Pten constrains centroacinar cell expansion and malignant transformation in the pancreas

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Summary

To determine the role of the phosphatidylinositol 3-kinase (PI3-K) pathway in pancreas development, we generated a pancreas-specific knockout of *Pten*, a negative regulator of PI3-K signaling. Knockout mice display progressive replacement of the acinar pancreas with highly proliferative ductal structures that contain abundant mucins and express Pdx1 and Hes1, two markers of pancreatic progenitor cells. Moreover, a fraction of these mice develop ductal malignancy. We provide evidence that ductal metaplasia results from the expansion of centroacinar cells rather than transdifferentiation of acinar cells. These results indicate that *Pten* actively maintains the balance between different cell types in the adult pancreas and that misregulation of the PI3-K pathway in centroacinar cells may contribute to the initiation of pancreatic carcinoma in vivo.

Introduction

Pancreatic adenocarcinoma is among the most lethal human tumors, killing approximately 95% of patients within 5 years of diagnosis. Recent work has led to important advances in the understanding of pancreatic cancer biology. First, histologic evaluation of resected pancreatic adenocarcinomas has facilitated the morphologic classification of dysplastic lesions that represent the putative precursors of invasive carcinoma—pancreatic intraepithelial neoplasia (PanIN) and intraductal papillary mucinous neoplasia (IPMN). PanINs encompass a spectrum of pancreatic ductal epithelial changes characterized by enhanced mucin production, graded nuclear atypia, and loss of cell polarity (Hruban et al., 2001); IPMNs constitute another class of noninvasive precursor lesions that are histologically distinct from, but related to, PanINs (Hruban et al., 2004). Second, molecular genetic analysis has implicated several genes in the progression of pancreatic carcinoma: these include the *Kras*, *INK4A/ARF*, and *p53* genes, as well as elements of the EGF, TGFβ, Notch, and hedgehog signaling pathways (Jaffee et al., 2002; Miyamoto et al., 2003; Thayer et al., 2003). Third, several animal models of pancreatic cancer have been developed. Implantation of the carcinogen dimethylbenzanthracene (DMBA; Dissin et al., 1975) or overexpression of TGFα in exocrine tissue (Sandgren et al., 1990; Jhappan et al., 1990) causes a premalignant acinar-to-ductal metaplasia by a process that has been generally regarded to be transdifferentiation (Wagner et al., 1998, 2001; Schmid, 2002; Bockman et al., 2003). Newer models that incorporate the most frequent genetic alteration in human pancreatic cancer—an activated mu-

SIGNIFICANCE

Metaplasia—the replacement of one adult tissue type with another—is a harbinger of cancer in many tissues. It is unknown whether metaplastic cells arise by transdifferentiation or through the expansion of a minority cell type or a stem/progenitor cell. Pancreas-specific deletion of *Pten* has provided an unanticipated and informative view of this process by revealing a progressive ductal metaplastic process with infrequent malignant transformation. Molecular and histopathological analysis supports the view that expansion of centroacinar cells, rather than transdifferentiation of acinar cells, is the mechanism for metaplasia in this model. These results fortify the link between PI3-K signaling and pancreatic cancer and suggest that PI3-K may act by perturbing the balance between cell types in the adult pancreas.

tant *Kras* allele—faithfully reproduce the histologic features of invasive human disease (Hingorani et al., 2003; Aguirre et al., 2003).

In spite of this progress, critical gaps remain in our understanding of pancreatic cancer. On the molecular level, the interplay between different signaling pathways remains an area of active investigation. On the tissue level, the cell type that gives rise to ductal adenocarcinoma is not known. Proposed cellular origins for pancreatic carcinoma include duct cells (Cubilla and Fitzgerald, 1976; Hruban et al., 2001), acinar cells (Meszoely et al., 2001; Wagner et al., 2001; Bockman et al., 2003), islet cells (Yoshida and Hanahan, 1994; Pour et al., 2003), or rare undifferentiated precursor cells in the adult pancreas (Leach, 2004). Centroacinar cells have emerged as a candidate cell of origin based upon the persistent activation of the Notch pathway in these cells in adulthood (Miyamoto et al., 2003). Although centroacinar cells constitute the terminal cells of the ductal system and contain ultrastructural features of ductal cells (Ekholm et al., 1962), the precise lineage of centroacinar cells has not yet been elucidated.

The *Pten* tumor suppressor gene encodes a dual activity phosphatase that is frequently mutated in human tumors (Luo et al., 2003). As an antagonist of the phosphatidylinositol 3-kinase (PI3-K) signaling pathway, *Pten* occupies a central position in the response to signals from growth factor receptors governing cell proliferation and survival, among other biological processes. While point mutations in *Pten* occur rarely in pancreatic cancer (Sakurada et al., 1997; Okami et al., 1998), functional inactivation of PTEN through promoter methylation (Asano et al., 2004), loss of protein expression (Altomare et al., 2003), reduction of mRNA levels (Ebert et al., 2002), or loss of heterozygosity (LOH) of linked markers (Hahn et al., 1995; Okami et al., 1998) occurs with high frequency. Studies in pancreatic cancer cell lines have demonstrated that PI3-K signaling is required for growth and survival of tumor cells (Perugini et al., 2000; Shah et al., 2001; Bondar et al., 2002). Furthermore, amplification or activation of AKT2 kinase, a major target of the PI3-K complex, occurs in up to 60% of pancreatic cancers (Cheng et al., 1996; Ruggeri et al., 1998; Altomare et al., 2003; Schlieman et al., 2003), supporting the participation of an activated PI3-K-AKT axis in this disease. Nevertheless, the role of activated PI3-K signaling in the initiation or progression of pancreatic carcinoma in vivo has not been evaluated on the genetic level.

Here, to study the biological impact of pancreatic PI3-K activation, we examined the consequences of pancreas-specific *Pten* gene deletion. Serial analyses revealed progressive pancreatic abnormalities, with the gradual replacement of the entire exocrine pancreas with ductal structures lined by mucin-producing epithelium, and eventual tumor formation. This mouse model provides insights into the origin of preneoplastic metaplasia in the pancreas.

Results

Generation of mice with pancreas-specific *Pten* deletion

Immunohistochemistry was performed to determine the normal expression pattern of PTEN in the pancreas. In agreement with a previous report (Perren et al., 2000), strong PTEN staining was observed in the pancreatic ducts and islets, but not acinar cells (Figures 1A, 1B, and 1D and data not shown). In addition, PTEN staining was observed in cells that occupied the distinctive centroacinar position (Figure 1B, arrowhead and inset).

Mice carrying a "floxed" allele of the *Pten* gene (Groszer et al., 2001) were bred to *Pdx1-Cre* transgenic mice (Gu et al., 2002). Compound *Pdx1-Cre;Pten$^{lox/lox}$* mice were born at the expected Mendelian ratio and exhibited normal growth and development through weaning. Deletion of the *Pten* gene within the pancreas was confirmed by several methods. Cre activity was confirmed by crossing mice from the *Z/AP* reporter strain (Lobe et al., 1999) into the *Pdx1-Cre;Pten$^{lox/lox}$* background. As reported previously (Gu et al., 2002), widespread expression of human placental alkaline phosphatase (HPAP)—reflecting excision of a transcriptional stop sequence in the Z/AP transgene—was observed in all pancreatic lineages of adult mice (Figure 1C). Pancreas-specific deletion of *Pten* was confirmed by PCR for the recombined allele (Figure 6A and data not shown). Next, immunohistochemistry for PTEN and phospho-AKT (P-AKT) was performed. As expected, PTEN staining was lost in ducts of *Pdx1-Cre;Pten$^{lox/lox}$* mice (Figure 1E). The AKT kinase lies downstream of PI3-K, and its phosphorylation status reflects the activity of the PI3-K pathway. *Pdx1-Cre;Pten$^{lox/lox}$* but not wild-type ducts exhibited strong P-AKT staining, reflecting kinase activation (Figures 1D and 1E). Finally, cytoplasmic redistribution of the forkhead transcription factor FKHR reflects active PI3-K/AKT signaling (Brunet et al., 1999). In contrast to nuclear staining in wild-type, cytoplasmic staining was observed in duct cells of *Pten*-deficient mice (see Figure S4 in the Supplemental Data available with this article online). We infer that *Pten* was deleted in all pancreatic lineages with resulting constitutive activation of the PI3-K pathway in compartments that normally express *Pten*, namely islets, ducts, and centroacinar cells.

Pancreatic PTEN deficiency causes an islet phenotype and early expansion of the ductal lineage

The pancreata of newborn *Pdx1-Cre;Pten$^{lox/lox}$* mice appeared normal upon gross inspection. However, knockout mice displayed abnormal endocrine development, marked by the variable appearance of enlarged and irregularly shaped islets (Figures 1F and 1G). These abnormalities in islet morphogenesis did not translate into dysregulated glucose homeostasis, as fed and fasting blood glucose levels and glucose tolerance were identical in *Pten$^{lox/lox}$* and *Pdx1-Cre;Pten$^{lox/lox}$* mice (Figure 1H and data not shown). With the exception of this islet phenotype, pancreata from newborn *Pdx1-Cre;Pten$^{lox/lox}$* mice appeared histologically normal.

BrdU labeling was performed to determine whether *Pten* deficiency alters the proliferation rate in either islets or ducts. Preweaned *Pdx1-Cre;Pten$^{lox/lox}$* mice exhibited an approximately 4-fold increase in BrdU incorporation in the acinar pancreas (Figure 2A, lower panels, and data not shown), in areas that were otherwise histologically normal (Figure 2A, upper panels). Closer examination of the BrdU staining pattern revealed that a sizeable fraction of the labeled cells occupied the centroacinar position (Figure 2A, inset). A smaller and more variable enhancement of BrdU incorporation was detected in histologically normal epithelial cells present within large ducts (Figures S1A and S1B); large ducts appeared otherwise normal (data not shown).

We assessed the ductal lineage with the lectin Dolichos

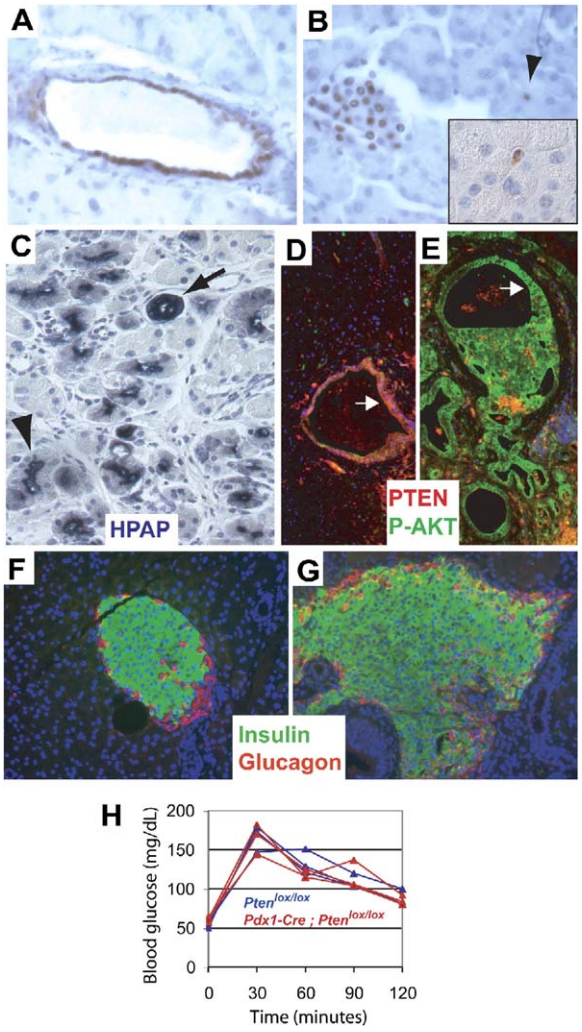

Figure 1. Expression and deletion of *Pten* in the pancreas

A and B: Expression of PTEN in pancreatic duct cells (**A**), islet cells (left portion of **B**), and a centroacinar cell (arrowhead; 800×). Centroacinar cells were also visualized with Nomarski optics (inset).

C: Evidence for Cre-mediated deletion in the pancreas. Sections of the pancreas from a *Pdx1-Cre;Pten$^{lox/lox}$; Z/AP* mouse, stained for human placental alkaline phosphatase (HPAP; dark blue) and counterstained with hematoxylin (pale blue). HPAP expression, reflecting recombination in the *Z/AP* locus, is seen in acini (arrowhead) and ducts (arrow). The HPAP reporter protein is localized to apical membranes in acinar cells (400×).

D and E: Expression of PTEN (red) and phospho-AKT (green) in the pancreas of wild-type (**D**) and *Pdx1-Cre;Pten$^{lox/lox}$* (**E**) littermates at 4 weeks of age. Nuclei are stained pale blue with DAPI. Arrows point to ductal epithelium. Note PTEN expression in wild-type ducts and scattered mesenchymal cells, but not in acinar tissue (cells in top half of **D**). PTEN expression persists in mesenchymal tissue surrounding the ducts in the mutant (scattered non-epithelial red-staining cells; 400×).

F and G: Pancreatic *Pten* deficiency causes islet hyperplasia. Immunofluorescence staining of insulin (green) and glucagon (red) in typical islets from the pancreas of *Pten$^{lox/lox}$* (**F**) and *Pdx1-Cre;Pten$^{lox/lox}$* (**G**) littermates.

H: Glucose tolerance test on *Pten$^{lox/lox}$* and *Pdx1-Cre;Pten$^{lox/lox}$* littermates (age 2 months). Despite an abnormal islet morphology, *Pdx1-Cre;Pten$^{lox/lox}$* mice responded normally to a glucose challenge.

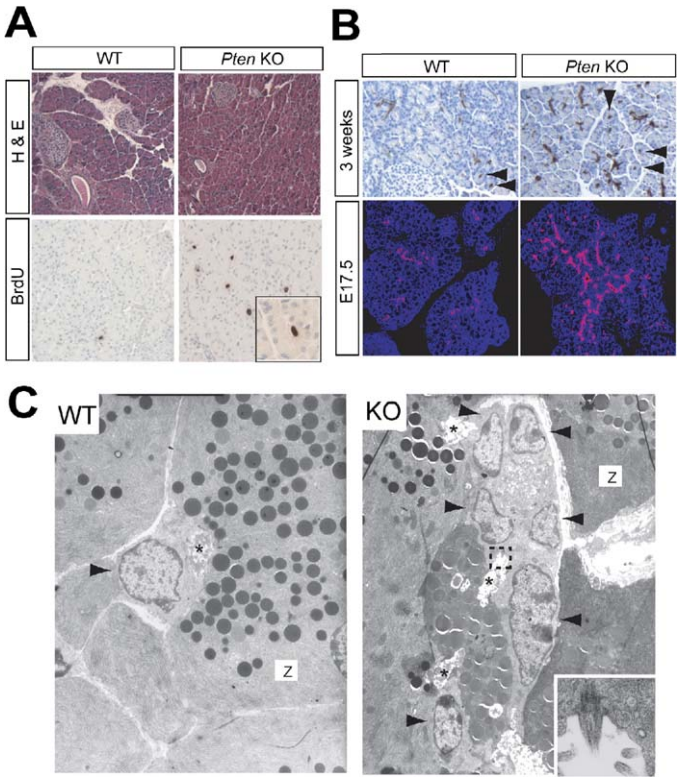

Figure 2. Increased proliferation and centroacinar cell expansion in *Pten*-deleted pancreata

A: *Pdx1-Cre;Pten$^{lox/lox}$* mice exhibit increased proliferation in the acinar compartment. Mice were injected with BrdU 2 hr prior to sacrifice. *Pdx1-Cre;Pten$^{lox/lox}$* mice (*Pten* KO) had an increased number of BrdU^{+} cells (brown) compared to *Pten$^{lox/lox}$* (WT) littermates (lower panels), despite normal acinar and ductal H&E staining (upper panels; 200×). BrdU^{+} cells tended to occupy a centroacinar position in *Pdx1-Cre;Pten$^{lox/lox}$* mice (inset; 400×).

B: *Pdx1-Cre;Pten$^{lox/lox}$* mice exhibit enhanced ductal staining with the lectin DBA. Upper panels: DBA lectin staining of 3-week-old mice (brown) showing enhancement of ductal and centroacinar (arrowheads) staining in *Pdx1-Cre;Pten$^{lox/lox}$* (*Pten* KO) mice (160×). Lower panels: E17.5 embryos stained with DBA lectin (red) and DAPI (blue), also demonstrating enhanced staining in *Pten* KO embryos (100×).

C: Electron micrographs demonstrating an increase in centroacinar cells in *Pdx1-Cre;Pten$^{lox/lox}$* mice (KO) compared to *Pten$^{lox/lox}$* mice (WT). Centroacinar cells (arrowheads) are surrounded by zymogen-rich acinar cells (Z), with which they share a lumen (asterisks; 2,950×). The basal body of a cilium (boxed area) is shown (inset; 11,500×).

biflorus agglutinin (DBA), a marker of ductal cells and some pancreatic progenitor cells (Murtaugh et al., 2003). In wild-type pancreas, DBA staining was evident in interlobular and intralobular pancreatic ducts. DBA-labeled cells were also present in acinar regions, where staining was again observed in a centroacinar pattern (Figure 2B, upper left). *Pdx1-Cre;Pten$^{lox/lox}$* mice displayed a dramatic expansion in DBA staining throughout the exocrine pancreas (Figure 2B, upper right). This increase in DBA staining was first apparent during embryogenesis, with a significant expansion of the DBA-positive population as early as E17.0 (Figure 2B, lower panels). The area stained by DBA in *Pdx1-Cre;Pten$^{lox/+}$* and *Pdx1-Cre;Pten$^{lox/lox}$* embryos occupied 3.2% ± 1.3% and 7.7% ± 3.7% of the tissue, respectively ($p = 0.0003$).

The pattern of increased BrdU and DBA staining raised the possibility that pancreatic centroacinar cells were affected by *Pten* deletion. To look directly at the centroacinar cell compartment, we analyzed pancreata from wild-type and *Pdx1-Cre; Pten$^{lox/lox}$* mice by electron microscopy. In wild-type mice, centroacinar cells were found by carefully screening pancreas sections. As described previously (Ekholm et al., 1962), these cells were smaller than acinar cells and had a low cytoplasmic density with sparse endoplasmic reticulum (Figure 2C). Centroacinar cells were found in much greater abundance in *Pdx1-Cre;Pten$^{lox/lox}$* pancreata and were present in groups of four to eight cells, contrasting dramatically with the appearance of single or doublet centroacinar cells in wild-type pancreata. Some of the centroacinar cells in mutant animals contained cilia, confirming a duct-like phenotype (Figure 2C, inset). Thus, islet hyperplasia and expansion of DBA$^+$ centroacinar cells precede other phenotypes associated with *Pten* deficiency.

Ductal metaplasia and carcinoma in *Pten*-deficient pancreata

To determine whether *Pten* deficiency results in age-dependent phenotypes, a cohort of *Pdx1-Cre;Pten$^{lox/lox}$* mice and age-matched littermate controls was followed into adulthood. Starting at approximately 3 weeks of age, mutant pancreata exhibited multifocal architectural changes. The basic lesion consisted of a ductular metaplasia of the acini, with neoductules surrounded by loose spindle mesenchyme (Figure 3A). Examination under high magnification revealed the presence of structures containing both acinar and ductular cells, suggestive of an active metaplastic transition (Figure 3B, arrow). Acini in early stages of the transition exhibited localized expansion of small cells, continuous with an enlarged acinar lumen (Figure 3B, arrowheads). More advanced metaplastic lesions exhibited a lobular distribution, with lobules exhibiting metaplasia positioned adjacent to lobules that appeared preserved (Figure 3C). These pancreata exhibited extensive ductular complexes that completely replaced the acinar parenchyma but spared the endocrine islets (Figure 3D).

Because *Pdx1* promoter activity is largely confined to the islets of adult pancreas (Figure 4B), recombination in other compartments of the pancreas was likely to have occurred during embryonic development. To further test this, *Pdx1-CreER^{TM}; Pten$^{lox/lox}$* mice (Gu et al., 2002) were injected with tamoxifen (TM) at 2–3 weeks of age and examined 8–10 weeks later (n = 10 mice). These mice exhibited islet hyperplasia but no ductal abnormalities or increase in DBA staining (data not shown), suggesting that the ductal phenotype in *Pdx1-Cre; Pten$^{lox/lox}$* mice is caused by embryonic *Pten* loss.

At approximately 2–3 months of age, *Pdx1-Cre;Pten$^{lox/lox}$* mice began to show signs of illness, with wasting, respiratory distress, and frequent death before 6 months of age. Necropsy of several animals revealed the presence of a cystic mass occupying the pancreatic space. Histologically, some tubular complexes took on a cystic appearance (Figure 3E, asterisks) and exhibited mucinous metaplasia (Figure 3F). An inflammatory infiltrate with extension of inflammation into the ductular epithelium was noted in some samples.

Malignant transformation was observed in two *Pdx1-Cre; Pten$^{lox/lox}$* animals (out of 14 adult mice that were examined histologically), both of which exhibited bloody ascites and a firm pancreatic mass. In one 11-week-old animal malignant transformation to a papillary ductal adenocarcinoma was observed; the tumor contained exuberant micropapillary projections, with marked nuclear atypia, mitoses, and apoptosis (Figure 3G). The tumor stained with antibodies that recognize the mucin Muc5AC, and some areas exhibited small glandular crowding that was suspicious for invasion (Figure S2 and data not shown). Regions of continuity were observed between the mucinous and carcinomatous epithelia (Figure S2), suggesting that the tumor had arisen directly from the metaplastic epithelium. A second 13-month-old *Pdx1-Cre;Pten$^{lox/lox}$* animal developed a pancreatic ductal adenocarcinoma that invaded into the duodenum and exhibited a desmoplastic stroma (Figure 3H

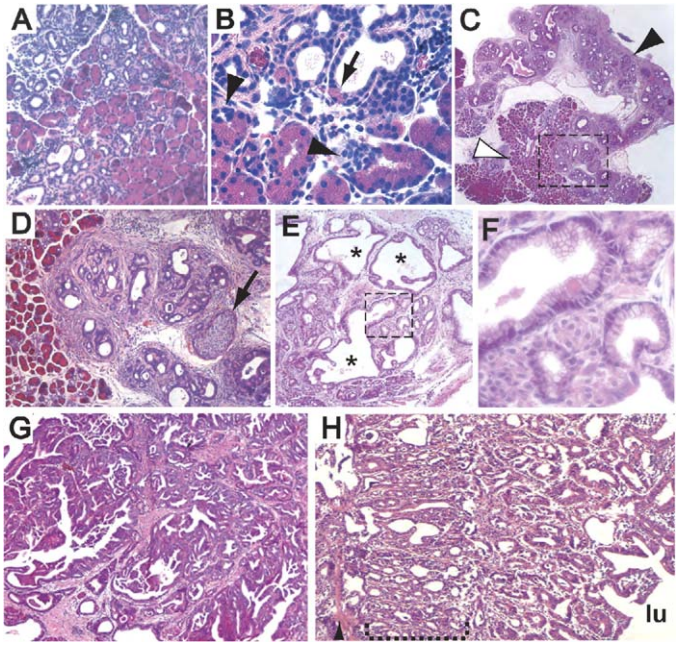

Figure 3. *Pten* deficiency leads to progressive ductal metaplasia and malignant transformation

A: *Pdx1-Cre;Pten$^{lox/lox}$* pancreas, 4 weeks. A zone of transition is seen, with diffuse metaplasia of acinar tissue to a ductal phenotype (160×).

B: High-magnification view from **A**. Structures having both acinar (arrow) and ductal features are readily seen. Some acini display an increased number of small cells in continuity with an enlarged acinar lumen (arrowheads; 640×).

C: *Pdx1-Cre;Pten$^{lox/lox}$* pancreas, 17 weeks. The body of the pancreas has been replaced by ductal structures (black arrowhead). A few lobules containing normal-appearing acinar parenchyma remain intact (white arrowhead; 20×).

D: Magnified view of the boxed area in **C**. A lobule that has undergone metaplastic transformation is shown adjacent to normal acinar tissue. An islet within the metaplastic region appears normal (arrow; 100×).

E: *Pdx1-Cre;Pten$^{lox/lox}$* pancreas, 11 weeks, showing cystic dilation of neoductules (asterisks). A hyalinized stroma is visible between the ducts (100×).

F: Magnified view of boxed area in **E** showing tall mucin-producing epithelial cells within the neoductules (400×).

G: Papillary ductal adenocarcinoma arising in an 11-week-old *Pdx1-Cre; Pten$^{lox/lox}$* animal. The ductal epithelium is characterized by proliferative epithelial fronts (100×).

H: Duodenum invaded by adjacent pancreatic ductal adenocarcinoma. The duodenal lamina propria is replaced by well-differentiated pancreatic adenocarcinoma (dashed line) that has invaded through the muscularis mucosae (arrowhead). The villous duodenal surface (lu) is preserved (100×).

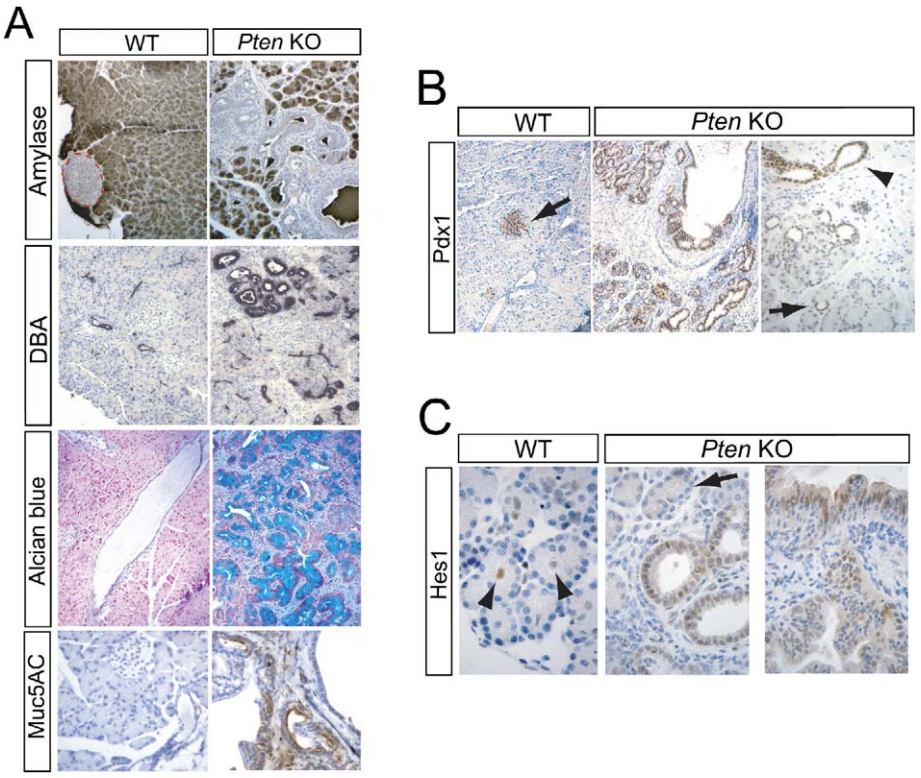

Figure 4. Features of the *Pten*-deficient metaplastic epithelium

A: Wild-type (WT; left) or *Pdx1-Cre;Pten*$^{lox/lox}$ (*Pten* KO; right) pancreata (age 9 to 11 weeks) stained by the indicated method. Metaplastic ductules demonstrate loss of amylase expression and retention of DBA staining. Alcian blue reveals abundant acid mucins in the *Pten* KO pancreas. Immunohistochemistry for Muc5AC shows staining in the metaplastic epithelium. (Muc5AC staining 400×; all others 200×).

B: Pdx1 immunohistochemistry. Left panel: strong Pdx1 staining in adult wild-type mice is limited to islets (arrow). Middle panel: Pdx1 is highly expressed in the metaplastic ductules. Right panel: structures containing acinar and duct-like cells demonstrate Pdx1 staining in emerging ductal elements (arrow); staining intensity increases in more well-formed ductal structures (arrowhead; 200×).

C: Hes1 immunohistochemistry. Left panel: Hes1 staining is observed in centroacinar cells of wild-type mice (arrowheads). Middle and right panels: Hes1 is expressed in the abnormal epithelium of *Pdx1-Cre;Pten*$^{lox/lox}$ mice, but not in adjacent acinar cells (arrow; 400×).

and Figure S2). Macrometastatic disease was not observed in either animal.

A homozygous mutant allele of *p53* was introduced into the *Pdx1-Cre;Pten*$^{lox/lox}$ background to determine whether loss of this tumor suppressor gene altered the spectrum or kinetics of tumor formation. Two *Pdx1-Cre;Pten*$^{lox/lox}$*;p53*$^{-/-}$ animals (out of five examined) developed papillary adenocarcinomas (age 4 and 6 months) that were histologically indistinguishable from the papillary ductal adenocarcinoma described above (data not shown). In addition, one (out of five) *Pdx1-Cre;Pten*$^{lox/lox}$*; p53*$^{-/-}$ animals developed a small acinar carcinoma (data not shown).

Features of the metaplastic epithelium

To better understand the mechanism of ductal metaplasia, pancreata of 9- to 11-week-old *Pdx1-Cre;Pten*$^{lox/lox}$ mice were stained for a variety of markers. Staining was initially performed for amylase, which marks acinar cells, and the lectin DBA, which marks centroacinar cells (Figure 2), ductal cells, and embryonic pancreatic progenitor cells. Most of the tubular complexes were positive for DBA and negative for amylase (Figure 4A, upper panels), although numerous structures containing both acinar and ductal features could be observed (Figures 3B and 5B). Mucinous metaplasia of the ductal epithelium was confirmed by Alcian blue staining and ranged from a few cells to complete ductular replacement; widespread epithelial expression of Muc5AC (Figure 4A, lower panels) but not Muc2 (data not shown) was observed.

We next examined the mutant epithelium for markers expressed during pancreatic development, as developmental programs have been proposed to play a role in pancreatic carcinogenesis (Meszoely et al., 2001). The mutant epithelium expressed Pdx1 (Figure 4B, middle panel), a marker of early foregut and pancreatic progenitor cells (Offield et al., 1996; Gu et al., 2002) that in the normal adult pancreas is expressed in islets (Figure 4B, left panel). Areas containing both acini and neoductules (henceforth referred to as "transitional areas") demonstrated low levels of Pdx1 in new duct-like cells and higher levels of expression in more mature-appearing ductal structures (Figure 4B, right panel). No increase in Pdx1 staining was observed in the normal-appearing areas of *Pdx1-Cre; Pten*$^{lox/lox}$ mice (Figure 4B and data not shown). Expression of the Notch pathway target gene *Hes1*, another marker of early pancreatic progenitor cells, was also assessed. Consistent with prior reports (Miyamoto et al., 2003), Hes1 expression in normal adult pancreas was limited to centroacinar cells (Figure 4C, left panel) and some duct cells (Figure S3). In contrast, Hes1 was expressed throughout the mutant epithelium of *Pdx1-Cre;Pten*$^{lox/lox}$ mice (Figure 4C, middle and right panels). Nkx6.1, another marker of pancreatic progenitors (Hald et al., 2003), was absent (data not shown).

Although ductal metaplasia was the predominant phenotype observed in *Pdx1-Cre;Pten*$^{lox/lox}$ mice, we also examined *Pdx1-Cre;Pten*$^{lox/lox}$ mice for duct-specific phenotypes. Recently, consensus criteria have been established for "mouse PanINs" that account for the greater frequency of ductal metaplasia in mouse pancreas compared to human. Specifically, mouse PanINs must meet the cytologic criteria for human PanINs plus an additional standard—the lesions must arise in the setting of a preexisting duct and not a metaplastic duct (R.H. Hruban, N.V. Adsay, J. Albores-Saavedra, M.R. Anver, A.V. Biankin, G.P. Boivin, E.E. Furth, T. Furukawa, A. Klein, D.S.

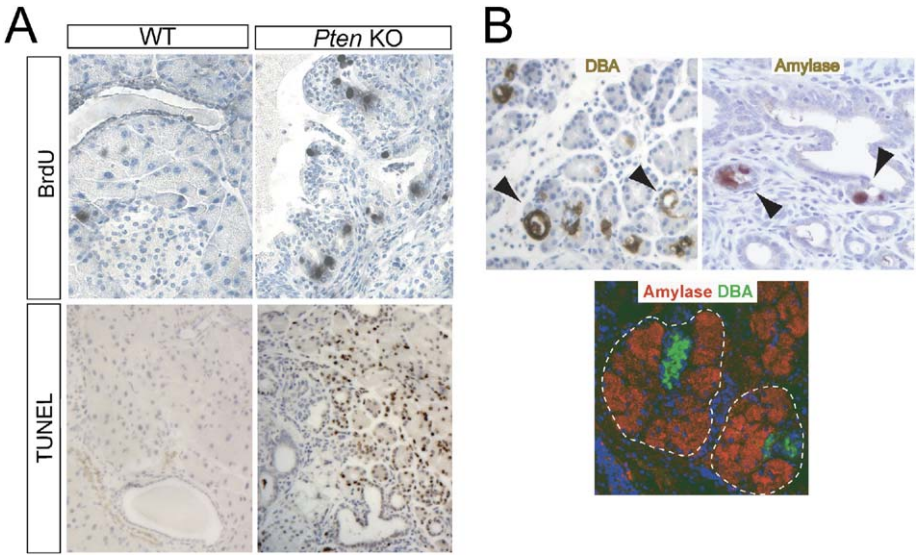

Figure 5. Analysis of "transitional areas" suggests that ductal metaplasia occurs by replacement, rather than transdifferentiation, of acinar cells

A: Epithelial hyperproliferation and acinar cell death accompany ductal metaplasia. Upper panels: BrdU staining (gray) demonstrates hyperproliferation of metaplastic ductules (*Pten* KO; right) compared to wild-type ducts (WT; left) in mice injected with BrdU 3 hr prior to sacrifice. Lower panels: TUNEL staining (brown) demonstrates little or no apoptosis in wild-type pancreas (left) and extensive death in an area of ductal metaplasia in a *Pten*-deficient pancreas (right). Substantially less TUNEL staining is observed in the tubular complexes of the mutant compared to the acini.

B: Transitional areas lack cells that coexpress acinar and ductal markers. During the early stages of metaplasia, transitional areas with mixed acinar-ductal structures are abundant (see also Figure 3B). Such structures contain duct-like cells (DBA-labeled; left) and acinar cells (amylase$^+$; right) within a tubular complex. Double-stained sections were analyzed by confocal microscopy (lower panel). Shown are two acini in transition to duct-like epithelium (dashed lines). No cells exhibiting costaining with amylase and DBA were observed, as would have been predicted if DBA-labeled cells were derived from acinar cells.

Klimstra, G. Klöppel, G.Y.L., D.S. Longnecker, J. Lüttges, A. Maitra, G.J.A. Offerhaus, L. Pérez-Gallego, M. Redston, and D.A. Tuveson, unpublished data). Two *Pdx1-Cre;Pten$^{lox/lox}$* animals (out of 14 examined) exhibited ducts with tall mucin-producing columnar cells, and a third animal exhibited a more advanced ductal phenotype characterized by nuclear pseudostratification and atypia (Figure S1). These changes were distinct from the changes observed in the metaplastic ductules and occurred in the setting of preexisting ducts. Therefore, pancreatic deletion of *Pten* also results in mouse PanINs, even when the restrictive criteria established by the consensus panel are utilized.

Metaplasia occurs by replacement, rather than transdifferentiation

As ductal metaplasia and/or PanIN formation precede pancreatic carcinogenesis, we sought to determine the cellular origins of these lesions. We reasoned that metaplasia might occur by one of two mechanisms: (1) expansion of a ductal lineage cell with parallel acinar cell loss, or (2) transdifferentiation of acinar cells into ductal cells, as proposed previously (Wagner et al., 1998; Schmid et al., 1999). We performed several experiments to distinguish between these two mechanisms. If metaplasia occurs through expansion and replacement, we would predict that cellular proliferation would be detectable prior to histologic changes, and that increased cell death would accompany the loss of acinar tissue. In accordance with this model, BrdU labeling of young *Pdx1-Cre;Pten$^{lox/lox}$* mice had demonstrated that centroacinar cells are highly proliferative before the onset of metaplasia (Figure 2A). This hyperproliferative state was present at an even greater level following metaplastic changes (Figure 5A, upper panels). Furthermore, TUNEL staining of *Pdx1-Cre;Pten$^{lox/lox}$* pancreata revealed a significant increase in the apoptosis of acinar cells in transitional areas (Figure 5A, bottom panels). Amylase/TUNEL coimmunostaining revealed the presence of numerous double-positive cells within the lumens of the neoductules, suggesting that acinar cell bodies had been deposited into the lumen (data not shown).

One prediction of an acinar-to-ductal transdifferentiation model is that intermediate cell types should be detectable. We focused our search on transitional areas, in which structures containing both ductal and acinar elements had been observed (Figure 5B, upper panels). Examination of multiple areas by confocal microscopy failed to reveal any cells that displayed both amylase immunoreactivity and DBA staining (Figure 5B, lower panel), and thus we find no evidence for an intermediate cell type.

Neoductules do not arise from acinar cells

Transdifferentiation and expansion/replacement models for metaplasia also differ with respect to the cell of origin. A transdifferentiation model entails a direct conversion from acinar to ductal fate (Schmid et al., 1999), and thus by definition the cell of origin would be acinar. By contrast, an expansion/replacement model would entail a nonacinar cell type overtaking the acinar pancreas. To further test these models, we took advantage of the Cre system to delete *Pten* in acinar cells.

Transgenic mice were generated in which a cDNA encoding a tamoxifen (TM)-dependent Cre recombinase (CreER^{TM}; Danielian et al., 1998; Gu et al., 2002; Dor et al., 2004) was placed under the control of the elastase gene enhancer (Ornitz et al., 1987; Swift et al., 1989). *Elastase-CreER^{TM}* mice were bred with *Z/AP* reporter mice to test the acinar specificity of this deleter strain; upon intraperitoneal injection of the synthetic estrogen agonist TM, *Elastase-CreER^{TM};Z/AP* mice exhibited widespread HPAP activity in acini but not large ducts or centroacinar cells (Figure 6 and Figure S6). Further confirmation that the *Elastase-CreER^{TM}* strain mediates recombination in acinar (but not centroacinar) cells was achieved by breeding *Elastase-CreER^{TM}* mice to *Z/EG* reporter mice, which express EGFP in the progeny of cells that have undergone Cre-mediated recombination. Confocal microscopy of TM-injected *Elas-*

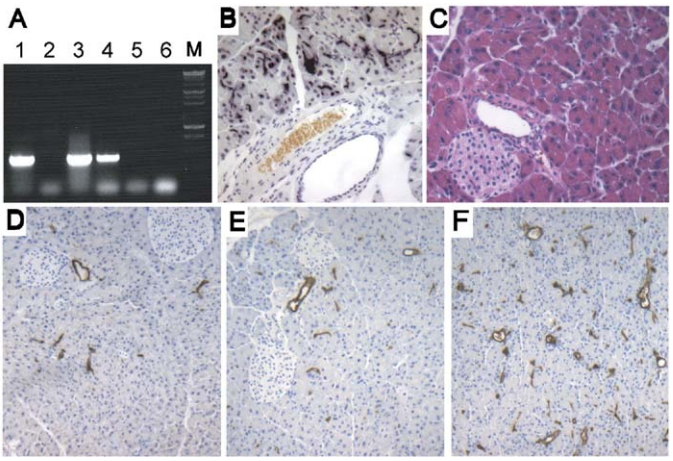

Figure 6. Deletion of *Pten* in acinar cells does not result in ductal metaplasia or enhanced DBA staining

A: PCR for the deleted *Pten* allele (Δ5; product size 300 bp) showing robust deletion of *Pten* with the *Elastase-CreER*^{TM} strain. The following DNA templates were used. Lanes 1 and 2: pancreas (lane 1) or lung (lane 2) from a *Pdx1-Cre;Pten*$^{lox/lox}$ mouse. Lane 3: pancreas from an *Elastase-CreER*^{TM}*;Pten*$^{lox/+}$ pancreas 1 week after three injections with tamoxifen. Lanes 4 and 5: tail DNA from a *Pten*$^{lox/\Delta 5}$ (lane 4) or *Pten*$^{lox/lox}$ (lane 5) mouse. Lane 6: no DNA. M, molecular weight standard.

B: Cre-mediated recombination in acinar cells. Staining for human placental alkaline phosphatase (HPAP) in the pancreas of a 12-week-old *Elastase-CreER*^{TM}*;Pten*$^{lox/\Delta}$*;Z/AP* mouse after a series of tamoxifen (TM) injections. HPAP is sorted to the apical membrane of acinar cells. Ducts and blood vessels (bottom half of the image) are not labeled (320×).

C: Normal pancreas histology in *Elastase-CreER*^{TM}*;Pten*$^{lox/\Delta}$ mice at 12 weeks of age, 1 month after TM injection (400×).

D–F: Wild-type (**D**) and TM-injected *Elastase-CreER*^{TM}*;Pten*$^{lox/\Delta}$ mice (**E**) display equivalent levels of DBA lectin staining. For comparison, increased DBA staining is evident in *Pdx1-Cre;Pten*$^{lox/lox}$ mice (**F**) (200×).

tase-CreER^{TM}*;Z/EG* mice revealed efficient labeling of acinar cells without evidence for centroacinar cell labeling (Figure S6).

Elastase-CreER^{TM}*;Z/AP* mice were then bred into the *Pten* mutant background, resulting in pancreatic deletion of the *Pten* gene (Figure 6A). To achieve more efficient deletion of *Pten* in the acinar pancreas, we used mice in which one *Pten* allele had already been deleted in the germline (*Pten*$^{lox/\Delta}$; Groszer et al., 2001). Despite widespread recombination in the acinar pancreas 4 weeks after multiple TM injections (Figure 6B), *Elastase-CreER*^{TM}*;Pten*$^{lox/\Delta}$*;Z/AP* pancreata exhibited no ductal metaplasia (Figure 6C) and no enhanced staining for DBA compared to wild-type, unlike *Pdx1-Cre;Pten*$^{lox/lox}$ pancreata (Figures 6D–6F). We conclude that ductal metaplasia in *Pdx1-Cre;Pten*$^{lox/lox}$ mice results from the deletion of *Pten* in a cell type other than mature acinar cells.

Discussion

In this study, mice with a pancreas-specific deletion of the *Pten* gene exhibited widespread ductal metaplasia with increased mucin production and developed PanIN lesions and malignant transformation with low frequency. We have examined the process of metaplasia in detail and conclude that centroacinar cells, the terminal cells of the pancreatic ductal system, are instrumental in initiating the process. These results strengthen the association between the activation of PI3-K and pancreatic carcinogenesis and give further insight into the cellular origins of preneoplastic lesions in the pancreas.

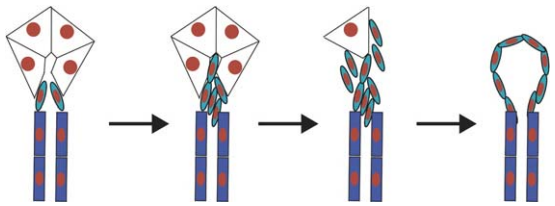

Figure 7. Model for *Pten* deletion and ductal metaplasia

Pten-deficient centroacinar cells (green ovals) begin to proliferate during development. At a few weeks of age, centroacinar cell expansion leads to apoptosis of acinar cells (white triangles) due to loss of acinar integrity or survival signals. Progeny of these centroacinar cells form tubular complexes with mucinous metaplasia. See text for details.

Model for ductal metaplasia

Metaplasia—the widespread interconversion within a tissue of one cell type into another—is observed in association with cancers of the esophagus, stomach, lung, and bladder (Tosh and Slack, 2002) and has also been reported in association with pancreatic cancer in both humans and animal models (Parsa et al., 1985; Bockman et al., 2003). Metaplastic transformation could occur through one of two mechanisms: transdifferentiation or the expansion of a precursor cell that displaces the normal tissue. Transdifferentiation has been proposed as a possible mechanism of metaplasia in transgenic mice that misexpress TGFα in the exocrine pancreas (Wagner et al., 1998), although it should be noted that this interpretation was based mainly on the expression of duct markers by cells within the tubular complexes. Although we cannot completely exclude the possibility of transdifferentiation in our *Pdx1-Cre;Pten*$^{lox/lox}$ model, we provide evidence of a distinct path to ductal metaplasia marked by a series of steps that includes centroacinar cell expansion and acinar cell death (Figure 7).

This model is based on several lines of evidence. First, PTEN is normally expressed in pancreatic ducts, centroacinar cells, and islets, but not in acinar cells. As PTEN acts cell autonomously to regulate PI3-K function, a loss of function phenotype is therefore likely to be primarily related to the cell types that express the gene, namely ductal, centroacinar, and islet cells. Second, a proliferative expansion of centroacinar cells is seen prior to any overt histological changes in the pancreas. Third, "transitional areas" undergoing an apparent acinar-to-ductal conversion exhibit widespread apoptosis of acinar cells. Fourth, we failed to find direct evidence of acinar-to-ductal transdifferentiation; no cells coexpressing the ductal marker DBA and exocrine marker amylase could be found in any of the transitional areas surveyed. Fifth and finally, *Elastase-CreER*^{TM}*;Pten*$^{lox/\Delta}$ mice, in which *Pten* was deleted within acinar cells but not ductal or centroacinar cells, did not phenocopy the *Pdx1-Cre;Pten*$^{lox/lox}$ phenotype. It is possible that these mice did not exhibit metaplasia because *Pten* deletion was performed postnatally or because mice were followed for only 1 month. However, *Pdx1-Cre*-mediated *Pten* deletion causes early increases in DBA staining, which we did not observe in *Elastase-CreER*^{TM}*;Pten*$^{lox/\Delta}$

mice. Taken together, our results indicate that acinar cells are indifferent to changes in PTEN activity and support replacement rather than transdifferentiation as the mechanism of ductal metaplasia.

Centroacinar cells are very similar to duct cells and exhibit only subtle differences in apical membrane features (Ekholm et al., 1962). Such similarities are consistent with centroacinar, intercalary, and intralobular duct cells all representing a homologous cell type (Ekholm et al., 1962). Thus, it is reasonable to postulate that the neoductules detected in *Pdx1-Cre;Pten$^{lox/lox}$* mice are the direct descendents of this expanded centroacinar pool (Bardeesy et al., 2002). Supporting this idea, the metaplastic epithelium retains expression of the centroacinar cell marker Hes1. Importantly, Hes1 is also upregulated in human PanIN lesions and pancreatic adenocarcinomas (Miyamoto et al., 2003) and mouse PanIN lesions (Hingorani et al., 2003; see discussion below). We acknowledge, however, that Hes1 expression per se does not necessarily reflect a centroacinar origin of metaplastic lesions; a formal proof will await direct lineage tracing experiments.

The postnatal onset of metaplasia is consistent with a physiologic trigger acting in concert with the genetic defect. The basis for acinar cell death is unclear but may be related to a "mass effect" of the expanding ductal system, leading to accumulation of digestive enzymes in acinar lumens. Widespread acinar cell death leading to expansion of duct-like epithelium has also been described after surgical ligation of the pancreatic duct (Scoggins et al., 2000). It is possible that acinar cells provide growth inhibitory signals that normally keep the ductal compartment in check; loss of these signals upon acinar cell death may accelerate the metaplastic process, accounting for the increased proliferation rate detected in metaplastic lesions. It is therefore tempting to speculate that this may be one mechanism by which chronic pancreatitis elevates the risk of pancreatic adenocarcinoma (Ghadirian et al., 2003).

Implications for pancreatic neoplasia

As one of the major effector arms of KRAS signaling, PI3-K is well situated to play a role in pancreatic adenocarcinoma. The downstream AKT kinase is activated in a large percentage of human pancreatic tumors (Cheng et al., 1996; Ruggeri et al., 1998; Altomare et al., 2003; Schlieman et al., 2003), and there is evidence that reduced expression of *Pten* forms the basis of pathway activation (Asano et al., 2004). Moreover, PI3-K signaling is required for tumor cell growth in vitro (Bondar et al., 2002; Shah et al., 2001; Perugini et al., 2000). Consistent with these reports, genome-wide analyses of copy number alterations point to common amplification or deletion of key components in the PI3-K pathway (Aguirre et al., 2004 and data not shown). Viewed in the context of these reports, our finding that *Pten* deletion leads to ductal malignancies suggests that PI3-K plays an important role in the natural history of pancreatic cancer.

Activation of a mutant *Kras* gene (*Kras^{G12D}*) in the pancreas leads to the development of premalignant PanIN lesions and infrequent progression to pancreatic adenocarcinoma (Aguirre et al., 2003; Hingorani et al., 2003). Further analysis of *Pdx1-Cre;LSL-Kras^{G12D}* mice suggests that the phenotypes resulting from *Kras* activation and *Pten* deletion share several important features. First, Hes1 expression is observed in the early PanIN lesions of *Pdx1-Cre;LSL-Kras^{G12D}* mice, raising the possibility that these lesions may arise from centroacinar cells or duct cells that express Hes1 (Figure S3; Hingorani et al., 2003). Second, *Pdx1-Cre;LSL-Kras^{G12D}* mice exhibit an activated PI3-K pathway, as evidenced by the cytoplasmic relocalization of FKHR (Figure S4). Third, the early lesions that arise in both *Pdx1-Cre;Pten$^{lox/lox}$* and *Pdx1-Cre;LSL-Kras^{G12D}* mice express EGFR (Aguirre et al., 2003; Figure S5 and data not shown). These similarities suggest that PI3-K activation and centroacinar cell expansion may constitute common early events in pancreatic adenocarcinoma.

Nevertheless, there are marked differences between the phenotypes resulting from pancreatic *Pten* deletion and *Kras* activation. Specifically, activation of mutant *Kras^{G12D}* results predominantly in ductal PanIN lesions with relative sparing of acini. High-grade PanIN lesions occur almost exclusively after 5 months of age in *Kras^{G12D}* mice (Hingorani et al., 2003), while we frequently observe complete replacement of the *Pten*-deficient acinar pancreas with metaplastic ductules prior to this age. Several possible explanations may account for these discrepancies. First, the *Kras* mutant phenotype may not be limited to ducts, as ductal metaplasia that is histologically similar to that seen in *Pten* knockout animals also occurs in *Pdx1-Cre; LSL-Kras^{G12D}* mice (see, for example, Figure 1C in Aguirre et al., 2003). In addition, the use of different methodologies creates a bias in the pools of cells that are susceptible to different mutations. For example, expression of the *LSL-Kras^{G12D}* locus is under the control of the endogenous *Kras* promoter (Jackson et al., 2001), and thus only those cell types that normally express *Kras* will express the mutant allele. To our knowledge, the pancreatic expression pattern of the endogenous *Kras* gene has not been investigated. In *Pten*-deficient mice, by contrast, cell types that normally express *Pten* will be most directly influenced by its loss. Thus, variations in phenotypes may be attributable to differences in the relative expression of *Kras* and *Pten* within centroacinar and duct cells.

The rapid progression of ductal metaplasia in *Pten*-deficient mice may further amplify these differences, as premature lethality prior to 5 months of age may preclude the emergence of PanIN lesions that appear mostly later in the *Kras* model (Hingorani et al., 2003). PTEN itself has functions that are independent of its activity as an antagonist of PI3-K, which may account for part of its pancreatic phenotype; among these is the ability of PTEN to bind and modulate the DNA binding activity of p53 (Freeman et al., 2003). Nevertheless, it remains likely that other signaling surrogates that lie downstream of Ras (such as Braf) may be necessary in addition to PI3-K for the "full" oncogenic properties of *Kras^{G12D}*. In particular, other Ras signals may protect acinar cells from the apoptosis that occurs in *Pdx1-Cre;Pten$^{lox/lox}$* mice, accounting for the greater ductal specificity of the *Pdx1-Cre;LSL-Kras^{G12D}* phenotype (Aguirre et al., 2003).

The phenotype of *Pdx1-Cre;Pten$^{lox/lox}$* mice resembles that of mice that misexpress TGFα in the exocrine pancreas (Sandgren et al., 1990; Jhappan et al., 1990). In both models, the tubular complexes exhibit similar lobular distribution, histologic appearance, expression of ductal markers, and *Pdx1* expression. Moreover, tubular complexes become apparent predominantly in the postweaning period in both *Pdx1-Cre;Pten$^{lox/lox}$* mice and TGFα transgenic mice. These phenotypic similarities suggest that at least part of the TGFα signal, following Ras activation, is mediated by PI3-K.

PTEN and pancreas development

We have shown that PTEN is necessary to maintain the proper balance of cell types within the pancreas. The expression of Pdx1 by metaplastic ducts was unexpected, as Pdx1 expression is normally limited to islets in the adult pancreas. In the embryo, *Pdx1* is required for early steps in pancreas development (Jonsson et al., 1994; Offield et al., 1996), and its expression marks a population of cells with the capacity to give rise to all pancreatic lineages (Gu et al., 2002). PDX1 is expressed by approximately 43% of human pancreatic cancers, and its expression is correlated significantly with poor patient outcome (Koizumi et al., 2003). Pdx1 is also expressed strongly in metaplastic ducts following TGFα misexpression (Song et al., 1999) and weakly in the PanIN lesions of *Pdx1-Cre;LSL-Kras^{G12D}* mice (Hingorani et al., 2003). The expression of Pdx1 in this array of premalignant lesions and cancers suggests that the immature cell type marked by Pdx1 plays an important role in pancreatic carcinogenesis. Together, these data are consistent with a postulated association between developmental pathways and cancer (Meszoely et al., 2001). We did not detect an increased number of Pdx1$^+$ cells in *Pten*-deficient pancreata prior to the onset of metaplasia. Pdx1$^+$ cells within the metaplastic ducts are therefore likely to arise from the "reacquisition" of *Pdx1* expression rather than the expansion of rare *Pdx1*$^+$ cells, although we cannot rule out the latter possibility. Furthermore, the lack of metaplasia in *Pdx1-CreER^{TM}; Pten$^{lox/lox}$* mice (when injected postnatally with TM) argues against a contribution of preexisting Pdx1$^+$ cells to ductal metaplasia.

Similarly, Notch signals are critical during embryonic development for maintaining pancreatic progenitor cells in an undifferentiated state (Murtaugh et al., 2003; Hald et al., 2003), but Notch signaling is absent in most adult pancreatic cell types. Normally, expression of the Notch target gene *Hes1* is limited to centroacinar cells and a subset of duct cells, but high levels of *Hes1* expression have been observed in PanINs and pancreatic cancers (Miyamoto et al., 2003). In this study, we found *Hes1* expression in the premalignant lesions of *Pdx1-Cre; Pten$^{lox/lox}$* and *Pdx1-Cre;LSL-Kras^{G12D}* mice, further supporting a role for Notch signaling during pancreatic carcinogenesis.

In the central nervous system, *Pten* regulates stem cell number (Groszer et al., 2001). It is possible that the Pdx1$^+$ cells that arise in the pancreata of *Pdx1-Cre;Pten$^{lox/lox}$* mice may retain the capacity to give rise to multiple differentiated lineages under appropriate conditions, and thereby act as multipotent progenitor cells in the adult pancreas. *Pten* is irreversibly inactivated through the action of Cre in our model, preventing us from determining whether these cells retain the capacity to differentiate upon withdrawal of the activated PI3-K signal. Mice engineered to enable regulated activation and extinction of PI3-K signaling will permit this hypothesis to be tested.

Experimental procedures

Mice

Mice were maintained in an SPF facility. The *Elastase-CreER^{TM}* construct was generated by fusing a 200 bp enhancer of the *Elastase* gene (gift of G. Swift and R. MacDonald; Swift et al., 1989) to a minimal *hsp68* promoter (a gift from M. Gannon) and placing the chimeric promoter upstream of a *CreER^{TM}* coding sequence (a gift from A. McMahon). Transgenic mice were generated by pronuclear injection. Two founders were identified that, when crossed with Z/AP mice, showed acinar-specific expression of HPAP upon TM injection. TM (Sigma; 20 mg/ml in corn oil) was injected intraperitoneally twice per week into 4-week old mice (five injections of 8 mg each). Glucose tolerance tests were performed by injecting 2 g/kg glucose intraperitoneally after overnight fasting. BrdU (Amersham cell proliferation kit; RPN20) was injected 2–3 hr prior to sacrifice. *Pten$^{lox/lox}$*, *Pten$^{lox/\Delta}$*, *Pdx1-Cre*, and *Pdx1-CreER^{TM}* mice have been described (Groszer et al., 2001; Gu et al., 2002). *Z/AP* mice were a gift from C. Lobe (Lobe et al., 1999). *p53* mutant mice (Jacks et al., 1994) and Z/EG mice (Novak et al., 2000) were obtained from the Jackson Laboratory (Bar Harbor).

Histology

Formalin-fixed, paraffin-embedded 6–8 μm sections of the pancreas were used. The following primary antibodies were used: mouse anti-PTEN (Cell Signaling; 1:50), rabbit anti-P-AKT (Cell Signaling; 1:500), mouse anti-BrdU (Amersham cell proliferation kit), guinea pig anti-Pdx1 (a gift from Chris Wright; 1:500), guinea pig anti-insulin (DAKO; 1:500), rabbit anti-glucagon (Linco; 1:250), rabbit anti-amylase (Sigma; 1:500), rabbit anti-FKHR (Cell Signaling; 1:50), rabbit anti-EGFR (Cell Signaling; 1:50), mouse anti-human Muc5AC (Chemicon; 1:1000), rabbit anti-Muc2 (Santa Cruz; 1:150), and rabbit anti-Hes1 (a gift from Tetsuo Sudo; 0.6 micrograms/ml). Biotinylated DBA lectin (Vector) was used at 1:250 in HEPES/NaCl. TUNEL staining was performed with the DeadEnd apoptosis detection kit (Promega) according to the manufacturer's instructions. Alcian blue staining was performed by staining sections with 1% Alcian blue 8GX (Sigma) in 3% glacial acetic acid for 30 min at room temperature (RT), followed by counterstaining with nuclear fast red (Vector). For the detection of PTEN, P-AKT, BrdU, Pdx1, and Hes1, antigen retrieval was performed by microwave heating the slides in citrate buffer (pH 6) prior to staining. Secondary antibodies used were from Jackson Immunoresearch. Staining for HPAP was performed as described (Lobe et al., 1999; Gu et al., 2002).

For electron microscopy, mice were perfused with 2% glutaraldehyde in 0.1 M sodium cacodylate, rinsed in PBS, then embedded in EPON for morphology. Blocks of tissue (0.1–1.5 mm^3) were osmicated in 1% OsO_4 in 0.1 M sodium cacodylate for 1 hr at RT, rinsed in cacodylate buffer, and then rinsed in distilled water before staining in 2% uranyl acetate/DDW for 1 hr. Sections were dehydrated in graded alcohols and placed in 100% propylene oxide. The blocks were infiltrated in EPON:PO 1:1 overnight at RT, then changed to 100% EPON and polymerized overnight. Sections (90 nm) were cut on a Reichert-Jung Ultracut E microtome, collected on slot grids, and stained with 2% urinal acetate and lead citrate before viewing on a Philips CM 10 electron microscope.

Supplemental data

The Supplemental Data include six figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/8/3/185/DC1/.

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