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

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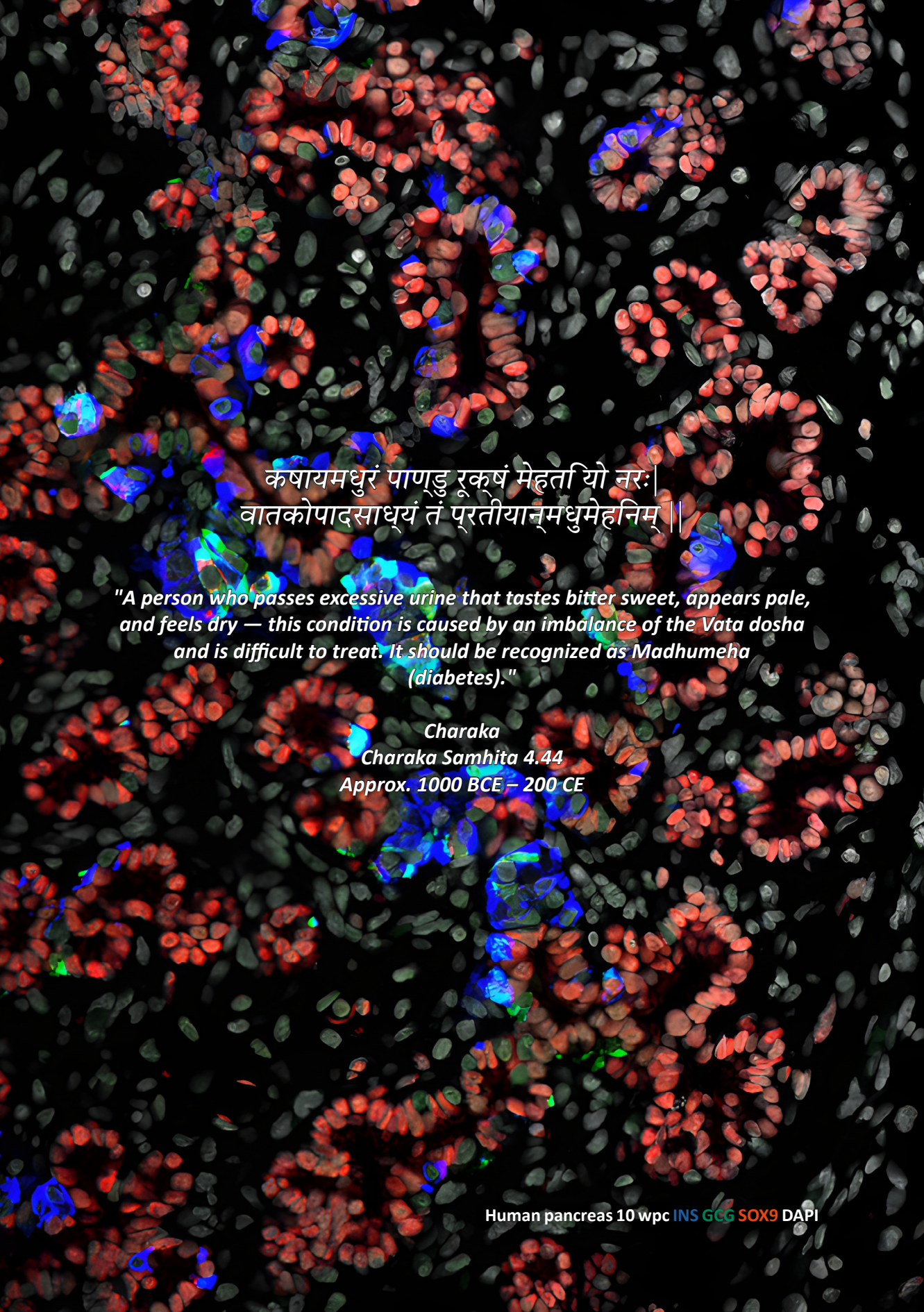

कषायमधुरं पाण्डु रूक्षं मेहति यो नरः|
वातकोपादसाध्यं तं प्रतीयान्मधुमेहनिम् ||

"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.

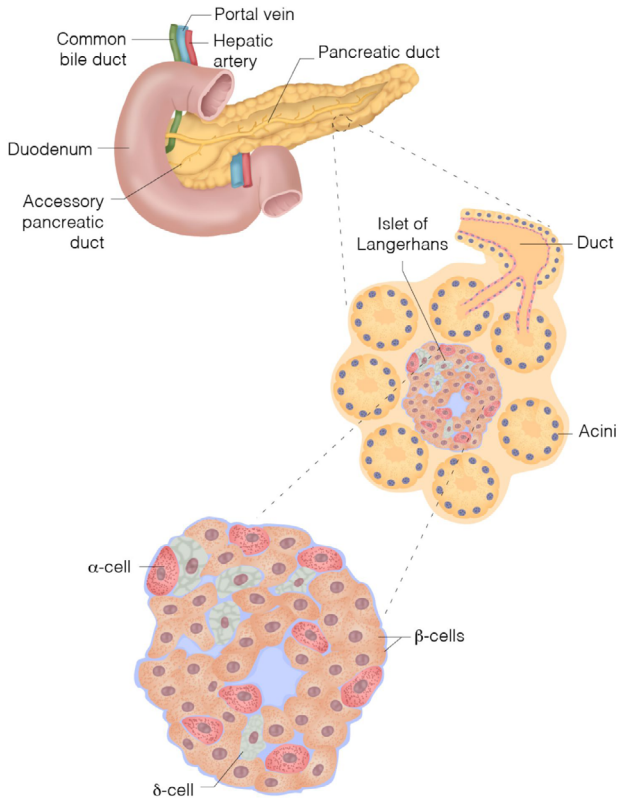

Figure 1. Pancreas anatomy and architecture of exocrine and endocrine tissue
Schematic overview of human pancreas location and showing the exocrine architecture formed by ducts and acinar cells. The endocrine gland is formed by the islets of Langerhans composed of different endocrine cells including 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].

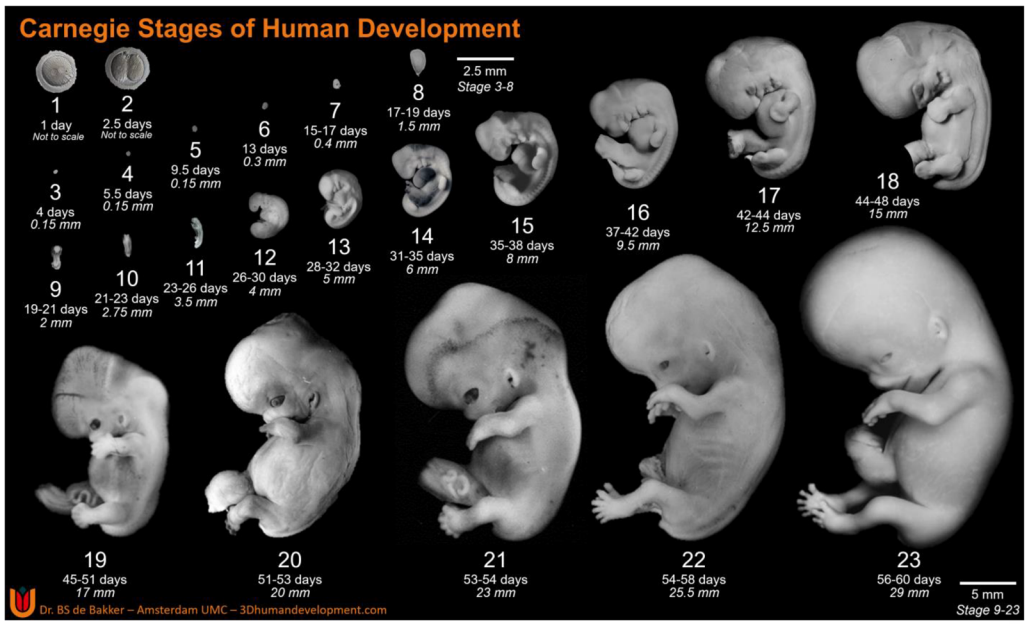

Figure 2. Carnegie stages of human development
Carnegie stages 3-10, dorsal view; stage 11 onwards, left lateral view (reprinted from Flierman et al. *Life*. 2023).

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.

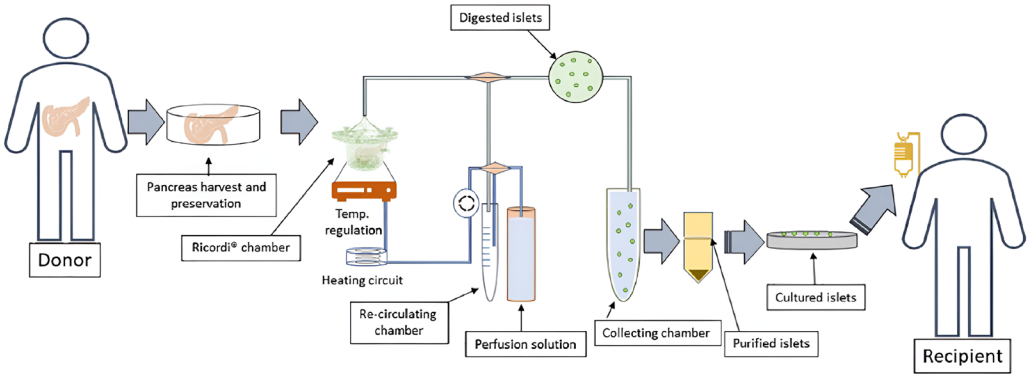

Figure 3. Islet isolation and transplantation process
Islet isolation and purification process using a pancreas and a Ricordi chamber to digest a human donor pancreas mechanically and enzymatically. The digest is then further purified using a gradient separation. Purified islets are cultured and afterwards 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.

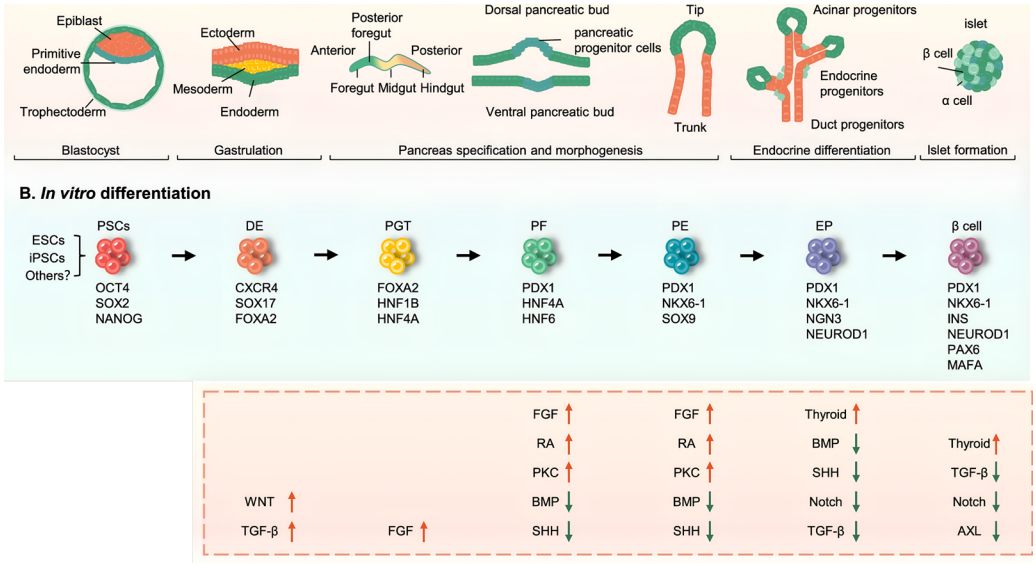

Figure 4. *In vitro* recapitulation of *in vivo* development using pluripotent stem cells and a stepwise differentiation protocol
Through mimicking ***in vivo*** pancreatic development, human pluripotent stem cells (PSCs) are differentiated into pancreatic lineage and eventually into beta cells. Examples 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.

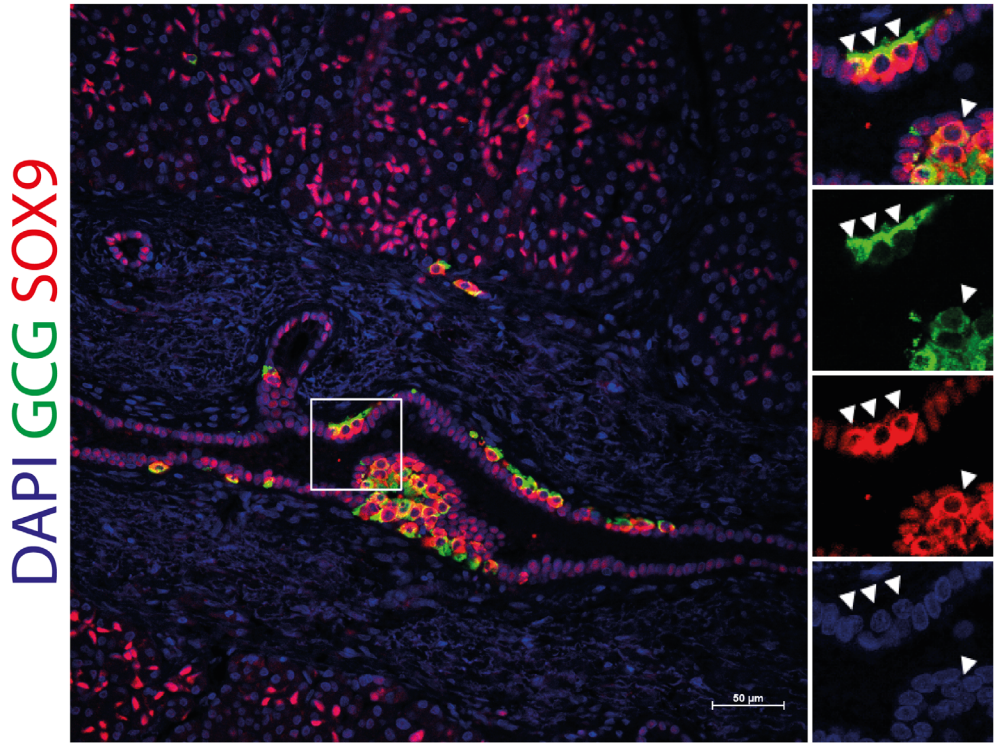

Figure 5. Human adult pancreatic tissue showing a duct with endocrine cells
Human adult pancreatic tissue with an immunostaining for SOX9 (red, duct marker) and glucagon (green, glucagon) and DAPI (blue, nuclear marker), showing endocrine cells in and around the duct. Scale: 50 um.

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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