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SUMMARY

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal human malignancies. To investigate the cellular origin(s) of this cancer, we determined the effect of PDAC-relevant gene mutations in distinct cell types of the adult pancreas. We show that a subpopulation of *Pdx1*-expressing cells is susceptible to oncogenic K-Ras-induced transformation without tissue injury, whereas *insulin*-expressing endocrine cells are completely refractory to transformation under these conditions. However, chronic pancreatic injury can alter their endocrine fate and allow them to serve as the cell of origin for exocrine neoplasia. These results suggest that one mechanism by which inflammation and/or tissue damage can promote neoplasia is by altering the fate of differentiated cells that are normally refractory to oncogenic stimulation.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the United States (Li et al., 2004). Identification of the cell(s) of origin of PDAC and defining the biochemical and biological changes that accompany their transformation are essential for the development of early detection and treatment tools.

The pancreas is composed of four parenchymal cell types: acinar, ductal, centroacinar (exocrine), and islet (endocrine) cells. The acinar cells synthesize and secrete zymogens into the ductal lumen. The ducts carry the enzymes to the duodenum and generate bicarbonate. The centroacinar cells (CACs) have been suggested to be progenitor cells in the adult pancreas. The islets of Langerhans are composed of endocrine cell types, which secrete insulin and other proteins that regulate glucose homeostasis (Bardeesy and DePinho, 2002). Rare cells expressing endocrine markers such as insulin can also be found associated with the acini and the ductal epithelium (Mills, 2007).

PDAC arises from in situ precursor lesions termed pancreatic intraepithelial neoplasia (PanIN) (Hruban et al., 2001). PanIN lesions include a spectrum of abnormal proliferative ductal structures (termed PanIN 1–3) that are recognized by transformation from a cuboidal to columnar epithelium, mucin production, and nuclear atypia.

Mutations that result in a constitutively active K-RAS are found in over 95% of PDACs and are believed to be an initiating event for this type of cancer (Jones et al., 2008; Rozenblum et al., 1997). *KRAS* encodes a small GTPase that in its active GTP-bound form promotes a wide range of cellular responses including proliferation, survival, migration, and metabolism (Shields et al., 2000). The *CDKN2A* locus encodes the *Ink4A* and *ARF* tumor suppressor genes and is also frequently mutated in PDAC. *INK4A* inactivation mutations are present in virtually all

SIGNIFICANCE

The identification of the cell type(s) from which PDAC originates and is maintained is essential for the development of new methods for PDAC diagnosis and treatment. In this paper, we demonstrate that oncogenic K-Ras can transform *Pdx1*-expressing cells in the adult pancreas of the mouse in the absence of tissue injury. In contrast, *insulin*-expressing cells require injury in combination with K-Ras activation for transformation. Our study emphasizes the role of both genetic and nongenetic events in PDAC initiation and provides additional mouse models to aid in the development of new treatment and diagnostic strategies.

sporadic PDAC, suggesting that INK4A/ARF normally constrains the malignant potential of mutant K-RAS. Other frequent alterations include loss-of-function mutations of the *SMAD4* and *p53* tumor suppressor genes (Jaffee et al., 2002). Moreover, developmental regulatory pathways, in particular the Notch, Sonic Hedgehog, and Wnt pathways, are often activated in PDACs (Berman et al., 2003; Miyamoto et al., 2003; Thayer et al., 2003; Wang et al., 2009).

PDAC is commonly believed to arise from transformation of ductal cells, although experimental studies have suggested other differentiated cells or progenitor/stem cells as potential origin(s) for this type of cancer. For instance, mouse models expressing various oncogenes in acinar cells during development, such as the *Elastase-Tgfα* (Wagner et al., 1998) and *Elastase-Kras^{G12D}* (Grippo et al., 2003) transgenic strains and the knockin *Mist1-Kras4B^{G12D}* animals (Tuveson et al., 2006), have implicated acinar cells as being susceptible to transformation and giving rise to tumors containing ductal elements. Transplantation of mouse islet cell cultures expressing the *polyoma virus middle T* oncogene into histocompatible mice resulted in the induction of pancreatic cancer with ductal histology (Yoshida and Hanahan, 1994), suggesting a potential endocrine origin for PDAC. A role of CACs has been proposed based on studies of mice that harbor a pancreas-specific deletion of the *PTEN* gene; these animals exhibit a proliferative expansion of CACs that can progress to carcinoma (Stanger et al., 2005). Nestin$^+$ exocrine progenitor cells have been shown to be highly responsive to K-Ras oncogenic activation and were suggested to represent a progenitor origin for PDAC (Carriere et al., 2007).

The close developmental relationships of the different pancreatic cell types and the capacity of endodermal lineages to transdifferentiate (Slack, 1995) add complexity to the PDAC cell-of-origin question. PanIN lesions can express markers of gastric and foregut differentiation (Prasad et al., 2005), suggesting that some degree of transdifferentiation occurs routinely during PDAC progression. This developmental plasticity is evident in some human PDACs that show focal expression of nonductal lineage markers, including endocrine and exocrine factors. More recently, Habbe et al. (2008) and De La O et al. (2008) have shown that activation of oncogenic K-Ras in acinar cells of the adult mouse leads to efficient mPanIN formation. These groups have also demonstrated in vivo acinar-to-ductal transdifferentiation by lineage tracing studies in these models (De La O et al., 2008; Habbe et al., 2008). Given this plasticity, it is possible that there is no exclusive cell of origin for PDAC and that specific genetic alteration(s) define the resulting malignant phenotype regardless of the cell in which they occur. Moreover, the physiological context, including tissue damage and inflammation, may alter a given cell's susceptibility to transformation. For example, Guerra et al. (2007) have reported that K-Ras activation in acinar cells (using the *K-Ras$^{+/LSLG12Vgeo}$;Elas-tTA/tetO-Cre* system) resulted in adult onset malignancy only following treatment with caerulein, which causes tissue injury and inflammation (Guerra et al., 2007).

In this study, we investigate whether PDAC arises from an exclusive cell of origin and whether a combination of genetic and nongenetic events can collaborate to induce neoplasia in a range of cell types. We describe the use of genetic manipulation of the mouse to systematically determine the effects of oncogenic K-Ras in distinct subsets of pancreatic cells of the adult mouse in a context-dependent manner.

RESULTS

Temporally Restricted Activation of Oncogenic K-Ras in Distinct Cell Populations of the Adult Pancreas

Activation of oncogenic K-Ras in the common pancreatic embryonic progenitor in the *Pdx1Cre;LSL-Kras^{G12D}* model results in mPanIN formation and spontaneous progression to PDAC (Aguirre et al., 2003; Hingorani et al., 2003). Because human PDAC is an adult-onset malignancy, we sought to engineer PDAC mouse models in which oncogenic K-Ras (Kras^{G12D}) is activated in the adult animal. We crossed the *LSL-Kras^{G12D}* strain to different cell-specific *CreER* lines and compared the phenotypic effect of Kras^{G12D} activation on these distinct cell populations in the adult pancreas (Figure 1A). The CreER lines used were: (1) the *Pdx1CreER^{TM}* transgenic strain (Gu et al., 2002), which marks the earliest pancreatic progenitor cell during development as well as adult endocrine β cells, some ductal, acinar cells, and possibly adult progenitor/stem cells (Koizumi et al., 2003; Stoffers et al., 1999; Swift et al., 1998; Wu et al., 1997); (2) the *RipCreER^{TM}* transgenic strain (Dor et al., 2004), which is selective for *insulin$^+$* cells; and (3) the acinar *procarboxypeptidase A1 CreER^{T2} (proCPA1CreER^{T2})* knockin strain (Zhou et al., 2007). The recombination specificity and efficiency of the different *CreER* strains was determined by crossing them to the *LSL-LacZ* reporter mouse and analyzing the X-gal-stained pancreata of their Tamoxifen (TM)-treated compound progeny (Figures 1B–1E and Table S1 available online). As shown in Figure 1B, in the *Pdx1CreER^{TM}* transgenic strain, recombination was achieved in a mosaic fashion in the islets, acinar, and ductal cells. The *proCPA1CreER^{T2}* strain exhibited recombination mainly in acinar cells and possibly CACs; however, recombination was evident also in a subset of ductal and islet cells. Recombination in this strain was variable, with half of the treated mice showing no evidence of recombination and the other half ranging from 0% to 49% in acinar cells (Figure 1C, Table S1, and data not shown). With TM administration, the *RipCreER^{TM}* transgenic strain was very specific and efficient in causing recombination in both insulin-producing β cells located in the islets of Langerhans and in single insulin$^+$ cells found scattered throughout the pancreas parenchyma, as shown by X-gal and coimmunofluorescent (CoIF) staining (Figures 1D and 1E, respectively, and Table S1).

Expression of Kras^{G12D} in different cell types of the adult pancreas was achieved by systemic TM administration in mice at 14–56 days of age. Because newly transformed cells would be expected to resemble their cell of origin both genetically and morphologically, we sought to define the earliest time point when transformed cells could be detected after TM administration. Therefore, mice were sacrificed at different times following the last dose, ranging from 4 to 240 days.

Kras^{G12D} Induces Transformation in *Pdx1$^+$* Cells of the Adult Pancreas

Pdx1CreER^{TM};LSL-Kras^{G12D} mice were treated with TM at day 14, 21, 24, 27, or 56 after birth and sacrificed after 4–120 days. mPanINs and ductal metaplasia were observed in all age groups

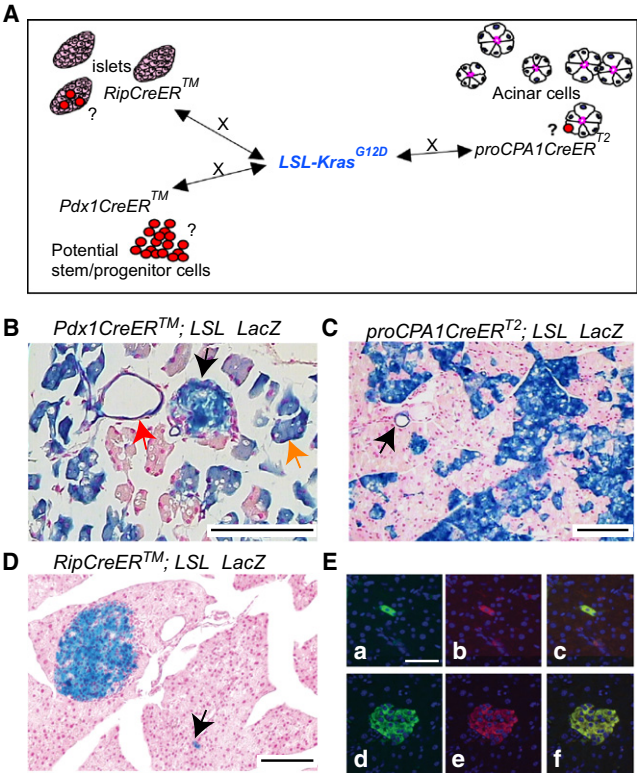

Figure 1. Kras^{G12D} Activation in Distinct Cell Populations of the Adult Pancreas

(A) Diagram describing the different mouse strains used in the study. The *LSL Kras^{G12D}* strain was crossed to the insulin^{+} cell specific *RipCreER^{TM}* strain (Dor et al., 2004); to the acinar *proCPA1CreER^{T2}* strain (Zhou et al., 2007); and to the putative stem/progenitor cell specific *Pdx1CreER^{TM}* strain (Gu et al., 2002).

(B D) X gal stained pancreata of TM treated *Pdx1CreER^{TM};LSL LacZ* (B), *proCPA1CreER^{T2};LSL LacZ* (C), and *RipCreER^{TM};LSL LacZ* (D). Note specific recombination in islet (indicated by black arrow), acinar (indicated by an orange arrow), and ductal (indicated by a red arrow) cells (B); in acinar cells (C) and a duct (C, indicated by an arrow); and in the islets of Langerhans (D) and scattered extrainsular endocrine cells (D, indicated by an arrow).

(E) LacZ and insulin CoIF staining of pancreas derived from a TM treated *RipCreER^{TM};LSL LacZ.* Note double staining of a single cell (top row) and of cells within the islets of Langerhans (lower row). (Ea) and (Ed) show insulin staining; (Eb) and (Ee) show LacZ staining; and (Ec) and (Ef) show a merge. Scale bars represent100 μm.

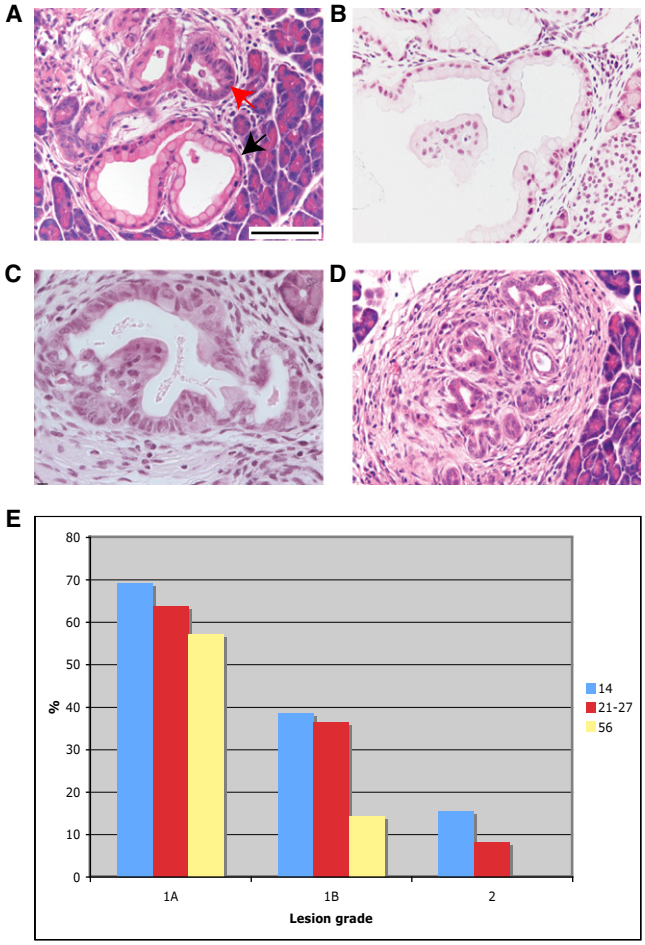

Figure 2. Kras^{G12D} Activation by *Pdx1CreER^{TM}* Causes mPanIN Formation and Ductal Metaplasia

(A D) H&E stained sections of *Pdx1CreER^{TM};LSL Kras^{G12D}* mice 20 (A) and 120 (B D) days after TM administration.

(A) mPanIN1A (black arrow). Note flat columnar epithelium with mucin accu mulation and basally oriented nuclei (red arrow indicates ductal metaplasia).

(B) mPanIN1B. Note papillary architecture with small, basally oriented nuclei.

(C) mPanIN2. Note papillary architecture, moderate nuclear pleomorphism, and partial loss of polarity.

(D) Ductal metaplasia. The scale bar represents 100 μm.

(E) Percentage of mice that developed different grades of mPanINs upon Kras^{G12D} activation at the described postnatal dates.

(Figure 2 and Table S2). Low-grade mPanIN 1A lesions were the most prevalent and were observed to a comparable extent across the three main age groups, whereas mPanIN 1B were less frequent in the 56-day-old group. Although mPanIN lesions were predominantly grade 1, grade 2 lesions were also identified, mostly at 120 days after TM administration and only in the 14- and 27-day-old treated mice (Table S2 and Figure 2E). These results suggest that the *Pdx1*$^{+}$ target cell for transformation is either less susceptible or less abundant in 56-day-old mice. mPanIN 3 was found only in one mouse that was analyzed 188 days after TM administration, and it was also the only mouse that developed PDAC. This result is consistent with observations in humans that it is unusual to find PanIN 3 in pancreata lacking invasive carcinoma (Mills, 2007).

TM-treated 14- or 27-day-old mice exhibited additional phenotypes (n = 23). One of the most notable was the appearance of acinar cells and ductal structures "embedded" in the islets of Langerhans as soon as 10 days after TM treatment (Figures 3A and 3B, respectively). The ductal structures grew over time (Figure 3C) and remained proliferative (data not shown). These lesions became elongated and produced mucin, reminiscent of mPanINs (Figure 3D). In contrast, some mPanIN in the exocrine compartment harbored cells that positively stained for endocrine markers such as glucagon, insulin, somatostatin, and PYY (Figures S1A and S1B and data not shown). Other histologic findings at 120 days after TM treatment included robust atrophy of the pancreas with a hypoplastic exocrine compartment, large cystically dilated areas, and strong stromal reaction

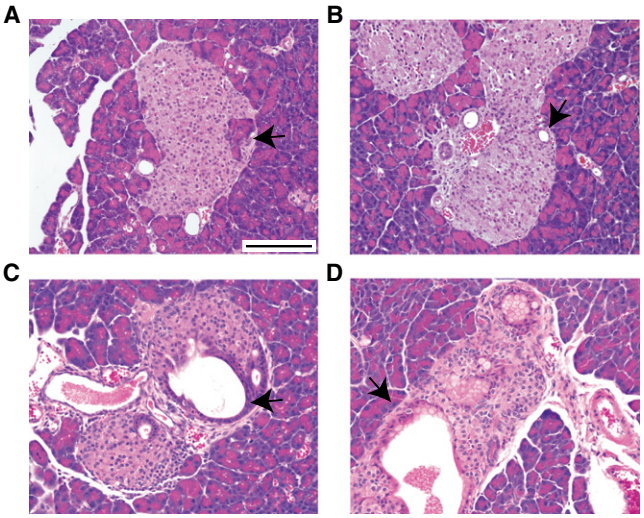

Figure 3. Kras^{G12D} Activation by *Pdx1CreER^{TM}* Causes Intraislet Ductal/Acinar Lesions

(A D) H&E stained sections of *Pdx1CreER^{TM};LSL Kras^{G12D}* mice 10 (A and B), 20 (C), and 40 (D) days after TM administration. Acinar cells (A, indicated by an arrow) and small ducts (B, indicated by an arrow) embedded within the islets are shown. Enlarged ducts within the islets (C, indicated by an arrow) are shown. Mucin producing elongated cells in ductal structures in the islets (D, indicated by an arrow) are shown. The scale bar represents 50 μm.

(Figures S1C and S1D). Importantly, no lesions arose in vehicle-treated mice (n = 15) (Table S2). These results exclude the possibility of leaky Cre recombinase activity and TM-independent K-Ras activation during development and/or adulthood. In addition, we observed no evidence of chronic pancreatitis in treated mice. Since TM is dissolved in corn oil, which by itself causes a mild form of peritonitis (Figure S1E), we treated *Pdx1CreER^{TM};LSL-Kras^{G12D}* mice with TM dissolved in carboxymethyl cellulose (CMC) by oral gavage. Dissolving TM in corn oil or CMC did not affect the specificity and efficiency of Cre-induced recombination as confirmed by X-gal staining in mice harboring a LacZ reporter (data not shown). As shown in Figure S1F, pancreata of mice treated with TM dissolved in CMC were free of peritonitis yet still developed grade 1 mPanINs (n = 5). This result precludes the possibility that the corn oil-induced peritonitis was essential for formation of mPanINs and ductal metaplasia in the *Pdx1CreER^{TM};LSL-Kras^{G12D}* model.

Activation of Kras^{G12D} in combination with mutations of *Ink4A/Arf* (Aguirre et al., 2003) or *Trp53* (Jonkers et al., 2001) in *Pdx1^{+}* cells in the adult mouse resulted in formation of moderately to poorly differentiated PDAC in 10/13 mice tested (Figure 4 and Table S2). Many of the tumors contained undifferentiated areas with sarcomatoid or anaplastic features, and extension beyond the pancreas was common. We observed direct invasion into the stomach and small intestine as well as metastasis to mediastinal lymph nodes, diaphragm, and peritoneal adipose tissue (Figure 4 and data not shown). We also identified a full range of mPanIN lesions in these mice, including grade 3 (Table S2 and Figures 4A and 4B). Thus, *Pdx1^{+}* cells, or at least a subpopulation of *Pdx1^{+}* cells, represent an excellent candidate to be the/a cell of origin for PDAC in the mouse and in humans.

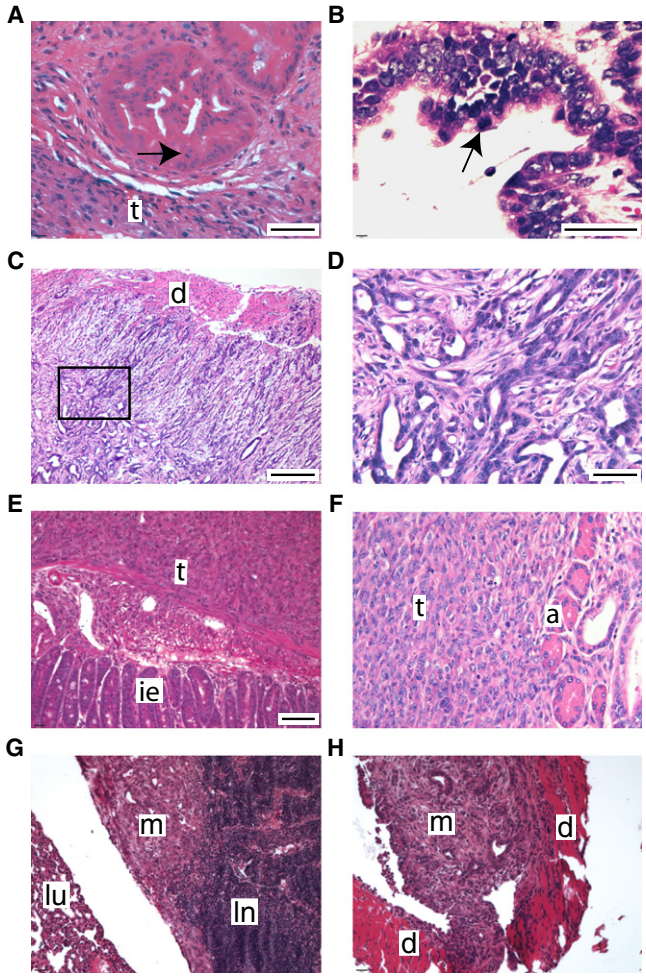

Figure 4. Kras^{G12D} Activation in Combination with p53 or Ink4A/Arf loss in *Pdx1^{+}* Cells of the Adult Pancreas Results in mPanIN3 and PDAC Development

(A E and G H) H&E stained sections of mPanIN3 and PDACs derived from *Pdx1CreER^{TM};LSL Kras^{G12D};Trp53$^{flox/flox}$* mice.

(A and B) mPanIN3 lesions. Note adjacent tumor (t) and high grade features, including cribriform architecture (A) and apical mitosis (B). Arrows point to mitotic figures.

(C and D) Invasive, moderately differentiated PDAC (note invasion through the duodenal wall [d] in [C]).

(D) Enlarged box in (C).

(E) Undifferentiated PDAC. Tumor (t) invades submucosa of the small intestine and abuts intestinal epithelium (ie).

(F) H&E stained section of undifferentiated PDAC (t) and residual normal acini (a) derived from a *Pdx1CreER^{TM};LSL Kras^{G12D};Ink4A/Arf$^{flox/flox}$* mouse.

(G and H) Metastatic PDAC.

(G) Metastasis (m) to mediastinal lymph node (ln) with adjacent lung (lu).

(H) Metastasis to diaphragm (d). The scale bars represent 100 μm in (A) and (B); 200 μm in (C); 50 μm in (D) and (F); and 200 μm (E), (G), and (H).

Targeted Expression of Kras^{G12D} in *proCPA1^{+}* Cells

Targeting the expression of Kras^{G12D} to pancreatic acinar cells and other cell types by treating *proCPA1CreER^{T2};LSL-Kras^{G12D}* mice with TM resulted in low grade mPanIN-1A formation in only 2/20 mice (Table S3 and Figure S2A). All mice were tested and confirmed for *LSL-Kras^{G12D}* recombination by PCR analysis of

DNA extracted from tissue sections (Table S3). These results suggest that *proCPA1*$^{+}$ cells are not efficiently transformed by Kras^{G12D} into mPanIN lesions in the adult mouse. It has been recently shown that Kras^{G12D} activation in acinar cells of the adult mouse leads to efficient mPanIN formation in the absence of tissue injury in the *Ela-CreERT2*$^{Tg/+}$, *Mist1*$^{CreERT2/+}$, and *ElaCreERT* strains (De La O et al., 2008; Habbe et al., 2008). The phenotypic diversity observed between these models is discussed below (see also Table S7).

To determine whether loss of p53 or Ink4A/Arf could affect the neoplastic potential of *proCPA1*$^{+}$ cells, we combined the *LSL-Kras*^{G12D} allele with the *Trp53*^{flox} or *Ink4A/Arf*^{flox} alleles on the background of the *proCPA1CreER*^{T2} strain. The pancreata derived from TM-treated *proCPA1CreER*^{T2}*;LSL-Kras*^{G12D}*;Ink4A/Arf*$^{flox/flox}$ mice (n = 7) showed no overt cancer phenotype when examined at 50, 83, 113, 158, 188, and 309 days after TM administration. However, one *proCPA1CreER*^{T2}*;LSL-Kras*^{G12D}*;Trp53*$^{flox/flox}$ mouse (n = 5) developed a moderately to poorly differentiated PDAC 81 days after TM injection at 53 days of age. The tumor contained undifferentiated areas, was locally invasive, and metastasized to the liver and diaphragm (Table S3 and Figure S2B). As described earlier, all mice in this study were analyzed for DNA recombination by PCR (Table S3). We conclude that highly aggressive PDAC can be initiated in *proCPA1*$^{+}$ cells in adult mice without chronic pancreatitis, albeit with low penetrance in this system.

In a previous study, Guerra et al. (2007) reported that selective expression of an endogenous K-Ras$^{(G12V)}$ oncogene in adult cells of the acinar/centroacinar lineage using the *K-Ras*$^{+/LSLG12Vgeo}$*; Elas-tTA/tetO-Cre* mouse model resulted in no phenotype unless the mice were pretreated with caerulein (Guerra et al., 2007). Caerulein induces chronic pancreatitis that results in regeneration of normal tissue and formation of metaplastic lesions of a ductal phenotype in the exocrine compartment (Strobel et al., 2007). The authors concluded that chronic pancreatitis can be a cofactor in the induction of PDAC by the K-Ras$^{(G12V)}$ oncogene in adult mice (Guerra et al., 2007). Because the *proCPA1CreER*^{T2}*; LSL-Kras*^{G12D} mice described above were largely refractory to Kras^{G12D}-induced transformation, we set out to test whether chronic pancreatitis could facilitate PDAC formation in these mice.

We treated 34- to 60-day-old mice (n = 9) with caerulein for a total of 44–193 days. One month after caerulein treatment initiation, the mice were treated with TM to activate Cre. In addition to caerulein-induced inflammation and ductal metaplasia, 3/9 *proCPA1 CreER*^{T2}*;LSL-Kras*^{G12D} and *proCPA1CreER*^{T2}*;LSL-Kras*^{G12D}*; LSL-LacZ* mice developed grade 1 mPanINs (Figure 5A and Table S4). We confirmed *LSL-Kras*^{G12D} recombination by PCR analysis of DNA extracted from laser-captured microdissected (LCM) lesions (Figure 5C and Table S4). Hence, the mPanINs arose from *ProCPA*$^{+}$ cells. Importantly, three out of six *proCPA1CreER*^{T2}*;LSL-Kras*^{G12D}*;p53*$^{flox/flox}$ developed PDAC when treated first with caerulein and then with TM (Figure S3B and Table S4). mPanIN grades 1 and 2 were observed, and the neoplastic lesions and advanced tumors were similar to those arising in *Pdx1CreER*^{TM} compound mutant mice with respect to histologic appearance, local invasion, and distant metastasis. In addition, a single *proCPACreER*^{tT2}*;LSL-Kras*^{G12D}*;Ink4A/Arf*$^{f/f}$ mouse that was treated first with TM and then with caerulein developed PDAC (Figure S3C and Table S4). To rule out false-positive results due to nonspecific Cre recombinase activity upon caerulein-induced injury, we treated *proCPACreER*^{T2}*;LSL-Kras*^{G12D}*; LSL-LacZ* mice (n = 3) with caerulein without prior TM administration for 8, 15, and 22 days. LacZ^{+} cells were not observed in these mice (data not shown). Thus, the *proCPA CreER*^{T2} allele remains tightly controlled under chronic pancreatitis conditions. Immunofluorescence (IF) for CPA revealed that most mPanIN cells in the *proCPA1CreER*^{T2}*; LSL-Kras*^{G12D} mouse model failed to express this marker (Figure 6D). This result supports the hypothesis that *proCPA*$^{+}$ cells can undergo injury-induced transdifferentiation and give rise to mPanIN formation in the context of Kras^{G12D} activation. In addition, our results provide evidence that pancreatic injury synergizes with Kras^{G12D} in transforming *proCPA*$^{+}$ cells and initiating PDAC.

Targeted Expression of Kras^{G12D} in *insulin*$^{+}$ Cells

Targeting the expression of Kras^{G12D} to *insulin*$^{+}$ cells by treating *RipCreER*^{TM}*;LSL-Kras*^{G12D} mice with TM did not result in mPanIN formation in any of the different age groups (n = 30), even after 8 months (Table S3). Although these results suggest that *insulin*$^{+}$ cells are not targeted for transformation by Kras^{G12D}, we nonetheless combined the *Kras*^{G12D} allele with the conditional *Trp53* or *Ink4A/Arf* alleles on the background of the *RipCreER*^{TM} strain. None of the *RipCreER*^{TM}*;LSL-Kras*^{G12D}*; Trp53*$^{flox/flox}$ mice or the *RipCreER*^{TM}*;LSL-Kras*^{G12D}*;Ink4A/ Arf*$^{flox/flox}$ mice developed mPanINs or PDAC (n = 14). As described above, recombination of *LSL-Kras*^{G12D} was confirmed by PCR analysis of DNA extracted from tissue sections of all TM-treated mice in this study (Table S3). These results strongly suggest that *insulin*$^{+}$ cells are highly refractory to transformation by multiple oncogenic mutations.

We next set out to test the effect of chronic pancreatitis on PDAC formation in the *RipCreER*^{TM}*;LSL-Kras*^{G12D} mice. Two *RipCreER*^{TM}*;LSL-Kras*^{G12D}*;LSL-LacZ* mice were treated with caerulein for a total of 84 and 89 days. One month after caerulein treatment initiation, the mice were treated with TM to activate Cre. Interestingly, grade 1 mPanINs were detected in one of these mice (Figure 5B and Table S4). We confirmed *LSL-Kras*^{G12D} recombination in the pancreas of the mouse that did not show mPanIN formation as well as in mPanINs derived from the second mouse by PCR analysis of DNA extracted from tissue section and of DNA extracted from LCM lesions, respectively (Figure 5C and Table S4). Hence, the mPanINs observed arose from cells in which the transgenic rat insulin promoter was active. Importantly, all three *RipCreER*^{TM}*;LSL-Kras*^{G12D}*;p53* $^{flox/flox}$*;LSL-LacZ* mice developed poorly and undifferentiated PDAC (n = 2 and n = 1, respectively) when treated first with caerulein and then with TM (Figure S3D and Table S4). As in the *Pdx1CreER*^{TM}*;LSL-Kras*^{G12D}*;p53* $^{flox/flox}$ mutant mice, these were highly aggressive tumors that exhibited a capacity for local invasion and distant metastasis.

One interpretation of these results (as well as those of Guerra et al. [2007]) is that caerulein treatment or inflammation caused a change in differentiated cells that made them susceptible to K-Ras activation. To investigate this possibility, we crossed the *RipCreER*^{TM}*;LSL-Kras*^{G12D} mice to the *LacZ* reporter strain and

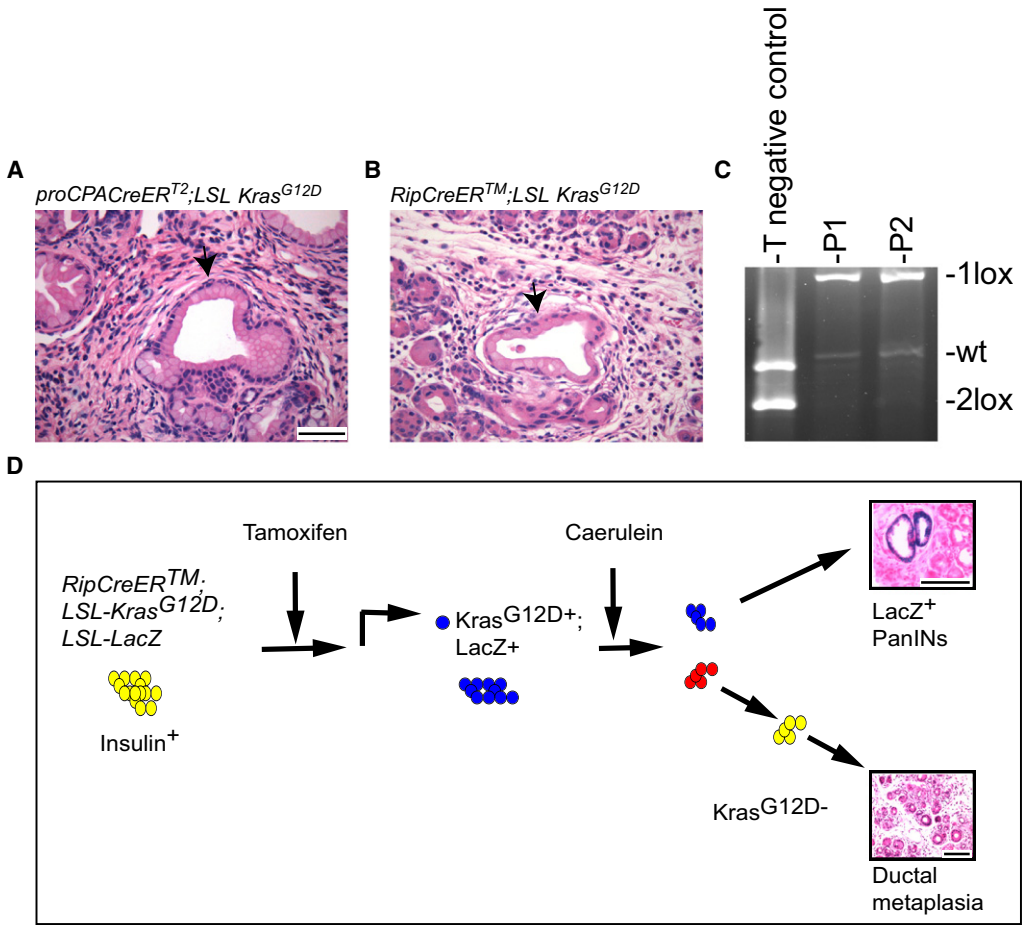

Figure 5. Chronic Pancreatitis Promotes mPanIN Formation in Mouse Models Largely Refractory to Kras^{G12D} Activation Alone

(A and B) H&E staining of pancreata derived from caerulein/TM treated *proCPA1CreER^{T2};LSL Kras^{G12D}* (A) and *RipCreER^{TM};LSL Kras^{G12D}* mice (B). Note mPanIN formation (arrows). The scale bar represents 50 μm.

(C) PCR of tail (T) and laser captured microdissected mPanINs DNA from caerulein/TM treated *proCPA1CreER^{T2};LSL Kras^{G12D}* (P1) and *RipCreER^{TM};LSL Kras^{G12D}* (P2) mice. The recombined allele (1lox) is present in the mPanINs DNA but not in the tail DNA of compound mutant mice.

(D) Suggested model of caerulein/inflammation induced mPanIN formation in TM treated *RipCreER^{TM};LSL Kras^{G12D};LSL LacZ* mice. TM administration results in recombination induced activation of Kras^{G12D} and LacZ expression in insulin^{+} cells of the adult mouse. This recombination is permanent and marks both the cells and their progeny. Upon caerulein treatments, if insulin^{+} cells are targeted for transformation by Kras^{G12D}, they will give rise to LacZ^{+} mPanINs. Otherwise, only LacZ caerulein induced metaplasia will form. The scale bar represents 50 μm in (A) and (B) and represents 100 μm in (D).

pulsed the triple compound progeny of these mice with TM 7–14 days prior to caerulein treatment initiation (n = 6) (Table S4). In this way, differentiated insulin^{+} cells and their progeny would be marked by the LacZ expression and their fate could be identified after caerulein treatment by X-gal staining (Figure 5D). Strikingly, LacZ^{+} mPanINs were found in 4/6 pancreata of these mice (Figure 6A and Table S4). In the remaining two mice mPanINs were not observed, which might be explained by the small pancreatic area analyzed (less than 2 mm^2; Table S6). In addition, two mice developed LacZ^{+} poorly to undifferentiated tumors with local invasion to adjacent tissues. These results suggest that Kras^{G12D}-expressing insulin^{+} cells can be transformed and give rise to mPanINs and PDAC specifically in the context of chronic pancreatitis.

To explore the possibility of nonspecific Cre recombinase activity upon caerulein-induced injury, we treated *RipCreER^{TM};LSL-Kras^{G12D};LSL-LacZ* mice (n = 5) with caerulein without prior TM administration. Surprisingly, despite the absence of TM, LacZ^{+} cells were observed in the islets of Langerhans and in scattered cells throughout the parenchyma as early as 2 days after caerulein treatment initiation (data not shown). Thus, although the *RipCreER^{TM}* strain is tightly controlled and does not normally show leaky expression of the Rat insulin promoter in insulin^{-} cells or TM-independent activation of Cre recombinase (Table S1), Cre activity was more promiscuous under chronic pancreatitis conditions. This result raised the possibility that the mPanIN lesions observed in the experiment described above arose in non-insulin-expressing cells following inappropriate Cre activation during the caerulein treatment. To address this possibility directly, we treated *RipCreER^{TM};LSL-LacZ* mice (n = 5) with TM for 5–7 days, treated them with caerulein for 65 days, and subsequently analyzed them for coexpression of LacZ and insulin by CoIF (Table S5). In all cases, LacZ^{+}insulin^{+} cells were observed within the islet of Langerhans. In addition,

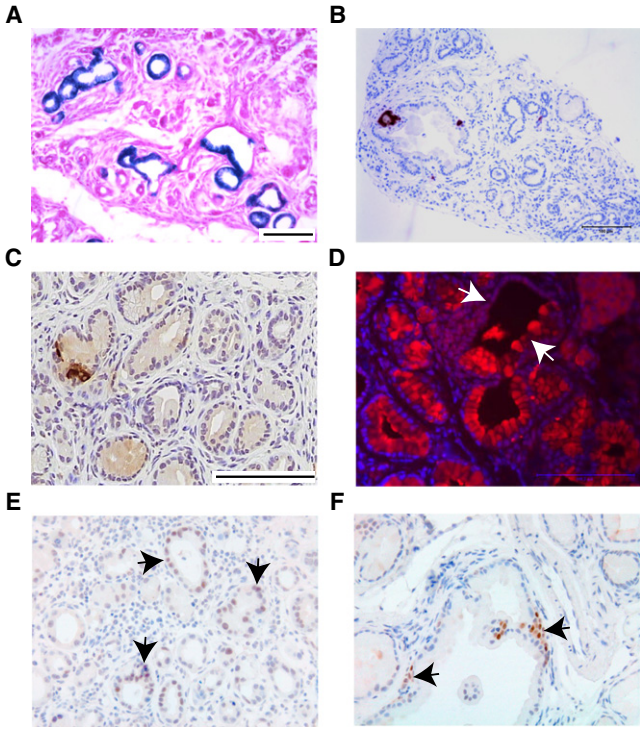

Figure 6. Characterization of mPanINs Derived from TM and then Caerulein Treated *proCPA1CreER^{T2};LSL Kras^{G12D}* and *RipCreER^{TM};LSL Kras^{G12D}* Mice

(A C and E) X gal (A), insulin (B and C), and Pdx1 (E) staining of pancreata derived from *RipCreER^{TM};LSL LacZ;LSL Kras^{G12D}* mice. Note X gal (A) and Pdx1 (E, indicated by black arrows) positive staining in mPanINs and negative staining for insulin in most mPanINs (B and C).

(D and F) IF staining for CPA1 and IHC staining for Pdx1 of pancreata derived from *proCPA1CreER^{T2};LSL Kras^{G12D}* mice. Note positive and negative CPA1 staining in the same mPanIN (D, white arrows) and positive staining for Pdx1 (F, black arrows). Bars: A and B, 100 μm; C F, 100 μm.

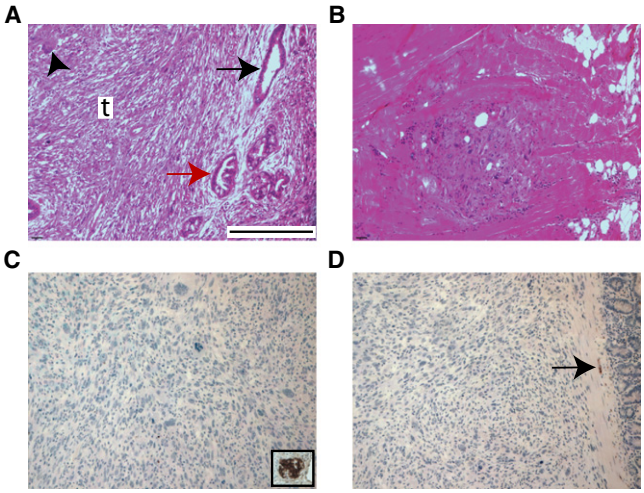

Figure 7. Kras^{G12D} Activation and p53 Loss Followed by Chronic Pancreatitis Results in mPanIN2 3 and PDAC Development from Insulin^{+} Cells

Pancreata derived from *RipCreER^{TM};LSL Kras^{G12D};Trp53$^{flox/flox}$* mice treated with TM followed by caerulein.

(A) Multiple mPanINs (grade 2 and 3 [black and red arrows, respectively]) and undifferentiated carcinoma (t). The arrowhead indicates a giant cell of invasive carcinoma.

(B) Metastatic carcinoma in the diaphragm.

(C) Negative immunostain for insulin in carcinoma. The inset shows a positive insulin immunostain in nonneoplastic islet.

(D) Negative immunostain for synaptophysin in carcinoma. Note positive staining in axon located in the smooth muscle of the small intestine (arrow). The scale bar for (A) (D) represent 400 μm.

we scored a total of 83 LacZ^{+}insulin^{+} and 6 LacZ^{+}insulin^{-} single cells scattered throughout the pancreata. We then calculated the frequency of mPanIN lesions per mm^2 in the TM/caerulein-treated *RipCreER^{TM};LSL-Kras^{G12D};LSL-LacZ* and *RipCreER^{TM};LSL-Kras^{G12D}* mice. Importantly, the frequency of mPanIN lesions in these mice was 6-fold higher than the frequency of LacZ^{+}insulin^{-} cells in TM/caerulein-treated *RipCreER^{TM};LSL-LacZ* mice ($p = 0.04$ by Wilcoxon Rank Sum Test) (Tables S5 and S6). Thus, a minority of the mPanINs observed in the TM- and caerulein-treated *RipCreER^{TM};LSL-Kras^{G12D};LSL-LacZ* and *RipCreER^{TM};LSL-Kras^{G12D}* mice might have arisen from insulin^{-} cells. However, based on this statistical analysis, it is likely that the vast majority of the mPanINs can be attributed to transformation of insulin^{+} cells. Thus, our results provide evidence that insulin^{+} cells can be made susceptible to Kras^{G12D} and give rise to mPanIN and PDAC formation following tissue injury and inflammation. Interestingly, immunohistochemistry (IHC) revealed that most mPanIN cells in this model failed to express insulin (Figures 6B and 6C). Although the mechanism through which mature insulin-producing cells undergo this fate change is unclear, it is notable that these mPanINs harbored Pdx1^{+} cells and cells that positively stain for the Notch intracellular domain (Figure 6E and data not shown).

To further study transformation of insulin^{+} cells, we treated four *RipCreER^{TM};LSL-Kras^{G12D};p53$^{flox/flox}$;LSL-LacZ* mice with TM and 14–40 days later with caerulein for additional 42–52 days. These mice developed high-grade mPanIN and poorly differentiated to undifferentiated carcinoma capable of local invasion and distant metastasis (Figures 7A and 7B and Table S4). The undifferentiated areas were morphologically identical to those observed in *Pdx1CreER^{TM};LSL-Kras^{G12D};Trp53$^{flox/flox}$* mice and also resembled human undifferentiated pancreatic carcinoma (Hoorens et al., 1998). Tumors in these mice were negative for insulin as well as the neuroendocrine marker synaptophysin by IHC (Figures 7C and 7D). Furthermore, no preneoplastic lesions were observed in the islets of these mice (data not shown). In conclusion, our results provide evidence that in combination with pancreatic injury, Kras^{G12D}-expressing insulin^{+} cells of the endocrine lineage, which are refractory to transformation under normal conditions, can also serve as a cell of origin of PDAC, a malignancy with an exocrine phenotype.

DISCUSSION

We have studied the susceptibility of adult pancreatic cells to transformation by Kras^{G12D}. We show that *Pdx1^{+}* cells can efficiently give rise to mPanIN and ductal metaplasia and, in combination with *p53* or *Ink4A/Arf* deletion, can develop into PDAC. In

contrast, in the absence of tissue injury and inflammation, *insulin*$^+$ cells (which also express *Pdx1*) did not show any overt phenotype, even in the context of tumor suppressor gene mutation. Likewise, *proCPA1*$^+$ cells were inefficiently transformed. Thus, we conclude that under noninflammatory conditions, a subpopulation of *Pdx1*$^+$ cells of the adult pancreas have heightened sensitivity to the Kras^{G12D} oncogenic effects and represent a cell of origin of PDAC. Of note, Habbe et al. (2008) have reported that *Pdx1*$^+$ cells are relatively resistant to Kras^{G12D}-induced transformation in 42-day-old mice. Our data suggest that younger mice are more susceptible to transformation of this cell type. It is also possible that the different observations are attributable to background differences between the mice used in both studies.

Four Cre lines have been used to target the expression of oncogenic K-Ras to adult acinar cells: *K-Ras*$^{+/LSLG12Vgeo}$(KRas^{G12V})*; Elas-tTA/tetO-Cre* (Guerra et al., 2007); *Ela-CreERT2*$^{Tg/+}$ and *Mist1*$^{CreERT2/+}$ (Habbe et al., 2008); *ElaCreERT* (De La O et al., 2008); and the *proCPA1CreER*^{T2} studied here. Different phenotypes, ranging from no lesion to high-grade mPanIN-3 formation, were observed in these strains (Table S7). The phenotypic diversity might be explained by several factors, including: (1) the *K-Ras*$^{+/LSLG12Vgeo}$ strain lacks the 3′ UTR element, which contains several regulatory sequences, including binding sites for the let-7 miRNA family that regulates both N-Ras and K-Ras (Johnson et al., 2005), and has been suggested to function in tumor suppression (Lee and Dutta, 2007; Mayr et al., 2007; Takamizawa et al., 2004; Yu et al., 2007). Thus, the more abundant lesion formation in the 10-day-old *K-Ras*$^{+/LSLG12Vgeo}$*; Elas-tTA/tetO-Cre* mice compared to the *proCPA1CreER*^{T2}*; LSL-Kras*^{G12D} may result from more tightly controlled translational repression of Kras^{G12D} versus K-Ras$^{(G12V)}$. (2) The models utilize distinct *K-ras* activating mutations (G12V versus G12D), which might have an effect on the oncogenic potency of K-Ras (Bardeesy and Sharpless, 2006; Collado et al., 2005; Seeburg et al., 1984). (3) Recombination efficiency differences between the different strains may exist, either because of differences between Cre-driving promoters, per se, mouse background, or different TM and doxycycline administration protocols. (4) The different mouse models may reflect a previously uncharacterized heterogeneity of acinar cells with respect to function and/or susceptibility to transformation. Nevertheless, the collective observations provide evidence that there are acinar cells in the adult pancreas sensitive to oncogenic K-Ras activation that can progress to at least mPanIN in the absence of overt tissue injury and that highly aggressive PDAC can be initiated from *proCPA1*$^+$ cells, albeit with low penetrance.

When injury was induced before recombination, the *proCPA1 CreER*^{T2}*;LSL-Kras*^{G12D} and *RipCreER*^{TM}*;LSL-Kras*^{G12D} mice became sensitive to Kras^{G12D} activation. Importantly, when we activated Kras^{G12D} first and then induced chronic pancreatitis, *RipCreER*^{TM}*;LSL-Kras*^{G12D} mice still developed mPanINs. We found that under caerulein-only treatment conditions Cre recombinase activity was not detectable in the *proCPA1CreER*^{T2} strain, although, surprisingly, it could be detected in insulin$^+$ and rare insulin$^-$ cells in the *RipCreER*^{TM} strain. This result might reflect a differential sensitivity to caerulein of CreER^{T2} (Indra et al., 1999) in the *proCPA1CreER*^{T2} versus CreER^{TM} (Danielian et al., 1998) in the *RipCreER*^{TM} strain. Alternatively, an increased amount of Cre protein might be produced by the *RipCreER*^{TM} transgene in comparison with the *proCPA1CreER*^{T2} knockin strain that might result in its greater sensitivity to caerulein. Regardless of the effects of caerulein, the vast majority of the mPanINs can be attributed to transformation of insulin$^+$ cells, although it is possible that a few mPanINs are derived from insulin$^-$ cells as well. Furthermore, 2/6 of the *RipCreER*^{TM}*; LSL-Kras*^{G12D}*;LSL-LacZ* and all *RipCreER*^{TM}*;LSL-Kras*^{G12D}*; p53* $^{flox/flox}$*;LSL-LacZ* mice (n = 4) developed high-grade mPanIN and tumors that resembled the most aggressive PDAC that arose in *Pdx1CreER*^{TM} and *proCPA1CreER*^{T2} compound mice. These tumors exhibited no morphologic or immunohistochemical evidence of neuroendocrine differentiation. In addition, the preinvasive changes in these mice were of an exocrine phenotype (i.e., mPanIN) rather than endocrine. Taken together, these results show that insulin$^+$ cells of the endocrine lineage of the adult pancreas can transdifferentiate and give rise to highly aggressive exocrine neoplasia (PDAC). Of note, LacZ$^+$ cells were not detected in ductal metaplasia in caerulein/TM-treated *RipCreER*^{TM}*;LSL-LacZ* mice (data not shown), suggesting that the transdifferentiation of the insulin$^+$ cells requires both injury and oncogenic K-Ras. Similar results were observed by Strobel et al. (2007) in *RipCreER*^{TM};Z/AP mice. Interestingly, oncogenic K-Ras activation is rarely observed in human endocrine tumors (Jonkers et al., 2007). This may in part be explained by reprogramming of endocrine cells to an exocrine fate upon injury and K-Ras activation.

The transdifferentiated cells in the *RipCreER*^{TM}*;LSL-Kras*^{G12D} mice may be insulin$^+$ cells that reside either in and/or outside the islets of Langerhans. The physiological role of the insulin$^+$ cells that reside outside of the islets is largely unknown. However, the mPanINs in the caerulein/TM-treated mice were always observed outside the islets, suggesting that these cells may be more susceptible to Kras^{G12D} than those that reside in the islets. Interestingly, neoplastic ducts were found within the islets of TM-treated *Pdx1CreER*^{TM}*;LSL-Kras*^{G12D} mice but not when Kras^{G12D} was activated during development with the nonconditional *Pdx1Cre* strain (Hingorani et al., 2003). This may reflect a developmental compensation mechanism or result from mouse background differences.

Caerulein might promote Kras^{G12D}-induced transformation by several mechanisms. It is possible that a rare population of *proCPA1*- and/or *insulin*-expressing progenitor cells that are normally susceptible to Kras^{G12D} oncogenic effect needs to be amplified by tissue injury for the appearance of the phenotype. Another possibility is that injury-induced regeneration or the secretion of inflammatory cytokines leads to the proliferation of stem cell/facultative stem cells that are most probably Pdx1$^+$. Upon the initiation of differentiation of such cells, a subset might transiently express either *proCPA1CreER*^{T2} or *RipCreER*^{TM} and, thus, activate Kras^{G12D} upon TM administration. However, our results provide evidence that *proCPA1*- and *insulin*-expressing cells can form mPanINs, favoring the hypothesis that inflammation and tissue injury facilitate PDAC development by promoting reprogramming of differentiated cells rather than by stem cell mobilization. The mechanism through which such a reprogramming event might occur requires further investigation. However, our study and those of others suggest a role for the Notch signaling pathway in this process. Caerulein-derived acinar-ductal

metaplasia is associated with Notch pathway upregulation (Gomez et al., 2004; Jensen et al., 2005; Siveke et al., 2008). We also found Notch to be activated in mPanINs derived from caerulein/TM *RipCreER^{TM};LSL-Kras^{G12D}* mice. Reactivation of the Notch signaling pathway, which is important for cell-fate decisions and maintenance of undifferentiated stem/progenitor cells during embryogenesis (Artavanis-Tsakonas et al., 1999), may be a mechanism by which differentiated cells can revert to a stem/progenitor fate or to convert to a cell type that is sensitive to Kras^{G12D} activity. It has been recently shown that Notch and K-Ras reprogram acinar cells to mPanINs (De La O et al., 2008). The precise mechanism of, and requirement for, interaction between chronic pancreatitis, Notch, and K-Ras activation for mPanIN formation from different pancreatic cells still needs to be determined.

These data add to the increasing body of evidence pointing to a remarkable plasticity of pancreatic adult differentiated cells (De La O et al., 2008; Habbe et al., 2008; Shen et al., 2000; Zhou et al., 2008) and question the existence of a pancreatic "stem cell" as well as its relevance for PDAC. Our findings suggest that Kras^{G12D} regulates the differentiation status of pancreatic epithelial progenitor/differentiated cells to a certain extent and that in combination with nongenetic stress such as inflammation this is further accelerated. More specifically, a multipotent *Pdx1^{+} Insulin^{-}* cell may exist in the adult pancreas. This cell type may be the source of the endocrine cells within mPanINs and ductal metaplasia observed in the TM-treated *Pdx1CreER^{TM};LSL-Kras^{G12D}* mice and the endocrine cells arising from the ductal lining after pancreatic duct ligation (Xu et al., 2008). It is possible that this cell type is activated only in certain settings such as injury and neoplasia and may be particularly susceptible to Kras^{G12D}-induced proliferation and differentiation. It might also be the source of the ductal structures located in the islets, if it resides both in the ductal lining as well as the islets or if it migrates to the islets under certain conditions. Alternatively, these structures may arise from a differentiated cell, such as the carbonic anhydrase II^{+} cell that gives rise to endocrine and exocrine cells after birth and injury (Inada et al., 2008), upon Kras^{G12D} activation.

Our study reveals the involvement of different cell types in PDAC initiation and provides a series of models to explore the biology and treatment of PDAC. With the advances in the ability to reprogram adult pancreatic cells in a controlled manner (Zhou et al., 2008), we expect that a better understanding of the reprogramming and transforming events attributed to Kras^{G12D} activation in combination with chronic inflammation will lead to the development of new therapeutic modalities to prevent PanIN formation or to repair the diseased/damaged tissue.

EXPERIMENTAL PROCEDURES

Mouse Strains

All animal studies and procedures were approved by the Massachusetts Institute of Technology Institutional Animal Care and Use Committee. Research was conducted in compliance with the Animal Welfare Act Regulations and other Federal statutes relating to animals and experiments involving animals and adheres to the principles set forth in the Guide for Care and Use of Laboratory Animals, National Research Council, 1996. The *LSL Kras^{G12D}* strain (Jackson et al., 2001) was crossed to the following strains: *Pdx1CreER^{TM}* (Gu et al., 2002), *proCPA1CreER^{T2}* (Zhou et al., 2007), *RipCreER^{TM}* (Dor et al., 2004), *Trp53^{flox}* (Jonkers et al., 2001), and *Ink4A/Arf^{flox}* (Aguirre et al., 2003). Primers used for genotyping by PCR are listed in Table S8 and details of reactions are available upon request. Cre strains were crossed to *R26 LSL LacZ* mice (Jackson Laboratories) to determine Cre expression patterns.

Histopathology, Immunohistochemistry, and Immunofluorescence

Histopathologic analysis of pancreata was carried out by two pathologists (G.C.C. and E.L.S.). mPanIN lesions and PDAC were graded according to consensus criteria for mouse models of pancreatic cancer (Hruban et al., 2006). We carried out IHC and IF analyses according to manufacturers' recommendations, typically with a modified citric acid unmasking protocol followed by standard detection with 3,3 diaminobenzidine with a kit from Vector Laboratories. In some cases, secondary antibodies were conjugated to AlexaFluor 594 (Invitrogen) and nuclei were counterstained with 4,6 diamidino 2 phenylindole. We used the following primary antibodies: insulin (Zymed), glucagon (Abcam), PYY (RDI), Pdx1 (gift of C.V. Wright), CPA1 (Chemicon), and β galactosidase (AbD Serotec). IF staining was performed as described previously (Jackson et al., 2001). Triple color microscopy and imaging were performed with a Nikon Eclipse E600 and a Spot cooled CCD camera and software. Images were processed with Adobe Photoshop.

β-Galactosidase Histochemistry

Pancreata were dissected and fixed in 4% PFA, incubated in 0.5 M Sucrose, and frozen in OCT. β galactosidase activity was assayed as described previously (Paratore et al., 2002).

Tamoxifen Treatment

Mice were treated with TM (Sigma) by intraperitoneal injections (i.p.) of 9 mg/40 g body weight in corn oil, one every other day for a total of three injections. For oral delivery, TM was dissolved in a 0.5% CMC, 0.4% Tween 80, and 0.9% NaCl solution.

Caerulein Treatment

Mice were i.p. injected with 0.1 ml of a 50 μg/ml caerulein (Sigma) in saline solution five times per week.

Pancreata Size Measurement

Pancreata areas were determined with Bioquant Image Analysis software in manual measurement mode.

Molecular Analysis

For verification of Cre mediated recombination, DNA was prepared from wild type tails and LCM mPanINs of caerulein/TM treated *proCPA1CreER^{T2};LSL Kras^{G12D}* and *RipCreER^{TM};LSL Kras^{G12D}* mice. PCR was performed with primers flanking the Lox Stop Lox cassette (sequences available in Table S8 and on http://web.mit.edu/jacks lab/protocols table.html). Wild type *K ras*, *2Lox*, and *1Lox K ras^{G12D}* alleles were detected, yielding 620 bp, 510 bp, and ~1100 bp products, respectively. LCM and DNA isolation were performed with the Veritas Microdissection System and the PicoPure DNA Extraction Kit from Molecular Devices, respectively.

SUPPLEMENTAL DATA

Supplemental Data include three figures and eight tables and can be found with this article online at http://www.cell.com/cancer cell/supplemental/S1535 6108(09)00338 9.

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