Abstract

K-ras is the most commonly mutated oncogene in pancreatic cancer and its activation in murine models is sufficient to recapitulate the spectrum of lesions seen in human pancreatic ductal adenocarcinoma (PDAC). Recent studies suggest that Notch receptor signaling becomes reactivated in a subset of PDACs, leading to the hypothesis that Notch1 functions as an oncogene in this setting. To determine whether Notch1 is required for K-ras-induced tumorigenesis, we used a mouse model in which an oncogenic allele of K-ras is activated and Notch1 is deleted simultaneously in the pancreas. Unexpectedly, the loss of Notch1 in this model resulted in increased tumor incidence and progression, implying that Notch1 can function as a tumor suppressor gene in PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive forms of human cancer. Pathogenesis of PDAC is thought to evolve through progression of precursor lesions, termed pancreatic intraepithelial neoplasias (PanIN). The PanINs are classified into four subgroups (1A, 1B, 2, and 3) and eventually evolve into invasive carcinoma. The most commonly mutated oncogene in PDAC is K-ras, with activating mutations found in more than 90% of human cases. Recently developed animal models have further underscored this point, as expression of a mutant activated K-ras allele in the pancreas is sufficient to induce the formation of both premalignant and malignant lesions in a mouse model, faithfully recapitulating the human disease (1).

Materials and Methods

Mouse strains

The LSL-K-ras<sup>G12D</sup> (13), Notch<sup>lox/lox</sup> (14), and PDX-1-Cre (1) mice have been previously described.

K-ras<sup>G12D</sup> and Notch1 allele recombination PCR assays

The LSL-K-ras<sup>G12D</sup> allele was analyzed by PCR as described (13). Notch1 allele recombination PCR assay was performed as described, without multiplexing (15).
Histology and immunohistochemistry

Formalin-fixed paraffin-embedded murine pancreatic tissue was processed by standard methods or subjected to immunohistochemical staining, using citrate buffer antigen retrieval. Antibodies were rat anti-Ki67 (1:400; Dako), rabbit anti-cleaved caspase-3 (1:200; Cell Signaling), rabbit anti-Hey1 (1:125; Abcam), rabbit anti-Hes1 (1:500; B. Stanger, University of Pennsylvania, Philadelphia, PA), and mouse anti-β-catenin (1:200; BD Biosciences).

Quantitative PCR

Pancreatic tissue samples were snap-frozen. Total RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel) and reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen). cDNA transcripts were amplified by quantitative real-time PCR using SYBR Green (Applied Biosystems). Detection/quantitation was done on ABI Prism 7000 (Applied Biosystems). Each gene was normalized to 18S rRNA. Notch1 primer sequences were fwd-TGGATGTCAATGTTCGAGGA and rev-C ACTGCAGGAGGAATCAT.

Western blot analysis

Tissues or cells were homogenized in radioimmuno-precipitation assay buffer. Primary antibodies were rabbit anti-Notch1 (1:500; Epitomics), rabbit anti-Notch2, anti-Notch3, and anti-Notch4 (all 1:200; Santa Cruz), mouse anti-β-catenin (1:1,000; BD Biosciences), mouse anti–active β-catenin (1:1,000; Millipore), rabbit anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10,000; Sigma), and mouse anti-tubulin (1:8,000; Sigma).

Figure 1. Histologic analysis of pancreata. A, H&E-stained sections of pancreata at 20 wk from PDX-1-Cre:Notch1lox/lox mice (i, islet), PDX-1-Cre:LSL-K-rasG12D mice (arrowhead, tubular complexes), and PDX-1-Cre:LSL-K-rasG12D:Notch1lox/lox mice (arrows, ducts exhibiting PanIN 1B-2 changes). Bar, 400 μm. B to D, detailed characterization of pathology exhibited in PDX-1-Cre:LSL-K-rasG12D:Notch1lox/lox pancreata. B, PanIN1B; papillary ductal lesions without significant loss of polarity or nuclear atypia (arrows). C, PanIN1B transitioning to PanIN2, revealing moderate nuclear atypia and a loss of polarity. D, PanIN1B showing intraepithelial polymorphonuclear leukocytes (arrowheads). Bar, 200 μm.
Isolation and culture of primary pancreatic ductal cells

Primary pancreatic ductal cells (PDCs) were derived as previously described (16). For siRNA experiments, PDX-1-Cre:K-rasG12D:Notch1lox/lox PDCs were plated at 1 × 10⁴ per well in a 12-well plate on day −1, then transfected on day 0 with either β-catenin ON-TARGETplus SMART pool siRNA or ON-TARGETplus Nontargeting siRNA pool#3 (Dharmacon), using DharmaFect 2 transfection reagent (Dharmacon) following the manufacturer’s protocol.

Statistical analysis

Ki67-positive nuclei comparisons were assessed by a standard unpaired t test. Error bars represent the SD of triplicate counts (P < 0.05 was considered significant). For real-time PCR, mean ± SD are shown.

Results and Discussion

Loss of Notch1 in the context of activated K-ras leads to increased PanIN incidence and progression

To test whether Notch1 is required for K-ras–induced pancreatic tumorigenesis in vivo, we used a mouse model of PDAC (1). The simultaneous expression of an oncogenic K-ras allele and the deletion of both Notch1 alleles was achieved by interbreeding mice harboring both a conditional activated K-ras allele (LSL-K-rasG12D; ref. 13) and conditional Notch1lox/lox knockout alleles (14) with PDX-1-Cre transgenic mice that express Cre-recombinase as early as day 8.5 of embryonic development in progenitors of all major pancreatic cell types (17).

We compared the pancreatea of PDX-1-Cre:LSL-K-rasG12D; Notch1lox/lox mice to those of PDX-1-Cre:LSL-K-rasG12D mice. At the 20-week time point, the PDX-1-Cre:LSL-K-rasG12D pancreatea displayed predominantly a combination of very early changes: acinar to ductal metaplasia (ADM), tubular complexes (TC), and PanIN1A lesions (Fig. 1A). Approximately 50% of the mice displayed ADM/TC, 30% displayed PanIN1A, and less than 20% displayed a lesion classified as PanIN1B (Fig. 2A), consistent with previous findings in this model (1). In contrast, 100% of PDX-1-Cre:LSL-K-rasG12D; Notch1lox/lox pancreata were classified histologically as PanIN1B, transitioning to PanIN2 (Figs. 1A and 2A). All mice in this cohort displayed a tissue architecture that was significantly altered. The majority of ducts exhibited PanIN1B-PanIN2 changes, with only between 10% and 40% of normal acinar, ductal, and islet tissue remaining (Figs. 1B–D and 2A).
appearance of intraepithelial and stromal polymorphonuclear leukocytes, a cell type often present in subtypes of human pancreatic cancer and thought to play a role in carcinogenesis, was noted (Fig. 1D). Thus, the PDX-1-Cre:LSL-K-ras\textsuperscript{G12D}:Notch1\textsuperscript{lox/lox} mice displayed both increased numbers and more advanced-stage PanIN lesions than the PDX-1-Cre:LSL-K-ras\textsuperscript{G12D} mice. The PDX-1-Cre:Notch1\textsuperscript{lox/lox} pancreata displayed no signs of abnormalities (Figs. 1A and 2A), consistent with recently published findings (18). Likewise, PDX-1-Cre pancreata appeared unremarkable (not shown). To verify whether the expected Cre-mediated recombination events occurred, we isolated genomic DNA from the pancreata and tails of mice from the various genotypes and performed PCR reactions to monitor recombination events. DNA isolated from the pancreas, and not the tails, verified recombination of both alleles (Supplementary Fig. S1).

Additionally, mice that are heterozygous for the floxed Notch1 allele (PDX-1-Cre:LSL-K-ras\textsuperscript{G12D}:Notch1\textsuperscript{lox/+}) exhibited an intermediate phenotype. PanIN lesions were predominantly graded as PanIN1B, but a much greater proportion of normal acinar, ductal, and islet tissue remained than when compared with the PDX-1-Cre:LSL-K-ras\textsuperscript{G12D}:Notch1\textsuperscript{lox/lox} cohort (not shown).

To compare the proliferative index of the preneoplastic lesions in the PDX-1-Cre:LSL-K-ras\textsuperscript{G12D} and PDX-1-Cre:LSL-K-ras\textsuperscript{G12D}:Notch1\textsuperscript{lox/lox} pancreata, we performed Ki67 immunohistochemical staining and scored the number of positive cells per duct (Fig. 2B). Ki67-positive nuclei were more frequent in similar-grade PanINs from PDX-1-Cre:LSL-K-ras\textsuperscript{G12D}:Notch1\textsuperscript{lox/lox} pancreata compared with PDX-1-Cre:LSL-K-ras\textsuperscript{G12D} pancreata (Fig. 2C). This indicates that in similar-grade lesions, more cells were actively proliferating in the PDX-1-Cre:LSL-K-ras\textsuperscript{G12D}:Notch1\textsuperscript{lox/lox} pancreata. To rule out that

Figure 3. Expression of Notch family members and targets in pancreata from cohorts. A, relative expression levels of Notch1 in pancreatic tissue determined by quantitative PCR (normalized to 18S rRNA). B, Western blot analysis of Notch1 in whole pancreatic lysates. Both full-length Notch1 (250 kDa) and cleaved Notch1 intracellular domain (120 kDa) are detected. Three independent samples are shown for each genotype. PKN, PDX-1-Cre:LSL-K-ras\textsuperscript{G12D}:Notch1\textsuperscript{lox/lox}; PK, PDX-1-Cre:LSL-K-ras\textsuperscript{G12D}; PN, PDX-1-Cre:Notch1\textsuperscript{lox/lox}; P, PDX-1-Cre; GAPDH, loading control. C, expression of Notch2, Notch3, and Notch4 in whole pancreatic lysate determined by Western blot analysis. Three independent samples are shown for each genotype. GAPDH, loading control. D, immunohistochemical analysis of Hes1 and Hey1 in similar-grade PanIN lesions. Bar, 40 μm.
decreased rates of apoptosis lead to a more severe phenotype in PDX-1-Cre:LSL-K-rasG12D:Notch1lox/lox, we stained sections for cleaved caspase-3. No difference was evident when compared with PDX-1-Cre:LSL-K-rasG12D pancreata (Fig. 2D). These studies show that the loss of Notch1 in the context of activated K-ras results in increased proliferation rates of pancreatic ductal cells in vivo, increased PanIN incidence, and progression. This implies that Notch1 possesses tumor suppressor–like function in a mouse model of K-ras–induced PDAC.

In a recent report, conditionally coexpressing activated Notch and K-ras in mouse pancreata induced synergy in PanIN formation (12). This was interpreted as an indication of Notch1 functioning to inhibit the normal differentiation of the tumor-initiating cells in the pancreas. It is possible that differences in target cells and/or timing of recombination events might account for the differences between our findings. In addition, Notch1 expression and activation are highly regulated and overexpression of a constitutively active form of Notch1 could lead to nonphysiologic phenomena. Finally, a recent report which catalogued core signaling pathways in human pancreatic cancer suggests that the expression levels of Notch-family members and downstream targets were not upregulated in primary tumor samples and cell lines, when compared with normal pancreatic ductal epithelium (19).

**Activation of Notch family members is not responsible for accelerated tumorigenesis in PDX-1-Cre:LSL-K-rasG12D:Notch1lox/lox mice**

To assess whether other Notch family members (Notch2–Notch4) are induced to compensate and substitute for the loss of Notch1, we examined the expression and activation of the Notch-family receptors. Employing quantitative PCR to verify loss of Notch1 expression, we find, as expected, that expression of Notch1 mRNA was reduced in both the PDX-1-Cre:Notch1lox/lox and the PDX-1-Cre:LSL-K-rasG12D:Notch1lox/lox pancreata, when compared with control PDX-1-Cre

---

**Figure 4.** Expression and activation state of β-catenin in pancreata from cohorts. Expression and localization of total β-catenin (A; bar, 40 μm) by immunohistochemical staining in pancreata from PDX-1-Cre, PDX-1-Cre:LSL-K-rasG12D, and PDX-1-Cre:LSL-K-rasG12D:Notch1lox/lox mice. B, expression levels of total β-catenin in whole pancreatic lysates by Western blot analyses. Four independent samples are shown for PDX-1-Cre:LSL-K-rasG12D:Notch1lox/lox (PKN) and PDX-1-Cre:LSL-K-rasG12D (PK) cells, three independent samples for PDX-1-Cre:Notch1lox/lox (PN) cells, and two independent samples for PDX-1-Cre (P) cells. GAPDH, loading control. C, Western blot analysis of total and activated β-catenin expression in PDCs. Tubulin, loading control. Results shown are representative of two independent PDC lines tested for each genotype. D, proliferation of PDX-1-Cre:LSL-K-rasG12D:Notch1lox/lox PDCs untreated or treated with nontargeting siRNA pool (Ntg siRNA) or β-catenin siRNA pool (β-cat siRNA). Values shown are the mean of three independent samples. Western blot analysis of β-catenin knockdown in PDCs treated with siRNA on days 2, 3, and 4. Tubulin, loading control.
pancreata. In the PDX-1-Cre:LSL-K-ras<sup>G12D</sup> pancreata, the expression of Notch1 mRNA seemed to be slightly upregulated when compared with PDX-1-Cre (Fig. 3A). Additionally, we analyzed Notch1 protein levels by western blotting, confirming loss of Notch1 in PDX-1-Cre:LSL-K-ras<sup>G12D</sup>:Notch1lox/lox and PDX-1-Cre:Notch1lox/lox pancreata (Fig. 3B). Western blot analysis of Notch2, Notch3, and Notch4 in extracts prepared from pancreas tissue, as above, indicates no significant differences in levels of expression between the cohorts (Fig. 3C). To further establish whether the activity of Notch family members might be increased in the pancreata of PDX-1-Cre:LSL-K-ras<sup>G12D</sup>,Notch1lox/lox mice, we analyzed the expression of established Notch downstream target genes, Hes1 and Hey1, by immunohistochemistry. Expression of Hes1 and Hey1 was detected at similar levels in comparable-grade lesions of PDX-1-Cre:LSL-K-ras<sup>G12D</sup>:Notch1lox/lox mice compared with PDX-1-Cre:LSL-K-ras<sup>G12D</sup> (Fig. 3D). Collectively, our data indicate that Notch1 has been effectively deleted in the pancreata of PDX-1-Cre:LSL-K-ras<sup>G12D</sup>,Notch1lox/lox mice, and that activation of other Notch family members is not likely to account for the observed acceleration of tumorigenesis in these mice.

**Activation of β-catenin in pancreata of PDX-1-Cre:LSL-K-ras<sup>G12D</sup>:Notch1lox/lox mice**

To investigate potential mechanisms underlying the observed tumor-suppressive function of Notch1 in K-ras–induced PDA, we examined the status of β-catenin, which has been identified as a target of the Notch1 tumor suppressor function in the skin (4, 7). In normal adult pancreas, localization at the cell membrane serves as an indication of inactivity, whereas cytoplasmic and nuclear localization of β-catenin are commonly regarded as indicators of active canonical Wnt/β-catenin signaling (20). In PDX-1-Cre acinar and ductal cells, total β-catenin was restricted to the cell membrane (Fig. 4A). In PDX-1-Cre:LSL-K-ras<sup>G12D</sup>, β-catenin was observed mostly in ductal cells at the cell membrane and faintly in the cytoplasm (Fig. 4A). In contrast, intense β-catenin staining was observed in the membrane and cytoplasm in PDX-1-Cre:LSL-K-ras<sup>G12D</sup>,Notch1lox/lox ductal cells, implying that β-catenin levels are induced and possibly activated (Fig. 4A). Importantly, total β-catenin levels were similar in pancreatic cells of all genotypes (Fig. 4B).

To further assess β-catenin activation directly in pancreatic ductal cells, we isolated primary PDCs from each of the different genotypes. Western blot analysis revealed no increase in either total or activated β-catenin (dephosphorylated on Ser37 and Thr41) in PDCs derived from PDX-1-Cre:LSL-K-ras<sup>G12D</sup>,Notch1lox/lox mice compared with the other genotypes (Fig. 4C). Finally, to functionally determine if β-catenin is required for proliferation of PDX-1-Cre:LSL-K-ras<sup>G12D</sup>:Notch1lox/lox PDCs, β-catenin was knocked down in these cells using an siRNA-based approach. Cells treated with β-catenin siRNA displayed a significant reduction in β-catenin levels; however, this knockdown did not affect the proliferative capacity of these cells when compared with untreated cells or cells treated with nontargeting siRNA (Fig. 4D).

These findings suggest that β-catenin repression might not represent the putative tumor-suppressive function of Notch1 in our mouse model of K-ras–induced PDAC. This conclusion is based on studies in PDCs, and it is possible that these findings do not reflect the complex interactions occurring in vivo.

In conclusion, we show that loss of Notch1, in the context of activated K-ras, leads to acceleration of tumor progression and an increase in PanIN numbers in a mouse model of PDAC. This implies that Notch1 can function as a tumor suppressor in K-ras–induced PDAC, and additional studies are required to determine which downstream effectors of Notch1 signaling are essential for this activity.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank M. Wescott and A. Panikkar for technical assistance, I. Coban and D. Altinel for assistance with the analyses of the pancreatic pathology, and Denise DiFrancesco, director Wistar Animal Facility.

**Grant Support**

Pennsylvania Department of Health, the W.W. Smith Charitable Trust (J.L. Kissil), and NIH grants CA124495 (J.L. Kissil), DK056645 (A.K. Rustgi), and CA83736 (A.J. Capobianco).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 12/22/2009; revised 03/19/2010; accepted 04/01/2010; published OnlineFirst 05/18/2010.

**References**


Notch1 Functions as a Tumor Suppressor in a Model of K-ras–Induced Pancreatic Ductal Adenocarcinoma

Linda Hanlon, Jacqueline L. Avila, Renée M. Demarest, et al.

Cancer Res Published OnlineFirst May 18, 2010.