Determining the Mechanisms Underlying Islet Dysfunction and Clinical Heterogeneity in Type 1 Diabetes (T1D)

By

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DEDICATION

To my father,
whose story motivates me to bridge the gap
between research and medicine
and who always taught me to question and persevere

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LIST OF ABBREVIATIONS

ADI	Alberta Diabetes Institute IsletCore
AHN	Allegheny Health Network
ANOVA	Analysis of variance
ARX	Aristaless-related homeobox
AUC	Area under the curve
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
Cav1	Caveolin-1
CD31	Cluster of differentiation 31, endothelial cell marker
CGM	Continuous glucose monitor
ColIV	Collagen IV
Dapi	4’6-diamidino-2-phenylindole
DiViD	Diabetes Virus Detection study
DPT-1	Diabetes Prevention Trial-Type 1
ECM	Extracellular matrix
EC	Endothelial cell
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum
ESC	Embryonic stem cell
Ex4	Exendin-4
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FOXA2	Forkhead box A2
FPIR	First-phase insulin response
GATA4	GATA binding protein 4
GATA6	GATA binding protein 6
GCG	Glucagon
GCK	Glucokinase
GFP	Green fluorescent protein
GLP-1	Glucagon-like peptide 1
GLUT	Glucose transporter
GSIS	Glucose-stimulated insulin secretion
HbA1C	Glycated hemaglobin
HIRN	Human islet research network
HNF1A	Hepatic nuclear factor 1 alpha
HNF4A	Hepatic nuclear factor 4 alpha
IAPP	Islet amyloid polypeptide

Iba1	Ionized calcium-binding adaptor molecule 1, macrophage marker
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IIAM	International Institute for the Advancement of Medicine
IIDP	Integrated Islet Distribution Program
INS	Insulin
iPSC	Induced pluripotent stem cells
IVGTT	Intravenous glucose tolerance test
MAFA	V-maf musculoaponeurotic fibrosarcoma oncogene homolog A
MAFB	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B
MMTT	Mixed meal tolerance test
MODY	Maturity-onset diabetes of the young
ND	Normal Donors
NDRI	National Disease Research Interchange
NEUROG3	Neurogenin 3
NKX2.2	Nirenberg and Kim 2 homeobox 2
NKX6.1	Nirenberg and Kim 6 homeobox 1
NOD	Non-obese diabetic mouse model
nPOD	Network for Pancreatic Organ Donors with Diabetes
NSG	NOD-*scid*-*IL2rγ*^{null}
NSG-DTR	*Nod*-*SCID*-*IL2Rγ*^{null}; Rat Insulin Promoter-Human Diphtheria Toxin Receptor$^{tg/tg}$
OGTT	Oral glucose tolerance test
PAX6	Paired box 6
PDX1	Pancreatic and duodenal homeobox 1
PP	Pancreatic polypeptide
RFX6	Regulatory Factor X 6
RNA-Seq	RNA-Sequencing
RPKM	Reads per kilobase per million mapped reads
RT-PCR	Quantitative reverse transcription polymerase chain reaction
SOC	Store-operated channel
SOX9	SRY (sex-determining region Y)-box 9
STIM	Stromal interaction molecule
SUR	Sulfonylurea receptor
TX	Transplant
T1D	Type 1 diabetes
T2D	Type 2 diabetes
VDCC	Voltage-dependent calcium channels
VEGFR2	Vascular endothelial growth factor receptor 2

CHAPTER I

BACKGROUND AND SIGNIFICANCE

Pancreatic Islets of Langerhans

Anatomy

The pancreas is a mixed organ of endocrine and exocrine tissues. Exocrine tissue makes up nearly 98% of the organ mass and is responsible for the production of digestive enzymes such as proteases, amylases, lipases, and nucleases necessary for the breakdown of macromolecules in food[1]. Acinar cells produce and release these enzymes into a ductal system that empties into the duodenum of the small intestine during digestion (**Figure 1**).

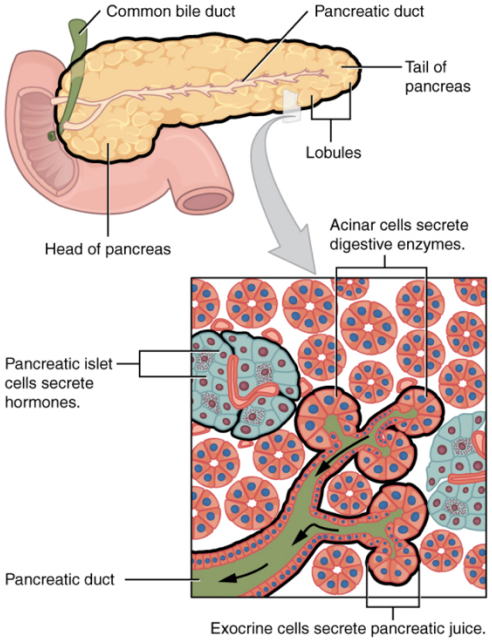


Figure 1. Anatomy of the pancreas. The pancreas is a mixed endocrine and exocrine organ. Exocrine function is to secrete digestive enzymes into the small intestine via the pancreatic duct while endocrine function involves the secretion of hormones from the pancreatic islet. Image adapted from OpenStax Anatomy & Physiology (https://cnx.org).

Interspersed in the exocrine tissue are cell clusters known as the islets of Langerhans that secrete endocrine hormones necessary for the regulation of blood glucose (**Figure 1**)[2,3]. There are five primary cell types that make up the islet, and unlike exocrine cells, they secrete hormones directly into the bloodstream. β cells secrete insulin in response to increases in blood glucose to signal to the muscle, liver and fat to uptake glucose for utilization. In contrast, α cells counter the actions of insulin by secreting glucagon, which signals to the liver to increase glucose production through glycogenolysis or gluconeogenesis. The somatostatin-producing δ cells are important paracrine regulators of both α and β cells. Even rarer are the PP cells which secrete pancreatic polypeptide and ε cells that secrete ghrelin. The pancreatic islet coordinates hormone secretion with the rest of the body by receiving a rich vascular supply, up to 20% of the total pancreatic blood source,[1,4-7] and extensive innervation by cholinergic and adrenergic nerve branches[8-10] allowing rapid, accurate sensing of blood glucose and insulin delivery to the peripheral circulation.

Islet development and islet-enriched transcription factors

Both the endocrine and exocrine compartment, though distinct in terminal cell function, are derived from common progenitors residing in the embryonic foregut endoderm. Much of our understanding of pancreatic development comes from studying the developing mouse embryo and transgenic mouse models with recent advances from characterizing human pancreatic development and endocrine cell differentiation of human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs).

Development of the human pancreas is first evident at gestational day 26 (G26d) and begins with fusion of the ventral and dorsal bud upon gut rotation. The majority of the pancreas arises from the dorsal bud to form the three regions of the pancreas known as the head, body and tail[11]. By G33d, the human pancreatic buds are composed of stratified epithelium containing multipotent pancreatic progenitor cells (MPCs) expressing transcription factors pancreas and duodenum homeobox 1 (PDX1), forkhead box A2 (FOXA2), GATA transcription factor 4 (GATA4), GATA transcription factor 6 (GATA6), SRY (sex-determining region Y)-box 9 (SOX9), and Nirenberg and Kim homeobox 6.1 (NKX6.1)[12-15]. In mouse, tip-trunk compartmentalization of the pancreatic epithelium defines the regions that eventually give rise to endocrine cells (i.e. trunk) and acinar cells (i.e. tip)[16,17]. The compartmentalization of pancreatic epithelium in human has not yet been well defined.

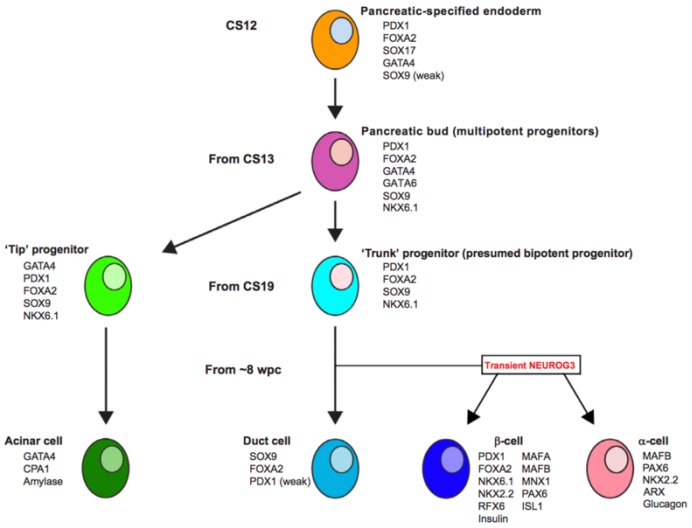


Figure 2. Expression of islet-enriched transcription factors during human pancreas development. Timely expression of transcription factors and other key markers identify different stages of pancreatic islet development during specification and subsequent lineage commitment. These factors were identified using immunohistochemical analysis of the developing human pancreas. CS refers to the Carnegie Stages of development. 8wpc: 8 weeks post conception. Image adapted from Jennings et al., 2015.

Islet cell differentiation is a complex series of cell specification events regulated by the dynamic and successive expression of transcription factors (**Figure 2**)[11,13]. Expression of Neurogenin 3 (NEUROG3) defines the endocrine progenitor cell population[11,18]. Signals from the vasculature and innervation during development are also important in establishing pancreatic islet architecture[10,19-23]. Insulin-producing cells are the first endocrine cells detectable in the human pancreas and make up the majority of the

endocrine cells at birth after which post-natal development continues to redefine and organize islet cells into the adult pancreatic islet[12,13].

Transcription factors and their appropriate co-regulators regulate gene expression in response to environmental cues through binding to specific enhancer sequences and regulatory elements within DNA. In the adult islet, cell-specific expression of many transcriptional regulators found in early pancreatic progenitor cells are maintained and form a regulatory network necessary for maintenance of mature islet cell identity and function[24,25]. In humans, adult β cells are defined by a transcription factor profile of PDX1[26,27], NKX6.1[28-30], SIX3[31] and MAFA[32-34] while mature α cells selectively express ARX[35,36], IRX2[37,38], and MAFB[39-41]. Notably, some human β cells also express low levels of MAFB[41,42]. Pan-endocrine markers shared by α and β cells are NKX2-2[43,44], RFX6[45-47], PAX6[48-50], HNF1A[51-53], and HNF4A[54] among others.

Loss of or changes to this transcription factor profile can have important implications in disease. Indeed, metabolic stress can impact this tightly regulated system[40,55,56], variants associated in these transcription factors have been identified in genome-wide association studies for Type 2 diabetes (T2D)[57], and pathogenic variants in many of these genes are responsible for monogenic forms of diabetes[25].

Glucose homeostasis and diabetes

Glucose homeostasis refers to the maintenance of blood glucose levels in a normal range despite internal and external changes (**Figure 3**). The secretion of insulin and glucagon from the endocrine compartment of the pancreas is central to this balance. Islet endocrine cells sense and integrate signals from blood glucose levels, hormones, neurotransmitters, and other nutrients like amino acids and these regulate hormone secretion. The pancreatic islet works in coordination with other organ systems, such as the brain, liver, and muscle to regulate blood glucose levels. For example, the autonomic nervous system integrates internal and external environmental signals to

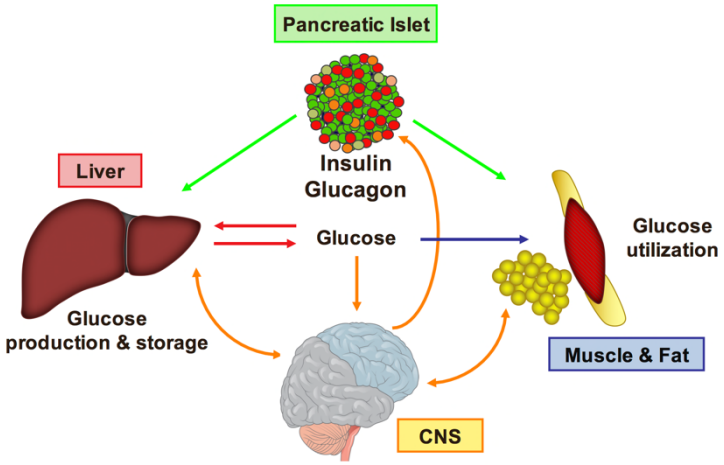


Figure 3. Pancreatic islet is central to glucose homeostasis. Glucose homeostasis is regulated by coordinated effort between multiple organ systems in the body. Defects in the pancreatic islet result in most forms of diabetes.

regulate pancreatic islet hormone release. Glucose-sensing neurons of the autonomic nervous system located at distinct anatomical sites, mainly the brainstem and hypothalamus, are activated by changing levels of blood glucose and signal to organs involved in glucose homeostasis like the liver, muscle, fat tissue, and the endocrine cells of the pancreas[58].

Diabetes results from either destruction or dysfunction of the β cells leading to an inability to maintain a normal range of blood glucose. Diabetes affects more than 400 million people worldwide with an estimated increase to 500 million by 2030[59]. The social and economic burden of diabetes is substantial within the U.S. costing more than $245 billion a year and responsible for the seventh leading cause of death (Center for Disease Control 2017). While often thought of as a single disorder, diabetes is increasingly recognized as a heterogeneous condition.

The most common form of diabetes (~90-95% of all cases) is type 2 diabetes (T2D), which can be complex and heterogeneous in presentation. In very early T2D, glucose tolerance remains nearly normal because increased insulin secretion from the β cell compensates for insulin resistance; however, as disease progresses the pancreatic islet cannot sustain this high insulin demand and impaired glucose tolerance develops. T2D results from impaired insulin secretion and action in the peripheral tissues, increased glucagon levels and hepatic glucose production, and abnormal fat metabolism. Genetics and environmental factors, such as a sedentary lifestyle and excessive sugar and fat consumption that lead to obesity and visceral adiposity, contribute to the development and pathogenesis of T2D[60,61]. Genome-wide association studies have identified common variants that predispose to type 2 but account for less than 10% of the overall estimated genetic contribution[57]. Furthermore, ethnicity can impact an individual's risk of developing T2D with onset occurring on average at an earlier age in certain ethnic groups (Asian, African, and Latin American) likely due to poorly understood differences in pathophysiology[61]. Depending on the extent of β cell dysfunction and/or insulin resistance, patients with T2D can control their blood glucose levels with a combination of therapeutic options and lifestyle changes (ex. weight reduction). Oral glucose-lowering therapies such as biguanides (metformin), which reduce hepatic glucose production and enhance peripheral tissue sensitivity, can be effective as monotherapy early in disease or in combination with drugs that act to stimulate β cell insulin secretion such as sulfonylureas (glyburide, glimepiride) and glucagon-like peptide-1 (GLP-1) receptor agonists (exenatide, liraglutide)[62] or decrease blood glucose by preventing intestinal absorption of glucose (α-glucosidase inhibitors) or increasing glucose urinary excretion (sodium glucose transporter-2 (SGLT-2) inhibitors)[63]. However, if lifestyle modifications and oral medications fail to meet glycemic targets exogenous insulin therapy is indicated[64].

Type 1 diabetes (T1D) contributes to 5% of cases and is caused by an autoimmune destruction of pancreatic β cells leaving individuals insulin-deficient. These patients require exogenous insulin by injection or a pump to survive. Pathophysiology and complications of T1D will be discussed further in section **Type 1 Diabetes**. Less commonly, diabetes can result from genetic mutations that result in impaired insulin production, such as neonatal diabetes mellitus or maturity-onset diabetes of the young or even be secondary to other medical conditions such as surgery, medication, infection, cystic fibrosis, pancreatitis, etc.

In an individual without diabetes, plasma glucose concentrations are normally maintained within a relatively narrow range (60 – 160 mg/dL) despite fluctuations in the supply and demand of glucose. This ensures a continuous supply of glucose to the brain, which is essential for function as the brain is unable to store glucose. Individuals with diabetes experience highs and lows of blood glucose outside of this normal range that can have deleterious effects. The effects of high blood glucose, i.e. hyperglycemia, have been correlated with increased risk for diabetes associated co-morbidities such as retinopathy, autonomic neuropathy, and nephropathy. In turn, the inability to recover from hypoglycemia, i.e. low blood glucose, has acute effects such as impaired cognitive functions, adrenergic symptoms, lethargy, seizures, and if not corrected, permanent damage/death.

Similarities and differences between rodent and human islets

Our understanding of pancreatic islet function comes primarily from mouse models that provide insight into islet development, genetics and physiology. However, studies using rodent model systems have some limitations in translation to human due to considerable physiological differences in human and mouse pancreatic biology and response to disease [2,3,9,42,65,66]. For example, islet composition and organization differs between mouse and human islets. In the mouse, β cells exist primarily at the core of the islet and are mantled by α and δ cells. In contrast, β cells in adult human islets are found intermingled with α cells and δ cells resulting in increased contacts between these cell types[2,3,67]. Regarding composition, β cells can account for 28-75% of the islet endocrine cells in humans unlike in rodent islets where there is little variability and β cells make up 61-81% of the endocrine cells (**Figure 4A** and **4C**). Importantly, α cells contribute more significantly to the endocrine compartment in humans making up anywhere from 10-65% of the islet in contrast to rodents (9-31%) (**Figure 4**)[2,3]. Furthermore, differences in the islet micro-environment exist between the two species. Unlike in mouse islets where nerve branches extend into the islet and make direct contacts to endocrine cells, human islets are sparsely innervated and instead

innervation contacts smooth muscle cells of the vasculature suggesting an indirect mechanism of autonomic regulation via changes in blood flow[9,10,68]. Similarly, the vasculature of human islets is also different where human islets exhibit fewer blood vessels than the mouse islet[65].

Moreover, distinctions from mouse in gene expression and electrical signaling result in differences in human islet physiology. For example, in contrast to mouse, human islets secrete insulin at glucose concentrations as low as 3 mM, which likely correlates with lower fasting glucose levels in humans[42,69,70]. Human β cells primarily express the facilitated diffusion glucose transporter GLUT1 (*SLC2A1*), which has a lower K_m than the mouse β cell low-affinity GLUT2 (*Slc2a2*), and have differences in voltage-gated ion channel expression allowing insulin secretion at lower glucose concentrations[71-74]. Furthermore, β cell heterogeneity of gene expression has been described in both humans[75-77] and mice[78] but interestingly does not overlap and likely has functional implications [79,80].

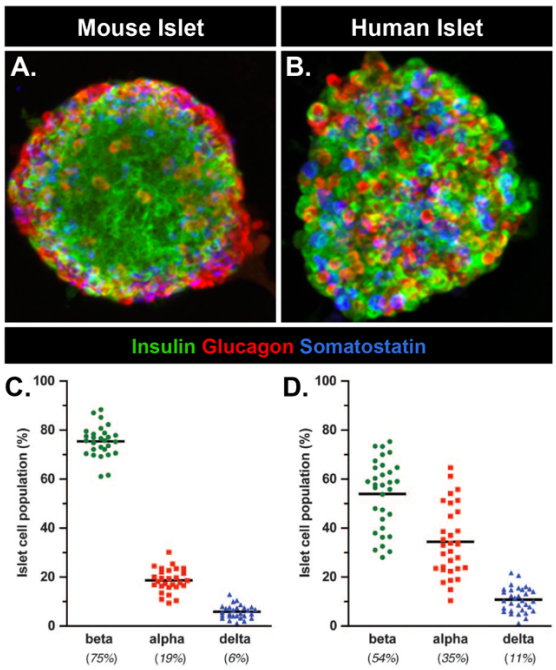


Figure 4. Islet composition and morphology varies between human and mouse. (**A**) Mouse and (**B**) human islets labeled for insulin (green), glucagon (red), and somatostatin (blue). Endocrine composition of (**C**) mouse islets, n=28, and (**D**) human islets, n=32, determined by analysis of optical sections taken throughout entire islets. Human islet composition differed significantly ($p<0.0001$) across all endocrine cell populations examined. Horizontal bar represents the mean of each cell population. Image adapted from Brissova et al., 2005.

Though less understood, reported disparities between mouse and human α cells suggest important species-specific differences in α cell biology as well. While near ablation (98%) of rodent α cells had little to no effect on overall mouse glucose homeostasis [81], the increased proportion of α cells in human islets indicates *both* β and α cell function are essential to glucose homeostasis in man. Morphologically, the higher β:α cell ratio in humans allows increased β-to-α cell contacts, suggesting paracrine signaling between these cell types are important in islet function, and human α cells are also uniquely arranged in close contact to islet vasculature[67]. Advances in transcriptomic analysis of human α cells from single cell and bulk RNA-sequencing reveal differences in α cell heterogeneity, proliferative capacity, and gene expression that were previously unknown and begin to provide insight into human α cell function[38,82-85].

Pancreatic β and α cell function

Cell-specific transcription factors regulate a set of genes in pancreatic β cells and α cells to allow reciprocal hormone release and coordinated regulation of blood glucose (**Figure 5**)[86]. The β cell secretes insulin in response to elevation of

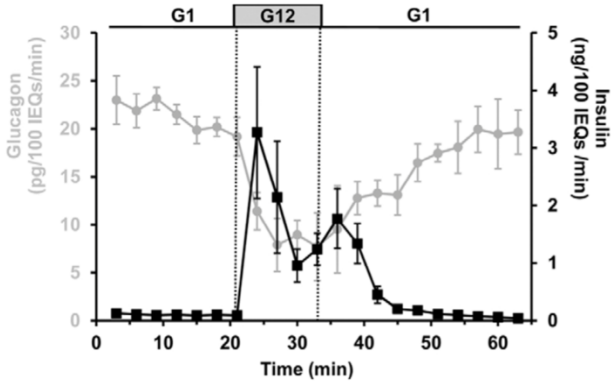


Figure 5. Glucose has reciprocal effects on insulin and glucagon hormone secretion from perifused mouse islets. Isolated islets were exposed to low glucose (1mM; G1) for 30 minutes prior to this experiment. Insulin (black) and glucagon (gray) responses were measured in response to low glucose (G1) followed by high glucose (12 mM; G12) and then back to low glucose (G1). The experiment was repeated three times with 450 islets from 6 mice. Error bars represent the standard error of the mean. All hormone release was measured per islet equivalent (IEQ), which normalizes for islet size. Figure from Marchand et al., 2012.

blood glucose. Glucose enters β cells through glucose transporters, immediately undergoes phosphorylation by glucokinase, and enters oxidative metabolism resulting in elevated ATP levels. This increase in the β cell ATP-to-ADP ratio results in the closure of ATP-sensitive K^+ channels, made up of pore forming Kir6.x subunits and four sulfonylurea receptor subunits (SUR1), and membrane depolarization (V_m). Sufficient V_m depolarization triggers calcium entry through voltage dependent calcium channels (VDCCs, primarily L-type channels)[87]. Elevated intracellular calcium levels interact with exocytotic machinery of the insulin granules leading to vesicle fusion with the cell membrane and granule exocytosis (**Figure 6B**).

While fasting conditions inhibit insulin secretion (**Figure 6A**), glucagon is secreted from the α cell in response to hypoglycemia and adrenergic stimulation and to increased levels of amino acids. Although low glucose stimulation of α cell glucagon secretion is well established, the mechanisms underlying glucose-regulated glucagon secretion are less clear. Similar to β cells, α cells share expression of glucose sensing and secretory machinery important to hormone secretion[82,88]; however, important differences in their utilization of this machinery impact function (**Figure 6**)[89]. For example, α cell glucagon secretion appears to be highly dependent on high-voltage activated P/Q-type VDCCs. In addition, Na^+ channels participate more in the upstroke of the α cell action potential compared to β cells. Finally, whether α cells can intrinsically sense changes in glucose[88,90] or whether they rely primarily on paracrine signaling[91] is still controversial.

Because there is conflicting evidence for the effects of glucose on membrane potential in the α cells, there are two prevailing models of α cell glucagon secretion. Both models postulate α cells are able to intrinsically sense changes in energy status through ATP levels. One model suggests that α cells share β cell K_{ATP}-dependent membrane depolarization in high glucose, but that this depolarization inactivates the critical Na^+-voltage gated channels and P/Q-type Ca^{2+} channels needed for glucagon secretion[92] (**Figure 6D**). In low glucose, the K_{ATP} currents are intact permitting the potential needed for Na^+ channel-facilitated action potentials and P/Q Type VDCC opening[92-94] (**Figure 6C**). In contrast, the second model advocates for a depolarizing effect of low glucose. Low ATP levels within the cell lead to depleted endoplasmic reticulum (ER) Ca^{2+} stores and activation of store-operated channels (SOC). Activation of SOC occurs when ER-resident Ca^{2+} sensor stromal interaction molecule (STIM) proteins interact with and open plasma membrane ORAI Ca^{2+} channels producing a depolarizing current. This mechanism of membrane depolarization activates the necessary channels to result in an action potential[95-98]. Evidence for α cell heterogeneity suggests that the secretory mechanisms may not be identical in all α cells contributing to two prevailing theories.

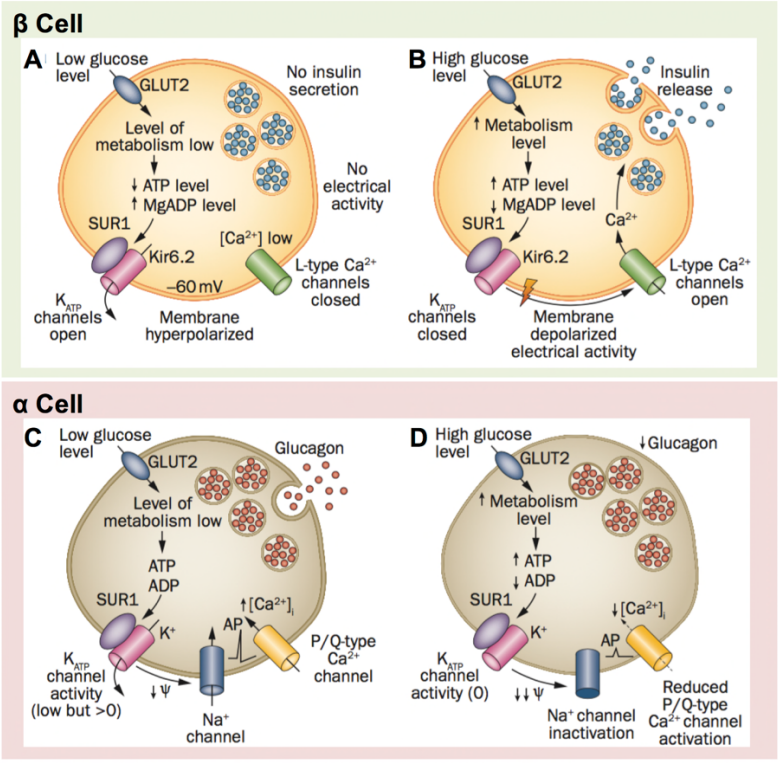


Figure 6. K_{ATP} modulation of hormone release in β and α cells. Insulin secretion from β cells is inhibited in (**A**) low glucose and stimulated in (**B**) high glucose. Only in cases of high glucose does glucose metabolism increase the ratio of ATP to ADP in the cell leading to closure of the KATP channel, membrane depolarization, and opening of L-type calcium channels allowing for insulin exocytosis as depicted in **B**. Conversely, in the α cell, glucagon secretion is stimulated in low glucose (**C**) and inhibited in high glucose (**D**). Because β and α cells share similar machinery, this model postulates open KATP channel activity during low glucose keeps the membrane depolarized such that the appropriate Na channels are open producing an action potential to open P/Q-type calcium channels necessary for glucagon release. High glucose then would inhibit KATP channels similar to the β cell, but this depolarizes the membrane beyond the optimal potential for Na channel activation, limiting an action potential resulting in low opening of the P/Q-type calcium channels. Figure adapted from Ashcroft and Rorsman 2013.

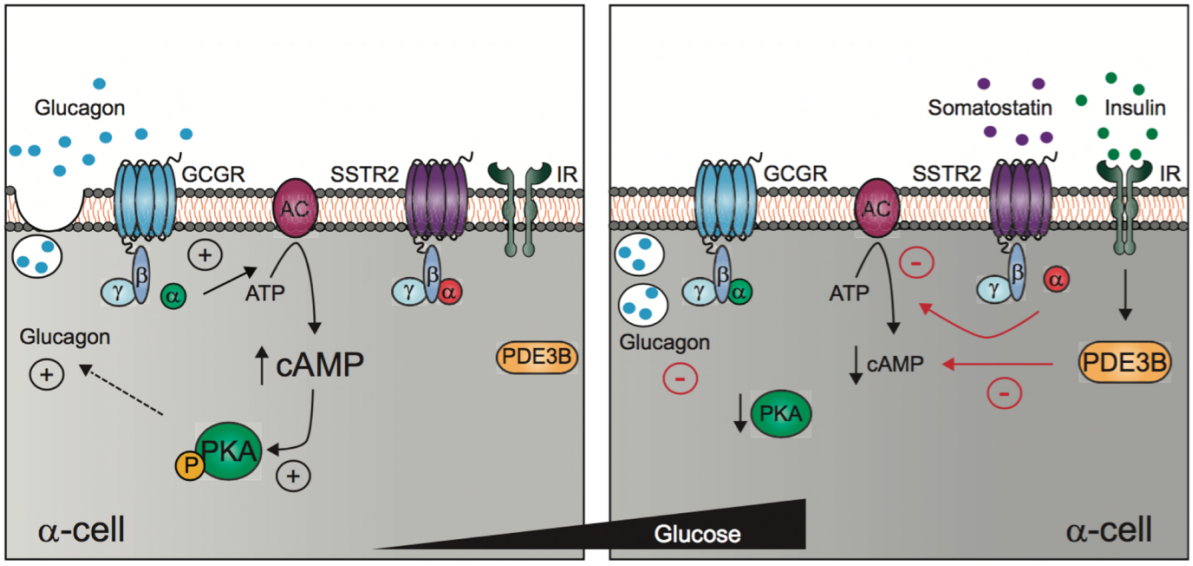


Figure 7. Schematic of paracrine regulators of α cell secretion by somatostatin and insulin at high and low glucose. Under low glucose conditions, glucagon stimulates α cell glucagon release in an autocrine fashion through cAMP-mediated mechanisms. However, under high glucose conditions, the inhibitory effects of somatostatin and insulin can lower cAMP signaling by decreasing PKA activity. PKA: protein Kinase A; GCGR: glucagon receptor; P: phosphorylated PKA; PDE3B: phosphodiesterase 3B; AC: adenylyl cyclase; SSTR2: somatostatin Receptor 2; IR: insulin receptor; cAMP: cyclic adenosine monophosphate. Figure from Elliott et al., 2015.

Other factors are also likely important in regulating α cell glucagon release. Autonomic innervation of the islet via parasympathetic and sympathetic nerve fibers can release neurotransmitters that inhibit or stimulate glucagon secretion. In addition, paracrine signaling by factors secreted from both β and δ cells have been implicated in modulating α cell glucagon secretion and likely play a prominent role in regulating α cell function (**Figure 7**)[91,99].

Type 1 Diabetes (T1D)

Epidemiology, diagnosis, and treatment

Type 1 diabetes (T1D) is a chronic illness resulting from an autoimmune process that produces autoantibodies to β cell specific markers and selective destruction of insulin-producing β cells thought to be mediated primarily by cytotoxic T cells. Both genetic and environmental factors play a role in disease initiation and progression. T1D affects approximately 1.25 million individuals in the United States. The current prevalence of T1D in U.S. youth is 2.13/1000 following an increase by 21.1% from 2001 to 2009 and is projected to increase 144% by the year 2050[100].

T1D usually presents in childhood or adolescence with classic signs and symptoms of hyperglycemia i.e. polyuria, polydipsia, and weight loss. Diabetic ketoacidosis, hyperglycemia and ketoacidosis, more commonly presents in younger children (under 6 years of age) with similar but more severe symptoms including neurologic findings such as drowsiness and lethargy. Diagnostic criteria for all forms of diabetes mellitus is based upon one of the four parameters of abnormal glucose metabolism: fasting plasma glucose ≥126 mg/dL more than once, random venous plasma glucose ≥200 mg/dL in a patient with symptoms of hyperglycemia, plasma glucose ≥200 mg/dL two hours after an oral glucose tolerance test (OGTT), and glycated hemoglobin (HbA1C) ≥6.5%[60]. There is no diagnostic test to consistently distinguish between type 1 and type 2 diabetes, so clinical characteristics like body habitus (non-obese), age of onset (childhood/adolescence), insulin sensitivity, and family history of T1D in conjunction with testing for the presence of autoantibodies and C-peptide levels can be helpful in confirming T1D[60]. Despite these guidelines, nearly 30% of patients may not clearly fit typical criteria and require further testing[101].

Normally, insulin is released by β cells in a pulsatile fashion in response to food intake. Individuals with T1D require physiological replacement of insulin and mostly rely on either biosynthetic human insulin (e.g. neutral protamine hagedorn (NPH), regular) or analogs of insulin such as glargine or lispro. Because human insulin pharmacokinetics do not replicate endogenous basal and postprandial insulin secretion, insulin analogs are often preferred for insulin-deficient individuals with T1D. Generally, someone with T1D will be placed on an “intensive insulin therapy” designed to achieve near-normal glycemia (blood glucose)[102]. In this regimen, the individual is prescribed both rapid-acting insulin analogs, which are faster onset and shorter duration to replicate insulin response to a meal, and long-acting insulin analogs, which have a longer time course for basal coverage. Because insulin is injected subcutaneously i.e. into the peripheral circulation and not into the portal vein, insulin analogs are modified to either favor or reduce native hexamer formation allowing modulation of absorption, onset, and duration of action relevant to the type of analog (i.e. rapid-acting or long-acting) [103].

Management of T1D by intensive insulin therapy can often be complicated with the competing risk of hypoglycemia. To avoid this, technological advances in insulin therapy have provided improved therapeutic options for T1Ds. Optimal glycemic control is dependent on frequent monitoring of blood glucose[104,105]. Historically, patients manually measure blood glucose, but the development of subcutaneous glucose sensors, which continuously monitor glucose levels within the interstitial fluid, provide real-time self blood glucose supervision. Better control can be achieved when continuous glucose monitors (CGM) are used in conjunction with insulin pumps, which provide a continuous subcutaneous insulin infusion of a rapid-acting insulin at a basal rate (~0.5-1 U/hour)

and boluses as needed with meals. Calibration and close monitoring are required for most CGMs and insulin pumps; however, true “closed-loop” systems are currently being implemented, which will automate the communication between the CGM and pump and serve as an “artificial pancreas” [106-109]. Interestingly, testing of a bi-hormonal pancreas (i.e. delivery of both insulin and glucagon) is also under development with evidence for improved blood glucose control[110,111]. Finally, islet transplantation has proven efficacy in T1Ds with recurrent life-threatening hypoglycemia but is limited by immunosuppressive drugs, poor long-term efficacy and donor islet availability[112-116]. Advances in cell replacement therapies and microencapsulation strategies provide promise for cell therapy without immunosuppression[117-119].

Although these innovations in glucose monitoring and insulin administration have improved outcomes, current therapeutic interventions still do not accurately restore glucose homeostasis. Accordingly, exogenous insulin therapy does not entirely eliminate diabetes associated co-morbidities including cardiovascular disease, retinopathy, nephropathy, and neuropathy and is complicated by an increased risk of hypoglycemia in individuals with T1D. Further study of the mechanisms behind islet dysfunction and pathophysiology in T1D will help improve existing therapy and develop targeted therapeutic strategies.

Emerging concepts in the pathophysiology of T1D

In a paradigm developed by the late George S. Eisenbarth, T1D is thought to be a chronic progressive autoimmune disease that occurs in stages and results in “total” diabetes with complete β cell loss (**Figure 8**)[120]. Notably, the rate of progression from onset of β cell autoimmunity to glucose intolerance and symptomatic disease is variable and could last anywhere from months to decades[121]. Under this model, it was hypothesized that immune intervention administered in this time period either at onset or prior to onset could prevent or delay clinical disease. Studies conducted as early as the 1980s have attempted to do this with immunomodulatory drugs like anti-thymocyte globulin, anti-CD5 immunotoxin, and steroids[122]. Many have also championed antigen-based therapies would be a safe and specific way to provide immune modulation in T1Ds[122] though most of these trials failed to show a beneficial effect. For example, the hypothesis that prophylactic insulin use could prevent disease was tested in the Diabetes Prevention Trial-Type 1 (DPT-1). DPT-1, which began in 1993, was a large multicenter, randomized-controlled clinical trials to determine whether daily doses of parenteral or oral insulin cold delay or prevent clinical T1D. Even though these trials were also therapeutically unsuccessful[123], the trial demonstrated the ability to perform studies on large-scale cohorts and that diabetes risk in relatives of individuals with T1D could be predicted with genetic evaluation and autoantibody testing.

This set the stage for Type 1 Diabetes TrialNet, which evolved from DPT-1, and is an international consortium of clinical research centers aimed at continuing to identify ways to prevent or delay T1D[124]. TrialNet enables screening of at-risk individuals (i.e. 15,000 children and young adults who are first- or second-degree relatives of individuals with T1D) allowing continued monitoring to understand disease progression, testing therapies for prevention of T1D, and interventions to decrease β cell loss[125]. Data emerging from TrialNet demonstrates that individuals with two or more autoantibodies eventually develop T1D[126,127] allowing islet autoantibody screening as an opportunity to identify individuals who would best benefit from intervention. Recognizing the disease begins prior to its symptomatic manifestations has had a profound impact on how we clinically diagnose T1D[128]. Furthermore, while T1D is considered a disease of children and adolescents, recent data report adults are as likely to develop T1D as children with more than 40% of T1D cases occurring after the age of 30 years[129]. The stages of this revised paradigm are briefly summarized below:

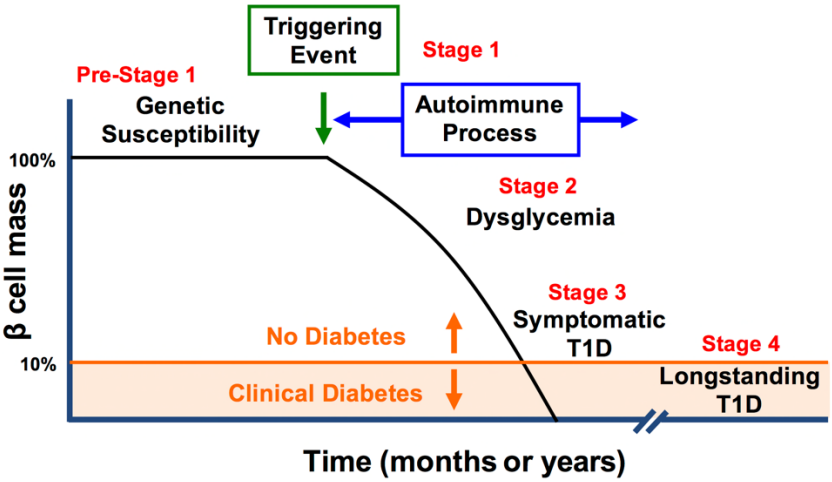


Figure 8. Stages in the development of type 1 diabetes (T1D). As described by George Eisenbarth in 1986, it is thought the pathophysiology of T1D occurs in successive stages that can be described in relation to hypothetical β cell mass plotted against time from birth. Adapted from Eisenbarth 1986 and Insel et al., 2015.

Pre-Stage 1: Genetic Susceptibility and Risk of T1D

GWAS studies of T1D revealed the majority of disease-associated loci are associated with immune regulation[130]. Susceptibility loci within the human leukocyte antigen (HLA) region, such as DRB1 0401, DRB1 0402, DRB1 0405, DQA1 0301, DQB1 0302 or DQB1 0201 alleles, have been reproducibly linked to T1D and are most common in Europeans or those with European ancestry[131,132]. The highest risk for T1D is the heterozygous DR3/4 genotype. Interestingly HLA class II DRB1 1501 and DQA1 0102-DQB1 0602 confer disease resistance[128]. In addition, risk loci in approximately 50 non-HLA genes such as *INS, CTLA4, PTPN22,* and *IL2RA* have also been identified and associated with T1D[133]. Many of these non-HLA genes also contribute to susceptibility to other autoimmune diseases[130].

To determine avenues of prevention in the pre-symptomatic stage of disease, genetic risk scores have the potential of using genetic information to accurately predict the development of T1D[134]. Genetic scores applied to The Environmental Determinants of Diabetes in the Young (TEDDY) study, which prospectively followed genetically susceptible children at 3- to 6-month intervals from birth for a period of 6-14 years, provided evidence for the utility of combining genetic information from multiple risk loci to improve prediction of T1D[135,136].

Stage 1: Autoimmunity+/Normoglycemia/Presymptomatic T1D

Stage 1 is defined by individuals who have developed two or more T1D-associated islet autoantibodies but remain normoglycemic[128]. There is still little we understand about the initiation of islet autoimmunity. It is theorized that an environmental trigger such as diet, viral infection, route of neonatal delivery, antibiotics, and host microbiome can activate the immune system in a genetically susceptible host [137-139]. Long-term studies following children born in Germany, Finland, and Colorado carrying high-risk HLA revealed islet autoantibody seroconversion occurs in the first years of life, often many years prior to disease onset, and is an important prognostic factor for the development of disease (**Figure 9**)[126]. Autoantibodies form to β cell specific antigens with the most common and often first being insulin (IAA) followed by glutamic acid decarboxylase (GAD65), protein tyrosine phosphatase (IA2) and zinc transporter 8 (ZnT8)[128]. The pattern of autoantibody presentation is informative to disease progression and risk factors[140]. The rate of progression to symptomatic disease is dependent on the number of islet autoantibodies present, the age of autoantibody seroconversion, type of autoantibody, and the magnitude of the autoantibody titer[126,141-143].

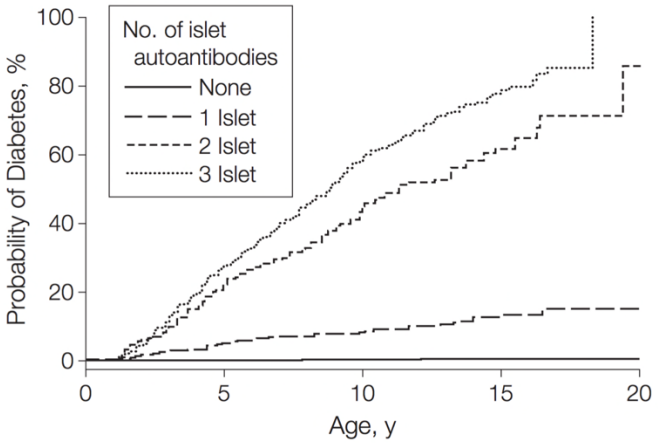


Figure 9. Seroconversion occurs early in life in children most likely to develop T1D. Prospective cohort studies conducted in Colorado, Finland, and Germany following 13,377 children at-risk for developing T1D were undertaken to investigate the natural history of T1D. These studies identified children who developed multiple antibodies had a higher risk of developing diabetes and the median age at seroconversion in the children that developed multiple islet autoantibodies was 2.1 years (range, 0.5-16 years). Figure from Zeigler et al., 2013.

Significant research efforts have attempted to understand the molecular features of immune cells, in specific T lymphocytes, at this stage. Evaluation of peripheral blood mononuclear cells (PBMCs) from children during this time period revealed that there is a pro-inflammatory cluster of immune cells that precedes autoimmunity identifiable by a

divergence of T cell responses to common autoantigens[144]. In addition, naïve T cells from children in this stage of asymptomatic autoimmunity have a profound impairment of regulatory T cells, important in regulating peripheral T cell tolerance[145]. Identification of individuals during this time period and improving our understanding of the immune defects could provide an opportunity for intervention targeting dysfunctional components of the immune system[146].

Stage 2: Autoimmunity+/Dysglycemia/Presymptomatic T1D

This stage of T1D includes individuals with two or more autoantibodies, but who also have developed signs of glucose intolerance or dysglycemia. This has primarily been defined by features of abnormal glucose tolerance such as impaired fasting plasma glucose levels of ≥100 mg/dL. In addition, decreased first phase insulin response has been reported within a year before disease onset[147-150]. In the DPT-1 study, a 2-hr OGTT best predicted progression but did not have notable changes until ~0.8 years before diagnosis[149]. Cross-sectional analysis of pancreatic islets from autoantibody-positive, non-diabetic donors demonstrates normal β cell mass[151-153] and suggests that substantial β cell loss may primarily occur within the year leading up to symptomatic disease presentation[153].

Stage 3: Onset of Symptomatic T1D

As β cell mass can vary significantly from person to person independent of age or gender[154], it is estimated an individual develops clinical symptoms of T1D when they reach about 10-30% of their original β cell mass. At this point, individuals require exogenous insulin to regulate blood glucose[155]. While significant β cell loss is present at disease onset, recent evidence has challenged the notion of total β cell destruction in T1D. Many studies now show that β cells persist and secrete C-peptide in T1D patients, even after many years of disease [156,157]. These clinical studies correlate with the presence of insulin-positive islets within the first ten years of disease[152].

In this early stage of disease, evidence for an impaired α cell response to changes in glucose have been reported[158-160]. Insulitis, i.e. islet inflammation, in human T1D is much less robust compared to animal models[161] but can persist in this time period often found in insulin-positive islets[152]. Analysis of pancreatic tissue collected from six living patients at the onset of T1D showed 5-58% of the insulin-containing islets met the criteria for insulitis with 36% of all islets still containing insulin[162]. These studies imply β cell loss continues to occur within the first ten years after clinical presentation.

Stage 4: Longstanding Symptomatic T1D

Many individuals with disease for 10 years of more still have some C-peptide production[156,157,163]. While it is very rare to identify insulin-positive islets, insulin-positive cells are found interspersed in the exocrine parenchyma (**Figure 10**)[156]. Evaluation of the long-standing pancreas reveals islet morphology and architecture is significantly altered. Notably, changes to the whole pancreas are evident. Systematic review of imaging studies from patients with T1D determined pancreatic volume is decreased by nearly 47% compared to matched controls and declines with disease duration[164]. There is evidence that these changes in weight may occur at and/or prior to disease onset[165]. Because the endocrine compartment makes up only 2% of the total pancreas, loss of islet β cells cannot fully account for this difference suggesting T1D pathogenesis could include the exocrine pancreas[166-168].

In this stage, patient care focuses on well-controlled blood glucose and preventing the progression of chronic complications associated with hyperglycemia. This is complicated by an increased risk in long-standing patients for severe hypoglycemia, in part due to repeated episodes of hypoglycemia and the development of hypoglycemia unawareness[169-175].

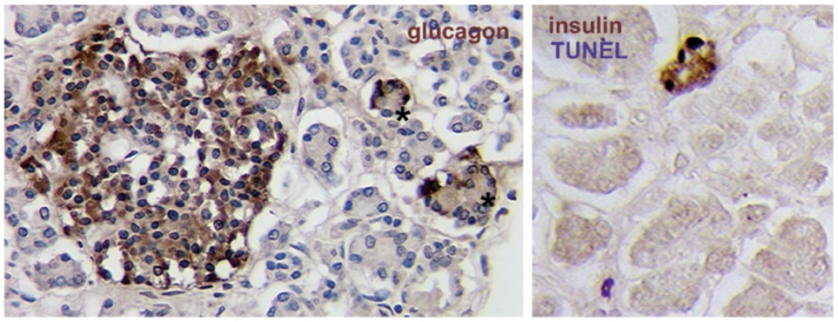


Figure 10. Histological findings in pancreas from individuals with T1D for 50 years or more. By studying the pancreas from individuals who had T1D for 50 years or more, the majority of islets in the pancreas were devoid of β cells staining positive for glucagon (**A**) with occasional insulin positive clusters (**B**) or single cells found scattered in the exocrine parenchyma. Images from Keenen et al., 2010.

Evidence for pancreatic islet cell dysfunction in T1D

T1D β cells

Since T1D results from the targeted destruction of β cells, researchers and clinicians have attempted to probe the mechanisms behind T1D β cell destruction and loss. Unfortunately, the T1D research community is limited by the paucity of T1D β cells for analysis and the technical challenges involved in their study. For this reason, histological analysis of pancreatic samples and clinical studies have attempted to dissect the mechanisms behind changes to the β cell during the stages before and after overt T1D. The creation of a nation-wide network to collect and study human pancreatic T1D tissue, Network for Pancreatic Organ Donors with Diabetes (nPOD; **http://www.jdrfnpod.org**), has provided insight into β cells in T1D.

Pancreatic β cell profile and function prior to disease onset

Evidence for metabolic abnormalities in β cell function prior to disease initiation have been reported. Through TrialNet and other programs, clinical studies evaluating autoantibody-positive individuals who are assessed prior to developing overt diabetes have reported increased fasting plasma glucose and impaired glucose tolerance[147-150] and that these changes often become evident only within a year prior to disease onset (**Figure 11**)[149]. Moreover, reports of an increased blood proinsulin-to-C-peptide ratio preceded disease onset in high-risk subjects and could be detected at least 12 months prior to diagnosis[176]. Interestingly, first-phase insulin response (FPIR), a measurement of insulin secretion within the first 3 minutes of an intravenous glucose tolerance test (IVGTT), is reduced during β cell autoimmunity in most individuals who progress to T1D (**Figure 12**)[150]. However, decreased FPIR may correlate more strongly with autoantibody positivity as many autoantibody positive individuals maintain low FPIR for years without developing disease underscoring the variability in these markers for disease progression (**Figure 13**)[148].

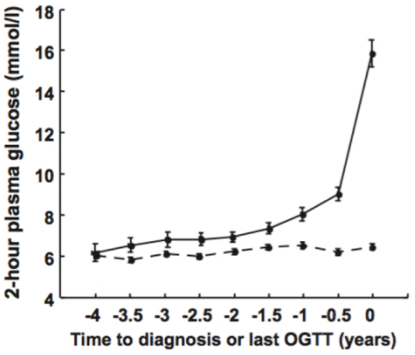


Figure 11. Impaired glucose tolerance becomes most apparent within a year of disease onset. 2-hr plasma glucose concentrations after sequential oral glucose tolerance test (OGTT) in antibody positive cases (avg. age of 11 yrs.) revealed most progressors (n=52, solid line) had a biphasic pattern compared to the monophasic response seen in non-progressors (dashed line) over this time course. Figure from Ferrannini et al., 2010.

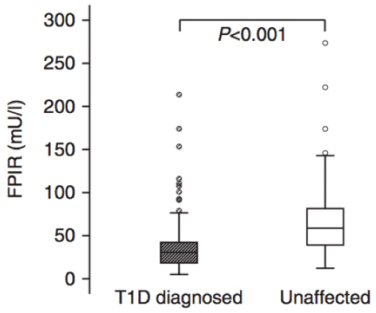


Figure 12. Autoantibody positive individuals that progressed to T1D have lower levels of first phase insulin response to glucose stimulation. First phase insulin response (FPIR) to an intravenous glucose tolerance test (IVGTT) evaluated during β cell autoimmunity in a total of 218 subjects found decreased FPIR in the 151 patients who progressed to T1D (T1D diagnosed) compared to the 67 who remained non-diabetic (unaffected). *P* values for Mann-Whitney *U* Tests. Figure from Siljander et al., 2013.

Access to samples from autoantibody-positive individuals without disease has provided unique insights into the T1D β cell. Studies from these samples have demonstrated evidence for altered β cell insulin processing that could correlate with decreased first phase insulin secretion[153]; however, preservation of β cell mass by insulin positivity in these individuals further suggesting that the majority of β cell loss occurs close to disease onset (**Figure 14**)[153].

Pancreatic β cell profile and function after overt disease

β cells persistent in the T1D pancreas especially in the first few years of disease; however, there is little knowledge about the functional and molecular features of these remnant β cells. With the development of ultra-sensitive C-peptide assays, we have learned that the majority of patients with long-duration

T1D are C-peptide microsecretors and a subset can respond to stimulation with a mixed meal tolerance test (**Figure 15**)[156,157,163]. This suggests these remnant insulin-positive cells have insulin secretory function. Review of histological specimens showed proinsulin protein and insulin mRNA in remnant T1D β cells but decreased expression of the proconvertase PCSK1 suggesting incomplete processing of proinsulin after disease onset[177].

Thus far, *ex vivo* T1D β cell function has been evaluated in very rare occasions. In 2000, Marchetti et al. assessed insulin release in islets isolated from a 14-year-old female with T1D for 8 months and found reduced glucose-stimulated insulin secretion, as measured as a percent of insulin content, that improved to normal with culture[178]. More recently, pancreatic islets collected from individuals at the onset of disease available through the DiViD study (10-20 islets per case) showed evidence for maintained glucose stimulated insulin secretion that also improved with culture in some donors when measured by total insulin secretion[179]. Both of these studies suggest defects of insulin secretion are quantitative in nature (i.e. resulting from an overall decrease in β cell mass) and may be compounded by a hostile native pancreatic islet environment.

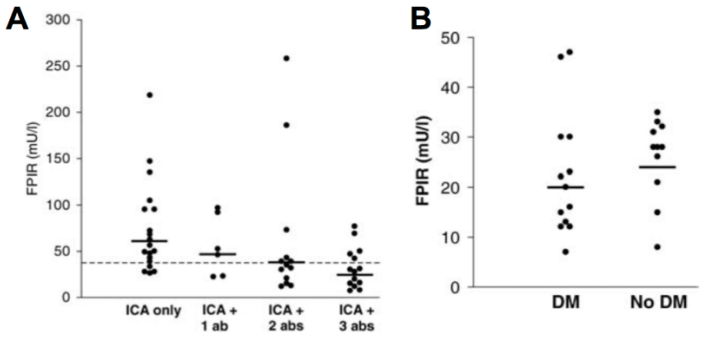


Figure 13. Decreased FPIR may more closely reflected autoantibody status than disease progression. (**A**) Low FPIR in this study was defined by a value under the 5th percentile (38 mU/l) of FPIRs of 20 healthy children 1.3 to 4.9 years of age (represented by dashed line). Increasing number of autoantibodies (abs) correlated with subnormal FPIR in children (1-5 years old). (**B**) Many autoantibody positive individuals with low FPIR who did not develop T1D (No DM) had similar FPIR levels to progressors (DM). The difference between the two groups did not reach significance (p=0.209). Figures from Keskinen et al., 2002.

Remaining Questions

While these studies have provided great insight into T1D β cells, many questions still remain: Are the changes in glucose responsiveness a result of decreasing β cell mass or evidence for an inherent defect in the β cell? What mechanisms of insulin secretion are maintained in the T1D β cell? Do they retain expression of markers important for β cell function and identity? Are specific β cell populations selectively targeted during the autoimmune response that leads to overt diabetes? The work included in this Dissertation will address many of these questions.

T1D α cells

Individuals with T1D have two glucagon secretory defects. During hypoglycemia they are unable to mount a counterregulatory glucagon secretory response[158,160], but at the same time they seem to secrete a relative excess of glucagon in response to amino-acid mediated nutrient stimulation, which correlates with progressive β cell loss[159,180]. To understand how inappropriate glucagon secretion in response changes in blood glucose levels can profoundly affect overall glucose homeostasis, the normal role of α cells will be discussed first.

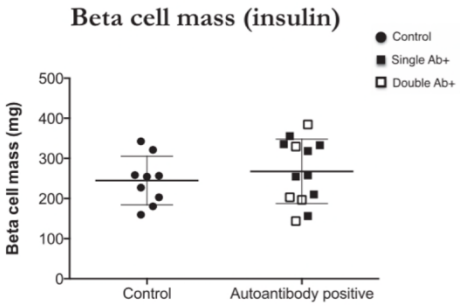


Figure 14. Normal β cell mass measured in autoantibody-positive histological pancreas sections. Evaluation of control (n=9) and single antibody positive (n=8) and double antibody positive (n=5) donor pancreatic sections for β cell mass calculated as total pancreas weight multiplied by insulin area showed no difference. Image from Rodriguez-calvo et al., 2017.

Under normal conditions, the pancreatic α cell participates in a systemic glucose counterregulatory response to defend against hypoglycemia (**Figure 16**). Glucose counterregulation is the sum of processes that prevent hypoglycemia to ensure a continuous glucose supply to the brain, which requires but does not store glucose. When blood glucose drops, pancreatic islet insulin secretion decreases leading to an increase of glucagon secretion by the α cell. In the absence of an increase in glucagon, an increase in glucose-raising hormones such as epinephrine, which can directly act on the α cell, as well as cortisol and growth hormone that signal to the liver lead to an increase in blood glucose. In addition, some of these hormones prompt the body to ingest carbohydrates by producing sympathetic symptoms (i.e. palpitations, tremor, sweating, hunger)[58,160]. Many of these counterregulatory hormones are also evoked in exercise to acutely stimulate endogenous glucose production and avoid glucose depletion from the sympathetic decrease of insulin release[181]. Conversely, when postprandial blood glucose rises, i.e. glucose levels after a meal, glucagon secretion is suppressed by a concurrent increase in intra-islet insulin and somatostatin coupled with inherent energy sensing mechanisms of the α cell[9,58,160,182].

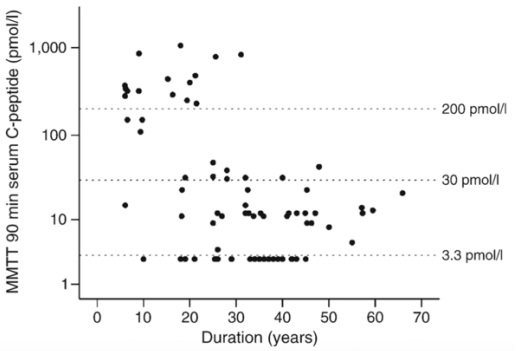


Figure 15. The majority of patients with long-duration T1D are insulin-microsecretors and can respond to stimulation. Serum C-peptide was analyzed in individuals with varying durations of T1D (n=74) in response to a mixed-meal tolerance test (MMTT). Lower-limit of detection assays allowed measurement of response in low-secreting individuals (below 30 pmol/l). Figure from Oram et al., 2013.

The counterregulatory response becomes important in T1D because exogenous insulin therapy renders individuals with T1D prone to episodes of hypoglycemia. This is exacerbated with the fact that these individuals have compromised defenses against hypoglycemia increasing the risk of hypoglycemic episodes, a major barrier to achieving adequate glycemic control[160]. As described below, clinical studies have probed the mechanisms behind impaired counter-regulation to determine whether it results from systemic changes in the nervous system or an inherent defect in the α cell.

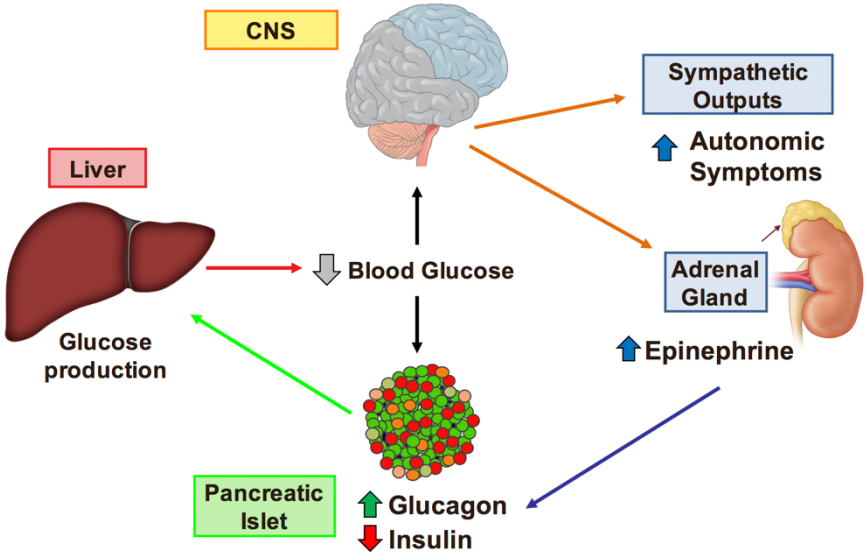


Figure 16. Glucose counterregulation to hypoglycemia results from a coordinated, systemic response involving the pancreatic islet. Decreases in blood glucose signal to the pancreatic islet to decrease insulin and increase glucagon which stimulates the liver to release glucose into the blood by glucose production. Low glucose is also counteracted by sympathetic responses from the central nervous system (CNS) to produce autonomic symptoms and release glucose-raising hormones, like epinephrine.

Pancreatic α cell response to hypoglycemia in T1D

Insulin-induced hypoglycemia in individuals with T1D of varying duration (2 weeks to 42 years) failed to produce an increase in glucagon despite intact responses of growth hormone and cortisol[158,169,183,184]. Because repeated episodes of hypoglycemia leads to attenuation of sympathetic outputs, causing hypoglycemia unawareness[172,173,185], Bolli and colleagues compared plasma glucagon and epinephrine level responses to an insulin infusion in individuals with different durations of T1D. Interestingly, they found that the loss of α cell responsiveness preceded the defects in the sympathetic nervous system evident with longer duration T1D (**Figure 17**)[184,186-188]. This defect did not correlate with classical autonomic neuropathy[160,172] and did not improve with avoidance of hypoglycemia[160,189]. Interestingly, episodes of hypoglycemia resulting from exercise are also more common in T1D but are primarily mediated through other mechanisms, such as adrenergic dysfunction[190,191], increased sensitivity to exogenous insulin[192] and repeated episodes of hypoglycemia[181], as glucagon secretion in response to exercise in T1D is intact[190,191,193,194].

Pancreatic α cell response to postprandial glucose in T1D

In addition to an impaired glucagon response to hypoglycemia, improper α cell glucagon secretion is thought to contribute to postprandial hyperglycemia. For example, T1Ds have a three times greater glucagon response to a mixed meal (protein, carb and fats) and exhibit an early rapid increase in plasma glucagon levels reaching maximal levels 30 minutes after eating (**Figure 18**)[159,180,195]. Somatostatin, which can directly inhibit glucagon secretion, decreased postprandial glucose levels suggesting glucagon contributes to the excessive rise in plasma glucose[180]. Others have confirmed these effects by showing glucose-induced hyperglycemia is unable to suppress plasma glucagon in children with T1D[196].

Remaining Questions

Multiple hypotheses have been proposed to explain defective glucagon secretion in T1D. Clinical and rodent studies point to an intrinsic defect for α cells[158,186,197,198]. It is possible that defective intracellular mechanisms result in aberrant transmission of the glucose signal in the α cell. Impaired glucagon secretion may also be specific to glucose-responsiveness, as arginine-coupled glucagon stimulation is still intact indicating a defect in the secretory apparatus alone is unlikely[158]. Studies assessing the molecular, transcriptional and functional profile of T1D pancreatic α cells in this Dissertation provide unique insights into the mechanisms of an inherent α cell defect.

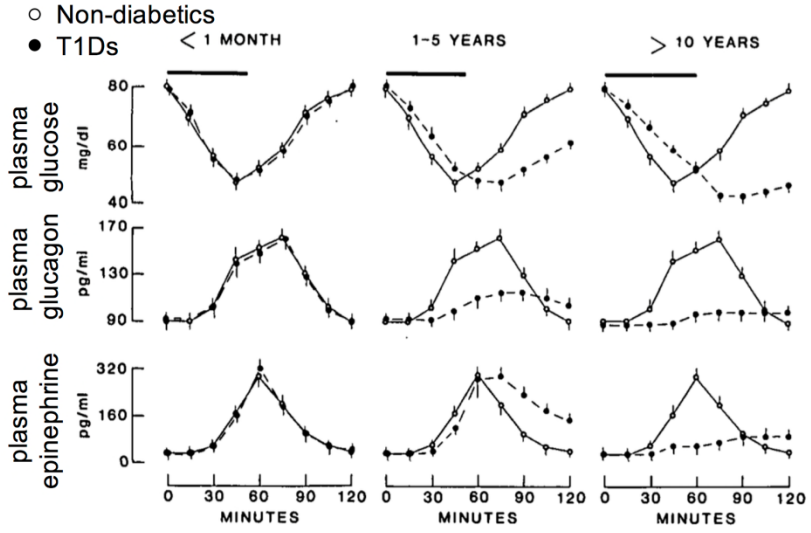


Figure 17. Impaired glucagon release to hypoglycemia precedes defects in epinephrine. During insulin-induced hypoglycemia, where insulin was infused from 1-60 minutes as indicated by black bars, individuals with less than one month of T1D (n=5), 1-5 years of T1D (n=11), and over 10 years of T1D (n=5) achieved hypoglycemia similar to controls (n=10, open circles) but had impaired recovery that became evident and more severe with increasing disease duration. Notably, glucagon secretion was impaired in individuals with disease for 1-5 years and accompanied by a compensatory increase in epinephrine. By 10 years of disease, loss of responsiveness for both glucagon and epinephrine could be seen. Figure adapted from Bolli et al., 1983.

Moreover, loss of neighboring β cells and subsequent paracrine signaling may be an important contributor to impaired α cell function[199]. Interestingly, dysregulated glucagon secretion to a mixed meal closely correlated with declining β cell function when measured within the first year of T1D diagnosis implicating decreasing intra-islet insulin

in the pathophysiological mechanism[159]. Intrahepatic islet transplantation into long duration T1D (average 27 years) only partially restored glucagon and epinephrine responses to an insulin-induced hypoglycemic clamp further supporting a role for the intact islet environment[114]. In addition, rodent studies have shown that extreme β cell loss (>99%) led to the spontaneous reprogramming of α cells into β cells[200]. Yet, study of remnant T1D β cells in cross-sectional tissue sections for proliferation or apoptosis showed no evidence of β cell turn over or neogenesis via ductal cells or α cells indicating limited native plasticity[201]. By assessing human T1D islets in an *in vivo* model in this Dissertation, we probe the translation of this model further.

Impaired glucagon secretion may also be a result of reduced sympathetic innervation and impaired neural sensing[175,202]. Recent episodes of hypoglycemia could impair CNS metabolic regulation, specifically of the ventromedial hypothalamus, decreasing whole-body glucose sensing[203,204]. Rodent models of T1D show islet sympathetic nerves are decreased as a result of the autoimmune process[205-207] and was reported altered in human T1D cases[208]. However, analysis of direct sympathetic innervation of pancreatic islets from individuals with T1D revealed no changes based on disease duration (**Figure 19**, unpublished) contradicting previous findings. Furthermore, loss of extrinsic neural input does not abolish glucagon secretion[209-211]. The contribution of altered islet innervation and neural sensing to impaired glucagon secretion in T1D requires further exploration.

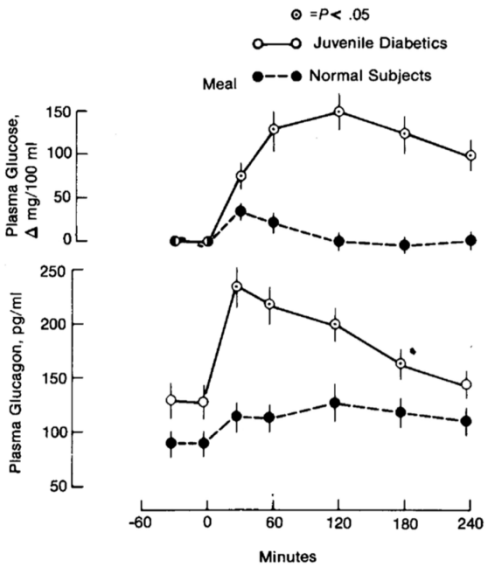


Figure 18. Abnormal post-prandial glucagon secretion in T1Ds. Response of glucose and glucagon were measured to a standard meal in T1Ds with disease duration from 1-17 years (n=12, open cirlces) and normal subjects (n=12, closed circles). Post-prandial hyperglycemia occurred in T1Ds accompanied by an abnormal release of glucagon. Figure adapted from Gerich et al., 1975.

As with the T1D β cell, mechanisms of T1D α cell dysfunction are poorly understood. Fortunately, unlike the T1D β cell, the T1D α cell is more available to investigation as these cells survive the autoimmune attack. Investigation of this cell type, as will be discussed in this Dissertation, can provide answers to questions such as: What are the properties of the T1D α cell? Do they share the plasticity for α-to-β cell reprogramming described in mouse models of profound β cell loss[200,212]? Can α cells be targeted to improve T1D therapy by preventing hypoglycemia unawareness?

Clinical Heterogeneity in T1D

T1D has historically been a clinical diagnosis, but recent observations have highlighted the heterogeneity in the T1D pancreas. Type 2 diabetes is regarded as a heterogeneous set of disorders, but the heterogeneity of T1D, thought to be an autoimmune disease, is increasingly being recognized[152]. For example, phenotypic differences in the disease can result from variables such as age of onset, ethnicity, and genetics[213-215]. Monogenic forms of diabetes, which make up 1-5% of all cases of diabetes, contribute to this heterogeneity but are poorly understood and subsequently significantly underdiagnosed[216]. Unfortunately, this impacts medical management as many forms of monogenic diabetes respond to alternative treatments to insulin therapy.

Monogenic Diabetes

Monogenic diabetes is broadly classified into three forms: neonatal diabetes (presenting within the first 6 months of life), maturity-onset diabetes of the young (MODY), and syndromic forms of diabetes that include other clinical features (ex. Wolfram Syndrome). Currently, over 20 genes are associated with monogenic forms of diabetes often encoding proteins that play a critical role in pancreatic β cell development and/or function; however, improvements in next generation sequencing and increased availability of targeted molecular genetics reveal the spectrum of disease phenotypes associated with known and unknown gene variants continues to expand[217-219].

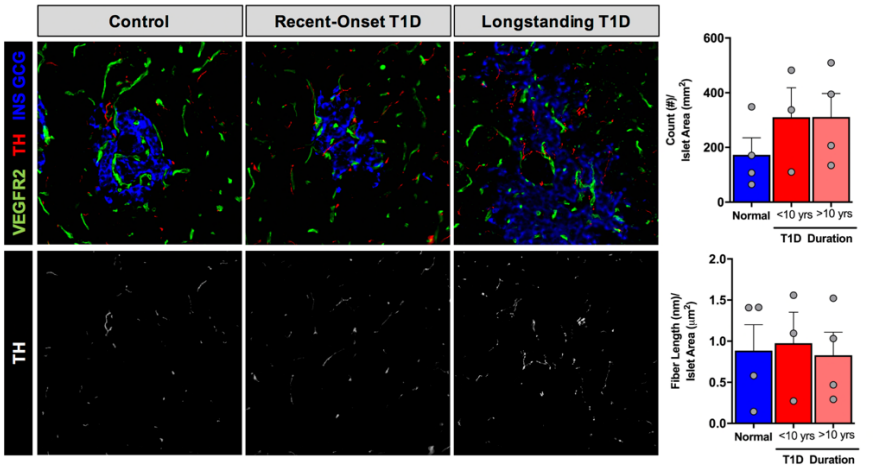


Figure 19. Islet sympathetic nerve fibers do not change with T1D. Pancreatic sections from the head, body and tail regions of control (n=4), recent-onset T1D (n=3; donors #1,2,4 **Table 1**) and longstanding T1D (n=4; donors #7, 8, 10, and 11) were evaluated for islet sympathetic nerve fiber density and length by tyrosine hydroxylase (TH) staining. One-Way ANOVA with Multiple comparisons for fiber density and fiber length were not significant (p = 0.9517).

Because MODY presents during early and late adolescence, it can sometimes be mistaken for T1D. For instance, 6.5% of autoantibody-negative individuals with T1D have pathogenic MODY gene variants[220]. MODY is characterized by autosomal dominant inheritance, a young age of onset (before 25 years of age) and pancreatic β cell dysfunction. Mutations in transcription factor hepatic nuclear factor (HNF)-1α is the most common cause of MODY in the US accounting for 50% of MODY subtypes

followed by mutations in glucokinase (GCK) and hepatic nuclear factor (HNF)-4α[221,222]. Due to limited availability of these samples and poor translatability of relevant animal models, we do not fully understand the mechanisms behind islet dysfunction in many forms of MODY.

Tools to Study Human Pancreatic Islet Physiology and Pathophysiology

To understand the pathogenesis of human disease, analysis of human samples is critical, but these are often difficult to collect and may provide mostly static data. To overcome this, the scientific community developed experimental models, which have delivered critical contributions to our understanding of human physiology and disease. However, we continue to learn that some model systems may not translate into clinically relevant information and may even inadvertently be misleading[223-226].

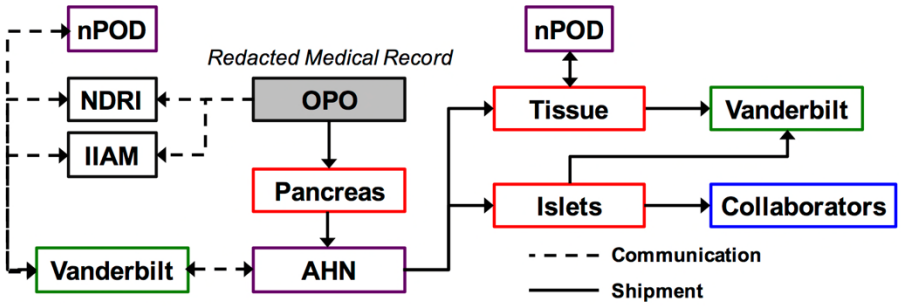


Figure 20. Infrastructure for studying human pancreas and islets. Infrastructure established by the Powers group and colleagues to collect organ-donated quality tissue for analysis. OPO: Organ Procurement Organizations; NDRI: National Disease Research Interchange; IIAM; International Institute for the Advancement of Medicine; nPOD: Network for Pancreatic Organ Donors with Diabetes; AHN: Allegheny Health Network.

The critical role of the pancreatic islet in human diabetes mellitus is incompletely defined partly due to the risk of live-biopsy of the pancreas and challenges in viable post-mortem tissue processing resulting from auto-digestion of exocrine tissue. Furthermore, our understanding of molecular changes in the pancreatic islet and its cell types in human diabetes has primarily relied on studies in animal models, *in vitro* systems, and analysis of limited post-mortem samples. Finally, rodent and cellular models of diabetes, including some forms of human monogenic diabetes, may not reflect the altered glucose homeostasis in humans, leaving the pathophysiologic impact of disease and/or genetic variants on the human pancreatic islet incompletely understood.

For example, two spontaneous animal models of T1D, the non-obese diabetic (NOD) mouse strain where overt hyperglycemia occurs in only 80% of females and 20% of male mice and the Biobreeding rat where 60% of an animal cohort manifest T1D, have not completely translated to human T1D. Many successful interventions in the NOD model (>500) have not proven effective in T1D patients[227,228]. Therapies directed at T

cells (Anti-CD3 and thymoglobulin), B cells (anti-CD20 rituximab), and adaptive immunity co-stimulation (CTLA-4-Ig abatacept), all of which had efficacy to prevent or reverse T1D in mouse models, were unsuccessful in preventing C-peptide decline in early-onset T1D[229-234]. Genetic discrepancies, evolutionary differences in the adaptive and innate immune systems, and distinct disease kinetics between mouse and human are likely important contributors to poor clinical adaptation of these models[229]. For this reason, programs were developed to collect and study rare samples from individuals with T1D, such as nPOD, Diabetes Virus Detection (DiViD) study, Integrated Islet Distribution Program (IIDP) and Human Islet Research Network (HIRN)-funded programs. These programs have increased focus on the use of human samples in understanding islet biology[235,236].

To bridge this gap in knowledge, we have developed infrastructure to collect pancreatic islets and tissue from the same individual in conjunction with the donor's de-identified medical record and to characterize in new ways the pancreatic islets in both normal and diseased samples (**Figure 20**). By studying the human pancreata in this way, we hope to integrate findings from model systems and primary human tissue to understand the mechanisms of islet function and dysfunction as it relates to disease (**Figure 21**).

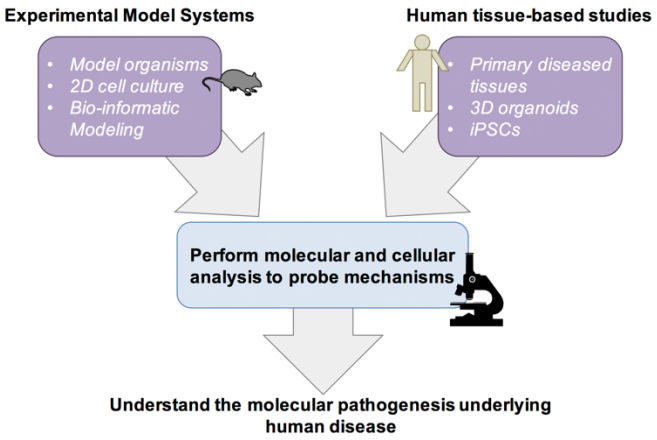


Figure 21. Schematic for understanding mechanisms of disease in primary human tissues. iPSC: induced pluripotent stem cells.

Aims of Dissertation

The primary goal of the research included in this Dissertation is to advance our understanding of the molecular mechanisms behind pancreatic islet dysfunction in human T1D and investigate how clinical heterogeneity contributes to insulin-deficient diabetes.

In **Chapter III**, we describe our establishment and use of an integrative approach to study the native pancreas and isolated islets from eight human donors with varying disease duration. When studying islet function, β cells maintained a nearly normal insulin secretory pattern, however α cells had a blunted glucagon secretory response compared to controls. Through histological analysis of the native pancreatic tissue, the endocrine cell molecular profile of donor pancreata was assessed. The remnant β cells

maintained their expression of markers of β cell identity, but α cells had altered expression of transcription factors constituting α and β cell identity. The T1D islets were further studied *in vivo* by placing them into a non-autoimmune and normoglycemic environment. However, this removal from an inhospitable environment and additional exposure to a proliferative stimulus, the GLP-1 analogue Exendin-4 (Ex4), did not lead to β cell expansion or α-to-β cell transdifferentiation, but did result in partial recovery of α cell specific markers. Transcriptional profiling of the T1D α cell revealed changes in genes important for α cell identity and function. Our data suggest that remnant T1D human β cells appear to be essentially normal, while T1D human α cells exhibit features of an intrinsic defect, which could help explain the aberrant response to hypoglycemia seen clinically in T1D patients.

As part of studies of T1D pancreata, two out of ten donors diagnosed clinically with diabetes had atypical features on histology and substantial insulin secretion. Comprehensive analysis of the pancreas and islets from one individual revealed that this donor carried a disease-causing variant in hepatocyte nuclear factor 1 alpha (*HNF1A),* leading to a form of monogenic diabetes. Analysis demonstrated HNF1A dysfunction leads to insulin deficiency and monogenic diabetes by impacting β cell transcriptional networks and processes critical for glucose-stimulated insulin secretion. For the other donor, a heterozygous intronic mutation of unknown significance in the glucokinase (*GCK*) gene was identified; however, whether this mutation was responsible for the donor's hyperglycemia appears unlikely. These first direct studies of human pancreatic islets with mutations in genes associated with monogenic diabetes are described in **Chapter IV** and highlight the heterogeneity of pancreatic phenotypes that exists within clinically diagnosed T1D.

Finally, to investigate the molecular mechanisms of α cell dysfunction in T1D, we developed an approach to create a system of α cell targeting where human islet dispersion and re-aggregation using a modified hanging droplet method generates human pseudoislets that morphologically and functionally closely resemble native islets. The characterization and application of this system to investigate primary human islet physiology and pathophysiology are reported in **Chapter V**.

The materials and methods used to conduct these studies are described in **Chapter II**, and the significance of these findings and future directions are presented in **Chapter VI**.

CHAPTER II

MATERIALS AND METHODS

Some methods in this chapter have been published in Brissova, Haliyur, Saunders, and Shrestha et al., 2018[237]

Experimental Model and Subject Details

Animals

Immunodeficient 10-12-week old NOD-*scid-IL2rγ*^{null} (NSG) male mice or NSG-DTR (*Nod-SCID-IL2Rγ*^{null}; Rat Insulin Promoter-Human Diphtheria Toxin Receptor$^{tg/tg}$) male or female mice were used for human islet transplantation studies. Animals were maintained by Vanderbilt Division of Animal Care in group-housing in sterile containers within a pathogen-free barrier facility housed with a 12hr light/12hr dark cycle and access to free water and standard rodent chow. All animal procedures were approved from by the Vanderbilt Institutional Animal Care and Use Committees.

Primary cell cultures

Primary human islets were cultured in CMRL 1066 media (5.5 mM glucose, 10% FBS, 1% Pen/Strep, 2 mM L-glutamine) in 5% CO_2 at 37°C for 24-72 hours prior to reported studies.

Cell lines

A non-islet cell line, HeLa [238], and pancreatic islet cell line, MIN6 [239], were cultured in a monolayer under conditions described previously for use in studies for *HNF1A*. Human endothelial cells derived from induced pluripotent stem cells (iPSCs) were purchased from Cell Dynamics (ECC-100-010-001) and cultured using endothelial cell medium supplement (ECM-100-030-001) in a monolayer as recommended and with human islet cells in pseudoislets.

Human specimens

Pancreata and islets from normal donors and donors clinically diagnosed with T1D were obtained through a partnership with the International Institute for Advancement of Medicine (IIAM), National Disease Research Interchange (NDRI), Integrated Islet Distribution Program (IIDP), Alberta Diabetes Institute (ADI) and Network for Pancreatic Organ Donors with Diabetes (nPOD). Most pancreata from normal donors were

processed either for islet isolation or histological analysis (described below). In most diseased pancreatic organs, islets and tissue specimens were procured from the same organ. For a number of controls, human islets were obtained through IIDP (**Table 2**). Donor demographic information and phenotype of all donors clinically diagnosed with T1D is summarized in **Table 1**. The Vanderbilt University Institutional Review Board declared studies on de-identified human pancreatic specimens does not qualify as human subject research.

Methods

Human pancreatic islet procurement

Pancreas processing and islet isolation were performed by Dr. Rita Bottino at Allegheny Health System. Pancreata from normal and T1D-diagnosed donors were received within 18 hours from cross clamp and maintained in cold preservation solution on ice until processing. Pancreas was then cleaned from connective tissue and fat, measured and weighed. Prior to islet isolation, multiple cross-sectional slices of pancreas with 2-3 mm thickness were obtained from the head, body and distal tail (**Figure 26A**). Pancreatic slices were further divided into four quadrants and then either snap frozen or processed for cryosections.

Tissue specimens processed for cryosections were fixed in 0.1 M phosphate buffered saline (PBS) containing 4% paraformaldehyde (Electron Microscopy Sciences) for 3 hours on ice with mild agitation, washed in four changes of 0.1 M PBS over 2 hours, equilibrated in 30% sucrose/0.01 M PBS overnight and embedded in Tissue-Plus O.C.T. compound (Fisher Scientific). Pancreatic organs were processed for islet isolation using an approach previously described{Balamurugan:tp}.

Briefly, depending on the size of pancreatic duct, 18G or 22G catheters were inserted into the main pancreatic duct (one catheter towards head and the other one towards tail). Accessory duct and main pancreatic duct were clamped at the points where sections were collected to prevent leakage of collagenase solution during infusion. Collagenase solution consisting of collagenase NB1, (1600 U/isolation, Crescent Chemical), neutral protease NB1 (200U/isolation, Crescent Chemical), and DNase I (12000U/isolation, Worthington Biochemical Corporation) was pre-warmed to 28ºC and delivered intraductally using a Rajotte's perfusion system and then maintained at 37ºC for approximately 20min. The inflated tissue was then transferred to a Ricordi's chamber apparatus for combined mechanical and enzymatic digestion, which was maintained at 36ºC for 5-15 minutes prior to warm and cold collection. The digest was incubated in cold RPMI media (Mediatech) supplemented with heat inactivated 10% Fetal Calf

Serum (Life Technologies) for 1 hour on ice. If post-digestion tissue pellet was larger than 2 mL and islets were distinguishable from exocrine tissue by dithizone staining (Sigma), a purification step consisting of density gradient (Biocoll, Cedarlane) centrifugation on a COBE 2991 Cell Processor (Gambro-Terumo) was used to separate islets from exocrine tissue. Islets were re-suspended in CMRL 1066 medium (Mediatech) supplemented with 10% heat-inactivated Fetal Calf Serum (Life Technologies), 100 units/mL Penicillin/0.1mg/mL Streptomycin (Life Technologies), 2 mmol/L L-glutamine (Life Technologies).

On average, islet-enriched fraction contained from 30,000 (T1D pancreas) to 90,000 islet equivalents (IEQs) (normal pancreas) with 25 – 50% purity. For the HNF1A donor (donor #9, **Table 1**), the pancreas weighed 75.4 g and the islet-enriched digestion fraction contained 54,200 islet equivalents (IEQs) with 70% purity. For the donor with a GCK variant (donor #6, **Table 1**), the pancreas weighed 59.1g and the islet-enriched digestion fraction contained 32,000 IEQs with 65% purity. Islets were cultured for 12 – 24 hours and then shipped from Pittsburgh to Vanderbilt University and/or University of Massachusetts for further analysis following shipping protocols developed by IIDP. Subsequent assays with isolated islets were set within 24 hours of islet arrival.

DNA sequencing

DNA sequencing was performed by Dr. Louis Philipson and colleagues at the University of Chicago. DNA samples were sequenced using a custom designed next-generation sequencing (NGS) targeted panel that includes 148 genes implicated in monogenic forms of diabetes (neonatal diabetes and MODY), insulin resistance, lipodystrophy, obesity, rare syndromic forms of diabetes, and diabetes candidate genes[240]. The targeted NGS approach was based on the SureSelect enrichment (Agilent Technologies) protocol followed by MiSeq Illumina NGS. Data quality was assessed using FastQC. Variants were called using The Genome Analysis Toolkit (GATK) HaploTypeCaller v3.3 and assigned to the transcripts of interest. Variants were then annotated in regards to their positions in transcripts of interest, position relative to the coding sequence, consequence for the protein or mRNA and a collection of direct and indirect evidentiary tools and databases including NCBI dbSNP, 1000 Genomes Project, Exome Sequencing Project (ESP), GERP, Conseq, PolyPhen-2, SIFT and the Human Gene Mutation Database (HGMD). All variants were interpreted according to the guidelines of the American College of Medical Genetics. All likely pathogenic variants identified by NGS were confirmed by Sanger sequencing. DNA Sequencing results for all donors clinically diagnosed with T1D are in **Table 3**.

Measurement of islet endocrine cell populations by flow cytometry

Intracellular flow cytometry was performed by David Harlan and colleagues at the University of Massachusetts Amherst. Islet dissociation and intracellular antibody staining used a previously described protocol[82]. Anti-insulin (Gallus Immunotech), anti-chicken allophycocyanin, (Jackson ImmunoResearch), anti-glucagon (Sigma-Aldrich) conjugated with Zenon Pacific Blue (Invitrogen), and anti-somatostatin (LSBio) conjugated with Zenon Alexa Fluor 488 (Invitrogen) were used to stain β, α, and δ cells, respectively (**Tables 4** and **5**). Dissociated islet cell preparations were analyzed using a BD Biosciences FACS Aria II Cell Sorter (University of Massachusetts Medical School Flow Core Laboratory). Cellular debris was eliminated from islet preparations using a forward scatter versus side scatter size gate.

Assessment of pancreatic islet function in vitro

Islet perifusions were performed either by Powers research group or Vanderbilt's Islet Procurement and Analysis core. Function of islets from diseased donors and normal controls (**Tables 1** and **2**) were studied in a dynamic cell perifusion system at a perifusate flow rate of 1 mL/min[241]. The effluent was collected at 3-minute intervals using an automatic fraction collector. Insulin and glucagon concentrations in each perifusion fraction and islet extracts were measured by radioimmunoassay (insulin, RI-13K, Millipore; glucagon, GL-32K, Millipore). Area under the curve (AUC) above baseline hormone release was calculated with the trapezoidal method by GraphPad Prism 7.0. For some experiments, *in vitro* function was assessed by consecutive static incubation of primary human islets and/or pseudoislet islets for 1-hr in DMEM based media containing 1.7 mM glucose (low) followed by 16.7 mM glucose (high), and 16.7 mM glucose with IBMX. Islets were then lysed with acid-ethanol solution to extract the total hormone content. Insulin and glucagon from supernatants and islet extracts were quantified using the aforementioned assays.

In vitro calcium imaging

Whole islet calcium imaging was performed by Dr. David Jacobson and colleagues at Vanderbilt University. Following overnight culture, native islets or pseudoislets were loaded with Fura-2 AM (Molecular Probes) for 20 minutes followed by incubation in KRB with 5.6mM glucose (basal), 16.7mM glucose (high), 16.7mM glucose with diazoxide, and 16.7mM glucose with diazoxide and KCl. Intracellular calcium imaging was performed as previously described[242].

β and α cell sorting by FACS for RNA-sequencing

Human islet cell sorting was performed by Diane Saunders and Jack Walker within the Powers group. Human islets were dispersed using a modified protocol as described previously [243]. Briefly, 0.025% trypsin was used to disperse cells and reaction was quenched with modified RPMI medium (10% FBS, 1% Penn/Strep, 5 mM glucose). Cells were washed in the same medium and counted on a hemocytometer, then transferred to FACS buffer (2 mM EDTA, 2% FBS, 1X PBS). Indirect antibody labeling was completed via two sequential incubation periods at 4C, with one wash in FACS buffer following each incubation. Primary and secondary antibodies, listed in **Tables 4** and **5**, have been characterized previously by our group and others and used to isolate high-quality RNA from β and α cells [75]. Appropriate single color compensation controls were run alongside samples. Prior to sorting, propidium iodide (0.05 ug/100,000 cells; BD Biosciences, San Jose, CA) was added to samples for non-viable cell exclusion. Flow analysis was performed using an LSRFortessa cell analyzer (BD Biosciences), and a FACSAria III cell sorter (BD Biosciences) was used for FACS. Analysis of flow cytometry data was completed using FlowJo 10.1.5 (Tree Star).

Site directed mutagenesis

Site directed mutagenesis was performed by Xin Tong (Stein Lab) at Vanderbilt University. Point mutation of HNF1α-T260M expressing plasmid was generated by QuickChange II Site-Directed Mutagenesis Kit (Agilent) based on a Myc-tagged human HNF1α expressing plasmid (FR_HNF1A, Addgene #31104).

Electrophoretic Mobility Shift Assay (EMSA) and Western blot

EMSA and western blots were performed by Xin Tong (Stein Lab) at Vanderbilt University. Western blots of pseudoislets were performed by Erick Spears. HeLa or MIN6 cells were transfected with either wildtype (WT) or mutant (T260M) Myc-tagged HNF1α plasmid. Forty-eight hours post transfection, nuclear extract preparation and DNA binding reactions were performed as described previously[244]. Briefly, 10μg of nuclear extract and 400fmol (150000-200000cpm) of ^{32}P labeled double strand DNA probe (WT Oligo: 5'-TCGACTTGGTTAATAATTCACCAGAG-3') were mixed with a 20μl reaction system containing 10 mM HEPES (pH 7.9), 10% glycerol, 120 mM NaCl, 2 mM DTT, 1 mM of EDTA and 0.8μg of poly(dI-dC). The nuclear extract was pre-incubated in buffer for 20 min on ice with antibody as appropriate then the ^{32}P labeled DNA probe was added and incubated for an additional 20 min on ice. The un-labeled DNA oligo competitors were added together with the probe at 20-fold molar excess (Comp Oligo: 5'-TCGACTTGCGGACGACGGCACCAGAG-3'). EMSA reactions were separated on

5% or 6% native acrylamide gels in 0.5% Tris borate-EDTA buffer (TBE) at 200 V for 1.5 h. The protein:DNA complexes were visualized by autoradiography. Western blot analysis was performed as described previously[245]. Primary antibodies used in EMSA and western blots are listed in **Table 4**.

Luciferase Reporter Assays

Luciferase reporter assays were performed by Xin Tong (Stein Lab) at Vanderbilt University. HeLa cells were co-transfected with the WT or site mutant (MAFA R3 M2G^{-7816}) of the mouse *MAFA* Region 3 (R3) enhancer driven pFox-Prl-Firefly Luciferase plasmid[244], the phRL-TK Renilla luciferase internal control plasmid, and CMV-driven HNF1A^{WT} and/or HNF1A^{T260M} expression plasmids. Transcriptional activity was evaluated 48-hrs after transfection using a dual-luciferase assay according to the manufacturer's protocol (Promega). Each transfection was repeated at least three times. Firefly luciferase measurements were normalized to the Renilla internal control (phRL-TK). HNF1A^{WT} and HNF1A^{T260M} were also shown to be produced at similar levels by immunoblot analysis (**Figure 35G**).

Pseudoislet formation

In-house lentiviral packing were performed by Erick Spears in the Powers group. Human islets isolated from the pancreas of organ donors were obtained through IIDP, our collaboration with the Allegheny Health Network (AHN), or Alberta Diabetes Institute (ADI) Isletcore and hand-picked to purity. Islets were dispersed into single cells with a 0.025% Trypsin-EDTA solution at room temperature while pipetting up and down for 10 minutes. The islet cell suspension was evaluated for cell count and viability. At this stage, cells could be live-FACS sorted for a specific islet cell population as described above, treated with virus, or re-aggregated with human iPSC derived endothelial cells. For viral transduction, dispersed islet cells were transduced at 37C for 2.5-hrs with the appropriate lentivirus in combined media containing 4 µg/mL polybrene. Islet cells were washed with media twice before continuing with the re-aggregation protocol. Lentiviral construct plasmids were purchased from VectorBuilder and packaged into lentivirus. If appropriate, endothelial cells were added at a ratio of 3 islet cells to 1 endothelial cells. Droplets of 40 µL with 2500 cells were formed using a modified hanging droplet system (GravityPLUS System, Perkin Elmer ISP-06-010). Droplet pseudoislets reaggregation occurs for a period of 72 to 96-hrs before additional media and centrifugation allow collection of each droplet into individual wells of a 96-well plate. Wells are designed to allow for daily medium exchange without disturbing the pseudoislet. Pseudoislets were collected for analysis six to seven days post re-aggregation. Pseudoislets and intact

native islets from the same donor were cultured in parallel in a 1:1 combination of islet re-aggregation media (CMRL 1066 medium (5.5 mM glucose) containing 20% FBS, 100 ug/mL Penn/Strep, 2mM GlutaMAX, 2mM HEPES, and 1mM sodium pyruvate) and VascuLife Endothelial Cell medium (LL-0003). The demographic information for all donors in which islets were procured for pseudoislet formation are listed in **Table 7** with the specific experiments performed for pseudoislet characterization in **Table 8**.

Islet transplantation and assessment of grafts

Surgical transplantation of human islets into the both the ACE and kidney capsule were performed by Powers Group Senior Research Specialist Greg Poffenberger.

T1D islets

T1D islets were transplanted under the kidney capsule of immunodeficient 10-12-week old NSG male mice. Human islets from three normal donors with an average age of 37 years (range from 20 to 53 years) and average BMI of 25.1 (range from 21.7 to 28.2) were obtained through IIDP (**Table 2**). NSG mice were transplanted beneath the renal capsule with 500 – 600 normal or T1D islet equivalents (prepared as described above). Each set of islets was transplanted into 7-8 mice. Islets were allowed to engraft for 1 month and then mice were randomized for an additional 1-month treatment with either phosphate buffered saline (PBS) or exendin-4 (Ex), which were administered using an Alzet minipump (model 1004 with a pump volume of 100 μL, Durect Corporation). Ex-4 (California Peptide) was reconstituted in PBS without Ca^{2+} and Mg^{2+} and loaded into Alzet pump at concentration of 0.9 mg/mL. Ex-4 in plasma was measured by EIA (Phoenix Pharmaceuticals) and reached concentration of 4.4 ± 0.2 ng/mL (n=8). Insulin secretion in transplanted human islets was assessed by intraperitoneal administration of glucose (2 g/kg of body weight) and arginine (2 g/kg of body weight) prior to and after Ex-4/PBS treatment. Human insulin in plasma was measured by species-specific radioimmunoassay (Millipore).

Pseudoislets

Anywhere from 75 to 100 human native islets and pseudoislets were transplanted into the anterior chamber of the eye (ACE) of male and female NSG-DTR mice as previously described[246]. After 2 weeks of engraftment, endogenous mouse β cells were ablated by a single, 300 μL i.p. injection of 5 ng diphtheria toxin (DT) (List Biological Laboratories Inc., Cat#150). Control NSG-DTR mice were treated with an equal volume of 1X PBS (Sigma Aldrich) allowing studies to occur either under normoglycemic or hyperglycemic conditions for an additional period of 2 weeks. *In vivo* human insulin was assessed after a 6-hour fast in mouse serum collected retro-orbitally. Mice were enucleated after 1 month of engraftment for immunohistochemical analysis.

Immunohistochemical Analysis

Immunohistochemical analysis of pancreas was performed on serial 5-μm cryosections from multiple blocks from head, body and tail regions as described [247]. Kidneys bearing islet transplants were collected and then 5-μm cryosections from 5-6 different depths of each graft were labeled for immunofluorescence as described [247]. Analysis of pseudoislets were performed on islet whole mounts and/or with islets embedded in collagen IV gels. Primary antibodies to all antigens and their concentrations are listed in **Table 4** and were visualized using the appropriate secondary antibodies listed in **Table 5**. Digital images were acquired with a Zeiss LSM510 META laser scanning confocal microscope (Carl Zeiss) or Fluorescent ScanScope (Aperio). Apoptosis was assessed by TUNEL stain (Millipore, S1675). Tyramide Signal Amplification (Perkin Elmer) was used to visualize ARX and NKX2.2 labeling.

Quantification and Statistical Analysis

Quantification of nuclear protein expression

Histopathology reviews were conducted on the whole slide digital images. Protein expression of nuclear factors in α and β cells was quantified using MetaMorph 7.1 imaging software (Molecular Devices) using manual cell counting [42]. For the studies on T1D tissue, an average of 351±73 α cells and 861±141 β cells were counted per normal donor (n=7, **Table 2**), and average of 718±50 α cells and 45±17 β cells were counted per T1D donor (n=4, **Table 1**) for each transcription factor. For the HNF1A donor, an average of 141 α cells (HNF1A) and 554±205 β cells (for PDX1, NKX2.2, NKX6.1 and HNF1A) were counted per normal donor (n=5, **Table 2**), and an average of 382 α cells and 1169±318 β cells were counted for the HNF1A donor (n=1, **Table 1**) for each transcription factor. Ki67 and TUNEL in glucagon and insulin positive cells were quantified using a Cytonuclear v3 algorithm (Indica Labs).

Quantification of islet cell mass

To quantify endocrine cell area, 1-2 entire pancreatic sections from four blocks of the head, body, and tail regions of the donor pancreas and normal controls (n=7 donors, **Table 2**) were imaged using a Fluorescent ScanScope (Aperio). The ratio of hormone-positive cells for each hormone (insulin, glucagon, or somatostatin) over the total number of cells discovered by DAPI nuclear stain was measured using imaging software Cytonuclear v3 algorithm (Indica Labs) and represented the fraction of total pancreatic parenchyma for each hormone (i.e. hormone-positive area). Donor islet

density and size did not differ from controls. Cells calculated as double-positive for hormone were excluded in this analysis. Endocrine cell mass was determined as a product of the fraction of hormone-positive area and the measured pancreas weight taken before islet isolation (1g weight = 1cm^3 volume).

Structural analysis

Structural Analysis of HNF1A was performed in collaboration with Raymond D. Blind at Vanderbilt University. The X-ray structure of human HNF1A protein (PDB ID-1IC8) was retrieved from the PDB database (https://www.rcsb.org/). PyMOL Molecular Graphics System (Schrodinger, LLC; https://sourceforge.net/projects/pymol/) was used to visualize the DNA-protein complex to analyze interactions between the WT protein, mutant protein (T260M) and DNA.

qRT-PCR of isolated pancreatic islets

qRT-PCR was performed by Chunhua Dai within the Powers group. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using the primer-probe approach from Applied Biosystems (Life Technologies) with *18S* and *ACTB* endogenous controls using Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines as described[247]. All primers are listed in **Table 6**. Gene expression in recent-onset T1D donors was compared to normal controls (**Tables 1** and **2**). We were able to detect *INS* mRNA in T1D islets by RT-PCR and found that it was reduced to 19±7% compared to controls.

RNA-Sequencing and analysis

RNA-sequencing was performed at the HudsonAlpha Institute for Biotechnology in Huntsville, AL, in collaboration with Shristi Shrestha and Nripesh Prasad. Sorted α and β cells (5,000-125,000) were added to 200 µL lysis/binding solution in the RNAqueous micro-scale phenol-free total RNA isolation kit (Ambion) as previously described [237]. RNA integrity was evaluated (Agilent 2100 Bioanalyzer; control α cells, 8.36±0.22 RIN, n=5; T1D α cells, 7.97±0.32 RIN, n=3; HNF1A α cells, 8.3 RIN; control β cells, 7.86±0.67 RIN, n=5, and HNF1A β cells, RIN 7.40) and high-integrity total RNA was amplified (Ovation system; NuGen Technologies) per standard protocol as described previously[247]. Amplified cDNA was sheared to target 200bp fragment size and libraries were prepared using NEBNext DNA Library Prep (New England BioLabs). 50bp Paired End (PE) sequencing was performed on an Illumina HiSeq 2500 using traditional Illumina methods[248] to generate approximately 50 million reads per sample. Raw reads were mapped to the reference human genome hg19 using TopHat v2.1[249]. Aligned

reads were then imported onto the Avadis NGS analysis platform (Strand life Sciences) and filtered based on read quality followed by read statistics to remove duplicates. Transcript abundance was quantified using the TMM (Trimmed Mean of M-values) algorithm[250,251] as the normalization method. Genes with normalized expression values less than 25 were removed prior to differential expression analysis between controls and T1D α cells or HNF1A α or β cells. Fold change (cutoff ≥ ±1.5) was calculated based on *p*-value estimated by z-score calculations (cutoff 0.05) as determined by Benjamini Hochberg false discovery rate (FDR) correction of 0.05. Differentially expressed genes were further analyzed through Ingenuity Pathway Analysis (IPA, Qiagen) and Gene Ontology (GO) analysis using DAVID v6.8[252].

Statistical analysis

To compare global differences in perifusion outcomes in T1D donors and controls, two-way ANOVA with Sidak's multiple comparisons test was used. Data were expressed as mean ± standard error of mean. Two-tailed Student's *t* test was used for analysis of statistical significance. For the HNF1A studies, values are shown as mean ± standard error of the mean (SEM) for control samples. Data from a sample size of n=1 for the donor precluded formal statistical analysis. Two-tailed Student's *t* test was used for analysis of statistical significance for two-group comparisons between donor and controls when assessing transcription factor expression within multiple pancreatic islets. Statistical analysis was performed using GraphPad Prism software. Statistical details of experiments are described in the figure legends and Results section. A *p*-value less than 0.05 was considered significant.

Table 1. Demographic information and phenotype of donors with diabetes

Donors	Age (years)	T1D Duration (years)	Ethnicity/ Race	Gender	BMI	Cause of Death	High-risk HLA*	AutoAb	C-peptide (ng/ml)	HbA1C**
1	12	3	Caucasian	F	26.6	Anoxia	DR3, DQ2	mIAA	0.05	9.8
2	13	5	Caucasian	M	19.1	Anoxia	DR4 DQ2, DQ8	IA2A mIAA	<0.02	N/A
3 nPOD Case #6342	14	2	Caucasian	F	24.3	Anoxia	DR4	IA2A mIAA	0.26	9.2
4	20	7	Caucasian	M	25.5	Anoxia	DR4 DQ2, DQ8	IA2A	0.43	N/A
5 nPOD Case #6323	22	6	Caucasian	F	24.7	Anoxia	DR3 DR4, DQ2	GADA IA2A	<0.02	6.6
6	22	8	Caucasian	M	25.7	Anoxia	DR4, DQ2 DQ8	ND	0.06	11.9
7	27	17	Caucasian	M	18.5	Anoxia	DR4 DQ2, DQ8	ND	<0.02	N/A
8	30	20	Caucasian	M	29.8	Anoxia	DR4, DQ8	ND	<0.02	N/A
9	33	16	Caucasian	M	25.8	Head Trauma	DR4, DQ8	ND	0.4	8.9
10	58	31	Caucasian	M	21.7	Anoxia	DR4	N/A	N/A	8.8
11	63	44	Caucasian	M	24.3	Anoxia	DR4	N/A	<0.02	N/A

BMI – Body mass index, AutoAb – Autoantibodies, mIAA – Insulin autoantibody, IA2A – Autoantibody to transmembrane protein of the protein tyrosine phosphatase family, GADA – Glutamic acid decarboxylase autoantibody, HbA1C – Hemoglobin A1C, N/A – Not available, ND – Non-detectable. The nature of T1D pancreas, organ scarcity, and logistics of organ procurement and processing precluded us from collecting the entire data set on each T1D donor. All data from T1D donors except for donor #11 are reported in **Chapter III**. Donors #6 and #9 were unusual cases that are described in **Chapter IV**. Perifusion – Donors #1,4,5,6,9; Islet endocrine cell composition by FACS – Donors #1,4,5,6,9,10; Histology – Donors #1,2,5,6,9,10; Islet transplantation – Donors #1,5,6,8; whole islet RNA-sequencing – Donors #4,5,6. Islet cell purification and RNA-sequencing – Donors #3,7,8,9.

*HLA typing provided by Organ Procurement Organization.

**HbA1C collected from donor's redacted medical chart.

Table 2. Demographic information of normal donors in Chapters III and IV.

Donors	Age (years)	Ethnicity/ Race	Gender	BMI	Cause of Death	Tissue/Islet Source
Normal Controls for Islet Perifusion	7	Caucasian	M	26.8	Respiratory arrest	NDRI
	8	Caucasian	F	16.1	Intracerebral hemorrhage	IIAM
	8	African American	M	17.2	Anoxia	NDRI
	9	Caucasian	M	15.5	Head Trauma	NDRI
	11	African American	M	18.3	Anoxia	IIAM
	16	African American	M	23.2	Head Trauma	IIAM
	19	Caucasian	M	20.1	Head Trauma	NDRI
	19	Caucasian	M	21.2	Anoxia	NDRI
	21	Caucasian	M	21.7	Head Trauma	IIDP
	52	African American	M	29.2	Stroke	TDS
	55	African American	F	24.2	Stroke	TDS
Normal Controls for Islet Transplants	20	African American	M	21.7	Head Trauma	IIDP
	39	N/A	F	28.2	N/A	IIDP
	53	Native Hawaiian or Other Pacific Islander	F	25.4	N/A	IIDP
Normal Controls for qRT-PCR	11	Caucasian	M	22.7	Anoxia	NDRI
	20	Hispanic/ Latino	F	24.6	Anoxia	IIDP
	29	Hispanic/ Latino	M	27.5	Head Trauma	IIDP
Normal Controls for Histology	8	African American	M	17.2	Anoxia	NDRI
	10	Caucasian	M	19.3	Head Trauma	NDRI
	19	Caucasian	M	20.1	Head Trauma	NDRI
	19	Caucasian	M	21.2	Anoxia	NDRI
	20	Hispanic/ Latino	M	19.4	Head Trauma	IIAM
	24	Caucasian	M	35.5	Head Trauma	IIAM
	35	Caucasian	M	26.8	Head Trauma	IIAM
	55	African American	M	35.6	Stroke	IIAM
Normal Controls for RNA-seq	26	Hispanic/Latino	F	35.9	Anoxia	IIDP
	35	Caucasian	F	23.6	Anoxia	IIDP
	49	Caucasian	F	31.6	Stroke	IIDP
	50	African American	M	30.2	Stroke	IIDP
	55	Caucasian	M	27.8	Stroke	IIDP

*All NDRI, IIAM, and TDS islets were isolated by Rita Bottino at Allegheny Health Network in Pittsburgh, PA, USA. NDRI – National Disease Research Interchange; IIAM – International Institute for the Advancement of Medicine; IIDP – Integrated Islet Distribution Program; TDS – Tennessee Donor Services

	Gene	Chr	Transcript	Nucleotide	Amino Acid Change	dbSNP ID	MAF	POLY Score
1	*AKT2*	19	NM_001626.5	c.*9C>T	-	rs79275829	0.004	0
	CYP27B1	12	NM_000785.3	c.963+2T>G	-	-	0	0
	CYP27B1	12	NM_000785.3	c.963+7T>G	-	-	0	0
	FOXP3	X	NM_014009.3	c.403A>C	p.Thr135Pro	-	0	0
	IFIH1	2	NM_022168.3	c.1641+1G>C	-	rs35337543	0.007	0
	SIRT1	10	NM_012238.4	c.110C>T	p.Pro37Leu	-	0	0.013
	GLP1R	6	NM_002062.3	c.1347G>A	p.Ala449Ala	rs201020486	0	0
2	*AIRE*	21	NM_000383.3	c.1411C>T	p.Arg471Cys	rs74203920	0.006	0.997
	ALMS1	2	NM_015120.4	c.69_77del	p.Glu27_Glu29del	-	0	0
	HSD11B1	1	NM_005525.3	c.219+6G>A	-	rs202219444	0	0
	LRBA	4	NM_006726.4	c.1536A>G	p.Ser512Ser	-	0	0
	POMC	2	NM_001035256.1	c.706C>G	p.Arg236Gly	rs28932472	0.004	1
	PTPN22	1	NM_015967.5	c.1508A>G	p.Tyr503Cys	rs371916399	0	0.004
	TBC1D4	13	NM_014832.3	c.2913A>T	p.Gly971Gly	rs184774790	0	0
3	*BBS5*	2	NM_152384.2	c.620G>A	p.Arg207His	rs35487251	0.006	0.833
	BLM	15	NM_000057.3	c.2119C>T	p.Pro707Ser	rs146077918	0.001	0.018
	EIF2AK3	2	NM_004836.5	c.-201A>G	-	rs144057685	0.005	0
	HFE	6	NM_000410.3	c.845G>A	p.Cys282Tyr	rs1800562	0.02	0
	HNF4A	20	NM_175914.4	c.1314C>G	p.Leu438Leu	-	0	0
	PIK3R1	5	NM_181523.2	c.1176C>T	p.Phe392Phe	rs3730090	0.308	0
4	*ALMS1*	2	NM_015120.4	c.12278G>A	p.Arg4093His	-	0	0
	FBN1	15	NM_000138.4	c.3294C>T	p.Asp1098Asp	rs140587	0.008	0
	GLP1R	6	NM_002062.3	c.59G>A	p.Arg20Lys	rs10305421	0.007	0.643
	IFIH1	2	NM_022168.3	c.1075G>C	p.Val359Leu	-	0	0.996
	MKS1	17	NM_017777.3	c.1528C>T	p.Arg510Trp	-	0	0.976
5	*BLM*	15	NM_000057.3	c.2268A>G	p.Lys756Lys	rs146013879	0.001	0

	CLEC16A	16	NM_015226.2	c.2945G>A	p.Ser982Asn	rs72650689	0.004	0.967
	HFE	6	NM_000410.3	c.187C>G	p.His63Asp	rs1799945	0.084	0
	LRBA	4	NM_006726.4	c.7597A>C	p.Thr2533Pro	rs62346982	0.003	0.167
	KCNK16	6	NM_032115.3	c.165G>A	p.Leu55Leu	rs138076469	0.002	0
	LMNA	1	NM_005572.3	c.612G>A	p.Leu204Leu	rs12117552	0.004	0
6	*CDKN1C*	11	NM_000076.2	c.543_554del	p.Ala191_Pro194del	NA	0	0
	CYP27B1	12	NM_000785.3	c.963+7T>G	-	NA	0	0
	EIF2AK3	2	NM_004836.5	c.-201A>G	-	rs144057685	0.005	0
	FBN1	15	NM_000138.4	c.3294C>T	p.Asp1098Asp	rs140587	0.005	0
	GCK	7	NM_000162.3	c.209-8G>A	-	rs144798843	0.001	0
7	*ABCC8*	11	NM_000352.4	c.-430C>T	-	-	0	0
	BBS2	16	NM_031885.3	c.155T>A	p.Val52Asp	-	0	0.016
	EIF2AK3	2	NM_004836.5	c.-201A>G	-	rs144057685	0.005	0
	MKKS	20	NM_018848.3	c.1015A>G	p.Ile339Val	rs137853909	0.001	0.008
	NTRK2	9	NM_006180.4	c.483T>G	p.Thr161Thr	rs199849633	0	0
	VPS13B	8	NM_017890.4	c.8978A>G	p.Asn2993Ser	rs28940272	0.002	0.997
8	*ABCC8*	11	NM_000352.4	c.2176G>A	p.Ala726Thr	rs138687850	0.001	0.02634
	AKT2	19	NM_001626.5	c.1110G>T	p.Pro370Pro	rs41309435	0.001	0
	CFTR	7	NM_000492.3	c.1521_1523del	p.Phe508del	rs199826652	0.006	0
	LRBA	4	NM_006726.4	c.217-10del	-	-	0	0
	VPS13B	8	NM_017890.4	c.1832G>A	p.Arg611Lys	rs61754109	0	0.02634
9	*ALMS1*	2	NM_015120.4	c.2041C>T	p.Arg681*	-	0	0
	DYRK1B	19	NM_004714.1	c.*9C>G	-	rs370237703	0	0
	FBN1	15	NM_000138.4	c.5343G>A	p.Val1781Val	rs140649	0.003	0
	HNF1A	12	NM_000545.6	c.779C>T	p.Thr260Met	rs886039544	0	1
	IFIH1	2	NM_022168.3	c.1491G>A	p.Thr497Thr	-	0	0
	INS	11	NM_000207.2	c.-414C>A	-	-	0	0
	KCNJ11	11	NM_000525.3	c.-179C>T	-	-	0	0
	LRBA	4	NM_006726.4	c.217-10del	-	-	0	0

	PCNT	21	NM_006031.5	c.1754G>A	p.Arg585Gln	-	0	0
	PTEN	10	NM_000314.4	c.579G>A	p.Leu193Leu	-	0	0
	SLC29A3	10	NM_018344.5	c.300+10del	-	-	0	0
	WFS1	4	NM_006005.3	c.2052G>A	p.Ala684Ala	rs71539668	0.002	0
10	*AKT2*	19	NM_001626.5	c.945G>A	p.Glu315Glu	rs150000674	0.002	0
	CYP27B1	12	NM_000785.3	c.963+2T>G	-	-	0	0
	CYP27B1	12	NM_000785.3	c.963+7T>G	-	-	0	0
	FXN	9	NM_000144.4	c.-7G>A	-	rs145006100	0.011	0
	IFIH1	2	NM_022168.3	c.1641+1G>C	-	rs35337543	0.007	0

Chr – Chromosome, MAF – Minor allele frequency; DNA isolated from pancreatic samples of T1D donors was subjected to DNA sequencing covering coding regions and splice junctions of 148 genes associated with monogenic diabetes. *nonsense mutation

Table 3. DNA sequencing of donors with clinically diagnosed T1D for variants associated with monogenic diabetes.

Table 4. Sources and concentrations of primary antibodies

Antigen	Host Species	Working Dilutions			Vendor	Catalog #
		Cryo-sections	Western Blot; EMSA	Flow Cytometry		
Glucagon	Rabbit	1:200	-	-	Cell Signaling	2760
Glucagon	Mouse	1:500	-	-	Abcam	ab10988
Glucagon-Pacific Blue	Mouse	-	-	1:600	Sigma-Aldrich	G2654
Insulin-647	Rabbit	1:65	-	-	Cell Signaling	9008
Insulin	Guinea pig	1:1000	-	-	Dako	A0564
Insulin	Chicken	-	-	1:10	Gallus Immunotech	ABI
C-peptide (human)	Rat	1:100	-	-	DSHB	GN-ID4
Somatostatin	Goat	1:500	-	-	Santa Cruz	sc-7819
Somatostatin - AlexaFluor 488	Mouse	-	-	1:200	LS Bio	LS-C169129-100
Ghrelin	Mouse	1:1000	-	-	Abcam	57222
Pancreatic Polypeptide	Rabbit	1:1000	-	-	Peninsula Laboratories	T-4088
Ki67	Mouse	1:5000	-	-	Dako	M7240
CD45	Rabbit	1:100	-	-	Dako	A0452
Iba1	Rabbit	1:500	-	-	Wako	019-19741
VEGFR2	Goat	1:200	-	-	R&D Systems	AF644
Caveolin-1	Rabbit	1:2000	-	-	Abcam	ab2910
Laminin	Rabbit	1:1000	-	-	Sigma-Aldrich	L9393
Collagen IV	Rabbit	1:1000	-	-	Abcam	ab6586
TH	Rabbit	1:1000	-	-	Millipore	AB152
Synapsin	Rabbit	1:2000	-	-	Synaptic Systems	106 002
HNF1A	Rabbit	1:1000	-	-	Abcam	ab204306
HNF1A	Mouse	-	1:2000	-	Thermo Fisher	GT4110
HNF1A	Rabbit	-	N/A; 1:40	-	Proteintech	22426-1-AP
c-Myc	Mouse	-	1:2000; 1:40	-	Santa Cruz	9E10
β-Actin	Rabbit	-	1:4000	-	Cell Signaling	4967
Vinculin	Mouse	-	1:500	-	Sigma	V9131
GAPDH	Rabbit	-	1:10,000	-	Millipore	ABS16
NKX2.2	Mouse	1:100	-	-	Developmental Studies Hybridoma	74-5A5

NKX6.1	Rabbit	1:2000	-	-	BCBC/Palle Serup	N/A
PDX1	Rabbit	1:5000	-	-	C. V. E. Wright	N/A
PDX1	Goat	1:5000	-	-	C. V. E. Wright	N/A
ARX	Rabbit	1:1000	-	-	Beta Cell Biology Consortium (BCBC)/Patrick Collombat	N/A
ARX	Sheep	1:1000	-	-	R&D Systems	AF7068
MAFB	Rabbit	1:3000	-	-	Gift from Roland Stein, Vanderbilt University	BL1228
PAX6	Rabbit	1:5000	1:5000	-	Biolegend	901301
RFX6	Rabbit	-	1:500	-	Sigma Life Sciences	HPA037696
RFX6	Rabbit	1:500	-	-	Gift from Gérard Gradwohl	N/A
HIC3-2D12 (Hpa3)	Mouse	-	-	1:200	Gift from Dr. Philip Streeter	N/A
HIC0-4F9 (Hpi1) - Biotin	Mouse	-	-	1:100	Novus	NBP1-18872B
CD39L3	Mouse	-	-	1:100	Gift from Jean Sévigny	N/A

BCBC – Beta Cell Biology Consortium, N/A – not applicable

Table 5. Sources and concentrations of secondary antibodies

Host Species	Primary Ab Species	Fluorophore /Chromogen	Working Dilutions			Vendor	Catalog #
			Cryo-sections	Paraffin-embedded sections	Flow Cytometry		
Donkey	Chicken	APC	-	-	1:25	Jackson Immuno-Research	703-136-155
Donkey	Goat	Cy5	1:200	-	-	Jackson Immuno-Research	705-605-003
Donkey	Guinea pig	Cy2	1:500	-	-	Jackson Immuno-Research	706-225-148
Donkey	Guinea pig	Cy5	1:200	-	-	Jackson Immuno-Research	706-175-148
Donkey	Mouse	Cy5	1:200	-	-	Jackson Immuno-Research	715-175-151
Donkey	Rabbit	Cy3	1:500	-	-	Jackson Immuno-Research	711-165-152
Goat	Guinea pig	Biotin		1:300	-	Vector	BA-7000
Goat	Mouse	AP	-	1:500	-	Biocare Medical	MALP521L
Goat	Mouse	HRP	-	1:500	-	Biocare Medical	MHRP520L
Goat	Rabbit	HRP	-	1:500	-	Biocare Medical	RHRP520L
Goat	Mouse	PE			1:1000	BD Bio-sciences	550589
Goat	Mouse	Streptavadin BV421			1:500	BD Bio-sciences	563259

Table 6. Primers for quantitative real-time RT-PCR

Gene Symbol	TaqMan Assay ID
18S	Hs99999901_s1
ACTB	Hs99999903_m1
ARX	Hs00292465_m1
GCG	Hs00174967_m1
INS	Hs02741908_m1
MAFA	Hs01651425_s1
MAFB	Hs00534343_s1
NKX2.2	Hs00159616_m1
NKX6.1	Hs00232355_m1
PDX1	Hs00236830_m1

Table 7. Demographic information for islets used to form pseudoislets

Unique Identifier	Age/Gender	Ethnicity	BMI	Islet Source	Culture Time (prior to re-agg)
AEIS203	35yM	Hispanic	24.3	SC	4 days
AEI1395	48yM	White	23.8	SL	6 days
AEJE412	55yM	White	30.1	SL	5 days
AEJT193	51yM	White	29	UW	4 days
AEJR491	38yM	White	33	UM	6 days
AEKA111	43yM	White	35	SC	5 days
AFAE017	32yM	White	26.2	UW	5 days
R252	26yF	N/A	25.4	ADI	5 days
R253	57yM	N/A	25.6	ADI	5 days
AFBI329	7yF	Hispanic	14.9	AHN	4 days
AFBK273	49yM	Hispanic	34	SC	4 days
R260	73yM	N/A	26.9	ADI	4 days
AFBM114	28yM	White	34.7	SL	7 days
AFCD032	56yM	Asian	33.1	SC	7 days
AFCD035	45yM	Hispanic	26.6	SC	7 days
R264	44yM	N/A	33.8	ADI	5 days
AFCU387	14yM	White	33.8	AHN	5 days
AFCU134	39yF	White	34.8	HPAP	5 days
R268	48yF	N/A	29.2	ADI	4 days

Islet Isolation Center Abbreviations: AHN – islets were isolated by Rita Bottino at Allegheny Health Network in Pittsburgh, PA; HPAP – Human Pancreas Analysis Program (HIRN); SC – Southern California; SL – Sharp Lacy; ADI – Alberta Diabetes Institute; UW – University of Wisconsin. With the exception of HPAP, AHN, and ADI, all islet isolation centers are part of the Integrated Islet Distribution Program; N/A – not available

Table 8. Demographic information for donors used in pseudoislet characterization

Donors	Age (years)	Ethnicity/ Race	Gender	BMI	Unique Identifier	Islet Source
Islet Perifusion	26	Caucasian	F	25.4	R252	ADI
	28	White	M	34.7	AFBM114	SL
	44	African American	M	33.8	R264	ADI
	57	Caucasian	M	25.6	R253	ADI
	73	African American	M	26.9	R260	ADI
Static Incubation	39	White	F	34.8	AFCU134	HPAP
	45	Hispanic	M	26.6	AFCD035	SC
ACE Transplant	48	N/A	F	22.7	R268	ALB
	56	Asian	M	27.5	AFCD032	SC
IHC Analysis	7	Hispanic	F	14.9	AFBI329	AHN
	14	White	M	33.8	AFCU387	AHN
	26	N/A	F	25.4	R252	ADI
	28	White	M	34.7	AFBM114	SL
	32	White	M	26.2	AFAE017	UW
	39	White	F	34.8	AFCU134	HPAP
	49	Hispanic	M	34	AFBK273	SC
	57	N/A	M	25.6	R253	ADI
	73	N/A	M	26.9	R260	ADI
Calcium Imaging	45	Hispanic	M	26.6	AFCD035	SC
α-cell only	7	Hispanic	F	14.9	AFBI329	AHN
	39	White	F	34.8	AFCU134	HPAP
Viral Transduction	26	Caucasian	F	25.4	R252	ADI
	32	White	M	26.2	AFAE017	UW
	43	White	M	35	AEKA111	SC
	45	Hispanic	M	26.6	AFCD035	SC
	48	N/A		29.2	R268	ADI
	56	Asian	M	27.5	AFCD032	SC
	57	Caucasian	M	25.6	R253	ADI

Islet Isolation Center Abbreviations: AHN – islets were isolated by Rita Bottino at Allegheny Health Network in Pittsburgh, PA; HPAP – Human Pancreas Analysis Program (HIRN); SC – Southern California; SL – Sharp Lacy; ADI – Alberta Diabetes Institute; UW – University of Wisconsin. With the exception of HPAP, AHN, and ADI, all islet isolation centers are part of the Integrated Islet Distribution Program; N/A – not available

CHAPTER III

α CELL FUNCTION AND GENE EXPRESSION ARE COMPROMISED IN TYPE 1 DIABETES

The text and data in this chapter have been published in Brissova, Haliyur, Saunders, Shrestha et al., 2018[237]

Introduction

The events related to T1D pathophysiology in humans are poorly defined. For example, we do not understand the initiating trigger for T1D, how β cell loss proceeds, whether the loss is inevitable or can be abrogated, or the potential for residual β cell recovery. The longstanding view of T1D pathogenesis was that autoimmune β cell destruction resulted in complete loss of pancreatic insulin secretion. The improved sensitivity of C-peptide detection as well as studies using pancreatic specimens have recently led to the realization that many individuals with T1D have insulin-secreting cells, even 50 years after diagnosis[156,157]. Additionally, little is known about the properties of the glucagon-producing α cells in the T1D pancreas and whether they share the plasticity recently described in mouse models of profound β cell loss[212,253]. Moreover, it is unclear why T1D α cells have impaired glucagon secretion[158,186,195], which contributes to hypoglycemia susceptibility.

To comprehensively define the functional and molecular properties of T1D α cells, we used an approach that allows study of the pancreas and isolated islets from the same organ donor. Using this approach, we describe molecular and functional properties of the α and β cell from individuals with T1D. Our findings show that remnant β cells appear to maintain several features of regulated insulin secretion. In contrast, glucagon secretion was significantly compromised, and the levels of essential α cell transcription factors and their downstream targets involved in α cell electrical activity were reduced. Moreover, an important β-cell-enriched transcription factor was misexpressed in T1D α cells. These results provide insight into the functional and molecular profile of α cells in T1D.

Results

Procurement of pancreatic islets and tissue from the same organ donor allows for multifaceted phenotypic analysis of T1D islets

Our methodology for islet isolation and tissue procurement from the same pancreas allowed coupling of islet functional and molecular analysis with histological assessment of islets in the native organ (**Figure 26A**). In this way, we were able to study 5 donors

with recent-onset T1D (<10 years of T1D duration) and 3 donors with long-standing T1D (>10 years of T1D duration) receiving continuous insulin therapy compared to the appropriate non-diabetic controls (**Tables 1** and **2**). Experimental approaches used for analysis of each T1D donor are indicated in **Table 1** and labeled accordingly in figure legends. Due to clinical heterogeneity of T1D, we confirmed disease status by DNA sequencing[254] as described in the Supplemental Experimental Procedures. DNA sequencing covering coding regions and splice junctions of 148 genes associated with monogenic diabetes did not detect variants associated with monogenic diabetes [240] (**Table 3**). By flow cytometry analysis, recent-onset T1D islets contained 7-fold more α cells than β cells, and the β cell fraction was reduced approximately 6-fold compared to normal islets[82] (**Figures 26B** – **26D**).

T1D β cells have regulated insulin secretion and express key transcriptional regulators

Next, we analyzed the secretory function of the T1D islets in a dynamic cell perifusion system and compared it with islets from normal donors[241]. We found that the few remaining T1D β cells responded to glucose, cAMP-evoked stimulation, and KCl-mediated depolarization with a similar pattern as controls (**Figure 22A** and **1B**). The biphasic glucose-stimulated insulin secretion in islets at T1D onset was also shown recently by Krogvold and colleagues[179]. As expected, insulin secretion by T1D islets was diminished when normalized to overall islet cell volume (expressed in islet equivalents, IEQs) (**Figure 22A**) due to the greatly reduced β cell number (**Figure 26C**). However, insulin secretion normalized to islet insulin content (reflecting β cell number) by T1D islets nearly overlapped in terms of magnitude with the secretory response of controls (**Figure 22B**). Consistent with flow cytometry data in **Figure 26C**, the T1D β cell population was 4-6-fold less than in control islets when adjusted to islet insulin content (**Figure 22C**). Furthermore, the expression of transcription factors critical for β cell identity *PDX1*[27] and *NKX6.* [30] was not changed in either isolated T1D islets or by protein analysis of the native pancreatic tissue. Even in the 58-year-old T1D donor with longstanding T1D, these transcription factors were expressed in rare insulin+ cells found scattered in the exocrine parenchyma (**Figures 22D**, **22E**, **22F**, and **27**). However, *MAFA*[40], a transcription factor known to be required for murine β cell maturation, was reduced in the T1D islet (**Figure 22D**) and there were fewer NKX2.2-expressing T1D β cells compared to controls (**Figures 22G** and **27**) even though islet *NKX2-2* mRNA was unchanged (**Figure 22D**). These studies allowed us to directly access multiple pathways of insulin secretion and suggest that the T1D β cells appear to maintain several functional features of normal β cells, supporting the notion that T1D is a disease primarily of β cell loss. Due to very few T1D β cells available for deeper analyses, we focused our efforts on comprehensive characterization of the most abundant endocrine cell type in T1D islets, the α cell (**Figure 26C**).

T1D α cells are functionally impaired and have altered expression of transcription factors constituting α and β cell identity

Surprisingly, in spite of T1D islets containing 2-fold more α cells than normal islets (**Figure 26C**), their glucagon secretion was not significantly increased compared to controls when normalized to overall islet cell volume (expressed as islet equivalents, IEQs) (**Figure 23A**). The response was reduced when normalized to islet glucagon content (**Figure 23B**), and lacked the appropriate increase at low glucose following 30-minute high glucose inhibition (**Figure 23B,** inset). Marchetti and colleagues observed a similar defect in glucagon secretion in islets isolated from a single T1D donor 8 months after the disease onset[178]. These functional changes in T1D α cells were accompanied by reduced mRNA expression of two *bona fide* α cell regulators *ARX*[36] and *MAFB* [40] in isolated islets (**Figure 23D**). Notably, histological analysis of native tissues further revealed that most α cells from T1D donors did not express MAFB and ARX (**Figures 23E**, **23F**, and **28**), but did express low levels of NKX6.1, which is normally only found in β-cells (**Figures 23G** and **28**). A similar pattern has been seen in a mouse model with extreme β cell loss[212,253]. To test if there was evidence of α-to-β cell conversion in the T1D donor pancreas, we searched for, but did not find, islet cells co-expressing insulin and glucagon (data not shown). This observation differs from the recently described α-to-β cell conversion in a mouse model of 99% β cell loss[212,253].

Non-autoimmune, normoglycemic environment does not promote conversion of T1D α cells into β cells

To determine if human T1D α cells following extreme β cell loss can give rise to β cells when placed in a normoglycemic, non-autoimmune environment, we transplanted islets from the same T1D donors into immunodeficient *Nod-SCID-IL2Rγ^{null}* (NSG) mice[247] (**Figure 24A**). After one-month engraftment, mice were treated with either PBS or exendin-4[255], a GLP-1 analogue reported to promote β cell maturation or proliferation for an additional 1 month. At the end of the treatment, *in vivo* insulin secretion was stimulated by a bolus of high glucose and arginine. Although a species-specific assay readily detected a rise in mouse plasma insulin levels, human insulin was undetectable (data not shown), indicating the absence of functional human β cells in T1D islet grafts. Similar to native tissue, graft immunocytochemistry showed that β cells were very rare and did not detect insulin/glucagon co-expression (**Figure 24B**, and data not shown). Since there were no significant phenotypic differences between PBS and exendin-4 treated groups, these treatment groups were combined to assess α cell transcription factor expression. After transplantation, the number of α cells expressing ARX in T1D islet grafts was greater (**Figure 24D**) with a decrease in the number of NKX6.1+ α cells

(**Figure 24E)** compared to α cells in the native T1D pancreas, suggesting that the normoglycemic, non-autoimmune environment allowed for partial recovery of α cell identity marker expression.

Genes critical to α cell identity and function are differentially expressed between T1D and control α cells

T1D and control islet α cells were purified by FACS (**Figure 29A**). RNA-sequencing analysis (RNA-seq) performed on these cells indicated significant differences in the gene expression profiles (**Figures 25A, 25B, and 29B**). Ingenuity pathway analysis (IPA) and Gene Ontology (GO) term analysis (**Tables 9 and 10**, and **Figures 29C and 29D**) identified differences in processes associated with protein synthesis and handling, immune-activated signaling, and cell stress response pathways. Specifically, T1D α cells had increased expression of genes important in the unfolded protein response and formation of tight and adhesive junctions. Conversely, T1D α cells had significantly reduced expression of genes recently identified by single cell RNA-seq as α cell-enriched, such as *KLHL41*, *LOXL4* and *PTGER3*[38,84]. Our RNA-seq analysis further confirmed dysregulated expression of several islet-enriched transcription factors in T1D α cells, that we initially detected by RT-PCR in whole islets and at a protein level in pancreatic tissues (*MAFB*, *ARX*, and *NKX6-1*) (**Figure 23**). Among islet-enriched transcription factors, *RFX6*, which lies upstream o*f MAFB, ARX* and *NKX6-1* in endocrine cell differentiation[45,46], had the most reduced expression (7.2 fold). In mature mouse and human β cells, *RFX6* directly controls expression of P/Q and L-type voltage-gated calcium channels (*CACNA1A*, *CACNA1C*, *CACNA1D*), and the KATP channel subunit sulfonylurea receptor 1 (*ABCC8*)[46,47] that associates with Kir6.x pore-forming subunits[256]. T1D α cells also had altered expression of potassium and sodium ion channels, vesicle trafficking proteins, and cAMP signaling molecules, which collectively point to altered T1D α cell electrical activity and impaired glucagon exocytosis (**Figure 25D**).

Discussion

These results show the utility and advantages of an experimental approach that studies the pancreatic tissue and isolated islets from the same T1D individual and incorporates the *in vitro* and *in vivo* analysis of islets removed from the autoimmune, hyperglycemic environment. This approach also allowed us to directly test multiple pathways of hormone secretion and uncouple effects of decreased β cell mass and β cell dysfunction not possible in clinical studies *in vivo*. We found that the rare β cells in the pancreas present not only in recent-onset T1D, but also many years after T1D diagnosis, maintained features of regulated insulin secretion and/or produced key

transcriptional regulators known to play a critical role in the maintenance of β cell fate and function.

In contrast, T1D α cells, while highly abundant, were functionally impaired. Impaired glucagon secretion by T1D islets was associated with altered expression of multiple nuclear regulators (e.g., *ARX*, *MAFB*, and *RFX6*) and their downstream targets suggesting that these changes directly and indirectly impact glucagon secretory pathways by altering expression of potassium and sodium ion channels, vesicle trafficking proteins, and cAMP signaling molecules. Abnormal glucagon secretion is a common complication of T1D, including impaired counterregulatory response of glucagon to hypoglycemia[158] and an inappropriate rise in circulating glucagon in response to a mixed meal challenge[195]. Defects in neural glucose sensing, impaired islet innervation, or intra-islet insulin deficiency have been proposed to explain these abnormalities in glucagon secretion[257]. The current analysis provides a new explanation and molecular mechanism for the dysregulated glucagon secretion in T1D, namely an intrinsic α cell defect (**Figure 25D**). Our observation that the changes in α cell gene expression partially resolved when T1D islets were transplanted into a normoglycemic, non-autoimmune environment suggests that interventions might be developed to improve α cell gene expression and glucagon secretion in T1D (**Figure 30**).

These data provide insight and raise important questions about the molecular and functional changes in human T1D α cells. After massive β cell loss in mice, β cells can be gradually and partially replenished by a sustained α-to-β cell reprogramming[212,253]. Unlike in mice, the current analysis did not identify cells co-expressing insulin and glucagon in the native pancreas or after transplantation into a normoglycemic, non-autoimmune environment further supporting the notion that α-to-β cell conversion in humans is a very rare event [258]. Our findings do suggest that T1D α cells have reduced key molecular regulators (*ARX*, *MAFB*) and express a transcription factor, NKX6.1, that is usually β cell specific, raising the possibility of partial change toward a β cell phenotype. Perhaps an additional stimulus or multiple stimuli may be required for human α cell reprogramming. Lineage tracing studies of human α cells are needed to investigate the plasticity of human α cells.

These results stimulate a number of questions about the molecular and cellular changes in T1D islets:

- Are the α cell changes the result of the autoimmune attack on the β cells also affecting α cells, the lack of α cell-β cell contact, the diabetic milieu of hyperglycemia, or reduced intra-islet insulin?
- Have the remnant β cells, which have a number of features of normal β cells, somehow escaped the autoimmunity?

- Do T1D β cells comprise a specific subset of β cells[75], or do they represent an incomplete regenerative attempt, arising via de novo neogenesis from facultative pancreas progenitors[259], β replication[247,260,261], and/or transdifferentiation of acinar cells[262] or other islet endocrine cell types such as α cells[212,253]?

Additional studies of isolated T1D islets and T1D pancreatic tissue are needed to better understand the phenotype and possible heterogeneity of T1D α and β cells.

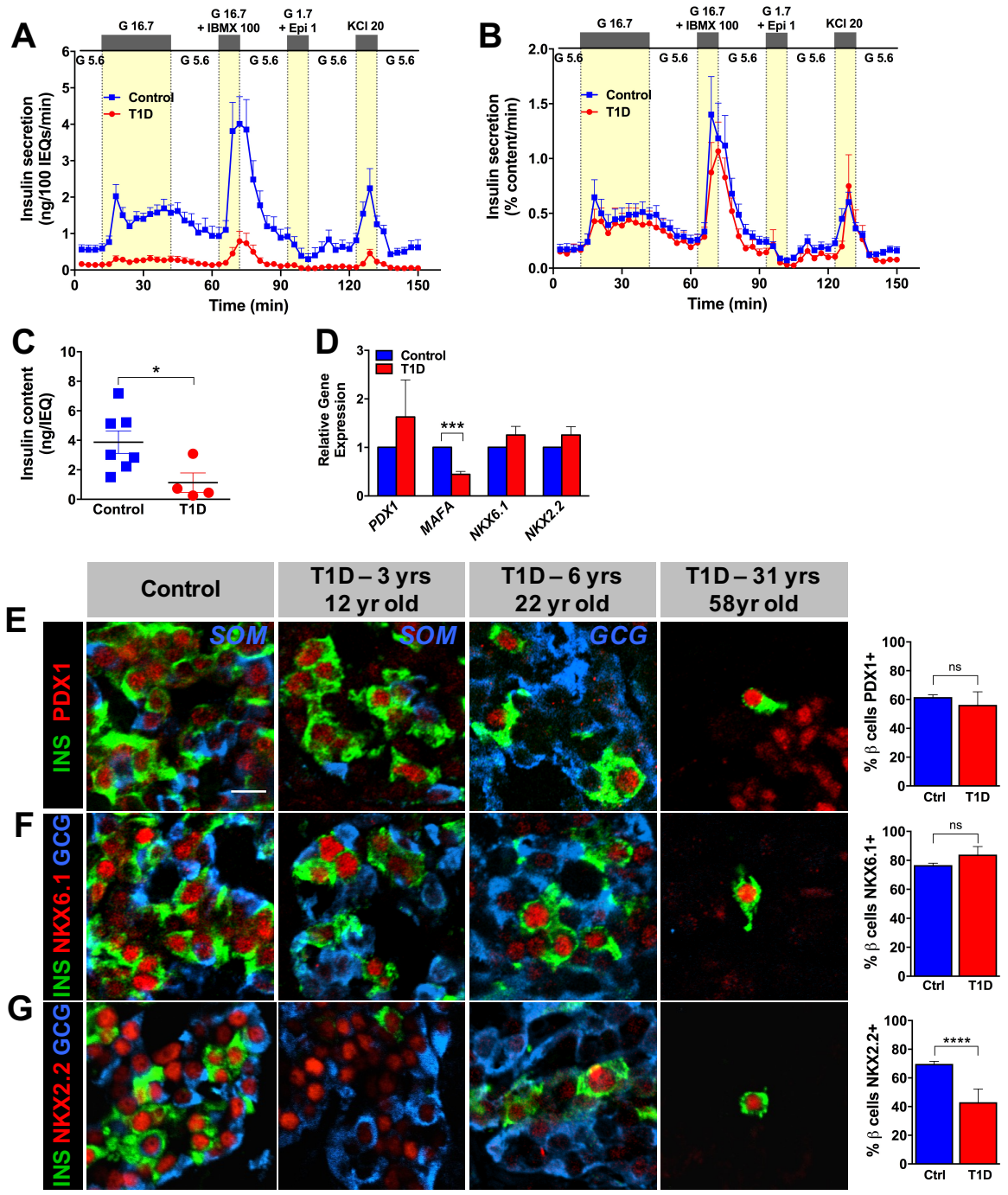


Figure 22. T1D β cells in recent-onset T1D retain secretory properties and gene expression pattern similar to normal β cells. (**A**, **B**) Insulin secretion was assessed in islets isolated from donors with recent-onset T1D (n=4; ages 12-22yrs, donors #1,3,4,5) and compared to normal controls (n=7; ages 7-21yrs); G 5.6–5.6 mM glucose, G 16.7–16.7 mM glucose, G 16.7+IBMX 100–16.7 mM glucose+100 μM isobutylmethylxanthine (IBMX), G 1.7 + Epi 1–1.7 mM glucose +1 μM epinephrine, KCl 20–20 mM potassium chloride. (**A**) Insulin secretion normalized to overall islet cell volume (expressed as islet equivalents, IEQs); ****, p<0.0001. (**B**) Insulin secretion normalized to islet insulin content; ***, p=0.0005. Data in **A** and **B** was compared by two-way ANOVA. (**C**) Insulin content of control (3.873±0.763 ng/IEQ) and T1D islets (1.131±0.660 ng/IEQ); p=0.0394. (**D**) Expression of β cell-enriched transcription factors by qRT-PCR in whole T1D islets (n=3 donors; ages 12-22yrs, donors #1,4,5) and controls (n=3 donors; ages 11-29yrs) was normalized to endogenous control and *INS* expression; ***, p<0.0007. (**E**–**G**) Expression of β cell-enriched transcription factors in the native pancreatic tissue from donors with recent-onset T1D (n=2; ages 12-22yrs, donors #1,5) was compared to 58-year-old donor with 31 years of T1D duration (donor #10) and controls (n=7; ages 8-55yrs). The pancreas of 58-year-old T1D donor did not have any insulin+ islets; only rare β cells were found in exocrine parenchyma. T1D β cells (n=3 donors; ages 12-58yrs, donors #1,5,10) had normal expression of β cell-enriched transcription factors PDX1 (**E**) and NKX6.1 (**F**) but decreased expression of NKX2.2 (**G**) compared to controls (n=7 donors; ages 8-55yrs); ****, p<0.0001. Data in **C**–**G** was compared by two-tailed Student's *t* test. Scale bar in **E** is 10 μm and also corresponds to **F** and **G**. See also **Figures 26** and **27**.

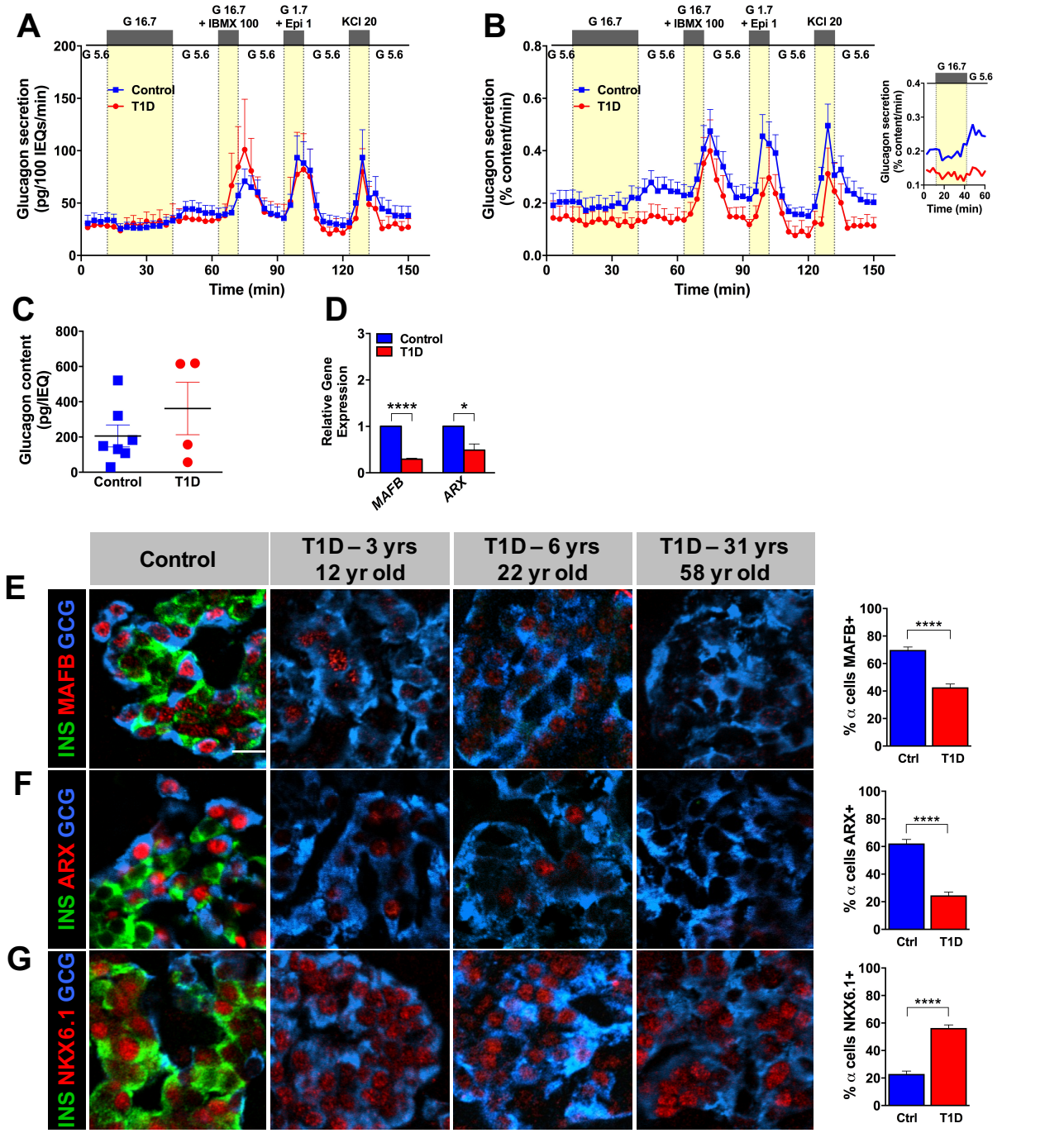


Figure 23. T1D α cells in recent-onset T1D have reduced glucagon secretion and dysregulated gene expression. The same sets of islets shown in **Figures 22A** and **22B** were simultaneously analyzed for glucagon secretion. The same non-diabetic controls were used as **Figure 22**. The labeling of islet stimuli is identical to that in **Figure 22**. (**A**) Glucagon secretion normalized to overall islet cell volume (expressed as islet equivalents, IEQs); p=0.2470. (**B**) Glucagon secretion normalized to islet glucagon content; ****, p<0.0001. Data in **A** and **B** was compared by two-way ANOVA. Inset shows mean glucagon response to low glucose following the 30-minute inhibition with high glucose. (**C**) Glucagon content in control (206±62 pg/IEQ) and T1D islets (362±149 pg/IEQ); p=0.2831. (**D**) Expression of α cell-enriched factors by qRT-PCR in whole T1D islets (n=3 donors; ages 12-22yrs, donors #1,4,5) and controls (n=3 donors; ages 11-29yrs) was normalized to endogenous control and *GCG* expression; ****, p<0.0001; *, p=0.0184. (**E**–**G**) Analysis of native pancreatic tissue for expression of islet-enriched transcription factors. T1D α cells (n=4 donors; ages 12-58yrs, donors #1,2,5,10) expressed β cell marker NKX6.1 (**G**) and lost *bona fide* α cell markers MAFB (**E**) and ARX (**F**) in most T1D α cells compared to controls (n=7 donors; ages 8-55yrs); ****, p<0.0001. Data in **C**–**G** was compared by two-tailed Student's *t* test. Scale bar in **E** is 10 μm and also corresponds to **F** and **G**. See also **Figures 26** and **28**.

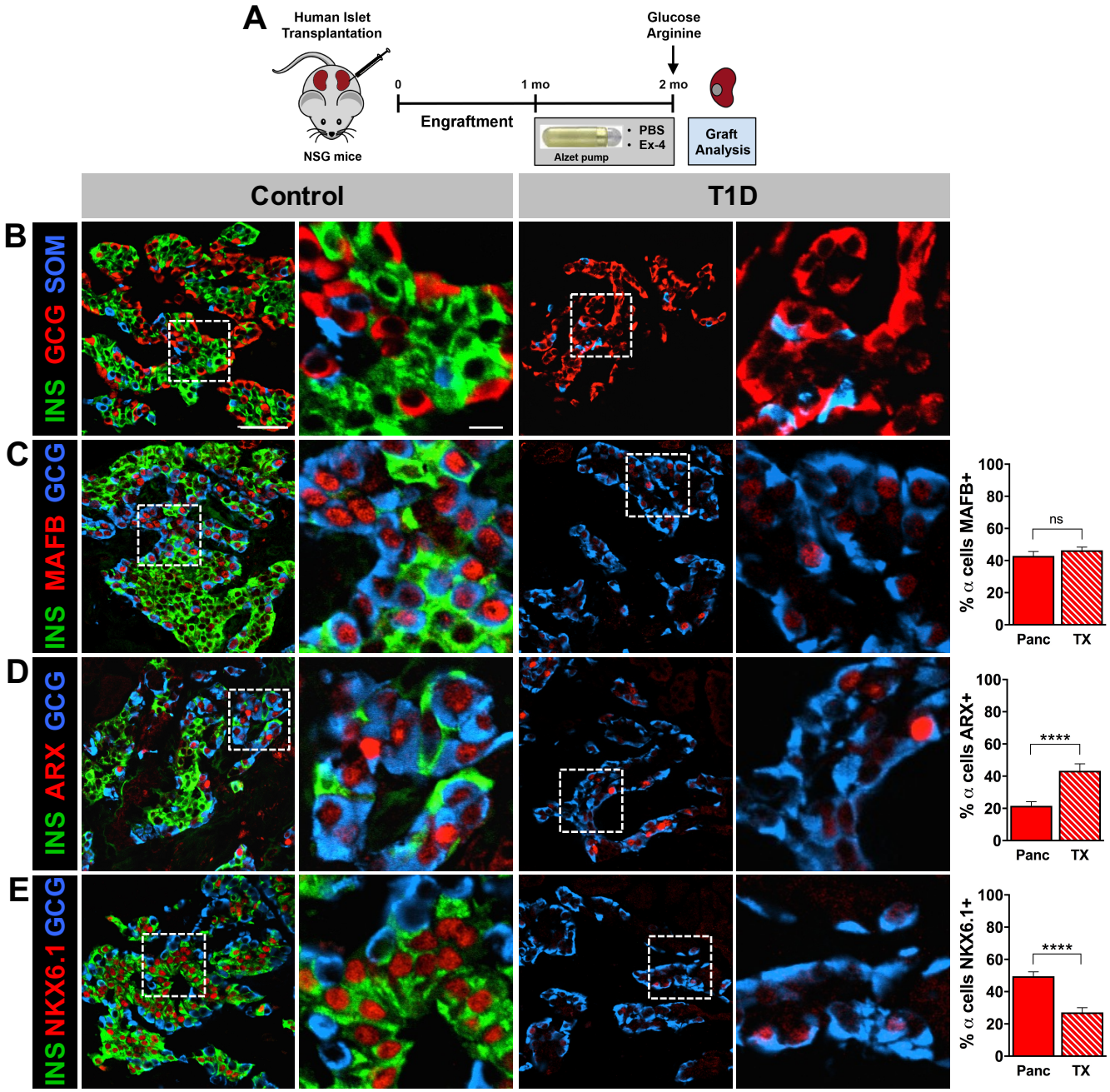


Figure 24. T1D α cells do not show evidence of α-to-β cell reprogramming in normoglycemic, non-autoimmune environment. (**A**) Islets from donors with recent-onset and longstanding T1D (n=3 donors; 12-58yrs, donors #1,5,10) depicted in **Figures 22** and **23** were transplanted into NSG mice. After 1-month engraftment, mice were treated with either PBS or Ex-4 for an additional 1 month. Representative images of islet grafts are from the 12-year-old individual with 3-year T1D duration (donor #1). In Control and T1D columns, regions denoted by the dashed line in images on the left in panels **B**–**E** (scale bar in **B** is 50 μm) are displayed on the right (scale bar is 10 μm). (**B**) Insulin (INS) and glucagon (GCG) double-positive cells were not detected in either type of T1D islet grafts (PBS or Ex-4). (**C**–**D**) As there were no phenotypic differences between PBS and Ex-4 treatment groups, representative images were taken from both cohorts and analyzed for α cell transcription factor expression. Change in number of GCG+ cells expressing MAFB, ARX, and NKX6.1 in transplanted T1D islets (TX) relative to donor's native pancreas (Panc). ****, p<0.0001. Data in **C**–**E** was compared by two-tailed Student's *t* test.

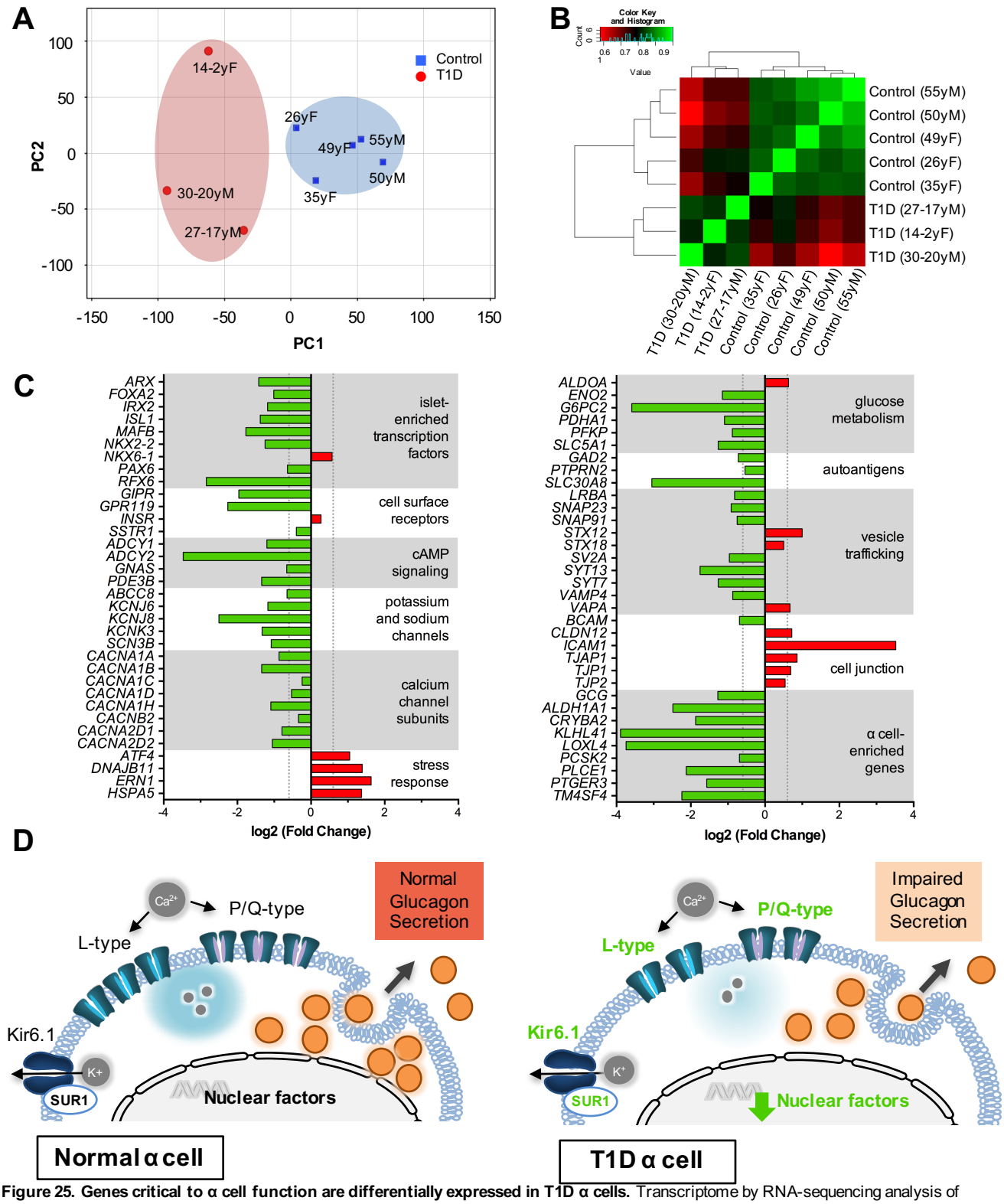


Figure 25. Genes critical to α cell function are differentially expressed in T1D α cells. Transcriptome by RNA-sequencing analysis of purified human α cells from T1D donors (n=3; ages 14-30yrs, donors #3,7,8) and controls (n=5; ages 26-55yrs). (**A**) Principal component analysis (PCA) plot shows clustering of α cell samples from control and T1D donors. (**B**) Heat map of the pairwise correlation between all samples based on the Spearman correlation coefficient. Perfect correlation is indicated by 1. (**C**) Genes associated with α cell identity and function are significantly downregulated in the T1D α cells with increased expression of stress response factors and cell-cell contact proteins. Vertical dotted lines represent point of significance for FC=1.5x threshold analysis; $p<0.05$ for all values shown. (**D**) Proposed model for disrupted glucagon secretion in T1D α cells. Normal α cell function is maintained by islet-enriched transcription factors, which regulate α cell machinery necessary for glucagon synthesis and secretion (left panel). Altered expression of transcription factors likely leads to reduced α cell glucagon production, disrupted calcium signaling and electrical activity that results in impaired glucagon secretion (right panel, green font indicates downregulation). See also data in **Figure 29,** and **Tables 9** and **10**.

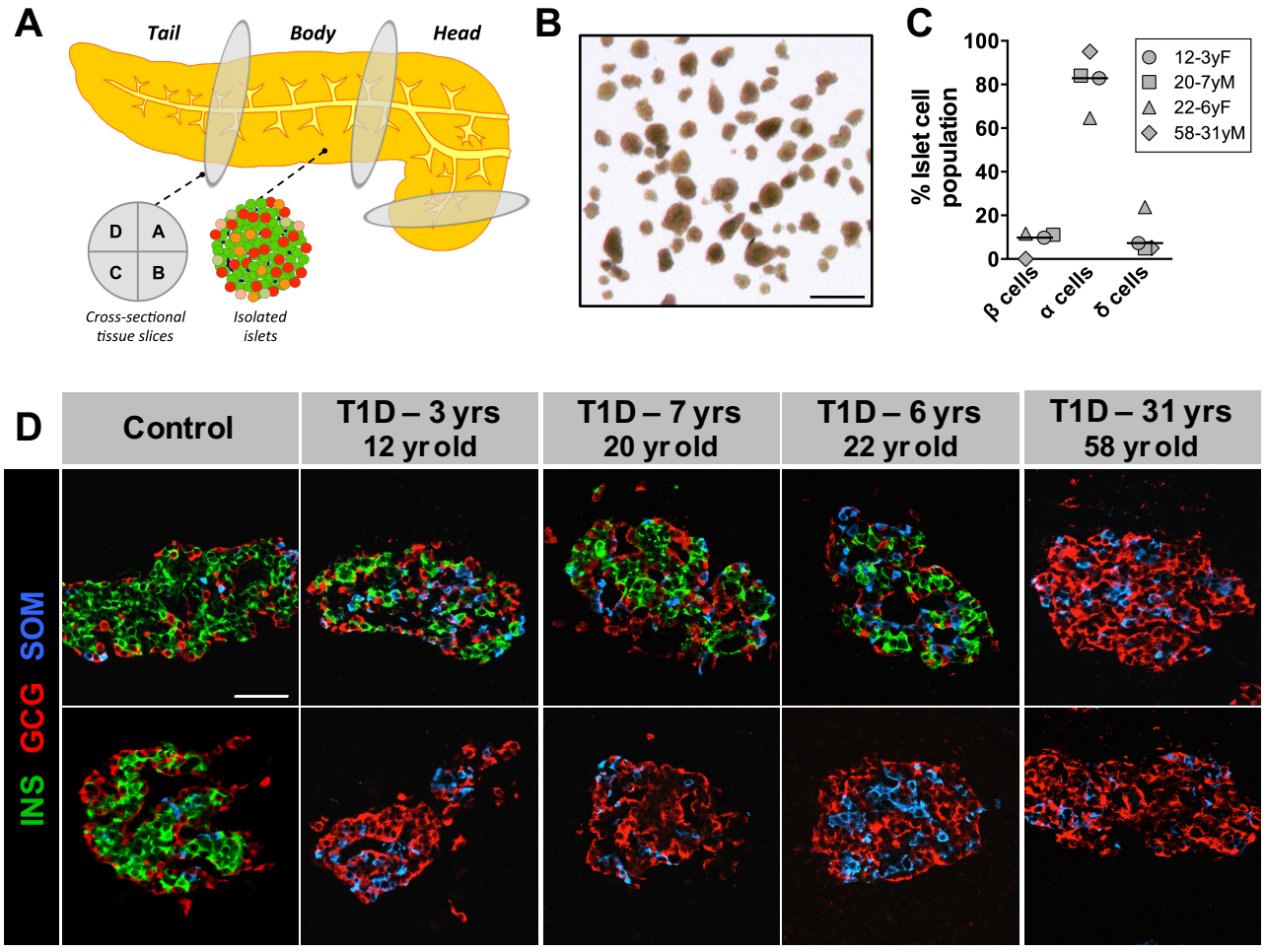


Figure 26. Related to Figures 22 and 23. Composition and morphology in T1D islets. (**A**) Schematic of islet isolation and tissue procurement from the same pancreas. Prior to islet isolation, multiple cross-˘sectional slices of pancreas with 2-3 mm thickness were obtained from the head, body and tail. Pancreatic slices were further divided into four quadrants (A,B, C, D) and processed for histology. (**B**) Pancreatic islets procured from a 12-year-old individual with 3-year T1D duration (donor #1). (**C**) Endocrine cell populations in dispersed isolated islets from 3 donors (#1,4,5) with recent-onset T1D contained 10.8±0.5% β cells, 77.1±6.3% α cells, and 12.0±5.9% δ cells. The donor with longstanding T1D (donor #10) had 0% β cells, 95% α cells, and 5% δ cells. For comparison, islets from normal individuals (n=28) with average age of 36±2 years (range 16 – 63 years) assessed by this approach had 53.4±2.6% β cells, 38.5±2.7% α cells, and 7.5±0.9% δ cells (Blodgett et al., 2015). (**D**) Morphology of T1D islets in the native pancreas. On average 250 islets from pancreatic head, body and tail of each T1D donor were analyzed for the presence of β cells. An islet was categorized as insulin+ even if it had only one insulin-˘positive cell. The number of insulin+ islets varied in 4 donors (#1,3,4,5) with recent-onset T1D (17.8±15.5%), but no insulin+ islets were found in the pancreatic sections of our longstanding cases (donors #7,8,10). If donors had insulin+ islets, representative islets are displayed in row 1. INS–insulin, GCG–glucagon, SOM–somatostatin. Scale bar is 50 µm.

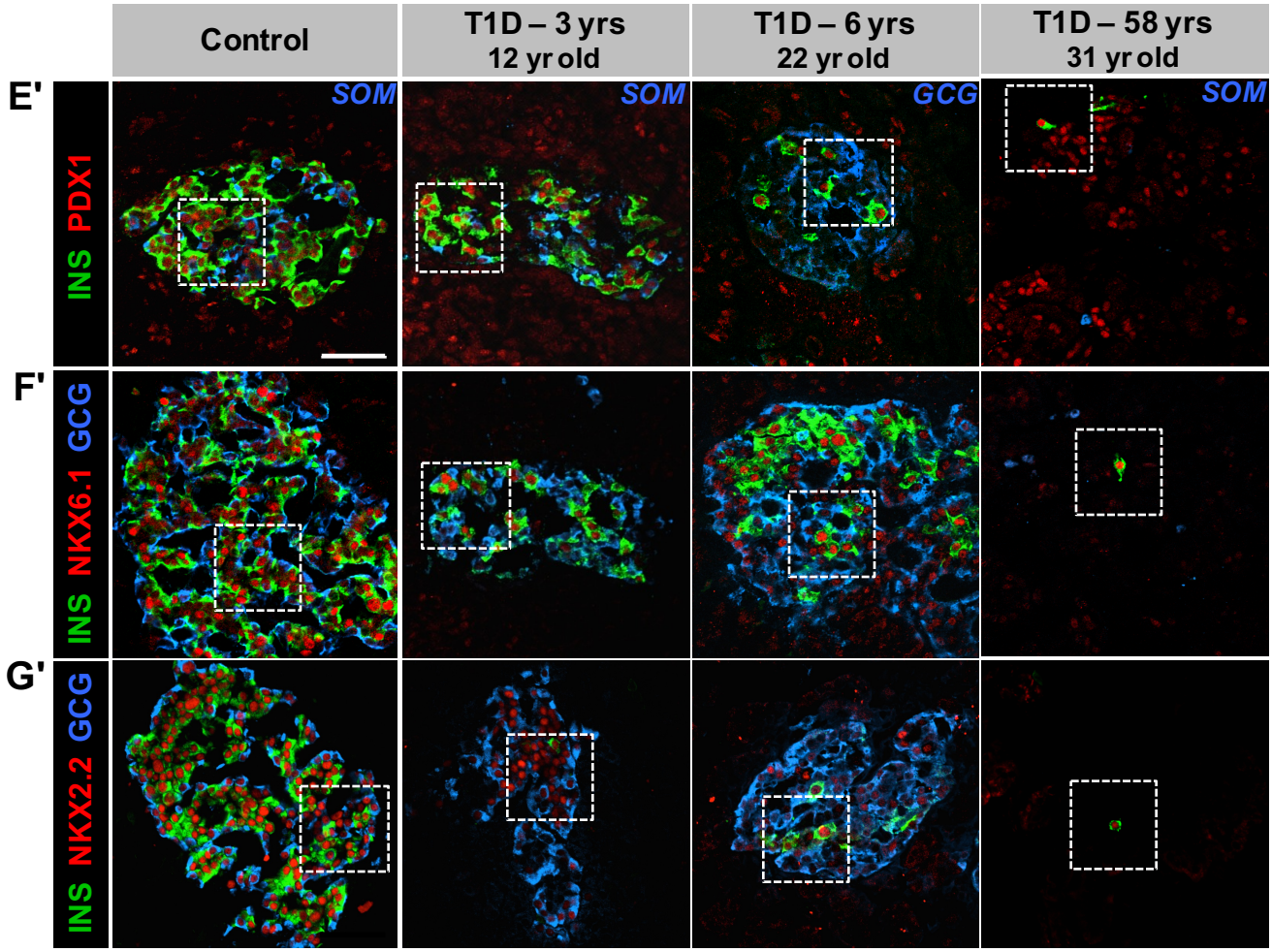


Figure 27. **Related to Figure 22**. **Expression pattern of PDX1, NKX6.1, and NKX2.2 in T1D β cells.** Expression of β cell-enriched transcription factors in the native pancreatic tissue from donors with recent-onset T1D (donors #1,5) was compared to 58-year-old donor with 31 years of T1D duration (donor #10) and controls. INS–insulin, GCG–glucagon, SOM–somatostatin. Regions denoted by the dashed line in panels **E**'–**G**' are displayed in panels **E**–**G** in **Figure 22**, respectively. Scale bar in **E**' is 50 µm and also corresponds to panels **F**' and **G**'.

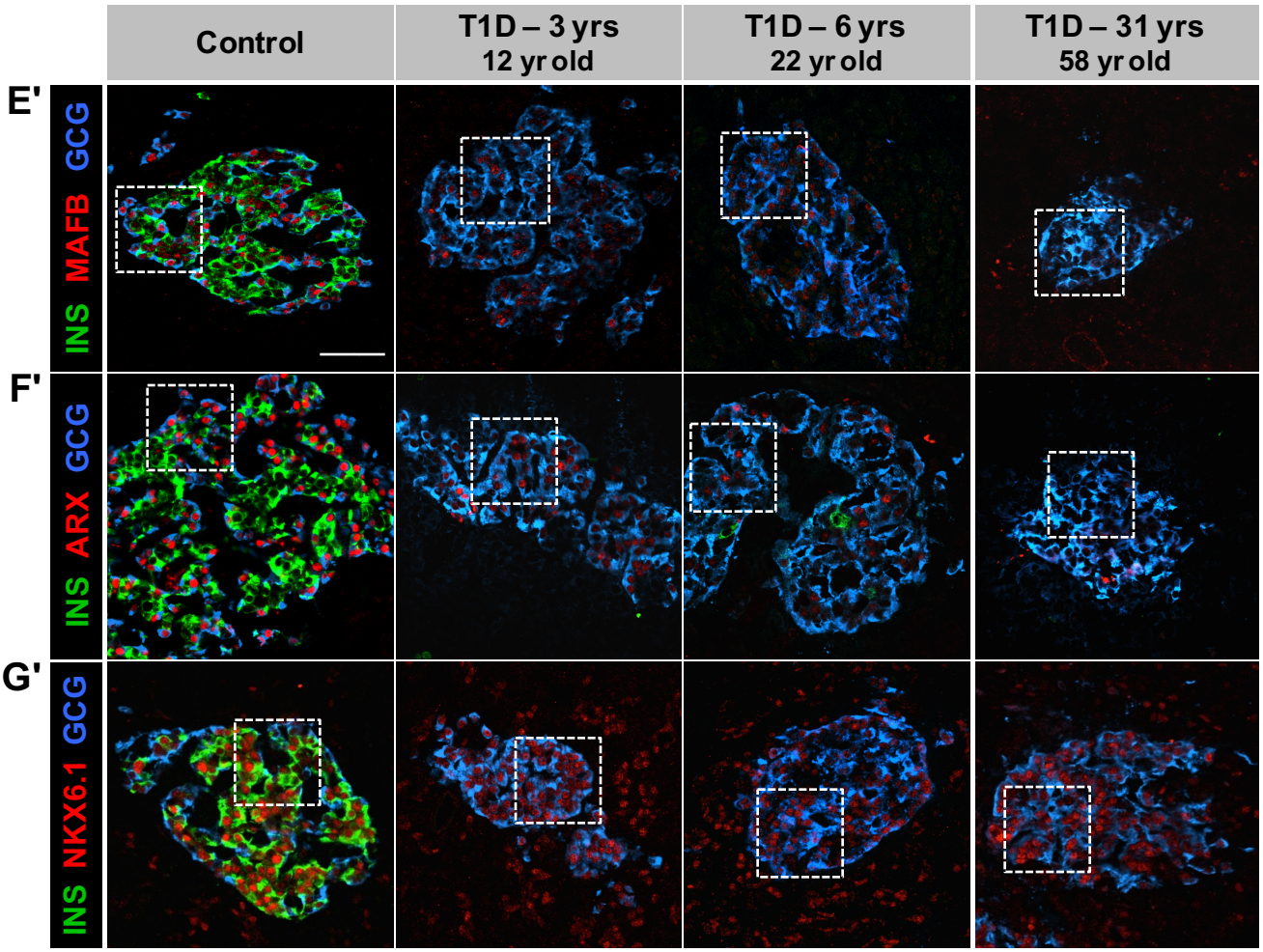


Figure 28. Related to Figure 23. Expression pattern of MAFB, ARX, and NKX6.1 in T1D α cells. Expression of α cell-enriched transcription factors in the native pancreatic tissue from donors with recent-onset T1D (donors #1,2,5) was compared to 58-year-old donor with 31 years of T1D duration and controls (donor #10). GCG–glucagon, SOM–somatostatin. Regions denoted by the dashed line in panels **E**'– **G**' are displayed in panels **E**– **G** in **Figure 23**, respectively. Scale bar in **E'** is 50 µm and also corresponds to panels **F**' and **G**'.

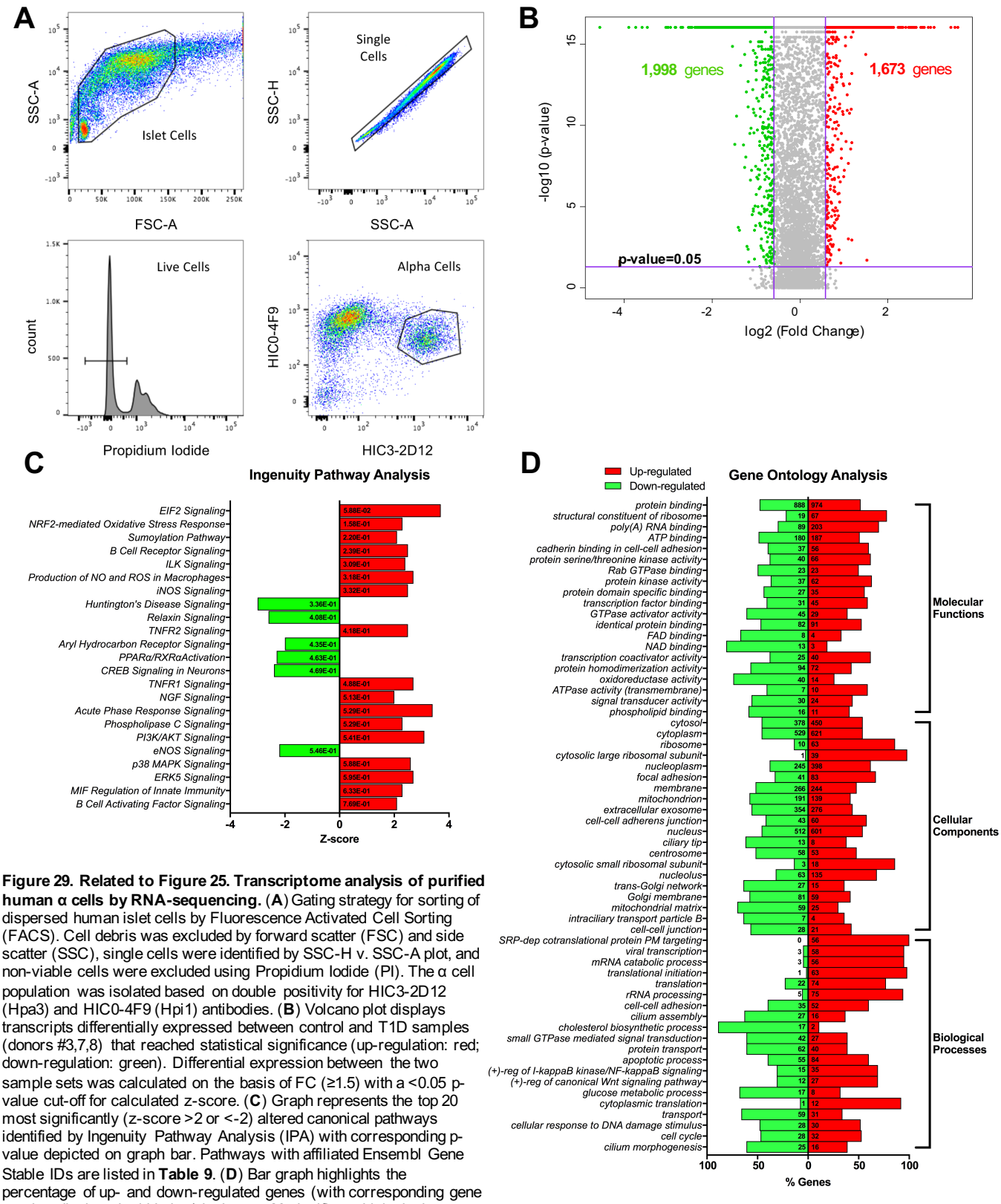


Figure 29. Related to Figure 25. Transcriptome analysis of purified human α cells by RNA-sequencing. (**A**) Gating strategy for sorting of dispersed human islet cells by Fluorescence Activated Cell Sorting (FACS). Cell debris was excluded by forward scatter (FSC) and side scatter (SSC), single cells were identified by SSC-H v. SSC-A plot, and non-viable cells were excluded using Propidium Iodide (PI). The α cell population was isolated based on double positivity for HIC3-2D12 (Hpa3) and HIC0-4F9 (Hpi1) antibodies. (**B**) Volcano plot displays transcripts differentially expressed between control and T1D samples (donors #3,7,8) that reached statistical significance (up-regulation: red; down-regulation: green). Differential expression between the two sample sets was calculated on the basis of FC (≥1.5) with a <0.05 p-value cut-off for calculated z-score. (**C**) Graph represents the top 20 most significantly (z-score >2 or <-2) altered canonical pathways identified by Ingenuity Pathway Analysis (IPA) with corresponding p-value depicted on graph bar. Pathways with affiliated Ensembl Gene Stable IDs are listed in **Table 9**. (**D**) Bar graph highlights the percentage of up- and down-regulated genes (with corresponding gene number displayed within bar) in the top 20 significant biological processes, cellular components, and molecular functions identified by Gene Ontology (GO) term analysis. Corresponding p-values, Ensembl Gene Stable IDs and process GO accession numbers are listed in **Table 10**.

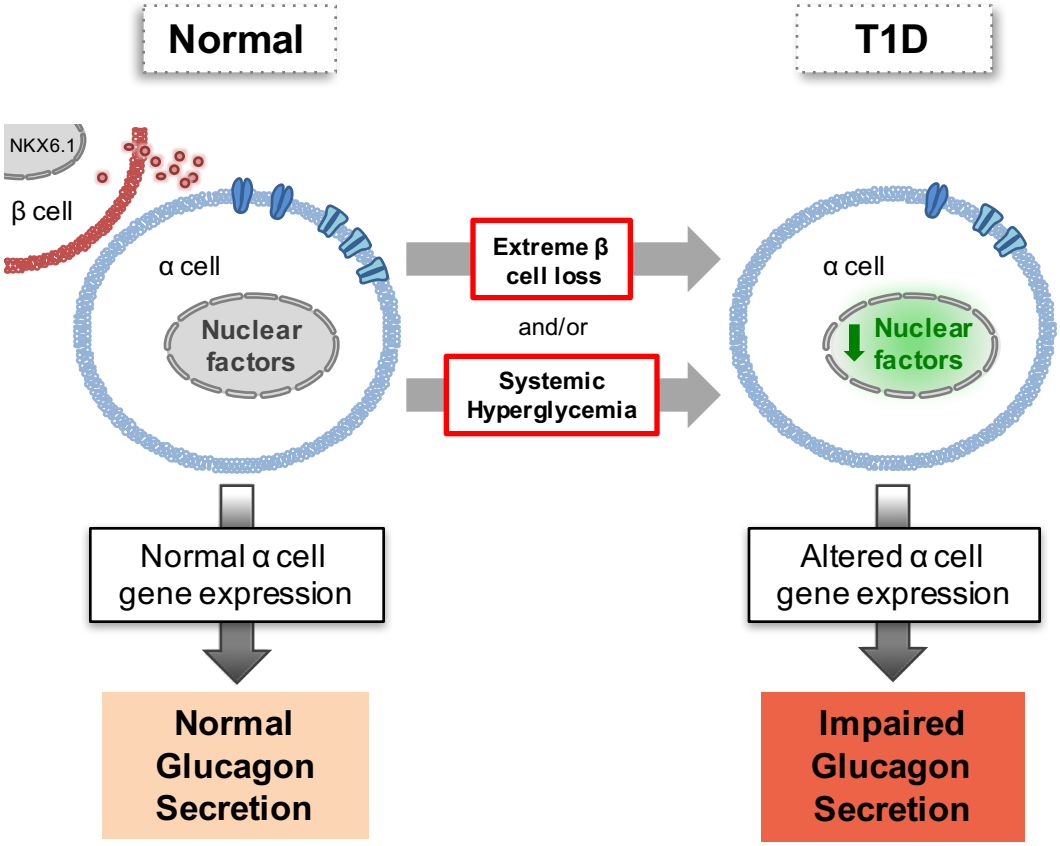


Figure 30. Pancreatic α cells in T1D. The islet microenvironment is rich with paracrine signals (red circles) that stimulate the expression of α cell-specific transcription factors that allow gene expression to produce necessary machinery for normal glucagon secretion. In the context of extreme β cell loss and/or systemic hyperglycemia, such as in T1D, we hypothesize that α cells lose these environmental cues and respond with decreased expression of necessary α cell nuclear factors leading to reduced α cell glucagon production, disrupted calcium signaling and electrical activity that results in impaired glucagon secretion.

CHAPTER IV

HETEROGENEITY IN CLINICALLY DIAGNOSED TYPE 1 DIABETES

Text and data in this chapter have been adapted from Haliyur et al., 2018 (manuscript under review) and Haliyur et al., 2018 (manuscript in preparation).

Introduction

The clinical diagnosis of diabetes, reflected by hyperglycemia, is straightforward; however, identifying the underlying molecular mechanism(s) is often challenging and often not possible [263]. These challenges are further confounded by the heterogeneity of type 2 diabetes and heterogeneity of type 1 diabetes (T1D) increasingly being recognized [216,264]. Partly, this is because the molecular defect(s) for most forms of diabetes are not known and partly because the molecular phenotyping of tissues involved in human diabetes is inadequate and limited. Critical human tissue and cellular samples relevant to diabetes are challenging to collect, sometimes are not accessible, or are limited by tissue processing that precludes functional analysis and the application of new technologies. For example, technical barriers prevent sampling of the human pancreas, as it cannot be safely and routinely biopsied in living individuals and rapidly undergoes auto-digestion post-mortem, hindering adequate molecular diagnostic and clinical phenotyping of the human pancreatic islet in all forms of diabetes.

To overcome such limitations, experimental models have been used to discover critical contributions to our understanding of human physiology and disease. However, in several instances, widely used model systems appear limited in their translation into clinically relevant information and may even inadvertently be misleading. For example, gene expression responses to inflammation in mice appear to have incomplete predictive clinical value and correlate with only a minority of human gene expression changes [223]. Similarly, some rodent models of human monogenic diabetes do not fully reflect the altered glucose homeostasis observed in humans. For example, heterozygous mutations in the key pancreatic islet HNF1A transcription factor, which causes the most common form of monogenic diabetes, does not mimic the human disease in mouse models, leaving the pathophysiologic effect of HNF1A genetic variants on the human pancreatic islet incompletely understood [51,52,265].

To overcome these translational barriers and improve preclinical modeling of human disease, renewed emphasis and new approaches to study human tissue have led to the development of collaborative human tissue repositories or accessible databases such as the Network of Pancreatic Organ donors with Diabetes (nPOD), the Genotype Tissue Expression Project (GTEX), and the Human Islet Research Network (HIRN). Studies of human pancreatic islets show human islets have illustrated similarities and differences

from rodent islets in endocrine cell composition and arrangement, innervation, vasculature, and function [2,9,42].

Using an infrastructure to study in new ways pancreatic islets and tissue from donors with diabetes in conjunction with the donor's de-identified medical record, investigators are working to better understand the changes in the pancreatic islet in T1D [237,266,267]. In this report, we describe unexpected functional and molecular findings from the pancreas of an individual with the clinical diagnosis of T1D, thus highlighting how systematic analysis of rare human samples can provide critical insight into human disease and potentially lead to new approaches to therapy.

Human Pancreatic Islets Expressing HNF1A Variant Have Defective β cell Transcriptional Regulatory Network

Case Summary

- Caucasian 33-year-old male died due to head trauma secondary to a motorcycle accident
- BMI: 25.8 kg/m^2
- Clinical history was significant for 17-year duration of Type 1 Diabetes (T1D) treated with insulin (HbA1C of 8.9%) and described as poorly controlled; he was reported to experience "tingling in feet and hands."
- He had concurrent hypertension diagnosed at age 16 and a kidney infection at age 20
- Social history included cigarettes (4/7 pack-years), alcohol use (1x/week for 4 years), intravenous drug abuse, and daily THC-use (5 years) prior to death.
- 4-day terminal hospital admission in the intensive care unit with respiratory support and treated with corticosteroids, diuretics, antibiotics, and vasopressors.
- His blood glucose ranged from 105-582 mg/dL and was treated with intravenous insulin
- He had a family history of diabetes (mother, maternal aunt, cousins, and grandmother).
- Carried high-risk HLA haplotypes (HLA Class II DR4, DQA1 03; DR4-DQ8)
- Measurable C-peptide (0.4 ng/mL)
- T1D-associated autoantibodies were negative at time of death.
- Renal (Cr 1.07 mg/dL) and hepatic (AST 26 u/L, ALT 24 u/L, ALP 53 u/L) function were normal.

Results

As part of studies of the pancreas and islets from individuals with T1D[237,266,267], we were surprised to find that analysis of pancreatic sections from the head, body and tail regions of one donor showed that all islets contained β cells (**Figure 31A, Figure 34A,** and **Tables 1** (donor #9)**, 2, 4,** and **5**), but lacked insulitis typical of T1D (infiltration of CD45+ cells)[268].

Donor pancreas had normal β cell mass, but β cells were functionally impaired.

The donor pancreas had β and δ cell mass within the normal range with slightly elevated α cell mass (**Figure 31B** and **34B**) and an increased α:β cell ratio (**Figure 34C, G-H**). No β cell apoptosis (TUNEL) or proliferation (Ki67) was detected (**Figure 34D**). In a dynamic perifusion system, isolated donor islets had normal insulin content but had higher basal insulin secretion, lacked biphasic glucose-stimulated insulin secretion (GSIS), and had a decreased secretory response to KCl-mediated membrane depolarization (**Figure 31C-D** and **inset**). Despite the lack of GSIS, the donor's islets responded normally to high glucose coupled with the phosphodiesterase inhibitor, IBMX. Moreover, glucagon secretion from donor islet α cells had an abrogated response to potent α cell stimuli such as low glucose (1.7 mM) and epinephrine (1 μM) and, strikingly, showed an inhibitory response to membrane depolarization by KCl (**Figure 34E-F**). Donor pancreas islet innervation and vasculature, important for coordinated islet function *in vivo*[6,9]*,* were normal (**Figure S1I**). Due to these unexpected histological and functional findings, we sequenced the donor DNA for variants associated with monogenic diabetes and uncovered a heterozygous, disease-associated variant in a conserved region of the POU_H DNA binding domain of hepatocyte nuclear factor 1 alpha (*HNF1A*: c.779C>T, p.Thr260Met)[269,270] (**Table 3**, **Figure 35A**). Variants in *HNF1A* comprise the most common form of Maturity-Onset Diabetes of the Young 3 (termed MODY3)[271,272].

$HNF1A^{T260M}$ variant displayed compromised DNA binding.

Nuclear HNF1A protein was detected in both the exocrine and endocrine compartments of the donor pancreas with normal expression in donor β cells and α cells (**Figure 32A** and **35A-B**). The DNA binding capacity of the altered $HNF1A^{T260M}$ protein as assessed by electrophoretic mobility shift assay (EMSA) was severely compromised compared to $HNF1A^{WT}$ protein (**Figure 32B** and **35C-E**). As expected, $HNF1A^{T260M}$ had little to no ability to stimulate *MAFA* Region 3 enhancer-driven reporter activity in relation to $HNF1A^{WT}$ co-transfection assays (**Figure 35F-G**). Notably, each of these proteins were expressed at similar levels, and wildtype activation was dependent on HNF1A site binding, as described earlier [244]. Furthermore, $HNF1A^{T260M}$ decreased $HNF1A^{WT}$

activation in a dose-dependent manner, providing evidence that the dominant negative action of HNF1A^{T260M} is due to dimerization with HNF1A^{WT} (**Figure 35F-G).** Moreover, protein modeling predicted that disrupted DNA binding results from the missing hydrogen donor at position 260 in the variant protein, which destabilizes the direct DNA binding residue Arg-263 residue (**Figure 32C**), yet leaves the dimerization domain of the transcription factor intact [273].

HNF1A$^{+/T260M}$ β cells have preserved markers of β cell identity, but changes in processes critical for glucose-stimulated insulin secretion.

RNA-sequencing and transcriptional profiling of purified HNF1A$^{+/T260M}$ β cells (**Figure 33A-B**) showed relatively preserved expression of *INS* mRNA and transcription factor markers of β cell identity and maturity (*PDX1*, *NKX2.2*, and *NKX6.1*), which was confirmed by protein expression analysis (**Figure 35H**). However, decreased expression of other transcription factors associated with mature β cell function (i.e. *MAFA*[244], *SIX3*[31], *FOXA2*[274], and *RFX6*[47]) suggests that the HNF1A^{T260M} variant impacts transcriptional regulatory networks required for β cell function rather than maintaining identity and maturity. Decreased expression of known (ex. *MLXIPL*, *HNF4A*, *PKM*, *OGDH*, *PPP1R1A*, *G6PC2, TMEM27*)[53] and previously undescribed HNF1A targets (*IAPP*, *ABCC8*, *KCNJ11, TMEM37, SYNGR4, FOXRED2)* likely contributes to the loss of glucose-dependent insulin release identified by islet perifusion (**Figure 33C**)*.* Notably, most voltage-gated calcium channels, such as L-Type and P/Q-Type, were not changed in HNF1A$^{+/T260M}$ β cells, but ATP-sensitive channels were decreased (*ABCC8*, *KCNJ11*, *KCNJ8*, *FXYD2*). Pathway analysis of HNF1A$^{+/T260M}$ β cells revealed changes in glucose metabolism and ATP production important in glucose-mediated insulin secretory processes as well as in core cellular pathways such as gene transcription, intracellular protein transport (i.e. synthesis, ubiquitination, and exocytosis), cell stress response, and cell signaling (**Figure 36D**). Approximately 50% of the genes differentially expressed in HNF1A$^{+/T260M}$ β cells were also in donor α cells (**Figure 36A-G**) suggesting that HNF1A dysfunction is a common effector in both cell types. We also noted other processes such as amino acid nutrient sensing and metabolism, cell cycle regulators, and cell adhesion/motility were altered in HNF1A$^{+/T260M}$ islet cells (*SLC38A4*, *GLUL, IGFBP5, CREB3L1*).

Discussion

Using an integrated approach for molecular and functional analysis of pancreatic samples, we report the first direct studies of human islet morphology, function, and gene expression in an individual with a heterozygous, missense variant in the *HFN1A* locus (T260M). We show the HNF1A$^{+/T260M}$ donor had a relatively normal β cell mass with maintained key markers of β cell identity but lacked an insulin secretory response to

glucose challenge. This insulin secretory deficit was accompanied by alterations in genes encoding pathways of glucose metabolism and ATP production, which were also coupled with changes in core metabolic functions, such as gene transcription, protein synthesis and degradation, unfolded protein response, and intracellular and cell-cell communications in HNF1A$^{+/T260M}$ β cells. Our findings indicate that the HNF1A^{T260M} variant leads to insulin-insufficient diabetes by impacting pathways critical for β cell glucose-stimulated insulin release.

This report highlights how molecular and functional findings in unique human samples, even in a single case, can contribute to our understanding of physiology and disease pathogenesis. Levels of *HNF1A* gene transcript in the human pancreas are substantially less[275] compared to mouse such that mouse models of heterozygous *HNF1A* do not phenocopy the human disease[51,52,265]. Missense mutations in the *HNF1A* dimerization and DNA binding domains account for the majority of described pathogenic *HNF1A* variants [276]. Our modeling predicted that the T260M change would impair DNA binding of HNF1A rendering this transcriptional factor nonfunctional, which was demonstrated by EMSA analysis. The dose-dependent decrease in transcriptional activity in wild type HNF1A dependent *MAFA* gene activation by HNF1A^{T260M} suggests the dimerization between these proteins leads to impaired DNA binding activity and reduced HNF1A target gene regulation in individuals carrying this variant. From this dataset, we propose this class of loss-of-function variants in *HNF1A* lead to insulin-insufficient diabetes not by significant loss of β cell mass but rather by impacting β cell transcriptional regulatory networks (*HNF4A, MAFA, RFX6, SIX3, FOXA2, MLXIPL*) that results in impairment of β cell pathways necessary for a normal insulin response to glucose (**Figure 33D**).

Furthermore, by investigating hormone secretion in isolated pancreatic islets, we discovered depolarization by KCl, which directly stimulates hormone secretion by activating voltage-dependent calcium channels (VDCC), was impaired in HNF1A$^{+/T260M}$ α and β cells, in contrast to results from mouse models[51]. Interestingly, elevated basal insulin secretion was observed in islets from this donor, consistent with decreased expression of genes associated with glucose sensitivity of insulin secretion (*G6PC2, SLC37A4*) [277]. Our data revealed a previously unrecognized role for HNF1A in α cell function as HNF1A$^{+/T260M}$ impacted expression of many shared genes involved in hormone regulated secretion (**Figure 36C**). Transcriptome analysis of HNF1A$^{+/T260M}$ also uncovered HNF1A regulated gene targets in β cells, such as *PPP1R1A* and *RFX6,* and pathways, like protein synthesis and amino acid metabolism. In addition, a number of genes differentially regulated in HNF1A$^{+/T260M}$ β cells included those identified in β cell subpopulations by Dorell and colleagues (*HCN4*, *SPP1*, *KCNJ8*, *RFX6*, *SIX3*, *PPP1R1A*, *FAM159B*, *G6PC2*), suggesting that HNF1A may regulate the development of these β cell populations[75].

Preserved β cell mass in a pancreas with 17 years of MODY3 highlight the importance of clinical identification and intervention even years after diagnosis. Low-dose sulfonylurea therapy produces effective glycemic control in some individuals with MODY3[278] by stimulating this existing β cell reservoir; however, our data provides rationale for a therapeutic alternative to current treatment. Sulfonylureas likely have clinical efficacy because these agents initiate membrane depolarization with potassium channel closure and bypass effects from impaired ATP production not possible with KCl alone, producing insulin responses comparable to control subjects[279] (**Chapter I**). However, hypoglycemia, a common, but unexplained adverse effect from sulfonylurea therapy in MODY3, limits this therapy and may result from impaired glucagon secretion related to α cell depolarization (**Figure 34E**). The islet perifusion data from this donor suggests targeting cAMP-dependent pathways of insulin secretion, such as with glucagon-like-peptide 1 (GLP-1) agonists, would be advantageous to sulfonylureas as this pathway of insulin secretion is preserved and accompanied by an intact glucagon response thus lowering the risk of hypoglycemia in such MODY3 patients[280] (**Figure 31C** and **Figure 34E**).

Clinical and pathogenic heterogeneity in clinically diagnosed T1D is now increasingly apparent with the ability to study affected human pancreatic tissue[237,254,268,281]. Clinical features of many MODY phenotypes, which make up 1-5% of all diabetes cases, can be easily mistaken for T1D. Lack of islet-related humoral autoantibodies, significant family history of insulin-deficient diabetes, and/or concurrent kidney dysfunction should prompt genetic testing for *HNF1A* and other monogenic forms of diabetes. Because of this, in collaboration with the Vanderbilt Institutional Review Board (IRB), we are working to communicate our findings to the de-identified donor's family and recommend diagnostic MODY genetic testing in potentially affected family members. Overall, this report shows how integrating clinical information with molecular and cellular analyses identified what appeared to be T1D was in fact part of a broader spectrum of insulin-deficient diabetes and provides translational insight into an incompletely understood human disease.

Unexpected findings in the integrated analysis of the pancreas and islets from a 22-year-old male with 8 years of Type 1 Diabetes

Case Summary

- Caucasian 22-year-old male died due to anoxic brain injury secondary to cardiac arrest from drug intoxication
- BMI: 25.7 kg/m^2
- Clinical history was significant for 8-year duration of Type 1 Diabetes (T1D) treated with Novolog and Lantus but described as "non-compliant with care" (HbA1C of 11.9%)
- Social history included cigarettes (1/2 pack-year), alcohol use (1x/week for 4 years), intravenous drug abuse, and daily THC-use (5 years) prior to death.
- 4-day terminal hospital admission in the intensive care unit with respiratory support and treated with corticosteroids, diuretics, antibiotics, and vasopressors.
- Admission glucose was 719 mg/dL and was treated with intravenous insulin (ranged 112–219 mg/dL).
- No family history of diabetes was reported in the redacted medical chart.
- Carried high-risk HLA haplotypes (HLA Class II DR4, DQ2, and DQ8)
- Non-fasting C-peptide (0.06 ng/mL) suggests absolute insulin deficiency
- T1D-associated autoantibodies were negative at time of death.
- Renal (Cr 0.6 mg/dL) and hepatic (AST 14 u/L, ALT 38 u/L, ALP 88 u/L) function were normal.

Results

Normal in vitro insulin secretion levels and considerable β cell mass in an individual with 8 years of T1D

We examined pancreatic tissue and islets recovered from this individual in collaboration with the International Institute for the Advancement of Medicine (IIAM) (donor #6, **Table 1**) and compared these to normal (n=7; 10-55 yrs of age, **Table 2**) and other relatively recent-onset T1Ds (n=5; 12-22y of age, 2-7 years of T1D duration, **Table 1**)[237]. By perifusion, pancreatic islet function had normal insulin and glucagon levels, a surprising observation in a T1D donor with serum C-peptide (0.06 ng/mL) similar to recent-onset T1Ds (n=5; 0.02 – 0.43 ng/mL) (**Table 1**, **Figure 37C-D**, **inset**, **Figure 39C-D)**. Notably, the first phase of glucose-stimulated insulin secretion was slightly blunted (**Figure 37C-D**, **inset**) and glucagon secretion above normal to cAMP-potentiated high glucose (G 16.7 + IBMX) (**Figure 39C-D**). Evaluation of isolated islet endocrine composition by flow cytometry (**Figure 39B**) and islet hormone content (**Figure 39E-F**) were in the range of normal.

Surveying the head, body and tail regions of the donor pancreas revealed reduced β cell mass to normal, but as many as 68.6% of the islets contained abundant numbers of β cells, in stark contrast to recent-onset T1D (17.8±15.5%)[143,228] (**Figure 37A-B**, **Figure 39A**). Furthermore, 4.2% of pancreatic islets (n=71 islets) examined from six blocks encompassing the pancreas head, body and tail regions demonstrated mild CD3+ infiltration measured as 15 or more CD3+ cells within the islet or at the islet periphery (**Figure 39G**)[161,162].

DNA sequencing identified a heterozygous intronic variant of unknown significance in glucokinase gene (GCK)

Because of the unexpected islet insulin content and islet histology, we sequenced the donor DNA for variants associated with monogenic diabetes, which identified a previously reported variant in the intronic region of the glucokinase (*GCK*) gene (c.209-8G>A, **Table 3**)[282,283]. Expressed preferentially in glucose sensing tissues such as the pancreatic islet cells and the liver, glucokinase phosphorylates glucose to glucose-6-phosphate (G6P). Low affinity for glucose and lack of end-product inhibition distinguish GCK from ubiquitous hexokinase isoforms and permits glucose sensing as the rate of glucose phosphorylation is proportional to glucose concentration. Over 600 variants have been reported in GCK and can have variable effects on protein function[284]. For example, activating variants in GCK lead to hypoglycemia from hyperinsulinism while inactivating variants result in either mild or severe forms of diabetes[285,286]. Heterozygous loss of function variants in GCK result in Maturity-onset diabetes of the young 2 (MODY2), with mild hyperglycemia that can be managed by diet[284,286]. This clinical presentation conflicts with our donor who required insulin (0.06 ng/mL C-peptide) and had an average blood glucose of 295 mg/dL (HbA1C of 11.9%). Previous reports of the identified variant (rs144798843) noted it did not segregate with the diabetes phenotype across generations[282,283] and consider it a variant of unknown significance.

Donor islets have differential gene expression in processes associated with GCK function and inflammation

To further investigate the role of GCK in this donor, we performed whole-islet RNA-sequencing, which showed a two-fold decrease in *GCK* transcript and altered islet gene expression (**Figure 37E**, **Figure 40B**). Predicted to be benign by *in silico* splicing analysis, this variant did not result in truncated GCK isoforms (**Figure 40A**). Tissue-specific isoforms of GCK result from alternative splicing of the *GCK* mRNA transcript; however, the most abundant isoform found in controls (n=3; 24-55yrs of age), i.e. the primary pancreatic islet isoform, was reduced in this donor (**Figure 40A**). Furthermore,

RNA-sequencing identified reduced expression of genes important in glucose and G6P metabolism coupled with increased expression of compensatory proteins such as lactate dehydrogenase (*LHAL6A*) and other hexokinases and their accessory proteins (*HK1*, *HK2*, and *ADPGK*). These gene expression changes were accompanied by decreased expression of genes important to β cell function and secretion (**Figure 37E**). Notably, many immune-associated genes and pathways were up-regulated (**Figure 40C-D**).

Donor islet cell molecular identity recovered in normoglycemia and non-autoimmunity

Evaluation of the pancreatic tissue revealed protein expression of transcription factors important to α cell identity was partially altered similar to the T1D α cell with misexpression of NKX6.1 (**Figure 38B-D, Figure 41B-D**). Interestingly, when we normalized the donor's glucagon trace to total content we saw decreased α cell glucagon secretion comparable to T1D with a diminished glucagon response when moving from high (G 16.7) to low (G 5.6) glucose and, unlike T1D, impaired KCl-mediated membrane depolarization (**Figure 39H**)[237]. Transplantation of donor islets into a normoglycemic, non-autoimmune environment yielded normal *in vivo* islet function (**Figure 39I**) and was followed by treatment of either the GLP-1 agonist exendin-4 (Ex-4) or PBS (**Figure 38A**). Assessment of the graft 8-weeks post transplantation revealed no difference in treatment groups, but that the molecular profile of α and β cell-specific transcription factors had recovered (**Figure 38C, Figure 41C**).

Discussion

By integrating pancreatic islet histology, function, and molecular analysis correlated with clinical information, we report unexpected findings in the pancreas of an individual clinically diagnosed with T1D. Decreased pancreatic β cell mass and altered protein expression in the native pancreatic tissue likely contributed to insulin-insufficiency in this donor. Our data implicates the native islet environment in the disease process as isolated islets had normal *in vitro* function and normal gene expression when transplantated into a normoglycemic, non-autoimmune environment. Yet, the contribution of the immune system and/or the identified intronic mutation in *GCK* to this donor's hyperglycemia is still uncertain.

Further studies examining the mechanism of this donor's diabetes are necessary to elucidate the processes contributing to hyperglycemia. For example, whole-exome sequencing of the donor could identify other potential genetic causes of diabetes. Because there were no significant effects of this variant on *in vitro* islet function, it is possible this particular *GCK* variant impacts glucose sensing by other organs such as

the brain or liver. Expression of this variant in human hepatocyte and β cell *in vitro* systems could allow us to better investigate this question. It is also possible that this donor had a mild or abrogated form of type 1 diabetes resulting in partial β cell loss (**Figure 37A-B**) and the reported changes in *GCK* gene expression were secondary to hyperglycemia of reduced β cells in this donor. These atypical cases have been reported previously [287-289] and could be elucidated by evaluating donor DNA for a T1D genetic risk score [134,136,290,291]. Additionally, assessment of *GCK* and its pathways in engrafted normal human islets in NSG-DTR mice that experience either normoglycemia or hyperglycemia would be informative.

Furthermore, this case provides interesting insight into changes described in the T1D α cell. In a donor with significant hyperglycemia but incomplete β cell mass, we saw only a partial T1D α cell profile with features of α cell dysfunction[237]. This provides further evidence that a hostile native islet environment contributes to the defects described in T1D α cells, but that both significant β cell loss and hyperglycemia are likely necessary result in the described T1D α cell phenotype. While we do not have donor α cell transcriptomic information to probe this further, future work evaluating how β cell loss and hyperglycemia effects human α cells will elucidate this. This case provides unique insights into the complex mechanisms of insulin-deficiency and illustrates the heterogeneous pancreatic phenotype that comprises clinically diagnosed T1D.

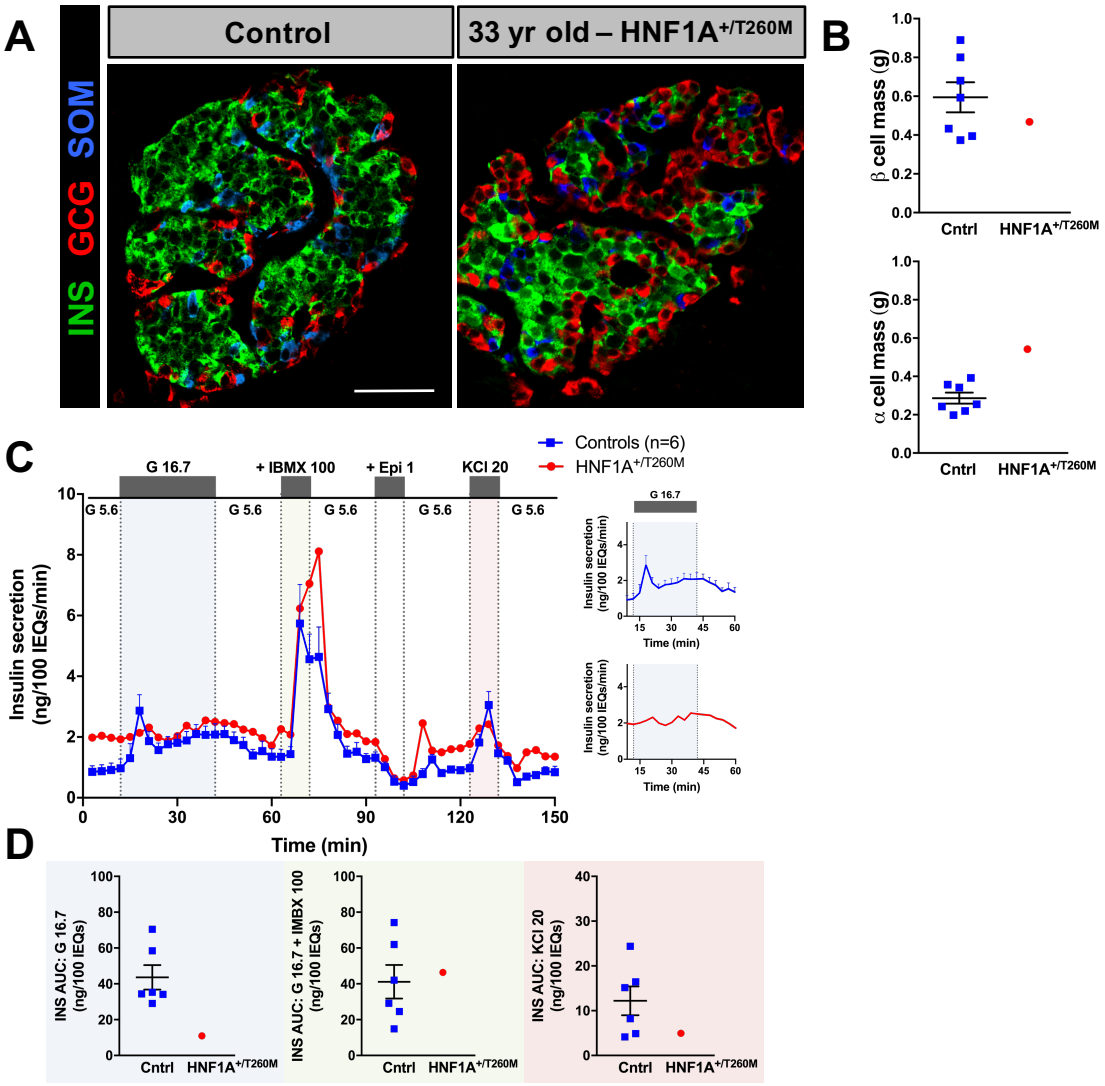


Figure 31. Histological and functional analysis of HNF1A$^{+/T260M}$ pancreas and islets. (**A**). Expression of insulin (INS), glucagon (GCG), and somatostatin (SOM) in the donor's native pancreatic tissue compared to control. Scale bar represents 50 μm. (**B**) β cell mass (grams) and α cell mass (grams) in HNF1A$^{+/T260M}$ pancreas compared to controls (n=7; ages 10-55yrs). Each data point represents the average mass across the combined pancreatic head, body and tail regions of each donor. (**C**) Insulin secretion measured in islets isolated from the HNF1A$^{+/T260M}$ pancreas compared to normal controls (n=6; ages 8-55yrs) and normalized to overall islet cell volume (expressed as islet equivalents, IEQs); G 5.6 – 5.6 mM glucose; G 16.7 – 16.7 mM glucose; G 16.7 + IBMX 100 – 16.7 mM glucose + 100 μM isobutylmethylxanthine (IBMX); G 1.7 + Epi 1 – 1.7 mM glucose + 1 μM epinephrine; KCl 20 – 20 mM potassium chloride. Insets shows average insulin response of controls and HNF1A$^{+/T260M}$ donor to 30-minute stimulation with 16.7mM glucose. (**D**) Integrated insulin secretion was calculated as area under the curve (AUC) for the following secretagogues G 16.7, G 16.7 + IBMX 100, and KCl 20 (shaded to correspond to color-matched regions of perifusion trace in panel **C**). Results of the control samples are expressed as mean ± standard error of the mean.

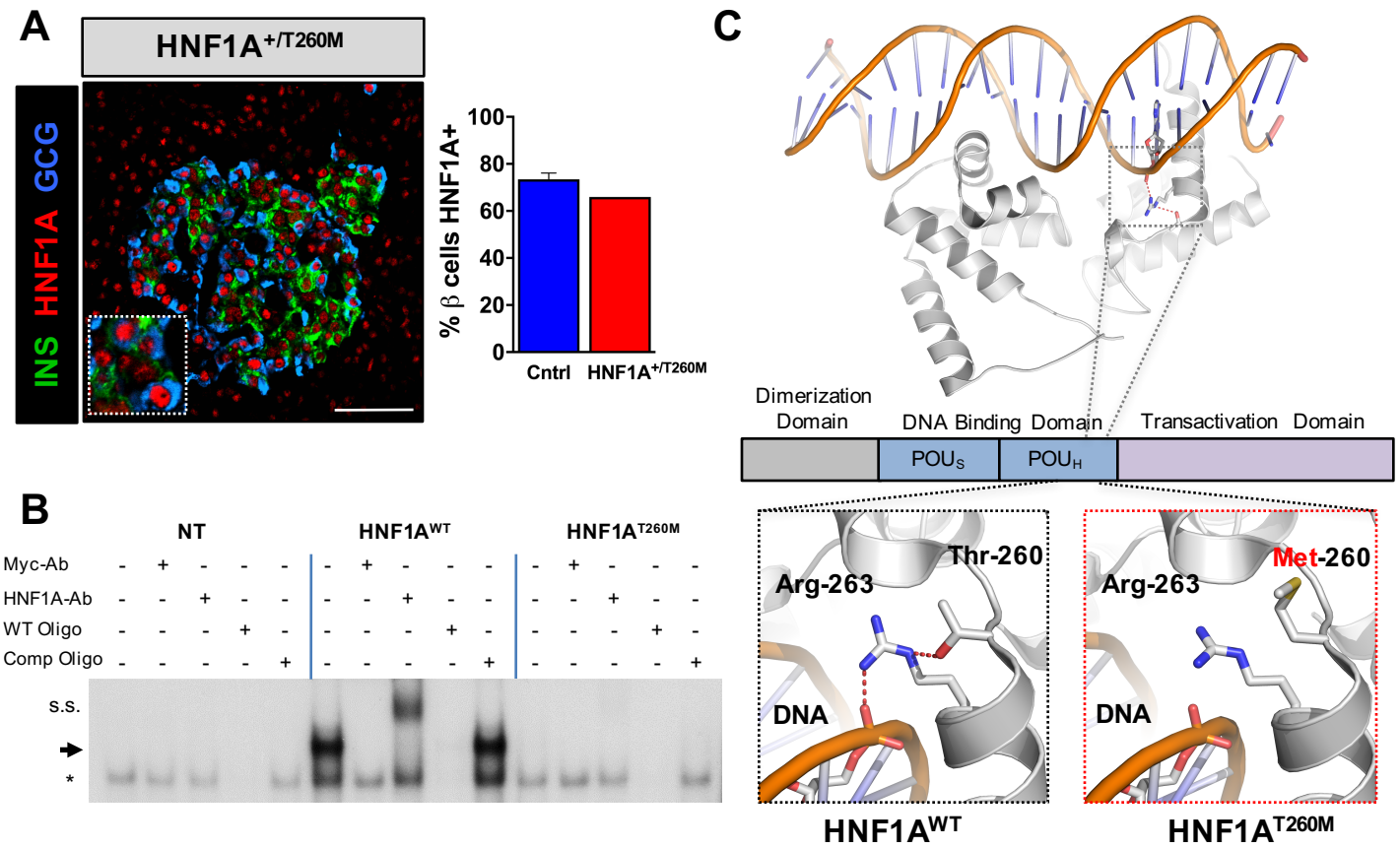


Figure 32. Expression and functional characterization of HNF1A^{T260M} variant. (**A**) Analysis of the donor's native pancreatic tissue for expression of HNF1A compared to controls (n=3; ages 10-55yrs) revealed HNF1A protein expression in donor β cells and pancreas. Scale bar represents 50 μm. (**B**) Electrophoretic Mobility Shift Assay (EMSA) shows that HNF1A^{T260M} variant has impaired DNA binding, with loss of the HNF1A-specific DNA binding complex (arrow) in myc-tagged HNF1A^{T260M} transfected HeLa cells compared to myc-tagged HNF1A^{WT}. Specificity of this complex (arrow) was shown by exclusive elimination of these species by adding either Myc-antibody (Myc-Ab) or unlabeled oligonucleotide (WT Oligo) containing the HNF1A consensus recognition motif, but not a mutated form of this oligonucleotide (Comp Oligo). Moreover, HNF1A-antibody (HNF1A-Ab) only supershifted (s.s.) this complex. All samples in **B** include oligonucleotide labeled with ^{32}P as described in the Star Methods. WT— wildtype; arrow – HNF1A-DNA complex; s.s. – supershift of HNF1A-DNA complex; asterisk – nonspecific complexes; Comp Oligo – mutated HNF1A consensus recognition motif; NT – non-transfected HeLa cells. (**C**) Molecular modeling of the HNF1A^{T260} variant in PyMOL predicts the hydroxyl group (red) on the T260 residue stabilizes R263 by hydrogen bonding to nitrogen (blue). R263 hydrogen bonds to the DNA backbone of the 5th adenosine of the HNF1A consensus recognition motif (5'CTTGGTTAATAATTCACCAGA-3), in control conditions(Chi et al., 2002). A missense mutation from threonine to methionine at position 260 is predicted to result in the loss of this interaction by de-stabilizing R263 and subsequently DNA binding.

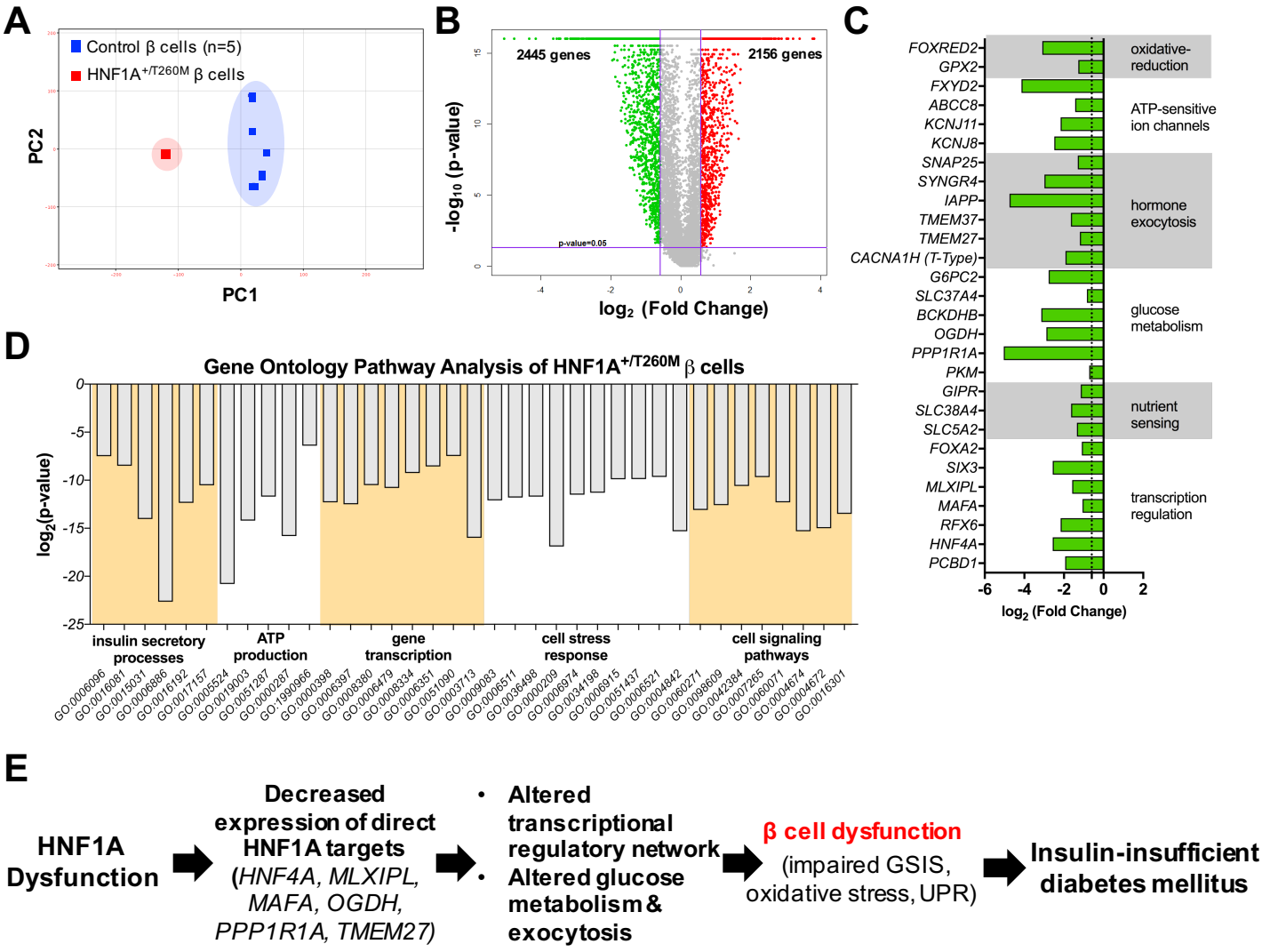


Figure 33. Transcriptomic analysis of HNF1A$^{+/T260M}$ β cells. (**A**) Principal component analysis (PCA) plot depicts clustering of control (n=5; ages 26-55yrs) β cells separate from HNF1A$^{+/T260M}$ β cells. (**B**) The volcano plot demonstrates transcripts differentially expressed between control and HNF1A$^{+/T260M}$ β cells (red – up-regulated gene expression; green – down-regulated gene expression). Differential expression between the two sample sets was calculated on the basis of FC (≥1.5) with a <0.05 p-value cut-off for calculated z-score. (**C**) Genes of interest and HNF1A targets are significantly down-regulated in HNF1A$^{+/T260M}$ β cells. The vertical dotted line represents a fold change (FC) =-1.5x threshold; p<0.05 for all values shown. (**D**) Significant processes identified by Gene Ontology (GO) term enrichment analysis grouped and displayed by their p-value in log2 scale. (**E**) From these results, we propose the following model for the disrupted β cell function in HNF1A-associated diabetes: Dysfunction of HNF1A leads to decreased expression of direct targets, which encompass both enzymatic and gene regulatory products, producing broad changes in transcriptional regulation, glucose metabolism, and insulin secretion. These processes ultimately lead to β cell dysfunction and result in clinical manifestation of insulin-insufficient diabetes mellitus. GSIS – glucose stimulated insulin secretion; UPR – unfolded protein response.

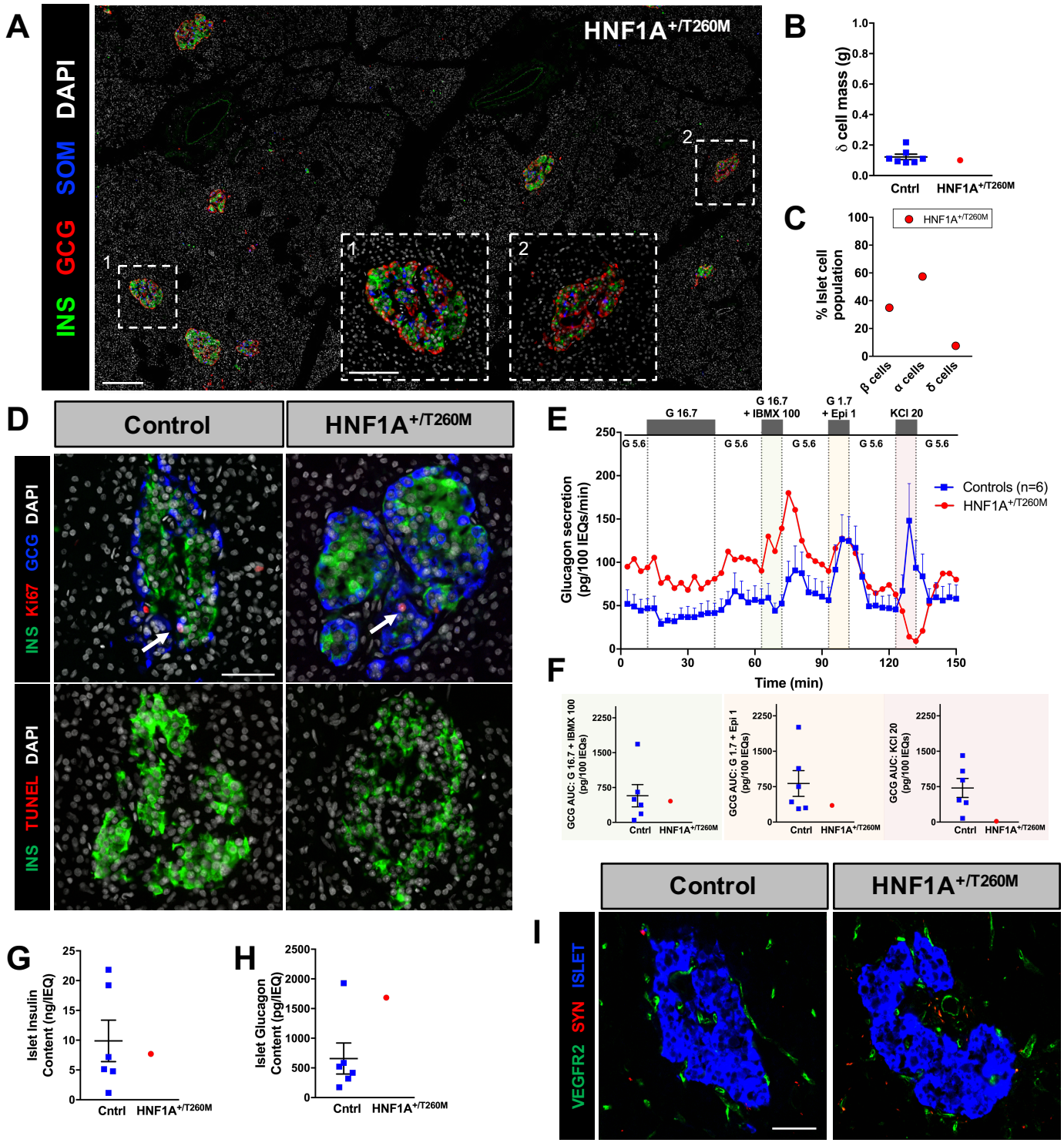


Figure 34. Related to Figure 31. Immunohistochemical and functional analysis of HNF1A$^{+/T260M}$ pancreas. (**A**) Pancreatic tissue section of HNF1A$^{+/T260M}$ donor (donor #9, **Table 1**) stained for insulin (INS), glucagon (GCG), and somatostatin (SOM) and counterstained with nuclear marker DAPI. All islets identified in this donor were insulin+. Scale bar is 250 μm Insets represent islet heterogeneity and are numbered accordingly. Scale bar is 100 μm. (**B**) δ cell mass was calculated as described in **Figure 31**. (**C**) Endocrine cell populations in dispersed isolated islets from HNF1A$^{+/T260M}$ contained 34.9% β cells, 57.4% α cells, and 7.6% δ cells. Normal control islets collected by this method had a range of 53.4±2.6% β cells, 38.5±2.7% α cells, and 7.5±0.9% δ cells (Blodgett et al., 2015). (**D**) The native pancreatic tissue from the HNF1A$^{+/T260M}$ donor was assessed for proliferative marker Ki67 and apoptotic signal TUNEL. Scale bar is 50 μm. Arrow depicts Ki67+ islet cells. (**E**) The same islets shown in **Figure 31C** were simultaneously analyzed for glucagon secretion and normalized to overall islet cell volume (expressed as islet equivalents, IEQs). As in **Figure 31**, **F** depicts integrated glucagon release as area under the curve from basal glucagon release for secretagogues G 16.7 + IBMX, G 1.7 + Epi 1, and KCl 20 (corresponding to shaded color-matched regions of perifusion trace). Insulin (**G**) and glucagon (**H**) content in control and HNF1A$^{+/T260M}$ islets. (**I**) Normal innervation (synapsin) and vasculature (VEGFR2) were observed in islets (detected by insulin and glucagon) in donor. SYN – synapsin. Scale bar is 50 μm.

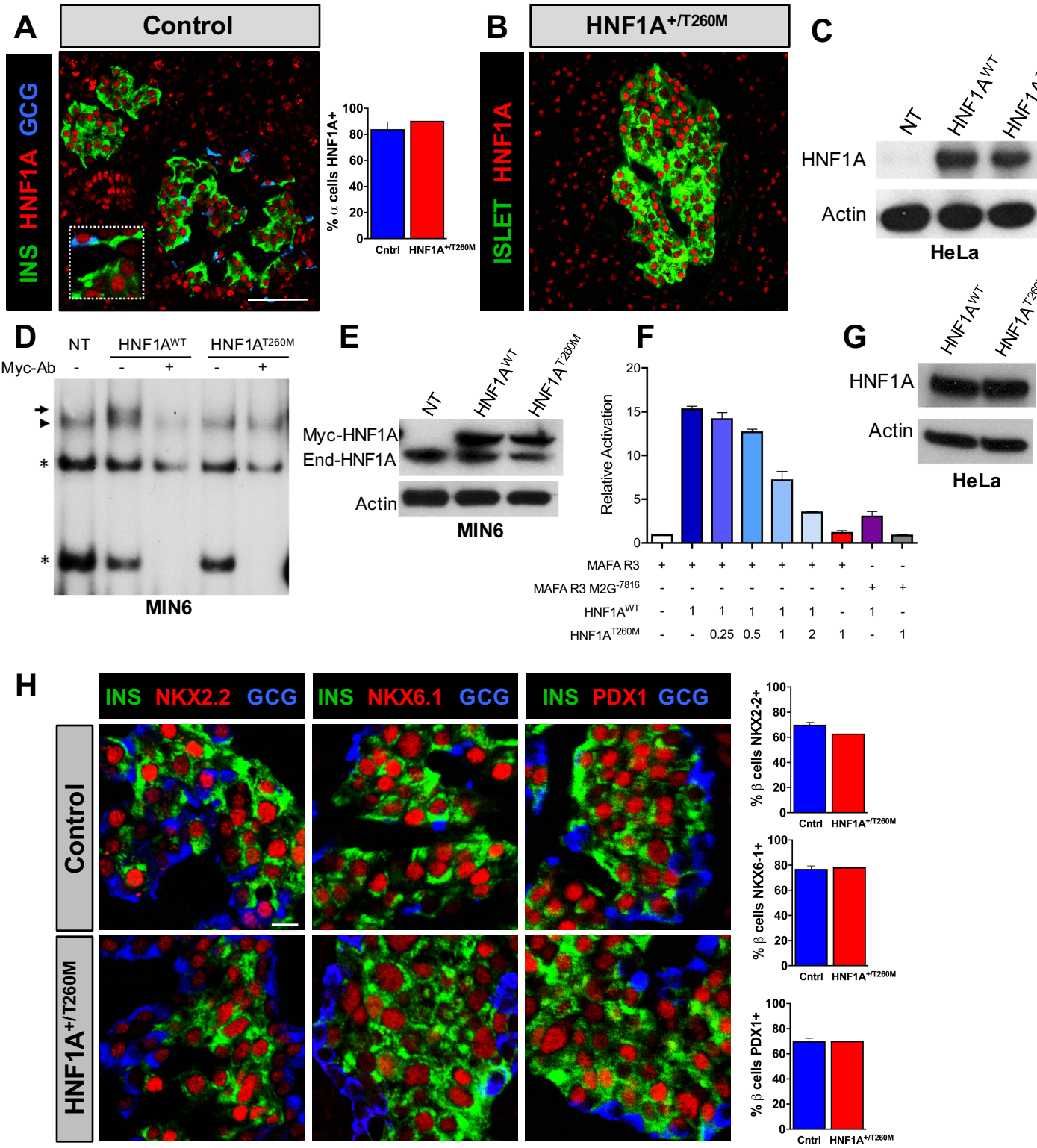
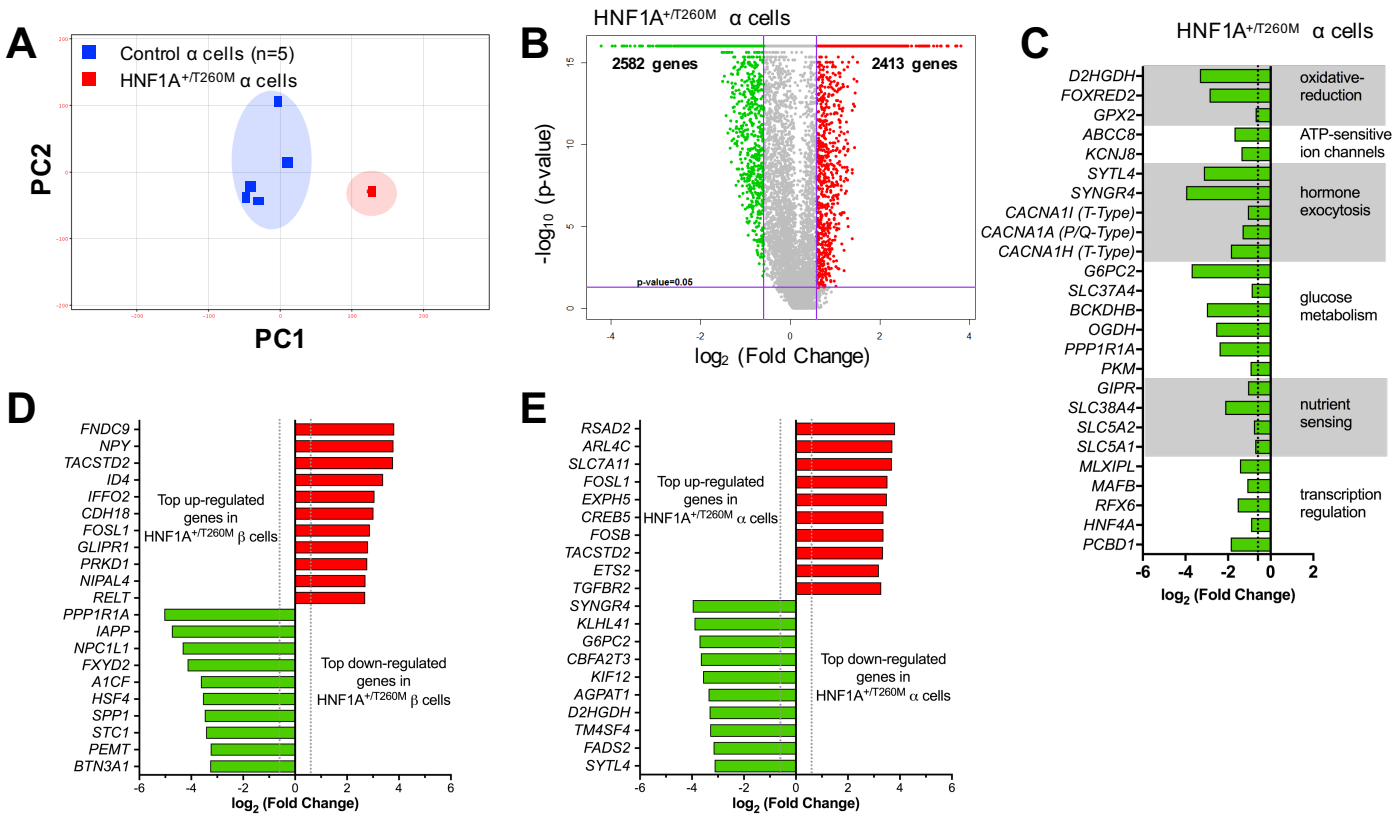
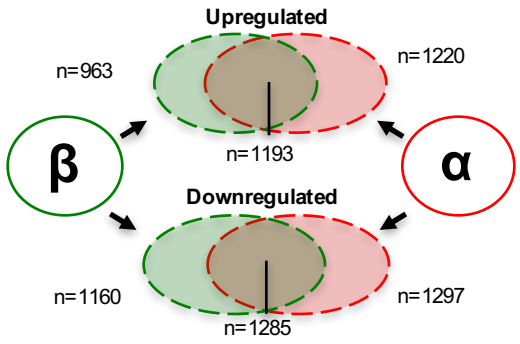


Figure 35. Related to Figure 32. Expression and functional characterization of HNF1A^{T260M} variant. (**A**) Example of control staining for **Figure 32A** with quantification of HNF1A protein expression in α cells. Scale bar represents 50 μm and is applied to **B**. (**B**) HNF1A nuclei associated with hormone-positive endocrine cells in donor islets, marked here with insulin, glucagon, somatostatin, and ghrelin on the same channel. (**C**) Corresponding western blot of HeLa cells without transfection and transfection with either wildtype (WT) or T260M mutant Myc-tagged HNF1A from **Figure 32B**. EMSA of HNF1A^{WT} or HNF1A^{T260M} transfected MIN6 cells confirmed the effects of the mutant protein on DNA-binding in an immortalized β cell line (**D**) with corresponding western blot (**E**). Specific elimination of the Myc-tagged HNF1A-DNA complex with Myc antibody (arrow) was observed only in WT transfected MIN6 cells, but not in Myc-tagged HNF1A^{T260M} expressing conditions. The endogenous HNF1α-DNA complex remained. Arrow – Myc-tagged HNF1A-DNA complex; arrow head – endogenous HNF1A-DNA complex in MIN6 cells; asterisk – nonspecific complexes; Myc-HNF1A – Myc-tagged HNF1A; End-HNF1A – Endogenous HNF1A of MIN6 cells; NT – non-transfected cells. (**F**) HNF1A^{T260M} reduced *MAFA* R3-driven reporter activity in a dose and *cis*-element site dependent manner in HeLa cells. (**G**) Immunoblot analysis of HeLa cells transfected with only HNF1A^{WT} or HNF1A^{T260M}, which corresponds to the blue and red bar conditions of panel **F**. (**H**) Analysis of native pancreatic tissue from HNF1A^{WTT260M} donor for expression of β cell-enriched transcription factors. HNF1A$^{+/T260M}$ β cells expressed β cell markers NKX2.2, NKX6.1, and PDX1 similar to controls (n=7; ages 8-55yrs). Scale bar represents 10 μm and corresponds to all panels in **H**.





G

Top 10 Common DAVID GO Analysis Molecular Functions in HNF1A+/T260M α and β cells	α cells p-value	β cells p-value
GO:0005515:protein binding	7.07E-29	5.40E-32
GO:0044822:poly(A) RNA binding	1.01E-13	7.04E-11
GO:0005524:ATP binding	4.84E-10	5.48E-07
GO:0000287:magnesium ion binding	3.68E-05	1.76E-05
Go:0046872:metal ion binding	2.57E-06	3.28E-05
GO:0004672:protein kinase activity	3.11E-05	2.99E-05
GO:0004674:protein serine/threonine kinase activity	4.20E-06	2.56E-05
GO:0016301:kinase activity	1.39E-05	8.90E-05
GO:0051287:NAD binding	6.61E-05	2.93E-04
GO:0098641:cadherin binding involved in cell-cell adhesion	2.40E-08	3.48E-04

Figure 36. Related to Figure 33. Transcriptome analysis of purified HNF1A+/T260M β and α cells by RNA-sequencing. (**A**) PCA plot and (**B**) volcano plot of HNF1A+/T260M α cells as described in **Figure 33**. (**C**) Graph represents down-regulation of HNF1A associated-targets in α cells from the HNF1A+/T260M donor compared to controls. Graphs represent the top 10 most significantly up- and down-regulated genes in the HNF1A+/T260M β cells (**D**) and α cells (**E**) compared to controls (n=5; ages 26-55yrs). The same set of control donor islets were used for α and β cell transcriptomic analysis. (**F**) Venn diagrams depict the number of genes up- and down- regulated in HNF1A+/T260M α and β cells highlighting approximately 50% overlap of up- and down-regulated genes between cell types. (**G**) Table represents the top 10 common Molecular Functions identified by DAVID GO term analysis.

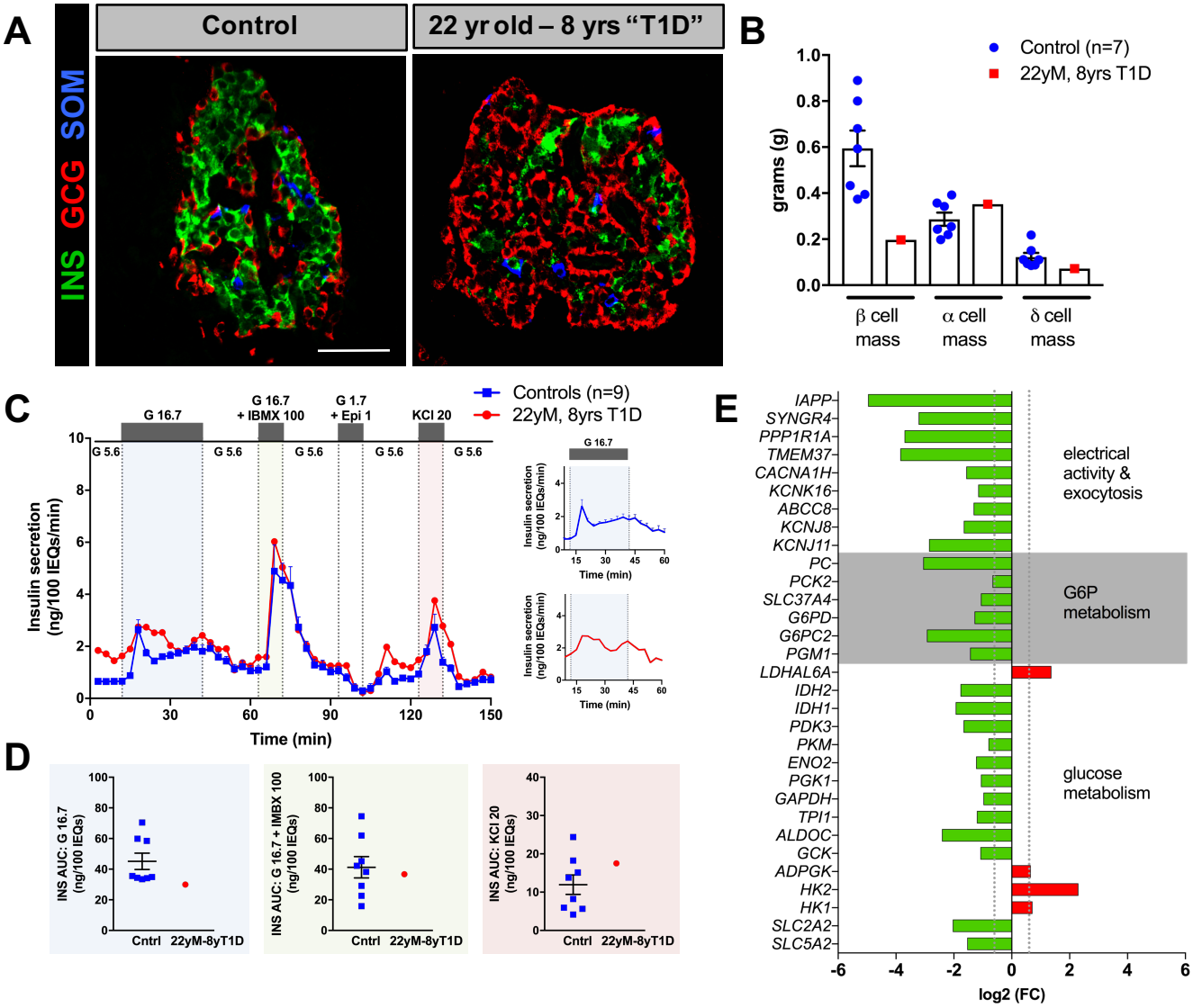


Figure 37. Histological, functional and transcriptional analysis of 22-year-old donor with 8 years of T1D pancreas and islets. (**A**). Expression of insulin (INS), glucagon (GCG), and somatostatin (SOM) in the donor's (donor #6, **Table 1**) native pancreatic tissue compared to control. Scale bar represents 50 μm. (**B**) β cell, α cell and δ cell mass (grams) in donor pancreas compared to controls (n=7; ages 10-55yrs). Each data point represents the average mass across the combined pancreatic head, body and tail regions of each donor. (**C**) Insulin secretion measured in islets isolated from donor #6 pancreas compared to normal controls (n=9; ages 7-19yrs) and normalized to overall islet cell volume (expressed as islet equivalents, IEQs); G 5.6 – 5.6 mM glucose; G 16.7 – 16.7 mM glucose; G 16.7 + IBMX 100 – 16.7 mM glucose + 100 μM isobutylmethylxanthine (IBMX); G 1.7 + Epi 1 – 1.7 mM glucose + 1 μM epinephrine; KCl 20 – 20 mM potassium chloride. Insets shows average insulin response of controls and donor #6 to 30-minute stimulation with 16.7mM glucose. (**D**) Integrated insulin secretion was calculated as area under the curve (AUC) for the following secretagogues G 16.7, G 16.7 + IBMX 100, and KCl 20 (shaded to correspond to color-matched regions of perifusion trace in panel **C**). Results of the control samples are expressed as mean ± standard error of the mean. (**E**) Genes of interest important in glucose and glucose-6-phosphate (G6P) metabolism and β cell function are significantly down-regulated in donor #6 whole islets. The vertical dotted line represents a fold change (FC) =±1.5x threshold; $p<0.05$ for all values shown.

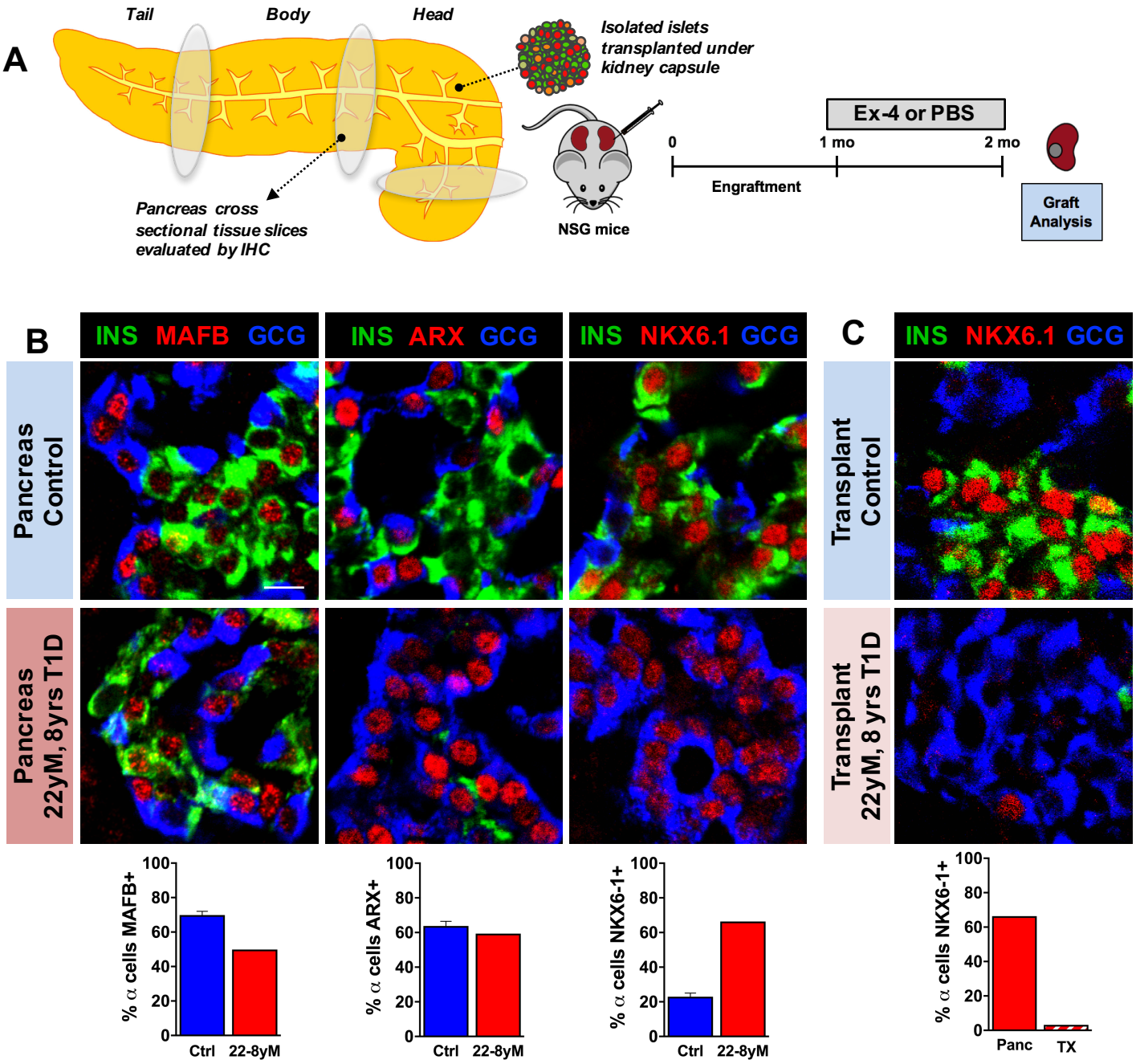


Figure 38. Normoglycemia and non-autoimmunity recovers α and β cell-specific transcription factor protein expression in donor islets. (**A**) Native pancreatic tissue cross-sectional tissue slices from the head, body and tail regions of the pancreas (donor #6) were evaluated by immunohistochemistry (IHC). Islets isolated from the remainder of the pancreas were transplanted into NSG mice (n=4 mice). After 1-month engraftment, mice were treated with either osmotic pumps carrying either exendin-4 or PBS. Graft tissue was collected for analysis after 2-months engraftment. (**B**) Expression of nuclear markers PDX1, MAFB, and NKX6.1 were evaluated in the native pancreas (solid fill) and (**C**) NKX6.1 was also evaluated in transplants (hashed) and quantified compared to the appropriate controls (**Table 2**). The quantified bar graphs of **B** refer to native tissue (panc) and **C** refers to native tissue and transplants (TX) from donor #6, i.e. 22-8yM. Scale bar in B is 10 μm.

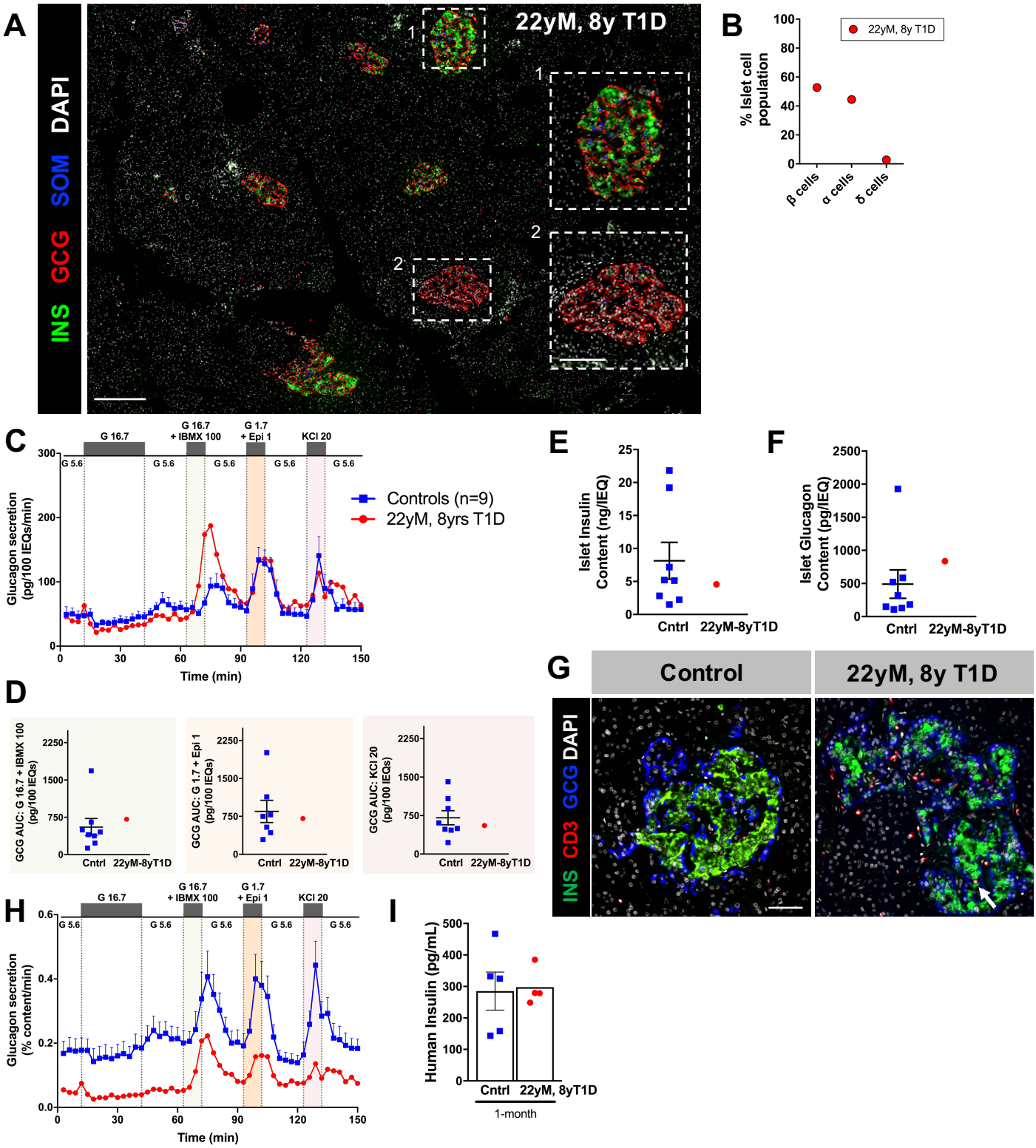
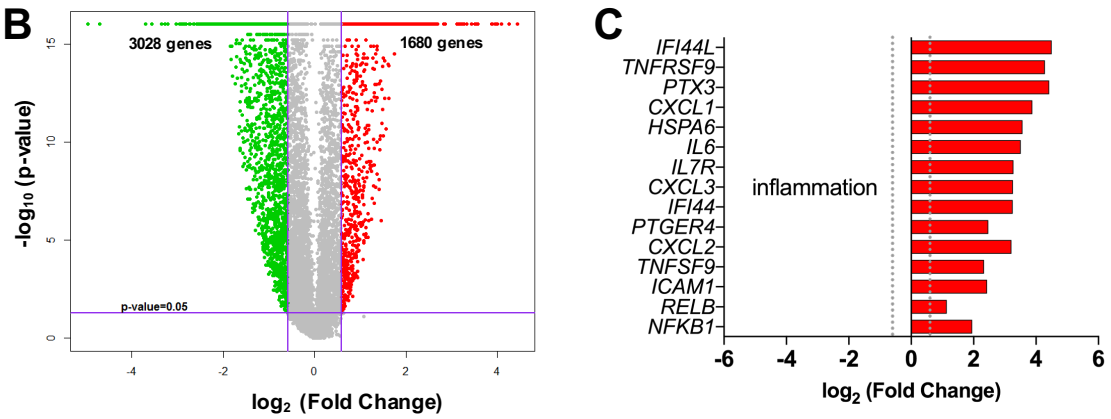


Figure 39. Related to Figure 37. Immunohistochemical and functional analysis of donor pancreas. (**A**) Pancreatic tissue section of 22-year-old male with 8 years of T1D (donor #6) stained for insulin (INS), glucagon (GCG), and somatostatin (SOM) and counterstained with nuclear marker DAPI. All islets identified in this donor were insulin+. Scale bar is 200 μm. Insets represent islet heterogeneity and are numbered accordingly. Scale bar is 100 μm. (**B**) Endocrine cell populations in dispersed isolated islets from donor #6 islets contained 52.7% β cells, 44.4% α cells, and 2.7% δ cells. Normal control islets collected by this method had a range of 53.4±2.6% β cells, 38.5±2.7% α cells, and 7.5±0.9% δ cells (Blodgett et al., 2015). (**C**) The same islets shown in **Figure 37C** were simultaneously analyzed for glucagon secretion and normalized to overall islet cell volume (expressed as islet equivalents, IEQs). As in **Figure 37**, **D** depicts integrated glucagon release as area under the curve from basal glucagon release for secretagogues G 16.7 + IBMX, G 1.7 + Epi 1, and KCl 20 (corresponding to shaded color-matched regions of perifusion trace). Insulin (**E**) and glucagon (**F**) content in control and donor #6 islets. (**G**) The native pancreatic tissue from the donor was assessed for T cell marker CD3. Scale bar is 50 μm. Arrow depicts intra-islet CD3+ cell. (**H**) Glucagon secretion normalized to islet glucagon content. (**I**) Human insulin measured from mouse serum of NSG mice transplanted with either control or donor islets under fasting conditions. Results of the control samples are expressed as mean ± standard error of the mean.

A

Isoform	NCBI ENS ID	Type	Transcript length	# of Exons	RPKM		Unique transcript reads		Total transcript reads	
					Control	Donor	Control	Donor	Control	Donor
1	**ENST00000403799**	**Islet**	**2729**	**10**	**1.39**	**0.21**	**11.7**	**3**	**143.7**	**21**
2	ENST00000345378	Liver (major)	2421	10	0.12	0.04	0.0	0	11.0	4
3	ENST00000395796	Liver (minor)	2539	11	0.36	0.45	2.3	9	35.0	42
6	ENST00000336642	N/A	1311	3	0.21	0.08	0.0	0	10.7	4
N/A	ENST00000437084	N/A	1385	10	0.13	0.02	0.7	0	6.7	1
N/A	ENST00000473353	no protein	404	3	0.05	0.07	0.3	0	0.7	1
N/A	ENST00000459642	no protein	1641	2	0.36	0.15	1.7	1	22.3	9
N/A	ENST00000476008	no protein	553	2	0.1	0.19	2	4	2	4



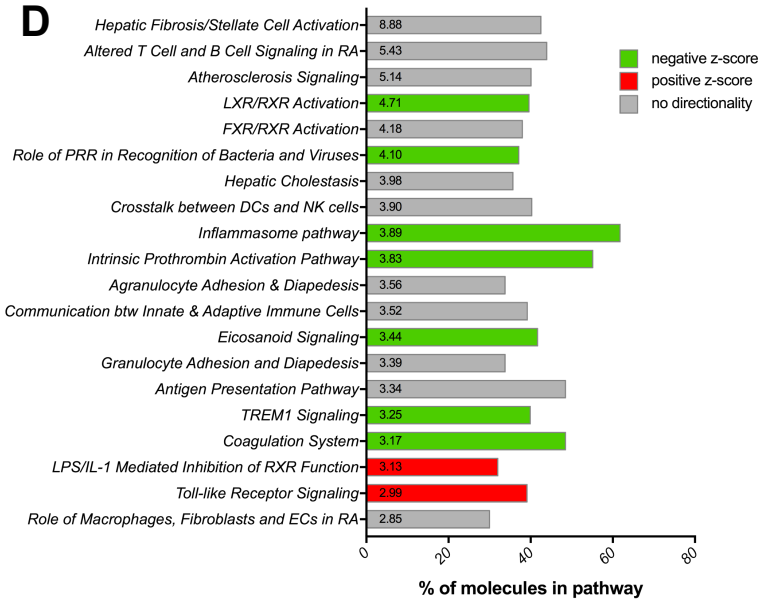


Figure 40. Related to Figure 37. Effect of *GCK* intronic variant c.209-8G>A on *GCK* RNA transcript and whole islet transcriptome. (A) Table of GCK isoforms and their expression in whole islets from GCK$^{+/c.209\text{-}8G>A}$ donor (donor #6) versus controls (n=3; 24-55 years of age). Bolded row indicates the GCK isoform predominately expressed in the pancreatic islet. Ensembl IDs are provided for each transcript. RPKM – Reads per kilobase of transcript. (**B**) The volcano plot demonstrates transcripts differentially expressed between control and donor islets (red – up-regulated gene expression; green – down-regulated gene expression). Differential expression between the two sample sets was calculated on the basis of FC (≥1.5) with a <0.05 p-value cut-off for calculated z-score. Genes (**C**) associated with inflammation are up-regulated in donor islets and pathways (**D**) related to the immune system were identified by Ingenuity pathway analysis (IPA). In **D**, the percent of differentially expressed molecules and the –log(p-value) is reported for each pathway. A green column indicates a negative z-score, red indicates a positive z-score, and gray identifies no directionality. RA: Rheumatoid Arthritis; DCs: Dendritic Cells; NK cells: Natural Killer cells; ECs: Endothelial Cells; PRR: Pattern Recognition Receptor.

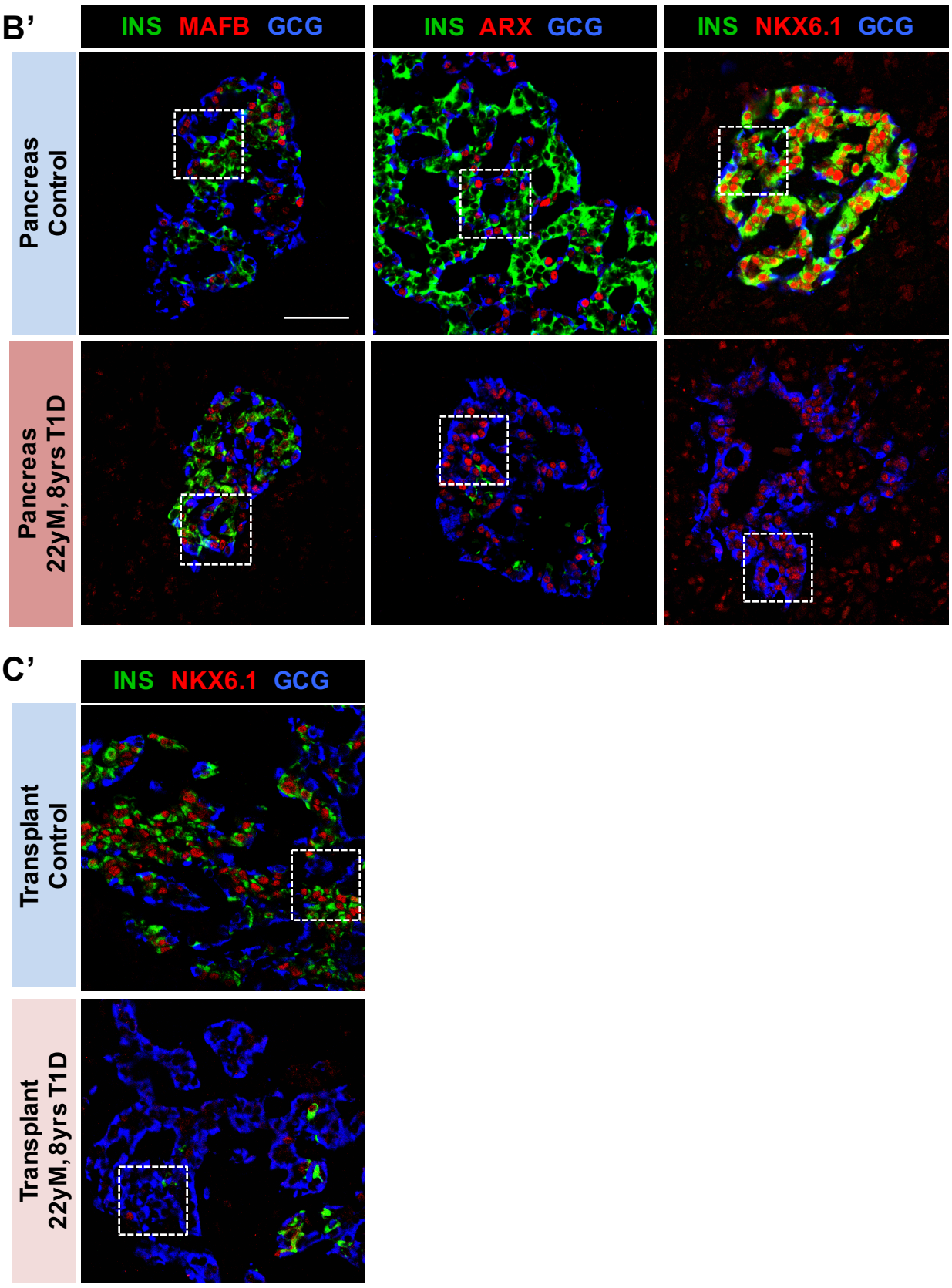


Figure 41. Related to Figure 38. Expression pattern of PDX1, MAFB, and NKX6.1 in donor β and α cells. Expression of β and α cell-enriched transcription factors in the native pancreatic tissue from controls and donor (**B'**) was compared to expression of donor islets after transplantation into normoglycemia and non-autoimmunity for 2-months (**C'**). INS–insulin, GCG–glucagon, SOM–somatostatin. Regions denoted by the dashed line in panels **B'**–**C'** are displayed in panels **B**–**C** in **Figure 38**, respectively. Scale bar in **B'** is 50 µm and also corresponds to **C'**.

CHAPTER V

PANCREATIC PSEUDOISLET SYSTEM ALLOWS FOR GENETIC MANIPULATION AND MICROENVIRONMENT MANIPULATION TO STUDY HUMAN ISLET CELL BIOLOGY

Introduction

Our understanding of the molecular mechanisms in diabetes mellitus has been advanced by studies in animal models and *in vitro* systems. However, technical limitations make studies of the human pancreas and islets a challenge. Recent advances in human pancreatic procurement and islet isolation strategies and the development of programs like the integrated islet distribution program (IIDP) have allowed scientists and clinicians to begin to gain new insights into the physiology and pathophysiology of human islet biology. However, challenges to the study of isolated human islets still remain. For example, tools to genetically manipulate human islet cells are inadequate. Viral delivery of shRNA constructs, manipulation of gene expression, or lineage tracing in intact, multicellular isolated human islets is ineffective or not developed. For example, viral delivery penetrates only 1-2 peripheral cell layers deep[292-294]. Other complicating factors include human donor islet heterogeneity and limited viability of long-term culture of primary cells. In addition, the spherical, 3D nature of the pancreatic islet, which fosters intimate connections between neighboring cells and the extracellular matrix, is important in islet cellular function and signaling limiting the translation of existing islet cell lines or studying human islets in a dispersed state[86,295-297].

To address these challenges, we sought to develop a system that would allow investigation of molecular mechanisms in primary human islets to further understand human islet cell biology and how it changes in the context of disease. By adapting and enhancing existing systems[298,299], we have been able to reproducibly create “pseudoislets”. Using a modified hanging droplet culture approach, islet cells are dispersed into a single cell state, where they can be modified for cell composition or gene expression, and re-aggregated to generate human pancreatic pseudoislets. Remarkably, pseudoislets resemble native islets in their morphological and functional features, but in contrast to native islets, pseudoislets allow much more effective construct delivery and cell-type specific manipulation. Here, we use this system to demonstrate knockdown of islet-enriched transcription factors, modified islet cell composition, and use of pseudoislets in *in vivo* systems. We show how the human pancreatic pseudoislet system allows investigation of molecular mechanisms of primary human islet physiology and pathophysiology.

Results

Pseudoislets closely resemble intact human islets in function and molecular properties

Hand-picked human islets isolated from the pancreas of organ donors were dispersed and re-aggregated using a modified hanging droplet method (**Figure 42A**) as described in **Chapter II**. Notably, native human islets were obtained from multiple islet isolation centers such as the Integrated Islet Distribution Program (IIDP), Alberta Diabetes Institute (ADI) IsletCore, or in collaboration with our colleagues at Allegheny Health Network in Pittsburgh. Pseudoislets consistently formed from these different preps with morphology and dithizone uptake similar to intact parent human islets cultured in parallel (**Figure 42B-C**). Pseudoislet morphology had less overall cell density possibly from cell loss during re-aggregation, but increased uniformity compared to native islets. Immunohistochemical analysis of hormone composition of the pseudoislets compared to their intact native islets after 7 days of culture revealed that the proportion of insulin+, glucagon+ and somatostatin+ cells in the endocrine compartment was similar (**Figure 42D**). Interestingly, islet architecture of both native islets and pseudoislets revealed insulin+ β cells existed primarily on the islet periphery with glucagon+ α cells existing in the layer underneath (**Figure 42B-C**, **Figure 44A**).

Functional analysis by dynamic perifusion showed that pseudoislets maintained pathways of regulated hormone secretion such as biphasic insulin response to high glucose, suppression of glucagon secretion by high glucose, and co-secretion of both hormones in response to cAMP-evoked stimulation and KCl-mediated depolarization (**Figure 42E-F**). Total hormone content was similar with a slightly lower overall islet insulin content per islet equivalent (IEQ) in pseudoislets (**Figure 42G-H**, $p=0.0317$). Intracellular calcium response in whole pseudoislets to changes in glucose, diazoxide and KCl-mediated depolarization was also similar to normal islets (**Figure 42I**).

Accompanying preserved function, markers specifically expressed in human β cells (PDX1, NKX6.1) and α cells (MAFB, ARX) as well as markers expressed in both cell types (PAX6, NKX2.2) were maintained at the protein level in both groups (**Figure 43A-C**). Importantly, we did not see misexpression of α and β cell markers in the opposite cell type or bihormonal cells (**Figure 43**). However, we did notice nuclei positive for markers PAX6, ARX, or MAFB that did not stain for either glucagon or insulin within both groups (**Figure 43**, white arrows). To determine whether they would belong to other endocrine cell types, such as PP+ or Ghrelin+ cells, we stained for these markers but found they were relatively rare and could not account for this cell population. We next looked for co-expression of these nuclei and found that many of these hormone-negative nuclei overlapped (**Figure 43D**). Quantification revealed that co-labeled PAX6 and ARX positive cells that were not positive for either insulin or glucagon were more

frequent in pseudoislets (24.2±3.9%) than native islets (15.7±4.0%). Further investigation of this nascent islet cell population within the pseudoislets is on-going.

Long-term culture results in a core of extracellular matrix proteins and endothelial cells in native islets and pseudoislets

While the endocrine compartment of the pseudoislet resembled native islets, there were clusters of hormone-negative, DAPI-positive cells that frequently formed a core in both islet cohorts (**Figure 44A**). To further characterize the human pseudoislet platform, islets embedded in a collagen I matrix were fixed and stained for other markers part of the islet microenvironment such as extracellular matrix (ECM) proteins (collagen IV, Laminin), exocrine markers (α-amylase), endothelial cells (VEGFR2, Caveolin-1), and islet macrophages (Iba1) (**Figure 44A**). Due to high purity of hand-picked islets, there was very little exocrine staining in the pseudoislets. Interestingly, we identified Iba1+ macrophages remained within some native islets and reincorporated into some pseudoislets. The DAPI-positive regions at the core of pseudoislets and native islets most often stained positive for ECM proteins and endothelial cell markers (**Figure 44A**). To evaluate overall pseudoislet composition, we quantified the percent area of the pseudoislet made up of hormone (insulin and glucagon), extracellular matrix protein (collagen IV), and other cells (dapi-positive nuclei) (**Figure 44B**), which revealed 23% of the pseudoislet, about 10% more than in the native islet, was made up of this population of “other” cells. We predict a population of these DAPI-positive cells are other endocrine cells and immune cells, but it is also possible another cell type is contributing. Work to identify these cells is still on-going.

We next assessed the 7-day culture paradigm on islet viability by staining for markers of cell proliferation (Ki67) and death (TUNEL) (**Figure 44C**). Despite no significant difference in proliferative markers between groups, native islets and pseudoislets both showed increased α cell Ki67+ expression compared to recently isolated islets[300], but in the range of normal compared to α cell proliferation in native tissue[301]. Conversely, there were very few hormone-positive TUNEL+ cells identified in both groups (<1%). Notably, the cells that stained positive for these markers were often found in the core region suggesting turnover of endothelial cells and other cell types, such as fibroblasts or other mesenchymal cells, producing supportive proteins.

Pseudoislet assembly allows introduction of viral vector, alteration of gene expression, and modification of islet cell composition

We next evaluated how to use this system to study mechanisms of α cell dysfunction identified in T1D (**Chapter III**)[237]. Human islet-specific transcription factors form a remarkably interconnected network. RNA-seq analysis of T1D α cells revealed among

islet-enriched transcription factors, *RFX6*, a transcription factor that lies upstream of other T1D dysregulated transcription factors and regulates the expression of necessary hormone secretory machinery in β cells, was most significantly reduced (**Figure 25**); however, the role of RFX6 in human α cell function is unknown. Native human islets were dispersed and treated with a lentivirus (LV) carrying either shRNA to *RFX6* or a scramble (Sc) sequence under a ubiquitous promoter (U6) (**Figure 45A**). Pseudoislets formed as seen previously indicating normal morphology and DTZ uptake regardless of treatment group (**Figure 45B-C**). Visualization of GFP expression in formed pseudoislets and confocal z-stacks through the islet indicated viral delivery occurred throughout the islet in contrast to the 1-2 peripheral cell layers described previously (**Figure 45B-C** and data not shown). Western blot analysis of pseudoislets showed knockdown of RFX6 protein and its reported target, PAX6 (**Figure 45D**). Importantly, scramble lentiviral (LV:Sc) treated pseudoislets maintained regulated insulin and glucagon secretion by perifusion (data not shown). As a part of this islet-specific transcription factor network, we next evaluated the role decreased PAX6 plays in T1D α cell biology (**Figure 45E-F**). Knockdown of PAX6 in human pseudoislets (**Figure 45G**) led to a significant reduction in glucose-stimulated insulin secretion evaluated *in vitro* by static incubation, but no change in glucose-stimulated glucagon inhibition (**Figure 45H**). These studies demonstrate the utility of pancreatic pseudoislets to test pathways of normal islet signaling and dysregulated hormone release identified in disease.

Observations with transplanted T1D islets indicate that the T1D environment affects the phenotype of T1D α cells and that extreme loss of neighboring β cells may be necessary to induce changes in the α cell (**Figure 24** and **Figure 30**). Evidence from human islet studies suggest intra-islet cell-to-cell communication is important for α cell function[91,297]. Furthermore, murine studies show α cells undergo transcriptional changes in response to β cell loss[212]. To study human α cells in the context of extreme β cell loss, we performed fluorescence activated cell sorting (FACS) on human islets for α cells and re-aggregated them using our pseudoislet platform (**Figure 46A-B**). We found that pseudoislets consistently formed and were enriched with α cells (95.7%) compared to other endocrine cell types evaluated (**Figure 46C**). Future studies of α cell only pseudoislets by *in vitro* analysis will determine whether loss of intra-islet insulin and β cell contacts lead to changes in glucagon secretion to changes in glucose and cAMP-evoked stimulation. Evaluation of the molecular profile of α cell pseudoislets will also help further elucidate the plasticity of human α cells and how it compares to mouse.

Pseudoislet in vivo function and transcription factor expression profile persisted despite hyperglycemia

From our studies of the T1D pancreas, we hypothesized that systemic hyperglycemia produced metabolic stress in islet cells and was a likely contributor to the T1D islet

dysfunction described (**Figures 24** and **30**). To address this, we transplanted pseudoislets and native islets into the anterior chamber of the eye (ACE) of normoglycemic NSG-DTR mice where hyperglycemia can be induced by selective ablation of endogenous β cells expressing the diphtheria toxin receptor (DTR) at a dose of diphtheria toxin (DT) non-toxic to the human graft[56]. Pseudoislets or native islets engrafted for a period of two weeks and then half of the mice were treated with DT to induce hyperglycemia (**Figure 47A**). Approximately 75 pseudoislets or native islets were transplanted per mouse (**Figure 47B**). Multiple cohorts of pseudoislet ACE transplants indicated preserved *in vivo* human insulin secretion by grafts similar to native islets (**Figure 47C**) and graft recovery (**Figure 47D**) prior to hyperglycemia. After two weeks of hyperglycemia (**Figure 47E**), fasting human insulin levels were higher in the serum of hyperglycemic mice (**Figure 47F**). Grafts were evaluated for the expression of transcription factors reported as differentially expressed in the T1D α cells (**Figure 24**)[237]. Despite nearly 14 days of systemic blood glucose levels over 500 mg/dL, expression of these markers was not different from normoglycemic grafts. It is likely longer exposure to hyperglycemia is necessary to see molecular changes. We next quantified area of insulin and glucagon to determine whether changes to cell hormone expression occurred in the context of hyperglycemia. While we did not see differences between the two groups, we identified a decrease in the ratio of β to α cells (0.789) in the engrafted pseudoislets compared to pseudoislets immediately post-formation (1.155) (**Figure 42D** and **Figure 47H**). Although some hormone negative ARX+ nuclei (**Figure 47G**, white arrows) were identified, they were less common compared to pseudoislets evaluated 7-days after re-aggregation. This suggests the *in vivo* environment caused the hormone-negative ARX+, MAFB+, PAX6+ cells to regress or to trans-differentiate into another cell type, such as glucagon-positive α cells.

Discussion

Development of a human pancreatic pseudoislet system

This work demonstrates the feasibility and application of a pseudoislet system using native human islets. Pseudoislets closely resemble intact human islets in function and molecular properties in both *in vivo* and *in vitro* settings. Here, we report how assembly of pseudoislets allow for cell manipulation such as introduction of viral vectors and modification of islet cell composition. These results demonstrate ways in which the human pseudoislet system allows testing of hypotheses on how gene expression and cell composition impacts human islet cell function.

While formation of rodent and human pseudoislets has been known, little is understood about how to adapt this system to studying human islet biology. For example, described

applications have been limited to improving homogeneity, viability and function of human islets for transplantation and/or generating uniformity necessary for high-throughput drug screens[299,302-308]. Here, we describe the development and characterization of a reproducible and scalable human pseudoislet system which we predict will have multiple applications for mechanistic studies of all islet cell types.

To develop a platform for broad purposes, we adapted and optimized existing pseudoislet protocols to preserve function and viability of human pseudoislets. Unlike previous reports using agarose-based microwells, micromolds, or hydrogel beads[305-307], use of a modified hanging droplet allowed self-assembly of pseudoislets free of synthetic materials, which likely preserved the natural interactions necessary for coordinated hormone release. As previously described in native human islets[67], α cells tend to exist in close proximity to vasculature and was maintained in pseudoislets. In contrast to traditional hanging-drop culture methods[298,299], our modified approach allowed daily media exchanges after the critical window of early cell re-aggregation enhancing pseudoislet viability by providing further support to pseudoislets as they continued to coalesce. Furthermore, nutrient-rich media supplemented with growth factors important in endothelial cell survival was optimized for long-term culture and used during pseudoislet formation. This media was designed to provide nutrient support to easily susceptible human β cells and allow the production of scaffold proteins necessary for cohesive islet formation.

Interesting human islet biology emerging from study of human pseudoislets

Re-arrangement of primary islet cells and microenvironment in pseudoislets

Pseudoislets had an inverted architecture with insulin+ cells on the periphery of the pseudoislet followed by glucagon+ cells surrounding a core of scaffold proteins and cells including collagen IV, laminin, and endothelial cells. While this has been reported previously in pseudoislets[305,308], interestingly, this unusual architecture was identified even in the intact native islets cultured in parallel. Even more interesting, this re-arrangement of islet cells did not disrupt regulated hormone release (**Figure 42E-F** and **I**).

Natural determinants of islet cell arrangement include signals and cell-to-cell communications between islet cells and secreted ECM proteins, endothelial cells, nerves, and immune cells. Interactions between islet cells and the ECM are well known to be important in regulating islet cell physiology related to cell survival, proliferation, and hormone secretion[247,309-312]. Both collagen IV and laminin, primarily secreted by fibroblasts and endothelial cells, make up the basement membrane in islets and

communicate with islet cell types by cell surface receptors such as integrins. We predict extracellular matrix and vasculature within both native islets and pseudoislet collapse during the 7-day culture to provide a scaffold for islet support[313], but continue to communicate with islet cells through these receptor-ligand interactions to form the structures described. Evaluation of integrin receptor expression of pseudoislets and cultured native islets and whether inhibition of these pathways prevent pseudoislet formation or islet cell re-arrangement would provide insight into the signals re-arranging the islet structure.

Components of the pseudoislet formation media, which contains growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF), likely stimulate the development of this core of scaffold proteins and cells. Optimization of the media to modify the contribution of some of these factors could prevent the development of large scaffold cores. Additionally, smaller pseudoislets (i.e. lower cell seeding density) could require less supportive elements and result in an increase of the islet cell compartment of pseudoislets.

Intriguingly, α cell pseudoislets, which did not contain these supportive cells or have evidence for hormone-negative regions, still formed pseudoislet structures. This could imply human α cells secrete proteins necessary for developing islet cell contacts and a spheroid structure. Further analysis of α cell only pseudoislets for the presence of matrix proteins and islet function would provide more insight. Additionally, real-time visualization of cell rearrangements and restructuring during pseudoislet formation would elucidate the interesting mechanisms behind islet cell re-aggregation.

Unidentified cells of an endocrine cell lineage in pseudoislets

Moreover, while bi-hormonal cells and/or misexpression of key-islet enriched transcription factors were not evident 7 days after re-aggregation, we did identify hormone-negative nuclei positive for α cell lineage markers (MAFB and ARX) and the pan-endocrine marker PAX6, many of which co-localized with one another. After pseudoislet engraftment *in vivo*, we saw a decrease in the β to α cell ratio and fewer hormone-negative MAFB+ and ARX+ nuclei.

Is this subset of cells in the pseudoislet intermediate or de-differentiated cells of an endocrine cell lineage? Interestingly, studies by Spijker et al. reported β to α cell transdifferentiation in their human pseudoislet system[305]. Furthermore, Lam and colleagues recently described proliferative cells of an alpha-cell lineage (ARX+) that exist *in situ* in the pancreas that could be the origin of this population[301]. Evaluation of pseudoislets and native islets with a pan-endocrine marker such as synaptophysin or

chromogranin A would provide more insight into whether these transcription factor positive cells maintain an endocrine lineage without hormone expression[24]. Because these cells can be identified in both native islets and pseudoislets, it is possible that factors in the media, such as insulin-like growth factor (IGF) and high protein concentration (20% fetal bovine serum), are providing signals for islet cell turnover (**Figure 44C**) or de-differentiation. Formation of pseudoislets in the presence of incorporating markers of proliferative cells such as bromodeoxyuridine (BrdU) would elucidate whether cell turnover is contributing to this cell population. Human β and α cells express receptors for IGF, but downstream signaling and effects on cell differentiation is unknown[314]. To address the contribution of IGF, pseudoislets formed in the presence of IGF1R inhibitors could be evaluated for these cells. Further studies using lineage tracing techniques are required to investigate the origin of these cells and whether their loss *in vivo* is due to cell regression or differentiation into an endocrine cell type, such as glucagon-positive α cells, due to additional environmental cues.

Further characterization and optimization

While significant progress has been made in characterization of this pseudoislet system, further work is required. Areas for future optimization include:

- Optimization and effective lentiviral transduction of pseudoislets: All described studies used an shRNA construct with a GFP reporter driven from a separate, ubiquitous U6 promoter. Improved transduction efficiency requires optimized multiplicity of infection and construct design. The miR-30 shRNA lentiviral backbone that allows the reporter to run under the same promoter as the shRNA will allow direct evaluation of transduction efficiency and knockdown. It also allows expression of multiple shRNA likely enhancing the consistency of knockdown between preps.
- Assess the presence and role of immune cells within both groups.
- Evaluate pseudoislet α and β cell transcriptome using single cell sequencing
- Further optimize components of pseudoislet size and media as described above to decrease scaffold proteins and other cell populations and increase the islet endocrine compartment.

Applications of Pseudoislet System

The development of a human pancreatic pseudoislet system opens several new avenues of investigation and discovery. We have begun to use this platform to ask questions previously not possible in traditional human islet studies, such as:

- Investigate mechanisms of human islet physiology

- Use of cell-specific promoters with pseudoislets will allow us to dissect mechanisms of human α and β cell biology.
- Investigate the plasticity of human islet cells and compare potential for dedifferentiation and/or transdifferentiation of α cells versus β cells
- Determine the contribution and implications of the described hormone-negative but α cell-specific transcription factor positive cells in the pseudoislet and native islet groups.
- By creating pseudoislets with islets isolated from donors of different ages or diseased states, we could ask a number of interesting questions: Do we see increased plasticity in juvenile islets? Is islet dysfunction in diseased islets maintained in pseudoislets? Do interactions of young islets or diseased islets with the endothelial cells and extracellular matrix different result in different pseudoislet architecture? Do we see differences in the proliferative capacity of young islets in pseudoislet formation?

- Investigate mechanisms of human islet pathophysiology
  - With targeted viral vectors, pseudoislets can be used to test the effect of genes identified by transcriptional profiling of human T1D α cells[237] and T2D α and β cells[84] (and unpublished) to determine their functional significance which can be evaluated by hormone secretion, intracellular calcium signaling, molecular properties and *in vivo* analysis
  - By manipulating islet composition, studies probing the effects of different β to α cell ratios on islet function could explore the impact of islet composition with respect to different disease states (i.e. type 1 and type 2 diabetes).
  - The formation of α cell only pseudoislets would allow researchers to evaluate how β cell loss effects human α cell gene expression and function in the context of T1D (**Figure 30**).
  - To further evaluate the T1D environment's impact on α cells, α cell only pseudoislets transplanted into an *in vivo* setting using hyperglycemic NSG-DTR would help us determine the effect of a high glucose environment on changes in α cells with and and without β cells present (**Figure 30**).
  - Determine whether pseudoislet formation of T1D α cells with control donor β cells can recover α cell gene expression and function
- Investigate processes of pseudoislet formation to ask questions about human β and α cell biology and islet microenvironment
  - With cell-type specific labeling, real-time imaging of pseudoislet formation will provide insight into processes that guide human islet formation
  - Study the role of specific cell types (endothelial cells, fibroblasts, α and β cells, immune cells) in pseudoislet formation

    - Test which signals are necessary for islet cell re-aggregation and re-arrangement such as cell-to-cell contacts, integrin signaling, ECM deposition, etc.
- Therapeutic Strategies for Human Islet Transplantation
    - In contrast to α cell only pseudoislets, generation of β cell only pseudoislets has been difficult and may require the addition of a supportive cell type. For example, pseudoislet formation of all islet cell types with iPSC derived endothelial cells was successful (data not shown) and could be applied to generating β cell only pseudoislets. Functional and molecular studies of β cell only islets will provide information about human β cell function and the implication of replacement of only β cells in the context of therapy in T1D.
    - Re-aggregation of human induced pluripotent stem cells (iPSCs) derived human islet cells has been reported to help in the final steps of differentiation. Can the pseudoislet platform improve this process?
    - The ability to genetically manipulate and reform functional pancreatic islet *in vitro* holds potential for clinical transplantation for individuals with different forms of diabetes.

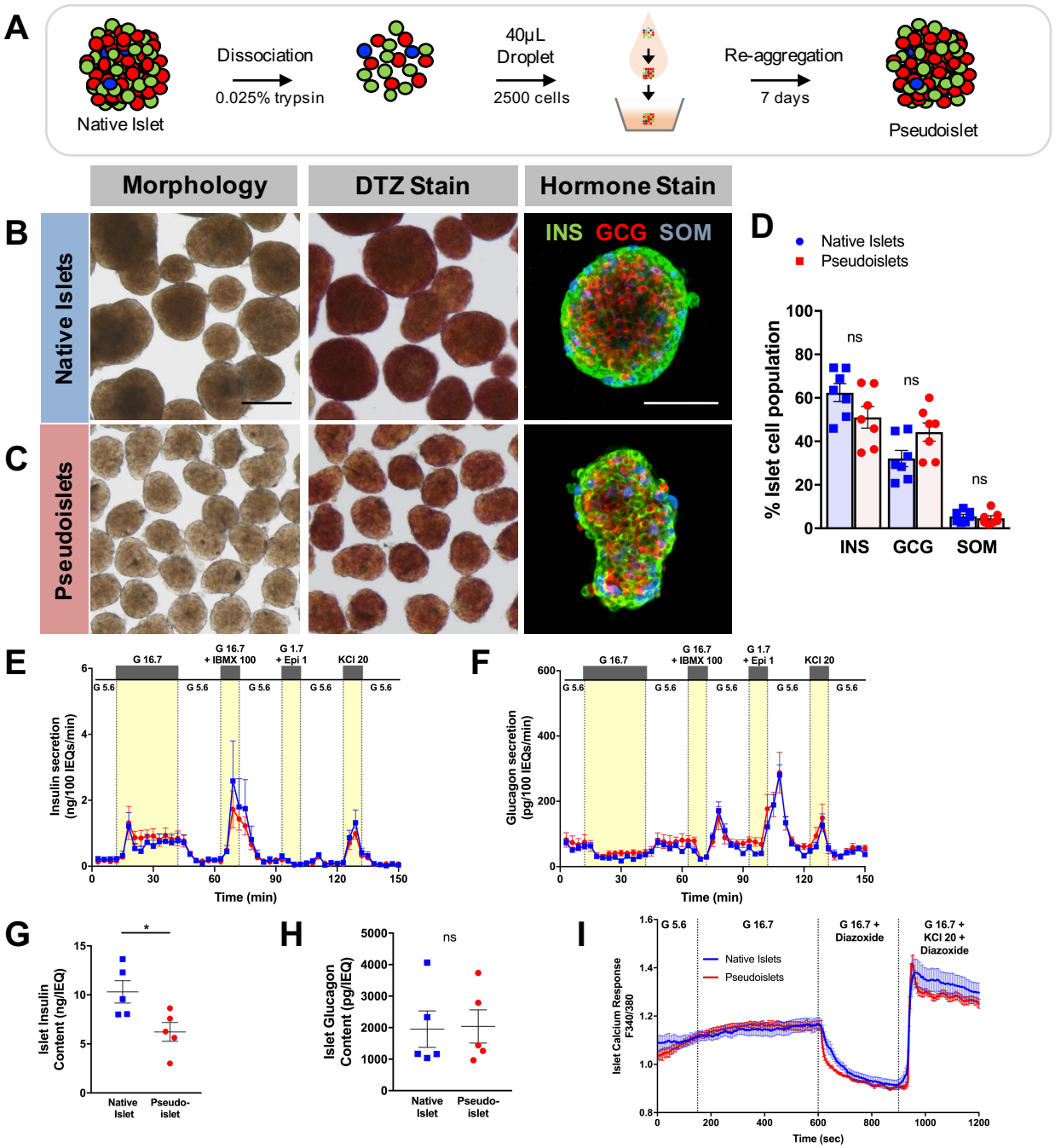


Figure 42. Pseudoislets closely resemble native human islets in hormone composition and function. (**A**) Schematic of human islet dispersion and re-aggregation for pseudoislet formation. Native human islets are dispersed with 0.025% tryspin and re-aggregated in 40 µL droplets with a seeding density of 2500 cells. Pseudoislets form in droplets for a period of 72-96-hrs before they are recovered in wells. Medium exchange continues daily for an additional three days in wells before pseudoislets are harvested for analysis resulting in a total of 7 days in culture. Analysis are performed either on the day of or day after harvest. Islet morphology, dithizone (DTZ) uptake, and hormone composition for (**B**) native islets or (**C**) pseudoislets that have been cultured in parallel. Scale bar for bright field images is 200 µm. Scale bar for confocal images of hormone is 100 µm. (**D**) Quantification of the % insulin+, glucagon+, and somatostatin+ cells in the endocrine compartment of native islets and pseudoislets (n=7; ages 7-73 yrs). Native islets had 62.4±4.1% INS, 32.2±3.7% GCG, and 5.5±1.0% SOM while pseudoislets had 51.1±5.0% INS, 44.2±4.3% GCG, and 4.6±1.1% SOM. (**E**) Insulin and (**F**) glucagon secretion measured by perifusion of native islets and pseudoislets normalized to islet volume (per islet equivalent (IEQ)). (**G**) Insulin content of native (10.31±1.14 ng/IEQ) and pseudoislets (6.22±0.96 ng/IEQ);*, p = 0.0317, and (**H**) glucagon content of native (1952±577 pg/IEQ) and pseudoislets (2038±527.3 pg/IEQ) per islet equivalent of islets perifused in **E** and **F**. (**I**) Intracellular calcium dynamics of native islets and pseudoislets in response to basal glucose, high glucose, high glucose with diaxozide, followed by the addition of 20mM KCl. INS – Insulin; GCG – Glucagon; SOM – Somatostatin; G 5.6 – 5.6 mM glucose; G 16.7 – 16.7 mM glucose; G 16.7 + IBMX 100 – 16.7 mM glucose with 100µM isobutylmethylxanthine (IBMX); G1.7 + Epi 1 – 1.7 mM glucose and 1µM epinephrine; KCl 20 – 20mM of potassium chloride (KCl); ns – not significant.

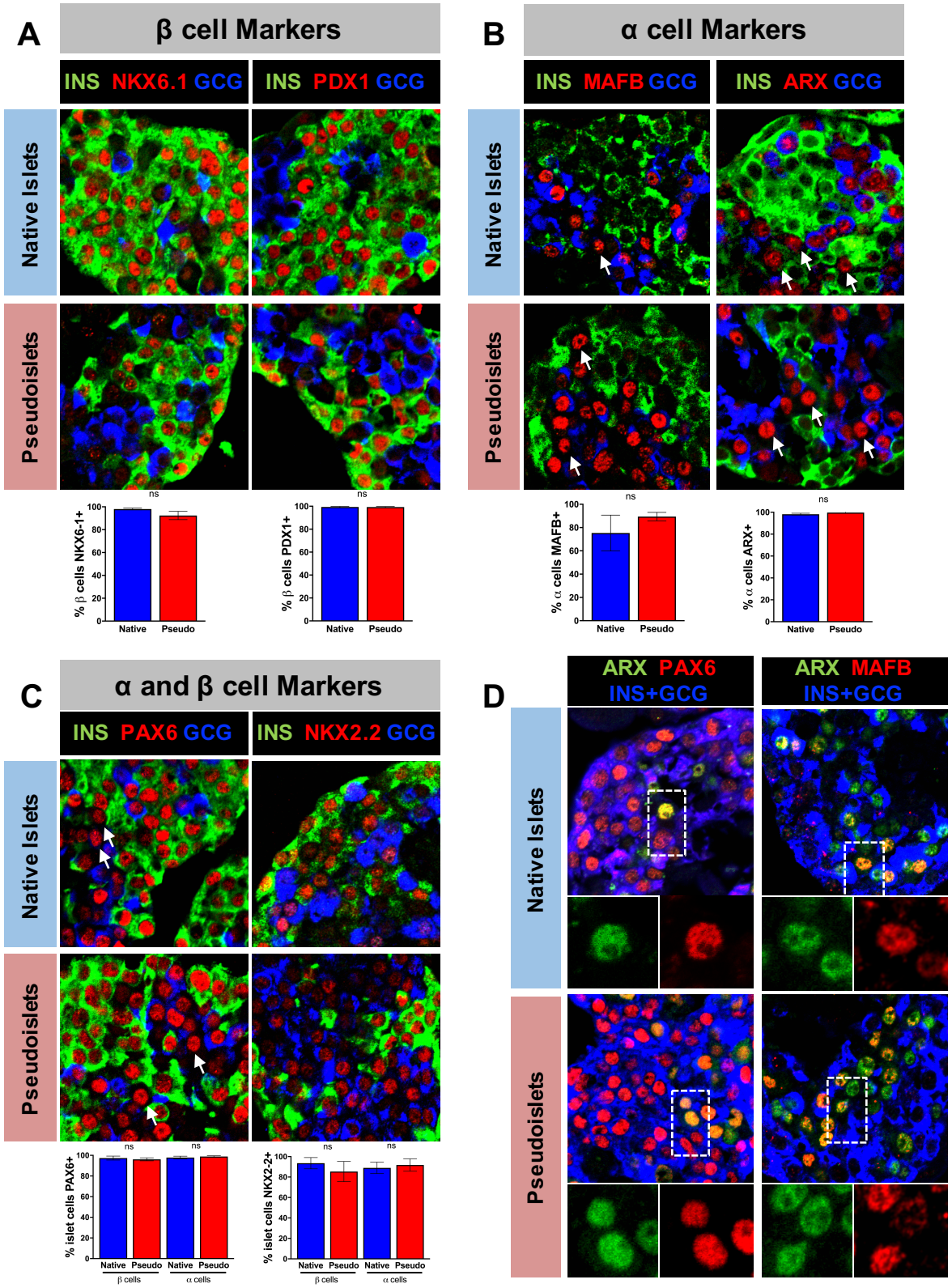


Figure 43. Expression of key islet-enriched transcription factors is maintained in pseudoislets. Expression of (**A**) β cell markers NKX6.1 and PDX1, (**B**) α cell markers MAFB and ARX, and (**C**) α and β cell markers PAX6 and NKX2.2 in insulin-positive and glucagon-positive cells of both natives and pseudoislets from the same donors (n=3 ages 28-73yrs). Data in **A–C** was compared by a two-tailed Student's *t* test. White arrows depict transcription factor positive nuclei (MAFB, ARX, and PAX6) not associated with either an insulin-positive or glucagon-positive cell. (**D**) Co-expression of transcription factors in hormone-negative cells suggest these nuclei may represent cells of an α cell lineage that are not expressing primary endocrine hormones. Notably, not all ARX+ cells co-localized with MAFB.

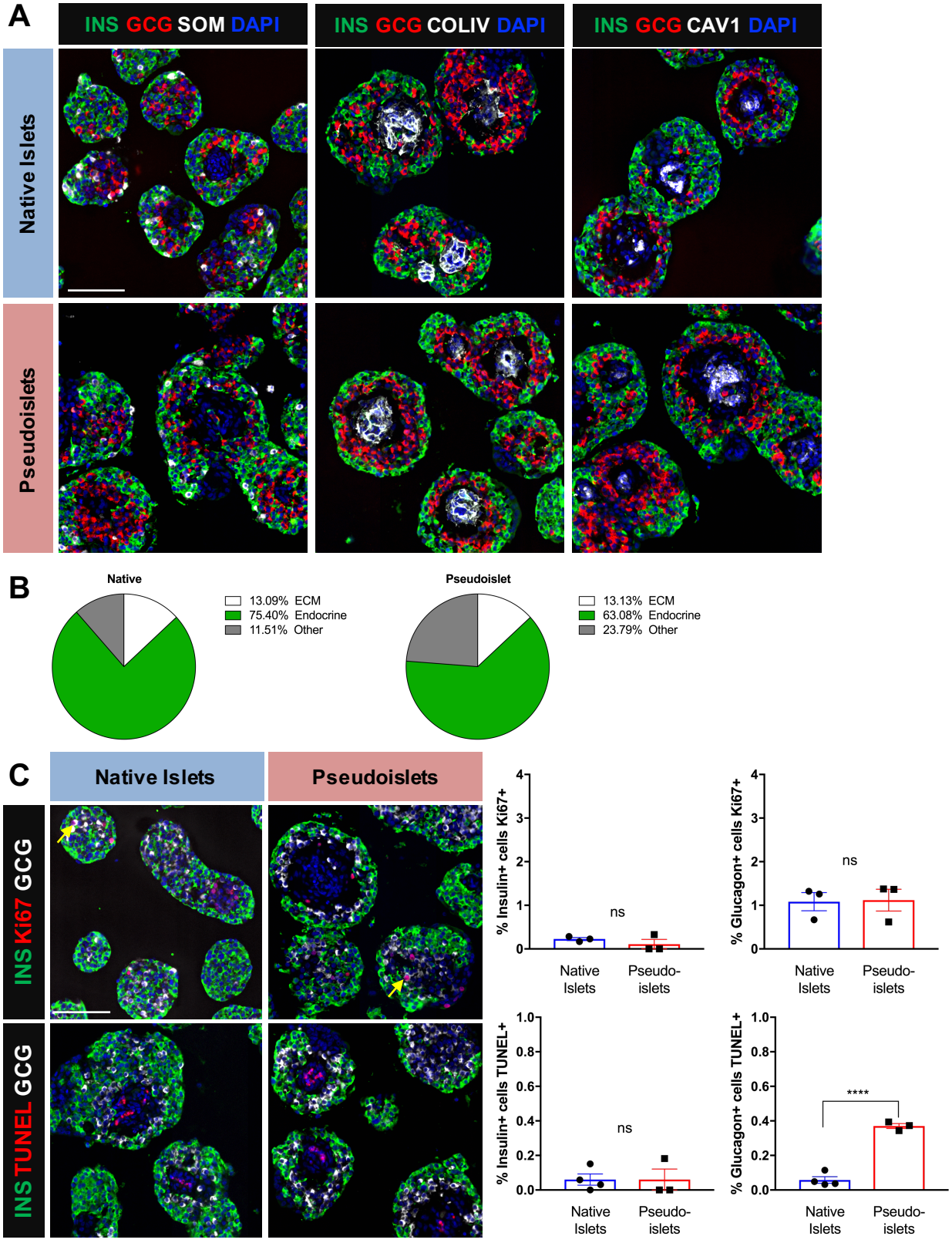


Figure 44. Native islets and pseudoislets form a core of extracellular matrix proteins and endothelial cells in long-term culture. (**A**) Gel-embedded native and pseudoislets were stained for hormones, extracellular matrix (ECM) proteins (collagen IV: COLIV), and endothelial cells (Caveolin-1: CAV1) and counterstained with the nuclear marker DAPI. Scale bar is 100 µm and applies to **C**. (**B**) Pie charts depict the percent of native islet and pseudoislet total area for collagen IV (ECM), insulin and glucagon (endocrine), and DAPI positive cells (other). (**C**) Quantification of percent of insulin (INS) positive and glucagon (GCG) positive cells expressing the nuclear proliferative marker Ki67+ (top row) and apoptotic marker TUNEL (bottom row) in both native islets and pseudoislets; ****, $p<0.0001$. Data in **C** was compared by a two-tailed Student's *t* test. SOM – Somatostatin.

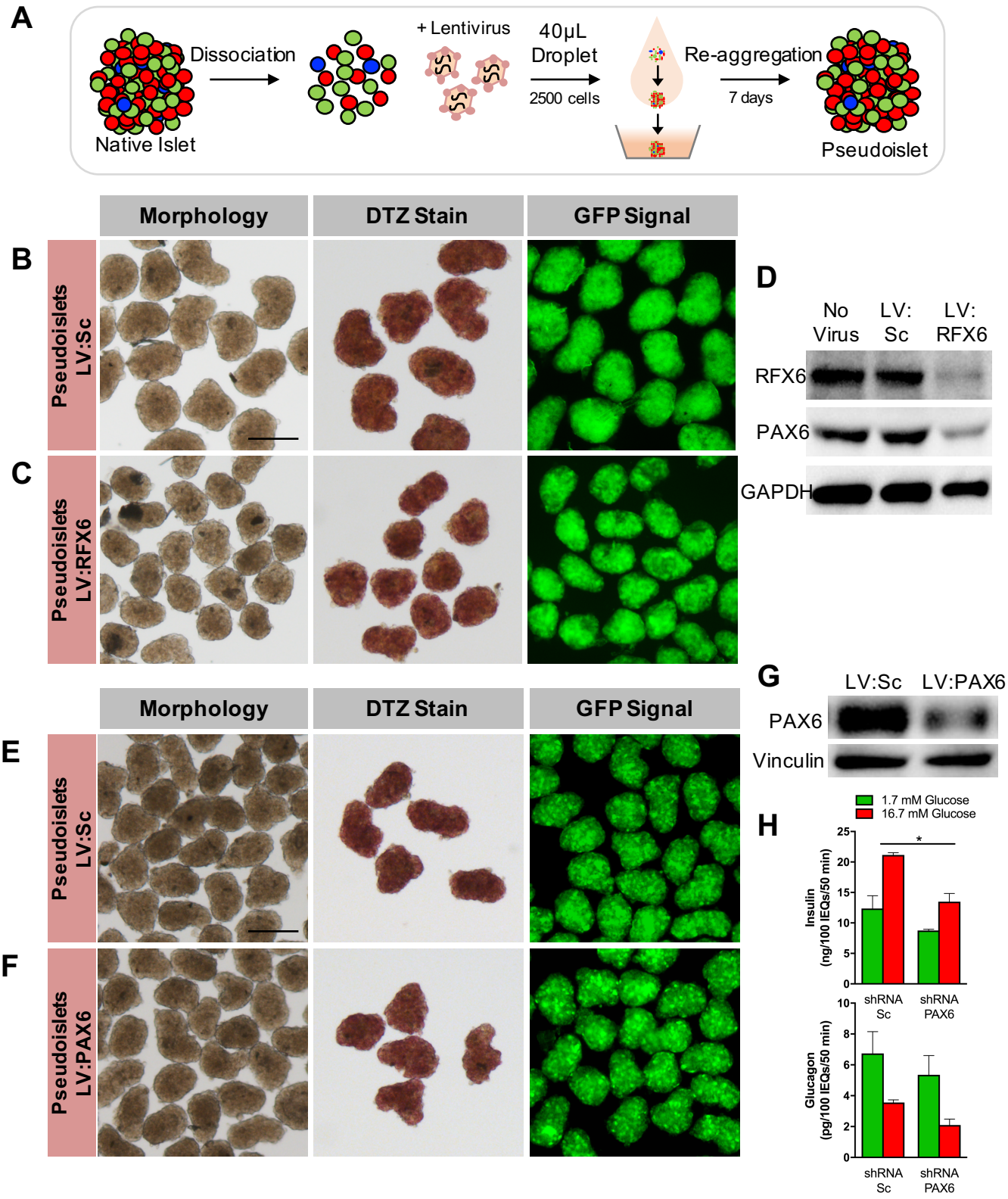


Figure 45. Knockdown of islet-specific transcription factors is possible in pseudoislets. (**A**) Schematic of lentiviral (LV) transduction (2.5hrs) of dispersed islet cells that are the washed and re-aggregated into pseudoislets. Morphology, dithizone (DTZ) uptake, and GFP expression of (**B**) scramble (Sc) treated and (**C**) shRNA to RFX6 treated pseudoislets. Scale bar is 200 μm. (**D**) Corresponding western blot of whole pseudoislets treated with no virus, LV:Sc, or LV:RFX6 demonstrating knockdown of target RFX6 and downstream target PAX6 with loading control GAPDH in pseudoislets formed from one donor (43 yrs of age). (**E-G**) Images represent pseudoislets treated with lentiviral shRNA targeting PAX6 as described in **B-D**. Vinculin was used as a loading control in **G**. (**H**) Static incubation was performed on pseudoislets formed from one donor (45 yrs of age) in 3 technical replicates. Data in **H** was compared with a two-way ANOVA; *, $p<0.05$.

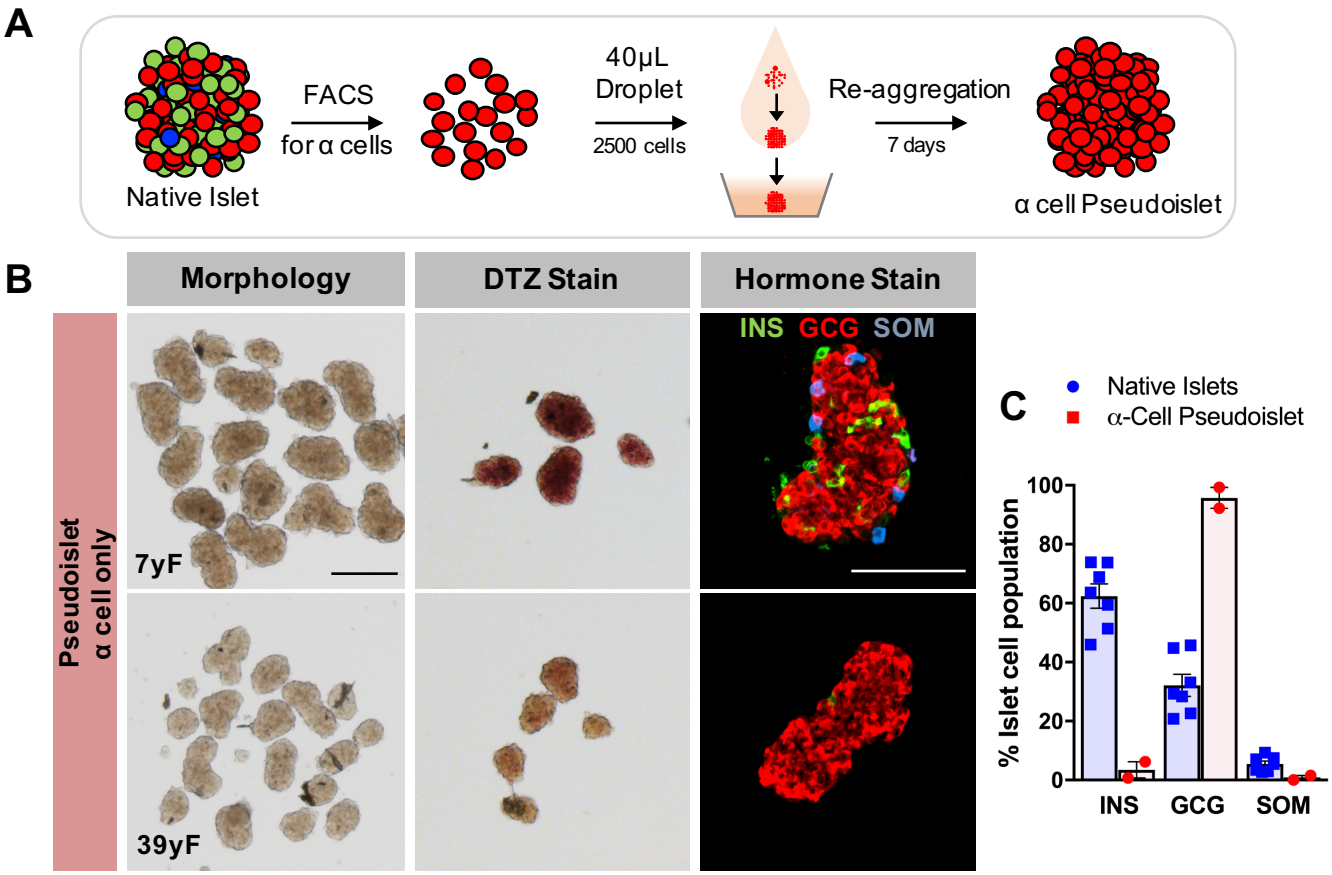


Figure 46. Pseudoislet technique allows modification of islet cell composition. (**A**) Native islets were dispersed and FACS sorted for α cells. The pseudoislet protocol was applied and α cell pseudoislets were formed. (**B**) Morphology and dithizone (DTZ) uptake were variable between the two donor, but showed consistent α cell pseudoislet formation. Scale bar is 200 μm for bright field images and 100 μm for confocal images. (**C**) Evaluation of hormone composition by confocal z-stacks revealed that the α cell pseudoislets (n=2, ages 7 and 39 yrs) were enriched with glucagon quantified as 95.7±3.5% GCG, 3.4±2.8% INS, and 0.8±0.8% SOM. The same controls from **Figure 42** are displayed in **C**. INS – Insulin, GCG – Glucagon, SOM – Somatostatin.

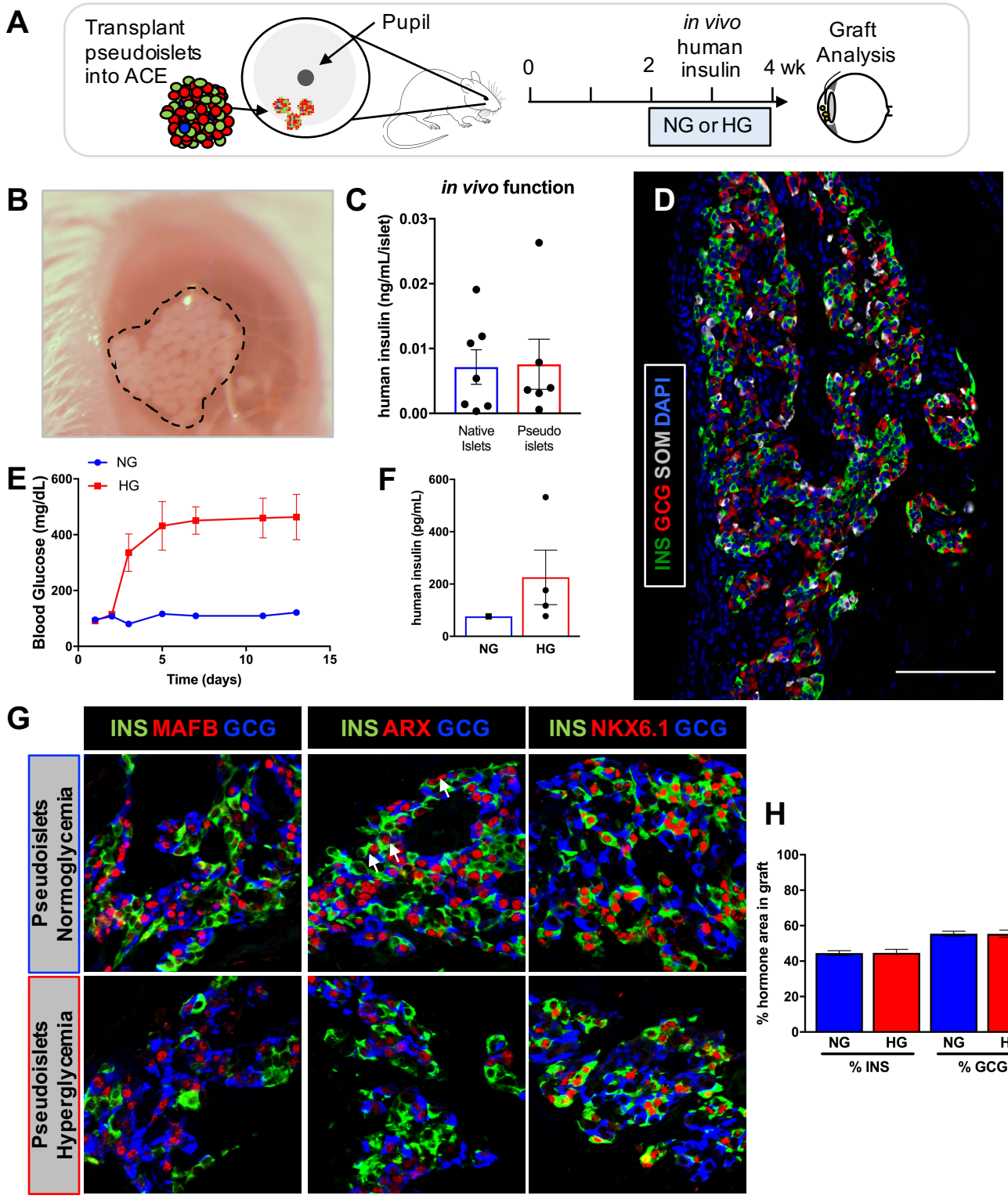


Figure 47. Anterior chamber of the eye (ACE) transplanted pseudoislets retain *in vivo* function. (A) Schematic showing how after formation, pseudoislets are transplanted into the ACE of normoglycemic (NG) immunodeficient NSG-DTR mice. 2-weeks post engraftment half of the mice are injected with diphtheria toxin to generate a cohort of hyperglycemic (HG) mice. Grafts are collected 4 weeks after transplantation for analysis. (**B**) 75 pseudoislets transplanted into the ACE of an NSG-DTR mouse. (**C**) human insulin levels measured in mouse serum from ACEs transplanted with native islets or pseudoislets. (**D**) Immunohistochemical staining of pseudoislet graft for hormones insulin (INS), glucagon (GCG), and somatostatin (SOM) counterstained with DAPI. Scale bar is 200μm. (**E**) Random blood glucose levels of NSG-DTR mice after PBS or DT injection that were normoglycemic (NG) (n=1 mouse) or hyperglycemic (HG) (n=4 mice). (F) Serum human insulin levels in mice after 2 weeks of NG or HG. (**G**) Expression of transcription factors MAFB, ARX, and NKX6.1 in insulin or glucagon positive cells of pseudoislet grafts in normoglycemia (top row) or hyperglycemia (bottom row). (**H**) Quantification of % insulin (INS) and glucagon (GCG) over total area of INS and GCG in grafts in either NG or HG.

CHAPTER VI

SIGNIFICANCE AND FUTURE DIRECTIONS

Summary

The primary goal of this Dissertation was to discover features of human islet biology and advance our understanding of functional and molecular features of the α and β cells in the pancreas of individuals with type 1 diabetes and how these profiles contribute to islet dysfunction and disease. To accomplish this, we obtained organ-donated pancreatic tissue and isolated islets from the same T1D individual and integrated *in vitro* and *in vivo* analysis of islets removed from the autoimmune, hyperglycemic environment. This approach allowed us to directly test multiple pathways of hormone secretion and uncouple effects of decreased β cell mass and β cell dysfunction not possible in clinical studies *in vivo*. We found that the rare β cells in the pancreas present not only in recent-onset T1D, but also many years after T1D diagnosis, maintained features of regulated insulin secretion and/or produced key transcriptional regulators known to play a critical role in the maintenance of β cell fate and function. Surprisingly, T1D α cells, while highly abundant, had an abnormal glucagon secretion accompanied by altered gene expression of important regulators of α cell function and identity. Furthermore, unlike in mice, significant β cell loss did not result in co-expressing insulin and glucagon cells in native tissue or after transplantation into a normoglycemic, non-autoimmune environment. Instead, transplantation showed recovery of T1D α cell-specific transcription factor expression. These findings describe important roles for β and α cells in the pathophysiology of type 1 diabetes (**Figure 48**, reproduced here) and open many doors for future directions as discussed below.

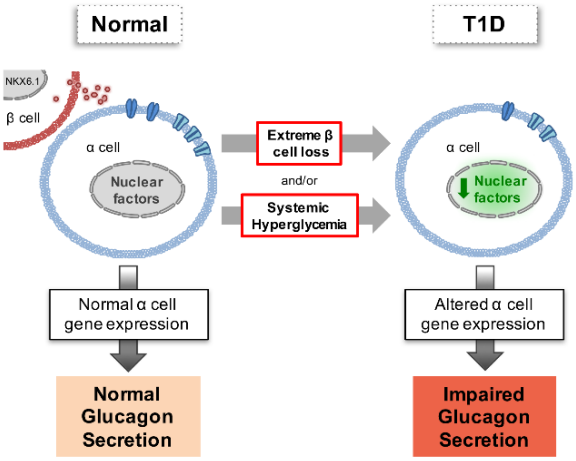


Figure 48. Pancreatic α cells in T1D. Model depicting relationship between T1D environment and α cell gene expression and function.

In our analysis of the T1D pancreas, we also identified cases with a clinical T1D phenotype but unexpected pancreatic pathology. For the first time, we provide functional, histological and transcription studies of the human pancreas and islets in the most common form of monogenic diabetes (HNF1A) and a functional rationale for a clinical alternative to current therapy. In addition, we identified a donor with considerable β cell mass despite 8 years of T1D where analysis could not fully explain the cause of

diabetes. These studies provided new understanding to the mechanisms responsible for human β and α cell function and highlight the heterogeneity in clinically diagnosed T1D. The functional and clinical implications of these findings warrant future exploration and are discussed below.

Lastly, to be able to mechanistically study the changes described in the T1D pancreas in human islets, we developed a system to investigate human islet biology *in vitro* and *in vivo* to understand islet physiology and pathophysiology. We adapted and enhanced existing methodology to create human pancreatic pseudoislets. Below, we discuss unique human islet biology discovered in the process of making pseudoislets, areas of further optimization, and potential applications of this system moving forward.

Implications, Limitations, and Future Directions

Role of pancreatic β cells in the pathophysiology of T1D

Our findings that the remnant T1D β cells maintained features of regulated insulin secretion emphasizes that T1D is primarily a disease of β cell loss rather than β cell dysfunction. This aligns with the very rare cases in which *ex vivo* β cell function has been evaluated[178,179], which reported glucose-stimulated insulin secretion present in recent-onset cases. We corroborate these findings and, for the first time, report preserved pathways of insulin release to cAMP-evoked stimulation and KCl-mediated depolarization. This raises interesting questions about how these β cells, which appear to be normal β cells, escaped the autoimmune process. For example, do these β cells represent a specific subset of β cells[75], or a regenerative attempt from *de novo* neogenesis of facultative pancreas progenitors[259], β cell replication[247] and/or transdifferentation of acinar cells[262] or other islet endocrine cells[200,212]? Single-cell RNA-sequencing of human T1D islets would provide critical insight into the contribution of reported α and β cell heterogeneity on the phenotypes reported in **Chapter III**[237]. Further understanding of the properties of these remnant β cells, and how they compare to normal β cell heterogeneity[75], could help in the design of clinical trials aimed to prevent and/or reverse β cell autoimmunity and destruction. Also, recognizing β cells are relatively normal in T1D supports initiatives for T1D prevention when disease may still be asymptomatic to preserve β cell mass[146].

Mechanisms and implications of an inherent defect in α cell biology in T1D

Our results demonstrate that α cell-intrinsic defective intracellular mechanisms, such as altered expression of transcription factors that regulate machinery important in secretion, are responsible for impaired glucagon secretion in T1D. The most down-

regulated key islet-enriched transcription factor identified in our transcriptomic analysis of T1D α cells was Regulatory Factor X 6 (RFX6). Homozygous mutations in RFX6 cause Mitchell-Riley syndrome, which is characterized by neonatal diabetes due to absent mature hormone-producing endocrine cells and intestinal atresia[45], and recently, heterozygous polymorphisms in RFX6 have been implicated as a potential form of Maturity-onset diabetes of the young (MODY)[315]. In mature mouse and human β cells, RFX6 has been shown to directly control expression of P/Q and L-Type voltage gated calcium channels and the K_{ATP} channel subunit sulfonylurea receptor 1[46,47], all of which were also downregulated in T1D α cells. Interestingly, transcript levels of RFX6 is more abundant in human α cells compared to β cells[82,84] (and unpublished), yet the role of RFX6 in α cell function and identity has not been studied. Future studies genetically manipulating RFX6 in human α cells will help us understand how RFX6 regulates α cell identity (expression of GCG and α cell-specific transcription factors like ARX and MAFB) and signaling pathways important in α cell function (intracellular calcium and cAMP production). Notably, α cell dysfunction described in patients with T1D and seen in our functional analysis is pronounced with changes in glucose levels. These proposed studies will target RFX6's role in glucose-dependent α cell glucagon secretion to determine how RFX6 is contributing to the described clinical phenotype. By comparing these molecular and functional results to the data generated from studies of T1D α cells, we can better interpret the role of decreased RFX6 in impaired α cell hormone secretion in T1D.

With partial recovery of T1D α cell-specific transcription factor expression when transplanted into a normoglycemic, non-autoimmune environment, we hypothesize that loss of neighboring β cells and a hostile native islet environment are important contributors to the defects described in T1D α cells. Supporting this hypothesis is evidence for α cell dysfunction in a 22-year-old male with 8 years of atypical T1D characterized by partial β cell mass and hyperglycemia described in **Chapter IV**. Intra-islet communication has been shown to be important for α cell function[67,91,99], yet the molecular mechanisms of this interaction are not fully understood. Additionally, reduced intra-islet insulin has been implicated in both axes of impaired glucagon counterregulation in T1D[158,159,180]. Furthermore, hyperglycemia in T1D produces metabolic stress on islet cells and could be an important contributor to islet dysfunction. By studying loss of α-to-β cell contacts and systemic hyperglycemia separately and in combination, we can independently define the roles of these two variables on α cell dysfunction (see **future directions of pseudoislet system**). By creating α cell only islets, we can evaluate the effect of β cell loss on α cell gene expression and function. This can be compared to *in vivo* function of α cell only islets in the context of normoglycemia and hyperglycemia by transplantation into the NSG-DTR mouse model. As reported in this Dissertation, 2-weeks of hyperglycemia was not sufficient to induce

changes to the α cell molecular profile. Future studies will test whether longer exposure to hyperglycemia (4 weeks) is necessary to see an effect. Finally, though difficult to study, an important component of the T1D islet microenvironment is autoimmunity. While we know that the inflammation of human T1D islets is much less robust than mouse models[152,161], the autoimmune attack on the β cells could be facilitating changes of α cells in T1D. Studies evaluating how α cell gene expression and function respond to immune-mediators would provide insight on the contribution of autoimmunity to our findings.

To determine whether defects in islet innervation could be contributing to α cell dysfunction *in situ*, we evaluated the sympathetic innervation of T1D pancreatic tissue. Unlike a previous report[208], we found no difference in sympathetic innervation of the pancreatic islet (**Figure 19**). Technical limitations due to tissue processing, antigen retrieval or the use of different TH antibodies could explain the differences in these results. Interestingly, differences in islet microvasculature such as vessel diameter and density were recently described in the T1D islet[316]. Recognizing that human islet sympathetic nerve fibers primarily innervate the islet vasculature[9], future studies evaluating the alignment of the sympathetic fibers to vasculature in T1D would provide mechanistic insight to possible defects in islet neural sensing.

These studies will help us understand how we can clinically target α cells to avoid insulin-induced hypoglycemia, an important complication to the management of T1D. For example, if α cell dysfunction occurs primarily due to loss of intra-islet insulin and β cell contacts, then this would direct islet transplantation replacement strategies to replace not only β cells but intact islets that allow α to β cell communication. If α cells become dysregulated in response to systemic hyperglycemia, then early identification and rigorous blood glucose control in T1D could delay the development of α cell changes. Likewise, if RFX6 is implicated in T1D α cell dysfunction, many of it's downstream effectors are therapeutically targetable (K_{ATP} channels and calcium channels). As it is likely that more than one component is involved in α cell dysfunction, these studies will elucidate strategies that could be used in combination to counter α cell dysfunction. In addition, more clinical studies are required to understand α cell dysfunction in T1D. For example, does α cell dysfunction present before the onset of clinical disease? Does impaired glucagon secretion to insulin-induced hypoglycemia correlate with residual C-peptide levels?

Clinical Heterogeneity in T1D

Nearly 30% of cases of T1D present with an unusual phenotype that is difficult to characterize within the current paradigm of T1D[101]. For example, this could include

individuals with late or adult-onset T1D, autoimmune-negative T1D that do not harbor known genetic variants associated with MODY, autoimmune T1D in the context of obesity, individuals with T1D who are poorly controlled despite adherence to insulin treatment, etc. Many clinical studies are performed in high-risk groups (for example, Finnish population), which provide important insight, but do not fully reflect the variability in phenotypes seen in the main population (age of onset, % of individuals carrying high-risk HLA haplotypes, autoantibody presentation). In our evaluation of 11 donors with T1D (**Table 1**), we identified two donors who clinically were diagnosed and treated for type 1 diabetes, but upon evaluation of the pancreatic islets and tissue had considerable β cells and insulin.

In one case, we were able to identify a pathogenic variant in the Maturity Onset-Diabetes of the Young 3-associated gene *HNF1A* that was responsible for this individual's diabetes. Molecular and functional analysis of the pancreas suggests this class of loss-of-function variants in *HNF1A* lead to insulin-insufficient diabetes not by significant loss of β cell mass but rather by impacting β cell transcriptional regulatory networks (*HNF4A, MAFA, RFX6, SIX3, FOXA2, MLXIPL*) that results in impairment of β cell pathways necessary for a normal insulin response to glucose. We also report impaired glucagon secretion related to α cell depolarization that may contribute to the unexplained adverse side-effect of hypoglycemia in sulfonylurea therapy in MODY3[317,318].

These findings have important clinical implications in the care of individuals with similar variants in *HNF1A*. The islet perifusion data from this donor suggests targeting cAMP-dependent pathways of insulin secretion, such as with glucagon-like-peptide 1 (GLP-1) receptor agonists, would be advantageous to sulfonylureas as this pathway of insulin secretion is preserved and accompanied by an intact glucagon response thus lowering the risk of hypoglycemia in such MODY3 patients (**Figure 31C** and **Figure 34E**). A 6-week double-blind, randomized, cross-over clinical trial demonstrated glucose control and reduced episodes of hypoglycemia in MODY3 patients taking GLP-1R agonists (Liraglutide) compared to standard of care with a sulfonylurea (glimepiride) [280]. Similar studies evaluating longer use of GLP-1R agonists and subsequent glucagon secretion would provide further insight. Future work to support this clinical application could determine whether cAMP-mediated mechanisms of hormone release are independent of HNF1A regulation in the β and α cell.

In the second case, the primary cause of diabetes is less clear. We identified normal insulin secretion and content in the isolated islets, but pancreatic histology revealed overall reduced β cell mass with evidence of mild islet-inflammation both by RNA and the presence of CD3+ cells within islets. To interpret our findings, we performed DNA

sequencing and identified a heterozygous variant of unknown significance in glucokinase (GCK), which would be expected to produce mild clinical symptoms[284,286] contrasting the significant hyperglycemia reported in this donor (HbA1C 11.9%). However, at the RNA level, the pancreatic isoform of GCK transcript was reduced and had differential expression of GCK-related processes.

The organ characteristics were similar to T1D with reduced pancreas size (59.1g), but no difficulty in islet isolation. While insulin-positive islets may have been more easily isolated and identified for analyses and transplantation, this donor carried considerable insulin-positive islets compared to reported cases of T1D for 8-years duration[152]. Future studies are necessary to understand the cause of diabetes in this 22-year-old male with 8 years of T1D. For example, whole-exome sequencing of the donor could identify other potential genetic causes of diabetes. Because there were no significant effects of this variant on *in vitro* islet function, it is possible this particular *GCK* variant impacts glucose sensing by other organs such as the brain or liver. Expression of this variant in human hepatocyte and β cell *in vitro* systems could allow us to better investigate this question. It is also possible that this donor had a mild or abrogated form of type 1 diabetes resulting in partial β cell loss and the reported changes in *GCK* gene expression were secondary to hyperglycemia of reduced β cells. These atypical cases have been reported previously [287-289] and could be elucidated by evaluating donor DNA for a T1D genetic risk score [134,136,290,291]. Assessment of *GCK* and associated pathways in engrafted human islets in NSG-DTR mice that experience either normoglycemia or hyperglycemia would provide insight.

Human Pseudoislet System

As described in **Chapter V**, we demonstrate the feasibility and application of a pseudoislet system using native human islets. Pseudoislets closely resemble intact human islets in function and molecular properties in both *in vivo* and *in vitro* settings. We report how assembly of pseudoislets allows for cell manipulation such as introduction of viral vectors and modification of islet cell composition. These results demonstrate ways in which the human pseudoislet system allows testing of hypotheses on how gene expression and cell composition impacts human islet cell function.

Emerging human islet biology from evaluation of pseudoislets and native islets

Pseudoislets had an inverted architecture with insulin+ cells on the periphery of the pseudoislet followed by glucagon+ cells surrounding a core of scaffold proteins and cells including collagen IV, laminin, and endothelial cells. While this has been reported previously in pseudoislets[305,308], interestingly, this unusual architecture was identified

even in the intact native islets cultured in parallel. Even more interesting, this re-arrangement of islet cells did not disrupt regulated hormone release (**Figure 42E-F** and **I**).

Natural determinants of islet cell arrangement include signals and cell-to-cell communications between islet cells and secreted ECM proteins, endothelial cells, nerves, and immune cells. Interactions between islet cells and the ECM are well known to be important in regulating islet cell physiology related to cell survival, proliferation, and hormone secretion[247,309-312]. Both collagen IV and laminin, primarily secreted by fibroblasts and endothelial cells, make up the basement membrane in islets and communicate with islet cell types by cell surface receptors such as integrins. We predict extracellular matrix and vasculature within both native islets and pseudoislet collapse during the 7-day culture to provide a scaffold for islet support[313], but continue to communicate with islet cells through these receptor-ligand interactions to form the structures described. Evaluation of integrin receptor expression of pseudoislets and cultured native islets and whether inhibition of these pathways prevent pseudoislet formation or islet cell re-arrangement would provide insight into the signals re-arranging the islet structure.

Components of the pseudoislet formation media, which contains growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF), likely stimulate the development of this core of scaffold proteins and cells. Optimization of the media to modify some of these factors could prevent the development of large scaffold cores. Additionally, smaller pseudoislets (i.e. lower cell seeding density) could require less supportive elements and result in an increase of the islet cell compartment of pseudoislets.

Intriguingly, α cell pseudoislets, which did not contain these supportive cells or have evidence for hormone-negative regions, still formed pseudoislet structures. This could imply human α cells secrete proteins necessary for developing islet cell contacts and a spheroid structure. Further analysis of α cell only pseudoislets for the presence of matrix proteins and islet function would provide more insight. Additionally, real-time visualization of cell rearrangements and restructuring during pseudoislet formation would elucidate the interesting mechanisms behind islet cell re-aggregation.

Moreover, while bi-hormonal cells and/or misexpression of key-islet enriched transcription factors were not evident 7 days after re-aggregation, we did identify hormone-negative nuclei positive for α cell lineage markers (MAFB and ARX) and the pan-endocrine marker PAX6, many of which co-localized with one another. After

pseudoislet engraftment *in vivo*, we saw a decrease in the β to α cell ratio and fewer hormone-negative MAFB+ and ARX+ nuclei.

Is this subset of cells in the pseudoislet intermediate or de-differentiated cells of an endocrine cell lineage? Interestingly, studies by Spijker et al. reported β to α cell transdifferentiation in their human pseudoislet system[305]. Furthermore, Lam and colleagues recently described proliferative cells of an alpha-cell lineage (ARX+) that exist *in situ* in the pancreas that could be the origin of this population[301]. Evaluation of pseudoislets and native islets with a pan-endocrine marker such as synaptophysin or chromogranin A would provide more insight into whether these transcription factor positive cells maintain an endocrine lineage without hormone expression[24]. Because these cells can be identified in both native islets and pseudoislets, it is possible that factors in the media, such as insulin-like growth factor (IGF) and high protein concentration (20% fetal bovine serum), are providing signals for islet cell turnover (**Figure 44C**) or de-differentiation. Formation of pseudoislets in the presence of incorporating markers of proliferative cells such as bromodeoxyuridine (BrdU) would elucidate whether cell turnover is contributing to this cell population. Human β and α cells express receptors for IGF, but downstream signaling and effects on cell differentiation is unknown[314]. To address the contribution of IGF, pseudoislets formed in the presence of IGF1R inhibitors could be evaluated for these cells. Further studies using lineage tracing techniques are required to investigate the origin of these cells and whether their loss *in vivo* is due to cell regression or differentiation into an endocrine cell type, such as glucagon-positive α cells, due to additional environmental cues.

Further characterization and optimization

While significant progress has been made in characterization of this pseudoislet system, further work is required. Areas for future optimization include:

- Optimization and effective lentiviral transduction of pseudoislets: All described studies used an shRNA construct with a GFP reporter driven from a separate, ubiquitous U6 promoter. Improved transduction efficiency requires optimized multiplicity of infection and construct design. The miR-30 shRNA lentiviral backbone that allows the reporter to run under the same promoter as the shRNA will allow direct evaluation of transduction efficiency and knockdown. It also allows expression of multiple shRNA likely enhancing the consistency of knockdown between preps.
- Assess the presence and role of immune cells within both groups.
- Evaluate pseudoislet α and β cell transcriptome using single cell sequencing

- Further optimize components of pseudoislet size and media as described above to decrease scaffold proteins and other cell populations and increase the islet endocrine compartment.

Applications of Pseudoislet System

The development of a human pancreatic pseudoislet system opens several new avenues of investigation and discovery. We have begun to use this platform to ask questions previously not possible in traditional human islet studies, such as:

- Investigate mechanisms of human islet physiology
    - Use of cell-specific promoters with pseudoislets will allow us to dissect mechanisms of human α and β cell biology.
    - Investigate the plasticity of human islet cells and compare potential for dedifferentiation and/or transdifferentiation of α cells versus β cells
    - Determine the contribution and implications of the described hormone-negative but α cell-specific transcription factor positive cells in the pseudoislet and native islet groups.
    - By creating pseudoislets with islets isolated from donors of different ages or diseased states, we could ask a number of interesting questions: Do we see increased plasticity in juvenile islets? Is islet dysfunction in diseased islets maintained in pseudoislets? Do interactions of young islets or diseased islets with the endothelial cells and extracellular matrix different result in different pseudoislet architecture? Do we see differences in the proliferative capacity of young islets in pseudoislet formation?
- Investigate mechanisms of human islet pathophysiology
    - With targeted viral vectors, pseudoislets can be used to test the effect of genes identified by transcriptional profiling of human T1D α cells[237] and T2D α and β cells[84] (and unpublished) to determine their functional significance which can be evaluated by hormone secretion, intracellular calcium signaling, molecular properties and *in vivo* analysis
    - By manipulating islet composition, studies probing the effects of different β to α cell ratios on islet function could explore the impact of islet composition with respect to different disease states (i.e. type 1 and type 2 diabetes).
    - The formation of α cell only pseudoislets would allow researchers to evaluate how β cell loss effects human α cell gene expression and function in the context of T1D (**Figure 30**).
    - To further evaluate the T1D environment's impact on α cells, α cell only pseudoislets transplanted into an *in vivo* setting using hyperglycemic NSG-DTR would help us determine the effect of a high glucose

environment on changes in α cells with and and without β cells present (**Figure 30**).
    - Determine whether pseudoislet formation of T1D α cells with control donor β cells can recover α cell gene expression and function
- Investigate processes of pseudoislet formation to ask questions about human β and α cell biology and islet microenvironment
    - With cell-type specific labeling, real-time imaging of pseudoislet formation will provide insight into processes that guide human islet formation
    - Study the role of specific cell types (endothelial cells, fibroblasts, α and β cells, immune cells) in pseudoislet formation
    - Test which signals are necessary for islet cell re-aggregation and re-arrangement such as cell-to-cell contacts, integrin signaling, ECM deposition, etc.
- Therapeutic Strategies for Human Islet Transplantation
    - In contrast to α cell only pseudoislets, generation of β cell only pseudoislets has been difficult and may require the addition of a supportive cell type. For example, pseudoislet formation of all islet cell types with iPSC derived endothelial cells was successful (data not shown) and could be applied to generating β cell only pseudoislets. Functional and molecular studies of β cell only islets will provide information about human β cell function and the implication of replacement of only β cells in the context of therapy in T1D.
    - Re-aggregation of human induced pluripotent stem cells (iPSCs) derived human islet cells has been reported to help in the final steps of differentiation. Can the pseudoislet platform improve this process?
    - The ability to genetically manipulate and reform functional pancreatic islet *in vitro* holds potential for clinical transplantation for individuals with different forms of diabetes.

Closing Remarks

In this Dissertation, we establish how the study of clinically-relevant samples and the development of model systems to study primary human islets can provide unique insights into human islet physiology and pathophysiology. By integrating clinical information with functional, histological and transcriptional analyses, we made discoveries that provide new understanding into the mechanisms of islet dysfunction in type 1 diabetes and the heterogeneity that contributes to insulin-deficient diabetes. Overall, the results presented in this Dissertation further our understanding of T1D and provide a foundation for future research investigating potential therapeutic targets for improved care in individuals with T1D.

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