Pten is a Major Tumor Suppressor in Pancreatic Ductal Adenocarcinoma and Regulates an NF-κB–Cytokine Network
ABSTRACT
Initiation of pancreatic ductal adenocarcinoma (PDAC) is driven by oncogenic KRAS mutation, and disease progression is associated with frequent loss of tumor suppressors. In this study, human PDAC genome analyses revealed frequent deletion of the PTEN gene as well as loss of expression in primary tumor specimens. A potential role for PTEN as a hallmarks of tumor suppressor is further supported by mouse genetic studies. The mouse PDAC driven by oncogenic Kras mutation and Pten deficiency also sustains spontaneous extinction of Ink4a expression and shows metastatic capacity. Unbiased transcriptomic analyses established that combined oncogenic Kras and Pten loss promotes marked NF-κB activation and its cytokine network, with accompanying robust stromal activation and immune cell infiltration with known tumor-promoting properties. Thus, PTEN/phosphoinositide 3-kinase (PI3K) pathway alteration is a common event in PDAC development and functions in part to strongly activate the NF-κB network, which may serve to shape the PDAC tumor microenvironment.

SIGNIFICANCE: Detailed molecular genetics studies established that PTEN operates as a hallmark tumor suppressor to promote metastatic PDAC development. The strong activation of the NF-κB-cytokine program in Pten-deficient tumors provides additional avenues for targeted therapies in tumors with altered PI3K regulation. Cancer Discovery. 1(2); OF1-OF12. ©2011 AACR.

INTRODUCTION
Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the United States, with a dismal 5-year survival rate ranging between 3% and 5% (1). Clinically, >3% of PDAC patients present with advanced primary disease, often with metastases primarily to the liver and peritoneum (1). Pathologically, PDAC exhibits a predominantly ductal-like glandular pattern with varying degrees of differentiation that typically arise from precursor ductal lesions termed pancreatic intraepithelial neoplasia (PanIN; ref. 1). A hallmark pathologic feature of primary PDAC is desmoplasia characterized by the presence of an exuberant stroma consisting of fibroblasts and inflammatory cells (2). PDAC desmoplasia functions not only to promote tumor development (3) but also to impair cancer drug delivery by decreasing tumor vascular density and thereby contributing to poor therapeutic response (4).

The pathways and mechanisms shaping the PDAC tumor microenvironment and immune cell infiltration during tumor genesis and progression are not well understood, although paracrine signals involving Tgfβ1 and Hh have been shown to moderate aspects of the protumorigenic communication between tumor cells and surrounding stroma (5, 6).

The key genetic alteration driving PDAC genesis is activating KRAS mutations, found in >90% of cases, and it occurs as an early genetic event driving PanIN formation (7, 8). Although activation of KRAS (G12D) is the critical event driving PDAC genesis, its precise signaling surrogates and interactions with other signaling pathways remain an area of active investigation. In most tumor cell types, the classic signaling circuits emanating from activated KRAS(G12D) include RAF-mitogen–activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), and Ral GDS pathways. However, in human PDAC, mounting genomic evidence has indicated the presence of additional mutations targeting components of the PI3K pathway, suggesting that KRAS(G12D) activation of the PI3K pathway is under active repression. Alterations in the PI3K pathway include activating mutations in the catalytic PI3K subunit (PIK3CA), mutations in the regulatory PI3K subunit (PIK3R1/p85α), and amplification of the PI3K downstream effector (AKT2; refs. 9–11). Decreased PTEN expression and accompanying elevations in PI3K/AKT signaling have been observed in pancreatic tumor cell lines, although deletion or loss-of-function mutations targeting PTEN have not been detected with significant frequency in human PDAC (12) and is widely assumed to be a less relevant tumor suppressor in human PDAC. However, previous mouse studies showing that pancreatic deletion of Pten or expression of constitutive active Akt leads to expansion of central acinar cells, putative pancreatic progenitors, and formation of PDAC in a small percentage of mice (13, 14), supporting the view that PTEN serves as a
RESULTS

The frequent genomic alterations of PI3K pathway components in diverse tumor types prompted analysis of PTEN and AKT status in human PDAC. Tissue microarray (TMA) analysis of PTEN protein and measurement of AKT phosphorylation at S473 were performed in a collection of 54 human PDAC samples (Fig. 1A). Relative to surrounding stroma, we observed low or no PTEN expression in 70% of PDAC samples (38 of 54; Fig. 1Ai and ii), findings consistent with major tumor suppressor in murine, and possibly human, PDAC development.

In this study, using genetically engineered mouse models, we documented strong cooperative interactions of \( \text{Kras}^{G12D} \) and \( \text{Pten} \) loss in promoting metastatic PDAC. Furthermore, molecular analysis indicated that Pten-deficient tumors exhibit classic PDAC genetic lesions as well as robust activation of NF-κB and its downstream cytokine pathway, which is associated with inflammatory responses in the tumor microenvironment.

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<tr>
<th>Group</th>
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<tr>
<td>pAKT low</td>
<td>8</td>
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Figure 1. PTEN is deleted/downregulated in human pancreatic cancer, and Pten inactivation cooperates with Kras\(^{G12D}\) to induce PDAC. A, representative IHC images of PTEN and pAKT staining from the TMA analysis showing samples with low or no PTEN staining and moderate to high pAKT (i, ii) or high PTEN staining and low pAKT (iii). Statistical analysis of the TMA data is shown in the bottom panel.

- pAKT, phospho-AKT.
- B, aCGH heat map detailing patterns of PTEN deletion and AKT2 gain/amplification in primary PDAC tumor specimens. Regions of amplification and deletion are denoted in red and blue, respectively. The arrowhead indicates the sample with concomitant PTEN deletion and AKT2 amplification.
- C, Kaplan–Meier overall survival analysis for mice of indicated genotypes. Cohort size for each genotype is indicated.
- D, typical ductal adenocarcinoma observed in a Pax1-Cre \( LSL-\text{Kras}^{G12D} \) \( \text{Pten}^{+/+} \) animal showing well-differentiated glandular tumor cells (i) that are positive for the ductal marker, cytokeratin-19 (ii), and are negative for the acinar marker, amylase (iii), and the endocrine marker, insulin (iv). CK19, cytokeratin-19. Scale bars, 100 μm.
previous reports of absent PTEN expression in human PDAC (12, 15). Analysis of phospho-AKT revealed moderate to high AKT phosphorylation in 68.5% of samples (37 of 54), with most tumors exhibiting moderate levels of AKT activation (30 of 54; Fig. 1A). Statistical analysis revealed significant negative correlation between PTEN expression and AKT phosphorylation (P = 0.02, Fisher exact test; P = 0.03, χ² test; Fig. 1A). Correspondingly, high-resolution array comparative genomic hybridization (aCGH) analysis of 61 samples of epithelial-enriched primary tumor cells or primary xenografts from PDAC patient samples showed deletion of 1 or 2 copies of the PTEN locus in 9 of 61 (15%) of tumor samples (Fig. 1B). PTEN deletion events were typically associated with large regions of 10q chromosome loss, occasionally the whole chromosome arm—a pattern similar to that in other tumor types (16). In addition, the AKT2 locus was subject to gain/amplification in 12 of 61 (20%) samples (Fig. 1B), in line with a previous report (11). Although only 1 sample harbored both genetic events, PTEN deletion or AKT2 gain/amplification occurred in 32.8% of PDAC samples (20 of 61; Fig.1B). Together, these observations implicate aberrant activation of the PI3K pathway by either PTEN loss or AKT2 activation in a significant subset of human PDACs.

Although P13K is regarded as a major surrogate of KRAS signaling, Pdx1-Cre KrasG12D PanINs showed a notable lack of robust Akt activation (Supplementary Fig. S2Aiii). The above genomic data prompted us to hypothesize that PTEN may be actively suppressing the activation of the PI3K pathway in KrasG12D-driven lesions. To test this hypothesis, we examined the cooperative interaction of activated KrasG12D and Pten loss, using mice engineered with Pdx1-Cre LSL-KrasL/+ mice (L/+). In contrast to Pdx1-Cre LSL-KrasG12D mice, the Pdx1-Cre LSL-KrasG12D Pten−/− mice (n = 40) presented with highly invasive pancreatic tumors and a median survival of 17 weeks (Fig. 1C). End-stage histopathologic analysis revealed most mice (28 of 40) with adenocarcinoma exhibiting well-differentiated to moderately differentiated glandular structures that were CK19 positive and devoid of acinar (amylase) or endocrine (insulin) markers (Fig. 1D). These morphologic features are typical of human PDAC (20). In contrast, all 11 Pdx1-Cre LSL-KrasG12D Pten−/− mice presented with rapidly progressive acinar-to-duetal metaplasia (ADM) and PanIN formation and exuberant stromal reaction throughout the pancreas, with occasional invasive cancers (Supplementary Fig. S1A). None of these mice survived beyond 3 weeks, presumably as a result of severe pancreatic insufficiency. Consistent with a previous report (14), Pdx1-Cre Pten−/− mice progressively develop ADM, whereas the Pten-heterozygous pancreas is morphologically normal (Supplementary Fig. S2B and C), more profound stromal reaction (Supplementary Fig. S2Aii and v), increased epithelial proliferation (Supplementary Fig. S2Aiv and viii), and moderately elevated Akt phosphorylation (Supplementary Fig. S2Aii and vii). The lesions of both genotypes exhibited similarly strong ERK (MAPK1) activation (Supplementary Fig.S2Aii and vi). Together, these data suggest that, in the context of KrasG12D-initiated pancreatic neoplasms, Pten can repress PI3K signaling and constrain malignant progression in vivo.

One prominent and consistent feature of Pdx1-Cre KrasG12D Pten−/− ductal lesions was a pronounced stromal reaction with inflammatory cell infiltration comparable to that in human PDAC (1). When compared with Pdx1-Cre KrasG12D littersmates, 6-week-old Pdx1-Cre KrasG12D Pten−/− animals already exhibited more exuberant reactive stroma surrounding the metaplasia and PanIN lesions that was highly enriched with smooth muscle actin (SMA)–positive pancreatic stellate cells and S100A4-positive fibroblasts (Fig. 2Ai–iii and vi–viii). In addition, fluorescence-activated cell sorting (FACS) analysis of Pdx1-Cre LSL-KrasG12D Pten−/− pancreata showed dramatically increased CD45+ leukocyte infiltration compared with that in wild-type or Pdx1-Cre KrasG12D pancreata (Supplementary Fig. S3). Specifically, in the Pdx1-Cre LSL-KrasG12D Pten−/− pancreata, CD11b+Gr-1+ myeloid cells accounted for the majority of the inflammatory cell infiltration, especially Gr-1–low myeloid-derived suppressor cells (Fig. 2B), and significant increases in CD25+Foxp3+ regulatory T cells (Treg) were also documented (Fig. 2B). These FACS results aligned with immunohistochemical (IHC) analysis showing obvious myeloid and Treg infiltration surrounding metaplastic and PanIN lesions in Pdx1-Cre LSL-KrasG12D Pten−/− specimens (Fig. 2Aiv, v, ix, and x).

Pdx1-Cre LSL-KrasG12D Pten−/− tumors also showed extensive invasion of adjacent organs (Fig. 2C). Invasion and metastases into the lymphatic system were also frequently detected (Fig. 2Ciii), and multifocal CK19-positive macro- or micro-metastases to liver were observed in 12 of 35 (34%) of tumor-bearing animals with lesions resembling primary tumors (Fig. 2Civ and v). In contrast, microscopic lung metastasis was rare (1 of 35; Fig. 2Cvi). Overall, the distribution pattern of liver and regional lymph node metastases is similar to that observed in the human disease (1).

Given the capacity of PTEN to operate as a haploinsufficient tumor suppressor in other settings (21–23), we next examined the status of the remaining wild-type allele of Pten in the Pdx1-Cre LSL-KrasG12D Pten−/− tumors. Early-passage cell lines derived from the murine PDACs were used to avoid the presence of contaminating normal cells. Allele-specific PCR genotyping revealed that 3 of 5 tumors retained the wild-type Pten allele as well as Pten protein expression (Fig. 3A and B; Supplementary Fig. S4A), and sequence analysis of the reverse transcriptase PCR (RT-PCR)–generated open reading frame (ORF) revealed wild-type Pten sequences (Supplementary Table S1).

Beyond PTEN analysis, we also audited the status of other key PDAC tumor suppressor genes: Ink4a/Arf, p53, and Smad4. Analysis of the Ink4a/Arf locus revealed loss of p16INK4a and p19INK4B expression in all Pdx1-Cre LSL-KrasG12D Pten−/− PDAC cells derived from end-stage tumors between 11 and 24 weeks of age (n = 5) relative to expression in premalignant pancreatic ductal epithelial cells (PDEC) derived from 6-week-old Pdx1-Cre KrasG12D Pten−/− or Pdx1-Cre KrasG12D mice (Fig. 3C). The loss of p16INK4a expression resulted from gene deletion (1 of 5;
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Figure 2. Pten deficiency induces strong stromal reaction with immune cell infiltration and promotes the development of invasive and metastatic pancreatic tumors in cooperation with Kras<sup>G12D</sup>. A, six-week-old pancreata from Kras<sup>G12D</sup> (i–iv) and Kras<sup>G12D</sup> Pten<sup>L/+</sup> (v–viii) stained with H&E (i, vi), or with antibodies to SMA (ii, vii), S100A4 (iii, viii), Gr-1 (iv, ix), and FoxP3 (v, x). B, left, analysis of myeloid cells. Bar graph showing percentage of CD4<sup>+</sup>CD11b<sup>+</sup>Gr-1<sup>-low</sup> (white) and Gr-1<sup>+</sup> (black) cells in total live cells of wild-type (n = 3), Kras<sup>G12D</sup> (n = 3), and Kras<sup>G12D</sup> Pten<sup>L/-</sup> (n = 3) pancreata. Right, Treg analysis. Percentage of CD25<sup>+</sup>FoxP3<sup>+</sup> cells within CD4<sup>+</sup> T cells in pancreas of wild-type (n = 2), Kras<sup>G12D</sup> (n = 2) and Kras<sup>G12D</sup> Pten<sup>L/+</sup> (n = 2) mice.*, P < 0.05; **, P < 0.01. C, tumor invasion and metastases. Gross picture shows liver metastases (i). H&E staining shows local invasion into duodenum wall (ii), lymph node metastasis (iii), liver metastasis (iv), and microscopic lung metastasis (v). Liver metastasis is stained with cytokeratin-19 antibody (v). Scale bars, 100 μm.

Supplementary Fig. S4B, lane 2) or methylation-associated epigenetic silencing (4 of 5) of the p16<sup>INK4a</sup> promoter region (Supplementary Fig. S4B, lanes 1, 3–5). Examination of the p53 tumor suppressor revealed physical and functional integrity, as evidenced by strong induction of p53 and its downstream target p21<sup>CDSP</sup> upon doxorubicin treatment of 5 Pdx1-Cre Kras<sup>G12D</sup> Pten<sup>L/+</sup> tumor cell cultures (Fig. 3D). All 5 samples tested retained Smad4 expression, and sequence analysis of RT-PCR–generated Smad4 ORF confirmed the wild-type Smad4 status (Fig. 3C; Supplementary Table S1).

Thus, in this model, Pten can function as a haploinsufficient tumor suppressor and extinction of Ink4a/Arf remains a critical cooperative event, as classically observed in the human disease (2).

To understand the molecular basis of how oncogenic Kras<sup>G12D</sup> and Pten deficiency might cooperate to promote the aforementioned tumor biological features, we performed an unbiased transcriptomic profiling of Pdx1-Cre Kras<sup>G12D</sup> PDECs versus Pdx1-Cre Kras<sup>G12D</sup> Pten<sup>L/+</sup> PDECs derived from respective 6-week-old animals. We used primary PDECs, as opposed to tumor cells, to better discern the proximal molecular actions of Kras<sup>G12D</sup> and Pten loss on premalignant epithelium. The differentially expressed genes (215 genes; fold change ≥1.3; P < 0.05) were further analyzed for enrichment of specific binding elements to discern key regulators driving these changes, as reported previously (18). These <i>in silico</i> promoter analyses showed significant enrichment of NF-κB binding elements (Supplementary Table S2, <i>P</i> = 0.03). The observations gained added support in light of NF-κB induction by an activated PI3K/AKT pathway (24, 25). Accordingly, Pdx1-Cre Kras<sup>G12D</sup> Pten<sup>L/+</sup> PDECs exhibited increased nuclear accumulation of NF-κB transcription factor subunit p65 (also known as RelA; Fig. 4A) and higher NF-κB transactivation activity (Fig. 4B) relative to Pdx1-Cre Kras<sup>G12D</sup> controls. In addition, IHC analysis confirmed markedly elevated levels of phosphorylated nuclear NF-κB in Pdx1-Cre Kras<sup>G12D</sup> Pten<sup>L/+</sup> PanINs relative to those morphologically similar lesions in Pdx1-Cre Kras<sup>G12D</sup> tissue (Fig. 4C), suggesting that activation of NF-κB is not likely the consequence of a difference in lesion size between the 2 genotypes. Of interest, although Pdx-Cre Pten<sup>L/L</sup> mice develop progressive ADM, no significant nuclear accumulation of NF-κB was observed in the ductal lesions (Supplementary Fig. S5).
indicating that both Kras mutation and Pten deficiency are required for activation of the NF-κB pathway.

On the functional level, treatment of Pdx1-Cre Kras<sup>G12D</sup> Pten<sup>−/+</sup> PDECs with a PI3K inhibitor, LY294002 (LY), attenuated nuclear RelA levels and NF-κB transactivation activity (Fig. 4A and B). Conversely, short hairpin RNA (shRNA)-mediated knockdown of Pten in Kras<sup>G12D</sup> PDECs induced Akt phosphorylation and NF-κB nuclear accumulation (Fig. 4D), indicating that NF-κB activation is PI3K dependent. The IKK pathway plays essential roles during NF-κB activation (26). Moreover, IκBα mutant expression decreased nuclear IκBα/β levels and abolished cytokine and chemokine expression in numerous cell types, including epithelial cells (29), multiple cytokines and chemokines—including IL-6, and IL-23) showed significant upregulation in human PDAC. To that end, we analyzed publicly available expression profiles (30) in Oncomine and documented that 4 of the 5 cytokines (CCL20, CXCL1, IL-6, and IL-23) showed significant upregulation in human PDAC compared with normal pancreas and that PTEN expression was significantly downregulated in tumor samples

that PI3K/Pten-dependent NF-κB pathway activation. NF-κB activation was associated with induction of various cytokines and chemokines, which may contribute to the prominent inflammatory cell infiltrates in the Pdx1-Cre KrasG12D PtenL/+ tumors. In addition, suppression of the NF-κB pathway inhibited Pdx1-Cre KrasG12D PtenL/+ tumor growth in vivo, indicating an important role in tumor maintenance and hence a potential therapeutic target in tumors with altered PI3K regulation.

The lack of Akt activation in KrasG12D PanIN and metaplastic lesions implied active suppression of the PI3K signaling axis. The activity of the PI3K pathway is constantly regulated on many levels involving multiple nodes in feedback loops. This circuitry is in part reflected by coexisting alterations targeting multiple PI3K pathway components in the course of tumor progression, such as the occurrence of KRAS and PTEN mutations in colorectal cancers (32). These observations may align with a recent report that p85α, the regulatory subunit of PI3K, directly interacts with PTEN and enhances its phosphatase activity (33). In this context, it is possible that p85α induction of Pten activity in the presence of mutant Kras keeps PI3K signaling in check and that disruption of such regulation by genetic loss or reduction of Pten enables KrasG12D to drive malignant progression of PDAC. Similarly, “reinforcing” events in PDAC, such as mutations

**Figure 4.** Pten deficiency activates the NF-κB pathway. A, cytoplasmic (C) or nuclear (N) extract was prepared from KrasG12D (lanes 1–4) or KrasG12D PtenL/+ (lanes 5–12) PDECs and blotted for NF-κB p65. Lamin A/C and caspase 3 were used as markers for nuclear and cytoplasmic fraction, respectively. KrasG12D PtenL/+ PDECs were also treated with either vehicle (Ctr) or LY, 20 μM, for 24 hours before collection. B, KrasG12D or KrasG12D PtenL/+ PDECs were treated with either vehicle (Ctr) or LY, 20 μM, for 24 hours before being collected for luciferase activity assay. *, P < 0.05; **, P < 0.01. C, six-week-old pancreata from KrasG12D (top) and KrasG12D PtenL/+ (bottom) mice were stained with phospho–NF-κB p65 (pRelA). Scale bars, 100 μm. D, KrasG12D PDECs were infected with pSuper (Ctr) or pSuper-shPten (shPten) and blotted for Pten, pAkt, and Akt (lanes 1–4). Cytoplasmic (C) or nuclear (N) extract was blotted for NF-κB p65 (lanes 5–12).

**DISCUSSION**

In this study, high-resolution analysis of human PDAC genomes has uncovered frequent loss of at least 1 copy of the PTEN gene. In mouse genetic studies, combined genomic, genetic, and functional studies establish a key role for the Pten tumor suppressor in the progression of Kras-initiated PDAC development in the mouse. Although recent studies have shown Kras and Pten cooperation in the development of mouse PDAC (31), our study is distinguished in showing that Pten can function as a haploinsufficient tumor suppressor, aligning well with human genomic profiles, as evidenced by retention of Pten heterozygosity in the majority of mouse tumors.

Further characterization also established frequent liver metastases. In both mouse and human PDAC, genome scale analysis and confirmatory biochemical studies reveal (Supplementary Fig. S8). Notably, with the exception of CSF3, the upregulated expression of the 4 cytokines showed strong negative correlation with PTEN level (Supplementary Fig. S8). These studies suggest that functional genetic interactions between PTEN and the NF-κB–cytokine network appear operative in both mouse and human PDAC, although we acknowledge that multiple genetic mechanisms beyond NF-κB may regulate cytokine expression in human PDAC.
Pten is a Haploinsufficient Tumor Suppressor for PDAC

**Figure 5.** Suppression of NF-κB inhibits tumorigenic activity. **A**, PDAC from KrasG12D PtenL/+ animals was stained for phospho–NF-κB p65 (pRelA) Scale bar, 100 μm. **B**, colony formation assay for KrasG12D PtenL/+ PDAC cell lines infected with pBabe (Vec) or pBabe-IκBαM. Quantification of colony numbers was shown on the right. **C**, KrasG12D PtenL/+ PDAC cell lines were infected with pBabe (Vec) or pBabe-IκBαM and injected s.c. into nude mice with Vec in the left flank and IκBαM in the right flank, respectively. Tumor volumes were measured in **D**. *P < 0.05; **P < 0.01.

**Figure 6.** Pten deficiency induces cytokine expression. **A**, expression of indicated cytokines and chemokines was measured by quantitative PCR (qPCR) in KrasG12D (n = 3) or KrasG12D PtenL/+ (n = 5) PDECs. **B**, expression of indicated cytokines and chemokines was measured by qPCR in KrasG12D PtenL/+ PDECs infected with pBabe (Vec) or pBabe-IκBαM. **C**, expression of indicated cytokines and chemokines was measured by qPCR in KrasG12D PtenL/+ PDECs treated with vehicle (Ctr) or LY-LY, 20 μM, for 24 hours. **D**, expression of indicated cytokines and chemokines was measured by qPCR in KrasG12D PDECs infected with pSuper (Ctr) or pSuper-shPten (shPten). *P < 0.05; **P < 0.01.
of p85α or PIK3CA or amplification of AKT2, would be expected to disrupt negative regulation to ensure the full activation of PI3K signaling in tumorigenesis. The genetic and biological observations of our study justify the need for a more comprehensive mutational analysis of these components in a large human PDAC sample set and assessment of these profiles with modulation of PI3K signaling and NF-κB network activation.

The NF-κB pathway is constitutively active across many different types of tumors, including pancreatic cancer (2, 34). NF-κB activation in RAS-transformed cells is mediated through both MAPK and PI3K pathways (35). In agreement, our data showed that Pten deficiency cooperates with KrasG12D to induce NF-κB activity through the PI3K pathway. Similarly, NF-κB activity is found to be upregulated by an elevated PI3K/Akt pathway in several human PDAC cell lines and is important for the viability of the tumor cells (12). Of interest, PTEN expression itself is suppressed by the NF-κB pathway (36). Therefore, PI3K activation via Pten loss and NF-κB may form a powerful positive feedback loop downstream of oncogenic Kras during PDAC development. It was recently shown that loss of p53 also cooperates with KrasG12D to activate the NF-κB pathway in lung adenocarcinoma (37), suggesting that additional mechanisms may contribute to NF-κB activation in tumors driven by oncogenic Kras. Our data that p53 remains intact in Pdx1-Cre KrasG12D Pten−/− tumor cells and p19ARF expression is maintained in premalignant ductal cells (Fig. 3C and D) suggest that the p53 axis is not prominently involved in activation of the NF-κB pathway in Pten-deficient PDECs. At the same time, p53 inactivation through loss of p19ARF expression in PDAC cells may further contribute to NF-κB activation in more advanced invasive tumors.

Many in vivo studies have established a central role of NF-κB during tumor development (38). Selective inactivation of IKKβ in either intestinal epithelial cells or myeloid cells suppressed tumor formation in inflammation-induced colorectal carcinoma model, indicating that the NF-κB pathway functions in both a cell-autonomous and non–cell-autonomous fashion (39). Suppression of NF-κB by a dominant-negative IkBa mutant inhibits the tumorigenic activity of Pdx1-Cre KrasG12D Pten−/− PDAC cells and suggests that the NF-κB pathway may function in a cell-autonomous way in established tumors. Meanwhile, our data showing that the NF-κB pathway is activated in Pdx1-Cre KrasG12D Pten−/− PDECs to induce various cytokines and chemokines reveal that NF-κB may also function through paracrine mechanisms during PDAC development to modulate the interaction between tumor cells and their microenvironment, a supposition that will require further study in a defined genetic model.

An anatomic and functional component of cancer, the composition of the tumor microenvironment shows many characteristics observed in chronic inflammation (40). A recent animal study has indicated that induction of chronic pancreatitis dramatically accelerated KrasG12D-induced pancreatic cancer formation, pointing to the importance of a permissive environment during Kras-mediated PDAC development (41). However, the underlying mechanisms that orchestrate cooperation between tumor cells and their inflammatory milieu are less understood. In this article, we provided additional evidence that the PTEN/PI3K pathway may cooperate with endogenous KrasG12D to induce the expression of various cytokines and chemokines in premalignant epithelial cells in NF-κB–dependent manner. Among the induced cytokines, IL-23 and Ccl20 were particularly noteworthy, given their well-established roles as chemotactants for lymphocyte subsets, including Tregs (42, 43); furthermore, Cxcl1 and Csf3 are integral in the chemotaxis of granulocytes and macrophages to promote tumor progression (44). Such cytokine induction in KrasG12D Pten−/− ductal epithelial cells supported the hypothesis that PI3K activation in combination with oncogenic Kras in the epithelial compartment may facilitate the recruitment of various protumor cell types within tumor stroma. Not only may the highly enriched cytokines and chemokines in the tumor microenvironment suppress antitumor immunity, but also the infiltrating inflammatory cells, as well as other stromal cells, produce various growth factors to further amplify the PI3K pathway output in Pten-deficient cells and support tumor growth. An inbred PDAC model will be critical to further clarify such tumor microenvironment communication.

Tumors harboring oncogenic Kras mutations are often associated with therapeutic resistance and poor prognosis, and mutant Kras is, until now, still regarded as an “undruggable” target (45). Notably, using a Kras mutant animal lung cancer model, a recent in vivo study showed that the combination of a dual PI3K/mTOR inhibitor and a MAP/ERK kinase (MEK) inhibitor induced drastic tumor shrinkage, indicating that targeting Kras surrogates is a feasible therapeutic strategy (46). The strong cooperation between Pten deficiency and oncogenic Kras during PDAC development, along with activation of the NF-κB pathway within such a context, further endorses clinical trials for combined therapies with MEK inhibitors and PI3K or NF-κB inhibitors. Alternatively, directly targeting the cytokine networks may diminish immune sequestration in PDAC and enhance immunotherapeutic strategies.

**METHODS**

**Primary Tumors, Tumor Cell Enrichment, and aCGH Profiling**

TMA samples of human PDAC were obtained from Brigham and Women’s Hospital Department of Pathology tumor bank (Boston, Massachusetts). For aCGH profiling, fresh-frozen specimens of primary PDAC were obtained from Massachusetts General Hospital Center for Pancreatic Research tumor bank (Boston, Massachusetts). To overcome 2 problems typical for biorepositories—namely, low tumor cellularity and insufficient size of a specimen to perform genomics studies—the following 2-step protocol has been developed. First, samples were enriched for epithelial content, using EpCAM-coated Dynabeads (Invitrogen). About 10-mm² chunks of frozen tissue embedded with OCT were disintegrated by Medimachine (BD Biosciences) using 50-μm Medicon (BD Biosciences) and syringe-type 50-μm Filcon (BD Biosciences) in the buffer containing PBS, 1% fetal calf serum, 0.8% sodium citrate, and 10 mM EGTA. Bead bounding and cell isolation were performed according to the Dynabeads Epithelial Enrich protocol (Invitrogen). The purity of the enriched epithelial cells was >80%. The yield of epithelial cells from frozen tissue corresponded to approximately 40% of the yield from fresh tissue according to our pilot experiments (A. Protopopov, unpublished observations). Immediately after the enrichment, cells were subjected to DNA isolation using QIaAmp DNA Micro Kit (Qiagen). Next, DNA
was treated according to GenomePlex whole-genome amplification technology (Sigma-Aldrich). aCGH profiling was performed as described (11, 47) on the Agilent Human CGH 244K platform, with the following modification: 2 μg of whole-genome amplification product per hybridization was labeled chemically.

**Mice**

Pdx1-Cre, KrasG12D and Pten- mice have been described previously (17–19). Mice were interbred and maintained on a FvB/C57Bl6 hybrid background in pathogen-free conditions at Dana-Farber Cancer Institute (Boston, Massachusetts). Mice were injected i.p. with BrdU at 60 mg/kg every 12 hours for 48 hours before necropsy.

Cancer Institute (Boston, Massachusetts). Mice were injected i.p. with BrdU at 60 mg/kg every 12 hours for 48 hours before necropsy. All manipulations were performed with Institutional Animal Care and Use Committee (IACUC) approval.

**Xenograft Studies**

For xenografts, 1 × 105 cells suspended in 100 μL of Hanks buffered saline solution were injected s.c. into the lower flank of NCr nude mice (Tacomic). Tumor volumes were measured every 3 days, starting from day 4 postinjection. All xenograft experiments were approved by the IACUC.

**IHC and Western Blot Analysis**

Tissues were fixed in 10% formalin overnight and embedded in paraffin. IHC analysis was performed as described (48). The visualization of primary antibodies was accomplished with the horserodish peroxidase system (Vectorstain ABC Kit; Vector). The primary antibodies used for IHC were as follows: cytokeratin 19 (TROMA-III; DSHB), amylose (AB273; Sigma-Aldrich), insulin (A0564; Dako), SMA (NB600-531; Novus), Gr-1 (550291; BD Biosciences), FoxP3 (ab54501; Abcam), caspase-3 (9665; Cell Signaling), IKKαNF-κB p65 (ab7970; Abcam), lamin A/C (2032; Cell Signaling), p19 Arf (sc-1615; Santa Cruz Biotechnology), p16Ink4a (sc-1207; Santa Cruz Biotechnology), Smad4 (sc-7966; Santa Cruz Biotechnology), Pten (9559; Cell Signaling), BrdU (ab1293; Abcam), Pdx1-Cre, KrasG12D and Pten+ mice have been described previously (17–19).

**Expression Profiling and Bioinformatics Analysis**

mRNA expression profiling was performed at the Dana-Farber Microarray Core facility, using the Mouse Genome 430 2.0 Array (Affymetrix). A Student t test was used to identify differentially expressed genes according to expression profiles. Promoter analysis was performed as described (18). Complete profiles are deposited on the Gene Expression Omnibus website under superseries accession number GSE25828. Oncomine (Compendia Bioscience, Ann Arbor, MI) was used for analysis of the human PDAC expression profile. Gene expression correlation was calculated with a Pearson correlation analysis.

**Statistical Analysis**

Tumor-free survivals were analyzed by Graphpad Prism 4. The nonparametric Mann–Whitney test was used for statistical analyses. Significance of enrichment in the promoter analysis was computed based on Poisson distribution with Bonferroni correction. Correlation analysis was performed with the Fisher exact test and the χ2 test. Other comparisons were performed using the unpaired Student t test. For all experiments with error bars, the standard deviation was calculated to indicate the variation within each experiment and data; values represent mean ± SD.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Haoqiang Ying, Kutlu G. Elpek, Anant Vinjamoori, et al.

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