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Authors

Butler, Alexandra E
Kirakossian, David
Gurlo, Tatyana
et al.

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In the Setting of Beta Cell Stress, the Pancreatic Duct Gland Transcriptome Shows Characteristics of an Activated Regenerative Response

Running Title: Pancreatic duct glands

Alexandra E. Butler[1], David Kirakossian[1], Tatyana Gurlo[1], Fuying Gao[2], Giovanni Coppola[2], Peter C. Butler[1]

[1]Larry L. Hillblom Islet Research Center, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California [2]Department of Psychiatry, Semel Institute for Neuroscience and Human Behavior, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California

CORRESPONDENCE and reprint requests to:

Peter C. Butler, MD

Larry L. Hillblom Islet Research Center

UCLA David Geffen School of Medicine

900 Veteran Ave, 24-130 Warren Hall

Los Angeles CA 90095-7073, USA

Tel +1 310 794 7645

Fax +1 310 206 5368

Email: pbutler@mednet.ucla.edu

ABSTRACT

The pancreatic duct gland (PDG) compartment has been proposed as a potential stem cell niche based on its coiled tubular structure embedded in mesenchyme, its proliferation and expansion in response to pancreatic injury, and the fact that it contains endocrine and exocrine epithelial cells. Little is known of the molecular signature of the PDG compartment in either a quiescent state or the potentially activated state during beta cell stress characteristic of diabetes. To address this, we performed RNA sequencing on RNA obtained from PDGs of wild type versus pre-diabetic HIP rats, a model of type 2 diabetes. The transcriptome of the PDG compartment, compared to a library of 84 tissue types, placed PDGs midpoint between the exocrine and endocrine pancreas and closely related to seminiferous tubules, consistent with a role as a stem cell niche for the exocrine and endocrine pancreas. Standard differential expression analysis (permissive threshold $p<0.005$) identified 245 genes differentially expressed in PDGs from HIP rats versus WT rats, with overrepresentation of transcripts involved in acute inflammatory responses, regulation of cell proliferation, and tissue development, while pathway analysis pointed to enrichment of cell movement-related pathways. In conclusion the transcriptome of the PDG compartment is consistent with a pancreatic stem cell niche that is activated by ongoing beta cell stress signals. The documented PDG transcriptome provides potential candidates to be exploited for lineage tracing studies of this as yet little investigated compartment.

NEW & NOTEWORTHY

The pancreatic duct gland (PDG) compartment has been proposed as potential stem cell niche. Transcriptome analysis of the PDG gland placed it midpoint between exocrine and endocrine tissues with adaptation towards response to inflammation and increased cell movement in a model of type 2 diabetes with ongoing beta cell apoptosis. These findings support the proposal that PDGs may act as a pancreatic stem cell niche.

KEYWORDS

Pancreatic duct glands, regeneration, pancreas, endocrine, diabetes

GLOSSARY

LCM, laser capture microdissection; OCT, optimum cutting temperature; PDGs, pancreatic duct glands; PEN, polyethylene naphthalate; RNase, ribonuclease; RNA Seq, RNA Sequencing

INTRODUCTION

It has been known for many years that there are glandular-like structures arising from larger pancreatic ducts that undergo proliferation following pancreatic injury with an increase in cells expressing the transcription factor Pdx-1, and containing occasional endocrine cells (22, 23). More recently, these glandular structures were proposed as being a distinct anatomic compartment with molecular features consistent with a stem cell niche, and named pancreatic duct glands (PDGs) (20). PDGs are crypt-like invaginations off the pancreatic ductal tree, embedded in mesenchyme and as such are anatomically reminiscent of the gastric, ileal and colonic crypt stem cell niches (Figure 1) (3). Similar glandular structures (peribiliary glands) are present as crypt-like outpouches off the biliary tree and have been reported to bear multiprogenitor cells with a potential ductal epithelium or endocrine fate (8). PDGs, like ileal crypts, have zones of increased replication, consistent with a transit amplifying zone, and a predominance of exocrine epithelial cells with a minority of endocrine cells. The PDG compartment is expanded with increased proliferation in humans with both type 1 diabetes (17) and type 2 diabetes (19).

Relatively little is known about the molecular signature of PDGs. Thus far, molecular characterization of PDGs has relied on candidate immunohistochemistry or RT-PCR in rats or mice (20, 22, 23) and immunohistochemistry in humans (17). To date this approach has revealed Pdx-1, nestin, mucin-6, Hes1 and Ngn3 expression in rodent PDGs and Sox9, GATA4, NKx6.1, NKx2.2 and chromogranin A in humans PDGs.

Powerful new tools are available to characterize the molecular signatures of tissues of interest based on RNA and/or protein profiling. Laser capture microdissection (LCM) is a technique that enables isolation of RNA and/or protein from a compartment of interest within an organ. After identification of the compartment by microscopy, laser dissection is used to procure a sample of the cells of interest to permit subsequent gene expression and/or proteome profiling (11, 21). In the present study we employed that approach to procure high quality RNA by a protocol validated for PDGs (7). We then characterized the transcriptional profile of these samples using RNA sequencing (RNA-seq) to establish the molecular identity of the PDG compartment in an unbiased manner. We used these data 1) to compare the molecular signature of PDGs to that of multiple tissues; 2) to identify possible markers overrepresented in the PDG compartment; and 3) to compare PDG samples from a rat model of type 2 diabetes (HIP rat) compared to wild type controls, with the goal of identifying the signaling networks and pathways altered in PDGs in the context of diabetes.

MATERIALS AND METHODS

Rats. The generation of the human IAPP transgenic rats (HIP) has been described in detail previously (5). Wild type rats were littermates of HIP rats. Rats were bred at the University of California, Los Angeles (UCLA) animal housing facility and subjected to a standard 12-h light-dark cycle and were fed Rodent Diet 8604 (Harlan Teklad, Madison, WI) ad libitum. All experimental procedures were approved by the UCLA Institutional Animal Care and Use Committee. HIP rats develop overt diabetes between 9 and 12 months of age with islet pathology similar to humans with type 2 diabetes, specifically

ongoing beta cell apoptosis with a progressive defect in beta cell mass (5). HIP rats used for this study were ~6 months of age, and so prediabetic with ongoing beta cell apoptosis but without the confounding secondary actions of hyperglycemia on gene expression (15). WT rats were age-matched.

Tissue procurement and LCM. On the day of study, animals were anesthetized by inhalation of isoflurane (Abbott Laboratories, Chicago, IL). Rat pancreas was rapidly dissected, divided into two portions (head and body of pancreas, and tail of pancreas), and cryopreserved in Optimal Cutting Temperature (OCT) compound (7). 10-15 Sections were cut from the head of the pancreas for each LCM experiment. In brief, eight micrometer sections were mounted on UV irradiated polyethylene naphthalate (PEN) membrane slides and stored at -80^{o}C. Right before use, slides were fixed in alcohol and stained with hematoxylin (solutions contained RNA inhibitors).

The PDG compartment was identified morphologically. As described in (20, 24), the PDG compartment is readily identifiable in mouse and human pancreas due to its unique architecture distinct from the ductal epithelium. We confirmed previously that similar to humans and mice, PDGs in rat pancreatic tissue sections appear as coiled structures embedded in the mesenchyme surrounding the main duct, and are readily stained with Alcian blue and p-aminosalicylic acid, and also characterized by the increased proliferation rate relative to normal ductal cells (12). For current study we set out to collect only PDGs in the substantial layer of mesenchyme where, based on

126 extensive experience (6, 12, 17, 19), they are readily and rapidly identified, ensuring
127 specificity and quality of collected RNA.
128
129 The PDG compartment was cut into 0.5 ml tube cap (Axygen Scientific Inc, Union City,
130 CA) filled with 10 µl of extraction buffer (PicoPure® RNA Isolation Kit, KIT0204) and 0.5
131 µl RNase inhibitor (1 U/µl) (SUPERase●IN™, AM2694; Ambion® Carlsbad, CA). To
132 avoid RNA degradation, slides were processed one at a time, and staining and
133 dissection from each slide was finished within 20 min. The LCM procedure was
134 performed using a LMD7000 Laser Microdissection system (Leica; Wetzlar, Germany)
135 at the California Nano Systems Institute Advanced Light Microscopy/Spectroscopy
136 Shared Resource Facility at UCLA.
137
138 ***Quality control and LCM selectivity.*** RNA quality was tested with a 2100 Bioanalyzer
139 using a RNA 6000 Pico LabChip Kit (Agilent Technologies, Santa Clara, CA). To
140 validate the LCM selectivity, RNA was isolated from LCM-derived samples from PDGs
141 and islets and tested by RT-PCR for the abundance of transcripts known to be
142 expressed in PDGs or islets. mRNA expression of PDG/ductal cell protein Cytokeratin-
143 19 (CK-19) and islet hormones insulin and glucagon were analyzed (7). By this
144 approach, CK-19 was highly expressed in PDGs but not islets. Insulin and glucagon
145 transcripts were abundantly expressed in islets and at low levels (~1,000 fold lower than
146 islets) in PDGs, consistent with occasional endocrine cells in this compartment. In this
147 study, we ranked expression levels of all 15,679 rat probes included in the RNA-seq
148 annotation and calculated the corresponding expression percentile. Cytokeratin 19

(*Krt19*) ranked 6/15,679 (<0.1th percentile), insulin (*Ins1*), ranked 19, 0.1% percentile), *Ins2* ranked #126, 0.7th percentile, and glucagon ranked 13,682 (87th percentile). While Cytokeratin 19 and Glucagon levels were consistently at the top and bottom of the list, respectively, insulin values were more variable across replicates, suggesting variability across our samples. Since we did not run RNAseq on islet tissue for this experiment we cannot assess expression relative to islet tissue.

RNA Seq. RNA-seq was performed in the UCLA Neuroscience Genomics Core (UNGC, http://www.semel.ucla.edu/ungc). Between 5-20 ng of total RNA were extracted per tissue. After quantification and quality check, 5 ng of total RNA were amplified at UNGC using the NuGEN Ultralow Library System kit (NuGEN), which is optimized for downstream Illumina library preparation. We extracted RNA from 6 samples: 3 from HIP and 3 from WT animals. Illumina RNA-seq libraries were then prepared according to manufacturer's instructions. Sequencing was performed using the Illumina HiSeq 2500 sequencer and the v3 Illumina chemistry. We barcoded multiple samples and ran them over multiple lanes, in order to minimize batch effects (2). We ran the equivalent of 3 samples per lane, with paired-end 100bp read length, corresponding to 2 HiSeq 2500 lanes.

Between 83 and 187 million, 100 basepair long, paired-end reads were obtained and aligned to the rat genome (rn5) using the STAR spliced read aligner (10). 65-72% reads mapped uniquely to the rat genome, and ~50% of the genes in the rat genome were detected as present by at least 100 mapping reads. Samples were clustered using

hierarchical clustering and multidimensional scaling (MDS), and no outliers were detected.

Data Analysis. An RNA-Seq pipeline is established in the UCLA Informatics Center for Neurogenetics and Neurogenomics (https://github.com/icnn/RNAseq-PIPELINE.git). Initial analysis steps included: 1) Quality analysis, alignment to reference genome using STAR (10), and filtering of reads not uniquely mapping or mapping to repetitive regions; 2) Mapping of reads to exons, untranslated regions (UTRs) and intron-exon junctions using STAR, and generation of RefSeq isoform counts; 3) Normalization and differential expression analysis by tissue and condition using the software DEseq (1) and edgeR (18); 4) Data upload onto our web-based gene expression database.

Multidimensional scaling (MDS) was used to cluster PDG samples with samples obtained from an atlas of gene expression in human tissues. Briefly, the human GNF database was downloaded from the bioGPS website (http://biogps.org), probes targeting transcripts shared across the two platforms (Affymetrix, used in the GNF database and RNA-seq, used in this study) were normalized jointly using quantile normalization and MDS plots were generated to organize samples in a 2-dimensional space, based on the expression of the top 1000 most variable genes. Differential expression analysis was performed to compare HIP and WT samples, using the edgeR package and setting p-value threshold of 0.005. Gene ontology and pathway analysis were performed using DAVID (http://david.abcc.ncifcrf.gov/) and Ingenuity Pathway Analysis (ingenuity.org).

RESULTS

Identification of the PDG compartment. PDGs share many properties with human ileal crypts (Figure 1). Both are crypt like structures embedded in mesenchyme. Ileal crypts and PDGs are composed primarily of gut or pancreas epithelium respectively but with occasional endocrine cells (Figure 1A, 1B). Both ileal crypts and PDGs have zones of increased replication (Figure 1C, 1D). In human and rat pancreas PDGs were readily identified in hematoxylin-stained tissue sections based on their unique anatomic location as invaginations off main pancreatic ducts embedded as coiled structures within the mesenchyme surrounding main pancreatic ducts (Figure 1, 2). In keeping with prior descriptions (12, 19, 20, 24), PDGs were notable for abundant mucins as detected by Alcian Blue, increased frequency of proliferating cells (Figure1A, 1B) and the presence of occasional insulin positive cells (Figure 1B, 2B).

RNA sequencing-based gene expression analyses in dissected PDG samples. Having previously established a method to obtain RNA by LCM from PDGs of a suitable quality and confirmed to reflect the PDG compartment based on RT-PCR of sentinel genes (7), we applied this approach here to obtain RNA from PDGs to perform RNA-seq so as to obtain an unbiased genetic expression profile of the PDG compartment. With these data, we then compared the PDG transcriptome to an atlas of transcriptional data including 84 tissues. MDS analysis (see Methods, Figure 3 legend) clustered related tissues (e.g. brain regions, or blood cell types) together due to similarity of their transcriptome. Our PDG samples were placed in a region including other pancreatic

samples (both exocrine and endocrine), further supporting their source tissue of origin, but also testis (including seminiferous tubules, which host actively replicating cells with pluripotent potential), and pituitary gland (Figure 3).

We then proceeded to identify the markers most specific to the PDG compartment. Briefly, after normalization we compared the all six PDG samples to the average of all other tissues in the GNF database. This analysis identified 22 transcripts as putatively overexpressed in PDG cells relative to the average expression levels of 84 tissues ($p<0.005$, Table 1). Finally, we compared PDG expression profiles between HIP and WT rats (Table 2). Standard differential expression analysis at a permissive threshold ($p<0.005$) identified 245 genes (Figure 4A, 4B), which were differentially expressed across the three replicates. Gene Ontology analysis (Figure 4C) highlighted an overrepresentation of transcripts involved in acute inflammatory response, regulation of cell proliferation, and tissue development. Pathway analysis pointed to enrichment of cell movement-related pathways, and the top 2 networks (Figure 5A, 5B) included *ADIPOQ*, playing an important role in type 2 diabetes, and the known therapeutic target *PPARG*.

In conclusion, unbiased transcriptional analyses support the notion that the PDG compartment includes a unique transcriptional niche, similar to both the tissue of origin (pancreas) and to replicating cells, and that a signal related to diabetes is detectable in a rat model of type 2 diabetes.

DISCUSSION

While the existence of blind pouches from main pancreatic ducts bearing stem cell markers has been appreciated for many years, only recently has this anatomical compartment been named the pancreatic duct gland (PDG) compartment and been proposed as a stem cell niche responsible for repair of pancreas following acute injury (20).

Features of the PDG compartment that are consistent with a stem cell niche include a tubular crypt like structure embedded in mesenchyme, expression of stem cell markers such as Hes-1 and proliferation with expansion in response to inflammation and/or injury (20). Other foregut derived tissues such as the duodenum and proximal ileum have well-characterized stem cell niches that are also crypt-like, located at the base of the intestinal villi embedded in mesenchyme. These well-defined stem cell niches generate cells that transition through proliferative transit amplifying zones to generate sufficient cells to replace the short-lived epithelial cells that migrate up the villi and are discarded after several days. A small subset of cells (~1%) derived from the intestinal crypt are transdifferentiated into endocrine cells under the induction of Ngn-3 signaling (9). This raises the possibility that the PDG compartment might not only serve to repair exocrine ductal tissue as already reported, but also be a potential source of pancreatic endocrine cells, although no effective beta cell formation from the PDG compartment was identified in humans with type 1 or 2 diabetes (17, 19).

The turnover of pancreatic duct epithelial cells is much less frequent than that of the cells that form intestinal villi. However, in common with intestinal crypts, PDGs have a zone of increased replication compared to that of the duct epithelia, and this is enhanced in response to injury or the known growth factor GLP-1 (12) (19). The unbiased gene expression studies by RNA-seq of PDGs presented in this manuscript reveal a molecular signature intermediate between the exocrine and endocrine pancreas as well as the well-defined stem cell niche in the testis, consistent with a pancreatic stem cell niche that may serve both the exocrine and endocrine pancreas.

There has been controversy as to whether endocrine cells arise from pancreatic duct epithelium in postnatal life (so called ductal neogenesis) (13). The postulate that they do was initially rendered based on the adjacency of islets and pancreatic duct epithelium commonly found in pancreas (4). However, most lineage studies have failed to demonstrate endocrine cells arising from pancreatic ductal or acinar cells. One possible explanation for this is that the lineage markers employed represent those of definitive ductal epithelium rather than that of the putative pancreatic stem cell niche. One of the purposes of the present study was to further characterize the molecular identity of the PDG compartment to offer investigators potential candidates to lineage trace the derivatives of the compartment. Several lines of investigation suggest that there is ongoing beta cell formation in the adult pancreas that cannot be attributed only to replication of existing beta cells (16).

Modeling studies applied to the HIP rat model of type 2 diabetes revealed an adaptive increase in beta cell formation not attributable to beta cell replication (14). Therefore, we selected this model of type 2 diabetes to investigate the PDG transcriptome for evidence of adaptive changes that might be expected in a relevant stem cell niche. Differential gene expression analysis between PDGs from HIP and WT rats were consistent with tissue stem cell response to injury in a relevant compartment. For example the most significant alteration was in genes engaged in inflammatory responses. This implies communication between injured pancreatic islets and PDGs, presumably either through the known intrapancreatic portal venous system or through the rich intrapancreatic neural network. Also, given the well-recognized role that inflammatory pathways play in the induction of tissue repair, it is of interest that PDGs in HIP rats not only apparently sense and respond to pancreatic islet inflammation, but also that regulation of cell proliferation and tissue development genes are also highly represented in the PDG transcriptome of HIP rats compared to non-diabetic WT rats. Pathway analysis also pointed to enrichment of cell movement-related pathways, and the top 2 networks (Figure 5A, 5B) included *ADIPOQ*, a gene with known linkage to obesity and type 2 diabetes, and the therapeutic target in type 2 diabetes, *PPARG*. The PDG compartment has been reported to be expanded with increased proliferation in humans with both type 1 and 2 diabetes, and this is reproduced in the HIP rat model (17, 19). Here we now report the transcriptome of the PDG is consistent with a tissue stem cell niche serving both the exocrine and endocrine pancreas, and undergoing anticipated adaptive changes in response to inflammatory signals arising from stressed beta cells.

If the PDG compartment is a potential source of new beta cells, the question arises, why are beta cells not restored in type 1 and 2 diabetes? Tissue stem cell niches recapitulate the development of the host tissue, and so endocrine cells would be expected to be a minority of new cells formed by a pancreatic stem cell niche. Moreover, in the face of ongoing beta cell autoimmunity in type 1 diabetes, and misfolded protein stress in type 2 diabetes, presumably beta cell loss would likely match any beta cell formation. On the other hand if the PDG compartment can serve as a source of new beta cells, it is plausible that the relative fate of newly forming cells might be therapeutically manipulated towards an endocrine rather than exocrine fate to enhance new cell formation.

As with all studies, the present studies have limitations. The samples sizes are relatively small, constrained by the costs of the dissection protocol and RNA seq. The studies are limited to rodent pancreas, as efforts to procure consistently high quality RNA from human pancreas samples unfortunately were unsuccessful. Nonetheless the data that has been established implies that the PDG compartment may indeed serve as a pancreatic stem cell niche, and provides some insights that might be exploited to establish lineage dynamics in genetic models.

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GRANTS

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DISCLOSURES

The authors have no conflicts of interest.

AUTHOR CONTRIBUTIONS

AEB and DK performed the experiments, AEB, TG, GC and PCB designed the studies and helped write the manuscript, FG and GC performed the analysis of the RNA-seq pathways and networks.

FIGURE LEGENDS

Figure 1. Comparison of pancreatic duct gland and ileal crypt.

Human ileal crypts and PDGs share many properties. Both are crypt like structures embedded in mesenchyme (A, B). Ileal crypts and PDGs are composed primarily of gut or pancreas epithelium respectively but with occasional endocrine cells (A, inset stained brown, chromogranin) and (B, stained pink, insulin). Both ileal crypts and PDGs have zones of increased replication (Ki67 C, D) compared to surrounding structures, consistent with a role as a transit amplifying zone. The variability in frequency of proliferating cells in PDG compartments was detected.

Figure 2. Pancreatic duct gland histology.

A. Section through a large duct in pancreas from a wild type [WT] rat demonstrating the pancreatic duct gland [PDG] compartment present in the mesenchyme surrounding the large duct, with PDGs connecting directly with the large duct lumen (arrows). B. Section through a large duct in pancreas from a human IAPP transgenic [HIP] rat demonstrating the extensive pancreatic duct gland [PDG] compartment relative to WT rats. Sections are stained for insulin [DAB] with hematoxylin counterstain. Images were taken at 10x (100x magnification). ★indicates large duct lumen. The inset (B) shows a PDG epithelial cell staining for insulin. Scale bar= 200μm.

Figure 3. Multidimensional scaling plot of tissue samples human expression atlas (http://biogps.org/). Each dot represents the relative location of gene profile from one of the 176 samples from 84 surveyed tissues. Dots are color-coded by tissue group or

system. Samples cluster based on similarity. Validity of the analytical approach is illustrated by the clustering of expression profiles obtained for brain or blood (shades areas). In red, RNA-seq-derived genetic profiles obtained from PDGs are reassuringly close to each other. Also, PDG gene expression profiles are placed between a classical stem cell profile (germ cells) and stem cell niche profile (Leydig cells) in one dimension and then close to the two pancreas compartments (islet and exocrine pancreas).

Figure 4. Differential expression analysis comparing PDG samples from HIP and WT rats. (A) Number of up- (red bar) and down-regulated (green) transcripts when comparing HIP vs. WT; (B) heatmap representing ratios of the 245 dysregulated transcripts. Individual genes are in rows, samples are in columns. Each cell represents a ratio (each HIP sample vs. the average of WT). Shades of red: upregulation, shades of green: downregulation; (C) overrepresentation of gene ontology categories within the differentially expressed gene set. Within each category, green represents the proportion of downregulated and red the portion of upregulated transcripts when comparing HIP vs. WT samples.

Figure 5. Top two networks identified by Ingenuity Pathway Analysis as overrepresented within the list of 245 genes ($p<0.005$) dysregulated in HIP vs. WT. Symbols in shades of green or red denote up- and down-regulated transcripts, respectively. Symbols in gray are not significantly differentially expressed in this dataset, but known to be part of the pathway or network. Solid lines denote direct

interaction, dotted lines indirect interaction, such as an alteration in expression levels, or post-translational modification.

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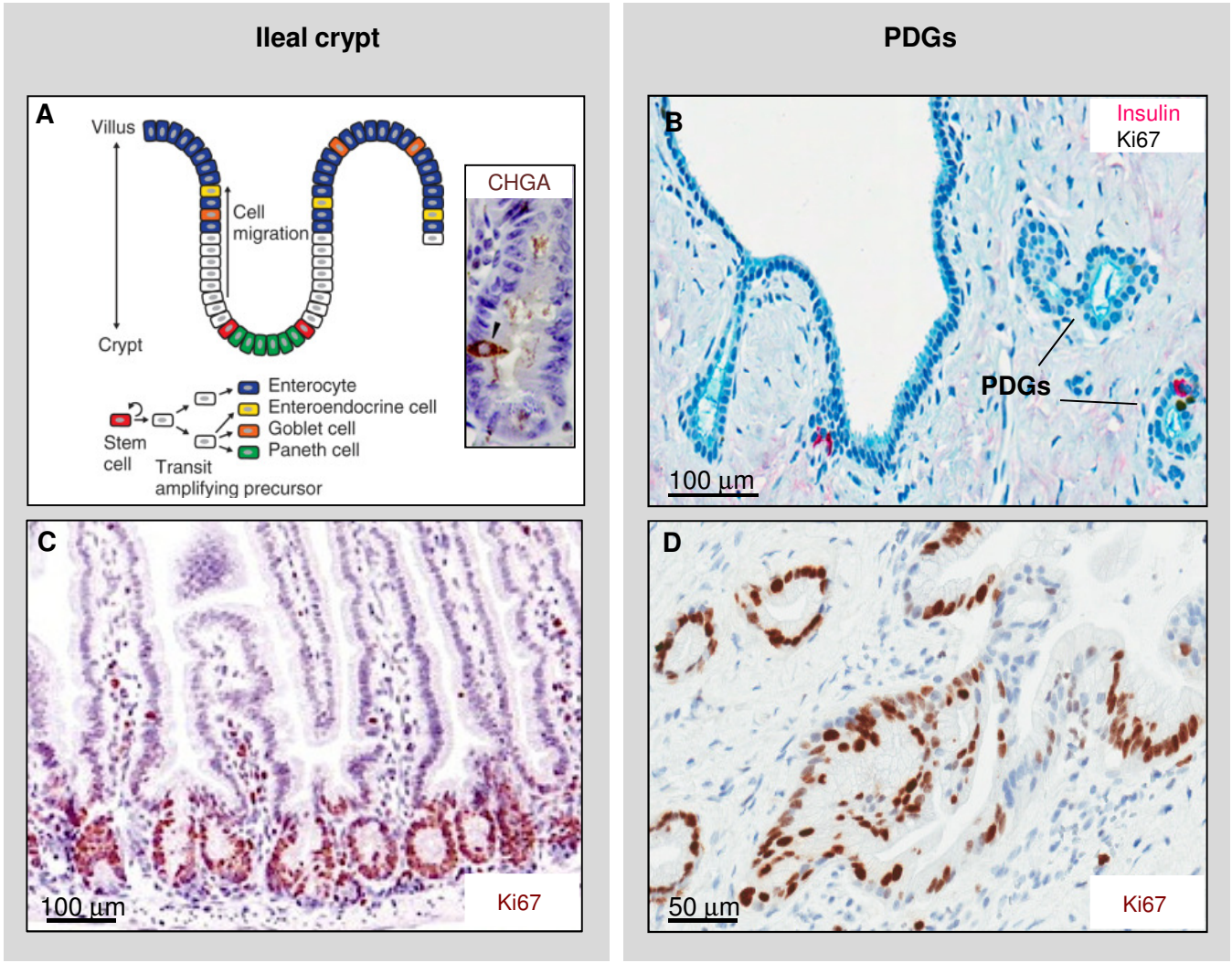
SUPPLEMENTAL DATA

Table 1: List of 22 differentially expressed genes when comparing the 6 PDG samples vs. the average expression levels across 84 human samples in the Human GNF database. Fold changes are expressed after log2 transformation.

Table 2: List of 245 differentially expressed genes when comparing the PDG samples from the HIP rat model of type 2 diabetes vs. WT animals. Fold changes are expressed after log2 transformation.

Figure 1



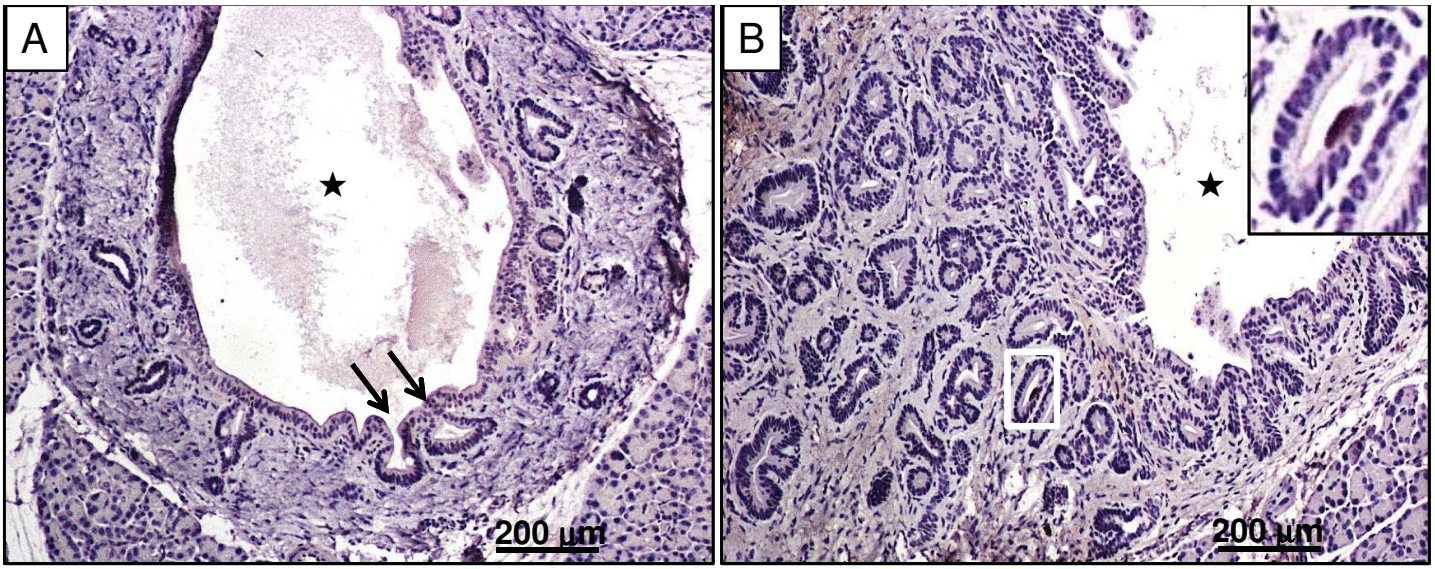


Figure 2