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Regulation of the development of pancreatic diseases by cell intrinsic and stromal influences

Author
Vander Laan - Greer, Renee L.

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Regulation of the development of pancreatic diseases by cell intrinsic and stromal influences

by

Renee L. Vander Laan - Greer

DISSERTATION

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Abstract

Diseases of the pancreas affect a large portion of the world's population; pancreatic ductal adenocarcinoma (PDA) is one of the leading causes of cancer deaths and type 2 diabetes is now reaching epidemic levels. Mouse models of pancreatic diseases have been instrumental in defining our understanding of the initiation and progression of these diseases. My studies examined various aspects of proper function of pancreatic cells and the development of disease, including the role of Numb in regulation of acinar cell dedifferentiation and metaplasia, and the role of stromal changes in the development of both PDA and regulation of glucose homeostasis.

Studies of mouse models of PDA have indicated that the most common cell type in the pancreas, the acinar cell is capable of giving rise to precursor lesions and PDA under the direction of activated Kras. However, even when Kras is activated in a majority of acinar cells, only a small percentage of cells go on to result in precursor lesions and disease in the absence of additional stresses such as additional mutations and/or inflammation. Here I demonstrate that Numb, a protein with a multitude of functions in regulation of signaling pathways, cell junction formation, endocytosis and asymmetric division, restrains acinar cell dedifferentiation and acinar to ductal metaplasia and promotes cell viability.

In addition to cell intrinsic changes, alterations in the stromal microenvironment are well documented in pancreatic disease. Specifically, changes in inflammatory cells have been well documented in PDA development, but how these different cell types influence PDA development is unclear. My work reveals that neutrophils are important

for propagating pancreatic damage and acinar dedifferentiation, but are not instrumental in the formation of initiating lesions that lead to PDA development.

Finally, the pancreatic mesenchyme is critical for pancreatic development, but how these cells contribute to tissue homeostasis is unknown. My studies demonstrate that pancreatic mesenchymal cells are found throughout the adult pancreas and that they are essential for maintenance of acinar cell organization and glucose homeostasis, partially through regulation of the extracellular matrix.

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CHAPTER 1

Introduction to the pancreas and diseases of the pancreas

Structure and function of the pancreas

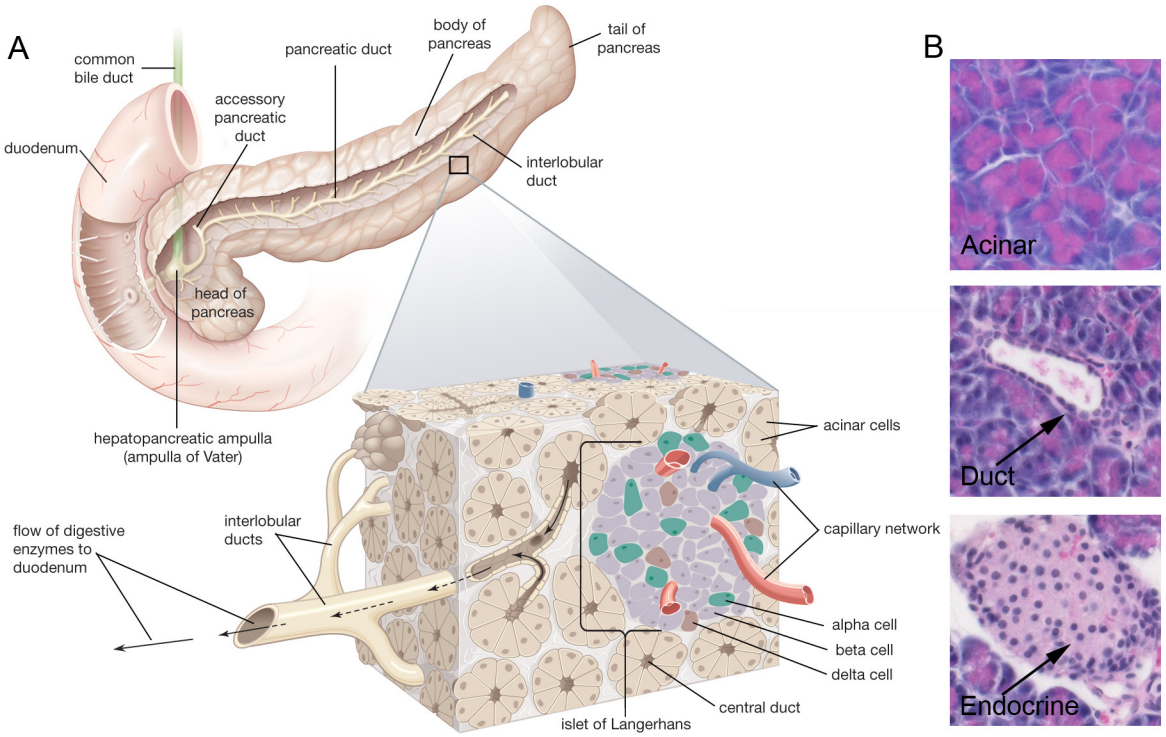
The pancreas is a lobular organ located in the retroperitoneal space, with the head adjacent to the duodenum and the tail extending to the spleen (Figure 1.1A) (Go, 1993). It has two primary functions: it is a part of the digestive tract, producing enzymes that are released into the duodenum to aid in digestion, and it is a component of the endocrine system, producing a variety of hormones that regulate blood sugar and metabolism. Corresponding to its two functions, there are two main epithelial compartments of the pancreas, called the exocrine and endocrine pancreas.

The exocrine pancreas

The exocrine pancreas is comprised of clusters of acinar cells connected to each other and subsequently to the duodenum through a ductal tree (Figure 1.1A). The acinar cell is the primary functional cell type of the exocrine pancreas. This cell derives its name from its organizational structure, as cells are arranged in an acinus around a central lumen (Figure 1.1B) (Go, 1993). Acini are separated from each other by a distinct basal lamina (Go, 1993). Acinar cells are highly productive cells with large numbers of mitochondria and extensive Golgi and endoplasmic reticulum networks. They also contain a high density of zymogen granules that house a variety of digestive enzymes such as amylase, trypsin, chymotrypsin and lipase and are polarized and undergo directed secretion of these enzymes into the lumen (Pictet, 1972). Acinar lumens connect into the ductal tree, which is generated by the other main cell type of the exocrine pancreas, the ductal cell (Figure 1.1B). These intercalated ducts connect each acinus to the larger ductal tree and eventually to the main pancreatic duct for secretion into the duodenum (Figure 1.1A) (Go, 1993; Pictet, 1972). The centroacinar cell is a small, spindle shaped cell that appears

Figure 1.1: Pancreas structure and organization

(A) The pancreas is located in the peritoneum and is arranged between the stomach, duodenum and spleen. It is comprised of enzyme producing acinar cells arranged in a rosette structure around a central lumen that connect to a ductal system created by duct cells. This ductal tree connects acini and transports digestive enzymes from the pancreas into the duodenum. The endocrine compartment is composed of multiple types of endocrine cells (α, β, δ, and pp) arranged in structures called islets of Langerhans, which are embedded in the exocrine tissue. (B) H&E staining demonstrating examples of the three classes of epithelial cells.



Adapted from Encyclopædia Britannica Online, 2003

to bridge the acinar and ductal border and has been identified as the exclusive domain of Notch activity in the adult pancreas based on expression of *Hes1*, a transcription factor expressed in response to activation of the Notch signaling pathway (Miyamoto, 2003). They are also marked by aldehyde dehydrogenase (ALDH1) activity and are enriched in their expression of *Sca1, Sdf1, c-Met* and *Nestin* (Rovira, 2010). Centroacinar cells have been proposed to be a stem-like cell in the pancreas due to the observations that they lie on the border of two distinctly different cell types, proliferate in response to various types of damage such as acinar damage due to caerulein pancreatitis (Strobel, 2007) and β-cell death due to streptazoticin (Nagasao, 2003) and are capable of forming self-renewing spheres in culture (Rovira, 2010), but whether or not they are a true stem cell population remains an area of active debate.

The endocrine pancreas

The endocrine pancreas is organized into structures called the islets of Langerhans, which are clusters of hormone-producing endocrine cells including the insulin-producing β-cells, glucagon-producing α-cells and somatastatin-producing δ cells (Figure 1.1A, B). These 3 major cell types within the islet and the hormones that they produce serve different functions in the sensing and regulation of glucose levels in the blood. Insulin is released in response to increased blood glucose and acts to induce the uptake of glucose from the blood by cells in the liver, muscle and fat and increases glucose conversion into glycogen for storage in the liver. In conditions of low blood glucose, the release of glucagon is induced which promotes glycogen metabolism to glucose in the liver to restore normoglycemia. Somatostatin can act locally to regulate the release of insulin and glucagon under varying conditions; it can also suppress the

secretion of gastrointestinal hormones. Additionally, pp-cells produce hormones that regulate food intake and digestion including ghrelin and pancreatic polypeptide.

Pancreatic specification and morphogenesis

Pancreas specification from endoderm

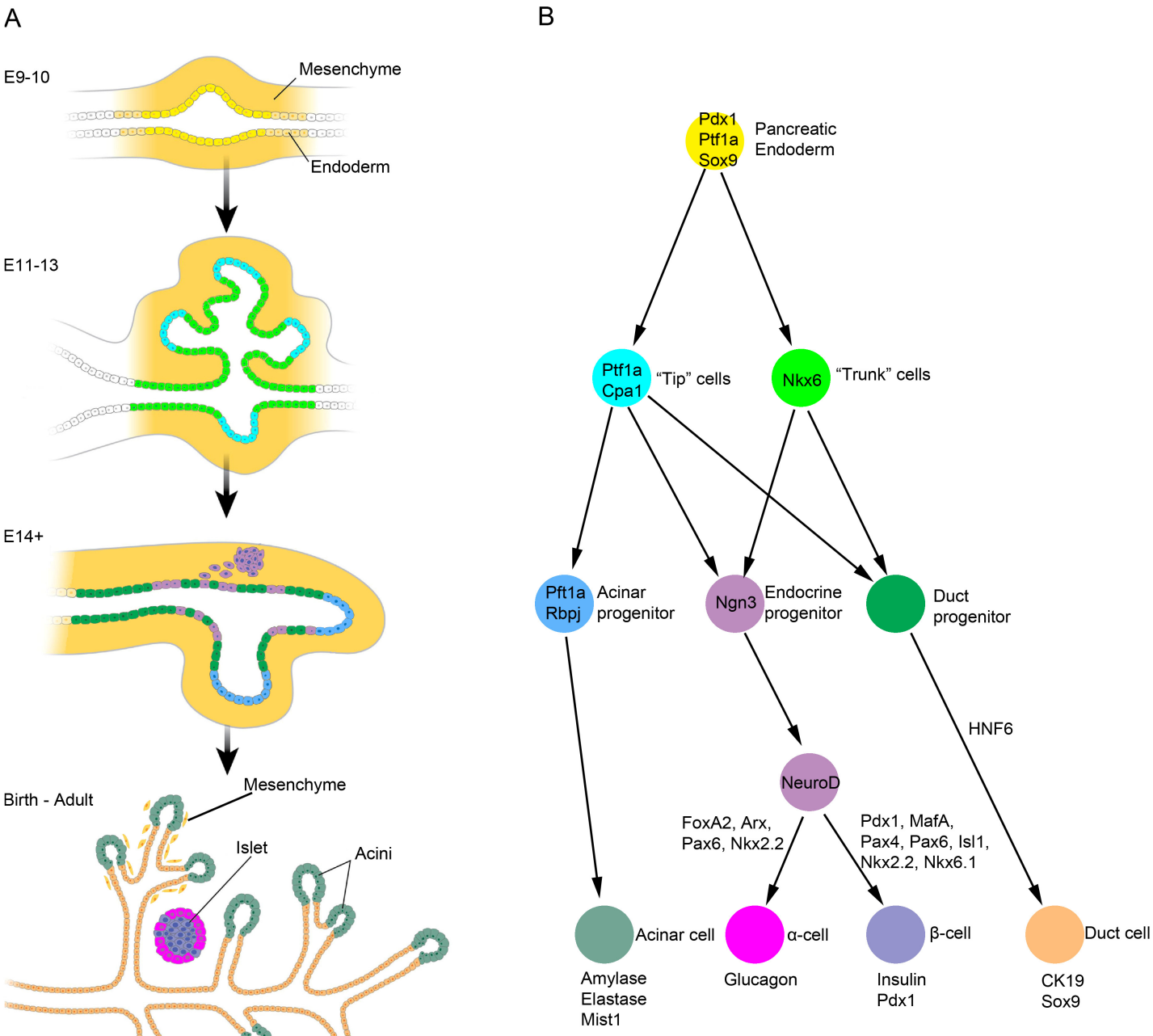
The pancreas is an endodermally derived organ whose initiation begins around embryonic day 9.5 (e9.5) in the mouse, corresponding to the 26th day of human gestation. 3 distinct pancreatic buds (1 dorsal and 2 ventral, one of which will eventually regress) originate from the foregut endoderm from e9.0-10 in response to signals from the surrounding tissues, first from the notochord and subsequently from the dorsal aorta and cardiac mesoderm. After e10.5, the pancreas expands and forms tubular structures to generate the characteristic branching organization of the pancreas (Villasenor, 2010). At the time of branching, cell specification begins to occur, with multipotent progenitor cells (MPCs) located at the "tip" of each branch, and bipotent progenitor cells, with duct and endocrine potential only, located in the "stalk" of the branch (Figure 1.2A) (Zhou, 2007). Between e12-13 (gestational days 37-42 in humans), gut rotation occurs that brings the dorsal and ventral buds in close proximity and fusion occurs (reviewed in Cano, 2007; Gittes, 2009; Jorgensen, 2007).

Molecular control of pancreas specification

Pancreas development requires orchestration of a complex network of signaling pathways, which will be discussed only briefly here. In the dorsal pancreas, retinoic acid (RA) from the somites is critical for the initial specification and expression of the pancreas specific transcription factor *Pdx1* (Martin, 2005) and fibroblast growth factors (FGFs) and activin from the notochord are required to suppress Sonic Hedgehog (Shh) in

Figure 1.2: Pancreatic development

(A) The pancreas buds from the endoderm at e9-10. A mesenchymal layer, represented by solid yellow, encapsulates the developing pancreas and instructs its growth and differentiation. The developing pancreas goes through a series of branching and morphology changes to eventually develop into the mature adult organ consisting of acinar tissue connected by a ductal network and embedded with islets of endocrine cells. (B) A complex transcriptional network controls pancreatic progenitor cell differentiation. At e11-13, "tip" cells have the capacity to give rise to all three epithelial lineages whereas "trunk" cells can give rise to only duct and endocrine cells. By e13-14, "tip" cells have become dedicated acinar progenitors. Ngn3+ cells located in the "trunk" cell population are the progenitor cells for the endocrine lineage.



Adapted from Cano et al 2007

the region of the pancreatic bud (Hebrok, 1998). Specification of the ventral pancreas is controlled by expression of FGFs and bone morphogenic proteins (BMPs) in the cardiac mesoderm and lateral plate mesoderm, respectively (reviewed in (Mastracci, 2012; Oliver-Krasinski, 2008; Puri, 2010). The activation of the Notch signaling pathway contributes to the maintenance of the undifferentiated state of progenitors and its repression is required for the continued differentiation of the endocrine lineage (Murtaugh, 2003). Finally, Wnt signaling has been implicated in numerous stages of pancreas development, although results are to some degree conflicting and the exact function(s) of Wnt in this process remain to be clearly elucidated (reviewed in Oliver-Krasinski, 2008; Puri, 2010).

Mesenchymal influence

Following initial specification of the pancreas from the endoderm, pancreatic contacts with previous sources of signaling molecules (notochord, dorsal aorta, cardiac mesoderm) are lost. At this time, the emerging pancreas becomes encapsulated with a developing and expanding mesenchymal layer (Figure 1.2A). This early mesenchyme will continue to provide critical signals to the developing pancreas. In early experiments, it was demonstrated that the pancreatic anlage cannot survive following physical separation from the mesenchyme. However, mesenchyme from alternative, non-pancreatic sources could also compensate, although with varying effects of final cellular differentiation (Golosow, 1962). Further work used a sophisticated system of mesenchymal cell ablation to show that the mesenchyme is required for pancreatic progenitor expansion throughout pancreas development (Landsman, 2011a). The specific signals the mesenchyme contributes to control pancreas development remains unclear,

but some signals have been implicated as being mesenchymal in origin, such as Fgf10. Fgf10 is critical for pancreatic epithelial expansion between e9.5-11.5 and the source of this growth factor at that time point is the pancreatic mesenchyme (Bhushan, 2001).

Cell fate decisions and pancreatic differentiation

The signaling pathways and transcription factors involved in cell fate decisions and differentiation of the epithelial cell types of the pancreas are quite well characterized. The pancreatic-duodenal homeobox domain protein *Pdx1* is one of the earliest transcription factors to mark the pancreatic lineage; its expression begins at e8.5 and it is required for pancreas differentiation (Offield, 1996). Subsequent expression of pancreas specific transcription factor 1, or *Ptf1a* (*p48*) defines the MPC domain (Kawaguchi, 2002), and the SRY-box containing protein 9, *Sox9,* is coexpressed with *Pdx1*, indicating that it marks a progenitor cell population (Figure 1.2B) (Seymour, 2007).

The first wave of primitive hormone expressing cells, called the primary transition, can be observed as early as e9.5, but these cells are not well characterized and make little to no contribution to the adult endocrine cell population. The bulk of adult endocrine cells will originate during the secondary transition beginning at e13.5 (reviewed in Gittes, 2009). Endocrine cells emerge from the *Neurogenin3* (*Ngn3*) positive population located in the trunk region of the developing pancreatic branches (Zhou, 2007). These cells delaminate from the newly forming ductal structure and begin to cluster (Figure 1.2A) (reviewed in Jorgensen, 2007). This process is accompanied by the expression of numerous more specific endocrine genes, many of which are known targets of *Ngn3*, including *Pax* and *Nkx* gene family members, *NeuroD*, *Arx* and *Islet1*, among others (Figure 1.2B). The expression of these genes will in turn determine the

ultimate differentiation of specific endocrine cell types (reviewed in Oliver-Krasinski, 2008; Puri, 2010), but these specifics will not be discussed further here.

The pathways and factors required for exocrine cell specification are less well understood than those involved in endocrine specification. By e14, MPC tip cells lose their multipotency and become acinar progenitor cells (Figure 1.2A,B) (Zhou, 2007). Although *Ptf1a* is expressed in the pancreatic progenitor cell pool for both the exocrine and endocrine lineages, some evidence suggests that levels of this transcription factor contribute to the exocrine versus endocrine cell fate decision. Recent studies indicate that higher levels of *Ptf1a* instruct the exocrine, and specifically acinar, lineage (Dong, 2008; Hesselson, 2011; Zhou, 2007). Additionally, the transcription factor *Mist1* and a balance of Notch and Wnt signaling are important for acinar cell differentiation and maturation, although these understanding these mechanisms requires further study (reviewed in Benitez, 2012).

Markers for the duct progenitor cell are also not well elucidated, but it is believed that they lie within the trunk structure of the branching epithelium, interspersed among the previously described *Ngn3*+ endocrine progenitors (Figure 1.2A) (Zhou, 2007). Recent studies have implicated the transcription factors *HNF1β* and *HNF6* in the establishment of ductal characteristics (Haumaitre, 2005; Pierreux, 2006; Zhang, 2009).

Diseases of the pancreas

Pancreatitis and pancreatic regeneration

Pancreatitis is an inflammatory condition of the pancreas. In humans it can be characterized as acute, which resolves itself, or chronic, which causes the acinar cell population to degrade gradually and be replaced by fibrotic tissue. Causes of pancreatitis

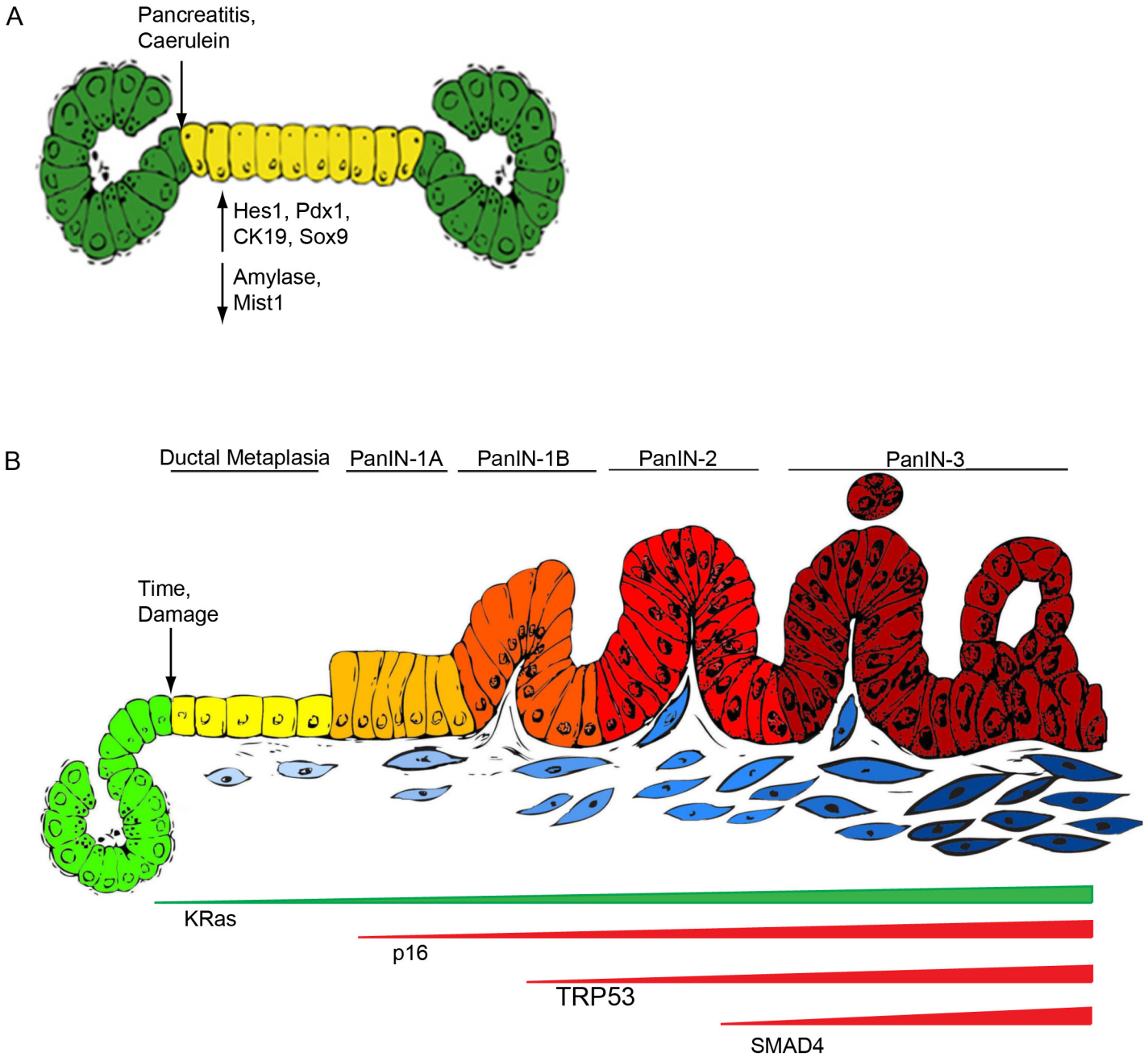
include gallstones, which can block the release of digestive enzymes into the duodenum, as well as alcohol consumption. Mutations in the trypsinogen gene that cause inappropriate activation of digestive enzymes are the primary cause of familial pancreatitis.

Acute pancreatitis can be modeled in rodents by supramaximal treatment with the cholecystokinin (CCK)-analogue caerulein. This treatment stimulates the release of digestive enzymes and activation of trypsin within the pancreas resulting in autodigestion and inflammation (Halangk, 2002). This model demonstrates the remarkable capacity for regeneration in the pancreas. Following caerulein treatment, there is a nearly complete loss of acinar cell function and severe disruption of pancreas organization, but within one week of treatment, acinar function and organization are restored (Figure 1.3A).

Closer analysis of the damaged acinar cells observed after caerulein treatment reveals the transient adoption of a duct-like organization. Accompanying this morphological change is the reduction of acinar markers, such as amylase and *Mist1* expression (Jensen, 2005) and an increased expression of duct markers such as *Sox9* and *CK19* (Morris, 2010a). Interestingly, these cells also activate signaling pathways typically associated with development such as Notch and Wnt signaling pathways (Jensen, 2005; Morris, 2010a; Siveke, 2008) and express transcription factors normally reserved for pancreatic progenitor cells such as *Pdx1* (Jensen, 2005). These cells are therefore often referred to as transiently dedifferentiated duct-like intermediates. The activation of Notch and Wnt signaling pathways is required for the proper regeneration of these dedifferentiated cells (Miyamoto, 2003; Morris, 2010a; Siveke, 2008).

Figure 1.3: Pancreatic regeneration and pancreatic ductal adenocarcinoma

(A) The acinar compartment of the pancreas has a remarkable capacity for regeneration. In response to damage, such as that induced by caerulein treatment, acinar cells dedifferentiate and assume a more ductal and progenitor-like state. They reduce expression of acinar markers and increase expression of duct markers and factors associated with pancreatic development. By seven days after treatment, mature acinar cells are nearly fully regenerated. (B) Pancreatic ductal adenocarcinoma progresses through a series of precursor lesions called pancreatic intraepithelial neoplasias (PanINs) of increasing levels of dysmorphia. A characteristic series of mutations is associated with increasing severity of these lesions as well as increasing amounts of desmoplasia.



Line drawings adapted from Hruban et al. 2000 by Jimmy Chen

The enzyme Elastase 1 (chymotrypsin-like elastase family member 1, *Cela1*) is specifically expressed in acinar cells during development and throughout adulthood and has been used to generate vital tools for analysis of adult acinar cells such as the tamoxifen inducible *Ela*Cre^{ERT2} (Desai, 2007; Stanger, 2005)**.** Lineage tracing using *Ela*Cre^{ERT2} demonstrates that preexisting acinar cells and not a dedicated stem cell population, that are responsible for repair and repopulation of the acinar compartment following caerulein pancreatitis damage (Desai, 2007; Strobel, 2007), indicating that this transiently dedifferentiated duct-like cell population serves as the facultive stem cell-like cell population. This evidence demonstrates the plasticity of the pancreatic acinar cell and further characterization of this state and regulation of acinar cell differentiation will provide valuable insights into transdifferentiation and metaplastic processes.

Pancreatic cancer

Pancreatic adenocarcinoma (PDA) is the most prevalent type of cancer of the pancreas and is one of the leading causes of cancer deaths in the United States. For those diagnosed with this disease, the outlook is bleak: diagnosis at the distant metaplastic stage has a 6-year survival of less than 2%. Even diagnosis at the early local stage has a 6-year survival of only 23% (American Cancer Society, 2011). A large portion of PDA cases are not detected until later stages, primarily due to a lack of symptoms with early disease as well as the inaccessibility of the pancreas for imaging and biopsy.

Other cancers of the pancreas include acinar cell carcinoma, solid pseudopapillary tumors and pancreatic neuroendocrine tumors (pNETs) such as insulinoma. These types of diseases will not be discussed in greater detail here and the remainder of this section will focus on PDA.

Epidemiology of pancreatic ductal adenocarcinoma

PDA was named as such due to the glandular nature of the lesions, which bear similarity to the pancreatic ducts. Because of this fact, PDA is often assumed to originate from the ducts. However, this is an active area of research, and much evidence suggests that the acinar cell is an important cell of origin for this disease (see section on "Cell of origin"). PDA does have some familial basis, although familial cases comprise only around 7-10% of diagnosed cases (Petersen, 2006). Genetic abnormalities associated with increased PDA risk include mutations in *CDKN2A* (*INK4A/ARF*), *BRCA2*, *PALB2* and *LKB1* (reviewed in Bardeesy, 2002; Vincent, 2011).

The most significant environmental risk factor observed to date is smoking, which doubles PDA risk and is associated with approximately 20% of PDA cases (Hassan, 2007; Iodice, 2008). Additionally, pancreatitis, an inflammatory condition of the pancreas, has been associated with an increased risk for PDA. Germline mutations in the trypsinogen gene *PRSS1* that result in inappropriate activation of the enzyme are associated with a 53-fold increased risk of development of PDA (Lowenfels, 1997; Whitcomb, 1996).

Diagnosis and treatment of pancreatic cancer

As previously discussed, detection is a major challenge in treatment of PDA, as the disease is often asymptomatic until late stages and the positioning of the pancreas makes imaging and biopsy of the pancreas technically difficult. Currently, computed tomography (CT) is the preferred imaging and diagnostic tool and is used to classify patients into resectable, locally advanced and metastatic disease stages to better determine course of treatment (Varadhachary, 2006), (reviewed in Vincent, 2011).

Additional tools such as fine-needle aspiration and endoscopic ultrasound can then be used to rule out alternate diagnoses such as chronic pancreatitis. Biomarkers for pancreatic cancer are currently not very beneficial for detection, but high levels of CA 19-9 can sometimes be useful in determination of resectability (Maithel, 2008).

In addition to late detection issues, a lack of effective treatment is another contributing factor to the poor prognosis of pancreatic cancer diagnosis. For resectable tumors, standard of care is adjuvant fluoruracil chemotherapy, and neoadjuvant chemoradiotherapy for patients determined to have borderline resectable tumors (reviewed in Vincent, 2011). A recent study has indicated that treatment with gemcitabine following surgery increases 5-year survival from 9.0% to 21% (Neuhaus, 2008). In cases of locally invasive disease, chemoradiotherapy combined followed by gemcitabine is standard; a second line treatment of capecitabine is recommended for metastatic disease. Unfortunately, while these treatments do provide some benefit for patients, they are only modest improvements. Additionally, a variety of treatment options, including erlotonib, bevacizumab, paclitaxel, PARP inhibitors and Hedgehog pathway inhibitors, among many others, are in various stages of clinical trials (reviewed in Vincent, 2011). Limited success with current therapy regimens demonstrates that new drug development is required and variable responses to current standard treatment suggest that a more customized and target specific approach would benefit treatment of pancreatic cancer.

Pathology of pancreatic cancer

Pancreatic ductal adenocarcinoma is proposed to proceed through a series of characteristic precursor lesions termed pancreatic intraepithelial neoplasias, or PanINs,

ranging in grade from 1-3 before progression on to invasive disease (Figure 1.3B) (reviewed in Feldman, 2008c, Hruban, 2000). PanIN-1a is the lowest grade lesion described and these lesions are present in a majority of people at late stages of life, unassociated with additional advanced lesions and invasive disease (Andea, 2003; Cubilla, 1976; Luttges, 1999). PanIN-1a are described as being flat ductal lesions composed of mucinous, columnar cells with basally localized nuclei. PanIN-1b are not significantly different than 1a, but have begun to develop more papillary characteristics. PanIN-2 present with some nuclear atypia, and are primarily mucinous and papillary in nature. PanIN-3, also called carcinoma in situ, are composed of highly atypical cells with multiple nuclear abnormalities and frequent mitoses. Additionally, cribiforming and budding off of cells into the lumen are frequently observed, but invasion through the basement membrane is absent (Figure 1.3B) (Hruban, 2004). It is important to note that while this entire range of lesion grades have been observed in association with PDA, there is currently no direct experimental evidence to indicate that any given cell or lesion must progress through these stages in a linear fashion in order to develop into PDA.

In addition, intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs) are alternative potential pathways to development of frank PDA. IPMNs are described as involving the pancreatic duct system, are often resectable and typically have a higher survival rate than PDA associated with PanINs (reviewed in Hruban, 2004). One contributing factor to this more favorable prognosis is a higher rate of early detection of IPMN lesions due to their large, cystic nature (reviewed in Vincent, 2011).

Molecular basis of pancreatic cancer

An accumulating number of stereotypical mutations occur in the progression of PanINs (Figure 1.3B). Typically, activating mutations of the GTPase domain of Kras are the earliest and most frequent mutations in PDA, occurring in nearly 100% of invasive PDAs (Rozenblum, 1997). Progressive mutations include inactivation of *CDKN2A,* a dual gene locus consisting of two tumor suppressors, p16 and p19 (*INK4A/ARF*), followed frequently by mutations in *TRP53,* generally associated with the PanIN-2 stage. Common later stage mutations, found frequently in PanIN-3 and invasive disease, include loss of *SMAD4* which is deleted in approximately 30% of PDA cases (Hahn, 1996) and *BRCA2* loss of heterozygosity for patients already carrying a germline mutation (Goggins, 2000). Finally, reactivation of telomerase activity and shortened telomeres are associated with invasive disease (Kobitsu, 1997; Suehara, 1997).

Mouse models of PDA and the question of the cell of origin

Many sophisticated animal models have been developed to reproduce the human disease and these models have been critical in our understanding of almost every aspect of PDA. Most broadly used is a model utilizing a conditional, oncogenic form of Kras (LSL-$Kras^{G12D}$) expressed under its endogenous promoter (Jackson, 2001) and activating its expression specifically in the pancreas by use of Cre recombinase under the control of the *Pdx1* promoter (*Pdx1-Cre*). This model faithfully recapitulates the full spectrum of PanIN lesions and results in the development of PDA in some mice at 1-2 years of age (Hingorani, 2003). Adding a deletion or mutation of one copy of p53 can dramatically increase the frequency and speed of development of PDA (Bardeesy, 2006a; Hingorani, 2005). Additionally, *Ptf1a-Cre* (Kawaguchi, 2002) is also commonly used with oncogenic Kras to model pancreatic cancer. These models have been used to test the

contributions of numerous genes and pathways to the development of PDA. Homozygous deletion of *p16* and *p19* in the context of the *Pdx1-Cre;Kras*^{G12D} mouse model changes the tumor type spectrum, resulting in an increase in the frequency of development of anaplastic tumor histology (Bardeesy, 2006a) and *SMAD4* deficiency in this model promotes development of IPMN lesions and PDA (Bardeesy, 2006b). These studies provide vast knowledge about the interplay of common mutations found in PDA and how timing and mutation combinations can affect the clinical presentation of the disease. However, since *Pdx1-Cre* is active in nearly every epithelial cell in the pancreas during development, this model is unable to provide insight into the originating cell compartment of this disease. Due to the ductal nature of PanINs and PDA, it was commonly assumed that they originate from ductal cells. However, many studies have now attempted to express activated Kras in specific cell compartments to directly address this question. This work has demonstrated the remarkable plasticity of epithelial cell types in the pancreas and suggests that multiple cell types can give rise to PanINs and PDA given the right circumstances and environment.

Duct cells remain poorly categorized, with few specific markers to allow for analysis and specific gene alteration. Cytokeratin 19 (*Krt19*, also called *CK19*) is expressed in duct cells, but not acinar and endocrine cells (Brembeck, 2001). Initial studies on duct cell contribution to PDA formation utilized an active form of Kras (Kras^{G12V}) expressed directly under the control of the *Krt19* promoter. This resulted in the development of ductal hyperplasia, but no advanced lesions or tumor formation were reported (Brembeck, 2003). Similarly, activation of Kras with an inducible *Krt19-Cre* (*CK19*^{CreERT}) leads to the formation of PanIN-1 lesions, but progression was not observed

in the time frame of this study and these mice suffer from the development of other significant diseases, including lung adenomas and oral papillomas (Ray, 2011). Use of the newly developed inducible Cre lines using the duct specific genes, Hepatocyte nuclear factor 1 homeobox B (*HNF1βCre*^{ERT2}) (Solar, 2009) and Carbonic anhydrase II (*CAII-CreER*^{TM}) (Inada, 2008) may help to clarify the ability of duct cells to give rise to PDA.

Even insulin positive cells have been suggested to have the capability to give rise to PanIN lesions given the right environment. Activation of Kras with *RIPCreER*^{TM}, a transgene based on the rat insulin promoter, does not result in lesion formation under steady state conditions, but PanINs are observed following inflammatory stress due to pancreatitis (Gidekel Friedlander, 2009). However, due to a low level of expression of the *RIPCreER*^{TM} in acinar cells, this study requires further validation (Blaine, 2010).

The acinar cell has been an area of great focus, and it is now clear that this cell type is a major player in the development of PDA. Acinar to ductal metaplasia (ADM) has been frequently documented as a histological feature often observed in conjunction with PDA. However, this provides no evidence of the ability of these ADM lesions to go on to form PanIN lesions and PDA. Acinar cell-specific Cre lines have now provided significant evidence that this metaplasia can in fact progress on to PanINs and invasive disease. Activation of Kras in the developing acinar cell population by the use of *Mist1-Kras*^{G12D} induces the formation of rapid and severe pancreatic cancer of mixed differentiation status, indicating that, at least when Kras is activated during development, acinar cells have the capability to give rise to pancreatic cancer (Tuveson, 2006). It is important to note that in this study, because Kras was not expressed under its endogenous

promoter, the level of expression may be different than those that occur in many of the other models described. Additionally, levels of *Mist1* may be reduced in this model, which may complicate interpretation, as knockout of *Mist1* disrupts acinar polarity and results in mild acinar to ductal metaplasia (Pin, 2001). Either or both of these factors could help to account for the rapidity of disease development and mixed histology.

Activation of Kras specifically in adult acinar cells provides further evidence that acinar cells contribute to the formation of PDA. Guerra et al used a tetracycline inducible system (K-Ras$^{+/LSLG12Vgeo}$;Elas-tTA/tetO-Cre) to activate Kras specifically in acinar cells. When expression of Kras is induced during late embryogenesis, development of PanIN lesions characterized as acinar to ductal metaplasia occurs at 1-3 months of age (Guerra, 2007). However, adult acinar cells appeared to be refractory to any Kras induced acinar to ductal metaplasia without additional injury. Addition of the inflammatory insult induced by caerulein pancreatitis induces rapid formation of mucinous ductal lesions, PanINs and eventually invasive disease that originates from adult acinar cells (Guerra, 2007). These findings are confirmed by the use of the *ElaCre*^{ETR2} to express Kras^{G12D} in adult acinar cells, which results in the development of significant numbers of PanINs following caerulein treatment and a small number of PanIN lesions even in the absence of caerulein pancreatitis (De La, 2008).

Taken together, the most convincing evidence points to the acinar cell as the likely cell of origin of pancreatic ductal adenocarcinoma, although ductal cells and β-cells both appear to have the capacity to form PanINs given the right set of conditions. These findings also reinforce the dramatic plasticity of the acinar cell and its capacity not only for regeneration but also for transformation and metaplasia. While it is clear that

inflammation and pancreatitis creates a permissive environment for metaplasia, many questions remain about what the exact nature of this environment is and what regulates acinar differentiation state.

Key signaling pathways in pancreatic cancer

Mouse models that faithfully replicate the human disease have allowed for identification of pathways that are involved in pancreatic cancer. Interestingly, pancreatic cancer displays activation of many signaling pathways that have also been shown to be critical to pancreatic development as well as pancreatic regeneration. This again underlies the strong ties between the plasticity of the acinar cell state and transformation.

Notch signaling plays many roles in the pancreas. As previously discussed, Notch signaling is important for maintaining the undifferentiated state of pancreatic progenitor cells during development, its inhibition is required for endocrine differentiation and it is required for acinar regeneration. It is therefore logical that Notch could be involved in ADM and PDA development. Indeed, by using a Notch gain of function allele, it was shown that Notch and Kras cooperate to promote metaplasia (De La, 2008). Additionally, Notch activation is found in a high percentage of human tumors and its inhibition by gamma secretase inhibitors (Plentz, 2009), or by deletion of the Notch2 receptor (Mazur, 2010) inhibits the development of carcinoma in a mouse model of PDA. Therefore, Notch is required both for metaplasia and progression of PDA.

Hedgehog (Hh) signaling plays a complicated role in the pancreas and in pancreatic cancer. Hh ligand expression is detected in around 75% of human PDA samples and expression can be found even in early PanINs and throughout progression in both mouse and human (Hingorani, 2005; Morton, 2007; Thayer, 2003). Because both

Hh ligand and activation of the Hh pathway are observed in tumor cells, it was assumed that the ligand was acting in an autocrine fashion. Indeed, treatment of xenograft tumors and mouse models with inhibitors of the pathway does decrease tumor burden (Feldmann, 2007; Feldmann, 2008a; Feldmann, 2008b; Olive, 2009). However, when the pathway activation is specifically inhibited by deletion of the Hh receptor, Smoothened (SMO), no significant effect on tumor development was observed (Nolan-Stevaux, 2009). Evidence now points to the tumor fibroblasts as the primary target for Hh ligand, with the pathway being activated in a ligand independent fashion, likely directly through Kras, in the tumor cells themselves (Tian, 2009; Yauch, 2008). The role of Hh signaling in the stroma will be discussed in more detail in the next section on tumor microenvironment. Although it is now clear that Hh signaling plays an important role in promotion of PDA, it is not sufficient to drive tumor formation alone, as constitutive activation of the pathway in pancreatic epithelium via a dominant active GLI2 transgene does not result in PDA, but tumors of an undifferentiated nature, with or without the presence of activated Kras (Pasca di Magliano, 2006).

Wnt/β-catenin signaling is yet another common developmental signaling pathway that has come to be recognized for its importance in PDA. Unlike in other types of cancer where Wnt signaling is known to be involved, such as colorectal carcinoma, mutations in negative regulators of the pathway such as APC are not common in PDA (Gerdes, 1999; Seymour, 1994) and activation of the pathway by deletion of APC or forced stabilization of β-catenin is insufficient to drive PDA formation in the mouse (Heiser, 2008; Strom, 2007). Although Wnt is not universally activated in PDA, cytoplasmic and nuclear

expression of β-catenin, indicating activation of the pathway, can be observed in PanINs and PDA (Morris, 2010a; Pasca di Magliano, 2007; Zeng, 2006).

β-catenin appears to play different roles in PDA development at different disease stages. Work from our lab shows that while β-catenin is required for acinar regeneration in response to caerulein treatment, enforced activation of the pathway blocks acinar to ductal metaplasia (Morris, 2010a). This finding is in agreement with the fact that activation of β-catenin in the context of $Kras^{G12D}$ results in a distinctly different tumor called solid pseudopapillary neoplasm (SPN) (Heiser, 2008), indicating that activation of the Wnt pathway prevents the adoption of the correct differentiation state for development of the PanIN/PDA lineage. However although activation of Wnt signaling appears to be preventative to PanIN development, it is capable of supporting PDA tumor cell proliferation and survival (Pasca di Magliano, 2007).

The tumor microenvironment in PDA

A strong desmoplastic response, including infiltration and expansion of fibroblasts and immune cells and significant matrix deposition, is one of the defining characteristics of pancreatic ductal adenocarcinoma (Chu, 2007). It has been hypothesized that the dense stromal deposition contributes to the failure of current therapies because it is poorly vascularized and may therefore block the access of the drug to the tumor cells.

Even at early stages of the disease, the importance of the microenvironmental contribution has been documented. The role of the inflammatory microenvironment induced by pancreatitis clearly contributes to acinar to ductal metaplasia and PanIN formation, indicating that immune cells likely contribute factors to promote this process

(Carriere, 2009; Guerra, 2007; Morris, 2010a). Additionally, proliferation and activation of the mesenchymal compartment, including pancreatic stellate cells (PSCs) and infiltration of immune cells can be observed even around the earliest lesions. Conversely, isolated PSCs are capable of stimulating the growth and invasion of tumor cells, providing evidence for a mutually supportive relationship between the tumor cell and the stroma (Hwang, 2008; Vonlaufen, 2008).

Much cross talk occurs between the tumor cells and the stroma, involving transforming growth factor β (TGF-β), hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and matrix metalloproteinases (MMPs) among many others (reviewed in Neesse, 2010). One notable pathway that has repeatedly been shown to regulate desmoplasia is the Hedgehog signaling pathway. As discussed previously, high levels of Hh ligand are observed in precancerous lesions and PDA, but the tumor cells themselves do not appear to respond to ligand (Tian, 2009; Yauch, 2008). However, cancer associated fibroblasts are highly responsive to ligand mediated Hh pathway activation, which induces the proliferation of pancreatic stellate cells (Bailey, 2008). Activation of Hh in fibroblasts in turn promotes the growth of tumor cells, as abrogation of Hh signaling in fibroblasts by treatment with Hh antagonists or deletion of the receptor Smoothened delays growth of tumor xenografts (Yauch, 2008). Excitingly, recent evidence indicates the dependence of CAFs may be taken advantage of to improve drug treatment. Olive et al treated PDA mice (both human tumor xenografts as well as the *Pdx1Cre*;*Kras*^{G12D} mouse model) with a synthetic Smoothened inhibitor combined with the standard chemotherapy agent for PDA, gemcitabine. Following combination treatment, extent of desmoplasia was reduced and vascularity was increased compared to

gemcitabine alone. Most importantly, survival was increased compared to gemcitabine alone (Olive, 2009). These studies demonstrate that the PDA stroma both directly supports tumor growth and indirectly inhibits drug access to the tumor and therefore it is a viable target for therapy.

Diabetes Mellitus

Diabetes now affects 25.8 million people in the United States, 8.3% of the population, and nearly 80 million people have been documented to be in a prediabetic state. It is the 7th leading cause of death in the United States. In addition to the loss to life, it is also one of the most expensive diseases, with 174 billion dollars being spent on diagnosed diabetes patients in 2007 (Centers for Disease Contrl and Prevention, 2011). Diabetes is the generic term for multiple types of specific disorders of blood glucose regulation. The primary types are type 1 diabetes mellitus (T1DM), an autoimmune disorder that results in loss of β-cells, and type 2 diabetes mellitus (T2DM), a complex metabolic disorder influences by lifestyle, environment and genetic factors.

β-cell function and maturation

Insulin secretion by β-cells is now fairly well understood and is dictated by membrane potential due to calcium influx into the cell. Glucose in the blood is taken up by pancreatic β-cells via the glucose transporter GLUT1 in humans (Glut2 in rodents) and is metabolized to generate ATP. This induces the closure of ATP sensitive potassium channels, which in turn allows the opening of voltage-gated calcium channels. The influx of calcium then stimulates the exocytosis of insulin granules into the bloodstream.

Maintenance of β-cell identity is critical for their continued function. Aberrant activation of multiple signaling pathways such as Hedgehog signaling and hypoxia

signaling pathways can induce dedifferentiation of β-cells as marked by a decrease in transcription factors known to be associated with maturity and a down-regulation of critical glucose sensing and insulin secretion components (Landsman, 2011b; Puri, 2009). It is still unclear what contribution β-cell dedifferentiation makes to the development of diabetes in humans.

Type 1 Diabetes Mellitus

T1DM is a chronic autoimmune disease that is typically diagnosed early in life; it is also commonly known as juvenile diabetes. It is believed to result from a complex mix of genetic and environmental factors (Knip, 2005). It eventually results in the near complete destruction of β-cells, but because patients remain insulin tolerant, blood glucose can typically be well controlled with insulin injections. Additionally, attempts have been made to partially prevent β-cell destruction with immunosuppression. This work is still ongoing and long-term effects are not yet clear (reviewed in Bluestone, 2010).

Type 2 Diabetes Mellitus

T2DM, often referred to as adult-onset or maturity-onset diabetes, develops later in life compared to T1DM. It is clear that there are many environmental and lifestyle factors involved, the most apparent being diet and obesity, although increased body fact is not a prerequisite to the disease. T2DM is primarily a disease of reduced β-cell function. Loss of β-cell function is compounded by peripheral insulin resistance, where the secondary organs such as the liver and muscle have reduced ability to take up glucose from the blood stream in response to insulin secretion. Obesity can induce insulin resistance, but additional hits on the function of β-cells are likely required to lead to the

development of full-blown diabetes. Global gene analysis studies have identified a large number of single nucleotide polymorphisms (SNPs) that are associated with development of T2DM, but much work is still needed in this field, as the functional contribution to diabetes development of a majority of these mutations is still unclear (reviewed in Ashcroft, 2012).

Monoallelic Disease

While both T1DM and T2DM develop due to complex interactions of multiple genes and environmental, an increasing number of single gene mutations have also discovered to lead to diabetes. This category of disease is termed maturity-onset of diabetes in the young, or MODY and it encompasses a wide range of disease presentations (reviewed in Ashcroft, 2012). Some genes identified as "MODYs" have obvious function in β-cells, such as components of the glucose sensing and secretion machinery Glut2, Kir6.12, Sur1 and even insulin itself (Gloyn, 2004; Hattersley, 2005; Stoy, 2010; Yoo, 2002) and transcription factors important for β-cell development such as Pdx1 (Stoffers, 1997). These types of genes typically have a very early onset of disease and may present with other complications such as developmental delay and pancreatic agenesis. Other known MODYs include transcription factors known to be important in maintenance of β-cell function, such as HNF1α, HNF1β and HNF4α (Servitja, 2004), which tend to have a later and more gradual onset. These studies of MODY genes can inform our knowledge of β-cell function and possibly provide a better understanding the complicated mix of genetic and environmental factors involved in T2DM development.

CHAPTER 2

Numb regulates mouse acinar cell dedifferentiation and survival during pancreatic damage and acinar to ductal metaplasia

Summary

Background and Aims: Pancreatic ductal adenocarcinoma (PDA) is a leading cause of cancer deaths. The pancreatic acinar cell has been shown to be capable of giving rise to pancreatic intraepithelial neoplasia (PanIN), the most common PDA precursor, through the process of acinar to ductal metaplasia (ADM). However, even when Kras is activated in a majority of acinar cells, ADM and development of PanINs is inefficient in the absence of additional stresses. The factors that restrain the ability of Kras to induce metaplasia are currently unclear. **Methods:** Here we utilize mouse models of pancreatic regeneration and PDA combined with loss-of-function alleles of *Numb*, a multifunctional protein that regulates multiple signaling pathways through protein degradation and regulation of localization, internalization and recycling, to determine its role in pancreatic regeneration and ADM. **Results:** Deletion of Numb promotes dedifferentiation of acinar cells in response to injury upon treatment with the cholecystokinin analog caerulein and interferes with the acinar cell regeneration. Numb regulates signaling pathways in acinar cells during caerulein-induced pancreatitis, including Notch and cell adhesion signaling. Elimination of Numb also accelerates and destabilizes acinar to ductal metaplasia in the context of oncogenic Kras. **Conclusions:** Our results reveal Numb as a critical regulator of acinar cell differentiation and stability. Elimination of Numb reveals a propensity of dedifferentiated acinar cells to undergo apoptosis upon pancreatitis injury, a process that is not efficiently mitigated by oncogenic Kras. Summarily, our findings identify Numb as a novel and important regulator of acinar dedifferentiation and ADM.

Introduction

Pancreatic ductal adenocarcinoma (PDA) is one of the most lethal of human malignancies. Pathogenesis is believed to occur through the progression of precursor lesions, the most well described of which are pancreatic intraepithelial neoplasias (PanINs) (Feldmann, 2008c). Understanding the mechanisms of formation and progression of these lesions is critical for development of early detection techniques, preventative measures and therapeutic intervention.

Mutations in the small GTPase Kras are nearly universal in human PDA (Almoguera, 1988), and are often detected in early PanIN lesions. The notion that Kras is the primary oncogene in PDA is supported by mouse models in which the expression of an activated form of Kras in the pancreas under the endogenous Kras promoter ($Kras^{G12D}$) results in the development of the full spectrum of precursor PanIN lesions and PDA (Hingorani, 2003). Although PanINs and PDA appear ductal in nature and express markers of pancreatic ductal cells, prior studies have shown that a variety of cell types in the pancreas can give rise to PanIN lesions. In particular, acinar cells can undergo a reprogramming process termed acinar-to-ductal metaplasia (ADM), to form metaplastic duct and PanIN lesions (De La, 2008; Guerra, 2007; Habbe, 2008; Morris, 2010a; Zhu, 2007). While oncogenic Kras is sufficient for transformation of adult acinar cells, this is a stochastic process as some cells retain their acinar fate and resist rapid dedifferentiation (Habbe, 2008; Hingorani, 2003). This suggests that other changes are required in order to generate a permissive environment for Kras to exert its effects. For example, environmental insults, including pancreatitis and inflammation have been shown to

promote PanINs in mice expressing mutant Kras (Carriere, 2009; Guerra, 2007; Morris, 2010a).

Caerulein-induced pancreatitis has been used as a tool to study the plasticity of the adult acinar cell. In response to acute doses of caerulein, mature acinar cells change their differentiation state resulting in decreased expression of acinar markers, inappropriate expression of ductal markers, a transition to a duct-like morphology (Habbe, 2008; Strobel, 2007), and re-expression of factors associated with embryonic pancreas development (Jensen, 2005; Siveke, 2008). Additionally, previous studies have demonstrated that acinar cells undergo dramatic changes in cell adhesion and morphology upon caerulein treatment, including dissociation and subsequent reassembly of E-cadherin and ß-catenin (Lerch, 1997) at the adherens junction. Integrin signaling also maintains acinar differentiation. For example, ß1 integrin is required for the maintenance of acinar cell polarity and in its absence, acinar cells become disorganized, and have increased sensitivity to caerulein treatment (Bombardelli, 2010).

In wild type mice, the dedifferentiated acinar state is transient and reversible as demonstrated by lineage tracing experiments in which damaged acinar cells re-differentiate into fully functional acinar cells (Strobel, 2007). However, increasing evidence suggests that this transient cell state is permissive for the disruptive activity of oncogenic Kras that diverts the regenerative process towards permanent acinar to ductal metaplasia and the subsequent formation of PanINs (Morris, 2010a). De-differentiation induces re-activation of embryonic signaling pathways and markers of embryonic progenitor cells, including Pdx1 (Miyatsuka, 2006) and Hes1 (Miyamoto, 2003), a downstream mediator of the Notch signaling cascade. Expression of these factors is

sustained throughout neoplasia and cancer formation, indicating that they play important roles in disease formation and progression. Thus, determining the mechanisms that regulate acinar cell dedifferentiation can provide insights into the nature of the permissive environment that allows for full activation of oncogenic Kras and initiation and progression of PDA.

The Numb protein plays important roles in cell division, progenitor cell maintenance (Petersen, 2002) and regulating cell fate decisions (Petersen, 2004; Zhong, 1996), in large part through its negative regulation of Notch (McGill, 2003; McGill, 2009). Numb also regulates a number of other pathways, including Wnt through degradation and regulation of localization of β-catenin (Kwon, 2011; Wang, 2009) and Hedgehog signaling through the degradation of Gli1 (Di Marcotullio, 2006) and also promotes p53 stabilization through the suppression of Mdm2 (Colaluca, 2008). Furthermore, Numb is capable of interacting with other proteins that influence epithelial cell polarity such as cadherins and catenins to regulate adherens junctions (Rasin, 2007; Wang, 2009) and localization and recycling of integrins (Nishimura, 2007).

Given its known functions in regulating cell-cell junctions, integrins and the activity of embryonic signaling pathways, we speculated that Numb may be involved in controlling acinar cell fate and morphology during caerulein-induced pancreatitis. By using conditional deletion of Numb (Zhong, 2000) combined with mouse models for pancreatic regeneration, acinar to ductal metaplasia and PanIN initiation, we have uncovered a role for Numb in controlling these processes. We show that Numb restrains acinar cell dedifferentiation, as its elimination results in rapid and extensive duct-like cell formation in response to caerulein treatment. Numb further promotes cell survival during

pancreatic regeneration and massive pancreatic atrophy occurs in its absence. Interestingly, Numb also restrains acinar-ductal metaplasia promoted by oncogenic Kras. Summarily, our findings point to Numb as a critical regulator of the acinar differentiation state upon pancreatitis injury.

Results

Numb is required for pancreatic regeneration

We first determined the expression pattern of Numb in pancreatic epithelial cells. Given a lack of antibodies suitable for immunostaining in pancreas, we developed a FACS isolation procedure that allows enrichment for acinar and duct cells from adult murine pancreas (Morris, in preparation). This method yields enrichment of acinar markers into the CD49f+;CD133- negative population and duct markers in the CD49f+;CD133+ population (Supplementary Figure A1.1B). Additionally, markers of active Notch signaling are enriched in the duct population (Supplementary Figure A1.1C), indicating that centroacinar cells are sorted into this population.

Numb, but not the highly related protein Numb-Like, was detected in isolated acinar cells by quantitative PCR (Supplementary Figure A1.2). In order to determine whether Numb plays a role in controlling the extent of pancreatic damage and acinar regeneration, we we eliminated *Numb* in pancreas epithelium by crossing conditional *Numb* mutant mice (*Numb*$^{f/f}$) with a pancreas Cre line (Zhong, 2000). We have previously demonstrated that the *p48Cre* line leads to specific recombination in the majority of acinar cells and a subset of duct cells during pancreatic development (Heiser, 2008). At 6 weeks of age, *p48Cre;Numb*$^{f/f}$ mice showed no pancreatic abnormalities in pancreas size, morphology and histology. Expression of cell type specific markers E-cadherin, Amylase and CK19 was unaffected (Supplementary Figure A1.2) while QT-PCR analysis from total pancreas RNA confirmed that Numb transcript levels were dramatically reduced. The remaining transcript observed in *p48Cre;Numb*$^{f/f}$ tissue likely originates from subsets of exocrine or endocrine cells, as well as non-epithelial cells within the pancreas that are

not targeted by *p48Cre*. Western blot on total pancreatic lysate confirmed that protein levels were also dramatically reduced in the pancreas (Supplementary Figure A1.2).

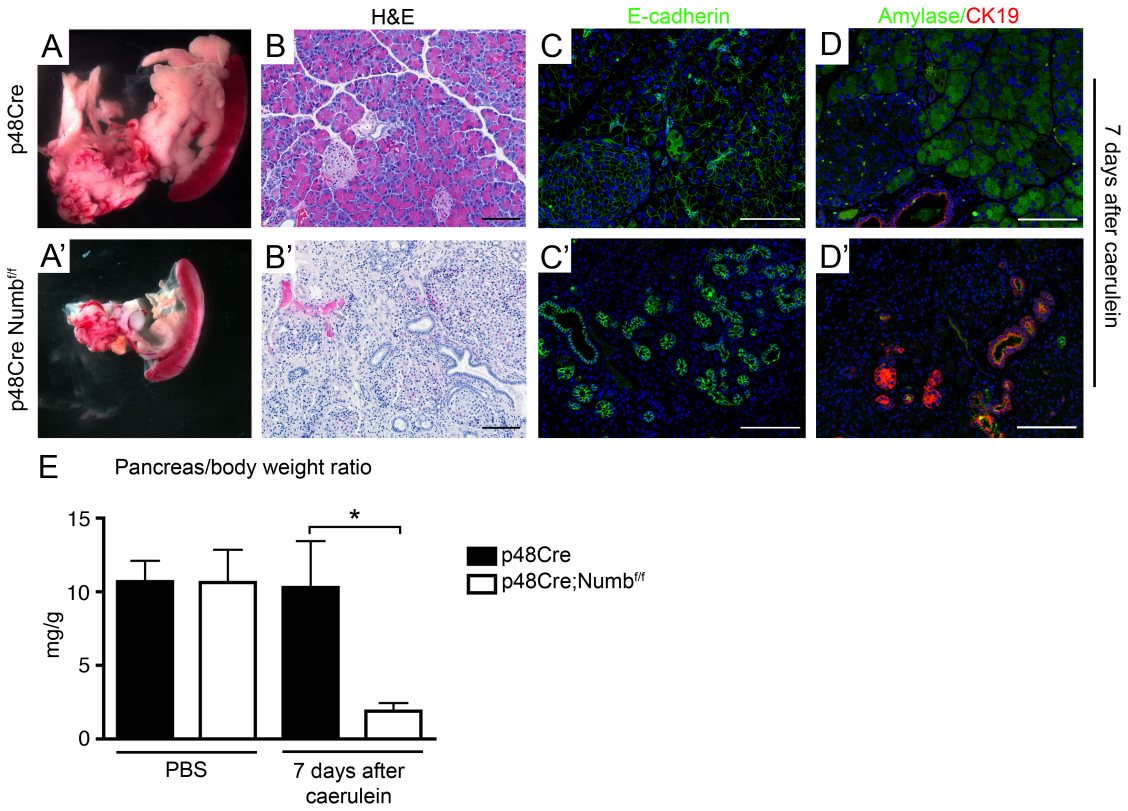
When pancreatitis is induced in the adult pancreas by treatment with caerulein, dedifferentiation of acinar cells is transient and rapidly resolved. As expected, 7 days post caerulein treatment, regeneration and repair of damage was nearly complete in *p48Cre* controls as indicated by morphology, histology and immunostaining of E-cadherin and Amylase/CK19 (Figure 2.1*A-D*). In contrast, massive pancreatic atrophy was observed at this time point in mice lacking Numb, resulting in severe reduction in the pancreas mass to body ratio (Figure 1*E*). The remaining pancreas tissue contained considerable non-epithelial tissue, duct structures and few amylase-expressing acinar cells (Figure 2.1*A'-D'*). Pancreas mass to body weight ratio was severely reduced (Figure 2.1*E*). Therefore, Numb is required for regeneration of acinar tissue in response to caerulein-induced pancreatitis.

Numb restrains acinar cell dedifferentiation

Numb has previously been shown to inhibit the activity of several signaling pathways, including Notch signaling. The Notch pathway controls acinar dedifferentiation and we therefore tested whether Numb elimination affects the early stages following acinar injury. *p48Cre* and *p48Cre;Numb$^{f/f}$* mice were injected with caerulein and pancreatic tissue was examined shortly after cessation of treatment (days 1 and 2 post injection). At these time points, *p48Cre* controls showed the expected early signs of acinar dedifferentiation, including some expansion of the central lumen in acinar clusters, changes in the expression pattern of Amylase, and expression of low levels of

Figure 2.1: Acinar cells lacking Numb are incapable of regeneration and have reduced metaplastic capacity

Seven days after caerulein, acinar regeneration is perturbed in *p48Cre;Numb*$^{f/f}$ mice. (*A*) Gross morphology images demonstrates smaller pancreas size of *p48Cre;Numb*$^{f/f}$, (*B*) H&E staining reveals lack of acinar tissue. (*C*) Immunostaining for E-cadherin (green), counterstained with DAPI (blue) shows reduction of E-cadherin positive cells, most of which have a duct-like organization. (*D*) Immunostaining for Amylase (green) and CK19 (red), counterstained with DAPI (blue) demonstrates an absence of amylase expressing acinar cells and the presence of some highly CK19 positive ductal structures in *p48Cre;Numb*$^{f/f}$. Scale bars represent 100μm. (E) Pancreas mass:body weight ratio of PBS and caerulein treated mice at day 7. * $P<0.05$, N=3-5.


H&E
E-cadherin
Amylase/CK19
A
B
C
D
p48Cre
A'
B'
C'
D'
p48Cre Numb f/f
7 days after caerulein
E
Pancreas/body weight ratio
15
10
5
0
mg/g
*
p48Cre
p48Cre;Numb f/f
PBS
7 days after caerulein

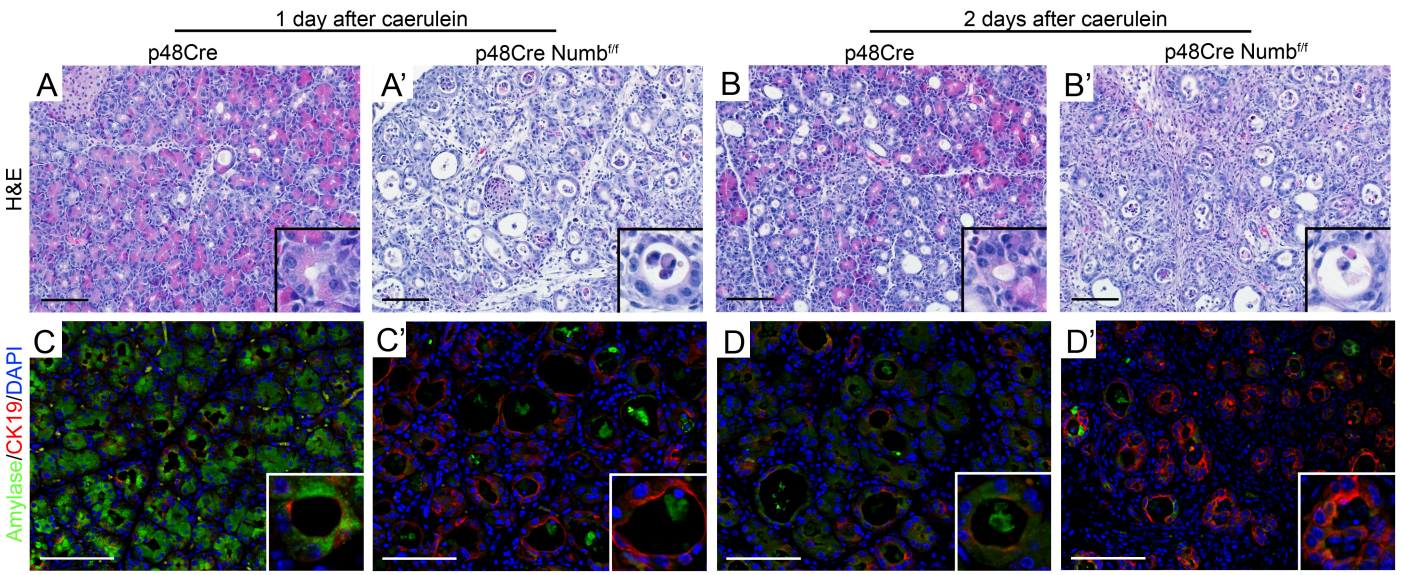
CK19, a marker for duct cells (Figure 2.2*A,C*). *p48Cre;Numb$^{f/f}$* pancreata presented with similar, but more extensive acinar dedifferentiation, with increased lumen expansion, more dramatic loss of Amylase and more frequent and higher expression of CK19 as compared to controls (Figure 2.2*A',C'*). Significantly increased activation of the Notch pathway was also observed in *p48Cre;Numb$^{f/f}$* pancreata, as indicated by elevated transcript levels of the Notch targets Hes1, Hey1, Hey2 and HeyL (Figure 2.2*E*). Additionally, *p48Cre;Numb$^{f/f}$* pancreata showed more apparent stromal expansion and immune cell infiltration, as measured by increased presence of α-Smooth Muscle Actin and CD45 positive cells, respectively (Supplementary Figure A1.3). Notably, Numb ablation did not prevent proliferation of dedifferentiated acinar cells at these time points (Supplementary Figure A1.4). These data indicate that Numb elimination makes acinar cells more susceptible to pancreatitis cues that initiate dedifferentiation events.

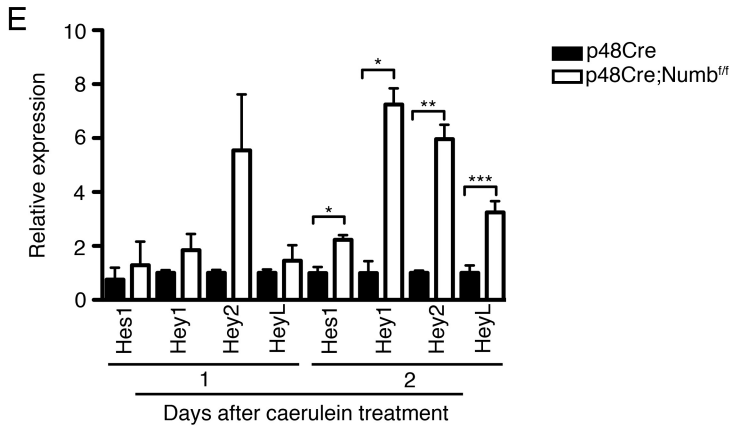
Numb maintains cell adhesion in response to pancreatic damage

In addition to its modulating activities on embryonic signaling pathways, Numb has been also shown to affect cell adhesion through regulation of integrins, cadherins and catenins (Nishimura, 2007; Rasin, 2007; Wang, 2009). Notably, deletion of β1 integrin in the pancreas also results in loss of polarity, misdirection of enzyme secretion, increased sensitivity to caerulein-induced damage, and progressive loss of pancreatic tissue mass (Bombardelli, 2010). Therefore, we examined signaling pathways associated with cellular stress and adhesion in *p48Cre;Numb$^{f/f}$* pancreata following caerulein treatment. Binding of integrins to basement membrane proteins activates Focal Adhesion Kinase (FAK) through phosphorylation. Phospho-FAK is present in control pancreata 1 and 2 days

Figure 2.2: Acinar cells lacking Numb undergo more rapid and extensive dedifferentiation, increased expression of ductal markers, and elevated Notch signaling

(*A-B*) H&E staining shows extensive ductal structures in *p48Cre;Numb*$^{f/f}$ pancreata at days 1 and 2 following caerulein treatment. (*C-D*) Amylase (green) and CK19 (red) immunostaining, counterstained with DAPI (blue) at days 1 and 2 following caerulein reveals increased expression of CK19 in *p48Cre;Numb*$^{f/f}$ and greater loss of amylase expression. (*E*) Transcriptional analysis of Notch target genes 1-2 days after caerulein treatment shows elevated expression of target genes compared to controls. * $P<0.05$, ** $P<0.01$, *** $P<0.005$. N=3-4.


1 day after caerulein
2 days after caerulein
p48Cre
p48Cre Numb f/f
p48Cre
p48Cre Numb f/f
A
A'
B
B'
H&E
C
C'
D
D'
Amylase/CK19/DAPI

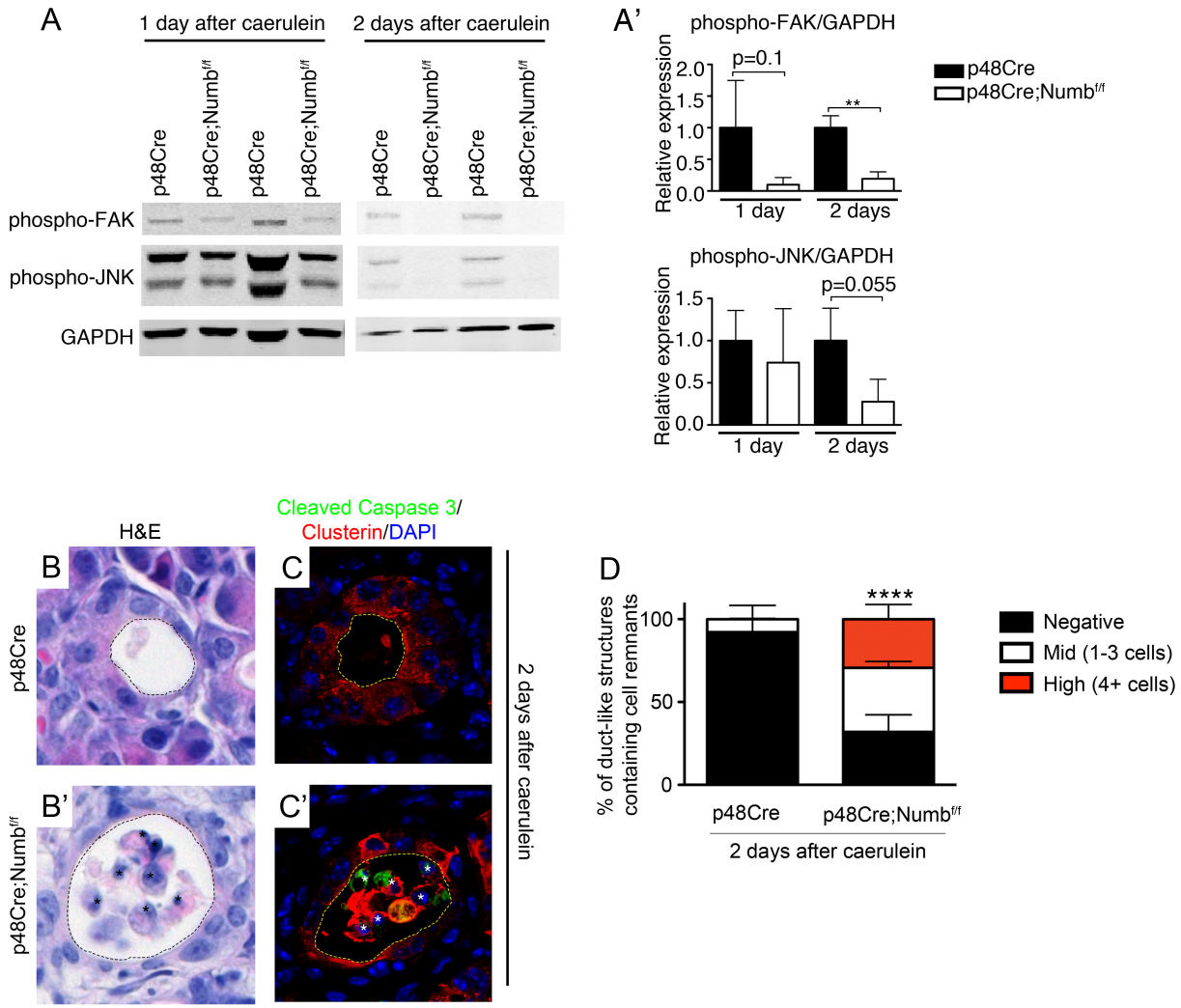

E
Relative expression
10
8
6
4
2
0
Hes1
Hey1
Hey2
HeyL
Hes1
Hey1
Hey2
HeyL
1
2
Days after caerulein treatment
p48Cre
p48Cre;Numb f/f
*
**
***
*

after caerulein, but absent from pancreata lacking Numb, (Figure 2.3*A*, GAPDH served as loading control). Similarly, integrin-mediated activity of Janus-activated kinase (JNK) is reduced in *p48Cre;Numb$^{f/f}$* pancreata 1 day after caerulein treatment and abolished 2 days after caerulein treatment (Figure 2.3*A*). Depending on context, JNK activation responds to cellular stress and can serve as a pro-survival signal. As massive pancreatic tissue loss occurs in *p48Cre;Numb$^{f/f}$* mice by day 7 following caerulein, this data suggests that, JNK is activated by the cellular stress of caerulein-induced pancreatitis and may act as a survival signal for dedifferentiated wild type acinar cells. In the absence of Numb, this pro-survival stress response pathway is lost, which may contribute to the elimination of acinar-derived cells.

Furthermore, disruption of integrin-mediated epithelial cell binding to the extracellular matrix can result in the separation of cells from the epithelial sheet and induction of anoikis, cellular death caused by the loss of cell contact to the basement membrane. Indeed, remnants of extruded cells are observed in the lumens of duct structures in *p48Cre;Numb$^{f/f}$* mice at day 2 after caerulein treatment (Figure 2.3*B'*), indicating that cells are being extruded from the epithelium. Quantification of the percentages of structures with none, mid (1-3) and high (4+) numbers of cells per lumen reveals a significant increase in the percentage of both the mid and high populations (Figure 2.3*D*). Cells located in the lumen are often, but not always, positive for Cleaved Caspase 3 and co-express the acinar stress marker Clusterin (Figure 2.3*C'*). This suggests that metaplastic cells are undergoing the process of apoptosis while being extruded into the ductal lumen.

Figure 2.3: Deletion of Numb alters cell adhesion signaling and results in cell extrusion and death

(*A*) Little to no phosphorylated FAK and JNK are detected by Western blot in *p48Cre;Numb$^{f/f}$* mice at 1 and 2 days after caerulein treatment. (A*'*) Quantification of Western blots, normalized to GAPDH. (*B, B'*) Extensive numbers of cells located in the ductal structure lumen in *p48Cre;Numb$^{f/f}$* are visible by H&E staining. (*C, C'*) Immunostaining of luminally localized cells for Cleaved Caspase 3 (green) and Clusterin (red), counterstained with DAPI (blue) reveals cell death occurring in the lumen of ductal structures of *p48Cre;Numb$^{f/f}$* mice. Ductal lumen are outlined with dashed lines, cell remnants contained in lumens are marked with (*). Images in B and C, original magnification 400x. (D) Quantification of percent of ductal structures containing either no cells, mid (1-3), or high (4+) numbers of cells within the lumen. Data represented as mean ± SD. ** $P<0.01$, **** $P<0.001$. N=3-4.


A
1 day after caerulein
2 days after caerulein
p48Cre
p48Cre;Numb f/f
p48Cre
p48Cre;Numb f/f
p48Cre
p48Cre;Numb f/f
p48Cre
p48Cre;Numb f/f
phospho-FAK
phospho-JNK
GAPDH
A'
phospho-FAK/GAPDH
Relative expression
p=0.1
**
2.0
1.5
1.0
0.5
0.0
1 day
2 days
p48Cre
p48Cre;Numb f/f
phospho-JNK/GAPDH
Relative expression
p=0.055
1.5
1.0
0.5
0.0
1 day
2 days
H&E
Cleaved Caspase 3/
Clusterin/DAPI
B
C
p48Cre
B'
C'
p48Cre;Numb f/f
2 days after caerulein
D
% of duct-like structures containing cell remnants
****
100
50
0
p48Cre
p48Cre;Numb f/f
2 days after caerulein
Negative
Mid (1-3 cells)
High (4+ cells)

Cell death in pancreata lacking Numb is p53 independent

Anoikis can progress either in a p53 dependent or independent manner and to further analyze this process, we examined p53 protein levels by immunostaining. We observed increased numbers of p53-positive epithelial cells within duct-like structures at day 1 following caerulein treatment in *p48Cre;Numb$^{f/f}$* animals compared to controls (Supplementary Figure A1.5*A*). We also examined transcriptional expression of genes known to be involved in apoptosis. The expression of pro-apoptotic p53 target gene Noxa is significantly increased at day 1 and expression of p53 target genes Apaf1 and Puma follows at day 2 (Supplementary Figure A1.5*B*). Additionally, the pro-apoptotic, non-p53 target gene Bim is significantly upregulated (Supplementary Figure A1.5*C*) while levels of another common pro-apoptotic gene, Bax, were not increased (data not shown). These data suggest that Numb blocks the apoptotic response upon pancreatitis injury in part through regulating the temporal stability of p53 and the expression its pro-apoptotic targets in dedifferentiated acinar cells.

To assess the requirement for p53 in the process of cell death during extrusion, we generated compound transgenic *p48Cre;p53$^{f/f}$;Numb$^{f/f}$* mice characterized by the simultaneous loss of Numb and p53 in pancreatic cells. While the deletion of p53 alone did no appear to alter regenerative capacities of acinar cells (Supplementary Figure A1.5*D,E*, *p48Cre;p53$^{f/f}$;Numb$^{f/wt}$* controls), the combined loss of p53 and Numb did not rescue the dramatic reduction in pancreatic tissue characteristic of Numb ablation 7 days after caerulein treatment (Supplementary Figure A1.5*D,E*). Thus, although p53 accumulation and induction of target genes is increased in *p48Cre;Numb$^{f/f}$* mice, the induction of cell death in mice lacking Numb can proceed independent of p53 function.

Deletion of Numb accelerates Kras-mediated acinar to ductal metaplasia

We previously observed that Numb restrains acinar dedifferentiation in response to caerulein pancreatitis. Given these findings, we sought to determine whether Numb also regulates the acinar to ductal metaplasia that occurs in response to expression of oncogenic Kras. To address this question, we crossed a LSL-activated form of $Kras^{G12D}$ (where LSL refers to "lox site-transcription stop-lox site") (Jackson, 2001) with *p48Cre* and $Numb^{f/f}$ to generate $p48Cre;Kras^{G12D};Numb^{f/f}$ mice.

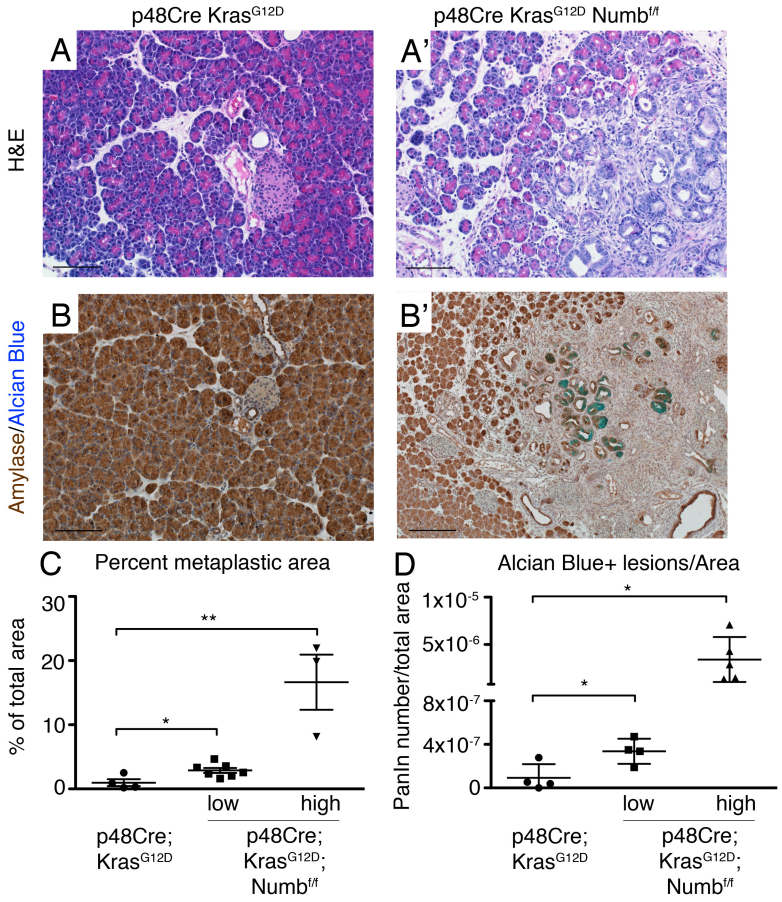
At 3 weeks of age, the pancreata of $p48Cre;Kras^{G12D}$ control mice are grossly normal, with only very few regions of ADM observed (Figure 2.4*A*) and few Alcian blue positive lesions (Figure 2.4*B*). However in $p48Cre;Kras^{G12D};Numb^{f/f}$, entire lobes of tissue have already undergone metaplasia, and a significant amount of ADM and numerous Alcian blue positive lesions are present (Figure 2.4*A',B'*). While we have observed a high degree of variability between samples with regard to the extent of the metaplastic and Alcian blue areas (Figure 2.4*C,D*), even the low penetrance groups were significantly more impacted than the control tissues in which oncogenic Kras is expressed in the context of normal Numb. This data demonstrates that Numb regulates ADM that occurs in response to Kras activation.

PanINs and PDA develop in the absence of Numb but pancreatic tissue is lost during progression

Because metaplasia is initiated earlier and more broadly in $p48Cre;Kras^{G12D};Numb^{f/f}$ mice, we anticipated a continued acceleration of PanIN

Figure 2.4: *p48Cre;Kras^{G12D};Numb$^{f/f}$* mice undergo rapid metaplasia

(*A,A'*) H&E staining shows regions of acinar tissue replaced by ADM in *p48Cre;Kras^{G12D};Numb$^{f/f}$* mice at 3 weeks of age. (*B,B'*) Amylase and Alcian blue staining at 3 weeks of age marks loss of acinar cells and increased number of PanINs, respectively. Scale bars represent 100μm. *p48Cre;Kras^{G12D};Numb$^{f/f}$* have significantly higher (*C*) percent metaplastic and (*D*) alcian blue positive lesions per total area analyzed. Data represented as individual mice analyzed, overlaid with mean ± SD. * $P<0.05$, ** $P<0.01$.


p48Cre KrasG12D
p48Cre KrasG12D Numbf/f
A
A'
H&E
B
B'
Amylase/Alcian Blue
C
Percent metaplastic area
% of total area
30
20
10
0
**
*
low
high
p48Cre; KrasG12D
p48Cre; KrasG12D; Numbf/f
D
Alcian Blue+ lesions/Area
PanIn number/total area
1x10-5
5x10-6
8x10-7
4x10-7
0
*
*
low
high
p48Cre; KrasG12D
p48Cre; KrasG12D; Numbf/f

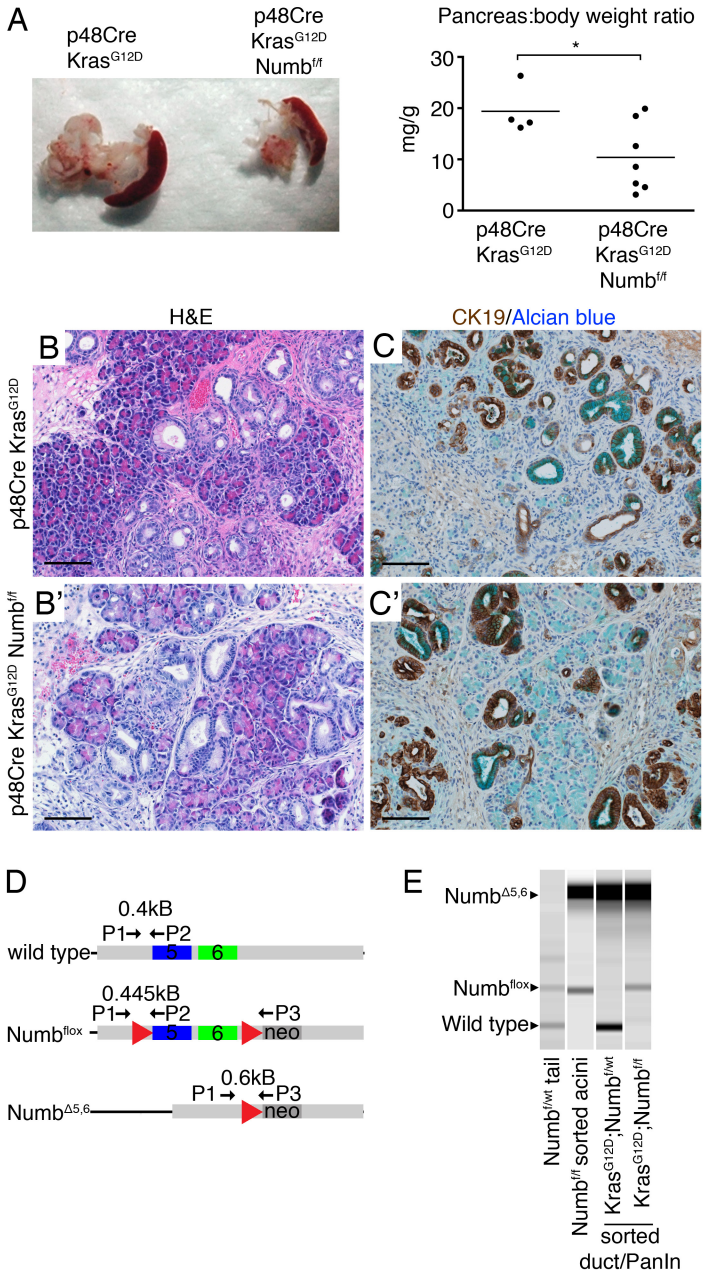
formation at later time points. Surprisingly, histological evaluation revealed no differences with regard to ADM and PanIN formation in mutant and control mice at 10 weeks of age (Figure 2.5*A,B*). However, pancreata of *p48Cre;Kras^{G12D};Numb$^{f/f}$* mice were significantly smaller than controls as demonstrated by gross morphology and analysis of pancreas to body weight ratio (Figure 2.5*C*). These findings suggest that in addition to restraining early stages of acinar dedifferentiation and metaplasia, Numb further promotes the survival of acinar cells undergoing neoplastic transformation. Additionally, ablation of Numb does not appear to prevent proliferation of metaplastic ducts and PanINs at 6 and 10 weeks of age (Supplementary Figure 3C',D'). Importantly, the profound loss of pancreas tissue in *p48Cre;Kras^{G12D};Numb$^{f/f}$* mice upon injury indicate that the pro-survival signals provided by oncogenic Kras cannot fully compensate for the loss of Numb function. However, loss of Numb does not appear to preclude PanIN formation should a cell manage to overcome pro-apoptotic signals that are present during dedifferentiation and metaplasia.

Although *p48Cre;Kras^{G12D};Numb$^{f/f}$* acinar cells are more readily able to undergo ADM, the process appears to be unstable, therefore we hypothesized that PanINs present in Numb$^{f/f}$ mice may have retained Numb function by escaping recombination of both *floxed* alleles of Numb. To address this question, we isolated DNA from the PanIN/duct cell population at 10 weeks. By using primers specific for the wild type, floxed and recombined Numb alleles as diagrammed in Figure 5D, we analyzed the presence of allelic variation. Notably, we do not observe increased intensity of the unrecombined floxed band (Figure 2.5*E*; Numb^{flox}) in *p48Cre;Kras^{G12D};Numb$^{f/f}$* ducts/PanINs when compared to signal obtained from *p48Cre;Numb$^{f/f}$* acinar cells (Figure 5*E*). These data

indicate that PanIN formation can occur in the absence of Numb. In other words, loss of Numb does not appear to preclude PanIN formation should a cell manage to overcome pro-apoptotic signals that are present during dedifferentiation and metaplasia.

Figure 2.5: Deletion of Numb reduces the ability of acinar cells to complete acinar to ductal metaplasia and form PanIns

(*A, A'*) H&E staining (*B,B'*) CK19 (brown) and Alcian blue (blue) staining reveals similar histology and presence of ADM and PanINs between the two genotypes. Scale bars represent 100μm. (*C,C'*) Gross morphology and pancreatic weight, normalized to body weight indicate that pancreata of *p48Cre;Kras^{G12D};Numb$^{f/f}$* mice are significantly smaller than controls at 10 weeks of age. (*D*) Schematic of PCR for detection of Numb recombination. Primers 1 and 2 generate 0.4kB products from the wild type and unrecombined Numb^{flox} alleles, with the latter being 45 base pairs longer. Primers 1 and 3 generate a 0.6kB product from the recombined Numb$^{\Delta 5\text{-}6}$. The red bar between primer arrows indicates the replication product produced for each respective allele. (*E*) PCR analysis of recombination of the Numb alleles in FACS isolated duct/PanIn cells. Note the similar intensity levels of Numb^{flox} bands in *p48Cre;Kras^{G12D};Numb$^{f/f}$* and *p48Cre:Numb$^{f/f}$*, suggesting that there is no significant selection against the Numb$^{\Delta 5\text{-}6}$ allele in PanINs in *p48Cre:Numb$^{f/f}$* mice. Images are representative of 3 individuals analyzed per genotype. Pancreas weight data represented as individuals, overlaid with mean ± SD. * $P<0.05$.


A
p48Cre
KrasG12D
p48Cre
KrasG12D
Numbf/f
Pancreas:body weight ratio
*
30
20
10
0
mg/g
p48Cre
KrasG12D
p48Cre
KrasG12D
Numbf/f
H&E
CK19/Alcian blue
B
C
p48Cre KrasG12D
B'
C'
p48Cre KrasG12D Numbf/f
D
0.4kB
P1→ ←P2
wild type
5
6
0.445kB
P1→ ←P2
←P3
Numbflox
5
6
neo
0.6kB
P1→
←P3
Numbδ5,6
neo
E
Numbδ5,6
Numbflox
Wild type
Numbf/wt tail
Numbf/f sorted acini
KrasG12D;Numbf/wt
KrasG12D;Numbf/f
sorted
duct/PanIn

Discussion

Increasing evidence indicates that upon injury, acinar cells (Morris, 2010b) undergo a process of dedifferentiation during which they reduce expression of mature markers and upregulate expression of factors commonly found in non-acinar cell types, including embryonic pancreas progenitor cells. Understanding the mechanisms underlying acinar cell dedifferentiation is important, as, in the presence of oncogenic Kras, this process is known to give rise to the precursors to pancreatic ductal adenocarcinoma through the process of acinar to ductal metaplasia. In the present study we have uncovered a role for the multifunctional protein Numb in the regulation of acinar cell regeneration and acinar to ductal metaplasia.

Numb regulates acinar cell dedifferentiation and regeneration and promotes Notch signaling

Acinar cells respond to caerulein pancreatitis injury with rapid, transient dedifferentiation. Cessation of injury results in quick regeneration of the damaged cells through reactivation of the acinar differentiation state in wild type mice (Morris, 2010a; Strobel, 2007). Our data demonstrate a critical role for Numb in acinar dedifferentiation. Caerulein-induced pancreatitis is enhanced in mutant mice, as indicated by an increased number of duct-like structures, reduced expression of acinar cell markers and increased expression of duct markers. Thus, our findings suggest that Numb contributes to the maintenance of the acinar differentiation state by restricting the acinar cell response to pancreatic damage. In addition, our data demonstrate that Numb also governs the regenerative capacity of acinar cells in response to damage. Not only does deletion of

Numb prevent acinar regeneration, loss of the factor results in massive pancreatic atrophy following caerulein pancreatitis, indicating that Numb normally functions to stabilize the dedifferentiated state, allowing for proper regeneration of acinar cells.

Reactivation of signaling pathways mainly present during embryogenesis has been demonstrated upon acinar injury (Jensen, 2005) and Numb is known to govern the activity of these pathways in other tissues (McGill, 2003; McGill, 2009). For example, Notch signaling has been shown to promote the transdifferentiation of acinar cells towards duct-like cells (Miyamoto, 2003). We find that Numb elimination increases Notch signaling during acinar cell dedifferentiation. Thus, it is likely that deregulation of Notch signaling during caerulein-induced pancreatitis in mice lacking Numb enhances the dedifferentiation of acinar cells and the adoption of the duct- and progenitor-like cell state. Additionally, Numb does not affect all Notch receptors equally; different combinations of Numb isoforms and Notch receptors determine the signaling output (Beres, 2011). Therefore it is possible that in addition to increasing overall pathway activity levels, deletion of Numb alters the ultimate signaling outcome of the Notch pathway in dedifferentiated acinar cells.

The contribution of overactive Notch signaling to the loss of cell viability remains unclear. Some evidence demonstrates that excessive Notch signaling can lead to cell death, for example aberrant Notch activation in murine neural progenitor cells promotes apoptosis. This effect was restricted to the dividing progenitor cells, as activation of Notch in post-mitotic neurons did not lead to cell death (Yang, 2004). We speculate that the dedifferentiated acinar cell state may be sensitive to fluctuations in Notch signaling, requiring some pathway activation, but hindered by excessive levels. Our data may be yet

another instance demonstrating the requirement for a delicate balance of signaling pathway activation in regulating acinar dedifferentiation and regeneration, as has already been demonstrated for Wnt signaling (Morris, 2010a).

Numb regulates cell stress responses and cell adhesion during caerulein-induced pancreatitis

We have also gathered evidence that Numb ablation extends beyond Notch regulation and includes perturbed cell adhesion signals, which may be mediated via abrogation of integrin signaling. The reduction of JNK signaling indicates a failure to respond to cellular stress. The lack of FAK activation points to a disruption of cell adhesion and connection of the cell to the basement membrane, which can result in induction of cell death by a process known as anoikis. Numb is known to be required for the maintenance of adherens junctions as well as trafficking of integrins (Nishimura, 2007; Rasin, 2007), and these activities could be essential for maintenance of tight cell-cell and cell-matrix interactions. Our data support recent findings illustrating the connection between cell-cell interactions and pancreatic cancer. For example, loss of β1 integrin has been demonstrated to disrupt acinar cell polarity, lead to acinar to ductal metaplasia and result in the loss of pancreatic tissue mass over an extended period of time (Bombardelli, 2010). However, this study only manipulated a single component of the integrin signaling cascade. Deletion of Numb potentially affects localization and recycling of multiple components of the integrin complexes, thus possibly enhancing the phenotypes of increased acinar cell sensitivity to caerulein and overall tissue atrophy observed upon the loss of β1 integrin alone.

Numb regulates and stabilizes Kras mediated acinar to ductal metaplasia

In the absence of additional stresses such as inflammation due to caerulein pancreatitis, Kras is fairly inefficient at reprogramming acini into PanIN lesions. However, the roadblocks that exist to restrain this metaplasia are unclear. In this study, we have revealed that Numb provides such a barrier, as its ablation accelerates metaplasia on acinar cells due to deregulated Kras activity. Because Numb deletion alone does not appear to increase Notch activity levels in the absence of additional stresses, it is unlikely that acceleration of metaplasia is simply due to premature activation of Notch, although increased Notch signaling in metaplastic cells could promote progression, as Kras and Notch have been documented to cooperate during metaplasia (De La, 2008; Mazur, 2010; Sawey, 2007).

Notably, Numb's effects on cell are also observed in the presence of a constitutively active, oncogenic form of Kras. Although ablation of Numb accelerates the process of ADM and PanIN formation that occurs under the control of activated Kras, the viability of the metaplastic cells is reduced and tissue mass decreases significantly over time. Thus, even the strong neoplastic cues provided by mutant Kras are not sufficient to stabilize the malleable, dedifferentiated state generated in the absence of Numb. This is an exciting finding, as it poses possibilities for ways to dampen the transformative properties of Kras. One possible mechanism for destabilization of cell viability during ADM is changes in the Notch signaling pathway. Previous work has shown that a shift from Notch1 to Notch2 promotes PanIN progression (Mazur, 2010). Because Numb has varying affinities for the different Numb receptors, perhaps its absence causes a change in

the balance of signaling from these different receptors, thus affecting PanIN development. Nonetheless, some PanIN formation still occurs in the absence of Numb. The notion that PanIN development can proceed under these conditions, albeit with reduced viability during the metaplastic stage, strongly suggests that the pro-apoptotic cues generated in the absence of Numb can be overcome. Further analysis of the cell survival signals present in these remaining PanINs could provide important insight into critical mechanisms promoting PanIN maintenance and progression.

These findings also indicate that subsequent development of PDA could occur in the absence of Numb. Future studies will address whether this is indeed the case and explore whether Numb elimination in PDA might change the properties of tumor cells with regard to their malignant properties. One might speculate that changes in cell adhesion and increases in the activity levels of various signaling pathways that may be detrimental to a cell undergoing metaplasia may actually enhance the tumorigenicity of a fully transformed cell, thus influencing tumor growth, invasion and metastasis. Although no known mutations of Numb have been documented in pancreatic cancer in the COSMIC Database (Forbes, 2010), Numb expression is decreased in PDA compared to microdissected duct cells (Jones, 2008). More in depth analysis is required to fully determine the changes in Numb expression and activity in PDA and how these changes may affect tumorigenicity.

Material and Methods

Mouse lines

Experimental animals were generated by crossing p48Cre (gift of Chris Wright, Vanderbilt University, Nashville, Tennessee, USA) with LSL-Kras^{G12D} (gift of Dave Tuveson, Cancer Research UK Cambridge Research Institute, Cambridge, United Kingdom) and Numb$^{f/f}$ (Gift of Yuh Nung Jan, University of California, San Francisco, California, USA). All mice experiments were performed with approval from the UCSF Institutional Use and Care of Animals Committee (IACUC). Littermate controls were used.

Caerulein treatment

Acute pancreatitis was induced in p48Cre, p48Cre;Numb$^{f/f}$, p48Cre;Kras^{G12D} and p48Cre;Kras^{G12D};Numb$^{f/f}$ mice at 5-6 weeks after birth with a typical weight range of 22-25g by caerulein injection as previously described (Jensen, 2005). Briefly, mice received 8 hourly i.p injections of caerulein (American Peptide Company), 2ug/injection on 2 consecutive days. Day 0 is counted as immediately following the final caerulein injection.

Immunohistochemistry and immunofluorescence

For histological, immunohistolochemical and immunofluorescence analysis, dissected pancreatic tissues were fixed with zinc buffered formalin (Anatech) overnight and embedded in paraffin wax. Heat mediated antigen retrieval was performed with Citra antigen retrieval solution (BioGenex) prior to staining. Tissue sections were stained with the following primary antibodies: mouse anti-α Smooth Muscle Actin (1:200, Sigma-Alrich), rabbit anti-Amylase, (1:200 Sigma-Aldrich), rat anti-CD45 (1:400, BD BioSciences), rat anti-CK19 (TROMAIII, 1:200, University of Iowa Hybridoma Bank),

rabbit anti-Cleaved Caspase 3 (1:200, Cell Signaling Technologies), goat anti-Clusterin (1:400, Santa Cruz BioTechnologies), mouse anti-E-cadherin (1:400, BD BioSciences), rabbit anti-p53 (1:400, Vector). For immunofluorescence, AlexaFluor tagged secondary antibodies (1:400, Invitrogen) were used and slides were mounted using DAPI-containing Vectashield media (Vector). For CK19 and CD45 immunohistochemistry biotinylated goat anti-rat secondary (1:400, Vector) was used. 3-3'-Diaminobenzidine tetrahydrochloride was used as a chromagen. For CK19, sections were counterstained with 1% Alcian Blue 8GX (Sigma) in 3% acetic acid solution and Meyer's Hematoxylin (Sigma). For CD45 immunohistochemistry, sections were counterstained with hematoxylin.

Fluorescent images were acquired using a Zeiss AxioImager microscope. Brightfield images were acquired using a Zeiss Axio Imager D1 microscope.

Western blotting

Immunoblotting was performed by homogenizing tissue in RIPA buffer with protease and phosphatase inhibitors (Roche) and electrophoresing samples on a 4-20% Tris-HCl gel (BioRad). The following primary antibodies were used: rabbit anti-Numb (Pan) (1:1000, Chemicon), rabbit anti-phospho (Tyr-397) FAK (1:1000, Invitrogen), mouse anti-GAPDH (1:5000, Santa Cruz BioTechnology), rabbit anti-phospho (Thr183/Tyr185) JNK (1:1000, Cell Signaling Technologies). Secondary antibodies used were goat anti-rabbit IR800 (1:15,000, LI-COR) and goat anti-mouse IR680 (1:15,000, LI-COR). Imaging was performed on a LI-COR Odyssey scanner and quantification was performed by normalizing fluorescence intensity of each individual band to the corresponding intensity of GAPDH.

Flow cytometry

A single cell suspension from freshly dissected pancreata was prepared as described by Morris et al (Morris, in preparation). Pancreata were digested with a series of reagents: 1) 2.5mg/ml Collagenase D (Roche) with 0.1ng/ml DNase I (Sigma-Aldrich) in Hank's buffered saline solution, 2) 0.05% Trypsin-EDTA, 3) 2U/ml Dispase (Invitrogen) in HBSS. Single cells were stained with FITC-conjugated anti-CD45 (1:400, eBioscience), PE-conjugated anti-CD49f (1:50, eBioscience) and Biotin-conjugated CD133 (Prominin1, 1:50, eBioscience). APC-conjugated streptavidin (1:100, eBioscience) was used as a secondary antibody. DAPI was used to exclude dead cells from analysis. Cells were isolated using a FACS Aria (BD BioScience).

Quantitative PCR

Fresh whole pancreatic tissue was stored for at least 12 hours in RNA Later (Ambion) at -80C. RNA was extracted from whole tissue and sorted pancreatic cells using Trizol and RNeasy kit (Qiagen). Reverse transcription was performed using SuperScript III (Invitrogen). Expression of all genes was analyzed using Taqman assays (Applied Biosystems) and normalized to cyclophilin expression (Primers: GGCCGATGACGAGCCC, and TGTCTTTGGCTTTGTCTGCAA, probe: TGGGCCGCGTCTCCTTCGA)

DNA analysis of sorted cells

DNA was extracted from FACS isolated cells using RecoverAll Total Nucleic Acid Isolation kit (Ambion). Deletion of exons 5-6 during recombination was detected as previously described (Zhong, 2000). Primers 1:

TCAGCAGTTTCTGAGTTCAGTTCCC, primer 2: TAAAAACGCAGTCGAGAAAC, primer 3: ACGAGTTCTTCTGAGGGGATCGGC.

Quantification

For quantification of duct structures with luminal cells, percent metaplastic area and Alcian blue postive lesion count, 5-10 non-overlapping 100x H&E images were taken per sample and score by hand. For luminal cell counting, duct structures were categorized into 3 groups (no, mid (1-3) and high (4+)). Metaplastic area, in pixels, was selected by hand in Adobe Photoshop and normalized to total area in pixels calculated by Metamorph. Alcian blue positive lesions were counted by hand and normalized to total area in pixels calculated by Metamorph.

Statistical Analysis

Results are shown as the mean ±SD. Paired data were evaluated using a two-tailed Student's *t*-test. Luminal cell count data was evaluated by Chi squared test.

Acknowledgements

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CHAPTER 3

Pancreatic mesenchyme is required for pancreatic homeostasis

Summary

Pancreatic mesenchyme is required for pancreatic development and changes in the pancreatic microenvironment, including proliferation and activation of pancreatic stellate cells, are known to be associated with pancreatic cancer formation. However, the contribution of the pancreatic mesenchyme to adult tissue homeostasis is unknown. Here we show that pancreatic mesenchymal cells (PaMCs) are found throughout the adult pancreas and are in contact with all types of pancreatic epithelial cells. Specifically, they are found embedded in acinar tissue, surrounding pancreatic ducts and creating a network surrounding the islets of Langerhans. Depletion of these cells results in disruption of acinar morphology and reduction of amylase expression. Additionally, depletion of PaMCs induces hyper-secretion of endocrine hormones leading to loss of regulation of glucose levels, and disruption of β-cell maturity. Finally, ECM molecules produced specifically by PaMCs are responsible, at least in part, for the maintenance of β-cell maturity. These results indicate that pancreatic mesenchyme is required for continued normal function of the pancreas and suggest that mutations that affect these cells could affect disease development and progression.

Introduction

The pancreatic mesenchyme is critical for proper growth and development of the pancreas. Additionally, alterations in the pancreatic microenvironment are common characteristics of diseases such as pancreatic cancer and pancreatitis (reviewed in Duner, 2011) and some evidence now suggests that changes in the microenvironment are also associated with development of diabetes (Hayden, 2008; Wang, 2003).

The most well characterized resident fibroblast population in the adult pancreas is the pancreatic stellate cell (PSC). This cell surrounds acini and is characterized by the expression of Desmin and Vimentin and the presence of Vitamin A lipid droplets (Apte, 1998; Bachem, 1998). It has been shown to proliferate and become activated during the development of PDA and contribute to development of the disease (Hwang, 2008; Vonlaufen, 2008). Work from our lab recently demonstrated that the pancreatic mesenchyme surrounds developing islets and supports expansion of the pancreatic epithelium throughout development (Landsman, 2011a).

Diabetes is disease characterized by loss of regulation of blood glucose levels, either due to destruction of the β-cell population or loss of β-cell function and peripheral tissue insulin sensitivity. It is a costly disease that affects around 8% of the population of the United States (Centers for Disease Control and Prevention, 2011). Glucose levels are regulated by a tightly controlled system of insulin and glucagon release by β-cells and α-cells, respectively. In order to respond to changes in blood glucose levels, these cells must maintain expression of proteins involved in sensing glucose levels and translating changes into signals to secrete insulin. A well-characterized set of transcription factors is responsible for keeping the cell in this mature and functional state (Bernardo, 2008).

Additionally, signals from neighboring cell types have been shown to play a role in maintaining the functionality of β-cells, such as GLP-1 secreted from L-cells in the gut and extracellular matrix secreted from endothelial cells within the islet (Drucker, 2006; Nikolova, 2006). However, the contribution of the pancreatic mesenchyme in maintaining the functionality of β-cells is unknown.

By using a transgenic system to drive gene expression specifically in the pancreatic mesenchyme, we have been able to label, analyze and deplete these cells in the adult pancreas in order to study their function. Here we demonstrate for the first time, that pancreatic mesenchymal cells contribute to the adult tissue and interact with the pancreatic epithelium, making close contact with acini, ducts and islets. We find that PaMCs express typical markers associated with fibroblasts and respond to signals during pancreatitis by upregulating inflammatory and activation markers. We demonstrate that PaMCs aid in maintenance of acinar cell differentiation and organization. Most strikingly, we find that PaMCs are required for regulated insulin and glucagon secretion and maintenance of β-cell differentiation state, and therefore are required for blood glucose regulation. This effect is, in part, mediated by production of ECM components by PaMCs.

Results

Pancreatic mesenchyme surrounds the epithelium in the adult

The transcription factor Bapx1 (Nkx3.2) is expressed in the embryonic pancreatic, gut and stomach mesechyme as well as in the skeletal somites (Tribioli, 1997). Expression of Cre recombinase under the control of this promoter (*Nkx3.2Cre*) (Verzi, 2009) allows for gene expression and deletion specifically in the mesenchyme, but not in other cell types in the pancreas (Landsman, 2011a). Here we utilize *Nkx3.2Cre* to induce recombination of a reporter allele, *R26-YFP*^{LSL}, therefore labeling the Nkx3.2 positive pancreatic mesenchyme and all of its progeny. In adult mice (6-8 weeks of age), we observed cells labeled by *Nkx3.2Cre;R26-YFP*^{LSL}, (henceforth referred to as Nkx3.2/YFP^{+} cells) throughout the pancreas, including around acinar cells, ducts and blood vessels (Figure 3.1F,G, 3.2B). Expression of known fibroblast markers Desmin, Vimentin and Fibronectin confirms the mesenchymal nature of these Nkx3.2/YFP^{+} cells (Figure 3.1A-F). Finally, a majority of these cells also express the surface marker PDGFRα, which can be detected by FACS (Figure 3.1H).

Fibroblast populations have been described in the exocrine tissue, but their presence has not been well documented around islets. Interestingly, Nkx3.2/YFP^{+} cells are observed surrounding islets (Figure 3.2B). Further analysis by live imaging of isolated islets revealed that they form a network both surrounding and extending into islets and are in close contact with endocrine cells (Figure 3.2D). These Nkx3.2/YFP^{+} cells comprise about 11% of the total cell numbers in isolated islets (Figure 3.2C). Importantly, we also observe close interactions between VIMENTIN positive cells and the endocrine cells of the islet in the human pancreas, indicating that fibroblasts also

Figure 3.1. Nkx3.2/YFP$^+$ cells are fibroblasts found throughout the pancreas

(A,**C**) Nkx3.2/YFP$^+$ cells co-express known fibroblast markers. Pancreatic tissue from *Nkx3.2*-Cre;YFP adult mice was immunostained with antibodies against YFP (green) and Desmin/Vimentin (red). (**B,D,E**) Nkx3.2/YFP$^+$ cells co-express known fibroblast markers. RNA was isolated from bulk pancreatic tissues or FACS-sorted YFP$^+$ cells isolated from *Nkx3.2*-Cre;YFP pancreata. Expression levels of indicated genes were analyzed by qPCR and normalized to total pancreatic tissue. N=4. Data represent the mean ± SD. (**F**) Nkx3.2/YFP$^+$ cells are localized among acinar cells. Pancreatic tissues from *Nkx3.2*-Cre;YFP adult mice were immunostained with antibodies against YFP (green) and Amylase (red). (**G**) Nkx3.2/YFP$^+$ cells are found surrounding blood vessels. Pancreatic tissues from *Nkx3.2*-Cre;YFP adult mice were immunostained with antibodies against YFP (green) and PECAM1 (red), and counterstained with DAPI (blue). Insert shows higher magnification. (**H**) A majority of pancreatic PDGFRa$^+$ cells are Nkx3.2/YFP$^+$ cells. Flow cytometry analysis of digested *Nkx3.2*-Cre;YFP pancreas stained with an APC-conjugated anti-PDGFRa antibody. Red boxes mark PDGFRa$^+$Nkx3.2/YFP$^+$ (upper) and PDGFRa$^+$Nkx3.2/YFP$^-$ (lower) cell populations. Numbers indicate the percentage of the relevant population out of PDGFRa$^+$ total cells.

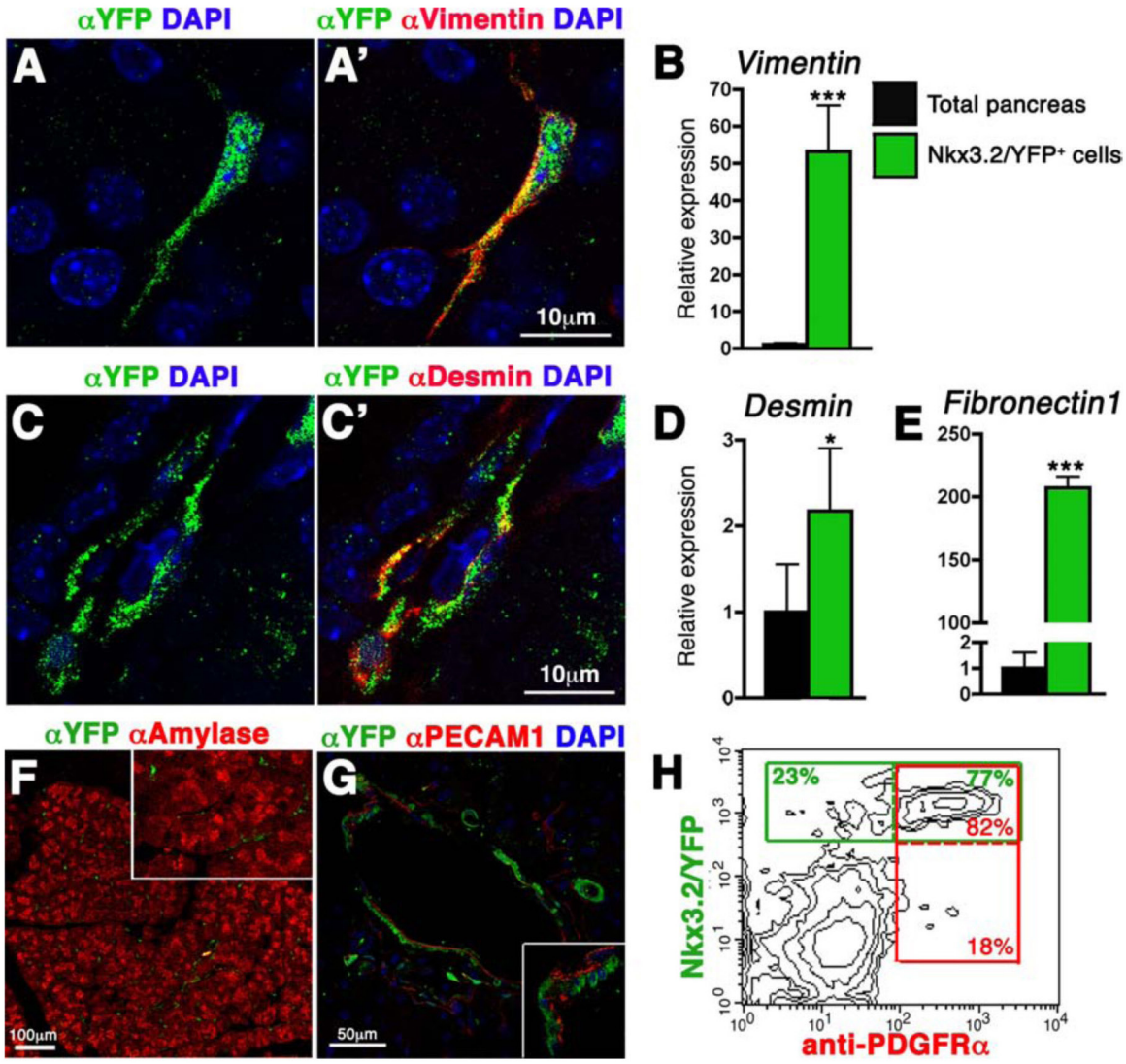
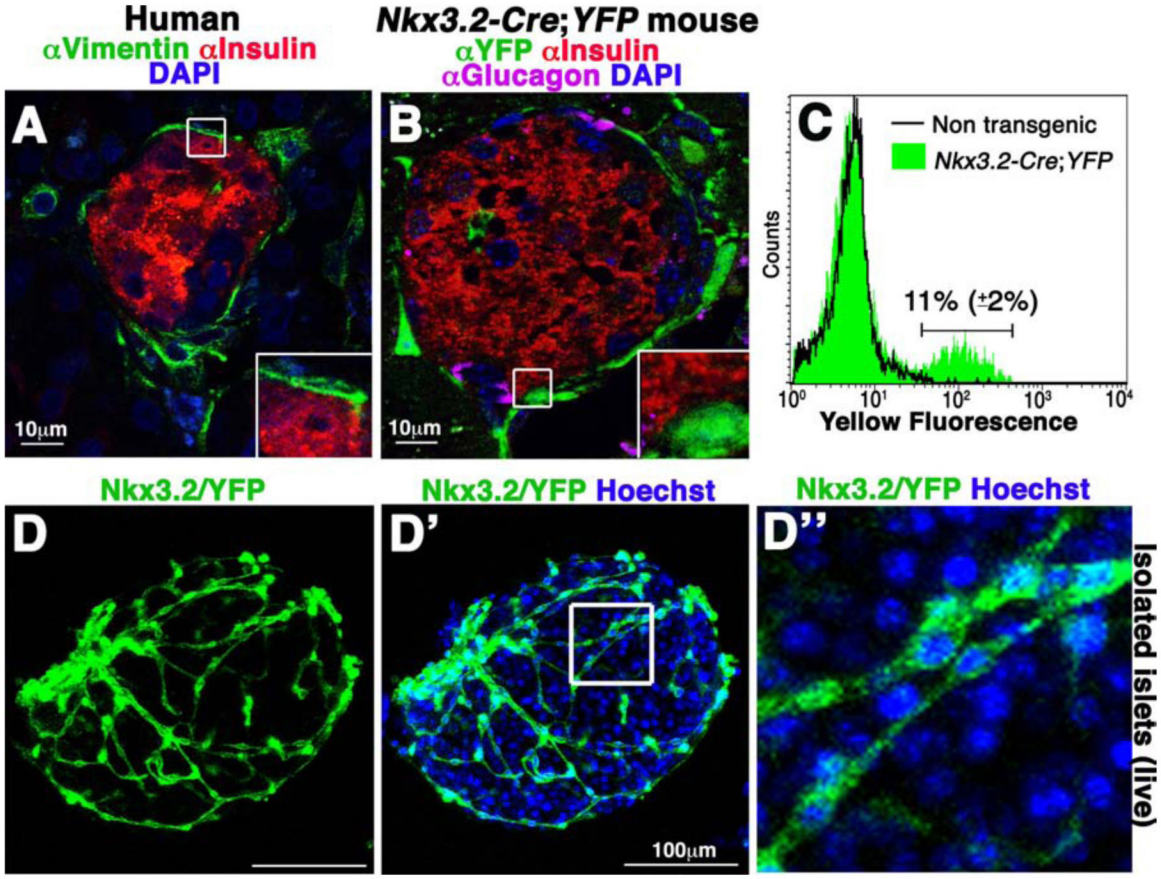

αYFP DAPI
αYFP αVimentin DAPI
A
A'
10μm
B
Vimentin
Relative expression
***
Total pancreas
Nkx3.2/YFP+ cells
αYFP DAPI
αYFP αDesmin DAPI
C
C'
10μm
D
Desmin
Relative expression
*
E
Fibronectin1
***
αYFP αAmylase
αYFP αPECAM1 DAPI
F
100μm
G
50μm
H
23%
77%
82%
18%
Nkx3.2/YFP
anti-PDGFRα

Figure 3.2: Nkx3.2/YFP$^+$ cells form a network around islets and make close contact with β-cells

(**A**) Vimentin-expressing cells are localized around human islets. Fixed pancreatic tissue from a non-diabetic human donor was stained with antibodies against Vimentin (green), Insulin (red), and counterstained with DAPI (blue). Insert shows higher magnification of the area framed in white box. (**B**) Nkx3.2/YFP$^+$ cells are found in close proximity to β-cells in transgenic mouse pancreas. Fixed pancreatic tissue from a *Nkx3.2-Cre;YFP* mouse was stained with antibodies against YFP (green), insulin (red), glucagon (fuchsia) and counterstained with DAPI (blue). Insert shows higher magnification of the area framed in white box. (**C**) Flow cytometric analysis for YFP in dissociated *Nkx3.2-Cre;YFP* islets, in comparison to non-transgenic (non tg) islets. Number represents the percentage of gated cells (marked with a bar) out of total live cell population in transgenic islets. N = 3. Data represent the mean ± SD. (**D**) Nkx3.2/YFP$^+$ cells form a network around islets. Fresh isolated islets from *Nkx3.2-Cre*;*YFP* mice were imaged for YFP (green) (D) and Hoechst (blue)(D'). (D'') Higher magnification of the area framed in white box in D'. Picture represents projections of multiple images taken in different focal planes.


Human
αVimentin αInsulin
DAPI
Nkx3.2-Cre;YFP mouse
αYFP αInsulin
αGlucagon DAPI
A
B
C
10μm
10μm
Non transgenic
Nkx3.2-Cre;YFP
Counts
11% (±2%)
Yellow Fluorescence
Nkx3.2/YFP
Nkx3.2/YFP Hoechst
Nkx3.2/YFP Hoechst
D
D'
D''
100μm
Isolated islets (live)

surround human pancreatic islet (Figure 3.2A). Although these Nkx3.2/YFP^{+} cells do appear around islets and express some markers known to be found in stellate cells, whether or not these cells are true stellate cells, is a unique population, or is a mixed population, remains unclear, therefore we will refer to them as pancreatic mesenchymal cells, or PaMCs.

PaMCs respond to pancreatic damage

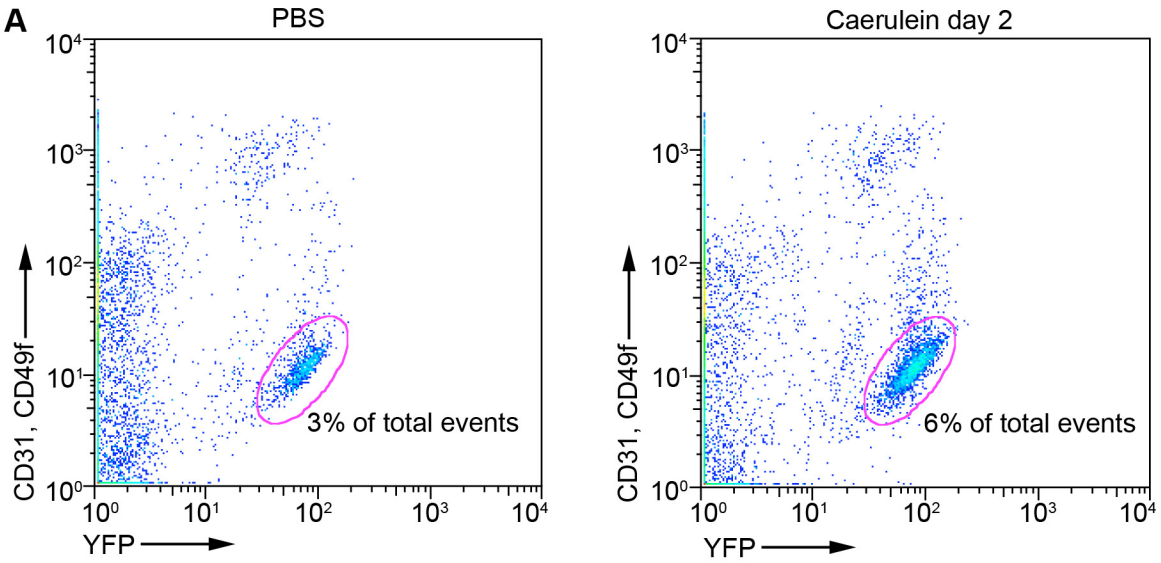
Pancreatic stellate cells are known to become activated in response to pancreatic damage and disease (reviewed in Apte, 2012; Duner, 2011). Caerulein is a cholosystekinin analog that induces hypersecretion of acinar cells, resulting in pancreatic damage and acute pancreatitis. Symptoms of this damage include infiltration of immune cells, activation and expansion of fibroblast cell populations and dedifferentiation of acinar cells to a more ductal and progenitor-like state. We used microarray analysis to identify changes in gene expression in PaMCs cells in response to caerulein treatment. Two days after caerulein treatment, the PaMC population has doubled in size compared to PBS control, indicating that these cells do respond to pancreatic damage (Figure 3.3A). Microarray analysis reveals that during pancreatitis, PaMCs respond by increasing expression of many types of genes, primarily those involved in production of, and interaction with, the extracellular matrix (Figure 3.3B,C).

Depletion of PaMCs results in disruption of acinar cells

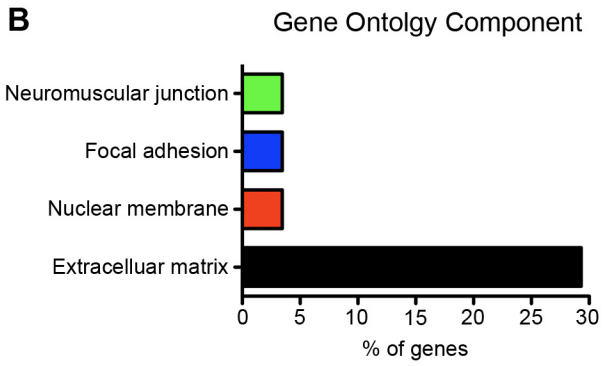
To study the potential role of PaMCs in regulation of pancreas homeostasis, we utilized a well-characterized system of cell ablation based on conditional expression of the Diptheria Toxin (DT) receptor. Uptake of DT into a cell causes to rapid inhibition of protein synthesis, which leads to apoptosis (Saito, 2001). Because mouse cells lack a

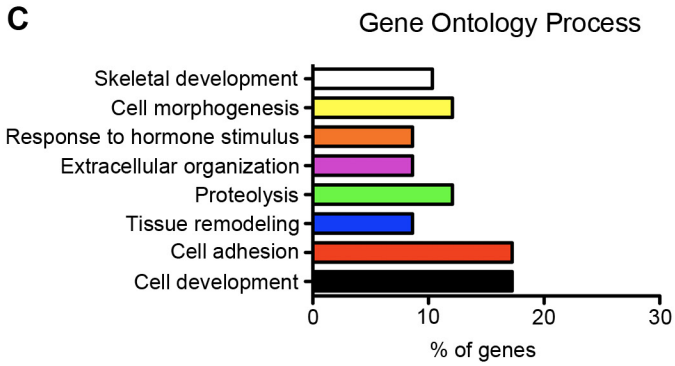
Figure 3.3: Nkx3.2/YFP$^+$ cells respond to caerulein pancreatitis

(**A**) Nkx3.2/YFP$^+$ population expands in response to caerulein treatment. Flow cytometry of PBS and caerulein treated (day 2) dissociated pancreata from *Nkx3.2Cre;YFP* mice. (**B,C**) Nkx3.2/YFP$^+$ cells respond to caerulein treatment by changing gene expression. Microarray analysis of Nkx3.2/YFP$^+$ cells isolated by flow cytometry from PBS and caerulein treated (day 2) dissociated pancreata from *Nkx3.2Cre;YFP* mice. Genes included in analysis are upregulated >2 fold, $p<0.01$. N=3.



Upregulated genes in caerulein day 2 compared to PBS



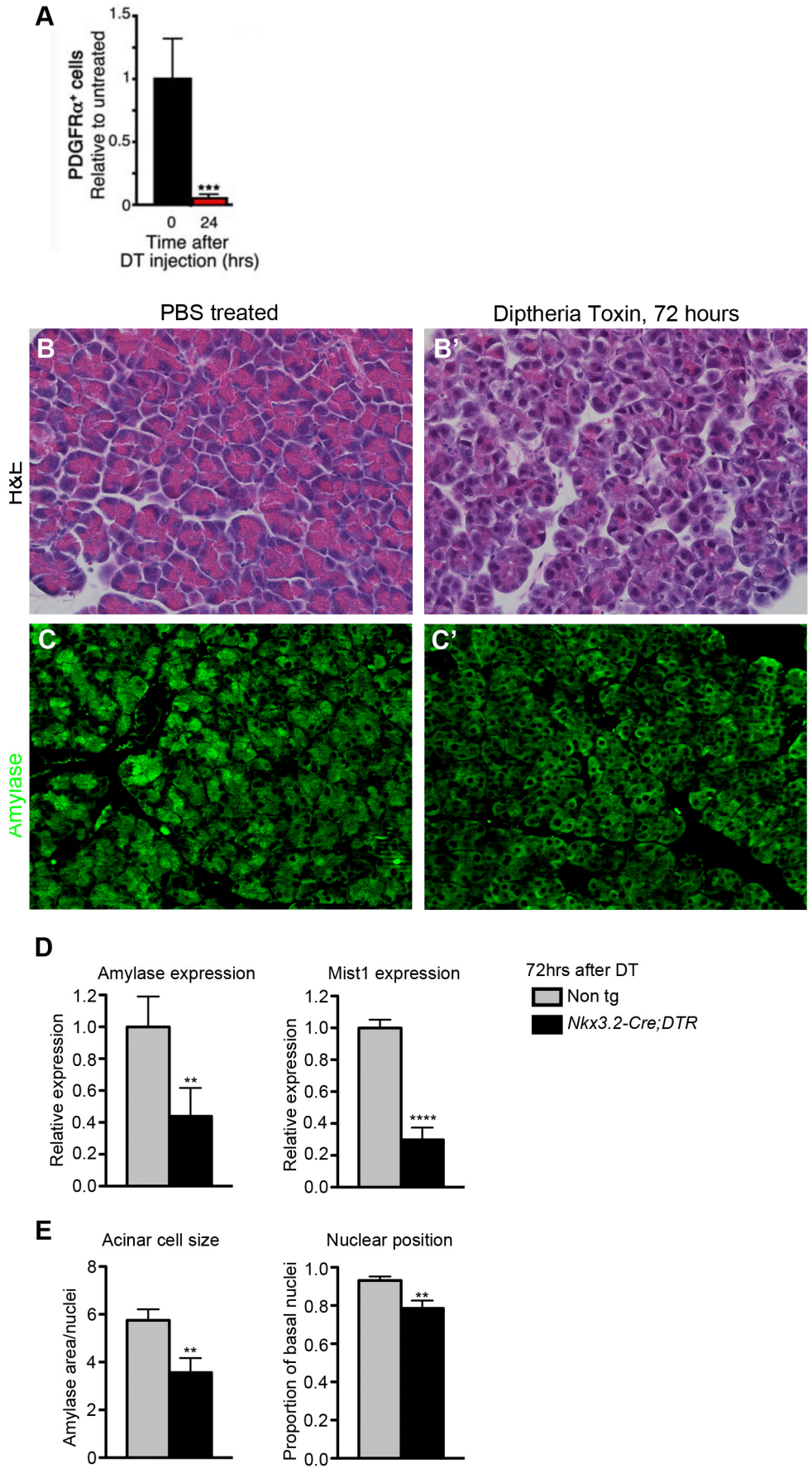


specific receptor for the toxin, they are resistant to DT-induced apoptosis, but expression of a transgene encoding an allele of human hbEGF (DTR) renders them sensitive to the toxin. By crossing a Cre-inducible, *Rosa26-DTR*^{flox}, also known as iDTR, (Buch, 2005) with the *Nkx3.2-Cre*, we can specifically ablate PaMCs at any time following expression of the Cre. In the adult, PaMCs are effectively depleted by 24 hours after treatment with a single dose of DT, as demonstrated by the absence of PDGFRα+ cells (Figure 3.4A).

72 hours after DT administration, mice presented with reduced activity and body temperature and were euthanized. During development, *Nkx3.2* is also expressed in the stomach and gut mesenchyme as well as the skeletal somites (Tribioli, 1997). Thus tissues that develop from other *Nkx3.2* positive regions, such as cells of the rib cage and joints, are also expected to be sensitive to DT treatment and loss of these cells may be contributing to an overall decline in mouse health. Therefore, the majority of our analysis restricted to within the first 48 hours after treatment, when mice still remain in good overall condition.

24-48 hours after depletion, little difference is observed in overall pancreatic morphology and histology (data not shown). However, 72 hours after DT administration, acinar cell organization appears disrupted. By H&E, acinar cells appeared disorganized and showed reduced eosin staining, suggesting changes in granularity (Figure 3.4B). Additionally, the expression of amylase appeared reduced and its distribution within the cell was altered, appearing more condensed due to reduced cell size and less granular (Figure 3.4C). Amylase transcript (*Amy2*) expression was also significantly reduced, as was expression of the acinar cell specific transcription factor *Mist1* (Figure 3.4D). Acinar cells appear constricted, both by histology and amylase staining, and also are reduced in

Figure 3.4: PaMC depletion leads to disorganization and disruption of acinar tissue (**A**) Effective depletion of PaMCs 24hrs after DT treatment. FACS analysis of $PDGRF^{+}$ cells in the pancreas of untreated transgenic controls compared to transgenic animals 24 hours after DT injection. Numbers are expressed as a percentage of live cells. N=3. (**B**) Acini appear disorganized 72hrs after depletion. H&E staining of non-transgenic and transgenic animals 72hrs after treatment with DT. (**C**) Tissue from non-transgenic and transgenic animals 72hrs after treatment with DT stained with an antibody against amylase (green). (**D**) Reduction expression of amylase specific genes. RNA was isolated from whole pancreas 72 hours after treatment with DT and expression of *Amy2* and *Mist1* was analyzed by qPCR. (**E**) Acinar cell size is reduced and acinar polarity is disrupted following PaMC depletion. Acinar cell size was quantified by $Amylase^{+}$ area/total nuclei in $Amylase^{+}$ area. Nuclear localization was scored as either basal or non-basal. N=3. *P < 0.05, **P < 0.01, ***P < 0.005, NS = non-significant, as compared to treated non-transgenic mice. Data represent the mean ± SD.


A
PDGFRα+ cells
Relative to untreated
1.5
1
0.5
0
***
0
24
Time after
DT injection (hrs)
PBS treated
Diptheria Toxin, 72 hours
B
B'
H&E
C
C'
Amylase
D
Amylase expression
Mist1 expression
Relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
****
72hrs after DT
Non tg
Nkx3.2-Cre;DTR
E
Acinar cell size
Nuclear position
Amylase area/nuclei
8
6
4
2
0
**
Proportion of basal nuclei
1.0
0.8
0.6
0.4
0.2
0.0
**

size (Figure 3.4E). Finally, the basal localization of acinar cell nuclei is disrupted, indicating changes in acinar cell polarization (Figure 3.4E). These data indicate that PaMCs are required for maintaining acinar cell polarity and maturity.

PaMCs are required for regulation of hormone secretion

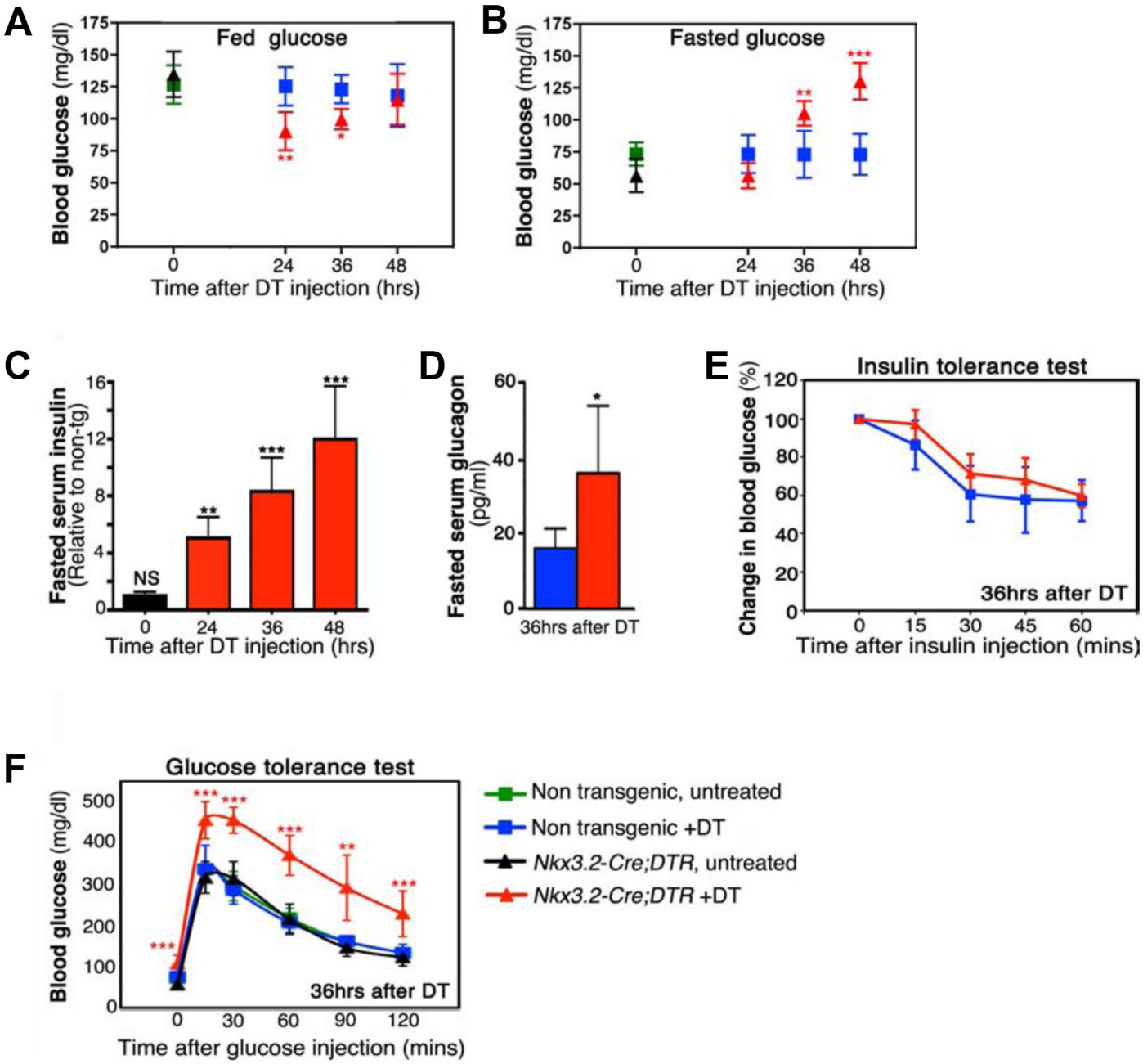
Because PaMCs are observed surrounding and interacting with islets, we examined the effects of PaMC depletion on glucose homeostasis and endocrine function. 24 hours after DT treatment, transgenic mice were initially hypoglycemic compared to DT treated non-transgenic controls. However, this phenotype reverted to normoglycemia by 48 hours after treatment (Figure 3.5A). Interestingly, the opposite phenotype is observed following an overnight fast, with DT treated transgenic mice presenting with hyperglycemia at 36 and 48 hours (Figure 3.5B). Unexpectedly, these fasted, hyperglycemic mice had elevated serum insulin at all time points examined (Figure 3.5C). This seemingly contradicting data could be explained by a few possibilities, including insulin insensitivity and elevation of glucagon. An insulin tolerance test demonstrated that PaMC depleted mice retain insulin sensitivity (Figure 3.5E), but do have elevated serum glucagon levels (Figure 3.5D), which is a possible explanation for the increase in blood glucose levels despite elevated serum insulin. Overall, these data indicate that in the absence of PaMCs, proper regulation of hormone secretion is lost, resulting in a lack of regulation of blood glucose levels.

Hypersecretion of hormones may be a result of lack of regulation of secretion or of cell death resulting in the release of cellular contents. Depletion of PaMCs did not alter islet morphology (Supplementary Figure A2.1A,B) or islet cell size (Supplementary Figure A2.1E,F) and did not result in increased apoptosis of β-cells as indicated by

Figure 3.5: Blood glucose and hormone secretion are deregulated upon PaMC depletion

Nkx3.2-Cre;DTR and age-matched non-transgenic controls were either left untreated (green and black, respectively) or injected with 4 ng/gr body weight Diphtheria Toxin (DT; blue and red, respectively).

(**A**,**B**) Blood glucose was measured in 9-12 wks old transgenic and control males (A) Transgenic mice were hypoglycemic under fed conditions 24 and 36 hrs after treatment. N=4. (B) Following an overnight fast, transgenic mice were hyperglycemic 36 and 48 hrs after treatment. N = 6-10. (**C**) Increased serum insulin upon PaMC depletion. Serum was collected following an overnight fast and insulin was measured by ELISA. To accommodate for changes between experiments, the ratio between transgenic and similarly treated non-transgenic (non-tg) controls is shown. N = 4. (**D**) Increased serum glucagon levels upon PaMC depletion. Serum was collected after an overnight fast from control and transgenic mice at 36 hrs after DT treatment, and analyzed by RIA. N = 4-5. (**E**) Mice are not insulin resistant following PaMC depletion. After an overnight fast, control and transgenic mice (36 hrs after DT treatment) were injected i.p. with 1 U/kg body weight insulin and blood glucose levels were measured at indicated time points. N = 8. (**F**) Mice are glucose intolerant following PaMCs depletion. After an over night fast, untreated and DT-treated mice (36 hrs after treatment) were injected i.p. with 2 mg/gr body weight Dextrose and blood glucose levels were measured at indicated time points. N = 8. *P < 0.05, **P < 0.01, ***P < 0.005, NS = non-significant, as compared to untreated transgenic mice (A) or treated non-transgenic control (B-G). Data represent the mean ± SD.


A
Fed glucose
Blood glucose (mg/dl)
Time after DT injection (hrs)
B
Fasted glucose
Blood glucose (mg/dl)
Time after DT injection (hrs)
C
Fasted serum insulin (Relative to non-tg)
NS
Time after DT injection (hrs)
D
Fasted serum glucagon (pg/ml)
36hrs after DT
E
Insulin tolerance test
Change in blood glucose (%)
36hrs after DT
Time after insulin injection (mins)
F
Glucose tolerance test
Blood glucose (mg/dl)
36hrs after DT
Time after glucose injection (mins)
Non transgenic, untreated
Non transgenic +DT
Nkx3.2-Cre;DTR, untreated
Nkx3.2-Cre;DTR +DT

TUNEL staining. Arrows mark the remainder of dying PaMCs (Supplementary Figure A2.1C,D). Importantly, vasculature also does not appear to be disrupted (Supplementary Figure A2.2). Therefore, hypersecretion is likely the result of defects in β-cell function.

Depletion of PaMCs results in β-cell dysfunction

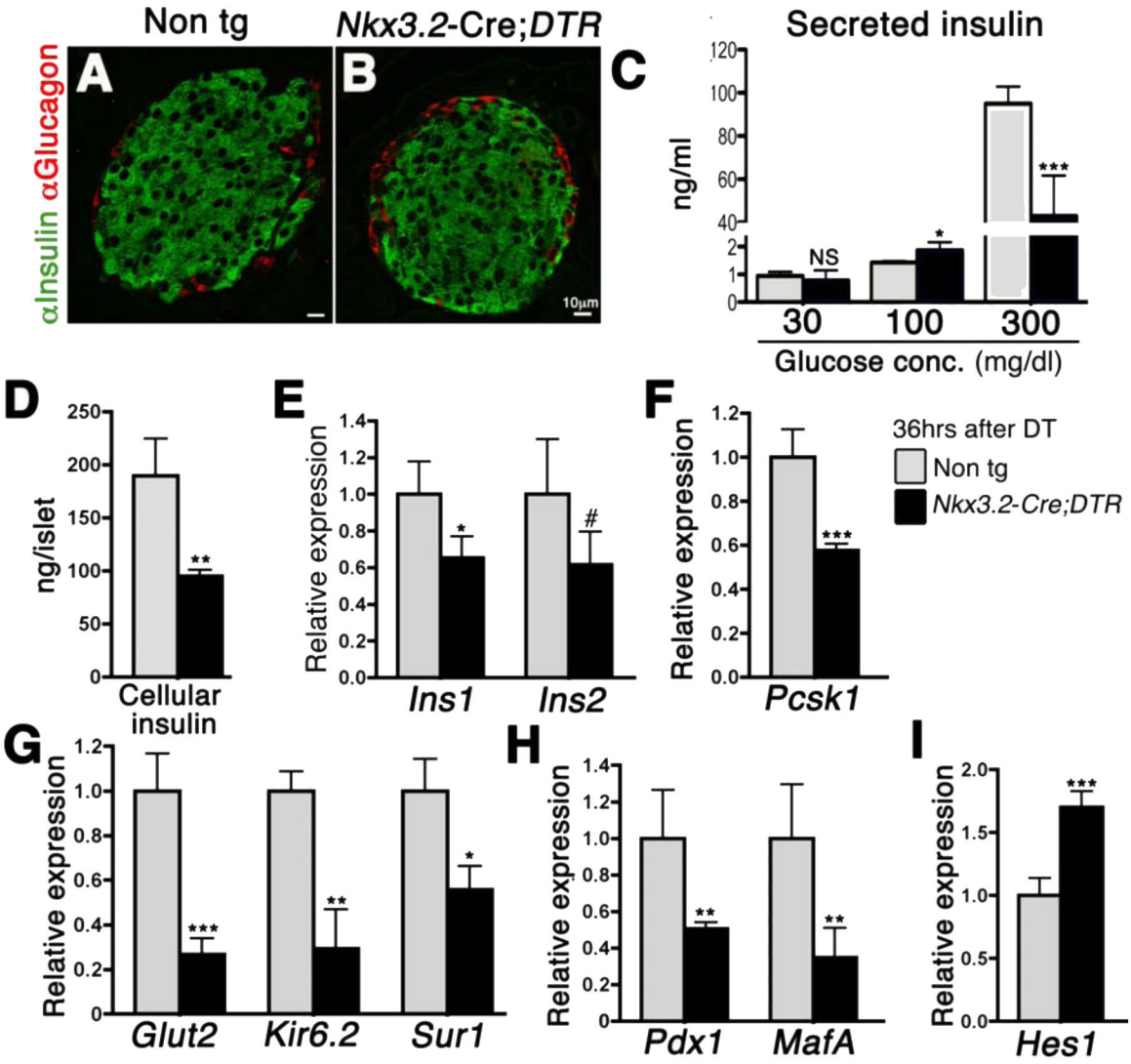
In order to test for defects in β-cell function, glucose tolerance tests were performed. DT treated mice are glucose intolerant and are incapable of responding properly to increases in blood glucose (Figure 3.5F). In confirmation of *in vivo* findings, under conditions mimicking normoglycemia (100mg/dl glucose), depleted islets secrete more insulin than controls, but under high glucose conditions (300mg/dl glucose), they do not respond by increasing their insulin secretion, unlike controls (Figure 3.6C). This lack of insulin secretion may be due to insufficient insulin production in combination with reduced protein levels in the cell due to earlier hypersecretion. In fact, expression of *Ins1* is significantly reduced and *Ins2* expression is decreased, although this decrease does not reach statistical significance (Figure 3.6E). Additionally, expression of glucagon (*Gcg*) is also significantly reduced (Supplementary Figure A2.3B), indicating a disruption of hormone production in α-cells as well as in β-cells. Reduced expression of the proprotein convertase 1/3 (PC1/3) that converts pro-insulin to insulin (Goodge, 2000) provides additional evidence for disruption of insulin production as a result of PaMC depletion (Figure 3.6F).

The reduction of overall quality of health of DT treated mice may contribute to changes in endocrine function. To address these concerns, isolated islets from untreated transgenic mice were isolated and treated with DT *in vitro.* Insulin expression is also reduced under these conditions (Supplementary Figure A2.3A), indicating that defects in

Figure 3.6: Loss of ß-cell maturity upon depletion of PaMCs

9-12 week old *Nkx3.2-Cre;DTR* (black) and non-transgenic (non tg) control (gray) were injected i.p. with DT and analyzed 36 hrs after treatment.

(**A**, **B**) Normal endocrine cell distribution in transgenic islets. Pancreatic tissues from transgenic (B) and control (A) mice were stained with antibodies against insulin (green) and glucagon (red). (**C**) Elevated insulin secretion in islets isolated from DT-treated transgenic mice. Isolated islets were exposed to 30, 100 or 300 mg/dl glucose for one hour and secreted insulin levels were measured by ELISA. N = 4. (**D**) Reduced insulin content in islets isolated from 36 hour DT-treated transgenic animals compared to nontransgenic controls, measured by ELISA. N = 4. (**E-I**) Reduced expression of b-cell specific genes and elevated expression of *Hes1* in islets isolated from DT-treated transgenic mice as compared to non-transgenic controls. RNA was extracted from freshly isolated islets and expression of indicated genes was analyzed by qPCR. N = 4. *P < 0.05, **P < 0.01, ***P < 0.005, NS = non significant, as compared to non-transgenic control. Data represent the mean ± SD.


Non tg
Nkx3.2-Cre;DTR
αInsulin αGlucagon
A
B
10μm
C
Secreted insulin
ng/ml
NS
*
***
30
100
300
Glucose conc. (mg/dl)
D
ng/islet
**
Cellular insulin
E
Relative expression
*
#
Ins1
Ins2
F
Relative expression
***
Pcsk1
36hrs after DT
Non tg
Nkx3.2-Cre;DTR
G
Relative expression
***
**
*
Glut2
Kir6.2
Sur1
H
Relative expression
**
**
Pdx1
MafA
I
Relative expression
***
Hes1

insulin production are due to depletion of PaMCs, not changes in overall mouse health. Summarily, these data indicate that PaMCs are required for maintenance of proper hormone production and glucose-dependent secretion.

β-cell dedifferentiation underlies defects in glucose homeostasis

In order to better understand the underlying causes for β-cell dysfunction, we characterized the expression of critical components of the glucose sensing machinery, *Glut2, Kir6.2* and *Sur1* (Ashcroft, 2005; Thorens, 2001). Expression of all three of these factors was significantly reduced (Figure 3.6G). Additionally, β-cell dedifferentiation is often a key contributing factor to β-cell dysfunction. The transcription factors *Pdx1* and *MafA*, which are critical for establishing the mature β-cell state (Ahlgren, 1998; Zhang, 2005), are also significantly reduced in islets isolated from DT treated mice (Figure 3.6H). Finally, expression of the Notch pathway transcription factor *Hes1*, which is normally excluded from β-cells and is inappropriately expressed in β-cells in other systems of dedifferentiation and dysfunction (Bar, 2008; Kopinke, 2011; Landsman, 2011b), is significantly increased (Figure 3.6I), indicating that β-cells in PaMC depleted islets are undergoing dedifferentiation. Reduction of the α-cell specific transcription factor *Arx* (Supplementary Figure A2.3B) suggests that this dedifferentiation phenotype is not restricted to β-cells, but is an underlying cause of dysfunction of in α-cells as well. Overall, this data suggests that PaMCs support the functionality of endocrine cells by promoting their mature differentiation state.

PaMCs produce Laminin α2 which helps maintain β-cell differentiation

PaMCs may be impacting β-cells through a number of mechanisms ranging from the production of secreted factors and signaling molecules and direct cell-cell

interactions. Microarray analysis of the PaMC population revealed that these cells produce many components of the ECM that may be important for β-cell function, including laminins. Laminin consists of three chains, two of which (β and γ) are broadly expressed and one, α, that provides specificity. Interestingly, it has been documented that β-cells do not produce extracelluar matrix, instead relying on other cell types, such as endothelial cells (Huang, 2012; Kragl, 2011; Nikolova, 2006), to produce a basement membrane for them. For example, Laminins α4 and α5 are produced by endothelial cells and promote insulin expression (Nikolova, 2006). Expression of all α chains are decreased following PaMC ablation, but most interestingly, Laminin α2 expression is nearly completely lost (Figure 3.7A). PaMCs, but not endothelial cells, express the transcript, suggesting that PaMCs are the primary source of Laminin α2 in the pancreas (Figure 3.7B). Immunostaining shows the localization of the α2 chain surrounding islets (Figure 3.7C). Following depletion, a significant number of islets have lost the Laminin α2 layer that surrounds them (Figure 3.7D), although presence of Laminin α2 is still observed around some islets (Supplementary Figure A2.4). Laminins are known to have a long half-life (Urich, 2011), so it is possible that even islets that still appear encapsulated are being affected by subtle changes in protein structure and organization (Naom, 2000) may be occurring at earlier time points, although overall protein is not yet lost.

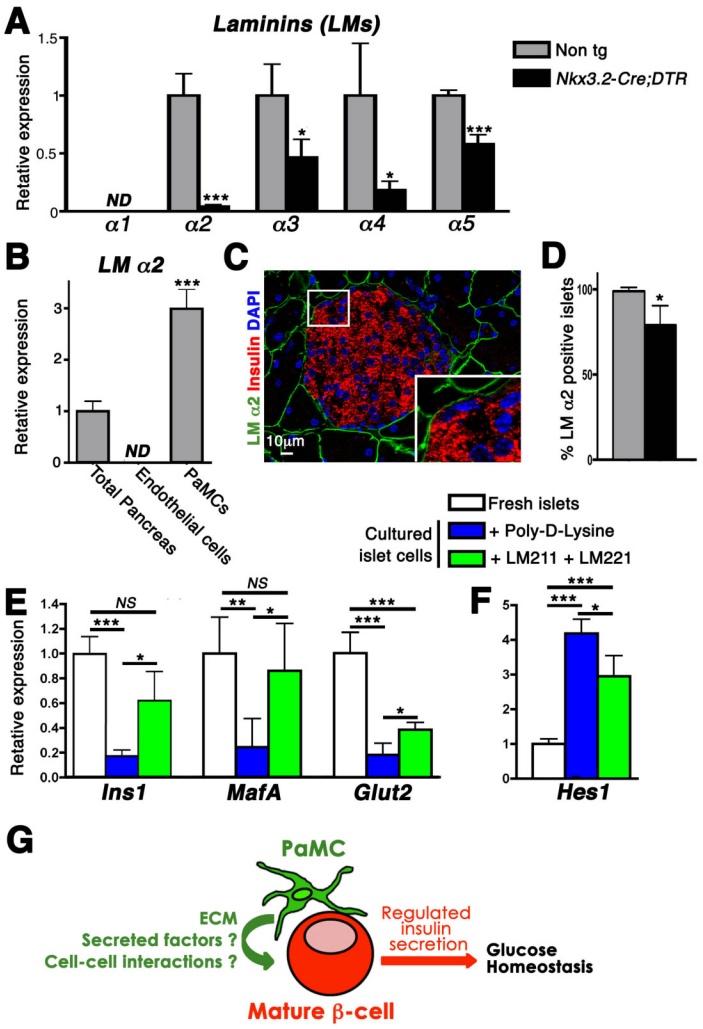
To test if Laminin α2 produced by PaMCs promotes β-cell function functionality and maturity, islets were isolated from PaMC depleted mice, dispersed into single cells and cultured on Laminins 211/221 or poly-D lysine as a control. This type of culture induces the dedifferentiation of β-cells, as observed by decreased expression of *Ins1*, *MafA* and *Glut2* and increased expression of *Hes1* compared to fresh islets (Figure 3.7E,

F, poly-D lysine controls). However, culture on LM211/221 significantly reduced the extent of dedifferentiation that occurred and cells cultures on LM211/221 expressed significantly higher levels of *Ins1*, *MafA* and *Glut2* compared to poly-D lysine controls (Figure 3.7E), with the levels of *Ins1* and *MafA* being comparable to that of isolated islets. Induction of *Hes1* expression was also partially blocked by culture on LM211/221 (Figure 3.7F). Thus, expression of Laminin α2 by PaMCs is partially responsible for maintenance of β-cell maturity, and therefore functionality.

Figure 3.7: Laminin a2 is specifically produced by PaMCs and partially protects β-cells from culture-induced dedifferentiation

(**A**) Expression of various Laminin (LM) a-chains is reduced due to PaMC depletion. RNA was extracted from pancreatic tissues of non-transgenic (gray bars) or *Nkx3.2-Cre*;*DTR* (black bars) mice, 24hrs after DT treatment and gene expression was analyzed by qPCR. Laminin a1 *(Lama1)* transcript was not detected. N=4. *P < 0.05, ***P < 0.005 as compared to total untreated pancreata. ND= Not detected. (**B**) *Lama2,* encoding the LM a2 chain, is expressed by PaMCs. RNA was isolated from bulk pancreatic tissues ("total pancreas"), pancreatic endothelial cells ($PECAM1^+$ cells, isolated by flow cytometry from total pancreata) and PaMCs ($Nkx3.2/YFP^+$ cells, isolated by flow cytometry from *Nkx3.2-Cre*;*YFP* total pancreata). *Lama2* expression was analyzed by qPCR. Importantly, *Lama2* transcript was not detected in endothelial cells. N=4. ***P < 0.005 as compared to total untreated pancreata. ND= Not detected. (**C**) LM a2 encapsulates islets. Fixed pancreatic tissue was stained with antibodies against Laminin a2 chain (green), insulin (red), and counterstained with DAPI (blue). Insert shows higher magnification of the area framed in white box. (**D**) Reduced number of islets surrounded by LM a2 chain in transgenic islets (black) as compared to control (gray), 36 hrs after DT treatment. Islets were stained as described for C and percentage of LM-positive islets was determined. N=5 mice (n>70 islets/mouse). *P < 0.05 as compared to non-transgenic controls. Data represent the mean ± SD. (**E**,**F**) LMs 211 and 221 partially inhibit b-cell dedifferentiation induced by culture. RNA was extracted from freshly isolated islets (white) or from trypsin-dispersed islet cells after 3 days of culture on plates coated with either Poly-D-Lysine (blue) or human Merosin (a mixture of LM211 and LM221, green).

N=4. Data show one representative of two independent experiments with comparable results. *P < 0.05, **P < 0.01, ***P < 0.005, NS=non significant, as compared to freshly isolated islets. (**G**) A model for PaMC function in glucose regulation. PaMCs (green) secrete ECM components, including Laminins, to support b-cell (red) maturity and functionality. PaMCs likely also provide additional secreted factors and possibly direct interactions to support endocrine cells.


A
Laminins (LMs)
Retative expression
Non tg
Nkx3.2-Cre;DTR
ND
α1
α2
α3
α4
α5
B
LM α2
Retative expression
ND
Total Pancreas
Endothelial cells
PaMCs
C
LM α2 Insulin DAPI
10µm
D
% LM α2 positive islets
Fresh islets
Cultured islet cells
+ Poly-D-Lysine
+ LM211 + LM221
E
Retative expression
NS
Ins1
MafA
Glut2
F
Hes1
G
PaMC
ECM
Secreted factors ?
Cell-cell interactions ?
Regulated insulin secretion
Glucose Homeostasis
Mature β-cell

Discussion

In this study, we identified and characterized a population of fibroblasts, PaMCs, which originates from the Nkx3.2+ mesenchyme. These cells make close interactions with acini, ducts and islets within the pancreas and are capable of responding to changes in the pancreatic environment, such as inflammatory damage due to caerulein pancreatitis. Importantly, this work provides evidence for the role of PaMCs in pancreas homestasis, both in the exocrine and endocrine compartments. In the absence of PaMCs, acinar cells become disorganized and lose expression of enzymes and transcription factors that are critical for their function. The factors regulating these changes, as well as the effects on this disorganization on exocrine function, remain to be determined. In the absence of PaMCs, endocrine function is severely disrupted. PaMCs are required for regulation of hormone secretion, and in their absence, hypersecretion occurs, resulting in a disruption of both fasting and fed blood glucose regulation. Underlying this lack of blood glucose regulation is β-cell dysfunction and dedifferentiation, as indicated by decreased expression of insulin, insulin processing components, glucose sensing machinery and β-cell specific transcription factors. We demonstrate that PaMCs are the source of Laminin α2 in the pancreas, which can promote β-cell functionality and maturity.

PaMCs are likely providing many factors influencing different aspects of β-cell biology. The connection between the hypersecretion and dedifferentiation phenotypes is currently unclear. It is possible that both are direct effects of the loss of signals from PaMCs; alternatively, deregulation of secretion could in turn force the reduction of insulin production and induce β-cell dedifferentiation as a protective mechanism. Further

characterization and dissection of the communication between PaMCs and β-cells is required in order distinguish between these possibilities.

A further question is the relationship between fibroblasts and the development of diabetes. β-cell dysfunction is a key step in the development of this disease (Marchetti, 2010), and it has been noted to occur alongside pancreatic cancer and pancreatitis (Wang, 2003). It will be interesting to examine if changes in the fibroblast population that occur during these diseases can induce β-cell dysfunction. Additionally, a high number of mutations have been found to be associated with the development of diabetes, but their contribution of the disease is currently unclear (Doria, 2008). It may be possible that some of these mutations affect the relationship between β-cells and fibroblasts. Ultimately, this study reveals PaMCs as a possible player in the development of diabetes and provides evidence that may connect various pancreatic diseases.

Methods

Mice

Mice used in this study were maintained according to protocols approved by the Committee on Animal Research at the University of California, San Francisco. *Nkx3.2 (Bapx1)*-Cre mice were described previously (Verzi, 2009). *R26-YFP*^{flox} (*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$) and *iDTR*, (*Gt(ROSA)26Sor*$^{tm1(HBEGF)Awai}$*)* mice were obtained from Jackson Laboratories, and wild-type C57BL mice from Charles Rivers laboratories.

Caerulein treatment

Acute pancreatitis was induced at 5-6 weeks, weight range 22-25g of age in p48Cre, p48Cre;Numb$^{f/f}$, p48Cre;Kras^{G12D} and p48Cre;Kras^{G12D};Numb$^{f/f}$ mice as previously described (Jensen, 2005). Briefly, mice received 8 hourly i.p injections of caerulein (American Peptide Company), 2ug/injection on 2 consecutive days. Day 0 is counted as immediately following the final caerulein injection.

Cell depletion

Mice were i.p. injected with a single dose of 4 ng Diphtheria Toxin (Sigma) per gram body weight.

Immunohistochemistry

For human tissue, fixed human pancreatic tissue (Prodo) was embedded in OCT (TissueTek), cryopreserved and cut into 10mm thick sections. For mouse tissue, dissected mouse pancreas was fixed with Z-fix (Anatech) overnight, embedded in paraffin wax and cut into 5mm thick sections. Prior to Laminin a2 staining, dissected tissues were fixed for

4 hrs with Z-fix, embedded in OCT (TissueTek), cryopreserved and cut into 10mm thick sections.

For immunofluorescence (except PECAM1), paraffin-embedded tissues underwent heat-mediated Citra antigen retrieval (BioGenex). Prior to PECAM1 staining, tissues were treated with 4mg/ml Proteinase K (Roche). Tissue sections were stained using the following primary antibodies: rabbit anti-Amylase (1:200, Sigma), mouse anti-Desmin (1:200, Dako), rabbit anti-Glucagon (1:200, Linco), guinea pig anti-Insulin (1:200, Linco), rat anti-Laminin a2 (1:200, Enzo), rat anti-PECAM1 (1:200, BD), mouse anti- mouse Vimentin (1:200, Sigma) mouse anti- human Vimentin (1:50, Calbiochem) and chicken anti-YFP/GFP (1:400, Abcam) followed by staining with AlexaFluor tagged secondary antibodies (1:500, Invitrogen) and mounting with DAPI-containing Vectashield media (Vector).

For TUNEL analysis, ApopTag Plus In Situ Peroxidase Apoptosis Detection kit (Millipore) was used according to manufacturer's protocol on paraffin-embedded tissues, followed by counterstaining with Meyer's Hematoxylin (Sigma).

For histological analysis, tissue sections were stained with Meyer's Hematoxylin followed by Eosin using standard protocols.

Images were acquired using Zeiss ApoTome and Leica SP5 microscopes.

Islet imaging and culturing

Islets were isolated by the UCSF Islet Isolation core, according to standard protocols. Freshly isolated islets were incubated with Hoechst 33342 (Sigma) and analyzed using an inverted Leica SP5 confocal microscope. For ECM analysis, isolated islets were dispersed into single cells by 5-10 min incubation with 0.25% Trypsin (Gibco)

and filtered. Cells were incubated for 3 days either on poly-D-Lysine coated-plates (Millipore) or on plates coated overnight with 5 mg/ml human Merosin (Millipore), which is comprised of a mixture of LM211 and LM221.

Flow cytometry

Isolated islets were incubated in 0.25% Trypsin for 5-10 min at 37^0C. Dissected pancreata were digested in 0.4 mg/ml Collagenase P (Roche) and 0.1 ng/ml DNase (Sigma) diluted in HBSS for 30 min at 37^0C and filtered through a 40 mm filter. Followed staining (Table S1), cell analysis was performed using FACS caliber (BD), and cell isolation using FACS Aria (BD).

Microarray analysis

Nkx/YFP$^+$ cells were purified from pancreata by flow cytometry as described above. RNA was isolated with the RNeasy Micro kit (Qiagen). Microarray analysis was performed by the UCSF Gladstone Genomics Core using Affymetrix Mouse Exon 1.0 ST Array.

Hormone detection

Insulin levels were determined using mouse ELISA kit (Mercodia) and Glucagon content using RIA kit (Millipore), according to manufacturers' protocols.

Quantitative PCR

RNA was extracted using RNeasy kit (Qiagen), followed by a reverse transcription reaction with SuperScript II (Roche). Expression of *Ins1* (primers: GGGTCGAGGTGGGCC and CTGCTGGCCTCGCTTGC), *MafA* (primers: GCTGGTATCCATGTCCGTGC and TGTTTCAGTCGGATGACCTCC) and *Arx* (primers: GGCCGGAGTGCAAGAGTAAA and GCTCCCAGAAGCCTCATTTTG)

was analyzed using SYBR green assay (Applied Biosystems) and normalized to *Cyclophilin* (primers: TGCCGCCAGTGCCATT and TCACAGAATTATTCCAGGATTC). Expression of additional genes was analyzed using Taqman assays (Applied Biosystems) and normalized to *Cyclophilin* (Primers: GGCCGATGACGAGCCC, and TGTCTTTGGAACTTTGTCTGCAA, probe: TGGGCCGCGTCTCCTTCGA).

Blood vessels imaging

Dextran (70 kDa) conjugated to Texas Red dye (Invitrogen) was i.v. injected to mice. Mice were euthanized 30 min after injection and their pancreatic tissue was embedded in OCT and fresh frozen. Unfixed tissue sections were analyzed using Zeiss AxioImager microscope.

Statistical analysis

Results are shown as the mean ±SD. Paired data were evaluated using a two-tailed Student's *t*-test.

CHAPTER 4

Neutrophils regulate pancreatic damage, but do not significantly contribute to development of PDA

Summary

Dramatically expanded and altered stroma, including fibroblasts, immune cells and endothelial cells, is a hallmark of pancreatic cancer. Acinar cells undergoing metaplasia induce expression of cytokines and other damage signals that lead to an inflammatory response very early on the disease process. The inflammatory milieu also changes over time, shifting from mostly suppressor cells to more pro-tumorigenic cell types. Neutrophils have been shown to promote the severity of pancreas and lung damage that is induced in response to caerulein treatment. Pancreatitis-associated damage of acinar cells creates a permissive environment to allow for these cells to undergo ductal metaplasia. Surprisingly, although pancreatic damage and acinar dedifferentiation is attenuated in the absence of neutrophils, formation of precursor lesions called pancreatic intraepithelial neoplasias (PanINs) and progression to PDA is not significantly affected.

Introduction

Tumorigenesis is a process that is both intrinsic to the initiating cell, as well as impacted and influenced by the surrounding microenvironment, including the immune system. Inflammatory cells may play dual roles in the development and progression of cancer, acting through immuno-surveillance to identify and destroy tumor cells (reviewed in Pardoll, 2003), but also produce pro-tumorigenic factors that can promote tumor cell proliferation, angiogenesis and metastasis among others (reviewed in Coussens, 2002; Tlsty, 2006). A number of cell types including, but not limited to, B cells, macrophages, and mast cells have been shown to be involved in development and promotion of various tumors (Coussens, 1999; de Visser, 2005; Nowicki, 1996).

Pancreatic ductal adenocarcinoma (PDA) is a tumor type that consists of significant stromal infiltration, both of fibroblasts and a variety of inflammatory populations. The immune response throughout disease progression has been characterized. Infiltration into the pancreas occurs early in the process, significant numbers of CD45+ cells are found even infiltrating as soon as precursor lesions, known as pancreatic intraepithelial neoplasias (PanINs), are observed. This infiltrate consists of a large portion of innate immune cells, especially macrophages, and slightly later in disease progression, myloid derived suppressor cells (MDSCs) (Clark, 2007). Neutrophils do not appear to be a significant component of invasive PDA, but often associate with some types of precursor lesions such as MCNs and IPMNs (Reid, 2011). However, neutrophils have been strongly associated with pancreatitis, a condition that increases risk for development of pancreatic cancer. In fact, neutrophils promote the activation of pancreatic enzymes during caerulein pancreatitis and suppression of neutrophils can

reduce the severity of symptoms of pancreatitis such as excessive release of pancreatic enzymes into the bloodstream and associated lung injury (Abdulla, 2011; Bhatia, 1998; Pastor, 2006; Sandoval, 1996). Additionally, release of proteinases, specifically MMP-9, from neutrophils, can regulate the angiogenic switch in a mouse model of a pancreatic neuroendocrine tumor (PNET) and in PDA xenografts (Bausch, 2011; Nozawa, 2006). Therefore, we asked whether neutrophils were involved in acinar dedifferentiation in response to caerulein treatment and acinar to ductal metaplasia that occurs in the context of activation of Kras.

Interestingly, expression of the granulocyte colony stimulating factor (G-CSF or CSF3) is detected in human PDA cell lines and also detected at increasing levels in the blood of PDA mice, indicating that PDA may be inducing the production and recruitment of neutrophils. In order to determine if neutrophils are involved in pancreatitis and acinar to ductal metaplasia, we utilized a mouse carrying a deletion of the granulocyte colony stimulating factor receptor (G-CSFR, also known as CSF3R). This receptor is required for the maturation of granulocytes and these mice have severely reduced numbers of peripheral neutrophils (Liu, 1996). As suggested by previous work, these mice are partially resistant to acinar dedifferentiation in response to caerulein-induced pancreatitis, confirming the role of neutrophils in promotion of pancreatic damage. However, PanIN and PDA formation appeared unaffected, indicating that these processes are not exclusively dependent on the presence of neutrophils.

Results

G-CSF and G-CSFR expression is increased in pancreatic ductal adenocarcinoma

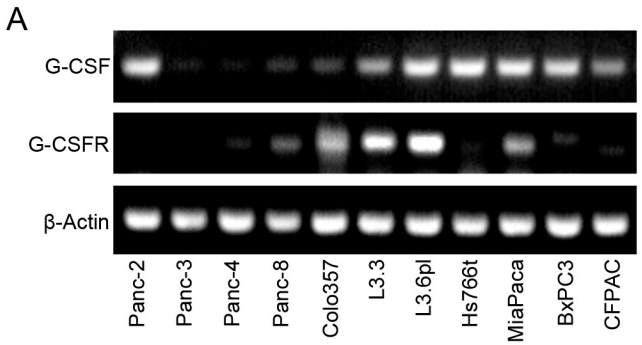
A panel of human PDA cell lines was assayed for their expression of G-CSF. G-CSF expression by tumors could contribute to increasing neutrophil production and release from the bone marrow. A number of cell lines were found to express G-CSF, although this was not universal (Figure 4.1A). Additionally, a smaller portion of cell lines express the receptor, G-CSFR, indicating that some tumors may have the capability to respond to G-CSF in a cell autonomous fashion (Figure 4.1A). In order to assess the expression of the receptor *in vivo*, normal acinar cells and tumor cells from *p48Cre;Kras^{G12D};p53$^{f/wt}$* PDA were isolated by FACS. Very limited expression of the receptor can be detected in normal acinar cells, and a slightly increased expression can be detected in mouse PDA cells, although levels still remain very low (Figure 4.2B). Elevated levels of G-CSF can also be detected *in vivo* in the serum of *p48Cre;Kras^{G12D};p53$^{f/wt}$* mice, with levels increasing with disease progression (Figure 4.1C). By 11 weeks, many mice have developed invasive PDA. However, this data does not directly indicate production of G-CSF by the tumor cell itself, but does suggest that neutrophil production and release in these mice may be enhanced.

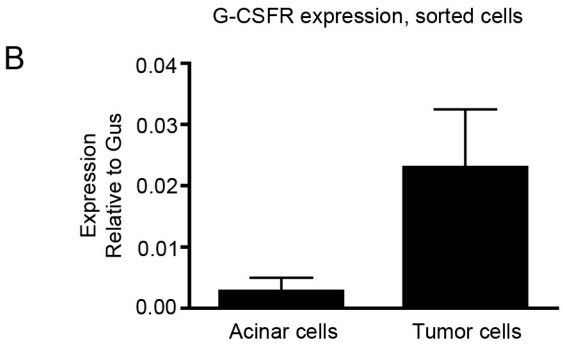
Neutrophils promote pancreatic damage and acinar cell de-differentiation associated with caerulein-induced pancreatitis

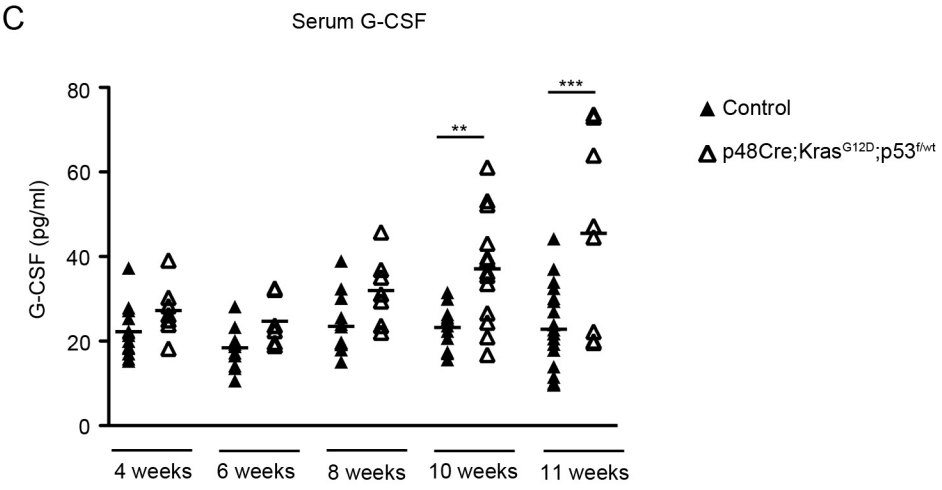
In order to determine if neutrophils play a role in caerulein-induced pancreatitis, control and G-CSFR$^{-/-}$ mice were treated with caerulein and analyzed 2 days later, when significant acinar dedifferentiation is observed in controls. G-CSFR$^{-/-}$ mice have reduced acinar damage as demonstrated by retention of amylase and reduction expression of the

Figure 4.1: G-CSF and G-CSFR expression in the pancreas and PDA

(A) G-CSF and G-CSFR expression was analyzed in a panel of human PDA cell lines by semi-quantitative PCR and compared to β-Actin as a control. (B) Acinar cells from wild type mice and duct/tumor cells from p48Cre;$Kras^{G12D}$;$p53^{f/wt}$ were isolated by FACS. Expression of G-CSFR was determined by quantitative PCR. (C) G-CSF protein was detected at elevated levels by ELISA in the serum of PDA mice.


A
G-CSF
G-CSFR
β-Actin
Panc-2
Panc-3
Panc-4
Panc-8
Colo357
L3.3
L3.6pl
Hs766t
MiaPaca
BxPC3
CFPAC


G-CSFR expression, sorted cells
B
Expression Relative to Gus
0.04
0.03
0.02
0.01
0.00
Acinar cells
Tumor cells


C
Serum G-CSF
G-CSF (pg/ml)
80
60
40
20
0
**
***
4 weeks
6 weeks
8 weeks
10 weeks
11 weeks
Control
p48Cre;KrasG12D;p53f/wt

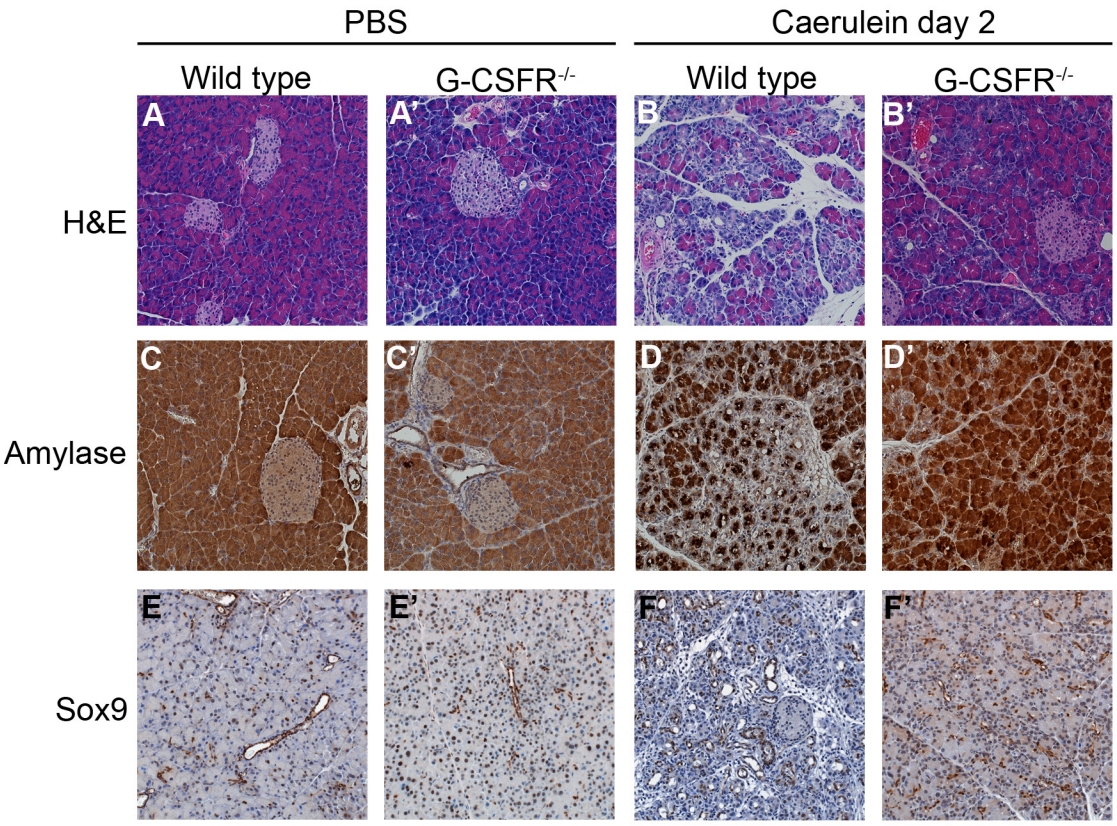
ductal and de-differentiation marker Sox9 compared to wild type and G-CSFR$^{+/-}$ controls (Figure 4.2B,D,F). Amylase positive area is significantly higher in G-CSFR$^{-/-}$ pancreata compared to controls (Figure 4.2G). This is consistent with previous studies showing that neutrophils are important for chemical pancreatitis and mis-activation of pancreatic enzymes associated with damage. In observed regions of damage, deletion of G-CSFR did not affect overall immune infiltration as indicated by the number of CD45+ cells (Figure 4.3B). Additionally, cell proliferation and vasculature as indicated by Ki67+ and CD31+ cells, respectively were not significantly affected (Figure 4.3A,C). This suggests that neutrophils contribute to promotion of acinar dedifferentiation associated with caerulein pancreatitis, but are not necessarily required.

Absence of neutrophils does not impact PanIn formation or progression to PDA

Acinar dedifferentiation creates a permissive state for oncogenic Kras to act in order to drive acinar to ductal metaplasia (ADM). Since neutrophils promote the adoption of this state during caerulein pancreatitis, we next asked if neutrophils promote ADM in response to Kras. At 6 weeks of age, similar numbers of PanINs and comparable metaplastic area are observed in *p48Cre;Kras^{G12D}* and *p48Cre;Kras^{G12D};G-CSFR$^{-/-}$* mice, suggesting that neutrophils do not play a significant role in induction of PanIN lesions (Figure 4.4A-D). Alternatively, given the longer time frame of this study compared to caerulein pancreatitis, another cell type could have compensated for the lack of neutrophils. Additionally, progression to PDA is not affected, as both genotypes developed advanced PanIN lesions and invasive disease within the same time frame (Figure 4.4E-I).

Figure 4.2: Neutrophils promote acinar dedifferentiation in response to caerulein pancreatitis

(A,C,E) Pancreata appear normal in G-CSFR$^{-/-}$ mice by H&E, Amylase and Sox9 staining. (B,D,F) H&E, Amylase and Sox9 staining of wild type and G-CSFR$^{-/-}$ tissue at day 2 following caerulein treatment. Note significant retention of eosin and amylase staining area, an indication of mature acinar cells as well as reduced appearance of Sox9+ cells. (G) Quantification of amylase positive area at day 2.


PBS
Caerulein day 2
Wild type
G-CSFR-/-
Wild type
G-CSFR-/-
H&E
Amylase
Sox9
A
A'
B
B'
C
C'
D
D'
E
E'
F
F'

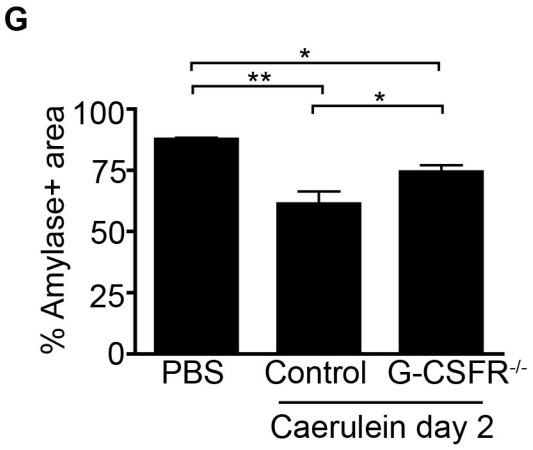

G
*
**
*
% Amylase+ area
100
75
50
25
0
PBS
Control
G-CSFR-/-
Caerulein day 2

Figure 4.3: Absence of neutrophils does not affect cell proliferation and damage response in damaged areas

Quantification performed only on areas of damage in both control and G-CSFR$^{-/-}$ at day 2, as determined by cellular morphology. (A) Quantification of cell proliferation in areas of damage as determined by expression of phospho-Histone H3. (B) Quantification of recruitment of CD45+ cells into areas of damage. (C) Quantification of number of endothelial cells in damaged areas.

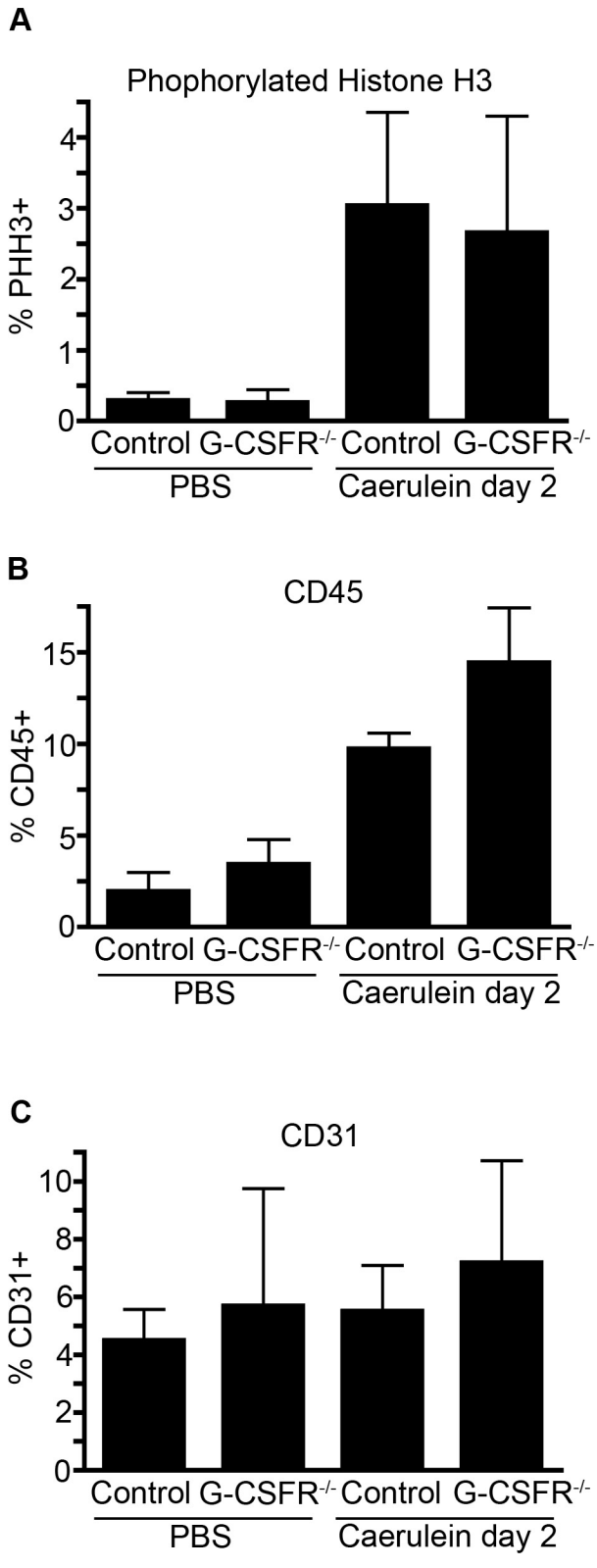
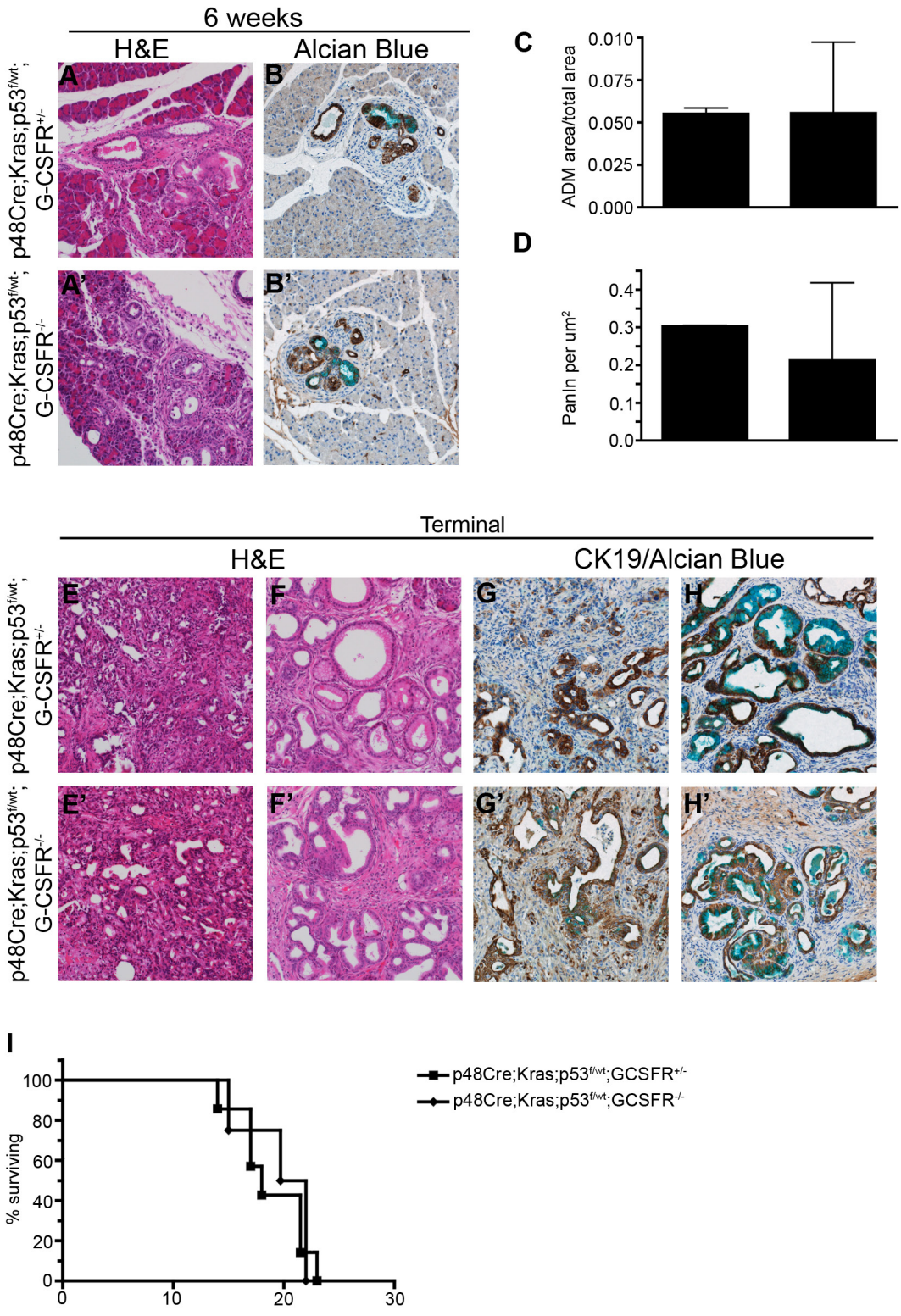

A
Phophorylated Histone H3
% PHH3+
0
1
2
3
4
Control
G-CSFR-/-
Control
G-CSFR-/-
PBS
Caerulein day 2
B
CD45
% CD45+
0
5
10
15
Control
G-CSFR-/-
Control
G-CSFR-/-
PBS
Caerulein day 2
C
CD31
% CD31+
0
2
4
6
8
10
Control
G-CSFR-/-
Control
G-CSFR-/-
PBS
Caerulein day 2

Figure 4.4: Neutrophils are not required for PanIN formation and PDA development

(A) H&E staining of 6 week pancreata from *p48Cre;Kras^{G12D};G-CSFR$^{+/-}$* and *p48Cre;Kras^{G12D};G-CSFR$^{-/-}$* mice showing presence of metaplasia and PanINs in both. (B) CK19/Alcian blue staining of 6 week pancreata from *p48Cre;Kras^{G12D};G-CSFR$^{+/-}$* and *p48Cre;Kras^{G12D};G-CSFR$^{-/-}$* mice showing presence of metaplasia and PanINs in both. (C) Quantification of percent metaplastic area of total pancreatic area. (D) Quantification of number of PanINs per area. (E, F) H&E staining of terminal stage *p48Cre;Kras^{G12D};p53$^{f/wt}$;G-CSFR$^{+/-}$* and *p48Cre;Kras^{G12D};p53$^{f/wt}$;G-CSFR$^{-/-}$* mice showing presence of invasive PDA (E) and large numbers of PanINs (F) in both. (G,H) CK19/Alcian blue staining of terminal stage *p48Cre;Kras^{G12D};p53$^{f/wt}$;G-CSFR$^{+/-}$* and *p48Cre;Kras^{G12D}; p53$^{f/wt}$;G-CSFR$^{-/-}$* mice showing presence of invasive PDA (G) and large numbers of PanINs (H). (I) Kaplan-Meier survival curve of *p48Cre;Kras^{G12D};p53$^{f/wt}$;G-CSFR$^{+/-}$* and *p48Cre;Kras^{G12D};p53$^{f/wt}$;G-CSFR$^{-/-}$* mice demonstrates a similar time course of disease.


6 weeks
H&E
Alcian Blue
A
B
A'
B'
p48Cre;Kras;p53f/wt; G-CSFR+/-
p48Cre;Kras;p53f/wt; G-CSFR-/-
C
ADM area/total area
0.010
0.075
0.050
0.025
0.000
D
PanIn per um2
0.4
0.3
0.2
0.1
0.0
Terminal
H&E
CK19/Alcian Blue
E
F
G
H
E'
F'
G'
H'
I
% surviving
100
80
60
40
20
0
0
10
20
30
p48Cre;Kras;p53f/wt;GCSFR+/-
p48Cre;Kras;p53f/wt;GCSFR-/-

Discussion

Pancreatitis is known to be an inflammatory condition, but the contribution of specific immune populations to the pathology of the disease remains unclear. We have provided evidence to confirm the role of neutrophils in the propagation of pancreatic damage, and specifically in the dedifferentiation of acinar cells, during pancreatitis. Absence of neutrophils decreases the extent of pancreatic injury and acinar dedifferentiation, indicating that neutrophils provide signals that enhance the damaging imflammatory environment. Surprisingly, although neutrophils do promote pancreatic damage and acinar dedifferentiation, they do not appear to enhance acinar to ductal metaplasia. In the absence of neutrophils, ductal reprogramming of acinar cells by Kras occurs at the same rate and frequency as in controls and progression to PDA is unaffected. However, this finding does not indicate that neutrophils play no role in PDA initiation and progression, only that in their absence, additional cell types may compensate for any signals neutrophils may normally provide to promote PDA development. Further work is required to elucidate the complicated immune environment in PDA and how inflammation affects development of the disease.

Materials and Methods

Mouse lines

Experimental animals were obtained: *p48Cre* (gift of Chris Wright, Vanderbilt University, Nashville, Tennessee, USA) with *LSL-Kras*^{G12D} (gift of Dave Tuveson, Cancer Research UK Cambridge Research Institute, Cambridge, United Kingdom) and *G-CSFR*$^{-/-}$ (gift of Daniel C. Link, Washington University, St. Louis, Missouri, USA). All mice experiments were performed with approval from the UCSF Institutional Use and Care of Animals Committee (IACUC). Littermate controls were used.

Caerulein treatment

Acute pancreatitis was induced at 5-6 weeks, weight range 22-25g of age in wild type, G-CSFR$^{+/-}$ and G-CSFR$^{-/-}$ mice as previously described (Jensen, 2005). Briefly, mice received 8 hourly i.p injections of caerulein (American Peptide Company), 2ug/injection on 2 consecutive days. Day 0 is counted as immediately following the final caerulein injection.

G-CSF detection

Blood from mice tail vein was collected into a serum separator tube (BD) and centrifuged at 8000rpm for 5min to collect serum. G-CSF expression was detected using G-CSF ELISA (Ray Biotech) according to manufacturer's protocol.

Immunohistochemistry and immunofluorescence

For histological, immunohistolochemical and immunofluorescence analysis, dissected pancreatic tissues were fixed with zinc buffered formalin (Anatech) overnight and embedded in paraffin wax. For CD31 staining, tissue sections were treated with proteinase K (prediluted, DAKO) for 30 seconds. For all other antibodies, heat mediated

antigen retrieval was performed with Citra antigen retrieval solution (BioGenex) prior to staining. Tissue sections were stained with the following primary antibodies: rabbit anti-Amylase, (1:200 Sigma-Aldrich), rat anti-CD31 (1:200 BD Biosciences), rat anti-CD45 (1:400, BD BioSciences), rat anti-CK19 (TROMAIII, 1:200, University of Iowa Hybridoma Bank), rabbit anti-phospho Histone H3 (1:200 BD Biosciences), rabbit anti-Sox9 (1:400, Millipore). For all antibodies, biotinylated goat secondary antibodies (1:400, Vector) were used. 3-3'-Diaminobenzidine tetrahydrochloride sections was used as a chromagen. For CK19, sections were counterstained with 1% Alcian Blue 8GX (Sigma) in 3% acetic acid solution and Meyer's Hematoxylin (Sigma). For all other antibodies, sections were counterstained with hematoxylin.

Brightfield images were acquired using a Zeiss Axio Imager D1 microscope.

Flow cytometry

A single cell suspension from freshly dissected pancreata was prepared as described by Morris et al (Morris, in preparation). Briefly, pancreata were digested with a series of reagents: 1) 2.5mg/ml Collagenase D (Roche) with 0.1ng/ml DNase I (Sigma-Aldrich) in Hank's buffered saline solution, 2) 0.05% Trypsin-EDTA, 3) 2U/ml Dispase (Invitrogen) in HBSS. Single cells were stained with FITC-conjugated anti-CD45 (1:400, eBioscience), PE-conjugated anti-CD49f (1:50, eBioscience) and Biotin-conjugated CD133 (Prominin1, 1:50, eBioscience). APC-conjugated streptavidin (1:100, eBioscience) was used as a secondary antibody. DAPI was used to exclude dead cells from analysis. Cells were isolated using a FACS Aria (BD BioScience).

Quantitative PCR

RNA was extracted from cell lines and sorted pancreatic cells using Trizol and RNeasy kit (Qiagen). Reverse transcription was performed using SuperScript III (Invitrogen). For quantitative PCR, expression of all genes was analyzed using Taqman assays (Applied Biosystems) and normalized to GUS expression (Primers: GGCCGATGACGAGCCC, and TGTCTTTGGCTTTGTCTGCAA, probe: TGGGCCGCGTCTCCTTCGA)

Quantification

For quantification of amylase area, PanINs number and metaplastic area, 5-10 non-overlapping 100x H&E images were taken per sample. Amylase area and total area in pixels were determined using Metamorph. Metaplastic area, in pixels, was selected by hand in Adobe Photoshop and normalized to total area in pixels calculated by Metamorph. Alcian blue positive PanINs were counted by hand and normalized to total area in pixels calculated by Metamorph. For quantification of PHH3+, CD45+ and CD31+ cells, quantification was performed on an Aperio Slide Scanner system.

CHAPTER 5

Insights for the future study on pancreatic ductal adenocarcinoma and diabetes

In the previous chapters I have discussed three distinct projects providing insight into pancreatic disease, including pancreatitis, pancreatic ductal adenocarcinoma and diabetes. While much is known about many aspects of these diseases, significant work is needed in each field to continue expanding our understanding and to provide new avenues for disease prevention, detection and treatment. I would like to highlight a few areas of specific future work as well as a few more general topics that I believe are of future interest.

Polarity and cell adhesion in pancreatic cancer

My work suggests that maintenance of cell adhesion is critical for cell differentiation and viability during acinar dedifferentiation and metaplasia. What remains to be understood is the mechanism(s) behind this requirement. The lack of FAK signaling suggests that perhaps integrins are involved and signaling downstream of them may be required to promote cell survival. Tools to better survey integrin signaling during acinar dedifferentiation, regeneration and acinar to ductal metaplasia could greatly enhance our understanding of the roles of integrins in these processes.

Additionally, the loss of cell viability in response to the absence of Numb during ADM precludes a thorough study of the role of Numb in PDA itself, although limited data (not shown) suggests that PDA can indeed form in the absence of Numb. One continuation of this work would be to better determine the role of Numb in later stages of PanIN progression and development of PDA. This could be done in a combination of ways, including the generation of cell lines from Numb null PDA mice and modulation of Numb expression in human tumor cell lines. However, while both of these approaches have their uses, they are limited in their ability to recreate a true tumor environment. The

generation of a mouse that allows for deletion of Numb through Cre independent means (ex. Tet inducible) would allow for many interesting studies on the role of Numb in PDA as well as allowing for the separation of the acinar cell dedifferentiation phenotype from the cell viability phenotype. Use of a Cre independent system for modulation of Numb expression would allow for ablation of Numb expression at various time points throughout PDA progression, instead of the model I have currently used, which requires concomitant deletion of Numb and activation of Kras, as both are under the control of the same Cre recombinase.

Given that a majority of PDA cases have mutations in p53, the p53 independence of cell death in the absence of Numb is also of great interest. My studies indicate that both acinar cells and early metaplastic cells under the control of activated Kras are both sensitive to anoikis induced by the deletion of Numb. However, it is important to determine if cells continue to be sensitive to this mechanism of cell death at later stages of progression. The studies described above would also be useful in this matter as well. If PDA cells remain sensitive to anoikis due to the deletion of Numb, this could have interesting therapeutic implications, as it may provide a possible route for inducing cell death specifically in metaplastic cells, even in the absence of functional p53.

In general, an exploration of the role of integrins could also provide additional insight into how the changing cancer microenvironment regulates PDA progression. The desmoplastic response in PDA has been well documented and accumulation of stroma, including matrix deposition, occurs from the earliest metaplastic stage. Additionally, multiple studies have shown that cancer cells express different integrin types than normal pancreatic cells and they are often expressed across the entire cell membrane instead of

localized to the basal surface (reviewed in Grzesiak, 2007). However, little is known about the changes in expression of integrins at earlier time points such as in ADM and PanINs. It would be interesting to determine if changes in integrin expression occur at these stages, if these changes correlate with changes in the type of matrix present and to determine how/if this impacts disease progression.

Changes in the type of matrix present also significantly impact the stiffness of the tissue; these changes in mechanical force have the capability to alter downstream signaling. Studies in breast cancer have demonstrated that the increasing stiffness of the tissue and increases in focal adhesions promotes tumor formation (Levental, 2009). Similar studies with PDA lines and models would likely provide interesting evidence for further connection between the increasing matrix deposition and cancer development. This information could also have therapeutic implications. Hedgehog inhibitors have been shown to decrease the degree of stroma in mouse models of PDA, which improves chemotherapy delivery (Olive, 2009). However, reduction of the stromal compartment could dramatically influence tumor cell survival and behavior through changes in tissue stiffness and alterations in integrin signaling as well. A thorough study of the tumor cell response to changes in the surrounding matrix could influence the potential use of Hedgehog inhibitors in PDA treatment in the future.

Epithelial tissue is highly organized and polarized, a characteristic that is often lost during cancer development. The effects of loss of cell polarity have been studied intensively in flies, and these studies have indicated that polarity can help to restrain cell proliferation. Disruption of polarity by deletion of single polarity complex genes often leads to hyperplasia and cell masses reminiscent of tumors (reviewed in Tanos, 2008;

Wodarz, 2000). The big question is – how does this relate to cancer in mammals? Epithelial to mesenchymal transition is well documented in advanced stage cancers, suggesting that the loss of apical-basal polarity may be a contributing factor in the transition to invasive and metastatic disease. However, what remains unclear is the role of polarity in early metaplastic events, for example, in acinar to ductal metaplasia, which involves the transition from one polarized cell state to another.

Although data from flies clearly demonstrates a relationship between loss of cell polarity and cellular hyperplasia, this link has not been definitively made in mammals. Cancer development in mammals generally requires a second hit, such as activation of an oncogene. My data supports this notion by demonstrating that loss of an indirect polarity regulator, Numb, alone does not lead to pancreatic defects or disease, but enhances the phenotype resulting from caerulein pancreatitis and the activation of Kras. My work suggests an important role for cell adhesion in maintaining cell viability during acinar cell metaplasia, but does not directly address the role of apico-basal cell polarity in these processes. During caerulein pancreatitis, the adherens junction is disassembled and reassembled (Lerch, 1997). However, it would be interesting to determine if changes in tight junctions also occur during this process and how cell polarity proteins may be involved. For example, the Par complex (Par3/Par6/atypical Protein Kinase C (aPKC)) is required to establish and maintain tight junctions and separating the apical and basal surfaces of the cell. If this complex is disrupted during acinar dedifferentiation and metaplasia, it may interfere with reestablishment of polarity in regenerating acinar cells. Additionally, a component of the Scribble complex, Lethal Giant Larvae (Lgl2), which is required for esablishment of the basal domain, is expressed in early stage PanINs, but lost

in PanIN3 and PDA (Lisovsky, 2010). This indicates that loss of Lgl2 may promote transformation and invasiveness. Would deletion of Lgl2 at early stages promote metaplasia and transformation, or is maintenance of polarity required during initiation and progression of early lesions? The generation and use of additional tools specifically altering polarity, such as creation of conditional alleles of genes directly involved in establishing and maintaining polarity (ex. aPKC, Par6, Lgl2) would further our understanding of the cooperation between Kras and polarity in the pancreas and during the development of pancreatic cancer.

Other types of cell-cell connections such as adherens junctions and desmosomes are also of great interest. In fact, desmosomes have been shown to regulate invasiveness in a mouse model of pancreatic neuroendocrine tumor (Chun, 2010). Epithelial cells of the pancreas express E-cadherin at adherens junctions and it is often used as a marker during progression. Its loss is often documented in advanced cancers, including those of the pancreas, but its role(s) in preventing and/or promoting disease are relatively poorly understood. These junctions serve many roles in the epithelial cell that may be important in regulating metaplasia. Given the requirement for tight regulation of Wnt signaling during pancreatic regeneration and ADM, it is easy to speculate how changes in E-cadherin expression and adherens junction assembly may affect these processes in this manner, as the extent of complexing of β-catenin into junctions affects the pool of β-catenin available for signaling (reviewed in Jeanes, 2008). E-cadherin is also capable of restricting the activity of growth factor signaling, such as restricting response to EGF ligand (reviewed in Jeanes, 2008). As Ras/MEK/ERK signaling is activated in pancreatic regeneration, it could be speculated this type of negative regulation may be important for

restoring proper acinar function and that disruption of this interaction may lead to continued pathway activation and possibly metaplasia. In depth analysis of cell junctions as well as the status of E-cadherin localization, cycling and function in pancreatic regeneration, metaplasia and PDA will be important for our understanding of role of these cell features in disease. Further disruption of E-cadherin and other cell-cell junction components will provide valuable information on how cell-cell junctions are impacting disease progression.

Mesenchymal involvement in diabetes and pancreatic cancer

Our work demonstrates that pancreatic mesenchymal cells (PaMCs) are required for maintenance of glucose homeostasis, partially through the production of ECM components. However, it is likely that ECM production is just one of many mechanisms for how PaMCs interact with endocrine cells. Better analysis of the expression profile of these cells could yield clues to secreted factors PaMCs may provide and cell-cell interactions they may make with endocrine cells. This could allow for disruption of these interactions, given the right tools, to uncover additional mechanisms of glucose regulation. Additionally, depletion of PaMCs is a blunt tool for examining their function. Our work currently uses a Cre line that is only expressed during a very short window of time during development to irreversibly mark cells that derive from Cre positive tissue. The limitation of this is that it does not allow for analysis of many specific genes during adulthood because their deletion in the embryo often interferes with pancreatic development. The next step for this work would be to find a way to specifically alter gene expression in this cell population in the adult, for example, the generation of a Cre^{ER} or Tet line with expression in adult PaMCs. Analysis of the expression array could

potentially reveal PaMC specific genes that would allow for generation of such a line. Either of these types of lines would permit normal development and allow for specific gene modulation at the time of our choosing. Production of a Tet line is of specific interest, as it could be used in combination with the Cre driven models of pancreatic diseases.

Many SNPs and mutations have been linked to diabetes, but the expression patterns of these genes and how these mutations contribute to the development of diabetes is unclear. It would be interesting to look for expression of these genes in PaMCs, which may give indications as to how these genes may influence disease development as well as additional mechanisms by which PaMCs may regulate blood glucose. Development of the Cre^{ER} or Tet line as described above would then allow us to determine if these genes are truly involved in the regulation of blood glucose and β-cell function.

In addition to being involved in blood glucose regulation, it will be exciting to examine if PaMCs are involved in PDA development. An increasing desmoplastic response, including significant numbers of fibroblasts, is a hallmark of pancreatic cancer, but the origin of these fibroblasts is unclear. Generation of a Tet line that allows for the marking of PaMCs in combination with a Cre driven model of PDA would be very informative in determining what extent of fibroblasts in PDA originate from PaMCs. Excitingly, this system could also allow for specific gene alteration in tumor-associated fibroblasts, which would revolutionize the field of PDA-stroma interaction research.

Finally, it has been observed that defects in blood glucose regulation are frequently associated with advanced stages of PDA, even though no obvious changes are

observed in the islets. Our data indicating that changes in ECM can affect β-cell function, which could be a possible explanation the correlation between PDA formation and the development of hyperglycemia. If the balance of the ECM is disrupted by the presence of PDA, could this induce the development of hyperglycemia, possibly leading to diabetes? In depth studies examining blood glucose regulation in mice with PDA combined with analysis of changing ECM surrounding islets could help to further our understanding of these connections.

Immunoregulation of PDA development

My work demonstrates that neutrophils contribute to pancreatic damage associated with caerulein pancreatitis, but that their deletion does not significantly affect PanIN and PDA development. One caveat of this work is the method used to delete neutrophils, germ-line deletion of G-CSFR, is chronic. This type of deletion may allow for compensation of function of these cells by other cell types that do not require G-CSFR for their development. A possible continuation of this project would be to use alternate methods of neutrophil depletion that are more acute, such as Gr1 antibody depletion and diptheria toxin mediated depletion. Both of these methods would result in loss of a slightly different population of cells, but may give a better overall picture of the role of neutrophils pancreatic damage, regeneration and ADM. However, since these are temporary depletions, a study of the effects of PDA development would not be feasible.

The role of the immune system in cancer development is currently an extremely active field. However, not much is know specifically about immunoregulation of PDA. Our lab has demonstrated that deletion of a critical immune modulator, Stat3, in the pancreas, results in a reduction of ADM, which is accompanied by changes in the

immune environment (Fukuda, 2011). Recent work has also shown that dendritic cells promote cell viability during caerulein pancreatitis (Bedrosian, 2011). Additional studies combining the loss of specific immune populations with pancreatic regeneration, ADM and PDA mouse models would further our understanding of how the immune system affects pancreatic regeneration and PDA development.

Medical Implications

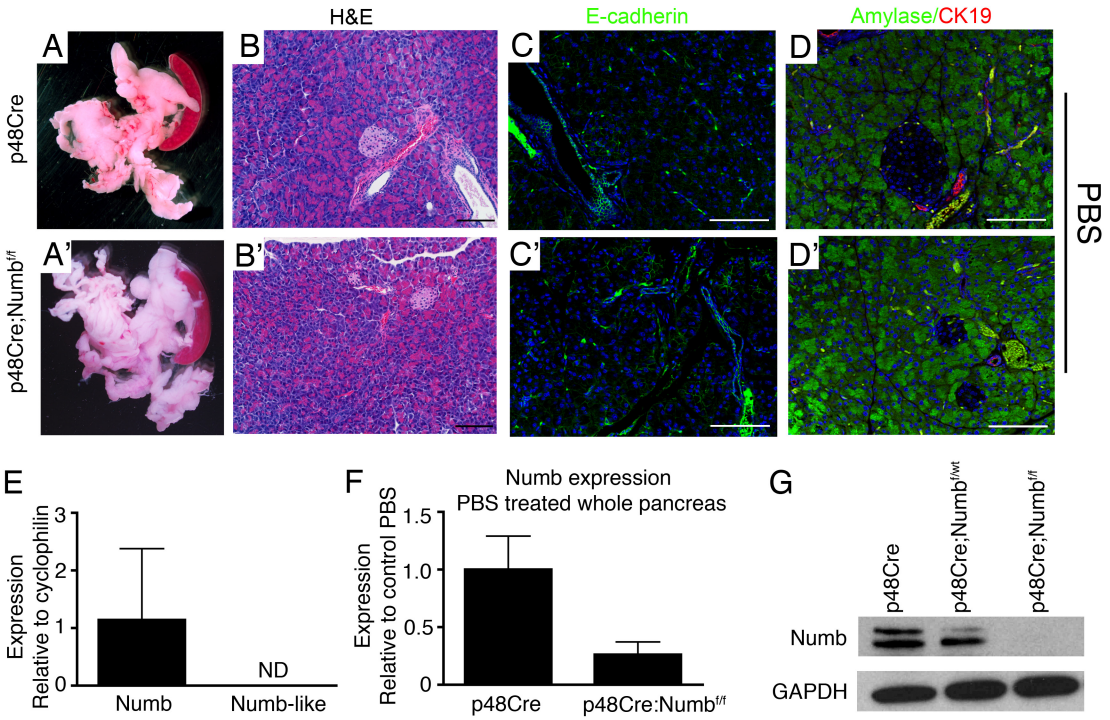
One of the primary reasons for researching disease is to contribute to the medical knowledge in hopes of bettering the outlook for patients. My studies on the regulation of ADM, a very early stage of PDA development, will hopefully one day inform our knowledge on how to prevent and detect disease. If we can understand what additional factors accelerate the activities of Kras, perhaps we can distinguish between early lesions that are more likely to go on to become cancerous as opposed to those that will likely remain benign. Of course, this type of distinction will have to be combined with early detection methods, an area that sorely needs development. Additionally, following up on my studies on pancreatic mesenchyme could benefit our knowledge of treatment of PDA and diabetes. If we know how the mesenchyme is promoting PDA, or how changes in the mesenchyme may disrupt endocrine cell function, perhaps new therapies can be developed that take these aspects of the disease into account.

APPENDIX 1

Supplemental Figures for Chapter 2

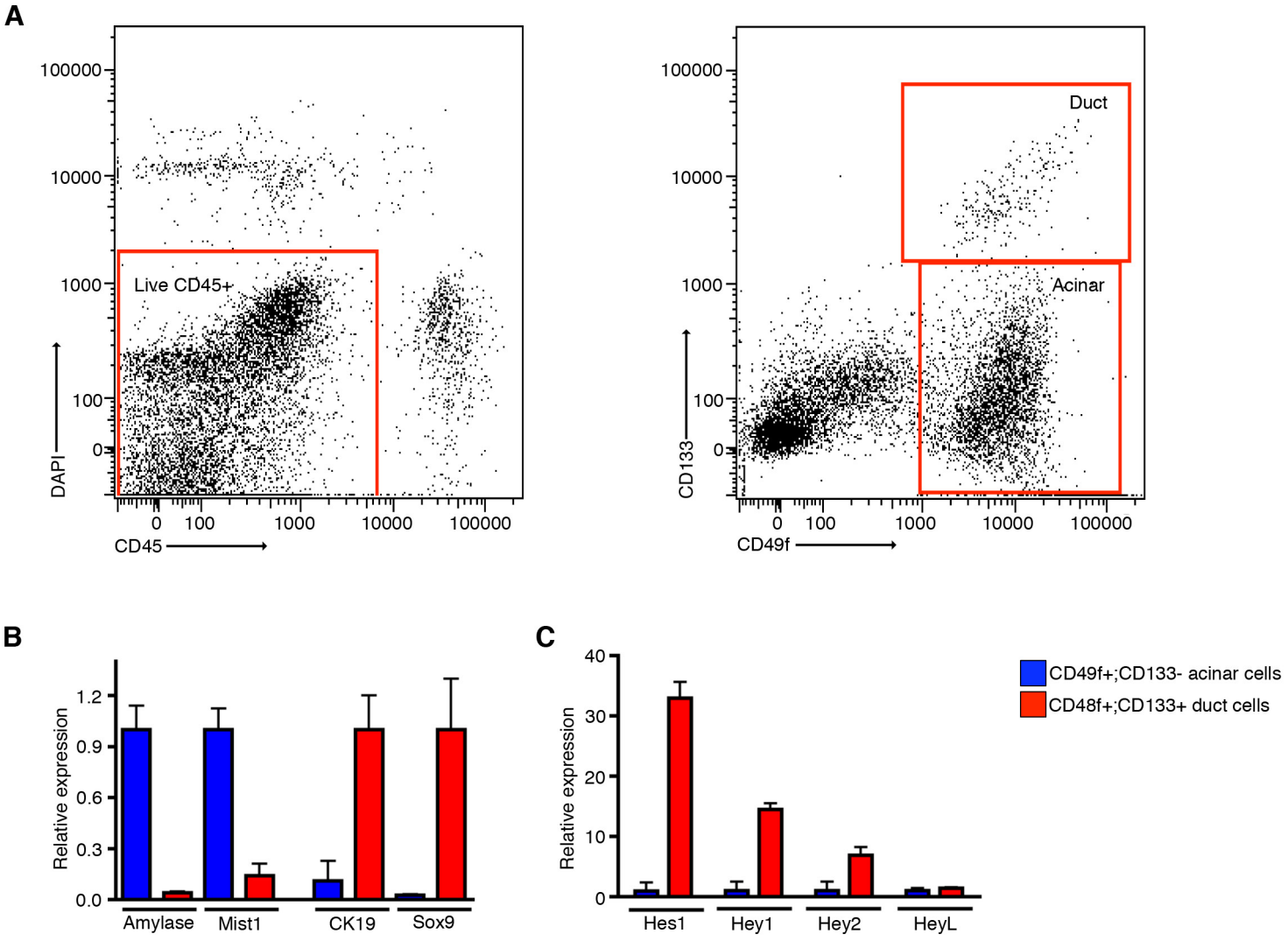
Supplementary Figure A1.1: A sorting method for enrichment of acinar and ductal cell populations

(A) Marker and gating strategy for FACS isolation of acinar and ductal cells. The live, CD45- cell population is gated for on CD49f and CD133. CD49f+;CD133- population contains acinar cells and CD49f+;CD133+ population contains duct cells. (B) qPCR confirmed enrichment of acinar and ductal specific genes into their respective populations. (C) Analysis of transcriptional targets of Notch signaling indicates that centroacinar cells are sorted into the duct population. qPCR is represented as mean ± SD, N=3.


H&E
E-cadherin
Amylase/CK19
A
B
C
D
p48Cre
A'
B'
C'
D'
p48Cre;Numb^f/f
PBS
E
Expression
Relative to cyclophilin
3
2
1
0
ND
Numb
Numb-like
F
Numb expression
PBS treated whole pancreas
Expression
Relative to control PBS
1.5
1.0
0.5
0
p48Cre
p48Cre:Numb^f/f
G
p48Cre
p48Cre;Numb^f/wt
p48Cre;Numb^f/f
Numb
GAPDH

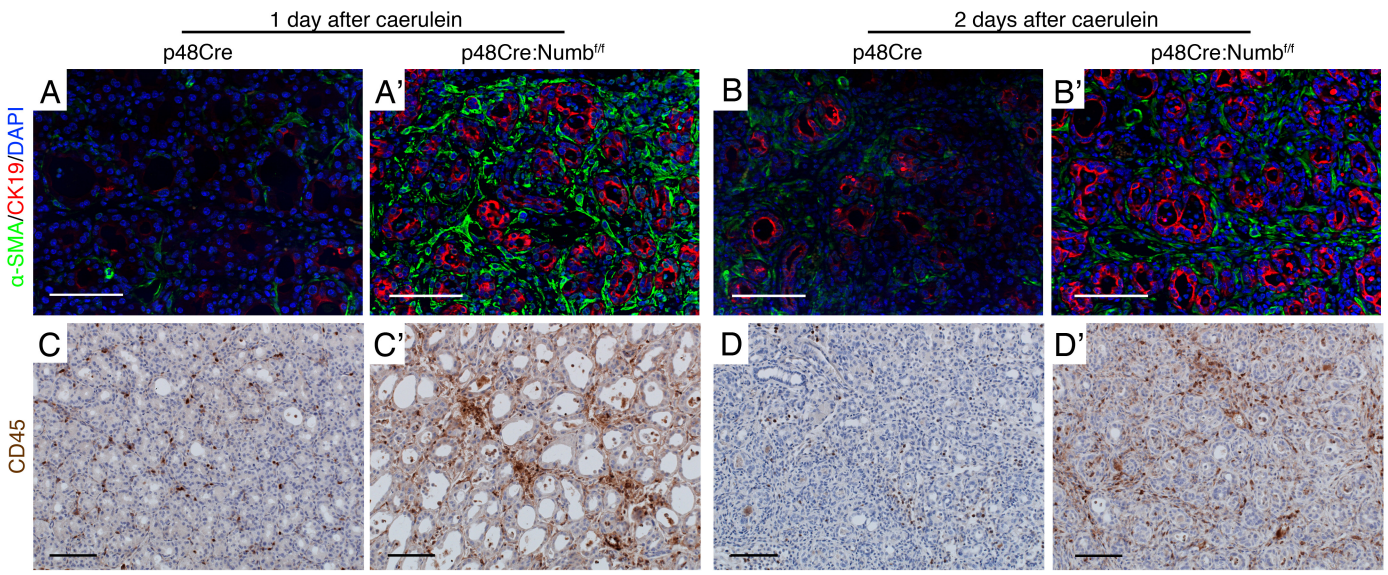
Supplementary figure A1.2: Verification of Numb expression and deletion

Deletion of Numb does not affect normal pancreas development as indicated by (*A*) Gross morphology, (*B*) H&E staining (*C*) immunostaining for E-cadherin (green), counterstained with DAPI (blue) and (*D*) immunostaining for Amylase (green) and CK19 (red), counterstained with DAPI (blue) in 6 week old, PBS treated animals. (*E*) Numb, but not Numb-Like expression is detected by qPCR in FACS isolated acinar cells. (*F*) Numb is transcript level is reduced in *p48Cre;Numb*$^{f/f}$ mice as determined by qPCR on total pancreas RNA and (*G*) Numb protein is not detected by Western blot detect of total pancreatic lysate *p48Cre;Numb*$^{f/f}$ mice. qPCR data expressed as mean ± SD. Western blot image representative of N=3 per genotype.


A
100000
10000
1000
100
0
DAPI
Live CD45+
0 100 1000 10000 100000
CD45
100000
10000
1000
100
0
CD133
Duct
Acinar
0 100 1000 10000 100000
CD49f
B
Relative expression
1.2
0.9
0.6
0.3
0.0
Amylase
Mist1
CK19
Sox9
C
Relative expression
40
30
20
10
0
Hes1
Hey1
Hey2
HeyL
CD49f+;CD133- acinar cells
CD48f+;CD133+ duct cells

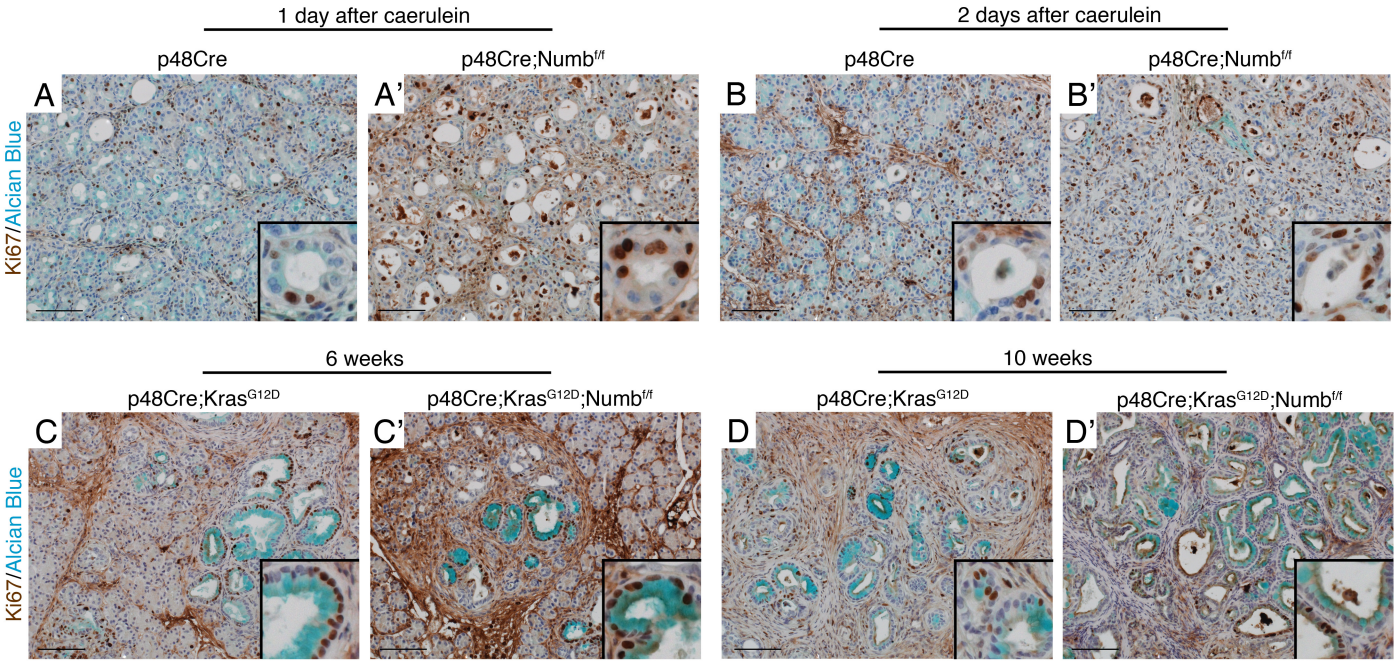
Supplementary Figure A1.3: Enhanced and accelerated stromal and immune cell recruitment following caerulein treatment in Numb deleted mice

(*A,B*) Immunostaining for α-Smooth Muscle Actin (green) and CK19 (red), counterstained with DAPI (blue) at days 1 and 2 following caerulein treatment. (*C,D*) Immunostaining for CD45 (brown), counterstained with hematoxylin (blue) at days 1 and 2 following caerulein. Scale bars represent 100μm.


1 day after caerulein
2 days after caerulein
p48Cre
p48Cre:Numb f/f
p48Cre
p48Cre:Numb f/f
A
A'
B
B'
α-SMA/CK19/DAPI
C
C'
D
D'
CD45

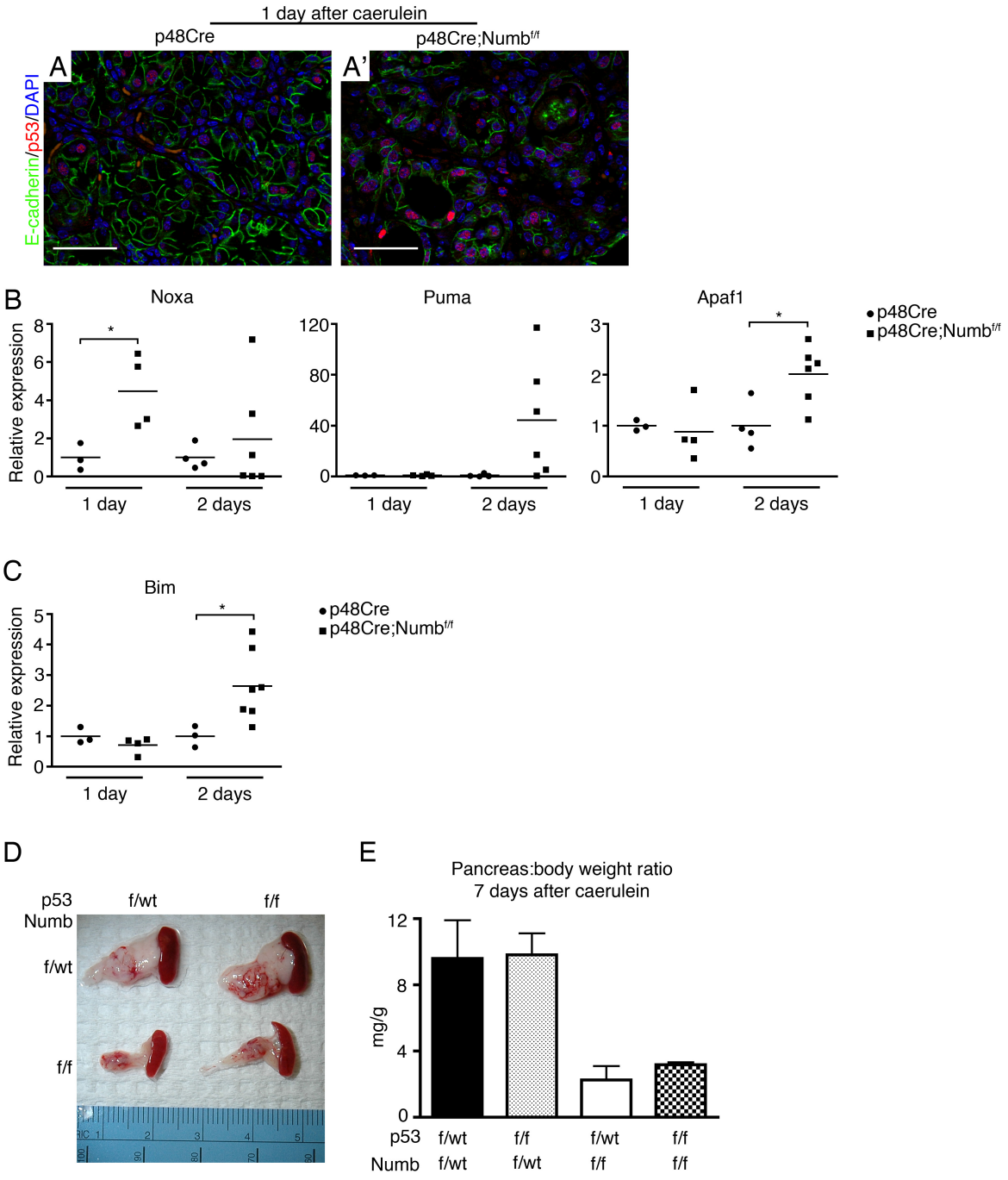
Supplementary Figure A1.4: Cells lacking Numb retain proliferative capacity

(*A-D*) Immunostaining for Ki67 (brown), counterstained with Alcian Blue. Scale bars represent 100μm.


1 day after caerulein
2 days after caerulein
p48Cre
p48Cre;Numb$^{f/f}$
p48Cre
p48Cre;Numb$^{f/f}$
A
A'
B
B'
Ki67/Alcian Blue
6 weeks
10 weeks
p48Cre;Kras^{G12D}
p48Cre;Kras^{G12D};Numb$^{f/f}$
p48Cre;Kras^{G12D}
p48Cre;Kras^{G12D};Numb$^{f/f}$
C
C'
D
D'
Ki67/Alcian Blue

Supplementary Figure A1.5: Caerulein-induced apoptosis of acinar cells in p48Cre;Numb$^{f/f}$ mice is p53 independent

(*A,B*) Immunostaining for p53 protein (red) and E-cadherin (green), counterstained with DAPI (blue) 1-2 days after caerulein treatment in *p48Cre* and *p48Cre;Numb$^{f/f}$* mice. (*C*) Quantitative PCR analysis detects increased expression of some pro-apoptotic genes, including p53 targets Noxa, Apaf1 and Puma as well as Bim. (*D*) No difference in pancreas size of *p48Cre;Numb$^{f/f}$* and *p48Cre;Numb$^{f/f}$;p53$^{f/f}$* pancreata at day 7 following caerulein treatment. (*E*) Pancreas mass to body weight ratio at day 7. Scale bars for (A-B) represent 50μm. qPCR data represented as individuals, overlaid with mean ± SD. * $P<0.05$.


1 day after caerulein
p48Cre
p48Cre;Numb f/f
A
A'
E-cadherin/p53/DAPI
B
Noxa
Puma
Apaf1
Relative expression
1 day
2 days
p48Cre
p48Cre;Numb f/f
C
Bim
D
p53
Numb
f/wt
f/f
E
Pancreas:body weight ratio
7 days after caerulein
mg/g

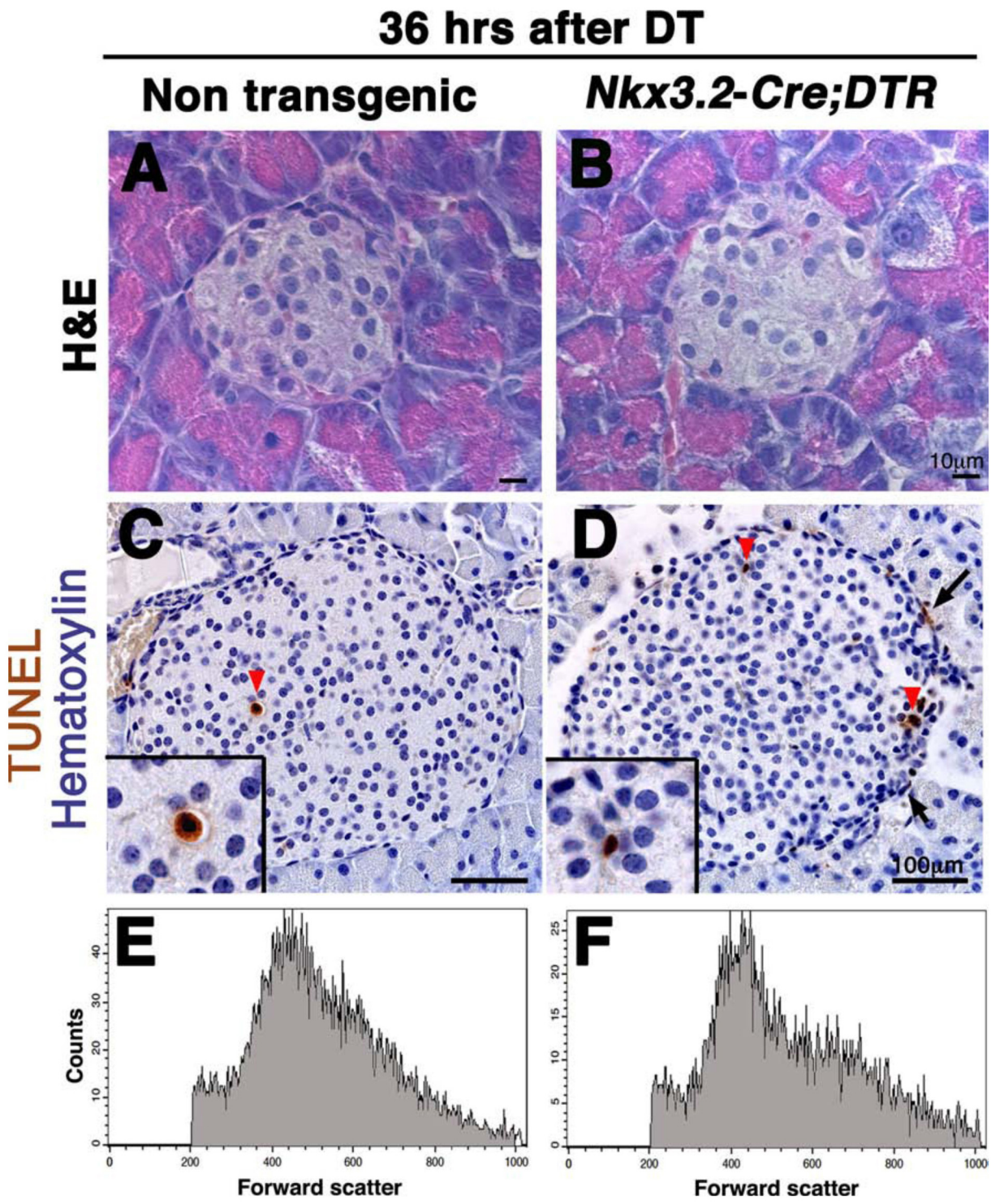
APPENDIX 2

Supplemental Figures for Chapter 3

Supplementary Figure A2.1: Islet morphology is normal following PaMC depletion

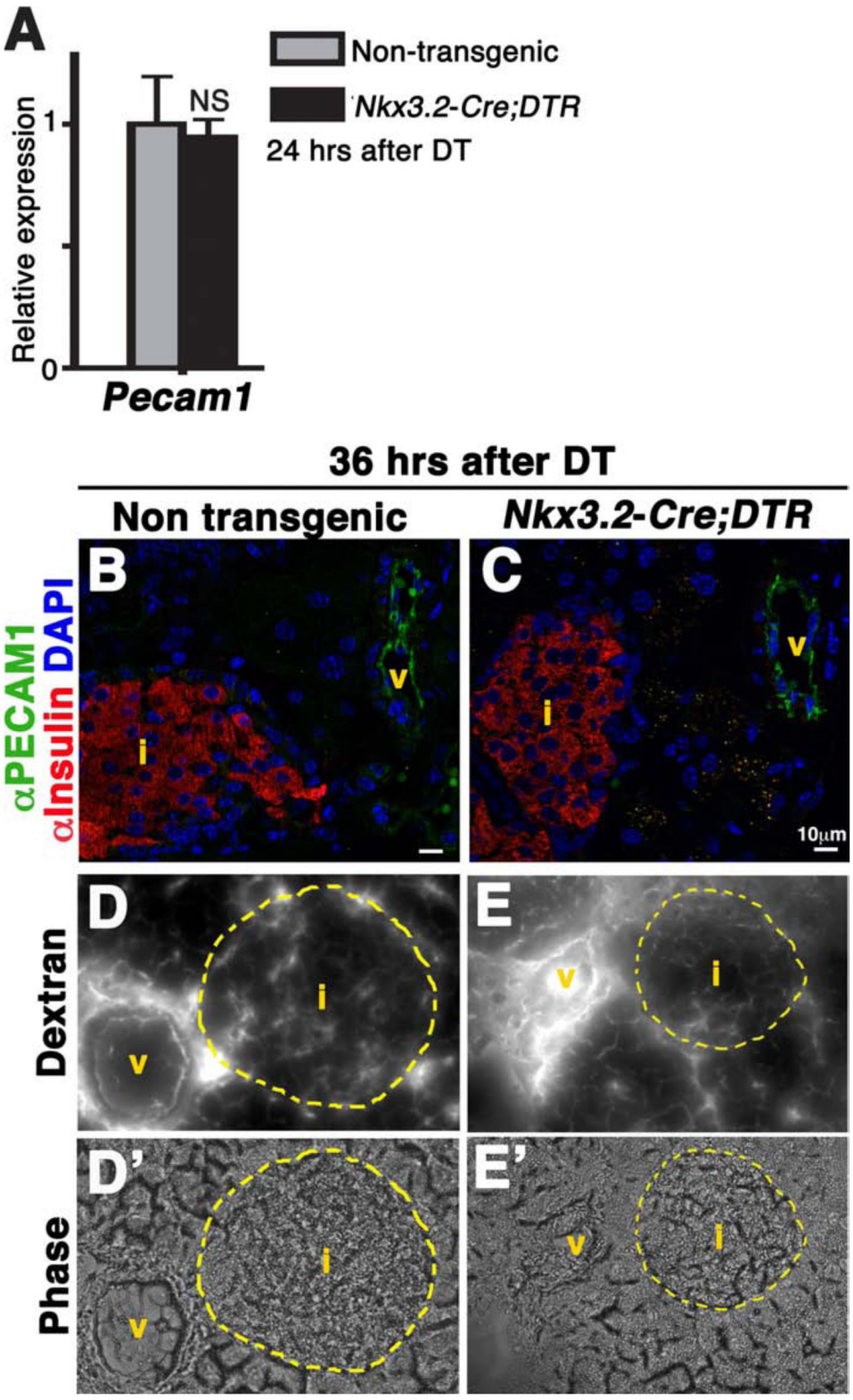
Nkx3.2-Cre;DTR and non-transgenic control were analyzed 36 hrs after DT treatment. Images show representative islets.

(**A,B**) Islet morphology is not disrupted. Pancreatic tissue from transgenic (B) and control (A) mice, 36 hours after DT treatment, was stained by H&E. (**C,D**) No increase in apoptosis is observed in islets. TUNEL staining (brown) of transgenic (D) and nontransgenic control (C) 36 hours after DT treatment, counterstained with hematoxylin. Black arrows mark $TUNEL^+$ cells in islet periphery (likely representing dying PaMCs) and red arrowheads mark $TUNEL^+$ cell within the islets. Inserts show a higher magnification of $TUNEL^+$ cells. (**E,F**) Islet cell size is not altered upon fibroblast depletion. Flow cytometic analysis of isolated islets from transgenic (f) and control (e) mice. Analysis shows forward scatter, indicative of cell size. Representative histogram, 3 mice analyzed per group.


36 hrs after DT
Non transgenic
Nkx3.2-Cre;DTR
A
B
H&E
10μm
C
D
TUNEL
Hematoxylin
100μm
E
F
Counts
Forward scatter
Forward scatter

Supplementary Figure A2.2: Pancreatic vasculature is not disrupted by PaMC depletion

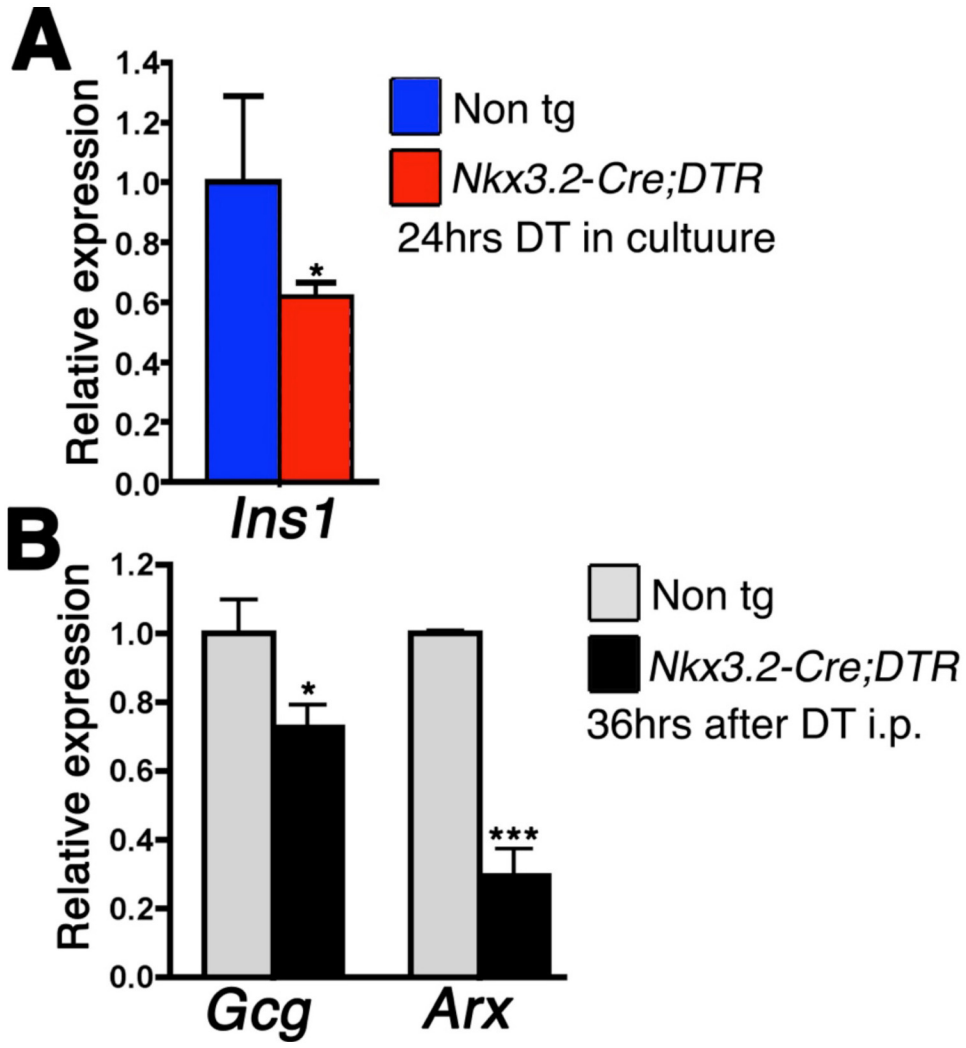
(**A**) Pancreatic PECAM1 expression is maintained in DT-treated mice**.** RNA was isolated from bulk pancreatic tissues of non-transgenic (gray) and Nkx3.2-Cre;DTR (black) mice, 24 hrs after DT injection. Expression levels were analyzed by qPCR and normalized to untreated control. N=4. Data represent the mean ± SD. (**B,C**) Blood vessel morphology is not perturbed in proximity to islets. Fixed pancreatic tissues from transgenic (C) and control (B) mice, 36 hrs after DT treatment, were immunostained with antibodies against insulin (red) and PECAM1 (green) and were counterstained with DAPI (blue). i = islet, v = vein. Images show representative islets. (**D,E**) Islet blood flow is maintained in treated transgenic mice. 36 hrs after DT treatment, transgenic (E) and control (D) mice were injected with fluorescently labeled Dextran into the tail vein and euthanized 30 min after injection. Fluorescent imaging (D,E, "dextran") on pancreatic tissue sections revealed presence of functional vessels and bright field analysis (E',D', "phase") indicated normal tissue architecture. Yellow dashed lines demarcate islets. i = islet, v = vein. Images show representative islets.


A
Non-transgenic
Nkx3.2-Cre;DTR
24 hrs after DT
Relative expression
1
0
NS
Pecam1
36 hrs after DT
Non transgenic
Nkx3.2-Cre;DTR
αPECAM1
αInsulin DAPI
B
C
v
i
10μm
Dextran
D
E
Phase
D'
E'

Supplementary Figure A2.3: Changes in endocrine gene expression upon PaMCs ablation

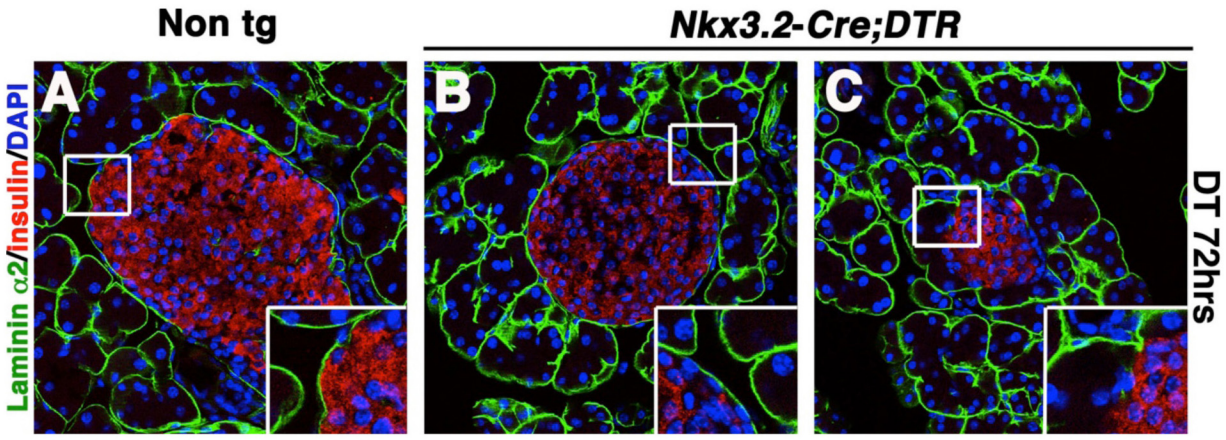
(**A**) Direct effect of *ex vivo* PaMC ablation on *Ins1* expression levels. Islets isolated from untreated *Nkx3.2-Cre;DTR* (red bars) and non-transgenic (blue bars) animals were cultured in the presence of 1 mg/ml DT for 24 hrs. RNA was extracted and gene expression analyzed by qPCR. N=4. Data shows one representative of two independent experiments with comparable results. (**B**) Reduced expression of a-cell specific genes, *Glucagon (Gcg)* and *Arx*, in transgenic islets. RNA was extracted from islets isolated from DT-treated transgenic (black bars) and non-transgenic (gray bars) mice and expression of indicated genes was analyzed by qPCR. N=4.

*P < 0.05, ***P < 0.005, NS = Non significant, as compared to non-transgenic controls. Data represent the mean ± SD.


A
Relative expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Ins1
Non tg
Nkx3.2-Cre;DTR
24hrs DT in cultuure
B
Relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
***
Gcg
Arx
Non tg
Nkx3.2-Cre;DTR
36hrs after DT i.p.

Supplementary Figure A2.4: Laminin a2 presence in DT-treated *Nkx3.2*-Cre;DTR pancreata

Fixed pancreatic tissues from non transgenic (**A**) and transgenic (**B**, **C**) mice 72 hours after DT treatment were stained for Laminin a2 (green), Insulin (red), and counterstained with DAPI (blue). Images show representative islets. Inserts show higher magnifications.


Non tg
Nkx3.2-Cre;DTR
A
B
C
Laminin α2/Insulin/DAPI
DT 72hrs

References

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