Inactivation of Smad4 Accelerates Kras$^{G12D}$-Mediated Pancreatic Neoplasia


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Abstract
Pancreatic ductal adenocarcinoma (PDAC) is one of the most fatal human malignancies, with an overall 5-year survival rate of <5%. Genetic analysis of PDAC patient samples has shown that specific disease-associated mutations are correlated with histologically defined stages of neoplastic progression in the ductal epithelium. Activating mutations in KRAS are almost uniformly present in early-stage disease, with subsequent inactivating mutations in p16$^{INK4A}$, p53, and SMAD4 occurring in more advanced lesions. In this study, we have tested whether the loss of Smad4 would cooperate with an activating Kras$^{G12D}$ mutation to promote progression to PDAC using the Pdx1-Cre transgenic system to activate Kras$^{G12D}$ and delete Smad4 in all pancreatic lineages including the ductal epithelium. Analysis of double-mutant mice showed that loss of Smad4 significantly accelerated the progression of pancreatic intraepithelial neoplasias (mPanIN) and promoted a high incidence of intraductal papillary mucinous neoplasia and active fibrosis compared with Pdx1-Cre;Kras$^{G12D}$ or Pdx1-Cre;Smad4$^{flox/flox}$ mice. Occasionally, double-mutant mice progressed to locally invasive PDAC with little evidence of metastases by 6 months of age and without the detectable loss of p53 or p16$^{INK4A}$ expression or function. The loss of Smad4 only seemed to promote disease progression in the presence of the activated Kras$^{G12D}$ allele because we observed no abnormal pathology within the pancreata of 23 Pdx1-Cre;Smad4$^{flox/flox}$ animals that were analyzed up to 8 months of age. This indicates that Smad4 is dispensable for normal pancreatic development but is critical for at least partial suppression of multiple Kras$^{G12D}$-dependent disease-associated phenotypes. [Cancer Res 2007;67(17):8121–30]

Introduction
Infiltrating pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the United States. It is almost uniformly fatal, with an overall 5-year survival rate of <5% (1, 2). The extremely poor prognosis associated with PDAC is primarily due to the advanced stage of disease at the time of clinical diagnosis and the refractory nature of PDAC to conventional chemotherapy and radiotherapy regimens.

Preinvasive neoplasias affecting the pancreatic ductal epithelium have been grouped into three major pathologic categories that include pancreatic intraepithelial neoplasias (PanIN), which are primarily observed in the small ducts, intraductal papillary mucinous neoplasms (IPMN), which occur in the main pancreatic duct or in branch ducts, and mucinous cystic neoplasms, where cystic lesions are lined by a mucin-producing, tall columnar epithelium with an associated ovarian-type stroma.

Histologic and molecular genetic characterization of ductal lesions before invasive PDAC has led to the development of a staged progression model wherein the presence of specific disease-associated mutations is correlated with defined histologic abnormalities in the ductal epithelium (3, 4). Neoplasias representing PanIN range from PanIN-1A and PanIN-1B to PanIN-2 and PanIN-3, with each stage showing increased cellular and nuclear atypia (5, 6). PanIN-3 is frequently found in association with invasive PDAC and likely represents a precursor lesion to invasive disease.

A number of mutations have been associated with specific PanIN stages, including the activation of the KRAS2 and HER-2/NEU genes and the loss of the tumor suppressor proteins CDKN2A/p16$^{INK4A}$, p53, DPC4/SMAD4, and BRCA2 (7). The presence of an activating mutation in the KRAS2 locus was detected in ~40% of early PanIN-1A and PanIN-1B lesions and is found in virtually 100% of advanced PDAC cases (8–12). Analysis of mice where a Pdx1-Cre transgene was used to activate expression of a constitutively active allele of Kras (Kras$^{G12D}$) in the ductal epithelium showed the full spectrum of preinvasive PanIN-like lesions (mPanIN), which supports the model that Kras activation is a critical initiating step in progression to PDAC (13, 14). Although loss of p16$^{INK4A}$ tumor suppressor expression can be detected in PanIN-1A and PanIN-1B lesions, it is more frequently associated with PanIN-2, wherein the ductal epithelium exhibits nuclear atypia and papillary architecture (15). Invasive PDAC, more than 90% of cases show loss of p16$^{INK4A}$ function (12, 16–19). In mice, loss of either p16$^{INK4A}$ or p53 in conjunction with constitutive Kras activity promotes progression to PDAC, which indicates that both the retinoblastoma (Rb) and p53 pathways are involved in suppression of PDAC progression (20, 21). However, the observation that p53 remains intact in a Pdx1-Cre;Kras$^{G12D}$;p16$^{INK4A}$−/− genetic background that progresses to PDAC and that p16$^{INK4A}$ remains intact in Pdx1-Cre;Kras$^{G12D}$;p53−/− ductal epithelia with PDAC indicates that inactivation of both pathways may not be necessary for fully penetrant disease in the mouse model.

Finally, inactivating mutations in the gene encoding the downstream transforming growth factor β (TGF-β) effector protein, deleted in pancreatic cancer 4 (DPC4/SMAD4/MADH4), have been found in association with late stages of PDAC progression when there is evidence of carcinoma in situ and invasive disease in ~55% of PDAC cases (22, 23). The loss of DPC4/SMAD4 has also been associated with familial juvenile polyposis (24) and
with ~15% of colorectal tumors (25). In mice, haploinsufficiency of Smad4 was sufficient to initiate gastric polyf formation and progression to invasive carcinoma that was associated with loss of the wild-type Smad4 allele in older animals (26). In a number of human PDAC cell lines that lack SMAD4, TGFβ signaling through the receptor-associated SMADs, SMAD2 and SMAD3, seems to be intact in that both proteins are serine phosphorylated and localized to the nucleus (27, 28). It has been proposed that the loss of SMAD4 may abrogate TGFβ-induced cell cycle arrest but maintain TGFβ-dependent, SMAD4-independent signaling pathways that function in tumor promotion (29).

Mutational analyses of IPMN samples have revealed similar genetic abnormalities to what has been observed in PanIN lesions, with a high incidence of activating KRAS mutations and loss of p16 and p53 expression (30–32). Loss of SMAD4 is not typically found in association with IPMN (32, 33).

In this study, we have tested whether the loss of Smad4 would cooperate with an activating KrasG12D mutation to promote progression to PDAC using the Pdx1-Cre transgenic system (34, 35). Analysis of double-mutant mice showed that loss of Smad4 significantly accelerated the progression of mPanIN lesions, the loss of acinar tissue, and the appearance of active fibrosis compared with single-mutant Pdx1-Cre;KrasG12D/ Lox/Lox or Pdx1-Cre;Smad4flox/Lox mice. Pdx1-Cre;Smad4flox/Lox mice showed no gross histologic abnormalities in the pancreas up to 8 months of age. A high frequency of double-mutant mice (14 of 16) also exhibited IPMN-like lesions of the gastric type and displayed duct ectasia with an associated pancreatitis. Lesions resembling mucinous cystic neoplasm were not observed in any of the animals and there was no evidence of ovarian-type stroma as determined by histologic examination and by negative staining for progesterone and estrogen receptor. These results indicate that, in conjunction with KrasG12D, the loss of Smad4 may stimulate PDAC progression through multiple factors including accelerated fibrosis, enhanced acinar cell loss, and more rapid promotion of neoplastic changes resembling mPanIN and IPMN within the ductal epithelium.

Materials and Methods

Mouse strains. The Pdx1-Cre transgenic strain originally generated on an ICR background was kindly provided by Doug Melton (35) and was backcrossed to C57BL/6 for four generations. The LSL-KrasG12DERTK knock-in strain on a C57BL/6×129SvJae background was purchased from the Mouse Models of Human Cancer Consortium (National Cancer Institute-Frederick) and was backcrossed to C57BL/6 for five generations. The floxed Smad4 strain on a C57BL/6 background was a kind gift from Chu-Xia Deng (36). The genotypes Pdx1-Cre;Smad4flox/flox and KrasG12DERTK;Smad4flox/flox were intercrossed to generate double-mutant mice (Pdx1-Cre;KrasG12DERTK;Smad4flox/flox). Genomic and recombination screens were done by PCR (primer sequences are given in Supplementary Methods).

Histology and immunohistochemistry. Tissues were fixed either in 70% ethanol/10% neutral buffered formalin overnight or in 4% parafomaldehyde in PBS for 4 h, and embedded in paraffin. Routine H&E staining was done with Quick Start Bradford Protein Assay (Bio-Rad Laboratories). Protein concentration was measured with a pull-down assay to quantify the level of Ras-GTP expression of activated Kras G12D within pancreatic tissue was assessed using a pull-down assay to quantify the level of Ras activity and expression of activated Kras G12D within pancreatic tissue was assessed using a pull-down assay to quantify the level of Ras activity. Protein detection was done with SuperSignal West Femto Maximum Sensitivity Substrate kit (Pierce Biotech). The primary antibodies used were Smad4 (B-8, Santa Cruz Biotechnology), p15 (Cell Signaling), p16 (M-156, Santa Cruz Biotechnology), p16 (H-140, Santa Cruz Biotechnology), p21 (SXX16, BD PharMingen), CK19 (BA17, DakoCytomation), and γ-actin (A5441, Sigma-Aldrich). The secondary antibodies used were goat anti-mouse IgG-horseradish peroxidase (HRP) and goat anti-rabbit IgG-HRP from SuperSignal West Femto Maximum Sensitivity Substrate kit. Positive controls were 3T3 lysates (for p16, p15, p21, and Smad4), purified phospho-Rb-C (for Rb), and WR19L cell lysate (for p53). For Ras activity assay, pancreatic tissues were digested with 4 mg/mL each of collagenase for 20 min at 37°C. Digested samples were then lysed in MgCl2 lysis buffer (Ras Activation Assay kit, Upstate Cell Signaling Solutions) and 1 mg of each lysate was used to measure Ras activity.

Results

Loss of Smad4 accelerates development of mPanIN lesions induced by oncogenic KrasG12D. To determine whether the loss of Smad4 would cooperate with a constitutively active allele of Kras to promote progression of PDAC, we generated F1 progeny by crossing Pdx1-Cre;Smad4flox/flox and KrasG12DERTK;Smad4flox/flox animals (Fig. 1A). Activation of the inducible KrasG12D allele occurs by Cre-mediated deletion of transcriptional and translational stop sequences that were inserted upstream of the first coding exon of the Kras locus that also contained a knocked-in G12D mutation (13, 37). Genotyping of animals was done by PCR, whereas expression of activated KrasG12D within pancreatic tissue was assessed using a pull-down assay to quantify the level of Ras-GTP (Fig. 1A). Deletion of floxed sequences within the Smad4 locus results in the loss of exon 8 and generates a null Smad4 allele in all endocrine and exocrine cells due to expression of the Pdx1-Cre transgene in pancreatic progenitor cells (Fig. 1A; refs. 34–36). Double-mutant animals (Pdx1-Cre;KrasG12DERTK;Smad4flox/flox) were
generated at the expected Mendelian frequencies, indicating that Pdx1-Cre–mediated inactivation of Smad4 was not lethal.

Histopathologic analysis of pancreatic tissue within single-mutant (KrasG12D/ or Smad4lox/lox alone) or double-mutant animals at both early (1.5–2.5 months) and late (6–8 months) time points showed normal exocrine glandular components and islets in Pdx1-Cre control (n = 5 at 1.5–2.5 months, n = 7 at 6–8 months) and in Pdx1-Cre;Smad4lox/lox animals (n = 3 at 1.5–2.5 months, n = 10 at 6–8 months; Fig. 1B). Pdx1-Cre;KrasG12D/;Smad4lox/lox mice (n = 4 at 1.5–2.5 months, n = 5 at 6–8 months) had low frequencies of low-grade PanIN-like lesions (mPanIN) at 1.5 to 2.5 months and more advanced preinvasive lesions and focal acinar-ductal metaplasia at later time points as previously reported (Fig. 1B; refs. 13, 14). Analysis of Pdx1-Cre;KrasG12D/+;Smad4lox/lox mice showed significantly accelerated progression of more advanced mPanIN (mPanIN-2) and the appearance of active fibrosis as
compared with age-matched Pdx1-Cre;Kras<sup>G12D/+</sup> mice (Fig. 1B and C). Only 1 of 17 double-mutant animals lived beyond 33 weeks of age, which limited comparative analyses beyond this point. Due to the near-complete loss of acinar tissue, which was mediated in part through acinar-ductal metaplasia (Fig. 1D, left), accurate assessment of mPanIN was often difficult in older animals and was typed in younger double-mutant animals with less severe acinar loss. Low-power examination of H&E-stained double-mutant pancreata used for Fig. 1C is shown in Supplementary Fig. S1; mouse nos. 490, 523, and 506, with mouse no. 484 not being used for analysis due to extensive acinar cell loss. In addition, Pdx1-Cre;Kras<sup>G12D/+;Smad4<sup>lox/lox</sup></sup> mice commonly exhibited ductulo-insular complex involvement in both young and older animals (Table 1; Fig. 1D, right).

**Pdx1-Cre;Kras<sup>G12D/+;Smad4<sup>lox/lox</sup></sup> mice progress to locally invasive PDAC with infrequent instances of metastases.** Further characterization of older Pdx1-Cre;Kras<sup>G12D/+;Smad4<sup>lox/lox</sup></sup> mice showed that animals between 23 and 33 weeks of age progressed to either locally invasive PDAC without metastases (Fig. 2A–C, three of five cases) or, more rarely, to adenocarcinoma with sarcomatoid features that had metastasized to the liver (Fig. 2D and E; Table 1; mouse no. 239). One younger animal also exhibited invasive, moderately differentiated adenocarcinoma (Fig. 2F; Table 1, mouse no. 484). All older Pdx1-Cre;Kras<sup>G12D/+;Smad4<sup>lox/lox</sup></sup> mice had very limited normal endocrine or exocrine tissue that remained, and the overall size of the pancreas was significantly expanded (Table 1; Supplementary Fig. S1).

**Pdx1-Cre;Kras<sup>G12D/+;Smad4<sup>lox/lox</sup></sup> mice exhibit IPMN-like lesions with duct ectasia and chronic pancreatitis.** In the majority of both young and older Pdx1-Cre;Kras<sup>G12D/+;Smad4<sup>lox/lox</sup></sup> mice (14 of 16 moribund animals, Table 1), we observed cystic lesions resembling IPMN of the gastric/foveolar subtype that occurred with or without evidence of papillae (Fig. 2G–I; refs. 6, 8, 38, 39). In most cases, the IPMN was an intraductal papillary mucinous adenoma (IPMA) that involved the branch ducts, which presented without significant architectural atypia and without papillae formation (Fig. 2G). IPMA was often accompanied by pancreatitis with duct ectasia and the occasional presence of inflammatory cells within the ducts (Supplementary Fig. S1, mouse no. 456, and data not shown). In cases of IPMN with papillae formation (Fig. 2H–L), we observed rare instances where fibrovascular cores contained endocrine cells (Fig. 2H and I). A papilla composed of acinar cells was also noted in one case (Fig. 2J). Mucinous ducts stained positive for Muc1 and were Muc2 negative (Supplementary Fig. S2A). The surrounding stroma lacked expression of both progesterone and estrogen receptor (Supplementary Fig. S2B) and did not exhibit "wavy" appearing nuclei, which are a characteristic of stroma associated with mucinous cystic neoplasm in humans. The presence of cystic lesions was also evident on gross examination of the pancreas, where large nodules were clearly evident within the expanded parenchyma (Supplementary Fig. S3). We did not observe cystic lesions in Pdx1-Cre;Smad4<sup>lox/lox</sup> animals but did note focal IPMA in two of six older Pdx1-Cre;Kras<sup>G12D/+;Smad4<sup>lox/lox</sup></sup> mice that was not associated with the loss of Smad4 based on immunohistochemical staining (ages 24 and 30 weeks; Fig. 2M–P and data not shown). Pancreata from these mice also showed ductulo-insular complex involvement (Fig. 2Q and R), indicating that these structures may be derived from Smad4-independent changes occurring downstream of Kras<sup>G12D</sup> expression.

**Immunohistochemical characterization of Pdx1-Cre;Kras<sup>G12D/+;Smad4<sup>lox/lox</sup></sup> mice.** Further characterization of Pdx1-Cre;Kras<sup>G12D/+;Smad4<sup>lox/lox</sup></sup> and Pdx1-Cre;Smad4<sup>lox/lox</sup> animals at 53 weeks of age showed that Smad4 expression was lost in ductal epithelial cells (Fig. 3, top row, arrowheads) and in acinar cells, whereas high-level expression remained evident in the stroma (two right columns, arrows). Because Pdx1 is expressed in pancreatic progenitor cells during development, Pdx1-Cre would not be expected to delete Smad4 within the stromal compartment in Pdx1-Cre;Smad4<sup>lox/lox</sup> mice. Ductal epithelia that lacked Smad4 showed nuclear phospho-Smad2 expression, which indicates that certain arms of the TGFβ signaling pathway remain intact in the absence of Smad4 (Fig. 3). Ductal lesions were composed of epithelial cells, as determined by CK19 positivity (see Fig. 3, arrowheads) and expressed mucin based on Alcian blue staining (Fig. 3). Ki-67 staining of the ductal epithelium showed that Kras<sup>G12D</sup> expression enhanced ductal proliferation ~2-fold in histologically normal ducts (as compared with Pdx1-Cre control ducts) and was significantly increased in high-grade mPanIN lesions as previously described (Supplementary Table S1; Supplementary Fig. S4; ref. 13). The loss of Smad4 had a modest, but statistically significant, effect in promotion of Kras<sup>G12D</sup>-associated ductal proliferation in early-stage mPanIN-1A lesions (P < 0.05; Table 2). Higher frequencies of proliferating ductal cells in more advanced mPanIN lacking Smad4 were noted but were not statistically significant. There was no increase in Ki-67 staining in the apparently normal ductal epithelium of Pdx1-Cre;Smad4<sup>lox/lox</sup> animals compared with Pdx1-Cre control mice.

**Loss of Smad4 in the context of Kras<sup>G12D</sup> promotes progression to PDAC without the loss of p16<sup>INK4A</sup> or p53.** Because human PDAC is associated with the loss of the p16<sup>INK4A</sup> and p53 tumor suppressors in ~90% and 75% of cases, respectively, we analyzed four independent pancreatic ductal epithelial cell lines that were clonally derived from each of two moribund Pdx1-Cre;Kras<sup>G12D/+;Smad4<sup>lox/lox</sup></sup> mice to determine if histopathologic progression might be due to the loss of either p16<sup>INK4A</sup> and/or p53 function (see Supplementary Fig. S5 for representative morphology of murine ductal cell lines compared with a human ductal epithelial cell line and for CK19 expression). Western blot analysis using an equivalent amount of protein from a pancreatic cell line derived from a Pdx1-Cre;Kras<sup>G12D/+</sup> animal (Fig. 4, Bas) or from representative early-passage cell lines clonally isolated from Pdx1-Cre;Kras<sup>G12D/+;Smad4<sup>lox/lox</sup></sup> mice (mouse nos. 162 and 337, Table 1) showed that both p53 and the p53 target genes, p21/Waf1/Cip1, were highly induced by γ-irradiation (10 Gy using a cobalt source). Sequencing of a PCR product amplified from the p53 locus also revealed that no point mutations had occurred within the p53 coding sequences (data not shown). These results suggest that the p53 signaling pathway remained intact in the presence of the Kras<sup>G12D</sup> and Smad4<sup>lox/lox</sup> mutations. Because p16<sup>INK4A</sup> is known to regulate cdk4 and cdk6 phosphorylation of the Rb protein, we analyzed for expression of both p16<sup>INK4A</sup> and phosphorylated Rb (pRb) in Kras<sup>G12D</sup>Smad4<sup>lox/lox</sup> mutant cells. Figure 4 shows that both phosphorylated forms of Rb were present in Kras<sup>G12D</sup>Smad4<sup>lox/lox</sup> mutant cells as was p16<sup>INK4A</sup>. These results indicate that significant acceleration of pancreatic neoplasia in Pdx1-Cre;Kras<sup>G12D/+;Smad4<sup>lox/lox</sup></sup> mice can occur in the presence of p16<sup>INK4A</sup> and p53. Previous studies have shown that TGFβ signaling via a Smad4-dependent pathway can induce transcription of both p15<sup>INK4B</sup> and p21/Waf1 (40–42); thus, we tested whether p15<sup>INK4B</sup> expression was affected in Smad4-deficient cells. Western blot
analysis showed that p15Ink4B was expressed at relatively high levels in Smad4-deficient cell lines as compared with the line derived from a Pdx1-Cre;KrasG12D/+ animal, which indicates that other factors can maintain p15Ink4B expression in the absence of Smad4 within the ductal epithelium.

Pdx1-Cre;KrasG12D;Smad4lox/lox mice exhibit squamous cell carcinoma in the esophagus/stomach and polypoid lesion formation in the duodenum. One limitation of the Pdx1-Cre transgenic system is expression of Pdx1 in regions outside of the pancreas during embryogenesis, including cells that give rise to the antral stomach and rostral duodenum (34). Although we noted no gross abnormalities in the pancreas of Pdx1-Cre;Smad4lox/lox mice up to 6 months of age, all Pdx1-Cre;Smad4lox/lox mice examined (n = 10) displayed significant polyp formation and had occasional cellular atypia in the duodenum that was not observed in age-matched Pdx1-Cre;KrasG12D/+ animals (Fig. 5, compare A and B with C and D). Polyps in Pdx1-Cre;Smad4lox/lox mice seemed to cause moderate obstruction and occupied as much as 50% to 70% of the intestinal lumen. This level of obstruction was not severe enough to cause significant weight loss in any of the Pdx1-Cre;Smad4lox/lox mice that were examined up to 14 months of age (data not shown). Cells lining the duodenum of age-matched Pdx1-Cre;KrasG12D/+ animals (n = 5) were mildly hyperplastic and showed cystic changes compared

Table 1. Characterization of moribund Pdx1-Cre;KrasG12D/+;Smad4lox/lox mice

<table>
<thead>
<tr>
<th>ID</th>
<th>Age (wk)</th>
<th>Weight loss*</th>
<th>Bloody ascites</th>
<th>% Abnormal PA ¹</th>
<th>PA</th>
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<th>ST</th>
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<tr>
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<td>100</td>
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<td>Polypoid obstruction</td>
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<td>N</td>
<td>N</td>
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<td>Early polypoid lesions</td>
<td>Polypoid lesions; squamous cell carcinoma from esophagus</td>
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<tr>
<td>523</td>
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<td>Y</td>
<td>N</td>
<td>50</td>
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<td>Early polypoid lesions</td>
<td>Early squamous cell carcinoma from esophagus</td>
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<tr>
<td>506</td>
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<td>N</td>
<td>30</td>
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<td>Early polypoid lesions</td>
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<td>Normal</td>
<td>Squamous cell carcinoma from esophagus</td>
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<td>N</td>
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<td>Y</td>
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<td>Polypoid obstruction; carcinoma in situ</td>
<td>Normal</td>
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</table>

NOTE: Only highest grade lesions observed in each mouse are listed.
Abbreviations: PA, pancreas; DU, duodenum; ST, stomach; Y, yes; N, no; IPMN-like, lesions resembling human gastric type IPMN; N/A, not available.
*Defined by >5% weight loss between 3-wk intervals where weight was examined.
¹Relative percentage of abnormal structure (mPanIN, neoplasia, proliferative stroma) to the whole pancreas.
with control animals (Fig. 5B and data not shown). Hyperplastic mucosae in the duodenum of Pdx1-Cre;Kras<sup>G12D/+</sup> animals were previously noted by others (13). In Pdx1-Cre;Kras<sup>G12D/+;Smad4<sup>lox/lox</sup></sup> mice, more pronounced neoplastic polypoid lesions were present in the duodenum along with evidence of carcinoma in situ by 15 weeks of age (Table 1, Fig. 5E and F). Gross examination frequently showed an enlarged duodenum (Fig. 5G, arrow) along with expansion of pancreatic tissue (asterisk).

Analysis of the stomach and esophagus in moribund Pdx1-Cre;Kras<sup>G12D/+;Smad4<sup>lox/lox</sup></sup> mice showed that 4 of 16 animals had squamous cell carcinoma that arose either from the stomach or from the esophagus as early as 6 weeks of age (Table 1; Fig. 5K–M). In two instances, carcinoma was noted to extend into the stomach and esophagus (Fig. 5I more subtle polypoid and hyperplastic foveolar cell changes in both the stomach and esophagus (Fig. 5I and data not shown). Characterization of Pdx1-Cre;Smad4<sup>lox/lox</sup> animals at 30 weeks of age revealed polypoid lesions in the stomach of two of four animals (Fig. 5J). The abnormal changes associated with the stomach/esophagus in Pdx1-Cre;Smad4<sup>lox/lox</sup> animals did not result in any death in animals observed beyond 14 months of age (n = 8). The complex phenotypes arising in moribund Pdx1-Cre;Kras<sup>G12D/+;Smad4<sup>lox/lox</sup></sup> mice often made it difficult to ascertain the precise cause of death, which occurred with a mean survival of 16 weeks (n = 16; Table 1; Supplementary Fig. S6 for Kaplan-Meier survival curve). However, it was clear that more than half of all moribund Pdx1-Cre;Kras<sup>G12D/+;Smad4<sup>lox/lox</sup></sup> animals had very little normal pancreatic tissue, which would have contributed significantly to the pathology observed in the animals, and other mice clearly showed evidence of advanced carcinomas/sarcomas that in all likelihood caused the death of the animals.

**Discussion**

Homozygous deletions and intragenic mutations at the SMAD4 locus on human chromosome 18q21.1 occur predominantly in pancreatic cancer and at reduced frequencies in colorectal and gastric cancers (22, 25, 43, 44). The specific association of loss of SMAD4 with defined cancer types supports the importance of TGFβ signaling in suppression of these malignancies. In PDAC, loss of SMAD4 occurs late in disease progression at a stage typically associated with invasive disease and subsequent to loss of the tumor suppressor genes, p16<sup>INK4A</sup> and p53, and activating mutations in KRAS (23). In our studies, homozygous deletion of Smad4 in the context of an activating Kras<sup>G12D</sup> mutation significantly accelerated neoplastic changes in the ductal epithelium that resembled both mPanIN and IPMN of the gastric/foveolar type and promoted active fibrosis that was associated with significant acinar cell loss. These changes occurred without the loss of p53 or p16<sup>ink4a</sup> (Fig. 4). Interestingly, the loss of Smad4 only seemed to promote disease progression in the presence of the Kras<sup>G12D</sup> mutation because we observed no abnormal pancreatic histology or illness in 23 Pdx1-Cre;Smad4<sup>lox/lox</sup> animals that were...
analyzed up to 14 months of age. This indicates that Smad4 is dispensable for normal pancreatic development but is critical for at least partial suppression of multiple KrasG12D-dependent disease-associated phenotypes.

Previous studies using the Pdx1-Cre transgenic model in mice have shown that homozygous loss of either p53 or p16INK4A in conjunction with the KrasG12D mutation results in the development of lethal pancreatic malignancies by 8 and 18 weeks of age, respectively (21). Deletion of both p16 Ink4A and p19Arf (in the presence of wild-type p53) accelerated lethality to 8 to 11 weeks, which indicates that both the p53 and pRb pathways are critical for suppression of PDAC progression (14, 21). In the context of Pdx1-Cre;KrasG12D/+;Smad4lox/lox mice, death occurred with a mean latency of 16 weeks, although death was likely due to tumor formation in the gastric epithelium and not to pancreatic abnormalities in ~25% of cases (Table 1). However, all moribund animals showed significant histopathologic abnormalities affecting both the pancreatic tissue and the stroma at the time of sacrifice that were substantially more progressed than age-matched Pdx1-Cre;KrasG12D/+ mice (Fig. 2B and Supplementary Fig. S1). Although Pdx1-CreKrasG12D/+;Smad4fl/fl mice progressed to locally invasive PDAC (Fig. 2A–C), their less aggressive pancreatic phenotype compared with Pdx1-CreKrasG12D/+ animals with homozygous deletions in p53 or p16INK4A supports observations in human disease where these mutations (particularly loss of p16 INK4A expression) occur in association with SMAD4 inactivation (11).

The observation that p53, p16INK4A, and p15INK4B continue to be expressed at apparently wild-type levels in ductal epithelial cell lines cultured from moribund Pdx1-Cre;KrasG12D/+;Smad4lox/lox mice might explain the less aggressive disease presentation,

Table 2. Percentage Ki-67–positive ductal cells

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>mPanIN 1A</th>
<th>mPanIN 1B</th>
<th>mPanIN 2</th>
<th>mPanIN 3</th>
<th>mPDAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pdx1-Cre</td>
<td>2.2% (699)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Pdx1-CreKrasG12D/+</td>
<td>5.9% (223)</td>
<td>10.4% (128)</td>
<td>24.1% (191)</td>
<td>69.4% (280)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Pdx1-Cre;Smad4fl/fl</td>
<td>1.0% (513)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Pdx1-CreKrasG12D/+;Smad4fl/fl</td>
<td>—</td>
<td>16.5% (240)</td>
<td>31.4% (212)</td>
<td>81.9% (275)</td>
<td>90.6% (178)</td>
<td>92.5% (65)</td>
</tr>
</tbody>
</table>

NOTE: Percentage of Ki-67–positive ductal cells within preinvasive ductal lesions in mice of the defined genotypes. The total number of ductal cells counted from at least three independent mice per genotype is shown in parentheses. Statistically significant differences in the frequencies of proliferating ductal cells were noted in mPanIN-1A ducts typed from Pdx1-CreKrasG12D/+ animals versus Pdx1-CreKrasG12D/+;Smad4fl/fl mice (P < 0.05) but not at other mPanIN stages, although all stages showed a trend toward increased proliferation with loss of Smad4.
especially with respect to metastasis, as compared with Kras<sup>G12D</sup>-expressing animals that lack p53 or p16<sup>ink4A/p19Arf</sup>. In contrast to previous murine studies where mPanIN was observed in association with Kras<sup>G12D</sup> expression, the loss of Smad4 in the context of the Kras<sup>G12D</sup> mutation resulted in the development of preneoplastic IPMN-like lesions that were evident in the majority (14 of 16) of both young and older Pdx1-Cre;Kras<sup>G12D</sup>+/Smad4<sup>lox/lox</sup> mice (Fig. 2G–I; Table 1). These observations contrast with human IPMN cases, which exhibit activated KRAS expression but typically not the loss of Smad4 (32, 33). This suggests that development of IPMN may occur through distinct genetic pathways in mice as compared with humans. Alternatively, IPMN-like changes associated with Smad4 loss in mice may be occurring due to the continued presence of p16<sup>ink4A</sup> and/or p53, which may be altering the normal genetic progression pathways associated with development of IPMN and mPanIN. This suggestion is supported by a recent study from the DePinho group, where they elegantly show that loss of p16<sup>ink4A</sup>/p19<sup>Arf</sup> results in a decreased incidence of IPMN in the context of either Pdx1-Cre;Kras<sup>G12D</sup>++;<sup>Smad4</sup><sup>lox/lox</sup> or Ptf1a-Cre;Kras<sup>G12D</sup>++;<sup>Smad4</sup><sup>lox/lox</sup> mice (45). With respect to both the subtype and incidence of IPMN and to overall survival and pancreatic histopathology, our observations of Pdx1-Cre;Kras<sup>G12D</sup>+/Smad4<sup>lox/lox</sup> mice are consistent with this recent study. Significantly, we also noted rare focal, gastric-type IPMN in two of six older Pdx1-Cre;Kras<sup>G12D</sup>+/Smad4<sup>lox/lox</sup> mice that was not associated with the loss of Smad4 (Fig. 2M–P), although the appearance of IPMN was extremely rare in Pdx1-Cre;Kras<sup>G12D</sup>+/ mice. This suggests that IPMN in mice may develop by a Smad4-independent pathway, as is presumably the case for human pancreatic tumors exhibiting IPMN. In another study, IPMN was not noted in the context of Ptf1a-Cre;Kras<sup>G12D</sup>++;<sup>Tgfbr2</sup><sup>lox/lox</sup> mice, where rapid lethality and PDAC arise in the context of mPanIN and desmoplasia (46). The contrasting ductal phenotypes associated with Kras<sup>G12D</sup> expression and the loss of either Smad4 or the type II TGFβ receptor could reflect a loss of responsiveness to other receptor signaling pathways that use Smad4, like activin or BMP. Alternatively, these differences may highlight the importance of Smad4-independent pathways in rapid tumor promotion in the Ptf1a-Cre;Kras<sup>G12D</sup>++;<sup>Tgfbr2</sup><sup>lox/lox</sup> model. Interestingly, mucinous cystic neoplasms and not IPMN was observed in another very recent study of Ptf1a-Cre;Kras<sup>G12D</sup>++;<sup>Smad4</sup><sup>lox/lox</sup> mice (47). Differences between our observations and this study could be due to modifying effects of the genetic background, which was more closely C57BL/6J in our analysis. In addition, development of mucinous cystic neoplasms may be favored over IPMN in the context of Smad4 haploinsufficiency that progresses through loss of the wild-type Smad4 allele, whereas IPMN may develop in association with rapid and complete loss of Smad4. Because another study noted IPMN and mPanIN in the context of Ptf1a-Cre (45), it is unlikely that differences in cell type– or stage-specific expression of Cre from the Pdx1 or Ptf1a transgene are causing this difference in neoplastic presentation. The complete absence of ovarian-type stroma and the lack of progesterone and estrogen receptor positivity, as well as the clear involvement of cystic lesions throughout the pancreas in association with the branch (and sometimes the main) pancreatic ducts (see Supplementary Fig. S1, mouse nos. 490 and 523 as examples), strongly rule out mucinous cystic neoplasms in the context of this study.

It is presently unclear what drives the significant histopathologic changes that are observed in Pdx1-Cre;Kras<sup>G12D</sup>++;<sup>Smad4</sup><sup>lox/lox</sup> mice. One prominent feature of Smad4 loss is the marked expansion of reactive stroma and enhanced acinar cell loss that may have been due, at least in part, to acinar-to-ductal metaplasia stimulated by Kras<sup>G12D</sup> expression in acinar tissue, as noted in previous transgenic and knock-in models (Fig. 1D; refs. 13, 48, 49). However, acinar-to-ductal metaplasia occurring in Pdx1-Cre;Kras<sup>G12D</sup>++;<sup>Smad4</sup><sup>lox/lox</sup> mice was much more focal and less pronounced than in models where Kras<sup>G12D</sup> was expressed using acinar-specific transgenes or Cre-deleter strains. The
Figure 5. Effect of Smad4 inactivation on duodenum and stomach/esophagus. A to F, Smad4 inactivation results in development of proliferative, mucinous crypts (D, arrowhead), and polyploid lesions in the duodenum of both Pdx1-Cre;Smad4lox/lox and Pdx1-Cre;KrasG12D/"+";Smad4lox/lox mice (age of representative mice: A, 33 wks; B, 30 wks; C and D, 33 wks; E and F, 33 wks). Carcinoma in situ (F, asterisk) was only observed in Pdx1-Cre;KrasG12D/"+', Smad4lox/lox mice (A–C and E, ×200; D and F, ×600). G, gross anatomy of a 33-wk-old Pdx1-Cre;KrasG12D/"+', Smad4lox/lox mouse illustrates significantly enlarged duodenum (arrow) and pancreas (asterisk). H to J, relatively normal stomach architecture observed in Pdx1-Cre-control, Pdx1-Cre;KrasG12D/"+', and Pdx1-Cre;Smad4lox/lox mice at 30 wks of age (∼200). K and L, squamous cell carcinoma (L, asterisk) in 12-wk-old Pdx1-Cre;KrasG12D/"+', Smad4lox/lox esophagus and stomach (K, ×200; L, ×600). M, squamous cell carcinoma (asterisk) in the esophagus of an 18-wk-old Pdx1-Cre;KrasG12D/"+', Smad4lox/lox animal invading into the muscular parenchyma (×100).

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References

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