Duct cells in development, regeneration, and transplantation: charting a path to new islets

Balak, J.R.A.

DUCT CELLS IN DEVELOPMENT, REGENERATION, AND TRANSPLANTATION

CHARTING A PATH TO NEW ISLETS

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Charting a Path to New Islets

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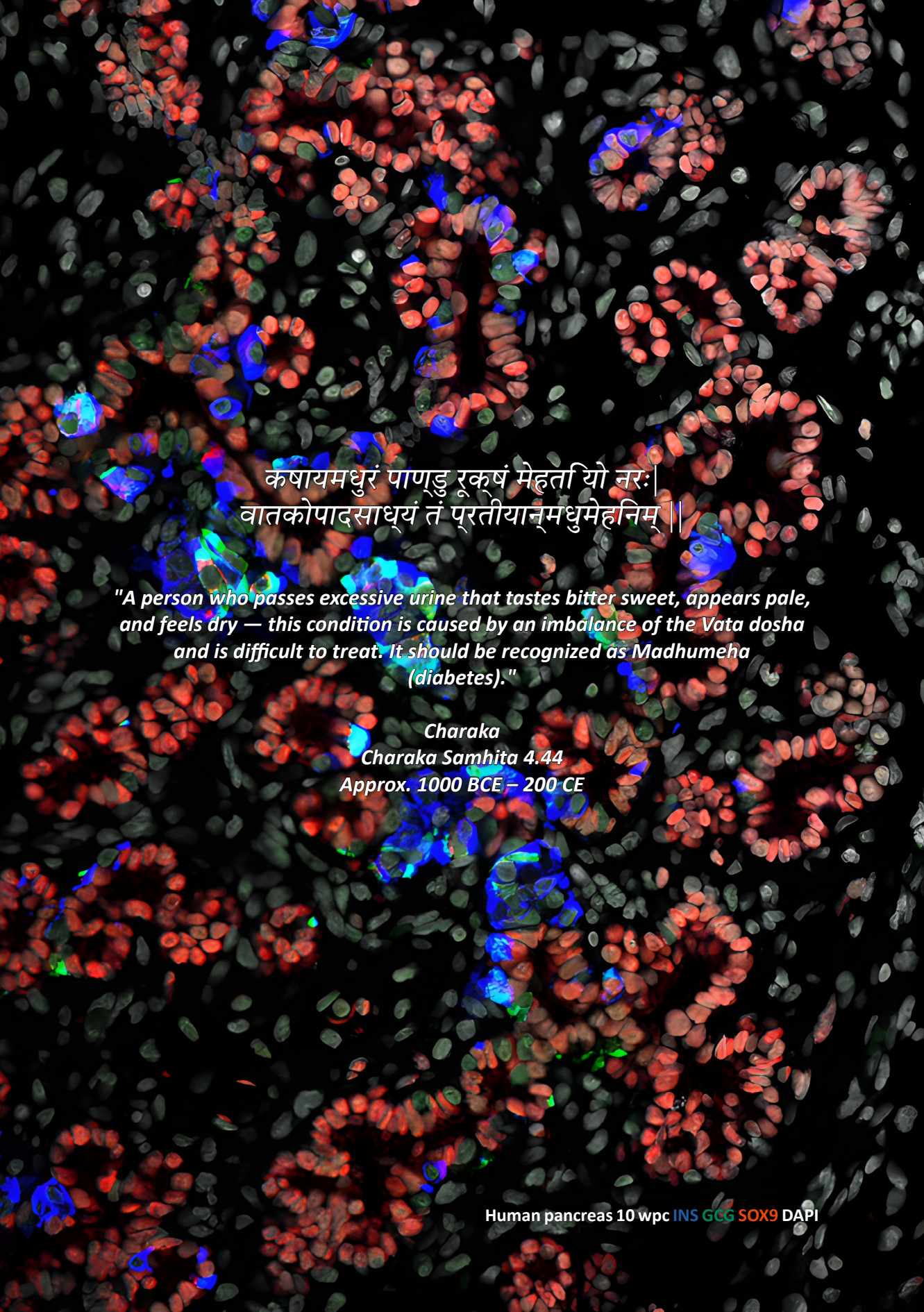

कषायमधुरं पाण्डु रूक्षं मेहति यो नरः|
वातकोपादसाध्यं तं प्रतीयान्मधुमेहनिम् ||

"A person who passes excessive urine that tastes bitter sweet, appears pale, and feels dry — this condition is caused by an imbalance of the Vata dosha and is difficult to treat. It should be recognized as Madhumeha (diabetes)."

Charaka
Charaka Samhita 4.44
Approx. 1000 BCE – 200 CE

Human pancreas 10 wpc INS GCG SOX9 DAPI

CHAPTER| 1

General Introduction and Aims of This Thesis

General introduction

Diabetes mellitus type 1 (T1DM) is characterised by an autoimmune-mediated destruction of insulin-producing beta cells[1]. The hyperglycemia associated with T1DM can result in devastating vascular complications, leading to significant disability and an increased risk of premature death[2]. The discovery of insulin as a therapy for diabetes more than 100 years ago was a major breakthrough, marking the end of imminent premature death for patients with T1DM[3]. However, insulin is no cure and can merely alleviate complications of diabetes.

Replenishment of lost insulin-producing beta cells via transplantation provides superior glycemic control in T1DM patients compared to insulin therapy[4-6]. However, shortage of donor organs prevents widespread use of this therapy, prompting numerous studies focused on pancreas development and homeostasis in an effort to find new therapeutic options.

One attractive alternative approach is to use adult progenitor cells to generate new beta cells for replacement therapy. This thesis is focused on investigating the possible role of pancreatic ductal cells in beta cell regeneration. The introductory chapter offers a concise overview of pancreas anatomy, function, development, and disease. In addition, beta cell replacement therapy, alternative cell sources for beta cell replacement therapy, beta cell regeneration and the outlines of this thesis are presented.

The pancreas

The pancreas is an abdominal organ and essential for digestion and glucose homeostasis. Although they did not understand its function, the ancient Greeks were the first to identify the pancreas as a distinct organ. Rufus of Ephesus (100 A.D.) coined the term *'pancreas'* from the Greek *'pan'* (all) and *'kreas'* (flesh or meat), most likely due to the relatively uniform composition and consistency of the organ without bone or cartilage[7]. The pancreas has historically been overlooked, probably due to its somewhat hidden retroperitoneal location behind the stomach. Abdominal surgeons in the early 20th century referred to the pancreas as the '*hermit organ*', because operations on it were rare.

Anatomy and function

The pancreas is located in the retroperitoneal space of the upper abdomen, weighs approximately 50-100 grams and has a length of 14-18 cm. It can be divided into a head, body and tail region; the head of the pancreas is located in the inner curvature of the duodenum and the tail ends near the hilum of the spleen (**Figure 1**)[8,9]. It is a mixed gland composed of two morphologically and functionally distinct structures. The exocrine gland is composed of acinar and ductal cells and forms the majority of the pancreas, whereas the remaining 1-2% represents the endocrine gland formed by scattered endocrine cell clusters known as the islets of Langerhans[8].

The exocrine pancreas produces, secretes, and transports digestive enzymes and bicarbonate in a fluid composition known as pancreatic juice, of which a human makes approximately 2.5 liters per day[9]. Clusters of 15-100 acinar cells form an acinus, which produces digestive proteins such as

inactive enzyme precursors (zymogens) and active enzymes (amylase and lipase). Small intercalated ducts drain these acini, which then merge distally to form intralobular ducts. These intralobular ducts drain into larger interlobular ducts, which then merge to form the main pancreatic duct. This duct connects the entire exocrine gland to the duodenal lumen via the hepatopancreatic ampulla, known as the ampulla of Vater[8,10]. In the intestinal lumen, the pancreatic juice neutralizes gastric acid and breaks down carbohydrates, proteins and fat for absorption. The pancreatic ducts not only serve as conduits for the protein-rich fluid produced by the acinar cells, but also play an important role in modifying its content by synthesis and secretion of a bicarbonate-rich fluid. The importance of this process is demonstrated in cystic fibrosis (CF), a common lethal genetic disease characterised clinically by progressive pancreatic and pulmonary insufficiency due to a defect in bicarbonate secretion, which results in thickened secretions that create obstructive complications in the ducts[11].

The endocrine part is formed by approximately 500,000 - 1,000,000 islets of Langerhans that are dispersed throughout the organ, and play a crucial role in glucose homeostasis by the secretion of hormones in the blood. Human islets are endocrine cell clusters formed by approximately 60% of beta cells that secrete insulin, 30% of alpha cells that secrete glucagon, 10% of delta cells that secrete somatostatin, and finally a small percentage of pancreatic polypeptide cells and epsilon cells that secrete pancreatic polypeptide and ghrelin, respectively (**Figure 1**)[12,13].

In human islets, pancreatic endocrine cells are organised in a unique arrangement allowing multiple complex regulatory mechanisms, such as humoral, cell-cell, and neural communication, to tightly control hormone synthesis and secretion[14-16]. To further facilitate this, islets are richly perfused by structured fenestrated capillaries, allowing communication between cells and the rapid release of insulin by beta cells upon glucose stimulation[17-19]. In addition, islets are well innervated by both the sympathetic and parasympathetic branches of the autonomic nervous system[20].

Pancreatic diseases are diverse and can affect both exocrine tissue (*e.g.*, pancreatitis, cystic fibrosis, pancreatic adenocarcinoma) and endocrine tissue (*e.g.*, diabetes mellitus, rare neuroendocrine tumours). Pancreatic cancer is associated with high mortality rates, attributed to the fact that most treatment options are rendered ineffective due to the typically late-stage detection of disease that has already metastasized[21]. Compared to other gastrointestinal malignancies where diagnostic and therapeutic advances have improved survival rates, survival for pancreatic adenocarcinoma remains poor[22,23]. This illustrates why a thorough understanding of pancreas development is crucial for a complete understanding of pancreas (patho)physiology, as well as the advancement of diagnostic and treatment strategies for disease.

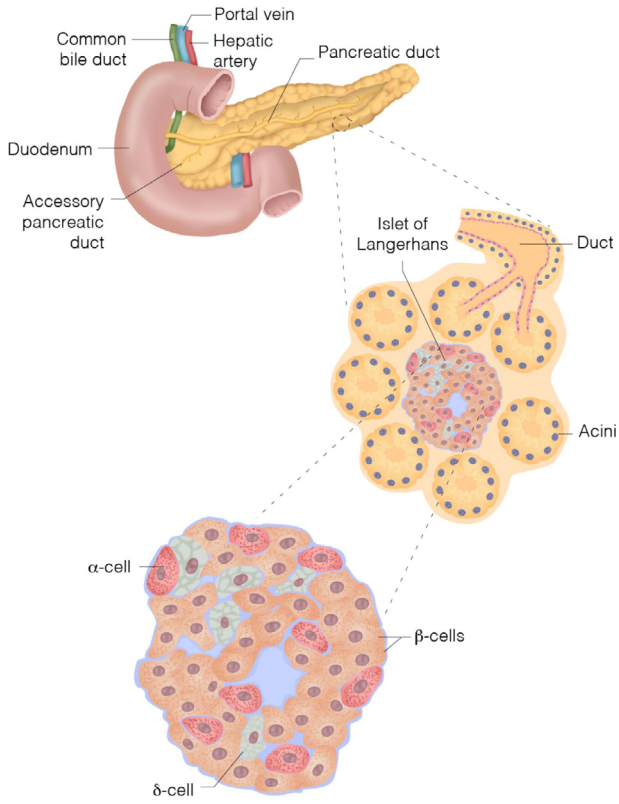

Figure 1. Pancreas anatomy and architecture of exocrine and endocrine tissue
Schematic overview of human pancreas location and showing the exocrine architecture formed by ducts and acinar cells. The endocrine gland is formed by the islets of Langerhans composed of different endocrine cells including the insulin producing beta cells (reprinted from Al-Hasani et al. *Signal Transduction and Targeted Therapy.* 2022).

Pancreas development

The study of human pancreas development, especially early embryonic events like foregut patterning and pancreatic bud formation, is limited due to sample availability, ethical considerations, and legal regulations[24,25]. As a result, the vast majority of our current understanding of pancreas development is based on data from studies on pancreas development in animal models[26-29]. Most of these data were generated using mouse models, largely due to their accessibility and the ease with which they can be genetically modified.

It is reassuring that the limited knowledge derived from scarce histopathological human samples suggest that the transcriptional regulatory mechanisms during development between mice and humans are largely conserved, although important differences do exist[30,31]. Over the last decade, powerful new strategies have been developed that enable the in-depth study of human pancreas

development. These include efficient pancreatic differentiation protocols for pluripotent stem cells, expansion of progenitor cells using organoid culture techniques, and high-resolution analytical tools such as single-cell transcriptomics.

Transcriptional regulation of pancreas development

One of the most fascinating aspects of multicellular life is cellular differentiation in composition, organisation, and function. It is truly remarkable that cells with such vastly different roles during life are generated from only a few progenitor cells. The developmental process that generates the various types of specialised tissue requires signaling cues from the cellular niche and surrounding environment, initiating a stepwise differentiation process orchestrated by the spatiotemporal expression of unique sets of proteins[32]. These proteins, known as transcription factors, are gene regulatory proteins that dictate cell specification and function by controlling gene transcription[33-37]. During differentiation towards a terminally differentiated cell, the plasticity of progenitor cells becomes progressively restricted.

The significance of these regulatory transcription factors and the impact of mutations has been demonstrated in studies using transgenic mice. These models allow for the manipulation of gene expression to observe the effects on pancreatic development and function, leading to the identification of several key transcription factors required for pancreatic development.

For example, pancreatic agenesis in rodents is observed upon deletion of the pancreatic key transcription factors *pancreatic and duodenal homeobox 1* (PDX1)[38,39] and *pancreas-associated transcription factor 1a* (PTF1A)[40,41], highlighting the importance of these genes in the differentiation of cells into the pancreatic lineage. These transgenic models correlate, at least partially, with human transcription factor function, as demonstrated by monogenic forms of diabetes. In these forms of diabetes, mutations in pancreatic transcription factors result in pancreatic dysgenesis or beta cell dysfunction[33]. Crucial genes for early pancreas specification identified in humans include PDX1[42,43] and PTF1A[44,45]. However, disparities in the essential genes involved in pancreas development between humans and rodents have also been identified. In humans, *GATA binding protein 6* (GATA6) mutation leads to pancreatic agenesis[46], whereas in GATA6-knockout mice the pancreas is still able to develop unless *GATA binding protein 4* (GATA4) is simultaneously deleted[47]. Key transcription factors essential for endocrine development have also been identified. Among them, *neurogenin 3* (NEUROG3) is required for endocrine differentiation, NEUROG3-knockout mice lack all islet endocrine cells[48], and in humans NEUROG3 mutations contribute to diabetes[49,50]. Other transcription factors that do not result in pancreatic agenesis but are important for proper endocrine differentiation are mutations in genes linked to neonatal diabetes; these include *GLIS family zinc finger 3* (GLIS3), *regulatory factor X6* (RFX6), *neuronal differentiation 1* (NEUROD1), *NK2 homeobox 2* (NKX2.2), *motor neuron and pancreas homeobox 1* (MNX1), and *paired box 6* (PAX6)[34,35].

Formation endoderm

During the first 8 weeks after ovulation, the developing human organism is referred to as an embryo. This period is divided into 23 stages, known as Carnegie Stages (CS), based on specific external and internal morphological characteristics (**Figure 2**). After the embryonic period the developing human is referred to as a fetus[24,51].

Based on data extrapolated from rodent studies, the specification of internal organs such as the pancreas in early human embryonic development begins after gastrulation at CS 7. Gastrulation is the process that generates the three germ layers: ectoderm (which forms the nervous system and skin), mesoderm (which forms muscles such as the heart and the mesenchymal tissue), and endoderm. The endoderm develops into the majority of internal organs, including the respiratory and gastrointestinal tracts, as well as related organs such as the liver and pancreas. These organs are essential for a variety of homeostatic processes within the body, such as nutrient absorption (intestines), gas exchange (lung), detoxification (liver), and glucose homeostasis (pancreas).

At CS 9, the endodermal sheet folds at the head, tail and flank regions of the embryo to form the primitive gut tube[30]. Rodent studies have shown that signaling cues from adjacent structures like the notochord, endothelium, and mesenchyme regulate the spatiotemporal expression of transcription factors in the primitive gut tube. This creates an anterior-posterior patterning that divides it into the anterior foregut (which will form the esophagus, forestomach, lung, and trachea); posterior foregut (which will form the antral stomach, duodenum, liver, gallbladder, and pancreas); midgut (which will form the duodenum and small intestine); and hindgut (which will form the colon)[52].

At CS 10, the anterior foregut invaginates to form the anterior intestinal portal (AIP) that marks the boundary between the posterior foregut and midgut. The AIP will eventually develop into the yolk stalk[30]. In addition, the AIP is also adjacent to the region of the posterior foregut of which the pancreas develops. The pancreas will develop from two buds that emerge on the ventral and dorsal sides of the posterior foregut, that initially differentiate independently but later fuse together to form a single organ[30,53].

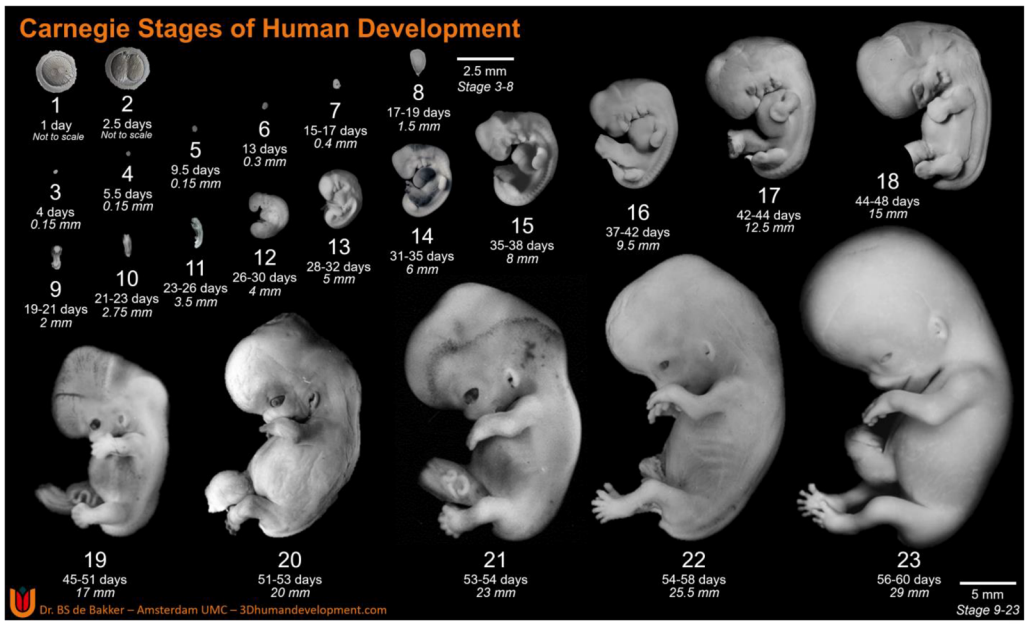

Figure 2. Carnegie stages of human development
Carnegie stages 3-10, dorsal view; stage 11 onwards, left lateral view (reprinted from Flierman et al. *Life*. 2023).

Pancreas specification

First, the dorsal pancreatic bud is formed by signaling cues from the adjacent notochord and dorsal aortae. This occurs at CS 10, when the notochord is temporarily located in proximity to the dorsal foregut endoderm. By CS 12, the fusion of the dorsal aortae disrupts the contact between the notochord and the endoderm[24,25,30]. Data from chick and mouse studies suggest that fibroblast growth factor 2 (FGF2) and activin signaling from the adjacent notochord inhibit *sonic hedgehog* (Shh) expression in the dorsal foregut endoderm, thereby permitting the expression of PDX1[54-56]. Furthermore, in humans SHH is absent from the dorsal foregut endoderm, followed by the expression of PDX1 in dorsal posterior foregut cells at CS 12. Subsequently these cells evaginate from the dorsal posterior foregut and form the dorsal pancreatic bud[30].

The ventral pancreatic bud is formed from the ventral posterior foregut. In addition to the ventral pancreas, the liver and the extrahepatic biliary system (gallbladder, cystic duct, and common bile duct) are also derived from this endodermal region. In rodents, signaling cues from the adjacent cardiac mesoderm and septum transversum mesenchyme affect transcription factor expression in this region, leading to PDX1 expression and the evagination of cells that form the ventral bud[26].

At CS 13 the rounded dorsal and ventral pancreatic bud are clearly visible and are composed of multipotent progenitor cells expressing PDX1, *SRY-box transcription factor 9* (SOX9), *NK6 homeobox 1* (NKX6.1) and GATA[30]. The buds consist of stratified epithelium and are connected to the primitive gut tube through a primary central lumen, and in the buds micro-lumens also form.

At CS 13 the dorsal pancreatic bud is separated from the dorsal aortae by splanchnic mesoderm[30].

From CS 14 to CS 18 the pancreatic epithelium is embedded in loose mesenchyme with dense peripancreatic mesenchyme. In rodents, signals from the nearby cardiac mesoderm and septum transversum mesenchyme influence transcription factor expression in this area, resulting in PDX1 expression and the formation of the ventral bud through cell evagination[24]. Moreover, it starts to form the ductal tree-like epithelial network by branching morphogenesis, which includes fusion of the micro-lumens into an immature, highly interconnected tubular plexus. What exactly drives this morphogenesis is unknown. In rodents, it is suggested that Notch, retinoic acid and BMP signaling play a role at this stage, however human data on these signaling pathways at this stage are lacking[53].

At CS 18 the ventral and dorsal bud lie adjacent to each other after a rotation of the primitive gut in the longitudinal axis called gut rotation, allowing both buds to start fusing to form a single organ[30].

Lineage specification

The next step of the pancreatic development is the lineage specification of the different pancreatic compartments, *i.e.,* the duct, acinar, and endocrine cells. At CS 19, a tip-trunk segregation of progenitor cells in the branching epithelium develops. Bipotent trunk cells that will form ductal and endocrine cells lose GATA4 expression, whereas GATA4 is retained in the peripheral tip cells that will form the future acinar cells[30]. This tip-trunk segregation is completed at 10-14 weeks post conception (wpc). At that stage, tip cells also express the acinar marker *carboxypeptidase A1* (CPA1) and have lost the common pancreatic progenitor markers NKX6.1 and SOX9, which are still expressed in the epithelial trunk[24,25].

The endocrine compartment of the pancreas starts forming after the embryonic period of development. Endocrine progenitor cells are demarcated by NEUROG3 expression which starts at 8 wpc, peaks between 10-14 wpc, declines from 18 wpc onwards and is probably switched off at 26-28 wpc[57,58].

Once NEUROG3 cells differentiate towards endocrine cells, they cease to proliferate, suggesting that endocrine cell allocation *in utero* is specified between 8-28 wpc[24]. The NEUROG3 expression is transient, and endocrine cells will only develop if a certain NEUROG3 expression threshold is passed. What determines the commitment of NEUROG3-expressing cells to a specific endocrine cell type remains unknown. There might be a temporal effect of NEUROG3 expression as the first endocrine cells to develop in humans are insulin-positive cells at 8 wpc, followed by glucagon cells at 9 wpc[30,37,59-61].

In mice, endocrine cells are formed only from the trunk regions, after which they migrate into the surrounding mesenchyme via a process called epithelial-to-mesenchymal transition (EMT), eventually coalescing into the islets of Langerhans[26]. In humans, by 10 wpc, beta cell clusters are vascularized, and by 14 wpc, islets are formed that contain all endocrine cell types[30,62]. During development, the morphology of islets changes. At 14 wpc, islets have a beta cell core surrounded

by alpha cell mantle, as also observed in small human islets and rodents. However, at 21 wpc, cell types are intermingled in humans, and it is believed that this specific islet morphology is essential for human endocrine cells to attain their fully mature functional state.

Diabetes mellitus: a pancreas disease of the endocrine cells

Diabetes mellitus (DM) is a heterogeneous group of metabolic disorders characterised by loss or dysfunction of the beta cells in the pancreas, resulting in elevated glycemia. It can be classified in four broad categories[63]. The two most prevalent forms of diabetes have a polygenetic and multifactorial etiology; type 1 diabetes mellitus (T1DM), which accounts for 5-10% of all cases, and type 2 diabetes mellitus (T2DM), which accounts for approximately 90% of cases. Gestational diabetes is a third form, characterised by a temporary relative insulin deficiency that occurs in up to 10% of pregnancies. Gestational diabetes also has a polygenic and multifactorial etiology, and is a strong risk factor for the later development of T2DM[64,65]. A fourth category includes rare forms such as monogenic diabetes syndromes, caused by single-gene mutations that impair beta cell development and function—examples include maturity-onset diabetes of the young (MODY) and neonatal diabetes mellitus (NDM)[66-69]. In addition, secondary diabetes may occur as a consequence of other medical conditions or interventions, such as exocrine pancreatic disease (*e.g.,* cystic fibrosis, pancreatitis) or drug-/chemical-induced diabetes (*e.g.,* glucocorticoid-induced diabetes).

The growing epidemic of DM makes it one of the most important and serious health challenges in the 21st century. Since 1980, almost all regions of the world have observed a rapid increase in prevalence of DM, in particular due to a rise in cases of T2DM. Recent reports estimate that approximately 8.8% (425 million adults) of the global total adult population suffers from DM, and this percentage is projected to increase to 9.9% (693 million adults) in 2045[70].

Type 1 diabetes mellitus

T1DM is a chronic disease in which a T-cell-mediated autoimmune destruction of the beta cells creates an absolute insulin deficiency. While it is unknown what exactly triggers this beta cell targeted autoimmunity process, evidence indicates it is the result of polygenic susceptibility influenced by environmental factors[71]. Autoantibodies against proteins associated with the secretory granules of the beta cells are used as biomarkers of the disease.

T1DM is the most prevalent form of diabetes in children, who rely on lifelong insulin injections for their survival. New insulin administration approaches, including insulin pumps, continuous glucose monitoring, and hybrid closed-loop systems, are being developed to enhance glycemic control through precise glucose monitoring and insulin delivery.

Although exogenous insulin alleviates DM-associated metabolic abnormalities, it does not address the underlying beta cell deficit, underscoring the need for new therapeutic approaches to achieve a cure for the disease. In parallel, research is also focused on preventing the onset of T1DM. Years of intensive investigation have led to an increased understanding of glucose metabolism

and the immune pathogenesis of T1DM, that will hopefully lead to the further development of innovative preventive strategies, such as immunotherapy targeting the autoimmune response (*e.g.,* therapy targeting T cells)[72,73]. A new concept even suggests that beta cells are not passive victims of the immune system, but instead play an active role in their destruction by the immune system[74]. However, the complex and heterogeneous immunopathology of T1DM—still not fully understood—poses major challenges for the development of novel therapies, and the design of effective clinical trials[71].

Type 2 diabetes mellitus

T2DM is a multifactorial disease involving genetic and environmental factors that cause an increasingly impaired insulin secretion from beta cells, typically superimposed on a background of peripheral insulin resistance, resulting in a relative insulin deficiency[75,76]. T2DM has also been linked with decreased beta cell mass[77-80].

Ethnicity and a family history of diabetes, combined with obesity, poor dietary habits, and limited physical activity, are primary risk factors for T2DM. For individuals at high risk, intensive lifestyle changes can effectively delay or even prevent the onset of T2DM. In addition, there is a wide variety of antidiabetic drugs, including those that suppress hepatic glucose production (*e.g.,* metformin), increase insulin secretion (*e.g.,* sulfonylureas or meglitinides), increase insulin sensitivity (*e.g.,* thiazolidinediones), modulate GLP-1 (*e.g.,* DPP-4 inhibitors, GLP-1 receptor agonists), or decrease intestinal and renal glucose absorption (*e.g.,* SGLT-2 inhibitors and alpha-glucosidase inhibitors). When oral medication is insufficient for glycemic control, insulin injections are used as therapy.

Symptoms and complications of diabetes

Insulin deficiency results in elevated glycemia, which may cause symptoms such as polyuria, polydipsia, nocturia, blurred vision, and weight loss. Furthermore, acute metabolic derangements can lead to life-threatening emergencies, such as the possibility of diabetic ketoacidosis. This condition occurs due to the breakdown of lipids into ketones caused by an absolute insulin deficiency and can lead to coma in patients.

In T1DM the onset of symptoms is rapid (days to weeks), and the majority of patients are diagnosed when they seek medical attention for their symptoms. In contrast, the onset of T2DM is slower, and the majority of patients are identified by screening. Biochemical tests such as elevated levels of plasma glucose or glycated hemoglobin (HbA1c) are used to diagnose diabetes.

Both T1DM and T2DM are associated with increased morbidity and mortality due to serious vascular complications, that can be divided in microvascular and macrovascular complications. Particularly cell types that share the inability to decrease the rate of glucose transport when exposed to hyperglycemia, such as the capillary endothelial cells in the retina, mesangial cells in the renal glomerulus, and neurons and Schwann cells in peripheral nerves, are susceptible

to damage. The hyperglycemia creates an overproduction of superoxide and oxidative stress, resulting in the diabetes-specific microvascular complications: retinopathy, nephropathy, and neuropathy[81]. Diabetic retinopathy is one of the largest contributors to vision loss globally[82]. Diabetic nephropathy is characterised by urinary albumin excretion (albuminuria) in the absence of other renal abnormalities and is the leading cause of chronic kidney disease worldwide.

Coronary artery disease, peripheral artery disease, and cerebrovascular events like stroke, are examples of macrovascular complications. These conditions significantly contribute to reduced quality of life, increased disability, and premature death in individuals with diabetes[83].

Beta cell replacement therapy

Currently the most promising treatment to cure diabetes is to restore insulin secretion by replenishment of beta cell mass. The goal of transplantation is to alleviate some of the burdens of the disease by achieving insulin independence and reduction of acute metabolic derangement (*i.e.,* hypoglycemic events) or other complications. Transplantation options include whole-organ pancreas transplantation (PT) or transplantation of the isolated islets, known as islet transplantation (IT). Both methods of transplantation are most suited for people with diabetes and end-stage renal disease, who already require immunosuppressive medicine for a kidney graft. Compared to whole-organ transplantation, IT offers a less invasive solution for the replenishment of lost beta cell mass. IT is associated with a lower complication rate and better overall survival while producing similarly effective outcomes, making it suitable for a wider range of patients[5,6].

Clinical islet transplantation

IT begins with the extraction of islets of Langerhans from donor organs through a complex digestion and purification process, followed by transplantation into the liver (**Figure 3**). After transplantation in T1DM patients, these islets can restore physiological endogenous insulin secretion, providing near-perfect control of blood glucose and offering hope that improved glycemic control will also prevent the long-term complications of diabetes. Because the cells are percutaneously injected into the liver through the portal vein under radiographic guidance, the transplant procedure eliminates the need for major surgery. As a result, over 90% of patients can be discharged after a brief recovery period[84]. Allogeneic IT is currently recognised as a key strategy for treatment of T1DM patients with hypoglycemic unawareness or problematic hypoglycemic episodes. Recent reports show that 30% of patients are insulin independent five years after IT, 60% have achieved optimal glycemic control (HbA1c < 53 mmol/mol Hb), and more than 90% no longer experience severe hypoglycemic episodes[85,86]. In addition, beta cell replacement therapy attenuates progression of diabetic complications and improves quality of life[87,88]. These outcomes have not been achieved yet by optimal insulin therapy, and a randomized trial comparing insulin treatment versus IT supports these findings[4].

Although IT is an established treatment with beneficial effects, there are still hurdles that

prevent widespread use of IT as potential cure for T1DM. These include a limited supply of donor organs, suboptimal engraftment, and the need for lifelong immunosuppression. Intensive efforts are focused on further optimisation of the islet isolation and transplantation procedure, and the immunosuppressive regimen used in IT. In addition, these hurdles might partially be overcome by use of alternative sources for the generation of beta cell replacement therapy.

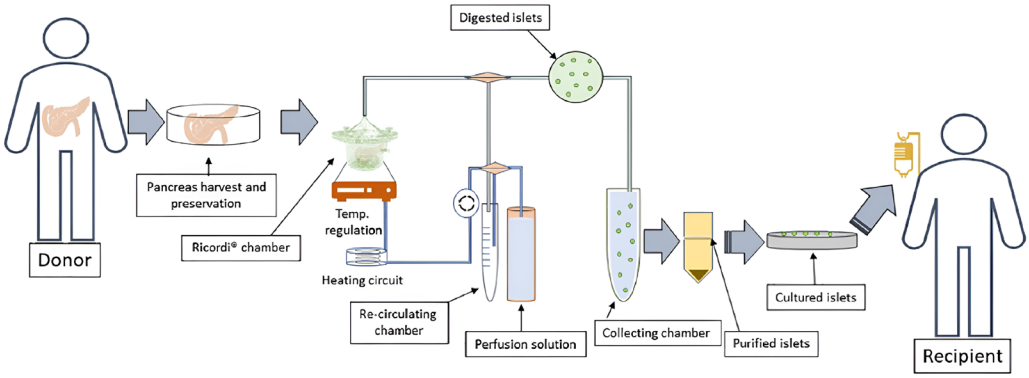

Figure 3. Islet isolation and transplantation process
Islet isolation and purification process using a pancreas and a Ricordi chamber to digest a human donor pancreas mechanically and enzymatically. The digest is then further purified using a gradient separation. Purified islets are cultured and afterwards transplanted in recipients (reprinted from Pathak et al. *Clinical Medicine Insights: Endocrinology and Diabetes*. 2019).

Alternative sources for beta cells

Significant efforts have been made to find new regenerative medicine strategies for diabetes therapy. In 1998 embryonic stem cells (ESC) were successfully cultured[89], offering the potential to generate any differentiated cell type. However, the pluripotency of these cells is a double-edged sword, as this plasticity makes it difficult to control and direct ESCs towards the desired cell type[90]. The generation of beta cells from ESC requires *in vitro* recapitulation of the important processes that occur during pancreatic development. The first protocols successful in the generation of insulin-positive cells were based on numerous developmental studies in animals, which had mapped out key steps and critical signaling events required for beta cell development[91-93]. The commitment of a human embryonic stem cell (hESC) towards a beta cell was guided in a stepwise manner, using combinations of small compounds or growth factors to modulate key signaling pathways involved in beta cell differentiation (**Figure 4**). However, the insulin-positive cells generated resembled immature beta cells, displaying poor glucose responsiveness and co-expressing multiple hormones[94-97]. Current protocols have been improved, to the extent that glucose-responsive beta cells are generated, and the protocols can be applied to multiple pluripotent stem cell lines. However, for the development of fully functioning beta cells, these protocols still rely on *in vivo* transplantation for the final maturation step[98-100]. Despite these encouraging findings, ethical concerns[101] and the risk of teratoma formation

by potentially undifferentiated cells, still hinder clinical application[102].

Another major advance for the pluripotent stem cell field occurred in 2006, when researchers discovered that by overexpression of specific transcription factors, adult somatic cells could be reprogrammed into pluripotent stem cells making induced pluripotent stem cells (iPSC)[103], providing an attractive alternative strategy to generate patient-specific cell products. Compared to hESC, iPSC-derived beta cells have less ethical and immunological obstacles for clinical applications. Glucose-responsive beta cells can be generated from iPSCs. However, similar to those derived from hESCs, they remain immature, which limits their immediate application in patient-specific beta cell replacement therapy.

Recent clinical trials, using enhanced differentiation protocols, coupled with alternative transplantation strategies—such as cell delivery devices or implantation at non-traditional sites—have shown the potential to establish functional beta cell masses capable of improving glucose control[104-106]. Future innovations in this field hold great promise for addressing current limitations and advancing beta cell replacement therapy.

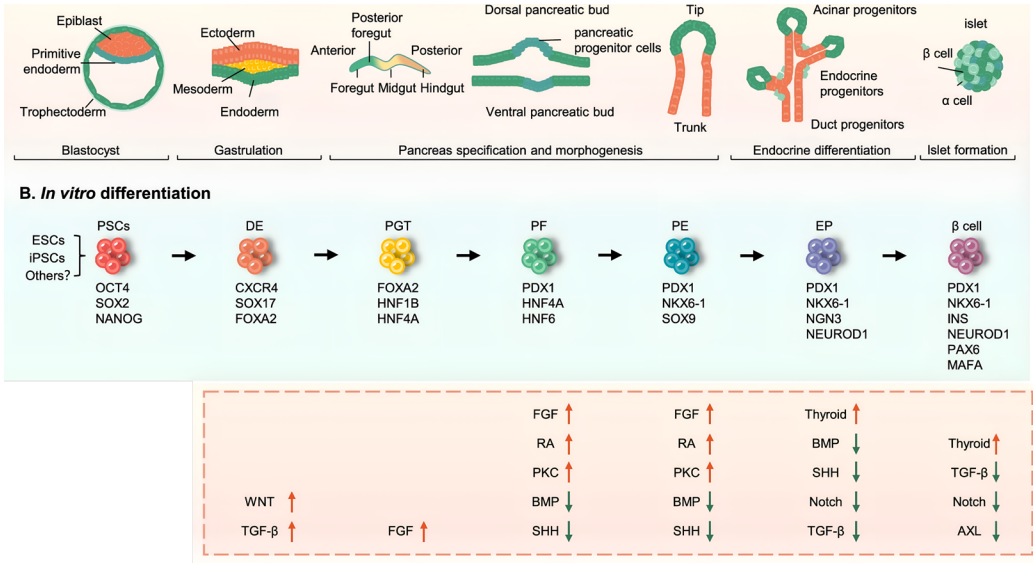

Figure 4. *In vitro* recapitulation of *in vivo* development using pluripotent stem cells and a stepwise differentiation protocol
Through mimicking ***in vivo*** pancreatic development, human pluripotent stem cells (PSCs) are differentiated into pancreatic lineage and eventually into beta cells. Examples of pathways that are commonly manipulated for the stepwise differentiation using small molecules are depicted (reprinted and adapted from Jin and Jiang. ***Cell Regeneration***. 2022).

Beta cell regeneration

Observations of beta cell turnover in the human postnatal pancreas have encouraged exploration into alternative approaches for the generation of new beta cells that could be used for beta cell

replacement therapy. Endogenous pancreatic cells are of special interest, due to their potential ability to even facilitate endogenous regeneration of beta cell mass[107]. A variety of cells and options have been studied, such as proliferation of existing beta cells, conversion of other endocrine cells into beta cells, or differentiation from putative progenitors, such as exocrine cells[107-110.]

Homeostatic control of human beta cell mass

Pancreatic beta cell mass is maintained by the closely regulated balance of beta cell birth and growth (due to replication, neogenesis, and hypertrophy) and beta cell loss (due to death or atrophy). In humans, new beta cells are differentiated from endocrine progenitor cells during development, while postnatal beta cell expansion is primarily driven by proliferation of beta cells, which peaks the first two years after birth, with proliferation rates ranging from 1-3%. Beta cell proliferation is rapidly reduced in early childhood. In adulthood, replication rates are less than 0.1%[62,111-114]. This low proliferation rate reflects the long half-life of beta cells, which rarely undergo cell death under normal conditions, although it has been proposed the proliferation could be underestimated owing to a post-mortem decline of replication markers[115]. Other methods, however, that have evaluated postnatal beta cell mass longevity and expansion, confirm that total beta cell mass is determined and stable before 20-30 years[116,117]. In addition, experimental data derived from rodent and human islets also show an age-dependent decrease in beta cell replication capacity[118-120].

Multiple mechanisms contribute to the resistant nature of human beta cells to proliferate, such as nuclear accumulation of cell cycle inhibitors, while key cell cycle regulatory molecules required for proliferation (such as cyclins and cyclin-dependent kinases), generally reside in the cytoplasm[121,122]. It is postulated that the inhibition of quick beta cell turnover protects against hyperinsulinemia and potential hypoglycemic lethality[123].

While adult beta cell turnover is uncommon under normal conditions, compensatory beta cell mass expansion has been observed in post-mortem samples of individuals with prolonged metabolic states with high insulin demand, such as pregnancy and obesity[78,124]. In addition, some T1DM individuals show evidence of residual functional beta cells after prolonged periods following the onset of their disease, suggesting continuous beta cell turnover[125-128]. However, it is unclear from which cellular compartment these new beta cells originate. Unfortunately, human beta cell turnover is difficult to study using only post-mortem samples, which has led to the use of rodent models in an effort to identify the origin of expanded beta cell mass.

Rodent models of beta cell regeneration

Beta cell turnover has been intensively studied in rodent injury models that stimulate pancreas or beta cell regeneration, such as partial pancreatectomy, pancreatic duct ligation, or chemical ablation of beta cells. These models are often applied to transgenic mice, making it possible to determine the source of newly formed beta cells via lineage tracing. Unfortunately, this experimental approach has delivered a plethora of contradictory results, creating even more uncertainty about the cellular

origin and mechanisms underlying beta cell regeneration[107,110].

For example, while studies using the pancreatic duct ligation model in mice suggest that new beta cells originate from ductal cells[129,130], other studies show that beta cells are not derived from duct cells[131,132], that beta cells are derived from replicating pre-existing beta cells[133], or that no beta cell regeneration occurs at all[134]. Even lineage tracing studies using the same marker (SOX9) in a pancreatic duct ligation model have reported SOX9 to be an adult progenitor marker[135], while others conclude that SOX9 is not a progenitor marker[132]. Furthermore, these rodent models have also produced evidence showing that self-replication of beta cells is the major mechanism for beta cell replenishment in normal homeostasis or in tissue regeneration response[136-138], but new beta cells converted from other mature endocrine cells have also been described[139,140].

These conflicting results could in part be due to heterogeneity in the type of rodent strain used or due to variability in age of the studied rodents[141]. In addition, variability in the injury models (*e.g.,* inflammation in the pancreatic duct ligation model due to exocrine cell death versus beta cell ablation with less inflammation), and the markers used for lineage tracing, combined with differences in efficiency and specificity of lineage tracing, could very well contribute to the variation of results obtained from different studies[142,143]. Additionally, growing evidence shows a previously unknown plasticity of terminally differentiated pancreatic cells that are able to convert or dedifferentiate[144]. This plasticity is even more present during dynamic processes that are present in these injury models, such as those induced by stress or genetic manipulation[145,146].

Thus, negative lineage tracing results do not necessarily imply the absence of a certain (progenitor) cell type, as it just provides a snapshot of the temporal expression of a few genes and may not even label all the cells in a compartment, especially in highly dynamic states such as obtained with pancreatic injury models. Furthermore, recent data collected with novel antibodies or techniques such as single-cell RNA sequencing show that pancreatic compartments are composed of heterogeneous cell populations, with even the same cell types exhibiting different signatures[147,148]. This makes it difficult to correctly interpret lineage tracing studies, which might be the reason for the large variation in reported results and conclusions.

Moreover, our increased understanding of the considerable differences between human and mouse beta cell homeostasis challenge the translational relevance of these findings. There are notable distinctions between human and rodent islets and beta cells, such as variations in islet distribution throughout the pancreas, differences in the composition and organisation of islets, and differences in islet vasculature and neural innervation, as well as in normal physiology and diabetes reversal[8,12,149,150].

While lineage-tracing experiments are considered to be the 'gold standard' to elucidate the progenitor cell capacity of cells, these insights highlight the limitations of animal models in accurately reflecting human beta cell regeneration, as well as the critical need for studies with human material to draw trustworthy conclusions on beta cell regeneration that could be used for therapeutic strategies.

Human beta cell replication

The limited replication capacity of primary human beta cells and the large number of documented differences in signaling mechanisms modulating the proliferation between human and rodent islet cells make it difficult to understand human beta cell proliferation[151-153]. Despite these challenges, screening large compound libraries using high-throughput methods have revealed a limited set of molecules that are able to promote human beta cell proliferation *in vitro* and *in vivo*[154]. The most promising target involved in beta cell replication is the dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A). It has been observed that small molecules inhibiting DYRK1A (*e.g.,* harmine analogues) stimulate human beta cell proliferation[155-157]. However, even with this new class of drugs, beta cell replication rates continue to remain low (1-3%)[123]. Furthermore, DYRK1A is not only expressed in beta cells making these DYRK1A inhibitors unspecific, raising safety concerns which might limit the therapeutic potential of these drugs[158]. Further research is needed to identify more potent and more beta cell specific mitogens to increase beta cell specific replication rates while limiting off-target adverse effects.

Human endocrine cell conversion

Another possible method for the production of new beta cells includes the conversion of other endocrine pancreatic cells. Currently, most evidence has been collected on the conversion of alpha cells towards beta cells, although limited reproducibility has made this a controversial topic. Transplantation of human islets under the kidney capsule of mice treated with the neurotransmitter γ-aminobutyric acid (GABA) resulted in a reduction of alpha cells and a concomitant increase in beta cells within the grafts, indicating a potential conversion of alpha cells to beta cells[159,160]. In addition, stimulation with GABA also increased insulin secretion of human islets[160]. It is postulated that the mechanism behind this conversion in human islets involves the inactivation of the glucagon-specific transcription factor *aristaless related homeobox* (ARX), which is shuttled to the cytoplasm upon GABA stimulation[160]. These experiments with human islets were performed after initial findings in rodents, where extreme STZ-mediated beta cell ablation led to an age-dependent conversion of alpha or delta cells towards beta cells[140,161]. In transgenic rodents, *in vivo* downregulation of ARX also resulted in alpha-to-beta cell conversion, demonstrating that ARX is a master regulatory transcription factor essential for the maintenance of alpha cells[162]. Subsequently, compounds were used to inhibit ARX expression in rodents and zebrafish, promoting alpha-to-beta cell conversion[159,160]. However, after the initial reports, multiple groups were unable to replicate these findings. Therefore, while the nucleocytoplasmic shuttling of ARX induced by exogenous GABA signaling, in combination with already clinically approved drugs, is an intriguing concept, further investigations are needed to assess its potential therapeutic value for human islets[163].

Differentiation of human exocrine tissue

Human exocrine tissue remaining after islet isolation procedures (~ 98% of the pancreas) would be

an abundant attractive source for new beta cells that could be used for replacement therapy. Multiple exocrine cells (ductal, acinar or centroacinar cells) have been proposed as putative progenitor cells. The majority of current data derived from human material indicates that the ductal compartment is a possible source of beta cell progenitor cells. However, obtaining direct evidence for exocrine-derived beta cell neogenesis in humans has been challenging, and as previously discussed, rodent studies show contradictory results.

The theory of a putative progenitor in the pancreatic ductal epithelium has already been around for nearly 40 decades, after histological observations of islets growing from human ductal epithelium[164]. The theory is further supported by the ontogeny of human endocrine cells, that during development are derived from the pancreatic duct epithelium. Furthermore, post-mortem studies of individuals, such as those with compensatory beta cell mass increases, reveal the presence of small islets and single hormone-positive cells within ductal structures or near ducts, suggesting that these ducts may give rise to newly formed endocrine cells (**Figure 5**)[78,124,165,166].

The first studies used duct cells derived from islet-depleted material that were expanded *in vitro*, and could subsequently be differentiated towards islet cells in low frequencies[167,168]. However, the heterogeneous starting material in these experiments could not rule out residual beta cell contamination. Consequently, subsequent studies employed various strategies to either deplete beta cells or label/enrich for ductal cells before differentiation experiments. These methods included *in vitro* treatment with STZ[169], lentiviral labelling of ductal cells[170], or the use of duct-specific cell surface markers, such as carbohydrate antigen 19-9 (CA19-9)[171-173]. These studies also demonstrated the generation of sporadic insulin-expressing cells, some of which even expressed duct markers[171].

Additional investigations have also focused on where the putative progenitor cells might be located in the duct compartment. It has long been understood that the ductal compartment is a heterogeneous cell population[10], which has been made more evident with single-cell RNA-sequencing[174]. This technique also revealed that the ductal cell population derived from human islet-depleted tissue might harbor a progenitor cell[175], however spatial resolution is lacking raising the question where this cell is exactly located (*e.g.,* the peripheral small ducts or the larger centrally located ducts).

Several groups have utilized cell surface markers to enrich for ductal subpopulations with possible progenitor capacity. The marker CD133 and CD49F were found to enrich for NEUROG3 progenitor cells in human fetal tissue[176], and CD133 was also used to isolate for ductal cells from human islet-depleted tissue[177]. However, genetic manipulation by overexpression of beta cell specific transcription factors was required for these expanded CD133-positive ductal cells to acquire a beta cell phenotype. Other groups also have used methods to overexpress transcription factor in an effort to create beta cells from ductal cells[178-180].

More recently, a subpopulation of human ductal cells characterised by expression of PDX1 and activin A receptor, type 1 (ALK3) could be isolated using the P2Y purinoceptor 1 (P2RY1) as alternative surface marker for PDX1, and was capable of forming colonies upon stimulation with bone morphogenetic protein 7 (BMP-7). After expansion and BMP-7 withdrawal, cells were capable of

differentiation in various pancreatic lineages, including beta cells[181,182]. Further immunohistochemical analysis demonstrated that the sorted ductal subpopulation was predominantly located in the major ducts and pancreatic duct glands, which are gland-like structures protruding from the walls of larger ducts[182]. These glands were first described in 196[183], and were recently identified as a distinct compartment in the pancreatic duct[184,185]. The blind-ending outpouches express a unique set of transcription factors, and are reminiscent of the intestinal crypts that harbor the intestinal stem cells, so it is not surprising that these glands have been postulated to harbor progenitor cells. Rodent experimental data shows that this compartment was responsible for epithelial repair upon injury[186], and also in T1DM patients increased cell proliferation was observed in these glands[187].

In conclusion, although the evidence pointing towards a ductal origin of beta cell progenitors is interesting, direct evidence to support this theory is limited due to scarcity in techniques and protocols for human ductal cells.

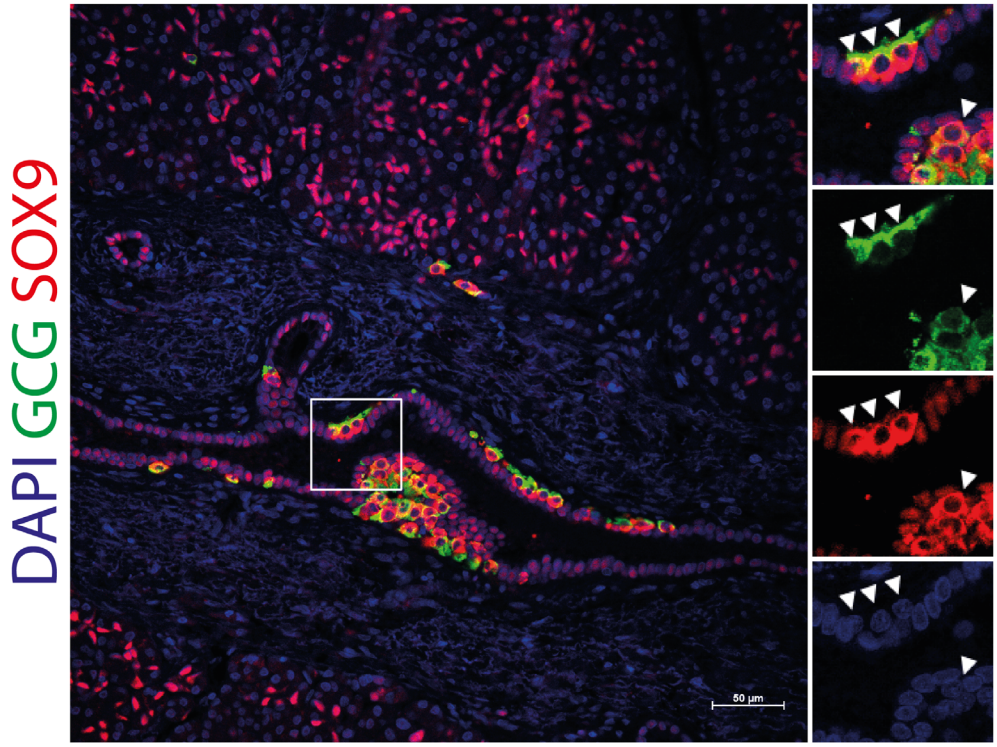

Figure 5. Human adult pancreatic tissue showing a duct with endocrine cells
Human adult pancreatic tissue with an immunostaining for SOX9 (red, duct marker) and glucagon (green, glucagon) and DAPI (blue, nuclear marker), showing endocrine cells in and around the duct. Scale: 50 um.

Aims of this thesis

The key question leading to this thesis was: can we use the human adult exocrine tissue, in particular

ductal cells, as a source for new beta cells that could be used for replacement therapy for diabetes mellitus and also provide insight into the regenerative processes involved in beta cell turnover. In this thesis, we aimed to answer the following questions:

1. Is there a progenitor cell in the human exocrine compartment that can develop into a beta cell and how can we provide direct evidence for this?
2. How can we expand this progenitor cell *ex vivo*?
3. What is the most efficient way to differentiate this progenitor cell into a beta cell *ex vivo*?

In **Chapter 2**, we investigate how the presence of exocrine cells in the final cell preparations, including ductal cells, affects metabolic outcomes of clinical islet transplantation. In **Chapter 3**, we review the potential value of a new 3D culture technique for primary human pancreas cells to expand progenitor cells in structures reminiscent of mini-organs called organoids. In **Chapter 4**, we use this novel organoid culture system on human islet-depleted tissue chunks to generate pancreatic organoids harboring endocrine progenitor cells. In **Chapter 5,** we systematically compare several methods to improve lentiviral transduction of primary human ductal cells which can be used to genetically manipulate these cells. In **Chapter 6**, we study the effects of several differentiation protocols including agents stimulating beta cell neogenesis in order to improve differentiation of primary human ductal cells. In **Chapter 7**, we evaluate the cytoplasmic expression of SOX9 during human pancreatic development, which is required for proper endocrine differentiation.

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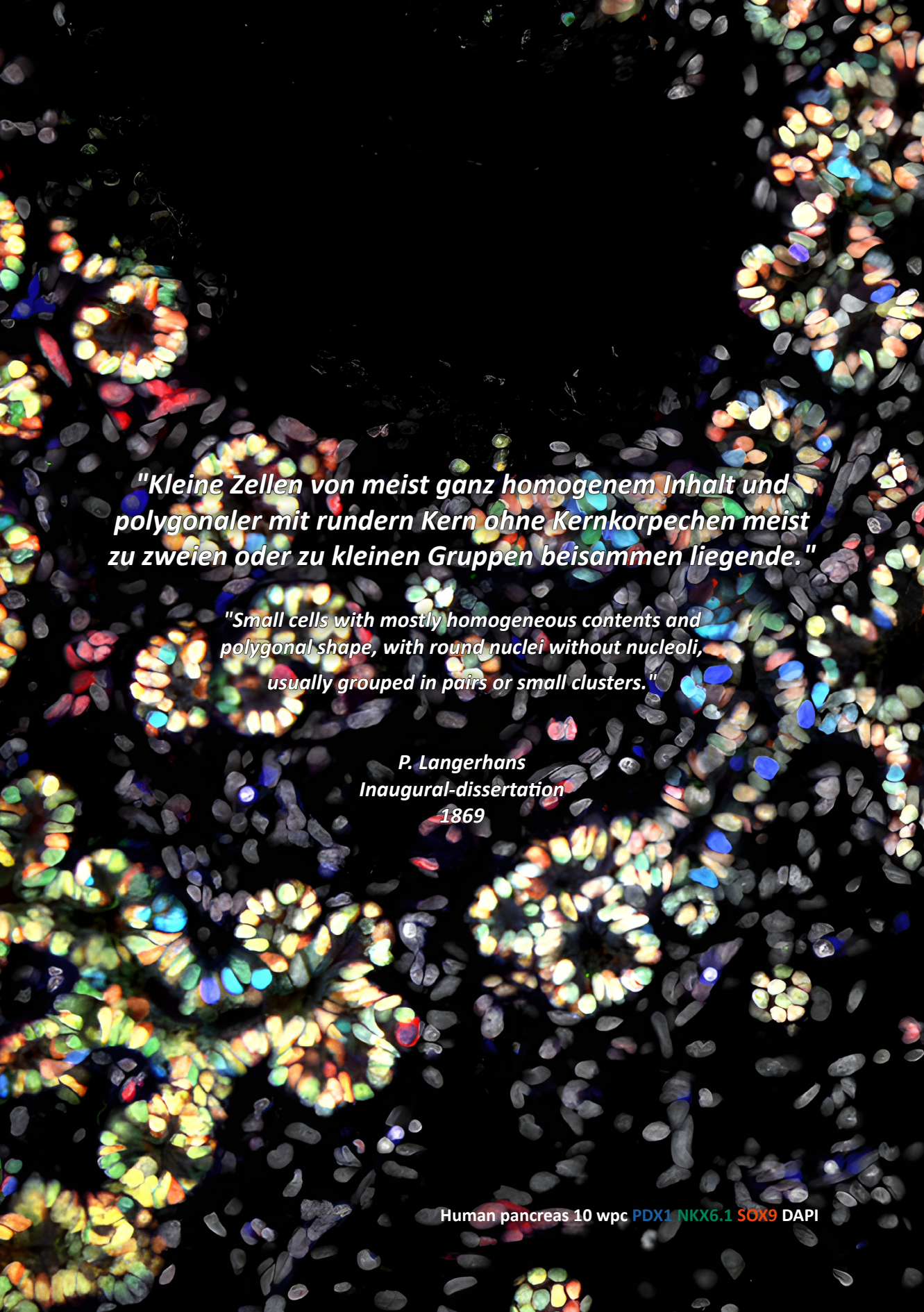

"Kleine Zellen von meist ganz homogenem Inhalt und polygonaler mit rundern Kern ohne Kernkorpechen meist zu zweien oder zu kleinen Gruppen beisammen liegende."

"Small cells with mostly homogeneous contents and polygonal shape, with round nuclei without nucleoli, usually grouped in pairs or small clusters."

P. Langerhans
Inaugural-dissertation
1869

Human pancreas 10 wpc PDX1 NKX6.1 SOX9 DAPI

CHAPTER| 2

Impact of Islet Purity on Graft Function in Islet Allotransplantation

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In preparation

Abstract

The effect of islet purity on metabolic outcomes in islet transplant recipients with severe beta cell deficiency is still unclear due to imprecise measurements of islet purity and small cohorts. We evaluated the impact of islet purity on short- and long-term graft function using digitized images of the islet preparation, combined with a web-based, objective, and reproducible computerized method to calculate islet purity (IsletNet). The cohort (n=41) was divided into tertiles based on IsletNet-assessed islet purity. Graft function was evaluated using C-peptide and glucose measurements from mixed meal tolerance tests. Purity in the low, intermediate, and high group was 27%, 49%, and 65%, respectively. The $AUC_{C\text{-}peptide}/AUC_{glucose}$ at three months showed a positive correlation with purity. However, no difference was found between the purity groups up to 5 years after transplantation. In conclusion, islet purity is not associated with long-term islet graft function. Further investigations are required to understand the impact of non-islet cells on long-term islet function.

Introduction

Allogeneic islet transplantation is an established therapy for patients with complicated type 1 diabetes mellitus (T1D)[1]. Providing a sufficient number of islets is important for achieving favorable metabolic outcomes, but to maximize the safety of the transplantation procedure the islets should be delivered in a limited volume[2]. Therefore, islet purity - defined as the proportion of dithizone (DTZ) stained islets to unstained (exocrine) cell clusters in the islet preparation - is considered an important factor in islet transplantation[1,3-6]. The current range for islet purity in clinical islet transplantation typically falls between 53% and 66%[7,8]. Studies indicate that lower purity islets are associated with short-term negative metabolic outcome[9,10], but long-term positive metabolic outcome[11,12]. However, these studies had several limitations including small cohorts, imprecise measurements of islet purity, and indirect measurements of graft function such as daily insulin use. As a result, the impact of non-islet cells on graft function remains unclear.

Pancreatic duct cells constitute about 30-40% of the human pancreas[13], and islet grafts used for clinical islet transplantation contain up to 20-30% ductal cells[11,14]. Islets derive from the ductal cell compartment during human development, and ductal cells may be relevant for islet regeneration in the adult pancreas[15-18]. It has been hypothesized that the reported beneficial long-term outcomes after lower purity islet transplantation could be attributed to beta cell neogenesis from transplanted beta cell progenitors, specifically the duct cells[18,19]. Other hypotheses for the favorable long-term effect include the secretion of supportive growth factors by exocrine cells[20,21].

Understanding the effects of islet purity on graft function and clinical outcomes is crucial for making optimal use of scarce donor tissue, refining transplantation protocols, and improving patient outcomes and potentially future stem cell-derived islet transplantation strategies. Here we employed a robust, objective, and computerized tool (IsletNet), that eliminates operator bias to quantify islet purity, and evaluated the effect of islet purity on graft function with mixed meal stimulation tests in a cohort of patients with severe beta cell deficiency who underwent their first

allogeneic islet transplantation.

Materials and methods

Study design and participants

We performed a longitudinal analysis on the impact of islet purity on clinical outcomes in patients with severe beta cell deficiency who underwent a first islet transplantation. All patients were referred to the transplantation center of the Leiden University Medical Center (Leiden, The Netherlands) for beta cell replacement therapy.

Organ procurement

Donor pancreas were allocated to patients on the islet transplantation waiting list according to Eurotransplant rules. Important reasons to decline a donor pancreas were a history of diabetes mellitus, an HbA1c level exceeding 6.5% (48 mmol/mol Hb), or indications of impaired islet functionality such as suspected pancreatic injury, suspected ischemic injury, or signs of infection. In case of donation after circulatory death (DCD) procedures, the donor pancreas was declined if the total warm ischemia time exceeded 120 minutes or functional warm ischemia time exceeded 30 minutes. The total warm ischemia time was defined as the interval between switch-off and starting cold perfusion. The functional warm ischemia time was defined as the interval between inadequate organ perfusion after switch off (mean arterial pressure [MAP] <50 mmHg and/or O_2 saturation < 80%) until start cold perfusion[22].

Islet isolation procedure

Islet isolations were performed at the good manufacturing practice (GMP) facility of the Leiden University Medical Center following standard operating procedures as previously described[22,23]. Briefly, peripancreatic tissue was removed and a blend of Collagenase NB1 and Neutral Protease NB1 (Serva Electrophoresis, Germany) was infused via the main pancreatic duct. After infusion, the pancreas was cut into pieces and transferred to a digestion chamber (Ricordi Isolator, Biorep, USA) connected to a closed-loop system for mechanically-assisted enzymatic digestion. The digested pancreatic tissue was collected, washed and islets were purified by density gradient centrifugation using a COBE 2991 cell separator (TerumoBCT, USA).

Assessment of islet purity and IEQ

DTZ staining was performed on a small sample of a homogeneous cell suspension from the islet preparation. Microscopic images were taken directly after the DTZ staining with an Axiovert 25 microscope (Zeiss, Germany).

Visual assessment of islet purity based on the photographic images of the final islet preparation was performed by an experienced member of the islet isolation team and verified by a

second member. If two donors were used for one islet infusion, the purity was evaluated per donor. In this study, we retrospectively analysed the image of the islet preparation by uploading the images to the website of IsletNet, a web-based application that employs deep learning technology for fully automated analysis of DTZ-based images of islets[24-26]. The measurements include the number of cell clusters, the size and number of clusters stained red (islets), and the size and number of islets with >50% of their circumference surrounded by exocrine tissue (embedded islets). Based on these measurements, average islet size, purity (the total number of cell clusters divided by the number of islets), islet index (the ratio between total islet volume and the number of islets, with an index <1 indicating an average islet size of <150 μm and vice versa), and the percentage of embedded islets were calculated. For islet transplants comprising islet preparations from two separate donors, the microscopic image of the islet preparation was analysed individually per donor. Subsequently, the mean characteristics of the combined islet transplant were calculated by correcting purity, islet index and percentage embedded islets for the volume contributed per donor to the transplanted graft. This resulted in a single calculated purity, islet index and percentage embedded islets for each combined islet transplant that was infused. Based on the (combined) purity assessed using IsletNet, the cohort was categorized into tertiles. For the relationship between purity of the islet transplant and stimulated C-peptide after transplantation, the combined purity was used in case of two donors for one islet infusion. The individual purity per islet preparation was used only for correlation of visual and calculated purity. IEQ was calculated using volume and islet purity with the formula: volume (in μL) x islet purity (in percentage) x 1000 / 230.

Islet transplantation procedure

The islet transplantation procedure was performed as previously described[23]. In thirty-four recipients induction was performed using lymphocyte-depletion with alemtuzumab (Genzyme Europe, the Netherlands) which was given subcutaneously twice, 15 mg on the day before transplantation and 15 mg on the day of the islet infusion and steroids. In seven recipients induction therapy consisted of the IL-2 receptor blocker Basiliximab (Novartis Pharma, Switzerland), administered 1 hour before and 96 hours after the islet infusion and steroids. The standard maintenance immunosuppressive therapy consisted of low dose prednisolone, the calcineurin inhibitor tacrolimus and the antimetabolite mycophenolate mofetil. To achieve optimal glycemic control at least 48 hours after transplantation, a continuous intravenous insulin infusion in combination with frequent blood glucose measurements and/or a continuous glucose monitor was performed to maintain glucose levels in the euglycemic range.

The islet transplant was infused into the liver via the portal vein. Using ultrasound guidance and local anesthesia, an interventional radiologist advanced a catheter into the main portal vein via smaller branches. Heparin at a dose of 35 IU/kg was infused directly into the portal vein and heparin 35 IU/kg was also added to the islet transplant. Portal vein pressure was monitored before, during and after infusion. Every patient underwent abdominal ultrasound to exclude portal vein

thrombosis the day after the procedure.

Islet graft function

C-peptide (nmol/L) and glucose (mmol/L) levels were measured before transplantation and at specific time points after transplantation: 1 hour, 4 hours, 1 day, 4-6 weeks, 7-10 weeks, and 3 months. Islet graft function after transplantation was evaluated using a mixed meal test at 3 months, one year, and annually afterwards, as described previously[27]. In short, patients arrived for the mixed meal test in a fasted state and, if possible, refrained from taking exogenous insulin. If this was not possible only long-acting insulin or a basal pump infusion rate was allowed. C-peptide and glucose levels were measured at -10, 0, 15, 30, 60 and 120 minutes after ingestion of BOOST® (50 g of carbohydrates, 22.7 g of protein, and 9 g of fat) or Nutridrink® (49.7 g of carbohydrates, 15.9 g of protein, and 15.7 g of fat). The $AUC_{C\text{-}peptide}$ and $AUC_{glucose}$ were calculated and corrected for the total IEQ transplanted. Mixed meal tests were no longer performed in patients with C-peptide concentrations <0.03 nmol/L during follow-up or no significant C-peptide stimulation during the previous mixed meal test.

Statistical analysis

The primary outcome measure was islet graft function based on $AUC_{C\text{-}peptide}/AUC_{glucose}$ derived from a mixed meal test up to 5 years after transplantation. All statistical analyses were performed with GraphPad Prism 5.01. Results are expressed as medians (interquartile range, IQR) for continuous variables, and as frequencies for categorical variables. Relationships between variables were checked for linearity using the Pearson correlation coefficient (r). Comparisons of non-paired quantitative parameters were performed using Student's t-test or ANOVA. Percentage comparisons were performed with Chi-square or Fisher's exact tests. In analysis including more than two groups, the data were compared using a one-way ANOVA test.

Results

Donor and islet preparation characteristics

Pancreas from 54 donors were used. The median age of the donors was 48 (IQR 42 - 56) years with a BMI of 27 (IQR 25 - 30) kg/m^2. The majority of donors (72%) were from donation after brain death (DBD) procedures (**Table 1**). Thirteen islet transplants involved islet preparations from two donors, while the remaining infusions were composed of islets from a single donor.

The median purity by visual assessment was 65 (IQR 55 - 71) percent and the median purity by IsletNet assessment of photographic images was 48 (IQR 33 - 59) percent (**Supplemental Figure 1a**). Islet purity was positively associated with the islet size index (R^2=0.45, p<0.0001) (**Figure 1a**), negatively associated with the percentage of embedded islets (R^2=0.55, p<0.0001) (**Figure 1b**), and negatively associated with transplant volume (R^2=0.22, p=0.002) (**Figure 1c**).

Table 1. Baseline characteristics of pancreas donors

	Complete cohort	Low purity ≤38%	Intermediate purity 39-58%	High purity ≥59%	*p*-value*
n	54	18	20	18	NA
Age, years	48 (42- 56)	49 (40- 58)	50 (43- 56)	48 (42- 56)	0.882
Sex, female (n, %)	19 (35%)	7 (18%)	8 (20%)	4 (16%)	0.598
BMI, kg/m^2	28 (25- 30)	27 (25- 30)	27 (23- 30)	29 (27- 32)	0.435
Type of donation (DBD/DCD)	39/15	10/8	17/3	12/4	0.124

Values depicted as median (Q1- Q3) unless otherwise noted
BMI = body mass index; DBD = donation after brain death; DCD = donation after cardiac death
**p*-value represents a comparison between the low, intermediate and high purity group

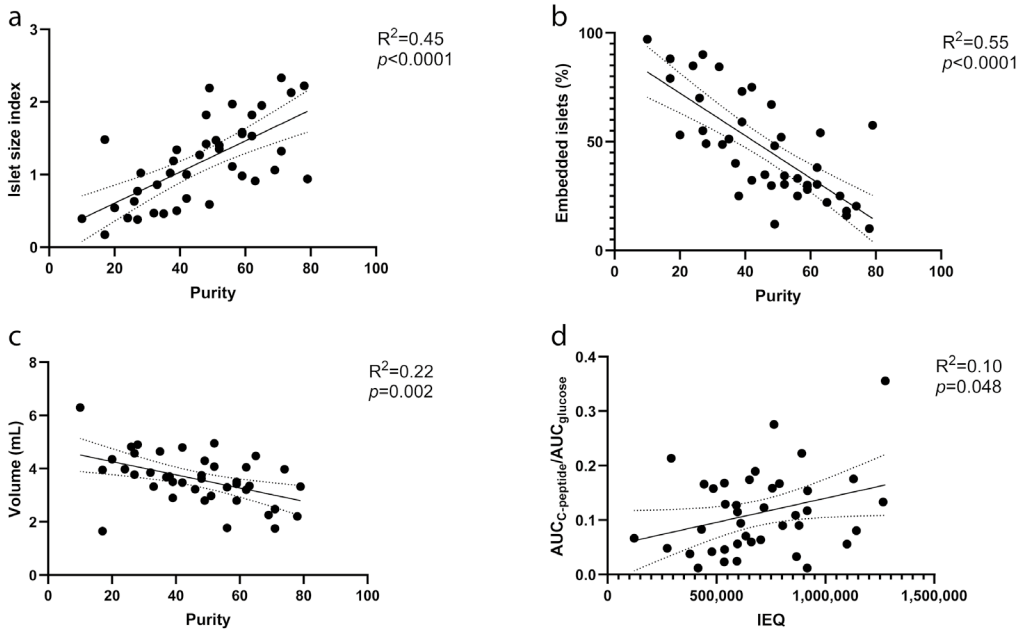

Figure 1. Characteristics of islet preparations
(**a**) Correlation of islet index with purity of the final transplant. Islet index and islet purity were assessed using IsletNet (islet index <1 indicates a mean islet diameter < 150 µm and vice versa).
(**b**) Correlation of the percentage of embedded islets and purity of the final transplant. The percentage of embedded islets and islet purity was assessed with IsletNet.
(**c**) Correlation of volume and purity of the final transplant. Islet purity was assessed with IsletNet.
(**d**) Correlation of IEQ and $AUC_{C\text{-}peptide}/AUC_{glucose}$ from the mixed meal tests at three months post-transplantation. IEQ was calculated with the IsletNet-assessed purity.

Recipients

A total of 41 patients with severe beta cell deficiency underwent a first-time islet transplantation (nine patients islet-alone, 30 patients islet-after-kidney, two patients islet-after-lung). The median age was 56 (IQR 47 - 61) years and the duration of diabetes mellitus was 36 (IQR 27 - 46) years with a HbA1c of 63 (IQR 53 - 73) mmol/mol Hb. Other baseline characteristics are depicted in **Table 2.**

Table 2. Baseline characteristics of islet recipients

	Complete cohort	Low purity ≤38%	Intermediate purity 39-58%	High purity ≥59%	*p*-value*
n	41	14	14	13	NA
Age, years	56 (47- 61)	49 (40- 59)	58 (54- 66)	55 (45- 61)	0.014
Sex, female (n, %)	16 (39%)	6 (43%)	5 (36%)	13 (38%)	0.927
BMI, kg/m^2	24 (22- 26)	23 (20- 29)	24 (21- 26)	24 (23- 27)	0.536
Duration of diabetes mellitus, years	36 (27- 46)	30 (25- 45)	41 (31- 50)	36 (29- 43)	0.151
HbA1c, mmol/mol Hb	63 (53- 73)	66 (56- 74)	61 (52- 72)	67 (53- 68)	0.333
Anti-GAD65, IU/mL	6 (1- 94)	7 (2- 242)	8 (1- 36)	6 (0- 19)	0.602
Serum creatinine, µmol/L	126 (84- 166)	113 (77- 140)	143 (99- 175)	114 (91- 166)	0.527
eGFR, mL/min/1.73m^2	54 (35- 63)	58 (40- 90)	43 (35- 60)	55 (37- 60)	0.186
IAK or IAL/ITA, n	32/9	11/3	13/1	8/5	0.145

Values depicted as median (Q1- Q3) unless otherwise noted
BMI = body mass index; GAD65= glutamic acid decarboxylase 65, eGFR = estimated glomerular filtration rate; IAK = islet after kidney; IAL = islet after lung, ITA = islet transplantation alone
**p*-value represents a comparison between the low, intermediate and high purity group.

Transplanted IEQ and graft outcome

The median transplanted IEQ, assessed by IsletNet, was 659,674 (IQR 536,630 - 867,830) with a median purity of 48 (IQR 33 - 59) percent, in a median volume of 3.6 (IQR 3.2 - 4.1) mL.

There was a positive correlation between IEQ and $AUC_{C\text{-}peptide}/AUC_{glucose}$ obtained during a mixed meal tolerance test at 3 months (R^2=0.10, p=0.048) (**Figure 1d**). No correlation was found when glucose was not taken into account (**Supplemental Figure 2a**). There was no correlation between IEQ and $AUC_{C\text{-}peptide}/AUC_{glucose}$, or IEQ and $AUC_{C\text{-}peptide}$ when IEQ was calculated using visually assessed purity (**Supplemental Figure 1b, 1c**). Next, we evaluated the role of purity as a determinant of graft outcome.

Purity and graft function

Purity showed a positive correlation with the $AUC_{C\text{-}peptide}/AUC_{glucose}$ obtained with a mixed meal tolerance test at 3 months (R^2=0.16, p=0.011) (**Figure 2a**). No correlation was found between purity

and $AUC_{C\text{-peptide}}$ alone at 3 months (**Supplemental Figure 2b**).

In islet isolation practice there is often a custom to broadly classify islet preparations as preparations with low, intermediate and high purity. Therefore, we divided islet preparations into tertiles, yielding groups with low (≤38%), intermediate (39-58%), and high (≥59%) islet purity (**Table 1 and Table 2**). The median IEQ transplanted was 519,130 (415,544 - 591,196) in the low purity group, 708,098 (IQR 596,522 - 867,283) in the intermediate purity group, and 878,587 (IQR 746,087 - 1,098,478) in the high purity group. Median purity in the low, intermediate and high islet purity group was 27%, 49% and 65%, respectively. No difference was found between the groups in C-peptide/glucose ratio (CP/G) during these first 3 months (**Figure 2b**). Furthermore, yearly evaluation of $AUC_{C\text{-peptide}}/AUC_{glucose}$ during the mixed meal test showed no significant difference between the 3 purity groups up to 5 years after transplantation (p=0.23) (**Figure 2c**), also when glucose values were not taken into account during the mixed meal tests by using only the $AUC_{C\text{-peptide}}$ (**Supplemental Figure 2c**).

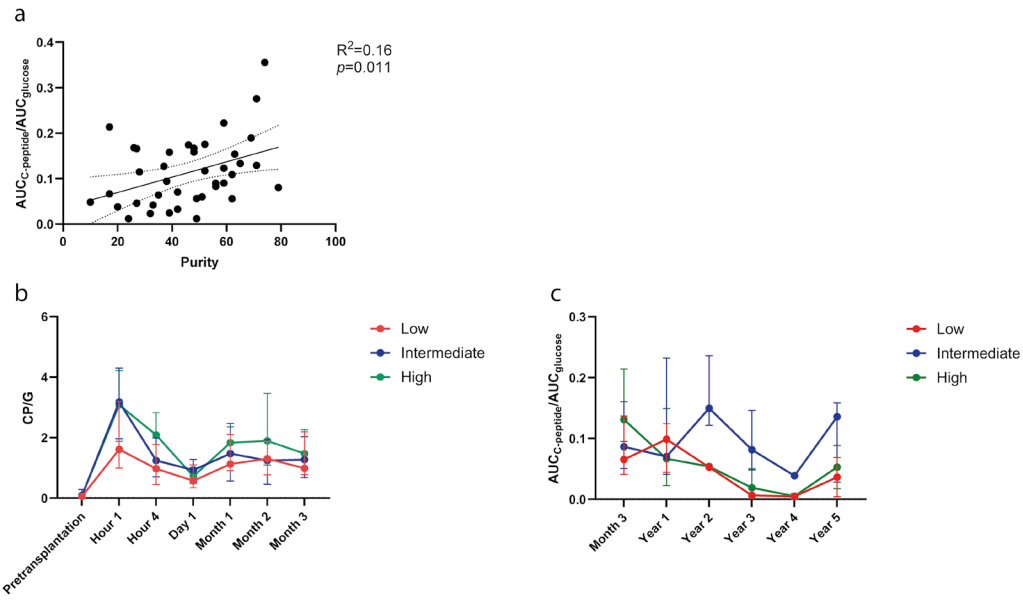

Figure 2. Purity and graft function

(**a**) Correlation of purity of the final transplant and $AUC_{C\text{-peptide}}/AUC_{glucose}$ from the mixed meal test at three months post-transplantation. Islet purity was assessed with IsletNet.

(**b**) The C-peptide/glucose ratio (CP/G) up to 3 months after transplantation. Glucose and C-peptide concentrations were taken from random blood samples.

(**c**) The $AUC_{C\text{-peptide}}/AUC_{glucose}$ annually up to 5 years after transplantation.

Values are depicted as median (IQR).

Discussion

The main findings of our study indicate that islet purity has no major effect on metabolic outcomes in

first-time islet transplant patients with severe beta cell deficiency up to 5 years after transplantation. Previous clinical studies showed that islet transplants with more exocrine tissue result in superior metabolic outcomes up to 5 years after transplantation[11,12]. However, the limitations of those studies include small cohort sizes, imprecise measurements of islet purity, and indirect metabolic outcome measurements such as daily insulin requirement. In contrast, here we applied a robust objective method to calculate islet purity and used mixed meal tests with C-peptide and glucose measurements to evaluate islet graft function. Cell composition of islet clusters and its implication for long-term graft outcome is also an issue in current discussions related to stem cell-derived islet preparations[28,29].

Here we observed that higher purity islet transplants contained less embedded islets and larger islets and were transplanted in less volume suggesting a better digestion and islet isolation procedure. In addition, we observed that islet purity was overestimated by visual assessment despite our experienced operators, particularly in cases with lower IsletNet-calculated purity. Previous reports have also described that visual estimation after DTZ-staining overestimates islet purity when compared with precise quantification techniques such as electron microscopy or DNA content[14,30]. We only found a significant correlation between IEQ and $AUC_{C\text{-}peptide}/AUC_{glucose}$ at three months when IsletNet purity was used to calculate IEQ. These findings underscore the importance of precise and reproducible determination of islet purity in optimizing the outcomes of islet transplantation procedures.

In addition, we found that higher purity islets provide a significant metabolic benefit at three months post-transplantation, although this advantage diminished over a five-year period, resulting in no observable difference in long-term outcomes between the purity groups. During the initial period following transplantation, islets depend on diffusion for their oxygenation, as the revascularization process is not yet complete[31]. The liver microenvironment is characterised by a low oxygen tension, which makes islets more susceptible to hypoxia-induced cell death[32]. Non-islet tissue, primarily composed of exocrine cells, may further compromise the already problematic oxygenation of islets by competing for oxygen and nutrients. This competition could result in a higher rate of islet apoptosis or dysfunction, leading to the poorer metabolic outcomes observed in the low purity group at three months. In time, the impact of non-islet tissue on islet survival and function appears to change. Ductal cells are known to secrete angiogenic factors, such as vascular endothelial growth factor (VEGF), which can promote the vascularization of islets[20,33]. Better vascularization of islets increases the delivery of oxygen and nutrients, which can prevent beta cell dysfunction and beta cell loss[34-36].

Furthermore, it could be hypothesized that lower purity islet transplants are more resistant to injury and subsequent inflammation, possibly due to increased protection of non-islet cells during and after infusion. Inflammation in the early phases of islet transplantation has been recognised as a major contributor to poor long-term graft survival[37]. During islet infusion blood comes in contact with islet surface molecules such as tissue factor, which induces activation of coagulation, complement, and an innate immune response known as the instant blood-mediated inflammatory reaction (IBMIR)[38]. This reaction can rapidly destroy transplanted islet cells, reducing effective islet

cell mass by 50-60%[3,39]. As a result, most patients require additional transplantation of donor islets to achieve clinical goals[40]. Evidence for possible protection of non-islet cells can be observed in autologous islet transplantation, where unpurified islets are transplanted. Compared to allogeneic islet transplantation, the acute inflammatory response following transplantation is reduced, potentially due to the protective effects of non-islet tissue[41].

Whether ductal cells are a source of islet progenitors that could turn into functional insulin-producing cells is a matter of long-term debate[18]. In addition to *in vitro* research which indicates that a progenitor cell might be present in the ductal compartment[19], indirect evidence includes the detection of insulin-positive cell clusters in ductal structures or close to ducts in individuals with increased metabolic demand, such as pregnancy or obesity[15-19]. The authors of a study that discovered that lower purity islet transplantation improves long-term islet graft function suggested that this beneficial effect might be mediated via ductal-to-endocrine cell differentiation[12]. In our cohort, however, we did not observe better long-term metabolic outcome in the lower purity group as a sign of beta cell neogenesis from transplanted exocrine cells.

It is important to acknowledge the limitations of our study. Although our study is one of the larger studies investigating the effect of islet purity on transplantation outcomes to date, the sample size was limited especially for long-term islet outcomes, given that patients who received a second islet infusion were excluded from further analysis. Additional studies with larger sample sizes and longer follow-up periods are necessary to validate and extend our findings.

In conclusion, the comparable long-term metabolic outcomes observed between purity groups in our study indicate that islet purity is not associated with long-term islet graft function. These findings could have implications for optimizing donor islet transplantation protocols, cell composition of stem cell-derived islets in novel islet replacement strategies, and call for further research to better understand the effect of non-islet cells on long-term graft function.

Abbreviations

BMI, body mass index; CA19-9, Carbohydrate antigen 19-9; CP/G, C-peptide/glucose ratio; HbA1c, hemoglobin A1c; IEQ, islet equivalents

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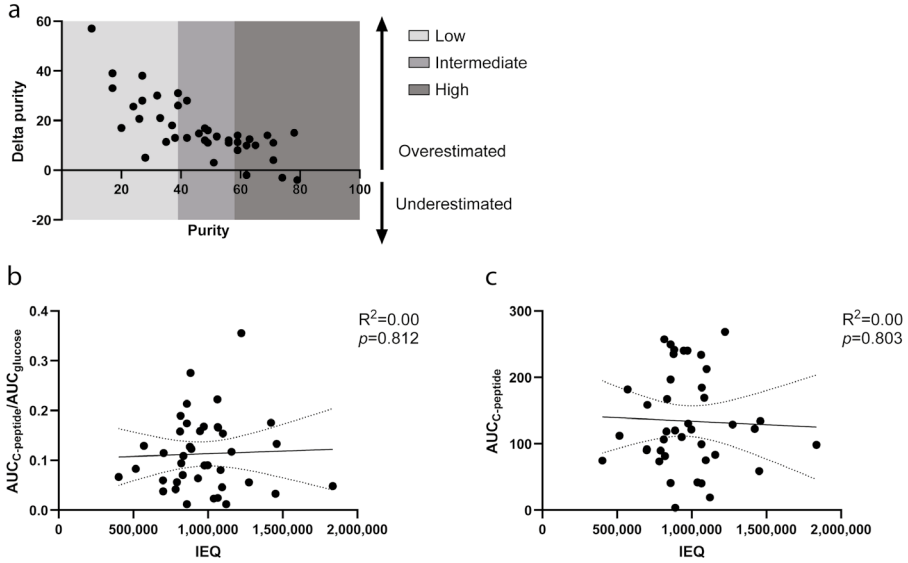
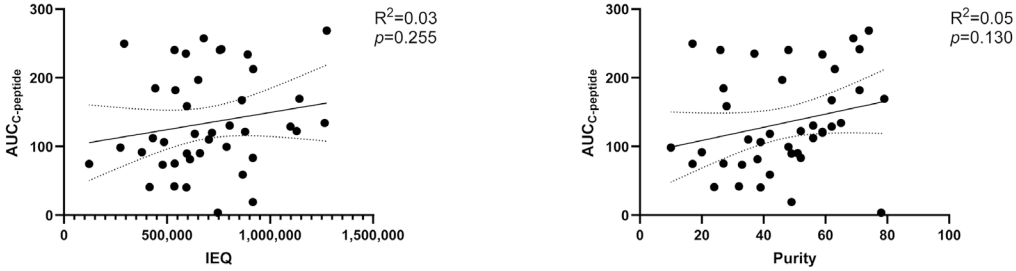
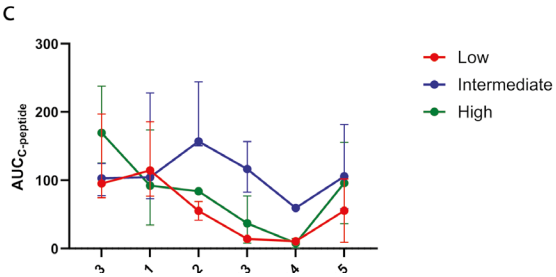
Supplemental information



Supplemental Figure 1. Visually assessed islet purity

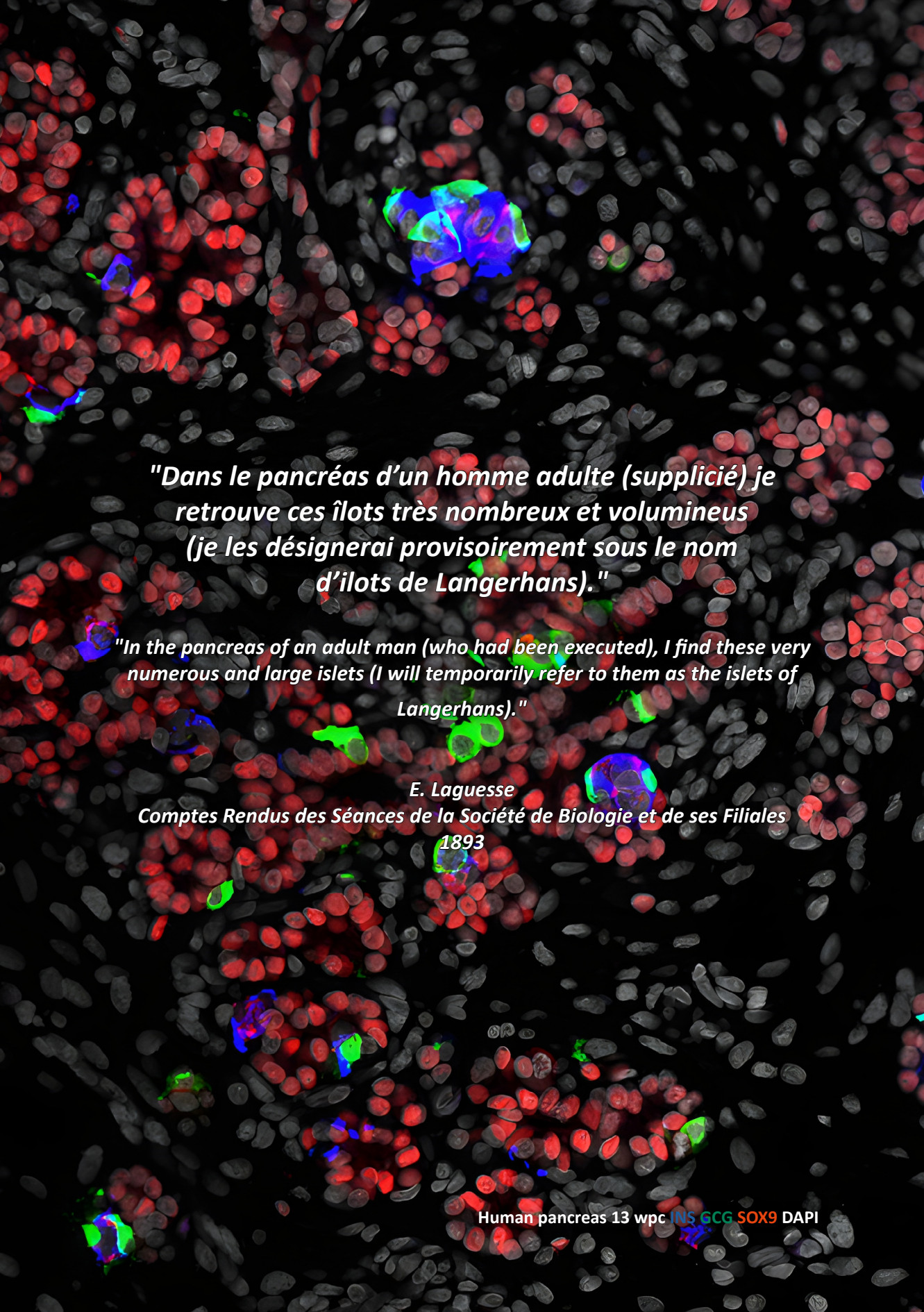
(**a**) The difference between visually assessed islet purity and IsletNet assessed islet purity. The delta purity is visually assessed purity – IsletNet assessed purity. (**b**) Correlation of IEQ and $AUC_{C\text{-}peptide}$/ $AUC_{glucose}$ from the mixed meal tests at three months post-transplantation. IEQ was calculated with the visually assessed islet purity. (**c**) Correlation of IEQ and $AUC_{C\text{-}peptide}$ from the mixed meal tests at three months post-transplantation. IEQ was calculated using the visually assessed islet purity.





Supplemental Figure 2. $AUC_{C\text{-}peptide}$ and $AUC_{glucose}$ during follow-up

(**a**) Correlation of IEQ and $AUC_{C\text{-}peptide}$ from the mixed meal tests at three months post-transplantation. IEQ was calculated with the IsletNet assessed purity. (**b**) Correlation of purity and $AUC_{C\text{-}peptide}$ from the mixed meal test at three months post-transplantation. Islet purity was assessed using IsletNet. (**c**) The $AUC_{C\text{-}peptide}$ annually up to 5 years after transplantation. Values are depicted as median (IQR).


"Dans le pancréas d'un homme adulte (supplicié) je retrouve ces îlots très nombreux et volumineux (je les désignerai provisoirement sous le nom d'ilots de Langerhans)."
"In the pancreas of an adult man (who had been executed), I find these very numerous and large islets (I will temporarily refer to them as the islets of Langerhans)."
E. Laguesse
Comptes Rendus des Séances de la Société de Biologie et de ses Filiales
1893
Human pancreas 13 wpc INS GCG SOX9 DAPI

CHAPTER| 3

Organoids From the Human Fetal and Adult Pancreas

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Abstract

Purpose of review

Novel 3D organoid culture techniques have enabled long-term expansion of pancreatic tissue. This review comprehensively summarizes and evaluates the applications of primary tissue-derived pancreatic organoids in regenerative studies, disease modelling, and personalized medicine.

Recent findings

Organoids derived from human fetal and adult pancreatic tissue have been used to study pancreas development and repair. Generated adult human pancreatic organoids harbor the capacity for clonal expansion and endocrine cell formation. In addition, organoids have been generated from human pancreatic ductal adenocarcinoma in order to study tumour behaviour and assess drug responses.

Summary

Pancreatic organoids constitute an important translational bridge between *in vitro* and *in vivo* models, enhancing our understanding of pancreatic cell biology. Current applications for pancreatic organoid technology include studies on tissue regeneration, disease modelling, and drug screening.

Introduction

The pancreas is a mixed endocrine and exocrine gland that plays a pivotal role in digestion and metabolic homeostasis. The endocrine pancreatic compartment includes the alpha and beta cells, which secrete the hormones glucagon and insulin, respectively, that are required for normal glycemic control. The exocrine compartment is composed of acinar cells, which store and secrete digestive enzymes that are drained into the duodenum by a highly branched, tubular epithelial tree-like duct network within the pancreas.

Pancreas biology has been intensively studied, in an effort to understand pancreas development and function, to obtain insight into pathophysiological processes, to identify disease-associated markers, and to find better treatment options for devastating pancreatic diseases associated with high mortality and healthcare costs, such as diabetes mellitus, pancreatic adenocarcinoma (PDAC), pancreatitis, and cystic fibrosis. Studying the normal human pancreas at the cellular level is difficult. The high risk for complications of pancreatic biopsies more or less precludes this option to generate cross-sectional and longitudinal data of normal human pancreatic tissue at the cellular level in most patients. So we are dependent on generating cross-sectional data from surgical specimens, being aware that the pathological process for which the surgery was indicated could affect the 'normal' pancreatic tissue surrounding the pathological process, or from donor pancreas that is not (entirely) used for pancreas or islet transplantation. *In vivo* models, such as rodents, do not have this limitation but are time-consuming and expensive. Moreover, results obtained from *in vivo* animal models are not always translatable to humans[1]. Although characterised by simplicity and good controllability, two-dimensional (2D) culture models using immortalized cell lines or primary cells limit the study of crucial aspects such as cell polarity, cell-to-cell contacts,

three-dimensional self-organisation, and interaction with stromal cells and other extracellular matrix components[2-6]. Moreover, adaptation of primary cells or cell lines during monolayer culture conditions fundamentally changes cell behaviour so that findings are often no longer translatable to the primary tissue[7-9].

Recent breakthroughs in three-dimensional (3D) culture methods have led to the development of organoid culture platforms, which allow researchers to perform translational research on long-term *in vitro* cultures not limited by barriers present in 2D culture or animal models[10-13]. Organoids are three-dimensional cellular structures created through self-organisation of cells that can closely mimic the architecture and functionality of the native organ from which the cells were originally derived.

Long-term organoid cultures were originally established by the identification of conditions that recapitulate the *in vivo* intestinal stem cell niche *in vitro*. The field was spearheaded by a report that organoids could be made from isolated mouse intestinal crypts cultured in Matrigel and supplemented with growth factors that stimulated Wnt signaling, reflecting the high degree of Wnt signaling in intestinal crypts[14]. One of the key components in organoid culture systems is the 3D environment in which cells behave most optimally. Cells are cultured within an extracellular matrix, such as Matrigel, which prevents attachment of cells to the surface of the tissue culture plates and aims to mimic *in vivo* mechanical and biochemical stimuli dictating cell polarization and autonomous reorganisation[15-18]. In addition to the extracellular matrix, organoids are cultured in a medium that usually contains growth factors that are known to stimulate proliferation during organogenesis and tissue homeostasis, such as Wnt activators (Wnt3a, R-spondin), receptor tyrosine kinase ligands (EGF, FGF10), BMP inhibitors (Noggin), and TGF-beta inhibitors[19]. The starting material of organoids can either be fragmented or dissociated primary tissue, or pluripotent cells such as embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC) that are pre-differentiated *in vitro*. One of the additional advantages of organoids is the large amount of biomass that can be expanded with just one or a few progenitor cells.

In this review, we discuss the application of human fetal and adult tissue-derived pancreatic organoids in regenerative studies and their use as patient-specific tools for disease modelling.

Generation of organoids from fetal pancreatic tissue: fetal pancreatic organoids

Pancreas organogenesis is a complex process requiring a dynamic spatiotemporal interplay of multiple cells and their surrounding niche. Deciphering the molecular mechanisms underlying morphogenesis and cell specification is essential for a better understanding of pancreas development and regeneration. While the use of transgenic animal models has greatly increased our knowledge of the molecular basis of pancreatic lineage decisions and cell specification, the mechanisms governing morphogenesis are not well understood. Thus, a different approach for the study of fetal development is necessary. Because there are currently no cell lines available that can act as multipotent pancreatic

progenitor cells, an alternative approach to overcome this hurdle is the culture of primary fetal tissue.

Due to the limited availability of human fetal tissue, rodent fetal tissue has initially been used for *in vitro* studies. Some of the early insights into rodent pancreatic differentiation arose from the culture of rat fetal pancreas explants, with the tissue being cultured with or without the surrounding mesenchyme. A marked increase in endocrine cells was observed in cultures without mesenchymal cells[20,21].

While rodent explants at least partially recapitulate endocrine differentiation *in vitro*, they are limited by their short-term maintenance and the contribution of multiple cell types, which makes it difficult to characterize and study the *bona fide* fetal pancreatic stem cells. Prior studies indicated that cells expressing SOX9 during early pancreas development can give rise to both endocrine and exocrine cells making this a suitable candidate marker for multipotent progenitors[22]. With that in mind, SOX9 progenitor cells that were negative for the endocrine progenitor marker Ngn3 were isolated by flow cytometry from E11.5 SOX9-eGFP, NGN3-tdTomato mouse pancreas and embedded in Matrigel for clonal expansion[23]. These single cells generated heterogeneous spheres with Sox9(+), Ngn3(-) cells, and a small amount of Sox9(-), Ngn3(+), C-peptide(+), and glucagon(+) cells *in vitro*, showing a degree of multipotency which was maintained for up to three passages. Additional shRNA-based loss-of-function screening of endocrine developmental genes was performed on these spheres to demonstrate that these spheres recapitulate several key aspects of endocrine differentiation *in vitro*, thus establishing a possible model for the study of differentiation of multipotent progenitor cells *in vitro*.

A similar method was applied in an effort to investigate the morphogenesis of murine pancreatic duct development[24]. Cells expressing Sox9 were isolated from E10.5 SOX9-eGFP mice and cultured in Matrigel. Depending on the specific combinations of growth factors used in the culture medium two different types of organoids could be generated—hollow spheres which maintained a higher contribution of progenitor cells that were similar to the previously described cells[23], or complex organoids containing more differentiated cells (**Figure 1**). These complex organoids developed a branching ductal network of polarized cells and showed tip-trunk segregation reminiscent of pancreas morphogenesis *in vivo*. These organoids were shown to grow out from their tip-like structures forming tubular networks, the branching of which is thought to be caused by strong local inhibitory signaling[25,26]. As such, the tips of these organoids formed acinar cells, whereas the center of the organoids contained Hnf1β(+), Sox9(+), and Pdx1(+) progenitor-like cells and a small number of endocrine cells. Furthermore, endocrine cell formation in these organoids could be increased through inhibition of mesenchymal signaling by the removal of FGF1 from the culture medium or by blocking FGF signaling via small molecule inhibitors[24]. To study the interplay between mesenchymal signaling and organoid differentiation, a co-culture method was developed in which E10.5 mouse organoids were tightly enveloped by their native mesenchyme[27]. Using these organoids, it was shown that the gene NFIA acts as a regulator of Notch activation through blockade of Dll1 endocytosis leading to an endocrine fate[27]. Interestingly, these results obtained with organoids are in line with the results from rat fetal explant cultures, in which the presence of mesenchyme was

associated with reduced endocrine cell specification.

In an effort to obtain insight into human fetal pancreas development, Bonfanti et al.[28] used human fetal pancreatic tissue to create a model for the study of pancreas development in a 3D culture system. The authors described efficient expansion of small fragments of human fetal tissue that, similar to their mouse counterparts, grew as hollow spheres containing polarized cells. These fetal organoids also expressed the key transcription factors of pancreatic progenitor cells (PDX1, NKX6.1, SOX9). By using a growth factor combination of the Wnt agonist R-Spondin1 (RSPO1), FGF10 and EGF, they were able to keep these progenitors in expansion for up to 5 months. Interestingly, in the absence of EGF in the culture medium, endocrine differentiation was promoted at the expense of proliferation.

Although large steps have been taken in the study of pancreatic development through the use of fetal organoids, limited accessibility and ethical considerations hamper the use of human fetal tissue. Nevertheless, the use of human fetal tissue in organoid culture is a valuable method to study signaling pathways and conditions necessary for normal pancreas morphogenesis and cell specification.

Generation of organoids from adult pancreatic tissue: adult pancreatic organoids

Recent technological achievements have revealed that adult tissues may contain a more abundant source of tissue stem cells than previously anticipated, allowing organoid cultures from multiple types of tissues to study differentiation, tissue stem cell maintenance, and disease. A breakthrough in adult primary tissue culture was the discovery of Lgr5(+) adult stem cells in the intestinal crypts of mice that generated organoids *in vitro*[14,29]. Since then, organoid culture methods have been established for stomach, liver, pancreas, brain, lungs, and many other organs and tissues[30]. This led to the hypothesis that the culture of putative tissue stem cells from the adult human pancreas could prove useful for studies into pancreas regeneration, including regeneration of pancreatic islets which could be potentially relevant for beta cell replacement therapy.

Human pancreatic ducts have been shown to exhibit a capacity for *in vitro* expansion and formation of 3D hollow structures when cultured in collagen or Matrigel, hence showing potential as tools for regenerative studies[31]. Initially the short-term expansion and differentiation capacity of adult human and mouse pancreatic cells was demonstrated in suspension culture, where single islet and duct cells formed spheres expressing both neural and progenitor markers. Single spheres from both origins showed capacity for differentiation towards C-peptide-positive beta-like cells and neurons, demonstrating that a clonally expandable progenitor pool could be present in the adult human and mouse pancreas[32,33]. To zoom in on the putative progenitor cell in the murine pancreas, Jin et al. used flow cytometry to isolate and culture a subpopulation of ductal cells that formed hollow ductal spheres in a methylcellulose and Matrigel-containing semisolid medium. These cells showed capacity for differentiation towards an endocrine cell fate after being treated with R-Spondin1,

which induced the formation of dense organoids with increased expression of endocrine progenitor markers[34]. Huch et al.[35] used similar culture conditions but applied them on small ductal fragments isolated from mice. These organoids formed hollow spheres of ductal cells that generated budding structures and demonstrated unlimited expansion while maintaining genetic stability. Although these structures retained ductal characteristics *in vitro*, they were able to form endocrine-like cells when co-transplanted with mouse embryonic pancreatic cells under the kidney capsule of mice, demonstrating that these organoids harbor bipotent progenitors.

Multiple groups have reported on the generation of pancreatic organoids derived from the adult human pancreas[36,37]. These organoids formed mostly hollow spheres with no capacity for the formation of complex tip-trunk structures or any spontaneous endocrine cell differentiation, which was observed in fetal tissue-derived organoids (**Figure 1**). Human adult tissue-derived organoids could, however, still be coerced into differentiation towards a beta cell-like phenotype by the overexpression of key transcription factors regulating beta cell formation[36]. Recently, we described a protocol for the generation of more complex organoids derived from human pancreatic islet-depleted tissue fragments[38] (**Figure 1**). Without genetic modification, these organoids showed increased expression of aldehyde dehydrogenase (ALDH) in their budding structures and showed formation of de novo insulin(+) cells upon xenotransplantation into immunodeficient mice. Additionally, we showed that increased ALDH activity in human pancreatic organoids could be used to enrich for cells with colony forming capacity.

Organoids derived from the adult human pancreas differ from fetal pancreatic organoids in multiple aspects, such as morphology and endocrine cell formation, most of which are likely to be explained by the apparent absence of a multipotent progenitor in the adult organ or the lack of an optimal culture medium or enrichment strategies. They are, however, important tools to study regeneration by identification of the cell population responsible for organoid formation and of factors or cell-to-cell interactions involved in this process.

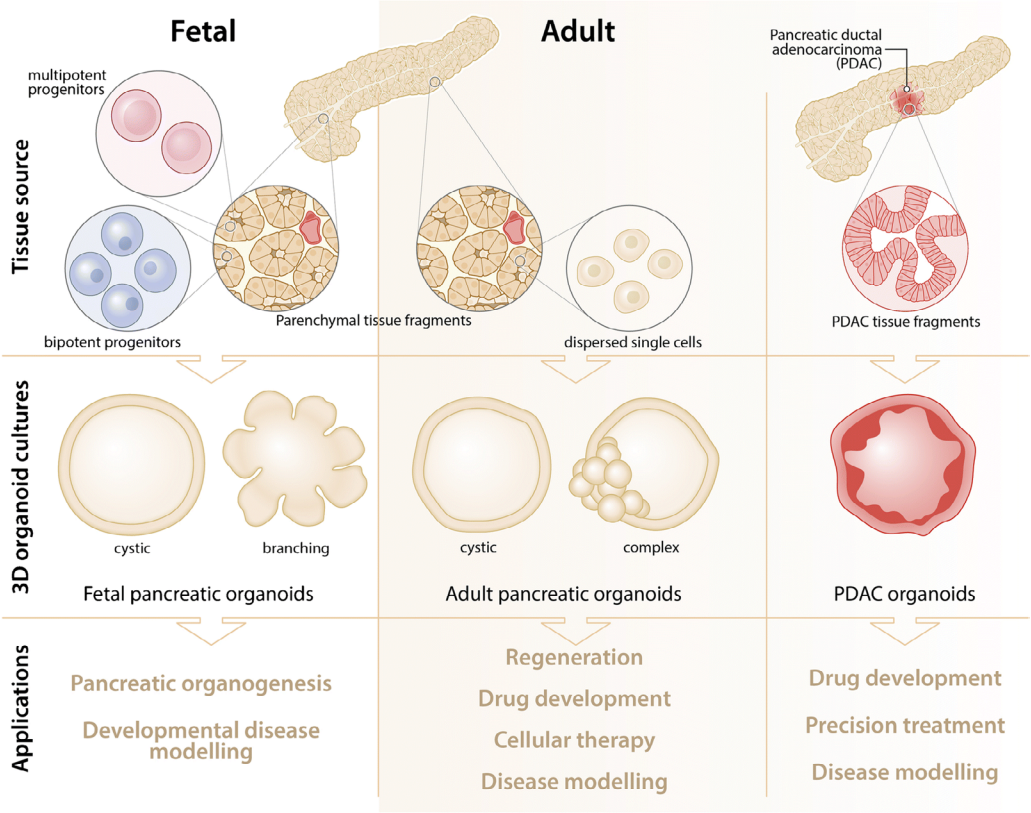

Figure 1. Pancreatic organoid generation and its applications
By using 3D culture conditions, it is possible to generate organoids from primary fetal and adult pancreatic tissue or from pancreatic ductal adenocarcinoma (PDAC). Depending on the starting tissue/cell source and the culture conditions, there are differences in organoid morphology and cellular composition. More branching fetal organoids can be generated from fragments of fetal pancreatic parenchymal tissue. Dispersed single cells from these fetal tissue fragments can give rise to cystic organoids with multipotent progenitors also generating branching organoids. They permit the study of developmental cues and morphogenesis. Similarly, adult pancreatic parenchymal tissue fragments can lead to the formation of more complex organoids while dispersed single cells generate more cystic organoids, allowing the study of regenerative mechanisms, drug development, modelling of disease, or differentiation towards endocrine lineages. Tissue biopsies from patients with pancreatic ductal adenocarcinoma (PDAC) can be used to produce patient-specific PDAC organoid lines that can be used for disease modelling, drug development, and person-centered treatment options (precision medicine).

Which pancreatic cell types generate the pancreatic organoids?

Unlike the small intestine, there is limited information on the presence or identity of progenitor cells in the adult pancreas[29], and whether in their absence regeneration and homeostasis is orchestrated through the replication and transdifferentiation of mature cells. The matter is further complicated

by contradictory results from animal models. We know that animal models do not fully recapitulate human physiology, which is known for its low cell turnover and limited regenerative capacity[39-45], whereas animal models demonstrate increased pancreatic regeneration, beta cell proliferation, and islet neogenesis[22,46-51]. Nevertheless, human beta cell plasticity has been observed when there is increased insulin demand, such as in pregnancy or obesity[52,53]. Apparently this regenerative capacity fails in patients with type 2 diabetes that have a decreased beta cell mass, despite their increased insulin demand[53-55]. Additionally, patients suffering from pancreatitis, an inflammation of the pancreas, show signs of exocrine metaplasia and ductal cell proliferation[56], indicating that the human pancreas has at least some degree of regenerative capacity. Unlocking that potential could lead to effective treatment options for diseases such as diabetes and pancreatitis. However, to gain a better understanding of human pancreatic regeneration, it is imperative to understand the mechanisms underlying organ maintenance, starting with the cell types involved and the signaling pathways responsible.

Long-term organoid cultures of adult pancreatic tissue fragments largely consist of cells expressing ductal markers such as KRT19, SOX9, and MUC1, suggesting that the organoid-forming cells are derived from the pancreatic duct compartment or through transdifferentiation of other pancreatic cells. But what subpopulation of cells exactly forms complex organoids is still unclear. Multiple groups have used cell surface markers or other isolation techniques in an effort to find adult pancreatic exocrine cells capable of clonal expansion in 3D culture. Initially the pan-epithelial cell surface marker EpCAM in combination with the fluorescent chelator TSQ, which binds endocrine granules, was used on adult mouse pancreas to sort EpCAM-positive, non-endocrine epithelial cells that were capable of long-term clonal expansion in 3D culture[35]. By using transgenic mice to lineage trace and sort SOX9 and PTF1a, the authors were able to demonstrate that only SOX9-positive ductal cells were capable of long-term clonal expansion of single cells, whereas PTF1A-positive acinar cells failed to clonally expand over prolonged time.

The cell surface marker CD133 (prominin-1), initially recognised as a surface marker for hematopoietic and neural stem cells[57,58], has been successfully used to enrich for cells with stem cell or cancer-initiating characteristics from multiple organs[59-63]. In the human fetal and adult pancreas, CD133 has been identified as a cell surface marker labelling ductal cells[64,65]. Lee et al.[36] used human pancreata to sort CD133-positive single ductal cells that could subsequently be clonally expanded as cystic structures in Matrigel with expansion medium supplemented with Wnt signaling factors. During expansion, these 3D cultures retained some of the characteristics of the primary human pancreatic ducts, such as cell polarity and KRT19 expression. Although *in vitro* differentiation protocols were applied, expanded ductal cells could not be converted to beta-like cells without adenoviral-mediated overexpression of the beta cell-specific transcription factors MAFA, PDX1, NGN3, and PAX6 followed by a 2-week culture for maturation. Other groups have used CD133 in combination with other cell surface markers to enrich for ductal subpopulations from adult mouse pancreas. For example, CD133(+) cells combined with CD71(low) positivity enriched for an adult mouse pancreas ductal cell subpopulation which could be expanded as cystic structures in 3D

culture. When transplanted in diabetic mice, these cells yielded ductal cells, acinar cells, and insulin and glucagon monohormonal cells[66,67].

The exocrine compartment of the pancreas is for a large part composed of acinar cells. There is limited evidence from rodent studies that suggests that the adult acinar compartment can generate new exocrine and endocrine cells during pancreatic injury[68,69]. Wollny et al. used cellular size to separate acinar cells from ductal cells in the adult mouse pancreas, and applied a sorting strategy to isolate doublets and triplets of acinar cells from mouse pancreas. They identified a progenitor-like acinar cell subpopulation capable of generating organoids that underwent an acinar-to-ductal metaplasia with a loss of amylase expression and gain of KRT19 expression, indicating that although most acinar cells cannot proliferate or exhibit a limited capacity for expansion *in vitro*, there are a small subset of acinar cells that retain a long-term but unipotent capacity for expansion[70].

Altogether, these results indicate that enrichment for cell subpopulations with progenitor cell characteristics from the heterogeneous pancreatic exocrine compartment can yield cells with an increased capacity for clonal expansion. However, the exact location of these subpopulations and their role in organ maintenance during homeostasis or injury has not been elucidated.

Can pancreatic organoid cells differentiate towards insulin-producing cells?

Since the organoid culture methods outlined here allow the long-term culture of pancreatic cells, it is now possible to use these as a model to further study the mechanisms involved in adult pancreas regeneration. As only 1-2% of the pancreas is composed of endocrine cells and the remaining 98-99% of exocrine cells, insight in the processes that allow endocrine cell specification from exocrine cells could be beneficial for the creation of novel beta cells—this could be used for beta cell replacement therapy for patients with diabetes mellitus, a treatment which is currently hampered due to the shortage of donor tissue. We have demonstrated that the complex organoids derived from human adult islet-depleted tissue fragments harbor cells that have the potential for endocrine differentiation as they can develop into a limited number of hormone-positive cells when transplanted *in vivo* under the kidney capsule of mice[38]. The required signals that are necessary for this differentiation are not clear, and *in vitro* endocrine differentiation of human pancreatic organoids is still inefficient.

In order to improve our understanding of the signals required for progenitor cell growth and differentiation, more defined culture medium in organoid models should be used. A serum-free, conditioned medium-free 3D culture model for adult murine pancreatic progenitors was developed by using 7 defined growth factors and small molecules in culture, although extracellular matrix was formed by a mix of methylcellulose and Matrigel. These growth factors combined with ECM were shown to support self-renewal and *in vitro* differentiation of cells that resemble ductal, acinar, and endocrine cells on the gene expression level, indicating tri-lineage differentiation potential of these multipotent cells[71].

Investigations into the underlying molecular mechanism that enhance endocrine

differentiation could provide additional insights into the required factors for endocrine differentiation. Azzarelli et al.[72] used adult mouse pancreatic ductal organoids and demonstrated that the inhibition of post-translation NGN3 protein phosphorylation enhanced stability of the transcription factor, thus promoting increased expression of its downstream target genes, known to drive endocrine differentiation.

The current protocols for the formation of mature and functional beta cells from adult pancreatic organoids are still far from clinical application due to limitations in efficiency, reproducibility, and scalability. Nonetheless, the *in vitro* platform that organoid culture offers will allow researchers in the field to further decipher the pathways and mechanisms controlling exocrine and endocrine differentiation, which could subsequently aid in the development of future clinical applications such as beta cell replacement therapy. One could envisage that patients undergoing islet autotransplantation after total pancreatectomy due to benign pancreatic disease will receive an additional infusion procedure using their own expanded and differentiated organoids as the isolated islet preparation itself is often insufficient for insulin independence. Naturally this requires more robust expansion and differentiation protocols and also sufficient evidence of genomic stability of these expanded cells before and after transplantation.

Drug responses and toxicity screening using pancreatic organoids

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal forms of cancer with 5-year survival rates less than 8% and is projected to be the second most common cause of cancer-related deaths by 2030[73,74]. The high mortality rate of PDAC is largely due to difficulties in early detection, aggressive late-stage metastasis, and large disease heterogeneity resulting in a mixed response to pharmacological treatment[75,76]. These poor clinical outcomes illustrate the need for new tools to rapidly and accurately identify effective therapies for patients.

Although cell lines, rodent models, and patient-derived xenograft models have generated valuable insights, organoid models have emerged as a superior tool for studying PDAC behaviour and assessment of the response to potential therapeutic molecules or screening for drug sensitivities *in vitro*[77-79]. Romero-Calvo et al.[80] performed detailed comparative analyses by histopathological profiling and thorough genomic characterization by deep sequencing of primary human PDAC tissue, organoids derived from the PDAC tissue and PDAC tissue transplanted in mice. They demonstrated that human PDAC organoids show strong concordance at the structural and genetic level with the primary tumour, and that the genetic composition of PDAC organoids remained constant over multiple passages. Moreover, tumour-specific drug responses could be evaluated in assays, with an *in vivo* differential drug response to drug treatments of patient-derived xenografts that could be recapitulated *in vitro* with matched PDAC organoids[80]. These comparative results are in line with findings obtained by other groups that have compared PDAC organoids with the primary tumour and patient-derived xenograft models[37,81], and drug sensitivity studies performed by other

groups on mouse and human PDAC organoids[37,82,83]. Novel tools have also been developed to assess drug response of PDAC organoids. Walsh et al.[82] use optimal metabolic imaging (OMI), a novel and non-destructive imaging tool, to quantify drug-induced changes in cellular metabolism in order to evaluate the effect of several anti-cancer treatments on PDAC organoids.

Genomic analysis has identified molecular subtypes of pancreatic cancer and PDAC subtypes, as well as their differential response to therapy[84,85]. PDAC organoids have also been used to study tumour biology and heterogeneity of a single pancreatic tumour. A recent PDAC organoid study used CRISPR gene editing to demonstrate that Wnt independence is acquired over time by genetic and epigenetic mechanisms[86]. This heterogeneity is reflected in differences in the PDAC organoid culture medium composition, with some groups using culture conditions rich in Wnt[37] and groups using culture conditions without the addition of Wnt[82,83]. These differences illustrate the necessity for continuous improvements of PDAC organoid culture methods in order to compare results[86].

Besides the potential to study tumour behaviour and drug responses in human PDAC organoids, another advantage is the possibility to generate large amounts of human PDAC organoids from small amounts of tissue. Using endoscopic ultrasound fine needle aspiration (EUS-FNA) to collect tumour tissue, it has been demonstrated that PDAC organoids can be robustly generated with high rates of success with this technique[82,87]. This enhances the clinical application potential and increases the number of patients that could possibly benefit from drug screening and personalized medicine.

Pre-stages of PDAC have also been studied using organoid models. Transplantation of human PDAC organoids into immunodeficient mice resulted in PDAC precursor lesions called pancreatic intraepithelial neoplasms (PanINs)[37]. This allows us to study the disease progression of these precursor lesions to PDAC and could enable us to identify novel biomarkers and diagnostic opportunities for early stages of invasive PDAC.

In summary, these recent studies demonstrate that large numbers of PDAC organoids can be derived from small tissue samples and that PDAC organoids show great promise for the recapitulation of PDAC tumour characteristics *in vitro*. A current limitation of the PDAC organoid cultures is the limited recapitulation of tumour microenvironment *in vitro*, which is provided by stromal cells, blood vessels, nerves, and immune cells *in vivo*. This tumour microenvironment in PDAC is highly dynamic; promotes tumour progression, metastatic niche formation, and therapeutic resistance; and thus has impact on clinical outcome[88]. Future PDAC organoid studies can address this issue by developing co-culture systems with aforementioned supporting cells[89,90]. Little information is available on organoid formation from other types of pancreatic tumours. Ultimately investigations into pancreatic cancer organoids will result in better understanding of pancreatic cancer biology and the development of novel, personalized treatment and diagnostic approaches for this disease.

Pancreatic organoids are also a promising tool for screening of toxic effects of drugs and other compounds, such as alcohol, on pancreatic cells. Alcohol is a major cause of acute and chronic pancreatitis in man, but little information is available on the molecular mechanisms that underlie this

pancreatic disease. Currently, the effects of alcohol have been largely studied on cell lines that exhibit features of acinar cells or on primary acinar cells isolated from rodents[91,92]. However, these screens are labor-intensive and largely limited by quantity of non-expanded primary tissue. Organoids that mimic exocrine functionality can potentially serve as a substitute as they allow for scalability for toxicity screening of large numbers of compounds on organoids with different genetic backgrounds.

Modelling monogenetic diseases with pancreatic organoids

Pancreas disease modelling in a 3D culture system can also be performed using induced pluripotent stem cells from subjects with monogenetic diseases. Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the cystic fibrosis membrane conductance regulator (CFTR), affecting anion transport and fluid secretion in the pancreas and ultimately leading to chronic pancreatitis and pancreatic insufficiency. Also, diabetes often occurs in patients with CF and pancreas exocrine insufficiency, which is the result of a complex cascade of events that has not been elucidated. Unfortunately, most of the animal models of CF do not fully recapitulate important disease aspects of human CF[93]. Hence, there is a clinical need for better understanding of the effects of CF on the pancreas.

Primary human intestine and rectal tissue from patients with CF have already been cultured as organoids, which could be used for quantitative assays of CFTR function and for the testing of new drugs that modulate CFTR protein function[94,95]. For the generation of pancreatic organoids with a CFTR gene mutation, iPSC-derived from a patient with CF were differentiated to a pancreatic progenitor stage and cultured in Matrigel. In Matrigel, these cells rapidly formed cystic organoids that expressed a wide range of ductal markers, such as KRT19, CFTR, and SOX9 when cultured with FGF2 and nicotinamide. These organoids also showed ductal and acinar cell function, as measured by carbonic anhydrase II (CAII) and enzymes (amylase, trypsin, elastase), and could be used to study the effect of the gene mutation on the structure and behaviour of iPSC pancreatic organoids. These models could be applied to further investigate exocrine pancreatic diseases such as cystic fibrosis.

Conclusion

In conclusion, human pancreatic organoids constitute an important translational bridge between *in vitro* 2D monolayers composed of a single-cell-type and *in vivo* animal models. While the expansion and differentiation protocols are still being improved, studies performed with human pancreatic organoids have increased our understanding of pancreas development, homeostasis, and benign and malignant pancreatic diseases. Their potential as a platform for developmental studies, disease modelling, toxicity screening, and personalized treatments for PDAC is already evident, and we envision that they will have a substantial clinical impact in the near future.

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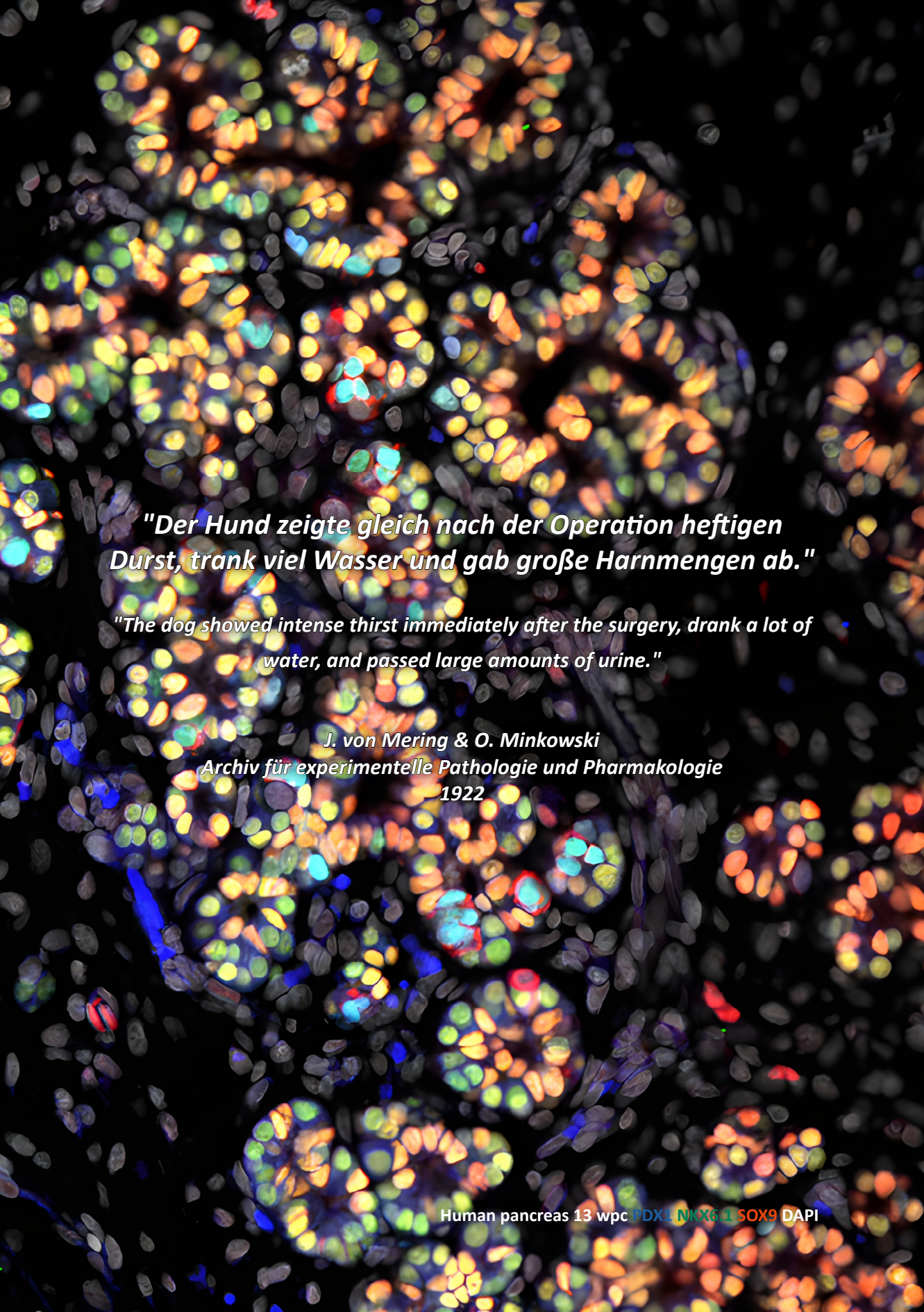
"Der Hund zeigte gleich nach der Operation heftigen Durst, trank viel Wasser und gab große Harnmengen ab."
"The dog showed intense thirst immediately after the surgery, drank a lot of water, and passed large amounts of urine."
J. von Mering & O. Minkowski
Archiv für experimentelle Pathologie und Pharmakologie
1922
Human pancreas 13 wpc PDX1 NKX6.1 SOX9 DAPI

CHAPTER| 4

Expansion of Adult Human Pancreatic Tissue Yields Organoids Harboring Progenitor Cells With Endocrine Differentiation Potential

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Summary

Generating an unlimited source of human insulin-producing cells is a prerequisite to advance beta cell replacement therapy for diabetes. Here, we describe a 3D culture system that supports the expansion of adult human pancreatic tissue and the generation of a cell subpopulation with progenitor characteristics. These cells display high aldehyde dehydrogenase activity ($ALDH^{hi}$), express pancreatic progenitor markers (PDX1, PTF1A, CPA1, and MYC), and can form new organoids in contrast to $ALDH^{lo}$ cells. Interestingly, gene expression profiling revealed that $ALDH^{hi}$ cells are closer to human fetal pancreatic tissue compared with adult pancreatic tissue. Endocrine lineage markers were detected upon *in vitro* differentiation. Engrafted organoids differentiated toward insulin-positive (INS^{+}) cells, and circulating human C-peptide was detected upon glucose challenge 1 month after transplantation. Engrafted $ALDH^{hi}$ cells formed INS^{+} cells. We conclude that adult human pancreatic tissue has potential for expansion into 3D structures harboring progenitor cells with endocrine differentiation potential.

Highlights

- A 3D culture system can support the expansion of adult human pancreatic tissue
- An $ALDH^{hi}$ cell subpopulation is identified in these organoids
- $ALDH^{hi}$ cells, and not $ALDH^{lo}$ cells, are capable of forming new organoids
- $ALDH^{hi}$ cells show endocrine differentiation potential

In the context of beta cell replacement therapy for diabetes, de Koning and colleagues describe a 3D culture platform that supports *ex vivo* expansion of human pancreatic tissue as organoids. These organoids harbor a subpopulation of $ALDH^{hi}$ cells that display proliferative capacity and can differentiate to an endocrine fate.

Introduction

Beta cell replacement therapy is an attractive therapy to achieve normoglycemia in patients with diabetes mellitus due to severe beta cell failure[1,2]. The shortage of organ donors severely limits the number of patients that are eligible for current beta cell replacement therapy, *i.e.*, pancreas or islet transplantation. Mature human beta cells cannot be expanded *in vitro* without complex dedifferentiation and redifferentiation processes[3,4]. Thus, there is an unmet clinical need to generate insulin-producing cells from alternative cell sources to make this therapy more widely available.

Several types of cells have been studied as possible sources of insulin-producing cells, including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (iPSCs). While the phenotype of these cells has long been characterised by immature maturation[5], recently more glucose-responsive cells have been generated from human pluripotent stem cells *in vitro*[6,7], but safety remains a major concern for any regenerative strategy using hESCs or iPSCs[8,9]. An attractive alternative could be the use of putative progenitor cells from adult human pancreas that give rise to

the endocrine lineage. Histological studies of human pancreas indicate that neogenesis of insulin-producing cells is associated with the ductal tree in obesity and pregnancy[10,11]. Other studies have also shown that some insulin-producing cells can be generated from cultured human pancreatic ductal tissue[12-15]. We recently showed that *in silico* analysis of single-cell transcriptome profiles of human adult pancreatic cells using a StemID algorithm predicts a distinct subpopulation of ductal cells with multipotential differentiation potential[16]. In mice, the existence of postnatal endocrine progenitors within the pancreatic ductal population has become controversial, with lineage-tracing experiments showing contradictory results. Although several studies were able to detect endocrine cells derived from the ductal lineage postnatally or after injury[17-20], others did not find this[21-23].

At present, expansion of human pancreatic cells in a standard, 2D culture system is hampered by the transition of both islet[4,24] and duct cells[25-27] to a mesenchymal cell-like phenotype during passaging. This approach does not provide the natural 3D environment of tissues, and thus important information of cell orientation and polarity for proliferation, growth, and differentiation are lost. In fact, proper alignment and polarization of progenitor cells is known to be required for successful differentiation of fetal pancreatic progenitor cells[28,29], and 3D culture of fetal murine pancreatic progenitors can be used to unravel and mimic niches important in pancreas development[30]. Thus, it is tempting to hypothesize that 3D culture of adult human pancreatic tissue may provide a microenvironment that enhances expansion and differentiation of pancreatic progenitors.

A Matrigel-based 3D culture system was developed in our institute that yields organoids from stem cells in different organs, with the capacity for long-term expansion and generation of functional differentiated organ-specific cells[31-33]. Single isolated adult mouse pancreatic progenitor cells can be expanded by forming colonies or organoids in a Matrigel-based system[30,32,34]. We observed that these progenitor cells are derived from the ductal tree, express the stem cell marker leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5) in culture and are able to differentiate toward the endocrine lineage[32].

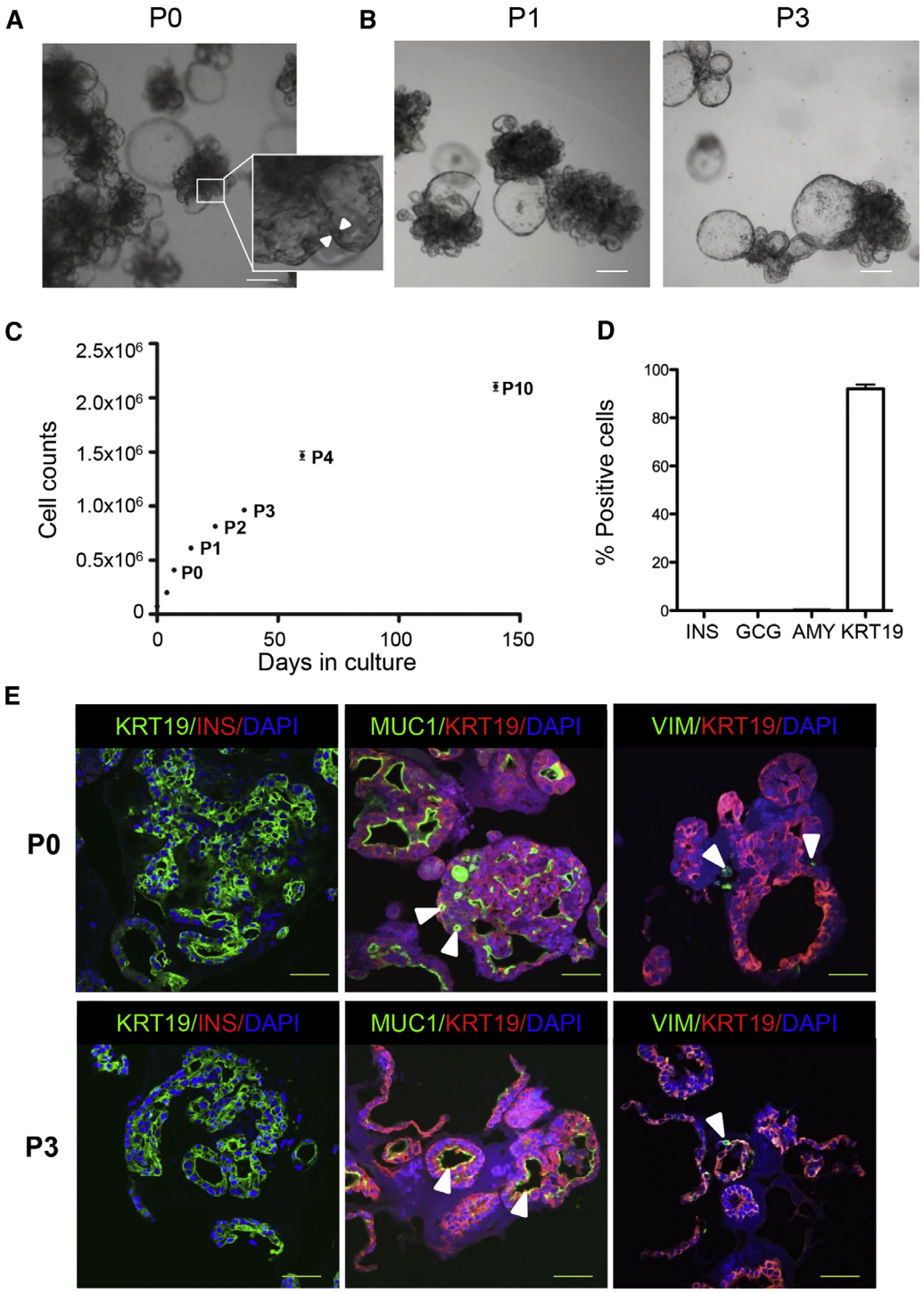
Results

Human pancreatic tissue expands as budding organoids

Islet-depleted pancreatic tissue after collagenase digestion was obtained from 35 non-diabetic organ donors (age 53.6 ± 12.1 years and BMI 24.7 ± 4.0 kg/m^2) and one organ donor with a history of type 1 diabetes (age 48 years, BMI 21 kg/m^2). After mechanical dissociation the small clumps of tissue were embedded in Matrigel and supplied with an epidermal growth factor/Noggin/R-spondin-based expansion medium. More than 90% of small pancreatic cell clusters formed budding structures within 3 days (**Movie S1**), and expanded with a cauliflower-like appearance by day 7 (**Figure 1A**). Some larger cyst-like structures were present in the organoids (**Figure 1A**). Organoids could be passaged without macroscopic changes in phenotype (**Figure 1B**), and maintained in culture for at least 10 passages (**Figure 1C**). The calculated rate for cell doubling was approximately 67 hr during passage 0 (P0) (n = 3). Growth rate slightly diminished upon passaging (**Figure 1C**). The

proportion of small budding structures and cyst-like structures varied among donors (**Figure S1A**). Organoids could also be generated from pancreatic tissue from an organ donor with a history of type 1 diabetes (**Figure S1B**).

Next, we analysed the cellular composition of organoids in the expansion phase. At day 7 (P0), 92.3% ± 5.4% of organoid cells were positive for the epithelial marker keratin 19 (KRT19), indicating a ductal phenotype (**Figure 1D and 1E**). Also at passage 3 (P3), the vast majority of the cells still had a ductal phenotype (87.2% ± 8.4% of organoid cells were $KRT19^+$). No insulin-positive (INS^+), glucagon-positive (GCG^+), or amylase-positive (AMY^+) cells were detected in the organoids at day 7 of the expansion phase (P0) (**Figure 1D and 1E**). The organoids were organised as $KRT19^+$ cells lining cystic or smaller elongated luminal spaces (**Figure 1E**). In both early and late passages, cells appeared polarized with positive mucin-1 (MUC1) staining at the luminal side of the $KRT19^+$ epithelial lining (**Figure 1E**). Although human pancreatic cells in 2D culture systems acquire a mesenchymal cell-like phenotype[4,25-27,35], few vimentin-positive cells (<2%) were observed in our organoid cultures at P0 or P3 (**Figure 1E**). Thus, small adult human pancreatic cell clusters can be expanded and passaged in 3D culture, generating polarized ductal budding structures.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% Positive cells
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80
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20
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INS
GCG
AMY
KRT19
E
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P3
KRT19/INS/DAPI
MUC1/KRT19/DAPI
VIM/KRT19/DAPI

Figure 1. Characterization of human pancreatic organoids during expansion in a 3D Culture system

(**a**) Bright-field image of pancreatic organoids (P0), expanded for 7 days in a 3D Matrigel-based culture system, reveals extensive growth with multiple budding structures. Some larger buds have a cyst-like appearance. The insert shows a close-up image of the cell lining (between arrowheads) in a budding structure. Scale bar, 100 µm. (**b**) Passaged organoids (P1 and P3 are shown) expanded in similar conditions as P0 (A), also forming budding structures in a cauliflower-like configuration. Scale bar, 100 µm. (**c**) Growth curve of pancreatic organoids cultured for 140 days. Cell numbers were counted on days 0, 4, and 7 (P0), and at the end of subsequent passages. Cells in five wells per time point were counted (mean ± SEM). The expansion curve is representative of n = 3 donors. (**d**) Quantification of number of cells positive for INS, GCG, AMY, or KRT19 by immunohistochemistry in organoids on day 7 of expansion culture (n = 8 donors; at least 10 organoids/donor were counted; mean ± SEM). DAPI was used as nuclear counterstain. (**e**) Non-passaged organoids (P0, upper panels) have similar features compared with passaged organoids (P3, lower panels). Left panels: the majority of the cells are KRT19$^+$ (green), with no INS$^+$ cells (red) detected during the expansion phase. Middle panels: MUC1 (green) staining at the apical cell border indicates polarization of the duct cells. Small lumena are visible within the organoids (arrowheads). Right panels: few vimentin-positive (VIM$^+$) cells (green) are present in the organoids (arrowheads). n = 5 donors; >15 organoids/donor were stained. DAPI was used as nuclear counterstain. Scale bars, 50 µm. See also **Figure S1**.

Human pancreatic organoids display pancreatic progenitors clustered toward the tips of the budding structures

Since extensive growth by budding was observed, we determined the proliferative capacity of the budding structures. Quantification showed that 27.3% ± 8.7% of the cells within an organoid were Ki67$^+$ at day 7 of expansion (**Figure 2A**). Only a few Ki67$^+$ cells were negative for the duct marker KRT19 (1.3% ± 0.8% of Ki67$^+$ cells). Ki67$^+$ cells were mainly observed in tips of buds and were not frequently observed in trunk regions (**Figure 2B**). Also, budding structures with wide tips and narrow trunks were present in the organoids (**Figure 2B and 2C**). Labelling with the nucleoside analog 5-ethynyl-2'-deoxyuridine that incorporates into newly synthesized DNA further confirmed the location of the proliferative cells toward the tip region (data not shown).

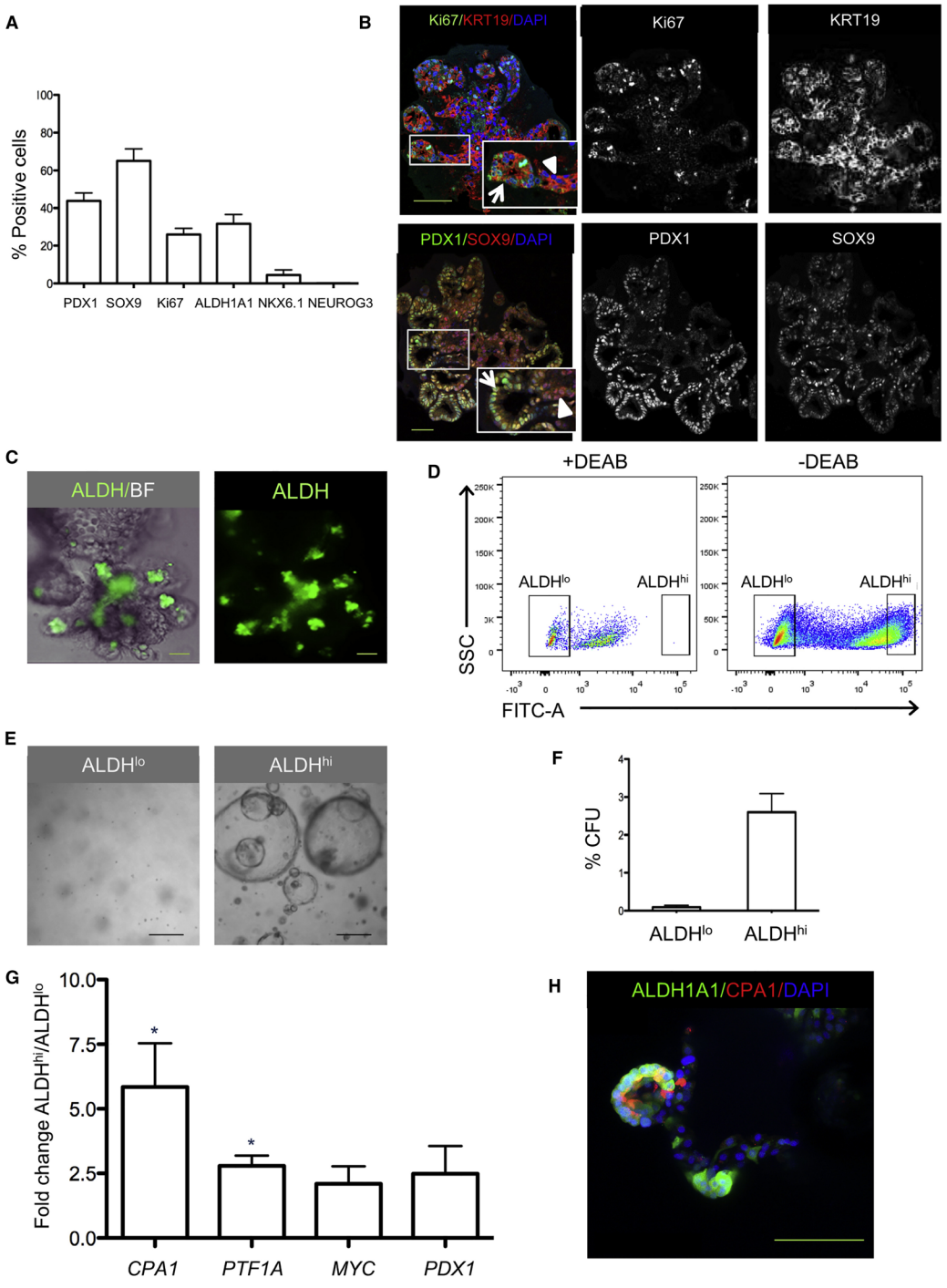
Based on the configuration of the budding structures, we hypothesized that the tips of the budding structures would be enriched for pancreatic progenitor cells, as has been reported for mouse fetal pancreatic development[36,37]. Immunostaining of the organoids for the pancreatic progenitor markers *pancreatic and duodenal homeobox 1* (PDX1) and *SRY (sex-determining region Y) box 9* (SOX9) (**Figure 2B**) showed high PDX1 expression in the budding structures of the organoids, particularly in tip regions, while SOX9 was more homogeneously distributed (**Figure 2B**). SOX9 and PDX1 gene expression increased during expansion (**Figure S2A**). Furthermore, gene expression of LGR5 increased during this time (**Figure S2B**), and a subset of cells in budding structures clearly expressed LGR5 mRNA, as assessed by smFISH (**Figure S2C**). No *neurogenin-3*-positive (NEUROG3$^+$) cells were observed in organoids in the expansion phase (**Figure 2A**). Next, we exposed the organoids to a fluorescent reagent (Aldefluor) that identifies progenitor cells based on their increased aldehyde dehydrogenase (ALDH) activity. Pancreatic progenitors with high

expression of ALDH1 isoforms have recently been identified in both developing and adult mouse pancreas[38,39]. Cells concentrated in the tips of the budding structures showed high ALDH activity (**Figure 2C**). The Aldefluor reagent is optimised to detect enzyme activity of ALDH1 isoforms, and we found that ALDH1A1 immunostaining co-localized with both KRT19^{+} and KRT19^{-} cells in the tips of the organoid buds, indicating the presence of heterogeneous populations of ALDH1A1^{+} cells (data not shown). High ALDH activity arose during organoid culture as no ALDH^{hi} cells were found in the d0 islet-depleted tissue (n = 3; data not shown). LGR5 gene expression was higher in sorted ALDH^{hi} compared with ALDH^{lo} cells (**Figure S2D**).

Single ALDH^{hi} cells show progenitor characteristics

To determine whether ALDH^{hi} cells have characteristics of progenitor cells, organoids were dispersed into single cells, labelled with Aldefluor, and sorted by fluorescence-activated cell sorting (FACS) (ALDH^{lo} cells 34.0% ± 7.4% and ALDH^{hi} cells 25.4% ± 6.0%, n = 5) (**Figure 2D**). A subpopulation of ALDH^{hi} cells derived from organoids was able to generate small cyst-like colonies in 3D culture (2.2% ± 0.8% of ALDH^{hi} cells), in marked contrast to ALDH^{lo} cells that formed no colonies (**Figure 2E, 2F, and S3A**). When organoids derived from single-sorted ALDH^{hi} cells were immunostained for ALDH1A1, both ALDH1A1^{+} and ALDH1A1^{-} cells were observed, indicating the generation of a heterogeneous cell population from a single ALDH^{hi} cell (**Figure S3B**). When these labelled organoids were sorted once more, yet again only ALDH^{hi} cells showed colony-forming potential in 3D culture (data not shown). Interestingly, complex budding structures were not observed when single ALDH^{hi} cells were expanded, which could indicate the requirement of supporting cells for self-assembly into a ductal-tree-like configuration (**Figure S3A**).

Next, we characterised the ALDH^{hi} cell population for the presence of markers previously described for multipotent progenitor cells in mouse pancreatic organogenesis[36]. Gene expression levels of the markers carboxypeptidase A1 (CPA1) and pancreas-specific transcription factor, 1a (PTF1A) were significantly upregulated in ALDH^{hi} cells compared with the ALDH^{lo} fraction (**Figure 2G**). Immunoreactivity for CPA1 was found in ALDH1A1^{+} cells in the tips of organoid budding regions, but some ALDH1A1^{+} cells were CPA1^{-} (**Figure 2H**). Importantly, these ALDH1A1^{+} cells were negative for the acinar cell marker amylase (**Figure S3C**). Many ALDH1A1^{+} cells in the tips of budding regions buds also co-stained for PDX1 (**Figure S3D**). Genes that are known to be upregulated in mouse centroacinar (CAC) cells, such as HES1, SOX9, SCA1, MET, NES, and HEY1[39,40], were analysed in sorted ALDH^{hi} and ALDH^{lo} cells. No upregulation of these markers was present in ALDH^{hi} compared with ALDH^{lo} cells (**Figure S3E**). HES1^{+} cells were present in expanded organoids after 7 days, but their distribution was not well defined (**Figure S3F**). Thus, a subpopulation of ALDH^{hi} cells within human pancreatic organoids has colony formation capacity and expresses pancreatic progenitor markers.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B
Ki67/KRT19/DAPI
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PDX1/SOX9/DAPI
PDX1
SOX9
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+DEAB
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10.0
7.5
5.0
2.5
0.0
CPA1
PTF1A
MYC
PDX1
H
ALDH1A1/CPA1/DAPI

Figure 2. Tips of budding structures of pancreatic organoids contain cells with progenitor cell characteristics

(**a**) Quantification of cells positive for pancreatic progenitor and proliferation markers in organoids on day 7 of expansion culture. Organoids derived from four to six donors were analysed (at least ten organoids/donor). Data are expressed as mean ± SEM. (**b**) Confocal image of pancreatic organoids on day 7 of expansion. Top: organoids were stained for the proliferation marker Ki67 (green) and ductal marker KRT19 (red). Organoid budding structures show a narrow trunk region (arrowhead) and a wider tip region (arrow). Bottom: organoids were stained for SOX9 (red) and PDX1 (green). $SOX9^+$ cells were present both in the buds and in the trunk region of the organoids (arrowhead). $PDX1^+$ cells (green), which frequently co-expressed SOX9 (yellow), were mainly located in the budding structures with strong staining for PDX1 often observed in cells at the outermost tip regions (arrow). Both overlay and individual channels are depicted. DAPI was used as nuclear counterstain. Scale bars, 100 µm. (**c**) Organoids in expansion phase for 7 days were labelled with the Aldefluor fluorescent reagent system, marking progenitor cells characterised by high ALDH activity. Most $ALDH^{hi}$ cells (green) were located in the tips of organoid buds. Scale bar, 50 µm. (**d**) FACS analysis of dispersed organoid cells (after 7 days in expansion culture) labelled with Aldefluor with and without the ALDH inhibitor DEAB. The FACS plot shows how $ALDH^{hi}$ cells (***i.e.***, cells that express high ALDH activity) and $ALDH^{lo}$ cells (***i.e.***, cells that express low ALDH activity) are selected. Representative plot from n = 10 donors. (**e**) Bright-field images of sorted and expanded single $ALDH^{lo}$ cells (left panel) and $ALDH^{hi}$ cells (right panel) in Matrigel for 14 days. Scale bar, 100 µm. (**f**) Proportion of sorted $ALDH^{lo}$ and $ALDH^{hi}$ cells with colony-forming (organoid-forming) potential. Data represent mean ± SEM (n = 3 donors). (**g**) Gene expression of CPA1, PTF1A, MYC, and PDX1 in sorted $ALDH^{lo}$ and $ALDH^{hi}$ cells derived from organoids expanded for 7 days. The graph shows the gene expression ratio in $ALDH^{hi}$ to $ALDH^{lo}$ cells for the different markers. Mean ± SEM (n = 3 donors) $*P < 0.05$. (**h**) Whole-mount immunostaining for ALDH1A1 and CPA1 of organoids expanded for 7 days. Confocal images show $ALDH1A1^+$ cells (green) and $CPA1^+$ cells (red) in the tip of the budding structures. Some cells co-express the two markers. Scale bar, 50 µm. CFU, colony-forming unit. See also **Figures S2** and **S3**.

Transcriptional profiling shows clustering of $ALDH^{hi}$ cells from adult and fetal pancreatic organoids

Since the self-assembly of adult human pancreatic tissue into organoids in our 3D culture system resembles budding structures previously described in pancreatic development, we compared characteristics of organoids derived from adult pancreatic tissue (adult pancreatic organoids) with human fetal pancreatic tissue and organoids derived from this fetal pancreatic tissue (fetal pancreatic organoids). We observed that fetal pancreatic organoids displayed a similar morphology to adult pancreatic organoids under the same culture conditions (**Figure 3A**). $ALDH^{hi}$ cells were predominantly located in budding structures (**Figure 3A**). This observation strengthened our hypothesis that $ALDH^{hi}$ cells that are concentrated in tip regions have pancreatic progenitor characteristics. We set out to determine the level of similarity of these putative $ALDH^{hi}$ progenitors isolated from adult pancreatic organoids with $ALDH^{hi}$ cells derived from fetal pancreatic organoids. Global gene expression analysis revealed that $ALDH^{hi}$ cells from adult pancreatic organoids are transcriptionally closer to $ALDH^{hi}$ cells isolated from fetal pancreatic organoids than to the adult exocrine tissue they originated from (**Figure 3B and 3C**). In contrast, $ALDH^{hi}$ cells from fetal pancreatic organoids retained their more primitive identity, clustering closely to fetal pancreatic

tissue (**Figure 3B**). Furthermore, expression levels of the multipotent progenitor markers CPA1, PTF1A, PDX1, and MYC in ALDH^{hi} cells from adult pancreatic organoids were comparable with those in ALDH^{hi} cells from organoids derived from fetal pancreas (no significant difference, $p < 0.05$; data not shown).

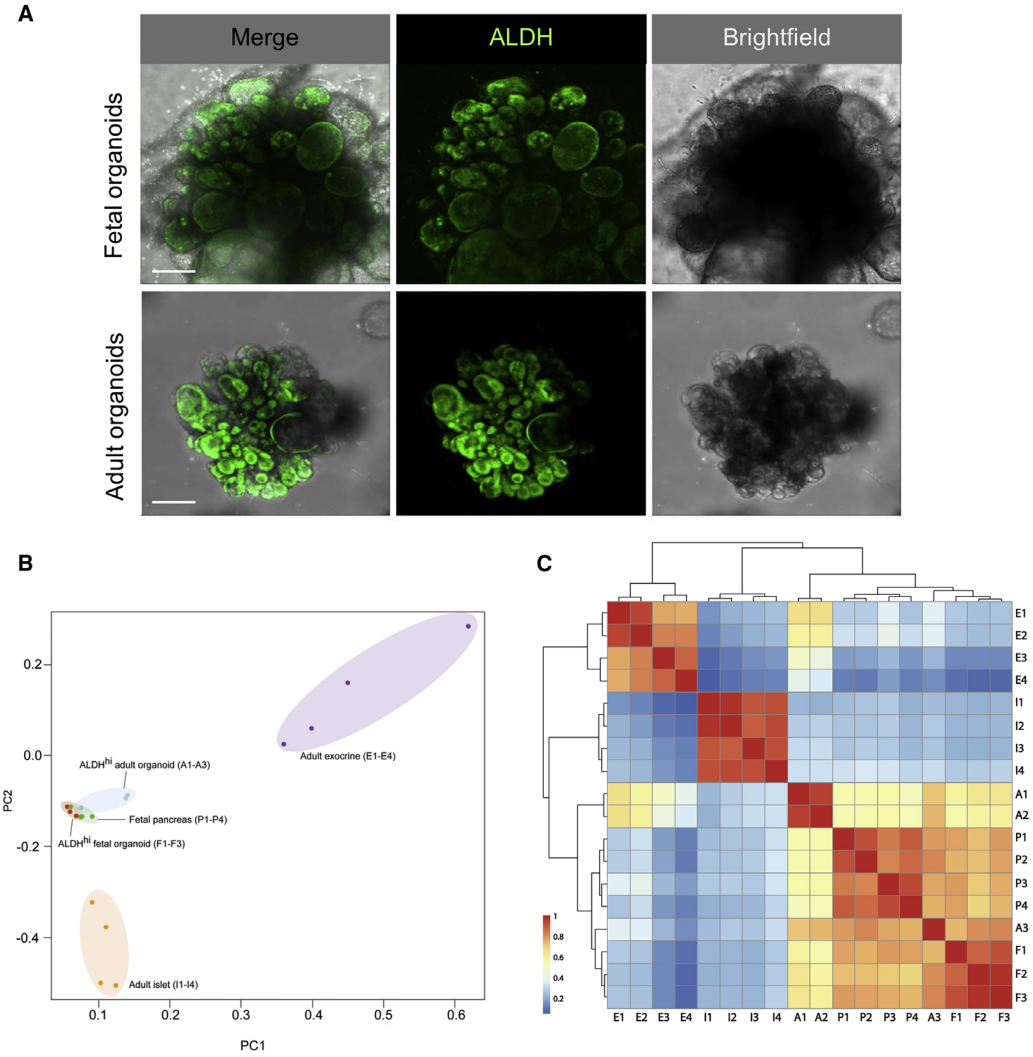


Figure 3. Transcriptional profiling shows clustering of ALDH^{hi} cells from adult and fetal pancreatic organoids

(**a**) Aldefluor labelling of adult and fetal human pancreatic organoids expanded for 7 days. Maximum projection (confocal imaging). Scale bar, 100 µm. (**b**) Principal-component analysis for gene expression profiles of the following samples: A1-A3: ALDH^{hi} cells sorted from organoids after 7 days expansion derived from human adult pancreatic tissue (age 50.0 ± 18.7 years, BMI 22.0 ± 4.0 kg/m2). F1-F3: ALDH^{hi} cells sorted from organoids after 7 days expansion derived from human fetal pancreatic tissue (gestational age: F1 9 weeks, F2 20 weeks, and F3 22 weeks). P1-P4: primary fetal pancreatic tissue (gestational age: P1 9 weeks, P2 18 weeks, P3 14 weeks, and P4 10 weeks). I1-I4: adult human islets (age 34.5 ± 17.3 years, BMI 23.8 ± 4.5 kg/m^2). E1-E4: adult exocrine (islet-

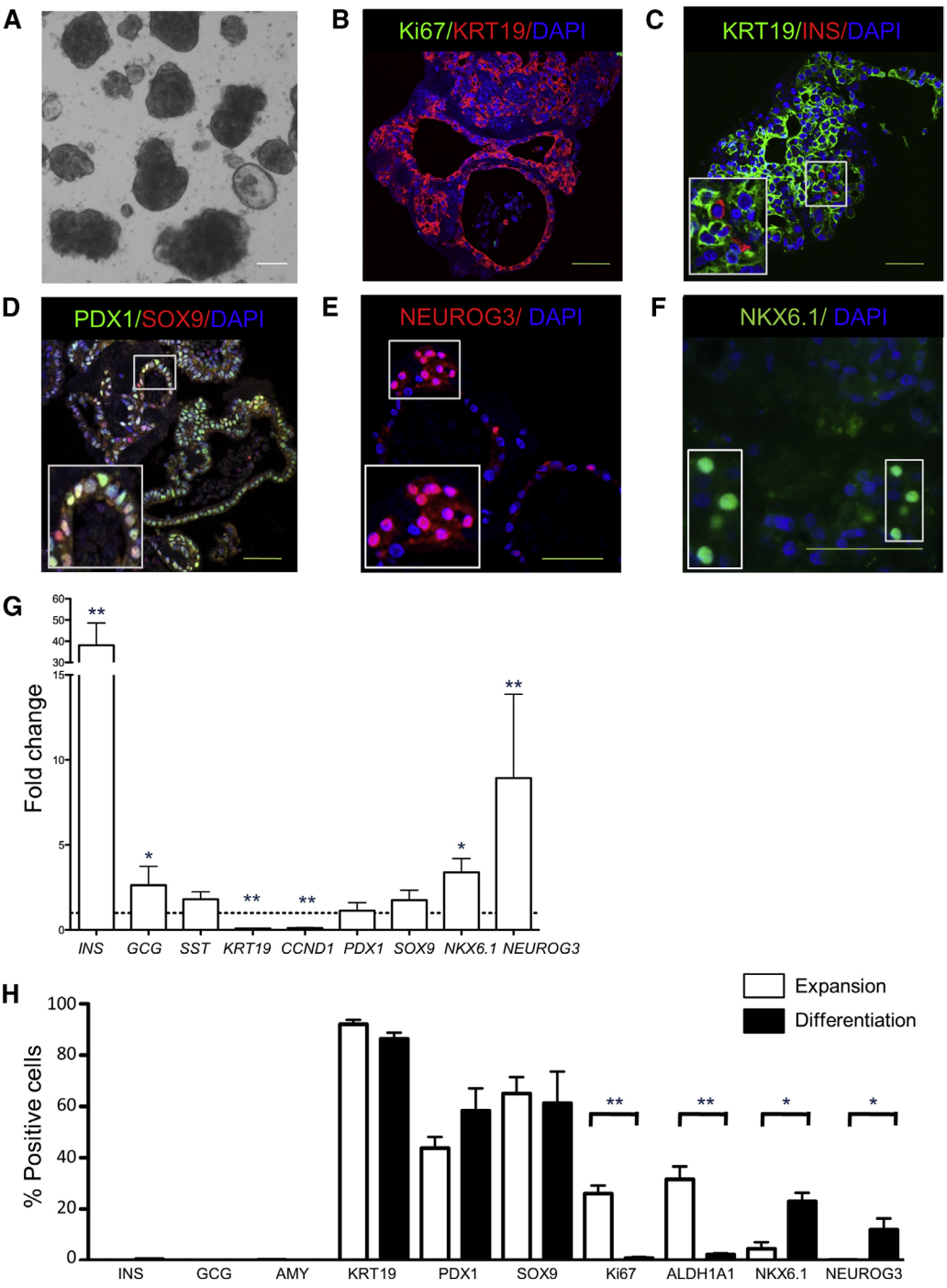
depleted) pancreatic tissue (age 63.5 ± 5.7 years, BMI 25.5 ± 3.7 kg/m^2). (**c**) Correlation cluster analysis for the gene expression profiles of the samples described in (B).

Pancreatic organoids can differentiate toward an endocrine fate *in vitro*

Since pancreatic organoids expanded from adult human exocrine tissue express progenitor markers during expansion, their differentiation potential was studied using culture conditions reported to differentiate human duct cells[13,41]. When P0 organoids were transferred to low attachment plates containing differentiation medium, their budding structure appeared to collapse and a rounded shape was seen after 7 days (**Figure 4A**). By then, growth had almost completely stopped as indicated by the near absence of Ki67$^+$ cells (0.9% ± 0.4% of total cells; **Figure 4B and 4H**) and reduced Cyclin D1 (CCND1) gene expression (**Figure 4G**). Furthermore, the proportion of ALDH1A1$^+$ cells decreased considerably from 27.0% ± 14.5% (P0 expansion day 7) to 1.4% ± 1.3% of total cells (differentiation day 7) (**Figure 4H**).

A marked increase in insulin gene expression after 7 days of differentiation was observed when compared with the end of the expansion phase (**Figure 4G**). Despite the considerable increase in insulin gene expression with differentiation, few INS$^+$ cells were observed by immunostaining (0.52% ± 0.22%; **Figure 4C and 4H**). GCG gene expression was only slightly increased between expansion and differentiation (**Figure 4G**), and no GCG$^+$ cells were present in the differentiation phase (**Figure 4H**). To exclude the possibility that insulin was taken up from the medium, we confirmed that INS$^+$ cells were also positive for human C-peptide (**Figure S4A**). No AMY$^+$ cells were observed and the majority of cells remained KRT19$^+$ during differentiation (83.4% ± 14.5% of cells; **Figure 4H**), even though gene expression of KRT19 decreased (**Figure 4G**).

Gene expression of the endocrine progenitor marker NEUROG3 and beta cell marker NKX6 homeobox 1 (NKX6.1) were significantly upregulated during the differentiation phase (**Figure 4G**). NEUROG3$^+$ cells could also be identified by immunostaining in the 'collapsed' organoids (**Figure 4E and 4H**). In addition, more cells expressed NKX6.1 in the differentiation phase compared with the expansion phase (**Figure 4F and 4H**). Conversely, no change was found in gene expression of the pancreatic progenitor markers PDX1 and SOX9 upon differentiation (**Figure 4G**) or the number of PDX1$^+$ and SOX9$^+$ cells (**Figure 4D and 4H**). Thus, upon *in vitro* differentiation, pancreatic progenitors can be directed along the endocrine lineage.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Ki67/KRT19/DAPI
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Fold change
INS
GCG
SST
KRT19
CCND1
PDX1
SOX9
NKX6.1
NEUROG3
H
% Positive cells
Expansion
Differentiation
INS
GCG
AMY
KRT19
PDX1
SOX9
Ki67
ALDH1A1
NKX6.1
NEUROG3

Figure 4. Endocrine cell markers in pancreatic organoids after 7 days of *in vitro* differentiation
(**a**) Bright-field image of human pancreatic organoids initially expanded for 7 days followed by differentiation culture for 7 days. DAPI was used as nuclear counterstain. Scale bar, 100 µm. (**b**) Confocal image of pancreatic organoids immunostained for Ki67 (green) and KRT19 (red). The majority of cells were KRT19$^+$. No cells were Ki67$^+$. DAPI was used as nuclear counterstain. Scale bar, 50 µm. (**c**) Confocal image of pancreatic organoids immunostained for KRT19 (green) and INS (red). Few INS$^+$ (0.52%) cells were present. DAPI was used as nuclear counterstain. Scale bar, 50 µm. (**d**) Confocal image of pancreatic progenitor markers PDX1 (green) and SOX9 (red). DAPI was used as nuclear counterstain. Scale bar, 50 µm. (**e**) Confocal image of pancreatic progenitor marker NEUROG3 (red). DAPI was used as nuclear counterstain. Scale bar, 50 µm. (**f**) Confocal image of pancreatic progenitor marker NKX6.1 (green). DAPI was used as nuclear counterstain. Scale bar, 50 µm. (**g**) Gene expression of several markers in organoids on day 7 of differentiation compared with organoids on day 7 of expansion. Gene expression in expansion organoids were set to 1 (dotted line). Mean ± SEM, n = 8 donors. *$P < 0.05$ and **$P < 0.01$. (**h**) Percentage of cells immunostained for different markers on day 7 of expansion (white bars) compared with day 7 of differentiation (black bars) using confocal images. More than 10 organoids per donor were assessed. Mean ± SEM, n = 4-6 donors. *$P < 0.05$ and **$P < 0.01$. See also Figure S4.

Human pancreatic tissue can be cryopreserved without losing expansion or differentiation capacities

With therapeutic purposes in mind, we explored the possibility of cryopreserving pancreatic tissue before organoid expansion. Freshly retrieved tissue (day 0) was compared with cryopreserved tissue (day 0) from the same donors (n = 4). Growth of organoids with budding structures was observed from both groups (**Figure S4B**), but the organoids grown from cryopreserved starting material had more cystic appearance than organoids grown from freshly isolated material. When subjected to *in vitro* differentiation, no significant differences in gene expression were found (**Figure S4C**). Thus, organoids can be grown from cryopreserved primary human adult pancreatic tissue.

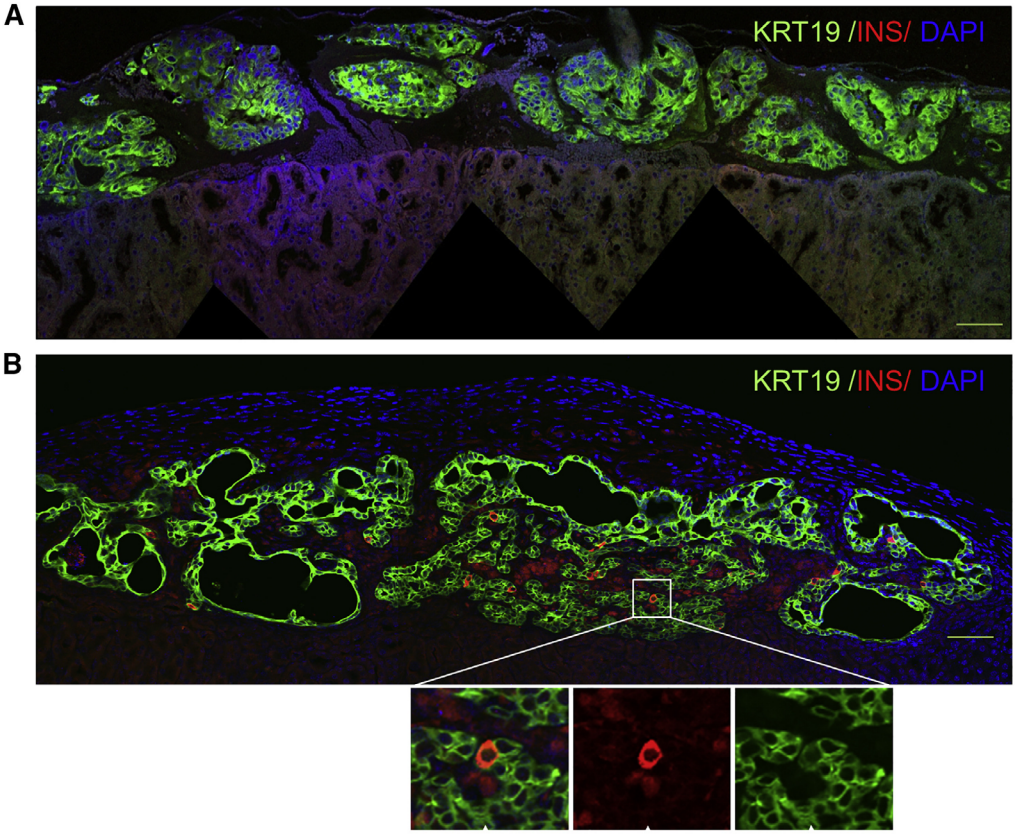
Human pancreatic organoids generate insulin-producing cells *in vivo*

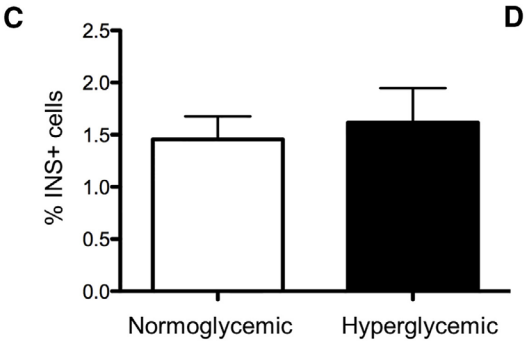
While the *in vitro* differentiation experiments indicated differentiation toward an endocrine lineage based on gene expression analysis, only a few INS$^+$ cells were observed. It is well known that human ESC differentiate into insulin-producing cells after implantation into mice[42]. Therefore, we tested the capacity of pancreatic organoids to further differentiate *in vivo* after transplantation under the kidney capsule of immunodeficient mice. One day after transplantation the majority of grafted cells were KRT19$^+$ and no INS$^+$ cells were observed (**Figure 5A**). However, pancreatic organoids from the same donor 1 month after transplantation showed INS$^+$ cells within the ductal lining (**Figure 5B**). Organoids derived from each donor (n = 8) were able to generate 1.5% ± 0.2% INS$^+$ cells within the ductal lining (n = 8; **Figure 5B**). No difference in the proportion of INS$^+$ cells was observed in the hyperglycemic animals (**Figure 5C**). Production of insulin was confirmed by immunostaining of C-peptide in INS$^+$ cells (CPEP) (**Figure S5A**). Furthermore, insulin was co-expressed with several functional endocrine markers (PDX1, IAPP, NKX6.1, and SYP) but not with GCG (**Figure S5B-S5E**).

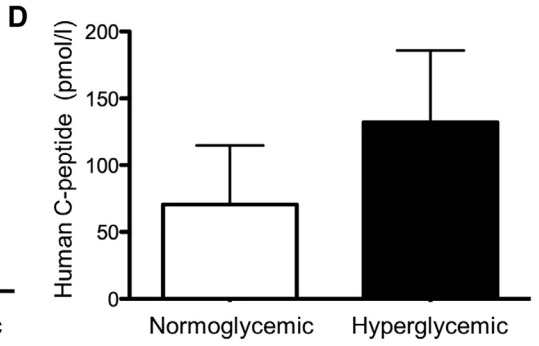
The differentiation phase *in vitro* was necessary for the appearance of INS^+ cells as transplantation of organoids 1 week after expansion yielded either no grafts or only large cystic structures with no hormone-positive cells (data not shown).

Human C-peptide could be readily measured in normoglycemic and hyperglycemic mice after a glucose challenge indicating that human insulin was released into the circulation upon a common beta cell stimulus (**Figure 5D**). Basal human C-peptide was also present in normoglycemic mice before the glucose challenge (89.2 ± 59.9 pmol/L, n = 8). Blood glucose of grafted mice did not decrease significantly in either hyperglycemic mice (26.5 ± 3.9 mmol/L at day 0 versus 24.3 ± 3.8 mmol/L at day 30, n = 8) or in the normoglycemic group (8.6 ± 1.3 mmol/L at day 0 versus 6.7 ± 2.4 mmol/L at day 30, n = 8). Thus, graft function was detectable but not sufficient to restore normoglycemia within 1 month after organoid transplantation in hyperglycemic mice.

Sorted $ALDH^{hi}$ cells derived from organoids expanded for 7 days were also differentiated for 1 week *in vitro* and subsequently transplanted under the kidney capsule of normoglycemic immunodeficient mice. After 50 days, retrieved grafts contained both $KRT19^+$ and C-$peptide^+$ cells (**Figure 5E**).


A
KRT19 /INS/ DAPI
B
KRT19 /INS/ DAPI


C
% INS+ cells
2.5
2.0
1.5
1.0
0.5
0.0
Normoglycemic
Hyperglycemic


D
Human C-peptide (pmol/l)
200
150
100
50
0
Normoglycemic
Hyperglycemic

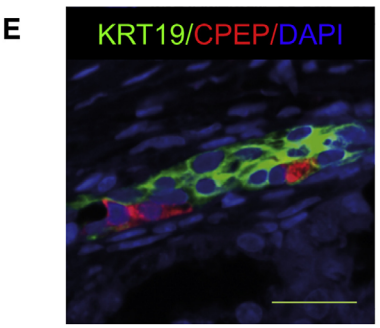

E
KRT19/CPEP/DAPI

Figure 5. Human pancreatic organoids generate insulin-producing cells *in vivo*
Grafts composed of *in vitro* differentiated organoids derived from islet-depleted exocrine pancreatic tissue were transplanted under the mouse kidney capsule after 7 days of expansion and 7 days of differentiation. (**a**) Day 1 after transplantation. Immunostaining for KRT19 (green) and INS (red). No INS^+ cells are present. DAPI (blue) was used as nuclear counterstain. Scale bar, 50 µm. (**b**) Day 30 after transplantation. Transplanted organoids formed a ductal network (KRT19, green) and many INS^+ cells (red) are present within the ductal lining (arrows indicate INS^+ cells). DAPI (blue) was used as nuclear counterstain. Scale bar, 50 µm. (**c**) Percentage of INS^+ cells in the ductal lining 1 month after transplantation. For each donor, grafts were transplanted in both normoglycemic and hyperglycemic mice, and two mice were transplanted per donor. No significant difference in the number of INS^+ cells between the two groups of mice was found. Mean ± SEM, n = 8 donors. (**d**) Human C-peptide concentration after a glucose challenge in mice 1 month after organoid transplantation. Grafts were transplanted in either normoglycemic mice or hyperglycemic mice, and two mice were transplanted per donor. No significant difference in human C-peptide concentration between the two groups of mice was observed. Mean ± SEM, n = 8 donors. (**e**) Confocal image of a graft of sorted $ALDH^{hi}$ cells isolated from organoids after expansion for 7 days. The $ALDH^{hi}$ cells were differentiated for 7 days in vitro before transplantation under the kidney capsule of normoglycemic mice (n = 3 donors, 1 mouse per donor). The graft was retrieved after 50 days and contained both $KRT19^+$ and $CPEP^+$ cells. Cells co-expressing KRT19 and C-peptide indicate a transition stage from a duct (-like) phenotype to a beta (-like) cell phenotype. DAPI (blue) was used as nuclear counterstain. Scale bar, 50 µm. See also **Figure S5**.

Discussion

Our data indicate that adult human pancreatic tissue can be expanded as 3D organoids and long-term expansion can be achieved by passaging these pancreatic organoids. Cryopreservation of the pancreatic tissue is possible without losing these characteristics. A subpopulation of cells from the organoids have progenitor characteristics and give rise to endocrine cells after transplantation.

Progress to expand human adult pancreatic tissue and thus exploration of its capacity for endocrine cell differentiation has been hampered by epithelial-to-mesenchymal transition in 2D culture systems. The 3D culture system we have developed here provides an environment that allows substantial growth of human pancreatic cells starting from minced tissue. Recently we showed that adult mouse pancreatic organoids can also be expanded using similar culture conditions[32]. We and others described Matrigel-based culture methods for expansion of dispersed exocrine cells from adult mouse[32,34] and human[43] pancreas and for expansion of genetically modified human pancreatic ductal cells[44]. The striking aspect of our expansion protocol is the self-organisation of the tissue into organoids with complex budding structures. These organoids display a remarkable $KRT19^+$ epithelial tree-like structure with apical-basal polarity. This configuration has been shown for mouse embryonic pancreas tissue *in vivo* and *in vitro*[30,36], and is present during human pancreatic organogenesis[45]. Similar culture systems enabled the expansion of human intestine and stomach in complex cell configurations, also called organoids, that appear to recapitulate organogenesis and tissue regeneration[31,46].

During embryogenesis, the tip regions of the expanding ductal trees in the developing pancreas harbor progenitor cells[21,36,45]; and, in adult human tissue, we predicted, from single-cell

transcriptome data, that the progenitor population in human adult pancreatic cells resides among the ductal population[16]. The morphology of the pancreatic organoids (derived from adult pancreatic tissue) in our study allowed us to search for putative progenitors in the budding structures. ALDH^{hi} cells had differentially high expression of the progenitor markers PDX1, CPA1, MYC, and PTF1A[36]. In addition, we established organoid culture from human fetal pancreatic tissue. These fetal pancreatic organoids also contained ALDH^{hi} cells in budding structures. Interestingly, transcriptional profiling revealed that ALDH^{hi} cells from adult pancreatic organoids were closer to ALDH^{hi} cells from fetal pancreatic organoids than to adult exocrine tissue (*i.e.*, the islet-depleted tissue before expansion). This indicates a reprogramming of a subset of adult pancreatic cells to a progenitor-like stage.

Organoids expanded from human pancreas and transplanted in immunodeficient mice gave rise to insulin-producing cells after 1 month. The location of the insulin-producing cells in the grafts, *i.e.*, in close proximity of or within the ductal lining, and the increased number of insulin-positive cells after engraftment compared with the initial cell population, support the concept that these cells were newly generated. However, while human C-peptide could be detected in the circulation the number of beta cells was not sufficient to restore normoglycemia in diabetic mice 1 month after transplantation. In a recent study by Lee et al.[44], the authors also showed that a subpopulation of expanded ductal cells could differentiate to insulin-secreting cells using an alternative approach of adenovirus-mediated expression of pro-endocrine factors and co-transplantation with mouse embryonic fibroblasts. In summary, although culture conditions need to be optimised to increase graft efficacy, our study provides a proof-of-concept that this 3D culture system can be used to expand primary human ductal cells that can differentiate to an endocrine fate without the need for genetic modification.

The origin of the ALDH^{hi} cells in our system is unclear. An ALDH^{hi} progenitor cell population has been identified in a subset of adult ductal/CAC cells[39] and in the developing pancreas[38] of mice, and these cells had the ability to self-renew and to differentiate into both endocrine and exocrine cells.

Notably, several studies have recently attributed a functional role to ALDH1 isoforms during pancreas development in humans[47], mice[48], and zebrafish[49]. Our data indicate that the specific culture conditions we developed support this progenitor state. Whether these ALDH^{hi} cells have a CAC origin or not warrants further investigation. At present this question is challenging as there is a lack of markers specific for human adult CACs. In mature murine pancreas, HES1 expression marks terminal ductal or CACs, whereas it identifies MPCs in early embryonic pancreas (until e11.5), and exocrine-restricted progenitors from e13.5 until birth[40]. Here, we found that HES1 was slightly upregulated at the mRNA level in ALDH^{hi} cells, but HES1 protein expression was not restricted to the tips of the budding structures where the majority of ALDH^{hi} cells reside. Finally, the ALDH^{hi} population is heterogeneous, with only a small proportion of cells able to expand *in vitro*. Therefore, additional cell surface markers will be necessary to efficiently enrich and characterize the progenitor subpopulation from this heterogeneous population of ALDH^{hi} cells.

It has been unclear whether an endocrine/multipotent progenitor population exists in the

adult human pancreas. Indeed, classical definitions of what constitutes an adult stem cell population are under debate[50]. However, we provide a proof-of-concept for the existence of a $ALDH^{hi}$ population of human pancreatic cells (most likely $KRT19^{+}$ cells) that appears under specific culture conditions, and that has the capacity to differentiate into an endocrine cell phenotype. Aldefluor has been used to detect $ALDH^{hi}$ stem/progenitor cells in multiple tissues[51]. Here, we show that this cell population expresses the pancreatic progenitor markers CPA1, PDX1, MYC, and PTF1A. Furthermore, the gene expression profile of adult pancreatic $ALDH^{hi}$ population presents a high degree of similarity with fetal pancreatic $ALDH^{hi}$ cells.

Finally, since these cells are derived from the adult human pancreas and, thus, have already committed to a pancreatic fate, differentiation to the beta cell lineage may be easier to achieve than differentiation of other adult stem/progenitor cells. In current allogeneic and autologous islet transplantation contaminating non-islet cells from the human pancreas are always co-transplanted with islets into human recipients without adverse effects[52]. Therefore, expanded and differentiated adult human pancreatic cells are likely to be relatively safe compared with embryonic or induced pluripotent cell lines for future beta cell replacement therapy programs.

Experimental procedures

Human primary tissue

Adult Pancreatic Tissue Islet-depleted pancreatic tissue and human islets that could not be used for clinical transplantation were used in the studies according to national laws and if research consent was available.

Fetal Pancreatic Tissue Fetal pancreatic tissue from elective abortions was used after written (parental) informed consent was provided. Collection and use of human fetal tissue for research was approved by the Medical Ethics committee of the LUMC.

Generation of human and fetal pancreatic organoids

Human adult islet-depleted pancreatic tissue was obtained after human islet isolation procedures. For culture of adult pancreatic organoids, small pieces of exocrine tissue were plated and expanded in Matrigel (BD Biosciences). Human fetal pancreatic tissue was obtained from fetuses with a gestational age of 9, 20, and 22 weeks. For culture of fetal pancreatic organoids, small pieces of human fetal pancreatic tissue were plated and expanded in Matrigel similar to adult pancreatic organoids. A progenitor population within the organoids was identified using the Aldefluor fluorescent reagent system (STEMCELL), which is based on detection of high ALDH activity in progenitor cells. $ALDH^{hi}$ and $ALDH^{lo}$ cell populations were sorted and plated in Matrigel before analysis. Human and fetal pancreatic organoids were analysed by immunohistochemistry, RNA sequencing, qPCR, and smFISH. For a detailed description, see Supplemental Experimental Procedures.

In vitro and *in vivo* differentiation of human pancreatic organoids

After expansion, adult pancreatic organoids were retrieved from the Matrigel and analysed, cryopreserved in liquid nitrogen, or transferred to a differentiation culture medium (3 mL), characterised by absence of growth factors, in six-well hydrophobic plates (Corning). Organoids were cultured for 7-18 days before analysis or transplantation under the kidney capsule in normoglycemic or hyperglycemic immunodeficient mice. Human C-peptide was measured and grafts were analysed up to 1 month after transplantation. For a detailed description, see Supplemental Experimental Procedures.

Acknowledgement

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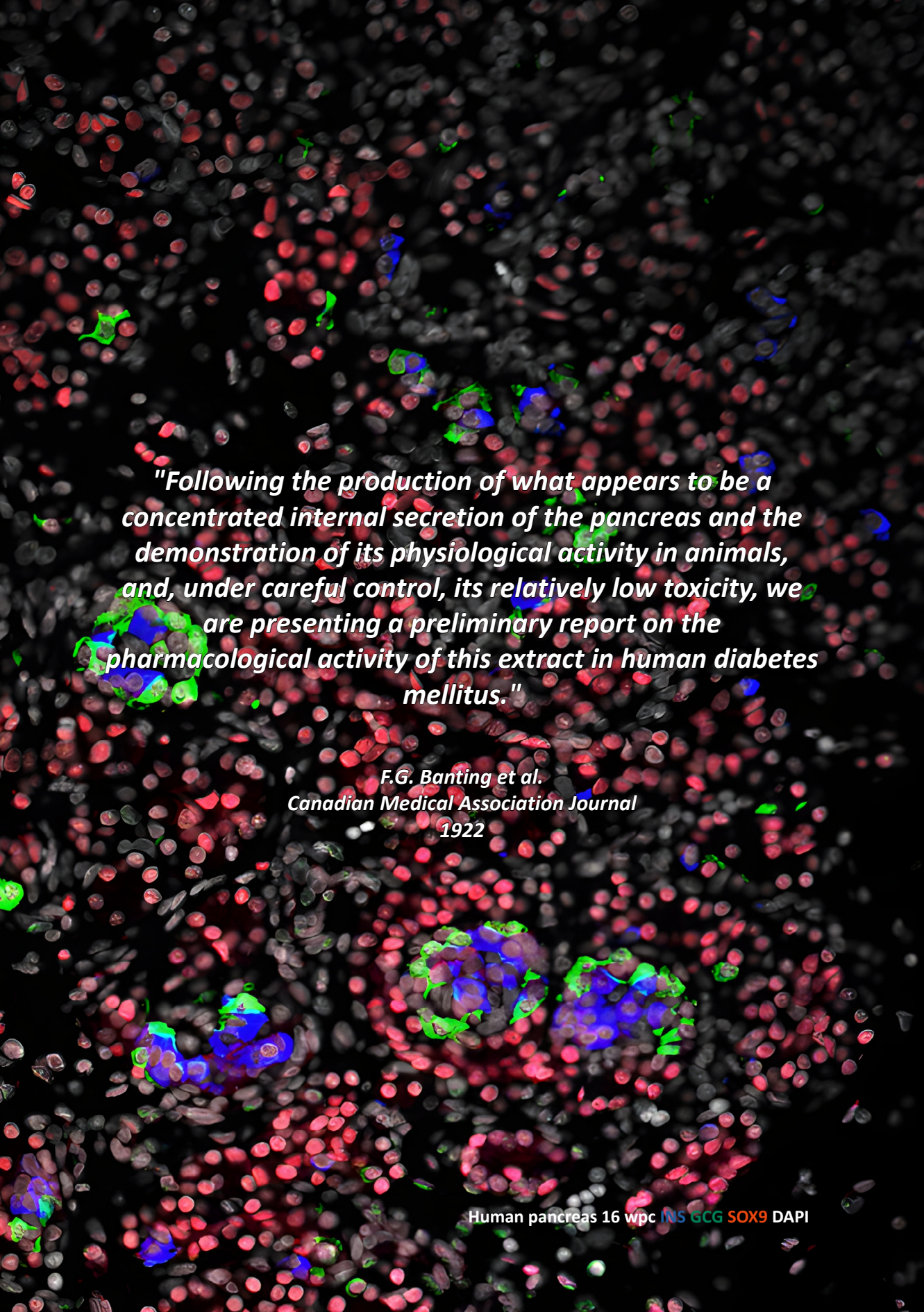
Supplemental information


"Following the production of what appears to be a concentrated internal secretion of the pancreas and the demonstration of its physiological activity in animals, and, under careful control, its relatively low toxicity, we are presenting a preliminary report on the pharmacological activity of this extract in human diabetes mellitus."
F.G. Banting et al.
Canadian Medical Association Journal
1922
Human pancreas 16 wpc INS GCG SOX9 DAPI

CHAPTER| 5

Highly Efficient *Ex Vivo* Lentiviral Transduction of Primary Human Pancreatic Exocrine Cells

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Abstract

The lack of efficient gene transfer methods into primary human pancreatic exocrine cells hampers studies on the plasticity of these cells, and their possible role in beta cell regeneration. Therefore, improved gene transfer protocols are needed. Lentiviral vectors are widely used to drive ectopic gene expression in mammalian cells, including primary human islet cells. Here we aimed to optimise gene transfer into primary human exocrine cells using modified lentiviral vectors or transduction conditions. We evaluated different promoters, viral envelopes, medium composition and transduction adjuvants. Transduction efficiency of a reporter vector was evaluated by fluorescence microscopy and flow cytometry. We show that protamine sulfate-assisted transduction of a VSV-G-pseudotyped vector expressing eGFP under the control of a CMV promoter in a serum-free environment resulted in the best transduction efficiency of exocrine cells, reaching up to 90% of GFP-positive cells 5 days after transduction. Our findings will enable further studies on pancreas (patho)physiology that require gene transfer such as gene overexpression, gene knockdown or lineage tracing studies.

Introduction

Beta cell replacement therapy is a promising treatment for patients with type 1 diabetes mellitus (T1DM)[1]. However, the lack of donor organs prevents the widespread use of this treatment. It is hypothesized that the exocrine compartment of the human pancreas may harbor a progenitor cell population that can develop towards beta cells[2]. Rodent models of diabetes and pancreatic injury, however, demonstrate contradictory results with regards to the exocrine-derived beta cell neogenesis capacity of the adult pancreas[3-8], and the findings obtained in rodent studies are often not confirmed with human cells[9]. Such discrepancies highlight the need for studies on primary human pancreas cells. Single-cell transcriptomics data of human adult pancreatic cells indicates the duct compartment as a potential source for beta cell progenitor cells[10]. In addition, we previously reported long-term expansion of adult human exocrine tissue generating organoids harboring progenitor cells with endocrine differentiation potential[11]. Yet, the lack of reliable lineage tracing systems hampers studies on the identification of the origin of these newly formed endocrine cells, as endocrine contamination in heterogeneous starting populations cannot be fully excluded. Therefore, the development of efficient gene transfer methods into primary human pancreatic exocrine cells is crucial to allow the assessment of the plasticity of these cells and their possible role in human beta cell regeneration.

Several virus-based gene transfer methods into pancreatic cells are available. Adenoviral vectors have been used previously in primary pancreatic cells[12-17], but this approach is limited by the non-integrating nature of these viruses, which result in a transient effect of the transduction. Moreover, the large amounts of viral particles necessary for efficient transduction is associated with increased cytotoxicity[13]. Lentiviral vectors have the potential to transduce a wide variety of both dividing and non-dividing cell types with long-term and stable expression of transgenes, including

cells that are difficult to transfect such as neuronal and glial cells[18-20]. Also in clinical studies, lentiviral gene modification of autologous haematopoietic stem cells has demonstrated small successes in the treatment of monogenetic blood diseases[21-24]. With regards to pancreatic cells, lentiviral vectors have been successfully used to transduce primary human islet cells, enabling studies focused on human beta cell physiology and plasticity[3,25-31]. However, little is known about lentiviral-mediated gene transfer to the primary human pancreatic exocrine cells[32].

In this study, we optimised the transduction conditions of primary human adult exocrine cells with a HIV-1 (human immune deficiency virus 1)-based, replication-deficient, lentiviral reporter vector. This novel protocol can be used for improved transgene expression in primary human exocrine tissue, which will enable further investigations on the putative role of these cells in beta cell regeneration.

Materials and methods

Generation of HIV-1-based SIN lentiviral vectors

Third-generation self-inactivating HIV-1 based lentiviral vectors were produced as described previously[20]. In short, three helper plasmids (pMDLg-RRE (gag-pol), pRSV-REV and the envelope plasmid) along with the transgene encoding the lentiviral vector were co-transfected overnight into 293T cells using polyethylenimine. The lentiviral vector was pseudotyped with the surface glycoproteins pCMVAXF, pHCMV-G, pHCMV-LCMV-GP, pRRVpcDNA3.1Zeo+ and pHCMV-RabiesG, coding for the MLV4070Aenv (amphotropic murine leukemia virus), the VSV-G (vesicular stomatitis virus), the LCMV-GP (lymphocytic choriomeningitis virus), the RRV-GP (Ross River virus) and the RV-G (rabies virus) surface glycoproteins, respectively. The LCMV-GP plasmid was kindly provided by Dr. von Laer (Innsbruck Medical University, Innsbruck, Austria), the pcDNA3.1Zeo+ plasmid was kindly provided by Dr. Sanders (Purdue University, West-Lafayette, United States) and the MLV4070Aenv, RRV-GP, RV-G plasmids were kindly provided by Dr. Renner (University of Veterinary Medicine, Vienna, Austria). The medium was refreshed and viral supernatant was harvested after 48 hours and 72 hours and filtered using a 0.45 µm filter (Pall Corporation). For concentration, lentiviruses were concentrated by one round of ultracentrifugation at 50,000 g for 120 minutes at 4 °C. After removal of the supernatant, the remaining pellet was resuspended in 1 mL of T50N130E1 buffer (50 mM Tris-Cl, 130 mM NaCl and 1 mM EDTA; pH 7.8) by shaking overnight at 4 °C. Virus was quantified by antigen capture ELISA measuring HIV p24 levels (Zeptometrix) and stored at -80 °C.

Dissociation and purification of human pancreatic islet-depleted tissue

Pancreatic tissue was obtained from human organ donor pancreata. Islets and exocrine tissue were only studied if they could not be used for clinical purposes and if research consent was present. According to national law, ethics approval is not required for research on donor tissues that cannot be used for clinical transplantation. All methods were carried out in accordance with relevant

guidelines and regulations. Mean age of the donors was 51.3 years (range 23-71 years), mean BMI was 25.4 kg/m^2 (range 21-31 kg/m^2), and none of the donors had a medical history of diabetes mellitus (**Table S1**). The donor pancreata were processed at the Good Manufacturing Practice facility of our institute according to the Ricordi method as described previously[33]. The donor pancreata were enzymatically and mechanically digested followed by islet purification using a Ficoll gradient. The islet-depleted tissue contained <5% islets as determined by dithizone staining and was cultured in culture bags containing 4 mL of tissue in 200 mL Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Bodinco), 100 U/mL penicillin (Invitrogen) and 100 µg/mL streptomycin (Invitrogen). After overnight culture, islet-depleted tissue was dissociated with trypsin and filtered using 70 µm filters (Miltenyi Biotech). For duct purification, magnetic-activated cell sort (MACS) was performed on dissociated islet-depleted tissue using the ductal cell surface marker CA19-9 (Carbohydrate antigen 19-9) as described previously[34,35]. Briefly, cells were incubated with an antibody against CA19-9 (1:200; Invitrogen) for 30 minutes and afterwards with anti-mouse IgG1 microbeads (1:250; Miltenyi Biotech), before magnetic sorting with LS-columns and a QuadroMACS separator (Miltenyi Biotech).

Lentiviral transduction of human pancreatic exocrine cells

After assessment of cell viability with trypan blue, viral supernatant was added to the cells in an ultra-low attachment plate or dish (Costar, Corning) and cells were incubated with viral supernatant for 4 hours, 8 hours or overnight. For the default transduction condition a vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped cytomegalovirus-green fluorescent protein (CMV-GFP) vector was used at a multiplicity of infection (MOI) of 2, and transduction was performed in serum-rich medium supplemented with polybrene, unless specified otherwise. Serum-free transductions were performed in DMEM supplemented with 0.1% human albumin (Sanquin) and 1× Insulin-Transferrin-Selenium (ITS) (Sigma Aldrich). The polycation agents used were polybrene 8 µg/mL, protamine sulfate 4 µg/mL or DEAE-Dextran 8 µg/mL. The transduction volume can be of influence on the transduction efficiency, therefore the transduction volume was kept uniform throughout all experiments, with a volume of 135.5 µL/cm^2 of growth area of the well/dish the transduction took place in, and with a minimum of 50% of the transduction volume consisting of fresh medium. Transduction efficiency was evaluated of primary pancreatic exocrine cells that were first expanded for 5-6 days in monolayer culture in treated tissue-culture flasks (Corning) prior to transduction. At confluency the cells in monolayer were detached and incubated with viral supernatant in suspension in ultra-low attachment plates. After incubation cells were spun down by centrifuge, and after removal of the viral supernatant cells were washed with PBS and subsequently cultured in suspension in ultra-low attachment plates (**Figure 1a** 'MC-T-SC'). For the two other conditions, fresh islet-depleted tissue was used after a post-isolation overnight culture in culture bags. Cells were dissociated and incubated with viral supernatant in ultra-low attachment plates in suspension. After incubation cells were spun down by centrifuge, and after removal of the viral supernatant cells were

washed with PBS and cultured in suspension in ultra-low attachment plates (**Figure 1a** 'T-SC'), or as monolayer on treated tissue-culture flasks (**Figure 1a** 'T-MC'). Cells in all conditions were cultured in Endothelial Cell Basal Medium-2 (EBM-2) (Lonza) supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin and 100 µg/mL streptomycin for 5-6 days before analysis. Medium in all conditions was refreshed every two days.

Flow cytometry

Cells in suspension culture were washed and dissociated with trypsin. For monolayer cultures, cells were detached from the plastic with non-enzymatic cell dissociation solution (Sigma). Dissociated cells were washed with PBS and afterwards filtered with a 40 µm filter (Miltenyi Biotec). After fixation with formalin and permeabilization with 0.1% saponin, cells were incubated with antibodies against keratin 19 (1:100; Dakocytomation) with the secondary antibody anti-mouse Alexa-Fluor 647 (1:500; Molecular Probes). Endogenous GFP was used for analysis of GFP gene expression. Flow cytometry was performed on a FACScalibur (BD Biosciences) or LSR II (BD Biosciences) and flow cytometry data was analysed with FlowJo X (FlowJo).

Immunostaining and microscopy

Cells were fixed with formalin and centrifuged at high speed in fluid agar. Agar-containing cell pellets were subsequently embedded in paraffin blocks which were cut into 4 µm sections. Primary antibodies against keratin 19 (KRT19, 1:100; Dakocytomation, 1:250; Abcam) and GFP (1:500; Roche and Molecular Probes) were used. Secondary antibodies were Alexa-Fluor 488 and Alexa-Fluor 568 anti-mouse or anti-rabbit (1:1000; Molecular Probes), 4',6-diamidino-2-phenylindole (DAPI) (Vector) was used for nuclear counterstaining. Immunofluorescent images of paraffin slides were acquired with a DM5500 microscope (Leica). The GFP expression of live cells was evaluated with a CK40 microscope (Olympus) using endogenous GFP expression. Images were processed using Zen Lite software (Zeiss).

Results

Transduction efficiency of islet-depleted tissue is higher in freshly isolated exocrine cells as compared to monolayer expanded cells

We first tested the standard protocol for lentivirus transduction of mammalian cells that we successfully used on primary human beta cells in previous experiments[30]. Because it is hypothesized that the putative progenitor cell subpopulation involved in beta cell regeneration is located in the ductal compartment of the pancreas, initial experiments were performed on human pancreatic exocrine cultures that were enriched for ductal cells by monolayer culture on tissue-culture-treated plastic, to which the pancreatic ductal cells selectively adhere[36]. Because these preliminary experiments indicated that transduction of human exocrine cells during monolayer expansion was

very poor (<1% of GFP-positive cells, data not shown), we tested whether transduction efficiency of human pancreatic exocrine cells could be improved by transduction performed on cells in suspension after they had first been expanded as monolayer culture for 5-6 days. Confluent monolayers were detached, and dispersed cells were transduced with a CMV-GFP vector in standard conditions (in suspension, MOI 2, serum-containing medium supplemented with polybrene overnight or for 8 hours). After medium refreshment, cells were transferred to ultra-low attachment plates for suspension culture and spontaneously aggregated (**Figure 1a** 'MC-T-SC'). The transduction efficiency was low. Immunostainings showed only sporadic expression of the GFP reporter gene (**Figure 1b**), and only up to 1.2% of cells were GFP-positive as quantified by flow cytometry (**Figure 1f** 'MC-T-SC', n = 3), suggesting a resilience of primary human ductal cells to lentiviral transduction.

Next, we investigated whether the transduction efficiency could be improved if fresh islet-depleted tissue was used, with only a post-isolation overnight recovery culture in culture bags. Dissociated islet-depleted tissue was transduced with a CMV-GFP in standard conditions (in suspension, MOI 2, serum-containing medium supplemented with polybrene overnight or for 8 hours). After transduction, the transduced cells were then either maintained in suspension culture (**Figure 1a** 'T-SC') or transferred to monolayer culture (**Figure 1a** 'T-MC'). Transduction of islet-containing fractions under T-SC condition resulted in a large fraction of GFP-positive cells after transduction, but with only a minor fraction of these GFP-positive cells co-expressing KRT19 (**Figure 1c**), confirming a resilience of ductal cells to transduction. Similarly, exocrine cells transduced under these conditions showed an increase in the fraction of GFP-positive cells, limited to 5% of GFP-positive cells 5 days post-transduction (**Figure 1d-f** 'T-SC', n = 2). Transduced cells transferred to monolayer culture after transduction were able to proliferate and showed up to 30% of cells GFP-positive 5 days after transduction (**Figure 1d-f** 'T-MC', n = 2). Overall, these results show that transduction efficiency is improved when freshly isolated exocrine cells are transduced in suspension, but the efficiency is still limited.

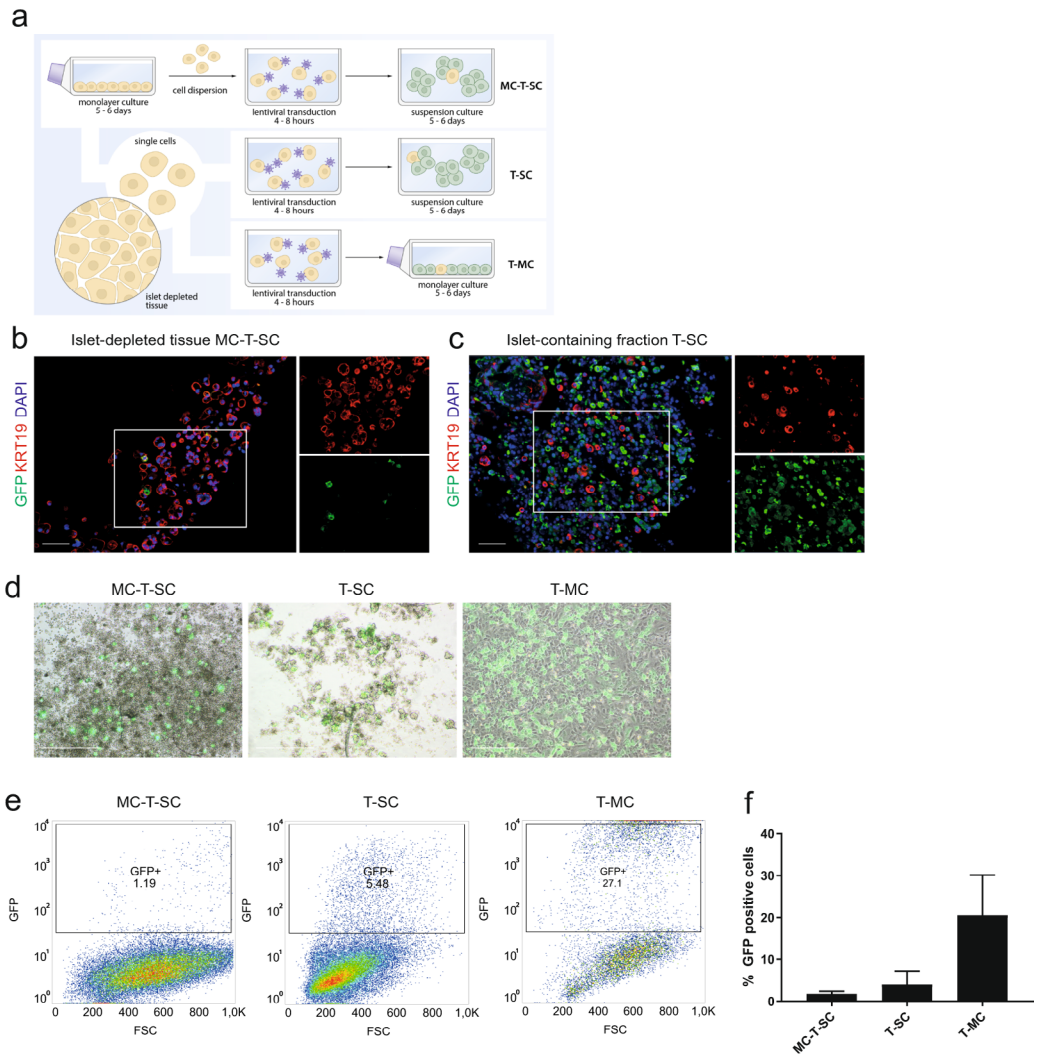

Figure 1. Human islet-depleted lentiviral transduction efficiency can be improved by transducing cells before culture

(**a**) Illustration of the transduction with cultured or freshly isolated human islet-depleted tissue. (**b**) Immunofluorescent staining for KRT19 (ductal marker, red) and GFP (green) of human dissociated islet-depleted tissue after monolayer expansion transduced with CMV-GFP in the standard condition prior to suspension culture ('MC-T-SC') at day 5 post-transduction, demonstrating sporadic expression of GFP^+ cells. Scale bar = 100 µm. (**c**) Immunofluorescent staining for KRT19 (ductal marker, red) and GFP (green) of human dissociated islet-containing fractions (30% purity) transduced with CMV-GFP in the standard condition prior to suspension culture ('T-SC') at day 5 post-transduction, showing a large proportion of GFP^+KRT19^- cells. Scale bar = 100 µm. (**d**) Brightfield images with GFP overlay of human dissociated islet-depleted tissue transduced with CMV-GFP in the standard condition at different time points during culture at day 5 post-transduction. Scale bar 'MC-T-SC' and 'T-SC' = 500 µm. Scale bar 'T-MC' = 200 µm. (**e**) Representative flow cytometry plots showing GFP versus forward scatter of human dissociated islet-depleted tissue transduced with

CMV-GFP in the standard condition at different time points during culture at day 5 post-transduction. (**f**) Flow cytometry quantification of the GFP-positive fraction of human dissociated islet-depleted tissue transduced with CMV-GFP in the standard condition at different time points during culture at day 5 post-transduction, showing the highest fraction of GFP-positive cells in the condition with cells transduced before monolayer expansion (n = 2-3; mean ± SEM).

CMV is the strongest constitutive promoter for the transduction of primary human exocrine cells

We then examined whether the choice of promoter (CMV) could partly explain the low frequency of GFP-positive cells. We tested two other constitutively active promoters of non-viral origin that are often successfully used in lentiviral vectors, the elongation factor 1 alpha (EF1a) and the phosphoglycerate kinase (PGK) promoter. Dissociated islet-depleted tissue was transduced with a promoter-GFP vector in standard conditions (in suspension, MOI 2, serum-containing medium supplemented with polybrene overnight or for 8 hours), and subsequently cultured in suspension (**Figure 1a** 'T-SC'). The GFP intensity was evaluated with fluorescence microscopy (**Figure 2a**), showing brightest GFP expression in cells transduced with the CMV-GFP vector. Quantification with flow cytometry indicated a slightly higher frequency of GFP-positive cells upon transduction with the PGK-GFP vector (**Figure 2b**), but the intensity of the signal was lower than for CMV-GFP, as illustrated by the difference in mean fluorescence intensity (MFI) (**Figure 2c**, n = 3). The EF1a promoter was the weakest promoter, with only sporadic cells expressing GFP. Thus, with CMV being the strongest promoter with brightest GFP-expression and highest MFI in our target cells, this promoter was used for subsequent experiments and optimisation of the transduction protocol.

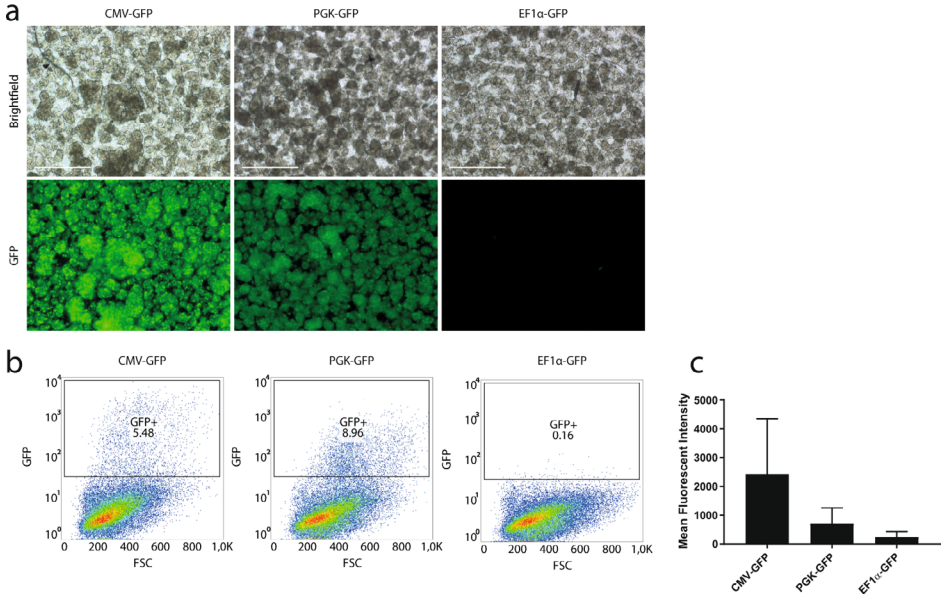

Figure 2. The constitutive CMV promoter yields the brightest GFP-positive cells
(**a**) Brightfield and GFP images of human dissociated islet-depleted tissue transduced with CMV-GFP, PGK-GFP or EF1a-GFP in the standard condition prior to suspension culture ('T-SC') at day 5 post-transduction, demonstrating brightest GFP expression in cells transduced with CMV-GFP. Scale bar = 200 µm. (**b**) Representative flow cytometry plots showing GFP versus forward scatter of human dissociated islet-depleted tissue transduced with CMV-GFP, PGK-GFP or EF1a-GFP in the standard condition prior to suspension culture ('T-SC') at day 5 post-transduction, showing the highest fraction of GFP-positive cells in the culture transduced with PGK-GFP. (**c**) Mean Fluorescent Intensity (MFI) of the GFP-signal of human dissociated islet-depleted tissue transduced with CMV-GFP, PGK-GFP or EF1a-GFP in the standard condition prior to suspension culture ('T-SC') at day 5 post-transduction, showing the highest MFI in cells transduced with CMV-GFP (n = 3, mean ± SEM).

An increased MOI does not increase the transduction efficiency

To investigate whether a higher MOI could increase transduction efficiency, we compared several MOIs in suspension and monolayer culture. In this experiment, dissociated islet-depleted tissue was first enriched for ductal cells by using MACS for the cell surface marker CA19-9.

After isolation, exocrine cells were transduced with a CMV-GFP lentivirus at a MOI of 2, 7 or 13 in standard condition (in suspension, serum-containing medium supplemented with polybrene overnight or for 8 hours), with subsequent culture in suspension (**Figure 1a** 'T-SC'). Evaluation with microscopy showed that cultures transduced with a higher MOI had fewer aggregates and more single cells (**Supplementary Figure 1a**). Also, flow cytometry analysis showed an increase of a side population of cells in the FSC/SSC plot (**Supplementary Figure 1b**, n = 1), most likely representing unhealthy cells. In addition, no difference in GFP-expression was observed with fluorescence microscopy or flow cytometry, with approximately 6% of cells GFP-positive 5 days post-transduction with a MOI of 2, 7 or 13.

In the monolayer setup islet-depleted tissue was enriched for CA19-9 cells by MACS. After isolation, cells were plated as monolayer culture and transduced while in monolayer expansion at approximately 40% confluency with a CMV-GFP with a MOI of 2, 4 or 6 in standard conditions (serum-containing medium supplemented with polybrene overnight or for 8 hours). During expansion, these cells showed an increase in cell detachment with increased MOIs, suggesting increased cytotoxicity (**Supplementary Figure 1c**, n = 1). Subsequent flow cytometry analysis did not show an increase in the fraction of GFP-positive cells, and moreover indicated a decrease in the KRT19-positive cell fraction of the monolayer cultures transduced with a MOI of 4 or 6 (**Supplementary Figure 1d**, n = 1). Because transduction efficiency did not increase with a higher MOI but did seem to have cytotoxic effect, a MOI of 2 was selected as standard MOI for future experiments.

Increased transduction efficiency with the adjuvant protamine sulfate and serum-free transduction environment

Another potential factor that may affect the transduction efficiency of primary cells is the

adjuvant used during the transduction and the presence of serum. We first tested the effect of the commonly used adjuvants polybrene and DEAE-dextran on dissociated islet-depleted tissue during standard transduction before suspension culture (**Figure 1a** 'T-SC'). There was no improvement in GFP-expression with polybrene or DEAE-dextran compared to the control without adjuvant (**Supplementary Figure 2a-c**, n = 2-3). Next, we tested the effect of protamine sulfate as adjuvant and a serum-free transduction environment on the transduction efficiency. In this experiment, CA19-9 cells were isolated from dissociated islet-depleted tissue with MACS. After isolation, the adjuvants polybrene, protamine sulfate and serum-free transduction without adjuvant were compared on exocrine cells transduced with a CMV-GFP in standard conditions (in suspension, MOI 2, serum-containing medium for 4 hours), with subsequent culture in suspension (**Figure 1a** 'T-SC'). The adjuvant protamine sulfate in the presence of serum, or a serum-free transduction environment without adjuvant, appeared to increase the percentage of GFP-positive cells to 15% of the ductal cells (**Figure 3a-c**, n = 2), and the same effect was also seen on non-purified dissociated islet-depleted tissue (data not shown, n = 2). Subsequently, we investigated the effect of these transduction conditions combined on dissociated islet-depleted tissue prior to enrichment for ductal cells by monolayer culture on tissue-culture-treated plastic. The combination of protamine sulfate combined with serum-free transduction on non-purified dissociated islet-depleted tissue transduced before monolayer culture (**Figure 1a** 'T-MC') further increased the number of GFP-positive cells up to 50% (**Figure 4a-b** 'VSV-G (V)', n = 4). Therefore, this method was used for subsequent experiments.

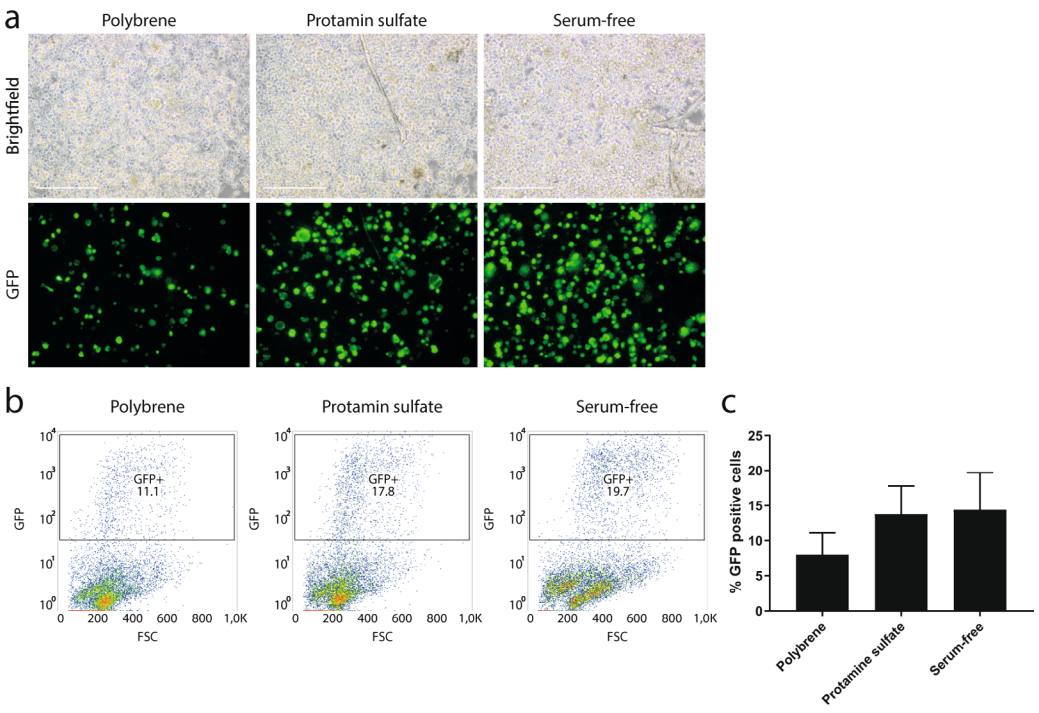

Figure 3. Improved transduction efficiency with the adjuvant protamine sulfate and a serum-free environment without adjuvant

(**a**) Brightfield and GFP images of ductal cells isolated from human dissociated islet-depleted tissue, transduced with CMV-GFP in presence of polybrene, protamine sulfate or in absence of serum and adjuvant prior to suspension culture ('T-SC') at day 5 post-transduction. Scale bar = 100 µm. (**b**) Representative flow cytometry plots showing GFP versus forward scatter of ductal cells isolated from human dissociated islet-depleted tissue, transduced with CMV-GFP in presence of polybrene, protamine sulfate or in absence of serum and adjuvant prior to suspension culture ('T-SC') at day 5 post-transduction, showing a small increase in the fraction of GFP-positive cells when protamine sulfate or a serum-free condition without adjuvant was used. (**c**) Quantification of GFP-positive cells by flow cytometry of ductal cells isolated from human dissociated islet-depleted tissue, transduced with CMV-GFP in the presence of polybrene, protamine sulfate or in the absence of serum and adjuvant with subsequent suspension culture ('T-SC') at day 5 post-transduction, showing an increase to 15% of GFP-positive cells (n = 2, mean ± SEM).

Pseudotyping with the envelope of vesicular stomatitis virus yields the highest transduction efficiency

To investigate whether cell entry of the lentivirus vector in primary exocrine cells could be a limitation for efficient transduction we tested envelopes from other viruses. The envelopes tested were derived from Ross River virus, rabies virus, lymphocytic choriomeningitis virus, amphotropic murine leukemia virus, and were compared to the most commonly used vesicular stomatitis virus envelope. Dissociated islet-depleted tissue was transduced with a pseudotyped CMV-GFP vector in serum-free medium supplemented with protamine sulfate for 4 hours, with subsequent monolayer expansion (**Figure 1a** 'T-MC'). We observed a large difference in the fraction of GFP-positive cells, with 47% of GFP-positive cells when the VSV-G pseudotyped lentiviral vector was used compared to only sporadic GFP-expressing cells when one of the other pseudotyped vector was used (**Figure 4a-b**, n = 3). Based on these results, lentiviral vectors for the primary exocrine cells were pseudotyped with the VSV-G envelope for future experiments.

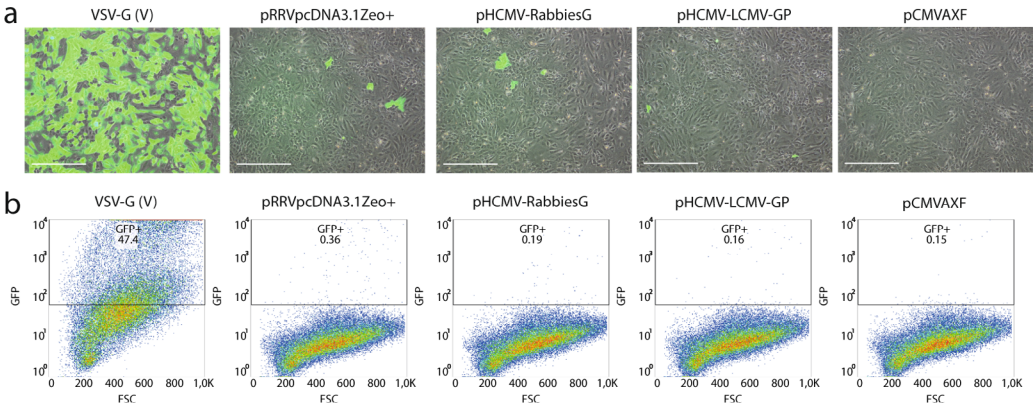

Figure 4. Pseudotyping with the surface glycoproteins of vesicular stomatitis virus yields the highest transduction efficiency
(**a**) Brightfield images with GFP overlay of human dissociated islet-depleted tissue, transduced in serum-free medium in the presence of protamine sulfate with CMV-GFP pseudotyped with the VSV-G, RR-GP, RRV-GP, LCMV-GP or MLV4070Aenv with subsequent monolayer culture ('T-MC') at day 5 post-transduction, with the most GFP-positive cells observed with the VSV-G pseudotyped vector and sporadic GFP-positive cells with the other pseudotyped vectors. Scale bar = 200 µm. (**b**) Flow cytometry data showing GFP versus forward scatter of human exocrine cells transduced in serum-free medium in the presence of protamine sulfate with CMV-GFP pseudotyped with the VSV-G, RR-GP, RRV-GP, LCMV-GP or MLV4070Aenv with subsequent monolayer culture ('T-MC') at day 5 post-transduction. A large fraction of GFP-positive cells was observed when the VSV-G pseudotyped vector was used (n = 3).

Ultracentrifugation of viral supernatant further increases the efficiency of transduction

In order to further deplete the lentiviral transduction conditions of serum and other potentially inhibiting factors that might have been secreted by the lentivirus-producing cell line, viral supernatant was concentrated by ultracentrifugation and the supernatant containing medium and serum was removed. Dissociated islet-depleted tissue was transduced with serum-containing virus supernatant or serum-depleted virus supernatant at a MOI of 2 in serum-free medium supplemented with protamine sulfate for 4 hours, prior to monolayer expansion (**Figure 1a** 'T-MC). The use of serum-depleted CMV-GFP viral supernatant increased transduction efficiency 0.6-fold to a maximum of 90% of GFP-positive cells, compared to a maximum of 51% of GFP-positive cells with the CMV-GFP serum-containing viral supernatant (**Figure 5a-c** 'CMV-GFP (serum-containing) and CMV-GFP (serum-depleted)' n = 3).

Finally, in an attempt to develop a ductal-cell-specific lineage tracing system, we compared specificity and efficiency of various promoters from genes known to be specifically expressed in ductal cells in the human pancreas: 380 bp mouse KRT19 promoter[37], 732 bp human KRT19 promoter[38], 2952 bp human KRT19 promoter[39], and a human carbonic anhydrase II (hCAII) promoter[4,40]. The 380 bp mouse KRT19 (mKRT19) promoter displayed the most promising results with regards to transduction efficiency and specificity on KRT19-positive and KRT19-negative cell lines (**Supplemental Figure 3** and data not shown). Serum-containing viral supernatant of the mKRT19-GFP lentivirus vector was subsequently compared to the mKRT19-GFP serum-depleted viral supernatant on primary human exocrine tissue. The frequency of GFP-positive cells was lower compared to the strong ubiquitous CMV promoter, but we could increase the transduction efficiency 3.4-fold from a maximum of 8% of GFP-positive cells with the serum-containing viral supernatant, to a maximum of 40% of GFP-positive cells using serum-depleted viral supernatant (**Figure 5a-c**, n = 3). Importantly, only 1.2% of GFP-positive cells were KRT19-negative, suggesting a good specificity of the construct in these islet-depleted preparations. Next, we tested the specificity of these construct on islet-containing fractions, which also contain endocrine cells next to the

exocrine cells. These additional experiments showed a higher frequency (up to 9%) of GFP-positive cells, that were not ductal cells (KRT19-negative) when mKRT19-GFP and hCAII-GFP constructs were used (**Supplemental Figure 4a-c**, n = 3). We therefore considered these promoters to be insufficiently specific for application in lineage tracing studies. Yet, altogether our data indicate that ultracentrifugated viral supernatant combined with serum-free transduction condition and protamine sulfate constitute optimal conditions to efficiently transduce primary human exocrine cells.

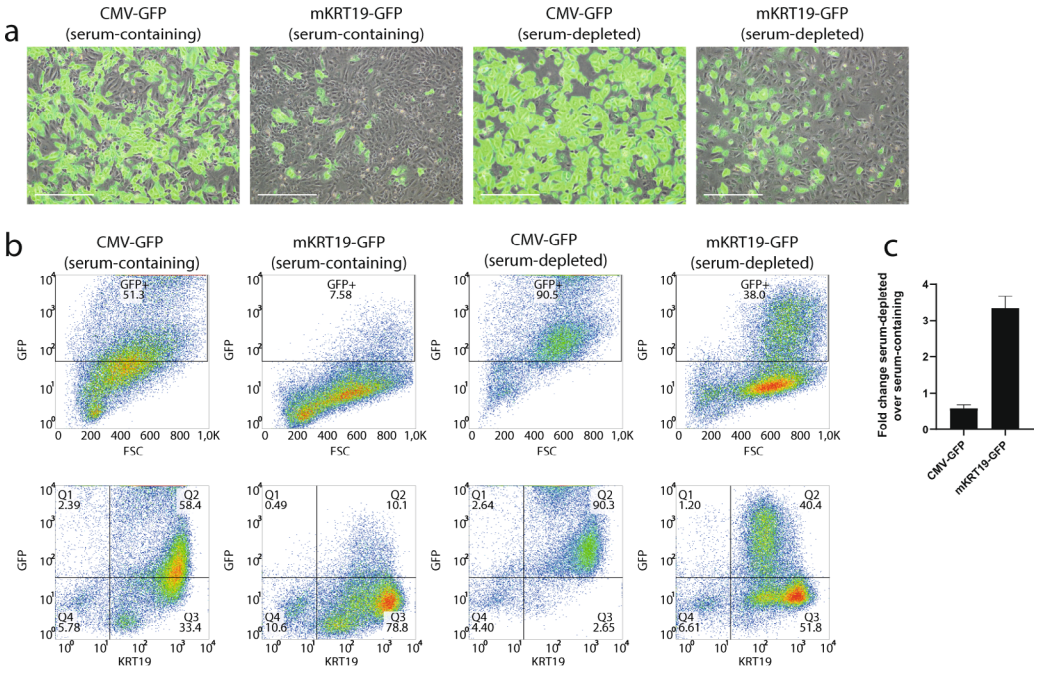

Figure 5. Higher transduction efficiency by serum-depletion of viral supernatant
(**a**) Brightfield and GFP overlay images of human dissociated islet-depleted tissue transduced with CMV-GFP or mKRT19-GFP serum-depleted or serum-containing viral supernatant at a MOI of 2 in serum-free medium in presence of protamine sulfate with subsequent monolayer culture ('T-MC') at day 5 post-transduction. Scale bar = 200 µm. (**b**) Flow cytometry plots showing GFP versus forward scatter, and GFP versus KRT19 (ductal marker), of human dissociated islet-depleted tissue transduced with CMV-GFP or mKRT19-GFP serum-depleted or serum-containing viral supernatant at a MOI of 2 in serum-free medium in presence of protamine sulfate with subsequent monolayer culture ('T-MC'), at 5 days post-transduction showing an increase up to 90% of GFP-positive cells when serum-depleted CMV-GFP viral supernatant was used. (**c**) Fold change of the GFP-positive fraction when human dissociated islet-depleted tissue was transduced with a serum-depleted as compared to serum-containing viral supernatant of a CMV-GFP or a mKRT19-GFP construct at a MOI of 2 in presence of protamine sulfate with subsequent monolayer culture ('T-MC') at 5 days post-transduction (n = 3, mean ± SEM).

Discussion

An alternative source of beta cells for beta cell replacement therapy in type 1 diabetes mellitus is urgently needed as the availability of human pancreas donor material is too limited. Pancreatic ductal cells are an attractive alternative source for the generation of new beta cells, but it remains unclear whether human pancreatic ductal cells can act as beta cell progenitor cells due to the lack of an efficient lineage tracing system. In contrast to primary human islet cells, primary human ductal cells are highly resilient to lentiviral transduction by standard protocol. Various factors can strongly influence lentiviral transduction. Here we systematically tested various factors such as the timing of transduction, promoters (both ubiquitous and cell-type-specific), pseudotyping of the lentivirus vector, MOI and the presence of serum or an adjuvant.

First, we looked at host factors that could be of influence on transduction efficiency. We found that cellular state during culture was a critical point of influence on transduction efficiency, with the highest transduction efficiency obtained by using fresh exocrine cells transduced in suspension. Possible reasons for this difference in transduction efficiency might be a change in cellular receptors after adhesion and expansion of these cells, limiting viral entry or post-entry events. Cellular adhesion and timing of transduction are also known to play a role in the cellular events controlling viral entry and/or integration in hematopoietic stem cells, which is reflected in transduction efficiency differences depending on the timing, type and pre-stimulation of the culture[41-45].

Secondly, we looked at the lentiviral vector characteristics that could be important for efficient transduction. We found that the best lentiviral vector for our target cells included a CMV-promoter to drive the expression of the transgene, combined with a vesicular stomatitis virus glycoprotein for cell entry. Since the strength and expression efficiency of a constitutive promoter is variable and dependent on the type of cell to be transduced, it is necessary to compare different promoters in order to select the strongest and most efficient promoter for the cell of interest, which has been illustrated by several published systematic promoter comparisons[46-49]. In an attempt to develop a lineage tracing system we also evaluated several promoters of genes known to be specific for the pancreatic ductal cells (KRT19 and CAII), but specificity of these promoters was not specific enough for a lineage tracing system to investigate duct cell fate during different culture conditions. In addition, the envelope which allows the entry of the virus into the cells could also limit transduction efficiency. Pseudotyping of lentiviral vectors by adding surface glycoproteins of other viruses can alter the tropism of a lentiviral vector and potentially increase the amount of specific cellular targets that can be transduced[50]. Our data indicate that transduction efficiency of our target cells was not improved when lentiviral vectors pseudotyped with different envelopes were used, as compared to the standard VSV-G envelope. Finally, we identified the adjuvant protamine sulfate in combination with serum-free medium and ultracentrifugated lentiviral supernatant as the best environment for the transduction of exocrine cells. The presence of positively-charged polycation agents reduces electrostatic repulsion forces between the negatively charged cell and lentiviral particles, and thus improves transduction efficiency. The effect of these adjuvants is dependent on the type of cell that

is transduced, and the lentiviral construct that is used[51,52]. We also observed the variability of this adjuvant effect in our experiments, with no effect on transduction efficiency when the widely used polybrene and DEAE-dextran were evaluated as adjuvant for our target cells. In addition, the presence of serum and other unknown factors that might have been secreted by the lentivirus producing cell lines during the transduction can also affect the efficiency of transduction. Postulated mechanisms include an attenuated effect of positive polycation agents by the negatively charged proteins present in serum and medium, or virus particle aggregation due to proteins, resulting in viral aggregates with less infectious capacity compared to single virus particles[53]. For example, hematopoietic stem cell transduction efficiency is negatively affected when transduction is performed in conditions supplemented with serum[42,54].

Altogether, our access to primary human tissue from our islet isolation facility enabled us to systematically evaluate various transduction conditions to improve lentiviral transduction of these exocrine cells. Although shortage of donor tissue limited the number of biological replicates we could include for all the different conditions tested, our data provide lentiviral protocol optimisations that enable more efficient gene transfer into primary human pancreatic exocrine cells. These findings will allow future investigations on the (patho)physiology of pancreatic diseases and could stimulate further studies on their possible *ex vivo* gene therapy options, in addition to enabling studies on the plasticity of human pancreatic exocrine cells and their role in beta cell regeneration.

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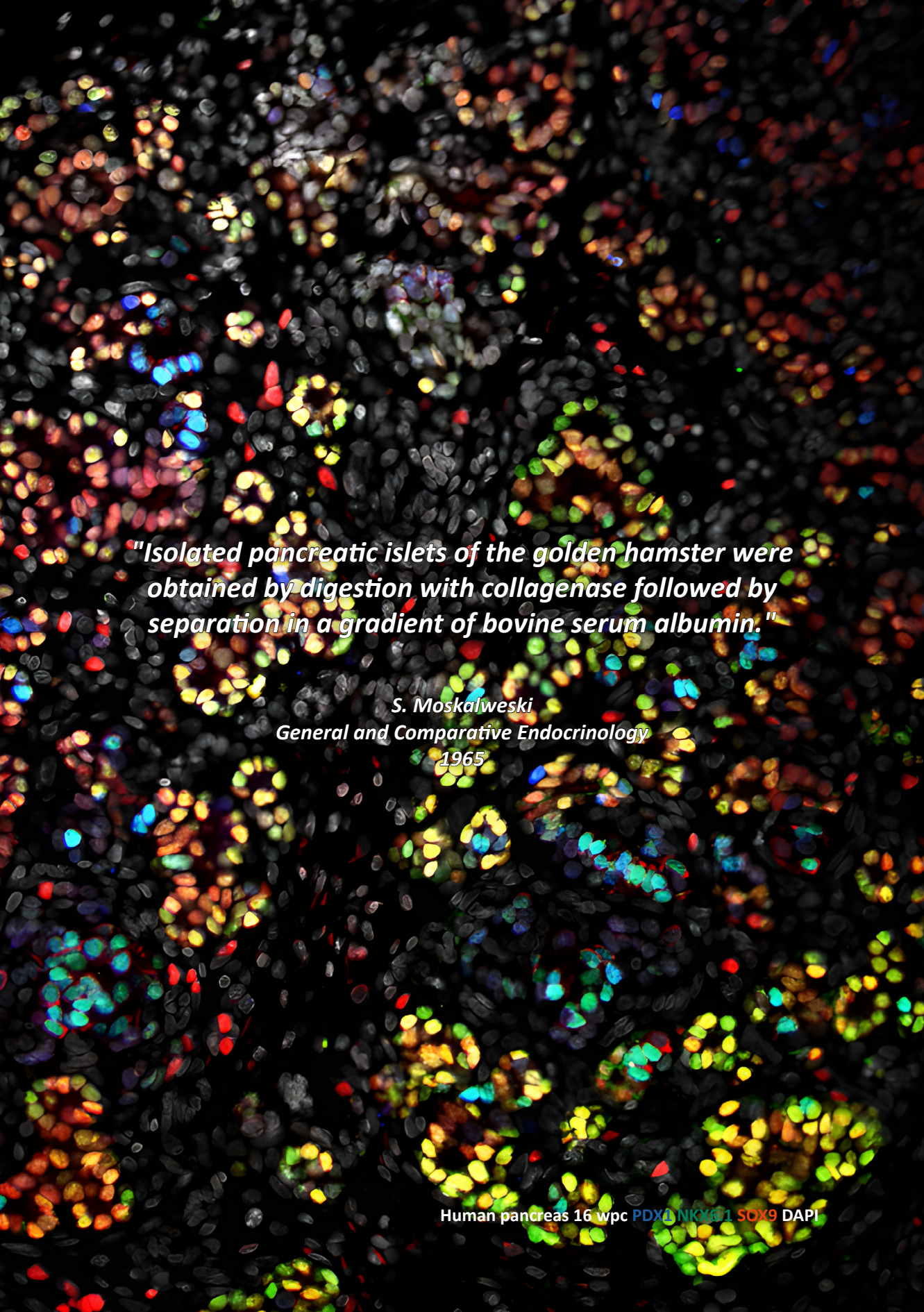
Supplemental information


"Isolated pancreatic islets of the golden hamster were obtained by digestion with collagenase followed by separation in a gradient of bovine serum albumin."
S. Moskalweski
General and Comparative Endocrinology
1965
Human pancreas 16 wpc PDX1 NKX6.1 SOX9 DAPI

CHAPTER| 6

Differentiation of Human Pancreatic Ductal Cells Towards a Beta Cell Phenotype Using INGAP, FGF7 and a GLP-1R Agonist

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Abstract

Putative progenitors in the human adult duct compartment of the pancreas are an attractive alternative cell source for beta cell replacement therapy. Potential candidates to improve differentiation protocols are compounds associated with duct-derived beta cell neogenesis. Here we aim to explore the activation of GLP-1R, INGAP and FGF7 pathways as triggers for beta cell differentiation of primary human islet-depleted tissue. Ductal cells were expanded from human islet-depleted tissue as monolayer culture and subsequently differentiated in suspension culture. We evaluated a multistage protocol based on the use of INGAP, the GLP-1R agonist liraglutide and FGF7, in comparison with a basic serum-free differentiation medium. Upon *ex vivo* differentiation, gene expression of endocrine hormone markers was induced up to 15- to 75-fold in the multistage protocol. In the multistage protocol 6% of the aggregated cells were PDX1^{high}-positive and 60% of these cells also co-expressed NKX6.1. After transplantation in mice PDX1^{high}NKX6.1^{+}Insulin^{+} cells were observed in the graft. Remarkably, differentiated beta cells were found outside duct structures suggesting a delamination process. In conclusion, this study provides evidence that pancreatic endocrine progenitor cells can be formed *ex vivo* from primary human exocrine cells.

Introduction

Type 1 diabetes mellitus (T1DM) is a chronic disease characterised by an autoimmune destruction of the insulin-producing beta cells located in the islets of Langerhans, resulting in hyperglycemia[1]. T1DM is treated by administration of exogenous insulin. In a small group of T1DM patients beta cell replacement (pancreas or islet transplantation) is performed. A lack of donor organs is one of the factors why this treatment occurs rarely. One of the main challenges in making beta cell replacement therapy accessible to a broader group of patients is identifying a renewable source of beta cells.

Alternative sources for beta cells include pluripotent stem cells such as human embryonic stem cells (hESC) or induced pluripotent stem cells (iPSC). Recent developments in this field are encouraging with development of multistage differentiation protocols, yielding pure populations of beta cells[2,3]. However, clinical use of these pluripotent cells is currently limited by ethical concerns and by the risk of malignant formation by residual undifferentiated stem cells when transplanted *in vivo*[4].

Another option would be the use of adult pancreas-specific progenitor cells. It is hypothesized that the adult pancreas harbours cells that are able to develop towards a beta cell phenotype[5-9]. During embryonic development the pancreatic ductal epithelium is able to generate all pancreatic cell types, including the beta cells[10,11]. However, whether the duct cells retain this capacity in the postnatal pancreas is still controversial[12-15]. Human autopsy studies have indicated that in altered metabolic states such as pregnancy or obesity there is a significant increase in the number of beta cells[16-18]. These newly formed beta cells are in close proximity or located in the ducts of the pancreas, indicating that the origin of these cells is in the ductal compartment. Moreover, we recently reported that a novel computational method to exploit single-cell transcriptome data (StemID)

identified pancreatic progenitor cells to be located in the human pancreatic duct cell population[19,20].

In an effort to find compounds that can induce beta cell neogenesis several animal models of diabetes and pancreatic injury models have been developed, inducing regeneration of the pancreas[21-23]. These models have resulted in the discovery of compounds associated with duct-derived beta cell neogenesis. One of these models is the pancreatic duct obstruction model in hamsters, in which new beta cells were formed in close proximity of the duct[24]. The bioactive peptide islet neogenesis associated protein (INGAP) was isolated from the regenerating pancreas and has demonstrated to reduce hyperglycemia in diabetic rodents and to improve glucose homeostasis in patients with diabetes mellitus in clinical trials[25-27]. Also, *in vitro* experiments with a pancreatic ductal cell line demonstrated differentiation into endocrine cells when treated with this peptide[28]. Another neuropeptide is glucagon-like peptide-1 (GLP-1), an incretin endogenously produced by L-cells in the intestine. GLP-1 receptor agonists (GLP-1 RA) are widely used for its insulinotropic properties in patients with type 2 diabetes mellitus[29]. In several rodent models of diabetes, administration of GLP-1 or its long-acting receptor agonist exendin-4, significantly increased beta cell mass. Individual beta cells were found in ducts and in small clusters outside islets, next to an increased proliferation of duct and acinar tissue, suggesting GLP-1 has an effect on both the proliferation and differentiation of pancreatic cells[30,31]. Moreover, *in vitro* studies using the pancreatic ductal cell lines ARIP and PANC-1 showed increased beta cell differentiation with GLP1-R agonist treatment[32]. Fibroblast growth factor 7 (FGF7) is known for its regulation of proliferation and differentiation of cells. During development it is expressed in the pancreatic mesenchyme where it promotes growth, morphogenesis and differentiation of pancreatic exocrine cells by activation of the NOTCH pathway[33,34]. *In vitro* studies with pluripotent cells demonstrate that FGF7 promotes differentiation of PDX1-expressing pancreatic progenitor cells[35].

The vast majority of our understanding of beta cell neogenesis is derived from research performed with animals or cell lines due to limited access to primary human tissue. For development of a potentially translatable therapeutic approach of duct-derived beta cell neogenesis, it is essential to determine the effects of these agents on human tissue. A differentiation protocol that included INGAP, FGF7 and a GLP-1R agonist was successfully used to differentiate human embryonic stem cells towards insulin-producing beta cell[36]. When applying this protocol, the differentiating cells underwent a phase in which the majority of cells expressed KRT19 (ductal marker), prior to endocrine differentiation. We hypothesized that the differentiation of putative progenitors located in the ductal compartment of the human pancreas can also be triggered by using these agents that stimulate islet neogenesis. Here, we evaluated the effects on primary human islet-depleted tissue of three compounds associated with duct-derived beta cell neogenesis *in vivo*, *i.e.,* INGAP, GLP-1, and FGF7.

Materials and methods

Expansion of islet-depleted tissue

Human donor pancreata were processed at in the Good Manufacturing Practice facility of our institute according to the Ricordi method[37]. Tissue was only used if consent for research was indicated on the Eurotransplant organ donor forms. Organ donors were between 14 and 61 years of age, had a BMI between 20 and 34 kg/m^2 and did not have a medical history of diabetes mellitus (**Table 1**). The donor pancreata were enzymatically and mechanically digested followed by islet purification using a Ficoll gradient. Islet purity was directly assessed after digestion using dithizone (DTZ) staining and islet-depleted tissue fractions were cultured overnight in culture bags containing 4 mL of tissue and 200 mL Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin. Next, islet-depleted tissue was washed with PBS and single cells were obtained by trypsinisation and filtered using 70 µm filters. Samples of single cells were collected for RNA isolation and formalin fixation. Single cells were plated in tissue-culture-treated T75 tissue culture flasks (Corning) at a density of 15 x 10^6 cells per flask in Endothelial Basal Medium-2 (EBM-2) (Clonetics) supplemented with 10% FCS, 100U/mL penicillin and 100 µg/mL streptomycin. The cells were cultured at 37 °C under a humidified condition of 95% air and 5% CO_2 until confluency for 5-7 days. The medium was changed the day after plating and subsequently every 2 days.

Table 1. Donor characteristics

Donor number	Sex	Age (years)	BMI (kg/m^2)	Cause of death
1	M	36	27	Cardiac arrest
2	F	14	20	Trauma
3	F	20	25	Trauma
4	M	51	34	Stroke
5	F	61	23	Trauma
6	M	17	20	Stroke
7	F	47	26	Stroke
8	F	42	22	Stroke
9	M	46	25	Suicide
10	F	45	24	Brain tumour
11	M	27	30	Suicide

Ex vivo differentiation of expanded islet-depleted tissue

The cells in monolayer were washed with PBS before incubation in 8 mL of non-enzymatic cell dissociation solution (NCDS) (Sigma) to detach the cells from the plastic surface. Cells were collected in DMEM containing 10% FCS, 100U/mL penicillin and 100 µg/mL streptomycin. After

washing, cells were counted and samples for RNA isolation and formalin fixation were obtained. The rest of the cells were used for differentiation by 3D culture in suspension in 6-well low attachment plates (Corning).

For differentiation, two protocols were compared (**Supplemental Figure 1**). The control protocol was based on differentiation culture medium described and used for primary human ductal cells and consisted of three weeks of culture in serum-free DMEM (Life technologies) supplemented with 24 mM glucose, 1 µg/mL insulin, transferrin, selenium (ITS, Sigma), 10 mM nicotinamide, 2 g/L human albumin (Cealb, Sanquin) and 200 U/mL penicillin with 200 µg/mL streptomycin[38]. This protocol was compared to a multistage protocol based on a human embryonic stem cell differentiation protocol, starting with 2 weeks of culture with DMEM/F12 (Life technologies) supplemented with 17 mM glucose, 1 µg/mL insulin, transferrin, selenium (ITS, Sigma), 10 mM nicotinamide, 2 g/L human albumin (Cealb, Sanquin), 200 nM INGAP (Sequence: IGLHDPSHGTLPNGS, synthesized at the peptide facility of our institute), 10 nM Liraglutide (GLP-1R agonist, Novo Nordisk) and 10 ng/µL fibroblast growth factor 7 (FGF7, R&D), followed by 1 week of culture with RPMI1640 (Life technologies) with B27 1× (Life technologies) and 10 mM nicotinamide[36]. Medium for both protocols was refreshed every two days.

Transplantation of *ex vivo* differentiated aggregates

All animal experiments were approved by the committee for animal ethics of our institute. After 3 weeks of *ex vivo* differentiation aggregates were washed with PBS and cell pellets of approximately 200 µL were transplanted under the kidney capsule of normoglycaemic 8-12 weeks old NOD/SCID mice. After one month of *in vivo* maturation the kidneys with the grafts were removed and processed for immunohistochemistry.

Quantitative real-time PCR

RNA was extracted using the RNeasy mini kit (Qiagen) following the manufacturer's protocol. The amount and quality of the RNA extracted was assessed using a Nanodrop ND-1000 spectrometer (NanoDrop technologies). 1 µg RNA was used for reverse transcription into cDNA using M-MLV RTase (Life technologies). PCR reactions were run in duplicate using a mix of 1/20 of the cDNA, 1 mM forward and reverse primer, 6.25 µL SYBR Green Master Mix (Qiagen) and sterile water up to a total volume of 12.5 µL. The primer sequences can be found in **Table 2**. The PCR reactions were performed on a LightCycler 480II (Roche, Basel, Switzerland). Acquired Ct values were normalized against the housekeeper gene GAPDH. The normalized Ct values were quantified using the delta-delta Ct method. Gene expression is presented relative to gene expression in cells after expansion.

Table 2. Human primer sequences

Gene	Forward primer	Reverse primer
CK19	CTACAGCCACTACTACACGAC	CAGAGCCTGTTCCGTCTCAAA
FOXA2	GGGAGCGGTGAAGATGGA	TCATGTTGCTCACGGAGGAGTA
GAPDH	TTCCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG
GCG	CAAGGCAGCTGGCAACGT	CTGGTGAATGTGCCCTGTGA
HNF6	CGCTCCGCTTAGCAGCAT	GTGTTGCCTCTATCCTTCCCAT
INS	GCAGCCTTTGTGAACCAACA	TTCCCCGCACACTAGGTAGAGA
MAFA	CTTCAGCAAGGAGGAGGTCATC	GCGTAGCCGCGGTTCTT
NKX6.1	CTGGCCTGTACCCCTCATCA	CTTCCCGTCTTTGTCCAACAA
PDX1	CCATGGATGAAGTCTACCAAAGCT	CGTGAGATGTACTTGTTGAATAGGAACT
PTF1A	AGGCCCAGAAGGTCATC	AGGGAGGCCATAATCAGG
SOX9	AGTACCCGCACTTGCACAAC	ACTTGTAATCCGGGTGGTCCTT

Tissue fixation and immunohistochemistry

Single cells or aggregates were fixed with 4% formalin (Klinipath) for 30 minutes. The mouse kidney containing the transplanted graft was fixed with 4% formalin overnight. After fixation the samples were washed with PBS and suspended in a 2% agar solution, before being processed and embedded in paraffin for sectioning of 4 μm slides. Immunofluorescence staining was performed using the following primary antibodies: 1:1000 mouse anti Synaptophysin (Millipore), 1:250 rabbit anti KRT19 (Abcam), 1:5 goat anti PDX1 (R&D systems), 1:500 mouse anti NKX6.1 (DSHB Hybridoma Bank), 1:200 guinea pig anti Insulin (Santa Cruz), 1:200 rabbit anti Glucagon (Vector Laboratories). The secondary antibodies that were used: 1:1000 goat anti mouse Alexa Fluor 568 (Molecular Probes), 1:1000 goat anti guinea pig Alexa Fluor 647 (Molecular Probes), 1:200 streptavidin Alexa Fluor 488 (Invitrogen), 1:400 TRITC anti guinea pig (Jackson ImmunoResearch), 1:500 goat anti rabbit Pacific Blue (Molecular Probes), 1:1000 goat anti rabbit Alexa Fluor 488 (Molecular Probes). Antibodies were diluted in PBS with 1% lamb serum (Invitrogen). Antigen retrieval was performed by boiling the slides in 0.01 M citrate buffer. Nuclei were stained with a 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Microscopic images were taken with a Leica DM5500 microscope (Leica Inc., Germany) and confocal microscopy was performed on a Zeiss LSM 7 MP (Zeiss, Germany). Fluorescent images were processed and analysed using ImageJ (http://rsbweb.nih.gov/ij) and Zen Lite software (Zeiss, Germany).

Flow cytometry

Dissociated tissue was fixed with 4% formalin for 15 minutes at room temperature and permeabilised with 0.1% saponin for 30 minutes. Cells were washed with 1% BSA/0.1% saponin and filtered with 40 μm filters (BD Bioscience). Single cells were incubated with 1:100 mouse anti KRT19 (DakoCytomation) and subsequently with 1:1000 goat anti mouse Alexa Fluor 488 (Invitrogen).

Flow cytometry was performed on a BD FACSCalibur (BD Bioscience). Flow cytometry data was analysed with FlowJo X (Tree Star).

Statistical analysis

Data are represented as means ± SEM. Statistical significance was determined using a one-tailed Student's t-test or two-way ANOVA followed by a Bonferroni multiple comparison test where appropriate. Differences were considered to be statistically significant when $P \leq 0.05$. All statistical analyses was performed with GraphPad Prism 6.0 (GraphPad Software Inc).

Results

Monolayer expansion of human islet-depleted tissue yields KRT19-positive ductal cells

Crude islet-depleted pancreatic tissue was cultured overnight. Prior to cell dissociation the crude islet-depleted tissue contained 20-30% of KRT19-positive cells (keratin 19, a marker for ductal cells) (**Supplemental Figure 2A, Supplemental Figure 2B**)[39]. The tissue was then dispersed to single cells and plated on tissue culture-treated plastic. The day after plating, cells attached to the plastic surface to a confluency of approximately 10%, whereas non-adherent cells and other dead cells were discarded with medium change. Cells cultured as monolayer expanded rapidly reached confluency after 5-6 days and showed a cobblestone morphology characteristic of ductal cells (**Figure 1A, Supplemental Figure 3**). Immunostainings showed that post-expansion 90% of the cells were KRT19-positive, a marker for ductal cells, and no cells were positive for the beta cell marker C-peptide despite the low amount of C-peptide-positive and synaptophysin-positive cells (2.9%) immediately after cell dispersion before expansion (**Figure 1B, Supplemental Figure 2A, Supplemental Figure 2B**). No acinar cells were found by amylase immunostainings (data not shown). Also, gene expression of endocrine and acinar markers decreased markedly after expansion in contrast to the enrichment for KRT19 (**Figure 1C-F**).

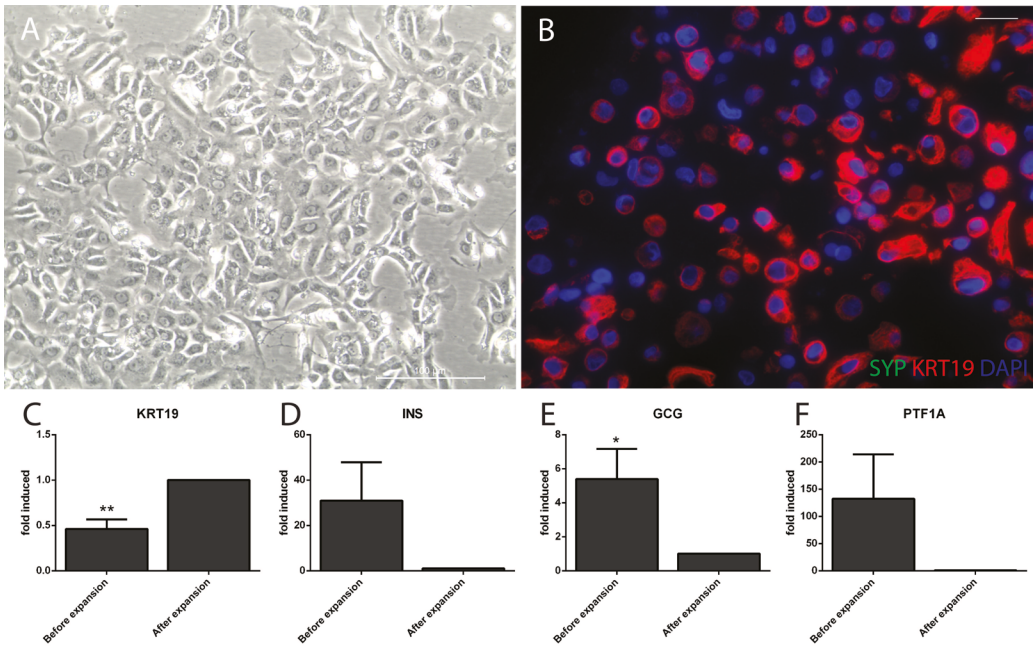

Figure 1. Adherent culture of human islet-depleted tissue leads to enrichment of KRT19-positive cells

(**a**) Islet-depleted tissue cultured for 5 days in monolayer showing cobblestone morphology typical for ductal cells. Scale bar = 100 µm. (**b**) Immunofluorescent staining of the cell population after expansion for keratin 19 (KRT19; ductal marker, red) and synaptophysin (SYP; endocrine marker, green) demonstrating most cells were KRT19-positive and no SYP-positive cells were observed. Scale bar = 20 µm. (**c-f**) Gene expression of islet-depleted tissue before and after expansion. After expansion there is enrichment for KRT19 (duct marker) and a decrease in INS and GCG (insulin and glucagon; endocrine hormones) and a decrease in PTF1A (acinar marker). Values are fold induction (after expansion=1). (n=3 donors) (*$P \leq 0.05$ vs after expansion, **$P \leq 0.01$ vs after expansion).

Differentiation with the multistage protocol yields cells with a more endocrine phenotype

Expanded cells could be efficiently detached from the plastic surface. The next step was to trigger differentiation of these cells towards an endocrine phenotype using a selection of compounds associated with islet neogenesis in the multistage protocol. In a first set of preliminary experiments, the single compounds GLP-1R agonist and INGAP were added to the medium and compared to medium in which both compounds were added for one week. These experiments indicated a higher induction of endocrine marker gene expression when compounds were combined (**Supplemental Figure 4**) (n=3 donors); therefore a complete protocol for multiple weeks was designed. With this protocol cells autonomously formed aggregates within 2-3 days (**Figure 2A**) (n=9 donors). Larger and more aggregates were found in the multistage protocol compared to the control protocol.

Immunostaining for Ki67 showed the presence of proliferating cells in the first two weeks when cells were treated with the multistage protocol (**Figure 2B-2D**), whereas proliferating cells were only incidentally found in the control protocol (**Supplemental Figure 5**). Upon *ex vivo* differentiation, gene expression of the duct marker KRT19 decreased (**Figure 3I**). PTF1A gene expression (a marker of early progenitor cells and restricted to acinar cells in the adult pancreas) and NGN3 (endocrine progenitor marker) initially increased during the first week, and decreased the second and third week of differentiation in the multistage protocol (**Figure 3D-E**). Pancreatic progenitor markers SOX9, FOXA2, HNF6 were upregulated throughout the three weeks of differentiation (**Figure 3A-C**). Gene expression of the beta cell markers PDX1, NKX6.1, MAFA were all upregulated in the multistage protocol (**Figure 3F-H**), as well as genes for the endocrine hormones insulin and glucagon that were induced up to 15- to 75-fold in the multistage protocol after three weeks (**Figure 3J-K**). No major differences were observed at the transcriptional level between the multistage protocol and the control protocol (**Figure 3A-K**). Differentiation was also examined at the protein level. In both protocols, approximately 6% of the cells were highly positive for the beta cell marker PDX1 (PDX1^{high}) (**Figure 4A-E**). Interestingly, 60% of these PDX1^{high} cells also expressed the beta cell marker NKX6.1 in the multistage protocol; in contrast to only 20% in control aggregates (**Figure 4G**). Only sporadic insulin-positive cells were found with the multistage protocol at this stage. Cells that were differentiated towards an endocrine cell fate lost their KRT19 expression. Overall, these findings suggest that the multistage protocol is able to trigger the differentiation of human islet-depleted tissue towards an endocrine phenotype.

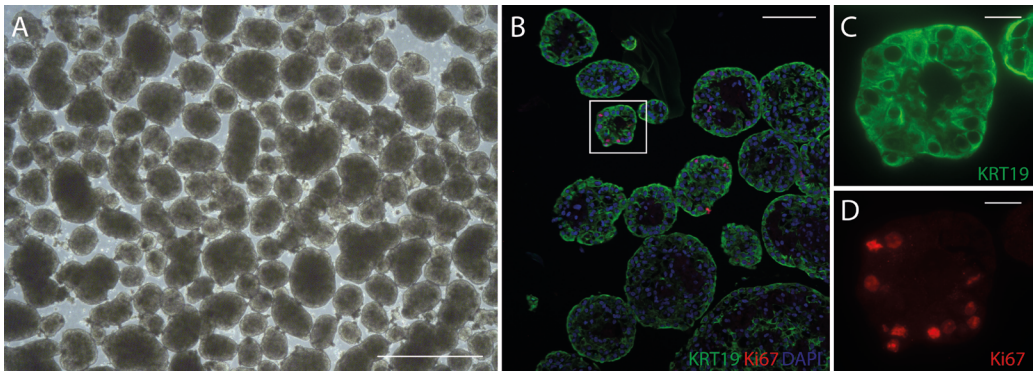

Figure 2. Cells cultured in suspension with the multistage protocol yield aggregates with proliferating cells

(**a**) Brightfield image of aggregates at 1 week of differentiation with the multistage protocol demonstrating heterogeneous aggregates. Scale bar = 100 µm. (**b**) Immunofluorescent staining of aggregates differentiated for 2 weeks with the multistage protocol for: KRT19 (ductal marker, green), Ki67 (proliferation marker, red) and DAPI (nuclear marker, blue), demonstrating proliferating ductal cells. Scale bar = 100 µm. (**c-d**) Magnification of B, showing single channel KRT19-positive cells and Ki67-positive cells. Scale bar = 20 µm. (n=3 donors).

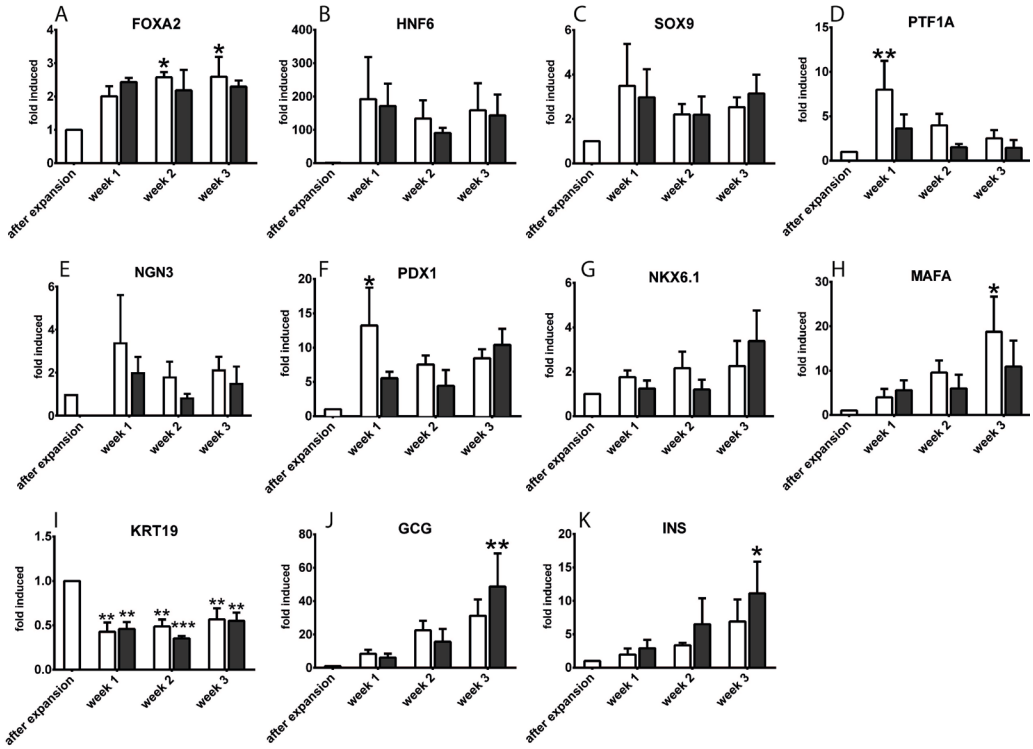

Figure 3. Gene expression during differentiation using the multistage protocol
(**a-c**) Increase in the SOX9, FOXA2 and HNF6 (pancreatic progenitor markers) during differentiation. (**d-e**) Initial increase and then decrease of PTF1A (progenitor marker in progenitor cells, restricted to acinar cells in adult cells) and NGN3 (endocrine progenitor marker). (**f-h**) Increase in the beta cell transcription factors PDX1, NKX6.1, MAFA. (**i-k**) Decrease in KRT19 (ductal marker) and increase in GCG and INS (endocrine hormones). Control aggregates depicted as white bars, multistage protocol aggregates as black bars. Values are fold induction (after expansion=1). (N=3 donors). (**P* ≤ 0.05 vs after expansion, ***P* ≤ 0.01 vs after expansion, ****P* ≤ 0.001 vs after expansion).

In vivo differentiation of *ex vivo* pre-differentiated cells

Prior studies have shown that maturation of progenitor cells can occur after transplantation *in vivo*[2,38,40]. In order to further differentiate the cells from the multistage differentiation protocol towards a beta cell phenotype, pre-differentiated aggregates were transplanted under the kidney capsule of NOD/SCID mice for a month. Immunostainings of the grafts showed that the transplanted aggregates had formed large ductal structures (**Figure 5A**). Up to 5% of the graft consisted of PDX1^{high}NKX6.1^{+}Insulin^{+} cells, most differentiated cells were found in the duct lining (**Figure 5A**). Interestingly, PDX1^{high}NKX6.1^{+}Insulin^{+} cells were also found in close proximity but separate from the duct structure, suggesting that delamination had occurred (**Figure 5B**). Serum was harvested at 1 and 4 weeks post-transplantation for a C-peptide ELISA (Mercodia). The amount of C-peptide in the serum was undetectable for the detection level of the assay (data not shown)

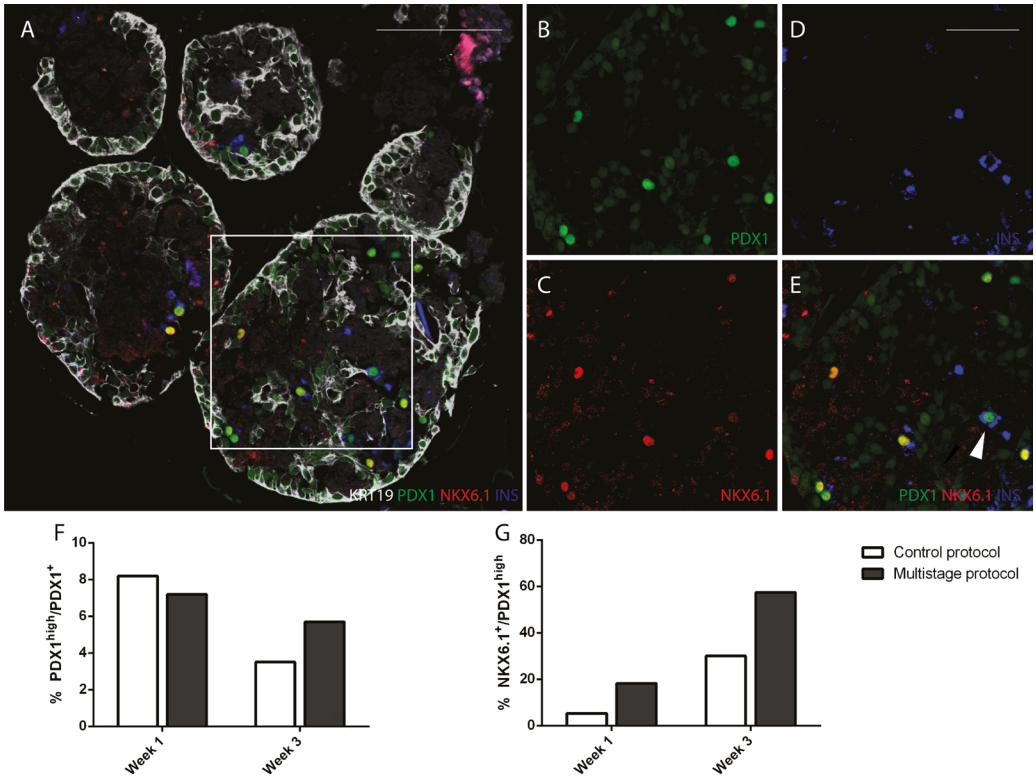

Figure 4. Aggregates differentiated for three weeks with the multistage protocol yield more pancreatic progenitor cells

(**a**) Immunofluorescent staining of aggregates after three weeks of differentiation for KRT19 (ductal marker, white), NKX6.1 (beta cell marker, red), PDX1 (beta cell marker, green) and insulin (beta cell hormone, blue). Scale bar = 100 µm. (**b-e**) Magnification of figure A showing single channel fluorescent images of PDX1^{high}, NKX6.1^{+} and Insulin^{+} cells. Arrowhead in E indicates a PDX1^{high}NKX6.1^{+}Insulin^{+}. Scale bar = 50 µm. (**f-g**) Quantification of PDX1^{high}-positive cells and PDX1^{high}/NKX6.1^{+} cells, indicating more PDX1^{high}NKX6.1^{+} cells in the multistage protocol (800 cells quantified in control aggregates, 1000 cells in multiprotocol aggregates). (n=1 donor)

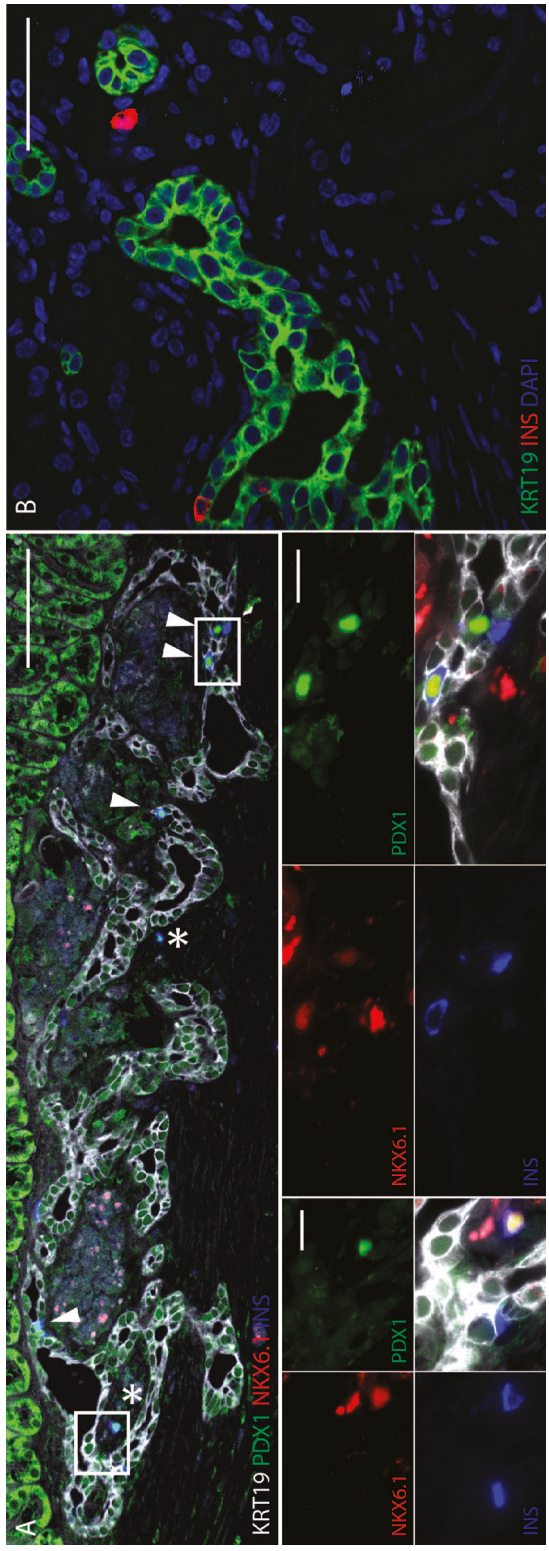
A
KRT19 PDX1 NKX6.1 INS
NKX6.1
PDX1
NKX6.1
PDX1
INS
INS
B
KRT19 INS DAPI

Figure 5. After one month of *in vivo* maturation cells pre-differentiated towards pancreatic progenitor cells become PDX^{high}NKX6.1^{+}insulin^{+} cells
(**a**) Immunofluorescent staining of graft retrieved after one-week maturation under the kidney capsule for KRT19 (ductal marker, white), NKX6.1 (beta cell marker, red), PDX1 (beta cell marker, green) and insulin (beta cell hormone, blue). Triple positive cells are inside and outside the duct lining. Arrowheads in A indicate PDX1^{high}NKX6.1^{+}Insulin^{+} cells in the duct structure and the asterisk indicate delaminating PDX1^{high}NKX6.1^{+}Insulin^{+} cells. Scale bar = 100 µm. Magnification shows single channel fluorescent images. Scale bar = 20 µm. (**b**) graft retrieved after one month ***in vivo*** maturation showing a delaminating insulin-positive cell. Scale bar = 50 µm (n=1 donor transplanted in 5 mice).

Discussion

Islet transplantation is a promising treatment for patients with T1DM, but a lack of donor tissue is one of the major hurdles for widespread use. Therefore, there is an urgent need for an alternative source of beta cells. There have been very few studies published on beta cell neogenesis and primary human exocrine tissue. The differentiation medium we used as control in this study is derived from a protocol published in 2007[38]. A more recent study showed improved differentiation of human duct cells to beta-like cells, but upon genetic manipulation (overexpression of transcription factors)[41]. The goal of our study was to improve the current differentiation protocols using a non-genetic approach by using beta cell neogenesis agents that have been demonstrated to stimulate endocrine differentiation from ductal cells in rodent models of diabetes, ductal cell lines, and human embryonic stem cells. Here we developed a multistage protocol that allows the generation of pancreatic endocrine progenitor cells (PDX1^{high} NKX6.1^{+}) from human islet-depleted tissue using a non-genetic approach using beta cell neogenesis agents that have been demonstrated to stimulate endocrine differentiation in rodent models of diabetes, ductal cell lines, and human embryonic stem cells. Upon maturation *in vivo*, differentiated beta cells were found outside duct structures suggesting a delamination process.

The co-expression of PDX1 and NKX6.1 is a crucial stage in the formation of beta cells. During embryonic development pancreatic endocrine progenitor cells co-express PDX1 and NKX6.1, a combination of transcription factors that becomes restricted to beta cells in the adult pancreas. In the hESC field it has been demonstrated that only cells co-expressing PDX1 and NKX6.1 (pancreatic progenitor cells) are capable of differentiating into mature beta cells[2,40]. Human adult duct cells normally do not co-express these transcription factors. However, after three weeks of *ex vivo* pre-differentiation we find an increase in cells co-expressing both transcription factors, indicating that our protocol with beta cell neogenesis agents can trigger the differentiation of human adult primary exocrine tissue towards pancreatic progenitor cells *ex vivo.* Moreover, pancreatic progenitor markers such as SOX9, HNF1B, HNF6 were upregulated. PTF1A, a progenitor marker restricted to acinar cells in the adult pancreas and NGN3, an endocrine progenitor marker, were transiently upregulated before endocrine differentiation, suggesting a dedifferentiation process prior to differentiation. By transplanting these progenitor cells *in vivo* we confirm that these progenitor cells can differentiate further into insulin-positive cells.

Additionally, we observed insulin-positive cells outside the duct lining after one month of *in vivo* maturation, indicating that these cells have delaminated. During development endocrine cells are formed within the progenitor ductal epithelium, and subsequently delaminate from the epithelium and migrate to the surrounding mesenchyme where they cluster together and form the islets. In prior studies with pre-differentiated crude duct aggregates and *in vivo* maturation under the kidney capsule newly formed beta cells were only found in the ductal lining[38]. Even though clusters of insulin-positive cells indicating early islet formation were not observed yet, the fact that single insulin-positive cells were delaminated from the ductal epithelium indicates that the novel multistage protocol triggers a higher degree of endocrine maturation of the cells.

The number of endocrine cells that are formed is currently too limited for sufficient clinical impact, although it cannot be excluded that *in vivo* differentiation for a longer time period than a month would increase this frequency. Up to 5% of the cells were found insulin positive after *in vivo* maturation. These insulin-positive cells maintained PDX1^{high}NKX6.1^{+}, indicative of maturity. The origin of the differentiated cells with an endocrine phenotype in our samples is unclear, as our starting population is crude exocrine tissue. The pancreatic duct compartment is a heterogeneous compartment, consisting of smaller and larger sized ducts[42]. Evidence points towards different intrinsic properties of the cells lining these bigger and smaller ducts, and it is hypothesized that the progenitor capacity is only restricted to a small subpopulation of ductal cells[43,44]. Efficient selection and expansion using cell surface markers of a possible progenitor population might result in a higher yield of pancreatic progenitor cells and eventually more beta cells. Due to lack of current knowledge on which cell subpopulation would be the most prone to differentiate to beta cells and the required cell surface markers to isolate for these populations, we chose to start expanding cells from an unsorted cell population (*i.e.*, islet-depleted tissue) in contrast to other studies[41]. The expansion method we use has been used by other groups to selectively expand ductal cells[45,46]. After expansion, 90% of the cells we harvest are KRT19-positive in our study. However, by using this crude and unbiased approach we cannot exclude the possibility of a contaminating cell population (*e.g.*, acinar cells) to contribute to the endocrine cell formation.

Moreover, our current basic understanding of the precise spatiotemporal cues that play a role during embryonic development is insufficient to develop a complete protocol for efficient, proper beta cell specification from adult human ductal cells. The exact pathways activated by GLP-1R agonist and INGAP are not completely elucidated. Prior studies indicate that activation of the GLP-1R in hESC leads to differentiation by activation of the hedgehog, cyclic adenosine monophosphate (cAMP) and phosphatidylinositol-3-kinase (PI3K) signaling pathways[47,48]. Also , INGAP has shown to generate endocrine cells from KRT19^{+} cells by activation of the PI3K pathway[49,50]. A downstream activator of PI3K signaling pathway is Akt1, which is a regulator of neurogenin3 (NGN3, key transcription factor for endocrine differentiation) expression, indicating that this pathway is involved in endocrine formation[51]. This is supported by other studies demonstrating that the Akt1 pathway is necessary for beta cell growth and differentiation *in vitro* and *in vivo*[52-54]. Further studies on islet biology and discovery of additional signaling pathways involved in beta cell specification are

needed to optimise the selection of compounds to further increase the efficiency of differentiation of adult human ductal cells to beta cells.

Finally, promising breakthroughs are made in the hESC field, where new protocols are developed for formation of islets from pluripotent cells. These newly formed islets are getting close to fully functional beta cells, and are capable of physiological insulin secretion in response to glucose[55]. However, there is still a risk of undifferentiated pluripotent cells forming a teratoma when transplanted *in vivo*. Interestingly the pluripotent cells in this culture undergo an intermediate stage in which they acquire a ductal phenotype, expressing KRT19, followed by a stage in which they express other pancreatic progenitor markers such as PDX1 and NKX6.1[2,36], as in our study. Building upon these protocols should allow the further development of differentiation protocols for primary human adult ductal cells, a cell type already committed to a pancreatic cell fate, thus likely making the differentiation process shorter and with a lower risk of tumour formation when transplanted *in vivo* due to limited pluripotency.

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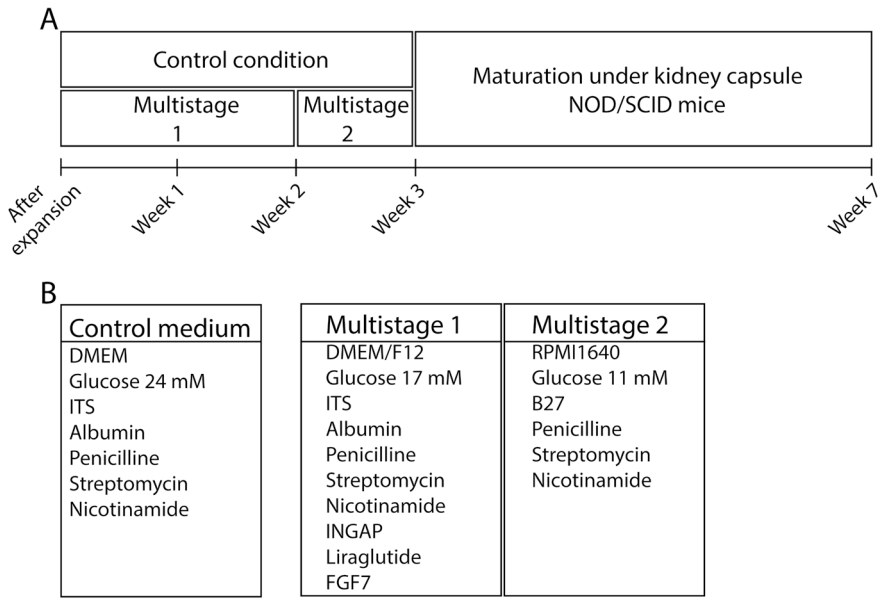
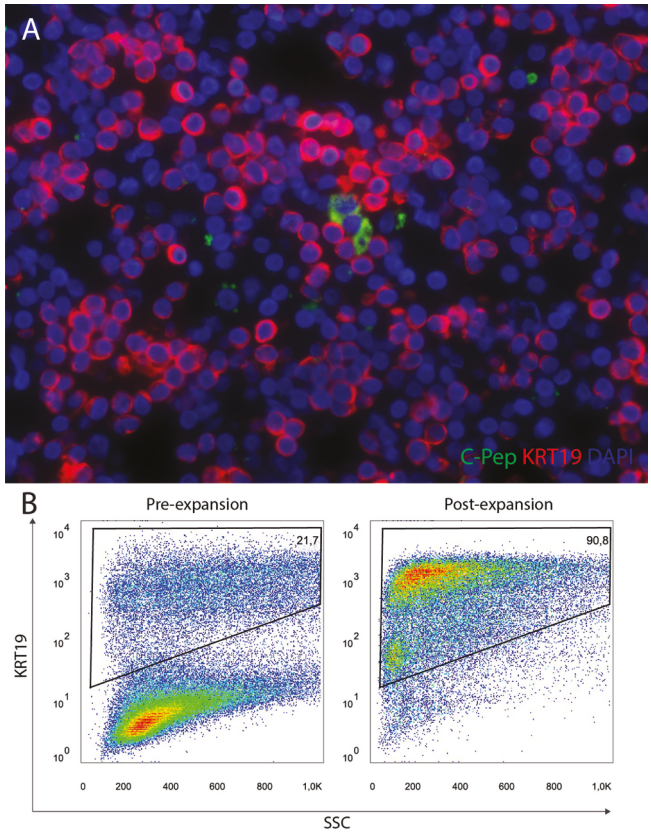
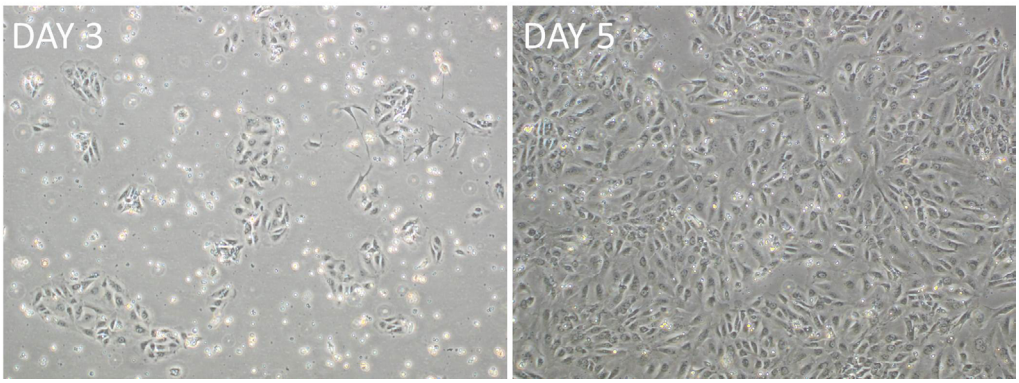
Supplemental information



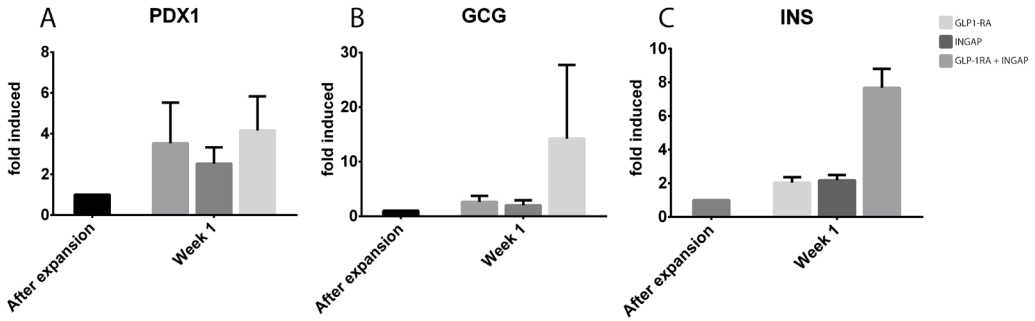
Supplemental Figure 1. Experimental setup
(**a**) Timeline of *ex vivo* differentiation and *in vivo* maturation. (**b**) Medium composition during different stages.



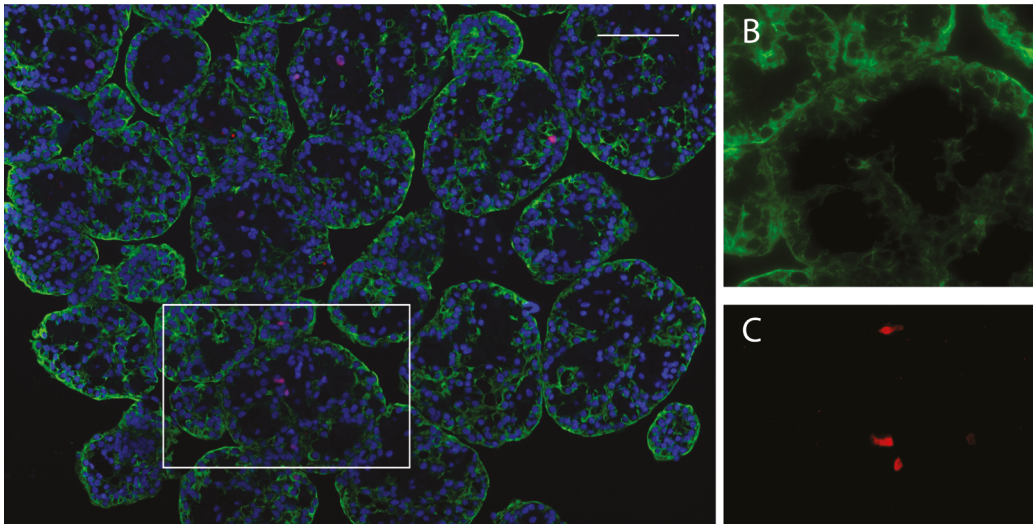
Supplemental Figure 2. Quantification of KRT19+ cells pre-expansion and post-expansion
(**a**) Immunofluorescent staining of the cell population before expansion for keratin-19 (KRT19; ductal marker, red) and synaptophysin (SYP; endocrine marker, green) demonstrating most cells were KRT19 positive and incidentally SYP positive cells were observed. Scale bar = 20 µm. (**b**) Flow cytometry showing 20% KRT19+ cells pre-expansion and 90% KRT19+ cells post expansion. SSC: Side Scatter.



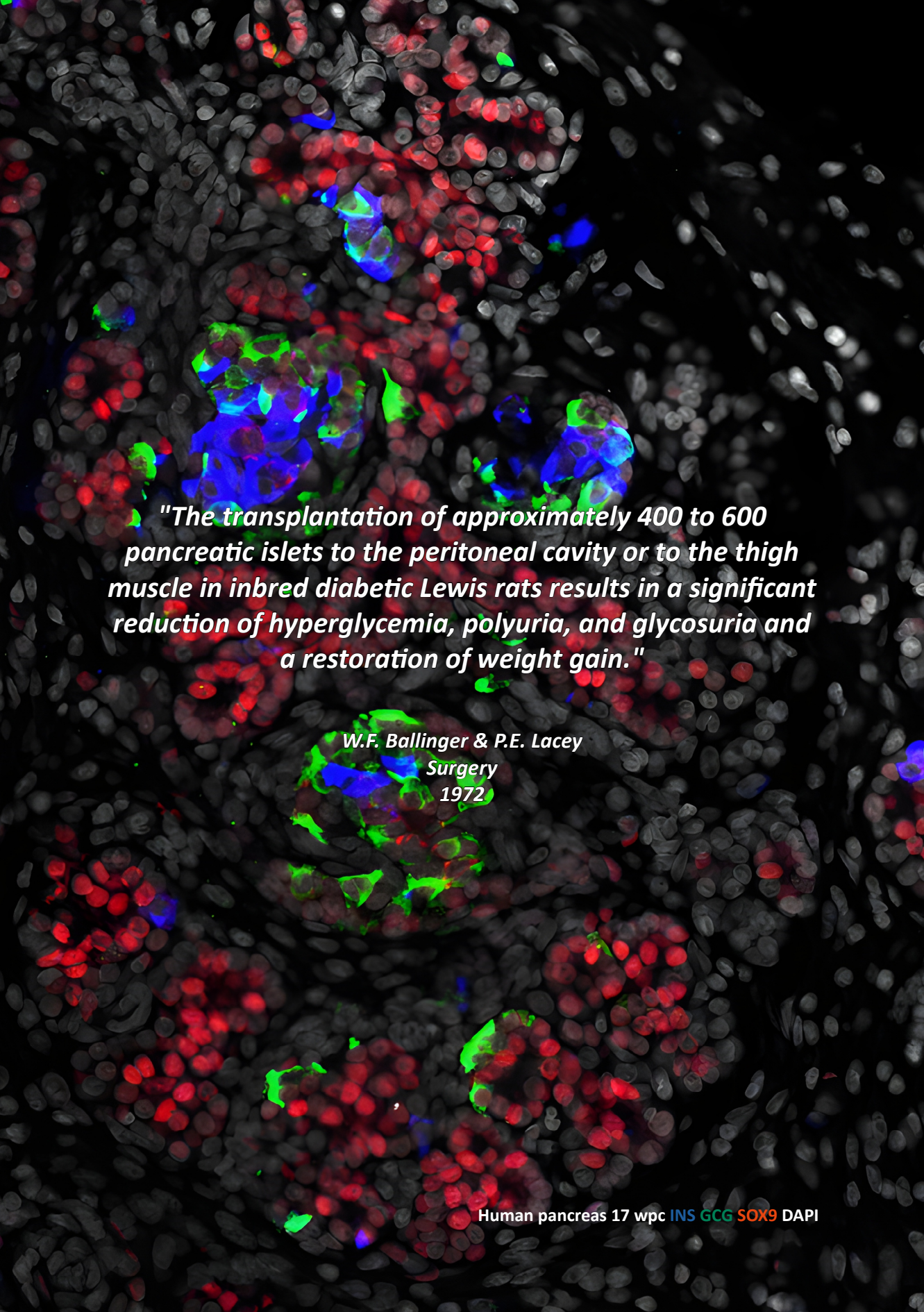
Supplemental Figure 3. In monolayer culture cells selectively attach to the plastic surface and rapidly expand
Brightfield image of a representative monolayer culture at 3 and 5 days after plating.



Supplemental Figure 4. Upregulation of endocrine transcription factors compared between single compounds and combined compounds after one week.
(**a-c**) Combining the compounds in the multistage medium induced a higher upregulation of endocrine gene expression. Values are fold induction (after expansion=1). (N=3 donors).



Supplemental Figure 5. Cells cultured in suspension with the control protocol yield proliferating cells
(**a**) Immunofluorescent staining of aggregates differentiated for 2 weeks with the control protocol for: KRT19 (ductal marker, green), Ki67 (proliferation marker, red) and DAPI (nuclear marker, blue), demonstrating a proliferating ductal cell. Scale bar = 100 µm. (**b-d**) Magnification of B, showing single channel KRT19 positive cells and Ki67 positive cells. Scale bar = 20 µm.


"The transplantation of approximately 400 to 600 pancreatic islets to the peritoneal cavity or to the thigh muscle in inbred diabetic Lewis rats results in a significant reduction of hyperglycemia, polyuria, and glycosuria and a restoration of weight gain."
W.F. Ballinger & P.E. Lacey
Surgery
1972
Human pancreas 17 wpc INS GCG SOX9 DAPI

CHAPTER| 7

Cytoplasmic SOX9 Expression in Human Pancreas Development

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In preparation

Abstract

Deciphering the transcriptional regulation of human pancreatic islet development will facilitate the generation of novel regenerative strategies for the treatment of diabetes mellitus. Recently, a proliferative subset of adult human alpha cells was identified displaying cytoplasmic expression of SOX9 (SOX9^{cyto}), a transcription factor typically localized to the nucleus of pancreatic progenitor cells during development and to exocrine duct cells in the adult pancreas. Nucleocytoplasmic shuttling of SOX9 is an essential mechanism by which SOX9 regulates cell fate, but its potential implication in human pancreas development is unknown. We performed histological assessment of first and second trimester human pancreatic samples. We confirmed the expression of SOX9^{cyto} restricted to a subset of alpha cells in the adult human pancreas. In contrast, we identified a subpopulation of SOX9^{cyto} cells among fetal beta cells located both in the ductal epithelium and in endocrine cell clusters, and present throughout the development. These findings suggest that nucleocytoplasmic shuttling of SOX9 might play a role in islet cell specification during human pancreas development. This knowledge may ultimately contribute to further optimisation of strategies for beta cell replacement therapy from alternative cell sources.

Introduction

An incomplete understanding of the transcriptional regulation of human islet development hampers the formation of fully functional beta cells from pluripotent stem cells[1]. Recent investigations initially focused on finding the origin of residual beta cells in T1D individuals, led to the identification of a highly proliferative islet cell subset in perinatal, adolescent, and young adult pancreata[2]. Islet cell proliferation was comparable in healthy and T1D individuals, suggesting a normal physiological process involved in islet cell plasticity rather than a regenerative response to beta cell deficiency. The proliferating islet cells expressed alpha cell markers (GCG, ARX) together with SOX9, that was unexpectedly expressed in the cytoplasm.

The nuclear expression of SOX9 in pancreatic progenitor cells is required for maintenance of pancreatic progenitor cells, but also for endocrine cell formation[3]. Individuals affected by the lethal SOX9 haploinsufficiency syndrome campomelic hypoplasia show endocrine hypoplasia[4], and transgenic rodent models show that SOX9 haploinsufficiency results in decreased number of NEUROG3-positive cells and islet hypoplasia[5].

Sex-determining region Y (SRY)-box 9 protein (SOX9) is a member of the SOX family of transcription factors, which are developmental regulators defined by the presence of a highly conserved high-mobility group (HMG) domain that mediates DNA binding[6]. These unique binding properties, in addition to other complex regulatory mechanisms at the level of transcription, translation, post-translation modifications, and cofactor interaction, enables highly dynamic, flexible, and accurately controlled expression of SOX9, resulting in stage-specific functions in a multitude of tissues and cells[6]. The access of SOX proteins to the nucleus via nucleocytoplasmic shuttling is known to be a key driver of developmental switches and programmed cell differentiation, and is

regulated by posttranslational modifications such as acetylation, phosphorylation, sumoylation, and ubiquitination[7,8].

Although the requirement of nuclear SOX9 for normal pancreas organogenesis and endocrine development has been well established, the presence of SOX9^{cyto} in human pancreas development has not been described. Here we report the identification of a subset of human fetal beta cells displaying the cytoplasmic expression of SOX9, which - given the versatile function of SOX9 - might play an important process by which endocrine cell specification is regulated in the pancreas.

Materials and methods

Human fetal and adult pancreas collection

The collection and use of fetal material were carried out following ethical approval from the Medical Ethics committee in the LUMC. Human fetal tissue ranging from 7 to 20 wpc (weeks post conception) (n=14) was collected with informed consent after termination of pregnancy performed by vacuum aspiration in an elective abortion clinic. Human adult pancreatic tissue was derived from the clinical islet transplantation program at the Leiden University Medical Center and was only used when research consent was present.

Immunohistochemistry and microscopy

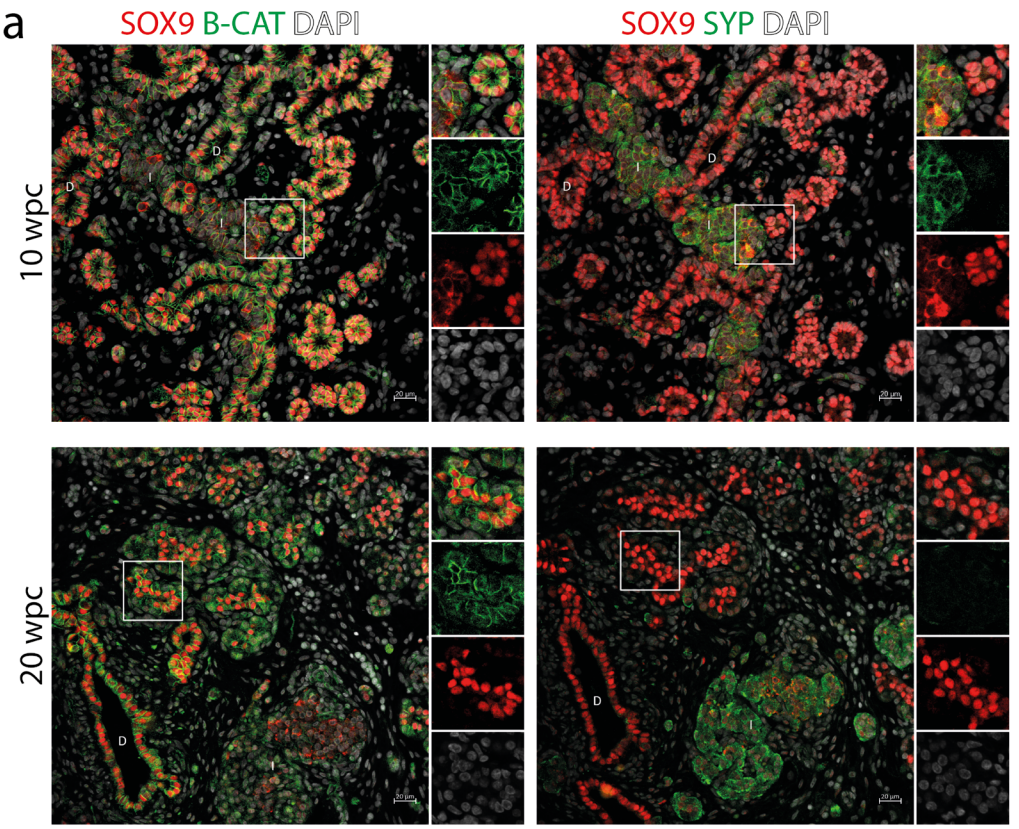
Tissue was processed and sectioned as previously described[9]. Heat-induced epitope retrieval was performed with a pressure cooker using a citrate buffer (0.01 M citric acid, pH 6.0). After blocking with normal donkey serum, slides were incubated with the primary antibodies for one hour at room temperature or overnight at 4 °C, for immunofluorescent detection slides were incubated for one hour with an appropriate secondary antibody (**Supplemental Table 1**). ARX was visualized using a Tyramide Signal Amplification set according to manufacturer's protocol (Perkin Elmer). Nuclei were counterstained with DAPI (Vector laboratories). Images were recorded using an LSM 7 MP confocal microscope (Zeiss) and were processed using the Zen Lite software (Zeiss). Quantification was performed by point counting positive cells by hand using ImageJ (National Institute of Health). A minimum of 1500 cells were quantified per fetal donor (average of 4300 cells/donor).

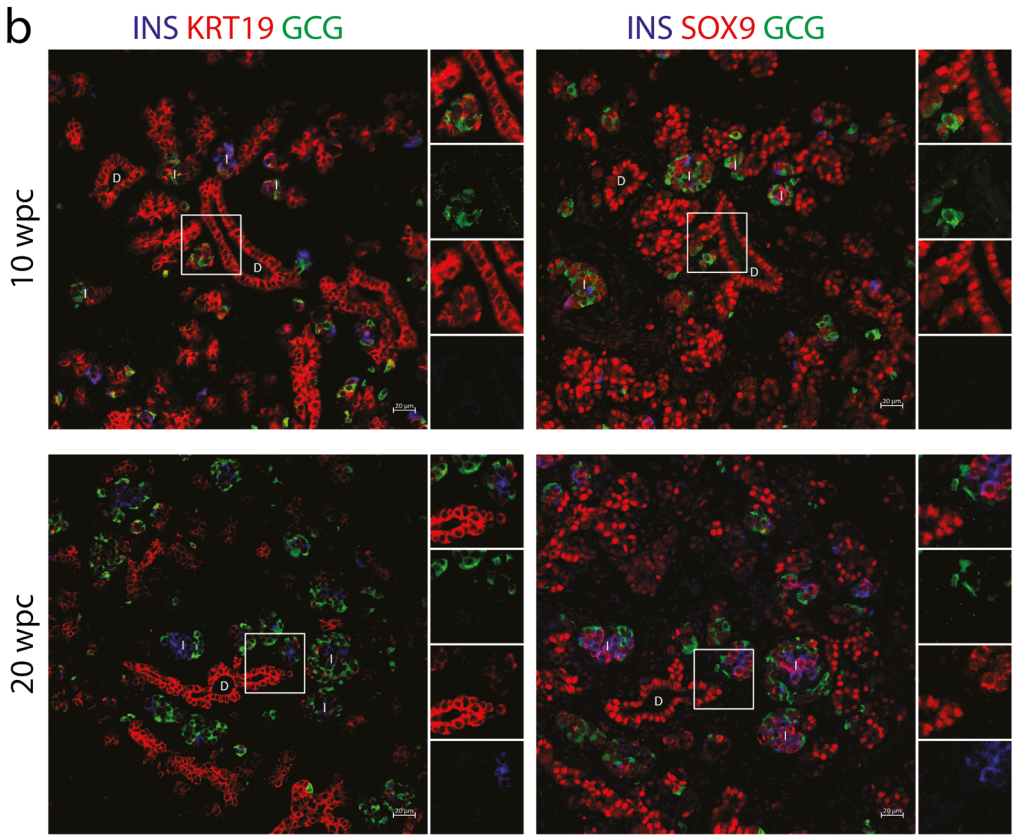
Results

SOX9^{cyto} is expressed in the human pancreas during development

Differentiated structures such as ducts and endocrine cell clusters could be identified in first and second trimester samples using β-catenin (epithelial marker), keratin 19 (duct marker), SOX9 (pancreatic progenitor and duct marker) and synaptophysin (pan-endocrine marker) (**Figure 1a-b**). Faint KRT19 expression was observed in endocrine cell clusters in early fetal samples and decreased with advancing gestational age, as previously described[10,11]. Nuclear SOX9 was abundantly co-expressed with PDX1 in the duct epithelium throughout development (**Figure 1c**)[4]. In order

to examine SOX9 subcellular expression, specifically in the fetal endocrine compartment, we performed co-immunostainings for SOX9 and synaptophysin (**Figure 1a**) or insulin and glucagon (**Figure 1d**). Strikingly, SOX9^{cyto}-positive cells were found in and around the endocrine cell clusters, showing that SOX9^{cyto} is expressed in a subset of endocrine cells in human pancreatic fetal tissue.

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INS KRT19 GCG
INS SOX9 GCG
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D
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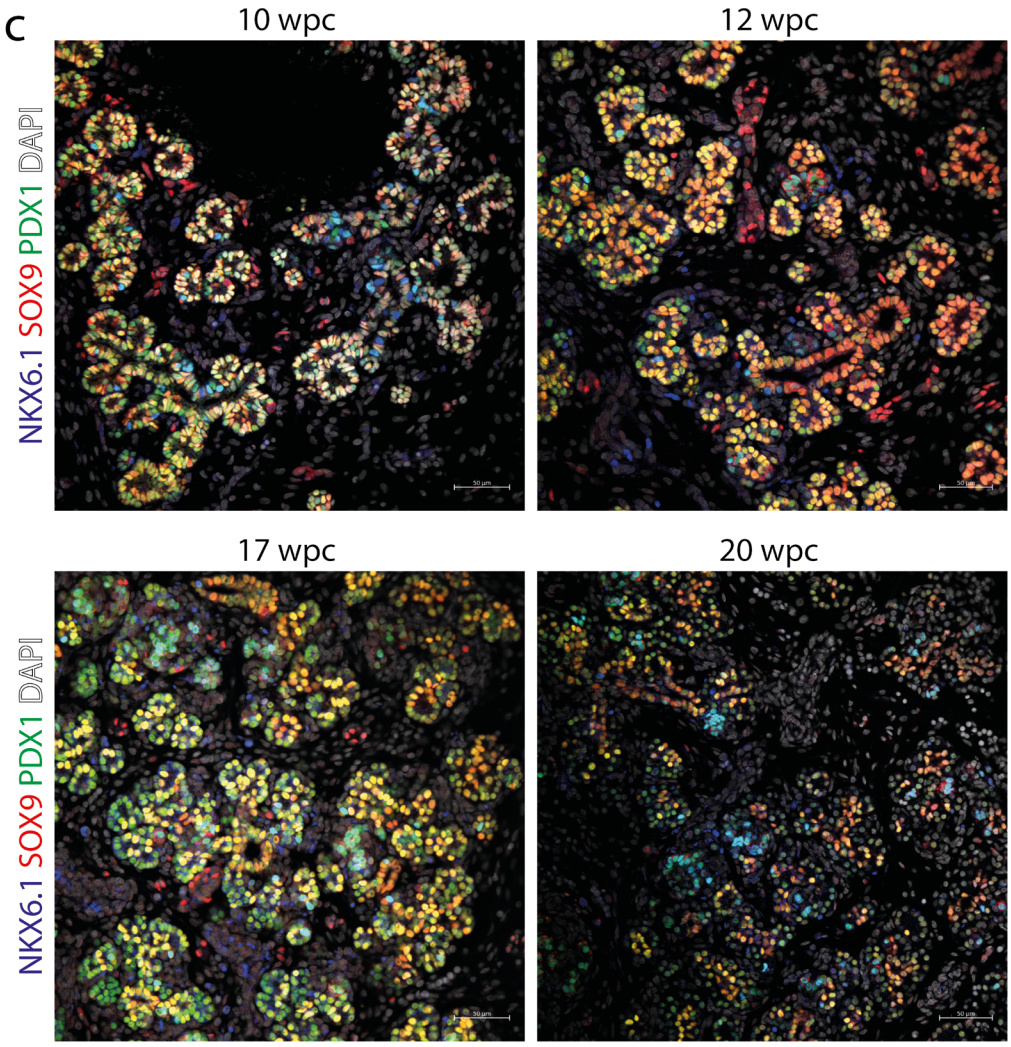
C
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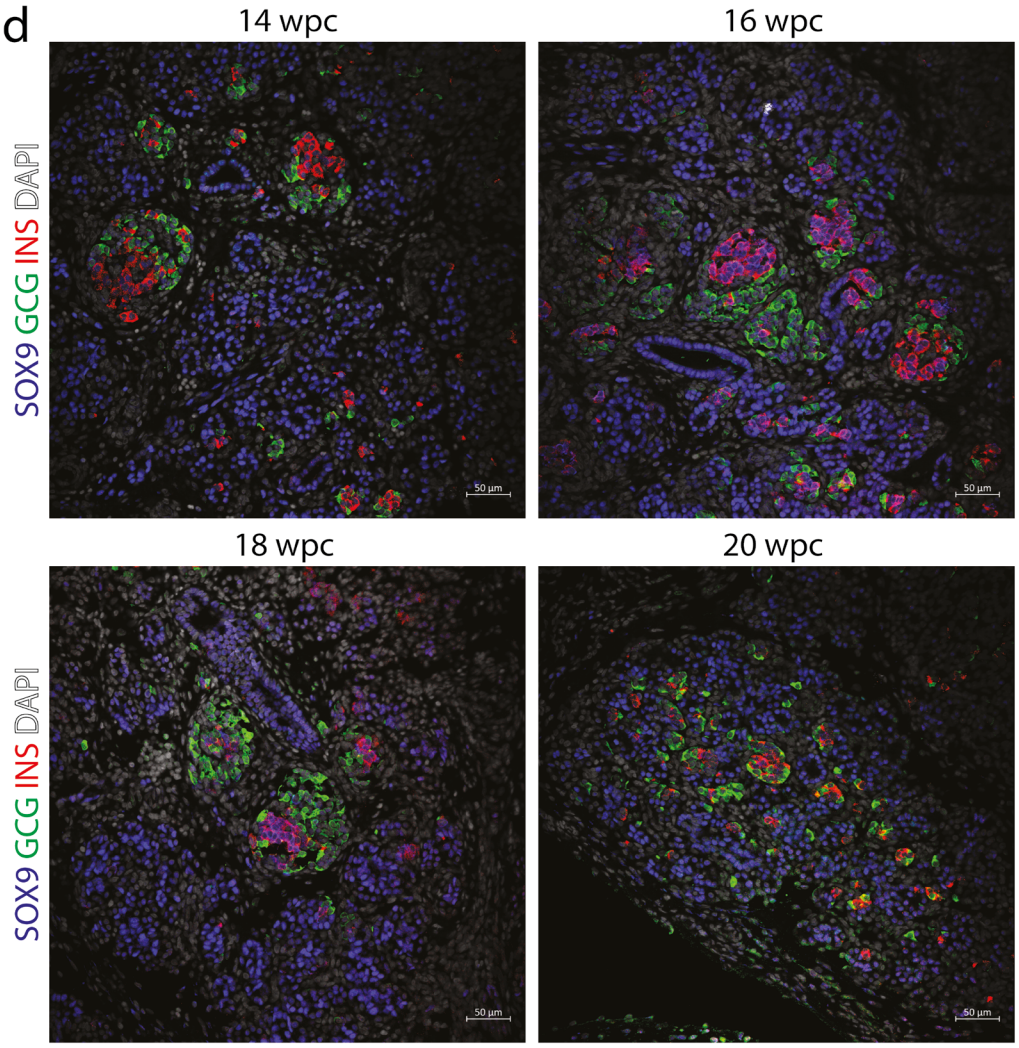
12 wpc
17 wpc
20 wpc
NKX6.1 SOX9 PDX1 DAPI
NKX6.1 SOX9 PDX1 DAPI


d
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18 wpc
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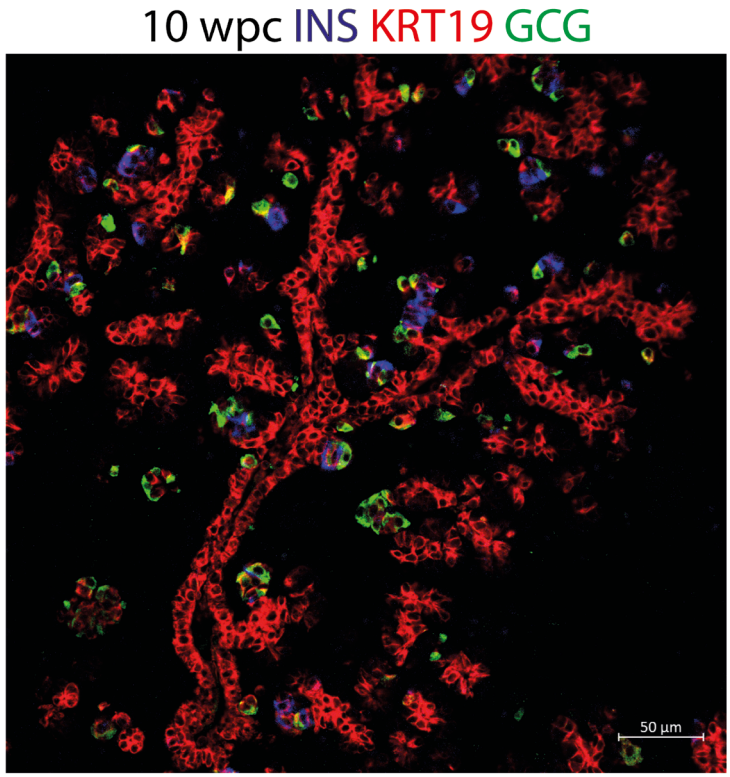
SOX9 GCG INS DAPI
SOX9 GCG INS DAPI
50 µm
50 µm
50 µm
50 µm

e



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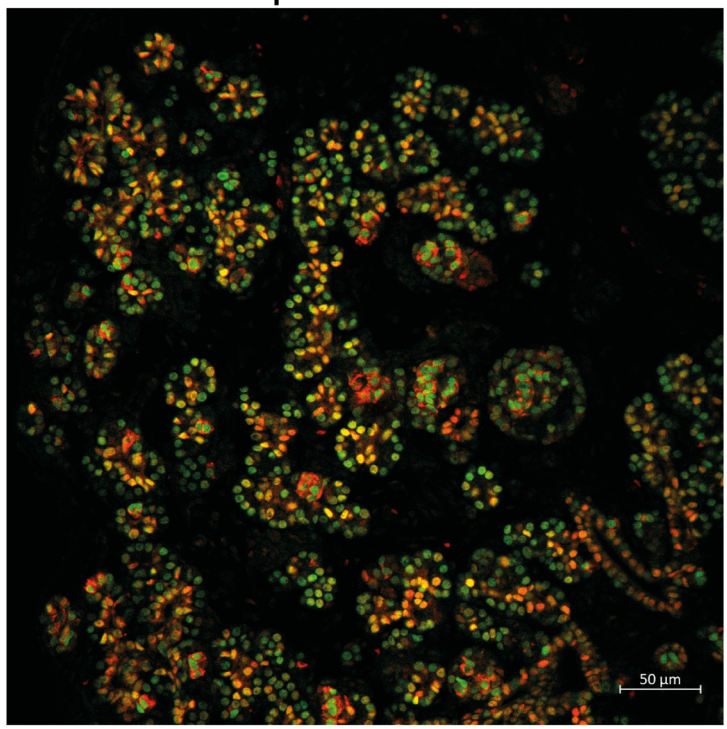


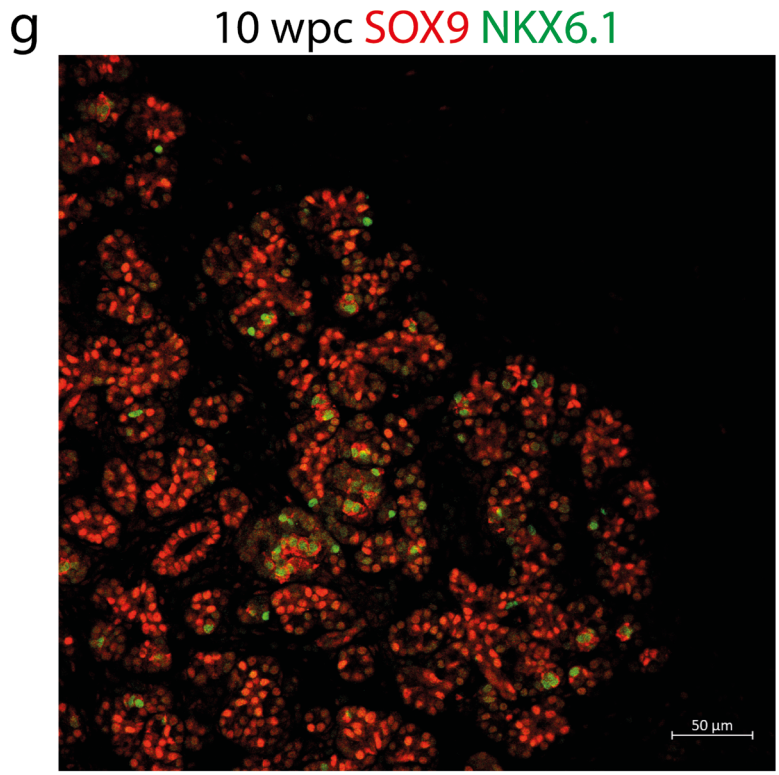


Figure 1. Identification of differentiated pancreatic structures during human development
(**a**) Representative sections of human fetal tissue of 10 wpc and 20 wpc with immunofluorescent staining for β-CAT (β-catenin, epithelial marker, green), SOX9 (ductal marker, red); and SYP (synaptophysin, endocrine cell marker, green), SOX9 (red), showing bright expression of β-catenin in ductal cells. The fetal endocrine cell clusters have a faint expression of β-catenin and also express synaptophysin. D=duct, I= islet. Scale bar = 20 µm. (**b**) Immunofluorescent staining for GCG (glucagon, alpha cell marker, green), KRT19 (keratin 19, ductal marker, red), INS (insulin, beta cell marker) showing expression of KRT19 in the pancreatic duct and a faint staining in the endocrine cell clusters. D=duct, I= islet. Scale bar = 20 µm. (**c**) Sections of human fetal tissue of 10 wpc, 12 wpc, 17 wpc and 20 wpc with immunofluorescent staining for NKX6.1 (beta cells marker, blue), SOX9 (ductal marker, red), PDX1 (beta cell marker, green) showing expression of NKX6.1 /PDX1-double-positive cells in the ductal epithelium and fetal islets throughout gestation. Scale bar = 50 µm. (**d**) Sections of human fetal tissue of 14 wpc, 16 wpc, 18 wpc and 20 wpc with immunofluorescent staining for SOX9 (duct marker, blue), GCG (glucagon, alpha cell marker, green), INS (insulin, beta cell marker, red) showing fetal endocrine cells in islets, also expressing cytoplasmic SOX9. Scale bar = 50 µm. (**e-g**) Human fetal tissue of 10 wpc with immunofluorescent staining for INS (insulin, beta cell marker, blue), KRT19 (keratin 19, duct cell marker, red), GCG (glucagon, alpha cell marker, green); SOX9 (duct marker, red), PDX1 (beta cell marker, green); and SOX9 (duct cell marker, red) and NKX6.1 (beta cell marker, green), showing the branching ductal epithelium and developing islets. Scale bar = 50 µm.

Proliferating fetal pancreatic cells rarely express SOX9^{cyto}

We next assessed whether cytoplasmic expression of SOX9 was related to proliferation, as reported

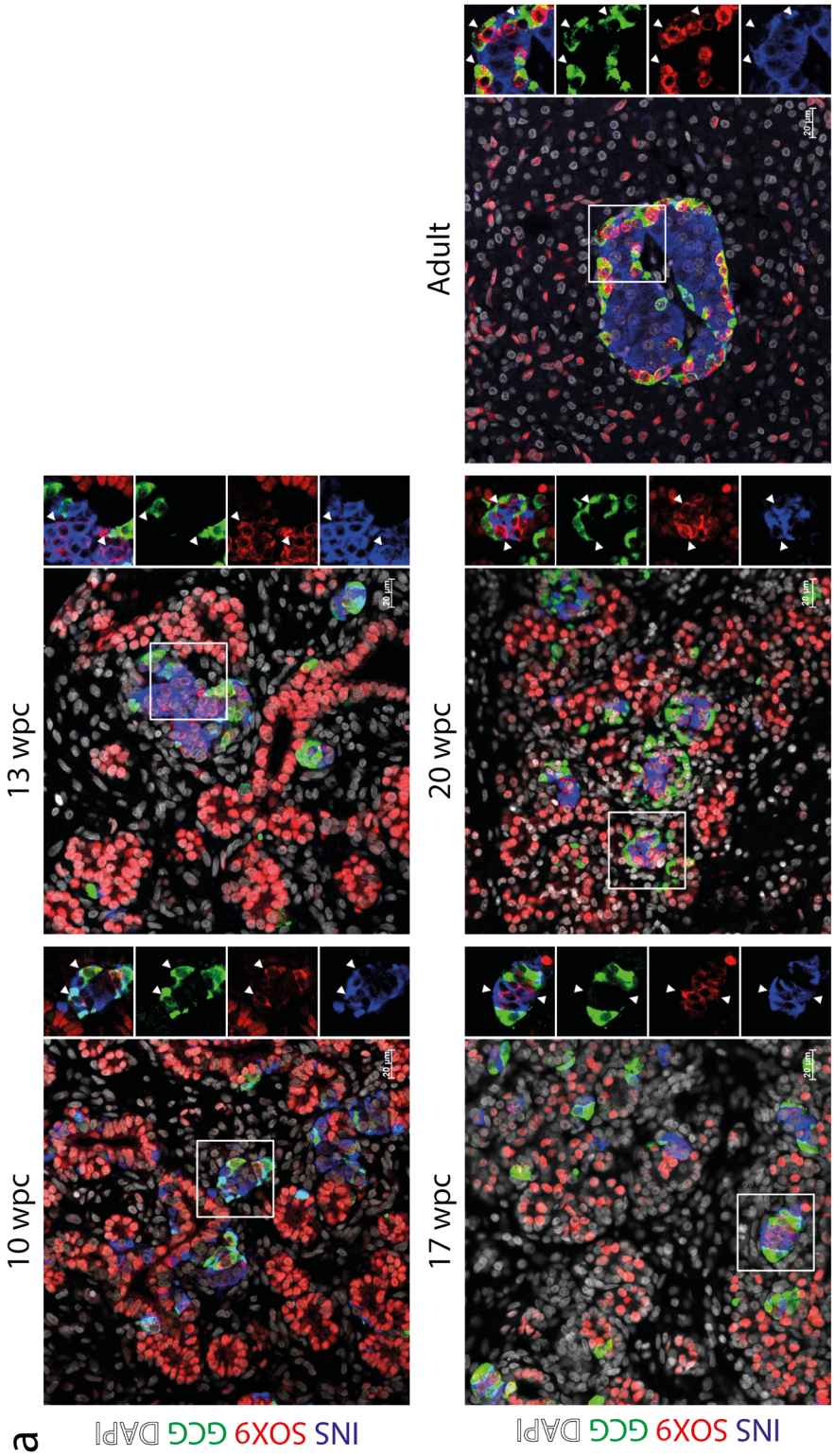
for the subset of islet cells identified in the early postnatal and adult pancreas[2]. We performed co-immunostainings for Ki67 and SOX9 in samples of a 17 wpc pancreas, and found that the majority of proliferating cells were in the mesenchyme and pancreatic ducts, while only sporadic SOX9^{cyto} cells expressed Ki67 **(Supplemental Figure 1)**. This indicates that SOX9^{cyto}-positive cells do not represent a proliferative cell population during pancreatic development.

SOX9^{cyto} is expressed in endocrine cells during human pancreas development

We next examined which endocrine cell type predominantly expressed SOX9^{cyto}, focusing on alpha and beta cells, as these contribute most to the endocrine compartment during human pancreatic development and in the postnatal pancreas (**Figure 2a**)[10,12,13]. The fraction of SOX9^{cyto}-positive cells relative to the total number of SOX9-positive cells (SOX9^{nuc} and SOX9^{cyto}) increased with gestational age (**Figure 2b**), reaching up to 13% in the second trimester samples. We found SOX9^{cyto} to be expressed mainly in insulin-positive cells: at 10 wpc, 25% of SOX9^{cyto}-positive cells expressed insulin and the fraction of cells co-expressing insulin and glucagon was 29% (**Figure 2c**), while only 4% of SOX9^{cyto}-positive cells expressed glucagon only. Polyhormonal cells have been described both in immature endocrine cells during early human pancreatic development and in pluripotent stem cell-derived endocrine cells differentiated *in vitro*[14-18]. The proportion of polyhormonal SOX9^{cyto}-positive cells declined during development, decreasing to 11% between 13 and 18 wpc, and further dropping to 2% by 20 wpc. This reduction was accompanied by a concomitant increase in SOX9^{cyto}-expression in insulin-only-expressing cells, with 65% of SOX9^{cyto}-positive cells co-expressing insulin at 14 wpc. Throughout development, we observed a fraction of SOX9^{cyto}-positive cells negative for both insulin and glucagon, which ranged from 41% at 13 wpc, decreased to 15% at 14 wpc, and then increased up to 39% at later gestational ages.

We next evaluated the distribution of SOX9-positive cells in the insulin-positive cell population, and found an average of 21% of insulin-positive cells expressed SOX9^{cyto} throughout pancreas development (**Figure 2d**). In contrast, only 8% of glucagon-positive cells were SOX9-positive, excluding the 10 wpc sample, which contained a high fraction of insulin/glucagon-double-positive cells (**Figure 2a**). From these SOX9-positive insulin or glucagon-positive cells, the large majority of the cells expressed SOX9 in the cytoplasm. Yet, in the early gestational age samples we also observed some hormone-positive cells with SOX9 expression in the nuclear, possibly representing cells in transition from duct to endocrine cells (**Figure 2d-e**).

Altogether, our data demonstrate that the cytoplasmic expression of SOX9 is present in endocrine cells during fetal development and the majority of these cells are polyhormonal-positive for insulin and glucagon at early gestational age, and monohormonal insulin-positive at a later gestational age. This is in contrast with postnatal pancreatic cells, where cytoplasmic SOX9 expression was observed exclusively in a subset of alpha-like cells (**Figure 2a 'Adult'**)[2].


a
INS SOX9 GCG DAPI
INS SOX9 GCG DAPI
10 wpc
13 wpc
17 wpc
20 wpc
Adult
20 µm

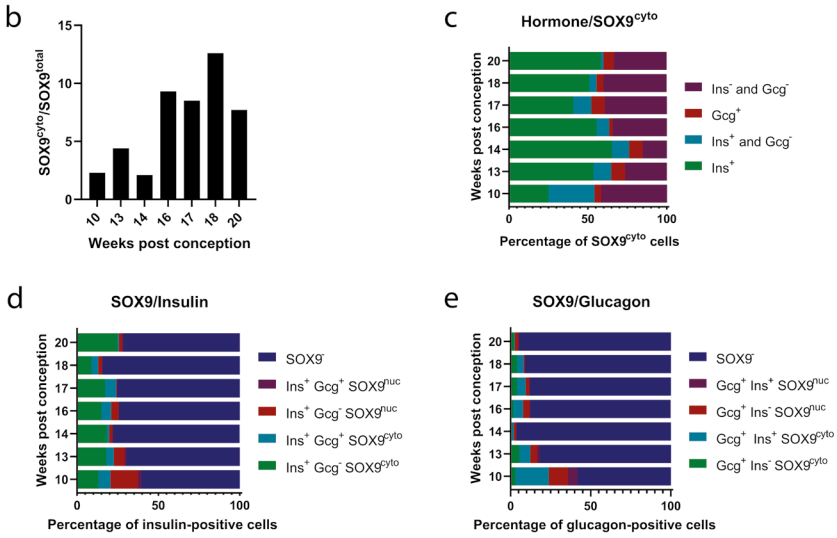


Figure 2. The cytoplasmic expression of SOX9 in hormone-positive cells during human pancreas development

(**a**) Immunofluorescent staining of human fetal pancreatic tissue for INS (insulin, beta cell marker, blue), SOX9 (ductal marker, red), GCG (glucagon; alpha cell marker, green), DAPI (nuclear marker, white), showing the cytoplasmic expression of INS/GCG-double-positive cells at 10 wpc and subsequently INS-positive cells at 13, 17 and 20 wpc. In human adult pancreatic tissue cytoplasmic expression of SOX9 is restricted to GCG-positive cells. Arrowheads point to $SOX9^{cyto}$-positive cells. Scale bar = 20 µm. (**b**) Quantification of immunofluorescent stainings for INS/GCG/SOX9 showing the fraction $SOX9^{cyto}$ of $SOX9^{total}$ ($SOX9^{nuc}$ and $SOX9^{cyto}$) cells in human fetal pancreatic tissue, showing an increase in the fraction $SOX9^{cyto}/SOX9^{total}$ in second trimester samples. (**c**) Quantification of immunofluorescent stainings for INS/GCG/SOX9 showing $SOX9^{cyto}$ subpopulations, $SOX9^{cyto}$ cells mainly colocalize with insulin-positive cells. In the first trimester there is a large fraction of polyhormonal cells observed, which decreased in the second trimester. (**d**) Quantification of immunofluorescent stainings for INS/GCG/SOX9 showing the fraction of $SOX9^{cyto}$ cells in the total insulin-positive cell population, the $SOX9^{cyto}$-negative cell fraction of insulin-positive cells is stable around 73% throughout development. (**e**) Quantification of immunofluorescent stainings for INS/GCG/SOX9 showing the fraction of $SOX9^{cyto}$-positive cells in total glucagon-positive cell population, the percentage of $SOX9^{cyto}$-negative cells is around 90% during development, with the exception of 12 wpc due to a high fraction of polyhormonal cells.

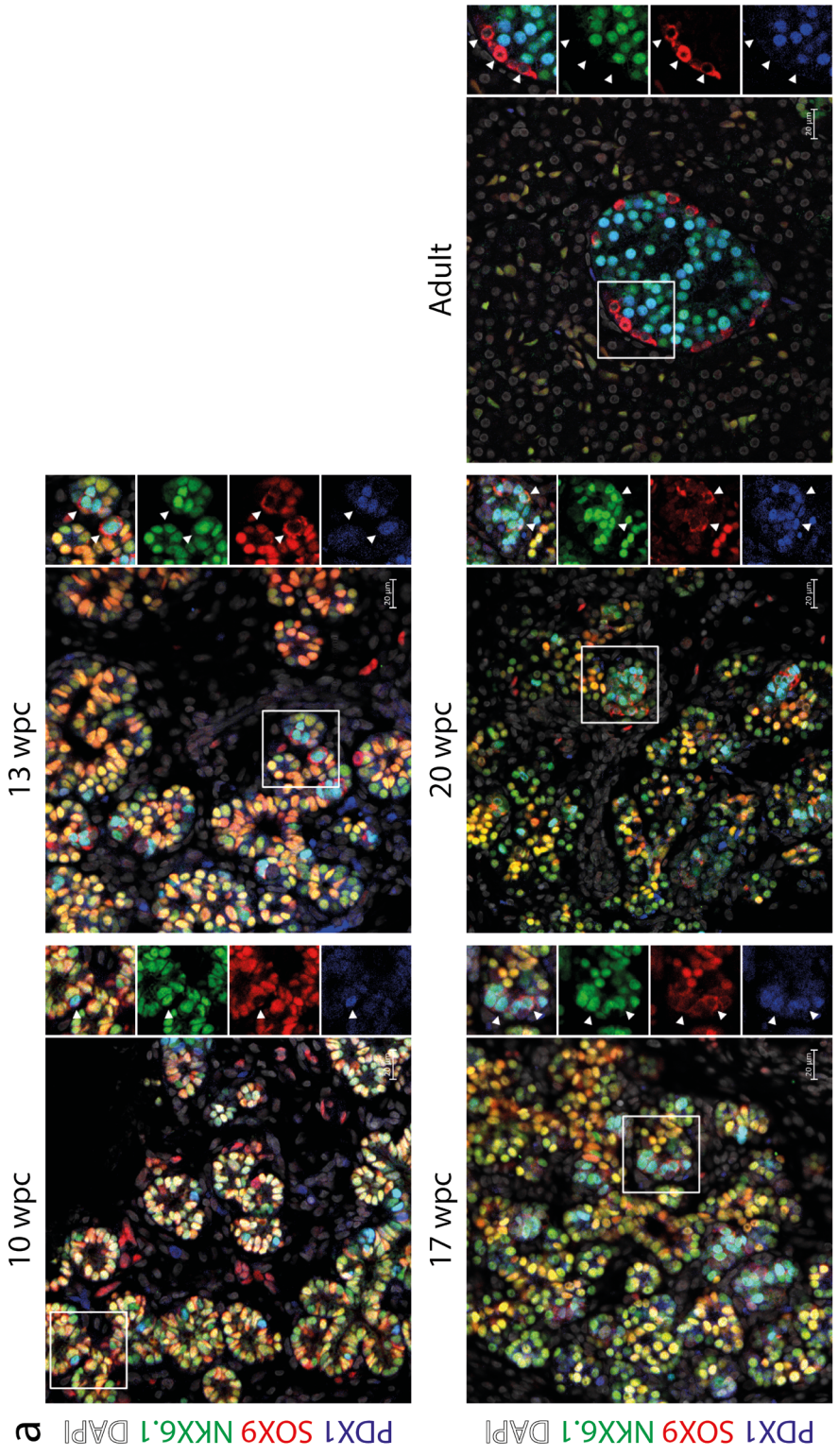
$SOX9^{cyto}$ is expressed in a subset of fetal beta cells

We next wondered whether cytoplasmic expression of SOX9 could play a role in endocrine cell specification in pancreatic progenitor cells (**Figure 3a, Figure 3c-g**). At 6 wpc, pancreatic progenitor cells start to organize in a tip-trunk segregation, after which the endocrine cells will form from SOX9/PDX1-double-positive trunk cells, whereas PDX1/CPA-double-positive tip cells will form exocrine cells[19]. The percentage of $SOX9^{nuc}$-positive cells expressing PDX1 was 60-80% (**Figure 3c**). The $SOX9^{cyto}$-positive cell population was composed for more than 50% of PDX1 and/or NKX6.1-

positive cells at all gestational ages (**Figure 3d**). At 10 wpc, 33% of $SOX9^{cyto}$ cells were PDX1-positive and NKX6.1-negative, whereas 11% of cells was double-positive for PDX1 and NKX6.1. During the later stages of development, the majority of $SOX9^{cyto}$ cells was double-positive for NKX6.1 and PDX1, reaching up to 47% cells in the later gestational ages.

The percentage of $SOX9^{cyto}$-positive cells of all PDX1-positive cells increased during gestation up to 8% at 18 wpc (**Figure 3e**). The majority of these cells was also positive for NKX6.1. For the NKX6.1-positive cell population, only 1% of NKX6.1-positive cells was $SOX9^{cyto}$-positive at 10 wpc, increasing to 50% at 18 wpc (**Figure 3f**). More than 90% of these cells co-expressed PDX1. When looking at the total amount of NKX6.1/PDX1-double-positive cells, more than half was $SOX9^{cyto}$-positive with the exception at 10 wpc (**Figure 3g**).

Because $SOX9^{cyto}$ in the postnatal pancreas is mostly expressed in a subset of alpha-like cells, we also performed co-immunostainings with the alpha cell lineage-specific transcription factor ARX. The expression of ARX was observed in cells expressing glucagon, however we rarely found ARX-cells co-expressing $SOX9^{cyto}$ **(Figure 3b, Figure 3h).** In contrast, in adult tissue we found ARX-positive cells co-expressing glucagon and $SOX9^{cyto}$, as previously reported (**Figure 3b** 'Adult')[2].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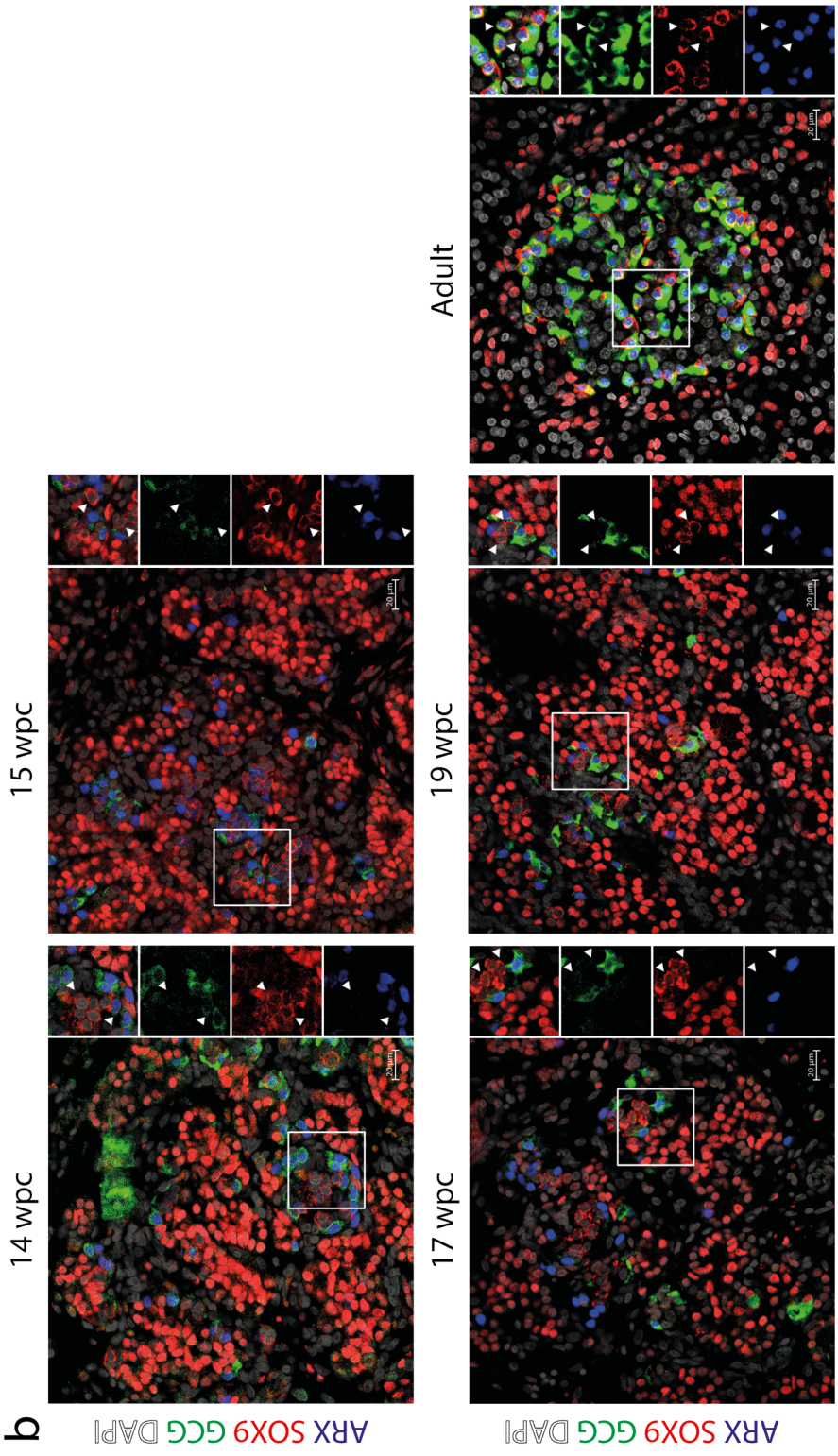
PDX1 SOX9 NKX6.1 DAPI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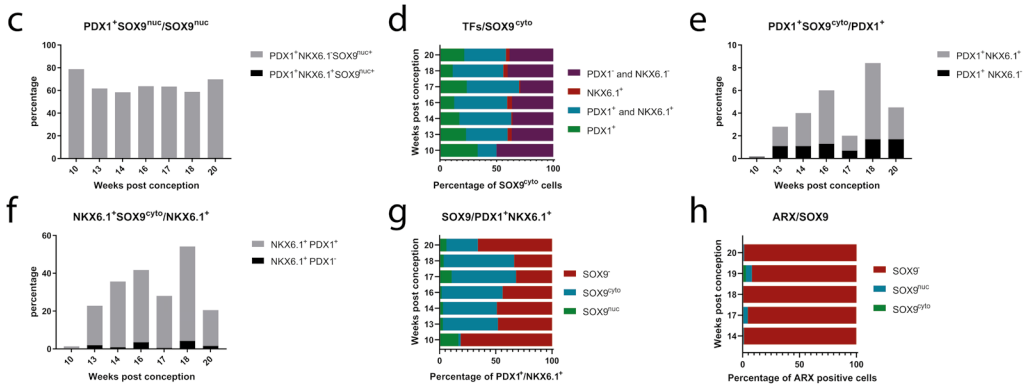


Figure 3. SOX9^{cyto} is expressed in cells expressing only beta cell markers in fetal tissue, but restricted to cells with only alpha cell markers in adult tissue

(**a**) Immunofluorescent staining of human fetal pancreatic tissue for PDX1 (beta cell marker, blue), SOX9 (ductal marker, red), NKX6.1 (beta cell marker, green), DAPI (nuclear marker, white), showing SOX9^{cyto} expression in PDX1/NKX6.1-positive cells throughout development. In human adult pancreatic islets SOX9^{cyto} expression is restricted to PDX1/NKX6.1-double negative cells. Arrowheads point to SOX9^{cyto}-positive cells. Scale bar = 20 µm. (**b**) Immunofluorescent staining of human fetal pancreatic tissue for ARX (alpha cell marker, blue), SOX9 (ductal marker, red), GCG (alpha cell marker, green), DAPI (nuclear marker, white), showing islets with SOX9^{cyto}-expression in ARX/GCG-double negative cells throughout development. In adult tissue SOX9^{cyto} is expressed in ARX/GCG-double-positive cells. Arrowheads point to SOX9^{cyto}-positive cells. Scale bar = 20 µm. (**c**) Quantification of immunofluorescent stainings for PDX1/NKX6.1/SOX9, showing that the percentage of PDX1/SOX9^{nuc}-double-positive cells of the SOX9^{nuc}-population is between 60-80% during development. The fraction of PDX1/SOX9^{nuc}-double cells also positive for NKX6.1 is <1%. (**d**) Quantification of immunofluorescent stainings for PDX1/NKX6.1/SOX9, indicating that throughout development 50-70% of SOX9^{cyto} cells is positive for PDX1 and/or NKX6.1. (**e**) Quantification of immunofluorescent stainings for PDX1/NKX6.1/SOX9, showing that the percentage of SOX9^{cyto}-positive cells in the PDX1-positive population increased during gestation, up to 8% at 18 wpc. The majority of PDX1/SOX9^{cyto}-double-positive cells also express NKX6.1. (**f**) Quantification of immunofluorescent stainings for PDX1/NKX6.1/SOX9 showing the percentage of SOX9^{cyto}-positive cells in the NKX6.1-positive population. There is a large increase in the percentage of SOX9^{cyto}-positive cells during development, at 18 wpc almost 55% of NKX6.1- positive cells expresses SOX9^{cyto}, and almost all cells also co-express PDX1. (**g**) Quantification of immunofluorescent stainings for PDX1/NKX6.1/SOX9, showing there is a large increase of SOX9^{cyto} cells in the PDX1/NKX6.1-double-positive population during development. (**h**) Quantification of immunostainings for ARX/SOX9/GCG, indicating that <1% of ARX-positive cells expressed SOX9^{cyto}.

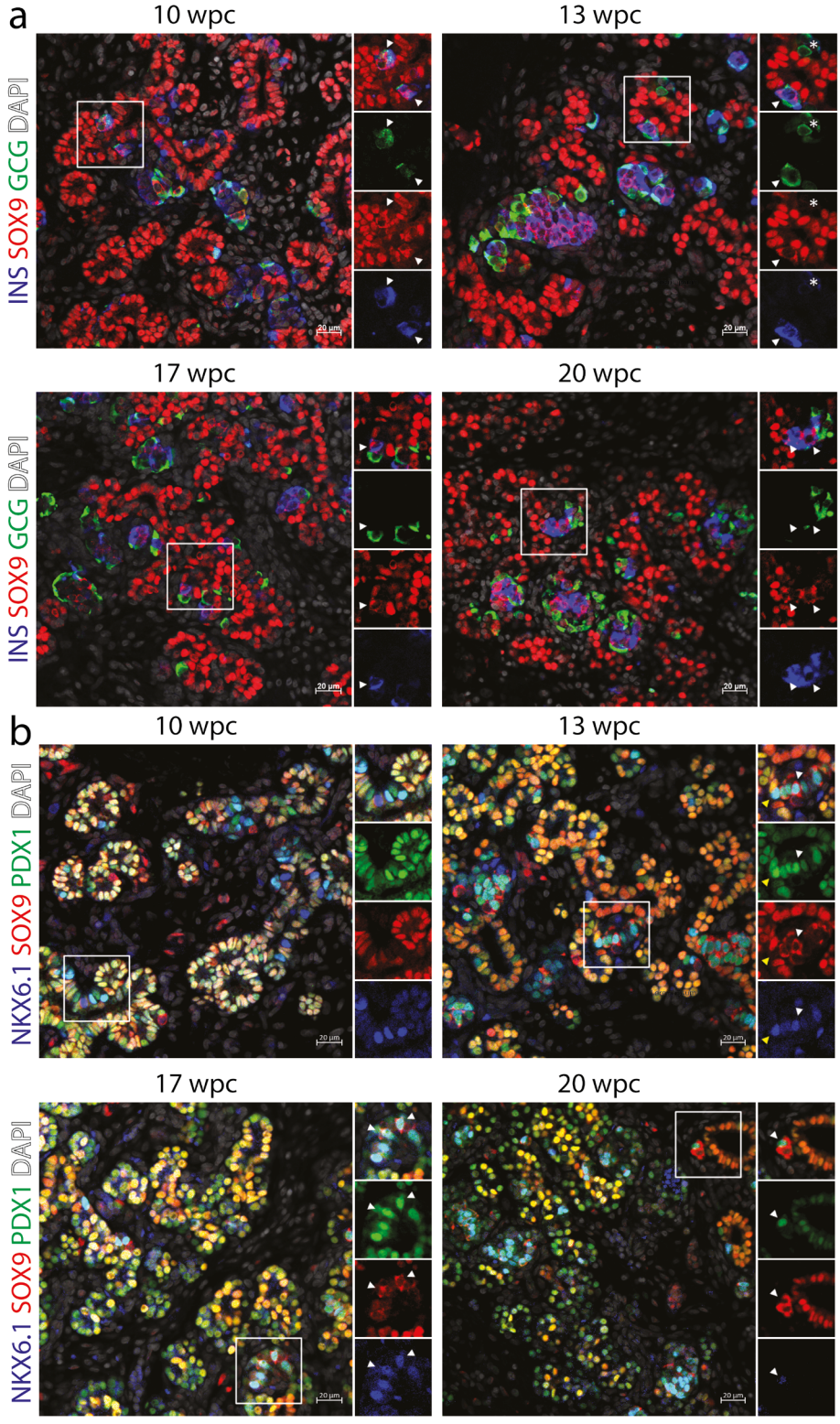
SOX9^{cyto}-positive cells are located in endocrine cell clusters as well as the ductal epithelium

To evaluate whether SOX9 is expressed in the cytoplasm of progenitor cells before differentiation, delamination, and migration into the endocrine cell clusters, we examined the location of SOX9^{cyto}-positive cells. We identified insulin/SOX9^{cyto}-double-positive cells and PDX1/NKX6.1/SOX9^{cyto}-

triple-positive cells in and around the ductal epithelium, as well as in endocrine cell clusters (**Figure 4a-b**). In addition, we observed SOX9^{cyto}-negative/insulin-positive cells, suggesting a transient SOX9^{cyto} expression and heterogeneity in the beta cell and beta cell progenitor population.

We further examined the NKX6.1-positive cell population. While this marker can be indicative for pancreatic endocrine progenitors[20], we found that almost all NKX6.1/SOX9^{cyto}-double-positive cells were also insulin-positive, indicative of a beta cell phenotype instead (**Figure 4c**). Of note, we also identified a subpopulation of PDX1/NKX6.1^{bright}-double-positive cells in the ducts that were insulin/SOX9-double-negative (**Figure 4c**). Furthermore, we observed SOX9^{cyto}/glucagon-positive cells located in and around a large duct in the adult pancreas (**Figure 4d**).

In conclusion, a subset of fetal beta cells positive for insulin, PDX1 and NKX6.1 express SOX9^{cyto}, and although the majority of cells can be found in the endocrine cell clusters there are also sporadic cells in the ductal epithelium lining. In adult tissue SOX9^{cyto} can be found in duct structures co-localizing with glucagon.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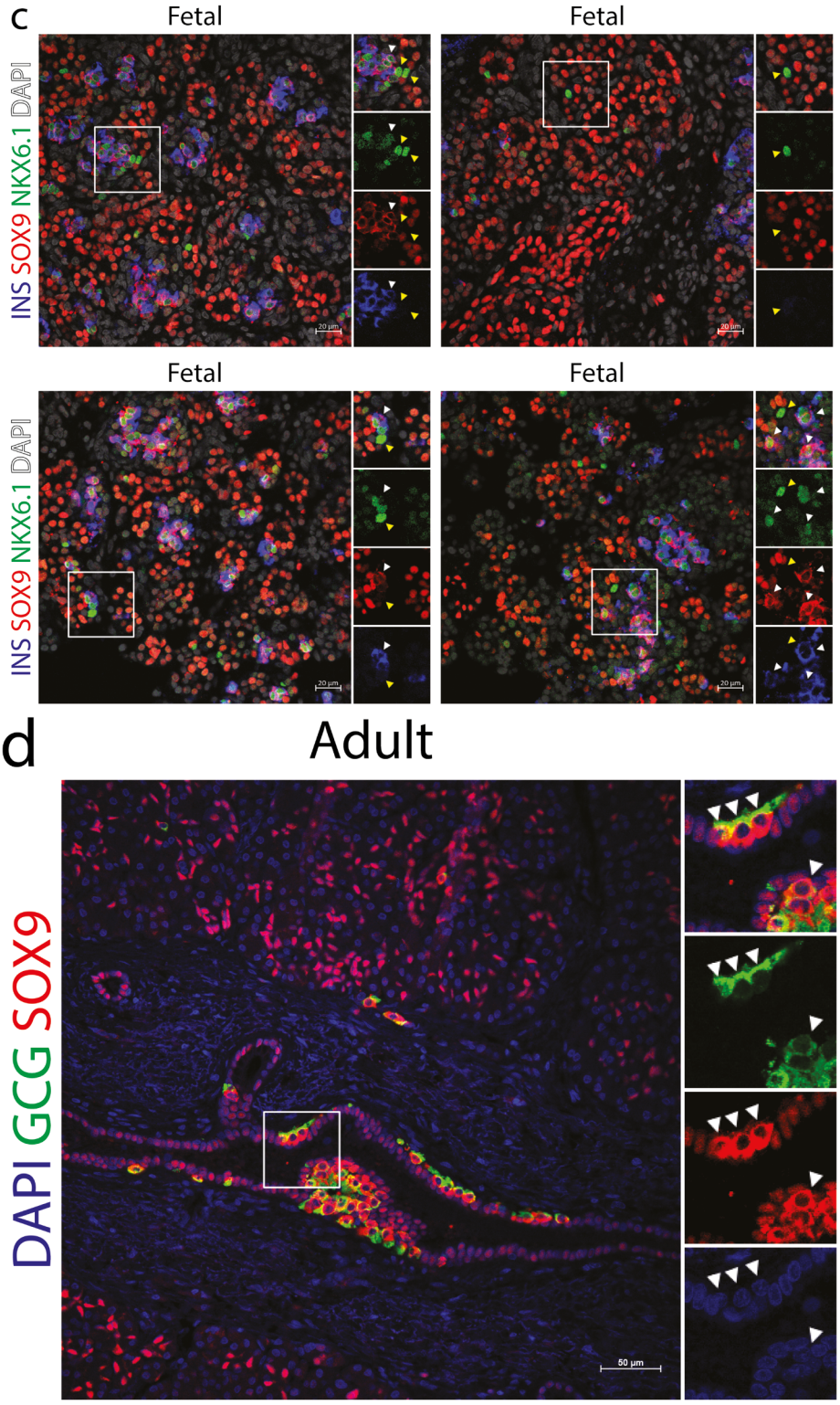
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Fetal
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Fetal
Fetal
INS SOX9 NKX6.1 DAPI
d
Adult
DAPI GCG SOX9
50 µm

Figure 4. SOX9^{cyto}-positive cells in human fetal pancreatic tissue are located in endocrine cell clusters as well as ductal structures
(**a**) Immunofluorescent staining for INS (insulin, beta cell marker, blue), SOX9 (ductal cell marker, red) and GCG (glucagon, alpha cell marker, green). White arrows point to hormone-positive cells located in or near the ductal epithelium that are also SOX9^{cyto} positive. The asterisk indicates a glucagon-positive cell that is insulin and SOX9 negative. Scale bar = 20 µm. (**b**) Immunofluorescent staining for PDX1 (beta cell marker, blue), SOX9 (ductal marker, red), and NKX6.1 (beta cell marker, green). White arrowheads point to PDX/NKX6.1-double-positive cells in the ductal epithelium that are also positive for cytoplasmic SOX9, yellow arrowheads points to PDX/NKX6.1-double-positive cells in the ductal epithelium that are SOX9^{cyto} negative. Scale bar = 20 µm. (**c**) Immunofluorescent staining for INS (insulin, beta cell marker, blue), SOX9 (ductal cell marker, red) and NKX6.1 (beta cell marker, green). White arrowheads point to insulin-positive cells with a faint NKX6.1-expression that are also SOX9^{cyto}-positive. Yellow arrowheads point to NKX6.1^{bright}-positive cells that are insulin and SOX9-negative. Scale bar = 20 µm. (**d**) Immunofluorescent staining of human pancreatic adult tissue for DAPI (nuclear marker, blue), SOX9 (ductal marker, red), GCG (alpha cell marker, green), white arrowheads showing SOX9^{cyto}-expression in cells that also express GCG. Scale bar = 20 µm.

Discussion

Deciphering the transcriptional mechanisms underlying endocrine cell formation during human pancreatic development is essential for the optimisation of beta cell regeneration strategies. Here we show that SOX9 is expressed in the cytoplasm of a subset of human fetal beta cells, which has never been reported before, and is in contrast to the SOX9^{cyto}-expression observed in a subset of adult pancreatic alpha cells[2]. Advanced insights have led to an increased understanding of human islet cell heterogeneity and plasticity, and it has been postulated that the SOX9^{cyto}-expression displayed in the adult pancreas might be indicative of a previously unknown islet cell plasticity[2]. It has been observed that under certain stimuli human endocrine cells can convert their identity[21]. Furthermore, human alpha cells display a plastic epigenomic state supporting a conversion to beta cells[22]. However, our findings based on SOX9^{cyto}-expression in fetal tissue suggest the opposite, *i.e.,* adult alpha cells might be derived from beta cells. There have been several reports showing evidence that beta cells can change identity to alpha cells, but why this would occur in postnatal pancreata of healthy subjects is unknown[23-26]. Moreover, it is unclear what could explain the discrepancy between the different hormone positive SOX9^{cyto} cells in fetal and postnatal pancreas, it could be that plasticity potential of these endocrine cells at these stages is different, or that these are two different cells expressing SOX9^{cyto}. Lack of lineage tracing strategies for human fetal post-mortem samples prevents the collection of direct evidence for this.

In addition to the fetal endocrine cell clusters, we find SOX9^{cyto}-positive cells expressing beta cell markers in the fetal ductal epithelium, while in the adult pancreas SOX9^{cyto} is expressed in glucagon-positive cells that are located within the pancreatic duct. Could this mean that SOX9 nucleocytoplasmic shuttling is important for endocrine specification from pancreatic progenitor cells? Controlled access of proteins to the nucleus is a key driver of developmental switches and programmed cell differentiation. In mammalian gonadal development, nuclear translocation of SOX9 is the prime activator of testis differentiation, while cytoplasmic expression contributes to

ovary differentiation[27,28]. Defects in the nucleocytoplasmic shuttling process of SOX transcription factors in humans results in the male-to-female sex reversal syndrome[8,29,30]. In the intestinal epithelium, Wnt-signaling-stimulated upregulation of SOX9 represses differentiation genes such as CDX2 and MUC2, thereby maintaining a progenitor phenotype[31]. Human limbal epithelial stem/progenitor cells (LEPCs) have SOX9^{cyto} expression in basal LEPCs that is required for maintenance and quiescence of these stem cells, whereas nuclear translocation of SOX9 parallels proliferation and early differentiation[32]. It could be that loss of nuclear SOX9 prevents SOX9 from maintaining pancreatic progenitor identity and enables cells to differentiate towards an endocrine cell type. But if SOX9^{cyto} is involved in endocrine cell differentiation, it is unclear why we observe this discrepancy in fetal beta cells and adult alpha cells. It is unknown what exactly regulates the segregation of endocrine progenitor cells into the specific endocrine lineages during human development, but in-depth analysis of NEUROG3 endocrine progenitor cells in rodents show a temporal heterogeneity of endocrine progenitor cells during development[33], and lineage tracing studies show that adult beta cell heterogeneity originates from endocrine progenitor heterogeneity during development[34]. Thus, endocrine progenitor heterogeneity might explain the discrepancy between fetal beta- and adult alpha cells, and it might be possible that a temporal stage-specific aspect (*i.e.,* fetal and postnatal) trigger differentiation of a heterogeneous endocrine progenitor population into a specific endocrine lineage.

We found no link between proliferation and expression of SOX9^{cyto} in human fetal pancreatic cells, in contrast to the previous findings in adult tissue. This could be a reflection of normal endocrine cell formation in humans, which is reported to be the result of endocrine progenitor cell differentiation during development, whereas, postnatally replication is the main contributor to new endocrine cells in humans[35]. Other postnatal states where SOX9^{cyto} is linked to proliferation are aggressive cancers. Human pancreatic ductal adenocarcinoma (PDAC) expressing tumour protein 53 (TP53) is associated with cytoplasmic SOX9 expression in ductal cells and is clinically associated with poor disease-free survival and higher tumour grade. *In vitro* lentiviral-mediated expression of TP53^{R175H} in human PSC-differentiated exocrine organoids induced SOX9^{cyto} expression, whereas in control mCherry or KRAS^{G12V} organoids nuclear SOX9 was observed, demonstrating SOX9^{cyto} expression can be induced in specific (patho)physiological conditions[36]. Cytoplasmic SOX9 is also a marker of higher tumour grade and poor prognosis in other malignancies, such as breast cancer and oral squamous carcinoma[37,38]. Additionally, *in vitro* treatment of breast cancer cells with Trichostatin A, an epigenetic modifier, led to translocation of SOX9 to the nucleus, which induced growth arrest of these cells[39].

To our knowledge, we are the first to describe the expression of SOX9^{cyto} in the human developing pancreas. The majority of previous reports that identified the crucial role of nuclear expression of SOX9 in pancreatic multipotent progenitors and ductal cells have generated data using rodent models[40]. However, it has become increasingly evident that islet biology and development, including transcriptional regulation, have interspecies differences[41], which could explain why SOX9^{cyto} in the fetal pancreas has not been described before. Moreover, it could be that the current

murine antibodies do not detect the same (post-transcriptionally modified) form of SOX9 as the human antibody.

Although the mechanism of action of SOX9^{cyto} remains unclear, a strength of our study is that we were able to identify a novel SOX9^{cyto} population in human fetal pancreas development in early gestational age. It would be interesting to see from which embryonic age SOX9 shuttling occurs, and whether SOX9^{cyto} activity can be observed in earlier pancreatic progenitor cells perhaps prior to endocrine cell specification which would indicate its requirement for endocrine cell specification. In addition, it would be interesting to evaluate whether pancreata from older fetal donors also show alpha cells with cytoplasmic SOX9, similar to the early postnatal pancreas, to determine if there is a temporal aspect to SOX9^{cyto} expression in relation to endocrine cell specification. However, limitations due to current legislations, ethical review, and codes of practice limits the study of certain pancreatic development periods.

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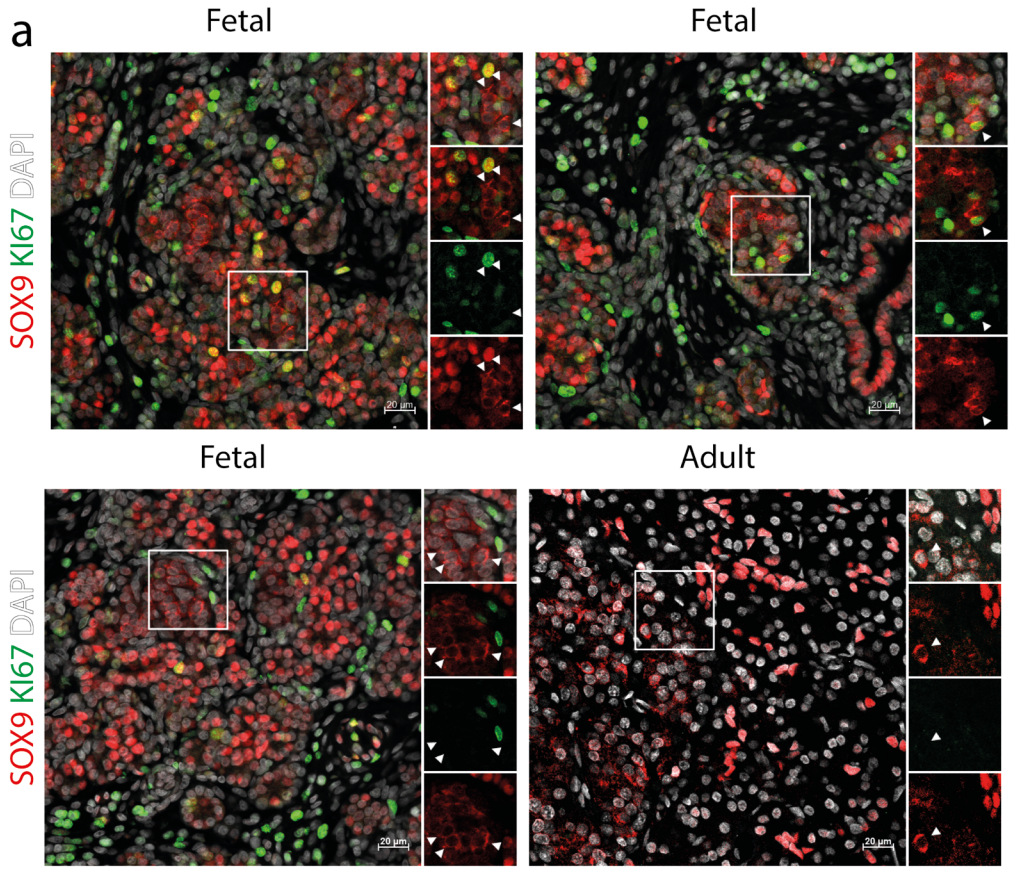
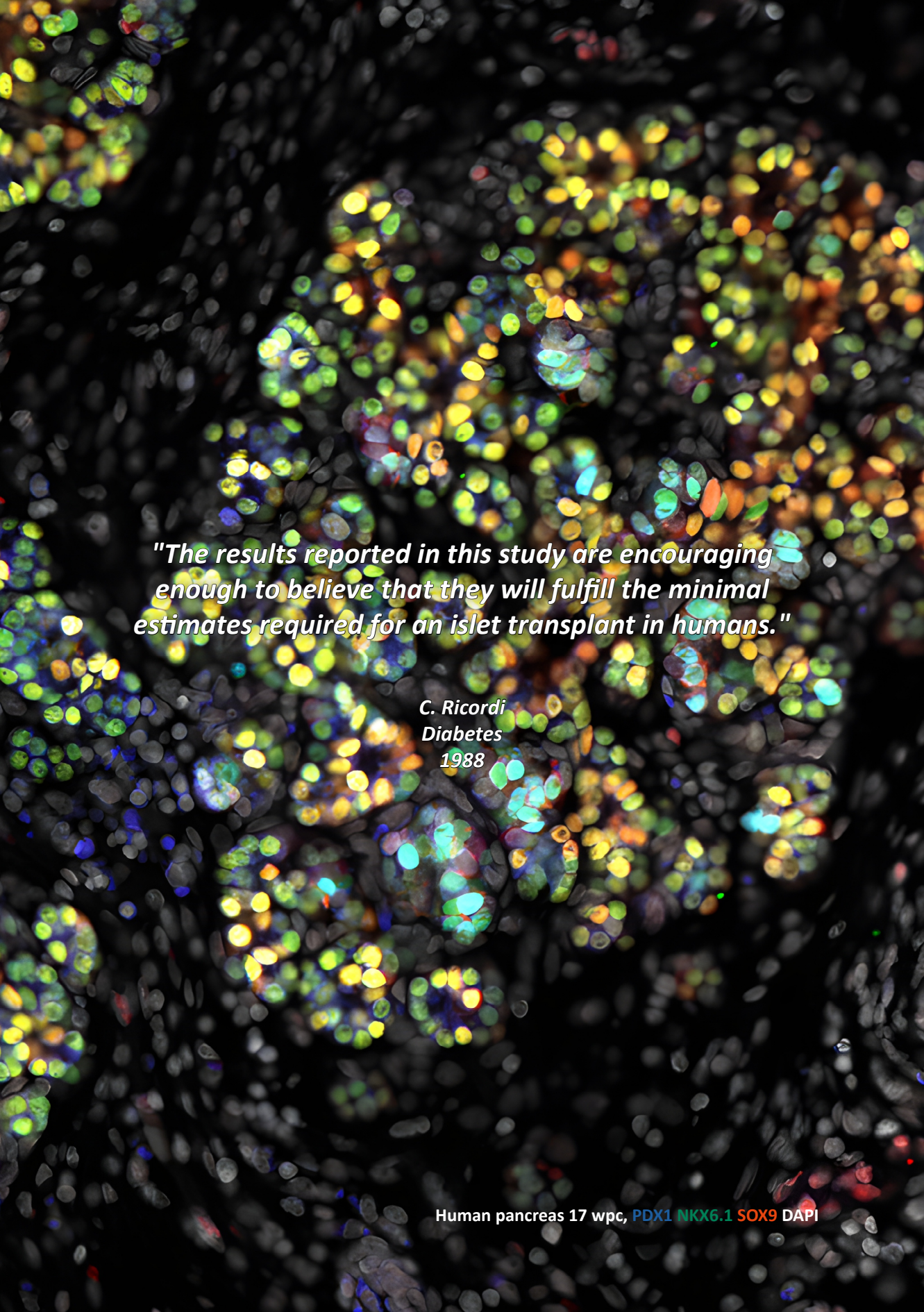
Supplemental information

Supplemental Table 1. List of primary antibodies used

Marker	Host	Provider	Cat number	Dilution
ARX	Sheep	R&D	AF7068	1:1000
Glucagon	Mouse	Sigma	G2654	1:200
Insulin	Guinea pig	Abcam	AB7842	1:200
Synaptophysin	Mouse	Millipore	MAB5258	1:1000
Ki67	Mouse	BD Pharmingen	556003	1:200
KRT19	Rabbit	Abcam	AB52625	1:250
KRT19	Mouse	Cell Signaling	4558	1:200
NKX6.1	Mouse	DSHB	F55A12	1:1000
PDX1	Goat	R&D	BAF2419	1:10
SOX9	Rabbit	Millipore	AB5535	1:500
SOX9	Rabbit	Abcam	AB36748	1:100



Supplemental Figure 1. Ki67 is rarely expressed in SOX9^{cyto} cells in fetal and adult pancreata
(**a**) Immunofluorescent staining of human fetal pancreas (19 wpc) and human adult pancreas for SOX9 (ductal marker, red), Ki67 (proliferation marker, green), DAPI (nuclear marker, white), showing sporadic SOX9^{cyto}/Ki67 double-positive cells. No SOX9^{cyto} proliferating cells were observed in adult tissue. Arrowheads point to SOX9^{cyto} positive cells. Scale bar = 20 µm.


"The results reported in this study are encouraging enough to believe that they will fulfill the minimal estimates required for an islet transplant in humans."
C. Ricordi
Diabetes
1988
Human pancreas 17 wpc, PDX1 NKX6.1 SOX9 DAPI

CHAPTER| 8

General Discussion and Future Directions

General Discussion

Diabetes mellitus poses a significant threat to global health, impacting millions of people worldwide. The hallmark of type 1 diabetes mellitus (T1DM) is the autoimmune-mediated destruction of the insulin-producing beta cells residing in the pancreatic islets of Langerhans[1]. Patients with T1DM require lifelong administration of exogenous insulin for their survival. However, achieving optimal glycemic control remains challenging despite advances in insulin therapy and glucose monitoring technologies, leading to complications from hyperglycemia or hypoglycemia, in addition to a substantial self-care burden[2].

Currently, the most promising approach to cure diabetes is to restore insulin secretion by replenishment of beta cell mass, which can be performed with a whole-organ pancreas transplant (PT) or an islet transplant (IT)[3]. IT is associated with a lower complication rate and better overall survival while producing similarly effective outcomes compared to PT, making it suitable for a wider range of patients[4,5]. However, its widespread adoption is limited by several factors, including shortage of donor tissue[6]. Therefore, finding alternative sources for insulin-producing beta cells is an important goal in IT.

The existence of a beta cell progenitor in the pancreas has been a topic of debate for over a century[7,8]. Early studies suggested that the pancreatic ductal epithelium may harbor progenitor cells, supported by histological observations in human samples and lineage tracing studies in animal models. However, conflicting findings from various animal models and challenges in directly studying human pancreatic tissue have made it difficult to formulate definite answers[7,9]. The central question this thesis aimed to address was:

Can human pancreatic duct cells be used as a source for beta cell regeneration, thereby offering a novel approach to the treatment of diabetes?

The research described in this thesis involved multiple specific objectives: i) evaluation of the effect of islet purity in allo-islet transplantation, ii) the identification and characterization of potential progenitor cells within the human pancreas, iii) the development of novel methods to expand and differentiate these cells *ex vivo*, and iv) clarifying the role of key transcription factors in the differentiation of progenitor cells into beta cells. The findings from this thesis provides important insights into these areas. However, several challenges and limitations remain, highlighting the need for further research.

Evidence for beta cell regeneration in allo-islet transplantation

Our study explored the association of islet purity and metabolic outcome in patients undergoing islet transplantation. Demonstrating improved metabolic outcomes using lower-purity islets could provide evidence for beta cell regeneration from non-islet cells, such as duct cells, and potentially lead to compelling alternative strategies in clinical islet transplantation. These strategies could reduce

the reliance on high-purity islets and optimise the utilisation of donor material, including the non-islet portion. This area of research is important due to the limited availability of donor islets and the hypothesis that duct cells may form beta cells.

Islet purity is a key consideration in islet transplantation for two reasons, namely i) the delivery of a sufficient number of islets is considered essential for favorable metabolic outcomes post-transplantation, and ii) the procedure's safety depends on the ability to deliver these islets within a constrained volume to prevent complications such as portal vein thrombosis[10,11]. Therefore, islet isolation procedures include a purification step to address these concerns. However, purifying islets is challenging and often results in preparations that, despite containing a significant number of islets, are not transplanted due to insufficient purity[12,13]. Moreover, it is unclear how beneficial this purification is for graft function, as the influence of non-islet cells within the graft on metabolic outcomes remains uncertain. Interestingly, a few studies have reported that lower-purity islets are associated with improved long-term metabolic outcomes compared to high-purity islets[14,15]. The authors of these studies hypothesize that this effect might be attributable to beta cell neogenesis originating from ductal cells. However, these studies have small sample sizes and use inconsistent methods to measure islet purity, which complicates the interpretation of the impact of non-islet cells in IT.

In **Chapter 2** we examined the impact of islet purity on graft function in our patient cohort, using robust and reproducible methods to quantify islet purity and graft function. We found that high-purity islets resulted in better graft function three months post-transplant, but long-term graft function did not significantly differ across purity levels. This indicates that islet purity is not a critical determinant of long-term graft outcome. These findings have important implications. First, it is essential to use reproducible and unbiased methods to measure islet purity to accurately evaluate its impact on graft function, and to enable comparisons between different studies. In addition, our findings suggest that islet purity may be less critical for long-term outcomes than previously thought, potentially shifting the focus away from purity as an important factor when considering metabolic outcome in islet transplantation. This finding is also relevant in the ongoing discussions about the cellular composition of stem cell-derived islets, in which the impact of non-islet cells on function and safety after transplantation is debated[16]. Although our analysis included a large number of patients, long-term follow-up was limited, particularly due to exclusion of patients after receiving a second islet transplantation. This highlights the need for additional studies to validate our findings and to better understand the role of non-islet cells in graft function over time. In conclusion, our findings could help to update transplantation protocols and guide future research aimed at further improvement of the beneficial effects of IT. Importantly, the absence of improved long-term metabolic outcomes in lower-purity islets prevented us from finding any evidence of beta cell regeneration from duct cells in IT.

Investigating beta cell progenitors in the human adult pancreas

Previous work exploring the role of pancreatic ductal cells in beta cell regeneration has largely relied on rodent models, resulting in a wide range of contradictory findings, and shifting focus away from the investigations into the possible progenitor capacity of ductal cells[17,18]. However, rodent models are suboptimal for translating beta cell turnover findings to humans due to significant differences in beta cell physiology, highlighting the need for experimental studies using human tissues[19,20].

Progress in finding direct evidence for beta cell regeneration from ductal progenitors in humans has been modest, largely due to the limited availability of normal primary human pancreatic tissue for research. Early studies conducted over 20 years ago provided some of the first insights, showing that duct cells could be expanded from islet-depleted human pancreatic tissue[21-23]. These studies also demonstrated that, under specific culture conditions or genetic manipulations, expanded duct cells could acquire a beta cell phenotype, providing insights into the microenvironment and specific signaling pathways required for the differentiation of these cells into endocrine cells. However, the differentiation efficiency was low, and the highly heterogeneous starting population made it challenging to identify the origin of the generated insulin-positive cells. Subsequent studies used cell surface markers that could be used to enrich for duct cell subpopulations, to create a more homogeneous starting population. For example, duct cells expressing the surface marker carbohydrate antigen 19-9 (CA19-9) could be isolated and expanded on a plastic surface[24,25]. After expansion, the cells were cultured in suspension and proliferation-stimulating agents were withdrawn. This process resulted in the aggregation of ductal cells and their spontaneous differentiation into insulin-producing cells, though this occurred with low efficiency. Another cell surface marker, prominin-1 (CD133), was identified in fetal mouse pancreas labelling endocrine progenitor cells[26]. This marker was also used to isolate human adult pancreatic duct cells, which could be clonally expanded in 3D culture using Matrigel[27]. However, for differentiation towards beta cells these cells required genetic manipulation.

In **Chapter 4**, we optimised organoid culture techniques to expand human islet-depleted pancreatic tissue. After expansion, the duct cells exhibit characteristics such as self-renewal in clonal expansion experiments, and demonstrate similarities to fetal cells. We focused on cells expressing the stem cell marker ALDH, and found that ALDH^{hi}-positive ductal cells isolated after expansion were capable of clonal expansion and showed more similarities to ALDH^{hi}-positive cells derived from human fetal pancreatic organoids compared to human islet-depleted tissue, suggesting a more primitive state. In addition, we confirmed the potential for endocrine differentiation of expanded duct cells without genetic manipulation. The organoids derived from human islet-depleted tissue were capable of generating insulin-expressing cells *in vivo*, although the differentiation efficiency was still low, with only about 1.5% of the cells becoming insulin-positive. The low differentiation efficiency highlights the need to further refine the isolation and expansion protocols for ductal progenitor cells, as well as optimisation of the conditions that that promote their differentiation into functional beta cells. Despite the low number of insulin-positive cells generated in our experiments, our findings are significant as they confirm the progenitor potential of adult human duct cells.

One of the primary limitations of our study remains the heterogeneity of the islet-depleted tissue that was used to expand, which complicates the identification of the origin of the endocrine cells that are formed. However, creating a single-cell suspension from human islet-depleted tissue and plating it in Matrigel under the same organoid culture conditions used for human islet-depleted tissue chunks, did not result in the formation of similar complex organoids as can be generated with human fetal pancreatic progenitor cells. This highlights the importance of the progenitor cell niche required for the development of ductal cells into complex organoids. Similar challenges have been observed in Lgr5-positive intestinal stem cell cultures, in which self-renewal and proliferation is dependent on direct cell-cell contact of Lgr5-positive cells and supporting cells in their niche, such as Paneth cells[28]. Methods for culturing Lgr5-positive intestinal stem cells in complex organoids without the typically required niche have been developed, by using synthetic matrices and specific growth factors. These cultures can be maintained for a long time and directed to differentiate into various intestinal cell types[29]. Future research will have to determine whether the use of cell surface markers to isolate duct cells in combination with the culture additives can result in improved (clonal) expansion of duct cells directly after isolation, which could provide strong evidence for the progenitor capacity of duct cells.

Improved differentiation of primary human ductal cells

In the context of beta cell replacement therapy, the discovery of beta cell progenitor cells alone is insufficient without the parallel development of efficient endocrine differentiation protocols. While significant progress has been made in the differentiation of pluripotent stem cells (PSCs) into beta cells[30], similar protocols tailored for human ductal cells remain underdeveloped. One interesting finding from PSC differentiation studies is that cells commonly undergo a duct-like phenotype before further differentiation into insulin-producing cells, recapitulating islet formation as observed in human embryonic development[31]. Therefore, we hypothesized that the protocols used in these PSC procedures would serve as a valuable starting point for the development of new beta cell differentiation protocols for adult ductal cells. Besides the agents derived from PSC differentiation protocols, a range of other compounds that were able to induce beta cell neogenesis in animal or *in vitro* studies have been identified. These compounds, including factors like glucagon-like peptide-1 receptor agonists (GLP-1RA) or other small peptides such as islet neogenesis associated protein (INGAP), hold additional potential for inducing beta cell neogenesis, although their effect has yet to be verified in human ductal cells[32-35].

In **Chapter 5**, we aimed to address this gap by adapting an established differentiation protocol originally designed for PSC[36]. This protocol was modified to include several agents identified from PSC differentiation protocols and animal studies or *in vitro* experiments studying beta cell regeneration. We hypothesized that by combining insights from both fields, we could develop a more effective protocol for the generation of insulin-positive cells from primary human ductal cells. As proof-of-concept, we compared our adapted protocol with a traditional basic medium

used for spontaneous differentiation of ductal cells[24]. Our modified protocol, which included fibroblast growth factor 7 (FGF7), GLP-1RA and INGAP, increased the number of insulin-positive cells, showing that human duct cell differentiation can be improved using adapted differentiation protocols. However, even with our adapted protocol, differentiation efficiency remained low, forming only 5% of insulin-positive cells after *in vivo* maturation. Further understanding of the molecular mechanisms and pathways required for the differentiation process of adult ductal cells is essential for further development and optimisation of these protocols.

Gathering evidence for duct cell plasticity using lineage tracing

In the regenerative medicine field, clonal expansion and lineage tracing studies are key to identifying cells with progenitor characteristics. Lineage tracing refers to a collection of techniques used to track the fate of individual cells and their progeny with minimal disruption to their normal function[37,38]. This approach is useful for investigating the pluripotency potential of cells both *in vivo* and *in vitro*. It has been successfully applied in various contexts, including studies with human pancreatic cells[39,40]. Therefore, implementing effective and specific lineage tracing methods could provide an alternative strategy to show conclusive evidence regarding the plasticity and/or progenitor capacity of pancreatic ductal cells.

In **Chapter 6**, we optimised lentiviral transduction of primary human exocrine cells, achieving up to 90% efficiency using a VSV-G-pseudotyped vector with eGFP under a CMV promoter, with addition of protamine sulfate in a serum-free transduction environment. Despite this success, our attempts to create a duct-cell specific lineage tracing system using the duct cell specific promoters keratin 19 (KRT19) and carbonic anhydrase II (CAII) were unsuccessful due to a lack of specificity. Ongoing efforts testing other promoters are expected to yield duct-specific lentiviral constructs, which will enable precise and efficient labelling of these cells with our optimised protocol. This technique could be employed to trace the fate of adult duct cells in culture, or to facilitate genetic manipulations aimed at elucidating the signaling pathways required for duct cell proliferation and differentiation.

Further investigations into the transcriptional mechanism underlying beta cell differentiation

Understanding the transcriptional mechanisms that drive endocrine cell development is essential for the improvement of differentiation protocols. In the context of pancreas formation, transcription factors such as PDX1, NEUROG3, and SOX9, have been widely studied for their roles in directing progenitor cells toward specific endocrine lineages[41]. Recent investigations aimed at identifying proliferative islet cells in the adult human pancreas have uncovered a highly proliferative subset of alpha cells that also exhibited cytoplasmic expression of SOX9[42]. SOX9 is traditionally known as a nuclear transcription factor in the pancreas, where it plays an important role during the organ formation and the differentiation of progenitor cells into endocrine lineages[43,44]. Interestingly, the

ability of SOX9 to shuttle between the nucleus and cytoplasm (nucleocytoplasmic shuttling) is identified as a crucial process in regulating gene expression required for proper organ formation and function, such as in sex differentiation[45]. The discovery of cytoplasmic SOX9 in adult endocrine cells suggests that nucleocytoplasmic shuttling might be involved in the regulation of endocrine cell specification or function. However, this process has not yet been investigated in the context of human pancreatic development.

In **Chapter 7** we found that SOX9^{cyto} is present during human pancreas development, and is initially expressed in both insulin-positive and polyhormonal cells during early gestation, but later becomes restricted to monohormonal insulin-positive cells. It did not mark a highly proliferative cell population. This pattern contrasts with cytoplasmic expression observed in adult proliferative alpha cells. These findings indicate that pancreatic biology and endocrine cell differentiation are more complex than previously understood, possibly involving spatiotemporal nuclear-cytoplasmic shuttling of transcription factors to regulate cell specification. This suggests the potential for enhancing beta cell differentiation protocols by manipulating the subcellular localization of transcription factors like SOX9. Future research should focus on understanding the dynamic regulation and localization of SOX9, as well as its function in endocrine cell specification. This deeper understanding of the transcriptional mechanisms that drive endocrine cell differentiation might eventually lead to new strategies or protocols for the development of new beta cells for cell-based diabetes therapies.

Future Directions

The challenge of addressing the massive global burden of diabetes underscores the need for novel therapies that are both cost-effective and scalable. Over the past two decades, significant advances have been made in differentiating human PSCs into functional pancreatic beta cells[46,47]. These methods have shown great promise, with ongoing clinical trials demonstrating their potential to replace lost beta cells in patients with type 1 diabetes[48,49]. However, challenges such as immune rejection, the risk of tumourigenesis, and difficulties in ensuring the long-term survival and functionality of transplanted cells persist in stem cell-based therapies[50-52].

While these technologies are an important step toward a potential cure, they have also shifted some attention away from the search for beta cell progenitors in the pancreas. However, identifying these putative progenitor cells is still vital, not only for our further understanding of beta cell turnover, but also for the development of new therapeutic strategies for diabetes. For instance, enhancing the possible endogenous regenerative capacity of the native pancreas through beta cell differentiation or replication mechanisms could overcome some of the challenges associated with cell therapies, such as time-consuming and expensive culture methods[19,53]. Therefore, it remains essential to obtain direct evidence confirming the existence of a progenitor cell in the human adult pancreas.

Characterization of the heterogeneous duct compartment

The pancreatic duct tree is an extensive system composed of a heterogeneous cell population, comprising various-sized ducts with distinct phenotypic and functional characteristics[54]. It has been hypothesized that only a subpopulation of these cells has progenitor capacity, but the specific locations of these cells—whether in large ducts, small ducts, or even in centroacinar cells that connect ducts to acini—remains unclear.

New high-resolution analytical tools offer enhanced opportunities to better understand pancreatic duct heterogeneity. Single-cell transcriptomics has become the leading method for studying heterogeneity in cell populations, reconstructing developmental pathways, and modelling transcriptional dynamics[55]. This approach is applied to a variety of cells, including those derived from PSC cultures, organoids, and other tissues[56,57]. Application of single-cell transcriptomics to the pancreatic duct tissue has revealed a previously unknown level of heterogeneity within the ductal tree[58-62]. This new information can be used to distinguish between duct subpopulations and identify the populations with progenitor capacity. Some approaches have already enriched duct subpopulations with progenitor potential[63-65]. Further investigations using high-resolution tools, particularly studies involving human fetal tissue where the pancreas is still rich in progenitor cells developing into endocrine cells, are crucial for deepening our understanding of normal pancreatic development and the characteristics of progenitor cells[66]. Ultimately, this research could provide spatial insights into the progenitor cell distribution along the pancreatic ductal tree in the human adult pancreas, in addition to the identification of cell surface markers that could be used to isolate these progenitor cells. This would facilitate additional studies on the plasticity of these cells and their differentiation potential into beta cells, paving the way for new therapeutic strategies.

Further optimisation of expansion and differentiation

Expanding and differentiating primary human pancreatic progenitor cells into beta cells *ex vivo* remains a complex process with significant challenges. One of the main challenges is the low efficiency of differentiation, with only a small fraction of progenitor cells successfully converting into hormone-positive cells. Future research should focus on optimizing the differentiation process by refining the combination of growth factors and peptides used to guide progenitor cells towards beta cells. Advances in bioengineering, such as the development of biomimetic scaffolds that mimic the extracellular matrix in the natural niche of progenitor cells, or dynamic culture systems like microfluidic devices, could also improve the expansion and differentiation of these pancreatic cells by providing a more physiologically relevant environment[67-69].

For example, organ-on-chip systems are microfluidic devices designed to replicate the physical and biochemical conditions of organs offering a more physiologically accurate model than traditional culture methods[70]. These systems can simulate the dynamic environment of the pancreatic ductal system, recreating fluid flow, mechanical stress, and nutrient gradients that are required for cell differentiation and proper function. This approach could be particularly beneficial

for optimizing the differentiation of pancreatic progenitor cells, as it closely replicates the *in vivo* conditions they would encounter within the body.

Future research should further refine these systems, exploring the optimal combinations of growth factors, extracellular matrix components, and dynamic culture conditions, that best support the differentiation and maturation of progenitor cells into functional beta cells. Such advancements are crucial not only for validating the potential of duct cells as a reliable source for beta cell replacement therapies, but also for enhancing our understanding of the mechanisms that regulate their differentiation.

Concluding Remarks

In summary, this thesis has provided insights into the potential of human pancreatic duct cells to serve as a source for beta cell regeneration, laying the groundwork for future research in regenerative therapies for diabetes. While there are challenges and gaps that remain to be addressed, the work outlined in this thesis offers new methodologies and insights that advance our understanding of beta cell regeneration and the role of pancreatic duct cells in this process. By exploring the potential of these cells as a source for beta cell replacement therapy, this research contributes to the broader field of diabetes research and opens up new avenues for therapeutic development.

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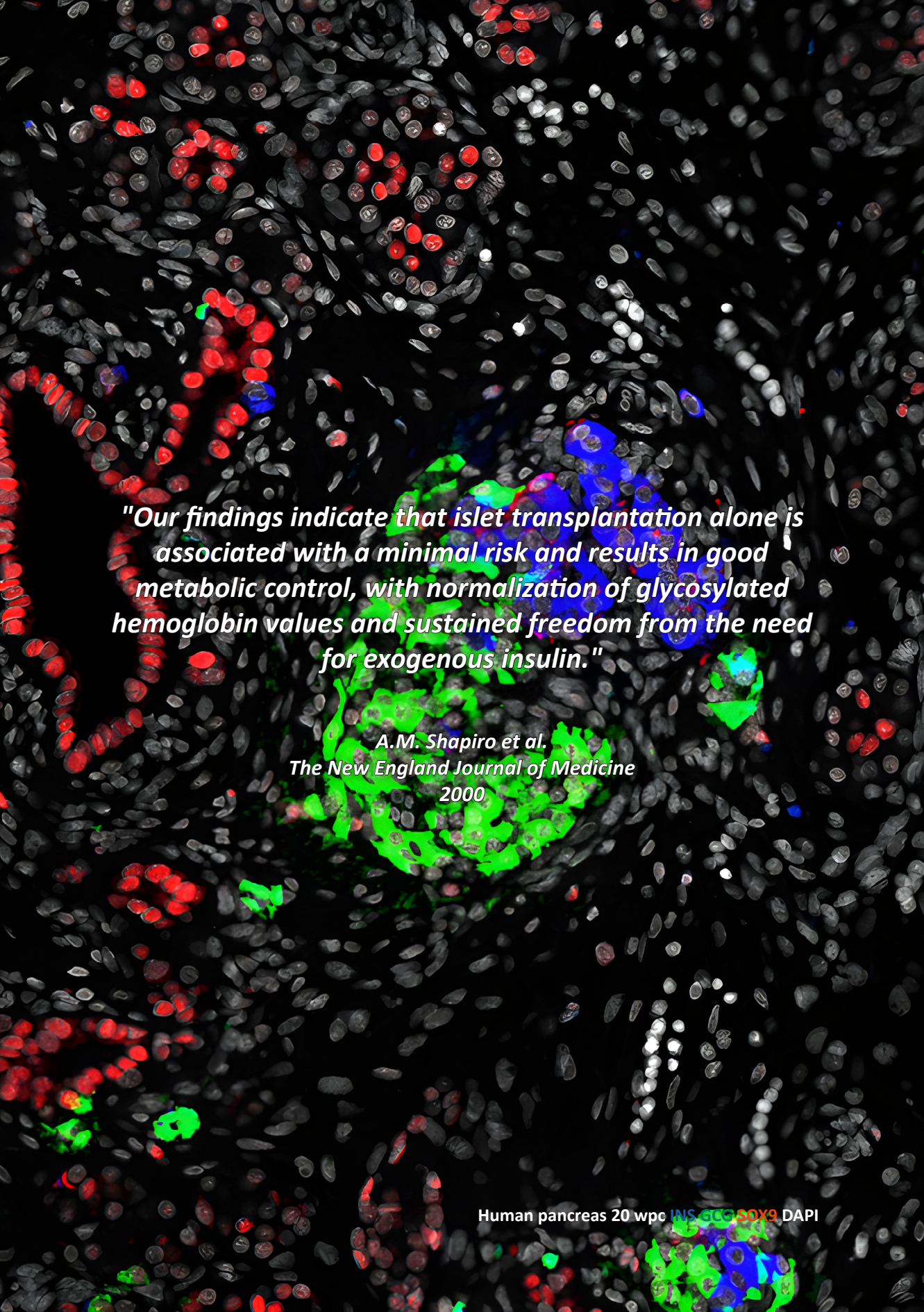
"Our findings indicate that islet transplantation alone is associated with a minimal risk and results in good metabolic control, with normalization of glycosylated hemoglobin values and sustained freedom from the need for exogenous insulin."
A.M. Shapiro et al.
The New England Journal of Medicine
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Human pancreas 20 wpc INS GCG SOX9 DAPI

CHAPTER 9

Summary

Summary

Restoring beta cell mass as a functional cure for type 1 diabetes mellitus (T1DM) through pancreas or islet transplantation is a well-established treatment, with islet transplantation offering fewer complications. However, its widespread use is limited due to donor tissue shortages. This thesis investigates whether human pancreatic duct cells could serve as a source for beta cell regeneration.

In **Chapter 2**, we focus on the impact of islet purity on graft function in islet allotransplantation in recipients with severe beta cell deficiency. Previous studies have suggested that lower-purity islet transplants might offer a beneficial long-term effect on metabolic outcomes, potentially due to the differentiation of progenitor cells within the non-islet cell population into beta cells[1,2]. In particular duct cells, which constitute about 30-40% of the human pancreas, and up to 20-30% of transplanted islet grafts, have been suggested as a potential progenitor cell[2-4]. However, the impact of non-islet cells on metabolic outcomes in islet transplantation remains unclear, due to limitations such as small cohort sizes and unreliable methods for assessing islet purity.

In this study, we employed a robust and reproducible method to quantify the purity of transplanted islets, alongside evaluation of graft function using C-peptide and glucose levels obtained during mixed meal tolerance tests. Our findings indicated that high-purity islets were associated with improved graft function at three months post-transplantation. However, in the long-term, no significant differences in graft function were observed across islet grafts with different purity. Similar long-term metabolic outcomes across the purity groups demonstrate that islet purity is not an important determinant of long-term graft function. Importantly, these results provided no clear evidence to support the hypothesis that pancreatic duct cells can serve as a source for beta cell regeneration.

Organoids are three-dimensional structures that replicate the architecture and functionality of the tissues from which they are derived, making them a powerful tool for studying cell differentiation, disease modelling, and regenerative medicine[5,6]. In **Chapter 3**, we comprehensively summarize and evaluate the application of primary tissue-derived pancreatic organoids in regenerative studies, disease modelling, and personalized medicine. We explore how these organoids have advanced our understanding of pancreas biology and their potential as a platform for developing new treatments.

In **Chapter 4**, we optimised the organoid culture system for primary human adult and fetal pancreatic cells, in order to study the role of pancreatic progenitor cells in the context of beta cell regeneration. By refining the organoid culture protocol for human islet-depleted tissue, we demonstrated that human islet-depleted tissue can efficiently form organoids composed of duct cells that can be expanded and maintained over time. Aldehyde dehydrogenase (ALDH) is widely recognised as a marker of stem cells and progenitor cells across various tissues, including the pancreas[7,8]. We found ALDH expression in the organoid-expanded duct cell population, and discovered that ALDH^{hi} duct cells were effective in self-renewal and showed capacity for clonal expansion. In addition, gene expression profiling revealed that these ALDH^{hi} duct cells more closely resembled ALDH^{hi}-positive duct cells derived from fetal pancreatic tissue than adult pancreatic

tissue. In addition, pancreatic organoids were able to differentiate into insulin-expressing cells upon engraftment into the kidney capsule of mice. Although the differentiation efficiency was relatively low, with only 1.5% of cells turning insulin-positive, this shows the potential for endocrine differentiation in cultured duct cells.

The differentiation of progenitor cells into functional beta cells is a complex process involving various signaling pathways, transcription factors, and environmental factors[9,10]. Experiments with human embryonic stem cells (hESCs) have offered valuable insights and helped identify the stimuli needed to differentiate pluripotent stem cells into beta cells. In **Chapter 5**, we explored whether differentiation of primary human duct cells towards beta cells could be improved using beta cell neogenesis agents discovered in pluripotent stem cell protocols, animal models or *in vitro* studies. Through a series of experiments using various growth factors and small molecules, we discovered that a combination of INGAP (islet neogenesis-associated protein), FGF7 (fibroblast growth factor 7), and a GLP-1R (glucagon-like peptide-1 receptor) agonist induced the differentiation of duct cells into insulin-expressing cells, exceeding the results achieved with a basic medium that allows for spontaneous differentiation[11]. After *in vivo* maturation in mice, the number of insulin-positive cells increased further up to 5%, although the differentiation efficiency and functionality, including glucose responsiveness and insulin secretion, remained limited. These findings reveal how additional agents could enhance the efficiency of beta cell differentiation protocols for primary human duct cells. This is an important step toward the development of effective cell-based therapies for diabetes or endogenous beta cell regeneration.

Lineage tracing techniques track the fate of individual cells and their progeny with minimal disruption to their normal function, useful for studying the pluripotency of progenitor cells[12,13]. While effective lineage tracing, especially through genetic manipulation, could confirm the endocrine potential of duct cells, our initial experiments faced difficulties in targeting primary human exocrine cells with lentiviral transduction, complicating the development of a duct cell-specific tracing system. In **Chapter 6**, we addressed the challenge of efficiently transducing primary human exocrine cells with lentiviral vectors. By systematically comparing various transduction conditions—including different promoters, viral envelopes, medium compositions, and transduction adjuvants—we optimised the lentiviral transduction process. By using a VSV-G pseudotyped vector expressing eGFP under a CMV promoter, with protamine sulfate assistance in a serum-free transduction environment, we achieved up to 90% GFP-positive cells at 5 days post-transduction. This highlights the critical role of the culture environment in gene transfer. We also found that freshly isolated exocrine cells exhibited higher transduction efficiency compared to expanded cells, suggesting that the cellular state at the time of transduction significantly influences transduction efficiency. Furthermore, we attempted to develop a pancreatic duct-cell specific lineage tracing system using various duct-cell specific promoters, such as keratin 19 (KRT19) and carbonic anhydrase II (CAII). However, both the KRT19 and CAII promoters tested lacked sufficient specificity for reliable application in duct cell lineage tracing, as they also labelled insulin-positive cells in islet cultures. Continued efforts to identify a more specific duct cell promoter will ultimately provide a valuable tool for further exploration of a

duct cell plasticity and their potential for beta cell regeneration.

The transcription factor SOX9 has been shown to be essential for the proper formation of the pancreas during embryonic development, and its expression is required for the maintenance and differentiation of progenitor cells into endocrine lineages[14]. In **Chapter 7**, we provide new insights into the role of SOX9 in human pancreatic development, specifically focusing on the cytoplasmic expression of SOX9 in fetal pancreatic cells and its implication for islet cell specification. We first identified expression of $SOX9^{cyto}$ in a subset of human fetal pancreatic cells. Initially, $SOX9^{cyto}$ is present in both insulin-positive and polyhormonal cells during early gestation, with a shift towards monohormonal insulin-positive cells later in development. This expression of $SOX9^{cyto}$ in a subset of fetal beta cells contrasts with its known nuclear expression in pancreatic progenitor cells and its cytoplasmic expression in a subset of human adult alpha cells[15]. In addition, we found that $SOX9^{cyto}$ cells in the fetal pancreas are not highly proliferative, which also contrasts with earlier findings in adult tissues[15]. This suggests that the role of $SOX9^{cyto}$ may differ between developmental and adult stages. Understanding the mechanisms by which $SOX9^{cyto}$ influences cell fate during development could be critical for advancing strategies to generate functional beta cells for diabetes treatment[16]. Our findings suggest that the nucleocytoplasmic shuttling of SOX9 could be an important factor in the specification of endocrine cells from progenitor cells.

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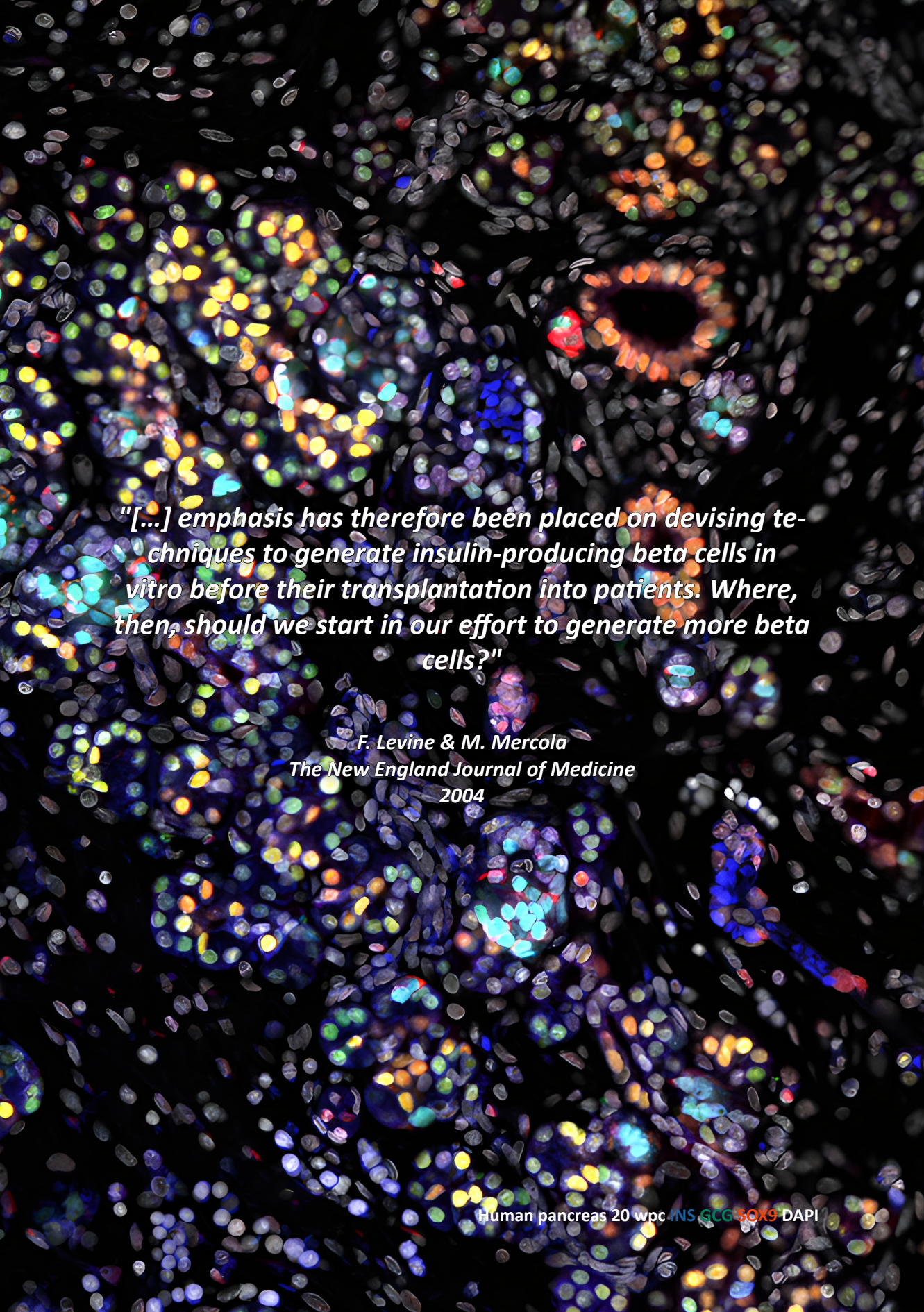
"[...] emphasis has therefore been placed on devising techniques to generate insulin-producing beta cells in vitro before their transplantation into patients. Where, then, should we start in our effort to generate more beta cells?"
F. Levine & M. Mercola
The New England Journal of Medicine
2004
Human pancreas 20 wpc INS GCG SOX9 DAPI

CHAPTER| 10

Nederlandse Samenvatting
Curriculum Vitae
List of Publications

Nederlandse Samenvatting

Diabetes mellitus type 1 (T1DM) is een auto-immuunziekte waarbij insuline producerende bètacellen in de pancreas worden vernietigd. Dit leidt tot ernstige complicaties en vereist levenslange insulinetherapie. Bètacel regeneratie therapie in de vorm van bijvoorbeeld een eilandjestransplantatie (transplantatie van de geïsoleerde eilandjes van Langerhans) kan de fysiologische insuline secretie herstellen. Echter, de beperkte beschikbaarheid van donorweefsel vormt een belemmering voor een brede toepassing van deze therapie. Daarom worden alternatieve strategieën voor bètacelregeneratie onderzocht, waaronder het gebruik van voorlopercellen als mogelijke bron voor nieuwe bètacellen. Dit proefschrift onderzoekt of humane ductale cellen uit de pancreas zouden kunnen dienen als een potentiële bron voor bètacelregeneratietherapie.

In **hoofdstuk 2** onderzochten we hoe de zuiverheid van eilandjes-transplantaten de functie beïnvloedt na transplantatie bij patiënten met ernstige bètaceldeficiëntie. Zuiverheid verwijst naar de verhouding tussen geïsoleerde eilandjes van Langerhans en andere cellen in het transplantaat. Een hoog zuiverheidspercentage betekent dat een transplantaat voornamelijk endocriene cellen bevat, terwijl een transplantaat met een lagere zuiverheid ook andere cellen bevat, zoals ductale- en acinaire cellen. Eerdere studies suggereren dat minder zuivere eilandjes de transplantaatfunctie op lange termijn verbeteren, mogelijk doordat voorlopercellen, zoals ductale cellen, zich tot bètacellen ontwikkelen. Echter, door kleine cohorten en onbetrouwbare methoden voor zuiverheidsbepaling, blijft de impact van niet-endocriene cellen op metabole uitkomsten na eilandjestransplantatie onduidelijk. In deze studie hebben we een objectieve en reproduceerbare methode toegepast om de zuiverheid van getransplanteerde eilandjes te kwantificeren. Daarnaast hebben we de eilandjesfunctie geëvalueerd met een robuuste methode op basis van gestimuleerde C-peptide- en glucosewaarden. Onze resultaten tonen aan dat een hoge zuiverheid van het eilandjestransplantaat na drie maanden een betere functie geeft, maar op de lange termijn geen significante invloed heeft op metabole uitkomsten. Dit suggereert dat eilandjeszuiverheid geen bepalende factor is voor de lange termijn functie van het transplantaat. Daarnaast vonden we geen overtuigend bewijs dat potentiële voorlopercellen bijdragen aan de vorming van nieuwe bètacellen in de setting van een klinische eilandjestransplantatie.

Organoïden zijn driedimensionale gekweekte celstructuren met complexe cel interacties die *in vitro* de menselijke biologie nabootsen. Hierdoor is het mogelijk om organoïden te gebruiken voor medisch onderzoek. Zo kunnen organoïden bijvoorbeeld dienen als ziektemodellen, door cellen te laten groeien met genetische mutaties of bloot te stellen aan ziekteverwekkers. Daarnaast kunnen organoïden gebruikt worden voor medicijntesten en onderzoek in de regeneratieve geneeskunde. In de context van diabetesonderzoek worden pancreas organoïden gebruikt als een mogelijke bron voor nieuwe insuline producerende bètacellen. In **hoofdstuk 3** geven we een uitgebreide samenvatting en evaluatie van de toepassing van pancreas organoïden afkomstig van primair humaan donor materiaal in regeneratieve studies, ziektemodellering en gepersonaliseerde geneeskunde. We onderzoeken hoe deze organoïden onze kennis over de pancreasbiologie hebben vergroot en hun potentie als platform voor de ontwikkeling van nieuwe behandelingen.

In **hoofdstuk 4** optimaliseerden we het kweeksysteem voor organoïden uit humaan pancreasweefsel afkomstig van zowel volwassenen als foetussen, met als doel het bestuderen van de potentiële bètacel vorming uit voorloper cellen. We toonden aan dat organoïden efficiënt kunnen worden gekweekt uit exocrien weefsel, voornamelijk uit epitheliale ductale cellen bestaan en langdurig in kweek kunnen worden gehouden. Om potentiële bètacel voorlopercellen te identificeren, onderzochten we de expressie van aldehyde dehydrogenase (ALDH) in de organoïden. ALDH is een stamcel- en voorloper cel marker in verschillende weefsels waaronder de pancreas. We vonden ALDH-expressie in de organoïden en ontdekten dat ALDH^{hi} ductale cellen zich efficiënt konden vernieuwen, en daarnaast ook andere stamcel eigenschappen hadden zoals de capaciteit voor clonale expansie. Genexpressieanalyse liet zien dat deze ALDH^{hi} ductale cellen meer overeenkomsten vertoonden met ALDH^{hi}-positieve ductale cellen afgeleid van foetaal pancreas weefsel dan van volwassen pancreas weefsel. Verder konden de pancreas organoïden differentiëren in insuline-positieve cellen na transplantatie in muizen. Hoewel de differentiatie-efficiëntie laag was, met slechts 1,5% insuline-positieve cellen, toont dit onderzoek de potentie van dit kweeksysteem.

De differentiatie van voorlopercellen naar functionele bètacellen is een complex proces dat afhankelijk is van omgevingsfactoren voor de juiste activering van signaaltransductieroutes en transcriptiefactoren. Studies met menselijke embryonale stamcellen (hESC's) hebben waardevolle inzichten opgeleverd en geholpen bij het identificeren van stimuli die noodzakelijk zijn om pluripotente cellen te ontwikkelen tot bètacellen. In **hoofdstuk 5** onderzochten we of de differentiatie van primaire humane ductale cellen naar bètacellen kon worden verbeterd met behulp van stimuli die zijn ontdekt in pluripotente stamcelprotocollen, diermodellen of *in vitro* studies. Door middel van een reeks experimenten met verschillende groeifactoren en kleine moleculen ontdekten we dat een combinatie van INGAP (islet neogenesis-associated protein), FGF7 (fibroblast growth factor 7) en een GLP-1R (glucagon-like peptide-1 receptor) agonist de differentiatie van ductcellen in insuline-producerende cellen induceerde, met betere resultaten dan spontane differentiatie in basaal medium. Na *in vivo* rijping in muizen nam het aantal insuline-positieve cellen toe tot 5%. Hoewel de differentiatie-efficiëntie en cel functionaliteit, waaronder glucoseresponsiviteit en insulinesecretie, beperkt bleven, toonden de resultaten aan dat aanvullende stimuli de efficiëntie van bètaceldifferentiatie uit primaire humane ductale cellen kan verbeteren.

Cell lineage tracing of celafstammingstracering maakt het mogelijk het lot van individuele cellen en hun nakomelingen in vivo of in vitro te volgen met minimale verstoring van hun normale functie. Hierbij worden cellen permanent gemerkt om bijvoorbeeld hun nakomelingen te identificeren, of de origine van gedifferentieerde cellen te identificeren. Deze benadering zou nuttig kunnen zijn voor het bestuderen van het voorloper potentie van ductale cellen, door deze bijvoorbeeld specifiek en permanent te markeren voordat ze gebruikt worden in differentiatie experimenten. In initiële experimenten bleek het echter moeilijk om humane pancreas ductale cellen te transduceren met lentivirale vectoren, wat het ontwikkelen van een lineage tracing-systeem bemoeilijkte. In **hoofdstuk 6** ontwikkelden we een efficiënt transductie protocol voor primaire humane exocriene cellen. Door systematisch verschillende transductiecondities te vergelijken

- waaronder verschillende promotoren, virale enveloppen, mediumcomposities en transductie-adjuvanten - hebben we het lentivirale transductieproces geoptimaliseerd. Door gebruik te maken van een VSV-G gepseudotypeerde vector die eGFP tot expressie brengt onder controle van een CMV-promotor, met protaminesulfaat in een serumvrij kweek, bereikten we tot 90% GFP-positieve cellen vijf dagen na transductie. Dit benadrukt de cruciale rol van de kweekomgeving in gentransfer. Verder probeerden we een pancreas ductale cel-specifiek lineage tracing-systeem te ontwikkelen door gebruik te maken van verschillende ductale cel-specifieke promotoren, zoals bijvoorbeeld keratine 19 (KRT19) en carbonic anhydrase II (CAII). Echter, zowel de KRT19- als de CAII-promotoren vertoonden onvoldoende specificiteit voor betrouwbare toepassing in ductale cel lineage tracing. Verdere inspanningen om een specifiekere ductale cel promotor te identificeren zullen uiteindelijk een waardevol hulpmiddel opleveren voor verder onderzoek naar de plasticiteit van ductale cellen en hun potentie in bètacelregeneratie.

De transcriptiefactor SOX9 is essentieel voor pancreas formatie tijdens de ontwikkeling, waarbij SOX9 verantwoordelijk is voor het behoud en de differentiatie van voorloper cellen naar endocriene cellen. In **hoofdstuk 7** rapporteren we nieuwe inzichten in de rol van SOX9 in de humane pancreas ontwikkeling, met een specifieke focus op de cytoplasmatische expressie van SOX9 in foetale pancreascellen en de implicaties daarvan voor de ontwikkeling van endocriene cellen. Eerst beschrijven we de expressie van $SOX9^{cyto}$ in een subset van pancreascellen; in het begin van de zwangerschap is $SOX9^{cyto}$ aanwezig in zowel insuline-positieve als polyhormonale cellen, met een verschuiving naar monohormonale insuline-positieve cellen later in de ontwikkeling. Deze expressie van $SOX9^{cyto}$ in een subset van foetale bètacellen staat in contrast met de bekende nucleaire expressie in foetale stamcellen tijdens ontwikkeling, en de cytoplasmatische expressie die eerder beschreven is in een subset van menselijke volwassen alfacellen. Daarnaast hebben we ontdekt dat de $SOX9^{cyto}$-cellen in de foetale alvleesklier niet sterk prolifereren, wat ook verschilt van eerdere bevindingen in volwassen weefsels. Dit suggereert dat de rol van $SOX9^{cyto}$ tussen de ontwikkelings- en volwassen stadia kan variëren. Het begrijpen van de mechanismen waarmee $SOX9^{cyto}$ de differentiatie beïnvloedt tijdens de ontwikkeling kan van cruciaal belang zijn voor het bevorderen van strategieën om functionele bètacellen te genereren voor de behandeling van diabetes. Onze bevindingen suggereren dat het nucleocytoplasmatische transport van SOX9 een belangrijke factor kan zijn in de specificatie van endocriene cellen uit voorloper cellen.

Curriculum Vitae

Jeetindra Balak was born on August 3, 1989, in Leiden, the Netherlands. After completing his pre-university education at Bonaventura College in Leiden, he began his medical training at Leiden University in September 2007.

During his studies, Jeetindra actively engaged in both academic and extracurricular pursuits. He was involved in student organisations dedicated to medical education, including the Medische Faculteit der Leidse Studenten (MFLS) and the International Federation of Medical Students' Associations-Leiden (IFMSA-Leiden). He also served as the first president of the Spoedeisende Hulp Studenten Organisatie (SEHSO)-Leiden, an organisation focused on emergency medicine education for students. On an international scale, he held the role of Secretary-General of the European Medical Students' Association (EMSA), advocating for cross-border collaboration in medical education. Additionally, he was a member of the Leiden University Student Council. Academically, Jeetindra participated in two prestigious Honours Classes at Leiden University: the Honours Class of 2009 - *Psychiatric Disorders Across the Lifespan* (Faculty of Medicine), where he contributed as an editor for the final publication, and the Honours Class 2010 - *For God's Sake* (Faculties of Medicine, Law, and Humanities), an interdisciplinary exploration of healthcare, ethics, and law.

His first steps into research came through an elective summer research project, *Breakpoint Analysis of Novel Deletions Causing Thalassemia*, at the Hemoglobinopathies Laboratory (Department of Human and Clinical Genetics, LUMC). Under the mentorship of Dr. C. Harteveld, he investigated genomic deletions responsible for thalassemia, sparking his fascination with basic research. This passion led him to a scientific internship in the laboratory of Prof. Dr. E.J.P. de Koning, where he focused on beta cell regeneration. His growing interest in translational medicine ultimately drove him to pursue a PhD at the same lab, focused on human pancreas duct cell plasticity and the possible role of these cells in beta cell regeneration. During his PhD traineeship he mentored multiple medical and biomedical science students during their internships.

While advancing his research, Jeetindra also continued his clinical training, earning his master's degree in Medicine in December 2016. In January 2017, he began his residency in internal medicine at the Haga Hospital in The Hague under the guidance of Dr. M. van Buren and Dr. Ir. J. Lagro. In his second year of residency, he won the prestigious *Heilige Huisjes* session at the annual conference of the Dutch Society of Internal Medicine with his presentation: *First, Do No Harm: No Insulin in the Treatment of the Hyperosmolar Hyperglycaemic Syndrome*. He was also actively involved as a member of the editorial board for *Het Acute Boekje*, a key resource for internal medicine doctors in the Netherlands.

In 2020, he transitioned to Leiden University Medical Center (LUMC) to complete his internal medicine training under the mentorship of Prof. Dr. J.W. de Fijter and Prof. Dr. N.M. Appelman-Dijkstra. He then pursued a nephrology specialization under the supervision of Prof. Dr. J.I. Rotmans and Drs. A. Gaasbeek. During his nephrology residency, Jeetindra participated in the Nephrology Partnership for Advancing Technology in Healthcare (N-PATH), the first official

European Advanced Training Course in Diagnostic and Interventional Nephrology, implementing a multidisciplinary approach funded by Erasmus+ Knowledge Alliance. In this project he received advanced training from leading European nephrologists, together with fellow nephrology residents.

Upon completing his nephrology training in 2023, he joined the nephrology department at the LUMC as an internist-nephrologist. In November 2024, Jeetindra has come full circle—returning to the Haga Hospital, where he first developed his clinical skills. Through his continued clinical practice, research and education contributions, he remains dedicated to advancing nephrology and internal medicine to improve outcomes for patients.

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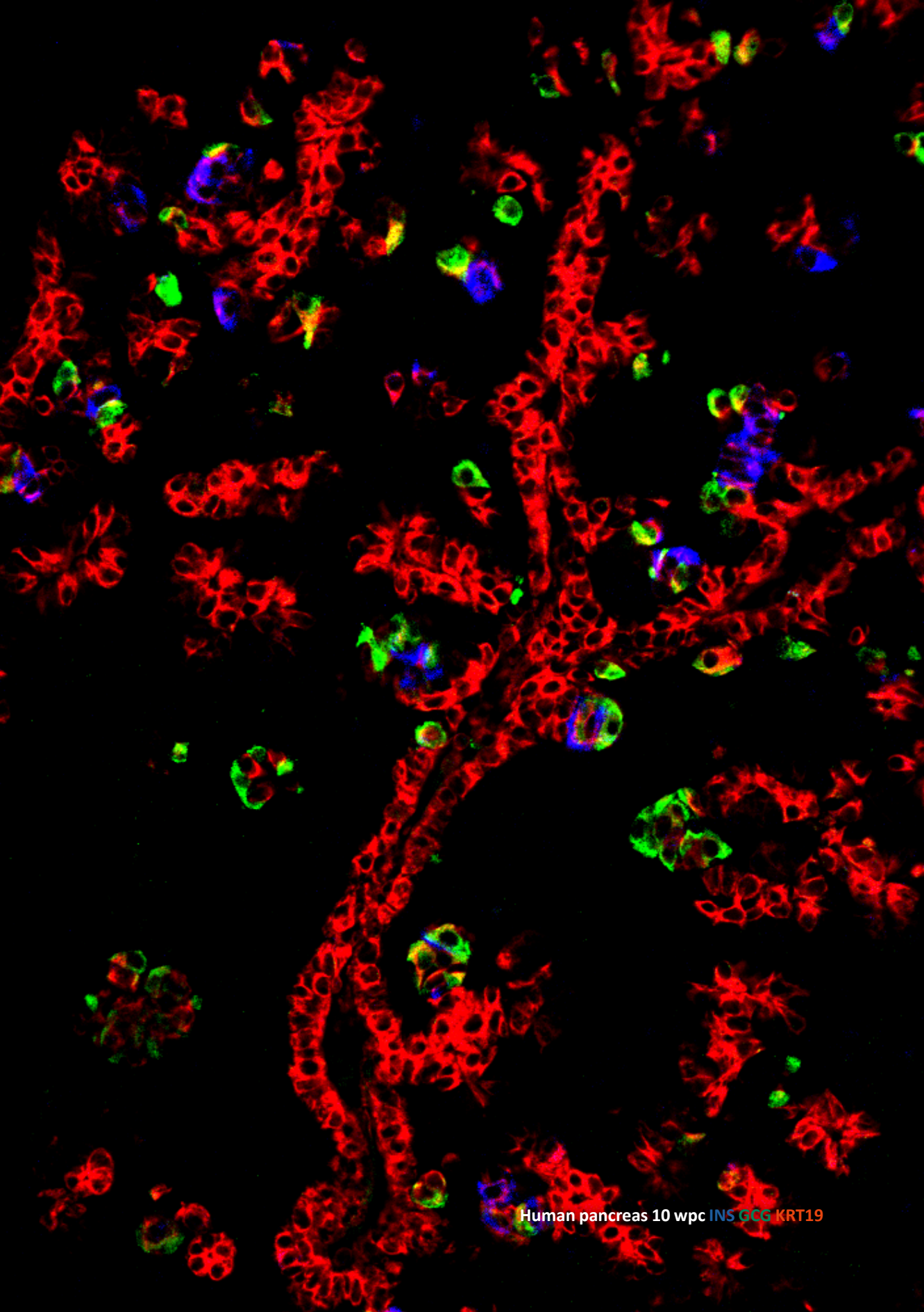
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भूर्भुवस्स्वः
तत्सवितुर्वरेण्यं
भर्गो देवस्य धीमहि
धियो यो नःप्रचोदयात्

All

Earth Intermedium Space
That light I utilize

On that extremely pure energy I concentrate
To lead my attentions towards bright clear rightnesses

Rigveda 3.62.10