

# Differentiation of Pancreatic Ductal Epithelial Cells into Insulin Like Cell Clusters in Chronic Pancreatitis

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## Abstract

**Background/Aim:** Islet regeneration in chronic pancreatitis (CP) is relevant for managing the associated loss of endocrine function. Because ductal epithelial cells were earlier demonstrated to differentiate into pancreatic endocrine mass, we evaluated their proliferation and differentiation in chronic pancreatitis. **Methods:** Pancreatic ducts were obtained from surgically resected pancreata of 12 patients with chronic pancreatitis and 15 control subjects. CK19 positive ductal cells were evaluated for their proliferating and differentiating abilities upon immunostaining with Ki 67 and hormone positivity for insulin and glucagon, apart from monitoring Pdx 1 expression. **Results:** In comparison to the controls, a greater number of proliferating pancreatic ductal epithelial cells (PDECs) were observed under conditions of CP. The increase in Pdx1 expressing PDECs (22%) and proliferating Pdx1 expressing PDECs (30%) was significant ( $P < 0.04$ ). Number of cells expressing insulin/glucagon in the exocrine ducts increased significantly in CP as compared to controls ( $P < 0.02$ ) and  $\beta$  cell mass adjacent to the ducts increased by 28%. **Conclusion:** Enhanced capability of PDECs to proliferate and differentiate into endocrine mass suggests that PDECs form a source of progenitors for cell based therapy in chronic pancreatitis.

## Keywords

Chronic Pancreatitis, Ductal Epithelial Cells, Islet Like Clusters, Cell Differentiation

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## 1. Introduction

Chronic pancreatitis (CP) is an inflammatory disease of multifactorial etiology resulting in progressive and irre-

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versible destruction of exocrine pancreas, followed by endocrine dysfunction and replacement of exocrine tissue with fibrous tissue leading to clinical manifestations typical of “end-stage” disorder of pancreatic function [1]. Reduction of pancreatic volume and  $\beta$  cell area in CP [2] along with enhanced destruction of islets necessitates exogenous insulin for maintenance of glucose homeostasis [3]. Since autologous islet cell transplantation after total pancreatectomy (TPIAT) is reported to improve glycemic status in CP patients [4], cell based therapy appears to be a viable option for treatment of diabetes. The possible sources of islets/progenitors for cell-based therapy in the treatment of diabetes include embryonic and mesenchymal stem cells [5] [6], induced pluripotent stem cells [7], intra-islet nestin positive cells [8], intra-pancreatic progenitors [9]-[11] and extra-pancreatic cells of endodermal origin [12]. While all these cells regenerate into islets by neogenesis, lineage tracing experiments denote that self replication of pre-existing islets is a major source of restoration of  $\beta$ -cell mass [13]-[15].

Among various islet progenitors, the ductal epithelial cells of adult pancreas were reported to regress [16], maintain plasticity [17] and were hypothesized to form a source of progenitors for normal renewal process. A recent study demonstrated that  $\beta$ -cell progenitors, located in the ductal lining, could be activated in adult mouse pancreas and differentiation of these cells into glucose responsive  $\beta$  cells was shown to be Ngn3 (neurogenin 3) dependent [18]. Upon observing transient expression of Pdx-1 both in pancreatic ductal cells of mouse and in regenerating pancreas of rats after partial pancreatectomy [19] [20], Bonner-Weir had conducted studies to demonstrate that purified ductal cells from adult human pancreas (PDEC) can differentiate to insulin-producing cells (IPC) [21]. Interestingly, yet another study demonstrated that Cdk4 (Cyclin dependent kinase 4) regulates recruitment of ductal epithelial progenitors to reconstitute  $\beta$  cell mass [22]. However, all of such studies were conducted employing either animal models or ductal cells cultured under controlled conditions. Existence of pancreatic progenitors/facultative stem cells is yet to be tested and confirmed under normal physiological conditions. Further, it is not clear as to whether differentiation of ductal cells into endocrine mass occurs under conditions of CP. Thus, the present study was conducted to examine proliferation of ductal epithelial cells and their differentiation into hormone positive cells by immunohistochemistry. Obtaining insights into such events in CP and comparing the data with controls would yield valuable information not only for the management of CP but also towards 1) cell based therapy in the treatment of diabetes and for 2) employing ductal cells as progenitors of islets for autologous transplantation in patients who need to undergo partial/total pancreatectomy.

## 2. Materials and Methods

**Patients:** Pancreatic tissues were obtained from surgical wards of Asian Institute of Gastroenterology, Hyderabad, India during 2012-2013 for this study upon pancreaticoduodenectomy/partial pancreatectomy/distal pancreatectomy/lateral pancreaticojejunostomy. Informed consent was obtained from patients and study protocols were approved by Institutional Review Board. Since it is not feasible to obtain pancreatic tissue from healthy controls, patients who had undergone partial pancreatectomy for pancreatic adenoma or extra pancreatic tumors without history of any CP formed the control group (n = 15, 12 males, 3 females). Pancreatic tissue 2 cm away from the benign tumor was considered to be the control tissue. Patients diagnosed for early stage CP based on clinical, biochemical as well as radiological investigations and confirmed by histopathological examination formed the study group (n = 12, 10 males, 2 females). All the patients were in the early stage of the disease those with pancreatic malignancies and endocrine tumors were excluded.

**Resection of ducts:** Resected pancreatic tissues (1 - 3 g) were collected in RPMI 1640 nutrient medium (Sigma Chemicals, St. Louis, MO, USA) and processed immediately. Ductal regions were dissected out from the pancreatic specimens, cleared of the adjacent tissue and washed thrice with RPMI 1640 nutrient medium. Crude ducts and pancreatic tissues were fixed in 40% formaldehyde and embedded in paraffin blocks. The paraffin blocks were then sectioned (~0.5  $\mu$ m) and immunostained for CK19 (ductal marker), Ki67 (proliferation marker), Pdx1 (transcription factor), insulin and glucagon.

**Immunohistochemistry:** Paraffin-embedded blocks of crude ducts and ductal regions of the pancreatic tissues were sectioned (1  $\times$  1 cm) and immunostained as per the protocol of Hagman *et al.* [23] with minor modifications. Tissue sections (~0.5  $\mu$ m) were mounted and antigen retrieval was conducted using 10 mM sodium citrate at 95°C for 21 seconds. After cooling the sections to room temperature, they were washed twice with PBS, blocked with 5% FBS for 1 h at room temperature and then incubated overnight at 4°C with primary antibodies rabbit anti-PDX-1 at 1:100 dilution; guinea pig anti-insulin at 1:200 dilution; Sigma Aldrich, (mouse anti CK19, guinea pig antiKi67, mouse anti-PDX-1 at 1:100 dilution; guinea pig anti-insulin at 1:200 dilution; Sigma, St.

Louis, USA), mouse anti glucagon antibodies followed by incubation with secondary antibodies (goat anti mouse Alexa 546 for PDX-1, glucagon, CK19, goat anti-guinea pig Alexa 488 for insulin and Ki67; Invitrogen, CA, USA) for 1 h at room temperature. Nuclei were stained with 4', 6-diamidino-2-phenylindole. Fluorescence images were captured using a Bioimager using 40× objective (CARV II, BD BioSciences, CA, USA) using software (IP LAB; BD Bio Sciences, CA, USA). Cells expressing CK19, CK19 + Ki67 (proliferating DEC/progenitors), Pdx1, Pdx1 + Ki67, (proliferating Pdx1 positive cells), insulin and glucagon positive cells were counted.

Determination of number of cells expressing CK19, Pdx1, Ki67 and differentiating into hormone positive cells: Ducts were located based on their appearance, and presence of single layer of cuboidal epithelial cells surrounding a lumen upon staining with hematoxylin & eosin, and immunostaining for CK19. Ten ducts in 5 fields were counted to identify proliferating CK19 + cells, Pdx1 expressing cells and their ability to differentiate into hormone positive cells in the same regions. Each field comprised of 50  $\mu\text{m}$  for a field view (22 mm under 10× eye piece). Both CK19, Pdx1 positive cells were counted in the imaged pictures for co-expression of Ki67 to examine their proliferative ability and expressed as percentage of ductal cells that were proliferating, expressing Pdx1 and differentiating into hormone positive cells.

Statistical analysis: The results were subjected to ANOVA analysis using SPSS version 13.0 and expressed as mean  $\pm$  SD. P value < 0.05 was considered significant

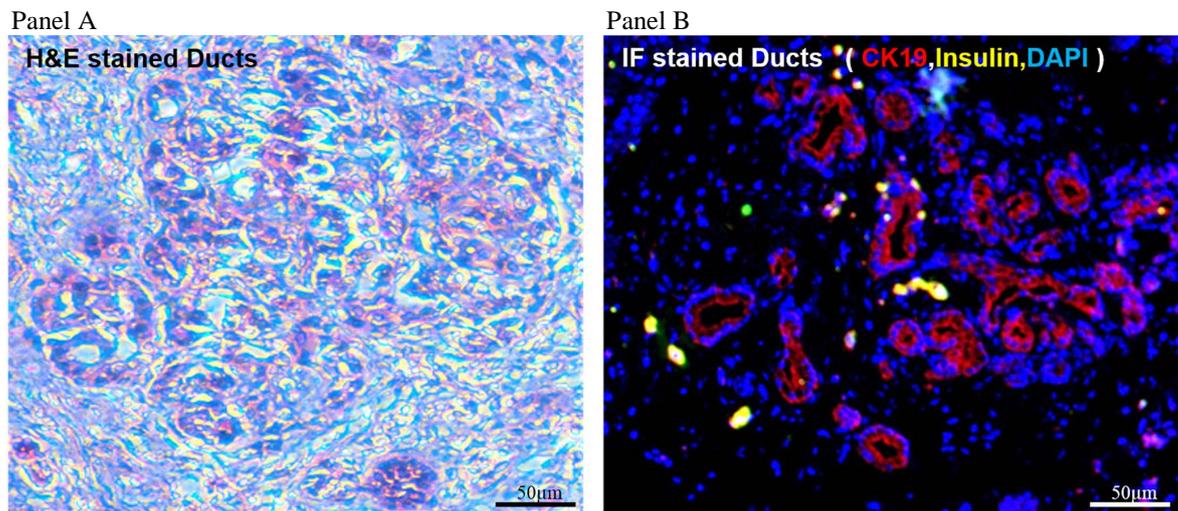
### 3. Results

Clinical and demographic details of the study groups are given in **Table 1**. Control group (n = 15; male: 12, age 45  $\pm$  15 years) were patients with complaints of abdominal pain, loss of appetite and vomiting. Computed tomography scans of this group revealed pancreatic mass and pancreatic tissue surgically resected from them had normal appearance with no ductal dilatations. Histopathology confirmed benign lesion (adenoma). Patients with CP (n = 12; male: 10, age 33  $\pm$  18.4 years) were experiencing debilitating, recurrent abdominal pain. Computed tomography scans of patients with CP revealed increased ductal wall echoes and ductal dilatations on endoscopic retrograde cholangiopancreatography (ERCP) and endoscopic ultrasonography (EUS). Surgical specimen obtained upon partial pancreatectomy from CP patients showed inflammation, calcifications, degenerative changes and fibrosis. Histopathology confirmed intra and inter lobular fibrosis consistent with chronic pancreatitis.

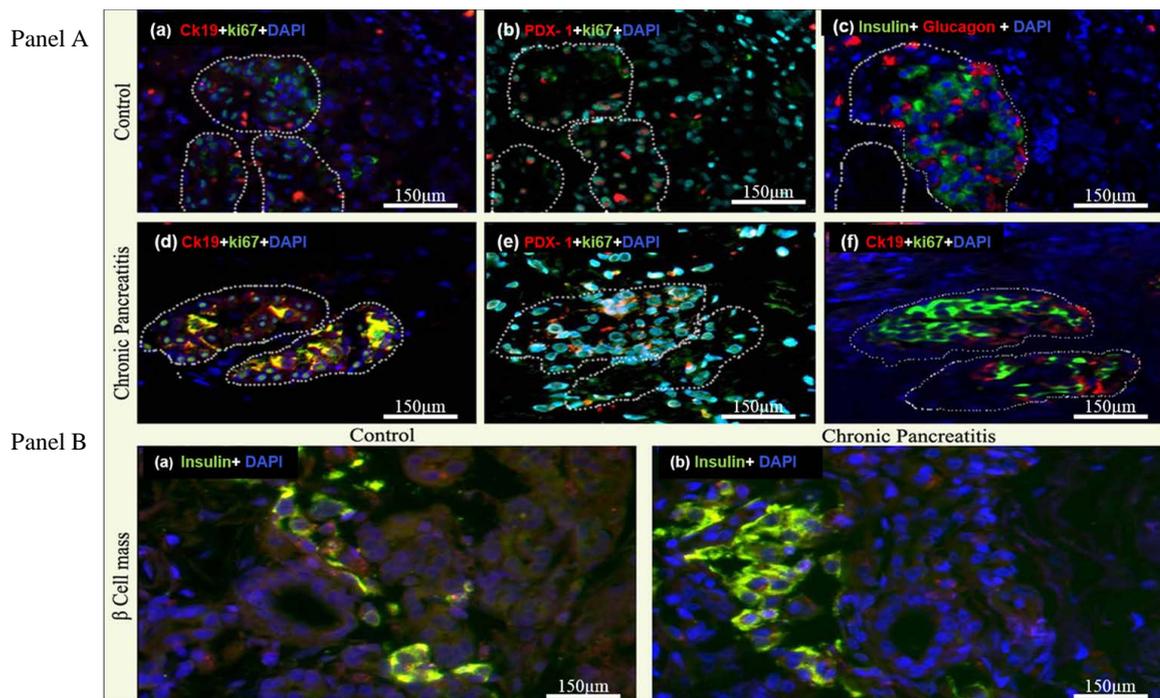
CK19, Pdx1 and Ki67 expression and  $\beta$  cell mass: Ducts could be located based on their appearance on hematoxylin and eosin staining as well as immunostaining for CK19 **Figure 1**. In each patient ten ducts were examined in 5 fields for proliferating ductal epithelial cells. In a field view of 10× magnification with a field number of 22 mm, 27  $\pm$  9 proliferating CK19 + PDECs were observed in control group and 31  $\pm$  10 in CP as shown in **Figure 2** (Panel A) & **Figure 3** (Panel A). In comparison to control group, the number of PDECs expressing pdx1 increased by 22% and Ki67 positive pdx1 expressing PDECs (proliferating pdx1 PDECs) increased by 30% in patients with CP as shown in **Figure 2**. The increase in Pdx1 expression and proliferating pdx1 PDECs was significant (P < 0.04) as shown in **Figure 3**. Insulin and glucagon positive cells also increased significantly (P < 0.02) in CP as shown in **Figure 2** (Panel B).  $\beta$ -cell mass measured as insulin positive area was also increased by 28% in CP as compared to control and the increase was significant (P < 0.05) as shown in **Figure 3**, Panel B.

**Table 1.** Patient demographics.

	Control subjects n = 15	Chronic pancreatitis patients n = 12
Age (Y)	45 $\pm$ 15.7	33 $\pm$ 18.4
Sex (M/F)	12/3	10/2
Diabetes (yes/no)	1/15	3/12
Fasting glucose (mg/dl)	113 $\pm$ 19	105 $\pm$ 36
Ductal morphology	No ductal dilatation	Noted ductal dilatation



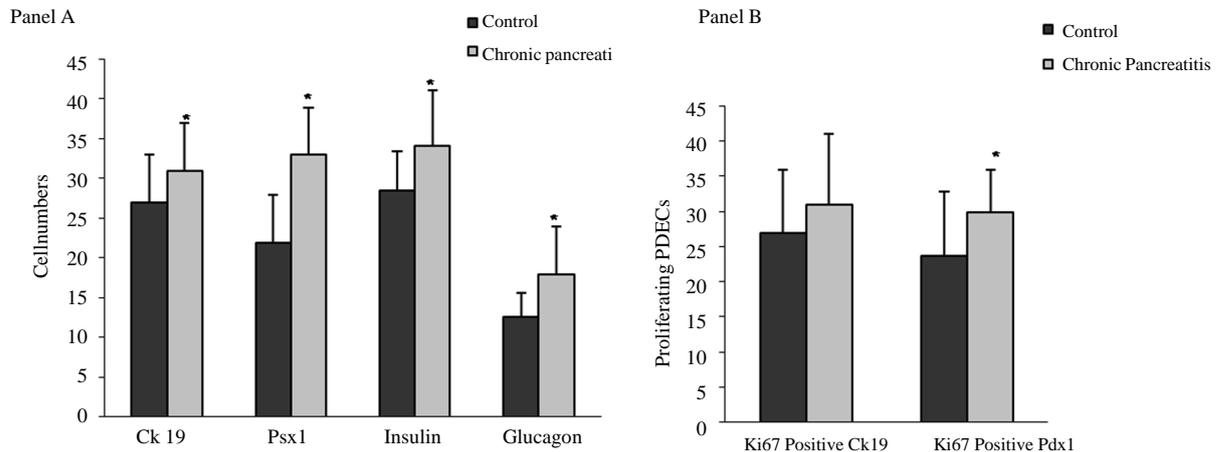
**Figure 1.** Paraffin embedded pancreatic tissues from control subjects. Panel A: Shows purple color ductal epithelial cells in H&E staining. Panel B: Shows immunofluorescent (IF) study of same tissue were red color indicate CK19 positivity, greenish yellow color indicates Insulin positive cells. Blue color represent cell nuclei (DAPI).



**Figure 2.** Conversion of epithelial cells into insulin producing cells is higher in CP patients than controls. Panel A: (a) and (d) shows CK19 and Ki67 positive cells is control and CP patients (b) and (e) shows Pdx1 and Ki67 positive cells is control and CP patients, (c) and (f) shows insulin and Glucagon positive cells is control and CP patients respectively. Panel B: Proliferation of Insulin producing cells higher in CP patients than controls (a) and (b) shows immunostaining of pancreatic tissues demonstrating increased  $\beta$  cell mass adjacent to ducts in CP than the controls.

#### 4. Discussion

The present study was conducted primarily to monitor the proliferation and differentiation of ductal epithelial cells in chronic pancreatitis. The obtained results showed increased proliferation and differentiation of PDECs to hormone positive cells indicating that regenerative ability of ductal epithelium is retained in chronic pancreatitis and that regeneration of endocrine mass may indeed originate in the ductal epithelium.



**Figure 3.** Panel A: Quantitative representation of CK19, Pdx1, Insulin and Glucagon cells. Quantitative representation of number of proliferating CK19, pdx1, insulin and glucagon positive cells. Significant increase is seen in proliferating Pdx1 positive cells and insulin, glucagon positive cells. \* indicates  $P < 0.05$ . Panel B: Quantitative representation of CK19 and Pdx1 expressing cells positive for Ki67. Quantitative representation of number of proliferating pancreatic ductal epithelial cells positive for Ki67 and expressing CK19 and Pdx1. Significant increase was observed in Ki67 positive Pdx1 expressing cells. \* indicates  $P < 0.05$ .

Ducts obtained from resected pancreatic tissues did not vary in the numbers in between control subjects and patients with CP. Interesting finding in the present study is that, though the number of proliferating (Ki67 + ve) PDECs did not show significant increase there was a significant increase in the number of Pdx1 + ve cells that co express Ki67 ( $P < 0.04$ ) in CP patients indicating that endocrine mass does regenerate from ductal epithelium. This result is in agreement with earlier reports which noted the presence of higher number of endocrine cells in the exocrine ducts and suggested that endocrine islets in patients with CP may be increasingly regenerated from ductal epithelium [24]. It was also reported earlier that purified ductal epithelial cells do proliferate and differentiate into insulin positive cells [21]. On the contrary, Shradder *et al.*, did not find any difference in the percentage of Ki67 positive cells and insulin expression in exocrine ducts which they explained could be due to lack of use of ductal marker (2). In continuation of these observations, the present study documents increased number of Pdx-1 expressing CK19 positive PDECs from CP patients having differentiation ability to restore the endocrine mass in CP for the first time. The observed increase in proliferation of Pdx1 positive cells may be occurring to compensate the loss of endocrine mass and decreased number of islets in chronic pancreatitis. Significant increase in the number of PDECs with simultaneous expression of Pdx1 and CK19 in the same location within the ducts of CP patients suggests that CK19 positive PDECs do express Pdx1 and differentiate into hormone positive cells, probably to compensate loss of islets due to inflammatory conditions prevalent in the disease. Earlier studies demonstrated differentiation of affinity purified CK19 + PDECs to insulin positive cells. Our study shows presence of both glucagon and insulin positive cells in the islet like clusters confirming that CK19 positive PDECs that express Pdx1 maintain plasticity and differentiate into both insulin positive and glucagon positive cells. This observation is also supported by the observed expression of insulin and glucagon genes in the pancreatic ductal tissue explants.

## 5. Conclusion

In conclusion, results of this study indicate that replicating CK19 positive PDECs form the pool of progenitors for normal regeneration of pancreatic endocrine mass in CP. Identifying endogenous and exogenous agents that stimulate CK19 positive PDECs towards differentiation into hormone positive cells in early stage of CP may offer a means to alleviate deficiency of availability of islets for cell based therapy in the treatment of diabetes.

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