC lesions, which were defined by the consensus criteria of the pancreatic mouse modeling community (13). Histologic examination showed evidence of accelerated PDAC development in \(\text{Pdx1-Cre}^{+}\cdot\text{Kras}^{\text{G12D}/+}\cdot\text{Pten}^{\text{lox/lox}}\) mice compared with \(\text{Pdx1-Cre}^{+}\cdot\text{Kras}^{\text{G12D}/+}\) littermate controls. Whereas \(\text{Pdx1-Cre}^{+}\cdot\text{Kras}^{\text{G12D}/+}\) mice exhibited limited early-stage mPanIN lesions within an otherwise normal pancreas parenchyma at 3 months, \(\text{Pdx1-Cre}^{+}\cdot\text{Kras}^{\text{G12D}/+}\cdot\text{Pten}^{\text{lox/lox}}\) mice showed an almost complete loss of normal gland architecture and were involved by invasive ductal tumors histologically similar to human PDACs [Fig. 1A (right) and B (bottom)]. Invasive cancers were defined as the presence of malignant ductal epithelial cells, identifiable by CK19 staining, which had penetrated through the basement membrane into the surrounding SMA-positive stroma (Fig. 1B). Twenty-one of 25 \(\text{Pdx1-Cre}^{+}\cdot\text{Kras}^{\text{G12D}/+}\cdot\text{Pten}^{\text{lox/lox}}\)
mice alive past 2 months of age developed well-to-moderately differentiated invasive ductal cancers. The spleen, liver, and lungs of tumor-bearing mice were examined, with only rare microscopic metastasis to the lungs noted (n = 2; data not shown). The metastatic lung lesions histologically resembled the primary pancreatic tumor. The infrequent rate of metastasis indicates that the mice rapidly succumb to their primary cancers before...
the time needed to develop metastatic lesions and/or that other genetic events are necessary for metastasis.

Mice with \( \text{Kras}^{G12D} \) activation and homozygous Pten deletion had an even earlier onset of lethal PDAC. \( \text{Pdx1-Cre}^+; \text{Kra}^{G12D/\beta}; \text{Pten}^{lox/\beta} \) mice were moribund and either naturally succumbed or had to be euthanized before weaning (red line in Fig. 1A; \( n = 26 \)). Histopathologic examination of the pancreata of the \( \text{Pdx1-Cre}^+; \text{Kra}^{G12D/\beta}; \text{Pten}^{lox/\beta} \) mice from postnatal days 1 to 17 (P1–P17) showed pancreatic enlargement (Supplementary Fig. S1A, left) and severe exocrine pancreatic atrophy and edema. In addition, the presence of mPanINs, metaplasias, ducto-insular complexes (endocrine cells that contain aberrantly proliferating ductules; ref. 13), and cancer formation was observed (Supplementary Fig. S1B). Taken together, these data suggest that Pten and PTEN-controlled signaling pathways may serve as an important "brake" for \( \text{Kras}^{G12D} \)-initiated PDAC progression. The rapid onset and lethality observed in the \( \text{Pdx1-Cre}^+; \text{Kra}^{G12D/\beta}; \text{Pten}^{lox/\beta} \) mice precluded any detailed mechanistic analysis on the initiation and progression of PDAC, which led us to focus our attention on the \( \text{Pdx1-Cre}^+; \text{Kra}^{G12D/\beta}; \text{Pten}^{lox/\beta} \) mice.

\( \text{Pdx1-Cre}^+; \text{Kra}^{G12D/\beta}; \text{Pten}^{lox/\beta} \) mice recapitulate the pathologic features of human PDACs

The hallmarks of human pancreatic cancers are an abundance of neoplastic cells expressing ductal markers, such as CK19 and DBA lectin (14), as well as their invasive behavior (12). Similar to human pancreatic cancers, the PDAC lesions in \( \text{Pdx1-Cre}^+; \text{Kra}^{G12D/\beta}; \text{Pten}^{lox/\beta} \) pancreata were positive for DBA and PDX1, a transcription factor associated with early pancreatic development and known to be upregulated in human PDAC (Fig. 1B; refs. 15, 16). Using double immunofluorescence analysis, we observed that the majority of tumor cells in \( \text{Pdx1-Cre}^+; \text{Kra}^{G12D/\beta}; \text{Pten}^{lox/\beta} \) pancreata are CK19 positive and invasive, penetrating through the basement membrane into the stroma (Fig. 1B, white arrow in the bottom right), consistent with phenotypes observed in human PDAC. Taken together, these findings show that the tumors in \( \text{Pdx1-Cre}^+; \text{Kra}^{G12D/\beta}; \text{Pten}^{lox/\beta} \) PDAC mice resemble human PDAC on the histologic level and can be used to study the molecular and genetic events, leading to human PDAC initiation and progression.

PTEN haploinsufficiency determines the onset of mPanIN, but complete PTEN loss is required for PDAC progression

Of the many different types of lesions thought to be the precursors to PDAC, PanIN lesions have been the best characterized (12). For this reason, we decided to first determine if Pten haploinsufficiency could accelerate mPanIN development by following three cohorts of mice (\( \text{Pdx1-Cre}^+; \text{Pten}^{loxp/+} \), \( \text{Pdx1-Cre}^+; \text{Kra}^{G12D/+} \), and \( \text{Pdx1-Cre}^+; \text{Kra}^{G12D/+}; \text{Pten}^{lox/+} \)) from 1 to 6 months. We examined multiple sections from each pancreas (see Materials and Methods) and scored each animal for the presence of mPanIN lesions of various grades or PDAC (Supplementary Fig. S2A). At 1 month of age, neither \( \text{Pdx1-Cre}^+; \text{Pten}^{loxp/+} \) nor \( \text{Pdx1-Cre}^+; \text{Kra}^{G12D/+} \) mice (Fig. 2A, white or gray columns, respectively) showed mPanIN development (\( n = 5 \) each cohort), whereas two of six \( \text{Pdx1-Cre}^+; \text{Kra}^{G12D/+}; \text{Pten}^{lox/+} \) mice (Fig. 2A, black columns) showed mPanIN lesions. At 3 months of age, all \( \text{Pdx1-Cre}^+; \text{Kra}^{G12/+}; \text{Pten}^{lox/+} \) mice examined (\( n = 12 \)) had mPanIN lesions, whereas only one of seven \( \text{Pdx1-Cre}^+; \text{Kra}^{G12/+} \) mice had mPanINs.

Having established that Pten haploinsufficiency led to earlier development of mPanIN lesions, we next examined if PDAC development was accelerated as well. At 3 months of age, 8 of 12 \( \text{Pdx1-Cre}^+; \text{Kra}^{G12/+}; \text{Pten}^{lox/+} \) mice showed the development of PDACs (Fig. 2B, black columns), whereas \( \text{Pdx1-Cre}^+; \text{Pten}^{loxp/+} \) or \( \text{Pdx1-Cre}^+; \text{Kra}^{G12/+} \) mice of the same age (\( n = 5 \) and 7, respectively) showed no evidence of PDAC development (Fig. 2B, white or gray columns, respectively). Ultimately, all \( \text{Pdx1-Cre}^+; \text{Kra}^{G12/+}; \text{Pten}^{lox/+} \) mice that we examined at the 6-month time point (\( n = 6 \)) showed evidence of PDAC (Fig. 2B, black columns) compared with 2 of 11 \( \text{Pdx1-Cre}^+; \text{Kra}^{G12/+} \) mice (Fig. 2B, gray columns). Therefore, the Pten/Pik3/Akt and Ras/Raf/MAPK pathways synergize at both initiation and progression steps of PDAC development.

We next determined if the synergy between the PTEN/Pik3/Akt and Ras/Raf/MAPK pathways in PDAC development requires complete loss of PTEN function or loss of heterozygosity (LOH). For this, we first evaluated the status of phosphorylated p44/42 MAPK (P-ERK), Akt (P-Akt), and S6 (P-S6), surrogate markers for dysregulated Ras/ Raf/MAPK and PTEN/Pik3/Akt pathways, respectively, in the mPanINs and PDACs of \( \text{Pdx1-Cre}^+; \text{Kra}^{G12/+} \) and \( \text{Pdx1-Cre}^+; \text{Kra}^{G12/+}; \text{Pten}^{lox/+} \) mice. Immunohistochemistry showed positive staining of P-ERK, but not P-Akt or P-S6, in the stage-matched mPanIN lesions of both \( \text{Pdx1-Cre}^+; \text{Kra}^{G12/+} \) and \( \text{Pdx1-Cre}^+; \text{Kra}^{G12/+}; \text{Pten}^{lox/+} \) mice (Fig. 2A, right), indicating that it was unlikely that LOH had happened at this stage in lesion development. On the other hand, PDACs in 6-month-old \( \text{Pdx1-Cre}^+; \text{Kra}^{G12/+}; \text{Pten}^{lox/+} \) mice showed positive staining for P-ERK, P-Akt, and P-S6, strongly indicating that Pten LOH may have occurred in these lesions (Fig. 2B, right).

To confirm if Pten LOH indeed happened during PDAC progression, we used LCM to separate PDAC lesions from adjacent mPanINs in \( \text{Pdx1-Cre}^+; \text{Kra}^{G12/+}; \text{Pten}^{lox/+} \) mice (\( n = 5 \); Supplementary Fig. S2B, left) and performed PCR analysis using DNA isolated from the microdissected tissues. As shown in Supplementary Fig. S2B (right), all of the PDAC lesions showed the presence of a band corresponding to the Cre-mediated Pten deletion product, whereas four of five showed absence of the WT Pten allele, indicating that Pten LOH had occurred in these samples (the WT band in sample 3 could have come from stromal cell contamination). In contrast, all adjacent mPanIN lesions showed the presence of the WT allele (Supplementary Fig. S2B, right, bottom, outlined in red). Taken together, these findings suggest that whereas Pten LOH contributes to PDAC development in the \( \text{Pdx1-Cre}^+; \text{Kra}^{G12/+}; \text{Pten}^{lox/+} \) model, Pten haploinsufficiency, as observed in other systems (17), is sufficient to cause accelerated mPanIN initiation.
ADM development precedes mPanIN formation in Pdx1-Cre<sup>+</sup>:Kras<sup>G12D</sup>+/Pten<sup>lox/lox</sup> mice

When we examined the pathology of the Pdx1-Cre<sup>+</sup>:Kras<sup>G12D</sup>+/Pten<sup>lox/lox</sup> mice between 1 and 3 months of age, we observed, in addition to traditional mPanIN lesions, a large number of ADMs (Fig. 3A, top left, white arrow), similar to the metaplasias (12) we had observed in the Pdx1-Cre<sup>+</sup>:Pten<sup>lox/lox</sup> model (9). These ADMs were concentrated in the “transition zone” between morphologically normal pancreatic structures and mPanIN and PDAC lesions and could be easily identified on H&E-stained sections, where ductal, tubular structures, often with mucinous cytoplasm, emerged from acinar cells. These structures can be numerous, replacing the normal acinar parenchyma, and are thought to possibly arise from either acinar (18) or centroacinar cells (9). H&E-stained sections from resected human tumors revealed similar pathology in areas adjacent to PDAC (Fig. 3A, white arrow). Double immunofluorescence confirmed that these lesions in fact contained duct-like cells (pan-cytokeratin labeled; green) within acinar structures (amylase; red; Fig. 3A, white arrow). ADMs were also observed in the Pdx1-Cre<sup>+</sup>:Kras<sup>G12D</sup>+/Pten<sup>lox/lox</sup> mice but were not observed in WT controls (Fig. 3B).
To decipher the relationship between ADM and mPanINs, we first investigated the chronological appearance of the two lesions. Although no mPanIN lesions were detected in the pancreata of Pdx1-Cre+;KrasG12D/+ and Pdx1-Cre+;Ptenlox/+ mice at 1 month of age, one of five Pdx1-Cre+;KrasG12D/+ mice and three of six Pdx1-Cre+;KrasG12D/+;Ptenlox/+ mice showed ADM alone, and another two of six Pdx1-Cre+;KrasG12D/+;Ptenlox/+ mice showed both metaplasias and mPanIN lesions (Fig. 3B). Interestingly, none of the animals developed mPanIN lesions only, suggesting that (a) metaplasias are the earliest pathologic lesions identified in the Pdx1-Cre+;KrasG12D/+ and Pdx1-Cre+;KrasG12D/+;Ptenlox/+ mouse model, (b) KRAS and PTEN/PI3K pathways collaborate to promote metaplasia development, and (c) ADMs likely serve as the precursors of mPanINs or parallel with mPanIN in pancreatic cancer development.

ADM may serve as the precursor of PDAC in Pdx1-Cre+;KrasG12D/+;Ptenlox/+ mouse model and human cancers

To investigate the functional significance of metaplasias in pancreatic cancer development, we quantified the proliferation of metaplasia and mPanIN lesions on stage-matched Pdx1-Cre+;KrasG12D/+ and Pdx1-Cre+;KrasG12D/+;Ptenlox/+ mice (see Materials and Methods; Fig. 4; n = 4). The proliferation index of metaplasia lesions was significantly higher than that of the mPanIN lesions in both the Pdx1-Cre+;KrasG12D/+ (7.4% versus 2.4%) and Pdx1-Cre+;KrasG12D/+;Ptenlox/+ cohorts (11.4% versus 6.2%; Fig. 4). Moreover, there was a significant increase in the proliferation rates of both types of lesions in Pdx1-Cre+;KrasG12D/+;Ptenlox/+ mice compared with Pdx1-Cre+;KrasG12D/+ mice. This shows not only that ADMs precede the development of mPanINs but also that Pten haploinsufficiency makes the already more rapidly dividing ADMs even more proliferative, which could contribute to accelerated disease initiation and progression.

Increase in CD44-positive cells in murine and human metaplastic lesions

CD44 is a cell surface glycoprotein known to be upregulated in human PDACs (19) and has been used as a marker to identify a stem-like subpopulation in the pancreas (20). CD44-positive cells were present in the early ADMs in the Pdx1-Cre+;KrasG12D/+;Ptenlox/+ model as well as in human samples (Fig. 5A, top row; Supplementary Fig. S3B) but almost absent in the nearby mPanINs (Fig. 5A, bottom row) or in normal acinar and ductal cells (Supplementary Fig. S3A). Interestingly, we observed high CD44 expression in the invasive PDAC lesions in Pdx1-Cre+;KrasG12D/+;Ptenlox/+ mice (Fig. 5B, top row), which matches the CD44 staining pattern observed in human PDAC samples (Fig. 5B, bottom row). The level of CD44 staining was much less intense in the PDAC lesions in Pdx1-Cre+;KrasG12D/+ mice (Supplementary Fig. S3A). Taken together, these data suggest that Pten/Pi3K and KRAS pathways collaborate in accelerating ADM development and in turn promote CD44-positive PDAC formation.
Discussion

PTEN and pancreatic cancer development

Even with improvements in surgical techniques, the overall survival rate for patients with PDAC remains dismal. There is still much to be learned about the origin and development of PDAC, but unfortunately, most of our knowledge about this disease comes from biopsies of patients with late-stage tumors, making it difficult to determine which cell types, and more importantly which molecular changes, are important for disease onset and progression. Animal models that faithfully recapitulate both the histology and molecular profile of the human disease have been an indispensable tool, which has allowed us to dissect out the role various genetic events play in the initiation and progression of PDAC. Mutations in KRAS resulting in its constitutive activation occur in >90% of PDACs (21). However, mice expressing KrasG12D from its endogenous locus progress to invasive cancers only after a prolonged latency (2) unless coupled with other genetic alterations commonly found in human PDAC (21). Previous studies using the KrasG12D mouse model, where median survival is 15 months (2), have shown that homozygous deletion of both the p16Ink4A and p19Arf loci decreased survival to 8 weeks (3). Likewise, KrasG12D mice with either p53, type II TGF-β receptor gene (Tgfbr2), or Smad4 inactivation show accelerated development of PDACs with median survival times of 5, 4, or 2 months, respectively (4–6). Our study shows that loss of PTEN function causes accelerated cancer development and decreased survival in the KrasG12D mouse model to either 3.5 months in the case of heterozygous Pten or 3 weeks with homozygous Pten deletion (Figs. 1 and 2). Although point mutations or LOH in PTEN rarely occur in human PDAC (7, 8), there is evidence to support that loss of PTEN function or alteration of PTEN-controlled signaling pathways can occur through alternative mechanisms, including promoter methylation (22), increased expression of AKT2 (23), or reduction of mRNA levels (24). In addition, the expression of several signaling pathways linked to PDAC development, including RAS (25), TGF-β (26, 27), and insulin-like growth factor-1 (28), has been shown to downregulate the expression of PTEN or inactivate the catalytic activity of PTEN, leading to reduced apoptosis (25), increased cell motility (26, 27), and increased cell proliferation and invasion (28) in pancreatic cancer cells. Therefore, although PTEN itself is not mutated frequently in the human disease, PTEN and PTEN-controlled pathways are being modulated in ways that are critical for disease development.

Pdx1-Cre+:KrasG12D+/Ptenlox/lox mice recapitulate human PDAC

Histologic and immunohistochemical analysis verified that the cancers in Pdx1-Cre+:KrasG12D+/Ptenlox/lox mice recapitulated the pathologic features of human PDACs (Fig. 1B). Despite their rapid tumor onset, which is similar in latency to mice with p16Ink4A and p19Arf deletion, Pdx1-Cre+:KrasG12D+/Ptenlox/lox mice do not show the development of a large percentage of sarcomatoid tumors, a tumor type that makes up only a small portion of the human disease but is prevalent in the Pdx1-Cre+:KrasG12D+/Ink4a/Arflox/lox model (3). In this respect, the Pdx1-Cre+:KrasG12D+/Ptenlox/lox mice are similar to...
the Ptf1a-Cre⁺;KrasG12D/+;Tgfbr2 flox/flox mice that developed well-differentiated ductal adenocarcinomas with no evidence of sarcomatoid features (4). The drastic acceleration of PDAC development and early lethality seen in these two models may explain why metastases are only seen in a few cases when animals live well beyond the median survival age. The two metastases found in our Pdx1-Cre⁺;KrasG12D/+;Ptenlox/+ model occurred in mice at 20 and 24 weeks of age (data not shown). This pattern of staining was also observed in areas adjacent to PDACs from human tumors that contained metaplasias and mPanINs. Magnification, ×400. B, top row, H&E staining shows the morphology of a PDAC (magnification, ×400) in a 6-mo-old Pdx1-Cre⁺;KrasG12D/+;Ptenlox/+ mouse. Immunofluorescence on a serial section confirms the presence of invading ductal epithelium (right, CK19; red/SMA staining, green). Bottom, immunohistochemistry shows that these invasive tumors show CD44 staining, matching the expression pattern of CD44 seen in human PDAC samples. Magnification, ×400. Scale bars, 50 μm.

**Figure 5.** Mice with Pten heterozygosity and mutant KrasG12D activation show an increase in CD44-positive cells. A, immunohistochemistry on Pdx1-Cre⁺;KrasG12D/+ and Pdx1-Cre⁺;KrasG12D/+;Ptenlox/+ mice at 3 mo of age showed that the cells of metaplasias (top row), but not mPanINs (bottom row), showed expression of CD44. This pattern of staining was also observed in areas adjacent to PDACs from human tumors that contained metaplasias and mPanINs. Magnification, ×400. B, top row, H&E staining shows the morphology of a PDAC (magnification, ×400) in a 6-mo-old Pdx1-Cre⁺;KrasG12D/+;Ptenlox/+ mouse. Immunofluorescence on a serial section confirms the presence of invading ductal epithelium (right, CK19; red/SMA staining, green). Bottom, immunohistochemistry shows that these invasive tumors show CD44 staining, matching the expression pattern of CD44 seen in human PDAC samples. Magnification, ×400. Scale bars, 50 μm.
changes not only in the epithelial cell compartment but also in the surrounding desmoplastic stroma, again recapitulating the nature of the human disease. Pdx1-Cre;KrasG12D/+;Ptenlox/+ mice showed a significant stromal component (as marked by SMA-positive staining) surrounding both mPanINs and PDACs (Fig. 1B). While investigating the nature of the desmoplastic stroma seen in the tumors of Pdx1-Cre;KrasG12D/+;Ptenlox/+ mice, we observed increased staining for PDGFRβ in the stroma of Pdx1-Cre;KrasG12D;Ptenlox/+ mice compared with that of Pdx1-Cre;KrasG12D alone. This staining pattern matched what was observed in the human samples (Supplementary Fig. S3A), as human PDACs are characterized by induction of PDGFRβ expression in the stromal compartment (29). The presence of cells positive for PDGFRβ has been found to aid tumor angiogenesis through pericyte recruitment (30), and it is believed that targeting vascular endothelial growth factor receptor (VEGFR) and PDGFRβ may be capable of preventing the growth of some types of pancreatic cancers (31). This strongly suggests that combined alteration of the PTEN/PI3K and KRAS pathways in the epithelial cells can elicit a protumorigenic response from the surrounding microenvironment through non–cell-autonomous mechanisms.

**Kras**G12D activation and *Pten* haploinsufficiency are sufficient for accelerated lesion development

Our results show that *Pten* LOH corresponded only with PDAC development in this mouse model (Supplementary Fig. S2B). This raises the question of what is driving earlier mPanIN or ADM development in the Pdx1-Cre;KrasG12D/+;Ptenlox/+ mice because we cannot detect *Pten* LOH in either of these precursor lesions (Supplementary Figs. S2B and S3C). This earlier onset could be brought about by the increased cell proliferation that results from the synergy between the KRAS and PI3K/AKT pathway alterations. Both ADMs and mPanINs in Pdx1-Cre;KrasG12D/+;Ptenlox/+ mice showed an increased rate of proliferation compared with their Pdx1-Cre;KrasG12D/+ littermates (Fig. 4). In addition, these early ADMs and PDACs in Pdx1-Cre;KrasG12D/+;Ptenlox/+ mice showed intense staining of cyclin D1 (Supplementary Fig. S3A), which has been shown to be regulated by PTEN (29). The presence of cells positive for PDGFRβ has been found to aid tumor angiogenesis through pericyte recruitment (30), and it is believed that targeting vascular endothelial growth factor receptor (VEGFR) and PDGFRβ may be capable of preventing the growth of some types of pancreatic cancers (31). This strongly suggests that combined alteration of the PTEN/PI3K and KRAS pathways in the epithelial cells can elicit a protumorigenic response from the surrounding microenvironment through non–cell-autonomous mechanisms.

Recent work has shown that KrasG12D mice with alterations in SMAD4 (5) and TGF-β signaling (4) have an increase in ADM formation accompanying tumor acceleration. Likewise, expression of the KrasG12D oncogene in the acinar/centroacinar lineage results in ADMs, preceding the development of mPanINs and invasive PDAC (18). In addition, although mice with KrasG12D activation and NOTCH gain of function did not progress to PDAC, a significant acceleration of ADM reprogramming leading to mPanIN formation was observed (37). Our analysis indicates that *Pten* haploinsufficiency in KrasG12D mice is also sufficient to accelerate the development of ADM lesions (Figs. 2 and 3), adding to the growing lines of evidence (18, 37, 38) that show that these ADMs are the earliest precursor lesions seen in the KrasG12D mouse models. The development of metaplasias precedes mPanINs in both the Pdx1-Cre;KrasG12D/+ and

**Figure 6.** KrasG12D activation and PTEN loss of function drives ADM and mPanIN development with complete PTEN loss of function promoting PDAC development.
Pdx1-Cre;Kras\(G^{12D}/\)\(;Pten^{lox/}\) mice (Fig. 3B), suggesting that although PTEN dosage plays a critical role in promoting metaplasia development, ADMs are likely the actual precursor lesions that give rise to the neoplastic ducts seen in PDAC regardless of PTEN function. Our hypothesis, which will require vigorous experimental testing using more sophisticated methodologies such as lineage tracing, is that ADMs, independent of mPanIN development, are capable of giving rise to PDAC. We have also observed mPanIN/ADM hybrid lesions, as reported by other studies, which have linked metaplasia with mPanIN development (18, 38). Taken together, these observations indicate that some lesions traditionally identified as mPanINs most likely developed from the preexisting metaplasias. The fact that we see more proliferation in ADMs than mPanINs (Fig. 4) could indicate that mPanINs may be a more quiescent, intermediate state between ADM and PDAC (Fig. 6). Alternatively, it is possible, as others have suggested (37), that mPanINs may represent a “dead-end” stage for highly proliferative precursor lesions that would have otherwise immediately progressed to PDAC. Given their earlier appearance and hyperproliferative features (Fig. 4), it is possible that most ADMs generated in this model due to Pten haploinsufficiency could develop directly into PDACs, bypassing the mPanIN stage. Interestingly, no evidence of P-AKT was detectable by immunohistochemistry in early metaplasias (Supplementary Fig. S3C). Likewise, our data showed that similar to mPanINs, ADMs showed no evidence of Pten LOH (data not shown). Further studies using isolated lesion populations will have to be performed to uncover the exact mechanism through which Pten haploinsufficiency leads to earlier ADM.

Regulation of cancer stem cells?

In our study, we also observed that the ADMs contained more CD44-positive cells than mPanINs and PanINs (Fig. 5A). Expression of CD44 has recently been used as a marker for isolating cancer stem cells in many tumors (39, 40), and its expression has been linked to poor prognosis and gemcitabine resistance in pancreatic cancer (41). Moreover, metaplastic, highly aggressive, chemoresistant tumors in the breast (42) have been shown to have an increase in CD44-positive cell populations as well as a high frequency of mutation, amplification, and activation of PI3K/AKT pathway components (43). It will be interesting to test whether (a) metaplasias contain stem cell–like properties, (b) PTEN loss leads to the enrichment of tumor-initiating/stem cells, and (c) CD44 upregulation found on lesion initiation may aid tumor survival through PI3K/AKT-dependent pathways. Previous work with mice with only Pten deficiency in the pancreas (9) showed that loss of PTEN function in Pdx1-Cre; Pten\(^{lox/}\) mice led to centroacinar cell expansion, which produced metaplasias similar to what is seen in Pdx1-Cre; Kras\(G^{12D}/;Pten^{lox/}\) mice. However, although both models exhibit metaplasia, the tumors in Pdx1-Cre;Pten\(^{lox/}\) mice are more papillary in nature, whereas Pdx1-Cre;Kras\(G^{12D}/;Pten^{lox/}\) tumors seem identical to Pdx1-Cre;Kras\(G^{12D}/\) mice. This could indicate that the phenotype in the Pdx1-Cre;Pten\(^{lox/}\) mice might reflect the consequence of Pten loss in the centroacinar cell compartment, whereas the phenotype in the Pdx1-Cre;Kras\(G^{12D}/;Pten^{lox/}\) mice mimics that of the human disease. This model provides a valuable resource for elucidating how loss of PTEN function aids tumor development and for determining whether targeting PI3K/AKT signaling is a viable therapeutic strategy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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