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A Pro-Endocrine Pancreatic Islet Transcriptional Program Established During Development Is Retained in Human Gallbladder Epithelial Cells

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SUMMARY
Insulin-producing cells are present in the epithelial lining of developing, as well as adult, mouse and human gallbladders. Although not physiologically relevant, these cells possess the capacity to transcribe and translate insulin. These cells were not destroyed in a mouse model of type 1 diabetes.

BACKGROUND & AIMS: Pancreatic islet β-cells are factories for insulin production; however, ectopic expression of insulin also is well recognized. The gallbladder is a next-door neighbor to the developing pancreas. Here, we wanted to understand if gallbladders contain functional insulin-producing cells.

METHODS: We compared developing and adult mouse as well as human gallbladder epithelial cells and islets using immuno-histochemistry, flow cytometry, enzyme-linked immunosorbent assays, RNA sequencing, real-time polymerase chain reaction, chromatin immunoprecipitation, and functional studies.

RESULTS: We show that the epithelial lining of developing, as well as adult, mouse and human gallbladders naturally contain interspersed cells that retain the capacity to actively transcribe, translate, package, and release insulin. We show that human gallbladders also contain functional insulin-secreting cells with the potential to naturally respond to glucose in vitro and in situ. Notably, in a non-obese diabetic (NOD) mouse model of type 1 diabetes, we observed that insulin-producing cells in the gallbladder are not targeted by autoimmune cells. Interestingly, in human gallbladders, insulin splice variants are absent, although insulin splice forms are observed in human islets.

CONCLUSIONS: In summary, our biochemical, transcriptomic, and functional data in mouse and human gallbladder epithelial cells collectively show the evolutionary and developmental similarities between gallbladder and the pancreas that allow gallbladder epithelial cells to continue insulin production in adult life. Understanding the mechanisms regulating insulin transcription and translation in gallbladder epithelial cells would help guide future studies in type 1 diabetes therapy. (Cell Mol Gastroenterol Hepatol 2022;13:1530–1553; https://doi.org/10.1016/j.jcmgh.2022.01.008)

Keywords: Islets; Insulin; Splice Variants; Gallbladder Development; Differentiation.

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of insulin in the gallbladder/biliary duct epithelium, thymus, and the brain. The gallbladder and pancreas arise from the foregut endoderm and share transcription factors during embryonic development. The occurrence of β-like cells in mouse biliary ducts was reported previously, and the inhibition of Hes1 in mouse gallbladder epithelial cells improved insulin production. A recent study also implicated their therapeutic potential by forced expression of pancreatic transcription factors to differentiate gallbladder-derived cells from individuals with type 1 diabetes (T1D) into insulin-producing cells.

The development of the pancreas and its close neighbors (the liver and gallbladder) has been studied in mouse models. However, gallbladder development has not been reported as vastly as in the pancreas and the liver. Similarly, pancreas development in both rodents and human beings has not been compared with that of gallbladder. Here, using mouse models and human tissue/samples spanning embryonic to post-natal/adult life, we show a comparative molecular and functional analysis of insulin-transcribing cells in the gallbladder and pancreas. Although expression of genes and their splice variants across human tissues is fundamental to the understanding of tissue specificity and function, current tissue expression data sets, such as the Genotype-Tissue Expression (GTEx) project, lack gene expression data from the gallbladder. Our work adds new knowledge while showing the gallbladder to be an important and interesting naturally occurring source of insulin-producing cells. Considering the developmental similarity with the pancreas, natural occurrence of insulin-producing cells, and tissue availability, the gallbladder presents an alternative for cell-replacement therapy. Although the insulin secretion from these cells is physiologically not relevant, our data support the need for future work on understanding cellular and molecular mechanisms regulating insulin production in this functionally diverse tissue.

Results
Insulin Expression in Developing Mouse Gallbladder Cells

The gallbladder originates from the pancreatic bud that also gives rise to the ventral pancreas (Figure 1A) during embryonic development. We profiled key endocrine pancreatic gene transcripts in developing mouse pancreas and gallbladder on embryonic day (E)15.5, E16.5, and E18.5. The developing pancreas contained more copies of Ins1, Ins2 (Figure 1B), and Gcg, Sst (Figure 1C) gene transcripts compared with those in the gallbladder. Ins1, which is known to be more specific to mouse pancreas (but not the brain), was significantly higher in the developing pancreas at all of the embryonic time points (Figure 1B). Ins2 expression levels were significantly different from those in the gallbladder closer to birth. Interestingly, the master regulatory pancreatic transcription factor Pdx1 was expressed in gallbladder cells at all times, and at levels similar to those in the pancreas closer to birth (E16.5 and E18.5) (Figure 1B). The expression of other gene transcripts (Hnf1b, Sox9, Hnf4a, and Hhx) did not show significant differences between developing pancreas and gallbladder (Figure 1C) during the time points assessed.

Developing mouse gallbladders also contain immunoreactive insulin protein, albeit at significantly lower levels than those in the developing pancreas (Figure 1D). Using a Pdx1GFP/+ reporter mouse (Figure 1E), we confirmed that cells within the prospective gallbladder and pancreatic buds show Pdx1 gene promoter activity (Figure 1F). As expected, wild-type gallbladder cells do not contain any GFP+ cells (Figure 1F, right bottom). We then performed bulk RNA sequencing (RNA-seq) on adult mouse pancreas and gallbladders to assess similarities and differences in pancreatic gene transcript levels across these functionally different adult tissues. Pancreatic hormone transcripts were significantly higher (≥2-fold difference; P < 0.05) in the adult pancreas, whereas Hes1, a negative regulator of the pro-endocrine gene Neurog3 (also known as Ngn3) was expressed at significantly higher levels in gallbladder cells (Figure 1G). In line with the embryonic data (Figure 1B), the expression of Pdx1 was similar (Figure 1G) between adult gallbladders and the pancreas. Validation of RNA-seq data using real-time quantitative polymerase chain reaction (qPCR), confirmed similar Pdx1 expression in both adult tissues (Figure 2A). Relatively lower levels of Neurog3 transcripts in adult gallbladder presented with a significantly higher level of Hes1 in the qPCR-based validation (Figure 2A). Pancreatic islets and gallbladder epithelial cells of Pdx1GFP/+ mice had insulin- and as well as Pdx1-co-expressing cells (Figure 2B). Pancreatic β-cells had predominantly nuclear Pdx1 expression, whereas gallbladder epithelial cells from the same mice presented with cytoplasmic localization of Pdx1 (Figure 2B). The continued expression of Pdx1 in mouse gallbladder cells is intriguing. Pdx1 gene expression is controlled by 4 conserved sequence domains: area I (2761/-2457 base pairs [bp]), area II (-1253/-1923 bp), area III (-1879/-1600 bp), and area IV (-6529/-6047 bp). Chromatin immunoprecipitation (ChIP) analysis at the Pdx1 gene promoter region showed that these sites are active in the gallbladder (Figure 2C). Using flow cytometry and another mouse model (Mouse Insulin Promoter driving Green Fluorescent Protein; MIP-GFP), we confirmed mouse Ins1 promoter activity in adult mouse gallbladder cells. Here, we observed GFP-positive cells in both pancreas and gallbladder of MIP-GFP mice, with gallbladder tissue harboring approximately 9-fold less insulin-containing cells (Figure 2D). We then probed if the insulin produced by mouse gallbladder epithelial cells was packaged. Immune-electron microscopy of mouse gallbladder epithelial cells confirmed the presence (albeit a lower number) of electron-dense insulin secretory vesicles (Figure 2E) within gallbladder epithelium. Although the number and density of these granules was significantly lower, their existence in gallbladder is promising. Together, these studies show pancreatic endocrine gene and protein expression in embryonic and adult mouse gallbladder epithelium.
Pancreatic Endocrine Gene Expression in Developing Human Gallbladder

We extended our mouse studies to developing human pancreas and gallbladders accessible from 11 fetuses at early (16–20 weeks gestational age [WGA]; n = 5) or late stages (>20 WGA; n = 6) (Figure 3A) of pregnancy. In comparison with pancreatic tissue, all the islet hormones (INS, GCG, and SST) were expressed at significantly lower abundance (P < .001) (Figure 3B) in gallbladder tissues throughout the embryonic development. Differences between islet and gallbladder (pro)hormone transcript levels were significant across early (16–20 WGA; P < .01; n = 5) (Figure 3B), but not later, stages (>20 WGA; n = 6) (Figure 3B). Histologic (Figure 3C and D) and confocal microscopy analysis (Figure 3E and F) of >20 WGA human pancreas and gallbladders confirmed tissue-specific morphology and the presence of hormone-containing cells. Although insulin-producing cells are localized mostly to cell clusters within pancreatic islets, they are interspersed among columnar epithelial cells lining the gallbladder. We
then compared the expression of pancreas-enriched transcription factors and receptors between matched pairs of gallbladder and pancreas samples from 4 human fetal donors (Figure 3G). Unsupervised bidirectional hierarchical clustering separated fetal pancreas and fetal gallbladders into 2 distinct groups (Figure 3G). We also validated the expression of selected pancreatic genes in remaining human pancreatic and gallbladder fetal tissues. Unlike mouse development, the significantly lower \(P = .04\) level of human \textit{NEUROG3} in developing gallbladder cells was not associated with any differences in \textit{HES1} expression (Figure 3H). Although levels of \textit{GCK} gene transcripts were significantly higher in the developing pancreas \(P = .02\) (Figure 3I), GLP1R and the glucose transporter \textit{GLUT2} (also known as \textit{SLC2A2}) were similar across these developing tissues (Figure 3I). Several other genes (\textit{CDH1}, \textit{HB9}, \textit{NEUROD1}, \textit{PAX6}, and \textit{PCSK2}) were expressed at significantly higher levels in the developing human pancreas as compared with their levels in gallbladders (Figure 4). No significant differences were observed across the expression of zinc finger transcription factors (\textit{GATA4} and \textit{GATA6}) (Figure 3H), islet integrin αv (\textit{ITGAV}), and the histone deacetylases (\textit{HDAC1-3}) (Figure 4).

**Similarities in the Developing Human Pancreas and Gallbladders Are Retained in Adult Life**

Bright-field and confocal microscopy of adult human pancreatic islets and gallbladder epithelial cells confirmed that the morphology and islet-specific protein production observed in fetal stages is retained in adult human gallbladder cells (Figure 5A). Adult human islets contain mature, hormone-producing cells that also present with Pancreas and Duodenal Homeobox gene 1 (PDX1)-immunopositivity, which is largely localized to the nuclei. Similar to isolated islets, adult human gallbladder epithelial cells isolated as epithelial sheets (see the Methods section) form hollow spheres of epithelial cells, which are immunopositive for C-peptide and PDX1 (Figure 5A), confirming the capacity of the gallbladder epithelial cells to process the prohormone into mature insulin and C-peptide. Similar to human pancreatic islets,\textsuperscript{14} gallbladder-derived epithelial cell clusters show immunopositivity for E-cadherin and β-catenin (Figure 5A). Bulk RNA-seq in freshly isolated adult human islets and gallbladder epithelial cells confirmed a large number of genes that are significantly different across these functionally diverse tissues (Figure 5B). RNA-seq coverage maps confirm that adult human gallbladder transcripts for \textit{INS}, \textit{GCG}, \textit{SST}, and \textit{PDX1} mapped to the same genomic regions (Figure 5C) as in islets, although with a lot fewer copies in gallbladder cells. Validation in a different set of the freshly isolated adult human islet and gallbladder samples using TaqMan-based real-time qPCR (Figures 5D and E, and 6A) confirmed that islet hormones (\textit{INS}, \textit{GCG}, and \textit{SST}) were at significantly higher (several hundred- to thousand-fold) levels in human islets, relative to those in gallbladder epithelium, while the expression of \textit{GLUT2} and \textit{INSR} were comparable (Figure 5D). Pancreatic transcription factors (including \textit{PDX1} and \textit{MAFA}), but not \textit{NEUROG3}, were significantly more abundant in pancreatic islets (Figure 5E). We compared our human gallbladder RNA-seq data with a publicly available RNA expression data set of human laser capture microdissected β-cells from non-diabetic individuals (E-GEOD-20966).\textsuperscript{15} Although a large number of gene transcripts were significantly different, interestingly, several gene transcripts retained similar expression levels across these 2 functionally diverse tissues (Figure 6B, Supplementary Table 1). Gene ontology (GO) analysis of gallbladder transcripts filtered for β-cell expression indicated several GO terms that are relevant to insulin packaging and secretion (Figure 6C), cellular development/differentiation (Figure 6D), vesicle transport (Figure 6E), mitochondrial structure and function (Figure 6F), as well as carbohydrate metabolism including glucose metabolic processes (Figure 6G). Parallel with these analyses, we probed if gallbladder-enriched genes were expressed in human pancreatic islet cell subsets. We mapped the expression of 21 highly abundant human gallbladder-enriched gene transcripts identified through our RNA-seq data sets

![Figure 1.](https://example.com/figure1.png) (See previous page). Mouse gallbladder and pancreas development. (A) Mouse pancreatic gut schematic showing developing stomach (St), liver (L), dorsal pancreatic bud (DP), and the ventral pancreatic bud (VP) that gives rise to the gallbladder (Gb) and ventral pancreas. TaqMan-based real-time qPCR for rodent insulin genes \textit{Ins1}, \textit{Ins2}), the master regulatory transcription factor \textit{Pdx1} (B) as well as other genes (C) in developing mouse pancreas and gallbladder tissues harvested on E15.5, E16.5, and E18.5. Data in B and C represent means ± SD from 3 different litters of Friend Virus B NIH Jackson (FVB/NJ) mice, each with at least 6–7 embryos/litter. Transcript abundance was analyzed using 2-way analysis of variance with the Sidak adjustment for multiple comparisons. Each point in the scatter bar graph presents data from a single litter. (D) Insulin content in developing mouse pancreas and gallbladder tissues harvested at E16.5, E18.5, and neonatal day 1 pups. Data were obtained from pooled tissues from 3 different litters of FVB/NJ mice, each with at least 6–7 embryos/pups and presented after normalizing to total protein. Aligned dot plots present means ± SEM. Significance was calculated using 2-way analysis of variance with the Fisher’s Least Significant Difference (LSD) test. (E and F) \textit{Pdx1} \textit{GFP} \textit{w/w} reporter mouse embryos were obtained from timed pregnancies (E10.5, E13.5, and E15.5) and GFP fluorescence (arrowheads) was used to identify pancreatic buds. Representative flow cytometry plots of pancreas and gallbladder tissues from \textit{Pdx1} \textit{GFP} \textit{w/w} and \textit{Pdx1} \textit{GFP} \textit{w/w} (wild-type) mice are presented with proportions of \textit{GFP} \textit{w} cells as indicated. Acquisition and gating parameters were identical across WT and \textit{GFP} \textit{w} tissues. Experiments were repeated at least 3 times with tissues pooled from 6 to 7 embryos. (G) Volcano plot for bulk RNA-seq data from the adult mouse gallbladder (n = 6) and pancreas (n = 6). Normalized read count difference between pancreas and gallbladder is plotted on the X-axis and statistical significance (\(-\log_{10} P\) value) is presented on the Y-axis. The dashed horizontal line represents \(P = .05\), and the dashed vertical lines represent a 2-fold normalized DEseq value difference. Significantly altered \((P \leq .05 \text{ and } >2\text{-fold difference})\) transcripts are presented in red. Selected important pancreatic genes on the volcano plot are labeled and highlighted in purple. Throughout the figure, bars/dots for the pancreas are in red and for the gallbladder are in green. * \(P < .05\), ** \(P < .01\).
(Figure 7A) to publicly available pancreatic single-cell RNA-seq data sets (Figure 7B). Intriguingly, 17 of the 21 gene transcripts were present in acinar and/or ductal cells, while approximately 50% (10 of 21) of the gallbladder-enriched gene transcripts also were transcribed by pancreatic α- and/or β-cells (Figure 7C). In summary, our data show that the inherent capacity of human gallbladder epithelial cells for pro-endocrine gene transcript and protein expression (albeit at low levels), is retained in the post-natal stage and that the adult human α- and β-cells transcribe several of the gallbladder-enriched gene transcripts.

### Adult Human Gallbladder Epithelial Cells Can Proliferate and Differentiate In Vitro

We established a unique protocol to isolate gallbladder epithelium without enzymatic digestion via gentle scraping of gallbladder epithelial cells using a sterile scalpel blade (Figure 8A). The epithelial sheets obtained by scraping, close on themselves to form hollow, epithelial clusters of cells (Figure 8B), which migrate and proliferate in vitro as mesenchymal-like cells (Figure 8C). Actual phase-contrast micrographs of this process are shown in Figure 8D. We exposed freshly isolated human gallbladder epithelial cells
(as shown in Figure 8B or day 0 of 8D) to 2 consecutive pulses of different thymidine analogs; 5-chloro-2-deoxyuridine (CldU) and 5-iodo-2-deoxyuridine (IdU), added sequentially to cell culture media before their detection using specific antibodies. Using this unbiased, dual-thymidine, analogue-based, cell lineage tracing technique,14,16 we detected the presence of CldU⁺, IdU⁺, and C-peptide⁺ cells (Figure 8E) in gallbladder epithelial cells, propagating under optimal cell culture conditions (Figure 8F). This confirmed that insulin-producing cells in gallbladder epithelium contribute to the proliferating subsets of gallbladder-derived cells. Eventually, all proliferating populations of gallbladder epithelial cells acquire a mesenchymal-like phenotype and begin expressing mesenchymal gene transcripts (Figure 8G) and proteins such as smooth muscle actin and vimentin (Figure 8H). Interestingly, freshly isolated epithelial clusters had lower expression levels of these mesenchymal genes that significantly increased in abundance from passage 0 to passage 5 (Figure 8G). Flow cytometry-based analysis of passage 5 gallbladder-derived cells showed the presence of typical surface antigens expressed by mesenchymal stem cells such as for CD29, CD44, CD90, and CD105 (Figure 8I). We then assessed the chromatin landscape at the insulin gene in freshly isolated human islets and gallbladder clusters. At 2 different sites of insulin gene (-275 and +1318), human islets showed open chromatin conformation as confirmed by higher levels of H3H4Ac and H3K9Ac, histone modifications associated with active genes along with lower levels of H3K9 trimethylation, associated with silenced/inactive genes. In the freshly isolated gallbladder epithelial cells, these chromatin modifications were not significantly different from those observed in adult human islets (Figure 9A). Analysis of pancreatic gene promoters in passages 5–10 of gallbladder-derived mesenchymal cells showed a more inactive chromatin conformation (H3K9-Me3 and H3K9-Me2) at insulin and neurogenin 3 promoters, whereas an open/accessible chromatin conformation (significantly higher H3K4-Me2) was seen at the HES1 gene promoter (Figure 9B). Interestingly, the PDX1 gene promoter retained an active/open promoter conformation in these cells (Figure 9B). These data corroborate the lack of insulin expression in gallbladder-derived mesenchymal cells and support the potential for re-expression of insulin gene transcripts through chromatin conformational changes or via repressing HES1 expression.

We, therefore, assessed endocrine differentiation of gallbladder-derived mesenchymal cells using small molecules that are known to be DNA methyltransferase inhibitors or histone deacetylase inhibitors17–19 and/or via forced expression of a dominant-negative (ΔHES1, or of the pro-endocrine transcription factors (PDX1, MAFA, and NEUROG3). Although differentiation to insulin-expressing islet-like clusters was observed over 14 days in the serum-free differentiation medium (Figure 9C), none of the small molecules (sodium butyrate, trichostatin A, valproic acid, 5-aza-2′-deoxycytidine, and dexamethasone) induced significantly higher insulin expression compared with vehicle controls (Figure 9D). On the other hand, we obtained a consistent and significant increase in INS transcript expression using both (ΔHES1 and pro-endocrine transcription factor) overexpression strategies (Figure 9E). Our data support the view that gallbladder/biliary duct cells can be differentiated to promote insulin expression.

Functional Analyses of Insulin-Producing Human Gallbladder Cells

To extend our understanding of insulin secretion from gallbladder epithelial cells, we planned a series of experiments using freshly isolated adult human gallbladder epithelial cells. We compared genes known to be important in insulin sensing and exocytosis using bulk RNA-seq data of adult human islet and gallbladder epithelial cells. Although gene transcripts of islet hormones, chromogranin A, prohormone processing enzymes (PCSK1, PCSK2), and the glucose sensor (GCK) were significantly lower in gallbladder epithelial cells, the transcripts of the 2 glucose transporters
were either similar (for SLC2A1) or significantly higher (for SLC2A2) in adult gallbladder epithelial cells (Figure 10A) than those in adult human islets. Except for pancreatic adenosine triphosphate–sensitive K+ channel ABCC8 (or SUR1) and the transmembrane protein synaptotagmin-7 (SYT7), gallbladder epithelial cells and human islets contained similar levels (Figure 10B) of gene transcripts for KCNJ11 (or Kir6.2), the L-type calcium channel CACNA1C (encoding CAV1.3 protein), the islet syntaxins and binding proteins (STX1A, STX3, and STXB2), the synaptosomal-associated protein (SNAP25), and gene transcripts encoding the vesicle-associated membrane proteins (VAMP2 and VAMP8). These comparisons (Figures 5G and 10B) indicate that gallbladder epithelial cells express the set of genes necessary for insulin exocytosis. Freshly isolated gallbladder epithelial cells (Figure 10C) contain insulin protein and also release insulin/C-peptide in response to glucose (Figure 10D), indicating successful processing and secretion of insulin after glucose exposure in vitro. We then transplanted freshly isolated adult human gallbladder epithelial cells under the kidney capsule of immunocompromised Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (Figure 10E). A functional assessment performed on day 30 using intraperitoneal glucose injection
resulted in detectable levels of human insulin in mouse circulation, which was not seen immediately after transplantation/day 0 (Figure 10F). To understand in situ insulin secretion from human gallbladder cells, we collected blood from the median cubital vein (peripheral) and cystic vein (gallbladder) at 30 minutes after intravenous glucose injection from individuals who were enrolled for cholecystectomy. Intriguingly, insulin levels in the cystic vein were higher than those in the cubital vein for 80% (n = 4 of 5) individuals assessed in this study (Figure 10G). Overall, we show that naturally occurring insulin-secreting cells within the gallbladder have relevant insulin secretory machinery and can respond to physiological changes in glucose concentrations. However the amount of insulin secreted from these cells (Figure 10C) is not physiologically relevant to maintain normal glucose concentrations, and is significantly lower than that reported in human islets.20

**Insulin-Producing Gallbladder Epithelial Cells in Diabetes**

To understand if insulin-containing gallbladder epithelial cells also elicit an immune-mediated attack during diabetes progression, we examined immune infiltration in gallbladder and islets from female NOD mice. Because NOD females show significant infiltration at 12–14 weeks of age, we obtained gallbladder and pancreas from NOD mice at 4, 14, 16, and 18 weeks of age (Figure 10H). Predictably, the pancreas from these mice showed increasing infiltration of immune cells (Figure 10H) at 14–18 weeks of age, but no infiltration was observed in their gallbladders. Immuno-staining for islet hormones confirmed the decreasing number (quantitative data not shown) of insulin-producing cells in the pancreas as plasma glucose increased (Figure 10I, yellow horizontal line for normoglycemic levels). Insulin-immunoreactive cells were present in gallbladder epithelial cells even when NOD mice showed high glucose levels at 18 weeks (Figure 10H), indicating that gallbladder epithelial cells can potentially escape immune recognition during type 1 diabetes progression in NOD mice. This interesting observation could be owing to a different autoimmune antigenic repertoire between islet β-cells and gallbladder insulin-producing cells. Analysis of splice variants in gallbladder (GSE152419, n = 7) and human islet (GSE152111, N = 66; and GSE134068, N = 18) RNA-seq data sets indicate a high differential splice index for human insulin variant (ENST00000250971) in islets (splicing index, 0.14, N = 66 islets or splicing index, 0.17, N = 18 islets) compared with no differential splicing observed in human gallbladder epithelial cells (ENST00000250971; splicing index, 0.0; n = 7) (Figure 11).

**Discussion**

The origin/proximity of the gallbladder endoderm to the developing pancreas is evolutionarily conserved across lower vertebrates.21,22 The toadfish has a single primary islet located in the dorsal region of the gallbladder,21 with
smaller islets embedded in the mesenteric part of the gut. Zebrafish has one of its pancreatic lobes along the intestine–gallbladder–spleen tissue axis,\(^{22}\) while in Lake Van fish, pancreas is observed next to the gallbladder and bile duct.\(^{23}\) Ectopic pancreas in gallbladder;\(^{24}\) somatostatinomia in human extrahepatic biliary tract;\(^{25}\) and glucagon expression in
Figure 6. Gene expression pathways enriched in adult human gallbladder epithelial cells. (A) An unsupervised bidirectional hierarchical cluster of 51 genes known to be associated with or necessary for normal pancreas development/function, profiled in adult human pancreatic islets (red, n = 21) and human gallbladder epithelial cells (green, n = 18) was plotted using Euclidean distance metric and average linkage. The heat map representing normalized qPCR Ct values (color bar) for each gene (listed on right Y-axis) with low Ct values/high expression in orange–red color and higher Ct values/low expression in shades of yellow to white. Data consist of measurement on ViiA7 (INS, GCG, MAFA, NEUROG3, HES1, and PDX1) or using a custom TaqMan OpenArray platform. (B) Venn diagram showing the number of genes common between our bulk gallbladder RNA-seq (n = 6) and the publicly available data set of human islet β-cells (see the Methods section). (C–G) The most significant and relevant Biological Process GO categories enriched in gallbladder cells are presented. Data obtained from RNA-seq was used to identify the gallbladder-expressed genes and total gallbladder transcripts were filtered for β-cell–expressed transcripts obtained from E-GEOD-20966. The X-axis represents $-\log_{10} P$ value, the dotted vertical line represents the significant $P$ value = .05, and relevant pathways are provided on the Y-axis. Pathways involved in (C) endocrine pancreatic β-cell function, (D) development and differentiation, (E) vesicle transport, (F) mitochondrial function, and (G) carbohydrate metabolism are presented. ATP, adenosine triphosphate; COPII, Coat protein complex II; NADH, Nicotinamide adenine dinucleotide phosphate.
liver and bile ducts are reported. All of these studies suggest evolutionary conserved common lineage of pancreas and gallbladder.

Insulin hormone immunoreactivity has been reported in body fluids other than blood. The presence of insulin in human gallbladder was reported in the 1960s, when insulin immunoassays were available to reliably measure insulin. These studies indicated significantly higher levels of insulin in the bile from the gallbladder, suggestive of a selective reabsorption or active production of insulin by

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**Figure 7. Human pancreatic single-cell RNA-seq shows the expression of gallbladder-enriched genes.**

(A) Gene ID, gene symbol, and average read count of 21 highly abundantly human gallbladder-enriched gene transcripts. (B) Uniform Manifold Approximation and Projection (UMAP) plot generated using Seurat (version 0.2.1) from human pancreatic single-cell RNA-seq (Panc8) data (see the Methods section for details). The single cells were clustered into 13 different pancreatic cell types, and each cluster is highlighted with a different color. (C) Violin plots showing the expression of each gene listed in panel A across all human pancreatic cell types presented in panel B. The Y-axis represents read counts (expression) of the gene transcript presented in each panel for the subset of human pancreatic cell type shown in panel B. The shape of the violin plot represents the density of individual data points and extends to minimum/maximum values. The color of the violin plot reflects the color and cell type from the UMAP plots in panel B.
gallbladder epithelium. Our quantitative studies (PCR and enzyme-linked immunosorbent assay [ELISA]) show that insulin is indeed produced in gallbladder epithelial cells. Immunostaining is a qualitative technique that provides a relative measure of immunopositive cells and the intensity of fluorescence depends on the acquisition settings. Our immunostaining data show that insulin protein is present in the gallbladder epithelium, while the quantitative data emphasize that it is significantly lower than in the islets. However, in a smaller number of individuals (N = 5) the presence of insulin in the cystic duct after glucose administration (Figure 10G) corroborates earlier observations of insulin immunoactivity within the gallbladder.

A significant body of evidence has shown that biliary-/gallbladder-derived stem/progenitor cells can produce insulin after recombinant PDX1, adenovirus-mediated transcription factor overexpression, or exposure to small molecules/growth factors. Several groups have reported their experience in propagating gallbladder epithelial cells from a range of species including mouse, rabbit, guinea pig, dog, bovine, and human beings. We used a nonenzymatic approach to isolate gallbladder epithelial cells. In line with previous reports, the overexpression of key pancreatic transcription factors (PDX1, MAFA, and NEUROG3) or HES1 inhibition in gallbladder-derived cells induced insulin expression.

Our studies in NOD mice, which naturally develop immune-mediated T1D, indicate that gallbladder epithelial cells do not show immune infiltration in T1D and that these cells may continue to produce small amounts of insulin. Considering recent reports of mechanisms contributing to autoimmunity in diabetes, a lower abundance of insulin, as well as the absence of alternate insulin splice forms (Figure 11) in the gallbladder, is encouraging. Genetic or environmental factors leading to endoplasmic reticulum (ER) stress are of critical importance in regulating the expression of alternative splice variants via alternative reading frames of insulin. Metabolic or inflammatory stress may lead to the generation of novel splice forms via recognition of a newly created splice site and the generation of defective ribosomal peptides. Such defective ribosomal peptides may act as neoantigens, to which central immune tolerance is absent. Another possibility is high levels of the taurine-conjugated bile acid tauroursodeoxycholic acid in the gallbladder, which is known to reduce ER stress and protect islet β-cells in T1D mouse models.

The present study had several strengths. This study collectively showed the interspecies and age-related similarities across 2 functionally diverse, but developmentally related, organs: the gallbladder and the pancreas. We present a comparison between the gallbladder and pancreatic cells/samples using multiple techniques to show similarities in chromatin conformation (ChIP-PCR), gene transcription (bulk/single-cell RNA-seq, TaqMan qPCR, gene reporter analyses), insulin production (confocal microscopy, ELISA, immune-electron microscopy), and insulin release (animal models, human cells, and clinical in situ measurements). We also present direct analysis of insulin-producing cells in gallbladder and pancreas from NOD mice.

A limitation was that our studies do not explain the possible mechanisms underlying the biological variation observed in insulin transcript abundance across different samples. None of the gallbladder donors had type 1 or type 2 diabetes, although we do not have data on prediabetes or undiagnosed type 2 diabetes (hemoglobin A1c levels) in these individuals. Future studies would need to assess proinsulin and insulin ratios in the same gallbladder epithelial cell samples to understand the concentrations of processed and mature insulin and be statistically powered to understand if differences are related to ethnic, genetic, disease, or environmental components. There is a consistent presence of Pdx1 transcripts in the majority of gallbladder cells; while variable levels of insulin suggest that in some cells the endocrine differentiation has progressed selectively, while it is inhibited in other cells. Single-cell sequencing technologies for PDX-1 and PDX-1Igalbladder cells would be informative to understand heterogeneity and to tease out regulatory pathways and molecules during gallbladder development. Although the differentiation of gallbladder-derived cells was not the principal aim of this study, differentiation strategies need improvement to enhance insulin production in gallbladder-derived progenitor cells followed by long-term functional validation in animal models of diabetes.

In summary, we show the inherent property of mouse and human gallbladder cells to transcribe, translate, package, and release insulin in response to glucose, although not at physiologically relevant levels. The capacity for insulin gene transcription in gallbladder epithelium appears to be driven by the developmental similarity, which programs pancreatic islet epigenetic and transcriptomic signatures in the gallbladder. The demonstration that this intrinsic insulin production escapes autoimmune damage during T1D progression renews interest in this functionally diverse organ.

Methods

Animals

Friend Virus B NIH Jackson (FVB/NJ) mice were used for embryonic gene expression and insulin content; CD1 mice were used for immune-gold labeling studies at the University of Bath; C57BL/6J mice were used for RNA-seq, qPCR, or ChIP studies; and Pdx1-GFP, MIP-GFP, and NOD
mice were used for gene reporter and immunostaining studies. All animals were maintained at the experimental animal facilities in India, the United Kingdom, or Australia according to guidelines outlined by the respective institute’s animal care and use committee. Ethical approvals for the study were obtained from animal ethics committees at the National Centre for Cell Science in India, St. Vincent’s Hospital in Melbourne, and the University of Sydney. Breeding pairs were set, and pregnancy was confirmed by observing vaginal smears. Pregnant females and newborn mice were killed at predefined intervals and pancreatic buds or pancreas, as well as gallbladder tissue, was dissected carefully without cross-contamination using a stereomicroscope. Tissue samples at each of these time
points were used for RNA isolation (in TRlZol, Thermo Fisher Scientific, Waltham, MA) and immunostaining (in 4% freshly prepared paraformaldehyde). Animal strain, numbers, age, and comparison groups for each experiment are provided in the respective Figure legends as per Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines.

**Human Tissue Collection**

Adult gallbladder, pancreas, and islet samples were obtained after human research ethics committee approvals from the Sydney Local Health District and St. Vincent’s Hospital in Melbourne. Fetal gallbladder and pancreas were obtained after informed consent from prospective parents who consented to fetal tissue for research after elective termination of pregnancy (<20 WGA) or after late abortions/miscarriage (>20 WGA) as per human research ethics committee approvals from the Shree Seva Medical Foundation (India) and the National Centre for Cell Science (India). Human cadaveric non-diabetic pancreas and islet samples were obtained as part of the research consented tissues through the Australian Islet Transplantation Program (at Westmead Hospital in Sydney and at St. Vincent’s Institute in Melbourne). Human gallbladders were obtained as surgical waste tissues (noncancerous) after cholecystectomies from the surgical teams (at Strathfield Private Hospital, Royal Prince Alfred Hospital [Sydney] and St. Vincent’s Hospital [Melbourne], and the National Centre for Cell Science [Pune, India]). Tissue samples were stored for RNA isolation (in TRlZol), immunostaining (in 4% freshly prepared paraformaldehyde), or processed for cell culture as detailed later. For in situ glucose stimulation study, we consented 5 individuals undergoing cholecystectomies for blood collection at the Asian Institute of Gastroenterology.

Blood was collected before and after the glucose challenge. The study was performed according to the Declaration of Helsinki II. Study participants provided written informed consent before inclusion as per the ethical committee approval from the Asian Institute of Gastroenterology in India.

**Gallbladder Cell Culture**

Gallbladder tissues were collected in transport medium (M199 with 25 mmol/L HEPES and 2 mmol/L glutamine medium with 2× antibiotics) and processed in the laboratory. The gallbladder sample was washed thoroughly with the serum-free medium (M199 with 25 mmol/L HEPES and 2 mmol/L glutamine + Ham’s F12k) containing 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Carlsbad, CA) and fungizone (Gibco) until bile was removed. The inner surface of the gallbladder then was scraped off gently with a disposable aseptic scalpel blade to isolate the epithelial lining. These isolated cells were washed with the wash medium (described earlier), centrifuged at 1000g for 2 minutes, and the cell pellet was resuspended and plated in serum-containing medium (10% fetal bovine serum + M199 with 25 mmol/L HEPES and 2 mmol/L glutamine + Ham’s F12k) with penicillin and streptomycin (Gibco). This medium is referred to hereafter as a growth-promoting medium or serum-containing medium. Cells were maintained in an incubator at 37°C and with humidified 5% CO2 in the air and passaged 1:2 when confluent using trypsin (Gibco) + EDTA. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to understand the medium composition supporting the maximum viability and metabolic activity of isolated cells. Briefly, 50,000 cells were seeded in each well of 96-well plates and allowed to adhere overnight. Different media and serum concentrations were formulated and added to cells (24 conditions) in quadruplets. After 24 hours and 48 hours, MTT reagent (Sigma-Aldrich, St. Louis, MO) was added to each well, and after 2–3 hours conversion of MTT
to formazan was measured using a spectrophotometer. The experiment was repeated with 3–4 different gallbladder preparations.

**In Vitro Differentiation of Human Adult Gallbladder-Derived Cells**

Human adult gallbladder-derived mesenchymal-like cells are trypsinized and differentiated after an optimized protocol in our laboratory.\(^{50-52}\) Briefly, these cells are plated on day 0 in serum-free medium (day 0 SFM) that consists of Dulbecco’s modified Eagle medium:F12 + 1% bovine serum albumin (BSA) +1\(\times\) insulin-transferrin-selenium. Single cells on day 0 start to aggregate into islet-like clusters (ICAs). On day 1, the medium was changed with day 0 SFM to remove dead cells. On days 4 and 7, ICAs were exposed to day 4 SFM that contained 0.3 mmol/L taurine + day 0 SFM. On day 10, ICAs were exposed to day 10 SFM that contained nicotinamide (1 mmol/L) and exendin-4 (100 nmol/L), along with day 4 SFM. On day 14 these ICAs were harvested for RNA isolation. In some experiments, DNA methyltransferases (DNMT) and histone deacetylase (HDAC) inhibitors were added during the entire time of differentiation at the following final concentrations: 1 mmol/L sodium butyrate, 100 nmol/L trichostatin A, 1 mmol/L valproic acid, 2 \(\mu\)mol/L 5-aza-2'-deoxycytidine, 5-Aza, 5-aza-2'-deoxycytidine; Dex, dexamethasone; GB, gallbladder; SB, sodium butyrate; TSA, trichostatin A; VPA, valproic acid.
and 1 μmol/L dexamethasone. Vehicle control (phosphate-buffered saline [PBS] or dimethyl sulfoxide) was used for comparison. Adenoviral vectors for PDX1, NEUROG3, and MAFA were used at 2 different multiplicities of infection in gallbladder-derived cells. Transfections were performed as described earlier.53 Transduced cells were differentiated following the protocol detailed earlier and GFP-alone adenovirus-transduced cells were used for gene expression comparisons.

**RNA Isolation, Complementary DNA Synthesis, and Real-Time qPCR**

RNA isolation from fresh human and mouse tissues at different stages (embryonic, adult) was performed using TRIzol (Invitrogen, Carlsbad, CA). RNA quality and quantity were measured on a ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Complementary DNA synthesis was performed using a High-Capacity Complementary DNA Reverse-Transcription Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s recommendations. Real-time qPCR was performed with TaqMan primer and probes mix (Thermo Fisher Scientific, Foster City, CA) for genes listed in Supplementary Table 2 and TaqMan Fast Universal PCR Master Mix (Thermo Fisher Scientific, Foster City, CA) in a 5-μL reaction in 96-well optical clear plates. The cycle threshold (Ct) values of all the genes were normalized to the housekeeping gene 18s ribosomal RNA. Transcript abundance was calculated using normalized Ct values and the following formula: 

\[ \text{abundance} = 2^{(39-\text{Ct value})} \]

where 39 is the limit of detection on the real-time PCR system.54 The TaqMan Low-Density Array (TLDA) cards were designed for selected pancreatic genes and housekeeping genes (Supplementary Table 3) and were obtained from Thermo Fisher Scientific (Waltham, MA). TLDA cards were used to assess gene expression in human fetal pancreas and gallbladder tissues using the manufacturer’s protocol standardized for TLDA cards on the 7900 HT system (Thermo Fisher Scientific, Waltham, MA). Normalized Ct values were used to plot hierarchical cluster heatmaps. A customized OpenArray Human Messenger RNA panel (Thermo Fisher Scientific, Waltham, MA) was designed to analyze the 45 selected pancreatic gene transcripts and housekeeping control genes in adult human islets and gallbladder (Supplementary Table 4). The customized panels were used following the manufacturer’s protocol standardized for gene expression using OpenArray on the QuantStudio 12K Flex Real-Time PCR platform (Thermo Fisher Scientific, Waltham, MA). Normalized Ct values were used to plot hierarchical cluster heatmaps.

**Bulk RNA Sequencing**

RNA-seq was performed on mouse tissues as detailed by Williams et al55 using the Ion Total RNA-Seq Kit v2 and the Ion OneTouch 200 Template Kit v2 DL (Thermo Fisher Scientific, Waltham, MA), and sequenced on the Ion Torrent PGM Instrument using the Ion PGM 316 Chip and the Ion PGM 200 Sequencing Kit v2 (Thermo Fisher Scientific, Waltham, MA). Adult human pancreatic islets and gallbladder epithelial cell samples (gallbladder data set GSE152419, n = 7; and human islet data set GSE152111, N = 66) were sequenced as 150 paired-end reads on the HiSeq4000 platform (Illumina, Singapore) as detailed elsewhere.20 Poly-T oligo-attached magnetic beads were used to purify the messenger RNA from total RNA for these samples.

**Bulk RNA-Seq Analysis**

The Strand next-generation sequencing version 2.5 software (Strand LS, Bengaluru, India) was used to analyze the RNA-seq data. For mouse samples, reads were aligned to the mouse mmu10 University of California Santa Cruz (UCSC) transcriptome and genome together with novel splice variants, using the Ensembl genes and transcript model. However, for human samples, reads were aligned to the human genome version 38 (hg38) transcriptome and genome together (with novel splice variants) using the Ensembl genes and transcript model. Raw reads were aligned with a minimum of 90% identity, a maximum of 5% gaps, and a minimum match length of 25 bps. The output of read-pairs with more than 5 valid matches was not reported. Quality trimming was applied to trim the 3’ end with an average quality less than 10 and on a poorly aligned portion at the 3’ end post alignment. After alignment, raw reads were filtered to remove reads with an average base quality below 20 and reads that have failed quality control. For mouse samples, an average of 2.57 million clean reads per sample were aligned to the reference transcriptome/gene (with novel splice variants; GSE152419). For human samples, an average of 29 million clean reads were generated through bulk RNA-seq of gallbladder epithelial cells (GSE152419, n = 7) and human islets (GSE152111, N = 66) and 78 million clean reads for another set of human islet samples (GSE134068, N = 18). DEseq was used to quantify and normalize the aligned reads, with the threshold normalized count set at 1.56. Baseline transformation was not applied to the preprocessing of the aligned read input data.

**Pancreatic Single-Cell RNA Sequencing Analysis**

Panc8 single-cell sequencing data (N = 14,890) were extracted from public data sets (GSE84133, GSE85241, EMTAB-5061, GSE83139, and GSE81608). It was analyzed via R studio version 1.2.5033 (RStudio, Boston, MA) (built under R 3.6.1; R Foundation for Statistical Computing, Vienna, Austria) using SeuratData (version 0.2.1), Seurat, ggplot2, and cowplot packages. The original Seurat Pance8 package contains 8 different pancreas single-cell RNA-seq data sets from across 5 technologies (inDrop, CEL-Seq1, CEL-Seq2, Smart-Seq2, and Fluidigm C1). To improve the integrity of the data, low-read-count data sets obtained through inDrop technology were excluded. Analytical workflow included data preprocessing and feature selection, dimension reduction, and identification of anchor correspondences between data sets; filtering, scoring, and weighting of anchor correspondences; and data matrix correction or data transfer across experiments as described elsewhere.
**Differential Splicing Analysis**

Differential splicing was analyzed using the Strand next-generation sequencing software. Differential splicing of the gene is based on changes in transcript proportion across the samples. The splice index of a gene refers to the variation associated with the proportion of transcript between each sample. The index is between 0 and 1, and a larger index indicates more differential expression/splicing.
**Immunostaining**

Tissue sections/cells were immunostained as described by Joglekar and Hardikar. For immunostaining, tissue sections or freshly isolated epithelial clusters, islets, or cultured gallbladder cells were fixed in 4% paraformaldehyde (PFA). Tissues were embedded in paraffin and 5- to 10-μm sections were placed on slides for further processing. H&E staining was performed on tissue sections using standard protocol after deparaffinization. For immunohistochemistry, permeabilization was performed in chilled methanol (50% v/v in water) or 0.5% Triton X-100 (Thermo Fisher Scientific, Waltham, MA) followed by blocking with 4% normal donkey serum (Sigma-Aldrich). Incubation with the primary antibody was at a dilution of 1:100 or 1:200 overnight at 4°C. Cells then were washed 5 times with 1× PBS containing Ca²⁺ and Mg²⁺ (Gibco) and incubated with secondary antibody at 37°C. Cells then were washed thoroughly and mounted in Vectashield mounting medium (Vector Laboratories, Peterborough, UK) containing Hoechst 33342 (Invitrogen). The primary antibodies used were rabbit polyclonal antibody to human C-peptide and guinea pig anti-insulin (both from Linco Research, Inc, St. Charles, MO), rabbit antisomatostatin (Dako, Santa Barbara, CA), mouse antiguclidean, mouse anti-GFP, mouse anti-smooth muscle actin, and rabbit anti-slug (all from Sigma-Aldrich), goat anti-Pdx1 (Abcam, Cambridge, UK), mouse anti-E-cadherin and mouse anti-β-catenin (BD Biosciences, Franklin Lakes, NJ), and mouse monoclonal antivimentin (Chemicon Int, Inc, Temecula, CA). Alexa-Fluor 488, 546, and 633 secondary antibodies (Invitrogen) were used at 1:200 dilution. Antibody concentrations and incubation times were identical across both tissue types (islets and gallbladder) and all samples. Images were scanned and assessed separately for islets and gallbladder cells using a Zeiss LSM 510 laser scanning confocal microscope (Zeiss, Oberkochen, Baden-Württemberg, Germany). Current voltage applied to the photomultiplier tube, and laser intensities were identical across all samples within a tissue type and were adjusted so as to have below-saturation thresholds. Significantly lower laser power was needed to achieve below-saturation signal in the islet samples.

**Immunoelectron Microscopy**

CD1 mouse gallbladder and pancreas isolated from the same animal were fixed in 0.5% glutaraldehyde, 4% paraformaldehyde (Agar Sciences, Stansted, Essex, England), and 2.5 mmol/L CaCl₂ (Thermo Fisher Scientific, Waltham, MA) in 0.1 mol/L sodium cacodylate (Agar Sciences, Stansted, Essex, England) buffer (SCB) at 4°C overnight, rinsed the next day in a solution of 0.1 mol/L SCB and 2.5 mmol/L CaCl₂ for 10 minutes, with 4 changes at room temperature. Samples were dehydrated in ethanol (70%, 90%, 100% dry X 2) for 15 minutes at room temperature, infiltrated with London resin (LR) white (Agar Sciences, Stansted, Essex, England) for 1 hour at room temperature, overnight at 4°C, and again at room temperature for 1 hour. Each tissue was transferred to a gelatin capsule (size 0; Agar Sciences, Stansted, Essex, England) and the capsule was filled completely with LR white resin and allowed to polymerize at 50°C for 24 hours. Sections (100-nm) were cut and mounted on a formvar-carbon-coated nickel slot grid (Agar Sciences, Stansted, Essex, England) and immunolabeled by floating the mounted grids on respective solutions. Sample (grids) were exposed to 0.05 mol/L glycine (Sigma) in PBS to block aldehyde sites for 15 minutes, rinsed briefly with PBS, blocked in blocking solution with 10% normal goat serum, 0.5% BSA in PBS for 15 minutes, rinsed briefly, and then incubated in guinea pig anti-insulin (Linco Research, Inc) at a dilution of 1:500 overnight at 4°C. Grids then were washed with PBS 3 times for 10 minutes each, and incubated in secondary antibodies (gold-tagged goat anti-guinea pig IgG; British Biocell, Cardiff, UK) at 1:50 dilution for 2 hours at room temperature, covered, and washed in 0.1 mol/L SCB 3 times. The grids then were postfixed with 1% glutaraldehyde in 0.1 mol/L SCB for 15 minutes, rinsed twice with distilled water for 5 minutes each, and then dried. The grids then were stained in 2% osmium tetroxide and poststained with 1% uranyl acetate and 1% lead citrate. Sections were then washed in distilled water before embedding in LR white resin. Grids were examined with a Zeiss EM 109 transmission electron microscope (Zeiss, Oberkochen, Germany). Scale bars: 200 nm. *P < .05, **P < .01, ***P < .001, and ****P < .0001. GB, gallbladder.
Figure 11. Analysis of INS splice variants in human islet and gallbladder samples. Differential splicing was analyzed on the INS gene separately for human islet samples (GSE152111, N = 66) and gallbladder samples (GSE152419, n = 7). (A) The 5 INS transcript variants, along with partition coverage for INS gene (orange). The INS transcript splice variant profile is shown across (B) human islet samples and (C) gallbladder samples. (B and C) The 5 INS transcript splice variants, INS-201 (ENST00000250971), INS-202 (ENST00000381330), INS-203 (ENST00000397262), INS-204 (ENST00000421783), and INS-205 (ENST00000512523), along with the INS gene (red line, as per color legion), are shown. Data are presented in read density, which is the proportion of all reads associated with the gene computed for the quantification allocated to each transcript of the gene fractionally.
uranyl acetate for 10 minutes, covered with lead citrate for approximately 4 minutes in a NaOH-containing chamber, washed with fresh distilled water, dried, and then imaged using transmission electron microscopy (Jeol 1200EX transmission electron microscopy, University of Bath, UK).

**Flow Cytometry**

Pdx1-GFP and MIP-GFP mice were killed at predetermined embryonic and adult stages. Pancreas and gallbladder tissues were stored on ice, finely chopped, followed by collagenase digestion (3 mg/mL, 10 minutes at 37°C) to generate a single-cell suspension. Cells were strained through a 70-μm cell strainer and then resuspended in fluorescence-activated cell sorting (FACS) buffer (Ca²⁺, Mg²⁺-free PBS +2% fetal calf serum) and acquired on a BD FACS Calibur (BD Biosciences, Franklin Lakes, NJ). Gallbladder-derived mesenchymal-like monolayer cells at passage 5 were harvested using trypsin. Cells were washed twice with FACS buffer, blocked with 5% BSA in 1× PBS (Ca²⁺ and Mg²⁺ free) for 30 minutes, incubated with fluorescence-conjugated antibodies for 1 hour, washed twice with FACS buffer, fixed with 4% PFA, and then acquired using FACS Calibur. The antibodies used were phycoerythrin-labeled rat IgG2 (isotype control), anti-CD29, anti-CD44, anti-CD90, and anti-CD105 (all phycoerythrin-labeled; BD Biosciences, Franklin Lakes, NJ) at dilutions recommended by the manufacturer. The data were analyzed using CellQuest Pro software (BD Biosciences, Franklin Lakes, NJ). Isotype control or wild-type tissues were used to set up gating. Propidium iodide (PI)⁺ (dead) cells were kept unchanged for the same type of tissue or cells.

**Cell Lineage Tracing**

To follow the propagation of the insulin-positive cells in vitro, we used 2 thymidine analogues, CldU and IdU (Sigma-Aldrich), as described previously.14 Freshly isolated gallbladder epithelial cells were seeded on Lab-Tek chamber slides (Nunc, Rochester, NY). CldU was added to freshly isolated cells at a concentration of 10 μmol/L and was washed with fresh medium with 1% BSA for 3 hours postsurgery after scraping of the epithelial layer (Figure 8B, and day 0 of 8D). They were kept in serum-free medium with 1% BSA for 3–4 hours before being handpicked under a phase-contrast microscope. These then were washed with Krebs–Ringer bicarbonate HEPES buffer containing 0.1% BSA (Sigma) and exposed in quadruplicate to basal (2.5 mmol/L glucose) or stimulated (25 mmol/L glucose) buffer, for 1 hour at 37°C. At the end of the incubation/exposure, cells were settled down or centrifuged at 300g for 1 minute to pellet. The supernatant was collected and assayed for insulin/C-peptide. The insulin content in the tissues/cells was measured by sonicating them in 200–500 μL acid ethanol, depending on the size of a tissue or cell pellet. The total protein concentration was measured using the Bradford assay (for mouse samples) or the Thermo Fisher Scientific (Waltham, MA) Qubit protein assay (for human samples). Insulin or C-peptide concentrations were

**ChIP**

Adult mouse gallbladder and pancreas, freshly isolated epithelial cells from the adult human gallbladder, freshly isolated human islets, and gallbladder-derived mesenchymal cells in culture at passage 5 were used in ChIP assay as described previously.50 Freshly isolated gallbladder epithelial cells (Figure 8B, and day 0 of 8D) from 3 to 5 donors had to be pooled in 2 experiments for ChIP to meet the requirement of high cell numbers. Briefly, cells were cross-linked, washed with buffers containing protease inhibitor cocktail (Sigma-Aldrich), and sonicated in lysis buffer to generate DNA fragments of 200–400 bps. Chromatin was immunoprecipitated using 2 μg specific dimethyl and trimethyl antibodies for H3K4 and H3K9, as well as for acetylation of H3K9 and H3 and H4 (Millipore, Billerica, MA). Precipitation cocktails included protein A/G plus beads (Pierce, Pittsburgh, PA), sonicated salmon sperm DNA (Amersham Biosciences, Pittsburgh, PA), and BSA (USB Corporations, Cleveland, OH). Rabbit and mouse IgG (Upstate, Millipore) were used as isotype controls. Chromatin was eluted using 2% sodium dodecyl sulfate, 0.1 mol/L NaHCO₃, and 10 mmol/L dithiothreitol. Cross-links were reversed by incubating the eluted chromatin in 4 mol/L NaCl overnight at 65°C. This was followed by proteinase-K digestion and DNA extraction using phenol–chloroform–isoamyl alcohol. Input, immunoprecipitated, and isotype control DNA was resuspended in nuclease-free water and used for SYBR green or TaqMan qPCR with the primers listed in Supplementary Table 5 and with Fast SYBR green or TaqMan Fast master mix (Thermo Fisher Scientific, Waltham, MA) on the ViiA7 Real-Time PCR System platform (Thermo Fisher Scientific, Waltham, MA).

**Glucose-Stimulated Insulin Secretion**

Gallbladder epithelial clusters were obtained within 12–24 hours postsurgery after scraping of the epithelial layer (Figure 8B, and day 0 of 8D). They were kept in serum-free medium with 1% BSA for 3–4 hours before being handpicked under a phase-contrast microscope. These then were washed with Krebs–Ringer bicarbonate HEPES buffer containing 0.1% BSA (Sigma) and exposed in quadruplicate to basal (2.5 mmol/L glucose) or stimulated (25 mmol/L glucose) buffer, for 1 hour at 37°C. At the end of the incubation/exposure, cells were settled down or centrifuged at 300g for 1 minute to pellet. The supernatant was collected and assayed for insulin/C-peptide. The insulin content in the tissues/cells was measured by sonicating them in 200–500 μL acid ethanol, depending on the size of a tissue or cell pellet. The total protein concentration was measured using the Bradford assay (for mouse samples) or the Thermo Fisher Scientific (Waltham, MA) Qubit protein assay (for human samples). Insulin or C-peptide concentrations were
measured by an ELISA kit (Mercodia, Winston Salem, NC). Circulating human insulin was measured using the same kits (Mercodia) after plasma separation. Plasma was separated by centrifugation at 1000g for 10 minutes and stored at -80°C before performing insulin ELISAs. Blood glucose was measured using the Accu-Chek glucometer (Roche Diagnostics, Basel, Switzerland).

**Transplantation of Human Gallbladder Epithelial Cells**

Transplantation of freshly isolated gallbladder epithelial cells was performed on 8- to 12-week-old male NOD/SCID mice to assess their function in response to glucose stimulation as described previously. During this procedure, animals were placed on their back after anesthesia with isoflurane. A total of 500–700 freshly isolated gallbladder epithelial clusters were added into approximately 20 µL animal blood (obtained from the tail) to form a blood clot, which then was transplanted under the kidney capsule of NOD/SCID mice without losing any of the cell clusters. Animals were opened by making a left lateral incision to expose the left kidney. The kidney gently was pulled out and a superficial cut was made in the kidney capsule. The blood clot containing gallbladder cells was placed below the kidney capsule. The capsule then was massaged gently to close the cut. The kidney then was placed back in its original position. The incision was closed using 3–4 absorbable sutures (Davis-Geck, Manati, PR) and an autoclip wound clipper (BD Biosciences). Topical ointment (Soframycin; Sanofi-aventis, NSW, Australia) was applied over the sutured wounds after surgery and animals were administered analgesics (0.05 mg/kg buprenorphine every 12 hours for 3 days). On the 30th day after surgery, blood was collected at 30 minutes after a 2 g/kg body weight glucose load.

**Pathway Analysis**

To analyze enrichment for β-cell pathways, lists of gallbladder-expressed genes (GSE152419) were compared with β-cell–expressed genes (from E-GEOD-20966) using GO analysis on Pantherdb.org. Pre-analytic workflows included cleaning up entries not mapping to protein-coding gene symbols. Gene lists of 16,584 gallbladder transcripts and 13,164 β-cell transcripts were compared for overlap in transcripts using Venn diagrams (https://bioinfogg.cnb.csic.es/tools/venny/index.html), identifying 7227 genes present in both the gallbladder and β-cell expression data set. GO analysis was performed using the list of 7227 gallbladder transcripts also present in β-cells and compared with the list of all human genes as reference. Statistical over-representation was calculated using the Fisher exact test, using Bonferroni correction for multiple testing.

**Statistical Analysis**

Statistical analyses were performed using GraphPad Prism 8.4.1 (GraphPad Software, San Diego, CA) or R software (version 3.6.2; R Foundation for Statistical Computing, Vienna, Austria), SPSS Statistics 27 (Chicago, IL), or Microsoft Excel (version 2016; Microsoft, Redmond, WA). R software was used to perform unsupervised hierarchical clustering maps using heatmap.2 function in gplots. Add-on and other R packages XLconnect (with ActivePerl software) and RColorBrewer were used for data set import and visualization along with R package gplots. GraphPad Prism was used to perform all remaining analyses using appropriate statistical tests and corrected for multiple comparisons if required. Details of each statistical test, the number of replicates, and the number of animals/biological preparations are provided in the respective Figure legends. Split violin and spider/radar plots were created using the BioVinci data visualization package (BioTuring Inc, San Diego, CA). The Kolmogorov–Smirnov test was used to check for data normality in SPSS. The F-test was performed to check for variance in Excel. For non-normally distributed data, a 2-tailed Mann–Whitney test was used to calculate the P value with no ties computed as performed in R. For normally distributed data with equal or unequal variance, a 2-tailed Student or Welch t test was used to calculate the P value in Excel, respectively.

All authors had access to the study data and reviewed and approved the final manuscript.

**Data and Materials Availability**

The Biospecimen Reporting for Improved Study Quality guidelines was followed. Single-cell sequencing (Panc8) data sets (N = 14,890) were extracted from already available data sets (GSE84133, GSE85241, E-MTAB-5061, GSE81076 and GSE86469). Our data on bulk RNA-seq for the human gallbladder data set (GSE152419, n = 7) and human islet data sets (GSE152111, N = 66 and GSE134068, N = 18), have been uploaded to the Gene Expression Omnibus database. Data for mouse gallbladder and pancreas bulk RNA-seq also are available through GSE152419.

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The authors disclose no conflicts.

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### Supplementary Table 2. Gene Assay IDs Used for qPCR

| Target species | Assay ID          | Gene symbol | Gene name                                                                 | Dye         |
|----------------|-------------------|-------------|---------------------------------------------------------------------------|-------------|
| Human          | Hs02741908_m1     | INS         | Insulin                                                                  | FAM-MGB     |
| Human          | Hs00174967_m1     | GCG         | Glucagon                                                                 | FAM-MGB     |
| Human          | Hs00174949_m1     | SST         | Somatostatin                                                             | FAM-MGB     |
| Human          | Hs01651425_s1     | MAFA        | V-Maf (v- musculoaponeurotic fibrosarcoma) avian musculoaponeurotic fibrosarcoma oncogene homolog A | FAM-MGB     |
| Human          | Hs00360700_g1     | NGN3        | Neuronogenin 3                                                           | FAM-MGB     |
| Human          | Hs00172878_m1     | HES1        | Hairy and enhancer of split 1 (hairy and enhancer of split family basic helix-loop-helix (BHLH) transcription factor 1) | FAM-MGB     |
| Human          | Hs03003631_g1     | 18S         | Eukaryotic 18S ribosomal RNA                                             | VIC-MGB     |
| Mouse          | Mm01259683_g1     | Ins1        | Insulin-1                                                                | FAM-MGB     |
| Mouse          | Mm00731595_gH     | Ins2        | Insulin-2                                                                | FAM-MGB     |
| Mouse          | Mm00801712_m1     | Gcg         | Glucagon                                                                 | FAM-MGB     |
| Mouse          | Mm00436671_m1     | Sst         | Somatostatin                                                             | FAM-MGB     |
| Mouse          | Mm00437606_s1     | Neurog3     | Neurogenin 3                                                             | FAM-MGB     |
| Mouse          | Mm01342805_m1     | Hes1        | Hairy and enhancer of split 1                                             | FAM-MGB     |
| Mouse          | Mm00435565_m1     | Pdx1        | Pancreatic and duodenal homebox 1                                        | FAM-MGB     |
| Mouse          | Mm00447452_m1     | Hnf1b       | Hepatocyte nuclear factor 1-β                                            | FAM-MGB     |
| Mouse          | Mm00448840_m1     | Sox9        | Sex-determining gene on the Y chromosome (SRY)-box transcription factor 9 | FAM-MGB     |
| Mouse          | Mm00433964_m1     | Hnf4a       | Hepatocyte nuclear factor 4-α                                            | FAM-MGB     |
| Mouse          | Mm00468656_m1     | Hlx         | H2.0-like homeobox                                                       | FAM-MGB     |
| Mouse          | Mm03302249_g1     | Gapdh       | Glyceraldehyde-3-phosphate dehydrogenase                                 | FAM-MGB     |

**NOTE.** List of TaqMan primer/probe assays selected for real-time qPCR on the ViiA7 platform. rRNA, ribosomal RNA. FAM-MGB, Fluorescein amidites-minor groove binder;
## Supplementary Table 3. List of TaqMan Primer/Probe Gene Expression Assays for Real-Time qPCR on the TLDA Platform

| Assay ID   | Gene symbol | Gene name                                                                 |
|------------|-------------|---------------------------------------------------------------------------|
| Hs00169631_m1 | INSR        | Insulin receptor                                                          |
| Hs00218236_m1 | FOXJ2       | Forkhead box J2                                                           |
| Hs00174949_m1 | SST         | Somatostatin                                                              |
| Hs00607978_s1 | CCR4        | C-X-C (Cysteine-X-cysteine) motif chemokine receptor 4                     |
| Hs00426835_g1 | ACTA2       | Actin α2, smooth muscle                                                    |
| Hs00183740_m1 | DKK1        | Dickkopf WNT signaling pathway inhibitor 1                                 |
| Hs00175619_m1 | PCSK1       | Proprotein convertase subtilisin/kexin type 1                             |
| Hs00707120_s1 | NES         | Nestin                                                                    |
| Hs00355773_m1 | INS         | Insulin                                                                    |
| Hs00174967_m1 | GCG         | Glucagon                                                                  |
| Hs00240792_m1 | FGFR2       | Fibroblast growth factor receptor 2                                       |
| Hs00274931_s1 | GAL         | Galanin and GMAP (galanin message associated peptide) prepropeptide       |
| Hs00174139_m1 | CD44        | CD44 molecule (Indian blood group)                                        |
| Hs00171403_m1 | GATA4       | GATA binding protein 4                                                    |
| Hs00264887_s1 | POU3F4      | POU class 3 homebox 4                                                     |
| Hs00240871_m1 | PAX6        | Paired box 6                                                              |
| Hs00185584_m1 | VIM         | Vimentin                                                                  |
| Hs00165775_m1 | GLUT2 (SLC2A2) | Solute carrier family 2 member 2                                        |
| Hs00153380_m1 | CCND2       | Cyclin D2                                                                 |
| Hs00232018_m1 | GATA6       | GATA binding protein 6                                                    |
| Hs99999901_s1 | 18S         | Eukaryotic 18S ribosomal RNA                                               |
| Hs00606262_g1 | HDAC1       | Histone deacetylase 1                                                     |
| Hs00173014_m1 | PAX4        | Paired box 4                                                              |
| Hs00360700_g1 | NEUROG3     | Neurogenin 3                                                               |
| Hs00232355_m1 | NKX6-1      | NK6 homeobox 1                                                            |
| Hs00232128_m1 | HLXB9       | Motor neuron and pancreas homeobox 1                                      |
| Hs00192380_m1 | SERPIN1     | Serpin family 1 member 1                                                  |
| Hs00170285_m1 | ICAM5       | Intercellular adhesion molecule 5                                          |
| Hs00168575_m1 | P4HA1       | Prolyl 4-hydroxylase subunit α 1                                          |
| Hs00164438_m1 | ENG         | Endoglin                                                                   |
| Hs00159922_m1 | PCSK2       | Proprotein convertase subtilisin/kexin type 2                             |
| Hs00179829_m1 | FGFR3       | Fibroblast growth factor receptor 3                                       |
| Hs00210096_m1 | KRT23       | Keratin 23                                                                |
| Hs00247426_m1 | DKK3        | Dickkopf WNT (Wingless-related integration site) signaling pathway inhibitor 3 |
| Hs00187320_m1 | HDAC3       | Histone deacetylase 3                                                     |
| Hs00170423_m1 | CDH1        | Cadherin 1                                                                 |
| Hs00606991_m1 | MKI67       | Marker of proliferation Ki-67                                              |
| Hs00167155_m1 | SERPINE1    | Serpin family E member 1                                                  |
| Hs00159598_m1 | NEUROD1     | Neuronal differentiation 1                                                |
| Hs00231032_m1 | HDAC2       | Histone deacetylase 2                                                     |
| Hs00169777_m1 | PECAM1      | Platelet and endothelial cell adhesion molecule 1                          |
| Hs99999905_m1 | GAPDH       | Glyceraldehyde-3-phosphate dehydrogenase                                  |
| Hs00234422_m1 | MMP2        | Matrix metallopeptidase 2                                                 |
| Hs00157705_m1 | GLP1R       | Glucagon-like peptide 1                                                  |
| Hs00169851_m1 | NCAM1       | Neural cell adhesion molecule 1                                           |
| Hs00158126_m1 | ISL1        | ISL LIM homeobox 1                                                         |
| Hs00277220_m1 | GCK         | Glucokinase                                                                |
| Hs00233790_m1 | ITGAV       | Integrin subunit α V                                                       |

**NOTE.** All assays use FAM-MGB (Fluorescein amidites-minor groove binder) dye at 20× stock concentration. 18S is the housekeeping gene.
### Supplementary Table 4. List of TaqMan Primer/Probe Gene Expression Assays for Real-Time qPCR on the OpenArray Platform

| Assay ID         | Gene symbol | Gene name                                                                 |
|------------------|-------------|---------------------------------------------------------------------------|
| Hs00173014_m1    | PAX4        | Paired box 4                                                              |
| Hs00271378_s1    | MAFB        | V-Maf (v- musculoaponeurotic fibrosarcoma) avian musculoaponeurotic fibrosarcoma oncogene homolog B |
| Hs00359592_m1    | NOVA1       | Neuro-oncologic ventral antigen 1                                           |
| Hs00240858_m1    | PAX2        | Paired box 2                                                              |
| Hs03003631_g1    | 18S         | Eukaryotic 18S ribosomal RNA                                               |
| Hs00240871_m1    | PAX6        | Paired box 6                                                              |
| Hs00268388_s1    | SOX4        | SRY (sex-determining gene on the Y chromosome)-box transcription factor 4 |
| Hs01001343_g1    | SOX9        | SRY (sex-determining gene on the Y chromosome)-box transcription factor 9 |
| Hs00603586_g1    | PTF1A       | Pancreas associated transcription factor 1a                                 |
| Hs00167041_m1    | HNF6 (ONECUT1) | Hepatocyte nuclear factor 6 (1 cut homeobox 1)                           |
| Hs01001602_m1    | HNF1b (TCF2) | Hepatocyte nuclear factor 1-β (transcription factor 2)                     |
| Hs00167041_m1    | HNF1a (TCF1) | Hepatocyte nuclear factor 1-α (transcription factor 1)                     |
|                  | HNF4α       | Hepatocyte nuclear factor 4-α                                               |
| Hs00541450_m1    | GLIS3       | GLIS family zinc finger 3                                                  |
| Hs00232355_m1    | NKX6.1      | NK6 transcription factor related, locus 1                                  |
| Hs00159616_m1    | NKX2.2      | NK2 transcription factor related, locus 2                                  |
| Hs00892941_m1    | PROX1       | Prospero-related homeobox 1                                               |
| Hs01367669_g1    | HES3        | Hairy and enhancer of split 3 (Hes family BHLH (basic helix-loop-helix) transcription factor 3) |
| Hs01922995_s1    | NEUROD1     | Neuronal differentiation 1                                                |
| Hs00892681_m1    | GLUT1 (SLC2A1) | Solute carrier family 2 (facilitated glucose transporter), member 1      |
| Hs01096908_m1    | GLUT2 (SLC2A2) | Solute carrier family 2 (facilitated glucose transporter), member 2      |
| Hs01564555_m1    | GCK         | Glucokinase                                                               |
| Hs00846499_s1    | UCN3        | Urocortin 3                                                               |
| Hs00158126_m1    | ISL1        | ISL LIM homeobox 1                                                         |
| Hs00171403_m1    | GATA4       | GATA binding protein 4                                                    |
| Hs00232018_m1    | GATA6       | GATA binding protein 6                                                    |
| Hs00292465_m1    | ARX         | Aristalless related homeobox                                              |
| Hs01005963_m1    | IGF2        | Insulin-like growth factor 2                                               |
| Hs00649887_s1    | POU3F4      | POU class 3 homeobox 4                                                    |
| Hs00703572_s1    | BHLHA15 (MIST1) | Basic helix-loop-helix family member A15                                 |
| Hs00907365_m1    | HB9 (MNX1)  | Homeobox HB9 (motor neuron and pancreas homeobox 1)                       |
| Hs00158750_m1    | LMX1.2 (LMX1B) | LIM homeobox transcription factor 1β                                      |
| Hs00892663_m1    | LMX1.1 (LMX1A) | LIM homeobox transcription factor 1α                                      |
| Hs01708080_m1    | CDX2        | Caudal type homeobox 2                                                    |
| Hs00793699_g1    | GSX1        | Genomic Screened homeobox 1                                               |
| Hs00370195_m1    | GSX2        | Genomic Screened homeobox 2                                               |
| Hs01116195_m1    | EXO1        | Exonuclease 1                                                             |
| Hs0170171_m1     | REG3A       | Regenerating family member 3α                                              |
| Hs01551078_m1    | TLR3        | Toll-like receptor 3                                                       |
| Hs00358111_g1    | PPY         | Pancreatic polypeptide                                                    |
| Hs00230829_m1    | AIRE        | Autoimmune regulator                                                      |
| Hs01074053_m1    | GHRL        | Ghrelin and obestatin prepropeptide                                        |
| Hs01026107_m1    | PCSK1       | Proprotein convertase subtilisin/kexin type 1                             |
| Hs00159922_m1    | PCSK2       | Proprotein convertase subtilisin/kexin type 2                             |
| Hs00232764_m1    | HNF3α (FOXA2) | Hepatocyte nuclear factor 3α (forkhead box A2)                            |

**NOTE.** All assays use FAM-MGB (Fluorescein amidites-minor groove binder) dye at 20x stock concentration. 18S is the housekeeping gene.
**Supplementary Table 5. List of Primer Sets Used for ChIP DNA qPCR**

| Species | Gene locus | Forward primer | Reverse primer | Probe |
|---------|------------|----------------|----------------|-------|
| Mouse   | Pdx1 A-I   | CCAGTATCAGGGAGGACTATCA | TACCCAGCCATTAGGCAAGA |       |
| Mouse   | Pdx1 A-III | ACCGTGTCAACAGTCTCAACCC | AGAGCCACCTGTGCCCGTCAA |       |
| Mouse   | Pdx1 A-IV  | CTCTTCTGTACCTCACTGAGTC | AACTAAGTGCTCTGGGCTCTG |       |
| Human   | INS pro    | GTGAAAAGTGTAGTTAGGTGAGGT | ACCTGCTTGTGGCCCTCTCTGT |       |
| Human   | PDX1 pro   | CACACAACGAAATCCACAGGTTTCG | ACTGATCTTCAGAGGAAACCCACA |       |
| Human   | NEUROG3 pro| AAGAGAGGCGATGAAACACCAGGA | AACTCTCGGTTCCTCAAAGAGGCT |       |
| Human   | HES1 pro   | TCCTTCTCCCCATTGGCTGAAGT | TTGGTGATCAGTAGGCGCTGTCGA |       |
| Human   | INS -275   | TGTGAGCAGGGACAGGTCTG | TCCTCAGGACCAGCGGG | 6FAM-CCACCGGGCCTGGTAAAGACTCTA |
| Human   | INS +1318  | CAGCTGGAGAATCTGCACTAGA | GCTGGTTCAAGGGCTTTATTCC | 6FAM-CCGCTCTGGCACCGAGAGA |

**NOTE.** Sequences start from 5’ to 3’. Gene loci for which a probe sequence is not provided were performed using SYBR green chemistry. TaqMan qPCR was used for human INS-275 and INS+1318. Pro, promoter.