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Causes and effects of epithelial morphogenesis in pancreatitis and pancreatic cancer

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By

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Abstract of the Dissertation

Causes and effects of epithelial morphogenesis in pancreatitis and pancreatic cancer

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Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal disease due to lack of early detection, making the study of initiating events, intrinsic and extrinsic, invaluable. An aberrant epithelial structure consistently associated with PDAC development is the metaplastic ductal lesion (MDL), formed as a result of acinar to ductal metaplasia (ADM) and hypothesized to be pre-neoplastic. Through lineage tracing and immunohistochemistry I have determined that MDLs derive from acinar cells that have converted to structures resembling the developmentally related biliary duct gland. Consistent with this process, MDLs co-express the transcription factors PDX1 and SOX17, a molecular signature reserved for pancreatobiliary progenitor cells during organ development. Additionally, I have found that tuft cells (TCs) are consistently associated with MDLs, a characteristic of the normal bile duct. To probe the importance of pancreas-to-biliary metaplasia, I utilized a transgenic SOX17 overexpression murine model. I found that SOX17 overexpression is sufficient to drive a metaplastic, pancreatitis-like disease state, complete with TC transdifferentiation. While genetic mutation is sufficient to induce pancreatitis, a risk factor for PDAC, extrinsic factors are considered more common effectors. In attempt to discern what environmental stimuli are sufficient to induce pancreatitis, I utilized models of *Salmonella enterica* serovar Typhimurium infection and found that infection is sufficient to induce pancreatitis, including ADM, a possible source of neoplasia. Simulation of infection through lipopolysaccharide-treatment of genetically engineered mouse models suggests that infection is pro-tumorigenic and requires epidermal growth factor receptor (EGFR). My analysis of ADM reveals that both cell intrinsic programming and extrinsic environmental stimuli are sufficient to induce both inflammation and ADM, considered early events in PDAC development.

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List of Abbreviations

ADM	acinar to ductal metaplasia
BMP	bone morphogenetic protein
CAII	carbonic anhydrase II
CAC	centroacinar cell
CD3	cluster of differentiation 3
CDX	caudal-related homeodomain transcription factor
C/EBPβ	CCAAT-enhancer-binding protein
CFTR	cystic fibrosis transmembrane receptor
CFU	colony forming unit
CK19	cytokeratin 19
CNS	central nervous system
COX1/2	cyclooxygenase 1/2
CP	chronic pancreatitis
CSF-1R	colony stimulating factor-1 receptor
CT	computed tomography
CTL	cytotoxic T lymphocyte
CXCR4	C-X-C chemokine receptor type 4
DBA lectin	dolichos biflorus agglutinin
DCLK1	doublecortin and calmodulin like kinase 1
DCS	diffuse chemosensory system
ECM	extracellular matrix
EGF(R)	epidermal growth factor (receptor)
EMT	epithelial-mesenchymal transition
EpCAM	epithelial cell adhesion molecule
EphB2/3	ephrin type-B receptor 2/3
ERBB2	erythroblastic leukemia viral oncogene homolog 2
ERK	extracellular-signal-regulated kinases
F4/80	mouse restricted macrophage receptor
FGF	fibroblast growth factor
FOXA1/2	forkhead box protein A1/2
GATA4/6	zinc-finger transcription factor
Gfi-1β	growth factor independent 1beta transcription repressor
GFP	green fluorescent protein
GLUT2	glucose transporter type 2
GM-CSF	granulocyte-macrophage colony-stimulating factor
GOF	gain of function
H&E	hematoxylin and eosin
HAND	heart- and neural crest derivatives-expressed protein
HB9	homeodomain transcription factor
HB-EGF	heparin-binding EGF-like growth factor
HES1	hairy and enchancer of split-1
HHEX	hematopoietically-expressed homeobox protein
HNF1β	hepatocyte nuclear factor 1 beta
HNF6	hepatocyte nuclear factor 6

HPGDS	hematopoietic prostaglandin D synthase
IPMN	intrapapillary mucinous neoplasm
Kras	Kirsten rat sarcoma viral oncogene homolog
LGFR5	leucine-rich repeat-containing G-protein coupled receptor 5
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
Ly6B.2	polymorphonuclear cell antigen
Mac2	mature murine macrophage marker
MafA	cell-specific insulin gene transcription factor
MAPK	mitogen-activated protein kinase
MEF2	myocyte enhancer factor-2
MDL	metaplastic ductal lesion
MMP	matrix metalloproteinases
MRI	magnetic resonance imaging
NCAM	neural cell adhesion molecule
NF-κB	nuclear factor kappa B
NGN3	neurogenin 3
NKT	natural killer T cell
NKX2.5	NK2 transcription factor related, locus 5
OCT	optimal cutting temperature
OCT4	octamer-binding transcription factor 4
P21	cyclin dependent kinase inhibitor
PAMP	pathogen associated molecular pattern
PanIN	pancreatic intraepithelial neoplasia
PAX8	paired box 8
PBG	peribiliary gland
PDAC	pancreatic ductal adenocarcinoma
PDG	pancreatic duct gland
PDX1	pancreatic and duodenal homeobox 1
PSC	pancreatic stellate cells
PTF1A	pancreas transcription factor 1 subunit alpha
PTS1	pancreatic secretory cationic trypsinogen inhibitor
PVDF	polyvinylidene difluoride
RT-PCR	real time polymerase chain reaction
SCC	solitary chemosensory cell
SHH	sonic hedgehog
SOX	sex determining region Y box
SRC	sarcoma kinase
STAT3	signal transducer and activator of transcription 3
TAM	tumor associated macrophage
TBX	T-box transcription factor
TC	tuft cell
TGFα/β	transforming growth factor α/β
Th	helper T cell
TLR4	toll like receptor 4
TMA	tissue microarray

TNFα	tumor necrosis factor alpha
TRPM5	transient receptor potential cation channel subfamily M member 5
TTX	homeobox transcription factor
VAV-1	vav 1 guanine nucleotide exchange factor
Wnt	wingless signaling
YFP	yellow fluorescent protein

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Publications

Ali WH, Chen Q, **DelGiorno KE**, Su W, Hall JC, Crawford HC, Kanaho Y, Di Paolo G, and Frohman MA. Altered PA localization leads to abnormal actin dynamics in innate immune cells from PLD knock out mice. *PLoS One* (In revision)

Ardito CM, Gruner BM, Takeuchi KK, Lubeseder-Martellato C, Teichmann N, Mazur, PK, **DelGiorno KE**, Carpenter ES, Halbrook C, Hall J, Pal D, Briel T, Herner A, Trajkovic-Arsic M, Sipos B, Liou G, Storz P, Murray NR, Threadgill DW, Sibilia M, Washington MK, Wilson CL, Schmid RM, Raines EW, Crawford HC and Siveke JT. EGF Receptor is required for Kras-induced Pancreatic Tumorigenesis. *Cancer Cell* 2012;22:304-17.

Bombardelli L, Carpenter ES, Wu AP, Alston N, **DelGiorno K**, and Crawford HC. Pancreas-specific ablation of beta1 integrin induces tissue degeneration by disrupting acinar cell polarity. Gastroenterology 2010;138:2531-40, 2540 e1-4.

Chapter 1: INTRODUCTION

Pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarincoma (PDAC) is a highly lethal disease with a median survival period of 6 months post-diagnosis and an abysmal five-year survival rate of just 6%[1]. These statistics are due to late detection; absence of early diagnostics results in diagnosis of PDAC at an already incurable stage. Unlike breast or colon cancers, in which diagnostics and therapies are now readily available, the retroperitoneal location of the pancreas prevents easy preventative screening, meaning that the tissue may be regularly exposed to environmental factors like infection, alcohol, and dietary components, and go unchecked. Treatment options for PDAC have changed little in the last 30 years; people are contracting the disease at the same rate and face a short list of treatment options topped by curative resection, which, for those eligible, only extends median survival to 14-20 months and the 5-year survival rate to 25%[2-5].

There is a clear need for early diagnostics, which requires a deep understanding of initiating events in early tumorigenesis. From the human condition we have learned that pre-cancerous pancreatic intraepithelial neoplastic (PanIN) lesions are associated with PDAC, are ductal in nature, and largely replace the acinar cell epithelium. These lesions are consistently associated with a highly reactive, non-transformed ductal epithelium hypothesized to derive from normal acinar tissue in a process termed acinar to ductal metaplasia (ADM). Acinar and ductal cells make up the exocrine component of the pancreas; acinar cells produce digestive enzymes, which empty into ducts that drain into the duodenum. When damaged, acinar cells convert to duct-like cells, at which time they proliferate, migrate, and redifferentiate to functional acinar cells. In the context of an oncogenic *Kras* mutation, it is thought that metaplastic cells are unable to redifferentiate and, instead, progress stepwise into PanINs and, subsequently, PDAC. Determining what molecular factors are required for ADM to take place may point to detectable changes in the pancreas. Understanding what environmental factors initiate ADM may allow for at risk patients to take preventative measures.

Metaplasia

Metaplasia is a broad pathology term for the conversion of one tissue type into another and is often confused with transdifferentiation, a subtype of metaplasia, which is

defined as the irreversible switch of one differentiated cell type into another[6, 7]. A differentiated cell is a functional cell, typically identifiable under the light microscope; examples include a neuron, an intestinal absorptive cell and a cartilage cell[7]. Transdifferentiation was originally described by Okada and Eguchi to describe the conversion of pigmented epithelial cells to lens fibers during lens regeneration in the newt and was demonstrated *in vitro* utilizing a clonal cell culture system[6, 8]. The term “metaplasia” appeared over a decade after “transdifferentiation” in response to the unforeseen discovery of foreign tissue in ectopic sites, and has since expanded to include any switch in cell type regardless of pathway, including stem cell tissue switching[6, 9, 10]. The classical definition of a stem cell is a type of undifferentiated, pluripotent cell that is both self-renewing and can generate one or more differentiated cell types given exposure to appropriate environmental stimuli[6]. Compared to embryonic stem cells, adult stem cells are believed to have a more limited differentiation potential, typically restricted to a particular lineage. The definition of an adult stem cell contradicts the definition of metaplasia as applied to adult stem cell plasticity, examples of which include adult bone marrow conversion to liver, kidney, lung, and pancreas, demonstrating a metaplastic switch outside of a single lineage[6, 11-14]. Progenitor cells derive from stem cells and are a committed, intermediate stage of differentiation, which proliferate and give rise to differentiated cells[6]. The term “plasticity” has been suggested as an alternative to transdifferentiation and metaplasia, but is typically used in the context of nuclear reprogramming, as applied to regenerative medicine[6, 15]. In some cases, selective outgrowth of the minor cell types originally contained in a given organ may be described as metaplasia; however, a true metaplastic event refers to tissue type switching of adult stem cells or the conversion of preexisting differentiated cells[6].

Clinical significance of metaplasia

Elucidating the intrinsic players and environmental causes of metaplasia is critical, as many metaplasias are considered to be precursors to neoplasia and may be regarded as the first step in tumorigenesis[7, 16]. Epithelial metaplasias generally arise in the context of chronic tissue regeneration and inflammation, in response to injury caused by repeated trauma, infection, or abnormal hormonal stimulation[7, 9]. Tissue damage causes a need for

stem-cell niches to be repopulated with new cells, which gives a metaplastic focus the opportunity to expand to visible size[7]. In this state the epithelium becomes proliferative and dedifferentiates, producing a population of cells with transformative potential. Esophageal adenocarcinoma is derived from patches of intestinal or gastric metaplasia in Barrett's esophagus, where the lower end of the esophagus becomes damaged due to reflux of the acid contents of the stomach[17]. During regeneration of damaged esophageal stratified squamous epithelium from esophageal stem cells, foci of intestinal-type tissue are induced. Epidemiological studies of individuals with intestinal metaplasia in the stomach reveal that metaplasia carries 10-fold increased risk of developing gastric cancer[18, 19]. In smokers, the lung bronchi columnar epithelium undergoes a squamous metaplasia, which correlates to lung cancer development[20]. Vaginal adenosis is the appearance of aberrant glandular metaplasia in the cervico-vaginal squamous epithelium of women who were exposed *in utero* to the synthetic steroid agonist diethylstilbestrol (DES), which was prescribed to prevent miscarriage from the 1940s to 1960s. Formation of these metaplastic islands leads to the otherwise rare development of vaginal adenocarcinoma (clear cell carcinoma)[21]. Finally, it is from acinar to ductal metaplasia (ADM) in the pancreas that PDAC is hypothesized to develop. An understanding of the molecular mechanisms underlying metaplasia may provide a way to antagonize harmful transformative states and prevent tumorigenesis.

Metaplasia is also considered clinically significant due to its relation to the field of cell-based therapies[6]. An understanding of tissue switching allows for human manipulation and the regeneration of diseased or damaged organs, necessary due to shortage of organs for transplantation. The ability to transdifferentiate or differentiate tissue from stem cells allows tissue engineers to treat degenerative diseases, such as Parkinson's disease, diabetes, and heart disease.

Metaplasia as defined by molecular developmental biology

Metaplasia in a given organ is often composed of a developmentally related tissue type, one that arose as an adjacent rudiment in the embryo[6, 9, 22]. This is exemplified in Barrett's esophagus, where the normal squamous epithelium is replaced with intestinal metaplasia, including goblet, enteroendocrine, and secretory cells. Intestinal metaplasia

in the stomach can be either 'complete' and consist of absorptive, Paneth, and goblet cells, or 'incomplete', comprised of columnar and goblet cells[23]. The intestine and stomach develop from adjacent territories of the endoderm in the early embryo[7]. Metaplasia in the bladder in cystidis glandularis is colonic-like as both tissues are derived from neighboring endoderm in the embryo[7, 24].

During embryogenesis, different organs arise due to the coordinated activation and suppression of regulatory genes that, in combination, form a 'code' for that body part, giving rise to the activation of the relevant set of differentiation genes[7]. For example, the heart is encoded by a combination of NKX2.5, MEF2, GATA, TBX, and HAND factors, while the thyroid requires TTX1, TTX2, and PAX8[25, 26]. Tissues that develop as neighboring rudiments in a common cell sheet will have similar combinations of transcription factors and may only be defined as separate tissues by the expression of only one or a few factors, making those genes prime candidates for "master switch" genes[10, 16]. It is the molecular signature, or combination of expressed regulatory genes, which define what region of the embryo will become a particular organ, and it is a change in the expression of "master switch" genes in the adult that directs the tissue type assumed in metaplasia[6, 7].

Caudal-related homeobox transcription factor 2 (CDX2) is considered a master switch gene for intestinal tissue; in the adult, CDX1 and CDX2 expression is restricted to the epithelial layers of the small intestine and colon and, in the adult, is required for development, differentiation, and maintenance of stem cell fate[27]. Homozygous ablation of *Cdx2* in developmental models is embryonic lethal because *Cdx2* is essential for the function of the trophoblast. Heterozygotes are viable and develop metaplastic foci of keratinized, stratified, squamous epithelium resembling esophageal epithelium[7, 28]. These lesions reveal suppression of the remaining *Cdx2* allele, representing local inactivation of this homeotic gene[16]. As determined by two independent groups, transgenic mice that express *Cdx2* in the gastric mucosa under the control of stomach-specific promoters develop ectopic intestinal tissue; all gastric mucosal cells except for enterochromaffin-like cells are completely replaced with intestinal metaplasia, including goblet, enteroendocrine, and absorptive cells[29, 30]. Intestinal metaplasia in the adult, in both the stomach and in Barrett's esophagus, aberrantly expresses both CDX1 and CDX2,

indicating that ectopic expression of *Cdx* genes is causal to the development of metaplasia[7, 27, 31].

Development of the pancreas

Pancreas and duodenal homeobox factor 1 (PDX1) is considered the "master switch" gene for the pancreas. Homozygous deletion of *Pdx1* in mice results in complete depletion of pancreatic tissue, demonstrating the requirement of *Pdx1* for pancreas development[32]. The pancreas, liver, and biliary tract arise from a shared multipotent cell population in the posterior ventral foregut endoderm expressing transcription factors HNF6, HB9, FOXA2, HNF1β, HHEX, PDX1, C/EBPβ, and SOX17[33, 34]. SRY-related HMB-box-17 (SOX17) is required for the formation and maintenance of gut endoderm, vascular endothelium, and fetal hematopoietic stem cells in many vertebrate species, including the mouse, and ablation of *Sox17* in development is embryonic lethal[35-37]. SOX17 expression is driven by embryonic stem cell factor OCT4 and promotes differentiation in ES cells; without it, embryonic stem cells fail to differentiate into extraembryonic cell types and maintain expression of pluripotency factors OCT4, Nanog, and SOX2[38, 39]. SOX17, in turn, drives transcription of endodermal factors, like HNF1β, which is expressed throughout the early endoderm and drives expression of HNF6 in the gut endoderm. HNF6 regulates FOXA2, which, in turn, drives PDX1 expression during pancreas specification[40]. Subsequent activation and repression of transcription factors leads to segregation of PDX1 to pancreatic progenitors, CEBPβ to the liver tissue, and SOX17 to the biliary tract. Due to the developmental relationship and regenerative capacity of these tissue types, it is possible to transform one into another through overexpression or ablation of master switch genes. For example, overexpression of PDX1 in hepatocytes will induce pancreatic cell transdifferentiation through suppression of CEBPβ, which is required for the expression of liver-specific proteins. Ectopic expression of PDX1 in tadpoles and human hepatoma cells will induce a pancreatic phenotype in the liver, including both exocrine and endocrine cells[41].

Of these three tissue types, it was determined by Spence et al. that the extrahepatobiliary tract is most closely related to the pancreas embryologically, meaning that the biliary tract and pancreas have more regulatory genes in common. The common

pancreatobiliary progenitor is PDX1$^+$/SOX17$^+$ at embryonic day 8.5 (E8.5); PDX1 will segregate into the ventral pancreas, while SOX17 directs formation of the biliary primordium[33]. Acting as a master-switch gene, deletion of *Sox17* at E8.5 leads to agenesis of biliary structures and the formation of ectopic pancreatic tissue in the liver bud and common duct, while over-expression of *Sox17* suppresses pancreas development and promotes ectopic biliary-like tissue throughout the PDX1$^+$ domain[33]. SOX17 acts in concert with Notch signaling, activating HES1 in a feedback loop to restrict SOX17 expression to the ventral gut. Loss of *Hes1* at E9.5 results in gall bladder agenesis, severe hypoplasia of extrahepatic bile ducts, and ectopic pancreatic tissue in the common duct[42, 43]. By E10.5 in *Hes1*$^{-/-}$ animals, both SOX17 and PDX1 are no longer detectable in the ventral endoderm, and the ventral pancreas and biliary primordium do not develop into distinct structures.

Downregulation of SOX17 from the ventral pancreas allows for PDX1 to direct formation of pancreas-specific tissue types including exocrine acinar and ductal cells, as well as endocrine islet cells, in response to Hedgehog and Notch signaling, along with cues from the mesenchyme. At E9.5, the pancreas appears as a cluster of cells budding from the dorsal aspect of the gut endoderm, and the ventral bud appears about a day later. The endoderm cells making up these buds rapidly divide and form a branching epithelium surrounded by mesenchyme[44]. The mesenchyme is constituted by the notochord, lateral plate mesoderm, and the vasculature and influences pancreatic development and growth through the temporal release of dosage-regulated signaling of secreted members of the fibroblast growth factor (FGF), transforming growth factor β (TGFβ), and bone morphogenetic protein (BMP) signaling pathways[34, 45, 46]. FGF10 impacts the size of the pancreas progenitor cell population through regulation of Notch signaling; loss of FGF signaling in development blocks epithelial proliferation[47-49]. Activin, a member of the TGF signaling pathway, released from the surrounding mesenchyme regulates appropriate epithelial development; later activation of the TGFβ pathway regulates the endocrine-exocrine fate decision[50, 51]. FGF and BMP signals from the septum transversum mesenchyme pattern the ventral foregut by promoting hepatic fate, while suppressing pancreatic fate[52-54]. It is the concerted effort of highly regulated

external cues that induces transcriptional activity within the foregut epithelium, directing lineage commitment.

Development of pancreatic tissue types

Around E13 in the mouse, the embryonic pancreas undergoes a dramatic transformation in a synchronized wave of differentiation termed the secondary transition[44]. Endocrine islet cell development is driven by PDX1 expression, which persists into adulthood and directly modulates expression of insulin, GLUT2, and glucokinase, which are required for β-cell function[55]. PDX1 and HNF6 expression, as well as inactivation of Notch signaling (to allow for differentiation), is followed by transient expression of neurogenin 3 (NGN3), which initiates β-cell differentiation[56, 57]. Animals lacking NGN3 expression are devoid of islets and die shortly after birth due to hyperglycemia; ectopic expression of NGN3 will cause some cells to exit the cell cycle and express some endocrine markers[57-59]. MafA, and another wave of PDX1 expression, drive insulin production, signaling differentiated, functional, adult β-cells[60, 61]. The exocrine lineage develops in response to a lack of pro-endocrine transcription factors and by the presence of permissive signals from the pancreatic mesenchyme, including Wnt/-catenin signaling, laminin-1, and follistatin[51, 62-67].

The exocrine compartment constitutes more than 90% of the pancreas and consists of acinar and ductal cell epithelia[46]. Adult pancreatic ductal cells line channels that deliver acinar cell digestive enzymes to the duodenum, secrete electrolytes and mucins, and may be identified through expression of functional markers cytokeratin 19 (CK19), cystic fibrosis transmembrane receptor (CFTR), carbonic anhydrase II (CAII) and DBA lectin. Ductal cells develop from $PDX1^+$ cells between E9.5 and E11.5 and express regulatory proteins HNF1β, HNF6, and SOX9[58]. Ablation of transcription factor HNF6 in mice, both globally and specifically from the pancreas, inhibits formation of primary cilia, and cysts are formed instead of ducts[68, 69]. Additionally, mice with pancreas-specific ablation of *Hnf6* present with increased ductal cell proliferation and metaplasia, as well as additional characteristics of pancreatitis[69]. Transcription factor HNF1β (also known as TCF2) is upstream of HNF6 and is also expressed in adult pancreatic ductal cells. As *Hnf1b*$^{-/-}$ mice die before gastrulation, Haumaitre et al.

generated a chimeric mouse by tetraploid aggregation, which displayed agenesis of the ventral bud and rudimentary development of the dorsal pancreas[70]. Lineage tracing reveals that HNF1^{+} cells (E11.5-E13.5) give rise to all pancreatic lineages, but expression persists in ductal and endocrine cells shortly after E13.5 and becomes constrained to ductal cells by E18[71]. SOX9 is yet another critical transcription factor in pancreas development that continues to be expressed in adult ductal cells. SOX9 is essential for the maintenance of a pancreatic progenitor cell pool, and SOX9^{+} cells contribute to all three pancreatic lineages as late as E16.5. Lynn et al. determined that SOX9 regulates expression of HNF1β, HNF6, and FOXA2 *in vitro*. In turn, SOX9 expression is regulated by HNF1β and FOXA2, demonstrating feedback circuits and a possible role for SOX9 coordination of the transcriptional network in pancreatic progenitor cells[44]. Due to its role in progenitor cell maintenance, mice with pancreas-specific ablation of *Sox9* present with hypoplasia of the gland[72]. Lineage tracing demonstrates that 7 days post-birth (P7), SOX9 is expressed in ductal and centroacinar cells, suggesting that this population maintains the exocrine compartment[63, 73].

The second major component of the exocrine pancreas is the acinar cell compartment. Adult acinar cells produce and secrete digestive enzymes and may be identified by their expression of PTF1A and Mist1, as well as functional markers amylase and lipase. At E9.5, PDX1 activates Pancreas-specific transcription factor 1a (PTF1A, also known as p48), which contributes to all three pancreatic cell lineages, but becomes restricted to acinar progenitor cells in the mouse by E13.5. Notch signaling antagonizes PTF1A function and must be suppressed by this stage to allow for acinar cell differentiation[74]. By E15, histologically distinct acinar and ductal cells appear, and by E16.5, acinar cells separate from the central ducts[75-77]. Ablation of *Ptf1a* in the murine embryo leads to absence of the exocrine pancreas, forming a severely abrogated pancreatic rudiment containing ductal and endocrine cell types, with islets largely displaced to the spleen, which is where the endocrine compartment resides in some lower vertebrates[76, 78]. This inactivation switches the character of pancreatic progenitors such that their progeny proliferate in and adopt the normal fates of duodenal epithelium, including its stem cell compartment[78]. Wells et al. determined that β-catenin signaling is

critical for PTF1^{+} cell proliferation and may function in part to maintain exocrine, but not endocrine, precursors in an undifferentiated state[79].

Mist1, another key transcriptional regulator in the terminal differentiation of acinar cells, maintains acinar cell lineage in the developing pancreas by limiting its proliferation. Acinar cells from mice with a gene deletion in *Mist1* exhibit a higher proliferative index; ectopic expression of *Mist1* induces the expression of cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$, implying that Mist1 promotes terminal differentiation of the acinar cell by affecting its cell cycle[75, 80]. Differentiating and mature acinar cells lose PDX1, but retain expression of PTF1A and Mist1, and express digestive enzymes such as elastase-1 and amylase. Kawaguchi et al. propose that PDX1 expression marks a territory of endoderm with intrinsic foregut competence, where co-expression of PDX1 and PTF1A are required for proper pancreatic determination and subsequent proliferation and differentiation[78].

Biliary development

The biliary tree can be divided into two portions, the intraheptatic bile ducts and the extrahepatic bile ducts, the latter of which consists of the left and right hepatic ducts, the common hepatic duct, the gallbladder and cystic duct, the bile duct (choledochus) and the hepatopancreatic ampulla[81]. As in the pancreas, the biliary tree is derived from the ventral foregut endoderm; the cranial portion of the hepatic diverticulum gives rise to the liver and intrahepatic biliary tree, while the caudal portion forms the extrahepatic biliary tract[82]. The intra- and extrahepatic biliary tracts develop separately and through different mechanisms. While the pancreas, liver, and biliary tract share a progenitor population expressing the transcription factors HNF6, HB9, FOXA2, HNF1, HHEX, PDX1, C/EBPβ, and SOX17, the extrahepatic biliary tract distinguishes itself through persistent expression of 'master switch' gene *Sox17*[33]. As previously mentioned, SOX17 directs transcription of HNF6, which is required for formation of pancreatic ducts. Analysis of the biliary tract in *Hnf6*$^{-/-}$ mice reveals abnormal extrahepatic biliary ducts and a complete loss of the gallbladder[83].

Peribiliary glands

A unique feature of both the large intrahepatic bile ducts and the extrahepatic biliary system is the presence of tubule-alveolar peribiliary glands (PBGs) with mucinous and serous glandular acini in the deeper tissue of the duct walls near the fibromuscular layer[81]. Cells located *in situ* in the glands of the biliary tree are able, *in vitro* and *in vivo*, to differentiate into hepatocytes, cholangiocytes, and pancreatic endocrine cells[84]. These pools are phenotypically heterogeneous and express transcription factors, as well as surface and cytoplasmic markers, for stem/progenitors of the liver (SOX9, SOX17), pancreas (PDX1), and endoderm (SOX17, EPCAM, NCAM, CXCR4, LGR5, OCT4), but not mature markers (albumin, secretin receptor, insulin)[81]. The dedifferentiated nature of PBGs and their expression of stem and progenitor factors suggests they act as a stem cell reservoir to the liver, pancreas, and bile duct in a manner similar to intestinal crypts in the intestinal tract.

Adult pancreas plasticity and regeneration

Both the endocrine and exocrine compartments of the pancreas have been shown to possess regenerative capacity; Brockenbrough et al. demonstrated ample regeneration in partially pancreatectomised rats only 8-10 weeks following removal of 90% of the pancreas[85]. It was determined that this response requires recruitment of a stem cell population residing in the ductal system and the proliferation of acinar cells[6, 86]. In another model, recapitulating the autoimmune destruction of islets in type I diabetes, it was determined that islets rapidly regenerate through proliferation and differentiation of ductal cells in a process closely resembling embryonic islet development[6, 87].

To determine how closely pancreatic regeneration recapitulates elements of embryonic development, Nygaard et al. performed comparative histology for adult and embryonic pancreatic markers in murine pancreata from experimental pancreatitis models and controls. They found that surviving pancreatic exocrine cells repress the terminal exocrine gene program and induce expression of proteins normally associated with the undifferentiated pancreatic progenitor cell: PDX1, E-cadherin, β-catenin, and Notch components Notch1, Notch2, and Jagged2[88]. Pinho et al. isolated murine acinar cells, cultured them in suspension, and analyzed molecular changes as compared to

experimental pancreatitis. They found that cultured acinar cells acquire a dedifferentiated phenotype reminiscent of pancreatic embryonic progenitor cells, expressing PTF1A, PDX1, SOX9, and HNF1β and demonstrating reactivation of the Notch pathway[89]. Additionally, it was determined that a senescence program associated with Ras and MAPK activation limits the proliferative capacity of these cells[89].

As in developmental studies, tissue switching to developmentally related tissues has also been recapitulated in the adult pancreas. In a model developed by Rao et al., rats were fed a copper-depleted diet, containing the copper-chelating agent triethylenetetramine tetrahydrachloride, leading to vast destruction of pancreatic acinar cell epithelium. Examined over the course of a recovery period, while being fed a normal diet, was the occupancy of more than 60% of pancreatic volume by albumin-expressing hepatocytes[6, 90]. Under these experimental conditions, proliferation of both ductal and oval cells led to the probable transdifferentiation of hepatocytes. Krakowski et al. have shown that overexpression of keratinocyte growth factor in pancreatic islets is sufficient to induce hepatocyte differentiation[91]. *In vitro* models have been employed to determine the likelihood of this transdifferentiation event. It has been shown in the amylase-expressing, rat pancreatic tumor cell line AR42J that treatment with the synthetic glucocorticoid dexamethasone and oncostatin M can induce conversion to an albumin-expressing hepatocyte phenotype by induction of transcription factor C/EBPβ[92]. Transfection of C/EBPβ into AR42J-B13 cells provokes transdifferentiation, while introduction of the dominant negative form of C/EBPβ prevents this event[92]. This phenomenon has been naturally observed in a subset of human pancreatic tumors[6, 93]. Conversely, ectopic pancreatic tissue has been found in the liver of rats treated with polychlorinated biphenyls, in fish liver tumors induced by carcinogens, and in the liver of a human patient with hepatic cirrhosis; most cases of ectopic pancreatic heterotopia consist of exocrine cells[6, 94-98]. Experimentally overexpressing PDX1 can drive transdifferentiation of adult hepatocytes into pancreatic cells by repressing C/EBPβ, which leads to the reduced expression of mature hepatocyte genes, such as albumin, alcohol dehydrogenase 1β, and glucose-6-phosphate and increased expression of the immature hepatic cell marker α-fetoprotein[99, 100]. The authors determined that dedifferentiation had to occur before PDX1 could initiate the pancreatic gene program

and induce these dedifferentiated cells to enter a new lineage. In many cases, pancreatic metaplasia is observed in hepatic tumorigenesis or injury, such as hepatocellular carcinomas (derived from hepatocytes), cholangiolar neoplasms (derived from the cholangiocytes of the biliary tract), or adenofibrosis[96].

Studies in regenerative medicine have also demonstrated the capacity of cell type switching between different pancreatic cell lineages. Zhou et al. demonstrated that adenoviral vector delivery of early progenitor and islet cell transcription factors PDX1, NGN3, and MafA by injection directly into the tail of the pancreas *in vivo* is sufficient to induce insulin expression and β-cell morphology in acinar cells, as demonstrated by lineage tracing[101]. Pancreatic ductal cells have also been shown to be a source of β-cells. In the rat, it was found that ductal ligation fosters islet cell transdifferentiation, and human ductal tissue can be expanded *in vitro* to produce insulin-positive cells[102, 103]. Exocrine to islet cell transdifferentiation has been demonstrated *in vitro* through treatment of cultured adult exocrine pancreatic cells with leukemia inhibitory factor (LIF) and epidermal growth factor (EGF)[104, 105]. Thorel et al. demonstrated in a β-cell regeneration model that the near complete destruction of insulin-producing β-cells *in vivo* forces glucagon-producing pancreatic α-cells to transdifferentiate to replace them[99, 106].

Pancreatic acinar to ductal metaplasia

It has been shown that in the setting of acute or chronic pancreatitis, or oncogenic *Kras*, pancreatic acinar cells have the capacity to undergo metaplasia to a pancreatic ductal cell phenotype, demonstrating an important link to PDAC[63]. In response to injury, the pancreas activates regenerative processes and dedifferentiates to a ductal epithelium consisting of 'facultative progenitor cells' that express early developmental factors[75]. Siveke et al. demonstrated reactivation of the Notch signaling pathway following tissue injury in experimental pancreatitis[107]. Notch is also activated in the setting of *Kras*-induced metaplasia[108]. Fendrich et al. discovered that Hedgehog signaling is upregulated in acinar cells after induction of experimental pancreatitits and that its blockade, genetically or pharmacologically, allows for ADM, but prevents acinar cell redifferentiation[109]. Morris et al. demonstrated that damage-induced β-catenin signaling is required for acinar cell redifferentiation and stabilized β-catenin signaling antagonizes

the ability of Kras to reprogram acini[110]. ADM may result from reprogramming of a progenitor population, direct conversion of acinar to ductal cells, or dedifferentiation via an intermediate cell type[63].

Metaplasia is often observed in human disease by the appearance of aberrant tissue structure; however, this analysis is conducted histologically from static snapshots, creating skepticism as to cell of origin. It is for this reason that Eguchi and Kodama created criteria to define a metaplastic event. The first is that **the two cell states before and after the transition must be clearly defined structurally and molecularly,** and the second is that **the cell lineage relationship must be established through lineage tracing**[8]. Pancreatic ADM meets the criteria outlined by Eguchi and Kodoma. First, acinar cells are tightly polarized and may be denoted structurally by dense packing of zymogen granules of digestive enzymes at the apical surface; molecularly, acinar cells are $PTF1A^{+}Mist1^{+}PDX1^{low}HES1^{-}SOX9^{-}$[75]. Metaplastic ductal cells are identified by their arrangement into tubular structures and expression of CK19, while, molecularly, they are $PTF1A^{-}Mist1^{-}PDX1^{high}Hes1^{+}SOX9^{+}$. Lineage tracing demonstrates that ectopic ductal tissue in the diseased pancreas arises from metaplasia in models of acute and chronic pancreatitis, meeting the second criterion[111].

EGFR signaling is required for ADM

Molecularly, development of ADM involves a number of pathways, particularly EGFR signaling[63]. Metaplastic acinar structures, retaining some acinar characteristics, over-express EGF signaling components, including EGFR and ERBB2, while metaplastic ductal structures also express active downstream MAPK signaling[112, 113]. Transgenic mice overexpressing EGFR ligands TGFα or heparin-binding EGF-like growth factor (HB-EGF) develop extensive ductal metaplasia and fibrosis in the exocrine compartment[114, 115]. EGFR-driven ductal metaplasia can be mimicked *in vitro* by treating acinar cell explants with TGFα; lineage tracing demonstrates direct derivation of cystic ductal epithelial structures from acinar cells[116, 117]. Recently, Navas et al. demonstrated that pancreas-specific ablation of EGFR through the acinar cell-specific elastase promotor blocks *Kras*-driven and pancreatitis-associated metaplasia[118]. Ardito et al. demonstrated that EGFR ablation inhibits required MAPK signaling[113, 118]. Pancreatic

plasticity has also been demonstrated in human acinar cells through lineage tracing. After one week in culture, surviving human acinar cells were shown to express ductal cell markers; this effect could be partially abrogated by inhibiting MAPK signaling[119].

Pancreatic developmental factors and ADM

Transgenic mouse models demonstrate that maintenance of the transcriptional 'code' identifying adult acinar cells is necessary to prevent metaplasia. Miyatsuka et al. demonstrated that gain of expression of the master switch gene *Pdx1* in the acinar cell compartment is sufficient to induce ADM through STAT3 activation[120]. Overexpression of ductal marker HNF6 is sufficient and necessary to repress acinar markers and to induce ADM both *in vitro* and *in vivo*. Metaplastic conversion of acinar cells is inhibited in both *Hnf6*$^{-/-}$ and *Sox9*$^{-/-}$ mice[121]. Loss of *Mist1* both *in vitro* and *in vivo*, with transgenic mice expressing a dominant-negative Mist1, results in ADM[122]. Additionally, it has been shown that Notch signaling (HES1) is required for TGFα-mediated, EGFR signaling effects and suppresses the acinar cell phenotype[112, 123]. These studies demonstrate a concerted effort between EGFR signaling and alteration of key transcription factors for the induction of ADM. Collectively, these data suggest a model where pancreatic damage induces developmentally related pathway signaling (Notch, SHH, and β-catenin), which allows for ADM through activation of the EGFR pathway and acinar cell dedifferentiation through a change in regulatory genes: a loss of acinar cell genes and a gain in expression of ductal genes. In this dedifferentiated state, the epithelium can proliferate and redifferentiate. However, in the setting of an oncogenic *Kras* mutation, ADM may lead to PanIn formation.

Inflammation and cancer development

Inflammation is an essential immune response required to fight off infection and for the healing process; however, inflammatory responses may be also be detrimental as persistent inflammation can lead to tissue injury and plays a role in several stages of tumor development, including initiation, promotion, malignant conversion, invasion, and metastasis[124, 125]. Inflammation is typically composed of four components: Inducers, such as infection or tissue damage, sensors (inflammatory cells), mediators (cytokines), and

the target tissue. An inducer, for example infection with *S*. Typhimurium, is detected by receptors of the innate immune system, such as toll-like receptor 4 (TLR4), expressed on tissue-resident macrophages. This activation induces production and release of pro-inflammatory cytokines, chemokines, and prostaglandins (mediators), which will act on the target tissue to induce vasodilation, extravasation of neutrophils, and leakage of plasma into the infected tissue. Recruited neutrophils, macrophages, and mast cells seek and destroy pathogens, allowing for clearance of infection[124, 126]. In the case of tissue damage, an inducer, such as tissue damage due to excess alcohol intake, induces tissue damage in the pancreas (target tissue) causing release of molecules from dying cells (such as extracellular ATP) and possible breakdown of products from the extracellular matrix (ECM). Tissue damage is sensed by both tissue-resident macrophages and pain receptors, inducing inflammatory and reparative responses and pain sensation (mediators), respectively[124, 127]. The acute inflammatory response is terminated once the triggering insult is eliminated, the infection is cleared, and tissue repair is accomplished. Resolution involves a switch from pro-inflammatory prostaglandins to anti-inflammatory lipoxins, which leads to a switch from neutrophil to monocyte recruitment resulting in clearance of dead cells and debris, and initiation of tissue repair[128]. If the inflammatory trigger is not eliminated and persists, a chronic inflammatory state may result. This may be the result of chronic infection, such as long-term exposure to *S.* Typhimurium, or unrepaired tissue damage, such as that due to exposure to digestive enzymes due to gallstone blockage of the pancreatic duct. The chronic inflammatory response is typically localized to the site where the inducer is localized and results in different types of local tissue remodeling, such as granuloma formation or generation of tertiary lymphoid organs[124].

Inflammation of the pancreas is referred to as pancreatitis, and has been determined to be a risk factor for pancreatic cancer both in humans and in mouse models[129, 130]. This may seem counterintuitive as inflammation is required for the healing process, but it has been shown to play numerous roles in cancer development, due not only to tissue disturbance, but also due to the nature of the inflammatory response. Immune mediators and modulators, as well as the abundance and activation state of different cell types in the tumor microenvironment, determine whether the inflammatory response is tumor promoting or anti-tumorigenic[125, 131, 132]. The inflammatory cell

component associated with advanced tumors is largely tumor promoting and consists mainly of tumor-associated macrophages (TAMs) and T cells. High TAM content correlates with poor prognosis and TAMs are thought to be required for angiogenesis, invasion, and metastasis, exerting pro-tumorigenic effects through immunosuppression and production of cytokines, chemokines, proteases, growth factors, and angiogenic factors[125, 133, 134].

There are several subsets of T cells, namely $CD8^+$ cytotoxic T cells (CTLs) and $CD4^+$ helper T (Th) cells, which can be further divided into Th1, Th2, Th17, and T regulatory cells, as well as natural killer T cells (NKT). Increased infiltration of T cells, particularly of CTLs, capable of directly lysing cancer cells, and Th1 cells, known to aid CTLs in tumor reduction, correlates with better survival in a number of cancers, including pancreatic cancer, indicating that these populations are anti-tumorigenic[125]. Tcell deficiency or disruption of specific cytotoxic mechanisms has been found to increase susceptibility to oncogenic transformation in animal models[135]. Conversely, several T cell populations have been shown to be pro-tumorigenic, including CTLs, Th1 cells, Th2 cells, and Th17 cells; a pro-tumorigenic role for NKT cells has yet to be found[136-141]. Even T regulatory cells, which are thought to be largely pro-tumorigenic, through suppression of anti-tumorigenic inflammatory mediators, have been determined to have anti-tumorigenic function in some cases[142, 143]. As with TAMs, it is not the inflammatory cell type itself, but the cytokine and chemokine effectors released by said cells, that determines the pro- or anti-tumorigenic effects of the inflammatory response.

Macrophages can also be classified into subtypes; however, this is largely reflective of the milieu of cytokines released, reflecting plasticity as opposed to the lineage pathways that differentiate Th1 and Th2 T cells[144]. M1 macrophages are activated by interferon-γ and microbial products and express high levels of pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-12 or IL-23), major histocompatibility complex molecules, and inducible nitric oxide synthase. These macrophages are largely considered anti-tumorigenic due to their ability to kill pathogens and prime an anti-tumor response. In contrast, TAMs are largely M2 macrophages, which are “alternatively” activated by IL-4, IL-10, and IL-13, down-regulate major histocompatibility complex molecules, and show increased expression of the anti-inflammatory cytokine IL-10,

scavenger receptor A, and arginase. The M2 phenotype promotes tumor angiogenesis and tissue remodeling[125, 144]. In addition to macrophages and T cells, neutrophils can have both anti-tumorigenic effects, by directing cytotoxicity and regulating CTL responses, and pro-tumorigenic effects, through the production of cytokines, proteases, and reactive oxygen species[125].

Acute and chronic pancreatitis

Chronic pancreatitis, a condition marked by metaplasia, inflammation, and desmoplasia, has been identified as a risk factor for PDAC and has been shown to significantly accelerate *Kras*-driven pancreatic disease in animal models[129, 130]. Acute pancreatitis can progress to chronic pancreatitis in humans, often under conditions of persistent environmental insult, and has been shown in mouse models to accelerate *Kras*-driven PDA[110, 145]. Acute pancreatitis can range from mild interstitial pancreatitis to a severe condition associated with necrosis and concomitant multiorgan failure[146]. Most patients have mild attacks, but up to 25% experience a severe attack with a mortality rate between 30 and 50%[147]. Acute pancreatitis is typically rapid in onset and criteria for diagnosis include having two of the following three manifestations: characteristic upper abdominal pain, elevated levels of pancreatic enzymes, and findings of ultrasonography, CT, or MRI suggesting acute pancreatitis[148]. In murine models, acute pancreatitis is induced by short-term exposure to a damaging agent, such as the cholecystokinin analog ceruelin, and is measured by the presence of edema, acinar cell death, and an acute inflammatory response consisting of macrophages and neutrophils[149].

Chronic pancreatitis results from persistent insult to the pancreas and is typically asymptomatic, relying on diagnosis as a result of complications like jaundice, diabetes, or anemia. Diagnosis can often be made based on one or more of the following criteria: pancreatic calcifications evidenced by x-ray, CT, ultrasonography or endoscopic ultrasonography, moderate to marked pancreatic ductal lesions on endoscopic retrograde or intraoperative pancreatography, or typical histology on an adequate pancreatic specimen[150]. As a murine model appropriately reflecting human chronic pancreatitis has yet to be identified, instead, chronic pancreatitis-like disease is induced in mice through multiple exposures to damaging agents like cerulein. In this case, chronic pancreatitis-

like disease is identified by the presence of a stromal reaction, acinar to ductal metaplasia, and the presence of an adaptive immune response consisting of T cells.

Studies show there has been an increase in incidence of pancreatitis, from 33.2 cases to 43.8 cases/100,000 adults from 1994 to 2001, due almost entirely to gallstones, resulting from increasing obesity rates[151]. Gallstone, or biliary, pancreatitis is caused by passage of gallstones into the common bile duct where they can obstruct the biliary or pancreatic ducts, leading to backflow of digestive enzymes and epithelial damage. Alcohol is a well-established cause of pancreatitis and has the highest associated risk of overall mortality; odds of death are increased 90% as compared to biliary pancreatitis[152]. Less common are the inherited mutations associated with the disease. Mutation in cystic fibrosis transmembrane receptor (CFTR) may be present in up to 10% of patients[153]. Hereditary pancreatitis is a rare disease and is caused by mutation in the pancreatic secretory cationic trypsinogen inhibitor (PTS1) gene; cumulative incidence of pancreatic cancer in a person with this mutation is 40% by age 70[154-156]. Infection-associated pancreatitis is also rare and is mainly published in case reports. A number of parasites, viruses, and bacteria, namely *Mycoplasma*, *Legionella*, *Leptospira*, and *Salmonella*, have been associated with disease induction[157, 158]. Pediatric cases may result from multisystem disease or systemic infection[159]. It is important to note that between 10 and 30% of cases are idiopathic in nature[160]. These statistics imply that there are a number of unknown causes of pancreatitis, prohibiting preventative measures. If the causes of disease are not discovered and the condition goes untreated, the risk of recurrence is 40% within 6 years, further increasing the risk of developing PDAC[161].

ADM and the stromal reaction

Pancreatic ADM and presence of a reactive stromal compartment, consisting of immune cells, stellate cells, endothelial cells, nerve cells, and the extracellular matrix (ECM), are components of pancreatitis in both human disease and mouse models[162]. They occur seemingly simultaneously in conditions of damage, as well as tumor progression. More recently it has been discovered that stromal activation is not just a bystander effect of disease progression, but an active contributor known to affect tumorigenesis, angiogenesis, therapy resistance, and possibly the metastatic spread of

tumor cells[162]. Desmoplasia, or fibrotic stroma, is variable among patients and has been researched as a potential prognostic marker[163]. Desmoplasia results from activation of pancreatic stellate cells (PSC), which normally reside in the periacinar space and extend along the base of adjacent acinar cells. Their ability to secrete acetylcholine suggests an intermediate role in the nerve-acinar cell interaction, influencing exocrine secretion[164, 165]. Studies of the interaction between desmoplasia and PDAC have determined that PSC are activated in response to oxidative stress and through the secretion of cytokines and growth factors from cancer cells[164, 166-168]. Fibrosis accumulates throughout disease progression and supports tumor growth by increasing chemoresistance and impacting tumor vascularity[167, 169]. Metaplasia, itself, is pro-inflammatory and secretes both cytokines and growth factors, as evidenced by expression of active STAT3 signaling and up-regulation of EGFR ligands[113, 170]. Fendrich et al. determined that acinar cell-specific overexpression of Sonic Hedge Hog (SHH) was sufficient to induce both metaplasia in the epithelial compartment and stromal expansion[171]. Colby et al. demonstrated this effect through overexpression of pro-inflammatory prostaglandin synthase cyclooxygenase-2 (COX2) in the epithelium[172]. This suggests that pro-inflammatory changes occurring during ADM are activating stellate cells; the epithelium is not only providing a potential source of tumor, but also a pro-tumorigenic microenvironment.

In addition to PSC activation, ADM expression of pro-inflammatory cytokines may also encourage inflammatory cell infiltration, an important link between pancreatitis and PDAC development[129]. Recently, it has been discovered that early PanINs express and release granulocyte-macrophage colony-stimulating factor (GM-CSF), which regulates infiltration of immature myeloid cells, inhibiting the cytotoxic T cell response and protecting advancing tumors from the immune response[173, 174]. GM-CSF is released from cancer cells and has been shown to induce EGFR ligand HB-EGF expression in macrophages and neutrophils[175]. A number of additional studies have shown that infiltrating lymphocytes express EGFR ligands, which, as previously mentioned, are sufficient to induce metaplasia and are required for *Kras*-induced tumor initiation[113, 115]. For example, it has been demonstrated that T lymphocytes associated with ovarian and breast tumors express HB-EGF and that tumor associated macrophages (TAMs), but not carcinoma cells, express EGF[176-178]. Goswami et al. used an *in vivo* invasion assay to

demonstrate a paracrine signaling loop between TAMs and breast carcinoma cells. Blocking the CSF-1 receptor, which is expressed on TAMs, inhibited TAM chemotaxis, and blocking the EGF receptor inhibited invasion of breast carcinoma cells[179]. Collectively, these data suggest that ADM is not only a likely pre-neoplastic lesion evolving to PDAC, but also promotes a pro-tumorigenic microenvironment by eliciting stromal and inflammatory responses. While the lesion itself is not readily detectable, signals from the resulting microenvironment may provide and should be evaluated as a diagnostic of advancing pancreatic disease.

ADM as a precursor to neoplasia

Pancreatic ductal adenocarcinoma (PDAC) is named for the morphologic appearance of the disease and is attributed to misplaced attempts by neoplastic cells to recapitulate normal ductal structures within the pancreas[180]. This terminology suggests that PDAC arises *de novo* and forms ductal-like epithelium, rather than deriving from ductal precursor lesions. There are, however, several reports bolstering the idea that PDAC develops through the progression of pancreatic intraepithelial neoplasias (PanINs), which are non-invasive, ductal, precursor lesions derived from the pancreatic epithelium[181-183]. As in ADM, PanINs express a number of developmentally related pathways and show increased EGFR signaling[112, 113].

Due to the ductal nature of PanINs and their association with PDAC, it is not surprising that early reports on this subject purported the idea of a PDAC cell-of-origin arising from the normal pancreatic ductal epithelium[184]. Attempts to express oncogenic *Kras* in the $CK19^+$ ductal epithelium in the pancreas, however, failed to yield a neoplastic phenotype, though it was designed so that the mutant allele was not expressed under endogenous regulatory elements[185]. Studies of chemical carcinogenesis-induced PDAC in Syrian hampsters and rats advanced the idea that PDAC derives from the endocrine and acinar compartments, respectively; however, induction of widespread damage in the pancreas and lack of lineage tracing prohibits determination of cell of origin in these models[186, 187]. Additional reports noted the heterogeneity of metaplastic and early neoplastic lesions, consisting of both acinar and ductal cells, pointing towards an acinar cell of origin[112].

Specific cell type targeting of oncogenic *Kras* became possible with the advancement of *Cre*-lox technology, where $Kras^{G12D}$ or $Kras^{G12V}$ is conditionally activated by promoter-specific Cre recombination during development[188, 189]. As oncogenic *Kras* is knocked into its own endogenous regulatory elements, physiologic expression levels mimic those observed in human disease. These mice develop murine PanINs (mPanINs), which progress to PDA in combination with additional genetic mutation[190-192]. Many of these models express oncogenic *Kras* in either the *Pdx1* or *Ptf1a* domains during development. Activation of *Kras* in the *Pdx1Cre;LSL-*$Kras^{G12D}$ model results in mPanIN formation and spontaneous progression to PDAC[188, 190]. PDX1 is not cell type-specific, however, and is expressed not only in the progenitor pool that seeds the pancreas, but also the biliary tract, stomach, and duodenum. PTF1A is restricted to adult acinar cells; however, in development, PTF1A is expressed in progenitor cells that seed all three pancreatic cell types. Several groups have utilized models targeting oncogenic *Kras* to the *Elastase*-expression domain[189, 193]. Guerra et al. demonstrate development of PanINs and metastatic adenocarcinoma in this model, in the setting of chronic pancreatitis[129]. While more specific, the *Elastase* domain still targets both mature acinar cells and progenitor-like centroacinar cells (CACs)[194].

It is the more recent advancement of inducible *Kras* models that has allowed for cell-type specific targeting in adult mice, eliminating developmental induction of the oncogene. In a study conducted by Habbe et al., *Kras* was activated in 6-week-old mice in a tamoxifen-inducible system in both $Elastase^{+}$ and $Mist^{+}$ driven models, limiting expression to adult acinar cells. Analysis revealed spontaneous mPanIn formation, proceeded by extensive ADM, in the setting of aberrant Notch activation[180]. Lineage tracing studies with *Rosa26*R reporter mice confirmed that mPanIns in this model were derived from adult acinar cells[180]. Shi et al. utilized this system to simultaneously activate oncogenic *Kras* and knockout *Mist1* from adult acinar cells, which significantly accelerates mPanIn development. It is possible that pancreatic dysplasia induced by the knockout could allow for ductal cells to proliferate into PanIns; however, lineage tracing reveals that acinar cells themselves directly participate, which would indicate that a loss of acinar cell identity, or ADM, is involved in tumorigenesis[195].

Although acinar cells and ADM are a source of pancreatic disease, they may not be the only cell type contributing to disease progression. To discern which pancreatic cell population is most likely the source of tumor development, Gidekel Friedlander et al. expressed oncogenic *Kras* in inducible adult *Pdx1CreER*^{TM} mice (targeting mainly ductal and islet cells), *RipCreER*^{TM} mice (insulin^{+} islet cells), and *proCPA1CreER*^{T2} (acinar cell specific) mice[58, 196-198]. Upon induction of oncogenic *Kras* expression, *RipCreER*^{TM} mice developed mPanINs only under conditions of chronic pancreatic injury. All of the *Pdx1CreER*^{TM} mice developed mPanINs and ductal metaplasia without tissue damage, while, in contrast to previously mentioned reports, disease induction was seen in only a small fraction of *proCPA1CreER*^{T2} mice[196]. These results suggest that pancreatic ductal cells are a more likely source of PanIN formation; however, it may be that *Kras* more easily transforms them, as they do not have to first undergo ductal metaplasia. While these results indicate ductal, acinar, and to some extent, islet cells in pancreatic disease, they underscore that adult acinar cells undergo ADM, which, as in Barrett's esophagus, should be considered a precursor to neoplasia and the first step in tumorigenesis[7, 16].

Mouse models

The following mouse models were employed in these studies:

LSL-Kras$^{G12D/+}$***;Ptf1a***$^{Cre/+}$: As over 90% of human pancreatic ductal adenocarcinoma (PDA) samples have been determined to harbor an activating mutation in the *Kras* gene, PDA is modeled in mice harboring one *LSL-Kras*$^{G12D/+}$ conditional allele[182, 199]. Widespread expression of *Kras*^{G12D} causes embryonic lethality, requiring these mice to have one wild type *Kras* allele and a second conditionally expressed allele[200]. In the presence of Cre recombinase, the lox-stop-lox sequence preceding the mutated *Kras* allele is removed and *Kras*$^{G12D/+}$ is expressed. In order to direct expression of *Kras*$^{G12D/+}$ to the pancreas, Cre recombinase is expressed from the *Ptf1a* promotor (a knock-in to the *Ptf1a* locus), which is pancreas specific and expressed from embryonic day 9.5, seeding all pancreatic cell lineages[74].

MT-Tgfα: In this model, TGFα cDNA has been inserted downstream of an inducible metallothionein-1 promoter, which is expressed throughout the mouse[115]. In the presence of Zn^{2+}, which is often administered in drinking water, TGFα expression is activated during development and is overexpressed in many adult tissues[201, 202]. Overexpression of TGFα results in selective organ hyperplasia, particularly in the liver, intestines, and pancreas[115].

***LSL-Kras*$^{G12D/+}$*;Ptf1a*$^{Cre\text{-}ERTM/+}$**: As in the *LSL-Kras*$^{G12D/+}$*;Ptf1a*$^{Cre/+}$model, this model results in pancreas specific *Kras*^{G12D} expression. However, to restrict expression temporally, Cre activity is made inducible by fusion to the hormone-binding domain of a mutant mouse estrogen receptor (ER^{TM}), which fails to bind the naturally occurring ligand 17β-estradiol at normal concentrations, but retains relatively high affinity for the synthetic ligand 4-hydroxytamoxifen (4-OHT)[203]. Estrogen receptor fusion proteins are inactive, but are released upon binding of ligand, meaning that Cre-recombinase is active upon tamoxifen admistration[203]. To ensure that Cre expression is acinar cell specific, *Ptf1a*$^{Cre\text{-}ERTM/+}$ mice were used (a knock-in to the *Ptf1a* locus); originally generated by Kopinke et al. through recombinase-mediated cassette exchange, inserting the Cre-ERTM coding region into the first exon of *Ptf1a*[203, 204]. Although expressed at E9.5 in the mouse embryo, PTF1A expression is restricted to acinar and centroacinar cells in the adult mouse, meaning that tamoxifen administration in an adult *LSL-Kras*$^{G12D/+}$*;Ptf1a*$^{Cre\text{-}ERTM/+}$ mouse will result in acinar and centroacinar-specific expression of Cre-recombinase and, thus, *Kras*$^{G12D/+}$expression [74].

***Dclk1*$^{\Delta/\Delta}$*;LSL-Kras*^{G12D}*;Ptf1a*$^{Cre/+}$**: To knock-out DCLK1 from the tumor-bearing pancreas, I utilized the *Dclk1*$^{\Delta/\Delta}$ mouse. Created by Koizumi et al., this mouse has LoxP sites flanking exon 3 of the *Dclk1* gene[205]. Homologous recombination occurs upon expression of Cre-recombinase, which leads to excision of exon 3 and ablation of DCLK1 expression from Cre-expressing cells. To ensure DCLK1 ablation would be pancreas specific, I crossed these mice to *Ptf1a*$^{Cre/+}$ mice, which will express Cre-recombinase in all pancreatic lineages from E9.5[74]. To induce tumorigenesis, these mice were crossed to *LSL-Kras*^{G12D} mice.

***Pdx1$^{tTa/+}$;tetO-SOX17*:** Overexpression of Sox17 in the pancreas was accomplished by utilizing a Pdx1-driven tetracycline transactivator mouse (*Pdx1$^{tTA/+}$*) and a tetracycline regulated Sox17 transgene (*tetO-Sox17*)[33]. The Sox17 transgene was created by Park et al. by injecting fertilized zygotes with a plasmid construct consisting of cDNAs encoding full length Sox17, internal ribosome entry site (IRES) sequence, and green fluorescent protein, regulated by a tetracycline-responsive promoter element (TRE), cloned upstream of a CMV minimal promoter[206]. For pancreas-specific Sox17 expression, these mice were crossed to the *Pdx1$^{tTA/+}$* mouse created by Holland et al., which has the coding region of a tetracycline-regulated transactivator (tTA_{off}) knocked into the coding region of the endogenous *Pdx1* gene[207]. The substitution of tTA_{off} for *Pdx1* places tTA_{off} expression under the control of all endogenous Pdx1 transcriptional regulatory sequences, ensuring that the transgene is expressed in cells normally expressing Pdx1 (such as in the pancreas)[207]. Therefore when tetracycline or doxycycline is absent, endogenous Pdx1 transcriptional regulatory sequences drive tTA expression, which can then bind to the TRE of the Sox17 transgene and activate transcription. In the presence of tetracycline or doxycycline, tTA cannot bind to the TRE and expression from the target gene remains inactive[208].

ROSA$^{tTa/+}$;Ptf1a$^{Cre/+}$;tetO-SOX17: As an alternative to the *Pdx1$^{tTa/+}$;tetO-SOX17* mouse, the tetracycline regulated Sox17 transgenic (*tetO-Sox17*) mouse was crossed to the *Gt(ROSA)26Sortm1(tTA)Roos/J* mouse, which has a loxP-flanked stop cassette preventing transcription of a downstream optimized/modified tetracycline-controlled transactivator protein (tTA), combining both the Cre-lox and Tet-off technologies[209]. In this model, Cre-recombinase is required to remove the stop sequence preceding tTA to allow for expression. In the presence of Cre recombinase (Ptf1a-Cre allows for expression in all pancreatic lineages), tTA is expressed and in the absence of tetracycline or doxycline, tTA is available to bind the TRE of the Sox17 transgene and activate transcription.

ROSA**$^{tTa/+}$**;Ptf1a**$^{Cre\text{-}ERTM/+}$**;tetO-SOX17: To overexpress Sox17 specifically in adult pancreatic acinar cells, I combined the tetracycline regulated Sox17 transgene with the Cre-regulated ROSA tTa and employed the tamoxifen-inducible Ptf1a-Cre[203, 206, 209]. In this model, tamoxifen treatment activates the estrogen receptor fusion Cre protein specifically in Ptf1a^{+} cells, which are the only pancreatic acinar and centroacinar cells in the adult mouse, which can now excise the stop cassette preventing tTA transcription. In the absence of tetracycline or doxycycline, tTA can now bind and activate the TRE of the *Sox17* transgene, inducing expression.

NRAMP**$^{+/+}$C57BL/6*:** Different strains of mice show different levels of susceptibility to *Salmonella* infection[210]. In the mouse, the gene *Nramp1*, which is restricted to cells of the monocyte/macrophage lineage, controls a significant component of innate resistance/susceptibility to infection with *S.* Typhimurium by affecting the capacity of the host to control intracellular replication of microorganisms[211-214]. C57BL6/J mice have a single nucleotide polymorphism that renders *Nramp*1 non-functional, making these mice inherently susceptible to *S.* Typhimurium infection, causing them to die just a few days post-infection due to uncontrolled bacterial replication[215]. As such, mice bearing wild type *Nramp1* were used to study long-term (chronic) infection with *S.* Typhimurium as they are less susceptible to infection and are able to chronically carry *S.* Typhimurium up to a year post-infection[216].

C57BL/6J x 129X1/svJ: 129X1/svJ mice have wild type *Nramp1* (are *Nramp1*-sufficient) and are thus naturally more resistant to *S.* Typhimurium infection than C57BL/6J mice, making them a favorable model for persistent/chronic *Salmonella* infection[216]. As a large body of work with *S.* Typhimurium has been conducted in C57BL/6J mice, F1 generation C57BL/6J x 129X1/svJ mice were bred to take advantage of the autosomal dominant resistance of 129X1/svJ mice to *S.* Typhimurium, as well as the body of literature on infection in C57BL/6J mice[217].

***Egfr**$^{\Delta/\Delta}$**;LSL-Kras**^{G12D}**;Ptf1a**$^{Cre/+}$*: To knock-out EGFR from the tumor-bearing pancreas, I utilized *EGFR*$^{\Delta/\Delta}$*;LSL-Kras*^{G12D}*;Ptf1a*$^{Cre/+}$mice as previously reported[113]. To

conditionally knock out EGFR, Lee et al. inserted LoxP sites flanking exon 3 of the *Egfr* gene[218]. Homologous recombination occurs upon expression of Cre-recombinase, which leads to excision of exon 3 and ablation of EGFR expression from Cre-expressing cells. For pancreas-specific EGFR ablation, these mice were crossed to *Ptf1a*$^{Cre/+}$ mice. To induce tumorigenesis, these mice were crossed to *LSL-Kras*^{G12D} mice.

CHAPTER 2: K-ras-induced pancreatic epithelial metaplasia represents a biliary transdifferentiation event

INTRODUCTION

The necessity for discovery of early markers of tumorigenesis strongly advocates for a focus on aspects of early disease progression, such as acinar-to-ductal metaplasia (ADM), a transformative event resulting in the presence of highly reactive ductal epithelia. As previously mentioned, epithelial metaplasia is a hallmark of inflammatory and neoplastic disease in several organs, such as the presence of Barrett's metaplasia leading to development of esophageal adenocarincoma. In the pancreas, ADM is generally described as the replacement of pancreatic acinar cells with pancreatic duct cells.

One cell type absent from the murine exocrine pancreas that is found commonly in the developmentally related intestine and biliary tract is the tuft cell, a type of solitary chemosensory cell (SCC). SCCs are part of the diffuse chemosensory system (DCS) and are analogous to taste cells, though they do not aggregate in buds. SCCs are thought to link chemosensation of intraluminal content to local control of absorptive and secretory processes, as well as central nervous system (CNS) activity[219]. Expressing taste cell receptors, SCCs in a healthy individual may function in the detection of irritants and toxins, releasing effectors that stimulate the nervous system to protect the body by inducing apnea or by slowing gastric emptying[219, 220]. Information about the distribution, morphology, and proteome of this normal cell type is constantly expanding, but functional data are scarce, and a role for the DCS in human disease has not been clearly demonstrated.

Tuft cells are characterized by microvilli accompanied by deep actin rootlets and a well-developed tubulovesicular system in the supranuclear cytoplasm[221]. Distinct from enteroendocrine cells, which are also thought to play a role in chemosensation, tuft cells can be distinguished by co-expression of DCLK1, COX1, COX2, hematopoietic prostaglandin-D synthase (HPGDS), and SOX9; the limited HPGDS-COX1 marker signature has been demonstrated to be sufficient for unambiguous identification[222]. Physical features, as well as expression of taste cell signaling components and effectors, indicate that this cell type acts as a signal integrator with the potential to interpret external information and alter the tissue microenvironment[219]. Stem-like characteristics, such as expression of stem and endoderm progenitor factors, however, suggest a possible

role as a multipotent progenitor cell, although simultaneous expression of differentiation markers of various tissues has made this controversial[222, 223].

In my analysis of pancreatic metaplasia, I have discovered that tuft cells are common in pancreatic disease both in human patients and murine models, with the highest proportion found in ADM. Lineage tracing studies show that tuft cells transdifferentiate from adult acinar cells and express a full array of markers associated with mature tuft cells found in other tissues. To determine if pancreatic metaplasia represents conversion to a biliary-like phenotype, I assayed for biliary markers and found that ADM and murine PanINs (mPanINs) consistently contained a PDX1^{+}/SOX17^{+} cell population reminiscent of the common pancreatobiliary progenitor[33]. Forced expression of SOX17 in the pancreas resulted in a pancreatitis-like phenotype, marked by tuft cell rich ADM, fibrosis, and an adaptive immune response. I conclude that pancreatic ADM found in PDAC represents transdifferentiation to a biliary phenotype and is an active contributor to disease progression due to both a pancreatobiliary progenitor phenotype and the potential activity of SCC tuft cells.

MATERIALS AND METHODS

Mouse strains

LSL-Kras$^{G12D/+}$, *Ptf1a*$^{Cre/+}$, *MT-TGF*α, *Ptf1a*$^{Cre\text{-}ERTM/+}$, *tetO-SOX17*, *Pdx1*$^{tTa/+}$, and *ROSA*$^{tTa/tTa}$ strains have been described previously and were genotyped accordingly[78, 115, 206, 224-226]. *ROSA*^{YFP} and *DCLK1*$^{\Delta/\Delta}$ mice were obtained from Jackson Laboratories. 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.

Genotyping

Genotyping of transgenic mice was accomplished by PCR from tail DNA using gene specific primers described below:

Strain	Primers
LSL-Kras$^{G12D/+}$	F: CGCAGACTGTAGAGCAGCG R: CCATGGCTTGAGTAAGTCTGC
Ptf1a^{Cre}	F: TCGCGATTATCTTCTATATCTTCAG R: GCTCGACCAGTTTAGTTACCC
MT-TGFα	F: TGTGGCCCTGGCTGTCCTCA R: GGCACTGCCAGGGGTGTTGT
ROSA^{YFP}	F: ACATGGTCCTGCTGGAGTTC R: GCGATGCAATTTCCTCATTT
DCLK1$^{\Delta/\Delta}$	F: CAGGACACAGATGGGGAACT R: AGTGAGATGGTTTACAGGCAAG
tetO-SOX17	F: CGAGCTGGACGGCGAGCTAA R: GCTGTTGTAGTTGTACTCCAGCT
ROSA$^{tTa/+}$	F: CCTGGACAAGTCCATCAACTCCGCCC R: CCTCCTTGGCCACGTGCTCCTGGTCC
Pdx1$^{tTa/+}$	F: TAGAAGGGGAAAGCTGGCAAG R: TCCAGATCGAAATCGTCTAGCG

Mouse Tissue Microarrays

Mouse tissue microarrays (TMAs) were a generous gift of Dr. Kenneth Olive. Custom 5-mm TMAs were assembled by a hand corer and pre-cast recipient molds. Pancreatic ductal adenocarcinoma from 10 *LSL-Kras*^{G12D}*;P53*$^{R172H/+}$*;PDX1*$^{Cre/+}$ mice were included. Adjacent PanIN-containing tissues were included for 5 of the tumors, while distant metastases from several organ sites were included for the 5 other tumors.

Induction of experimental metaplasia *in vivo*

To induce pancreatitis-associated metaplasia, wild-type aged-matched mice of either sex were injected intraperitoneally twice daily with 250 g/kg cerulein (Sigma-Aldrich, St. Louis, MO) for two weeks followed by 24 hours recovery. Metaplasia was induced in the MT-TGFα strain by administration of 25 mM $ZnSO_4$ in drinking water for either 3, 6, or 9-10 months.

Human samples

Distribution and use of all human samples were approved by the Institutional Review Boards of Vanderbilt University Medical Center and the Mayo Clinic. Human tissue microarrays were staged by Dr. Kay Washington.

Immunostaining

Tissues were harvested and fixed overnight in 4% paraformaldehyde. Immunohistochemistry was performed as previously described[227]. Slides were counterstained with hematoxylin and photographed on an Olympus BX41 light microscope (Olympus, Tokyo, Japan).

Pancreata from *LSL-Kras*^{G12D};*Ptf1a*$^{Cre/+}$ mice aged 4-6 months were prepared for immunofluorescence by perfusion with 10 ml of 0.1 M PBS, followed by 50 ml of 4% PFA in 0.1 M PBS. Pancreata were excised and fixed for three hours in 4% PFA, followed by three 5 minute 0.1 M PBS washes and an overnight float in 30% sucrose. Pancreata were incubated in a 1:1 mixture of 30% sucrose and OCT compound, mixed for 30 min, embedded in OCT and frozen at -80°C. Seven µm sections were permeabilized with 0.1% Triton X-100 in 10 mM PBS and blocked in 10 mM PBS, 5% normal donkey serum, 1% BSA for 60 min at RT. Sections were then incubated with primary antibodies diluted in 10 mM PBS, 1% BSA, 0.1% Triton X-100 overnight at RT. Slides were then washed three times with 0.1% Triton X-100/PBS and incubated with Alexafluor488 and/or Alexafluor594-conjugated secondary antibody (Invitrogen, Carlsbad, CA). Stained slides were washed three 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).

Picrosirius Red Stain Kit (Polysciences, Inc., 24901) was used for collagen staining. A Periodic Acid Stain Kit (Sigma Aldrich, 395B) was used for mucin staining.

Primary Antibodies

Primary antibodies used were as follows: anti-DCLK1 (ab37994; 1:200), anti-GATA6 (ab22600), anti-CD3 (ab5690, 1:200), anti-CXCR4 (ab2074, 1:100), and anti-Ki67 (ab15580; 1:200) were from abcam. Anti- F4/80 (MCA497R, 1:200) and anti LY6B.2 (MCA7716) were from AbD Serotec. Anti-EpCAM was from BD Pharmingen (552370, 1:500). Anti-HPGDS (160013, 1:200) was from Cayman Chemical. Anti-phosho-SRC(Y416) (2101s; 1:100), anti-phospho-44/42(T202/Y204) (9101s; 1:100), anti-VAV-1 (2502s; 1:100), anti-HSP90 (4874), and anti-phospho-μ-opioid receptor(S375) (3451s, 1:200) were obtained from Cell Signaling Technology. Anti-phospho-EGFR (Y1068) (1727-1, 1:200), and anti-Cyclin D1 (ab134175) was from Epitomics. Anti-β-endorphin (20063; 1:2000) was from Immunostar. Anti-TRPM5 (NB100-98867; 1:500), anti-LGR5 (NBP1-40567, 1:200), and anti-TGFα (NB100-91993; 1:200) were obtained from Novus Biologicals. Anti-neurofilaments (heavy) (AB5539; 1:100), anti-SOX9 (AB5535, 1:400), and anti-Musashi (AB5977) were obtained from Millipore. Anti-SOX17 (AF1924; 1:100), anti-EphB2 (AF467), and anti-EphB3 (AF432) were obtained from R&D Systems. Anti-FOXA2 (HNF-3, sc-6554), anti-FOXA1 (sc-6553), anti-GATA4 (sc-9053), anti-COX1 (sc-1754; 1:200), anti-COX2 (sc-1747; 1:200), anti-villin (sc-7672; 1:200), anti-Gfi1b (sc-8559) and anti-G-α-gustducin (sc-395; 1:200) were purchased from Santa Cruz Biotechnology, Inc. Anti-acetylated-α-tubulin (T7451; 1:200) was obtained from Sigma-Aldrich. Anti-PDX-1 was obtained from Dr. Christopher Wright (Vanderbilt University). Texas Red Phalloidin (A12381; 1:250) was obtained from Invitrogen.

Electron Microscopy

Tissue was prepared for EM by perfusion of mice with 2% paraformaldehyde/2.5% EM grade glutaraldehyde in 0.1M PBS, pH 7.4. Samples were viewed with a FEI Tecnail2 BioTwinG^2 transmission electron microscope at 80 kV. Digital images were acquired with an AMT XR-60 CCD Digital Camera System.

Tuft cell quantitation

DCLK1 immunohistochemistry was performed on paraffin-embedded tissue from 11 *LSL-Kras*^{G12D}*;Ptf1a*$^{Cre/+}$ mice ranging in age from 4 months to 1 year, as previously described. A minimum of twenty images at 40x were acquired per slide and lesions staged. Tuft cells were quantitiated as DCLK1^{+} cells per number of nuclei per lesion. For quantitiation in MT-TGFα mice, 10 images at 40x were taken from 9 mice treated with $ZnSO_4$ for 3-10 months, tuft cells were quantitiated as DCLK1^{+} cells per number of nuclei per lesion.

Lineage Tracing

Recombination was induced in *LSL-Kras*$^{G12D/+}$*;ROSA*^{YFP}*;Ptf1a*$^{Cre\text{-}ERTM/+}$ mice with one daily intraperitoneal (i.p.) injection of 3 mg of tamoxifen (Sigma-Aldrich) for 5 d. Tumorigenesis was accelerated by a daily i.p. injection of 250 g/kg of cerulein for 5 d. Mice were sacrificed 9 weeks later and tissue was prepared for immunofluorescence.

Cell Culture

Human PDA cell lines were purchased from the American Type Culture Collection (ATCC) and maintained at 37°C in 5% CO_2 in the ATCC-recommended medium, supplemented with 10% fetal bovine serum and 0.5 g/mL gentamicin.

Western Blotting

Pre-confluent cells were harvested in ice cold RIPA buffer supplemented with PhosStop phosphatase inhibitor and cOmplete EDTA-free protease inhibitor (Roche, Indianapolis, IN). Protein (75 μg) was run on a 7% SDS-gel and blotted to a PVDF membrane for antibody incubation.

RESULTS

Tuft cells are a consistent component of epithelial metaplasia in mouse models of pancreatic tumorigenesis

The *LSL-Kras*$^{G12D/+}$*;Ptf1a*$^{Cre/+}$ murine model of pancreatic tumorigenesis presents with mainly ductal metaplasia and early murine PanIns (mPanINs), up to ~1 year of age, when later stage mPanINs and, occasionally, PDAC, is found. The EGFR pathway has long been associated with PDAC progression and, recently, Ardito et al. demonstrated that activity is required for the induction of pancreatic tumorigenesis[113, 228]. This discovery led me to assess EGFR pathway activity by immunofluorescence (IF) in this model. I observed significant heterogeneity within metaplastic structures and while EGFR activity was elevated in metaplastic structures overall, I identified isolated cells strikingly positive for pY1068 EGFR, pY416 Src, pT202/pY204 ERK and the EGFR ligand TGFα, at levels above those of the surrounding epithelia (Figure 1A). This staining pattern was never observed in the ducts of wild type control pancreata (data not shown), but could readily be identified in the pancreatobiliary tract, previously shown to be densely populated with tuft cells in the rat[221]. Using phalloidin to costain for F-actin, I noted that these cells had a unique arrangement of microfilaments, marked by a perpendicular orientation to the apical membrane, typical of a tuft cell[221]. IF for villin and acetylated α-tubulin confirmed the presence of both prominent microvilli and the tubulovesicular system, respectively (Figure 1B). Using the unique microfilament arrangement as a guide, electron microscopy of a four-month-old *LSL-Kras*$^{G12D/+}$*;Ptf1a*$^{Cre/+}$pancreas confirmed that tuft cells were commonly integrated into ADM (Figure 1C). Quantitation of co-IF for pY1068 EGFR and phalloidin showed that 100% (n=300) of tuft cells have activated EGFR.

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 thought to represent a terminally differentiated cell population[222, 229, 230]. 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 1D). The remaining 28% of DCLK1 positive cells that were not obviously tuft cells were possibly misidentified due to section planarity or represent a non-tuft cell population, such as an adult stem cell population identified previously[229].

Tuft cells associated with pancreatic metaplasia express markers of differentiated SCCs

Normal tuft cells in tissues such as the intestine and bile duct express several signaling molecules consistent with the SCC functions of chemosensation of intraluminal content and localized control of absorptive and secretory processes[219]. Mature SCCs can be identified by expression of taste cell signaling components; such as G-protein coupled receptor G-α-gustducin and ion channel TRPM5[219, 231-234]. TRPM5 has been shown to comprise a ubiquitous signaling component in chemosensory cells, which, in intestine, are primarily tuft cells[235, 236]. Identified through IF co-staining with phalloidin, I determined that 88.5% of metaplasia-associated tuft cells are G-α-gustducin positive (n=339) and 97.5% are TRPM5 positive (n=300) (Figure 2A,B). In line with a terminally differentiated cell, I found that 99.5% are Ki67 negative (n=200) and 72.5% of tuft cells are Cyclin D1 negative (n=300), demonstrating that most tuft cells are not in cell cycle and may be considered differentiated, although this is also consistent with a slowly cycling stem cell (Figure 2C,D).

Tuft cells associated with pancreatic metaplasia express stem cell markers

The literature currently contains conflicting accounts of what cell types are marked by DCLK1 expression, with some groups reporting it as a marker of differentiated tuft cells in the intestines, while other groups report it as a stem cell marker in the pancreas[222, 229]. As previously mentioned, I have determined that about 72% of DCLK1$^+$ cells were definitively tuft cells. Recently, it has been reported that intestinal tuft cells harbor transcripts of proteins that are typically localized to the stem compartment of the villus: LGR5, EphB2, EphB3, and Musashi[223]. To assess the differentiation status of pancreatic metaplasia-associated tuft cells, I conducted co-IF for phalloidin and the aforementioned markers. LGR5 was expressed throughout metaplasia,

but at particularly high levels in tuft cells. EphB3 was not expressed in tuft cells (data not shown), while EphB2 was ubiquitous throughout metaplasia, to include tuft cells, and particularly high in the stroma. Musashi appeared to be tuft cell specific (Figure 3). While the presence of these proteins may indicate that tuft cells are stem-like, it is also important to note that they are also functional proteins and may be required for tuft cells to express functional components of the ectoderm (inflammatory), endoderm (epithelium), and mesoderm (neuronal).

Tuft cells express inflammatory cell effectors with the potential to alter the tissue microenvironment

Expression of taste cell signaling components suggested a potential role for ADM-associated tuft cells as SCCs, meaning they likely produce relevant environmental effectors. In the normal gastrointestinal tract, tuft cells express inflammatory cell markers that include both isoforms of cyclooxygenase (COX-1 and COX-2), as well as hematopoietic prostaglandin D synthase (HPGDS)[222]. As determined by co-staining with phalloidin, I found that 96.5% of pancreatic tuft cells express COX-1 (n=311), 95.2% express COX-2 (n=315) and 98.7% express HPGDS (n=304) (Figure 4A). Expression of these critical components of prostaglandin synthesis strongly suggests that metaplastic tuft cells are capable of contributing to remodeling of the inflammatory microenvironment. In addition, I have found that tuft cells constitute the sole epithelial source of the Rac-GEF VAV-1, previously thought to be inflammatory cell-specific and noted to be correlated with poor survival in PDA; 100% of metaplastic tuft cells express VAV-1 (n=300) (Figure 4A)[237]. In line with both an inflammatory cell and a progenitor cell phenotype, pancreatic tuft cells specifically express transcription factor Gfi-1β, a progenitor cell marker of various hematopoietic cell lineages, also suggested to be involved in intestinal lineage decisions (Figure 4B)[238]. It is important to consider that while Gf1-1β is a progenitor marker, it is also a transcription factor and may be necessary for this epithelial cell type to express markers typical to other germ layers.

Tuft cells express neuronal cell effectors with the potential to alter the tissue microenvironment

In addition to inflammatory cell components, tuft cells have also been described to express neuronal-specific effectors such as β-endorphin, an opioid neurotransmitter known to mediate multiple functions in gastrointestinal mucosa physiology, including regulation of gastric emptying, gut motility, and intestinal secretion[222, 239]. β-endorphin has been described as a potent analgesic, 12-33 times more potent than synthetic morphine, requiring TRPM5 for release[240, 241]. Neurotransmitter expression together with SCC close proximity to nerve fibers in the murine stomach and nasal cavity has suggested a neuronal signaling role for these cells. Indeed, co-staining for β-endorphin with phalloidin demonstrated that 66.7% of pancreatic tuft cells were β-endorphin positive (n=300) (Figure 5A). I also confirmed that tuft cells are in close proximity to nerve fibers in *LSL-Kras*^{G12D}*;Ptf1a*$^{Cre/+}$ pancreata by co-staining for neurofilament proteins and phalloidin (data not shown). Co-IF for active phosphorylated μ-opioid receptor, a receptor for β-endorphin, showed that nerves near tuft cells had activated this analgesic pathway, implicating tuft cells in the opioid-dependent masking of pain associated with early stage pancreatic cancer (Figure 5B)[242, 243].

Tuft cells are most commonly associated with metaplasia in pancreatic disease

To determine the association of tuft cells with disease progression, I quantitiated 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 lesions (15% of ductal cells) and decreased throughout disease progression, constituting 11% of the epithelium in mPanIN-1, 8% in mPanIN-2 and 3% in mPanIN-3, whereas metastatic disease was devoid of tuft cells (Figure 6A). Analysis of pancreata from the *LSL-Kras*^{G12D}*;P53*$^{R172H/+}$*;Pdx-1*$^{Cre/+}$ model of PDAC revealed that tuft cells are also associated with this model of pancreatic disease (Figure 6B).

As tuft cells are most abundant in metaplasia, I analyzed pancreata from MT-TGFα transgenic mice, which form extensive metaplasia after months of TGFα expression[115]. Identified and quantitated by DCLK1 expression, I determined that 73.5% of metaplastic ducts in this model contained tuft cells. Within tuft cell-containing ducts,

tuft cells constituted 14.4% of the epithelium, demonstrating that chronic EGFR activation was sufficient to induce the formation of tuft cell-containing metaplastic ducts (Figure 7A). Tuft cell identity was confirmed by electron microscopy (Figure 7B).

Examining another model of pancreatic metaplasia, I used cerulein treatment to induce a chronic pancreatitis-like state. DCLK1 IHC revealed that tuft cells were rare under these conditions (Figure 7C). Tuft cell genesis being induced by chronic *Kras*^{G12D} or TGFα expression, but not by relatively short term cerulein treatment, from which the murine pancreas is known to recover, suggests that tuft cells mark a stable disease state, rather than a transient wound-healing response.

Metaplastic tuft cells suggest adoption of a biliary phenotype

Tuft cell localization has been previously described within the normal murine biliary tract and intestine; however, in my experiments, I never observed these cells in random sections of wild type pancreata[222, 244]. To definitively determine if the normal main pancreatic duct harbored a tuft cell population, I dissected the main pancreatic duct away from the common duct of the biliary tract and the pancreatobiliary duct (the segment of duct following the intersection of the main pancreatic duct and the bile duct, prior to fusion with the duodenum) and examined them histologically. As expected, the murine biliary tract, which was composed of a central duct, decorated along its length by ancilliary peribiliary glands (PBGs), was lined with columnar epithelium, including numerous tuft cells, identified by DCLK1 IHC (Figure 8)[81]. In contrast, the murine main pancreatic duct was composed of low cuboidal epithelium, lacked PBGs and was entirely devoid of tuft cells (Figure 8). The pancreatobiliary duct was morphologically similar to the bile duct, including PBGs and numerous tuft cells (Figure 8). I conclude that, while present in the pancreatic ducts of more advanced organisms like the rat, the murine pancreatic duct is devoid of tuft cells, making it a good model in which to study tuft cell function[233].

Pancreatic tuft cells transdifferentiate from the exocrine compartment of the epithelium

Although absent from the main pancreatic duct, tuft cells are commonly associated with the biliary tract, particularly the PBGs associated with the common duct and the pancreatobiliary duct. Hypothesized to be a source of progenitor cells for the liver, biliary tract, and pancreas, the PBGs have been shown to be mucinous and express a number of stem and progenitor markers[81]. Strobel et al. have labeled similar structures pancreatic duct glands (PDGs), describing them as a potential source of pancreatic disease. The Strobel study suggests that tuft cell-containing ADM may directly emanate from the PDGs, with the alternative being that normal pancreas transdifferentiates to the PDG-like phenotype[245]. To distinguish these possibilities, I 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) in PTF1A^{+} adult acinar cells upon tamoxifen induction of CRE activity[226]. YFP fluorescence was found in 68.7% (n=300) of tuft cells, indicating that these tuft cells were derived from the acinar cell compartment (Figure 9). YFP-negative tuft cells may represent derivation from normal pancreatic epithelia, or, more likely, incomplete recombination of the ROSA locus. I conclude that while *Kras*-induced metaplasia was not derived from PDGs, it does take on a PDG-like phenotype.

DCLK1 is not required for tuft cell formation or tumor progression in Kras$^{G12D/+}$*;Ptf1a*$^{Cre/+}$ *mice*

As doublecortin-like kinase 1 (DCLK1) expression is characteristic of tuft cells in a number of normal tissue types and serves as a tubulin polymerization serine/threonine kinase in a cell type with an extensive tubulin network, I hypothesized that ablation of DCLK1 expression would eliminate tuft cell formation in *Kras*^{G12D}-induced tumorigenesis[222, 230]. *DCLK1*$^{\Delta/\Delta}$ mice were crossed to *Kras*$^{G12D/+}$*;Ptf1a*$^{Cre/+}$ mice and aged to 6 weeks. Immunohistochemical analysis revealed that, as compared to controls, knock-out mice had approximate levels of tumorigenesis, populated by tuft cells, determined by COX1 IHC (Figure 10A)[222]. To accelerate tumorigenesis, cerulein was administered to *DCLK1*$^{\Delta/\Delta}$*;Kras*$^{G12D/+}$*;Ptf1a*$^{Cre/+}$ mice and controls. Analysis revealed that

lack of DCLK1 expression did not inhibit tumorigenesis or tuft cell formation *in vivo* (Figure 10B).

Pancreatic metaplasia takes on a pancreato-biliary progenitor phenotype

Metaplasia has been described as a developmental reversion, taking on the phenotype of cells normally confined to other developmentally related organs[7]. It has been determined that the murine biliary tract is most closely related to the pancreas developmentally, sharing a common progenitor cell population[33]. The observation that ADM resembles tuft cell-containing PBGs led me to hypothesize that ADM adopts other characteristics of the biliary phenotype. To test this, I 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 (IPMNs)[33, 246].

The pancreas and biliary tract share a common progenitor cell population characterized by co-expression of PDX1 and SOX17[33]. In the adult, however, epithelial SOX17 expression is restricted to the biliary tract and is no longer expressed in either the acinar or ductal cells of the pancreas, though endothelial SOX17 can be found lining blood vessels in the pancreas (Figure 11A). Co-IF for SOX17 and PDX1 revealed two cell types within metaplastic ducts: a $PDX1^{+}SOX17^{-}$ population and a $PDX1^{+}/SOX17^{+}$ population, reminiscent of the common pancreatobiliary progenitor cell (Figure 11B). 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, regardless of tumor grade (Figure 11C). Overall, SOX17 was expressed at modest but consistent levels in metaplasia, was highly expressed in a subset of cells in mPanINs and was absent from invasive disease.

Lining the $SOX17^{+}$ biliary tract are PBGs, which are hypothesized to serve as progenitor/stem cell reservoirs for the liver, biliary tract, and pancreas[81]. These mucinous, tuft cell-containing glands express stem cell factors such as OCT4 and LGR5, and endoderm-specific markers SOX9, SOX17, EPCAM, CXCR4, and FOXA2[81]. Upon tamoxifen treatment of *LSL-Kras*^{G12D}*;ROSA*^{YFP}*;Ptf1a*$^{Cre\text{-}ERTM/+}$ mice, I found that acinar cell-derived pancreatic metaplasia widely expressed mucins, SOX9, PDX1, EPCAM, and

CXCR4, and as previously mentioned, stem cell marker LGR5, consistent with a biliary phenotype (Figure 12). IHC for CDX-2 was conducted in the *LSL-Kras$^{G12D/+}$;Ptf1a$^{Cre/+}$* model to ensure that pancreatic metaplasia was not becoming intestine-like in nature, as that seen in Barrett's esophagus[10, 29]. Expression was observed periodically, but was not widespread in pancreatic tumorigenesis, and was also found in the wild type pancreatobiliary duct (data not shown).

Human pancreatic disease assumes a biliary-like phenotype

To determine if human pancreatic disease phenocopies progression seen in murine models, I assessed human pancreatic tissue and cell lines for biliary markers. To analyze the possible association of tuft cells, human CP, PanIN, and PDA samples were assessed immunohistochemically for tuft cell markers. Co-IF for the tuft cell markers COX-1 and phospho-EGFR in human tissue microarrays demonstrated that tuft cells were consistently associated with early PanINs (28/50 samples; 21/36 PanIN1 samples), but were less commonly associated with invasive disease (14/47 PDA samples) (Figure 13A). Unlike the murine model, tuft cells were associated with normal ducts (9/36 samples) and were commonly found in pancreatitis-associated metaplasia (18/58 CP samples). To confirm SOX17 expression in PDAC, a panel of pancreatic cancer cell lines was assessed for SOX17 expression by western blot. While expression levels of SOX17 isoforms varied, 9/12 PDAC cell lines examined expressed detectable levels (Figure 13B).

SOX17 expression drives a pancreatitis-like disease state in the pancreas

To determine how assumption of a biliary phenotype may contribute to pancreatic disease progression, I utilized both the *ROSA$^{tTa/+}$;Ptf1a$^{Cre/+}$;tetO-SOX17* and *PDX1$^{tTa/+}$;tetO-SOX17* gain of function mouse models[33]. Mice from both models were aged to 6 weeks and overexpression of SOX17 in the pancreas was confirmed by western blot (Figure 14A). *ROSA$^{tTa/+}$;Ptf1a$^{Cre/+}$;tetO-SOX17* mice expressed higher levels of SOX17 than the *PDX1$^{tTa/+}$;tetO-SOX17* mice and were also significantly smaller than aged-matched wild type mice (Figure 14B). This reduced body weight may be due to malnourishment resulting from formation of a significantly smaller pancreas, as compared to aged-matched mice (Figure 14C).

PDX1$^{tTa/+}$;tetO-SOX17 mice have been previously described and express SOX17 in PDX1 positive cell populations, exhibiting expansion of ductal structures at the expense of acinar and islet compartments by 6 weeks of age in the absence of doxycycline[33]. I found that, accompanying aberrant duct formation, ducts from 6-8 week old *PDX1$^{tTa/+}$;tetO-SOX17* mice were rich in DCLK1 and COX1 positive tuft cells, consistent with ADM (n=6) (Figure 15). Also consistent with ADM, aberrant ducts were Ki67^{+} (Figure 15). Unexpectedly, *PDX1$^{tTa/+}$;tetO-SOX17* pancreata showed a robust stromal reaction, as determined by picrosirius staining and IHC for macrophages (F4/80^{+}) and T cells (CD3^{+}), consistent with an adaptive inflammatory reaction (Figure 15). Analysis for markers of de-differentiation revealed increased expression of both PDX1 and SOX9 in *PDX1$^{tTa/+}$;tetO-SOX17* mice, as compared to wild type mice (Figure 16). Assaying for expression of known SOX17 endodermal target genes revealed increased expression of GATA6 and FOXA1, but not GATA4 and FOXA2 (Figure 16)[247]. Taken together, these data indicate that epithelial overexpression of SOX17 is capable of driving a stable phenotype recapitulating all major aspects of chronic pancreatitis, a known risk factor for pancreatic tumorigenesis, in the absence of overt tissue damage.

As SOX17-GOF mice mis-express SOX17 beginning in development, I sought to determine whether the observed pancreatitis was a result of SOX17 overexpression or the end result of developmental defects. To accomplish this, *PDX1$^{tTa/+}$* mice were bred to *tetO-SOX17* mice in the presence of doxycycline, so that developing pups would express only the endogenous *Sox17*. *PDX1$^{tTa/+}$;tetO-SOX17* mice remained on doxycycline until 4-6 weeks of age, at which time they were placed on doxycycline-free chow for 4 weeks. Analysis of pancreata from adult *PDX1$^{tTa/+}$;tetO-SOX17* mice revealed SOX17 expression in centroacinar cells and a lack of pancreatitis-associated symptoms, including metaplasia and inflammation (n=3) (Figure 17). The lack of epithelial disturbance may be due to the very slight increase in SOX17 expression, suggesting that PDX1 expression may have to be induced in the acinar cell epithelium to drive greater levels of SOX17 expression.

This led me to examine the *ROSA$^{tTa/+}$;Ptf1a$^{Cre/+}$;tetO-SOX17* model of pancreatic SOX17 mis-expression, which has not been previously described. In this model, SOX17 expression is driven by PTF1A, which is widely expressed throughout the pancreas

during development, but is restricted to acinar and centroacinar cells in the adult pancreas. In the absence of doxycycline, pancreata from 6 week old mice exhibit an even greater replacement of the pancreatic acinar and endocrine compartments with ductal epithelium than the *PDX1*$^{tTa/+}$*;tetO-SOX17* model, which is consistent with a greater expression level of SOX17 (n=4) (Figure 18). Although symptoms of pancreatitis are present, these mice exhibit a milder pancreatitis-like phenotype where tuft cells, as determined by DCLK1 and COX1 IHC, and collagen deposition, as determined by picrosirius staining, are present to a lesser extent (Figure 18). However, the epithelium is still proliferative, as determined by Ki67 expression, and inflamed, as determined by F4/80 IHC for macrophages and CD3 IHC for T cells (Figure 18). Analysis of de-differentiation markers indicated reduced PDX1 and SOX9 expression, as compared to the *PDX1*$^{tTa/+}$*;tetO-SOX17* model (Figure 19). Expression of SOX17 target genes GATA6 and FOXA1 is elevated as compared to the wild type, whereas GATA4 and FOXA2 are not (Figure 19).

To determine whether SOX17 overexpression is responsible for pancreatitis induction in this model, I compared 6 week old *ROSA*$^{tTa/+}$*;Ptf1a*$^{Cre/+}$*;tetO-SOX17* mice to litter mates placed on doxycycline at 3 weeks of age (SOX17-OFF) and aged to 6 weeks. IHC analysis reveals that the pancreas is still largely composed of ducts, but they are SOX17 negative; ablation of SOX17 expression did not cause an expansion of the pancreatic acinar cell compartment (n=6) (Figure 20). Ducts present appear much larger in size, as compared to 6-week-old SOX17-ON mice, and the inflammatory response becomes largely composed of macrophages (F4/80$^+$) with a decrease in T cells (CD3$^+$), suggesting that SOX17 expression may be necessary for maintenance of the inflammatory response. To determine if the SOX17-OFF phenotype persists, 9-week-old SOX17-ON mice (n=5) were compared to 9-week-old mice that expressed SOX17 until three weeks of age, at which time SOX17 was turned off for 6 weeks (n=4). I found that the phenotype did indeed persist, with no significant increase in acinar cell epithelia (Figure 20).

To determine if this phenotype is the result of SOX17 over-expression, or the result of a developmental defect, *ROSA*$^{tTa/+}$*;Ptf1a*$^{Cre/+}$*;tetO-SOX17* mice were bred while on doxycycline, so that pups *in utero* were exposed to only endogenous levels of SOX17.

Progeny were aged to 4 weeks at which time doxycycline chow was removed and SOX17 was turned on in the pancreas for an additional 4 weeks. Analysis of pancreata from these mice revealed various levels of SOX17 expression in the pancreas and a lack of pancreatitis symptoms (n=3) (Figure 21). These data suggest that induction of tissue damage or SOX17 and PDX1 expression in concert may be required for metaplasia.

Concurrent to these experiments, $ROSA^{tTa/+}$*;tetO-SOX17* mice were crossed into the aforementioned tamoxifen-inducible $Ptf1a^{Cre\text{-}ERTM/+}$ model, which will over-express SOX17 in adult acinar and centroacinar cells upon tamoxifen induction of CRE activity. These mice were aged to 8 weeks at which time SOX17 expression was induced and mice were aged an additional 4 weeks. Compared to untreated controls, analysis of pancreata revealed SOX17 expression and extensive tuft cell-containing ADM, as determined by DCLK1 and COX1 IHC (Figure 22). These mice have a notable stromal reaction, including significant macrophage (F4/80) and T cell (CD3) infiltration, indicating pancreatitis (Figure 22). Analysis of de-differentiation markers indicated increased PDX1 expression, as compared to wild type (Figure 22).

DISCUSSION

Pancreatic metaplastic ductal lesions represent an early transdifferentiation event in pancreatic disease progression and are hypothesized to be pre-neoplastic. My work demonstrates that ADM results in a phenotype where the epithelium assumes several characteristics of the developmentally related bile duct, such as the presence of numerous tuft cells and expression of SOX17. Through marker analysis, I have shown that metaplastic tuft cells are very similar to normal tuft cells in other tissues, but are absent from the normal murine pancreas. While tuft cells are relatively rare in the murine intestine, constituting only 0.4% of the epithelium, they are common within the biliary tract, established to have a developmental relationship to the pancreas[33, 221, 222]. Meeting the criteria of Eguchi and Kodama, *K-ras* induced ADM is an acinar-to-biliary duct cell-like metaplasic event[8]. Again, acinar cells are identified by the presence of zymogen granules and expression of digestive enzymes and are PTF1A^{+}Mist1^{+}PDX1^{low}SOX17^{-}HES1^{-}SOX9^{-}. Metaplastic biliary-like ductal cells form tubules, are CK19^{+}, and are PTF1A^{-}Mist1^{-}PDX1^{high}SOX17^{+}HES1^{+}SOX9^{+}. To address the second criterion and demonstrate that ectopic biliary tissue was the result of metaplasia and not cell migration, I utilized a murine model where constitutively active *Kras*^{G12D} and YFP are induced in adult PTF1A^{+} acinar cells. Lineage tracing revealed that SOX17^{+} epithelium, as well tuft cells, in ADM were derived from adult acinar cells, meeting the second criterion and identifying *Kras*-induced metaplasia as a biliary-like transformation event. This tissue-switching phenomenon, such as that found in Barrett's esophagus, constitutes a previously undescribed state in the pancreas, but reinforces the well-established idea that metaplasia is often composed of a tissue type normally derived from a neighboring region of the embryo[7]. Interestingly, the biliary-like pancreatic metaplasia closely resembles a structure recently labeled a "duct gland" by Strobel et al., described to be a potential source of disease[245].

Tuft cells were found to be consistently associated with early pancreatic disease in multiple murine models, as well as with human tumors. Although attractive as a putative source of disease markers, tuft cells express a number of pro-tumorigenic factors, indicating that tuft cells have the potential to drive disease progression through modification of the tumor microenvironment. Exploring their similarities to normal

intestinal cells, I confirmed that metaplastic tuft cells express COX1, COX2, and HPGDS known to produce prostaglandins involved in normal physiological processes, such as mucosal integrity and inflammation[222, 248]. Aberrant emergence of this cell population in the context of pancreatic tumorigenesis results in a novel source of pro-inflammatory prostaglandins in the epithelium, which, in turn, are known to drive the disease state[249, 250]. Epidemiological studies have identified chronic inflammation as a risk factor for PDA; both acute and chronic pancreatitis have been shown to strongly enhance PDA in adult mice[129, 250]. Possibly critical for homeostasis in a number of normal tissues, metaplastic tuft cell prostaglandins in the pancreas could significantly disrupt a tissue in which they are not normally found, with the net effect of altering the tissue microenvironment and exacerbating tumorigenesis.

Metaplasia-associated tuft cells have also been determined to constitute the sole epithelial source of β-endorphin, an additional secretory product with the potential to drastically alter the tumor microenvironment. Survival rates for PDA are grim in large part due to late detection resulting from a lack of early, diagnosable symptoms[242]. I have found tuft cell transdifferentiation to be an early event in tumorigenesis resulting in the production of β-endorphin, an opioid considered to be 18-33 times more potent than synthetic morphine[240]. I have also found the β-endorphin receptor, μ-opioid receptor, to be active in nerves associated with pancreatic disease, suggesting an active signaling pathway[239]. Sevcik et al. determined that endogenous opioids, β-endorphin for example, inhibit early stage pain in a murine model of pancreatic cancer[242]. While endogenous opioid production is consistent with normal homeostatic processes such as peristalsis, a local source in early pancreatic disease is consistent with the lack of pain-related symptoms seen in patients.

Noting that anomalous emergence of tuft cells may dramatically alter the tumor microenvironment, I sought to elucidate what impact, if any, other characteristics of biliary transdifferentiation have on tumorigenesis. To determine the role of aberrant SOX17 expression in $Kras^{G12D}$-induced pancreatic metaplasia, I utilized the $PDX1^{tTa/+}$*;tetO-SOX17* and $ROSA^{tTa/+}$*;*$Ptf1a^{Cre/+}$*;tetO-SOX17* SOX17 gain of function mouse models. As previously reported, there is extensive replacement of both the endocrine and exocrine compartments with ductal epithelium[33]. I found, however, that

these ducts are not mimicking normal pancreatic ducts, but are tuft-cell containing, pro-inflammatory ducts accompanied by a robust desmoplastic response, much like that found in *Kras*^{G12D}-induced metaplasia. To determine if SOX17 expression is sufficient to induce this phenotype in adult animals, I removed mice from the doxycycline regimen. Both models showed various levels of SOX17 mis-expression, but were phenotypically normal. When SOX17 was mis-expressed in the *ROSA*$^{tTa/+}$*; Ptf1a*$^{Cre\text{-}ERTM/+}$*;tetO-SOX17* model, however, mice were phenotypically similar to those mis-expressing SOX17 from development. A major difference between this model and the aforementioned models is the persistent inflammatory response resulting from intraperitoneal injection of corn oil, in which the tamoxifen is suspended. It may be that metaplasia is occurring in response to SOX17 expression in the developmental models due to pro-inflammatory ducts that formed during development, and it is a lack of inflammation in the adult gain-of-function model that prohibits acinar cells from undergoing metaplasia. Inducing mild damage with cerulein in adult gain-of-function models and assaying for pancreatitis will rectify this discrepancy.

The adaptive nature of the inflammatory response seen in the developmental and tamoxifen-inducible models is reflective of chronic pancreatitis seen in humans. An abundance of fibrotic stroma is a typical feature of PDA in humans and typically originates around pre-cancerous lesions[162]. The stroma itself not only physically shields tumors from chemotherapeutic intervention, but the extracellular matrix produced by stellate cells influences growth, survival, differentiation, and motility of cancer cells[162]. The finding that SOX17 expression, in the context of an inflammatory microenvironment, is sufficient to drive formation of tuft cell-containing, pro-inflammatory, ductal lesions accompanied by a dramatic stromal response, suggests that both epithelial SOX17 and the accompanying biliary transdifferentiation promote pancreatic tumorigenesis and tumor progression.

FUTURE DIRECTIONS

To begin to address whether the appearance of tuft cells in pancreatic metaplasia is merely a consequence of biliary transdifferentiation, or has functional consequences, the cell type will have to be ablated and thus absent upon tumor induction in *Kras*$^{G12D/+}$;*Ptf1a*$^{Cre/+}$ mice. My attempt to deplete tuft cells with ablation of DCLK1 proved to be unsuccessful. If the literature is correct and transcription factor Gfi-1b is required for tuft cell differentiation in the intestine, it is possible that knocking out this protein in the *Kras*^{G12D} model will ablate tuft cells[238]. Additionally, if the cell type itself cannot be removed, it is possible that functionality could be blocked or inhibited by ablation of SCC signaling components. G-α-gustducin knockout mice are available and exhibit decreased behavioral sensitivity to bitter and sweet stimuli[251]. TRPM5, a second SCC signaling protein, has been shown to be required for β-endorphin release, and TRPM5 ablation, therefore, may knockout any β-endorphin function in addition to SCC function[241, 252].

SOX17 over-expression induces a pancreatitis-like phenotype, but the mechanism of action has yet to be elucidated. SOX17 is a transcription factor with few known targets apart from interaction with β-catenin signaling[247, 253, 254]. Pancreatic cancer cell line analysis for active β-catenin signaling in the context of both SOX17 shRNA and SOX17 transfection may illuminate any role for this relationship in the context of the pancreas. To further explore the role of SOX17 in *Kras*^{G12D}-induced metaplasia, and subsequent tumorigenesis, I am currently crossing the SOX17 gain-of-function mice into both the *LSL-Kras*$^{G12D/+}$;*Ptf1a*$^{Cre/+}$ and *LSL-Kras*$^{G12D/+}$;*Ptf1a*$^{Cre-ERTM/+}$ models. I hypothesize that, given that SOX17 is present in tumor, but methylated in lung, gastric, and breast cancers, that over-expression of SOX17 in this model will accelerate PanIN formation, but will inhibit EMT[255-257]. To further explore the role of SOX17, *SOX17*$^{\Delta/\Delta}$ mice will be crossed into the *LSL-Kras*$^{G12D/+}$;*Ptf1a*$^{Cre/+}$ model. It is possible that ablation of SOX17 will slow ductal formation; however, due to the presence of 29 additional SOX proteins in the mouse, it is also possible that compensation mechanisms exist.

CHAPTER 3: *Salmonella enterica* serovar Typhimurium infection as a causative agent of pancreatic metaplasia and pancreatitis, a risk factor for PDA

INTRODUCTION

Infection has been linked to acute pancreatitis in several case studies; however, direct causation has yet to be demonstrated. As acute pancreatitis is sufficient to agonize oncogenic *Kras*-driven tumorigenesis, the ability of bacterial infection to induce pancreatitis would make it a risk factor for PDA development[110, 145]. This suggests that infection is sufficient to induce both inflammation and possible tissue damage leading to metaplasia. Studies of *Helicobacter pylori* infection in the stomach identify the bacterium as one of the major aetiological factors contributing to the development of intestinal metaplasia. Persistent gastric mucosal irritation caused by infection leads to intestinal metaplasia, which is believed to arise due to the differentiation of gastric stem cells toward cells of an intestinal cell type rather than a gastric phenotype[258]. *H. pylori* has been established as a carcinogen and, as previously mentioned, intestinal metaplasia in the stomach carries a significantly increased risk of developing gastric cancer, the second most common cancer globally[18, 19, 259, 260]. Other infections have been linked to cancer, such as Hepatitis C to liver carcinoma and schistosomiasis to bladder and colon carcinoma[261].

Case reports have linked pancreatitis to infection with a variety of pathogens, including viruses, bacteria, parasites, and fungal agents[157]. Infection with several bacterial gastrointestinal pathogens, including *Salmonella enterica* serovar Typhi, *Campylobacter jejuni*, *Yersinia enterocolitica*, and *Y. pseudotuberculosis*, has been determined to be causal to pancreatitis development[262-273]. Pancreatitis has also been associated with disease progression in leptospirosis, legionellosis, brucellosis, and infection with *Actinomyces* and *Nocardia*[157, 274-285]. Infection with *Mycobacterium tuberculosis* or *M. avium* has been correlated to micro- and macroabscesses and granulomas in the pancreas, which are not characteristic of pancreatitis, but are thought to result from direct seeding of bacteria in the tissue through hematogenous spread or through direct extension from regional lymph nodes[286-291]. This demonstrates the ability of pathogens to directly infect pancreatic tissue. Although not a primary site of infection, analysis of the regular distribution pattern for *Samonella* Enteritidis in mouse studies indicates infection of the pancreas, subjecting the tissue to possible infection-induced damage and associated inflammation[292].

Lipopolysaccharide (LPS) is a structural component of the outer membrane of Gram-negative bacteria, such as *Salmonella*, and is considered a pathogen-associated molecular pattern (PAMP). During infection, LPS acts as an endotoxin in hosts, eliciting a strong immune reaction. Vaccaro et al. have shown that LPS directly affects pancreatic acinar cells, inducing apoptosis and the secretion of cytokines, such as TNFα, IL-1, and IL-8, in AR4-2J cells *in vitro* and inducing structural cell damage, nuclear alterations, and high expression of pancreatitis-associated protein (PAP) *in vivo*[293]. Recent work by Daniluk et al. demonstrates that LPS treatment in the mouse is capable of agonizing pancreatic tumorigenesis in the context of an activating *Kras* mutation, but does not induce symptoms of pancreatitis in wild type animals[294]. The possibility of infection, including exposure to PAMPs, inducing pancreatic damage and eliciting an immune response suggests a causative role in the development of pancreatitis and suggests the possibility of infection as a risk factor for pancreatic cancer.

Salmonella enterica serovar Typhiumurium (*Salmonella* Typhimurium) is a Gram-negative, non-spore forming, motile, facultative intracellular pathogen[295]. Infection in humans via the fecal-oral route from contaminated food or water may present as gastroenteritis, enteric fever, bacteremia, or an asymptomatic carrier state[295]. Symptoms range from mild to cholera-like and may include fever, abdominal pain, diarrhea, nausea, vomiting, and chills. *Salmonella* Typhimurium is a non-typhoid serovar, and with other serovars, makes up about 2.3% of gastroenteritis cases in the United States, or 2-4 million cases annually[295].

Several serovars of *Salmonella* have been identified as causative agents of acute pancreatitis in a number of case reports[265, 266, 295-304]. Additionally, two retrospective studies have reported a frequency of hyperamylasemia of 50%, referring to abnormal levels of serum amylase, and a frequency of clinical pancreatitis ranging from 28-62% in patients with *Salmonella* infection[267, 305]. In contrast, Pezzilli et al. conducted a prospective study of 30 patients infected with *Salmonella* for acute pancreatitis and found that none of the patients developed pancreatitis. They did find, however, that *Salmonella* infection (with serovars Typhimurium and Enteritidis) significantly increases serum lipase, but not serum amylase, indicating that infection with *Salmonella* has an effect on the pancreas[306].

Although a link between infection and pancreatitis has been reported in patients, reports are contradictory and direct causation studies are lacking. In agreement with Daniluk et al., I found that treatment with *S.* Typhimurium LPS does agonize *Kras*-induced tumorigenesis and does not induce pancreatitis-like symptoms in wild type mice[294]. However, I also discovered that exposure to LPS induces influx of a substantial population of macrophages into the pancreas, suggesting that the inflammatory system, and not just the LPS itself, may be agonizing *Kras*-induced tumorigenesis. LPS treatment was not found to induce an adaptive inflammatory response or to activate pro-tumorigenic EGFR signaling in wild type pancreata. To determine whether infection would induce pancreatitis, and pro-tumorigenic pathway activation, I examined pancreata from mice infected with *Salmonella* Typhimurium, due to its reported role in pancreatitis induction. My analysis of infected pancreata revealed that this common pathogen is capable of inducing both acute and chronic pancreatitis. Acute infection is sufficient to induce an innate immune response, as well as EGFR pathway activation. Chronic infection induces metaplasia and fibrosis, as well as an adaptive immune response. Analysis of pancreata infected with *Francisella tularensis* or *Yersinia pseudotuberculosis* demonstrated the ability of several Gram-negative pathogens to induce pancreatitis. Organ burden assay revealed a direct interaction between the bacteria and the epithelium. These data demonstrate that infection with *S.* Typhimurium induces pancreatitis in mouse models and the resulting metaplasia, inflammation, and EGFR activation reveals its potential as a risk factor for pancreatic cancer development.

MATERIALS AND METHODS

Mouse strains

LSL-Kras$^{G12D/+}$, *Ptf1a*$^{Cre/+}$, *Ptf1a*$^{Cre\text{-}ERTM/+}$, and *EGFR*$^{\Delta/\Delta}$ strains have been described previously and were genotyped accordingly[78, 218, 224, 226]. C57BL/6J, 129X1/SvJ, and *NRAMP*$^{+/+}$C57BL/6 mice were used in experiments performed by the van der Velden laboratory; C57BL/6J and 129X1/SvJ mice were purchased from Jackson Laboratories and *NRAMP*$^{+/+}$C57BL/6 and F1(C57BL/6J x 129X1/svJ) mice were bred at Stony Brook University. 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.

Genotyping

Genotyping of transgenic mice was accomplished by PCR from tail DNA using gene specific primers described below:

LSL-Kras$^{G12D/+}$	F: CGCAGACTGTAGAGCAGCG
	R: CCATGGCTTGAGTAAGTCTGC
Ptf1a^{Cre}	F: TCGCGATTATCTTCTATATCTTCAG
	R: GCTCGACCAGTTTAGTTACCC
EGFR$^{\Delta/\Delta}$	F: CTTTGGAGAACCTGCAGATC
	R: CTGCTACTGGCTCAAGTTTC

Immunostaining

Tissues were harvested and fixed overnight in 4% paraformaldehyde. Immunohistochemistry and immunofluorescence were performed as previously described[227]. Picrosirius Red Stain Kit (Polysciences, Inc., 24901) was used for collagen staining.

Primary antibodies

Anti-CD3 (ab5690) was obtained from Abcam. Anti-F4/80 (MCA497R) and anti-LY6B.2 (MCA7716) were obtained from AbD Serotec. Anti-Mac2 (CL8942AP) was obtained from Cedarlane Labs. Anti-Troma III (CK19) was obtained from the Developmental Studies Hybridoma Bank (Iowa City, Iowa). Anti-Cyclin D1 (ab134175)

and anti-EGFR (1902-1) were obtained from Epitomics. Anti-COX-2 (sc-1747; 1:200) was obtained from Santa Cruz.

Lipopolysaccharide treatment

Animals were intraperitoneally injected at 8-10 weeks of age with 5 mg/kg LPS, *Salmonella* Typhimurium S-form (Enzo Life Sciences), every other day for 10 days, and were allowed to recover for 1 hour, 24 hours, or 7 days.

Infection protocol

Infection of mice was performed by Jason W. Tam in the van der Velden laboratory at Stony Brook University. To accomplish an acute *S.* Typhimurium infection, 8-12 week old C57BL/6J and F1(129X1/svJ x C57BL/6J) mice were infected once either intravenously or by oral gavage with IR715, a nalidixic-resistant strain of *Salmonella* Typhimurium (5000 colony forming units (CFU) and $5x10^7$ CFU, respectively) and were sacrificed 10 days post infection; for IF assay, an IR715-GFP strain was used. Chronic *S.* Typhimurium infection was conducted in 8-12 week old 129X1/svJ or *NRAMP*$^{+/+}$ C57BL/6 mice infected once either intravenously or by oral gavage with IR715 (5000 CFU and $5x10^7$ CFU, respectively); mice were sacrificed up to 60 days post infection. For IF, an IR715 rpsM::GFP (constitutively expressed) strain was utilized.

Acute *Francisella tularensis* infection was conducted in 8-12 week old C57BL/6J mice by one intradermal infection with $5x10^5$ CFU *Francisella tularensis* live vaccine strain for 7 days. Acute *Yersinis pseudotuberculosis* infection was conducted in 8-12 week old C57BL/6J mice by oral gavage, once, of strain IP2777 ($5x10^7$ CFU) for 7 days.

Organ burden assay

Organ burden assay was conducted by Jason W. Tam in the van der Velden laboratory at Stony Brook University. Pancreata were removed from 8-12 week old C57BL/6J mice acutely infected with *S.* Typhimurium strain IR715 ($5x10^7$ CFU by oral gavage), meaning one infection allowed to persist for 7 days. Organs were placed in 0.2% NP40 alternative detergent (Calbiochem) and homogenized using a PowerGen 125 homogenizer with the PowerGen generator (Saw-Tooth, SS; Stator Dia. x L: 7 x 95 mm). The homogenate

was diluted and plated on nalidixic acid sodium salt (Sigma)-containing lysogeny broth plates for viable CFU.

RESULTS

LPS treatment induces macrophage infiltration and influences pancreatic tumorigenesis

S. Typhimurium has been identified as a causative agent of pancreatitis in several case reports and LPS has been determined to agonize *K-ras*-induced tumorigenesis[294, 306]. To determine whether treatment with *S.* Typhimurium LPS would induce pancreatic disease, I treated both $Kras^{G12D}$ and wild type mice and analyzed pancreata histologically. As previously reported, I found that LPS treatment agonized tumorigenesis in $Kras^{G12D}$ mice, compared to saline-treated animals (n=3), demonstrated by histology revealing replacement of acinar cell epithelium with tumor, and by an increase in pancreatic mass (Figure 23)[294]. Increased tumorigenesis was accompanied by increased infiltration of inflammatory cells, particularly macrophages ($F4/80^{+}$) (Figure 23). Also in agreement with Daniluk et al., I found that *S.* Typhimurium LPS-treatment of wild type mice did not induce pancreatitis; histological analysis revealed a lack of epithelial damage or edema (n=8) (Figure 23)[294]. In contrast to this report, however, I found that LPS treatment does affect the normal pancreas by inducing significant infiltration of macrophages into the epithelium, which persists up to a week post-treatment (Figure 23). While a substantial macrophage response was evident, the chosen recovery time point may be too late to observe a neutrophil response, and no T cell response was evident (Figure 23).

To determine whether the LPS-induced inflammatory cell infiltrate is pro- or anti-tumorigenic, I utilized the inducible $LSL\text{-}Kras^{G12D/+};Ptf1a^{Cre\text{-}ERTM/+}$ murine model. Mice were treated with LPS, to induce the macrophage response seen in wild type mice, followed by *K-ras* activation through tamoxifen administration. As compared to saline-treated animals, LPS pre-treated animals had fewer tumors and more areas of macrophage accumulation, determined by expression of F4/80 and Mac2 (Figure 24). To determine what effect, if any, macrophage infiltration has on pancreatic acinar cells in wild type mice, I analyzed the epithelium histologically for markers of proliferation, cell death, and EGFR pathway activation. The lack of response (data not shown) suggests that LPS-induced macrophage infiltration may affect $Kras^{G12D}$ and wild type pancreata differently.

Infection with Salmonella serovar Typhimurium induces pancreatitis

S. Typhimurium-specific LPS treatment induces inflammatory cell infiltration, but not pancreatitis. To determine the ability of the actual bacteria to induce this pro-tumorigenic condition, I analyzed pancreata from mice infected with *S.* Typhimurium by Jason Tam in collaboration with the van der Velden laboratory. Acute infection was induced by either intravenous injection of *S*. Typhimurium into C57BL/6J x 129X1/svJ mice or through the orogastric route in C57BL/6J mice and pancreata were collected 10 days later. Acute bacterial infection induced pancreatitis regardless of model employed, including edema and macrophage infiltration (n=6, Figure 25). As seen with LPS treatment, there was a minimal T cell response (Figure 25). Chronic infection was induced intravenously in *NRAMP*$^{+/+}$C57BL/6 mice or through the orogastric route in C57BL/6J x 129X1/svJ mice, and pancreata were collected 60 days later. Chronic infection with *S.* Typhimurium in both models induced chronic pancreatitis, including epithelial damage, metaplasia, fibrosis, and an adaptive inflammatory cell response, demonstrated by T cell infiltration (CD3^{+}) (n=11, Figure 25). The ability of *S.* Typhimurium infection to induce tissue damage and metaplasia, which are pro-tumorigenic, suggests a possible role in tumorigenesis.

Various Gram-negative bacteria have the ability to induce pancreatitis

To determine if induction of pancreatitis is a *S.* Typhimurium-specific response, I analyzed the pancreata from mice infected with Gram-negative *Francisella tularensis*, the causative agent of tularemia, and *Yersinia pseudotuberculosis*, the causative agent of pseudotuberculosis in animals, by Jason Tam in collaboration with the van der Velden laboratory. Histological analysis revealed that acute infection, or a one-time infection allowed to persist for 7 days, with either agent was sufficient to induce pancreatitis (Figure 26). Severity of pancreatitis varied among mice infected with *F. tularensis*; however, all mice (n=4) demonstrated macrophage infiltration, and more severely affected mice experienced acinar cell disturbance and T cell infiltration (Figure 26). Signs of infection with *Y. pseudotuberculosis* were milder than those with *F. tularensis*, but acute infection still led to pancreatitis as determined by macrophage infiltration (n=4,

Figure 26). All infected mice exhibited macrophage infiltration throughout the pancreas; however, there were often focal points of accumulation associated with tissue disturbance.

Infection with S. Typhimurium induces pro-tumorigenic signaling pathways

The discovery that infection with Gram-negative bacteria induces pancreatitis, a risk factor for pancreatic cancer, led me to analyze pancreata from mice infected with *S.* Typhimurium for activation of pro-tumorigenic signaling pathways. Picrosirius staining revealed extracellular matrix deposition in the pancreata of mice with acute or chronic *S.* Typhimurium infection (Figure 27). Collagen density has been shown to promote mammary tumor initiation and progression[307]. Sandgren et al. established that activation of EGFR signaling through over-expression of TGFα is sufficient to induce epithelial hyperplasia and pancreatic metaplasia, and Ardito et al. demonstrated that EGFR is required for both induction of experimental pancreatitis and Kras-induced pancreatic tumorigenesis[113, 115]. Noting the requirement for EGFR up-regulation and pathway activation for induction of pancreatic disease, I analyzed the pancreata from mice infected with *S.* Typhimurium histologically for pathway activation. Analysis of mice with an acute infection revealed EGFR expression to be upregulated in acinar cell epithelium, but signaling was not activated to the extent that downstream effectors COX2 (a pro-inflammatory marker that can also be upstream) and Cyclin D1 (a cell cycle marker) were activated (Figure 27)[308, 309]. In contrast, mice with a chronic infection, regardless of infection protocol or mouse model, demonstrated upregulation of EGFR protein expression within acinar cells, as well as heightened expression of COX2 and nuclear expression of Cyclin D1 (Figure 27). These data reveal that infection with *S.* Typhimurium is sufficient to induce EGFR up-regulation and, in the setting of chronic infection, pathway activation that may be sufficient to induce metaplasia. These factors together suggest that infection has a pro-tumorigenic effect on the epithelium.

S. Typhimurium localizes to the pancreas during infection

As infection with *S.* Typhimurium, but not *S.* Typhimurium-specific LPS, is capable of inducing pancreatitis and pro-tumorigenic EGFR signaling, I analyzed infected pancreata for direct interaction of the bacterium with the pancreatic epithelium.

In collaboration with the van der Velden laboratory, Jason Tam induced acute *S. typhimurium* infection orogastrically in C57BL/6J mice with a green fluorescent protein (GFP) expressing strain of *Salmonella* and pancreata were examined 5 days later. Examination of the pancreata by confocal microscopy revealed that the bacterium was present in the pancreatic epithelium (Figure 28). Quantitative analysis through an organ burden assay was performed by Jason Tam and demonstrated the consistent presence of bacteria in the pancreas as a result of acute infection (Figure 28). Infection of the pancreas may explain why inflammatory cells tend to accumulate in infected animals and suggests that tissue damage might be due to direct interaction of the bacteria with the epithelium.

EGFR is required for LPS-induced tumorigenesis

As LPS has been determined to agonize *Kras*-induced tumorigenesis and EGFR has been determined to be required for *Kras*-induced tumorigenesis, I utilized the $EGFR^{\Delta/\Delta}$; $Kras^{G12D}$; $Ptf1a^{Cre/+}$ murine model to determine if EGFR is required for LPS-induced tumorigenesis[113, 294]. As compared to LPS-treated $EGFR^{\Delta/+}$; $Kras^{G12D}$; $Ptf1a^{Cre/+}$ animals, LPS-treated $EGFR^{\Delta/\Delta}$; $Kras^{G12D}$; $Ptf1a^{Cre/+}$ animals have a dramatically reduced tumor burden (n=4), equivalent to that of saline-treated $EGFR^{\Delta/\Delta}$; $Kras^{G12D}$; $Ptf1a^{Cre/+}$ mice (Figure 29A). The tumor that did form in treated $EGFR^{\Delta/\Delta}$; $Kras^{G12D}$; $Ptf1a^{Cre/+}$ mice, as determined by H&E and cytokeratin 19 (CK19) IHC, was found to be EGFR-positive, indicating areas of the epithelium with incomplete recombination (Figure 29A). Analysis of macrophage infiltration reveals that, in response to LPS treatment, macrophage infiltration in $EGFR^{\Delta/\Delta}$; $Kras^{G12D}$; $Ptf1a^{Cre/+}$ mice is similar to that seen in wild type mice, indicating that EGFR expression in the epithelium is not required for macrophage infiltration and that, without EGFR, this infiltrate is not inducing tumorigenesis (Figure 29B).

DISCUSSION

Treatment with the Gram-negative bacterial outer membrane component lipopolysaccharide (LPS) has been shown to agonize *Kras*-induced pancreatic tumorigenesis in murine models[294]. My work demonstrates that LPS not only agonizes *Kras*, but also induces a pancreatic inflammatory response with the potential to be either pro- or anti-tumorigenic. LPS treatment of wild type mice induces pancreatic infiltration of F4/80^{high}, Mac2^{low} macrophages, suggesting an abnormal accumulation of immature myeloid cells, which in the tumor microenvironment are thought to play a critical immunosuppressive role[310-312]. This is consistent with LPS treatment of the *LSL-Kras*^{G12D}*;Ptf1a*$^{Cre/+}$ murine model, where there is a LPS-induced inflammatory cell infiltrate; however, it does not appear to limit tumorigenesis. This suggests that the tumor itself may be inducing an immunosuppressive inflammatory response. When the pancreas is pre-treated with LPS, before *Kras*^{G12D} activation is induced, the inflammatory infiltrate appears to, instead, contain tumorigenesis. This could be due to different infiltrating populations of inflammatory cells, as suggested by the Mac2 IHC, where the treated *Kras*^{G12D} pancreas is associated with pro-tumorigenic M2 macrophages, which have increased expression of the anti-inflammatory cytokine IL-10 and promote tumor angiogenesis and tissue remodeling[125, 144]. It is possible that activation of mutant *Kras* following LPS-treatment also programs the inflammatory cell infiltrate, but in such a way that an anti-tumorigenic M1 macrophage response is induced. M1 macrophages express high levels of pro-inflammatory cytokines and are considered anti-tumorigenic due to their ability to kill pathogens and prime an anti-tumorigenic response[125]. This idea is consistent with LPS-pretreatment of the pancreas containing tumorigenesis. Infectious disease has been proposed as an environmental modifier of autoimmunity in both human populations and mouse models, and these data are consistent with the idea that acute infection does not cause cancer, but instead agonizes the effects of an oncogenic mutation and that infection without genetic mutation may be, in fact, anti-tumorigenic[262, 313].

Through this work I have also shown that infection with *S.* Typhimurium can, in fact, induce pancreatitis, confirming infection as a causative agent of pancreatic disease. Infection has been linked to acute pancreatitis, which in murine models is sufficient to agonize oncogenic *Kras*-driven tumorigenesis[110, 145]. This suggests that in the context of

an oncogenic mutation in the pancreas, that infection could rapidly accelerate disease progression. While humans do not typically harbor a chronic *S.* Typhimurium infection, the ability of Gram-negative bacterium to induce tissue damage and metaplasia, from which neoplasia is thought to derive, suggests that infection may induce a pro-tumorigenic microenvironment within the pancreas. It is possible that, in a manner similar to *H. pylori*-induced gastric irritation, that persistent pancreatic irritation (or multiple bouts of infection) due to direct pancreatic infection with Gram-negative bacteria could induce oncogenic mutation. While infection is considered a minor risk factor for pancreatitis, gallstones are considered a major risk factor. Crawford et al. have shown that certain serovars of *Salmonella* are capable of forming biofilms on gallstones, suggesting that infection may accompany pancreatitis more often than previously considered[314]. This suggests that infection may be a significant risk factor for pancreatic cancer and is deserving of further investigation.

Ardito et al. reported that EGFR is required for cerulein-induced pancreatitis and $Kras^{G12D}$-induced tumorigenesis; I have shown here that it is also required for LPS-induced tumorigenesis[113]. Up-regulation of EGFR pathway components in both acute and chronic models of *S.* Typhimurium infection suggests a mechanism for infection-induced pancreatic disease through EGFR-mediated signaling. Further investigation will reveal the value of EGFR inhibitor administration in human patients following bouts of serious infection, to prevent pancreatic metaplasia that could lead to neoplastic transformation.

FUTURE DIRECTIONS

LPS treatment of mice before or after activation of oncogenic *Kras* appears to determine whether the inflammatory response will be pro- or anti-tumorigenic. The next step is to identify what inflammatory cells constitute the immune reaction to LPS treatment in wild type mice, in mice harboring an activating *Kras* mutation, and in mice that have been pre-treated with LPS, followed by *Kras* activation. Identifying the inflammatory infiltrate through flow cytometry and marker analysis will suggest whether each model is experiencing an M1 or M2 heavy macrophage response. Analyzing the macrophage infiltrate for the milieu of cytokines released will suggest whether these cells are pro- or anti-tumorigenic. For example, pro-inflammatory, anti-tumorigenic M1 macrophages typically express TNF-α, IL-1, IL-6, IL-12, and/or IL-23. Immunosuppressive, pro-tumorigenic M2 macrophages show increased expression of IL-10, scavenger receptor A, and arginase[125, 144]. Determining what constitutes the inflammatory reaction in each model may elucidate the role for infection in tumorigenesis and suggest a way to “reprogram” the immune reaction from immunosuppressive to anti-tumorigenic.

Infection of mice with *S.* Typhimurium induces pancreatitis, which is considered a major risk factor for the development of pancreatic cancer. The next step is to treat infected mice with antibiotics to determine if pancreatitis is resolved. This would determine whether or not infection induces irreparable, pro-tumorigenic damage in the pancreas. To determine if *S.* Typhimurium infection agonizes *Kras*-induced tumorigenesis, infection will be induced in the $LSL\text{-}Kras^{G12D};Ptf1a^{Cre/+}$ murine model. The inducible $LSL\text{-}Kras^{G12D/+};Ptf1a^{Cre\text{-}ERTM/+}$ murine model may also be infected, before or after induction of oncogenic *Kras*, to determine if infection prior to oncogenic mutation has a protective effect. In addition, the $EGFR^{\Delta/\Delta}; Kras^{G12D};Ptf1a^{Cre/+}$ murine model will be infected to determine if EGFR is required for infection-induced pancreatitis and subsequent tumorigenesis.

It was determined, through this work, that EGFR is required for LPS-induced pancreatic tumorigenesis in $Kras^{G12D}$ mice. LPS activates toll-like receptor 4 (TLR4), which activates ADAM17 to cleave TNF-α. Ardito et al. showed that ADAM17 signaling through EGFR is required for *Kras*-driven tumorigenesis[113]. Daniluk et al.

demonstrated that LPS treatment activates Ras signaling in the pancreas through an NF-κB-mediated positive feedback loop that can be blocked through inhibition of COX-2[294]. Recently, McElroy et al. demonstrated in the small intestinal crypt cell line IEC-6 that LPS activates TLR4, which activates MAPK p38 and matrix metalloproteinases, transactivating EGFR to activate COX-2[308]. Overall this suggests a mechanism for LPS-induced (and possibly infection-induced) tumorigenesis where TLR4 activation leads to ADAM17, NF-κB and MAPK activation that must signal through EGFR to further activate NF-κB through AKT signaling and activate oncogenic *Kras* and downstream COX-2. This mechanism may be worked out in acinar cell explants by treating cells with LPS to determine if EGFR signaling and COX-2 are activated. $Kras^{G12D}$;$Ptf1a^{Cre/+}$ and $EGFR^{\Delta/\Delta}$; $Kras^{G12D}$; $Ptf1a^{Cre/+}$explants may be treated with LPS to confirm that LPS agonizes Kras and to determine if this effect is blocked in the EGFR KO model. It may also be determined if NF-κB and p38 activation requires EGFR by IHC analysis of LPS-treated tissue from both models, to determine if activation of these pathways are upstream or downstream of EGFR.

IV: Figures

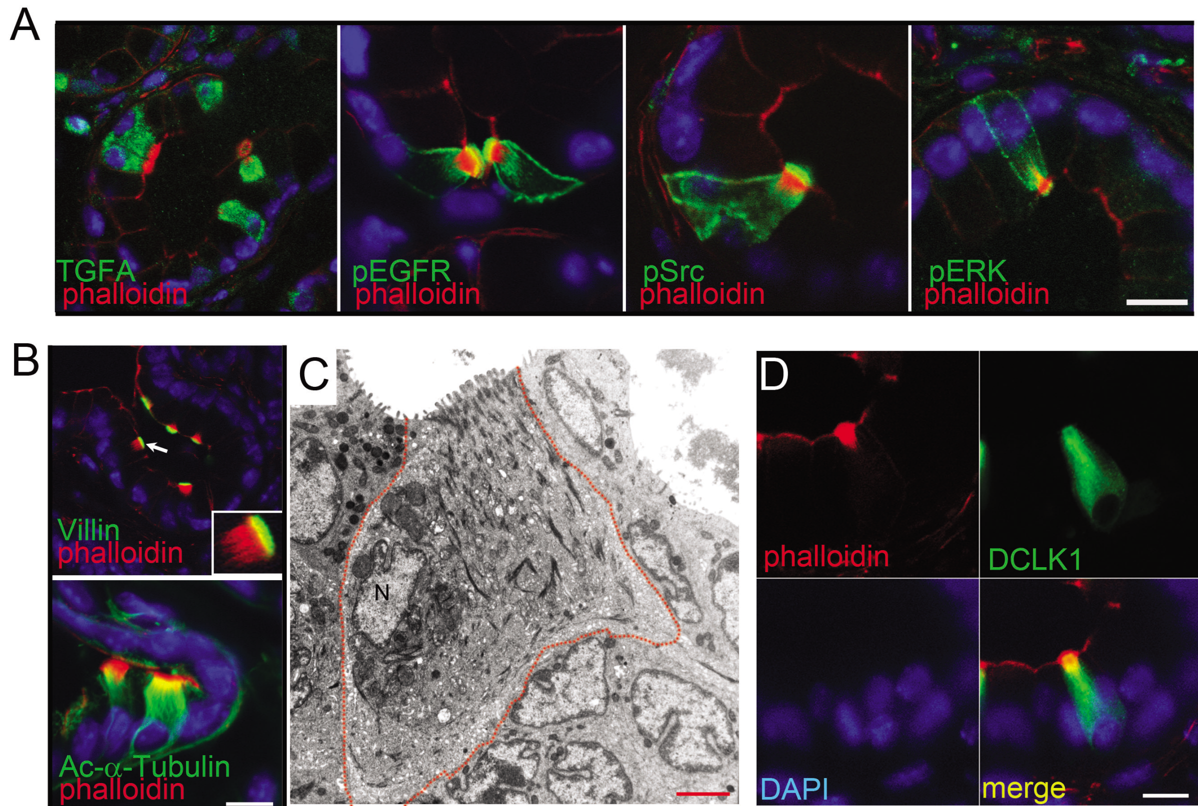


Figure 1. Tuft cells in pancreatic metaplasia. Co-immunofluorescence staining in 4-6 month old *LSL-Kras*$^{G12D/+}$*;Ptf1a*$^{cre/+}$ mice including (A) TGFα, phosphor-EGFR (pY1068), phosphor-SRC (pY416), or phosphor-ERK (pT202/pY204) (green) with phalloidin (red). (B) Tuft cell structural components villin and acetylated alpha tubulin (green) with phalloidin (red). 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-immunofluorescence for DCLK1 (green) and phalloidin (red). Nuclei are stained with DAPI.

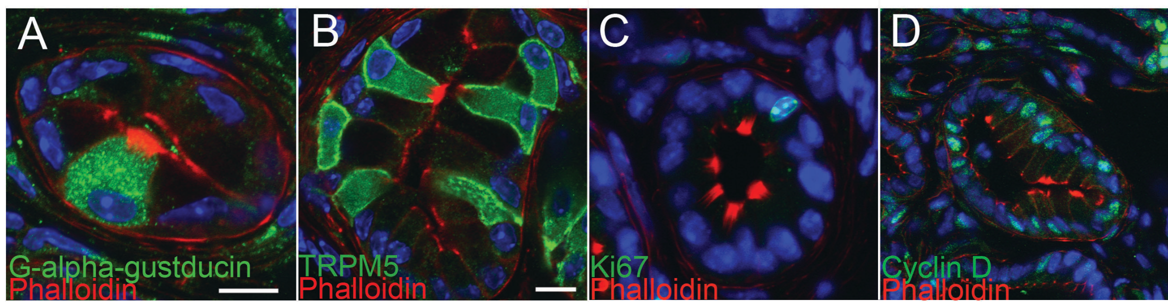


Figure 2. Pancreatic tuft cells as solitary chemosensory cells. Co-immunofluorescence staining for (A) G-α-gustducin, (B) TRPM5, (C) Ki67, and (D) Cyclin D1 (green) and phalloidin (red). Scale bars = 10 μm. Nuclei are stained with DAPI. All IF were done in 4-6 month old *LSL-Kras*$^{G12D/+}$*;Ptf1a*$^{cre/+}$ mice.

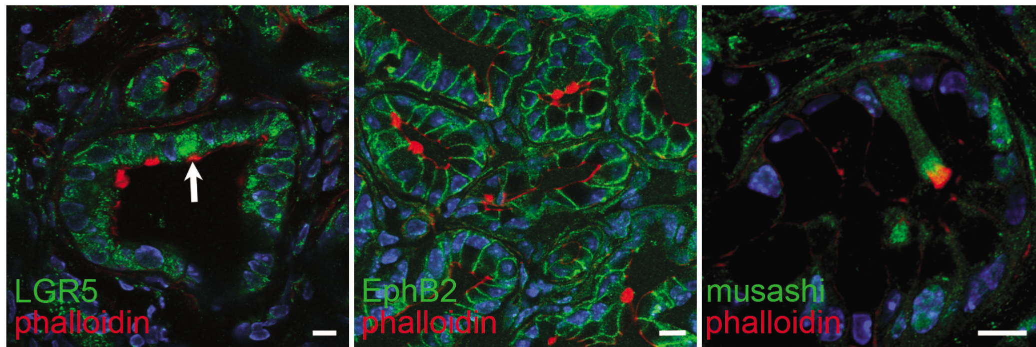


Figure 3. Pancreatic tuft cells express stem cell markers. Co-immunofluorescence staining for LGR5, EphB2, or Musashi (green) and phalloidin (red). Scale bars = 10 µm. Nuclei are stained with DAPI. All IF were done in 4-6 month old *LSL-Kras$^{G12D/+}$;Ptf1a$^{cre/+}$* mice.

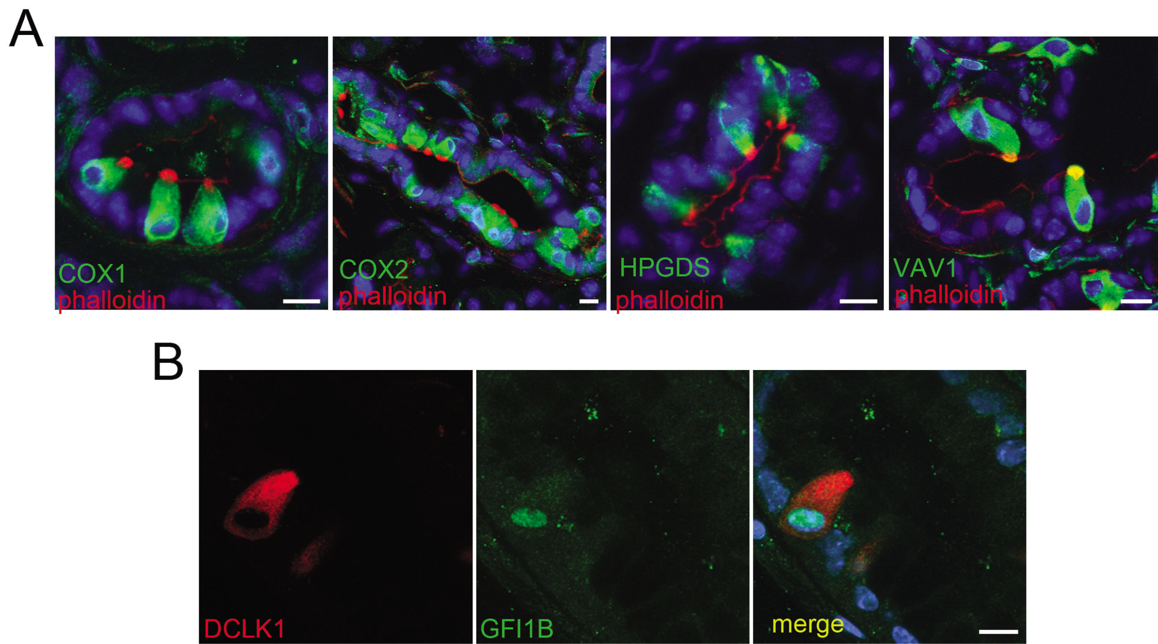


Figure 4. Pancreatic tuft cells express inflammatory cell markers. Co-immunofluorescence staining for (A) COX1, COX2, HPGDS, or VAV1 (green) and phalloidin (red). (B) DCLK1 (red) and GFI1B (green). Scale bars = 10 µm. Nuclei are stained with DAPI. All IF were done in 4-6 month old *LSL-Kras*$^{G12D/+}$*;Ptf1a*$^{cre/+}$ mice.

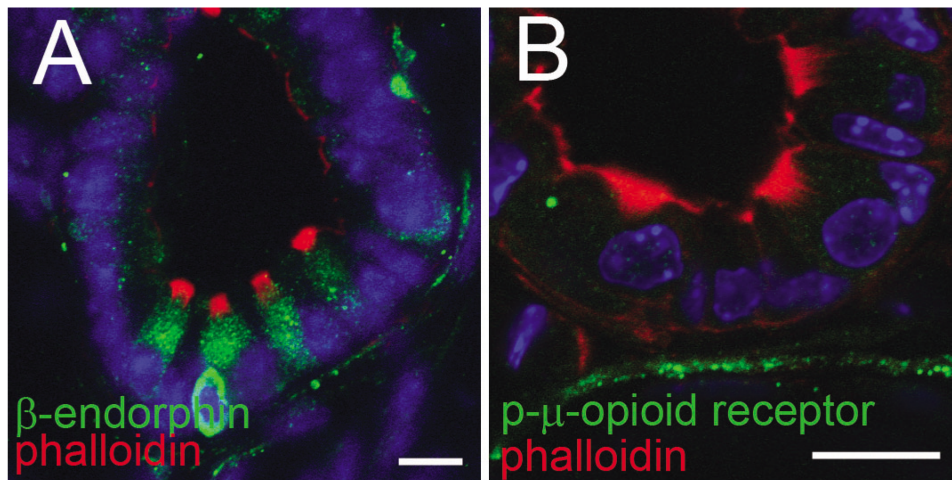


Figure 5. Pancreatic tuft cells and nerve cell signaling. Co-immunofluorescence staining for (A) Beta-endorphin (green) and phalloidin (red). (B) phospho-mu-opioid receptor (green) and phalloidin (red). Scale bars = 10 μm. Nuclei are stained with DAPI. All IF were done in 4-6 month old *LSL-Kras*$^{G12D/+}$*;Ptf1a*$^{cre/+}$ mice.

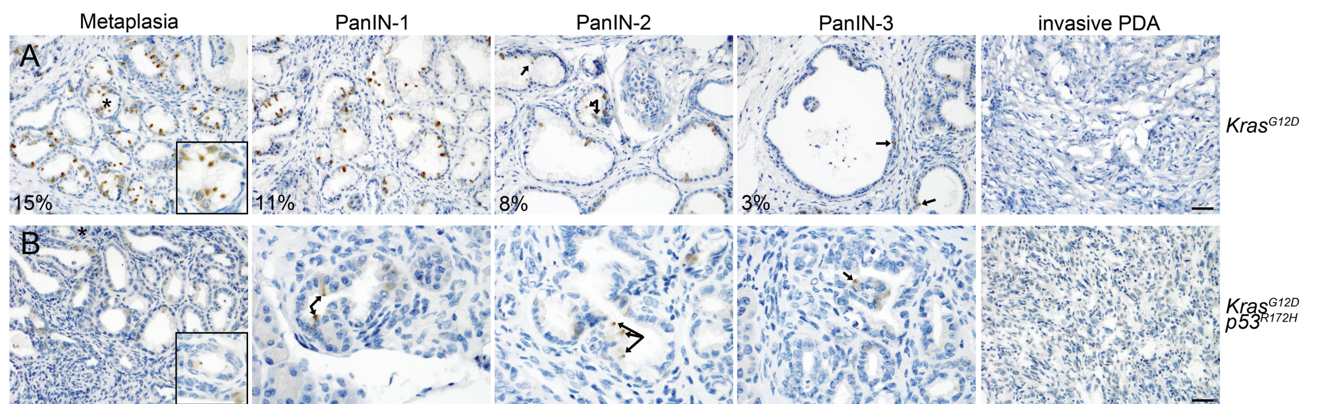


Figure 6. Tuft cells and pancreatic tumor progression. Immunohistochemistry for DCLK1 in either (A) *LSL-Kras$^{G12D/+}$;Ptf1a$^{cre/+}$* or (B) *LSL-Kras$^{G12D/+}$;P53$^{R172/+}$* mice in metaplasia, PanIN1-3 and invasive PDA. Percentages represent the proportion of a lesion made up by tuft cells. Scale bars = 10 μm.

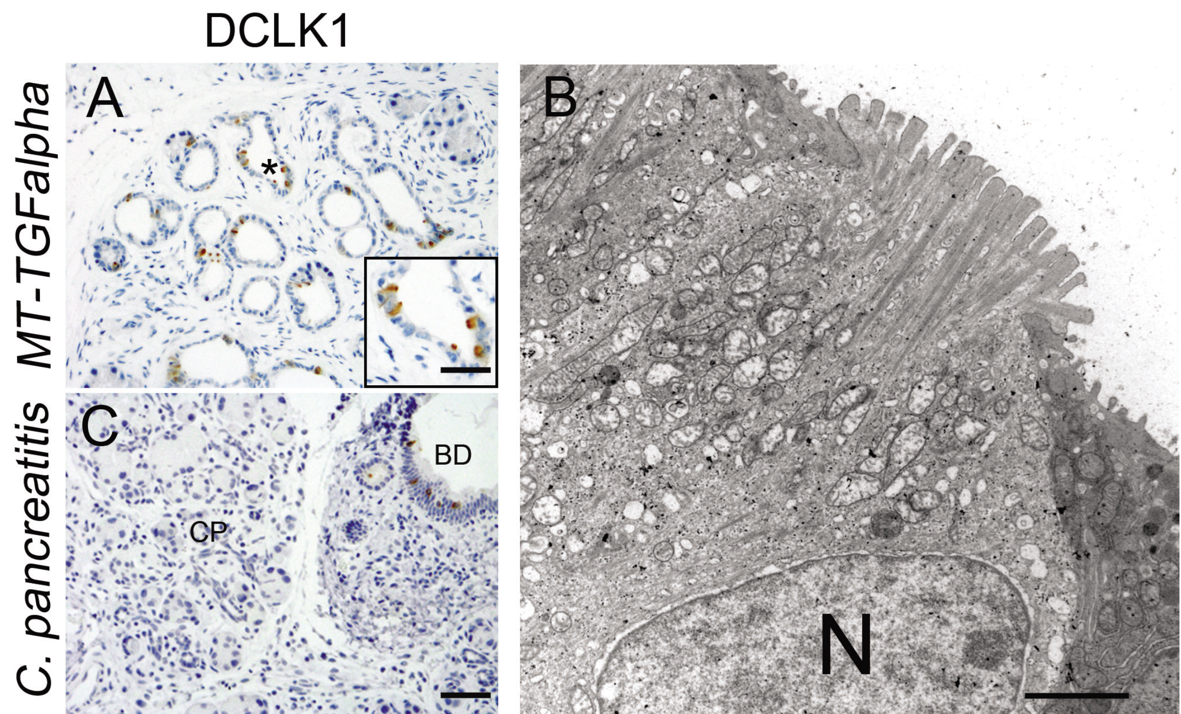


Figure 7. Tuft cells in multiple models of metaplasia. (A) Immunohistochemistry for DCLK1 in *MT-TGF*α mice. (B) Electron microscopy of a metaplastic tuft cell in a *MT-TGF*α mouse, scale bar = 2 μm. (C) Immunohistochemistry for DCLK1 in a wild type mouse with cerulein-induce chronic pancreatitis. CP denotes chronic pancreatitis and BD denotes bile duct. Immunohistochemistry scale bars = 100 μm.

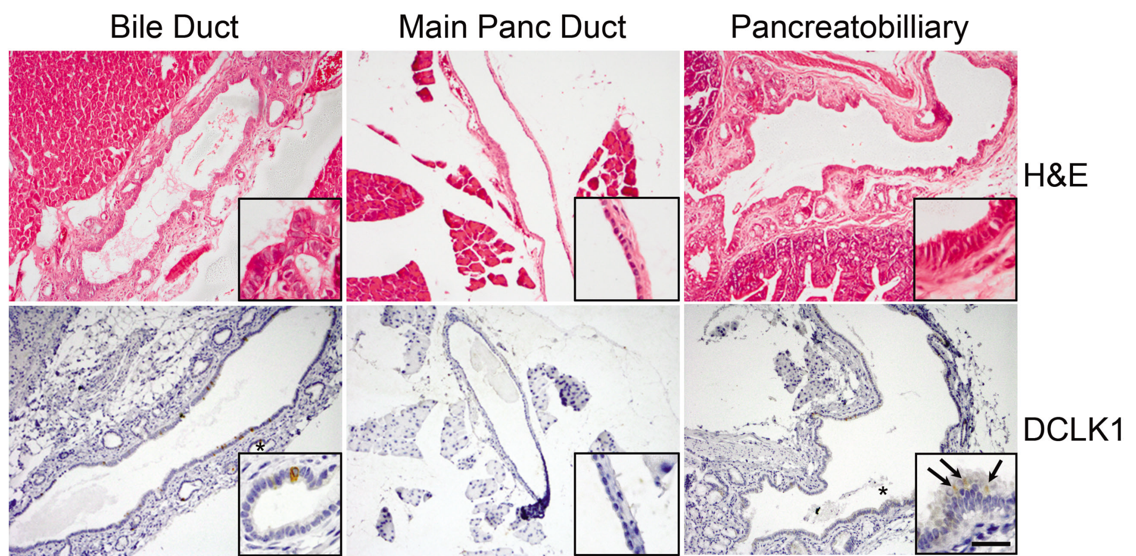


Figure 8. Tuft cells are absent from the normal pancreatic duct. Histological analysis of the wild type murine bile duct, pancreatic duct, and pancreatobiliary duct by hematoxylin and eosin analysis and DCLK1 immuohistochemistry reveals tuft cells are normally absent from the pancreas, but are commonly found in the biliary tract. Scale bar = 100 μm for all panels, 25 μm for insets.

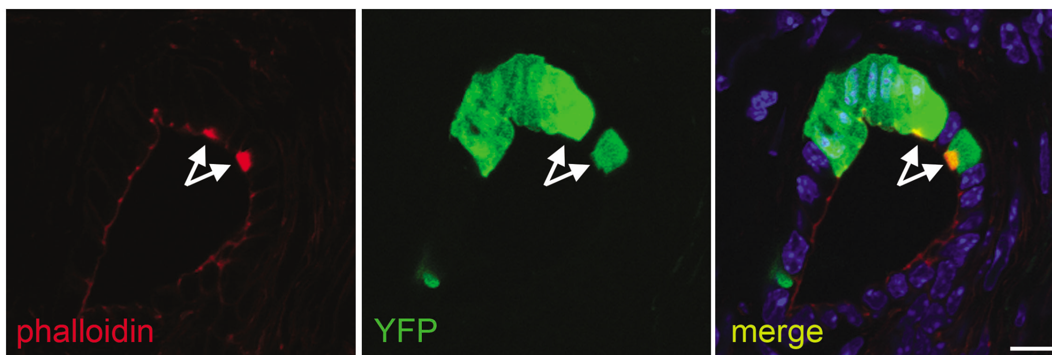


Figure 9. Pancreatic tuft cells transdifferentiate from PTF1A⁺ epithelium. Lineage tracing and immunofluorescent analysis in *LSL-Kras*$^{G12D/+}$;*ROSA*^{YFP};*Ptf1a*$^{Cre\text{-}ERTM/+}$ mice by YFP (green) and phalloidin (red) demonstrates that a significant number of tuft cells derive from the PTF1A^{+} pancreatic epithelium. Nuclei are stained with DAPI. Scale bar = 10 μm.

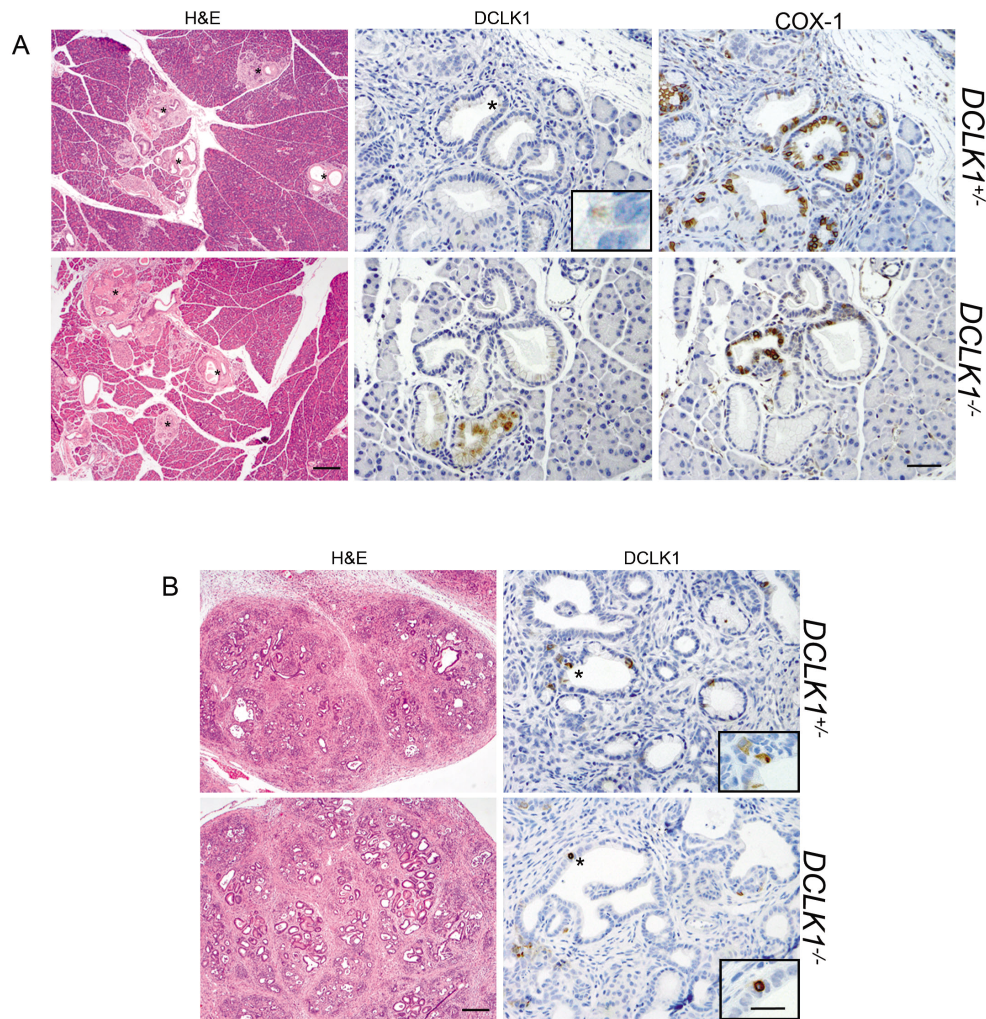


Figure 10. DCLK1 is not required for tuft cell formation or tumorigenesis. (A) Histological analysis of tumorigenesis in 6 week old $DCLK1^{\Delta/\Delta};LSL\text{-}Kras^{G12D/+};Ptf1a^{cre/+}$ and $DCLK1^{\Delta/+};LSL\text{-}Kras^{G12D/+};Ptf1a^{cre/+}$ mice by hematoxylin and eosin (H&E) staining. Immunohistochemistry (IHC) for tuft cell markers DCLK1 and COX1 reveals that DCLK1 is not required for tuft cell formation. (B) Histological analysis of tumor in cerulein treated $DCLK1^{\Delta/\Delta};LSL\text{-}Kras^{G12D/+};Ptf1a^{cre/+}$ and $DCLK1^{\Delta/+};LSL\text{-}Kras^{G12D/+};Ptf1a^{cre/+}$ mice by H&E and IHC for DCLK1. Despite a lack of DCLK1 in $DCLK1^{\Delta/\Delta};LSL\text{-}Kras^{G12D/+};Ptf1a^{cre/+}$ mice, there is an equivalent amount of tumor formation. Scale bars, all H&E = 200 μm, all IHC = 100 μm, and all insets = 25 μm.

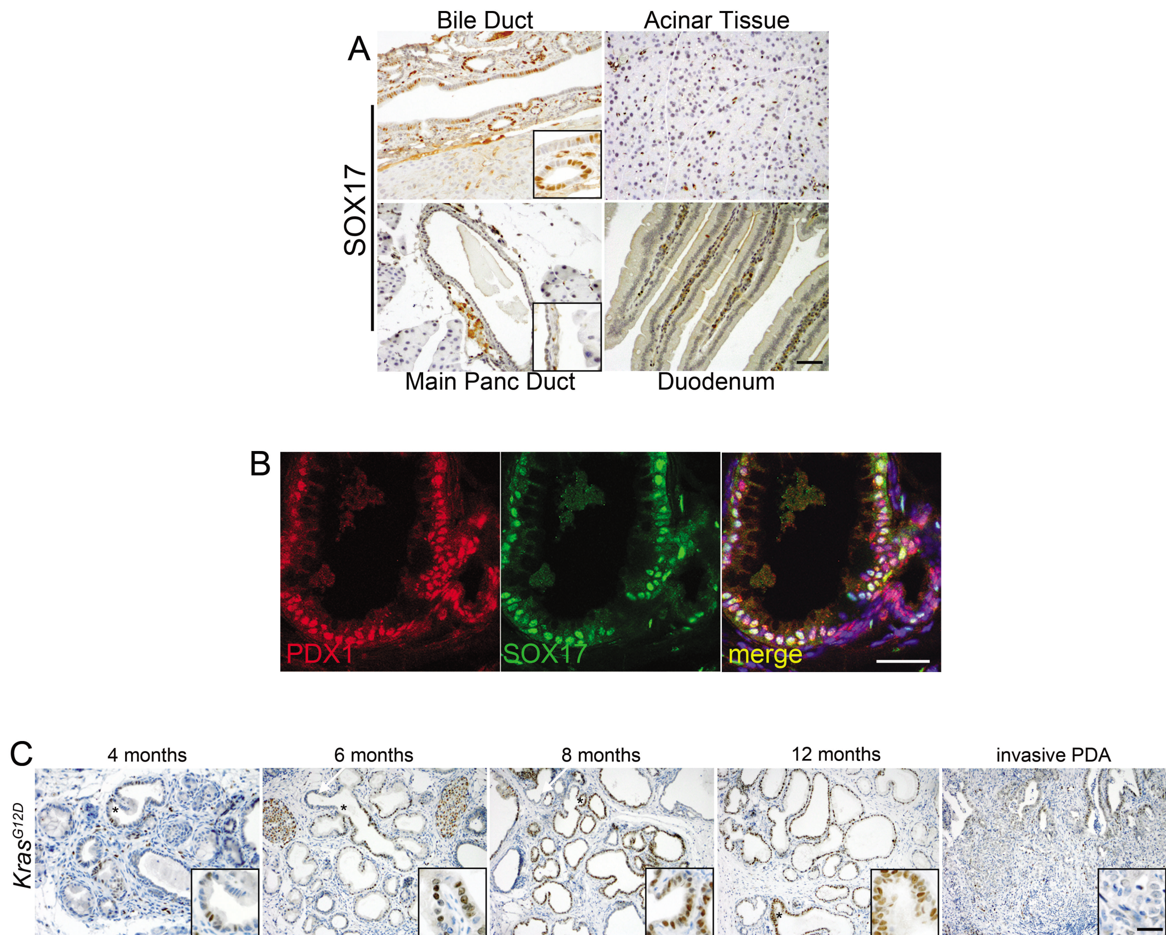


Figure 11. SOX17 is absent from the wild type pancreas, but is expressed during pancreatic tumorigenesis. (A) Immunohistochemistry for SOX17 is positive in the wild type bile duct, but not the pancreas (acinar or ductal compartments), nor the duodenum. Scale bar = 100 μm for Bile Duct panel, 50 μm for all other panels and 25 μm for insets. (B) Co-immunofluorescence for SOX17 (green) and PDX1 (red) in a six month old *LSL-Kras*$^{G12D/+}$;*Ptf1a*$^{Cre/+}$ pancreas reveals that pancreatic metaplasia phenocopies the pancreatobiliary progenitor cell. Nuclei are stained with DAPI. Scale bar = 50 μm. (C) Immunohistochemistry for SOX17 in 4-12 month old *LSL-Kras*$^{G12D/+}$;*Ptf1a*$^{Cre/+}$ mice demonstrates that SOX17 expression increases over time, regardless of tumor grade, but is absent from invasive disease. Scale bar = 50 μm for all panels, 25 μm for insets.

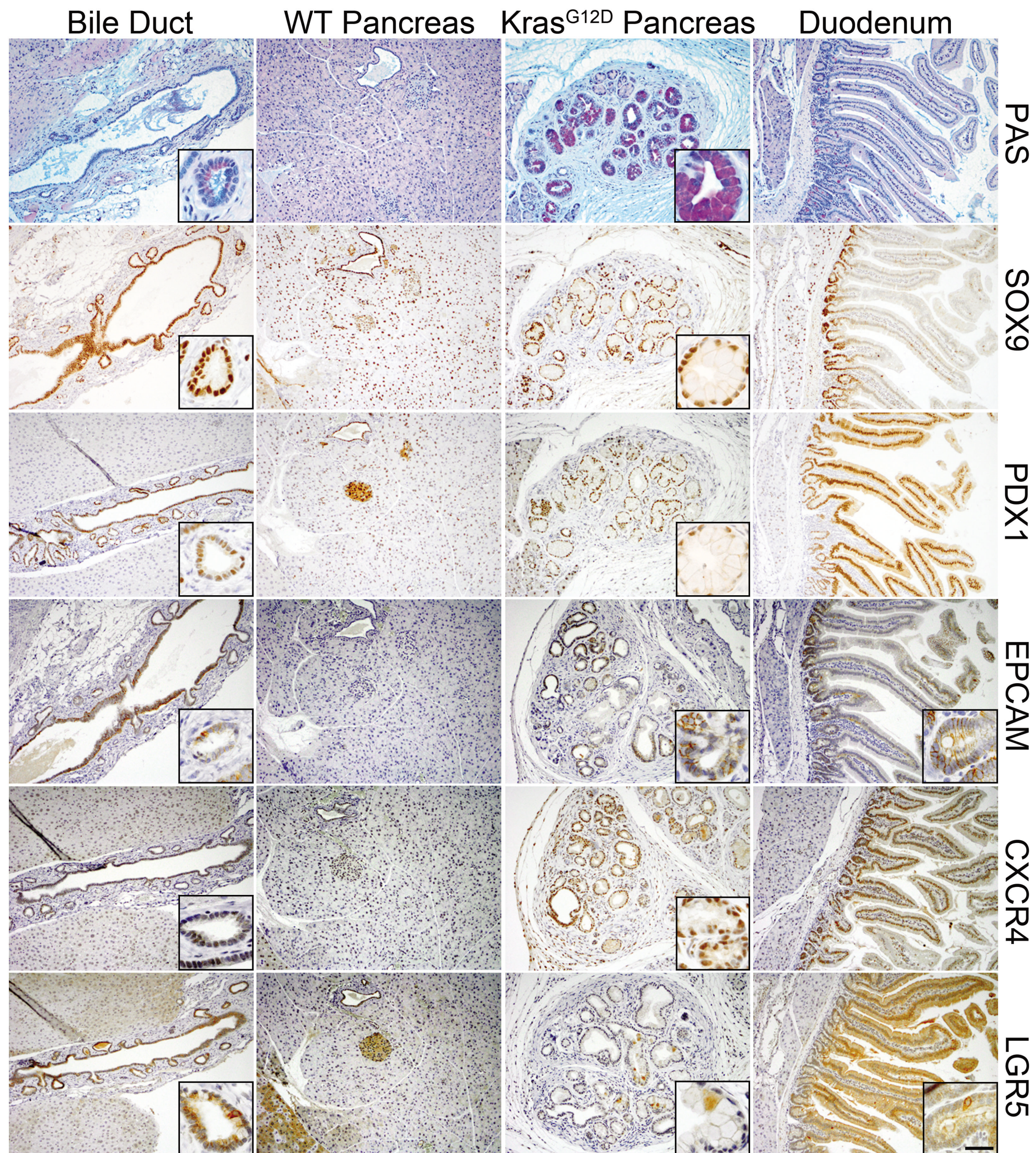
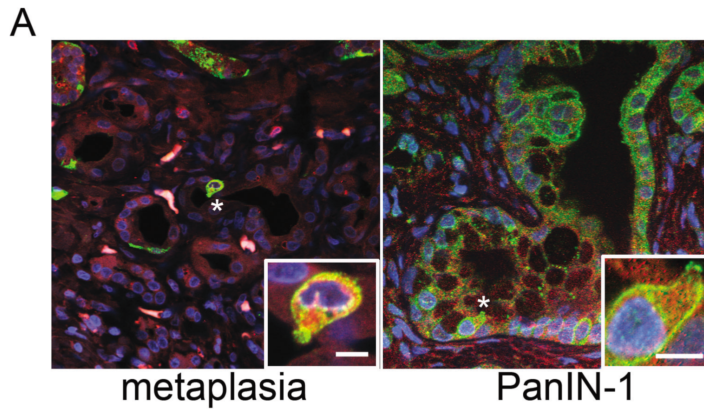


Figure 12. Pre-neoplastic pancreatic metaplasia phenocopies normal biliary duct glands. Histological comparison of the bile duct, wild type pancreas, *Kras*$^{G12D/+}$*;ROSA*^{YFP}*;Ptf1a*$^{Cre\text{-}ERTM/+}$*,* and duodenum reveals molecular similarity between the wild type bile duct and diseased pancreas. Known to express stem and progenitor cell markers, biliary duct glands are mucinous (PAS) and express markers SOX9, PDX1, EpCAM, and CXCR4, as well as stem cell marker LGR5. Upon induction of *Kras*, the epithelium transdifferentiates to a ductal state expressing biliary duct gland markers. Scale bar = 100 μm for all panels, 25 μm for insets.



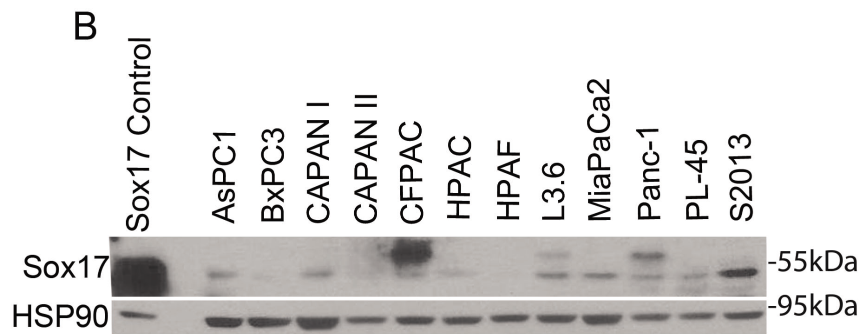


Figure 13. Human pancreatic disease assumes a biliary phenotype. (A) Co-immunofluorescence for phospho-EGFR (Y1068) (green) and COX1 (red) on human pancreatic tissue arrays reveals the presence of tuft cells in metaplasia and early PanIN lesions. Nuclei are stained with DAPI. Scale bars = 20 μm for both panels, 80 μm for insets. (B) Western blot analysis of a panel of human pancreatic cancer cell lines reveals detectable levels of SOX17 in 9/12 cell lines. Molecular weight was confirmed by transfection of MiaPaCa2 cells with mSox17(164/623)IRESGFP-pTRE (Sox17 control).

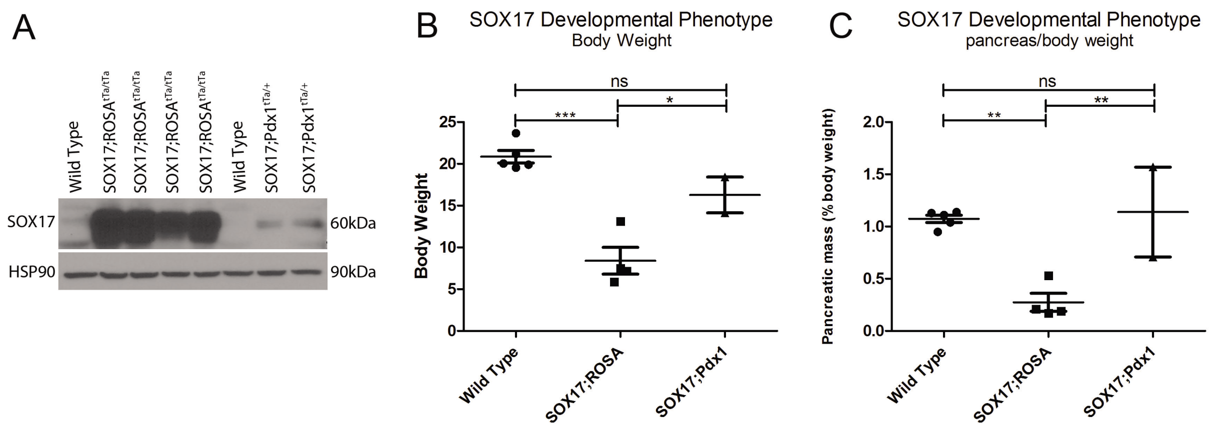


Figure 14. Overexpression of SOX17 in murine pancreata. Overexpression of SOX17 in the murine pancreas was accomplished by utilizing murine models *ROSA*$^{tTa/tTa}$*;Ptf1a*$^{Cre/+}$*;tetO-SOX17* and *PDX1*$^{tTa/+}$*;tetO-SOX17*. Mice were aged to 6 weeks, at which time overexpression of SOX17 was confirmed by western blot (A). Higher levels of SOX17 expression in the *ROSA*$^{tTa/tTa}$*;Ptf1a*$^{Cre/+}$*;tetO-SOX17* model correlate to stunted body weight (B) and pancreas mass (C). Statistics were accomplished using the one-way Anova test; one star represents a confidence interval of less than 0.05, two stars is less than 0.01, and three stars is less than 0.005.

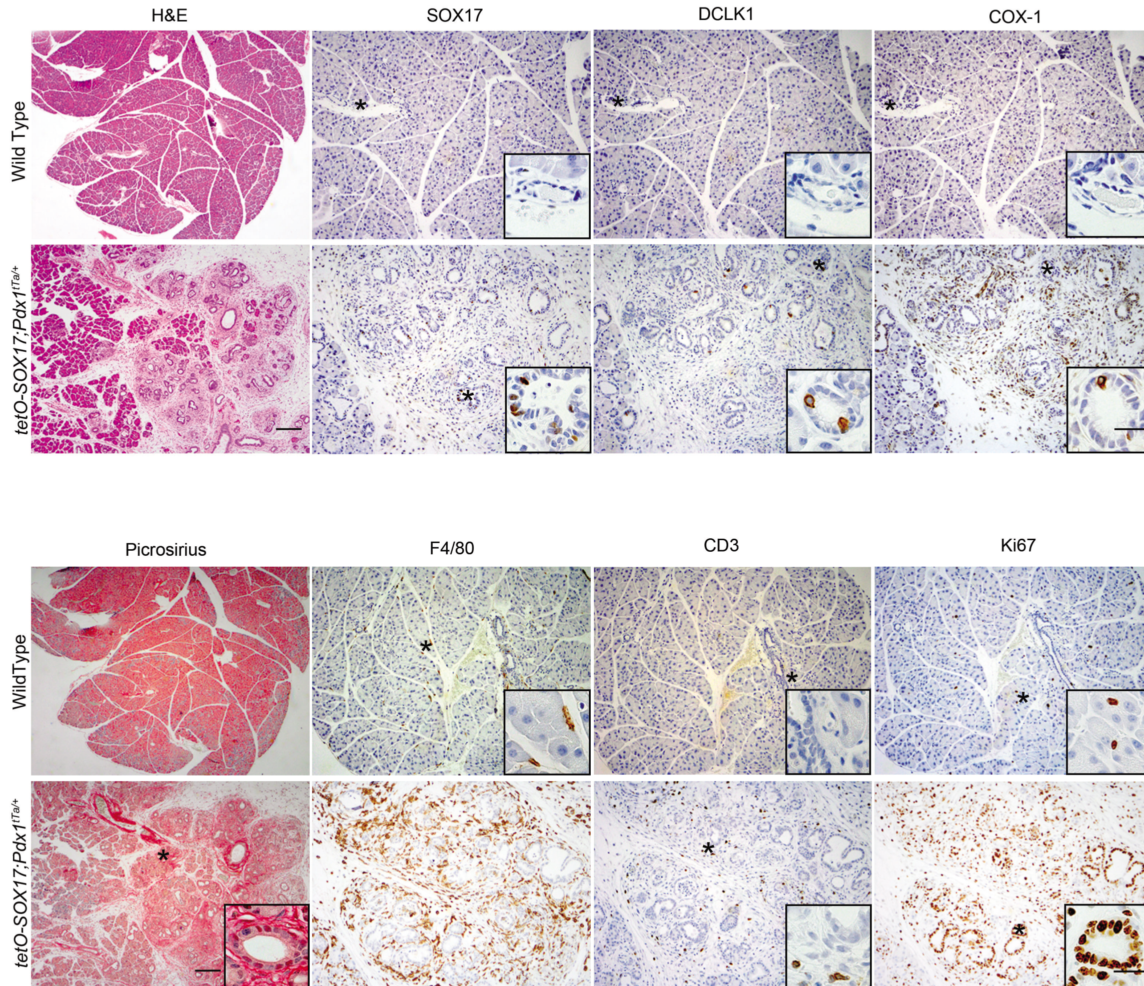


Figure 15. Overexpression of SOX17 in *PDX1$^{tTa/+}$;tetO-SOX17* pancreata results in a pancreatitis-like phenotype. Analysis of pancreata from six week old *PDX1$^{tTa/+}$;tetO-SOX17* (n=5) mice reveals replacement of acinar and endocrine epithelium with SOX17^{+}, tuft cell-containing (DCLK1^{+}, COX1^{+}), metaplasia-like ducts. This response is accompanied by pancreatitis-like symptoms, such as fibrosis (picrosirius), macrophage (F4/80) and T cell (CD3) infiltration, and proliferative epithelium (Ki67). Scale bars for H&E and picrosirius = 200 μm, all IHC = 100 μm, and all insets = 25 μm.

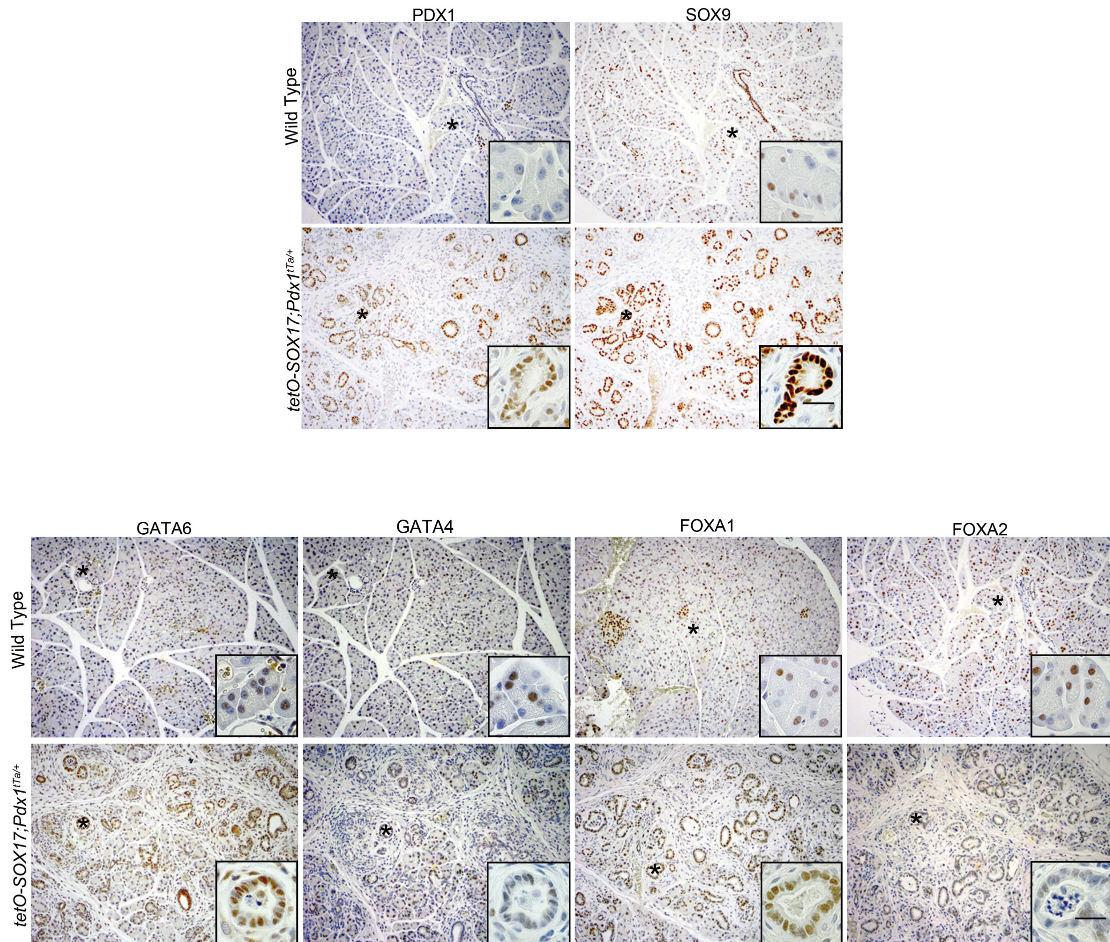


Figure 16. Overexpression of SOX17 in *PDX1$^{tTa/+}$;tetO-SOX17* mice results in expression of pancreas progenitor markers. Immunohistochemical analysis of pancreata from six week old *PDX1$^{tTa/+}$;tetO-SOX17* (n=5) mice, as compared to wild type, reveals increased expression of pancreatic progenitor markers PDX1 and SOX9. Increased expression of SOX17 target genes GATA6 and FOXA1, but not GATA4 and FOXA2, is also evident. Scale bars for H&E and picrosirius = 200 μm, all IHC = 100 μm, and all insets = 25 μm.

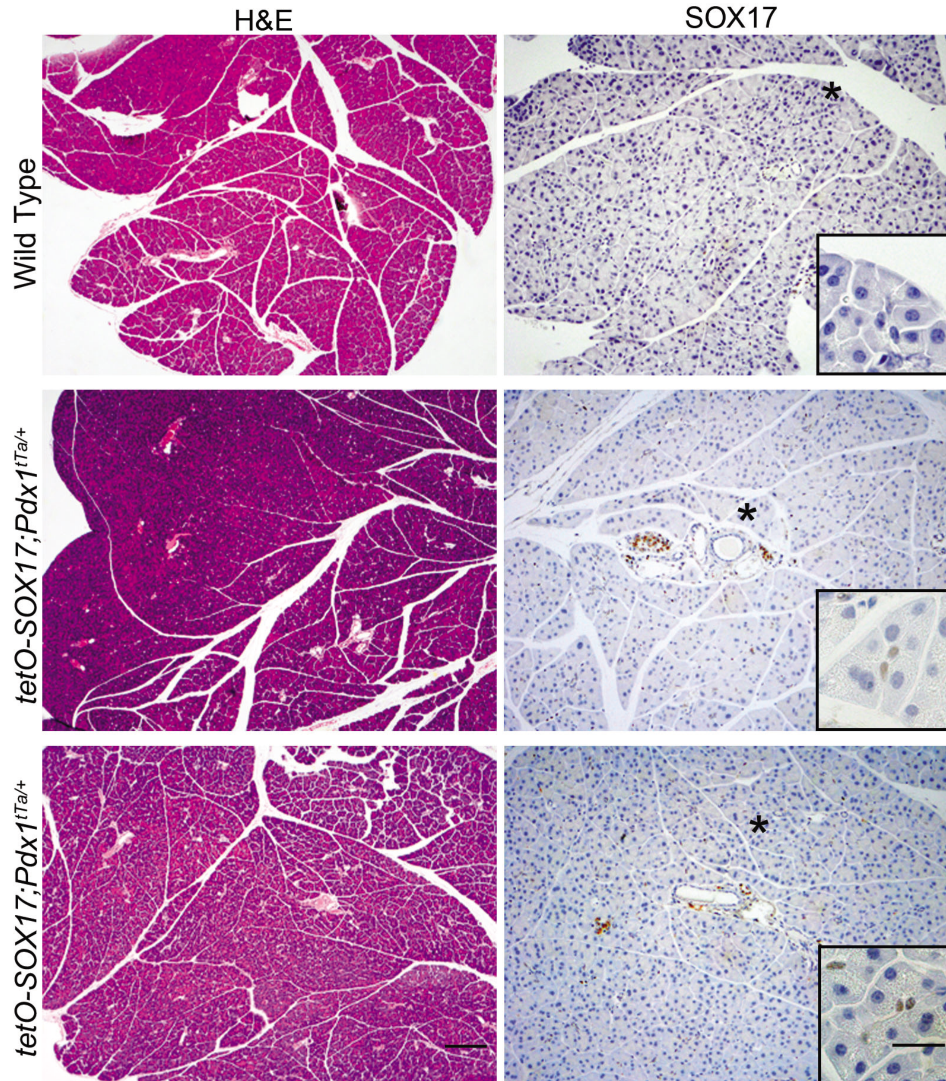


Figure 17. Induction of SOX17 in adult *PDX1$^{tTa/+}$;tetO-SOX17* mice. SOX17 induction in adult mice 4-6 weeks of age (n=3), by removal of doxycycline chow for four weeks, resulted in minimal expression of SOX17, confined to centroacinar cells, and no overt phenotype. Scale bars for H&E = 200 μm, all IHC = 100 μm, and all insets = 25 μm.

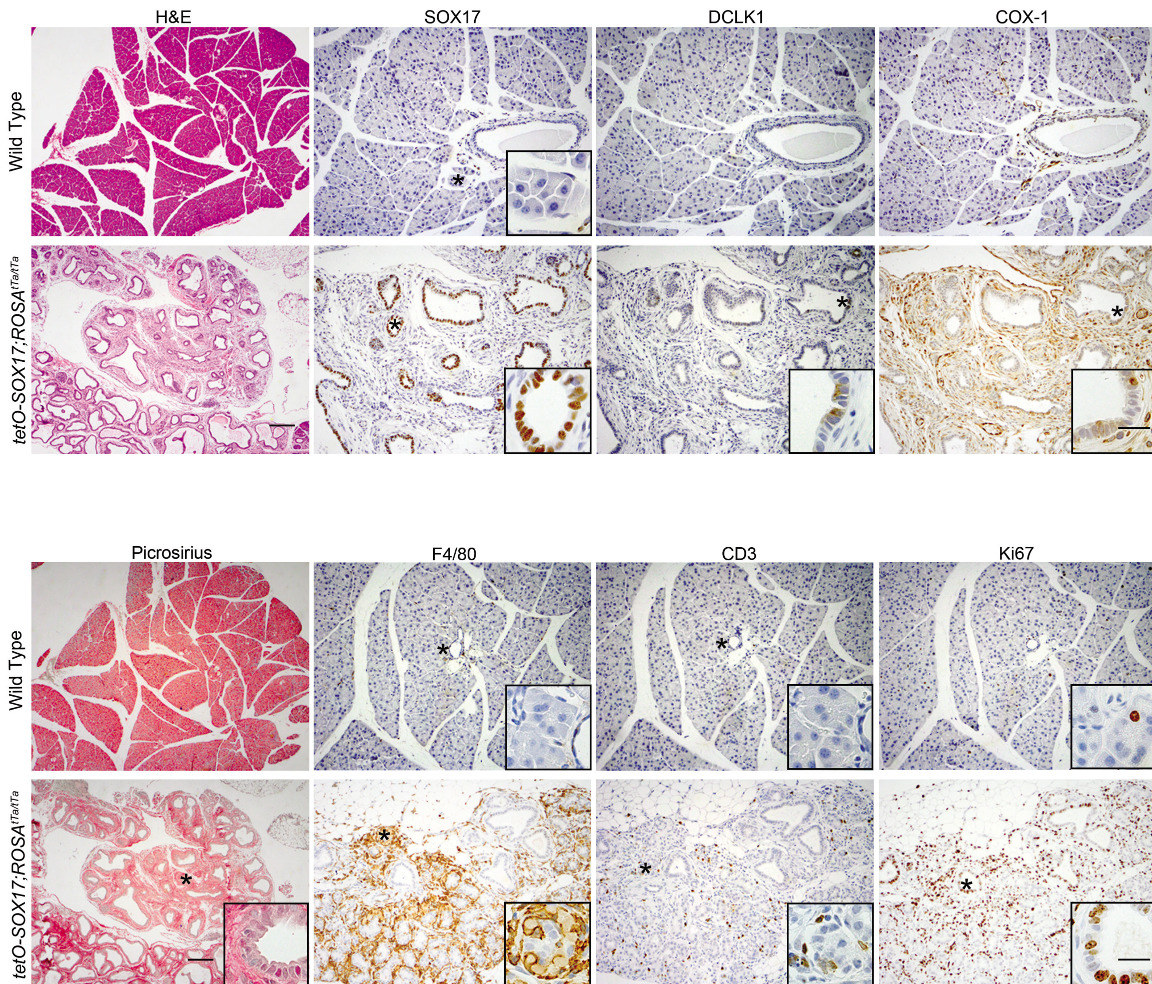


Figure 18. Overexpression of SOX17 in *ROSA*$^{tTa/+}$*;Ptf1a*$^{Cre/+}$*;tetO-SOX17* mice results in a pancreatitis-like phenotype. Analysis of pancreata from six week old *ROSA*$^{tTa/+}$*;Ptf1a*$^{Cre/+}$*;tetO-SOX17* mice (n=4) reveals even greater replacement of acinar and endocrine epithelium with ductal epithelia than the *PDX1*$^{tTa/+}$*;tetO-SOX17* model. While tuft cell containing (DCLK1^{+}, COX1^{+}), metaplasia-like ducts are present, there is an inverse correlation to increased SOX17 expression. This response is accompanied by pancreatitis-like symptoms, such as fibrosis (picrosirius), macrophage (F4/80) and T cell (CD3) infiltration, and proliferative epithelium (Ki67). Scale bars for H&E and picrosirius = 200 μm, all IHC = 100 μm, and all insets = 25 μm.

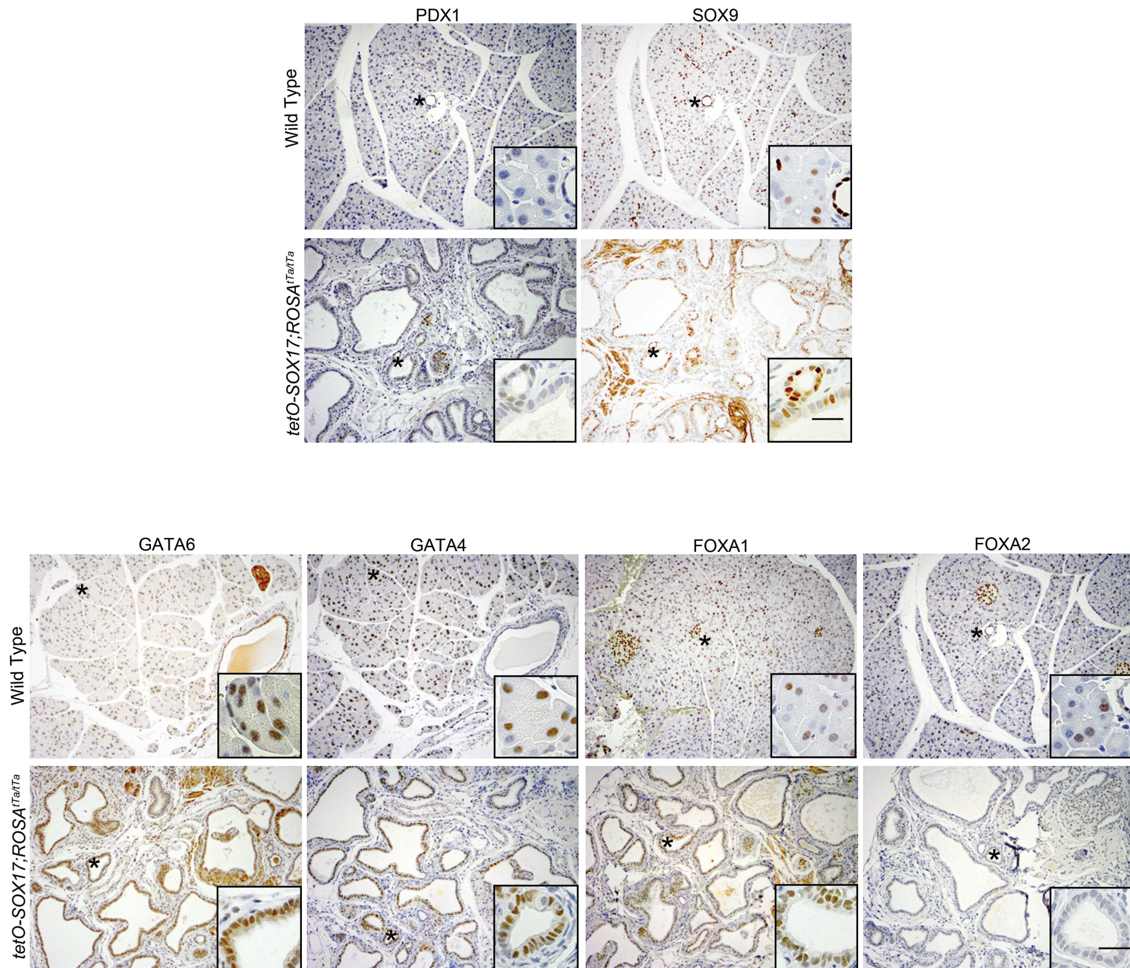


Figure 19. Overexpression of SOX17 in *ROSA$^{tTa/+}$;Ptf1a$^{Cre/+}$;tetO-SOX17* mice results in a more differentiated ductal transdifferentiation event. Immunohistochemical analysis of pancreata from six week old *ROSA$^{tTa/+}$;Ptf1a$^{Cre/+}$;tetO-SOX17* mice (n=4) reveals increased expression of pancreatic progenitor markers PDX1 and SOX9, as compared to wild type, but far less expression than seen in the *PDX1$^{tTa/+}$;tetO-SOX17* model, suggesting a more differentiated ductal epithelium. Increased expression of SOX17 target genes GATA6 and FOXA1, but not GATA4 and FOXA2, is also evident. Scale bars for all IHC = 100 μm, and all insets = 25 μm.

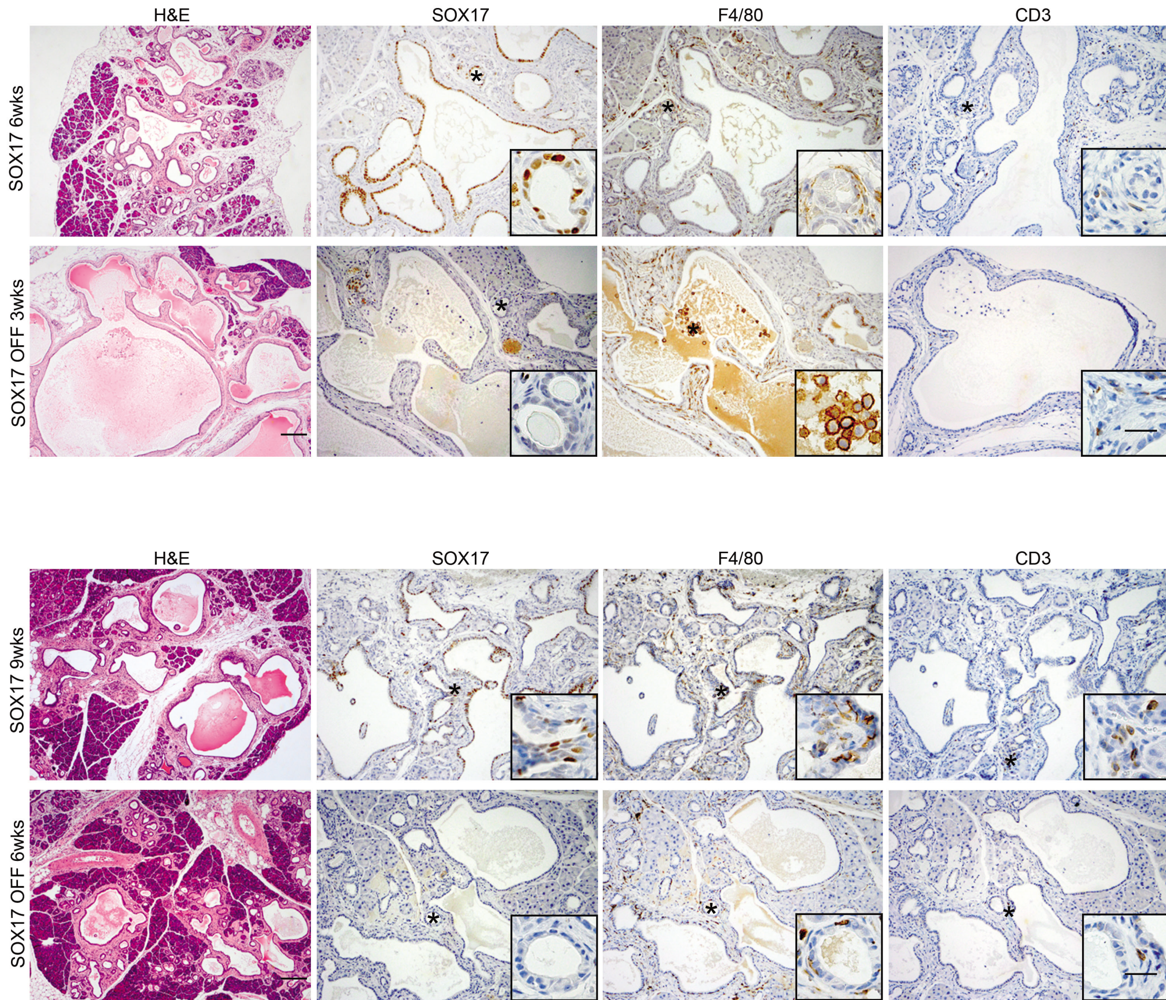


Figure 20. Halting SOX17 expression does not result in expansion of acinar cell epithelium. Eliminating SOX17 expression, through administration of doxycycline chow, in 3 week old *ROSA*$^{tTa/+}$*;Ptf1a*$^{Cre/+}$*;tetO-SOX17* mice for either 3 (n=6) or 6 (n=4) weeks results in persistence of now SOX17^{-} ducts, confirmed by immunohistochemistry. While the chronic inflammatory response persists, as determined for IHC for macrophages (F4/80) and T cells (CD3), the response may be dampened in SOX17-OFF mice due to inhibition of further transdifferentiation. Scale bars for H&E = 200 μm, all IHC = 100 μm, and all insets = 25 μm.

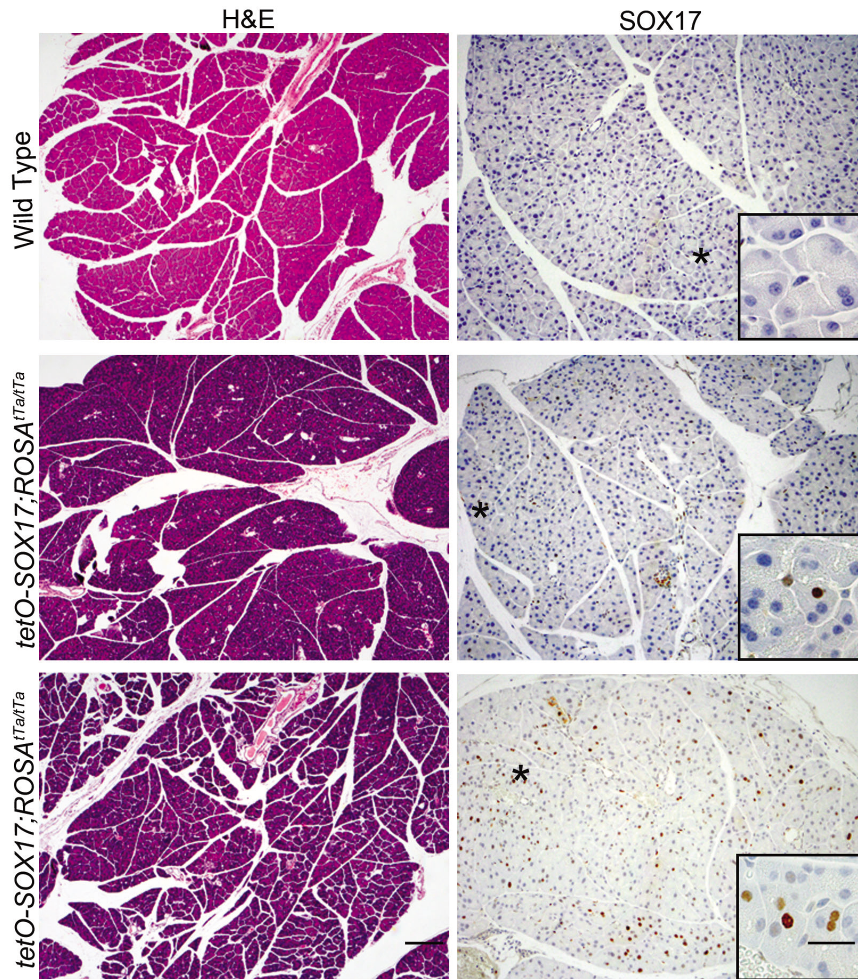


Figure 21. Induction of SOX17 in adult *ROSA$^{tTa/+}$;Ptf1a$^{Cre/+}$;tetO-SOX17* mice. SOX17 induction in adult mice at 4 weeks of age (n=3), by removal of doxycycline chow for four weeks, resulted in varied levels of SOX17 expression throughout the pancreas, with no overt phenotype. Scale bars for H&E = 200 μm, all IHC = 100 μm, and all insets = 25 μm.

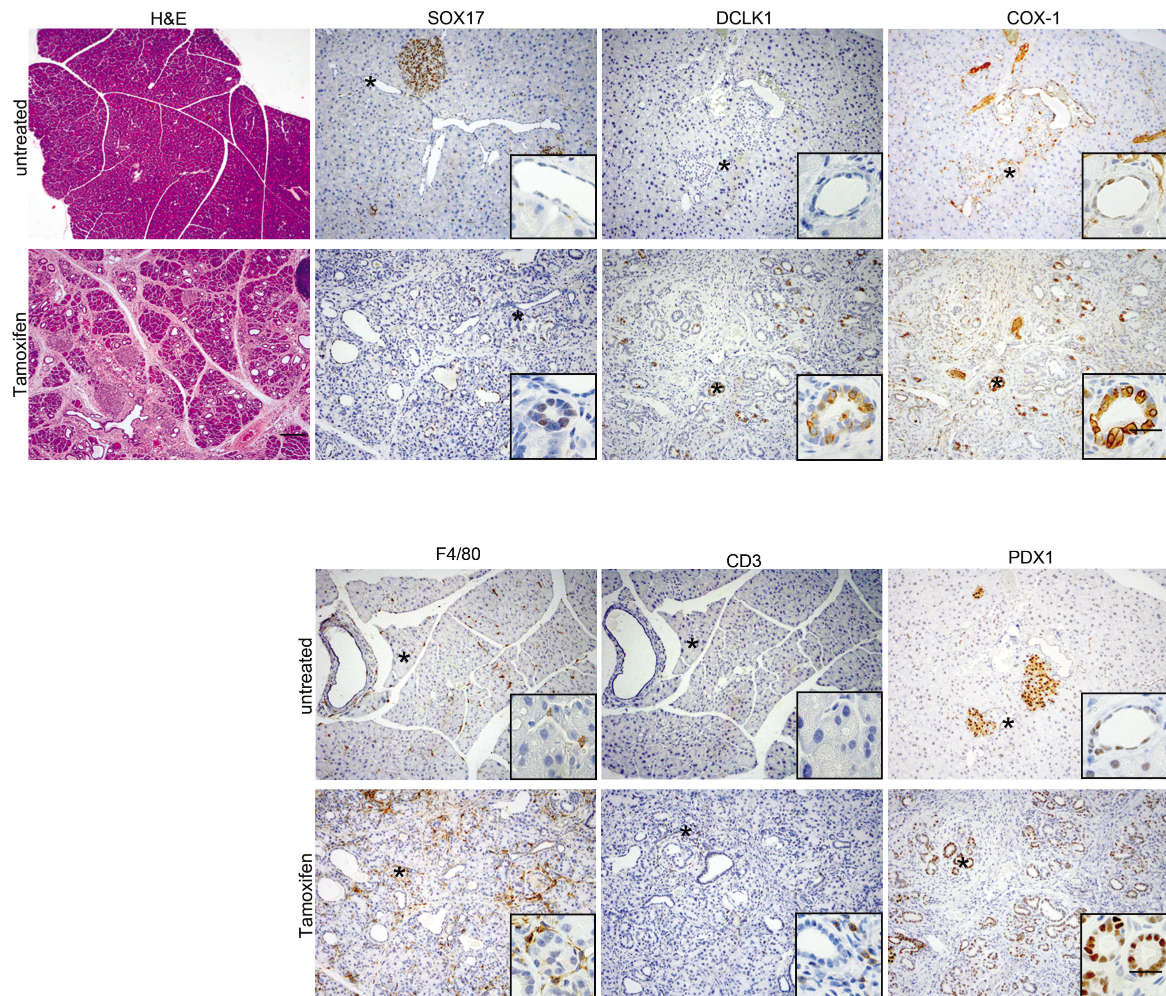
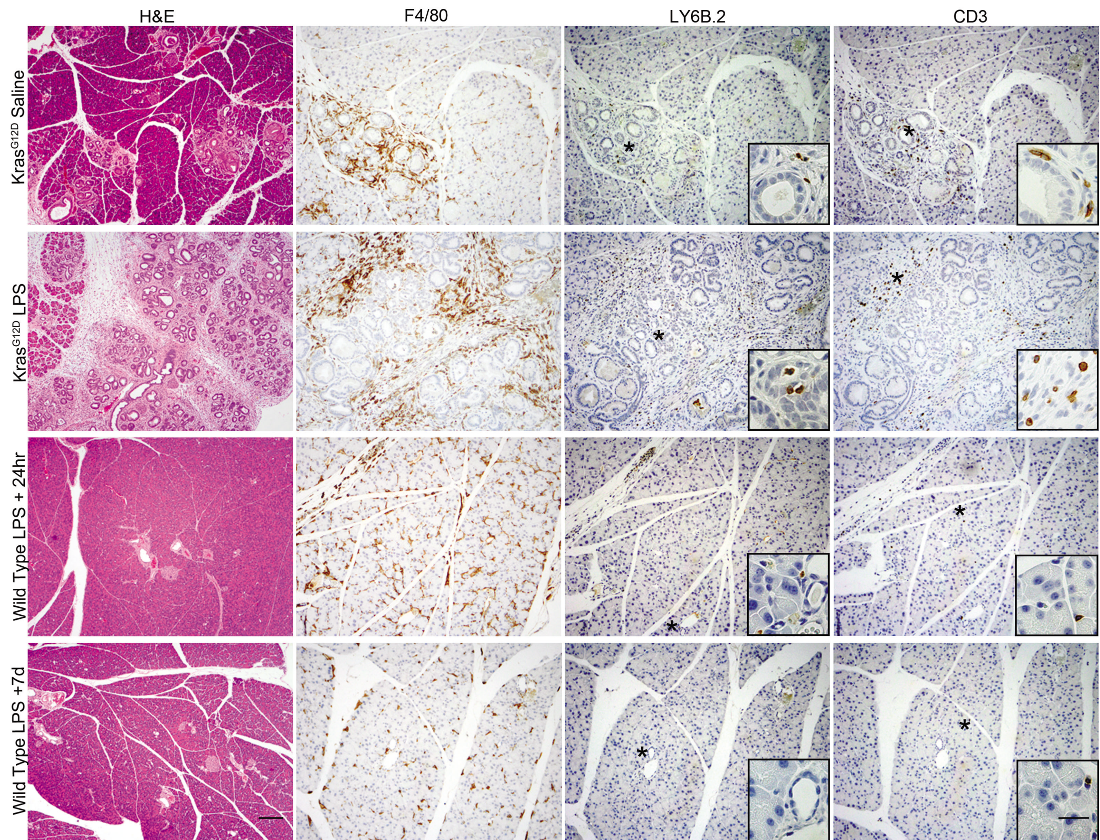


Figure 22. Induction of SOX17 in adult *ROSA$^{tTa/+}$;Ptf1a$^{Cre-ERTM/+}$;tetO-SOX17* mice results in a pancreatitis-like phenotype. SOX17 induction in adult mice at 8 weeks of age, through administration of tamoxifen, resulted in replacement of acinar and endocrine epithelium with SOX17^{+}, tuft cell-containing (DCLK1^{+}, COX1^{+}), metaplasia-like ducts. This response is accompanied by pancreatitis-like symptoms, such as macrophage (F4/80) and T cell (CD3) infiltration, and proliferative epithelium (Ki67). Scale bars for H&E = 200 μm, all IHC = 100 μm, and all insets = 25 μm.



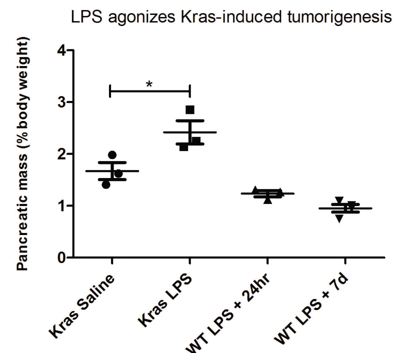


Figure 23. Lipopolysaccharide treatment agonizes *Kras*-driven tumorigenesis and induces macrophage infiltration. As compared to saline-treated animals, lipopolysaccharide (LPS) enhances tumorigenesis, determined by histological analysis and pancreatic mass. Statistics were accomplished using the one-way Anova test; one star represents a confidence interval of less than 0.05. Tumorigenesis is accompanied by an inflammatory response made up of macrophages (F4/80), neutrophils (Ly6B.2), and T cells (CD3). Scale bars for H&E = 200 μm, all IHC = 100 μm, and all insets = 25 μm.

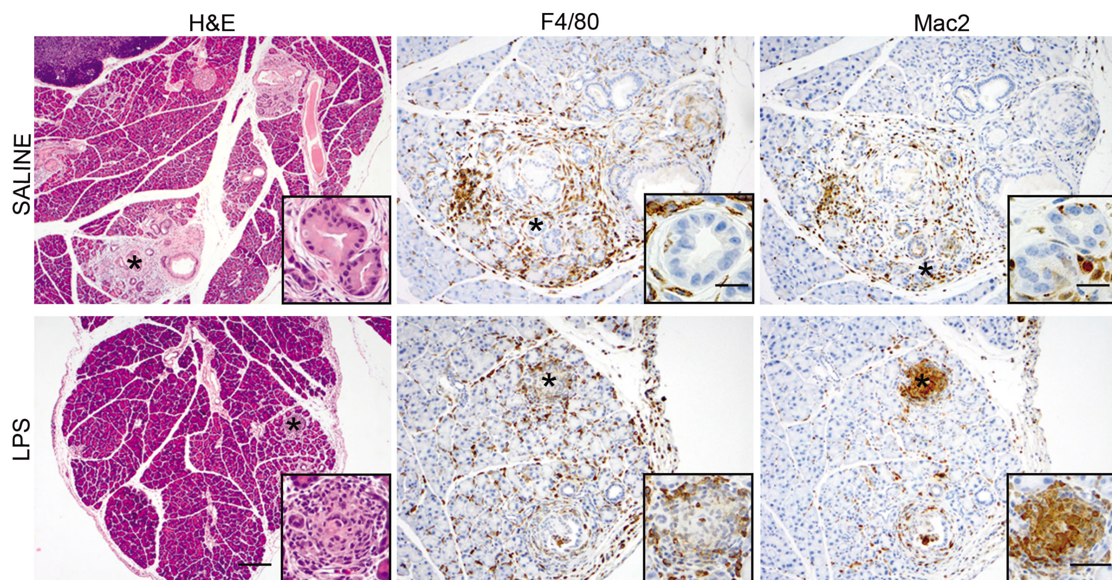


Figure 24. Pre-treatment with LPS alters the composition of the inflammatory cell infiltrate. Inducible *Kras*^{G12D};*Ptf1a*$^{Cre\text{-}ERTM/+}$ mice were pre-treated with either saline or LPS, to induce macrophage infiltration, at which time *Kras*^{G12D} activity was induced by tamoxifen treatment. As compared to saline treated animals LPS pre-treated mice had less tumor (A) and greater bolus-like accumulation of F4/80^{+}(B) and Mac2^{+} (C) macrophages. Scale bars for H&E = 200 μm, for IHC = 100 μm, for saline-treated insets = 20 μm, and for LPS-treated insets = 50 μm.

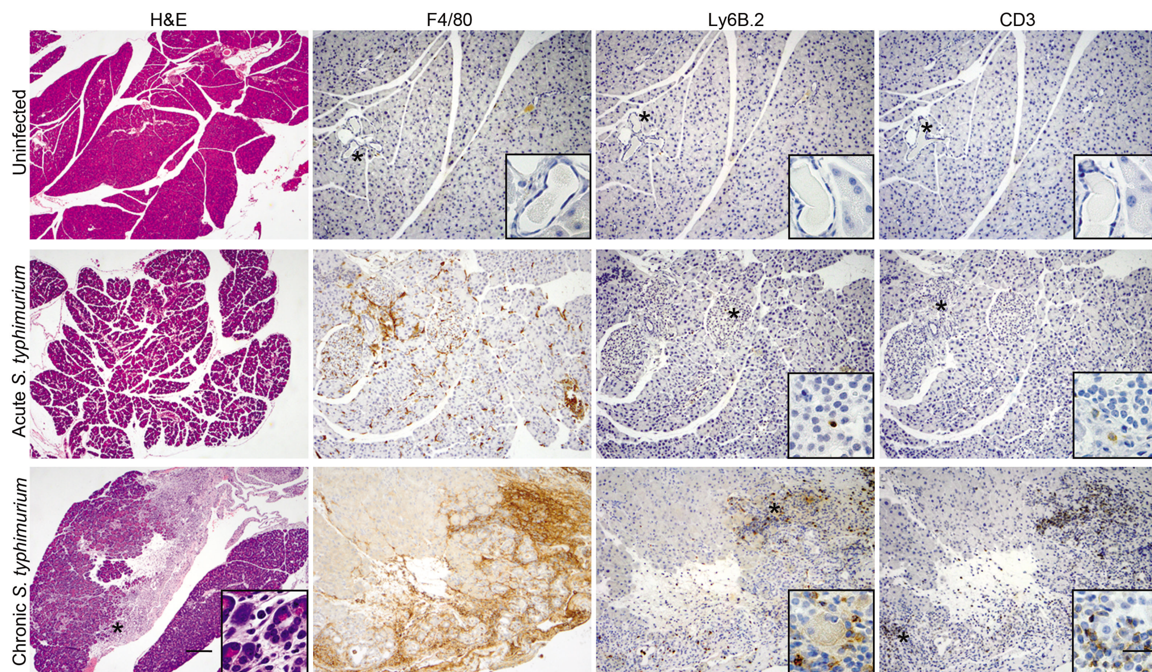


Figure 25. Infection with *Salmonella* serovar Typhimurium induces pancreatitis. As compared to uninfected controls, mice with an acute *S.* Typhimurium infection, induced intravenously in C57BL/6J x 129X1/svJ mice, develop acute pancreatitis as determined by edema (H&E) and macrophage infiltration (F4/80). Mice with a chronic *S.* Typhimurium infection, induced intravenously in $NRAMP^{+/+}$C57BL/6 mice, develop chronic pancreatitis, identified by acinar-to-ductal metaplasia (H&E), and a mixed immune response composed of macrophages (F4/80), neutrophils (Ly6B.2) and T cells (CD3). Scale bars for H&E = 200 μm, for IHC = 100 μm, and for insets = 25 μm.

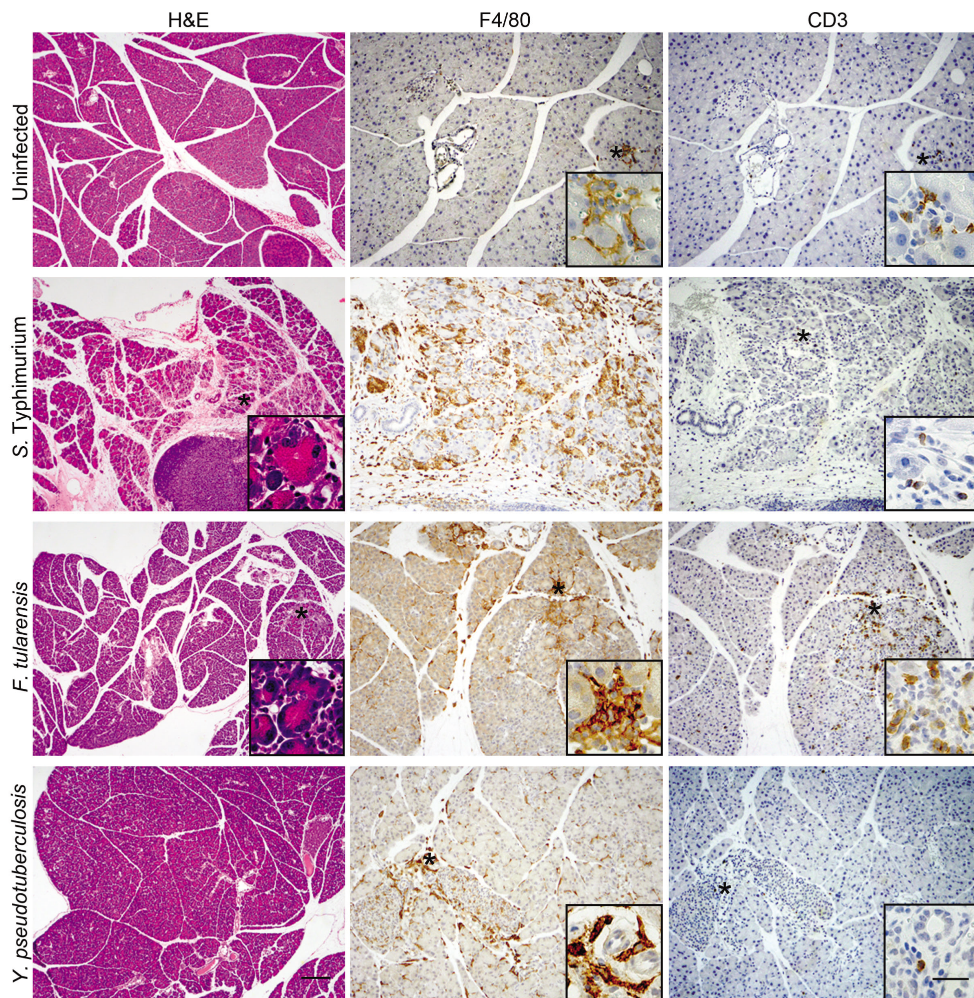


Figure 26. Various Gram-negative bacteria have the ability to induce pancreatitis. Like *S.* Typhimurium, *Francisella tularensis* and *Yersinia pseudotuberculosis* are capable of inducing pancreatitis. Acute infection with *Francisella tularensis* is substantial enough to induce acinar cell disturbance (H&E) and an immune infiltrate composed of macrophages (F4/80) and T cells (CD3). Acute infection with *Yersinia pseudotuberculosis* induces an immune infiltrate largely composed of macrophages. Scale bars for H&E = 200 μm, for IHC = 100 μm, and for insets = 25 μm.

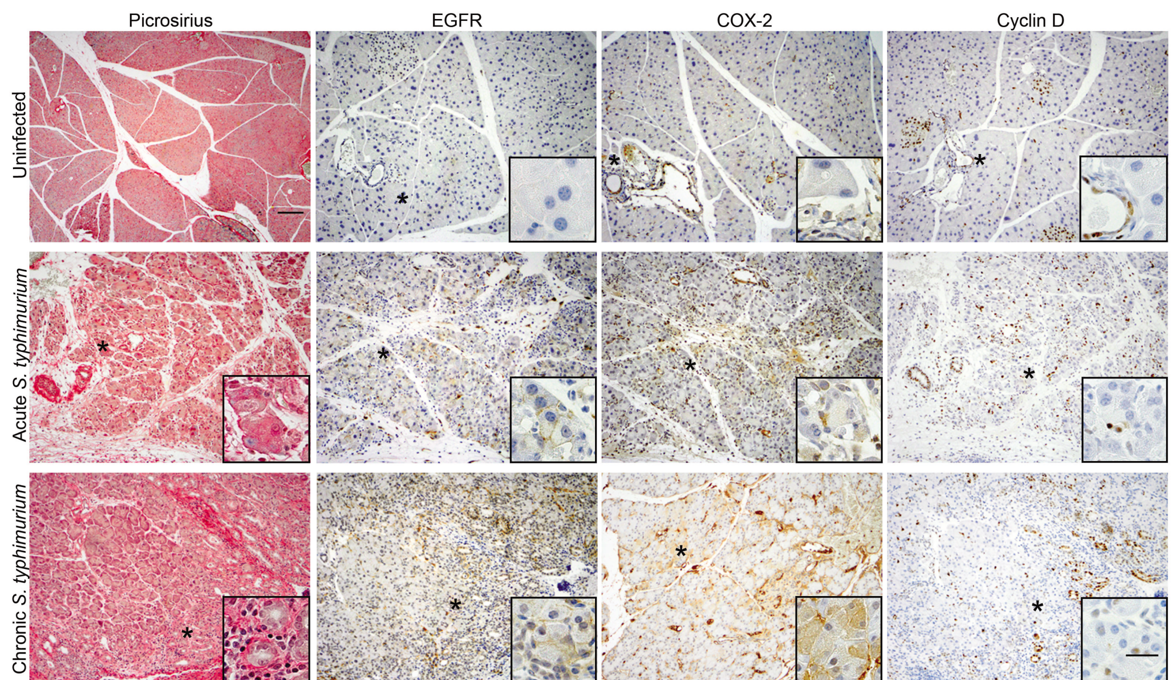
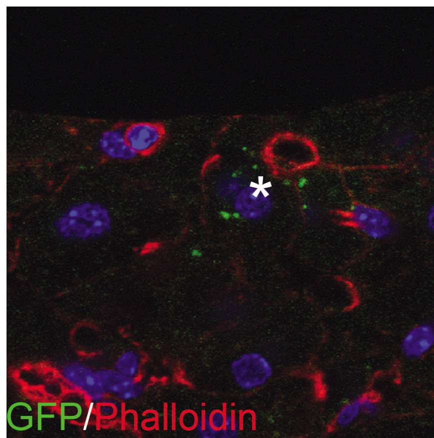


Figure 27. Infection with *S.* Typhimurium induces pro-tumorigenic signaling pathways. Histological analysis of pancreata from mice with either an acute or chronic *S.* Typhimurium infection reveals a substantial stromal response and activation of pro-tumorigenic EGFR signaling. Mice harboring an acute infection exhibit collagen deposition (picrosirius) and activation of EGFR, but not downstream signaling components COX2 and Cylin D1. Mice with a chronic infection, regardless of model analyzed, have a greater abundance of collagen (picrosirius) and EGFR pathway activation in acinar cells leading to both COX2 and Cyclin D1 expression. Acute infection was induced orogastrically in C57BL/6J mice. Chronic infection was induced either intravenously in *NRAMP*$^{+/+}$C57BL/6 mice or orogastrically in C57BL/6J x 129X1/svJ mice. Scale bar for picrosirius in the uninfected mouse = 200 μm, for infected picrosirius and all IHC = 100 μm, and for insets = 25 μm.



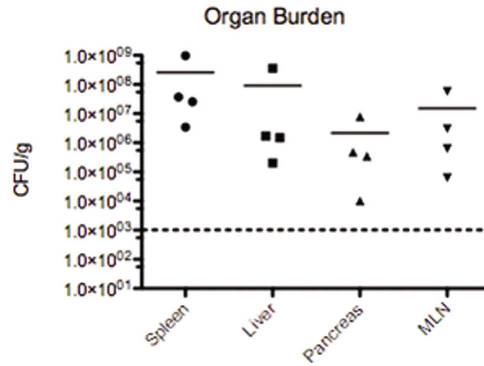


Figure 28. *S.* Typhimurium localizes to the pancreas during infection. Confocal microscopy analysis of pancreata from C57BL/6J mice infected orogastrically with a GFP-positive strain of *S.* Typhimurium demonstrates localization of the bacteria to the pancreas 5 days post-infection. Organ burden analysis confirms that the bacteria directly inhabit the pancreas, exposing the epithelium to PAMPs.

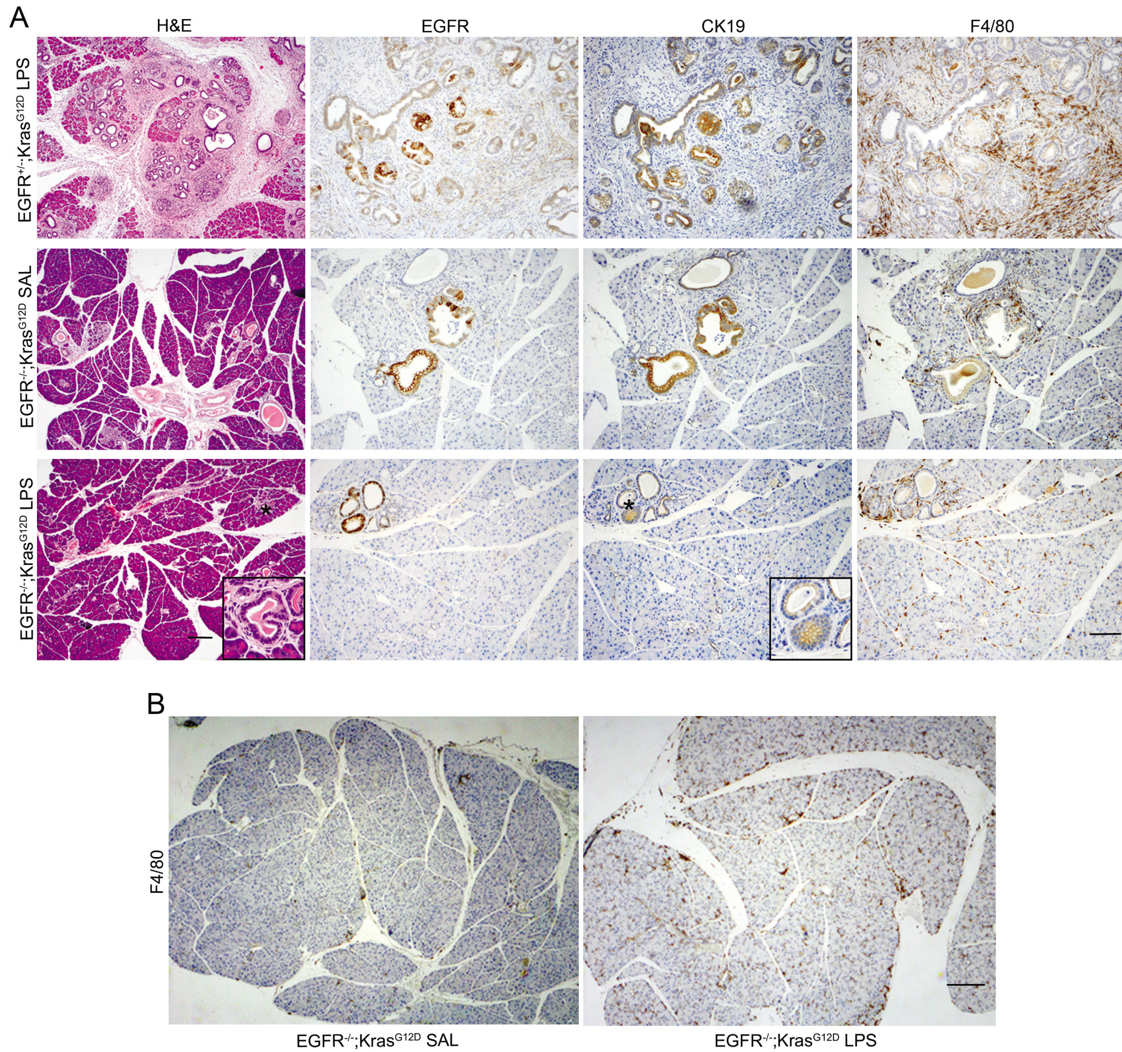


Figure 29. EGFR is required for LPS-induced tumorigenesis. (A) As compared to LPS-treated *EGFR*$^{\Delta/+}$*;Kras*^{G12D}*;Ptf1a*$^{Cre/+}$ mice, LPS-treated EGFR$^{\Delta/\Delta}$;Kras^{G12D};Ptf1a$^{Cre/+}$ mice develop significantly less tumor, comparable to that of saline treated mice (H&E). The tumor that does develop, identified by CK19, is EGFR positive, indicating incomplete recombination. Macrophage infiltration is reduced in LPS-treated EGFR$^{\Delta/\Delta}$ mice as compared to LPS-treated *EGFR*$^{\Delta/+}$mice, but (B) is elevated compared to saline treated animals, reflective of LPS treatment.

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