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Cell and Molecular Regulation of Pancreas Regeneration

Johnathan Smid

This thesis is submitted as a
partial fulfillment of the Ph.D. program in
Cellular and Molecular Medicine

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Faculty of Graduate and Postdoctoral Studies
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Abstract

Although pancreatic regeneration has been well documented, the cellular source and inducing factors remain unknown. To elucidate the cellular source of regeneration cells from the adult resting pancreas expressing Stem cell antigen 1 (Sca1) were assessed as a candidate stem cell population. Sca1-expressing cells represented a heterogeneous population with only a very small percentage capable of differentiating to the β-cell lineage. After expansion in culture Sca1-expressing cells lost their ability to differentiate and uniformly expressed the mesenchymal markers of activated pancreatic stellate cells (PSCs). To elucidate factors which stimulate regeneration the remnants following partial pancreatectomy (PPx) in adult C57BL/6J mice were examined. An early proliferation of mesenchymal cells producing a stroma was observed. Therefore, it was hypothesized that this mesenchymal stroma played a role in facilitating regeneration. Directly injecting mesenchymal cells into the pancreas of adult SCID-BEIGE mice was sufficient to induce pancreatic regeneration including tubular complex formation, ductal Pdx1 and Ngn3 expression. To determine specific factors that initiate the regeneration process a microarray screen was performed on the tip of the remnant following PPx. The secreted protein periostin was highly expressed in this regenerating tip. Injecting periostin directly into the pancreas stimulated the proliferation of resident mesenchymal cells to create a stroma. Following stromal formation, proliferating tubular complexes formed and both Ngn3 and Pdx1 were expressed. Periostin null mice had reduced stromal accumulation after PPx which impaired regeneration. Therefore, the formation of a

mesenchymal stroma is necessary and sufficient for the induction of regeneration in the pancreas. Thus, inducing pancreatic regeneration by stimulating mesenchymal cells provides a novel approach for the treatment of diseases such as diabetes.

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List of Abbreviations

Apoe	apolipoprotein E
bFGF	basic fibroblast growth factor
BMP4	bone morphogenic protien 4
CACs	centroacinar cells
cDNA	complementary dioxy-ribonucleotide
Ck7	Cytokeratin 7
Col1a2	procollagen, type I, alpha 2
Col3a1	procollagen, type III, alpha 1
C-terminal	carboxy-terminal
DAPI	4',6-diamidino-2-phenylindole
DMBA	7,12-dimethylbenz(a)anthracene
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
E	Embryonic day
Ecad	E-cadherin; Cadherin 1
ECM	extracellular matrix
EDTA	ethyl diamine-tetra-acetic acid
EGF	epidermal growth factor
EMT	epithelial to mesenchymal transition
ES	embryonic stem cell
FACS	fluorescent-activated cell sorting
fas1	fasciclin-1 domain
FBS	fetal bovine serum
FDG	fluorescein digalactoside
FITC	fluorescein (isothiocyanate)
GAPDH	glyceraldyde-3-phosphate dehydrogenase
HCO_3^-	hydrogen carbonate

HSCs	hematopoietic stem cells
ICD	Intercalating Ducts
IDDM	Insulin-Dependent Diabetes Mellitus
iPS cells	induced pluripotent stem cells
Isl-1	mouse Islet-1 transcription factor
Kb	kilobase
kDa	kilodalton
Ki-67	proliferation-related Ki-67 antigen
LB	Luria broth
Ly6a	lymphocyte antigen 6 complex, locus A
Lyzs	mouse lysozyme gene
MEM	Minimal essential medium
MMP	matrix metalloproteinase
Mmp2	matrix metallopeptidase 2 gene
mRNA	messenger RNA
MSCs	mesenchymal stem cells
NaCl	sodium chloride
NIDDM	Non-Insulin Dependent Diabetes Mellitus
NIPs	nestin-positive islet derived progenitor cells
NLS	nuclear localization sequence
NOD mice	non-obese diabetic mice
N-terminal	amino-terminal
OFP	Orange Fluorescent Protein
PanIN	pancreatic intraepithelial neoplasia
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDAC	Pancreatic Ductal Adenocarcinoma
PDL	partial duct ligation
Pdx1	pancreatic and duodenal homeobox 1 gene

PFA	paraformaldehyde
PMPs	pancreas-derived multipotent precursors
Postn	periostin, osteoblast specific factor gene
PPx	partial pancreatectomy
PSCs	pancreatic stellate cells
Pten	mouse phosphatase and tensin homolog gene
RA	Retinoic Acid
Rbp1	retinol binding protein 1 gene
RNA	ribonucleic acid
RT	reverse-transcription
Runx2	runt related transcription factor 2 gene
Shh	sonic hedgehog
SMA	α-smooth muscle actin
SP cells	side-population cells
Sparc	secreted acidic cysteine rich glycoprotein
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TC1	Type 1 tubular complexes
TC2	Type 2 tubular complexes
TCs	Tubular Complexes
Tgfbi	transforming growth factor, beta induced gene
Vim	vimentin
Wt	Wild-type

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Chapter 1 - General Introduction

1.1. The Pancreas

The pancreas is a unique vertebrate organ with two contrasting roles in metabolism, carried out by either exocrine or endocrine cells. Pancreatic endocrine cells secrete hormones into the blood stream. Conversely, exocrine cells secrete digestive enzymes into the intestinal tract. The endocrine tissue is comprised of clusters of hormone secreting cells known as the Islets of Langerhans. The exocrine tissue is made of enzyme secreting acinar cells connecting to a branching network of ducts which transport digestive molecules to the duodenum. The epithelium of these distinct tissue groups is surrounded by mesenchymal cells including pancreatic stellate cells (**Fig. 1**).

1.1.1. The Endocrine Pancreas: Islets of Langerhans

A vital but small percentage (1-2%) of the pancreatic mass makes up the endocrine tissue, which secretes hormones into the blood stream (Rahier et al., 1983). These cells are clustered into tiny spheres or islets called the 'Islets of Langerhans', named after the German pathologist who discovered them in 1869. The majority of cells within islets are insulin secreting β-cells (Stefan et al., 1982). In mice a β-cell core is surrounded by glucagon secreting α cells as well as cells that secrete somatostatin and pancreatic polypeptide. Although these cell types are also found in human islets they are not confined to the periphery of islets, as in the mouse, but have a more random distribution (Brissova et al., 2005; Cabrera et al., 2006). The most recent endocrine cell type found within islets expresses ghrelin, although they represent less than 1% of the cells in islets (Wierup et al., 2002). In the average human islet, insulin secreting cells make up 67% of endocrine cells, pancreatic polypeptide 19%, glucagon 10%, and somatostatin 3% (Stefan et al., 1982).

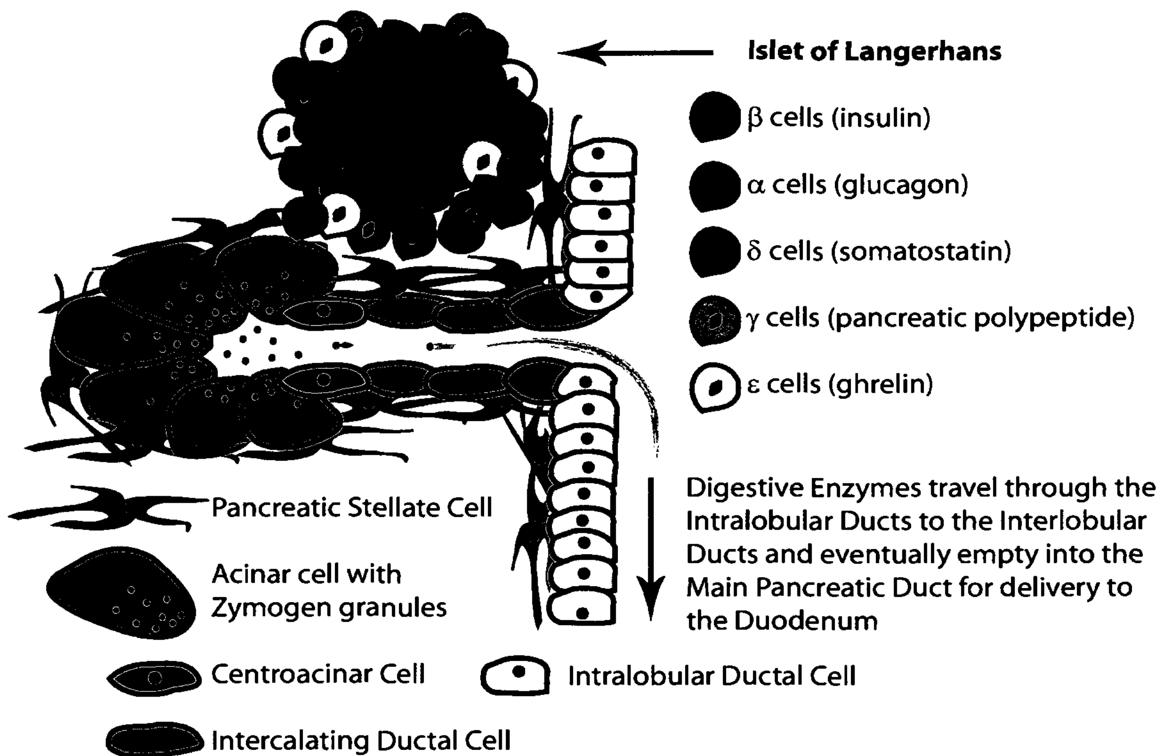

Figure 1. Localization and function of cells found in the adult pancreas. The endocrine compartment comprises only 1-2% of cells in the adult pancreas. Endocrine cells (green outlines) secrete hormones into the blood stream. They are found in small clusters, known as the Islets of Langerhans, which are scattered throughout the pancreas. In mice the islet core is made of insulin secreting β-cells (green) surrounded by α cells secreting glucagon (red). Additional hormone secreting cells are found on the periphery of islets, but in much smaller numbers, including δ (magenta), γ (yellow), and ε (light blue) cells which secrete somatostatin, pancreatic polypeptide and ghrelin, respectively. Pancreatic stellate cells (red) surround clusters of acinar cells, as well as the pancreatic ducts. Acinar cells (dark brown) release zymogen granules (purple) into the acinar lumen. The lumen contains centroacinar cells (orange) and connects to the intercalating ducts (brown). Digestive enzymes (purple) are released from the zymogen granules and pass through the intercalating ducts (brown) to the intralobular ducts (light brown). The intralobular ducts empty into the interlobular ducts (not shown) and eventually through the main pancreatic duct to the duodenum. In addition the pancreas contains endothelial, lymphatic and nerve tissues (not shown).

1.1.2. Exocrine: Acinar and Ductal cells

The function of the exocrine tissue is to secrete molecules into the intestine to facilitate digestion. The majority of cells in the pancreas are dedicated to the process of either producing digestive enzymes or hydrogen carbonate and delivering them to the duodenum. Digestive enzymes help break down food, while hydrogen carbonate aids in the neutralization of hydrochloric acid found in stomach acid. The secreted digestive molecules are delivered to the duodenum via a branched ductal network.

Acinar cells, found at the end of the ductal branches, produce digestive enzymes such as proteases, nucleases and lipases. Amylase and elastase are two of the common enzymes used to mark pancreatic acinar cells. Acinar, derived from the Latin word for berry, describes the arrangement of the cells on the distal ends of the ductal tree.

Pancreatic ductal cells form the epithelial lining in the branched network of tubes that delivers digestive enzymes and hydrogen carbonate into the duodenum. The main pancreatic duct which empties into the duodenum is made of columnar epithelial cells and is surrounded by connective tissue (Grapin-Botton, 2005). From the main pancreatic duct stems interlobular ducts which join the different lobes of the pancreas. The interlobular ducts are connected to intralobular ducts within each lobe. The intralobular ducts are then connected to intercalated ducts which drain the acinar lumens of enzymes released through the secretion of zymogen granules (Grapin-Botton, 2005). The differences and similarities between each of the different ductal cell types within the branched network of cells have yet to be fully resolved.

In addition to forming a network that delivers digestive enzymes produced by the acini, ductal cells produce hydrogen bicarbonate HCO_3^- to neutralize stomach acidity (Githens, 1988; Hollander and Birnbaum, 1952). Production of bicarbonate is controlled by secretin when the pH within the duodenum drops below 4.5 (Bayliss and Starling, 1902). The production of bicarbonate by ductal cells makes carbonic anahydrase a useful marker of pancreatic ductal cells (Inada et al., 2006).

Ductal cells have cilia that are potentially important for flow sensing in the pancreatic ducts (Calvet, 2003). Unlike other exocrine glands the pancreatic ductal system does not contain myoepithelial cells around the ducts to aid in secretion. Goblet cells, which contribute to mucin production, are intermingled among ductal cells making up about 2% of cells in larger ductal structures (Grapin-Botton, 2005).

At the interface of acinar clusters and the ductal system, reside spindle shaped cells known as centroacinar cells (CACs) (Stanger et al., 2005). Interestingly, unlike acinar or ductal cells, CACs maintain active notch signaling (Miyamoto et al., 2003). This is significant as notch signaling has been shown to be critical in maintaining pancreatic progenitor cells in an undifferentiated state (Apelqvist et al., 1999; Murtaugh et al., 2003). Several groups have suggested that centroacinar cells are a precursor to endocrine cells (Nagasao et al., 2003; Suzuki et al., 2003). In addition, centroacinar cells are a putative pancreatic cancer stem cell (Stanger et al., 2005). Therefore, centroacinar cells could more specifically define the ductal cells that represent the cellular source of regeneration.

1.1.3. Pancreatic Stellate Cells

Pancreatic stellate cells (PSCs) were first identified as resident cells within the pancreas containing storage depots of vitamin A. PSCs undergo a transformation from 'quiesence' to an 'activated' state either *in vivo* following pancreatic injury (Apte et al., 1999; Haber et al., 1999) or *in vitro* after several days of cultivation (Apte et al., 1998; Bachem et al., 1998). Following this transformation, PSCs lose their vitamin A containing fat droplets and begin to express the intermediate filament α-smooth muscle actin (SMA) (Apte et al., 1998; Bachem et al., 1998). The 'activated' state of PSCs is also characterized by an increase in proliferation and expression of extracellular matrix (ECM) synthesis (Apte et al., 1998; Bachem et al., 1998; Gressner and Bachem, 1995; Haber et al., 1999). In addition, PSCs also express the mesenchymal markers vimentin and nestin (Lardon et al., 2002).

It has been shown that PSCs play a central role in pancreatic fibrosis (Apte et al., 1999; Haber et al., 1999; Luttenberger et al., 2000; Mews et al., 2002; Schmid-Kotsas et al., 1999). Progressive fibrosis accompanies pancreatic cancer and chronic pancreatitis resulting in the accumulation of ECM (Etemad and Whitcomb, 2001; Kloppel and Maillet, 1993; Yen et al., 2002). The progressive fibrosis initiated by pancreatic cancer is known as the desmoplastic reaction (Apte et al., 2004). The end result is the creation of a stroma that supports the growth and metastasis of pancreatic cancer cells (Hartel et al., 2004). In addition to synthesizing ECM, activated PSCs have been shown to produce both cytokines and chemokines (Andoh et al., 2000; Gressner, 1995; Kruse et al., 2000). Therefore, the neighboring epithelial cells, with exocrine and endocrine function, have the potential to be strongly influenced by the activation of pancreatic stellate cells.

1.2. Embryonic Development

The pancreas develops from the innermost germ layer, the endoderm. At 8.5 days of mouse gestation (E8.5) or 3 weeks in humans, the pancreas is induced in an area of the gut tube that lacks sonic hedgehog (*Shh*) gene expression (Hebrok et al., 1998). *Shh* expression is repressed by activin-related signaling from the notochord (Hebrok et al., 1998; Kim et al., 1997). At this time, the cells which will become the pancreas are marked by the expression of the transcription factor pancreatic and duodenal homeobox 1 (Pdx1) (Miyatsuka et al., 2006; Zhou et al., 2007). All endocrine, ductal and acinar cells derive from this common Pdx1-expressing progenitor (Fishman and Melton, 2002). Pancreatic β-cells are the only adult cells to maintain expression of Pdx1. Therefore, expression of Pdx1 in the adult pancreas, outside of pancreatic β-cells, has been used as a marker of regeneration (Bonner-Weir et al., 1993).

Following specification, there is an expansion of the mesenchyme surrounding Pdx1-expressing cells fated to become the pancreas. At E9.5 epithelial cells begin to bud into the surrounding mesenchyme (Oliver-Krasinski and Stoffers, 2008). The mesenchyme is necessary for this budding to occur (Ahlgren et al., 1996; Golosow and Grobstein, 1962; Spooner et al., 1970). Fibroblast growth factors 7 and 10, expressed by the mesenchyme, are two of the factors required for the proliferation and branching of the early pancreatic epithelium (Ye et al., 2005). Mice lacking *Isl-1*, a transcription factor expressed in pancreatic mesenchymal cells, have a deficiency of mesenchyme and fail to develop a dorsal pancreatic bud (Ahlgren et al., 1997). Thus, the mesenchyme plays a vitally important role in embryonic development.

The pancreas is produced from two separate areas of the gut tube; dorsal and ventral pancreatic buds. These buds will eventually merge to form the complete pancreas. In the rat pancreas, the formation of the dorsal bud precedes that of the ventral by 12 hrs (Stefan et al., 1982). The ventral bud contributes to merely 10% of the adult tissue, leaving the dorsal bud to form the bulk of the pancreas. However, even within the same species there is variability in how the two buds combine (Klein and Affronti, 2004).

The pancreatic buds develop into a branching network of epithelium. At the tips of the branching network reside multipotent pancreatic progenitors expressing Pdx1, Ptf1a, Cpa1 and high levels of cMyc. Lineage tracing experiments have shown that these 'tip cells' give rise to endocrine, ductal, and acinar cells *in vivo* (Zhou et al., 2007). As the tip cells divide they leave behind differentiated cells, which form the trunk of the branching network, as it pushes forward into the embryonic mesenchyme. At E13.5 endocrine progenitors express the transcription factor Neurogenin3, Ngn3 (**Fig. 2**). Ngn3-expressing progenitors give rise to α, β, δ, ε and PP cells (Jorgensen et al., 2007).

To maintain homeostasis postnatally, expansion of β-cell mass is required to meet the demands of the body. In pregnancy β-cell mass doubles, in part due to the action of the hormone prolactin (Karnik et al., 2007). Hyperplasia of islets due to endocrine proliferation is also observed in multiple endocrine neoplasia type 1 (MEN1), a simultaneous cancer occurring in the endocrine pancreas and thyroid. Most cases arise from a mutation in the *Men1* gene, which encodes the protein Menin (Larsson et al., 1988). Similarly, Menin negatively regulates β-cell proliferation during pregnancy and overexpression results in gestational diabetes (Karnik et al., 2007). Therefore, β-cell division is regulated after embryonic development and throughout adult life.

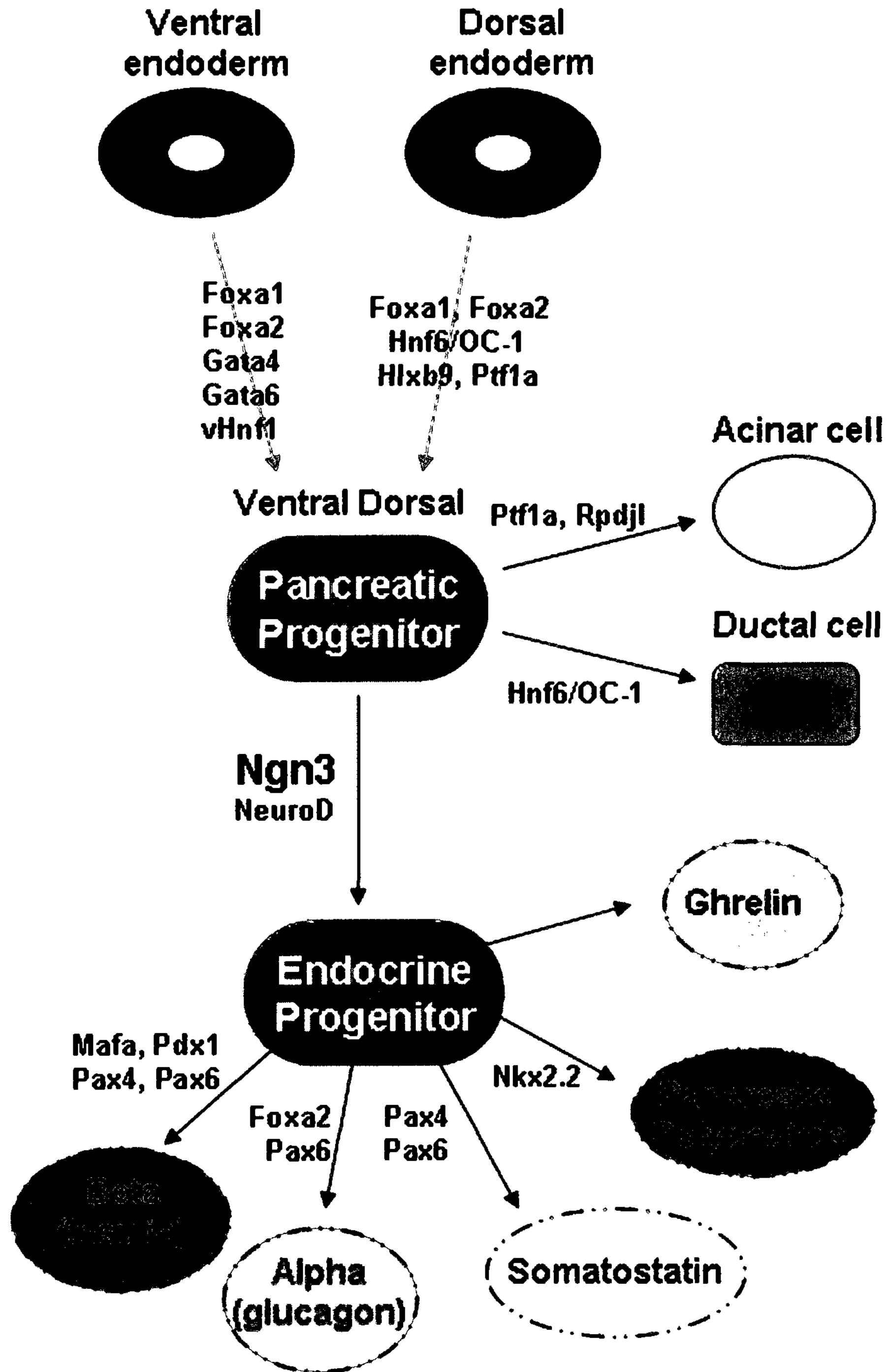

Figure 2. The endoderm lineage during pancreatic development. The key transcription factors involved in pancreatic development. Ngn3 is a key factor in the determination of endocrine progenitors.

1.3. Pathology of the Pancreas

1.3.1. Pancreatitis

Acute pancreatitis is caused by an inappropriate activation of enzymes which in turn leads to a build up of fluid, inflammation and necrosis of the pancreas in severe cases. The leading cause of acute pancreatitis is gallstones which can obstruct the pancreatic ducts (Mitchell et al., 2003).

Multiple cases of acute pancreatitis lead to chronic pancreatitis where permanent damage results from progressive inflammation. Mutations in the gene cystic fibrosis transmembrane conductance regulator (CFTR) are often associated with chronic pancreatitis. This gene, which is defective in cystic fibrosis, is important for bicarbonate secretion (Hug et al., 2003). Without the appropriate bicarbonate secretion to aid flow to the intestine there is a build up of digestive proteins within the pancreas. This eventually leads to the early activation of the digestive enzymes which results in damage to the pancreas causing inflammation and fluid build up. In some cases, pancreatitis can lead to pancreatic cancer. Chronic pancreatitis is essential to produce Pancreatic Ductal Adenocarcinoma (PDAC) in adult mice induced to express the oncogene K-Ras^{G12V} (Guerra et al., 2007). Although it is unclear how this process occurs, the increased stroma observed in pancreatitis may contribute to cancer progression by altering the microenvironment of cells that initiate pancreatic cancer (Erkan et al., 2008; Farrow et al., 2008). This stroma is produced by the activation of pancreatic stellate cells (PSCs) in pancreatitis (Apte et al., 1999; Haber et al., 1999). Furthermore, PSCs were suggested to make an early contribution to the regeneration observed following acute pancreatitis in humans (Zimmermann et al., 2002).

1.3.2. Pancreatic Cancer

Understanding the similarities and differences between pancreatic cancer and regeneration is extremely valuable in elucidating the cellular and molecular mechanisms involved in each. Characterization of a pancreatic stem cell could pinpoint the cellular source of both pancreatic cancer and regeneration. Morphological similarities between regeneration and cancer suggest that perhaps the latter is a result of the regeneration process gone wrong. This is evident in the tubular complexes that are observed during regeneration and cancer progression (Bockman, 2008).

The most common pancreatic cancer is Pancreatic Ductal Adenocarcinoma (PDAC). PDAC is an extremely lethal disease, ranking fourth in cancer-related deaths with a 5 year survival rate of less than 5 per cent (Jemal et al., 2006). This poor survival is often due to a late diagnosis at which time the patient is at an unresectable stage of treatment, currently the only 'cure' (Wagner et al., 2004). Surgery is currently the best treatment as pancreatic tumors are often resistant to chemotherapy and other more conventional therapies. In addition, even if the patient is diagnosed in time for resection there is often a recurrence of the cancer following the surgery. Considering these factors it is evident that much research is required in order to help individuals being diagnosed with PDAC in the future.

Invasive PDAC results after progression through several non-invasive stages. Small lesions (<5mm) within the pancreatic ducts termed pancreatic intraepithelial neoplasia (PanIN) are a hallmark of the pre-invasive state of PDAC (Hruban et al., 2001). The progression of pancreatic cancer from PanIN to PDAC is morphologically characterized into three stages. Throughout the progression, normally cube shaped

pancreatic ductal cells become tall and column shaped. They begin to have abundant aggregates of protein within the nucleus, they become crowded with enlarged nuclei and increase their mitotic rate (Hruban et al., 2000). Beyond these stages cells break away from their duct-like structures and become invasive.

Many mutations are found within the genome of PDAC, the earliest and most common is an activating point mutation in the K-ras proto-oncogene. The activating K-ras mutation is found within PanIN lesions and the later stages of more than 90% of PDAC (Lemoine et al., 1992). Following K-ras mutation there is often telomere shortening and p16 loss (Caldas et al., 1994). In later events of cancer progression there is p53 loss (Maitra et al., 2003), followed by SMAD4 loss (Wilentz et al., 2000). These mutations have been found at varying rates such as 75% for p53 loss and SMAD4 loss in nearly half of the cases observed (Rozenblum et al., 1997).

PDAC is characterized by a dense stroma termed desmoplasia, which surrounds pancreatic cancer cells (Mahadevan and Von Hoff, 2007). This stroma is a source of many factors influencing cancer progression including Transforming growth factor-β1 (TGF-β1) (Apte and Wilson, 2007; Chu et al., 2007; Mahadevan and Von Hoff, 2007; Sethi et al., 1999). Pancreatic stellate cells are thought to be the source of the desmoplastic reaction (Apte et al., 2004), which supports tumor growth (Yen et al., 2002). Therefore, they are likely the source contributing to the high levels of TGF-β1 in pancreatic cancers (Lohr et al., 2001).

TGF-β1 contributes to the invasiveness of cancer cells themselves by inducing an epithelial to mesenchymal transition (EMT) of cancer cells (Bates and Mercurio, 2005; Zavadil and Bottinger, 2005). EMT has been described in embryonic development,

chronic fibrotic disorders as well as cancer progression (Thiery, 2003). In cancer, EMT is characterized as a reversible conversion of static epithelial cells into a highly motile fibroblast like cell (Thiery and Sleeman, 2006). At the molecular level EMT is marked by a decrease in cell-cell adhesion molecules such as E-cadherin and a down regulation of epithelial differentiation markers typical of that tissue (Fujimoto et al., 2001; Takano et al., 2007). At the same time, mesenchymal markers such as vimentin and fibronectin are upregulated (Bates and Mercurio, 2005). The end result is a cell phenotype that is more motile and capable of spreading into the surrounding tissue.

The pancreas specific *Pten* knockout mouse has been shown to develop pancreatic cancer, although at a low frequency (Stanger et al., 2005). In this model of pancreatic cancer, rather than PanIN formation, there is a loss of exocrine cell mass and a metaplastic ductal phenotype prior to cancer formation (Stanger et al., 2005). It is suggested that the cellular source is not from the ducts but from centroacinar cells (Stanger et al., 2005). Therefore, the cellular source contributing to pancreatic cancer remains unresolved.

1.3.3. Diabetes: Type1 vs. Type2

Diabetes mellitus is a devastating disease reducing the quality of life in more than 150 million people worldwide (Soria, 2001). The main characteristic of Type 1 diabetes (T1D) is a lack of insulin to meet the demands of the body, due to a self-destructive loss of β-cells. Type 2 diabetes (T2D) is the inability of the islets to produce enough insulin to meet the demands required by the body. T2D is often due to a decrease of insulin

sensitivity in the cells which require it; therefore more insulin is required to overcome this barrier.

Increasing the amount of insulin produced by generating new β-cells would be beneficial to the treatment of Type 1 diabetes only if the autoimmune attack on the newly formed cells could be prevented. If β-cells were formed *in vitro* it may be possible to genetically manipulate these cells so that antigen that triggers the immune attack is lost. Alternatively, newly formed β-cells could be implanted within restrictive containers that prevent immune attack but allow insulin release. However, this is difficult as β-cells need close contact with the vascular system in order to function properly.

Type 2 diabetes would benefit from increased insulin production but in some cases the amount of insulin required could not be achieved by generating new β-cells. When insulin sensitivity is too low it would no longer be feasible to simply produce more β-cells and therefore treatments which combine increasing both insulin production and sensitivity would be required. Therefore, to be successful as a therapeutic for Type 1 or Type 2 diabetes, β-cell regeneration would require accompanying treatments.

1.4. Models of Regeneration in the Pancreas

In mammals, the liver and pancreas are known to have a strong potential for regeneration (Zaret and Grompe, 2008). Experimental models of regeneration have been developed to further our understanding of pancreatic regeneration (Bonner-Weir et al., 1981; Bonner-Weir et al., 1983; Hartmann et al., 1989; Jensen et al., 2005; Rosenberg et al., 1983). Understanding the similarities and differences between each of these models is important for elucidating the mechanisms involved in the regeneration process.

1.4.1. Chemically induced regeneration

Chemically inducing regeneration in the pancreas is challenging. Sufficient insult is required to initiate regeneration, however too much results in severe morbidity or mortality. In addition, chemicals tend to target either the exocrine compartment (Jensen et al., 2005) or the endocrine compartment (Bonner-Weir et al., 1981) and trigger primarily endocrine or exocrine regeneration. Therefore, chemically inducing regeneration is convenient, but does not necessarily induce complete regeneration.

Insult to the pancreas can be induced to the endocrine compartment by targeting β-cells with toxins such as alloxan (Jacob, 1977) or streptozotocin (STZ) (Fernandes et al., 1997; Zhang et al., 2002). Low dose STZ administration, 50 mg/kg body weight, in neonatal male rats induces enough damage to result in diabetes. However, seventy percent of these male rats spontaneously recover from this diabetes after 15 weeks (Hartmann et al., 1989). At higher levels of STZ neonatal rats do not recover suggesting that in the STZ-induced regenerative model some of the endocrine pancreas must remain intact for the repair to occur. Therefore, this regeneration may not involve islet neogenesis but rely on β-cell division. However, the use of STZ to induce permanent diabetes is a convenient model for testing potential therapies.

In the caerulein model of regeneration, insult to the pancreas is induced to the exocrine compartment by the injection of caerulein (Gomez et al., 2001; Jensen et al., 2005). High dosages of caerulein prematurely activates trypsinogen within the acini of the pancreas resulting in auto-digestion (Halangk et al., 2002). Although most cells of the exocrine pancreas are initially lost, full pancreatic function is restored demonstrating the robust regenerative potential of the exocrine pancreas. However, a complete

regeneration of the pancreas is not observed as endocrine cells remain intact. Therefore, neither STZ nor caerulein can induce complete pancreatic regeneration.

1.4.2. Partial duct obstruction

Ligation of the pancreatic duct has been performed in experimental studies since the early 20th century, in order to investigate pancreatitis that is caused by obstruction within the ducts of the pancreas (Kirkbride, 1912). Atrophic acini, fibrosis and a change in islets are all characteristics observed in these ligation models (Arvanitakis and Folscroft, 1978; Kirkbride, 1912; Walpole and Innes, 1946). In addition, obstruction of the pancreatic duct in humans has been shown to result in ductal hyperplasia (Cubilla and Fitzgerald, 1975). The location and degree of ligation alter the morphological outcome similar to what is observed in human duct obstruction.

Partial duct obstruction of the hamster pancreas, by cellophane wrapping, has been shown to induce proliferation of ductal cells and islet cell regeneration including new islet formation (Rosenberg et al., 1983). The new formation of islet cells was also observed in streptozotocin-induced diabetes (Rosenberg et al., 1988; Rosenberg and Vinik, 1989). Newly formed islet cells were observed to originate from precursor cells associated with the ductal epithelium (Rosenberg et al., 1983; Rosenberg et al., 1988). The newly developed islets were shown to contain the different endocrine cell types (Rosenberg et al., 1989). In addition, islet neogenesis-associated protein (INGAP) was found to be expressed in the partially obstructed pancreas and was shown to stimulate proliferation of ductal cells in culture (Rafaeloff et al., 1997; Rafaeloff et al., 1996).

1.4.3. Pancreatectomy

Regeneration following partial pancreatectomy (PPx) has been demonstrated in humans (Schlegel et al., 2000) and in many animal models (Bonner-Weir et al., 1993; Brockenbrough et al., 1988; Morisset et al., 2000). Similar to partial duct ligation, PPx induces the formation of new islets (Bonner-Weir et al., 1993). In addition to the formation of new islets, following PPx, the exocrine compartment is induced to regenerate (Bonner-Weir et al., 1983; Brockenbrough et al., 1988). This pathway of regeneration in the rat pancreas involves the proliferation and differentiation of precursor cells in the ductal epithelium in a manner that recapitulates embryonic development (Bonner-Weir et al., 1993). *In vivo* pulse labelling demonstrated that proliferation was first observed in the common pancreatic duct, followed by smaller ducts in focal areas of proliferation. Three days after PPx, most BrdU labelling is within these focal areas, however they disappear by one week in the rat pancreas (Bonner-Weir et al., 1993).

Partial pancreatectomy can induce regeneration in STZ-induced diabetic mice (Hardikar et al., 1999) or in rats (Finegood et al., 1999). This evidence strongly supports the idea that the diabetic pancreas maintains an islet stem cell population that can be induced by PPx to give rise to new islets. *Intra peritoneal* injections of cytosolic extracts of the regenerating pancreas were found to reverse diabetic status (Hardikar and Bhonde, 1999). Therefore, identifying the neogenic factors that initiate this regeneration would lead to future treatments of diabetes.

1.5. The cellular sources of regeneration

1.5.1. Stem Cells vs. β-cell Division

The contribution of adult pancreatic stem cells to regeneration has remained elusive, although several groups have shown multipotent precursors reside in the pancreas (Seaberg et al., 2004; Suzuki et al., 2004). Other groups have shown cells from outside the pancreas; bone marrow (Ianus et al., 2003) or spleen (Kodama et al., 2003), to infiltrate the pancreas and differentiate into functioning β-cells. However, such reports have remained controversial and repeating the experiments with bone marrow (Hess et al., 2003; Lechner et al., 2004) or spleen (Chong et al., 2006) have not had the same results. However, it is suggested that external cells can aid pre-existing pancreatic cells to regenerate (Hess et al., 2003). Furthermore, it has been clearly shown that if a stem cell does not reside in the adult pancreas, there is at least a progenitor to β-cells present in response to injury (Xu et al., 2008). Therefore, it would be advantageous to make use of this facultative progenitor as a β-cell source for the treatment of diabetes.

Dor and co-workers have suggested that the β-cells are the major cellular source facilitating their own regeneration by self duplication (Dor et al., 2004). These results emphasize the importance of β cell division, but do not dismiss the ability of the pancreas to generate new islets or the existence of progenitors in response to pancreatic injury (Xu et al., 2008). Finally, the results they describe allow for de-differentiation of islet cells prior to self duplication. The results do however show that under normal physiological conditions the turnover of cells within the pancreas does not rely on the contribution of stem cells at detectable levels.

1.5.2. Nestin expressing cells and the dedifferentiation of β-cells

Due to the similarities between pancreatic islets and neuronal cells, researchers hypothesized that the intermediate filament protein nestin could be used as a putative stem cell marker in the pancreas as well as in the central nervous system (Lendahl et al., 1990). Hunziker and Stein found a subset of cells expressing nestin within the pancreatic islets (Hunziker and Stein, 2000). Similarly, a potential progenitor cell within human and canine islets has been identified based on its quiescence and unique small cell phenotype (Petropavlovskaia and Rosenberg, 2002). The recent ability to expand these cells *in vitro* has allowed them to be further characterized (Petropavlovskaia et al., 2007).

Culturing human islets generates a monolayer of cells called NIPs, nestin-positive islet derived progenitor cells (Zulewski et al., 2001). It is unknown if these cells are derived from the proliferation of progenitor cells residing in islets or dedifferentiated endocrine cells. However, it has been demonstrated that cultured human islets can dedifferentiate to duct-like structures expressing cytokeratin (Jamal et al., 2005; Yuan et al., 1996). TGFβ is important in the process of dedifferentiation (Hanley and Rosenberg, 2007). α-, σ- and PP-cells have been shown to contribute equally to the duct-like cells while β-cells have minimal contribution (Hanley et al., 2008). However, the β-cells did contribute significantly to nestin-expressing cells *in vitro*. Although this process has been clearly demonstrated *in vitro* it has yet to be shown *in vivo*. Therefore, it is currently unclear whether endocrine cell dedifferentiation occurs during pancreatic regeneration. Moreover, nestin-expression is unlikely to represent a stem cell marker in the pancreas. Regardless, further understanding this process will lead to potential strategies for islet cell expansion for the treatment of diabetes.

1.5.3. Pancreatic Ductal Cells and Tubular Complexes (TCs)

It has been suggested that the pancreatic duct contains cells that can give rise to islets. Newly formed islets are found closely associated with ductal epithelium (Rosenberg, 1998) and *in vitro* islet-like structures have been generated from ductal extracts (Ramiya et al., 2000). A pathway of regeneration in the rat pancreas involving proliferation and differentiation of duct-like precursor cells has been identified (Bonner-Weir et al., 1993). *In vivo* pulse labelling revealed proliferation, first in the common pancreatic duct, and then sequentially in smaller ducts, which are suggested to give rise to focal areas of proliferation. Three days after PPx, most BrdU labelling is found within these focal areas; however they disappear after one week. It has been suggested that this pathway resembles the pattern observed in the embryonic development of ductal proliferation followed by differentiation (Bonner-Weir et al., 1993).

Lineage tracing studies have provided direct evidence for the contribution of ductal cells to regeneration (Inada et al., 2008). In these studies, transgenic mice expressing Cre recombinase (Cre) under the human carbonic anhydrase II (CAII) promoter were crossed with ROSA26 LacZ reporter mice. This cross was used to demonstrate that ductal cells expressing CAII give rise to new islets and acini after birth and after ductal ligation (Inada et al., 2008). This is the first lineage tracing study to identify a differentiated pancreatic cell type to become a facultative *in vivo* progenitor. However, this interpretation becomes problematic if expression of the CAII transgenic promoter is not specific for differentiated ductal cells. Furthermore, it does not specify which ductal cell-type gives rise to these pancreatic β-cells.

Tubular complexes (TCs) were first observed prior to the induction of adenocarcinoma by implantation of 7,12-dimethylbenz(a)anthracene (DMBA) (Bockman et al., 1978). TCs consist of a monolayer of flattened duct-like cells which form a cylindrical tube often with a wide lumen (Lechene de la Porte et al., 1991; Willemer and Adler, 1989). These structures have also been observed in human pancreatitis (Bockman et al., 1982; Willemer and Adler, 1989), pancreatic cancer (Bockman et al., 2003; Longnecker et al., 1983), and in animal models of chemical (Lechene de la Porte et al., 1991) and surgical pancreatic injury (Tokoro et al., 2003). Tubular complexes have been shown to be a source of regeneration in the BB rat which spontaneously develops diabetes (Wang et al., 2005). It was also suggested that TCs could give rise to new acinar tissue (Lechene de la Porte et al., 1991; Tokoro et al., 2003) or that they represent early cancer precursors (Bockman et al., 2003; Wagner et al., 1998). The significance of TCs was controversial until a recent study demonstrated endogenous progenitors exist within these ductal like structures after partial duct ligation (PDL) of the adult mouse pancreas (Xu et al., 2008). Using the expression of the embryonic endocrine progenitor transcription factor neurogenin 3 (Ngn3) as a guide, pancreatic progenitors were located in and around ductal structures (Xu et al., 2008). Isolated Ngn3-expressing cells could differentiate into β-cells when co-cultured with fetal pancreata (Xu et al., 2008). However, Ngn3-expressing cells are not present in the adult resting pancreas. Therefore, it is uncertain which cells in the adult resting pancreas give rise to these progenitors.

Lineage tracing studies have been employed to determine whether or not the cellular source of tubular complexes arises from the transdifferentiation of acinar cells (Desai et al., 2007; Strobel et al., 2007). Crossing mice expressing Cre recombinase

under the elastase promoter, with a LacZ reporter revealed Type 1 Tubular Complexes (TC1) are derived from acinar cells. TC1 were described as circular with a widened lumen, and are composed of only 4-5 cells. A Type 2 Tubular Complex (TC2) is not of acinar origin. These tubular complexes exhibit very extensive branching patterns. TC2 often cover entire areas in which branching complexes are surrounded by a thick stroma. While proliferation is only occasionally detected in TC1, Ki-67 is frequently expressed in TC2 cells (Strobel et al., 2007). Therefore, the facultative progenitors found within TC2 are not derived from acinar cells but likely from a ductal source (**Fig. 3**).

1.6. Periostin

1.6.1. Cloning and Discovery

Periostin was originally cloned from a mouse osteoblastic cell line (Ismail et al., 2000; Takeshita et al., 1993) and was named for its expression in the periosteum of bone and periodontal ligament cells (Horiuchi et al., 1999). The Periostin gene, *Postn*, is also expressed in embryonic endocardial cushions (Kruzynska-Frejtag et al., 2001; Norris et al., 2004). Based on western blot analysis multiple isoforms of periostin are predicted, the highest molecular weight is predominant early during embryonic development and the lower before birth (Kruzynska-Frejtag et al., 2004). The isoforms are a product of alternate splicing which occurs at the C terminus (Gillan et al., 2002) (**Fig. 4**).

Periostin is a homolog of the Drosophila protein, fasciclin I, which contains four conserved domains that facilitate axonal guidance, cell sorting and adhesion during insect nervous system morphogenesis (Bastiani et al., 1987). There are four mammalian genes with fasciclin domains: *Postn*, *Tgfbi*, *stabilin-1* and *stabilin-2* (Lindsley et al., 2005).

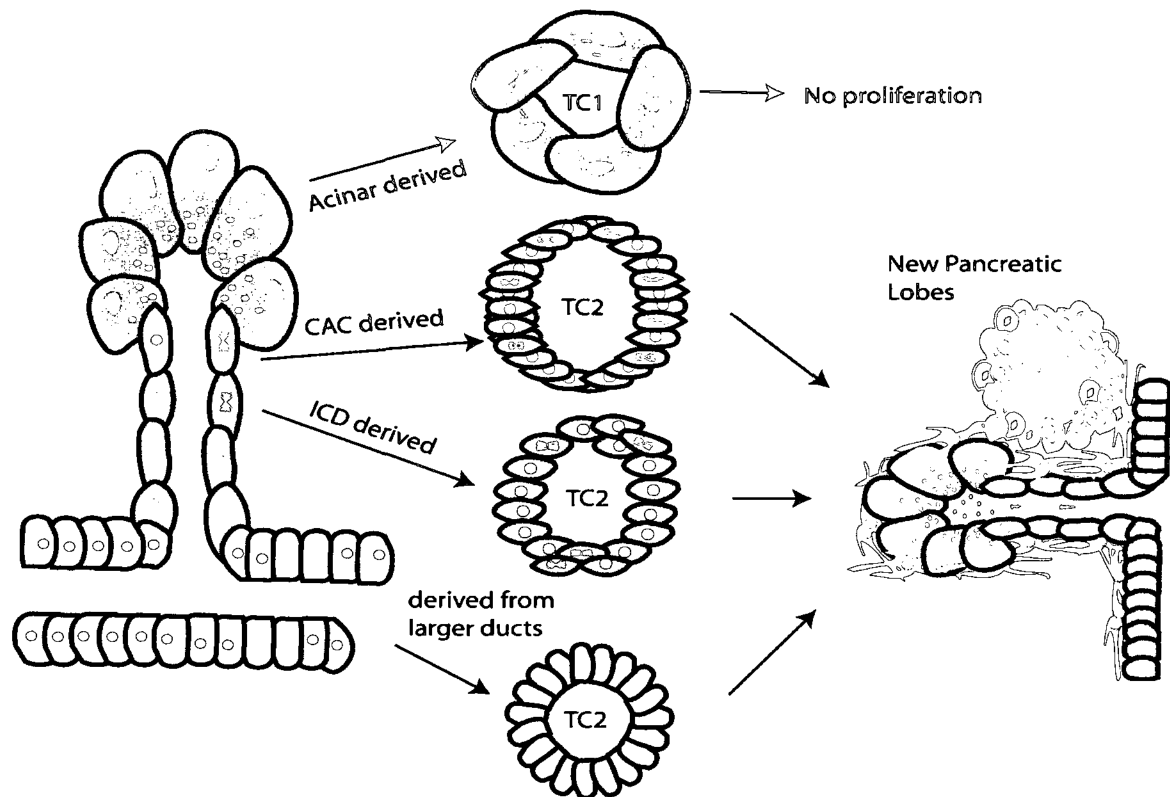

Figure 3. The cellular sources of Type 1 and Type 2 tubular complexes.

Type 1 tubular complexes (TC1) are derived from elastase secreting acinar cells (dark brown) and have minimal proliferation. Type 2 tubular complexes (TC2) are derived from non-elastase secreting cells making the ductal cells as a candidate source. This includes centroacinar cells (CACs) and intercalating ductal cells (ICD). Cells from larger ducts may also contribute to TC2 formation including intralobular ducts, interlobular or the main pancreatic duct. TC2 may also contain a mixing of ductal cell types or arise from a specialized stem/progenitor cell yet to be characterized.

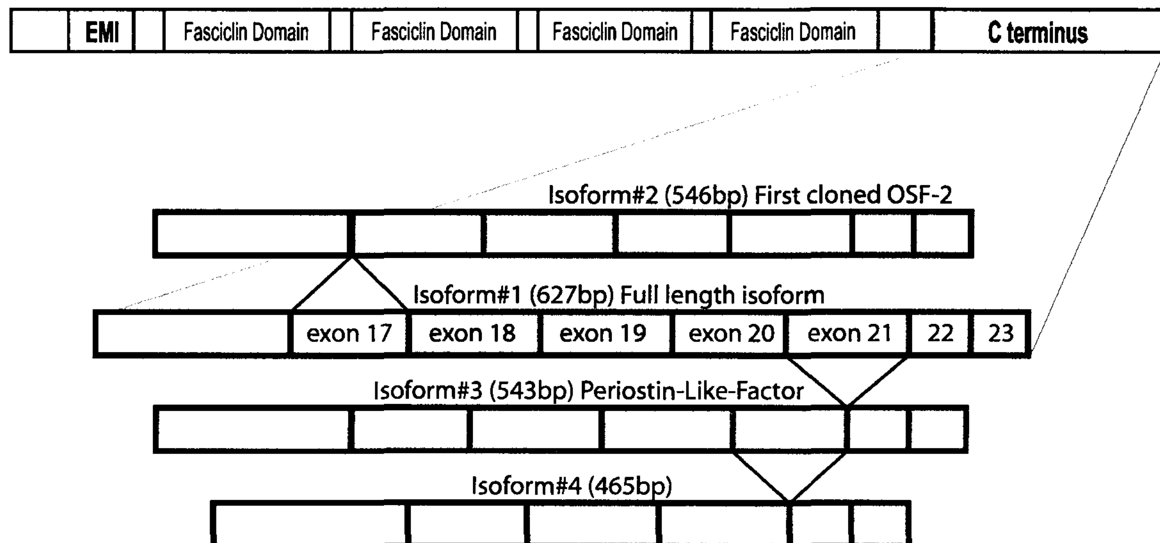

Figure 4. Domains of Periostin and known isoforms in the mouse .

The EMI and Fasciclin Domains of Periostin are shown to scale with the C terminus. Alternative splicing within the C terminus produces the four known isoforms of periostin in the mouse (Horiuchi et al., 1999).

Postn and *Tgfbi* contain the four conserved fasciclin domains, while stabilins are more divergent from fasciclin I and contain seven of the conserved domains. In addition, both are secreted proteins, are upregulated by TGFβ, and are known to bind integrins (Ferguson et al., 2003; Gillan et al., 2002; Lindsley et al., 2005). *Tgfbi* (transforming growth factor, beta induced gene) was identified in a screen of a human lung adenocarcinoma cell line after treatment with TGFβ (Skonier et al., 1992). *In vitro* studies suggest that *Tgfbi* may mediate cell adhesion (Kim et al., 2000; LeBaron et al., 1995). *Tgfbi* is increased during cardiac development in a pattern complementary to *Postn* (Lindsley et al., 2005).

1.6.2. Role in Cardiac Repair

Periostin has also been shown to promote cardiac repair following myocardial infarct (Dorn, 2007; Kuhn et al., 2007; Oka et al., 2007; Shimazaki et al., 2008). The adult heart expresses *Postn* after pathological insult (Oka et al., 2007). In an *in vitro* screen of factors, *Postn* was discovered to promote the proliferation of differentiated cardiac myocytes (Kuhn et al., 2007). Inducible overexpression of *Postn* (Oka et al., 2007) or introduction of the recombinant protein (Kuhn et al., 2007) promoted cardiac repair following myocardial infarction. In addition, overexpression of *Postn* in the heart induced spontaneous hypertrophy with aging (Oka et al., 2007). Although there was no morphological cardiac phenotype in *Postn* null mice, cardiac regeneration was greatly impaired following myocardial infarct (Shimazaki et al., 2008).

Although *Postn* has a been shown to be a regulator of cardiac remodelling the evidence that there is a direct effect on the proliferation of cardiomyocytes has been recently challenged (Lorts et al., 2009). Lorts and colleagues have shown that although

Postn is expressed in the heart following injury it does not stimulate DNA synthesis, mitosis, or cytokinesis of cardiomyocytes *in vitro* or *in vivo* (Lorts et al., 2009). However, it still promotes cardiac repair. Therefore, *Postn* may play a role in cardiac remodelling by regulating cells other than cardiomyocytes, such as the mesenchymal stroma or interstitial cells.

1.6.3. Role in Cancer

Although *Postn* is not normally expressed in the pancreas, transcription is markedly over expressed in pancreatic cancer (Baril et al., 2007). Increased levels of *Postn* in pancreatic cancer patients has been correlated to a shortened survival time (Baril et al., 2007; Tilman et al., 2007). Periostin has also been shown to promote invasiveness and survival of pancreatic cancer cells (Baril et al., 2007). In addition, it has been shown that *Postn* sustains fibrogenic stellate cell activity in an autocrine manner to create a tumor supportive microenvironment in the pancreas (Erkan et al., 2007). These stroma cells have been identified as the main source of periostin (Kanno et al., 2008). Furthermore, pancreatic stellate cells themselves have been suggested as this source (Erkan et al., 2007; Kanno et al., 2008).

Epithelial ovarian cancer cells have been reported to secrete Periostin (Ismail et al., 2000). Furthermore, secretion of Periostin in these cells has been shown to promote cell motility by binding to integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Gillan et al., 2002). Clinical studies have shown that increased expression of *Postn* closely correlated with metastasis (Sasaki et al., 2001; Sasaki et al., 2003; Shao et al., 2004). Initially *Postn* was thought of as just a marker for mesenchymal cells that had undergone epithelial to mesenchymal

transformation, such as during embryonic heart valve formation (Kruzynska-Frejtag et al., 2001). However, *Postn* has been shown to be more than just a marker for mesenchymal transition and has the ability to induce cell invasiveness (Yan and Shao, 2006).

1.7. Hypothesis and Specific Aims

Although pancreatic regeneration has been well documented, the cellular source and the factors which stimulate this regeneration remain unknown. Therefore, we hypothesised that if we could understand the cellular source or molecular factors involved in pancreatic regeneration novel therapeutics for diseases such as diabetes could be created. First, to address the cellular source of regeneration, a putative stem cell population within the pancreas was assessed. Second, factors which stimulate pancreatic regeneration were elucidated, uncovering the importance of a mesenchymal stroma. Finally, the ability of the secreted protein Periostin to generate a mesenchymal stroma to stimulate pancreatic regeneration was investigated.

1.7.1. Characterization of cells expressing Sca1 in the pancreas

We first explored resident Stem cell antigen 1 (Sca1) expressing cells as the source of pancreatic regeneration. Based on experiments performed in other tissues, we hypothesized Sca1-expressing cells represent a resident pancreatic stem cell and thus could be induced to differentiate into pancreatic β-cells. Therefore, we FACS purified Sca1-expressing cells for the purpose of *in vitro* characterization. In addition, we examined Sca1 expression during regeneration following pancreatectomy.

1.7.2. Mesenchymal cells contribute to pancreatic regeneration

Resection of the pancreas has been shown to induce regeneration, but the factors involved are unknown. Therefore, identification of such factors would uncover triggers that could be used to stimulate such regeneration. Following pancreatectomy we found an early expansion of mesenchymal cells creating a stroma. Therefore, we hypothesized the injection of cultured mesenchymal cells into the pancreas could induce pancreatic regeneration.

1.7.3. Periostin stimulates pancreatic regeneration

The importance of the mesenchymal stroma for pancreatic regeneration leads to the possibility of inducing stromal formation which in turn could stimulate regeneration. If resident mesenchymal stem cells were stimulated *in vivo*, then a stroma would be produced that could stimulate regeneration. Therefore, we hypothesized Periostin could be used to stimulate a pancreatic stroma and induce regeneration.

Chapter 2 – Sca1 expression in the adult pancreas

Characterization of Stem Cell Antigen 1 Expression in the Adult Resting and Regenerating Pancreas.

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Running Title: Sca1 expression in the adult pancreas

Abstract

Background: Although the adult pancreas has the ability to regenerate the cellular source of this regeneration has been unresolved. In addition, the existence of multipotent precursors within the adult mouse pancreas has been demonstrated; however, their localization within the pancreas remains unclear. In mice the *Ly6A* gene encodes the cell surface protein Stem cell antigen 1 (Sca1), which has been shown to be expressed on the adult stem cells of bone marrow and muscle. Taken together, our objective was to identify the cellular source of regeneration and localize multipotent precursors by examining cells that express Sca1 in the resting and regenerating pancreas.

Methods and Findings: Sca1-expressing cells were isolated by flow cytometry using fluorescently tagged antibodies specific to Sca1. Co-culturing freshly isolated Sca1-expressing cells with a fetal pancreas induced a small percentage of cells to differentiate towards a pancreatic β-cell lineage. Co-localization with CD31 revealed a large portion of $Sca1^+$ cells were endothelial accounting for many of the cells that failed to convert to the pancreatic β-cell lineage. Although $Sca1^+CD31^+$ cells were not viable, $Sca1^+CD31^-$ cells proliferated in culture but lost their potential to differentiate to pancreatic β-cells. Cultured $Sca1^+CD31^-$ cells had similarities to mesenchymal stem cells and could differentiate to an oesteogenic lineage. In addition, cultured $Sca1^+CD31^-$ cells uniformly expressed the mesenchymal markers of activated pancreatic stellate cells including; α-smooth muscle actin, Nestin, Vimentin, Desmin, GFAP, and PDGFRβ. Furthermore, during regeneration following partial pancreatectomy the expression of Sca1 was widespread due to the proliferation of PSCs and *de novo* expression in the ducts, acinar cells and regenerating tubular complexes.

Conclusion: Sca1 expressing cells represent a dynamic population in the adult pancreas. The variability of Sca1 expression prevents the localization of the small β-cell precursor population found in the freshly isolated cells. During regeneration Sca1 is expressed on ductal cells, acinar cells and subsequently tubular complexes. In the resting pancreas Sca1 is mainly expressed on endothelial and mesenchymal cells including PSCs. Culturing $Sca1^{+}CD31^{-}$ cells results in the activation of PSCs and the ensuing population of mesenchymal cells uniformly expresses activated PSC markers.

Introduction

Diabetes mellitus is a rising concern in healthcare as it reduces the quality of life of more than 150 million people worldwide [1]. Therefore, the ability to generate new insulin producing cells or initiate the process of pancreatic regeneration would have an immense clinical application. The main characteristic of Type 1 (autoimmune) diabetes is a lack of insulin to meet the demands of the body, due to a self destructive loss of β-cells. The Edmonton protocol has shown that β-cell replacement therapy can be successful in reversing Type 1 diabetes [2]. However, this protocol uses the islets isolated from at least two human cadavers. If this therapy is going to be available to more than just a few patients with diabetes, then new sources of insulin-producing cells have to be identified. Furthermore, the ability to regenerate new β-cells from resident cells within the pancreas is an attractive alternative approach to the development of a cure for diabetes.

Several methods of physical insult to the pancreas induce regeneration such as partial pancreatectomy (PPx) [3], partial duct ligation (PDL) [4] or cellophane wrapping [5]. Although several sources have been suggested the location of the cellular source responsible for this regeneration is unknown. In addition, several groups have isolated multipotent precursors from the uninjured pancreas showing *in vitro* differentiation down several pathways [6,7]. However, the *in vivo* localization is unknown, as is the degree to which these cells contribute to regeneration.

A cellular source of regeneration outside the pancreas has also been suggested and groups have shown cells from bone marrow [8] or spleen [9] infiltrate the pancreas and differentiate into functioning β-cells. These reports, however, have remained controversial and repeating the experiments with bone marrow [10,11] or spleen [12] have not had the same results. In all cases the percentage of differentiation reported is low and no report showing significant contribution to pancreatic regeneration is given. However, it is suggested that external cells can aid pre-existing pancreatic cells within the pancreas to regenerate [10]. Furthermore, it has been clearly shown that if a stem cell does not reside in the adult uninjured pancreas, there is a facultative progenitor present that is responsive to injury [13]. However, the cellular source of this facultative progenitor is unknown.

It has been suggested that the pancreatic duct contains cells that can give rise to islets. Newly formed islets are found closely associated with ductal epithelium [5] and *in vitro* islet-like structures have been generated from ductal extracts [14]. A pathway of regeneration in the rat pancreas involving proliferation and differentiation of cells in the ductal epithelium has been elucidated [3]. *In vivo* pulse labelling has shown proliferation starts in the common pancreatic duct and then sequentially in smaller ducts, which give rise to focal areas of proliferation. Three days after PPx, most BrdU labelling is within these focal areas, however these foci disappear after one week. The authors suggest that this pathway resembles the pattern observed in embryonic development of ductal proliferation followed by differentiation [3]. Therefore, the ductal epithelium may contain the cellular source of regeneration.

Dor et al have suggested that β-cells are the major cellular source facilitating their own regeneration by self-duplication [15]. These results do not dismiss the ability of the pancreas to generate new islets and existence of pancreatic progenitors in response to pancreatic injury [13]. Finally, de-differentiation of islet cells to provide a facultative stem cell remains a possibility. The results do, however, show that under normal physiological conditions the turnover of cells within the pancreas does not necessarily rely on the contribution of stem cells.

Due to the similarities between pancreatic islets and neuronal cells researchers hypothesized that the intermediate filament protein nestin, used as a marker for a multipotent stem cell population in the central nervous system [16], would also be found in the pancreas. Although it was previously suggested that pancreatic stem cells might be found in the ducts, Hunziker and Stein found a subset of cells expressing nestin within the pancreatic islets [17]. Zulewski et al. discovered that by culturing human islets they were able to generate a monolayer of nestin-positive islet derived progenitor cells, NIPs [18]. Lechner and coworkers have shown that 2.1% of these cells exclude Hoechst 33342 dye [19], which defines the pluripotent side population of haematopoietic stem cells [20]. These cells are called side population (SP) cells because they are found off to the side of the main population of Hoechst stained cells when analyzed by FACS. When exposed to verapamil, an inhibitor of multi-drug resistance-like proteins, they can no longer exclude Hoechst dye. A population of SP cells directly isolated from the pancreas has yet to be characterized. However, it has been shown that in the resting pancreas nestin is not a marker of pancreatic stem cells but of pancreatic stellate cells and angiogenic cells [21].

Stem cell antigen-1 (Sca1) is a small cell surface glycoprotein that provided one of the first epitotes of the isolation of murine HSCs [22,23]. The use of Sca1 antibody provided a 100-fold enrichment of HSCs [23,24]. The Sca1 epitote used for this enrichment is encoded by the *Ly6A* gene [25]. Since the discovery that Sca1 enriches HSCs, it has been used for the isolation of stem cells from additional tissues such as muscle [26] and mammary epithelium [27]. Therefore, Sca1 is a putative marker for resident stem cells in all adult tissues of the mouse.

The overall aim of our research was to evaluate the use of Sca1 as a marker for the cellular source of pancreatic regeneration and multipotent precursors in the resting pancreas. Given the therapeutic value, particular interest was given to the ability of Sca1-expressing cells to differentiate towards a mature pancreatic β-cell. Although a small percentage of these cells could differentiate towards a mature β-cell, we found Sca1 to be a poor marker for an exclusive resident pancreatic precursor as it represented a very dynamic heterogeneous population. Although a vast majority of Sca1-expressing cells are not the direct cellular source of regeneration, they likely play an indirect role in facilitating regeneration as they represent endothelial, perivascular and pancreatic stellate cells.

Results

Isolation of putative stem cells from the adult resting pancreas

To examine the cellular source of pancreatic regeneration the complete adult pancreas was digested into a single cell solution and incubated on ice with fluorescently tagged antibodies specific for Sca1 (see Table 1). Fluorescent activated cell sorting (FACS) was then performed on the single cell solution with Sca1FITC (n=3), Sca1PE (n=3) or Sca1APC (n=5). The Sca1APC antibody labeled a distinct population, representing close to 10% of the cells in the adult pancreas (Fig. 1A). Unspecific labeling was quantified using the same concentration of Rat IgG2a isotype control antibody (see Table I) revealing a background of only 0.06% (Fig. 1A).

In an alternative method the differential efflux of Hoechst dye was employed to purify a putative pancreatic stem cell population. The cells which efflux the Hoechst dye have been shown to have stem cell properties in bone marrow [28] and muscle [20]. These cells are represented in the side population, SP, which is distinct from the main population on a FACS histogram (Fig. 1B). Just over 1% of the cells in the pancreas are found in the SP population (n=6). Verapamil blocks the efflux of the dye and is used as a control for the SP population (Fig. 1B).

Staining cells for Sca1 following the Hoechst procedure revealed Sca1 to be enriched 10-fold in the SP population (n=3). However, cells isolated with the Hoechst protocol were not viable as they require a prolonged incubation at 37 degrees, during which time digestive enzymes released by the exocrine cells resulted in cell death. This difficulty makes the isolation of viable SP cells uniquely problematic in the pancreas. Therefore, we focused on the isolation and characterization of $Sca1^{+}$ cells.

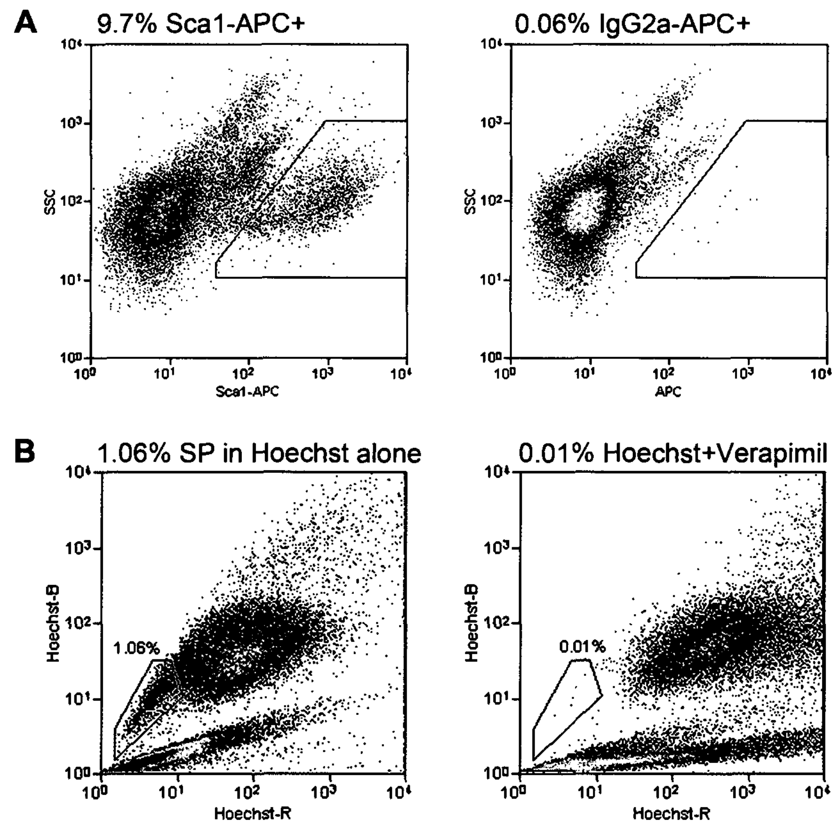

Figure 1. Isolation of putative adult stem cells from the pancreas.

A) The isolation of Sca1$^+$ cells using a monoclonal antibody specific for mouse Sca1 directly conjugated to Allophycocyanin (APC). Gating for the positive population was set to ensure less than 0.1% contaminating cells using an APC isotype control antibody.

B) Isolation of an SP population of cells from the pancreas using the differential efflux of the Hoechst dye. Gating was set using verapamil as a control as it inhibits the transporters responsible for the efflux of the dye.

Induced differentiation towards a mature β-cell

To investigate the ability of differentiation to β-cells, $Sca1^+$ cells were isolated from mice ubiquitously expressing GFP and co-cultured with pancreatic cell lines, islets and fetal pancreata. Although co-culture with pancreatic cell lines or islets did not induce the expression of Pdx1 after either three days or one week (Fig. 2A), co-culture with fetal pancreata (E17.5) induced 5% of $Sca1^+$ donor cells to express Pdx1 (Fig. 2B). Pdx1 was not seen in $Sca1^+$ cells isolated at the same time but cultured alone. When the experiment was repeated (n=3) $Sca1^+$ cells differentiated into $Pdx1^+$ cells, but with a reduced efficiency (3%, <1%). The use of inserts with 0.4 μm pores to separate the fetal pancreas from donor cells (n=3) or conditioned media (n=4) failed to induce the differentiation of $Sca1^+$ cells.

Pdx1 is expressed in progenitors during pancreatic development and expression is maintained only within β-cells in the adult. To determine if the induced differentiation was towards an early progenitor or towards a mature insulin expressing β-cell we set out to determine if these differentiated cells were expressing insulin. As it is difficult to determine if insulin is produced or taken up by cells in culture, we made use of the transgenic mouse line that expresses GFP under the mouse insulin promoter (MIPGFP). Isolation of $Sca1^+$ cells from MIPGFP mice confirmed that β-cells do not express Sca1 (Fig. 2C). The $Sca1^+MIPGFP^{-ve}$ fraction of cells were isolated and co-cultured with a fetal pancreata (n=5). A small percentage, less than 1%, of freshly isolated $Sca1^+$ cells became GFP^+ when co-cultured with fetal pancreata. Moreover, the use of the MIPGFP mouse allowed a mechanism to ensure GFP cells did not appear due to contamination during sorting.

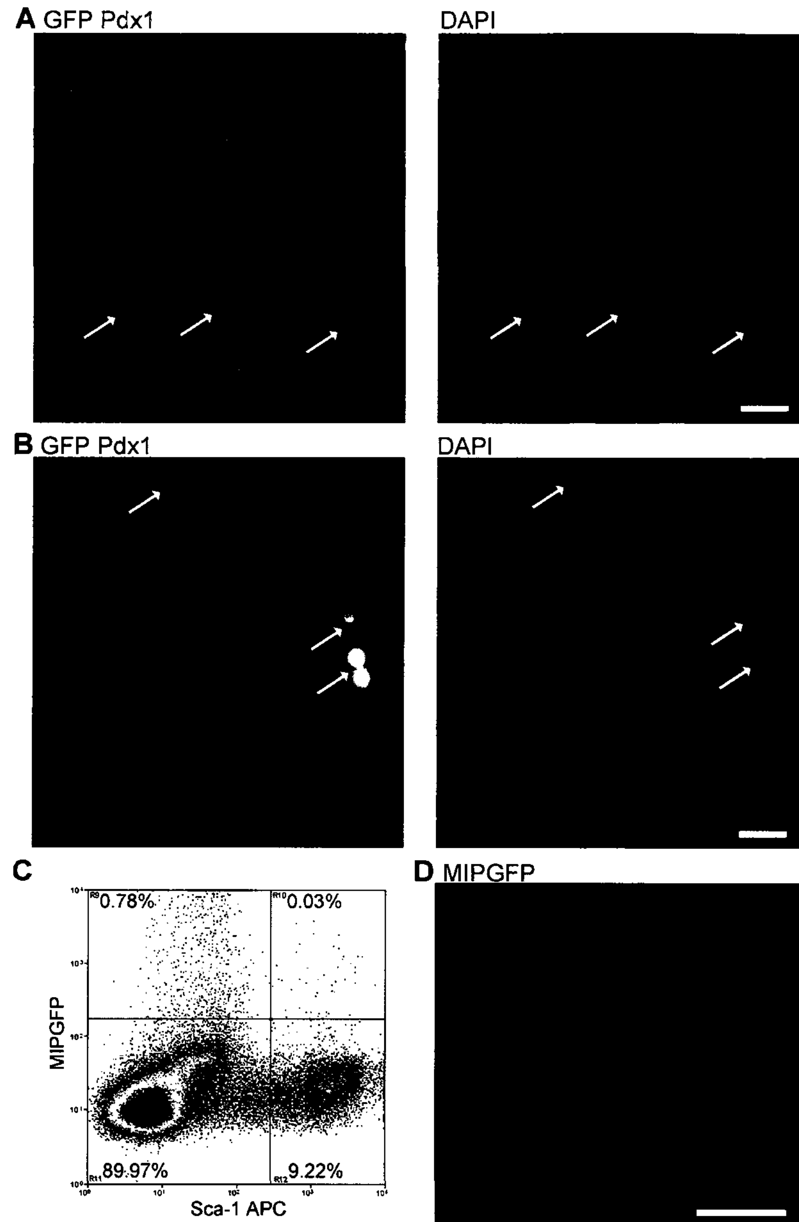

Figure 2. Freshly isolated Sca1^{+} cells can differentiate to pancreatic β-cells. A) When cultured with islets Sca1^{+} cells isolated from mice ubiquitously expressing GFP do not express Pdx1 (n=3). B) When co-cultured with an E17.5 fetal pancreas Sca1^{+} cells isolated from the same mouse express Pdx1 (n=4). C) When Sca1^{+} cells are isolated from mice expressing GFP under the mouse insulin promoter (MIPGFP) the GFP^{+ve} β-cells (0.78%) are Sca1^{-ve} (n=3) D) When Sca1^{+}MIPGFP^{-ve} cells are isolated and cultured with a fetal pancreas a small percentage start expressing GFP (n=3). Scale bars represent 50μm.

Localization of Sca1 expression in the adult pancreas

To determine which cells in the pancreas express Sca1, immunohistochemistry was performed on pancreatic sections of adult C57BL/6J mice 8 to 10 weeks old. Sca1 expression was observed scattered throughout the pancreas (Fig. 3A) by direct labeling with an antibody for Sca1 bound to the fluorochrome Allophycocyanin (APC). Minimal fluorescence was observed when the same concentrations and conditions were carried out with the rat IgG_{2a} isotype control antibody (Fig. 3B).

CD31, also known as PECAM-1 (Platelet Endothelial Cell Adhesion Molecule-1), is expressed constitutively on the surface of mature adult endothelial cells [29]. Co-localization with CD31 showed an overlap of Sca1 with mature endothelial cells (Fig. 3C,D). However, cells surrounding the $CD31^{+}$ vasculature contained $Sca1^{+}CD31^{-ve}$ cells (arrowheads; Fig. 3C,D). In addition, $Sca1^{+}CD31^{-ve}$ cells were found scattered throughout the exocrine tissue (arrows; Fig. 3C,D) and surrounding the pancreatic ducts (*; Fig. 3C,D). These $Sca1^{+}CD31^{-ve}$ cells localized to areas within the pancreas that contain pancreatic stellate cells; such as surrounding acinar and ductal structures.

To determine what fraction of $Sca1^{+}$ cells marked the endothelium we labelled a single cell solution with antibodies against both Sca1 and CD31 and performed a double FACS analysis (n=6). Although the Sca1PE antibody did not give as discrete a $Sca1^{+}$ population as the Sca1APC antibody (Fig.1A vs. Fig. 3D; left), the percentage of cells isolated was comparable. Analysis of the Sca1PE positive cells with CD31APC revealed close to two thirds of isolated $Sca1^{+}$ cells were mature endothelial cells (Fig. 3D; right).

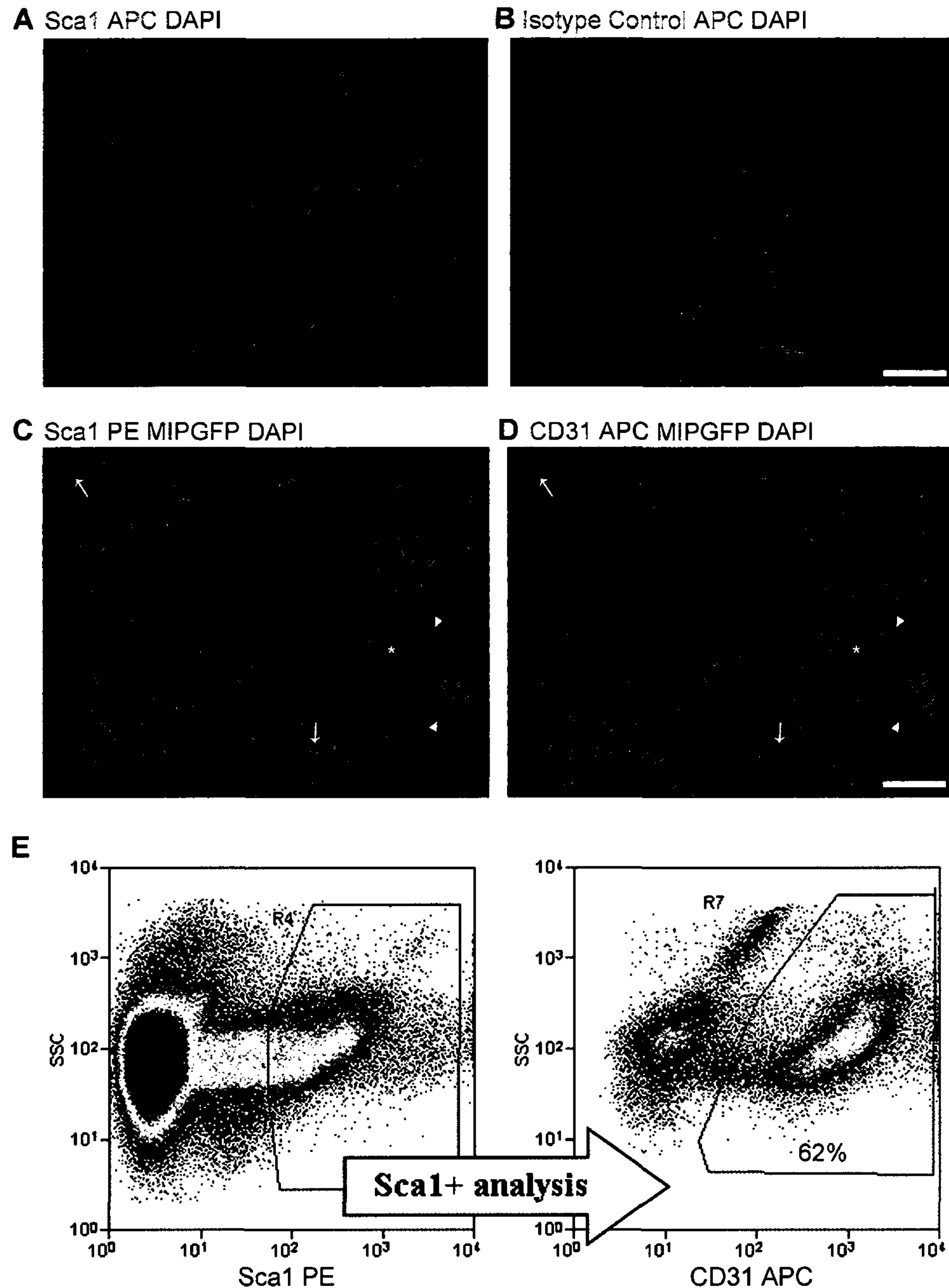

Figure 3. Immunohistochemical localization of Sca1 in the adult pancreas.

A) Sca1APC reveals labeling of cells scattered throughout the pancreas, which is not observed by the isotype control antibody (B). Scale bar represents 100um.

C,D) Sca1^{+}CD31^{-} cells are found scattered throughout the pancreas (arrows) as well as surrounding pancreatic ducts (*) and the vasculature (arrowheads). Scale bar 100um.

E) Representative FACS analysis of the Sca1 population revealing close to two thirds of the population are mature endothelial cells that express CD31 (n=6).

Proliferation of Sca1$^+$CD31$^-$ cells in vitro

Localization of Sca1 in tissue sections showed an overlap with the endothelium, therefore our efforts were directed towards Sca1$^+$CD31$^-$ cells. Moreover, Sca1$^+$CD31$^+$ cells died in culture while Sca1$^+$CD31$^-$ cells could be cultured *in vitro* with RPMI and 10% FBS either attached (Fig. 4A) or as spheres (Fig. 4B) when forced to grow in suspension on Poly(2-hydroxyethyl methacrylate) plated culture dishes. Therefore, following FACS purification a higher percentage of cells were viable when isolating Sca1$^+$CD31$^-$ cells as opposed to Sca1 alone. However, the actual number of viable cells per pancreata was the same as when isolating Sca1 alone. Therefore, co-culturing Sca1$^+$CD31$^-$ cells with fetal pancreata did not increase the percentage of surviving cells converting to Pdx1$^+$ cells. Furthermore, after passaging Sca1$^+$CD31$^-$ cells the ability to differentiate to Pdx1 or Insulin positive cells was lost.

Addition of 1 ng/ml of FGF had a significant effect on the growth of Sca1$^+$CD31$^-$ cells after one week in culture (n=3). There was an increase in growth rate (Fig. 4C) relative to culture in RPMI with just 10% FBS (Fig. 4D). In addition, significant changes were observed in cell morphology. Cells appeared to fuse together and form large spindle like structures (Fig. 4C). It should be noted, however, that this may have been a result in the high confluency of the cells due to the increased growth rate. Culturing the Sca1$^+$CD31$^-$ cells with FGF prior to inducing them to differentiate to Pdx1$^+$/MIPGFP$^+$ cells with a fetal pancreas was unsuccessful. The β-cell precursors within the freshly isolated Sca1$^+$CD31$^-$ cell population are lost either due to cell death or overgrowth of a competing cell type.

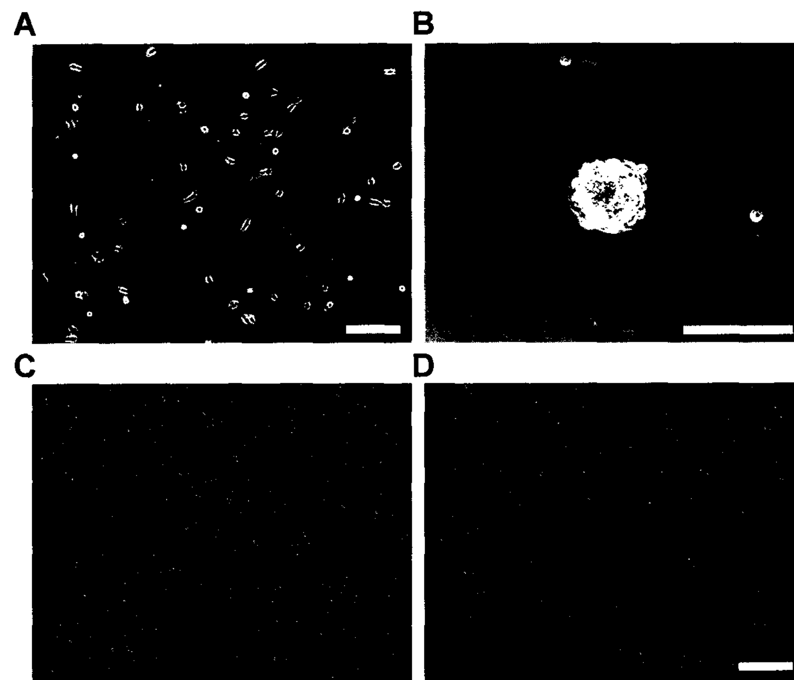

Figure 4. The proliferation of $Sca1^{+}CD31^{-}$ cells is enhanced by bFGF.

A) $Sca1^{+}CD31^{-}$ cells proliferate *in vitro* in RPMI + 10% FBS and attach to the culture dish. Morphology varies from rounded to stellate shaped.

B) When forced to grow in suspension $Sca1^{+}CD31^{-}$ cells grow in spheres.

C) Cells cultured for one week with the addition of 1 ng/ml of basic Fibroblast Growth Factor (bFGF) and D) when cultured in RPMI and serum alone. C,D) DAPI staining taken at the same magnification. All scale bars represent 200um.

Freshly isolated Sca1$^+$CD31$^-$ cells contain endothelial progenitors

The localization of Sca1$^+$CD31$^-$ cells surrounding vasculature suggested that this population of cells may contain endothelial progenitors. To test this hypothesis Sca1$^+$CD31$^-$ cells were cultured on matrigel in an assay known to induce the formation of endothelial like structures (n=5). Sca1$^+$CD31$^-$ cells showed the capacity to differentiate to an endothelial lineage as they formed capillary like structures when grown on matrigel (Fig. 5A). Sca1$^+$CD31$^+$ cells did not form these structures when cultured on matrigel (n=3). In fact similar to their inability to be maintained in culture with RPMI and 10% FBS, we were also unable to culture Sca1$^+$CD31$^+$ cells on matrigel (Fig. 5B). Further proof for the existence of endothelial progenitors within the Sca1$^+$CD31$^-$ population was observed when the cells were grown to confluence (n=3). When grown to confluence small colonies of cells began spontaneously expressing CD31 (Fig. 5C). To determine if these cells were circulating progenitors we cultured Sca1$^+$CD31$^-$CD45$^+$ cells on matrigel and we found that they did not form the vascular structures produced by Sca1$^+$CD31$^-$ cells (Fig. 5D). Therefore, Sca1$^+$CD31$^-$ cells likely contain a resident endothelial progenitor population, similar to the c-kit$^+$CD45$^-$ cells previously reported in the liver [30], and muscle [31].

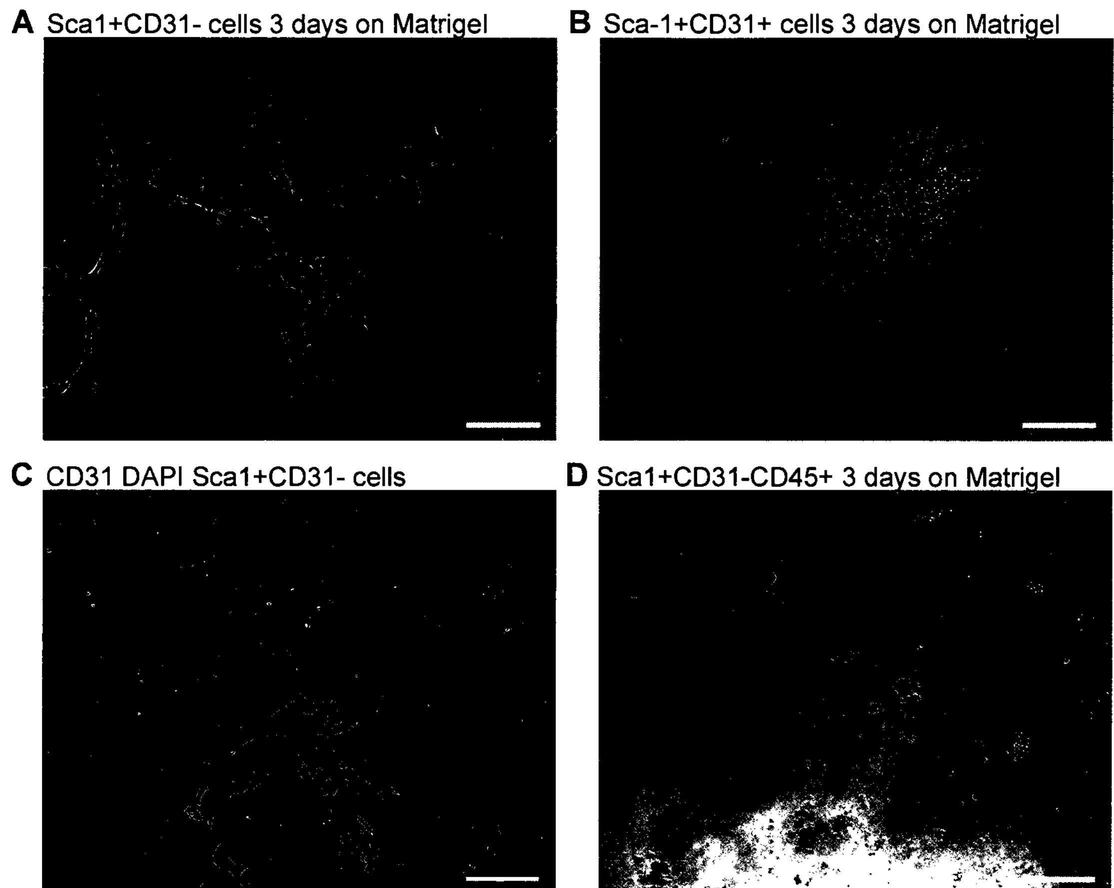

Figure 5. Freshly isolated Sca1$^+$CD31$^-$ cells contain endothelial progenitors. A) After 3 days on matrigel Sca1$^+$CD31$^-$ cells form capillary like structures (n=5). B) Sca1$^+$CD31$^+$ cells do not survive or grow capillaries when cultured on matrigel. C) When Sca1$^+$CD31$^-$ cells are cultured and allowed to proliferate into a confluent monolayer small scattered colonies are found that spontaneously express CD31 (n=3). D) The cells within the Sca1$^+$CD31$^-$ population that differentiate into mature CD31 positive endothelium are not circulating endothelial progenitors as they do not express CD45 as shown when Sca1$^+$CD31$^-$CD45$^+$ cells fail to grow vascular tubes in culture. Scale bars represent 100μm.

Cultured Sca1^{+} cells exhibit characteristics of mesenchymal stem cells

The isolation of mesenchymal stem cells (MSCs) from the pancreas has been previously described [32]. Localization of Sca1 suggests Sca1^{+}CD31^{-} cells may contain MSCs. BMP4 has been shown to induce MSCs to an oesteogenic lineage. Therefore, BMP4 was added to Sca1^{+}CD31^{-} cultures and the expression of oesteogenic markers was assessed (n=3). Alkaline phosphatase has been shown to be a marker of oesteogenic differentiation. As predicted the addition of BMP4 to Sca1^{+}CD31^{-} cultures increased alkaline phosphatase (Fig. 6A). *Runx2*, a master regulator in oesteoblasts, was expressed after the addition of BMP4 (Fig. 6B). In addition, there was a loss of α-smooth muscle actin corresponding to a change in cell phenotype (Fig. 6B).

Previous reports have described the ability to induce the differentiation of mesenchymal cells isolated from the pancreas into islet-like clusters [18,33]. Therefore, Sca1^{+}CD31^{-} cultures were tested using these published protocols. Confluent monolayers of Sca1^{+}CD31^{-} cells isolated from MIPGFP mice were covered with matrigel (n=6). As was previously reported clusters of cells, called islet-like clusters, emerged from the monolayer and grew into the matrigel (Fig. 6C). As these cells were isolated from MIPGFP mice, the presence of GFP could be used to verify insulin expression. Although these clusters appeared GFP positive (Fig. 6D), the Rhodamine channel (Fig. 6E) revealed most of the fluorescence observed was not from GFP but from auto-fluorescence as shown by merging the two images (Fig. 6F). However, some small areas within only a few larger islet-like clusters did express GFP (arrows; Fig. 6D to F). Therefore, insulin expression was both rare and if present only represented a small portion of the islet-like cluster.

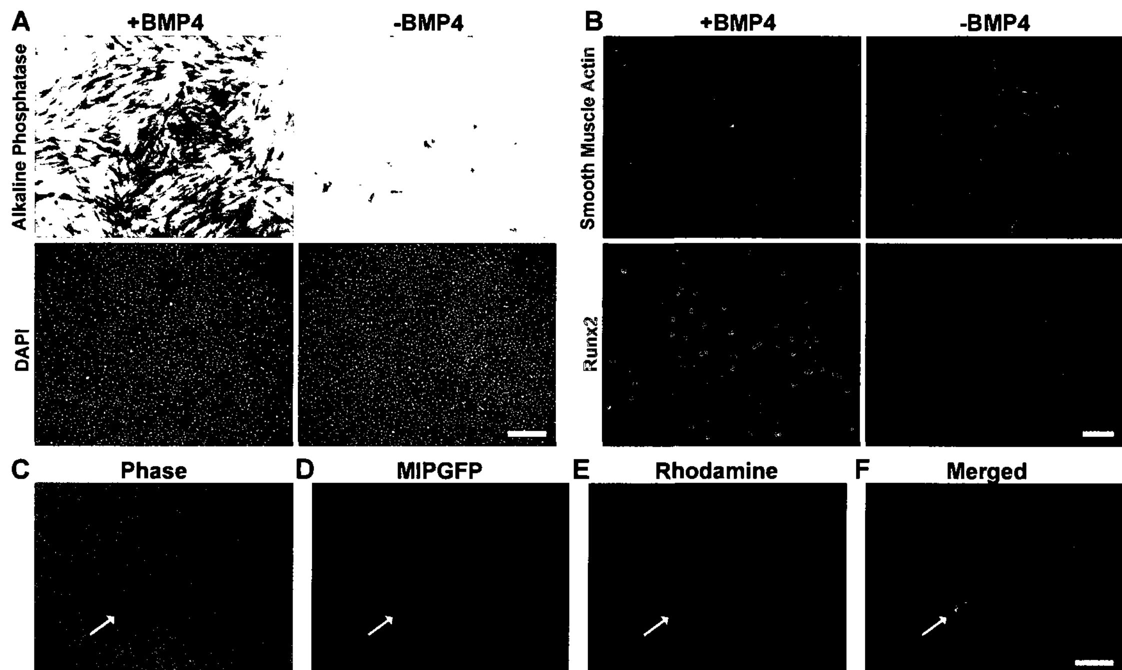

Figure 6. Differentiation of Sca1^{+}CD31^{-} cells following prolonged culture.

A) Addition of BMP4 to Sca1^{+}CD31^{-} cells increased alkaline phosphatase. Scale 500μm.

B) BMP4 reduced α-smooth muscle actin (top; green) and increased the expression of *Runx2* (bottom; red), a master regulator of osteoblast differentiation. Scale bar 100μm.

C) Islet-like clusters formed after culturing Sca1^{+}CD31^{-} cells, isolated from MIPGFP mice, under matrigel as previously reported for differentiation to islet-like clusters.

D) GFP fluorescence was observed in the islet-like clusters, however, a majority of the fluorescence was also observed in the rhodamine channel (E) indicating that only a small portion of GFP is expressed within islet-like clusters, as shown in the merged images of GFP and rhodamine channels (F). C-F) Scale bar represents 500μm.

Sca1 as a marker of pancreatic stellate cells

Pancreatic stellate cells (PSCs) are resident cells with long cytoplasmic processes wrapping around exocrine acinus, vasculature and ducts of the pancreas [34,35,36,37]. Similar tissue localization was observed for $Sca1^{+}CD31^{-}$ cells (Fig. 3A). Therefore, we examined cultured $Sca1^{+}CD31^{-}$ for known markers of pancreatic stellate cells. Upon activation, or when cultured *in vitro,* PSCs have increased expression of the intermediate filament α-smooth muscle actin (SMA) and form stress fibers [36,37]. After two weeks of culture $Sca1^{+}CD31^{-}$ cells showed SMA^{+} stress fibers (Fig. 7A). Additional markers for PSCs include Vimentin [37], Nestin [21], GFAP [36] and in a smaller percentage of PSCs Desmin [36,37]. The expression of Vimentin (Fig. 7B), Nestin (Fig. 7C) and GFAP (Fig. 7D) was observed in cultured $Sca1^{+}CD31^{-}$ cells. However, Desmin (Fig. 7E) was present in fewer than half of the cultured $Sca1^{+}CD31^{-}$ cells as reported for PSCs [36,37]. Platelet-derived growth factor receptor type β (PDGFR β) has been reported as a marker for PSCs and their activation in pancreatic fibrosis [38]. We found $Sca1^{+}CD31^{-}$ cultured cells also expressed PDGFRβ (Fig. 7F), another marker for PSCs [38]. Therefore, in the resting pancreas PSCs express Sca1, creating a convenient marker for their isolation. Isolation and culture of Sca1-expressing cells results in the activation of pancreatic stellate cells giving rise to proliferating mesenchymal cells.

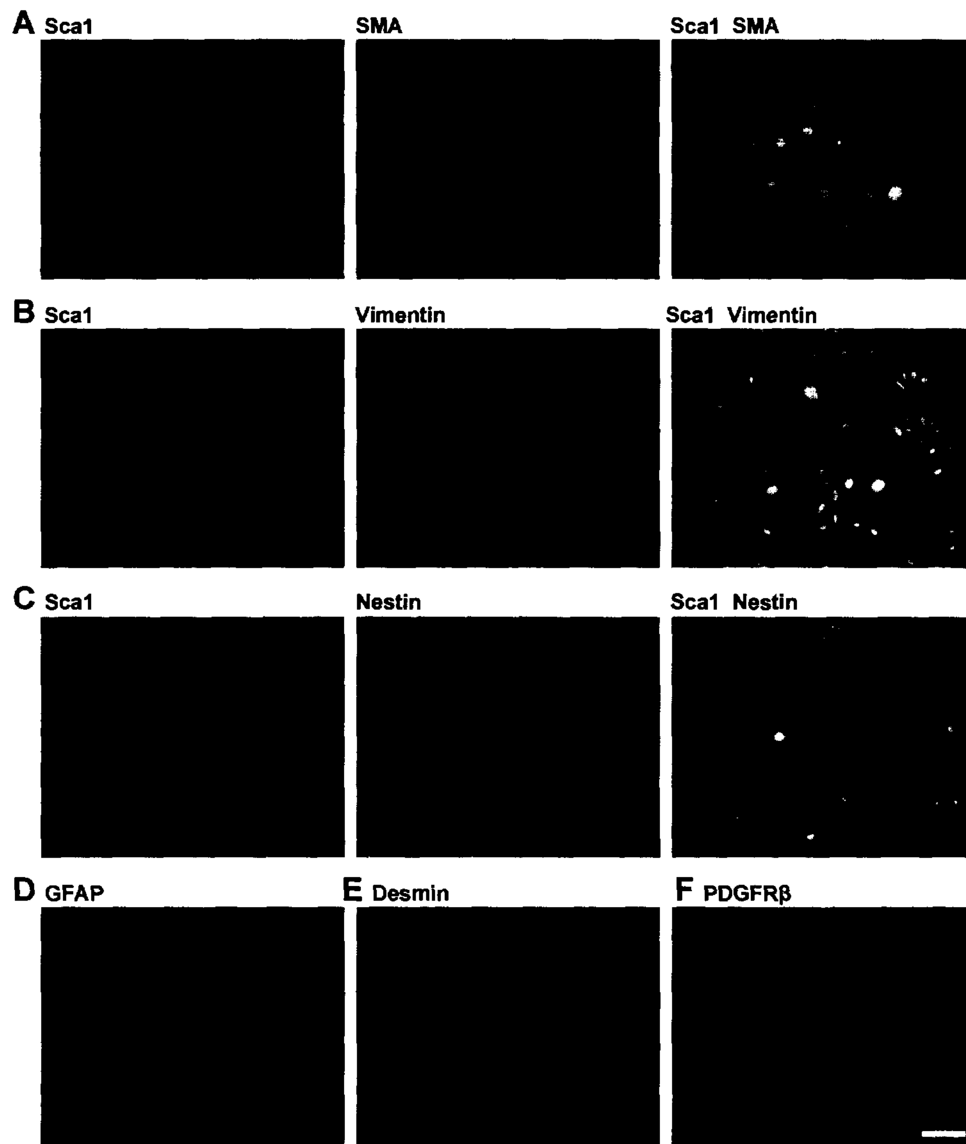

Figure 7. Sca1$^+$CD31$^-$ cells express markers of pancreatic stellate cells.

Sca1$^+$CD31$^-$ cells express α-smooth muscle actin (A), Vimentin (B) and Nestin (C) while maintaining the expression of Sca1 in culture. D) GFAP is also expressed in Sca1$^+$CD31$^-$ cells but due to the antigen retrieval required could not be co-stained with Sca1.

E) Less than 50% of cultured Sca1$^+$CD31$^-$ cells expressed Desmin. The morphology of Desmin$^+$ cells was flat and spread out compared to the stellate morphology of other cells.

F) Nearly all the cells also expressed the cell surface marker PDGFRβ, also reported to be expressed on pancreatic stellate cells. Scale bar 100um.

Sca1 expression during pancreatic regeneration

To further investigate the cells that directly contribute to regeneration we looked at the expression of Sca1 during pancreatic regeneration. Towards the base of the regenerating pancreatic remnant Sca1 marked stellate shaped cells (Fig. 8A). In addition, the base of the pancreas resembled a normal pancreatic morphology with a majority of tissue consisting of exocrine cells expressing amylase (Fig. 8B). Merging Sca1 expression with amylase (Fig. 8C) revealed they are exclusively expressed on distinct cell types. This Sca1 expression pattern was comparable to that observed in the resting pancreas (Fig. 3A).

Sca1 expression increased towards the tip of the regenerating pancreas and was present on a larger number of cells (Fig. 8D). The regenerating tip contained fewer exocrine cells expressing amylase (Fig. 8E). Merging Sca1 expression with amylase revealed Sca1 was expressed on exocrine tissue at the pancreatic tip during regeneration (Fig. 8F). Therefore, the broad expression of Sca1 found at the regenerating tip was due to both the proliferation of pancreatic stellate cells and novel expression from the acinar cells.

In areas containing tubular complexes, first observed three days following pancreatectomy, Sca1 was widely expressed with intense expression on clusters of cells (Fig. 8G). These clusters of cells co-localized with E-Cadherin (Fig. 8H,I). In addition, intense Sca1 expression (Fig. 8J) co-localized with tubular complexes expressing cytokeratin 7 (Fig. 8K,L). In the resting pancreas Sca1 is not found on epithelial cells expressing E-Cadherin or ductal cells expressing cytokeratin 7. Therefore, Sca1 expression is induced within these cell types at the focal area of regeneration.

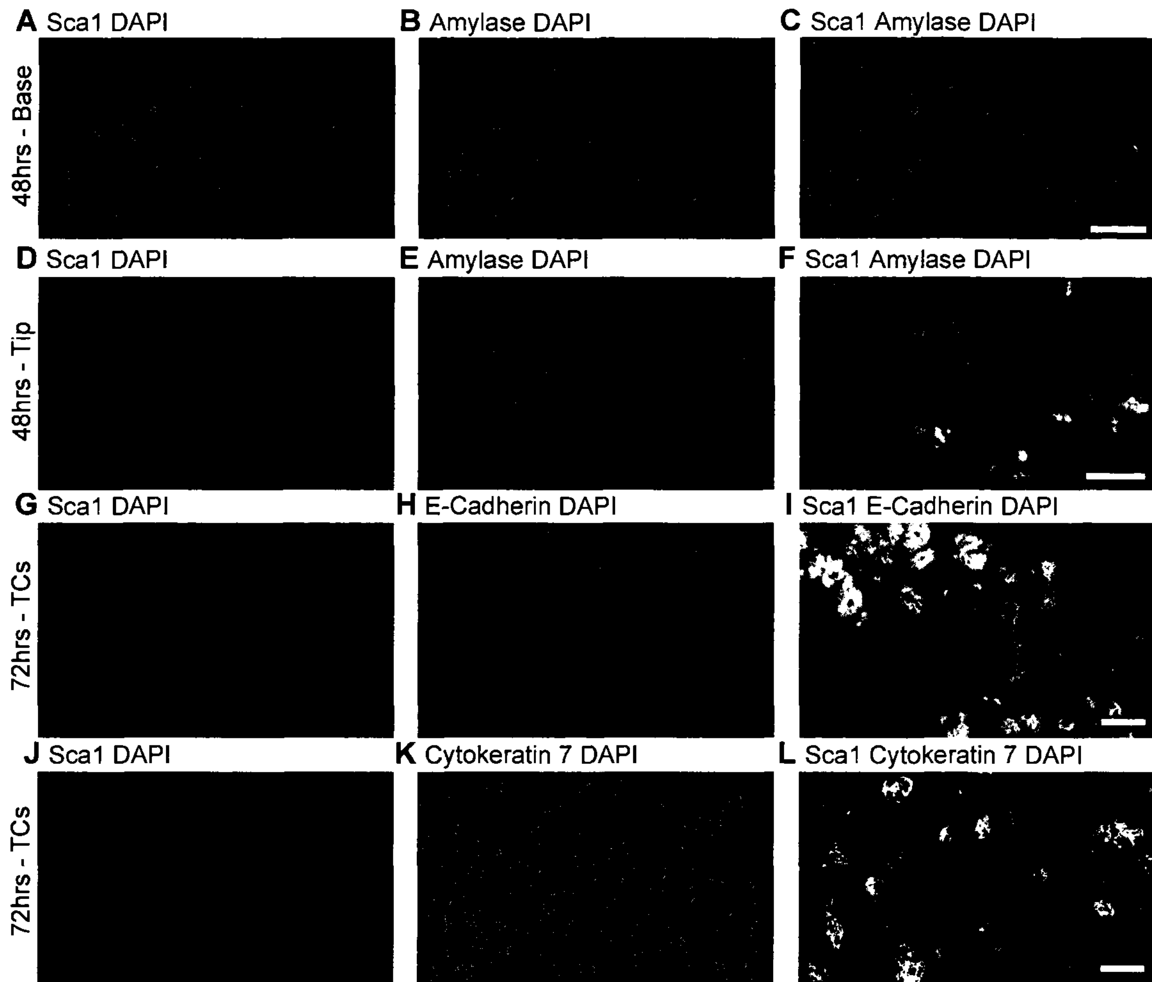

Figure 8. Sca1 is widely expressed during pancreatic regeneration.

A) Towards the base of the pancreatic remnant Sca1 (green) marks pancreatic stellate cells (PSCs) that surround B) amylase (red) expressing exocrine cells. C) Merging A,B) reveals Sca1 is exclusively expressed on PSCs surrounding the exocrine clusters. D) Sca1 (green) expression is increased close to the regenerating tip prior to tubular complex formation and E) amylase expression (red) is reduced. F) Merging D) and E) reveals amylase secreting exocrine cells are expressing Sca1 in the regenerating tip. G) Sca1 (green) expression is maintained as H) E-cadherin (red) expressing tubular complexes are formed. I) Merging the images shows Sca1 co-localizes with E-cadherin. J) Sca1 (green) expression on and surrounding K) tubular complexes expressing Cytokeratin 7 (Ck7). L) Merged images showing Sca1 expression on tubular complexes. A-L) Nuclei are stained with DAPI (blue). Scale bars represent 500μm.

In addition to the irregular expression of Sca1 at the regenerating pancreatic tip, Sca1 was also found to be expressed within the ducts soon after pancreatectomy. Twelve hours after pancreatectomy Sca1 was observed within areas of the ducts (arrow; Fig. 9A). The epithelial expression of E-cadherin, however, was maintained (Fig. 9B) and the cells were tightly clustered (Fig. 9C). One day following pancreatectomy Sca1 expression was increased within the ducts (arrowhead; Fig. 9D) and adjacent cells as they began to proliferate as shown by Ki-67 staining (Fig. 9E). The strongest Sca1 expression was observed on the non-proliferative type 1 tubular complexes (arrows; Fig. 9F) and expression was reduced on the more proliferative type 2 tubular complexes (arrowheads; Fig. 9F). Ki-67 staining clearly demonstrates the distinction between type 1 and type 2 tubular complexes (Fig. 9G,H). Therefore, Sca1 is expressed on proliferating tubular complexes in the focal area of regeneration; however, the broad expression observed hinders use of Sca1 as a marker of the cellular source of regeneration.

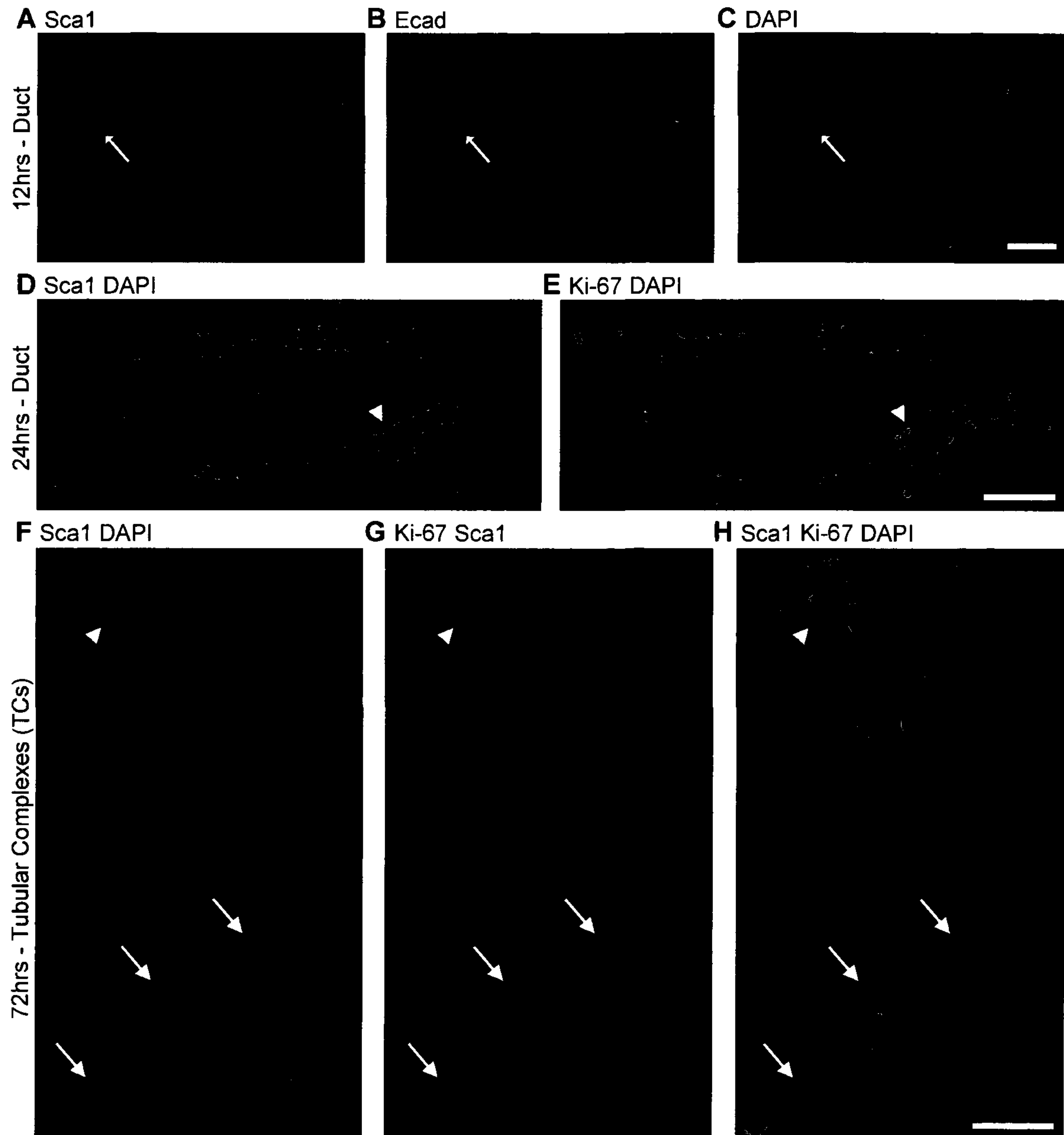

Figure 9. Sca1 is expressed on ducts and tubular complexes during regeneration. A-C) 12 hours after partial pancreatectomy (PPx) A) Sca1 is expressed in ducts (arrow), which also express B) E-cadherin (Ecad). C) Nuclei stained with DAPI. Scale bar 10μm. D,E) 24 hours after PPx, Sca1(red) is widely expressed in the ducts and nearby cells, as both begin to proliferate as demonstrated by Ki-67(green) (arrowhead). Scale bar 100μm. F-H) Sca1 (green) is expressed on the proliferative type 2 tubular complexes (arrowhead) as shown by Ki-67 (red). Sca1 is highly expressed on the non-proliferative type 1 tubular complexes (arrows). Nuclei are stained with DAPI (blue). Scale bar represent 500μm.

Discussion

The use of Sca1 as a stem cell marker in the pancreas is problematic as it defines a large population of cells in the resting pancreas and an even larger population in the regenerating pancreas. The presence of a putative stem cell within the heterogeneous Sca1 population was evident after a small percentage of cells, isolated based on Sca1 expression, could differentiate into Pdx1/insulin positive cells. During regeneration Sca1 marked a very dynamic population of cells and was observed in ducts and acinar cells shortly after pancreatectomy. Taken together, the isolation procedure itself could induce Sca1 expression in ductal cells, allowing them to mix in with $Sca1^{+}CD31^{-}$ purified cells. This is further supported by the observation of a small Cytokeratin 7 positive colony for every thousand freshly isolated $Sca1^{+}CD31^{-}$ cells (data not shown). Interestingly, early FACS purifications might have provided more time for Sca1 to be expressed in ductal cells allowing the higher percentage of differentiation to the β-cell lineage.

A large portion of the Sca1 expressing cells was accounted for after they were found to co-localize with CD31 expressing endothelial cells. However, isolation of $Sca1^{+}CD31^{-}$ cells did not increase the percentage of differentiating cells as $Sca1^{+}CD31^{+}$ cells already did not grow in culture. $Sca1^{+}CD31^{-}$ grew well in culture and proliferation was increased by the addition of FGF. Disappointingly, culturing $Sca1^{+}CD31^{-}$ cells diminished the capability of differentiation to the pancreatic β-cell lineage. $Sca1^{+}CD31^{-}$ cells localized to areas in the adult resting pancreas that are known to contain mesenchymal cells. These include pericytes surrounding the vasculature and pancreatic stellate cells surrounding the ducts and acinar cells.

We found cultured $Sca1^{+}$ cells express the mesenchymal markers reported for activated pancreatic stellate cells. In addition, FGF has been shown to induce the proliferation of pancreatic stellate cells [39]. Therefore, the effect FGF has on $Sca1^{+}CD31^{-}$ cells is further evidence that these cells represent PSCs in the pancreas. It is unlikely that freshly isolated $Sca1^{+}CD31^{-}$ cells exclusively represent PSCs as a fraction of the $Sca1^{+}CD31^{-}$ cells became CD31 positive in culture and readily grew capillary-like structures on matrigel. Therefore, $Sca1^{+}CD31^{-}$ cells contain a population of resident endothelial progenitors in addition to PSCs. Markers for endothelial progenitors overlap with PSCs [21], making it difficult to determine the number of endothelial progenitors present or if the two populations are mutually exclusive.

Similar markers exist between activated pancreatic stellate cells and mesenchymal stem cells. Therefore, $Sca1^{+}CD31^{-}$ cells could represent a mesenchymal stem cell population. To test this hypothesis BMP4 was added to cultured $Sca1^{+}CD31^{-}$ cells. Just as BMP4 induces the formation of bone in MSCs, it also induced the differentiation of $Sca1^{+}CD31^{-}$ cells. However, the differentiation of $Sca1^{+}CD31^{-}$ cells towards a pancreatic β-cell was much more difficult, with a very limited number of cells differentiating. This could be attributed to a difference between cultured $Sca1^{+}CD31^{-}$ cells and the mesenchymal cells previously published [33] or uncontrollable variations between culture conditions such as differences in media, FBS and how the cells were passaged.

After culture, $Sca1^{+}CD31^{-}$ cells appear to consist entirely of pancreatic stellate cells based on staining of PSC markers. It is difficult to determine the heterogeneity of this population as PSCs themselves have various characteristics. This is displayed in the variation of Desmin that is observed in only a fraction of the population [36,37,38,40].

The $Sca1^{+}CD31^{-}$ cells that expressed Desmin had a flat pancake-like shape and much larger cell body then the stellate shaped cells that are present in the rest of the PSC population. It is possible that the variation of Desmin observed represents distinct lineages of PSCs or is simply a variation of cell cycle or activation status. Nevertheless, PSCs are not uniform themselves making it difficult to validate the purity of the population. Previous isolation protocols relied only on gradient separation techniques. However, here we present a unique method for the isolation of PSCs.

Although $Sca1^{+}CD31^{-}$ cells fail to embody the direct cellular source of regeneration, they represent a cellular compartment valuable to pancreatic function, maintenance and regeneration. The idea of pancreatic stellate cells contributing to regeneration in the human has already been speculated [41]. In addition, pancreatic stellate cells have been shown to give rise to the desmoplastic reaction in pancreatic cancer, which influences cancer growth [42,43]. Furthermore, PSCs have been shown to be activated following duct obstruction [44]. This activation may be a key requirement for the emergence of $Ngn3^{+}$ progenitors observed following duct ligation [13]. Moreover, the expression of Sca1 itself could play a role in regeneration as it is expressed on ductal cells and tubular complexes in the regenerating pancreas. Therefore, further study of Sca1 expression in the adult pancreas will advance our understanding of the process of pancreatic regeneration.

Materials and Methods

Cell Isolation. The pancreas was removed, minced and resuspended in 3,000units (12 mg) of collagenase type I (sigma) in 4 ml RPMI + 10% FBS. The solution was shaken for 6mins in a 15 ml falcon. Digestion was stopped by adding 10 ml of cold DMEM + 10% FBS. The digested pancreas was spun down and resuspended in 2 ml of 2.5% Trypsin for 3 min, then 12 ml of cold DMEM + 10% FBS was added and the cells were spun down and resuspended in culture media with the appropriate antibodies.

Flow Cytometry. Single cells isolated from the pancreas were incubated on ice for 20min with the D7 monoclonal antibody specific for Sca1 (eBioscience; 17-5981) directly conjugated to Allophycocyanin (APC) at a concentration of 1:200. As a negative control the same the same procedure was followed with a Rat IgG2a isotype control antibody (eBioscience; 17-432). Following incubation cells were separated on a MoFlo cytometer (DakoCytomation). Dead cells and debris were removed by gating on forward vs. side scatter profiles. Gating for Sca1 expressing cells was set up so that less than 0.1% of cells were present within the gate for cells incubated with the rat IgG2a isotype control antibody. The protocol for isolation of SP cells was followed as previously described for bone marrow [28] and muscle [20]. $Sca1^{+}MIPGFP^{-ve}$ cells were isolated by first gating on the MIPGFP negative population before gating on the Sca1 expressing cells. All other antibodies used for FACS are listed in Table I.

Cell Culture and Differentiation Assays. FACS purified cells were cultured in RPMI with 10% FBS and 1% penicillin streptomycin. Islets were hand picked from partially digested pancreata of adult male C57BL/6J mice using Dithozone as a marker as previously described [45]. Fetal pancreata were isolated from embryos at E17.5. For the

growth as spheres Sca1^{+}CD31^{-} cells when grown on Poly 2-hydroxyethyl methacrylate (Sigma Cell Culture; P-3932) prepared as per manufacturers directions. Proliferation of attached cells was enhanced by the addition of 1 ng/ml of human recombinant, basic Fibroblast Growth Factor, bFGF (BioShop; F028). For the formation of vascular tubes Sca1^{+}CD31^{-} cells were cultured on 500ul of Matrigel (VWR; 47743) in one well of an 8 well slide (VWR; 62405-178). Alternatively, a confluent monolayer of Sca1^{+}CD31^{-} cells was covered with matrigel as previously described for the differentiation of mesenchymal cells to islet-like clusters [33]. Differentiation of cells to the oesteogenic lineage was induced by the addition of 5 ng/ml human BMP-4 (Cedarlane; cat#314-BP-010). Alkaline phosphatase staining was performed using NBT/BCIP tablets (Roche; 1 697 471) as per manufacturer's instructions.

Experimental animals and standard surgical procedures. Eight- to nine-week-old male C57BL/6J, GFPNagy and MIPGFP mice were housed under standard conditions and allowed free access to standard mouse chow and water. All studies were approved by the Animal Care Committee at the University of Ottawa. One hour before surgery mice were given a dosage of 0.05 mg/kg Buprenorphine subcutaneously. Anesthesia was induced in an anesthetic box with isoflurane gradually increased to 5%. The anesthetic was delivered by an Ohio Forane vaporizer (induction box) and an isoflurane vaporizer (mask). Once the mice were anesthetized they were transferred to a face mask with isoflurane at 1.5% where they are maintained throughout surgery, increasing or decreasing the percentage as necessary to keep the animal sedated but breathing normally. The surgical area was shaved and cleaned with Endure soap, rinsed with sterile water and surgically prepared with chlorahexseptic solution. BNP eye ointment was

placed in the animals eyes to protect them from drying. 1 ml of sterile saline was administered subcutaneously prior to surgery. Once the surgery was complete mice are placed on oxygen until they recovered and started to move.

Partial Pancreatectomy. To remove pancreatic tissue access to the abdominal cavity was obtained by performing a midline incision. First, a 1 to 1.5cm incision was made through the skin in the middle of the abdomen using a No.10 scalpel blade. Using forceps the skin was gently separated from the abdominal wall to reveal the midline of the abdomen. The midline was lifted with rat tooth forceps and a small cut less than 1cm was made with scissors through the body wall. Once located the splenic pancreatic lobe was lifted through the incision with forceps. The entire splenic lobe and distal portions of the gastric and duodenal pancreatic lobes were removed by gentle abrasion with forceps and a cotton applicator to ensure no major veins or arteries were broken. If excessive bleeding was observed the site of bleeding was clamped for several minutes to promote clotting. Once removed only a small portion (~30%) of the pancreas remained along the duodenum. The pancreas that was surgically removed was approximately 70% of the total pancreas. The body wall was closed with silk surgical sutures (Johnson&Johnson) using two to three discontinuous sutures. The skin was closed with two to three surgical staples (Fisher). Once the surgery was complete the mice were placed on oxygen for approximately one minute and then returned to their cage as soon as they began to move. Blood glucose levels were analyzed every other day checking blood sugar levels for increased glucose. In addition, mice were given 0.05 mg/kg Buprenorphine subcutaneously every day following surgery for the first week. For the administration of BrdU 100ul of 1 mg/ml BrdU in saline was placed in the abdominal cavity prior to

closing. For continuous BrdU labeling, drinking water containing 0.8 mg/ml was given to animals following surgery and changed every other day thereafter.

Immunocytochemistry. Cultured $Sca1^{+}CD31^{-}$ cells were fixed with 4% PFA, permeabilized with 0.2% TritonX and stained for markers of pancreatic stellate cells (see Table II). For staining of cell surface antibodies such as Sca1 the antibody was incubated on live cells in media at 4 degrees prior to fixation. All antibodies used for immunocyctochemistry are listed in Table II.

Immunohistochemistry. Pancreata were embedded in optimal cutting temperature compound, O.C.T. (Tissue-Tek) : 20% sucrose solution (2:1) and frozen by emersion into 95% ethanol/dry ice. Cryosections (8um) were cut using a cryostat (Leicia CM 1850). The antibodies used are outlined in Table III. Nuclei were counter-stained with DAPI (Sigma). Images were taken with a Carl Zeiss Axioplan 2 Microscope with an AxioCam HRm b/w (Zeiss) camera using Axiovision v3.2 (Zeiss) acquisition software.

Table I: Antibodies used for fluorescent activated cell sorting, FACS.

Primary Antibodies	Dilution	Company	Cat#
APC Rat IgG2a anti-mouse Sca1	1:200	Cedarlane (ebioscience)	17-5981-83
APC Rat IgG2a Isotype Control	1:200	Cedarlane (ebioscience)	17-4321-81
CD31-APC	1:200	BD Bioscience	551262
CD31-PE	1:200	BD Bioscience	553373
CD45-FITC	1:200	BD Bioscience	01114A
Sca1 PE	1:200	BD Bioscience	553108

Table II: Antibodies used for immunofluoresent labeling of cultured cells.

Primary Antibodies	Dilution	Company	Cat#
CD31 PE	1:200	BD Bioscience	553373
Cytokeratin 7 (msIgG1)	1:200	BD Bioscience	550507
Desmin (msIgG1)	1:150	Dako	M0760
GFAP cocktail (msIgG2b)	1:200	BD Bioscience	556330
Nestin (msIgG1)	1:500	BD Bioscience	556309
PDGFRβ (rat IgG2a)	1:200	ebioscience	14-1402-82
Pdx1 (rabbit)	1:5,000	Gift from Chris Wright	
Runx2 (msIgG2b)	1:150	MBL Ltd. Japan	D130-3
Sca1 APC	1:200	Cedarlane (ebioscience)	17-5981-83
Sca1 PE	1:200	BD Bioscience	553108
Vimentin D547	1:200	Abcam	38873
α smooth muscle actin (msIgG1)	1:1,000	Sigma	A5228
Secondary Antibodies			
anti-Mouse IgG1 A488	1:2,000	Invitrogen - Molecular Probes	A21121
anti-Mouse IgG1 A568	1:2,000	Invitrogen - Molecular Probes	A21124
anti-Mouse IgG2a A488	1:2,000	Invitrogen - Molecular Probes	A21131
anti-Mouse IgG2b A647	1:1,000	Invitrogen - Molecular Probes	A21242
anti-Rabbit A546	1:2,000	Invitrogen - Molecular Probes	A11035
A488 donkey anti-rat IgG (H+L)	1:1,000	Invitrogen - Molecular Probes	A21208

Table III: Antibodies used for immunofluorescent labeling of frozen sections.

Primary Antibodies	Dilution	Company	Cat#
Amylase (rabbit)	1:500	Sigma	A8273
CD31 PE	1:200	BD Bioscience	553373
Cytokeratin 7 (msIgG1)	1:200	BD Bioscience	550507
E-Cadherin (msIgG2a)	1:500	BD Bioscience	610182
Ki-67 (msIgG1)	1:200	BD Bioscience	550609
Sca1 APC (Rat IgG2a)	1:200	Cedarlane (ebioscience)	17-5981-83
Sca1 APC Isotype Control	1:200	Cedarlane (ebioscience)	17-4321-81
Sca1 FITC	1:500	BD Bioscience	01164a
Secondary Antibodies			
anti-Mouse IgG1 A568	1:2,000	Invitrogen - Molecular Probes	A21124
anti-Mouse IgG2a A546	1:2,000	Invitrogen - Molecular Probes	A21133
anti-Rabbit A546	1:2,000	Invitrogen - Molecular Probes	A11035

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Chapter 3 – Mesenchymal cells facilitate regeneration

Mesenchymal cells facilitate pancreatic regeneration by producing a supportive stroma.

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Abstract

Background: The adult resting pancreas has been suggested to undergo growth and self-renewal very slowly by duplication of pre-existing cells. However, partial pancreatectomy in the rat induces regeneration through rapid proliferation in duct-like tubular complexes. The aim of this study was to first localize these proliferating areas in the mouse, and second to identify novel factors within these regenerative foci using a microarray screen.

Methodology/Principal Findings: Early proliferation after 70% pancreatectomy in the adult mouse was observed within mesenchymal cells. Within one day proliferation was most prominent surrounding pancreatic ducts. From two to four days following pancreatectomy proliferation became localized to patches of duct-like cells near the site of excision. At five days prominent foci of regeneration, located distal to the duodenum, created a regenerating tip that was distinct from the base of the pancreatic remnant. These distinct foci were characterized as having significantly increased amounts of BrdU incorporation and Ki67 expression. In addition, the regenerating tip contained an increased number of Cytokeratin7-expressing tubular complex structures, as well as Ngn3-expressing pancreatic progenitors. To identify novel factors contributing to the regeneration process RNA expression in the early focal areas of regeneration was compared to that of resting pancreatic tissue by microarray analysis. A significant increase was found in a wide variety of factors in the regenerating pancreatic tip compared to the resting tissue, some of which were expressed by the mesenchymal stroma. Importantly, the transplantation of activated pancreatic stellate cells into the pancreas was sufficient to produce a mesenchymal stroma which facilitated regeneration.

Conclusions/Significance: Pancreatic stellate cells produce a mesenchymal stroma to facilitate regeneration in the pancreas following pancreatectomy. The formation of this stroma precedes the appearance of tubular complexes and Ngn3-expressing progenitor cells. Transplantation of stroma is sufficient to induce the formation of tubular complexes. Therefore, understanding the factors produced by this stroma will uncover the mechanism through which neogenesis occurs during pancreatic regeneration.

Introduction

Studying regeneration following partial pancreatectomy provides the opportunity to uncover factors with potential therapeutic use for the treatment of both Type 1 and Type 2 diabetes. In Type 1 diabetes pancreatic β-cells are attacked and destroyed by the immune system. If regeneration could be initiated within the pancreas of a diabetic patient, and the immune system is suppressed, they could replace lost insulin producing cells. In Type 2 diabetics not enough insulin can be produced to meet the demand required by the body. If the number of β-cells could be increased, in combination with increasing insulin sensitivity, the demand required for insulin could be met and the symptoms of Type 2 diabetes would be eliminated.

The pancreas consists of E-cadherin expressing epithelial cells and vimentin expressing mesenchymal cells including pancreatic stellate cells (PSCs) (Apte et al. 1998; Bachem et al. 1998). PSCs are resident cells of the pancreas that are present in the periacinar space. Their long cytoplasmic extensions stretch out to give them their stellate or star-like shape. In addition to surrounding acinar clusters PSCs are also found within the perivascular (Ikejiri 1990) and periductal regions (Apte et al. 1998; Bachem et al. 1998) of the pancreas. Recently, PSCs have been found to be a key player in pancreatic diseases. Pancreatic cancer is surrounded by a dense stroma, called desmoplasia (Mahadevan and Von Hoff 2007). This stroma is a source of many factors influencing cancer progression (Sethi et al. 1999; Apte and Wilson 2007; Chu et al. 2007; Mahadevan and Von Hoff 2007), and PSCs are thought to be the source of the stroma supporting tumor growth (Yen et al. 2002; Apte et al. 2004). Similarly PSCs are thought to be the

source of fibrosis in the pancreas resulting in pancreatitis (Wells and Crawford 1998; Haber et al. 1999).

Increased islet neogenesis has been reported following the administration of exendin-4 (Xu et al. 1999) or Betacellulin (Li et al. 2003a), when interferon-γ (Gu and Sarvetnick 1993) or TGF-α (Wang et al. 1993) was overexpressed in transgenic mice, and after pancreatectomy (Bonner-Weir et al. 1983; Brockenbrough et al. 1988; Bonner-Weir et al. 1993; Montana et al. 1994) or partial duct ligation (Hultquist et al. 1979; Rosenberg 1998; Li et al. 2003b; Bonner-Weir et al. 2008). Pancreatic duct ligation has been shown to induce Ngn3 precursors to emerge (Xu et al. 2008). Furthermore, the differentiation of these Ngn3 precursors into islets has been demonstrated (Xu et al. 2008). Although Ngn3 was not observed following 50% pancreatectomy (Lee et al. 2006), the presence of these cells following 70% pancreatectomy has yet to be determined. Regeneration within the rat pancreas following 90% pancreatectomy has been well documented (Bonner-Weir et al. 1993). In this model regeneration is accomplished by the replication of pre-existing cells as well as the formation of new lobes of the pancreas (Bonner-Weir et al. 1993). The new lobes first appear as patches of proliferating ducts two and a half days following pancreatectomy (Bonner-Weir et al. 1993). The ductal cells within these distinct foci of regeneration transiently express Pdx1 (Sharma et al. 1999).

The direct cellular source of regeneration remains controversial. The substantial growth of islets postnatally suggests the existence of adult progenitors (Bonner-Weir 2000). In addition, studying regeneration following ductal ligation has shown islet neogenesis closely associating with the ducts (Rosenberg 1998; Bonner-Weir et al. 2008).

Furthermore, *in vitro* studies of the ductal epithelium have shown ductal cells to be a pool for pancreatic progenitors (Bonner-Weir et al. 2000). By irreversibly marking ductal cells based on their expression of Carbonic anhydrase II recent lineage tracing studies have shown these cells to be progenitors for both the endocrine and exocrine pancreas after birth (Inada et al. 2008).

The overall aim of our study was to examine regeneration following 70% pancreatectomy in the mouse with particular interest in pancreatic β-cells given the therapeutic potential. Two major mechanisms account for the increase in β-cells observed during regeneration. The first is the replication of pre-existing β-cells by self duplication (Dor et al. 2004). The second includes the process of post-natal islet neogenesis (Bonner-Weir et al. 1993; Inada et al. 2008). To locate focal areas of regeneration that would contain areas of islet neogenesis we examined the proliferation and histology of the pancreas following pancreatectomy. We first observed proliferation of PSCs which created a mesenchymal stroma accommodating a second wave of proliferation within duct-like tubular complexes. Furthermore, a microarray screen revealed novel factors within the regenerative foci, some of which are produced by the mesenchymal stroma. The transplantation of activated PSCs into the pancreas is sufficient to create a mesenchyme facilitating the emergence of Ngn3 progenitors and tubular complex formation. Therefore, we hypothesize PSCs to play an important role in producing a mesenchymal stroma that is sufficient to initiate the emergence of Ngn3 precursors and tubular complex formation.

Results

Mesenchymal cells form a stroma prior to the initiation of regeneration.

To temporally and spatially localize proliferation during regeneration pancreata were examined at 12 hour increments following partial pancreatectomy (PPx). Mock surgeries were performed (n=15) to determine the percentage of pancreas removed in the surgical procedure to be approximately 68% (Supplementary Figure 1). Resection of this quantity resulted in nearly 100% survival of mice. Increasing the degree of PPx reduced the viability of mice following surgery. Less than 50% of mice survived when approximately 80% of the pancreas was removed with even fewer surviving after 90% pancreatectomy. The most common mortality was due to internal bleeding based on necropsy.

To obtain a baseline of proliferation Ki67 expression was examined in mice that had not undergone pancreatectomy (n=3). In the absence of PPx proliferating cells expressing Ki-67 were rarely observed in pancreatic epithelial cells that express E-cadherin (Fig. 1a). This includes the epithelial cells of the islets, exocrine tissue and pancreatic ducts. In addition, proliferation was extremely rare or absent in non-epithelial cells that do not express E-cadherin (Fig. 1a). Within twelve hours following pancreatectomy (n=3) proliferation was observed surrounding the ductal epithelial cells but still rarely within the epithelium (Fig. 1b). A disruption within the epithelium was observed twenty-four hours after pancreatectomy (Fig. 1c). At this time proliferation continued around the ducts (arrowheads; Fig. 1c) and within selective areas of the ducts (arrows; Fig. 1c).

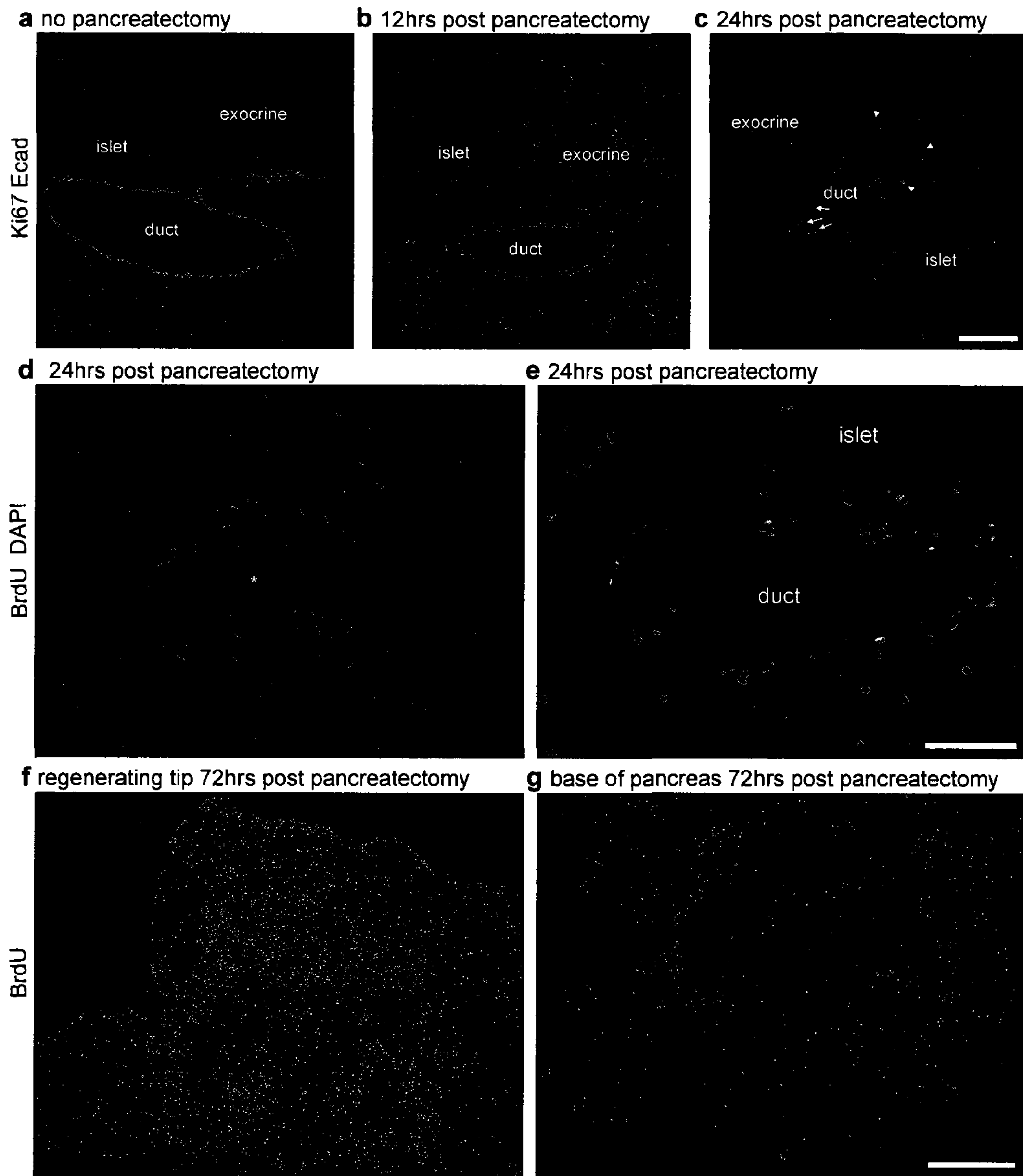


Figure 1. Early proliferation of mesenchymal cells after partial pancreatectomy.
a) Proliferating cells expressing Ki67 (red) are rarely observed in the absence of PPx.
b) 12 hours following PPx, Ki67 marks proliferating cells surrounding the ducts.
c) 24 hours following PPx proliferation initiates within the ducts (arrows) and continues surrounding the ducts (arrowheads). Scale bar for a-c represents 100μm.
d,e) Within the first 24 hours cells surrounding arteries (* in d) and pancreatic ducts (e) incorporate BrdU (red). Nuclei stained with DAPI (blue). Scale bar represents 100μm.
f,g) Three days following pancreatectomy BrdU accumulation is greater at the tip of the pancreatic remnant (f) compared to the base (g). Scale bar represents 500μm.

To trace the proliferating cells BrdU was administered at the time of surgery and continuously post-surgery in the drinking water. Cells that incorporated BrdU within the first twenty-four hours were observed around the vasculature of arteries (asterisk; Fig. 1d) and the pancreatic ducts (Fig. 1e) verifying the observations made with Ki67. However, the distribution of BrdU incorporated cells (Fig. 1e) extended further from the ducts then Ki67 expression (Fig. 1c). This suggested that proliferating cells were migrating away from the ducts, a hypothesis that was strengthened when greater BrdU accumulation was found at the tip of the pancreatic remnant (Fig. 1f) compared to the base (Fig. 1g) three days following pancreatectomy. The area of accumulated BrdU positive cells was distal to the head of the pancreas at the site of excision.

To further examine the proliferating non-epithelial cells we investigated the expression pattern of vimentin a known marker for mesenchymal cells including pancreatic stellate cells (PSCs). Prior to pancreatectomy vimentin marked PSCs surrounding ducts and scattered throughout the exocrine tissue, while epithelial structures expressed E-cadherin (Fig. 2a). Early after pancreatectomy, twelve hours, the vimentin expressing PSCs co-expressed the proliferation marker Ki67 (Fig. 2b). The proliferating PSCs resulted in an expansion of the mesenchymal stroma surrounding the pancreatic ducts. Twenty-four hours following pancreatectomy an increase in the number of cells expressing vimentin were observed surrounding ducts (Fig. 2c). Two days following pancreatectomy the stroma spread throughout the exocrine tissue (Fig. 2d) away from ducts and toward the pancreatic tip.

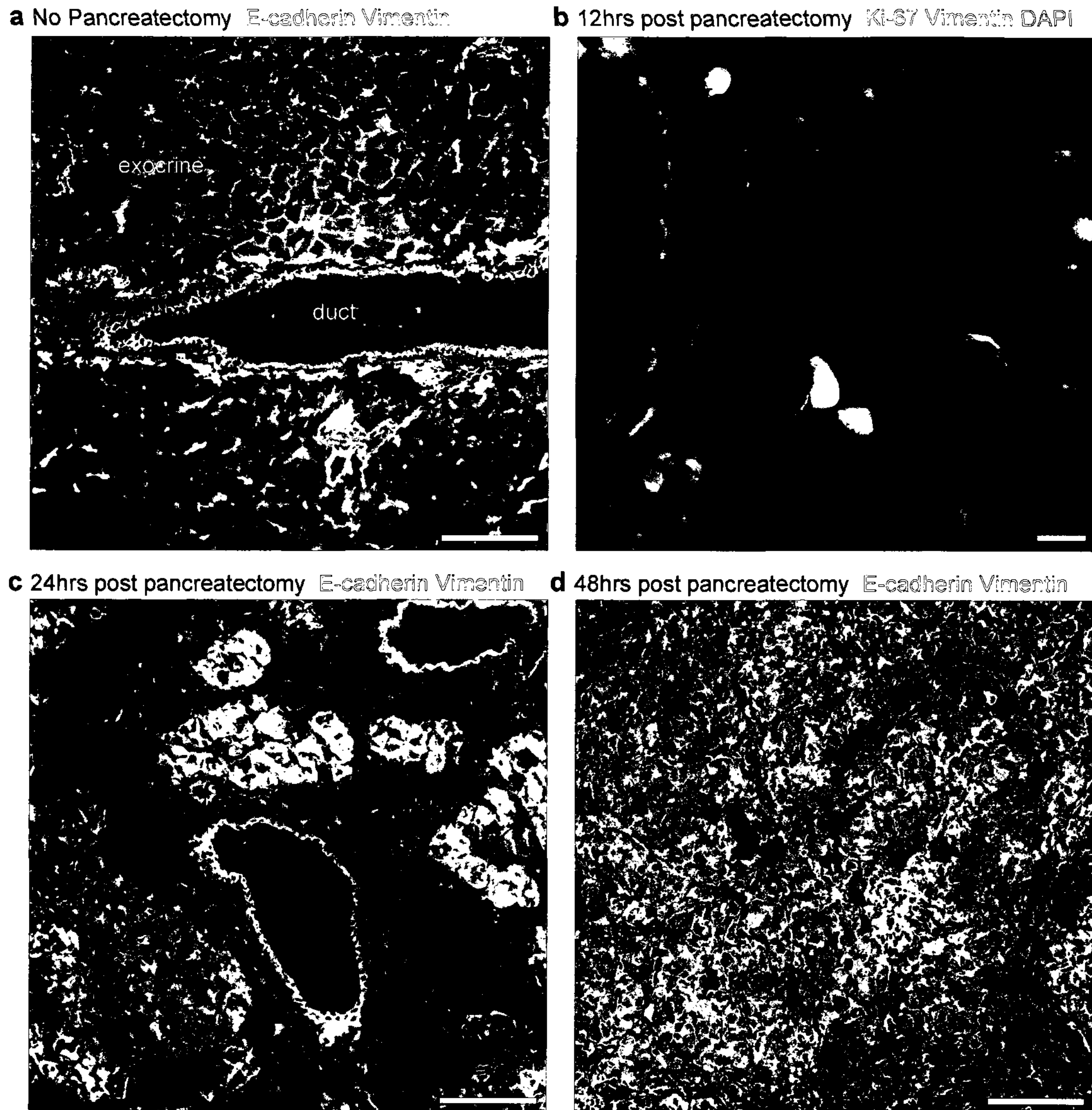


Figure 2. Mesenchymal cells form a stroma early after partial pancreatectomy.

a) Prior to partial pancreatectomy (PPx) vimentin (red) marks the mesenchymal pancreatic stellate cells (PSCs) that surround epithelial structures of the ducts and exocrine tissue that express E-cadherin (green). Scale bar represents 100μm.

b) Vimentin$^+$ (red) PSCs proliferate near ducts (left) 12 hours after PPx and express Ki67 (green). Nuclei stained with DAPI (blue). Scale bar represents 10μm.

c) 24 hours following pancreatectomy an increase in vimentin expressing mesenchymal cells are first observed surrounding ducts. Scale bar represents 100μm.

d) Two days following pancreatectomy mesenchymal cells spread throughout the exocrine tissue. Scale bar represents 100μm.

Ngn3⁺ progenitors emerge following the expansion of mesenchymal cells.

Neurogenin 3 (Ngn3) expression has been shown to mark progenitor cells within the pancreas following partial duct ligation (Xu et al. 2008). Therefore, we examined regenerating pancreata for expression of Ngn3. Although Ngn3 expression was not found within the resting pancreas, Ngn3-expressing progenitors emerged during regeneration induced by partial pancreatectomy (Fig. 3a). These Ngn3-expressing progenitors were not derived from the proliferating mesenchymal cells as they co-expressed the epithelial marker E-cadherin (Fig. 3b). In addition, Ngn3 was localized to the nucleus (Fig. 3c) in tightly clustered cells. The Ngn3-expressing progenitors were observed following the activation of PSC and resulting proliferation of mesenchymal cells but did not require a full stroma to be formed. This is evident as E-cadherin is still widely expressed in areas where Ngn3 expression was observed (Fig. 3a). However, the migrating mesenchymal cells had started to penetrate into the epithelium disrupting the tight expression of E-cadherin that is observed in the absence of pancreatectomy (Fig. 1a and Fig. 2a) or even early after pancreatectomy (Fig. 2b).

Tubular complexes form within a mesenchymal stroma following pancreatectomy.

Three days following pancreatectomy tubular complexes first appeared near the outer edge of the pancreatic remnant (right side of Fig. 4a). Tubular complexes are known to express ductal markers such as Cytokeratin 7 (Fig. 4). Although tubular complexes first appeared at the regenerating tip three days after pancreatectomy, they were not fully abundant until five days (lower panel; Fig. 4a). At this time the area of the pancreas distal to the head (or duodenum) contained a large focal area of regeneration

a Ngn3 E-cadherin

b Ngn3 E-cadherin

c DAPI E-cadherin

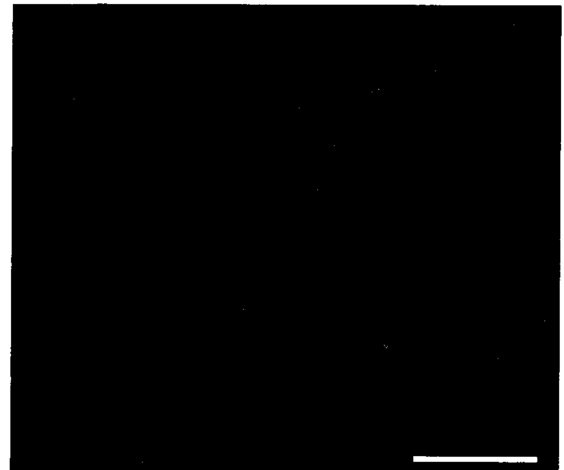


Figure 3. Ngn3^{+} cells emerge following the expansion of mesenchymal cells.

a) Following the expansion of mesenchymal cells Ngn3^{+} (red) progenitors begin to emerge within E-cadherin (green) expressing cells. Scale bar represents 100μm.

b) Inset from (a) showing a closer magnification of Ngn3 (red) expressing cells that also co-express the epithelial marker E-cadherin (green). Scale bar represents 100μm.

c) Inset from (a) with epithelial cells represented by anti-E-cadherin antibody (green) and nuclei stained with DAPI (blue). Scale bar represents 100μm.

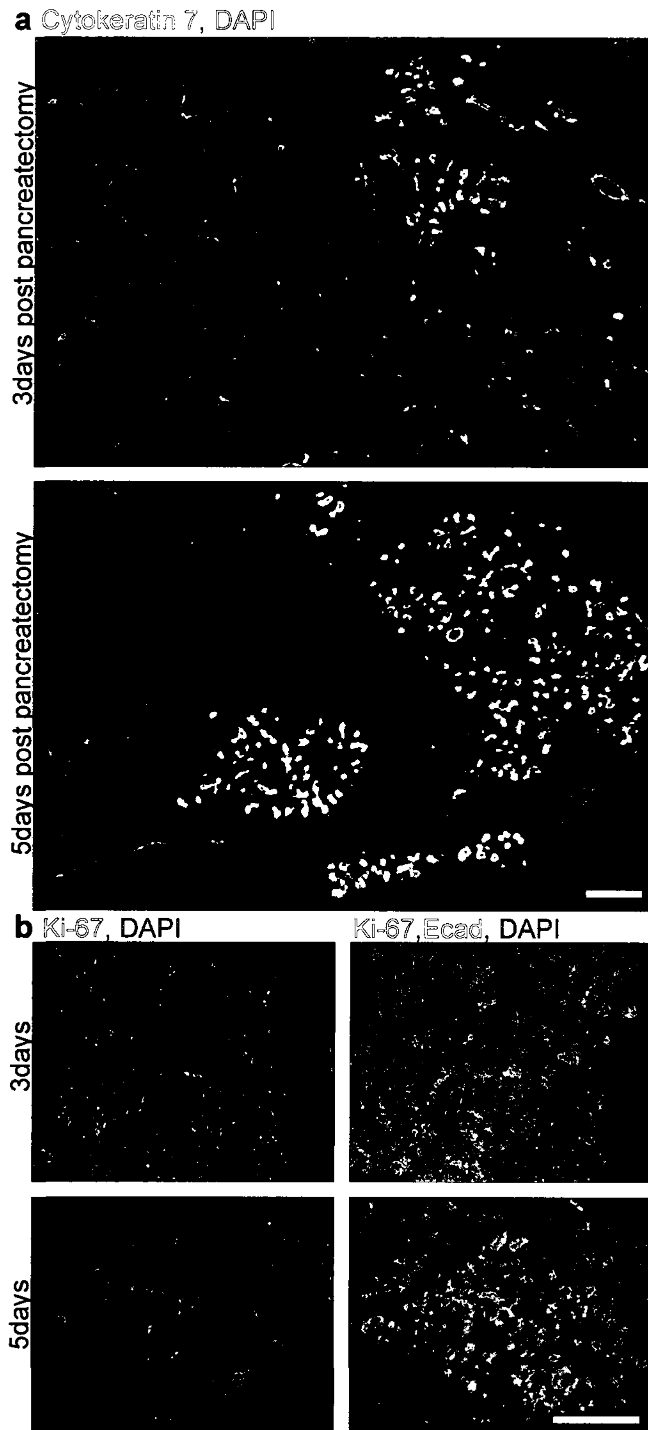


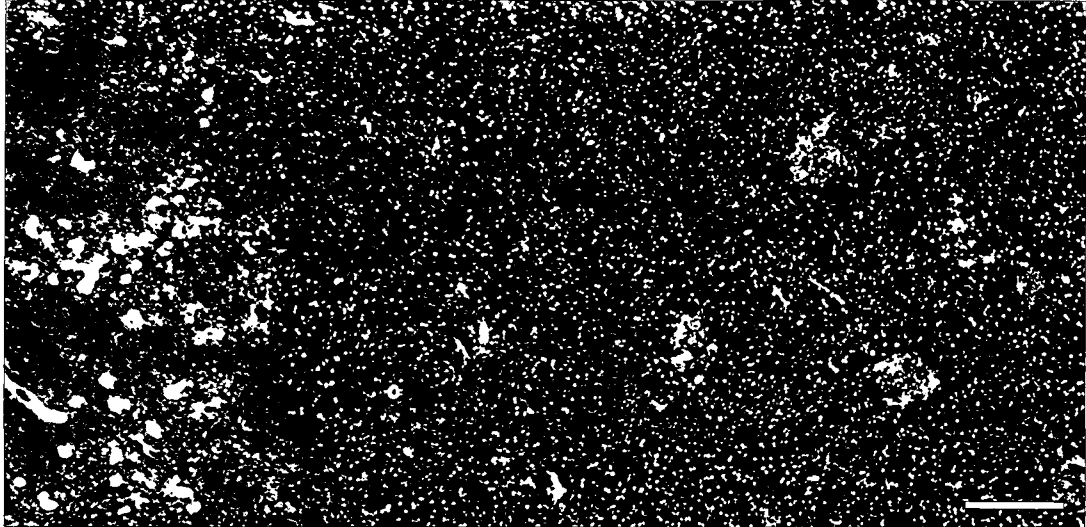
Figure 4. Focal area of regeneration in the mouse following a partial pancreatectomy.
a) Tubular complexes (green) begin to emerge near the site of excision 3 days after partial pancreatectomy (PPx), but a distinct focal area of regeneration is not developed until 5.
b) Three days after PPx proliferation is labelled by Ki-67(red) found in E-cad$^+$ (green) and non-epithelial cells. Five days after PPx Ki-67 is confined to epithelial cells. Nuclei are stained with DAPI (blue). Scale bars represent 20µm.

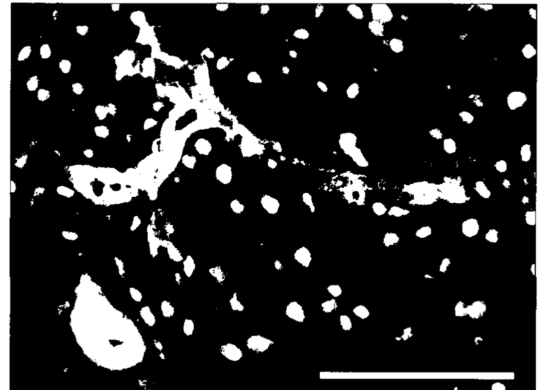
abundant with proliferating tubular complexes (Fig. 4b). Three days after PPx in areas surrounding the early foci of regeneration proliferation was still observed within mesenchymal cells (Fig. 4b). However, five days following pancreatectomy proliferation became confined to epithelial cells expressing E-cadherin (Fig. 4b).

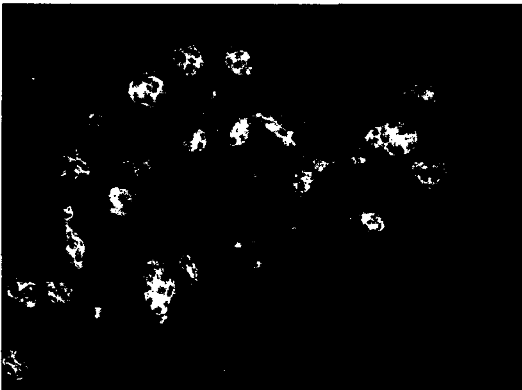
Focal areas of regeneration that contained tubular complexes were distinct from the base of the pancreatic remnant (left; Fig. 5a). Within focal areas of regeneration proliferation was localized to tubular complexes (Fig. 5b,c) and not within the surrounding mesenchymal stroma. In addition, within focal areas of regeneration tubular complexes (Fig. 5d) were the only cells co-expressing E-cadherin (Fig. 5e).

Microarray screen for factors initiating Ngn3 precursors and TC formation.

To elucidate factors initiating regeneration we isolated RNA from the location where foci of regeneration were first observed three days after PPx. From the histological studies already described (Fig. 1f, 4a) this area was determined to be distal from the duodenum at the site of resection. Therefore, tissue from this area was used for RNA extraction in a microarray screen compared to an uninjured pancreas. Genes present in both samples (P value < 0.05) but with increased expression in the early focal area of regeneration are listed in Table I. The known genes with at least a 3-fold increase in expression are listed in order of their relative fold increase. The genes expressed in the early focal area of regeneration but absent in the resting pancreas are listed in Table II. All genes listed in Table II were called present with a P value < 0.05 and have at least a 4 fold increase in signal intensity relative to the resting pancreatic tissue. Factors known to be expressed in mesenchymal cells are shown in bold in both tables.







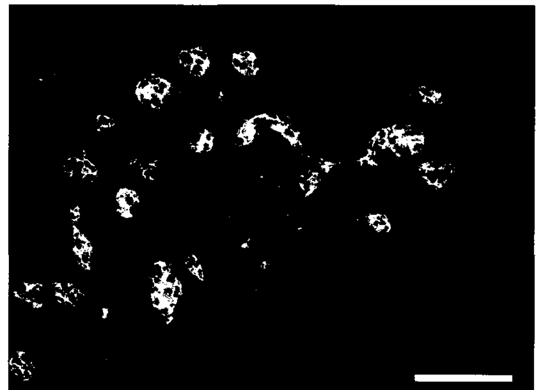


Figure 5. Proliferation within Tubular Complexes surrounded by a stroma. a) Three days after PPx the first TCs appear (left) and express Cytokeratin 7 (green). b) Ck7 (green) expressing TCs proliferate, as shown by Ki67 (red). d) Ck7 (green) expressing TCs are the only cells to express the epithelial marker E-cadherin (e; red). Nuclei are stained with DAPI (blue; a,c). Scale bar represents a) 200μm c,e) 100μm.

Table I. Genes with increased expression in the early focal area of regeneration. Genes detected as present in both samples and with at least a 3-fold increase in signal intensity are listed in order of increase over resting pancreatic tissue.

Symbol	Gene Title	Rgn Signal	Resting Signal	Increase Fold	PValue
Col3a1	**procollagen, type III, alpha 1**	**87667.6**	**9258.5**	**9.85**	**0.00002**
Lyzs	lysozyme	34559.1	4053.8	7.46	0.00002
Col1a2	**procollagen, type I, alpha 2**	**27619.8**	**3816**	**6.50**	**0.00002**
Sparc	**secreted acidic cysteine rich glycoprotein**	38294.1	6417	6.50	0.00002
Lzp-s	P lysozyme structural	79879.5	6900.7	5.66	0.00004
Apoe	apolipoprotein E	19736.4	3630.4	5.28	0.00002
Serping1	serine peptidase inhibitor, clade G, member 1	10942.1	1759.5	4.92	0.00002
Basp1	brain abundant, membrane attached signal protein 1	5391.9	946.5	4.59	0.00035
C3	complement component 3	23708.3	6417.2	4.59	0.00004
Tmsb10	thymosin, beta 10	36476.6	9107.2	4.29	0.00002
Ccl6	chemokine (C-C motif) ligand 6	12644.3	3066.1	4.00	0.00002
Lrg1	leucine-rich alpha-2-glycoprotein 1	10727.3	3039.8	3.73	0.00004
Vim	**vimentin**	**8152.9**	**1737.2**	**3.73**	**0.00003**
Klk1b24	kallikrein 1-related peptidase b24	27469.6	7269.6	3.48	0.00002
Prg1	proteoglycan 1, secretory granule	14853.7	4716.4	3.48	0.00019
Anxa2	annexin A2	14442.2	6006.9	3.03	0.00027
S100a8	S100 calcium binding protein A8 (calgranulin A)	12058.5	3106.1	3.03	0.00003

Table II. Genes expressed in the early focal area of regeneration.

Genes were detected as absent in the resting pancreas but present in the regenerating pancreas with a P value < 0.05. Genes with at least a 4 fold increase in signal intensity are listed in order of their relative increase over the signal achieved from the resting pancreatic tissue.

		Regeneration		Resting	Relative
Symbol	Gene Title	Signal	PValue	Signal	Increase
Saa3	serum amyloid A 3	28926	0.00024	243.1	111.43
Ccl6	chemokine (C-C motif) ligand 6	5477.3	0.00122	103.2	84.45
Lcn2	lipocalin 2	51105	0.00024	1302	36.76
Hexb	hexosaminidase B	2282.6	0.00415	57.1	34.30
C1qc	complement component 1, q , C chain	5238	0.01074	141.1	22.63
Ms4a6d	membrane-spanning 4-domains, A, 6D	2570.8	0.01074	132.9	19.70
Ccl8	chemokine (C-C motif) ligand 8	15467	0.00073	830	18.38
Msn	moesin	4461.3	0.04614	238.9	18.38
Postn	**periostin, osteoblast specific factor**	**6136.1**	**0.01856**	**311.3**	**18.38**
V1rd1	vomeronasal 1 receptor, D1 olfactory receptor	1566.4	0.02905	42	18.38
Mfap5	microfibrillar associated protein 5	5282.6	0.01856	352.3	17.15
Zfp207	zinc finger protein 207	1461.5	0.04614	80.2	17.15
Ptgis	prostaglandin I2 (prostacyclin) synthase	3153.8	0.03760	96.8	16.00
Gm1960	dendritic cell inflammatory protein	2629.3	0.00293	200.5	14.93
Ms4a11	membrane-spanning 4-domains, A, 11	3218.5	0.00024	204.7	13.93
Ctla2b	cytotoxic T lymphocyte-associated protein 2beta	1857.3	0.01074	78.9	13.00
Slc37a3	solute carrier family 37	2712.8	0.01856	152	13.00
Col5a2	**procollagen, type V, alpha 2**	**6317.5**	**0.00024**	**560.3**	**11.31**
Hmgcs2	3-OH-3-methylglutaryl-Coenzyme A synthase 2	7516.4	0.00024	608.6	11.31
Fcgr2b	Fc receptor, IgG, low affinity IIb	1952.3	0.00073	130.4	10.56
Rbp1	**retinol binding protein 1, cellular**	**4066.1**	**0.00415**	**362.6**	**10.56**
Serpinh1	serine peptidase inhibitor, clade H, 1,3	4762.2	0.00293	281.3	10.56
S100a10	S100 calcium binding protein A10 (calpactin)	3862.8	0.01856	329	9.19
Tmsb10	thymosin, beta 10	4009.1	0.00415	515	9.19
Ugt1a2	UDP glucuronosyltransferase 1, A2	3187.1	0.00293	291.7	9.19
Col1a1	**procollagen, type I, alpha 1**	**12350**	**0.00024**	**1708.3**	**8.57**
Fstl1	**follistatin-like 1**	**4737.9**	**0.00806**	**563.7**	**8.57**
Gpx3	glutathione peroxidase 3	7917.8	0.04614	891.2	8.57
Bgn	biglycan	9934.5	0.00806	1041.9	8.00
S100a9	S100 calcium binding protein A9	11381	0.01856	1822.8	8.00
Plac8	placenta-specific 8	6278	0.00073	966.8	7.46
Cd68	CD68 antigen	2910.7	0.01074	70.7	6.96
Tgfbi	**transforming growth factor, beta induced**	**2492.4**	**0.02393**	**495.9**	**6.96**
Slpi	secretory leukocyte peptidase inhibitor	14503	0.00073	2111.2	6.06
Ms4a6b	membrane-spanning 4-domains, A, 6B	2378.5	0.00415	706.2	5.66
Fn1	**fibronectin 1**	**12318**	**0.00195**	**1729.4**	**5.28**
Sparc	secreted acidic cysteine rich glycoprotein	18548	0.00024	4279.3	5.28
Tyrobp	TYRO protein tyrosine kinase binding protein	7214.6	0.00073	1069.4	5.28
Ctss	cathepsin S	14447	0.00415	1946.2	4.92
Spop	speckle-type POZ protein	2331	0.04614	193.8	4.92
Tspan5	tetraspanin 5	1812.6	0.01856	188.3	4.92
Cotl1	coactosin-like 1 (Dictyostelium)	2473.8	0.00073	509.7	4.59
Mmp2	**matrix metallopeptidase 2**	**3763.6**	**0.00195**	**642.2**	**4.59**
Cfh	complement component factor h	1672.4	0.04614	93.7	4.29
Loxl2	lysyl oxidase-like 2	2812.4	0.00122	368.1	4.29
Col3a1	**procollagen, type III, alpha 1**	**6579.3**	**0.00586**	**973.3**	**4.00**
Nudt21	nudix-type motif 21	2202.1	0.04614	531.6	4.00
Scoc	short coiled-coil protein	2916.9	0.00586	694.6	4.00

Injection of cultured PSCs induces Ngn3 expression and TC formation.

Previously we have shown that mesenchymal cells including pancreatic stellate cells, PSCs, can be isolated with flow cytometry based on Sca-1 expression. Following isolation Sca-1$^+$ cells cultured *in vitro* uniformly express the mesenchymal marker vimentin in addition to other markers of activated pancreatic stellate cells including α smooth muscle actin. To determine if these mesenchymal cells were sufficient to induce pancreatic regeneration we transplanted them into the pancreas of SCID-BEIGE mice. To track the cells *in vivo* we used the pancreas of mice ubiquitously expressing GFP (cmvGFP mice). The pancreas from cmvGFP mice was digested into a single cell solution and cells were isolated using flow cytometry with anti-Sca-1 antibody (Fig 6a). Isolated cells were expanded in culture for two weeks then directly implanted into the pancreas of SCID-BEIGE mice. GFP expression was observed in recipient mice three days following the transplant (n=2) representing the integration of the donor cells into the recipient pancreas (Fig.7a). After one week (n=3) cells were found scattered throughout the exocrine tissue (Fig. 7b,c). Donor cells were also observed scattered within localized areas of the pancreas two weeks after implantation (Fig. 6b arrow,d,e) or in aggregates (Fig 6b,c). The scattered donor cells that disrupted the pre-existing epithelium resulted in reduced E-cadherin expression (Fig. 7d) compared to the resting pancreas (Figures 1a, 2a). The donor cells stimulated tubular complex formation from an endogenous E-cad$^+$ cell that started to co-express Ngn3 one week after implantation (Fig. 7e). Two weeks following implantation areas containing cmvGFP donor cells contained tubular complexes (Fig. 7f) that expressed Pdx1 (Fig. 7g). Therefore, mesenchymal cells are sufficient to generate Ngn3$^+$ progenitors and tubular complexes to facilitate regeneration.

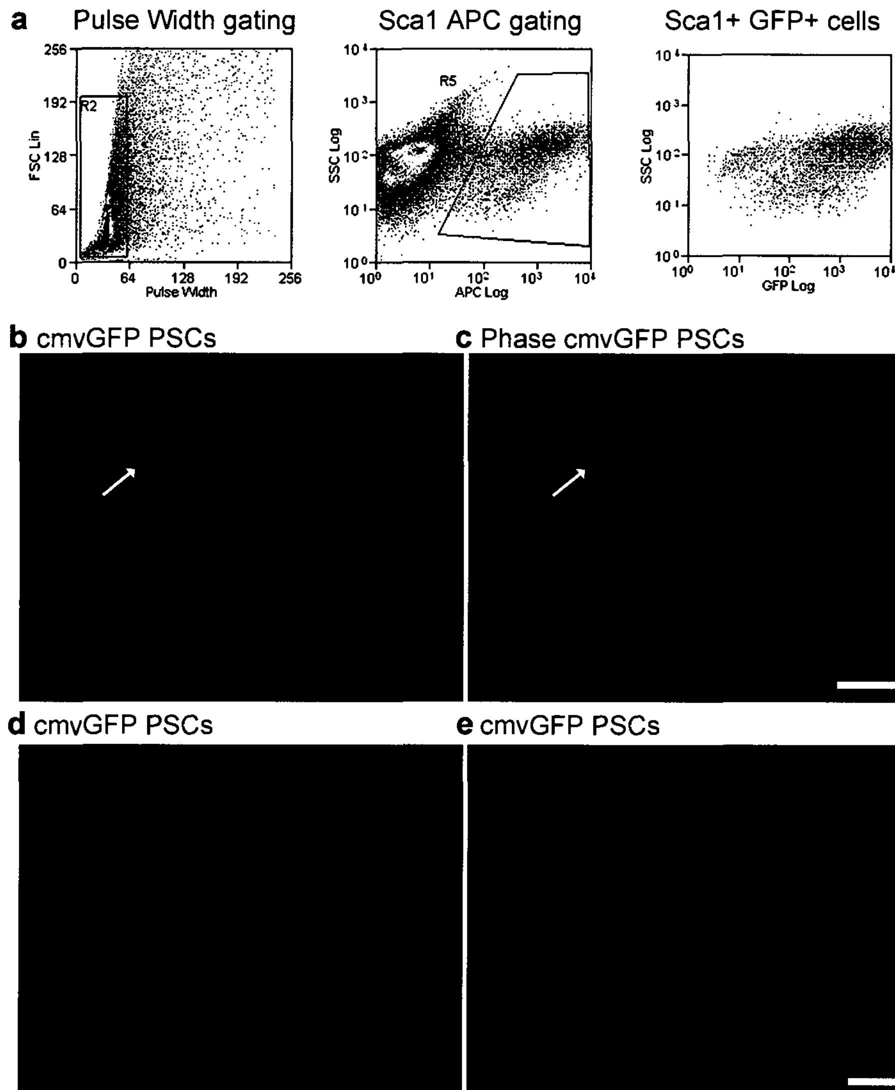


Figure 6. The isolation of Sca1$^+$ cells and transplantation following culture.

a) Gating used for the isolation of Sca-1$^+$ cells. Pulse width gating selects for single cells (left). Analysis of the isolated Sca1 cells shows most express GFP (right).

b) GFP$^+$ donor cells reside within the recipient tissue (arrow) or in large aggregates near the edge of the pancreas as shown in c) a with phase contrast. Scale bar 2 mm.

d,e) Higher magnification of area shown by the arrow in (b), at two focal points. Scale bar represents 500μm.

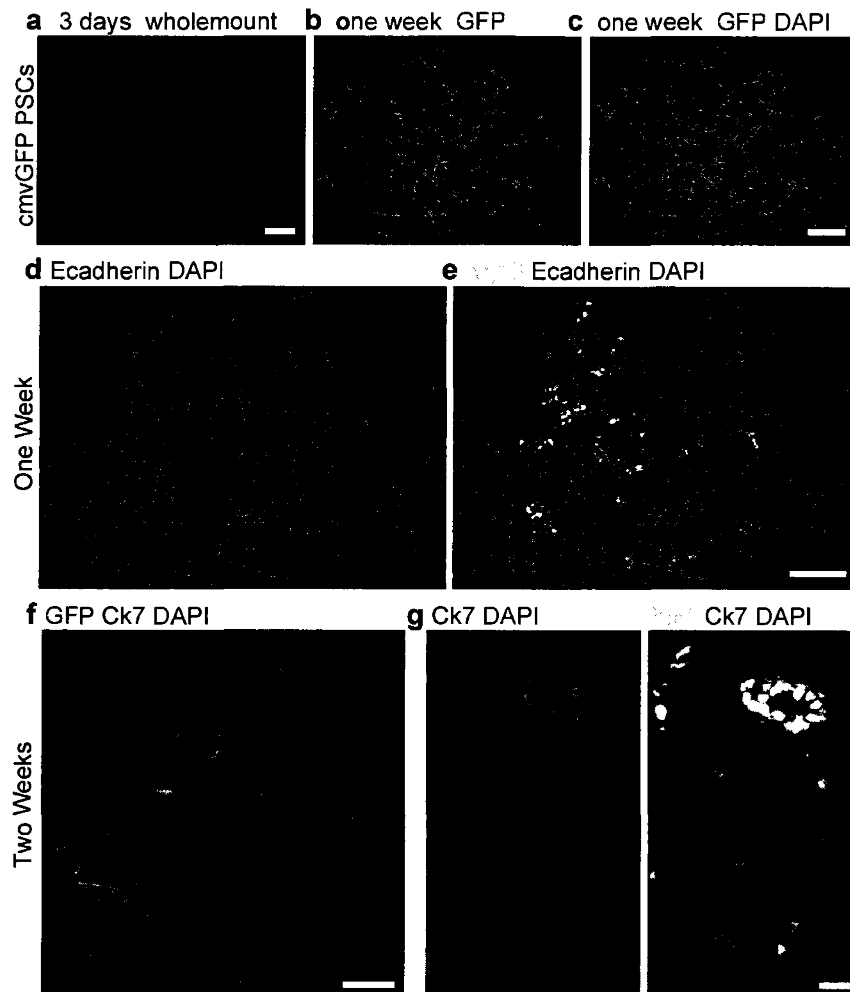


Figure 7. Mesenchymal cells are sufficient to induce tubular complex formation. The pancreas of mice ubiquitously expressing GFP was used to isolate mesenchymal cells that were cultured and directly implanted into the pancreas of SCID-BEIGE mice. a) Whole mount showing GFP expression of donor cells residing within the recipient pancreas three days after implantation. Scale bar 500μm. b,c) One week after transplant donor cells are detected with anti-GFPAlexa488 antibody (green). Scale bar 100μm. d) Pre-existing E-cadherin^{+} (red) epithelium is disrupted by the donor mesenchymal cells and e) expresses the pancreatic progenitor marker Ngn3 (yellow). Scale bar 100μm. f) Two weeks after implantation GFP labeled cells (green) are found surrounding duct-like tubular complexes structures that express Cytokeratin 7 (red). Scale bar 20μm. g) Cytokeratin7 (red) tubular complexes co-express Pdx1 (yellow). Scale bar 20μm.

Discussion

We have shown that pre-existing resident mesenchymal cells proliferate to give rise to a pancreatic stroma during regeneration (Fig. 1b,c and Fig. 2b). The initial proliferation observed within vimentin expressing cells led us to believe pancreatic stellate cells, PSCs, were the main source of this stroma. However, we cannot rule out the contribution that pericytes or cells derived from the bone marrow may provide to this stroma. The observation of BrdU incorporation in cells surrounding the vasculature (Fig. 1d) provides support to the idea of a contribution from such cells. However, the same observation was made surrounding the ducts (Fig. 1e). Regardless of the source we have shown that a stroma is created three days following pancreatectomy which leads to the formation of focal areas of regeneration located distal to the head of the pancreas. In addition, the transplantation of activated pancreatic stellate cells is sufficient to provide a stroma that induces regeneration.

The delay observed following mouse pancreatectomy in comparison to that previously reported in the rat may arise from species variation or the inability to achieve the quantity of resection performed in the rat. Performing a 90 to 95% resection in the mouse pancreas proved to be extremely difficult with a high mortality rate. This is likely due to the smaller size of the mice or a variation in anatomy between the two species. In particular the localization of the vasculature makes excision of the duodenal lobe very difficult to perform without tearing major veins or arteries. However, removal of the splenic lobe along with gently removing a portion of the duodenal lobe allowed the removal of roughly 70% of the pancreas. This was sufficient to induce the formation of tubular complexes and the expression of Ngn3 and Pdx1.

Aside from duplication of pre-existing β-cells, ductal cells have been suggested as the major source of β-cell regeneration (Bonner-Weir et al. 2008). Intercalated ductal cells and centroacinar cells have been speculated to be the source of islet neogenesis (Hayashi et al. 2003). Here we show that five days following pancreatectomy proliferation is most prominent within tubular complexes that resemble ductal like cells which express Cytokeratin 7, suggesting ductal cells provide the cellular source of epithelial regeneration. However, it is unclear which ductal compartment gives rise to tubular complexes within the focal area of regeneration. Previously it has been suggested that the cells within the main duct are the first ductal cells to proliferate and eventually give rise to the proliferating ducts within focal areas of regeneration (Bonner-Weir et al. 1993). Similarly we observed early proliferation within larger ducts (Fig. 1c). However, prior to this proliferation we observed the proliferation of mesenchymal cells around the ducts. The activation of these pancreatic stellate cells may initiate the subsequent proliferation of the larger ducts. Moreover, as mesenchymal cells migrate to the distal area of the pancreatic remnant they create a stroma which could initiate proliferation of distal intralobular, intercalating ductal or centroacinar cells resulting in tubular complex formation. Even though the cellular source giving rise to new epithelium is unclear, here we suggest that mesenchymal cells initiate the proliferation of and support the expansion of pancreatic progenitor cells.

To elucidate factors that facilitate regeneration we performed a microarray screen on newly forming focal areas of regeneration that are present three days following pancreatectomy. Among the most significant increases observed were several genes known to be expressed by mesenchymal cells including pancreatic stellate cells (see

genes highlighted in tables I and II). Activated pancreatic stellate cells are known to secrete extracellular matrix proteins such as collagen types I, III and fibronectin (Bachem et al. 1998; Apte et al. 1999; Schmid-Kotsas et al. 1999; Casini et al. 2000) all of which were found in the microarray screen. Similarly, Matrix metallopeptidase 2 (*Mmp2*) and Retinol binding protein 1 (*Rbp1*) have been shown to be expressed by pancreatic stellate cells (Buchholz et al. 2005). Periostin (*Postn*) has been shown to be expressed in pancreatic stellate cells but not within hepatic stellate cells (Buchholz et al. 2005). We also found *Tgfbi*, the homologue of *Postn* (Horiuchi et al. 1999), to be highly expressed during regeneration. Further study of such genes may elucidate factors which may stimulate pancreatic regeneration. Additional microarray screens would be beneficial to understanding the expression patterns of these genes throughout the course of regeneration. Furthermore, a full microarray comparison in triplicate may elucidate specific signaling pathways during regeneration.

Although a second pathway of regeneration has been described within the adult pancreas little is known regarding the factors which facilitate that pathway. Here we suggest that mesenchymal cells create a stroma that facilitates regeneration and is a source of such factors. Furthermore, we anticipate the microarray screen to contain factors provided by the mesenchymal stroma that are vital for pancreatic regeneration. Further study of genes expressed by mesenchymal cells including activated pancreatic stellate cells will elucidate factors that contribute to pancreatic regeneration.

Materials and Methods

Experimental animals. Eight- to nine-week-old male C57BL/6J, cmvGFP and SCID-BEIGE mice were housed in the barrier facility at the University of Ottawa. SCID-BEIGE (Taconic # CBSCBG-MM) mice were given slightly acidified water. All other mice were treated under standard conditions and allowed free access to standard mouse chow and water. All studies were approved by the Animal Care Committee at the University of Ottawa. cmvGFP mice were created by crossing B6.C-Tg(CMV-cre)1Cgn/J (Jackson Stock#006054) with Z/EG reporter mouse. CMV-cre mice are a transgenic strain that can delete *loxP*-flanked genes in all tissues including germ cells. The Z/EG reporter mouse constitutively expresses *lacZ* under the control of the CMV enhancer/chicken actin promoter. When crossed with the CMV-cre strain, *lacZ* expression is replaced with enhanced GFP expression in all tissues. Therefore, the resulting mouse ubiquitously expressed GFP in pancreatic mesenchymal cells.

Partial Pancreatectomy. To remove pancreatic tissue access to the abdominal cavity was obtained by performing a midline incision. First, a 1 to 1.5cm incision was made through the skin in the middle of the abdomen using a No.10 scalpel blade. Using forceps the skin was gently separated from the abdominal wall to reveal the midline of the abdomen. The midline was lifted with rat tooth forceps and a small cut, less than 1cm, was made with scissors through the body wall. Once located the splenic pancreatic lobe was lifted through the incision with forceps. The entire splenic lobe and distal portions of the gastric and duodenal pancreatic lobes were removed by gentle abrasion with forceps and a wet cotton applicator if necessary to ensure no major veins or arteries

were broken. If excessive bleeding was observed the site of bleeding was clamped for several minutes to promote clotting. Once removed only a small portion (~30%) of the pancreas remained along the duodenum. The pancreas that was surgically removed was approximately 70% of the total pancreas, as was confirmed by weighing the removed and remnant portions during a pilot study without recovery (see supplementary figure 1). The body wall was closed with silk surgical sutures (Johnson&Johnson) using two to three discontinuous sutures. The skin was closed with two to three surgical staples (Fisher). Once the surgery was complete the mice were placed on oxygen for approximately one minute and then returned to their cage as soon as they began to move. Blood glucose levels were analyzed every other day checking blood sugar levels for increased glucose. A 70% pancreatectomy resulted in no variation of blood glucose. In addition, mice were given 0.05 mg/kg Buprenorphine subcutaneously every day following surgery for the first week. For the administration of BrdU 100ul of 1 mg/ml BrdU in saline was placed in the abdominal cavity prior to closing. For continuous BrdU labelling, drinking water containing 0.8 mg/ml was given to animals following surgery and changed every other day thereafter.

RNA isolation and microarray screen. The pancreas remnant or the corresponding tail section of a resting pancreas was harvested and quickly frozen in liquid nitrogen. The tissue was ground with a mortar and pestle and while still frozen RNA was isolated using Trizol (Invitrogen) followed by DNase treatment (Quiagen). RNA integrity was confirmed by Agilant 2100 Bioanalyzer (Agilant Technologies). Total RNA was hybridized on the Mouse Genome 430 v2.0 GeneChip® (Affymetrix). Raw data files

were processed with Affymetrix GeneChip® Operating Software (GCOS) to derive signals and present/marginal/absent calls for each sample. Pair wise comparisons were made between the regenerating and the resting pancreas to produce log(fold) ratio estimates of change and increase/no change/decrease calls. Processed results were exported from GCOS and imported into Excel and Access (Microsoft) for further analysis.

Immunohistochemistry. Pancreata were embedded in optimal cutting temperature compound, O.C.T. (Tissue-Tek) : 20% sucrose solution (2:1) and frozen by emersion into 95% ethanol/dry ice. Cryosections (8um) were cut using a cryostat (Leicia CM 1850). The antibodies used are outlined in Supplementary Table S1. BrdU detection was performed by following the protocol outlined in the BrdU in situ detection kit (BD Biosciences). Nuclei were counter-stained with DAPI (Sigma). Images were taken with a Carl Zeiss Axioplan 2 Microscope with an AxioCam HRm b/w (Zeiss) camera using Axiovision v3.2 (Zeiss) acquisition software.

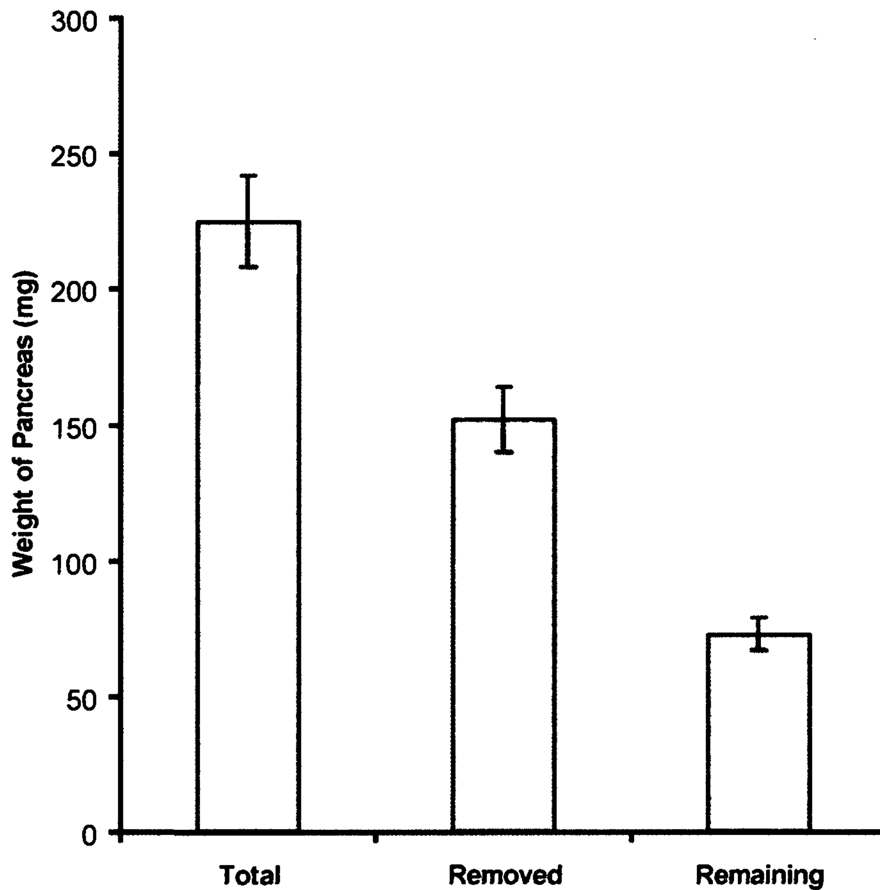
Isolation of mesenchymal cells from the pancreas. The pancreas was removed, minced and resuspended in 3,000 units (12 mg) of collagenase type I (sigma) in 4 ml RPMI + 10% FBS. The solution was shaken for 6mins in a 15 ml falcon. Digestion was stopped by adding 10 ml of cold DMEM + 10% FBS. The digested pancreas was spun down and resuspended in 2 ml of 2.5% Trypsin for 3 min, then 12 ml of cold DMEM + 10% FBS was added and the cells were spun down and resuspended in media containing an antibody for Sca1 (1:200) directly conjugated to APC (BD Pharmingen). Cells were

separated on a MoFlo cytometer (DakoCytomation). Dead cells and debris were removed by gating on forward vs. side scatter profiles. Doublets were removed by pulse width gating (see Fig. 6a). After isolation $Sca1^{+}$ cells were cultured in RPMI with 10% FBS for a minimum of 2 weeks or 3 passages before transplantation.

Transplantation of mesenchymal cells into the pancreas. The splenic lobe of the pancreas was exposed as previously described for pancreatectomy. 10,000 cells suspended in 10μl were directly injected into the splenic lobe of the pancreas using a 10μl Hamilton syringe (Hamilton; #701RN) equipped with a 33 gauge needle (Hamilton; point style 4). Following injection the body wall was closed with two to three discontinuous sutures and the skin was closed with two to three surgical staples.

Supplementary Information

Supplementary Figures



Supplementary Figure I. The amount of pancreas removed in mock pancreatectomies (n=15) to determine the percentage of pancreatectomy achievable in the mouse.

Supplementary Tables

Supplementary Table I. Antibodies used in the study.

Primary Antibodies	Dilution	Company	Cat#
BrdU (msIgG1)	1:200	BD Bioscience	347580
Cytokeratin 7 (msIgG1)	1:200	BD Bioscience	550507
E-Cadherin (msIgG2a)	1:500	BD Bioscience	610182
E-Cadherin FITC	1:200	BD Bioscience	612131
GFP (rabbit) A488	1:1,000	BD Bioscience	A21311
Ki-67 (msIgG1)	1:200	BD Bioscience	550609
Neurogenin 3 (msIgG1)	1:1,000	Developmental Studies Hybridoma Bank	F25A1B3-c
Pdx1 (rabbit)	1:5,000	Gift from Chris Wright (Vanderbuilt)	
Sca-1 APC	1:200	Cedarlane (ebioscience)	17-5981-83
Vimentin D547	1:200	Abcam	38873
Secondary Antibodies			
anti-Mouse IgG1 A488	1:2,000	Invitrogen - Molecular Probes	A21121
anti-Mouse IgG1 A568	1:2,000	Invitrogen - Molecular Probes	A21124
anti-Mouse IgG1 A647	1:2,000	Invitrogen - Molecular Probes	A21240
anti-Mouse IgG2a A546	1:2,000	Invitrogen - Molecular Probes	A21133
anti-Rabbit A647	1:2,000	Invitrogen - Molecular Probes	A21443

Supplementary Methods

Standard surgical procedures. One hour before surgery mice were given a dosage of 0.05 mg/kg Buprenorphine subcutaneously. Anesthesia was induced in an anesthetic box with isoflurane gradually increased to 5%. The anesthetic was delivered by an Ohio Forane vaporizer (induction box) and an isoflurane vaporizer (mask). Once anesthetized the mice were transferred to a face mask with isoflurane at 1.5% where they were maintained throughout surgery, increasing or decreasing the percentage as necessary to keep the animal sedated but breathing normally. The surgical area was shaved and cleaned with Endure soap, rinsed with sterile water and surgically prepared with chlorahexseptic solution. BNP eye ointment was placed in the animals eyes to protect them from drying. 1 ml of sterile saline was administered subcutaneously prior to surgery. Once the surgery was complete mice are placed on oxygen until they recovered and started to move.

Acknowledgments

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Chapter 4 – Periostin stimulates pancreatic regeneration

Periostin stimulates pancreatic regeneration including islet neogenesis

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SUMMARY

To identify factors regulating pancreatic regeneration, gene expression analysis of regenerating tissue was performed following pancreatectomy. This analysis revealed high levels of a novel periostin isoform were induced during regeneration. Notably, mice lacking periostin were defective in pancreatic regeneration. Furthermore, injection of recombinant periostin into the pancreas was sufficient to induce regeneration as indicated by the presence of tubular complexes expressing Ngn3 and Pdx1. This regeneration was preceded by the proliferation of pancreatic stellate cells which accumulated to create a stroma. Therefore, periostin plays a fundamental role in pancreatic regeneration by creating the stroma required for regeneration to take place. In addition, regeneration could also be stimulated in STZ-induced diabetic mice suggesting it is not dependent on the presence of pre-existing pancreatic β-cells. Therefore, the stimulation of endogenous pancreatic regeneration by periostin is a novel approach to develop a cure for diabetes.

INTRODUCTION

The ability of the pancreas to regenerate was first published soon after the discovery of insulin, during attempts to investigate the ability of insulin to prolong the life of pancreatectomized dogs. Fisher found new pancreatic tissue emerging if the entire pancreas was not completely removed[1]. Since this discovery other groups have shown that both partial pancreatectomy[2-4] and partial duct ligation[5-7] can induce extensive pancreatic regeneration. Regeneration occurs by both replication of pre-existing differentiated cells[8] and proliferation and differentiation of duct-like epithelial cells[2-4,9]. Preceding the formation of new pancreatic lobes, focal areas of regeneration are observed abundant with loose connective tissue and small proliferating 'ducts in foci' that resemble tubular complexes which are observed preceding pancreatic cancer[10]. Tubular complexes have been shown to be a source of islet neogenesis[11]. However, as tubular complexes are not present in the resting adult pancreas it is unclear where they originate from, although their resemblance to ductal cells is highly suggestive. In addition, the factors that initiate tubular complex formation are unknown.

Several groups have reported to have isolated stem cells from the pancreas showing *in vitro* differentiation down several pathways[12-16]. Recently it has been demonstrated that endogenous facultative progenitors expressing Ngn3 exist in and around duct-like structures of the ligated adult mouse pancreas[6]. Although, Ngn3 is not expressed in the resting pancreas it was found within structures resembling tubular complexes[11]. Using the expression of Ngn3 as a guide, Xu and colleagues found these pancreatic progenitors could differentiate into β-cells when cultured with fetal pancreata.[6] It is currently unclear what specific extracellular factors induce Ngn3-expressing cells to

emerge, proliferate and differentiate into pancreatic β-cells. If such factors were uncovered it would allow the possibility to introduce them into the pancreas to stimulate regeneration with the aim of reversing diabetes.

Periostin (*Postn*) was originally cloned from a mouse osteoblast cell line and initially named OSF-2[17,18]. It was later renamed for its expression in periosteum of bone and periodontal ligament cells[19]. Periostin is a 90 kDa protein made of a signal peptide and four fasciclin-1 (fas1) domains homologous to the Drosophila protein that facilitates axonal guidance[20]. *Postn* has also been shown to increase cell invasiveness by inducing epithelial to mesenchymal transitions (EMT)[21]. Four known isoforms created by alternative splicing at the C-terminus have been identified in mice[22]. *Postn* has also been implicated in repair of the adult heart[23-26]. The adult heart expresses *Postn* in response to pathological insults[25]. In an *in vitro* screen, *Postn* was discovered to promote the proliferation of differentiated cardiac myocytes[24]. Inducible over expression of *Postn* [25] or introduction of the recombinant protien[24] promoted cardiac repair following myocardial infarction.

Although only very low levels of periostin expression are detected in the pancreas, mRNA is over expressed in pancreatic cancer[27]. Increased levels of periostin in pancreatic cancer patients have been shown to correlate with a shortened survival time[27,28]. In addition, it has been shown that periostin sustains fibrogenic stellate cell activity to create a tumor supportive microenvironment in the pancreas[29]. Pancreatic stellate cells have been suggested to be the main source of periostin[29,30].

Given the therapeutic benefit to be gained from revealing the factors contributing to pancreatic regeneration a microarray analysis was performed following partial

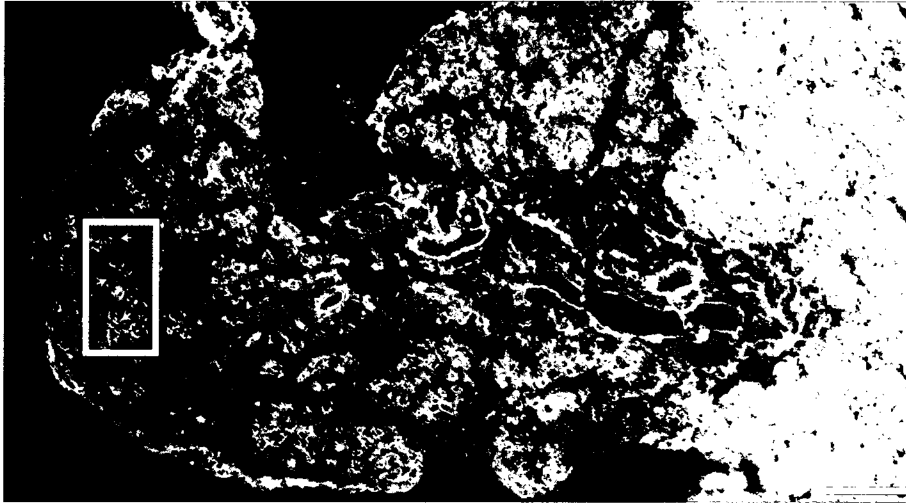
pancreatectomy. The analysis revealed a novel isoform of periostin was over expressed during regeneration. Here we have investigated the role periostin plays in pancreatic regeneration. Absence of periostin reduced the ability of the pancreas to regenerate following partial pancreatectomy. Furthermore, injecting recombinant periostin into the pancreas was sufficient to induce regeneration as shown by the formation of tubular complexes expressing Pdx1 and Ngn3. This regeneration was preceded by the proliferation of pancreatic stellate cells which accumulated to create a mesenchymal stroma. Therefore, periostin induced pancreatic stellate cells to create a mesenchymal stroma which stimulates regeneration and islet neogenesis. Furthermore, periostin induced regeneration was independent of diabetic status. These results show that the introduction of periostin into the pancreas induces endocrine regeneration, creating a novel therapeutic approach for the treatment of diabetes.

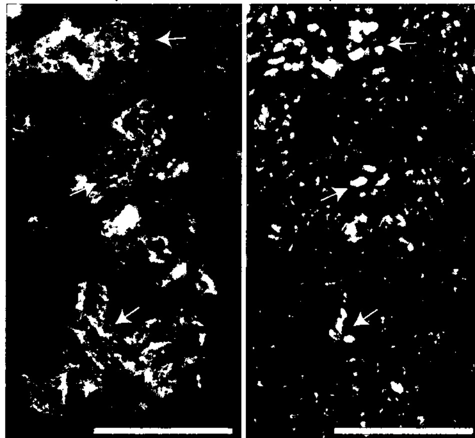
RESULTS

A novel isoform of periostin is expressed during pancreatic regeneration

To elucidate factors that contribute to pancreatic regeneration a microarray screen was performed between resting pancreatic tissue and the focal area of regeneration observed three days follow partial pancreatectomy[2]. One of the most significant increases found was the gene periostin (*Postn*). *Postn* is known to induce epithelial to mesenchymal transitions (EMT)[21]. Previously we found an increase of mesenchyme and a reduction in epithelial cells during regeneration. Therefore, further investigation was carried out to examine the role of *Postn* in the EMT observed during pancreatic regeneration.

To validate our finding real-time quantitative RT-PCR was performed relative to the resting pancreas to reveal that *Postn* is increased three fold during fetal development and nearly ten fold three days following pancreatectomy (**Fig. 1c**). To confirm the presence of Periostin protein in the focal area of regeneration immunohistochemistry, with an anti-periostin antibody, was performed on sections of regenerating pancreata (**Fig. 1a**). During regeneration Periostin is localized in the regenerating tip of the pancreas surrounding proliferating E-cadherin-expressing tubular complexes (**Fig. 1a,b**). Three days following pancreatectomy tubular complexes were revealed as the cellular source of proliferation within the focal area of regeneration by immunohistochemistry with an antibody specific for the proliferation marker Ki-67 (**arrows; Fig. 1a,b**). These proliferating tubular complexes were closely bordered by Periostin (**Fig. 1b**).





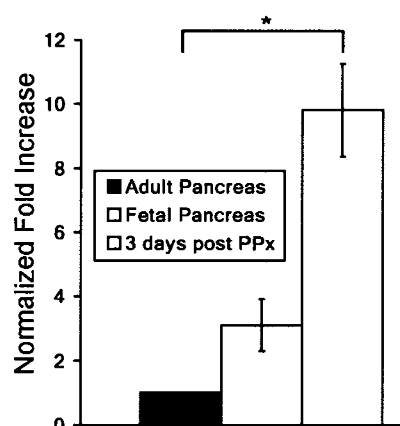


Figure 1 Periostin is specifically expressed during pancreatic regeneration.

(**a**) Tubular complexes found within the focal area of regeneration five days after partial pancreatectomy (PPx) express E-cadherin (green) and are surrounded with Periostin (red) which is found predominately in the regenerating tip. Scale bar represents 200um.

(**b**) Magnification of box shown in (**a**) with E-cadherin (green) tubular complexes (left panel) surrounded with Periostin (red). In the same field Ki-67 (yellow) reveals proliferation within tubular complexes (right panel). Scale bars represent 100um.

(**c**) Real Time QPCR of mRNA expression in fetal pancreatic development (grey) and in the third day of regeneration (white) relative to the resting pancreas (black). Data shown with SEM and * indicates a p-value of <0.05.

Four isoforms of periostin have been reported in the literature[19]. Therefore, to determine which isoforms are expressed during pancreatic regeneration we employed RT-PCR with primers flanking the C-terminus of the protein. Only one major PCR product was observed and sequencing of this product revealed it to be a novel isoform of periostin (**Supplementary Fig. 1**). This novel isoform differs from those previously reported as it is missing both exon 17 and 21 (**Supplementary Fig. 2**).

Periostin is required for pancreatic regeneration

To determine if periostin is required for pancreatic regeneration, pancreatectomies were performed on mice lacking periostin expression[31]. Periostin knockout (*Postn*-/-) mice have the translation start site and first exon replaced with the *lacZ* reporter gene. Although the pancreata of the *Postn*-/- mice are normal, regeneration was severely impaired as compared to wild-type controls (n=5). After five days of regeneration the *Postn*-/- mice showed a 50% reduction in pancreatic weight (**Fig. 2a**) and a reduced size of the regenerating tissue (**Fig. 2b**). Immunohistochemistry of *Postn*-/- regenerating pancreata showed markedly reduced stromal accumulation compared to wild-type controls (**Fig. 2c**). Paralleling the reduction in stroma was a dramatic decrease in the formation of tubular complexes in the periostin knockout pancreas (**Fig. 2c,d**). In four of the five *Postn*-/- mice there was no tubular complex formation, with only one mouse showing limited formation (**Fig. 2d**). However, the pancreatic progenitor marker Ngn3 and tubular complex formation was frequent in wild-type controls (**Fig. 2e**).

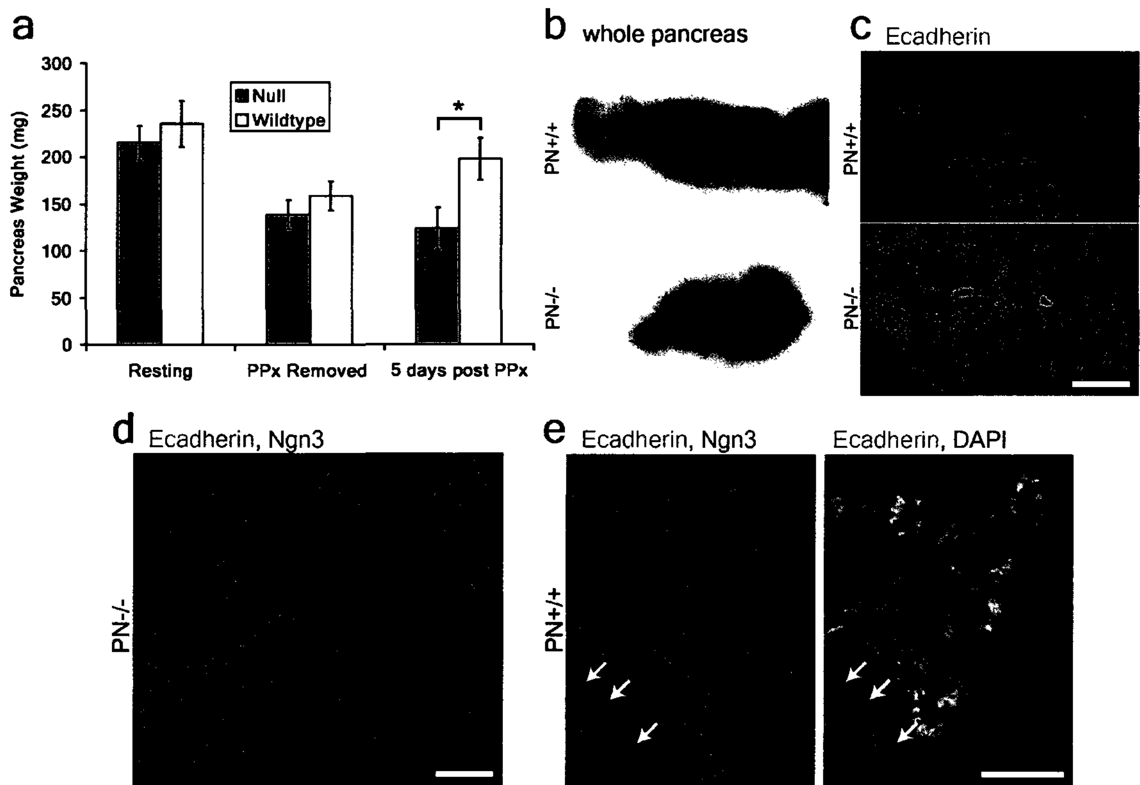


Figure 2 Periostin is required for pancreatic regeneration after pancreatectomy. (**a**) Although similar amounts of the pancreas were removed the weight of the pancreatic remnant from the periostin knockout (PN-/-) mouse (dark bar) is less than the wild-type (grey bar) after five days of regeneration (n=5). Data shown with SEM and * indicates a p-value of <0.05. (**b**) Five days following partial pancreatectomy the size of the pancreas from the PN-/- mouse (lower) is much smaller than that from wild-type mice (upper). (**c**) Although there is some decrease in E-cadherin (green) in the PN-/- regenerating pancreas (lower) the amount of mesenchymal accumulation is much less than in the wild-type pancreas five days following pancreatectomy (upper). Scale bar represents 200μm. (**d**) E-cadherin (green) the formation of tubular complexes is reduced in the PN -/- mouse and $Ngn3^{+}$ (red) cells were not observed. Scale bar represents 30μm. (**e**) Tubular complexes in the wild-type pancreas are abundant and contain cells which express Ngn3 (arrows). Scale bar represents 30μm.

Periostin stimulates and is secreted by pancreatic mesenchymal cells

To determine if periostin could induce regeneration the recombinant protein was injected directly into the pancreas (n=3). Periostin induced widespread proliferation in the pancreas 24 hours after being injected **(Fig. 3a)** compared to a saline injection **(Fig. 3b)**. This proliferation was not within the epithelial islets, ducts or acinar cells expressing Amylase **(Fig. 3c)**. Proliferation was localized to mesenchymal stellate cells expressing vimentin which surrounded islets and ducts and were scattered throughout the exocrine tissue **(Fig. 3d)**.

To further examine the effect of periostin on mesenchymal cells we isolated them from the pancreas and added 500 ng/ml recombinant periostin. Within one day the vimentin-expressing cells changed their morphology (**Supplementary Fig. 3**). Phase contrast imaging revealed periostin-treated cells becoming organized into elongated structures compared to untreated cells resembling a transformation to an advanced mesenchymal phenotype (**Supplementary Fig. 3**). The morphological change observed is characteristic of cells with enhanced migratory ability. To determine if periostin enhanced the migration of pancreatic stellate cells *in vivo* the recombinant protein was injected with Orange Fluorescent Protein (OFP). When OFP was injected alone it stayed within a contained area at the site of injection for up to three days. However, when OFP was injected with Periostin, single OFP^+ cells were found migrating away from the area of injection **(Supplementary Fig. 3)**. Therefore, the Periostin-induced stimulation of mesenchymal cells involves initiation of both proliferation and migration.

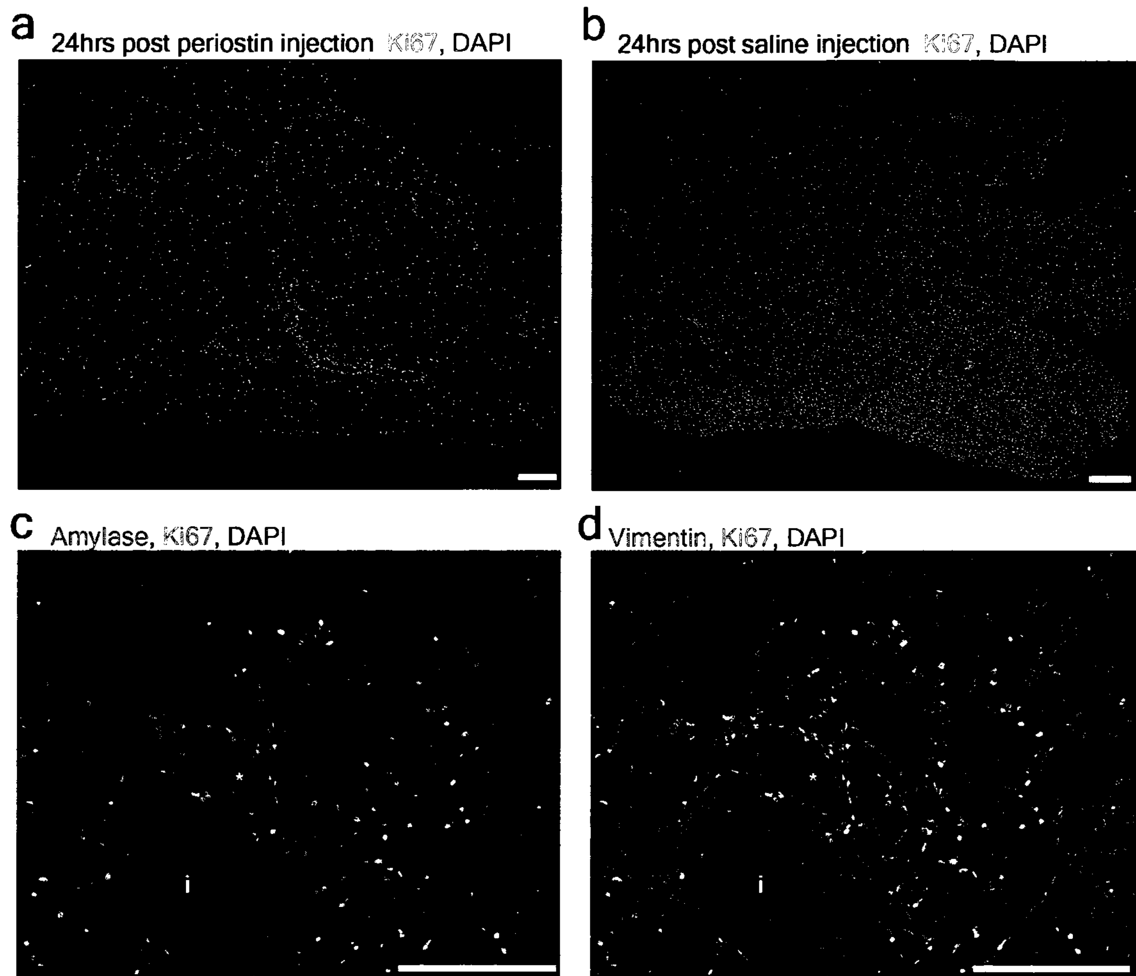


Figure 3 Periostin stimulates the proliferation of pancreatic stellate cells.
(**a**) Widespread proliferation within the pancreas injected with Periostin revealed by Ki67 (green) staining. (**b**) Proliferation is minimal in the saline injected control as shown by the reduced number of Ki67 (green) expressing cells. (**c**) Within the Periostin injected pancreas Ki67 (green) is not found within epithelial cells such as ducts (*), islets(i), or exocrine cells which express amylase (red). (**d**) Proliferation is observed in the surrounding mesenchymal cells as indicated by the yellow overlap of Ki67 (green) with Vimentin (red). Scale bars represent 200μm.

Mesenchymal pancreatic stellate cells have been shown to express *Postn* in pancreatic cancer[30]. Furthermore, previous work has shown that cultured $Sca1^+$ cells isolated from the resting pancreas expressed markers of mesenchymal cells including activated pancreatic stellate cells (**Supplementary Fig. 4**). Within the resting pancreas, Sca1 expression was localized to the pancreatic stellate cell niche surrounding the acinar cells (**Supplementary Fig. 5b**). Therefore, we extracted mRNA from freshly isolated Sca1-expressing cells (**Supplementary Fig. 5a**) and determined expression of periostin by RT-PCR. As hypothesized periostin expression in the pancreas was limited to Sca1-expressing cells (**Supplementary Fig. 5c**). In addition, $Sca1^+$ cells were isolated from heterozygous mice with β-galactosidase inserted into the periostin gene. These $Sca1^+$ cells were able to convert fluorescein digalactoside (FDG) into fluorescein, a process which requires β-galactosidase, confirming $Sca1^+$ cells express periostin (**Supplementary Fig. 6**). Therefore, Periostin stimulates the proliferation and migration of mesenchymal cells in the pancreas during the initial phase of regeneration. Mesenchymal cells including pancreatic stellate cells express Periostin allowing the protein to function in an autocrine manner.

Accumulation of stroma provides a milieu for regeneration

Cell types within the pancreas can be divided into two predominant classes; epithelial or mesenchymal. The epithelial cells all express E-cadherin and comprise of the acinar cells, ductal cells and endocrine cells. The mesenchymal compartment is made of cells expressing vimentin which includes pancreatic fibroblasts, vascular smooth muscle, pancreatic stellate cells and endothelial cells. Normally, epithelial cells form

tight junctions leaving little room for the mesenchymal stroma, however, three days following periostin injection the number of cells expressing vimentin had increased substantially **(Fig. 4a,c)**. This increase was localized to areas that contained tubular complexes **(Fig. 4c; top)**, while other areas of the pancreas expressed normal levels of vimentin and E-cadherin **(Fig. 4c; bottom)**. However, three days following periostin injection proliferation within vimentin expressing cells was less common **(Fig. 4a)** while cytokeratin7 expressing tubular complexes began to proliferate **(Fig. 4d)**. In addition, these areas contained ductal cells that expressed E-cadherin **(Fig. 4b)** and displayed increased proliferation as shown by increased Ki67 immunofluorescent staining **(Fig. 4b; lower left)**. These ductal cells also expressed the pancreatic progenitor marker Pdx1 **(Fig. 4b; lower right)**. Furthermore, areas with increased stroma contained $Ngn3^+$ cells both within **(Fig. 4c; arrows)** and around tubular complexes **(Fig. 4c)**. In areas distal to the periostin injection and tubular complex formation $Ngn3^+$ cells were absent **(Fig. 4c; bottom)**. Thus, the Periostin induced stroma is sufficient to facilitate the formation of tubular complexes which contain endogenous pancreatic progenitors expressing Ngn3.

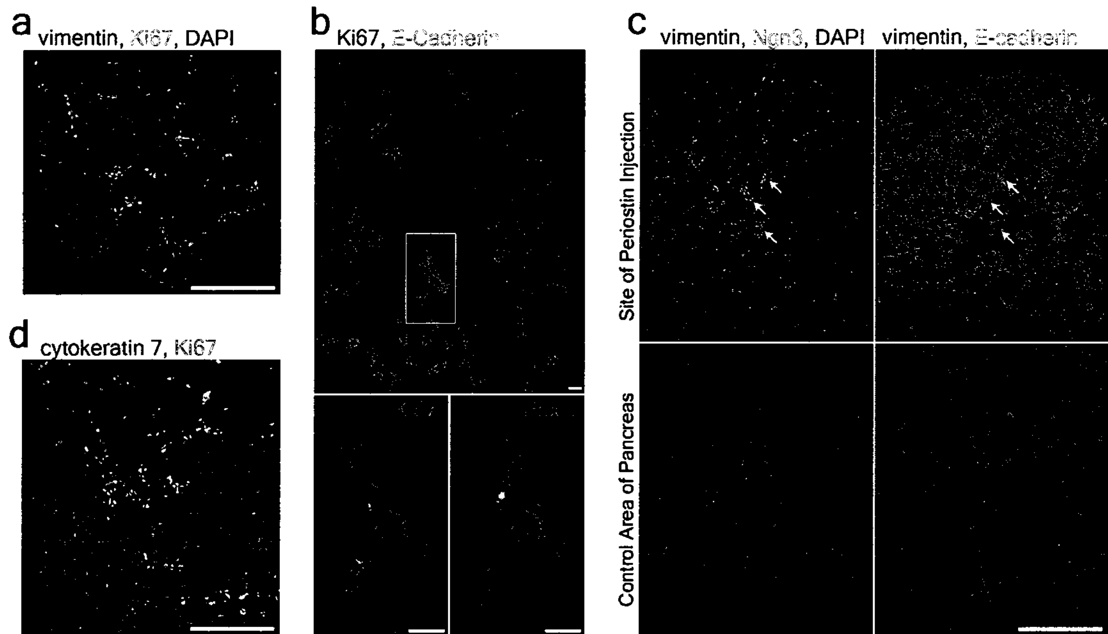


Figure 4 Acquired stroma facilitates early signs of regeneration.

(**a**) Proliferation is no longer within vimentin (red) expressing cells as shown by the lack of overlap with Ki67 (green). Nuclei are stained with DAPI (blue). Scale bar is 200μm. (**b**) Ki67 (red) is present within E-cadherin (green) ductal cells, magnified on the bottom left. The bottom right reveals Pdx1 (red) expression. The Scale bars represent 20μm. (**c**) Accumulations of Vimentin (red) expressing cells surround tubular complexes that express Ngn3(green; left) and E-cadherin(green; right). Normally E-cadherin (green) expression is unbroken and vimentin (red) detection is limited to a much smaller fraction of cells as observed in areas distal to the sight of injection (lower). Scale bar is 200μm. (**d**) Proliferation occurs within cytokeratin7^+ (red) tubular complexes as shown by the yellow overlap with Ki67 (green). Scale bar represents 100μm.

Vast proliferation of epithelial cells follows tubular complex formation

One week after injection of periostin a pancreatic stroma was visible as an accumulation of cells not expressing E-cadherin relative to saline injected pancreata (**Fig. 5a**). Although only a few tubular complexes remained in the periostin injected pancreata, proliferation was widespread as compared to the saline injected controls (**Fig. 5a**). Proliferation was detected within E-cadherin expressing cells (**Fig. 5b**) and not exclusively within tubular complexes as was observed three days following regeneration. Proliferation was within the amylase expressing acinar cells and absent in the surrounding stroma (**Supplementary Fig.7**). Proliferation of the surrounding stroma during the first few days of regeneration was confirmed by administering BrdU in the drinking water following the injection of periostin. The surrounding stroma showed accumulation of BrdU (**Fig. 5c**) and varied in size from 10 μm to 300 μm in width (**Supplementary Fig. 7**). The largest accumulations of stroma still contained some E-cadherin positive tubular complexes which also incorporated BrdU (**Supplementary Fig. 7**). However, tubular complexes were far less abundant as they were three days following periostin injection.

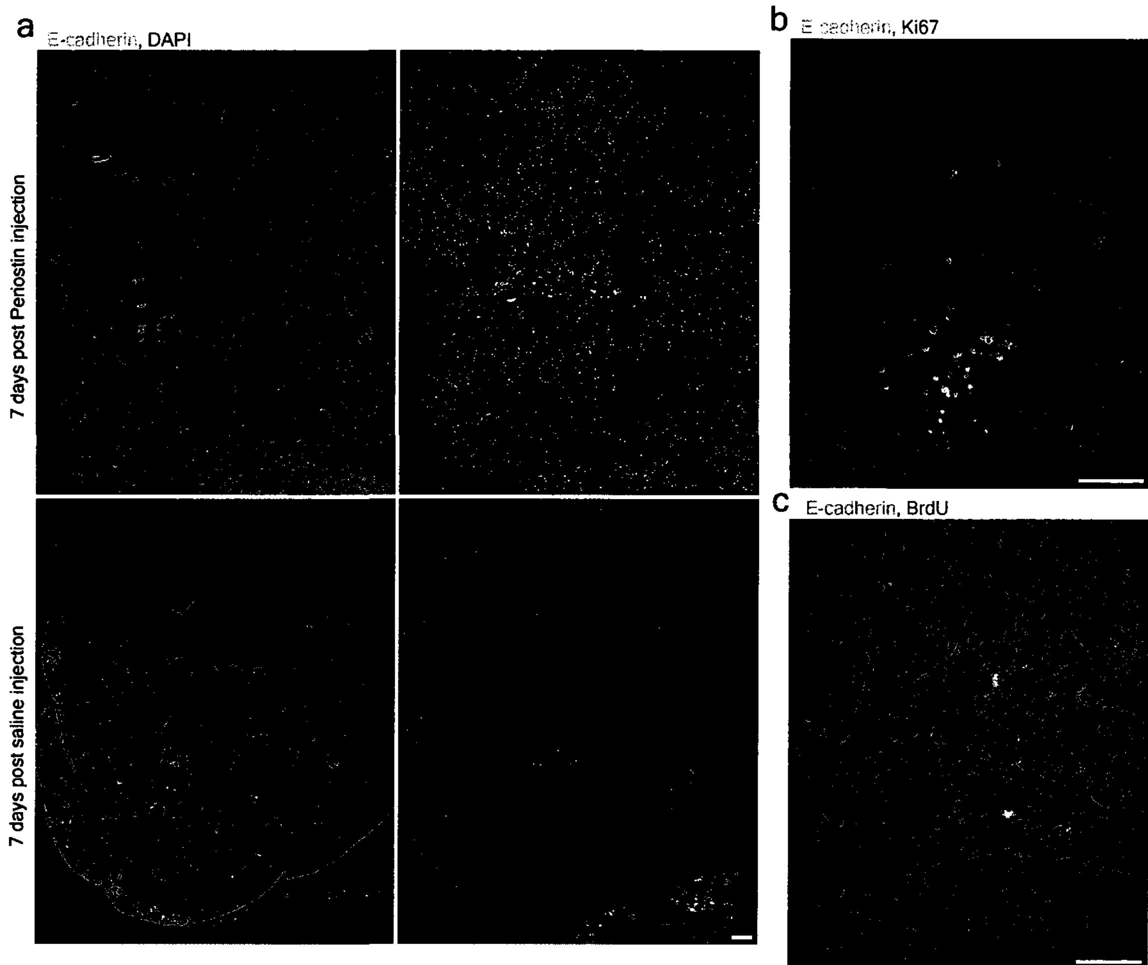


Figure 5 Epithelial proliferation follows Periostin induced stromal accumulation.

(**a**) E-cadherin (green) expressing cells are broken up by a mesenchymal stroma. In a second widespread wave of proliferation Ki67 (yellow) is observed in the periostin injected pancreas but not in the saline injected control, although Ki67 is observed in the highly proliferative spleen as expected (bottom right).

(**b**) Ki67 (red) is observed within the E-cadherin (green) expressing epithelial cells but not within the stroma at one week after injection.

(**c**) Varying degrees of BrdU (red) accumulation occurs within mesenchymal areas which are negative for Ecadherin (green). Scale bars represent 100μm.

Periostin induces regeneration in diabetic mice

To determine if periostin could induce regeneration independent of diabetic status, the recombinant protein was directly injected into the pancreas of mice with streptozotocin (STZ)-induced diabetes (n=9). Even in mice with hyperglycemia greater than 600 mg/dL Ngn3$^+$ pancreatic precursors were found following injection of recombinant periostin (**Fig. 6a**). One week following the injection insulin expression was observed in cytokeratin7$^+$ tubular complexes (**Fig. 6b**). Although cytokeratin7 was expressed in the ducts of saline injected controls as expected there was no mesenchymal accumulation, tubular complex formation, or insulin expression within the ducts (**Fig. 6c**). Four weeks following the direct injection of periostin into pancreas of diabetic mice insulin expression was frequently found in small clusters within (**Fig. 6d**) and surrounding ducts (**Fig. 6e**). These clusters contained both insulin positive cells (**Fig. 6f**) as well as glucagon positive cells which still expressed Ngn3 (**Fig. 6g**). Therefore, the regeneration induced by periostin is independent of diabetic status as we see islet neogenesis in STZ-induced diabetic mice.

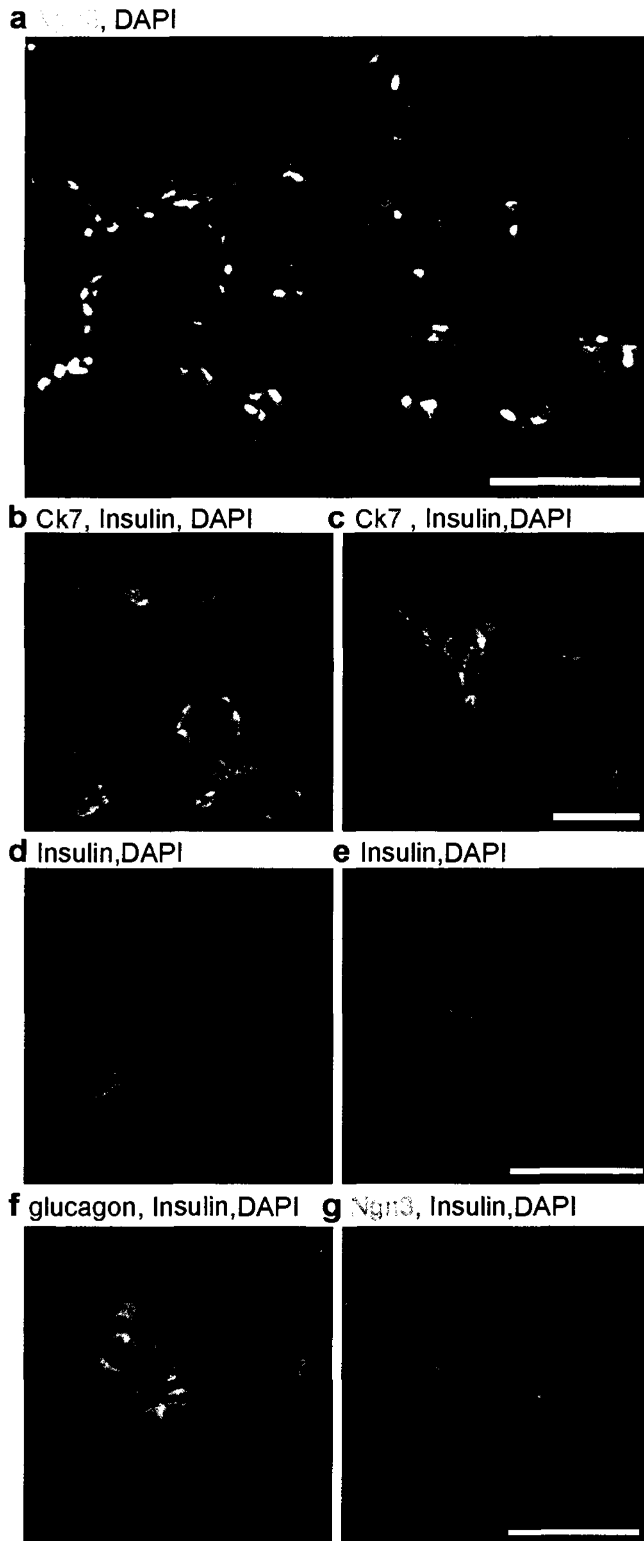


Figure 6 Periostin induces islet neogenesis in STZ-induced diabetic mice.
(**a**) One week following injection of Periostin into STZ-induced diabetic mice, Ngn3-expressing cells appear near the sites of injection. Scale bar is 100μm. (**b**) Ductal structures do not express insulin in saline controls (**c**) Insulin staining is found in Cytokeratin7-expressing tubular complex structures one week after Periostin injection, while tubular complexes are not found in saline controls (**b**). Scale bar is 100μm. (**d,e**) Four weeks following Periostin injection insulin staining is observed in (**d**) and around (**e**) pancreatic ductal structures of STZ-induced diabetic mice. The Scale bar represents 100μm. Insulin-expressing clusters contain cells that express Glucagon (**f**) and Ngn3 (**g**) suggesting they are still immature islets. Scale bar is 40μm.

DISCUSSION

It has been suggested that the pancreatic duct contains cells that can give rise to β-cells as newly formed islets are found closely associated with ductal epithelium[2,5] and in vitro islet-like structures have been generated from ductal extracts[32]. Dor et al have challenged this by showing β-cells as the major cellular source facilitating their own regeneration by self duplication[8]. However, more recent proof has been given for the existence of facultative pancreatic progenitors in Ngn3-expressing tubular complexes in response to pancreatic injury[6]. These structures have been observed in human pancreatitis[33,34], pancreatic cancer[35,36], and in animal models of chemical[37] and surgical pancreatic injury[6,38]. It has been suggested that tubular complexes can give rise to new islets[11] or acinar tissue[37,38] and that they represent early cancer precursors[35,39].
In this study we observe tubular complex formation three days following the injection of recombinant periostin. Although the cellular source of tubular complexes remains unknown these cells express the progenitor marker Ngn3 as previously reported following ductal ligation[6] and in diabetic prone rats[11]. Therefore, periostin is an extracellular factor that initiates a route by which these facultative progenitors emerge. Here we suggest this route is through the stimulation of pancreatic stellate cells to develop a mesenchymal stoma responsible for initiating tubular complex formation.

It is hypothesized that pancreatic stellate cells and their mesenchymal progeny play an important role in regeneration. In periostin null mice we see greatly reduced mesenchymal cell accumulation. This strongly supports the assertion that periostin is required for initiating and maintaining the proliferation of stellate cells to provide the

mesenchymal stroma required for the induction of tubular complex formation. Similarly, periostin has been shown to modify pancreatic cancer by influencing pancreatic stellate cells to create a tumor supportive microenvironment[29]. Therefore, due to reduced mesenchymal stroma, tubular complex formation is greatly impaired in the periostin null mice. Furthermore, the effect of periostin on pancreatic stellate cells and their mesenchymal progeny has been shown by directly injecting the recombinant protein into the pancreas. Twenty four hours after direct injection of periostin mesenchymal cells proliferate to provide the necessary microenvironment for regeneration to occur.

Once pancreatic stellate cells have established a stroma, proliferation occurs within type 2 tubular complexes. These complexes express the pancreatic progenitor markers Pdx1 and Ngn3. Isolating Ngn3-expressing cells from injured pancreata and culturing them with fetal pancreata have shown their ability to differentiate to the β-cell lineage[6]. Because Ngn3-expressing cells are not found in the resting adult pancreas it cannot be used as a marker to isolate adult stem cells. It is unknown whether the Ngn3-expressing cells are derived from an adult pancreatic stem cell or a facultative stem cell acquired from trans-differentiation of a mature cell type. Although the origin of this cell type is unknown, lineage tracing experiments indicate that these cells are not derived from acinar[40] or islet cells[41], leaving ductal or centroacinar cells the likely candidates. Nevertheless, it is only after injury that the Ngn3-expressing cells are found, therefore, an additional signal is required to activate Ngn3 expression. Here we propose that pancreatic stellate cells play an important role in this process.

Pancreatic stellate cells express Sca1 in the resting pancreas and can be activated by the secreted protein periostin. It has been shown both in vivo and in vitro that

periostin can activate pancreatic stellate cells residing in the pancreas. Once activated these cells give rise to quickly dividing mesenchymal cells that provide the stroma for regeneration to occur. This stroma potentially provides scaffolding, support and growth factors to stimulate regeneration. This stroma induces tubular complex formation in which Pdx1 and Ngn3-expressing progenitor cells reside. In areas missing stroma accumulation tubular complex formation is absent. The idea of pancreatic stellate cells contributing to regeneration in the human has already been speculated[42]. In addition, pancreatic stellate cells have been shown to give rise to stromal expansion in pancreatic cancer which influences cancer growth[43,44].

What initially activates pancreatic stellate cells following partial pancreatectomy is unknown, but once activated the secretion of periostin from stellate cells themselves would result in their own auto-regulation as has already been shown to occur in pancreatic cancer[29]. Therefore, the discovery of additional factors, in addition to periostin, will be useful in initiating full regeneration that recapitulates pancreatic development. Several candidates can already be speculated such TGFβ which is known to induce periostin expression[22,45,46].

Interestingly, Ngn3-expressing cells have not been found in pancreatectomies when only 50% of the pancreas was removed[47]. However, in the studies performed here using periostin injection or pancreatectomies of at least 70% we see Ngn3-expressing pancreatic precursors. It would be interesting to determine if a 50% pancreatectomy has reduced mesenchymal accumulation resulting in reduced tubular complex formation and therefore no generation of Ngn3-expressing pancreatic progenitors. This would reiterate the importance of mesenchymal stroma for induction of pancreatic progenitors. In the

absence of a full stromal development following a 50% partial pancreatectomy, self-duplication might be the main source for regeneration and homeostasis of the pancreas. Therefore, the ability to develop a mesenchymal stroma might explain the differences observed following a 50% or a 70% partial pancreatectomy.

Identifying the factors contributing to pancreatic regeneration would be a tremendous asset in developing a cure for both Type 1 and Type 2 diabetes. To uncover such factors a microarray analysis was performed on regenerating pancreatic tissue. This analysis revealed the high expression of a novel isoform of periostin, which lead us to examine the role periostin, plays in pancreatic regeneration. Absence of periostin impaired pancreatic regeneration following partial pancreatectomy. Furthermore, injecting recombinant periostin into the pancreas was sufficient to induce the formation of tubular complexes expressing the progenitor markers Pdx1 and Ngn3. The emergence of these pancreatic progenitors was preceded by the proliferation of pancreatic stellate cells which created a mesenchymal stroma. By inducing stellate cells to create a stroma, periostin is able to stimulate pancreatic regeneration, creating a novel approach towards a cure for diabetes.

EXPERIMENTAL PROCEDURES

Experimental animals and standard surgical procedures. Eight- to nine-week-old male C57BL/6J, MIPGFP and periostin null mice were housed under standard conditions and allowed free access to standard mouse chow and water. All studies were approved by the Animal Care Committee at the University of Ottawa. One hour before surgery mice were given a dosage of 0.05 mg/kg Buprenorphine subcutaneously. Anesthesia was induced in an anesthetic box with isoflurane gradually increased to 5%. Once the mice were anesthetized they were transferred to a face mask with isoflurane at 1.5% where they are maintained throughout surgery, increasing or decreasing the percentage as necessary to keep the animal sedated but breathing normally. The surgical area was shaved and cleaned with Endure soap, rinsed with sterile water and surgically prepared with chlorahexseptic solution. BNP eye ointment was placed in the animals eyes to protect them from drying. 1 ml of sterile saline was administered subcutaneously prior to surgery. Once the surgery was complete mice are placed on oxygen until they recovered and started to move.

Partial Pancreatectomy. To remove pancreatic tissue access to the abdominal cavity was obtained by performing a midline incision. First, a 1 to 1.5cm incision was made through the skin in the middle of the abdomen using a No.10 scalpel blade. Using forceps the skin was gently separated from the abdominal wall to reveal the midline of the abdomen. The midline was lifted with rat tooth forceps and a small cut less than 1cm was made with scissors through the body wall. Once located the splenic pancreatic lobe was lifted through the incision with forceps. The entire splenic lobe and distal portions of the gastric and duodenal pancreatic lobes were removed by gentle abrasion with forceps

and a cotton applicator to ensure no major veins or arteries were broken. If excessive bleeding was observed the site of bleeding was clamped for several minutes to promote clotting. Once removed only a small portion (~30%) of the pancreas remained along the duodenum. The pancreas that was surgically removed was approximately 70% of the total pancreas, as was confirmed by weighing the removed and remnant portions during a pilot study. The body wall was closed with silk surgical sutures (Johnson&Johnson) in two to three discontinuous sutures. The skin was closed with two to three surgical staples (Fisher). Once the surgery was complete the mice were placed on oxygen for approximately one minute and then returned to their cage as soon as they began to move. Blood glucose levels were analyzed every other day checking blood sugar levels for increased glucose. In addition, mice were given 0.05 mg/kg Buprenorphine subcutaneously every day following surgery for the first week.

Periostin injections. Periostin (BioVendor LLC, Candler, NC) was administered directly (500 ng). Direct injection was performed by exposing the pancreas with a midline incision, as outlined above, and injecting 10ul recombinant periostin solution (50 ng/ul) directly into the pancreas with a Hamilton syringe. Vehicle-treated animals received the same amounts of buffer diluted with saline. Following injection into the pancreas the body wall was closed with sutures and the skin with wound clips, as was done following pancreatectomy. Mice were monitored daily and given 0.05 mg/kg Buprenorphine subcutaneously every day following the first week of surgery. Following the surgery BrdU (sigma) was administered in the drinking water at 0.8 mg/ml to continuously label of dividing cells.

Streptozotocin induced Diabetes. Streptozotocin (Sigma), which selectively targets and destroys pancreatic β-cells to create diabetes, was injected into adult mice. More specifically, one *intra peritoneal* injection of 100 mg/kg of Streptozotocin (STZ) was given every other day until the mouse became diabetic as described previously in mice[48] and within the C57BL/6J background being used[49]. Following the first STZ injection (Day 0) blood sugar levels were taken daily to determine the diabetic status of mice.

RNA isolation and microarray screen. The pancreas remnant or the corresponding tail section of a resting pancreas was harvested and quickly frozen in liquid nitrogen. The tissue was ground with a mortar and pestle and while still frozen RNA was isolated using Trizol (Invitrogen) followed by DNase treatment (Quiagen). RNA integrity was confirmed by Agilant 2100 Bioanalyzer (Agilant Technologies). Total RNA was hybridized on the Mouse Genome 430 v2.0 GeneChip® (Affymetrix). Raw data files were processed with Affymetrix GeneChip® Operating Software (GCOS) to derive signals and present/marginal/absent calls for each sample. Pair wise comparisons were made between the regenerating and the resting pancreas to produce log(fold) ratio estimates of change and increase/no change/decrease calls. Processed results were exported from GCOS and imported into Excel and Access (Microsoft) for further analysis.

Quantitative real-time PCR validation. Total RNA from both the resting and regenerating pancreas was reverse transcribed using the RNA PCR Core Kit (PerkinElmer) following the manufacturer's instructions using random hexamer primers. RT reactions were diluted in 10 mM Tris, pH 8.0 to achieve a ct value of approx. 20 cycles for the control housekeeping gene GAPDH. Once diluted the same concentration

of RT products were used for all PCR reactions. Each real-time PCR reaction included: 2 μl of diluted reverse transcription product from the master mix, 10μl of 2x iQSYBR Green SuperMix (Bio-Rad Laboratories), 30nM ROX passive reference dye (Stratagene), 50nM forward and 50nM reverse PCR primer. Primers were designed using OligoPerfect™ Designer (Invitrogen). If possible forward and reverse primers were designed to span introns and therefore avoid amplification from contaminating DNA. Real-time data was gathered using the MX3000 RT-PCR system (Stratagene). The starting conditions are 40 cycles (30 s at 94°C, 60 s at 58°C, and 30 s at 72°C) followed by a denaturation curve from 54 to 94°C in 30 second increments of 0.5°C to ensure amplification specificity. If multiple peaks were observed in the denaturation curves then products were run out on a gel to look at band specificity and if necessary new primers were designed. Ct values were calculated with the MX3000 software by using the moving window averaging with an adaptive baseline. Relative fold changes and all other calculations were performed in Excel (Microsoft). All reactions were performed in triplicate. The threshold cycle (CT) for periostin was compared with the CT for the internal control (GAPDH). Fold effects were determined assuming each cycle represented a 2 fold difference in number of initial transcripts.

Immunohistochemistry. Pancreata were embedded in optimal cutting temperature compound, O.C.T. (Tissue-Tek) : 20% sucrose solution (2:1) and frozen by emersion into 95% ethanol/dry ice. Cryosections (8um) were cut using a cryostat (Leicia CM 1850). The directly conjugated antibodies used are listed in **Supplementary Table SI.** The primary antibodies used are listed in **Supplementary Table SII** and the corresponding secondary antibodies in **Supplementary Table SIII.** BrdU detection was performed by

following the protocol outlined in the BrdU in situ detection kit (BD Biosciences). Nuclei were counter-stained with DAPI (Sigma). Images were taken with a Carl Zeiss Axioplan 2 Microscope with an AxioCam HRm b/w (Zeiss) camera using Axiovision v3.2 (Zeiss) acquisition software.

Isolation of single cells from the Pancreas. The pancreas was removed, minced and resuspended in 3,000units (12 mg) of collagenase type I (sigma) in 4 ml RPMI + 10% FBS. The solution was shaken for 6mins in a 15 ml falcon. Digestion was stopped by adding 10 ml of cold DMEM + 10% FBS. The digested pancreas was spun down and resuspended in 2 ml of 2.5% Trypsin for 3min, then 12 ml of cold DMEM + 10% FBS was added and the cells were spun down and resuspended in culture media.

Flow Cytometry. Single cells were isolated from the pancreas then stained with an antibody for Sca1 (1:200) directly conjugated to APC (BD Pharmingen). Cells were separated on a MoFlo cytometer (DakoCytomation). Dead cells and debris were removed by gating on forward vs. side scatter profiles. To quantify the number of insulin$^+$ cells in the pancreas mice expressing GFP under the insulin promoter were employed. One week following an i.p. injection of Periostin the pancreata were digested into single cells and the number of GFP$^+$ cells were quantified using a MoFlo cytometer (DakoCytomation).

Identification of cells expressing LacZ. Fluorescein di-β-D-galactopyranoside (FDG) is useful in detecting cells expressing β-galactosidase in LacZ knock-in mice. Non-fluorescent FDG is hydrolyzed by β-galactosidase, first becoming fluorescein monogalactoside (FMG) and then the highly fluorescent fluorescein. A 20 mM stock solution of FDG (invitrogen) was created by dissolving 5 mg of FDG in 381ul of DMSO

(sigma). A working solution was then created by diluting the stock solution 10x in sterile deionized water at 37 degrees Celsius. Equal portions of working solution were mixed with growth medium and placed on cells in culture. The combined mixture was incubated for 1min at 37 degrees Celsius then diluted 10x with ice cold growth medium. Cells were then observed on an Observer.Z1 (Zeiss) microscope after 30 to 60 minutes incubation on ice.

***In vitro* cell culture.** Mesenchymal cells were cultured from total pancreatic cell isolations in RPMI with 10% FBS. 500 ng/ml of Recombinant Periostin protein (BioVendor LLC, Candler, NC) was added for 24 hours to study the effect of periostin on cell morphology. Alternatively to study the effect of Periostin on growth rate mesenchymal cells were infected with Periostin in a pLenti6 lentiviral construct (invitrogen). FACS purified $Sca1^+$ cells were cultured under the same conditions and fixed with 4% PFA, permeabilized with 0.2% TritonX and stained for markers of pancreatic stellate cells. The antibodies used are listed in **Supplementary Table SI-SII**.

Data analysis. Data in graphs are provided as mean ± SEM and were analyzed using a two-tailed student's t test (*$P<0.05$).

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SUPPLEMENTARY INFORMATION

SUPPLEMENTARY FIGURES

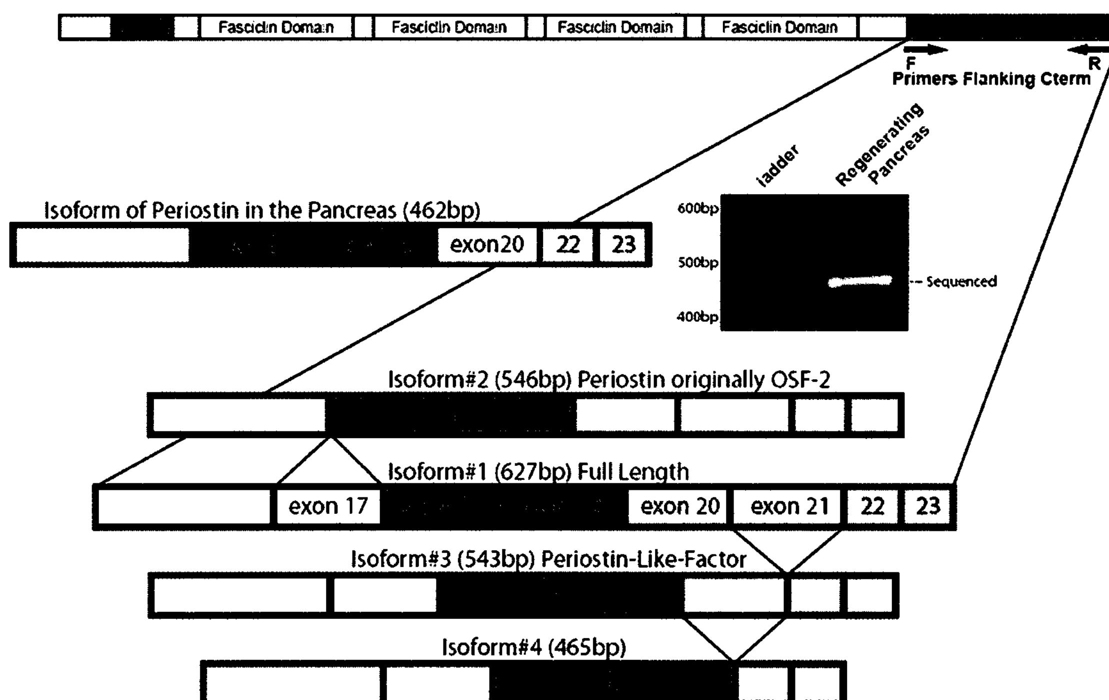

Figure S1. Identification of periostin isoform expressed during regeneration. The EMI and Fasciclin Domains of Periostin shown to scale with the varying C terminus to create the four known isoforms. A fifth and novel isoform of periostin was sequenced from the regenerating pancreas using primers flanking the C terminus.

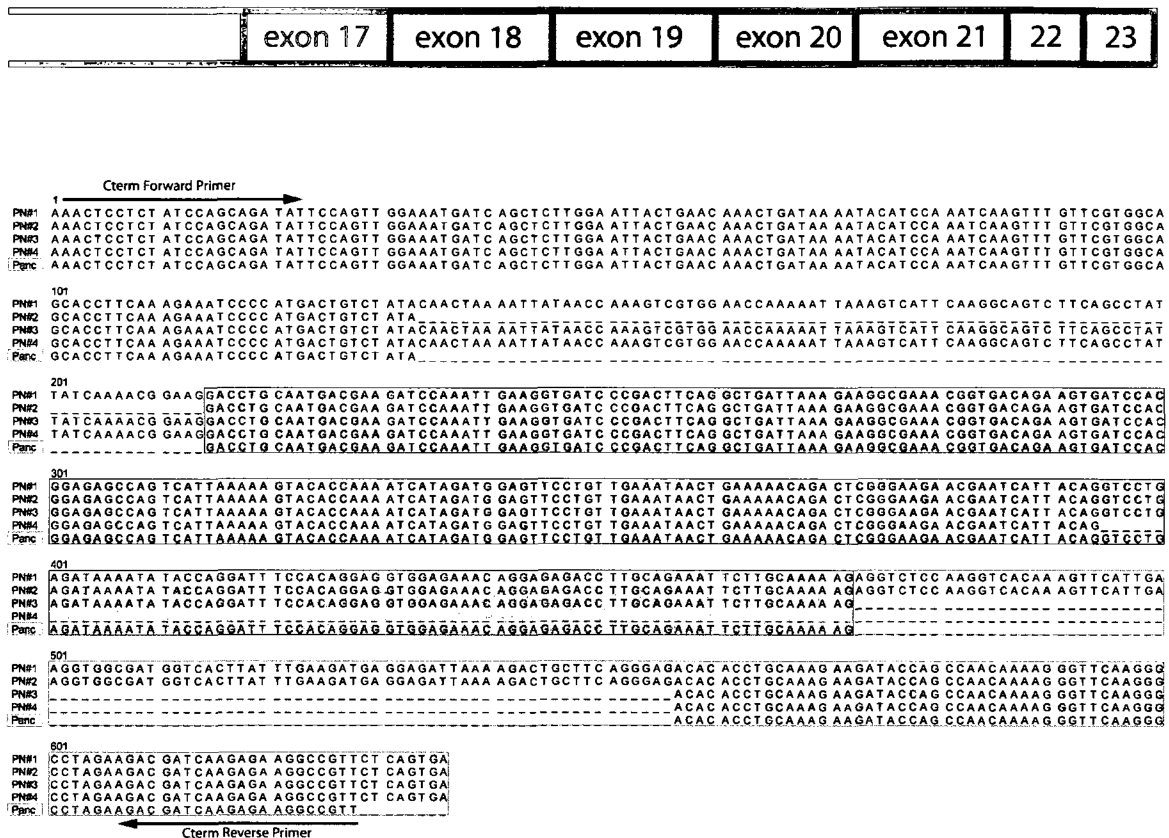

Figure S2. Sequencing of periostin isoform expressed during regeneration. Sequences of known isoforms of mouse periostin (PN#1, PN#2, PN#3 and PN#4) aligned with the sequence of the major isoform present during pancreatic regeneration (Panc). The major RT-PCR product was excised from an agarose gel, purified and sequenced with primers as shown. The full length isoform (PN#1) contains exons 17 to 23. The original isoform of periostin that was first cloned (PN#2) is missing exon 17 (yellow). Periostin-like factor (PN#3) is missing exon 21 (orange). The final known isoform (PN#4) is missing exon 20 (green) and 21 (orange). The periostin isoform isolated from the pancreas is missing exon 17 (yellow) and 21 (orange).

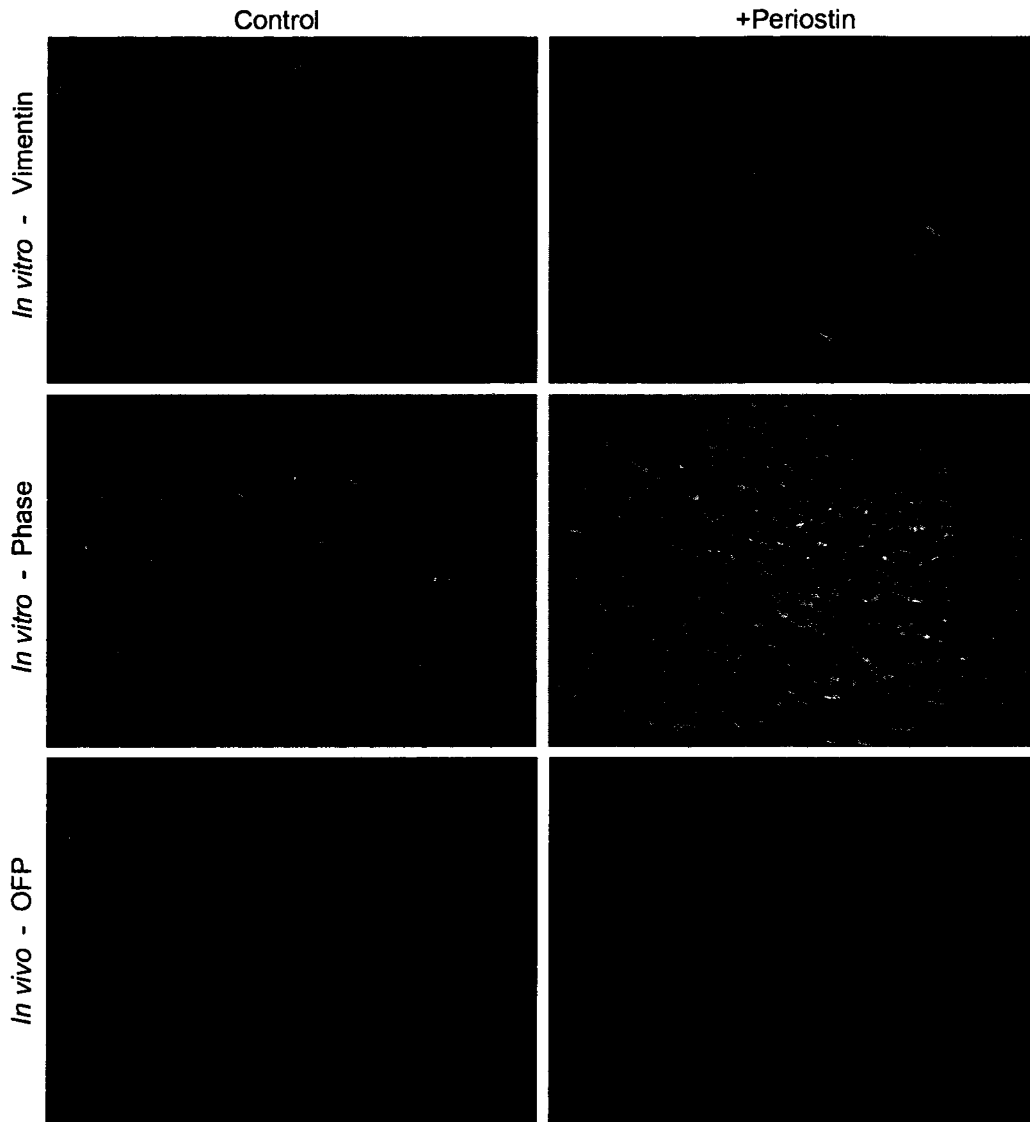

Figure S3. Periostin changes morphology of pancreatic mesenchymal cells. Cultured mesenchymal cells express vimentin (top left), addition of recombinant periostin resulted in significant changes in morphology (right). Changes in morphology were also observed using phase contrast (middle). To determine if a change in morphology represented increased migration *in vivo*, Orange Fluorescent Protein (OFP) was injected with Periostin. When injected alone OFP remained within the area of injection, however, when injected with Periostin OFP migrated away from the area of injection after three days (bottom).

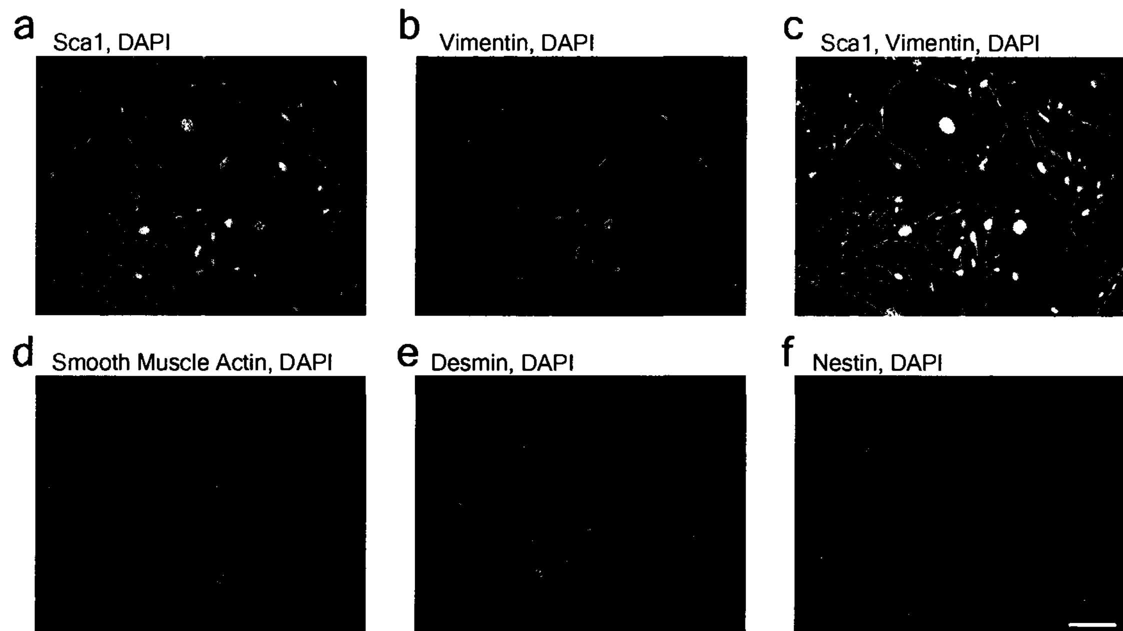

Figure S4. Sca1^{+} cells express markers of pancreatic stellate cells.

Sca1^{+} cells isolated from the pancreas and cultured express Sca1 (a) and Vimentin (b) within the same cell as observed in the merge images (c). These cells also express Smooth Muscle Actin (d) Desmin (e) and Nestin (f) all previously described to be markers of pancreatic stellate cells. As previously described Desmin is not expressed in all pancreatic stellate cells. Scale bar represents 100μm.

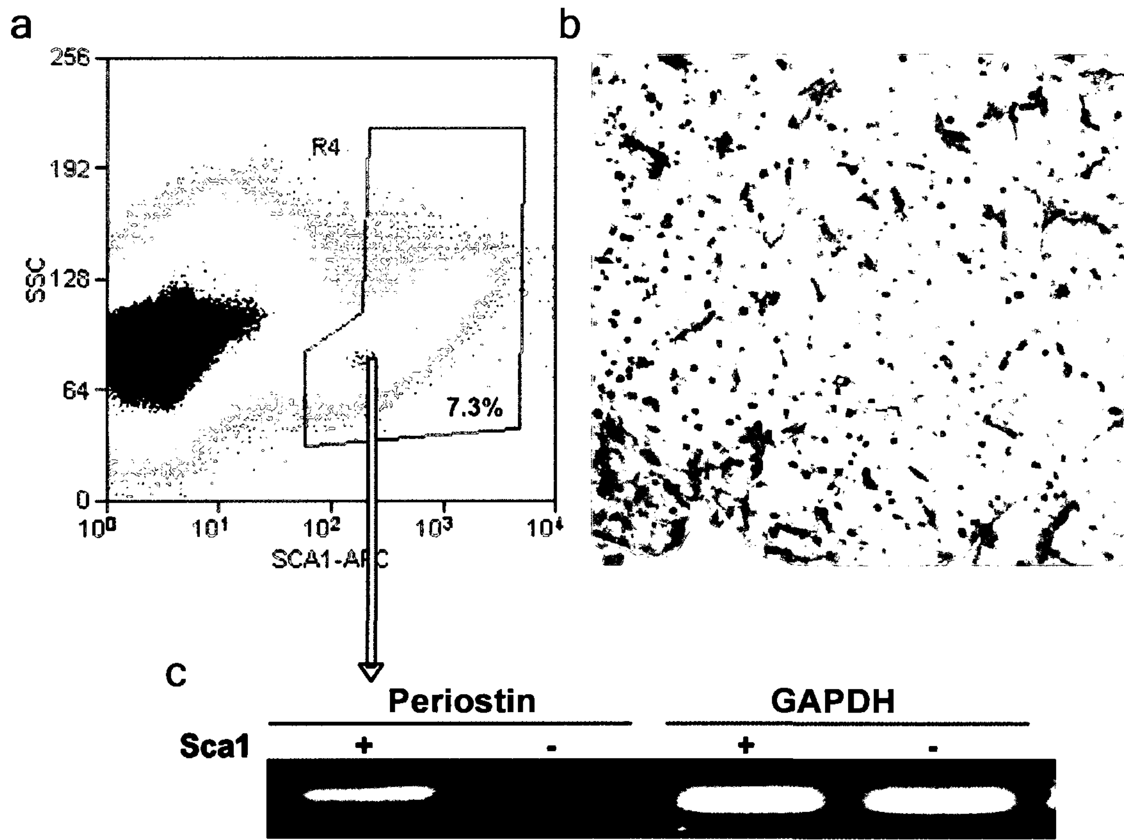

Figure S5. Periostin expression in the resting pancreas is limited to Scal$^+$ cells.

a) Scal labels approx. 7% of the cells in the adult pancreas, corresponding to the number of pancreatic stellate cells and fibroblasts previously reported within the pancreas. b) These Scal$^+$ cells (brown) were present surrounding the exocrine tissue in the same location as pancreatic stellate cells. c) mRNA for periostin was only detected within the Scal$^+$ fraction of cells suggesting pancreatic stellate cells are the source of periostin in the pancreas.

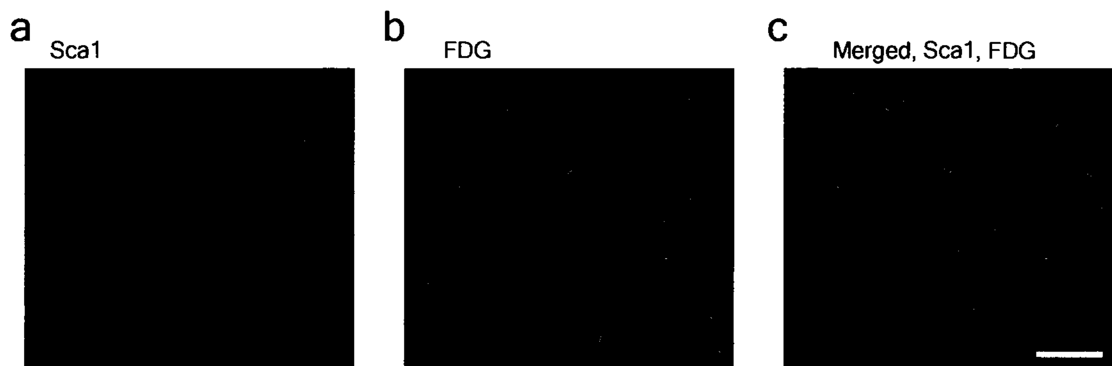

Figure S6. Cultured Scal$^+$ cells express periostin.

Cultured Sca-1$^+$ cells express β-galactosidase when isolated from heterozygous knock-in mice expressing β-galactosidase under the periostin promoter.

a) FACS purified Scal$^+$ cells were cultured and immunostained with anti-Scal antibody directly conjugated to PE (red).

b) Prior to the addition of Scal antibody FDG was introduced into the cells and cells expressing β-galactosidase convert FDG into fluorescein (green).

c) A merged image showing a majority of the Scal expressing cells co-express β-galactosidase. Scale bar represents 200um.

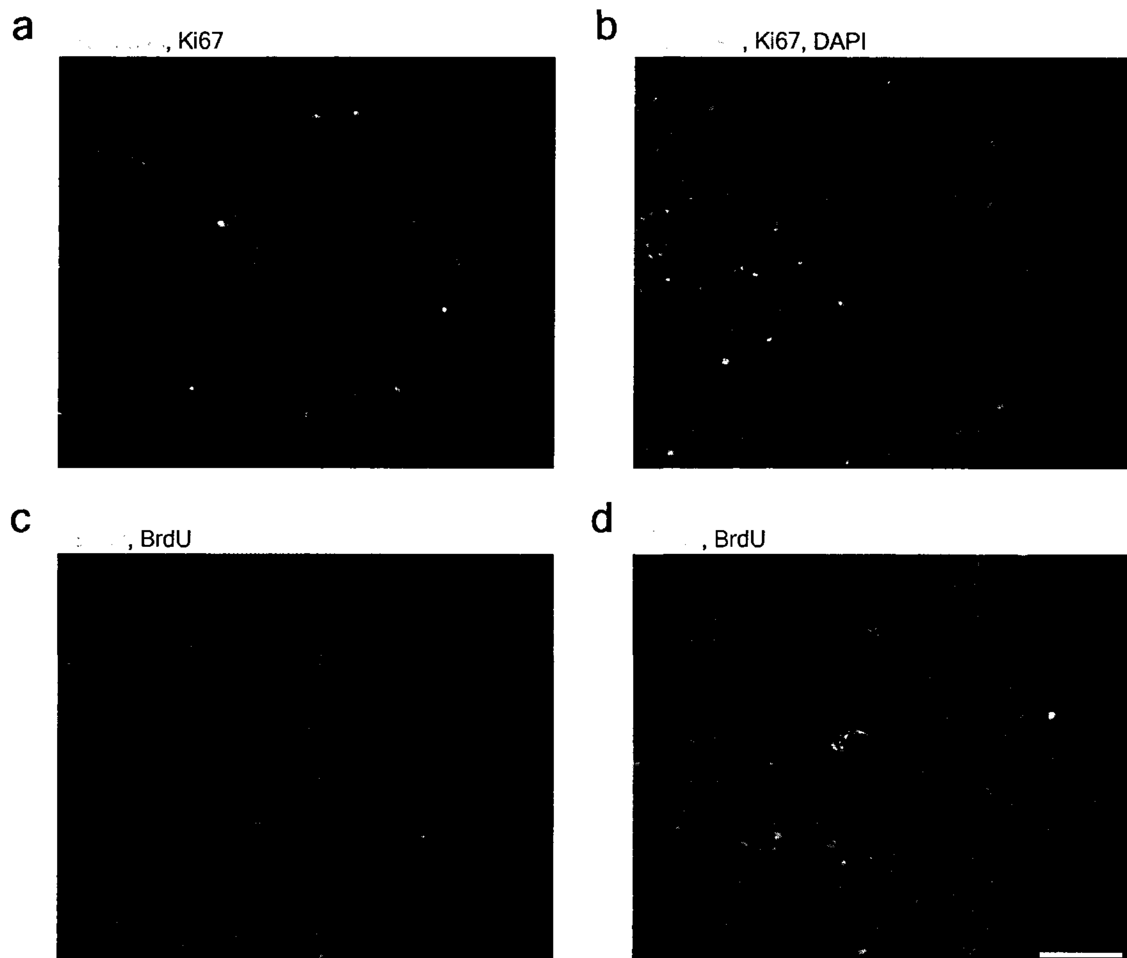

Figure S7. Proliferation one week following periostin

Proliferation no longer occurred within the mesenchymal stroma but within mature epithelial cells one week after the direct injection of periostin into the pancreas. a) Proliferation occurred within cells expressing amylase but not within the surrounding stroma (b). c) BrdU accumulated in the stroma due to proliferation within the first few days of regeneration. The accumulation of stroma varied in size from roughly 50um (c) to 300um (d) after one week. d) The largest accumulations still contained some E-cadherin positive tubular complexes. Scale bar represents 100µm.

SUPPLEMENTARY TABLES

Supplementary Table SI: Directly conjugated antibodies used in study.

Primary Antibodies	Dilution	Company	Cat#
E-Cadherin FITC	1:200	BD Bioscience	612131
Sca-1 APC	1:200	Cedarlane (ebioscience)	17-5981-83
Sca-1 FITC	1:200	BD Bioscience	01164a
Sca-1 PE	1:200	BD Bioscience	01165b
Vimentin D547	1:200	Abcam	38873

Supplementary Table SII: Primary antibodies used in the study.

Primary Antibodies	Raised In	Dilution	Company	Cat#
Amylase	Rabbit	1:500	Sigma	A8273
BrdU	Mouse IgG_1	1:200	BD Bioscience	347580
Cytokeratin 7	Mouse IgG_1	1:200	BD Bioscience	550507
Desmin	Mouse IgG_1	1:500	Dako	M0760
E-Cadherin	Mouse IgG_{2a}	1:500	BD Bioscience	610182
Glucagon	Rabbit	1:200	Dako	A0565
Insulin	Guinea Pig	1:200	Dako	A0564
ki 67	Mouse IgG_1	1:200	BD Bioscience	550609
Nestin	Mouse IgG_1	1:500	BD Bioscience	556309
Neurogenin 3	Mouse IgG_1	1:1,000	DSHB	F25A1B3-c
Pdx1	Rabbit	1:5,000	C. Wright (Vanderbuilt)	
Periostin	Rabbit	1:5,000	Abcam	14041
α smooth muscle actin	Mouse IgG_1	1:1,000	Sigma	A5228

Supplementary Table SIII: Secondary antibodies used in the study.

Secondary Antibodies	Dilution	Company	Cat#
anti-Guinea Pig	1:1,000	Resarch Diagnostics Inc	706765148
anti-Mouse IgG_1 A488	1:2,000	Invitrogen - Molecular Probes	A21121
anti-Mouse IgG_1 A568	1:2,000	Invitrogen - Molecular Probes	A21124
anti-Mouse IgG_1 A647	1:2,000	Invitrogen - Molecular Probes	A21240
anti-Mouse IgG_{2a} A488	1:2,000	Invitrogen - Molecular Probes	A21131
anti-Mouse IgG_{2a} A647	1:2,000	Invitrogen - Molecular Probes	A21241
anti-Rabbit A488	1:2,000	Invitrogen - Molecular Probes	A21206
anti-Rabbit A546	1:2,000	Invitrogen - Molecular Probes	A11035
anti-Rabbit A647	1:2,000	Invitrogen - Molecular Probes	A21443

Chapter 5 – General Discussion

5.1. Overview of Findings

Although the pancreas has the ability to regenerate, the cell and molecular mechanisms that regulate this regeneration are unknown. Presently, we have demonstrated that pancreatic regeneration requires a mesenchymal stroma, produced in part by the activation of pancreatic stellate cells. In the resting pancreas, mesenchymal cells, including pancreatic stellate cells, express Stem cell antigen 1 (Sca1), creating a convenient marker for their isolation. However, following injury to the pancreas, exocrine and ductal cells also express Sca1, resulting in widespread expression in the regenerating pancreas. Furthermore, this dynamic expression provides the opportunity for cells to start expressing Sca1 during the isolation process. Thus, the diversity of cells within the freshly isolated population is increased. Nevertheless, culturing Sca1 cells *in vitro* produces a population of cells uniformly expressing mesenchymal markers, including those of activated pancreatic stellate cells (PSCs). Once activated, the mesenchymal cells, produced by PSCs, outgrow other cell types within the isolated population. Although this mesenchymal cell type is not the direct source for pancreatic regeneration it plays a key role in the regeneration process.

Following partial pancreatectomy there is an early proliferation of resident mesenchymal cells, partly arising from the activation of pancreatic stellate cells. Proliferation of these cells leads to the development of a mesenchymal stroma that facilitates the regeneration process. Once formed, the mesenchymal stroma houses proliferating tubular complexes, including Pdx1 expressing duct-like cells and facultative Ngn3-expressing pancreatic precursor cells. These complexes represent the cellular source which will form the new pancreatic lobes.

Isolation and culture of $Sca1^{+}CD31^{-}$ cells produce a uniform population consisting of activated pancreatic stellate cells. Injecting these cells into the pancreas was sufficient to produce a stroma which induced regeneration, similar to that observed following partial pancreatectomy. An important step in understanding this regeneration process will be uncovering factors provided by the mesenchyme that supports tubular complex formation, proliferation and differentiation.

The secreted protein periostin was discovered in a microarray screen as a factor highly expressed during regeneration following partial pancreatectomies. Periostin expression was localized to the regenerating tip of the pancreas in the stroma surrounding proliferating tubular complexes. Based on previous reports (Yan and Shao, 2006), we initially hypothesized that periostin may induce an epithelial to mesenchymal transition (EMT) during pancreatic regeneration. To test this hypothesis we injected the recombinant protein directly into the pancreas. Although we did not observe any epithelial cells undergoing EMT, twelve hours after the injection pancreatic stellate cells were activated giving rise to a widespread proliferation of resident mesenchymal cells. These mesenchymal cells accumulated within areas that subsequently showed distinct features of regeneration, such as tubular complex formation, Pdx1 and Ngn3 expression. Therefore, periostin activates pancreatic stellate cells to produce a stroma, which in turn facilitates regeneration. These findings have exciting therapeutic value for the treatment of diseases in which pancreatic tissue has been lost, such as diabetes.

5.2. Biomedical Implications: Towards the Treatment of Diabetes

The ability to control pancreatic regeneration would launch exciting new approaches for therapeutics, particularly for the treatment of diabetes. We have shown that a mesenchymal stroma is capable of initiating the regeneration process whether it is created by directly injecting mesenchymal cells, or by activating resident pancreatic stellate cells with periostin. In each case, the results are similar with the formation of tubular complexes and the expression of Ngn3 and Pdx1 in these duct-like cells. Thus, by controlling the pancreatic stroma we can control pancreatic regeneration.

5.2.1. Injection of mesenchymal cells for the treatment of diabetes

Injection of mesenchymal cells for the treatment of diabetes involves several potential risks which need to be fully explored. Firstly, expansion of these cells could lead to cytogenetic abnormalities resulting in tumor development when used for treatment (Miura et al., 2006; Tolar et al., 2007). Secondly, mesenchymal cells often maintain the ability to differentiate into adipose or oesteogenic tissue which would be an unwanted result if this differentiation occurred after transplantation (Breitbach et al., 2007). Finally, the *in vitro* culture of mesenchymal cells is currently done with fetal calf serum which could lead to an immune reaction if the cells were to be implanted. However, it is possible that human serum (Spees et al., 2004) or platelet lysates (Doucet et al., 2005) could be used instead to avoid this problem. Aside from these challenges, the use of mesenchymal cell transplantation is a novel approach to the treatment of diabetes.

5.2.2. Injection of periostin for the treatment of diabetes

The activation of resident pancreatic stellate cells by injecting recombinant periostin would overcome several of the challenges that would exist if mesenchymal cells themselves were injected. However, care must still be taken to ensure that the induced stromal formation will not initiate pancreatic cancer. This is a concern as a link between chronic pancreatitis and pancreatic cancer has already been suggested (Guerra et al., 2007). Additionally, pancreatitis is known to result in the activation of pancreatic stellate cells (Madro et al., 2004). Therefore, consideration should be given to the amount of activation that would be required for diabetes to be reversed. Reducing the amount of activation required would lower the chances of any potential unwanted side effects.

Identification of factors that promote β-cell differentiation would enhance the effectiveness of periostin treatment. Currently, the formation of a stroma by either injecting activated PSCs or periostin induces a complete regeneration of both exocrine and endocrine tissue. In therapeutic approaches for diabetes, it would only be necessary to induce β-cell neogenesis. If downstream mechanisms could be targeted that specifically trigger endocrine regeneration, the efficiency of islet neogenesis could be improved. This is important as unnecessary side effects may occur from inducing a complete regeneration of the pancreas. This could include the generation of an excess of digestive enzymes which leads to pancreatitis and potentially pancreatic cancer. In addition, directly targeting endocrine regeneration would ensure enough insulin producing cells are produced to lower blood sugars and fully reverse diabetes.

Therefore, it is crucial that we understand what drives facultative progenitors toward the pancreatic β-cell lineage. Treatment with periostin or mesenchymal cell

injections results in the emergence of these facultative progenitors; however, we do not yet understand how to drive these progenitors exclusively into mature pancreatic islets. Alternatively, if we elucidated the factors involved in the differentiation to exocrine or ductal tissue we could block this pathway, resulting in increased endocrine regeneration. Therefore, further understanding the differences between exocrine and endocrine regeneration will allow for better targeting of the desired regeneration pathway.

5.2.3. In vitro differentiation of pancreatic β-cells from stem cells

Understanding the basic biology of endocrine regeneration would aid alternative approaches such as the *in vitro* differentiation of pancreatic β-cells from a stem cell source. Many groups have created differentiation protocols to produce pancreatic β-cells from stem cells. However, no single differentiation process is yet perfected, and each would benefit from elucidating the processes involved in the *in vivo* differentiation of β-cells during regeneration. Factors secreted by mesenchymal cells may prove to be beneficial. Therefore, we can extend our study to provide insight into the differentiation of stem cells to generate islets *in vitro* for the treatment of diabetes.

The pluripotency of embryonic stem (ES) cells makes them an attractive source for the differentiation of pancreatic β-cells. The first successful differentiation of ES cells to pancreatic ß cells, which were capable of reversing STZ-induced diabetes, was performed using a cell-trapping system (Soria et al., 2000). Subsequently, a five-stage protocol to differentiate ES cells into β-cells was created (Lumelsky et al., 2001). Other groups have improved this five-stage induction protocol by adding growth inhibitor LY294002 (Hori et al., 2002), or over-expressing the pax4 gene (Blyszczuk et al., 2003).

However, repeating the five-stage protocol revealed insulin was absorbed from the culture medium, not produced by the ES cells (Hansson et al., 2004). Therefore, specific induction factors need to be determined to conclusively differentiate ES cells.

Selected for their importance developmentally, activin A and all-trans retinoic acid (RA) have shown potential for inducing embryonic stem cells to differentiate into insulin secreting cells (Shi et al., 2005). Interestingly, pancreatic stellate cells secrete activin A (Ohnishi et al., 2003) and also process retinol to produce RA (Bachem et al., 1998). These factors could be provided by PSCs to facilitate regeneration. This would strongly suggest similarities between embryonic development and adult regeneration, an idea already put forward by others (Bonner-Weir et al., 1993; Jensen et al., 2005).

Considerable effort has been applied to the search for a pancreatic stem cell, as it would provide a sustainable cellular source for the *in vitro* generation of β-cells. Most efforts have not used a clonal isolation of single adult pancreatic precursor cell, making it difficult to determine if such cells are truly multi-potent. However, single cells have been isolated from the adult pancreas and determined to be multi-potent as their progeny differentiate into mature neurons, pancreatic β-cells, α-cells, δ-cells and exocrine cells. In addition, the β-like cells were functional and released insulin in a glucose-dependent manner (Seaberg et al., 2004). Directing differentiation of these pancreas-derived multipotent precursors (PMPs) may be possible as the factors involved in their *in vivo* differentiation are uncovered. Furthermore, identifying the location of these cells *in vivo* may aid in facilitating endogenous regeneration.

Reprogramming mature somatic cells has revolutionized the way we view regenerative medicine. Introducing the key factors Oct3/4, Sox2, c-Myc, and Klf4

demonstrated that adult fibroblasts can be reprogrammed into induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006). However, to differentiate iPS cells into pancreatic β-cells we still need to understand the factors required to induce this differentiation. Therefore, understanding the *in vivo* process of pancreatic β-cell regeneration is extremely valuable. Understanding this process will enable the differentiation of iPS cells to generate β-cells *in vitro* for autologous transplantation. Alternatively, exocrine cells have been reprogrammed to secrete insulin *in vivo* using Ngn3, Pdx1 and Mafa without reversion to a pluripotent stem cell state (Zhou et al., 2008). However, these reprogrammed β-cells do not form into islets, nor do they fully reverse diabetes. Consequently, this reprogramming approach may benefit from the activation of PSCs; hence, none of the therapeutic approaches are mutually exclusive.

5.3. The role of mesenchymal cells in pancreatic regeneration

Whether implanted or activated by periostin, mesenchymal cells have been shown to play a vital role in regeneration. Although the mechanisms by which mesenchymal cells facilitate regeneration have not been identified, several hypotheses can be made based on previous studies. Furthermore, it is likely that mesenchymal cells participate in multiple roles within the process of regeneration.

When pancreatic stellate cells are activated, they give rise to mesenchymal cells that secrete extracellular matrix (ECM) (Bachem et al., 1998). Therefore, introducing these cells into the pancreas or activating endogenous cells creates an ECM in which regeneration can take place. The importance of the ECM to regeneration was clearly identified in a recent study showing the ability of cells to rebuild an entire rat heart when

given only the matrix of a heart depleted of myocytes (Ott et al., 2008). Therefore, extending this observation we can hypothesize that generating the appropriate ECM in the pancreas would facilitate regeneration.

The formation of a mesenchymal stroma provides a physical scaffold for regeneration to take place. The use of silicone *in vivo* has demonstrated that creating a 'free space' promotes regeneration via tubular complexes in the pancreas (Kitamura et al., 2005). In addition, physically controlling the size and geometry of cells by changing the tissue microenvironment has been shown to alter cell fate (Geisse et al., 2009). Therefore, in this manner a mesenchymal stroma could alter the tissue microenvironment to facilitate pancreatic regeneration.

Mesenchymal cells are a known source of growth factors which could directly influence pancreatic regeneration. In pancreatic cancer, stromal cells have been shown to support cancer growth (Hwang et al., 2008). Therefore, in regeneration it is also likely that the stromal cells are a source for the secretion of growth factors. Moreover, fetal mesenchyme is important in pancreatic development. Implanting adult pancreatic ductal epithelium with fetal mesenchyme, demonstrated the ability of the mesenchyme to induce differentiation of the adult ductal cells into islet-like structures (Dudek and Lawrence, 1988). If mesenchyme alone or duct epithelium alone were implanted, there was no evidence of differentiation (Dudek et al., 1991). The fetal mesenchyme used in these studies might be comparable to the stroma created when PSCs are activated during regeneration. Interestingly, the cultured $Sca1^{+}CD31^{-}$ cells described in Chapter 2 express the gene *Isl-1* (data not shown), which was previously shown to be expressed in the fetal mesenchyme and is essential for embryonic development (Ahlgren et al., 1997).

5.4. Facultative stem cells within the pancreatic ductal cells

In vivo lineage tracing analysis has revealed that the cellular source of regeneration is likely found within the ducts (Inada et al., 2008). Knocking out *Pten* has suggested the cellular source in pancreatic cancer is specifically within centroacinar cells (Stanger et al., 2005). Although tubular complexes are not normally observed in the pancreas, they are a precursor in many forms of pancreatic regeneration (Lechene de la Porte et al., 1991; Wang et al., 2005) and cancer (Bockman, 1981; Bockman et al., 1978). Tubular complexes, first named based on their morphological description, can now be divided into Type 1 or Type 2 tubular complexes. Although, Type 2 was identified as the cellular source of regeneration both expressed Sca1. Therefore, during regeneration Sca1 does not discriminate between the two types of tubular complexes.

5.5. The contribution of Periostin to stromal formation

It has been shown that periostin stimulates mesenchymal cells to proliferate, which eventually produces a mesenchymal stroma. In addition, we have shown that the mesenchymal cells themselves produce periostin. Therefore, periostin likely works in an autocrine manner to sustain proliferation and activation. The factors initiating PSCs to produce periostin following pancreatectomy have yet to be determined. One likely candidate is Transforming growth factor-β1 (TGF-β1), as it has already been shown to induce *Postn* expression (Horiuchi et al., 1999). In addition, TGF-β1 contributes to the invasiveness of cancer cells by inducing an epithelial to mesenchymal transition (EMT) of cancer cells (Bates and Mercurio, 2005; Zavadil and Bottinger, 2005). EMT has been described in embryonic development, chronic fibrotic disorders, as well as cancer

progression (Thiery, 2003). In cancer, EMT is characterized as a reversible conversion of static epithelial cells into a highly motile fibroblast-like cell (Thiery and Sleeman, 2006). At the molecular level, EMT is marked by a decrease in cell-cell adhesion molecules such as E-cadherin and a down regulation of epithelial differentiation markers typical of that tissue (Fujimoto et al., 2001; Takano et al., 2007). At the same time mesenchymal markers such as vimentin and fibronectin are expressed (Bates and Mercurio, 2005). The end result is a cell phenotype that is more motile and able to spread into surrounding tissue, thus making treatment difficult. TGF-β1 could have the same effect on PSCs and enhancing their migratory ability. In addition, TGF-β1 could be activating periostin to facilitate this process (see Figure 1).

5.6. Conclusions

Activation of resident mesenchymal cells within the pancreas is necessary and sufficient for pancreatic regeneration. The formation of a mesenchymal stroma could be a common denominator among the many forms of pancreatic regeneration previously described. By controlling the mesenchymal stroma, pancreatic regeneration can be controlled. Elucidating the factors such as Wnts, BMPs or Activins known to be important during development, and provided by this mesenchymal stroma, could result in targeting regeneration more efficiently, or specifically to the β-cell lineage. The production of a mesenchymal stroma to induce endogenous pancreatic regeneration is a novel approach towards the treatment of diseases such as diabetes.

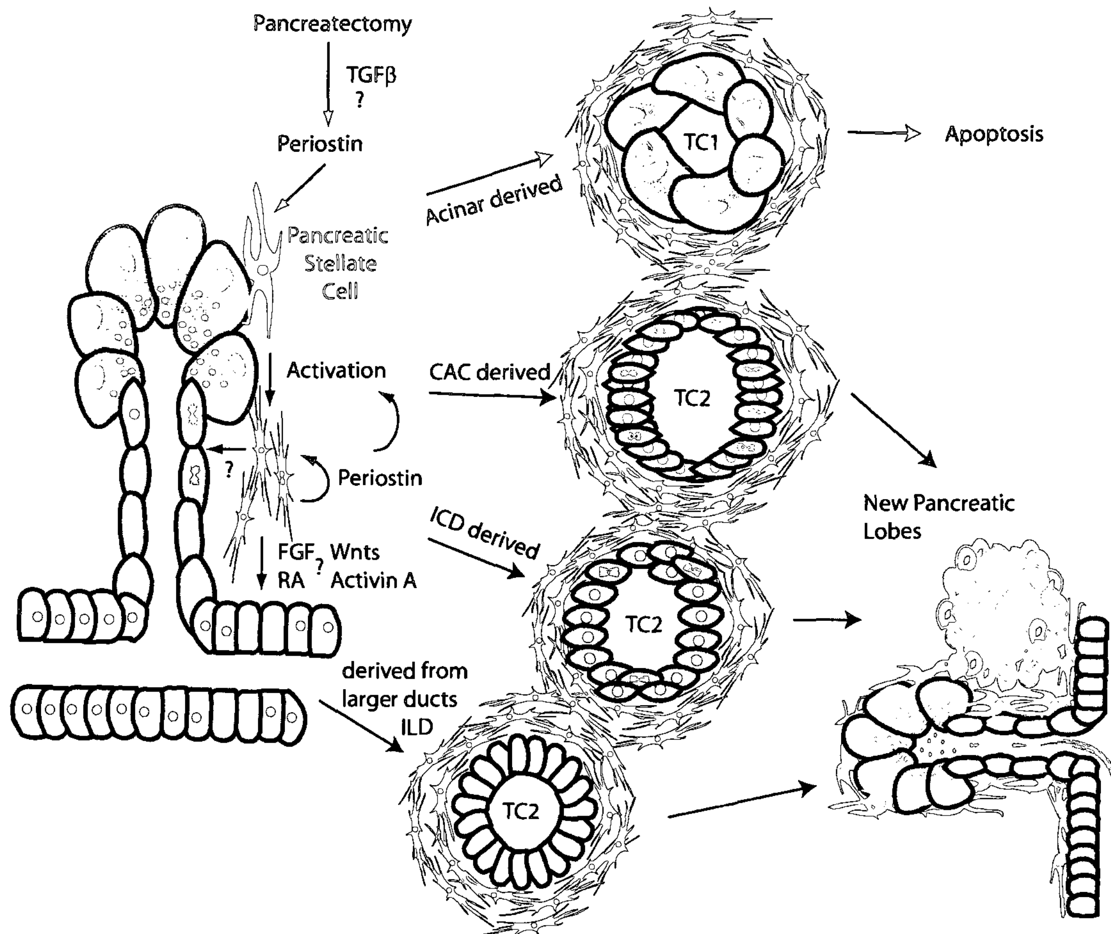

Figure 1. Regulation of regeneration in the adult pancreas.

Partial pancreatectomies (PPx) or direct injection of Periostin leads to the activation of pancreatic stellate cells (PSCs) creating a mesenchymal stroma. Following PPx, TGFβ1 is a candidate responsible for inducing the expression of periostin and activating PSCs leading to the formation of a mesenchymal stroma. This stroma then results in the formation of tubular complexes. Type 1 tubular complexes (TC1) are formed by acinar cells. TC1 show little proliferation and have been shown to have minimal contribution to regeneration, and therefore are likely to undergo apoptosis later in the regeneration process. Type 2 tubular complexes (TC2) are derived from duct-like cells that do not express elastase. TC2 are potentially derived from centroacinar cells (CACs), intercalating (ICD) or intra (or inter) lobular (ILD) ductal cells. Tubular complexes are surrounded by a mesenchymal stroma secreting factors to promote the proliferation. These factors are unknown but may share similarities to factors involved in embryonic development such as FGFs, Wnts, Retinoic Acid (RA) or Activin A.

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Appendix A – Periostin delivery to reverse diabetes

SUMMARY

Previously we have shown that direct injection of Periostin into the pancreas induced the formation of immature islets in STZ-induced diabetic mice. However, these mice failed to survive long enough for blood sugar levels to be reversed. Therefore, given the therapeutic benefit of reversing diabetes we attempted several alternative methods of Periostin delivery into STZ-induced diabetic mice. In a less invasive approach we administered periostin by *intra peritoneal* (IP) injection. IP injections did not induce noticeable stromal formation, $Ngn3^+$ pancreatic precursors or tubular complex formation. However, there was a noticeable increase in the amount of GFP fluorescence observed when injected into MIPGFP mice. If Periostin was injected shortly after STZ treatment diabetes could be prevented. However, if periostin was injected after diabetes was established it could not be reversed. Previous experiments were performed using high doses of STZ (100 mg/kg body weight) administered every other day until mice had blood sugars greater than 600 mg/dL or 33.3 mmol/L. This severe hyperglycemia inhibits islet neogenesis; therefore, we used a lower dose of STZ (50 mg/kg body weight) with the aim of reducing hyperglycemia in STZ-induced diabetic mice. Low dose STZ only slightly reduced hyperglycemia but did extend the life of STZ-induced diabetic mice, however, diabetes was not reversed. Therefore, we used an alternative delivery approach of electroporating Periostin into the pancreas, which provided the ability to delivery the specific mouse periostin isoform over a prolonged period of time. Electroporation of periostin reversed diabetes when electroporated into STZ-induced diabetic mice with moderate hyperglycemia but did not reverse diabetes in mice with severe hyperglycemia.

INTRODUCTION

Given the therapeutic benefit to be gained by reversing diabetes, stimulating endogenous pancreatic regeneration is a potential approach for the replacement of lost pancreatic β-cells. Previously we have shown that Periostin can stimulate pancreatic stellate cells (PSCs) creating a stroma to induce islet neogenesis. However, these islets remained immature and did not produce enough insulin to lower blood sugars. This failure to mature may be due to the severe hyperglycemia exhibited by these mice which has been shown to inhibit islet maturation (Guz et al., 2002). These immature islets are therefore comparable to neonatal islets which have been shown to secrete less insulin and are unable to sense changes in extracellular glucose concentrations (Navarro-Tableros et al., 2007).

Diabetes can be induced in mice by targeting β-cells with toxins such as alloxan (Jacob, 1977) or streptozotocin (STZ) (Fernandes et al., 1997; Zhang et al., 2002). A low dose STZ administration, 50 mg/kg body weight, in neonatal male rats induces enough damage that results in diabetes. However, seventy percent of these male rats spontaneously recover from this diabetes after 15 weeks (Hartmann et al., 1989). In adult mice the same low dose induces diabetes with moderate glucose levels; however, recovery from diabetes is rare. At higher levels of STZ (100 mg/kg body weight) mice have severe hyperglycemia and a shortened lifespan.

Electroporation has successfully delivered plasmid DNA into cells of mice *in vivo* (Titomirov et al., 1991). The electroporation method provides a means of delivering a protein (via plasmid DNA) over a longer period of time in comparison to a single

injection of a recombinant protein. In addition, electroporation enables the expression of a specific isoform to be expressed, such as that which was expressed during regeneration. Therefore, the electroporation of periostin into the pancreas has several benefits over the direct injection of the recombinant protein.

Periostin induced regeneration was shown to be independent of diabetic status. These results suggest that administration of periostin directly into the pancreas induces endocrine regeneration, creating a novel treatment of diabetes. However, direct injection of the recombinant protein is an invasive approach to drug delivery and failed to fully reverse diabetes. Therefore, we explored alternative approaches of delivering periostin by *intra peritoneal* or *intravenous* injections or electroporation directly into the pancreas.

RESULTS

A less invasive *intra peritoneal* (IP) injection of Periostin

To determine if periostin could be administered in a less invasive approach but still induce regeneration the recombinant protein was administered by *intra peritoneal* (IP) injection as opposed to being directly injected into the pancreas as done previously. 24 hours following the Periostin IP injection of 500 ng, 1.5 μg, 2 μg or 3 μg there was not the noticeable proliferation of pancreatic stellate cells that was observed after direct injection. Similarly there was not an accumulation of stroma and tubular complex formation after 3 days. However, one week following periostin IP injection there appeared to be an increase in the amount of GFP fluorescence under the mouse insulin promoter, MIPGFP (**Fig. 1a**). Quantification by FACS analysis revealed nearly a two fold increase in the total number of GFP expressing pancreatic β-cells. In addition, increased uptake of BrdU (red) was observed in large islets of Periostin injected pancreata (**Fig. 1b**). The increase in BrdU uptake correlated with increased proliferation within islets as shown by Ki67 staining (**Fig. 1c**), compared to saline injected controls (**Fig. 1d**). To determine if periostin could prevent STZ induced diabetes it was injected shortly following STZ injection (**Fig. 2**). Eleven of fifteen sets of mice were able to maintain normal blood sugar levels when injected with STZ and Periostin, while littermate controls given STZ alone became diabetic (**Fig. 2,3**). Plotting the dosage of periostin given per kg body weight revealed the experiments using 50-70 μg/kg were most successful (**Fig. 3**). However, this dosage of periostin could not reverse diabetes when given to mice once they were already diabetic (n=6) nor could delivery of periostin by *intra venous* injection (n=6).

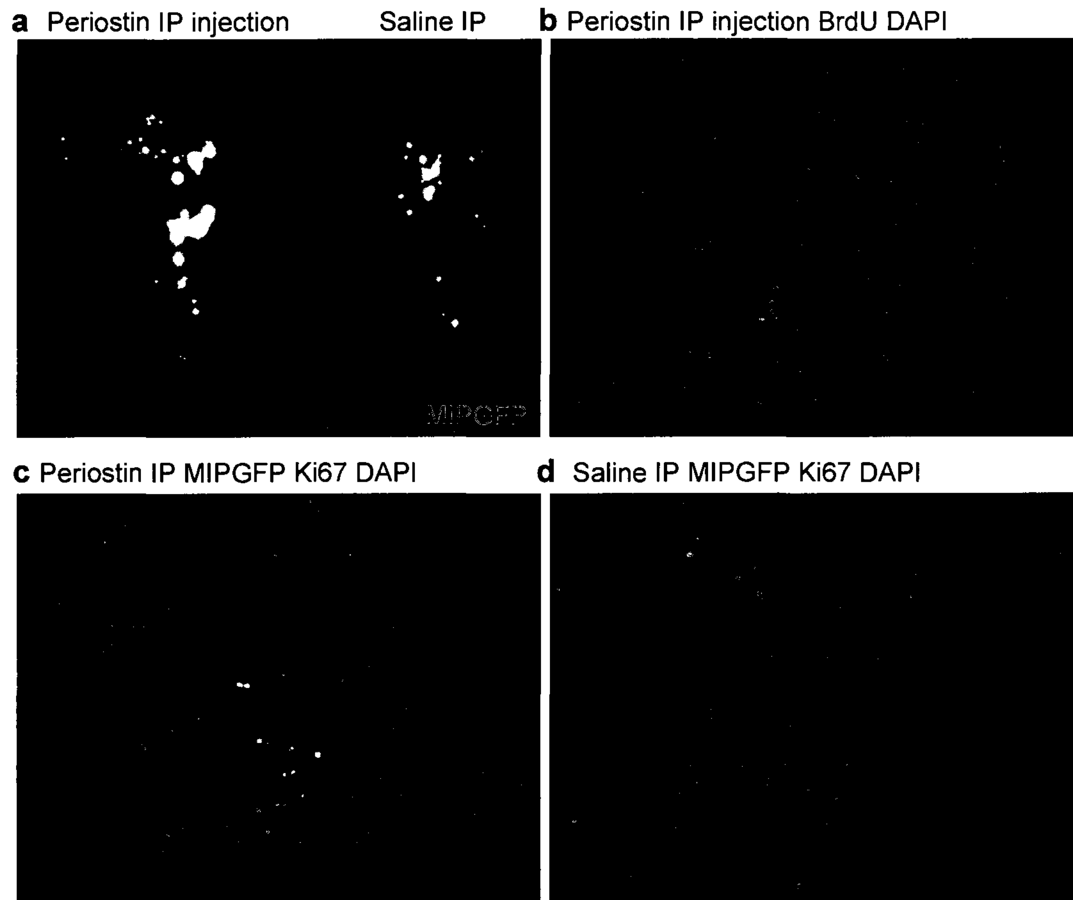

Figure 1. Proliferation is increased in islets following Periostin IP injection.
a) One week following Periostin IP injection there was an increase in the area of the pancreas expressing GFP under the insulin promoter (MIPGFP). The complete periostin injected pancreas (left) was compared immediately following removal to the saline injected control (right) using a dissection microscope equipped with fluorescence.
b) Increased uptake of BrdU (red) was observed in islets of Periostin injected pancreata.
c) The increase in BrdU uptake correlated with an increase in proliferation revealed by Ki67 expression (red) observed within islets (MIPGFP; green) three days following periostin injection relative to saline injected controls (d).

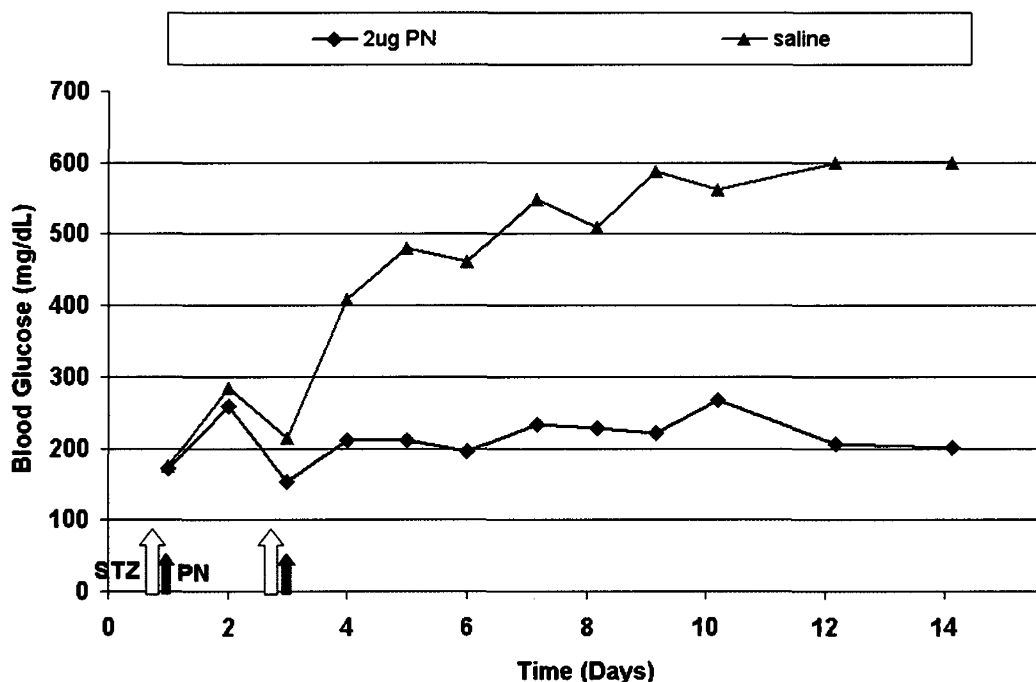

Figure 2. Example of single experiment performed to test the effect of periostin. One of fifteen experiments conducted to determine the effect IP injections of Periostin had on blood glucose following high dosage treatment of STZ treatment (100 mg/kg). A single IP injection of periostin (2 μg) or saline was administered 3 hours following STZ treatment on the 1st and 3rd day. By the 5th day blood glucose in the saline control was greater than 400 mg/dL and therefore a third treatment of STZ was not given. Blood glucose was greater than 400 mg/dL after two weeks in the saline control but remained within the normal range in the periostin treated animal. Periostin injections successfully kept mice within a normal blood glucose range in 11 of 15 experiments while the saline controls became diabetic (see Figure 3).

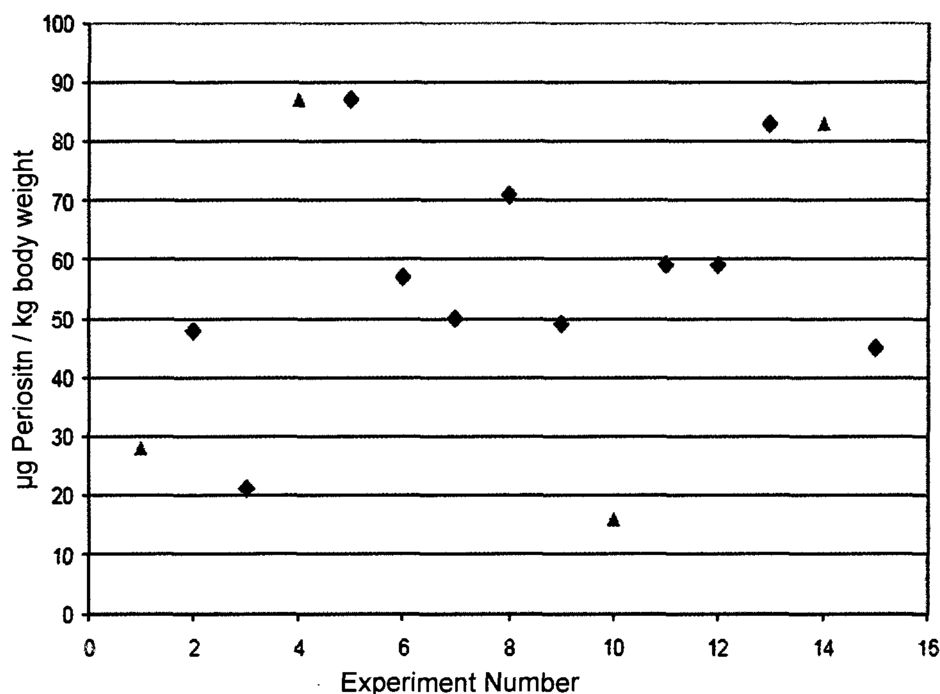

Figure 3. Summary of Periostin IP trial to prevent STZ-induced diabetes in mice. Eleven of fifteen animals were able to maintain normal blood sugar levels when injected with Periostin following a high dosage STZ treatment (100 mg/kg) compared to saline following STZ injected littermate controls that became diabetic. These mice are represented by a green diamond while red triangles represent experiments where periostin injected mice became diabetic. Plotting experiments based on the amount of periostin given per kg of body weight reveals a nominal dosage around 55µg Periostin / kg body weight.

Direct injection of Periostin into low dose STZ-induced mice

Previous experiments to induce diabetes in mice relied on high dosages of STZ (2 or more doses of 100 mg/kg). This high dosage resulted in the complete destruction of pancreatic β-cells and blood glucose levels higher then 33.3 mmol/L. These mice could only survive one month before they had to be euthanized due to high levels of morbidity. Low dosage STZ treatment (50 mg/kg daily injections for 5 days) has been shown to create diabetic mice that have moderately high blood glucose levels, reduced morbidity and can live longer in a diabetic state. Therefore, we attempted to reverse diabetes in mice that had low dosage treatment of STZ using a direct injection of Periostin. In diabetic mice with moderately high blood glucose levels we injected saline (n=3) or 100 ng of Periostin (n=3). Although mice survived longer blood sugars remained high in both treatment groups (**Fig. 4**). Immunohistochemistry revealed small islets that had not fully developed even after 8 weeks. Similarly, directly injecting 10 ng, 500 ng, 1 μg or 10 μg of Periostin could not reverse diabetes (data not shown).

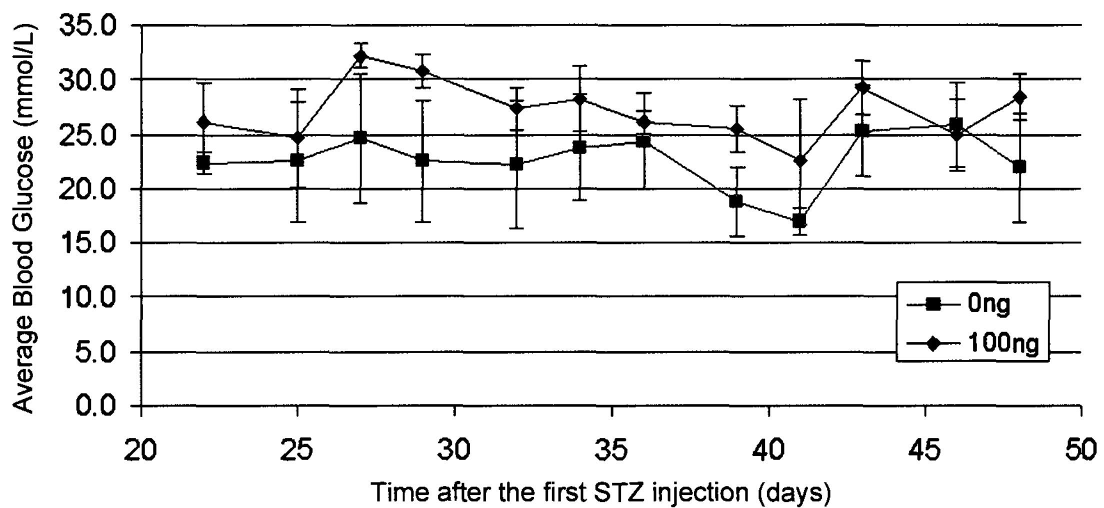

Figure 4. Blood glucose in low dose STZ-diabetic mice injected with Periostin. Periostin was directly injected into the pancreas 24 days after the first STZ injection. 48 days after direct injection of Periostin blood glucose remained high with 100 ng periostin injection (n=3) or without (n=3). Data given as mean ± SEM.

Electroporation of Periostin into the Pancreas

Electroporation provides a means of genetically delivering a protein over a longer period of time then can be done with a single injection of a recombinant protein. In addition, electroporation enables the expression of a specific isoform to be expressed, such as that which was expressed during regeneration. Therefore, plasmid DNA containing the periostin isoform expressed during pancreatic regeneration under control of the cmv promoter was electroporated directly into the pancreas (n=3). As a negative control saline was used in a mock electroporation (n=3), however, one died within 24hrs from complications to the surgical procedure leaving only two negative controls. The STZ-induced diabetic mice had much lower starting blood glucose levels compared to previous experiments. However, after two months blood glucose levels were lower in periostin electroporated pancreata relative to saline controls (**Fig. 5**).

Six weeks after electroporation of periostin into STZ-induced diabetics, mice were challenged in a glucose tolerance test. Mice electroporated with the pancreatic isoform of Periostin (n=3) showed a faster glucose response time relative to the saline controls (n=2) (**Fig. 6**). However, the recover period was extended beyond the two hours in which time mice would normally return to baseline blood glucose levels.

Due to the previous success, the electroporation experiment was repeated with a larger group of mice. Eight weeks following periostin electroporation the blood sugars of low dose STZ-diabetic mice remained high (n=7). In fact, blood sugar levels remained higher than the saline controls (n=6) following the electroporation (**Fig.7**). However, starting blood glucose levels were higher than what was observed in the previous electroporation experiment.

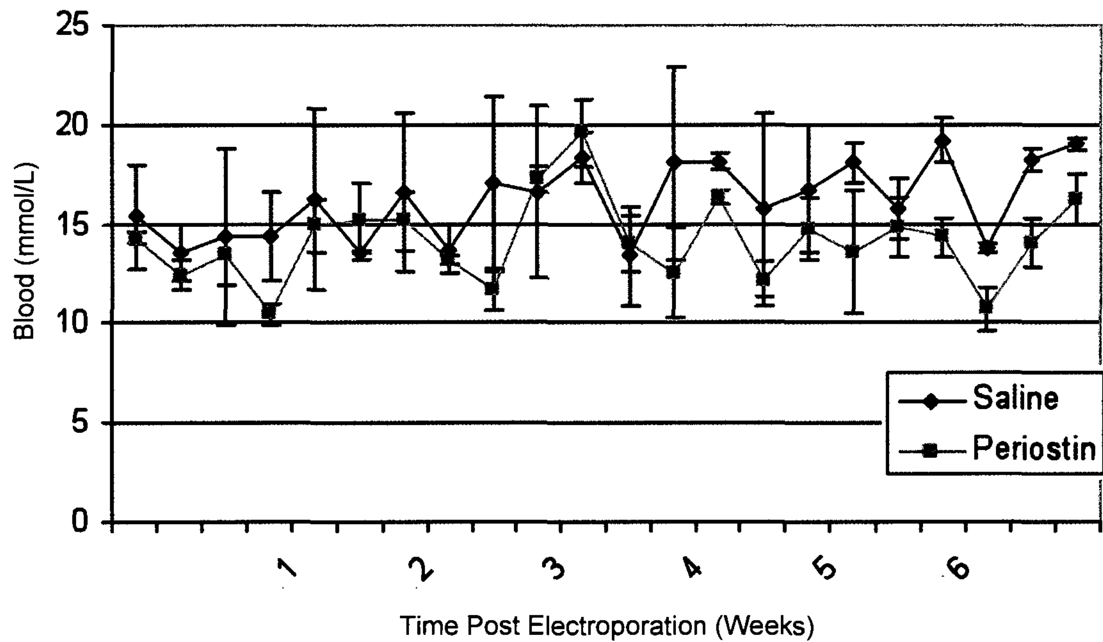

Figure 5. Blood glucose levels in low dose STZ diabetic mice.

At six weeks mice electroporated with periostin (n=3) have lower blood glucose levels than the saline controls (n=2). Data given as mean ± SEM.

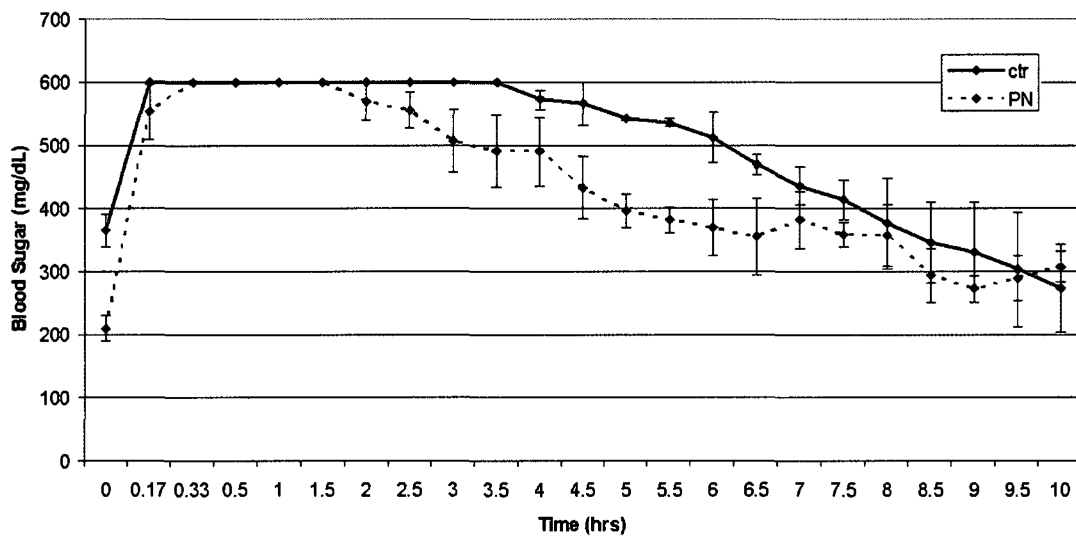

Figure 6. Glucose tolerance test of Periostin vs. Saline electroporated mice. Six weeks after electroporation into STZ-diabetic mice were challenged by direct injection of saline. Mice that were electroporated with the pancreatic isoform of Periostin (n=3) showed a faster glucose response relative to saline controls (n=2). However, the response to glucose was slower than normal. Data given as mean ± SEM.

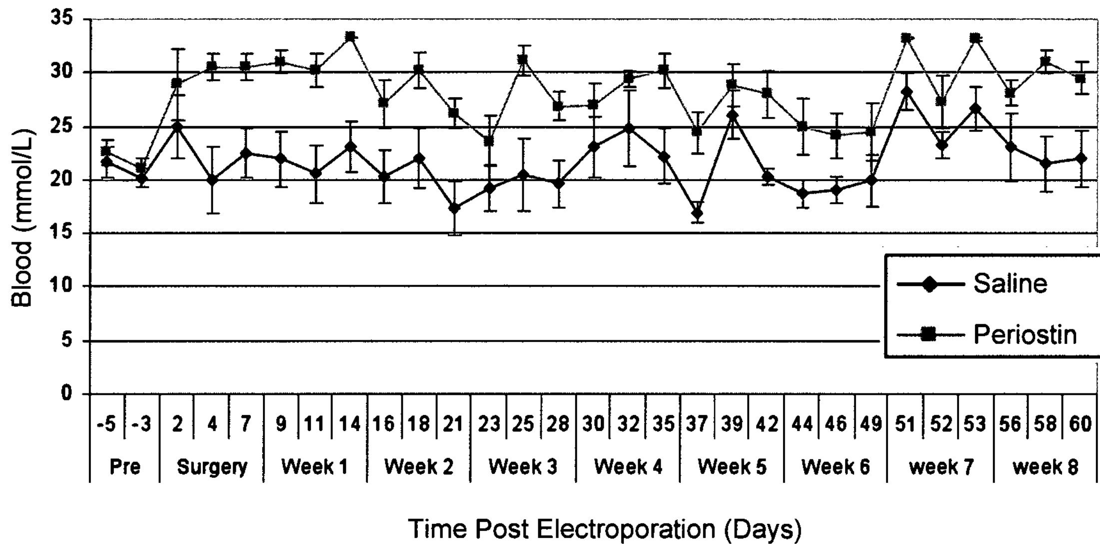

Figure 7. Blood glucose levels in STZ diabetic mice following electroporation. Eight weeks following electroporation into low dose STZ-diabetic mice, mice electroporated with periostin (n=7) have higher blood glucose levels than the saline controls (n=6). Data given as mean ± SEM.

DISCUSSION

The ability of periostin to induce regeneration is dependant on the method by which it is introduced into the pancreas and the degree to which diabetes is established in mice. An *intra peritoneal* (IP) injection of periostin has been shown to increase β-cell mass but may not result in β-cell neogenesis. Unlike directly injecting periostin into the pancreas an IP injection shows no signs of regeneration such as tubular complex formation or Ngn3 expression.

Direct injection of periostin induces pancreatic stellate cells (PSCs) to proliferate resulting in an accumulation of stroma for the induction of tubular complex formation. The regeneration process is then clearly visible when tubular complexes are expressing progenitor cell markers Pdx1 and Ngn3. Because of the widespread proliferation of stellate cells and only the localized areas of regeneration induced by direct injection it is likely that stellate cells migrate through the pancreas and collect in areas which eventually induce a localized regeneration. What causes PSCs to migrate to a particular location is unknown. This could be another difference between direct and IP injection. While in IP injection there is no direct contact with the pancreas a direct injection may induce minor damage in the pancreas and stellate cells stimulated by periostin may then migrate to this damaged area. Minor epithelial disruption in saline injected pancreata has been observed after sectioning through the complete pancreas to find the site of injection. However, unlike periostin injected mice, the saline injected controls do not exhibit the widespread activation of pancreatic stellate cells, the subsequent stromal formation nor the initiation of regeneration.

Although no clear activation of PSCs cells was observed after an IP injection of Periostin. It is possible that Periostin activates a small number of PSCs to migrate to islets and induce the proliferation of endogenous β-cells. The induction of proliferation by bone marrow and/or a mesenchymal helper cell has been previously suggested (Hess et al., 2003; Ohtake et al., 2008; Urban et al., 2008). A direct effect of Periostin on pancreatic β-cells is possible; however, no effect was observed when FACS purified pancreatic β-cells were cultured with or without Periostin (data not shown).

Although periostin IP was able to prevent STZ-induced diabetes, the inability to rescue mice once diabetes is established (even at the highest dosage levels of periostin given) suggests that β-cells must be present for an IP injection of periostin to increase β-cell mass. However, unlike an IP injection, direct injection of periostin has been shown to induce full regeneration with tubular complex formation containing $Ngn3^+$ pancreatic progenitors. Therefore, it is to be expected that a direct injection of periostin would be required as a therapeutic approach for the treatment of diabetes in which all β-cells have been lost.

The degree of hyperglycemia also plays a role when periostin is delivered directly to the pancreas. High levels of hyperglycemia inhibit islet neogenesis and the maturation of islets (Guz et al., 2002). This is evident when comparing the first and second electroporation experiments. In the first electroporation experiment the average starting blood glucose level was 15 mmol/L and in the second electroporation the average starting blood glucose level was 22 mmol/L. In addition, in the second experiment the blood glucose levels of the periostin electroporated mice quickly climbed higher than 30 mmol/L and at these high levels of hyperglycemia islet neogenesis could be inhibited.

Therefore, the severity of hyperglycemia is an important factor to consider when inducing endogenous pancreatic regeneration.

EXPERIMENTAL PROCEDURES

Experimental animals and surgical model. Eight- to nine-week-old male C57BL/6J mice and periostin null mice were housed under standard conditions and allowed free access to standard mouse chow and water. All studies were approved by the Animal Care Committee at the University of Ottawa. One hour before surgery mice were given a dosage of 0.05 mg/kg Buprenorphine subcutaneously. Anesthesia was induced in an anesthetic box with isoflurane gradually increased to 5%. The anesthetic was delivered by a Ohio Forane vaporizer (induction box) and a isoflurane vaporizer (mask). Once the mice were anesthetized they were transferred to a face mask with isoflurane at 1.5% where they are maintained throughout surgery, increasing or decreasing the percentage as necessary to keep the animal sedated but breathing normally. The surgical area was shaved and cleaned with Endure soap, rinsed with sterile water and surgically prepared with chlorahexseptic solution. BNP eye ointment was placed in the animals eyes to protect them from drying. 1 ml of sterile saline was administered subcutaneously prior to surgery. Once the surgery is complete mice are placed on oxygen until they recover and start to move.

Periostin injections. Periostin (BioVendor LLC, Candler, NC) was administered by direct (10 ng, 100 ng, 500 ng, 1 μg or 10 μg) or *intra peritoneal* (500 ng, 1.5 μg, 2 μg or 3 μg) injection. Direct injection was performed by exposing the pancreas with a midline incision and injecting 10ul recombinant periostin solution, in the appropriate concentration, directly into the pancreas with a Hamilton syringe. Vehicle-treated animals received the same amounts of buffer diluted into saline.

Streptozotocin induced Diabetes. Streptozotocin (Sigma), which selectively targets and destroys pancreatic β-cells to create diabetes, was injected into adult mice. More specifically, in high dose experiments one IP injection of 100 mg/kg of Streptozotocin (STZ) was given every other day until the mouse became diabetic as described previously in C57BL/6J mice. Following the first STZ injection (Day 0) blood sugar levels were taken daily to determine the diabetic status of mice. In an alternative protocol to improve the health and reduce the severity of hyperglycemia a lower dose was administered by IP injection of 50 mg/kg for 5 days into C57BL/6J mice.

Immunohistochemistry. Pancreata were embedded in optimal cutting temperature compound (Tissue-Tek) : 20% sucrose solution (2:1) and frozen by emersion into 95% ethanol/dry ice. Cryosections (8um) were stained with the msIgG1 anti-Ki67 antibody (BD Bioscience; 550609). BrdU detection was performed by following the protocol outlined in the BrdU in situ detection kit (BD Biosciences). The secondary antibodies used were Alexa488 and Alexa568 anti-mouse IgG1 (Invitrogen). Nuclei were counter-stained with DAPI.

Electroporation of Periostin into the pancreas. STZ treated mice were anaesthetized with isoflurane as previously described. The pancreas was exposed via midline incision into the abdomen and 2-needle array electrodes (Fisher; cat#BTX533) were inserted into the splenic lobe of the pancreas with as little manipulation of the pancreas as possible. Immediately following insertion of the electrodes 50 μl of periostin plasmid DNA (1 μg/μl) in saline or saline alone was directly injected into the pancreas between the electrodes. Immediately following injection of plasmid DNA electric stimulation was performed using six 100vpulses 20ms in length at an interval of 200ms. The pancreas

was removed from the electrodes and the abdominal wall was sutured. The skin closed with wound clips.

Glucose Tolerance Test (GTT). Mice were fasted overnight (16-18 hours) with free access to drinking water prior to the GTT. A 75 mg/ml glucose stock solution was prepared by dissolving 0.75 g of D-glucose, anhydrous in 10 ml of sterile, distilled water. The solution was sterilized by passing it through a 0.2um filter. Prior to the GTT, body weight and baseline glucose levels were measured. Mice were challenged with 1.5 mg glucose/gram body weight. Blood glucose was determined after 10, 20 and after ever 30 minutes up to 10 hours.

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