Cell Intrinsic Role of COX-2 in Pancreatic Cancer Development

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Abstract

COX-2 is upregulated in pancreatic ductal adenocarcinomas (PDAC). However, how COX-2 promotes PDAC development is unclear. While previous studies have evaluated the efficacy of COX-2 inhibition via the use of nonsteroidal anti-inflammatory drugs (NSAID) or the COX-2 inhibitor celecoxib in PDAC models, none have addressed the cell intrinsic versus microenvironment roles of COX-2 in modulating PDAC initiation and progression. We tested the cell intrinsic role of COX-2 in PDAC progression using both loss-of-function and gain-of-function approaches. Cox-2 deletion in Pdx1+ pancreatic progenitor cells significantly delays the development of PDAC in mice with K-ras activation and Pten haploinsufficiency. Conversely, COX-2 overexpression promotes early onset and progression of PDAC in the K-ras mouse model. Loss of PTEN function is a critical factor in determining lethal PDAC onset and overall survival. Mechanistically, COX-2 overexpression increases p-AKT levels in the precursor lesions of Pdx1+/K-rasG12D/+; Pten haploinsufficient mice in the absence of Pten LOH. In contrast, Cox-2 deletion in the same setting diminishes p-AKT levels and delays cancer progression. These data suggest an important cell intrinsic role for COX-2 in tumor initiation and progression through activation of the PI3K/AKT pathway. PDAC that is independent of intrinsic COX-2 expression eventually develops with decreased FKBP5 and increased GRP78 expression, two alternate pathways leading to AKT activation. Together, these results support a cell intrinsic role for COX-2 in PDAC development and suggest that while anti-COX-2 therapy may delay the development and progression of PDAC, mechanisms known to increase chemoresistance through AKT activation must also be overcome. Mol Cancer Ther; 11(10); 2127–37. ©2012 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC), the fourth leading cause of cancer-related deaths in the United States, has a 5-year survival rate of 4% (1). The poor outcome of PDAC has been attributed to late detection, the aggressive nature of the disease, and poor response to local and systemic therapies. A better understanding of the underlying mechanisms that lead to PDAC initiation, progression, and chemoresistance would help the development of more effective treatment regimens.

COX-2, also known as prostaglandin (PG) endoperoxide synthases, are enzymes essential in the conversion of arachidonic acid to prostaglandins. COXs exist as 2 isoforms, COX-1 and COX-2 (2). COX-1 is constitutively expressed in most mammalian tissues and is responsible for mediating various normal physiologic processes. COX-2, on the other hand, is low or undetectable in most normal tissues, but is induced in response to a wide range of stimuli in many cell types including epithelial cells, endothelial cells, and macrophages (3).

COX-2 levels are often elevated in lung, breast, esophageal, bladder, prostate, and pancreatic cancers (4). Substantial data suggest that COX-2 expression is not simply a by-product, but is a causal factor of tumor development. COX-2 overexpression in transgenic mice led to breast cancer formation, a phenotype greatly reduced by the use of the COX-2 inhibitor celecoxib (ref. 5; Fig. 1). Likewise, tumor growth in pancreatic cancers initiated by either COX-2 overexpression (6, 7) or mutant K-ras activation (8), and prostate cancers initiated by the SV40 large T antigen (9), was significantly reduced by celecoxib treatment. Importantly, celecoxib significantly reduced premalignant colon polyp formation in patients at risk for colon cancer (10). However, the addition of celecoxib to gemcitabine therapy, the current standard-of-care for treatment of PDAC did not show significant improvement in the survival of patients with metastatic disease (11), suggesting that the efficacy of celecoxib may be cancer- or cancer stage-dependent.
We previously showed that K-ras activation and Pten haploinsufficiency cooperate to activate p-AKT in precancerous lesions and accelerate tumor development in our mouse model of PDAC (12), suggesting that PTEN loss-of-function is a critical factor for determining the onset of lethal tumor development. Although PTEN mutation is not commonly found in human PDAC, loss of at least one copy of PTEN or gain/amplification of AKT2 has been reported in 32.8% of the PDAC xenografts derived from primary patient samples (13). Moreover, elevated p-AKT was observed in 68.5% of PDAC tissue microarray samples (13), suggesting that mechanisms other than PTEN deletion or AKT2 gain/amplification contribute to the elevated p-AKT levels seen in PDAC. In addition, our recent integrative, survival-based, molecular profiling of pancreatic progenitor cells showed that dysregulation of the PI3K/AKT pathway is regulated in PDAC development through activation of the PI3K/AKT pathway during tumorigenesis. These data provide strong rationale for further studies showing that dysregulation of the PI3K/AKT pathway is a critical factor for determining the onset of lethal tumor development.

We hypothesized that COX-2 expression, which suppresses PTEN activity in cell culture (15), could play a significant role in tumor development through activation of the PI3K/AKT pathway in PDAC. To investigate this hypothesis, we conditionally deleted or overexpressed Cox-2 in the Pdx1+ pancreatic progenitor cells. We recently showed that COX-2 overexpression and conditional knockout (Cox-2 KO) mice has been reported previously (17, 18). To conditionally overexpress COX-2 in the pancreas, we crossed the Pdx1-Cre line to the floxed Cox-2 COE transgenic line (17). Primers designed to detect the Cox-2 transgene were used for genotyping (17). The Pdx1-Cre+Cox-2COE line was further crossed to Pdx1-Cre;K-rasG12D;Ptenlox/+ mice (12) to produce Pdx1-Cre;K-rasG12D;Ptenlox/lox;Cox-2COE animals and littermate controls on a mixed C57/B6J/BL/6;129/BALB/c background. Likewise, Cox-2 KO mice (18) were used to produce Pdx1-Cre;K-rasG12D;Ptenlox/lox;Cox-2KO mice. All studies were conducted under the regulation of the Division of Laboratory Animal Medicine at the University of California at Los Angeles (UCLA; Los Angeles, CA).

Histology and immunohistochemistry

Immunohistochemical analysis was conducted on formalin-fixed, paraffin-embedded tissue. Antigen retrieval was conducted by heating the slides at 95 °C in citrate buffer (pH 6.0) or Tris-EDTA buffer (pH 8.0) for 15 minutes before staining. The following primary antibodies were used: phospho-AKT(Ser473) (Cell Signaling; 1:50), Cytokeratin 19 (ab15463, Abcam; 1:100), COX-2 (SP21; Thermo Scientific, ready-to-use), GRP78 (11587-1-AP, ProteinTech Group; 1:50), and FKBP5 (14155-1-AP, ProteinTech Group, 1:50). Blocking of GRP78 expression with an excess of synthetic GRP78 peptide (1084, Cell Signaling) was done by adding twice the volume of peptide as volume of antibody used for primary incubation. Only unidentifiable human tumor samples, collected by UCLA Clinical Microarray Core Laboratory at the UCLA under the approval of the UCLA Institutional Review Board, were used in this study. All pathology was reviewed at the UCLA under the approval of the UCLA Institutional Review Board, were used in this study. All pathology was reviewed at the UCLA.

Laser-capture microdissection and LOH analysis

Laser-capture microdissection of hematoxylin and eosin (H&E)-stained sections was conducted using a Leica LMD7000. Tissue (100,000 μm²) from acinar-to-ductal metaplasia (ADM) or PDACs in Pdx1-Cre;K-rasG12D;Ptenlox/lox;Cox-2COE and Pdx1-Cre;K-rasG12D;Ptenlox/lox;Cox-2KO mice were collected and extraction was done following protocols from Qiagen. Four-microliter aliquots of DNA were used for the PCR analysis. PCR was conducted for detection of wild-type, floxed, and recombinant alleles of Pten using previously described primers (12).

Statistical analysis

Array analysis was done by the UCLA Clinical Microarray Core on the Affymetrix Mouse 430 2.0 array. Microarray data are available at the National Center for Biotechnology Information Gene Expression Omnibus (GSE38988). Data normalization, filtering, and hierarchical
clustering were done using dChip software. For each gene, its expression in each genotype group was represented by the geometric average of the biologic replicated samples (n = 4). The log ratio between a pair of 2 genotypes was then calculated. The error bar represents ±SEM. Animal survival was determined by the Kaplan–Meier survival method. P < 0.001, log-rank test, for each pairwise combination.

Results

**Targeted Cox-2 deletion delays tumor onset in the K-ras/Pten PDAC model**

Increased COX-2 expression is observed in chronic pancreatitis, pancreatic intraepithelial neoplasias (PanIN), and PDACs (20). To address the effect of cell intrinsic loss of COX-2 activity on tumor formation, we genetically eliminated Cox-2 by crossing the Pdx1-Cre⁺;K-rasG12D/++;Ptenlox/lox PDAC model (12) to mice carrying a floxed, conditional Cox-2 deletion allele (Cox-2lox/; ref. 18). Pdx1 + pancreatic progenitor cells can give rise to endocrine, exocrine, and ductal tissue, but not to cells in the microenvironment, such as macrophages, or cells associated with blood vessels (21). Because conditional K-ras activation, Pten deletion, and Cox-2 deletions are all dependent on Cre activity driven by the Pdx1 promoter in these mice, Cox-2 will be deleted in the same cells in which mutant K-rasG12D activation and Pten deletion take place.

We first examined how Cox-2 deletion affected the survival of Pdx1-Cre⁺;K-rasG12D/++;Ptenlox/lox mice, which succumb to tumor burden by 3 weeks of age (ref. 12; Fig. 2A; left, green line). Removal of endogenous COX-2 expression in Pdx1-Cre⁺;K-rasG12D/++;Ptenlox/lox;Cox-2lox/lox mice extended the median survival time to 35 days (Fig. 2A; left, brown line).
At 2 weeks of age, Pdx1-Cre<sup>−/+</sup>;K-ras<sup>G12D/+;Pten<sup>lox/lox</sup></sup> mice developed PDAC (Fig. 2B, top). In contrast, removal of intrinsic COX-2 activity in Pdx1-Cre<sup>−/+;K-ras<sup>G12D/+;Pten<sup>lox/lox</sup></sup>; Cox-2<sup>lox/lox</sup> mice resulted in a predominantly normal pancreas parenchyma (Fig. 2B, bottom). Although lesions in the Pdx1-Cre<sup>−/+;K-ras<sup>G12D/+;Pten<sup>lox/lox</sup></sup>; Cox-2<sup>lox/lox</sup> mice appeared much later once the tumors developed the phenotypes resembled those of Pdx1-Cre<sup>−/+;K-ras<sup>G12D/+;Pten<sup>lox/lox</sup></sup> mice (Fig. 2C, left).

To confirm that Cox-2 is indeed deleted in tumors from Pdx1-Cre<sup>−/+;K-ras<sup>G12D/+;Pten<sup>lox/lox</sup></sup>; Cox-2<sup>lox/lox</sup> mice, we evaluated COX-2 expression by immunohistochemistry and double immunofluorescence analyses. Compared with the robust COX-2 staining observed in cytokeratin-19 positive (CK19<sup>+</sup>) neoplastic cells of Pdx1-Cre<sup>−/+;K-ras<sup>G12D/+;Pten<sup>lox/lox</sup></sup> mice (Fig. 2C, red arrow, top), no COX-2 expression can be detected in tumors from Pdx1-Cre<sup>−/+;K-ras<sup>G12D/+;Pten<sup>lox/lox</sup></sup>; Cox-2<sup>lox/lox</sup> mice (Fig. 2C, red arrow, bottom) except CK19<sup>+</sup> cells in the microenvironment (Fig. 2C, green arrow, bottom). These observations suggest that, although genetic deletion of Cox-2 in the Pdx1+ pancreatic progenitor cells is not sufficient to fully abrogate tumorigenesis caused by alterations of the RAS/RAF/MAPK and PTEN/P13K pathways, there is an important cell intrinsic role for COX-2 in pancreatic tumorigenesis; thus, without the presence of Cox-2, PDAC development is significantly delayed.

**COX-2 overexpression alone is insufficient to promote PDAC development**

Having established that loss of cell intrinsic COX-2 expression delays PDAC development, we next sought to determine whether cell intrinsic COX-2 overexpression alone had an effect on PDAC development. To examine this question, we crossed mice carrying a conditional Cox-2 overexpression allele (Cox-2COE; ref. 17) with the Pdx1-Cre<sup>+</sup> line (22) to generate Pdx1-Cre<sup>+</sup>; Cox-2COE mice. Examination of H&E-stained sections of pancreata from Pdx1-Cre<sup>+</sup>; Cox-2COE mice revealed structures that have the appearance of ADM, a preneoplastic ductal structure with the appearance of ADM, a preneoplastic ductal structure with the appearance of ADM, a preneoplastic ductal structure with the appearance of ADM, a preneoplastic ductal structure with the appearance of ADM, a preneoplastic ductal structure with the appearance of ADM, a preneoplastic ductal structure, which suggests that, although genetic deletion of Cox-2 in the Pdx1+ pancreatic progenitor cells is not sufficient to fully abrogate tumorigenesis caused by alterations of the RAS/RAF/MAPK and PTEN/P13K pathways, there is an important cell intrinsic role for COX-2 in pancreatic tumorigenesis; thus, without the presence of Cox-2, PDAC development is significantly delayed.

**COX-2 overexpression promotes earlier onset of PDAC development and reduces survival of Pdx1-Cre<sup>−/+;K-ras<sup>G12D/+;Pten<sup>lox/lox</sup></sup> mice**

Consistent with our finding that COX-2 overexpression alone is not sufficient to promote PDAC development, Guerra and colleagues (23) showed that injury-induced pancreatitis, in which Cox-2 overexpression plays an essential role, cannot cause PDAC unless a K-ras activation mutation is present. Given this precedent, we tested whether intrinsic COX-2 overexpression would accelerate tumorigenesis in a mouse model capable of developing PDACs.

Mice expressing K-ras<sup>G12D</sup> from its endogenous locus generate PDACs only after a prolonged latency (22), unless coupled with other genetic alterations commonly found in human PDAC, including loss of Ink4a/Arf (24), TGF-β receptor type 2 (Tgfbr2; ref. 25), Smad4 (26), mutation in p53 (27), or haploinsufficiency for Pten (12). COX-2 overexpression leads to significant reduction of median survival time in the Pdx1-Cre<sup>−/+;K-ras<sup>G12D</sup></sup> mice from 525 to 410 days (Fig. 4A, compare black and green lines).

When one allele of Pten was removed from the Pdx1-Cre<sup>−/+;K-ras<sup>G12D/+;Pten<sup>lox/lox</sup></sup> mice, we observed a substantial reduction in the overall survival in the Pdx1-Cre<sup>−/+;K-ras<sup>G12D/+;Pten<sup>lox/lox</sup></sup> mice (Fig. 4A, blue line). Of note, the Pdx1-Cre<sup>−/+;K-ras<sup>G12D/+;Pten<sup>lox/lox</sup></sup>; Cox-2COE mice have similar median survival times (Fig. 4A, blue and red line, respectively). This genetic evidence suggests that although COX-2 overexpression can collaborate with RAS/Raf/MAPK pathway activation, the biologic effects of COX-2 overexpression may overlap with PI3K/AKT activation in promoting PDAC development.

To further investigate the effects of COX-2 overexpression and RAS/Raf/MAPK and PI3K pathway activations, we examined the pancreata of each model between the age of 1 and 3 months (n = 12 for each cohort) and scored the presence of ADMs, mPanINs, or PDACs. As summarized in Fig. 4B, Pdx1-Cre<sup>−/+;K-ras<sup>G12D/+;Pten<sup>lox/+</sup></sup> and Pdx1-Cre<sup>−/+;K-ras<sup>G12D/+;Pten<sup>lox/+</sup></sup>; Cox-2COE mice developed a similar spectrum of lesions. Although Cox-2 overexpression had no significant impact on the incidence of early ADM and mPanIN1a lesions, it promoted the progression of these early lesions as evidenced by the larger and higher grade lesions found in Pdx1-Cre<sup>−/+;K-ras<sup>G12D/+;Pten<sup>lox/+</sup></sup>; Cox-2COE mice when compared with age- and genetically background-matched Pdx1-Cre<sup>−/+;K-ras<sup>G12D/+;Pten<sup>lox/+</sup></sup>; Cox-2COE mice (Figs. 4B and C). Only 3 of 12 Pdx1-Cre<sup>−/+;K-ras<sup>G12D/+;Pten<sup>lox/+</sup></sup>; Cox-2COE mice developed mPanIN2-3s during this time frame. Pdx1-Cre<sup>−/+;K-ras<sup>G12D/+;Pten<sup>lox/+</sup></sup>; Cox-2COE mice also displayed an increased incidence of invasive PDACs as quantified in Fig. 4B and shown in the lower panel of Fig. 4C. The fact that the early progression observed histopathologically does not seem to have a major impact on the overall survival of the 2 cohorts suggests that the kinetics of disease progression from the early lesions to lethal disease may not be linear because of the timing and nature of secondary mutations acquired by localized lesions in each cohort. Because metastatic incidences are rare in both cohorts (<5%), it is difficult to quantitatively compare the rates and onset of these lesions.
AKT activation is associated with COX-2–mediated tumor progression

Because Pdx1-Cre⁺;K-rasG12D/++;Ptenlox/+ mice and Pdx1-Cre⁺;K-rasG12D/++;Ptenlox/+;Cox-2COE mice have a similar median survival time, we reasoned that COX-2 overexpression may functionally mimic Pten LOH, an event critical for PDAC progression in the Pdx1-Cre⁺;K-rasG12D/++;Ptenlox/+ model (12). To test this hypothesis, we assessed both the Pten genomic and functional status in ADM and PDAC lesions in the Pdx1-Cre⁺;K-rasG12D/++;Ptenlox/+;Cox-2COE and Pdx1-Cre⁺;K-rasG12D/++;Ptenlox/+;Cox-2lox/lox mice. PTEN functional status was determined by p-AKT immunohistochemical analysis, and Pten genomic status was investigated by genomic PCR, using laser-captured lesion tissues. AKT is significantly activated in the ADM lesions of Pdx1-Cre⁺;K-rasG12D/++;Ptenlox/+;Cox-2COE mice, but not in similar lesions of either Pdx1-Cre⁺;K-rasG12D/++;Ptenlox/+;Cox-2COE mice (Hill and colleagues 2010; and data not shown) or Pdx1-Cre⁺;K-rasG12D/++;Ptenlox/+;Cox-2lox/lox mice (Fig. 5A; comparing left and right). In contrast, PDAC lesions from COX-2 overexpressing and Cox-2 deleted mice have comparably enhanced p-AKT staining, further supporting our hypothesis that AKT activation is a limiting step in ADM to PDAC progression (12).

We then extracted genomic DNA from laser-captured ADM and PDAC lesions from cohorts of Pdx1-Cre⁺;K-rasG12D/++;Ptenlox/+;Cox-2COE and Pdx1-Cre⁺;K-rasG12D/++;Ptenlox/+;Cox-2lox/lox mice (n = 4) and conducted PCR analysis. The WT Pten allele (Fig. 5B; in red) is maintained at near-equal molar ratio to the Pten-deleted allele (Fig. 5B; in blue) in both precancer (ADM) and cancer (PDAC) lesions, indicating that majority of cells in these lesions did not acquire Pten LOH. This result suggests that COX-2 overexpression, together with Pten haploinsufficiency,
promotes AKT activation—a necessary step for the progression of precursor lesions to PDAC (12). The presence of elevated COX-2 activity, therefore, removes the selective pressure for \textit{Pten} LOH that is observed in the Pdx1-\textit{Cre}^+;K-ras\,G12D^+/+;\textit{Pten}^loxp/+ model (15).

**Upregulated GRP78 expression correlates with p-AKT activation in the precursor lesions**

Upregulation of GRP78, a member of the Hsp70 protein family, is associated with poor prognosis of a number of human cancers (28, 29). While GRP78 is known to be expressed in the cytoplasm, the recent discovery of GRP78 on the cell surface and its role in activating the AKT (28, 29) prompted us to investigate whether GRP78 expression is involved in initiation and progression in human PDAC. While no GRP78 expression was observed in the ducts of wild-type murine samples (Supplementary Fig. S1A, green arrow in left), GRP78 expression is significantly upregulated in the ductal structures of human ADM and PDAC lesions (Fig. 6A, top; Supplementary Fig. S1A, right, low power magnification). Importantly, GRP78 is strongly expressed in both the cytoplasm and the membranes of these human lesions (Fig. 6A, top).
lesions in the absence of epithelial COX-2 expression, PDAC
GRP78 and to subsequent AKT activation. However, even lead to upregulated expression of membrane-associated 5A, 3rd panel from left) can be observed in the ADM (bottom left) and nearly undetectable AKT activation (Fig. 6B). Immunohistochemical analysis revealed strong FKBP5 staining in PDACs of Pdx1-Cre+;K-rasG12D/+;Ptenlox/lox;Cox-2COE mice (Fig. 7B). Furthermore, enhanced membrane-associated p-AKT correlates with reduced FKBP5 staining in PDACs of Pdx1-Cre+;K-rasG12D/+;Ptenlox/lox;Cox-2lox/lox mice (Fig. 7C, region indicated by green arrow, Supplementary Fig. S2). These results suggest that, in the absence of Pten LOH, downregulation of FKBP5 could

We then analyzed GRP78 expression in our murine models. Similar to the human disease, both ADM and PDAC lesions in the Pdx1-Cre+;K-rasG12D/+;Ptenlox/lox;Cox-2COE mice have strongly upregulated GRP78 expression, especially its membrane-associated form (Fig. 6A, middle), corresponding to the AKT activation (Fig. 5A, I and II panel from left). The specificity of this staining was confirmed by showing that excess GRP78 peptide can block labeling with this antibody (Supplemental Fig. S1B). In contrast, only low cytosolic GRP78 expression (Fig. 6A, bottom left) and nearly undetectable AKT activation (Fig. 5A, 3rd panel from left) can be observed in the ADM lesions from mice with the targeted Cox-2 deletion.

These data suggest that COX-2 overexpression may lead to upregulated expression of membrane-associated GRP78 and to subsequent AKT activation. However, even in the absence of epithelial COX-2 expression, PDAC lesions in the Pdx1-Cre+;K-rasG12D/+;Ptenlox/lox;Cox-2lox/lox mice also showed upregulated GRP78 expression (bottom right of Fig. 6A; Supplementary Fig. S1B), which colocalizes with enhanced p-AKT in the membrane (Fig. 6B), suggesting that GRP78 and AKT can be upregulated and activated in a COX-2-independent manner as the disease progresses.

Decreased FK506-binding protein 5 (FKBP5) negative feedback leads to enhanced AKT activation in PDAC of Pdx1-Cre+;K-rasG12D/+;Ptenlox/lox;Cox-2lox/lox mice

Although Cox-2 conditional deletion led to increased median survival of Pdx1-Cre+;K-rasG12D/+;Ptenlox/lox mice (65 days; data not shown), all Cox-2-deleted animals ultimately succumbed to aggressive PDAC without Pten LOH (Fig. 5A), suggesting that resistance to COX-2-directed therapy is likely to occur in patients.

Decreased expression of scaffold protein FKBP5 has been identified as one of the mechanisms that enhances AKT activity and causes PDAC chemoresistance (30). FKBP5, by acting as a scaffolding protein for AKT and PH domain and leucine-rich repeat protein phosphatase 1 (PHLPP), promotes PHLPP dephosphorylation of AKT at amino acid S473 (31), thereby suppressing AKT activity (30, 32). We examined the expression level of FKBP5 mRNA and found that FKBP5 is downregulated significantly in PDACs of Pdx1-Cre+;K-rasG12D/+;Ptenlox/lox;Cox-2lox/lox mice (Fig. 7A; P = 0.01; 2-tailed t test, determined as the Log2 ratio of the FKBP5 expression of knockout [Pdx1-Cre+;K-rasG12D/+;Ptenlox/lox;Cox-2lox/lox] over Pdx1-Cre+;K-rasG12D/+;Ptenlox/lox] and overexpressed [Pdx1-Cre+;K-rasG12D/+;Ptenlox/lox;Cox-2COE over Pdx1-Cre+;K-rasG12D/+;Ptenlox/lox]).

Immunohistochemical analysis revealed strong FKBP5 staining in PDACs of Pdx1-Cre+;K-rasG12D/+;Ptenlox/lox;Cox-2COE and Pdx1-Cre+;K-rasG12D/+;Ptenlox/lox;Cox-2lox/lox mice, whereas FKBP5 expression was reduced to almost undetectable levels in Pdx1-Cre+;K-rasG12D/+;Ptenlox/lox;Cox-2lox/lox mice (Fig. 7B). Furthermore, enhanced membrane-associated p-AKT correlates with reduced FKBP5 staining in PDACs of Pdx1-Cre+;K-rasG12D/+;Ptenlox/lox;Cox-2lox/lox mice (Fig. 7C, region indicated by green arrow, Supplementary Fig. S2). These results suggest that, in the absence of Pten LOH, downregulation of FKBP5 could
cause activation of AKT in PDAC with Cox-2 deletion or, in a clinical context, as a result of celecoxib treatment, leading to chemoresistance.

Discussion

Using both loss- and gain-of-function genetic approaches, our study establishes a cell intrinsic role for COX-2 in the onset and progression of PDAC. COX-2 overexpression cooperates with Pten haploinsufficiency to activate AKT, a gate-keeping event in PDAC progression, leading to accelerated tumor development. Mechanistically, AKT activation may occur as a result of increased membrane-associated GRP78 expression, induced as a consequence of enhanced COX-2 signaling (Fig. 7D, left). Alternatively, AKT can be activated by decreased FKBP-PHLFp-AKT negative feedback circuitry associated with Cox-2 abrogation (Fig. 7D, right) or, perhaps, in response to NSAID or celecoxib treatment. Our findings suggest that, while anti-COX-2 therapies may delay PDAC onset and progression, tumors undergoing NSAID or celecoxib treatment may acquire changes that lead to AKT activation and therapeutic resistance. These alternate mechanisms that lead to AKT-mediated resistance must also be overcome to achieve therapeutic efficacy (33–35).

Cell intrinsic role for COX-2 in pancreatic tumor development

Previous studies have evaluated the efficacy of COX-2 inhibition, via the use of NSAIDs or celecoxib treatment, in PDAC models (6–8). However, there are no previous studies that have been able to discriminate between the effect of COX-2 inhibition in both the tumor cells and the microenvironment, and COX-2 inhibition in only the tumor cells. The murine models we have generated provided an opportunity to elucidate the cell intrinsic function of COX-2 in PDAC development.

Unlike previous studies, which showed that cytokeratin-5–driven expression of COX-2 led to the development of PDAC, (6), we observed that COX-2 expression in Pdx1+ progenitor cells alone is not sufficient for the development of PDAC in Pdx1-Cre+;Cox-2COE mice. This result raises the question of what is preventing tumor development in Pdx1-Cre+;Cox-2COE mice or in mice with injury-induced pancreatitis, where COX-2 plays a critical role in subsequent tumor development (23). Recent studies indicate K-ras mutation is essential for the progression of premalignant lesions to PDAC in mice with injury-induced pancreatitis (23, 36). Consistent with these observations, our genetic study suggests that the cross-talk between COX-2 and RAS/MAPK signaling pathway is essential in promoting PDAC development. Furthermore, the median survival of Pdx1-Cre+;K-rasG12D/+;Cox-2COE mice was significantly longer than that of Pdx1-Cre+;K-rasG12D/+;Ptenlox/+;Cox-2COE mice, establishing the importance of PTEN pathway function in determining both PDAC progression and overall survival. The similar survival trends of Pdx1-Cre+;K-rasG12D/+;Ptenlox/+;Cox-2COE and Pdx1-Cre+;K-rasG12D/+;Ptenlox/+ mice also support the hypothesis that the effects of COX-2 expression overlap with the PI3K/AKT pathway and suggest that COX-2 is a key modulator of PI3K/AKT activation in pancreatic tumorigenesis, without the need for Pten loss of heterozygosity.

The PTEN pathway and PDAC progression

Tumors in the K-ras;Pten mice recapitulate the pathologic features of human PDACs. Moreover, these mice
model the loss of PTEN function, which is now recognized as a frequent, major contributor in human PDAC progression. While the loss of both alleles of PTEN is infrequent in PDAC, elevated p-AKT was frequently observed in the PDAC tissue microarray samples (13), suggesting that PDACs invariably developed in all K-rasG12D;Ptenlox/þ mice. A, FKBP5 mRNA expression in PDACs of Pdx1-Cre; K-rasG12D;Ptenlox/þ; Cox-2lox/þ mice. B, representative immunohistochemical analyses for FKBP5 in PDACs in the 3 mutant murine lines. C, immunohistochemistry for p-AKT and FKBP5 in consecutive sections in a PDAC from a Pdx1-Cre; K-rasG12D;Ptenlox/þ; Cox-2lox/þ mouse. Scale bars, 50 mm. D, model of COX-2-mediated activation of p-AKT in tumors with and without COX-2 expression. Left, Pdx1-Cre; K-rasG12D;Ptenlox/þ; Cox-2COE mice have earlier PDAC onset because early p-AKT activation, possibly mediated by increased GRP78 expression, cooperates with Pten haploinsufficiency to initiate tumorigenesis. Right, p-AKT activation in tumors of Pdx1-Cre; K-rasG12D;Ptenlox/þ; Cox-2lox/þ mice could be the result of loss of FKBP5-regulated AKT suppression, which is reduced in the absence of COX-2 expression.

Figure 7. Decreased FKBP5 negative feedback leads to enhanced AKT activation in Pdx1-Cre; K-rasG12D;Ptenlox/þ; Cox-2lox/þ mice. A, FKBP5 mRNA expression in PDACs of Pdx1-Cre; K-rasG12D;Ptenlox/þ; Cox-2lox/þ mice. K-rasG12D;Ptenlox/þ; Cox-2COE (OE, n = 4) mice. B, representative immunohistochemical analyses for FKBP5 in PDACs in the 3 mutant murine lines. C, immunohistochemistry for p-AKT and FKBP5 in consecutive sections in a PDAC from a Pdx1-Cre; K-rasG12D;Ptenlox/þ; Cox-2lox/þ mouse. Scale bars, 50 mm. D, model of COX-2-mediated activation of p-AKT in tumors with and without COX-2 expression. Left, Pdx1-Cre; K-rasG12D;Ptenlox/þ; Cox-2COE mice have earlier PDAC onset because early p-AKT activation, possibly mediated by increased GRP78 expression, cooperates with Pten haploinsufficiency to initiate tumorigenesis. Right, p-AKT activation in tumors of Pdx1-Cre; K-rasG12D;Ptenlox/þ; Cox-2lox/þ mice could be the result of loss of FKBP5-regulated AKT suppression, which is reduced in the absence of COX-2 expression.

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FKBPs expression, we suggest that celecoxib treatment could result in loss of FKBPs-mediated suppression of AKT activation.

Therapeutic implications

Our results and those of others (8) show that therapeutic regimens designed to target COX-2 expression may be most useful for delaying both precursor lesions and PDAC initiation in patients at high risk for pancreatic cancer. Recent studies have shown that dissemination of pancreatic epithelial tumor cells can occur much earlier than previously thought and that these cells can seed distant metastasis (43). Given the fact that PDAC can grow at an exponential rate with micrometastasis developing from small primary tumors before surgical resection (44), it is possible that therapeutics, which delay precursor lesion formation, like COX-2 inhibitors, could be administered in this previously unappreciated therapeutic window to significantly delay/prevent seeding of metastases in patients at high-risk for PDAC development.

Moreover, our results emphasize the importance of AKT activity in the effectiveness of anti-COX-2 therapies. Recent studies have shown that COX-2 inhibition in lung and colon cancer cells was ineffective at eliciting cell death unless COX-2 inhibitors were used in combination with PPARγ ligand ciglitazone (45) and atorvastatin (46). Induction of cell death, in both studies, was associated with decreased p-AKT activation. We identified increased GRP78 and decreased FKBP5 as 2 mechanisms through which pancreatic tumors could activate AKT. The lack of increased therapeutic benefit from combination celecoxib–gemcitabine treatment of metastatic PDACs (11) could be the result of increased therapeutic resistance resulting from AKT activation. We suggest, therefore, that pathways that cause AKT activation will likely have to be targeted to enhance therapeutic efficacy. Cotargeting the COX-2, RAS/RAF/MEK, and PI3K/AKT pathways may be necessary to slow progression of late-stage PDACs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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