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Strategies to prevent islet cell damage by targeting micro-environmental stress - Implication for clinical islet transplantation

By

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Shadab Abadpour

Table of selected abbreviations

ASC	Adipose-derived stem cell
ATF6	Activating transcription factor 6
cAMP	Cyclic adenosine monophosphate
CHOP	C/EBP-homologous protein
COX	Cyclooxygenase
CXCL	Chemokine (C-X-C motif) ligand
DBD	Donor after brain death
DCD	Donor after cardiac death
DM	Diabetes mellitus
eIF2α	Eukaryotic initiation factor 2 alpha
EP3	prostaglandin E receptor 3
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated protein degradation
FFA	Free fatty acid
GDNF	Glial cell line derived neurotrophic facto
GFL	GDNF-family ligand
GFR	GDNF family receptor
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide-1
GPR44	G-protein coupled receptor 44
GRO- α / β	Growth-regulated oncogene - α / β
GSIS	Glucose stimulated insulin secretion
HbA1c	Hemoglobin A1c
hESC	Human embryonic stem cell
HGF	Hepatocytes growth factor
HS	Human serum
HSA	Human serum albumin
HVEM	Herpesvirus entry mediator
IAPP	Islet amyloid polypeptide
IBMIR	Instant blood-mediated inflammatory reaction
IEQ	Islet equivalent
IGF	Insulin growth factor
IL	Interleukin
INF-γ	Interferon- gamma
iNOS	Inducible nitric oxide synthase
IP-10	Interferon gamma-induced protein-10
iPSC	Induced pluripotent stem cell
IRE-1α	Inositol requiring enzyme-1alpha
IVGTT	Intravenous glucose tolerance test
JNK	c-Jun N-terminal kinase
LIGHT	Homologous to lymphotoxin, exhibits inducible expression and competes with HSV glycoprotein D for binding to herpesvirus entry mediator, a receptor expressed on T-lymphocytes
LTβR	Lymphotoxin beta receptor
mafA	V-maf muculoaponeurotic fibrosarcoma oncogene homolog
MAPK	Mitogen-activated protein kinase
MCP-1	Monocytes chemoattractant protein 1
NeuroD1	Neurogenic differentiation 1
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor

NO	Nitric oxide
NSAID	Nonsteroidal anti-inflammatory drug
OGTT	Oral glucose tolerance test
Pdx-1	Pancreatic and duodenal homeobox 1
PERK	Protein kinase RNA-like endoplasmic
PG	Prostaglandin
PI3K	Phosphoinositol 3-kinase
PKA	Protein kinase A
ROS	Reactive oxygen species
RRP	Readily releasable pool
STAT	Signal transducer and activator of transcription
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TF	Tissue factor
Tg	Thapsigargin
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor- alpha
TXA2	Thromboxane A2
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
XBP-1	X-box binding protein 1

List of publications

Article I

Abadpour S, Göpel SO, Schive SW, Korsgren O, Foss A, Scholz H.

Glial cell-line derived neurotrophic factor protects human islets from nutrient deprivation and endoplasmic reticulum stress induced apoptosis.

Sci Rep. 2017 May 8, doi: 10.1038/s41598-017-01805-1.

Article II

Abadpour S, Halvorsen B, Sahraoui A, Korsgren O, Aukrust P, Scholz H.

Interleukin-22 reverses human islet dysfunction and apoptosis triggered by hyperglycemia and LIGHT.

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Article III

Shadab Abadpour, Stanko Skrtic, Simen W. Schive, Charlotte Wennberg Huldt, Peter Gennemark, Tina Rydén-Bergsten, David M. Smith, Olle Korsgren, Björn Tyrberg, Hanne Scholz and Maria Sörhede Winzell.

Inhibition of prostaglandin D2-GPR44 axis improves human islet function and survival.

1. Introduction

1.1. The human pancreas

The pancreas is an elongated gland that is located behind and below the stomach above duodenum [1]. The normal weight of the adult human pancreas has a range between approximately 60-100 grams and contains both exocrine and endocrine tissue [2]. About 98 % of a pancreas is exocrine cells. These cells are organized into acini and secrete pancreatic fluids into duodenum through common bile duct. Pancreatic fluids consist of two main components: 1) pancreatic enzyme and 2) aqueous alkaline solution [1]. Both components are responsible for neutralization of stomach acidity and assisting digestion and absorption of nutrients in the small intestine. Endocrine tissue, which is approximately 1-2 % of a pancreas volume, is organized into clusters of endocrine cells and responsible for glucose homeostasis of the body, called islets of Langerhans. An adult human islet contains at least five different endocrine cell types [3], which are listed below in Table 1.

Table 1. Cell types in an adult human islet [4]

Cell type	Alpha-cell	Beta-cell	Delta-cell	PP-cell	Epsilon cell
Peptide hormone	Glucagon	Insulin	Somatostatin	Pancreatic Polypeptide	Ghrelin
Molecular weight	3500	5800	1500	4200	3400
Number of amino acids	29	51	14	36	28
Volume % (adult)	15-20	50-80	5-10	15-25	≤1

1.1.1. Islets of Langerhans

Islets of Langerhans were first discovered in 1868 by a German pathological anatomist Paul Langerhans in histological sections of a pancreas [5]. The size of the adult islets varies between 20-800 µm [6] with the ability to monitor the nutrient status in the body and secrete hormones in

order to regulate glucose metabolism [7]. The most abundant cells in an islet are beta cells (50 - 80 % of an islet) [8]. Beta cells secrete insulin, which is a 51-amino-acids peptide, responsible for tightening the blood glucose within the physiological range. The insulin mRNA is translated to a single chain precursor called preproinsulin. Removal of the signal peptide from preproinsulin during its insertion into endoplasmic reticulum (ER) generates proinsulin. Proinsulin is biologically inactive and splits into three parts. Part A and B remains connected by sulfur bridges and form the biological active insulin molecules. Part C, called C-peptide is released together with insulin in a 1:1 molar ratio [9]. In addition to insulin, beta cells also secrete a 37-amino-acid peptide called islet amyloid polypeptide (IAPP). Under pathological conditions such as in type 2 diabetes and in insulinoma, IAPP may polymerize and form intra-islet amyloid deposits [10].

The second most abundant cell type in an islet is alpha cell. This type of cell secretes a 29-amino acids peptide with hyperglycemic action, called glucagon. Glucagon is derived from proglucagon (180-amino acid peptide) through proteolytic cleavage [11]. The number of alpha cells is estimated to be between 15-20 % in an islet. However, this number can be varied among islets [12].

The third abundant cell type within an islet is delta cell, which secretes somatostatin. This hormone has a very potent stimulatory effect on insulin secretion and inhibitory effect on glucagon. Delta cells form 5-10 % of an islet volume [13].

The less known cell type in an islet is PP cell, which secretes PP hormone. In a human pancreas, PP cells are predominantly located in the head of the pancreas [14, 15].

Epsilon cell is the latest cell type found in the islet of Langerhans. These cells secrete a hormone called ghrelin. An adult human islet contains less than 1% epsilon cells. The exact role of ghrelin hormone is not known but it is thought to have an impact on the metabolic regulation and energy balance of the islets [16].

1.1.2. Regulation of insulin secretion

Insulin is a hormone specifically secreted by pancreatic beta cells within an islet and is required for normal glucose metabolism. Insulin secretion is mainly stimulated by glucose. In addition, some amino acids such as combination of glutamine and leucine [17], free fatty acids (FFAs) (in case of insulin resistance and type 2 diabetes) [18, 19] as well as incretin hormones such as gastric inhibitory polypeptide (GIP) or glucagon-like peptide-1 (GLP-1) [20, 21] could also regulate insulin secretion. In order to properly sense nutritional status, pancreatic islets are connected to the vasculature system and receive 10 times more blood than cells in the exocrine part [22]. Pancreatic beta cells absorb circulating glucose through glucose transporters. These transporters are membrane proteins that are mainly expressed on beta cells but to the lesser extend expressed on the liver, renal and intestinal cells. Entering to beta cells, glucose is phosphorylated by glucokinase enzymes and proceeds to mitochondria to produce ATP [22]. Elevation in the level of ATP production leads to an increase in ATP / ADP ratio and closure of ATP-sensitive potassium (K_{ATP}) channel. This procedure further depolarizes plasma membrane and opens the voltage dependent Ca^{2+} channel that activates the exocytosis of insulin-containing granules and insulin secretion [23] (Figure 1).

Insulin secretion consists of two steps: transient first phase followed by a sustained second phase [24]. At the time of exocytosis, the small fraction of granules, called readily releasable pool (RRP), are available for release and contribute to the rapid insulin secretion. The remaining granules belong to the reserve pool with the ability to refill the empty RRPs and deplete insulin [25]. The first phase of exocytosis from RRPs is nutrient independent and could occur in the absence of nutrients. However, the second phase of insulin release and RRP refill from the reserve pool is highly nutrient dependent [26]. Cyclic adenosine monophosphate (cAMP) could also amplify exocytosis of insulin granules through direct activation of low affinity Ca^{2+} sensor [27, 28]. Influx of Ca^{2+} activates production of cAMP through an increase in adenylyl cyclases. cAMP formation triggers protein kinase A (PKA), which further mediates cAMP action in the beta cells.

PKA also facilitates calcium release mechanism leading to insulin secretion [29]. One mechanism to control and reduce formation of cAMP and insulin secretion is through prostaglandin (PG) receptors [30]. Therefore, the inhibition of PG receptors and/ or PG signaling pathways could restore insulin secretion [31-33].

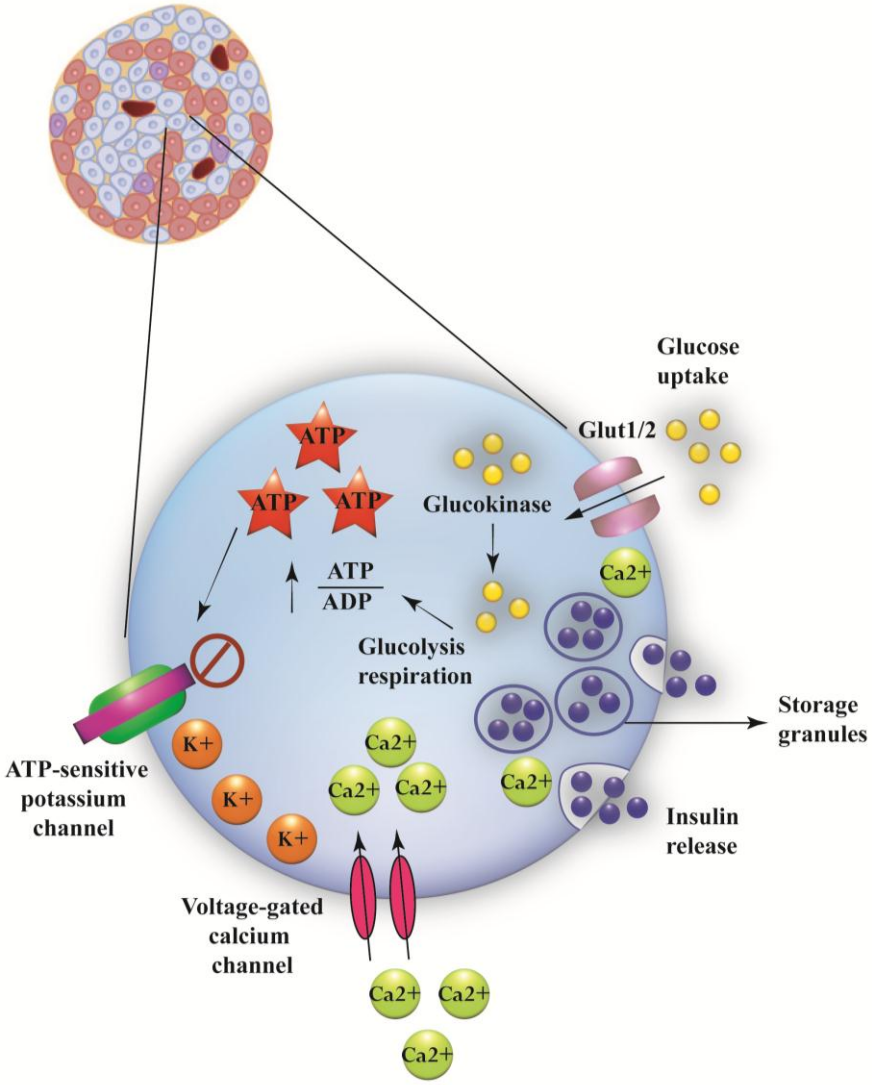

Figure 1: Mechanism of insulin secretion in pancreatic beta cells. Glucose enters beta cells through transmembrane glucose transporter, GLUT 1/2 receptors located on cell membrane. Inside the beta cells, glucose gets phosphorylated by glucokinases, which further increases ATP: ADP ratio. Enhanced ATP level inactivates the potassium channel and depolarizes the beta cell membrane causing the calcium channel to open up and allow the calcium ions to follow inwards. Increase in the cytoplasmic level of calcium leads to exocytosis of insulin from the storage granules [7].

1.2. Diabetes mellitus

Diabetes mellitus (DM) is a metabolic disorder caused by inherited and/or acquired deficiency in insulin production by the pancreas, or by ineffectiveness of the produced insulin and it is associated with prolonged period of high blood glucose, known as hyperglycemia. High level of blood glucose is toxic to the body system and damages in particular the blood vessels and nerves [34]. In 2015, 415 million people lived with diabetes worldwide and this number is estimated to increase to approximately 642 million in 2040 [35]. Much of this increase will occur in the developing countries due to population growth, aging, unhealthy diet, sedentary life style and obesity [34]. Diabetes and the related vascular complications are estimated to be the seventh leading cause of death. DM is mainly divided into two forms, type1 and type 2 diabetes.

1.2.1. Type 1 diabetes

Type 1 diabetes (T1D), which is mainly referred as the insulin-dependent disease, results from an autoimmune destruction of the beta cells within the pancreatic islets [36]. This type of diabetes is also known as juvenile diabetes since it is usually diagnosed in children and young adolescence. This disease has two phases: 1) Insulitis, when mixed populations of leukocytes invade the islets, and 2) Diabetes that is the state in which most of the beta cells are destroyed and no longer able to produce sufficient amount of insulin to control blood glucose levels of the body. This results in hyper- and hypoglycemic episodes in patients [37]. Although T1D is an old described disorder back to ancient Egyptian and Greek writing, we still lack enough knowledge about the occurrence and progression of this disorder [37]. Both genetic disposition and environmental factors are involved in autoimmune chain reactions inducing the beta cell destruction. Recently, the increasing knowledge in genome-wide screening and genotyping technology has identified more than 40 different genes that are involved in this type of diabetes. However the molecular mechanisms contributing to insulitis and beta cell destruction remain unclear [38]. Interestingly, two genetically-regulated pathways are identified to have role in the early stage of T1D and insulitis. These regulatory pathways are involved in recognition of the viruses by the innate

immune system and also regulation of type 1 interferons. These receptors could make beta cells susceptible to pro-apoptotic stimuli and induce an inadequate responses to viral infections [39]. Regarding the environmental factors associated with pathophysiology of T1D, viral infections and in particular enteroviruses are currently the main candidates supported by animal studies. The presence of this type of viruses has been found in type 1 diabetic patients [40, 41]. Animal studies together with virus detection in pancreas of type 1 diabetic cases have revealed the direct infection of the pancreatic beta cells leading to insulitis and activation of pro-inflammatory reactions in pancreatic tissue [42, 43]. Insulitis results in the exposure of beta cells to secreted pro-inflammatory cytokines including, Interleukin (IL)-1β, Tumor necrosis factor-alpha (TNF-α) and Interferon-gamma (INF-γ) and nitric oxide (NO) [44]. Long-term exposure to these pro-inflammatory cytokines in particular, IL-1β and INF-γ induce sequence of reactions regulated by transcription factors, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription 1 (STAT1) and leads to production of various pro-inflammatory cytokines by beta cells and consequently beta cell apoptosis via activation of mitogen-activated protein kinases (MAPKs) signaling pathway, endoplasmic reticulum (ER) stress and release of mitochondrial death signals [43]. Increase in secretion of various pro-inflammatory cytokines could also amplify immune attack of islet cells, mainly through activation of macrophages, T and B cells, dendritic cells (DCs), natural killer (NK) cells and recruitment of antigen presenting cells (APCs) into pancreatic tissue [45, 46]. APCs act as regulatory cells with the ability to remove apoptotic cells and maintain beta cell tolerance [47]. The removal of apoptotic beta cells is crucial. However, in T1D, the presence of viral infection together with the mutation in genes responsible for recognition of viral attack activates various pro-inflammatory reactions in pancreatic tissue and beta cells. This consequently leads to an increase in autoimmune attacks and chronic unresolved apoptotic beta cells [48].

1.2.2. Type 2 diabetes

Type 2 diabetes (T2D) characterizes by progressive beta cell dysfunction and insulin resistance. T2D is a multi-factorial and the most prevalent type of diabetes worldwide [34, 49]. This type of diabetes is largely due to obesity, physical inactivity, aging, sedentary life style and unhealthy diet [50]. In pre-diabetic phase, pancreatic beta cells respond to insulin resistance and ineffective insulin production by increasing their mass (beta cells hyperplasia) and insulin secretion (hyperinsulinemia). When the anatomical and functional expansion of beta cells fail to compensate for insulin resistance, relative insulin deficiency develops and over time leads to T2D [51]. The progression of T2D is driven by chronic low-grade inflammation and could prompt by obesity and unhealthy life style. Overfeeding together with an increase in circulating glucose elevate pancreatic beta cell workload, which further make the islets more susceptible to ER and oxidative stress [52, 53]. In addition, obesity and increased free fatty acid storage in adipocytes make these cells more prone to secrete pro-inflammatory cytokines including IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), IL-1β and TNF-α. Elevated level of pro-inflammatory molecules in the circulation activate T-cells, B-cells and macrophages and consequently results in beta cell loss [54]. Reduction in beta cell mass could be explained by both beta cell apoptosis and dedifferentiation of pancreatic beta cells [51, 55, 56]. In line with this, a significant decrease in beta cell mass has been reported from diabetic patients compared to the BMI-matched controls [55]. Treatment of T2D involves diet control and physical activity along with blood glucose lowering medications. In some cases, patients become insulin-dependent and require exogenous insulin treatment [57].

1.3. Treatment option for type 1 diabetes

A hurdle in T1D management is the life-long patient dependence on insulin therapy in order to stay euglycemic [58]. Therefore, both long-term and repeated period of hyper- and hypoglycemia are known as leading factors inducing diabetes complications such as retinopathy, nephropathy, neuropathy, cardiovascular disease, which thereby increases the mortality rate of patients

suffering from this disease [59]. The quality of glycemic control relies on accurate personalized treatment for patients based on the duration and stage of the disease as well as individual risk of acute and/or late-stage complications [60]. Insulin discovery in 1921 and the first medical administration of insulin to a T1D patient in 1922 were clearly the most significant therapeutic events in the history of diabetes [61]. Continuous administration of exogenous insulin by daily multiple insulin injections or through subcutaneous insulin infusion stands are the main approaches to control blood glucose in T1D [62]. Nowadays, advanced insulin pumps are able to calculate the amount of insulin a person needs based on the expected carbohydrate intake and the history of blood glucose concentration. Therefore, insulin pumps provide an advantage of avoiding unpleasant multiple self-injections and repeated daily glucose monitoring. Insulin pumps have been reported to provide better glycemic control, fewer episodes of hypo- and hyperglycemia and improved hemoglobin A1c (HbA1c) compared to the multiple insulin injections [63, 64].

1.4. Beta cell replacement therapy

Undoubtedly, insulin discovery resulted in a remarkable achievement in the field of diabetes therapy by reducing the mortality rate of diabetes from around 85% in 1897-1914 to less than 1% in 1950s. Exogenous administration of human recombinant insulin has greatly improved life expectancy in diabetic patients; however it does not work as efficient as endogenous insulin regarding precise regulation of blood glucose and therefore cannot overcome the high morbidity and mortality associated with diabetes [64]. One alternative to exogenous insulin delivery to patients with T1D with the aim to restore endogenous beta cell mass is beta cell replacement therapy. Nowadays, beta cell replacement therapy includes transplantation of the solid pancreas organ or isolated islets from deceased organ donors to patients suffering from severe T1D.

1.4.1. Pancreas transplantation

Beta cell replacement therapy has proven to restore glycemic control with the ability to improve the quality of life for T1D patients [65, 66]. Since the first pancreas transplantation in 1966 at the

University of Minnesota, more than 50,000 solid pancreas organs have been transplanted worldwide [67]. Analysis of pancreas transplants from the International Pancreas Transplant Registry, performed from 1984 to 2009 showed 73% and 56% of total 5-years and 10-years pancreas graft function respectively for simultaneous pancreas-kidney transplant. These numbers were around 64% and 38% for pancreas after kidney and 53% and 36% for pancreas transplantation alone respectively [68]. Given the improvement in the outcome of pancreas transplantation and significant benefits on patient quality of life, pancreas transplantation could be performed for T2D patients and older (age ≥ 60 years) recipients [69]. Although, pancreas solid organ transplantation has clearly reduced diabetic complications and improved patient survival, there are major drawbacks associated with this technique. This includes the risk of doing major surgery, and the use of high intensity immunosuppressive medication that could increase the risk of infection and malignancy [70-75]. While these drawbacks could be minimized with proper donor selections and improvement in surgical techniques, newer beta cell replacement therapies are under investigation that could be more beneficial and less invasive for diabetic patients.

1.4.2. Islet transplantation

A less invasive procedure of beta cell replacement therapy is allo-islet transplantation, which has proven to be a safe and effective treatment offering significant benefits in, i) lowering daily insulin requirement, ii) improving the level of HbA1c, iii) reducing the incident of unaware hypoglycemia, iv) reducing diabetes complications and v) improving quality of life [76-79]. From the surgical perspective, islet transplantation is considered less invasive with 20-fold lower morbidity risk compared to the whole pancreas transplantation. It consequently favors recipients at cardiovascular disease risk with the potential for secondary complications [76]. First attempts to reverse diabetes in rodents and large animal models using isolated islets led to further investigation for autologous and allogeneic clinical islet transplantation [80-84] and has made a transition in islet transplantation from being an experimental therapy to a routine clinical procedure with predictable efficacy for selected patient population with T1D. In 1990, the first

successful clinical human islet allo-transplantation was reported with the use of steroid-free immunosuppressive regimen. This allowed prolonged insulin independence status in type 1 diabetic recipients [85, 86]. The use of antioxidant and insulin therapy together with immunosuppression protocol improved human islet transplantation outcome and increased insulin independence rate to 30% 1 year after transplantation [87, 88]. In 2000, Shapiro et al. demonstrated a ground-breaking achievement on insulin independence after 12 months post transplantation in seven type 1 diabetic patients with uncontrolled blood glucose and severe hypoglycemia (A condition refers to a brittle type 1 diabetes) using a protocol that is now well-known as Edmonton protocol [78]. This protocol consists of steroid-free immunosuppression and transplantation of large islet mass (> 11000 islet equivalent (IEQ) / kg body weight) [78, 89]. Although, this protocol has achieved viable islet engraftment, insulin independence tends to be lost by fifth year post transplantation and only 10% of patients remain insulin independent by this time [90]. Edmonton protocol launched islet transplantation as an alternative to conventional insulin therapy. Islets transplantation has been improved over the years and it has led to 50% insulin independence 5 years after allogenic islet transplantation with 70% maintained graft function as measured by the protection from severe hypoglycemic episodes in recipients [76]. Furthermore, islet transplantation has now being used to prevent surgically induced insulin dependent diabetes in non-diabetic patients that require total or partial pancreatectomy due to malignant or non-malignant disease. Transplanting back the patient own islets, which is known as auto islet transplantation, cloud have significant impact on the quality of life after pancreatectomy in these patients [91, 92].

1.5. The islet isolation procedure

First attempts for islets isolation from pancreas are back to 1902, when the Russian doctor Leonid W. Sobolew suggested the idea of physically separating the exocrine tissue from the endocrine part of the pancreas [84]. In 1965, bacterial collagenase was first used to dissociate guinea pig islets from pancreatic tissue [93]. This method was not improved until 1985, when rodent islets

were isolated using intra-ductal injection of collagenase, followed by enzymatic destruction of pancreatic tissue and hand-picking isolated islets under a microscope [94] [95, 96]. Since hand-picking isolated islets were not feasible for large-scale islet isolation, density gradient purification method was developed. The first density gradient was based on sugar or albumin. However, Ficoll solution, which is a high molecular weight polymer of sucrose, was introduced later on and improved islet purification from exocrine tissue [96, 97]. A breakthrough in the field of islet isolation and transplantation was the invention of the Ricordi chamber in 1988. The Ricordi chamber is a dissociation-filtration chamber with a conical upper part and a lower cylindrical part, where pancreatic tissue is placed in the lower part and digested by the combination of enzymatic digestion at 37 ºC and gentle mechanical agitation of the chamber. This is followed by filtration of the released islets through the mesh located at the upper part of the chamber [82]. This method has been successful in the field of islet isolation and currently been used as a gold standard for human and large animal islet isolation around the world. On the same year, the introduction of COBE 2991 processor, which was originally used to process bone marrow, allowed large-scale purification of human islets from exocrine part of the pancreas using Ficoll solution in a sterile system. This method is also currently in use for islet isolation from large animals and humans [98].

A brief overview of clinical human islet isolation and transplantation is illustrated in Figure 2. In this procedure, pancreas is usually procured from a donor after brain-death (DBD) or cardiac-dead (DCD) and transported to a sterile good manufacturing practice (GMP) facility as soon as possible to avoid prolonged cold storage of pancreas (Figure 2, part 1). The pancreas undergoes enzymatic and mechanical digestions, which allow dissociation of islets from surrounding tissue (Figure 2, part 2). This is followed by islets purification from exocrine and non-islet cells using a density gradient processor (Figure 2, part 3). The functionality, viability and morphology of the purified islets are assessed before they are infused by an ultrasound guided percutaneous cannulation into the portal vein of the recipient liver, where they engraft and start producing insulin (Figure 2, part

4) [99]. Another advantage of this procedure is that it allows the *ex vivo* manipulation of isolated islets before transplantation. This could open a window of opportunity for improving the isolated islets in order to achieve superior transplantation outcome [100].

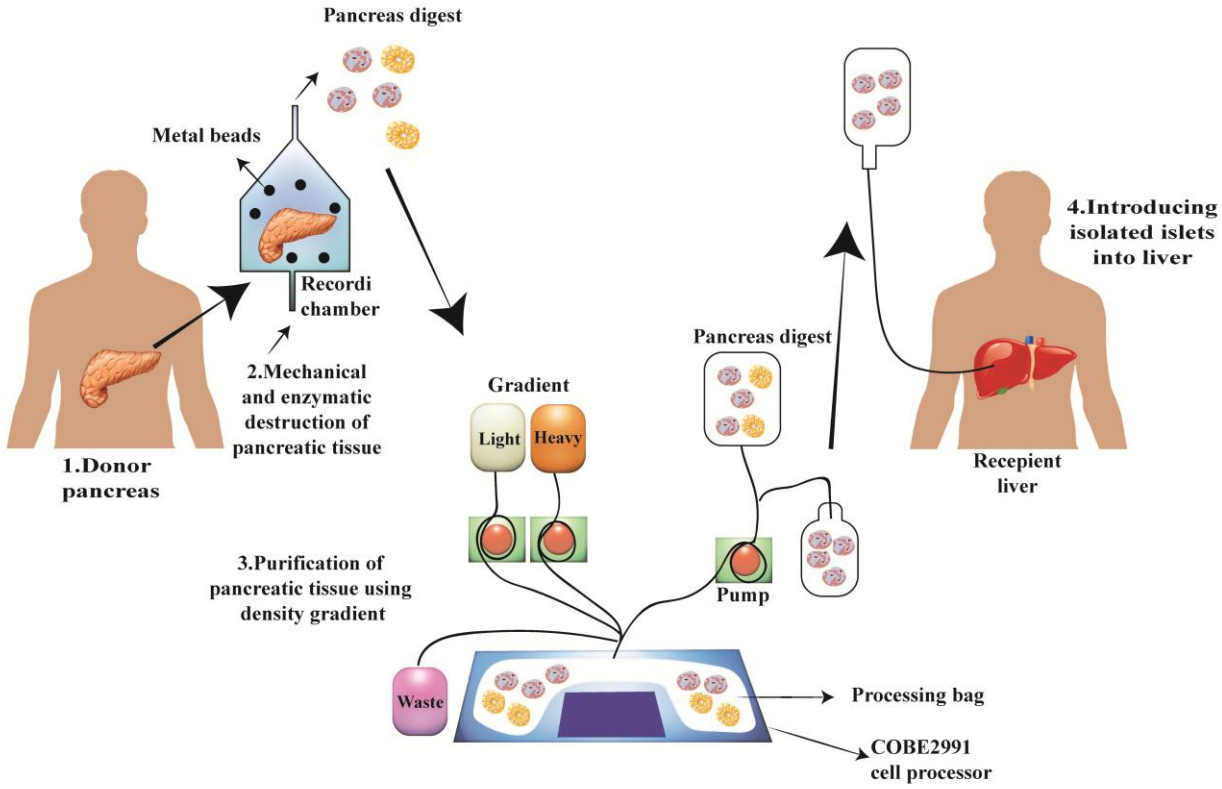

Figure 2: Overview of the islet isolation and purification procedure. 1. Pancreas obtains from a DBD or DCD organ donor and transports to an approved cell isolation facility. 2. Digestion of the pancreatic tissue is performed by using temperature controlled enzymatic and mechanical destruction of tissue in a specialized chamber called Ricordi chamber. 3. Digested pancreatic tissue is purified using continuous density gradient. 4. Purified islets that are passed quality controls could be transplanted into the recipient liver of T1D patients, where they engraft and secrete insulin in response to blood glucose.

Islet transplantation has proven to be a safe and effective treatment with the ability to provide glycemic control and hypoglycemia awareness for selected patients with T1D [76, 101-106]. However, there are risks and barriers associated with this technique that hinder the widespread application of this procedure [107].

1.6. Challenges for clinical islet transplantation

Efforts into clinical and basic research to overcome the barriers for the beta cell replacement therapy by means of islet transplantation could help this promising therapy into a more robust cure for patients with T1D. The two main challenges for islet transplantation that have been defined are the limitation of available donor organs and the need for life-long immunosuppressive therapy. A donor pancreas needs to meet certain criteria to be able to use as a donor pancreas and generate high yield of good quality islets for transplantation. Several attempts to generate donor criteria score system has been developed, but lack of reproducibility among the different donor cohort has revealed no clear independent factor such as donor age, cause of the death, BMI, duration of ischemia and parenchymal fat infiltration that could predict the clinical outcome of islet transplantation [107]. However, increasing the donor pool is necessary and more centers have now developed islet isolation from DCD donors [108, 109] and even using islets from partial pancreatectomized living donors [110, 111].

Examination of T1D progress revealed that an individual could stay euglycemic with only 10% of beta cells mass. Beyond this threshold, patients tend to have deteriorated glucose homeostasis leading to diabetes [112, 113]. Therefore, theoretically one donor should provide enough islets to make one patient (or maybe more than one patient) insulin free after transplantation. However, the necessity of using multiple donors to achieve insulin independence in T1D patients suggests a combination of various factors that could affect successful islet transplantation outcome. The factors influencing islet quality prior and post transplantation include instant blood-mediated inflammatory reaction (IBMIR), autoimmune reactions mediating islet destruction, allo-immune rejections, toxicity of long-term use of immunosuppressive medication, release of pro-inflammatory molecules by islets and immune cells at the transplantation site and activation of non-immune stress mediators within transplanted islets [114].

Brain dead donors are the major source for clinical islet transplantation. Pro-inflammatory molecules such as TNF-α and IL-6 are released at the onset of brain death and induce an adverse

effect on organs including islets [115, 116] (Figure 3, part 1). Genome-wide transcriptional studies obtained either by laser-captured micro dissection of whole pancreas or isolated islets from donor pancreas showed an up-regulation in the expression of pro-inflammatory molecules particularly, IL-8, MCP-1, GCP-2, MEC, CXCL-1/GRO-α, CXCL-2/GRO-β, IL-1ß and CXCL-12 in islets [117-119]. This elevation in pro-inflammatory mediators not only could deteriorate isolated islet survival prior to transplantation, but also impair transplanted islet graft function [117, 120].

Currently, the preferable site for islet transplantation is the intraportal route, although several studies has shown the disadvantage of this site, since it could led to > 60 % destruction of the islets due to innate immune responses [107]. The direct contact of newly transplanted islets with blood elements mediates activation of IBMIR, which is considered as one of the major reasons for islet loss in clinical islet transplantation (Figure 3, part 3) [121]. IBMIR is characterized by rapid activation of the platelets, coagulation and complement systems together with the infiltration of leukocytes to the islets [122, 123] that could trigger production of pro- inflammatory mediators such as tissue factor (TF) and MCP-1 by islets. Production of these mediators is associated with low outcome for clinical islet transplantation [124, 125]. The effect of IBMIR on islet damage and loss has been established using both *in vitro* and *in vivo* models of intraportal pig islet transplantation into mice [126] or into monkey [127, 128]. IBMIR was reported in 9 patients who had clinical allogenic islet transplantation evidenced by an increase in TF and thrombin anti-thrombin (TAT) complexes after islet infusion, followed by clothing process and islet damage [124, 129]. This increase in IBMIR reaction was correlated with deteriorated C-peptide secretion in patients 7 days post transplantation [129]. IBMIR response could create cytokine storm and an environment that enhance allogenic response and consequently loss of newly transplanted islets [130].

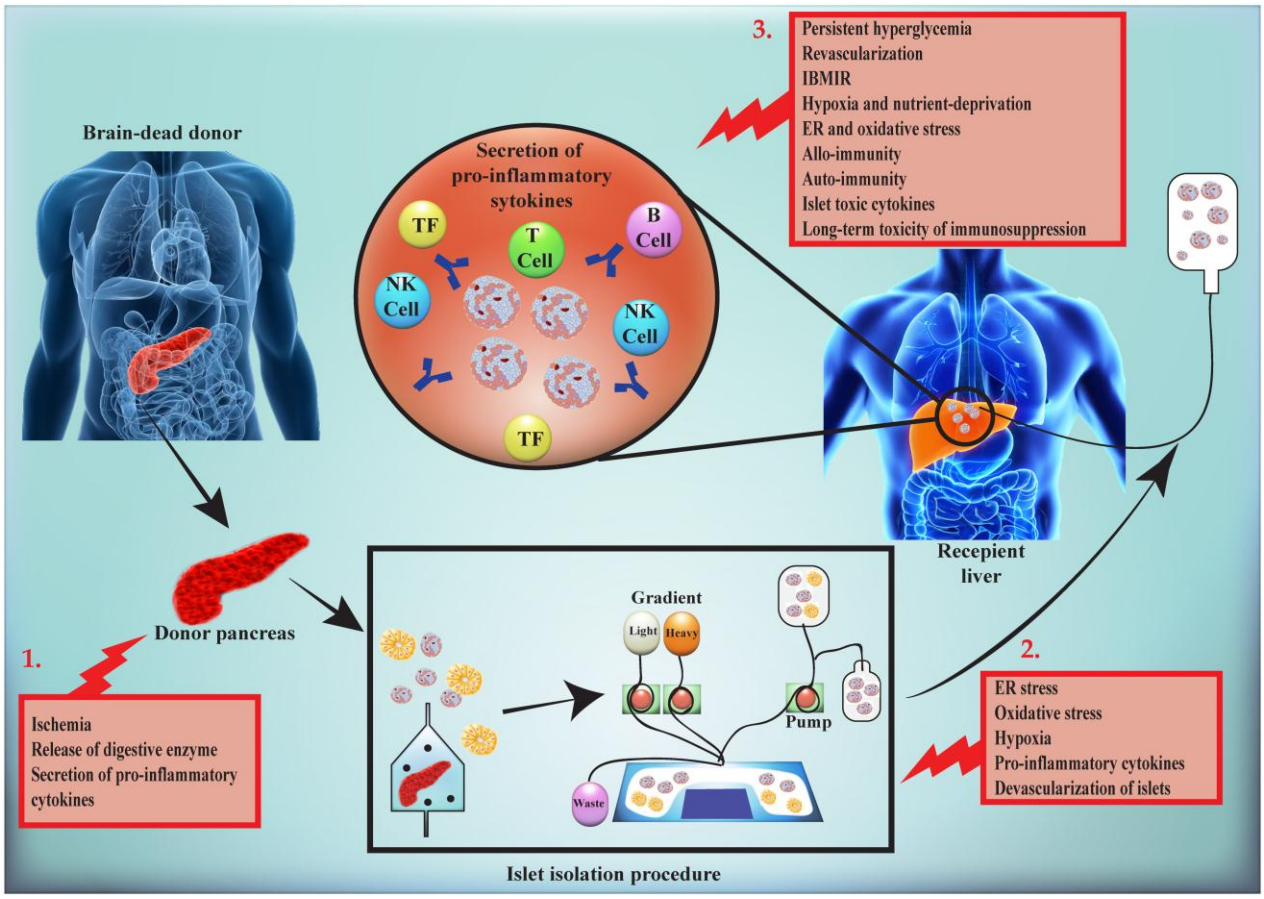

Figure 3: Obstacles in the process of islet transplantation. An overview of immunological and non-immunological stress conditions associated with islet loss and dysfunction in islet isolation and transplantation procedure.

The other main concern regarding a successful outcome of islet transplantation is immunosuppressive therapy to avoid allo-immune rejection of newly transplanted islets. Unfortunately, many of the compounds used as immunosuppression medication are toxic to islets. Corticosteroids are one of these compounds that were used before the Edmonton protocols as immunosuppression regimen after islet transplantation [131, 132]. One of the main strengths of Edmonton protocol was the avoidance of corticosteroids and its replacement with Sirolimus (mTOR inhibitor), Tacrolimus (a calcineurin inhibitor) and Daclizumab [78]. This protocol has its own side effects, as Tacrolimus might induce neuro- and nephrotoxicity in addition to beta cell damage [133, 134]. Moreover, by using this protocol, long-term insulin independence was not achieved and patients returned to modest amount of exogenous insulin administrations by 5 years

post transplantation. Substantial improvement in long-term insulin independence (> 5 years) have been made through induction agents such as Alemtuzumab in conjugation with low dose Tacrolimus / Mycophenolate Mofetil that induce the process of T-cells depletion [135]. The long-term success of islet transplantation is dependent on the successful establishment of immunosuppressive regimen that selectively target donor reactive T-cells while expanding population of regulatory T-cells [136].

Not only prevention of allo-immunity is an essential challenge for improving islet transplantation outcome, but also reoccurrence of auto-immunity is another barrier affecting the successful clinical islet transplantation in T1D patients (Figure 3, part 3). Reoccurrence of auto-immunity with selective beta cell destruction has been reported after islet transplantation [137, 138]. Although, detection of auto-immune antibodies such as anti-GAD65, anti-ZnT8A and anti-IA2A could only weakly predict the outcome of islet transplantation, positive recipient for auto-antibodies showed earlier loss of functional transplanted beta cells compared to the recipients who were negative for auto-antibodies prior to transplantation [139, 140]. These observations about the predictive role of auto-antibodies clearly confirm the importance of auto-immunity reoccurrence in the loss of functional islets post transplantation [141]. The factors that could influence auto-antibody changes post islet transplantation are still not fully understood, but immunosuppression medication, anti-thymocyte globulin (ATG) and Mycophenolate Mofetil treatment could positively associate with the risk of an increase in the levels of auto-antibodies [140, 142].

Despite the fact that allo-immunity and presence of auto-antibodies post islet transplantation are essential factors influencing islet graft function, evidence such as absence of islet auto-antibodies 1 year post transplantation in recipient who lost insulin independence [143] as well as gradual loss of glycemic control in patients received their own islets due to pancreatitis [144, 145] has suggested a progressive impairment in islet function and insulin production due to non-immune factors affecting long-term islet transplantation [146].

1.7. Influences of non-immune factors on islet function and survival

The exact cause of massive islet loss in the immediate phase after transplantation is not fully understood. Human islets are exposed to variety of non-immune stress responses due to mechanical and chemical destruction of pancreatic tissue and dissociation from their natural microenvironment during islet isolation procedure and after transplantation (Figure 3, part 2) [147-150]. These non-immune factors include prolonged hyperglycemia and blood glucose fluctuation, metabolic stress such as ER and oxidative stress, inflammatory reactions induced by pro-inflammatory molecules together with hypoxia and nutrient deprivation mainly due to complete loss of islet vasculature system during islet isolation procedure and revascularization after transplantation [121, 151, 152].

Islet transplant recipients demonstrate a decrease in first phage glucose stimulated insulin secretion, elevated level of fasting blood glucose and progressive loss of glycemic control compared to the healthy individuals [153, 154]. Prolonged high level of blood glucose in islet transplant recipients activates ER and oxidative stress, induces islet inflammation and could lead to beta cell death post transplantation [155]. Presence of hyperglycemia not only affects islet cells but also induces adverse effect on islet endothelium [155] and pro-inflammatory changes in monocytes [156]. Activation of inflammatory reaction within the newly transplanted islet graft due to not only hyperglycemia but also IBMIR reaction elevates secretion of pro-inflammatory cytokines by islets and infiltration of macrophages to the site of transplantation [146, 157, 158]. All these factors that could happen during islet isolation procedure and also post transplantation create a vicious cycle for islets and deteriorate long-term success of islet transplantation. These issues are discussed in detail in the following chapters.

1.7.1. Endoplasmic reticulum stress in pancreatic islets

The ER is a cellular organelle that is involved in protein and lipid synthesis, folding of newly synthetized proteins and post translational modifications. Appropriate protein folding is mandatory for their proper function. Therefore, ER contains molecular chaperones as well as high

concentration of Ca^{2+} that are required for functional protein folding [159]. However, an increase in the load of newly synthetized proteins to the ER and/or a decrease in the ER chaperones lead to accumulation of unfolded and misfolded proteins and activation of ER stress. A cellular adaptive response signaling pathway, which gets activated by ER stress with the aim to restore ER homeostasis is called unfolded protein response (UPR) [160]. UPR signaling network is initiated by activation of the three main transmembrane proteins: protein kinase R-like endoplasmic reticulum kinase (PERK), inositol- requiring enzyme-1α (IRE-1α) and activating transcription factor 6 (ATF6). These proteins participate in reducing protein translation as well as increasing the biosynthesis capacity of ER through elevation of molecular chaperones [159-161] (Figure 4).

Pancreatic beta cells are more sensitive to ER stress because of the high rate of insulin biosynthesis in response to glucose regulation. Short-term and mild ER stress activates adaptive UPR signaling pathway. Adaptive UPR secures ER homeostasis through transient reduction in global protein translation, increase in molecular chaperones and protein folding enzymes and ER-associated degradation (ERAD) machinery [162, 163]. Prolonged and unresolved ER stress switches the adaptive UPR to the apoptotic UPR through increase in transcription factor, C/EBP homologous protein (CHOP) and caspase cascades [164, 165]. Increase in the levels of ER stress transducers, such as phosphorylated eukaryotic initiation factor-2α (peIF2α), spliced x-box binding protein-1 (XBP-1) and phosphorylated c-Jun N-terminal kinase (p-JNK) as well as caspase 3/7 immediately after isolation but not in donor pancreatic tissue indicates the activation of apoptotic part of UPR pathway due to isolation procedure [151].

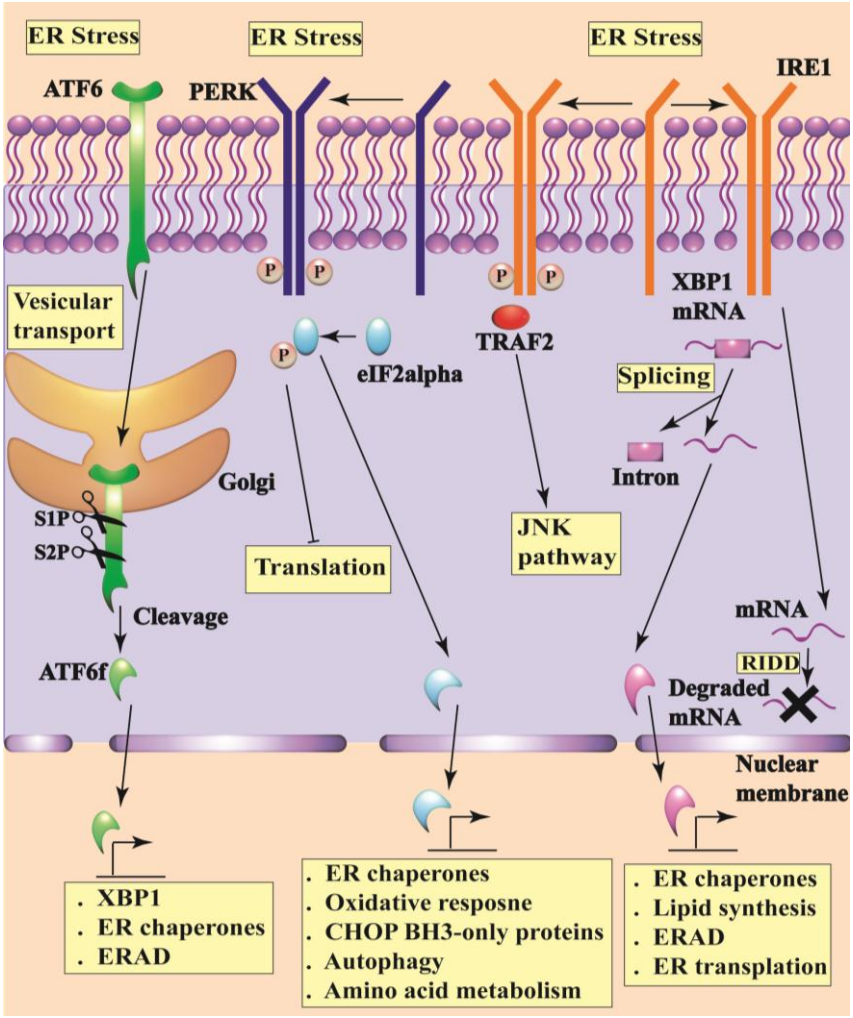

Figure 4: Description of ER stress and UPR signaling pathways. Accumulation of unfolded proteins in the ER activates IRE1, ATF6 and PERK. Activated IRE1 induces mRNA splicing and activation of XBP-1 followed by XBP-1 translocation to nucleus, where it regulates the genes involved in protein folding and ERAD pathway. IRE1 operates independent of XBP-1, by activation of TNF receptor-associated factor 2 (TRAF2) leading to induction of JNK and modulation of autophagy and apoptosis. ER stress induces translocation of ATF6 to the Golgi, where it is cleaved by site 1 protease (S1P) and S2P. Fragmented ATF6 (ATF6f) acts as a transcription factor and encodes genes required for ERAD and protein folding. Activated PERK phosphorylates eIF2α, which results in inhibition of total protein translation except ATF4. ATF4 translocates into the nucleus and encodes the expression of ER chaperones as well as genes involved in autophagy, redox control, amino acid metabolism and apoptosis including CHOP.

Islet transplantation in diabetic patients increases the metabolic demands for protein synthesis in transplanted human islets. This makes the transplanted islets susceptible to ER stress and activation of UPR. Elevated UPR mediators together with CHOP and caspase 3/7 have been reported in human islets transplanted under kidney capsule of hyperglycemic and marginally

normoglycemic mice [151]. This could suggest the involvement of apoptotic UPR induced by elevated glucose level in subjects long-term post islet transplantation [151]. ER stress and consequently apoptotic UPR could be one the main contributors involved in islet loss during islet isolation procedure and post transplantation [147, 151, 163]. Therefore, strategies to interfere with apoptotic UPR mechanism could improve the long-term success of islet transplantation outcome.

1.7.2. Oxidative stress in pancreatic islets

Reactive Oxygen Species (ROS) are a group of free radicals and molecules derived from oxygen by mitochondria and play an important role in physiology and pathology of insulin producing beta cells [166, 167]. Under physiological condition, all the cells including beta cells use several antioxidant systems in order to neutralize ROS and maintain their homeostasis status [168]. For many years, ROS has been thought as an unfortunate byproduct of respiratory energy production in mitochondria with extremely deleterious effect. However, ROS has been shown recently to modulate many physiological processes including gene transcription, ion transport and protein phosphorylation [52]. An enhanced ROS formation decreases cell antioxidant defense. This could lead to oxidative stress and apoptosis induced by the intrinsic and extrinsic mitochondrial signaling pathways [169]. It is well-known that the islets are sensitive to ROS not only due to their low levels of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalases [170-174], but also as a result of high glucose metabolism that could increase the ROS formation [167]. In addition, pancreatic islets have a poor DNA repair capacity [175], which could make them susceptible to loss of function and cell death [176, 177].

Enzymatic destruction of islet microvascular environment during isolation procedure together with the slow revascularization of transplanted graft make islets ischemic and susceptible to oxidative stress from the time of isolation to the period required for graft revascularization in the recipient [150]. Considering the low levels of antioxidant enzymes within an islet, prolonged oxidative stress has major deleterious effect on isolated islets and it is one of main reasons for islet loss post transplantation. One strategy to defeat oxidative stress in isolated islets is addition

of antioxidant compounds to the islet culture post isolation. Enhanced antioxidant capacity of islets has been shown to improve islet transplantation outcome in animal model [146, 178]. Nicotinamide (NA) is a well-established cyto-protective compound that could ameliorate islet injury induced by free radicals and pro-inflammatory molecules. Supplementing human islets with NA during isolation has been reported to significantly increase islet yield, reduce production of pro-inflammatory molecules and pro-coagulation factor tissue factor (TF) by islets without inducing any negative impact on insulin secretion post isolation [179]. Although, NA is currently added to clinical islet culture [180], more comprehensive studies are required to provide efficient protocols using combination of different antioxidants in order to reduce oxidative stress and enhance islet survival.

1.7.3. Hypoxia and nutrient deprivation in pancreatic islets

Pancreatic islets are highly vascularized cluster of cells and despite consisting only 1-2 % of an adult pancreas, islets receive 20 % of the pancreas blood flow [181]. In fact, studies on the morphology of intra-islet vascularization show that each cell within the islet is not more than one cell away from the blood circulation [182]. Pancreatic islets require highly oxygenated blood (pO_2 of 40 mmHg) and nutrient rich environment in order to facilitate their rapid response to blood glucose and allow the secreted hormones to exert an effect quickly [183]. The enzymatic and mechanical destructions of pancreatic tissue during islet isolation procedure destroy cellular and non-cellular compartments of the pancreas to make islets free from their surrounding tissue. Therefore, isolated islets lose their vascularized capillary and consequently, become more prone to hypoxia and nutrient deprivation [184]. This increase in hypoxia and nutrient deprivation in human islets have been reported to induce central necrosis combined with apoptotic features such as DNA fragmentation and activation of intrinsic apoptosis signaling pathway induced by pro-apoptotic member of Bcl-2 family [152, 184-186]. Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), poly (ADP-ribose) polymerase (PARP) and MAPK signaling pathways, the three major pathways responsible for cellular stress, were shown to be up-regulated during

islet isolation procedure in human islets and increase the release of IL-6, IL-8 and MCP-1 by the isolated islets [147, 187]. Elevated oxidative stress in isolated human islets [188], increased autophagy in human and rodent islets exposed to nutrient deprivation [189] and reduced ER-to-Golgi protein trafficking in mouse islets due to hypoxia [190] confirm the detrimental effects of low oxygen and nutrient deprivation on islet health and function.

Intraportal transplanted human islets require up to 14 days to start the re-vascularization process and even longer time for full vascular remodeling in patients with long-standing diabetes [191]. During this period, transplanted islet graft only receives oxygen and nutrients from passive diffusion [192]. Therefore, islets are exposed to immediate hypoxia post transplantation specially islets with more than 200 µm in diameter. These islets are nearly anoxic in center [193]. Partial oxygen pressure (pO_2) in liver portal circulation is between 10-15 mmHg, which is already lower than what islets require to maintain their homeostasis [194]. This oxygen deficiency is magnified by a reduction in the ability of transplanted islets to re-vascularize and absorb oxygen [195]. Oxygen pressure for newly transplanted islets is around 8-10 mmHg [183, 196]. Human and mouse islets implanted under kidney capsule of nude mice showed 50% less vascular density accompanied with reduced pO_2 in comparison with intact mouse and human islets one month post transplantation [197]. Reduced vascularization due to hypoxia and amyloid development were observed in human islet grafts one month after intraportal infusion in a nude mice models suggesting that the degree of islet cell death is an important factor for the engraftment [150]. In addition, elevated necrotic and apoptotic cells together with reduced total islet mass were reported in transplanted human islets into the liver of nude mice 30 days post transplantation [198]. Decrease in transplanted islet vasculature suggests poor and slow engraftment as one of the reasons for absence of insulin independence in long-term post islet transplantation [197].

1.7.4. Hyperglycemia and inflammation in pancreatic islets

Short-term (from minutes to few hours) exposure of islets to physiological level of glucose stimulates protein biosynthesis with preferential effect on proinsulin [199, 200]. Glucose regulates

several genes involve in ER and oxidative stress response [201]. It also increases Ca^{2+} pumping into the ER lumen [202], which is required for refilling insulin cellular stores and maintaining islet responsiveness for the next glucose challenge. Besides, the acute regulatory mechanism of glucose on pancreatic islets, long-term glucose regulation is responsible for maintenance of islet function through expression of specific beta cell transcription factors [203-205]. These transcription factors include pancreas duodenum homeobox-1 (PDX-1), V-maf-musculoaponeurotic fibrosarcoma oncogene homologue A (mafA) and neurogenic differentiation 1 (NeuroD1), which all are essential for maintenance of insulin gene expression and regulation of the genes involved in insulin secretion, mitochondrial respiratory chain and fatty acid biosynthesis [206].

While physiological concentration of glucose is essential for maintenance and proper function of pancreatic islets, prolonged and repeated exposure to elevated level of glucose creates a hyperglycemic milieu inducing a toxic effect on beta cells, which is defined as glucotoxicity [207, 208]. Loss of balance between the level of insulin biosynthesis and the required insulin release result in elevation of proinsulin to insulin ratio, activation of ER and oxidative stress responses that could emerge as dysfunction and apoptotic beta cells [43, 209]. Hyperglycemia could induce inflammatory responses in islets and recruitment of immune cells such as macrophages to the pancreatic islets. Thus, presence of prolonged hyperglycemia could create a vicious cycle leading to an increase in pro-inflammatory mediators within pancreatic tissue [7, 210], which is the hallmark in both type 1 and type 2 diabetes [211-214] (Figure 5).

In addition, platelet hyperactivity has been reported in both type 1 and type 2 diabetic patients and associated with i) an increase in platelet volume, ii) elevated adhesion and aggregation capacity and circulating level of platelet-specific proteins including platelet glycoproteins [215-218]. Activated platelets release factors including CD40 ligand (CD40L), chemokine (C-C motif) ligand 5 (CCL5, known as RANTES), tumor necrosis factor superfamily member 14 (TNFSF14,

known as LIGHT), CD36 and etc. which all could expand and amplify platelet activity and promote the release of cytokines and chemokines [215, 216].

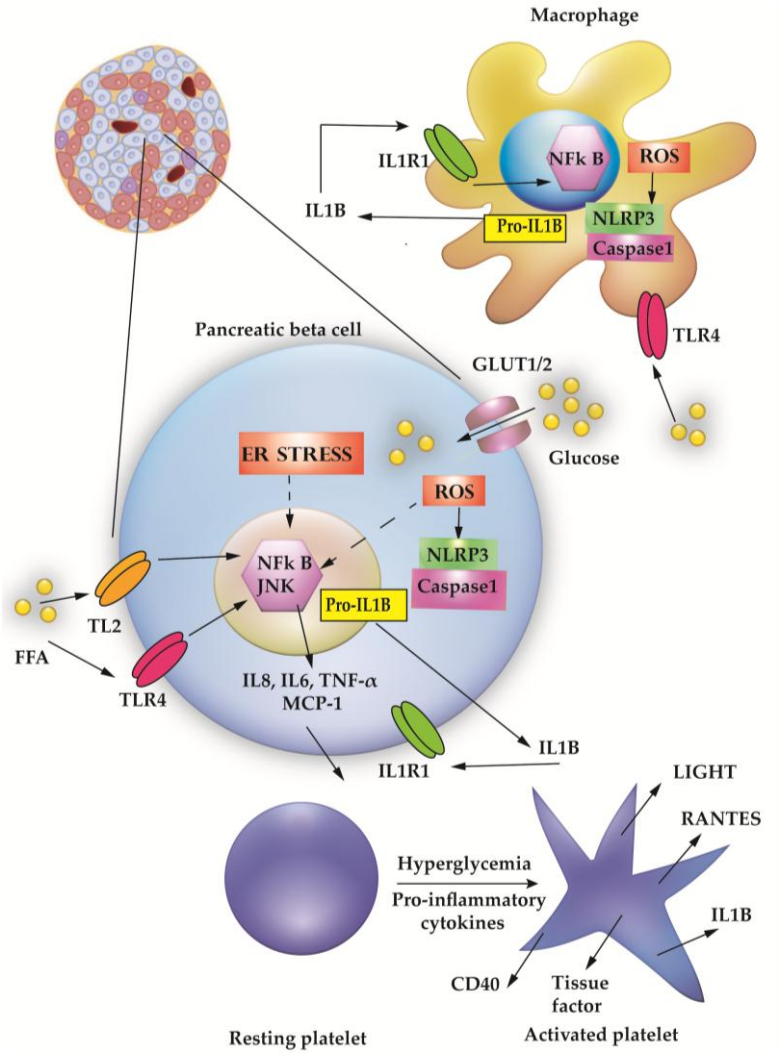

Figure 5: The effects of hyperglycemia and inflammation on pancreatic beta cells. Although physiological level of glucose is required for maintenance of beta cell function, prolonged and repeated exposure to elevated level of glucose induces an adverse effect on beta cell survival and function. This is partly through activation of ER and oxidative stress that could lead to upregulation of NFκB and JNK signaling pathways and mediate production of the pro-inflammatory molecules such as IL-6, IL-8, MCP-1, TNF-α and IL-1β by islets. Hyperglycemia not only activates apoptosis and inflammatory responses within the islets, but also attracts macrophages to the pancreatic islets. In addition, hyperglycemia mediates platelet activation. This could result in an increase in production and secretion of pro-inflammatory mediators such as LIGHT, RANTES, CD40, TF and IL1β by platelets. All these molecules could create a vicious cycle inducing beta cell loss of function and apoptosis [7].

Newly diagnosed type 2 diabetic patients with postprandial hyperglycemia were detected with platelet hyperactivity evidenced by increased urinary 11-dehydro-thromboxane (TX) B2, a marker of *in vivo* platelet activation [219]. Prolonged hyperglycemia is suggested as a causative factor for abnormal Ca^{2+} homeostasis in platelets that could lead to their hyperactivity [220].

After islet transplantation, the islets are often exposed to fluctuation in blood glucose levels, and activation of platelets by the non-immune inflammatory responses [114, 146, 157]. In addition, blood glucose fluctuation, hyperglycemia and release of various pro-inflammatory cytokines and chemokines not only attract host macrophages to the site of transplanted islets [156, 158], but could also activate residual macrophages within pancreatic islets. These macrophages are important mediators inducing islet injury mainly through production of IL-1β in response to pro-inflammatory cytokines TNF-α and INF-γ [221, 222]. Therefore, these pro-inflammatory responses create a harsh stress environment for the newly transplanted islets.

Furthermore, an study focusing on the effect of hyperglycemia on transplanted islet graft function revealed that transplanting mouse islets under hyperglycemic condition using diabetic mouse model induced down-regulation in the level of glucose transporter (GLUT)-2 [223] and an increase in the level of apoptosis and necrosis in transplanted islet graft [209]. Another study also showed down-regulation in the expression of genes involved in differentiation and maintenance of mature islets including Pdx-1, Neurod1, Nkx6.1 and Pax6 in marginal mass of rat islets transplanted under kidney capsule of diabetic rat model [224]. Islet injury induced by hyperglycemia may persist in transplanted islets, as impaired insulin secretion and reduced islet function have been reported in human islet graft transplanted in diabetic mice two weeks post transplantation [225]. Therefore, understanding the mechanism involves in irreversible adverse effect of hyperglycemia on islets could minimize beta cell loss and improve graft survival post transplantation [145, 209].

1.7.5. Inflammatory responses in pancreatic islets

In diabetic milieu, pro-inflammatory cytokine production and release by transplanted islets could induce apoptosis and necrosis within islet cells (autocrine effect). It could also attract other cell types such as infiltrated immune cells to the site of transplantation (paracrine effect), inducing detrimental effects on islet survival and function [146]. Pro-inflammatory cytokines such as IL-1β and TNF-α could activate NFκB and MAPK leading to NO production and activation of JNK signaling pathway. This was evidenced by enhanced NFκB activity, NO-induced oxidative stress and JNK-induced apoptosis in isolated human islets cultured with IL-1β, TNF-α and INF-γ [147, 157, 226-228].

IL-1β is a pro-inflammatory cytokine with a significant adverse effect on islet function and survival [229], through the inhibition of glucose stimulated insulin secretion and elevation of beta cell death [230]. Human islet exposure to elevated level of glucose could initiate IL-1β production and secretion. This is followed by NFκB activation, up-regulation of tumor necrosis factor receptor superfamily 6 (TNFRSR6-Fas), DNA fragmentation, and impaired beta cell function [231]. Residual macrophages within islets and infiltrated immune cells are also the major source of IL-1β that could amplify beta cell failure and apoptosis [232, 233]. In a rat model for intrahepatic islet transplantation, elevated IL-1β release together with increased TNF-α and NO production from transplanted islets are reported to involve in macrophages activation and reduction in islet engraftment [234].

The effect of IL-1β on infiltration of immune cells is through chemokine release by islets. One of these chemokines is MCP-1 that is reported to up-regulate by IL-1β [235] and involved in recruiting monocytes and macrophages to pancreatic islets [236, 237]. MCP-1 is elevated in both T1D and T2D [238, 239] and is associated with impaired islet allograft outcome [240]. Other chemokines such as IL-8 and interferon gamma-induced protein-10 (IP-10) could also regulate inflammatory reactions inducing islet graft failure post transplantation. Abundant expression of

IP-10 and IL-8 were reported in pancreatic islets shortly after intrahepatic islet infusion [119] and elevated level of IP-10 was associated with initial influx of T cells into the transplanted mice islet allograft [241].

IL-1β could also deteriorate insulin secretion through activation of cyclooxygenase (COX) enzyme leading to the increase in PG production [242]. COX enzymes include a constitutively active COX1 and stress induced COX2. Although, COX2 is induced under stress conditions in most cell types [243], in pancreatic islets, COX2 expresses under both basal and stimulated conditions and its expression predominates over COX1 [244]. Both T1D and T2D are associated with inflammatory reactions leading to activation of COX enzymes not only in pancreatic beta cells but also in macrophages and monocytes [243, 245]. COX2 production has been shown to increase in monocytes of type 1 diabetic patients as well as in those with higher risk of developing disease compared to the healthy individuals. This increase has been correlated with low insulin secretion suggesting the role of COX enzyme in pathogenesis of T1D [246]. COX2 promoter has binding sites for NFκB and IL-6, which could result in production of various pro-inflammatory mediators [244, 247]. Both COX1 and COX2 use arachidonic acid as a substrate to generate biologically active PGs [248]. PGs are lipid-derived molecules that signal through G-protein coupled receptors and are important modulators of pancreatic islet function, insulin secretion and inflammatory reactions [249]. PGE_2 is a member of PG family that has been studied in most detail compared to the other member of prostaglandin family in pancreatic islets and reported to inhibit glucose induced insulin secretion in both human and rodent islets [242]. Up-regulation of PGE_2 and its receptor prostaglandin E receptor 3 (EP3) were reported in mouse model for obesity-induced diabetes. The increase in the level of EP3 was also confirmed in human islets isolated from pancreas of type 2 diabetic organ donors and correlated with reduced cAMP production and impaired glucose induced insulin secretion [250].

In conclusion, inflammatory reactions initiated during islet isolation and transplantation procedure have negative impacts on islet health and function. Therefore, modulation of these inflammatory reactions could improve clinical islet transplantation outcome.

1.8. Strategies to reduce cell damage and modulate islet microenvironment

Pre-transplant *in vitro* culture of human islets is a time period when the quality of isolated islets is analyzed as well as immunotherapy is initiated in diabetic recipients [251, 252]. This pre-transplant time period is an opportunity to preserve islet function and survival post isolation and improve islet engraftment post transplantation. Many islet transplantation centers including the Nordic Network for Clinical Islet Transplantation with the islet isolation facility located at Uppsala University Hospital in Sweden and Oslo University Hospital in Norway, culture isolated islets between 24-72 hours before transplantation [253]. Previous studies focusing on comparing the survival and function of freshly isolated islets to the cultured islets were reported a reduction in JNK activity as well as improvement in ATP content and islet response to glucose in nearly fresh isolated islets (only 6 hours culture time) compared to the 48 hours cultured islets [254, 255]. Although, culture time might contribute to islet loss in pre-transplantation phase, adding favorable supplements such as NA could have substantial impacts on islet viability and function post isolation [179]. Supplementing NA to isolated islet culture has been reported to reduce production of TF and MCP-1 by islets, which consequently could decrease IBMIR reaction after portal islet infusion [179, 256]. In addition, administration of JNK inhibitory peptide (JNKI) to the islet culture was shown to reduce islet apoptosis and improve isolated islet yield and graft function post transplantation [257, 258].

Another beneficial supplement that is added to the islet culture for improving the survival of islets after isolation procedure is serum. Supplementing islet culture medium with 5% human serum albumin (HSA) not only reduced caspase 3 activity 24 hours after isolation, but also improved insulin secretory capacity of the islets compared to fetal calf serum, which is commonly in use as cell culture supplement [259]. In fact, upgrading islet pre-transplant culture further with amino

acids, vitamins, hormones and growth factors could be one strategy to enhance the quality of isolated islets [260, 261].

Pancreas oxygenation and cold-preservation could also improve isolated islet yield and quality. University of Wisconsin (UW) solution has been the standard pancreas preservation solution for the clinical islet transplantation since 1980s [262]. There is general agreement among the major islet transplantation centers that islet yield and quality could be improved by the use of cold-storage techniques during pancreas procurement. The cold-storage two-layer method (TLM) for human pancreas preservation reported to improve islet quality and yield [263, 264]. Islet oxygenation to avoid hypoxia-mediated injury using oxygenated perfluorocarbon during pancreas digestion was also reported to improve islet yield and quality [265, 266]. However, in a recent large-scale clinical study, the Edmonton group showed no beneficial effect of TLM with preoxygenated perfluorocarbon on islet isolation and transplantation outcome [267]. In addition, Uppsala group by using results from 200 islet isolations also reported no effect of TLM on islet isolation outcome from pancreas of elderly donors [268]. Therefore, more sophisticated methods might be required to replace cold pancreas storage in order to improve islet yield and quality.

Other factors such as temperature and culture density could have impact on islet viability post isolation. The favorable temperature for the isolated islets to have the best viability is still debated. In one hand, many laboratories have been reported that the low-temperature (22-24 ºC) decreases the level of necrotic cells, islet metabolic rate, endothelial cell activity, graft rejection and enzymatic activity of pancreatic tissue [269]. Culturing islets at 24 ºC has also been reported to prolong islet survival and improve the response to glucose concentration [270]. On the other hand, there are other reports claiming increased apoptosis and degradation [271] together with impaired insulin secretion in isolated islets cultured at low-temperature [272, 273]. Although, islet culture at higher temperature (37 ºC) has beneficial impact on insulin secretion and islet integrity, lower temperature (22-24 ºC) preserves islet survival and reduces necrosis and apoptosis within

the cells. Subsequently, perhaps the best approach is to use the combination of the two temperatures as our islet isolation lab has adopted islet culture at 37 ºC for the first 24 hours post isolation followed by 22 ºC until transplantation day to gain the advantages of both temperature.

1.8.1. The effect of growth factors on pancreatic islets

Growth factors are known for their beneficial impact on islet health and function. Various types of growth factors have been tested on isolated islets including i) the use of growth factors as supplements to islet culture medium, ii) viral delivery of growth factors to the islets and iii) co-culturing islets with mesenchymal stem cells (MSCs), which are the known source for growth factors and cyto-protective elements including hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), transforming growth factor-B (TGF-β). Diabetic mice transplanted with non-human primate islets that were transduced with mouse HGF showed complete control over blood glucose accompanied with improved graft function up to 42 days post transplantation [274]. Transducing rat epithelial cells with VEGF and their co-transplantation with rat islets under kidney capsule of diabetic rats accelerated islet graft revascularization accompanied with improved graft function post transplantation [275]. Human islets transfected with vectors containing VEGF and IL-1β receptor antagonist reported a reduction in caspase cascade activity, apoptosis, necrosis, NO production together with improved insulin release in response to glucose in the presence of inflammatory cytokines [276]. Administration of insulin growth factor (IGF) to pancreatic islets as a pre-transplant supplement increased islet survival and reduced islet toxicity as a result of creating supportive micro-environment for transplanted islets [277]. IGF was also shown to up-regulate the expression of VEGF, which could be particularly advantageous for revascularization and engraftment of the transplanted islet graft post transplantation [278]. This beneficial effect of IGF was reported to induce through activation of phosphoinositol 3-kinase (PI3K) signaling pathway [279]. Co-culturing Streptozotocin (STZ)-damaged rat islets with bone marrow-derived MSCs increased islet survival and viability through elevated expression of TGF-β [280]. A newly published study revealed the beneficial effect of

allogeneic islets and BM-MSCs co-transplantation into non-obese diabetic mice on improving blood glucose control and increasing vascular density of islet graft [281, 282].

Neurotrophic factors

Pancreatic beta cells share several biological characteristics with neuronal cells evidenced by identification of multipotent precursors from pancreatic islets and ductal population that co-express both neuronal and pancreatic precursor markers as well as co-expression of neuronal and pancreatic transcription factors within islets. After differentiation of precursors to beta cells, these cells showed glucose responsiveness and insulin release [283]. In addition, upon injury in Schwann cells, the glial cells of the peripheral nervous system (PNS), and rodent pancreatic islets either by STZ or autoimmune destruction of the cells, the damaged neurons and beta cells were shown to secrete nerve growth factor (NGF) and neurotrophin to eliminate cell injury [284]. Both, rodent and human beta cells express NGF receptor. NGF secreted by beta cells is biologically active since it could protect beta cells by inducing anti-apoptotic effect [285]. NGF withdrawal using a neutralizing monoclonal anti-NGF antibody revealed PI3K/AKT as a survival signaling pathway involved in NGF protective effect [285]. Both pre-treatment of islets with NGF prior to islets transplantation [286, 287] and intravenous administration of this growth factor to STZ-induced diabetic mice, accelerated normoglycemia achievement and improved glucose tolerance [288]. However, synergistic transplantation of NGF pre-treated mice islets in portal vein of STZ-induced diabetic mouse model not only failed to improve glucose responsiveness and angiogenesis, but also showed an increase in hypoxia and TUNEL positive cells within these transplanted islets [289]. The glial cell line derived neurotrophic factor (GDNF) has recently been found to have numerous similarities and potential overlaps with NGF regarding the effect on development of nervous system [290]. GDNF together with neuroturin (NTN), artemin (ART) and persephin (PSP) is part of the GDNF-family ligand (GFL). GDNF was reported to promote neuronal survival and suggested as a potential therapeutic agent for neurodegenerative diseases [291]. Addition of either, GDNF or neuroturin, to rat enteric neuron precursors supported the

survival and proliferation of these cells [292]. All GFLs signal through GDNF family receptors (GFRs). GFRs are linked to the plasma membrane through glycosyl phosphatidylinositol (GPI) anchor. GDNF activates the downstream signal pathway through binding to GFRα-1 followed by GDNF-GFRα-1 complex binding to the receptor tyrosine kinase (RET) [293]. Due to the mentioned similarities between the pancreatic islets and neuronal cells, the survival effect of GDNF has been investigated on beta cells. Presence of GDNF receptor; GFRα-1 was confirmed on mice pancreatic beta cells and over-expression of GDNF in pancreatic glial cells improved glucose tolerance and cell survival [294]. Isolated human islets treated with GDNF showed a reduction in beta cell apoptosis and improvement in glucose induced insulin secretion up to 10 days post isolation *in vitro*. Transplanting these human islets under kidney capsule of diabetic mice restored normoglycemia post transplantation [295]. The effect of trophic factors on angiogenesis and engraftment of transplanted islets were reported after co-culture of islets with MSCs prior to transplantation in diabetic mice [296]. In addition, adipose-derived stem cells (ASCs) were shown recently to be able to secrete GDNF and the secreted GDNF works independent of VEGF with the ability to stimulate the capillary network formation and improve angiogenesis [297]. Therefore, GDNF not only enhances the survival and viability of pancreatic islets but also improves the engraftment and vascularization of islet graft post transplantation.

1.8.2. The effect of anti-inflammatory molecules on pancreatic islets

Considerable efforts have been invested on understanding the mechanisms involve in pro-inflammatory cytokines deteriorating pancreatic beta cell function and viability in diabetes. However, much less attention has been focused on the role of anti-inflammatory cytokines as cyto-protective agents that could improve islet function and health. Anti-inflammatory cytokines are secreted by a number of different immune cell subtypes such as T-helper (Th) cells, macrophages, mast cells, regulatory B-cells and T-cells [298]. Anti-inflammatory cytokines normally induce antagonistic effects on their pro-inflammatory counterparts and are able to diminish inflammatory responses and protect the cells from cytotoxic insults. There is some

evidence showing the association of imbalance between pro- and anti-inflammatory cytokines with pathogenesis of T1D, T2D and islet graft dysfunction post transplantation [44, 146, 299]. The imbalance between Th1 and Th2 arms of cellular immune system producing pro-inflammatory cytokines (IL-2 and IFN- γ) and anti-inflammatory cytokines (IL-4 and IL-10) respectively was shown in T1D patients. In this study, stimulated peripheral blood mononuclear cells harvested from T1D patients produced less Th2 cytokines (IL-4, IL-10) followed by late increase in secretion of Th1 cytokines [213, 300]. Production of IL-13, another Th2 cell derived anti-inflammatory cytokine, was reported to decrease significantly in newly diagnosed T1D patients as well as in their first degree relatives with high risk for the disease [301]. Due to the fact that the production of anti-inflammatory cytokines are alleviated in diabetes and this reduction is accompanied with deteriorated islet function and survival, manipulation of these cytokines could serve as therapeutic agents against islet dysfunction. Combined administration of anti-inflammatory cytokines IL-4 and IL-10 using viral delivery system to non-obese diabetic mice [302] or as culture supplements to human islets [303] and rat insulin producing cell line [214] reduced infiltration and induced a direct anti-apoptotic effect on cultured islets. Evaluating the impact of IL-13 treatment in diabetes-prone non-obese mice was shown a significant reduction in insulitis and production of pro-inflammatory cytokines by splenic lymphoid cells of IL-13-treated mice [304]. IL-13 in rat insulinoma cell line was reported to signal through transcription factor, STAT3 [305]. This cytokines counteracted IL-1β on suppression of glucose oxidation but not insulin release in rat pancreatic islets [306]. However, IL-13 alone or in combination with IL-4 failed to reduce IL-1β stimulated NO production as well as initiation of oxidative and ER stress [306]. IL-10 is another anti-inflammatory cytokine that induces an inhibitory effect on production of pro-inflammatory cytokines through inhibition of transcription factor, NF-κB. This inhibition in activation of NF-κB leads to a reduction in TNF-α release and production of the pro-inflammatory cytokines [307]. IL-10 is produced by different type of immune cells such as T cells, dendritic cells and macrophages and it is involved in pathogen clearance and reduction of

inflammatory responses [308]. The expression of IL-10 has been shown previously to down-regulate in pancreatic islets of rodent model for brain dead. This could be partly as a result of an increase in macrophages-associated pro-inflammatory mediators (IL-1β, IL-6, MCP-1, TNF-α) and cytokine storm that occurs after brain dead [309]. Administration of IL-10 together with Rapamycin, which is an immunosuppressive agent inhibiting T cells and B cell activity, into diabetic mice after allogeneic islet transplantation was found to prevent allograft rejection and induced long-term graft survival post transplantation through activation of regulatory T cells (Treg) [310]. To further investigate the effect of IL-10 secreted by human Treg on preventing islet graft rejection, NOD-SCID mice were transplanted with porcine islets and received human Treg. These mice revealed blunted graft rejection that could highlight the effect IL-10 on reducing autoimmunity against transplanted graft [311]. Gene delivery is another methodology for introducing anti-inflammatory cytokines such as IL-10 to pancreatic islets [312]. Viral delivery of IL-10 to rat pancreatic islets did not interfere with total insulin content and insulin secretion in transfected islets. Transplantation of these transfected islets into STZ-induced diabetic rat model revealed improvement in graft function and survival [313]. This improvement was at least partly as a result of observed down-regulation in the level of pro-inflammatory molecules and reduced islet dysfunction and apoptosis [314]. Despite the protective effect of IL-10 on inflammation induced by pro-inflammatory mediators, administration of IL-10 to rat islets pre-treated with IL-1β, failed to reverse IL-1β-induced islet dysfunction and rather enhanced inhibitory effect of IL-1β on insulin secretion [315]. IL-10 has also been shown a paradoxical effect on early initiation of insulitis and T1D, which required more investigation. Transgenic expression of IL-10 in rodent pancreatic islets [316] or viral delivery of IL-10 together with IGF-1 to mice at the onset of diabetes [317] either accelerated autoimmune insulitis and diabetes onset [316] or induced no significant reduction on insulitis and elevated blood glucose [317].

Interleukin-22

IL-22 is an anti-inflammatory cytokine, which belongs to the IL-10 cytokine family. IL-22 is mainly produced by activated T cells such as Th22, Th17 and subset of innate lymphoid cells [318]. IL-22 requires the presence of two transmembrane receptor subunits, IL-22 receptor subunit 1 (IL-22R1) and IL-10 receptor subunit 2 (IL-10R2). Binding studies revealed a strong binding affinity of IL-22 to IL-22R1, followed by the IL-22 / IL-22R1 complex binding to IL-10R2. This suggests a two steps cytokine receptor binding to induce signal transduction within the cell [319, 320]. Despite IL-10, which has receptors on both immune and non-immune cell types to regulate growth and/or differentiation of both immune and endothelial cells [321], neither of resting nor activated immune cells express IL-22 receptor. In contrast, skin cells, digestive and respiratory systems and epithelial cells express IL-22 receptor [322]. This is absolutely an advantage due to the fact that modulating IL-22 / IL-22R system might not result in some of the immune-related side effects that could accompany with other cytokines [318]. IL-22 receptor is highly expressed in hepatocytes and over-expression of IL-22 in transgenic mouse model for liver injury showed an increase in hepatocyte survival and proliferation compared to their wild type [323, 324]. The expression of IL-22 receptor was found to up-regulate in mouse model for alcohol-induced liver injury and administration of exogenous IL-22 to hepatocytes not only down-regulated pro-inflammatory cytokines and fatty acid protein transports, but also increased expression of several antioxidants, anti-apoptotic and anti-microbial genes through activation of transcription factor, STAT3 [325]. IL-22 receptor is also highly expressed in pancreatic islets evidenced by immunofluorescent staining of pancreatic tissue for IL-22 receptor subunits. However, neither acinar cells nor duct cells stained positive for IL-22 receptor subunits [326]. IL-22 was reported to suppress oxidative and ER stress initiated by various pro-inflammatory cytokines in mouse pancreatic beta cell line and isolated human islets. Administration of this cytokine to rodent model for obesity modulated oxidative stress regulatory genes, suppressed ER stress and inflammation and importantly restored glucose stimulated insulin secretion [327].

Therefore, IL-22 could have a strong therapeutic advantage on islets exposed to diabetic environment and also it could induce beneficial effect on preventing islet dysfunction and loss after isolation and on early phase post transplantation.

1.8.3. The effect of prostaglandins on pancreatic islets

PGs are lipid molecules metabolized from arachidonic acids through COX enzyme activity. COX is an enzyme catalyzing the first step of PG formation by converting arachidonic acid to PGH_2. This procedure is followed by PGH_2 conversion to various PGs such as PGD_2, PGF_2, PGE_2, PGI_2 and thromboxane A_2 (TXA_2) [328]. While all PG members are synthetized by islets, there is very little known about the role of PGD_2, PGF_2, PGI_2 and TXA_2 in the context of pancreatic islets. PGE_2 is the only PG that has been studied in greatest detail in pancreatic islets. As it is discussed earlier, the inhibitory effect of PGE_2 on glucose induced insulin secretion was reported in pancreatic beta cell lines and human islets [329-331]. PGE_2 also could affect beta cells function *in vivo* as the increase in production of PGE_2 resulted in hyperglycemia and impaired glucose metabolism in transgenic mouse model [332]. PGE_2 could alter several mechanisms responsible for regulation of beta cell mass and apoptosis [249]. In rat islets, exogenous PGE_2 treatment resulted in a reduction in DNA synthesis, suggesting PGE_2 negative regulatory effect on beta cell mass [333]. In line with this study, an increase in production of PGE_2 using transgenic mice over-expressing COX2 and PGE synthase was found to decrease beta cells ratio per islets [332]. One possible mechanism for the inhibitory effect of PGE_2 on insulin secretion and reduction in beta cell mass could be through activation of forkhead protein box O-1 (FOXO-1) either by activation of JNK or inhibition of PI3K/AKT signaling pathway [334]. Inactivation of AKT leads to de-phosphorylation and activation of FOXO-1 followed by its translocation to nucleus, where it participates in nuclear exclusion of critical transcription factor, PDX-1. This transcription factor is essential for proper insulin secretion and maintenance of beta cell mass [249, 335-337].

Several lines of evidence indicate that the inhibition of PGE_2 receptor, EP3 reverses the negative effect of PGE_2 on insulin secretion and islet function. Inhibition of EP3 using EP3 antagonist

reported to increase glucose stimulated insulin secretion in pancreatic islets of T2D mouse model ($BTBR^{ob/ob}$) and in human islets isolated from donors with T2D [250]. However, due to the fact that the inhibition of EP3 did not affect insulin secretion in wild-type mice and non-diabetic human islets, it was suggested that the positive effect of EP3 inhibition might only be observed when the islet dysfunction is already present [250].

Hyperglycemia and pro-inflammatory cytokines including IL-1β play a crucial role on islet dysfunction and apoptosis though inducible nitric oxide synthase (iNOS) stimulation and activation of COX2 enzyme [249, 338, 339]. Over-stimulation of iNOS results in initiation of NO and COX2 activity, which further leads to production of pro-inflammatory PGs [340, 341]. Therefore, targeting IL-1β production and COX enzyme molecular signaling pathways inducing PGs are expected to enhance insulin secretion and improve pancreatic beta cell survival. Preventing IL-1β to up-regulate PGE_2 receptor and induce COX2 activity in isolated rat islets was found to improve glucose stimulated insulin secretion [342]. Culturing isolated mouse islets with IL-1β and a selective blocker for COX2 not only decreased the production of NO and PGE_2, but also reduced intracellular degradation of insulin [343, 344]. In addition, *in vivo* administration of COX2 inhibitor to rodents was reported to reduce hyperglycemia and restore insulin secretion [345]. Moreover, administration of IL-1 receptor antagonist (IL-1RA) and acetylsalicylic acid (AsA) that inhibits COX-2 enzyme activity, to the NOD mice transplanted with marginal rat islet mass alleviated graft dysfunction mainly through reduced intra-islet level of inflammation-related molecules IL-1β, TNF-α, iNOS, COX2 and chemokines MCP-1 and MIP-3 [346].

GPR44 receptor on human islets

PGD_2 was shown to express in pancreatic islets and hyperglycemia was reported to increase the production of PGD_2 in cultured rat islets [347, 348]. PGD_2 could signal through two receptors, prostaglandin D_2 receptor 1 (PD1) and PD2, which is also known as G-protein coupled receptor 44 (GPR44) [349]. GPR44 has been reported previously to express in CD4 positive T2 cells,

basophils and eosinophils during allergic inflammation where it mediates pro-inflammatory chemotaxin production [350, 351]. Therefore, antagonists for GPR44 receptor are under development for treatment of allergic reactions and asthma since the receptor is believed to up-regulate in these conditions [349]. GPR44 is shown to strongly express by pancreatic beta cells [352], with no or very low expression in other cell types including abdominal organs and lymphoid tissues [349]. Therefore, GPR44 is discovered recently as a beta cell specific biomarker using proteomics screening analysis [349, 352] and identified as a promising candidate for assessment of beta cell mass at the onset and during progression of T1D and T2D using positron emission tomography (PET) scan [353]. Immunohistochemistry staining of pancreatic tissue sections obtained from healthy individual and patients with T1D and T2D revealed that insulin-positive islets express GPR44 receptor [349]. In contrast, insulin-negative islets in patients with T1D both at new-onset and long-standing disease showed very low or negative GPR44 staining [349]. The expression of GPR44 on beta cells was reported recently to be species dependent since the expression of this receptor was presented on human, non-human primate and pig islets but not on rodent islet cells [354]. Although, GPR44 has been investigated for the purpose of developing islet-imaging biomarker, the role of PGD_2–GPR44 axis in pancreatic islets is still unknown.

2. Aims of the study

Inflammation and activation of stress signaling pathways contribute to beta cell loss during islet isolation procedure, culture and post transplantation. In addition, poorly vascularized transplanted islets in early phase post transplantation experience nutrient deprivation and activation of ER stress that could further enhance islet cell death and dysfunction. Comprehensive investigation regarding the activated destructive elements within islets and their signaling pathways will give us an opportunity to develop strategies for interrupting islet loss in pre- and post-transplant phase. The purpose of the presented work is to target islet damage due to stress and inflammatory reactions during islet isolation and transplantation and investigate possible strategies that could improve islet survival with implication for enhancing success of islet transplantation.

Specific Aims:

Article I: To test whether GDNF could recover isolated human islets from nutrient deprivation and ER stress induced apoptosis and consequently enhance islet function.

Article II: To investigate the adverse effect of hyperglycemia in combination with pro-inflammatory cytokine, LIGHT/TNFSF14 on human islets using *in vivo* and *in vitro* approaches and to test the potential protective effect of the anti-inflammatory cytokine, IL-22 on human islets.

Article III: To explore the effect and the potency of GPR44 inhibition on function and survival of human islets exposed to diabetic micro-environment both *in vitro* and post transplantation using a marginal mass islet transplant mouse model. In addition, we further studied the molecular signaling pathways that could be involved in GPR44 receptor mechanism in human islets.

3. Ethics

3.1. Human islets

Human islets were obtained from the JDRF award 31-2008-416 (ECIT Islet for Basic Research program) by the islet isolation facility of the Nordic Network for clinical islet transplantation after appropriate informed consent from relatives for multi-organ donation and for use in research. All experiments and methods using human islets were approved by and performed in accordance with the guidelines and regulations made by regional committee for medical and health research ethics central in Norway (2011/426).

3.2. Animal welfare

All animal experiment protocols were approved by the Norwegian National Animal Research Authority project license no FOTS ID 8588 (Article 1), 3017 and 4712 (Article 2), 7005 (Article 3). The animal experiments were performed in accordance to the European Directive 2010/63/EU and The Guide for the Care and Use of Laboratory Animals, 8th edition (NRC 2011, National Academic Press).

No more than 5 animals were housed in each cage and they were kept under the 12 hours light / dark cycle in an approved animal facility. Access to the water was never restricted; but the access to the food was restricted during the fasting time. In order to minimize the stress response in the animals, a skilled animal technician handled the animals including the administration of the treatments, performing the oral glucose tolerance test (OGTT), blood sampling and monitoring the animal welfare according to the guidelines designed by Oslo University Hospital.

4. Experimental methods

4.1. Human islet isolation and culture

Human islets were isolated according to semi-automated purification system [355] from 20/19 male/female brain-dead, heart-beating donors with mean age 52 years old (19-70 years old) and mean BMI 24 kg/m^2 (22-32kg/m^2) provided through the Nordic Network for Clinical Islets Transplantation (NNCIT) by the islet isolation facility of Uppsala University Hospital, Sweden and Oslo University Hospital, Norway after appropriate consent were given for multi-organ donation and for use in research. NNCIT includes the center in University Hospital in Gothenburg, Malmö, Stockholm and Uppsala in Sweden, University of Helsinki in Finland, Oslo University Hospital in Norway and Rigshospitalet in Denmark. Islet purity was judged by digital imaging analysis [356] or dithizone staining and islets with purity between 50-95% was used in the experiments. Dithizone is a red dye binding to zinc ions in pancreatic beta cells and can be easily used to distinguish islets from exocrine tissue and determine islet purity after isolation. Pancreatic islets were isolated with the purpose to use in clinical islet transplantation; but were released to use in research when their low quantity disqualified them for clinical islet transplantation. Fresh free floating isolated islets were cultured in CMRL 1066 (Corning, Manassas, VA, US) containing 10% human ABO-compatible serum and supplements, as it was published previously [180]. Isolated islets were kept at 37 °C with 5 % CO_2 first day after isolation. The entire culture medium was changed every 2-3 days and cells were maintained at 25 °C with 5 % CO_2 until they were manually handpicked to use in experiments. Handpicked islets for *in vitro* experiments were cultured at 37 °C with 5 % CO_2 during the incubation time in both control and intervention groups.

4.2. Static glucose stimulated insulin secretion assay

Pancreatic beta cells recognize extracellular glucose concentration and secrete insulin as required at a given time. Impaired insulin secretion in response to glucose is a key element for beta cell

dysfunction. Thus, performing glucose stimulated insulin secretion (GSIS) test *in vitro* gives one an opportunity to investigate islet function and the ability to control glucose homeostasis.

In order to perform this test, ten equally sized islets were handpicked and transferred into 24-transwells plate (Corning, NY, US) containing Krebs-Ringer Bicarbonate Buffer (11.5 mM $NaCl_2$, 0.5 mM KCl, 2.4 mM NaHCO3, 2.2 mM CaCl2, 1 mM MgCl2, 20 mM HEPES, and 2 mg/L albumin: all obtained from Sigma-Aldrich) supplemented with 1.67 mM glucose (low glucose). After 45 min incubation of islets at 37 °C in low glucose contained Krebs Buffer, transwells were switched to Krebs-Ringer Bicarbonate Buffer containing 20 mM glucose (high glucose) and incubated for another 45 min at 37 °C. Krebs Buffer supplemented with low and high glucose levels were harvested for insulin measurement secreted by islets in response to low (basal insulin) and high (stimulated insulin) glucose levels. Stimulation index (SI) was calculated as a ratio of stimulated over basal insulin levels.

4.3. Apoptosis detection assays

Apoptosis is a process of programmed cell death occurring in multicellular organism and it is involved in maintenance of organs and tissue. However, apoptosis is probably the main form of progressive beta cell death associated in T1D and T2D. Intensive investigations have been performed over the years using various technical approaches and methods to detect and understand the mechanism of apoptosis in pancreatic beta cells. Due to the fact that the specific detection of apoptotic cells is challenging and most of the apoptosis detection assays could also detect necrotic cells, usually different types of apoptosis detection assay is used in order to get clear information about the cell viability. Herein, the different methods that we have used for detection of apoptosis in beta cells are discussed:

Cell death detection ELISA assay: DNA in the nucleosome is tightly bound to the histones and protected from cleavage by endonucleases. However, apoptosis is associated with cell membrane disruption, condensation of cytoplasm and activation of endogenous endonucleases that lead to

the cleavage of double stranded DNA into mono- and oligo-nucleosomes. Herein, we analyzed apoptosis by detection of DNA-histone fragments in islet lysates using Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics, Mannheim, Germany) according to protocol offered and described by the manufacturer.

TUNEL assay: TUNEL assay detects the fragmented DNA in apoptotic cells by catalytically incorporating recombinant Terminal Deoxynucleotidyl Transferase (rTdT) to dUTPs presented at the 3'-hydroxyl (OH) end of fragmented DNA [357]. DeadEndTMFluorometric TUNEL system (Promega Biotech AB, Stockholm, Sweden) was performed on 60-80 handpicked and dispersed equally sized human islets according to the manufacturer protocol. A brief description of cell fixation and preparation has been explained in the method section of each article.

Although, TUNEL staining has been adopted to detect apoptosis, it is important to have in mind that this method is not limited to the detection of apoptotic cells and could detect all free 3'-OH DNA fragments regardless of the molecular mechanisms that have led to the development of these fragmented DNAs [358, 359]. Therefore, TUNEL staining might detect non-apoptotic cells including necrotic cells and cells undergoing DNA repair [357, 360]. In addition, proper quantification of the number of dead cells within the islets could be challenging due to the fact that there is few numbers of cells that stain positive for TUNEL and therefore, a large number of cells is required in order to get clear data about the viability status of islets.

FDA/PI viability assessment: FDA/PI is a fluorescence-based live / dead cells assay, which can be used to evaluate the viability of mammalian cells. Fluorescein diacetate (FDA) is a cell permeant esterase substrate that serves as a viability probe measuring both cell membrane integrity and enzymatic activity required for activation of FDA dye. Propidium iodide (PI) is a membrane impermeant dye and generally excludes from viable cells. PI binds to the DNA by interacting between bases and it is used for detection of dead cells [361]. FDA/PI staining was performed on handpicked islets using fluorescein diacetate (FDA) 20 µg/ml (Sigma-Aldrich

Norway AS, Oslo, Norway) and propidium iodide (PI) 100 µg/ml (Thermo Fisher Scientific, Oslo, Norway) for detection of live vs. dead cells.

Like other apoptosis detection assay, FDA/PI staining also has some limitations regarding the accuracy in scoring of viable versus damaged/dead cells. The use of high concentration of FDA or presence of extracellular esterase could generate a high background. The green emission peak of the FDA fluorescent product, fluorescein could also leak through red region of the spectrum and generate a false-positive PI staining. This could be resolved by using a very low final working concentration of FDA required for detection of live cells [361]. In addition, in this viability assay, it is assumed that the viable cells have esterase activity within their cytoplasm but dead or dying cells show little or no esterase activity. However, dead cells could also exhibit residual esterase activity which makes it complicated to evaluate dead cells [361].

Caspase 3/7 assay: The mechanism of apoptosis is fairly complex involving two distinct signaling pathways: (i) the extrinsic pathway or activation of death receptor pathways and (ii) the intrinsic pathway or the mitochondrial pathways [362]. However, there is now evidence showing both pathways are linked and have the same or execution pathway that initiates by cleavage of caspase 3 and results in DNA fragmentation [363]. In order to detect caspase 3 activity, the caspase 3/7 luminescent assay (Promega Biotech AB, NACKA, Sweden) was performed on the islets. The members of caspase family are cysteine proteases that sequestered in the cytoplasm in their inactive form and activated when they are cleaved by other caspases [358]. Presence of caspase 3 in the sample leads to cleavage of DEVD (Aspartate-Glutamate-Valine-Aspartate) provided in the assay substrate. Cleave of DEVD results in release of luminescence signal that could determine the amount of caspase 3 activity. To perform the assay, human islets were lysed and mixed with caspase3/7-Glu reagent at the equal ratio followed by caspase 3/7 measurements according to the protocol offered and described by the manufacturer. One of the main limitations that one needs to have in mind when performing this assay is the ability of other caspases than

caspase 3 to cleave the DEVD reagent [364]. Thus, it is important to use various types of assays and methods to detect apoptosis in the cells.

Assessment of intrinsic apoptosis signaling pathway: The intrinsic apoptosis signaling pathway consist of intracellular signals that result in activation of mitochondrial-initiated events [362]. Initiation of mitochondrial events leads to activation of the Bcl-2 family. Bcl-2 family consists of pro- and anti-apoptotic proteins that could determine whether the cells are committed to apoptosis or aborted the whole apoptosis process [365]. To date, a total of 25 genes have been identified in the Bcl-2 family. Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w and BAG are some of anti-apoptotic proteins in the Bcl-2 family and the pro-apoptotic proteins include Bcl-10, Bax, Bim, Bad, Bak, Bik [362]. Herein, Bioplex Pro RBM apoptosis assay (Bioplex, CA, USA) was used to investigate pro-apoptotic (Bad, Bim) and anti-apoptotic (Bcl-2, Mcl-1) proteins involved in an induction of intrinsic apoptosis signaling pathway. Multiplex immunoassay system is built upon detection of fluorescent dyed magnetic microspheres (beads), in which each bead has distinct color code to permit discrimination of individual molecules. In order to detect the protein level of these factors in the Bcl-2 family, cytosolic, nuclear and mitochondrial fractions of islets need to be provided. The islet fraction lysate was prepared using the reagents provided in the kit followed by the detection of pro- and anti-apoptotic proteins according to the protocol offered by the manufacturer (Biorad, CA, USA).

4.4. Detection of inflammatory mediators and cell signaling proteins

A multiplex assay has the ability to measure various analytes in one single run of the assay. This is an attractive rapid method to generate data for large number of analytes and require small sample volume compared to the measurement of one analyte at a time. Therefore, commercial multiplex bead-based luminex xMAP technology (BioRad, CA, USA or electrochemiluminescence immunoassay on MESO Quick analyzer (Meso Scale Diagnostics, MD, USA) was used for detection of the various pro-inflammatory cytokines and phospho-

proteins expression in mice plasma samples, cell-free supernatants or cell lysates according to the instruction provided by the manufacturer.

4.5. Immunofluorescent staining of human islets

Immunofluorescent staining is a widely used technique for detection of an antigen in cells or tissue sections. This technique uses specific antibody conjugated to a fluorescent dye in order to visualize the antigen with fluorescent microscope. In the performed studies included in this thesis, either handpicked and dispersed human islets or sectioned transplanted human islet grafts were subjected to labeling with antibodies conjugated to fluorescent dyes for detection of specific proteins after fixation and permeabilization of cells / tissues using Paraformaldehyde (PFA) and Triton-X100 respectively. Tissue sections require an additional unmasking step prior to fixation and permeabilization using antigen retrieval solution (citrate- or EDTA-based buffers) followed by boiling of the sections to facilitate antibody-antigen binding. Protein block serum free was used in order to increase the specification of antibodies to antigens and eliminates unspecific binding of antibody-antigen. List of antibodies as well as a brief description of cells/tissue preparation for immunofluorescent staining is explained in materials and methods section of each Article.

Limitations of this method include false negative results due to poor antibody penetration and binding. Auto-fluorescent property of a tissue could also result in an increase in the image background and consequently affect the detection of targeted antigen. False positive data induced by introduced artifacts to samples due to fixation and permeabilizing reagents is also another drawback for this method that could affect the accuracy and consistency of the protein expression analysis.

4.6. Protein immunoblot analysis

Protein immunoblotting commonly known as western blot is a method for detection of specific proteins in a cell and tissue. In this technique, proteins are separated based on their molecular

weight and produced a band representing each protein through a gel electrophoresis. Proteins are transferred to a membrane, where they are detected using primary antibodies followed by detection of the primary antibodies with secondary antibodies conjugated with peroxidase enzyme. In order to visualize labeled protein bands, membrane is incubated with chemiluminescent reagent and the peroxidase enzyme attached to the secondary antibody cleaves the chemiluminescent reagent. This cleavage produces luminescent light that could be detected by CCD camera. In our studies, human islet cells or transplanted islet grafts were lysed using cell lysis buffer supplemented with halt protease inhibitor followed by mechanical disruption of cells/tissue using sonication. Total protein concentrations were determined and equal amounts of total protein were separated on Biorad precast electrophoresis gels followed by protein band transfer to PVDF membrane. Unspecific antibody binding was blocked using skim milk or BSA according to the antibody data sheets. More detained protocol plus a list of primary and secondary antibodies used in different studies are explained in materials and methods of each Article.

Although, western blotting is one of the most common procedures to detect presence of a target protein, it is a non-quantitative method, which means it cannot tell researchers how much of the particular protein is present in a sample. In addition, a western blot can only be performed if a primary antibody is available against the protein of interest. This method is also highly dependent on the quality of antibodies and weak antibodies may not be able to detect the presence of a particular protein in a sample. Moreover, false-negative results could easily happen when large proteins are not given sufficient time to transfer to the membrane. On the other hand, antibodies could also generate false-positive results when they react with non-intended protein instead of binding specifically to the targeted protein. Therefore, choosing proper antibodies could have a huge impact of the quality and accuracy of western blot data.

Antibodies are available in two forms, polyclonal and monoclonal. Polyclonal antibody represents a collection of antibodies with the ability to recognize multiple epitopes of one antigen. A

polyclonal antibody is quick and inexpensive to produce. Since it can recognize multiple epitopes, it has a high affinity and sensitivity towards an antigen and using these antibodies are advantageous if particular antigens are present in a very low quantity. However, these antibodies have a very high potential for cross-reactivity due to the recognition of multiple epitopes. Also, there could be variations between batches produced in different animals at different times [366].

On the other hand, a monoclonal antibody only recognizes specifically one unique epitope of an antigen. Therefore, it shows a very low probability of cross-reactivity and batch-to-batch variability. However, these antibodies are more expensive to produce and small changes in the epitope make these antibodies enable to detect that particular epitope [366].

Finally, western blot is a costly method, since it requires numbers of equipment for running and detection of a particular protein. This method also requires precision in every step for proper identification of a specific protein and a minor error in protein isolation step, preparation of buffers and samples, running and transferring proteins on membrane, making antibody dilution, incubation time and condition could destroy the entire process.

4.7. Reverse transcription quantitative PCR (RTq-PCR)

Frozen harvested islet grafts were grinded and total RNA was isolated using Trizol / Chloroform (Qiagen, CA, USA) for phase separation followed by proceeding to the RNeasy Micro Kit (Qiagen, Hilden, Germany), which is designed for isolation of total RNA from small number of cells. Trizol is a monophasic solution of Phenol and Quanidinium Isothiocyanate, which solubilizes biological material and denatures proteins. Adding Chloroform after solubilization phase causes phase separation, where proteins are extracted in the organic phase, DNA resolves at the interface and RNA stays in the aqueous phase [367]. The concentration of total RNA samples was measured with NanoDrop ND-1000 UV/Vis spectrophotometer (Saveen Werner AB, Sweden) following by cDNA synthesis using the High-Capacity cDNA Archive Kit (Applied Biosystems, Forster City, CA, USA). Taqman primers and probes were used for analysis of the

targeted mRNA expression. Taqman probes are designed to anneal with targeted DNA that is amplified by the specific sets of primers. These probes consist of a fluorophore reporter and a quencher. When free-floating probe is not annealed to the template, reporter and quencher are in close proximity and quencher stops reporter to release fluorophore. After annealing to the template DNA and when Taqman polymerase extends the primer and synthetizes the nascent strand, the probe gets degraded from template DNA. Degradation of the reporter probe releases the fluorophore from it and breaks the inhibitory effect of quencher on reporter. The fluorescent signal is detected by quantitative PCR thermal cycler machine and it is directly proportional to the amount of DNA template that is present in PCR reaction [368].

4.8. Islet transplantation in mouse model

Small animal models have contributed greatly to the islet transplantation research. One of the widely used animal models for islet transplantation is chemical-induced diabetic model with the use of Alloxan or Streptozotocin (STZ) [369]. Alloxan is a pyrimidine derivative that was considered as a representative drug for diabetes by inducing necrosis to pancreatic beta cells in 1940s [370, 371]. STZ, which is a convenient drug to induce diabetes, was first used as a chemotherapeutic agent for cancer, based of its ability to inhibit DNA synthesis [372, 373]. Both Alloxan and STZ are glucose analogues. They could enter pancreatic beta cells through GLUT2 glucose transporter. GLUT2 is the dominant glucose transporter in rodents. Human islets use mainly GLUT1 and in less extent, GLUT3 to uptake glucose. Alloxan and STZ induce cell death in beta cells through different molecular mechanisms. Alloxan generates ROS and induce cell death due to the beta cell low anti-oxidative defense to defeat against oxidative stress. However, STZ is taken up by the beta cells and induces DNA fragmentation and cell death through its alkylating properties [374]. In order to mimic insulin dependent diabetes, we injected mice intravenously with one dose of 75 mg/kg of Alloxan. This concentration is within the required dose range to induce diabetes in mice (Alloxan dose range for mice is from 50-200 mg/kg depending on the mice strain and the route of administration). The definition of diabetes in our

animal model prior to transplantation is blood glucose ≥ 20 mmol/L for two consecutive days, which is the commonly reported blood glucose level for diabetic animal models for isle islet transplantation [375].

Equally sized human islets were handpicked prior to transplantation. Too big or too small islets as well as necrotic islets with dark core were avoided. The transplantation was performed blinded as the surgeon only did the operation and did not know the design of each experiment. In order to avoid autoimmune rejection of human islet grafts, we used either Balb/c Rag $1^{-/-}$ or NMRI nu/nu immunodeficient mouse as a recipient to allow human islet engraftment.

Mouse kidney capsule was chosen as a transplant site for human islets in our studies. Although, liver has been an approved site for clinical islet transplantation and it is favorable to investigate the intraportal islet transplantation in experimental rodent models, this transplantation site has several disadvantages that could have negative impacts on the consistency and quality of *in vivo* studies. Intraportal islet infusion requires major operation that carries 20% mortality rate compared to the kidney capsule with mortality rate around 6.7%. Islet infusion into the liver has a longer operative time (27.4 min per mouse) compared to the kidney capsule model with mean operative time of 6 min per mouse. Furthermore, the mean time for each animal to reach normoglycemic status is longer in intraportal model compared to kidney capsule model. Moreover, at the termination of study, transplanted grafts are easily accessible by nephrectomy for both histological analysis and proof of transplanted graft function [375].

In both Article 1 and 3, diabetes was introduced by administration of 75 mg/kg of Alloxan intravenously two days prior to transplantation of 500-800 human islets under kidney capsule of Balb/c Rag $1^{-/-}$ or NMRI nu/nu immunodeficient mice respectively. Transplanted human islets were either pre-treated with GDNF prior to transplantation or exposed to GPR44 antagonist by oral administration of the drug to mouse model started at the day of transplantation and continued throughout the study.

In Article 2, first normoglycemia was achieved by transplanting mouse islets under left kidney capsule of Alloxan-induced diabetic NMRI nu/nu immunodeficient mice (1^{th} graft). Second, minimal mass of human islets (250 islets) was transplanted under the right kidney capsule of each mouse (2^{nd} graft) followed by two weeks recovery time for the human islet grafts to revascularize before the nephrectomy of mouse islet grafts. More detailed explanation of the model is in Article 2.

After human islet transplantation, the recipient animals were followed up according to each study plan (explained in more detail in each study). Non-fasting blood glucose and animal weight were measured every second day in order to evaluate the glycemic status of animal post transplantation. OGTT or IVGTT was performed after 4 hours fasting and blood samples were collected via the saphenous or tail vein post glucose administration. IVGTT measures the direct effect of glucose stimulation inducing insulin release *in vivo*. However, OGTT reflects the effect of incretin hormones such as GLP-1 from the gut in augmentation of beta cell sensitivity to glucose and increasing secretion of insulin by pancreatic beta cells [376]. The acceptable amount and the frequency of blood sampling are dependent on the total blood volume in different species. In mice, only 0.75 % of total blood volume can be drained in single blood sampling every 24 hours. This amount will be 7.5 % and 10 % every 7 and 14 days respectively [377]. Therefore, blood sampling represents a great challenge when it comes to analyzing different parameters using different assays and molecular biology methods due to small amount of acceptable drained blood volume in every sampling.

4.9. Statistics analysis

GraphPad Prism software version 6.0 was used for statistical analysis and design of the graphs. In order to compare the difference between two study groups, either Mann Whitney U-test or Wilcoxon rank-signed test was used based on experimental design (paired vs. unpaired). One-way ANOVA with the Bonferroni post hoc test were performed to analyze differences among three experimental groups and the level of significance was set at $p<0.05$. In Article 1 and 3, data is

presented as mean ± SD and in Article 2, data is presented as mean ± SEM. Although mean ± SD is the most common descriptive analysis method, in Article 2, mean ± SEM was selected due to the journal style.

5. Summary of results

Article 1: Glial cell-line derived neurotrophic factor protects human islets from nutrient deprivation and endoplasmic reticulum stress induced apoptosis.

Although beta cell replacement therapy as islet transplantation is becoming a promising treatment for patients with brittle T1D, massive beta cell loss during islet isolation and post transplantation at least partly due to nutrient deprivation and activated ER stress, deteriorate long-term outcome of this therapy. In the present study, we mimicked nutrient deprivation and activated ER stress by *in vitro* culturing of isolated human islets with 0.5 % human serum alone or in combination with ER stress inducer, Tg and investigated the protective and anti-apoptotic effect of GDNF on pancreatic islets exposed to this stress environment.

We found a reduction in islet cell apoptosis and improvement in islet function, evidenced by enhanced insulin secretion in response to stimulated glucose concentration and increased insulin content in nutrient deprived human islets treated with GDNF. Improvement in insulin synthesis was at least partly through a reduction in the ratio of proinsulin over insulin in nutrient deprived human islets treated with GDNF. Moreover, GDNF reversed the elevated protein level of ER stress mediator, IRE-1α and molecular chaperone, BiP in cultured human islets treated with Tg *in vitro* and in human islet grafts transplanted in diabetic mice. Down-regulation in ER stress markers induced by GDNF was accompanied with elevated plasma level of human specific C-peptide and increase in the ratio of human C-peptide over fasting blood glucose in transplanted human islets pre-treated with Tg and GDNF prior to transplantation.

We also explored the molecular mechanisms involved in the beneficial effect of GDNF on human islets exposed to stress environment. We observed a reduction in phosphorylation of PI3K, AKT and GSK3β in isolated human islets treated with Tg *in vitro*. However, this effect was reversed by GDNF in Tg-treated human islets. Therefore, activation of survival signaling pathway PI3K/AKT and GSK3β could be at least partly involved in the protective effect of GDNF on human islets

exposed to nutrient deprivation and activated ER stress milieu. In conclusion, GDNF has the potential to protect human islets from ER stress responses and nutrient deprivation and consequently recover human islet function and viability. This data could suggest GDNF to function as a superior mediator to alleviate stress responses in isolated human islets.

Article 2: Interleukin-22 reverses human islet dysfunction and apoptosis triggered by hyperglycemia and LIGHT/TNSFS14.

Prolonged and repeated exposure to elevated level of glucose, defined as hyperglycemia, contributes to the initiation of stress responses within the islets. This could further result in activation of inflammatory reactions and induction of islet loss and dysfunction [7]. The molecular mechanisms involve in beta cell dysfunction and loss due to islet exposure to prolonged hyperglycemia is not fully understood. We have recently demonstrated the elevation of pro-inflammatory cytokine, LIGHT, which was correlated with elevated HbA1c in patients with T2D [378]. In the current study, a double islet transplantation model was used to investigate the effect of hyperglycemia on expression of LIGTH receptors (lymphotoxin beta receptor (LTβR) and herpes virus entry mediator (HVEM)) in engrafted human islets. We found that prolonged hyperglycemia up-regulated the expression of LTβR and HVEM in transplanted human islets. The adverse effects of prolonged hyperglycemia on transplanted human islets was evidenced by elevated TUNEL-positive staining within human islet grafts exposed to hyperglycemia as well as an increase in plasma level of mouse pro-inflammatory cytokines (IL-6, IP-10, MCP-1 and GRO-α) in these animals compared to the normoglycemic group.

To investigate the effect of combined hyperglycemia and LIGHT on human islets and whether IL-22 could protect the islets from this stress micro-environment, human islets were treated *in vitro* with high level of glucose (HG, 20mM) or human recombinant (hr) LIGHT alone and in combination with or without hrIL-22 for 48 hours. We confirmed an up-regulation in the protein levels of LTβR and HVEM in human islets treated with combination of HG and LIGHT. This was accompanied with an impaired insulin secretion in response to stimulated level of glucose and a decrease in insulin content in human islets exposed to HG and LIGHT. In this study, the observed human islet dysfunction could be at least partly due to an increase in secretion of pro-inflammatory molecules (IL-6, IL-8, IP-10, MCP-1) and TF by islets, activation of ER stress markers (IRE1-α and BiP) and elevation of pro- vs. anti- apoptotic mitochondrial signaling

pathway. Activation of all these stress signaling pathways could be involved in an observed increase in caspase activity and cell death in human islets.

In the presented study, human islets have been shown to express IL-22 specific receptor subunit, IL-22R1 and the expression of this receptor subunit increased in human islets exposed to combination of HG and LIGHT. Supplementing IL-22 to human islets treated with HG and LIGHT down-regulated the protein level of LTβR and HVEM receptors and enhanced insulin secretion in response to glucose stimulation. This improvement in human islet function could be at least partly as a result of IL-22 effect on reducing the level of pro-inflammatory cytokines as well as down-regulation in the protein expression of ER stress markers IRE1-α and BiP in human islets. Furthermore, IL-22 alleviated apoptosis mainly through activation of mitochondrial anti-apoptotic signaling pathway. Overall, the data suggest IL-22 as a powerful anti-inflammatory cytokine that could protect human islets from detrimental effect of hyperglycemia and LIGHT. Therefore, these results could support the strong beneficial effect of IL-22 on islet function and survival.

Article 3: Inhibition of prostaglandin D2-GPR44 axis improves human islet function and survival.

Prolonged hyperglycemia and elevated pro-inflammatory reactions induce an adverse effect on pancreatic islet function and survival [379, 380]. Pro-inflammatory cytokine IL-1β has been reported as one of the main inducers for prostaglandin formation [242, 381]. Pancreatic islets secrete PGD_2 and the level of the PGD_2 formation was found to increase in response to high level of glucose in rodent islets [382, 383].

We showed that PGD_2 is as potent as pro-inflammatory cytokines, IL-1β, TNF-α and INF-γ in inducing apoptosis in human islets. However, inhibition of GPR44 receptor using GPR44 antagonist reversed the apoptotic effect of PGD_2 on human islets. Anti-apoptotic effect of GPR44 antagonist was also observed in human islets exposed to the combination of high level of glucose (20mM, HG) and IL-1β. Inhibition of GPR44 not only reduced apoptosis, but also improved insulin secretion in response to glucose in cultured human islets exposed to combination of HG and IL-1β.

By using a diabetic mouse model in which, human islets were transplanted under kidney capsule and treated with GPR44 antagonist, fasting blood glucose was found to be reduced. This was accompanied with an improvement in glucose tolerance in mice treated with the GPR44 antagonist. The beneficial effect of GPR44 antagonist on transplanted human islet function was evidenced by elevated human C-peptide and increase in ratio of human C-peptide to fasting blood glucose. Interestingly, the beneficial effect of GPR44 antagonist on transplanted human islets was observed on early phase post transplantation. This was evidenced with an elevation in the levels of human C-peptide and the protein level of HGF, accompanied with a reduction in ratio of proinsulin to insulin and a decrease in secreted human pro-inflammatory cytokines, TNF-α and GRO-α in mice treated with GPR44 antagonist. Analysis of beta cell transcription factors revealed an up-regulation in the expression of PDX-1 and MAFA in transplanted human islets

treated with GPR44 antagonist compare to the untreated islets. Moreover, immunofluorescent labeling of transplanted human islets for insulin after termination of the study revealed that the inhibition of GPR44 could preserve transplanted human islets.

Mimicking diabetic micro-environment by *in vitro* islet culture with combination of HG and IL-1β showed a reduction in phosphorylation of AKT, GSK3β and FOXO-1, accompanied with a decrease in protein level of PDX-1. However, administration of GPR44 antagonist increased activation of AKT / GSK3β survival signaling pathway and elevated protein level of PDX-1.

To sum up, these data could support the beneficial effects of GPR44 inhibition on human islet function and mass, and could suggest GPR44 antagonist as an intervention to improve islet transplantation outcome.

6. Discussion

Diabetes is a chronic metabolic disease characterized by elevated levels of blood glucose, which as long-term problem causes serious damage to the heart, blood vessels, kidneys, eyes and nerves. According to the World Health Organization (WHO), the number of individuals with diabetes has risen from 180 million in 1980 to 422 million in 2014 with the global prevalence of 4.7 % in 1980 to 8.5 % in 2014 [384].

T1D has been determined to be an autoimmune disease involving the selective destruction of insulin producing pancreatic beta cells and results in progressive loss of insulin secretion and impaired glucose metabolism. T2D results in the body ineffective use of insulin due to the long-term exposure of pancreatic beta cell to elevated glucose, FFAs and low-grade inflammation.

Beta cell replacement therapy as islet transplantation has proven to be less invasive treatment compared to the whole pancreas transplantation and more effective alternative offering substantial benefits in (i) lowering daily exogenous insulin requirements, (ii) improving glucose control and (iii) reducing incidence of unaware hypoglycemia. However, the success rate for islet transplantation is constrained by limited long-term islet graft survival partly due to islet exposure to cytotoxic insults during isolation procedure and on early phase post transplantation [385]. In clinical islet transplantation, the pancreatic islets are directly infused into the portal vein, where they are exposed to low oxygen tension and nutrient deprivation due to their poor re-vascularization compared with the native islets in the pancreas. In addition, the transplanted islets are instantly exposed to blood mediated inflammatory reactions that initiate activation of complement system, coagulation pathways and induction of pro-inflammatory molecules [386]. Consequently, transplanted islet graft in early phase post transplantation gradually lose their function and mass due to mainly apoptosis induced by activated stress responses and pro-inflammatory reactions [118]. Despite all the improvement in islet isolation procedure and clinical

islet transplantation, this treatment still requires comprehensive research in order to improve isolated islet quality and reduce cell loss post transplantation.

6.1. Does the use of growth factors improve isolated islet quality and transplantation outcome?

Isolating islets from the pancreatic tissue opens windows of opportunities for *ex vivo* manipulation of islets prior to transplantation in order to prevent cell injury and improve transplantation outcome. Many research groups have tried to identify different growth factors and intracellular molecules, which would have the ability to increase islet function and survival prior and post transplantation. Among different growth factors, HGF and IGF have been shown promising results on increasing pancreatic islet survival and consequently reducing the number of islets required for successful transplantation [367, 387, 388]. However, most of these studies are restricted to rodent islets and more investigation on the effects of these factors on isolated human islets is required. Human islet isolation and culture deteriorate pancreatic beta cell function at least partly due to hypoxia, nutrient deprivation and activation of stress and inflammatory responses as well as activation of apoptotic signaling pathways [117, 151, 185]. Mwangi, et. al has reported recently the protective effect of GDNF on isolated human islets cultured for up to 10 days [295]. In Article 1, nutrient deprivation and activated ER stress response was mimicked by culturing human islets under low serum condition and ER stress inducer compound, Tg. Adding GDNF to this culture condition reduced apoptosis and improved isolated islet function. The observed protective effect of GDNF was at least partly through activation of survival signaling pathway, PI3K/AKT and GSK3β and reduction in the expression of ER stress sensor, IRE-1α and molecular chaperone, BiP. We also showed a decrease in ratio of proinsulin over insulin by GDNF. This ratio is considered as an evidence for overall improvement in insulin process and secretion as well as reduction in accumulation of unfolded proteins in isolated human islets.

Different delivery options have been suggested for inducing the expression of growth factors in pancreatic islet cells. Intraperitoneal administration of HGF to diabetic mice transplanted with

syngeneic marginal islet mass showed sustained glycemic control in transplanted animals [389]. However, considering a very short half-life of growth factors [390], which could increase the chance of their fast clearance from the circulation, continuous administration of growth factors might be required in order to reach therapeutics concentration. Nevertheless, continuous administration of growth factors might be not a safe and efficient approach due to the induction of tumorigenicity that could be associated with the effect of growth factors in various tissue types. This was evidenced previously by studies showing tumorigenesis in different tissue types in transgenic mouse model over-expressing growth factors [367, 388].

Pre-culturing islets with growth factors prior to transplantation allow the cells to recover from isolation procedure. It could also minimize islet loss and increase their survival post transplantation [295, 367, 388, 391]. As it is shown in Article 1, pre-culturing nutrient-deprived and ER stress-induced human islets with GDNF improved islet mass and function even 30 days post transplantation. This *in vivo* improvement observed in transplanted human islets is partly due to the overall reduction in activated ER stress responses within the transplanted graft.

Another interesting approach to deliver growth factors to the cells is the use of biomaterials that are designed to entrap or absorb growth factors to the biological scaffolds and cells [277, 392]. Rat islets seeded in a Polyglycolic Acid (PGA) scaffold and cultured in culture medium containing EGF, NGF and IGF showed full functioning islets after they were transplanted under kidney capsule of diabetic mouse model. This was evidenced by transplanted islet ability to secrete insulin, glucagon and somatostatin and also their ability to return diabetes-induced mice to the normoglycemic status [393].

Viral and non-viral transduction of isolated islets with growth factors represents another delivery option to enhance islet survival. The major advantage of non-viral vectors is their safety for clinical purposes. However, the low-efficiency transduction of non-viral vector is a significant disadvantage for this method to be used in pancreatic islets [354]. On the other hand, viral vectors

show high transduction efficiency, but the fact that they could stimulate the immune system represents a major drawback for this method to be used in clinical islet transplantation [394]. Considering a high amount of islet loss in immediate post transplantation phase mainly due to apoptosis and necrosis, another advantage of a gene therapy approach is that the islets would have a continuous production of anti-apoptotic growth factors before and in immediate phase after transplantation, which could improve their function and viability [277]. Regarding the safety of viral vectors delivering growth factors to the islets, one would claim that since the isolated islets are transduced *ex vivo* and any remaining viral vectors are washed off before transplantation, the likelihood of viral vectors mediating systemic response would be limited [364]. However, our knowledge regarding the potential life threatening use of genetically modified cells with viral vectors as well as continuous presence of a growth factor in islets and their effects on other tissue types are still limited and require further investigation.

The primary goal for islet transplantation is to achieve long-term normoglycemia in diabetic patients. One of the barriers that must be overcome in order to accomplish long-term islet graft survival is to improve isolated islet quality. Growth factors can be considered as promising candidates due to their ability to enhance function and survival of isolated islets. However, additional investigations are required regarding the efficiency and safety of using these molecules in the clinical islet transplantation, as well as optimal delivery approach of these molecules into islet micro-environment in order to achieve maximal beneficial effect of these factors in islets.

6.2. What are the effects of anti-inflammatory molecules on isolated human islet survival and function?

Islet injury during isolation and on early phase post transplantation has negative consequences on long-term transplantation outcome due to activation of pro-inflammatory mediators inducing detrimental effects on the islets [117-119]. Isolated islets secrete various pro-inflammatory molecules including IL-6, IL-8, IL-1β, MCP-1 and TF. Transplantation of isolated islets expressing pro-inflammatory molecules could further initiate various inflammatory responses

including IBMIR reaction, activation of complement system and platelet aggregation and activation. Therefore, successful clinical islet transplantation is directly related to the expression of pro-inflammatory mediators [124, 129]. Hyperglycemia and glucose fluctuation have been reported as an inducer for pro-inflammatory reactions and platelet activation [219, 220]. Thus, not only presence of TF and pro-inflammatory cytokines but also impaired glucose tolerance, which could be observed in recipients for islet transplantation, induces platelet activation and inflammatory reactions. Activated platelets secrete pro-inflammatory molecules including LIGHT [395]. The level of LIGHT was shown to increase in patients with T2D [378] and therefore suggested a link between LIGHT and pathogenesis of T2D. In Article 2, prolonged hyperglycemia was shown to up-regulate the protein level of LIGHT specific receptors. This was also accompanied with reduced islet function and insulin secretion in response to blood glucose in human islet grafts transplanted under the kidney capsule of diabetic mouse model. In addition, the adverse effect of prolonged hyperglycemia on human islets was evidenced by an increase in level of apoptosis in transplanted human islet grafts exposed to diabetic milieu. Pro-inflammatory cytokines particularly, IL-1β, INF-γ and TNF-α, were previously found to up-regulate LIGHT receptors in human islets and the combination of these cytokines and LIGHT impaired human islet response to high level of glucose [378]. Moreover, mimicking diabetic milieu by culturing isolated human islets with high level of glucose and LIGHT, we show that not only pro-inflammatory cytokines, but also presence of TF and activation of ER stress responses are involved in the adverse effect of hyperglycemia and LIGHT inducing islet dysfunction and loss.

Interventional studies targeting different pro-inflammatory molecules and their activated molecular signaling pathways have been explored and tested over the past years. One approach to reverse the adverse effects of pro-inflammatory cytokines in pancreatic islets is administration of specific blocker for particular pro-inflammatory molecules. The therapeutic option of NFκB inhibitor, Bortezomib was shown to reduce cellular stress responses and apoptosis together with decreased IP-10 release in isolated mouse islets *in vitro* [396]. IL-8 has been found to be highly

expressed by isolated human islets both in culture and in transplanted islet grafts. One approach on targeting elevated IL-8 is pharmacological inhibition of CXCL1/2 (IL-8 receptor) using Raparixin. This has been shown previously to improve transplantation outcome in T1D patients after an islet infusion [119]. The beneficial impact of Raparixin is on the basis of the promising preclinical results showing improved islet engraftment and delayed graft rejection in mouse model for intrahepatic islet transplantation [119]. Other pro-inflammatory mediators such as, TNF-α and IL-1β, are also secreted by the islets in response to stress environment and pro-inflammatory reactions. Therefore, pharmacological targeting of these pro-inflammatory molecules has also been investigated in the context of islet transplantation. The application of TNF-α inhibitor, Etanercept has become integrated into clinical practice for allogeneic islet transplantation. Therefore, several clinical studies reported insulin independence after single donor marginal islet mass transplantation when Etanercept was used in combination with T cells depletion agents [251, 397, 398]. Recently, the combination of Etanercept and IL-1β receptor antagonist (Anakinra) has been proposed to improve islet engraftment in comparison with Etanercept alone in a marginal mass model for islet transplantation [399]. Although, a combination of Anakinra and Etanercept plus T-cell depleting agents has been proposed as a safe approach for clinical islet transplantation, the performed study was limited to the small number of patients from a single institution. Thus, larger cohorts of patients from various centers would require in order to evaluate the efficacy of this treatment [399].

Despite the fact that considerable efforts have been invested on characterizing the pro-inflammatory molecules inducing beta cell loss and developing blockers to reverse their adverse effects on islets, much less attention has been paid to understand the role of anti-inflammatory cytokines as the potential cyto-protective agents on islets. Anti-inflammatory cytokines IL-4, IL-13 and IL-10 were reported to counteract elevation of pro-inflammatory molecules in diabetic mouse models [304], rat insulinoma-derived cell line [214] and isolated human islets [303]. However, all these anti-inflammatory cytokines and their specific receptors are expressed on both

endothelial and immune cells. Therefore, activation of their signaling pathways might induce undesirable immune-related side effects, which are not beneficial for the isolated islet health [316, 321, 400].

IL-22 is a newly discovered member of IL-10 family. IL-22 has been proposed recently as a suppressor of ER and oxidative stress responses in isolated human islets and mouse beta cell-line exposed to various pro-inflammatory mediators [327]. In Article 2, we have shown that human islets express IL-22 receptor, as it has also been previously reported [327]. In this study, the protective effect of IL-2 2 was observed in isolated human islets exposed to the combination of hyperglycemia and LIGHT. This was evidenced by not only down-regulation of LIGHT receptors, but also improvement in insulin secretion in response to glucose stimulation. The observed effect of IL-22 was at least partly as a result of a reduction in secreted pro-inflammatory cytokines and a decrease in activation of ER stress responses leading to the alleviation of islet loss. One possible mechanism for initiation of apoptosis is an increase in the ratio of pro- over anti-apoptotic elements in mitochondrial intrinsic pathway [401, 402]. In Article 2, the elevation of anti- over pro-apoptotic mitochondrial elements was observed upon IL-22 administration to the isolated human islets. This could explain the molecular mechanism involved in the anti-apoptotic and survival effects of IL-22 in the islets. One advantage of IL-22 over other anti-inflammatory cytokines is that IL-22 receptor is specifically expressed by endothelial cells including pancreatic islets and neither resting nor activated immune cells do not express this receptor [322]. In addition, IL-22 gene expression was found to be up-regulated in patients with T1D [403]. This could suggest IL-22 as a protective cytokine in islets exposed to activated pro-inflammatory and stress reactions in T1D milieu, however this requires further investigation. Therefore, modulating IL-22 / IL-22 receptor system on human islets exposed to stress environment could induce a strong beneficial effect on islets without undesirable activation of immune reactions [318]. IL-22 has presented a powerful anti-inflammatory and anti-apoptotic effect on islets. Considering the observed attenuation in pro-inflammatory molecules and TF release by islets upon IL-22

treatment, further investigations regarding the effect of IL-22 on activated IBMIR reaction during islet engraftment could open windows of possibilities to improve islet health and prolong graft survival post transplantation.

6.3. Could modulation of prostaglandin signaling pathway induce beneficial effects in islet transplantation setting?

PGs are lipid molecules generated by the action of COX enzymes and have an important impact on impaired islet function. IL-1β has been found to be a leading factor increasing COX enzyme activity through activation of transcription factor, NFκB. Activated NFκB translocates to nucleus, where it transcribes for pro-inflammatory molecules, iNOS and COX enzymes [404]. Therefore, attempts on ameliorating COX enzyme activity in order to reduce PG formation and improve islet function have been investigated over the years. Nonsteroidal anti-inflammatory drugs (NSAIDs), which are the most common drugs for inflammatory diseases, have been studied in the context of anti-diabetic medication. Aspirin, with the ability to block COX enzymes, has long been recommended for the diabetic patients with the high risk of cardiovascular disease [405]. Aspirin treatment was found beneficial for both T1D and T2D patients since it could reduce platelet activation and aggregation compared to the non-diabetic individuals [406]. Intraperitoneal administration of Aspirin to T2D rat model reported an improvement in glucose tolerance associated with increased insulin response in the islets. This effect is partly mediated by the anti-inflammatory properties of aspirin on reducing low-grade inflammation in T2D [407]. Aspirin has been reported to inhibit IL-1β from inducing NFkB activity, PGE_2 receptor (EP3) up-regulation and an increase in the gene expression of COX2. It is consequently prevented IL-1β from reducing glucose induced insulin secretion in rodent pancreatic islets [342]. Despite the observed beneficial effect of COX inhibitors on rodent pancreatic islets, these blockers has been found to have a number of side effects including reduced renal function, increased liver enzymes and gastrointestinal erosions in diabetic patients [408] and required further investigation regarding its beneficial effect on islet transplantation outcome.

Most of the prostaglandin receptors including EP1-4 (PGE_2 receptor) and IP (PGI_1 receptor) are not specifically expressed by pancreatic islets and has been found to express in liver, kidney, epididymal fat and muscle tissue [342]. In contrast, PGD_2 receptor, G-coupled protein GPR44 was recently discovered as a highly expressed receptor on pancreatic beta cells [353] and therefore identified as a human beta cell specific biomarker through proteomics screening analysis [352]. However, its molecular mechanism on human islets is still unknown. In Article 3, the inhibition of GPR44 using a GPR44 antagonist in human islets transplanted under kidney capsule of diabetic mouse model has been shown to enhance control over blood glucose level and improve transplanted islet function. Inhibition of GPR44 in transplanted human islets could also preserve the islet mass exposed to diabetic micro-environment *in vivo*. The beneficial effect of GPR44 inhibition on transplanted human islets that observed from early phase post transplantation included not only improvement in the level of C-peptide but also reduction in the ratio of proinsulin to insulin. A raised ratio of proinsulin to insulin could be a sign of elevated unfolded proteins as a result of stress environment and it is also considered as an early marker for islet dysfunction [9, 409]. Therefore, the effect of GPR44 inhibitor on ameliorating proinsulin to insulin ratio could suggest the beneficial effect of this blocker on transplanted human islet function and insulin synthesis.

Regarding the molecular mechanisms involved in the beneficial effect of GPR44 inhibition in human islets, a reduction in secreted human TNF-α and GRO-α was observed in human islets treated with GPR44 antagonist. It is well-known that enhanced inflammatory process and elevation of pro-inflammatory cytokines including IL-1β, TNF-α and INF-γ after islet transplantation are involved in cellular injury in the recently transplanted islets [234, 410]. Activation of these pro-inflammatory reactions could be at least partly due to the elevated level of IL-1β secreted not only by islets and but also by the activated macrophages [411] and neutrophils [412]. Elevated level of IL-1β could lead to activation and translocation of transcription factor, NF-κB to nucleus and result in production of various pro-inflammatory cytokines including TNF-

α by islets [413]. In addition, secretion of pro-inflammatory cytokine, GRO-α was also found to increase in isolated human islets [414]. In a study focusing on the effects of IBMIR on loss of transplanted human islets using a whole blood model for human allo-islet transplantation, GRO-α was reported to increase in human islets and this was associated with massive infiltration of neutrophils and monocytes to the transplanted islet graft [386]. In Article 3, the protein level of HGF was also found to be up-regulated in transplanted human islets treated with GPR44 inhibitor. Elevation in protein level of HGF was accompanied with an increase in phosphorylation of AKT/GSK3β, FOXO-1 and elevated proteins level of PDX-1 after administration of GPR44 antagonist to the human islets cultured in diabetic milieu. HGF has been suggested previously as a protective factor for human islets. This is due to the activation of PI3K/AKT survival signaling pathway that has been reported in islets upon HGF treatment [367, 368]. Elevated level of HGF and activation of AKT survival pathway upon GPR44 inhibition in human islets could therefore explain at least partly the molecular mechanism involved in the protective effect of GPR44 inhibition on human islets. Studies focusing on the role of PGD_2 on human islets are very limited. However, PGE_2 has been shown to alleviate islet function and insulin secretion through dephosphorylation of FOXO-1 leading to its translocation to nucleus, where it could participate in nuclear exclusion of beta cell transcription factor, PDX-1 [334-336]. Both PDX-1 and MAFA transcription factors are not only key factors for insulin secretion in beta cells but also regulate genes that are required for beta cell function [366, 415, 416]. Therefore, increase in phosphorylation of FOXO-1 and up-regulation of PDX-1 and MAFA as well as observed improvement in islet function and insulin secretion after inhibition of PGD_2-GPR44 axis in human islets could support the beneficial effect of GPR44 inhibition and explain its mechanism of action in human islets.

In islet isolation procedure for clinical islet transplantation, pancreas preservation and perfusion after organ retrieval play a key role on the quality of isolated islets. Currently, the most widely used solution for perfusion of the donor pancreas after organ retrieval is direct aortic flush of the

donor pancreas with cold UW solution [417]. Attempts in supplementing the cold storage solution with cyto-protective agents have been shown to improve the yield and the quality of isolated islet and the outcome of islet transplantation [265]. These agents include administration of NA to the islet culture medium to reduce production of pro-inflammatory molecules [256, 265] or inhibitor for JNK and p38 MAPK to reduce islet apoptosis in immediate phase after isolation [265]. In Article 3, the beneficial effects of GPR44 inhibition were reported not only on islet function, but also on preservation of the human islets transplanted into the diabetic mouse model. GPR44 has been shown to highly express in the endocrine part of the pancreas with almost no expression in the pancreatic exocrine tissue [353]. Therefore, supplementing the pancreas preservation solution or islet culture medium with GPR44 inhibitor could have the potential to induce a strong beneficial effect on isolated islet quality and yield after isolation and reduce stress micro-environment that islets experience during and after isolation procedure.

7. Future perspectives for beta cell replacement therapy

Despite impressive advances in the field of islet transplantation, scarcity of suitable donors for clinical islet transplantation as well as islet loss and dysfunction post transplantation due to activation of stress and inflammatory responses, hamper the widespread application of this procedure. In addition, in order to further understand the islet biology, develop treatments for diabetes and improve the current techniques for islet isolation procedure and transplantation, there is a need for pancreatic islets to be used in research. Continuous development of new strategies and improvement of the current procedures for *ex vivo* manipulation of isolated islets prior to transplantation have proven to induce beneficial impact on the outcome of islet transplantation. Improving islet health and function by applying growth factors and/or anti-inflammatory molecules to the islet culture prior to transplantation could play an essential role on defeating islet loss and dysfunction. Although, pro-inflammatory signature of islets after isolation has been well-described and interventional studies targeting these pro-inflammatory molecules have been reported promising outcome on reducing islet loss, targeting single pro-inflammatory axis seems not to be sufficient to achieve superior islet engraftment and function [340]. One strategy could be providing a cocktail of pro-inflammatory blockers and anti-inflammatory molecules that could target immune responses more efficiently and reduce islet insults post isolation and transplantation.

In order to target the shortage of pancreatic donors, alternative sources of islets including differentiation of stem cells and progenitor cells into insulin producing beta cells and the use of porcine islets for xenotransplantation have been investigated over the years [69]. Generating functional insulin producing beta cells from iPSC as a source for beta cell replacement therapy could address most of the limitations that the use of human or animal islets have. This not only includes shortage of suitable donors for pancreas and islet transplantation, but also life-long use of immunosuppressive medications as well as costly and intensive work for organ procurement and islet isolation procedure [418-420]. Unlike islet cells, iPSC could be cryopreserved and directly

differentiated to beta cells for clinical transplantation when it is needed [418, 419]. Current strategies are focusing on generating beta cells with the highest possible purity, either by *in vivo* approaches to induce terminal differentiation of iPSCs to beta cells [421, 422], or by making fully differentiated beta cells from iPSCs *in vitro* prior to transplantation into the animal models [418, 420, 423]. In addition, generation of more complex islet like structures from iPSCs including iPSC derived alpha cells are also under investigation [424, 425]. However, it remains to be seen if a beta cell graft derived from iPSCs could replicate the functional capacity of transplanted human islets or pancreas [426, 427]. iPSC derived beta cells by *in vitro* differentiation protocols using small molecules and growth factors have shown to reverse hyperglycemia in diabetic mouse model and prevent hyperglycemic status in diabetic prone mice. However, the insulin secretion capacity of these differentiated beta cells in response to glucose did not replicate human islets and is currently under investigation [420]. Therefore, more information regarding the functionality of iPSC derived beta cells in larger animals and human is lacking. In addition, the risk of tumor formation is another issue that needs to be addressed for clinical use of iPSCs derived beta cells. To explore this issue, iPSCs were encased into micro-encapsulated device and placed subcutaneously in mouse model, where they show mature beta cells functionality after transplantation [421, 428, 429]. However, the device acts a diffusion barrier preventing proper glucose sensing and insulin secretion. Thus, the satisfactory metabolic function of these differentiated beta cells has yet remained to be demonstrated [420, 426]. Moreover, a common barrier towards all mode of beta cell replacement therapy in T1D patients is autoimmune attack of the transplanted cells [142, 430, 431]. Immunomodulation of beta cells derived from iPSCs with the ability to release insulin in response to blood glucose but not get subjected to autoimmunity in T1D patients could be one promising approach for the use of these cells in this group of patients [432].

Another approach to overcome scarcity of donor islets is reprogramming the pancreatic exocrine tissue (acinar and duct cells) to insulin producing beta cells. Exocrine cells are discarded after

islet isolation in the current protocol. These cells have shown to have extreme plasticity with a strong potential to be reprogrammed to insulin producing beta cells [433-435]. However, more investigation is required for developing multistep protocols with the use of small molecules and growth factors in order to make this approach feasible and applicable for therapeutic advantages.

Porcine islet transplantation has similarities to allogenic islet transplantation and could benefit from years of experience in transplantation of allogenic islets into diabetic recipients. The first attempt for porcine islet transplantation is back to 1994, where the transplanted islets were shown to produce C-peptide and to survive in human under standard immunosuppression regimen [436]. One advantage of using porcine islets as beta cell replacement therapy is that pigs have a short gestation and rapid growth rate. Thus, they would facilitate a supply of islets required for islet transplantation [437]. Furthermore, with only a single amino acid difference, porcine insulin could bind to human receptors and secrete insulin in response to blood glucose [438]. Porcine islet transplantation is also facing various drawbacks that hinder the use of these cells for clinical islet transplantation. IBMIR reaction is one of the main barriers for transplanted porcine islets. This is due to the high level of endogenous circulating antibodies recognizing carbohydrate antigen that are present on porcine islets but not on human. Thus, a more aggressive immunosuppression regimen is required to prevent transplanted porcine islet rejection [127, 439-442]. Encapsulation of porcine islets is one strategy to avoid immune rejection of transplanted islets and also reduce or eliminate the use of intensive immunosuppression medications [443-446]. However, the insufficiency in metabolic function of these encapsulated porcine islets is one of the drawbacks that need to be addressed [447, 448]. Another strategy to avoid immune-rejection of porcine islets is to generate multi-transgenic and knock out (KO) pigs with islets that lack all identified carbohydrate antigens on islets [449, 450]. Nevertheless, creating such a multi-KO pig for all possible xeno-antigens is an expensive procedure with the use of conventional methods [451-453]. Although, xeno-islet transplantation is in a strong position to provide a large number of islets for widespread use of these cells in beta cell replacement therapy, advancement in gene

editing technologies and strategies to improve encapsulated islet quality are required in order to translate xeno-islet transplantation into clinical use for diabetic patients.

Since the first attempts of pancreatic islet transplantation on diabetic patients, the technique has moved forward and proven to be safe and effective treatment option with the ability to improve patient quality of life, protecting them from hypo- and hyperglycemic episodes and more importantly having a potential to make them independent of exogenous insulin administration. The ultimate goal for clinical islet transplantation is to have long-term survival of transplanted islet graft and make the patients suffering from diabetes independent of exogenous insulin therapy. However, this technique still facing uncertainties and challenges that needs to be addressed with the use of novel strategies in order to get maximum effectiveness from this treatment.

8. Conclusions

The main conclusions of the presented thesis are extracted as follows:

1. Nutrient deprivation and activated ER stress are involved in islet loss and dysfunction. By mimicking these conditions *in vitro*, GDNF showed improvement in human islet function accompanied with reduced apoptosis. Pre-treatment with GDNF prior to islet transplantation into diabetic mouse model enhanced islet graft function post transplantation. The protective role of GDNF was at least partly through an increase in phosphorylation of PI3K/AKT/GSK3B and alleviation of ER stress markers, IRE-1α and BiP (Figure 6, Article 1).
2. Hyperglycemia alone and in combination with the pro-inflammatory cytokine, LIGHT up-regulated the expression of LIGHT receptors in human islets. Combination of hyperglycemia and LIGHT impaired insulin secretion in response to glucose, which was at least partly as a result of an increase in pro-inflammatory mediators and TF, elevated ER stress markers, and apoptosis in human islets. Treatment with the anti-inflammatory cytokine IL-22 showed the capacity to counteract all these deleterious effects. We also found that human islets express the IL-22 receptor subunit 1 and this receptor subunit was up-regulated in response to the combination of hyperglycemia and LIGHT (Figure 6, Article 2).
3. Inhibition of the GPR44 receptor on human islets exposed to diabetic milieu *in vivo* using a mouse model, where minimal mass of human islets were transplanted under kidney capsule and treated with GPR44 antagonist, revealed improvement in islet function and preservation of transplanted islet mass. The protective effects of GPR44 inhibition on human islets include enhanced insulin secretion and reduced production of pro-inflammatory cytokines. Activation of survival pathway AKT/GSK3β together with up-regulation of PDX-1 and MAFA through deactivation of FOXO-1 was involved in the

molecular mechanisms inducing the protective effect of GPR44 inhibition on human islets (Figure 6, Article 3).

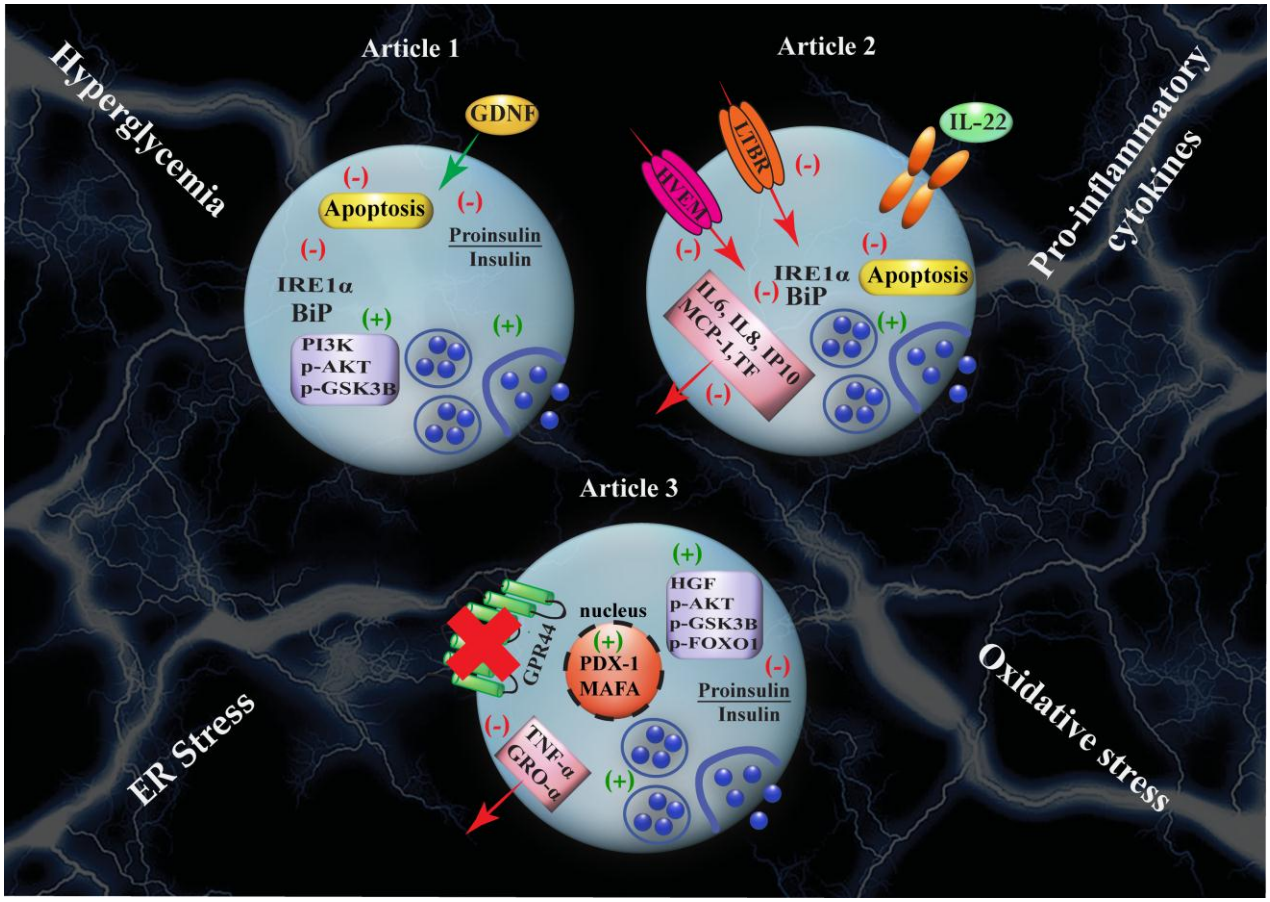

Figure 6: Summary of the main conclusions. Human islets are exposed to micro-environmental stress due to presence of hyperglycemia, ER stress, oxidative stress and pro-inflammatory cytokines. This stress milieu could deteriorate pancreatic islet health and function, which further leads to reduced islet survival and engraftment after islet transplantation. Herein, we show that targeting these stressors by treating human islets with GDNF (Article 1), anti-inflammatory IL-22 (Article 2) and inhibition of GPR44 using GPR44 antagonist (Article 3) could enhance isolated islet function and survival both *in vitro* and *in vivo*. In each study, we also investigated the molecular mechanisms that are involved in protecting islets from the selected stress milieu. We showed that activation of the PI3K/AKT signaling pathway and a decrease in production of pro-inflammatory mediators could lead to improvement in islet viability and survival.

9. References

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Glial cell-line derived neurotrophic factor protects human islets from nutrient deprivation and endoplasmic reticulum stress induced apoptosis

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One of the key limitations to successful human islet transplantation is loss of islets due to stress responses pre- and post-transplantation. Nutrient deprivation and ER stress have been identified as important mechanisms leading to apoptosis. Glial Cell-line Derived Neurotrophic Factor (GDNF) has recently been found to promote islet survival after isolation. However, whether GDNF could rescue human islets from nutrient deprivation and ER stress-mediated apoptosis is unknown. Herein, by mimicking those conditions *in vitro*, we have shown that GDNF significantly improved glucose stimulated insulin secretion, reduced apoptosis and proinsulin:insulin ratio in nutrient deprived human islets. Furthermore, GDNF alleviated thapsigargin-induced ER stress evidenced by reduced expressions of IRE1α and BiP and consequently apoptosis. Importantly, this was associated with an increase in phosphorylation of PI3K/AKT and GSK3B signaling pathway. Transplantation of ER stressed human islets pre-treated with GDNF under kidney capsule of diabetic mice resulted in reduced expressions of IRE1α and BiP in human islet grafts with improved grafts function shown by higher levels of human C-peptide post-transplantation. We suggest that GDNF has protective and anti-apoptotic effects on nutrient deprived and ER stress activated human islets and could play a significant role in rescuing human islets from stress responses.

Type-1 diabetes (T1D) results from autoimmune destruction of insulin producing pancreatic beta cells in islets of Langerhans, which is largely due to reactive T-cells[1]. The beta cells main function is to produce and secrete insulin to regulate the levels of glucose in the blood. Loss of beta cells function and mass increases the workload on the remaining fully functional beta cells[2,3]. Consequently, these cells are more prone to experience endoplasmic reticulum (ER) stress and activation of unfolded protein response (UPR)[4]. Although short-term and mild activation of UPR secures proper folding of newly synthetized proteins in beta cells[5,6], prolonged and unresolved UPR activation triggers programmed cell death, which is associated with an increase in inflammatory cytokines and apoptosis through activation of caspase cascades[7,8].

Beta cell replacement by islet transplantation to selected patients suffering from T1D is currently becoming an established therapy[9]. However, its success rate is constrained by limited long-term islets graft survival partly due to massive loss of islets caused by hypoxia and nutrient deprivation in poorly vascularized islet grafts and inability of the islets to tolerate long-term stress environment[10–14]. The islet isolation procedure itself prior to transplantation also destroys cellular and non-cellular compartments of the pancreas, which potentially plays a role in islets loss and apoptosis[10,12]. Newly isolated islets express high levels of ER stress sensors and activators (BiP, eIF2α, ATF4, sXPB1) as well as ER stress-associated apoptotic signals (JNK, CHOP, caspase3/7)[15]. Inositol-requiring

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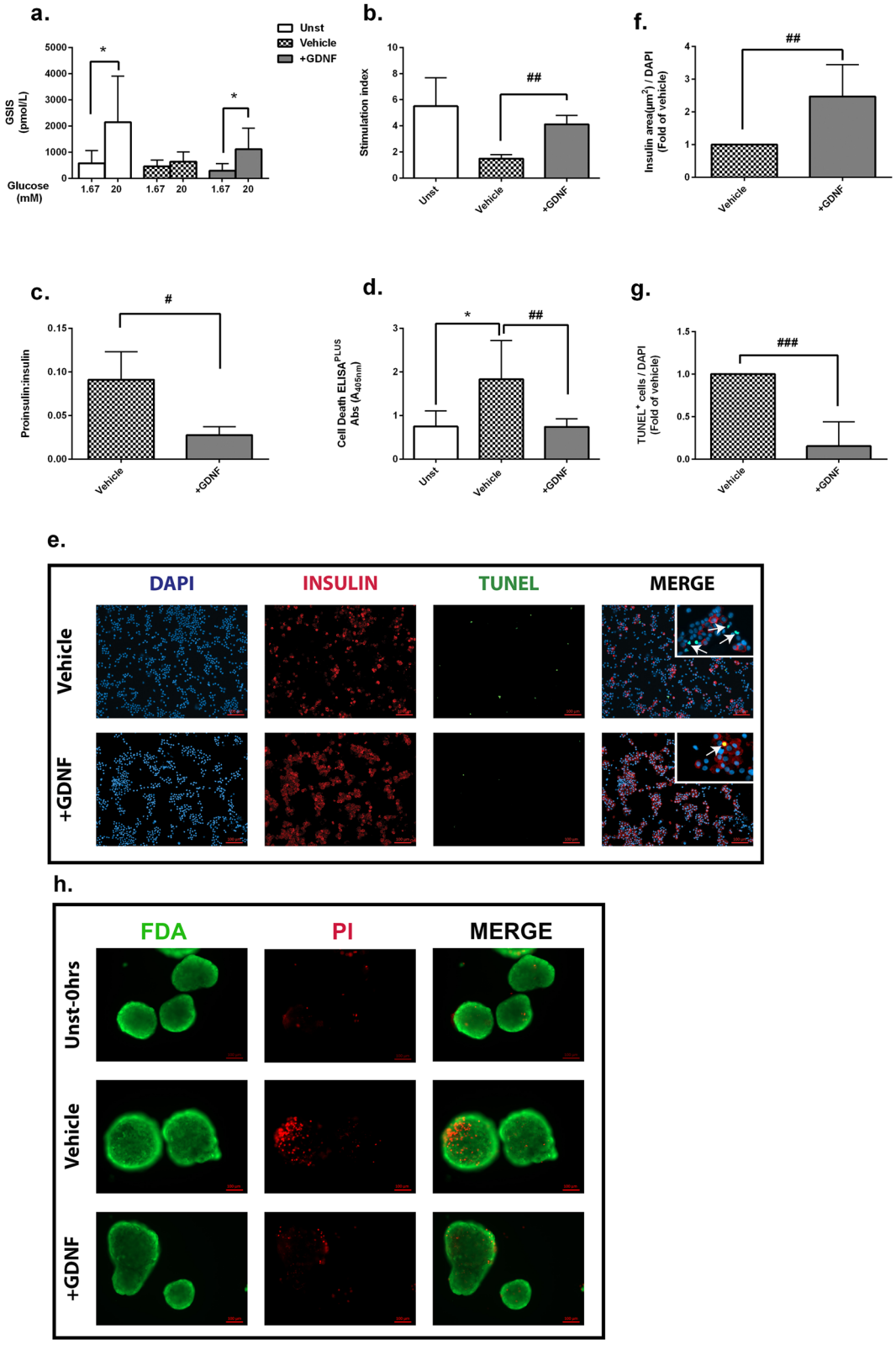

Figure 1. GDNF improves human islets function and viability under nutrient deprived culture condition. (**a**) Insulin secretion in response to basal (1.67 mM) and stimulated (20 mM) level of glucose (GSIS) (**b**) calculated as stimulation index in human islets prior to experiment start (0 hrs, unst islets) and after treatment for 72 hrs with or without GDNF (200 ng/nl) under nutrient deprived culture condition, n = 6. (**c**) Secreted insulin and proinsulin in culture medium were measured by EIA and presented as the proinsulin to insulin ratio, n = 4. (**d**) Apoptosis evaluated by cell death ELISA^{PLUS} in human islets, n = 6. (**e**) Representative images showing insulin (red) and TUNEL (green) with DAPI nuclear staining (blue) of dispersed human islets treated with or without GDNF. (**f**) Ratio of insulin area as well as (**g**) score of TUNEL^{+} cells over nuclear staining. Data is presented as a

fold of vehicle islets, n = 3, Five images were taken from every slide and minimum of 2000 cells were scored. (**h**) Representative images showing intact islets stained for PI (red) and FDA (green) prior to islets culture (0 hrs) and after 72 hrs culture of nutrient deprived islets treated with or without GDNF. Statistical analysis: p-values were analyzed by Wilcoxon matched-pairs test in (**a**), nonparametric ANOVA with Dunn's corrections in (**b**) (**d**), Mann-Whitney U-test in (**c**,**f**,**g**). For all analysis, data is presented as mean ± SD. *$p < 0.05$ vs.unst islets, $^{\#}0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. vehicle islets, *$p < 0.05$ vs stimulated (20 mM) glucose. Unst: unstarved islets. The n refers to the number of independent donors used for each experiment.

enzyme1α (IRE1α) is also one of the UPR mediators triggering inflammation and induces transition from physiological to pathological UPR[5,16,17]. Culturing murine islets with growth factors such as insulin growth factor (IGF) or nerve growth factor (NGF) reduces ER stress and consequently ER stress induced apoptosis through activation of the PI3K/AKT signaling pathway[18,19].

Glial cell-line derived neurotrophic factor (GDNF) produced by glial cells plays an important role in the development of the enteric nerve system[20,21]. GDNF signals through binding to GDNF-family receptor α-1 (GFRα-1) followed by GDNF-GFRα-1 complex binding to receptor tyrosine kinase (RET)[22–24]. Pancreatic beta cells share several biological characteristics with neuronal cells such as expression of neuronal transcription factors[25,26] and several findings link GDNF to beta cells survival and maintenance of beta cells function. Increased expression of GDNF has been reported in the proximity of pancreatic beta cells following islets injury suggesting involvement of GDNF in islets survival and repair[27]. Overexpression of GDNF in glial cells increases beta cell survival and improves glucose tolerance in transgenic mice[20]. *In vitro* pretreatment of human islets for 14 days in culture medium supplemented with human serum albumin, insulin growth factor -1 (IGF-1) and GDNF has also been shown to improve glycemic control and islet survival post transplantation in mice[28]. However, it is unclear whether or not GDNF can protect human islets against nutrient deprivation and ER stress induced apoptosis, which is detrimental in early phase after islet transplantation. By combining *in vitro* and *in vivo* approaches, we investigated the potential protective effect of GDNF on low-nutrient culture condition as well as ER stress induced apoptosis in human islets. Finally, we investigated the molecular signaling pathway by which GDNF protects against ER stress in human islets.

Results

GDNF improves function and viability of nutrient deprived human islets. In order to investigate the effect of GDNF on islet function and survival under nutrient deprivation, isolated human islets were cultured for 72 hrs under low concentration of serum (0.5%) with or without GDNF. Unstarved islets cultured in media supplemented with 10% human serum was also included as control. To evaluate the islet function, we performed GSIS assay, and stimulation index (SI) was calculated as described in methods section. Insulin secretion in response to stimulated level of glucose was significantly increased for unstarved islets and nutrient deprived islets treated with GDNF, but not for vehicle (Fig. 1a). Therefore, nutrient deprived islets treated with GDNF performed significantly better compared to the vehicle (mean SI 4.10 ± 1.20 vs. 2.20 ± 1.50, $p < 0.01$) (Fig. 1b). To further evaluate the protective effect of GDNF on islets dysfunction, we measured the secreted levels of proinsulin and insulin in GDNF-treated islets compared to vehicle; (proinsulin: 1981 ± 247.90 vs. 2562 ± 413.40 pmol/L, insulin: 4324 ± 64.12 vs. 2381 ± 145 pmol/L) that revealed significant reduction in proinsulin to insulin ratio in GDNF-treated islets compared to vehicle ($p < 0.05$) (Fig. 1c). The improved functionality by GDNF on nutrient deprived islets was followed by reduced apoptosis compared to vehicle measured by DNA fragmentation using Cell Death ELISA^{PLUS} assay (0.74 ± 0.07 vs. 1.83 ± 0.36, $p < 0.01$) (Fig. 1d), TUNEL assay (Fig. 1e,g) and FDA/PI staining (Fig. 1h). In addition, immunofluorescent analysis showed a 2.0-fold increase of insulin staining in the GDNF-treated islets compared to vehicle (Fig. 1e,f).

GDNF protects human islets from ER stress and consequently ER stress induced apoptosis. It is known that ER stress induces activation of UPR signaling pathways. Prolonged and unresolved activation of UPR leads to apoptosis and loss of beta cells function[5,29]. Protein folding is highly Ca^{2+}-dependent process and depleting the ER-Ca^{2+} stores by blocking sarco/endoplasmic Ca^{2+} -ATPase (SERCA) will thus cause unfolded proteins to accumulate in the ER and subsequently induces ER stress[30,31]. In order to investigate the effect of GDNF on human islets under ER stress, we cultured nutrient deprived islets with or without GDNF with the SERCA channel blocker, thapsigargin (Tg) for 48 hrs. Tg significantly increased the expressions of the UPR-mediators, IRE1α (3.0 fold of vehicle) and Binding immunoglobulin Protein (BiP) (4.0 fold of vehicle) as determined by western blotting. Importantly, treatment of Tg+GDNF almost completely blunted the upregulation of IRE1α (2.0 fold reduction) and BiP (2.5 fold reduction) compared to the islets treated with Tg alone (Fig. 2a–c). Furthermore, apoptosis measured by DNA fragmentation using Cell Death ELISA^{PLUS} was significantly reduced in Tg+GDNF compared to Tg alone (1.28 ± 0.21vs. 2.3 ± 0.29, $p < 0.05$) (Fig. 2d). Similarly, immunofluorescent double-staining of dispersed islet cells by TUNEL and insulin (Fig. 2e) showed not only less TUNEL positive cells (Fig. 2e,f), but GDNF also reversed the adverse effect of Tg on insulin staining in human islets (Fig. 2e,g). Lastly, we performed viability staining (FDA/PI) of intact islets and showed enhanced PI staining in the Tg treated islets compared to Tg+GDNF (Fig. 2h). Taken together, these results suggest that GDNF protects human islets from ER stress and consequently ER stress induced apoptosis.

GDNF reduces ER stress in human islets via PI3K/AKT signaling pathway. Since ER stress induced apoptosis is partly triggered by reduction of the PI3K/AKT/GSK3B signaling pathway[18], we investigated the effect of GDNF on phosphorylation of these phosphoproteins in ER stress induced human islets using an intracellular

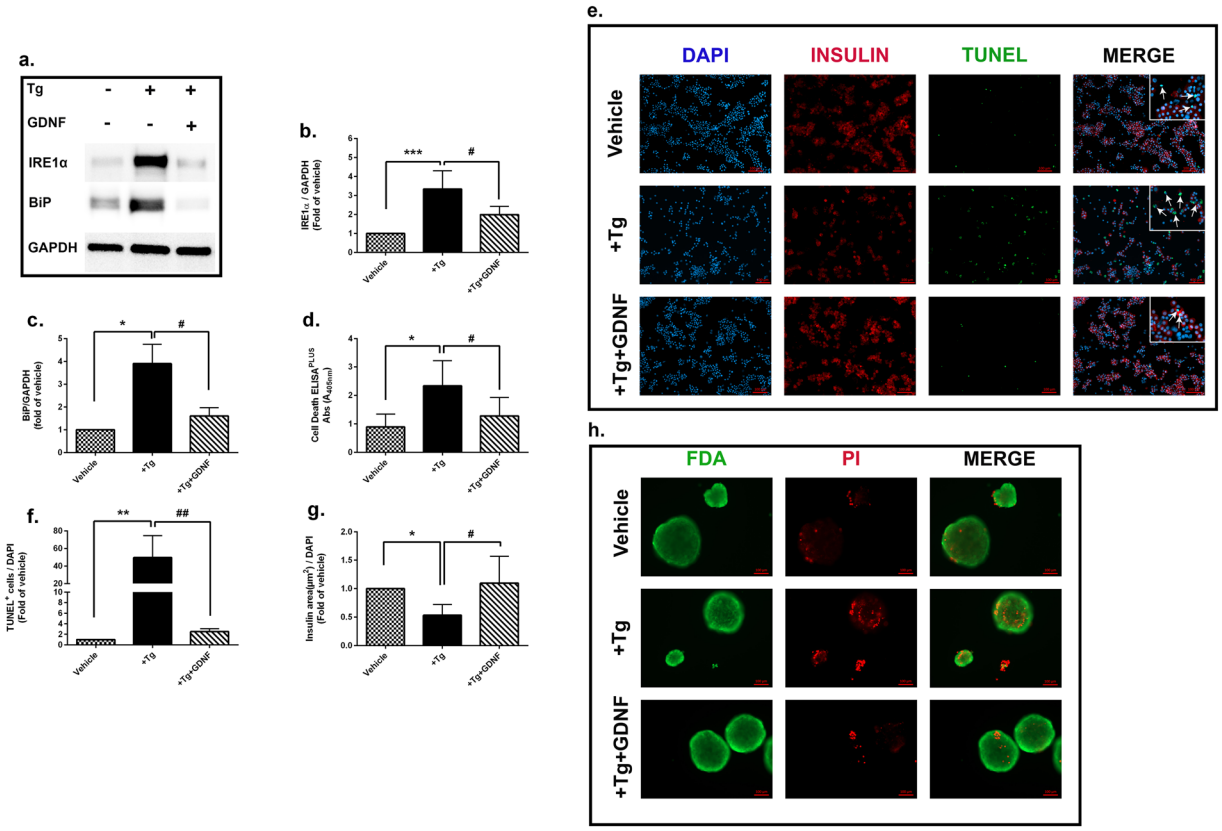

Figure 2. GDNF reduces ER stress and consequently ER stress induced apoptosis in human islets. (**a**) Representative western blot of IRE1α and BiP in human islets treated with Tg (1 μm) with or without GDNF (200 ng/ml) for 48 hrs. (**b**,**c**) Quantification of IRE1α and BiP band densities normalized to housekeeping gene GAPDH. Data is presented as a fold of vehicle islets, n = 6. (**d**) Cell death analysis by cell death ELISA^{PLUS} of human islets treated with Tg with or without GDNF for 48 hrs, n = 9. (**e**) Representative images showing insulin (red), TUNEL (green) and DAPI (blue) nuclear staining of dispersed human islets treated with Tg with or without GDNF for 48 hrs. (**f**) Score of TUNEL^{+} cells (**g**) and measurement of insulin area to DAPI nuclear staining in dispersed human islets. Data is presented as a fold of vehicle islets. n = 3, five images were taken from each slide and minimum of 2000 cells were scored. (**h**) Representative images showing intact islets stained for PI (red) and FDA (green). For all analysis, data is presented as mean ± SD and p-values were analyzed by nonparametric ANOVA with Dunn's corrections.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. vehicle islets, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. Tg-treated islets. Tg: Thapsigargin. The n refers to number of independent donors used for each experiment.

phosphoproteins signaling multiplex assay as described in the methods section. First, Tg treated islets reduced phosphorylation (p-) of PI3K (Fig. 3a), AKT (Fig. 3b) and GSK3B (Fig. 3c) compared to vehicle. Importantly, co-treatment of human islets with Tg+GDNF significantly recovered the levels of p-PI3K, p-AKT, p-GSK3B compared to Tg alone (Fig. 3), suggesting that GDNF protects human islets from ER-stress via activation of the PI3K/AKT signaling pathways.

Pre-treatment of ER stress induced human islets with GDNF not only improved islets graft function but also alleviated ER stress post transplantation. Finally, we investigated the possible protective effect of GDNF on nutrient deprived and ER stress induced human islets *in vivo*. Alloxan-induced diabetic male Rag1$^{-/-}$ mice were transplanted under kidney capsule with a minimal dose of human islets (800 islets) pre-cultured for 48 hrs under nutrient deprived culture condition with or without GDNF as well as Tg or Tg+GDNF and followed for 30 days post transplantation. Unstarved islets pre-cultured in complete medium supplemented with 10% human serum prior to transplantation was also included as control. Random blood glucose profile did not shown statistically significant differences among groups (Fig. 4a). Of the animals transplanted with unstarved islets or vehicle, 45–50% became euglycemic on day 3 and between days 7–13 respectively post transplantation. In contrast, none of the diabetic recipient mice transplanted with Tg pre-treated islets reached euglycemia in the follow-up period (Fig. 4b). However, Tg+GDNF recipients showed approximately 25% euglycemia achievement between day 7–17 post transplantation (Fig. 4b). As such, the levels of plasma C-peptide (Tg+GDNF: 814.06 ± 112.04 vs. Tg: 386.07 ± 60.86 pmol/L, $p < 0.01$) (Fig. 4c), as well as the ratio of human C-peptide to fasting blood glucose (Tg+GDNF: 129.06 ± 20.91 vs. Tg: 51.33 ± 13.70 $p < 0.05$) (Fig. 4d) were increased at day 30 post transplantation. In addition, GDNF recipients showed a tendency to increased plasma

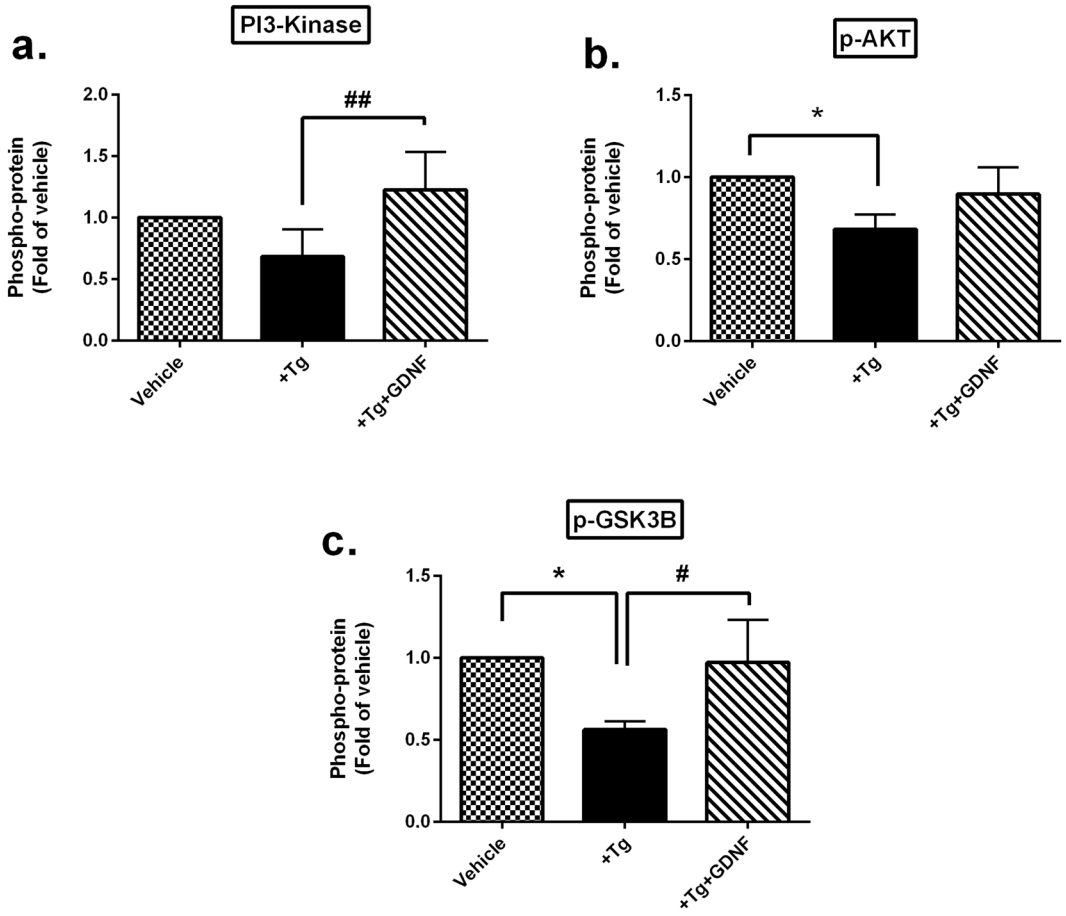

Figure 3. GDNF protective effect on ER stress induced human islets is through activation of PI3K/AKT signaling pathway. (**a–c**) Assessment of phosphoproteins in the PI3K/AKT/GSK3B signaling pathway by multiplex assay in human islets treated with Tg (1 μm) with or without GDNF (200 ng/ml) for 48 hrs. Data is presented as a fold of vehicle islets. n = 6. For all analysis, data is presented as mean ± SD and p-values were analyzed by nonparametric ANOVA with Dunn's corrections. *$p < 0.05$vs. vehicle islets, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. Tg-treated islets. Tg:Thapsigargin. The n refers to number of donors used for each experiment.

human C-peptide (GDNF: 765.05 ± 107.01 vs. vehicle: 490.06 ± 69.82 pmol/L, $p < 0.07$) (Fig. 4c) and human c-peptide to fasting blood glucose (136.08 ± 12.56 vs. 101.6 ± 15.09) (Fig. 4d) compared to the vehicle group. We further investigated the influence of GDNF on ER stress in transplanted islet grafts at day 30 post transplantation by immunoblot analysis of ER stress response proteins (Fig. 4e). We found a significantly decrease in protein expression of IRE1α (Fig. 4f) and BiP (Fig. 4g) in grafts containing islets pre-treated with Tg+GDNF compared to Tg alone (2.0 and 3.0 fold reduction, respectively). Taken together, these results suggest that GDNF protects human islets from ER stress and further contributes to improve islet grafts function post transplantation.

Discussion

In the current study, we have shown that GDNF could reverse the adverse effect of nutrient deprivation and SERCA channel blocker, Tg by improving human islets function and viability through reduction in ER stress induced apoptosis and activation of PI3K/p-AKT/p-GSK3B survival pathway. We have also shown that pre-treatment of nutrient deprived and ER stress induced human islets with GDNF prior to transplantation protected grafts function and mass through a reduction in expression of ER stress mediators.

Strategies to avoid massive beta-cells loss due to islets exposure to hypoxia, nutrient deprivation and activated ER stress during isolation as well as pre- and post- islets transplantation[10,32–35] have been of a great interest. In the present study, we mimicked nutrient deprivation and ER stress induced apoptosis by culturing islets under low serum condition and supplementing the ER stress inducer compound, Tg which has been shown previously to increase apoptosis, necrosis and autophagy in both mice and human islets and decreases beta-cell mass[36–38]. Although exposure to the low level of Tg mimics mild ER stress and consequently only disturbs ER Ca^{2+} filing but not islets function and insulin secretion[39], chronic exposure to elevated level of Tg (1 μM) inhibits insulin secretion as much as 90% and therefore, alleviates islets functionality[40].

Reducing activated ER stress response has been investigated by supplementing growth factors such as IGF, NGF to islets culture[19,41,42] or through viral delivery of HGF, VEGF and GDNF to islets[20,43–47]. Co-culturing islets with mesenchymal stem cells which are known as a source of growth factors and cyto-protective elements[48,49] have also been studies as a strategy for reducing islets loss. Although elevated islets function and viability as well

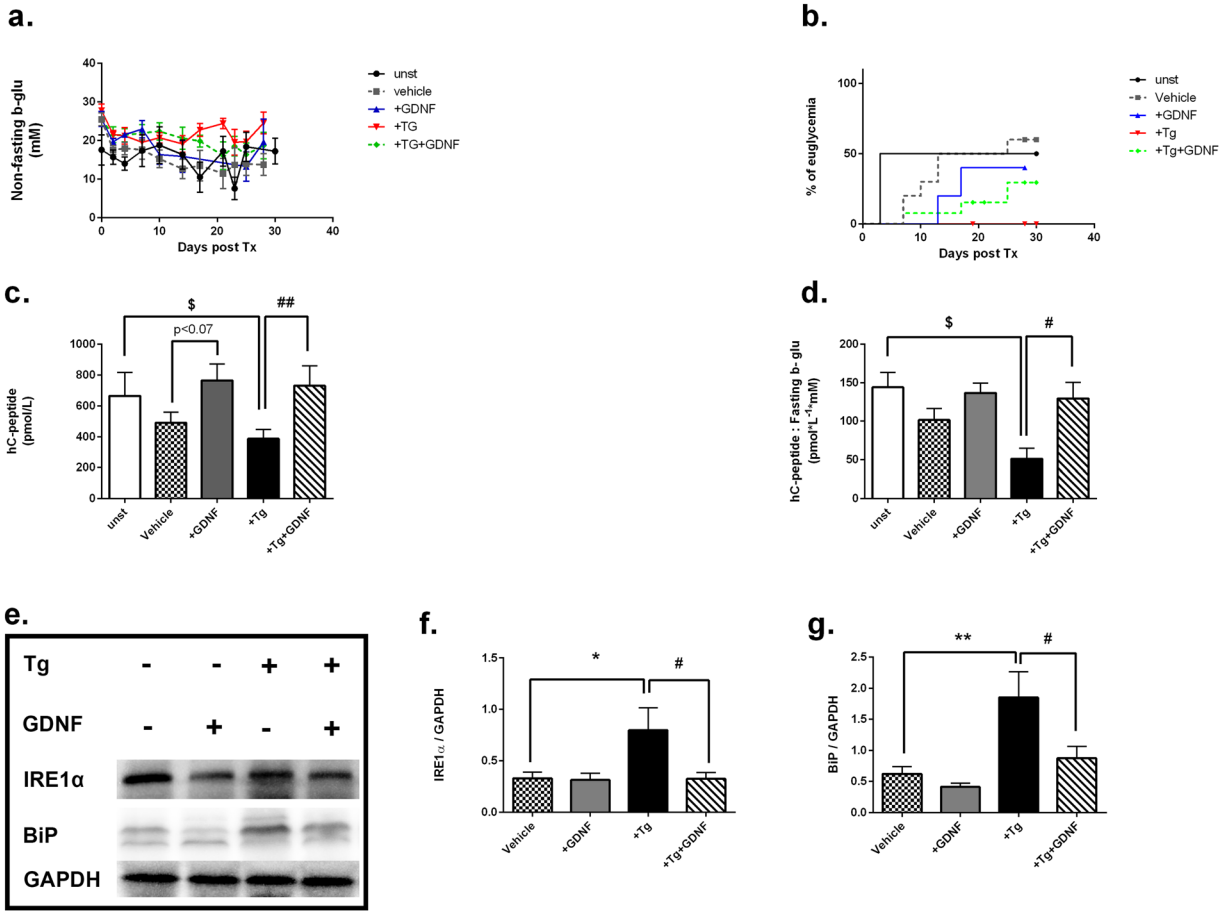

Figure 4. GDNF improves islet grafts function and reduces ER stress post transplantation. (**a**) Random blood glucose levels in diabetic mice transplanted with human islets pre-cultured for 48 hrs in complete medium (unst, n = 6), nutrient deprived medium (vehicle, n = 11) with GDNF (200 ng/ml, n = 6), Tg (1 μM, n = 8), or Tg+GDNF(n = 8). (**b**) Percentage of euglycemic mice post transplantation. (**c**) Circulating levels of hC-peptide measured by EIA in plasma at day 30 post transplantation (**d**) and presented as ratio of hC-peptide over fasting b-glu. (**e**) Representative western blot for protein expression of IRE1α and BiP in human islet grafts harvested on day 30 post transplantation. (**f**,**g**) Quantification of IRE1α and BiP bands densities normalized to housekeeping gene GAPDH. For all analysis, data is presented as mean ± SD and p-values were analyzed by nonparametric ANOVA with Dunn's corrections or Mann-Whitney U-test. The n refers to number of mice in each experimental group, $^{*}P < 0.05$, $^{**}p < 0.01$ vs. vehicle islets, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. Tg-treated islets. $^{\$}p < 0.05$ vs unstv group. Log-rank (Mantel-Cox) test was used to analyze the difference in percentage of euglycemic animals post transplantation. Unst: unstarved, Tg: Thapsigargin, B-glu: Blood glucose, hCpeptide: human C-peptide.

as improved islets graft revascularization have been shown, most studies are restricted to rodent or non-human primate islets. Recently, a combination of GDNF and IGF have been reported as a beneficial supplement to human islets transplantation medium for improving islets function and viability in culture as well as post transplantation[28]. However, human islets in that study were not exposed to stress responses that normally islets experience in the pre and post transplantation phase. In our study, we show that short-time exposure of human islets to GDNF in culture does not negatively impact human islets viability or functional potency. Importantly, we showed that GDNF recovered insulin secretion in response to stimulated level of glucose and increased total insulin content in nutrient deprived human islets. This was accompanied with a reduction in the ratio of proinsulin to insulin in GDNF-treated human islets. Elevation of proinsulin to insulin ratio reflects failed ER activity in folding newly synthetized proinsulin and processing to insulin[50,51]. Therefore, reduction in this ratio suggests an overall improvement in insulin process and secretion by GDNF in nutrient deprived islets.

There is a great interest in contribution of ER stress and nutrient deprivation to the failure of functional transplanted islets graft on early phase post transplantation as elevation of ER stress mediators have been reported in isolated and transplanted islets[10,15,52]. Herein, we have reported that pre-culturing ER stress-induced human islets with GDNF increased the percentage of euglycemia achievement in diabetic recipients, which was accompanied with significant improvement in human islets function and mass 30 days post transplantation evidenced by increased human C-peptide secretion and human C-peptide to fasting blood glucose ratio, respectively. However, we observed minor protective effect of GDNF compare to vehicle *in vivo*, which could be due to both milder stress

response induced by nutrient deprivation alone and shorter culture time in starvation condition prior to transplantation. Improved grafts function post transplantation in animals transplanted with Tg+GDNF pre-treated human islets was associated with significant reduction in expression of ER stress response proteins IRE1α and BiP. Elevations of ER stress sensor, IRE1α and molecular chaperone, BiP have been reported upon ER stress and in pancreatic islets of both type1 and type 2 diabetic patients[5,53,54]. An increase in expression of IRE1α is correlated with degradation of insulin mRNA[55,56]. Therefore, observed reduction in protein expressions of BiP and IRE1α associated with recovered islets mass and function suggest a protective role of GDNF through decreasing protein degradation, reducing ER stress induced apoptosis and also improving ER protein synthesis and folding efficiency. Recently, GDNF has been found as an angiogenic factor secreted by adipose-derived stem cells, which works independent of VEGF and mediates endothelial cells formation and angiogenesis[57]. This could also explain the improvement in transplanted graft function 30 days post transplantation found in our study.

The mechanism involves in the effect of GDNF on pancreatic islets under nutrient deprivation and activated ER stress is not fully understood. Nutrient deprivation induces activation of proinflammatory cytokines in islets[58]. Increased inflammation results in SERCA Ca^{2+} channel instability through activation of oxidative stress and NO production[59,60]. We induced both nutrient deprivation and ER stress by culturing islets in serum reduced culture medium together with SERCA channel blocker, Tg. Although, we have shown that GDNF reversed the adverse effect Tg through reduction in ER stress activity, it is unknown if GDNF could directly interfere with the SERCA channel or ER Ca^{2+} filing and we could not rule this out in the current study. In addition, GDNF has also been reported to induce protective effect on long-term human islets culture, which is a stress condition independent of nutrient deprivation and inhibition of SERCA channel[28].

Previous investigations on survival effect of neurotrophic factors such as NGF and GDNF on islets as well as enteric neurons, identified activation of survival pathways PI3K/AKT and glycogen synthase kinase-3β (GSK-3β) as possible candidates involved in cyto-protective effect of these neurotrophic factors[20,26,61]. Elevated PI3K/AKT correlates with suppression of ASK, its downstream kinase JNK and therefore reduced apoptosis in human islets[62]. We have demonstrated here that supplementing GDNF to ER stress induced human islets recovered phosphorylation and activation of PI3K, AKT and GSK3β. Therefore, PI3K/p-AKT/p-GSK3β is a possible signaling pathway by which GDNF could protect islets from different stress responses.

In conclusion, by mimicking nutrient deprivation and activated ER stress in isolated islets, we have shown that GDNF could recover human islets function and viability and consequently might be a superior mediator to alleviate stress responses within isolated islets.

Research Design and Methods

Human islets isolation and culture. Human islets were obtained from the JDRF award 31-2008-416 (ECIT Islet for Basic Research program) and were isolated according to semi-automated purification system[63] from male/female 10/8 brain-dead donors with mean age 55 years (19–70 years) provided by the islet isolation facility of the Nordic Network, Uppsala, Sweden, or Oslo University Hospital, Oslo, Norway after appropriate informed consent from relatives for multi-organ donation and for use in research. All experiments and methods using human islets were approved by and performed in accordance with the guidelines and regulations made by regional committee for medical and health research ethics central in Norway (2011/782). Islets purity was judged by digital imaging analysis[64] or dithizone staining and islets with purity between 50–95% was used in this study. Fresh free floating isolated islets were cultured in CMRL 1066 (Corning, Manassas, VA, USA) containing 10% human serum and supplements as previously described[65]. For experiments, human islets were manually picked and cultured in Sterilin petri dishes (Sterilin LtD, New Port, UK) with CMRL 1066 medium supplemented with 0.5% human AB-serum, 1% penicillin/streptomycin, 10 mM HEPES (Life Technologies AS, Oslo, Norway), with or without human recombinant GDNF (200 ng/ml) (a kind gift from Sven O. Göpel, AstraZeneca R&D, Molndal, Sweden) for 72 hrs at 37 °C (5% CO_2). In parallel experiments, handpicked human islets were cultured in CMRL medium supplemented with 0.5% human serum and treated with Thapsigargin (Tg) (1 μM) (Sigma Aldrich, Oslo, Norway) with or without GDNF for 48 hrs. Cells and supernatant were harvested as indicated and stored at −80 °C until further analysis.

Glucose stimulation insulin secretion assay. Ten equally-sized islets were handpicked and transferred into transwells plate (Corning, NY, USA) containing krebs-ringer bicarbonate buffer (1x stock buffer, 1 M $Cacl_2$, 1 M $Mgcl_2$, 1 M HEPES, 200 mg/ml human albumin) supplemented with 1.67 mM glucose and incubated for 45 min at 37 °C. Transwells were switched to krebs-ringer bicarbonate buffer containing 20 mM glucose and incubated for 45 min at 37 °C. Supernatants were harvested for insulin secretion analysis using human insulin ELISA kit (Mercodia AB, Uppsala, Sweden). Stimulation index (SI) was calculated as a ratio of insulin secreted in high concentration of glucose (20 mM) to insulin in low concentration of glucose (1.67 mM).

Proinsulin and insulin measurement. Levels of proinsulin and insulin were measured in cell-free supernatant using human insulin and proinsulin ELISA kit (Mercodia AB, Uppsala, Sweden).

Apoptosis assays. Programmed cell death was analyzed by detection of DNA-histone complexes in the cytoplasmic fraction of islets lysates using Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics, Mannheim, Germany) according to protocol offered and described by manufacturer.

TUNEL staining using DeadEndTMFluorometric TUNEL system (Promega Biotech AB, Stockholm, Sweden) was performed on 60–80 handpicked equally sized islets. Islets were dispersed into single cells using TrypLE Express (Life Technologies AS, Oslo, Norway) and proceed to universal 320 cyto-centrifuges (Hettich lab technology, Tuttlingen, Germany). Cytospin-made slides were fixed and permeabilized by 4% Paraformaldehyde (PFA) and 0.5% Triton-X100 in PBS respectively. Protein Block Serum Free (DAKO, Oslo, Norway) was used to block

non-specific staining. Slides were then incubated overnight at 4 °C with polyclonal Guinea Pig Anti-insulin 1:500 (DAKO, Oslo,Norway). After washing with 1x tris buffered saline plus Tween 20 (TBST), slides were incubated with goat-anti-guinea pig Alexafluor 594 1:300 (Life Technologies AS, Oslo, Norway) for 1 hr at room temperature followed by TUNEL staining according to protocol described by manufacturer. Nuclear staining was performed using SlowFade Gold antifade reagent with DAPI (Life Technologies AS, Oslo, Norway). Images were taken by Axio Observer Inverted Microscope (Carl Ziess AS, Germany) operates by ZEN lite software. Area of insulin positive cells and total number of TUNEL positive cells and nuclei per each image were measured and analyzed using Image J software (National Institute for Health, USA). Five images were taken from each slide and minimum of 2000 cells per slide were scored.

Viability assessment was performed on hand-picked islets using fluorescein diacetate (FDA) 20 µg/ml (Sigma-Aldrich Norway AS, Oslo, Norway) for detection of live cells and propidium iodide (PI) 100 µg/ml (Thremo Fisher Scientific, Oslo, Norway) for evaluating the degree of dead cells. Images were taken by Axio Observer Inverted Microscope (Carl Ziess AS, Germany) operates by ZEN lite software.

Phosphoproteins analysis of PI3K/AKT signaling pathway. 200 handpicked equally sized islets were collected and lysed using cell lysis buffer (BioRad, CA, USA) supplemented with 2 mM PMSF (Sigma Aldrich, Oslo, Norway). Protein lysate concentration was measured by Pierce BCA protein assay (Life Technologies AS, Oslo, Norway) and equal amount of protein lysate was added to each well of phosphoproteins Bio-Plex assay (171V50002M, 171V50007M, 171V500036M). Cell signaling assay was performed according to manufacturer protocol and analyzed using Bioplex 200 system (BioRad, CA, USA).

Western blot analysis. Cell lysis buffer (RIPA buffer supplemented with Halt protease inhibitor (Thermo scientific, Oslo, Norway) or tissue lysis buffer (RIPA buffer containing halt protease-phosphatase inhibitors and 1% sodium dodecyl sulfate) was added to human islets pellet (100 islets) or frozen graft-bearing kidney samples before proceeding to mechanical disruption using sonication. Samples were centrifuged and purified using QIAshredder purification column (QIAGEN, Hilden, Germany). Total protein concentrations were determined using Pierce BCA protein assay (Life Technologies AS, Oslo, Norway). Equal amounts of total proteins (20 µg) were separated on mini-PROTEIN GTX precast gels followed by proteins bands transfer to PVDF membrane (Bio-Rad, CA, USA). According to antibodies datasheet provided by manufacturer, membranes were blocked with 5% skim milk or 5% BSA in 1xTBST and incubated overnight at 4 °C with primary antibodies, IRE1α rabbit monoclonal antibody 1:1000, BiP rabbit monoclonal antibody 1:1000 (Cell Signaling, MA, USA), GAPDH goat polyclonal antibody 1:1000 (Santa Cruz Biotechnology, TX, USA). Bound antibodies were labeled with goat anti-rabbit IgG-HPR 1:10000 and donkey anti-goat IgG-HPR 1:10000 (Santa Cruz Biotechnology, TX, USA). Protein bands were visualized using clarity western ECL chemiluminescence substrate kit (Biorad, CA, USA) or super signal west femto (Thermo scientific, Oslo, Norway) followed by semi-quantitative measurement of band density using chemiDGC touch imaging system, (BioRad, CA, USA).

***In vivo* experimental model.** The experimental protocol was approved by the Norwegian National Animal Research Authority project license no FOTS id 8588. The animal experiments were performed in accordance with the European Directive 2010/63/EU and The Guide for the Care and Use of Laboratory Animals, 8th edition (NRC 2011, National Academic Press). Animals were housed under standard condition in an approved facility with free access to food and water except fasting time. Male Balb/c Rag $1^{-/-}$ immunodeficient mice (C.129S7(B6)-Rag1^{tm1Mom}/J, stock 003145, The Jackson Laboratory, Sacramento, California, USA) 8–10 weeks old mice were used as recipients. Diabetes was introduced by one intravenously injection of alloxan (Sigma Aldrich, St. Louis, Missouri, USA) (75 mg/kg body weight). Mice constantly showing non-fasting blood glucose above 20 mM for two consecutive days measured by glucometer (Accu-Chek Avia Nano, Rouche Diagnostics, Indiana, USA) were considered diabetic. Mice were divided into five groups and transplanted under kidney capsule with 800 human islets pre-cultured for 48 hrs either in culture medium containing 10% human serum as control (unst) (n = 6), or under nutrient starvation (0.5% human serum)(vehicle)(n = 11) with GDNF (200 ng/ml) (n = 6), Tg (1 µM) (n = 8), or Tg+GDNF (200 ng/ml) (n = 8) as previously described[66]. Random non-fasting blood glucose and weight were monitored every third day at 9 am until endpoint. At day 30 post transplantation, mice under anesthesia were sacrificed by heart puncture for blood samples and the graft-bearing kidney were harvest by snap frozen in liquid nitrogen. Plasma samples for analysis of human C-peptide (Mercodia, Uppsala, Sweden) together with harvested graft-bearing kidneys for analysis of ER stress mediators were stored at −80 C until use.

Statistical analysis. Data are presented as means ± SD and GraphPad Prism version 6.0. (La Jolla, CA, USA) was used for data analysis. Differences among three groups were evaluated by non-parametric ANOVA with Dunn's corrections. Mann-Whitney U-test and Wilcoxon matched-pairs test were performed based on experimental design (paired vs. unpaired) for difference analysis between two groups. Significance was set at $p < 0.05$.

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Author Contributions

Conceived and designed of the experiments: S.A., H.S. and S.O.G. Performed the experiments and analyzed the data: S.A., S.S. and H.S. Performed human islets isolation: O.K., H.S., S.A., S.S. Wrote the manuscript: S.A. and H.S. Edited the manuscript: S.A., H.S., S.S., S.O.G., A.F., O.K.

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Inhibition of the Prostaglandin D2-GPR44 axis improves human islet function and survival

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Abstract

GPR44 is a receptor for prostaglandin D_2 that recently identified to specifically express on human beta cells, however the function of this receptor has not been studied. Herein, the PGD_2-GPR44 axis was investigated using a selective GPR44 antagonist on human islets *in vitro* and *in vivo* by using a human islet transplantation mouse model. PGD_2 or pro-inflammatory cytokines (IL-1β, TNF-α, INF-γ) induced apoptosis in human islets and GPR44 inhibition reversed this effect in a dose dependent manner. Exposing human islets to high glucose plus IL-1β reduced insulin secretion, which was significantly reversed following the treatment with GPR44 antagonist. Administration of the GPR44 antagonist to diabetic mouse model transplanted with human islets resulted in reduced fasting blood glucose and enhanced glucose tolerance. Improved islet function was supported by increased hC-peptide levels compared to the vehicle treated recipients. GPR44 inhibition reduced plasma level of proinflammatory markers, TNF-α and GRO-α and increased HGF. GPR44 inhibition in human islets exposed to diabetic stressors *in vitro* resulted in activation of the AKT/GSK3β signaling pathway together with phosphorylation and inactivation of FOXO-1 and up-regulation of PDX-1. In conclusion, inhibition of GPR44 in human islets has the potential to improve islet function and survival under inflammatory and metabolic stress.

Introduction

GPR44, also known as DP2 or CRTh2, is a trans-membrane G-coupled protein receptor for prostaglandin (PG) D_2 that has recently been identified as a human beta cell specific biomarker through proteomics screening analysis (1). The expression of GPR44 is highly restricted to human pancreatic beta cells (2) and the receptor has recently been shown as a useful imaging biomarker for visualization of the human beta cell mass (3). However, the physiological role of GPR44 on human islet function and survival in the diabetic milieu remains unknown.

One hallmark of diabetes is the loss of functional beta cells mass, resulting in an insufficient release of insulin and development of hyperglycemia (4). Prolonged presence of hyperglycemia has an adverse effect on pancreatic beta cell function and mass due to, at least partly, activation of pro-inflammatory responses leading to beta cells apoptosis (4-6). Pro-inflammatory PGs are lipid molecules that derive from arachidonic acid (AA) in cell-membrane phospholipids and inflammatory molecules trigger their synthesis (7). Interleukin (IL)-1β, a pro-inflammatory cytokine that is known to induce beta cell injury and loss, has also been found to increase PG production in islets through activation of NF-κB (8, 9).

PGD_2 signals through both GPR44 and DP1 (10). However, only GPR44 is highly expressed in isolated human islets, while DP1 mRNA is barely detectable (1). Although, there is not much known about the role of PGD_2 and the regulatory mechanism of PGD_2-GPR44 in human islets, it has been demonstrated recently that the elevated level of glucose and IL-1ß could increase PGD_2 synthesis *in vitro* in human islets in a similar fashion as previously described for PGE_2 (11). Furthermore, PGD_2 reduces intracellular cAMP and inhibits glucose stimulated insulin secretion via GPR44 in human islets (7). The protein expression of GPR44 has been reported to significantly reduce in insulin negative human islets, demonstrated in pancreatic sections from patients with long-standing type 1

diabetes (12). In contrast, the expression of this receptor showed an up-regulation in sections from patients with type 2 diabetes compared to healthy individuals (12).

In the present study, we aimed to investigate the effect of GPR44 inhibition on the function and survival of human islets exposed to the diabetic milieu mimicked by high level of glucose and IL-1β. We used a diabetic immunodeficient human islet transplant mouse model to evaluate the *in vivo* effect of GPR44 inhibition. Finally, the molecular mechanisms involved in GPR44 inhibition on isolated human islets were explored.

Research Design and Methods

All experiments and methods using human islets were approved by and performed in accordance with the guidelines and regulations made by the regional committee for medical and health research ethics central in Norway (2011/426). The animal experiments were approved by the Norwegian National Animal Research Authority (FOTS ID 7005) and were performed in accordance with the European Directive 2010/63/EU and The Guide for the Care and Use of Laboratory Animals, 8th edition (NRC 2011, National Academic Press).

Compounds and *in vitro* pharmacology

The GPR44 antagonist, AZ8154 used in the present study is an analogue to the previously published GPR44 antagonist, AZD1981, which is a 4-(Acetylamino)-3-[(4-Chlorophenyl) Thio]-2-Methyl-1H-Indole-1-Acetic Acid (13). AZD1981 is a clinical drug candidate intended for treatment of asthma (14, 15). Both compounds were synthesized by AstraZeneca and came from two neighboring chemical series. Briefly, human EndoC-βH1 cells were plated in fibronectin- and extracellular matrix-coated 96-well plates for the *in vitro* pharmacology analysis including cAMP measurement and glucose-stimulated insulin secretion. The dose response curves for the two GPR44 antagonists were also compared and the IC50 values were found to be equal between the two compounds (Supplementary materials, Fig.S1 and Table S1).

Human islets culture and experiment condition

Human islets from non-diabetic donors were obtained from Prodo laboratory (Prodo Labs Inc., CA, USA). The cells were recovered overnight in standard Prodo Islet Media (PIM) (Prodo Labs, CA, USA) at 37 ºC with 5 % CO_2. On the day of experiment, islets were dispersed with TrypLE Express (Gibco, Paisley, UK) and aliquoted into a 96-well plate with 4000 cells/well in 80 µl of PIM before treatment with compounds. The cells were then incubated for 24 hrs with either a stable PGD_2-

analogue, 15(R)-15-methyl-PGD_2 (Cayman Chemicals, MI, USA) at the concentrations of 1, 10 or 100 nM or with a mix of the cytokines, IL-1β (10 ng/ml), IFNγ (50 ng/ml) and TNFα (50 ng/ml) (R&D systems, MN, USA) with or without addition of GPR44 antagonist (1, 3 or 10 μM) to evaluate the dose dependent effect of the ligand PGD_2 and the GPR44 antagonism on islet apoptosis.

For the rest of the experiments, human islets were obtained from the JDRF award 31-2008-416 (ECIT Islet for Basic Research program) and isolated according to semi-automated purification system (16) from male/female 7/3 non-diabetic brain dead donors with mean age 55 years (35-58 years) and mean BMI 24 kg/m^2 (23-30 kg/m^2) provided by the islet isolation facility of the Nordic Network for Clinical Islet Transplantation (Uppsala, Sweden and Oslo, Norway) after appropriate informed consent from relatives for multi-organ donation and for use in research. Islets purity was judged by digital imaging analysis (17) or Dithizone staining and islets with purity > 50% was used in this study. Fresh free floating isolated islets were cultured in CMRL 1066 (Corning, Manassas, VA, USA) containing 10 % human AB serum (Milan ANALYTICA AG, Switzerland) and supplements as previously published (18). Cell culture medium was changed the day after isolation and subsequently every 2-3 days until use in the experiments. In order to perform *in vitro* experiment, equally sized human islets were manually hand-picked and cultured at 37 °C with 5 % CO_2 for 48 hrs in petri dishes (Sterilin LtD, New Port, UK) with CMRL 1066 medium supplemented with 2 % human AB serum (Milan ANALYTICA AG, Switzerland), 1 % penicillin/streptomycin, 10 mM HEPES (Life Technologies AS, Oslo, Norway) without (untreated islets) or with a combination of high glucose (HG, 20 mM) and IL-1β (10 ng/ml) (R&D systems, MN, USA) with or without GPR44 antagonist (10 μM).

Cell death and apoptosis analysis

Programmed cell death was analyzed by detection of DNA-histone complexes in the cytoplasmic fraction of islet lysates using Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics, Mannheim, Germany) according to protocol offered and described by the manufacturer.

The level of caspase activity was determined by Caspase-Glo 3/7 assay according to the protocol provided by the manufacturer (Promega Biotech AB, Nacka, Sweden).

Glucose stimulation insulin secretion assay

20 equally-sized human islets were handpicked and transferred into a transwell plate (Corning, NY, USA) containing Krebs-Ringer Bicarbonate Buffer (11.5 mM $NaCl_2$, 0.5 mM KCl, 2.4 mM $NaHCO_3$, 2.2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, and 2 mg/L albumin: all from Sigma-Aldrich) supplemented with 1.67 mM glucose and incubated for 45 min at 37 °C. Transwells were switched to Krebs-Ringer Bicarbonate Buffer containing 20 mM glucose and incubated for 45 min at 37 °C. Supernatants were harvested for analysis of insulin secretion using human insulin ELISA kit (Mercodia AB, Uppsala, Sweden). Stimulation index (SI) was calculated as a ratio of the insulin secreted in high concentration of glucose (20 mM) to the insulin secreted in low concentration of glucose (1.67 mM).

Islet Transplantation

Male NMRI-nude mice (Harlan, IN, USA) 8-10 weeks old were intravenously administered with one dose of Alloxan Monohydrate 75 mg/kg (Sigma Aldrich, UK) 3 days prior to islet transplantation. Mice with blood glucose $\geq$ 20 mmol/L for 2 consecutive days were selected as diabetic recipients. 500 hand-picked human islets were transplanted under left kidney capsules as previously described (19).

In all experiments, the mice were started on the treatment with GPR44 antagonist one day prior to the human islet transplantation. In the first cohort, mice transplanted with human islets from one donor were administered orally twice daily with a high dose (100 mg/kg/day, n=8) (HD100) of GPR44 antagonist up to 16 days after transplantation. Control mice received vehicle, which was 0.5 % Hydroxypropyl Methyl Cellulose (HPMC, n=8).

In the second cohort, the mice were divided into three groups and a dose response study was performed with low dose (6 mg/kg/day, n=8) (LD6) and high dose (60 mg/kg/day, n=8) (HD60) of GPR44 antagonist, or vehicle (HPMC, n=8) until day 16 after transplantation.

In the third cohort, mice transplanted with human islets from two donors were treated with HD60 of GPR44 antagonist (n=14), or vehicle (n=14). This experiment was terminated 4 days after transplantation in order to study the early effects of GPR44 inhibition on graft function and to explore the molecular mechanism of GPR44 receptor in human islets. Fasted (4 hrs) blood samples were harvested on day 2, 4 and 10 after transplantation from the saphenous vein and on day 16 by hearth puncture after sacrificing the animals. Blood samples were collected into EDTA-coated or serum tubes (Becton, Dickinson and Company, Puls A/S, Oslo, Norway). Plasma was separated by centrifugation at 3000 rpm for 15 min and stored at -70 ºC awaiting assessment. The transplanted human islet grafts were harvested and fixed in 10 % formalin at termination of the second study cohort on day 16 after transplantation. Harvested grafts were embedded and sectioned for immunofluorescent staining of insulin. In the third study cohort, human islet grafts were collected at termination of the study on day 4 after transplantation. Harvested grafts were immediately snap-frozen in liquid nitrogen and stored at -70 ºC until further gene and protein expression analysis.

Glucose Measurements

Random non-fasting blood glucose and body weight were monitored every second day in the morning until termination of the study. In the second cohort of mice, the islet response to glucose was investigated with intravenous glucose tolerance test (IVGTT) on day 10, 4 hrs after administration of the GPR44 antagonist to the animals. Mice were fasted for 4 hrs before an intravenous D-glucose injection (1 g/kg body weight, Fresenius Kabi, Oslo, Norway) via a tail vein. Blood glucose was measured before (0), and 1, 5, 15, 45 min after glucose administration using glucometer (Accu-Chek Aviva Nano, Roche Diagnostics, IN, USA). To exclude the acute effect of the GPR44 antagonist, the second IVGTT was performed on day 15 without the morning administration of GPR44 antagonist.

Biochemical Measurements

Assessment of human specific C-peptide, proinsulin and insulin in plasma samples were performed using EIA assays (Mercodia, Uppsala, Sweden) according to the manufacturer protocol. Levels of pro-inflammatory cytokines, Tumor Necrosis Factor-α (TNF-α) and (C-XC motif) ligand 1 (CXCL-1/GRO-α) were measured in plasma samples using U-PLEX electrochemiluminescence immunoassay on MESO Quick analyzer (Meso Scale Diagnostics, MD, USA). The protein level of Hepatocytes Growth Factor (HGF), phosphorylated serine/threonine protein kinase B (PKB) AKT and Glycogen synthase kinase 3 beta (GSK3β) were measured in the human islet lysate utilizing multiplex immunoassay with magnetic beads and detected with multiplex analyzer (Biorad, CA, USA) according to the manufacturer instructions.

Immunofluorescent Staining

Tissue sections from paraffin-embedded human islet grafts were stained with guinea pig anti-insulin polyclonal antibody, 1:500 (DAKO, Oslo, Norway). Donkey-anti-guinea pig Alexafluor 594, 1:300 (Life Technologies AS, Oslo, Norway) was used as secondary antibody to detect expression of

insulin followed by nuclear staining using SlowFade Gold antifade reagent with DAPI (Life Technologies AS, Oslo, Norway) in transplanted islets. Slides were scanned and images were taken by slide scanner Axioscan Z1 (Carl Ziess AS, Germany) operated by ZEN lite blue software. Area of insulin-positive cells was analyzed using Image J software (National Institute for Health, USA).

Real-time qPCR

Frozen harvested islet grafts were grinded and total RNA was isolated using Trizol/Chloroform (Qiagen, CA, USA) for phase separation followed by proceeding to the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer guidelines. The concentration of total RNA samples was measured by NanoDrop ND-1000 UV/Vis spectrophotometer (Saveen Werner AB, Sweden). Following cDNA synthesis using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), mRNA expression was analyzed using the following TaqMan primers and probes: human v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A (MAFA) : Hs01651425_s1, human pancreatic and duodenal homeobox 1 (PDX-1) : Hs00426216_m1 with an ABI 7900HT standard Real-Time PCR System (Applied Biosystems, CA, USA). Results were normalized to the housekeeping gene human RPL-30: Hs00265497_m1. All primers were provided from Life Technologies AS, Oslo, Norway.

Western Blot Analysis

Cell lysis buffer (RIPA buffer supplemented with Halt protease inhibitor (Thermo scientific, Oslo, Norway)) was added to human islets before proceeding to mechanical disruption using sonication. Samples were centrifuged and purified using QIAshredder purification column (QIAGEN, Hilden, Germany). Total protein concentration was determined using Pierce BCA protein assay (Life Technologies AS, Oslo, Norway). Equal amount of total protein (20 μg) were analyzed by immunoblotting with primary antibodies phosphorylated FOXO-1 and total FOXO-1 rabbit

polyclonal antibodies (Cell Signaling, MA, USA), PDX-1 rabbit polyclonal antibody (Abcam, Cambridge, UK), and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) goat polyclonal antibody (Santa Cruz Biotechnology, TX, USA), all at the concentration of 1:1000. Bound antibodies were labeled with goat anti-rabbit IgG-HPR and donkey anti-goat IgG-HPR (Santa Cruz Biotechnology, TX, USA) 1:10000. Protein bands were visualized using clarity western ECL chemiluminescence substrate kit (Biorad, CA, USA) or super signal west femto (Thermo scientific, Oslo, Norway) followed by semi-quantitative measurement of band density using chemiDGC touch imaging system, (BioRad, CA, USA).

Statistical Analysis

Data are presented as mean ± SD. Differences among three groups were evaluated by one-way ANOVA with Bonferroni corrections. Mann Whitney U-test was performed for difference analysis between two groups. Significance was set at $p < 0.05$. Data were analyzed using the GraphPad Prism software, version 6.0 (La Jolla, CA, USA).

Results

GPR44 inhibition reduces apoptosis induced by PGD_2 and protects human islet function under diabetic stress conditions.

Dispersed human islets treated with the stable PGD_2 analogue, potently induced apoptosis, where maximal Caspase 3/7 activity was detected at 1 nM PGD_2 (Fig. 1A). Addition of the GPR44 antagonist (10μM) significantly reduced the Caspase 3/7 activity back to the basal level in all three tested concentrations (1, 10, 100 nM) of the PGD_2 analogue (Fig. 1A). Furthermore, treatment with GPR44 antagonist significantly reduced Caspase 3/7 activity, induced by the pro-inflammatory cytokines mix (IL-1β, TNF-α and INFγ) in a dose response manner with the highest concentration of GPR44 antagonist (10μM) as the most potent dose (Fig 1B). Moreover, we showed an increase in cell death, assessed by cell death detection $ELISA^{PLUS}$ assay in isolated human islets treated with a combination of high glucose (HG, 20mM) and IL-1β (10 ng/mL) for 48 hrs (2.30 ± 0.23 vs. 1.39 ± 0.23, $p<0.01$) (Fig. 1C). However, GPR44 antagonist (10μM) significantly reduced the level of cell death induced by HG and IL-1β (1.47 ± 0.10 vs. 2.30 ± 0.23, $p<0.05$) (Fig 1C).

To elucidate the potential functional consequences of GPR44 antagonist on human islets treated with HG and IL-1β, we examined insulin secretion in response to basal (1.67 mM) and stimulated (20 mM) glucose levels and calculated the SI (Fig. 1D). The impaired SI observed in human islet exposed to HG and IL-1β compared to untreated islets (0.95 ± 0.06 vs. 1.7 ± 0.21, $p<0.01$) was reversed by the GPR44 antagonist (1.76 ± 0.21 vs. 0.95 ± 0.06, $p<0.05$).

In vivo GPR44 inhibition improves glucose tolerance in diabetic mice transplanted with marginal mass of human islets.

Diabetic mice transplanted with human islets and treated with HD60 of GPR44 antagonist showed significant improvement in fasting blood glucose measured on day 2 (11.4 ± 2.3 mM vs. 24.1 ± 0.9 mM, n=8, $p<0.01$), day 10 (7.7 ± 1.8 mM vs. 24.6 ± 1.4 mM, $p<0.001$) and day 16 after

transplantation (13.8 ± 3.3 mM vs. 27.6 ± 1.2 mM, $p<0.01$) compared to the vehicle (Fig. 2A). In response to an intravenous glucose challenge examined on day 10, the mice treated with GPR44 antagonist showed a significant improvement in glucose tolerance compared to the vehicle group as shown by AUC quantitative analysis of the glucose excursion curves (594 ± 44 vs. 1387 ± 34 mM*min, $p<0.001$) (Fig. 2B). To exclude the observed acute effect of the GPR44 antagonist on improved glucose response in islets, IVGTT was performed on day 14 post transplantation after 18 hrs washout period of the GPR44 antagonist. The AUC corresponding to the level of blood glucose during the IVGTT showed a significant improvement in mice treated with HD60 of GPR44 antagonist compared to the vehicle (Fig. 2C) (767 ± 109 vs. 1449 ± 16 mM*min, $p<0.01$). The LD6 of GPR44 antagonist had neither effect on fasting blood glucose nor glucose tolerance in the IVGTT (Fig. 2).

***In vivo* GPR44 inhibition preserves transplanted human islet grafts.**

Blood samples taken on day 2 and 16 after transplantation demonstrated a significant improvement in islet function evidenced by an increase in the levels of hC-peptide in mice treated with HD100 of GPR44 antagonist compared to controls (Fig. 3A; Day 2: 378 ± 80 vs. 270 ± 53, Day 16: 803 ± 145 vs. 72 ± 28, $p<0.0001$). In addition, the ratio of hC-peptide/fasting blood glucose was significantly increased in mice treated with GPR44 antagonist compared to the vehicle (141 ± 36 vs. 11 ± 5 $pmol*L^{-1}*mM$, $p<0.001$) (Fig. 3B).

In the second cohort, human C-peptide levels were measured at day 2 and at the termination of the study on day 16. We found a significant increase in hC-peptide levels in mice treated with HD60 of GPR44 antagonist compared to the vehicle (Fig. 3C) calculated as the difference (Δ value) between day 2 and day 16 after transplantation. In addition, the ratio of hC-peptide over fasting blood glucose (Fig. 3D), which has been shown previously to correlate with the beta cell area in human islet (20) was also increased in mice treated with HD60 of the GPR44 antagonist compared to the vehicle on

day 16 after transplantation (8.28 ± 1.85 vs. 2.81 ± 0.22 pmol*L^{-1}*mM, $p<0.05$). The insulin protein levels by means of immunofluorescence insulin staining analyzed after termination of the study on day 16 in the human islet grafts (Fig. 3E), revealed a significant increase in insulin positive area in grafts from mice treated with HD60 of the GPR44 antagonist compared to vehicle (Fig. 3F, 394975 ± 37708 vs. 215900 ± 17658 μm^2, $p<0.0001$).

***In vivo* GPR44 inhibition protects human islets from inflammatory responses.**

We further investigated whether inhibition of GPR44 could induce a beneficial effect on transplanted human islets exposed to diabetic milieu on early phase after transplantation in severely diabetic mice. We found an increased level of plasma hC-peptide measured 4 days after transplantation in mice treated with the HD60 of GPR44 antagonist compared to the vehicle group (781 ± 102 vs. 400 ± 89 pmol*L^{-1}, $p<0.01$) (Fig. 4A). Increased ratio of proinsulin to insulin has been suggested as an early marker for islet dysfunction (21, 22). Herein, we showed a decrease in the ratio of proinsulin to insulin (Fig 4B) in mice treated with HD60 of GPR44 antagonist compared to the vehicle group. Furthermore, the levels of plasma pro-inflammatory cytokine, GRO-α were significantly reduced in GPR44 antagonist treated group (Fig. 4C) and this was accompanied with an increase in protein level of HGF compared to vehicle group (Fig. 4D). Interestingly, we found an increase in the mRNA level of beta cell specific transcription factors, PDX-1 (Fig. 4E) and MAFA (Fig. 4F) in human islet grafts from mice treated with HD60 of GPR44 antagonist compared to the vehicle group.

GPR44 inhibition in isolated human islets up-regulates PDX-1 via phosphorylation of AKT/GSK3β/FOXO-1.

To explore the mechanism involved in the protective effect of GPR44 inhibition in human islets exposed to a diabetic milieu, we assessed the phosphorylation and activation of the AKT survival pathway *in vitro* in isolated human islets treated with a combination of HG (20 mM) and IL-1β (10 ng/ml) with or without the GPR44 antagonist (10 μM) for 48 hrs. We found an approximately 2-fold

reduction in phosphorylation of AKT (Fig. 5A) and GSK3β (Fig. 5B) in human islets treated with HG + IL-1β compared to the vehicle. Administration of the GPR44 antagonist to HG + IL-1β- treated human islets significantly increased the phosphorylation of AKT and GSK3β by 2-fold (Fig. 5A-B). Western blot analysis of islets treated with HG + IL-1β revealed a 2-fold reduction in the phosphorylation of FOXO-1 (Fig. C, D) and PDX-1 (Fig. C, F) compared to the vehicle, whereas inhibition of GPR44 diminished these effects (Fig. C, D, and F). Of note, the protein level of total FOXO-1 stayed unchanged regardless of the treatment in each experimental group (Fig. C, E). These data suggest that GPR44 inhibition improved human islet function at least partly though activation of AKT/GSK3β, phosphorylation and inactivation of FOXO-1 and up-regulation of PDX-1.

Discussion

In the present study, we have demonstrated that specific inhibition of the PGD_2 receptor, GPR44 improves the survival and function of pancreatic human islets exposed to diabetic conditions. This involved a decrease in inflammatory status of the transplanted human islet and preserved beta cell mass, confirmed by using a diabetic mouse model transplanted with marginal mass of human islets and treated with GPR44 antagonist. The effect of inhibiting the PGD_2-GPR44 pathway in diabetic conditions involved an elevation in phosphorylation of AKT/GSK3β, FOXO1 and an increase in the expression of the transcription factors that are critical for beta cell maintenance.

Prostaglandins including PGD_2 are lipid molecules with the ability to regulate pancreatic beta cell function and insulin secretion (7). Hyperglycemia and systemic inflammation, which are the main hallmarks of diabetes, induce production of prostaglandin molecules through up-regulation of COX2 enzyme (7, 23-25). PGD_2 production was found to increase in pancreatic rat islets exposed to high level of glucose *in vitro* (26). Herein, we also found that PGD_2 is as potent as pro-inflammatory cytokines on inducing cell death in pancreatic human islets (27). By blocking the PGD_2 receptor, GPR44, using a specific GPR44 antagonist, the functionality and the survival of human islets were improved through preservation of the transplanted human islets in the diabetic environment.

PGE_2 and its G-coupled protein receptors have been studied with the greatest detail compared to the other members of prostaglandin family (7). *In vitro* studies in beta cell lines treated with PGE_2 demonstrated a reduction in insulin secretion in response to glucose (28). The *in vitro* data regarding the effect of PGE_2 on islets have been mirrored in the *in vivo* setting. Intravenous administration of PGE_2 to animal models or over-expression of PGE_2 using a transgenic mouse model decreased insulin secretion in response to glucose and induced hyperglycemic status in animal models (29-31). PGE_2 signals through four types of EP receptors (EP1-4), however EP3 has been found as the only PGE_2

receptor that couples to the inhibitory G-protein. PGE_2-EP3 reduced insulin secretion in response to glucose through a reduction in the intracellular cAMP (32, 33). Inhibiting EP3 receptor using EP3 receptor antagonist improved insulin secretion in response to glucose in MIN cell line and rat islets (34). EP3 inhibition could also enhance insulin secretion in human islets isolated from donors with type 2 diabetes (33). The PGD_2 receptor, DP1 was found to couple with the stimulatory G-protein leading to an increase in the level of intracellular cAMP and insulin secretion, whereas GPR44 couples to the inhibitory G-protein and decreases cAMP and insulin secretion (10). We showed that the administration of a GPR44 antagonist to Alloxan-induced diabetic mouse model transplanted with a marginal dose of human islets, improved the transplanted islet response to glucose and enhanced the plasma level of hC-peptide secreted by transplanted islet. Therefore, one possible hypothesis is that PGD_2 signals through the GPR44 receptor and PGD_2-GPR44 axis could not only impair insulin secretion due to the reduced level of cAMP, but also induce apoptotic signals that would damage the human islets. We confirm here that the *in vitro* incubation of human islets with PGD_2 resulted in an elevated level of apoptosis, to a similar degree as cytokines, and this effect could be blocked by addition of a GPR44 antagonist.

The ratio of hC-peptide to fasting blood glucose has been found recently to correlate with beta cell area in human islets (20) and we showed that the inhibition of GPR44 on transplanted human islets improved this ratio. In addition, insulin positive area evidenced by immunofluorescent labeling of transplanted human islet grafts for insulin (35) could further suggest a beneficial role of GPR44 inhibition in preserving human islet under diabetic conditions.

It is well known that pro-inflammatory cytokines are involved in pancreatic islet injury and islet graft destruction after transplantation (25). In particular, elevated level of the pro-inflammatory cytokines, IL-1β, TNF-α and INF-γ observed after islet transplantation and could mediate islet injury in recently transplanted islets (36). In addition, the production of the pro-inflammatory cytokines, GRO-α

together with IL-8 and IL-6 were found to increase 6 hrs after human islet transplantation in a whole blood model simulating allo-islet transplantation (37). This was followed by an increase in the level of INF-γ as well as neutrophil infiltration that could initiate graft loss around 48 hrs post transplantation (37). In our study, we found reduced plasma level of the human pro-inflammatory cytokines, GRO-α in mice treated with GPR44 antagonist, which could support an anti-inflammatory effect of GPR44 inhibition on human islets exposed to diabetic micro-environment. Moreover, we also observed a reduction in the ratio of proinsulin to insulin in animals treated with the GPR44 antagonist. Elevated secretion of proinsulin could be due to the presence of stress micro-environment and failing insulin synthesis (38, 39). Therefore, the effect of GPR44 inhibition on preserving transplanted human islets in diabetic animals could be as a result of the reduction in inflammatory reactions and the stress micro-environment within human islets.

To mimic the stress diabetic micro-environment induced by hyperglycemia and pro-inflammatory molecules and to explore the effect of GPR44 inhibition in this condition, isolated human islets were exposed to high glucose in combination with the pro-inflammatory cytokine, IL-1β. Herein, the inhibition of GPR44 receptor revealed an improvement in the function and survival of the human islets exposed to the diabetic milieu. IL-1β production is triggered by IL-1β itself and also by high concentration of glucose within islets in diabetic milieu (40). It is well established that the effect of IL-1β on islet function and insulin secretion is concentration-dependent, as islet culture with high concentration of IL-1β (5-20 ng/ml) for long period of time (> 24 hrs) has been shown to induce islet loss through metabolic and endoplasmic reticulum stress (41, 42).

There is lack of knowledge regarding the molecular mechanisms involved in the effect of PGD_2-GPR44 axis on pancreatic islet mass and survival. However, PGE_2 has been found to decrease DNA synthesis and islet survival in rat islets (43). This was suggested to be mediated through PGE_2

coupling to the EP3 receptor, leading to dephosphorylation of FOXO-1 either via activation of c-Jun N-Terminal Kinase (JNK1) or inhibition of phosphatidylinositol 3-Kinase (PI3K)/AKT survival signaling pathway (44, 45). This resulted in an increase in translocation of FOXO-1 to nucleus, where it could participate in nuclear exclusion of critical beta cell transcription factors, PDX-1 and MAFA (44, 46). MAFA and PDX-1 have been previously suggested not only as the key activators for insulin synthesis, but also master regulators of the genes implicated in the maintenance of beta cell function (47-49). Here, we found not only an increase in the gene expression of MAFA in transplanted human islets exposed to the GPR44 antagonist 4 days after transplantation, but we also observed an up-regulation in protein expression of HGF. HGF is known to improve islet survival and insulin secretion through mainly activation of PI3K/AKT signaling pathway (50). Secretion of HGF has been shown to increase in human islets exposed to the pro-inflammatory cytokines *in vitro*, suggesting a potential role of HGF in islet health and survival (51). Adenoviral delivery of HGF to non-human primate islets demonstrated a marked improvement in transplanted islet function and mass (52). This therefore, could support the protective effect of HGF on islets post transplantation. In the present study, we demonstrated that the inhibition of GPR44 on human islets could mediate the up-regulation of PDX-1, increase in phosphorylation of FOXO-1 and activation of AKT/GSK3β signaling pathway. Having in mind the essential effect of PDX-1 and MAFA on proper insulin secretion and maintenance of pancreatic beta cells, phosphorylation and deactivation of FOXO-1 accompanied with the activation of AKT and GSK3β upon GPR44 antagonist treatment could at least partly be involved in an up-regulation of PDX-1 and MAFA and consequently the improvement in human islet function and preservation of the transplanted graft mass post transplantation.

A recent investigation on GPR44 receptor as an imaging biomarker for visualization of beta cell mass has revealed the high selectivity of GPR44 antagonist for pancreatic beta cells. Radiolabeled GPR44 antagonist was shown to bind to insulin-positive islets in pancreatic sections obtained from healthy

individuals and patients with type 2 diabetes but not the insulin-negative islets in type 1 diabetic patients (3). Herein, our data support that the inhibition of GPR44 could be beneficial for human islet function with the possible implication to rescue human islets under diabetic conditions.

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Author Contribution

S.A, S.W.S, M.S-W and H.S did the research and analyzed the data. S.A, M.S-W and H.S wrote the manuscript and are the guarantor of this work, and as such have full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. O.K, H.S performed human islet isolation. S.A, H.S, T.R-B, D.M.S, O.K, B.T, S.S contributed to discussion and reviewed and edit the manuscript.

Prior Presentation

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Figure 1: Inhibition of GPR44 results in reduced apoptosis and improved function in human islets. (A) Caspase 3/7 activity in human islets treated with 15-methyl-15- PGD_2 (doses 1, 10 and 100 nM) with or without the GPR44 antag (10μM) for 24 hrs, n=4. (B) Caspase 3/7 activity in human islets treated with pro-inflammatory cytokines mix (IL-1β, TNF-α, and INF-γ) with or without the GPR44 antag in a dose dependent manner (doses 1, 3 and 10 μM) for 24 hrs, n=4. (C) Apoptosis evaluated in cytoplasmic fraction of isolated human islets treated with either GPR44 antag (10 μM) or HG (20 mM) + IL-1β (10 ng/ml) with or without the GPR44 antag (10 μM) for 48 hrs, n=7. (D) Insulin secretion in response to basal (1.67 mM) and stimulated (20 mM) levels of glucose was measured by EIA and calculated as stimulation index (SI) for human islets treated with either GPR44 antag (10μM) or HG (20mM) + IL-1β (10 ng/ml) with or without GPR44 antag (10μM) for 48 hrs, n=7. In all analysis, data is presented as mean ± SD and analyzed by one-way ANOVA with Bonferroni corrections. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. vehicle islets, * $p<0.05$ vs. HG + IL-1β-treated islets. GPR44 antag: GPR44 antagonist, PCM: pro-inflammatory cytokine mix; HG, high glucose; n, number of independent donors.

Figure 2: GPR44 inhibition enhances glucose tolerance of human islets transplanted in diabetic mice. (A) Fasting B-glu was measured on day 2, 10 and 16 in mice transplanted with human islets and treated with HD60 or LD6 of GPR44 antag or vehicle. B-glu levels during IVGTT and the corresponding AUC represents the difference in the level of B-glu in mice transplanted with human islets and treated with HD60 or LD6 of GPR44 antag or vehicle on day 10 after morning administration of GPR44 antag (B) and on day 14 without morning administration of GPR44 antag (C). Data is presented as mean ± SD. The differences in fasting B-glu and in AUC corresponding to the glucose levels during IVGTT were analyzed with one-way ANOVA with Bonferroni corrections. Differences in the level of B-glu were analyzed by IVGTT and calculated using multiple t-tests n=8 mice/each experimental group, ** $p<0.01$, *** $p<0.001$ vs. vehicle group. GPR44 antag: GPR44

antagonist, B-glu: Blood glucose, HD60: High dose 60 mg/kg/day, LD6: Low dose 6 mg/kg/day, IVGTT: intravenous glucose tolerance test, AUC: area under curve, POD: post operative day.

Figure 3: GPR44 inhibition improves transplanted human islet function and mass post transplantation. (A) Analysis of hC-peptide levels measured on day 2 and day 16 in mice transplanted with human islets and treated with HD100 GPR44 antag or vehicle. (B) The ratio of hC-peptide over fasting B-glu analyzed on day 16 in mice transplanted with human islet grafts and treated with HD100 of GPR44 antag or vehicle. (C) Assessment of difference (Δ value) in the level of plasma hC-peptide measured on day 16 and day 2 in mice transplanted with human islets and treated with LD6 or HD60 of GPR44 antag or vehicle. (D) The ratio of hC-peptide over fasting B-glu analyzed on day 16 in mice transplanted with human islet grafts and treated with LD6 and HD60 of GPR44 antag or vehicle. (E) Representative images showing immunofluorescent staining of insulin (red) together with DAPI-nuclear staining (blue) on day 16 and (F) quantification of the area of insulin-positive cells within transplanted islet grafts treated with LD6 and HD60 of GPR44 antag or vehicle. Magnification 10x with scale bar 200μm. In all analysis, data is presented as mean±SD and analyzed with one-way ANOVA with Bonferroni corrections. n=8 mice/each experimental group. * $p<0.05$, *** $p<0.001$, **** $p<0.0001$ vs. vehicle group. LD6: low dose 6 mg/kg/day, HD60: high dose 60 mg/kg/day, HD100: high dose 100 mg/kg/day, GPR44 antag: GPR44 antagonist, hC-peptide: human C-peptide, B-glu: blood glucose, POD: post operative day.

Figure 4: GPR44 inhibition improves islet function and ameliorates secretion of pro-inflammatory cytokines on early phase post transplantation. (A) Assessment of hC-peptide, (B) the ratio of human proinsulin over insulin and (C) human pro-inflammatory cytokines TNF-α and GRO-α measured on day 4 in plasma samples of mice transplanted with human islets and treated with HD60 of GPR44 antag or vehicle. (D) Assessment of HGF protein level and (E) mRNA level of PDX-1 and (F) MAFA in transplanted human islet lysates treated with HD60 of GPR44 antag or

vehicle on day4 after transplantation. n=14 mice/each experimental group. In all analysis, data is presented as mean±SD and analyzed with Mann Whitney U-test. * $p<0.05$, ** $p<0.01$ vs. vehicle group. hC-peptide: human C-peptide, HD60: high dose 60 mg/kg/day, GPR44 antag: GPR44 antagonist.

Figure 5: GPR44 inhibition increases protein level of PDX-1 via AKT/GSK3B/FOXO-1 signaling pathway in isolated human islets. Analyzing the Phosphorylation of (A) AKT and (B) GSK3β in human islets treated with either GPR44 antag (10μM) alone or HG (20mM) + IL-1β (10 ng/ml) with or without GPR44 antag (10μM) for 48 hrs. (C) Western blot analysis of phosphorylated FOXO-1, PDX-1, total FOXO-1 in human islets treated with either GPR44 antag (10μM) alone or HG (20mM) + IL-1β (10ng/ml) with or without GPR44 antag (10μM) for 48 hrs. Quantification of p-FOXO-1 (D), total FOXO-1 (E) and PDX-1 (F) band densities normalized to GAPDH and presented as fold of vehicle islets. For all analysis data is analyzed with one-way ANOVA with Bonferroni corrections and presented as mean ± SD, n=7. * $p<0.05$ vs. vehicle. * $p<0.5$, ** $p<0.01$ vs HG+IL-1β-treated human islets. HG, high glucose; GPR44 antag: GPR44 antagonist, n, number of independent donors.

Figure 1

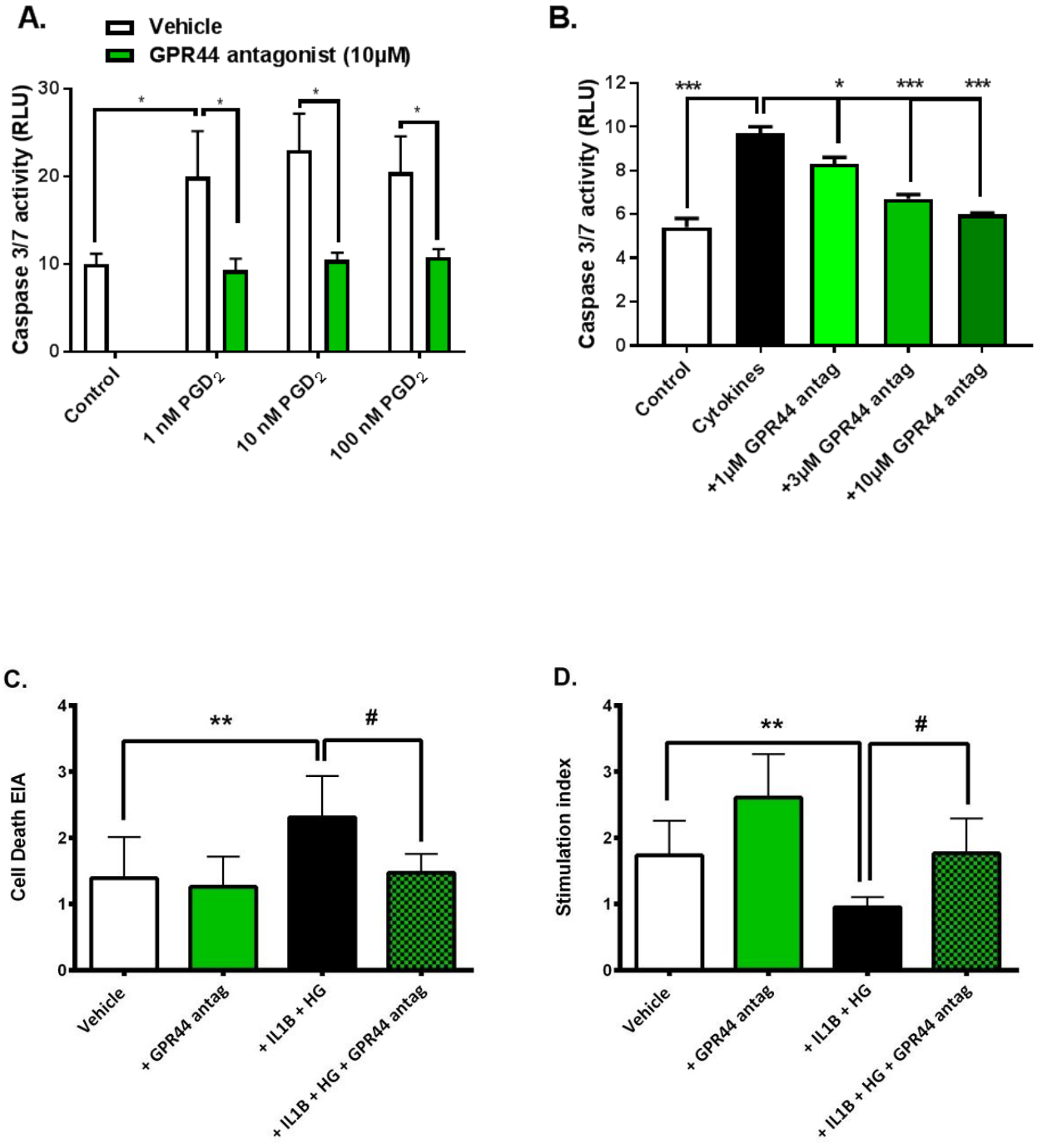

A.
Vehicle
GPR44 antagonist (10μM)
Caspase 3/7 activity (RLU)
Control
1 nM PGD2
10 nM PGD2
100 nM PGD2
B.
Caspase 3/7 activity (RLU)
Control
Cytokines
+1μM GPR44 antag
+3μM GPR44 antag
+10μM GPR44 antag
C.
Cell Death EIA
Vehicle
+ GPR44 antag
+ IL1B + HG
+ IL1B + HG + GPR44 antag
D.
Stimulation index
Vehicle
+ GPR44 antag
+ IL1B + HG
+ IL1B + HG + GPR44 antag

Figure 2

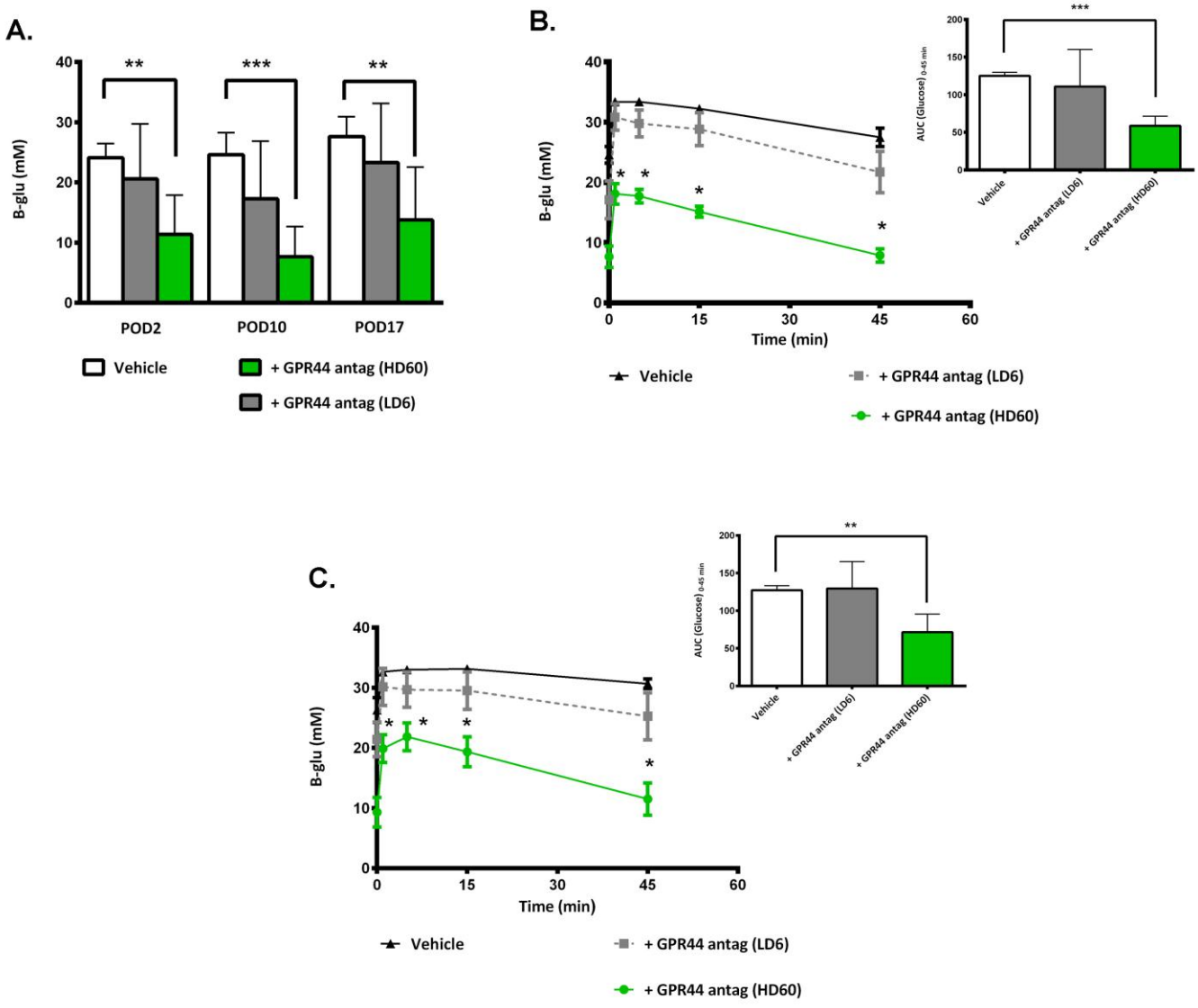

A.
B-glu (mM)
POD2
POD10
POD17
Vehicle
+ GPR44 antag (HD60)
+ GPR44 antag (LD6)
B.
B-glu (mM)
Time (min)
Vehicle
+ GPR44 antag (LD6)
+ GPR44 antag (HD60)
AUC (Glucose) 0-45 min
C.
B-glu (mM)
Time (min)
Vehicle
+ GPR44 antag (LD6)
+ GPR44 antag (HD60)
AUC (Glucose) 0-45 min

Figure 3

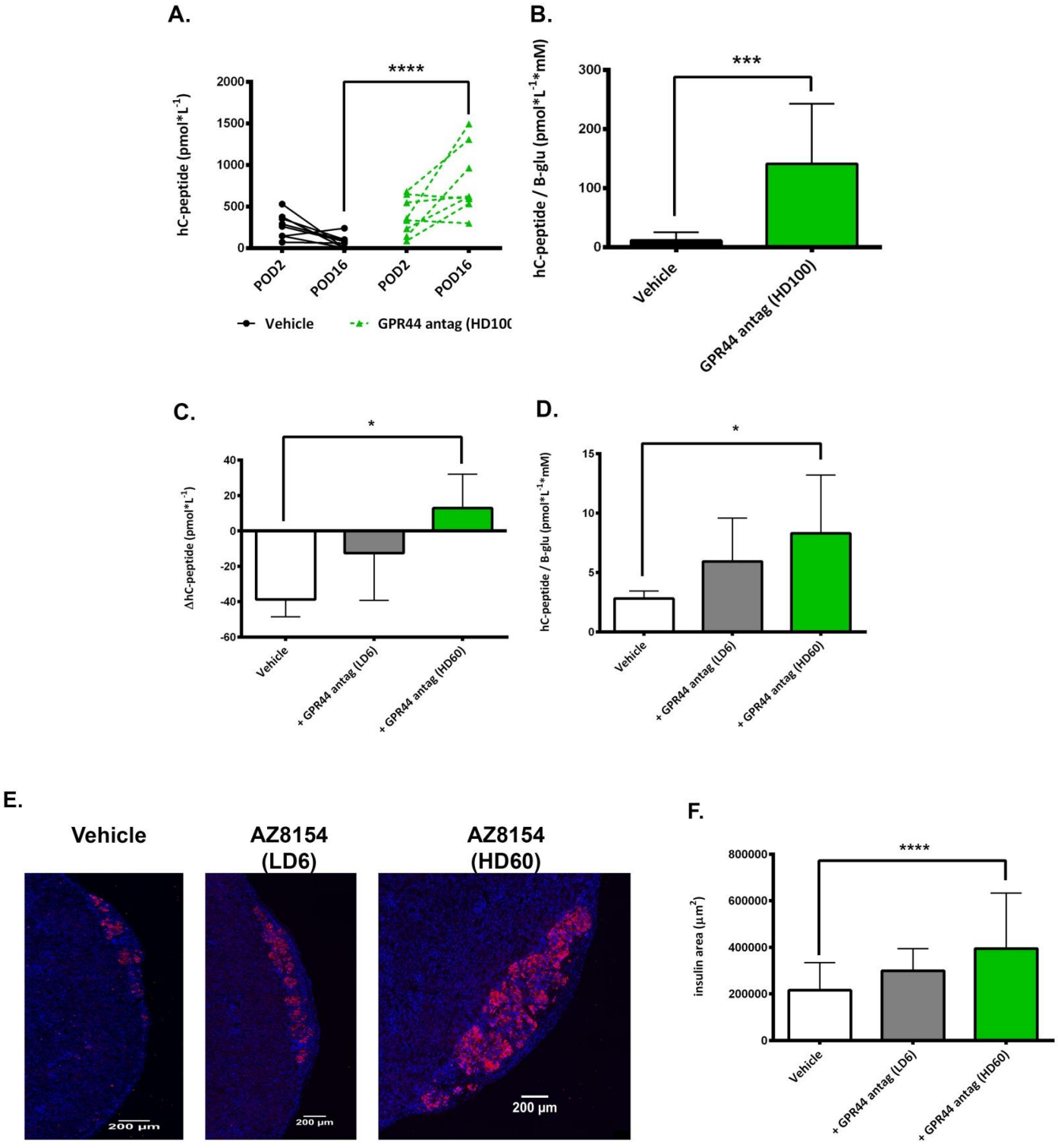

A.
hC-peptide (pmol*L-1)
2000
1500
1000
500
0
****
POD2
POD16
POD2
POD16
Vehicle
GPR44 antag (HD10(
B.
hC-peptide / B-glu (pmol*L-1*mM)
300
200
100
0
***
Vehicle
GPR44 antag (HD100)
C.
ΔhC-peptide (pmol*L-1)
40
20
0
-20
-40
-60
*
Vehicle
+ GPR44 antag (LD6)
+ GPR44 antag (HD60)
D.
hC-peptide / B-glu (pmol*L-1*mM)
15
10
5
0
*
Vehicle
+ GPR44 antag (LD6)
+ GPR44 antag (HD60)
E.
Vehicle
AZ8154 (LD6)
AZ8154 (HD60)
200 µm
200 µm
200 µm
F.
insulin area (µm2)
800000
600000
400000
200000
0
****
Vehicle
+ GPR44 antag (LD6)
+ GPR44 antag (HD60)

Figure 4

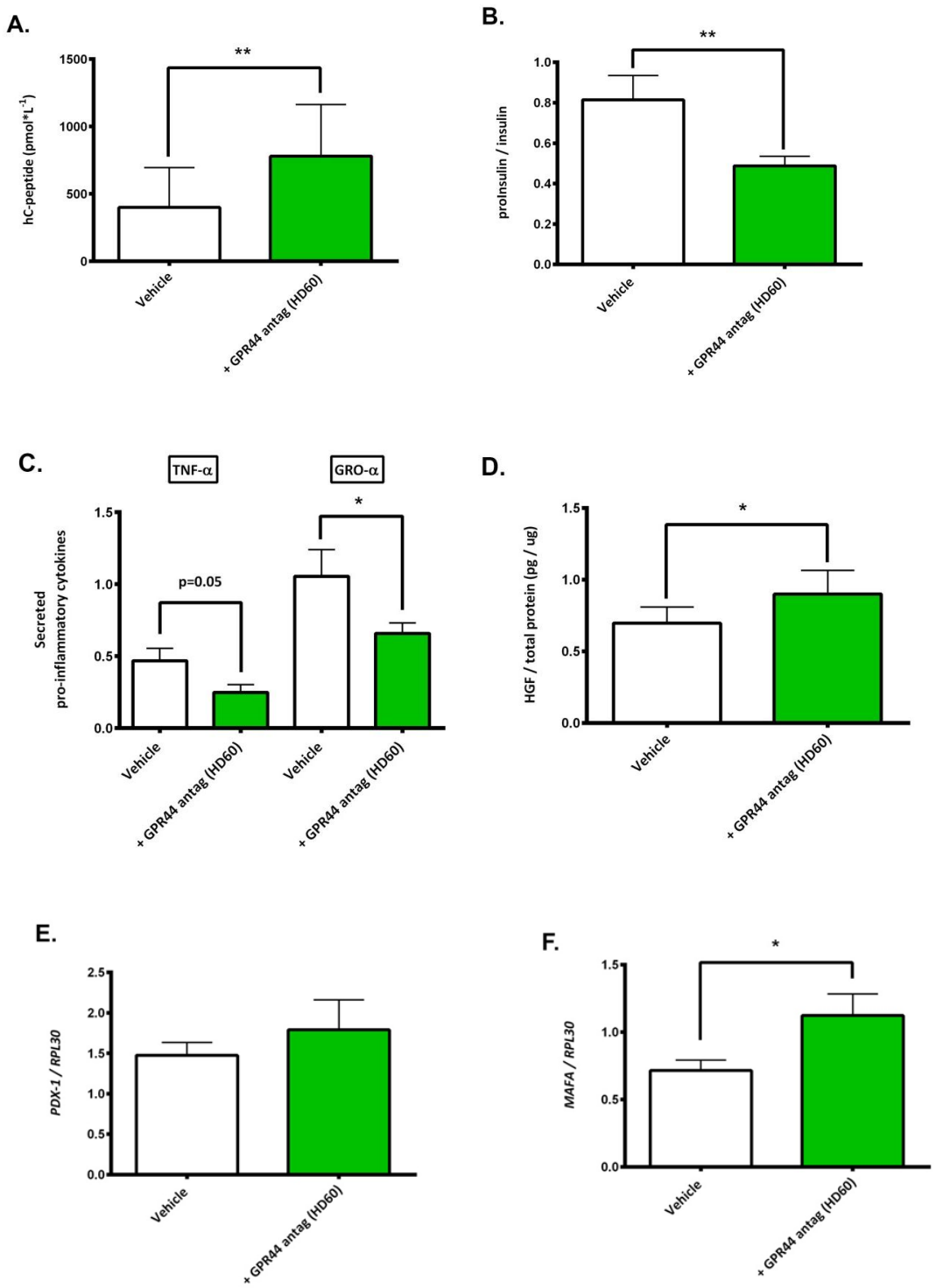
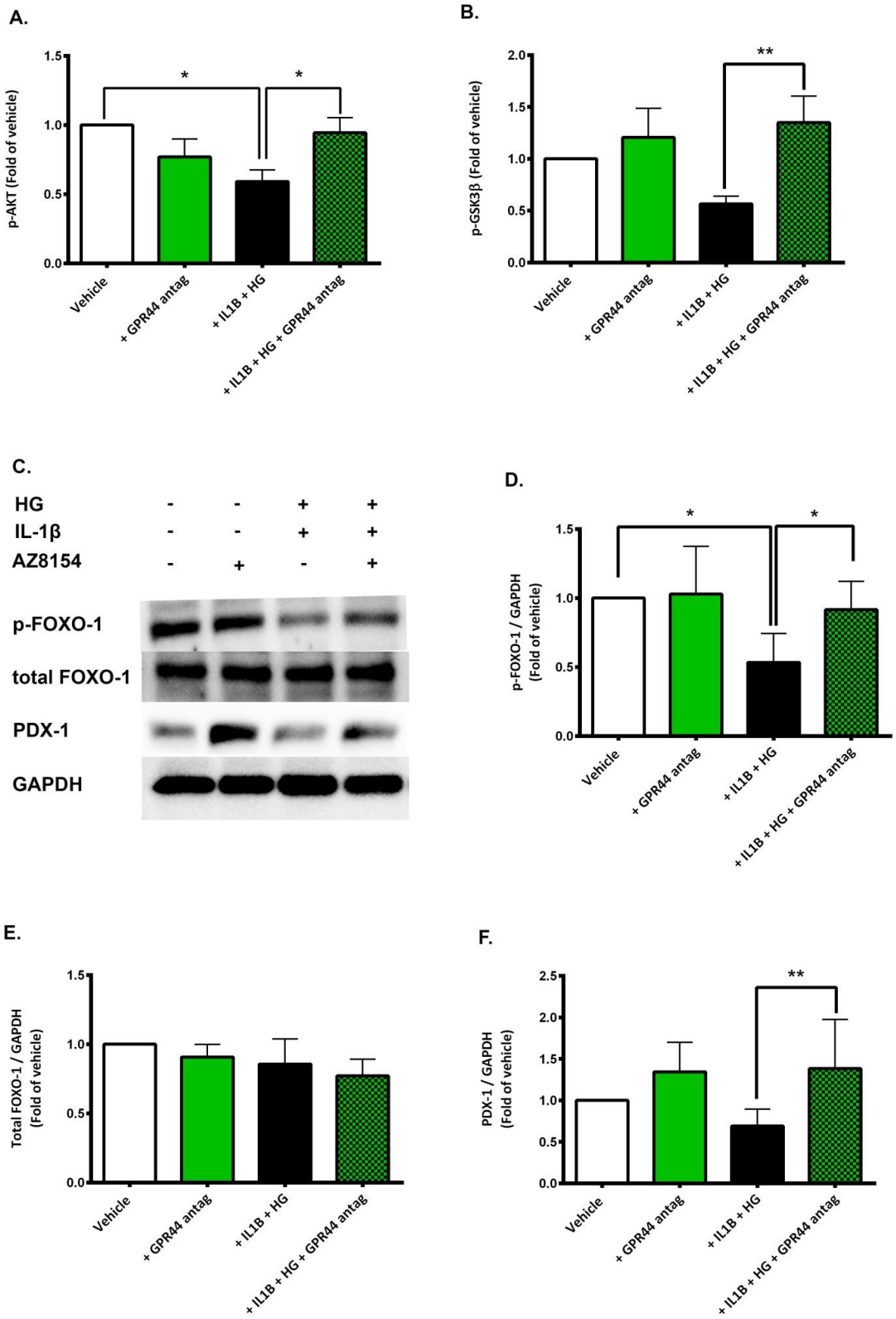

A.
hC-peptide (pmol*L-1)
1500
1000
500
0
**
Vehicle
+ GPR44 antag (HD60)
B.
proInsulin / insulin
1.0
0.8
0.6
0.4
0.2
0.0
**
Vehicle
+ GPR44 antag (HD60)
C.
TNF-α
GRO-α
Secreted pro-inflammatory cytokines
1.5
1.0
0.5
0.0
p=0.05
*
Vehicle
+ GPR44 antag (HD60)
Vehicle
+ GPR44 antag (HD60)
D.
HGF / total protein (pg / ug)
1.5
1.0
0.5
0.0
*
Vehicle
+ GPR44 antag (HD60)
E.
PDX-1 / RPL30
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
+ GPR44 antag (HD60)
F.
MAFA / RPL30
1.5
1.0
0.5
0.0
*
Vehicle
+ GPR44 antag (HD60)

Figure 5


A.
p-AKT (Fold of vehicle)
1.5
1.0
0.5
0.0
*
*
Vehicle
+ GPR44 antag
+ IL1B + HG
+ IL1B + HG + GPR44 antag
B.
p-GSK3β (Fold of vehicle)
2.0
1.5
1.0
0.5
0.0
**
Vehicle
+ GPR44 antag
+ IL1B + HG
+ IL1B + HG + GPR44 antag
C.
HG - - + +
IL-1β - - + +
AZ8154 - + - +
p-FOXO-1
total FOXO-1
PDX-1
GAPDH
D.
p-FOXO-1 / GAPDH (Fold of vehicle)
1.5
1.0
0.5
0.0
*
*
Vehicle
+ GPR44 antag
+ IL1B + HG
+ IL1B + HG + GPR44 antag
E.
Total FOXO-1 / GAPDH (Fold of vehicle)
1.5
1.0
0.5
0.0
Vehicle
+ GPR44 antag
+ IL1B + HG
+ IL1B + HG + GPR44 antag
F.
PDX-1 / GAPDH (Fold of vehicle)
2.5
2.0
1.5
1.0
0.5
0.0
**
Vehicle
+ GPR44 antag
+ IL1B + HG
+ IL1B + HG + GPR44 antag

Online Supplemental Materials

Insulin secretion assay with EndoC-βH1 cells

Human EndoC-βH1 cells were plated and pre-treated as described above for the cAMP measurements. Glucose-stimulated insulin secretion (GSIS) experiments were performed for 1 hr at 37 °C, 5 % CO_2 by incubating cells in KREBS buffer containing 0.2 % BSA, 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX, a phosphodiesterase inhibitor) and either 2.8 or 11.1 mM glucose, in the presence of serial dilutions of GPR44 antagonist (AZ8154 and AZD1981) at the concentration range of $3.81x10^{-11}$ M to 2.5 $x10^{-6}$ M, with or without EC_{80} (150 pM) of the GPR44 agonist 15(R)-15-methyl Prostaglandin D_2. At the end of the incubation period, cell-culture supernatants were collected and insulin levels determined by an HTRF®-based insulin assay from Cisbio Bioassays and read on a Paradigm plate reader (Molecular Devices) at 665 and 620 nm. Insulin standard curve was prepared according to the Cisbio protocol.

In other experiments, EndoC-βH1 cells were incubated with or without 150 pM 15(R)-15-methyl-PGD_2 and 11.1 mM glucose. The effect of the GPR44 antagonist (AZ8154 and AZD1981) on insulin secretion was determined at high glucose with 150 pM 15(R)-15-methyl- PGD_2. After 30 min incubation, insulin secretion was measured as described above.

cAMP measurements assay with EndoC-βH1 cells

Human EndoC-βH1 cells were plated in fibronectin- and extracellular matrix-coated 96-well plates at a density of $5x10^4$ cells per well and cultured for 2 days at 37 °C, 5 % CO_2. The maintenance medium was then replaced with low glucose (2.8 mM) medium and cells were cultured for another 16 hrs. Before stimulation of cAMP production, a 1 hr pre-incubation in KREBS buffer with 0.2 % BSA and 0.5 mM glucose was performed at 37° C, 5 % CO_2. Cyclic AMP experiments were then performed

for 30 minutes at 37 °C, 5 % CO_2 by incubating cells in KREBS buffer containing 0.2 % BSA, 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX, a phosphodiesterase inhibitor) and either 2.8 or 11.1mM glucose, in the presence of serial dilutions of the GPR44 antagonist (AZ8154 and AZD1981) at a concentration range of $3.81x10^{-11}$M to 2.5 $x10^{-6}$M, with or without EC_{80} (150 pM) of the GPR44 agonist 15(R)-15-methyl Prostaglandin D_2. At the end of the incubation period, cells were lysed and cAMP levels in the cell lysate was measured using an HTRF®-based cAMP assay from Cisbio Bioassays and read on a Paradigm plate reader (Molecular Devices) at 665 and 620 nm. cAMP standard curve was prepared according to the Cisbio protocol.

Supplementary figures

Supplementary figure 1: Both GPR44 antagonists, AZ8154 and AZD1981 were equally potent in blocking PGD_2 effects in human beta cells

(A) High glucose (11.1 mM) stimulated insulin secretion in the human beta cells, EndoC-βH, about 2-fold and this effect that was blocked by addition of 150 pM 15(R)-15-methyl- PGD_2. Addition of 100 nM AZD1981 or 100 nM AZ8154 completely restored the PGD_2 blunted GSIS response. (B) The same effects were observed when measuring the cAMP signal, with a significant inhibition of cAMP production by PGD_2 and complete restoration to stimulated levels by both AZD1981 and AZ8154.

The dose response curves for the two GPR44 antagonists were compared and the IC_{50} values were found to be similar between the two compounds with similar effects on GSIS (C) or cAMP production (D). The potencies of the compounds were measured multiple times and the results are summarized in Supplementary Table 1. In conclusion, both GPR44 antagonists demonstrated similar potencies in restoring PGD_2-induced inhibition of both cAMP production and GSIS on human beta cells.

Supplementary Table 1: Potency of AZ8154 and AZD1981 in inhibition of cellular responses mediated by GPR44 in human EndoC-βH1 cells

Assay readout	**IC_{50} AZ8154 (mean±SEM)**	**IC_{50} AZD1981 (mean±SEM)**
cAMP	16±2.1 (n=4)	21±8 (n=2)
Insulin secretion	21±5 (n=3)	24±11 (n=3)

Supplementary figures 1