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BASIC AND TRANSLATIONAL—PANCREAS

Identification and Manipulation of Biliary Metaplasia in Pancreatic Tumors

Kathleen E. DelGiorno,[1,2] Jason C. Hall,[2,3] Kenneth K. Takeuchi,[2] Fong Cheng Pan,[4] Christopher J. Halbrook,[2,3] M. Kay Washington,[5] Kenneth P. Olive,[6] Jason R. Spence,[7] Bence Sipos,[8] Christopher V. E. Wright,[4] James M. Wells,[9] and Howard C. Crawford[1,2]

Departments of [1]Molecular Genetics and Microbiology and [3]Pharmacological Sciences, Stony Brook University, Stony Brook, New York; [2]Department of Cancer Biology, Mayo Clinic, Jacksonville, Florida; Departments of [4]Cell and Developmental Biology and [5]Pathology, Vanderbilt University Medical Center, Nashville, Tennessee; [6]Departments of Medicine and Pathology, Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, New York, New York; [7]Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan; [8]Department of Pathology, University Hospital Tubingen, Tubingen, Germany; and [9]Department of Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio

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BACKGROUND & AIMS: Metaplasias often have characteristics of developmentally related tissues. Pancreatic metaplastic ducts are usually associated with pancreatitis and pancreatic ductal adenocarcinoma. The tuft cell is a chemosensory cell that responds to signals in the extracellular environment via effector molecules. Commonly found in the biliary tract, tuft cells are absent from normal murine pancreas. Using the aberrant appearance of tuft cells as an indicator, we tested if pancreatic metaplasia represents transdifferentiation to a biliary phenotype and what effect this has on pancreatic tumorigenesis. **METHODS:** We analyzed pancreatic tissue and tumors that developed in mice that express an activated form of Kras ($Kras^{LSL-G12D/+}$;$Ptf1a^{Cre/+}$ mice). Normal bile duct, pancreatic duct, and tumor-associated metaplasias from the mice were analyzed for tuft cell and biliary progenitor markers, including SOX17, a transcription factor that regulates biliary development. We also analyzed pancreatic tissues from mice expressing transgenic SOX17 alone ($ROSA^{tTa/+}$;$Ptf1^{CreERTM/+}$;*tetO-SOX17*) or along with activated Kras ($ROSAtT^{a/+}$;$Ptf1a\text{-}^{CreERTM/+}$;*tetO-SOX17*;$Kras^{LSL\text{-}G12D;+}$). **RESULTS:** Tuft cells were frequently found in areas of pancreatic metaplasia, decreased throughout tumor progression, and absent from invasive tumors. Analysis of the pancreatobiliary ductal systems of mice revealed tuft cells in the biliary tract but not the normal pancreatic duct. Analysis for biliary markers revealed expression of SOX17 in pancreatic metaplasia and tumors. Pancreas-specific overexpression of SOX17 led to ductal metaplasia along with inflammation and collagen deposition. Mice that overexpressed SOX17 along with $Kras^{G12D}$ had a greater degree of transformed tissue compared with mice expressing only $Kras^{G12D}$. Immunofluorescence analysis of human pancreatic tissue arrays revealed the presence of tuft cells in metaplasia and early-stage tumors, along with SOX17 expression, consistent with a biliary phenotype. **CONCLUSIONS:** Expression of $Kras^{G12D}$ and SOX17 in mice induces development of metaplasias with a biliary phenotype containing tuft cells. Tuft cells express a number of tumorigenic factors that can alter the microenvironment. Expression of SOX17 induces pancreatitis and promotes $Kras^{G12D}$-induced tumorigenesis in mice.

Keywords: Pancreatic Cancer; Pathogenesis; Mouse Model; Signal Transduction.

Pancreatic ductal adenocarcinoma (PDA) is currently the fourth leading cause of cancer death, with an overall 4-year survival rate of 6% and an abysmal median survival of just 4 to 6 months.[1] Because symptoms appear late in disease progression and metastasis has typically occurred by the time of diagnosis, earlier detection is likely to be invaluable. Elucidation of early detection markers requires a greater understanding of early disease pathology, such as pancreatic intraepithelial neoplasia (PanIN), a proposed precursor to PDA, and acinar-to-ductal metaplasia (ADM), a process that results in the formation of highly reactive metaplastic ducts. The consistent association of ADM with PDA suggests that lesions arise as a consequence of disruption of nearby normal tissue. Recent studies implicate them as a source of PanIN.[2,3] Consistent with this, metaplastic ducts are a hallmark of chronic pancreatitis, which may be part of the reason why chronic pancreatitis is a significant risk factor for PDA.

Epithelial metaplasia is a hallmark of inflammatory and neoplastic disease in several organs. In many tissues, normal epithelium is replaced by epithelium usually confined to a developmentally related organ; this

Abbreviations used in this paper: **ADM, acinar-to-ductal metaplasia; COX, cyclooxygenase; DAPI, 4′,6-diamidino-2-phenylindole; DCLK1, doublecortin-like kinase 1; EGFR, epidermal growth factor receptor; IF, immunofluorescence; IHC, immunohistochemistry; mPanIN, murine pancreatic intraepithelial neoplasia; PanIN, pancreatic intraepithelial neoplasia; PBG, peribiliary gland; PDA, pancreatic ductal adenocarcinoma; PDG, pancreatic duct gland; YFP, yellow fluorescent protein.**

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phenomenon occurs in adenocarcinoma of the esophagus in areas of Barrett's metaplasia, where the epithelium takes on gastric and intestinal characteristics marked by CDX2 expression, or cystitis glandularis, where bladder metaplasia becomes phenotypically colon-like.[4] In the pancreas, ADM is generally described as pancreatic acinar cells being replaced by pancreatic duct cells, with no prior description of their mimicry of epithelia of related tissues.

One cell type absent from the exocrine pancreas is the tuft cell. Tuft cells are a type of solitary chemosensory cell and are found in multiple organs but are prevalent in the developmentally related common bile duct and murine pancreatobiliary duct (the segment of duct following the intersection of the main pancreatic duct and the bile duct before fusion with the duodenum) and associated peribiliary glands (PBGs). Solitary chemosensory cells are part of the diffuse chemosensory system and are analogous to taste cells, although they do not aggregate in buds. Solitary chemosensory cells are believed to link chemosensation of intraluminal content to local control of absorptive and secretory processes as well as central nervous system activity.[5]

In our analysis of pancreatic metaplasia, we have discovered that the aberrant genesis of pancreatic tuft cells is common in $Kras^{G12D}$-induced pancreatic disease and is accompanied by epithelial expression of SOX17, a master control factor of biliary development and differentiation.[6] Lineage tracing showed that pancreatic tuft cells transdifferentiate from adult acinar cells and express a full array of markers associated with mature tuft cells found in other tissues. ADM and murine PanINs (mPanINs) consistently contained a $PDX1^{+}/SOX17^{+}$ cell population reminiscent of the common pancreatobiliary progenitor.[6] Forced expression of SOX17 in adult pancreas was sufficient to induce acinar cell transdifferentiation into a tuft cell–containing metaplasia, accompanied by a chronic pancreatitis–like phenotype, including fibrosis and an adaptive immune response. Transgenic expression of SOX17 in concert with oncogenic K-ras expression enhanced the degree of transformation of normal pancreas, suggesting that SOX17-induced metaplasia was fully susceptible to Kras-induced transformation. We conclude that pancreatic ADM found in PDA represents transdifferentiation to a biliary phenotype and contributes to disease progression through the assumption of a pancreatobiliary progenitor cell phenotype.

Materials and Methods

Mouse Strains

LSL-$Kras^{G12D/+}$, $Ptf1a^{Cre/+}$, $Ptf1a^{Cre\text{-}ERTM/+}$, *tetO-SOX17*, *MT-Tgfa*, and $ROSA^{tTa/+}$ strains have been described previously and were genotyped accordingly.[7–12] $ROSA^{YFP}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME). Experiments were conducted in accordance with the Office of Laboratory Animal Welfare and approved by the institutional animal care and use committees at Stony Brook University and the Mayo Clinic.

Mouse Tissue Microarrays

Custom 5-mm tissue microarrays were assembled by a hand corer and precast recipient molds. PDAs from 10 *LSL-*$Kras^{G12D}$*;*$P53^{R172H/+}$*;*$PDX1^{Cre/+}$ mice were included. Adjacent PanIN-containing tissues were included for 5 tumors, and distant metastases from several organ sites were included for 5 other tumors.

Human Samples

Distribution and use of all human samples was approved by the institutional review boards of Vanderbilt University Medical Center and the Mayo Clinic.

Induction of Experimental Pancreatitis

Cerulein-induced pancreatitis was achieved by treating mice twice daily with 250 μg/kg cerulein (Sigma-Aldrich, St Louis, MO) for 7 days and allowing mice to recover for 1 day. Metaplasia was induced in the *MT-Tgfa* strain by administration of 25 mmol/L $ZnSO_4$ in drinking water for 3, 6, or 9.5 months.

Overexpression of SOX17

Adult acinar cell–specific overexpression of SOX17 was accomplished by treating 6- to 12-week-old mice with 5 daily doses of 5 mg/kg tamoxifen administered through oral gavage to $ROSA^{tTa/+}$*;*$Ptf1a^{CreERTM/+}$*;tetO-SOX17* mice with 6 weeks of recovery. The identical protocol was used with $ROSA^{tTa/+}$*;*$Ptf1a^{CreERTM/+}$*;tetO-SOX17;*$Kras^{LSL\text{-}G12D;+}$ mice.

Tumor quantitation was performed by scanning 4 random H&E-stained slides from each pancreata with an Aperio slide scanner (Vista, CA). Aperio ImageScope software was used to delineate and quantitate areas flanked by fibrosis and containing metaplasia or neoplasia compared with total tissue area.

Immunostaining

Tissues were harvested and fixed overnight in 4% paraformaldehyde. Immunohistochemistry (IHC) was performed as previously described.[13] Slides were counterstained with hematoxylin and photographed on an Olympus BX41 light microscope (Olympus, Tokyo, Japan). Immunofluorescence (IF) was performed as previously described with some modifications (Supplementary Methods).[14]

Electron Microscopy

Tissue was prepared for electron microscopy by perfusion of mice with 2% paraformaldehyde/2.5% electron microscopy–grade glutaraldehyde in 0.1 mol/L phosphate-buffered saline, pH 7.4. Samples were viewed with a Tecnail2 BioTwinG[2] transmission electron microscope (FEI, Hillsboro, OR) at 80 kV. Digital images were acquired with an XR-60 CCD digital camera system (Advanced Microscopy Techniques, Woburn, MA).

Quantitation of Tuft Cells

DCLK1 IHC was performed on paraffin-embedded tissue from 11 *LSL-*$Kras^{G12D}$*;*$Ptf1a^{Cre/+}$ mice ranging in age from 4 months to 1 year using a Discovery XT autostainer (Ventana Medical Systems, Tuscon, AZ). A minimum of 20 images at 40× were acquired per slide and lesions staged. Tuft cells were quantitated as $DCLK1^{+}$ cells per number of nuclei per lesion. For quantitation in *MT-Tgfa* mice, DCLK1 IHC was performed

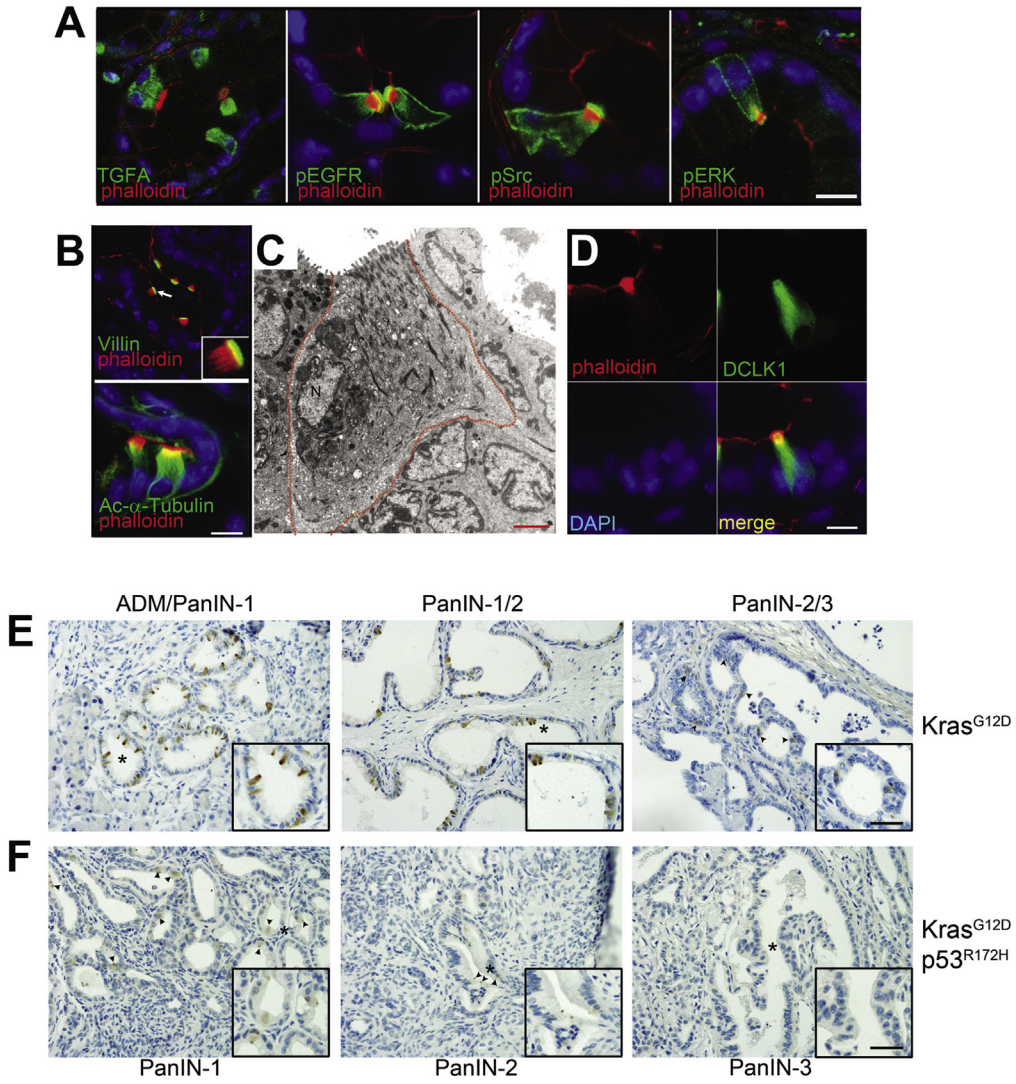

Figure 1. Tuft cells in pancreatic metaplasia. Co-IF staining in 4- to 6-month-old *LSL-Kras$^{G12D/+}$;Ptf1a$^{cre/+}$* mice including (*A*) TGFA, phospho-EGFR (pY1068), phospho-Src (pY416), or phospho-ERK (pT202/pY204) (*green*) with phalloidin (*red*). *Scale bars* = 10 μm for TGFA; 5 μm for pEGFR, pSrc, and pERK panels. (*B*) Tuft cell structural components villin and acetylated α-tubulin (*green*) with phalloidin (*red*). *Arrow* indicates cell depicted in the *inset*. *Scale bars* = 10 μm. (*C*) Electron microscopy of a metaplastic tuft cell in a 4-month-old *LSL-Kras^{G12D};Ptf1a$^{cre/+}$* mouse. *Scale bar* = 2 μm. (*D*) Co-IF for DCLK1 (*green*) and phalloidin (*red*). Nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI). *Scale bar* = 5 μm. (*E*) IHC for DCLK1 in either *LSL-Kras^{G12D};Ptf1a$^{cre/+}$* or (*F*) *LSL-Kras^{G12D};P53$^{R172H/+}$* mice in PanIN-1–3 and invasive PDA. *Asterisks* indicate areas depicted in the *insets*. *Scale bars* = 50 μm (*panels*) and 33 μm (*insets*).

on paraffin-embedded tissue from 9 mice treated with $ZnSO_4$ from 3 to 10 months. Ten images were taken at 40× per slide, and tuft cells were quantitated as DCLK1$^+$ cells per number of nuclei per metaplastic lesion.

Lineage Tracing

Recombination was induced in 8-week-old *Kras$^{G12D/+}$;ROSA^{YFP};Ptf1a$^{Cre\text{-}ERTM/+}$* mice with one daily intraperitoneal injection of 3 mg tamoxifen (Sigma-Aldrich) for 5 days. Tumorigenesis was accelerated by a daily intraperitoneal injection of 250 μg/kg cerulein for 5 days. Mice were killed 9 weeks later, and tissue was prepared for IF.

Cell Culture

Human PDA cell lines were purchased from American Type Culture Collection (Manassas, VA) and maintained at 37°C in 5% co_2 in American Type Culture Collection–recommended medium supplemented with 10% fetal bovine serum and 0.5 μg/mL gentamicin.

Western Blotting

Preconfluent cells were harvested in ice-cold RIPA buffer supplemented with PhosSTOP phosphatase inhibitor and cOmplete EDTA-free protease inhibitor (Roche, Indianapolis, IN). A total of 75 μg protein was run on a 7% sodium dodecyl sulfate gel and blotted to polyvinylidene difluoride membrane for antibody incubation.

Results

Tuft Cells Are a Consistent Component of Epithelial Metaplasia in a Mouse Model of Pancreatic Tumorigenesis

The *LSL-Kras$^{G12D/+}$;Ptf1a$^{Cre/+}$* murine model of pancreatic tumorigenesis presents with mainly ductal metaplasia and early mPanINs up to ~1 year of age, when later-stage mPanINs and occasionally PDA is found. The epidermal growth factor receptor (EGFR) pathway has been associated with progression of PDA, and recently we found that activity

was required for induction of tumorigenesis.[3,15] On examining EGFR pathway activity by IF in this model, we observed significant cellular heterogeneity within metaplastic structures. Although EGFR activity was elevated throughout the metaplastic epithelium, we found highly elevated positivity for pY1068 EGFR, pY416 Src, pT202/pY204 ERK, and the EGFR ligand transforming growth factor α within isolated cells of metaplastic ducts (Figure 1*A*). This staining pattern was never observed in ducts of wild-type control pancreata but could readily be identified in the nearby pancreatobiliary tract (data not shown). Using phalloidin to costain for F-actin, we noted that these phospho-EGFR–positive cells had a unique arrangement of microfilaments, marked by a perpendicular orientation to the apical membrane, typical of a tuft cell. IF for villin and acetylated α-tubulin confirmed the presence of both prominent microvilli and the tubulovesicular system, respectively (Figure 1*B*). Using the unique microfilament arrangement as a guide, electron microscopy of a 4-month-old *LSL-Kras*$^{G12D/+}$;*Ptf1a*$^{Cre/+}$ pancreas confirmed that tuft cells were commonly integrated into metaplastic ducts (Figure 1*C*).

Doublecortin-like kinase 1 (DCLK1), a tubulin polymerization serine/threonine kinase, has been proposed to be both a marker of quiescent stem cells in the pancreas as well as a marker of tuft cells in the stomach and intestine, where they are believed to represent a terminally differentiation cell population.[16–18] To address whether metaplastic tuft cells phenocopy mature tuft cells in other organs, DCLK1 expression was assessed relative to the expression level of other tuft cell markers. Co-IF with phalloidin revealed that 100% of tuft cells expressed DCLK1 (n = 300), whereas 72% of DCLK1-positive cells were identified unambiguously as tuft cells (n = 418; Figure 1*D*). The remaining 28% of DCLK1-positive cells that were not obviously tuft cells were possibly obscured due to section planarity or, alternatively, represent a legitimate non–tuft cell population, such as an adult stem cell population identified previously. Tuft cells of the normal biliary and intestinal tract have been shown to express an array of markers, including G-α-gustducin, TRPM5, β-endorphin, cyclooxygenase (COX)-1, COX-2, HPGDS, and Gfi1b (Supplementary Table 1). We found that metaplastic pancreatic tuft cells expressed each of these proteins at highly elevated levels, confirming their identity as bona fide tuft cells, indistinguishable from those found in normal tissue[16,19–21] (Supplementary Figure 1).

To determine the association of tuft cells with disease progression, we quantitated the number of DCLK1$^+$ tuft cells at different points in tumor progression using pancreata from *LSL-Kras*^{G12D};*Ptf1a*$^{Cre/+}$ mice of various ages. Tuft cells were most abundant in ADM (an average 15.0% of the epithelium per lesion) and became less frequent throughout disease progression, constituting 11.2% of the epithelium in mPanIN-1, 8.7% in mPanIN-2, and 2.9% in mPanIN-3, whereas invasive disease had no tuft cells (Figure 1*E*). Analysis of pancreata from the *LSL-Kras*^{G12D};*p53*$^{R172H/+}$;*Pdx1-Cre* model of PDA was consistent with these results, although the overall number of cells per lesion was generally reduced to 4.8% in PanIN-1s, 2.1% in PanIN-2s, and <1% in PanIN-3s, with several being entirely negative (Figure 1*F*). To determine if tuft cells accompanied nontumor models of pancreatic metaplasia, we examined DCLK1 expression in transforming growth factor α–driven and cerulein-induced metaplasia. Tuft cells were very commonly associated with metaplasia in *MT-Tgfa* mice, present in 73.5% of metaplastic lesions, constituting 14.4% of the epithelium (Supplementary Figure 2*A*). The presence of tuft cells was confirmed by electron microscopy (Supplementary Figure 2*B*). Interestingly, tuft cells were rarely found in cerulein-induced transient metaplasia (Supplementary Figure 2*C*).

Metaplastic Tuft Cells Suggest Adoption of a Biliary Phenotype

Tuft cells have been previously described within the normal murine biliary tract and intestine; however, in our experiments, we never observed these cells in random sections of wild-type pancreata.[16,22] To definitively determine if the normal main pancreatic duct harbored a tuft cell population, we dissected the main pancreatic duct away from the common duct of the biliary tract and the pancreatobiliary duct and examined them histologically. The murine biliary tract was composed of a central duct decorated along its length by ancillary peribiliary glands (PBGs), which were lined with columnar epithelium, including numerous tuft cells, identified by DCLK1 IHC.[23] In contrast, the murine main pancreatic duct was composed of low cuboidal epithelium, lacked PBGs, and was entirely devoid of tuft cells. The pancreatobiliary duct was morphologically similar to the bile duct, including PBGs and numerous tuft cells (Figure 2*A*).

Pancreatic Tuft Cells Transdifferentiate From Acinar Cells

PBGs associated with the common and pancreatobiliary ducts (Figure 2*A*) have been hypothesized to be a source of progenitor cells for the liver, biliary tract, and pancreas, in part due to their expression of several stem and progenitor markers.[23] Strobel et al have labeled related structures associated with the main pancreatic duct as "pancreatic duct glands" (PDGs), describing them as a potential source of pancreatic disease.[24] In this study, it is hypothesized that ADM may directly emanate from the expansion of PDGs, with a popular alternative being that acinar cells transdifferentiate to form metaplasia. To distinguish these possibilities in our system, we conducted lineage tracing in the *LSL-Kras*$^{G12D/+}$;*ROSA*^{YFP};*Ptf1a*$^{Cre\text{-}ERTM/+}$ murine model. This model initiates expression of both *Kras*^{G12D} and yellow fluorescent protein (YFP) exclusively in Ptf1a$^+$ adult acinar cells upon tamoxifen induction of CRE activity.[8] YFP fluorescence was found in 68.7% of tuft cells identified by phalloidin staining (n = 300) and 76.3% of DCLK1$^+$ cells (n = 300) (Figure 2*B*), indicating that they are commonly derived from transdifferentiation of acinar cells. YFP-negative tuft cells could represent partial derivation from normal PDGs, incomplete recombination of the ROSA locus, or ROSA promoter activity silencing, as has been observed

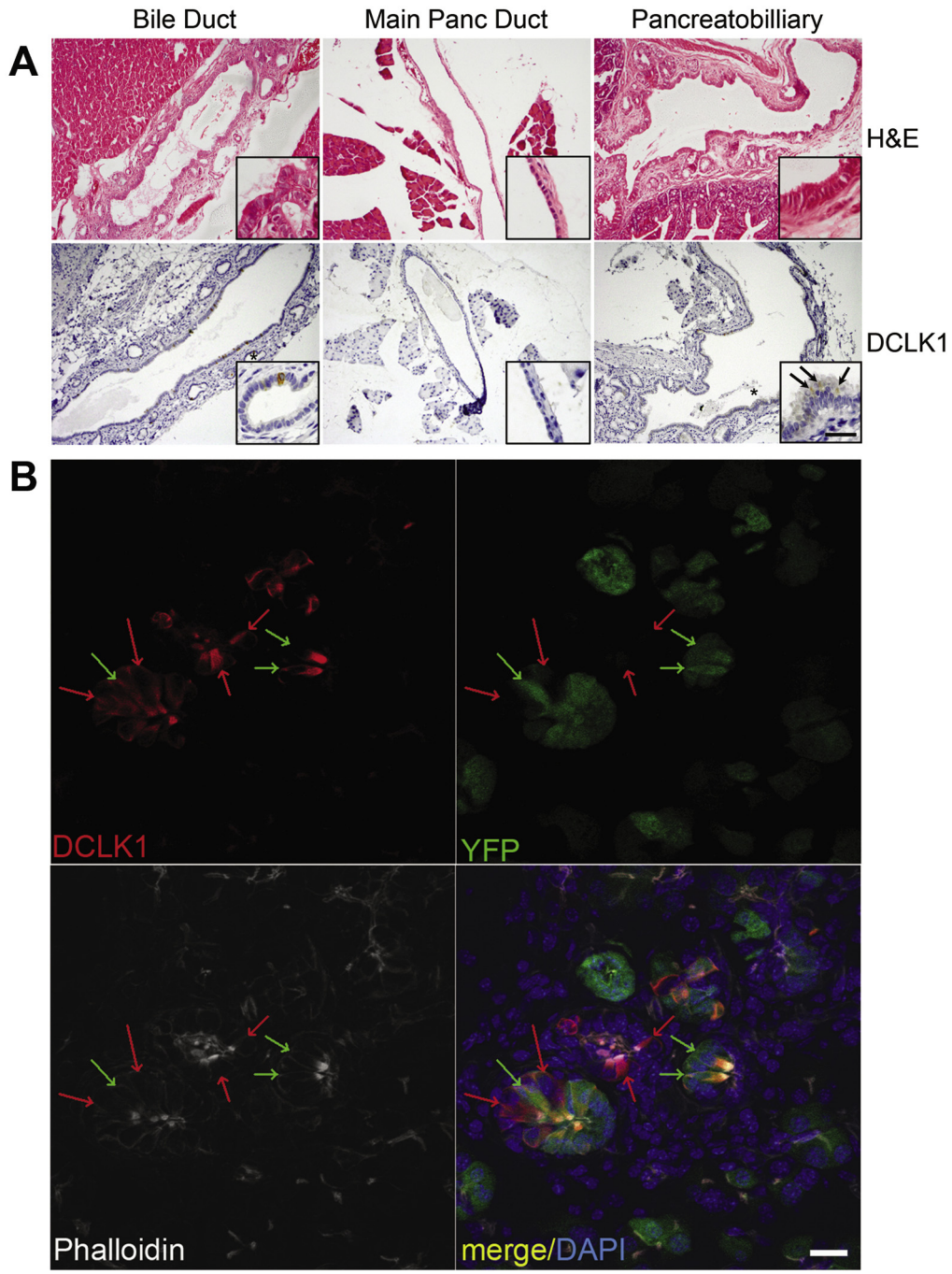

Figure 2. Pancreatic tuft cells are disease specific and transdifferentiate from Ptf1a^{+} epithelium. (*A*) Histological analysis of the murine bile duct, pancreatic duct, and pancreatobiliary duct by H&E analysis and DCLK1 IHC. *Scale bar* = 100 μm (*panels*) and 25 μm (*insets*). (*B*) Lineage tracing and IF analysis *in LSL-Kras^{G12D};ROSA^{YFP}; Ptf1a$^{Cre\text{-}ERTM/+}$* pancreata by DCLK1 (*red*), YFP (*green*), and phalloidin (*white*). Nuclei are stained with DAPI. YFP-negative tuft cells are indicated by *red arrows*, and YFP-positive tuft cells are indicated by *green arrows*. *Scale bar* = 20 μm.

in other adult tissues.[25] We conclude that while Kras-induced metaplasia is not primarily derived from PDGs, it does take on a PDG-like phenotype, including their possible regenerative function.

Pancreatic Metaplasia Takes on a Pancreatobiliary Progenitor Phenotype

Metaplasia has been described as a developmental reversion, taking on the phenotype of cells normally confined to other developmentally related organs.[4] Recent studies have shown that the murine biliary tract is developmentally more closely related to the pancreas, sharing a common progenitor cell population.[6] The observation that ADM resembles tuft cell–containing PBGs led us to hypothesize that ADM adopts other characteristics of the biliary tract. To test this, we examined the expression of SOX17, a transcription factor critical for bile duct development and recently described as being expressed in human intraductal papillary mucinous neoplasms.[26]

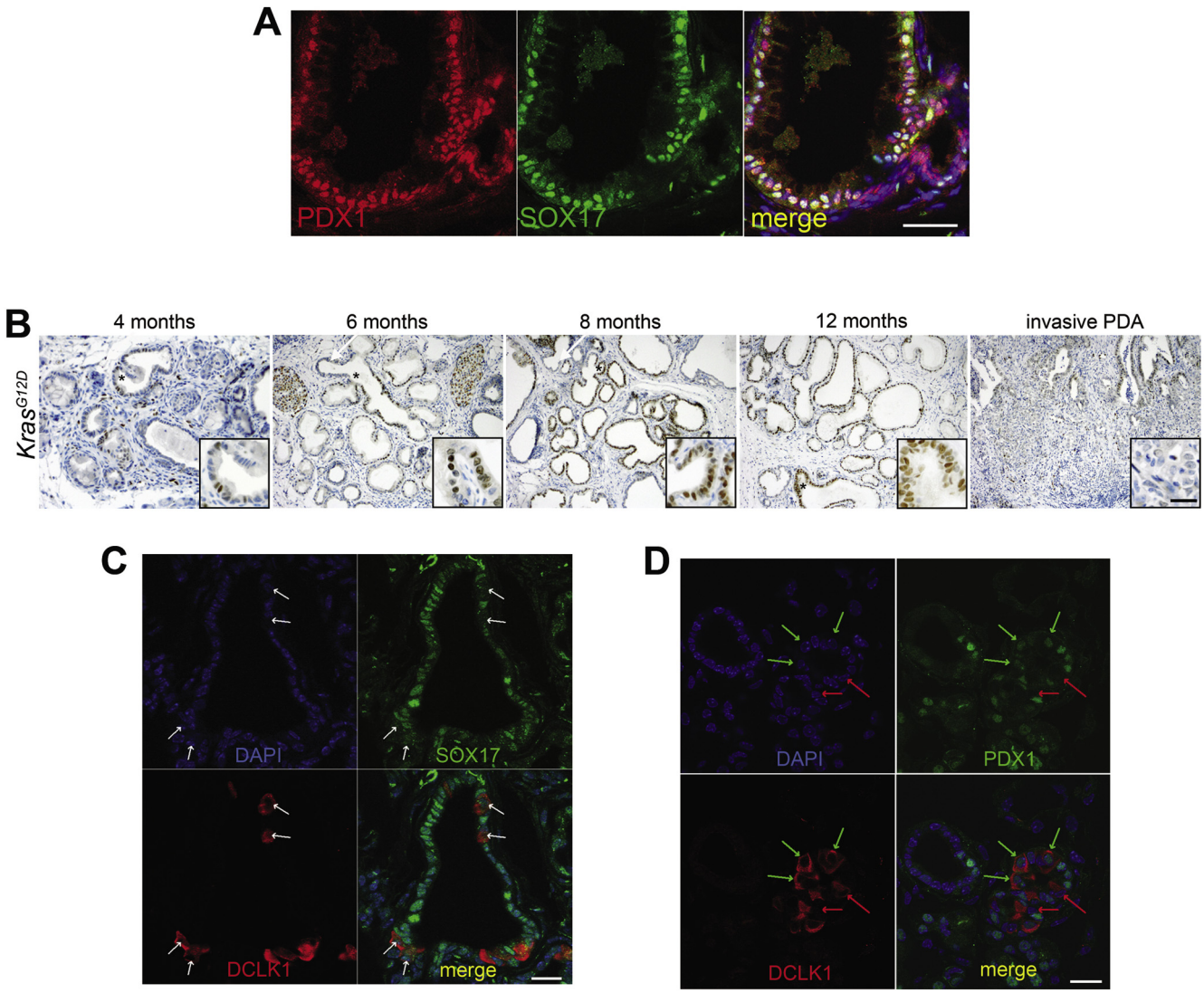

Figure 3. SOX17 is expressed during pancreatic tumorigenesis. (*A*) Co-IF for SOX17 (*green*) and PDX1 (*red*) in a 6-month-old *LSL-Kras*$^{G12D/+}$;*Ptf1a*$^{cre/+}$ pancreas. Nuclei are stained with DAPI. *Scale bar* = 50 μm. (*B*) IHC for SOX17 in 4- to 12-month-old *LSL-Kras*$^{G12D/+}$;*Ptf1a*$^{cre/+}$ mice. *Scale bar* = 50 μm (*panels*) and 25 μm (*insets*). *Asterisks* indicate areas depicted in the *insets*. (*C*) Co-IF for SOX17 (*green*) and DCLK1 (*red*). *Arrows* indicate several DCLK1-positive cells that have low SOX17 expression compared with nearby cells. Nuclei are stained with DAPI (*blue*). *Scale bar* = 20 μm. (*D*) Co-IF for PDX1 (*green*) and DCLK1 (*red*). *Green arrows* indicate co-positive cells, and *red arrows* indicate DCLK1-only positive cells. Nuclei are stained with DAPI (*blue*). *Scale bar* = 20 μm.

The pancreas and biliary tract share a common progenitor cell population characterized by coexpression of PDX1 and SOX17. In the adult, however, epithelial SOX17 expression is restricted to the biliary tract, although endothelial SOX17 can be found lining blood vessels[6] (Supplementary Figure 3). Co-IF for SOX17 and PDX1 revealed 2 cell subtypes within metaplastic ducts: a PDX1^{+}SOX17^{-} population and a PDX1^{+}/SOX17^{+} population (Figure 3*A*); the latter is reminiscent of the common pancreatobiliary progenitor cell.[6] To determine if this expression pattern persisted, *LSL-Kras*$^{G12D/+}$;*Ptf1a*$^{Cre/+}$ mice of various ages were assessed for SOX17 expression by IHC. The frequency and intensity of SOX17 expression increased throughout disease progression over time in PanINs of all stages. Like tuft cells, SOX17 was absent from invasive disease (Figure 3*B*). Interestingly, liver metastases from both the *Kras*$^{G12D/+}$;*Ptf1a*$^{Cre/+}$ and *LSL-Kras*^{G12D};*P53*$^{R172H/+}$;*Pdx1-Cre* models often expressed SOX17, suggesting reversion to a more differentiated phenotype (data not shown). SOX17 was not expressed in transient metaplastic ducts induced by long-term treatment with cerulein (data not shown).

The apparent inverse relationship between increasingly common SOX17 expression and the gradual loss of tuft cells in the mPanINs of *Kras*$^{G12D/+}$;*Ptf1a*$^{Cre/+}$ mice led us to examine their possible causal relationship more directly. We performed co-IF for SOX17 and DCLK1 on 4-month-old *Kras*$^{G12D/+}$;*Ptf1a*-$^{Cre/+}$ pancreata. Interestingly, DCLK1^{+} tuft cells consistently expressed relatively low levels of SOX17 compared with most other cells in the metaplastic epithelium (Figure 3*C*), indicating that elevated levels of SOX17 are not required and may have to

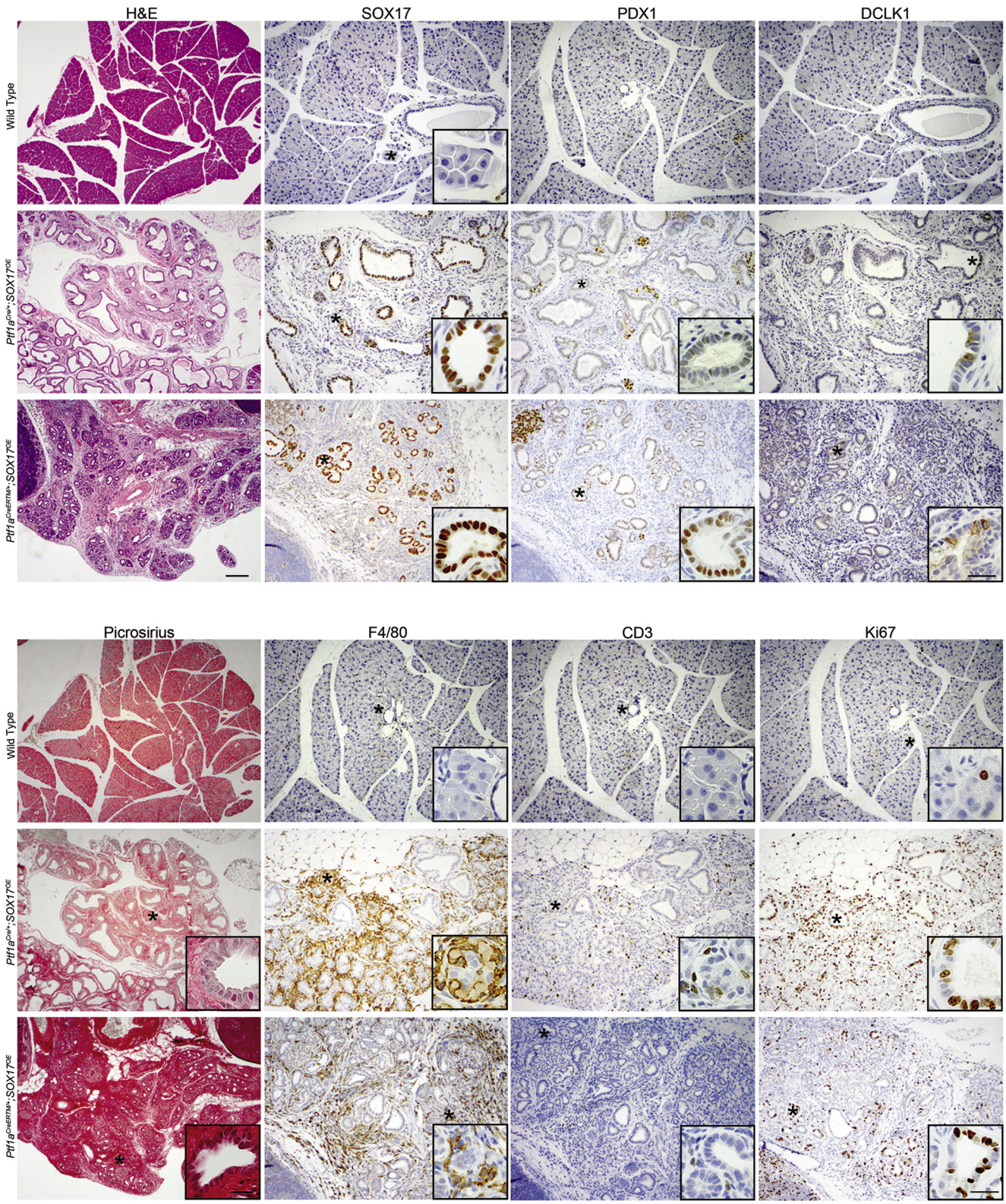

Figure 4. Transgenic expression of SOX17 induces a biliary phenotype and a pancreatitis-like disease state. Immunohistochemical comparison of the pancreata from a wild-type mouse to that of a *Ptf1a*$^{Cre/+}$*;SOX17*^{OE} mouse, where SOX17 has been overexpressed from development, and a *Ptf1a*$^{CreERTM/+}$*;SOX17*^{OE} mouse in which SOX17 expression was induced in the adult. The pancreata of both experimental models exhibit a biliary-like phenotype highly expressing SOX17 and DCLK1^{+} tuft cells. Consistent with biliary differentiation, PDX1 expression is low in the *Ptf1a*$^{Cre/+}$*;SOX17*^{OE} model. Consistent with metaplasia, the *Ptf1a*$^{CreERTM/+}$*;SOX17*^{OE} model highly expresses PDX1. Exhibiting characteristics of chronic pancreatitis, both models show an innate (F4/80) and adaptive (CD3) immune response accompanying a fibrotic response (picrosirius) and proliferative (Ki67) ducts. *Scale bars* = 200 μm (H&E and picrosirius), 100 μm (IHC), and 25 μm (*insets*). *Asterisks* indicate areas depicted in the *insets*.

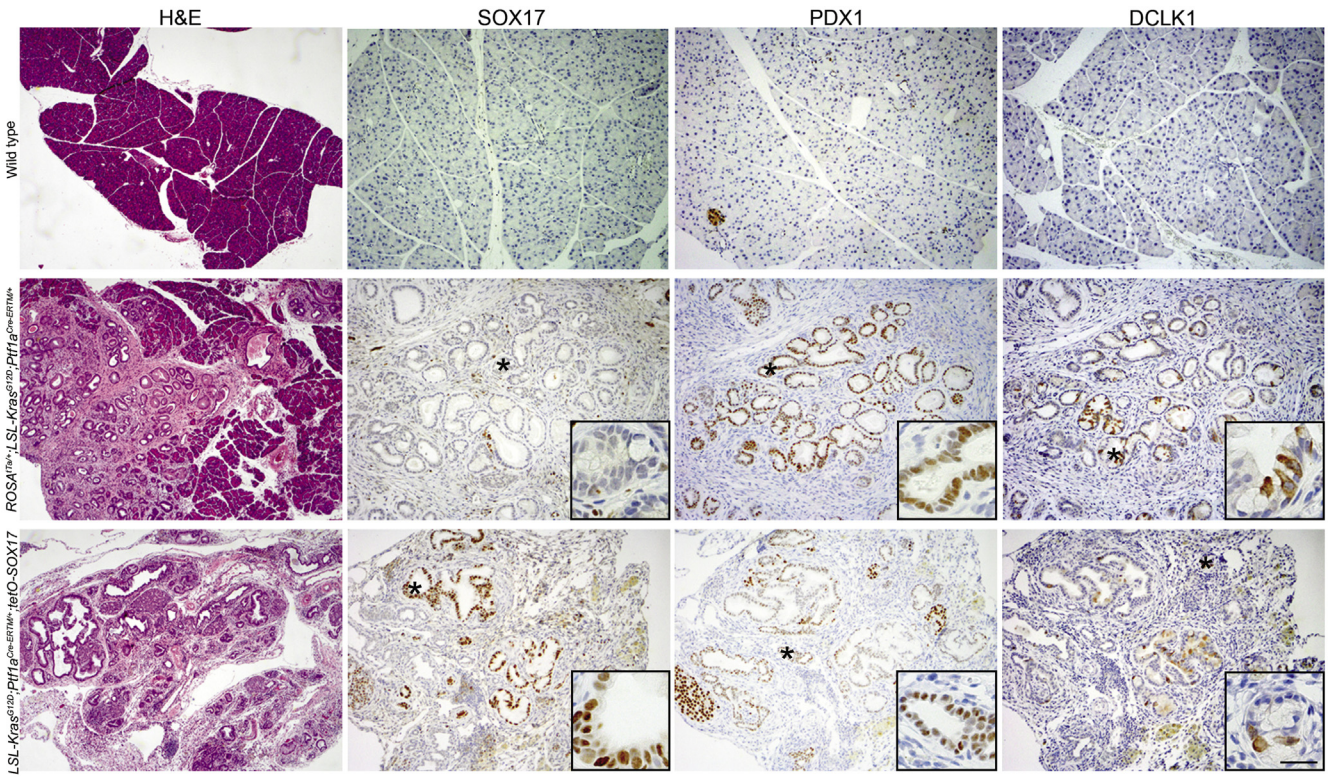

Figure 5. SOX17 expression facilitates $Kras^{G12D}$-driven tumorigenesis. Immunohistochemical comparison of the pancreata from a wild-type mouse to that of a $Kras^{G12D}$;$Ptf1a^{CreERTM/+}$;$ROSA^{tTa/+}$mouse and a $Kras^{G12D}$;$Ptf1a^{CreERTM/+}$;$SOX17^{OE}$ mouse treated with tamoxifen and allowed to recover for 6 weeks. In concert with $Kras^{G12D}$, overexpression of SOX17 leads to a greater degree of transformed tissue, as identified in H&E, characterized by PDX1 expression and DCLK1$^+$ tuft cells. *Scale bars* = 200 μm (H&E), 100 μm (IHC), and 25 μm (*insets*). *Asterisks* indicate areas depicted in the *insets*.

be suppressed for the derivation or maintenance of metaplastic tuft cells. PDX1 expression was found in some, but not all, DCLK1$^+$ tuft cells, suggesting no relationship with tuft cell maintenance (Figure 3*D*).

SOX17$^+$ PBGs are mucinous, tuft cell–containing glands that express stem cell factors such as OCT4 and LGR5 and endoderm-specific markers SOX9, PDX1, EPCAM, CXCR4, and FOXA2.[23] Many of these markers have been previously described to play a role in pancreatic ADM and tumorigenesis.[2,27–31] To examine the extent of PBG mimicry in acinar cell–derived metaplasia, we induced $Kras^{G12D}$ expression by treating 8-week-old *LSL-*$Kras^{G12D}$*;* $ROSA^{YFP}$;$Ptf1a^{Cre\text{-}ERTM/+}$ mice with tamoxifen to induce recombination and cerulein to accelerate tumorigenesis and examined their pancreata 9 weeks later. We found that this acinar cell–derived pancreatic metaplasia widely expressed SOX17, PDX1, SOX9, EPCAM, and LGR5, consistent with a PBG-like phenotype (Supplementary Figure 3).

SOX17 Expression Drives a Pancreatitis-like Disease State and Accelerates Kras-Driven Tumorigenesis

SOX17 is a known determinant of biliary development and differentiation. Its presence in pancreatic metaplasia suggests that it may play a causal role in acinar cell reprogramming during disease. It has been shown previously that SOX17 overexpression in PDX1$^+$ cells during early pancreatic development causes severe ductal expansion. Using a similar model that induces a more universal pancreatic expression of SOX17, $ROSA^{tTa/+}$;$Ptf1a^{Cre/+}$;*tetO-SOX17* ($Ptf1a^{Cre/+}$;$SOX17^{OE}$) and an adult acinar cell–specific inducible model of SOX17 overexpression, $ROSA^{tTa/+}$; $Ptf1a^{CreERTM/+}$;*tetO-SOX17* ($Ptf1a^{CreERTM/+}$;$SOX17^{OE}$), we set out to determine the full spectrum of downstream consequences of SOX17 expression in the pancreas. In the $Ptf1a^{Cre/+}$;$SOX17^{OE}$ model, mice express SOX17 in Ptf1a$^+$-positive progenitor cell populations and exhibit an expansion of PDX1-positive, tuft cell–containing ductal structures at the expense of the acinar and islet compartments by 6 weeks of age (Figure 4), consistent with previous findings.[6] We found that ductal structures were constituted by Ki67-positive epithelia and contained a subpopulation of DCLK1$^+$ tuft cells, consistent with Kras-induced ADM and the assumption of a biliary phenotype, respectively. Surprisingly, these pancreata showed a robust innate and adaptive inflammatory reaction, as determined by IHC for macrophages (F4/80$^+$) and T cells (CD3$^+$). Picrosirius red staining showed a vigorous fibrotic response (Figure 4).

To determine whether SOX17 was capable of reprogramming adult acinar cells, we induced SOX17 expression in the pancreata of adult mice using the $Ptf1a^{CreERTM/+}$; $SOX17^{OE}$ model. Five- to 10-week-old mice were treated with tamoxifen to induce acinar cell–specific CRE activity,

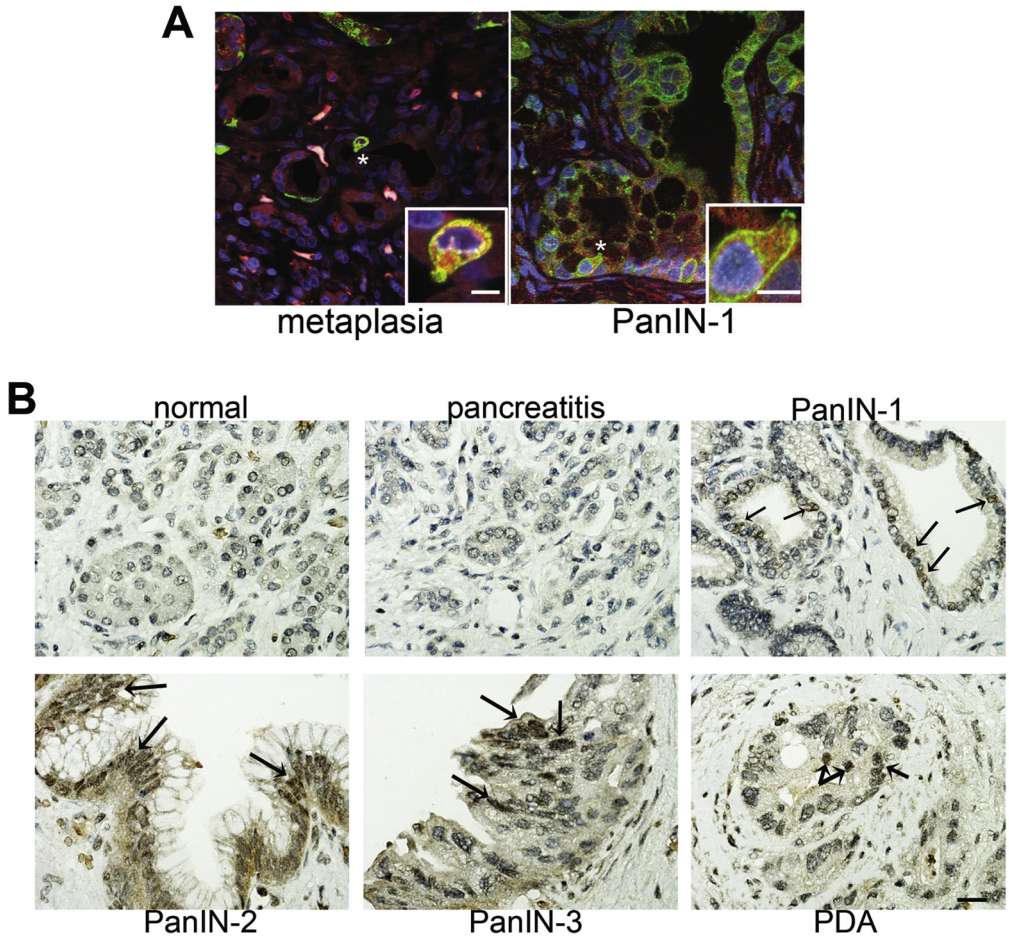

Figure 6. Human pancreatic disease assumes a biliary phenotype. (*A*) Co-IF for phospho-EGFR (Y1068) (*green*) and COX-1 (*red*) on human pancreatic tissue arrays reveals the presence of tuft cells in metaplasia and early PanIN lesions. Nuclei are stained with DAPI. *Scale bars* = 20 μm (*panels*) and 80 μm (*insets*). *Asterisk* indicates area depicted in the *inset*. (*B*) IHC for SOX17 in human pancreatic tissue microarray. Staining was found from PanIN-1 to PDA, with *arrows* indicating regions with positive staining in PanIN-3 and PDA. *Scale bar* = 20 μm. (*C*) Western blot analysis of a panel of human PDA cell lines reveals detectable levels of SOX17 in 9 of 12 cell lines. SOX17 control is lysate from MiaPaCa2 cells transfected with mSox17(164/623)IRESGFP-pTRE.

leading to tetracycline transactivator induction of SOX17 in these cells. Similar to what we observed with SOX17 expression initiated during organogenesis, expression of SOX17 for 6 weeks in adult acinar cells induced a PDX1$^+$ metaplasia in which 10.8% of the epithelium was composed of DCLK1$^+$ tuft cells, mimicking a pancreatobiliary progenitor cell phenotype and confirming a role for SOX17 in tuft cell genesis. The robust stromal reaction included innate and adaptive inflammatory responses (Figure 4) and fibrosis, consistent with a chronic pancreatitis phenotype, but no PanIN formation as determined by Muc5aC IHC (Supplementary Figure 4). Taken together, our data indicate that epithelial overexpression of SOX17 was sufficient to drive a stable phenotype recapitulating all major aspects of chronic pancreatitis, a known risk factor for pancreatic tumorigenesis, in the absence of overt tissue damage.

We then hypothesized that SOX17-induced ADM would collaborate with *Kras^{G12D}* expression, accelerating the relatively inefficient metaplastic process induced by Kras^{G12D} alone. To test this, we treated 5- to 10-week-old *ROSA$^{tTa/+}$;Kras$^{LSL\text{-}G12D/+}$;Ptf1a$^{CreERTM/+}$;tetO-SOX17* mice (*Kras^{G12D};Ptf1a$^{CreERTM/+}$;SOX17^{OE}*) with tamoxifen, which initiates coexpression of Kras^{G12D} and SOX17 in adult acinar cells, and allowed them to recover for 6 weeks. Compared with expression of Kras^{G12D} alone, which ranged from 5% to 12% of total tissue replacement by PanIN-containing regions, concomitant SOX17 expression led to a virtually complete replacement of the normal acinar cell compartment with

PanIN together with reactive epithelia and stroma. Tumors were characterized by PDX1 expression and DCLK1^{+} tuft cells (Figure 5). IHC for the PanIN marker, Muc5aC, confirmed that SOX17 expression did not affect the type of tumors formed in these mice (Supplementary Figure 4) and pathology showed no indication of advanced progression, with the majority of tumors in early PanIN-1/2 stage. These data indicate that SOX17-induced biliary metaplasia is highly susceptible to *Kras^{G12D}* transformation and, as such, can drive initiation of pancreatic disease.

Human Pancreatic Disease Assumes a Biliary-like Phenotype

To determine if human pancreatic disease resembles progression seen in murine models, we assessed human pancreatic tissue and cell lines for biliary markers. To analyze the possible association of tuft cells, human chronic pancreatitis, PanIN, and PDA samples were assessed by IHC for tuft cell markers. Finding that DCLK1 IHC was not robust in positive control human intestinal tissue, we examined factors with coexpression that was also definitive for tuft cells in the mouse: COX-1 and phospho-EGFR. Using co-IF in human pancreatic tissue microarrays, we found that tuft cells were associated with PanINs; 58% (29/50) of PanIN-1s, 40% (12/30) of PanIN-2s, and 33% (9/27) of PanIN-3s contained at least one tuft cell. Unlike the murine model, 30% of PDA samples (24/79) contained tuft cells (Figure 6*A*). Also distinct from the murine model, tuft cells were associated with ducts in 25% (20/79) of normal pancreas samples and 27% (21/78) of pancreatitis samples.

SOX17 expression was also examined in these pancreas tissue microarrays. Although staining in general was less intense compared with murine tissue, 78% (39/50) of PanIN-1s, 80% (24/30) of PanIN-2s, 93% (25/27) of PanIN-3s, and 30% of PDAs (14/47) expressed SOX17, although there were relatively few cells in the PDA samples (Figure 6*B*). Thirty-one percent of chronic pancreatitis samples had SOX17-positive cells, and 6% (5/79) of normal pancreas samples showed positivity confined to large normal ducts.

To confirm SOX17 expression in human PDA, a panel of PDA cell lines was assessed for SOX17 expression by Western blot. Although expression levels of the isoforms varied, 9 of 12 PDA cell lines expressed detectable levels of SOX17 (Figure 6*C*).

Discussion

Pancreatic metaplastic ductal lesions represent a transdifferentiation event and are hypothesized to be preneoplastic. Our work shows that Kras^{G12D}-induced ADM presents as an epithelium that assumes several characteristics of the developmentally related bile duct, marked by the presence of numerous tuft cells and SOX17 expression. This tissue-switching phenomenon, a previously unrecognized process in the pancreas, reinforces the well-established concept that metaplasia is composed of cell types phenotypically similar to those normally restricted to tissues derived from a neighboring region of the embryo.[4] We found that SOX17 expression alone, limited to adult acinar cells, was sufficient to induce a stable metaplastic change that included genesis of tuft cells and coexpression of PDX1, the latter being suggestive of reprogramming akin to a pancreatobiliary progenitor cell. SOX17 overexpression combined with expression of oncogenic Kras^{G12D} leads an enhancement of tumor initiation compared with Kras^{G12D} expression alone, consistent with the metaplastic change being a prerequisite for pancreatic tumorigenesis.

Although enhanced metaplasia is one explanation for accelerated tumorigenesis, SOX17 expression also induced a dramatic, concurrent stromal response reminiscent of chronic pancreatitis. Chronic pancreatitis is a known risk factor for PDA, and several studies have shown that the desmoplastic and inflammatory responses can contribute to pancreatic tumor progression.[32] The ability of SOX17 overexpression to induce this stromal reaction may contribute to the initial epithelial response. However, we favor a model in which the stromal response and susceptibility to transformation are consequences of biliary reprogramming rather than the converse. During examination of Kras^{G12D} or SOX17-induced metaplasia for proinflammatory signals, for instance, we found that key components of the prostaglandin synthesis pathway, including HPGDS, COX-1, and COX-2, the latter of which is a known contributor to pancreatic disease,[33] were expressed at very high levels in metaplastic tuft cells. The identification of these cells as chemosensory is suggestive of a role in detection of injury and initiation of a repair response. Consistent with this, tuft cells also express progenitor cell markers such as LGR5 and DCLK1 (Itzkovitz et al[34] and data not shown), which could aid in tissue repair. Interestingly, pancreatic tuft cells have been proposed to act as tumor-initiating cells when they are engineered to express oncogenic KRAS, which may usurp their putative regenerative function (Steven Leach, personal communication, June 2012).

The role of SOX17 in pancreatic metaplasia is somewhat reminiscent of what was recently observed in mice overexpressing SOX9, a related transcription factor.[2] In the previous study, SOX9 was not capable of hijacking acinar cell differentiation when expressed during organogenesis, as was SOX17, suggesting a unique reprogramming activity for SOX17 in pancreatic progenitors. Conversely, although SOX9 appears to be critical for stabilization of pancreatic metaplasia, we found that administration of doxycycline to turn off SOX17 expression after metaplasia forms did not lead to collapse of metaplastic ducts (data not shown). Thus, SOX17 and SOX9 appear to have similar but distinct functions in pancreatic disease.

We conclude that SOX17 expression itself is sufficient to drive formation of tuft cell–containing, proinflammatory, ductal reprogramming of pancreatic acinar cells in vivo. We found that this metaplasia is accompanied by a dramatic stromal response in the absence of further manipulation. Finally, when combined with Kras^{G12D} expression, SOX17 overexpression enhanced tumorigenesis, suggesting that SOX17 expression and the ensuing biliary transdifferentiation promotes tumor formation.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2013.08.053.

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Author names in bold designate shared co-first authorship.

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Reprint requests
Address requests for reprints to: Howard C. Crawford, PhD, 4500 San Pablo Road, Jacksonville, Florida 32224. e-mail: crawford.howard@mayo.edu; fax: (904) 953-6233.

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Conflicts of interest
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Supplementary Methods

IF

Pancreata from *LSL-Kras^{G12D};Ptf1a$^{Cre/+}$* mice aged 4 to 6 months were prepared for IF by perfusion with 10 mL 0.1 mol/L phosphate-buffered saline (PBS), followed by 50 mL 4% paraformaldehyde in 0.1 mol/L PBS. Pancreata were excised and fixed for 3 hours in 4% paraformaldehyde, followed by three 5-minute washes with 0.1 mol/L PBS and an overnight float in 30% sucrose. Pancreata were incubated in a 1:1 mixture of 30% sucrose and OCT, mixed for 30 minutes, embedded in OCT, and frozen at −80°C. Seven-micrometer sections were permeabilized with 0.1% Triton X-100 in 10 mmol/L PBS and blocked in 10 mmol/L 1× PBS, 5% normal donkey serum, and 1% bovine serum albumin for 60 minutes at room temperature. Sections were then incubated with primary antibodies diluted in 10 mmol/L 1× PBS, 1% bovine serum albumin, and 0.1% Triton X-100 overnight at room temperature. Slides were then washed 3 times with 0.1% Triton X-100/PBS and incubated with Alexa Fluor 488– and/or Alexa Fluor 594–conjugated secondary antibody (Invitrogen, Carlsbad, CA). Stained slides were washed 3 times, rinsed with deionized water, and mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). Images were acquired on a Zeiss 510LS Meta confocal microscope (Carl Zeiss MicroImaging, Inc, Thornwood, NY).

A picrosirius red stain kit (24901; Polysciences, Inc, Warrington, PA) was used for collagen staining, and a periodic acid stain kit (395B; Sigma-Aldrich) was used for mucin staining.

Antibodies

Primary antibodies used were as follows. anti-DCLK1 (ab37994; 1:200), anti-CD3 (ab5690; 1:200), and anti-Ki67 (ab15580; 1:200) were from Abcam (Cambridge, MA). Anti-F4/80 was from AbD Serotec (MCA497R; 1:200; Raleigh, NC). Anti-EpCAM was from BD Pharmingen (552370; 1:500; San Jose, CA). Anti-HPGDS (160013; 1:200) was from Cayman Chemical (Ann Arbor, MI). Anti–phosho-SRC(Y416) (2101s; 1:100) and anti–phospho-44/42(T202/Y204) (9101s; 1:100) were from Cell Signaling Technology (Danvers, MA). Anti–phospho-EGFRY1068 (1727-1; 1:200) was from Epitomics (Burlingame, CA). Anti–β-endorphin (20063; 1:2000) was from Immunostar. Anti-Sox9 (AB5535; 1:400) was from Millipore (Billerica, MA). Anti-TRPM5 (NB100-98867; 1:500), anti–transforming growth factor α (NB100-91993; 1:200), and anti-LGR5 (NBP1-40567, 1:200) were from Novus Biologicals (Littleton, CO). Anti-SOX17 antibodies were from R&D Systems (AF1924; 1:100; Minneapolis, MN) and a generous gift from Dr Jeffrey A. Whitsett (Cincinnati Children's Hospital). Anti–COX-1 (sc-1754; 1:200), anti–COX-2 (sc-1747; 1:200), anti-villin (sc-7672; 1:200), anti-Gfi1b (sc-8559; 1:100), and anti–G-α-gustducin (sc-395;1:200) were purchased from Santa Cruz Biotechnology, Inc (Dallas, TX). Anti–acetylated α-tubulin (T7451;1:200) was from Sigma-Aldrich (St Louis, MO). Rabbit and goat anti–PDX-1 was from Dr Christopher Wright (Vanderbilt University). Texas Red phalloidin (A12381; 1:250) was from Invitrogen (Carlsbad, CA).

Supplementary References

1. Gerbe F, van Es JH, Makrini L, et al. Distinct ATOH1 and Neurog3 requirements define tuft cells as a new secretory cell type in the intestinal epithelium. J Cell Biol 2011; 192:767–780.
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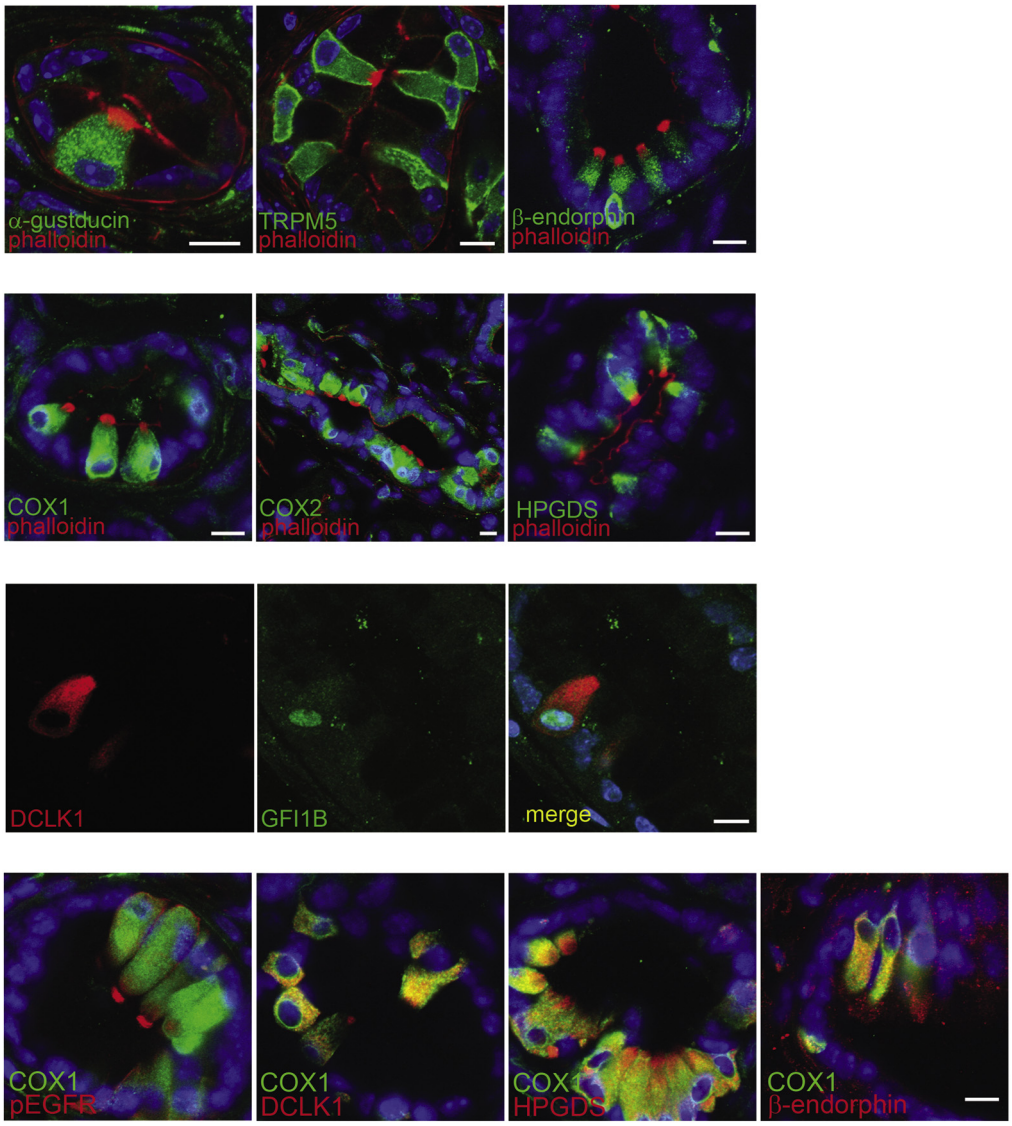
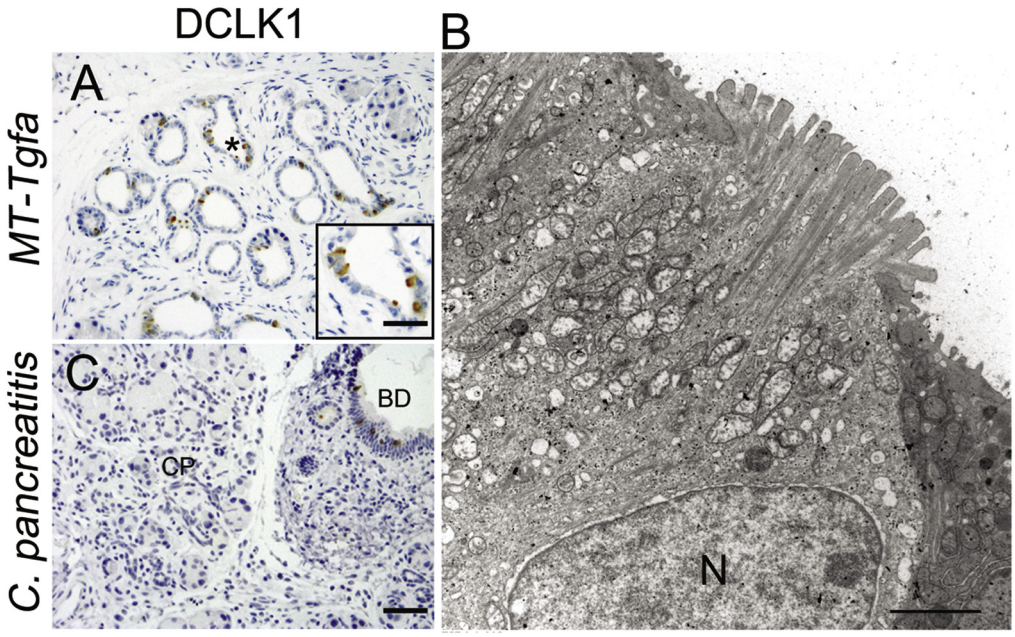
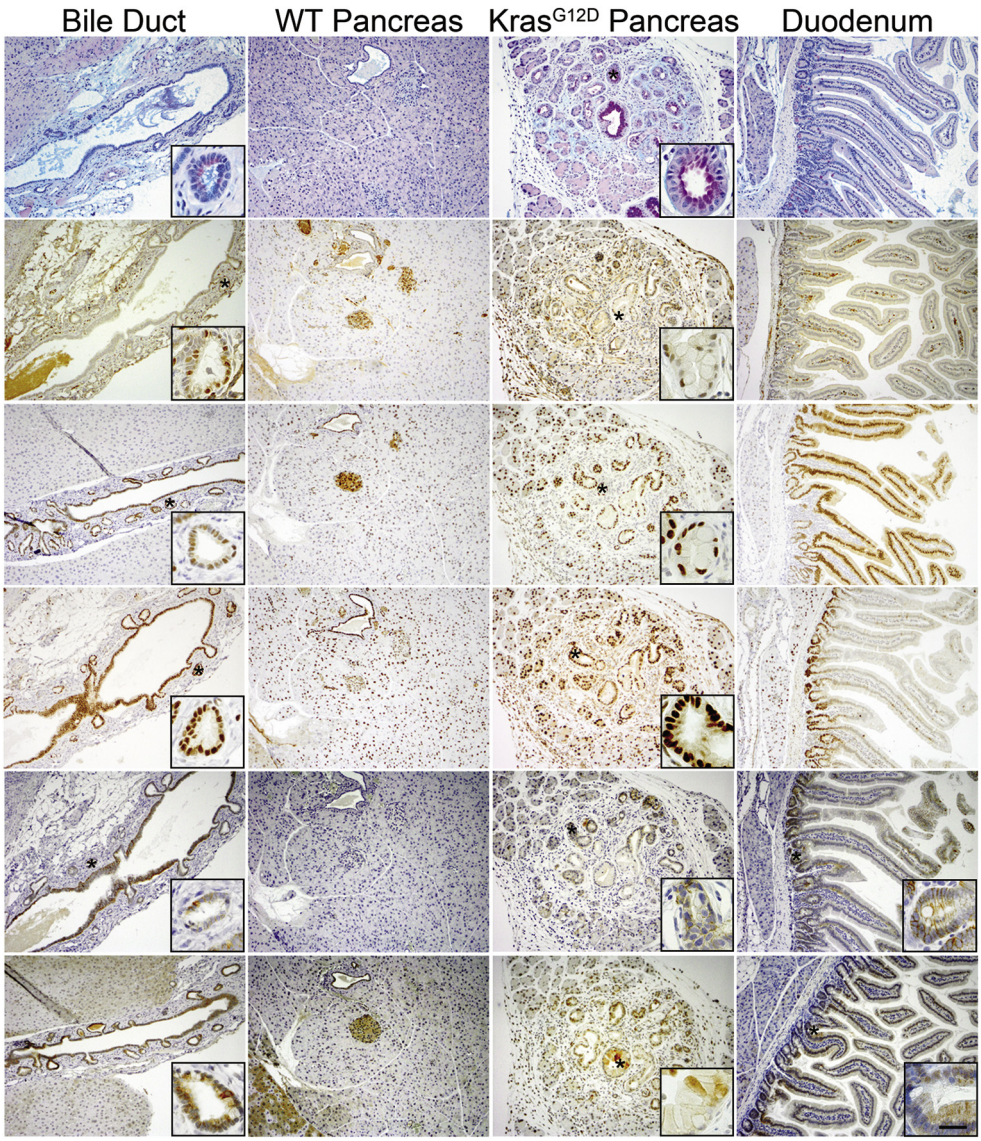


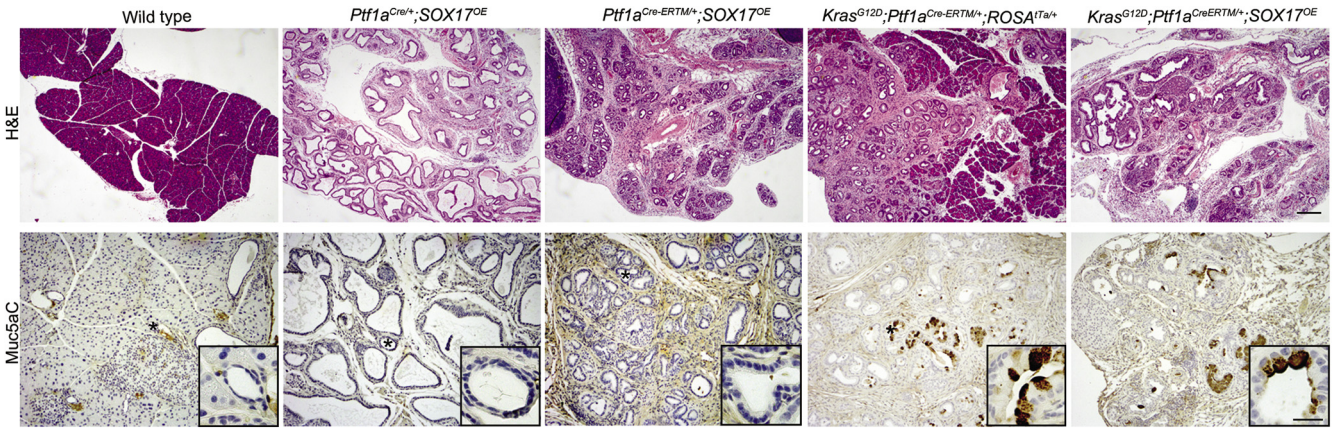
Supplementary Figure 1. Metaplastic pancreatic tuft cells express markers found in normal tuft cells. Co-IF for G-α-gustducin, TRPM5, β-endorphin, COX-1, COX-2, or HPGDS (*green*) and phalloidin (*red*), as well as Gfi1b (*green*) and DCLK1 (*red*), identifies previously described markers for normal tuft cells in pancreatic, metaplasia-associated tuft cells.[1–4] Co-IF for COX1 (*green*) and phospho-EGFR (Y1068), DCLK1, HPGDS, or β-endorphin (*red*) reveals that the same tuft cell population is undergoing active EGFR signaling and expresses DCLK1, prostaglandin synthases, and opioids. Staining was conducted in 4- to 6-month-old *LSL-Kras^{G12D};Ptf1a$^{cre/+}$* mice. Nuclei were stained with DAPI. *Scale bars* = 10 μm.



Supplementary Figure 2. Association of tuft cells with nontransformative models of pancreatic metaplasia. (*A*) IHC for DCLK1 identifies tuft cells in metaplasia resulting from treatment of *MT-Tgfa* mice with $ZnSO_4$. *Asterisk* indicates area depicted in the *inset*. (*B*) Electron microscopy of a tuft cell in the *MT-Tgfa* model. *Scale bar* = 2 μm. (*C*) IHC for DCLK1 in cerulein-induced pancreatitis. *Scale bars* = 100 μm (IHC) and 25 μm (*inset*).



Supplementary Figure 3. Acinar cell–derived pancreatic metaplasia phenocopies normal biliary duct glands. Histological comparison of the bile duct, wild-type pancreas, *Kras*^{G12D};*ROSA*^{YFP};*Ptf1a*$^{Cre\text{-}ERTM/+}$ pancreas, and duodenum reveals molecular similarity between the wild-type bile duct and the diseased pancreas. Known to harbor stem and progenitor cell markers, the biliary duct glands are mucinous and express SOX17, SOX9, PDX1, EPCAM, and LGR5. Upon induction of Kras^{G12D} expression with tamoxifen, the pancreatic epithelium transdifferentiates to a ductal state expressing biliary duct gland markers. *Scale bar* = 100 μm (*panels*) and 25 μm (*insets*). *Asterisks* indicate areas depicted in the *insets*.



Supplementary Figure 4. Overexpression of SOX17 alone induces metaplasia, whereas coexpression with $Kras^{G12D}$ induces PanIN. IHC for Muc5aC identifies $Kras^{G12D}$-driven metaplasia as preneoplastic. Lack of expression in ductal transdifferentiation due to SOX17 alone indicates nontransformed tissue. *Scale bar* = 200 μm (H&E) and 100 μm (IHC). *Asterisks* indicate areas depicted in the *insets*.

Supplementary Table 1. Characterization of Tuft Cell Markers in *$Kras^{G12D}$;Ptf1a-Cre* Pancreata

Protein	Frequency found in tuft cells (identified by phalloidin staining)	Function
pEGFR	100%	Receptor tyrosine kinase
DCLK1	100%	Regulator of microtubules
Acetylated α-tubulin	100%	Structural
G-α-gustducin	88.5%	Taste receptor signaling
TRPM5	97.5%	Taste receptor signaling
COX1	96.5%	Prostaglandin synthesis
COX2	95.2%	Prostaglandin synthesis
HPGDS	98.7%	Prostaglandin synthesis
Vav-1	100%	Rac GEF
β-endorphin	66.7%	Opioid receptor agonist
Ki67	0.5%	Proliferation
Cyclin D1	27.5%	Proliferation