Regulation of Glucose Metabolism via the Intra-Islet DPP4/Incretin Axis

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Abstract

Glycemic control in patients with type 2 diabetes (T2D) can be achieved through potentiation of the signalling by glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). Both incretin hormones have been traditionally characterized to be secreted by distinct enteroendocrine cells within the gut in response to nutrients. Signalling through the incretin receptors stimulates islet hormone release by potentiating glucose-stimulated insulin secretion from the β-cell and decreasing glucagon secretion from the α-cell. However, the bioactivity of GLP-1 and GIP is controlled by post-translational, N-terminal cleavage by the widely expressed serine protease dipeptidyl peptidase 4 (DPP4). As such, DPP4 inhibitors (DPP4i) have been successfully used to treat millions of patients with T2D. DPP4i target the catalytic active site of DPP4 and prevent the cleavage of the incretin hormones, thus prolonging their action.

Recently, studies in genetically modified mice have demonstrated that GLP-1 is not solely an intestinally-derived peptide hormone and proposed that islet-derived GLP-1 is required for proper glucose homeostasis. Therefore, with the current study, we sought to assess whether β-cell-derived DPP4 is an important target for the regulation of glycemia. Treatment of *Glp1r/Gipr*$^{\beta\text{-cell-/-}}$ mice with the DPP4 inhibitor sitagliptin demonstrated that β-cell incretin receptor signaling is required to mediate the beneficial effects of this class of drugs on glucose homeostasis. Additionally, *Dpp4*$^{-/-}$ mice exhibited a significant reduction in hepatic glucose production during hyperinsulinemic-euglycemic clamps. *Dpp4* mRNA, DPP4 protein and activity are present in isolated mouse islets, further supporting the islet as an important potential site of DPP4i action. In this study, we show that both DPP4i-treated wildtype islets and islets isolated from *Dpp4*$^{\beta\text{-cell-/-}}$ mice exhibit increased glucose-stimulated insulin secretion (GSIS) during perifusion after a high-fat diet feeding. Genetic elimination of *Dpp4* from islet β-cells also improved oral glucose tolerance and insulin sensitivity in female mice, but had no effects on circulating DPP4 or incretin levels. Finally, eliminating *Dpp4* from β-cells or the whole pancreas did not improve whole-body glucose tolerance, response to DPP4i, insulin tolerance, or body weight in male mice fed chow or a high-fat diet. Therefore, we provide evidence for islet-derived DPP4 to have a role in local hormone responses to glucose; however, its role in systemic glucose metabolism is shown to be sex-dependent.

Co-Authorship Statement

The execution of experiments, analysis, presentation of data, and preparation of this thesis was done by me, with the following exceptions:

Dr. Erin E. Mulvihill provided intellectual input and scientific expertise in the creation of the experimental design, hypothesis, and experiment execution.

University of Ottawa Heart Institute Animal Care Veterinary Services (ACVS) provided care for all animals used during the completion of this thesis.

Richard Seymour (ACVS) performed the pancreas inflation with collagenase required for islet isolation.

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Dr. Ilka Lorenzen-Schmidt assisted with breeding mouse colonies and genotyping mice.

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AAV	Adeno-Associated Virus
ADA	Adenosine Deaminase
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
AS160	Akt Substrate of 160kDa
ATP	Adenosine Triphosphate
BAD	BCL2-Associated Agonist of Cell Death
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
cAMP	Cyclic AMP; Cyclic Adenosine Monophosphate
CD4	Cluster of Differentiation 4
CNS	Central Nervous System
CPE	Carboxypeptidase E
DAPI	4′,6-diamidino-2-phenylindole
DASH	DPP4 Activity and/or Structure Homologue
DIRKO	Double Incretin Receptor Knockout
DNA	Deoxyribonucleic Acid
DPP4	Dipeptidyl Peptidase-4
DPP4i	Dipeptidyl Peptidase-4 Inhibitor
EC	Endothelial Cell
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
ER	Endoplasmic Reticulum
FAP	Fibroblast Activation Protein
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FFA	Free Fatty Acid
FOXO1	Forkhead Box O1
GABA	Gamma-Aminobutyric Acid
GCGR	Glucagon Receptor
GCSF	Granulocyte Colony-Stimulating Factor
GH	Growth Hormone
GH	Growth Hormone
GHIH	Growth Hormone-Inhibiting Hormone
GIP	Gastric Inhibitory Polypeptide

GIPR	Gastric Inhibitory Polypeptide Receptor
GLP-1	Glucagon-Like Peptide-1
GLP-1R	Glucagon-Like Peptide-1 Receptor
GLP-1RA	Glucagon-Like Peptide-1 Receptor Agonist
GLUT	Glucose Transporter
GRPP	Glicentin-Related Pancreatic Peptide
GSIS	Glucose-Stimulated Insulin Secretion
GSK3	Glycogen Synthase Kinase 3
HBSS	Hanks' Balanced Salt Solution
HFD	High-Fat Diet
i.p.	Intraperitoneal
IGF-1	Insulin-Like Growth Factor-1
IGF-2	Insulin-Like Growth Factor-2
IP2	Intervening Peptide-2
ipGTT	Intraperitoneal Glucose Tolerance Test
IR	Insulin Receptor
IRS	Insulin Receptor Substrate
IRS-2	Insulin Receptor Substrate-2
ITT	Insulin Tolerance Test
KRBH	Krebs-Ringer bicarbonate-HEPES buffer
MAPK	Mitogen Activated Protein Kinase
MIP1α	Macrophage Inflammatory Protein 1α
MMP	Metalloprotease
mRNA	Messenger RNA
mTOR	Mammalian Target of Rapamycin
NPY	Neuropeptide Y
oGTT	Oral Glucose Tolerance Test
PBS	Phosphate Buffered Saline
PC	Prohormone Convertase
PDGF	Platelet-Derived Growth Factor
PDK	3-Phosphoinositide Dependent Protein Kinase
PI3K	Phosphoinositide 3-Kinase
PIP3	Phosphatidylinositol-3,4,5-Triphosphate
PKB	Protein Kinase B
PP	Pancreatic Polypeptide
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
PYY	Peptide Tyrosine Tyrosine

RER	Rough Endoplasmic Reticulum
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RRP	Readily-Releasable Pool
SEM	Standard Error of the Mean
SGLT2	Sodium-Dependent Glucose Transporter-2
SHC	Src Homology 2 Domain-Containing
SNARE	SNAp Receptor; Soluble N-Ethylmaleimide–Sensitive Factor Attachment Protein Receptor
SNP	Single-Nucleotide Polymorphism
SST	Somatostatin
SSTR	Somatostatin Receptor
SU	Sulfonylurea
T2D	Type II Diabetes
TCA	Tricarboxylic Acid
TZD	Thiazolidinedione
UDPG	Uridine Diphosphate Glucose
VEGF	Vascular Endothelial Growth Factor
VGCC	Voltage-Gated Ca2+ Channel

1. Introduction

1.1 Diabetes

1.1.1 Global Burden of Disease

Diabetes prevalence continues to increase globally, affecting an estimated 7% of people and representing a heavy burden on the healthcare system worldwide (Hu, 2011; Khan et al., 2020). Diabetes is a chronic condition that impairs the body's ability to make insulin or to respond to the insulin secreted in response to food intake effectively. Type 2 diabetes (T2D) specifically is characterized by decreased insulin release and/or insulin resistance, decreased glucose uptake by the peripheral tissues, and elevated hepatic glucose production, all leading to persistent high blood sugar, or hyperglycemia (Bogardus et al., 1984; Samuel and Shulman, 2012). Over time, it can lead to significant microvascular complications affecting the function of the eyes, nerves, and kidneys. Furthermore, patients with T2D are at a significantly increased risk of developing coronary heart disease, ischaemic stroke, and many other cardiovascular complications (Sarwar et al., 2010). The risk of cardiovascular death in patients with T2D is so significant that the FDA mandated all new therapies undergo rigorous cardiovascular outcome trial. The continued rise in diabetes has been attributed to a complex interplay between a variety of factors, including growing urbanization, changing lifestyle habits, genetics, socioeconomic status, and the environment (Ali, 2013; Brown et al., 2004; Popkin and Gordon-Larsen, 2004; Prasad and Groop, 2015). As we continue to learn about the risks and possible causes of T2D, further understanding of the underlying mechanisms is essential for the development of effective treatments.

1.1.2 Physiological Regulation of Glucose Homeostasis

In healthy individuals, plasma glucose levels are maintained within a narrow range through a system of islet-derived hormones and neural pathways, that in turn regulate glucose transport proteins, glucose absorption, endogenous glucose production, and glucose uptake and release (Schwartz and Porte, 2005; Schwartz et al., 2013). Two major glucoregulatory hormones secreted by the endocrine pancreas are insulin and glucagon. Glucagon is released from the islet α-cell during the fasting state, and it works to increase low blood sugar. Glucagon binds to the glucagon receptor, a transmembrane G-protein coupled receptor expressed predominantly in the liver, with less found in the kidneys, heart, adipose tissue, and the adrenal gland (Svoboda et al., 1994). This binding promotes glycogenolysis in the liver and gluconeogenesis through the cyclic adenosine monophosphate (cAMP) messenger activation (Tengholm and Gylfe, 2017). Conversely, insulin is released from the islet β-cells in response to elevated blood sugar and food ingestion, and it exhibits a blood glucose-lowering effect through inhibiting endogenous glucose production and stimulating glucose uptake. By binding to its receptor on the surface of cells, insulin promotes the recruitment of the high-affinity glucose transporter 4 (GLUT4) to the surface of skeletal and fat cells, stimulating glucose transport across the membrane and subsequent glucose uptake (Bryant et al.,

2002). The disruption of these processes can lead to metabolic abnormalities, including blood glucose dysregulation and diabetes (Qaid and Abdelrahman, 2016).

Dietary carbohydrates can be used by the body for energy production and storage through several pathways. Glucose entering the small intestine after a meal is translocated from the intestinal lumen into the enterocytes through the sodium-dependent glucose transporter 1 (SGLT-1), and out into the interstitial space via the glucose transporter 2 (GLUT2) (Chen et al., 2016; Thorens, 1996). There, it is directly absorbed into the capillaries to be further delivered to and processed by many tissues, but mainly the liver and skeletal muscle, where glycogenesis and glycolysis take place (Chen et al., 2016). Glycogenesis, or the process of glycogen formation, utilizes glucose molecules from the circulation to synthesize glycogen as a form of energy storage. Glycogenesis starts with phosphorylation of a glucose molecule to G-6-P by hexokinases, followed by G-1-P formation through the transfer of a phosphate group from carbon 6 to carbon 1, and finally, uridine diphosphate glucose (UDPG) formation (Adeva-Andany et al., 2016; Blanco and Blanco, 2017). This transformation allows the glucose molecule to be added to the polymer backbone of the preexisting glycogen. In a combined hyperglycemic-hyperinsulinemic clamp study, the synthesis of muscle glycogen accounted for most of the total-body glucose uptake, and was the principal pathway of glucose disposal in both healthy and T2D subjects (Shulman et al., 1990). In addition to glycogenesis, liver and skeletal muscle contribute to post-prandial glucose disposal through glycolysis (Ferrannini et al., 1985; Kelley et al., 1988). Through a series of steps, glucose is converted to pyruvate, followed by its entry into the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, ultimately leading to the generation of adenosine triphosphate, or ATP.

In the fasted state, the liver plays a major role in glucose homeostasis by elevating blood glucose through two distinct mechanisms. Fasting induces a reduction in insulin signaling and an increase in glucagon, and catecholamine signaling initiates a cAMP-dependent pathway to activate glycogen phosphorylase and glycogenolysis, or the breakdown of glycogen (Han et al., 2016; Petersen et al., 2017). It is the predominant source of glucose released into systemic circulation in the early stages of fasting. Exercise or prolonged fasting resulting in glycogen depletion further leads to gluconeogenesis, or *de novo* glucose synthesis (Geisler et al., 2016). In this process, hepatocytes use gluconeogenic substrates such as pyruvate, lactate, glycerol, or amino acids to create glucose molecules, which are then released into the circulation and metabolized by extrahepatic tissues (Han et al., 2016). However, the dysregulation of these processes in patients with T2D leads to a significant reduction in postprandial glycogenesis, impaired inhibition of endogenous glucose production following a meal, and a reduction in skeletal muscle glucose disposal (Bokhari et al., 2009; Krssak et al., 2004), all contributing to impaired glucose metabolism and persistent hyperglycemia.

1.1.3 Diabetes Therapies

Current therapies available to patients with T2D are aimed at reducing the risks associated with the condition through managing hyperglycemia, as well as concurrent features such as dyslipidemia, hypertension, obesity, and insulin resistance. Intensive glycemic control in patients with diabetes mellitus has been shown to significantly delay the onset of diabetic retinopathy, nephropathy, and neuropathy (Ohkubo et al., 1995; UK Prospective Diabetes Study Group, 1998). Various formulations of insulin and insulin analogs continue to be developed for glycemia regulation for both type I and II diabetes. However, in addition to technical risks associated with regular subcutaneous injections, insulin-based therapies carry an increased risk of hypoglycemic episodes which are associated with increased morbidity and mortality (Dimeglio et al., 2004; Hanas and Ludvigsson, 2006; Pickup and Renard, 2008).

1.1.3.1 Metformin

Due to its high tolerability and ease of administration, metformin has been a popular early-phase antidiabetic agent since its introduction in the 1950s. Its primary glucoregulatory action is through a reduction in hepatic glucose output (Jackson et al., 1987; Perriello et al., 1994), while other studies suggest it may also increase glucose uptake into the peripheral tissues (Shaw et al., 2005), improve β-cell function (Diaz et al., 2017), and enhance insulin sensitivity (Gin et al., 1985). The liver-dominant mechanism of metformin is further supported by more recent clinical data, which demonstrated a pronounced reduction in hepatic glucose production without major changes in peripheral glucose disposal or insulin secretion (LaMoia and Shulman, 2020). However, a large number of patients taking metformin (11-26%) report gastrointestinal complications while taking both extended- and immediate-release preparations, with approximately 5% further developing metformin intolerance (Dujic et al., 2015; McCreight et al., 2016). This effect has been linked by other studies with the metformin-associated shifts in the intestinal microbiome composition, although the origin or contribution of this change to glucose-lowering remains unclear (LaMoia and Shulman, 2020). Additionally, although extremely rare, a serious and potentially fatal complication of metformin is metformin-associated lactic acidosis as a result of dehydration and acute renal failure (Vecchio and Protti, 2011).

1.1.3.2 Suflonylureas and Thiazolidinediones

Sulfonylurea (SU) and thiazolidinedione (TZD) drugs offer glycemic control through stimulating insulin secretion and increasing insulin sensitivity, respectively (Proks et al., 2002). SUs directly stimulate endogenous insulin secretion by binding to the ATP-sensitive potassium channels on insulin-producing β-cells, which causes membrane depolarization and release of insulin-containing secretory granules (Proks et al., 2002). As their glycemia-regulating effect depends on the presence of functional β-cells, SUs are most effective at the beginning stages of T2D, with over 50% of patients requiring additional therapy after 6 years (Wright et al., 2002). On the other hand, TZDs exert their glucose-lowering action by improving

insulin-mediated glucose uptake into the peripheral tissues and hepatic insulin sensitivity (Natali and Ferrannini, 2006). TZDs act as potent peroxisome proliferator-activated receptor gamma (PPARγ) ligands, although their exact mechanisms of action have not yet been described, as PPARγ is present in a variety of metabolic tissues (Soccio et al., 2014; Tonelli et al., 2004). Through the activation of PPARγ in the adipose tissue, TZDs were found to modulate gene expression, induce adipose tissue remodeling and adipocyte differentiation, thereby altering hormone signaling and mediating glucose disposal (Brunmair et al., 2004). Despite their potent insulin-sensitizing action, TZDs are now less popular in clinical use due to significantly increased risks of developing heart failure, macular oedema, and bone fractures (Motola et al., 2012; Soccio et al., 2014).

1.1.3.3 Sodium-Glucose Cotransporter-2 (SGLT2) Inhibitors

SGLT2 inhibitors are a new class of drugs that inhibit renal glucose reabsorption, allowing it to be excreted in the urine. SGLT2 protein expressed in the kidney accounts for 90% of filtered glucose reuptake (Hsia et al., 2017). The renal threshold for reabsorption of glucose is elevated in patients with T2D, causing a maladaptive response to glucose consumption. Selective SGLT2 inhibition can greatly reduce this threshold, increasing the excretion of glucose in urine and decreasing plasma glucose concentration without stimulating insulin release (Hsia et al., 2017). Clinical studies of SGLT2 inhibition have demonstrated additional beneficial effects, including weight loss and a significant reduction in blood pressure (Desouza et al., 2015; Monami et al., 2014). However, detectable glucose concentrations in urine resulting from SGLT2 inhibition were found to increase the incidences of genital mycotic infections, urinary tract infections, and adverse events related to osmotic diuresis (Davidson and Kuritzky, 2014). Lastly, canagliflozin, a type of SGLT2 inhibitor, was shown to increase the risk of lower extremity amputation, but no specific mechanism or at-risk group has yet been identified (Hsia et al., 2017)

1.1.3.4 Incretin therapies

Finally, two classes of anti-diabetic drugs that have emerged more recently are incretin-based drugs and DPP4 inhibitors, both of which employ the body's innate response to increases in blood glucose. Incretin hormones glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) exert a glucose-lowering effect through potentiating glucose-stimulated insulin secretion and inhibiting glucagon secretion from the pancreas (Drucker, 2006). They are, in turn, rapidly inactivated by ubiquitously expressed enzyme, dipeptidyl peptidase-4, or DPP4 (Mulvihill and Drucker, 2014). Incretin mimetics, such as GLP-1 receptor agonists (GLP-1RAs), are resistant to degradation by DPP4 *in vivo*, allowing them to exhibit a prolonged and more potent glucose-lowering effect than native GLP-1. From a clinical perspective, however, GLP-1 agonists can have tolerability issues due to common persistent gastrointestinal discomfort and daily or weekly subcutaneous injections (Lovshin and Drucker, 2009).

Pharmacological inhibition of DPP4 with DPP4 inhibitors (DPP4i), on the other hand, provides a valuable complementary approach for glycemia regulation. DPP4 inhibition prevents incretin degradation,

improves glucose tolerance, and enhances insulin response in patients with T2D (Mulvihill and Drucker, 2014). DPP4 inhibitors are generally well-tolerated, and short-term studies have not found any major increases in cancer or pancreatitis incidence in T2D patients (Monami et al., 2011). DPP4 inhibitors have demonstrated neither benefit nor harm after completion of cardiovascular outcome trials (Santamarina and Carlson, 2019). While concerns about long-term effects of DPP4 inhibitors, particularly their safety and the role of DPP4 inhibition in the progression of the disease have been raised, (Lovshin and Drucker, 2009; Santamarina and Carlson, 2019; Zhong et al., 2016) the completion of the cardiovascular outcome trials has not validated this unease.

1.2 The Endocrine Pancreas

1.2.1 Islet Architecture

Pancreatic islets, also known as the Islets of Langerhans, are highly vascularized clusters of endocrine cells within the pancreas that produce and secrete a variety of hormones to regulate metabolism. The major hormone-secreting cell types found in the islets are glucagon-producing α-cells, insulin-producing β-cells, somatostatin-secreting δ-cells, as well as less abundant ghrelin-secreting ε-cells and pancreatic polypeptide (PP) - producing cells (Andralojc et al., 2009; Brissova et al., 2005; Wang et al., 2013). The cytoarchitecture of islets has been found to vary greatly between species, which may correlate with differences in islet adaptability, and plasticity (Kim et al., 2009), although their endocrine function remains similar (Fig. 1).

In the non-diabetic, adult, human pancreas, the total number of islets is estimated to be between 3.6 and 14.8 million, with the number gradually increasing from the head to the tail section of the pancreas (Saito et al., 1978). While numerous, islets were found to take up a total volume of 0.544 – 1.311 cm^3, or 0.4-0.7% of the total volume of the pancreas (Saito et al., 1978). In humans, they are made up of approximately 40% α-cells and 50% β-cells, with the remainder accounting for other cell types (Brissova et al., 2005; Cabrera et al., 2006; Kim et al., 2009). Human islets exhibit heterogeneous organization, with different cell types distributed throughout the islet with no particular patterns and often apposed to blood vessels (Kim et al., 2009). This type of organization may allow for direct interaction and paracrine signalling between α- and β-cells independent of external factors.

Laboratory mice and rats are often used in islet biology research, and therefore it's important to acknowledge several key morphological differences between human and rodent islets. First, both mouse and rat islets have a well-defined islet structure, comprising of a "core" of β-cells surrounded by a "mantle" of α- and δ-cells (Cabrera et al., 2006; Kim et al., 2009). This organization has been suggested to play an important role in the synchronization of the β-cell activity in mice (Meissner, 1976; Ravier et al., 2005). The proportion of insulin-containing cells is 61-88% in a mouse islet, significantly higher than a human islet (Brissova et al., 2005). Furthermore, human and mouse islets exhibit distinct vascularization and innervation patterns, which may contribute to more functional differences between the species. Mouse islets

have increased vessel branching, vessel diameter, and total vessel network compared to human islets (Cohrs et al., 2017). Similarly, mouse islets are densely innervated, while human islet innervation was found to be sparse, with most endocrine cells lacking autonomic innervation (Rodriguez-Diaz et al., 2011).

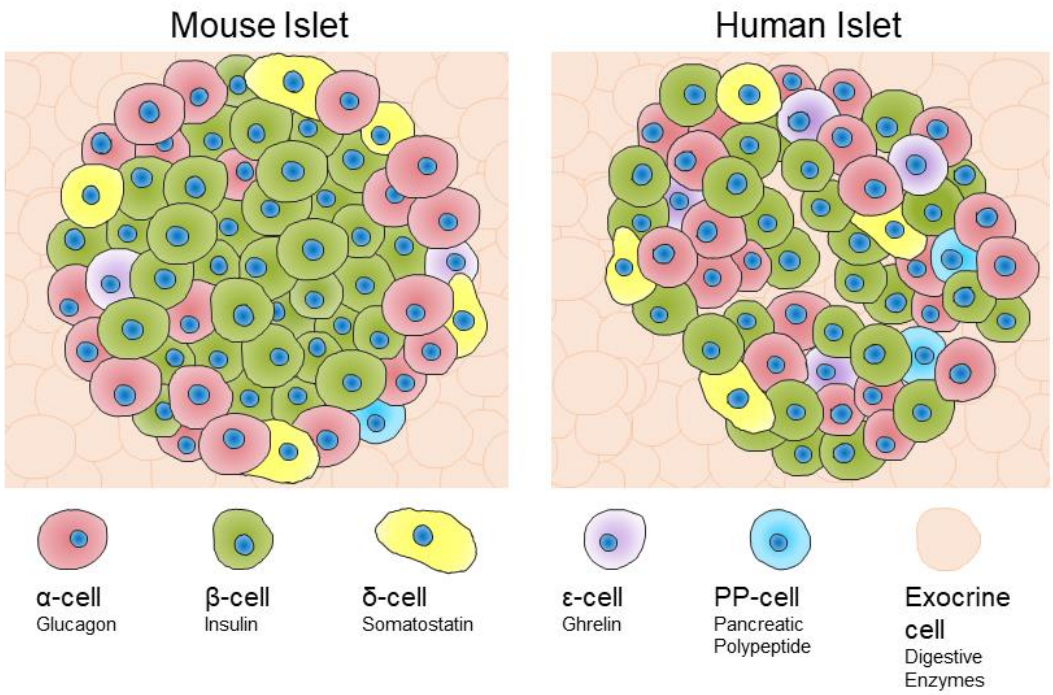

Figure 1. Islet Cytoarchitecture

Adapted from Brissova et al., 2005; Cabrera et al., 2006; Andralojc et al., 2008

1.2.2 β-Cells

Pancreatic islet β-cells maintain blood glucose homeostasis through the production and timely secretion of insulin. Insulin stimulates glucose uptake into skeletal cells through the recruitment of GLUT4 transporter to their surface, promotes fatty acid synthesis in the liver, and suppresses the breakdown of fat in adipose tissue (Carmen and Víctor, 2006; Manchester, 1972; Wong and Sul, 2010). At the cellular level, insulin secretion decreases glycogen breakdown and gluconeogenesis through modulating the activity of glycogen phosphorylase and 2,6 biphosphate kinase. Concurrently, it increases glycogenesis and glycolysis through dephosphorylating glycogen synthase and pyruvate kinase, respectively (Wilcox, 2005).

1.2.2.1 β-Cell Proliferation

During pancreatic development, the formation of new β-cells occurs through two distinct pathways: replication of already differentiated β-cells, and differentiation of new β-cells from stem cell precursors, also known as neogenesis (Swenne, 1992). The most studied factors regulating β-cell growth include glucose, which stimulates β-cell replication in both fetal and adult rodent islets, as well as insulin and insulin-like growth factor-1 (IGF-1), which stimulate β-cell replication in neonatal rodent pancreatic cells. Other factors, such as growth hormone (GH), prolactin and placental lactogen, insulin-like growth factor-2 (IGF-2), platelet-derived growth factor (PDGF), and several amino acids, have all been shown to stimulate β-cell replication *in vitro*, however, their effects remain to be examined *in vivo* (Reddy, 2000).

1.2.2.2 β-Cell Compensation

Total β-cell mass can also increase due to expansion of individual β-cells (Swenne, 1992). It is often initiated during the pre-diabetic insulin intolerance stage, in which islet β-cells hypersecrete insulin to cope with the increasing metabolic demand to maintain normoglycemia (Prentki and Nolan, 2006). Increased glucose utilization and oxidation, coupled with increases in free fatty acid (FFA) cycling and intestinal GLP-1 signaling promote β-cell proliferation and neogenesis while preventing apoptosis, leading to β-cell mass expansion, or hypertrophy (Prentki and Nolan, 2006). GLP-1 further enhances β-cell proliferation by activating the Insulin Receptor Substrate-2 (IRS-2) signaling pathway and deactivating the Forkhead Box O1 (FOXO1) transcription factor, thus increasing expression of the *PDX1* gene, a factor involved in β-cell proliferation and survival (Prentki and Nolan, 2006). Insulin and IGF-1 have also been proposed to play important roles in these processes. Similar to GLP-1, they act through the IRS-2 pathway via protein kinase B (PKB) phosphorylation, which inhibits proapoptotic proteins such as BCL2 associated agonist of cell death, or BAD (Prentki and Nolan, 2006).

1.2.2.3 β-Cell Apoptosis

Overtime, chronic exposure to elevated glucose and FFA levels negatively alters islet function and contributes to the progression from insulin intolerance to T2D. The heightened demand for β-cells to

produce insulin leads to increased proinsulin peptide biosynthesis, which causes an increase in rough endoplasmic reticulum (RER) protein flux (Donath and Halban, 2004). As RER flux is already high in β-cells compared with other cell types, this further increase may be contributing to the ER-stress-induced apoptosis (Donath and Halban, 2004). High glucose has been shown to generate reactive oxygen species (ROS), which are toxic to many cell types, and particularly to the β-cells (Ihara et al., 1999; Ross Laybutt et al., 2002). Furthermore, through a separate pathway, high glucose promotes the production of the proinflammatory cytokine IL-1β from the β-cell, which inhibits β-cell function and induces Fas-triggered apoptosis *in vitro* (Maedler et al., 2002). Both obese and lean patients with T2D exhibit decreased β-cell volume compared to age- and weight-matched non-diabetic patients (Butler et al., 2003). The rate of β-cell apoptosis was found to increase 10-fold in lean and 3-fold in obese T2D patients compared to the subjects without diabetes (Butler et al., 2003), suggesting that apoptosis is the mechanism underlying β-cell mass decrease in T2D.

1.2.2.4 Insulin

Insulin is encoded by the INS gene on chromosome 11 at position 15.5, and its biosynthesis occurs in a set of steps within the β-cell. First, the mRNA transcript is translated into a single-chain precursor, preproinsulin, consisting of the signal peptide, insulin A- and B-chains, and C-peptide (Liu et al., 2014b). The signal peptide guides the translocation of preproinsulin into the endoplasmic reticulum (ER) lumen, where it is cleaved by signal peptidase, forming proinsulin (Liu et al., 2014b). Within the ER lumen, ER chaperone proteins assist in proinsulin folding and disulfide bond formations, generating a direct insulin precursor with tertiary structure (Bass et al., 1998; Ozawa et al., 2005). Properly folded proinsulin is then trafficked through the Golgi apparatus and packaged into immature granules, which accumulate in the cytoplasm (Liu et al., 2014b). The final steps of proinsulin maturation occur in these granules through endoproteolytic cleavage by prohormone convertases (PC) 1/3, PC2, and exopeptidase cleavage by carboxypeptidase E (CPE). First, PC1/3 preferentially cleaves at the B-C junction, exposing a dibasic Arg31-Arg32 motif, which is removed by CPE to form an intermediate called des-31,32 proinsulin (Furuta et al., 1998; Rhodes et al., 1992). This is followed by similar processing by PC2 and CPE on the other side, converting proinsulin to mature insulin and a C-peptide (Liu et al., 2014b). Mature human insulin is composed of two peptide chains linked together by disulfide bonds. The A-chain consists of 21 amino acids, and the B-chain of 30 amino acids, with two interchain bonds formed between the cysteine residues at A7-B7 and A20-B19, and one intrachain bond at A6-A11 (Baker et al., 1988).

1.2.2.5 Insulin Secretion From the β-cell

The process of insulin secretion from the β-cell is primarily stimulated by a high concentration of blood glucose and occurs in two stages – rapid but transient early stage, followed by a less intense but more sustained second phase (Bratanova-Tochkova et al., 2002; Komatsu et al., 2013). This biphasic pattern is thought to occur due to the existence of several “pools” of insulin granules within the β-cell, that

can be characterized by their release competence and proximity to the plasma membrane (Rorsman and Renström, 2003). The readily-releasable pool (RRP) is found in direct contact with the plasma membrane and accounts for 1-5% of the total number of insulin granules within a cell, available for immediate secretion following stimulation (Eliasson et al., 1997; Olofsson et al., 2002; Rorsman and Renström, 2003). The depletion of RRP and the exocytotic capacity of the cell are recovered by the physical translocation of granules from the non-releasable pool to the membrane (Rorsman and Renström, 2003). Unlike the RRP, these granules must undergo a series of priming steps involving a formation of SNARE complexes (Xu et al., 1999) and granular acidification by a V-type H^+-ATPase (Barg et al., 2001) to gain release competence.

Glucose-stimulated insulin secretion, or GSIS, is initiated through membrane depolarization by the K_{ATP}-dependent pathway (Komatsu et al., 2013). First, glucose serves as a substrate for ATP generation through glycolysis and oxidative phosphorylation. This increase in ATP and accompanying decrease in ADP leads to a shift in the ATP/ADP ratio, which closes ATP-sensitive potassium (K_{ATP}) channels and leads to membrane depolarization. This, in turn, opens L-type voltage-gated Ca^{2+} channels (VGCCs), causing a Ca^{2+} influx and consequently triggering exocytosis of insulin secretory granules from the β-cell into the hepatic portal vein (Komatsu et al., 2013; Soria et al., 2004). Experiments in mouse islets have estimated first and second phase insulin secretion to amount to 0.14%/min and 0.05%/min of the total cell insulin content, respectively (Rorsman and Renström, 2003). In addition to glucose, amino acids like arginine, leucine, and alanine, as well as some neurotransmitters and hormones, have been found to stimulate β-cell membrane depolarization and insulin release either directly (through an accumulation of positive charges in the β-cell) or through Na^+ co-transporters (Charles et al., 1982; Henquin and Meissner, 1981; Hermans et al., 1987; Newsholme et al., 2006; Svendsen et al., 2018). However, most of them are unable to initiate this process independently and require the presence of glucose concentrations approaching the threshold for insulin secretion to be effective.

1.2.2.6 Insulin Receptor (IR)

Insulin produces its metabolic effects through binding to its receptor located in the cell membrane of target tissues, such as liver, adipose tissue, skeletal muscle, kidney, and the brain (Belfiore et al., 2009). The insulin receptor (IR) belongs to the receptor tyrosine kinase superfamily of proteins, and in humans it is encoded by a single gene, *INSR* (Ebina et al., 1985; Ullrich et al., 1985). Alternative splicing of the primary transcript results in the generation of either α- or β-subunit of the receptor (Belfiore et al., 2009). The mature IR protein is a heterotetramer, which contains two extracellular α-subunits and 2 transmembrane β-subunits, linked together by disulfide bonds (Belfiore et al., 2009; Lawrence et al., 2007).

1.2.2.7 IR Signal Transduction Pathway

Insulin binding to the extracellular α-subunit induces a conformational change in the IR, which allows the two β-subunits to associate, and leads to autophosphorylation of the kinase domains (Belfiore et al., 2009). The activated IR tyrosine kinase is then able to initiate a signalling cascade through recruitment

of several docking proteins, although most effects appear to be mediated through the insulin receptor substrates (IRS) 1 and 2, and the adapter Src homology 2 domain-containing protein (SHC) (Giorgetti et al., 1994; Giovannone et al., 2000; Taniguchi et al., 2006; Valverde et al., 1998; Versteyhe et al., 2009). Phosphorylation of these substrates further leads to the activation of two canonical IR signaling cascades – phosphoinositide 3-kinase (PI3K) pathway and the mitogen-activated protein kinase (MAPK) pathway (De Meyts, 2000). The MAPK signaling cascade is involved in insulin-mediated regulation of gene expression, mitogenesis, and differentiation (De Meyts, 2000). The pathway is activated exclusively through the IR/IRS interaction and is responsible for most metabolic effects of insulin (De Meyts, 2000).

Binding of IRS to the regulatory subunit of PI3K first generates phosphatidylinositol-3,4,5-triphosphate (PIP3) (Taniguchi et al., 2006). PIP3 then activates 3-phosphoinositide dependent protein kinase-1 and -2 (PDK1 and PDK2), which induce phosphorylation of protein kinase Akt/PKB at tyrosine-308 and serine-473, respectively, leading to PKB activation (Guo, 2014). Finally, the four major downstream targets of PKB facilitate glucose homeostasis through different mechanisms: mTOR (mammalian target of rapamycin) acts as a nutrient sensor and stimulates protein synthesis; GSK3 (glycogen synthase kinase 3) inhibits glycogen synthase; AS160 (Akt substrate of 160kDa) regulates the translocation of glucose transporter GLUT4 to increase glucose uptake; and the action of FoxO1 (forkhead box-containing protein O1) which normally activates gluconeogenesis, is inhibited (Guo, 2014; De Meyts, 2000).

1.2.3 α-Cells

Pancreatic islet α-cells contribute to glycemic regulation by secreting glucagon, a hormone that counteracts the effects of insulin. Glucagon is released in response to hypoglycemia to stimulate hepatic glucose synthesis and glycogenolysis, thus releasing glucose into the bloodstream and increasing blood glucose concentration (Quesada et al., 2008).

1.2.3.1 Glucagon Production

Glucagon is a peptide hormone derived from preproglucagon, a precursor protein encoded by the 180 base pair long *GCG* gene in humans (White and Saunders, 1986). In addition to the α-cells, the *GCG* gene is also expressed in the intestinal L-cells and the central nervous system (CNS). Preproglucagon is differentially cleaved by the dominant prohormone convertase (PC) subtype present in each tissue to produce different products. Within the islet, glucagon synthesis occurs in a series of intracellular events similar to insulin synthesis. Following its translation in the ER, the prohormone is transported into the Golgi apparatus for further modification and packaging, after which glucagon-containing granules migrate to the plasma membrane to be secreted (Guizzetti et al., 2014). The granules are released from the α-cells through Ca^{2+}-dependent exocytosis, with low glucose driving the influx of Ca^{2+} into the cell (Ramracheya et al., 2010).

1.2.3.2 Glucagon Receptor

Glucagon's glucose-lowering action is transduced through binding to the glucagon receptor (GCGR), a G-protein coupled receptor found mainly in the kidney and liver, and with lower expression in the heart, adipose tissue, spleen, gastrointestinal tract, and pancreas. Coupling of the GCGR to adenylyl cyclase allows it to activate the downstream signalling through increased production of cAMP and the PKA pathway (Charron and Vuguin, 2015). This leads to an increase in hepatic gluconeogenesis, glycogenolysis, and fatty acid oxidation (Charron and Vuguin, 2015).

In addition, glucagon acts as a local regulator of islet function, exerting both auto- and paracrine effects within the islet. Activation of the GCGR on the α-cell creates a positive feedback loop, upregulating *Gcg* transcription and stimulating more glucagon secretion through increasing exocytosis in isolated rodent islets (Leibiger et al., 2012; Ma et al., 2005). Interestingly, glucagon also stimulates insulin secretion from the islet β-cell in the presence of high glucose concentrations (Capozzi et al., 2019; Svendsen et al., 2018). The insulinotropic action of glucagon occurs primarily through the activation of its receptor, GCGR, on the islet β-cell. However, in the absence of functional β-cell GCGR, glucagon has been shown to activate GLP-1 receptors instead, resulting in insulin secretion similar to that of control mice (Capozzi et al., 2019; Svendsen et al., 2018).

The importance of glucagon/GCGR signalling on islet morphology and physiology has been established using mouse models with conditional *Gcgr* knockout and *Gcgr* overexpression. Ablation of GCGR signalling using *Gcgr*$^{-/-}$ mice was found to contribute to the α- and δ-cell hyperplasia, inhibit the maturation of α-cells, and delay the differentiation of β-cells in mice (Vuguin et al., 2006). Disruption of *Gcgr* was also associated with an increase in the number of pancreatic islets and alterations in the proportion of β- to α-cells in embryonic islets (Vuguin et al., 2006). On the other hand, overexpression of the *Gcgr* in insulin-producing β-cells lead to an increase in glucagon-stimulated insulin release and significantly contributed to the β-cell volume expansion (Gelling et al., 2009). Taken together, these findings emphasize glucagon as one of the essential regulators of islet function, and, more specifically, β-cell secretory responses.

1.2.4 Other Hormone-Producing Cell Types Within the Islet

1.2.4.1 δ cell

In addition to α- and β-cells that make up the majority of the cell populations within the islet, there are at least three other types of hormone-producing cells that have been identified. In humans, δ-cells make up around 10% of the islet cell mass and secrete somatostatin (SST), also known as growth hormone-inhibiting hormone (GHIH) (Cabrera et al., 2006). SST is also expressed in the hypothalamus and throughout the gastrointestinal tract. Glucagon and factors released from the β-cell, such as GABA and urocortin3, act as positive effectors of pancreatic SST release (Rorsman and Huising, 2018). In turn, SST

inhibits the release of glucagon and insulin from the islet through its binding to two distinct SST receptors – SSTR2 and SSTR5 (Cejvan et al., 2003; Strowski et al., 2000). This feedback loop provides a mechanism for the control of hormone secretion during hyperglycemia. In animal models, SST hypersecretion has been suggested as an explanation for impaired hypoglycemia-induced glucagon secretion – an effect that could be corrected by SST antagonists (Rorsman and Huising, 2018). In addition, pancreatic δ-cells were found to be critical in the regulation of insulin content, release, and neonatal blood glucose homeostasis through an SSTR-independent pathway, suggesting a role in glucose regulation for δ-cell-secreted factors other than SST (Li et al., 2018).

1.2.4.2 ϵ cell

Less than 1% of the mature islet cell population are ghrelin-producing ϵ-cells (epsilon cells). Most of the circulating ghrelin in adults originates within the gastrointestinal tract, although lower amounts are expressed in other tissues, including the pituitary gland, the lungs, the kidney, and the pancreas (Van Der Lely et al., 2004). Biological activities of ghrelin can be direct, through the ghrelin receptor binding, or mediated via the pituitary hormones and their efferent pathways (Van Der Lely et al., 2004). Ghrelin acts as a potent stimulant of growth hormone (GH) release, which contributes to a wide variety of physiological functions it exerts throughout the body (Schmid et al., 2005; Sun et al., 2004; Takaya et al., 2000). In addition to acting as a GH secretagogue, ghrelin is directly involved in regulating appetite and food intake, gastric motility and emptying, cell proliferation, cardiovascular function, glucose metabolism, and energy balance (Arta Korbonits et al., 2004; Van Der Lely et al., 2004; Levin et al., 2006; Mihalache et al., 2016; Sun et al., 2004; Thompson et al., 2004). However, the exact role of ghrelin in islet hormone secretion remains controversial. In several rodent studies, exogenous ghrelin administration leads to a reduction in glucose-stimulated insulin secretion in isolated rat pancreas, isolated mouse islets, and INS-1 cell lines (Colombo et al., 2003; Egido et al., 2002; Wierup et al., 2004). Conversely, other studies report ghrelin to increase insulin secretion in rat pancreas and isolated islets (Adeghate and Ponery, 2002; Date et al., 2002; Lee et al., 2002). Similar inconsistencies are seen in human studies, although a more sensitive approach with a steady-state ghrelin infusion by Tong et al. found that exogenous ghrelin reduces glucose-stimulated insulin secretion and glucose clearance in humans (Broglio et al., 2001; Lindqvist et al., 2020; Lucidi et al., 2005; Tong et al., 2010). Finally, a more recent study using mice with a ghrelin knockout reported intra-islet ghrelin signaling to not have any significant effects on insulin secretion (Gray et al., 2019).

Interestingly, pancreatic ghrelin-expressing cells were quite prominent in the human fetal pancreas from mid-gestation to the early postnatal period, comprising 10% of all endocrine cells and outnumbering those in the stomach (Wierup et al., 2002). This expression is greatly reduced in the adult pancreas, but still evident as single cells in islets, exocrine tissue, ducts, and pancreatic ganglia (Chanoine and Wong, 2004; Wierup et al., 2002). These data, combined with its role in regulating cell proliferation, may suggest that the ghrelin system is involved in islet cell maturation during embryonic development.

1.2.4.3 PP Cells

Pancreatic polypeptide (PP) – secreting cells, also known as γ-cells or F cells, are the least abundant islet cell type. The spatial distribution of PP cells in the human pancreas is uneven, with PP-cell-rich clusters restricted largely to the uncinate process within the head of the pancreas (Wang et al., 2013). PP is secreted postprandially, and it acts both as a neural and endocrine messenger within the pancreas-gut-brain axis (Holzer et al., 2012). It plays a role in appetite suppression through inhibition of gastric emptying, intestinal motor activity, and intestinal electrolyte and water secretion (Batterham et al., 2003; Fujimiya and Inui, 2000; Tough et al., 2006). It reduces food intake predominantly through direct stimulation of the anorexigenic α-Melanocyte-stimulating hormone (α-MSH) pathway via its binding to the Y4 receptor within the hypothalamus (Brereton et al., 2015; Katsuura et al., 2002; Lin et al., 2009). Data on the biological relevance of PP in the regulation of intra-islet hormone secretion are limited. In mouse islets, PP inhibited glucagon secretion through binding to PP receptor 1 on the α-cells (Aragón et al., 2015). However, intravenous infusion of PP did not affect insulin response to glucose and failed to improve glucose tolerance in a dog model of pancreatitis-induced diabetes (Bastidas et al., 1990).

1.2.5 Immune Cells Within the Islet

Endocrine (islet) and exocrine tissues of the pancreas contain small numbers of resident macrophages, each with unique properties and characteristics (Calderon et al., 2015). In islets, macrophages represent the only type of myeloid cell (Calderon et al., 2015). These macrophages are found in close contact with blood vessels, and have extensive filopodia-like structures that sample large areas of islets and blood contents (Ying et al., 2018; Zinselmeyer et al., 2018). These cells are highly effective at presenting β-cell antigens to CD4 T cells (Calderon et al., 2008). A feature of islet pathology in T2D is increased islet inflammation and macrophage infiltration, which induces β-cell dysfunction (Böni-Schnetzler and Meier, 2019; Butcher et al., 2014; Cucak et al., 2014; Eguchi and Manabe, 2013; Ehses et al., 2007). Under obese conditions, islet macrophages have shown to directly impair β-cell functions such as GSIS, as well as promote the adaptive expansion of β-cell mass associated with T2D (Xiao et al., 2014; Ying et al., 2018). Islets isolated from HFD-fed mice secreted more pro-inflammatory factors such as IL-6, IL-8, CXCL1, granulocyte colony-stimulating factor (GCSF) and macrophage inflammatory protein 1α (MIP1α) (Ehses et al., 2007). These results were replicated in islets isolated from healthy human donors, where treatment with palmitate increased secretion of pro-inflammatory cytokines and chemokines, mimicking a T2D profile (Eguchi and Manabe, 2013). Interestingly, increased islet macrophage presence in a model of HFD-induced T2D was not due to the recruitment from circulating monocytes. Instead, high-fat diet feeding and developing obesity induced a local proliferation of intra-islet macrophages (Ying et al., 2018).

1.3 Incretin Effect

Hormone secretion from the pancreas is tightly linked to food ingestion through the action of incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (Drucker, 2006). The discovery of incretin hormones followed an observation of enhanced insulin secretion in response to glucose ingested orally, as opposed to intravenous intake, even when the same plasma glucose concentration was achieved (Nauck and Meier, 2018; Perley and Kipnis, 1967). This suggested the presence of insulinotropic gut-derived factors, which were later isolated and identified as GLP-1 and GIP (Brown et al., 1975; Holst, 1994; Mojsov et al., 1987). Biologically active incretins inhibit glucagon secretion and augment insulin secretion, resulting in appropriate glucose uptake and utilization. Together, GLP-1 and GIP account for 50-70% of the postprandial rise in insulin in healthy subjects (Nauck et al., 1986). The incretin effect is greatly diminished in subjects with T2D, even in the very early stages with excellent glycemic control (Aulinger et al., 2016; Knop et al., 2012; Michaliszyn et al., 2014). Interestingly, only the insulinotropic action of GIP in the pancreas is reduced, whereas the response to GLP-1 is largely preserved (Nauck & Meier, 2016). Since the incretin action of the two hormones is additive, this reduction in GIP sensitivity is the likely reason for the reduced incretin effect. It can be calculated as the difference in insulin secretion during an oral glucose challenge versus one during an iso-glycemic glucose injection. In a recent study, young obese patients with established T2D, as well as age- and BMI-matched non-diabetic subjects, saw ~50% reduction in the incretin effect relative to the non-diabetic lean group (Aulinger et al., 2016). In the same cohort, only the T2D group exhibited impaired glucose tolerance and insulin secretion during the oral glucose tolerance test, while the lean and non-diabetic obese groups had comparable glucose excursions and β-cell function (Aulinger et al., 2016). As such, impairment of this measure has been proposed as an early marker of diabetes and β-cell dysfunction (Aulinger et al., 2016; Holst et al., 2011; Knop et al., 2012). The reduced responsivity to GIP is molecularly attributed to reduced GIPR expression and desensitization in models of T2DM (Lynn et al., 2001; Tseng and Zhang, 1998).

1.3.1 Glucagon-Like Peptide-1 (GLP-1)

1.3.1.1 Preproglucagon Peptides and Processing

Preproglucagon (*Gcg*) gene is expressed in the enteroendocrine L-cells of the intestine, neurons of the brainstem, and the α-cells of the endocrine pancreas. It encodes a 180 amino acid long proglucagon peptide, that is structurally identical in all cell types and is differentially cleaved based on the dominant subtype of the prohormone convertase (PC) expressed in the tissue (Fig.2). In the pancreatic α-cells, PC2 predominantly processes proglucagon to produce glucagon, GRPP, and major proglucagon fragment (Drucker et al., 2017; Sandoval and D'alessio, 2015). On the other hand, intestinal L-cells and CNS neurons have greater relative PC1/3 activity, resulting in the production of GLP-1, GLP-2, oxyntomodulin, IP2, and glicentin (Drucker et al., 2017; Sandoval and D'alessio, 2015). These differences in the regulation of the *Gcg* gene expression and post-translational modifications contribute to the diversity of actions exhibited by its products.

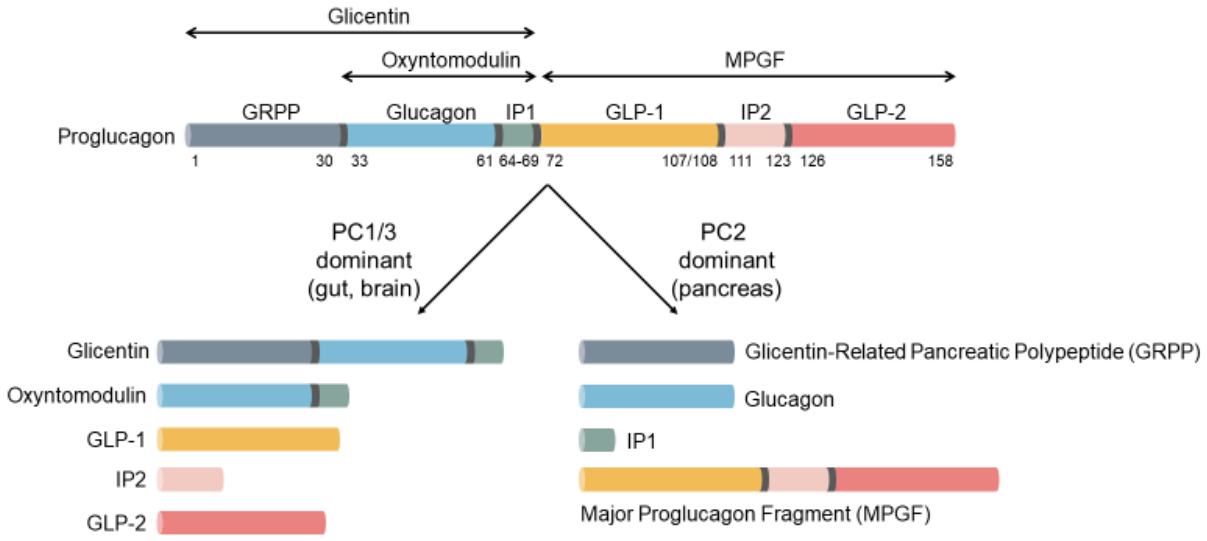

Figure 2. Post-Translational Processing of Proglucagon

Adapted from Sandoval and D'alessio, 2015; Drucker et al. 2017)

1.3.1.1.1 Preproglucagon in the Intestinal L-Cells

Gcg expression can be driven by a variety of physiological factors and chemical agents. Intestinal *Gcg* expression was found to decrease following a fast, while refeeding stimulated its production in rats (Hoyt et al., 1996). As such, nutrient ingestion is likely a primary physiological regulator of intestinal preproglucagon. Studies in intestinal cell cultures have demonstrated that preproglucagon gene expression is determined by the levels of intracellular cAMP and activation of the cAMP/PKA signaling (Baggio and Drucker, 2007). Thus, interactions with several effectors of the Wnt signaling pathway (a potential mediator of the PKA-dependent gene transcription) in the intestine, including TCF7L2, TCF-4 and β-catenin upregulates *Gcg* transcription (Yi et al., 2005; Yu and Jin, 2010). In addition, gastrin-releasing peptide and insulin both increased intestinal preproglucagon mRNA in mouse and rat intestinal cell cultures (Baggio and Drucker, 2007; Yi et al., 2008). Furthermore, a transcription factor Pax-6 is essential for preproglucagon gene expression in both intestine and pancreas, and adenoviral-mediated overexpression of Pax-6 in rat colonic epithelium cells was associated with enhanced endogenous preproglucagon expression *in vitro* (Hill et al., 1999; Trinh et al., 2003).

1.3.1.1.2 Preproglucagon in the Pancreas

In contrast to the intestinal L-cells, pancreatic preproglucagon gene expression is upregulated following fasting and hypoglycemia, and is inhibited by insulin (Baggio and Drucker, 2007; Phillippe, 1989). This inhibition is mediated via the interaction of insulin with the insulin-responsive DNA element within the preproglucagon gene promoter (Philippe, 1991). Furthermore, genetic inactivation of FoxO1, an insulin-dependent nuclear transcription factor, leads to a >40% reduction in pancreatic preproglucagon mRNA transcription, indicating FoxO1 as an important regulator of pancreatic *Gcg* expression (McKinnon et al., 2006). Factors stimulating preproglucagon mRNA transcription in the islet α-cells include activation of the PKC and PKA signaling pathways, cell membrane depolarization, and calcium influx (Baggio and Drucker, 2007). However, there is now more recent and increasing evidence of PC1/3 expression in the pancreatic α-cells and local production of GLP-1 within the islet, although at a lower level (Ellingsgaard et al., 2011; Kilimnik et al., 2010; Sandoval and D'alessio, 2015; de Souza et al., 2020). This intra-islet PC1/3 expression can be further increased under the conditions of hyperglycemia, hyperlipidemia, or metabolic stress such as pregnancy or inflammation (Kilimnik et al., 2010; Sancho et al., 2017; de Souza et al., 2020). As previous studies have localized GLP-1 expression mainly to colonic L-cells, these recent data has shifted the traditional paradigm of GLP-1 secretion and suggested that pancreatic islets are a significant source of GLP-1 secretion, highlighting the importance of intra-islet GLP-1 signalling for glucose homeostasis (Traub et al., 2017). The input from the islet-derived GLP-1 was found to be essential for maintaining glucose homeostasis, especially during metabolic stress induced by high-fat feeding (Capozzi et al., 2019; Chambers et al., 2017; Traub et al., 2017; Whalley et al., 2011). Alpha-cell-derived GLP-1 was required for β-cell adaptation to metabolic stress and increased secretory demand in mice, while prolonged α-cell ablation impaired insulin secretion and glucose tolerance (Traub et al., 2017). Furthermore, reactivation of

GLP-1 production in the gut of *Gcg*$^{-/-}$ mice did not affect their glucose metabolism, whereas reactivation of GLP-1 in the pancreas mimicked the positive responses seen in intact control mice (Chambers et al., 2017). These findings highlight the importance of intra-islet GLP-1 secretion and signaling in maintaining glucose homeostasis.

1.3.1.2 GLP-1, Insulin and Energy Metabolism

Glucagon-like peptide-1 (GLP-1) is a 30 amino acid-long peptide hormone liberated from the proglucagon peptide. Its two inactive forms, GLP-1[1-37] and GLP-1[1-36]NH_2 are cleaved into biologically active forms, GLP-1[7-37] and GLP-1[7-36]NH_2, via the action of PC1/3. The two peptides are equipotent and are a major source of the incretin effect (Edwards et al., 1999; Orskov et al., 1993).

GLP-1 release occurs rapidly following meal ingestion, but can be stimulated by individual nutrients, such as glucose and amino acids. Of note, the quick rise of plasma GLP-1 following food intake suggests the effect of neural factors promoting GLP-1 secretion, as the rise occurs well before the nutrients make direct contact with the L-cells (Drucker, 2006). The role of GLP-1 as an incretin hormone extends beyond the augmentation of nutrient-dependent insulin secretion. Its many actions implicated in glucose homeostasis include increasing insulin gene expression, stimulation of somatostatin secretion, inhibition of glucagon secretion, promoting β-cell health and neogenesis, inhibition of β-cell apoptosis, delaying gastric emptying, promoting satiety, and increasing peripheral glucose clearance (Baggio and Drucker, 2007; Donnelly, 2012). Under normal conditions, active GLP-1 is secreted from the L-cells as GLP-1 [7–37] and GLP-1[7–36]NH_2 via secretory granules, that fuse at the basolateral membrane and are then released into the bloodstream (Mulvihill and Drucker, 2014; Thurmond, 2009). Most GLP-1 signaling occurs through its binding to a single GLP-1 receptor (GLP-1R), a G protein-coupled receptor found within the pancreas, lung, adipose tissue, kidney, heart, smooth muscle, and parts of the CNS (Sandoval and D'alessio, 2015). Notably, active GLP-1 is subject to rapid cleavage and inactivation by dipeptidyl peptidase-4 (DPP4), a serine protease which limits the half-life of GLP-1 to several minutes and eliminates its glucoregulatory action due to a 100-fold decrease in its receptor's (GLP-1R) affinity (Deacon et al., 1995; Knudsen and Pridal, 1996). The binding of active GLP-1 to the adenylate cyclase-coupled GLP-1R present on the β-cells enhances the production of cAMP and thus activates the PKA pathway. As this pathway does not involve β-cell membrane depolarization and calcium influx, it is unable to initiate the release of insulin granules from the cell under hypoglycemic conditions. The insulinotropic action of GLP-1 is, therefore, correlated with the degree of hyperglycemia, increasing with greater glucose concentration (Nauck & Meier, 2018). There is also additional evidence that GLP-1 may act as a glucose sensitizer through mobilizing intracellular Ca^{2+} and facilitating glucose-dependent mitochondrial ATP synthesis (Holz IV et al., 1993; Tsuboi et al., 2003). In addition, it upregulates the expression of glucose transporters and glucokinases, thus enhancing glucose responsivity in previously resistant β-cells (Baggio and Drucker, 2007).

The role of GLP-1 on insulin gene transcription and insulin synthesis has also been studied extensively. It's been shown to act synergistically with glucose to stimulate proinsulin gene expression, cellular levels of proinsulin mRNA, and transcriptional biosynthesis of proinsulin (Drucker et al., 1987; Fehmann and Habener, 1992). Furthermore, incubation with GLP-1 specifically increased the capacity and affinity of insulin and proinsulin binding in a concentration-dependent manner in human monocytes and RINm5F cells (Ebinger et al., 2000).

Finally, considerable attention has been given to the role of GLP-1 in β-cell health and differentiation. Both GLP-1 and its agonist, exendin-4, induced Wnt signaling required for β-cell proliferation in INS-1 cell lines and isolated islets (Liu and Habener, 2008). Activation of GLP-1R inhibited H_2O_2-induced apoptosis, reduced DNA fragmentation, and improved survival in cultured mouse insulinoma cells via increased expression of anti-apoptotic proteins Bcl-2 and Bcl-xL through cAMP- and PI3K-dependent pathways. (Hui et al., 2003). GLP-1 demonstrated a potent protective effect from gluco-, lipo-, and glucolipotoxicity mediated by protein kinase B in human beta-cells and INS832/13 cells (Buteau et al., 2004). It directly reduced cytokine-mediated apoptosis in isolated rat β-cells after exposure to Il-1β, TNF-α, and IFN-γ (Li et al., 2003). *In vivo*, GLP-1 agonist exendin-4 significantly reduced streptozotocin-induced β-cell injury and apoptosis in mice (Li et al., 2003). Lastly, treatment of isolated human islet with GLP-1 preserved islet morphology, reduced the number of apoptotic cells, and enhanced intracellular insulin content compared to untreated controls, providing further support for the therapeutic use of GLP-1 and GLP-1 agonists in islet transplantation (Farilla et al., 2003).

1.3.1.3 Other Actions of GLP-1

Beyond its role in energy metabolism, GLP-1 has been reported to have biological activities in the cardiovascular system. Administration of GLP-1 after ischemia-reperfusion injury increased functional recovery, cardiomyocyte viability, and vasodilation, leading to reduced ischemic damage in mice (Ban et al., 2008). Interestingly, many of these effects were preserved in mice with genetic elimination of the GLP-1 receptor (*Glp1r*$^{-/-}$ mice), suggesting a mechanism of action independent of the known GLP-1 receptor. Similarly, a continuous intravenous infusion of GLP-1(7-36) and GLP-1(9-36) in a model of dilated cardiomyopathy in dogs significantly reduced left ventricular end-diastolic pressure, and increased myocardial glucose uptake without an increase in plasma insulin (Nikolaidis et al., 2005).

1.3.2 Gastric Inhibitory Polypeptide (GIP)

Mature, bioactive GIP(1-42) is a 42 amino acid-long peptide released from a larger proGIP precursor protein through post-translational cleavage by PC1/3 (Baggio and Drucker, 2007; Ugleholdt et al., 2006). GIP is synthesized within and is secreted from a distinct population of enteroendocrine K-cells, most of which are located in the duodenum and proximal jejunum (Buchan et al., 1978; Mortensen et al., 2003). Its secretion is primarily stimulated through nutrient ingestion and, more specifically, the rate of nutrient absorption, rising 10-15 fold within 15 minutes of food intake (Baggio and Drucker, 2007; Gribble

et al., 2018). Similar to GLP-1, GIP augments insulin secretion in the presence of normal and elevated plasma glucose concentrations, and is the other major contributor to the incretin effect (Gribble et al., 2018). However, it is important to note the interspecies differences in the regulation of GIP release, which may complicate translation from animal studies to human applications. In humans, fat is the more potent stimulator of GIP secretion, while carbohydrates were found to be more effective in rodents and pigs (Baggio and Drucker, 2007). Biological activity of GIP *in vivo* is also limited by its short half-life due to cleavage and inactivation by DPP4, which metabolizes over 50% of circulating GIP after 2 minutes in rodents and 5-7 minutes in humans (Deacon et al., 2000; Kieffer et al., 1995).

1.3.2.1 GIP, Insulin, and Energy Metabolism

There are several similarities between the mechanisms of action and effects of the two incretin hormones. In the pancreas, the actions of GIP are analogous to those of GLP-1, leading to enhanced nutrient-dependent insulin secretion (Baggio and Drucker, 2007; Gribble et al., 2018). Like the GLP-1R, the GIP receptor (GIPR) is a member of a G protein-coupled receptor superfamily, which exhibits its effects through elevation of cAMP and downstream signaling (Gribble et al., 2018). GIPR is expressed in a variety of tissues and organs, including the pancreas, stomach, adipose tissue, the heart, kidneys, and parts of the CNS. Binding of GIP to the GIPR on the β-cell increases cAMP, inhibits K_{ATP} channels, increases intracellular Ca^{2+}, and stimulates insulin exocytosis (Ding and Gromada, 1997; Gromada et al., 1998). These actions are mediated through cAMP-dependent activation of the PKA, PKB, and phospholipase A2 pathways (Baggio and Drucker, 2007).

In addition to its insulinotropic action, treatment with GIP was found to increase intracellular insulin content, biosynthesis, mRNA, and upregulation of genes of glucose sensors like GLUT1 and hexokinase I in insulin-producing cell lines (Wang et al., 1996). Furthermore, stimulation of INS-1 β-cells with GIP leads to a concentration-dependent activation of the PI3K/PKB/Foxo1 signaling pathway implicated in cell survival and proliferation (Kim et al., 2005). Concurrently, it suppressed Bcl-2-associated X protein (Bax) promoter activity, which is associated with apoptosis activation. Similar effects were observed in dispersed islet cells, where GIP reduced glucolipotoxicity-induced cell death, likely via down-regulation of Bax and up-regulation of Bcl-2 in pancreatic β-cells (Kim et al., 2005). Together, these results indicate GIP as an important mediator of pancreatic islet health and function.

1.4 DPP4 and DASH Proteins

Dipeptidyl peptidase-4 (DPP4, DPPIV), also known as CD26 (cluster of differentiation 26), is a widely expressed exopeptidase that modulates the bioactivity of over 40 identified substrates involved in energy metabolism, inflammation, and immune activation (Mulvihill and Drucker, 2014; Trzaskalski et al., 2020). It's found in many metabolically active tissues (including the liver, kidney, pancreas, spleen, and lungs), numerous cell types (endothelial cells, monocytes, macrophages, lymphocytes), and bodily fluids (blood, bile, urine, cerebral spinal fluid), suggesting a range of global and tissue-specific roles (Deacon,

2019; Mulvihill and Drucker, 2014; Mulvihill et al., 2017). DPP4 is a member of a larger serine peptidase family of proteins called subfamily 9b, which is characterized by their structure and function, and also includes fibroblast activation protein (FAP), DPP8, DPP9, DPP6, and DPP10 (Wagner et al., 2016). These enzymes are structural homologues of DPP4 and, with the exception of DPP6 and DPP10, display DPP4-like activity and inhibition profiles. Collectively, they are known as the DASH proteins (DPP4 activity and/or structure homologues) (Wagner et al., 2016). FAP, also referred to as seprase, exhibits the highest sequence identity and substrate similarity to DPP4, and is believed to have risen from a gene duplication. Co-localization of FAP and DPP4 in several cell types also leads to the creation of a heteromeric complex (Wagner et al., 2016). Lastly, both DPP8 and DPP9 exist as enzymatically active homodimers, however, unlike DPP4, lack the transmembrane domain and are therefore found intracellularly (Wagner et al., 2016).

1.4.1 Enzymatic Action of DPP4

DPP4 is a type II transmembrane protein with a typical C-terminal signal peptide, which concurrently targets the endoplasmic reticulum and serves as a membrane anchor (Röhrborn et al., 2015). The membrane-bound DPP4 protein consists of several distinct domains, including a short intracellular domain [residues 1-6], a transmembrane domain [7-28], followed by an extracellular flexible stalk, a glycosylated region, an adenosine deaminase (ADA) binding domain, fibronectin-binding domain, cysteine-rich domain, and the catalytic active site [507-766] (Mulvihill and Drucker, 2014). The extracellular part forms an eight-blade β-propeller and α/β-hydrolase domains responsible for binding to ADA and matrix proteins (Zhong et al., 2015). The catalytic activity of DPP4 within its C-terminal is accomplished via the classic catalytic triad (Ser630, Asp708, His740) and is highly conserved in prokaryotes and eukaryotes (Mulvihill and Drucker, 2014; Röhrborn et al., 2015). DPP4 selectively cleaves a dipeptide from the N-termini of its substrates, exhibiting a preference for proline or alanine in the penultimate position (Deacon, 2019; Mulvihill and Drucker, 2014). Alternate residues like hydroxyproline, dehydroproline, glycine, threonine, valine, or leucine can also be cleaved, albeit at a slower rate (Deacon, 2019; Mulvihill and Drucker, 2014).

The two principal isoforms of DPP4 are a 766 aa-long membrane-anchored form and a shorter soluble, circulating form (sDPP4, [39-766]) liberated from the plasma membrane and thus lacking the cytoplasmic and transmembrane domains (Mulvihill and Drucker, 2014). Both isoforms express catalytic activity, but the exact mechanism by which DPP4 is shed from the plasma membrane and is released into circulation remains unknown. *In vitro* studies suggest that this process is mediated by metalloproteases (MMPs), more specifically MMP1, MMP2, and MMP14 in shedding from vascular smooth muscle cells, and MMP9 in adipocytes (Röhrborn et al., 2014). Interestingly, shedding from different tissues seems to vary at different disease conditions. For example, plasma concentration of sDPP4 has been shown to increase in T2D, obesity, and NAFLD, but is decreased in rheumatoid arthritis (Anoop et al., 2017; Dawood et al., 2018; Sarkar et al., 2019; Sromova et al., 2015).

1.4.2 *DPP4* Expression and Regulation

DPP4 exists predominantly as a homodimer, but can form tetramers consisting of two soluble and two transmembrane proteins, which affects their interaction with proteolytic substrates and possibly mediates cell-cell contacts (Chien et al., 2004; Engel et al., 2003). A study using targeted mutation to disrupt the dimerization ability of DPP4 protein found that monomeric DPP4 exhibits a 300-fold decrease in catalytic activity, and that the C-terminal loop of DPP4 is essential for the dimerization process (Chien et al., 2004). The *DPP4* gene promoter region contains binding sites for several transcription factors, such as STAT1α, NFκB, SP-1, EGFR, and NF-1 (Röhrborn et al., 2015). Stimulation of B lymphocytic leukemia cells with IFNs phosphorylated STAT1α, increasing its affinity to the *DPP4* promoter region, which lead to nuclear translocation and increase in *DPP4* transcription, expression, and activity (Bauvois et al., 2000). In addition, stimulation with IL-1α, TNFα, and IL-12 has also been shown to regulate the expression of *DPP4* (Zhong et al., 2015).

1.4.3 DPP4 And the Incretin Hormones

DPP4 is the key enzyme regulating the bioactivity of the incretin hormones, and, therefore, glucose metabolism *in vivo*. Both GLP-1 and GIP are true physiological substrates of DPP4, and cleavage by DPP4 is the initial and primary route of their metabolism (Mentlein et al., 1993). DPP4 cleaves the bond between Ala2 and Glu3 in active GLP-1[7-37] or GLP-1[7-36]NH_2, generating GLP-1[9-37] or GLP-1[9-36]NH_2, and a dipeptide. Similarly, it liberates GIP[3-42] from GIP[1-42] by cleaving the bond after Ala2 (Mentlein et al., 1993). Cleavage by DPP4 inactivates the glucoregulatory effect of the incretin hormones due to a significant reduction in their respective receptors' affinities (Deacon et al., 1995, 2000; Knudsen and Pridal, 1996). As a result, circulating levels of active incretin hormones are extremely low, and only 10% of intact GLP-1 secreted into the circulation from the gut reaches the pancreas (Hansen et al., 1999; Hjøllund et al., 2011).

While the role of DPP4 in the regulation of incretin hormone action and glucose homeostasis is well documented, the exact cellular sites essential for the incretin degradation remain elusive due to the widespread expression of DPP4. Mice with whole-body deletion of DPP4 ($Dpp4^{-/-}$) are protected against high-fat feeding-induced obesity and insulin resistance, and display reduced glycemic excursion (Conarello et al., 2003; Mulvihill et al., 2017). Ablation of *Dpp4* is also associated with elevated plasma GLP-1, GIP, improved metabolic control, reduced islet hypertrophy, and protection from streptozotocin-induced β-cell death (Conarello et al., 2003; Mulvihill et al., 2017). In two recent studies, Mulvihill et al. and Varin et al. used mouse genetics and pharmacologic DPP4 inhibition to further elucidate the cellular origin of incretin-mediated glucoregulation by DPP4. Targeted *Dpp4* deletion was achieved by crossing double floxxed *Dpp4* mice ($Dpp4^{flox/flox}$) with lines expressing Cre recombinases under the control of tissue-specific promoters, or injecting with an adeno-associated virus (AAV) expressing Cre under the control of the thyroxin binding globulin promoter (Mulvihill et al., 2017; Varin et al., 2019a). Interestingly, ablation of *Dpp4* in the endothelial cells ($Dpp4^{EC-/-}$) lead to a significant increase in both fasting and glucose-induced active GLP-1 and GIP,

and improved glucose tolerance after high-fat feeding, revealing cells of vascular endothelial origin to be critical for the glucoregulatory action of DPP4 (Mulvihill et al., 2017). In contrast, while enterocyte-derived DPP4 contributed substantially to the intestinal DPP4 activity, *Dpp4*$^{gut-/-}$ mice did not display improved plasma active incretin levels or glucose tolerance (Mulvihill et al., 2017). Similarly, genetic disruption of *Dpp4* in adipocytes (*Dpp4*$^{adipo-/-}$) reduced plasma DPP4 activity and improved hepatic insulin sensitivity, but had no effect on incretin levels or glucose homeostasis (Varin et al., 2019a). Finally, hepatocyte-targeted *Dpp4* elimination (*Dpp4*$^{hep-/-}$) led to an increase in fasting levels of active incretins, but was not associated with improvements in glucose tolerance or increased levels of active incretins following glucose administration (Varin et al., 2019a). Collectively, these findings underline the unexpected complexity of DPP4 biology and extend the idea of selective, tissue-specific DPP4 bioactivity in the regulation of incretin hormone action (Summarized in Fig.3).

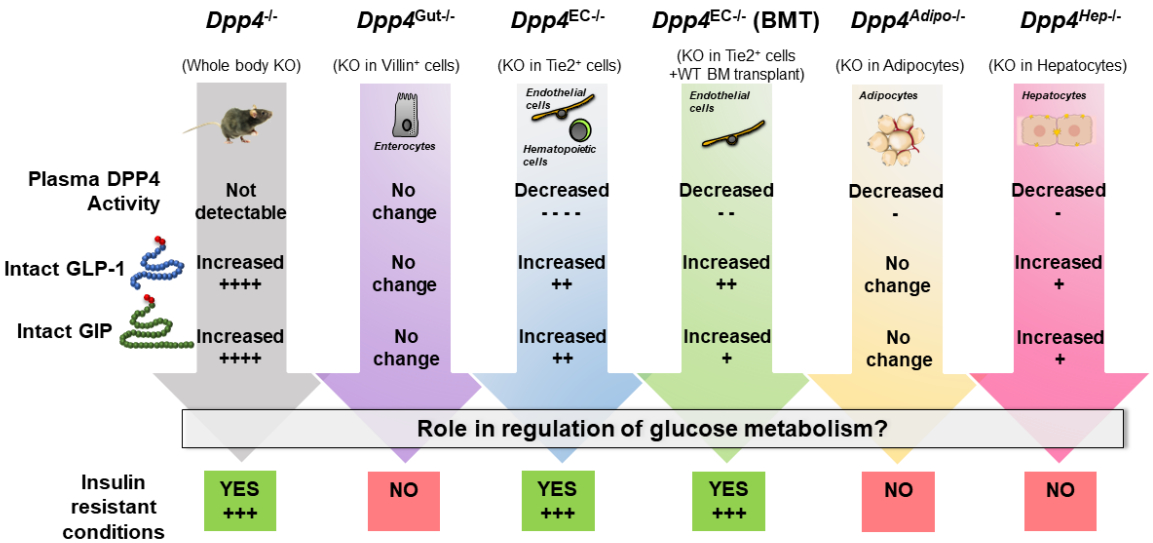

Figure 3. Cellular Sources of DPP4 and Control of the Incretin Axis

A graphical summary of the cellular sites of DPP4 action on incretin cleavage and glucose regulation in the insulin resistant state as determined by mouse genetics.

Adapted from Mulvihill et al., 2017; Varin et al., 2019.

1.4.4 Other Substrates of DPP4

Cleavage by DPP4 can inactivate peptides or generate new bioactive peptides, which allows for regulation of a wide range of biological processes. While many bioactive molecules have been demonstrated to be cleaved by DPP4 *in vitro*, very few of them are known to be physiological substrates *in vivo* (Mulvihill and Drucker, 2014). The challenges in identifying the physiological and pharmacological relevance of these peptides are many, and may include the inability to differentiate between cleaved and intact peptides due to the specificity or sensitivity of available immunoassays; absence of selective peptide antagonists; and difficulty in generating mouse knockout models for the receptors of interest (Mulvihill and Drucker, 2014). In addition to the incretin hormones, some other well-characterized substrates of DPP4 which may play a role in the islet include peptide tyrosine tyrosine (PYY), neuropeptide Y (NPY), and insulin-like growth factor-1 (IGF-1).

1.4.4.1 PYY

PYY, a 36 amino acid long peptide secreted from the intestinal L-cells has roles in satiety signaling, gastric emptying, gastric acid secretion, and fluid homeostasis (Gribble et al., 2018). PYY secretion is more effectively stimulated by lipids, and its plasma concentration is positively correlated with energy intake (Degen et al., 2005; Gibbons et al., 2013). Cleavage of PYY[1-36] by DPP4 generates the truncated peptide, PYY[3-36], which exhibits altered receptor selectivity. Unlike the full-length PYY[1-36] that binds to all Y receptor subtypes with equal affinity, the truncated PYY [3-36] stimulates an anorexigenic effect through high-affinity binding to the hypothalamic Y2 receptor, leading to a significant reduction in food intake (Batterham et al., 2002; Shi et al., 2013). Administration of exogenous PYY[3-36] during an intraperitoneal glucose tolerance test in mice has been shown to lower glycemia through GLP-1-mediated improvements in insulin secretion, while PYY[1-36] did not (Chandarana et al., 2013). Furthermore, PYY[1-36] was shown to inhibit glucose-stimulated insulin secretion in isolated islets, while PYY[3-36] had no effect (Chandarana et al., 2013). Finally, studies using PYY[1-36] and enzymatically stable PYY[1-36] analogues have demonstrated a significant increase in proliferation and protection from cytokine-induced apoptosis in treated BRIN BD11 and 1.1B4 β-cell lines (Lafferty et al., 2019). Collectively, these findings reveal the roles in metabolic processes for both intact PYY [1-36] and PYY[3-36], however, the biological relevance of the cleavage by DPP4 remains uncertain (Trzaskalski et al., 2020).

1.4.4.2 NPY

NPY[1-36] is a 36 amino acid long peptide, co-secreted with norepinephrine by sympathetic nerves and the adrenal medulla and involved in the regulation of mood, cardiovascular and immune functions, vasomotion, appetite, and neuroendocrine axis (Tan et al., 2018). It is implicated in the pathophysiology of several disease processes, and elevated plasma NPY is observed under stress conditions such as exercise, hypoxia, tissue injury, and ischemia (Tan et al., 2018). DPP4 cleaves NPY[1-36] to produce

NPY[3-36], a shorter peptide with a higher affinity for Y2 and Y5 receptors, which shifts its activity toward a pro-angiogenic profile (Li et al., 2011). Indeed, enhanced expression of DPP4 was found in migrating cells following cell wounding, and its enzymatic activity was required to mediate the angiogenic effects of NPY[3-36] in HUVEC cells (Ghersi et al., 2001). In a model of chronic myocardial ischemia, swine treated with NPY displayed a significant upregulation of both DPP4 and NPY receptors, with an elevated presence of factors like vascular endothelial growth factor (VEGF), endothelial Nitric Oxide Synthase (eNOS), and platelet-derived growth factors (PDGF). Moreover, treatment with exogenous NPY[3-36] improved myocardial function and increased angiogenesis by stimulating growth factor, pro-angiogenic receptor upregulation, and decreasing anti-angiogenic expression (Robich et al., 2010).

1.4.4.3 IGF-1

IGF-1 is a peptide hormone produced mainly by the liver and structurally similar to insulin (Wrigley et al., 2017). It acts downstream of the growth hormone (GH) and the growth hormone-releasing hormone (GHRH) to promote tissue growth and maturation via activating both MAP kinase and PI3K signaling pathways (Wrigley et al., 2017). IGF-1 was identified as a DPP4 substrate, however, *in vivo* DPP4 inhibition failed to increase active intact IGF-1 in plasma of pigs or male humans treated with DPP4 inhibitor sitagliptin (Bergman et al., 2006; Faidley et al., 2006; Frohman et al., 1989; Lin et al., 2010). Surprisingly, in a more recent study, treatment with sitagliptin enhanced stimulated GH secretion and free IGF-1 via GH receptor in women, but not men, potentially due to the sexual dimorphism of GH secretion (Wilson et al., 2018).

1.5 Incretin-Based Therapies: DPP4 Inhibitors and Incretin Mimetics

The great advancement of the incretin-based therapies is that they augment insulin secretion in a glucose-dependent manner, thus avoiding the risk of developing hypoglycemia if not taken properly. They are generally well-tolerated, do not induce weight gain and in addition to their glucoregulatory effect have been shown to attenuate the post-prandial rise in plasma triglycerides, free fatty acids, and intestinal lipoprotein production (Nauck et al., 2017). Incretin-based therapies aim to enhance the circulating concentrations of active incretin hormones and their receptor signaling, thus correcting the diminished incretin effect. Two classes of drugs, DPP4 inhibitors and GLP-1 receptor agonists (GLP-1RA), have now been established as therapeutic strategies for the treatment of T2D symptoms. DPP4 inhibitors work by selectively inhibiting the catalytic action of the DPP4 enzyme, preventing the degradation and inactivation of the incretin hormones and restoring glycemic control (Trzaskalski et al., 2020). DPP4 inhibitors have also been shown to improve glucose tolerance in mice with genetically eliminated GLP-1R (*Glp1r*$^{-/-}$), indicating DPP4i-mediated glucose regulation may occur via additional substrates or through a GLP-1-independent mechanism (Marguet et al., 2000). Another mechanism by which DPP4 inhibitors improve glycemic control is through inhibition of elevated glucagon secretion accompanied by a reduction in hepatic glucose production in T2D (Ahrén, 2007). As a result of the improved islet function, DPP4 inhibition may also improve insulin sensitivity, as observed through indirect measure of insulin sensitivity and hyperinsulinemic-

euglycemic clamp tests (Ahrén, 2007). DPP4 inhibitors currently approved for the clinical use have no reported effects on body weight, blood pressure, or heart rate, and were not found to increase major cardiovascular events or mortality (Kaneko and Narukawa, 2017; Nauck et al., 2019). DPP4 inhibitors tend to be the second-line therapy after metformin, and are often prescribed together to achieve euglycemia in patients with T2D. Patients on dual therapy experience the additive glucose-lowering effect of both drugs and a trend towards improved cardiovascular outcomes (Trzaskalski et al., 2020). Interestingly, DPP4 inhibitors also lowered the risk of developing autoimmune disease, including rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and systemic lupus erythematosus in patients with T2D compared to sulfonylurea combination therapy and thiazolidinediones combination therapy (Kim et al., 2015).

On the other hand, GLP-1RAs are structural homologues of endogenous GLP-1 that are resistant to degradation by DPP4 due to one amino acid substitution from Ala2 to Gly2. GLP-1RA treatment provides pharmacological levels of GLP-1, leading to enhanced glucose-dependent insulin secretion, decreased glucagon secretion, delayed gastric emptying, and increased satiety (Trujillo et al., 2015). In addition, they have been shown to reduce body weight, inflammation, and systolic blood pressure (Nauck et al., 2017). Unlike the DPP4 inhibitors, which are taken as oral tablets, GLP-1RAs are administered as subcutaneous injections. As such, their use may be limited by the adverse gastrointestinal effects, the need for the subcutaneous route of administration requiring self-administration education, and the cost of treatment (Trujillo et al., 2015). The effects of GLP-1 receptor agonists (GLP-1RAs) have also been assessed in humans for their cardioactive properties. GLP-1RAs were found to reduce blood pressure by 2 to 4 mmHg, reduce inflammation, and enhance vasorelaxation. Finally, two of the four GLP-1RAs (liraglutide and semaglutide) examined in CV outcome studies in humans demonstrated a significant reduction in major cardiovascular adverse effects, while another two (exenatide and lixisenatide) reported no changes (Holman et al., 2017; Mann et al., 2017; Marso et al., 2016b, 2016a; Pfeffer et al., 2015).

1.6 Rationale, Hypothesis, and Objectives

Pharmacological inhibition of DPP4 improves glycemia regulation through enhancing circulating levels of incretin hormones and potentiating insulin secretion in a glucose-dependent manner. Previous studies report DPP4 originating from endothelial cells, but not enterocytes, hepatocytes, or adipocytes to contribute substantially to circulating DPP4 activity, incretin degradation, and glucose control in vivo (Mulvihill et al., 2017; Varin et al., 2019). However, genetic elimination of DPP4 within endothelial cells could further be improved with systemic DPP4 inhibition, suggesting another cellular source of DPP4-mediated glucoregulation.

DPP4 expression has been observed in rodent, pig, and human islets, where it colocalized with the glucagon-secreting α-cells in humans and pigs, and β-cells in mice and rats. In addition, studies evaluating the role of islet-derived GLP-1 have found its input to be essential for maintaining glucose homeostasis, especially during metabolic stress induced by high-fat feeding. Therefore, due to its direct co-localization with the action site of the incretin hormones and GLP-1 secretion, it has been suggested that intra-islet DPP4 may be able to modulate β-cell function via a paracrine mechanism within the pancreatic islet.

This thesis will address the following hypothesis: islet-derived DPP4 is critical for the paracrine regulation of insulin secretion and glucose metabolism through intra-islet GLP-1 cleavage.

The aims of this thesis are as follows:

Objective 1: Localize and confirm DPP4 expression and activity in mouse pancreatic islets.

Objective 2: Describe dynamic hormone release and changes in local metabolic activity in islets isolated from mice with *Dpp4* specifically eliminated from islet β-cell (*Dpp4*$^{\beta\text{-cell-/-}}$) in response to perifusion with glucose (high/low concentration), KCl, GLP-1, GIP, glucagon, arginine, and DPP4 inhibitor sitagliptin.

Objective 3: Characterize incretin hormone levels, metabolic activity, and phenotype in mice with Dpp4 specifically eliminated from the islet β-cells (*Dpp4*$^{\beta\text{-cell-/-}}$) or the whole pancreas (*Dpp4*$^{Pan\text{-/-}}$) while on chow and after high-fat diet-induced obesity.

2. Materials and Methods

2.1 Animals, Diets, and Housing

Mice were housed under a 12-hour light/dark cycle at the University of Ottawa Heart Institute, and maintained on regular chow (Harlan Teklad, 2018, 18% kcal from fat) or HFD (Research Diets #D12451, 45% kcal from fat,) with free access to food and water, unless otherwise noted. For metabolic tests, food was removed at 8 am (5 hours prior to the tests), and replaced upon the completion of the test. Animals were cared for in accordance with the Canadian Guide for the Care and Use of Laboratory animals (CCAC). All experimental procedures were submitted approved under AUP #2909 and AUP #2020 by the University of Ottawa Animal Care and Veterinary Service (ACVS). *Dpp4*$^{-/-}$ mice were re-derived from a colony described previously (Marguet et al., 2000; Sauve et al., 2010). Flox *Dpp4* (*Dpp4*$^{fl/fl}$) mice (the LoxP sites span the catalytic serine in exon 22 of the *Dpp4* gene) were obtained from Merck Research Laboratories (Mulvihill et al., 2017). B6.Cg-Tg(Ins1-cre/ERT)1Lphi/J mice (Tamoxifen-inducible MIP-Cre) and B6.FVB-Tg(Pdx1-cre)6Tuv/J mice (PDX-Cre) were obtained from Jackson Laboratories (Bar Harbor, ME USA). B6;129S4-Gcgem1(cre/ERT2)Khk/Mmjax mice (GCG-Cre) were obtained from Jackson Laboratories (Bar Harbor, ME, USA).

2.2 Generating *Dpp4*$^{\beta\text{-cell-/-}}$ and *Dpp4*$^{Pan-/-}$ Mice

To generate β-cell specific *Dpp4* knockout mice, MIP-Cre-expressing mice were crossed with *Dpp4*$^{fl/fl}$ mice, resulting in *Dpp4*$^{fl/+}$ (loxP heterozygous) offspring. Intercrossing Cre+ and Cre- *Dpp4* loxP heterozygotes resulted in four genotypes: wildtype with no Cre (WT), wildtype expressing Cre (MIP-Cre), double floxxed *Dpp4* with no Cre (*Dpp4*$^{fl/fl}$) and double floxxed *Dpp4* mice expressing Cre (*Dpp4*$^{\beta\text{-cell-/-}}$). Tail or ear clips were collected from newly weaned mice, and the genomic DNA was isolated for genotyping. Primers used for genotyping are summarized in the table below (Table 1).

	Forward	Reverse
Dpp4$^{fl/fl}$	GAA TAT GAT CCT TGT CAG AGC AGC C	CTG CAC TCA GAA GTC TCA CTG
Cre	ATC CGA AAA GAA AAC GTT GA	ATC CAG GTT ACG GAT ATA GT

Table 1. Primers for Genotyping *Dpp4*$^{\beta\text{-cell-/-}}$ and Control Mice

MIP-Cre recombinase activity was induced by administering tamoxifen (Sigma-Adrich, #T5648) in corn oil (50 mg/mL) at 5 mg/mouse/day for 5 continuous days to all groups. Similarly, to obtain the pancreas specific *Dpp4* knockout, we crossed PDX-Cre-expressing mice with *Dpp4*$^{fl/fl}$ mice, generating *Dpp4*$^{fl/+}$ offspring. We then crossed Cre+ and Cre- *Dpp4*$^{fl/+}$ mice, which produced four genotypes: wildtype with no Cre (WT), wildtype expressing Cre (PDX-Cre), double floxxed *Dpp4* with no Cre (*Dpp4*$^{fl/fl}$) and double floxxed *Dpp4* mice expressing Cre (*Dpp4*$^{Pan-/-}$). The knockouts were assessed by tissue-specific DPP4 activity assay and immunofluorescent analysis of pancreatic and liver (control) sections with a DPP4-

specific antibody. Experiments were carried out in all groups described above as well as in full-body *Dpp4* knockout group (*Dpp4*$^{-/-}$). All experiments used age- and sex-matched littermates as control subjects.

2.3 Weight and Body Composition

Body weight was measured and recorded once every week. Body composition was measured by EchoMRI quantitative nuclear magnetic resonance (NMR) system (Houston, TX, USA) once every 4 weeks and before sacrifice. Fat (%) was calculated as 100% x (fat mass (g)) / (total mass (g)).

2.4 Glucose and Insulin Tolerance Tests

For glucose tolerance tests, mice were fasted for 5 hours, and then administered water or sitagliptin (Januvia, 10 mg/kg) by oral gavage. Thirty minutes later, mice were administered glucose in PBS (2 g/kg of body weight) by oral gavage (oGTT) or by intraperitoneal (*i.p.*) injection (ipGTT). For insulin tolerance tests, mice were injected *i.p.* with 0.6 IU/kg (Humalog, Lilly) in PBS. Blood for glucose measurements (Medisure glucometer) was collected via tail vein every 15 minutes up to 90 minutes post-glucose administration. Blood for active incretin measurements was collected 30 minutes prior to and 15 minutes after glucose administration in K^2EDTA coated tubes with the addition of 10% v/v DPP4 inhibitor (Aprotinin, Fisher BioReagents 9087-70-1).

2.5 Tissue Collection

Animals were euthanized by isoflurane inhalation and blood was collected by via cardiac puncture. The skin and abdomen were dissected, and the heart, kidney, liver, spleen, and the gut were removed, weighed, and flash-frozen in liquid nitrogen and stored at -80°C for future analyses. Separately, liver sections and the pancreas were removed, weighed, and fixed in 4% paraformaldehyde for 24-48 hours, and stored in 70% ethanol for histological analyses.

2.6 Gene Expression

RNA was isolated from tissues using Trizol reagent (Life Technologies, Mississauga, Ontario, Canada). First-strand cDNA was synthesized from total RNA using the SuperScript III synthesis system (Invitrogen). Specific mRNA abundances were measured by quantitative real-time PCR (qRT-PCR) on an ABI Prism Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. The standard curve method was used to determine mRNA concentrations, and each gene was normalized to cyclophilin (Ppia) expression.

2.7 Pancreas Perfusion and Isolation

Mice were fasted for 16 hours overnight, starting at the end of the light cycle prior to sacrifice. Animals were euthanized by isoflurane inhalation and cervical dislocation, and blood was collected via cardiac puncture. Mice were placed on a surgical board in the supine position and sterilized with 70% EtOH.

The skin and abdominal muscles were dissected to expose the viscera. The pancreatic duct was clamped with hemostatic forceps, and 5 mL of cold sterile collagenase solution (7.5 mg/mL collagenase (Sigma C7657) in HBSS (5 mM glucose, 1 mM $MgCl_2$) was injected via the common bile duct to distend the pancreas. The pancreas was then excised, placed into a 50 mL centrifuge tube containing 10 mL of the cold collagenase solution, and kept on ice until incubation.

2.8 Islet Isolation and Purification

Tubes with distended pancreata were incubated in a 37°C water bath for 12 minutes, after which they were manually shaken 15-20 times to disrupt the tissues. The digestion process was stopped with the addition of 25 mL of ice-cold HBSS to each sample (5 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$) and centrifugation at 1000 rpm for 1 minute. The supernatant was removed, and the wash process was repeated for a total of 3 times. The final pellet was resuspended in 5 mL of complete RPMI medium (10% FBS, 1% penicillin/streptomycin), and 5 mL of Histopaque (Sigma, 10771) were underlaid to each tube. Samples were centrifuged at 2400 rpm for 18 minutes, and the islets collected at the Histopaque-medium interface were collected into 10 cm petri dishes containing 6 mL of RPMI media. Islets were placed in the incubator at 37°C, 5% CO_2 for 10 minutes for recovery, and then handpicked into a new 3.5 cm dish using a P10 pipette. Handpicking was repeated to achieve purity of 98-100%, and islets were placed in a cell culture incubator at 37°C, 5%CO_2 for 16-24 hours to recover before perifusion experiments.

2.9 Islet Perifusion

Biorep Perifusion System (Biorep Technologies) was used to stimulate and collect the secretions of pancreatic islets *in vitro* for all perifusion experiments. All solutions were prepared in fresh KRBH buffer (115 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 24 mM $NaHCO_3$, 10 mM HEPES, and 1% BSA; pH = 7.4). After incubation, equal numbers of islets (50 or 75-80 islets per mouse) were handpicked and placed into tubes containing 2.8 mM glucose-containing KRBH buffer. Perifusion chambers were assembled according to the manufacturer's manual, and islet samples were placed between two layers of the polyacrylamide bead suspension (Bio-Gel P-4, Biorep). Islets were equilibrated for 48 minutes with the 2.8 mM glucose-containing buffer, and then perifused in intervals based on the experimental conditions. Flow rate (100 µl/min) and temperature (37°C) remained constant for every experiment, and the type of treatment is indicated at the top of each figure (Arginine, Sigma, #A5006; GLP-1 7-36, Bachem; GIP – (D-Ala^2)-GIP, Bachem #4054476; glucagon, Lilly). Effluent fractions were collected at 2-minute intervals into clear 96-well plates kept at 4-8°C during collection, which were then frozen and kept at -80°C for further assays.

2.10 BCA Assay

For use in the BCA and DPP4 activity assays, tissues were homogenized using a liquid nitrogen-cooled mini mortar and pestle set. Homogenization/lysis buffer (0.1mM EDTA, 1% v/v glycerol, 0.0067% v/v Brij-35) was added to powderized samples, which were then left on ice for 10 minutes to promote lysis.

Samples were then centrifuged at 10000 rpm for 10 minutes at 4°C, and the supernatant was collected and used in the assays immediately. The bicinchoninic acid (BCA) assay (Pierce Rapid Gold BCA Protein Assay Kit, Thermo Fisher) was used to quantify protein concentration in tissue and cell/islet samples according to the manufacturer's instructions. Briefly, 20 µL of sample or BSA standard were added per well of a clear 96-well plate, followed by 200 µL of working reagent mix. After 5 minutes of incubation at room temperature with gentle shaking, absorbance was measured on a Biotek Microplate Reader at 480 nm. The concentration of unknown samples was calculated based on the standard curve generated from BSA standards provided and expressed in µg/mL.

2.11 DPP4 Activity

DPP4 enzymatic activity in tissue lysates, cell lysates, and plasma samples was measured by a fluorometric assay. All samples were prepared as described for the BCA assay. Then, 10 µL of plasma or 25 µL of tissue/cell homogenate sample were incubated with 8 mM $MgCl_2$ and 50 µL of AMC substrate (H-Gly-Pro-AMC, Bachem, USA) in a black flat-bottom 96-well plate for 20 minutes at RT with gentle shaking. Fluorescence intensity was measured on a Biotek Microplate Reader at 450 nm, and the enzymatic activity of each sample was calculated from the standard curve generated based on the blank-corrected standards. Tissue DPP4 activity was normalized to total protein concentration in a sample and expressed as nmol AMC/minute/ng of protein (or nmol AMC/mL for plasma).

2.12 Circulating DPP4 Concentration

Mouse DPP4 ELISA (Mouse DPPIV/CD26 DuoSet ELISA, R&D Systems) was used to measure circulating plasma DPP4 concentrations in plasma without a DPP4 inhibitor according to the manufacturer's protocol. Samples were diluted 1:100 with reagent diluent (1% BSA in PBS) prior to the assay, and 100 µL of diluted sample or standard were added per well. Absorbance was measured on a Biotek Microplate Reader at 450 nm, and measurements at 540 nm were subtracted to correct for optical imperfections of the plate. Circulating DPP4 concentration was calculated using a standard curve generated from the standards of known concentrations and expressed in ng/mL.

2.13 Active GIP Measurements

Plasma was collected with a DPP4 inhibitor 15 minutes post-glucose administration and assayed using active GIP ELISA kit (CrystalChem, USA) according to the manufacturer's instructions. Briefly, 15 µl of sample was added to each well and incubated for 2 hours at room temperature with gentle shaking. The plate was washed, followed by a 1-hour incubation with HRP-labelled antibody. After adding the substrate and the stop solution, optical density was measured on a Biotek Microplate Reader at 460 and 630 nm, and measurements at 630 nm were subtracted to correct for optical imperfections of the plate. Active GIP concentration was calculated using a standard curve generated from the standards of known concentrations and expressed in pM.

2.14 Insulin Measurements

Mouse Ultrasensitive Insulin ELISA kit (ALPCO 80-INSMSU) or Mouse High Range Insulin ELISA kit (ALPCO 80-INSMSH) were used to measure insulin concentrations in mouse plasma and perifusion fractions, respectively. Briefly, 5 µL of sample or standard and 75 µL of conjugate solution were added to each well and incubated at room temperature at 800 rpm. The plate was then washed and incubated with 100 µL of TMB substrate per well for 15 minutes. 100 µL of stop solution was added, and absorbance at 450 nm was measured using Biotek Microplate Reader. Sample concentrations were calculated using a 5-parameter logistic curve fit.

2.15 Immunofluorescent Analysis

Tissues (pancreas, liver) were fixed in 4% paraformaldehyde for 24-48 hours at room temperature before paraffin wax embedding. Embedded tissues were sectioned into 5 µm-thick sections and deparaffinized using a series of xylene and alcohol washes at 5 minutes per wash. Heat-induced antigen retrieval was performed using sodium citrate buffer. Slides were heated in a 10mM sodium citrate buffer until boiling using a microwave, then allowed to cool for 15 minutes and washed with cold water. All sections were blocked with 10% horse serum in PBS for 2 hours at room temperature prior to staining. Sections were incubated with primary antibodies overnight at 4°C followed by secondary antibody incubation for 2 hours, and with DAPI for 5 minutes in the dark at room temperature. Fluorescent images were captured with Zeiss fluorescent microscope and processed with Zen software package.

Primary antibodies used: polyclonal goat anti-mouse DPP-4/CD 26 (1:50, R&D Systems, AF954), monoclonal rabbit anti-mouse insulin (1:200, Abcam, ab181547); monoclonal rabbit anti-mouse glucagon (1:200, Abcam, ab92517). Secondary antibodies used: Alexa Fluor 488 goat anti-rabbit antibody (1:1000, Abcam, ab6702), Alexa Fluor 555 donkey anti-goat antibody (1:1000, Abcam, ab150134), Alexa Fluor 488 donkey anti-goat antibody (1:1000, ab150129).

2.16 Statistical analysis

All data were expressed as the mean ± SEM, unless stated otherwise. Comparisons between groups were performed using One- or Two-Way ANOVA with post-hoc Tukey correction using GraphPad Prism 8. Area Under the Curve (AUC) analysis was performed using GraphPad Prism 8. Statistically significant differences are indicated as *$p \leq 05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$. For AUC graphs, different letters indicate statistical differences between groups.

3. Results

3.1 Activation of the GLP-1R and GIPR Within the β-cell Is Critical for Regulation of Glucose by DPP4i

Previous studies have demonstrated the glucoregulatory effects of the DPP4 inhibitor sitagliptin are dependant on the activation of GLP-1 and GIP receptors (Waget et al., 2011). However, doubts have been raised as to whether receptors in the islet mediate the entirety of the glucose-lowering response or if receptors within the portal circulation, enteric nervous system, or central nervous system also substantially contribute (Aulinger et al., 2020; Varin et al., 2019b). Here, we assessed the importance of intra-islet incretin receptors for DPP4i-dependent glucose-lowering using a murine model with selected elimination of both GLP1R and GIPR within β-cells: the Double Incretin Receptor Knock-Out (DIRKO). While both low (14 µg/mouse, selective enteral inhibition) and high (10 mg/kg body weight, systemic inhibition) concentrations of sitagliptin were able to reduce glycemia and total area under the curve in response to oral glucose gavage in control mice (Fig.3.1 A, C), it had no effect on glucose clearance in the DIRKO group (Fig.3.1 B, C). Since the expression of *Gipr* and *Glp1r* mRNA is known to be relatively high in the white adipose tissue (WAT) and the lung, respectively, compared to other tissues (Beaudry et al., 2019; Viby et al., 2013; Zhu et al., 2019), we analyzed the islet, jejunum, WAT, and lung tissue lysates by PCR to verify the β-cell-specific incretin receptor elimination. The levels of *Gipr* and *Glp1r* mRNA transcripts were significantly reduced in islets isolated from DIRKO mice compared to control (mixed group of MIP-Cre, $Dpp4^{fl/fl}$), with no differences detected in the relative expression of *Gipr* and *Glp1r* in the jejunum, epididymal white adipose tissue (eWAT), and the lung (Fig.3.1 D-F).

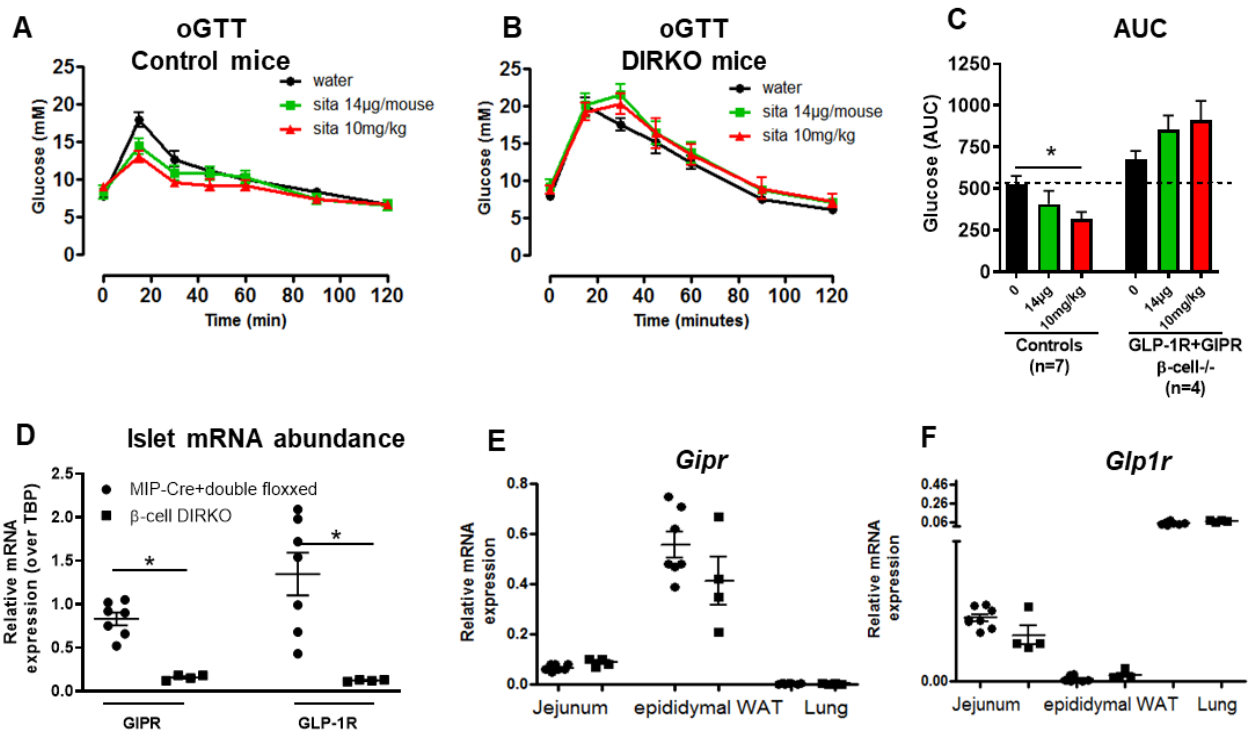

Figure 3.1. Activation of the GLP-1R and GIPR Within the β-cell Is Critical for Regulation of Glucose by DPP4i

(A) Oral glucose tolerance test (2 g/kg) in control (MIP-Cre and *Dpp4*$^{fl/fl}$) or (B) β-cell-specific double incretin receptor knockout (DIRKO) mice administered water or sitagliptin (14 µg/mouse or 10 mg/kg) by oral gavage 30 min prior to gavage of glucose. (C) Area under the curve depicting blood excursions during oGTTs in control (n=7) versus DIRKO (n=4) mice. (D) Abundance of *Gipr* and *Glp1r* mRNA in islets of control and β-cell-specific DIRKO mice (normalized to *Ppia*). (E) Relative expression of *Gipr* and (F) *Glp1r* mRNA transcripts in whole extracts of jejunum, epididymal white adipose tissue (WAT), and lung tissue (normalized to *Ppia*). Data are represented as the mean ± SEM, * indicates *p<0.05.*

3.2 Genetic Elimination of *Dpp4* Leads to Increased Pancreatic Insulin Content in HFD-Fed Mice, but Has No Significant Effects On Islet Function While On Regular Chow

Following our findings, we set out to investigate the role of endogenous DPP4 in islet physiology and function. First, we found no significant differences in islet *Dpp4* mRNA transcript levels between wildtype C57BL6J mice fed chow or a HFD (Fig.3.2 A). Interestingly, genetic whole-body *Dpp4* knockout resulted in significantly elevated pancreatic insulin content compared to WT controls in young mice fed a HFD, but had no effects while on regular chow (Fig.3.2 B). To evaluate the role of DPP4 in the dynamic hormone release from the islet, we isolated islets from 45-50 week-old chow-fed mice and sequentially perifused them with a buffer containing glucose, arginine, GLP-1, and KCl with and without the addition of DPP4 inhibitor sitagliptin. Interestingly, sitagliptin did not increase glucose-stimulated insulin secretion (GSIS) in islets isolated from chow-fed WT (Fig.3.2 C) or *Dpp4*$^{-/-}$ mice compared to their untreated controls (Fig.3.2 D). In addition, no significant differences in GSIS were detected between WT and *Dpp4*$^{-/-}$ islets (Fig.3.2 E), suggesting that intra-islet DPP4 is not a significant regulator of islet function in chow-fed mice.

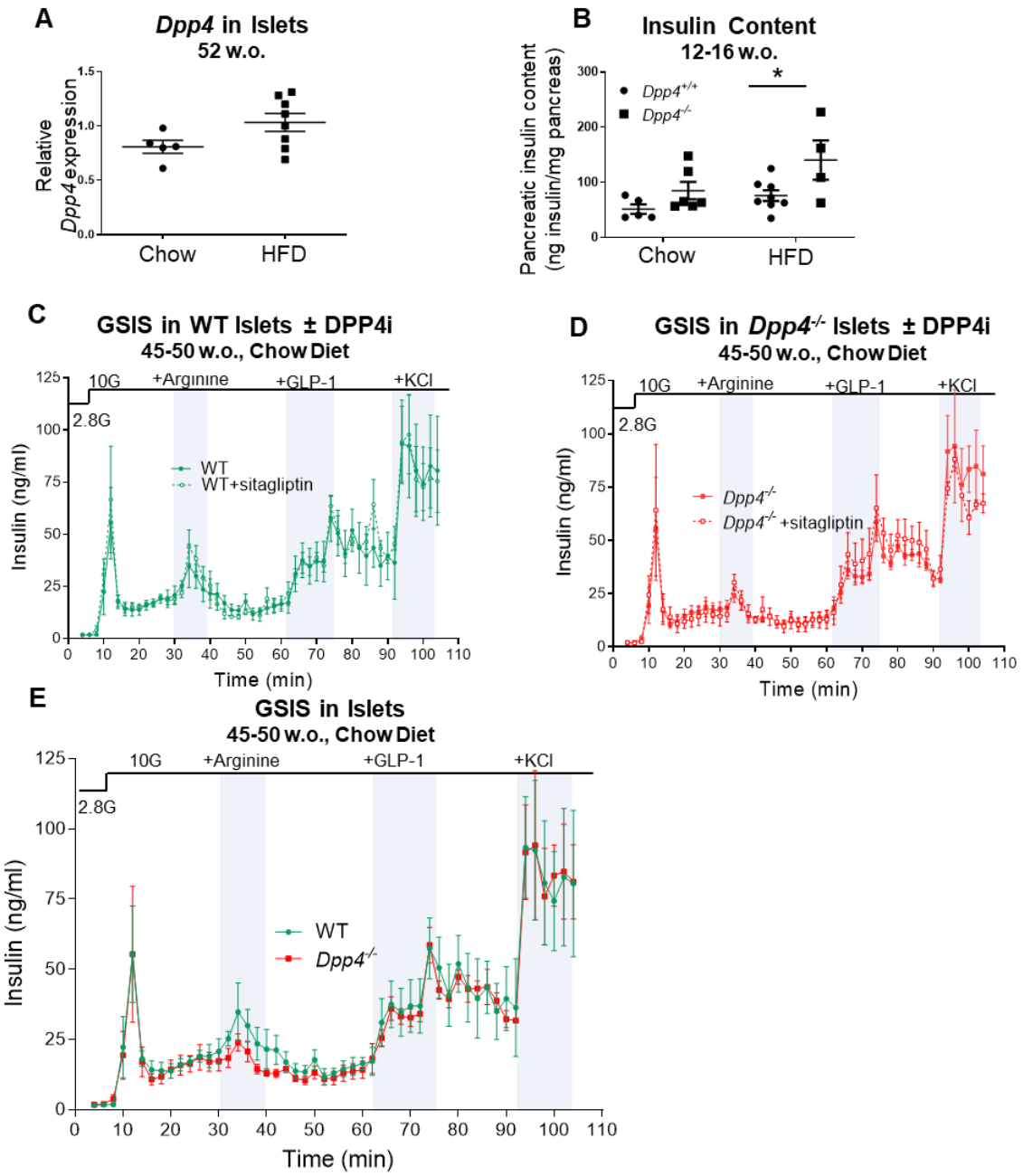

Figure 3.2. Elimination of *Dpp4* Leads to Increased Pancreatic Insulin Content in HFD-Fed Mice, but Has No Significant Effects On Islet Function While On Regular Chow.

(A) Relative expression of *Dpp4* mRNA in islets isolated from wildtype C57BL6J mice fed a chow diet or mice fed a high-fat diet for 52 weeks. (B) Insulin content measured in the pancreas of 12-week old *Dpp4*$^{+/+}$ or *Dpp4*$^{-/-}$ littermate controls fed chow or after continuing on a HFD (+4 weeks). (C-E) Insulin secretion measured in islets isolated from 45-50-week-old chow-fed mice in perifusion (n=4 per group). Following a 48-minute equilibration with 2.8 mM glucose-containing buffer, islets were sequentially perifused with 10 mM glucose buffer containing stimuli as indicated at the top of each graph. Sitagliptin (200nM) was added directly to the buffer solution. Data are represented as the mean ± SEM, * indicates $p<0.05$.

3.3 *Dpp4*$^{\beta\text{-cell-/-}}$ Mice Have Significantly Reduced Islet DPP4 Activity and Expression

Based on our findings in HFD-fed *Dpp4*$^{-/-}$ mice, we hypothesized that intra-islet DPP4 may have a distinct role in glucose regulation under the condition of higher metabolic demand. To evaluate its contribution to islet function, we created a β-cell-specific *Dpp4* knockout model using a *Dpp4*$^{fl/fl}$ line expressing a tamoxifen-inducible Cre under the control of Mouse Insulin Promoter (MIP-Cre). At 8-10 weeks, MIP-Cre recombinase activity was induced by oral administration of tamoxifen in corn oil (50 mg/ml) at 5 mg/mouse/day for 5 consecutive days to all groups (*Dpp4*$^{+/+}$, *Dpp4*$^{-/-}$, MIP-Cre, *Dpp4*$^{\beta\text{-cell-/-}}$, and *Dpp4*$^{fl/f}$). Cre-positive mice with homozygous floxxed *Dpp4* (*Dpp4*$^{fl/fl}$ Mip-Cre+) were considered *Dpp4*$^{\beta\text{-cell-/-}}$ (Fig.3.3 A) with recombination limited to β-cell. Mice expressing MIP-Cre and homozygous wildtype *Dpp4* allele (*Dpp4*$^{+/+}$ Mip-Cre+) were denoted as MIP-Cre and used as a Cre-positive control (Fig.2 A). Using a fluorometric DPP4 activity assay we found a significant decrease in DPP4 activity in islets isolated from chow-fed *Dpp4*$^{\beta\text{-cell-/-}}$ mice compared to their age- and weight-matched controls (*Dpp4*$^{+/+}$, MIP-Cre, *Dpp4*$^{fl/fl}$), with no significant changes observed in other tissues (Fig.3.3 B). The residual islet DPP4 activity measured by the assay could be attributed to DPP4 found in other cell types within the islet (α-cell, δ-cell), as well as the activity of DPP4-like (DASH) proteins (Wagner et al., 2016). We further confirmed the reduction in β-cell-derived DPP4 protein using fluorescence microscopy of paraformaldehyde-fixed pancreatic sections taken at sacrifice, with representative images shown in Fig.3.3 C. Paraffin blocks containing tissues of interest from 40-week-old HFD-fed male mice were sectioned to 5 μm-thick sections using a microtome, and stained with DPP4-specific antibody as described in methods. Wildtype mice exhibited uniform DPP4-positive staining within the islet (Fig.3.3 C, top left panel), while *Dpp4*$^{\beta\text{-cell-/-}}$ mice had greatly reduced expression (Fig.3.3 C, middle left panel). Confirming the tissue-specific knockout, no differences in DPP4 protein expression were observed in fixed liver sections of both groups (Fig.3 top right, middle right panels). Furthermore, no detectable DPP4 expression was found in the pancreas or liver of the whole-body *Dpp4* knockout (*Dpp4*$^{-/-}$) group (Fig.3.3 C bottom panels).

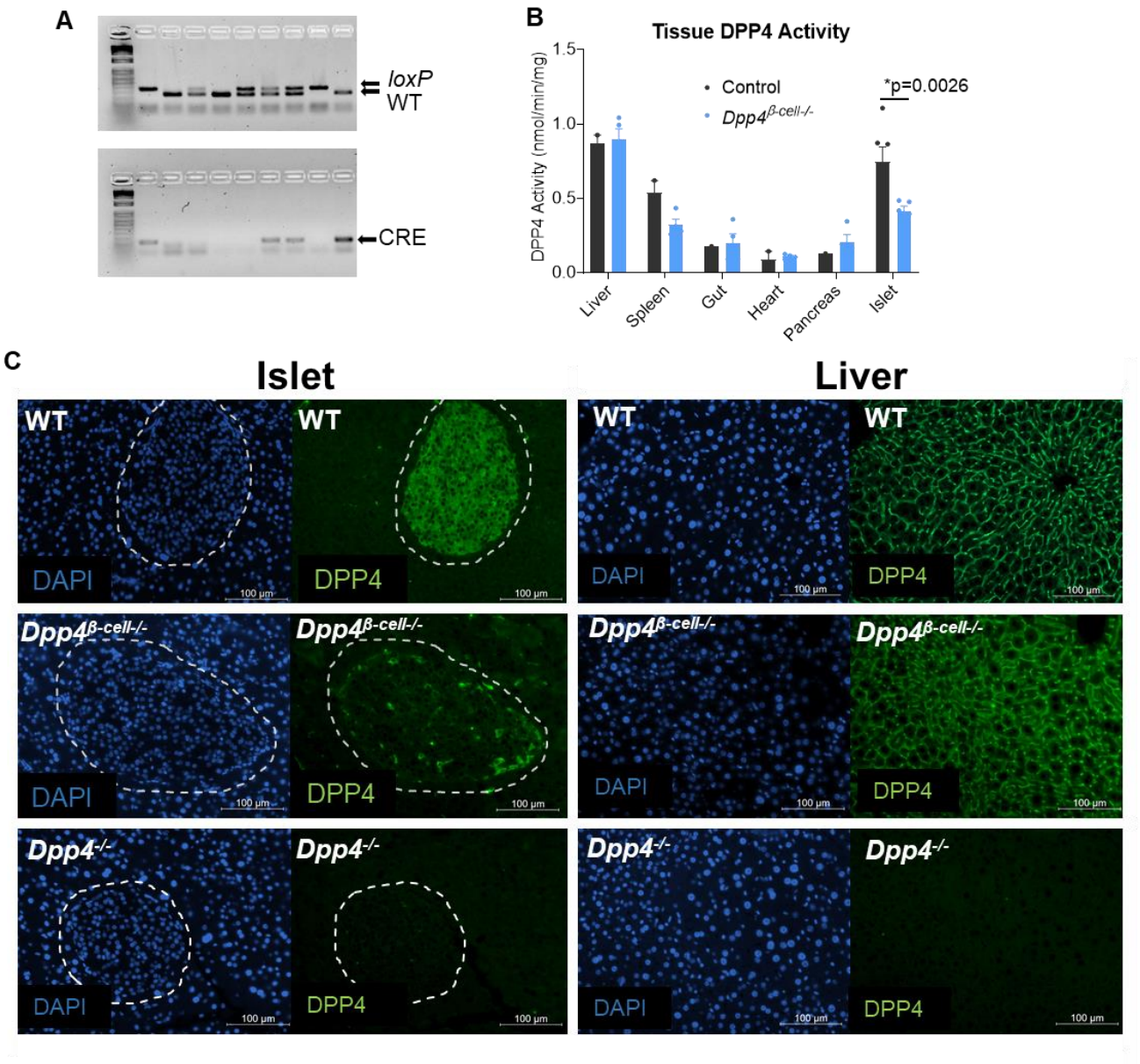

Figure 3.3. Islets from *Dpp4*$^{\beta\text{-cell-/-}}$ Mice Have Significantly Reduced Islet DPP4 Activity and Expression.

(A) Representative genotyping gel used to identify WT, *Dpp4*$^{wt/fl}$, and *Dpp4*$^{fl/fl}$ mice (top), as well as Cre+ or Cre- status (bottom). (B) DPP4 activity normalized to total protein measured in whole extracts of tissues (liver, spleen, gut, heart, pancreas, islets from control (WT, Mic-Cre, *Dpp4*$^{fl/fl}$) and *Dpp4*$^{\beta\text{-cell-/-}}$ young, chow-fed male mice (n=2-6 per group, except gut and pancreas where n=1-6). Data are represented as the mean ± SEM, * indicates *p<0.05.* (C) Immunofluorescence staining showing DPP4 expression (green) in paraffin-fixed mouse pancreatic sections (WT – top, *Dpp4*$^{\beta\text{-cell-/-}}$ - middle, *Dpp4*$^{-/-}$ - bottom). DAPI staining (blue) was used to identify nuclei; white dashed lines are used to outline islets; liver stained as a positive control.

3.4 Islets from HFD-Fed *Dpp4*$^{\beta\text{-cell-/-}}$ Mice Exhibit Increased Glucose-Stimulated Insulin Secretion in Perifusion

Once the knockout model was confirmed, we set out to evaluate the role of intra-islet DPP4 on islet secretory function *in vitro.* All mice were fed a HFD for 15 weeks and sacrificed at 40 weeks. Pancreatic islets were isolated from *Dpp4*$^{\beta\text{-cell-/-}}$ and control (*Dpp4*$^{+/+}$, MIP-Cre, *Dpp4*$^{fl/fl}$) mice using collagenase-aided digestion of the pancreas, followed by density gradient separation with Histopaque. While basal and KCl-induced insulin release remained similar between groups, a trend toward higher glucose-stimulated insulin secretion (GSIS) was observed in *Dpp4*$^{\beta\text{-cell-/-}}$ islets compared to control in response to perifusion with high (16.7 mM) concentration of glucose (Fig.3.4 A). In addition, total insulin released in response to stimulation, depicted by the area under the curve (AUC) analysis, was significantly higher in the *Dpp4*$^{\beta\text{-cell-/-}}$ mice compared to control (Fig.3.4 A).

To further investigate these differences, we compared GSIS in islets from the two groups with and without the addition of a highly potent and specific DPP4 inhibitor, sitagliptin (Sangle et al., 2012). Interestingly, when perifused with the same stimuli, addition of sitagliptin significantly increased both first- and second-phase GSIS, as well as total insulin secreted in islets from control mice (n=9), but had no measurable effects on islets with DPP4 eliminated from the β-cell (n=2) (Fig.3.4 B, C). Of note, a conclusive interpretation of these results is limited due to a small sample size of the *Dpp4*$^{\beta\text{-cell-/-}}$ group. However, the difference in GSIS between the control and *Dpp4*$^{\beta\text{-cell-/-}}$ islets (Fig.3.4 A), combined with the marked increase in GSIS of the sitagliptin-treated versus untreated control islets (Fig.3.4 B) strongly suggests that the catalytic action of DPP4 is a regulator of glucose-stimulated islet insulin secretion *in vitro.*

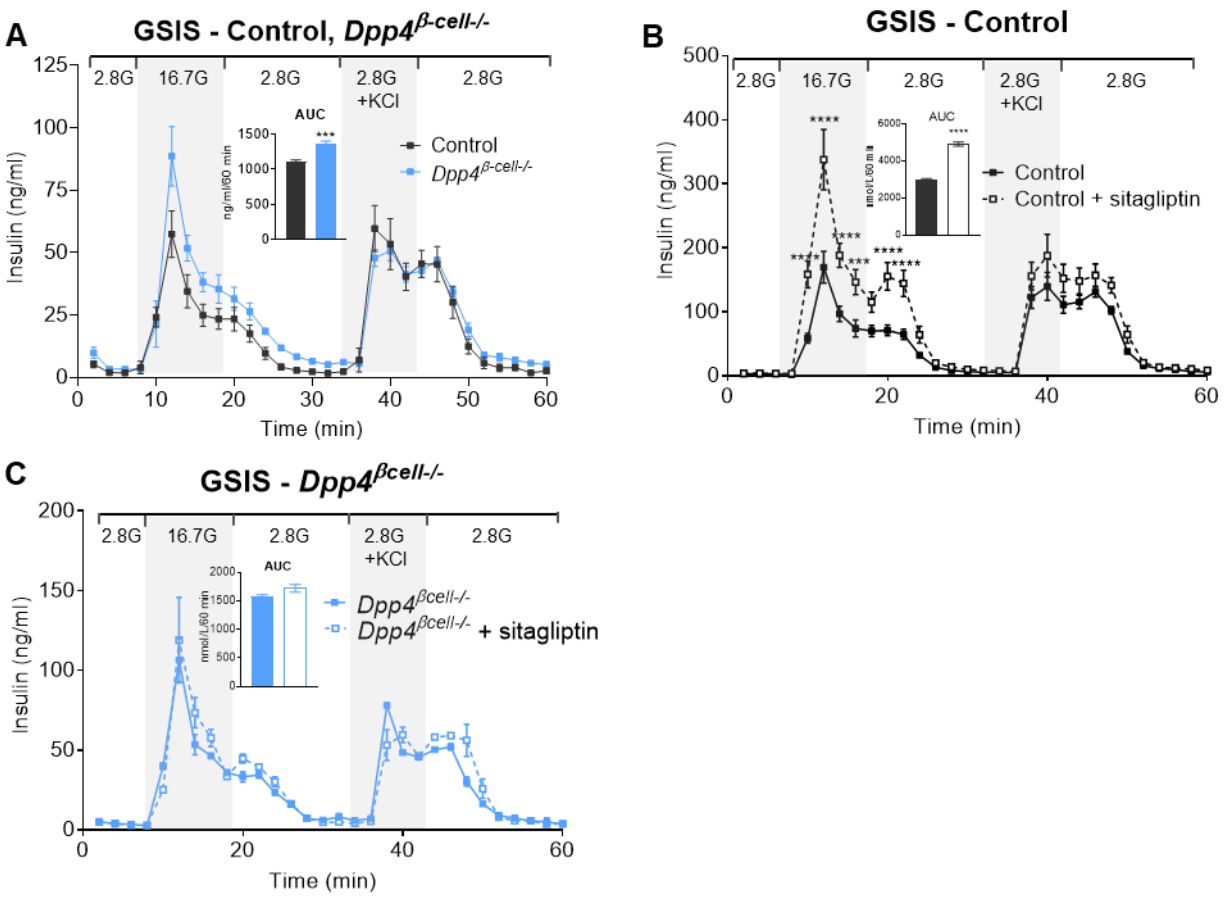

Figure 3.4. Islets from HFD-Fed *Dpp4*$^{\beta\text{-cell-/-}}$ Mice Exhibit Increased Glucose-Stimulated Insulin Secretion in Perifusion

(A) Glucose-stimulated insulin secretion (GSIS) measured in islets isolated from control (WT, *Dpp4*$^{fl/fl}$, and MIP-Cre, n=9) and *Dpp4*$^{\beta\text{-cell-/-}}$ (n=4) 40-week-old HFD-fed male mice in response to perifusion with buffer containing 2.8 mM glucose, 16.7 mM glucose, or 2.8 mM glucose + KCl. (B) GSIS measured in islets isolated from control (WT, *Dpp4*$^{fl/fl}$, and MIP-Cre, n=9) 40-week-old HFD-fed male mice in response to perifusion with glucose with and without DPP4 inhibitor sitagliptin. (C) GSIS in islets from *Dpp4*$^{\beta\text{-cell-/-}}$ 40-week-old HFD-fed male mice in response to perifusion with glucose with and without DPP4 inhibitor sitagliptin (n=2). Data are represented as the mean ±SEM. ***$p<0.001$, ****$p<0.0001$.

3.5 *Dpp4*$^{\beta\text{-cell-/-}}$ Male Mice on Chow Diet Exhibit Normal Glucose Tolerance and Retain Glucoregulatory Responses to Sitagliptin

To directly assess the importance of β-cell-derived DPP4 on glucose metabolism *in-vivo*, we subjected mice with MIP-Cre-targeted elimination of *Dpp4* (*Dpp4*$^{\beta\text{-cell-/-}}$) and littermate MIP-Cre-positive control mice (MIP-Cre) to a series of glucose and insulin tolerance tests while on regular chow or after being fed a HFD. Prior to the glucose tests, mice were gavaged with a high concentration of the DPP4 inhibitor sitagliptin (10 mg/kg of body weight) to induce systemic DPP4 inhibition, or an equivalent amount of water. MIP-Cre and *Dpp4*$^{\beta\text{-cell-/-}}$ mice exhibited similar glucose excursions in response to oral glucose gavage (2 g/kg), and intraperitoneal glucose injection (2 g/kg) on chow diet (Fig.3.5 A, B), with no significant differences in fasting glucose. Systemic DPP4 inhibition with sitagliptin prior to oral glucose test improved glucose excursion in both groups, leading to a 40% reduction in the initial glucose spike from baseline in the MIP-Cre and 45% in the *Dpp4*$^{\beta\text{-cell-/-}}$ group (Fig.3.5 A). Unsurprisingly, sitagliptin had no favourable effects on glucose metabolism during an *i.p.* glucose challenge, a setting in which plasma active incretin concentrations remain low (Fig.3.5 B). Lastly, insulin sensitivity was slightly decreased in the *Dpp4*$^{\beta\text{-cell-/-}}$ group, as demonstrated by the AUC analysis of total insulin response (Fig.3.5 C).

In a separate experiment, we collected plasma from this cohort following a 5-hour fast without adding a DPP4i. Unexpectedly, plasma DPP4 activity was elevated in the *Dpp4*$^{\beta\text{-cell-/-}}$ group compared to the age- and sex-matched MIP-Cre control group, but no significant differences were found in plasma DPP4 concentration between all groups fed a chow diet (Fig.3.5 D, E).

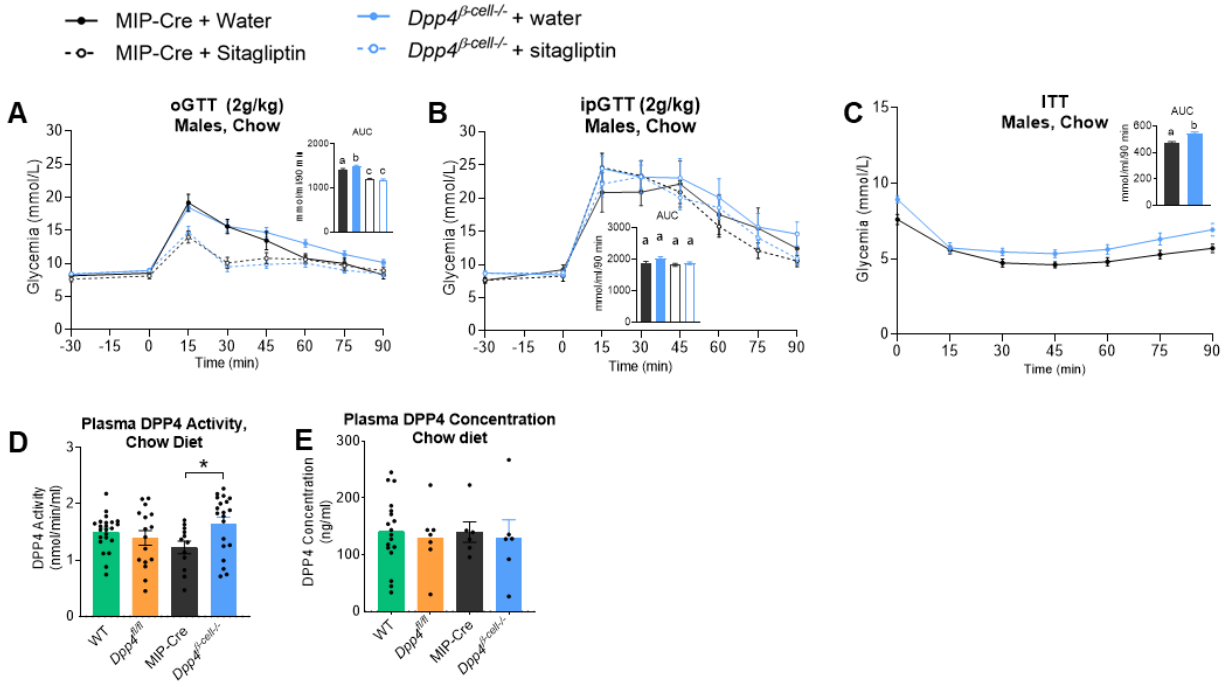

Figure 3.5. *Dpp4*$^{\beta\text{-cell-/-}}$ Male Mice on Chow Diet Exhibit Normal Glucose Tolerance and Retain Glucoregulatory Responses to Sitagliptin.

(A) Oral glucose, (B) *i.p.* glucose, and (C) insulin tolerance tests in chow-fed *Dpp4*$^{\beta\text{-cell-/-}}$ (n=21) and MIP-Cre (n=12) control mice. Sitagliptin was given by oral gavage 30 minutes prior to glucose tolerance tests. A solution of 30% glucose in PBS was given at 2 g/kg of body weight either orally or via an *i.p.* injection. Insulin was injected at 0.6IU/kg of body weight. Inset graphs represent area under the curve (AUC) for each group and treatment, and different letters indicate statistical differences between groups by ANOVA with post-hoc Tukey test. (D) DPP4 activity and (E) concentration measured in plasma of chow-fed wildtype, *Dpp4*$^{fl/fl}$, MIP-Cre, and *Dpp4*$^{\beta\text{-cell-/-}}$ mice. Data are represented as the mean ± SEM, * *p<0.05.*

3.6 Elimination of β-cell-Derived *Dpp4* Fails to Improve Glucose Tolerance or Prevent Incretin Degradation in HFD-fed Mice *in vivo*

Mice were put on HFD to facilitate weight gain and induce metabolic stress. After 5 weeks of HFD feeding both *Dpp4*$^{\beta\text{-cell-/-}}$ and MIP-Cre mice exhibited similar, elevated response to both oral and *i.p.* glucose administration (Fig.3.6 A, B). Again, consistent with findings in chow-fed mice, sitagliptin was able to reduce glucose excursion with oral, but not *i.p.* glucose challenge in both groups. While no significant differences were found in blood glucose in response to a 0.6 IU injection of insulin at every time point, overall HFD-fed *Dpp4*$^{\beta\text{-cell-/-}}$ mice demonstrated slightly improved overall insulin sensitivity compared to control (Fig.3.6 C). Consistent with previous findings by Mulvihill et al., (2017) systemic inhibition with sitagliptin prior to glucose tolerance tests increased the concentration of active circulating GIP (Fig.3.6 D, E), and insulin (Fig.3.6 F, G) 15 minutes post-glucose, however, no preferential increase was observed in HFD-fed *Dpp4*$^{\beta\text{-cell-/-}}$ compared to MIP-Cre mice. Circulating DPP4 activity and concentration remained similar between all groups on HFD (Fig.3.6 H, I).

Finally, to assess islet function *in vitro*, mice were sacrificed at 60-63 weeks after being on a HFD for 35-40 weeks. Islets were isolated and sequentially perifused with KRBH buffer containing (in order): 2.8 mM glucose, 10mM glucose, followed by the addition of arginine, GLP-1, GIP, and glucagon to the 10mM glucose-containing KRBH buffer. No significant differences in GSIS were observed in islets isolated from *Dpp4*$^{\beta\text{-cell-/-}}$ (n=4) or MIP-Cre control (n=4) mice (Fig.3.6 J). Taken together, these data demonstrate that elimination of DPP4 from the pancreatic β-cells may lead to enhanced insulin sensitivity, but is not sufficient to improve glucose tolerance or GIP levels achieved by the whole-body *Dpp4*$^{-/-}$ mice or systemic DPP4 inhibition with sitagliptin.

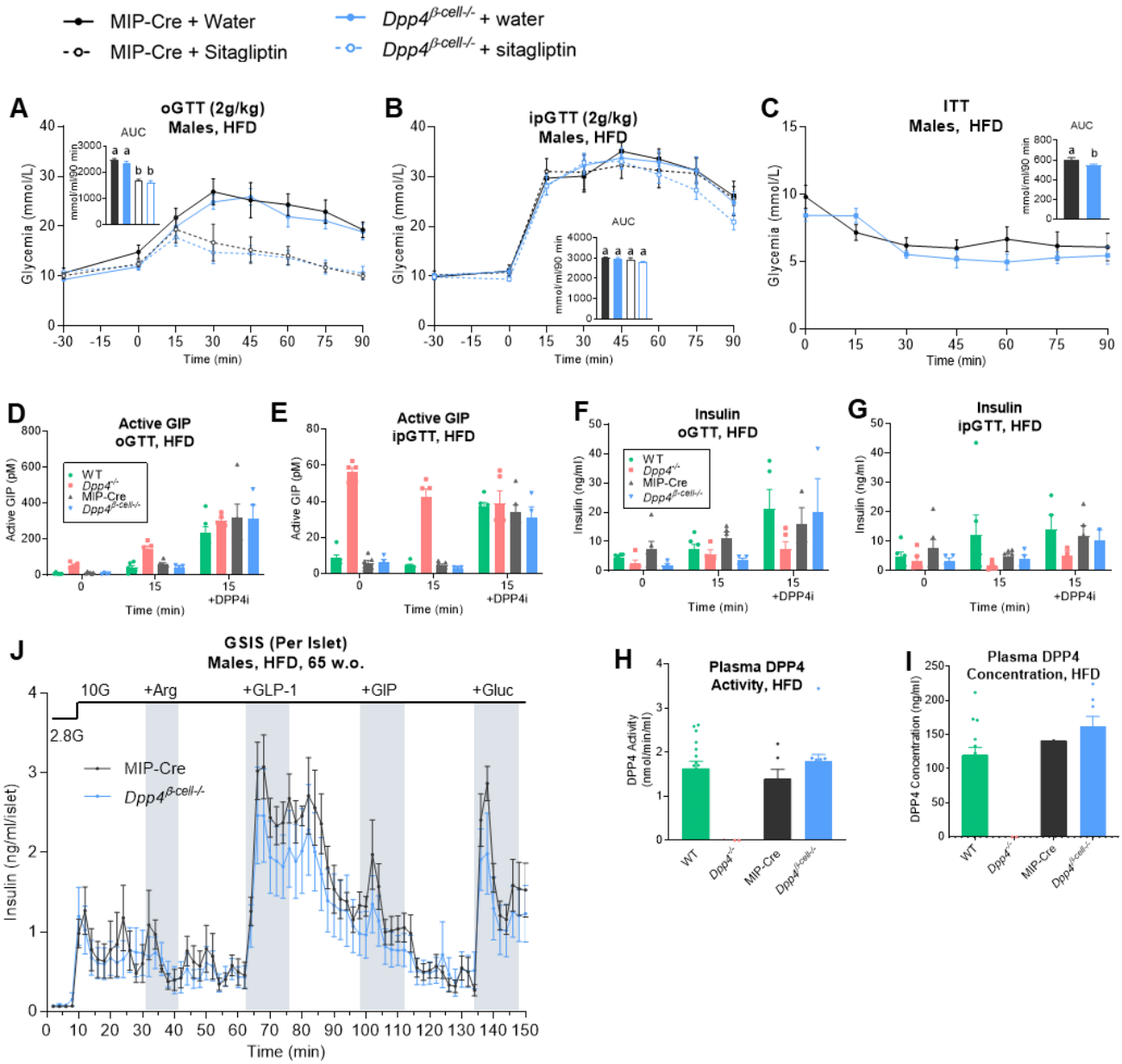

Figure 3.6. Elimination of β-cell-derived DPP4 Does Not Improve Glucose Tolerance, or Prevent Incretin Degradation *in vivo*.

(A) Oral glucose, (B) *i.p.* glucose, and (C) insulin tolerance tests in HFD-fed *Dpp4*$^{\beta\text{-cell-/-}}$ (n=13) and MIP-Cre control (n=6) mice. Sitagliptin was given by oral gavage 30 minutes prior to GTTs. A solution of 50% glucose in PBS was given at 2 g/kg body weight. Insulin was injected at 0.6 IU/kg of body weight. Inset graphs represent area under the curve (AUC), different letters indicate statistical differences between groups by ANOVA with post-hoc Tukey test. (D) Plasma active GIP measured in response to oral and (E) *i.p.* glucose in HFD-fed *Dpp4*$^{\beta\text{-cell-/-}}$ and control mice. (F) Plasma insulin measured in response to oral and (G) *i.p.* glucose in HFD-fed *Dpp4*$^{\beta\text{-cell-/-}}$ and control mice. (H) Plasma DPP4 activity and (I) concentration measured in plasma of HFD-fed *Dpp4*$^{\beta\text{-cell-/-}}$ and control mice without sitagliptin. (J) Insulin secretion measured during perifusion of islets isolated from 65-week old HFD-fed *Dpp4*$^{\beta\text{-cell-/-}}$ mice (n=4) and MIP-Cre control (n=4) mice with stimuli as indicated at the top of the graph. All data are represented as the mean ± SEM, *$p<0.05$.

3.7 β-Cell-Derived *Dpp4* Has a Role in Glucose Regulation in Female Mice Fed Chow Diet

To examine potential sex differences in the paracrine action of intra-islet DPP4 we also evaluated a female mouse model with DPP4 eliminated from the pancreatic β-cell (*Dpp4*$^{\beta\text{-cell-/-}}$) and assessed its ability to regulate glycemia *in vivo*. Unlike the male mice, female *Dpp4*$^{\beta\text{-cell-/-}}$ and their age- and weight-matched MIP-Cre-positive control exhibited different responses to an oral glucose gavage. Overall, the untreated MIP-Cre control group demonstrated the highest total rise in blood glucose during the oral glucose challenge as represented by the AUC analysis (Fig.3.7 A). This rise was also significantly reduced by sitagliptin treatment (Fig.3.7A). In contrast, sitagliptin had no further glucose-lowering effects on the *Dpp4*$^{\beta\text{-cell-/-}}$ group (Fig.3.7 A). In addition, glucose excursion of both sitagliptin-treated and untreated *Dpp4*$^{\beta\text{-cell-/-}}$ females was comparable to the sitagliptin-treated control group, suggesting a potential deletion of the DPP4 site important for glucose regulation in female *Dpp4*$^{\beta\text{-cell-/-}}$ mice. Unexpectedly, while MIP-Cre female mice were unaffected by sitagliptin treatment prior to *i.p.* glucose injection, *Dpp4*$^{\beta\text{-cell-/-}}$ group exhibited a modest decrease in glycemia during ipGTT as demonstrated by the AUC analysis (Fig.3.7 B). Lastly, no significant differences in insulin tolerance were observed between female *Dpp4*$^{\beta\text{-cell-/-}}$ and Cre-control mice (Fig.3.7 C).

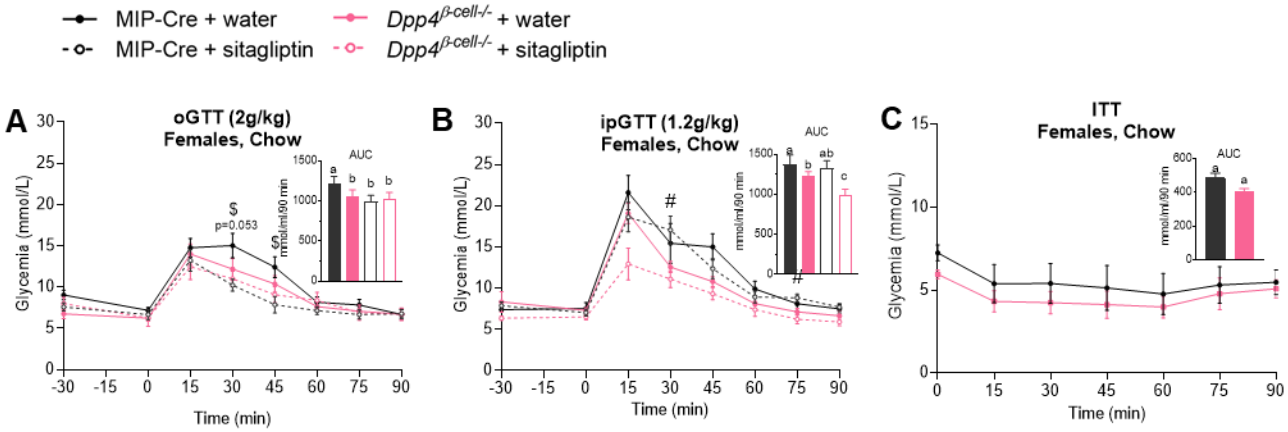

Figure 3.7. β-Cell-Derived *Dpp4* Has a Role in Glucose Regulation in Female Mice Fed Chow Diet.

(A) Oral glucose, (B) intraperitoneal glucose, and (C) insulin tolerance tests in young chow-fed female *Dpp4*$^{\beta\text{-cell-/-}}$ (n=7) and MIP-Cre control (n=10) mice. Sitagliptin was given by oral gavage 30 minutes prior to glucose tolerance tests. 30% glucose in PBS was given at 2 g/kg of body weight for oral glucose tolerance and at 1.2 g/kg of body weight for *i.p.* glucose tolerance tests. Inset graphs represent area under the curve (AUC), different letters indicate statistical differences between groups by ANOVA with post-hoc Tukey test. Data are represented as the mean ± SEM, *$p<0.05$.

$ indicates difference between sitagliptin-treated vs. water-treated MIP-Cre group; # indicates difference between sitagliptin-treated MIP-Cre and *Dpp4*$^{\beta\text{-cell-/-}}$ groups.

3.8 Elimination of β-cell-derived DPP4 Improves Oral Glucose Tolerance and Enhances Insulin Sensitivity in HFD-Fed Female Mice

After 5 weeks of HFD feeding to induce weight gain and metabolic dysregulation, we measured glycemic response to a gavage or an injection of glucose. Again, the untreated MIP-Cre control group exhibited the highest total rise in blood glucose when it was given orally, and the rise was significantly reduced by sitagliptin (Fig.3.8 A). HFD-fed *Dpp4*$^{\beta\text{-cell-/-}}$ group, however, saw no further improvements in glycemia with systemic DPP4 inhibition, providing further support for the intra-islet DPP4 being an important target for DPP4 inhibition in glycemia regulation in females. No significant differences in glucose excursions were found between groups with *i.p.* (1.2 g/kg of body weight) glucose administration (Fig.3.8 B), and treatment with sitagliptin was unable to significantly improve glycemia regulation in either group. Finally, *Dpp4*$^{\beta\text{-cell-/-}}$ mice exhibited a slight improvement in insulin sensitivity compared to control after 8 weeks of HFD-feeding as demonstrated by the AUC analysis (Fig.3.8 C).

Plasma collected during glucose tests was assayed for active GIP (Fig.3.8 D, E) and insulin (Fig.3.8 F, G) with no significant differences found between groups at any time point (baseline, 15 minutes post-glucose, or 15 minutes post-glucose with sitagliptin). Of note, systemic DPP4 inhibition achieved comparable increases in active circulating GIP levels in HFD-fed male and female mice (Fig.3.8 D, E, Fig.3.6 D, E). However, it resulted in approximately 1.5- to 2.5-fold increase in plasma insulin in females (unlike the 2- to 6-fold increase observed in males, Fig.6 F) during the OGTT. Finally, islets isolated from *Dpp4*$^{\beta\text{-cell-/-}}$ and MIP-Cre control mice exhibited similar glucose-stimulated insulin secretion in perifusion, with no significant differences observed during stimulation with arginine, GLP-1, or GIP (Fig.3.8 H).

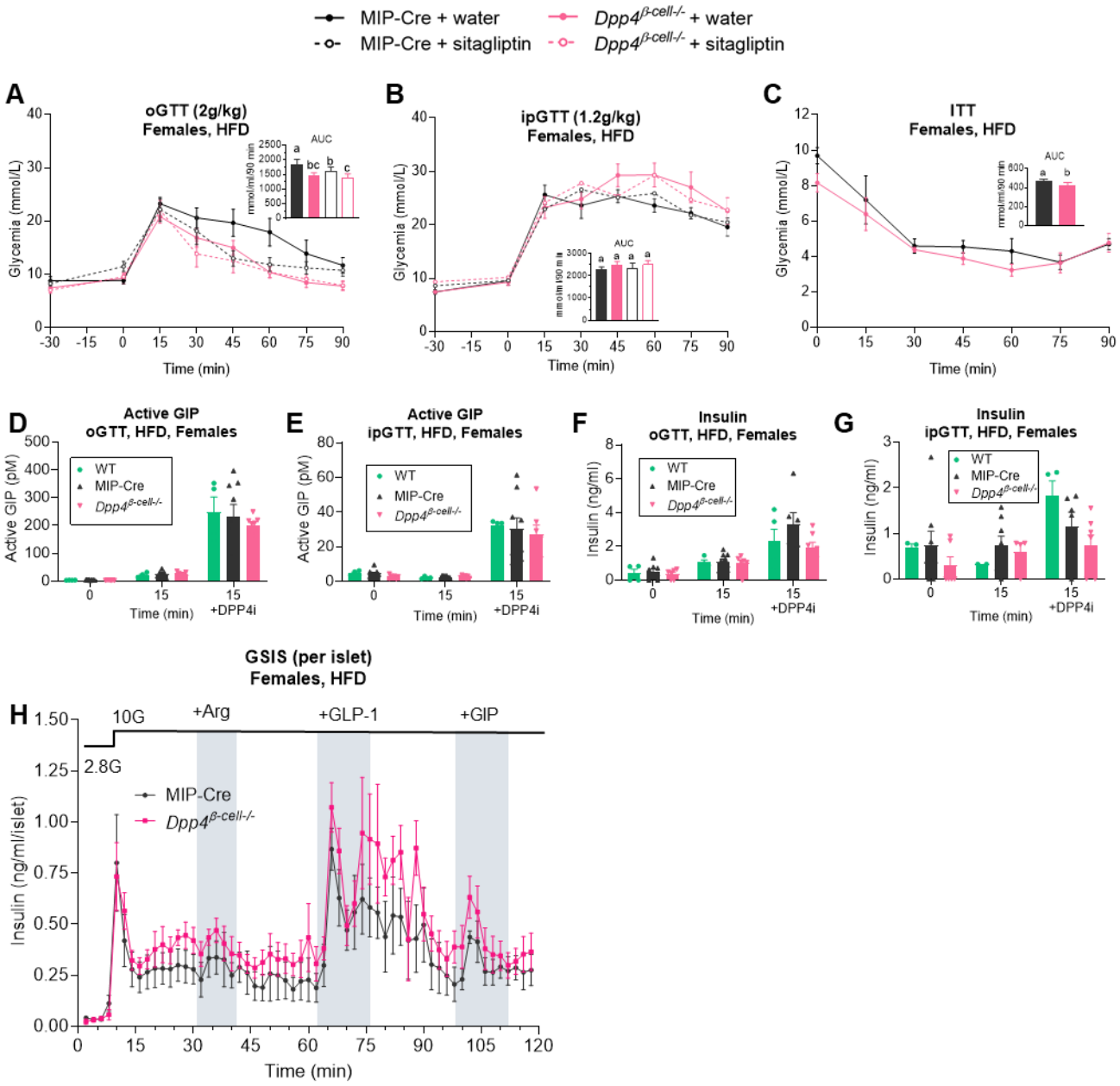

Figure 3.8. Elimination of β-cell-derived DPP4 Improves Oral Glucose Tolerance and Enhances Insulin Sensitivity in HFD-Fed Female Mice *in vivo*.

(A) Oral glucose, (B) intraperitoneal glucose, and (C) insulin tolerance tests in HFD-fed female *Dpp4*$^{\beta\text{-cell-/-}}$ and MIP-Cre control mice. 30% glucose (chow) or 50% glucose (HFD) in PBS was given at 2 g/kg of body weight for oral glucose tolerance and at 1.2 g/kg of body weight for *i.p.* glucose tolerance tests. n=7-10 per group. (D) Plasma active GIP measured at baseline and 15 minutes post-glucose in response to oral and (E) *i.p.* glucose tolerance tests in HFD-fed *Dpp4*$^{\beta\text{-cell-/-}}$ and control mice. (F) Plasma insulin measured at baseline and 15 minutes post-glucose during oral and (G) *i.p.* glucose tolerance tests in HFD-fed *Dpp4*$^{\beta\text{-cell-/-}}$ and control mice. (H) Stimulated insulin secretion measured during perifusion of islets isolated from HFD-fed *Dpp4*$^{\beta\text{-cell-/-}}$ (n=4) and MIP-Cre control (n=4) female mice with stimuli as indicated at the top of the graph. All data are represented as the mean ± SEM. *p<0.05.

3.9 Genetic Elimination of *Dpp4* in the Whole Pancreas (*Dpp4*$^{Pan-/-}$) Fails to Improve Glucose Tolerance in Mice Fed Normal Chow or a High-Fat Diet

Since our previous experiments found that elimination of β-cell-derived DPP4 had no consistent or significant effects on glucose metabolism and insulin sensitivity in male mice, we next examined whether whole pancreas-derived DPP4 could be overcompensating for this knockout. We generated a whole pancreas-specific *Dpp4* knockout mouse model (*Dpp4*$^{Pan-/-}$) using mice with the Cre recombinase expressed under the control of the mouse pancreatic duodenal homeobox (PDX1) promoter. Oral and *i.p.* glucose tolerance was compared to age- and sex-matched control groups that combined *Dpp4*$^{+/+}$ (wildtype), *Dpp4*$^{fl/fl}$, and PDX-Cre mice. Male *Dpp4*$^{Pan-/-}$ and their littermate controls exhibited similar glucose excursions in response to oral glucose gavage while on chow diet (Fig.3.9 A) and after HFD-feeding (Fig.3.9 C). Surprisingly, male *Dpp4*$^{Pan-/-}$ mice had significantly elevated plasma glucose levels after an *i.p.* injection of glucose versus control on regular chow (Fig.9 B), with a similar elevation also observed after switching to a HFD (Fig.3.9 D). Accordingly, area under the curve (AUC) analysis of glucose excursion over 90 minutes of the experiment demonstrated a significantly higher total rise in blood glucose during ipGTT on both diets. (Fig.3.9 B, D). Finally, female *Dpp4*$^{Pan-/-}$ mice displayed similar glucose excursions compared with controls regardless of route of glucose administration on a regular chow diet or after switching to HFD-feeding (Fig.3.9 E-H)

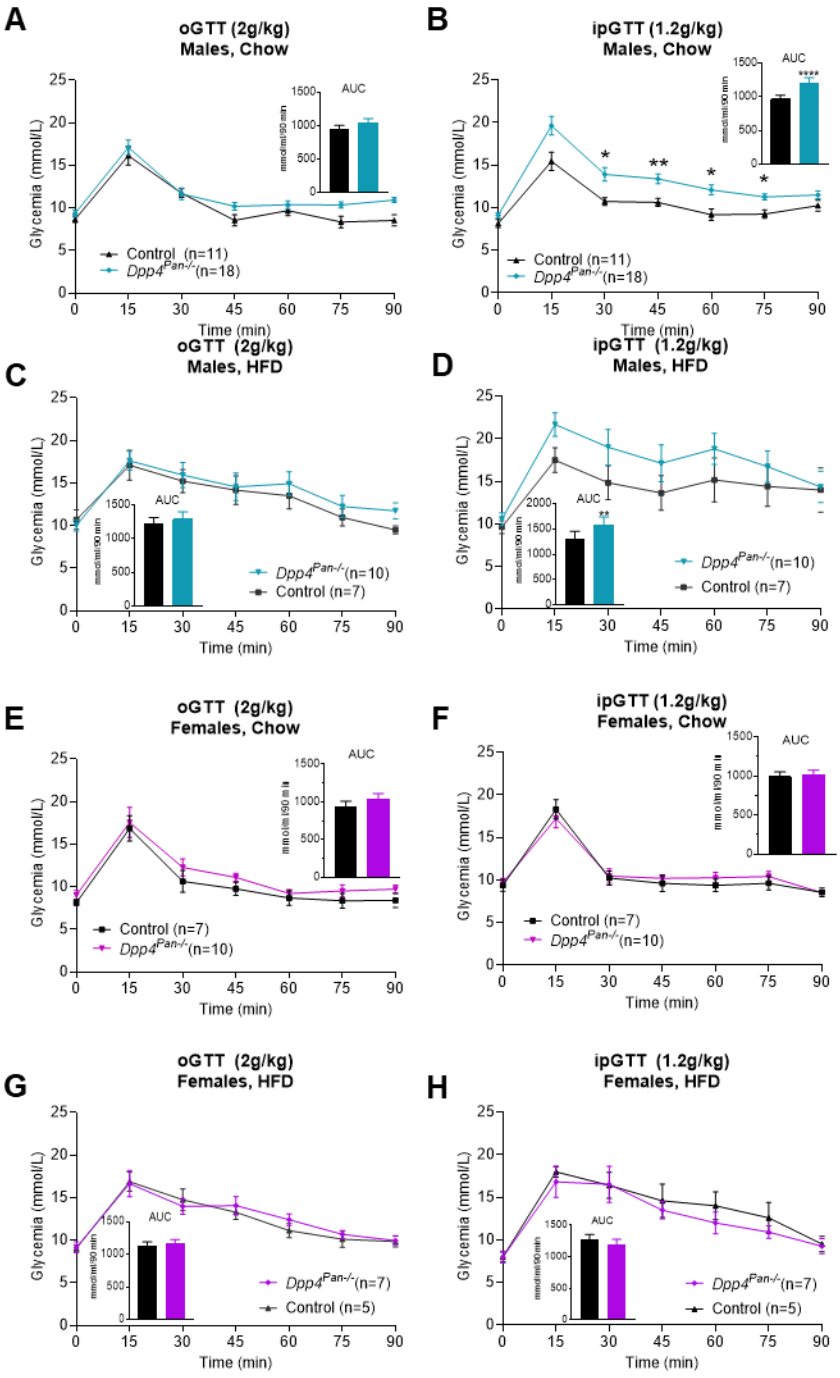

Figure 3.9. Genetic Elimination of *Dpp4* in the Whole Pancreas (*Dpp4*$^{Pan-/-}$) Fails to Improve Glucose Tolerance in Mice Fed Normal Chow or High-Fat Diet.

(A-D) Oral and *i.p.* glucose tolerance in male, chow-fed (A, B) or HFD-fed (C, D) *Dpp4*$^{Pan-/-}$ and control mice. (E-H) Oral and *i.p.* glucose tolerance in female, chow-fed (E, F) and HFD-fed (G, H) *Dpp4*$^{Pan-/-}$ and control mice. A solution containing 30% glucose (chow) or 50% glucose (HFD) in PBS was administered at 2 g/kg for oral and 1.2 g/kg for *i.p.* glucose tests at t=0. Inset graphs represent area under the curve (AUC) for each group. Control groups combined PDX-Cre, WT, and *Dpp4*$^{fl/fl}$. All data are represented as the mean ± SEM, **p<0.05.*

4. Discussion and Future Directions

4.1 Discussion

Previous studies have identified the gastrointestinal tract and vascular endothelial cells as the principal site of action for the acute glucoregulatory actions of DPP4 inhibitors (Mulvihill et al., 2017; Waget et al., 2011). However, despite a significant improvement in glycemia regulation and increased circulating incretin levels in HFD-fed mice with endothelial cell *Dpp4* knockout (*Dpp4*$^{EC-/-}$), these mice did not exhibit resistance to diet-induced obesity as seen in whole-body knockout *Dpp4*$^{-/-}$, highlighting the potential role for DPP4 inhibition in cell types beyond ECs (Mulvihill et al., 2017). Recently, studies in transgenic mice have identified pancreatic GLP-1 production as a requirement for glucose homeostasis during metabolic stress, and the deficiency in α-cell-derived GLP-1 could be rescued by DPP4 inhibitors (Traub et al., 2017). Therefore, in this project, we set out to evaluate the role of pancreatic and, more specifically, β-cell-derived DPP4 in the regulation of glycemia. The main findings of this thesis are summarized below.

Regulation of Glucose by DPP4 Inhibitors Is Dependent on The Activation of the Incretin Receptors on the β-Cell.

Under normal conditions, the binding of the incretin hormones GLP-1 and GIP to their receptors on the β-cell provides the essential stimulus for the nutrient-stimulated insulin secretion (Baggio and Drucker, 2007). Moreover, complete genetic elimination of the incretin receptors leads to impaired glucose tolerance and suppressed insulin response to oral glucose in mice (Ahrén et al., 2020; Hansotia et al., 2004). In line with previously published data (Mulvihill et al., 2017; Varin et al., 2019a; Waget et al., 2011), our data indicate that selective enteral inhibition of DPP4 with sitagliptin (14 µg/mouse) is sufficient to significantly improve post-prandial blood glucose regulation, and can be improved further with systemic (10 mg/kg) DPP4 inhibition in mice (Fig.1 A). However, compared to their wildtype counterparts, we found that mice with genetic deletion of both GIPR and GLP-1R (Double Incretin Receptor Knock Out, DIRKO) specifically within the β-cell exhibit elevated glucose excursion in response to oral glucose ingestion that could not be improved with either dose of DPP4 inhibitor sitagliptin (Fig.1 B). These data demonstrate that the activation of the incretin receptors GIPR and GLP-1R on the islet β-cell is essential for the glucose-lowering action by DPP4 inhibitors.

DPP4 Regulates Pancreatic Insulin Content and Glucose-Stimulated Insulin Secretion in Islets from HFD-Fed Mice *in vitro*

Previous studies have established that pancreatic DPP4 is localized mostly to glucagon-producing islet α-cells in humans and insulin-producing β-cells in rodents (Liu et al., 2014a). In humans, islet DPP4 activity and expression are reduced in T2D islets compared to islets from non-diabetic patients, and direct inhibition of islet DPP4 has been shown to improve β-cell function, survival, and insulin secretion (Bugliani et al., 2018; Omar et al., 2014). Conversely, DPP4 activity is increased in diet-induced obese mice, although its inhibition also results in potentiated insulin secretion in a GLP-1-dependent manner (Omar et al., 2014).

We showed here that genetic knockout of *Dpp4* can significantly increase pancreatic insulin content measured in pancreatic lysates of mice fed a high-fat, but not regular chow diet (Fig. 2C).

In order to further explore the link between islet-derived DPP4 and islet secretory function, we generated a line of tamoxifen-inducible β-cell-specific *Dpp4* knockout mice (*Dpp4*$^{\beta\text{-cell-/-}}$) using a Cre recombinase under the control of Mouse Insulin Promoter (MIP-Cre). The reduction in islet, but not liver DPP4 expression and activity, as demonstrated by a DPP4 activity assay and immunofluorescent analysis (Fig.3 B, C), confirmed the tissue-specific DPP4 knockout. Islets from both HFD-fed *Dpp4*$^{\beta\text{-cell-/-}}$ and control mice were isolated, and their response to glucose stimulation was assessed in perifusion (Fig.4 A-C). We found that *Dpp4*$^{\beta\text{-cell-/-}}$ islets have a modest, albeit not a significant increases in first- and second-phase insulin secretion in response to perifusion with 16.7mM glucose, resulting in significantly more overall insulin secreted after 60 minutes of stimulation (Fig.4 A). Concurrently, KCl-stimulated membrane depolarization resulted in comparable insulin secretion in both groups of islets, suggesting the initial difference observed was likely glucose-dependent (Brüning et al., 2017; Pizarro-Delgado et al., 2015). Therefore, to investigate whether this increase in GSIS occurred specifically due to a reduction in intra-islet DPP4 activity, we compared GSIS in these islets with and without the addition of a highly potent and specific DPP4 inhibitor, sitagliptin (Sangle et al., 2012). DPP4 inhibition resulted in a greatly increased GSIS in the control DPP4-positive islets (fig.4 B), however, did not further increase GSIS from the *Dpp4*$^{\beta\text{-cell-/-}}$ islets (Fig.4 C). This is in agreement with previously published findings reporting increased islet GLP-1 production in islets from patients with T2D, and the ability of DPP4 inhibitors to increase glucose-stimulated insulin granule exocytosis in T2D islets (Campbell et al., 2020; Ferdaoussi et al., 2020).Taken together, these results suggest that enzymatic cleavage by DPP4 is a significant regulator of glucose-stimulated insulin secretion *in vitro*. As such, pharmacological inhibition of intra-islet DPP4 may be an effective way so enhance insulin secretion from the islet.

DPP4 Expressed in MIP-Cre-Positive Cells (Islet β cells) Does Not Contribute to Glucose Regulation or Incretin Cleavage, but May Influence Insulin Tolerance in Male Mice.

The production of competent, active GLP-1 has been observed in both mouse and human islet α-cells, however, whether it is essential or dispensable for glucose regulation *in vivo* remains a debated topic (Chambers et al., 2017; Hansen et al., 2011; Marchetti et al., 2012; Song et al., 2019). It's been previously shown that treatment of islets from T2D patients with DPP4 inhibitor sitagliptin led to increased GLP-1 secretion, which was associated with improved β-cell health and survival (Bugliani et al., 2018; Buteau et al., 2004; Campbell et al., 2019; Farilla et al., 2003; Li et al., 2003). As such, we hypothesized that the deletion of intra-islet *Dpp4* could prevent the cleavage of GLP-1 by DPP4 within the islet, thus resulting in improved glucose tolerance via the augmented paracrine action on the β-cell.

While glucose excursion in response to oral glucose gavage remained comparable between groups throughout the experiment, overall glucose index, as shown by the AUC graph, was elevated in the *Dpp4*$^{\beta\text{-cell-/-}}$ mice (Fig.5 A). Accordingly, their circulating DPP4 activity, but not concentration was increased (Fig.5

D, E), which may explain the trend for slower glucose clearance during oGTT. In fact, recently published research suggests fluctuations in plasma DPP4 activity and protein concentration are dissociated, and it is the cellular origin of circulating DPP4 that determines whether one or both will be affected (Varin et al., 2019). More specifically, hepatocyte-derived circulating DPP4 was found to increase plasma DPP4 activity and promote insulin resistance, while adipocyte-derived DPP4 did not. Interestingly, we also saw a slight reduction in insulin sensitivity in *Dpp4*$^{\beta\text{-cell-/-}}$ mice on chow diet (Fig.5 C), an effect which could also be attributed to the increase in circulating DPP4 observed in these mice. However, the exact mechanism behind the observed differences remains to be explored.

Following the experiments on chow, we introduced HFD-feeding for a further 5 weeks to promote the development of the metabolic syndrome (Della Vedova et al., 2016). We hypothesized that diet-induced weight gain and metabolic stress may induce increased PC1/3 expression (Sancho et al., 2017) and subsequent increase in GLP-1 production in the islet, amplifying the potential differences in glucose tolerance or insulin sensitivity between groups. However, no differences in oral glucose tolerance were observed between the *Dpp4*$^{\beta\text{-cell-/-}}$ and Cre-positive control mice on HFD (Fig.6 A, B), suggesting that intra-islet DPP4 does not significantly regulate glucose metabolism *in vivo* under HFD conditions. Accordingly, our data demonstrate that intra-islet DPP4 does not contribute to circulating DPP4 concentration (Fig.6 I), activity (Fig.6 H), incretin cleavage (Fig.6 D, E) or insulin secretion (Fig.6 F, G) following glucose gavage or injection in mice fed a HFD. Here, DPP4 inhibition with sitagliptin improved glycemic excursion to an oral glucose gavage of both groups, but had no effects during an *i.p*. injection of glucose in either group regardless of the diet (Fig.5 B, Fig.6 B), providing further evidence for the gut-dominant incretin response in mice. In contrast to the results on chow diet, *Dpp4*$^{\beta\text{-cell-/-}}$ mice on HFD exhibited a slightly enhanced insulin sensitivity (Fig.6 C), however, this improvement was not correlated with any other parameter measured. As such, identifying a mechanism behind this effect becomes challenging. Finally, islet GSIS was without change in 65-week-old male control or *Dpp4*$^{\beta\text{-cell-/-}}$ mice fed a HFD for 40 weeks when perifused with 10 mM glucose-containing buffer or any other stimuli (Fig.6 J). Importantly, islet size was greatly and visibly increased in both groups at the age of takedown (65 weeks vs. 40 weeks shown in Fig.3 and Fig.4), indicative of extensive β-cell hypertrophy and hyperplasia (Ahrén et al., 2010). Our data, therefore, suggest that eliminating β-cell-derived DPP4 does not have a significant protective effect over chronic HFD feeding-induced β-cell dysfunction or islet function in male mice.

Female *Dpp4*$^{\beta\text{-cell-/-}}$ Mice Exhibit Improved Oral Glucose Tolerance and Insulin Sensitivity, but No Changes in Incretin Response or Insulin Secretion.

The protective effects of estrogen in diet-induced obesity and glucose homeostasis in female mice have previously been reported (Dakin et al., 2015; Hong et al., 2009; Pettersson et al., 2012; Stubbins et al., 2012). Following our findings in male mice, we set out to investigate the potential differences in islet function and glucose metabolism in female mice with induced *Dpp4* deletion from the islet β-cell. Here, chow-fed *Dpp4*$^{\beta\text{-cell-/-}}$ mice exhibited significantly lower total glucose response to oGTT compared to control,

and the glucose-lowering effect of DPP4i was absent, suggesting the site of DPP4 inhibition mediating the improvements in glycemia was no longer present. Curiously, when the glucose was administered *i.p.*, sitagliptin inhibited glucose response in *Dpp4*$^{\beta\text{-cell-/-}}$, but not in control mice. Since glucose is administered by an *i.p.* injection and bypasses the gut in the process, these data may suggest the presence of another gut-independent glucoregulatory mechanism sensitive to DPP4 inhibition in female mice.

Further validating intra-islet DPP4 as a target for DPP4i-mediated glucose regulation in females, HFD-fed *Dpp4*$^{\beta\text{-cell-/-}}$ females showed the lowest overall increase in plasma glucose, and no improvements in plasma glucose excursion in oGTT with the sitagliptin treatment (fig.8 A). However, *i.p.* glucose tolerance, as well as active circulating GIP and insulin measured 15 minutes after glucose injection (Fig.8 B, E, G) were not significantly improved in the *Dpp4*$^{\beta\text{-cell-/-}}$ females compared to Mip-Cre controls. Both groups remained sensitive to insulin as demonstrated by the insulin tolerance test (Fig.8 C), with a slight improvement in the *Dpp4*$^{\beta\text{-cell-/-}}$ mice. Overall, our findings show that female mice may have different mechanisms regulating their metabolic processes and glucose metabolism, and once again highlight the importance of researching as well as validating the therapeutic potential of drugs in both sexes.

Genetic Elimination of *Dpp4* in the Whole Pancreas Fails to Improve Glucose Tolerance in Mice Fed Normal Chow or a High-Fat Diet

Both human and mouse islets express active DPP4, but the localization pattern was found to be species-dependent: almost exclusively in α cells in the human islet, and mostly in β-cells in mouse islets (Liu et al., 2014a). However, recent findings using electron microscopy immunogold analysis demonstrated that approximately 25% of β-cells in human islets also express DPP4 (Bugliani et al., 2018). Our ability to generate an inducible α-cell specific *Dpp4* knockout, or a double α/β-cell *Dpp4* knockout was impeded by the linkage disequilibrium (LD) found between the *Dpp4* and *Gcg* genes (Appendix 1). LD mapping identified 3 linkage blocks within the recombination region, preventing homologous recombination and generation of our model of interest, or *Dpp4*$^{\alpha\text{-cell-/-}}$, by crossing *Dpp4*$^{fl/fl}$ line with a line where the *Gcg* promoter drives Cre expression. Therefore, in order to control for any remaining pancreatic or islet DPP4 activity in the *Dpp4*$^{\beta\text{-cell-/-}}$ mice, we generated a mouse model with a pancreas-specific DPP4 knockout (*Dpp4*$^{Pan\text{-/-}}$) and evaluated its response to glucose *in vivo.* Male *Dpp4*$^{Pan\text{-/-}}$ mice exhibited no changes in oral glucose tolerance on chow or HFD compared to their DPP4-positive controls (wildtype, *Dpp4*$^{fl/fl}$, PDX-Cre). Elimination of pancreatic DPP4 failed to improve glucose tolerance in female mice regardless of diet or route of glucose administration (Fig.9 E-H).

Unexpectedly, *Dpp4* deletion in the pancreas resulted in sustained elevated plasma glucose throughout the *i.p.* glucose challenge experiment in chow-fed male mice (Fig.9 B), and a trend toward elevated glycemia after HFD-feeding (Fig.9 D). In both experiments, *Dpp4*$^{Pan\text{-/-}}$ males showed significantly higher total glucose response as measured by the AUC analysis after 90 minutes of the experiment. This pattern was not expected, but several possibilities may be considered. First, the knockout mouse model used here was generated using mice expressing Cre recombinase under the control of the mouse

pancreatic and duodenal homeobox 1 (PDX1) promoter. PDX-Cre is active in the pancreatic epithelium, antral stomach and duodenum in neonates and in pancreatic β-cells in adults. Therefore, PDX-Cre-mediated deletion of the *Dpp4* sequences during the early stages of development may have resulted in unintended changes in pancreatic or gastrointestinal tract development, leading to impaired *i.p.* glucose tolerance in the $Dpp4^{Pan-/-}$ group. Indeed, PYY, a physiological substrate of DPP4, is highly expressed in the embryonic pancreas, and thus may be implicated in the endocrine cell differentiation (Bugliani et al., 2018; Jackerott et al., 1996). Furthermore, PYY was reported to be a negative regulator of bone mass, and its inhibition by DPP4 may be necessary for proper development in the early embryonic stages (Leitch et al., 2019). In addition, DPP4 binding to fibronectin and collagen, components of the extracellular matrix involved in cell adhesion and matrix remodelling (Piazza et al., 1989), was also disrupted in $Dpp4^{Pan-/-}$ mice. Lastly, while we made effort to only use Cre-positive mice in control groups, the sample size of the PDX-Cre-positive control groups was too small for any statistical analysis (n=2). This led us to combine their results with results from other DPP4-positive mice (wildtype, $Dpp4^{fl/fl}$), which may have skewed the final glucose curve (Song and Palmiter, 2018). Overall, this experiment recapitulates the findings from the $Dpp4^{\beta\text{-}cell-/-}$ mice, and the data suggest that targeted pancreatic deletion of *Dpp4* is not sufficient to improve glycemia in mice.

4.2 Future directions

In view of the failure of β-cell- or pancreas-specific deletion of *Dpp4* to improve glucose regulation in mice, there are now several themes that future studies could explore. First, our understanding of the biologically relevant substrates of DPP4 is currently very limited as many have been identified using *in vitro* approaches. Over the last several years, incretin-based therapeutic agents have been evaluated both in animals and humans, proving their safety, low rate of adverse effects, and efficacy in treating T2D symptoms. However, the implications of long-term pharmacological DPP4 inhibition in humans is an important area that remains to be investigated (Karagiannis et al., 2014). Moreover, as our results indicate, *Dpp4* may play an important role in the developing pancreas, and so the effect of embryonic, pancreas-specific *Dpp4* deletion should be explored.

Second, targeted inhibition of enzymatically active proteins in the DASH family, such as FAP, may provide an effective strategy to lower blood glucose as a single or dual therapy with other incretin-based interventions. DPP4 and FAP share a number of substrates, although with varied affinities, and FAP-mediated cleavage regulates other unique processes, such as ECM remodelling, cell migration, and cell signaling (Lay et al., 2019). Recently, FAP was found to regulate the bioactivity of human fibroblast growth factor 21 (FGF-21), a predominantly liver-derived protein involved in weight control, and glucose and lipid metabolism (Coppage et al., 2016). Interestingly, mouse FGF-21 lacks the FAP cleavage site and is therefore not affected by it (Coppage et al., 2016). Thus, studies evaluating selected FAP inhibition may lead to novel developments in treatment of T2D, however, would need to be carefully designed to accommodate any inter-species differences.

4.3 Limitations

There are several caveats to be considered with the findings presented in the thesis above, including the use of murine models of disease, and the use of Cre recombinases to induce genetic recombination. Although overall insulin signaling and glucose metabolism are mediated through similar mechanisms in mice and humans, important differences remain. Human pancreatic islet structure and cellular composition differs substantially from that of mouse. In addition, DPP4 colocalizes with α-cells in humans, and β-cells in mice. As our islet perifusion and DPP4 inhibition studies were limited to mouse islets, it remains to be determined whether intra-islet DPP4 has an effect in humans in the context of insulin secretion. Also, while the use of CreERT2/loxP system is a widely used tool to induce conditional genetic recombination in mice, its use also implies some major considerations. Several Cre strains have demonstrated unreported or unexpected deletion activity resulting from being expressed in off-target tissue types (Heffner et al., 2012). On the other hand, variability in Cre expression and activity may lead to Cre mosaicism and inconsistent or incomplete recombination in target tissues even between littermates (Heffner et al., 2012). Moreover, tamoxifen used to induce the Cre translocation was found to inhibit β-cell proliferation and induce acute fat loss, followed by *de novo* lipogenesis in mouse studies (Ahn et al., 2019; Ye et al., 2015). To mitigate these effects, all animals in the experiments reported here received the same dose of tamoxifen prior to metabolic experiments, followed by a 4-week washout period, and Cre-positive control mice were used whenever possible.

Separately, our research was interrupted due to the COVID-19 pandemic and the resulting quarantine, which severely limited our ability to conduct animal and laboratory experiments from March 16, 2020 to June 15, 2020. This included the use of any off-site facilities and equipment (access to Mesoscale Discovery Equipment for active GLP-1 measurements), age-matching male and female mice, tracking animal weights and food intake throughout the study, and a drastic reduction in the overall number of animals used.

4.4 Final conclusions

To examine the role of intra-islet DPP4, we generated two murine models with *Dpp4* genetically eliminated from the adult islet β-cell or embryonically through the entire pancreas using the Cre-loxP system under the control of mouse insulin promoter or the mouse pancreatic and duodenal homeobox 1, respectively. We evaluated the differences in their glucose regulation, insulin tolerance, incretin degradation, and circulating DPP4 activity and concentration. In addition, we subjected islets isolated from these animals to perifusion in order to assess their glucose-stimulated insulin secretion *in-vitro*. The results reported in this thesis have several important implications in the research of the physiological functions of DPP4 and incretin-based therapies. In summary, we have demonstrated that 1) the glucose-lowering action of DPP4 inhibitors requires activation of the incretin receptors on the islet β-cell; 2) Enzymatic cleavage by DPP4 is a significant regulator of glucose-stimulated insulin secretion *in vitro*; 3) Targeting pancreas- or β-cell-derived DPP4 fails to improve glucose metabolism or incretin cleavage, but may improve insulin sensitivity in male mice fed a HFD; and 4) β-cell-derived DPP4 may be an effective target of DPP4 inhibitors in regulating glycemia in female mice. As demonstrated above, we found sex to be an important variable in experiments involving metabolism and glucose regulation, however, the exact mechanisms underlying the improvements seen in female mice remain to be elucidated.

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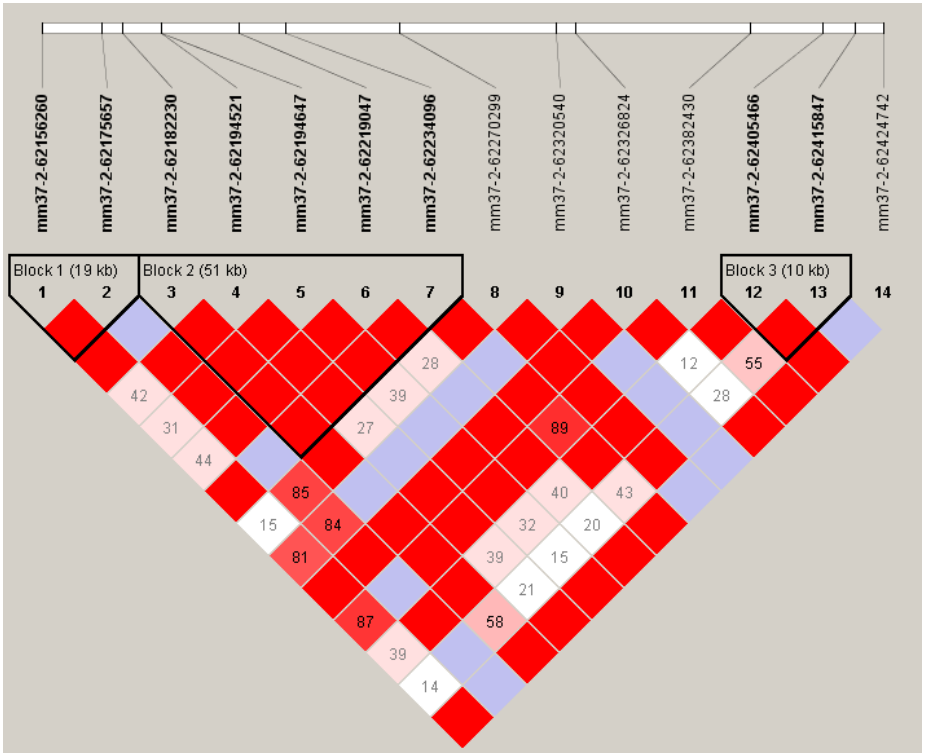

Appendix 1. Linkage Disequilibrium Between *Dpp4* and *Gcg* Interferes with the Creation of α-Cell-Specific *Dpp4 K*nockout (*Dpp4*$^{\alpha\text{-cell-/-}}$) Mouse.

Linkage disequilibrium (LD) mapping of fourteen SNPs identified in the C57BL6 mouse strain on chromosome 2 (from chr2:62 152 690-62 431 222) region containing *Dpp4*, *Gcg* and *Fap.* Three linkage blocks were identified within this region, thus preventing homologous recombination.