The Ambiguous beta-cell : On the loss of human pancreatic beta-cell identity

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ON THE LOSS OF HUMAN PANCREATIC β-CELL IDENTITY

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THE AMBIGUOUS β-CELL
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Veritas Christo et Ecclesiae
(Original Harvard University motto)

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CHAPTER 1

GENERAL INTRODUCTION

Diabetes mellitus is amongst the leading causes of morbidity and mortality worldwide. Insulin-producing pancreatic β-cells are central in establishing adequate glucose regulation and loss of functional β-cells results in the development of diabetes. Although it was previously thought that fully differentiated cells cannot change phenotype, recent murine studies indicated that mature β-cells can change identity into other islet cells under conditions of (metabolic) stress. It has been hypothesized that this process is associated with β-cell dysfunction and loss of β-cell mass that occurs in diabetes. Moreover, it was shown that islet cells can convert into functional β-cells, providing a possible source to obtain new β-cells. It is not known whether adult human β-cells can change identity and whether mechanisms of islet cell conversion play a role in human diabetes. The aim of this thesis is to explore the stability of adult human β-cell identity and to investigate whether loss of β-cell identity plays a role in the pathophysiology of diabetes.

DIFFERENT FORMS OF DIABETES MELLITUS

Diabetes mellitus is characterized by glycemic dysregulation, caused by an imbalance in the secretion of the pancreatic hormones insulin and glucagon in relation to blood glucose levels. Presenting clinical symptoms typically consist of polyuria, polydipsia, weight loss and sometimes polyphagia (1). Two main diabetes subtypes are distinguished based on the pathophysiology; type 1 diabetes mellitus (T1DM) and type 2 (T2DM). While glucose levels in the range of 3.5-6 mmol/l are considered normal, a fasting serum glucose level of ≥7 mmol/l is diagnostic for diabetes (2). Damage to macro- and microvasculature caused by dysregulation of glucose homeostasis, makes diabetes the leading cause of renal failure and blindness in developed countries, and increases the risk of stroke and lower-limb amputations (3-5). The number of diabetes patients increased globally from 153 million in 1980 to 347 million in 2008 (6). In the Netherlands, these numbers correspond to ~1 million diabetes patients, of which approximately 90% has T2DM.

T1DM is caused by a selective autoimmune destruction of the pancreatic β-cells, leading to insulin deficiency (7). Even though genetic predisposition and environmental factors are implied in the pathogenesis, the precise triggers eliciting the autoimmune reaction are not known. The disease predominantly occurs in younger people that are in direct need of lifelong exogenous insulin replacement therapy (8). The pathophysiology of T2DM results from a complex interplay of genetic factors and lifestyle behaviours that are associated with obesity. This interplay causes peripheral cells such as muscle or adipose tissue to respond inadequately to normal insulin levels, a process known as insulin resistance (9). Pancreatic islets will normally adapt to increased serum glucose levels by increasing the number of β-cells and increasing insulin secretion of individual β-cells (10). Failure of β-cell mass adaptation can arise from a lack of newly formed β-cells or by increased rates of apoptosis. Furthermore, when glucose concentrations are chronically elevated, first phase insulin secretion becomes blunted (11). Together, β-cell dysfunction, loss of β-cell mass and insulin resistance with increased hepatic glucose production lead to worsening of hyperglycemia.

Less often occurring diabetes subtypes include the distinct forms of Maturity-Onset Diabetes of the Young (MODY), caused by autosomal dominant inherited monogenic defects in essential β-cell genes (12). Furthermore, diabetes can be induced by pregnancy (gestational diabetes), certain drugs amongst which immunosuppressive drugs, exocrine pancreatic disorders such as cystic-fibrosis or severe pancreatitis, or in the context of syndromal disorders (1).

PANCREATIC ISLET PHYSIOLOGY

The pancreas consists of two glandular compartments that exert an exocrine and endocrine function. The exocrine gland plays a role in digestion by the secretion of digestive enzymes such as amylase or lipase and secretion of bicarbonates via the pancreatic duct system into the duodenum (13). The endocrine compartment occupies 1 to 2% of the pancreas and consists of approximately 1 million cell clusters that are scattered throughout the exocrine pancreas, so called islets of Langerhans.

Islet hormones

Pancreatic islets are highly vascularized micro-organs that secrete hormones directly in the bloodstream. The most frequent cell types in human islets are insulin-producing β-cells, representing 50-70% of islet cells, and glucagon-secreting α-cells (20-30%). The remaining islet cell types either produce somatostatin (δ-cells) or pancreatic polypeptide (PP-cells), while ghrelin-producing ε-cells can be found mainly during development and at lower frequency in adult human islets (14). The unique architecture of human islets has been emphasized by several studies (15-17). The majority of β-cells (~70%) are in direct contact with α-cells, and are in contact to the microvasculature to facilitate hormone secretion. In contrast to the human situation, mouse islets have a distinct architecture where β-cells comprise ~80% of islet cells and form the islet core surrounded by other islet cells (mainly α-cells) in the periphery (18).

Insulin protein is exclusively produced in β-cells. Upon transcription, the mRNA is translated into the protein preproinsulin. Post-translational processing in the endoplasmic reticulum includes proper folding of the protein and the formation of disulphide bonds between the A and B chains (19). Subsequently, the C chain will be cleaved off in the Golgi apparatus by prohormone convertase 1/3 (PC1/3), resulting in C-peptide and monomeric insulin (20). Mature insulin is then stored in secretory granules as stable hexamers surrounding 2 zinc atoms at a local pH 5.5. Under high glucose conditions, β-cells secrete insulin into the surrounding neutral pH leading to dissociation of the hexamers (19).

The prohormone proglucagon can be processed into different products following enzymatic cleavage. In α-cells, the abundance of PC2 results primarily in the 29 amino acid long peptide hormone glucagon. In intestinal L-cells, proglucagon is cleaved via PC1/3, resulting in incretin hormones such as glucagon-like peptide-1 (GLP-1), GLP-2 and glicentin (21).

Hormone secretion

Accurate glucose sensing and insulin secretion are essential to maintain glucose levels within a narrow physiological range. These processes are established by an intricate network of glucose transporters and enzymes in combination with the dense capillary network. During normal glucose levels, the β-cell membrane potential is negatively charged. Increased blood glucose concentrations lead to increased glucose uptake by β-cells, in animals via the glucose transporter Glut2 and in humans more likely via Glut1 (22). Glucose will be phosphorylated by glucokinase and used by mitochondria to produce ATP (23). The higher ATP/ADP ratio leads to closure of K_{ATP}-channels, which increases the membrane resistance and in turn leads to depolarization and opening of voltage-gated Ca^{2+}-channels. The increased intracellular calcium concentration triggers fusion of insulin granules with the plasma membrane inducing exocytosis (Fig. 1) (23). Circulating insulin binds the insulin receptor on its target cells and signals intracellularly via the insulin receptor substrate proteins (IRS) and phosphatidylinositol 3-kinase (PI3K) (24). Insulin signalling stimulates glucose uptake mainly in the liver, muscle and adipose tissue via activation of the glucose transporter Glut4, decreases hepatic glucose production (gluconeogenesis and glycogenolysis) and induces glycogen storage and lipid synthesis.

Glucagon secretion is generally triggered by low glucose levels, but can also be stimulated by circulating amino acids or lipids. Although the mechanisms of glucagon granule secretion are not fully understood, it is controlled by a combination of intrinsic K_{ATP}-channel dependent glucose sensing (hypoglycemia stimulates glucagon secretion), neuronal signalling (autonomic nervous system) and paracrine mechanisms (e.g. insulin, somatostatin and Zn^{2+}) (25;26). Similar to insulin secretion, glucagon exocytosis depends on electrical activity and is a Ca^{2+}-dependent process. However, using the K_{ATP}-channel stimulator diazoxide, it was shown that glucagon secretion is inhibited both when the K_{ATP}-channel activity is too low or too high and has a specific bell-shaped optimum (27). Circulating glucagon binds to the glucagon receptor, signalling inwards via cyclic AMP and protein kinase A (28). Glucagon works counterregulatory to insulin by stimulating glucose production in the liver via gluconeogenesis and glycogenolysis, but also exerts negative feedback on glucagon secretion itself and induces insulin secretion (29).

In conclusion, mature α- and β-cells are highly specialized endocrine cells that, in order to be fully functional, depend on a defined set of proteins that allow cell-specific actions such as hormone processing, glucose sensing and hormone secretion (including granule formation, transport and release). For the sake of this thesis, we will define functional islet cells as those that express and secrete hormones in a physiological manner to maintain normal glucose levels.

EMBRYONIC DEVELOPMENT OF THE PANCREAS

Pancreas development is first noticeable by the formation of endodermal outgrowths on the foregut endoderm, called the dorsal and ventral pancreatic buds. These branching epithelial structures start to arise at embryonic day (E) 9.5-10 in mice, comparable to

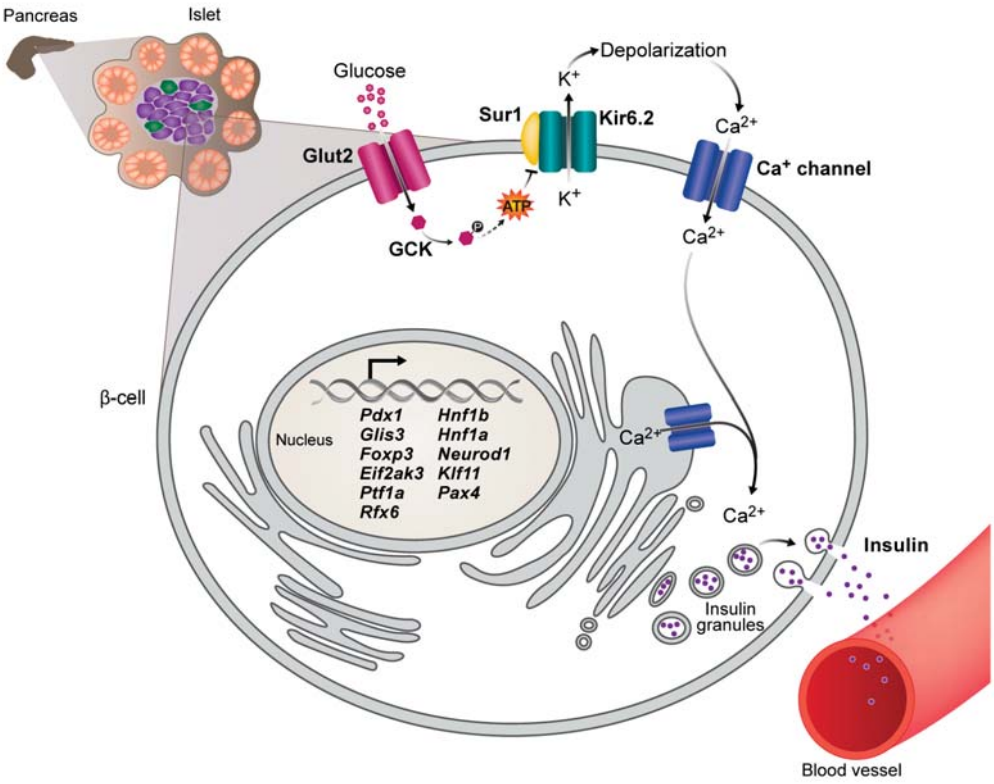

Figure 1. Glucose stimulated insulin secretion. In pancreatic β-cells, glucose stimulated insulin secretion starts by transportation of glucose into the cell via glucose transporters (Glut2). Glucose is phosphorylated by glucokinase (GCK) into glucose-6-phosphate and eventually converted into ATP. The increased ATP/ADP-ratio will trigger the closure of ATP-dependent potassium channels (subunits Sur1 and Kir6.2) which will lead to membrane depolarization and opening of calcium channels. The rise in intracellular calcium levels stimulates the exocytosis of insulin-containing granules into the bloodstream. Transcription factors that are essential for proper β-cell function are noted in the nucleus of the cell. *Figure adapted from Pagliuca and Melton with permission (113).*

a human embryonic age of 2-3 weeks (30;31). The early transition from definitive endoderm to pancreatic endoderm is mainly initiated by the transcription factor pancreatic duodenal homeobox-1 (Pdx1) (32), and null mutations in this gene result in pancreatic agenesis (33). Epithelial proliferation and remodelling follows a tip-trunk segregation. Acinar structures will derive from the cells in the tip of the proliferating epithelium, while ductal and endocrine cells are formed from bipotent progenitors in the trunk region starting at E11.5 (34). Expression of neurogenin3 (Ngn3) indicates commitment of trunk cells to the endocrine lineage (35). These precursor cells subsequently delaminate from the epithelium to form islet-like structures (36). In humans, vascularised structures in the parenchyme containing small islet aggregations appear from week 14 to 15, whereas adult-like islets containing all four endocrine cell types and a fine capillary network are observed by the beginning of the second trimester (31).

Lineage-specification within the endocrine compartment is controlled by the expression of specific transcription factors (Fig. 2) (35). Studies on genetically mutant mice have shown that the presence or absence of single transcription factors can skew endocrine progenitors towards a specific hormonal fate. For example, Nkx2.2 mutant mice show an increased number of ghrelin-containing ε-cells and a reduction in α-, β- and PP-cells (37). Deletion of Pax4 results in the loss of β-cells and an increased proportion of both α-cells and ε-cells, while the opposite holds true for Arx null mutant mice, in which the number of β-cells and δ-cells increases at the expense of α-cells (38;39). Finally, Arx/Pax4 double mutant mice show a massive number of δ-cells, but no α- or β-cells (40).

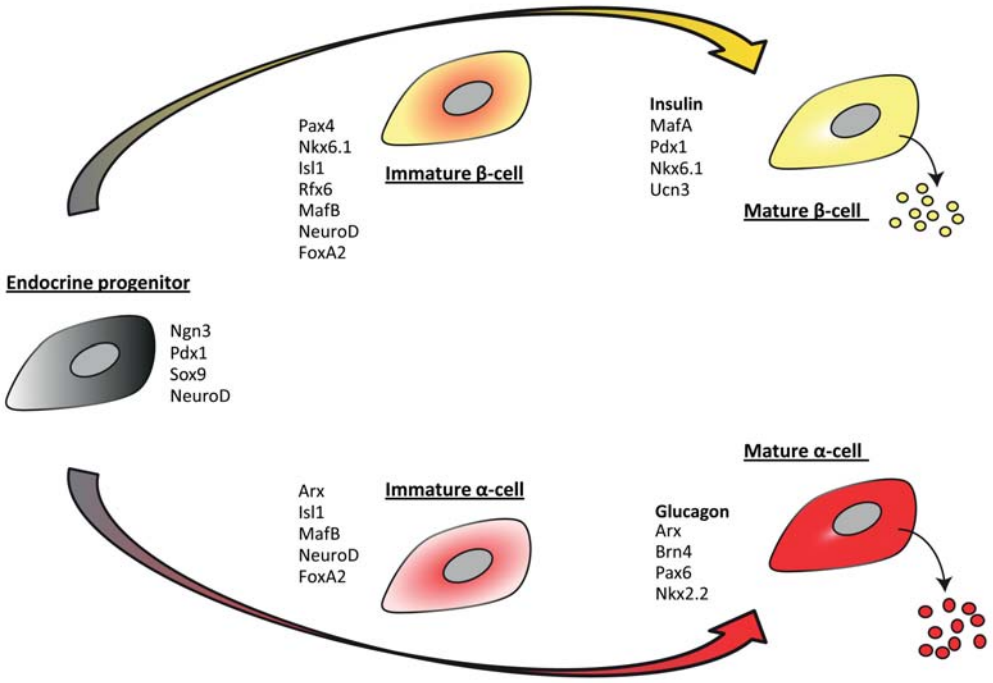

Figure 2. Lineage differentiation of α-cells and β-cells. Development of α- and β-cells is regulated by a complex interplay of specific transcription factors enabling functional maturation. Note that some factors (such as Pdx1) play a role in early cell development as well as in mature β-cells. Furthermore, despite their opposing functions in glucose metabolism, α- and β-cells share the expression of many transcription factors during development (such as NeuroD, FoxA2 and MafB).

Further maturation to form functional endocrine cells takes place after lineage specification. Although fetal human islets already secrete insulin during the first half of gestation (~week 17-20), implying the development of glucose sensing and hormone processing, the typical biphasic insulin secretion occurs in the early postnatal period (41;42). The developmental processes enabling glucose induced insulin secretion (GSIS) are not fully elucidated, but important roles have been identified for transcription factors

MafA, MafB, Pdx1, FoxA2 and NeuroD using genetically modified mouse models. High expression of MafA appears critical for the maturation of functional islet cells, also when the insulin-producing cells are derived from human embryonic stem cells (43). Accordingly, the loss of MafA during mouse development did not reduce the numbers of endocrine cells, but rendered them glucose-intolerant after birth (44). MafB is expressed both in α- and β-cells during development. During postnatal maturation in mice, MafB becomes restricted to α-cells, while MafA remains restricted to β-cells (45;46). While Pdx1 plays an essential role in the development of the early pancreas, its role is equally essential during further development and β-cell maturation. Both MafA and Pdx1 recognize specific binding sites that are present on promoters of essential β-cell genes such as *Insulin, Nkx6.1, Glut2*, *Glucokinase*, the zinc transporter *Slc30a8* and K_{ATP}-channels such as Kir6.2 (*Kcnj11)* (47;48). Accordingly, mice that lack Pdx1 specifically in β-cells become diabetic during adulthood and show impaired expression of both Glut2 and insulin (49). Foxa2 is essential in the maturation of both α- and β-cells. Initial islet cell specification is not perturbed in conditional *Foxa2*$^{-/-}$ mice (unconditional homozygous *Foxa2* null mice die during midgestation), but a 90% reduction in glucagon expression occurs in α-cell specific KO mice with a complete lack of the prohormone convertase PC2 (50). β-cell specific FoxA2 KO mice die shortly after birth because of (amongst others) the lack of K_{ATP}-channel expression (Kir6.2) and sulfonylurea receptor 1 (SUR1), resulting in hyperinsulinemic hypoglycemia (51;52). Finally, inactivation of NeuroD in β-cells during development resulted in severe glucose intolerance because of impaired GSIS, even though the islets still had half of the amount of insulin present (53).

Altogether, the development of mature islet cells depends on the complex interaction of specific transcription factors at specific time points. These factors will eventually activate the genes that constitute functional hormone secreting cells.

THE ROLE OF α- AND β-CELLS IN THE PATHOPHYSIOLOGY OF DIABETES

Whereas the architecture of pancreatic islet cells and cellular processes is delicately organized during normal development, it becomes disturbed in diseased state. In T1DM, islet pathophysiology is generally characterized by the autoimmune destruction of β-cells (Fig. 3) (54). However, recent studies indicate that T1DM is not a static disease after initial β-cell destruction. Pathological features of T1DM such as insulitis and HLA class I hyperexpression can still be present in patients with longstanding disease and usually occur in a multifocal pattern throughout the pancreas (55). Keenan et al showed that 67.4% of patients with longstanding diabetes (>50 years) have residual β-cell function defined as measurable serum C-peptide levels (56). Besides the primary profile of β-cell pathology in T1DM subjects, patients also have defects in α-cell regulation. Already in recent-onset patients, impairment in the suppression of glucagon levels in response to hyperglycemia is apparent, even though fasting plasma glucagon levels are similar to healthy controls (57). Moreover, the glucagon response to hypoglycemia can become blunted early in the course of type 1 diabetes (58). Possible explanations are that the local (intraislet)

insulin-mediated α-cell suppression is lost in T1DM because of the insulin deficiency or that the autoimmune process may change the microenvironment so that α-cells become unresponsive to paracrine insulin signalling (59).

Though less extreme than in T1DM, changes in islet architecture are distinct in T2DM (Fig. 3). An initial phase of β-cell mass compensation occurs following chronic exposure to high glucose load and insulin resistance, but final β-cell decompensation will result in glycemic dysregulation (11). Although β-cell dysfunction (disturbed GSIS) is central in the decompensation, marked changes in islet architecture can also be found. First, several studies on post-mortem sections or isolated islets of T2DM donors have shown a decreased β-cell mass and increased rates of β-cell apoptosis compared to non-diabetic controls (60-63). Although the importance in the etiology of T2DM is since long debated, the fact that β-cell mass is decreased in T2DM is now generally accepted (64;65).

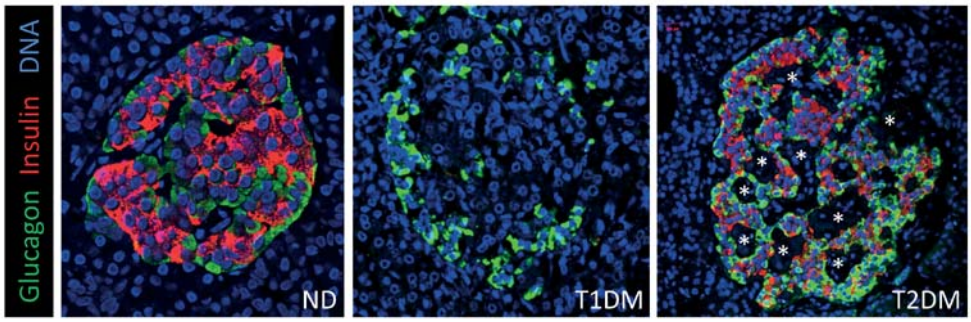

Figure 3. Changes in islet architecture related to diabetes. Representative images showing the islet architecture in a pancreatic biopsy of a non-diabetic donor (ND) and a donor with T1DM or T2DM stained for the hormones insulin (red) and glucagon (green). ND islets (left panel) display a mixed architecture of α- and β-cells containing a higher proportion of β-cells. Islets in T1DM (middle panel) are characterized by destruction of β-cells while α-cells remain. Islets in T2DM display a reduced number of β-cells and may show degradation of islet architecture by the formation of amyloid plaques (indicated by asterisks).

Glucagon dysregulation plays an important role in T2DM as well. On the islet level, although a relative increase in α-cells has been reported in T2DM (15;63;66;67), a recent study measuring absolute α-cell mass did not find this difference (29). Functionally, glucagon regulation is clearly abnormal under hyperglycemic conditions, and recent studies support that fasting plasma glucagon levels are elevated as well (68-71). The paradoxically elevated glucagon levels during hyperglycemia contribute to increased hepatic glucose production, thereby worsening hyperglycemia (72). Furthermore, the formation of islet amyloidosis is a hallmark of T2DM islets. While islet amyloid polypeptide (IAPP) is normally stored in insulin granules and co-secreted, its aggregation into β-sheets leads to the formation of islet amyloid plaques (73). Even though it is controversial whether amyloid is a cause or a consequence of β-cell dysfunction and apoptosis, there is a clear association with T2DM (66;74;75). Moreover, it has been shown that IAPP oligomers can be toxic *in vitro* and can elicit an inflammatory response (76;77).

In summary, though the pathophysiology of T1DM and T2DM are clearly different, islet architecture and islet cell function are affected in both types of diabetes. Moreover, both α-cell and β-cell function are impaired, resulting in hyperglycemia and a poor response to hypoglycemia.

DIABETES TREATMENT USING INSULIN REPLACEMENT OR β-CELL REPLACEMENT THERAPY

Since both types of diabetes are characterized by a failure of β-cells leading to hyperglycemia, current therapy is mainly focused on the replacement of β-cell function. The common goal for both T1DM and T2DM is to replace the (relative) insulin shortage to prevent shortterm dysregulation and longterm complications (78).

T1DM requires lifelong insulin replacement therapy. This can either be achieved by manual injection of long- and short-acting insulin or by insulin pump therapy. New developments that combine continuous glucose monitoring with smart algorithms can automatically titrate the required amount of insulin, thereby alleviating the burden of repeated glucose measurements (79).

Since the pathogenesis of T2DM reaches beyond the pancreatic islets, therapy is aimed at several targets altogether resulting in preservation of β-cell function and action. The action of clinically approved drugs include the inhibition of hepatic glucose production (biguanides), ameliorating peripheral insulin sensitivity (biguanides, thiazolidinediones), increasing insulin levels either irrespective of plasma glucose (sulphonylurea derivatives, exogenous insulin) or glucose-dependent (incretin-based therapy), or increasing glucose excretion (SGLT2 inhibitors) and often a combination of these modes of action (80).

Tight glucose regulation using sulphonylurea derivatives or insulin injections has the down side of an increased risk of hypoglycemia. The combination of attenuated glucagon and epinephrine responses causes the clinical syndrome of defective glucose counterregulation (81). Strict glycemic control therefore significantly increases the risk of hypoglycemic events both in patients with T1DM and T2DM that are under insulin therapy (78;82). The accompanying fear of hypoglycemia provides a psychological barrier that can have further negative impact on diabetes management (83).

Current β-cell replacement therapy; pancreas or islet transplantation

For patients that suffer from labile glucose regulation and its long-term complications despite optimization of insulin therapy, β-cell mass replacement is a therapeutical option. The concept of transplanting pancreas grafts or extracts is already under investigation since 1894 (84). Currently, β-cell replacement is achieved by transplantation of a whole donor pancreas or of isolated islets. In the latter case, the pancreas is first enzymatically digested and islets are separated from the non-islet tissue using a density gradient. The purified islet preparation can then be cultured and transplanted, commonly by infusion in the portal vein (85).

Whole pancreas transplantation can be performed as a simultaneous pancreas and kidney transplant (SPK), pancreas after kidney transplantation (PAK) or pancreas transplantation

alone (PTA). Graft survival rates after 3 years currently range from 75-85% with SPK showing the most favourable results (86). Besides replenishing the functional β-cell mass, pancreas transplantation normalizes glycemic control by restoring glucagon secretion, restoring the epinephrine response to hypoglycemia and by normalizing hepatic glucose production (87-89). Pancreas transplantation is an invasive procedure and the associated complications increase recipient morbidity and mortality. Amongst the most common complications requiring relaparotomy belong pancreas graft thrombosis, intraabdominal bleeding and deep wound infections (90).

For islet transplantation until the year 2000, only 12% of transplantations resulted in insulin independence for a time period of more than a week. It was only since the so-called Edmonton protocol was developed that intraportal islet transplantation was considered to be a successful and promising experimental therapy (91). The main reasons for the success of this protocol were found in the greater number of islets transplanted and the new immunosuppressive regimen that was applied. While high doses of glucocorticoids were commonly used (known to be diabetogenic), the Edmonton study developed a glucocorticoid-free regimen containing sirolimus, low-dose tacrolimus and daclizumab (a monoclonal antibody against the interleukin-2 receptor), inhibiting T-cell proliferation. After one year, insulin independence was achieved in 44% of patients, while 28% had partial graft function (92). More than 750 patients worldwide received an intraportal islet transplantation ever since, either as islet transplantation alone or following kidney transplantation (93). Criteria for patient eligibility include glycemic lability despite treatment optimization, recurrent hypoglycemic episodes and hypoglycemia unawareness. Follow-up data from the Edmonton cohort recently showed that 15% of transplanted patients was insulin independent after nine years (93). In >70% of these patients persistent C-peptide secretion and complete protection from hypoglycemic episodes was apparent, usually through the use of multiple (two or three) donors (93). A cohort from France showed similar results using the Edmonton protocol, reporting 57% insulin independence after 3.3 years (94). Furthermore, a recent islet-after-kidney transplantation cohort from Leiden showed the presence of C-peptide in 92% of patients after a 2-year follow-up period (95). Islet transplantation improves insulin sensitivity, is associated with restoration of hepatic glucose production and some but not all reports describe an improved counterregulatory reaction (96-99). The relatively uninvasive nature of percutaneous intraportal islet transplantation is associated with few procedure-related complications which include partial portal vein thrombosis and liver bleedings (92).

A current drawback of islet transplantation is the limited number of surviving islet cells directly following transplantation, estimations referring to 20-40% of the infused islet cell mass (100). The majority of this loss likely occurs directly following infusion in the portal vein due to hypoxia in the venous system, hyperglycemia and instant blood-mediated inflammatory reactions (101). Moreover, even though immunosuppressive regimens have improved, many of the drugs used are still harmful to islets. To improve survival of transplanted islets, several research groups focus on the production of encapsulation devices that serve as an impermeable barrier to immune cells while permitting nutrient diffusion and actively recruiting vascularization (102;103).

1 PRODUCING DE NOVO β-CELLS FROM STEM OR PROGENITOR CELLS

Considering the ~1 million diabetes patients, even the most promising scenario in which every offered donor organ in the Netherlands would be used to transplant one patient, the number of transplantations would not exceed ~250 per year. Clearly, the shortage of donor organs limits the widespread use of β-cell replacement therapy, and calls for novel sources of β-cells.

New β-cells could either be derived by directed differentiation of (pluripotent) stem cells or progenitor cells, transdifferentiation or lineage conversion of other cell types, or by replication of pre-existing β-cells (Fig. 4) (104). While β-cell replication is a potent mechanism in murine models (105), very low levels are present in mature human β-cells (60;106;107). For β-cell replication to become a promising therapeutic approach, mechanisms that prevent human β-cells from proliferating should first be uncovered and circumvented in a safe manner (108;109).

Pluripotent stem cells (PSC) have the potency to self-renew indefinitely and to differentiate into all 3 embryonic germ layers (endoderm, ectoderm and mesoderm). Commonly used PSC include human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC, reprogrammed adult somatic cells such as skin fibroblasts) (110;111). Since the differentiated state of PSC is comparable to that of the early embryo, differentiation protocols to obtain β-cells recapitulate embryonic development by the addition of specific growth factors and extracellular matrices (112;113). Standardized protocols initially lead to the formation of immature insulin-producing cells *in vitro* that become further maturated (i.e. glucose-responsive) within 3-4 months following transplantation into immunodeficient mice (43;114). Recently, extended differentiation protocols for hESC and hiPSC obtained cells that secreted insulin in comparable amount to adult β-cells in response to multiple glucose challenges *in vitro* and prevented or reversed hyperglycemia in mice (115;116). Remaining hurdles for clinical application include the ethical discussion (mainly on the use of hESC), the development of robustly defined protocols and safety issues concerning genetic stability and the risk of teratoma formation (117).

Adult stem cells or organ-specific progenitor cells are committed to differentiate into the cell types of the organ in which they reside. Typical examples of active adult stem cells reside in the intestine and the skin, where ongoing tissue renewal takes places (118). Several reports have used lineage tracing methods to show that cells from the pancreatic ductal compartment contribute to the replacement of functional β-cells following injury in adult mice (119;120). Upon injury by partial duct ligation, the adult stem cell marker *Lgr5* was expressed in regenerating pancreatic ducts and isolated cells could be expanded *ex vivo* (121). Since these studies were questioned using alternative lineage tracing strategies, the debate on the actual presence and location of pancreatic progenitor cells in adult pancreas is ongoing (34;122;123). However, studies on human cadaveric donor organs have noted more insulin-expressing cells in the ductal epithelium of pancreas from obese donors and pregnant women compared to controls (60;124). Moreover, culture of human islet-depleted tissue remaining after islet isolation formed islet-like structures that showed insulin expression (125-127).

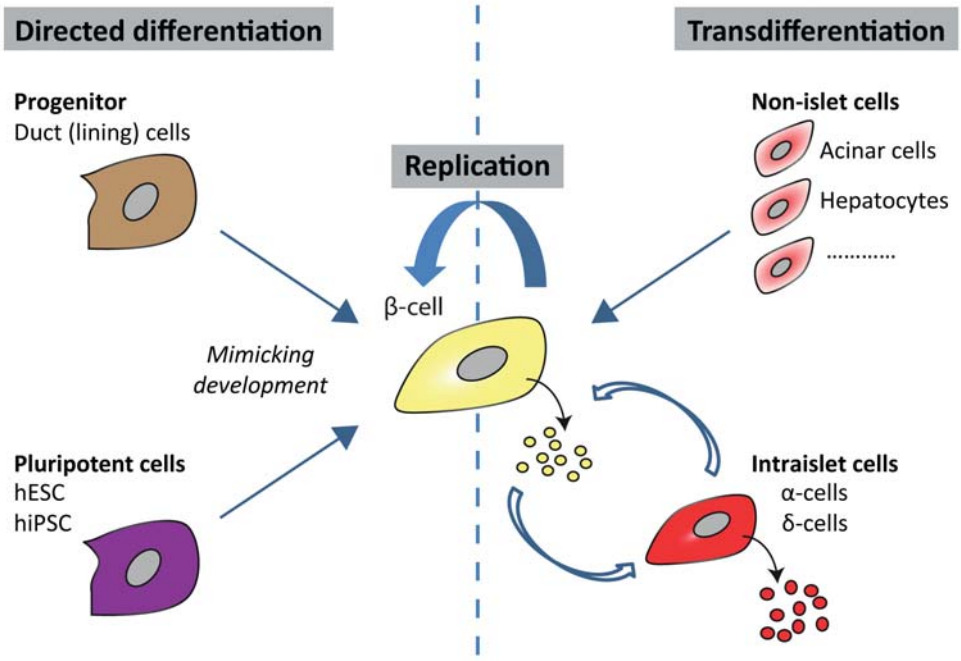

Figure 4. Strategies to generate new β-cells. Directions to generate new β-cells are divided as directed differentiation, replication or transdifferentiation. Directed differentiation makes use of pluripotent stem cells or adult progenitor cells. Differentiation protocols aim to mimic normal development by using combinations of growth factors, small molecules and matrix components to obtain new β-cells. Replication of pre-existing β-cells aims to identify small molecules that trigger proliferation of endogenous β-cell mass. Transdifferentiation relates to the reprogramming or conversion of terminally differentiated cell types. Conversion into insulin-producing cells has been achieved from related non-islet cells (acinar cells and hepatocytes) and from islet-derived cells (α- and δ-cells). hESC, human embryonic stem cells; hiPSC, human induced pluripotent stem cells.

Other groups reported the finding of rare stem cells that were isolated from adult mouse and human pancreas and differentiated into β-cells and neurons (128). Genetic lineage tracing revealed that these cells were derived from initially insulin-expressing cells (129), it is therefore not clear whether these cells represent true stem cells *in vivo* or β-cells that were dedifferentiated *in vitro* as shown previously (108;130).

Finally, mesenchymal stem cells (MSC) have been investigated to develop new β-cells. MSC are easily retrievable from bone marrow, adipose tissue or umbilical cord blood, can be expanded for multiple passages and can differentiate into blood, bone and adipose tissue cells (131). Initial reports described that MSC derived from bone marrow or splenocytes had the potential to differentiate into β-cells (132-134). Unfortunately, these studies could not be confirmed by several other groups (135;136). Current studies using MSC are focused on their angiogenic potential and evident role in immunomodulation in combination with allotransplantation (137;138).

Altogether, the application of stem cell technology holds great promise for future clinical application and important steps are being made especially in the differentiation of

pluripotent cells. Remaining hurdles including reproducibility of differentiation protocols and safety issues will need to be addressed in the near future.

DEDIFFERENTIATION AND TRANSDIFFERENTIATION

Stem cell differentiation has commonly been viewed as the ongoing commitment of a progenitor towards a terminally differentiated (unipotent) cell (139). This model is challenged by the finding that a fully differentiated cell can convert into another, a process called transdifferentiation or direct lineage conversion, or can take a step back to a less differentiated state, called dedifferentiation (140;141).

Dedifferentiation has been described in cardiac regeneration in zebrafish. After 20% removal of the zebrafish cardiac ventricle, regeneration occurred by dedifferentiation of cardiomyocytes, thereby enabling these cells to proliferate again before redifferentiating and restoring the healthy cardiomyocyte pool (142). A similar process takes place after Schwann cell damage in peripheral nerves. Dedifferentiation into a precursor cell type allows proliferation while subsequent redifferentiation into mature Schwann cells provide remyelination (143). Intriguingly, the salamander Ambystoma mexicanum (or axolotl) can reconstitute a fully functional limb after injury or amputation. Cells that are adjacent to the wound dedifferentiate and form a blastema that consists of tissue specific progenitor cells that proliferate and eventually redifferentiate to create a regenerated limb (144).

In these examples, the injured cell type itself provides regeneration via dedifferentiation and proliferation. Transdifferentiation or direct lineage conversion describes the process by which another differentiated cell type takes on the function of the injured cell type, either directly or via a dedifferentiation step (Fig. 5). Natural transdifferentiation has been described in the process of lens regeneration in adult newts. Damage to the lens results in dedifferentiation of pigment epithelial cells that normally reside in the dorsal iris, and subsequent transdifferentiation of these pigment cells regenerates the lens (145). In experimental models, transdifferentiation usually requires the genetic introduction of specific transcription factors or miRNAs in cells that are (in lineage) closely related to the desired cell type (146). In the pancreas, studies on transdifferentiation are either focused on obtaining new β-cells or understanding how β-cells maintain a stable identity.

Direct lineage conversion to obtain new β-cells

Several cell types have been used to study direct lineage conversion into β-cells. In mouse and isolated human hepatocytes, genetic activation of Pdx1 induced their conversion into insulin-producing cells that were functional *in vivo* (147;148). Zhou et al. accomplished to transdifferentiate murine pancreatic acinar cells *in vivo* into β-like cells using adenoviral delivery of 3 essential transcription factors; Ngn3, Pdx1 and MafA (141). More recently, this same cocktail of transcription factors was used to induce functional insulin-secreting cells from antral stomach cells (149). Also, rodent acinar cells were shown to differentiate into insulin-containing β-cells *in vitro* following cytokine treatment (150-152). Recent work added that a transient cytokine treatment *in vivo* could induce β-like cells from acinar cells and thereby reverse alloxan-induced hyperglycemia (153).

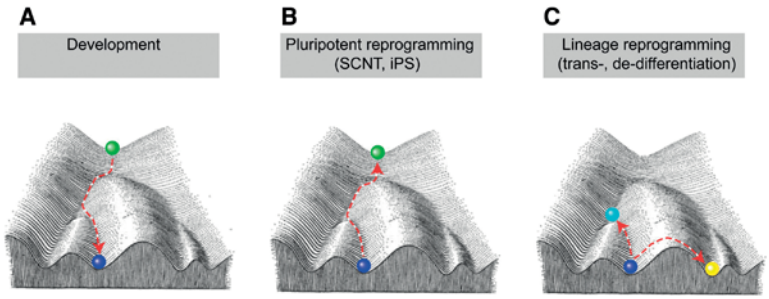

Figure 5. A landscape of development and reprogramming. *A:* In development, a pluripotent stem cell (green ball), rolls down bifurcating valleys, which represent all possible developmental paths. The cell differentiates into a mature cell (blue ball) at the bottom of the valley. *B:* During pluripotent reprogramming, including somatic cell nuclear transfer (SCNT) and formation of induced pluripotent stem (iPS) cells, the entire developmental process is reversed, and a differentiated cell is returned to a pluripotent state. This is represented by the ball rolling from the bottom of the valley backward to the top. *C:* Lineage reprogramming includes dedifferentiation and transdifferentiation, where a mature cell takes a step backward to a progenitor stage (cyan ball) or converts directly to another mature cell (yellow ball). *Figure adapted from Zhou and Melton with permission (141) .*

Intraislet cell conversion into β-cells has been observed in several lineage tracing studies in mice. Collombat et al. showed that overexpression of Pax4 in α-cells induced α-cell hyperplasia and conversion into β-cells, both during development and in adult mice (154;155). The ongoing α-cell to β-cell conversion resulted in a shortage of α-cells, that was in turn replenished by $Ngn3^{+}$ progenitor cells derived from the ductal epithelium, indicating that transdifferentiation and progenitor cell differentiation may act in concert (154). In addition, forced expression of Pdx1 in Ngn3-positive endocrine progenitors induced α- to β-cell reprogramming postnatally resulting in absence of α-cells (156). A report from Thorel et al. showed α-cell to β-cell transdifferentiation in a model of near-total β-cell ablation (157). In this study, the diphtheria toxin receptor was specifically overexpressed in β-cells so that administration of the diphtheria toxin lead to targeted β-cell destruction (>99%). Using α-cell lineage tracing, a large but variable fraction (~30-80|%) of the newly formed insulin-positive cells appeared to originate from a small subset (~5%) of α-cells (157). Using the same experimental model, it was recently shown that juvenile mice undergo massive reprogramming of δ-cells, but not α-cells, upon near total β-cell ablation, indicating that the transdifferentiation mechanism may differ with the age of the organism (158). Finally, partial α- to β-cell reprogramming was observed following treatment with a histone methyl transferases inhibitor, broadly affecting the epigenetic methylation signature (159).

These studies indicate that direct lineage conversion provides an opportunity to obtain new β-cells and can be achieved by shifting the balance of transcription factors in developmentally related cells. While all these studies are based on animal models, it is not known whether human islet cells have similar characteristics.

1 MAINTENANCE OF MATURE β-CELL IDENTITY

As β-cells are liable to loss of function under conditions of metabolic or inflammatory stress, it is essential for these cells to actively maintain functionality. Whereas embryonic β-cell development is a dynamic process that requires specific signals at specific time points, mature β-cells depend on a stable transcriptional program and supportive signalling from the microenvironment (113). The exact mechanisms that β-cells employ to maintain their differentiated identity are not fully understood, but recent studies have shed light on this issue.

Several experimental studies indicate that β-cell function becomes vulnerable when the genetic makeup is changed in the postnatal setting. Genetic loss of Pdx1 in β-cells from birth results in the development of diabetes with age, accompanied by impaired expression of the glucose transporter Glut2 and insulin, resembling MODY-4 (49). Moreover, it was shown that β-cell specific removal of Pdx1 during adulthood leads to derepression of MafB resulting in rapid conversion into α-cells (160). Deletion of Foxa1 and Foxa2 in mature β-cells affected both β-cell metabolism and insulin secretory mechanisms, leading to hyperinsulinemic hypoglycemia (161). On the post-transcriptional level, both RNA-binding proteins and several miRNAs have been shown to influence the turnover and translation of insulin synthesis and secretion (162;163). For example, miR-124a was shown to affect the expression of FoxA2 and Pdx1 (164). These studies show that subtle genetic changes may affect β-cell functionality.

Illustrating that active maintenance of β-cell identity is necessary to prevent complete transdifferentiation, several studies reported on the role of transcriptional repression of the α-cell transcription factor Arx in mature β-cells. These studies either used Arx overexpression or eliminated the transcriptional repression of Arx via (epi)genetic modifications, resulting in β-cell to α-cell conversion (38;165-167). Taken together, these studies strongly support that β-cell identity must be actively maintained and that alternative lineage repression is essential in this process.

That mature human β-cells depend on their microenvironment is illustrated by the process of epithelial-to-mesenchymal transition (EMT) that occurs within days following β-cell culture on plastic, resulting in a complete loss of the mature phenotype (168). This may in part be due to the lack of appropriate matrix and signalling molecules. Laminin 511 was previously identified in the vascular niche to promote insulin gene expression, likely via integrin β1 (169;170). Moreover, culture of purified human β-cells on laminin 511 partially blocked dedifferentiation via EMT (109). Mice with a mutated receptor for Bone Morphogenetic Protein-4 showed decreased expression of genes involved in glucose sensing, insulin processing and insulin secretion, and thereby developed diabetes (171). Although little is yet known in this field, these studies suggest that local signalling molecules and direct interaction between β-cells and the ECM are important substrates to sustain β-cell functional maturity.

Besides genetic alterations, pathological changes in the microenvironment can influence β-cell identity. Early studies in rats showed that chronic hyperglycemia following hemipancreatectomy was accompanied by a loss of β-cell transcription factors, which

likely lead to the loss of insulin expression and β-cell dysfunction (172). Similarly, β-cells that are cultured under conditions of oxidative stress lose expression of several essential transcription factors such as MafA and Pdx1 (173). Talchai et al. showed that mouse β-cells that genetically lack FoxO1 can dedifferentiate and convert into α-cells *in vivo*, but only under conditions of metabolic stress (induced by aging or multiparity) (174). The role of glucotoxicity or other stress factors on the stability of human β-cell identity is unknown.

The hypothesis that a differentiated cell encompasses a continuously active maintenance process, rather than being in a locked passive state, has been around for more than twenty years (175). Active maintenance of functional β-cell identity is under attention since rodent models suggest that β-cells can lose their identity which may result in diabetes. Much is still unknown about the exact triggers and mechanisms that influence the phenotype of mature β-cells and their relevance to human pathology has yet to become clear. However, it appears that β-cells are not passively locked cells and 'merely' produce insulin at the right moment, but are rather continuously active in a process to maintain their differentiated function.

AIM AND STRUCTURE OF THIS THESIS

Recent literature has highlighted the potential of intraislet cell conversion. However, most mechanistic studies are based on animal models while primary human islet material is scarce and studies on pancreatic tissue biopsies remain observational. The aim of this thesis is to explore the stability of the human β-cell phenotype and investigate whether loss of β-cell identity has a role in the pathophysiology of diabetes.

We first describe a case of intraportal islet transplantation in a patient with cystic fibrosis related diabetes in **Chapter 2,** illustrating the clinical benefit of β-cell replacement therapy. In **Chapter 3**, we present a novel agarose based microwell culture system that can be used for aggregate formation of human or rodent (islet) cells. We show that this platform provides reproducible results to study aggregation of primary human islet cells. In **Chapter 4**, we study the stability of human β-cells following islet cell reaggregration in the microwell culture system, using β-cell specific lineage tracing. We report that β-cells can spontaneously lose their identity and convert into glucagon-containing α-cells. In **Chapter 5**, we make use of human pancreatic tissue from donors with T2DM and matched controls to explore loss of β-cell identity in T2DM. We report that cells indicative of loss of β-cell identity are found more frequently in tissue samples from donors with a history of T2DM. In **Chapter 6**, we aim to inhibit the conversion process by studying the effects of Pax4 and GLP-1 receptor agonists in our model of β-cell conversion. We show that both factors can partially prevent loss of β-cell identity. **Chapter 7** provides a general discussion of the results described in this thesis and draws a model for the role of β-to-α cell conversion in diabetes.

1

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CHAPTER 2

ISLET-AFTER-LUNG TRANSPLANTATION IN A PATIENT WITH CYSTIC FIBROSIS RELATED DIABETES

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Cystic fibrosis-related diabetes (CFRD) is the most common comorbidity of cystic fibrosis (CF) (1). The incidence and prevalence of CFRD has increased due to life-saving advances, especially lung transplantation (2). In the pancreas, acinar fibrosis and fat cell infiltration are associated with decreased β-cell mass and β-cell dysfunction. Delayed gastric emptying contributes to dysregulation of glucose homeostasis (3). We report the case of a successful intraportal islet transplantation 6 years after bilateral lung transplantation in a patient with CFRD.

A 48-year old woman was referred to our clinic with CFRD since 30 years, complicated by severe gastrointestinal autonomic neuropathy with delayed gastric emptying. She had received a bilateral lung transplantation 7 years earlier. Immunosuppressive agents included mycophenolate mofetil (MMF, 1000 mg/day) and tacrolimus (6 mg/day). She had used steroids during the first 2 postoperative years. Lung function was stable (FVC 3140 mL and FEV1 2570 mL, 81.8% of FVC). Following lung transplantation, she suffered progressively from labile glucose regulation with frequent hypoglycemic episodes and hypoglycemia unawareness despite insulin pump therapy (0.7 IU/kg/day) and continuous glucose monitoring. HbA1c was 6.0-7.5% (42-58 mmol/mol) in the preceding year. Low basal insulin concentrations were present but no insulin secretory response to 1 mg glucagon occurred (basal C-peptide 0.13 nmol/L, stimulated C-peptide 0.14 nmol/L).

Pancreatic islets from 2 organ donors were isolated within 3 days, showing >3-fold induction of insulin secretion after glucose stimulation *in vitro*. Under local anesthesia, 4.95 ml islet tissue with a purity of 67.5% (1.4 million Islet Equivalents) was administered in the portal vein using ultrasound guidance. Induction immunosuppressive therapy consisted of basiliximab (20mg before transplantation and 1 day post-transplantation). As maintenance therapy, MMF and tacrolimus were continued in combination with prednisolone (6 weeks 10mg/day, 5mg/day afterwards). Additonal therapy included antibiotic prophylaxis, liraglutide and continuation of insulin pump therapy. The patient left the hospital 3 days post-transplantation without complications. Glucose control improved considerably within days without hypoglycemic episodes. She was hesitant to stop insulin pump therapy and slowly reduced the daily insulin dose. At six months insulin pump therapy was stopped. The HbA1c was 4.7% (28 mmol/mol). She underwent a mixed-meal challenge test showing a fasting plasma glucose concentration of 4.8 mmol/l and a maximal concentration of 6.5 mmol/l. Serum C-peptide concentration increased from 0.84 nmol/l to 1.25 nmol/l (Figure 1). After 1,5 years, long-acting insulin was restarted (3 IU/day) because of postprandial hyperglycemia while no episodes of hypoglycemia were reported. No donor-specific antibodies developed and pulmonary function remained stable.

Life expectancy for CF increases but extrapulmonary complications such as progressive β-cell failure and diabetes-related complications can occur. We report an islet-after-lung transplantation in a patient with CFRD, resulting in glycemic stability and absence of previously severe and disabling hypoglycemia. We propose that islet-after-lung transplantation is a safe and effective intervention for patients that could not benefit from simultaneous islet-lung or pancreas-lung allotransplantation (4;5). Although sufficient islet

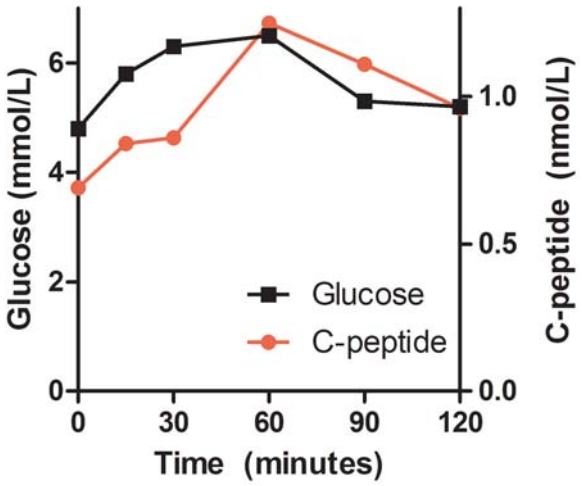

Figure 1. Plasma glucose (black squares) and C-peptide (red dots) concentrations in response to an orally consumed mixed-nutrient meal six months after islet transplantation. The peak glucose level is 6,5 mmol/L, accompanied by a noticeable C-peptide response.

mass may result in insulin independence, the main goal is to eliminate hypoglycemia-related problems and achieve stable glycemia, thereby improving quality of life.

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CHAPTER 3

CONTROLLED AGGREGATION OF PRIMARY HUMAN PANCREATIC ISLET CELLS LEADS TO GLUCOSE-RESPONSIVE PSEUDOISLETS COMPARABLE TO NATIVE ISLETS

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ABSTRACT

Clinical Islet transplantation is a promising treatment for patients with type 1 diabetes. However, pancreatic islets vary in size and shape affecting their survival and function after transplantation due to mass transport limitations. To reduce diffusion restrictions and improve survival the islet cells, the generation of islets with optimal dimensions by dispersion, and subsequent reassembly of islet cells, can help limit the length of diffusion pathways. This study describes a microwell platform that supports the controlled and reproducible production of three-dimensional pancreatic cell clusters of human donor islets. We observed that primary human islet cell aggregates with a diameter of 100-150 μm consisting of about 1000 cells best resembled intact pancreatic islets as they showed low apoptotic cell death (<2%), comparable glucose-responsiveness and increasing *PDX1*, *MAFA* and *INSULIN* gene expression with increasing aggregate size. The reassociated human islet cells showed an a-typical core shell configuration with β-cells predominantly on the outside unlike human islets, which became more randomized after implantation similar to native human islets. After transplantation of these islet cell aggregates under the kidney capsule of immunodeficient mice, human C-peptide was detected in the serum indicating that β-cells retained their endocrine function similar to human islets. The agarose microwell platform was shown to be an easy and very reproducible method to aggregate pancreatic islet cells with high accuracy providing a reliable tool to study cell-cell interactions between insuloma and primary islet cells.

INTRODUCTION

Although allogeneic islet transplantation offers a promising therapy for patients with type 1 diabetes, this method is still inefficient because of considerable islet loss shortly after transplantation (1;2). Upon isolation, the main islet vasculature is damaged or destroyed. Therefore, mass transport of relevant nutrients mainly depends on passive diffusion, which adversely affects the viability of isolated human islets (3). There are indications that small pancreatic islets (≤150 μm), of both human and rodent origin are superior to larger sized islets in terms of survival and insulin secretion since they are less susceptible to central necrosis (4-6). In a study on rat islet cell aggregates used for microencapsulation the authors showed that smaller aggregates (≤50 μm) performed better than native islets in terms of survival and function (5). Interestingly, when theoretical modelling is used to describe the optimal size for islets, based on oxygen consumption rate and nutrient diffusion throughout the islets it was found that a diameter of 100 μm is most optimal, while in fact native islets range from 50 to 400 μm and are often not spheroidal shaped (7). Generation of human islets with optimal dimensions by islet dispersion and subsequent reassembly of single islet cells into three-dimensional aggregates, so called 'pseudoislets', of a specific dimension may have beneficial effects on islet survival after transplantation and could help overcome nutrient diffusion limitations.

The creation of pseudoislets in which islets cells are assembled in a three-dimensional configuration may be equally important since several authors have demonstrated that β-cells require cell–cell contact to survive and properly function *in vitro*. The viability of MIN6 cells has been shown to improve when these cells were cultured in close contact with each other (8). Moreover, insulin secretion from these cells had clearly increased in three-dimensional cell aggregates compared to two-dimensional monolayer cultures (9). Improvement of insulin secretion is lost when such cell clusters are dispersed, and is regained upon re-aggregation (9-11). Similar results were obtained when using primary islet cells of both rodent and human origin, stressing the importance of cell–cell interactions and three-dimensional culture for islet function (12-15).

The most commonly used technique for creating three-dimensional cell aggregates are static suspension cultures on ultra-low attachment tissue culture plastic, resulting in a heterogeneous aggregate population with a large variety in aggregate dimensions and number of cells per aggregate. Another conventional method for cell aggregation involves the hanging drop method that is normally used for embryoid body formation when using embryonic stem cells, and has also been used for the generation of pseudoislets from dispersed pancreatic rat islets (16-18). Although the latter technique generates uniformly sized aggregates, the method is labour-intensive and therefore upscaling is limited. Several groups are developing alternative methods for controlled cell aggregation. Mendelsohn et al. showed the use of microcontact printing to form multilayered β-cell clusters on laminin-patterned surfaces (19;20). Although aggregate size can be controlled in this way, the cell clusters produced are only a few cell layers thick and cannot be removed from the substrate for further research or implantation afterwards. More recently, several microwell platforms have been developed and applied for controlled aggregation of

various cell types such as embryonic stem cells (21;22), fibroblasts (23), and chondrocytes (24) in a high-throughput manner. Recent studies have demonstrated the use of microwells for the controlled aggregation of MIN6 mouse β-cells, pancreatic progenitor cells, and dispersed rat islets of Langerhans (25-27). However until now, controlled aggregation of dispersed human islet cells to create islets with optimal dimensions has not yet been reported using aforementioned systems.

Our study describes a high-throughput microwell platform optimized for the generation of stable and uniformly sized aggregates of human islet cells, which serves two main purposes. First, the platform supports the formation of three-dimensional clusters of β-cells and dissociated human islets, of pre-defined dimensions while providing the cell-to-cell interaction that is required for these cells to survive and function properly. Secondly, controlling aggregate dimensions could help to reduce islet cell death and increase the reproducibility of experimental results since aggregates of equal size comprising an equal number of cells can be produced very accurately in high quantities. We hypothesize that there is an optimal aggregate size and cell number for three-dimensional assembly of human islet cells. Here, we report on the creation of primary human islet cell aggregates, pseudoislets, with various pre-defined dimensions and the evaluation of their viability and insulin secretion function. We investigated glucose-stimulated insulin expression, gene expression of β-cell specific markers in relation to size and cell number, and studied changes in morphology and the production of human C-peptide after transplantation of these human pseudoislets under the kidney capsule of mice.

RESEARCH DESIGN AND METHODS

Insulinoma cell culture

MIN6 clone B1 mouse insulinoma cells (kindly provided by Dr. P. Halban, University Medical Center, Geneva, Switzerland) (28) were cultured in high-glucose DMEM with 2.5 mM L-glutamine (Invitrogen, Bleiswijk, the Netherlands) supplemented with 10% fetal bovine serum, 100 U/ml streptomycin, 100 µg/ml penicillin and 70 µM freshly added β-mercaptoethanol. INS-1E rat insulinoma cells (kindly provided by Dr. B. Guigas, LUMC, Leiden, The Netherlands and Dr. P. Maechler, University Medical Center, Geneva, Switzerland) (29) were cultured in RPMI with 2.05 mM L-glutamine (Invitrogen) supplemented with 5% FBS, 100 U/ml streptomycin, 100 µg/ml penicillin, 10 mM HEPES, 1 mM sodium pyruvate, 50 µM freshly added β-mercaptoethanol. Cell cultures were maintained at 37°C in humidified air containing 5% carbon dioxide. Medium was refreshed every 3–4 days and cells were replated when 80% confluency was reached.

Primary human islet culture

Human islets of Langerhans not used for clinical islet transplantation were provided by the Leiden University Medical Center, Leiden, The Netherlands. Organs donors (4M/6F) had an average age of 50 ± 13 years and BMI of 23 ± 3 kg/m2 (Supplementary Table 1). Islets were dispersed into single cells by adding 0.025% trypsin solution containing 10 µ g/ml

DNase (Pulmozyme, Genentech, San Francisco, CA, USA) and seeded onto agarose microwells for controlled cell aggregation. Intact islets and human islet cell aggregates were cultured in CMRL 1066 medium (5.5 mM glucose) (Mediatech, Manassas, VA, USA) supplemented with 10% foetal calf serum, 20 µg/ml ciprofloxacin, 50 µg/ml gentamycin, 2 mM L-glutamine, 0.25 µg/ml fungizone, 10 mM HEPES and 1.2 mg/ml nicotinamide. Cell cultures were maintained at 37°C in a 5% CO2 humidified atmosphere. Medium was refreshed every 3–4 days.

Agarose microwell fabrication and cell aggregate formation

Non-adherent agarose microwells were aseptically fabricated as described previously (30). Briefly, microwell chips containing 2865 microwells with a diameter of 200 µm and chips containing 1585 microwells with a diameter of 400 µm were fabricated by pouring a 3% (w/v) UltrapureTM agarose (Invitrogen, Bleiswijk, the Netherlands) solution on negative moulds of polydimethylsiloxane (PDMS). After agarose solidification, the moulds were removed, covered with PBS and stored at 4°C until usage (see Fig. 1). Before cell seeding, the agarose chips were pre-incubated in culture medium overnight at 37°C. For cell aggregate formation, single cells were resuspended in fresh medium and seeded onto agarose chips at various densities resulting in aggregates consisting of 10, 25, 50, 100, 250, 500 and 1000 cells. Immediately after seeding, agarose chips were briefly centrifuged at 300 x g to allow the cells to settle down in the microwells. As a control, 1 x 10^5 cells were seeded onto ultra-low attachment plastic to allow spontaneous cell aggregation. Medium was refreshed every 1–2 days. Cell aggregates were cultured up to 7 days after which they were removed from the chips by upside down centrifugation (1 min. at 300 x g) or by medium flush, and used for further analysis. To measure the average aggregate diameter, microscopic images were taken and aggregate diameter was quantified using ImageJ (NIH image). For INS-1E cell aggregates, at least 50 aggregates were measured. For human islet cell aggregates, at least 40 aggregates derived from islet preparations of three different human donors were analysed.

Scanning Electron Microscopy

INS-1E cell aggregates were flushed out of the microwells, fixed in 4% (w/v) paraformaldehyde and embedded by mixing in 2% (w/v) agarose. Samples were prepared for scanning electron microscopy by dehydration in increasing concentrations of ethanol (1 hr each step) and dried using critical point dryer equipment (CPD 030; BAL-TECBalzers, Liechtenstein). The samples were sputter-coated with gold and imaged using a scanning electron microscope (XL30 ESEM-FEG, Philips, Eindhoven, the Netherlands).

Cell viability

Cell viability was assessed on day 7 by staining the cells with 6 µM ethidium homodimer and 1 µM calcein using a LIVE/DEAD Viability/Toxicity Kit (Invitrogen, NL) and visualized using fluorescence microscopy (Nikon Eclipse E600, NIKON, Amsterdam, the Netherlands).

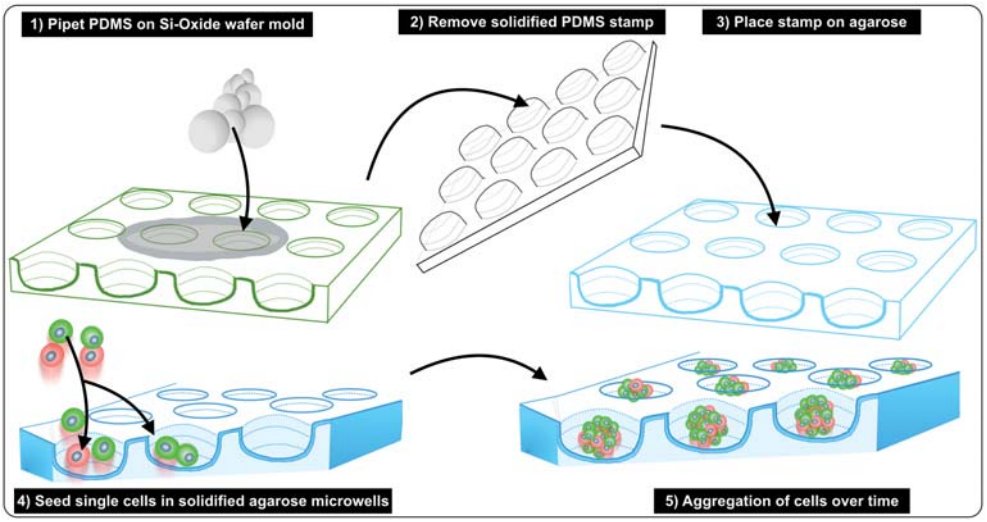

Figure 1. Schematic representation of agarose microwell fabrication and cell aggregation.

Viable cells were stained green, and DNA of dead cells was stained red. Staining was quantified as percentage of viable cells per total cell number, counting at least 100 cells per condition.

Histological analysis

Human islets, human islet cell aggregates and INS-1E cell aggregates were fixed in 4% (w/v) paraformaldehyde, washed in PBS, dehydrated in ethanol series, embedded in paraffin, and sectioned at 4–5 µm using a microtome (Microm HM355S; Thermo Scientific, Breda, the Netherlands). Sections were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol (100–96–90–80–70%) and rinsed in H2O and PBS.

For immunohistochemical labelling of insulin, blocking was done using dual endogenous enzyme block (DAKO, Glostrup,Denmark). Sections were incubated with rabbit anti-human insulin polyclonal antibody (1:400; Santa Cruz Biotechnology, Inc, Heidelberg, Germany for 1 hr at 21°C, followed by subsequent washing steps in PBS and 1% (v/v) bovine serum albumin/PBS and incubated with HRP-goat anti-rabbit IgG secondary antibody (1:100; DAKO, Glostrup Denmark) for 1 hr at 21°C. Sections were then rinsed in PBS and incubated for 4 min. with DAB liquid chromogen system (DAKO, Glostrup, Denmark), which yields a brown colour. Counterstaining was performed with haematoxylin (Gill's haematoxylin no. 3, Sigma-Aldrich, Zwijndrecht, the Netherlands) according to the manufacturer's protocol and samples were visualized using a Nikon Eclipse E600 microscope.

For fluorescent immunolabelling of insulin and glucagon, blocking was done with 0.1% (v/v) normal donkey serum/PBS for 1 hr and antibodies were diluted in 1% (v/v) lamb serum/PBS. Primary antibodies used were: 1:100 rabbit-anti-glucagon (Vector Labs Amsterdam, the Netherlands) overnight at 4°C, and 1:200 guinea pig-anti-insulin (Abcam) for 1.5

hrs at 21°C. Secondary antibodies used were: 1:200 biotin donkey anti-rabbit (Jackson ImmunoResearch), 1:200 streptavidin-Alexa488 Invitrogen, NL 1:400 rhodamine donkey anti-guinea pig (Jackson ImmunoResearch Suffolk, United Kingdom), all incubated for 1 hr at 21°C. Counterstaining was performed with 1 µg/ml 40-6-diamidino-2-phenylindole. Fluorescence was visualized using a Nikon Eclipse E600 microscope.

Apoptosis was assessed by TUNEL assay Roche, Woerden, the Netherlands. Sections were examined using confocal microscopy. The number of stained cells was quantified and expressed as percentage of positive cells per total cell number, counting at least 750 cells per donor for each condition.

Glucose-stimulated insulin secretion test

To test insulin secretory capacity, three groups of 50 human islets, human islet cell aggregates or INS-1E cell aggregates were hand-picked, transferred to an ultra-low attachment plate and incubated in a modified Krebs-Ringer bicarbonate buffer.

INS-1E cell aggregates were pre-incubated for 2 hrs in glucose-free culture medium, followed by a 30 min. pre-incubation in glucose-free incubation buffer. Subsequently, the INS-1E cell aggregates were incubated during three consecutive steps of 30 min. in low glucose buffer (2 mM), high-glucose buffer (20 mM) and low glucose buffer (2 mM) at 37°C. Human islets and human islet cell aggregates were pre-incubated for 1.5 hrs in glucose-free buffer followed by three successive incubation steps of 1 hr in low-glucose buffer (2 mM), highglucose buffer (20 mM) and low-glucose buffer (2 mM) at 37°C. Perifusion experiments were performed in a similar order using Suprafusion 1000 (Brandel, UK) at a flow of 0.2 ml/min., obtaining samples every 7.5 min. Islets and islet cell aggregates were sequentially incubated in low glucose (2 mM, samples 1–2), high glucose (20 mM, samples 3–10), and low glucose (2 mM, samples 11–20). Fold induction was expressed relative to the average value at the end of the lead-in period as base reference.

Medium samples were collected and the amount of secreted insulin was determined by ELISA according to the manufacturer's protocol (Mercodia, Sweden). Absorbance was analysed with Thermo Scientific Multiscan Go (450 nm). After glucose-stimulated insulin secretion test (GSIS), aggregates were collected and analysed for total DNA content using Quant-iT picogreen dsDNA assay kit (Invitrogen, NL). Fluorescence was analysed with a Perkin Elmer 1420 Multilabel counter (excitation 480 nm, emission 520 nm). Secreted insulin was normalized to the total DNA amount. Stimulation index (SI) was calculated as a ratio of released insulin after high-glucose stimulation divided by released after low glucose stimulation.

Quantitative PCR

Total RNA was extracted using RNeasy kit Qiagen Benelux BV, Venlo, the Netherlands according to the manufacturer's protocol. Total RNA (1 lg) was reverse transcribed using M-MLV reverse transcriptase (Invitrogen, Bleiswijk, the Netherlands). Quantitative PCR (qPCR) was performed with a Light Cycler 480-II Real-time PCR system (Roche, NL). Fold induction was calculated using deltaCT method with human b-actin as housekeeping gene.

C-peptide assay

Human C-peptide levels were measured in mouse serum using ultrasensitive C-peptide ELISA (Mercodia, Uppsala, Sweden) according to the manufacturer's protocol. Data are represented as average values (± SD) from two donors with 3–5 mice each donor.

Animal transplantation

The Leiden University Medical Center committee for animal ethics approved all animal experiments. Human islet cell aggregates (from 2 donors) were formed in vitro by 2-day aggregation of 1000 cells per microwell. Aggregates were harvested from the chips (2865 aggregates per chip) and transplantation was done with the yield of one chip under the kidney capsule of 7- to 15-week-old male NOD/SCID mice (n = 3), NOD.CB17-Prkdcscid/NcrCrl (Charles River, NL). After 14 days, the islet cell aggregate grafts were removed for histology and immunofluorescent labelling.

Statistical analysis

Statistical analyses were performed with one-way ANOVA and Bonferroni post-test. *P < 0.05, **P < 0.01, ***P < 0.001.

RESULTS

Fabrication and validation of microwell platform for controlled cell aggregation

Agarose microwell chips were fabricated using PDMS moulds. The chips are compatible with standard 12-well cell culture plates and contain 2865 non-adherent microwells with a diameter of 200 μm (Fig. 2A). When a single cell suspension is seeded onto these chips, controlled aggregation is induced and stable cell clusters are formed, as schematically shown in Figure 2B. To visualize cell aggregation inside these microwells, we measured the aggregate diameter of INS-1E cell aggregates in time. Figure 2C shows that single INS-1E cells started forming aggregates during the first 2 days of culture after which the aggregates remained stable in size until the end of the 7 days culture period. In addition, scanning electron microscopy demonstrated that the INS-1E cell aggregates do not disintegrate during harvesting from the chip, or during 7 days of suspension culture subsequently indicating solid integrity (Fig. 2D). INS-1E cell aggregates obtained by conventional suspension culture on ultra-low attachment plates showed a large size distribution with an average aggregate diameter of 88 ± 49 μm, whereas controlled aggregation of 500 cells per well in our microwell platform resulted in uniformly sized aggregates of 93 μm (SD ± 16; Fig. 2E).

Controlled generation of insulinoma and primary human islet cell aggregates

Controlled aggregation using these microwells was further optimized using MIN6 clone B1 and INS-1E insulinoma cell lines. These insulinproducing cell lines are widely used as a model for primary β-cells since they resemble most native β-cells (31;32). Cells were seeded onto microwell chips at various densities to generate aggregates of 10, 25, 50, 100,

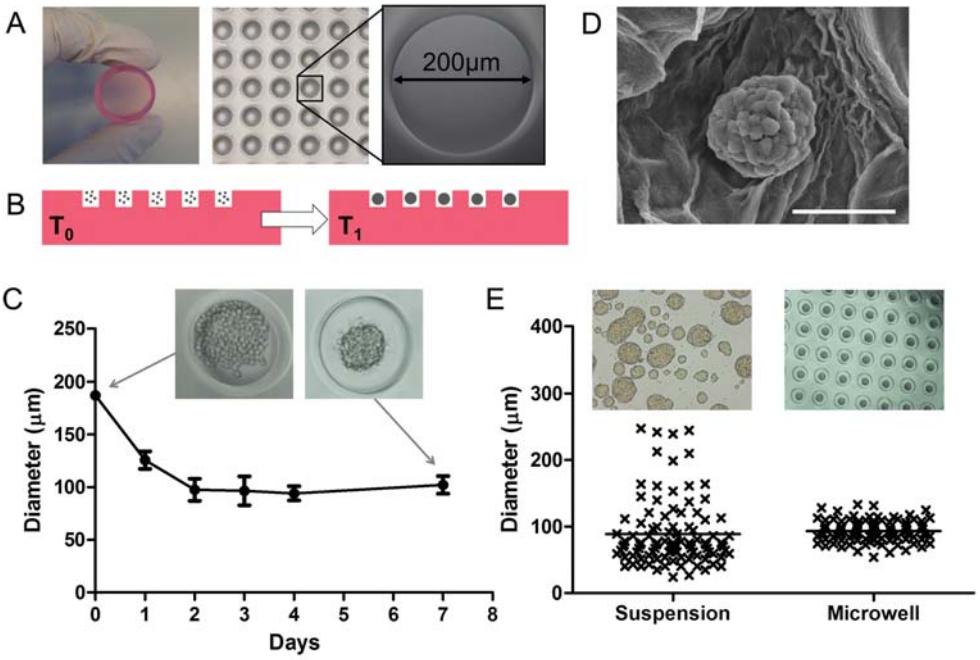

Figure 2. Fabrication and validation of a microwell platform for controlled production of uniformly-sized aggregates. *A:* Agarose microwell chip containing 2865 non-adherent wells with a diameter of 200 µm. *B:* Schematic representation of controlled cell aggregation in agarose microwell chips. Single cells are seeded in suspension onto agarose microwell chips and briefly centrifuged to allow the cells to settle down in the microwells (T0). In time, cells assemble and stable cell clusters are formed (T1). *C:* INS-1E cell aggregate formation in agarose microwells. Single INS-1E cells aggregate in the first 2 days of culture and form stable aggregates that were maintained up to 7 days of culture. *D:* SEM image of an INS-1E cell aggregate that was embedded in agarose gel. Scale bar = 50 µm. *E:* The microwell platform generates uniformly-sized INS-1E cell aggregates with less variability (SD=16) compared to conventional suspension culture (SD=49) (n=100).

250 and 500 cells, resulting in aggregates with welldefined dimensions. The aggregates could easily be removed from the chips by flushing out and mild centrifugation (Fig. 3A), after which they were used for further analysis. The average aggregate diameter correlates with the initial number of cells seeded per microwell, which ranged between 29 and 105 µm for INS-1E cells, and between 28 and 93 µm for MIN6 clone B1 cells, as shown in Figure 3B. Increasing the number of cells per microwell to 1000 resulted in unstable cell aggregates that were not suitable for further use. Next to controlled aggregation of MIN6 and INS-1E cell lines, our microwell platform supports the controlled reassembly of primary human islet cells. Human donor islets of Langerhans were dissociated into a single cell suspension (>95% viability, data not shown) and seeded at 100, 500 and 1000 cells per microwell to induce controlled aggregation similar to the insuloma cell lines. The resulting primary islet cell aggregates were uniform in size and remained intact after harvesting from the chips (Fig. 3C). After 7 days of culture, the islet cell aggregates had an average diameter of 80 ± 5.4, 105 ± 11.3 and 134 ± 15.3 µm respectively, depending on the initial cell number

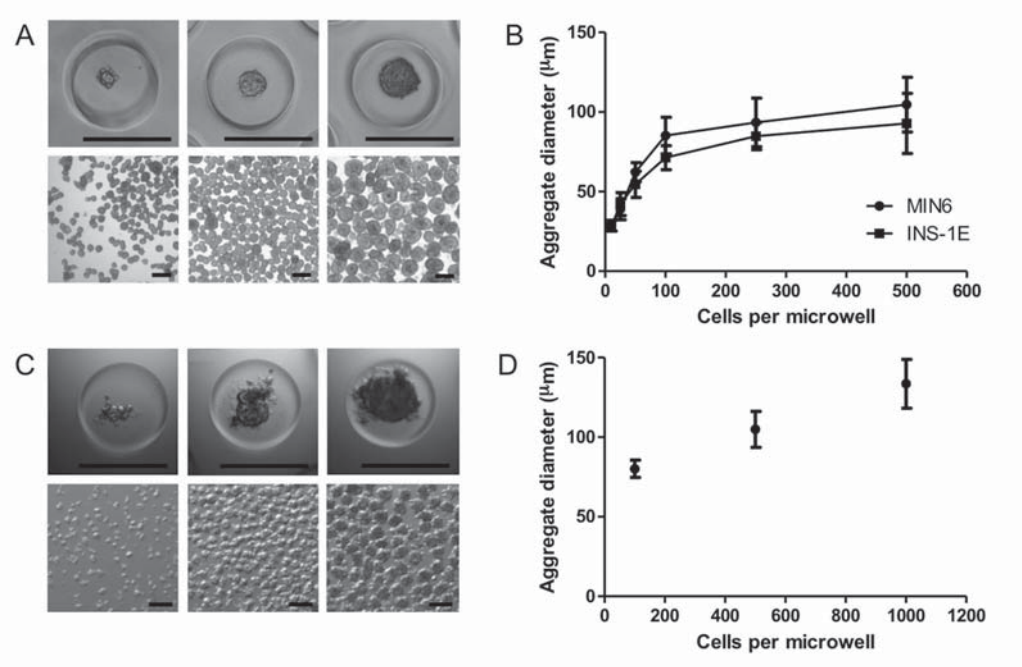

Figure 3. Seeding density determines aggregate size of insulinoma and human islet cell aggregates. *A:* INS-1E cell aggregates of 50, 100, and 500 cells after 7 days of culture inside agarose microwells (top panel) and after flushing out of the chip (bottom panel). Scale bar = 200 µm. *B:* Correlation between number of INS-1E and MIN6 cells per microwell and diameter of obtained aggregates after 7 days of culture. INS-1E and MIN6 cells were seeded at 10, 25, 50, 100, 250 and 500 cells per microwell. Each datapoint represents the average measurement of at least 50 aggregates per condition. Error bars represent ± SD. *C:* Primary human islets were dispersed and single islet cells were seeded at 100, 500 and 1000 cells per microwell. Figures represent human islet cell aggregates inside the microwells (top panel) and after flushing them out of the chip (bottom panel) after 7 days of culture. Scale bar = 200 µm. *D:* The diameter of human islet cell aggregates increased with increasing the number of cells seeded per well. Cells were seeded at 100, 500 and 1000 cells per microwell. At least 40 human islet cell aggregates were measured per condition. Datapoints represent the average values for 3 human donors. Error bars represent ± SD.

seeded (Fig. 3D). To study cell assembly kinetics, representative images were taken at different time-points during aggregate formation. We observed that aggregation occurs in the first 2–4 days after cell seeding, and stable aggregates are obtained after 7–14 days of culture (Supplementary Fig. 1). Since 200 µm diameter microwells cannot contain more than 1000 cells, we created wells with a diameter of 400 µm. Increasing the cell seeding density in these microwells to 2000, we observed that islet cells assembled uncontrollably into multiple smaller aggregates per well.

INS-1E insulinoma cell aggregates are viable and functional

To assess basic cell function within the aggregates in more detail, INS-1E cell aggregates were cultured in the agarose microwell platform up to 7 days after which cell viability, protein expression and insulin secretion was assessed. Live/dead staining was performed

on cells inside the microwell array and demonstrated a cell viability of at least 80% for all aggregate sizes (Fig. 4A). Insulin-specific immunolabelling showed that INS-1E cell aggregates of various sizes consisting of 50, 100 and 500 cells still expressed insulin (Fig. 4B). To test glucose-responsiveness, the aggregates were challenged with a high-glucose concentration. The INS-1E cell aggregates secreted insulin upon a high-glucose stimulus, and insulin secretion returned to baseline after a second stimulation with low glucose. Aggregates of various sizes all responded in the same way (average SI = 1.86 ± 0.7) (Fig. 4C).

Primary human islet cell aggregates are viable and functional *in vitro*

Human islet cell aggregates of various sizes were assessed for morphology, β-cell apoptosis, gene and protein expression and insulin secretion function. The a-typical architecture, with glucagon-positive α-cells located in the core, and insulin-positive β-cells at the periphery of aggregates that was previously published by our group (33), was present in both small (250 cells) and in large (1000 cells) human islet cell aggregates (Fig. 5A). TUNEL-insulin double staining showed few apoptotic cells, which comprise mainly non-β-cells (Fig. 5B). Quantification of TUNEL+ cells showed less than 2.5% overall cell apoptosis for small aggregates (100–250 cells) and less than 1% apoptotic cell death for aggregates consisting of 500 or 1000 human islet cells (Fig. 5C). We measured the gene expression for INSULIN, MAFA and PDX1 at day 7 of culture. The expression levels in human islet cell aggregates were lower compared to intact control islets of the same donor. However, we found that increasing the number of cells per aggregate from 100 to 1000 lead to increased expression of INSULIN, PDX1 and MAFA, which was still somewhat lower compared to intact cultured islets of the same origin (Supplementary Fig. 2). Importantly, glucose-stimulated insulin secretion assays demonstrated that primary human islet cell aggregates of 250 and 1000 cells responded similar to high-glucose as intact control islets of the same donor after 7 days of culture (Fig. 5D). No significant differences were observed between the stimulation indices of primary human islet cell aggregates of 250 or 1000 cells (SI = 2.1 and 1.8, respectively), and intact control islets (SI = 2.3) of the same donor. Similar results were obtained after dynamic GSIS. Although dynamic GSIS showed a more sustained secretion during high glucose in intact islets compared to aggregates, stimulation indices were found to be similar with some variation between the two donors (Fig. 5E).

Primary human islet cell aggregates are viable and functional *in vivo*

Following 2 days of in vitro aggregation in microwells primary human islet cell aggregates were transplanted for 14 days under the kidney capsule of NOD/SCID mice. Figure 6A shows that after 14 days in vivo, human islet cell aggregates exhibit a normal cellular architecture. Quantification of TUNEL+ cells showed negligible, 0.95 ± 0.75%, apoptotic cell death. Human C-peptide, around 200 pmol/l (average of 2 human donors) at day 7 and 14, was observed in serum of transplanted mice whereas the human C-peptide concentrations in non-transplanted mice were undetectable at day 7 and 14 post transplantation (Fig. 6B).

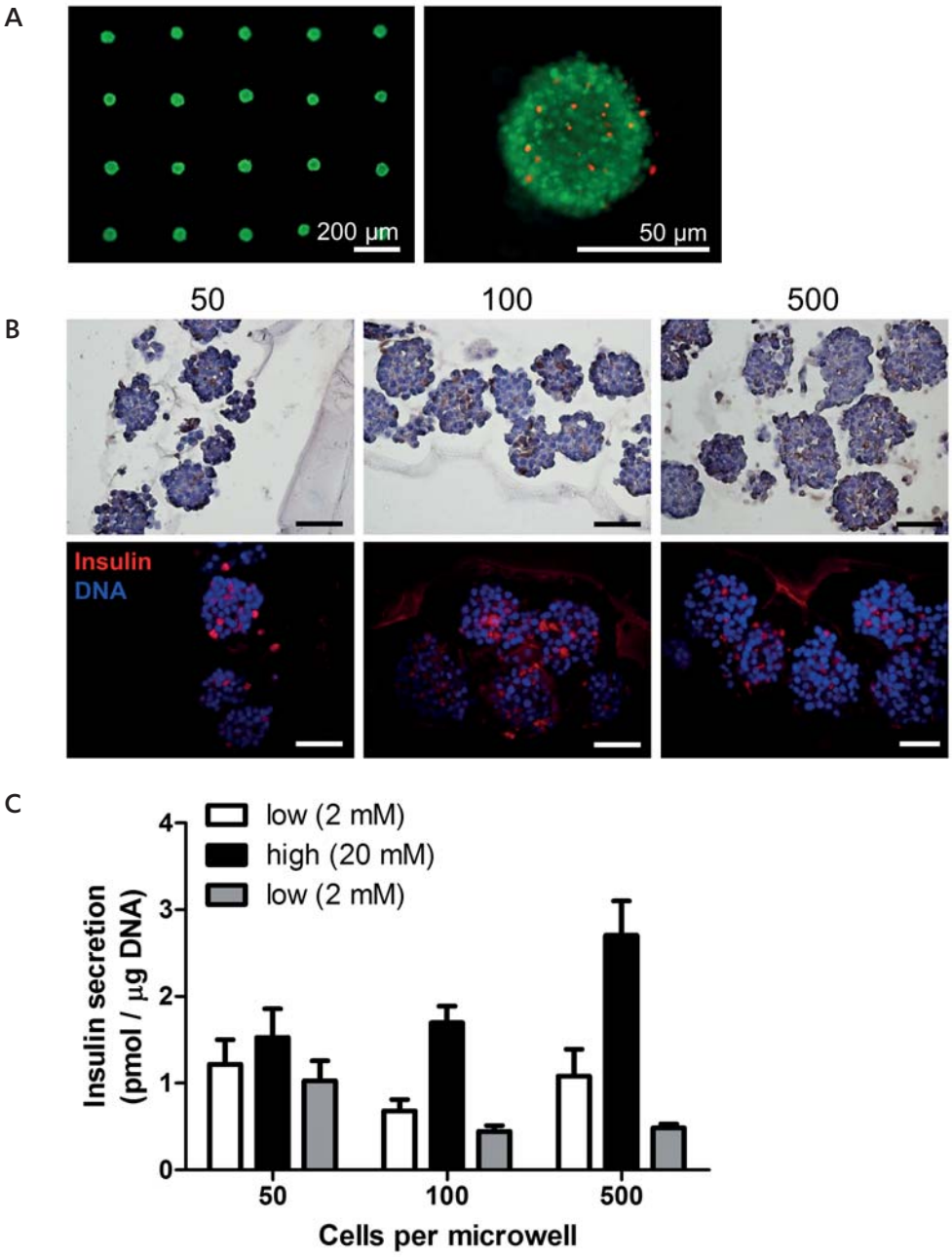

Figure 4. INS-1E cell aggregates of various sizes remain viable and functional up to 7 days of culture. *A:* INS-1E cell aggregates were formed using agarose microwells. After 7 days of culture, a viability assay was performed to visualize living cells (green) and dead cells (red). *B:* Histological evaluation of INS-1E cell aggregates of 50, 100 and 500 cells after 7 days of culture. Both DAB chromogen and fluorescent anti-insulin staining demonstrate that INS-1E cell aggregates of various sizes express insulin. Scale bars = 50 µm. *C:* Glucose-stimulated insulin secretion of INS-1E cell aggregates of various sizes measured after 7 days of culture (100 aggregates per condition, in triplicate). Error bars represent ± SD.

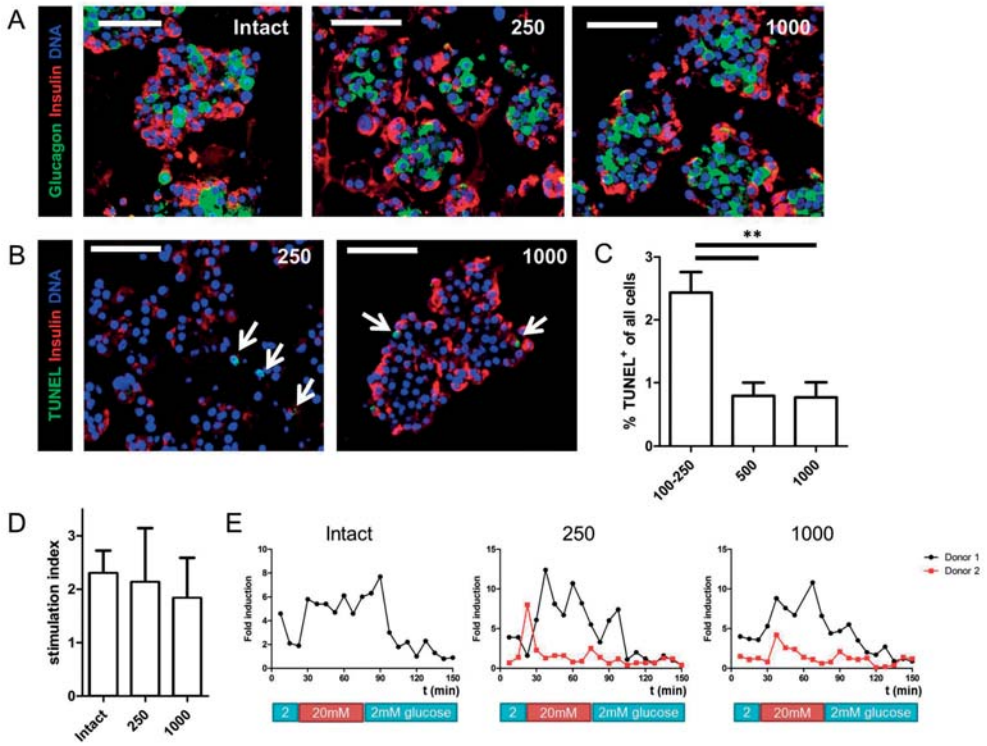

Figure 5. Human islet cell aggregates are viable and function *in vitro*. *A:* Human islet cell aggregates of all sizes show an architecture with beta-cells (red) located at the periphery and alpha-cells (green) in the core. In comparison, intact human islets show a heterogeneous distribution of alpha- and beta-cells. *B:* TUNEL staining showing only few apoptotic cells in both small (250 cells/aggregate) and large (1000 cells/aggregate) human islet cells aggregates (positive cells are indicated with an arrow). *C:* Quantification of TUNEL+ cells shows that 2.5% of all cells are apoptotic in small aggregates. Aggregates of 500 or 1000 cells contain less than 1% apoptotic cells (n=3-4 donors per condition). *D:* Static glucose stimulated insulin secretion (GSIS) test was performed on human islet cell aggregates of 250 and 1000 cells and compared with intact control islets. Islet cell aggregates of both sizes show a similar secretory response as intact control islets. Error bars represent ± SD. *E:* Dynamic GSIS shows more sustained insulin secretion during high glucose in intact islets compared to human islet cell aggregates, although the induction is similar. Scale bar = 100 µm.

DISCUSSION

The controlled assembly of primary human islet cells into predefined three-dimensional optimal aggregates, in which β-cells show appropriate endocrine function, may have beneficial effects on islet survival after transplantation and could help overcome nutrient diffusion limitations. We developed a microwell platform for the generation of human islet cell aggregates of pre-defined dimensions comprising equal cell numbers. We studied the controlled formation of primary human islet cell aggregates and investigated morphology, the apoptotic cell death and β-cell function in relation to their size. Human islet cell aggregates with a diameter of 100–150 µm showed little apoptotic cell death (<2%), an adequate insulin secretory response to a glucose, and MAFA, PDX1 and INSULIN

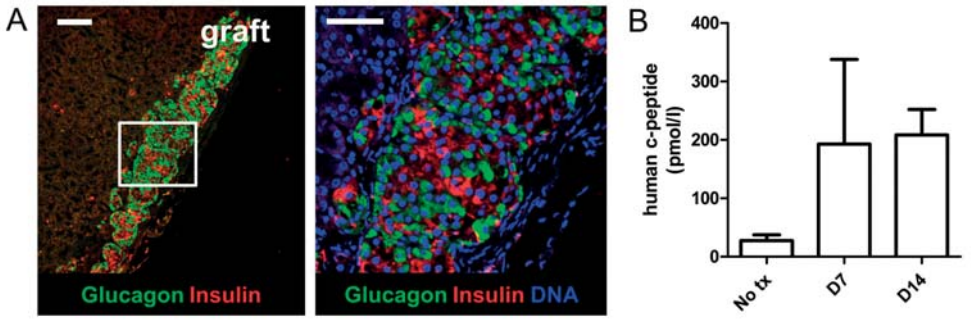

Figure 6. Human islet cell aggregates are viable and function *in vivo*. *A:* 14 days after transplantation under the kidney capsule of immune deficient mice, human islet cell aggregates (1000 cells/aggregate) show a heterogeneous cell architecture that is similar to intact human islets, with α- and β- cells randomly spread throughout the aggregate. Scale bar = 50 µm. *B:* Serum C-peptide levels seem higher in transplanted mice compared to control mice at day 7 and 14 after transplantation (n = 3-5 mice, 2 donors). Error bars represent mean ± SD.

gene expression similar to human islets. After reassociation of the primary human islet cells the aggregates constituted a specific core and mantle arrangement, in which the mantle comprised predominantly of β-cells, and the core of α-cells, which is a-typical compared to the native random dispersion normally found in human islets. These findings confirm our previous observations in a recent study on β- to α-cell transdifferentiation in which a similar observation was done (33). Others have demonstrated that dispersed rat islet cells reassemble in culture and form islet-like aggregates with a core mantle organization similar to that of native rodent islets, which indicates that the signals required for this specific organization are likely cell-mediated (34). It has been shown that differential expression of distinct cell adhesion molecules (CAMs), more specifically neural CAM (N-CAM), is responsible for the establishment and maintenance of rat islet architecture (35-37). Our findings suggest that in contrast to rodent islet cells, the islet cells themselves do not solely mediate the unique cellular organization of human islets. Despite their non-native architecture, the in vitro insulin secretory response of human islet cell aggregates of various sizes suggests that islet dispersion and reassembly does not affect their glucose-responsiveness.

We found that in vivo transplantation of primary human islet cell aggregates for 14 days under the kidney capsule of NOD/SCID mice resulted in an architecture in which α- and β-cells become more heterogeneously distributed throughout the islet graft, like is found in normal human islets, suggesting that external factors like revascularization, or cell-matrix interactions are involved in maintaining normal islet architecture and responsible for remodelling of the initial core mantle distribution observed. The trigger to induce migration could be the change in oxygen tension and nutrient availability because of in vivo re-vascularization, while in vitro the nutrient supply is solely dependent on mass transport by diffusion to the cells in the aggregate. The latter could mean that the cells in the aggregate core are exposed to less than optimal nutrient and oxygen supply.

The second possibility for in vivo aggregate remodelling is that cells can transdifferentiate, and therefore grafts change to a different architecture after transplantation. However, we do not have lineage tracing techniques that can trace α-cell fate available. We cannot therefor exclude, or support the hypothesis of α-cell to β-cell conversion. Although we have recently shown that β-cells can convert into α-cells in this relatively short time period, we do not see an increased percentage of β-cells in our grafts, suggesting migration is a more likely event (33).

Controlled cell aggregation in our microwell platform was optimized using MIN6 and INS-1E cell lines and resulted in uniformly sized cell aggregates with a small variability in diameter, compared to heterogeneous cell aggregation in conventional suspension culture. Using our microwells, aggregate dimensions could accurately be controlled by changing the initial cell seeding density, resulting in cell aggregates with pre-defined dimensions. This is in line with other studies demonstrating the use of poly(ethylene glycol) microwells for controlled aggregation of MIN6 β-cells and the aggregation of dispersed rat islet cells in glass micromoulds (25;27). Our wells were prepared in agarose, which is a polysaccharide that is cheap, non-toxic and easy to use. In addition, cells do not adhere to the material that supports cell aggregation. Since no special equipment is required, the microwells can be used in all basic research laboratories in standard tissue culture plates. In addition, the negative PDMS moulds can easily be varied to create microwells of various sizes and shapes, and are re-usable which makes for a fast and sustainable low-cost and reliable fabrication procedure. The maximum aggregate diameter that could be obtained using our platform was limited to approximately 150 μm, as increasing the number of cells above 1000 cells per aggregate resulted in unstable aggregates. We find that controlling cell aggregation using this microwell platform aids in reducing the variability and increases the reproducibility of experimental results, since it allows one to accurately control aggregate size by seeding a specific cell number per well. A major advantage of the agarose microwell cell aggregation method compared to conventional ultra-low attachment plates is the reproducibility of aggregate formation. Whereas cell aggregation in ultra-low attachment plates, as shown in Figure 2, results in aggregates, with heterogeneous size and shape between 40 and 250 μm, while aggregation in the agarose microwell system results in well-defined aggregates, between 90 and 110 μm ensuring reproducible results when comparing different conditions and repetitive experiments. In comparison to, for example, the more labour-intensive hanging drop method; the agarose microwell technique consists of a high-throughput format following standard simple cell culture procedures without the need for careful handling of samples.

Regarding their endocrine function, INS-1E cell aggregates showed an insulin secretory response upon stimulation with high glucose, indicating that the aggregates were glucose-responsive. This is in line with an extensive follow-up study by Merglen et al., reporting that INS-1E cells and primary rat islets share similar insulin secretory kinetics, underlining the potential of INS-1E cell aggregates as a valuable model for research purposes (29). We did not observe a correlation between aggregate size and function, which is in agreement with other studies using MIN6 cells (25).

In conclusion, our agarose microwell platform provides a platform to create primary human islet cell aggregates, pseudoislets, with pre-defined dimensions. This three-dimensional shape has been shown to be critical for optimal β-cell function (38). We showed that primary human islet cell aggregates with a diameter of 100–150 μm remain stable, viable and functional both in vitro and in vivo. We find that controlled reassembly of dissociated human islet cells into pre-defined aggregates leads to an initial atypical core mantle arrangement of β- and α-cells, which remodels after implantation under the kidney capsule during 14 days. Moreover, with increase in islet cell number and aggregate diameter β-cell specific gene expression and function increases to almost similar levels as native islets. The slight differences between islets cell aggregates and native islets seem to suggest a crucial factor is missing, an important factor could be the lack of appropriate β-cell and extracellular matrix interaction, which is known to play an important role in β-cell function, the cellular interaction with islet extracellular matrix is evidently lost after enzymatic dissociation of pancreatic islets (39). Future research could include the role of islets extracellular matrix proteins and their effect on β-cell function during islet cell aggregate assembly using agarose microwells for controlled aggregation to further elucidate this finding.

ACKNOWLEDGEMENTS

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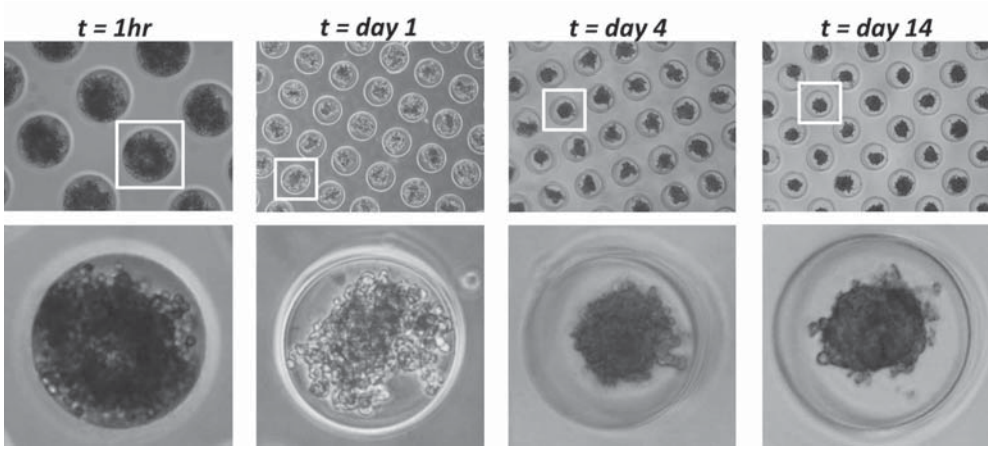
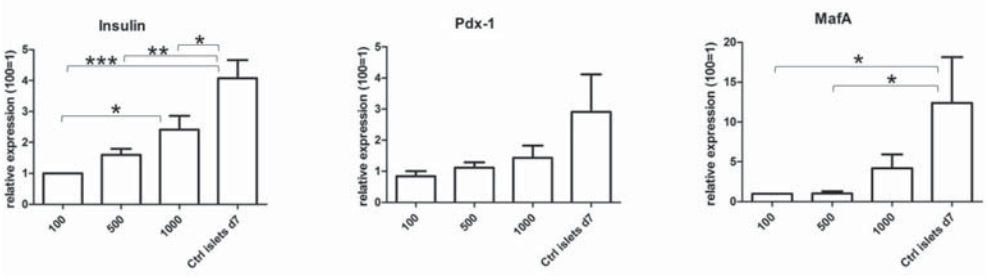
SUPPLEMENTARY FIGURES AND TABLES

Supplementary Table 1. Human pancreas donor information

Pancreas	Age (yrs)	Sex	BMI	Purity (%)
1	53	M	21	55
2	55	F	24	70
3	19	F	19	65
4	43	F	21	85
5	51	F	30	90
6	61	F	23	95
7	42	M	22	90
8	67	F	25	90
9	55	M	25	85
10	55	M	23	70
Average	50		23	80
LOWEST	19		19	55
HIGHEST	67		30	95



Supplementary Figure 1. Aggregation dynamics in agarose microwells. Primary human islets were dispersed and single islet cells were seeded at 1000 cells per microwell. Images represent human islet cell aggregates inside the microwells at various time points during 14 days of culture. Cell aggregation occurs during the first 2-4 days, and stable aggregates were maintained up to 14 days of culture.



Supplementary Figure 2. Gene expression of human islet cell aggregates. Relative mRNA levels for *INSULIN*, *PDX1* and *MAFA* expressed by intact control islets and human islet cell aggregates of 100, 500 or 1000 cells after 7 days of culture in microwell chips.

CHAPTER 4

CONVERSION OF MATURE HUMAN β-CELLS INTO GLUCAGON-PRODUCING α-CELLS

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ABSTRACT

Conversion of one terminally differentiated cell type into another (or transdifferentiation) usually requires the forced expression of key transcription factors. We examined the plasticity of human insulin-producing β-cells in a model of islet cell aggregate formation.

Here, we show that primary human β-cells can undergo a conversion into glucagon-producing α-cells without introduction of any genetic modification. The process occurs within days as revealed by lentivirus-mediated β-cell lineage tracing. Converted cells are indistinguishable from native α-cells based on ultrastructural morphology and maintain their α-cell phenotype after transplantation *in vivo*. Transition of β-cells into α-cells occurs following β-cell degranulation and is characterized by the presence of β-cell specific transcription factors Pdx1 and Nkx6.1 in glucagon$^+$ cells. Finally, we show that lentivirus-mediated knockdown of Arx, a determinant of the α-cell lineage, inhibits the conversion. Our findings reveal an unknown plasticity of human adult endocrine cells that can be modulated. This endocrine cell plasticity could have implications for islet development, (patho)physiology and regeneration.

The composition and architecture of human islets of Langerhans has been studied for years within their native environment, the pancreas. More recently, the development of islet transplantation as a novel therapeutic option for patients with severe β-cell loss has promoted the study of isolated human islets and single endocrine cells (1). The majority of pancreatic islets consist of two main cell types that together play a key role in glucose homeostasis: insulin-producing β-cells (50-70%) and glucagon-producing α-cells (20-30%) (1;2). Human islets display a unique architecture that favors contacts between β-cells and α-cells while both cell types remain in close relation to the vasculature (3).

α- and β-cells originate from a common neurogenin-3 (Ngn3) expressing endocrine progenitor (4). The balance between transcription factors Aristaless-related Homeobox (Arx) and Paired box4 (Pax4) likely determines the early fate restriction of α- and β-cells , respectively (5). Further maturation of β-cells is enabled by the expression of Nkx6.1 (6), while β-cell function is maintained in the adult pancreas by key transcription factors like Pdx1, MafA and FoxO1 (7).

Strategies to convert postnatal cells derived from the endodermal lineage into endocrine cells have gained much attention in recent years. Forced expression of key transcription factors in murine liver (8;9) or pancreatic cells (10-12) induce conversion into cells with a β-cell phenotype. Furthermore, in mice near-total loss of β-cell mass causes a small proportion of remaining α-cells to regenerate β-cell mass (13).

It is generally thought that human endocrine cells do not switch their hormone production once fully differentiated. Without using genetic modification of human islet cells we now show that β-cells spontaneously convert into glucagon-producing α-cells during islet cell reaggregation.

RESEARCH DESIGN AND METHODS

Human islet isolation and cell culture

Human islet isolations were performed in the GMP-facility of our institute according to the method described by Ricordi (14). Islets were dispersed into single cells by adding 0,025% trypsin solution containing 10 µg/ml DNase (Pulmozyme, Genentech) at 37 °C while pipetting up and down for 6-7 minutes. The islet cell suspension was plated onto 3% agarose microwell chips containing 2865 microwells per chip with a diameter of 200 µm per microwell(15). Suspension of $3x10^6$ cells per chip resulted in spontaneous reaggregation of approximately 1,000 islet cells per microwell. Islet cell aggregates and intact human islets (control) were cultured in CMRL 1066 medium (5.5 mM glucose) containing 10% FCS, 20 µg/ml ciprofloxacin, 50 µg/ml gentamycin, 2 mM L-glutamin, 0.25 µg/ml fungizone, 10 mM Hepes and 1.2 mg/ml nicotinamide.

Lentivirus vectors

pTrip-RIP405Cre-ERT2-DeltaU3 (RIP-CreERT2) and pTrip–loxP-NEO-STOP-loxP-eGFP-DeltaU3 (CMVstopGFP) were kindly provided by P. Ravassard(16). pTrip vectors were produced as 3rd generation lentivirus vectors by adding a Tat expressing vector to the regular

helper plasmids. The shRNA construct against Arx (shArx) was obtained from the MISSION library (Sigma-Aldrich, clone #6591, non-target control #SHC-002) and produced as described previously (17). For lineage tracing, transduction was performed as described (16). Briefly, dispersed islet cells were transduced overnight with a 1:1 mixture of the two lentiviruses at a multiplicity of infection of 2 in regular CMRL medium containing 8 ug/mL polybrene. In experiments using the shArx construct, a second round of transduction was subsequently performed for 8 hours. 4-Hydroxy-tamoxifen (Sigma-Aldrich, St.Louis, MO) was added to a final concentration of 1 μM in the evening. Following overnight incubation, the medium was refreshed and cells were seeded on the microwell. The start of reaggregation represents day 0 in our experiments.

RNA isolation and quantitative PCR

Total RNA was extracted using RNeasy kit (Qiagen) according to the manufacturer's protocol. 1 μg of total RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen). Quantitative PCR (qPCR) was performed on a Light Cycler 480-II Real-time PCR system (Roche). Fold induction was calculated using deltaCT method with human β-actin as housekeeping gene. Primers used are listed in Supplementary table 1.

Immunofluorescence staining

Formalin-fixed islet cell aggregates were washed in PBS and spun down at high speed in fluid agar. Agar containing cell pellets were embedded in paraffin. Blocks were cut into 4 μm sections. Primary antibodies against insulin (Linco, 1:200), C-peptide (Millipore, 1:1000), glucagon (Vector, 1:200; Invitrogen, 1:200), Pdx1 (R&D Systems, 1:5), Nkx6.1 (Developmental Studies Hybridoma Bank, clone F55A12, 1:1000), Ki67 (BD Pharmingen, 1:200) and GFP (Molecular Probes, 1:500; Roche, 1:500) were used. DAPI (Vector) was used as nuclear counterstaining. Secondary antibodies were TRITC-anti-guinea pig (Jackson, 1:400) and Alexa 488-, 568- and 647- anti-mouse or anti-rabbit when appropriate (1:1000). Apoptosis was assessed by terminal dUTP nick end labelling (TUNEL) assay (Roche). Sections were examined using confocal microscopy. Staining was quantified as percentage of positive cells per total cell number, counting at least 750 cells per donor for each condition.

Glucose Stimulated Insulin Secretion

Four groups of 30 aggregates per condition were incubated in a modified Krebs-Ringer Bicarbonate buffer (KRBH). Islets were successively incubated for 1 hour in KRBH with 2 mM and 20 mM glucose at 37°C. Insulin concentration was determined by ELISA (Mercodia, Sweden).

Electron microscopy

Islet cell aggregates were fixed in 1.5% glutaraldehyde and post-fixed in 1% osmium tetroxide for conventional transmission electron microscopy. 100 nm thin sections were

post-stained with uranyl acetate and lead citrate for ultrastructural analysis. For immunogold labelling, cells were fixed in 0.2% glutaraldehyde and 2% paraformaldehyde. Sections were stained using rabbit-anti-GFP (1:100), rabbit-anti-glucagon (1:200) and mouse-anti-C-peptide (Millipore, 1:1000) and binding was identified using protein A gold (10 or 15nm labels). Images were made on a FEI Tecnai 12 BioTwin transmission electron microscope. Automated stitching of electron micrographs was performed as described previously (18). Stitched images were quantified using imagescope software.

Animal transplantation

All animal experiments were approved by the LUMC committee for animal ethics. Human islet cell aggregates were transplanted under the kidney capsule of 7-12 weeks old male NOD/SCID mice. After 2 weeks, grafts were removed for histology.

Statistical analysis

Data are expressed as mean±S.E.M unless stated otherwise. Statistical significance of differences between groups were determined by an unpaired Student's *t*-test or by one-way ANOVA followed by Bonferroni's multiple comparisons test, as appropriate. $P<0.05$ was considered statistically significant.

RESULTS

Human islet cell aggregate formation results in an increase in glucagon$^+$ cells

Human islets were isolated from pancreas of organ donors (average age 52, range 19-71 years, Supplementary Table 2) to ≥70% purity as determined by dithizone staining (Supplementary Fig. 1*A*). The starting population of intact islets was further characterized by immunostaining and showed 30,1±3.9% β-cells, 8.1±1.9% glucagon-immunoreactive cells and 20,6±5.2% duct cells (Figure 1 and Supplementary Figure 1*B*). For functional and histological studies on islet cell aggregates with a predefined size, human islets were dispersed into single cells followed by reaggregation during culture in agarose-based microwells. One thousand islet cells were seeded per microwell resulting in the formation of human islet cell aggregates with a diameter of 131±10 μm (Fig. 1*A*). Compared to intact islets, human islet cell aggregates showed an unusual architecture after 7 days: insulin$^+$ cells were typically found at the rim and glucagon$^+$ cells were located in the center of the aggregates (Fig. 1*B*). Furthermore, within 4 days of reaggregation the percentage of insulin-immunoreactive cells was significantly decreased (Fig. 1*C*). This decrease was accompanied by a decrease in gene expression of *insulin*, *pdx1* and *mafa*. In line, immunolabeling for Pdx1 was decreased following reaggregation (Supplementary Fig. 1*C* and *D)*. In contrast, the percentage of glucagon-immunoreactive cells was significantly increased during the 14 day reaggregation period, accompanied by a tendency in increased gene expression (Fig. 1*C* and Supplementary Fig.1*C*). We first assessed whether these changes were due to β-cell loss and/or α-cell proliferation. Terminal dUTP nick end labelling (TUNEL) assay showed overall <2% apoptotic cells while

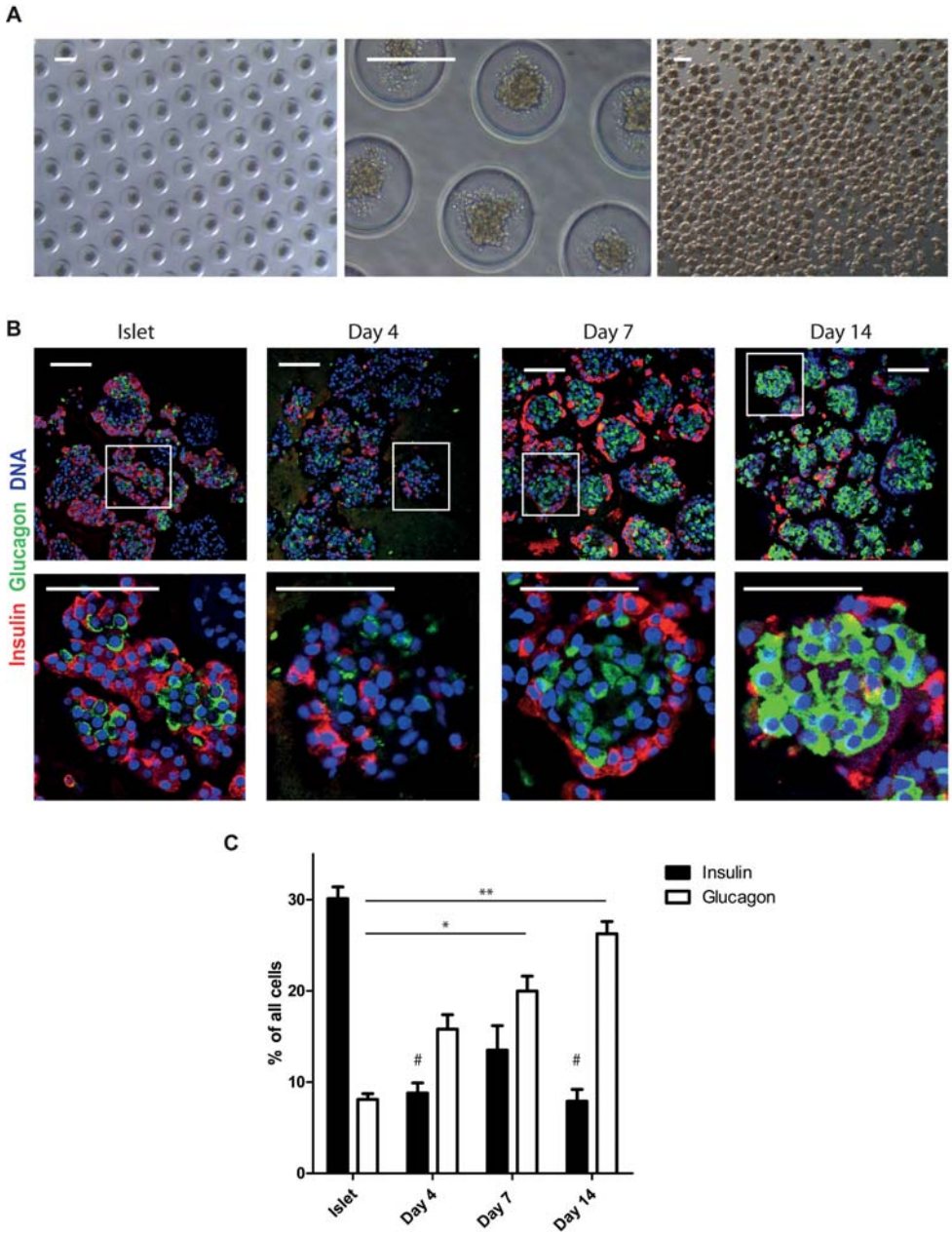
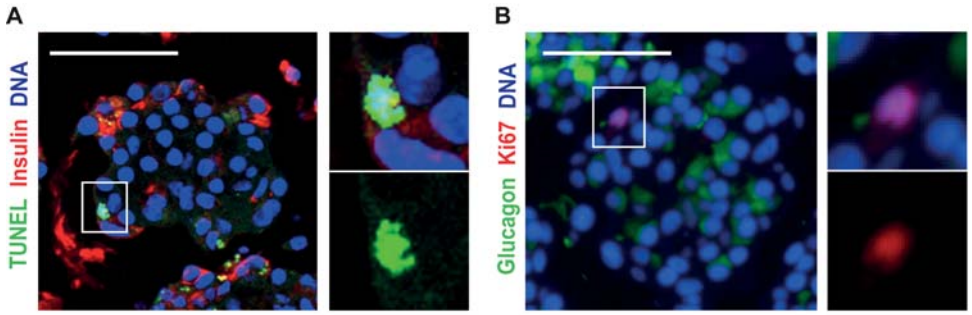


Figure 1. Human islet cell aggregate formation results in an increase in glucagon+-cells. A: Culture of dispersed islet cells in microwells (diameter 200 µm) results in homogenously sized islet cell aggregates that remain intact after flushing them out of the microwells. Scale bars: 200 µm. B: Human islet cell aggregate formation results in a distinct architecture following reaggregation: β-cells (insulin, red) are located at the rim and α-cells in the center (glucagon, green). Scale bars: 100 µm. C: Over time, an increased proportion of glucagon+-cells was present in islet cell aggregates (data are presented as mean ± S.E.M., n = 4-9 donors, *p<0.05, **p<0.005 vs. intact islets glucagon, #p<0.05 vs. intact islets insulin).

<1% cell proliferation (Ki67) was observed, of which only 4 out of 115 Ki67$^+$ cells were also glucagon immunoreactive. No significant differences in labeling between isolated islets and at 4, 7 or 14 days of reaggregation were detected that could explain the change in cell composition (Fig. 2).



C

	Donors	TUNEL+ (%)	Ki67+ (%)
Islet	5	50/6022 (0.77±0.34)	48/5665 (0.62±0.70)
Day 4	3	36/4979 (0.80±0.41)	29/6060 (0.47±0.45)
Day 7	5	58/6738 (0.94±0.36)	36/12339 (0.41±0.48)
Day 14	3	37/2663 (1.66±1.87)	2/2237 (0.07±0.12)

Figure 2. Change in endocrine hormone distribution is not due to apoptosis or proliferation. A and B: Representative picture of TUNEL assay combined with insulin staining (A) and Ki67 staining with glucagon (B). C: Quantification of apoptotic and proliferating cells defined by the number of positive nuclei in counted cells (data are presented as mean ± S.E.M., n = 3-6 donors).

Increased number of glucagon+ cells results from the conversion of β-cells

In order to explain the decrease in insulin$^+$ cells and increase in glucagon$^+$ cells, we hypothesized that a subpopulation of β-cells converts into α-cells. To trace β-cell fate directly, dispersed islet cells were transduced with RIP-Cre-ERT2 and CMV-loxP-NEO-STOP-loxP-eGFP lentiviral vectors as shown previously (16;19). Tamoxifen administration results in sustained GFP expression in cells that express insulin at time of the transduction (Fig. 3*A*, *B* & Supplementary Fig. 2*A*, *C* and *E*). We verified the efficiency and specificity of the lineage tracing system by immunostaining (Supplementary Fig. 2*D* and *E*). Importantly, less than 1% of GFP$^+$ cells expressed glucagon 1 day after tamoxifen administration (Supplementary Fig. 2*E*). After 7 days islet aggregate formation in the microwells, not all GFP$^+$ cells were positive for insulin or C-peptide (Fig. 3*C*). Intriguingly, up to 15% of GFP$^+$ cells co-expressed glucagon after 14 days (Fig. 3*D* and *E*). This was confirmed by

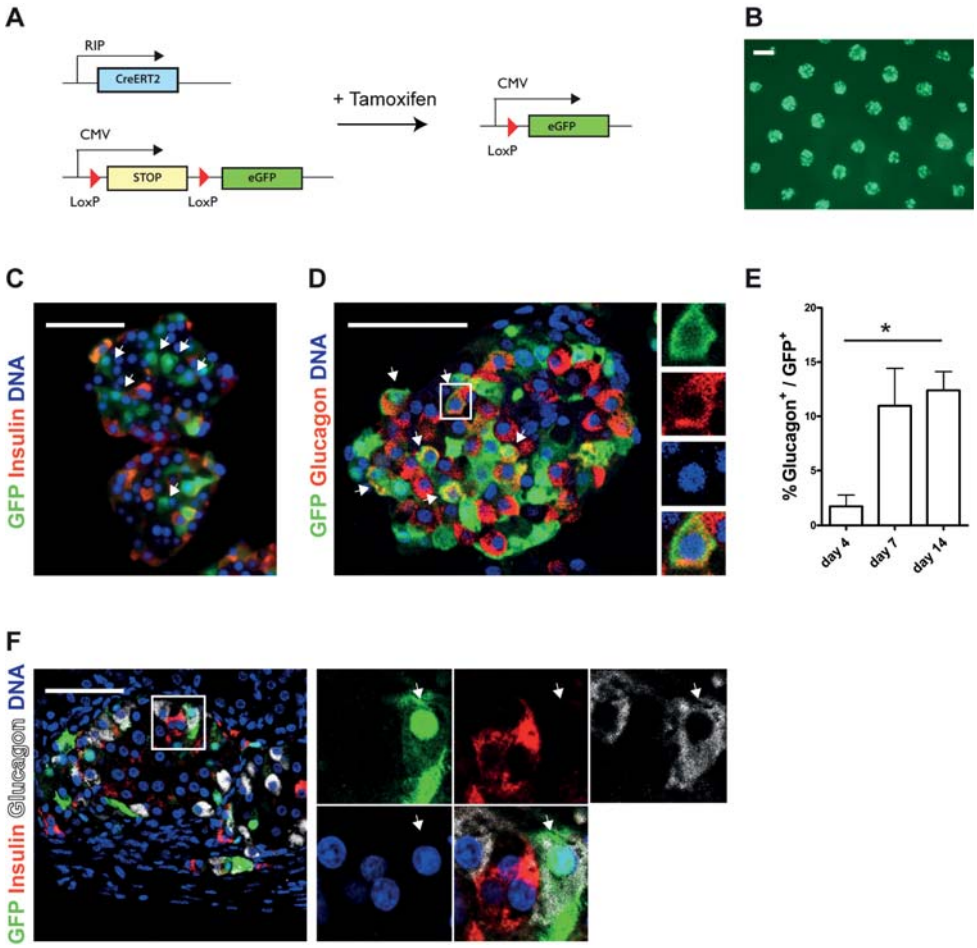


Figure 3. Increased number of glucagon+-cells results from the conversion of β-cells. A: Schematic representation of conditional β-cell specific lineage tracing using two lentiviral vectors. B: Live cell imaging of transduced human islet cell aggregates shows an equal distribution of epifluorescent GFP throughout the microwell chip. C: After 7 days reaggregation, GFP+-cells are observed that do not express insulin (arrows) D and E: Glucagon+/GFP+-cells (arrows) are present in human islet cell aggregates (D) with increasing numbers over time (E) (data are presented as mean + S.E.M., n = 3-6 donors, *p<0,05). F: Glucagon+/GFP+-cell (arrows) in a graft after transplantation under the kidney capsule of NOD/SCID mice. Scale bars: 50 µm, except for B: 200 µm.

whole mount confocal imaging to detect epifluorescent GFP in cells immunolabeled for glucagon, excluding GFP antibody artifacts (Supplementary Fig. 3*A*). The presence of GFP$^+$/glucagon$^+$ cells was further confirmed by the detection of glucagon$^+$ cells after fluorescence activated cell sorting (FACS) of the GFP$^+$ fraction following redispersion of the islet cell aggregates (Supplementary Fig. 3*B*). GFP$^+$/glucagon$^+$ cells remained present even after transplantation of the islet cell aggregates *in vivo* under the kidney capsule of NOD/SCID mice (Fig. 3*F*). The presence of GFP$^+$ cells did not significantly change over time during the lineage tracing experiments *in vitro* and *in vivo* (data not shown). Of note,

both the typical architecture and the cell conversion were observed after reaggregation in 6-well ultra-low attachment plates, excluding the possibility that the microwell culture system is involved directly (data not shown).

Converted cells have an α-cell phenotype based on ultrastructural morphology

To determine whether the converted β-cells, defined as GFP$^+$/glucagon$^+$ cells, have an α-cell ultrastructure, electron microscopy was performed. Secretory granules containing insulin can be distinguished from secretory granules containing other islet hormones based on their ultrastructure (20). Immunogold labelling for GFP confirmed co-localization with cells containing insulin granules but also demonstrated co-localization with cells containing more homogeneous, darker, dense granules typical of α-cells (Fig. 4*A* and *B*). Double immunogold labelling for GFP and glucagon showed that 51% of GFP$^+$ cells contained insulin granules while 29% contained typical, immunogold-labelled, glucagon granules after 14 days (Fig. 4*C*, Supplementary Fig. 4*A* and *B*). Granule morphology and localization were identical to that in the GFP-negative α-cells (Fig. 4*C* and *D*). All GFP$^+$ cells containing the homogeneous, darker, dense granules typical of α-cells were positive for glucagon immunogold, thereby confirming the preferential conversion of β-cells into α-cells. Furthermore, out of 254 counted cells 18% of GFP$^+$ cells were determined as degranulated (<10 granules) and 1 cell contained large amylase granules. Thus, in combination with the lineage tracing, our data demonstrate that mature human β-cells can convert into glucagon-producing α-cells within days of culture following dispersion and reaggregation.

β-cell degranulation occurs during human islet cell aggregate formation

To get an insight into the mechanism of β- to α-cell conversion, we studied in more detail the early events following dispersion and reaggregation. First of all, using immunostaining, no co-expression of glucagon with insulin (Fig. 1B) or C-peptide (data not shown) was detected 4, 7 or 14 days after reaggregation. To ensure the absence of bi-hormonal cells at the level of single granules, we confirmed these findings by electron microscopy performing double labelings for glucagon and C-peptide. (Supplementary Fig. 5). However, immunostaining within the first 5 days following reaggregation showed up to 50% of cells negative for insulin, but positive for the β-cell specific transcription factors Pdx1 or Nkx6.1, indicating β-cell degranulation (Fig. 5*A* and *B*, and data not shown). This was confirmed by ultrastructural analysis showing that 77% of β-cells contained less than 70 granules after 4 days reaggregation compared to only 32% of β-cells in intact islets (Fig. 5*C* and *D*). Altogether these data show that dispersion followed by reaggregation results in degranulated β-cells that may still contain specific transcription factors.

β-to-α transition is demonstrated by glucagon$^+$ cells that express β-cell transcription factors

Next we hypothesized that, during the transition phase, cells that lost insulin might co-express β-cell transcription factors and glucagon. Using β-cell lineage tracing, we

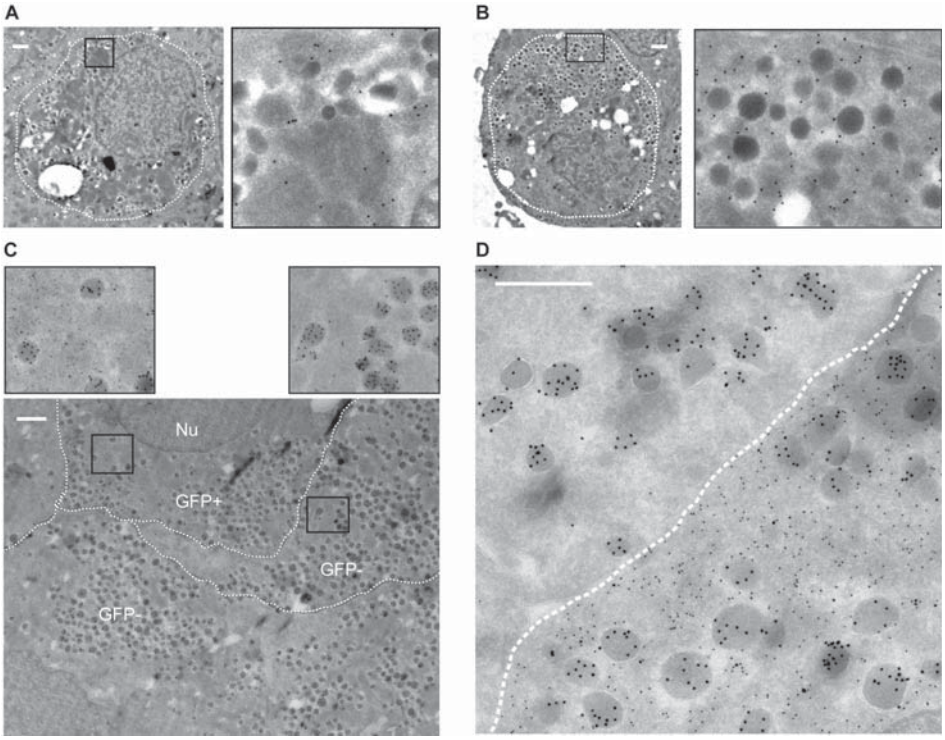


Figure 4. Converted cells are regular α-cells based on ultrastructural morphology. A and B: Immunogold labeling for GFP (15 nm gold particles) is present as black dots in both cells with granules with a typical crystalline structure containing insulin (A) and cells with more homogenous dark and dense non-insulin granules (B). C and D: Double immunogold labelling for GFP (10 nm gold particles) and glucagon (15 nm gold particles) shows the presence of α-cell granules in both GFP+ (converted) and GFP- cells (C). Immunogold labeling for GFP is absent within granules (D). The borders between the cells are marked manually by a broken line (nu = nucleus). All scale bars: 500 nm.

show the presence of cells expressing Nkx6.1 or Pdx1 together with glucagon and GFP (Fig. 6*A*). Interestingly, the percentage of cells co-expressing Pdx1 and glucagon was significantly increased up to 3% of glucagon$^+$ cells at 7 days reaggregation (Fig. 6*B*). In addition, using immuno electron microscopy we observed the presence of GFP$^+$ cells that contain only very few (<10) glucagon-labeled granules, suggesting recent transition after degranulation (Fig. 6*C*).

Arx knockdown inhibits the conversion of β-cells into α-cells

Arx gene expression was increased in human islet cell aggregates (Fig. 7*A*). To investigate the role of Arx in the conversion process, we knocked down *arx* gene expression using a lentivirus-mediated short hairpin RNA against *arx* (shArx). Knockdown was accompanied by a significant decrease in *glucagon* mRNA and a trend towards induction of *pax4* and *insulin* gene expression (Fig. 7*B*). β-cell specific lineage tracing revealed that *Arx*

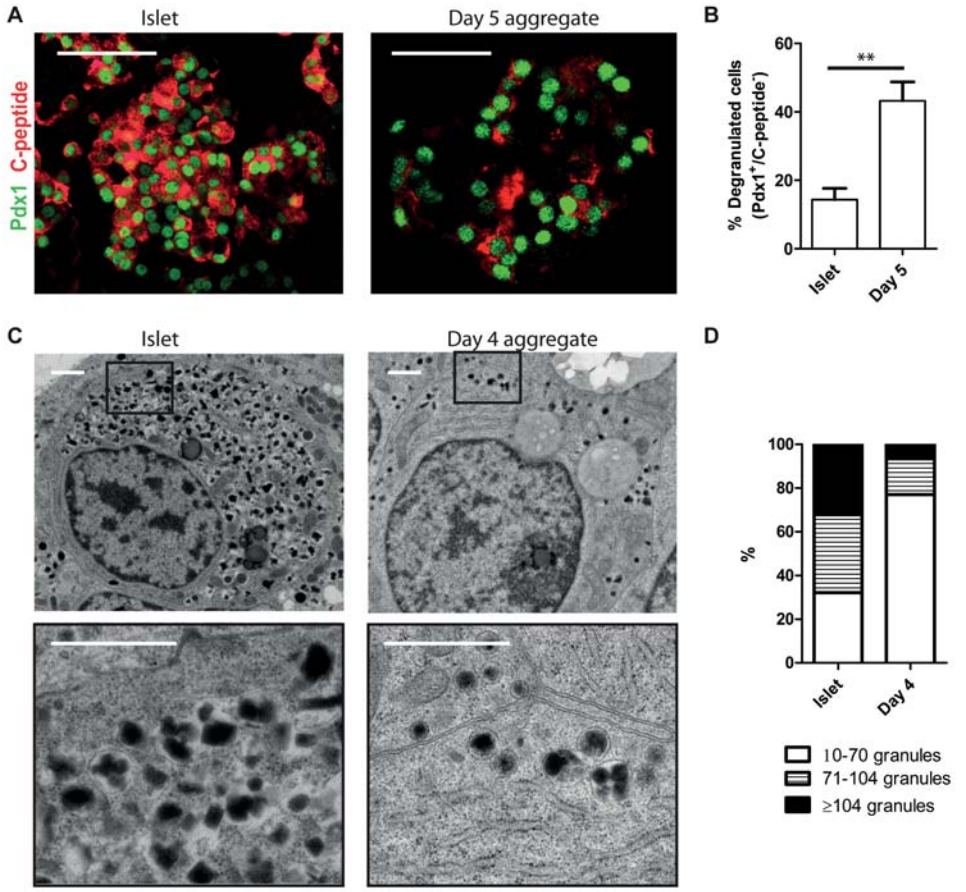


Figure 5. Reaggregation is accompanied by β-cell degranulation. A: Immunostaining for Pdx1 (green) and C-peptide (red) in intact islets and 5 days following reaggregation. Scale bars: 100 µm. B: Quantification of the number of Pdx1+/C-peptide--cells (data are presented as mean + S.E.M., n=3 donors, **p<0,005). C: Electron microscopy photographs showing insulin granules in intact islet β-cells and 4 days following reaggregation. Scale bars: 1 µm D: Distribution of the amount of insulin granules per β-cell in intact islets and 4 days following reaggregation (for representation of the distribution, granule number in intact islets was divided in tertiles, >50 cells were counted per condition).

knockdown increased the number of GFP$^+$/insulin$^+$ cells almost twofold, while the number of GFP$^+$/glucagon$^+$ cells decreased by 40% (Fig. 7*C-E*). Finally, shArx treated cells showed higher glucose-induced insulin secretion than cells treated and transduced with a non-target (control) shRNA (average stimulation index 5,5±2,5 for shCtrl versus 8,3±0,6 for shArx) in two independent experiments (Fig. 7*F*). These data demonstrate that the conversion of human β-cells into glucagon-producing α-cells can be modulated by changing the expression of a key transcription factor.

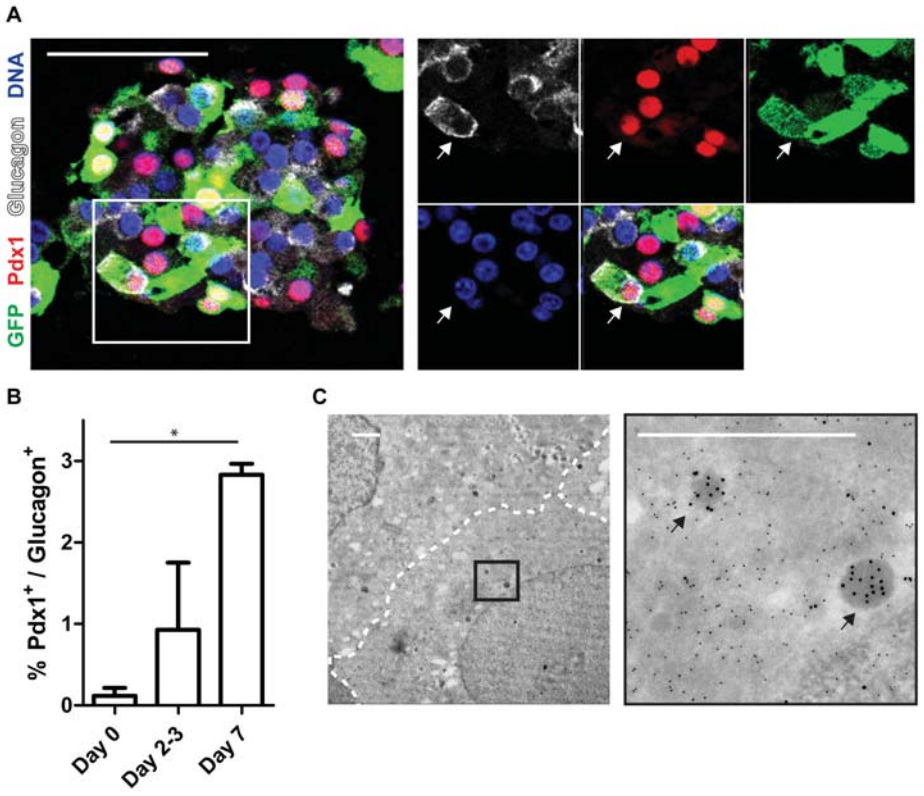


Figure 6. β- to α-cell transition is marked by the presence of glucagon+-cells containing β-cell transcription factors. A: Immunostaining for GFP (green), Pdx1 (red) and glucagon (green), (arrows in enlargement show triple positive cell). B: Quantification of Pdx1+/Glucagon+-cells shows an increase during reaggregation (n = 3 donors, *p<0,05). C: Double immunogold labelling for GFP (10 nm gold particles) and glucagon (15 nm gold particles) following reaggregation (arrows show single glucagon granules). Scale bars EM: 1 µm, IF = 100um

DISCUSSION

Mature human β-cells are typically considered as terminally differentiated cells that do not switch their hormone production once fully differentiated. Our results show that human β-cells can convert rapidly and preferentially into glucagon-producing α-cells without forced expression of exogenous transcription factors. Of importance, the conversion occurred in islets from pancreas of organ donors from different backgrounds, independent of donor factors such as age or gender.

To date only few examples of human cell transdifferentiation have been reported. This usually requires the forced expression of transcription factors or miRNAs to convert human fibroblasts into neurons, hematopoietic progenitors or brown fat cells (21) or human liver cells into β-cells (22). In our 3-dimensional culture system the dispersion into single cells followed by reaggregation into islet cell aggregates is likely to be a critical step for cell conversion. Previous lineage tracing studies in human β-cells following dispersion and

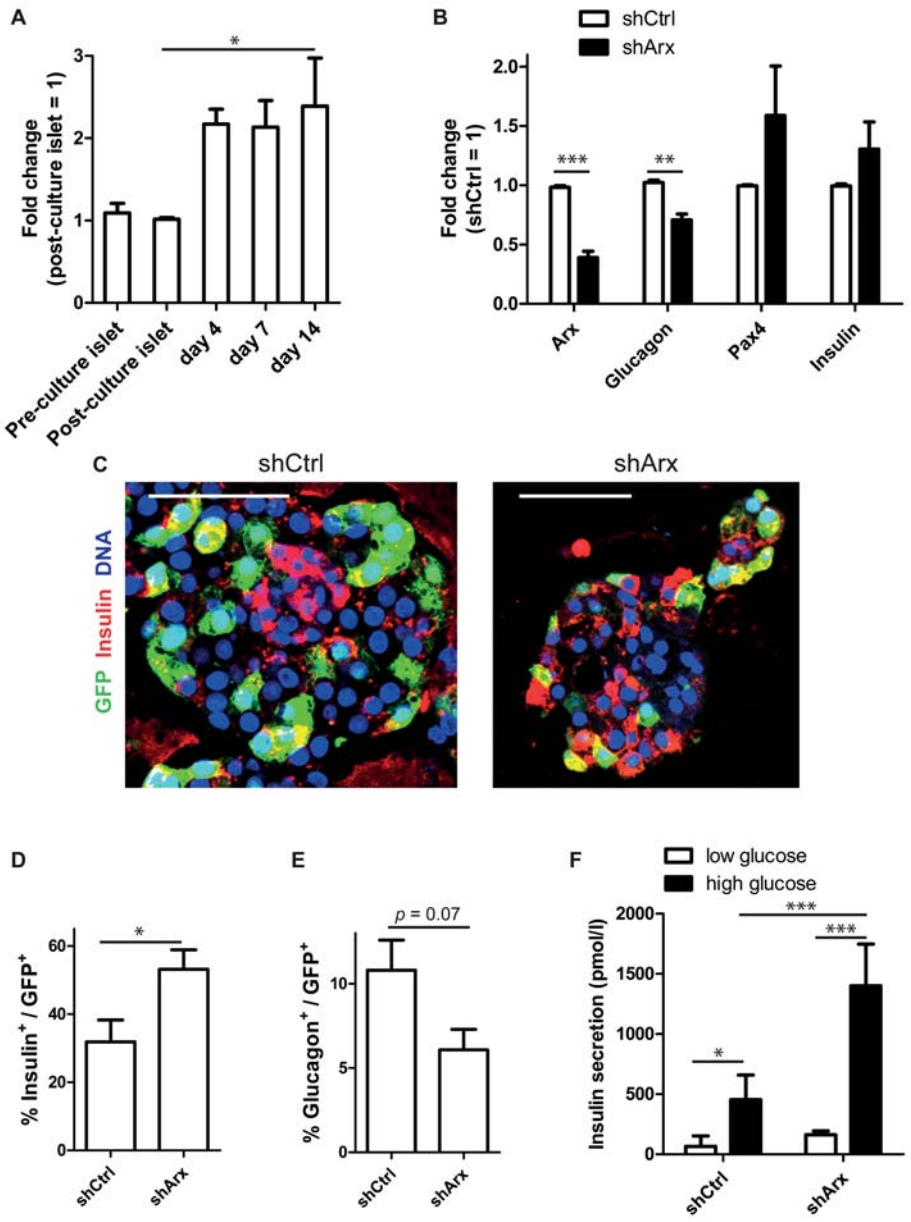


Figure 7. Arx knockdown inhibits the conversion of β-cells into α-cells. A: Arx gene expression in human islet cell aggregates compared to control intact islets (n = 3-6 donors). B: shRNA directed against Arx (shArx) results in knockdown of Arx mRNA that is accompanied by changes in glucagon, Pax4 and insulin mRNA levels (n = 4 donors). C: Combining β-cell lineage tracing and shRNA treatment shows a large proportion of GFP+/insulin- cells in shCtrl-treated islet cell aggregates, but an increase in GFP+/insulin+-cells in shArx-treated islet cell aggregates (D and E: The number of remaining β-cells is increased in shArx-treated aggregates compared to shCtrl (D) while the number of converted cells is decreased as represented by GFP+/glucagon+-cells (E) (n = 4 donors). F: Representative graph of a glucose stimulated insulin secretion assay that shows increased insulin secretion after treatment with shArx (mean ± SD of quadruplicates). All experiments were performed after 7-14 days reaggregation. Scale bars: 50 μm.

2-dimensional culture show rapid epithelial-to-mesenchymal transition and no generation of α-cells (16;23). In this context, the specific architecture of islet cell aggregates is of interest, as β-cells were mainly located at the periphery and α-cells in the center of the aggregates. Hence, cell-cell and cell-matrix interactions which are disrupted and reestablished, or the changed cell composition and paracrine signaling between islet cells within the aggregates could be mechanistically linked to our observations.

The mechanisms by which islet cell conversion occur include direct conversion from one endocrine cell type into another or dedifferentiation into a progenitor stage followed by the acquisition of a new endocrine cell phenotype (24). Although the short time period for the conversion to occur and the presence of cells co-expressing glucagon and β-cell specific transcription factors indicate a direct β- to α-cell conversion, we can't rule out a very transient dedifferentiation stage. Along this line, Talchai et al reported that murine FoxO1 deficient β-cells first dedifferentiate to a progenitor-like stage expressing Ngn3, Oct4, Nanog and L-Myc and subsequently convert into α-cells (25). In contrast, Thorel et al showed direct conversion of α-cells into β-cells following near-total β-cell ablation through the presence of bihormonal cells (glucagon^{+}/insulin^{+}) (13), thereby indicating that distinct mechanisms of islet cell conversion may underly these studies.

Maintenance of β-cell functional maturity is ascribed to two possible mechanisms, either passive or active control (7). Whereas the first option consists of transient activation of transcription factors that lock the cell in a mature state, the second theory relies on continuously active regulators that maintain a differentiated state. We now show that adult human β-cells display potential plasticity, favoring the second hypothesis**.** Accordingly, this plasticity is accompanied by the downregulation of key β-cell transcription factors such as Pdx1, Nkx6.1 and MafA. Moreover, knockdown of the α-cell factor Arx could block the conversion, indicating that β-cell plasticity depends on the presence or absence of active modulators. This is in line with recent data from transgenic mouse models (DNMT$^{-/-}$ and Nkx2.2$^{TNmut/TNmut}$) that indicate that β-cell identity is maintained by active repression of Arx gene transcription (26;27). We extend these observations by showing that Arx is pivotal in the maintenance of a β-cell phenotype in human cells as well, since the downregulation of Arx efficiently blocks the conversion of human adult β-cells.

The (patho)physiological relevance of our findings in humans is unclear. An increased α-cell mass has been reported in patients with type 2 diabetes (28), a condition which is also characterized by hyperglucagonemia, but this hypothesis has been challenged by a recent study in 50 patients (29). Interestingly, recent work from Talchai et al associates metabolic stress and type 2 diabetes in mice with β-cell dedifferentiation and conversion into α-cells (25). This is in line with studies on rats that suffer from chronic hyperglycemia following partial pancreatectomy, showing progressive loss of β-cell differentiation and a strong reduction in expression of β-cell transcription factors (30). In our human model, the substantial β-cell stress that is induced during the dispersion and reaggregation is likely to be an important factor as well. This could explain why near-total α-cell ablation in mice does not induce conversion of β-cells into α-cells. Since glucose homeostasis was hardly affected, it may be that β-cell stress in this experiment was limited (31).

Our data have potential relevance for regenerative strategies in diabetes. We show that without genetic modification conversion of one human islet cell type into another can be achieved. Identification of the signals that are relevant for maintenance and change of islet cell identity could be useful in the quest for generation of β-cells from alternative cell sources.

In conclusion, we provide a proof of principle that postnatal human β-cells can undergo a change in endocrine cell identity. It remains to be investigated whether other human endocrine cell types display the same plasticity and whether this phenomenon plays a role *in vivo*. Understanding the mechanisms by which adult β-cells maintain or change their identity can have important implications for understanding islet development, (patho) physiology and regenerative strategies in diabetes.

ACKNOWLEDGEMENTS

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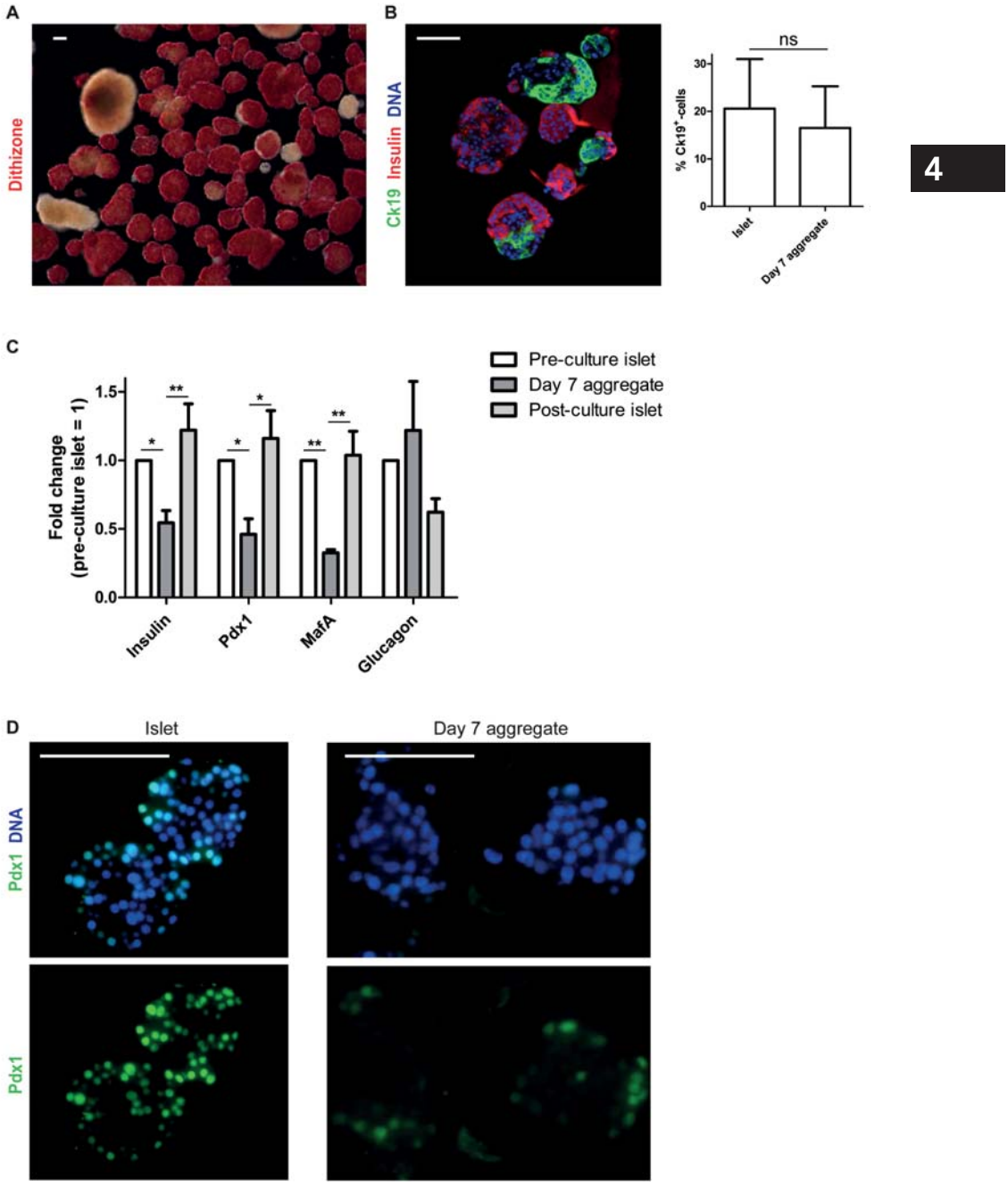
SUPPLEMENTARY FIGURES AND TABLES

Supplementary Table 1. qPCR primers and probes

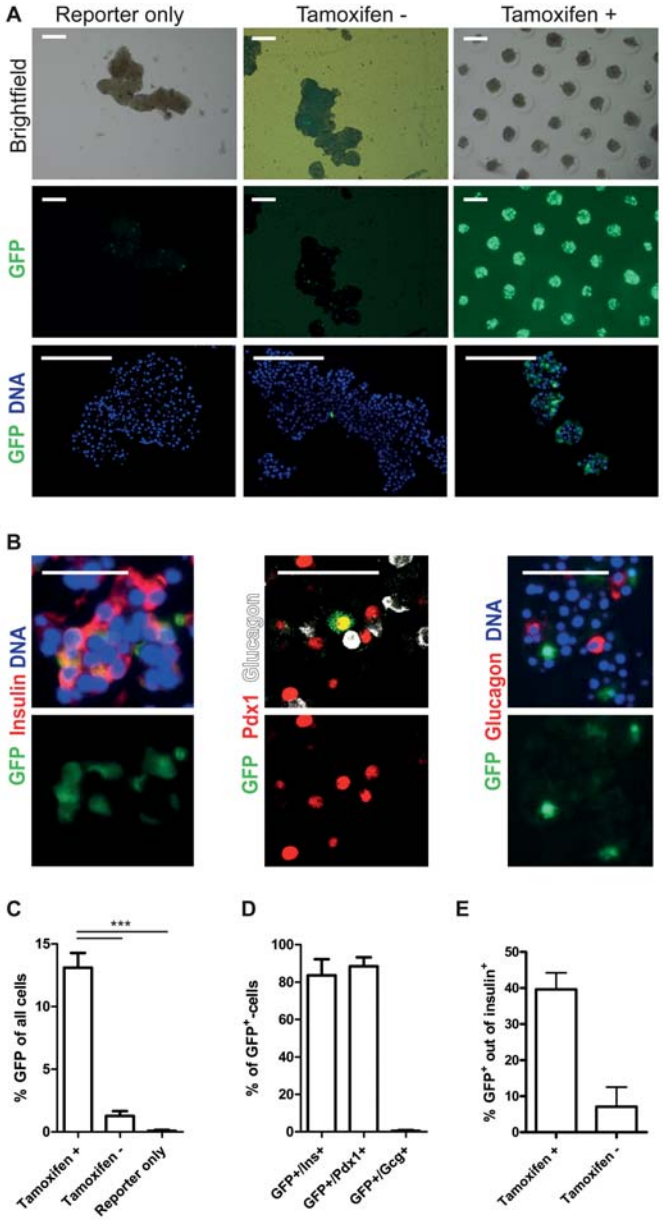
Gene	Probe / primer sequence
Glucagon	Hs00174967_m1
Insulin	Hs00355773_m1
Arx	Hs00292465_m1
Pax4	Hs00173014_m1
Glucagon	F 5'-CAAGGCAGCTGGCAACGT-3'
	R 5'-CTGGTGAATGTGCCCTGTGA-3'
Insulin	F 5'-GCAGCCTTTGTGAACCAACA-3'
	R 5'-TTCCCCGCACACTAGGTAGAGA-3'
MafA	F 5'-CTTCAGCAAGGAGGAGGTCATC-3'
	R 5'-GCGTAGCCGCGGTTCTT-3'
Pdx1	F 5'-CCATGGATGAAGTCTACCAAAGCT-3'
	R 5'-CGTGAGATGTACTTGTTGAATAGGAACT-3'

Supplementary Table 2. Donor list

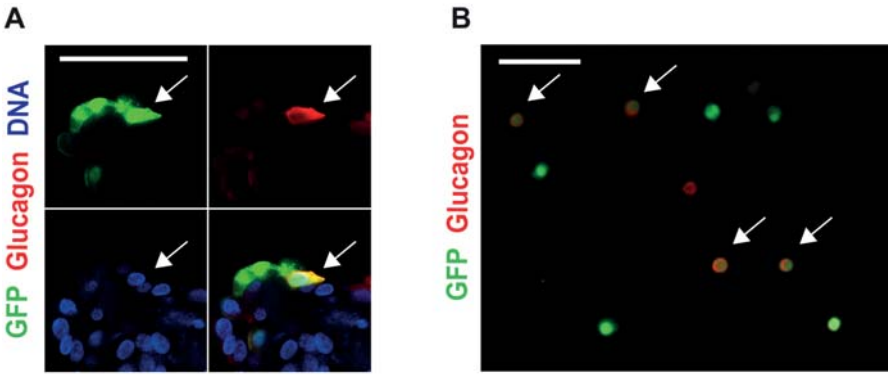
Pancreas number	age	sex	BMI
1	51	F	30
2	58	F	22
3	58	M	28
4	33	F	20
5	49	F	31
6	45	F	23
7	62	F	22
8	71	F	24
9	47	M	36
10	40	F	28
11	61	F	23
12	57	F	21
13	61	F	22
14	63	F	26
15	59	F	29
16	67	F	23
17	47	F	26
18	58	M	34
19	68	M	24
20	55	F	21
21	21	F	21
22	19	M	26
23	42	M	22
24	60	F	28
Average	52		25
LOWEST	19		20
HIGHEST	71		36



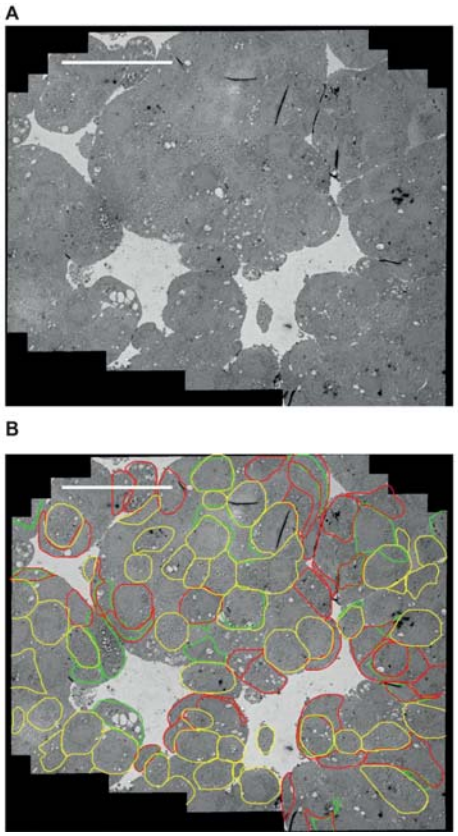
Supplementary Figure 1. Decrease in β-cells is accompanied by a decrease in expression of β-cell transcription factors. A: Freshly isolated purified human islets are discriminated from non-islet tissue using dithizone staining (red). B: Isolated human pancreatic islets stained for Ck19 (green) and insulin (red) and quantified before and after reaggregation (7 days) C: qPCR analysis of islet aggregates compared to intact islet before and after 7 days culture (* p<0,05, **p<0,01). D: Immunostaining for Pdx1 (green) and C-peptide (red) in intact and reaggregated islet cells. Scale bars: 100 μm.



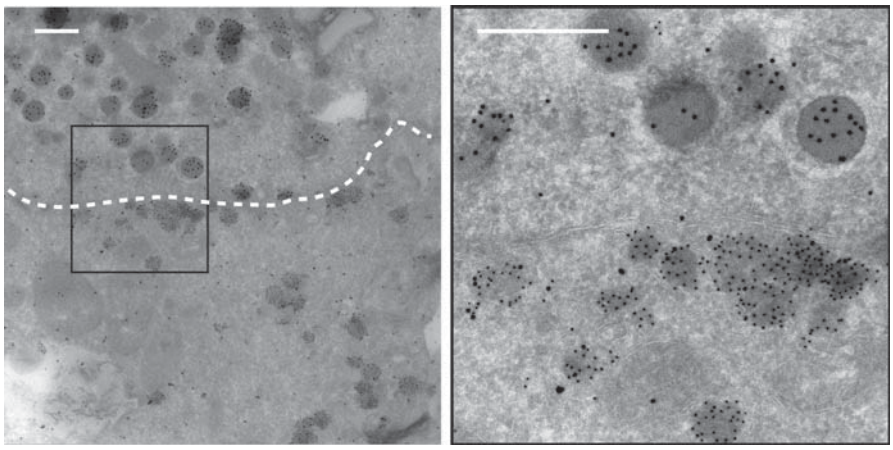
Supplementary Figure 2. β-cell lineage tracing specificity and confirmation of conversion. A: Live imaging and immunostaining shows few GFP+-cells in aggregates transduced with the reporter construct only (CMV-stop-GFP) or without tamoxifen compared to with tamoxifen administration. B: Immunostainings that combine GFP with either insulin, Pdx1 or glucagon were performed directly following tamoxifen-induced GFP expression (24-48 hours following dispersion) to assess specificity of the lineage tracing. C: Quantification of the percentage of GFP+-cells out of all cells. D Quantification of the percentage of GFP+-cells co-expressing either insulin (83.6±8.6%), Pdx1 (88.5±4.9%) or glucagon (<1%), showing the specificity of lineage tracing (arrows indicate double positive cells). E: Quantification of the percentage of insulin+-cells that express GFP showing the efficiency of the lineage tracing. Data are shown as mean ±S.E.M., ***p<0,001, n=2-4 donors. Scale bars A: 200 μm and B: 50 μm.



Supplementary Figure 3. A: Confocal image shows an optical section of epifluorescent GFP (green) and glucagon (red). B: FACSorted GFP+-cells immunostained for glucagon on cytospin. Scale bars: 50 μm.



Supplementary Figure 4. Quantification of electron microscopy analysis using digital stitching. A: Approximately 2500 electron microscopic photographs were stitched together to image a complete islet cell aggregate tissue section (150-250 cells per stitch) B: Annotations of a stitched image showing the localization of α-cells (yellow), β-cells (red) and GFP+-cells (green). Scale bars: 20 μm.



Supplementary Figure 5. Double immunogold labeling for glucagon and c-peptide. sDouble immunogold labeling was performed for glucagon (15 nm gold labels) and c-peptide (10 nm gold labels) after 4 days reaggregation. Borders between the cells are marked manually by a broken line on the left panel. Right panel shows higher magnification of the marked area. Scale bars: 500 nm

CHAPTER 5

LOSS OF β-CELL IDENTITY OCCURS IN TYPE 2 DIABETES AND IS ASSOCIATED WITH ISLET AMYLOID DEPOSITS

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ABSTRACT

Loss of pancreatic islet β-cell mass and β-cell dysfunction are central in the development of type 2 diabetes (T2DM). We recently showed that mature human insulin-containing β-cells can convert into glucagon-containing α-cells *ex vivo*. This loss of β-cell identity was characterized by the presence of β-cell transcription factors (Nkx6.1, Pdx1) in glucagon$^+$ cells. Here, we investigated whether loss of β-cell identity also occurs *in vivo* and whether it is related to the presence of (pre)diabetes in humans and non-human primates. We observed an 8x increased frequency of insulin$^+$ cells co-expressing glucagon in diabetic donors. Up to 5% of the cells that were Nkx6.1$^+$ but insulin$^-$ co-expressed glucagon, which represents a 5x increased frequency compared to the control group. This increase in bihormonal and Nkx6.1$^+$glucagon$^+$insulin$^-$ cells was also found in islets of diabetic macaques. The higher proportion of bihormonal cells and Nkx6.1$^+$glucagon$^+$insulin$^-$ cells in diabetic macaques and humans was correlated with the presence and extent of islet amyloidosis. These data indicate that loss of β-cell identity occurs in T2DM and could contribute to the decrease of functional β-cell mass. Maintenance of β-cell identity is a potential novel strategy to preserve β-cell function in diabetes.

Loss of pancreatic β-cell mass and β-cell dysfunction are central in the development of type 2 diabetes (T2DM) and, in combination with peripheral insulin resistance, lead to hyperglycemia (1). Whereas β-cells on the one hand fail to properly secrete insulin at a given glucose level, there is also a progressive decline in the number of β-cells (2;3). Loss of β-cell mass has been ascribed to increased apoptosis in T2DM (4). In patients with T2DM, β-cell mass can be up to 40-60% lower than in healthy controls (4-6). In addition, abnormal function of glucagon-producing α-cells leading to hyperglucagonemia is associated with T2DM (7). β-cell dedifferentiation and subsequent transition to other islet cell types was suggested as an alternative explanation for the loss of functional β-cell mass in mice (8;9). In this concept, β-cells lose insulin content and insulin secretory capacity followed by the production of other endocrine hormones such as glucagon (8). We recently showed that loss of β-cell identity with conversion of β-cells into glucagon-containing α-cells can occur in human pancreatic islets *ex vivo* (10).

A number of transcription factors have been identified to be essential for the development and maintenance of functional β-cells (11;12). Recent reports indicate that a selective loss of transcription factors MafA, Nkx6.1 and Pdx1 is associated with β-cell dysfunction and T2DM (13;14). Chronic hyperglycemia in rats is accompanied by the loss of β-cell transcription factors (15). Moreover, mouse β-cells that genetically lack FoxO1 can dedifferentiate *in vivo* under conditions of metabolic stress and subsequently convert (or transdifferentiate) into glucagon-producing α-cells, accompanied by hyperglucagonemia (8). In our *ex vivo* culture system, lineage tracing showed that mature human β-cells can lose their identity and convert into α-cells following dispersion and reaggregation (10). Conversion was characterized by a transition phase in which β-cell specific transcription factors (Nkx6.1, Pdx1) were expressed in glucagon-positive cells, a phenomenon also observed in the mice that genetically lack FoxO1 in β-cells (8;10). It is not clear whether loss of β-cell identity occurs in humans *in vivo* and whether this could contribute to a reduction in functional β-cell mass. We hypothesized that loss of β-cell identity (where β-cell identity is defined by the presence of specific transcription factors and insulin), is involved in the loss of functional β-cell mass that occurs in T2DM. Therefore we studied pancreas from humans and non-human primates to determine the relationship between metabolic status, pathological changes in islet morphology and loss of β-cell identity.

RESEARCH DESIGN AND METHODS

Human pancreas

Human cadaveric donor pancreas were procured through a multi-organ donor program. Pancreatic tissue was used in our study if the pancreas could not be used for clinical pancreas or islet transplantation, according to national laws and institutional ethical requirements, and if research consent was present. Tissue from organ donors who were previously diagnosed with T2DM (n=11) were compared to donors who had no history of diabetes (ND, n=9). Control donors were matched for age and BMI. More detailed information on all donors is summarized in Supplementary Table 1. Pancreatic tail

samples were fixed overnight in 4% formaldehyde (Klinipath), stored in 70% ethanol and subsequently embedded in paraffin. Blocks were cut into 4-µm thick sections.

Tissue from non-human primates

Fourteen male non-human primates (12 *Macaca mulatta* and 2 *M. fascicularis*) were selected from a larger colony that has extensively been characterized (16). The non-human primates were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and all protocols have been reviewed and approved by the university animal care and use committee. The animals were maintained on standard Lab Diet monkey chow (15% protein, 26% fat, and 59% carbohydrate, Purina, St Louis) that was provided *ad libitum*. Animals were divided into three groups based on the metabolic profile at sacrifice, being either normoglycemic and normoinsulinemic, normoglycemic and hyperinsulinemic (fasting immunoreactive insulin, IRI > 600 pmol/L) or overtly diabetic (fasting plasma glucose, FPG >7.8 mmol/L). The baseline characteristics (including β-cell and amyloid area) (17) for each group are provided in Table 1 and for each animal in Supplementary Table 2. Post-mortem samples were taken at sacrifice and processed for wax embedding. Some tissue blocks (in all 3 groups) showed poor quality of nuclear staining including DAPI (likely due to the fixation procedure). For the studies on non-human primates, staining quality was considered sufficient when nuclear Nkx6.1 labeling could be identified. This decision was made prior to analysis of > 20 islets. Tail samples were used except for 1 animal (animal Q in Supplementary Table 2) from which only a sample of the pancreas body was available.

Morphometry

β-cell area and α/β-ratio were determined as described previously (18;19). Briefly, sections were immunostained with primary antibodies against insulin (1:200, Millipore or Santa Cruz Biotechnology) and glucagon (1:200, Vector) for 1 hour followed by horseradish peroxidase (HRP)- or alkaline phosphatase (AP) conjugated secondary antibodies for 1 hour. Sections were developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) or

Table 1. Characteristics of the selected groups of non-human primates.

Group	NDM	NDM, HI	DM
Monkey (n)	4	4	6
Age (years)	10.9 ± 3.0	22.7 ± 2.7	23.3 ± 1.4
Body weight (kg)	7.6 ± 1.4	12.6 ± 1.8	11.3 ± 0.8
FPG (mmol/l)	3.9 ± 0.1	5.2 ± 0.9	13.7 ± 2.0
IRI (pmol/l)	299 ± 46	1278 ± 368	105 ± 19
Body fat (%)	26.4 ± 4.5	33.6 ± 2.8	37.3 ± 2.9
Amyloid/islet (%)	0	18.5 ± 7.7	56.5 ± 12.1
Islet with amyloid (%)	0	8.7 ± 4.1	82.6 ± 16.5

NDM = non-diabetic, HI = hyperinsulinemic, DM = diabetic IRI, immunoreactive insulin. FPG, fasting plasma glucose. Data are represented as mean ± SEM.

liquid permanent red (LPR, Dako) respectively, and counterstained with hematoxylin. Stained sections were completely imaged using a digital slide scanner (Panoramic MIDI, 3DHISTECH). β-cell and α-cell area were determined using an image-analysis program (Stacks 2.1, Leiden University Medical Center), and expressed as percentage relative to exocrine area after exclusion of large blood vessels and ducts, adipose tissue and lymph nodes. A threshold of 4 cells in a cluster was used in order to be included in the analysis. Scattered single cells or duct-associated single cells were not included.

Immunofluorescence

Following rehydration of tissue sections, antigen retrieval was performed by heating slides in citrate buffer (pH 6.0) using a pressure cooker. Primary antibodies against insulin (1:200; Millipore or Santa Cruz), glucagon (1:200; Vector or Invitrogen), Pdx1 (1:5; R&D Systems), Nkx6.1 (1:1,000; clone F55A12, Developmental Studies Hybridoma Bank and 1:250, Sigma), Nkx6.2 (1:50, Santa Cruz), MafA (1:200, LP9872, Betalogics Venture), Arx (1:1000, R&D Systems) and FoxO1 (1:200, Cell Signaling) were used. DAPI (Vector) was used as nuclear counterstaining. Secondary antibodies were TRITC–anti–guinea pig (1:400; Jackson) and Alexa Fluor 488-, 568-, and 647 anti-mouse or anti-rabbit when appropriate (1:1,000, Molecular Probes). Sections were imaged using a LSM 7 MP confocal microscope (Zeiss) and images collected for subsequent quantification. The investigators were blind to the donor background during quantification of the staining for Nkx6.1, glucagon and insulin. Labeling was analyzed by counting cells in at least 25 islets per donor, containing >10 hormone-positive cells per islet. Bihormonal cells in our study were defined as cells that were positive for both insulin and glucagon. The proportion of bihormonal cells is represented relative to the total number of insulin$^+$ cells, whereas the proportion of Nkx6.1$^+$glucagon$^+$insulin$^-$ cells is represented relative to the total number of Nkx6.1$^+$ cells.

To analyse the presence of glucagon$^+$/Nkx6.1$^+$ cells in contact with amyloid deposits, amyloid was labeled by incubation in 0.5% Thioflavin S in PBS for 2 minutes after antigen retrieval but before immunolabeling for the detection of glucagon and Nkx6.1. Amyloid area was determined from the Thioflavin S positive area in >20 islets for all donors. Islet areas (μm^2) and amyloid area were measured using ImageJ software (NIH). The islet amyloid percentage was calculated from the amyloid area and islet area.

Immuno Electron Microscopy

Pancreas samples from two T2DM subjects were processed for immunoelectron microscopic analysis. One tissue sample was embedded in LRgold resin as described previously (20). The second sample was fixed in 0.2% glutaraldehyde and 2% paraformaldehyde and prepared for cryo-immunogold labeling (21). Ultrathin sections from both samples were labeled in two consecutive steps. First, sections were incubated with monoclonal mouse anti–C-peptide (Millipore, 1:2,000 or 1:400 for cryosections or LRgold, respectively) followed by rabbit-anti-mouse IgG (1:200). In the second round, polyclonal rabbit-anti-glucagon (Vector, 1:400 or 1:30 for cryosections or LRgold, respectively) was used.. In both steps, antibody binding was identified using protein A gold (10- or 15-nm particle

size), while fixation in 1% glutaraldehyde was performed between both steps to avoid crossreactivity. Images were made on a FEI Tecnai 12 BioTwin transmission electron microscope at 120 kV.

Statistical Analysis

All data are expressed as means ± SEM unless stated otherwise. Statistical significance of differences between two groups was assessed by Mann-Whitney U test, for more than two groups Kruskal-Wallis was used followed by Dunn's multiple comparisons test and Spearman rank was used to test correlations. P <0.05 was considered to be statistically significant.

RESULTS

Subjects with T2DM show a higher α/β-ratio compared to matched controls

Age (61.2 ± 8.7 vs. 59.4 ± 8.7 years, ND vs. T2DM) and BMI (29.2 ± 4.3 vs. 30.0 ± 4.9 kg/m^2, ND vs. T2DM) were similar in both groups of organ donors (Supplementary Table 1). Immunohistochemical labelings for glucagon and insulin were performed to assess the relative areas of α- and β-cells, respectively (Supplementary Fig. 1*A*). The mean relative β-cell area was 25% lower in the T2DM subjects compared to the matched ND controls (2.04 ± 0.28 vs. 1.54 ± 0.37%, ND vs. T2DM, p < 0.05, Fig. 1*A*). The ratio of α-cell to β-cell areas (α/β-ratio) was significantly higher in subjects with T2DM compared to ND controls (0.50 ± 0.19 vs. 0.98 ± 0.24, ND vs. T2DM, p < 0.05, Fig. 1*B*), while overall islet size did not significantly differ between the two groups (Supplementary Fig. 1*B*). In line with

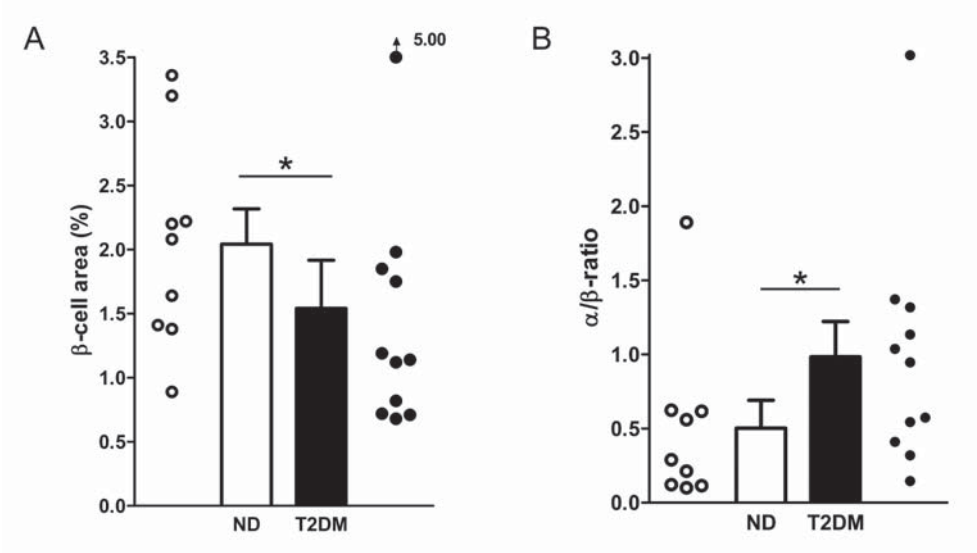

Figure 1. Increased α-cell proportion in T2DM. (*A* and *B*) The mean β-cell area (% of exocrine area) (*A*) and the ratio of α-cell to β-cell areas (*B*) in the pancreas of non-diabetic (ND) and diabetic (T2DM) donors. Panels represent both individual values (as scatter plots) and mean values ± SEM (as bars, *P < 0.05).

previously reported data (5), β-cell area was increased in the ND donors with the highest BMI (Supplementary Fig. 1*C*).

Increased frequency of insulin-positive cells co-expressing glucagon in T2DM

Confocal imaging of sequential optical sections (Z-stacks) through the 4-µm tissue section revealed the presence of insulin$^+$ cells that also expressed glucagon (i.e. bihormonal cells) (Fig. 2*A* and Supplementary Fig. 2*A*). These cells were present at a markedly increased frequency in the T2DM group (0.52 ± 0.18% vs. 4.05 ± 1.37, ND vs. T2DM, $p < 0.01$, Fig. 2*B*) and affected 33% of the islets in T2DM versus 13% in ND controls. The presence of these cells was confirmed by flow cytometry of dispersed human isolated islets (Supplementary Fig. 2*B*). Approximately half of bihormonal cells were negative for the β-cell specific nuclear transcription factor Nkx6.1 in both ND and T2DM groups (Fig. 2*A* and *B* and Supplementary Table 3). Nkx6.1 expression was exclusively present in the nuclei in ND donors, but localization also occurred in the cytoplasm in subjects with a history of T2DM (Supplementary Fig. 3*A*).

No significant correlation was found between bihormonal cell proportion and the α/β-ratio or donor factors such as BMI or age (Supplementary Fig. 2*C-F*). To identify bihormonal cells at the level of single granules, we performed immunoelectron microscopy. Double immunogold labeling showed the presence of distinct granules labeled for either glucagon or C-peptide in the same cell (Fig. 2*C*). Granule ultrastructure in bihormonal cells was similar to normal insulin or glucagon granules, showing homogenous and electron dense glucagon granules compared to a more crystalline structure of insulin granules (Fig. 2*C*). Lipofuscin bodies could be detected in the bihormonal cells (Fig. 2*C*).

Increased frequency of Nkx6.1$^+$ cells expressing glucagon, but not insulin, in T2DM

We observed that out of all nuclear Nkx6.1$^+$ cells that contained glucagon, 60% did not express insulin. We therefore quantified the presence of glucagon in cells containing nuclear Nkx6.1 that were insulin-negative as another indication of β-cell identity change. The percentage of Nkx6.1$^+$glucagon$^+$insulin$^-$ cells was 4.5-fold higher in T2DM compared to the ND controls (1.14 ± 0.36% vs. 5.11 ± 1.49%, ND vs. T2DM, $p < 0.01$, Fig. 3*A* and *B*). The percentage of Nkx6.1$^+$glucagon$^+$insulin$^-$ cells significantly correlated with the α/β-ratio (Fig. 3C), the percentage of bihormonal cells (Fig. 3*D*) and the duration of T2DM while no correlation was found with BMI or age (Supplementary Fig. 3*B-D*).

Expression of MafA, FoxO1 and Pdx1 in bihormonal and Nkx6.1$^+$glucagon$^+$ cells

To further characterize bihormonal and Nkx6.1$^+$glucagon$^+$ cells, we analyzed the presence of other β-cell transcription factors in these cells. First, we observed differences in the subcellular localization of the transcription factors MafA and FoxO1 between the ND and T2DM donors. Using DAB immunohistochemistry, we found that MafA was mainly expressed in the nucleus in all ND donors, while it was mainly expressed in the cytoplasm in 6 out of 11 T2DM donors. (Supplementary Fig. 4*A,B*). Using immunofluorescence, we

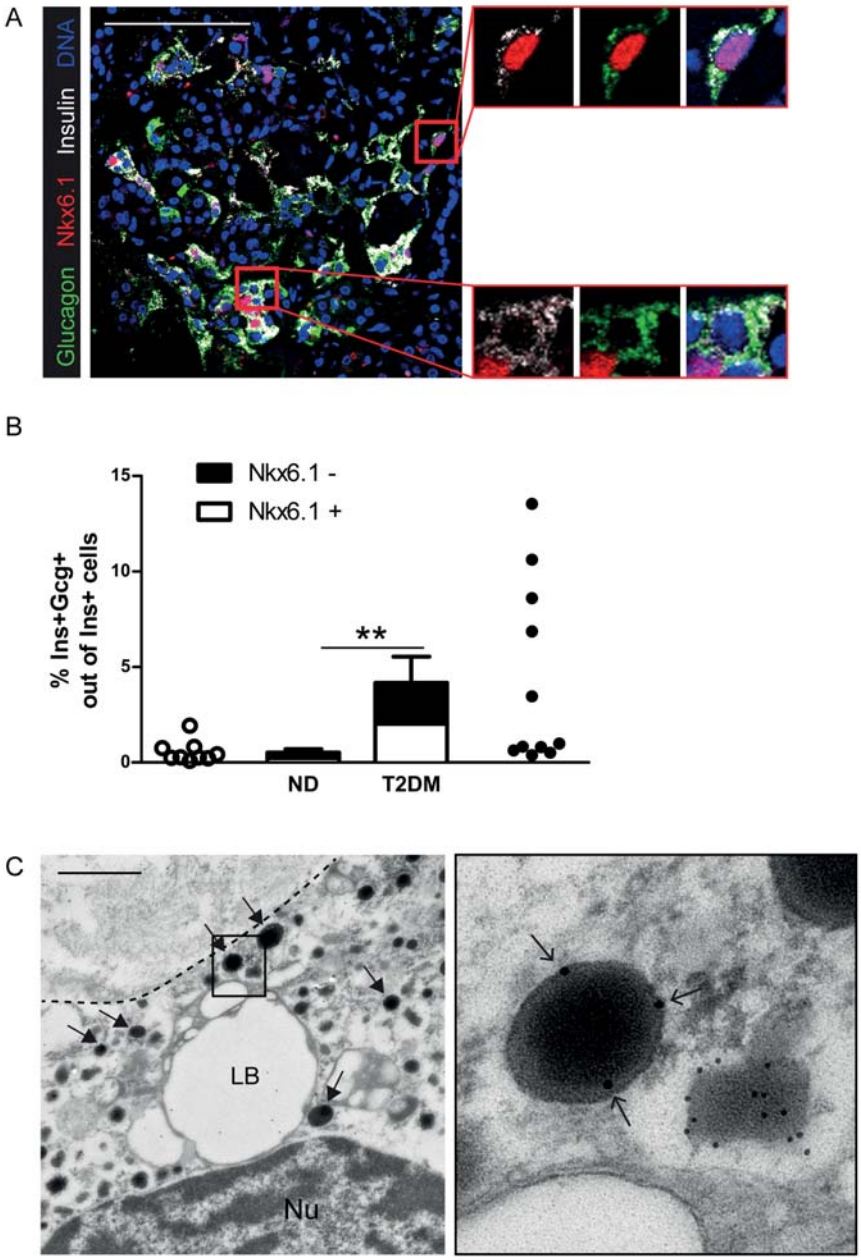

Figure 2. Increased percentage of bihormonal (glucagon$^+$insulin$^+$) cells in T2DM. (*A*) Representative labeled section of a pancreatic islet from a subject with T2DM showing a Nkx6.1$^+$glucagon$^+$insulin$^+$ cell (upper inset) and a Nkx6.1$^-$glucagon$^+$insulin$^+$ cell (lower inset). Scale bar: 100 µm. (*B*) Quantification of the percentage of glucagon$^+$insulin$^+$ cells out of all insulin$^+$ cells showing a significantly higher value in T2DM donors (mean ± SEM and individual values, ** $P < 0.01$). (*C*) Double immunogold labeling of pancreatic tissue from a T2DM donor for glucagon (granules with 15-nm gold particles, arrows) and C-peptide (10-nm gold particles) showing both types of granules within the same cell. A representative image of tissue embedded in LRgold resin is shown. The cell border is marked manually by a broken line (Nu = nucleus, LB = lipofuscin body). Scale bar: 1 µm

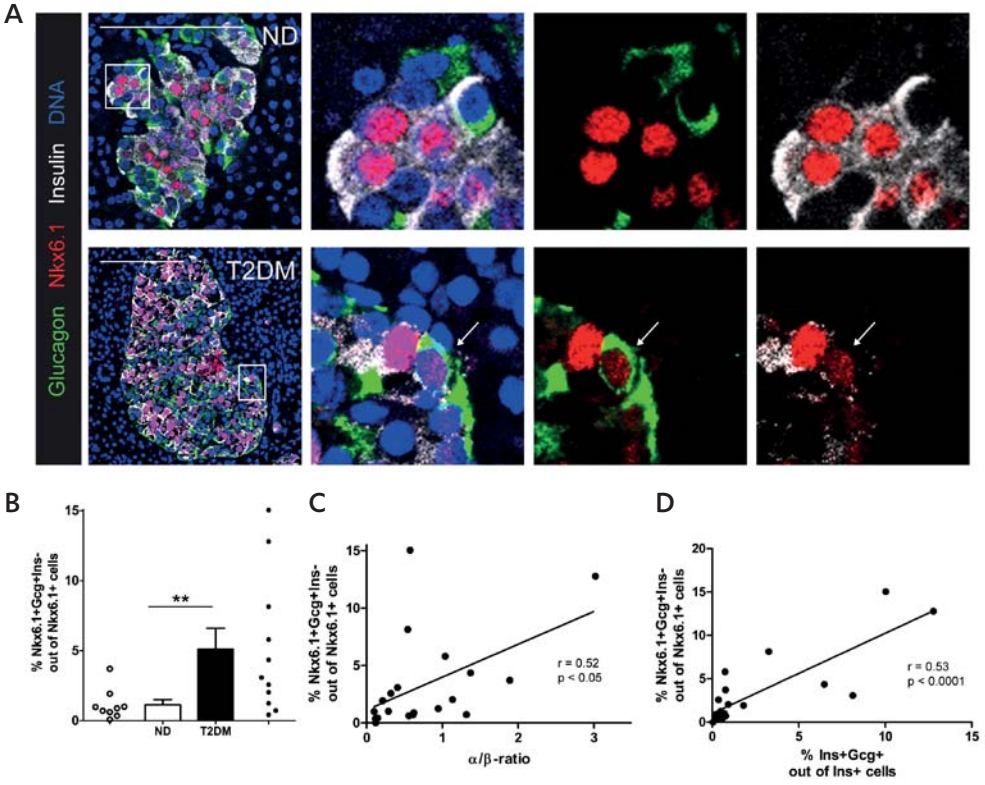

Figure 3. Increased percentage of Nkx6.1$^+$glucagon$^+$insulin$^-$ cells in T2DM. (*A*) Representative immunostaining of normal β-cells (Nkx6.1$^+$glucagon$^-$insulin$^+$) (upper panel) and a Nkx6.1$^+$glucagon$^+$insulin$^-$ cell (lower panel, arrow). Scale bars: 100 μm. (*B*) Quantification of the percentage of Nkx6.1$^+$glucagon$^+$insulin$^-$ cells out of all Nkx6.1$^+$ cells (mean ± SEM and individual values, ** $P < 0.01$). (*C* and *D*) The percentage of Nkx6.1$^+$glucagon$^+$insulin$^-$ cells positively correlates with the ratio of α- to β-cell areas (*C*) and the percentage of glucagon$^+$insulin$^+$ cells (*D*).

then examined the expression of MafA in bihormonal cells. Interestingly, MafA was either cytoplasmic or absent, but was rarely found to be nuclear in bihormonal cells even in donors that showed predominant nuclear expression (Fig. 4*A*). FoxO1 was expressed mainly in the cytoplasm in 7 out of 9 ND donors and localized predominantly in the nucleus in 7 out of 11 T2DM donor (Supplementary Fig. 4*B*). This pattern was maintained in bihormonal cells (Fig. 4*B*). Furthermore, nuclear Pdx1 was found to be expressed in a subset of glucagon$^+$Nkx6.1$^+$ cells (Fig. 4*C*). The α-cell marker Arx was also found to be expressed in the nucleus of bihormonal cells, but not all bihormonal cells expressed Arx (Fig 4*D*). Nkx6.2 labeling was negative in islets from both ND and T2DM donors (data not shown). Finally, we checked for the early endocrine marker Ngn3 in bihormonal cells. No Ngn3 was found in islets containing bihormonal cells (data not shown).

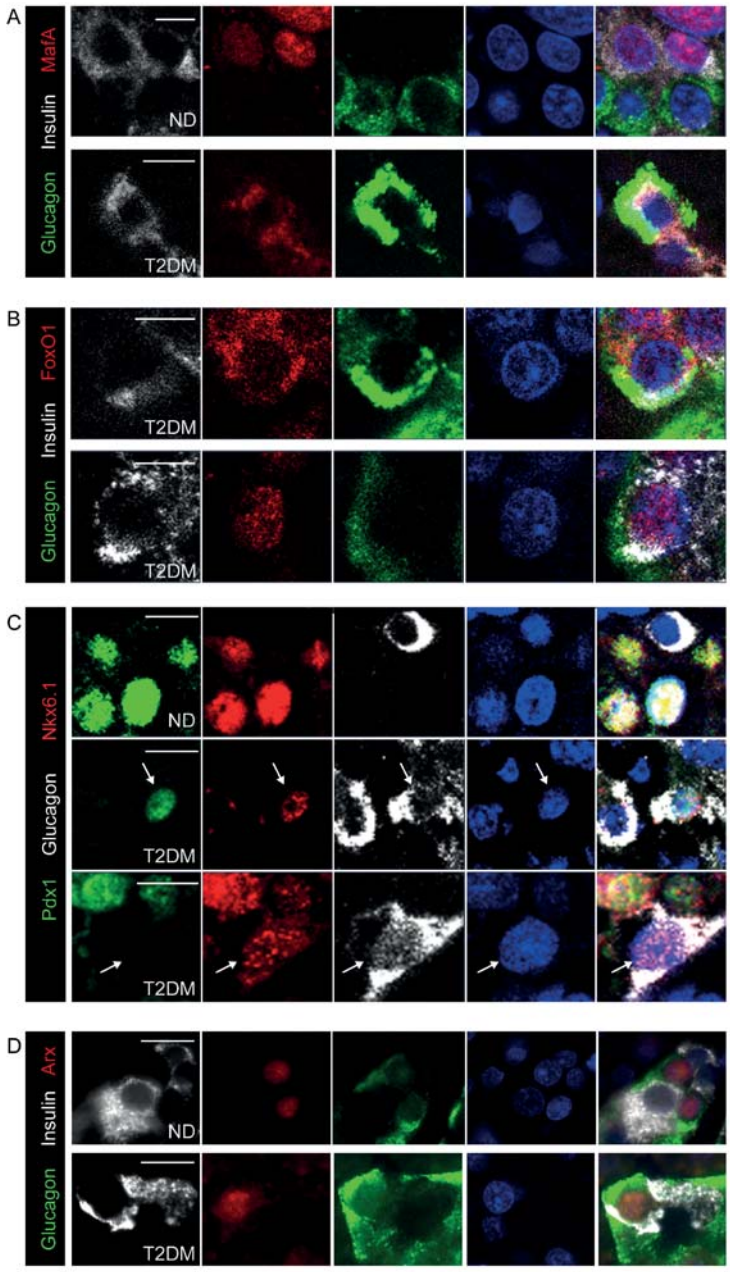

Figure 4. Expression of MafA, FoxO1 and Pdx1 in cells with a mixed phenotype. (*A*) Labeling for MafA (red) shows nuclear localization in ND pancreas (upper panel), restricted to insulin-containing (white) cells. The lower panels show a bihormonal glucagon$^+$insulin$^+$ cell in T2DM containing cytoplasmic MafA. (*B*) FoxO1 (red) can be localized either in the cytoplasm (upper panel) or in the nucleus (lower panel) in bihormonal cells. (*C*) Detection of nuclear Pdx1 (green) and Nkx6.1 (red) in a subset of glucagon$^+$ cells (white) in T2DM (arrows in the middle panel). Not all Nkx6.1$^+$glucagon$^+$ cells are labeled for Pdx1 (arrows in the lower panel). (*D*) Labeling for Arx (red) shows nuclear localization that is restricted to α-cells in ND but is also expressed in bihormonal cells in T2DM (lower panel). Blue labeling represents DNA in all panels. All scale bars: 10 μm.

Loss of β-cell identity occurs at an increased frequency in diabetic non-human primates

To determine whether the changes in islet cell identity occur before the onset of diabetes, we studied tissue from *Macaca mulatta* non-human primates (Table 1) that develop diabetes with similar characteristics to T2DM in humans, including a significantly decreased β-cell area (17), and that have been well characterized metabolically (16). Non-diabetic (ND) animals had an islet architecture with Nkx6.1 expression similar to human islets (Supplementary Fig. 5). In agreement with our human data, the proportion of bihormonal and Nkx6.1$^+$glucagon$^+$insulin$^-$ cells was higher in diabetic (DM) animals (bihormonal cells 0.24 ± 0.10 vs. 17.0 ± 4.4% and Nkx6.1$^+$glucagon$^+$insulin$^-$ cells 0.07 ± 0.07 vs. 13.8 ± 3.9% out of Nkx6.1$^+$, ND vs. DM, Fig. 5*A,B*). In contrast, the group of hyperinsulinemic (HI) animals, that are considered to be prediabetic, had a higher proportion of bihormonal and Nkx6.1$^+$glucagon$^+$insulin$^-$ cells compared to the ND group but this difference was not significant (bihormonal cells 0.26 ± 0.10 vs. 1.33 ± 0.29 out of insulin$^+$ and Nkx6.1$^+$glucagon$^+$insulin$^-$ cells 0.07 ± 0.07 vs. 0.74 ± 0.45 out of Nkx6.1$^+$, ND vs. HI, Fig. 5*A,B*).

β-cell conversion is associated with the presence of amyloid deposits in T2DM

Islet pathology in humans with T2DM and in diabetic non-human primates is characterized by the deposition of amyloid fibrils (22). Therefore we investigated whether a change in islet cell identity was related to the presence of amyloid deposits both in humans and in non-human primates.

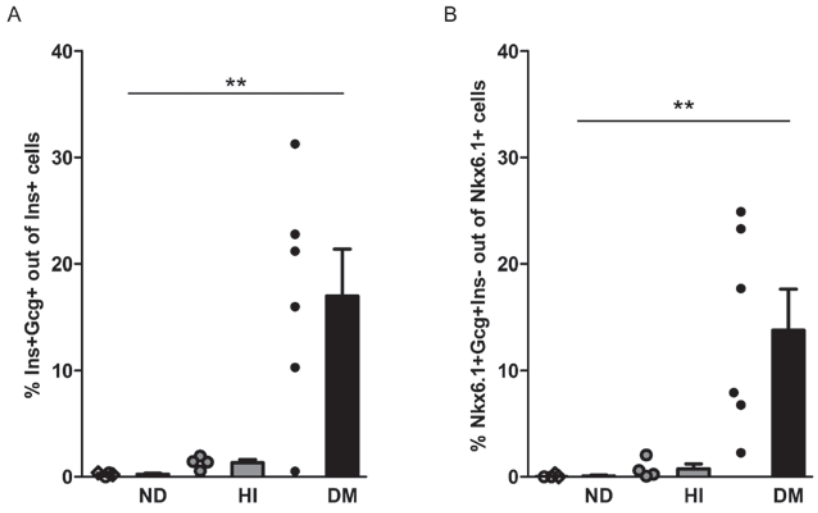

Figure 5. Nkx6.1$^+$glucagon$^+$insulin$^-$ cells and bihormonal cells occur more frequently in diabetic non-human primates. (*A* and *B*) Quantification of the percentage of glucagon$^+$insulin$^+$ out of insulin$^+$ cells (*A*) and Nkx6.1$^+$glucagon$^+$insulin$^-$ cells out of Nkx6.1$^+$-cells (*B*) in non-diabetic (ND), hyperinsulinemic (HI) and diabetic (DM) animals (dots represent individual *M. mulatta* and diamonds *M. fascicularis* in the ND group, bars show mean values ± SEM, ** $P < 0.01$).

In pancreas of human donors, islet amyloid was present in 6 out of 11 T2DM and in only 1 out of 9 control donors (Supplementary Table 1). In the macaques, 5 out of 6 DM, 3 out of 4 HI and none of the ND monkeys had islet amyloid (Supplementary Table 2). The presence of islet amyloid was positively associated with a higher percentage of Nkx6.1$^+$glucagon$^+$insulin$^-$ cells and bihormonal cells in both monkey and human pancreas (Supplementary Fig. 6*C-E*). Both the percentage of bihormonal cells and Nkx6.1$^+$glucagon$^+$insulin$^-$ cells correlated with the average percentage of islet amyloid area per human donor (Fig. 6*A,B*). Since the extent of islet amyloidosis was heterogeneous in different islets within a donor, we were able to investigate the relationship between the extent of islet amyloidosis and the presence of Nkx6.1$^+$glucagon$^+$ cells per islet (Fig.6*C*). Islets that had a higher degree of amyloidosis (>20%) showed a higher number of Nkx6.1$^+$glucagon$^+$ cells compared to islets from the same donor in which <1% of the area was comprised by amyloid fibrils (Fig. 6*D*).

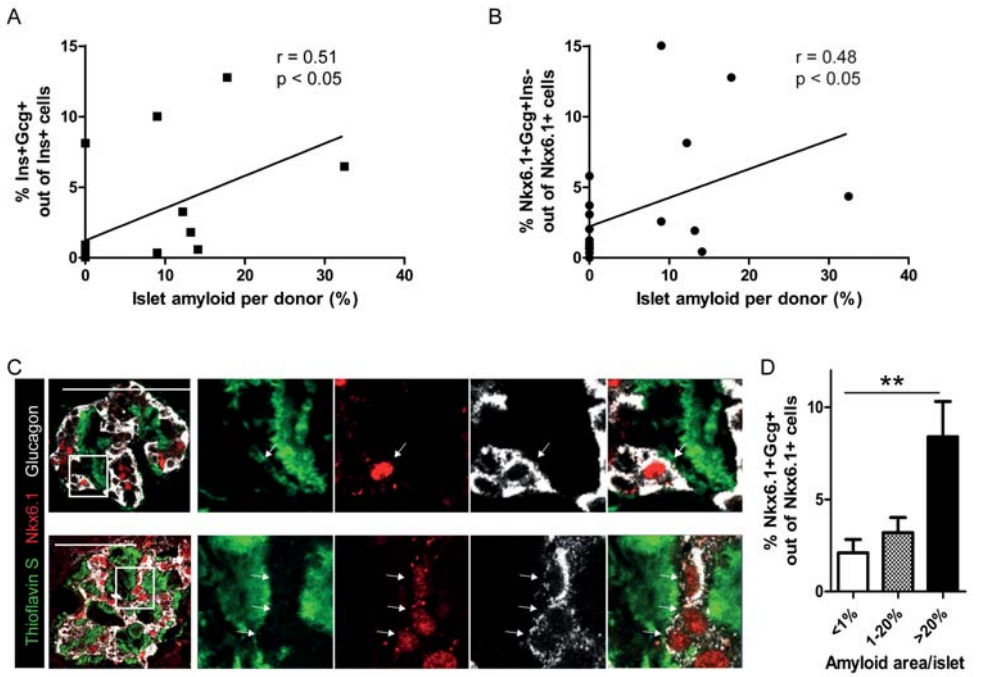

Figure 6. Nkx6.1$^+$glucagon$^+$ cells are more frequently present in islets with amyloid. (*A* and *B*) The degree of islet amyloid area per donor correlates positively with the presence of Nkx6.1$^+$glucagon$^+$insulin$^-$ cells (*A*) and glucagon$^+$insulin$^+$ cells (*B*). (*C*) Two representative images showing amyloid deposits by Thioflavin S staining (green) and cells co-expressing Nkx6.1 (red) and glucagon (white) by immunostaing (arrows indicate Nkx6.1$^+$glucagon$^+$ cells). The cells are adjacent to the amyloid deposits. Pancreatic sections from T2DM donors. Scale bars: 100 µm. (*D*) Nkx6.1$^+$glucagon$^+$ cells are present at a significantly higher frequency in ND and T2DM islets with higher amyloid area per islet (amyloid containing islets were divided in tertiles based on amyloid area/islet, mean ± SEM, ** $P < 0.01$).

DISCUSSION

We recently showed that mature human β-cells can lose their identity and convert into α-cells *ex vivo* (10). Here, we provide strong evidence that loss of β-cell identity in islets of humans and non-human primates *in vivo* is associated with diabetes mellitus and the presence of islet amyloidosis.

In our *ex vivo* conversion model, the transition from human β- to α-cell is characterized by the presence of β-cell transcription factors (Nkx6.1, Pdx1) in glucagon-expressing cells (10). Now, we report that these cells with a mixed phenotype are present at an increased frequency in the pancreas of subjects with T2DM. It is known that T2DM is associated with an increased α- to β-cell ratio (23-25). Interestingly, the proportion of converted cells in our study correlated with an increased α/β-ratio. Thus, although it cannot be excluded from crossectional data that conversion also occurs vice versa, we propose that the presence of these cells reflects ongoing β- to α-cell conversion. The model of β- to α-cell conversion is also supported by recent data from a mouse model in which conversion of β- to α-cells occurred following β-cell specific deletion of FoxO1 resulting in hyperglycemia (8). Furthermore, T2DM associated oxidative stress was recently associated with loss of β-cell identity (13). Consequently, we suggest that in addition to loss of β-cells by apoptosis in T2DM (4), loss of β-cell identity and subsequent conversion of β-cells into α-cells contributes to a decrease in β-cell mass.

An increasing number of reports has drawn attention to α-cell dysfunction in T2DM (26;27) and it is generally accepted that hyperglucagonemia plays a pivotal role in the dysregulation of glucose homeostasis (7;28). Since only ~5% of cells had a mixed phenotype, it is unlikely that hyperglucagonemia can be completely attributed to β- to α-cell conversion but a contributary role cannot be excluded.

It is not clear how changes in metabolism could affect β- to α-cell conversion and whether β- to α-cell conversion is involved in the onset and progression of diabetes. *Macaca mulatta* rhesus monkeys fed *ad libitum* develop diabetes in a similar fashion to T2DM in humans including the onset of obesity-associated insulin resistance, β-cell dysfunction and the presence of islet amyloidosis (17). Whereas metabolic data in human organ donors and information on donor organs is limited, in non-human primates in depth characterization of metabolic changes related to islet cell composition is possible. Overt diabetes mellitus in rhesus monkeys is preceded by a hyperinsulinemic stage that is characterized by increased insulin secretion to maintain normal glucose levels (29). A significantly increased proportion of converted cells (either bihormonal cells or glucagon-positive cells expressing β-cell transcription factors) was found in animals with overt diabetes, that were previously shown to have a significantly reduced β-cell area (17). Although a 15x increase in the frequency of cells with this mixed phenotype was found in obese, hyperinsulinemic animals that are considered to be pre-diabetic compared to non-diabetic control animals, the difference did not reach statistical significance possibly due to the small numbers of monkeys in each group (29). Nevertheless, these data are in agreement with our previous observations that the population of obese hyperinsulinemic monkeys did not exhibit a decrease in β-cell area, while this was evident in diabetic animals (17). A recent report on rhesus monkeys

that were fed a high fat/high sugar diet for 24 months, reported an increased ratio of α- to β-cell areas in the presence of normoglycemia (30). Both the difference in diet composition and the age of the hyperinsulinemic animals in our study (almost twice the age at sacrifice) may have contributed to the different outcome. In addition, no bihormonal cells were reported that could point at β- to α-cell transdifferentiation in those normoglycemic animals (30). These findings indicate that β- to α-cell conversion accompanies progression of diabetes and may worsen hyperglycemia but its potential contribution to events leading to early β-cell failure is still unclear.

Islet amyloid deposits derived from islet amyloid polypeptide (IAPP) are associated with a reduced β-cell mass in T2DM (22;31;32). The loss of β-cell mass has generally been attributed to increased rates of apoptosis (33;34). We found that the loss of β-cell identity in pancreatic islets from both monkeys and humans with diabetes correlated with the presence of islet amyloid deposits. In monkeys, converted cells were not only absent in non-diabetic animals that are characterized by the absence of islet amyloid, but islets in both the hyperinsulinemic and diabetic animals that were amyloid-free had a lower (animal A) or even undetectable (animal V) proportion of converted β-cells. Compared to humans, the diabetic animals had more severe islet amyloidosis (>50% of islet area in 100% of islets from diabetic animals compared to ~20% islet area in 70% of islets from humans with islet amyloid) which could have contributed to the increased presence of converted cells in the diabetic monkeys compared to the diabetic humans. Of note, whereas we matched human donors for age, the design of the monkey study did not allow matching for age and diabetic animals were generally older. The two non-diabetic human donors who had more than 1% converted cells either had islet amyloidosis (ND7) or had the highest BMI of the group (ND10, BMI 35). We cannot exclude that undiagnosed T2DM was present in these two organ donors.

Although there is variability in the degree of amyloidosis between islets within a human pancreas (35), it was evident that islets containing amyloid deposits showed a higher proportion of converted cells which were frequently found directly adjacent to the amyloid deposits. Aggregated IAPP can exert a direct toxic effect on β-cells (36;37) but it is not known whether IAPP fibrils can directly induce conversion of β-cells into α-cells. T2DM is thought to be associated with a low-grade inflammatory state (38) and IAPP oligomers can elicit activation of the inflammasome (39). It was also recently shown that overexpression of hIAPP induces β-cell inflammation and dysfunction in mice (40). Whether a pro-inflammatory local islet microenvironment may lead to both islet amyloid formation and β- to α-cell conversion or whether amyloid fibrils may in fact have a direct role in the cell conversion process is not known.

Conversion of human β-cells into α-cells *ex vivo* following dispersion and reaggregation is likely to be affected by β-cell stress induced by breaking down cell-cell and cell-matrix interactions (10). We speculate that a similar, but more gradually developing β-cell stress occurs in islets when T2DM develops and amyloid fibril formation occurs, leading to loss of β-cell identity and changes in islet cell composition (41). This hypothesis is supported by the changes in subcellular localization of the β-cell factors MafA and FoxO1,

while the α-cell factor Arx is in its functional nuclear compartment in bihormonal cells. A gradual conversion process *in vivo* could also explain the finding of bihormonal cells in the current study, in contrast to our *ex vivo* model that is characterized by severe β-cell degranulation in the days following islet dispersion. Bihormonal cells have recently been described in human pancreas from subjects with T2DM or insulin resistance, both in single or clustered β-cells and in islets in combination with mesenchymal markers (42-45). Since the ultrastructural morphology remained typical for glucagon and insulin granules, the storage of both hormones appears to remain distinct in bihormonal cells. In addition, the presence of lipofuscin bodies indicates that these cells have had a longer life span (46).

Loss of differentiated β-cells, characterized by a downregulation of key transcription factors, has been associated with β-cell dysfunction and diabetes in several studies (13-15;47;48). Whether the loss of β-cell transcription factors is directly responsible for or merely associated with β-cell dysfunction remains to be elucidated. We now add that human β-cells (as defined by β-cell specific transcription factors) can express both glucagon and insulin in T2DM; it is likely that these are dysfunctional β-cells (49). Even though our study was limited by the measurement of relative β-cell area, these data support the idea that conventional quantification of β-cell mass in T2DM, usually measured by insulin immunostaining, can overestimate the functional insulin-secreting β-cell mass (50;51). The evaluation of mechanisms that actively regulate functional β-cell maintenance could create new opportunities in the field of regenerative medicine, such as the induction of α-cell to β-cell conversion. In addition, identification of the exact mechanisms that trigger loss of human β-cell identity may contribute to strategies to prevent and delay the progression of β-cell dysfunction in diabetes.

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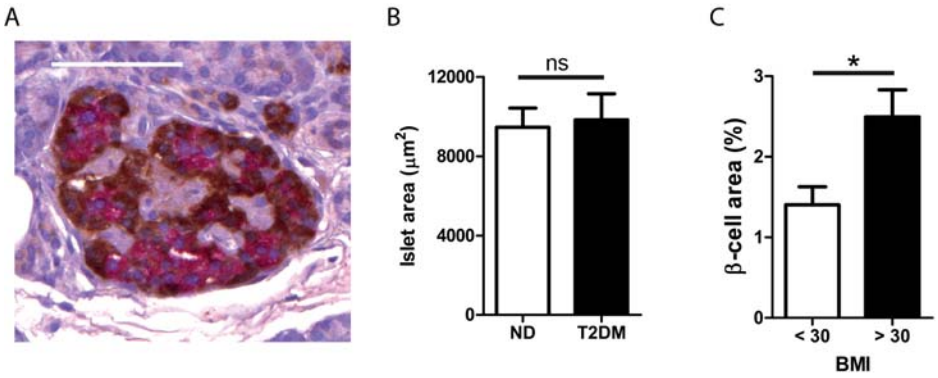
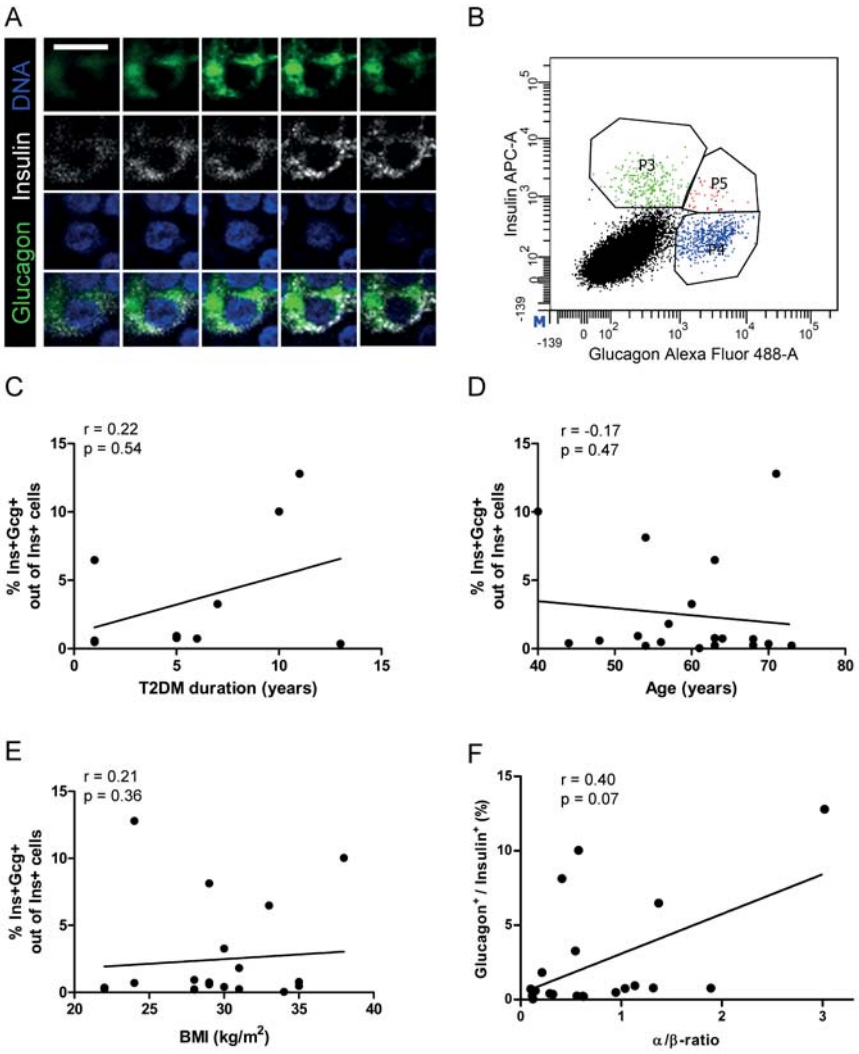
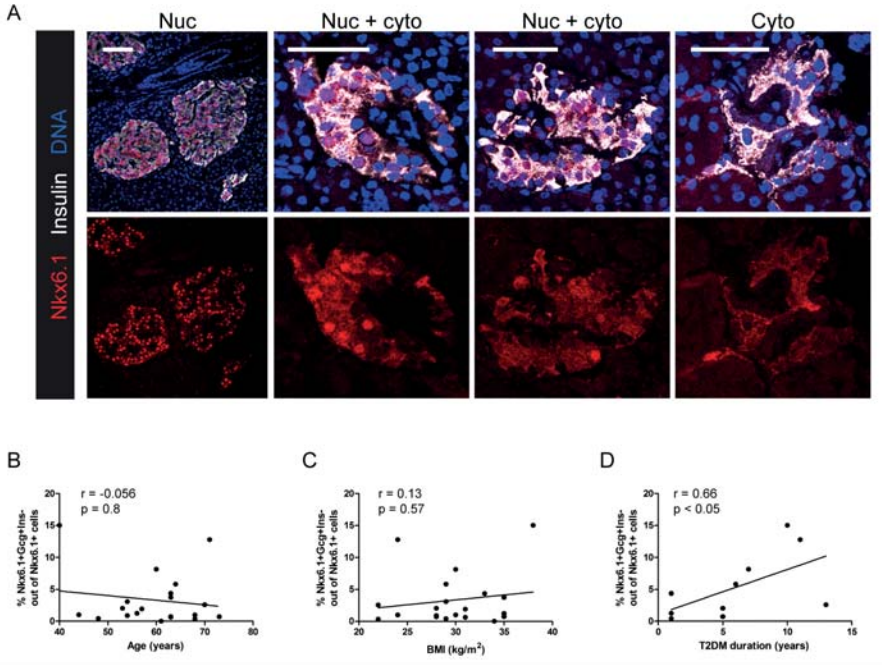
SUPPLEMENTARY FIGURES AND TABLES



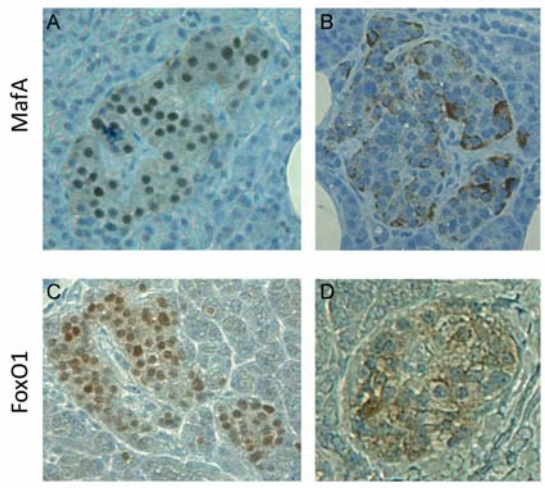
Supplementary Figure 1. Islet morphometry. (*A*) Representative image of immunohistochemical staining for glucagon (brown) and insulin (red) that were used to determine α- and β-cell area, respectively. Scale bar: 50 µm. (*B*) The average islet area (µm^2) is not different between non-diabetic donors (ND) and donors with type 2 diabetes (T2DM). (*C*) Non-diabetic donors with a BMI >30 kg/m^2 had a larger β-cell area than non-diabetic donors with a BMI < 30 kg/m^2. Data are represented as mean ± SEM (*P < 0.05).



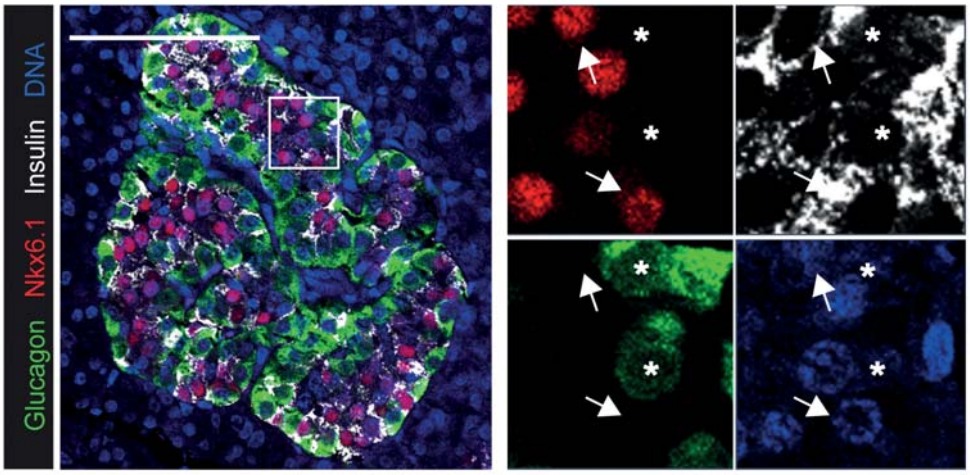
Supplementary Figure 2. Identification of bihormonal cells and correlations with donor and islet characteristic. (*A*) Z-stack gallery showing a series of 1,4 µm thin optical sections of a glucagon$^+$insulin$^+$ cell. Scale bar: 10 µm. (*B*) Representative FACS plot of dispersed human isolated islet cells labelled for insulin (green, P3) and glucagon (blue, P4) showing a population of glucagon$^+$insulin$^+$ cells (red, P5). (*C-F*) There is no significant correlation of the percentage of bihormonal (glucagon$^+$insulin$^+$) cells with T2DM duration (*C*), age (*D*), BMI (*E*) or the ratio of α- to β-cell area (*F*).



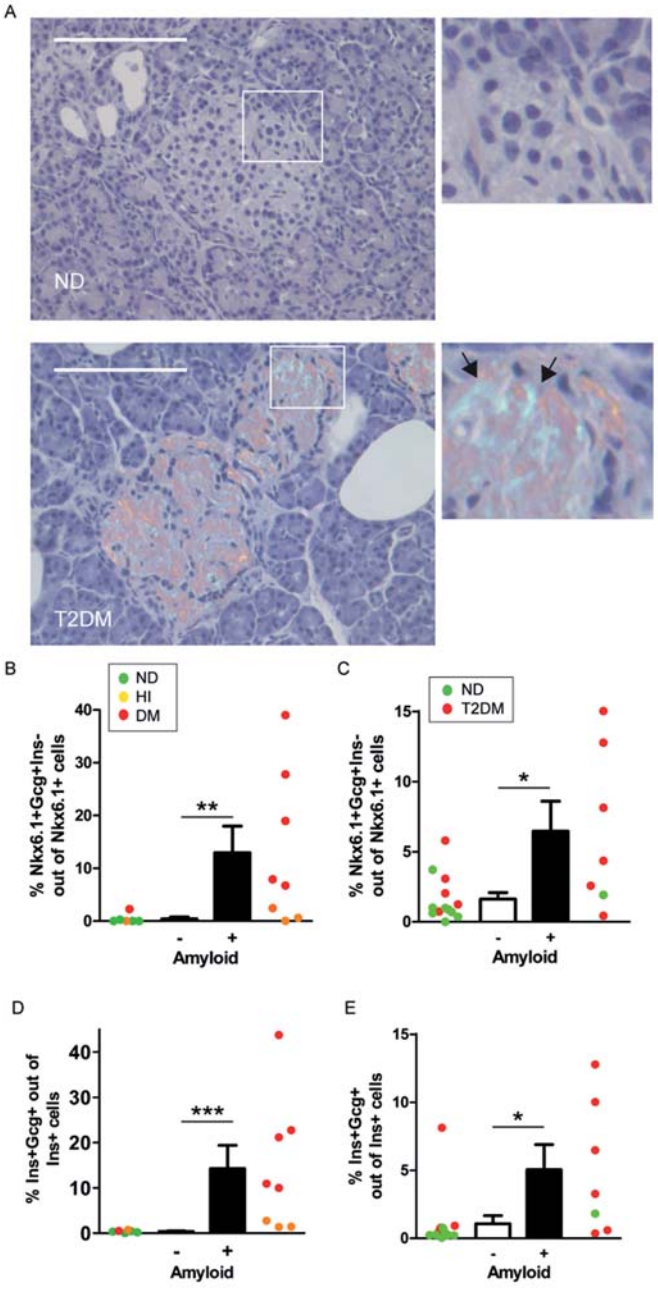
Supplementary Figure 3. Relationship between the presence of Nkx6.1$^+$glucagon$^+$insulin$^-$ cells and donor characteristics. (*A*) Representative pictures of islets showing the localization of Nkx6.1 (red) which is either nuclear (nuc), cytoplasmic (cyto) or both in subjects with T2DM. Scale bars: 50 µm. (*B* and *C*) The percentage of Nkx6.1$^+$glucagon$^+$insulin$^-$ cells does not correlate with donor characteristics such as age (*B*) or BMI (*C*). (*D*) A significant correlation is present between the percentage of Nkx6.1$^+$glucagon$^+$insulin$^-$ and T2DM disease duration.



Supplementary Figure 4. Immunohistochemistry for MafA and FoxO1. (*A* and *B*) Representative images of immunohistochemical stainings for MafA in the islet of a non-diabetic donor showing nuclear localization (*A*) or cytoplasmic localization in an islet of a T2DM donor *B*). (*C* and *D*) Immunhistochemical staining for FoxO1 showing either nuclear (*C*) or cytoplasmic localization (*D*).



Supplementary Figure 5. Islet architecture and transcription factor expression in non-diabetic non-human primates. Representative image of a pancreatic section from a non-diabetic monkey showing nuclear staining for Nkx6.1 (red) in insulin-positive cells (arrows), but not in glucagon-positive cells (asterisks). Note that the mixed cell composition is similar to that of human pancreatic islets. Scale bar: 100 μm.



Supplementary Figure 6. Amyloid deposits in pancreatic islets. (*A*) Representative pictures of pancreatic tissue sections stained with congo red, hematoxylin and eosin showing no amyloid deposits in an islet from a non-diabetic donor (upper panel) and severe islet amyloidosis in an islet from a donor with T2DM (lower panel). Green birefringence typical of islet amyloid deposits (arrows) was detected by using polarised light. Scale bars: 100 μm. (*B* and *C*) Nkx6.1^{+}glucagon^{+}insulin^{-} cells and bihormonal cells (*D* and *E*) are more frequent in the pancreas of monkeys (*B, D*) and humans (*C, E*) that contain islet amyloid deposits. Different colours indicate the subgroups. Data are represented as mean ± SEM (*P < 0.05, ** P < 0.01, *** P < 0.001).

Supplementary Table 1. General characteristics of human pancreas donors

Type 2 diabetes							Non-diabetic				
Donor	Age (years)	BMI (kg/m^2)	Duration T2DM (years)	Diabetes medication	Cause of death (DBD/DCD)	Amyloid	Donor	Age (years)	BMI (kg/m^2)	Cause of death (DBD/DCD)	Amyloid
T2DM1	70	22	13	Glimepiride	SAB (DBD)	+	ND1	68	22	Stroke (DBD)	
T2DM2	71	24	11	Metformin	Trauma (DBD)	+	ND2	68	24	SAB (DBD)	
T2DM3	64	29	6	Metformin, Pioglitazone	SAB (DBD)		ND3	73	28	Stroke (DBD)	
T2DM4	54	29	UNK	Insulin, metformin	Stroke (DBD)		ND4	54	28	SAB (DBD)	
T2DM5	53	28	5	Metformin	Stroke (DBD)		ND5	44	30	Cardiac (DCD)	
T2DM6	40	38	10	UNK	Stroke (DBD)	+	ND6	57	31	Cardiac (DCD)	+
T2DM7	60	30	7	Insulin	Suicide (DCD)	+	ND7	63	31	SAB (DBD)	
T2DM8	63	33	1	Metformin, Glimepiride	Stroke (DCD)	+	ND8	61	34	Stroke (DCD)	
T2DM9	56	35	1	Metformin, Glimepiride	Stroke (DBD)		ND9	63	35	Trauma (DCD)	
T2DM10	59	27	1	UNK	Stroke (DCD)	+					
T2DM11	63	35	5	Metformin	SAB (DCD)						
Average	59.4	30.0	6.0					61.2	29.2		
SD	8.7	4.9	4.3					8.7	4.3		

BMI, body mass index. DBD, donation after brain death. DCD, donation after cardiac death. SAB, subarachnoidal bleeding. UNK, unknown. SD, standard deviation.

Supplementary Table 2. General and metabolic characteristics of individual monkeys

Group	Monkey	Age (years)	Weight (kg)	FPG (mmol/l)	IRI (pmol/l)	Body fat (%)	Amyloid area (%)	Islets with amyloid (%)	β-cell area/islet area (%)
Non-diabetic	F	13.2	10.6	3.8	345	21.9	0	0	77.9
	Q	14.6	9.4	3.9	252	30.8	0	0	43.4
	CI	5	4.3	-	-	-	0	0	-
	CII	-	6.2	-	-	-	0	0	-
Hyperinsulinemic	K	17.5	8.5	6.8	792	32.1	28.6	8.0	-
	V	30.0	12.8	3.0	611	31.6	0	0	51.3
	L	20.3	12.0	4.4	1479	28.9	12.2	19.8	50.1
	J	22.8	17.1	6.4	2230	41.7	33.3	7.0	63.4
Diabetic	I	24.2	11.3	8.4	136	30.5	77.7	95.7	1.7
	U	26.0	9.8	9.1	65	46.5	49.5	100	0.1
	R	26.7	13.1	16.0	-	37.4	63.5	100	0.2
	Z	19.0	9.4	-	-	-	69.6	100	-
	T	24.8	9.8	17.0	79	39.7	78.5	100	-
	A	18.9	14.2	18.0	139	32.2	0	0	64.1

FPG, fasting plasma glucose. IRI, immunoreactive insulin.

Supplementary Table 3. Proportions of investigated phenotypes

	GCG+ INS+		GCG+ INS-		GCG+ INS+ NKX6.1 -		GCG+ INS+ NKX6.1 +	
Out of	ND	T2DM	ND	T2DM	ND	T2DM	ND	T2DM
Insulin$^+$ cells	0,52	4,05			0,29	2,02	0,23	2,04
Nkx6.1$^+$ cells	0,25	3,44	1,14	5,11				
Islets affected (%)	13	33			17	28		

ND, non-diabetic. T2DM, type 2 diabetic. GCG, glucagon. INS, insulin.

CHAPTER 6

GLUCAGON-LIKE PEPTIDE-1 RECEPTOR AGONISTS PREVENT LOSS OF β-CELL IDENTITY IN HUMAN ISLET CELL AGGREGATES

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ABSTRACT

Loss of insulin-secreting β-cell mass and function is central in the pathophysiology of type 2 diabetes (T2DM). We previously reported that mature human β-cells can lose identity and spontaneously convert into α-cells following islet cell dispersion and reaggregation *ex vivo* and that this conversion may be involved in β-cell failure in T2DM. Activation of the GLP-1 receptor is involved in β-cell differentiation and can prevent apoptosis. Therefore, we investigated whether activation of the GLP-1 receptor plays a role in maintenance of a β-cell phenotype. Using β-cell specific lineage tracing, we show that GLP-1 receptor agonists can prevent loss of human β-cell identity, as characterized by a higher percentage of insulin^{+}GFP^{+} out of GFP^{+} cells after 7 days of reaggregation. Incretin treatment was accompanied by an induction of endogenous Pax4 gene expression. The protective effects of GLP-1 signaling on human β-cells were mimicked by overexpression of hPax4 in human islet cell aggregates. Our results indicate a novel potential role for incretin-based therapies, targeting the maintenance of mature β-cells, possibly through induction of Pax4 gene expression.

INTRODUCTION

Dysfunction of insulin-secreting β-cells and loss of pancreatic β-cell mass are central in the development of type 2 diabetes (T2DM) (1). Reduced β-cell mass may result from increased loss via apoptosis or reduced cell renewal via proliferation or neogenesis (2). But β-cell loss could also arise due to dedifferentiation as recent data shows that adult murine β-cells depleted of FoxO1 lose their identity and even convert into α-cells under conditions of metabolic stress (3). Moreover, oxidative stress, associated with β-cell failure in T2DM, lowers the expression of transcription factors such as MafA and Nkx6.1 that promote insulin gene transcription (4). We previously used a lineage tracing approach to show that human β-cells can spontaneously convert into α-cells following human islet cell dispersion and reaggregation *ex vivo* (5). The β- to α-cell transition was marked by β-cell degranulation and glucagon-positive cells expressing β-cell transcription factors (Nkx6.1 or Pdx1), a similar phenotype as observed in the islets of the genetically manipulated FoxO1 model (3;5). In addition, we and others found that cells with such a mixed phenotype (glucagon$^+$/insulin$^+$ bihormonal cells or glucagon$^+$-cells co-expressing β-cell transcription factors) are more prevalent in donor pancreas from subjects with T2DM, indicating that β-cells may lose their identity during the progression of diabetes (6-9). Therefore, ways to prevent or reverse loss of β-cell identity may provide novel therapeutic opportunities in T2DM.

During embryologic development, endocrine cell types are formed from a common Neurogenin3-expressing progenitor (10-12). The subsequent segregation of the α- and β-cell lineages depends on the expression of specific transcription factors with Arx and Pax4 playing a central diverging role. Arx null mutant mice do not contain α-cells, while mice that lack Pax4 show large numbers of α-cells at the expense of β- and δ-cells (13). We have previously shown that knockdown of Arx can prevent loss of human β-cell identity *ex vivo* (5). In line with these observations, overexpression of Pax4 *in vivo* can protect mouse β-cells against streptozotocin induced hyperglycemia (14). The role of Pax4 in human β-cell (de)differentiation is unclear.

The incretin hormone glucagon-like peptide-1 (GLP-1) is a gut-derived peptide that enhances glucose-induced insulin secretion, and GLP-1 receptor agonists (GLP-1RAs) are currently used in clinical care. Moreover, GLP-1RAs were used in differentiation protocols for culture of stem and progenitor cells to obtain insulin-producing cells (15;16). It is known that GLP-1 can also reduce glucagon secretion and prevent β-cell apoptosis (17), and that GLP-1 induces Pax4 expression in human isolated islets (18). In this study, we investigated the effects of GLP-1RAs and Pax4 on the maintenance of human β-cell identity.

MATERIALS & METHODS

Human islet isolation and cell culture

Human islet isolations were performed in the Good Manufacturing Practice facility of our institute according to a modified protocol originally described by Ricordi et al. (19). Pancreatic tissue was used in our study if the pancreas could not be used for clinical

pancreas or islet transplantation, according to national laws, and if research consent was present. Islet cells were dispersed and left to reaggregate in microwell plates after lentiviral transduction (5). In short, islets were dispersed into single cells by adding 0.025% trypsin solution containing 10 mg/mL DNase (Pulmozyme, Genentech) at 37°C while pipetting up and down for 6–7 min. The islet cell suspension was plated onto 3% agarose microwell chips containing 2,865 microwells/chip with a diameter of 200 μm/microwell (20). Seeding of ~3*10^6 cells per chip resulted in spontaneous reaggregation of ~1,000 islet cells/ microwell. Islet cell aggregates and intact human islets (control) were cultured in CMRL 1066 medium (5.5 mmol/L glucose) containing 10% FCS, 20 mg/mL ciprofloxacin, 50 mg/mL gentamycin, 2 mmol/L L-glutamin, 0.25 mg/mL fungizone, 10 mmol/L HEPES, and 1.2 mg/mL nicotinamide, in the presence or absence of 10 nM exendin-4 (Sigma) while medium was refreshed every 24-48 hours.

Lentivirus vectors

pTrip-RIP405Cre-ERT2-DeltaU3 (RIP-CreERT2) and pTrip–CMV-loxP-Neo-STOP-loxP-eGFP-DeltaU3 (CMVstopGFP) were kindly provided by P. Ravassard (21). pTrip vectors were produced as third-generation lentivirus vectors by adding a Tat-expressing vector (gift from B. Berkhout, Amsterdam) to the regular helper plasmids and produced as previously described (22). Vector pcDNA3.1:pcDNAPax4 encoding human Pax4 (hPax4) was kindly provided by K. Nanjo (23). Human Pax4 cDNA was subcloned in a pRRL lentivirus vector (CMV-hPax4) (22). For lineage tracing, transduction was performed overnight as previously described and exendin-4 was added following overnight lentivirus incubation (5). In case of hPax4, a second round of transduction was performed for 8 hours during the following day. 4-hydroxy-tamoxifen (Sigma-Aldrich, St. Louis, MO) was added to a final concentration of 1 mmol/L in the evening. After overnight incubation, the medium was refreshed and cells were seeded on the microwell. The start of reaggregation represents day 0 in our experiments.

RNA isolation and quantitative PCR

Total RNA was extracted using RNeasy kit (Qiagen) according to the manufacturer's protocol. RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen). Quantitative PCR was performed on a Light Cycler 480-II Real-time PCR system (Roche). Fold induction was calculated using delta CT method with human β-actin as housekeeping gene. Taqman probes were used for glucagon (Hs00174967_m1), insulin (Hs00355773_m1), Arx (Hs00292465_m1) and Pax4 (Hs00173014_m1).

Immunofluorescence staining

Formalin-fixed islet cell aggregates were washed in PBS and spun down at high speed in fluid agar. Agar-containing cell pellets were embedded in paraffin. Blocks were cut into 4-μm sections. Primary antibodies against insulin (1:200; Linco), glucagon (1:200; Vector and Invitrogen), Ki67 (1:200; BD Pharmingen), and green fluorescent protein (GFP) (1:500; Roche and Molecular Probes) were used. DAPI (Vector) was used as nuclear

counterstaining. Secondary antibodies were TRITC–anti–guinea pig (1:400; Jackson) and Alexa Fluor 488-, 568-, and 647 anti-mouse or anti-rabbit when appropriate (1:1,000). Apoptosis was assessed by TUNEL assay (Roche). Sections were examined using confocal microscopy. Staining was quantified as percentage of positive cells per total cell number, counting at least 750 cells per donor for each condition.

Statistical analysis

Data are expressed as mean ± SEM unless stated otherwise. Statistical significance of differences between two groups were determined by an unpaired Student's t test. One-way ANOVA followed by Bonferroni multiple comparisons test was used if more groups were compared. $P < 0.05$ was considered statistically significant.

RESULTS

GLP-1RA treatment prevents β-cell dedifferentiation in human islet cell aggregates

Treatment with the GLP-1RA exendin-4 showed a significantly higher proportion of GFP^+ cells expressing insulin after 1 week compared to aggregation without exendin-4 treatment (25.3 ± 4.3% vs. 39.8 ± 4.8%, untreated versus GLP-1RA treated, $P < 0.05$, Fig. 1A,B). As the lineage tracing system was induced by a tamoxifen pulse, this indicates that loss of β-cell identity was prevented rather than new β-cells generated. Immunolabeling showed that the overall number of proliferating (Ki67) and apoptotic (TUNEL) cells was low (<1% and <2%, respectively), and did not differ between both groups (Fig. 2). The proportion of $glucagon^+GFP^+$ out of GFP^+ cells was not significantly lower in the exendin-4 treated aggregates (Fig 3A,B).

Pax4 overexpression prevents β-cell dedifferentiation in human islet cell aggregates

Exendin-4 treatment induced a 2-fold higher *Pax4* gene expression in aggregates after 7 days of culture compared to intact cultured islets or aggregates without exendin-4 treatment (Fig. 4A). Since endogenous *Pax4* gene expression is normally low or even absent in mature β-cells (24), we hypothesized that increased *Pax4* expression following exendin-4 treatment induced protection against loss of β-cell identity. To test whether the protective effect of GLP-1 could be mimicked by human Pax4 protein overexpression (hPax4OE), we transduced islet cells using the lentiviral vector CMV-hPax4. Pax4 overexpression resulted in a 50% increase in *insulin* gene expression after 7 days of reaggregation (Fig. 4B). Gene expression of the α-cell transcription factor *Arx* and hormone *Glucagon* was significantly lower following hPax4OE (Fig. 3C). Using β-cell lineage tracing, a significantly higher proportion of GFP^+ cells expressing insulin was observed in hPax4OE aggregates (Fig. 4C). $Glucagon^+GFP^+$ cells were observed in both groups, and 2 out of 3 donors showed a distinct decrease in the percentage of double positive cells (Fig. 3D).

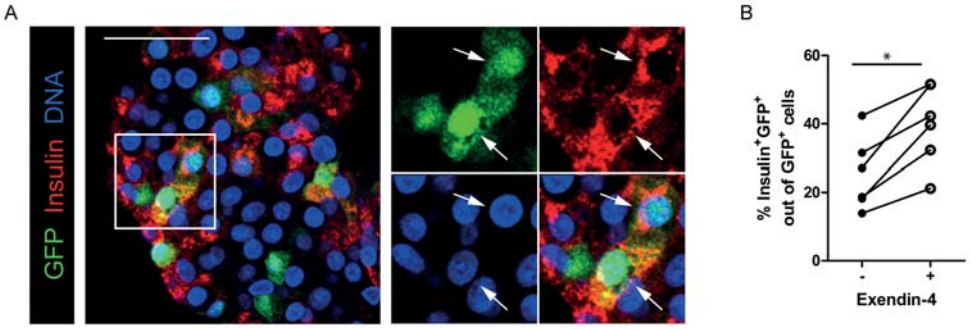

Figure 1. GLP-1RA administration yields a higher percentage of insulin$^+$GFP$^+$ cells. *A:* Representative immunostaining for insulin (red) and GFP (green) of a human aggregate after 7 days treatment with 10 nM GLP-1RA exendin-4. The right panel highlights the presence of Insulin$^+$GFP$^+$ cells (arrows). *B:* Quantification of the percentage of insulin$^+$GFP$^+$ out of all GFP$^+$ cells in 6 donors. Data derived from the same donors (with or without exendin-4 treatment) are indicated by connecting lines. * $P < 0.05$, scale bar: 50 μm.

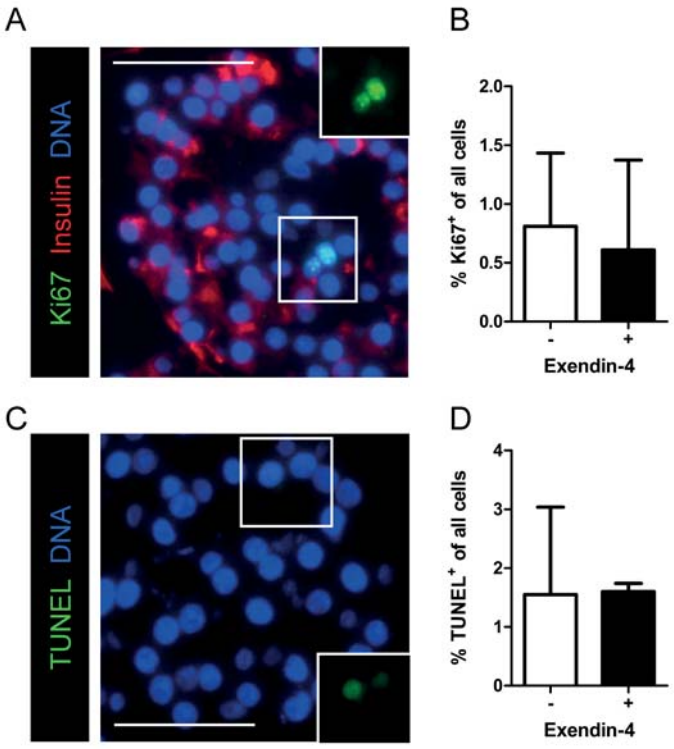

Figure 2. No difference in the proportion of proliferating (Ki67+) or apoptotic (TUNEL+) cells following treatment with exendin-4. *A:* Representative image of a human aggregate after 7 days reaggregation, immunostained for insulin (red) and Ki67 (green), inset shows two Ki67$^+$ cells. *B:* Quantification of the percentage of Ki67$^+$ cells out of all cells with or without GLP1-RA treatment. *C:* Representative image of TUNEL labelling (green). *D* Quantification of the percentage of TUNEL$^+$ cells out of all cells (n = 2, mean ± SD). Scale bars: 50 μm.

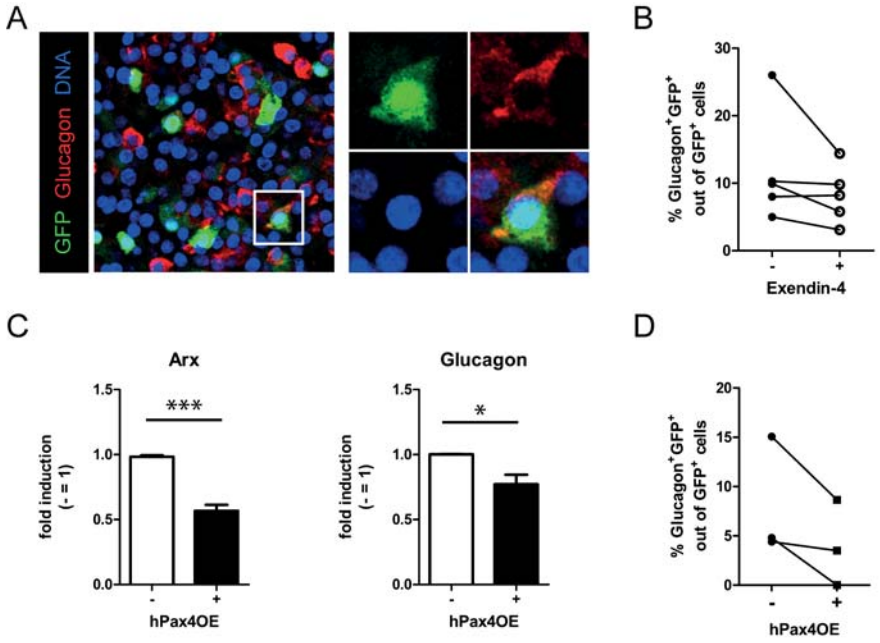

Figure 3. Human Pax4 overexpression lowers expression of α-cell genes *ARX* and *glucagon* but GLP1-RA or hPax4OE do not affect the proportion of glucagon$^+$GFP$^+$ cells. *A:* Immunostaining for glucagon (red) and GFP (green). *B:* Percentage of glucagon$^+$GFP$^+$ cells out of GFP$^+$ cells following exendin-4 treatment (n = 5, data from the same donors are indicated by connecting lines). *C:* hPax4OE in human islet cell aggregates results in a lower gene expression of the α-cell genes *ARX* and *glucagon* compared to controls. * $P < 0.05$, *** $P < 0.001$ (n = 6, mean ± SEM). *D:* Quantification of glucagon$^+$GFP$^+$ cells out of GFP$^+$ cells following hPax4OE (n = 3, data from the same donors are indicated by connecting lines).

DISCUSSION

We recently showed that loss of β-cell identity can occur in subjects with T2DM *in vivo* (7) and also in human pancreatic islets *ex vivo* following dispersion and reaggregation (5). Using our *ex vivo* culture system as a model for loss of human β-cell identity, we now add that both activation of the GLP-1 receptor as well as genetic overexpression of human Pax4 can promote the maintenance of β-cell identity.

GLP-1RAs are used in the clinic to improve glycemic control in patients with type 2 diabetes, mainly by stimulating glucose-dependent insulin secretion (25). Here, we show that activation of the GLP-1 receptor by exendin-4 can prevent loss of β-cell identity (characterized by a higher proportion of insulin$^+$GFP$^+$ out of GFP$^+$ cells after reaggregation) in human islet cell aggregates under normal glucose culture conditions. It has previously been shown that GLP-1 receptor signaling under normoglycemic conditions can increase insulin sensitivity and β-cell function (26) and can improve β-cell protection under lipid stress (27). The observation that approximately 50% of GFP$^+$ cells were insulin negative is in agreement with our previous finding that a large proportion of the cells is degranulated (5). Furthermore, β-cell conversion into α-cells still occurred since the proportion of

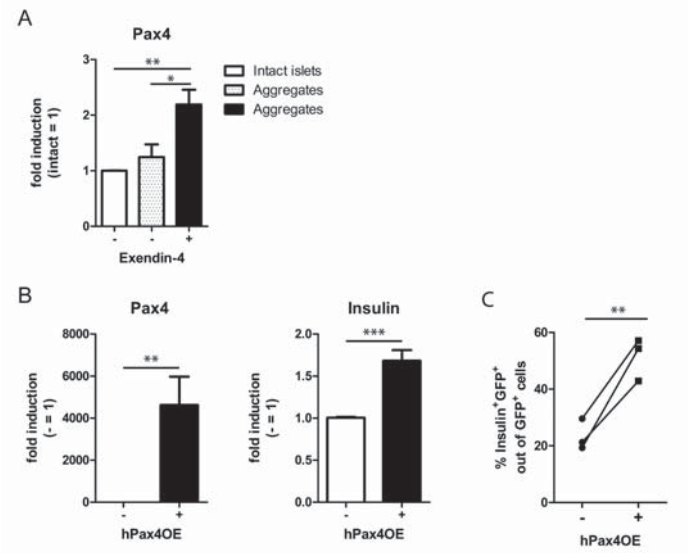

Figure 4. hPax4OE mimicks the effect of exendin-4 on human islet cell aggregates. *A:* Administration of 10 nM exendin-4 showing higher *PAX4* gene expression compared to aggregates or intact islets that were cultured for 7 days in the absence of exendin-4 (crude Ct-values ranging from 30-35 cycles). *B:* Lentivirus-mediated overexpression of human Pax4 (hPax4OE) in human islet cell aggregates results in higher *PAX4* and *insulin* gene expression after 7 days reaggregation (n = 6, mean ± SEM). *C:* hPax4OE shows a higher proportion of insulin$^+$GFP$^+$ out of GFP$^+$ cells as analysed by immunostaining (n = 3, data from the same donors are indicated by connecting lines). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

glucagon$^+$GFP$^+$ cells was not significantly changed. This lack of significance may be due to the small number of experiments, or it may indicate that GLP-1 receptor activation lowers the number of 'empty' GFP$^+$ cells (neither expressing glucagon nor insulin) by stimulating hormone expression. Altogether, our data suggest a novel benefit of GLP-1 receptor activation by providing maintenance of a β-cell phenotype.

GLP-1RA treatment in our study resulted in elevated *Pax4* gene expression levels. It was previously shown that inhibition of phosphatidylinositol-3 kinase (PI3-kinase) blunted this GLP-1 mediated effect on Pax4 (18). This suggests that the effect of GLP-1 on Pax4 is mediated by signaling via the epidermal growth factor receptor that activates PI3-kinase and AKT downstream (28). Whereas GLP-1RA treatment did not significantly affect α-cell markers (data not shown), Pax4 overexpression strongly diminished *Arx* and *Glucagon* gene expression. Furthermore, while GLP-1RA treatment had little effect on the proportion of glucagon$^+$GFP$^+$ cells, Pax4 overexpression showed a protective effect in 2 out of 3 donors. This difference may be explained by the higher levels of Pax4 expression that result from overexpression compared to the modest increase after GLP-1RA treatment.

Pax4 plays a pivotal role in the restriction of endocrine progenitors towards a β-cell fate in mice (29). In adult human β-cells, overexpression of mouse Pax4 has been shown to stimulate β-cell expansion and survival (30), while β-cell specific overexpression of wild-type Pax4, but not of mutated inactive Pax4, protected against streptozotocin-induced

apoptosis (14). Moreover, genetic polymorphisms in the human Pax4 gene have been associated with type 2 diabetes (23;31). We now add that hPax4 prevents loss of human β-cell identity, using a combination of hPax4 overexpression and β-cell lineage tracing. These data together suggest that Pax4 not only plays a crucial role in development, but is also involved in the protective response of β-cells against environmental (metabolic or inflammatory) stress (32). Interestingly, Pax4 gene expression is low or even absent in mature human islets (24), but is upregulated in a subset of subjects with T2DM (18). In light of our study, this could reflect activated protection against β-cell dedifferentiation.

Thus, we show that overexpression of Pax4 and GLP-1 receptor activation can prevent loss of human β-cell identity in an *in vitro* model of human islet cell reaggregation. We thereby identify a previously unknown function of these molecules, targeting maintenance of mature β-cells. Future research should investigate further by which mechanisms GLP-1 maintains β-cell identity.

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CHAPTER 7

GENERAL DISCUSSION

Diabetes is a chronic disease that has a major impact on patient quality of life and healthcare costs. Insulin-producing pancreatic β-cells are key players in the homeostasis of glucose metabolism and in the development of diabetes. While type 1 diabetes (T1DM) is characterized by autoimmune mediated β-cell destruction (1), type 2 diabetes (T2DM) reflects the interaction between β-cell dysfunction and decreased β-cell mass in a context of peripheral insulin resistance and obesity (2;3). Apart from the severe micro- and macrovascular complications, quality of life is strongly impacted by (the fear of) hypoglycemic episodes induced by current insulin therapies (4). Replacement of β-cell mass by whole pancreas or pancreatic islet transplantation is a promising therapy but is limited by the amount of donor organs. To obtain *de novo* functional β-cells from stem of progenitor cells, recent scientific efforts have focused on understanding β-cell development and differentiation. Although it was previously thought that fully differentiated cells cannot change phenotype, recent studies indicated that murine β-cells can change identity under conditions of metabolic stress or following genetic modification. It is not known whether human β-cells have similar changeable characteristics and whether this phenomenon plays a role in human disease. In this thesis, we provide evidence that mature human β-cells can change identity under specific culture conditions and that loss of β-cell identity may attribute to the pathophysiology of diabetes.

β-CELL REPLACEMENT THERAPY

To illustrate the major impact of diabetes and the promise of β-cell replacement therapy for individual patients, we first report a clinical case in chapter 2. We present a patient with severe diabetes related to cystic fibrosis, who suffered progressively from labile glucose regulation with frequent hypoglycemic episodes despite insulin pump therapy and continuous glucose monitoring. Successful intraportal islet transplantation resulted in more optimal glycemic control reducing (and transiently eliminating) the need for exogenous insulin. More importantly, no event of hypoglycemia was reported during the 18 months follow up period after transplantation. We propose that islet-after-lung transplantation is a safe and effective intervention for patients that could not benefit from simultaneous islet-lung or pancreas-lung allotransplantation. It was recently shown in patients with longstanding T1DM that the counterregulation in response to insulin-induced hypoglycemia (measured by insulin suppression, glucagon secretion and epinephrine secretion) was restored 6 months after islet transplantation (5). The accompanying fear of hypoglycemia is similarly reduced following successful islet transplantation (6). A recent islet-after-kidney transplantation cohort from Leiden showed the presence of C-peptide in 92% of patients while severe hypoglycemia was restricted to the only patient who had lost graft function (7). These features provide a rationale for the application of minimally invasive β-cell replacement therapy in a wider group of patients that suffer from labile glucose regulation.

Hurdles remain for islet transplantation to become common therapy. First of all, even though long term outcomes of islet transplantation have improved over the past years, the β-cell mass that remains after isolation and transplantation is often marginal and

function declines progressively (8). Secondly, transplantation of allogenic islet material requires lifelong immunosuppressive therapy to prevent rejection. Side effects of these drugs include nephrotoxicity, increased risk of infections and paradoxically diabetogenicity, parameters that are already affected in patients with longstanding diabetes (9). Even though this hurdle becomes less disturbing when islet transplantation follows kidney or lung transplantation, as patients are already on immunosuppressive drugs, the additional induction therapy accompanying islet transplantation increases this risk of side effects. Finally and perhaps most importantly, the current source of transplantable islets is limited due to the shortage of organ donors, thereby restricting widespread application.

The combination of knowledge in (bio)medical sciences and biomaterials creates an opportunity to overcome part of the above mentioned problems. Refining the islet isolation methods can minimize islet damage during isolation and optimize islet yield. Encapsulation of islets using biomaterials can provide protection from the (auto)immune reaction that follows transplantation, discarding the need for immunosuppressive drugs. Furthermore, coating of biomaterials with for example angiogenic drugs may facilitate homing and engraftment (10). Considering future transplantation of islets derived from stem or progenitor cells, devices are needed that enable controllable protocols for culture, differentiation and transplantation of islet-like cell clusters. Furthermore, the opportunity to selectively explant a biomaterial device in case of neoplastic cell growth offers an important safety measure after transplantation.

Controlled islet cell culture

To facilitate experimental islet cell culture and future stem cell differentiation, we present a novel microwell culture system and its application in the formation of islet cell aggregates of defined size (chapter 3). The agarose-based microwell chip contains 2865 wells with a diameter of 200 μm and is derived from a polydimethylsiloxane negative mould. Islets were enzymatically dissociated into single cells and subsequently allowed to reaggregate into islet cell aggregates of controlled size using the microwell chip. We show that this culture system enables a controllable and safe culture method to produce islet-like cell structures. Regular cell aggregation experiments use ultra-low attachment plates, resulting in aggregates with heterogeneous size and shape between 40 and 250 μm, while aggregation in the agarose microwell system resulted in well-defined aggregates between 90-110 μm. Homogenous aggregation can also be achieved using the hanging drop method, but this method is labour-intensive and therefore limited to smaller numbers of aggregates (11). The homogenous aggregation facilitates experimental reproducibility, which is essential when working with human donor islets, since each preparation already has a different genetic background. Moreover, the preparation of agarose microwells is cheap, non-toxic and easy to use compared to a previously published poly(ethylene glycol) microwell system (12). The formation of primary human islet cell aggregates with a diameter of 100–150 μm most closely resembled the function of intact isolated islets. However, the dissociation method itself may harm islet cell function, as cell-cell interactions and signals from the extracellular matrix are essential for efficient insulin secretion (13;14).

While others have shown that controlling aggregate size allows efficient production of clustered pancreatic endocrine precursors for *in vivo* transplantation (15), future research should demonstrate whether stem or progenitor cell differentiation will benefit from our microwell culture system.

Islet encapsulation

Transplantation of macroencapsulated islet cells using scaffolds is in progress. However, to design a device that provides immune protection for the transplanted islets contrasts with the need for direct contact with oxygen or nutrients and glucose sensing. A recent study reports the development of an oxygenated islet chamber comprised of immune-isolating alginate and polymembrane covers (16). Without systemic immunosuppression, function and survival of the allogeneic islets was persistent even 10 months following transplantation in a patient with T1DM (16). In immunocompromised mice, glucose-responsive insulin-secreting cells were retrieved from encapsulated human embryonic stem cells (hESC) 20 weeks after transplantation (17) and more recently for the first time from an immunocompetent animal model (18). Specific devices containing differentiated hESC are currently being tested in phase 1 trials in humans (19). The number of islets that can be transplanted within one of the abovementioned devices is yet insufficient to render patients insulin independent, emphasizing the need for future optimization.

Although β-cell replacement therapy is currently applied with success, the number of available donor organs limits the number of patients that can benefit in current clinical practice. Developing more efficient ways to isolate and preserve islets from a donor can provide more islets on the short term. However, approaches to expand the mass of isolated islets or find alternative sources of β-cells (e.g. xenotransplantation or stem cell-derived β-cells) are essential to extend care for a large group of diabetic patients in the future (20;21). In order to develop fully differentiated β-cells from stem cells and to maintain functionality after transplantation, it is essential to understand the mechanisms that maintain a functional β-cell phenotype.

INTRAISLET CELL CONVERSION

Triggers that elicit intraislet cell conversion

Intraislet cell conversion between α-, β-, and δ-cells has been reported in animals by a number of groups using lineage tracing approaches to substantiate their findings (Table 1) (22-33). In chapter 4, we use our microwell culture system to study human β-cell fate following islet dispersion and reaggregation. We observe a decreased percentage of β-cells after 4, 7 and 14 days of reaggregation while numbers of apoptotic cell death and proliferation were equal in the microwell culture system. Using lentiviral lineage tracing to track β-cells, we show that human β-cells can convert into glucagon-producing α-cells following islet reaggregation, explaining the decrease in the proportion of β-cells.

In our study, the conversion process occurred within a week. Studies that show a fast course of transition (within weeks or days) are mainly based on genetic knockout or

Table 1. Results of studies that show intra-islet cell conversion using lineage tracing techniques

	Intraislet cell conversion					
Authors	Cell conversion	Empty cells	Bihormonal	Intermediate phenotype	Time window	Mechanism
Thorel 2010	α → β		Yes	Yes	Months	β-cell ablation
Yang 2011	α → β	Few	Yes	Yes	Days-weeks	Pdx1 OE in progenitors
Dhawan 2011	β → α		Yes		Months	Dnmt1$^{-/-}$
Papizan 2011	β → α		Yes	Yes	Weeks	Nkx2.2$^{TNmut/TNmut}$
Talchai 2012	β → α	Yes	No	Yes	Months	FoxO1$^{-/-}$ and metabolic stress
Spijker 2013	β → α	Yes	No	Yes	Days	Reaggregation
Bramswig 2013	α → β		Yes	Yes	Days	Methyl transferase inhibitors
Al-Hasani 2013	α→ β		Rare	Rare	Days-weeks	Pax4 OE
Brereton 2014	β → α	Yes	Yes	Yes	Month	K_{ATP}-channel mutation
Wang 2014	β → α *				Months	K_{ATP}-channel mutation
Chera 2014	δ → β	Yes	Rare		Months	β-cell ablation
Gao 2014	β → α		Yes		Days	Pdx1 KO
Nishimura 2015	β → α	Yes	No		Months	MafA KO and db/db mice

* Only few events of transdifferentiation described.
KO: knock out; OE: over expression.

overexpression mouse models, targeting essential β-cell transcription factors such as Pax4 (32) or Pdx1 (25;30). Changes in β-cell identity in these studies are likely to be forced by strong intracellular signalling shifts. We hypothesize that the driving force in our study lies in the evoked stress from breaking down cell-cell and cell-matrix interactions, thereby affecting the transcription factor profile.

Reports that describe *in vivo* models of chronic metabolic stress (aging or multiparity in combination with FoxO1$^{-/-}$) or diabetes (*db/db* mice, near total β-cell ablation using diphtheria toxin mediated β-cell death or hyperglycemia due to a K_{ATP}-channel mutation) show more gradual islet cell changes over months (22;23;28;29;31). Since the driving force in these models likely comes from the surrounding environment (e.g. hyperglycemia, metabolic stress), we hypothesize that islet cells can initially still compensate but ultimately lose their identity and change phenotype.

We did not investigate whether epigenetic changes play a role in our model of intraislet cell conversion. Two murine studies investigated the effect of epigenetic changes on β-cell identity using either DNA methyltransferase 1 knockout mice or an inactivating mutation of Nkx2.2 (26;27). Both studies showed that loss of β-cell identity and subsequent conversion into α-cells occurred. Others have shown that *in vitro* treatment of human islets with the methyl transferase inhibitor adenosine dialdehyde (Adox) resulted in the presence of bihormonal cells (33). These findings support the hypothesis that maintenance of the epigenetic signature is essential to maintain a mature islet cell phenotype. While not

much is known about the relation between cell-cell signalling and epigenetics, it would need further investigation to determine whether reaggregation triggers β-cell conversion by changing epigenetic marks.

In our reaggregation model, a large proportion of β-cells was completely degranulated following the dispersion process, possibly due to dispersion method. This may have accelerated the conversion process and can explain why we did not observe bihormonal cells in this model. Several of the abovementioned studies that investigated intraislet cell conversion describe such 'empty' cells (23;25;28;29;34). In these studies, empty islet cells may indicate that longacting stressors result in β-cell exhaustion that precedes conversion. Consistently, these studies do not (or rarely) observe bihormonal cells, indicating that loss of the hormone storage may be a first event in the process of transdifferentiation.

Dedifferentiation versus direct lineage conversion

Differences in the mechanism behind intraislet cell conversion can also be found in the observation of islet cell dedifferentiation before lineage conversion occurs. Talchai et al. demonstrated β-cell dedifferentiation and subsequent conversion into other islet cells following metabolic stress (aging or multiparity) in the context of β-cell specific FoxO1$^{-/-}$ mice (23). As they observed markers specific for multipotent stem cells (Oct4, Nanog and Ngn3), the authors suggest that β-cell dedifferentiation into multipotent cells occurrs preceding the adoption of a new identity. Furthermore, Chera et al. showed in juvenile mice following near total β-cell ablation, that newly formed β-cells were derived from δ-cells that first dedifferentiated and underwent a proliferative stage, before the lineage switch into insulin-producing cells occurred (29). Although these two studies suggest a dedifferentiation process, a common feature of nearly all studies reporting on intraislet cell conversion is the observation of intermediate cell phenotypes between the original and eventual cell type (either bihormonal cells or costaining of for example α-cell hormone with β-cell transcription factors) (Table 1). This intermediate phenotype supports the idea that in most studies direct intraislet lineage conversion occurs, while both α- and β-cell markers are present in one cell. Further investigation will have to define whether dedifferentiation and direct lineage conversion may occur in parallel or depend on the experimental model.

MAINTENANCE OF β-CELL IDENTITY BY REPRESSION OF THE α-CELL PHENOTYPE

As stressed β-cells appear vulnerable to dysfunction, identity change or even cell death, active maintenance of β-cell identity is pivotal. Recent reports indicate that specific β-cell transcription factors are essential to maintain a functional phenotype. Gu et al. used an inducible knockout system to show that genetic loss of NeuroD in adult β-cells leads to glucose intolerance (35). Similarly, loss of Nkx6.1 or Rfx6 in adult β-cells caused glucose intolerance and disturbed insulin secretion, while Rfx6 also impaired glucose sensing (36;37). Finally, an inducible but not β-cell specific deletion of Pax6 induced severe diabetes in mice (38). While these models indicate that adult β-cell function depends

on a healthy cocktail of transcription factors, they do not explain how cellular conversion can occur.

As we report in Chapter 4 that β-to-α-cell conversion occurs following reaggregation, we further show that knockdown of the α-cell factor Arx during reaggregation inhibits conversion into α-cells. We hypothesize that when β-cell maintenance is disturbed, the transcriptional balance can be tilted towards the expression of α-cell factors. Several groups reported that activation of Arx in β-cells results in spontaneous β- to α- cell conversion. Dhawan et al showed that loss of the epigenetic regulator DNA methyltransferase 1 that normally maintains the genetic repression of Arx in β-cells, results in loss of β-cell identity and subsequent conversion into α-cells (26). In addition, transcription factor Nkx2.2 was shown to recruit a large repressive protein complex to the Arx promoter preventing Arx expression. An inactivating mutation of Nkx2.2 induced Arx expression and resulted in β- to α-cell conversion (27). Furthermore, ectopic expression of Arx in β-cells promoted conversion of fetal and mature β-cells into α-cells (39), while inactivation of Nkx6.1 converts β-cells to alternative endocrine lineages by the loss of Arx repression (40). Finally, the group of Stanger recently reported on β- to α- cell conversion following deletion of Pdx1 in adult β-cells (30). Although in this study the α-cell factor MafB (and not Arx) was induced, a similar change of phenotype was observed. Taken together, these data strongly support the concept that repression of the α-cell lineage is a fundamental principle by which β-cells are maintained.

Epigenetically, mature α-cells exhibit histone methylation profiles that are distinct from β-cells. Essential β-cell genes amongst which *Pdx1* and *MafA* are bivalently marked in α-cells, implying that both activating (H3K4me3) and repressive (H3K27me3) histone modifications are present (30). These bivalent marks occur more commonly in progenitor cells and usually resolve during differentiation while the marks render the genes inactive. In contrast, many of the genes marked bivalently in α-cells carry a monovalent activating or repressive mark in β-cells (30). These findings may support the hypothesis that β-cell maintenance mechanisms focus on repression of other lineages and that α-cells may serve as default phenotype.

LOSS OF β-CELL IDENTITY IN DIABETES

Whereas chapter 4 provides a proof of principle that the identity of adult human β-cells can be changed, the essential question remains whether this process plays a role in the pathophysiology of diabetes. In chapter 5 of this thesis, we investigate human post-mortem pancreatic tissue from non-diabetic donors and donors with a history of T2DM. In the T2DM group, a higher proportion of islet cells has a mixed phenotype compared to non-diabetic controls, expressing both glucagon and insulin or the hormone of one cell together with transcription factors specific to another (e.g. glucagon and Nkx6.1) (43). We conclude that the presence of these cells indicate that loss of β-cell identity occurs in type 2 diabetes and may represent a snapshot of β-cell dedifferentiation or conversion into α-cells.

Animal studies suggest loss of β-cell identity under diabetic conditions

Several recent murine studies support the hypothesis that loss of β-cell identity occurs in T2DM-like circumstances. Two independent groups used distinct mouse models with activating K_{ATP}-channel mutations resembling neonatal diabetes (28;31). In the Brereton study, a reduction in insulin-positive cells and an increase in glucagon-positive cells was observed without changes in rates of proliferation. Lineage tracing identified β-cells with a mixed phenotype similar to those in our study, expressing both insulin and glucagon or glucagon together with Pdx1, Glut2 or β-cell specific sodium-channels (28). The second neonatal diabetes model described β-cells that had lost insulin expression and regained the endocrine progenitor marker Ngn3 while only few events of conversion occurred (31). Furthermore, Nishimura et al. demonstrated β-cell dedifferentiation in two separate mouse models of diabetes, either *db/db* mice or multiple administrations of low-dose streptozotocin (22). The authors used a *MafB-GFP* construct to show that approximately 40% of β-cells in *db/db* mice expressed the α-cell factor MafB, compared with 1.5% in controls. Although the translation of murine studies towards human disease requires careful interpretation (41), these studies support the hypothesis that loss of β-cell identity occurs under diabetic conditions.

In our group of T2DM donors we show that FoxO1 was more often localized in the nuclear compartment of β-cells. It has previously been shown that FoxO1 translocates to the nucleus under conditions of metabolic stress, where it protects β-cell identity by activating transcription factors such as NeuroD and MafA (42). Talchai et al. investigated mice with β-cell specific FoxO1 depletion that were exposed to metabolic stress by means of aging or multiparity (23). $FoxO1^{-/-}$ β-cells dedifferentiated and eventually converted into other hormone-producing islet cells such as α-cells, as shown by lineage tracing (23). These data indicate that β-cells under conditions of metabolic stress normally protect their identity by factors such as FoxO1, whereas failure of these protective mechanisms may play a role in the development of hyperglycemia and β-cell dysfunction.

Loss of β-cell identity in humans

Studies indicating loss of β-cell identity in humans are hampered by their descriptive nature. Immunofluorescence studies investigating pancreas tissue from a small number of T2DM donors showed colocalization of the mesenchymal marker vimentin with α-cells (43), indicative of epithelial-to-mesenchymal transition (EMT), as well as bihormonal cells in donors with T2DM or insulin resistance (43-46). Although these studies may support β-cell conversion, cross-sectional data on human donor organs cannot identify whether cells dedifferentiate or actually represent α-cells becoming new β-cells. However, the proportion of cells with an intermediate phenotype in our study correlated with an increased α/β-ratio. Furthermore, we confirmed our data in a well characterized cohort of *M. mulatta* rhesus monkeys that show dramatic loss of β-cell mass and islet amyloidosis associated with diabetes. These observations together with the aforementioned mechanistic animal studies support our hypothesis that loss of β-cell identity and possibly β-cell conversion occurs in the natural course of T2DM development. Importantly, this hypothesis holds

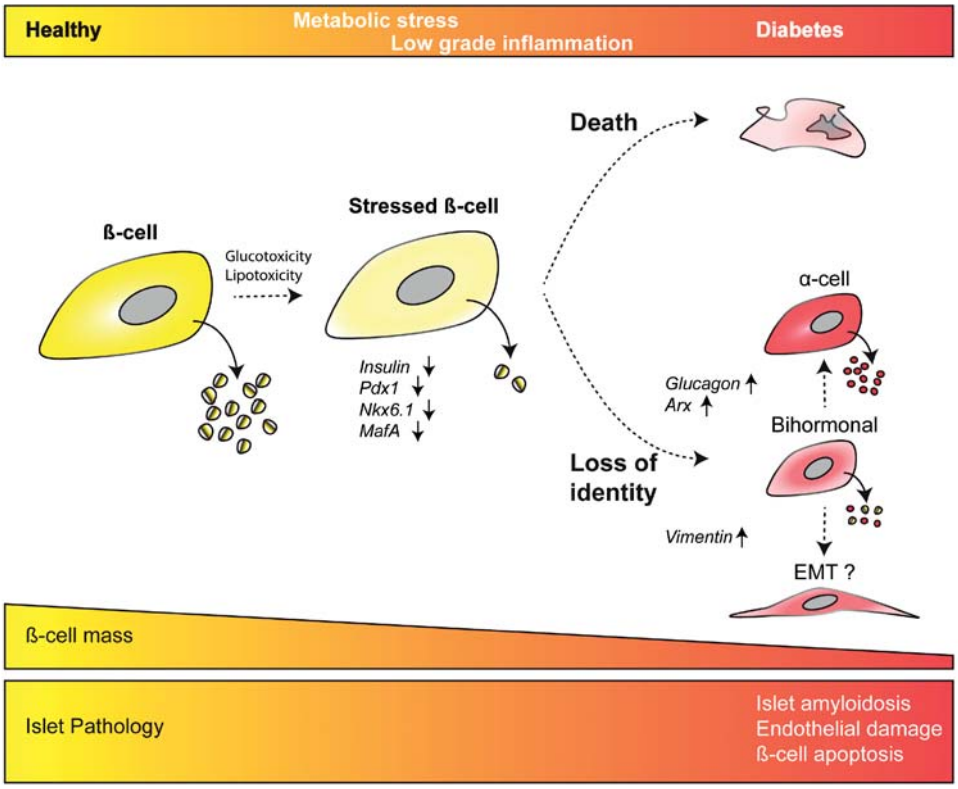

Figure 1. Model for loss of β-cell identity in diabetes. The development of diabetes gradually results in the loss of healthy and functional β-cells. Healthy β-cells are characterized by their stock of granules that can be readily secreted in response to raised glucose levels. At first, the islet microenvironment is affected by factors including amyloid fibril formation, low grade inflammation and metabolic stress in the context of gluco- or lipotoxicity. This will result in the loss of essential β-cell transcription factors such as Pdx1, MafA and Nkx6.1. Degranulation may occur and the secretory response to glucose will decline. Progression to overt diabetes may result in failure of β-cell maintenance, diminishing the repressive mark on α-cell specific gene promoters and resulting in upregulation of α-cell factors such as Arx and glucagon. Subsequent loss of β-cell identity may result in bihormonal cells with a mixed phenotype, transdifferentiation into glucagon-producing α-cells, epithelial to mesenchymal transition (EMT) or (apoptotic) cell death. Together, these events reduce functional β-cell mass thereby worsening glucose regulation.

with the current notion of β-cell dysfunction in T2DM as well as the decreased β-cell mass that could partly be explained by dedifferentiation and/or transdifferentiation, likely in combination with apoptosis (Fig. 1) (47). More research is needed to elucidate whether this process represents a bystander effect or directly contributes to the pathophysiology of T2DM. In addition, more sophisticated experimental models that mimic T2DM using human islets and their microenvironment should be developed in order to address these questions.

Possible factors triggering loss of β-cell identity in diabetes

The abovementioned studies indicate that certain stress factors in the context of diabetes impair the regulation of essential transcription factors and eventually impair β-cell function. Obvious candidates for cellular stress in T2DM are glucotoxicity, lipotoxicity or oxidative stress derived from increased serum glucose and/or lipid levels. Chronic hyperglycemia has been associated with a decrease in β-cell transcription factors in rats (48) and β-cell lines cultured in the presence of hydrogen peroxide (oxidative stress) lose expression of several essential transcription factors such as MafA and Pdx1 (49).

In chapter 5, we observe that loss of β-cell identity in T2DM is correlated to the presence and amount of islet amyloidosis. Amyloid can affect β-cells both through direct and indirect pathways. Direct contact of β-cells with amyloid fibrils has been shown to be toxic for β-cells and could therefore be the causative stressor (50). However, it has also been shown that amyloid can induce a local inflammatory response, inducing the pro-inflammatory cytokine interleukin-1β (51). This could in turn lead to stressed β-cells that are more prone to identity loss. Further research should address which stress factors truly induce loss of β-cell identity.

Hyperglucagonemia in T2DM

High plasma glucagon levels represent a feature of T2DM commonly explained by the loss of local (paracrine) α-cell inhibition because of decreased insulin secretion (52). Although we report conversion of β-cells into glucagon-producing cells (chapter 4) and detect cells with a mixed phenotype in T2DM suggesting conversion occurs in diabetes (chapter 5), we did not investigate the functionality of these glucagon-expressing cells. It is not unlikely that hormone secretion from these cells is significantly affected or differently regulated compared to mature α-cells. A hypothesis that may link β- to α-cell conversion to hyperglucagonemia is that converted (glucagon-producing) cells retain part of their β-cell machinery and thereby remain glucose-responsive. In this model, hyperglycemia would still elicit hormone secretion, but the wrong hormone (i.e. glucagon) would be secreted or both hormones would be secreted simultaneously. An observation supporting this hypothesis is that converted glucagon-positive cells still express β-cell specific Na^+-channels in the model of neonatal diabetes (28). More detailed investigation of the glucose sensing and secretion mechanisms of converting cells is needed to sustain this hypothesis, for example by measuring K_{ATP}-channel activity (53).

β-CELL MAINTENANCE AS A THERAPEUTICAL PERSPECTIVE

In chapter 6, we use our microwell model for islet cell reaggregation to investigate a small molecule that may prevent loss of β-cell identity. We stimulate β-cells by treatment with Glp-1 receptor agonists (Glp-1RA) during the reaggregation period. We show that activation of the Glp-1 receptor can prevent loss of β-cell identity as more cells co-expressed GFP (corresponding to β-cell lineage tracing) together with insulin following reaggregation under Glp-1RA treatment. We observe a higher expression of *Pax4* following Glp-1RA

treatment and can mimic the protective effect of Glp1-RA by lentiviral overexpression of Pax4. As the functions of Pax4 and Arx are known to be opposed, these data are in line with our findings in Chapter 4 that knockdown of Arx during reaggregation prevents conversion.

Glp-1 is known to signal via adenylate cyclases, increasing intracellular cyclic AMP levels and thereby activating protein kinase A and cAMP-regulated guanine nucleotide exchange factor II (54). Glp-1 can also activate phosphatidylinositol-3 kinase (PI3-kinase) via transactivation of the epidermal growth factor receptor (55). It has been shown that Glp1-RAs can increase Pax4 expression, an effect that is blocked by inhibition of PI3-kinase (56). Previous studies have shown in mice that Pax4 promotes β-cell survival and protects against streptozotocin-induced apoptosis (57;58). Therefore, the Glp-1 induced protection in our study may be mediated via stimulation of Pax4, possibly via PI3-kinase signalling.

Several recent studies demonstrate that loss of β-cell identity is a reversible process, suggesting that therapeutic options may be implicated. In the aforementioned mouse models in which specific K_{ATP}-channel mutations resemble neonatal diabetes, the loss of islet cell identity was prevented or reversed when blood glucose normalized following treatment with sulfonylurea derivatives or insulin (28;31). Blum et al. demonstrated that urocortin 3 is a marker of mature β-cells that becomes downregulated in diabetic mouse models (59). Using the *Ucn3* gene as a reporter to monitor β-cell dedifferentiation, a compound screen was performed showing that the small molecule inhibitor of the TGFβ receptor type I (Alk5) could restore β-cell maturation (59). Others have shown that increasing the expression of the antioxidant enzyme glutathione peroxidase-1 (Gpx-1), thereby protecting β-cells against reactive oxygen species, restored the nuclear localization of MafA and Nkx6.1 and improved glucose homeostasis in *db/db* mice (49;60). Similarly, use of the oral Gpx-1 mimetic ebselen improved fasting glucose and enhanced nuclear localization of Pdx1 and MafA in islets of Zucker diabetic fatty rats (61). Altogether, loss of β-cell identity appears a reversible process in murine models and further investigation will need to identify drug candidates targeting maintenance of human β-cell identity. Identification of the pathways that drive loss of β-cell identity are essential to screen drugs that may target this process in clinically meaningful ways.

FUTURE DIRECTIONS

In this thesis, we demonstrate that adult human β-cells can change their identity under specific culture conditions and propose that this mechanism plays a role in the pathophysiology of T2DM. Under diabetic conditions, β-cells have to remain functional in a complex microenvironment including the presence of hyperglycemia, lipotoxicity, endothelial dysfunction and (low grade) inflammation. We hypothesize that the pathological microenvironment leads to a loss of β-cell specific transcription factors such as Pdx1, Nkx6.1 or MafA, resulting in less functional β-cells as insulin production and secretion becomes impaired (Fig. 1). Moreover, the loss of transcription factors renders β-cells vulnerable to transdifferentiation, as these factors normally repress the phenotype of other islet cells, primarily α-cells.

As we find an association between loss of β-cell identity and islet amyloidosis, we suggest that amyloid plaques may be one of the responsible stressors, either directly or indirectly by eliciting an IL-1β mediated inflammatory response. Although mechanistic studies are needed to support this hypothesis, anti-inflammatory treatment in patients with T2DM using the IL-1 receptor antagonist anakinra resulted in improved glycated hemoglobin levels and reduced systemic inflammation (62;63). Whether this improvement reflects an improved function of individual β-cells remains to be identified. For this purpose, more suited experimental approaches that can monitor (de)differentiation of human β-cells *in vivo* and *ex vivo* are needed.

Maintenance of endogenous functional β-cell mass is essential. However, in current practice, profound loss of β-cell mass is usually evident at the time of diagnosis, especially in T1DM. Failure of endogenous β-cell mass urges the need for replacement of β-cell mass. Promising reports demonstrated that human stem cell-derived β-cells can secrete insulin in a glucose-regulated manner and ameliorate hyperglycemia in mice (18;64). While it has been shown that mouse α-cells can replenish β-cell mass (24), our findings using human islets may strengthen the effort to investigate human α-cells as an alternative renewable source to obtain β-cells. Developing a reliable lineage tracing system to track human α-cells is a prerequisite to this approach. However, if it appears feasible to obtain functional human β-cells from other islet cells or from non-islet pancreatic cells, this may provide the advantage to trigger these cells while remaining *in vivo*. Compound screens that expose α-cells to a series of small molecules may identify peptides that are capable of inducing α-cell transdifferentiation *in vivo*. While disturbance of glucagon signalling may be a potential concern, it has been shown that near total α-cell depletion does not influence the counterregulatory response in mice (60). More profound knowledge on the fundamental biology of islet cells and the islet microenvironment will provide a strong framework to estimate safety and feasibility of these new approaches to understand and treat diabetes.

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CHAPTER 8

ENGLISH SUMMARY

Diabetes mellitus is amongst the leading causes of morbidity and mortality worldwide. Insulin-producing pancreatic β-cells are central in establishing adequate glucose regulation and loss of functional β-cells is implicated in the development of both type 1 diabetes (T1DM) and type 2 diabetes (T2DM). Although it was previously thought that fully differentiated cells cannot change phenotype, recent murine studies indicated that mature β-cells can change identity into other islet cells under conditions of (metabolic) stress. It has been hypothesized that this process is associated with β-cell dysfunction and loss of β-cell mass in diabetes. Moreover, it was shown that other islet cells can convert into functional β-cells, providing a possible source to obtain new β-cells. It is not known whether adult human β-cells can change identity and whether mechanisms of islet cell conversion play a role in human diabetes. The aim of this thesis was to explore the stability of adult human β-cell identity and investigate whether loss of β-cell identity plays a role in the pathophysiology of diabetes.

In **chapter 2**, we first describe a case of β-cell replacement therapy in a 48-year-old patient with cystic fibrosis related diabetes, complicated by delayed gastric emptying and recurrent severe hypoglycemia. She underwent bilateral lung transplantation 7 years earlier and suffered from labile glucose regulation despite insulin pump therapy and continuous glucose monitoring. Pancreatic islets from two organ donors were infused intraportally under local anesthesia. The procedure was uncomplicated and the patient left the hospital 3 days posttransplantation. After 6 months, a mixed meal test showed excellent glucose regulation while insulin pump therapy was completely stopped. After 1.5 years, low dose long-acting insulin was restarted due to postprandial hyperglycemia, but no episodes of hypoglycemia were reported. Using this case report, we illustrate the impact of diabetes on quality of life and the relevance of minimally invasive β-cell replacement therapy in patients that suffer from labile glucose regulation.

Isolated human islets are heterogeneous three-dimensional mini-organs both in terms of size and shape. In **chapter 3**, we investigated a novel microwell culture system and its application in the formation of islet cell aggregates of defined size, designed to facilitate future islet and stem cell research. The culture system is derived from polydimethylsiloxane negative moulds and consists of agarose-based chips that contain 2865 microwells with a diameter of 200 μm each. The microwell platform enabled a highly reproducible aggregation culture for mouse and rat insulinoma cells and for dispersed primary human islet cells. Human islet cell aggregates with a diameter of 100–150 μm showed low apoptotic cell death and glucose-responsiveness comparable to intact isolated islets. After transplantation of islet cell aggregates under the kidney capsule of immunodeficient mice, human C-peptide was detected in the serum indicating that β-cells retained their endocrine function. We conclude that our novel microwell culture system provides a reproducible and 'easy to use' tool for islet cell research.

In **Chapter 4**, we used the microwell culture system to study the stability of human β-cells following islet cell reaggregration. We observed a marked reduction in the proportion of insulin-immunoreactive cells while the percentage of glucagon-containing cells was significantly higher during the 14-day reaggregation period. No differences were found

in the degree of β-cell apoptosis or α-cell proliferation that could explain the altered cell composition. We then introduced a β-cell specific lentiviral lineage tracing system before reaggregation. This lentivirus based technique contains a tamoxifen inducible Cre-Lox system dependent on the rat insulin promoter (RIPCreERT2). The Cre recombinase can remove the stop codon flanking a CMV promoter (CMV-stop-GFP) thereby enabling transcription of Green Fluorescent Protein (GFP). At the time of tamoxifen administration, the estrogen receptor bound Cre recombinase can shuttle to the nuclear compartment and remove the stop codon, marking only β-cells with GFP expression, independent of future phenotypic changes. Using this system, we show that a proportion of GFP^+ cells colocalizes with glucagon after reaggregation, indicating that adult human β-cells can undergo conversion into glucagon-producing α-cells. The conversion of β-cells into α-cells occurred following β-cell degranulation and was characterized by the presence of β-cell specific transcription factors (Nkx6.1 and Pdx1) in glucagon-containing cells. Finally, lentivirus-mediated knockdown of Arx, a determinant of the α-cell lineage, inhibited β-cell conversion. Altogether, this study revealed for the first time that adult human β-cells can lose their identity and convert into other endocrine islet cells.

As our findings in Chapter 4 were restricted to a specific experimental setup (dispersion and reaggregation), we addressed the relevance of these findings in the context of diabetes in **Chapter 5.** We hypothesized that loss of β-cell identity would be marked by the presence of β-cell transcription factors (Nkx6.1, Pdx1) in $glucagon^+$ cells, similar to the $Nkx6.1^+glucagon^+$ cells described in Chapter 4 following reaggregation. We investigated tissue biopsies from donors with T2DM and non-diabetic controls matched for age and BMI. We observed an eight times increased frequency of $insulin^+$ cells coexpressing glucagon (bihormonal cells) in donors with T2DM. Furthermore, up to 5% of the cells that expressed Nkx6.1 coexpressed glucagon instead of insulin, a five times increased frequency compared with the control group. We confirmed these findings in islets from a selected group of macaques that develop diabetes in a similar fashion to T2DM in humans and have been well characterized metabolically. In the bihormonal cells, the β-cell transcription factors MafA and FoxO1 showed a change of subcellular localization from nuclear to cytoplasmic, while the α-cell factor Arx was in its functional nuclear compartment. Finally, we observed that the presence of these cells with a mixed phenotype was correlated with the presence and extent of islet amyloidosis. We conclude that loss of β-cell identity occurs in T2DM and could contribute to the decreased functional β-cell mass.

As loss of β-cell identity may play a role in T2DM, the preservation of β-cell identity offers new therapeutic options. It is known that the incretin hormone GLP-1 can induce the transcription factor Pax4, is involved in β-cell differentiation and can prevent apoptosis. Overexpression of Pax4 can protect mouse β-cells against streptozotocin induced hyperglycemia. In **Chapter 6** we investigated the effect of GLP-1 receptor agonists (GLP-1RAs) on the maintenance of a β-cell phenotype. We used our model of islet cell reaggregation and β-cell specific lineage tracing in the presence or absence of GLP-1RAs. Stimulation of the GLP-1 receptor prevented loss of human β-cell identity,

as characterized by a higher percentage of insulin$^+$GFP$^+$ out of GFP$^+$ cells after 7 days of reaggregation. Incretin treatment was accompanied by an induction of endogenous Pax4 gene expression. Overexpression of Pax4 in human islet cell aggregates mimicked the protective effects of GLP-1 signaling. These results indicate a novel potential role for incretin-based therapies, targeting the maintenance of mature β-cells, possibly through induction of Pax4 gene expression.

CHAPTER 9

NEDERLANDSE SAMENVATTING

CURRICULUM VITAE

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NEDERLANDSE SAMENVATTING

Diabetes mellitus is wereldwijd een van de grootste oorzaken van ziekte en aan complicaties gerelateerde mortaliteit. Insuline-producerende β-cellen in de eilandjes van Langerhans in de alvleesklier waarborgen een gezonde glucose regulatie. Het verlies van functionele β-cellen staat centraal in de ontwikkeling van zowel type 1 diabetes (T1DM) als type 2 diabetes (T2DM). Voorheen werd aangenomen dat volledig gedifferentieerde cellen (zoals β-cellen) niet meer van fenotype kunnen veranderen. Recente dierproeven lieten zien dat volwassen β-cellen nog van identiteit kunnen wijzigen onder omstandigheden waarin (metabole) stress een rol speelt. Mogelijk is deze verandering van identiteit geassocieerd met de β-cel dysfunctie en het verlies van β-cel massa die beide kenmerkend zijn voor eilandjes van patiënten met type 2 diabetes. Het is bovendien aangetoond dat de overige endocriene eilandjescellen kunnen veranderen in β-cellen, een mogelijke bron van nieuwe β-cellen. Het is niet bekend of humane volwassen β-cellen van identiteit kunnen veranderen en of verlies van β-cel identiteit een rol speelt in de ontwikkeling van diabetes in mensen. Het doel van het onderzoek in dit proefschrift was om de stabiliteit van humane β-cellen te bestuderen en te onderzoeken of verlies van β-cel identiteit een rol speelt in de pathofysiologie van diabetes.

In **hoofdstuk 2** beschrijven we een casus van β-cel vervangingstherapie in een 48-jaar oude patiënt met cystische fibrose en daaraan gerelateerde diabetes, gecompliceerd door een vertraagde maaglediging en recidiverende ernstige hypoglykemie. Zij had reeds 7 jaar eerder een bilaterale longtransplantatie ondergaan en had een slecht gereguleerde diabetes ondanks insulinepomp therapie en continue glucose monitoring. Van twee donor alvleesklieren werden de geïsoleerde eilandjes geïnfundeerd in de vena portae onder lokale verdoving. De procedure verliep ongecompliceerd en de patiënt verliet na drie dagen het ziekenhuis. Na zes maanden werd een mixed meal test verricht waarbij sprake was van uitstekende glucose regulatie zonder enige insulinepomp therapie. Na 1,5 jaar werd een lage dosis langwerkende insuline herstart vanwege postprandiale hyperglykemie, er werd geen hypoglykemie meer gerapporteerd. Met deze casus illustreren we de impact van diabetes en recidiverende hypoglykemie en laten we zien hoe minimaal invasieve β-cel vervangingstherapie een uitkomst kan zijn voor patiënten die lijden aan een slecht gereguleerde diabetes.

Geïsoleerde humane eilandjes van Langerhans zijn heterogene 3-dimensionale mini-organen als het gaat om grootte en vorm. In **hoofdstuk 3** onderzochten we de toepassing van een nieuw microwell celkweeksysteem voor het maken van aggregaten van identieke grootte bestaande uit eilandjescellen. Het kweeksysteem is gebaseerd op een mal gemaakt van polydimethylsiloxaan waarmee agarose chips kunnen worden gemaakt die bestaan uit 2865 microwells met een diameter van 200 μm per microwell. Dit kweekplatform stelde ons in staat om op reproduceerbare wijze aggregaten te maken van insulinoom cellijnen van muis en rat en van gedispergeerde humane eilandjescellen. Aggregaten van menselijke eilandjescellen met een diameter van 100–150 μm lieten weinig apoptose zien en reageerden op glucose op een manier vergelijkbaar met intacte geïsoleerde eilandjes. Na transplantatie van de aggregaten onder het nierkapsel van de muis werd C-peptide

aangetroffen in het serum wat aangeeft dat de β-cellen nog functioneel waren na transplantatie. We concluderen dat het nieuwe microwell kweeksysteem een gemakkelijk te gebruiken instrument is waarmee op reproduceerbare wijze onderzoek kan worden verricht naar (humane) eilandjescellen.

In **hoofdstuk 4** gebruikten we het microwell celkweeksysteem om de stabiliteit van humane β-cellen te bestuderen na reaggregatie van de eilandjescellen. We namen een duidelijke afname waar in het aandeel van insuline-positieve cellen terwijl het percentage glucagon-producerende cellen significant was toegenomen tijdens de 14 dagen reaggregatie. Er was geen verschil in de mate van β-cel apoptose of α-cel proliferatie wat het verschil in compositie van de aggregaten zou kunnen verklaren. Om te onderzoeken waar de afname van β-cellen vandaan kwam gebruikten we vervolgens een β-cel specifiek 'lineage tracing' systeem tijdens de reaggregatie. Deze techniek is gebaseerd op het gebruik van twee lentivirussen waarbij een tamoxifen-induceerbaar Cre-Lox systeem onder controle staat van de rat insuline promoter (RIPCreERT2). Het Cre recombinase kan een stop codon verwijderen in het 2e lentivirale construct (CMV-stop-GFP) wat het mogelijk maakt om Green Fluorescent Protein (GFP) aan te maken. Wanneer tamoxifen aan het systeem wordt toegevoegd zal het Cre recombinase (gebonden aan de oestrogeenreceptor) zich verplaatsen naar de celkern en daar het stop codon verwijderen. Omdat deze activatie afhankelijk is van de insuline promoter zal het alleen in β-cellen plaatsvinden en is GFP-expressie onafhankelijk van toekomstige fenotypische veranderingen. Met dit systeem hebben we aangetoond dat een deel van de GFP-positieve cellen na reaggregatie glucagon bevat, wat betekent dat volwassen humane β-cellen conversie kunnen ondergaan tot glucagon-producerende α-cellen. De conversie van β-cellen naar α-cellen werd voorafgegaan door β-cel degranulatie en werd gekarakteriseerd door de aanwezigheid van β-cel specifieke transcriptiefactoren (Nkx6.1 en Pdx1) in glucagon bevattende cellen. Lentivirus-gemedieerde knockdown van Arx, een α-cel specifieke transcriptiefactor, verhinderde de β-cel conversie. Alles tezamen laat deze studie voor het eerst zien dat humane volwassen β-cellen hun identiteit kunnen verliezen en kunnen veranderen in andere hormoonproducerende eilandjescellen.

Aangezien onze bevindingen in hoofdstuk 4 waren beperkt tot een specifieke experimentele opzet, wilden we in **hoofdstuk 5** de relevantie hiervan onderzoeken in de context van diabetes**.** Onze hypothese was dat verlies van β-cel identiteit in diabetes zou worden gekenmerkt door de aanwezigheid van β-cel transcriptiefactoren (Nkx6.1, Pdx1) in glucagon-positieve cellen, vergelijkbaar met de Nkx6.1^{+}glucagon^{+} cellen die we beschreven na reaggregatie in hoofdstuk 4. Hiertoe onderzochten we biopten van donor pancreata van patiënten met T2DM en controles zonder diabetes die overeenkwamen qua leeftijd en BMI. We observeerden een acht keer toegenomen percentage van insuline-positieve cellen die tevens glucagon tot expressie brachten (bihormonale cellen) in donoren met T2DM. Bovendien bracht tot 5% van de cellen die Nkx6.1-positief waren, glucagon in plaats van insuline tot expressie. Dit percentage was vijf keer hoger vergeleken met de controle groep. We bevestigden deze bevindingen in eilandjes van een geselecteerde groep makaken, die in het verleden uitgebreid metabool

gekarakteriseerd zijn en diabetes ontwikkelen op een manier vergelijkbaar met ontwikkeling van diabetes bij mensen. In humane donoren van vonden we dat in bihormonale cellen de β-cel transcriptiefactoren MafA en FoxO1 waren veranderd van subcellulaire lokalisatie, van de celkern naar het cytoplasma, terwijl de α-cel factor Arx in de celkern aanwezig was. Tot slot konden we aantonen dat de aanwezigheid van cellen met een gemengd fenotype in een eilandje was gecorreleerd aan de aanwezigheid en mate van amyloidose in die eilandjes. We concluderen dat verlies van β-cel identiteit voorkomt in T2DM en dat het zou kunnen bijdragen aan het verlies van functionele β-cel massa in T2DM.

Indien verlies van β-cel identiteit een rol speelt in T2DM, dan biedt het behouden van β-cel identiteit nieuwe therapeutische opties. Het is bekend dat het incretine hormoon GLP-1 de β-cel transcriptiefactor Pax4 kan induceren en is betrokken in β-cel differentiatie en het voorkomen van apoptose. Verhoogde expressie van Pax4 kan β-cellen van muizen beschermen tegen streptozotocine geïnduceerde hyperglykemie. In **hoofdstuk 6** onderzochten we het effect van GLP-1 receptor agonisten (GLP-1Ras) op het behoud van het β-cel fenotype. We gebruikten ons model van reaggregatie van eilandjescellen tezamen met β-cel specifieke lineage tracing, ditmaal in de aan- of afwezigheid van GLP1-RAs. Stimulatie van de GLP-1 receptor voorkwam verlies van β-cel identiteit, gedefinieerd als een hoger percentage overgebleven insulin^{+}GFP^{+} cellen van alle GFP^{+} cellen na 7 dagen reaggregatie. Na de incretine behandeling was er tevens een verhoogde genexpressie van Pax4. Genetische overexpressie van Pax4 in humane eilandjescellen liet eenzelfde beschermend effect zien als na GLP-1RA behandeling. Deze resultaten laten een nieuwe mogelijke rol zien voor incretine-gebaseerde therapie, mogelijk gereguleerd via Pax4, waarbij de focus ligt op het behoud van mature β-cellen.

CURRICULUM VITAE

The author of this thesis, Harm Siebe Spijker, was born on the 10th of April 1984 in Hoek van Holland, The Netherlands. He completed secondary school in 2002 at the Gereformeerde Scholengemeenschap Randstad in Rotterdam. He studied Medicine at the Leiden University Medical Center, The Netherlands, and obtained his medical degree in 2009. In 2005, he started a simultaneous premaster program Biomedical Sciences, and conducted research in the department of pathology on the expression of the TGF-β signalling molecules Smad2 and Smad4 in cervical cancer under supervision of dr. J.N. Kloth and dr. A. Gorter. He performed a first master's research project in the department of Molecular Cell Biology on TGF-β/ALK1 induced activation of endothelial cells under supervision of dr. A. Gorter and prof. dr. P. ten Dijke. For his research on TGF-β signalling, he was nominated for the LUMC Student Research Award (2008) and the Leiden University Research Award (2009). A second master's project was performed at the department of Nephrology on the differentiation of pancreatic ductal cells under supervision of prof. dr. E.J.P. de Koning. He received his master's degree in Biomedical Sciences in 2010 (*cum laude*).

He started his PhD program, of which the results are described in this Thesis, in 2010 at the department of Nephrology under supervision of prof. dr. E.J.P. de Koning, prof. dr. A.J. Rabelink and dr. F. Carlotti. During this period he was awarded the NVE-Ipsen award for best basic science article in 2013 from the Dutch Society for Endocrinology. In 2014, he started his medical specialisation as internist at the Groene Hart Ziekenhuis in Gouda which he currently continues at the Leiden University Medical Center.

DANKWOORD

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