Pten is a major tumor suppressor in pancreatic ductal adenocarcinoma and regulates an NF-κB-cytokine network

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) initiation 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 haploinsufficient 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 pro-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/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 haploinsufficient tumor suppressor to promote metastatic PDAC development. The strong activation of NF-κB-cytokine program in Pten deficient tumors provides additional avenues for targeted therapies in tumors with altered PI3K regulation.
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%–5% (1). Clinically, over 30% of PDAC patients present with advanced primary disease, often with metastases primarily to 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) (1). A hallmark feature of primary PDAC pathology 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 impairs cancer drug delivery by decreasing tumor vascular density and thereby contributing to poor therapeutic response (4). A clear understanding of 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 mediate aspects of the pro-tumorigenic communication between tumor cells and surrounding stroma (5-6).

The key genetic alteration driving PDAC genesis is activating KRAS mutations found in greater than 90% of cases and occurs as an early genetic event driving PanIN formation (7-8). While activated 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 classical signaling circuits emanating from activated KRAS$^{G12D}$ include RAF-mitogen-activated 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 (PI3KCA), mutations in the regulatory PI3K subunit (PIK3R1/p85), and amplification of the PI3K downstream effector (AKT2) (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 major tumor suppressor in murine, and possibly human, PDAC development.

In this study, by using genetically engineered mouse models, we documented strong cooperative interactions of Kras$^{G12D}$ and 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 tumor microenvironment.

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 AKT phosphorylation at S473 were measured in a collection of 54 human PDAC samples (Fig.1A). Relative to surrounding stroma, we observed low/no PTEN expression in 70% of PDAC samples (38/54) (Fig.1A-i, ii), findings consistent with 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 (37/54), with most tumors exhibiting moderate levels of AKT activation (30/54) (Fig1A-i). Statistical analysis revealed significant negative correlation between PTEN expression and AKT phosphorylation (p=0.02, Fisher exact test; p=0.03, X² test) (Fig.1A). Correspondingly, high-resolution aCGH analysis of 61 samples of epithelial-enriched primary tumor cells or primary xenografts from PDAC patient samples showed deletion of one or two copies of the PTEN locus in 9/61 (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 those in other tumor types (16). In addition, the AKT2 locus was subject to gain/amplification in 12/61 (20%) samples (Fig. 1B), in line with a previous report (11). While only one sample harbored both genetic events, PTEN deletion or AKT2 gain/amplification occurred in 32.8% of PDAC samples (20/61) (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.

While PI3K is regarded as a major surrogate of KRAS signaling, Pdx1-Cre KrasG12D PanINs showed a notable lack of robust Akt activation (Fig.S2A-iii).
genomic data prompted us to hypothesize that PTEN may be actively suppressing the activation of PI3K pathway in Kras$^{G12D}$ driven lesions. To test this hypothesis, we examined the cooperative interaction of activated Kras$^{G12D}$ and Pten loss using mice engineered with LSL-Kras$^{G12D}$, Pten$^l$, and pancreatic-specific Pdx1-Cre alleles (17-19). In contrast to Pdx1-Cre LSL-Kras$^{G12D}$ mice, the Pdx1-Cre LSL-Kras$^{G12D}$ Pten$^{L/+}$ mice ($n=40$) presented with highly invasive pancreatic tumors and a median survival of 17 weeks (Fig.1C). End-stage histopathological analysis revealed most mice (28/40) with adenocarcinoma exhibiting well-to-moderately differentiated glandular structures which were CK19 positive and devoid of acinar (Amylase) or endocrine (Insulin) markers (Fig.1D). These morphological features are typical of human PDAC (20). In contrast, all 11 Pdx1-Cre LSL-Kras$^{G12D}$ Pten$^{L/L}$ mice presented with rapidly progressive acinar-to-ductal metaplasia (ADM) and PanIN formation and exuberant stroma reaction throughout the pancreas with occasional invasive cancers (Fig.S1A). None of these mice survived beyond 3 weeks presumably as a result of severe pancreatic insufficiency. Consistent with previous report (14), Pdx1-Cre Pten$^{L/L}$ mice progressively develop ADM while Pten heterozygous pancreas is morphologically normal (Fig. S1B).

To examine the impact of single copy Pten loss on evolving pancreatic tumors in the Kras$^{G12D}$ model, we conducted a detailed serial comparative analysis of Pdx1-Cre LSL-Kras$^{G12D}$ and Pdx1-Cre LSL-Kras$^{G12D}$ Pten$^{L/+}$ littermates at 4, 6, 8, and 10 weeks of age. Relative to the Pdx1-Cre Kras$^{G12D}$ model, the phenotype of the Pdx1-Cre Kras$^{G12D}$ Pten$^{L/+}$ pancreas was far more aggressive with significant increase in the number and size of ADM and PanIN lesions ($n=5$) (Fig.S2B&C), more profound stromal reaction (Fig.S2A-i&v), increased epithelial proliferation (Fig.S2A-iv&viii), and moderately
elevated Akt phosphorylation (Fig.S2A-iii&vii). The lesions of both genotypes exhibited similarly strong ERK (Mapk1) activation (Fig.S2A-ii&vi). Together, these data suggest that, in the context of Kras\textsuperscript{G12D}-initiated pancreatic neoplasms, Pten can repress PI3K signaling and constrain malignant progression in vivo.

One prominent and consistent feature of \textit{Pdx1-Cre Kras}\textsuperscript{G12D} \textit{Pten}\textsuperscript{L/+} ductal lesions was a pronounced stromal reaction with inflammatory cell infiltration comparable to that of human PDAC (1). When compared to \textit{Pdx1-Cre Kras}\textsuperscript{G12D} littermates, 6 week old \textit{Pdx1-Cre Kras}\textsuperscript{G12D} \textit{Pten}\textsuperscript{L/+} animals already exhibited more exuberant reactive stroma surrounding the metaplasia and PanIN lesions which was highly enriched with SMA-positive pancreatic stellate cells and S100A4-positive fibroblasts (Fig.2A-i,ii,iii,vi,vii,viii). In addition, FACS analysis of \textit{Pdx1-Cre Kras}\textsuperscript{G12D} \textit{Pten}\textsuperscript{L/+} pancreata showed dramatically increased CD45+ leukocyte infiltration compared to wild-type or \textit{Pdx1-Cre Kras}\textsuperscript{G12D} pancreata (Fig.S3). Specifically, in the \textit{Pdx1-Cre Kras}\textsuperscript{G12D} \textit{Pten}\textsuperscript{L/+} pancreata, CD11b+Gr-1+ myeloid cells accounted for the majority of the inflammatory cell infiltration, especially Gr-1-low myeloid derived suppressor cells (MDSCs) (Fig.2B), and significant increases in CD25+Foxp3+ regulatory T cells (Tregs) were also documented (Fig.2C). These FACS results aligned with IHC analysis showing obvious myeloid and Treg cell infiltration surrounding metaplastic and PanIN lesions in \textit{Pdx1-Cre Kras}\textsuperscript{G12D} \textit{Pten}\textsuperscript{L/+} specimens (Fig.2A-iv,v,ix,x).

\textit{Pdx1-Cre LSL-Kras}\textsuperscript{G12D} \textit{Pten}\textsuperscript{L/+} tumors also showed extensive invasion of adjacent organs (Fig.2D). Invasion and metastases into the lymphatic system were also frequently detected (Fig.2D-iii) and multifocal CK19-positive macro- or micro-metastases to liver were observed in 12/35 (34%) of tumor-bearing animals with
pathology resembling primary tumors (Fig.2D-iv&v). In contrast, microscopic lung metastasis was rare (1/35) (Fig.2D-vi). 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 PtenL/+ tumors. Early passage cell lines derived from the murine PDACs were used in order to avoid the presence of contaminating normal cells. Allele-specific PCR genotyping revealed that 3/5 tumors retained the wild-type Pten allele as well as Pten protein expression (Fig.3A&B, Fig.S4A), and sequence analysis of the RT-PCR-generated open reading frame (ORF) revealed wild-type Pten sequences (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 p19Arf expression in all Pdx1-Cre KrasG12D PtenL/+ PDAC cells derived from end stage tumors between 11-24 weeks of age (n=5) relative to expression in premalignant pancreatic ductal epithelial cells (PDECs) derived from 6 weeks old Pdx1-Cre KrasG12D PtenL/+ or Pdx1-Cre KrasG12D mice (Fig.3C). The loss of p16Ink4a expression resulted from gene deletion (1/5) (Fig.S4B, lane 2) or methylation-associated epigenetic silencing (4/5) of the p16Ink4a promoter region (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 p21CIP1 upon doxorubicin treatment of 5 Pdx1-Cre KrasG12D PtenL/+ 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; 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 KrasG12D and Pten deficiency might cooperate to promote the aforementioned tumor biological features, we performed an unbiased transcriptomic profiling of Pdx1-Cre KrasG12D PDECs versus Pdx1-Cre KrasG12D PtenL/+ PDECs derived from respective 6 week old animals. We employed primary PDECs, as opposed to tumor cells, to better discern the proximal molecular actions of KrasG12D 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 in silico promoter analyses showed significant enrichment of NF-κB binding elements (Table S2, p=0.03). These observations gain added support in light of NF-κB induction by activated PI3K/AKT pathway (24-25).

Accordingly, Pdx1-Cre KrasG12D PtenL/+ 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 KrasG12D controls. In addition, IHC analysis confirmed markedly elevated levels of phosphorylated nuclear NF-κB in Pdx1-Cre KrasG12D PtenL/+ PanINs relative to those morphologically similar lesions in Pdx1-Cre KrasG12D tissue (Fig.4C), suggesting the activation of NF-κB is unlikely the consequence of different lesion sizes between the two genotypes. Interestingly, although Pdx-Cre PtenL/L mice develop progressive acinar-to-ductal
metaplasia, no significant nuclear accumulation of NF-κB was observed in the ductal lesions (Fig.S5) indicating that both Kras mutation and Pten deficiency are required for activation of NF-κB pathway.

On the functional level, treatment of Pdx1-Cre Kras\textsuperscript{G12D} Pten\textsuperscript{L/+} PDECs with PI3K inhibitor, LY294002 (LY), attenuated nuclear RelA levels and NF-κB transactivation activity (Fig.4A&B) and, conversely, shRNA-mediated knockdown of Pten in Kras\textsuperscript{G12D} PDECs induced Akt phosphorylation and NF-κB nuclear accumulation (Fig.4D), indicating NF-κB activation is PI3K-dependent. IKK pathway plays essential roles during NF-κB activation (26). However, no obvious induction of IKKα/β activity was observed in Pdx1-Cre Kras\textsuperscript{G12D} Pten\textsuperscript{L/+} PDECs compared to Pdx1-Cre Kras\textsuperscript{G12D} cells and LY treatment exerted no inhibitory effect on IKKα/β activation as well as the downstream IκBα phosphorylation (Fig.S6). Therefore, our data supports the notion that PI3K-mediated NF-κB nuclear accumulation and activation is likely IKK-independent (24-25, 27). Finally, tumors from Pdx1-Cre Kras\textsuperscript{G12D} Pten\textsuperscript{L/+} mice also showed strong nuclear phospho-RelA signal (Fig.5A). To address the functional relevance of NF-κB activation in these established PDAC tumor cells, we assess tumorigenic activity following introduction of a well-known non-phosphorylatable, dominant-negative mutant of IκBα (IκBα\textsuperscript{M}) (28). As shown in Fig.5B, IκBα\textsuperscript{M} inhibited clonogenic activity of Pdx1-Cre Kras\textsuperscript{G12D} Pten\textsuperscript{L/+} PDAC lines in vitro, indicating cell-autonomous function of NF-κB activation. In addition, suppression of NF-κB also dramatically attenuated tumor xenograft growth in vivo (Fig.5C&D). Together, these data support the view that NF-κB
activation plays an important contributory role in the cooperative actions of Kras\textsuperscript{G12D} activation and Pten deficiency in PDAC development.

Consistent with NF-\kappa B control of cytokine and chemokine expression in numerous cell types including epithelial cells (29), multiple cytokines and chemokines including \textit{Il6}, \textit{Il23}, \textit{Cxcl1}, \textit{Ccl20}, and \textit{Csf3} were found to be significantly up-regulated in \textit{Pdx1-Cre Kras}\textsuperscript{G12D} \textit{Pten}\textsuperscript{L/+} PDECs and pancreata relative to controls (Fig.6A, Fig.7A). Moreover, \textit{I\kappa B\alpha} mutant expression decreased nuclear NF-\kappa B p65 levels and abolished cytokine and chemokine expression in the \textit{Pdx1-Cre Kras}\textsuperscript{G12D} \textit{Pten}\textsuperscript{L/+} PDECs (Fig.6B, Fig.7B). Consistent with PI3K-directed activation of the NF-\kappa B pathway, PI3K inhibitor, \textit{LY294002}, decreased cytokine and chemokine levels in \textit{Pdx1-Cre Kras}\textsuperscript{G12D} \textit{Pten}\textsuperscript{L/+} PDECs (Fig.6C). Conversely, shRNA-mediated Pten knockdown induced cytokine and chemokine levels in \textit{Pdx1-Cre Kras}\textsuperscript{G12D} PDECs (Fig.6D).

These murine observations prompted analysis of the relationship of PTEN status and cytokine and chemokine expression in human PDAC. To that end, we analyzed publically available expression profiles (30) in Oncomine\textsuperscript{TM} and, documented that 4 of the 5 cytokines (CCL20, CXCL1, IL6, IL23) showed significant upregulation in human PDAC compared to normal pancreas and that PTEN expression is significantly downregulated in tumor samples (Fig.S8). Notably, with the exception of CSF3, the upregulated expression of the 4 cytokines showed strong negative correlation with PTEN level (Fig.S8). These studies suggest that the functional genetic interactions between PTEN and the NF-\kappa B-cytokine network appears operative in both mouse and human PDAC, although we acknowledge that multiple genetic mechanisms beyond NF-\kappa B may regulate cytokine expression in human PDAC.
DISCUSSION

In this study, high resolution analysis of human PDAC genomes has uncovered frequent loss of at least one copy of the PTEN gene. In mouse genetic studies, combined genomic, genetic and functional studies establish a key role of the Pten tumor suppressor in the progression of Kras-initiated PDAC development in the mouse. While recent studies have shown Kras and Pten cooperation in mouse PDAC develop (31), our study is distinguished in demonstrating that Pten can function as a haploinsufficient tumor suppressor, aligning well with human genomic profiles, as evidence 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 demonstrate 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 Kras$^{G12D}$ Pten$^{L/+}$ tumors. In addition, suppression of NF-κB pathway inhibited Pdx1-Cre Kras$^{G12D}$ Pten$^{L/+}$ tumor growth in vivo, indicating an important role in tumor maintenance hence potential therapeutic target in tumors with altered PI3K regulation.

The lack of Akt activation in Kras$^{G12D}$ PanIN and metaplasia lesions implied active suppression of the PI3K signaling axis. The activity of 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 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/reduction of Pten enables Kras\(^{G12D}\) to drive malignant progression of PDAC. Similarly, ‘reinforcing’ events in PDAC such as mutations 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 justifies 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.

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 Kras\(^{G12D}\) to induce NF-κB activity through PI3K pathway. Similarly, NF-κB activity is found to be sustained by elevated PI3K/Akt pathway in several human PDAC cell lines and is important for the viability of the tumor cells (12). Interestingly, PTEN expression itself is suppressed by 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 Kras\(^{G12D}\) to activate NF-κB pathway in lung adenocarcinoma (37), suggesting that additional mechanisms may also contribute to
NF-κB activation in tumors driven by oncogenic Kras. Our data that p53 remains intact in Pdx1-Cre Kras\textsuperscript{G12D} Pten\textsuperscript{L/+} tumor cells and p19\textsuperscript{Arf} expression is maintained in premalignant ductal cells (Fig.3C&D) suggest that the p53 axis is not prominently involved in the activation of NF-κB pathway in Pten deficient PDECs. At the same time, p53 inactivation through loss of p19\textsuperscript{Arf} expression in PDAC cells may further contribute to NF-κB activation in the 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 NF-κB pathway functions both cell-autonomously and non cell-autonomously (39). Suppression of NF-κB by dominant negative IκBα mutant inhibits the tumorigenic activity of Pdx1-Cre Kras\textsuperscript{G12D} Pten\textsuperscript{L/+} PDAC cells suggests that NF-κB pathway may function cell-autonomously in established tumors. Meanwhile, our data that NF-κB pathway is activated in Pdx1-Cre Kras\textsuperscript{G12D} Pten\textsuperscript{L/+} PDECs to induce various cytokines and chemokines suggests that NF-κB may also function through paracrine mechanisms during PDAC development to modulate the interaction between tumor cells and its microenvironment, a supposition that will require further study in a defined genetic model.

As an anatomic and functional component of cancer, the composition of tumor microenvironment shows many characteristics observed in chronic inflammation (40). A recent animal study has shown that induction of chronic pancreatitis dramatically accelerated Kras\textsuperscript{G12V}-induced pancreatic cancer formation, pointing to the importance of permissive environment during Kras-mediated PDAC development (41). However, the
underlying mechanisms that orchestrate the cooperation between tumor cells and their inflammatory milieu are less understood. Here we provided additional evidence that the PTEN/PI3K pathway may cooperate with endogenous Kras\textsuperscript{G12D} to induce the expression of various cytokines and chemokines in pre-malignant epithelial cells in NF-κB-dependant manner. Among the induced cytokines, Il23 and Ccl20 were particularly noteworthy given their well-established roles as chemoattractants for lymphocyte subsets including regulatory T cells (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 \textit{Kras}\textsuperscript{G12D} \textit{Pten}\textsuperscript{L/+} ductal epithelial cells supported the hypothesis that PI3K activation in combination with oncogenic Kras in epithelial compartment may facilitate the recruitment of various pro-tumor cell types within tumor stroma. The highly enriched cytokines and chemokines in the tumor microenvironment may not only suppress anti-tumor immunity, but the infiltrating inflammatory cells as well as other stromal cells also 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 mutation 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 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 and the activation of NF-κB pathway under such context further endorses clinical trials for combined therapies with MEK inhibitors and PI3K or NF-κB inhibitors. Alternatively, direct targeting the cytokine networks may diminish immune sequestration in PDAC and enhance immunotherapeutic strategies for this disease.
MATERIALS AND METHODS

Primary tumors, tumor cell enrichment and Array-CGH profiling

Human PDAC TMA samples were obtained from Brigham and Women’s Hospital Department of Pathology tumor bank. For array-CGH profiling, fresh-frozen specimens of primary pancreatic ductal adenocarcinoma were obtained from Massachusetts General Hospital Center for Pancreatic Research tumor bank. To overcome two problems typical for bio-repositories, namely low tumor cellularity and insufficient size of a specimen to perform genomics studies, the following 2-step protocol has been developed. Firstly, samples were enriched for epithelial content using EpCAM-coated Dynabeads (Invitrogen). About 10 mm$^2$ size chunks of OCT frozen tissue were disintegrated by Medimachine (BD) using 50 μm Medicon (BD) and syringe-type 50 μm Filcon (BD) in the buffer containing PBS, 1% FCS, 0.8% Sodium Citrate, 10 mM EGTA. Bead bounding and cells isolation were done according to Dynabeads Epithelial Enrich protocol (Invitrogen). The purity of enriched epithelial cells was over >80%. The yield of epithelial cells from frozen tissue corresponded to approximately 40% of the yield from fresh tissue according to our pilot experiments (Protopopov, unpublished). 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). Array-CGH profiling was performed as described (11, 47) on Agilent Human CGH 244K platform with the following modification: 2 μg WGA product per hybridization was labeled chemically.

Mice
Pdx1-Cre, Kras^G12D and Pten^L mice have been described previously (17-19). Mice were interbred and maintained on FvB/C57Bl6 hybrid background in pathogen-free conditions at Dana-Farber Cancer Institute. For BrdU injection, mice were i.p. injected with BrdU at 60 mg/kg every 12 hours for 48 hours before necropsy. All manipulations were performed with IACUC approval.

Xenograft studies

For xenografts, 1x10^6 cells suspended in 100 μl Hanks Buffered Saline Solution were injected subcutaneously into the lower flank of NCr nude mice (Taconic). Tumor volumes were measured every three days starting from Day 4 post-injection. All xenograft experiments were approved by IACUC.

Immunohistochemistry and Western blot analysis

Tissues were fixed in 10% formalin overnight and embedded in paraffin. Immunohistochemical analysis was performed as described (48). The visualization of primary antibodies was performed with horseradish peroxidase system (Vectastain ABC kit, Vector). The primary antibodies used for immunohistochemistry were: Cytokeratin 19 (TROMA-III, DSHB), Amylase (A8273, Sigma), Insulin (A0564, Dako), SMA (NB600-531, Novus), Gr-1 (550291, BD), FoxP3 (ab54501, Abcam), phospho-RelA (3037, Cell Signaling), phospho-ERK (4376, Cell Signaling), BrdU (ab1293, Abcam), Pten (9559, Cell Signaling), Phospho-Akt^{Ser473} (3787, Cell Signaling). Images were captured using a Leica DM1400B microsystem and Leica FW4000 version 1.2.1. The primary antibodies used for Western blot were: Actin (sc-1615, Santa Cruz), p16^{Ink4a} (sc-1207, Santa Cruz), Smad4 (sc-7966, Santa Cruz), p19^{Arf} (ab80, Abcam), p21^{cip} (556431, BD), p53 (VP-
P956, Vector), NF-κB p65 (ab7970, Abcam), Lamin A/C (2032, cell signaling), Caspase 3 (9665, Cell Signaling), IKKα (2682, Cell signaling), IKKβ (2678, Cell signaling), phospho-IKKα/β (2697, Cell signaling), IκBα (sc-371, Santa Cruz), phospho-IκBα (2859, Cell signaling).

FACS analysis of inflammatory cell infiltration

Pancreas from mice were harvested and gently disrupted by forceps-teasing and incubated in digestion buffer containing RPMI, 0.2 mg/ml collagenase P (Roche), 0.1 mg/ml DNase I (Sigma) and 0.8 mg/ml dispase (Invitrogen) for 45 min at 37°C. Single cell suspensions were subjected to RBC-lysis using ACK buffer (Lonza Biowhittaker) and transferred into FACS buffer (PBS, 2 mM EDTA and 2% FBS) containing FcR-blocking (2.4G2) antibody. Cells were stained with fluorescently-labeled monoclonal antibodies specific for CD45 (30-F11), CD4 (RM4-5), CD11b (M1/70), CD25 (PC61), Gr-1 (RB6-8C5) or isotype controls. For analysis of regulatory T cells, cells were stained with anti-CD45, -CD4 and -CD25 antibodies, and intracellularly stained with anti-FoxP3-FITC (eBiosciences). Cells were analyzed using a FACS Aria (BD) and FlowJo Software (Tree Star).

Establishment of primary pancreatic ductal epithelial cell (PDEC) and pancreatic adenocarcinoma cell (PDAC) lines

Primary PDECs were established as described (49). Briefly, the pancreata from 6 week old mice were digested at 37°C with 1 mg/ml Collagenase V solution (Sigma) in DMEM containing 10% FBS with agitation for 20 minutes. The digested material was filtered through a 100 μm nylon cell strainer. Fragments trapped on the mesh were further digested with 0.25% trypsin-EDTA for 5 minutes. The ductal fragments were
plated on collagen I coated plates (BD) and maintained in DMEM/F12 medium supplemented with 5 mg/mL D-glucose (Sigma), 0.1 mg/mL soybean trypsin inhibitor type I (Sigma), 5 mL/L insulin-transferrin-selenium (ITS+; BD), 25 μg/mL bovine pituitary extract (Invitrogen), 20 ng/mL epidermal growth factor (Sigma), 5 nM 3,3',5-triiodo-L-thyronine (Sigma), 1 μM dexamethasone (Sigma), 100 ng/mL cholera toxin (Sigma), 1.22 mg/ml nicotinamide (Sigma), and 5% Nu-serum IV culture supplement (BD). All studies were done on cells cultivated for less than 8 passages. Establishment of primary PDAC lines were performed as described (48).

**Molecular analysis**

DNA and RNA isolation were performed as described. cDNA was reverse transcribed from RNA using Superscript II (Invitrogen). PCR primers were designed to amplify the full length open reading frame of *Pten* and *Smad4* and PCR products were subjected to direct sequencing.

For *Pten* cDNA sequencing, the primers were as follows:

- **Pten-F**, 5’- ATGACAGCCATCATCAAAAGAGATCGTTAG -3’;
- **Pten-R**, 5’- GGGTCAGACTTTTGTAATTTGTGAATGCTG -3’;
- **Pten-F1**, 5’- GACGGACTGGTGTAATGATTTGTGC -3’;
- **Pten-F2**, 5’- GGTAAATACGTTCTTCATACCAGGACC -3’;
- **Pten-R1**, 5’- GTCTCTGGTCCTTACTTCCCCAT -3’.

For *Smad4* cDNA sequencing, the primers were as follows:

- **Smad4-F**, 5’- CCGATGGACAATATGTCTATAACAAATACACC -3’;
- **Smad4-R**, 5’- TCAGTCTAAAGGCTGTGGGTCCGCAATG -3’;
- **Smad4-F1**, 5’- GAGCGGGTTGTTCTACCTGGAATTG -3’;
Smad4-F2, 5’- GAAGGTGACGTTTGGGTCAGGTG -3’;  
Smad4-R1, 5’- CTCATCCTTCACTAACATACTTGGAGC -3’;  
Smad4-R2, 5’- CCAGGTAGTAACTCTGTACAAAGACC -3’.

Methylation-specific PCR analysis was performed as described before (50). For DNA damage treatment, cells were treated with Doxorubicin at 0.4 μg/ml for 16 hours before collected for Western blot analysis.

Clonogenic assay

400 cells were seeded into each well of 6-well plate in duplicate and colonies were stained 7 days later with 0.2% crystal violet in 80% methanol.

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). Student’s 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 GEO website under super series accession number GSE25828. Oncomine™ (Compendia Bioscience, Ann Arbor, MI) was used for analysis of human PDAC expression profile. Gene expression correlation was calculated with Pearson correlation analysis.

Statistical analysis

Tumor-free survivals were analyzed using Graphpad Prism4. Statistical analyses were performed using nonparametric Mann_Whitney test. Significance of enrichment in the promoter analysis was computed based on Poisson distribution with Bonferroni correction. Correlation analysis was performed with Fisher exact test and Chi-square
Other comparisons were performed using the unpaired Student's t-test. For all experiments with error bars, standard deviation was calculated to indicate the variation within each experiment and data, and values represent mean ± s.d.

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REFERENCES


FIGURE LEGENDS

Figure 1. PTEN is deleted/down-regulated in human pancreatic cancer and Pten inactivation cooperates with \textit{Kras}^{G12D} to induce pancreatic ductal adenocarcinoma. (A) Representative immunohistochemical images of PTEN and phospho-AKT (pAKT) staining from the TMA analysis showing samples with low/no PTEN staining and moderate/high pAKT (i, ii) or high PTEN staining and low pAKT (iii). Statistical analysis of the TMA data is shown in the bottom panel. (B) Array-CGH 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. Arrowhead: 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 \textit{Pdx1-Cre LSL-Kras}^{G12D} \textit{Pten}^{L/+} animal showing well-differentiated glandular tumor cells (i) which are positive for ductal marker, cytokeratin-19 (CK19) (ii) and are negative for acinar marker, Amylase (iii), and endocrine marker, Insulin (iv). Scale bar: 100 μm.

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 \textit{Kras}^{G12D}. (A) 6 week old pancreata from \textit{Kras}^{G12D} (panels i-v) and \textit{Kras}^{G12D} \textit{Pten}^{L/+} (panels vi-x) stained with H&E (panels i, vi), or with antibodies to SMA (panels ii, vii), S100A4 (panels iii, viii), Gr-1 (panels iv, ix), and FoxP3 (panels v, x). (B) Analysis of myeloid cells. Bar graph showing percentage of CD45^{+}CD11b^{+}Gr-1^{low} (white)
and Gr-1<sup>high</sup> (black) cells in total live cells of wild-type (n=3), \( \text{Kras}^{G12D} \) (n=3) and \( \text{Kras}^{G12D} \text{Pten}^{L/+} \) (n=3) pancreata. (C) Regulatory T cells analysis. Percentage of CD25+FoxP3+ cells within CD4 T cells in pancreas of wild-type (n=2), \( \text{Kras}^{G12D} \) (n=2) and \( \text{Kras}^{G12D} \text{Pten}^{L/+} \) (n=2) mice. *: p<0.05. **: p<0.01. (D) 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 (vi). Liver metastasis is stained with Cytokeratin-19 antibody (v). Scale bar: 100 μm.

Figure 3. Molecular analysis of \( \text{Kras}^{G12D} \text{Pten}^{L/+} \) PDACs. (A) PCR analysis of the Pten locus in genomic DNA from primary murine PDAC cell lines (lanes 2, 4, 6, 8, 10) and control tail DNA from the same animal (lane 1, 3, 5, 7, 9). While all control tail DNA showed both wild-type (Pten<sup>+</sup>) and Pten lox (Pten<sup>L</sup>) alleles (lanes 1, 3, 5, 7, 9) and deleted Pten (Pten<sup>D</sup>) allele was present in all tumor DNA (lanes 2, 4, 6, 8, 10), three tumor lines maintain the wild-type Pten allele (lanes 4, 6, 8). (B) Pten immunohistochemistry staining in tumors with Pten LOH (top panel) or wild-type Pten allele (bottom panel). (C) Western blot analysis for p16<sup>Ink4a</sup>, p19<sup>Arf</sup> and Smad4 expression in early passage \( \text{Kras}^{G12D} \) (lanes 1-3) or \( \text{Kras}^{G12D} \text{Pten}^{L/+} \) (lanes 4-7) PDEC lines and \( \text{Kras}^{G12D} \text{Pten}^{L/+} \) PDAC lines (lanes 8-12). PDAC line from \( \text{Kras}^{G12D} \text{p53}^{L/+} \) was used as a control (lane 13). (D) \( \text{Kras}^{G12D} \text{Pten}^{L/+} \) PDAC cell lines were treated with Doxorubicin 0.4 μg/ml for 16 hours and collected for Western blot analysis for p53 and p21. \( \text{Kras}^{G12D} \text{p53}^{L/+} \) tumor line was used as a negative control (lane 11-12).
Figure 4. Pten deficiency activates NF-κB pathway. (A) Cytoplasmic (C) or nuclear (N) extract was prepared from \( \text{Kras}^{G12D} \) (lanes 1-4) or \( \text{Kras}^{G12D} \text{ Pten}^{L/+} \) (lanes 5-12) PDECs and blotted for NFκB p65. Lamin A/C and Caspase 3 were used as markers for nuclear and cytoplasmic fraction, respectively. \( \text{Kras}^{G12D} \text{ Pten}^{L/+} \) PDECs were also treated with either vehicle (Ctr) or LY-294002 (LY) 20 \( \mu\)M for 24 hours before collection. (B) \( \text{Kras}^{G12D} \) or \( \text{Kras}^{G12D} \text{ Pten}^{L/+} \) PDECs were transfected with NF-κB-luciferase reporter and \( \text{Kras}^{G12D} \text{ Pten}^{L/+} \) PDECs were treated with either vehicle (Ctr) or LY-294002 (LY) 20 \( \mu\)M for 24 hours before collected for luciferase activity assay. *: p<0.05; **: p<0.01. (C) 6 week old pancreata from \( \text{Kras}^{G12D} \) (top) and \( \text{Kras}^{G12D} \text{ Pten}^{L/+} \) (bottom) mice were stained with phospho-NF-κB p65 (pRelA). (D) \( \text{Kras}^{G12D} \) PDECs were infected with pSuper (Ctr) or pSuper-shPten (shPten) and blotted for Pten, pAkt and Akt (lane 1-4). Cytoplasmic (C) or nuclear (N) extract was blotted for NF-κB p65 (lane 5-12).

Figure 5. Suppression of NF-κB inhibits tumorigenic activity. (A) PDAC from \( \text{Kras}^{G12D} \text{ Pten}^{L/+} \) animals was stained for phospho-NF-κB p65 (pRelA). (B) Colony formation assay for \( \text{Kras}^{G12D} \text{ Pten}^{L/+} \) PDAC cell lines infected with pBabe (Vec) or pBabe-\( \text{IκBαM} \). Quantification of colony numbers was shown on the right. (C) \( \text{Kras}^{G12D} \text{ Pten}^{L/+} \) PDAC cell lines were infected with pBabe (Vec) or pBabe-\( \text{IκBαM} \) and subcutaneously injected into nude mice with Vec in left flank and \( \text{IκBαM} \) in right flank respectively. Tumor volumes were measured in (D). *: p<0.05; **: p<0.01.
Figure 6. Pten deficiency induces cytokine expression. (A) The expression of indicated cytokines and chemokines were measured with QPCR in $Kras^{G12D}$ (n=3) or $Kras^{G12D}$ $\text{Pten}^{L/+}$ (n=5) PDECs. (B) The expression of indicated cytokines and chemokines were measured by QPCR in $Kras^{G12D}$ $\text{Pten}^{L/+}$ PDECs infected with pBabe (Vec) or pBabe-IκBαM. (C) The expression of indicated cytokines and chemokines were measured by QPCR in $Kras^{G12D}$ $\text{Pten}^{L/+}$ PDECs treated with vehicle (Ctr) or LY-294002 (LY) 20 μM for 24 hours. (D) The expression of indicated cytokines and chemokines were measured by QPCR in $Kras^{G12D}$ PDECs infected with pSuper (Ctr) or pSuper-shPten (shPten). *: p<0.05; **: p<0.01.
A. | Kras | Kras; Pten\(^{L/+}\) 
---|---|---
LZ10 | LZ11 | 2880 | 3030 
C | N | C | N | C | N | C | N | C | N

B. 

Luciferase Activity

![Graph showing luciferase activity comparison](image)

C. 

![Immunohistochemistry images showing pRelA expression](image)

D. 

![Western blot images showing protein expression](image)
Pten Is a Major Tumor Suppressor in Pancreatic Ductal Adenocarcinoma and Regulates an NF-κB-Cytokine Network

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